Recurrent mTORC1-activating RRAGC mutations in follicular lymphoma

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Follicular lymphoma is an incurable B cell malignancy1 characterized by the (14;18) translocation and mutations affecting the epigenome2,3. Although frequent gene mutations in key signaling pathways, including JAK-STAT, NOTCH and NF-kB, have also been defined2–7, the spectrum of these mutations typically overlaps with that in the closely related diffuse large B cell lymphoma (DLBCL)6–13. Using a combination of discovery exome and extended targeted sequencing, we identified recurrent somatic mutations in RRAGC uniquely enriched in patients with follicular lymphoma (17%). More than half of the mutations preferentially co-occurred with mutations in ATP6V1B2 and ATP6AP1, which encode components of the vacuolar H+ -ATPase (V-ATPase) known to be necessary for amino acid–induced activation of mTORC1. The RagC variants increased raptor binding while rendering mTORC1 signaling resistant to amino acid deprivation. The activating nature of the RRAGC mutations, their existence in the dominant clone and their stability during disease progression support their potential as an excellent candidate for therapeutic targeting.

Follicular lymphoma is one of the commonest non-Hodgkin lymphomas (NHLs). Although the majority of affected individuals exhibit a characteristic protracted disease course with multiple relapses, others develop aggressive disease and histological transformation with shortened overall survival time. Genome-wide profiling studies have primarily focused on analyses at single time points or of the subset of patients who have undergone histological transformation to determine the genetic mediators of progression2,3. To gain insight into the genetic diversity of follicular lymphoma, we undertook temporal analyses on individuals diagnosed with follicular lymphoma who underwent several relapse episodes without transformation. These data uncovered recurrent mutations in components of the mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway specific to follicular lymphoma.

We performed exome sequencing on 24 tumors (from five patients, B2–B6) and matched constitutional DNA, with an average sequencing depth of 140x and 97.5% of the targeted bases covered by >10-fold (Online Methods and Supplementary Table 1). The clinical course from diagnosis to last follow-up ranged from 12.5 to 25 years (Supplementary Fig. 1 and Supplementary Table 2). We identified a median of 94 nonsynonymous mutations per tumor and validated mutations of interest by a combination of Sanger sequencing and tagged-amplicon sequencing (Supplementary Tables 3 and 4). Consistent with our earlier longitudinal study of paired follicular lymphoma and transformed follicular lymphomas2, tumors from the same individual confirmed a branched evolutionary pattern and demonstrated that all tumors evolve from a dominant ancestral clone (Supplementary Fig. 2). Moreover, mutations in KMT2D, CREBBP and MEF2B were present on the trunks of the phylogenetic trees in all five individuals, consistent with their existence in the dominant clone and their stability during disease progression. These findings support the hypothesis that recurrent RRAGC mutations in follicular lymphoma are the result of mTORC1 activation, a mechanism that could be targeted for therapeutic intervention.

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with the role of epigenetic deregulations as critical early events in the majority of follicular lymphomas.23,14,15.

Our data identified somatic non-silent mutations in the RRAGC gene, which encodes a Ras-related GTP-binding protein (RagC), occurring in four of the five cases. Notably, in cases B4 and B6, the RRAGC mutations (resulting in p.Pro118Leu and p.Lys74Arg substitutions) were conserved during disease progression, whereas in cases B2 and B3 we observed a convergent pattern of clonal selection, with different mutations occurring at different time points in disease evolution (Fig. 1a). We rarely observed copy number variation at the RRAGC locus (1p34.3) in both our current data and previous SNP array data sets (Supplementary Fig. 3). These findings, together with the RRAGC variant allele frequencies (VAFs), were consistent with heterozygous mutations (VAF range of 0.17–0.5), and clonality plots verified that the VAFs were comparable to those of early driver mutations, demonstrating that the RRAGC mutations reside within the dominant clone of the tumor biopsies (Fig. 1b).

