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Epigenetic regulation of the *Ink4a-Arf (Cdkn2a)* tumor suppressor locus in the initiation and progression of Notch1 driven T-ALL

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Abstract

Activating mutations of *NOTCH1* and deletion of the *INK4A-ARF (CDKN2A)* tumor suppressor locus are two of the most frequent genetic alterations in T cell acute lymphoblastic leukemia (T-ALL). In a murine model of T-ALL induced by the intracellular domain of Notch1 (ICN1), the genetic interaction between ICN1 signaling and *Arf* inactivation is developmentally stage-specific, with a more pronounced requirement for *Arf* deletion in thymocytes than in bone marrow precursors targeted for transformation. In the thymus, the target cell for transformation is a CD4 and CD8 double-negative progenitor that undergoes T cell receptor beta chain rearrangement, a cell type in which polycomb silencing of *Ink4a-Arf* is normally requisite. Epigenetic remodeling during tumor progression licenses *Arf* as a tumor suppressor and in turn provides the selective pressure for *Ink4a-Arf* deletion in clonal T-ALLs that emerge.

Keywords

Precursor T-cell Lymphoblastic Leukemia-Lymphoma; Epigenetic Processes; Tumor Suppressor Genes

Introduction

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy, which is fatal in 20% of pediatric patients and imposes significant morbidity on survivors

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[1]. Prominent genetic lesions that contribute to T-ALL include activating mutations of the Notch1 transmembrane receptor [2] and deletion of the *CDKN2A* tumor suppressor locus [3,4].

The Notch1 transmembrane receptor undergoes ligand-dependent proteolytic cleavage, releasing its intracellular fragment (ICN1), which relocates to the nucleus and directs transcriptional activation of target genes [5,6]. Notch1 signaling is required for the commitment of hematopoietic progenitor cells to the T lymphoid lineage and for the early stages of intrathymic T cell development [7–9]. Mutations that uncouple Notch1 signaling from ligand binding or that aberrantly prolong signaling by interfering with the degradation of ICN1 occur in more than 50% of T-ALL cases [2,10]. The *CDKN2A* locus encodes two tumor suppressor genes (*INK4A* and *ARF*) transcribed from alternative first exons (1 α and 1 β , respectively), whose RNAs are spliced to common second and third exon encoded sequences translated in different reading frames. Whereas p16^{INK4A} inhibits phosphorylation of the retinoblastoma protein by cyclin D-dependent kinases, p14^{ARF} (p19^{Arf} in mice) antagonizes the Mdm2 E3 ligase to activate p53 [11]. *NOTCH1* gain of function and *CDKN2A* inactivation contribute independently to T-ALL induction. Unlike many other hyperproliferative signals that trigger *Arf* activation, aberrant Notch1 signaling does not itself induce *Ink4a-Arf* expression in T cells, and, in some tumor settings, Notch1 activation can follow *Arf* engagement as a later event during tumor progression [12,13]. Although the factors that induce *CDKN2A* during T cell tumorigenesis remain unidentified, the deletion of *CDKN2A* in more than 70% of T-ALL cases at presentation [14] provides strong evidence that products of the previously intact locus act to suppress tumorigenesis at a stage in T cell development before frank clonal malignancies emerge.

T-ALL can be induced in lethally irradiated mice by retrovirus-mediated transduction of bone marrow progenitors with ICN1 [15]. Alternatively, when ICN1-transduced bone marrow or thymic progenitors are expanded *ex vivo* in a defined co-culture setting with supportive stromal cells, they give rise to mixed populations of lymphoblasts with immunophenotypes that reflect various stages of normal thymocyte development [16]. Whether derived from the bone marrow of donor mice exposed to 5-fluorouracil (5-FU) or from more mature lymphoid progenitors in the thymus, these cultured ICN1+ lymphoblasts rapidly produce T-ALL when infused into healthy nonirradiated syngeneic mice [17]. Both *Arf*^{+/+} and *Arf*^{-/-}, ICN1+ bone marrow cells rapidly generate fatal T-ALL with complete penetrance, but despite maintenance of an intact locus, all *Arf*^{+/+} tumors derived from ICN1+ bone marrow progenitors fail to express the p19^{Arf} protein. In contrast, only ICN1+, *Arf*^{-/-} thymocytes produce T-ALL with identical kinetics and lethality, whereas *Arf* expression in thymic T cell precursors prevented tumor formation [17].

