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Daley, Stephen R., Coakley, Kristen M., Hu, Daniel Y., Randall, Katrina L., Jenne, Craig N., Limnander, Andre, Myers, Darienne R., Polakos, Noelle K., Enders, Anselm, Roots, Carla, Balakishnan, Bhavani, Miosge, Lisa A., Sjollema, Geoff, Bertram, Edward M., Field, Matthew A., Shao, Yunli, Andrews, T. Daniel, Whittle, Belinda, Barnes, S Whitney, Walker, John R., Cyster, Jason G., Goodnow, Christopher, & Roose, Jeroen P. (2013) Rasgrp1 mutation increases naïve T-cell CD44 expression and drives mTOR-dependent accumulation of Helios+ T cells and autoantibodies. eLife, 2, Article number: e01020.

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https://doi.org/10.7554/eLife.01020
Rasgrp1 mutation increases naïve T-cell CD44 expression and drives mTOR-dependent accumulation of Helios+ T cells and autoantibodies

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Abstract Missense variants are a major source of human genetic variation. Here we analyze a new mouse missense variant, Rasgrp1 Anaef, with an ENU-mutated EF hand in the Rasgrp1 Ras guanine nucleotide exchange factor. Rasgrp1 Anaef mice exhibit anti-nuclear autoantibodies and gradually accumulate a CD44hi Helios+ PD-1+ CD4+ T cell population that is dependent on B cells. Despite reduced Rasgrp1-Ras-ERK activation in vitro, thymocyte selection in Rasgrp1 Anaef is mostly normal in vivo, although CD44 is overexpressed on naïve thymocytes and T cells in a T -cell-autonomous manner. We identify CD44 expression as a sensitive reporter of tonic mTOR-S6 kinase signaling through a novel mouse strain, chino, with a reduction-of-function mutation in Mtor. Elevated tonic mTOR-S6 signaling occurs in Rasgrp1 Anaef naïve CD4+ T cells. CD44 expression, CD4+ T cell subset ratios and serum autoantibodies all returned to normal in Rasgrp1 Anaef Mtor chino double-mutant mice, demonstrating that increased mTOR activity is essential for the Rasgrp1 Anaef T cell dysregulation.

DOI: 10.7554/eLife.01020.001

Introduction Positive and negative selection of thymocytes generates a population of T lymphocytes with a broad spectrum of antigen-specific T cell receptors (TCR) (Starr et al., 2003; Kortum et al., 2013). It was recognized early on that the small GTPase Ras plays a role (Swan et al., 1995). Three Ras guanine exchange factor (RasGEF) families can activate Ras: SOS, RasGRP, and RasGRF (Stone, 2011). Following TCR engagement, Son of Sevenless (SOS)-1 and -2 are recruited via a Grb2-phospho-LAT interaction. Simultaneously, the second messenger diacylglycerol (DAG), generated via PLCγ, directly recruits Ras guanine nucleotide releasing protein 1 (Rasgr1) to the plasma membrane.
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(Ebinu et al., 1998). Biochemically, Rasgrp1 and SOS1 synergize to induce high-level Ras activation (Roose et al., 2007) and Rasgrp1 serves a critical role in priming SOS1 via Rasgrp1-produced RasGTP (Das et al., 2009). Consequently, thymocyte development is severely impaired in Rasgrp1-deficient mice (Dower et al., 2000), and not compensated for by SOS RasGEFs. Additionally, there is only minimal compensation for loss of Rasgrp1 coming from Rasgrp3 or Rasgrp4 (Zhu et al., 2012; Golec et al., 2013). Rasgrp1-deficient mice exhibit a strong defect in positive selection and impaired ERK phosphorylation in thymocytes (Dower et al., 2000; Priatel et al., 2002). The importance of the canonical Rasgrp1-RasGTP-RAF-MEK-ERK pathway for developing thymocytes is further underscored by impaired positive selection in ERK-1 and -2 doubly deficient mice (Fischer et al., 2005).

Although Rasgrp1 plays a critical role in the activation of Ras, relatively little is known about its regulation in T lymphocytes or the in vivo importance of such regulation. In addition to membrane recruitment via its DAG-binding C1 domain (Ebinu et al., 1998), Rasgrp1’s GEF activity is enhanced by inducible phosphorylation of threonine 184 (Roose et al., 2005; Zheng et al., 2005). Phospholipase Cγ (PLCγ) not only generates DAG but also inositol 1,4,5-trisphosphate (IP3), which binds to IP3 receptors on the endoplasmic reticulum to activate the calcium pathway (Feske, 2007). Interestingly, Rasgrp1 also contains a pair of EF hands, motifs that often bind calcium, which induces conformational changes (Gifford et al., 2007). Rasgrp1 has been reported to bind calcium in vitro (Ebinu et al., 1998). In chicken DT40 B cells, the first EF domain enables the recruitment function of a C-terminal PT domain (plasma membrane targeting domain) that cooperates with the C1 domain to recruit Rasgrp1 to the membrane (Tazmini et al., 2009). Notably, the PT domain contribution is substantial in BCR-stimulated B cell lines, very modest in T cell lines, and negligible in fibroblasts (Beaulieu et al., 2007). Genetic deletion of Rasgrp1’s 200 C-terminal amino acids reduces the formation of mature thymocytes in Rasgrp1<sup>-/-</sup> mice (Fuller et al., 2012). Our recent structural studies revealed that Rasgrp1’s C terminus contains a coiled-coil dimerization domain (Iwig et al., 2013). Rasgrp1 dimerization plays
an important role in controlling Rasgrp1’s activity; the second EF hand of one Rasgrp1 molecule packs against the C1 domain of a second molecule in a manner that is incompatible with DAG-binding whereas calcium binding to the first EF hand is predicted to unlock this autoinhibitory dimer interface (Iwig et al., 2013). Lastly, it is unknown if Rasgrp1 may signal to pathways other than the canonical Rasgrp1-Ras-RAF-MEK-ERK cascade, although a link between Rasgrp1 and mTOR (mechanistic target of rapamycin) signaling has been proposed (Gorentla et al., 2011).

Older Rasgrp1-deficient (Coughlin et al., 2005) and Rasgrp1<sup>−−</sup> mice (Fuller et al., 2012) develop splenomegaly and autoantibodies. In these mouse models, the complete deletion or truncation of Rasgrp1 greatly decreases T cell development in the thymus (Dower et al., 2000; Fuller et al., 2012), resulting in peripheral T cell lymphopenia followed by accumulation of CD44<sup>hi</sup>CD62L<sup>lo</sup>CD4<sup>+</sup> T cells (Priatel et al., 2007; Fuller et al., 2012). Autoimmune phenotypes caused by these mutations have been attributed to compromised T cell selection in the thymus and compensatory expansion of peripheral T cells in response to lymphopenia and/or chronic infection. Hypomorphic missense alleles of the signaling molecules ZAP-70 and LAT also impair T cell development in the thymus and culminate in severe peripheral immune dysregulation. For example, an SKG allele of the kinase ZAP-70 has reduced binding-affinity for phospho-TCR<sub>ζ</sub> and leads to autoimmune arthritis in mice (Sakaguchi et al., 2003). Point mutations in ZAP70’s catalytic domain that reduce kinase activity to intermediate levels diminish thymic deletion and Foxp3<sup>+</sup> Treg differentiation but preserve peripheral T cell activation, resulting in autoantibody formation and hyper-IgE production (Siggs et al., 2007). Mutation of a single tyrosine in LAT (LAT<sup>Y<sub>136F</sub></sup>) results in hyperproliferative lymphocytes of a T<sub>1,2</sub> type (Aguado et al., 2002; Sommers et al., 2002). In each of these cases, peripheral T cell dysregulation is tied to, and potentially explained by, profound deficits in thymic T cell formation.

Single nucleotide variants that cause amino acid substitutions (missense variants; SNVs) or modify the level of gene expression rather than knocking out protein expression are a major form of human genetic variation: most people inherit ~12,000 missense gene variants (The 1000 Genomes Project Consortium, 2010). Given the emerging examples of missense alleles having very different immunological consequences from null alleles, mouse models that analyze the consequences of missense variants in key immune genes are needed to understand the pathogenesis of complex human immune diseases. Common tag SNVs near RASGRP1 are associated with susceptibility to autoimmune (Type 1) diabetes and to thyroid autoantibodies in Graves’ disease (Qu et al., 2009; Plagnol et al., 2011), while 13 unstudied RASGRP1 missense SNVs are currently listed in public databases. A fruitful approach for identifying missense gene variants that dysregulate immune function has been through N-ethyl-N-nitrosourea (ENU) mutagenesis (Nelms and Goodnow, 2001). Here we describe the analysis of a novel ENU-induced missense variant, Rasgrp1<sup>1<sub>Anaef</sub></sup> that reveals an important in vivo regulatory function of Rasgrp1’s EF hands. Rasgrp1<sup>1<sub>Anaef</sub></sup> is distinct from previously described autoimmune mutations in Rasgrp1, Zap70 or Lat, as Rasgrp1<sup>1<sub>Anaef</sub></sup> has no detectable effect on thymocyte development in mice with normal TCR repertoires, but results in peripheral accumulation of a distinct population of Helios<sup>+</sup> PD-1<sup>+</sup> T-helper cells and production of anti-nuclear autoantibodies. In contrast to Rasgrp1 deletion, the Rasgrp1<sup>1<sub>Anaef</sub></sup> missense variant increases tonic mTOR signaling in naïve CD4<sup>+</sup> T cells. Genetic reduction of mTOR function in Rasgrp1<sup>1<sub>Anaef</sub></sup> mice normalizes CD44 expression on naïve CD4<sup>+</sup> T cells and abolishes excessive accumulation of effector T cells and autoantibodies, demonstrating a central role for increased mTOR activity in driving immune dysregulation in Rasgrp1<sup>1<sub>Anaef</sub></sup> mice.

**Results**

**Identification of the Rasgrp1<sup>1<sub>Anaef</sub></sup> mouse strain with a mutated EF hand in Rasgrp1**

As part of a mouse genome-wide screen for immune phenotypes induced by ENU mutagenesis (Nelms and Goodnow, 2001), we identified a variant C57BL/6 (B6) pedigree displaying elevated frequencies of CD44<sup>hi</sup> CD4<sup>+</sup> cells (Figure 1A), elevated CD44 expression on naïve FOXP3<sup>+</sup> CD44<sup>hi</sup> CD4<sup>+</sup> cells (Figure 1B) and antinuclear antibodies (ANAs) staining with a homogeneous nuclear pattern (Figure 1C,D). The elevated frequency of CD44<sup>hi</sup> cells trait, which occurred at a frequency consistent with inheritance of a recessive gene variant (Figure 1A), was used to map the mutation in an F2 intercross to an interval between 114 and 121.2 Mb on chromosome 2 (Figure 1—figure supplement 1A).

