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Germline mutations in a G-protein identify signaling crosstalk in T cells

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Abstract

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Author contributions:

H.H. identified the interaction between active $G_{\alpha i2}$ and RASA2, performed experiments examining TCR-induced responsiveness of mutant $G_{\alpha i2}$ transduced T cells, TCR-induced signaling of patient T cells, transduced T cells, and RASA2-depleted T cells, validated *GNAI2* mutant allele (P1)-specific KO, and performed GST-pulldowns, coimmunoprecipitations, GAP assays, confocal microscopy, FLIM imaging, in vitro chemotaxis, and GTPase assays. H.J. performed experiments evaluating $G_{\alpha i2}$ variants by cAMP ELISA or reporter BRET assay and in vitro transwell chemotaxis, TCR-induced responsiveness of patient and KO T cells (*GNAI2* mutant allele (P1), RASA2, and ACs), and TCR-induced signaling of AC-KO T cells. I.T.L. identified and established the causal nature of patient *GNAI2* mutations, made initial observation of impaired chemotaxis, Ca^{2+} flux, and TCR-induced hyperresponsiveness, as well as performed experiments examining proximal and downstream signaling, in vitro transwell chemotaxis, chemokine induced Ca^{2+} flux, and $G_{\alpha i2}$ expression in patient and mutant $G_{\alpha i2}$ transduced T cells. I.T.L., J.N.M., R.N.G. studied chemotaxis in vivo using adoptive cell transfer. M.M.K. performed TCR-induced activation and signaling experiments of patient T cells. A.K., V.L.K. performed GTP binding and GTP hydrolysis by $G_{\alpha i}$ variants and analyzed data. Y.A.B., T.P.S. set up BRET assay to evaluate $G_{\alpha i2}$ mutant from P1 and examine the interaction of $G_{\alpha i2}$ and GPCR. D.E.A. performed mass spectrometry analysis to identify interacting proteins. K.M.D. performed RGS protein pull-down. M.K., S.R. performed cAMP measurements of $G_{\alpha i2}$ variants using Glo-sensor. D.B.K., D.L.F., X.X., W.S.K., T.J. performed EZ-TAXIScan experiments. K.A.Z. evaluated leukocyte migration into blisters. N.M.M. evaluated cell accumulation in oral mucosa. H.H.K. performed skin virome analysis. P.C. performed molecular modeling. M.M.K., J.D., and D.T. assisted in $G_{\alpha i2}$ expression, in vitro chemotaxis, GTPase assay, and processing patient samples. Ka.Me. performed coimmunoprecipitation of exogenously expressed RASA2 with $G_{\alpha i2}$ variants. M.F.R.Q. assisted with TCR-induced signaling experiments of patient T cells. S.G. and J.K. assisted with imaging capture and analyses. E.M.-F., R.B., C.C.G. generated and evaluated the *Gnai2* KI mice. V.J.W. generated the AC3 and AC6 double KO HEK293 cells and advised on cAMP assays. D.D.B. assisted in analyses of data for manuscript preparation.

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H.F.M. coordinated clinical study protocol and sample collection. H.C.S. planned and supervised the experimental work and data analyses. M.J.L. provided advice and assisted in supervising experimental work. Y.Z. performed, supervised, and coordinated genetic studies including Sanger sequencing mutation confirmations, WES re-analysis across the cohort and RNA-seq analyses. H.H., H.J., I.T.L., M.J.L., and H.C.S. prepared the manuscript. All authors read and discussed the submitted manuscript.

Competing interests: H.C.S. holds stock in Amgen and Eli Lilly. K.M. is an employee of GeneDx, a genetic testing laboratory. R.J.T. is an Illumina Inc employee and shareholder. J.P.S. was a member of a data monitoring committee for Leniolisib for SOBI and Pharming within the last 3 years. C.A.S. was a paid consultant for Lundbeck pharmaceuticals. C.A.S. is an inventor on patent applications US-20030022180-A1 held by NIH that covers PRKAR1A and Carney complex, US-20090005337-A1 held by NIH that covers PDE11A and adrenal disease, US-20170304405-A1 held by NIH that covers treatment of hormonal disorders of growth, and US-20190314459-A1 held by NIH that covers GPR101 function. The other authors declare that they have no competing interests.

Humans with monogenic inborn errors responsible for extreme disease phenotypes can reveal essential physiological pathways. We investigated germline mutations in *GNAI2*, which encodes $G_{\alpha i2}$, a key component in heterotrimeric G-protein signal transduction usually thought to regulate adenylyl cyclase-mediated cAMP production. Patients with activating $G_{\alpha i2}$ mutations had clinical presentations that included impaired immunity. Mutant $G_{\alpha i2}$ impaired cell migration and augmented responses to T cell receptor (TCR) stimulation. We found that mutant $G_{\alpha i2}$ influenced TCR signaling by sequestering the GTPase-activating protein RASA2, thereby promoting RAS activation and increasing downstream ERK/MAPK and PI3K-AKT S6 signaling to drive cellular growth and proliferation.

One-Sentence Summary:

Activating $G_{\alpha i2}$ mutations in a syndromic disorder reveal a cAMP-independent, $G_{\alpha i2}$ -RASA2-RAS pathway regulating T cell responsiveness.

Introduction

G-protein coupled receptors (GPCRs) are fundamental to mammalian physiology (1, 2). They direct cells to respond to diverse environmental cues including hormones, neurotransmitters, and chemokines. This is accomplished through a complex and highly regulated biochemical cycle mediated by associated heterotrimeric G-proteins ($G_{\alpha\beta\gamma}$). After GPCR ligation, the G_{α} subunit binds GTP, becomes active, and dissociates from both the $G_{\beta\gamma}$ complex and the GPCR. Dissociation causes both G_{α} -GTP and free $G_{\beta\gamma}$ to initiate downstream signals including generation of second messengers and ion fluxes. Ultimately, the GTPase activity of G_{α} hydrolyzes GTP into GDP to terminate signaling and allow reassembly of the heterotrimeric G-protein that can reassociate with a GPCR, completing the cycle and resetting cells to allow them to respond again to GPCR engagement (3).

The 16 human G_{α} subunits are grouped into four families ($G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$) having distinct expression patterns and effector partners (4). The $G_{\alpha i/o}$ family includes inhibitory isoforms of G_{α} that are thought to regulate biological responses by suppressing adenylyl cyclase (AC) production of the intracellular second messenger, cyclic AMP (cAMP) (5). AC exist as nine transmembrane isoforms expressed broadly, with different tissues expressing different levels of multiple isoforms (6, 7). Only group III (AC5, AC6) and to a lesser degree group I (AC1) ACs are sensitive to the inhibitory effects of $G_{\alpha i}$ (6, 7).

Humans with monogenic inborn errors responsible for extreme disease phenotypes can reveal essential physiological pathways. Germline mutations in $G_{\alpha i/o}$ family members (*GNAI1*, *GNAI3*, *GNAO1*, *GNAT1*, *GNAT2*) cause severe neurodevelopmental, craniofacial, or visual system defects (8–10). By contrast, the effect of germline *GNAI2* mutations in humans is not known. While $G_{\alpha i2}$ (encoded by *GNAI2*) is ubiquitously expressed, it has been implicated in normal functioning of the cardiovascular, nervous, endocrine, and immune systems (11, 12). Somatic *GNAI2* mutations that activate $G_{\alpha i2}$ have been identified in human ovarian and adrenal tumors (13). *GNAI2* variants of unknown significance were also identified in two individuals with neurodevelopmental defects (14, 15).

Since the roles of $G_{\alpha i2}$ in human physiology and development are not clear, we sought to identify individuals that harbor mutations in *GNAI2* to examine their clinical presentations and their underlying disease mechanisms.

***GNAI2* mutations found in humans inhibited the intrinsic GTPase activity**

We used whole exome/genome sequencing to discover 20 patients from 18 unrelated families of different ancestries worldwide, who had previously unreported or extremely rare heterozygous missense variants in *GNAI2*, which were also computationally predicted to be deleterious (CADD score > 25) (Fig. 1A, and table S1). The variants were absent in the Greater Middle Eastern Variome (16) and in the Genome Aggregation Database (gnomAD v3.1.2) (17), except for 3–50256263-G-A (GRCh38, p.Arg179His), which was found in 1 individual (minor allele frequency (MAF) 0.00002494 in the African/African American population) of unknown affection status. In our families, the variants segregated with a hitherto unrecognized autosomal dominant syndromic disorder, in which *de novo* (N = 12) mutations predominated. These mutations were detected in several tissues (fig. S1, A and B), suggesting they arose in germ cells or early during embryonic development. The patients' cells expressed equivalent levels of both mutant and wild-type (WT) transcripts, as well as normal levels of total $G_{\alpha i2}$ protein (fig. S1, C and D).

The mutations caused amino acid substitutions at residues that were evolutionarily constrained and highly conserved in all heterotrimeric G-protein superfamily members (Fig. 1B and fig. S1E). The altered residues were clustered in the Ras-like GTPase domain of G_{α} , especially within the highly conserved P-loop motif and switch regions that are critical for guanine-nucleotide binding and GTPase activity (Fig. 1B and fig. S1E). These mutations could interfere with binding of the GTP phosphate group and cofactors (Mg^{2+} ion, nucleophilic H_2O) that are necessary to hydrolyze bound GTP (Fig. 1C) or could indirectly influence GTP binding/hydrolysis (fig. S2, A to C), which would likely result in impaired signal termination (see Supplementary Text 1).

We tested the function of purified recombinant $G_{\alpha i2}$ variant proteins and compared their ability to bind and hydrolyze GTP with non-mutated WT $G_{\alpha i2}$ and a GTPase-mutant (Gln205Leu) known to be deficient in GTP hydrolysis (18). In general, the $G_{\alpha i2}$ mutants, except Arg179His, bound non-hydrolyzable GTP γ S more readily than WT (Fig. 1, D and E; fig. S2, D to F; and table S2) and all exhibited decreased GTP hydrolysis (Fig. 1, F and G; fig. S2, G to J; and table S2).

To substantiate the conclusion that the mutations in $G_{\alpha i2}$ impaired GTPase activity, we investigated their regulation by regulators of G-protein signaling (RGS). The intrinsic GTPase activity of $G_{\alpha i}$ is normally accelerated by RGS which act as GTPase activating proteins (GAPs) and are strictly dependent on binding to a G_{α} transition state conformation (18). GTPase-deficient G_{α} mutants, such as $G_{\alpha i1}$ -Gln204Leu, are generally resistant to RGS GAP activity (19). Accordingly, the GTPase activities of the patients' $G_{\alpha i2}$ mutants, except Arg179His, were not increased by adding RGS16 (Fig. 1G and fig. S2J). Furthermore, neither of two patient mutants we tested (Thr182Ala/Ile, nor Gln205Leu) bound RGS16 (fig. S2, K and L), similar to an RGS-insensitive Gly184Ser control (20). Taken together,

the faster GTP binding, decreased GTPase activity, and RGS insensitivity indicated that the patients' $G_{\alpha i2}$ mutant proteins could promote a GTP-bound form of $G_{\alpha i2}$, in this way delaying signal termination and prolonging $G_{\alpha i2}$ activity (table S2).

This constitutive activation of $G_{\alpha i2}$ would be expected to overstimulate downstream functions such as inhibition of cAMP production (5). Therefore, we measured forskolin (FSK)-induced AC synthesis of cAMP in the presence of overexpressed $G_{\alpha i2}$. Compared to WT, mutant $G_{\alpha i2}$ found in patients suppressed accumulated cAMP production (Fig. 1H, and fig. S3, A to D) or transient cAMP activity (Fig. 1I; fig. S3, E to H; and table S3). AC5 is a well-documented target of active $G_{\alpha i2}$ inhibition (5), and we observed similar effects of mutant $G_{\alpha i2}$ on AC5-induced cAMP production (fig. S3D). Finally, primary dermal fibroblasts isolated from several patients (bearing Thr182Ala, Lys46Thr, Arg209Trp mutations) showed decreased cAMP production (Fig. 1J). However, two mutants tested, including Arg209Trp, did not decrease cAMP production in the overexpression system (Fig. 1H and table S2). Mutations at Arg209, which is part of a highly conserved "Gly-Arg-Glu" triad found in all G_{α} family members, can disable G_{α} activation of effectors due its inability to dissociate from $G_{\beta\gamma}$ even in the GTP-bound conformation (Supplementary Text 2) (21).

Overall, our data suggested that the mutations in *GNAI2* that we identified in patients impaired the GTPase activity of $G_{\alpha i2}$ and enhanced the suppression of cAMP production, which would be consistent with them being pathogenic "activating" variants.