In primitive hematopoietic precursor cells and early stage thymocytes, polycomb group chromatin modifying complexes silence expression from the *Ink4a-Arf* locus [18–21]. Polycomb repressive complex 2 (PRC2), which is composed of EZH2, EED, SUZ12, and RbAp48, catalyzes the trimethylation of lysine 27 of histone H3 (H3K27Me3), a modification which denotes a silenced gene [22]. This histone mark, which is clustered near the transcriptional start site but may spread over the entire silenced gene, acts in opposition to activating histone modifications, most notably the histone H3 lysine 4 trimethyl mark (H3K4Me3) deposited through the action of trithorax complexes [23,24]. Genes that bear both marks are transcriptionally silent, but are considered to be poised to start transcription, given appropriate inductive signals. The H3K27Me3 mark recruits polycomb repressive complex 1 (PRC1), which monoubiquitinates histone H2A lysine 119 to enforce silencing. Targeted deletion of the PRC1 component *Bmi-1* causes early exhaustion of definitive hematopoietic stem cells [25] and a block in T lymphoid development at the transition from cells doubly negative for expression of CD4 and CD8 (DN cells) to cells positive for both

(DP cells) [26]. In mice, defects resulting from *Bmi-1* deletion can be partially rescued by co-deletion of *Ink4a-Arf* [18], demonstrating the importance of maintaining *Ink4a-Arf* silencing during these early developmental stages.

Together, these findings imply that T cell precursors targeted for transformation by aberrant Notch1 signaling are able to reverse the epigenetically silenced state of the *Cdkn2a* locus; this would enable *Ink4a-Arf* gene expression and, in turn, provide a strong selective pressure for the emergence of rare clones that bypass tumor suppression by deleting the locus. Experiments outlined below indicate that the epigenetic state of the target cell and susceptibility to *Ink4a-Arf* induction conspire to determine the developmental stages in T cell maturation at which aberrant Notch1 expression and *Ink4a-Arf* inactivation induce T-ALL.

Materials and Methods

Cell culture and T-ALL induction

Retroviral particles were produced [27] using a bicistronic murine stem cell virus vector encoding ICN1 and either green fluorescent protein (GFP) or mCherry fluorescent protein (CFP) to mark infected cells [17]. Thymocytes or bone marrow cells were isolated from syngeneic C57Bl/6J, *Arf*^{-/-} [28], or functionally *Arf*-null *Arf*^{Gfp} “knock-in” [29] donor mice, for bone marrow cells four days after the administration of 150 mg/kg 5-FU (Abraxis). Bone marrow cells and thymocytes transduced with ICN1+ were cocultured for 12 days on OP9 stromal feeder layers (ATCC) as described [17] and administered intravenously into healthy C57Bl/6J male recipient mice at a dose of 2×10^5 cells each. Recipients were monitored daily for clinical signs of leukemia, and humanely killed when moribund, under protocols approved by the Institutional Animal Care and Use Committee, and in accordance with NIH guidelines.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using a modified Millipore protocol. Whole cell extracts were prepared from washed cells after a 15 minute fixation in 1% paraformaldehyde. Pre-cleared aliquots of sheared chromatin equivalent to 2×10^6 cells were incubated with rabbit polyclonal antisera to H3K27Me3, EZH2 (Millipore), H3K4Me3 (Cell Signaling), JMJD3, or UTX (Abcam), or rabbit monoclonal antibody to EED (Millipore), or normal rabbit IgG (Cell Signaling) at 4°C overnight. Antibody-bound chromatin was recovered with blocked protein A agarose beads, and eluted after stringent washing. After reversal of crosslinks, the DNA was recovered using silica mini-spin columns (Qiagen), and was used as a template for quantitative PCR with SYBR green-based detection of amplicons (Qiagen). See Table for primer sequences. The percentage of input signal was calculated by comparing the cycle threshold (CT) values for 1% input, experimental precipitate (specific antisera), and background precipitate (normal IgG) using the delta-delta CT method [30].