Sequencing of the exons of Rasgrp1, the only gene within this interval with a known immune function, identified an A to G missense mutation in codon 519 within exon 13 (Figure 2A). Whole-exome capture,
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sequencing and computational analysis of DNA from an affected mouse (Andrews et al., 2012) identified this mutation as the only novel single-nucleotide variant within the interval of interest on chromosome 2 (data not shown). The mutant codon, located in Rasgrp1’s second EF hand (EF2), encodes a neutral amino acid glycine (G) instead of the normal arginine (R), a large polar molecule with a positive charge (Rasgrp1 R519G; Figure 2A,B). Rasgrp1’s arginine residue at 519 is also found in Rasgrp-2, and -4, and in EF3 of calcium and integrin binding protein (CIB) (Figure 2B). EF hands typically come in pairs separated by a linker and calcium binding subsequently alters the angle between helices E and F in proteins such as calmodulin (CaM) (Figure 2C) (Grabarek, 2006; Gifford et al., 2007). Unique to Rasgrp1, this linker is unusually short. Furthermore, biophysical studies revealed that Rasgrp1’s EF2 does not bind calcium, that the E helix is non-existent in EF2, but instead has evolved as a critical loop forming an autoinhibitory interface with the C1 domain (Figure 2D,E) (Iwig et al., 2013).
The ENU-generated allele was named ‘Rasgrp1\textsuperscript{Anaef}’ to reflect the combination of antinuclear antibody (ANA) production and the amino acid substitution in the EF hand. Genotyping of this mutation in multiple generations of B6 offspring (Figure 1—figure supplement 1B,C) demonstrated that inheritance of the \textit{Rasgrp1}\textsuperscript{Anaef} allele was well correlated with the immunological abnormalities described above and below. ANAs were present in 70% of homozygous \textit{Rasgrp1}\textsuperscript{Anaef/Anaef} mice and 35% of heterozygous \textit{Rasgrp1}\textsuperscript{Anaef/+} mice, compared to 5% of wildtype B6 mice, indicating a gene dosage effect. The R519G substitution caused an approximate 40% decrease in \textit{Rasgrp1} protein levels in homozygous \textit{Rasgrp1}\textsuperscript{Anaef/Anaef} thymocytes (Figure 2F). Since heterozygous \textit{Rasgrp1}\textsuperscript{Anaef/+} thymocytes do not bind calcium and the E helix has evolved into an autoinhibitory domain (Iwig et al., 2013), the \textit{Rasgrp1}\textsuperscript{Anaef} allele is caused by the specific R519G alteration and not simply by a reduction of \textit{Rasgrp1} protein levels. In the remainder of the manuscript we discuss the analysis of homozygous \textit{Rasgrp1}\textsuperscript{Anaef/Anaef} mice and refer to these as \textit{Rasgrp1}\textsuperscript{Anaef} mice.

\textbf{\textit{Rasgrp1}\textsuperscript{Anaef} preserves \textit{Rasgrp1} function for T cell selection in the thymus}

Analysis of \textit{Rasgrp1}\textsuperscript{Anaef} mice revealed a striking contrast to the published \textit{Rasgrp1}-deficient and \textit{Rasgrp1}\textsuperscript{d/d} mouse models, which have T cell developmental defects that result in low thymic T cell

\begin{figure}
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\caption{Mapping of the ENU mutation to the autoinhibitory second EF hand domain in \textit{Rasgrp1}. (A) Sequence trace of \textit{Rasgrp1}\textsuperscript{Anaef} exon 13 aligned to wildtype \textit{Rasgrp1} sequence. (B) Sequence comparison of CaM (calmodulin), CIB (calcium and integrin binding protein) and \textit{Rasgrp1}, -2, -3 and -4 EF hands with conserved residues highlighted. (Acidic, red; Basic, blue; Calcium-binding residues, highlighted in grey; ENU-mutated residue in green). (C and D) Model of a typical pair of EF hands with two calcium (Ca)-binding loops each flanked by N- and C-terminal \(\alpha\)-helices based on CaM (C). Model including the atypical second EF hand in \textit{RasGRP1} (D). \textit{RasGRP1}'s second EF hand does not bind calcium and the E helix has evolved into an autoinhibitory domain (Iwig et al., 2013). (E) Linear schematic of \textit{Rasgrp1} protein domains, phosphorylation site threonine 184, and position of R519G mutation in the second EF hand that evolved into a domain for autoinhibition. (F and G) Western blot for \textit{Rasgrp1} protein in thymocytes from \textit{Rasgrp1}\textsuperscript{Anaef/Anaef} (A/A), heterozygous \textit{Rasgrp1}\textsuperscript{Anaef/WT} (A/+), heterozygous \textit{Rasgrp1}\textsuperscript{Null/WT} (+/−), and wildtype (+/+). Blot was reprobed for \(\alpha\) tubulin as a loading control. Relative \textit{Rasgrp1} expression was calculated and is shown. Note the expression of \textit{Rasgrp1}'s typical doublet, thought to be due to alternative translation initiation (Poon and Stone, 2009). Representative blots of at least three independent experiments. DOI: 10.7554/eLife.01020.005}
\end{figure}
output (Dower et al., 2000; Priatel et al., 2007; Fuller et al., 2012). The frequency and number of mature CD4+ and CD8+ single positive (SP) thymocyte subsets was normal in Rasgrp1Anaef mice, whereas these subsets were markedly decreased in Rasgrp1−/− (knockout) mice analyzed in parallel (Figure 3A,B). Unlike the knockout allele, the Rasgrp1Anaef mutation did not decrease the frequency of CD69hi TCRβhi cells amongst CD4+CD8+ double positive (DP) thymocytes (Figure 3C) or the number of Foxp3+ CD4SP cells in the thymus (Figure 3B). Even in bone marrow chimeras reconstituted with a mixture of CD45.2+...
Rasgrp1\(^{Anaef}\) and CD45.1\(^{+}\) wildtype marrow, the Rasgrp1\(^{Anaef}\) thymocytes exhibited no competitive disadvantage as they matured from DP to SP cells (Figure 3D). Injection into mice of 5-bromo-2'-deoxyuridine (BrdU) to pulse-label a cohort of proliferating DP thymocytes followed by analysis on day 5 demonstrated that the kinetics of maturation into SP cells was unaffected by the Rasgrp1\(^{Anaef}\) mutation (Figure 3E, Figure 3—figure supplement 1A). There was also normal deletion of Vß5\(^{+}\) and Vß11\(^{+}\) SP thymocytes upon self-superantigen/I-E\(^{+}\) recognition in B10. Br mice (Figure 3F) and similar usage of TCRa segments in wildtype and Rasgrp1\(^{Anaef}\) CD4SP thymocyte populations (Figure 3G, Figure 3—figure supplement 1B). Thus, analysis of the thymus of Rasgrp1\(^{Anaef}\) mice with a diverse TCR repertoire revealed no abnormalities in positive selection, Foxp3\(^{+}\) T-Regulatory (T-reg) cell differentiation or clonal deletion, in striking contrast to previously described Rasgrp1 mutations.

### Anaef diminishes canonical Rasgrp1-Ras-ERK signaling in response to in vitro stimulation

Despite the normal thymic development in Rasgrp1\(^{Anaef}\) animals, there was a striking biochemical effect of the Rasgrp1\(^{Anaef}\) mutation on activation of the canonical Rasgrp1-Ras-ERK signaling pathway in a range of in vitro stimulation assays. GFP-tagged wildtype- or Anaef- Rasgrp1 was transiently expressed in RasGRP1-deficient Jurkat cells (JPRM441) (Roose et al., 2005), which were either left unstimulated or stimulated with a combination of PMA (a synthetic analog of diacylglycerol) and ionomycin (a calcium ionophore). Gating on cells with different GFP intensities (Figure 4—figure supplement 1A) revealed that Rasgrp1\(^{Anaef}\) was hypomorphic (partial loss of function) for activating the Ras-ERK pathway: in GFP\(^{+}\) cells expressing Rasgrp1\(^{Anaef}\) there was only low ERK phosphorylation (P-ERK) and this was only modestly increased when PMA and ionomycin were added (Figure 4A). By contrast, GFP\(^{+}\) cells expressing high levels of wildtype Rasgrp1 vector induced 5-times higher P-ERK spontaneously and this was doubled by PMA and ionomycin stimulation. Next, we stably reconstituted the JPRM441 cell line, which expresses residual wildtype RasGRP1 protein (Roose et al., 2005) with Rasgrp1\(^{Anaef}\) or Rasgrp1\(^{Wildtype}\) vectors and selected clones with Rasgrp1 expression levels similar to the parental Jurkat cell line (Figure 4—figure supplement 1B). Since JPRM441 cells do not express surface TCR (Roose et al., 2005), clonal cell lines were stimulated with PMA followed by RasGTP pull-down assays, which demonstrated that Rasgrp1\(^{Anaef}\) decreased PMA-induced GTP-loading of Ras to levels below that of the nontransfected JPRM441 cells (Figure 4B). In the same transfected cell lines, PMA-induced P-ERK responses were decreased in Rasgrp1\(^{Anaef}\) expressing cells, most notable with the lower dose of PMA (PMA MED; 5 ng/ml) and contrasted the effective induction of P-ERK signals in Jurkat and JPRM441-WT-Rasgrp1 cells (Figure 4C,D). Similarly, Rasgrp1\(^{Anaef}\) expressing cells demonstrated less potent synergy in P-ERK levels when ionomycin was combined with a very low PMA stimulus (2 ng/ml) (Figure 4E, Figure 4—figure supplement 1C). In fact, P-ERK responses in JPRM441-Rasgrp1\(^{Anaef}\) cells were more impaired than in the parental JPRM441 cells, indicating a dominant negative effect, which was also observed at the level of Ras activation (Figure 4B). We previously reported a dominant negative effect for ΔDAG-Rasgrp1, a form of Rasgrp1 lacking the DAG-binding C1 domain (ΔDAG) and we postulated that there may be competition with the residual ≈10% of wildtype RasGRP1 (Roose et al., 2005). As Rasgrp1 is regulated by DAG-driven membrane recruitment (Blinn et al., 1998; Roose et al., 2005) we examined this process for Rasgrp1\(^{Anaef}\). EGFP-tagged wildtype or Anaef Rasgrp1-transfected JPRM441 cells were FACS sorted on low GFP expression to avoid overexpression artifacts and cells were allowed to adhere to coated slides. PMA stimulation resulted in membrane recruitment and cytoplasmic clearing of wildtype Rasgrp1; whereas these events were decreased for Rasgrp1\(^{Anaef}\) (Figure 4F).