Patients with *GNAI2* mutations exhibited multiple clinical presentations

We next investigated the overall pathophysiological impact of the activating $G_{\alpha i2}$ mutations by in-depth clinical phenotyping (Data File S1 and Supplementary Text 3 and 4). The patients exhibited abnormal development characterized by intrauterine growth retardation, dysmorphism (Fig. 2A), bone dysostosis (Fig. 2B), neuroanatomical abnormalities (Fig. 2C), and birth defects in other organs (Fig. 2D). Midline structural defects, located along the body's central vertical axis and suggestive of abnormal development during blastogenesis, were observed (fig. S4A). These were most commonly congenital nasal septum deviation (Fig. 2B), dysgenesis of the corpus callosum (Fig. 2C), pituitary hypoplasia with growth hormone deficiency (Fig. 2C), Chiari I malformation (Fig. 2C), micropenis, sagittal cleft ("butterfly") vertebrae (Fig. 2B), and scoliosis (Fig. 2B). The occurrence of certain rare birth defects, such as subcortical band heterotopia (Fig. 2C), agenesis of olfactory bulbs (Fig. 2C), and coloboma, suggested defective neuronal migration during late embryogenesis/early fetal development, including that of olfactory and gonadotropin-releasing hormone neurons as well as retinal progenitor cells. Most patients exhibited postnatal abnormalities too, including short stature with neurodevelopmental delay, neurobehavioral deficits, and gastrointestinal dysfunction (Data File S1, and Supplementary Text 3 and 4).

Weighted analysis of clinical features affected in each patient revealed heterogeneity in the systems affected across the cohort (Fig. 2E). Nearly all patients (90%) had disease involvement in the immune system, characterized by recurrent, unusual, and/or severe infections (Data File S1 and Supplementary Text 3). However, the extent of disease involvement in the immune system was greater in patients mutated at residue Thr182 due

to their additional inflammatory or autoimmune complications (Fig. 2E). An attempt to knock-in (KI) the highly constitutively activating Thr182Ile mutation into mice failed to generate heterozygous embryos beyond the eight-cell stage, indicating *in utero* lethality (table S4).

Among the immune system presentations (Data File S1 and Supplementary Text 3), bacterial, superficial fungal, or unusually severe viral infections of the skin were striking, and included recurrent shingles, extensive warts, and rubella vaccine-associated skin granulomas (Fig. 2D and fig. S4, B and C). Respiratory, middle ear, and sinus infections were common with some developing bronchiectasis, and some had invasive bacterial infections (Fig. 2B and D; and fig. S4, B and C). Inflammatory or autoimmune complications included lymphocytic infiltration of organs, psoriasis, discoid lupus, autoimmune cytopenias with splenomegaly, Hashimoto thyroiditis, type I diabetes mellitus, colitis, or macrophage activation syndrome (Fig. 2D and fig. S4). Several had asthma or atopic dermatitis (fig. S4). Longitudinal laboratory testing revealed monocytosis and neutrophilia precipitated by acute infection, which persisted in some patients (fig. S5A, and Supplementary Text 5). T cell counts were initially low, with decreased recent thymic emigrants and paucity of naïve versus effector/memory T cells, but counts increased with age (fig. S5, B to D). Effector phenotype T cells showed cytokine perturbations including increased IL-17 expression (fig. S5E). Lymphocyte proliferation and activation appeared normal or even increased (fig. S6, and table S5). Dysgammaglobulinemia, characterized by low serum IgM and poorer vaccine titers, was accompanied by decreased B cell counts and atretic lymphoid follicles in secondary lymphoid organs (fig. S5, F and G; fig. S7; table S6; and Supplementary Text 6).

Overall, the patients' phenotypes indicated that mutant $G_{\alpha i2}$ caused multi-organ dysfunction, including life-threatening immunodysregulation and numerous birth defects. Their presentations underscore the importance of $G_{\alpha i2}$ in regulating diverse physiologic processes in humans.

$G_{\alpha i2}$ mutant proteins impaired immune cell migration

The patients' clinical features suggested impaired migratory behavior of immune cells for host defense, which are guided to sites of infection by chemokine receptors. Since heterotrimeric G-proteins transduce signals for all chemokine receptors (22), we tested whether hyperactive $G_{\alpha i2}$ altered cell migration. Both $CD4^+$ and $CD8^+$ T cells from several patients showed decreased chemotaxis toward multiple chemokines (Fig. 3A, and fig. S8, A to D) and reduced chemokine-induced Ca^{2+} fluxes mediated by free $G_{\beta\gamma}$ subunits in one tested patient (Fig. 3B, and fig. S8E), indicating defective proximal GPCR signaling. These defects were recapitulated by expressing all hyperactive $G_{\alpha i2}$ mutant proteins except Ile55Met in T cells from normal healthy donors, with intermediate and more variable effects of Leu38Arg and Arg179Cys (Fig. 3, C to E, fig. S8, F to H, and S9, A to E). Neutrophils from several patients also showed reduced directional migration in response to chemoattractants (fig. S10, A and B), and their defective migration was recapitulated by expressing several hyperactive $G_{\alpha i2}$ mutant proteins in the neutrophil-like HL60 cell line (fig. S10, C to F, and Movie S1–3). These data would be consistent with immune

cells having altered trafficking in patients with *GNAI2* mutations. Indeed, in one patient with periodontitis, leukocytes had impaired accumulation in the oral mucosa, or impaired migration into blisters after induction of sterile inflammation in the skin (fig. S10, G and H).

Furthermore, in two patients, splenic biopsies showed increased leukocyte numbers in red pulp with decreased white pulp (fig. S7 and Supplementary Text 6), suggesting that leukocyte migration into secondary lymphoid organs was also impaired. Hence, we tracked T cell migration in mice after adoptive transfer and found that cells expressing mutant $G_{\alpha i2}$ proteins migrated less well into lymph nodes and splenic white pulp (Fig. 3F, and fig. S9F). This might alter cell-cell interactions that normally take place within secondary lymphoid organs for developing immune cell functions.

Lastly, while suppressed cAMP could contribute to the impaired migration phenotype, the literature is contradictory (23). At least in T cells and neutrophils, pharmacologic manipulations that increase intracellular cAMP have been shown to inhibit chemotaxis (24, 25). Thus, we examined whether cAMP levels affected by $G_{\alpha i2}$ mutants impact T cell chemotaxis. We used CRISPR-Cas9-mediated gene editing to knockout (KO) *ADCYs* encoding major ACs expressed in human T cells (fig. S11, A and B). Ablation of AC3 or AC7 in healthy donor T cells had opposite effects on endogenous cAMP levels but did not affect chemotaxis, which instead segregated with the absence or presence of the mutant $G_{\alpha i2}$ (Fig. 3, G to I, and fig. S11, C to G). These results suggested that the impaired leukocyte chemotaxis in the patients did not result from $G_{\alpha i2}$ -mediated alterations in cAMP production.

Collectively, these findings showed that the patients' $G_{\alpha i2}$ mutants caused defective cell migration. We hypothesized that the patients' mutants, being in a "quasi-permanent" GTP-bound state of biochemical activation, might be mostly dissociated from GPCRs and therefore unable to transduce GPCR signals.

Using bioluminescence resonance energy transfer (BRET) to measure the interaction of $G_{\alpha i2}$ proteins with GPCRs overexpressed in living cells, we found that the Thr182Ala $G_{\alpha i2}$ mutant found in patients and Gln205Leu activating mutant control protein showed minimal steady state interaction with chemokine receptors (Fig. 3, J and K, and fig. S12, A to C). Even with ligand engagement across a broad range of concentrations, the already low BRET intensity remained unchanged, in contrast to the high BRET signal that rapidly declined to similarly low levels in cells overexpressing WT $G_{\alpha i2}$ (Fig. 3K, and fig. S12A). Increased dissociation from GPCRs also compromised chemokine-augmented suppression of transient cAMP production for several other patients' mutants (fig. S12, D and E). Hence, more potent "active" mutant $G_{\alpha i2}$ constitutively adopted a conformation that promoted decoupling from GPCRs, decreasing the pool of GPCR-WT $G_{\alpha i2}$ complexes able to recycle to active receptor complexes that can transduce GPCR signals for further biological responses.

Taken together, activating mutant $G_{\alpha i2}$ proteins had the seemingly paradoxical effect of impairing responsiveness to chemokines/chemoattractants because the mutant $G_{\alpha i2}$ proteins associate poorly to the receptors. This resulting impaired migration of immune cells can explain the patients' increased infection susceptibility.

T cells from patients with mutant $G_{\alpha i2}$ proteins were hyperresponsive to TCR stimulation

While impaired leukocyte migration could explain the patients' infection susceptibility, some patients also had life-threatening autoimmunity. Patients did not show decreased peripheral blood T_{reg} or increased $CD21^{lo}$ $CD38^{lo}$ B cell numbers that could account for their autoimmunity (fig. S5, D and F). However, we noticed that overall lymphocyte proliferative responses to mitogens and antigens were not decreased but appeared normal or even higher than expected (fig. S6 and Supplementary Text 5).

We hypothesized that T cell hyperresponsiveness might explain the autoimmunity in patients. Therefore, we examined T cell behavior in vitro under various T cell receptor (TCR)-stimulating conditions. Similar to our observations for gated T cells stimulated in peripheral blood mononuclear (PBMC) preparations (fig. S6), T cells purified from multiple patients (whether $CD4^{+}$ or $CD8^{+}$ T cells, or naïve or effector/memory T cells) exhibited enhanced induction of the activation markers CD69 and CD25 (interleukin-2 (IL-2) receptor α subunit) and increased proliferation upon stimulation with anti-CD3 plus anti-CD28 antibodies compared to control samples (Fig. 4, A to F, and fig. S13, A to L). These differential responses were also seen when cells were stimulated suboptimally (anti-CD3 antibodies only) but were not apparent when cells were treated with more potent stimulation (Beads, consisting of immobilized anti-CD2, anti-CD3, and anti-CD28 antibodies). The presence of exogenously provided IL-2 throughout the experiments and normal IL-2 production by ex vivo $CD4^{+}$ T cells (fig. S5E) suggested that the increased responses of the patient T cells did not result from increased IL-2 production.

To determine whether the increased T cell responsiveness was a direct effect of the mutant $G_{\alpha i2}$ protein, we designed a guide RNA sequence (named gMP) that specifically knocks out the mutant but not WT *GNAI2* allele of P1 using a CRISPR-Cas9 ribonucleoprotein system (fig. S14, A to C). Treatment with gMP restored activation and proliferation of patient T cells to normal levels indicating a positive role for the mutant protein in the increased T cell responsiveness (Fig. 4, G to I and fig. S14, D and E). Transduction of $CD4^{+}$ T cells from healthy normal donors with the Thr182Ala or Gln205Leu activating $G_{\alpha i2}$ mutants also increased TCR-induced responsiveness similar to patient T cells (Fig. 4, J and K, and fig. S13, M to Q), whereas partial KO of $G_{\alpha i2}$ in T cells from healthy donors failed to do so (fig. S14, F to H). Thus, activating- but not WT- $G_{\alpha i2}$ protein increased the stimulatory response to TCR engagement.

Proteins involved in TCR signaling showed increased distal phosphorylation in cells with mutant $G_{\alpha i2}$

T cell activation through the TCR initiates multiple signals that lead to rapid clonal expansion including activation of the RAS proteins (HRAS, KRAS, NRAS) facilitated by RAS guanyl-releasing proteins (RASGRPs) (26, 27). Activation of these small G-proteins in turn leads to activation of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, in parallel with the phosphatidylinositol

3-kinase (PI3K)-AKT-mechanistic target of rapamycin (mTOR) pathway (26, 27). Together, these signaling pathways promote a metabolic shift to aerobic glycolysis and an increase in protein synthesis for optimal cellular growth, proliferation, and differentiation. By contrast, overstimulation of these pathways can contribute to uncontrolled growth as seen in cancers, which have a high prevalence of somatic mutations in RAS, as well as certain leukoproliferative disorders associated with autoimmunity (28). G_{α} proteins are canonically thought to function by modulating production of the second messenger cAMP (2). They mediate GPCR signaling mainly at the inner leaflet of the plasma membrane (PM) and may themselves be regulated during TCR activation (29, 30). Therefore, we examined how the mutant activating $G_{\alpha i2}$ protein influences early events mediated by the TCR signaling complex at the PM. Previous work has shown that local cAMP at the PM of T cells stimulates the PKA-CSK pathway, which can counteract TCR signaling by deactivating the LCK non-receptor tyrosine kinase (29–31).

