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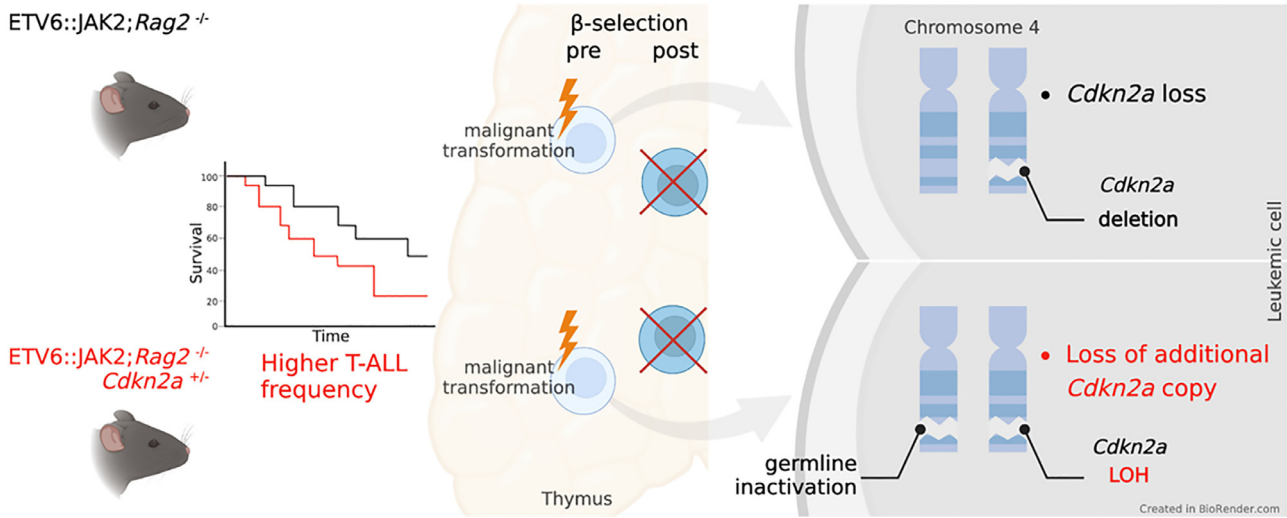

# *Cdkn2a* inactivation promotes malignant transformation of mouse immature thymocytes before the $\beta$-selection checkpoint

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## HIGHLIGHTS

- *Cdkn2a* deletions occur in T-ALL driven by the ETV6::JAK2 fusion protein when the pre−TCR is absent.
- Germline *Cdkn2a* inactivation favors malignant transformation of DN3 thymocytes.
- Mouse T-ALL originating from *Cdkn2a*$^{+/-}$ DN3 thymocytes often presents *Cdkn2a* loss of heterozygosity.
- Further secondary events occur in T-ALL originating from *Cdkn2a*$^{+/-}$ DN3 thymocytes.

*CDKN2A* deletion is the most frequent genetic alteration in T-cell acute lymphoblastic leukemia (T-ALL), occurring across all molecular and immunophenotypic subtypes. *CDKN2A* encodes two functionally unrelated tumor suppressor proteins, ARF and INK4a, which are critical regulators of cell cycle and proliferation. *Arf* has been reported to suppress T-ALL development in post−$\beta$-selection thymocytes, but whether *CDKN2A* acts as a tumor suppressor gene in immature, pre−$\beta$-selection thymocytes remains to be elucidated. Resorting to a *Rag2*-deficient model of T-ALL, driven by the ETV6::JAK2 fusion, we report that *Cdkn2a* haploinsufficiency at early stages of T-cell development facilitates leukemia development. ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL arose from pre−$\beta$-selection thymocytes because the thymocyte differentiation arrest caused by *Rag2* deficiency was maintained in preleukemic mice. Furthermore, somatic loss of the *Cdkn2a* wild-type allele was frequently observed in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells, suggesting a selective advantage for total inactivation of *Cdkn2a*. Both *Cdkn2a*-sufficient and *Cdkn2a*-defective T-ALL presented additional genetic alterations, such as *Notch1* mutations and gains of chromosomes 13 and 15. These data indicate that *Cdkn2a* acts as a gatekeeper for leukemogenesis from the most immature stages of thymocyte development.