To test TCR-induced Ras-ERK signaling in thymocytes, we first stimulated thymocytes from wildtype or Rasgrp1\(^{Anaef}\) mice with anti-CD3 crosslinking antibodies and probed lysates for tyrosine-phosphorylated proteins to examine the global biochemical effects of the Rasgrp1\(^{Anaef}\) mutation. Both thymocyte populations demonstrated similar induction of total phospho-tyrosine patterns and similar activating phosphorylation of Lck and Zap-70 that lie upstream of Rasgrp1 (Figure 5—figure supplement 1A). Rasgrp1’s GEF activity is also enhanced by phosphorylation on T184 (Roose et al., 2005; Zheng et al., 2005). Using a new monoclonal antibody specific for P-T184-Rasgrp1 (Figure 5—figure supplement 1B) we observed drastically impaired phosphorylation of T184-Rasgrp1, and reduced ERK phosphorylation in Rasgrp1\(^{Anaef}\) thymocytes (Figure 5A). By contrast, Rasgrp1\(^{-/-}\) thymocytes heterozygous for the null allele displayed readily detectable Rasgrp1- and ERK- phosphorylation (Figure 5B), demonstrating that the signaling defects in the Rasgrp1\(^{Anaef}\) thymocytes are much greater than when the amount of
Rasgrp1 is simply halved. Thymocyte subset-specific P-ERK analyses revealed reduced anti-CD3- and PMA-induced responses in DP, CD4SP, and CD8SP Rasgrp1Anaef thymocytes (Figure 5C, Figure 5—figure supplement 1C), echoing the cell line conclusion that the Anaef mutation results in a partial loss of function with respect to induced Ras-ERK signaling. When pressure was placed on TCR-Ras-ERK signaling for positive selection in vivo, by introducing any one of three rearranged TCR transgenes that are prematurely expressed at higher than normal levels on DP thymocytes, a small decrease in positive selection was revealed in Rasgrp1Anaef TCR-transgenic thymocytes compared to their wildtype controls (Figure 5—figure supplement 2). Collectively, these results lead to the surprising conclusion that the low affinity pMHC stimulation that drives physiological positive selection in vivo is remarkably robust to decreased Rasgrp1 activation of Ras-ERK.
Intrinsically dysregulated formation of Helios+ PD-1+ CD4+ T cells in Rasgrp1<sup>Anaef</sup> mice

Given the evidence above for normal thymic formation of T cells in Rasgrp1<sup>Anaef</sup> animals with normal TCR genes, we sought to define the peripheral CD4+ cell dysregulation that results in an expanded population of CD44<sup>hi</sup> CD4+ T cells. Total splenocyte numbers and CD4 subsets were within the normal range in young Rasgrp1<sup>Anaef</sup> animals, but between 50 and 150 days of age the frequencies of activated or memory CD44<sup>hi</sup> Foxp3− CD4+ cells increased, as did Foxp3+ CD4 cells, while the frequency of CD44<sup>lo</sup> Foxp3− naïve CD4+ cells decreased (Figure 6A–C).

Further resolution of CD4+ subsets based on intracellular cytokine staining revealed that interferon-γ producing cells were increased in frequency by a similar magnitude as CD44<sup>hi</sup> cells as a whole (Figure 6D and data not shown). Thus, there was no evidence that the Rasgrp1<sup>Anaef</sup> mutation skewed T-helper cells towards a Th1 phenotype, but simply increased the number of activated or memory/effector CD4+ cells. Staining for PD-1 and CXCR5, whose high expression on CD4+ cells identifies T follicular helper (T<sup>FH</sup>) cells (Ramiscal and Vinuesa, 2013) revealed a dramatic expansion of these cells in Rasgrp1<sup>Anaef</sup> mice (Figure 6E). However most of the increase in CD44<sup>hi</sup> Foxp3− CD4+ cells in Rasgrp1<sup>Anaef</sup> mice was due to a 600% increase in cells that expressed intermediate levels of PD-1 and CXCR5, and hence are unlikely to be T<sub>FH</sub> cells, but were distinguished by high expression of the Helios transcription factor (Figure 6E). Helios is highly expressed in Foxp3+ T-reg cells (Thornton et al., 2010), but in wildtype and Rasgrp1<sup>Anaef</sup> mice Helios is also upregulated in a subset of Foxp3− CD4+ cells, nearly all of which are CD44<sup>hi</sup> (Figure 6F). Rasgrp1<sup>Anaef</sup> greatly increased this Helios+ CD44<sup>hi</sup> Foxp3− CD4+ population,
Figure 6. Rasgrf1Anaef results in dysregulation of peripheral CD4 T cells. (A) Representative plots of wildtype or Rasgrf1Anaef CD4+ splenocytes subsetted into naïve (CD44lo FOXP3−), activated/memory (CD44hi FOXP3−), and T-reg (FOXP3+) populations. (B and C) Splenic cellularity and frequencies of the CD4+ subsets gated in (A) as a function of age in wildtype vs Rasgrf1Anaef mice; each dot represents one mouse (WT in white; Rasgrf1Anaef in black). Inset column graphs show the group mean ± SEM. Statistics obtained by unpaired Student’s t test. ***p<0.001, ****p<0.0001. (D) Representative intracellular labeling of IFNγ, IL-4, IL-2 or IL-17 on electronically gated Foxp3− CD4+ wildtype or Rasgrf1Anaef splenocytes that had been stimulated with PMA and ionomycin for 4 hr. Column graphs show mean ± SEM frequencies amongst all splenocytes. Statistical analysis of % IFNγ+ cells used an unpaired Student’s t test (n = 7 WT, 6 Anaef) **p<0.01. (E) Phenotype of CD4+ splenocytes showing a CXCR5+ PD-1hi gate for Figure 6. Continued on next page
which was also distinguished by high PD-1 expression in Rasgrp1<sup>Anaef</sup> (Figure 6F). Rasgrp1<sup>Anaef</sup> mice had normal frequencies of CD95 (Fas)<sup>hi</sup>, GL-7<sup>hi</sup> germline center T cells in the spleen (Figure 6G), consistent with the conclusion that the accumulating Helios<sup>hi</sup> CD4<sup>+</sup> T cells were a distinct type of activated CD4<sup>+</sup> cell but not fully differentiated T<sub>TH</sub> cells.

To test if the dysregulated accumulation of Helios<sup>hi</sup> CD4<sup>+</sup> T cells was profoundly suppressed (Figure 7A,B). Indeed, the Rasgrp1<sup>Anaef</sup>- driven distortion in relative frequencies of naïve, effector/memory and regulatory subsets of CD4 splenocytes was rectified by the absence of B cells in Rasgrp1<sup>Anaef</sup> Cd79a<sup>null</sup> mice (Figure 7C,D). By contrast, the elevated CD44 expression on naïve CD4<sup>+</sup> cells was still present (Figure 7E) indicating this is a constitutive effect of the Rasgrp1<sup>Anaef</sup> mutation.

The requirement for B cells could indicate they are needed as specialized antigen presenting cells, as is the case for T<sub>TH</sub> cells (Ramiscal and Vinuesa, 2013), or that the Anaef mutation also acts in B cells since B cells also express Rasgrp1 (Stone, 2011). To resolve these alternatives, we used bone marrow from Rasgrp1<sup>Anaef</sup> Cd79a<sup>null</sup> animals mixed with wildtype Rasgrp1<sup>+/+</sup> marrow to generate chimeric mice where the Rasgrp1<sup>Anaef</sup> mutation was excluded from B cells but present in most of the T cells (experimental group B in Figure 7F–I), and compared these with control chimeras where all hematopoietic cells were Rasgrp1<sup>Anaef</sup> or Rasgrp1<sup>WT</sup> (groups A, C and D in Figure 7). Despite having the Anaef mutation in the T but not B cells of group B mice, a high proportion developed antinuclear autoantibodies comparable to the control group D where both B and T cells carried the Anaef mutation (Figure 7G). Moreover, a high frequency and number of CD45.2<sup>+</sup> Rasgrp1<sup>Anaef</sup> CD4<sup>+</sup> T cells acquired a Helios<sup>hi</sup> PD-1<sup>+</sup> phenotype in Group B animals, unlike the co-resident CD45.1<sup>+</sup> wildtype T cells (Figure 7I). The accumulation of these activated CD4 T cells thus reflects a cell-autonomous effect of the Anaef mutation within the CD4 T cells and does not depend upon the Anaef allele being present in B cells.

**CD44 expression is a sensitive reporter of mTOR activity in naïve T cells**

Increased basal expression of the cell adhesion receptor, CD44, was a unique trait exhibited by naïve, CD62L-positive Rasgrp1<sup>Anaef</sup> T cells (Figure 1B). CD44 expression normally increases during differentiation of DP thymocytes into SP T cells, attains higher levels on naïve CD4 T cells than on naïve CD8 cells, and increases further on activated/memory T cells, but little was known about what determines the level of CD44 expressed. In cancer cells CD44 has been described as an mTOR target (Hsieh et al., 2012). In our ongoing peripheral blood screen of ENU mutagenized mouse pedigrees, we identified a strain, chino, with decreased CD44 expression on peripheral CD4<sup>+</sup> CD62L<sup>hi</sup> cells but relatively normal T cell numbers and subsets (Figure 8A). This unusual phenotype mapped to a single nucleotide change (T to G) in exon 5 of the mechanistic target of rapamycin (Mtor) gene, introducing serine in place of isoleucine at position 205 in the fifth predicted HEAT domain of the protein (Knutson, 2010) (Figure 8B). This mTOR isoleucine residue is entirely conserved from mammals to yeast (Figure 8—figure supplement 1). The mTOR HEAT-repeat domain forms a large superhelical structure that binds RAPTOR to recruit substrates such as S6 kinase for phosphorylation by the mTOR kinase domain (Kim et al., 2002; Adami et al., 2007).

As the absence of Mtor is embryonic lethal in mice (Gangloff et al., 2004; Murakami et al., 2004), the fact that Mtor<sup>chino/chino</sup> (hereafter referred to as Mtor<sup>chino</sup>) mice are viable but slightly smaller than wildtype (Figure 8C) indicates that the Mtor<sup>chino</sup> allele retains substantial function. Consistent with a
Figure 7. Role of B and T cells in Rasgrp1<sup>Anaef</sup>-induced Helios<sup>+</sup> PD-1<sup>+</sup> CD4<sup>+</sup> T cell and autoantibody formation. (A–E) B6.Cd79a<sup>−/−</sup> mice lacking B cells were intercrossed with B6.Rasgrp1<sup>Anaef</sup> mice to produce mice with the genotypes shown above the plots. Plots (left) display phenotype of (A and B) Foxp3<sup>+</sup> CD4<sup>+</sup> splenocytes and the gate used to define the PD-1<sup>+</sup> HELIOS<sup>+</sup> subpopulation and absolute numbers of these cells are shown on the column graphs (mean ± SEM). Statistical comparisons used unpaired Student’s t tests (n = 4 WT, 5 Anaef, 2 CD79a-null, and 4 Anaef.CD79a-null) *p<0.05. (C) CD4<sup>+</sup> splenocytes Figure 7. Continued on next page

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Table: donors and resulting chimeras

| Group | Donor bone marrow | Resulting chimeras |
|-------|-------------------|--------------------|
| A     | 20% WT 80% Rasgrp1<sup>WT</sup> Cd79a<sup>−/−</sup> | All cells are WT |
| B     | 20% WT 80% Rasgrp1<sup>Anaef</sup> Cd79a<sup>−/−</sup> | Anaef excluded from B cells |
| C     | None 100% Rasgrp1<sup>WT</sup> | All cells are WT |
| D     | None 100% Rasgrp1<sup>Anaef</sup> | Most B and T cells are Anaef |

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G: Number of chimeric mice

H: Phenotype of CD4<sup>+</sup> splenocytes in groups A and B

I: Comparison of HELIOS<sup>+</sup> PD-1<sup>+</sup> and Foxp3<sup>+</sup> CD4<sup>+</sup> splenocytes between groups A and B.
and the gates used to define FOXP3+ regulatory, CD44hi FOXP3− effector/memory, and CD44lo FOXP3− naive subsets. Absolute numbers of effector/memory splenocytes (D) and Relative CD44 expression on naive splenocytes (E) are shown in the column graphs (mean ± SEM). Statistical comparisons used unpaired Student’s t tests (n = 4 WT, 5 Aaef, 2 CD79a-null, and 4 Aaef CD79a-null) *p<0.05, **p<0.01. (F) Experimental design to delineate role of Rasgrp1Aaef in T and B cells. Irradiated mice received either 100% Rasgrp1WT marrow, 100% Rasgrp1Aaef marrow, or a 1:4 mixture of CD45.1+ wild-type marrow mixed with CD45.2+ marrow from either CD79aWT/Rasgrp1WT or CD79aAaef/Rasgrp1Aaef siblings. (G) Incidence of homogeneous nuclear ANA in blood plasma in chimeric mice collected 18 weeks after irradiation, measured by immunofluorescence on HEp-2 cells and scored in a blinded manner. (H) Representative plots (left), and (I) quantification of frequency (middle) and number (right) of PD-1+ T cells from mixed chimeras in groups A and B as described in (F) 45 weeks after irradiation. Lines in (I) connect measurements from individual mice. Statistical analysis used paired Student’s t tests within groups and unpaired t tests between groups. **p<0.01, ***p<0.001, ****p<0.0001.

