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Cutting Edge

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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.**

Ras is a small membrane-tethered GTP-binding protein that triggers activation of the MAPK and PI3K signaling pathways downstream of growth factor receptors in numerous cell types (1). In the T cell lineage, Ras signaling is essential for T cell development through the TCRβ and positive selection checkpoints and is required for T cell activation and differentiation in the periphery (2, 3). In contrast, excessive Ras signaling can result in T cell acute lymphoblastic leukemia/lymphoma (T-ALL) (4, 5). Ras cycles between inactive GDP-bound and active GTP-bound states. Ras guanine nucleotide exchange factors are recruited by growth factor receptors to membranes where they activate Ras by ejecting GDP from the Ras guanine nucleotide binding pocket, thereby permitting Ras to bind GTP (6). In T cells, Ras guanine nucleotide releasing protein 1 and mammalian son-of-sevenless have been defined as the most important Ras guanine nucleotide exchange factors (2, 3). Ras inactivation requires interaction with Ras GTPase-activating proteins (RasGAPs) that increase the ability of Ras to hydrolyze bound GTP by several orders of magnitude (7). Ten different RasGAPs have been identified in mammals. However, which RasGAPs function as regulators of Ras in the T cell compartment has remained unclear.

p120 RasGAP, also known as RASA1, and neurofibromin 1 (NF1) are two prototypical RasGAPs, both of which are expressed in T cells (7). Nonconditional gene knockout mice lacking expression of either RASA1 or NF1 die in midgestation as a result of abnormal cardiovascular development (8–10). Therefore, to investigate the roles of RASA1 and NF1 in the T cell compartment, we had previously generated T cell–specific NF1- and RASA1-deficient mice (11, 12). RASA1 and NF1 were found to be largely dispensable for normal T cell development in non-TCR transgenic mice, although subtle alterations in the efficiency of thymocyte positive selection were apparent on TCR transgenic backgrounds. Additionally, both RasGAPs were found to be dispensable as regulators of peripheral T cell activation induced by MHC peptides. However, it is conceivable that RASA1 and NF1 act as coregulators of Ras in the T cell lineage such that overt phenotypes would only become apparent when both RasGAPs are absent. For instance, in cardiovascular development, an overlapping function for RASA1 and NF1 was indicated by the finding that mice lacking both RasGAPs show more severe cardiovascular abnormalities and die at an earlier time point in gestation than do mice lacking either RasGAP alone (9). Therefore, to investigate whether RASA1 and NF1 have an overlapping function in T cells, we generated T cell–specific double RASA1- and NF1-deficient mice. These mice developed T-ALL, thus revealing a critical

*Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109; †Department of Anatomy, University of California, San Francisco, San Francisco, CA 94143; ‡Department of Developmental Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390; §Division of Molecular Medicine and Genetics, University of Michigan Medical School, Ann Arbor, MI 48109; and ¶Division of Hematology and Oncology, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109

Address correspondence and reprint requests to Dr. Philip D. King, Department of Microbiology and Immunology, University of Michigan Medical School, 6606 Medical Science II, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5620. E-mail address: kingp@umich.edu

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Abbreviations used in this article: DKO, double knockout; DN, double-negative; DP, double-positive; ICN, intracellular Notch; NF1, neurofibromin 1; qPCR, quantitative PCR; RASA1, p120 Ras GTPase-activating protein; RasGAP, Ras GTPase-activating protein; T-ALL, T cell acute lymphoblastic leukemia/lymphoma.

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

Materials and Methods

Mice

Rasa1$^{fl/fl}$ and *Nf1*$^{fl/fl}$ *pLck-Cre* mice have been described (11, 12). For this study, mice were crossed to generate compound *Rasa1*$^{fl/fl}$ *Nf1*$^{fl/fl}$ 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 × C57BL/6 background. Moribund 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 S473 (D9E) (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 IX70 fluorescence microscope.

