Immunodeficiencies and autoimmunity

Clinical

Research Article

Dysregulated RASGRP1 expression through RUNX1 mediated transcription promotes autoimmunity

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RasGRP1 is a Ras guanine nucleotide exchange factor, and an essential regulator of lymphocyte receptor signaling. In mice, *Rasgrp1* deletion results in defective T lymphocyte development. *RASGRP1*-deficient patients suffer from immune deficiency, and the *RASGRP1* gene has been linked to autoimmunity. However, how RasGRP1 levels are regulated, and if RasGRP1 dosage alterations contribute to autoimmunity remains unknown. We demonstrate that diminished Rasgrp1 expression caused defective T lymphocyte selection in C57BL/6 mice, and that the severity of inflammatory disease inversely correlates with Rasgrp1 expression levels. In patients with autoimmunity, active inflammation correlated with decreased *RASGRP1* levels in CD4+ T cells. By analyzing H3K27 acetylation profiles in human T cells, we identified a *RASGRP1* enhancer that harbors autoimmunity-associated SNPs. CRISPR-Cas9 disruption of this enhancer caused lower RasGRP1 expression, and decreased binding of RUNX1 and CBFB transcription factors. Analyzing patients with autoimmunity, we detected reduced RUNX1 expression in CD4+ T cells. Lastly, we mechanistically link RUNX1 to transcriptional regulation of *RASGRP1* to reveal a key circuit regulating RasGRP1 expression, which is vital to prevent inflammatory disease.

💻 Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Introduction

RasGRP1, a Ras guanine nucleotide exchange factor (RasGEF), is a key protein regulating effector kinases upon TCR signaling

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[1]. RasGRP1 expression levels regulate the output of lymphocyte receptor signaling in a dose-dependent manner [2,3]. Complete lack of *Rasgrp1* in mice results in a defect in positive selection of thymocytes (developing T cells), and immune deficiency, both in WT mice and mice expressing a transgenic TCR [4,5]. Recent studies described *RASGRP1* loss in patients with immune deficiencies [6,7], and RasGRP1 loss-of-function in autoimmune lymphoproliferative syndrome (ALPS) [8].

Dysregulation of RasGRP1 expression levels has been suggested to play a role as well in leukemia and autoimmunity. Increased expression of Rasgrp1 has been detected in murine models and patients with T-ALL [9,10], and some studies demonstrated aberrant expression levels of RasGRP1 in patients with autoimmunity. For example, patients with systemic lupus erythematosus with splice variants of RasGRP1 expressed decreased RasGRP1 protein levels [11], while in rheumatoid arthritis increased mRNA and a contrasting decrease in RasGRP1 protein was shown in total T cells [12]. Furthermore, single nucleotide polymorphisms (SNPs) in the *RASGRP1* locus have been linked to autoimmunity [13–15]. For instance, SNPs in coding regions have been shown to affect lymphocyte receptor signaling in cell lines [2], and in mice [16]. It has not been established if noncoding SNPs in *RasGRP1* affect its expression levels. In sum, it is unknown how RasGRP1 expression is regulated in T cells and if RasGRP1 dosage alterations may impact T cell function and immunological health. We set out to mechanistically understand how RasGRP1 expression levels are regulated and investigate if aberrant expression of RasGRP1 may contribute to inflammatory disease.

Results

Reduced RasGRP1 protein expression caused aberrant positive thymocyte selection

To test whether decreased Rasgrp1 expression levels can in principal cause autoimmunity, we assessed immune-phenotypes of WT (+/+), *Rasgrp1*-heterozygous (+/−), and *Rasgrp1*-KO (−/−) mice. All mice were on a C57BL/6 background, which is not prone to develop autoimmunity [17]. Thymocytes of *Rasgrp1* heterozygous mice express approximately half of normal Rasgrp1 protein levels (Fig. 1A). Analysis of stages of thymocyte development (Supporting Information Fig. S1A) by flow cytometry showed that aberrant Rasgrp1 expression impaired positive thymocyte selection. As reported previously [18], we observed that *Rasgrp1*$^{-/-}$ mice displayed an accumulation of CD44$^-$CD25$^+$ double negative (DN3) thymocytes, indicating defective β-selection. By contrast, *Rasgrp1*$^{+/-}$ mice did not show accumulation of this early T cell progenitor population directly prior to β-selection (Supporting Information Fig. S1B and D). The positive selection process shapes the TCR repertoire as assembled, mature TCRs are functionally tested in this selection process [19] and alterations in the repertoire can lead to self-recognition in the periphery and autoimmune diseases. We observed that intact Rasgrp1 expression is critical for positive selection; the numbers of TCRβ$^+$CD69$^+$ double positive (DP) thymocytes and CD4$^+$ SP cells were decreased in *Rasgrp1*$^{+/-}$ mice (Supporting Information Fig. S1C and E). As expected and reported [18], without any Rasgrp1 expression (in
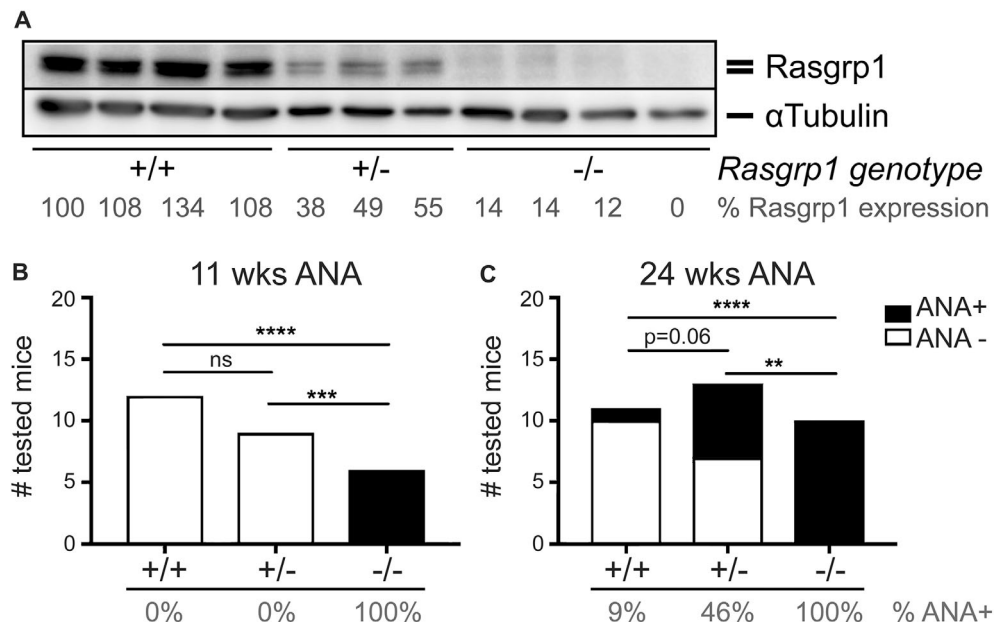

