# Stage-specific *Arf* tumor suppression in *Notch1*-induced T-cell acute lymphoblastic leukemia

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**Frequent hallmarks of T-cell acute lymphoblastic leukemia (T-ALL) include aberrant NOTCH signaling and deletion of the *CDKN2A* locus, which contains 2 closely linked tumor suppressor genes *(INK4A* and *ARF).* When bone marrow cells or thymocytes transduced with a vector encoding the constitutively activated intracellular domain of Notch1 (ICN1) are expanded ex vivo under conditions that support T-cell development, cultured progenitors rapidly induce CD4$^+$/CD8$^+$ T-ALLs after infusion into healthy syngeneic mice. Under these conditions, enforced ICN1 expression also drives formation of T-ALLs in unconditioned CD-1 nude mice, bypassing any requirements for thymic maturation. Retention of *Arf* had relatively modest activity in suppressing the formation of T-ALLs arising from bone marrow–derived ICN1$^+$ progenitors in which the locus is epigenetically silenced, and all resulting *Arf* $^{+/+}$ tumors failed to express the p19$^{Arf}$ protein. In striking contrast, retention of *Arf* in thymocyte-derived ICN1$^+$ donor cells significantly delayed disease onset and suppressed the penetrance of T-ALL. Use of cultured thymocyte-derived donor cells expressing a functionally null *Arf-GFP* knock-in allele confirmed that ICN1 signaling can induce *Arf* expression in vivo. *Arf* activation by ICN1 in T cells thereby provides stage-specific tumor suppression but also a strong selective pressure for deletion of the locus in T-ALL. (Blood. 2009;114: 4451-4459)**

## Introduction

T-cell acute lymphoblastic leukemia (T-ALL) accounts for approximately 20% of all pediatric lymphoblastic leukemias and comprises a significant proportion of cancers affecting children and adolescents.[1] Advances in treatment, focusing largely on dose intensification in multiagent therapeutic regimens, have produced remarkable cure rates, with overall survival of approximately 80%. Nonetheless, T-ALL is overly represented among relapsed ALL cases, and the dose-intensive therapy required to improve its cure imposes its own disease burden.

Activating mutations of *NOTCH1* have been found in 50% of T-ALLs, making it one of the most commonly mutated genes in this disease.[2] Notch is a cell-surface receptor expressed in many developing organ systems, in which Notch ligands function in conjunction with other morphogens, including Wnt, Hedgehog, and bone morphogenic proteins, to program cell fate decisions.[3] The Notch receptor is formed by intracellular proteolytic cleavage of a single polypeptide chain, the 2 resulting subunits undergoing dimerization and transport to the plasma membrane.[4] When bound by its ligands (members of the delta/jagged family), the transmembrane receptor is cleaved extracellularly by an ADAM protease and intracellularly by γ-secretase, thereby releasing the intracellular Notch (ICN) domain into the cytoplasm. After transport into the cell nucleus, the ICN complexes with DNA-binding partner proteins to activate target genes. Mutations in Notch may affect its heterodimerization domain, which reduces or eliminates ligand dependency of the receptor, or they may target the intracellular proline-, glutamic acid-, serine-, and threonine-rich domain, thereby stabilizing the active, intracellular signaling moiety.[2,4,5] These 2 classes of mutations can occur concomitantly in up-regulating Notch target gene expression.

Notch transcriptional activity directs virtually every stage of T-cell development, from the earliest commitment of bone marrow–derived progenitors to the T-lymphoid lineage through stages of thymocyte maturation to double-positive (DP) CD4$^+$/CD8$^+$ cells.[5,6] Although malignant thymocytes that typify Notch1-associated T-ALL are usually arrested at the DP stage, DP progenitors do not appear to be the tumor-initiating population. Instead, tumorigenic cells arise from more immature T-cell progenitors that ultimately generate monoclonal tumors expressing unique T-cell receptor (TCR)–β chains and diverse TCR-α chains.[7] Although leukemogenesis is independent of the pre-TCR, per se, ICN1 overexpression cannot induce leukemia in cells that lack pre-TCR signaling, implying that malignant transformation occurs after pre-TCR signaling but before completion of α chain rearrangement.[7-9] Although the extent to which *NOTCH1* mutations represent founding oncogenic events in T-ALLs has been debated,[10,11] robust mouse models of T-ALL driven by activated Notch1 implicate this pathway as an appealing target for therapeutic intervention.[4,5,11-13]

Occurring more frequently than mutation of *NOTCH1,* deletion of the *CDKN2A* (hereafter *INK4A-ARF*) locus, which encodes 2 functionally distinct but closely chromosomally linked tumor suppressors, p16$^{INK4A}$ and p14$^{ARF}$ (p19$^{Arf}$ in the mouse), affects more than 70% of T-ALL cases.[14,15] Most cases of T-ALL that exhibit *NOTCH1* mutations also sustain deletions of *INK4A-ARF,* arguing that functional interactions stemming from these different events may be an obligate part of the life history of the majority of T-ALLs. By inhibiting cyclin D–dependent kinases, p16$^{Ink4a}$ helps

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to maintain the retinoblastoma protein (RB) in its hypophosphorylated growth-suppressive form, thereby blocking entry into the DNA synthetic (S) phase of the cell-division cycle. Instead, $p19^{Arf}$ antagonizes the E3 ubiquitin ligase activity of the *Mdm2* gene product to activate a p53-dependent transcriptional program that triggers either cell-cycle arrest or apoptosis, depending upon the cell type and collateral signal inputs.[16] The products of the *Ink4a-Arf* locus are generally not expressed in normal mouse tissues but are activated by aberrantly elevated and sustained hyperproliferative stress signals. Subsequent expression of $p16^{Ink4a}$ and $p19^{Arf}$ triggers RB- and p53-dependent programs that eliminate incipient cancer cells. Conversely, deletion of the *Ink4a-Arf* locus simultaneously compromises the activities of both RB and p53 and is appreciated to be one of the most frequent events occurring in many forms of human cancer.[17,18]

The *Ink4a-Arf* locus is epigenetically silenced in bone marrow–derived adult hematopoietic stem cells,[19-24] but it is remodeled during lymphoid development, becoming poised to respond to oncogenic stress signals during the maturation of the T- and B-cell lineages.[25-27] Moreover, both *Ink4a* and *Arf* transcripts progressively accumulate in peripheral B-lymphoid cells as mice age, thus conferring resistance to lymphoid tumor development once the adaptive immune system has developed.[26,27] Deletion of the *Ink4a-Arf* locus in the mouse germline counters these age-related effects, and by increasing the self-renewal potential of lymphoid cells, strongly predisposes to the early development of T-cell neoplasms.[28,29] Using an adoptive transfer protocol that takes advantage of cultured Notch1-expressing T cells derived from progenitor pools enriched for different stages of the T-cell developmental program, we have now established a functional relationship between constitutive *Notch1* signaling and *Arf* deletion in the generation of T-ALL.

## Methods

### Expression vectors and retroviral production

A cDNA encoding the intracellular domain of human NOTCH1 (ICN1; amino acids 1761-2555 of the full-length protein) was expressed in a mouse stem cell virus–internal ribosome entry site–green fluorescent protein vector (MSCV-IRES-GFP)[30] provided by J. Opferman (St Jude Children's Research Hospital). A re-engineered vector that produces cherry fluorescent protein (CFP) in lieu of GFP was used where indicated. Packaging of replication-incompetent, ecotropic retroviral particles was achieved by transient cotransfection of HEK-293T cells with vector and helper plasmids as previously described.[31]

### Culture conditions

Marrow was obtained from the long bones of wild-type or syngeneic *Arf*-null C57BL/6 mice harboring targeted deletions of exon-1β[29] either 4 days after treatment with 150 mg/kg 5-fluorouracil (5-FU; Abraxis) or from untreated donors. Cells from 5-FU–treated mice were incubated for 2 days at 37°C in Stemspan SFEM medium (StemCell Technologies) supplemented with 20 ng/mL recombinant mouse interleukin-3 (IL-3), 50 ng/mL recombinant human IL-6, 50 ng/mL recombinant mouse stem cell factor and 100 ng/mL recombinant mouse thrombopoietin (all from R&D Systems). Bone marrow cells or single-cell suspensions prepared from the thymi of mice from the same genotypes were then infected with retrovirus immobilized on retronectin (Takara Bio). Cells exposed to virus for a period of 24 to 48 hours were either directly inoculated into irradiated mice or placed into coculture with OP9 stromal cells.[32,33] Cocultures on OP9 stroma were maintained in alpha-minimum essential medium (Invitrogen) supplemented with 10% fetal calf serum (Hyclone), 1mM sodium pyruvate (Sigma-Aldrich), penicillin/streptomycin, and recombinant mouse IL-7 and FMS-like tyrosine kinase 3 ligand (R&D Systems) both at 5 ng/mL. Cells were enumerated and passaged onto fresh stroma every 4 days. The immunophenotypes of GFP-positive cells in the cultures were determined by fluorescence-activated cell sorting (FACS) using fluorescently tagged antibodies directed to the mouse T-cell markers Thy1.2, TCRβ, CD4, and CD8.