Pre−T-cell receptor (pre−TCR) expression in CD4$^-$/CD8$^-$ double-negative (DN) thymocytes, following successful *Tcrb* locus rearrangements, is critical for the $\beta$-selection checkpoint. The pre−TCR is composed of a TCR$\beta$ chain paired with the invariant pT$\alpha$ protein and CD3 family proteins. Although pT$\alpha$ expression has been documented in a high proportion of human T-cell acute lymphoblastic leukemia (T-ALL) [1,2], its role in leukemogenesis has been a matter of debate. In several T-ALL mouse models [3,4], pT$\alpha$ or pre−TCR was found to be essential for leukemogenesis. In turn, although not found as essential, pre−TCR favored leukemogenesis induced by intracellular Notch1 (ICN1) transduction [5] or transgenic expression of Notch3 [6], Myc [7], and ETV6::JAK2 fusion [8]. Further indicating that pre−TCR expression can be a major driver of thymocyte malignant transformation, compensatory genetic alterations were observed in pre−TCR-deficient ETV6::JAK2-driven leukemias, including chromosome 15 gains and chromosome 4 microdeletions encompassing the *Cdkn2a* locus [8].

The *CDKN2A* locus encodes two distinct tumor suppressor proteins, ARF and INK4a, which are crucial to regulating cell survival and proliferation. *CDKN2A* deletions are common in solid and hematological malignancies, being frequent in T-ALL and specific subtypes of B-cell ALL [9]. Additionally, *Cdkn2a* epigenetic silencing was found in the ICN1-driven T-ALL mouse model [10], and inactivation of *Arf* and/or *Ink4a* genes accelerated T-ALL development in mouse models driven by expression of intracellular Notch1, Tal1, Lmo2, or Hmga1a proteins [11−14].

Arf expression was proposed to act as a fail-safe mechanism against aberrant pre−TCR-positive thymocytes by inducing cell death and impairing their maturation [15]. Similarly, Volanakis et al. [10] reported that *Arf* prevents the malignant transformation of thymocytes after $\beta$-selection when thymocytes undergo massive proliferation after TCR$\beta$ rearrangements. However, because *CDKN2A* loss has been detected across all maturation subtypes of human T-ALL [9,16], it is likely that *Arf* and/or *Ink4a* act as tumor suppressors not only after but also before $\beta$-selection.

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## METHODS

### Mice

E$\mu$SR$\alpha$-ETV6::JAK2 (B6.Cg-Tg(Emu-ETV6/JAK2)71Ghy) transgenic mice [8] were bred with *Rag2* (B6.129S6-Rag2tm1Fwa) and *Cdkn2a* (B6.129-Cdkn2a$^{tm1Rdp}$/NCI) knockout mice on the C57BL/6 background. The ETV6::JAK2 transgene was kept in hemizygosity. The *Cdkn2a* knockout mice [17] were obtained from the National Cancer Institute Mouse Repository (strain number 01XB1). Mice were bred and maintained at the i3S barrier animal facility (high efficiency particulate air filtration of incoming air, differential pressure, and disinfection or sterilization of room equipment and supplies) under 12:12-hour light:dark cycles and with food (2014S diet; Envigo) and water ad libitum. All experimental procedures were approved by the i3S ethics committee and Portuguese authorities (*Direção-Geral de Agricultura e Veterinária*) and followed recommendations from the European Commission (Directive 2010/63/UE) and the local Portuguese authorities (*Decreto-Lei* n°113/2013). Both female and male mice were used for all experiments. Mice were monitored for signs of disease (i.e., dyspnea, lethargy, enlarged lymph nodes, enlarged abdomen, and paraplegia) and euthanized by $CO_2$ inhalation upon reaching predefined experimental end points. Mice of different genotypes from the same litter were kept together in the same cages, and monitoring for signs of disease was performed blindly. Adult mice that were euthanized without leukemia were censored in Kaplan–Meier survival curves.

### Low-coverage Whole Genome Sequencing

For low-coverage whole genome sequencing, the Ion Xpress Plus Fragment Library kit (ThermoFisher Scientific) was used for library preparation, and sequencing was performed using the Ion S5XL system (ThermoFisher Scientific). For copy number analysis, sequencing data were aligned with the mouse reference genome (Genome Reference Consortium Mouse Build 38 or mm10 reference genome) and further analyzed using QDNAseq (RRID:SCR_003174) package, R software. A bin size of 30 kb was used.

