Figure S1.

A

CEPH line Coriel nr.	Numbering in this manuscript	Recognition phenotype	CEPH line Coriel nr.	Numbering in this manuscript	Recognition phenotype
10846	1	+	12156	62	+
12145	4	+	10838	66	+
12146	5	+	12003	68	+
12239	6	+	12004	69	+
7029	7	+	12005	70	+
7019	8	+	12006	71	+
6994	9	-	12753	76	+
7022	12	+	12762	79	+
6991	16	+	12763	80	+
7034	18	_	12801	81	+
6993	20	_	12802	82	+
10851	22	_	12812	83	_
11839	36	+	12814	86	+
11840	37	_	12864	88	+
10860	48	+	12865	89	+
10861	49	+	12872	90	+
11993	51	_	12873	91	+
11994	52	+	12875	93	_
11995	53	+	12878	94	+
12155	61	+	12892	96	+

B

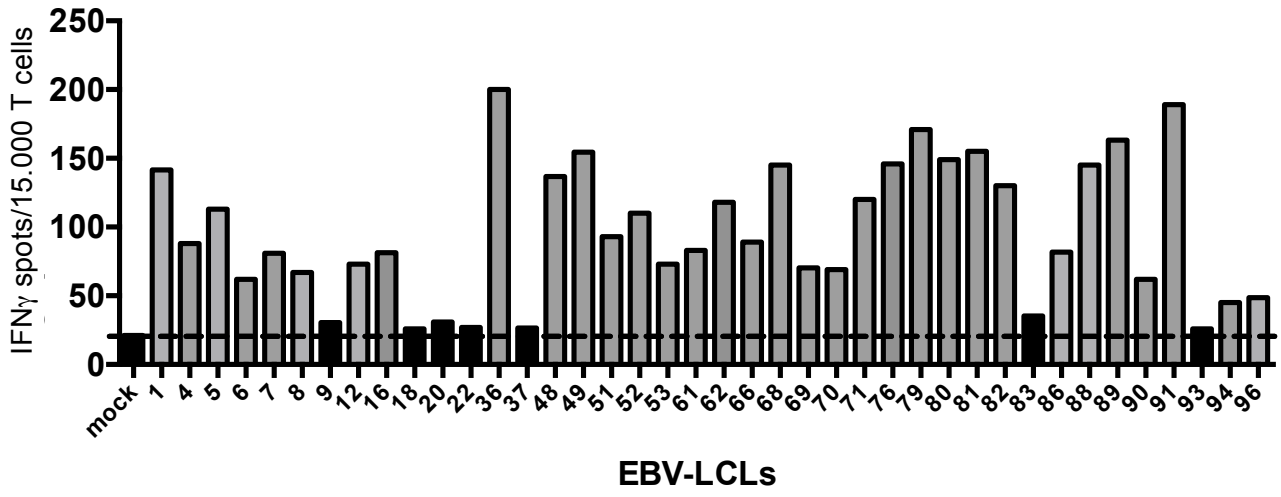

Figure S1. CEPH EBV-LCL lines used for identifying genetic loci associated with Vγ9Vδ2TCR-mediated recognition, related to Figure 1. (A) Recognition phenotype indicates whether EBV-LCL lines are recognized (+) or not (-) by Vγ9Vδ2 TCR+ T cells in three independent experiments. (B) Recognition phenotype of EBV-LCLs (black bars: not activating; grey bars: activating) was assessed by IFNγ-ELIspot assay, in which EBV-LCLs were used as targets against Vγ9Vδ2TCR+ T cells in the presence of ABP pamidronate. Figure shows the number of IFNγ spots of a representative experiment.

Figure S2.

