Dysregulated RasGRP1 expression promotes autoimmunity

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Summary

It is demonstrated that reduced Rasgrp1 expression causes autoimmunity in mice, and associates with active autoimmunity in humans. A key regulatory circuit of RASGRP1 transcription is revealed, consisting of an enhancer region containing autoimmunity-associated SNPs, and binding transcription factor RUNX1.

Abstract

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 in C57BL/6 mice that diminished Rasgrp1 expression caused defective T lymphocyte selection, and inflammatory disease with a severity that inversely correlated 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 the enhancer caused lower RasGRP1 expression, and decreased binding of RUNX1 and CBFB transcription factors. Lastly, knockdown of RUNX1 led to reduced *RASGRP1* transcription. Together, we revealed a key circuit regulating RasGRP1 expression, which is vital to prevent inflammatory disease.

Introduction

RasGRP1, a Ras guanine nucleotide exchange factor (RasGEF), is a key protein regulating effector kinases upon T cell receptor (TCR) signaling(Ksionda et al., 2013). RasGRP1 expression levels regulate the output of lymphocyte receptor signaling in a dose-dependent manner (Iwig et al., 2013; Vercoulen et al., 2017). Complete lack of *Rasgrp1* in mice results in a defect in positive selection of thymocytes (developing T cells), and immune deficiency, both in wildtype mice and mice expressing a transgenic TCR (Dower et al., 2000; Priatel et al., 2002). Recent studies described *RASGRP1* loss in patients with immune deficiencies (Salzer et al., 2016; Winter et al., 2018), and RasGRP1 loss-of-function in autoimmune lymphoproliferative syndrome (ALPS) (Mao et al., 2017).

RasGRP1 dysregulation 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 (Hartzell et al., 2013; Ksionda et al., 2015), 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 (Yasuda et al., 2007), while in rheumatoid arthritis increased mRNA and a contrasting decrease in RasGRP1 protein was shown in T cells (Golinski et al., 2015) .

Moreover, single nucleotide polymorphisms (SNPs) in the *RASGRP1* locus have been linked to autoimmunity (Farh et al., 2015; Huang et al., 2017; Onengut-Gumuscu et al., 2015). For instance, SNPs in coding regions have been shown to affect lymphocyte receptor signaling in cell lines (Vercoulen et al., 2017), and in mice (Daley et al., 2013). Non-coding SNPs that modulate expression levels of RASGRP1 could have a similar effect. 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 investigate how aberrant RasGRP1 expression levels can contribute to inflammatory disease.

Results and discussion

To assess whether decreased Rasgrp1 expression levels can in principal cause autoimmunity, we compared WT (+/+) to *Rasgrp1*-heterozygous (+/-) and *Rasgrp1*-KO (-/-) mice. All mice were on a C57BL/6 background, which is not prone to develop autoimmunity. Thymocytes of *Rasgrp1* heterozygous mice express approximately half of normal RasGRP1 protein levels (Fig 1A). Analysis of stages of thymocyte development (Fig 1B) by flow cytometry showed that aberrant Rasgrp1 expression impaired positive thymocyte selection. As reported previously (Golec et al., 2013), *Rasgrp1*$^{-/-}$ mice displayed an accumulation of CD44^{-}CD25^{+} double negative (DN3) thymocytes, which are very early T cell progenitors (Fig 1C, Fig 1-Suppl A), indicating defective β-selection. By contrast, *Rasgrp1*$^{+/-}$ mice did not show this accumulation. However, later in development at the positive selection stage, TCRβ^{+}CD69^{+} double positive (DP) thymocytes and CD4^{+} SP cells were decreased in *Rasgrp1*$^{+/-}$ mice (Fig 1D,E, Fig 1-Suppl B-C). As expected and reported (Golec et al., 2013), these two populations were nearly absent in *Rasgrp1*$^{-/-}$ mice (Fig 1D,E, Fig 1-Suppl B-C). 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.

Next, we analyzed serum for the presence of anti-nuclear antibodies, a commonly used indicator for autoimmune features (ANA, Fig 1-suppl D). In 11 weeks old mice, we observed that absence of Rasgrp1 led to ANA production, while *Rasgrp1*$^{+/-}$ and WT mice produced no ANA at all (Fig 1F). These results agreed with earlier studies performed in *Rasgrp1*$^{-/-}$ mice (Coughlin et al., 2005; Stone, 2011). By age 24 weeks however, we detected serum ANA in approximately half of the heterozygous *Rasgrp1*$^{+/-}$ mice, while only 1 out of 11 wildtype mice were ANA positive (Fig 1G). Thus, decreased expression of Rasgrp1 led to an increased risk of development of autoimmunity in C57BL/6 mice over time.