### Bone marrow transplantation and adoptive transfer of cultured cells

All mice were housed in an American Association of Laboratory Animal Care–accredited facility and were treated on protocols approved by St Jude Children's Hospital Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Recipient male C57BL/6 or CD-1 nude mice (8-12 weeks old) were lethally irradiated with 11 Gy split into 2 equal doses. Each recipient mouse was injected via tail vein with a mixture of $5 \times 10^5$ unmanipulated bone marrow cells and half of the ICN1-transduced bone marrow from one 5-FU–treated donor mouse. Irradiated mice were maintained on water supplemented with enrofloxacin (Bayer) for 3 weeks after transplantation.

Bone marrow–derived or thymocyte-derived cells cultured for 12 days on OP9 stroma were harvested, and the vector-infected percentage was determined by FACS for the GFP or CFP marker. Recipient animals were injected via tail vein with a dose calculated to deliver $2 \times 10^5$ fluorescent cells. All recipient mice were observed daily for clinical signs of illness and killed when moribund.

### Genomic PCR and immunoblotting

GFP-positive leukemic T cells were purified by FACS from single-cell suspensions prepared from the spleens or lymph nodes of moribund animals. Genomic DNA was subjected to polymerase chain reaction (PCR) for exon-1β and exon-2 of the *Cdkn2a (Ink4a-Arf)* locus. PCR reactions were carried out in 25-μL volumes using Taq polymerase, Q solution, and buffer (all from QIAGEN), 12.5 pmol forward and reverse primers, and 0.2mM deoxynucleotide triphosphates. The reaction mix was heated for 3 minutes at 94°C and then subjected to 30 cycles of 1-minute denaturation at 94°C, 1-minute annealing at 66°C, and 2-minute extension at 72°C. PCR products were visualized after electrophoresis on 2% agarose gels containing 0.5 ng/mL ethidium bromide. Primers for *Arf* exon-1β were (sense) 5′-AGTACAGCAGCGGGAGCATGG and (antisense) 5′-TTGAGGAGGACCGTGAAGCCG, and for *Ink4a-Arf* exon-2 were (sense) 5′-ATGATGATGGGCAACGTTC and (antisense) 5′-CCAATATCGCACGATGTC. Immunoblotting was performed as described.[31] In brief, detergent lysates were prepared from purified GFP-positive leukemic cells and protein concentration was quantified by bicinchoninic acid assay (Pierce). Samples (50 μg protein per lane) were electrophoretically separated on 4% to 12% Bis-Tris NuPAGE gels (Invitrogen), transferred to polyvinylidene fluoride membranes (Millipore), and detected using a highly sensitive rat monoclonal antibody to $p19^{Arf}$ (5C3-1).[34] Antibodies to nucleophosmin (NPM; Invitrogen) were used to control for protein loading.

### Southern blotting

Genomic DNA (20 μg/lane) from FACS-sorted, GFP-marked lymph node cells from moribund mice was digested with *Hin*dIII, separated on a 0.7% agarose gel, and transferred to Nytran SuPerCharge nylon membrane (Whatman Inc). The membrane was prehybridized for 1 hour at 65°C with PerfectHyb Plus (Sigma-Aldrich) containing 100 μg/mL salmon sperm DNA. Radiolabeled DNA probes prepared by random priming with [$\alpha^{32}$P] deoxyadenosine triphosphate (Roche Diagnostics) were then added, and hybridization was continued for 16 hours. TCRβ rearrangements were visualized using a radiolabeled 2.3-kb *Eco*RI Jβ2 fragment.[35] Membranes were briefly rinsed in 0-3M NaCl, 0.03M Na citrate containing

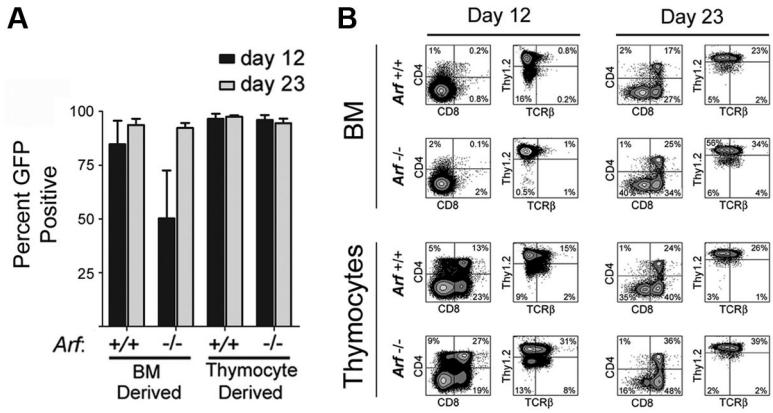


**Figure 1. Emergence of GFP⁺ cells and their immunophenotypes on day 12 and day 23 of culture.** (A) Cells derived from 5-FU–conditioned bone marrow (BM) or from thymocytes explanted from mice of the indicated *Arf* genotypes (labeled at bottom) were transduced with a vector encoding ICN1 and GFP in *cis* and cultured on OP9 stroma with cytokine support. Cultured cells expanded exponentially and underwent 25 to 30 population doublings over a period of 36 days (supplemental Figure 1). The percentage of GFP⁺ cells in the cultures was determined at day 12 and day 23. The results of 3 experiments are shown, and error bars indicate SDs. (B) After 12 and 23 days of culture (indicated at the top of the panels) the percentages of GFP⁺ cells that expressed CD4 and CD8 or Thy1.2 and TCRβ were determined using an automated cell sorter. The percentages of cells expressing each marker are indicated in at least 3 of the 4 quadrants of each panel. The origin of donor cells and their *Arf* genotypes are indicated to the left.

0.1% sodium dodecyl sulfate, followed by a stringent wash in 15mM NaCl, 1.5mM Na citrate with 0.1% sodium dodecyl sulfate for 30 minutes at 55°C. Dried membranes were exposed to Kodak BioMax film (Kodak) at −80°C.

## Results

### *Arf* inactivation modestly accelerates ICN1-induced T-cell maturation ex vivo

Established mouse models of Notch1-driven leukemia rely on transplantation of lethally irradiated recipient mice with primitive bone marrow–derived progenitors engineered to constitutively express gain-of-function Notch1 mutations.[8,11-13] Because the bone marrow transplantation (BMT) model of Notch1-driven leukemia is relatively, though not absolutely, dependent on the lethal irradiation of recipients, it diverges from the pathogenesis of the human disease, which arises in a hematologically and immune-competent host. Culturing of Bcr-Abl–transduced *Arf*-null bone marrow cells under conditions that favor the outgrowth of pre-B cells was previously revealed to efficiently enrich for leukemia-initiating cells (LICs) that rapidly induced fatal tumors in healthy, nonirradiated syngeneic recipient mice.[36,37] Following an analogous strategy, we transduced bone marrow progenitors or thymocytes from *Arf* $^{+/+}$ or *Arf* $^{-/-}$ mice with a vector encoding ICN1 and GFP and established short-term cocultures on OP9 stroma to support T-cell development through the DP stage.[32] Although mutant Notch1 alleles can drive ectopic T-cell development, those most commonly found in human T-ALL fail to initiate disease on their own in the mouse BMT model.[11] Therefore, to maximize the chances of inducing leukemia after infusion of cultured cells into naive recipient mice, we chose to use ICN1, a "strong" mutant allele that efficiently induces T-ALL in the BMT setting.

When transduced with the ICN1-GFP vector, cultured bone marrow and thymic progenitors passaged at 4-day intervals gave rise to T-cell populations that steadily expanded for at least a month (supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Although *Arf* $^{+/+}$ and *Arf* $^{-/-}$ bone marrow cells infected with a control vector encoding GFP alone were also able to proliferate, these populations expressed the B-cell markers B220 and CD19 and negligible levels of the T-cell marker Thy1.2. *Arf* $^{+/+}$ donor thymocytes transduced with the control vector were completely incapable of initiating cultures on OP9 stroma, whereas their *Arf* $^{-/-}$ counterparts produced poorly growing T-cell colonies at very low efficiencies (data not shown). Thus, expression of ICN1 was required to support T-cell development ex vivo.

After 23 days of culture, almost all of the cultured cells from mice of both *Arf* genotypes expressed GFP, implying that ICN1 coexpression provided them with a strong selective advantage (Figure 1A). However, GFP-marked *Arf* $^{-/-}$ bone marrow–derived cells did not gain as strong a proliferative advantage initially (Figure 1A, day 12), reflecting the propensity for *Arf* inactivation alone to facilitate the development of competing unmarked bone marrow–derived B-lineage cells that dominated the cultures at day 12 but were outcompeted by GFP-marked T cells by day 23. By day 12, 80% to 90% of GFP-marked cells expressed Thy1.2 (Figure 1B). Expression of TCRβ, CD4, and CD8 increased as the cells were passaged, highlighting ongoing maturation (Figure 1B). In age-matched cultures, *Arf*$^{-/-}$ cells reproducibly yielded a greater fraction of cells expressing TCRβ, CD4, and CD8 than did *Arf* $^{+/+}$ cells (Figure 1B), suggesting that *Arf* inactivation confers an increased propensity to generate DP thymocytes more rapidly.