DOI: 10.7554/eLife.01020.014

Rasgrp1Aaef exaggerates basal mTOR-S6 signaling and CD44 expression in T cells

Our finding that tonic CD44 expression on naïve T cells sensitively reports small changes in mTOR activity prompted further analysis of this pathway in Rasgrp1Aaef T cells. CD44 was elevated on Rasgrp1Aaef DP and SP thymocytes, in diametric contrast to decreased CD44 on these cells in Rasgrp1−/− knockouts (Figure 9A,B) (Priatel et al., 2007). A putative defect in regulatory T cells cannot explain the increased CD44 expression on naïve Rasgrp1Aaef CD4+ T cells, because in mixed chimeras bearing many wild-type Foxp3+ CD4+ cells, CD44 expression was still increased on Rasgrp1Aaef mice but not on co-resident wild-type CD62L+ CD4+ splenocytes (Figure 9C). The cell autonomous increase in CD44 expression was detectable on Rasgrp1Aaef SP and DP thymocytes (Figure 9—figure supplement 1A,B) and even on CD4+ cells expressing a transgenic TCR (Figure 9D). Elevated CD44 expression on Rasgrp1Aaef cells was selective: there were no distinguishable differences between wildtype and Rasgrp1Aaef cells in CD69 and TCRβ, which are markers of cumulative Ras signaling (D’Ambrosio et al., 1994; Genot and Cantrell, 2000; Starr et al., 2003), nor in CD5 expression, a sensitive reporter of TCR affinity and constitutive or tonic TCR signaling (Azzam et al., 1998; Mandl et al., 2013) (Figure 9E, Figure 9—figure supplement 1A,B), and rapamycin treatment in vivo selectively reduced the increased CD44 expression on Rasgrp1Aaef cells (Figure 8H) but did not impact TCR or CD69 expression (data not shown).

Consistent with the CD44 data, P-S6 levels in unstimulated TCRβ−/− (pre-selection) DP thymocytes were modestly increased in Rasgrp1Aaef mice whereas they were decreased in cells from Rasgrp1−/− mice (Figure 10A). CD44 levels gradually rise as T cells mature from DP to SP and peripheral naïve T cells, suggesting that CD44 expression may reflect basal or tonic signaling. Such signals can be visualized by constitutive tyrosine-phosphorylation of the TCRδ chains and other proteins (van Oers et al., 1993), basal levels of ZAP70 recruitment to phosphorylated zeta chains (van Oers et al., 1994; Stefanova et al., 2002), and tonic phosphorylation of ERK (Roose et al., 2003; Markegard et al., 2011). Furthermore, these signals dissipate when T cells are rested in vitro in non-stimulatory medium (van Oers et al., 1993; Stefanova et al., 2002). We investigated tonic mTOR–S6 signals and found substantial basal P-S6 levels in freshly isolated lymph node cells that decreased when cells were serum-starved in vitro
Figure 8. CD44 is a sensitive reporter of mTOR activity in immature and naïve CD4+ T cells. (A) CD44lo T cell phenotype upon which Mtorchino mice were identified. Phenotype of Foxp3− CD4+ splenocytes from Mtor+/+ (WT) and Mtorchino/ (chino) mice, showing the CD62Lhi subset on which CD44 expression was quantified and normalized to the mean of WT animals (mean ± SEM from three experiments shown below). (B) Schematic of mTOR protein showing functional domains and the chino I205S mutation in the fifth HEAT repeat. (C) Reduced body size in chino. Body mass vs age of WT (black dots; 10 female [top], 11 male [bottom]) or chino (unfilled dots; three female, two male) mice. Curves were fitted to the WT datasets using second order polynomial equations; dotted lines show 95% prediction bands (the area expected to enclose 95% of future WT data points; GraphPad Prism version 5.0d for MAC OS X). Straight lines connect multiple measurements of individual chino mice. (D) Splenocytes from WT or chino mice were left unstimulated or were stimulated with PMA (100 ng/ml) for 10 min, fixed, and stained for intracellular phosphorylated-S6 (P-S6). Histogram overlay shows P-S6 staining on CD4+ cells representative of two separate experiments. (E) Number of DP, CD4SP and CD8SP cells per thymus of WT (n = 7) or chino (n = 7) mice compiled from four experiments. (F) Selective reduction in CD44 expression on chino thymocytes. Histograms show CD44, CD5, CD69 or TCRb expression on gated DP, CD4SP or CD8SP thymocytes from WT (solid gray) vs chino (red overlay) mice, representative of four separate experiments (n = 7 mice per group in total). (G) Histogram (left) and column graph (right, mean ± SEM) shows CD44 expression on CD4+ CD3+ B220− peripheral blood lymphocytes from littermate mice of the indicated Mtor genotypes, compiled from five separate experiments using a total of 20 Mtor+/+, 49 Mtorchino and 7 Mtorchino mice. CD44 relative fluorescence intensity (RFI) was calculated by dividing by the mean for the Mtor+/+ group analyzed in the same experiment. Unpaired Student's t tests: ***p<0.0001. (H) CD44 Figure 8. Continued on next page
(Figure 10B). A recent study reported that naive CD4high T cells display a range of CD5 expression in which the CD5bright cells receive most tonic signal input and are most immune reactive (Mandl et al., 2013). We first sorted CD44low naive CD4high T cells into the most bright and most dim expression for CD5 and determined that CD5bright naive CD4high T cells have significantly more P-S6 than their CD44low counterparts (Figure 10C). Next, dividing CD5low and CD5high naive CD4high T cells in equal 50–50% splits revealed that basal P-S6 was increased in Rasgrp1Anaef, particularly in the CD5low subset compared to wildtype cells (Figure 10D). The exact origin of tonic signals in T cells and its function being either immune stimulatory or immune suppressive is still an area of debate (Polic et al., 2001; Smith et al., 2001; Bhandoola et al., 2002; Stefanova et al., 2002; Högquist et al., 2003), but at least part of the tone appears to be generated by low affinity TCR binding to self pMHC (Stefanova et al., 2002). To examine if self-peptide/MHCII recognition plays a role in the increased CD44 expression on Rasgrp1Anaef naive CD4high T cells, we adoptively transferred a mixture of wild-type and Rasgrp1Anaef splenocytes into wild-type or MHCII(H2-Aa)−deficient recipient mice (Figure 10—figure supplement 1). Maintenance of CD5 expression on T cells requires contact with self pMHC (Smith et al., 2001; Mandl et al., 2012) and, as expected, CD5 expression on wildtype CD45olah C62L-P Foxp3− T cells decreased in MHCII-deficient hosts (Figure 10E), consistent with the hypothesis that CD5 is a sensitive reporter of TCR signal strength. By contrast, CD44 levels were similar irrespective of MHCII expression in the adoptive hosts, and CD44 levels remained higher on Rasgrp1Anaef than co-transferred wild-type cells in both contexts (Figure 10E). These data reveal constitutively increased expression of two reporters of mTOR activity in Rasgrp1Anaef naive CD4high T cells: the well-established reporter P-S6 and the reporter clarified here, CD44. The fact that the Rasgrp1Anaef–driven increase in CD44 is retained in the absence of MHCII suggests that this tonic signal is at least partially independent of triggering of TCRs by self-pMHC.

**CD44high Helioshigh PD-1high CD4high T cell accumulation and autoantibodies in Rasgrp1Anaef mice are corrected by hypomorphic mTOR mutation**

To test the role of elevated mTOR signaling in the Rasgrp1Anaef–induced overexpression of CD44 in naïve T cells and in the accumulation of activated CD44high PD-1high CD4high cells and autoantibodies, Rasgrp1Anaef mice were intercrossed with the subtle loss-of-function MtorChimo strain. Using a CD62L−Foxp3− gate to resolve naive CD4high splenocytes, we found that the MtorChimo mutation abolished the Rasgrp1Anaef–driven increase in CD44 expression in these naïve T cells (Figure 11A). The MtorChimo mutation alone resulted in a decrease in numbers of splenocytes, including CD4high cells, as was observed in mice with reduced Mtor mRNA (Zhang et al., 2011), but the relative proportions of the CD4high subsets examined were normal (Figure 11B). Whereas Rasgrp1Anaef mice with normal mTOR accumulated a high frequency of CD44high Foxp3− CD4high splenocytes, including the prominent PD-1− Helios− subset, this was corrected down to normal numbers in Rasgrp1Anaef MtorChimo double mutants (Figure 11B,C). Moreover, in bone marrow chimeras bearing Rasgrp1Anaef MtorChimo double-mutant hematopoietic cells, the frequency of animals with antinuclear autoantibodies was corrected to the low frequency observed in control chimeras with wildtype Rasgrp1 and Mtor (Figure 11D). Collectively, these results establish that accumulation of CD44high Helios− PD-1− CD4high cells and autoantibodies induced by Rasgrp1Anaef is sensitive to small differences in mTOR signaling.

**Discussion**

The findings here reveal a new role for Rasgrp1 in the cell-intrinsic regulation of peripheral CD4high T cells. By analyzing a missense mutation in the Rasgrp1 EF-hand, the results dissociate this new function of Rasgrp1 from its well-known role in thymic positive selection. While the Anaef EF-hand mutation did decrease Rasgrp1 activation of Ras and ERK when thymocytes were stimulated acutely by antibodies to CD3 or with PMA in vitro, the activity of this pathway during physiological positive selection in vivo...
remained sufficient for normal numbers of single positive thymocytes and peripheral T cells to form even under competitive reconstitution conditions. T cell lymphopenia and sparse T cell repertoires secondary to defective positive selection potentially explain the autoantibodies observed in mice where Rasgrp1 is entirely absent or C-terminally deleted (Dower et al., 2000; Coughlin et al., 2005; Priatel et al., 2007; Fuller et al., 2012), and in animals with missense mutations in ZAP-70 or LAT (Aguado et al., 2002; Sommers et al., 2002; Sakaguchi et al., 2003; Siggs et al., 2007).