Based on these *in vivo* animal findings, 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 (Golinski et al., 2015). We set out to explore RASGRP1 expression in patients with oligoarticular Juvenile Idiopathic Arthritis (JIA), a relapsing/remitting form of autoimmunity. 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 (SF). 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 (Figs 2A, 2-Suppl A). These results suggest active maintenance of RASGRP1 expression levels in $CD4^+$ T cells and instances of decreased expression of RASGRP1 specifically under autoimmune inflammatory conditions.

To identify non-coding elements that could affect the *RASGRP1* gene, 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 upstream of the *RASGRP1* promoter, suggesting the presence of regulatory elements relevant to autoimmunity (Figs 2B, 2-Suppl B). 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 (Hnisz et al., 2013; Pott and Lieb, 2015). In human $CD4^+$ T cells, the H3K27ac signal was more pronounced in activated/stimulated T cells, and co-localized with the SNP clusters (Figs 2B). Notably, we identified a single H3K27ac peak marking a putative enhancer (autoimmunity-associated enhancer 1). Our 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.

To test whether the putative autoimmunity-associated enhancer 1 indeed regulates RASGRP1 expression, we perturbed this non-coding element with CRISPR-Cas9 editing in the Jurkat T cell lymphoma line (Schumann et al., 2015). First, we transfected cells with Cas9-Ribonucleoprotein (RNP) complexes targeting exon 2, and generated a *RASGRP1*-KO clone (RG1-KO), showing loss of RasGRP1 protein (Fig3A, 3-suppl A,B). An unedited clone (unedited-i) was used as a control. Having established this platform, we targeted autoimmunity-associated enhancer 1 (Fig 3, Suppl A). Analysis of selected single cell clones revealed two clones with deletions in different regions (Enh-i and Enh-ii), and an unedited clone (unedited-ii) as an additional control (Figs 3B, 3-Suppl A,B). qPCR analysis revealed reduced levels of RASGRP1 mRNA expression, with a concomitant reduction of RasGRP1 protein levels in clone Enh-ii (Figs 3C,D).

Next, we capitalized on a previously optimized platform to analyze RasGRP1 signaling through flow cytometry analyses of phosphorylated ERK (Vercoulen et al., 2017) (Phospho-flow), which allows quantitative detection of defects in ERK signaling such as in JPRM441, a Jurkat line expressing low levels of endogenous RasGRP1 (Roose et al., 2005). Phospho-flow analysis showed that parental Jurkat cells as well as the unedited control clones displayed robust induction of phosphorylated ERK (P-ERK) levels upon stimulation (Figs 3D, 3-Supplement C,D) and impaired induction of P-ERK in JPRM441, as previously reported (Roose et al., 2005). Notably, both the *RASGRP1*-knockout clone (RG1-KO) and clone Enh-ii demonstrated impaired induction of P-ERK (Figs 3D, 3-Supplement C,D).

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 (Makowski et al., 2016). The scrambled sequence of the 42 base pairs (Fig3-suppl E) that were deleted in clone Enh-ii, resulted in a loss of binding of CBFB and RUNX1 that together form a transcriptional heterodimer, as well as loss of binding of several ZBTB family members (Figs 3E).

We focused on RUNX1 because it 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 (Zhang et al., 2012). Upon short hairpin RNA-mediated knockdown of RUNX1 in Jurkat cells (35% knockdown, Fig 3-suppl F-H), a clear reduction of *RASGRP1* mRNA expression occurred (Fig 3F). 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.

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 peripheral $CD4^+$ T cells is specifically detected under circumstances of active autoimmunity in patients. In line with this, we identified a *RASGRP1* enhancer containing a cluster of autoimmunity-risk SNPs. Moreover, we show that transcription factor RUNX1 is essential for regulation of RASGRP1 expression levels. 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 (Figure 4).

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 which at least 30*106 PBMCs from both active disease as well as remission were available (N=8, Table 1). 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 wildtype, 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).