### Statistical Analysis

Statistical analysis was performed with GraphPad Prism 6.0 software (RRID:SCR_002798). Unpaired Student *t* test was used for comparisons between the two groups. Log-rank test was used to compare the survival of different groups. Sample numbers are indicated in the figure legends. $p < 0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

To determine whether *Cdkn2a* inactivation could act as a tumor suppressor in pre–$\beta$-selection thymocytes, we investigated a mouse model of T-ALL arising from thymocytes arrested at the DN stage. ETV6::JAK2;*Rag2*$^{-/-}$ mice develop late-onset thymic lymphomas (median of 9 months) with malignant cells expressing CD4 and CD8 [8]. Because *Rag2* deficiency imposes a strict arrest at the CD4$^-$/CD8$^-$/CD25$^+$/CD44$^-$ DN stage 3 of thymocyte development, we assessed if the arrest could be overcome by ETV6::JAK2 transgenic expression. Thymocyte cellularity of preleukemic ETV6::JAK2 and *Rag2*$^{-/-}$ mice remained very low, similar to that of *Rag2*$^{-/-}$ mice. Eight of nine mice retained the DN3 block (Figure 1A,B), indicating that the ETV6::JAK2 transgene did not systematically rescue the DN3 block caused by *Rag2* deficiency. One of nine ETV6::JAK2;*Rag2*$^{-/-}$ thymuses presented DN4 cells (CD4$^-$/CD8$^-$/CD25$^-$/CD44$^-$), but these expressed aberrantly high levels of the immature thymocyte marker CD24, indicating they were already transformed thymocytes. These data show that ETV6::JAK2 fusion protein does not bypass the $\beta$-selection block caused by *Rag2* deficiency and transforms malignant cells at DN3 or earlier stages of thymocyte development.

Because chromosome 4 deletions encompassing the *Cdkn2a* locus were previously described in pre–TCR-deficient ETV6::JAK2 T-ALL [8], we performed *Cdkn2a* locus quantitative PCR in ETV6::JAK2;*Rag2*$^{-/-}$ (pre–TCR-deficient) T-ALL. *Cdkn2a* copy number loss, encompassing all exons, was found in one of six cases (Supplementary Figure E1). Compiling these results with reported array comparative genomic hybridization data [8], we verified that *Cdkn2a* loss occurred only in pre–TCR-deficient and not in pre–TCR-proficient T-ALL (3/15 vs. 0/11 cases analyzed; Figure 1C). Together, these data hint that *Cdkn2a* inactivation can cooperate with the ETV6::JAK2 fusion protein in the malignant transformation of pre–$\beta$-selection thymocytes.

Next, to determine the impact of germline *Cdkn2a* genetic inactivation on pre–TCR-deficient T-ALL, we bred ETV6::JAK2;*Rag2*$^{-/-}$ mice with the *Cdkn2a* constitutive knockout allele to generate cohorts carrying normal or haploinsufficient *Cdkn2a* copy number. Although not statistically significant, ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice developed lymphoma/leukemia tendentially faster and with higher frequency than ETV6::JAK2;*Rag2*$^{-/-}$ mice (median survival of 53 weeks vs. 72 weeks and survival proportions of 19.7% vs. 49.2%, respectively; Figure 1D). ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice presented thymic lymphomas, reduced dissemination to the spleen and lymph nodes, and immature immunophenotype, that is, aberrant expression of CD4 and CD8, as well as high levels of CD24 and CD25, similar to that of ETV6::JAK2;*Rag2*$^{-/-}$ mice (Figure 1E, F). Of note, we could not investigate the impact of complete *Cdkn2a* deficiency on ETV6::JAK2;*Rag2*$^{-/-}$ T-ALL development, because *Rag2*$^{-/-}$;*Cdkn2a*$^{-/-}$ mice developed rapid B-cell precursor leukemia, a phenotype similar to that of *Rag2*$^{-/-}$;*Arf*$^{-/-}$ mice [16].

We surmised that the reduced latency of T-ALL in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice could be caused by a DN3 block bypass before leukemia onset. However, we found that *Cdkn2a* haploinsufficiency did not rescue the DN3 developmental block. Indeed, pre-leukemic ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice presented similar thymic atrophy, thymocyte hypocellularity, and DN3 thymocyte developmental arrest as *Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ or *Rag2*$^{-/-}$ mice (Supplementary Figure E2A,B).

