Cutting Edge: Codeletion of the Ras GTPase-Activating Proteins (RasGAPs) Neurofibromin 1 and p120 RasGAP in T Cells Results in the Development of T Cell Acute Lymphoblastic Leukemia

Beth A. Lubeck,* Philip E. Lapinski,* Jennifer A. Oliver,* Olga Ksionda,† Luis F. Parada,‡ Yuan Zhu,§ Ivan Maillard,¶ Mark Chiang,¶ Jeroen Roose,† and Philip D. King* 

Ras GTPase-activating proteins (RasGAPs) inhibit signal transduction initiated through the Ras small GTP-binding protein. However, which members of the RasGAP family act as negative regulators of T cell responses is not completely understood. In this study, we investigated potential roles for the RasGAPs RASA1 and neurofibromin 1 (NF1) in T cells through the generation and analysis of T cell–specific RASA1 and NF1 double-deficient mice. In contrast to mice lacking either RasGAP alone in T cells, double-deficient mice developed T cell acute lymphoblastic leukemia/lymphoma, which originated at an early point in T cell development and was dependent on activating mutations in the Notch1 gene. These findings highlight RASA1 and NF1 as cotumor suppressors in the T cell lineage. The Journal of Immunology, 2015, 195: 000–000.

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function for RASA1 and NF1 as cotumor suppressors in the T cell lineage.

Materials and Methods

**Mice**

*Rasα<sup>fl/fl</sup> and Nf1<sup>fl/fl</sup> pLck-Cre mice have been described (11, 12). For this study, mice were crossed to generate compound *Rasα<sup>fl/fl</sup> Nf1<sup>fl/fl</sup> pLck-Cre* mice with and without *pLck-Cre*. Mouse genotype was determined by PCR of tail genomic DNA using PCR primers described previously (11, 12). All mice were on a mixed 129S6/Sv background. Mice were euthanized and were recorded as end points in survival studies. All experiments were performed in compliance with University of Michigan guidelines and were approved by the University Committee on the Use and Care of Animals.

**Flow cytometry**

Single-cell suspensions from thymus and spleen, fresh T-ALL and T-ALL cell lines were stained with fluorochrome-labeled CD4 (GK1.5), CD8 (53-6.7) (BD Biosciences), and phospho-ERK1/2 (D13.14.4E) and phospho-AKT (Cell Signaling Technology) mAb as described (11, 12). Cell staining was analyzed by flow cytometry on a FACSCanto (BD Biosciences).

**Tissue staining**

Thymus and spleen were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer sections of tissues were stained with H&E. Sections were viewed on an Olympus BX60 fluorescence microscope.

**Quantitative PCR**

Genomic DNA was isolated from thymus tissue of mice with T-ALL (QiaGen). Efficiency of *Rasα* and *Nf1* gene disruption was determined by quantitative PCR (qPCR) using TaqMan primer/probe sets based in deleted exons (Mm00404879_cn and Mm00351296_cn; Life Technologies) and a 7500 Fast PCR machine (Applied Biosystems). A transferrin receptor primer/probe set was used as an internal control for all samples. The amount of intact wild-type *Rasα* and *Nf1* in T-ALL samples relative to thymi from *Rasα<sup>fl/fl</sup> Nf1<sup>fl/fl</sup> littermates was calculated as described (12).

**Notch mutation analysis**

To identify *Notch1* PEST domain mutations, genomic DNA was used as a template for PCR amplification of exon 34 of the *Notch1* gene (forward, 5'-CTAGTACACATGGCAGCCCGGGG-3'; reverse, 5'-CCGTTCGGCCAGG-CCCTGTTGGG-3'). PCR products were then analyzed by Sanger sequencing. To identify *Notch1* type 1 mutations, genomic DNA was PCR amplified using forward and reverse primers that flank exons 1 and 2 of the *Notch1* gene (forward, 5'-ATGTTGGAATGCCTACTTTGTA-3'; reverse 5'-CGTTTGGTGAGAAGAGATGCTTTAC-3') (13). A 500-bp product is generated only from a recombined *Notch1* allele (Supplemental Fig. 1F, 1G). To identify *Notch1* type 2 mutations, genomic DNA was sequenced by qPCR using primer/probe sets located in exons 23 and 31 (Mm00539165_cn and Mm00539178_cn; Life Technologies) (Supplemental Fig. 1F, 1G). A transferrin receptor primer/probe set was used as an internal control.