HEp-2 ANA Assays

HEp-2 assays were performed utilizing the Nova-Lite kit from INOVA diagnostics. See supplementary methods.

Cells, medium and reagents

PBMCs and SFMCs were extracted using Ficoll-Paque PLUS density gradient centrifugation (GE Healthcare Life Sciences), and stored frozen in FCS+10% DMSO. Jurkat and JPRM441 (low RasGRP1) cells were previously characterized and described (Roose et al., 2005; Roose et al., 2007) . RPMI1640 containing 10 mM HEPES, 2mM L-glutamine, 100 U/ml Penicillin-streptomycin, and 10% FCS was used as culture medium. Starvation was performed in plain RPMI1640 for 30 minutes. When indicated cells were stimulated with PMA (sigma, 20ng/ml), ionomycin (1 uM).

Flow cytometry

FACS buffer consisted of Phosphate buffered salt (PBS) with 2 mM EDTA, 2% FCS, and 0.1% NaN3. Cells were harvested, blocked with Fc block (1:200), normal mouse serum, normal rat serum, next cells were labeled with viability dye (eBiosciences eF780 or eF506 fixable viability dye, 1:1000), and extracellular staining was performed by incubation of cells with antibodies in FACS buffer for 15 minutes 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), or in 2 % paraformaldehyde in Phosphate buffered saline (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 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), according to the manufacturer instructions. Purity was assessed by flow cytometry staining, and manual gating in FacsDIVA (BD Biosciences).

Antibodies

MACS purity

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

Phospho-flow

P-ERK (clone 197G2, #4377 s, 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,).

Antibodies for analyzing thymocyte development:

BD Pharmingen 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)

BD Pharmingen™ PE Anti-mouse CD5 (clone 53-7.3; cat# 553023) (dil 1:100)

Tonbo APC anti-mouse CD25 (clone PC61.5; cat# 20-0251) (dil 1:100)

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

Tonbo 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 abs (violetFluor 450) (dil 1:100)

Tonbo Biosciences violetFluor 450 anti-human/mouse CD45R/CD19/b220 (clone RA3-6B2; cat# 75-0452-U100) ThermoFisher 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).

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.
Serum was applied to slides, stained with IgG-FITC (Jackson Labs) and DAPI (500ng/ml, LifeTechnologies). Slides were imaged on a Keyence BZ-X710 microscope.
Sera were scored blindly by 3 separate researchers as ANA negative or positive. Each slide contained a no serum negative control or a CD45 Wedge B6-129 F1 positive control serum (a gift from the Hermiston lab, UCSF).

CRISPR-Cas9 editing

Sequences of crRNA:
exon 2: GTCAATGAGATCGTCCAGGC, and AGCTGTCAATGAGATCGTCC,
Enhancer: TTATAAGAAGGGCTTACCGTGGG, TTTATAAGAAGGGCTTACCGTGG, CAAAACGGAGTTACATAGCAAGG, TGCTTGATCTCAGATTAAGCAGG, CTTAATCTGAGATCAAGCACAGG.

DNA and RNA

DNA and RNA were extracted from cells using either Blood & Cell Culture DNA Mini Kit and RNAeasy isolation kit (Qiagen), 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' TGCAGCTGTCAATGAGATCGT 3'; Enhancer: Fwd 5' GGATGGGCTGGTTGAGTCAA 3', Rev 5' ACAGTGTAGGTTCCTAGACCCT 3'. PCR fragments were sequenced and analyzed using TIDE (Brinkman et al., 2014). Subsequently PCR fragments were cloned into pJET (Thermo Scientific) and sequenced with primers from the pJET kit.

RNA purity and concentration were measured by NanoDrop 2000 (Thermo Scientific). cDNA was synthesized per 15 ng total RNA using the iScriptTM cDNA Synthesis Kit (Bio-Rad). Quantitative amplification of the cDNA templates was assessed by the SYBR® Select Master Mix (Thermo-Scientific) using the QuantStudio 12K Flex (Thermo-Scientific), or CFX384 Touch (Bio-Rad).

