SUPPLEMENTARY INFORMATION TO:

Cdkn2a inactivation promotes malignant transformation of mouse immature thymocytes before the β-selection checkpoint

Telmo A. Catarino,[a,b,c] Ivette Pacheco-Leyva,[a,b] Faiza Al-Dalali,[d] Marinella N. Ghezzo,[d,1] Mónica T. Fernandes,[d,2] Telma Costa,[a,b] and Nuno R. dos Santos[a,b,d]

[a]i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal.

[b]Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Porto, Portugal.

[c]Graduate Program in Areas of Basic and Applied Biology, Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

[d]Centre for Biomedical Research (CBMR), University of Algarve, Faro, Portugal.

Correspondence:

Nuno R. dos Santos, i3S - Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Rua Alfredo Allen, 208, 4200-135 Porto, Portugal; e-mail: nunos@ipatimup.pt

[1]Present address: Center for Research in Health Technologies and Information Systems (CINTESIS), Universidade do Algarve, 8005-139 Faro, Portugal.

[2]Present address: Escola Superior de Saúde, Universidade do Algarve; Algarve Biomedical Center Research Institute (ABC-RI), 8005-139 Faro, Portugal.

Supplementary Methods

Mice Genotyping

Rag2 knock-out mice were genotyped using the following primers: 5'-TGTCCCTGCAGATGGTAACA-3' and 5'-CCTTTGTATGAGCAAGTAGC-3' for WT allele and 5'-CTATTCGGCTATGACTGGG-3' and 5'-AAGGCGATAGAAGGCGATG-3' for the mutated allele. *Cdkn2a* knock-out mice were genotyped using 5'-GTGATCCCTCTACTTTTTCTTCTGACTT-3' primer in combination with 5'-CGGAACGCAAATATCGCAC-3' or 5'-GAGACTAGTGAGACGTGCTACTTCCA-3' for the detection of the WT or mutated alleles, respectively.

Flow cytometry

Single-cell suspensions were prepared from lymphoid organs using cell strainers, washed with FACS buffer (phosphate-buffered saline (PBS) with 3% fetal bovine serum (FBS) and 10 mM NaN_3) and stained for 30-45 min with fluorochrome-labeled antibodies in FACS buffer. The following Biolegend antibodies were used: CD25-fluoresceine isothiocyanate (FITC) (clone PC61), TCRβ-phycoerithrin (PE) (clone H57-597), CD44-PE/Cyanine5 (clone IM7), CD90.2-PE-Cyanine7 (clone 30-H12), CD24-allophycocyanate (APC) (clone M1/69), CD4-APC/Cyanine7 (clone GK1.5), CD8α-Pacific Blue (clone 53-6.7). Immunostained cells were washed twice with FACS buffer and incubated in PBS with 10 mM NaN_3. Cell viability was determined using the Zombie Aqua Fixable Viability Kit (Biolegend). Samples were acquired using BD FACS CANTO II and analyzed using FlowJo software.

Quantitative PCR

Genomic DNA was isolated from $2x10^6$ thymic cell pellet using GeneJet genomic DNA purification kit (Thermo Fisher Scientific) according to manufacturer's instructions. Quantitative PCR was performed using the SsoFast Evagreen Supermix (BioRad) under

the following conditions: denaturation and polymerase activation at 95°C for 3 minutes and 40 cycles of denaturation at 95°C for 1 min, annealing and elongation at 60°C for 30 sec. All samples were tested in triplicate and the results expressed as the mean plus SD. Quantitative PCR results were analyzed with BioRad CFX Manager Software, version 1.6, using the comparative CT method (2-ΔΔC (T) method). DNA copy number alterations were detected relative to the amplification of DNA from the *Vcpip* and *Spna2* loci (chromosomes 1 and 2, respectively) which were used as a normalization control. The following primers were used for each locus.