By contrast, the normal thymic development coupled with experiments in mixed bone marrow chimeras where wild-type and mutant T cells co-exist rules out this possibility for the $\text{Rasgrp1}_{\text{Anaef}}$ mutation, and shows that peripheral CD4 cells are intrinsically dysregulated. By a combination of biochemical and genetic studies, we identify overactive mTOR signaling within naïve CD4 T cells as a key component for $\text{Rasgrp1}_{\text{Anaef}}$ to drive two abnormalities: (1) a constitutive increase in CD44 expression in naïve CD4 T cells and (2) a gradual accumulation of peripheral Helios$^+$ CD44$^+$ CD4 cells and autoantibodies.

Figure 9. Selective and cell-autonomous increase in CD44 expression in $\text{Rasgrp1}_{\text{Anaef}}$ CD4+ T cells. (A) Representative CD44 expression on unstimulated CD4SP thymocytes from wildtype, $\text{Rasgrp1}_{\text{Anaef}}$ and $\text{Rasgrp1}^{-/-}$ mice. (B) Mean relative CD44 from 22 $\text{Rasgrp1}_{\text{Anaef}}$, 13 wildtype, and 3 $\text{Rasgrp1}^{-/-}$ mice compiled from seven experiments. In each experiment, the mean CD44 expression for the wildtype group was taken to be one, and values for individual mice were normalized to this. Unpaired Student’s t tests were used to compare $\text{Rasgrp1}_{\text{Anaef}}$ and $\text{Rasgrp1}^{-/-}$ with the wildtype group. **p<0.005, ***p<0.0005. (C) CD44 MFI on CD62L$^+$Foxp3$^-$ CD4$^+$ splenocytes from mixed bone marrow chimeras described in Figure 7F. Statistical comparisons used paired t tests within, and unpaired t tests between, groups of chimeras (n = 9 for both groups); ***p<0.001, **p<0.01. (D) CD44 MFI on TCR3A9$^+$ (clonotype positive) CD4$^+$Foxp3$^-$ splenocytes from B10.BR mixed bone marrow chimeras containing CD45.1$^+$ wildtype TCR3A9 plus either CD45.2$^+$ wildtype (n = 8) or CD45.2$^+$ $\text{Rasgrp1}_{\text{Anaef}}$ (n = 7) 3A9 TCR-transgenic hematopoietic cells. Statistical comparisons in (C) and (D) used paired t tests within groups of chimeras and unpaired t tests between groups of chimeras. p value symbols: ***p<0.001, **p<0.01, *p<0.05. (E) $\text{Rasgrp1}_{\text{Anaef}}$ increases CD44 expression levels but does not affect CD69 or CD5 expression. CD4SP thymocytes are analyzed from irradiated mice reconstituted with non-transgenic CD45.1$^+$ wildtype mixed with CD45.2$^+$ $\text{Rasgrp1}_{\text{Anaef}}$ bone marrow.

DOI: 10.7554/eLife.01020.017

The following figure supplements are available for figure 9:
Figure supplement 1. CD44 and P-S6 expression in thymocytes.
DOI: 10.7554/eLife.01020.018
Rasgrp1\textsuperscript{Anaef}'s effect on Ras/ERK signaling in vivo was much milder than expected from its effects in \textit{in vitro} assays. In response to relatively strong in vitro stimuli, the Rasgrp1\textsuperscript{Anaef} mutation results in impaired membrane recruitment and T\textsubscript{184} phosphorylation of Rasgrp1 as well as reduced activation of Ras-ERK, establishing that Rasgrp1\textsuperscript{Anaef} is a hypomorphic (partial loss-of-function) allele under these conditions. By contrast, in vivo, Rasgrp1\textsuperscript{Anaef} did not alter CD69 and TCR\textsubscript{β} induction or positive selection of thymocytes, unlike the C-terminally deleted (Rasgrp1\textsuperscript{d/d}) and knockout (Rasgrp1\textsuperscript{−/−}) alleles which caused moderate and severe decreases in these processes, respectively. This may indicate that the cumulative Ras-ERK signals required for these events are sufficiently buffered or robust that they tolerate a modest reduction in Rasgrp1's Ras activating activity. Only when a TCR transgene was
Figure 11. Mtor hypomorphic mutation corrects Rasgrp1\textsuperscript{Anaef}-induced increase in naive T-cell CD44 expression and accumulation of CD44\textsuperscript{+}Helios\textsuperscript{+}PD-1\textsuperscript{−}CD4\textsuperscript{+} cells and autoantibodies. (A) 6.6.Rasgrp1\textsuperscript{Anaef} mice were intercrossed with 6.6.Mtor\textsuperscript{chino} mice to generate the single and double-mutant mice. Representative CD44 histograms of CD62L\textsuperscript{+}FOXP3\textsuperscript{−}CD4\textsuperscript{+} splenocytes from these chino, Anaef, and Anaef.chino mutants were overlaid against wild-type cells and plotted. Below, CD44 MFI of mice was normalized against average CD44 MFI of wild-type mice across two independent experiments and graphed, with columns showing mean ± SEM. Significance indicated using a 1-way ANOVA and Tukey’s post-test at n = 13 WT, 5 chino, 15 Anaef, and 4 Anaef.chino. *p<0.05, **p<0.01, ***p<0.001. (B) Representative CD44 vs FOXP3 plots for these four genotypes from (A), which display CD4\textsuperscript{+} splenocytes gated into naïve (CD44\textsuperscript{lo}FOXP3\textsuperscript{−}), activated/memory (CD44\textsuperscript{hi}FOXP3\textsuperscript{−}), and T-reg (FOXP3\textsuperscript{+}) populations. Absolute numbers of these three subsets across all four genotypes is graphed below, with columns showing mean ± SEM. Significance indicated using a 1-way ANOVA and Tukey’s post-test at n = 13 WT, 5 chino, 15 Anaef, and 4 Anaef.chino. *p<0.05, **p<0.01. (C) Bone marrow cells from sibling mice described in (A) and (B) were used to reconstitute irradiated B6.SJL CD45\textsuperscript{1/1} mice, which were analyzed 28 weeks after irradiation. Plots show HELIOS vs PD-1 expression on FOXP3\textsuperscript{−}CD4\textsuperscript{+} splenocytes, representative of both nonchimeric and chimeric mice. Absolute number of HELIOS\textsuperscript{+}PD-1\textsuperscript{−}FOXP3\textsuperscript{−}CD4\textsuperscript{+} splenocytes per mouse is continued on next page.
CD44 expression on thymocytes is decreased in the complete absence of Rasgrp1 (Figure 9) (Priatel et al., 2007) and increased by oncogenic Ras (Kindler et al., 2008; Zhang et al., 2009; Wang et al., 2011) establishing that CD44 expression in thymocytes is positively regulated by Ras. T-cell CD44 expression is also sensitive to mTOR activity, being reduced by the partial loss-of-function Mtor<sup>chino</sup> (Figure 8) and Mtor<sup>pm11pmgr</sup> (Zhang et al., 2011) alleles, and dramatically decreased in the absence of Rictor (Delgoffe et al., 2011), a binding partner of mTOR. Rasgrp1<sup>Aααmel</sup> thymocytes and naïve CD4 T cells have increased CD44 and P-S6 expression, suggesting that the Anaaef mutation increases either Rasgrp1/Ras/ERK signaling or PI-3-kinase/mTOR/S6 signaling, or both. Our recent biophysical studies revealed that Rasgrp1’s EF hands keep the protein in an autoinhibited, dimeric state, and modeling indicates that calcium-binding to the EF domain would relieve autoinhibition (Iwig et al., 2013). Thus, Rasgrp1’s EF hands play both stimulatory- and inhibitory-roles that may result in the EF2 substitution in Rasgrp1<sup>Aααmel</sup> decreasing maintenance of the autoinhibited state in the absence of strong TCR stimuli and decreasing RasGRP1 activation during strong TCR stimulation. Evidence exists for multiple intersections between the RasGRP1/Ras/ERK/RSK and PI-3-kinase/mTOR/S6 pathways, including at the level of Ras with PI-3-kinase (Castellano and Downward, 2010). While the mechanism is currently unclear, the current evidence suggests that Rasgrp1<sup>Aααmel</sup>’s gain-of-function in naïve T cells in the absence of strong TCR stimulation—and apparently in the absence of MHCII ligands for the TCR (Figure 10E)—activates S6-CD44 more than it activates ERK-CD69.

In T cells, the mTOR pathway is activated by strong TCR stimulation (Gorencita et al., 2011) and is required for efficient differentiation of naïve CD4 cells into effector cells (Delgoffe et al., 2009). T-cell-specific deletion of Tsc1, a negative regulator of mTOR, results in increased levels of P-S6 and an exuberant response to TCR stimulation in naïve T cells (Yang et al., 2011). Increased mTOR stimulation by Rasgrp1<sup>ααmel</sup> may allow self-antigens to activate some naïve CD4 cells, resulting in the gradual accumulation of activated CD62L<sup>low</sup> CD44<sup>hi</sup> PD-1<sup>+</sup> HELIOS<sup>+</sup> T cells and antinuclear autoantibodies. Because accumulation of PD-1<sup>+</sup> HELIOS<sup>+</sup> T cells in Rasgrp1<sup>ααmel</sup> mice requires B cells (Figure 7), these T cells might require B cells as specialized APCs or they might require Fc receptor-dependent enhancement of antigen presentation by antibodies (Silva et al., 2011).

Given the huge number of missense variants in each person (The 1000 Genomes Project Consortium, 2010), patients with autoimmune diseases are more likely to have point mutations in various genes than complete loss of gene expression. This new Rasgrp1<sup>ααmel</sup> mouse model adds to an emerging category of animal models with point mutations in TCR signaling proteins, along with Zap70<sup>Y136F</sup> (Sakaguchi et al., 2003), Zap70<sup>tm1mod</sup> and Zap70<sup>tm1less</sup> hypomorphic alleles (Siggs et al., 2007), LAT<sup>1136f</sup> mice (Aguado et al., 2002; Sommers et al., 2002), and Card11<sup>immunodose</sup> mice (Jun et al., 2003), where a partial deficit in T cell signaling precipitates autoimmunity or allergy. RASGRP1 splice variants have been documented for patients with SLE (Yasuda et al., 2007) and abnormal microRNA-driven downregulation of Rasgrp1 expression may play a role in aberrant DNA methylation in Lupus CD4<sup>+</sup> T cells (Pan et al., 2010). In addition, RASGRP1-linked SNVs have been associated with autoimmune diabetes and thyroid disease (Qu et al., 2009; Plagnol et al., 2011). Of the 13 uncharacterized RASGRP1 missense SNVs currently known, rs62621817 is of particular interest here since it causes a missense variation in RasGRP1’s first EF hand, changing a conserved, negatively charged aspartic acid into a valine residue. We propose that Rasgrp1<sup>ααmel</sup> mice may provide a useful model system for further studies to help elucidate how RASGRP1 variants contribute to autoimmune disease, and to help target future efforts to modulate this pathway pharmacologically.
Materials and methods

Mice
Mice were housed in pathogen-free conditions and experiments approved by either the Australian National University Animal Ethics and Experimentation Committee (Goodnow group, A2011/46) or the Institutional Animal Care and Use Committee of the University of California, San Francisco (Roose group, AN084051-01). C57BL/6 (B6), C57BL/6.SJL (CD45.1), B10Br, B10Br.CD45.1, B10Br TCR3A9, Cd79a null (also called Cd79ama1ANU) and MHCII-deficient (H2-Aa1B1) mice were obtained from ANU Bioscience Services. The Rasgrp1Anaef and Mtorchino strains were established through ethylnitrosourea (ENU)-mediated mutagenesis of B6 mice at the Australian National University as previously described (Randall et al., 2009).