Samples contained 4 ng cDNA with primers for β-2-microglobulin (forward, 5' TGCTGTCTCCATGTTTGATGTATCT 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 GGTTCCTTGGTTCCCGGGCATAGGAAAGCTCATAGATTTCATCCTCAGTGTAGTAAAGAT CCAGGGATAACGTCAGCAAGTGTACCAAGTCCTTGTTAGCCTCCAAGG (exon 10). Each run included H2O 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 (Smits et al., 2013). Oligonucleotides for the DNA affinity purifications were ordered from IDT with the forward strand containing a 5' biotin moiety. DNA affinity purifications and on-bead trypsin digestion was performed as described (Makowski et al., 2016). Tryptic peptides from SNP variant pull-downs were desalted using Stage (stop and go extraction) tips (Rappsilber et al., 2007), and then subjected to stable isotope di-methyl labeling (Boersema et al., 2009) 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 (Cox and Mann, 2008) 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), for RUNX1 (TRCN0000338427, TRCN0000338490, and TRCN0000338489), and non-targeting shRNA control (SHC002). Jurkat cells were infected by spinoculation in the presence of 8µg/ml of polybrene for 3 hours at 22°C at 800rpm in a table-top centrifuge. Cells were selected with 1µg/ml of puromycin 24 hours 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 hour in 3% Bovine Serum Albumin (Roche) in TBS-T, membranes were incubated overnight at 4°C with primary antibodies for RasGRP1 (JR-E80-2(Hartzell et al., 2013), 1:1500), RUNX1 (ab23980, Abcam, 1:1000), or α-tubulin (T6074, Sigma, 1:2000) in blocking buffer, followed by secondary antibodies in TBS-T for 1 hour at RT. Proteins were visualized using enhanced chemiluminescence.

Author contributions

MB, TD, DS, DM, SB, KK, SZ, YV, conducted experiment, acquired and analyzed data. FvW, SdR, and AM provided resources and designed experiments. JR and YV designed the study, and wrote the first draft. All authors revised the manuscript.

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Supplemental material description.

Figure 1-supplement includes figures supporting figure 1

Figure 2- supplement A includes a table describing patient information of the JIA patients included in Figure 2A.

Figure 2- supplement B includes a table showing all autoimmunity-associated SNPs displayed in Figure 2B.

Figure 3-supplement consists of 2 pages of figures supporting the data in Figure 3.

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Legends

Figure 1. Limited RasGRP1 expression results in dose-dependent autoimmunity in mice.

(A) Western blot of RasGRP1 protein levels expressed by thymocytes of 11 weeks old mice with different genotypes: +/+ (wildtype), +/- (*Rasgrp1* heterozygote), -/- (*Rasgrp1* KO).

(B-D)Thymocytes were isolated from 5 weeks old mice and analyzed different stages of thymocyte selection by flow cytometry. Shown are average percentages ± S.D. of (B) $CD44^{-}CD25^{+}$ (DN3) of gated $CD4^{-}CD8^{-}$ cells (double negative, DN), (C)$TCR\beta^{+}CD69^{+}$ of $CD4^{+}CD8^{+}$ (double positive, DP) cells, and (D) $CD4^{+}CD8^{-}$ (CD4 single positive, SP) of total thymocytes. One-way ANOVA was used.

(E-F) Anti-nuclear antibody presence (ANA) was determined in sera isolated from mice at 11 weeks old (E, N=6, N=9, N=6), and 24 weeks old (F, N=7, N=13, N=10). Bars depict 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) RASGRP1 mRNA levels in $CD4^{+}$ T cells were isolated from juvenile idiopathic arthritis patients disease remission blood (JIA-Remission, N=8), active inflammatory disease blood (JIA-Active, N=8), or synovial fluid (JIA-Active SF, N=3), and healthy adult donors blood (N=6). Shown are averages ± S.D. 2^{-dCT} values of RasGRP1 corrected for B2M.

(B) RasGRP1 locus showing fine mapped disease-associated SNPs for Crohn's Disease (CD)(Huang et al., 2017) , Type 1 Diabetes (T1D)(Onengut-Gumuscu et al., 2015), and Rheumatoid Arthritis (RA)(Farh et al., 2015). The GWAS data was overlapped with histone 3 lysine 27 acetylation (H3K27Ac) tracks for different T cell types (Roadmap Epigenomics Project(Roadmap Epigenomics et al., 2015)): 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.

Figure 3. Transcription factor RUNX1 binds to autoimmunity-associated enhancer 1 and regulates RASGRP1 expression.