Vcpip1:

5'-AGTGACATGGGCCTTCAAAC-3'

5'-GGCCCAGATCCAGAATAACA-3';

Spna2:

5'-AGGAGGAGGCTTGGATCAAT-3'

5'-CGAGGGTACTGCCACTGTCT-3'

Cdkn2a exon 1a:

5'-ATCTGGAGCAGCATGGAGTC-3'

5'-CTCCTTGCCTACCTGAATCG-3'

Cdkn2a exon 2:

5'-TCAACTACGGTGCAGATTCG-3'

5'-TCGCACGATGTCTTGATGTC-3'

Cdkn2a exon 3:

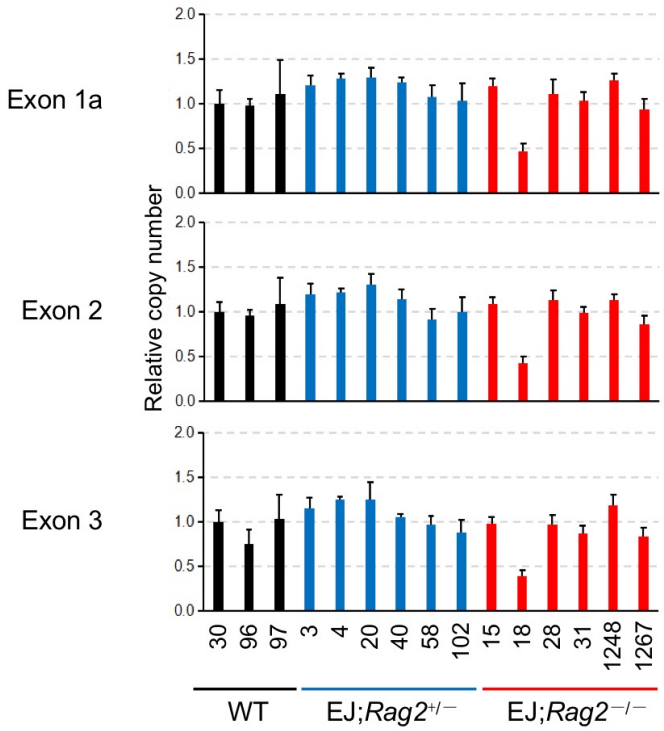
5'-GGTGGCTAACGGGTATCAGA-3'

5'-CTGAGGCCGGATTTAGCTCT-3'

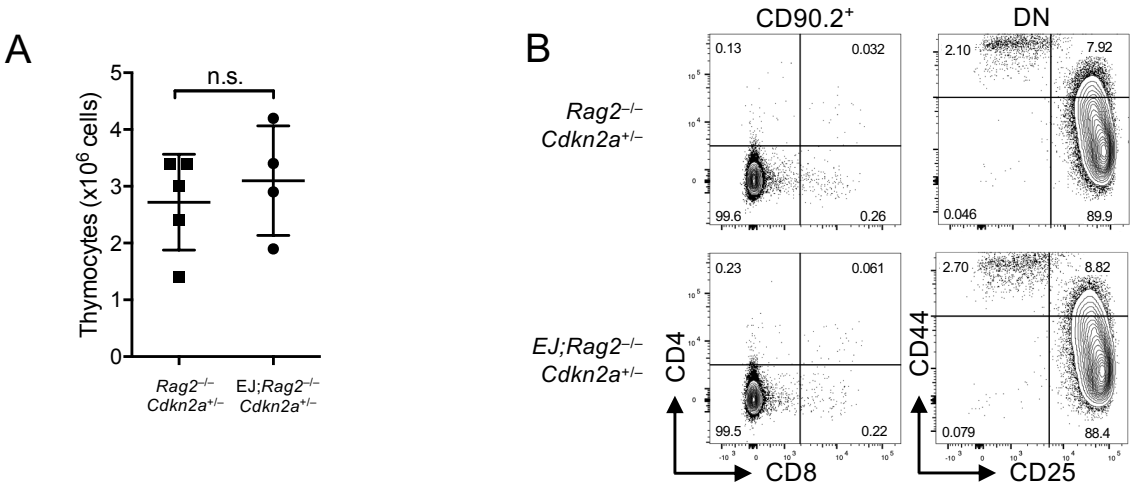
Notch1 **mutation detection**

Genomic DNA from mouse leukemia samples were used for PCR amplification of two segments of *Notch1* exon 34: primer pair 5'-GCTCCCTCATGTACCTCCTG-3' and 5'-TAGTGGCCCCATCATGCTAT-3', generating a predicted amplicon of 904 bp, and