To determine whether leukemogenesis was associated with somatic inactivation of *Cdkn2a*, we assessed the *Cdkn2a* copy number status in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemic cells. Indeed, six of 10 cases showed loss of the wild-type (WT) allele (Figure 2A), indicating a selective advantage for complete *Cdkn2a* loss. Because ETV6::JAK2;*Rag2*$^{-/-}$ leukemias presented increased frequency of DNA copy number alterations [8], we assessed the genome-wide copy number status of T-ALL from *Cdkn2a*-sufficient and -haploinsufficient mice by low-coverage whole genome sequencing. We observed recurrent chromosomal numerical alterations in both ETV6::JAK2;*Rag2*$^{-/-}$ (occurring in 83% of cases, most frequently chromosome 15 gains) and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL (found in 50% of cases, most frequently chromosome 4, 13, and

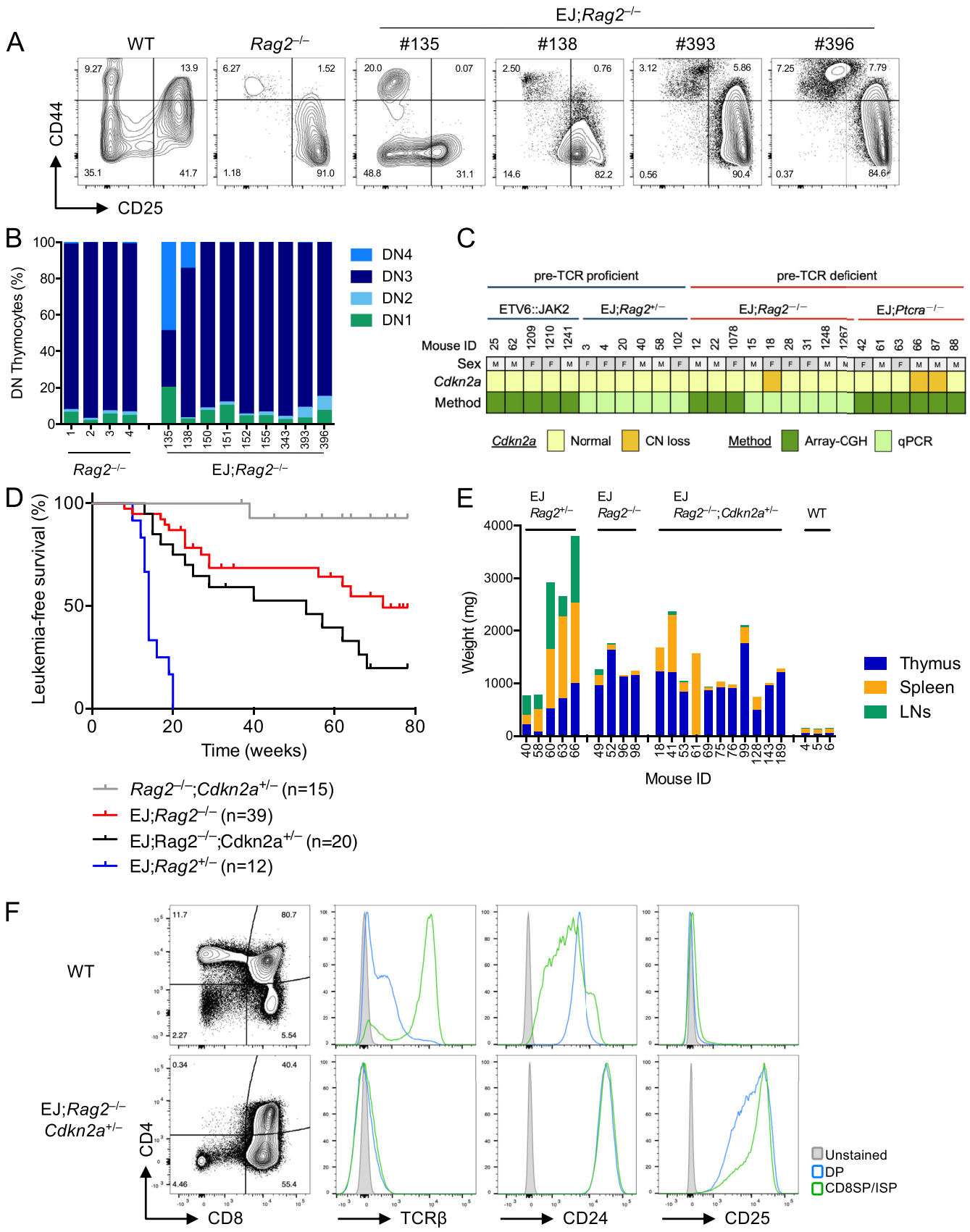


Figure 1 *Cdkn2a* genetic inactivation accelerates T-ALL development in ETV6::JAK2;*Rag2*$^{-/-}$ mice. **(A)** Flow cytometry analysis of DN thymocytes (CD90.2$^{+}$CD4$^{-}$CD8$^{-}$) from representative 4- to 6-month-old *Rag2*$^{-/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$ preleukemic mice.