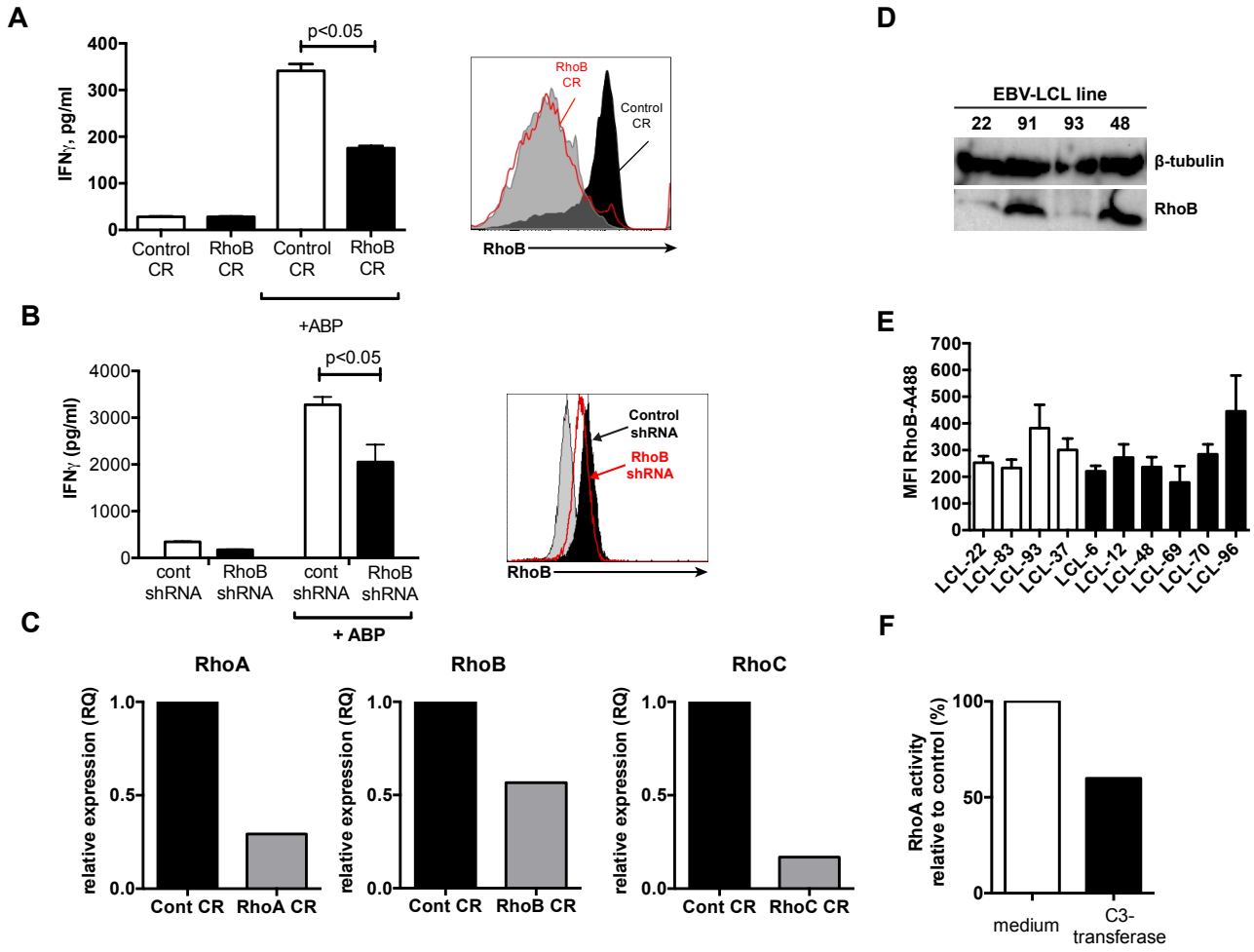

Figure S2. Additional data related to Figure 2. (A) RhoB was knocked out in 293 HEK cells using CRISPR/Cas system, and single cell clones were selected for stable complete knock out phenotype, and the effect of complete knock out on recognition by Vγ9Vδ2 TCR+ T cells was assessed by measuring IFNγ (left panel). A guide RNA targeting an irrelevant sequence was used as the control. Level of knock out was determined using intracellular flow cytometry (right panel). Data show mean±S.E.M of two independent experiments in duplicate samples, where Mann-Whitney test was used to analyze statistical significance. (B) Daudi cells were lentivirally

transduced with shRNA targeting RhoB, and the effect of RhoB knockdown on recognition by Vγ9Vδ2 TCR+ T cells was assessed by measuring IFNγ (left panel). Data show mean±S.E.M of three independent experiments in duplicate samples, where Mann-Whitney test was used to analyze statistical significance. A vector encoding an irrelevant shRNA served as negative control. Knock-down level of RhoB was determined by intracellular flow cytometry (right panel) (C) RhoA, B and C were knocked out using CRISPR/Cas system in 293 HEK cells. A guide RNA targeting an irrelevant sequence was used as control. Level of knock out was determined using qPCR. Figure shows a representative experiment. (D) RhoB protein levels were measured in the recognized EBV-LCL lines 48 and 91 and the non-recognized line 22 and 93 by western blot analysis. β-tubulin served as the loading control. Figure shows a representative experiment. (E) RhoB protein levels were measured in the recognized EBV-LCL lines 6, 12, 48, 69, 70 and 99 and the non-recognized line 22, 83, 93 and 37 by intracellular flow cytometry analysis. Data show mean±S.E.M of at least three independent experiments. (F) Rho inhibition of 293 HEK cells following C3-transferase treatment was determined by using G-Lisa. Figure shows a representative experiment of the relative inhibition of RhoA activity compared to untreated sample.

Figure S3.