### Cultured ICN1-expressing donor cells derived from bone marrow generate T-ALL in healthy nonirradiated animals

We next infused $2 \times 10^5$ GFP-positive cells derived from 12-day cultures of 5-FU–conditioned bone marrow into nonirradiated syngeneic mice. Recipients of these adoptively transferred ICN1-positive T cells developed a rapidly fatal T-ALL (Figure 2A), with bone marrow invasion, lymphocytosis, and bulky disseminated disease involving the spleen, liver, and lymph nodes, and variably invading the thymus, kidneys, and lungs (Figure 3A). Each of 15 recipients of ICN1⁺ *Arf* $^{-/-}$ donor cells and 18 of 20 recipients of ICN1⁺ *Arf* $^{+/+}$ cells succumbed to disease (Figure 2A). Median survival times for healthy mice that had received GFP-marked *Arf* $^{+/+}$ or *Arf* $^{-/-}$ bone marrow–derived cells were 34 and 29 days, respectively (Figure 2A). Notably, deletion of *Ink4a* in addition to *Arf* did not further accelerate disease onset (supplemental Figure 2A). Furthermore, these features paralleled the penetrance and latency periods observed in lethally irradiated recipient mice that received a transplant of acutely transduced donor cells together with an excess number of radioprotective, untransduced bone marrow progenitors (supplemental Figure 2B-C). The immunophenotype of the tumors confirmed their T-cell identity, with Thy1.2, CD4, CD8, and TCRβ all expressed (Figure 3B) and CD19, Mac-1, and Gr-1 absent (data not shown). Notably, the majority of leukemic cells were CD4⁺/CD8⁺ (Figure 3B) unlike the much less mature day-12 bone marrow–derived donor population that contained only approximately 1% DP cells (Figure 1B). The overall clinical signs of leukemic involvement in nonirradiated healthy recipients receiving cells by adoptive transfer were also similar to those observed in the BMT model (Figure 3C-D). The differences

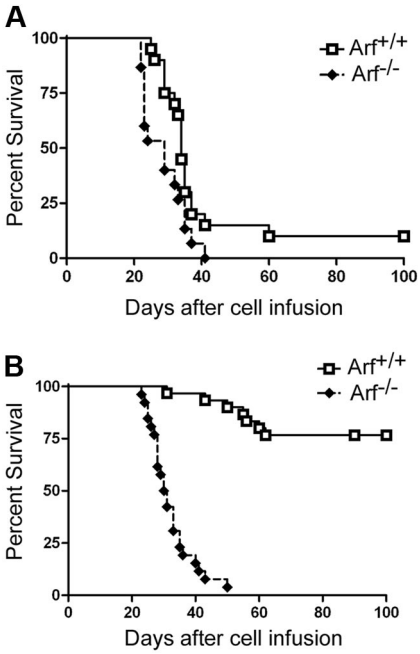


**Figure 2. Survival curves of recipient mice developing T-ALL.** Survival curves are shown for cohorts of healthy nonirradiated C57BL/6 mice injected with cultured (day-12) ICN1$^+$ cells derived from the bone marrow of 5-FU–conditioned mice (A) or unfractionated thymocytes (B) of the indicated *Arf* genotypes. (A) Twenty recipients of *Arf* $^{+/+}$ and 15 recipients of *Arf* $^{-/-}$ bone marrow–derived cells in 2 experiments had a median survival of 34 days and 29 days, respectively. The differences in latency are statistically significant ($P = .02$ by log-rank test). (B) In 3 experiments, all but 7 of 30 recipients of *Arf* $^{+/+}$ thymus-derived cells did not develop T-ALL, whereas 25 of 26 recipients of *Arf* $^{-/-}$ cells died of disease with a median survival of 30.5 days. Differences in latency are highly significant($P < .001$ by log-rank test).

were the tendency for more frequent bleeding and greater cytopenia in the BMT setting, reflected by significantly lower hemoglobin concentrations and platelet counts (Figure 3C).

Groups of 5 nonirradiated mice receiving cells derived from 5-FU–conditioned bone marrow infected with the control vector expressing GFP alone and cultured either on OP9 or OP9-DL1 stroma producing the delta ligand did not develop disease during a 6-month observation period. Moreover, although ICN1-positive cells derived from unconditioned (non–5-FU–treated) bone marrow cells also grew readily in culture and exhibited characteristics largely indistinguishable from cultures derived from 5-FU–treated marrow, such cells were unable to generate leukemias after adoptive transfer into a cohort of 10 healthy mice. Thus, by enriching for immature progenitors, 5-FU pretreatment before culture establishment increased the likelihood of endowing explanted bone marrow cells with detectable leukemogenicity, a dependency also observed using the BMT model.[12]

In short, the nearly complete penetrance of tumor development, the tissues involved, and the immunophenotype of T-cell tumors derived from adoptive transfer of cultured ICN1-expressing bone marrow cells were virtually identical to those seen after directly transplanting ICN1-transduced bone marrow into irradiated recipients. The principle virtue of this T-ALL model is that ex vivo culture maintains the survival of ICN1$^+$ leukemogenic cells that rapidly induce T-cell neoplasms in healthy unirradiated recipients. However, the significant but relatively modest acceleration of disease progression conferred by *Arf* inactivation in bone marrow progenitors, and the inability of the retained *Arf* gene to fully protect recipients from lethal disease, begs the question of why the *CDKN2A* locus is inactivated in human T-ALL.

### *Arf* strongly suppresses leukemia initiated by thymocyte-derived, ICN1-expressing T cells

When similar experiments were performed with cultured T cells derived from ICN1-transduced thymocytes, we observed a striking effect of *Arf* in preventing tumorigenesis. Whereas ICN1-transduced *Arf* $^{-/-}$ donor cells produced T-cell leukemias in healthy syngeneic recipients with a median survival of 31 days in 25 of 26 mice surveyed in 3 separate experiments, their *Arf*$^{+/+}$ counterparts initiated disease in only 7 of 30 mice observed for more than 4 months (Figure 2B). Considering only those recipients of thymocyte-derived ICN1$^+$ *Arf* $^{+/+}$ cells that succumbed to disease, their median survival was 56 days. *Arf* $^{-/-}$ thymocytes infected with a control GFP virus gave rise to Thy1.2-positive T cells when cultured on OP9-DL1 stroma expressing the delta ligand, but the GFP-marked cells were completely unable to initiate disease when infused into 10 recipients. Thus, *Arf* loss alone was incapable of producing T-ALL, even when the cells received developmental cues capable of directing T-cell differentiation through ligation of their endogenous Notch receptor.

Thymocyte-derived ICN1-expressing cells produced DP leukemic lymphoblasts with immunophenotypes and organ distributions indistinguishable from T-ALLs arising from cultured donor bone marrow cells (Figure 3A-D and other data not shown). The only consistent clinical difference between the T-cell neoplasms arising in mice receiving cultured ICN1-positive thymocyte-derived and bone marrow–derived cells was the tendency for *Arf* $^{-/-}$ thymocyte-derived cells to produce a more aggressive disease marked by unexpected deaths, hind limb paralysis, and ataxia. The latter signs indicated a greater propensity for central nervous system involvement in this group, which was documented at necropsy (supplemental Figure 3).

The intrinsic leukemogenic potential of thymocyte-derived T-ALL was confirmed by secondary transplantation of leukemic bone marrow from the affected animals. Bone marrow from 4 mice with thymocyte-induced *Arf* $^{-/-}$ T-cell neoplasms was infused into cohorts of 5 healthy, nonirradiated recipient mice at a dose of $10^5$ GFP-positive cells per mouse. This produced an even more rapidly fatal leukemia in all recipients (supplemental Figure 4), which phenotypically recapitulated the primary disease (data not shown). Although the majority of marked retransplanted donor cells were a clonally derived CD4$^+$/CD8$^+$ population enriched for tumor-initiating cells, the LICs might still derive from a minor fraction of less mature donor T cells.