We found that both freshly isolated and previously activated patient T cells showed normal, not increased, TCR proximal signaling (Fig. 5, A to D; fig. S15, A to D, and I to L; and fig. S16, A to D). However, the same cells showed enhanced distal phosphorylation of ribosomal S6 protein, a hallmark of growth and proliferation (Fig. 5, E to G; fig. S15, E, F, M, and N; and fig. S16, E and H to J). S6 phosphorylation is mainly regulated by ERK/MAPK and PI3K-AKT signaling pathways (Fig. 5G, right) (32). Indeed, we observed enhanced and prolonged phosphorylation of ERK1/2 and p90RSK as well as AKT and p70S6K, in patient T cells and in healthy donor CD4⁺ T cells transduced to express activating mutant $G_{\alpha i2}$ proteins (Fig. 5, G to L; fig. S15, G, H, O, and P; and fig. S16, F to J). Treating patient T cells with the PI3K/AKT/mTOR pathway inhibitor LY294002 and the mitogen-activated or extracellular signal-regulated protein kinase kinase (MEK) inhibitor U0126 normalized S6 phosphorylation by preventing hyperactivation of AKT and p90RSK, respectively (fig. S17). Thus, active $G_{\alpha i2}$ protein augments TCR-induced ERK/MAPK and PI3K/AKT/mTORC1 signaling pathways at a step unexpectedly downstream of proximal TCR signaling events.

Mutant $G_{\alpha i2}$ modulated TCR-dependent signaling independently of cAMP

We determined that mutant $G_{\alpha i2}$ proteins from the patients inhibited AC-mediated cAMP production and that patient fibroblasts produced less cAMP (Fig. 1, H to J, and fig. S3). However, intracellular cAMP, when raised, generally plays suppressive roles in immune cells (30). Therefore, suppression of cAMP production by mutant $G_{\alpha i2}$ proteins might cause increased T cell responsiveness. We found that patient T cells produced cAMP at levels within the range of healthy donor T cells (Fig. 6A). This would be consistent with the major ACs expressed in primary human T cells being AC3, AC7, and AC9 (fig. S11, A and B), and which are not expected to show substantial regulation by $G_{\alpha i2}$ (6, 7).

To investigate the role of cAMP in T cell hyperresponsiveness further, we investigated whether manipulating cAMP levels could mimic or rescue the patient T cell phenotypes. KO of AC3 or AC7 in healthy donor T cells had elevated or decreased endogenous cAMP levels respectively, but neither affected TCR-induced S6-regulatory signaling, cellular activation, or proliferation (Fig. 6, B to F; fig. S11, C and D; and fig. S18). Furthermore, an exogenously added cAMP analog failed to normalize the increased responsiveness of patient

T cells (fig. S19). Although these approaches did not specifically measure or manipulate local cAMP levels near the PM microdomain, the normal TCR proximal signaling in patient T cells suggests $G_{\alpha i2}$ may not affect local cAMP pools (Fig. 5, A to D; fig. S15, A to D and I to L; and fig. S16, A to D).

Thus, our findings would be consistent with $G_{\alpha i2}$ proteins regulating TCR signaling via cAMP-independent mechanisms.

The interaction between $G_{\alpha i2}$ and RASA2 regulated TCR-dependent responses

To determine how hyperactive $G_{\alpha i2}$ protein may regulate S6-regulatory signaling pathways through cAMP-independent mechanisms, we investigated $G_{\alpha i2}$ -interacting proteins by performing affinity pulldown followed by quantitative mass-spectrometry. For these experiments, we used $G_{\alpha i2}$ -Thr182Ala bound to $GTP_{\gamma}S$ as the bait protein, reasoning that relevant interactors might preferentially bind the active form of $G_{\alpha i2}$. We identified well-known and previously reported interactors of $G_{\alpha i2}$ (e.g., G_{β} subunits, RGS, G-protein signaling modulator proteins, RASA3), and previously unappreciated interacting proteins (including RASA2, PP2A-A α , PP2A-C α) (Fig. 6G and Data File S2). We did not detect any AC in our $G_{\alpha i2}$ -interacting proteins, which would support that the T cell hyperresponsive phenotype may be regulated by cAMP-independent mechanisms.

RASA2, a member of the RasGAP (Ras GTPase-activating protein) family, was an intriguing candidate because its target, the RAS proteins, are major upstream regulators for the ERK/MAPK and PI3K pathways (33, 34) that were augmented in the patients' T cells (Fig. 5). We confirmed the $G_{\alpha i2}$ -RASA2 interaction by coimmunoprecipitations from lysates of cells overexpressing RASA2 and $G_{\alpha i2}$ proteins (Fig. 6H), or of endogenously expressed proteins in patient T cells (Fig. 6I). Interactions of RASA2 with multiple different activating mutant $G_{\alpha i2}$ proteins were stronger than with WT $G_{\alpha i2}$, suggesting that RASA2 might be an effector target of $G_{\alpha i2}$ (Fig. 6, H and I, and fig. S20A). Purified WT $G_{\alpha i2}$ protein directly bound RASA2 when loaded with GDP, but the interaction was markedly strengthened when loaded with $GTP_{\gamma}S$ to "lock" $G_{\alpha i2}$ in its active state (Fig. 6J).

We found that depletion of RASA2 in T cells from healthy donors enhanced TCR-induced S6-regulatory signaling pathways, cellular activation, and proliferation (Fig. 6, K and L, and fig. S20, B to D). These observations phenocopied the effects of activating $G_{\alpha i2}$ proteins and indicated that RASA2 normally negatively regulates these responses. Gene editing of P1 T cells revealed that KO of RASA2 augmented the T cell hyperactivation, consistent with the mutant $G_{\alpha i2}$ having delayed (but not completely blocked) cycling upstream of RASA2 (Fig. 6M and fig. S21).

The levels of T cell hyperactivation in RASA2 KO T cells were comparable whether or not activated $G_{\alpha i2}$ was also present (Fig. 6M and fig. S21). As the expression of the $G_{\alpha i2}$ mutant did not further activate T cells in the absence of RASA2, it indicated that activated $G_{\alpha i2}$ regulated these T cell responses completely or largely via RASA2.

These observations suggested a model in which activating $G_{\alpha i2}$ proteins, by binding RASA2, might relieve RASA2's negative regulation of S6-regulatory signaling and T cell activation. Additionally, we identified and confirmed an interaction between active $G_{\alpha i2}$ with PP2A Ser/Thr phosphatase complex members, PP2A-A α and PP2A-C α (Fig. 6G, fig. S20, E to I, and Data File S2). As PP2A negatively regulates both ERK/MAPK and p70S6K pathways, this interaction might reinforce the negative regulatory effects of RASA2 (35).

Thus, our data suggest that active $G_{\alpha i2}$ enhances T cell activation in a cAMP-independent manner, by preventing negative regulation by RASA2 and/or the PP2A complex.

Mutant $G_{\alpha i2}$ proteins sequestered RASA2 resulting in augmented RAS activity in T cells

Like $G_{\alpha i2}$ and other G-proteins, RAS functions according to its GDP- or GTP-bound states, and RASA2 suppresses RAS activation by accelerating RAS GTPase activity (33, 34). Patients' T cells consistently showed enhanced activation of RAS and downstream ERK1/2 upon TCR stimulation, suggesting that active $G_{\alpha i2}$ limits RASA2 activity in T cells (Fig. 7A and fig. S22, A and B). To test whether RASA2's GAP activity towards RAS was inhibited by $G_{\alpha i2}$, we mixed purified recombinant RASA2 without or with active $G_{\alpha i2}$ (loaded with GTP γ S), HRAS, and GTP, then measured RAS GTPase enzymatic activity via consumption of GTP. As GTP levels were unchanged by including active $G_{\alpha i2}$, these data suggest that $G_{\alpha i2}$ did not directly inhibit RASA2's GAP activity toward RAS (Fig. 7B, and fig. S22C).

We then considered the possibility that active $G_{\alpha i2}$ could indirectly inhibit RASA2's GAP activity by altering its cellular location. Quantitative confocal imaging in patients' cells or in cells overexpressing activating $G_{\alpha i2}$ mutants revealed that active $G_{\alpha i2}$ redistributed RASA2 toward the PM (Fig. 7, C to F, and fig. S22, D and E). By performing Förster resonance energy transfer (FRET)-based fluorescence lifetime imaging, we examined whether this membrane recruitment of RASA2 is mediated by its interaction with $G_{\alpha i2}$ (Fig. 7G). Fluorescence lifetime of a WT $G_{\alpha i2}$ -mTFP1 FRET donor was quenched only in the presence of a YFP-RASA2 acceptor, indicating that the two proteins are closely apposed (within 10 nm) (Fig. 7, G to I). Quenching was mainly observed at the cell periphery near the PM, with FRET efficiency reciprocally also increased there (fig. S22F). The mTFP1 lifetime was dramatically shorter when the activating $G_{\alpha i2}$ mutant Gln205Leu was co-expressed with RASA2 (red vs. blue color in Fig. 7H), with FRET efficiency also increased under the same conditions (fig. S22F), supporting an increased association between these two proteins.

In T cells, exogenously expressed HRAS or NRAS localizes to both the PM and Golgi, but RAS activation predominates at the Golgi during TCR activation (26, 36). Therefore, we assessed whether RAS activation at the Golgi correlates with $G_{\alpha i2}$ -mediated RASA2 redistribution to the PM after TCR stimulation. Using Jurkat cells co-expressing fluorescently-tagged RAS, active-RAS sensor (RBD), and a Golgi marker (GalT), along with $G_{\alpha i2}$ proteins, we performed confocal microscopy colocalization analysis. We observed greatly enhanced RAS activation at the Golgi when cells expressed an activating mutant $G_{\alpha i2}$ vs. normal $G_{\alpha i2}$ (intensity ratio 2–2.8, vs. 1.7) (Fig. 7, J to L, and fig. S22, G to I).

PI3K-AKT activation is PM-restricted, so the enhanced PI3K-AKT signaling in the patient T cells implied that PM RAS activity was also increased (Fig. 5, I and K; fig. S15, H and P; fig. S16, G to I; and fig. S17) (37). Because both RAS and $G_{\alpha i2}$ at the PM undergo dynamic spatiotemporal regulation, $G_{\alpha i2}$ -mediated RASA2 sequestration might also enhance local RAS activity within the PM microdomains (29, 34). Overall, our findings suggest that active $G_{\alpha i2}$ enhances TCR-induced RAS activity by physically directing RASA2 away from RAS within cells.

Discussion

Previously, activating mutations in other G_{α} subunits have been reported in various types of human cancers and diseases, and investigations have focused on their augmented downstream signaling pathways (38, 39). By studying humans with activating *GNAI2* mutations, we find enhanced suppression of effector cAMP generation and establish a pathogenic role of chronic decoupling of active $G_{\alpha i2}$ from GPCRs. Chronic decoupling can result from multiple factors such as faster GTP binding, slower intrinsic hydrolysis, and RGS insensitivity. When compounded, they could disproportionately prolong the time G-proteins take to cycle from their active free form, back to their inactive GPCR-bound form capable of responding again to GPCR agonists. Rapid G-protein cycles may be required to respond to quickly changing environmental cues, such as for optimal spatiotemporal sensing of chemokine gradients through $G_{\alpha i}$ during cell migration. A similar requirement apparently underlies rapid photoreceptor deactivation to detect sudden changes in moving objects through $G_{\alpha t}$ during spatiotemporal visual signaling (40). Nevertheless, we do not exclude a possible additional contribution of active $G_{\alpha i2}$ through sequestering or otherwise interfering with free $G_{\beta\gamma}$ signaling (21, 41–44).

While we have established that the impaired migratory behavior of immune cells leads to the patients' infection susceptibility and immune dysregulation, their additional clinical features suggest that the migratory behavior of non-immune cells is similarly impaired during development. The patients' prominent midline anatomic defects may reflect abnormal neural crest cell migration, which proceeds along the anterior-posterior axis during embryogenesis to help form many tissues (45). Such migration requires CXCL12 signaling through the CXCR4 chemokine receptor, whose deficiency causes cerebellar and other anatomical abnormalities and is embryonically lethal in mice (46). Furthermore, partial knockdown of *Gnai2* in developing mouse embryos perturbed neuronal migration during corticogenesis, consistent with $G_{\alpha i2}$ regulating differentiated non-immune cell migration during development and paralleling defective leukocyte migration in *Gnai2* KO mice (15, 47). Unfortunately, we could not track migration of non-immune cells during development, as attempts to generate a patient-mimicking KI mouse model were unsuccessful.