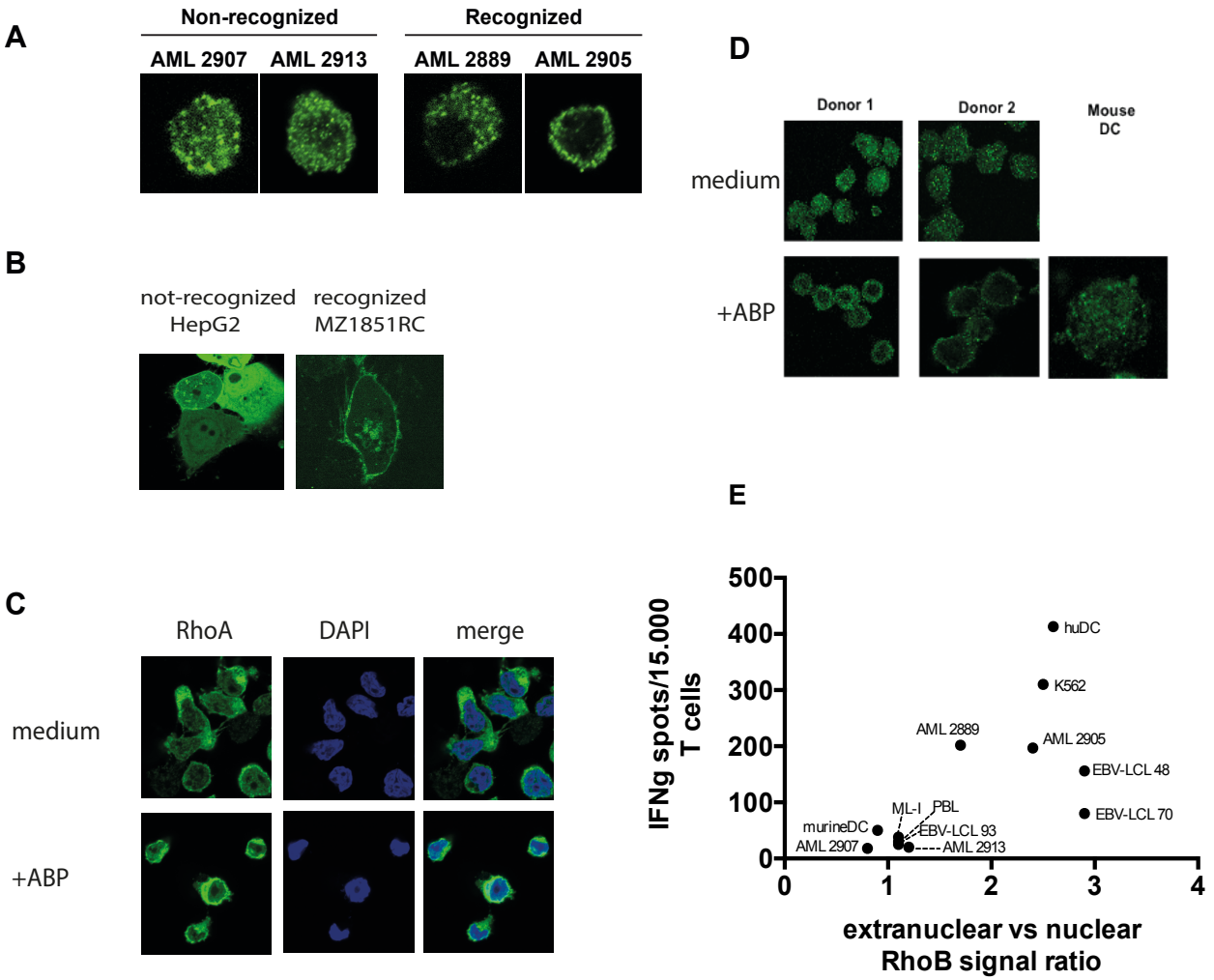

Figure S3. Additional data, related to Figure 3. (A) Representative images for the intracellular distribution of RhoB in recognized and non-recognized primary AML samples. (B) Vγ9Vδ2 TCR+ T cell-activating MZ1851RC and non-activating HepG2 tumour cell lines were transfected with RhoB-GFP constructs and intracellular distribution of RhoB was investigated via confocal microscopy. (C) The intracellular RhoA distribution in the presence or absence of ABP pamidronate was measured via confocal microscopy in EBV-LCL 48. (D) The intracellular RhoB distribution in the presence or absence of ABP pamidronate was determined in monocyte derived human dendritic cells from two different donors. Mouse bone marrow derived dendritic cells (>95% CD11c+) were treated with ABP pamidronate and used for intracellular labeling of RhoB. (E) The correlation of intracellular RhoB distribution to Vγ9Vδ2TCR T cell activation capacity of tumor target cells. Mean values of the extranuclear: nuclear RhoB intensity ratio in tumor cells were plotted against the number of IFNg spots when the same tumor cells were co-cultured with Vγ9Vδ2TCR T cells.

Figure S4.