The rates of T-ALL induction in healthy recipient mice receiving cultured ICN1$^+$ *Arf*$^{-/-}$ cells derived from bone marrow and from thymocytes (Figure 2) were not statistically different from one another (median survival of 29 and 31 days, respectively) and were similar to that observed in the BMT setting (33 days; supplemental Figure 2). This implies that the frequencies of leukemia-initiating cells in these different donor populations were comparable. Indeed, limiting dilution experiments performed with cultured ICN1$^+$ cells showed efficient leukemia-initiating capacity at a dose of $2 \times 10^4$ marked cells derived from either *Arf*$^{-/-}$ thymocytes or from *Arf* $^{+/+}$ bone marrow, but not at lower cell doses (supplemental Table 1). Hence, the number of leukemogenic progenitors generated from cultures of ICN1-expressing cells was lower than 1 in 2000. Southern blotting of genomic DNA from populations of T-ALLs derived from $2 \times 10^5$ ICN1$^+$ *Arf*$^{-/-}$ thymocytes harbored 2 to 6 rearranged TCRβ gene fragments, and in some experiments, multiple tumors derived from a single donor

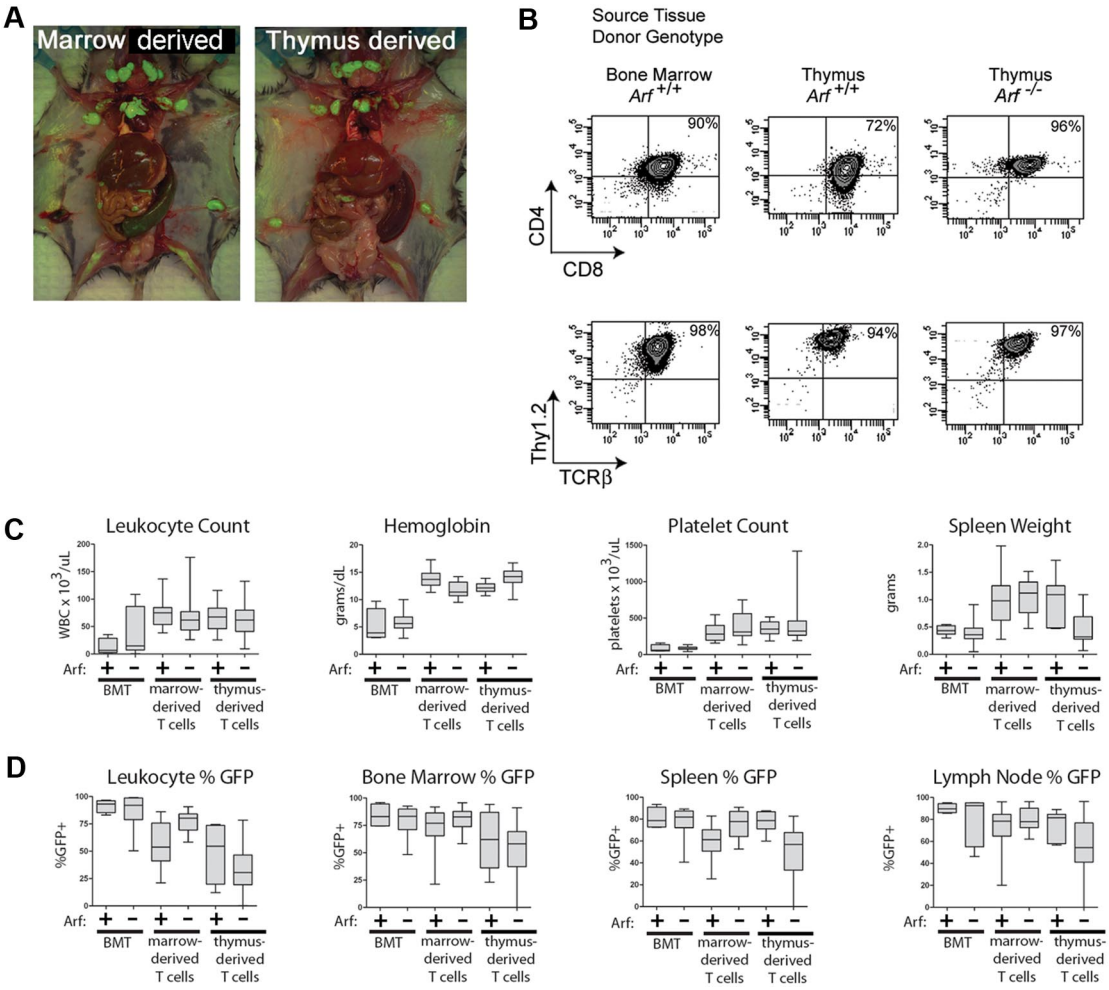


**Figure 3. T-ALLs arising from cultured ICN1$^+$ cells have similar disease phenotypes whether derived from bone marrow or thymocytes.** (A) Moribund mice were killed, dissected, and photographed. Images were captured with a Nikon Coolpix 5200 camera (Nikon) in ambient room light and through a Tiffen 15 deep yellow filter (Tiffen) under fluorescent illumination provided by an Illumatool light source (Light Tools Research). The images were merged using Photoshop 7.0 software (Adobe Systems). Recipient animals developed T-ALL with hepatosplenomegaly and extensive invasion of lymph nodes with GFP-marked leukemic cells. Representative recipients of marrow-derived cells (left panel) and of thymocyte-derived cells (right panel) are depicted. (B) Like irradiated animals that received bone marrow transplants of ICN1-transduced, but uncultured, donor cells, the T-ALLs arising in nonirradiated mice largely coexpressed CD4 and CD8, though some T-ALLs had a CD8 single-positive subpopulation, as well. No consistent variations in representative immunophenotypes of leukemic cells taken from different tissues or derived from *Arf* $^{+/+}$ or *Arf* $^{-/-}$ donors were seen. The remaining panels (C-D) compare clinical parameters of T-ALLs arising in irradiated animals undergoing conventional bone marrow transplantations (BMTs) with those of recipient mice that received cultured day-12 ICN1$^+$ bone marrow–derived or thymus-derived cells. Clinical hematologic measurements including a comparison of total white blood cell counts, hemoglobin concentration, platelet count, and spleen weights are shown in panel C, whereas the percentages of GFP-marked cells in various tissues are shown in panel D. The broad horizontal bars indicate median values, the gray bars delineate the upper and lower quartiles, and the whiskers indicate the overall range of readings.

culture shared the same bands (supplemental Figure 5). Together, these results argue that thymocyte-derived ICN1$^+$ *Arf*$^{-/-}$ donor cells require additional genetic events to convert them to LICs.

### The thymic microenvironment is dispensable for ICN1 induction of T-ALL

In the case of bone marrow–derived ICN1$^+$ donor cells, only early progenitors (from 5-FU–conditioned donors) were capable of inducing disease, although the resulting tumors reproducibly displayed the DP immunophenotype and expressed surface TCRβ. Thus, in agreement with previous BMT studies,[4,5,7,9,12] in vivo progression along the canonical T-cell developmental pathway to the DP stage appears to be a requisite feature of ICN1$^+$ leukemogenesis. There is some evidence that Notch-dependent T-lineage commitment can proceed at extrathymic sites after BMT into irradiated recipients.[38] Conceivably, constitutive ICN1 signaling coupled with cytokine and stromal support ex vivo might substitute for the thymic microenvironment in guaranteeing the development of malignant CD4$^+$/CD8$^+$ T cells. To address this issue directly, we repeated both BMT and adoptive transfer experiments using CD-1 nude recipient mice that lack a thymic epithelium and cannot support normal T-cell development. In these experiments, ICN1$^+$ cells again produced a rapidly fatal leukemia with involvement of the bone marrow, spleen, lymph nodes, and peripheral blood (Figure 4 and data not shown). As in syngeneic C57BL/6 recipients, an intact *Arf* locus provided a survival benefit in irradiated (Figure 4A) or nonconditioned (Figure 4B) recipients when ICN1$^+$ bone marrow donor cells were generated from 5-FU–treated mice. A relatively greater protective effect was seen using *Arf* $^{+/+}$ versus *Arf* $^{-/-}$ thymocyte-derived donor cells (Figure 4C), although the disease was fully penetrant in immunodeficient recipients. The immunophenotype of all T-ALLs, regardless of *Arf* genotype or source tissue, showed strong marking with Thy1.2 and TCRβ, and a predominance of DP cells (Figure 4D-5F). Constitutive ICN1