**T-ALL cell lines**

To establish T-ALL cell lines, thymocytes from T-ALL mice were cultured in RPMI 1640 with 10% FBS and IL-2 and IL-7 (R&D Systems), both at 10 ng/ml. Cell lines were propagated in the same medium without cytokines. Control murine T-ALL cell lines 55, 98, and 8946 that were not initiated by Ras activation were a gifts of M. Krummel and D. Felsher (14, 15).

**Ras activation**

Five million T-ALL cells were stimulated with 50 ng PMA for 3 min at 37°C before resuspension in lysis buffer containing 1% Nonidet P-40 with 0.5% n-dodecyl-β-D-maltoside. Lysates were rotated with GST-Raf1-RBD-coated agarose beads (Millipore) for 2 h at 4°C that were subsequently washed in lysis buffer. Bound Ras-GTP was detected by Western blotting using a Ras (BD Biosciences). Lysates were rotated with GST-Raf1-RBD-coated agarose beads (Millipore) for 2 h at 4°C that were subsequently washed in lysis buffer. Bound Ras-GTP was detected by Western blotting using a Ras (BD Biosciences) (Cell Signaling Technology) mAb as described (11, 12). Cell staining was analyzed by flow cytometry on a FACSCanto (BD Biosciences).

**Results and Discussion**

We showed previously that non-TCR transgenic *Rasα<sup>fl/fl</sup> pLck-Cre* and *Nf1<sup>fl/fl</sup> pLck-Cre* mice that lack expression of RASA1 or NF1, respectively, in the T cell lineage from the CD4<sup>+</sup>CD8<sup>+</sup> double-negative (DN)3 stage of development onward show only a minor T cell phenotype that is charac-

![FIGURE 1. T-ALL in pLCK-DKO mice. (A) Kaplan–Meier survival curves of *Rasα<sup>fl/fl</sup> Nf1<sup>fl/fl</sup> pLck-Cre* (pLCK-DKO) and littermate *Rasα<sup>fl/fl</sup> Nf1<sup>fl/fl</sup>* (control) mice (pLCK-DKO, n = 55; control, n = 50). (B) H&E-stained thymus and spleen sections of moribund pLCK-DKO mice and age-matched littermate control mice. Note loss of distinction between cortex (C) and medulla (M) in thymus and white (W) and red pulp (R) in spleen of the pLCK-DKO mouse. Scale bars, 400 μm. The same disorganized tissue architecture was observed in all additional examined pLCK-DKO mice (n = 4 total). (C) Two-color flow cytometry plots showing CD4 versus CD8 Ab staining of thymus and spleen from a moribund pLCK-DKO mouse and an age-matched littermate control mouse (6 mo of age). (D) Genomic DNA was prepared from thymocytes from wild-type (WT) mice and seven different moribund pLCK-DKO mice. Relative amounts of intact *Nf1* and *Rasα* genes were determined by qPCR. Values are expressed as a percentage of gene abundance in WT mice.
examples of murine T-ALL (16, 17). To confirm T-ALL, thymocytes from affected mice were adoptively transferred to sublethally irradiated recipients. Four to 8 wk after transfer, recipients became moribund and showed the same high numbers of abnormal T cells in spleen and lymph node (Supplemental Fig. 1E). T-ALL has never been observed in T cell–specific RASA1- or NF1-deficient mice at any age. Furthermore, qPCR analysis of genomic DNA extracted from thymocytes of mice with T-ALL using primer/probe sets located in regions that are excised upon Cre-mediated recombination showed near complete disruption of both Nf1 and Ras1 genes in all examined samples (Fig. 1D). Thus, development of T-ALL requires loss of both RasGAP genes.

Aside from the development of T-ALL, no other compound effects of the deletion of NF1 and RASA1 in T cells were noted. Thus, in preleukemic mice, no significant differences in the number and ratio of thymocyte DN subsets were observed, and although small reductions in the numbers of single-positive (SP) thymocytes and some DP subpopulations were noted in pLCK-DKO mice, these were comparable to those observed in T cell–specific NF1-deficient mice and T cell–specific RASA1-deficient mice reported beforehand (Supplemental Fig. 2A) (11, 12). The ability of peripheral T cells to synthesize cytokines in response to TCR stimulation was also not impaired in preleukemic pLCK-DKO mice (data not shown).