Besides establishing an important physiologic requirement in vivo for the normal cycling of $G_{\alpha i2}$ during cell migration, our patients with constitutively activating mutant $G_{\alpha i2}$ reveal a $G_{\alpha i2}$ -mediated but cAMP-independent RAS-regulatory pathway that controls the amplification of T cell responses through RASA2. We now place $G_{\alpha i2}$ immediately upstream of RASA2 at the nexus of GPCR and TCR signaling pathways. In healthy individuals, this pathway could operate physiologically when $G_{\alpha i2}$ is transiently activated

by chemokine receptors, to coordinate T cell migration with and optimize TCR-induced activation and proliferation. Such a model may be consistent with the previously reported co-stimulatory contribution of chemokine receptor signaling during T cell activation (48). In patients with activating *GNAI2* mutations, we propose that the resulting stronger TCR input breaks peripheral tolerance, predisposing to the autoimmunity and age-associated lymphocytosis seen in some individuals. Exploiting this $G_{\alpha i2}$ -RASA2-RAS signaling axis could facilitate development of T cell-based anti-tumor therapies. For example, improving expansion and activity of T cells expressing chimeric antigen receptor (CAR) could be achieved by fusing CAR to mutant $G_{\alpha i2}$ domains that preferentially scavenge RASA2 (49). Indeed, our observations support recent studies using CRISPR screens in primary T cells and mouse models of cancer immunotherapy that provide evidence of RASA2 as a negative regulator (50, 51). As KO of RASA2 in transgenic CD8⁺ T cells or CAR T cells increases antigen-specific tumor cell killing in vitro (50, 51), those results suggest that a similar approach targeting $G_{\alpha i2}$ upstream of RASA2 might also be promising.

There are several limitations to our study. First, we focused on the impact of the patients' *GNAI2* mutations on their $G_{\alpha i2}$ proteins and not on $G_{\beta\gamma}$ proteins. Their activating $G_{\alpha i2}$ mutants are expected to not only decouple from GPCR but also increase dissociated $G_{\beta\gamma}$. Free $G_{\beta\gamma}$ signaling regulates chemokine-mediated cell migration by modulating phosphoinositide-3-kinase γ (PI3K γ) and possibly AC activities (52). However, one of our patients had an Arg209Trp activating mutation in the "Gly-Arg-Glu" triad, which is required for $G_{\beta\gamma}$ dissociation from G_{α} for other G-proteins (21). This variant is predicted to have the opposite effect of decreasing free $G_{\beta\gamma}$, yet it was still associated with impaired leukocyte migration. Therefore, while the activating $G_{\alpha i2}$ mutants could also exert secondary effects through increased free $G_{\beta\gamma}$, this explanation does not account for our observations. Studies addressing the impact of the activating $G_{\alpha i2}$ mutants on $G_{\beta\gamma}$ biology, including downstream spatiotemporal effects on cell polarity, adhesion, and migration, will be needed to clarify these points.

A second limitation of our study is that while we have shown that activated $G_{\alpha i2}$ acts primarily through RASA2 to drive RAS-mediated T cell hyperresponsiveness, the effect conferred by RASA2 KO was greater than by activating $G_{\alpha i2}$ alone. Several factors may account for this difference. Constitutively activating $G_{\alpha i2}$ mutants are expected to have prolonged cycling but still pass through an inactive form, which binds less well to RASA2. In our experiments, we tested Thr182Ala, but other variants having different cycling times may show different relative effect sizes. Furthermore, the relative amounts of active $G_{\alpha i2}$ vs. RASA2 in the cells and the stoichiometry required for efficient sequestration away from the Golgi are unknown. Alternatively, $G_{\alpha i2}$ -independent factors could also regulate RASA2. Additional studies are needed to address these possibilities.

Finally, based upon their activated RAS-MAPK signaling, patients with germline activating *GNAI2* mutations can now be included within the RASopathy spectrum (53, 54). Indeed, our patients show clinical overlap, including autoimmunity, with patients having typical RASopathies such as Noonan syndrome (53, 55). The widespread tissue expression of $G_{\alpha i2}$ and RASA2, along with the diverse biological functions mediated by RAS proteins in cancer pathogenesis, raises the interesting possibility that the $G_{\alpha i2}$ -RASA2-RAS signaling axis

might broadly regulate growth, proliferation, and differentiation in the body. Consistent with this possibility, inhibitory roles of *RASA2* in cellular growth and proliferation have been reported in fibroblast and melanoma cells (56). Additionally, somatic activating *GNAI2* mutations have been identified in cancers including melanoma, adrenal cortical tumors, and ovarian sex cord stromal tumors (39, 57), paralleling loss-of-function *RASA2* mutations in melanomas and dysregulated RAS activity in various human cancers (33, 34, 56, 58). Thus, our discoveries also provide fresh insight into the molecular etiology and potential therapeutic targets to disrupt $G_{\alpha i2}$ for tumors with oncogenic *GNAI2* or RAS pathway mutations.

Materials and Methods

Study Participants and Human Sample Collection

The patients originated from diverse ethnicities/geographic regions (Latino/Admixed American, Non-Finnish European, European-Finnish, West Sub-Saharan African, Middle Eastern Arab). Age and sex of patients are contained in Supplementary Text 4. All enrolled subjects (patients, family members, healthy donors) provided written informed consent to participate in local Ethics or IRB-approved research protocols from various institutions (see Supplementary Materials for details). Patient or parent/legal guardian provided additional written authorization for publication of potentially identifiable facial photographs. Whole blood samples, fingernail clippings, skin punch biopsies, blister fluid, mouth washings, and skin swabs were obtained for experimental analyses in accordance with research protocols.

Mice

Animal housing, care, and experimental procedures of mice (*Mus musculus*) were performed under animal study protocols approved by the NIAID Animal Care Use Committee or the Garvan Institute of Medical Research/St Vincent's Hospital Animal Ethics Committee. Mouse euthanasia was performed by carbon dioxide inhalation followed by cervical dislocation. Wildtype (WT) B6 (C57BL/6J, strain# 000664), CD45.1 congenic B6 (B6.SJL-Ptprca Pepcb/BoyJ, strain# 002014), and Thy1.1 congenic B6 (B6.PL-Thy1a/CyJ, strain# 000406) were purchased from the Jackson Laboratory (Bar Harbor). Adoptive transfer experiments used female mice at 6 to 8 weeks of age. Generation of *Gnai2* KI embryos was as described in the Supplementary Methods.

Whole Exome Sequencing (WES), Whole Genome Sequencing (WGS), and Analyses

We conducted WES on the index patient (P1) and her healthy parents and sister. Patients 2 to 18 and 20 with mutations in the *GNAI2* gene were identified from WES or WGS data (14), either through GeneMatcher (phenotype-agnostic) or through other inquiries that were broadly immune phenotype driven (59). Exome or genome libraries from gDNA were generated, and variant calling and analysis performed using various platforms (see Supplementary Materials for details). The familial *GNAI2* mutation in P19 was identified by Sanger sequencing. In the patients, no other candidates besides *GNAI2* were shared under *de novo*, autosomal recessive (AR), or variable-penetrance autosomal dominant (AD) models of inheritance (table S1). All genomic variants in this manuscript are described according to Human Genome Variation Society recommendations (60), using GenBank

Reference Sequences [NC_000003.11](#)(gDNA), [NM_002070.2](#) (mRNA), and [NP_002061.1](#) (protein) based upon genome assembly Build GRCh37 unless otherwise indicated.

Characterization of G_{ai2} GTPase and RASA2 GAP Activities

For GTP binding and hydrolysis assays, recombinant G_{ai2} protein was mixed with BODIPY-FL-GTP or BODIPY-FL-GTP_γS (Thermo Fisher), and the kinetics of in vitro G_{ai2} protein activation measured (61). To examine RGS sensitivity, G_{ai2} was incubated with RGS16 before adding BODIPY-FL-GTP. Nucleotide-binding data were fit with one phase exponential equation $F = a - b e^{-kt}$, where F is a specific increase of fluorescence, to obtain k. GTP binding and hydrolysis curves were fit with the equation $F = (C_0 k_1 / (k_2 - k_1)) (e^{-k_1 t} - e^{-k_2 t})$ for the intermediate product in two sequential reactions (62). For RGS binding assays, recombinant His₆-G_{ai2} and GST-RGS16 were incubated together at 4 °C in the presence of a slurry of glutathione sepharose beads and either GDP or GDP plus aluminum magnesium fluoride (AMF) to mimic the transition state for GTP hydrolysis (18). Bound proteins were eluted from beads, resolved by SDS-PAGE, and immunoblotted. GAP activity of RASA2 toward RAS protein was measured according to manufacturer's instructions (Promega), with modifications. Unless stated otherwise, 1 μM His-tagged hRas (Cytoskeleton, Inc.), 0.25 μM maltose binding protein (MBP) / MBP-RASA2, and 2.5 μM GST / GST-G_{ai2} proteins, pre-loaded with either GDP or GTP_γS, were incubated with 5 μM GTP and 1 mM DTT in the provided GTPase/GAP Buffer. Levels of GTP remaining were measured.

Cells, Media, and Cell Culture

Human embryonic kidney 293T cells (293T), HEK293 cell line stably lacking AC3 and AC6 (HEK-ACΔ3/6) (63), Platinum-E cells, NIH/3T3, Jurkat T cells, and HL60 cells were cultured in DMEM or RPMI 1640 medium with supplements. Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood by Ficoll-Paque PLUS density gradient centrifugation (Cytiva). Pan-T cells or CD4⁺ T cells were isolated from PBMCs by negative selection (Miltenyi Biotec), or by fluorescence-activated cell sorting (FACS) using a BD FACSAria III cell sorter. For functional studies, purified T cells were used either immediately or activated using the T Cell Activation/Expansion Kit (Miltenyi Biotec). The latter were expanded in the presence of 100 U/mL recombinant human IL-2 for 2 to 3 weeks before use. Neutrophils were isolated by density gradient separation and used immediately for experiments. Fibroblasts were isolated from skin punch biopsies and cultured as described (64). Murine leukocytes isolated from spleens, inguinal and axillary lymph nodes were activated with plate bound anti-mouse CD3 (5 μg/mL) and soluble anti-mouse CD28 (1 μg/mL) and cultured in RPMI medium containing 100 U/mL recombinant human IL-2.

cAMP Measurements

293T cells (previously transfected with individual G_{ai2} plasmid using polyethylenimine) or human dermal fibroblasts were stimulated at 37 °C for 20 minutes with 5 μM forskolin (FSK) in the presence of 0.5 mM of the nonspecific inhibitor of phosphodiesterase 3-isobutyl-1-methylxanthine (IBMX). In some experiments, T cells (10 to 20 days after initial

activation) were stimulated with 50 μ M FSK/ 0.5 mM IBMX at 37 °C for 20 to 60 minutes. Cells were lysed and accumulated intracellular cAMP levels measured using the colorimetric cAMP ELISA Kit per manufacturer's instructions (Cell Biolabs). cAMP was alternatively measured using a YFP-Epac-rLuc cAMP biosensor (pcDNA3L-His-CAMYEL) in 293T cells previously transfected also with plasmids expressing $G_{\alpha i2}$ and CXCR4 (65). Cells were stimulated in BRET Buffer (0.5 mM $MgCl_2$ and 0.1% BSA fraction V in PBS) with FSK (0, 10^{-8} - 10^{-4} M) in the presence of 5 μ M Coelenterazine h for 10 minutes at room temperature. Luminescence and fluorescence readings were collected by sequential integration of the signals detected in the 480 ± 20 nm and 530 ± 20 nm windows for luciferase (Rluc) and YFP light emissions, respectively. Relative cAMP levels were indicated as 1/BRET (Rluc/YFP). In some experiments, 293T cells were transfected 24 hours previously with $G_{\alpha i2}$ plasmid and cAMP GloSensor reporter plasmid. Luminescence was measured at baseline and for 60 minutes after cells were treated with 2.5 μ M FSK.

Clinical Phenotyping and Analysis

Using criteria standardized across the patient cohort, clinical histories were coded into Human Phenotype Ontology (HPO) terms (66) and dysmorphology terms (defined at <https://elementsofmorphology.nih.gov/index.cgi>). For an individual patient, a value of “yes,” “no,” “ND” (not determined), or “NA” (not applicable, because of age or sex) was assigned to each HPO term (see table S4). Values were used to compute frequencies across the cohort for each HPO term. Selected midline (fig. S4A) or immune (fig. S4B) phenotypes were displayed as heatmaps, and phenotypes were also summarized at different level HPO categories.