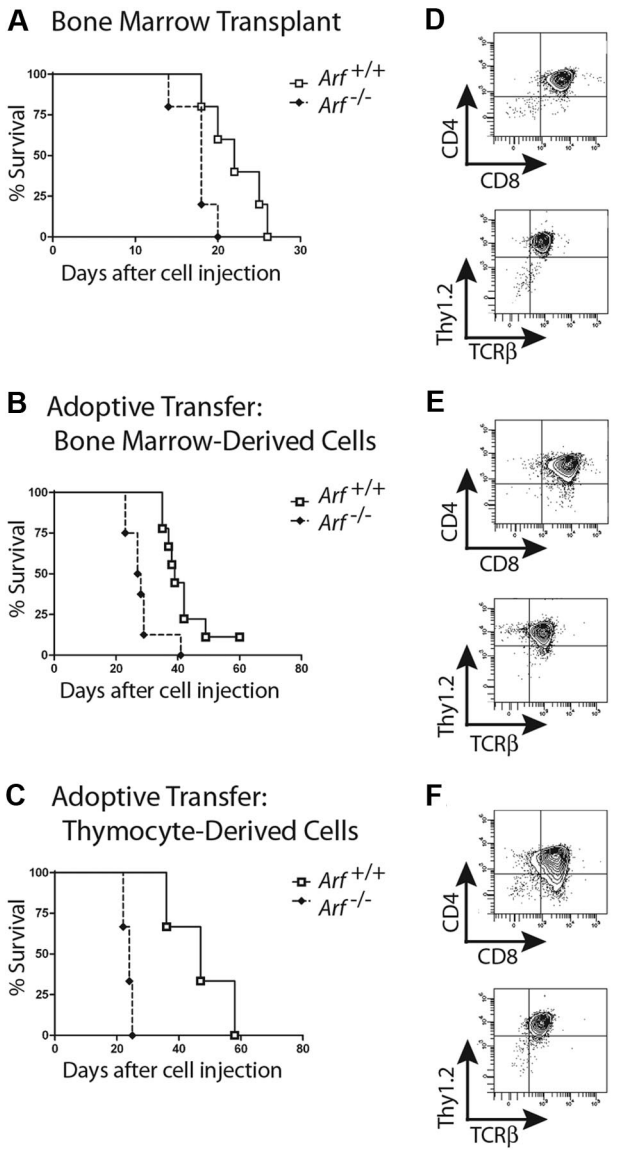


**Figure 4. Athymic nude mice develop CD4$^+$/CD8$^+$ T-ALLs when infused with ICN1-expressing cells.** Survival curves are shown for cohorts of irradiated nude mice infused with noncultured ICN1$^+$ bone marrow–derived cells (A; 5 mice/group; $P = .03$ by log-rank test), nonirradiated nude mice infused with ICN1$^+$ bone marrow–derived cells after 12 days of culture (B; 9 *Arf* $^{+/+}$, 8 *Arf* $^{-/-}$ recipient mice, $P = .002$), and nonirradiated nude mice infused with cultured ICN1$^+$ thymus-derived cells (C; 3 mice/group, $P = .02$). Representative immunophenotypes of T-ALLs arising in each experiment are depicted in panels to the right of the corresponding survival curves (D-F). Although all leukemias had a dominant CD4$^+$/CD8$^+$ population, several also had CD8 single-positive subpopulations.

expression is therefore sufficient to drive T-ALL–initiating cells to the DP stage in the absence of additional thymic function.

### Activation of *Arf* gene expression during T-cell leukemogenesis

The p19$^{Arf}$ protein must be expressed at least transiently in vitro or in vivo to limit the leukemogenicity of ICN1$^+$ *Arf* $^{+/+}$ thymocytes. Yet, we were unable to detect p19$^{Arf}$ expression in thymocytes cultured on OP9 stroma. Moreover, all T-ALLs that were induced by cultured ICN1$^+$ *Arf* $^{+/+}$ thymocyte-derived or bone marrow–derived cells and purified by FACS from moribund animals retained the *Arf* gene but failed to express the p19$^{Arf}$ protein (Figure 5). Thus, we assumed that any T cells that expressed *Arf* were rapidly eliminated, thereby forestalling tumor development, whereas those that did not were able to generate T-ALL.

In an attempt to establish that *Arf* can be induced during T-ALL development, we used donor T cells derived from a syngeneic mouse

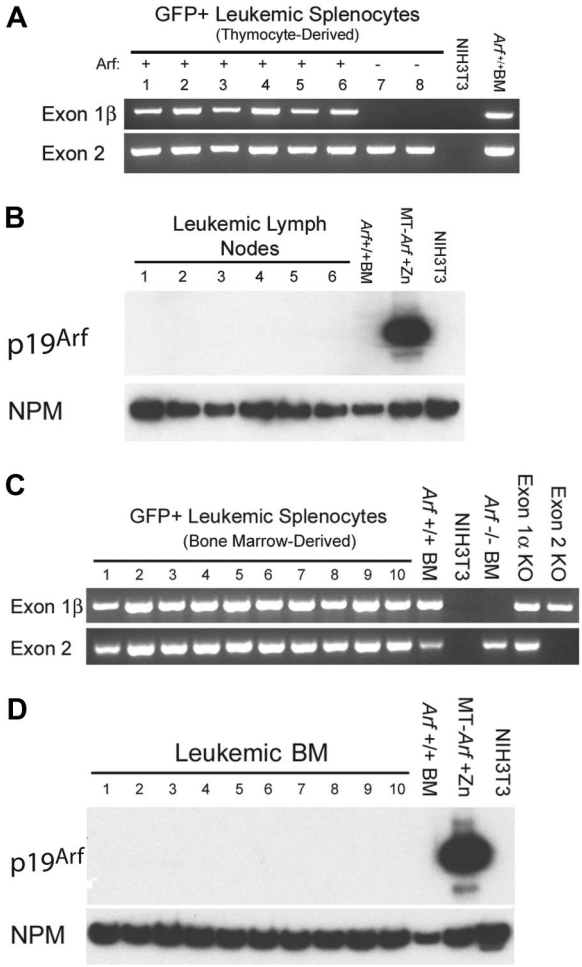


**Figure 5. T-ALLs arising from cultured ICN1$^+$ *Arf* $^{+/+}$ donor cells retain the gene but do not express p19$^{Arf}$ protein.** (A) Leukemic GFP$^+$ splenocytes from 8 mice that had received cultured (day-12) thymocyte-derived donor cells of the indicated *Arf* genotypes (top) were purified by FACS. Genomic DNA prepared from these cells was used as a template for PCR performed with primers specific for exon-1β and exon-2 of the *Ink4a-Arf* locus. Exon-1β encodes the unique N-terminus of p19$^{Arf}$, whereas exon-2 encodes C-terminal portions of p19$^{Arf}$ and p16$^{Ink4a}$ from alternative reading frames. DNAs extracted from unmanipulated *Arf* $^{+/+}$ bone marrow cells and from mouse NIH-3T3 cells that had deleted the *Ink4a-Arf* locus during the process of immortalization were used as the positive and negative controls, respectively. The fact that no exon-1β signal was revealed in samples 7 and 8 generated from *Arf* $^{-/-}$ donor cells indicates that the purified leukemic cells were uncontaminated by normal *Arf* $^{+/+}$ cells. (B) Although all genotyped leukemias retained the *Arf* locus, immunoblotting of electrophoretically separated proteins extracted from robustly GFP-positive lymph nodes taken from the same recipients of *Arf* $^{+/+}$ donor cells failed to reveal p19$^{Arf}$ expression. *Arf* expression is also silenced in normal bone marrow (BM) cells. Cells engineered to express *Arf* conditionally under the control of a metallothionein promoter (MT-Arf cells) were induced with zinc. The immunoblot was developed with a highly sensitive monoclonal antibody generated to mouse p19$^{Arf}$ (Bertwistle et al[34]), and the film was purposely overexposed in an attempt to reveal p19$^{Arf}$ expression. Nucleophosmin (NPM), an abundant nucleolar protein, was used as the loading control. (C) PCR analysis as in panel A. Samples 1 to 4 were taken from irradiated recipients that had received a transplant of uncultured ICN1-expressing bone marrow from 5-FU–conditioned donors. Samples 5 to 10 were taken from nonirradiated recipients that had received 5-FU–conditioned bone marrow transduced with ICN1 and cultured on OP9 stroma before their adoptive transfer. Control PCR reactions were performed using DNAs extracted from unmanipulated *Arf* $^{+/+}$ bone marrow cells, NIH-3T3 cells lacking the *Ink4a-Arf* locus, bone marrow cells from *Arf* $^{-/-}$ mice in which exon-1β is disrupted, bone marrow cells from p16$^{Ink4a}$-null mice in which the unique *Ink4a* exon-1α was disrupted, and from mice in which *Ink4a-Arf* exon-2 was disrupted. (D) Immunoblotting for p19$^{Arf}$ was performed on bone marrow taken from the mice analyzed in panel C.

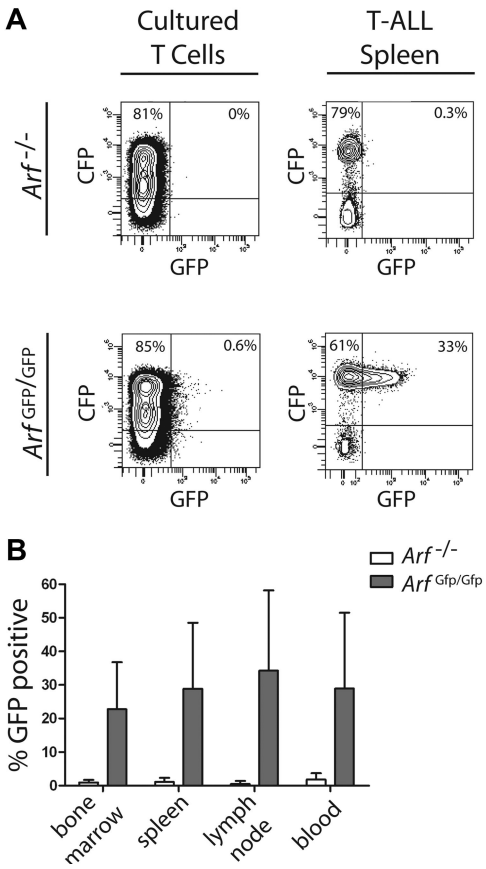


**Figure 6. Activation of the *Arf* promoter during T-ALL development.** Healthy syngeneic C57BL/6 mice received donor T cells derived from either control *Arf* $^{-/-}$ or "knock-in" *Arf* $^{Gfp/Gfp}$ (also functionally null) thymocytes infected with an ICN1-CFP vector. (A) Cells from day-12 cultured thymocytes (left panels) or from spleens of moribund mice (right panels) were studied by FACS for coexpression of vector-coded CFP (ordinate) and cellular *Arf*-encoded GFP (abscissa). (B) Hematopoietic tissues from 17 such mice were similarly analyzed and contained a significant proportion of CFP-marked cells that coexpressed GFP (ordinate). Error bars indicate the SD from the mean.