Quantitative PCR

Genomic DNA was isolated from thymus tissue of mice with T-ALL (Qiagen). Efficiency of *Rasa1* 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 *Rasa1* and *Nf1* in T-ALL samples relative to thymi from *Rasa1*$^{fl/fl}$*Nf1*$^{fl/fl}$ littermates was calculated as described (12).

Notch mutation analysis

To identify *Notch1* PEST domain mutations, thymus genomic DNA was used as a template for PCR amplification of exon 34 of the *Notch1* gene (forward, 5′-TGAGTACCAATGGCACGGGGG-3′; reverse, 5′-CCGTTCCCAAG-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′-ATGGTGGAATGCCTACTTTGTA-3′; reverse 5′-CGTTTGGGTAGAAGAGATGCTTTAC-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 screened 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 Ab (RAS10; Millipore).

Results and Discussion

We showed previously that non-TCR transgenic *Rasa1*$^{fl/fl}$ *pLck-Cre* and *Nf1*$^{fl/fl}$ *pLck-Cre* mice that lack expression of RASA1 or NF1, respectively, in the T cell lineage from the CD4$^-$CD8$^-$ double-negative (DN)3 stage of development onward show only a minor T cell phenotype that is characterized by small reductions in the numbers of CD4$^+$CD8$^+$ double-positive (DP) thymocytes and peripheral naive T cells (11, 12). To examine the effect of codeletion of RASA1 and NF1 in T cells, we generated *Rasa1*$^{fl/fl}$ *Nf1*$^{fl/fl}$ *pLck-Cre* mice, hereafter referred to as pLCK–double knockout (DKO) mice. Compared to control *Rasa1*$^{fl/fl}$ *Nf1*$^{fl/fl}$ littermate mice, pLCK-DKO mice showed early lethality that was first evident at 3 mo of age and affected 60% of mice by 20 mo of age (Fig. 1A). Postmortem analysis of deceased or euthanized moribund mice consistently revealed a grossly enlarged thymus and, in nearly all cases, enlarged peripheral lymphoid organs and liver (Supplemental Fig. 1A). Upon histological analysis, lymphoid organs were seen to comprise almost entirely lymphocytes and showed a loss of normal architecture (Fig. 1B). In liver, massive perivascular accumulations of lymphocytes were identified (Supplemental Fig. 1B). Higher power images of enlarged spleens showed a starry sky appearance consistent with T-ALL (Supplemental Fig. 1C).

As determined by flow cytometric analysis, essentially all thymocytes from affected pLCK-DKO mice were CD8$^+$ and expressed variable levels of CD4 (CD4^{var}; Fig. 1C). T cells with the same CD8$^+$CD4^{var} cell surface phenotype were found to comprise the vast majority of cells in enlarged peripheral lymphoid organs, and high numbers of T cells were also found in peripheral blood (Fig. 1C and data not shown). In lymphoid organs, the abnormal T cells expressed high levels of CD25 but were typically negative for CD44 and expressed low levels of TCRβ (Supplemental Fig. 1D). A similar cell surface phenotype has been reported in other