T cell Migration Assays

Migration of T cells from patients and healthy donors 12 to 24 days post-activation, or of $G_{\alpha i2}$ WT- or variant- transduced healthy donor T cells, was assessed in vitro using a standard Transwell system with 5 μ m membrane pore inserts. Recombinant human CXCL12 or CCL21 was added to the lower compartment with an equivalent number of CountBright Absolute Counting Beads (Thermo Fisher) to each well. After incubating at 37°C for 2 hours, the contents of the lower chamber were collected and stained with antibodies for flow cytometric analysis. The number of recovered cells was normalized to the number of CountBright Absolute Counting Beads collected. Migrated cells were expressed as % of the total number of cells collected from a well without a Transwell insert. Chemotaxis was calculated by subtracting random migration (determined by the wells without added chemokines). For in vivo assessment of T cell migration, wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor). Mouse CD45.1⁺Thy1.2⁺ and CD45.2⁺Thy1.2⁺ lymphocytes were activated and transduced with retroviral particles containing MSCV-GFP-T2A-*GNAI2* (WT) or MSCV-GFP-T2A-*GNAI2* (WT or variants), respectively. Transduced cells (mixed 1:1) were intravenously injected into CD45.2⁺Thy1.1⁺ recipient mice, followed by anti-CD5 PE/Cy7 one hour later to label leukocytes in the splenic red pulp and the blood (67), and euthanized 3 minutes later. T cells recovered from tissues and blood were identified as donor (Thy1.2⁺GFP⁺), WT $G_{\alpha i2}$ transduced cells were distinguished as CD45.1⁺Thy1.2⁺GFP⁺, and variant $G_{\alpha i2}$ transduced cells were CD45.2⁺Thy1.2⁺GFP⁺. The normalized ratios of variant to WT $G_{\alpha i2}$

transduced cells were calculated by normalizing for differences in transduction efficiencies of each donor cell prep, migration differences between the non-transduced CD45.1⁺Thy1.2⁺ and CD45.2⁺Thy1.2⁺ populations, and the WT G_{ai2} (CD45.1⁺Thy1.2⁺) vs. WT G_{ai2} (CD45.2⁺Thy1.2⁺) control group mice (average of these mice was set to a ratio of 1).

Neutrophil Migration Assays

Where neutrophils could be isolated freshly from patients and tested within 24 hours, their migration to buffer or N-formylmethionine leucyl-phenylalanine (fMLP) was measured ex vivo at 37 °C using EZ-TAXIScan instrumentation (Effector Cell Institute, Tokyo, Japan) as described (68). Alternatively, G_{ai2} WT- or variant- transduced HL60 cells were differentiated with 1.3% DMSO for 5 days, and their migration to fMLP, CXCL12, or leukotriene B4 (LTB4) was similarly assessed. Digital images of migrating cells were captured every 15 to 30 seconds for 30 minutes to 1 hour for quantitative analysis. In some experiments, migration to fMLP of DMSO-differentiated, G_{ai2}-transduced HL60 cells was also evaluated using the Transwell system. In vivo migration into cutaneous blister fluid of P1 and healthy donors was evaluated 16 hours after applying a suction blister device to skin as described (69), or in the oral cavity of subjects using a timed (10 seconds) oral rinsing procedure with 10 mL of sterile saline (0.9% Sodium Chloride) (70). The cell pellets from either blister exudate or oral cavity rinses were stained with a combination of anti-human antibodies for quantitative flow cytometric analysis.

Measurement of Chemokine Receptor - G_{ai2} Interactions

293T cells, previously transfected with CXCR4-YFP or CCR7-YFP acceptor plasmids and G_{ai2}-Rluc donor plasmid, were stimulated in BRET buffer for 5 minutes at 37°C with increasing amounts of CXCL12 or CCL21 before adding 5 µM Coelenterazine h. Luminescence and fluorescence readings were collected, and net BRET values were calculated by subtracting the background BRET signal from cells expressing only BRET donor (G_{ai2}-Rluc).

TCR Stimulation

Purified human T cells were stimulated in complete RPMI medium and 100 U/mL recombinant human IL-2, using 1 µg/mL soluble anti-human CD3 (α-CD3), 1 µg/mL soluble anti-CD3 and anti-CD28 antibodies (α-CD3/28), or beads with immobilized anti-CD2, anti-CD3, and anti-CD28 antibodies (Beads; at 1:1 bead to cell ratio; Miltenyi Biotec). In some cases, cells were previously stained with carboxyfluorescein succinimidyl ester (CFSE) or CellTrace Violet. Flow cytometry was used to analyze CD69 expression at 20 hours, or CD25 expression and CFSE dilution at 96 hours after stimulation. For transduced CD4⁺ T cells or Cas9/RNP transfected T cells, cells were stimulated with α-CD3/28 (0 to 1000 ng/mL), and CD69 and CD25 were examined at 18 to 20 hours later. For biochemical experiments, T cells were rested in serum-free RPMI at 37°C for one hour, incubated with 5 µg/mL α-CD3 in serum-free RPMI containing 0.5% bovine serum albumin (BSA) on ice for 10 minutes, followed by addition of 20 µg/mL of goat anti-mouse IgG antibodies at 37°C for 0 to 30 minutes. To stop stimulation, cells were washed with ice-cold PBS, and either lysed for immunoblot or active RAS pull-down, or fixed for flow cytometric intracellular staining.

In some experiments, inhibitors (3 μ M LY294002, 10 μ M U0126, or DMSO) were added one hour prior to, or the cAMP analog 8-CPT-cAMP 15 minutes prior to, T cell stimulation.

Gene knockout (KO) by CRISPR Cas9/RNP system

Cas9/RNP complexes were prepared according to manufacturer's instructions (IDT) (71) and transfected using a 4D nucleofector system into primary human T cells (P2 solution, program EH-100) or Jurkat T cells (SE solution, program CL-120). The total amount of transfected gRNA per nucleofection was kept constant by adding gNeg RNA as needed. CRISPR/Cas9-mediated KO efficiency was evaluated by immunoblotting or estimated via TIDE assay (72). For evaluation of the patient's mutant allele-specific KO, cDNA was isolated and subjected to Sanger dideoxy sequencing. Experiments were performed 6–7 days after transfection.

GST Pull-down Assays and Mass Spectrometry Analysis

Glutathione S-transferase (GST)-fused $G_{\alpha i2}$ proteins bound to glutathione (GSH)-agarose resin were loaded with 500 mM GDP or GTP $_{\gamma}$ S and washed before use. For pull-down assays, MBP fusion proteins were prepared in pull-down buffer (lysis buffer with 500 μ M GDP / GTP $_{\gamma}$ S and 20 mM MgCl $_2$), incubated with prepared GST fusion protein-bound resin, and interacting proteins were eluted for immunoblotting. Active Ras pull-down assays were performed according to manufacturer's instructions (Cytoskeleton, Inc.). For mass spectrometry analysis, clarified Jurkat T cell lysates prepared in pull-down buffer were incubated with GST- or GST- $G_{\alpha i2}$ (Thr182Ala)- bound resin in the presence of GTP $_{\gamma}$ S. Bound protein complexes were eluted and resuspended in acid extractable detergent. Samples were trypsin-digested and labeled with different isotopes using "reductive dimethylation" essentially as described for protocol C (73). Samples were mixed and a single long liquid chromatography with tandem mass spectrometry (LC-MS-MS) experiment was performed using the EASY-nLC 1000 Liquid Chromatograph interfaced with a Orbitrap FusionTM LumosTM TribridTM Mass Spectrometer (Thermo Fisher). Data were analyzed using MaxQuant (74) specifying 0 missed sites to decrease digestion difference based variation at an FDR of 1%. Mass spectrometry proteomics dataset was submitted to the ProteomeXchange Consortium via the PRIDE (75) partner repository (identifier PXD048980 and 10.6019/PXD048980).

Microscopy

Human CD4⁺ T cells or $G_{\alpha i2}$ KO Jurkat T cells transfected with plasmids expressing YFP-RASA2 and $G_{\alpha i2}$ were stained with CellBrite[®] Fix 555 plasma membrane dye (Biotium), fixed and permeabilized for endogenous RASA2 detection using polyclonal rabbit anti-RASA2 antibody (Novus Biologicals) /Alexa Fluor 488-conjugated goat anti-rabbit IgG, and DAPI-stained. RAS activation at the Golgi upon TCR stimulation was examined similarly as in (36). $G_{\alpha i2}$ KO Jurkat T cells were transfected with plasmids expressing mCherry-RBD, Cerulean-GalT, EGFP-HRAS / NRAS, and $G_{\alpha i2}$. Transfected cells were plated on non-treated or anti-CD3-coated chambered coverglasses. After 5 minutes, single cells expressing all fluorescent proteins in the field of view were imaged. Confocal images were acquired on either Airyscan-equipped LSM800 confocal (Zeiss) or SP8 confocal (Leica) microscopes. Images were taken with fixed acquisition settings, then analyzed and

automated using customized macro programs within ImageJ software. For fluorescence lifetime imaging microscopy (FLIM), 293T cells transfected with plasmids expressing mTFP1 and YFP were fixed and imaged on a Leica DMI 6000 SP5 confocal microscope. mTFP1 was excited at 805 nm with a femtosecond mode-locked (80 MHz repetition rate) Mai-Tai HP pulsed, multi-photon laser (Spectra Physics). Fluorescence was passed through a band-pass GFP filter at ET 525/50 (Chroma Technology Corp) and collected using a HPM100 Hybrid Detector R3809U-50 (Becker & Hickl; Hamamatsu Photonics). With SPC830 acquisition board, fluorescence decays were resolved by time-correlated single-photon counting. Acquired fluorescent transients were analyzed using SPCImage software according to single-life time decay and in ImageJ to determine FRET efficiencies in region of interest (ROI).

Supplementary Material

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Data and materials availability:

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. The WES datasets, where permitted under terms of patient informed consent for patients 1 to 6, 8 to 14, and 18 were deposited to the National Center for Biotechnology Information's database of Genotypes and Phenotypes (dbGaP) (77)

(accession number phs002817.v1.p1) under controlled access for qualified investigators and their institutions in accordance with potentially sensitive patient information. WES datasets for patients P7 and P15 are available to qualified investigators through inter-institutional data transfer agreements; requests should be made through the corresponding author. WGS datasets for P17 and P20 will be deposited to the Collecteur Analyseur de Données (CAD) under controlled and secured access for CAD Scientific and Ethics Committee (CSE)-validated research projects. P13 and P14 familial mutation was deposited in the ClinVar database under accession number SCV001955127. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (75) partner repository with the dataset identifier PXD048980 and 10.6019/PXD048980. Patient samples are available from H.C.S. via a Materials Transfer Agreement for Human Materials from the NIH.