(A)CRISPR-Cas9 RNP editing of exon 2 in Jurkat cells. Western blot showing RasGRP1 and α-tubulin, parental Jurkat cells, one non-edited clone (unedited-i), and one *RASGRP1*-KO clone (RG1-KO).

(B) Clones generated upon CRISPR-Cas9 targeting of autoimmunity-associated enhancer 1 in Jurkat cells. Displayed are sequences of two mutants and one unedited clone, showing the WT sequence (blue), deletions (black), and insertions (red), Het= heterozygous.

(B-C) RASGRP1 expression levels in parental Jurkat cells, unedited controls, and Enh1 targeted clones. (B) RASGRP1 mRNA expression, RASGRP1/B2M, relative to parent Jurkat cells: 2^{-ddCT}. Shown are averages ± S.D. One-way ANOVA was performed, significance is indicated, * $p<0.05$, ** $p<0.01$. (C) Western blot showing RasGRP1, and α-tubulin expression. (D) Phospho-flow P-ERK analysis of clones at baseline, and after 2 or 5 minutes of PMA/ionomycin stimulation. Shown is a heatmap of median P-ERK expression of a representative experiment. JPRM441 is a Jurkat line with low (~10%) RasGRP1 expression.

(E) Affinity-purification mass spectrometry analysis of proteins from Jurkat lysates binding wildtype (WT) versus mutant (42 bp scramble) enhancer sequence. Indicated in red are factors preferentially bound to WT. (F) RASGRP1 mRNA, corrected for B2M expression in Jurkat, comparing cells transduced with RUNX1 targeting shRNA or non-targeting (control) shRNA, relative to untransduced Jurkat cells (2^{-ddCT}). Shown are averages ± S.D. Two-tailed T-test was performed, significance is indicated, * $p<0.05$.

Figure 4. 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.