To determine the prevalence of RRAGC mutations, we performed Sanger sequencing, restricting our analyses to exons 1 and 2, in an additional 329 related mature B cell NHLs and 51 B cell lymphoma cell lines alongside an analysis of publically available sequencing data sets.10,12,13,16–20. RRAGC mutations were absent in other hematological malignancies, including myeloid and other mature B cell NHL entities (Table 1), with the exception of infrequent mutations in the closely related DLBCL. We found that RRAGC was rarely mutated in the non-hematological neoplasms (0.3% of nearly 10,000 samples) included in the Cancer Genomics database (cBioPortal)21, with the majority of substitutions mapping to residues beyond Pro118 (Supplementary Table 6). RRAGC mutations are therefore highly enriched in follicular lymphoma, with their nature and frequency suggesting that the changes are likely to be functionally relevant in this lymphoma.

RagC is one of four members of the Rag GTPase family in mammals, which form obligate heterodimers comprising RagA or RagB together with RagC or RagD.22,23. The Rag GTPases form a supercomplex on the lysosomal surface with Regulator, the V-ATPase and SLC38A9, with the majority of substitutions mapping to residues beyond Pro118 (Supplementary Table 6). RRAGC mutations are therefore highly enriched in follicular lymphoma, with their nature and frequency suggesting that the changes are likely to be functionally relevant in this lymphoma.
subject to genetic aberrations. This approach uncovered mutations in genes encoding two subunits of the V-ATPase complex, *ATP6V1B2* and *ATP6AP1*. The V-ATPase complex resides in intracellular compartments such as the lysosome and is composed of two domains, a cytosolic V₁ domain responsible for ATP hydrolysis and a transmembrane V₀ domain that enables proton translocation. *ATP6V1B2* is a non-catalytic subunit within the V₁ domain, and *ATP6AP1* is thought to be an accessory subunit that regulates the function of the V-ATPase complex. To assess the relationship between these V-ATPase subunit substitutions, *RRAGC* mutations and follicular lymphoma–associated genes, we resequenced our extension cohort of 141 follicular lymphoma and one case with only the transformed follicular lymphoma sample mutated. In cancer genomes restricted to follicular lymphoma and tFL pairs (Supplementary Table 9) and identified 257 genes differentially expressed between *RRAGC*-mutated and wild-type cases (Supplementary Fig. 5a). There was no difference in *RRAGC* expression between mutated and wild-type cases (Supplementary Fig. 5b). Gene set enrichment analyses showed that *RRAGC*-mutated cases were characterized by upregulated expression for gene sets involved in translation regulation, which are well-known targets downstream of mTOR (Supplementary Fig. 6 and Supplementary Table 10), implicating altered signaling as a consequence of these mutations.

The direct binding of Rag GTPase heterodimers to mTORC1 is a key event in the activation of mTORC1 by amino acids. Under these conditions, the active Rag heterodimer, composed of GTP-loaded RagA or RagB bound to GDP-loaded RagC or RagD, directly interacts with mTORC1 (Supplementary Fig. 4), a component of mTORC1 (ref. 25). We first assessed the effects of eight RagC variants detected in our follicular lymphoma series (p. Lys74Arg, p. Ser75Asn, p. Ser75Phe, p. Thr90Asn, p. Ile99Phe, p. Tyr115Arg, p. Asp116Gly and p. Pro118Leu) on raptor binding capacity by coexpressing each mutant sequence encoding RagC (RagCmut) together with wild-type RagB in HEK293T cells. These RagB–RagCmut heterodimers communoprecipitated substantially more endogenously bound raptor than a fully wild-type RagB–RagC heterodimer (Fig. 3a). The increased raptor binding was specific to the identified follicular lymphoma *RRAGC* mutations, as *RRAGC* mutations in other cancer types did not demonstrate the same capacity (Supplementary Fig. 7a). The increased raptor binding observed with the RagC mutants was similar to that seen with RagC Ser75Asn, a previously characterized variant with decreased affinity for GTP that therefore functions like a ‘GDP-bound’ mutant, mimicking the RagC conformation that is necessary for mTORC1 activation by amino acids (Supplementary Fig. 7a).

To understand the pathogenic role of *RRAGC* mutations, we examined RNA sequencing (RNA-seq) data in 13 follicular lymphoma cases (Supplementary Table 9) and identified 257 genes differentially expressed between *RRAGC*-mutated and wild-type cases (Supplementary Fig. 5a). There was no difference in *RRAGC* expression between mutated and wild-type cases (Supplementary Fig. 5b). Gene set enrichment analyses showed that *RRAGC*-mutated cases were characterized by upregulated expression for gene sets involved in translation regulation, which are well-known targets downstream of mTOR (Supplementary Fig. 6 and Supplementary Table 10), implicating altered signaling as a consequence of these mutations.