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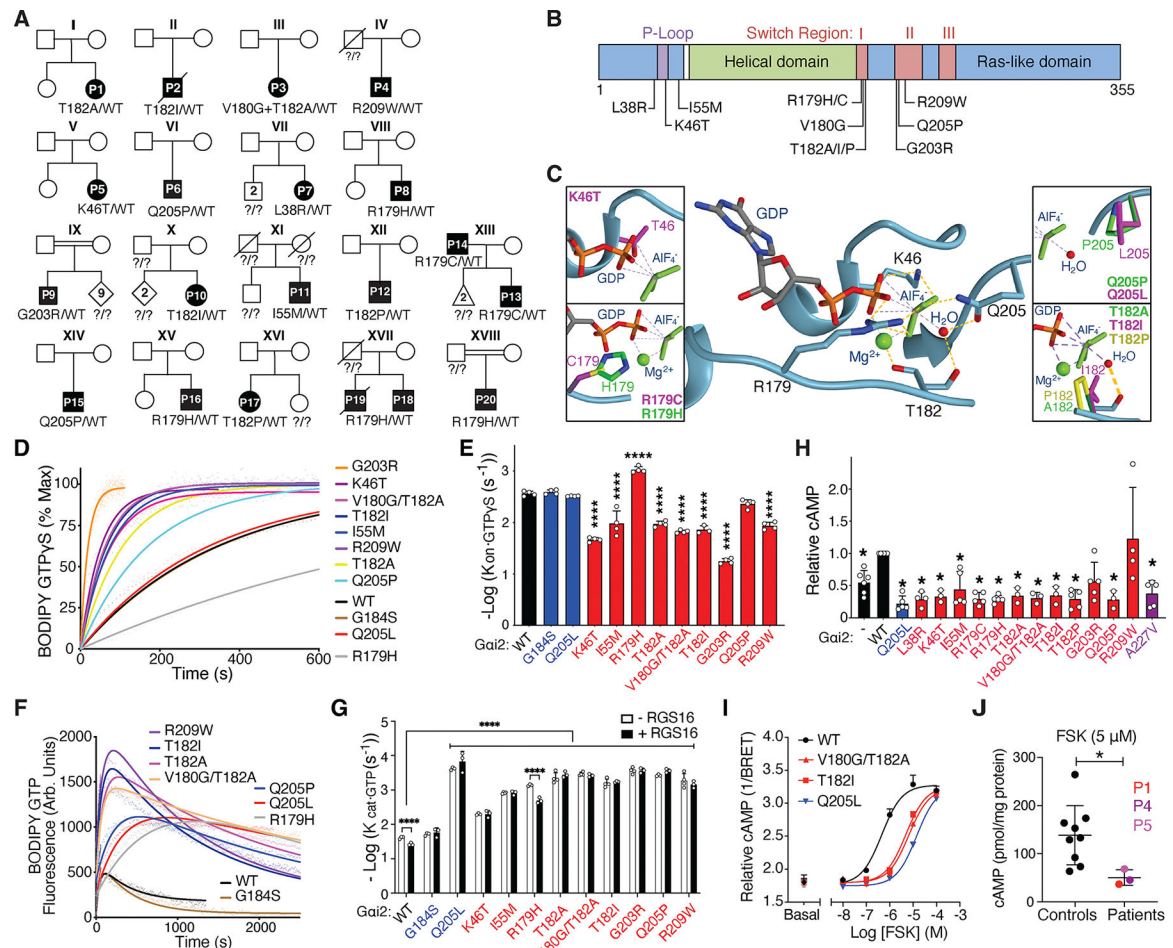
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(A) Patient pedigrees showing mutations and affected status. P8 was previously reported in a large cohort of individuals showing developmental disorders (14). (B) Location of mutations in the $G_{\alpha i2}$ protein, numbered according to the longest isoform that predominates across tissues including blood. (C) Structural model of $G_{\alpha i2} \cdot \text{GDP} \cdot \text{Mg}^{2+} \cdot \text{AlF}_4^-$ showing interactions in the GTPase catalytic site. AlF_4^- is a γ -phosphate mimic which acts as a transition state analog. Dashed lines, noncovalent bonds. Yellow, those mediated by $G_{\alpha i2}$. Insets show patient variants disrupting the interactions. The noncovalent bond between amino acid 182 and the nucleophilic H_2O is with the backbone amide oxygen on the residue. PDB: 1GFI. (D) GTP binding of non-hydrolyzable $\text{GTP}_{\gamma}\text{S}$ by purified recombinant $G_{\alpha i2}$ proteins. (E) Binding rate constants ($k_{on\text{GTP}\gamma\text{S}}$) from (D). Red, patients' variants. Blue, Q205L, GTPase-deficient positive control (18) and G184S, RGS-insensitive control (normal GDP/GTP exchange and intrinsic GTPase activity, but impaired RGS-mediated GTPase activity) (20). (F) GTPase assay. (G) Hydrolysis rate constants ($k_{cat\text{GTP}}$), with (black) or without (white) RGS16, from (F). (H) Forskolin (FSK)-stimulated cAMP in 293T cells after transfection with $G_{\alpha i2}$ variants, measured by ELISA. The increased cAMP in WT-transfected as compared to untransfected cells may reflect heterologous sensitization of AC (78). Purple, A227V, variant of unknown significance (15). (I) FSK-stimulated cAMP reporter activity in HEK293 cells

transfected with $G_{\alpha i2}$ variants and YFP-EPAC-RLuc reporter (and CXCR4). Relative cAMP was expressed as 1/BRET. **(J)** cAMP accumulation in primary fibroblasts from patients or healthy donors after FSK stimulation (see fig. S3I for basal levels). Data show representative (D, F, I) or mean $\pm$ SD (E, G, H, J) for 3–5 independent experiments (D–I), or for 3 patients (J). Individual points within graphs represent results from independent experiments (E, G, H, J). Statistical analyses were performed using one-way ANOVA with Tukey's multiple comparisons for comparing individual variants to WT for (E) and (G, without RGS); multiple t-tests using the Holm-Sidak method for comparison between with or without RGS16 for each variant (G, white vs. black); one-sample t-test with Two-stage step-up method of Benjamini, Krieger and Yekutieli with hypothetical value of 1 for (H); and unpaired t-test for (J). * $P < 0.05$; **** $P < 0.0001$.

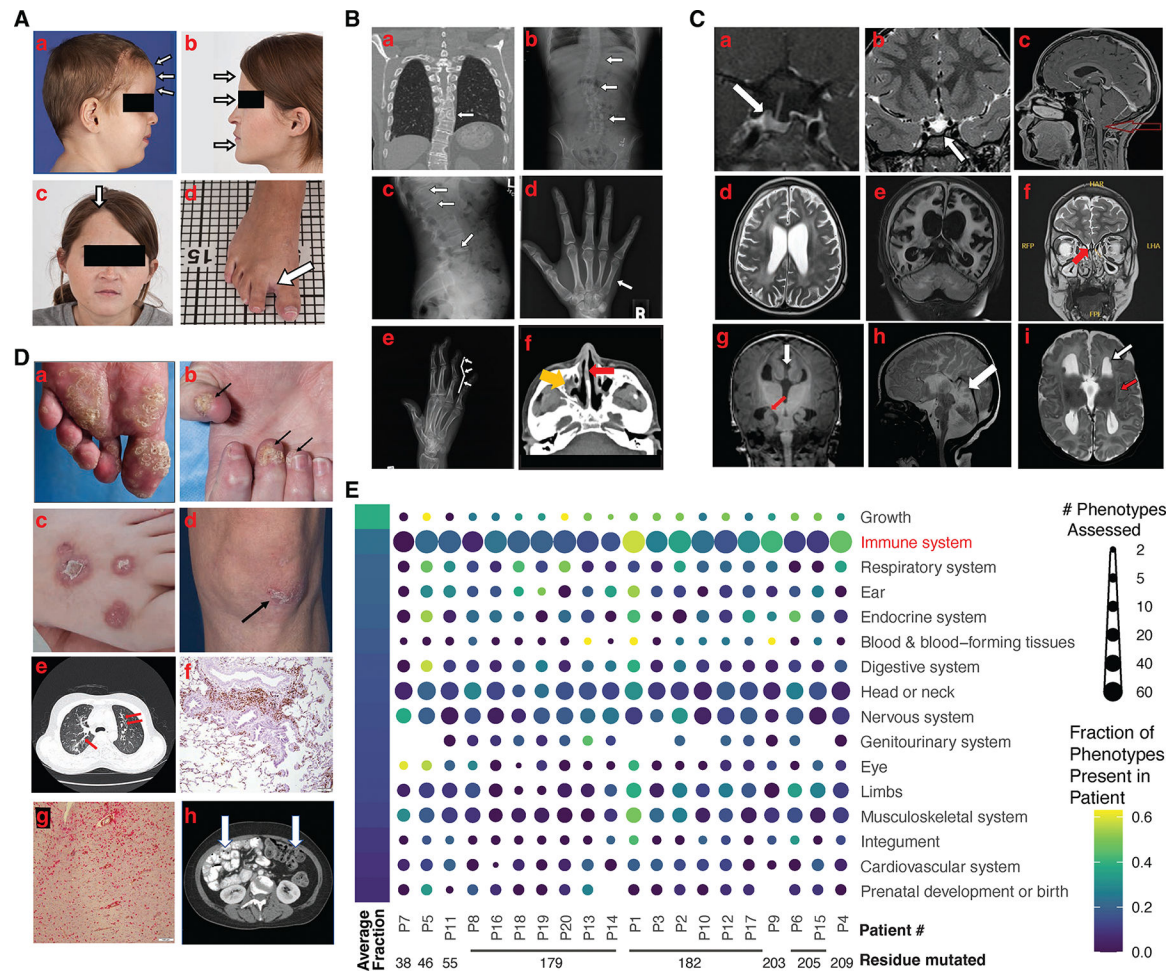


Figure 2. Selected clinical features of patients with activating *GNAI2* mutations.

(A) Dysmorphism: frontal prominence (a), flat face (b), high anterior hairline (c), sandal gap deformity (d). (B) Skeletal abnormalities: sagittal cleft vertebra (a), scoliosis (b), irregular vertebral endplates (c), brachydactyly type E (d), swan-neck deformity (e), deviated nasal septum (red arrow) with chronic sinusitis (yellow arrow) (f). (C) Neurological and associated midline defects: misshapen sella turcica (a), hypoplastic pituitary gland (b), Chiari I malformation (c), diffuse leukodystrophy (d) progressing to end-stage neurodegeneration (e), absence of olfactory bulbs (arrow shown for one side) (f), agenesis of the corpus callosum (white arrow) and hippocampus malrotation (red arrow) (g), cerebellar dysplasia (h), and polymicrogyria as well as subependymal (white arrow) and band (red arrow) heterotopia (i). (D) Infectious and inflammatory complications: persistent warts (a, b), rubella-vaccine induced skin granulomas (c), psoriasiform rash (d), bronchiectasis (e), T cell infiltrates in lung (brown) (f) or brain (red) (g) in absence of infection. Intestinal malrotation (h). (E) Human phenotype ontology (HPO) summarized at top-level categories for each patient. Size of circle, number of phenotypes assessed for a patient within each category. No circle, fewer than 2 phenotypes assessed. Color scale, fraction of those phenotypes confirmed in a patient. Top-level categories were sorted from top to bottom based on the average fraction across patients. The proportion of immune phenotypes present

was tested in patients having T182 mutations ([N = 6, sample median m = 0.315, SD = 0.145] compared to those without [N = 12, m = 0.194, SD = 0.131]; $t(16) = 1.788$, p-value = 0.046) by a one-tailed two-sample t-test. Patients 14 and 19 were removed from this analysis since patients 13 and 14 were related and patients 18 and 19 were related.

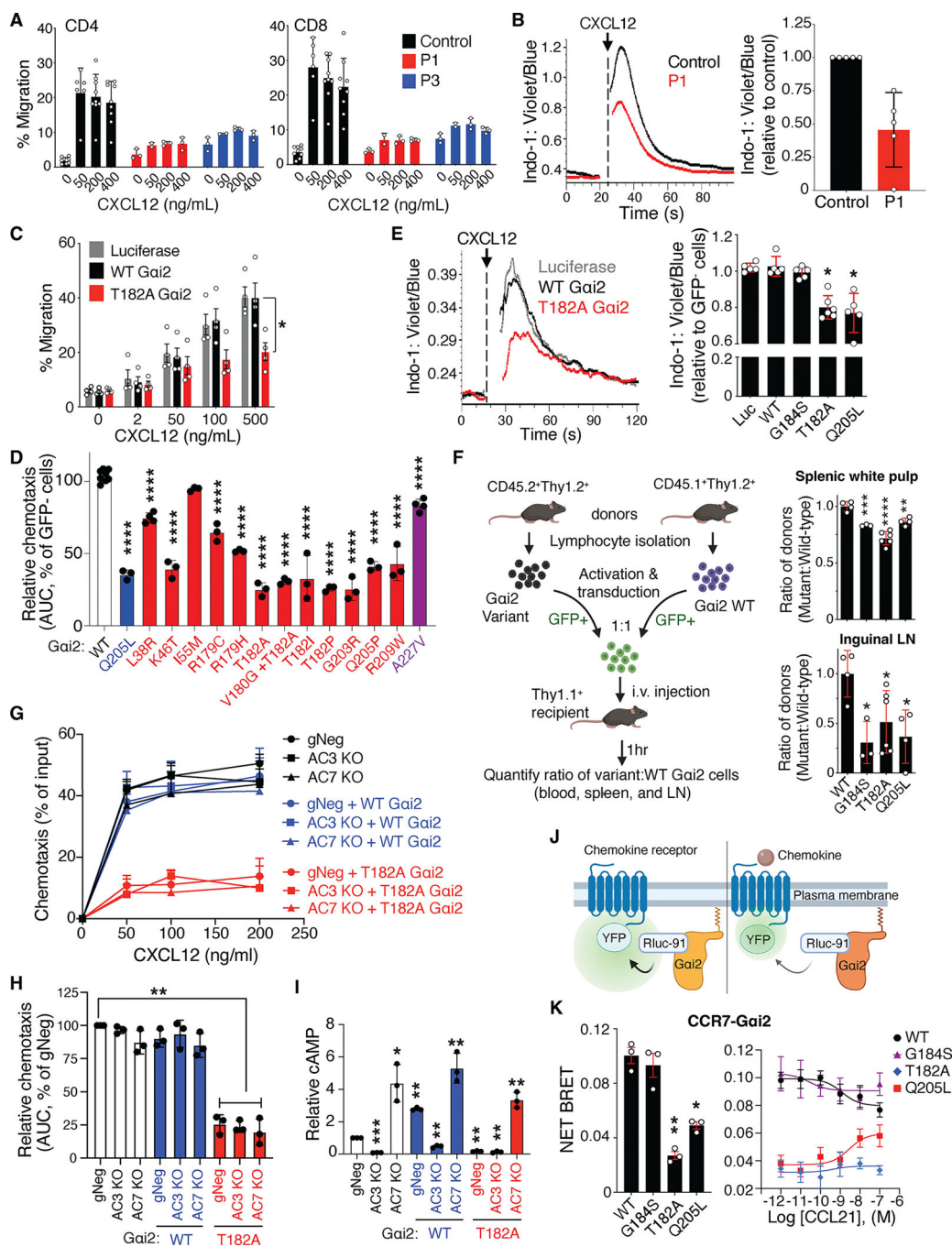


Figure 3. G_{αi2} mutants impair chemokine receptor signaling by decoupling from chemokine receptors.