strain in which a cDNA encoding GFP was knocked into the cellular *Arf* locus at the expense of p19$^{Arf}$ coding sequences.[39] The genomic *Arf-Gfp* allele is functionally null, so that homozygous *Arf* $^{Gfp/Gfp}$ thymocytes should be as susceptible as *Arf* $^{-/-}$ cells to ICN1-induced leukemogenesis. Importantly, the cellular *Arf* promoter and 5′ untranslated sequences remain intact and drive GFP expression in oncogene-induced tumors, but not in surrounding normal cells. We transduced thymocytes from *Arf* $^{Gfp/Gfp}$ mice with a retroviral vector expressing ICN1 and CFP, cultured the cells on OP9 stroma for 12 days, and infused $2 \times 10^5$ CFP-marked cells into nonirradiated syngeneic mice. Recipient animals developed CFP-positive T-ALLs that were indistinguishable from those previously generated using the ICN1-GFP vector. Notably, although less than 1% of ICN1-transduced T cells expressed *Arf-Gfp* after 12 days of culture, GFP was detected in a substantial percentage of CFP-marked T-ALL cells that arose in moribund animals (Figure 6A). Indeed, approximately one-third of CFP-marked T-ALL cells harvested from different hematopoietic tissues stably expressed *Arf-Gfp* (Figure 6B). These experiments provide direct evidence that the *Arf* promoter can be activated by ICN1 in a stage-specific manner during T-ALL development.

## Discussion

We have used short-term culture conditions to foster the outgrowth of ICN1-transduced progenitor cells that rapidly induce T-ALL in healthy syngeneic recipients. The cardinal characteristics of these T-ALLs were phenotypically indistinguishable from those induced with a standard BMT protocol,[12] highlighting the fact that ablating the hematopoietic and immune systems of recipient animals is not mandatory for T-ALL development. Moreover, transduction of ICN1 into T-cell progenitors not only can command the T-cell developmental program[32,33] but also is able to efficiently initiate peripheral CD4$^+$/CD8$^+$ T-ALLs in CD-1 nude mice that lack thymic function. These latter findings reinforce previous observations that Notch-dependent T-lineage commitment can proceed at extrathymic sites after BMT into irradiated recipients.[38]

Although activating Notch mutations and *CDKN2A* deletions commonly go hand in hand in human T-ALL, inactivation of *Arf* had no effect on disease frequency, phenotype, or clinical signs, and only a modest effect on disease latency when cultured ICN1-positive bone marrow–derived cells were used to trigger leukemogenesis. Inactivation of *Ink4a* in addition to *Arf* was without effect. Notably, although the *Ink4a-Arf* locus remained intact, p19$^{Arf}$ was not detectably expressed in leukemic blasts generated from these precursors. Abundant evidence indicates that the *Ink4a-Arf* locus is epigenetically silenced by polycomb group complexes in hematopoietic stem cells and in early progenitors that constitute the putative target cells in the donor bone marrow of 5-FU–treated mice.[19-24] The failure of the locus to undergo deletion in more mature DP T cells that made up the bulk of the induced tumors implies that, under the experimental conditions used, silencing of the locus was maintained during ICN1-driven tumor progression. Hence, in not expressing p19$^{Arf}$, these T-ALLs effectively phenocopied ICN1$^+$ *Arf* $^{-/-}$ leukemias.

In striking contrast, when cultures were initiated using ICN1-transduced thymocytes instead of 5-FU–conditioned bone marrow, T cells derived from *Arf* $^{+/+}$ mice were much less efficient than *Arf* $^{-/-}$ donor cells in inducing T-ALL. Because *Arf* restrains tumors initiated by cultured ICN1$^+$ *Arf* $^{+/+}$ thymocytes, p19$^{Arf}$ expression must efficiently cull out incipient tumor cells. Thus, once bone marrow progenitor cells home to the thymus and undergo further T-cell maturation, the *Arf* locus must be epigenetically remodeled to allow its induction, unmasking its tumor-suppressive activity. The polycomb components Bmi1 and Mel-18 initially silence the *Ink4a-Arf* locus and maintain Notch-induced Hes1 expression to allow the rapid expansion of viable double-negative (DN) CD4$^-$/CD8$^-$ thymic T cells through DN1 (CD44$^+$/CD25$^-$) to DN3 (CD44$^-$/CD25$^+$) stages of development.[19,40,41] Conversely, *Bmi1* deficiency in the thymus results in precocious p19$^{Arf}$ and p16$^{Ink4a}$ up-regulation. This promotes the p53-dependent death of activated pre-T cells and their complete failure to undergo a transition to the DP state,[25] the stage at which malignant transformation is thought to occur.[7-9] Notably, *Arf,* but not *Ink4a,* inactivation counters these effects of *Bmi1* loss of function in T cells and allows further development to proceed.[25] *Arf* inactivation would therefore be expected to compromise the p53 checkpoint, preventing the elimination of rare pre-T cells that harbor abortive receptor rearrangements[25] and predisposing to the generation of clonal DP T-ALLs triggered by abnormal Notch expression.

The few tumors that arose from ICN1-transduced *Arf* $^{+/+}$ thymocytes after a longer latency period might have been expected to sustain *Arf* deletions. Instead, like the T-ALLs induced by ICN1-expressing bone marrow progenitors, the locus was retained, and p19$^{Arf}$ was not detectably expressed. This subset of tumors may therefore have arisen from rare early DN cells present in the unfractionated thymocyte preparations used to initiate cocultures, in which epigenetic silencing of *Arf* is maintained. On the other

hand, when functionally null *Arf* $^{Gfp/Gfp}$ donor thymocytes transduced with an ICN1-CFP vector were used to initiate T-ALL, a significant fraction of the CFP-marked T-ALL cells coexpressed GFP, providing the first direct evidence that the cellular *Arf* gene can be activated by ICN signaling at this stage of disease progression. However, this does not imply that an ICN1-containing complex directly binds and transactivates the *Arf* promoter, and indeed, genome-wide chromatin immunoprecipitation and microarray analyses failed to provide evidence that NOTCH1 activates *CDKN2A*.[42] Moreover, although NOTCH1 induces c-*MYC*,[42,43] a well-established inducer of *ARF*,[31,39] Myc has not been demonstrated to bind directly to the *Arf* promoter either.[16] Thus, we infer that both ICN1 and Myc regulate *Arf* through indirect mechanisms.

Deletion of *Arf, Ink4a,* or both genes also accelerates the onset of leukemia and increases disease penetrance in *lck-tal1* transgenic mice, in which expression of the oncogenic Tal-1 transcription factor is limited to thymic and later stages of T-cell development.[44] However, in another transgenic mouse model, in which an activated Notch1 allele is conditionally expressed broadly in hematopoietic cells,[45] *Arf* is not induced.[46] These seemingly incompatible results are consistent with our findings that engagement of the Arf-p53 pathway upon oncogenic stress is developmentally stage specific. *Arf* is induced most readily in later thymic stages of T-cell development, and thus functions as an effective tumor suppressor in the *lck-tal1* system, whereas expression of ICN1 in more primitive hematopoietic cells generates leukemias that escape elimination by *Arf,* even though they can ultimately acquire characteristics of more differentiated T cells.

The very high frequency of *INK4A-ARF* deletion in human T-ALL implies that these tumors arise from similarly differentiated T-cell precursors, in which the *INK4A-ARF* gene is poised to respond to oncogenic insults. If silencing were maintained during the evolution of these tumors, as it is in murine bone marrow–derived T-ALL, no selective pressure would drive deletion of the locus. In further support of this hypothesis, human T-ALLs that delete *INK4A-ARF* are predominantly $CD4^+/CD8^+$, whereas an immature DN T-ALL subgroup characterized by expression of LYL1, CD45, and Bcl2 retains the locus.[14]

Most activated *NOTCH1* alleles detected in human T-ALL are only weakly oncogenic in murine bone marrow transplantation assays, suggesting that they may arise as secondary mutations in the majority of tumors that harbor them.[11] Nonetheless, γ-secretase treatment of murine T-ALLs driven primarily by mutant K-ras[11] or by ectopic Tal-1[47] abrogates tumor growth, indicating that Notch signaling is indispensable for tumor maintenance. In turn, constitutive Notch signaling per se may be insufficient to activate *INK4A-ARF* in the majority of human T-ALLs, in which other activated oncogenes might be required to contribute to its induction and eventual elimination. In contrast, the supraphysiologic Notch signal provided by ICN1 not only can drive T-cell development and maturation but also can elicit a fully penetrant neoplastic disease. Nonetheless, even in the case of ICN1 expression and *Arf* deletion, the emerging T-ALLs are monoclonal or oligoclonal, based on studies of TCRβ gene rearrangements, implying that additional genetic events are required to guarantee disease.

The adoptive transfer model of Notch1-driven T-ALL provides significant advantages as a tool both for developing therapeutics and understanding disease mechanisms. By producing disease in healthy, immune-competent mice, the model eliminates the potential for confounding findings resulting from the effects of radiation. Moreover, the thymocyte-derived disease closely phenocopies pediatric human T-ALL in its distribution, stage-specific cooperation between *Notch1* mutation and *Arf* deletion, and central nervous system involvement, a frequent cause of relapse in children.