Genetic mapping of the Anaef mutation
Affected Rasgrp1Anaef mice were crossed onto the CBA/J background to generate heterozygous F1 mice. F1 mice were intercrossed to yield mice homozygous for the Anaef mutation and carrying a mix of C57BL/6 and background CBA/J single nucleotide polymorphisms (SNPs). Genomic DNA samples isolated from both affected and unaffected mice were used as templates for SNP mapping at the Genomics Institute of the Novartis Research Foundation (San Diego, CA). SNP markers were spaced approximately every 3–5 Mbp throughout the genome. Once a defined interval was established, the Rasgrp1 encoding gene was sequenced from genomic DNA from both affected Anaef and WT mice. All exons were amplified by PCR with primers designed to include intronic RNA splice donor and acceptor sites. Exome enrichment using the SureSelect Mouse Exome kit (G7550A-001; Agilent, Santa Clara, CA), sequencing using the Illumina HiSeq 2000 (Illumina, San Diego, CA), and computational analysis to detect novel single-nucleotide variants were performed as described previously (Andrews et al., 2012).

Genotyping
Roose lab Anaef mice were genotyped using MS-PCR. Primers were combined in a single reaction with Taq, Taq buffer and dNTPs (all New England BioLabs, Ipswich, MA). Goodnow lab Anaef mice were genotyped by APF Genomics Services following the manufacturer’s instructions for Amplifluor PCR (SNP FAM/JOE; Millipore, Billerica, MA).

Transfections and stable cell lines
Transfections and creation of stable cell lines was performed as previously described (Roose et al., 2005).

Antinuclear antibody testing
Diluted mouse plasma was applied to HEp-2 slides (Inova, San Diego, CA). AlexaFluor488-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) was added and slides mounted with fluorescence mounting medium (Dako Australia). Photos were taken using an Olympus IX71 microscope and WIB filter with 20 × lens and exposure time of 1/25 s.

Flow cytometry
Suspensions of splenocytes (depleted of erythrocytes by brief osmotic lysis) or thymocytes were incubated with cocktails of anti-mouse antibodies specific for: CD44, CD4, CD45.1, CD45.2, CD5, CD62L, CD69, TCRβ, PD-1, TCR Vβ5, TCR Vβ8, TCR Vβ11, B220, CD95, GL-7, B220 (BD Pharmingen Franklin Lakes, NJ or BioLegend, San Diego, CA) or CD8 (BD Pharmingen and UCSF Monoclonal Antibody Core, clone YTS169.4) conjugated to AlexaFluor700, APC-780 or APCCy7, PE-Cy7, APC, PerCPCy5.5, FITC, PE, Pacific Blue or biotin. Biotinylated antibodies were detected in another incubation step with streptavidin conjugated to Qdot605 (Invitrogen) or BV605 (BioLegend). Cells expressing the 3A9 TCR transgene were detected using the 1G12 (mouse IgG1) antibody (ATCC, Manassas, VA) followed by another incubation in anti-mouse IgG1 (A85.1). To detect intracellular proteins, cells were fixed and permeabilized using a Foxp3 staining kit (eBioscience, San Diego, CA), then labeled with antibodies specific for Foxp3 (FJK-16s; eBioscience), Helios (clone 22F6; Biolegend), IFNγ, IL-4, IL-2 or IL-17 (all BD Pharmingen). Flow cytometry data was acquired on a FACSort (Becton Dickinson) or an LSR Fortessa system and analyzed with FlowJo v8 (Treestar, Ashland, OR).
Cell stimulations
Cells were stimulated using 25 ng/ml (HIGH), 5 ng/ml (MED), or 2 ng/ml (LOW) PMA (Calbiochem) with/without ionomycin (10 μM, Sigma, St. Louis, MO), or with 100 ng/ml PMA for Phospho-S6 assays. To mimic TCR engagement, cells were pre-labeled using anti-CD3 primary antibody (10 μg/ml, UCSF Monoclonal Antibody Core, clone 2C11) and crosslinking was achieved using goat anti-hamster antibody (10 μg/ml, Jackson ImmunoResearch). For intracellular cytokine detection by flow cytometry, splenocytes were stimulated in complete medium for 4 hr at 37°C with PMA (100 ng/ml; Sigma), ionomycin (500 ng/ml; Sigma) and GolgiStop (1/1000; BD), then labeled as described above.

Flow cytometry for phosphorylated proteins
Procedure was performed as described in Das et al., (2009). Cells were fixed using Cytofix Cell Fixative (BD Biosciences). Cells were permeabilized using 90% Methanol. Primary staining for phospho-Erk occurred using rabbit anti-mouse p-Erk antibody (#4377S; Cell Signaling) followed by staining with goat anti-Rabbit PE (Jackson ImmunoResearch, West Grove, PA). For analysis of tonic and PMA induced S6 phosphorylation by FACS, single-cell suspensions were prepared from thymus. Half of the cells were fixed in warm cytofix (BD Biosciences) immediately after harvesting and reserved for tonic signaling analysis. The remaining cells were then counted, and 10⁶ cells/sample were stimulated with PMA for 3 min, followed by fixation. Stimulated and unstimulated cells were then washed three times with cytoperm buffer (BD Biosciences) and incubated on ice in this buffer with a rabbit polyclonal antibody against phosphorylated S6 (cell signaling) for 45 min. The cells were then washed twice with cytoperm and incubated for an additional 45 min in cytoperm buffer containing an APC-conjugated goat anti-rabbit secondary antibody, as well as anti CD8-FITC, CD4, PE-Cy7 and TCRβ-PE. Cells were washed twice and analyzed in an LSR Fortessa system.

Ras pulldown
Activation of Ras was analyzed using a RasGTP pulldown assay (Upstate Biotechnology, Lake Placid, NY) as previously described (Roos et al., 2005).

Western blotting
Cells were lysed using Nonidet-P40 lysis buffer (1%) supplemented with protease and phosphatase inhibitors. Lysates were run on 10% acrylamide Bis-Tris gels and transferred onto PDVF filter (Millipore, Immobilon-P). Blots were probed for Rasgrp1 (M199; Santa Cruz, Dallas, TX), Alpha tubulin (Sigma), phospho-tyrosine (In house antibody prep, clone 4G10), phospho-Zap70 (Y493; Cell Signaling, Danvers, MA), phospho-PLCγ (Y783; Cell Signaling) and phospho-Lck (Y416; Cell Signaling), anti-ERK (pan-Erk) antibody (BD Transduction labs, clone 16/ERK). Rabbit anti-human Rasgrp1 (clone E80) was produced by Epitomics, Inc. (Burlingame, CA, USA). Mouse anti-Rasgrp1 p-T184 (clone JR-pT184RG1-4G7) was produced by AnaSpec (Fremont, CA, USA). Signal from primary antibodies detected using HRP conjugated secondary antibodies: Sheep anti-mouse HRP (GE Healthcare, Cleveland, OH) and goat anti-rabbit HRP (SouthernBiotech, Birmingham, AL). Blots were developed using Pierce ECL Western Blotting Substrate (ThermoScientific, Waltham, MA) and images recorded using a chemiluminescence imager (LAS-4000; Fuji).

Bone marrow chimeras
Bone marrow was collected, and in some experiments, depleted of T cells and NK cells by magnetic labelling using biotinylated anti-TCRβ and anti-NK1.1 and streptavidin microbeads followed by passage through a MACS LD column (Miltenyi Biotec, Germany). Recipient mice were irradiated with X-rays (2 doses of 4.5 Gy given 4 hr apart) then injected intravenously (i.v.) with 2 x 10⁶ bone marrow cells that were either from single or multiple donors as described in the text.

Rapamycin treatment
Suboptimal doses of rapamycin (Coenen et al., 2007; Araki et al., 2009) were prepared on day 0 in sufficient quantity for all injections in one experiment. The appropriate rapamycin stock volume was diluted to the indicated concentrations in DMSO (15.4%), Cremaphor (15.4%) and water (69.2%), and aliquoted in six equal portions (one aliquot per injection) and frozen. Mice were injected on days 0, 1, 2, 3, 5 and 7, and then sacrificed on day 8. Thymocytes were harvested and analyzed as before.
**BrdU labelling**

1 mg BrdU (BD) in PBS per mouse was injected i.p. to pulse label a cohort of dividing cells. Following surface staining of thymocytes as above, BrdU was detected following the BrdU Flow Kit (BD) protocol by fixing and permeabilizing cells with provided buffers, incubating for 1 hr at 37°C in DNase, then washing and staining with anti-BrdU antibody.

**CellTrace Violet (CTV) labelling**

CTV labeling was done at room temperature as described (Quah and Parish, 2010) with slight modifications. Splenocytes suspended at $10^8$ cells/ml in RPMI containing HI-FCS (10% vol/vol) were transferred to the base of a fresh 15 ml conical tube. 1 µl of CTV (Life Technologies, Carlsbad, CA) stock solution (10 mM) per ml of cell suspension was placed on the dry wall of the tubes, then tubes were capped, inverted and briefly vortexed (final CTV concentration 10 µM). After 5 min incubation in the dark, 10 ml of 10%FCS/RPMI was added, then cells were sedimented by centrifugation before another wash in 10 ml of the same medium. Cells were then resuspended in PBS and passed through a 70 µm cell strainer (BD) before i.v. injection (200 µl per mouse).

**Acknowledgements**

The authors would like to thank Drs Richard Glynne, Director of Genetics and Neglected Diseases at Novartis, for guiding the mapping project, Rich Lewis for discussion on calcium signals, and Michelle Hermiston for assistance with thymocyte FACS stainings. We thank Dr Jim Stone for sharing Rasgrp1 deficient mice and Dr Robert Barrington for sending these mice. We thank Debbie Howard, Nadine Barthel and Heather Domaschenz for expert technical assistance, and the animal services and genotyping teams at the Australian Phenomics Facility. We also thank the members of the Cyster, Goodnow, Vinuesa, and Roose labs and NHMRC Program members for helpful comments and suggestions.