Figure 1. Limited RasGRP1 expression results in dose-dependent autoimmunity in mice. (A) Western blot was performed to analyze RasGRP1, and α-tubulin (loading control) protein levels expressed by thymocytes of 11 weeks old mice with different genotypes: +/+ (WT, N = 4), +/− (*Rasgrp1* heterozygote, N = 3), −/− (*Rasgrp1* KO, N = 4), 1 experiment. (B and C) Anti-nuclear antibody presence (ANA) was determined by Hep2 assays in sera isolated from mice at 11 weeks old (B, N = 12, N = 9, N = 6), and 24 weeks old (C, N = 11, N = 13, N = 10), 2 separate experiments. Bars depict total numbers of mice tested positive (black), and negative (white), percentages of ANA positive samples are indicated. Fisher's exact test was used. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.
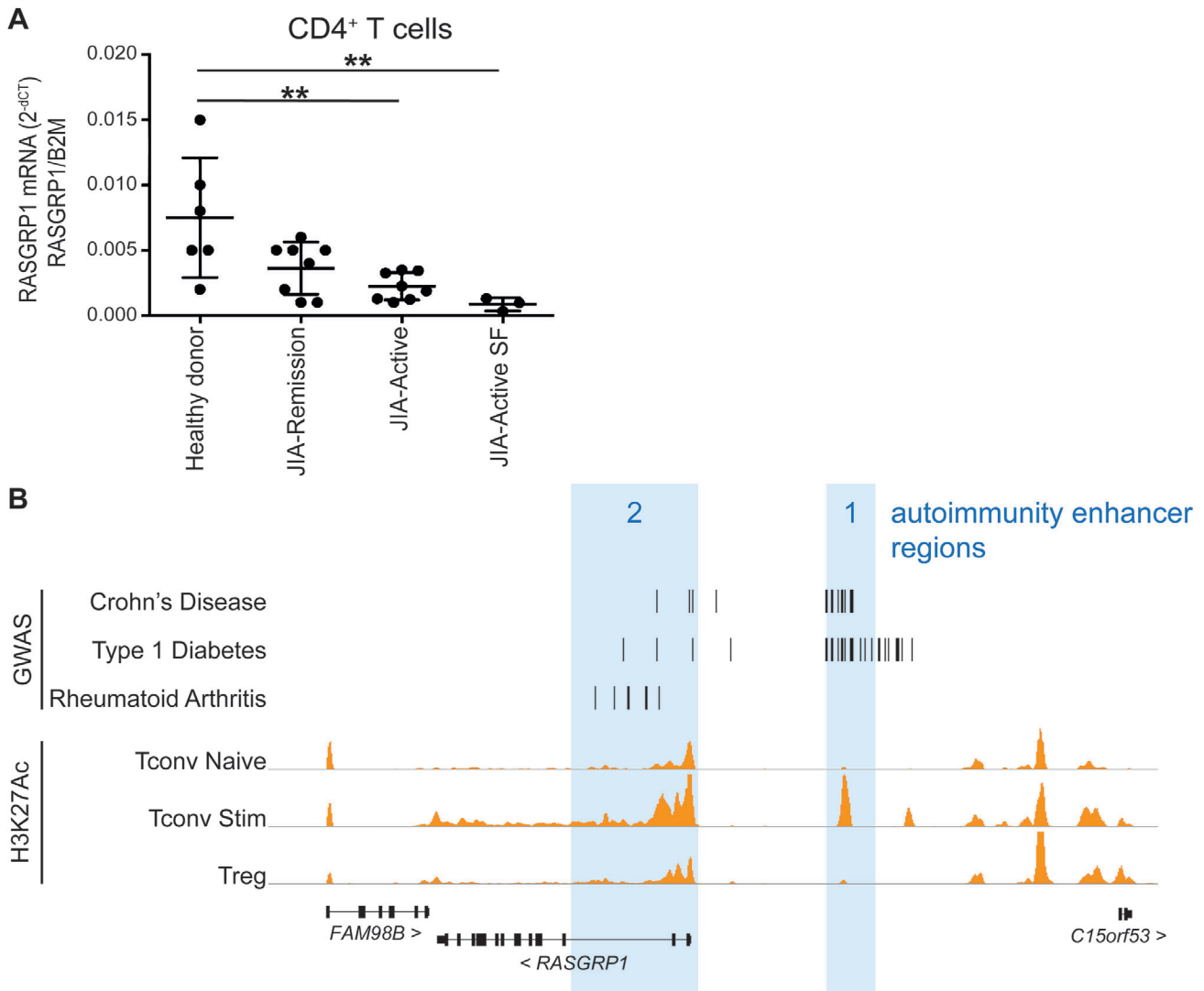
Figure 2. RASGRP1 levels and expression regulation are associated with human autoimmunity. (A) Displayed are qPCR analyses of *RASGRP1* mRNA levels, normalized to B2M in CD4$^+$ T cells that were isolated from juvenile idiopathic arthritis patients disease remission blood (JIA-Remission, $N = 8$, in three experiments of $N = 3$ or $N = 2$), active inflammatory disease blood (JIA-Active, $N = 8$, in three experiments of $N = 3$ or $N = 2$), or synovial fluid (JIA-Active synovial fluid, $N = 3$, one experiment), and healthy adult donors blood ($N = 6$, in two experiments of $N = 3$). Shown are averages $\pm$ SD. 2^{-dCT} values of *RASGRP1* corrected for *B2M*. One-way ANOVA, with Holm-Sidak's multiple comparisons test was used for statistical analysis, $^*p < 0.05$, $^{**}p < 0.01$. (B) *RASGRP1* locus showing fine mapped disease-associated SNPs for Crohn's disease (CD) [14], Type 1 diabetes (T1D) [15], and rheumatoid arthritis (RA) [13]. The GWAS data were overlapped with histone 3 lysine 27 acetylation (H3K27Ac) tracks for different T cell types (Roadmap Epigenomics Project [44]): Naïve conventional T cells (Tconv Naïve), anti-CD3/anti-CD28 stimulated conventional T cells (Tconv Stim), and CD4$^+$FOXP3$^+$ regulatory T cells (Treg). 2 regions with H3K27 signal and colocalizing autoimmune SNPs are highlighted as autoimmunity-associated enhancer regions.

Rasgrp1$^{-/-}$ mice), these two populations were nearly absent (Supporting Information Fig. S1C and E). These results showed an inverse effect of RasGRP1 expression on thymic positive selection; half the RasGRP1 protein dosage already resulted in less efficient positive selection.

Reduced RasGRP1 expression resulted in spontaneous antinuclear–antibody production