Southern blotting

Southern blotting was performed as described [17], using EcoRI-digested genomic DNA (20ug/lane) isolated from leukemic lymph node cells. The blot was probed with an [α^{32} P] radiolabeled *EcoRI-ClaI* fragment of the retroviral vector, encoding IRES-GFP, to detect proviral integrations, and subsequently stripped by immersion in boiling 0.1X SSC/0.5% SDS. The blot was then reprobed with a 2.3 kb *EcoRI* J β 2 fragment of the *Tcrb* gene to detect T cell receptor rearrangements.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was prepared using Qiagen RNeasy spin columns. Reverse transcription and PCR were performed with the Invitrogen Superscript III one-step RT-PCR kit (life technologies). Amplification signals generated with a previously described primer-probe set for *Arf* [31] were compared with reference to a commercially available TaqMan primer and probe set for 18S rRNA (Applied Biosystems).

Methylation sensitive restriction digest

Genomic DNA from bone marrow-derived, ICN1+ leukemia-initiating cultures or from leukemic lymph nodes from recipient mice was digested with Eco RI endonuclease and subsequently with either Msp I endonuclease, which is insensitive to CpG methylation, or to its isoschizomer Hpa II, which is blocked by CpG methylation (all from New England Biolabs). DNA was recovered on silica mini-spin columns (Qiagen) and subject to SYBR green-based quantitative PCR using primers that amplify a short segment of the *Arf* promoter, located in a CpG island and encompassing an Msp I/Hpa II cut site. The amplification signal generated from digested template was compared to input DNA using the delta-delta CT method, with reference to a second amplicon downstream of *Arf* Exon 1 β that lacks an Msp I/Hpa II restriction site.

Results

T cell developmental maturity restricts potential for malignant transformation

Bone marrow progenitor cells or thymocytes from C57BL/6 mice were transduced with a retroviral vector co-expressing ICN1 and GFP, and then cultured on OP9 stroma for 12 days, by which time transduced cells comprised the majority of cells [17]. GFP marked cells universally expressed the Thy1.2 T-lymphoid marker, and marrow-derived cells were primarily negative for both CD4 and CD8 (DN), though minor populations of cells singly positive for CD4 (CD4-SP) or CD8 (CD8-SP), or doubly positive for both markers (DP) were identified. Thymocytes gave rise to populations similarly heterogeneous for CD4 and CD8 staining, but with larger CD8-SP and DP fractions. Importantly, although many primary cell types cultured under adverse non-physiological conditions activate *Ink4a-Arf* expression [32], T cell progenitors grown with IL-7 and OP9 stromal support do not express p19^{Arf} or p16^{Ink4a} during the 12 day culture period.

Studies in several mouse T-ALL model systems have suggested that immature DN cells within the thymus are the most fertile source of leukemia-initiating cells, even though the clonal malignancies that ultimately emerge are of the more mature DP phenotype [13,33–37]. Given that our T-ALL model relies on the infusion of cultured ICN1+ progenitor cells into non-irradiated syngeneic mice, we wished to ascertain whether cultured thymocytes were similarly limited in leukemia-initiating potential by their stage of developmental maturity. ICN1-transduced *Arf*^{-/-} thymocytes were cultured on OP9 stroma for 12 days, by which time invariably greater than 95% of the cells expressed the GFP marker. The cells were separated into DN, CD8-SP, and DP fractions by FACS, and 10⁵ cells of each fraction were separately infused into non-irradiated recipient syngeneic mice. No difference in GFP fluorescence intensity was detected among the three fractions. Because the CD8-SP cells are primarily surface TCR-negative at this period of *ex vivo* expansion [38], they represent a step of development between DN and DP [39]. Unfractionated, pre-sorted cells infused in parallel produced T-ALL in recipients with the expected median survival of 29 days [17]. Recipients of the ICN1+ DN fraction experienced an accelerated course, with median survival of 23 days, while disease in recipients of DP cells was delayed (median survival of 50 days) and only half developed T-ALL (Figure 1). ICN1+ CD8-SP cells produced T-ALL with an intermediate median survival of 30 days. The significant differences in survival

among recipients of the sorted fractions (as per pairwise comparison by log rank test) thus reflected attenuation of leukemogenic potential with increasing T cell developmental maturity, presumably due to decreasing frequency of leukemia initiating cells in more mature fractions.