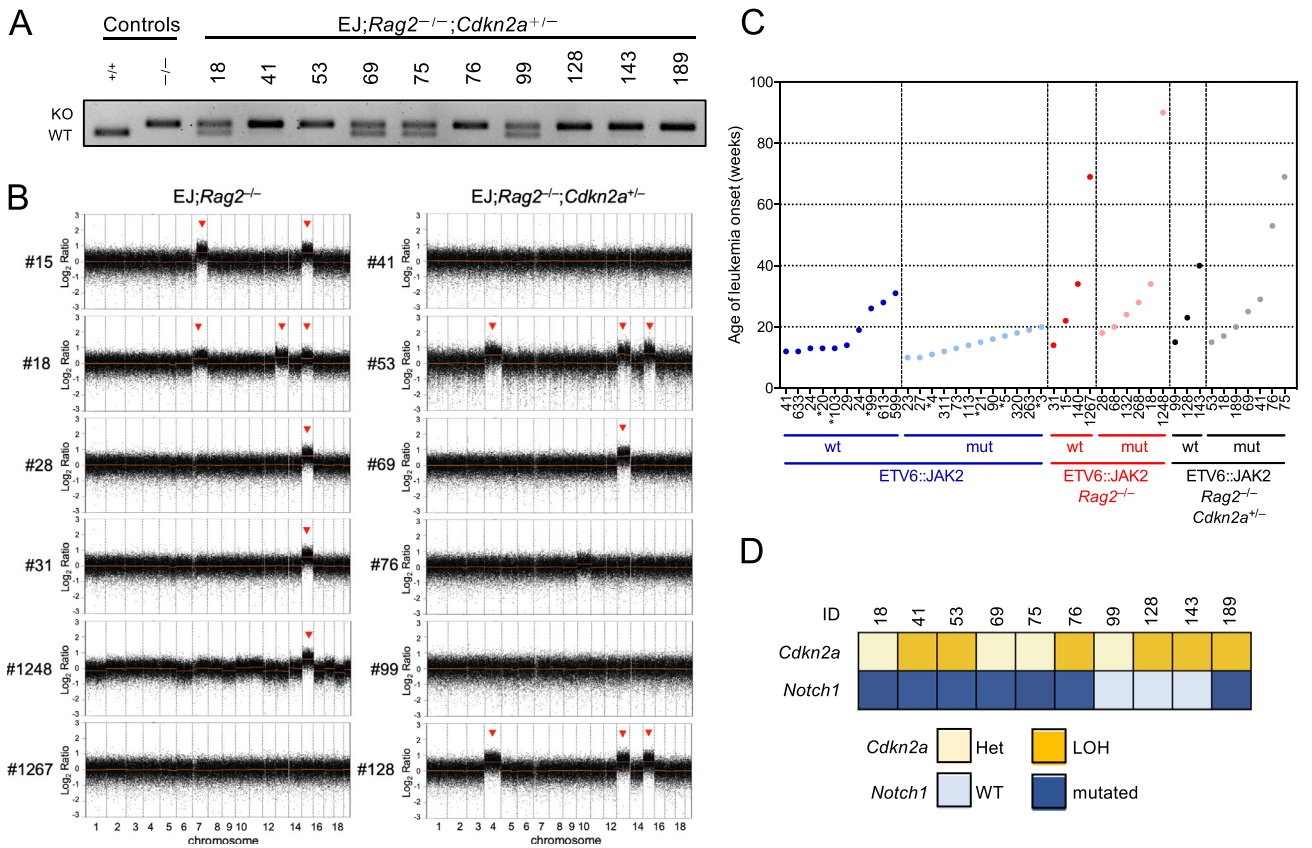


**Figure 2** Frequent *Cdkn2a* loss of heterozygosity and DNA copy number alterations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia. **(A)** PCR detection of wild-type (WT) and *Cdkn2a* knockout (KO) alleles in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells. Loss of the WT allele (loss of heterozygosity) was detected in 6 of 10 cases (numbers 41, 53, 76, 128, 143, and 189). **(B)** DNA copy number alterations assessed by low-coverage whole genome sequencing of T-ALL cells from *Cdkn2a*-sufficient and -haploinsufficient ETV6::JAK2;*Rag2*$^{-/-}$ mice. Recurrent copy number chromosomal alterations were found in both groups (chromosome 13 and 15 gains). Each graph represents DNA copy number distribution for each T-ALL case of the indicated genotype. Red arrowheads show chromosomal gains. **(C)** Schematic representation of the age of T-ALL onset in mice of the indicated genotypes with or without *Notch1* exon 34 PEST domain mutation (mut and wt, respectively). Each dot represents a mouse. Asterisks mark mice of ETV6::JAK2;*Rag2*$^{+/-}$ genotype **(D)** Schematic representation of detected *Cdkn2a* loss-of-heterozygosity (LOH) and *Notch1* mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cases. *Het*=heterozygous.

15 gains; Figure 2B). In two cases (nos. 53 and 128), *Cdkn2a* loss of heterozygosity (LOH) co-occurred with the gain of chromosome 4, which harbors the *Cdkn2a* locus. From the low-coverage sequencing, there was no indication of *Cdkn2a* deletion in these cases with chromosome 4 gain. These findings suggest that the chromosome carrying the WT *Cdkn2a* allele was lost, resulting in LOH, and the chromosome carrying the mutant allele was triplicated. This conjecture is reminiscent of uniparental isodisomies of chromosome 9 segments harboring *CDKN2A* detected in human T-ALL [18]. These data suggest that complete *Cdkn2a* inactivation favors ETV6::JAK2-induced leukemogenesis and that further genetic alterations are still required for the malignant transformation of pre−TCR-deficient thymocytes.