Next, we analyzed serum for the presence of antinuclear antibodies (ANAs) suggesting inflammation, which could indicate autoimmune features (Supporting Information Fig. S1F). In 11-week-old mice, we observed that absence of Rasgrp1 led to ANA production, while *Rasgrp1$^{+/-}$* and WT mice produced no ANA at all (Fig. 1B). These results agreed with earlier studies performed in *Rasgrp1$^{-/-}$* mice [20, 21]. However, by age 24 weeks we also detected serum ANA in approximately half of the heterozygous *Rasgrp1$^{+/-}$* mice, while only one of 11 WT mice were ANA positive (Fig. 1C). Together, decreased expression of Rasgrp1 led to defective positive thymocyte selection and a significant increase of total ANA production, suggesting an increased risk of development of autoimmunity in C57BL/6 mice over time.
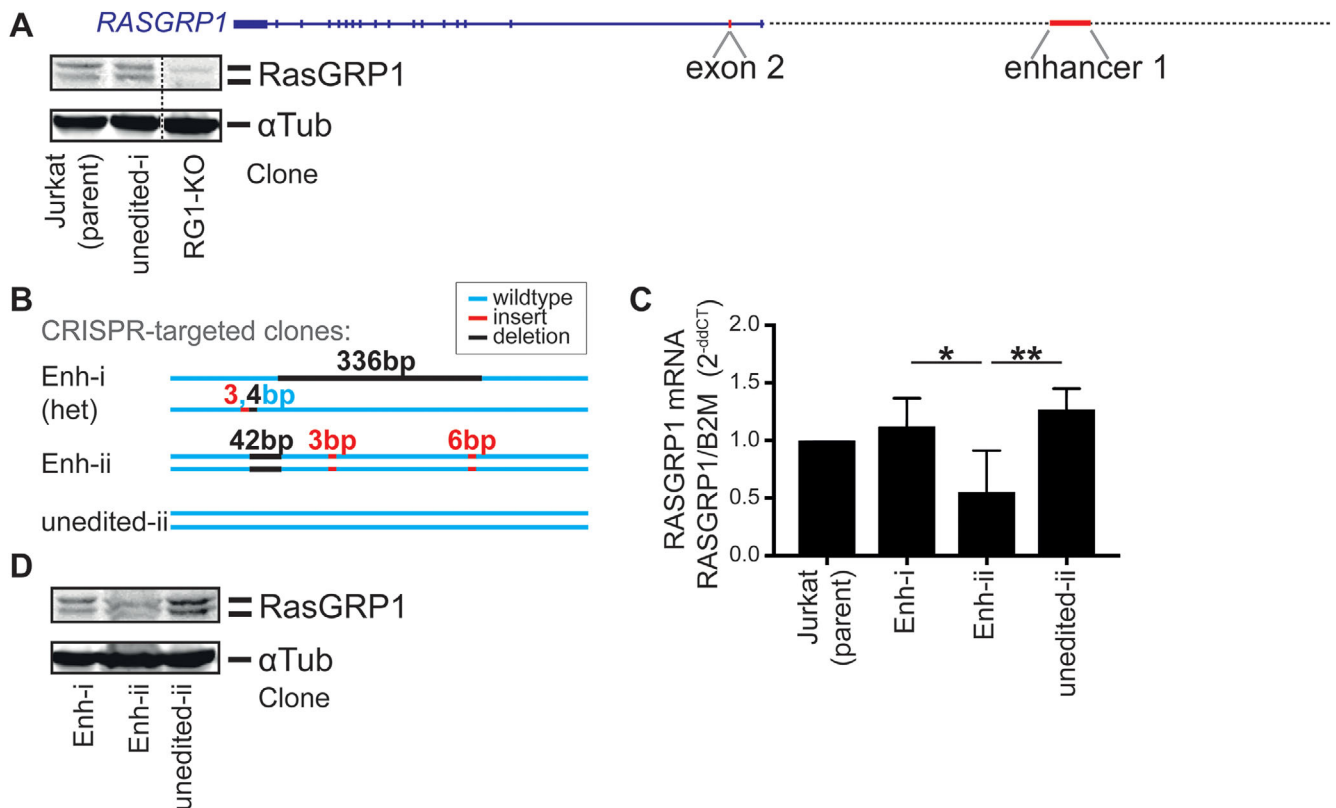
Figure 3. Autoimmunity-associated enhancer 1 regulates *RASGRP1* expression. (A) CRISPR-Cas9 ribonucleoprotein editing of exon 2 was performed in Jurkat cells. Displayed is a western blot showing RasGRP1 and α-tubulin (loading control), parental Jurkat cells, one non-edited clone (unedited-i), and one *RASGRP1*-KO clone (RG1-KO), 3 separate experiments. (B) CRISPR-Cas9 targeting of autoimmunity-associated enhancer 1 was performed in Jurkat cells. Displayed are the sequences of two mutants and one unedited clone, showing the WT sequence (blue), deletions (black), and insertions (red). Het = heterozygous. (C and D) RASGRP1 expression levels were analyzed in parental Jurkat cells, unedited controls, and Enh1 targeted clones. (C) *RASGRP1* mRNA expression, normalized to β2m, determined by qPCR ratio of *RASGRP1/B2M*, relative to parent Jurkat cells: 2^{-ddCT}. Shown are four samples averages ± SD. Two separate timepoints of RNA extractions, and duplicates were run per experiment. One-way ANOVA statistical test was performed, and significance is indicated: $^*p < 0.05$, $^{**}p < 0.01$. (D) Western blot was performed, showing RasGRP1, and α-tubulin (loading control) expression, three separate experiments.

RASGRP1 mRNA levels were decreased in patient T cells during active autoimmune inflammation

Based on these in vivo findings in C57BL/6 mice, we hypothesized that RasGRP1 expression could be dysregulated in human autoimmunity. Previous reports showed that peripheral blood total T cells (Both CD4^{+} and CD8^{+}) from patients with rheumatoid arthritis (RA) display higher *RASGRP1* mRNA levels than healthy donors, and the expression levels of *RASGRP1* inversely correlate with disease activity scores [12]. We set out to explore *RASGRP1* expression in patients with oligoarticular juvenile idiopathic arthritis (JIA), a relapsing/remitting form of autoimmunity in children. In mice, reduced rasgrp1 expression led to ANA production and reduced numbers of CD4^{+} SP thymocytes, suggesting a role for CD4^{+} T cells. Therefore, we analyzed *RASGRP1* expression in peripheral blood CD4^{+} T cells from patients who were either in remission or showed active inflammation, and compared this to healthy controls. Furthermore, we analyzed CD4^{+} T cells from the site of active inflammation; synovial fluid. We observed that *RASGRP1* mRNA expression was significantly decreased

in CD4^{+} T cells from patients with active autoimmune disease compared to healthy controls, and this was most pronounced in synovial fluid at the site of active inflammation (Fig. 2A). A possibly bimodal distribution is observed in the JIA patients in remission, it remains to be determined whether this is related to specific disease-course or SNPs in these patients. These results imply that there is active maintenance of *RASGRP1* expression levels in CD4^{+} T cells with instances of decreased expression of *RASGRP1* mRNA levels specifically under autoimmune inflammatory conditions, and motivated us to next investigate regulation of *RASGRP1* mRNA expression.

The *RASGRP1* locus contains regulatory enhancers with autoimmunity-associated SNPs

To identify noncoding elements that could affect *RASGRP1* gene expression in the context of autoimmunity, we analyzed the locations of candidate causal non-coding SNPs associated with autoimmunity. We found two distinct regions with clusters

of candidate causal SNPs associated with Crohn's disease, Type 1 Diabetes, and RA. One region was positioned upstream of the *RASGRP1* promoter, suggesting the presence of regulatory elements relevant to autoimmunity (Fig. 2B; Supporting Information Fig. S2B). Next, we analyzed histone 3 lysine 27 acetylation (H3K27ac), indicating transcriptional enhancers. Enhancers are essential to tune gene regulation, and contribute to cell identity and function in health and disease [22,23]. In human CD4+ T cells, the H3K27ac signal on *RASGRP1* elements was more pronounced in activated/stimulated T cells, and co-localized with the SNP clusters (Fig. 2B). Notably, we identified a prominent, single H3K27ac peak marking a putative enhancer (autoimmunity-associated enhancer 1), with a high local concentration of autoimmunity-associated SNPs. Another, larger, region contained multiple H3K27ac peaks (autoimmunity-associated enhancer 2). These data suggest that *RASGRP1* expression in CD4+ T cells is regulated by enhancers and that SNPs in these elements could contribute to RASGRP1 dysregulation and autoimmunity.

Autoimmunity-associated enhancer 1 regulates *RASGRP1* expression

To test whether the putative autoimmunity-associated enhancer 1 with the highest concentration of autoimmunity-associated SNPs indeed regulates RASGRP1 expression, we perturbed this non-coding element with CRISPR-Cas9 editing in the Jurkat T cell lymphoma line [24]. First, we transfected cells with Cas9-ribonucleoprotein complexes targeting exon 2 to generate a *RASGRP1*-KO clone with loss of RasGRP1 protein (Fig. 3A; Supporting Information Fig. S3A and B). An unedited clone (unedited-i) was used as control. Having established this platform, we targeted autoimmunity-associated enhancer 1 (Supporting Information Fig. S3A). Analysis of selected single cell clones revealed two clones with deletions in different regions (Enh-i and Enh-ii) that we selected as well as an unedited clone (unedited-ii) as additional control (Fig. 3B; Supporting Information Fig. S3A and B). Of note, none of the clones displayed additional mutations in the *RASGRP1* gene exons or untranslated regions (Supporting Information Table S1). qPCR analysis revealed reduced levels of *RASGRP1* mRNA expression with a concomitant reduction of RasGRP1 protein levels in clone Enh-ii, but not in clone Enh-i (Fig. 3C and D).