Leukemias produced by donor thymocytes displayed a DP phenotype with surface expression of the T cell receptor (Figure 1B), demonstrating that developmental maturation progresses *in vivo* and does not itself preclude leukemogenesis. However, analysis of viral integration patterns (Figure 1C, upper panel) and TCR beta chain rearrangements (lower panel) revealed that expansion of a single clone was responsible for disease in the majority of recipients, regardless of the developmental stage of the infused donor cells. The target cell for transformation was therefore limited to a rare subset of DN cells that proceeded to productively rearrange the TCR beta chain and mature to a DP phenotype.

The *Ink4a-Arf* locus is epigenetically silenced in cultured T-ALL leukemia-initiating cells

To determine the epigenetic state of the *Ink4a-Arf* locus in cultured cells, we performed ChIP, using antibodies to modified histone H3 and with PCR primer pairs flanking *Arf* exon-1 β and *Ink4a* exon 1 α (Figure 2A). Trimethylation of lysine 27 of H3 (H3K27Me3) is associated with epigenetic silencing, while trimethylation of H3 lysine 4 (H3K4Me3) is associated with transcriptional activity. Note that the amplicons surrounding exon 1 β of *Arf* were designed to fall outside the region disrupted in the generation of either *Arf*^{-/-} knockout and *Arf*^{Gfp} knockin mice [28,29], allowing analysis of tissues from both modified strains as well as *Arf*^{+/+} controls. A strong H3K27Me3 signal was present throughout the locus in *Arf*^{+/+} marrow-derived (Figure 2B), *Arf*^{-/-} marrow-derived (Figure 2C), and *Arf*^{-/-} thymocyte-derived (Figure 2D) ICN1+ cells, while H3K4Me3 was detected at the *Arf* promoter of all three cell types. Promoters bearing “bivalent” H3K27Me3 and H3K4Me3 marks are not transcriptionally active, but are thought to represent loci that are poised to begin transcription in response to appropriate stimuli. For comparison, parallel analysis of the locus in BCR-ABL-transduced pre-B cells that express high levels of p19^{Arf} [40] demonstrated no appreciable H3K27Me3 but very high H3K4Me3, particularly around *Arf* exon 1 β (Figure 2E). To confirm that silencing marks reflected PRC2 activity, we performed ChIP for the PRC2 components EZH2 and EED on thymocyte derived ICN1+ cells, and demonstrated occupancy in both promoter regions above background, as compared to occupancy at the beta actin promoter (Figure 2F).

To validate that the poised/silenced histone signature at the *Cdkn2a* locus correlates with diminished gene expression in these pre-leukemic cells, despite strong oncogene-driven mitogenic signals, we performed qRT-PCR on multiple independently initiated pools of ICN1-expressing marrow cells after 12 days of *ex vivo* expansion (Figure 2G). Only trace signal was detected in the *Arf*^{+/+} samples, consistent with either *Arf* expression in a very small subpopulation of cells or more widespread but trivial levels of transcription. DNA methylation of the *Arf* promoter presents a potential alternative or complementary mechanism for silencing in these pre-leukemic cells [41]. However, genomic DNA isolated from ICN1-expressing cultured *Arf*^{+/+} marrow cells was as readily cleaved by Hpa II CpG methylation-sensitive endonuclease as pre-B cells with a fully activated locus (Figure 2H.)

Maintenance of *Arf* silencing during progression to leukemia is favored in bone marrow-derived versus thymocyte-derived ICN1+ cells

Although *Ink4a-Arf* is silenced in cultured ICN1+ cells derived from bone marrow progenitors and thymocytes, the poised configuration of the *Arf* promoter suggests that silencing might be relieved in the course of tumor progression. Accordingly, we isolated lymph nodes from moribund recipients of marrow-derived ICN1+ cells, at which time T-

ALL blasts constituted >90% of the population. In all bone-marrow-derived T-ALLs tested, the *Ink4a-Arf* locus retained the silenced configuration with bivalent marking of the exon 1 β promoter (Figure 3A&B). These findings explain the observation that cultured ICN1+, *Arf*^{+/+} bone marrow progenitors efficiently induce T-ALLs that do not express p19^{Arf} while preserving an intact *Ink4a-Arf* locus.