Figure 1

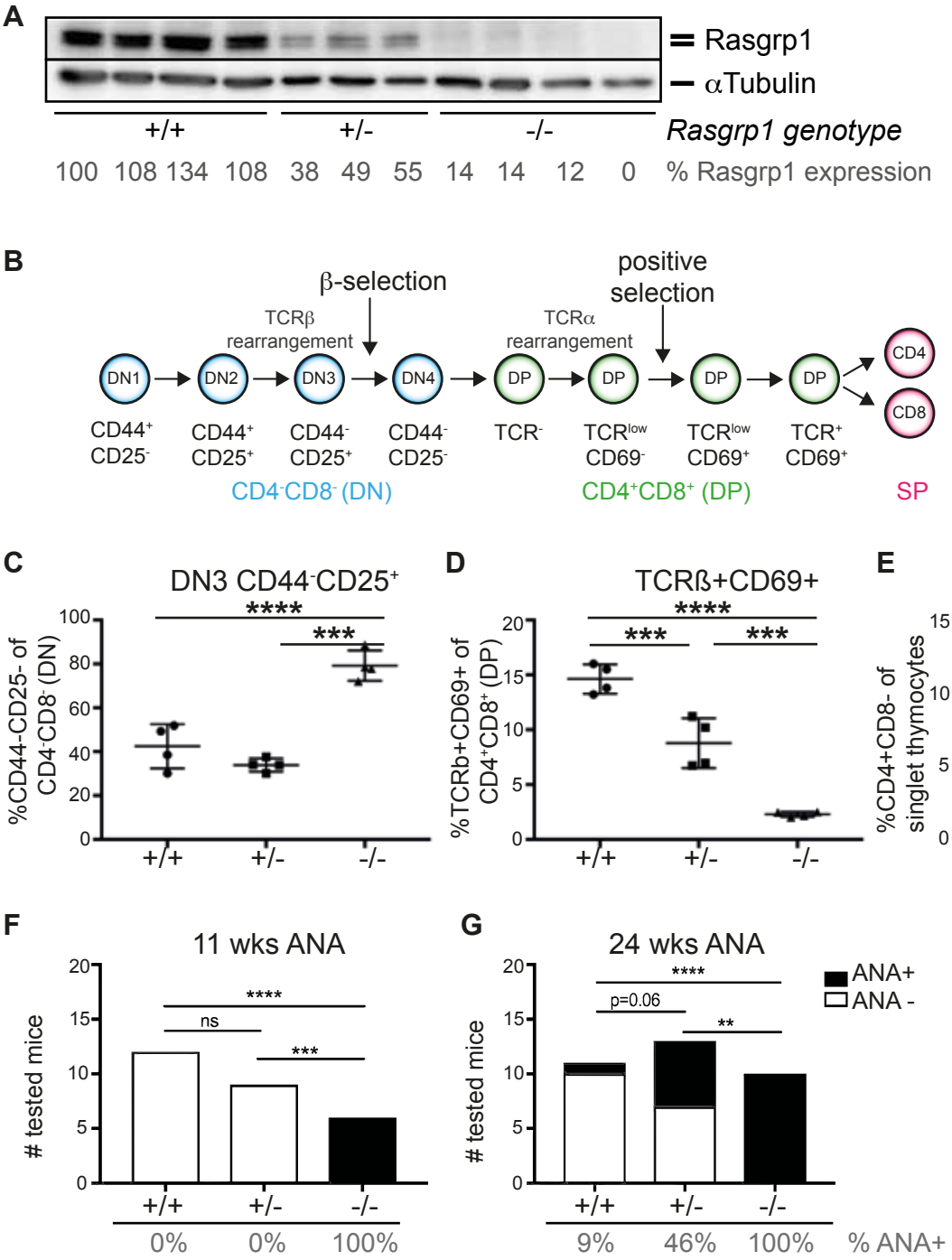

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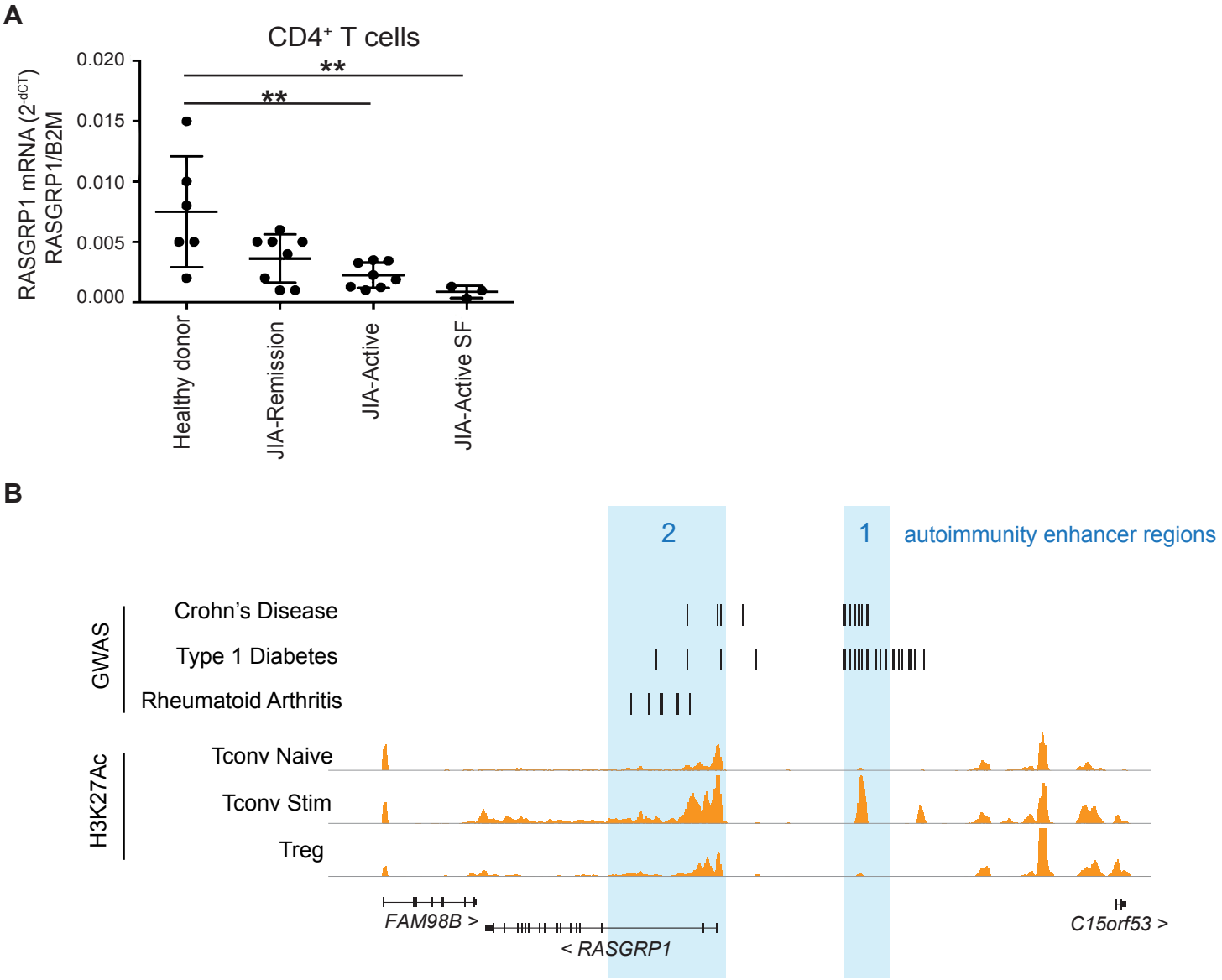
Figure 2.