Notch family molecules are type 1 transmembrane receptors that play pivotal roles in cell proliferation, differentiation, and survival, including during early thymocyte development (18). Upon recognition of Delta-like or Jagged ligands, Notch receptors are cleaved in a transmembrane region resulting in release of intracellular Notch (ICN) that translocates to the nucleus where in complex with CSL and Mastermind-like family proteins it activates transcription of Notch target genes. Gain-of-function mutations in the Notch1 gene are found in most cases of human and murine T-ALL, including T-ALL driven by oncogenic mutant forms of Ras that are resistant to the action of RasGAPs (13, 15, 19, 20). Therefore, we examined whether Notch1 mutations were present in T-ALL thymocytes from pLCK-DKO mice. Mutations of murine Notch1 that result in gain-of-function include PEST domain mutations and 5′ type 1 and type 2 deletions (Supplemental Fig. 1F) (13, 21–23). The Notch 1 PEST domain resides at the C terminus of the protein and regulates the stability of ICN. Mutations in the PEST domain include nonsense mutations and insertions or deletions that cause framemshifts and premature stop codons resulting in increased stability of ICN. Type 1 mutations are mediated by RAG proteins and involve deletion of the 5′ proximal promoter and exons 1 and 2 of Notch1. In contrast, type 2 mutations are RAG-independent and involve deletion of DNA upstream of exon 2 through exon 25 or 26. In the former type of deletion, transcription is initiated just 5′ of exon 26, whereas in the latter type of deletion, the site of transcription initiation is unaltered. However, in both types of deletion, translation is initiated from an internal methionine 1727 such that ICN is generated constitutively, independent of Notch1 ligand interaction.

Genetic analysis of T-ALL samples from 19 different pLCK-DKO mice indicated that all harbored type 1 mutations (Supplemental Fig. 1G, Table I). Additionally, 10 of 11 examined T-ALL samples contained PEST domain mutations. The PEST domain mutations were insertions or deletions or both, and in all cases mutations resulted in disruption of reading frame and premature stop codons (Supplemental Fig. 1G, Table I). Analysis of sequence traces indicated that PEST domain mutations were heterozygous and were present in all cells of each T-ALL sample (Supplemental Fig. 1G). Therefore, all T-ALL cells likely arise from a single precursor, that is, are monoclonal. Twelve of 15 examined T-ALL samples also contained heterozygous type 2 Notch mutations that were present in all cells as determined by qPCR (Supplemental Fig. 1G, Table I). Both of these samples also contained PEST domain mutations as well as the type 1 mutation. Because type 1 and type 2 mutations are mutually exclusive, they must be present on homologous chromosomes in these samples.

The occurrence of Notch1 mutations in all examined T-ALL samples from pLCK-DKO mice indicates an essential role for aberrant Notch1 signaling in the development of tumors. Furthermore, because all T-ALL contained type 1 mutations,

![Table I. Notch1 mutations in pLCK-DKO mice with T-ALL](image-url)

N, no; N/A, not applicable; Y, yes.
tumors must arise at the earliest from late DN2/early DN3 thymocytes and at the latest from prepositive selection DP thymocytes that corresponds to the window that RAG is expressed during T cell development (24). Additionally, the pLCK promoter that drives Cre expression is not active until late DN2/early DN3, which places an additional strict limit upon the earliest point of origin of tumors (25). To determine more precisely the population of origin of T-ALL, we examined sorted DN3, DN4, intermediate single-positive, and DP thymocytes from preleukemic pLCK-DKO mice for the presence of NT1 mutations using a qPCR strategy (Supplemental Fig. 2D). However, no NT1 mutations could be detected, indicating either that mutations had not yet occurred or that they were present in <0.8% of cells, which represented the limit of detection in this assay.