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To determine whether capacity to bind guanine nucleotides is affected in the RagC variants, we used a specific in vitro assay in

### Table 1 Frequency of *RRAGC* mutations in lymphoma and other hematological malignancies

| Tumor type                        | Occurrence (number/total number) | Frequency (%) |
|-----------------------------------|----------------------------------|---------------|
| Follicular lymphomas              | 25/141                           | 17.7          |
| Diagnostic                        | 13/94                            | 13.8          |
| Relapse                           | 12/47                            | 25.5          |
| Follicular lymphoma and tFL pairs | 6/32a                           | 18.8          |
| DLBCL                            | 3/174                            | 1.7           |
| GCB                               | 1/67                             | 1.5           |
| ABC                               | 1/43                             | 2.3           |
| PMBL                              | 1/29                             | 3.4           |
| U                                 | 0/35                             | 0             |
| DLBCLc                            | 1/185                            | 0.5           |
| B cell lymphomas                   |                                  |               |
| Burkitt lymphoma                  | 0/42                             | 0             |
| CLL/SLLb                          | 0/96                             | 0             |
| CLLc                              | 0/258                            | 0             |
| MCLc                              | 0/29                             | 0             |
| SMZLb                             | 0/48                             | 0             |
| Other B cell lymphomas             | 0/48                             | 0             |
| Cell lines (B cell NHL)            | 2/51                             | 3.9           |
| Other hematological malignancies   |                                  |               |
| AMLc                              | 0/200                            | 0             |
| CMLc                              | 0/129                            | 0             |
| MMc                               | 0/203                            | 0             |
| Benign reactive lymph nodes        | 0/10                             | 0             |
which nucleotide binding to Rag heterodimers could be assessed by purifying wild-type and mutant RagC in complex with a RagB mutant (Asp163Asn) that preferentially binds to xanthosine nucleotides. Using this RagB mutant allowed us to measure guanine nucleotide binding to the RagC variant only, even in the presence of RagB. Two classes of RagC variants emerged from this analysis. One class, including Ser75Asn and Ser75Phe RagC, had significantly decreased affinity for GTP in comparison to wild-type RagC and a preference for binding GDP over GTP (Fig. 4a,b). These mutants are analogous to the Ser17Asn Ras variant, where the substitution disrupts coordination of the magnesium cofactor, leading to decreased affinity for all nucleotides. This Ras mutant suppresses signaling, not through decreased GTP binding but rather through its high affinity for Ras guanine nucleotide exchange factors, thus preventing guanine nucleotide exchange on wild-type Ras. Further studies will be needed to uncover whether the same mechanism is present for the RagC mutants, as a guanine nucleotide exchange factor for RagC has yet to be identified. The second class of RagC variants, Thr90Asn and Trp115Arg, displayed a slight preference for binding GDP over GTP, without an overall decrease in GTP binding in comparison to wild-type RagC (Fig. 4a,b). Although the relative nucleotide affinity of these variants was biased toward GDP, this bias may not account for the signaling effects of these variants in cells, as intracellular GTP concentrations are 10−20 times higher than those of GDP. RagC amounts were consistent in our assays, indicating that the variation in nucleotide binding cannot be accounted for by differences in protein levels (Fig. 4c). Although this second class of variants may activate the mTORC1 pathway through mechanisms not involving changes in nucleotide loading, further work is needed to uncover the exact mechanism underlying their increased binding to raptor.

As the V-ATPase complex is functionally linked with the Rag GTPases and Ragulator in sensing amino acids and activating mTORC1 signaling, the coexistence of RagGC mutations with mutations in either ATP6V1B2 or ATP6AP1 raises the question of whether there is functional epistasis. ATP hydrolysis and the V-ATPase rotator conformation are crucial for relaying the amino acid signal from the lysosomal lumen to Rag GTPases, and our working hypothesis therefore is that mutations in these V-ATPase subunits help convey a ‘false’ amino acid sufficiency signal or alter interactions between the V-ATPase, Ragulator and Rag GTPases; this hypothesis requires experimental clarification.