Next, we capitalized on a previously optimized platform to analyze RasGRP1 signaling through flow cytometry analyses of phosphorylated ERK a kinase in the canonical Ras-MAPK pathway [2] (Phospho-flow). Phospho-flow allows quantitative detection of defects in ERK signaling such as in JPRM441, a Jurkat line expressing low levels of endogenous RasGRP1 [25]. Phospho-flow analyses revealed that parental Jurkat cells as well as the unedited control clones, and bulk Jurkat cells transfected with scrambled gRNA, displayed robust induction of phosphorylated ERK (P-ERK) levels upon stimulation (Supporting Information Fig. S3E).The *RASGRP1*-knockout clone demonstrated significantly impaired

induction of P-ERK, while JPRM441 showed decreased P-ERK induction, however not significantly in this statistical comparison of a large number of groups (Supporting Information Fig. S3C–E). Even though RasGRP1 expression was decreased in clone Enh-ii, the clone showed no significant decrease of induced P-ERK, which might be caused by changed "rewired" signal transduction in these clones when maintained in culture for a longer period of time.

Transcription factor RUNX1 binds and regulates *RASGRP1* autoimmunity-associated enhancer 1

We aimed to mechanistically understand how this enhancer region is impacting *RASGRP1* expression and we postulated that factors binding to the *RASGRP1* enhancer may be distinct in clones Enh-i and Enh-ii. To identify transcription factors regulating *RASGRP1* expression by binding autoimmunity-associated enhancer 1, we performed AP-MS (affinity purification mass spectrometry) analysis [26]. The scrambled sequence of the 42 base pairs (Supporting Information Fig. S4A) that were deleted in clone Enh-ii, resulted in a loss of binding of CBFB and RUNX1, which together form a transcriptional heterodimer, as well as loss of binding of several ZBTB family members (Fig. 4A). We focused on RUNX1 because this factor has been identified as an important transcriptional regulator in hematopoiesis [27], T cell development [28], and during T cell responses [29]. Mutations in RUNX1 have been described to play a role in T-ALL [30]. Furthermore, variants in RUNX1 have been associated with autoimmunity [31,32], and RUNX1 binding sites have been associated with super-enhancers in JIA [33].

To demonstrate the relevance of RUNX1 in CD4+ T cells in autoimmunity, we analyzed *RUNX1* expression levels in patients with JIA. We show a decreased *RUNX1* expression in all JIA patients compared to healthy donors that show variable expression. Again, decreased Runx1 expression was most pronounced in patients with active disease, in the synovial fluid, similar to *RASGRP1* expression (Fig. 4B and C). Upon short hairpin RNA-mediated knockdown of *RUNX1* in Jurkat cells (35% knockdown; Fig. 4D; Supporting Information Fig. S4B–D), a clear reduction of *RASGRP1* mRNA expression occurred (Fig. 4E). These data provide a novel link between RUNX1 and RasGRP1, and a mechanistic explanation for the reduced RasGRP1 expression in clone Enh-ii, because RUNX1 can no longer bind to the regulatory enhancer element that is key for *RASGRP1* transcription.

Discussion

We show for the first time that decreased expression levels of Rasgrp1 induce autoimmunity in mice through a reduction of positive thymocyte selection, and that decreased RASGRP1 in CD4+ T cells is specifically detected under circumstances of active autoimmunity in patients. In mice, reduced expression of RASGRP1 mildly disturbs T cell development, and results in a delayed onset of disease compared to complete deletion of RASGRP1. We detected

A

B

CD4+ T cells

C

CD4+ T cells

D

E
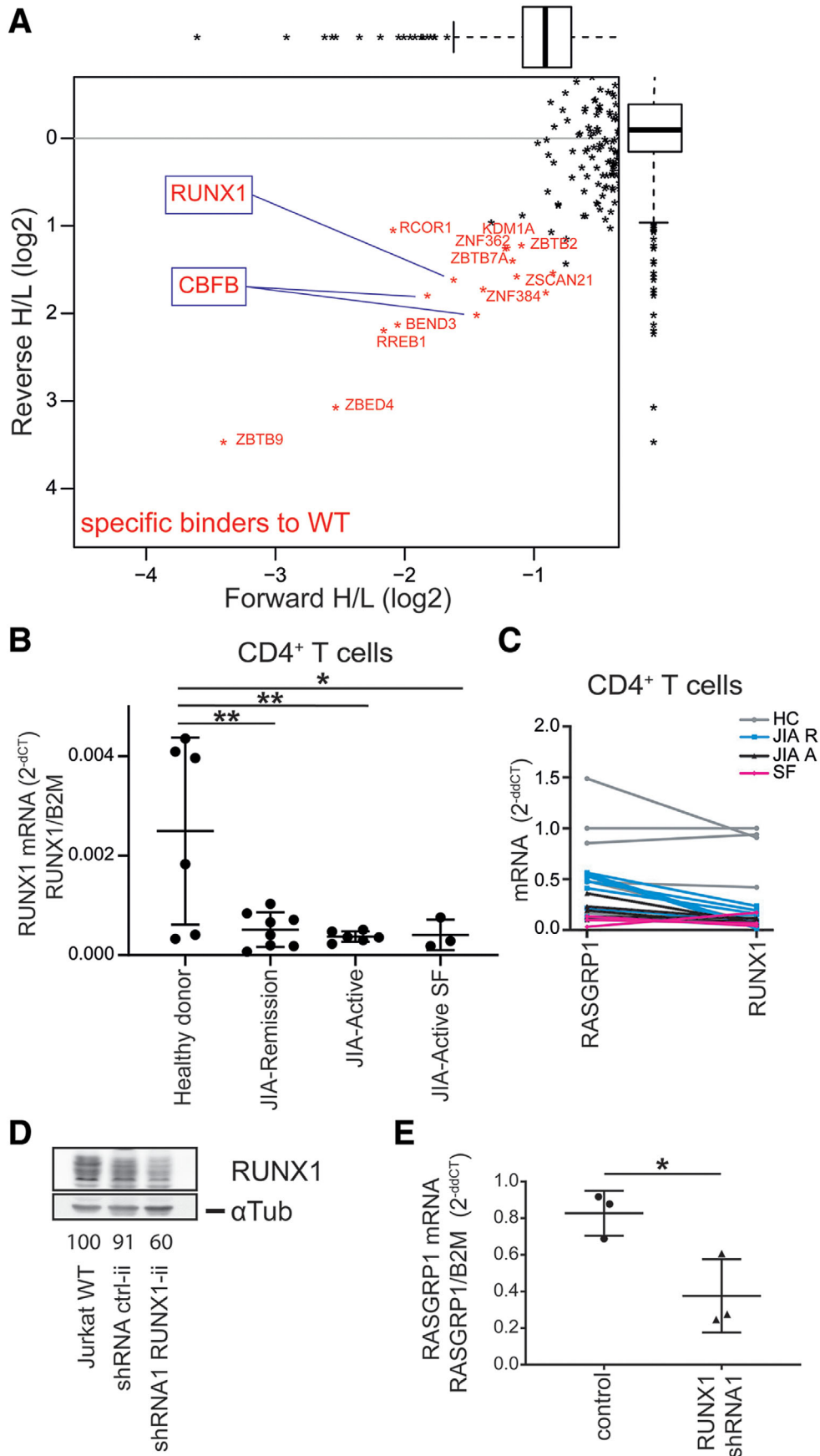
reduced levels of RASGRP1 in peripheral CD4+ T cells in patients, specifically in the synovial fluid at the site of active inflammation, suggesting that dysregulation of RASGRP1 expression occurs in activated T cells. In line with this, we identified a *RASGRP1* enhancer containing a cluster of autoimmunity-risk SNPs, that showed a higher H3K27ac signal in previously activated CD4+ memory T cells. While a study in RA patients confirmed reduced expression of RASGRP1 mRNA in patients with increased disease scores, the total RA patient population showed elevated RASGRP1 compared to healthy donors [12]. Analysis of the total T cell population may have caused this discrepancy with our study, which focuses on CD4+ T cells in patients.