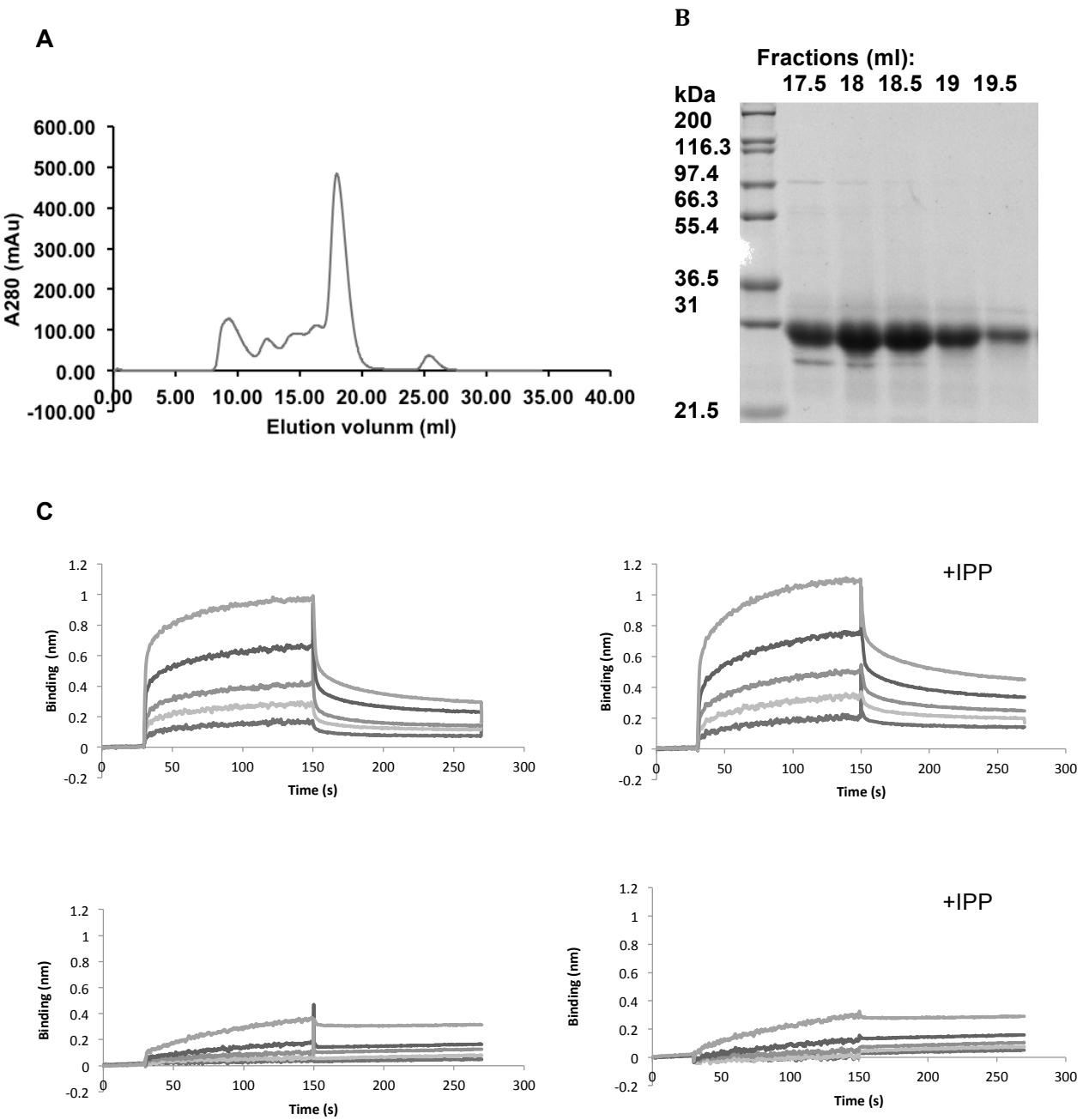

Figure S4. Additional data, related to Figure 5. (A) Gel filtration profile of RhoB GTPase expressed in *E coli*. The peak from 17.8 ml to 19.2 ml contained purified RhoB GTPase monomer. (B) SDS-PAGE showing the fractions (17.5-19.5 ml, 0.5ml/fraction) containing RhoB GTPase collected from the gel filtration experiment. (C) Concentration dependent binding of the full-length BTN3A1 intracellular domain (BFI) with RhoGTPase in the presence or absence of the phosphoantigen IPP (upper panels). Binding of BFI to RhoGTPase was measured using Biolayer Interferometry (BLI) either in the absence of IPP (left panel) or presence of IPP (1:1) (right panel). Concentrations of BTN3A1 BFI shown are 3.75, 7.5, 15, 30 and 60uM shown in grey. Same experimental setup but with recombinant BTN3A1 B30.2 domain, lacking the N terminal region connector to the transmembrane domain (lower panels). In the left panel, the interaction

was measured without IPP. Concentrations of BTN3A1 B30.2 shown were 3.75, 7.5, 15, 30 and 60uM shown in grey. In the right panel, the interaction was measured with IPP (1:1).

Table S1. Rate and affinity constants for binding interactions between RhoB GTPase and BFI or B30.2 domains, in the presence or absence of pAg related to Figure 5.

Interaction	KD (μM)	k_a (1/ Ms)	k_d (1/s)	Chi^2
RhoB-BFI	19.7	$2.2x10^3$	0.04351	$2.58x10^{-4}$
RhoB-B30.2	102	$2.02x10^3$	0.206	$4.81x10^{-5}$
RhoB-BFI-cHDMAPP	666	172	0.115	$5.66x10^{-5}$
RhoB-B30.2-cHDMAPP	N.A.	N.A.	N.A.	N.A.