In conclusion, our study identified frequent mutations in components of the lysosome-centric mTORC1 signaling cascade in follicular lymphoma. We demonstrated that RagGC mutants confer a gain-of-function mechanism by bypassing the amino acid deprivation state to activate mTORC1 signaling. Taken together, the emergence of frequent activating RagGC mutations that are clonally represented and maintained during progression is particularly valuable and might be exploited as a therapeutic target; however, the use of these mutations as a predictive biomarker of mTOR inhibitor sensitivity warrants further investigation.

URLs. cBioPortal for Cancer Genomics, http://www.cbioportal.org/; International Cancer Genome Consortium (ICGC) data portal, https://dcc.icgc.org/.
indicated proteins were treated and analyzed as in but not wild-type RagC leads to increased mTORC1 signaling in the absence of leucine in Karpas-422 cells. Karpas-422 cells stably expressing the indicated proteins were starved of leucine for 50 min and restimulated with leucine for 10 min. P-T389-S6K1, S6K1 phosphorylated at Thr389. (d) Immunoassay showing that stable overexpression of the indicated RagC mutants leads to an increase in mTORC1 signaling in the absence of arginine. HEK293T cells stably expressing the indicated proteins were starved of arginine for 50 min, restimulated with arginine for 10 min and analyzed as in (c). (e) Immunoassay showing that stable overexpression of the indicated RagC mutants but not wild-type RagC leads to increased mTORC1 signaling in the absence of leucine in Karpas-422 cells. Karpas-422 cells stably expressing the indicated proteins were treated and analyzed as in (b).

Figure 4 RagC mutants have altered nucleotide binding affinity. (a,b) Nucleotide binding assay by filter binding, with the indicated RagC heterodimer incubated with $[^3H]GDP$ (a) or $[^3H]GTP$ (b). RagBx is the specific Asp163Asn RagB mutant. Each value represents the normalized mean ± s.d. for $n = 3$ experiments. Statistical differences were assessed comparing each sample to the binding observed with the RagB-RagC wild-type heterodimer. Student’s $t$ test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$; NS, not significant. (c) SDS-PAGE analysis of aliquots of the purified Rag heterodimers used in the nucleotide-binding assays; the gel was stained with Coomassie.
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AUTHOR CONTRIBUTIONS
J.O. and J.F. conceived the study. J.O., D.M.S. and J.F. directed the study. C.M., G.P., P.J., A.D., J.C.S., L.W., L.W., B.M.C., L.E.-I., A.F., A.J., T.A.L., R.A., S.M. and J.G.G. provided patient samples and clinical data. M.C., A.J. and M.-Q.D. conducted pathological review of specimens. J.M. collated clinical information. S.I. prepared and processed samples. H.Q. provided cell line DNA. J.O., R.L.W., S.A., L.W., B.M.C., L.E.-I., A.F.A.S., A.C.E., C.B. and R.Z. performed experiments. J.W., J.A.G.-A., S.H.B. and C.C. performed the bioinformatic analysis. J.R. and R.S. coordinated and verified the ICGC data set. J.O., R.L.W., J.W., D.M.S. and J.F. analyzed and interpreted the data. J.O., R.L.W., D.M.S. and J.F. wrote the manuscript. All authors read and approved the final manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Patients and samples. Samples were obtained from individuals with follicular lymphoma and non-follicular lymphoma tumors following approval from the institutional review board and local ethics committee of all participating centers. Informed written consent was obtained from all individuals. The discovery cohort (Supplementary Fig. 1 and Supplementary Table 2) comprised five patients who had not undergone histological transformation and was selected on the basis of available fresh-frozen tumor lymph node biopsies, matched constitutional DNA and samples from multiple disease episodes. The clonality between tumor biopsies obtained from multiple disease episodes from an individual patient was confirmed by BCL2-IGH breakpoint analysis as previously described2. The follicular lymphoma validation cohort (Supplementary Table 11) comprised either diagnostic or relapse follicular lymphomas (n = 141 cases) or paired follicular lymphoma–transformed follicular lymphoma tumor biopsies (n = 32 cases) obtained from two centers (Barts Cancer Institute and University of Southampton). The clinical characteristics of the cohort are shown in Supplementary Table 12. Non-follicular lymphoma tumors for validation included DNA from 174 DLBCL, 96 chronic lymphocytic leukemia and 48 splenic marginal zone lymphoma tumors. Histology of all tumors was confirmed by pathological review.