**Additional information**

**Funding**

| Funder                                      | Grant reference number                                      | Author                          |
|---------------------------------------------|-------------------------------------------------------------|---------------------------------|
| Sandler Program in Basic Science            | K01CA113367, ARRA supplement, R56-AI095292                  | Jeroen P Roose                  |
| National Institutes of Health               |                                                                 |                                 |
| Wellcome Trust                              | 082030/B/07/Z                                               | Christopher C Goodnow           |
| Department of Innovation, Industry, Science, Research and Tertiary Education |                                                                 | Edward M Bertram, Christopher C Goodnow |
| Clive and Vera Ramasciotti Foundation Grant | GNT1035858                                                  | Anselm Enders, Christopher C Goodnow |
| National Health and Medical Research Council|                                                                 |                                 |
| National Institutes of Health               | 1R03AR062783-01A1                                           | Andre Limnander                 |
| National Institutes of Health               | R01-AI74847                                                 | Jason G Cyster                  |
| National Institutes of Health               | R01 AI52127, U54 AI054523                                  | Edward M Bertram, Christopher C Goodnow |
| National Health and Medical Research Council| Program Grant and Australia Fellowship                     | Christopher C Goodnow           |

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**Author contributions**

SRD, KMC, DYH, KLR, CNJ, AL, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; DRM, NKP, AE, CR, BB, LAM, GS, EMB, MAF,
YS, TDA, BW, SWB, JRW. Acquisition of data, Analysis and interpretation of data; JGC, Conception and design, Analysis and interpretation of data; CCG, JPR, Conception and design, Analysis and interpretation of data, Drafting or revising the article

Ethics
Animal experimentation: This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols of the University of California San Francisco (UCSF; approval number AN084051-03) and of the Australian Phenomics Facility and the Australian National University (ANU; approval number A2011/46). These protocols were approved by the Committee on the Ethics of Animal Experiments of UCSF and ANU.

References
Abarrategui I, Krangel MS. 2006. Regulation of T cell receptor-alpha gene recombination by transcription. Nature Immunology 7:1109–1115. doi: 10.1038/n1379.

Adami A, Garcia-Alvarez B, Arias-Palomino E, Barford D, Llorca O. 2007. Structure of TOR and its complex with KOG1. Molecular Cell 27:509–516. doi: 10.1016/j.molcel.2007.05.040.

Aguado E, Richelme S, Nunez-Cruz S, Miazek A, Mura AM, Richelme M, Guo XJ, Sainty D, He HT, Malissen B, Malissen M. 2002. Induction of T helper type 2 immunity by a point mutation in the LAT adaptor. Science 296:2036–2040. doi: 10.1126/science.1069057.

Andrews TD, Whittle B, Field MA, Balakishnan B, Zhang Y, Shao Y, Cho V, Kirk M, Singh M, Xia Y, Hager J, Winslade S, Spillemans G, Beutler B, Enders A, Goodnow CC. 2012. Massively parallel sequencing of the mouse exome to accurately identify rare, induced mutations: an immediate source for thousands of new mouse models. Open Biology 2:120061. doi: 10.1098/rsob.120061.

Araki K, Turner AP, Shaffer VO, Gangappa S, Keller SA, Bachmann MF, Larsen CP, Ahmed R. 2009. mTOR regulates memory CD8 T-cell differentiation. Nature 460:108–112. doi: 10.1038/nature08155.

Azzam HS, Grinberg A, Lui K, Shen H, Shores EW, Love PE. 1998. CD5 expression is developmentally regulated by T cell receptor (TCR) signals and TCR avidity. The Journal of Experimental Medicine 188:2301–2311. doi: 10.1084/jem.188.12.2301.

Beaulieu N, Zahedi B, Goulding RE, Tazmini G, Anthony KV, Omeis SL, De Jong DR, Kay RJ. 2007. Regulation of RasGRP1 by B cell antigen receptor requires cooperativity between three domains controlling translocation to the plasma membrane. Molecular Biology of the Cell 18:3156–3168. doi: 10.1091/mbc.E06-10-0932.

Bhandoola A, Tai X, Echhaus M, AUCHINLOSS H, Mason K, Rubin SA, Carbone KM, Grossman Z, Rosenberg AS, Singer A. 2002. Peripheral expression of self-MHC-II influences the reactivity and self-tolerance of mature CD4(+) T cells: evidence from a lymphopenic T cell model. Immunity 17:425–436. doi: 10.1016/S1074-7613(02)00417-X.

Bottema CD, Sommer SS. 1993. PCR amplification of specific alleles: rapid detection of known mutations and polymorphisms. Mutation Research 288:93–102. doi: 10.1016/0027-5107(93)90211-W.

Castellano E, Downward J. 2010. Role of RAS in the regulation of PI 3-kinase. Current Topics In Microbiology and Immunology 346:143–169. doi: 10.1007/978_2010_56.

Coenen JJ, Koenen HJ, Van Rijssen E, Kasran A, Boon L, Hilbrands LB, Joosten J. 2007. Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4+ CD25+ FoxP3+ T cells. Bone Marrow Transplantation 39:537–545. doi: 10.1038/sj.bmt.1705628.

Coughlin JJ, Stang SL, Dower NA, Stone JC. 2005. RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. Journal of Immunology 175:7179–7184.

D’Ambrosio D, Cantrell DA, Frati L, Santoni A, Testi R. 1994. Involvement of p21ras activation in T cell CD69 expression. European Journal of Immunology 24:616–620. doi: 10.1002/eji.1830240319.

Das J, Ho M, Zikherman J, Govern C, Yang M, Weiss A, Chakraborty AK, Roose JP. 2009. Digital signaling and hysteresis characterize ras activation in lymphoid cells. Cell 136:337–351. doi: 10.1016/j.cell.2008.11.051.

Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, Worley PF, Kozma SC, Powell JD. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity 30:832–844. doi: 10.1016/j.immuni.2009.04.014.

Delgoffe GM, Pollizzi KN, Waickman AT, Heikamp E, Meyers DJ, Horton MR, Xiao B, Worley PF, Powell JD. 2011. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. Nature Immunology 12:295–303. doi: 10.1038/n1205.

Dower NA, Stang SL, Bottorff DA, Ebini JO, Dickie P, Ostergaard HL, Stone JC. 2000. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. Nature Immunology 1:317–321. doi: 10.1038/79766.

Ebini JO, Bottorff DA, Chan SY, Stang SL, Dunn RJ, Stone JC. 1998. RasGRP, a Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Science 280:1082–1086. doi: 10.1126/science.280.5366.1082.

Feske S. 2007. Calcium signalling in lymphocyte activation and disease. Nature Reviews Immunology 7:690–702. doi: 10.1038/ni2152.

Fischer AM, Katayama CD, Pages G, Pouyssegur J, Hedrick SM. 2005. The role of erk1 and erk2 in multiple stages of T cell development. Immunity 23:431–443. doi: 10.1016/j.immuni.2005.08.013.
Fuller DM, Zhu M, Song X, Ou-Yang CW, Sullivan SA, Stone JC, Zhang W. 2012. Regulation of RasGRP1 function in T cell development and activation by its unique tail domain. Public Library of Science One 7:e38796. doi: 10.1371/journal.pone.0038796.

Gangloff YG, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz JF, Um SH, Brown EJ, Cereghini S, Thomas G, Kozma SC. 2004. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Molecular and Cellular Biology 24:9508–9516. doi: 10.1128/MCB.24.21.9508-9516.2004.

Genot E, Cantrell DA. 2000. Ras regulation and function in lymphocytes. Current Opinion In Immunology 12:289–294. doi: 10.1016/S0952-7915(00)00089-3.

Griffoldi JL, Walsh MP, Vogel HJ. 2007. Structures and metal-ion-binding properties of the Ca2+-binding loop and helix EF-hand motifs. Biochemical Journal 405:199–221. doi: 10.1042/BJ20070255.

Golec DP, Dower NA, Stone JC, Baldwin TA. 2013. RasGRP1, but not RasGRP3, is required for efficient thymic T cell development. Biochemical Journal 451:527–536. doi: 10.1042/BJ20130286.

Gorelka BK, Wan CK, Zhong XP. 2011. Negative regulation of mTOR activation by diacylglycerol kinases. Blood 117:4022–4031. doi: 10.1182/blood-2010-08-300731.

Grabarek Z. 2006. Structural basis for diversity of the EF-hand calcium-binding proteins. Journal of Molecular Biology 359:509–525. doi: 10.1016/j.jmb.2006.03.066.

Hogquist KA, Starr TK, Jameson SC. 2003. Receptor sensitivity: when T cells lose their sense of self. Current Biology 13:R239–R241. doi: 10.1016/S0960-9822(03)00161-1.

Hsieh AC, Liu Y, Edlind MP, Ingolia NT, Janes MR, Sher A, Shi EY, Stumpf CR, Christensen C, Bonham MJ, Wang S, Ren P, Martin M, Jessen K, Feldman ME, Weissman JS, Shokat KM, Rommel C, Ruggero D. 2012. The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature 485:55–61. doi: 10.1038/nature10912.

Iwig JS, Vercoulen Y, Das R, Barros T, Limnander A, Che Y, Pelton JG, Wemmer DE, Roose JP, Kuriyan J. 2013. Structural analysis of autoinhibition in the Ras-specific exchange factor Rap1GDP. eLife 2:e00813. doi: 10.7554/eLife.00813.

Jun JE, Wilson LE, Vinuesa CG, Lesage S, Blery M, Miosge L, Cook MC, Kucharska EM, Hara H, Penninger JM, Domachenz H, Hong NA, Glynne RJ, Nelms KA, Goodnow CC. 2003. Identifying the MAGUK protein Carma-1 as a dominant negative regulator of TCR signal transduction. Nature Immunology 4:751–762. doi: 10.1038/ni1091.

Kim DH, Sarbassov DD, Ali SM, King JE, Latek RR, Erdjument-Bromage H, Tempst P, Sabatini DM. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 113:161–175. doi: 10.1016/S0092-8674(02)00808-5.

Kindler T, Cornejo MG, Scholl C, Liu J, Leeman DS, Haydu JE, Frohling S, Lee BH, Gilliland DG. 2008. K-Ras12D-induced T-cell lymphoblastic lymphoma/leukemias harbor Notch1 mutations and are sensitive to gamma-secretase inhibitors. Blood 112:3373–3382. doi: 10.1182/blood-2008-03-147587.

Knutsen BA. 2010. Insights into the domain and repeat architecture of target of rapamycin. Journal of Structural Biology 170:354–363. doi: 10.1016/j.jsb.2010.01.002.

Kortum RL, Rouguette-Jazdanian AK, Samelson LE. 2013. Ras and extracellular signal-regulated kinase signaling in thymocytes and T cells. Trends in Immunology 34:259–268. doi: 10.1016/j.it.2013.02.004.

Manjil JN, Liou R, Klauschen F, Vrisekoop N, Monteiro JP, Yates AJ, Huang AY, Germain RN. 2012. Quantification of lymph node transit times reveals differences in antigen surveillance strategies of naive CD4+ and CD8+ T cells. Proceedings of the National Academy of Sciences of the United States of America 109:18036–18041. doi: 10.1073/pnas.1211717109.

Manjil JN, Monteiro JP, Vrisekoop N, Germain RN. 2013. T cell-positive selection uses self-ligand binding strength to optimize repertoire recognition of foreign antigens. Immunity 38:263–274. doi: 10.1016/j.immuni.2012.09.011.

Markegard E, Trager E, Yang CW, Zhang W, Weiss A, Roose JP. 2011. Basal LAT-diacylglycerol-RasGRP1 signals in T cells maintain TCRα gene expression. Public Library of Science One 6:e25540. doi: 10.1371/journal.pone.0025540.