Supplementary Experimental procedures

Cells and reagents

CEPH EBV-LCL lines (CEU population panel) were a kind gift from Tuna Mutis (UMC Utrecht, The Netherlands). Daudi, K562, SW480, HEK 293, HEK 293FT and Phoenix-Ampho cell lines were obtained from ATCC. LCL-TM (an EBV-LCL line separate from the CEPH panel) was kindly provided by Phil Greenberg (Seattle, U.S.A.). MZ1851RC was kindly provided by Barbara Seliger (University of Halle, Germany). Hek 293, Phoenix-Ampho, SW480, MZ1851RC cells were cultured in DMEM supplemented with 1% Pen/Strep (Invitrogen) and 10% FCS (Bodinco), all other cell lines in RPMI with 1% Pen/Strep and 10% FCS. Primary fresh PBMCs were isolated by Ficoll-Paque (GE Healthcare) from buffy coats supplied by Sanquin Blood Bank (Amsterdam, The Netherlands). Frozen primary acute myeloid leukemia (AML) samples were kindly provided by Matthias Theobald (Mainz, Germany) and were collected in compliance with GCP and Helsinki regulations.

The following reagents were used: pamidronate (Calbiochem), zoledronic acid monohydrate (zolidronate, Sigma-Aldrich), isopentenyl pyrophosphate (IPP) (Sigma-Aldrich), calpeptin (Rho activator II CN03, Cytoskeleton Inc), C3 transferase (Rho Inhibitor I CT04, Cytoskeleton Inc), the farnesyl transferase inhibitor (FTI) (Sigma-Aldrich), and the geranylgeranyltransferase inhibitor (GGTI) (Sigma-Aldrich).

Flow cytometry

Antibodies used for flow cytometry included: pan-γδTCR-PE (clone IMMU510, Beckman Coulter), CD4-FITC (eBioscience), CD8-APC (BD), unconjugated rabbit polyclonal RhoB (AbCam), goat-anti-rabiit Alexa Fluor 488 (Jackson ImmunoResearch). Mouse a-CD277 mAb (clone #20.1 and 103.2) were kindly provided by D. Oliver (INSERM U891, Marseille, France). Samples were processed with FACSCalibur and FACSCanto-II flow cytometers (BD) and analyzed with FACSDiva software (BD). Primary leukemic stem cells and healthy progenitor cells were sorted according to phenotypic markers as previously described (Terwijn et al., 2014).

Cells were sorted using a FACS Aria SORP (with red, blue, and violet solid-state lasers; BD Biosciences). Cells were kept on ice during the whole procedure. Cells were labelled with Anti-CD45RA Alexa Fluor 700, Anti-CD38APC, Anti-CD34 Horizon BV421, Anti-CD45 Horizon V500, all BD Biosciences, San Jose, CA, USA. CD34+CD38- stem cells were sorted based on CD45RA expression: CD45RA positive cells are neoplastic and CD45RA negative cells are normal hematopoietic stem cells. Above that, CD34+CD38+ progenitors were sorted.

Retroviral transduction of TCRs

The Vγ9Vδ2-TCR clone G115 (Allison et al., 2001) and a HLA-A*0201-restricted $WT1_{126-134}$-specific αβTCR (Kuball et al., 2007) were transduced into αβT cells as described (Marcu-Malina et al., 2011, Stanislawski et al., 2001). In brief, Phoenix-Ampho packaging cells were transfected with gag-pol (pHIT60), env (pCOLT-GALV) and pBullet retroviral constructs containing TCRγ/β-chain-IRES-neomycine or TCRδ/α-chain-IRES-puromycin, using Fugene6 (Promega). PBMCs preactivated with αCD3 (30ng/ml) (clone OKT3, Janssen-Cilag) and IL-2 (50U/ml) were transduced twice with viral supernatant within 48 hours in the presence of 50U/ml IL-2 and 4μg/ml polybrene (Sigma-Aldrich). Transduced T cells were expanded by stimulation with αCD3/CD28 Dynabeads ($0.5x10^6$ beads/10^6 cells) (Invitrogen) and IL-2 (50U/ml) and selected with 800μg/ml geneticin (Gibco) and 5μg/ml puromycin (Sigma-Aldrich) for one week. CD4+ TCR-transduced T cells were isolated by MACS-sorting using CD4-microbeads (Miltenyi Biotec).

Following transduction, transduced T cells were stimulated biweekly with 1μg/ml PHA-L (Sigma-Aldrich), 50U/ml IL-2 (Novartis Pharma), 5ng/ml IL-15 (R&D Systems), and irradiated allogeneic PBMCs, Daudi and LCL-TM cells. Fresh IL-2 was added twice a week. Transgenic TCR expression and purity of CD4+ populations was routinely assessed by flow cytometry.

Functional T cell assays

IFNγ ELISPOT was performed as previously described (Scheper et al., 2013, Marcu-Malina et al., 2011). Briefly, 15,000 Vγ9Vδ2 TCR-transduced or mock-transduced T cells and 50,000 target cells (ratio 0.3:1) were cocultured for 24 hrs in nitrocellulose-bottomed 96-well plates

(Millipore) precoated with anti-IFNγ antibody (clone 1-D1K) (Mabtech). Plates were washed and incubated with a second biotinylated anti-IFNγ antibody (clone 7-B6-1) (Mabtech) followed by streptavidin-HRP (Mabtech). IFNγ spots were visualized with TMB substrate (Sanquin) and the number of spots was quantified using ELISPOT Analysis Software (Aelvis).