Whole-exome sequencing and analysis. Whole-exome capture libraries were constructed from 2–3 μg of tumor or constitutional DNA after shearing, end repair, phosphorylation and ligation to a barcoded sequencing adaptor, using Human All Exon v5 SureSelect XT (Agilent Technologies). Enriched exome libraries were multiplexed and sequenced on the HiSeq 2500 instrument (Illumina) to generate 100-bp paired-end reads. Sequencing metrics are provided in Supplementary Table 1. The processing and analysis of whole-exome sequencing data were performed using our previously described pipeline2. Briefly, sequencing reads were aligned to the hg19 reference genome, using Burrows-Wheeler Aligner (BWA)48. Local alignments and base quality scores were adjusted using the Genome Analysis Toolkit (GATK)49 version 2.5.2.

Variant detection and mutation annotation. Somatic single-nucleotide variants and insertion–deletions were identified using the Strelka pipeline as previously described2. For each sample, the number of reads supporting the reference and variant alleles at each position was extracted. VAFs were calculated by dividing the number of reads supporting the variant allele by the total number of reads obtained. To improve variant calls across all tumors from the same patient, identified variants were further genotyped and verified across all tumors and matched normal samples using the VarScan2 multisample calling file to call variants. In brief, reads were aligned to hg19 using Bowtie2 (ref. 54). SAMTools55 was used to generate sorted BAM files, and the VarScan2 tool was used to examine the pileup file to call variants.

Deep tag-amplicon sequencing for the Rragc, Attp6v1b2 and Attp6ap1 genes. Universal adaptor sequences were tagged to the 5’ and 3’ ends of target-specific primers of ~200 ± 20 bp. On the basis of our initial experiments that showed clustering of variants within specific exons of the three genes, subsequent analyses were restricted to Rragc exons 1 and 2, Attp6v1b2 exons 11 and 12, and Attp6ap1 exons 9 and 10. Primer sequences are shown in Supplementary Table 13. Genomic DNA (100 ng) was amplified in 2- to 4-plex PCRs using non-overlapping tagged primers with the HotStartTaq Plus kit (Qiagen) under limited cycling conditions. Amplified PCR fragments were subsequently pooled in equimolar ratios by sample and prepared for sequencing with the attachment sample-specific indexes and Illumina adaptors sequences. Indexed libraries were pooled and sequenced on a single lane of an Illumina MiSeq platform using the v2 300-cycle MiSeq reagent kit (Illumina), generating 150-bp paired-end reads. Each sample was screened in duplicate. Variant calling and annotation are as described above.

DNA sequencing analysis. RNA-seq data for all 13 follicular lymphoma samples (five mutants and eight wild type) were downloaded from the ICGC data repository (see URLs). Details of the samples are summarized in Supplementary Table 9. Raw read counts for all annotated Ensembl genes across the 13 samples were extracted from the exp_seq.MALY-DE.tsv file in the ICGC data repository. Only genes that achieved at least one read count per million reads (CPM) in at least five samples were selected, with these criteria producing 22,126 filtered genes in total. After applying scale normalization, read counts were converted to log2 (CPM) using the voom function46 with associated weights ready for linear modeling. Analyses of differential gene expression between the mutant and wild-type groups were further performed using the limma R package, which powers differential gene expression analyses for RNA-seq and microarray data57. A double threshold of raw P < 0.01 and an absolute fold change > 2 were used to define significantly differentially expressed genes (Supplementary Fig. 5a). On the basis of the t statistic of filtered genes from the limma test, gene set enrichment analyses were performed against predefined curated gene sets (c2) acquired from the MSigDB collection48, including KEGG and Reactome gene sets. Top significantly enriched gene sets were selected on the basis of false discovery rate (FDR) q value < 0.05 (Supplementary Table 10).