In contrast, among *Arf*^{-/-} thymocyte-derived T-ALLs (Figure 3C), strong silencing marks were absent in some tumor samples (one of three shown). Thus, even after inactivation of the *Arf* gene, some thymocyte-derived ALL cells epigenetically remodeled the *Arf* promoter. Differential reactivation of *Arf* expression likely explains why ICN1+, *Arf*^{+/+} donor thymocytes, unlike ICN1+, *Arf*^{+/+} donor bone marrow cells, are poorly leukemogenic [17]. As with the cultured leukemia initiating cells, minimal levels of *Arf* transcript were detectable in marrow-derived T-ALL (Figure 3D), without DNA methylation of the *Arf* promoter (Figure 3E).

***Arf*-null T-ALL cells comprise epigenetically heterogeneous subpopulations**

We speculated that individual T-ALLs derived from thymocytes contained subpopulations with varying degrees of *Ink4a-Arf* silencing. Using a retroviral vector which encoded CFP (cherry) to mark productive viral integration, we induced T-ALL by injecting mice with cultured ICN1+ thymocytes from *Arf*^{Gfp} knock-in donors, in which a GFP expression cassette replaces the first exon of the cellular *Arf* gene [29]. Resulting T-ALLs are functionally *Arf* null but express GFP when the *Arf* cellular promoter is engaged. Importantly, although these tumors arose rapidly in the absence of functional *Arf*, *Arf* promoter activation during tumor progression need not be penetrant, because GFP expression is not selected *per se*.

As expected, the percentage of GFP-positive cells varied considerably among individual tumors. Cryopreserved, thymocyte-derived *Arf*^{Gfp} leukemic lymph node cells were thawed and placed in co-culture with OP9 stromal cells and continuously expanded in medium containing IL-7. Once the CFP+, *Arf*^{Gfp} T-ALL cells were established in culture, we used FACS to separate them into fractions with bright or absent GFP expression. In three of three T-ALL cultures, sorted GFP bright and negative tumor cell fractions gave rise to stable subcultures in which the percentage of GFP-expressing cells remained invariant over at least six subsequent passages (Figure 4A). This implies that remodeling of the locus occurs prior to the time that frank DP tumor cells emerge.

The persistent absence of transcriptional activity from the *Arf* promoter in the GFP-negative subcultures was paralleled by maintenance of silencing marks (Figure 3B) and PRC2 occupancy (Figure 4C – 4D) at the *Ink4a-Arf* locus. Interestingly, the silenced chromatin configuration was reproducibly clustered to the distal end of the locus, near the site of the *Ink4a* promoter. This may reflect prolonged time in culture, with co-selection against expression of wild type *Ink4a*. Nevertheless, this distribution of silencing marks was sufficient to maintain inactivity of GFP expression from the *Arf* promoter despite the presence of a strong H3K4Me3 signal. In contrast, silencing marks and PRC2 occupancy were absent throughout the locus in the GFP-positive subpopulation (Figure 4B – 3D). In these cells, the H3K4Me3 mark remained elevated at the *Arf* promoter, reflecting the strong expression of GFP. The balance of silencing and activating histone methylation marks is determined not only by histone methyltransferases but also by histone lysine demethylases, including JMJD3 and UTX [42]. In epithelial cells, JMJD3 is primarily responsible for demethylation of H3K27Me3 at *Ink4a-Arf* during cellular senescence in response to oncogenic Ras activation or the stress of continued tissue culture [43,44]. Similarly, JMJD3 but not UTX was recruited to the *Ink4a-Arf* locus in ICN1+ T-ALL cells that were

maintained in culture (Figure 4E). In all such experiments, then, the *Arf*^{Gfp} knock-in allele faithfully reported the epigenetic state of the *Ink4a-Arf* locus.