Alternatively, Vγ9Vδ2 TCR-transduced T cells and target cells were cocultured as above in round-bottom 96-well plates, and IFNγ levels in supernatants were measured by ELISA. Where indicated, target cells were pretreated with pamidronate (100 μM), IPP (15 μM), FTI (10 μM), GGTI (50 μM), calpeptin (2 μg/ml) or C3 transferase (20 μg/ml) prior to coincubation. For testing stimulation of WT1 αβTCR-transduced T cells, the HLA-A2+ cell lines EBV-LCL 48 and MZ1851RC were pulsed with 10 μM $WT1_{126-134}$ peptide.

Zygosity/SNP correlation analysis

Recognition of CEPH EBV-LCL lines (pretreated with either medium, pamidronate (100 μM) or IPP (15 μM) by Vγ9Vδ2 TCR-transduced CD4+ T cells was determined by ELISPOT. Mock-transduced T cells were included as effector controls, and any EBV-LCL line that elicited IFNγ production by mock-transduced cells were excluded from the analysis. Recognition of EBV-LCL lines by Vγ9Vδ2 TCR+ T cells in a single assay was defined as an at least two-fold increase in IFNγ spots compared to those produced in response healthy control target cells, irrespective of EBV-LCL pretreatment (i.e. medium, pamidronate or IPP). EBV-LCL line was defined as activating when recognized in at least three out of five independent experiments. Hypothetical zygosities for candidate genetic loci were deduced using classical Mendelian inheritance patterns within CEPH family pedigrees, where the influence of candidate alleles on Vγ9Vδ2 TCR-mediated recognition was assumed to be dominant. Correlations of predicted zygosities with Hapmap SNP genotypes of CEPH individuals were subsequently calculated with the software tool ssSNPer, as previously described (Spaapen et al., 2008). Proxy SNPs within 500 kb of SNPs produced by ssSNPer were collected by querying the SNP Annotation and Proxy Search (SNAP) tool (Johnson et al., 2008), using $r^2 = 0.8$ as a threshold for linkage disequilibrium. eQTL analysis of ssSNPer SNP and their proxies was performed using the Genevar (GENe Expression VARiation) tool (Yang et al., 2010).

shRNA and CRISPR/Cas genome editing

HEK 293FT cells were transfected using Fugene 6 (Promega) with lentiviral constructs containing shRNAs (Sigma-Aldrich) together with lentiviral helper constructs VSVG and pspax2, against candidate genes of interest. EBV-LCL 48 cells were transduced with viral supernatants four days prior to functional T cell assays. Knockdown of targeted genes was confirmed using real-time Q-PCR, or in the case of RhoB, by flow cytometry.

We employed the CRISPR/Cas9 system (van de Weijer et al., 2014) to knock out RHOA, RHOB, or RHOC from MZ1851RC cells. For this, we used lentiviral CRISPR/Cas9 vectors (Ref Weijer et al) co-expressing *S. pyogenes* Cas9, PuroR and a human U6 promoter driving expression of anti RHOA guideRNAs (gRNA). The gene-specific regions of the gRNA sequences were designed by the CRISPR design tool from the Zhang lab (http://crispr.mit.edu/) and their sequences were GAACTATGTGGCAGATATCG (RHOA), GTGGTGGGCGACGGCGCGTG (RHOB), and GAAAGAAGCTGGTGATCGT (RHOC). As control gRNA, we targeted the eGFP gene with GTGAACCGCATCGAGCTGAA.

Lentiviruses were generated using standard 3rd generation packaging vectors in 293T cells. MZ1851RC cells were transduced with indicated CRISPR/Cas9 lentiviruses, and cells were selected with 2 μg ml^{-1} puromycin. The efficiency of RhoB knockout was assessed using flow cytometry.

Western blot analysis

EBV-LCL lines 22, 48, 91 and 93 were treated with pamidronate overnight, and were lysed by lysis buffer containing NP-40. Lysates were centrifuged to remove cell debris and supernatants were separated by SDS-PAGE. Protein content was transferred to PVDF membranes (Millipore), blocked for 1 hr in blocking buffer (5% milk) and incubated overnight with rabbit polyclonal antibodies directed against RhoB (LifeSpan Biosciences) or β-tubulin (clone DM1A) (Sigma). Blots were subsequently incubated with HRP-conjugated secondary antibodies, and bands were visualized using Pierce ECL substrate (Thermpo Scientific).