Copy number variation of the Rragc locus. Data on copy number variation and copy-neutral loss of heterozygosity for the Attp6ap1, Attp6v1b2, Rragc and Tnfrsf14 gene loci were extracted from our previous SNP array analyses using the methodology previously described2. To detect copy number imbalances from our discovery whole-exome sequencing data, the VarScan2 ‘copynumber’ module was first applied, using a minimum read coverage of 20 and both mapping and base qualities of ≥20 for usable reads, to generate raw copy number calls. Raw calls were adjusted for GC content and centered to 0 on the basis of the modal log R value determined by kernel density estimates, using the VarScan2 ‘copyCaller’ module. Outliers were identified and modified using the data winsorizing procedure. The DNAcopy R Bioconductor package (R package version 1.40.0) was used to identify joint segments of log R values using the circular binary segmentation (CBS) algorithm. To identify regions of loss of heterozygosity variants (including SNPs and short insertion-deletions) with respect to the reference genome were first identified for paired normal and tumor samples using VarScan2. Next, B-allele frequency (BAF) files were created, allowing a minimum read depth of 10 for both tumor and normal samples. The ASCAT R package53 was then used to assess copy number alterations and loss-of-heterozygosity regions, using the log R and BAF files derived from VarScan variant calls, with the depth information normalized by dividing the depth of each variant by the median depth across all variants.

Targeted sequencing of Rag GTPase and mTorC1-associated genes. Target-specific primers for follicular lymphoma–associated genes2 and seven mTorC1-associated genes (RRAGA, RRAGB, RRAGC, RRAGD, MTOR, RPTOR and MLST8) were custom designed using Fluidigm’s D3 Assay design service. Targeted enrichment was performed by Access Array (Fluidigm) in a multiplex format using genomic DNA (50 ng) according to the manufacturer’s Multiplex Amplicon Tagging Protocol. The multiplexed library pools were sequenced on the Illumina MiSeq platform. All samples were screened in duplicate with the inclusion of normal tonsil DNA controls in each run. Variants were called and annotated as previously described2. In brief, reads were aligned to hg19 using Bowtie2 (ref. 54). SAMTools55 was used to generate sorted BAM files, and the VarScan2 tool was used to examine the pileup file to call variants.

Phylogenetic analyses. Evolutionary trees were reconstructed for each individual on the basis of the distance matrix between germline, follicular lymphoma and relapse follicular lymphoma samples derived from the numbers of somatic nonsynonymous variants for each biopsy, using the neighbor-joining algorithm52 implemented in the PHYLIIP package as previously reported2. Once the consensus phylogenetic tree was determined, it was redrawn starting from the germ line and leading to the putative common progenitor cell (CPC) and then to follicular lymphoma and subsequent relapse follicular lymphoma, with branch length proportional to the number of somatic changes, that is, genetic distance, between the samples.

Copy number variation of the Rragc locus. Data on copy number variation and copy-neutral loss of heterozygosity for the Attp6ap1, Attp6v1b2, Rragc and Tnfrsf14 gene loci were extracted from our previous SNP array analyses using the methodology previously described2. To detect copy number imbalances from our discovery whole-exome sequencing data, the VarScan2
Materials. Reagents were obtained from the following sources: horseradish peroxidase (HRP)-labeled anti-mouse and anti-rabbit secondary antibodies from Santa Cruz Biotechnology (sc-2031 and sc-2357, respectively); antibodies to S6K1 phosphorylated at Thr389, S6K1, mTOR and FLAG epitope from Santa Cruz Biotechnology (sc-2031 and sc-2357, respectively); antibodies to raptor from Santa Cruz Biotechnology (sc-2031 and sc-2357, respectively); antibodies to S6K1 phosphorylated at Thr389, S6K1, mTOR and FLAG epitope from Bethyl Laboratories (A190-208A); and antibody to raptor to from Sigma-Aldrich; XDP and XTP were from Jena Biosciences; 3H-labeled GTP and GDP were from PerkinElmer; DMEM was from SAF Biosciences; Complete Protease Cocktail was from Roche; Inactivated FCS (IFS) and simply blue stain were from Invitrogen; and amino acid-free RPMI was from US Biologicals.

Cell lines and tissue culture. HEK293T cells were cultured in DMEM supplemented with 10% IFS, 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Karpas-422, Raji, OCI-Ly7 and OCI-Ly8 cells were cultured in RPMI supplemented with 10% IFS, 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml). All cell lines were maintained at 37 °C and 5% CO2. All cell lines were obtained from the American Type Culture Collection (ATCC) and were free of mycoplasma contamination.