Wild-type (WT) thymocytes were analyzed as a control. **(B)** Percentage of DN1−DN4 thymocytes, gated in CD90.2$^{+}$CD4$^{-}$CD8$^{-}$ cells. Each column represents a mouse of the indicated genotype. **(C)** Summary of detected loss of *Cdkn2a* by quantitative PCR (Supplementary Figure E1) and array comparative genomic hybridization (Array-CGH) [8] in 3 of 15 pre−TCR-deficient and 0 of 11 pre−TCR-proficient T-ALL mice. **(D)** Kaplan−Meier leukemia-free survival curves for the indicated genotypes. Survival curve comparison of ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ and ETV6::JAK2;*Rag2*$^{-/-}$ mice yielded $p = 0.0585$ (log-rank test). The hazard ratio was 2.15, with a 95% CI of 0.97−4.73. Mice euthanized because of causes other than leukemia/lymphoma (e.g., solid tumors in old ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice) were censored in the analysis (tick marks). **(E)** Thymus, spleen, and lymph node (LNs) weights of mice with leukemia/lymphoma. Each column represents a mouse. Mouse no. 61 developed B-cell leukemia and splenic lymphoma without the involvement of other lymphoid organs. **(F)** Flow cytometry analysis of a representative ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemia showing surface expression of CD4, CD8, and CD25, high levels of CD24, and absence of TCR-$\beta$ expression. Expression of these markers in wild-type (WT) thymocyte subpopulations is shown as the control. *DP*=Double-positive; *SP*=single-positive; *ISP*=immature single-positive.

Because *Notch1* mutations occur frequently in human and mouse T-ALL, including ETV6::JAK2 mouse T-ALL [19], and have been associated with progression [20], we assessed whether the frequency of *Notch1* mutations was influenced by *Cdkn2a* allele loss. *Notch1* PEST domain (exon 34) mutations, which account for most *Notch1* mutations reported in murine T-ALL [21,22], were detected with similar frequency (~60%) among ETV6::JAK2, ETV6::JAK2;*Rag2*$^{-/-}$, and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ leukemias, with no correlation with age of onset (Figure 2C). *Notch1* PEST domain mutations in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL occurred independently of *Cdkn2a* copy number status because four cases carried both *Cdkn2a* LOH and *Notch1* mutation, two cases carried only *Cdkn2a* LOH, three cases carried only *Notch1* mutations, and one case neither alteration (Figure 2D and Supplementary Table E1). This indicates that *Cdkn2a* complete inactivation did not relieve the selective pressure for *Notch1* mutation acquisition and these two types of genetic alterations can complement each other in leukemogenesis.