Because the *Ink4a-Arf* locus can be induced by the stress of prolonged cell culture under nonphysiologic conditions [32], we wished to confirm that the heterogeneity of epigenetic state observed in cultured T-ALL cells could also be identified in uncultured tumor samples. Two independently derived, cryopreserved lymph node samples from recipients of *Arf*^{Gfp}, CFP-ICN1+ cells were separated by FACS into fractions with bright expression of both ICN1-CFP and GFP, or of ICN1-CFP alone, and directly subjected to ChIP for histone H3 modifications (Figure 5A and 5B). In both tumors, the GFP-negative fraction was marked by H3K27Me3 throughout the locus, and this mark was absent in the GFP-positive fractions of the same tumors. However, a bivalent H3K4Me3 mark was maintained in both tumors at the *Arf* promoter. The stark contrast in the histone code marking the locus in these otherwise identical tumor populations can be graphically represented by plotting the log-transformed ratio of the H3K4Me3 to H3K27Me3 signals (Figure 5C). Epigenetic heterogeneity within phenotypically homogeneous tumors indicates that relief of epigenetic silencing at the *Ink4a-Arf* locus is a feature of tumor progression *in vivo*.

Discussion

Cultured *Arf*^{-/-} thymocytes transduced with ICN1+ rapidly induce fatal T-ALL when infused into healthy syngeneic mice. Although the less mature ICN1+ DN subpopulation is considerably more efficient than DP cells in inducing disease, the T-ALLs that emerge have a DP phenotype and exhibit clonal retroviral insertions and TCR- β gene rearrangements. Thus, as in other mouse T-ALL models [13,33–37], the surface phenotype of the tumor is not indicative of the cell of origin. Analogous to the high frequency of *INK4A-ARF* (*CDKN2A*) deletion in human NOTCH1-driven T-ALL, *Arf* inactivation in mice is required for ICN1-induced leukemogenesis, although loss of *Ink4a* alone is without effect [17]. Before passage through mice, cultured ICN1+, *Arf*^{-/-} thymocytes exhibited a bivalent pattern of H3K27Me3 and H3K4Me3 modification at the *Ink4a-Arf* locus, as well as detectable binding of repressive PRC2 components (EZH2 and EED), consistent with an epigenetically silenced state and failure to express p19^{Arf} or p16^{Ink4a}. Yet, experiments using an *Arf*^{Gfp} knock-in allele that accurately reports the epigenetic state of the *Arf* promoter indicate that remodeling of the locus occurs during tumor progression *in vivo*. Observations that transformation occurs after rearrangement of the TCR beta chain and that the variable expression of the *Arf*^{Gfp} reporter becomes “fixed” by the time leukemic blasts reach the DP stage implies that epigenetic remodeling occurs at or near the DN to DP transition, and, indeed, normal immature thymocytes depend on silencing of *Ink4a-Arf* to successfully negotiate the late DN to DP stages of intrathymic development [26]. Subsequent engagement of the locus in response to hyperproliferative oncogenic signals would lead to tumor suppression, accounting for the inability of ICN1+, *Arf*^{+/+} donor thymocytes to generate tumors. However, rare cells that sustain *Ink4a-Arf* deletions would not be eliminated and would emerge as clonal malignancies.

In contrast to the relatively weak effects of NOTCH1 point mutations in human T-ALL [45], unbridled ICN1 activity might effectively model the multiple oncogenic genetic hits thought to be necessary for the pathogenesis of T-ALL [46]. The *CDKN2A* locus is not a direct target of NOTCH1 transcriptional regulation [13,47]; however, NOTCH1 is a potent inducer of Myc [47–49] and collaborates with K-Ras in leukemogenesis [45,50]. Both Myc and Ras are *Arf* inducers [11].

Recent reports have established that deletions and inactivating mutations of PRC2 component genes affect about a quarter of human T-ALL cases, implying that PRC2, like

CDKN2A, exerts a potent tumor suppressive function [51,52]. NOTCH1 upregulation in leukemic DP cells was associated with loss of H3K27Me3 marks from Notch target gene promoters. The correlation between aberrant Notch1 signaling and PRC2 inactivation suggests another manner by which ICN1 signaling would activate *CDKN2A* and, in turn, select for its deletion.