Virus production and viral transduction. The production of lentiviruses was achieved by transfection of viral HEK293T cells with pLJM60-FLAG-metap2 or pLJM60-FLAG-RagC (wild-type or mutant) constructs, with the VSV-G envelope and gag/pol packaging plasmids. Similarly, the production of retroviruses for infection of Karpas-422 cells was achieved by transfection of viral HEK293T cells with pMXs-RagC (wild-type or mutant) constructs, with the VSV-G envelope and gag/pol packaging plasmids. Twenty-four hours after transfection, the medium was changed to DMEM supplemented with 30% IFS. After another 24 h, the virus-containing supernatant was collected from the cells and passed through a 0.45-μm filter. Target cells were plated in six-well plates with virus-containing medium and 8 μg/ml polybrene. Spin infections were performed by centrifugation at 2,200 r.p.m. for 1 h. Twenty-four hours later, the medium was changed to fresh medium containing either puromycin (when cells were infected with the lentivirus) or blasticidin (when cells were infected with the retrovirus) for selection.

Cell lysis and immunoprecipitation. Cells were rinsed once with ice-cold PBS and immediately lysed with Triton lysis buffer (1% Triton, 10 mM β-glycerophosphate, 10 mM pyrophosphate, 40 mM HEPES (pH 7.4), 2.5 mM MgCl2, and one tablet of EDTA-free protease inhibitor (Roche) (per 25 ml of buffer)). The cell lysates were centrifuged at 13,000 r.p.m. in a microcentrifuge at 4 °C for 10 min. For anti-FLAG immunoprecipitations, FLAG-M2 affinity gel was washed three times with lysis buffer. Then, 30 μl of a 50% slurry of the affinity gel in lysis buffer was added to cleared cell lysates, and samples were rotated for 2 h at 4 °C. The beads were washed once with lysis buffer and three times with lysis buffer containing 500 mM NaCl after the incubation. Immunoprecipitated proteins were denatured by the addition of 50 μl of sample buffer and boiled for 5 min as described24, resolved by 8–16% SDS-PAGE and analyzed by immunoblotting.

For cotransfection experiments in HEK293T cells, 2 million cells were plated in 10-cm culture dishes. Twenty-four hours later, cells were transfected via the polyethylenimine method59 with the pRK5-based cDNA expression plasmids indicated in the figures in the following amounts: 800 ng of FLAG-Metap2 or 400 ng of FLAG-RagC (wild-type and mutant) plasmid and 400 ng of RagB (wild-type and mutant) plasmid. The total amount of plasmid DNA in each transfection was normalized to 5 μg with empty pRK5. Thirty-six hours after transfection, cells were lysed as described above.

For experiments that required amino acid, leucine or arginine starvation or restimulation, cells were treated as previously described35. Briefly, cells were incubated in amino acid-free, leucine-free or arginine-free RPMI for 50 min and then stimulated with amino acids, leucine or arginine for 10 min.

Nucleotide-binding assays. 40 pmol of FLAG-RagC (wild type or mutant)–HA-RagB Asp163Asn was loaded with either 8 μCi of [3H]GDP or 8 μCi of [3H]GTP (5–20 Ci/mmol) and also loaded with either 62.5 nM XDP or 62.5 nM XTP in a total volume of 80 μl of CHAPS buffer, supplemented with 2.5 mM DTT, 10 μg of BSA and 6.25 mM EDTA. The CHAPS buffer contained 0.3% CHAPS, 40 mM HEPES (pH 7.4) and 30 mM NaCl. The complexes were rotated for 10 min at room temperature, stabilized with 25 mM MgCl2, rotated for another 10 min at room temperature and then incubated on ice for 1 h to allow the binding reaction to occur. Samples of 10 μl were taken, in triplicate, and spotted onto nitrocellulose filters, which were washed three times with 1 ml of wash buffer (1.5% CHAPS, 40 mM HEPES (pH 7.4), 150 mM NaCl and 5 mM MgCl2). Filter-associated radioactivity was quantified using a TriCarb scintillation counter (PerkinElmer).

Statistical analysis. Fisher’s exact tests were used for comparison between two groups. For analysis of the nucleotide-binding assay groups, two-tailed t tests were used. P values of less than 0.05 were considered to indicate statistical significance.