Confocal microscopy and data analysis

For intracellular immunofluorescence staining of RhoB, cells were treated with pamidronate overnight (where indicated) and were allowed to attach onto coverslips precoated with poly-L-lysine (Sigma-Aldrich). Cells were subsequently permeabilized with Permeabilizing solution 2 (BD), blocked with blocking serum (50% pooled normal human serum in PBS), and stained with a rabbit polyclonal anti-RhoB antibody (AbCam) followed by a secondary Goat anti-Rabbit IgG Alexa Fluor 488-conjugated antibody (Jackson ImmunoResearch). Cells were washed with blocking serum, fixed with 4% paraformaldehyde, stained with DAPI (where indicated), and mounted onto microscopy slides using Mowiol. Images were acquired using a Zeiss confocal laser scanning microscope LSM 700. Ratios between nuclear and extranuclear signal of RhoB was determined using Volocity software (PerkinElmer), where DAPI staining was used, when available, to mark nuclei.

To determine colocalization between BTN3 molecules and the actin cytoskeleton, HEK 293 cells were grown onto poly-L-lysin-coated coverslips and pretreated with either calpeptin (2

µg/ml) or C3 transferase (20 µg/ml) prior to treating samples with pamidronate Cells were fixed, permeabilized and BTN3 and F-actin were stained with DyLight 680-conjugated BTN3 antibody (clone BT3.1, Novus Biologicals) and Fluorescein-coupled phalloidin (Sigma), respectively. The correlation coefficient between BTN3 and F-actin signal was determined as a measure of colocalization using Volocity software.

FRAP microscopy

FRAP analysis was performed as previously described (Harly et al., 2012, Sandstrom et al., 2014). In brief, HEK293FT cells expressing either EmGFP-fused CD277 were laid on **m**-slides (Ibidi) and analyzed using a Nikon A1 RS confocal microscope (60xNA 1.40 oil immersion objective). Selected rectangular areas were photobleached for 500 ms by using full power of laser intensity (> 90% of loss of fluorescence). Images were collected every 5 s, before (30 s) and after (120 s) bleaching using low laser intensity. Images were analyzed with Metamorph 7.5 (Molecular Devices, Universal Imaging) and NIS (Nikon) imaging software.The resulting curves were fitted using one-phase exponential equations.

Flow cytometry FRET

To study association of RhoB and BTN3 molecules, cells were permeabilized by using Permeabilization solution 2 (BD), then blocked with PBS containing 50% Human serum and labeled with rabbit polyclonal anti-RhoB antibody (AbCam). After washing with PBS, samples were labeled with Alexa594-conjugated Goat anti-Rabbit IgG (acceptor) (Jackson ImmunoResearch) and CD277-PE (donor) (BT3.1, Biollegend), respectively. The donor fluorescence was measured using a FACS Canto-II flow cytometer (BD) where donor fluorescence of the double-labeled samples was compared with that of samples labeled only with donor antibody. FRET efficiency was calculated from the fractional decrease of the donor fluorescence in the presence of the acceptor.

In order to determine homodimerization of CD277 molecules, cells were co-stained with equal amount of PE-conjugated anti-CD277 (donor) and Dyligth680-conjugated anti-CD277 (acceptor) and samples were measured using a FACS Canto-II flow cytometer (BD). FRET efficiency was calculated with equations according to Sebestyen and colleagues (Sebestyen et al., 2002) where donor fluorescence was excited at 488 nm and detected at 576 ± 26 nm, acceptor fluorescence was excited at 635 nm and detected at 780 ± 60 nm, whereas FRET intensity was excited at 488 nm and detected at 780 ± 60 nm. Correction factors for the spectral overlap between the different fluorescence channels were obtained from data measured on unlabeled and single-labeled cells.

Conformational change of BTN3 molecule was determined similarly as described in (Gaspar et al., 2001). Cells were labeled with 5ug/ml BODIPY-FL DPHE (donor) (Life Technologies) for 10 minutes on ice and then 10 minutes at 37C, then washed extensively with ice-cold PBS. Cells were subsequently labeled with mouse anti-CD277 mAbs (either clone #20.1 or #103.2) and Alexa594-conjugated Goat anti-Mouse Fab fragments (Jackson ImmunoResearch). After washing, cells were resuspended in ice cold PBS and measured immediately using a FACS Canto-II flowcytometer (BD). FRET efficiency was calculated from the fractional decrease of the donor fluorescence in the presence of the acceptor.

Proximity ligation assay

HEK 293FT cells were grown onto poly-L-lysine coated coverslips and pre-treated with 100uM pamidronate overnight prior to being fixed and permeabilized with Permeabilization buffer 2 (BD) for 15 minutes. Subsequently, cells were washed three times with PBS and blocked for 30 minutes at 22°C in PBS containing 50% human serum. After blocking, cells were incubated for 60 min at 22°C with rabbit anti-RhoB (AbCam) and mouse anti-CD277 (Novus Biologicals) in PBS containing 50% human serum. Cells were washed three times with PBST (0.05% Tween) and incubated with the secondary mouse PLUS and rabbit MINUS antibodies for 1.5 hours at 37°C in the dark. Cells were washed three times in PBST before detection of the probe with the *in situ* PLA detection kit (Abnova). Cells were analyzed with a 63× objective on a Zeiss LSM 710 fluorescence microscope.