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## Authorship

Contribution: E.J.V. designed and performed research, generated new reagents, analyzed data, and wrote the paper; R.T.W. designed research, analyzed data, and wrote the paper; and C.J.S. oversaw the project, designed research, analyzed data, and wrote the paper.

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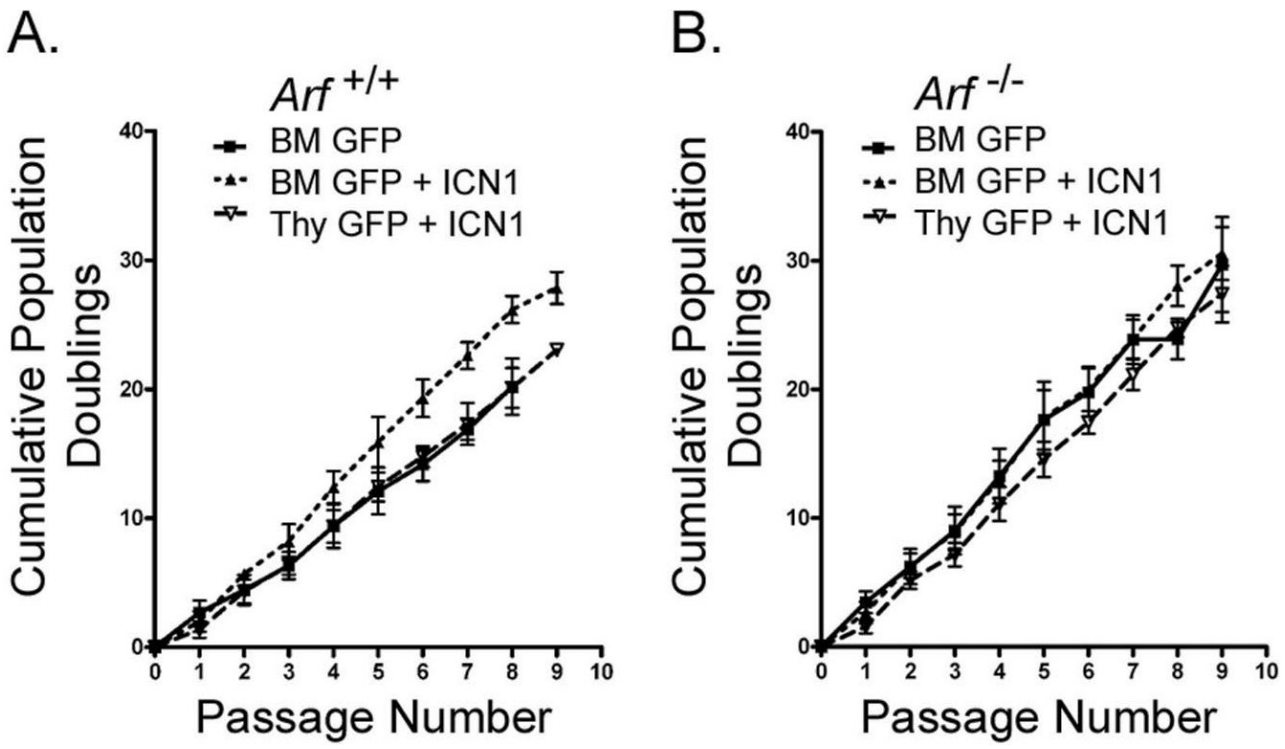


***Figure S1. Rates of proliferation in culture of transduced bone marrow cells and thymocytes.*** *$Arf^{+/+}$ and $Arf^{-/-}$ cells (panels A and B, respectively) derived from 5-FU–conditioned bone marrow (BM) or thymocytes (Thy) were transduced with a vector encoding GFP and ICN1 in cis or with a control vector encoding GFP alone, as indicated in both panels. Cells were counted and passaged every 4 days, and their cumulative population doublings were determined for 10 passages in three independent experiments. The bars indicate the standard errors of the mean of the recorded values.*

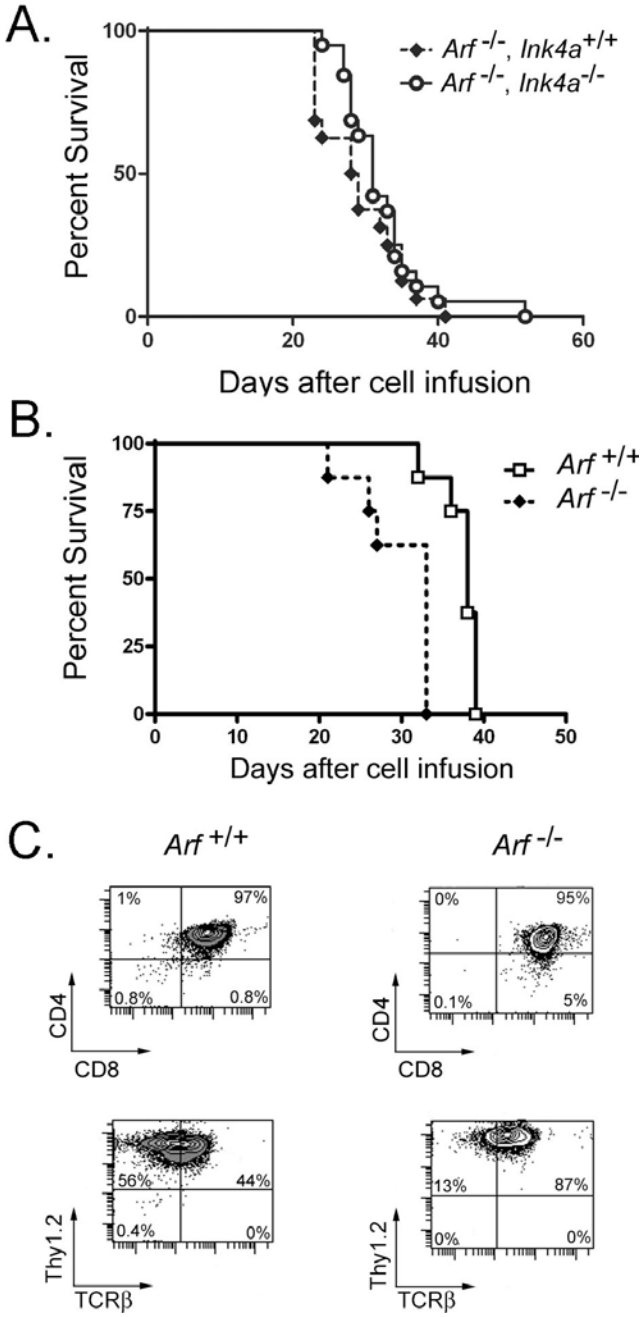


***Figure S2. Influence of Arf (and Ink4a) inactivation on bone marrow-derived leukemia.***
*(A) Survival curves for healthy non-irradiated C57BL/6 recipients of cultured (day 12) ICN1$^{+}$ cells derived from the bone marrow of 5-FU–conditioned mice harboring a targeted deletion of exon 1β (Arf$^{-/-}$, Ink4a$^{+/+}$) or exon 2 (Arf$^{-/-}$, Ink4a$^{-/-}$) of the Ink4a-Arf locus. Survival in the presence or absence of Ink4a was statistically indistinguishable (p = 0.34 by log-rank test). In two experiments, 16 recipients of Arf$^{-/-}$, Ink4a$^{+/+}$ cells died with a median latency of 29 days, compared to 31 days for 20 recipients of Arf$^{-/-}$, Ink4a$^{-/-}$ donor cells. (B) Survival curves are illustrated for lethally irradiated C57Bl/6 mice injected with retrovirally transduced, ICN1-expressing bone marrow cells from 5-FU treated donors of the indicated Arf genotype. Recipient mice also received a radio-protective dose of non-transduced bone marrow cells. Eight animals received Arf$^{+/+}$ donor cells, and nine received Arf$^{-/-}$ cells. GFP-positive T-ALLs arising from both Arf$^{-/-}$ and Arf$^{+/+}$ donor cells showed the same CD4$^{+}$/CD8$^{+}$ .β immunophenotype and were likewise positive for both Thy1.2 and TCR (C) A representative immunophenotypic FACS analysis of a T-ALLs arising in transplanted irradiated recipients is depicted.*