As RASA1 and NF1 are both RasGAPs, it is probable that T-ALL in pLCK-DKO mice is consequent to increased or dysregulated Ras signaling in one or more of these early thymocyte subpopulations. This would be consistent with the fact that oncogenic mutant forms of Ras drive T-ALL when expressed in hematopoietic cells and in a synergistic fashion with Notch mutations (4, 5, 13, 15, 19, 20). However, precisely how RasGAP loss and Notch mutation act together to promote T-ALL is uncertain. Small increases in the basal levels of activation of ERK MAPK and AKT (which lies downstream of PI3K) were observed in freshly isolated T-ALL cells from pLCK-DKO mice when compared with thymocytes from age-matched littermate controls (Fig. 2A). In contrast, no consistent increases in the basal or PMA-induced activation of Ras itself, MAPK, or AKT were apparent in T-ALL cell lines developed from pLCK-DKO mice compared with established murine T-ALL cell lines that were not initiated by activation of Ras signaling pathways (Fig. 2B, 2C and data not shown). This last finding indicates that increased Ras activation is unlikely to be necessary for the maintenance of tumors in pLCK-DKO mice, which is consistent with the observation that pharmacological inhibitors of MAPK and PI3K did not affect pLCK-DKO T-ALL cell line survival in vitro (data not shown).

To explore further the mechanism by which RasGAP loss and Notch1 mutation promote T-ALL, we retrovirally transduced ICN into purified DN thymocytes from preleukemic pLCK-DKO mice and controls. In these experiments, no increased expansion of ICN transduced thymocytes in pLCK-DKO cultures compared with control cultures was noted during 15 d of culture (Supplemental Fig. 2B). These findings support a model in which dual RasGAP loss acts prior to Notch mutation to drive T-ALL. Nonetheless, we were unable to detect increases in MAPK activation in early thymocyte populations (DN3, DN4, and DP) from preleukemic pLCK-DKO mice (Supplemental Fig. 2C). It is possible that subtle differences exist and contribute to transformation but are below the detection limit of our assays. However, we cannot at present exclude the alternative explanation that dual RasGAP loss promotes T-ALL as a consequence of dysregulation of distinct signaling pathways in early thymocytes. By whichever mechanism T-ALL develops in pLCK DKO mice, presumably single loss of either RasGAP is not sufficient to promote T-ALL, either because each RasGAP is able to compensate for the loss of the other or because any dysregulated signaling resulting from loss of single RasGAPs is below a threshold necessary to result in transformation.

**FIGURE 2.** Ras activation in pLCK-DKO T-ALL. (A) Flow cytometry plots showing phospho-ERK (pERK) and phospho-AKT (pAKT) staining in a freshly isolated T-ALL sample from a moribund pLCK-DKO mouse compared with DP thymocytes from an age-matched littermate control. Similar results were obtained with two additional freshly isolated T-ALL samples from independent pLCK-DKO mice and controls. (B and C) Three T-ALL cell lines derived from independent pLCK-DKO mice and three control murine T-ALL cell lines were stimulated with PMA for 3 min. (B) Ras activation was determined by Raf1-RBD pull down followed by Western blotting for Ras. L, lysates; P, pull down. (C) ERK and AKT activation was determined by phospho flow cytometry. Numbers to the right of plots indicate mean fluorescence intensity. The same results were obtained in repeat experiments.

In summary, we show in the present study that RASA1 and NF1 act as cotumor suppressors in the T cell lineage that guard against the development of T-ALL. NF1 is a recognized tumor suppressor. In humans, germline mutations of the NF1 gene cause the autosomal dominant disorder neurofibromatosis that is characterized by the development of cutaneous and plexiform neurofibromas and increased susceptibility to other neoplasms, including gliomas, pheochromocytomas, and juvenile chronic myelogenous leukemia (26). In mice, Schwann cell–specific disruption of the NF1 gene also results in the development of plexiform neurofibromas, whereas hematopoietic-specific disruption of the NF1 gene in mice results in the development of myeloproliferative disease (27, 28). In contrast, increased susceptibility to tumors has not previously been reported in either humans or mice with respective RASA1 or Rasa1 mutations (7). In this regard, the findings reported in the present study represent the first example of a function for RASA1 as a tumor suppressor. Which RasGAPs or combinations of RasGAPs regulate Ras activation in peripheral T cells in response to MHC peptide stimulation remains to be determined.

**Disclosures**

The authors have no financial conflicts of interest.
References