Murakami M, Ichisaka T, Maeda M, Oshiro N, Hara K, Edenhofe F, Kiyama H, Yonezawa K, Yamanaka S. 2004. mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Molecular and Cellular Biology 24:6710–6718. doi: 10.1128/MCB.24.15.6710-6718.2004.

Nelms KA, Goodnow CC. 2001. Genome-wide ENU mutagenesis to reveal immune regulators. Immunity 15:409–418. doi: 10.1016/S0976-7413(01)00199-6.

Pan W, Zhu S, Yuan M, Cui H, Wang L, Luo X, Li J, Zhou H, Tang Y, Shen N. 2010. MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1. The Journal of Immunology 184:6773–6781. doi: 10.4049/jimmunol.0904060.

Plagnol V, Howson JM, Smyth DJ, Walker N, Hafler JP, Bingley MJ, Bingley PJ, Gough SC, Todd JA. 2011. Genome-wide association analysis of autoantibody positivity in type 1 diabetes cases. Public Library of Science Genetics 7:e1002216. doi: 10.1371/journal.pgen.1002216.

Polic B, Kunkel D, Scheffold A, Rajewsky K. 2001. How alpha beta T cells deal with induced TCR alpha ablation. Nature Immunology 2:409–418. doi: 10.1038/350870a.

Poon HY, Stone JC. 2009. Functional links between diacylglycerol and phosphatidylinositol signaling systems in human leukocyte-derived cell lines. Biochemical and Biophysical Research 390:1395–1401. doi: 10.1016/j.bbrc.2009.11.004.

Daley et al. eLife 2013;2:e01020. DOI: 10.7554/eLife.01020
Priatel JJ, Chen X, Zewenlacz LA, Shen H, Harder KW, Horwitz MS, Teh H.S. 2007. Chronic immunodeficiency in mice lacking RasGRP1 results in CD4 T cell immune activation and exhaustion. The Journal of Immunology 179:2143–2152.

Priatel JJ, Teh SJ, Dower NA, Stone JC, Teh HS. 2002. RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. Immunity 17:617–627. doi: 10.1016/S1074-7613(02)00451-X.

Qua BJ, Parish CR. 2010. The use of carboxyfluorescein diacetate succinimidyl ester (CFSE) to monitor lymphocyte proliferation. Journal of Visualized Experiments 44:e2259. doi: 10.3791/2259.

Ramiscal RR, Vinuesa CG. 2013. T-cell subsets in the germinal center. Immunological Reviews 252:146–155. doi: 10.1111/imr.12031.

Randall KL, Lambe T, Johnson AL, Treanor B, Kucharska E, Domaschenz H, Whittle B, Tze LE, Enders A, Crockford TL, Bouriez-Jones T, Alizadeh AA, Botstein D, Brown PO, Weiss A. 2003. T cell receptor-independent basal signaling via Erk and Abl kinases suppresses DAG gene expression. Public Library of Science Biology 1:E55. doi: 10.1371/journal.pbio.0060053.

Reese ME, Killeen N, Weiss A. 1994. ZAP-70 is constitutively associated with tyrosine-phosphorylated Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. Blood 83:320–334.

Roose JP, Diehn M, Tomlinson MG, Lin J, Alizadeh AA, Botstein D, Brown PO, Weiss A. 2003. Dock8 mutations cripple B cell immunological synapses, germinal centers and long-lived antibody production. Nature Immunology 10:1283–1291. doi: 10.1038/ni.1820.

Roose JP, Mollenauer M, Gupta VA, Stone J, Weiss A. 2005. A diacylglycerol-protein kinase C-RasGRP1 pathway directs Ras activation upon antigen receptor stimulation of T cells. Molecular and Cellular Biology 25:4426–4441. doi: 10.1128/MCB.25.11.4426-4441.2005.

Roose JP, Mollenauer M, Ho M, Kurosiaki T, Weiss A. 2007. Unusual interplay of two types of Ras activators, RasGRP and SOS, establishes sensitive and robust Ras activation in lymphocytes. Molecular and Cellular Biology 27:2732–2745. doi: 10.1128/MCB.01882-06.

Rust S, Funke H, Assmann G. 1993. Mutagenically separated PCR (MS-PCR): a highly specific one step procedure for easy mutation detection. Nucleic Acids Research 21:3623–3629. doi: 10.1093/nar/21.16.3623.

Sakaguchi N, Takahashi T, Hata H, Nomura T, Tagami T, Yamazaki S, Sakihama T, MatsuTani T, Negishi I, Nakatsu S, Sakaguchi S. 2003. Altered thymic T-cell development due to a mutation of the ZAP-70 gene causes autoimmune arthritis in mice. Nature 426:454–460. doi: 10.1038/nature02119.

Salmond RJ, Emery J, Okkenhaug K, Zamoytka R. 2009. MAPK, phosphatidylinositol 3-kinase and mammalian target of rapamycin pathways converge at the level of ribosomal protein S6 phosphorylation to control metabolic signaling in CD8 T cells. Journal of Immunology 183:7388–7397. doi: 10.4049/jimmunol.0900294.

Siggs OM, Miosge LA, Yates AL, Kucharska EM, Sheahan D, Bridick T, Weiss A, Liston A, Goodnow CC. 2007. Opposing functions of the T cell receptor kinase ZAP-70 in immunity and tolerance differentially titrate in response to nucleotide substitutions. Immunity 27:912–926. doi: 10.1016/j.immuni.2007.11.013.

Silva DG, Daley SR, Hogan J, Lee SK, Teh CE, Hu DY, Lam KP, Goodnow CC, Vinuesa CG. 2011. Anti-islet autoantibodies trigger autoimmune diabetes in the presence of an increased frequency of islet-reactive CD4 T cells. Diabetes 60:2102–2111. doi: 10.2337/db10-1344.

Smith K, Seddon B, Purbho MA, Zamovytsa R, Fisher AG, Merkensch jogginger M. 2001. Sensory adaptation in naive peripheral CD4 T cells. Journal of Experimental Medicine 194:1253–1261. doi: 10.1084/jem.194.9.1253.

Sommers CL, Park CS, Lee J, Feng C, Fuller CL, Grinberg A, Hildebrand JA, Lacana E, Menon RK, Shores EW, Samelson LE, Love PE. 2002. A LAT mutation that inhibits T cell development yet induces lymphoproliferation. Science 296:2040–2043. doi: 10.1126/science.1069066.

Starr TK, Jameson SC, Hogquist KA. 2003. Positive and negative selection of T cells. Annual Review of Immunology 21:139–176. doi: 10.1146/annurev.immunol.21.120601.141107.

Stefanova I, Durman JR, Germain RN. 2002. Self-recognition promotes the foreign antigen sensitivity of naive T lymphocytes. Nature 420:429–434. doi: 10.1038/nature01146.

Stone JC. 2011. Regulation and function of the RasGRP family of Ras activators in blood cells. Genes Cancer 2:320–334. doi: 10.1177/1947660111408082.

Swan KA, Alberola-Ila I, Gross JA, Appleby MW, Forbush KA, Thomas JF, Perlmutter RM. 1995. Involvement of p21ras distinguishes positive and negative selection in thymocytes. The EMBO Journal 14:276–285.

Tazmin G, Beaulieu N, Woo A, Zahedi B, Goulding RE, Kay RJ. 2009. Membrane localization of RasGRP1 is controlled by an EF-hand, and by the GEF domain. Biochimica et Biophysica Acta 1793:447–461. doi: 10.1016/j.bbamcr.2008.12.019.

The 1000 Genomes Project Consortium, Abecasis GR, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, Hurles ME, McVean GA. 2010. A map of human genome variation from population-scale sequencing. Nature 467:1061–1073. doi: 10.1038/nature09534.

Thorton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. Journal of Immunology 184:3433–3441. doi: 10.4049/jimmunol.09004082.

van Oers NS, Kileen N, Weiss A. 1994. ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR zeta in murine thymocytes and lymph node T cells. Immunity 1:675–685. doi: 10.1016/1074-7613(94)90038-8.

Daley et al. eLife 2013;2:e01020. DOI: 10.7554/eLife.01020 25 of 26
van Oers NS, Tao W, Watts JD, Johnson P, Aebersold R, Teh HS. 1993. Constitutive tyrosine phosphorylation of the T-cell receptor (TCR) zeta subunit: regulation of TCR-associated protein tyrosine kinase activity by TCR zeta. Molecular and Cellular Biology 13:5771–5780.

Wang J, Liu Y, Li Z, Wang Z, Tan LX, Ryu MJ, Meline B, Du J, Young KH, Ranheim E, Chang Q, Zhang J. 2011. Endogenous oncogenic Nras mutation initiates hematopoietic malignancies in a dose- and cell type-dependent manner. Blood 118:368–379. doi: 10.1182/blood-2010-12-326058.

Yabas M, Teh CE, Frankenreiter S, Lal D, Roots CM, Whittle B, Andrews DT, Zhang Y, Teoh NC, Sprent J, Tze LE, Kucharska EM, Koffer J, Farell GC, Broer S, Goodnow CC, Enders A. 2011. ATP11C is critical for the internalization of phosphatidylinerse and differentiation of B lymphocytes. Nature Immunology 12:441–449. doi: 10.1038/ni.2011.

Yang K, Neale G, Green DR, He W, Chi H. 2011. The tumor suppressor Tsc1 enforces quiescence of naive T cells to promote immune homeostasis and function. Nature Immunology 12:888–897. doi: 10.1038/ni.2068.

Yasuda S, Stevens RL, Terada T, Takeda M, Hashimoto T, Fukae J, Horita T, Kataoka H, Atsumi T, Koike T. 2007. Defective expression of Ras guanyl nucleotide-releasing protein 1 in a subset of patients with systemic lupus erythematosus. The Journal of Immunology 179:4890–4900.

Zhang J, Wang J, Liu Y, Sidik H, Young KH, Lodish HF, Fleming MD. 2009. Oncogenic Kras-induced leukemogenesis: hematopoietic stem cells as the initial target and lineage-specific progenitors as the potential targets for final leukemic transformation. Blood 113:1304–1314. doi: 10.1182/blood-2008-01-134262.

Zhang S, Readinger JA, Dubois W, Janka-Junttila M, Robinson R, Pruitt M, Bliskovsky V, Wu JZ, Sakakibara K, Patel J, Parent CA, Tessarollo L, Schwartzberg PL, Mock BA. 2011. Constitutive reductions in mTOR alter cell size, immune cell development, and antibody production. Blood 117:1228–1238. doi: 10.1182/blood-2010-05-287821.

Zheng Y, Liu H, Coughlin J, Zheng J, Li L, Stone JC. 2005. Phosphorylation of RasGRP3 on threonine 133 provides a mechanistic link between PKC and Ras signaling systems in B cells. Blood 105:3648–3654. doi: 10.1182/blood-2004-10-3916.

Zhu M, Fuller DM, Zhang W. 2012. The role of Ras guanine nucleotide releasing protein 4 in Fc epsilonRI-mediated signaling, mast cell function, and T cell development. The Journal of Biological Chemistry 287:8135–8143. doi: 10.1074/jbc.M111.320580.