Mechanistically, we show that transcription factor RUNX1 is essential for regulation of RASGRP1 expression levels. RUNX1 has been identified as an important transcription factor in hematopoiesis and T cell development, and mutations in RUNX1 have been described to play a role in T-ALL [30]. To date, RUNX1 has not been linked to RASGRP1 transcriptional regulation, and the role of RUNX1 in autoimmunity remained ambiguous. For example, in patients with active immune thrombocytopenia RUNX1 mRNA expression was elevated [34], and RUNX1 has been suggested to promote maturation of CD4+ T cells in mice [35]. By contrast, another study showed that RUNX1 deletion in mice led to autoimmune lung disease [36]. Here, we provide evidence that decreased expression of RUNX1 leads to reduced RAS-GRP1 transcription, suggesting a role for RUNX1 in fine-tuning Ras-MAPK signaling in both developing T cells, and peripheral CD4+ T cells.

In conclusion, RasGRP1 expression regulates TCR-induced signaling and thymocyte selection in a dose-dependent manner. Tight regulation of RasGRP1 expression is vital to maintain immunological health and prevent diseases such as leukemia, autoimmunity and immune deficiency (Fig. 5).

Materials and methods

Patients and healthy donors

To investigate the differences in expression levels during disease course, patients with oligoarticular JIA (oJIA) were selected of whom at least 30×10^6 PBMCs from both active disease as well as remission were available ($N = 8$, Fig. S2A). In addition, three unpaired samples of oJIA SFMCs were selected. Healthy adult volunteers ($n = 6$) derived PBMCs, collected via the UMCU facility, served as a control group.

Mice

C57BL/6 mice WT, Rasgrp1$^{-/-}$ (provided by J. Stone), and Rasgrp1$^{+/-}$ were bred in house at UCSF.

Study approval

This study was conducted according to the principles expressed in the Declaration of Helsinki, and was approved by the UMCU medical ethical committee, study Pharmachild, number 11–499/C.

All donors provided written informed consent prior to inclusion in this study for sample collection and analysis, and all donors were numbered to anonymize the samples.

Mice were housed and treated in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of California, San Francisco (protocol number AN098375-03B).

Cells, medium, and reagents

PBMCs and SFMCs were extracted using Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare Life Sciences, Chicago, IL, USA), and stored frozen in FCS+10% DMSO. Jurkat and JPRM441 (low RasGRP1) cells were previously characterized and described [25,37]. RPMI1640 containing 10 mM HEPES, 2 mM L-glutamine, 100 U/mL Penicillin–streptomycin, and 10% FCS was used as culture medium. Starvation was performed in plain RPMI1640 for 30 min. When indicated cells were stimulated with PMA (Sigma-Aldrich, Zwijndrecht, the Netherlands, 20 ng/mL), ionomycin (Sigma-Aldrich, 1 μM).

Flow cytometry

Flow cytometry was performed adhering to the published guidelines [38].

FACS buffer consisted of PBS with 2 mM EDTA, 2% FCS, and 0.1% NaN$_3$. Cells were harvested, blocked with Fc block (1:200),

Figure 4. Transcription factor RUNX1 drives RASGRP1 expression, and is reduced in autoimmunity. (A) Affinity-purification mass spectrometry analysis was performed to identify proteins from Jurkat lysates binding WT versus mutant (42 bp scramble) enhancer sequence. Indicated in red are factors preferentially bound to WT, in black are non-significant changing binders. (B and C) *RUNX1* mRNA levels, normalized to β2m, in CD4+ T cells isolated from JIA patients' blood and during remission (JIA-R (emission), $N = 8$, in three experiments of $N = 3$ or $N = 2$) or active inflammatory disease (JIA-Active, $N = 8$, in three experiments of $N = 3$ or $N = 2$), or synovial fluid during active disease (SF, $N = 3$, one experiment), compared to healthy donors (HC, $N = 6$, two experiments). Three separate experiments were performed. Shown are averages ± SD. 2^{-dCT} values of *RUNX1*, corrected for B2M. (C) Plot comparing *RASGRP1* and *RUNX1* mRNA 2^{-ddCT} expression in each sample, connected by a line. (D) Western blot of RUNX1 and α-tubulin (loading control) showing a representative example of RUNX1 knockdown upon RUNX1 shRNA transduction in Jurkat cells ($N = 3$ separate transduction experiments). (E) *RASGRP1* mRNA, normalized to B2M expression in Jurkat, comparing cells transduced with RUNX1 targeting shRNA or non-targeting (control) shRNA, relative to untransduced Jurkat cells (2^{-ddCT}). $N = 3$ separate transduction experiments. Shown are averages ± SD. One-way ANOVA statistical test was performed in (B), Holm's-Sidak post-test, and Two-tailed T-test was performed in (E), significance is indicated, $^*p < 0.05$, $^{**}p < 0.01$.
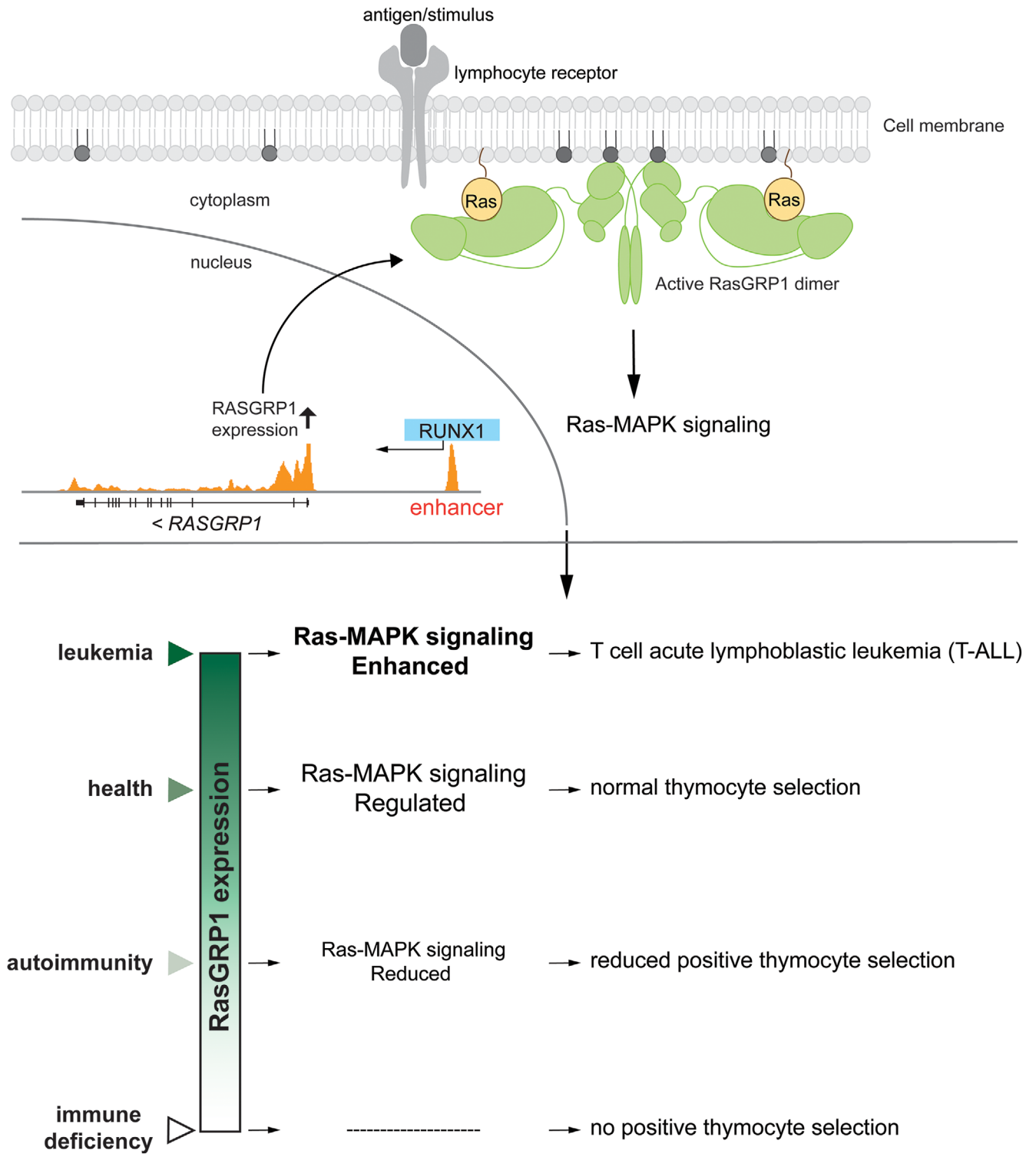
Figure 5. Tight regulation of RASGRP1 expression in T cells is vital to prevent disease. Top: *RASGRP1* transcription is regulated by enhancer 1 binding transcription factors (RUNX1/CBFB). T cell receptor signaling upon antigen/MHC binding induces activation of the RasGRP1 dimer, allowing membrane recruitment and binding to Ras. Next, RasGRP1 releases GDP bound to Ras, allowing GTP binding and active Ras-MAPK signaling. **Bottom**: The level of RASGRP1 expression and resulting Ras-MAPK signaling affects thymocyte selection and clinical outcome: Reduced RASGRP1 expression results in lower Ras-MAPK signaling, thus reduced positive thymocyte selection signals, and can cause autoimmunity. Absence of RASGRP1 results in a complete loss of positive thymocyte selection and immune deficiency, while increased levels of RASGRP1 expression and Ras-MAPK signaling have been shown in T-ALL. Together, tight regulation of RasGRP1 expression is essential to maintain healthy T cells and prevent disease.