Whereas *Arf* deletion is required for efficient leukemia induction by ICN1+ cultured thymic progenitors [17], T-ALLs induced by cultured ICN1+ bone marrow progenitors uniformly maintain silencing of the *Ink4a-Arf* locus, do not express p19^{Arf} or p16^{Ink4a}, and are therefore indifferent to *Arf* deletion. Thus, although ICN1+ bone marrow-derived progenitors ultimately give rise to T-ALLs that are clinically and pathologically identical to those induced by thymocytes, it remains paradoxical as to why these leukemia-initiating cells choose another means of bypassing *Arf* tumor suppression. In this model system, then, experiments with donor ICN1+ thymocytes best mimic the epigenetic regulation of tumor suppressors in human disease.

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Figure 1. Leukemia-initiating potential of ICN1+ thymocyte subsets

(A) Survival curves for recipients of *Arf*^{-/-} thymocytes transduced with ICN1 and expanded ex vivo. Equal cell doses were infused either from unsorted, cultured donor cells or after their separation by FACS on the basis of CD4 and CD8 surface expression. Data were compiled in two independent experiments, and comparison of the survival curves by the log rank test yielded $P = 0.002$ for all three pairwise combinations of the three cohorts of sorted cell recipients. (B) Representative immunophenotypic analysis of one T-ALL arising in a recipient of sorted DN cells. (C) Southern blot for viral integrations (upper blot) using a GFP probe or TCRβ chain rearrangements (lower blot) using a cloned fragment of the *Tcrb* gene. DNA was extracted from T-ALL developing in recipients of unfractionated or sorted cells, as indicated. The arrow indicates the germ line configuration of *Tcrb*.

Figure 2. Epigenetic state of the *Ink4a-Arf* locus in leukemia-initiating cells

(A) Map of the *Ink4a-Arf* genomic locus indicating the location of amplicons used to quantify DNA by qPCR, following either ChIP or methylation-sensitive restriction digest. Note that exon 1 β is replaced by a neo targeting cassette for *Arf*^{-/-} knockout mice [28] and by a Gfp/neo cassette for *Arf*^{Gfp} knock-in mice [29]. (B – E) ChIP for histone H3 modification of sheared chromatin prepared from *Arf*^{+/+} or *Arf*^{-/-} bone marrow cells (B and C respectively) or *Arf*^{-/-} thymocytes (D) transduced with ICN1 and expanded *ex vivo* on OP9 stromal cells for 12 days, and from p53-null pre-B cells (E) transformed with the p185 BCR-ABL oncogene. Plots represent mean values of at least 3 independent experiments, and error bars show standard deviations among those replicates. For each cell type and at each amplicon, values for the amplification signals associated with the two modifications were compared by two-tailed T-test assuming equal variance. (F) ChIP for PRC2 components EZH2 and EED performed on thymocyte-derived, ICN1 transduced cells. Representative data from one of three experiments is shown, and error bars delimit the standard deviation among three technical replicates. Values at amplicons A1 – A4 were compared to values at actin by two-tailed T-test assuming equal variance. (G) qRT-PCR for *Arf* transcript performed on total RNA isolated from three individually transduced and expanded pools of ICN1+, *Arf*^{+/+} or *Arf*^{-/-} bone marrow cells, normalized to p53-null pre-B cells transformed with BCR-ABL. Values for WT and *Arf*^{-/-} samples were aggregated and compared to each other and to control cells by two-tailed T-test assuming equal variance. (H) Fraction of intact genomic DNA remaining after restriction digest with Msp I or Hpa II endonucleases, determined by qPCR at amplicons A1 and A2 as mapped. Three independently transduced and expanded *Arf*^{+/+} marrow samples are compared to genomic DNA from p185 expressing pre-B cells, with or without Sss I methyltransferase treatment. For both HpaII and MspI digests, single factor ANOVA demonstrated a P value < 0.005, indicating significant variance between means. For G and H, representative data from one of two independent experiments is shown, and error bars indicate standard deviation of three technical replicates. Where indicated, * denotes P-value < 0.5 and ** P < 0.005.