(A) Transwell migration to chemokines of T cells from patients or healthy donors. (B) CXCL12-stimulated Ca²⁺ fluxes from a patient or healthy donor T cells (left), quantified as area under the curve (AUC) and normalized to healthy donors (right). (C) Same as (A) using healthy donor T cells stably expressing G_{αi2} variants or luciferase. (D) Same as (C) but quantified by normalizing AUC of transduced (GFP⁺) cells to untransduced (GFP⁻) cells. (E) Same as (B) using healthy donor T cells stably expressing G_{αi2} variants or

luciferase, but quantified by normalizing AUC of transduced (GFP⁺) cells to untransduced (GFP⁻) cells. **(F)** Migration after adoptive transfer of mouse T cells stably expressing G_{αi2}. Normalized ratio of variant to WT G_{αi2} transduced donor cells recovered from splenic white pulp or inguinal lymph node. **(G)** Transwell migration of AC3 or AC7 KO human T cells also stably expressing G_{αi2} variants or not. **(H)** AUC quantification of (G), normalized to gNeg-treated cells. **(I)** FSK-induced cAMP in cells from (G). **(J)** Schematized BRET reaction between G_{αi2}-RLuc91 and GPCR-YFP. Ligand binding (right) results in a reduction of pre-ligand (left) BRET signal (green). **(K)** Net BRET signal between G_{αi2}-RLuc91 and CCR7-YFP at basal conditions (**left**) or upon treatment with indicated chemokines (**right**). Gating strategies can be found in fig. S26A (for B), fig. S26B (for E), and fig. S26C (for F), and representative flow plots are presented in fig. S27A (for F). Data show representative (B, E left), means ± SEM (C, K) or means ± SD (A, B, E) for 3–6 (A–C, E) or 3 (K) experiments, or means ± SD of 2 independent experiments (F, total 3–5 mice/group), or means ± SD of 3 experiments from one of two different donor cell transductions (D, G–I). Combined results from multiple experiments are shown, with each individual point representing a different blood draw obtained longitudinally from a given patient (for A and B), or an individual recipient mouse (for F), or an independent experiment (C–E, H, I, K [left]). Statistical analyses were performed using Kruskal-Wallis test with Dunn's multiple comparisons for (C) and (E), or one-way ANOVA with Dunnett's multiple comparisons for (D), (F) and (K), or one-sample t-test with Two-stage step-up method of Benjamini, Krieger and Yekutieli for (H) and (I). **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001.

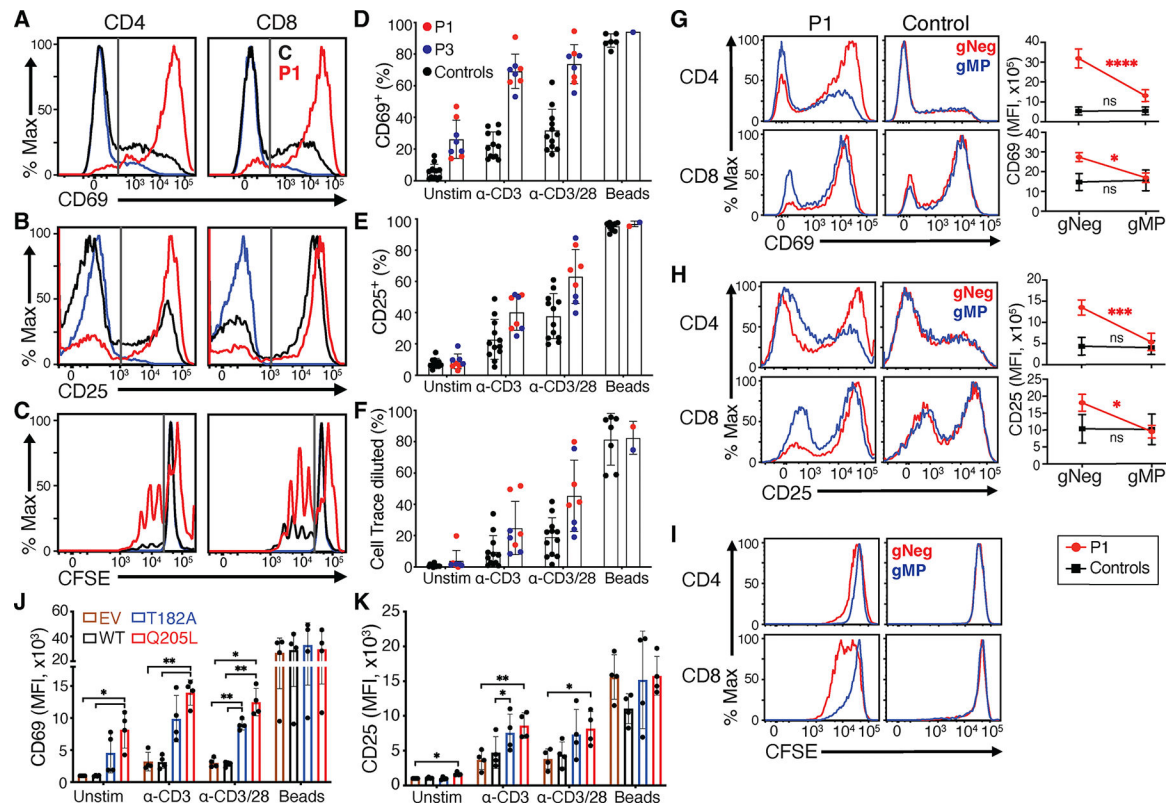


Figure 4. Activating $G_{\alpha i2}$ protein enhances T cell responses.

(A-F) TCR-induced surface expression of CD69 (A, D) and CD25 (B, E), and CFSE dilution (C, F) of naïve T cells purified from patient (P) or control (C) healthy donors. (A-C) Representative histograms of gated CD4⁺ (left) and CD8⁺ (right) T cells from P1 (red) and C (black) stimulated with soluble anti-CD3 and -CD28 antibodies (1 μ g/mL). Unstimulated C (blue). (D-F) Quantification of CD69⁺ or CD25⁺ cells as % of gated CD4⁺ T cells from 2 patients and 12 controls. Each dot represents a different experiment using a different blood draw collected longitudinally over a span of 6 years. Unstim: unstimulated. α -CD3 or α -CD3/28: soluble anti-CD3 or anti-CD3 and -CD28 antibodies (1 μ g/mL), Beads: bead-immobilized anti-CD2, -CD3, and -CD28 antibodies. (G-I) Same as (A-F) using P1 or control T cells treated with indicated Cas9/RNP and stimulated with anti-CD3 and anti-CD28 antibodies (0–1000 ng/mL; 100 ng/mL for representative histogram), except that Mean Fluorescence Intensity (MFI) was measured. MFI were plotted against doses of anti-CD3/28 (fig. S14D) to calculate AUC for each condition (G and H, right). gNeg is a non-specific guide RNA, and gMP targets the mutant *GNAI2* allele of P1. (J and K) Same as (A, B, D, E) using CD4⁺ T cells stably expressing $G_{\alpha i2}$ variants. EV: empty vector. Gating strategies can be found in fig. S26D (for A-I) and fig. S26E (for J and K). Data show representative flow plots alongside combined results with means $\pm$ SD for 4 (A-F, J and K), or 3 (G-I) experiments. Two-way ANOVA was performed with Sidak's multiple comparisons using cell type (Control or P1) and gRNA target (gNeg or gMP) as factors for (G) and (H), or one-way ANOVA with Tukey's multiple comparisons for (J) and (K). * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001; ns = not significant. See also fig. S13 and S14 for related data.

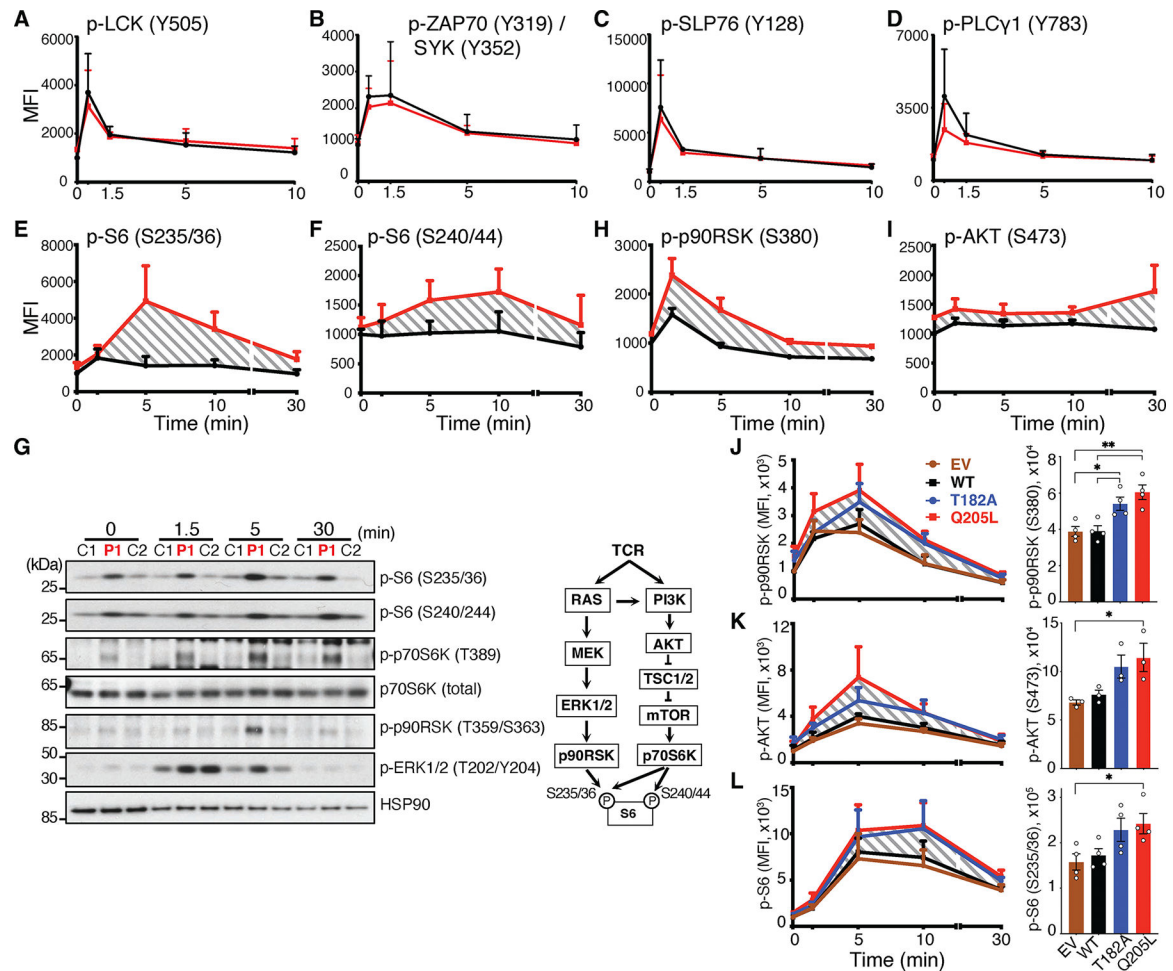


Figure 5. Active $G_{\alpha i2}$ enhances TCR-induced S6-regulatory pathways.

(A-I) Purified control (C) or P1 T cells were activated with anti-CD3 antibodies for the indicated times and samples subjected to flow-cytometry or immunoblot (G, left). **(A-F, H, and I)** Among the gated $CD4^+$ T cells, MFI was measured. **(G, right)** Simplified diagram of TCR-induced signaling pathways. **(J-L)** Similar to (A-I) using $CD4^+$ T cells from a healthy donor stably expressing $G_{\alpha i2}$ variants and calculating AUC. EV: empty vector. Gating strategies can be found in fig. S26F (for A-F, H, I) and fig. S26G (for J-L). Individual data points and representative flow plots are presented in fig. S27B (for A-F, H, I) and in fig. S27C (for J-L). Data show representative (G), means $\pm$ SEM (J-L, right), or means $\pm$ SD (rest), based upon 3 (A-H, K), 2 (I), or 4 (rest) experiments. One-way ANOVA was performed with Dunnett's multiple comparisons for (J), (K), and (L). * $P < 0.05$; ** $P < 0.01$.