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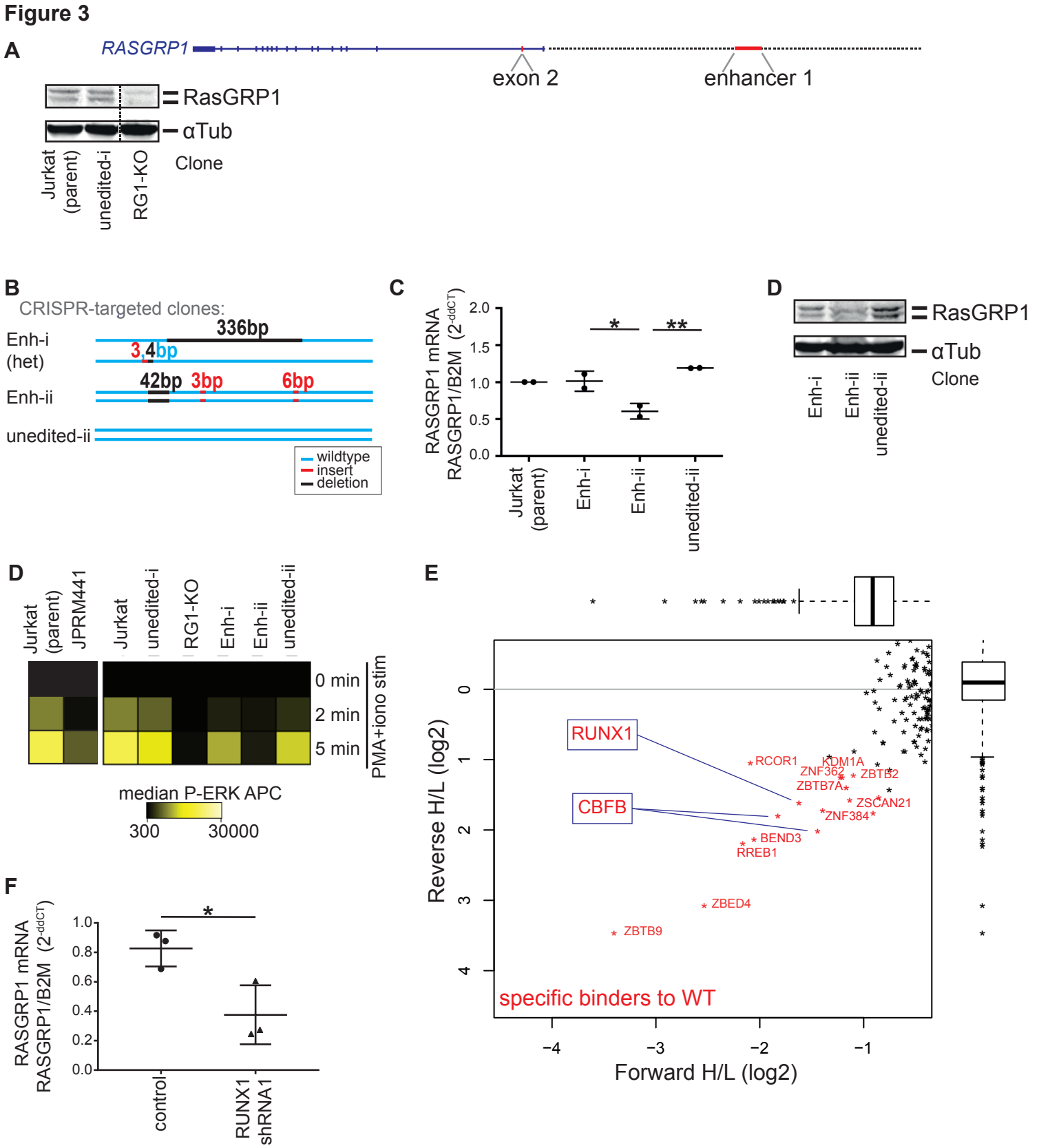