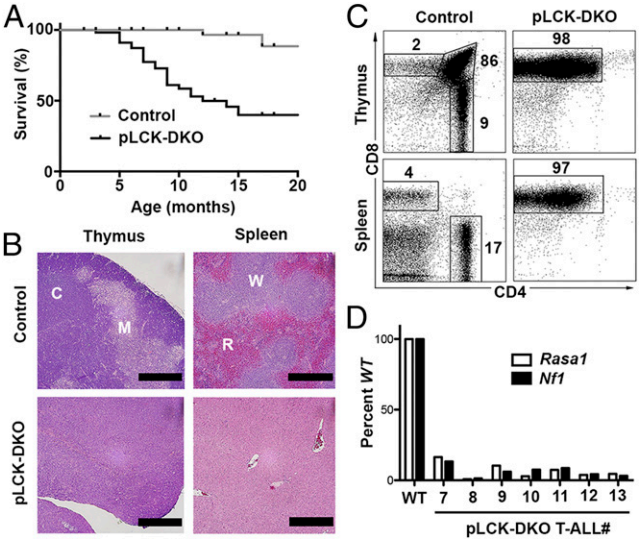

FIGURE 1. T-ALL in pLCK-DKO mice. (**A**) Kaplan–Meier survival curves of *Rasa1*$^{fl/fl}$ *Nf1*$^{fl/fl}$ *pLck-Cre* (pLCK-DKO) and littermate *Rasa1*$^{fl/fl}$ *Nf1*$^{fl/fl}$ (control) mice (pLCK-DKO, $n = 55$; control, $n = 50$). (**B**) H&E-stained thymus and spleen sections of moribund pLCK-DKO 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 *Rasa1* 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 *Rasa1* 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 frameshifts 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. Two 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

T-ALL	NT1	NT2	PEST Mutation	PEST Amino Acid
1	Y	N	c.7193_7194insAGAATAATA	pSer2397fsX3
2	Y	N	c.7194_7195insAGGG	p.Ala2399ArgfsX11
3	Y	N	c.7080_7081insGG c.7115_7118delCAGG	p.Leu2362GlyfsX128
4	Y	N	ND	ND
5	Y	Y	c.7051_7052insAA	p.Pro2351fsX39
6	Y	ND	ND	ND
7	Y	ND	c.7081_7082delG,insACC	p.Arg2361HisfsX35
8	Y	N	ND	ND
9	Y	N	ND	ND
10	Y	N	ND	ND
11	Y	N	c.7081_7082insAGGGGCCC	p.Arg2361GlnfsX129
12	Y	Y	c.7193_7194insCC	p.Ser2397fsX12
13	Y	N	ND	ND
14	Y	ND	ND	ND
15	Y	N	c.7081_7082delG,insCCC	p.Arg2361ProfsX36
16	Y	N	N	N/A
17	Y	N	c.7193_7194insAGGG	p.Ala2399ArgfsX92
18	Y	N	c.7081_7082insCC	p.Arg2361ProfsX36
19	Y	N	ND	ND

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.

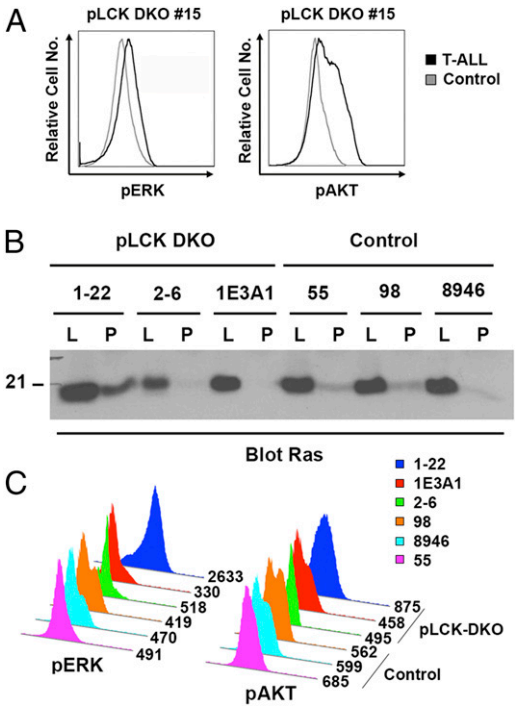

FIGURE 2. Ras activation in pLCK-DKO T-ALL. (**A**) Flow cytometry plots showing phospho-ERK (pERK) and phospho-AKT (pAKT) staining in a freshly isolated T-ALL sample from a moribund pLCK-DKO mouse compared with DP thymocytes from an age-matched littermate control. Similar results were obtained with 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 *Nf1* 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.

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