normal mouse serum, normal rat serum, next cells were labeled with viability dye (eBiosciences, San Diego, CA, USA, eF780 or eF506 fixable viability dye, 1:1000), and extracellular staining was performed by incubation of cells with antibodies in FACS buffer for 15 min on ice.

Phospho-flow: Cells were washed, resuspended in plain RPMI, seeded 0.4×10^6 per well in a 96-well round bottom plate, and starved in an incubator for 30 min. After stimulation, cells were fixated in prewarmed fixations buffer (Cytofix, BD Biosciences, San Jose, CA, USA), or in 2% paraformaldehyde in PBS, for 15 min at RT, washed in FACS buffer, and permeabilized in MetOH for 30 min on ice. After washing with FACS buffer, cells were incubated with anti-phospho-ERK, washed, and incubated with secondary antibody. Cells were then washed, stained with anti-cleaved caspase-3-Pacific Blue antibody, and run on a BD FACSCanto.

Magnetic cell isolation of CD4+ T cells

CD4+ T cells were magnetically purified using CD4+ T cell isolation kit and the autoMACS® Columns Pro Separator machine (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer instructions. Purity was assessed by flow cytometry staining, and manual gating in FacsDIVA (BD Biosciences).

Antibodies

The following antibodies were used for:

MACS purity control flow cytometry

CD3-BV510, clone OKT3, Biolegend; CD4-FITC, clone RPA-T4, eBioscience.

Phospho-flow stainings

P-ERK (clone 197G2, Cell Signaling Technology, Danvers, MA, USA, #4377s, diluted 1:50). AffiniPure F(ab')2 fragment Donkey-anti-Rabbit IgG, conjugated to APC (#711-136-152, 1:50) or PE (#711-116-152, 1:50, Jackson ImmunoResearch, West Grove, PA, USA). Cleaved-caspase 3-Pacific Blue (clone D3E9, Cell Signaling Technology #8788s, diluted 1:100).

Analysis of thymocyte development

Antibodies used for flow cytometry were:

BD Pharmingen (San Diego, CA, USA) PerCP-Cy5.5 anti-mouse CD4 (clone RM4-5; cat# 550954) (dil 1:800)

UCSF mAb core FITC anti-mouse CD8 (clone YTS169) (dil 1:800)

Tonbo Biosciences (San Diego, CA, USA) APC anti-mouse CD25 (clone PC61.5; cat# 20–0251) (dil 1:100)

Biolegend (San Diego, CA, USA) PE/Cy7 anti-mouse TCR-β (clone H57-597; cat# 109221) (dil 1:200)

Tonbo Biosciences PE/Cy7 anti-human/mouse CD44 (clone IM7; cat# 60-0441-U025) (dil 1:100)

BD Pharmingen APC anti-mouse CD69 (clone H1.2F3; cat# 560689) (dil 1:100)

Dump channel (violetFluor 450): Tonbo Biosciences violet-Fluor 450 anti-human/mouse CD45R/CD19/b220 (clone RA3-6B2; cat# 75-0452-U100) Thermo Fisher Scientific eFluor 450 anti-mouse Ter-119 (cat# 48-5921-82), Tonbo Biosciences violetFluor 450 anti-human/mouse CD11b (clone M1/70; cat# 75-0112-U100), Tonbo Biosciences violetFluor 450 anti-human/mouse CD11c (clone N418; cat# 75-0114-U025), Tonbo Biosciences violetFluor 450 anti-mouse Gr-1 (clone RB6-8C5; cat# 75-5931-U025). All antibodies were diluted 1:100.

Moreover, we used a viability dye: Live/dead fixable violet dead cell stain kit for 405 nm excitation, ThermoFisher Scientific (cat# L34955) (dil 1:1000)

HEp-2 ANA Assays

HEp-2 assays were performed utilizing the Nova-Lite kit from INOVA diagnostics (San Diego, CA, USA).

Serum was applied to slides, stained with IgG-FITC (Jackson Labs, Bar Harbor, ME, USA) and DAPI (500 ng/mL, Life Technologies, Carlsbad, CA, USA). Slides were imaged on a Keyence (Osaka, Japan) BZ-X710 microscope at 10× magnification.

All images were scored blindly by three separate researchers in two separate sessions as ANA negative or positive. At least of four of six scores were required for a "negative" or "positive" overall score. Each slide, containing 10 samples in total, contained a negative control (no serum) or a CD45 Wedge B6-129 F1 positive control serum (a gift from the Hermiston lab, UCSF).

CRISPR-Cas9 editing

Sequences of crRNA used:

exon 2: GTCAATGAGATCGTCCAGGC, AGCTGTCAATGA-GATCGTCC.

Enhancer: TTATAAGAAGGGCTTACCGTGGG, TTTATAAGAAGG-GCTTACCGTGG, CAAAACGGAGTTACATAGCAAGG, TGCTT-GATCTCAGATTAAGCAGG, CTTAATCTGAGATCAAGCACAGG.

DNA and RNA

DNA and RNA were extracted from cells using either Blood & Cell Culture DNA Mini Kit and RNAeasy isolation kit (Qiagen, Hilden Germany), or all-prep DNA/RNA kit (Qiagen). To assess genome editing, PCR amplification was followed by PCR-clean up and send out for sequencing, using the following primers: Exon 2: Fwd 5′ GAAACCTTCCCATGGCTGCA 3′, Rev 5′ TGCAGCTGT-CAATGAGATCGT 3′; Enhancer: Fwd 5′ GGATGGGCTGGTTGAGT-CAA 3′, Rev 5′ ACAGTGTAGGTTCCTAGACCCT 3′. PCR fragments were sequenced and analyzed using TIDE [39]. Subsequently PCR fragments were cloned into pJET (Thermo Fisher Scientific, Abcam) and sequenced with primers from the pJET kit.

To assess any other mutations in coding regions and untranslated regions of RASGRP1 DNA, PCR and subsequent sequencing was performed using primers in Supporting Information Table S1.