In summary, using the ETV6::JAK2-driven T-ALL mouse model, we show that *Cdkn2a* suppresses leukemogenesis in immature pre−$\beta$-selection thymocytes. This notion is supported by two main pieces of evidence: (1) germline loss of 1 *Cdkn2a* copy aggravated leukemogenesis in ETV6::JAK2;*Rag2*$^{-/-}$ mice without bypassing the DN3 thymocyte developmental block; and (2) *Cdkn2a* somatic allele loss was frequently observed in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cells, which suggests a selective advantage for total inactivation of *Cdkn2a*. The fact that 40% of cases retained the WT allele indicates that either *Cdkn2a* haploinsufficiency conferred a selective advantage for leukemic cells or *Cdkn2a* is inactivated through other mechanisms, such as epigenetic silencing, previously reported in Notch1-driven mouse T-ALL [10]. Although *Cdkn2a* genetic inactivation facilitated thymocyte leukemogenesis, *Cdkn2a*-defective leukemic cells still presented other genetic alterations, such as *Notch1* mutations and chromosomal gains. This indicates that *Cdkn2a* deletion does not fully compensate for the absence of a pre-TCR signaling complex, and secondary alterations, such as *Notch1*-activating mutations, are required for leukemogenesis.

Although *Cdkn2a* haploinsufficiency favored leukemogenesis, leukemia onset in ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ mice occurred much later than in pre−TCR-proficient ETV6::JAK2 transgenic mice (median survival of 14 weeks; $p < 0.0001$, log-rank test). The chance for the acquisition of genetic alterations in *Rag2*-deficient pre−$\beta$-selection thymocytes is purportedly very low, due to the absence of Rag-mediated mutagenic recombination events [23] and a low rate of cell division. Although at this stage we cannot determine which events in which order are required for leukemogenesis, we posit DN3 thymocytes expressing the ETV6::JAK2 fusion protein undergo malignant transformation upon acquisition of an odd oncogenic event that either inactivates *Cdkn2a* completely or cooperates with reduced INK4a/ARF tumor suppressive activity. Loss of Arf has been shown to promote leukemia self-renewal [13], so this might be a crucial event in pre−$\beta$-selection thymocytes. *Notch1* mutations or other events (e.g., chromosome 13 or 15 trisomy) may bolster the self-renewal or malignant phenotype of leukemic cells with haploinsufficient or complete inactivation of *Cdkn2a*. *Notch1* mutations in our model are likely secondary events, paralleling human T-ALL, where *NOTCH1* mutations are often found in minor subclones [18,20].

Our results support the notion that *Cdkn2a* acts as a brake for leukemogenesis at the early stages of thymocyte development before the formation of the pre-TCR complex. Other reports indicated that *Cdkn2a* is an important tumor suppressor in post−$\beta$-selection thymocytes [11], which undergo massive proliferation and are prone to genetic alterations. These data and the occurrence of *CDKN2A* deletions in all subtypes of human T-ALL [24] allow us to conclude that *Cdkn2a* acts as a gatekeeper for leukemogenesis from the most immature until the post−$\beta$-selection stages of thymocyte development.

## Conflict of Interest Disclosures

N.R.S. has received research funding from Gilead Sciences, Portugal, and *Fundação AstraZeneca*, Portugal, after applying in competitive calls for projects. The other authors do not have any conflicts of interest to declare in relation to this work.

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## Author Contributions

T.A.C. designed, performed, and analyzed experiments and created the figures and wrote the manuscript; I.P.-L., F.A.-D., M.N.G., M. T.F., and T.C. performed experiments; and N.R.S. designed the study, performed experiments, and wrote the manuscript.

## Data Availability

The data that support the findings of this study are available from the corresponding author upon request. Raw unaligned sequencing reads (fastq-format) that support the findings of this study have been deposited in the Sequence Read Archive under the accession number PRJNA843967.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.exphem.2022.10.001.