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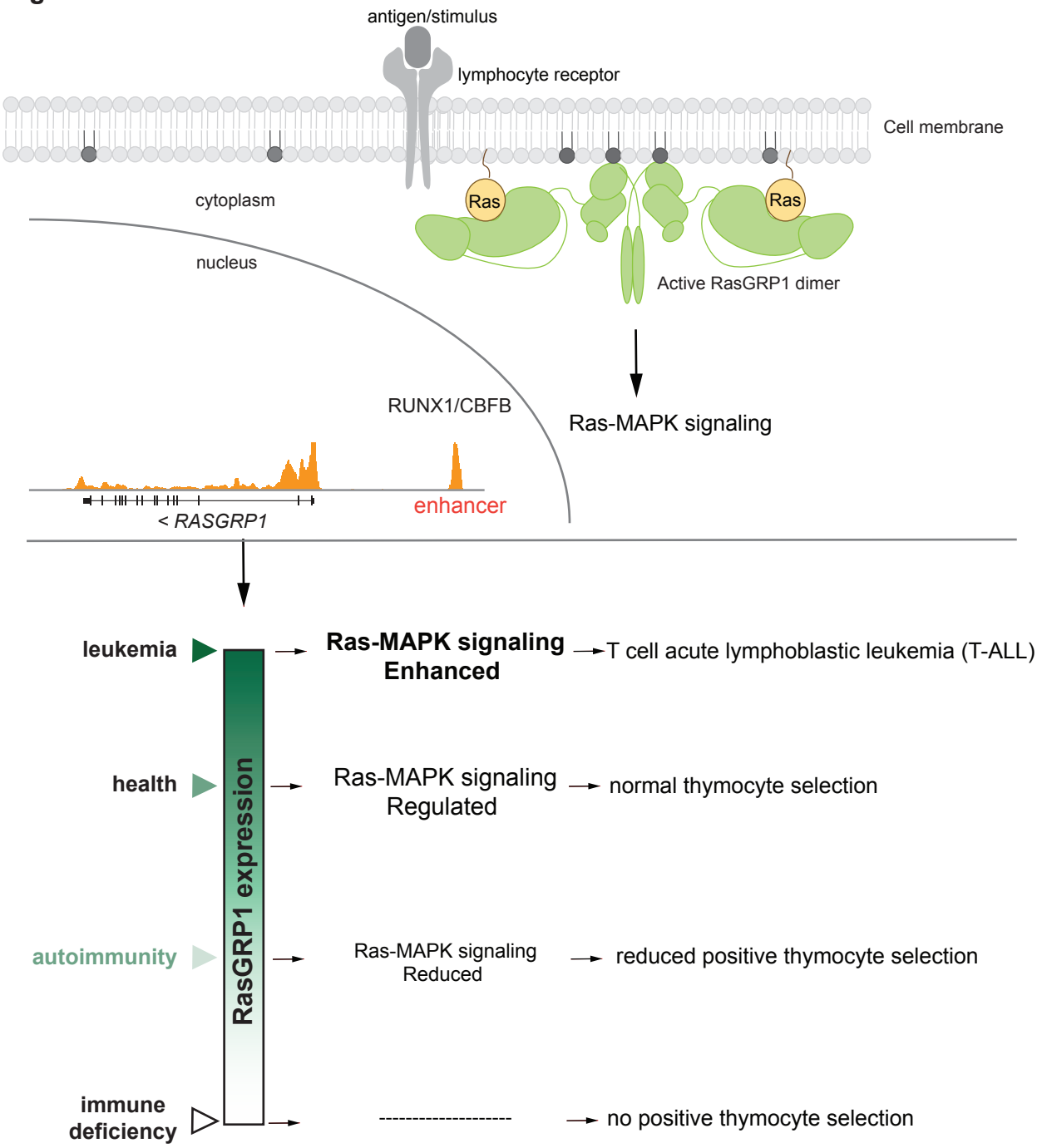

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