RNA purity and concentration were measured by NanoDrop 2000 (Thermo Fisher Scientific). cDNA was synthesized per 15 ng total RNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Quantitative amplification of the cDNA templates was assessed by the SYBR® Select Master Mix (Thermo-Scientific) using the QuantStudio 12K Flex (Thermo Fisher Scientific), or CFX384 Touch (Bio-Rad). Samples contained 4 ng cDNA with primers for $\beta 2m$ (forward, 5′ TGCTGTCTCCAT-GTTTGATGTATCT 3′; reverse 5′ TCTCTGCTCCCCACCTCTAAGT 3′), RUNX1 (forward, 5′ TGAGTCATTTCCTTCGTACC 3′; reverse 5′ TGCTGGCATCGTGGA 3′), or RasGRP1 (PrimePCR SYBR Green Assay RasGRP1 human, Bio-Rad, amplicon context sequence GGTTCCTTGGTTCCCGGGCATAGGAAAGCTCATAGATTTCATCC-TCAGTGTAGTAAAGATCCAGGGATAACGTCAGCAAGTGTACCAAG-TCCTTGTTAGCCTCCAAGG (exon 10). Each run included H_2O as negative control, and each sample was run in triplicate.

DNA affinity purification and LC–MS analysis

Nuclear extracts from Jurkat T cells were generated as described [40]. Oligonucleotides for the DNA affinity purifications were ordered from IDT (Newark, NJ, USA) with the forward strand containing a 5' biotin moiety. DNA affinity purifications and on-bead trypsin digestion were performed as previously described [26]. Tryptic peptides from SNP variant pull-downs were desalted using Stage (stop and go extraction) tips [41], and then subjected to stable isotope di-methyl labeling [42] on the Stage tips. Matching light and heavy peptides were then combined and samples were finally subjected to LC-MS and subsequent data analyses using MaxQuant [43] and R essentially as described.

shRNA transduction

Lentiviral particles were produced in HEK293T cells using third-generation packaging factors. shRNA plasmids were used from the Mission library (Sigma-Aldrich, Zwijndrecht, Netherlands), for RUNX1 (TRCN0000338427, TRCN0000338490, and TRCN0000338489), and nontargeting shRNA control (SHC002). Jurkat cells were infected by spinoculation in the presence of 8 μg/mL of polybrene for 3 h at 22°C at 800 rpm in a table-top centrifuge. Cells were selected with 1 μg/mL of puromycin 24 h after infection.

Western blot

Cells were lysed in SDS buffer, and lysates were run on SDS-page gel and transferred to a PVDF membrane. After blocking for 1 h in 3% BSA (Roche, Basel Switzerland) in TBS-T, membranes were incubated overnight at 4°C with primary antibodies for Ras-GRP1 (JR-E80-2 [9], 1:1500), RUNX1 (ab23980, 1:1000, Abcam, Cambridge, UK), or α-tubulin (T6074, Sigma-Aldrich, 1:2000) in blocking buffer, followed by secondary antibodies in TBS-T for 1 h at RT. Proteins were visualized using ECL.

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Conflict of interest: Jeroen Roose is a co-founder and scientific advisor of Seal Biosciences, Inc. and on the scientific advisory committee for the Mark Foundation for Cancer research. A.M. is a cofounder, member of the Boards of Directors and a member of the Scientific Advisory Boards of Spotlight Therapeutics and Arsenal Biosciences. A.M. has served as an advisor to Juno Therapeutics, was a member of the scientific advisory board at PACT Pharma, and was an advisor to Trizell. A.M. owns stock in Arsenal Biosciences, Spotlight Therapeutics and PACT Pharma. The Marson lab has received research support from Juno Therapeutics, Epinomics, Sanofi, GlaxoSmithKline, Gilead, and Anthem. D.R.S. is a co-founder of Beeline Therapeutics. The other authors have no commercial or financial interests.

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References

1 **Ksionda, O., Limnander, A. and Roose, J. P.**, RasGRP Ras guanine nucleotide exchange factors in cancer. *Front. Biol.* 2013. **8**: 508–532.

2 **Vercoulen, Y., Kondo, Y., Iwig, J. S., Janssen, A. B., White, K. A., Amini, M., Barber, D. L. et al.**, A Histidine pH sensor regulates activation of the Ras-specific guanine nucleotide exchange factor RasGRP1. *Elife* 2017. 6.

3 **Iwig, J. S., Vercoulen, Y., Das, R., Barros, T., Limnander, A., Che, Y., Pelton, J. G. et al.**, Structural analysis of autoinhibition in the Ras-specific exchange factor RasGRP1. *Elife* 2013. **2**: e00813.

4 **Dower, N. A., Stang, S. L., Bottorff, D. A., Ebinu, J. O., Dickie, P., Ostergaard, H. L., Stone, J. C.**, RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat. Immunol.* 2000. **1**: 317–321.

5 **Priatel, J. J., Teh, S. J., Dower, N. A., Stone, J. C. and Teh, H. S.**, RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. *Immunity* 2002. **17**: 617–627.

6 **Salzer, E., Cagdas, D., Hons, M., Mace, E. M., Garncarz, W., Petronczki, O. Y., Platzer, R. et al.**, RASGRP1 deficiency causes immunodeficiency with impaired cytoskeletal dynamics. *Nat. Immunol.* 2016. **17**: 1352–1360.

7 **Winter, S., Martin, E., Boutboul, D., Lenoir, C., Boudjemaa, S., Petit, A., Picard, C. et al.**, Loss of RASGRP1 in humans impairs T-cell expansion leading to Epstein-Barr virus susceptibility. *EMBO Mol. Med.* 2018. **10**: 188–199.

8 **Mao, H., Yang, W., Latour, S., Yang, J., Winter, S., Zheng, J., Ni, K. et al.**, RASGRP1 mutation in autoimmune lymphoproliferative syndrome-like disease. *J. Allergy Clin. Immunol.* 2017.

9 **Hartzell, C., Ksionda, O., Lemmens, E., Coakley, K., Yang, M., Dail, M., Harvey, R. C. et al.**, Dysregulated RasGRP1 responds to cytokine receptor input in T cell leukemogenesis. *Sci. Signal* 2013. **6**: ra21.

10 **Ksionda, O., Melton, A. A., Bache, J., Tenhagen, M., Bakker, J., Harvey, R., Winter, S. S. et al.**, RasGRP1 overexpression in T-ALL increases basal nucleotide exchange on Ras rendering the Ras/PI3K/Akt pathway responsive to protumorigenic cytokines. *Oncogene* 2015.

11 **Yasuda, S., Stevens, R. L., Terada, T., Takeda, M., Hashimoto, T., Fukae, J., Horita, T. et al.**, Defective expression of Ras guanyl nucleotide-releasing protein 1 in a subset of patients with systemic lupus erythematosus. *J. Immunol.* 2007. **179**: 4890–4900.

12 **Golinski, M. L., Vandhuick, T., Derambure, C., Freret, M., Lecuyer, M., Guillou, C., Hiron, M. et al.**, Dysregulation of RasGRP1 in rheumatoid arthritis and modulation of RasGRP3 as a biomarker of TNFalpha inhibitors. *Arthritis Res. Ther.* 2015. **17**: 382.

13 **Farh, K. K., Marson, A., Zhu, J., Kleinewietfeld, M., Housley, W. J., Beik, S., Shoresh, N. et al.**, Genetic and epigenetic fine mapping of causal autoimmune disease variants. *Nature* 2015. **518**: 337–343.

14 **Huang, H., Fang, M., Jostins, L., Umicevic Mirkov, M., Boucher, G., Anderson, C. A., Andersen, V. et al.**, Fine-mapping inflammatory bowel disease loci to single-variant resolution. *Nature* 2017. **547**: 173–178.