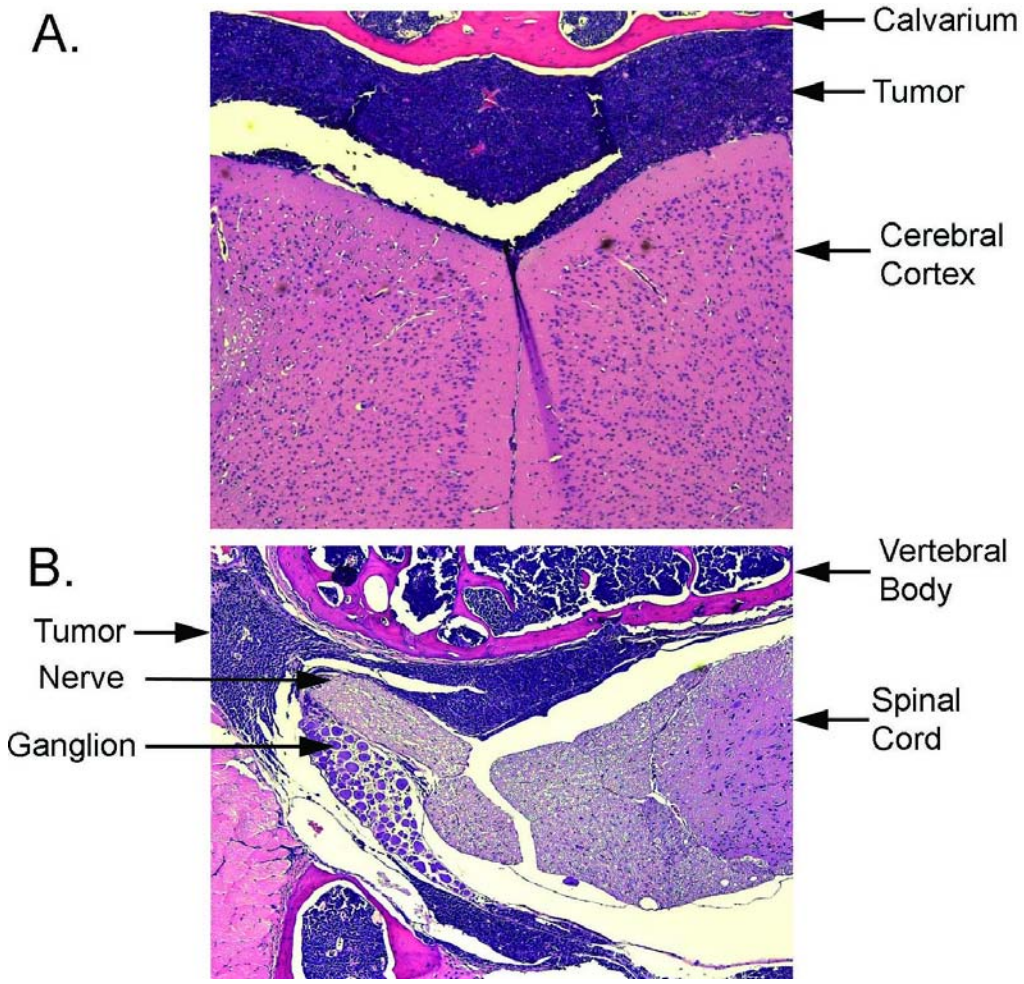


***Figure S3. T-ALLs arising from ICN1-positive Arf$^{-/-}$ thymocytes invade the central nervous system***
*(A) A coronal section of the cerebral hemispheres exhibits a leukemic infiltrate between the calvarium and brain. (B) A transverse section of the distal spine shows a leukemic infiltrate in the spinal canal. Tumor cells impinge on the spinal cord and the nerve root exiting the foramen. Images were acquired using Zeiss Axiovision software. Photos were produced using a Zeiss Axioskop-2 microscope and an AxioCam HRc camera. Magnifications are 5×.*

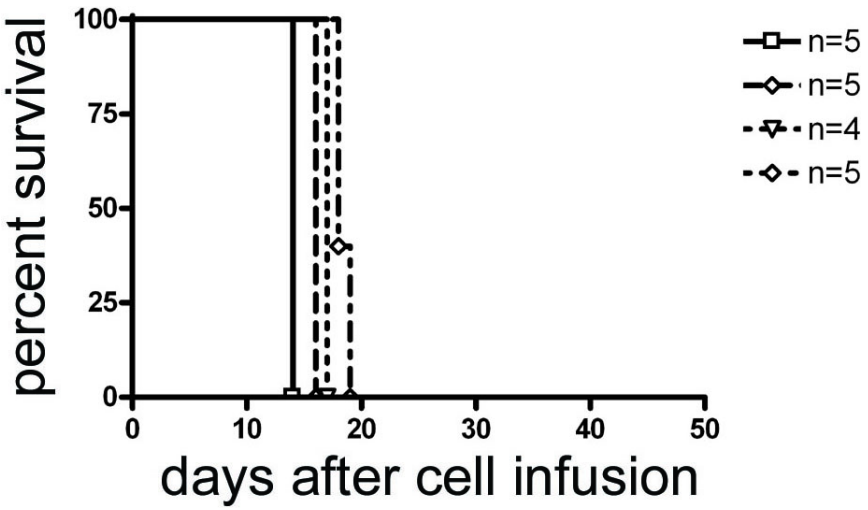


***Figure S4. Secondary transplantation of T-ALL cells.***
*GFP-marked bone marrow cells harvested from four moribund mice that had received ICN1$^{+}$ Arf$^{-/-}$ thymocyte-derived cells were each transfused into groups of five secondary recipients (1 × 10$^{5}$ marked cells per mouse), all of which developed T-ALLs that phenocopied the primary disease.*

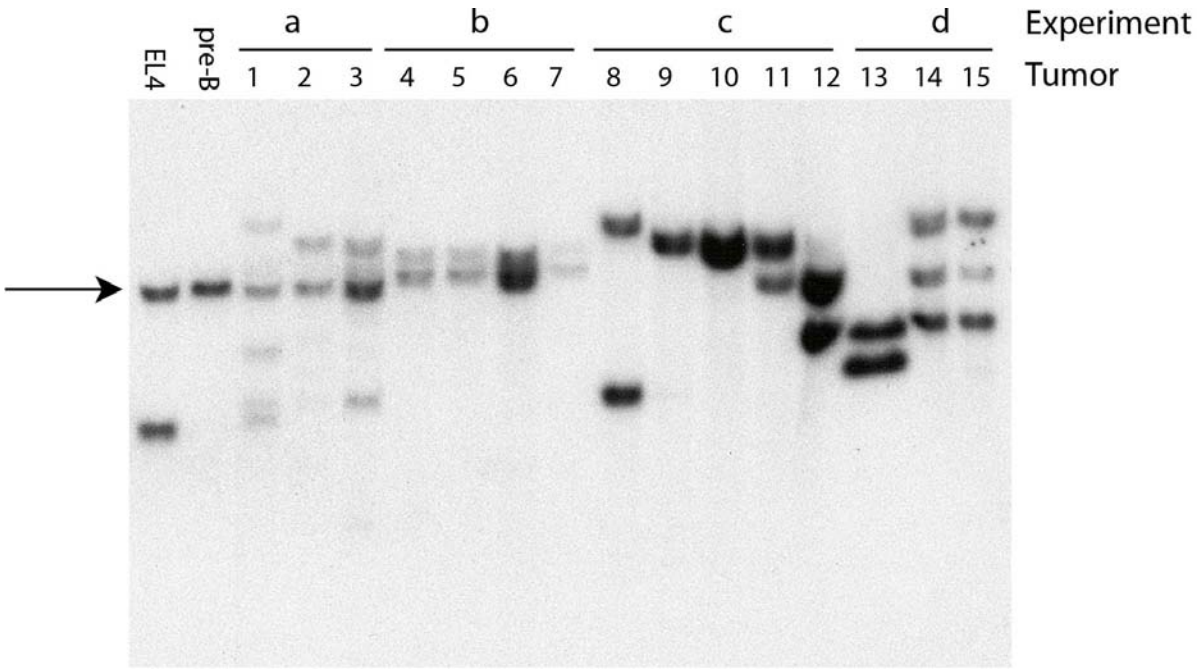


***Figure S5. Analysis of TCR rearrangements in T-ALLs arising from ICN1-positive Arf$^{-/-}$ thymocytes.*** *Genomic DNA from 15 thymocyte-derived T-ALLs generated in 4 independent experiments (labeled a–d), was digested with EcoRI, and the resulting Southern blot was probed with a radio-labeled 2.2 kb EcoRI fragment of TCR-Jβ2. DNA samples from the EL4 T lymphoma cell line and from a murine BCR-ABL positive pre-B leukemia are included as controls in the two left lanes. The germline band is indicated by an arrow. In some cases, tumors arising in individual mice that received cells from a single culture exhibited common rearrangements (a2 and a3, but not a1; b4–b7; c9 and c10, but not c8, c11, or c12; d14 and d15, but not d13).*

***Table S1: Estimation of the frequency of leukemia-initiating cells***

| | Donor cell type | |
|---|---|---|
| Cell dose | Cultured *Arf*$^{+/+}$ bone marrow (# T-ALLs/#recipients) | Cultured *Arf*$^{-/-}$ thymocyte (# T-ALLs/#recipients) |
| $2 \times 10^5$ | 9/10 | 9/10 |
| $2 \times 10^4$ | 4/9 | 9/12 |
| $2 \times 10^3$ | 0/10 | 2/10 |

Non-irradiated C57Bl/6 mice were infused with the indicated cell numbers of cultured ICN1-expressing cells derived from *Arf*$^{+/+}$ 5-FU conditioned bone marrow or *Arf*$^{-/-}$ thymocytes and sacrificed when moribund.