Figure 3. Epigenetic state of the *Ink4a-Arf* locus in ICN1-driven T-ALL

(A – C) ChIP for histone H3 modifications at the *Ink4a-Arf* locus in T-ALLs initiated in independent experiments by targeting *Arf*^{+/+} bone marrow cells (A), *Arf*^{-/-} bone marrow cells (B), or *Arf*^{-/-} thymocytes (C). For each tumor, values for the amplification signals associated with the two modifications at each amplicon were compared by two-tailed T-test assuming equal variance. (D) *Arf* expression in leukemic lymph nodes of recipients of ICN1+, *Arf*^{+/+} bone marrow cells. Values for WT and *Arf*^{-/-} T-ALLs were aggregated and compared to each other and to control cells by two-tailed t-test assuming equal variance. (E) Fraction of genomic DNA remaining intact after methylation sensitive restriction digest in T-ALL samples derived from ICN1+, *Arf*^{+/+} bone marrow cells. For both HpaII and MspI digests, single factor ANOVA demonstrated a P value < 0.005, indicating significant variance between means. Error bars show the standard deviation of three technical replicates. Panels D and E show representative data from one of two biological replicates. Where indicated, * denotes P-value < 0.5 and ** P < 0.005.

Figure 4. Epigenetic heterogeneity of the *Ink4a-Arf* locus in cultured tumor cell subpopulations (A) ICN1- expressing *Arf^{Gfp}* T-ALL cells adapted to culture were sorted into GFP-bright and GFP-negative fractions and separately propagated, and the percentage of GFP expressing cells was measured by flow cytometry with each passage. (B – E) ChIP performed on stably GFP negative or positive cells 5 passages after sorting to assess histone H3 modification (B), EZH2 occupancy (C), EED occupancy (D), and histone demethylases (E). All panels show results from one of three biological replicates, derived from independently generated primary T-ALLs. Error bars delimit standard deviation of three technical replicates. In panel (B) mean values for H3 modification were compared at each amplicon by two-tailed t-test assuming equal variance. In panels (C – E), the mean

occupancy of the indicated protein at each amplicon was compared to its occupancy at the beta actin promoter. Where indicated, * denotes P-value < 0.5 and ** P < 0.005.

Figure 5. Heterogeneity of epigenetic state of *Ink4a-Arf* in primary tumors

(A – B) ChIP for histone H3 modifications performed on the GFP negative (A) and GFP-bright (B) fractions of an *Arf^{Gfp}* T-ALL, separated by FACS of cryopreserved, uncultured lymph node cells. (C) Relative epigenetic marking of the locus as reflected by the log-transformed ratio of quantified H3K4Me3 to H3K27Me3 modifications depicted in (A) and (B). The experiment was performed twice with similar results. Error bars show the standard deviation of three technical replicates. Values for the amplification signals associated with the two modifications at each amplicon were compared by two-tailed T-test assuming equal variance. Where indicated, * denotes P-value < 0.5 and ** P < 0.005.

Table 1

Table Primers used for qPCR.

Orientation	Sequence	Location of amplicon
5'	GTGGGGGAAGGAGAAAAGG	804 bp upstream of exon 1beta of <i>Arf</i>
3'	GCGGAGCTAGGACTGACCAA	
5'	CTCCCTTTGCTACCCCTGAGAG	480 bp downstream of exon 1beta of <i>Arf</i>
3'	TTACTTATTTGCTCCCATCCAC	
5'	GTCCGATCCTTTAGCGCTGTT	181 bp upstream of exon 1alpha of <i>Ink4a</i>
3'	AGCCCGGACTACAGAAGAGATG	
5'	AGGCACCTTCCAGCCTTTTT	340 bp downstream of exon 1alpha of <i>Ink4a</i>
3'	TTCCCGTGAAACAAATGTGC	
5'	CCCAACACACCTAGCAAATTAGAACCAC	Mouse beta actin promoter
3'	CCTGGATTGAATGGACAGAGAGTCACT	