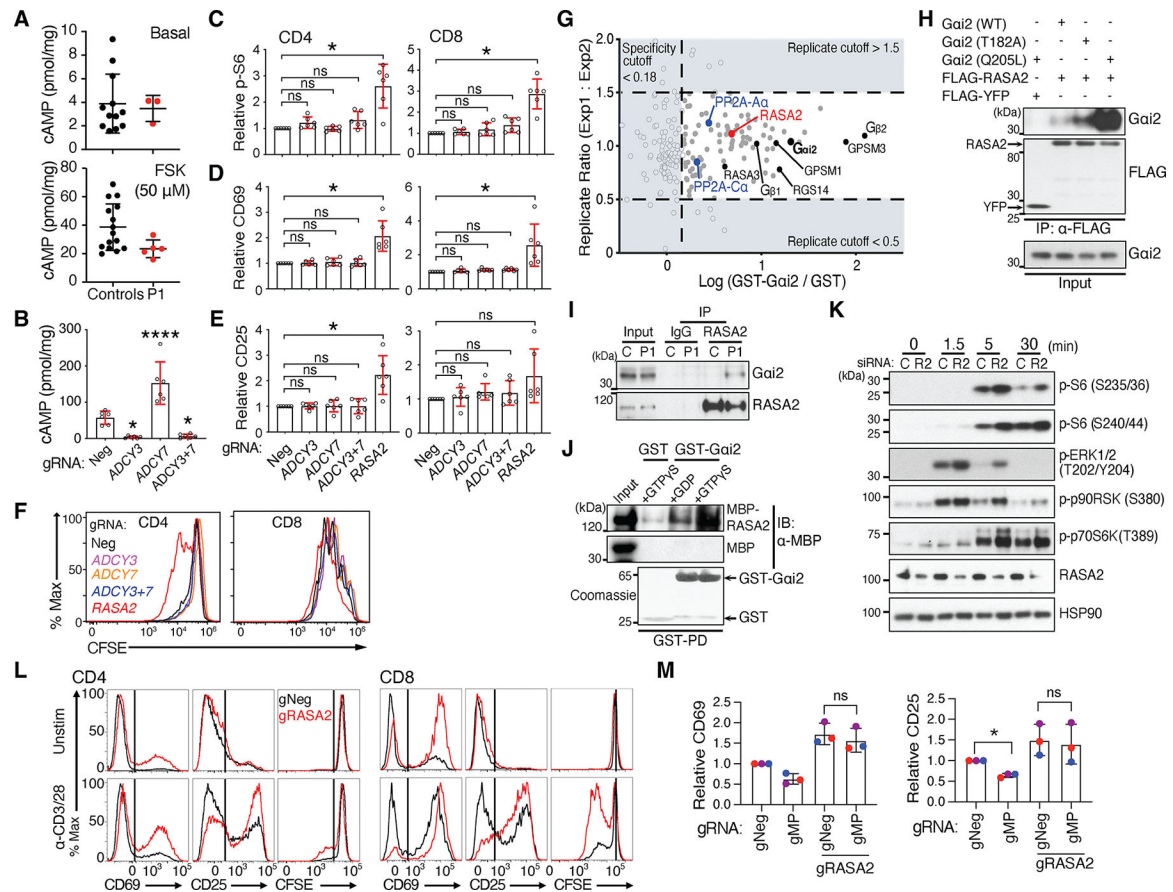


Figure 6. RASA2, a G_{ai2} effector target, constrains T cell responses.

(A) cAMP accumulation in control or P1 T cells at baseline (top), or upon stimulation with FSK (bottom). (B-F) T cells were transfected with indicated Cas9/RNP. (B) cAMP accumulation upon FSK treatment. (C-F) AUC quantification of TCR-induced S6 (S235/36) phosphorylation (C), surface expression of CD69 (D) or CD25 (E), and CFSE dilution (F), on gated CD4⁺ or CD8⁺ T cells, relative to gNeg control, after flow cytometric measurements of MFI. Cells were stimulated with anti-CD3 and anti-CD28 antibodies in (D and E; 0–1000 ng/mL) and in (F; 100 ng/mL). (G) Graphical representation of G_{ai2} -interacting proteins. Black: known interactors. Red and blue: candidate interactors. (H) 293T cells were transfected as indicated. FLAG was immunoprecipitated (IP) and immunoblotted (right-adjacent labels designate the specificity of antibodies used). (I) Immunoblot of RASA2 IP with T cell lysates from P1 or control (C). (J) Interaction between purified glutathione-S-transferase (GST)- G_{ai2} (loaded with either GDP or GTP γ S) and maltose binding protein (MBP) or MBP-RASA2 fusion proteins via GST pulldown (PD). Coomassie stain: GST-fusion proteins used in PD. (K) Healthy donor T cells transfected with control (C) or RASA2-targeting (R2) siRNAs were stimulated with anti-CD3 antibodies for varying times, and lysates immunoblotted for indicated proteins. (L) TCR-induced surface expression of CD69 or CD25, and CFSE dilution of CD4⁺ or CD8⁺ T cells treated with gNeg (black) or gRASA2 (red) Cas9/RNP. Cells were stimulated with anti-CD3 and anti-CD28 antibodies (100 ng/mL). (M) AUC quantification of CD69 (left) or CD25 (right) expression on gated CD4⁺ T cells, relative to gNeg control, after flow

cytometric measurements of MFI. Purified T cells from P1 were stimulated with increasing amounts of anti-CD3 and -CD28 antibodies (0–1000 ng/mL) after transfecting with the indicated Cas9/RNP9s (gNeg, gMP targeting mutant *GNAI2* allele of P1, gRASA2). Each colored dot indicates an experiment from a different blood draw. Gating strategies can be found in fig. S26F (for C), fig. S26D (for D-F), fig. S26H (for L and M). Representative flow plots are presented in fig. S27D (for C), fig. S27E (for D), fig. S27F (for E), and fig. S27P (for M). Combined results from multiple experiments are shown with each individual point representing an independent experiment (A-E, M). Data show representative (F, H-L) or means $\pm$ SD (rest), based upon 3 (A [top], H-K, M), 5 (L), 5 (A [bottom]), or 6 (B-F) experiments. (G) shows the analysis combined from two independent PD experiments. One-way ANOVA was performed with Dunnett's multiple comparisons for (B); one-sample t-test with Two-stage step-up method of Benjamini, Krieger and Yekutieli correction for multiple comparisons was performed with hypothetical value of 1 for (C-E); one-way ANOVA with Sidak's multiple comparisons was performed to compare gNeg vs. gMP, gNeg vs. gRASA2, gNeg vs. gRASA2+gMP, or gRASA2 vs. gRASA2+gMP in (M). * $P < 0.05$; **** $P < 0.0001$; ns (not significant).

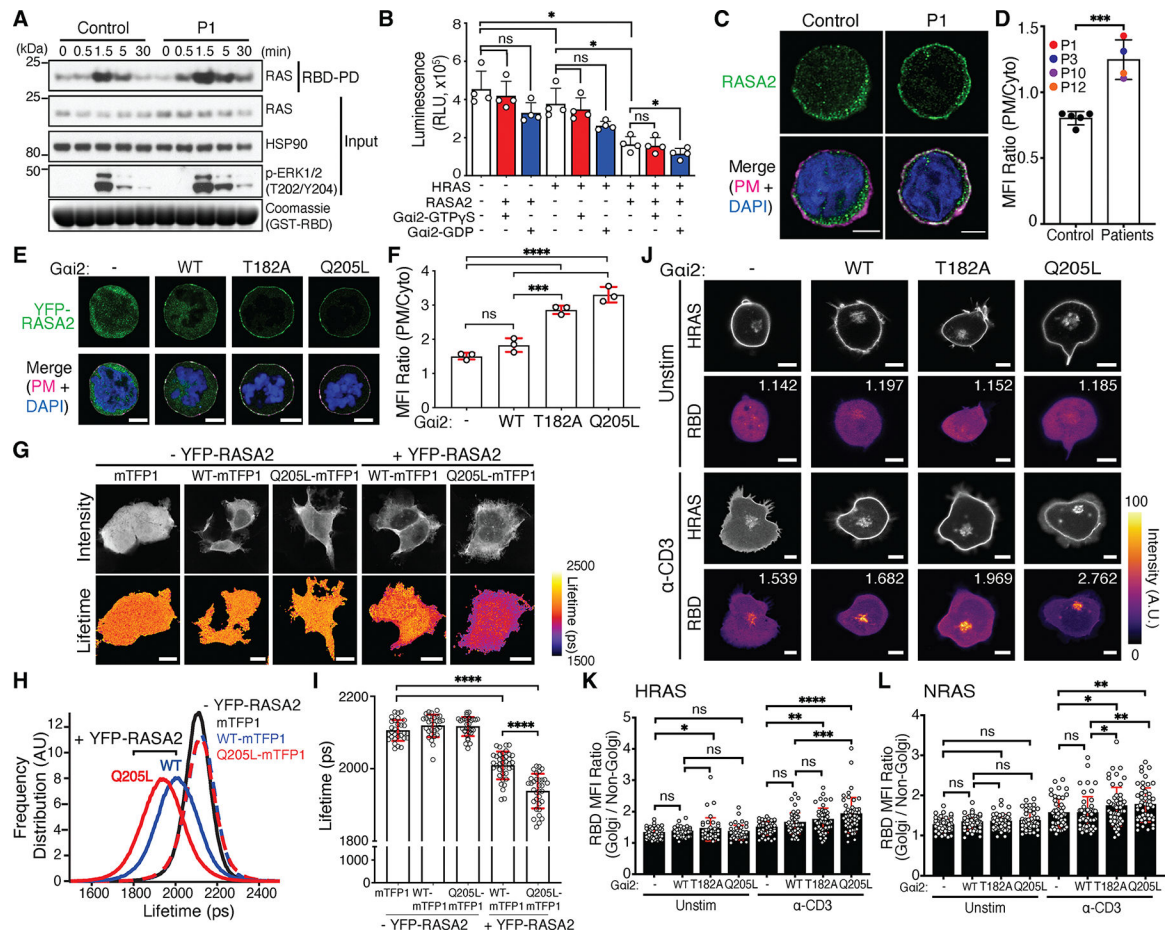


Figure 7. Active G α _{i2} promotes RAS activity by redirecting RASA2 to plasma membrane. (A) Control or P1 T cells were stimulated with anti-CD3 antibodies for varying times. Lysates were prepared for RBD pull down (PD) to detect active-RAS or for immunoblot for indicated proteins. Coomassie staining shows GST-RBD used in PD. (B) Purified HRAS, RASA2, and G α _{i2} proteins (preloaded with GTP γ S [non-hydrolyzable GTP analog that locks G α _{i2} in active state] or GDP) were incubated as indicated with an excess of GTP. GTP consumption was determined by measuring remaining GTP level (represented by relative light units [RLU]). (C) RASA2 distribution in CD4⁺ T cells from P1 or healthy donor control. PM: plasma membrane. (D) MFI of RASA2 ratio, at the PM relative to cytoplasm region (Cyto) in (C) and fig. S22D from 4 patients and 5 controls. (E) Distribution of YFP-RASA2 in G α _{i2} KO Jurkats co-expressing indicated G α _{i2} variants. (F) Quantification of (E) as done in (D). (G-I) Fluorescence lifetime imaging (FLIM) of mTFP1 or G α _{i2}-mTFP1 (WT, Q205L) with or without YFP-RASA2 expression in 293T cells. (G) Fluorescence intensity (top), and lifetime (bottom) of mTFP1. (H) mTFP1 Fluorescence lifetime distribution. (I) Quantification of mTFP1 mean fluorescence lifetime. (J-L) Confocal microscopy colocalization analysis of unstimulated or TCR-induced active RAS distribution in G α _{i2} KO Jurkats transfected with EGFP-HRAS (J and K) or NRAS (L), mCherry-RBD, and Cerulean-GalT along with G α _{i2} variants as indicated. Unstim: unstimulated. (J) Number on RBD image represents MFI ratio of RBD at Golgi (defined

by GalT stain) relative to non-Golgi region. **(K and L)** Quantification of RBD MFI ratio as in (J). Scale bar = 3 μm (C), 5 μm (E, J) or 10 μm (G). Data show representative or means $\pm$ SD for 3 (A, C-I, L) or 4 (B, J, K) experiments. Combined results from multiple experiments are shown (for B, D, and F), with each individual point representing an independent experiment (or a different patient for D). Each individual circle (for I, K, and L) corresponds to the value from a different cell, but with superimposed means $\pm$ SD from across multiple independent experiments. One-way ANOVA was performed with Tukey's multiple comparisons for (B) and (F); unpaired t-test for (D); two-way ANOVA was performed using mutant type and experiment as factors for (I); three-way ANOVA was performed using transfectant, stimulation, and experimental repeat as factors for (K) and (L) and Tukey multiple comparison conditional on the stimulation status. * $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$; ns = not significant.