primer pair 5’-ATAGCATGATGGGGCCACTA-3’ and 5’-CTTCACCCTGACCAGGAAAA-3’, generating a predicted amplicon of 893 bp. PCR products were Sanger sequenced using the same primers at i3S Genomics (Porto, Portugal) or CCMAR *Serviços de Biologia Molecular* (Faro, Portugal).



Supplementary Figure E1. *Cdkn2a* loss was found in *Rag2*-deficient T-ALL. Quantitative PCR of the indicated *Cdkn2a* exons in genomic DNA from leukemic cells of ETV6::JAK2;*Rag2*$^{+/-}$ (EJ;*Rag2*$^{+/-}$) and ETV6::JAK2;*Rag2*$^{-/-}$ (EJ;*Rag2*$^{-/-}$) mice and DNA from wild-type (WT) mice. One in 6 *Rag2*-deficient T-ALL cases showed loss of *Cdkn2a* (nº18). Relative copy number was determined by normalization to *Vcpip1* gene (chromosome 1), but similar results were obtained upon normalization to *Spna2* gene (chromosome 2; not shown). Bars represent standard deviation.



Supplementary Figure E2. *Cdkn2a* haploinsufficiency does not rescue the DN3 block caused by *Rag2* deficiency. (A) Thymocyte cellularity of 11-week-old, pre-leukemic *Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ (n=5) and ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ (n=4) mice (each symbol represents one mouse, mean and SD are shown). (B) Representative flow cytometry plots show CD90.2-positive thymocytes (left panels) and CD90.2-positive DN thymocytes (right panels) of the indicated genotype blocked at DN3 stage of differentiation.

Mouse ID	*Cdkn2a* copy number	*Notch1* exon 34	Chromosome alterations
ETV6::JAK2;*Rag2*$^{+/-}$			
3	wt	c.2351, Ins ATTA	n.d.
4	wt	c.2399, Ins CGGG	n.d.
20	wt	wt	n.d.
40	wt	n.d.	n.d.
58	wt	n.d.	n.d.
102	wt	n.d.	n.d.
ETV6::JAK2;*Rag2*$^{-/-}$			
15	wt	wt	+7 +15
18	Loss	c.280,Ins A	+7 +13 +15
28	wt	c.2398, Ins AGGGAT	+15
31	wt	wt	+15
1248	wt	c.2361, Del G, Ins CC	+15
1267	wt	wt	none
ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$			
18	het	c.2361, Del GG, Ins CCC; c.2364 Ins G	n.d.
41	LOH	c.2362, Ins GGG	none
53	LOH	c.2421, Ins GAC, Dup TGAGTGGGGAGCCCAGTCAGGCAGATGTACAAC	+4 +13 +15
69	het	Mut*	+13
75	het	c.2361, Ins CC	n.d.
76	LOH	c.2389, Del CC, Ins GAG	none
99	het	wt	none
128	LOH	wt	+4 +13 +15
143	LOH	wt	n.d.
189	LOH	c.2363, Ins GGCTCGG	n.d.

Supplementary Table E1. Summary of genetic alterations found in leukemic cells of the indicated genotypes. *Notch1* mutations occurred concomitantly with *Cdkn2a* loss in one ETV6::JAK2;*Rag2*$^{-/-}$ T-ALL case (no. 18) and with *Cdkn2a* loss of heterozygosity (LOH) in four ETV6::JAK2;*Rag2*$^{-/-}$;*Cdkn2a*$^{+/-}$ T-ALL cases (nos. 41, 53, 76 and 189). *Mouse no. 69 presented a *Notch1* exon 34 frameshift mutation but the altered sequence was not identified. n.d., not determined.