## REFERENCES

1. Asnafi V, Beldjord K, Boulanger E, et al. Analysis of TCR, pTα, and RAG-1 in T-acute lymphoblastic leukemias improves understanding of early human T-lymphoid lineage commitment. Blood 2003;101:2693–703.
2. Ivanyi P, Morgan M, Piao W, et al. Pre T-cell receptor alpha (pTα) expression patterns and functional analysis in human T-cell lymphoblastic leukemia. Cell Oncol 2010;32:101–8.
3. Winandy S, Wu L, Wang JH, Georgopoulos K. Pre-T cell receptor (TCR) and TCR-controlled checkpoints in T cell differentiation are set by Ikaros. J Exp Med 1999;190:1039–48.
4. Allman D, Karnell FG, Punt JA, et al. Separation of Notch1 promoted lineage commitment and expansion/transformation in developing T cells. J Exp Med 2001;194:99–106.
5. Campese AF, Garbe AI, Zhang F, et al. Notch1-dependent lymphomagenesis is assisted by but does not essentially require pre-TCR signaling. Blood 2006;108:305–10.
6. Bellavia D, Campese AF, Checquolo S, et al. Combined expression of pTα and Notch3 in T cell leukemia identifies the requirement of preTCR for leukemogenesis. Proc Natl Acad Sci U S A 2002;99:3788–93.
7. Smith DP, Bath ML, Harris AW, Cory S. T-cell lymphomas mask slower developing B-lymphoid and myeloid tumours in transgenic mice with broad haemopoietic expression of MYC. Oncogene 2005;24:3544–53.
8. dos Santos NR, Rickman DS, de Reynies A, et al. Pre-TCR expression cooperates with TEL-JAK2 to transform immature thymocytes and induce T-cell leukemia. Blood 2007;109:3972–81.
9. González-Gil C, Ribera J, Ribera JM, Genescà E. The yin and yang-like clinical implications of the *CDKN2A/ARF/CDKN2B* gene cluster in acute lymphoblastic leukemia. Genes 2021;12:79.
10. Volanakis EJ, Boothby MR, Sherr CJ. Epigenetic regulation of the Ink4a-Arf (Cdkn2a) tumor suppressor locus in the initiation and progression of Notch1-driven T cell acute lymphoblastic leukemia. Exp Hematol 2013;41:377–86.
11. Volanakis EJ, Williams RT, Sherr CJ. Stage-specific *Arf* tumor suppression in *Notch1*-induced T-cell acute lymphoblastic leukemia. Blood 2009;114:4451–9.
12. Shank-Calvo JA, Draheim K, Bhasin M, Kelliher MA. p16Ink4a or p19Arf loss contributes to Tal1-induced leukemogenesis in mice. Oncogene 2006;25:3023–31.
13. Treanor LM, Volanakis EJ, Zhou S, et al. Functional interactions between Lmo2, the *Arf* tumor suppressor, and Notch1 in murine T-cell malignancies. Blood 2011;117:5453–62.
14. di Cello F, Dhara S, Hristov AC, et al. Inactivation of the *Cdkn2a* locus cooperates with *HMGA1* to drive T-cell leukemogenesis. Leuk Lymphoma 2013;54:1762–8.
15. Miyazaki M, Miyazaki K, Itoi M, et al. Thymocyte proliferation induced by pre-T cell receptor signaling is maintained through polycomb gene product Bmi-1-mediated Cdkn2a repression. Immunity 2008;28:231–45.
16. Hauer J, Mullighan C, Morillon E, et al. Loss of *p19Arf* in a *Rag1*$^{-/-}$ B-cell precursor population initiates acute B-lymphoblastic leukemia. Blood 2011;118:544–53.
17. Serrano M, Lee HW, Chin L, et al. Role of the INK4a locus in tumor suppression and cell mortality. Cell 1996;85:27–37.
18. Karrman K, Castor A, Behrendtz M, et al. Deep sequencing and SNP array analyses of pediatric T-cell acute lymphoblastic leukemia reveal NOTCH1 mutations in minor subclones and a high incidence of uniparental isodisomies affecting CDKN2A. J Hematol Oncol 2015;8:1–9.
19. Ghezzo MN, Fernandes MT, Pacheco-Leyva I, et al. FoxN1-dependent thymic epithelial cells promote T-cell leukemia development. Carcinogenesis 2018;39:1463–76.
20. Albertí-Servera L, Demeyer S, Govaerts I, et al. Single-cell DNA amplicon sequencing reveals clonal heterogeneity and evolution in T-cell acute lymphoblastic leukemia. Blood 2021;137:801–11.
21. O'Neil J, Calvo J, McKenna K, et al. Activating *Notch1* mutations in mouse models of T-ALL. Blood 2006;107:781–5.
22. Lin Y-W, Nichols RA, Letterio JJ, Aplan PD. *Notch1* mutations are important for leukemic transformation in murine models of precursor-T leukemia/lymphoma. Blood 2006;107:2540–3.
23. Bianchi JJ, Murigneux V, Bedora-Faure M, Lescale C, Deriano L. Breakage-fusion-bridge events trigger complex genome rearrangements and amplifications in developmentally arrested T cell lymphomas. Cell Rep 2019;27:2847–58. e4.
24. Zhang J, Ding L, Holmfeldt L, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. Nature 2012;481:157–63.