15 **Onengut-Gumuscu, S., Chen, W. M., Burren, O., Cooper, N. J., Quinlan, A. R., Mychaleckyj, J. C., Farber, E. et al.**, Fine mapping of type 1 diabetes susceptibility loci and evidence for colocalization of causal variants with lymphoid gene enhancers. *Nat. Genet.* 2015. **47**: 381–386.

16 **Daley, S. R., Coakley, K. M., Hu, D. Y., Randall, K. L., Jenne, C. N., Limnander, A., Myers, D. R. et al.**, Rasgrp1 mutation increases naive T-cell CD44 expression and drives mTOR-dependent accumulation of Helios+ T cells and autoantibodies. *Elife* 2013. **2**: e01020.

17 **Sellers, R. S., Clifford, C. B., Treuting, P. M. and Brayton, C.**, Immunological variation between inbred laboratory mouse strains: points to consider in phenotyping genetically immunomodified mice. *Vet. Pathol.* 2012. **49**: 32–43.

18 **Golec, D. P., Dower, N. A., Stone, J. C. and Baldwin, T. A.**, RasGRP1, but not RasGRP3, is required for efficient thymic beta-selection and ERK activation downstream of CXCR4. *PLoS One* 2013. **8**: e53300.

19 **Bevan, M. J., Hogquist, K. A. and Jameson, S. C.**, Selecting the T cell receptor repertoire. *Science* 1994. **264**: 796–797.

20 **Coughlin, J. J., Stang, S. L., Dower, N. A. and Stone, J. C.**, RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. *J. Immunol.* 2005. **175**: 7179–7184.

21 **Stone, J. C.**, Regulation and function of the RasGRP family of ras activators in blood cells. *Genes Cancer* 2011. **2**: 320–334.

22 **Hnisz, D., Abraham, B. J., Lee, T. I., Lau, A., Saint-Andre, V., Sigova, A. A., Hoke, H. A. et al.**, Super-enhancers in the control of cell identity and disease. *Cell* 2013. **155**: 934–947.

23 **Pott, S. and Lieb, J. D.**, What are super-enhancers? *Nat. Genet.* 2015. **47**: 8–12.

24 **Schumann, K., Lin, S., Boyer, E., Simeonov, D. R., Subramaniam, M., Gate, R. E., Haliburton, G. E. et al.**, Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. *Proc. Natl. Acad. Sci. U. S. A.* 2015. **112**: 10437–10442.

25 **Roose, J. P., Mollenauer, M., Gupta, V. A., Stone, J. and Weiss, A.**, A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. *Mol. Cell. Biol.* 2005. **25**: 4426–4441.

26 **Makowski, M. M., Willems, E., Fang, J., Choi, J., Zhang, T., Jansen, P. W., Brown, K. M. et al.**, An interaction proteomics survey of transcription factor binding at recurrent TERT promoter mutations. *Proteomics* 2016. **16**: 417–426.

27 **Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G. and Downing, J. R.**, AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* 1996. **84**: 321–330.

28 **Kawazu, M., Asai, T., Ichikawa, M., Yamamoto, G., Saito, T., Goyama, S., Mitani, K. et al.**, Functional domains of Runx1 are differentially required for CD4 repression, TCRbeta expression, and CD4/8 double-negative to CD4/8 double-positive transition in thymocyte development. *J. Immunol.* 2005. **174**: 3526–3533.

29 **Lazarevic, V., Chen, X., Shim, J. H., Hwang, E. S., Jang, E., Bolm, A. N., Oukka, M. et al.**, T-bet represses T(H)17 differentiation by preventing Runx1-mediated activation of the gene encoding RORgammat. *Nat. Immunol.* 2011. **12**: 96–104.

30 **Zhang, J., Ding, L., Holmfeldt, L., Wu, G., Heatley, S. L., Payne-Turner, D., Easton, J. et al.**, The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature* 2012. **481**: 157–163.

31 **Helms, C., Cao, L., Krueger, J. G., Wijsman, E. M., Chamian, F., Gordon, D., Heffernan, M. et al.**, A putative RUNX1 binding site variant between SLC9A3R1 and NAT9 is associated with susceptibility to psoriasis. *Nat. Genet.* 2003. **35**: 349–356.

32 **Tokuhiro, S., Yamada, R., Chang, X., Suzuki, A., Kochi, Y., Sawada, T., Suzuki, M. et al.**, An intronic SNP in a RUNX1 binding site of SLC22A4, encoding an organic cation transporter, is associated with rheumatoid arthritis. *Nat. Genet.* 2003. **35**: 341–348.

33 **Peeters, J. G., Vervoort, S. J., Tan, S. C., Mijnheer, G., de Roock, S., Vastert, S. J., Nieuwenhuis, E. E. S. et al.**, Inhibition of super-enhancer activity in autoinflammatory site-derived T cells reduces disease-associated gene expression. *Cell Rep.* 2015. **12**: 1986–1996.

34 **Zhong, X., Wu, Y., Liu, Y., Zhu, F., Li, X., Li, D., Li, Z. et al.**, Increased RUNX1 expression in patients with immune thrombocytopenia. *Hum. Immunol.* 2016. **77**: 687–691.

35 **Hsu, F. C., Shapiro, M. J., Dash, B., Chen, C. C., Constans, M. M., Chung, J. Y., Romero Arocha, S. R. et al.**, An essential role for the transcription factor Runx1 in T cell maturation. *Sci. Rep.* 2016. **6**: 23533.

36 **Wong, W. F., Kohu, K., Nakamura, A., Ebina, M., Kikuchi, T., Tazawa, R., Tanaka, K. et al.**, Runx1 deficiency in CD4+ T cells causes fatal autoimmune inflammatory lung disease due to spontaneous hyperactivation of cells. *J. Immunol.* 2012. **188**: 5408–5420.

37 **Roose, J. P., Mollenauer, M., Ho, M., Kurosaki, T. and Weiss, A.**, Unusual interplay of two types of Ras activators, RasGRP and SOS, establishes sensitive and robust Ras activation in lymphocytes. *Mol. Cell. Biol.* 2007. **27**: 2732–2745.

38 **Cossarizza, A., Chang, H. D., Radbruch, A., Acs, A., Adam, D., Adam-Klages, S., Agace, W. W. et al.**, Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur. J. Immunol.* 2019. **49**: 1457–1973.

39 **Brinkman, E. K., Chen, T., Amendola, M. and van Steensel, B.**, Easy quantitative assessment of genome editing by sequence trace decomposition. *Nucleic. Acids. Res.* 2014. **42**: e168.

40 **Smits, A. H., Jansen, P. W., Poser, I., Hyman, A. A. and Vermeulen, M.**, Stoichiometry of chromatin-associated protein complexes revealed by label-free quantitative mass spectrometry-based proteomics. *Nucleic. Acids. Res.* 2013. **41**: e28.

41 **Rappsilber, J., Mann, M. and Ishihama, Y.**, Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat. Protoc.* 2007. **2**: 1896–1906.

42 **Boersema, P. J., Raijmakers, R., Lemeer, S., Mohammed, S. and Heck, A. J.**, Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. *Nat. Protoc.* 2009. **4**: 484–494.

43 **Cox, J. and Mann, M.**, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* 2008. **26**: 1367–1372.

44 **Roadmap Epigenomics, C., Kundaje, A., Meuleman, W., Ernst, J., Bilenky, M., Yen, A., Heravi-Moussavi, A. et al.**, Integrative analysis of 111 reference human epigenomes. *Nature* 2015. **518**: 317–330.

Abbreviations: **ALPS:** autoimmune lymphoproliferative syndrome · **ANA:** antinuclear antibody · **H3K27ac:** histone 3 lysine 27 acetylation · **JIA:** juvenile idiopathic arthritis · **RA:** rheumatoid arthritis · **RasGEF:** Ras guanine nucleotide exchange factor · **SNP:** single nucleotide polymorphism

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