In vitro protein expression and purification

The full length RhoB protein was cloned into the pET 28a vector with an C-terminal six-HIS tag followed by a thrombin cleavage site using restriction enzyme sites *NdeI* and *XhoI* (5' primer: CGCCATATGATGGCCGCCATCCG; 3' primer: CGCCTCGAGTTAGCAGCAGTTGATGCAGC). The C-terminal CKVL motif of RhoB was deleted to prevent improper prenylation in *Escherichia coli*. The construct was expressed in BL21 strain *Escherichia coli*. Cells were grown in Terrific Broth (TB) at 37 °C to OD600=0.6 and then transferred to room temperature (25°C). After 15min of recovery, the cells were induced with 1ml 1M Isopropyl β-D-1-thiogalactopyranoside (IPTG) per liter of culture for 12-16 hours. Protein was harvested and purified using Ni-NTA (Qiagen) IMAC chromatography in 20mM Tris pH8.0, 400mM NaCl, 20mM Imidazole, 5mM $MgCl_2$ and 4mM 2-mercaptoethanol (BME), washed first with 1mM ATP supplemented in the buffer mentioned above to dissociate potential chaperones from RhoB and then the buffer without ATP, and finally eluted with 20mM Tris pH8.0, 400mM NaCl, 250mM Imidazole, 5mM $MgCl_2$ and 4mM BME. The eluted fractions was desalted into 10mM Hepes 7.2, 150mM NaCl, 0.02% azide, 5mM $MgCl_2$ and 4mM BME using an Econo-Pac 10DG column (Biorad). Protein was further purified by gel filtration over a

Superdex 200 column (GE healthcare) in 10mM Hepes pH7.2, 150mM NaCl, 0.02% azide, 5mM $MgCl_2$ and 4mM BME. Protein concentration was measured by both BCA test and measuring A280 signal using ND-1000 spectrophotometer (NanoDrop Technologies, Inc.) using the theoretical extinction coefficient. The BTN3A1 B30.2 domain was expressed and purified as previously described (Sandstrom et al., 2014). The BTN3A1 full-length intracellular domain was cloned into pET28a with a 3C protease site followed by a carboxyl-terminus six-HIS tag using restriction enzyme sites *NcoI* and *XhoI* (5'primer: GCGCCATGGGGCAACAGCAGGAGGAAAAA; 3'primer: CGCTCGAGGGGCCCCTGGAACAGAACTTCCAGACCACCAGACGCTGGACAAATAGTC). The construct was expressed in BL21 strain *Escherichia coli*. Cells were grown to OD600=0.6 in Lysogeny Broth (LB) at 37 °C and induced with 1ml 1M IPTG per liter of culture for four hours at room temperature. Protein was harvested and purified using Ni-NTA (Qiagen) IMAC chromatography in 20mM Tris pH8.0, 400mM NaCl, 20mM Imidazole, 4mM BME, and eluted with 20mM Tris pH8.0, 400mM NaCl, 250mM Imidazole, 4mM BME and desalted into 10mM Hepes pH7.2, 150mM NaCl, 0.02% azide, 4mM BME using an Econo-Pac 10DG column (Biorad). Protein was cleaved overnight using 3C protease at 4 °C. Protein concentration was measured as mentioned above (Figure S4A and S4B).

Biolayer Interferometry (BLI)

The interactions between RhoB and BTN3A1 full-length intracellular domain (BFI) or BTN3A1 B30.2 domain were measured using Biolayer interferometry (BLItz, FortéBio). The BLI buffer used in baseline equilibration and the dissociation step was prepared with 10mM Hepes pH7.2, 150mM NaCl, 0.02% azide, 5mM $MgCl_2$, 4mM BME. RhoB, at a concentration of 2 mg/ml, was immobilized on the Ni-NTA biosensor hydrated with the BLI buffer using the basic kinetics method with the following parameters: 30s for baseline, 300s for association and 300s for dissociation. The RhoB-mounted biosensor was then blocked by 1mg/ml BSA and equilibrated with the BLI buffer using a similar method with the following parameters: 30s for baseline, 120s for association and 120s for dissociation. The buffer run served as a reference for the subsequent experiments. The interaction between RhoB and different concentrations of BFI (6.25, 12.5, 25, 50 and 100uM) or B30.2 domain (12.5, 25, 50, 100 and 200uM) were measured using the same method as mentioned above. The interaction between RhoB and different concentrations of BFI (3.75, 7.5, 15, 30 and 60uM) or B30.2 domain (3.75, 7.5, 15, 30 and 60uM) in the presence of (2E)-1-hydroxy-2-methylpent-2-enyl-pyrophosphonate (cHDAMPP) were also measured using the same method as mentioned above. The ratio between cHDMAPP and BFI or B30.2 domain was kept at 1:1 for these measurements. The rate and affinity constants for binding interactions were analyzed by Biaevulation (Biacore Life

Sciences). The data was truncated to 180s and the k_a and k_d were fitted simultaneously using 1:1 binding with drift baseline model.

G-Lisa analysis

RhoA activity was assayed using a G-LISA RhoA Activation Assay Biochem kit (cat. no. BK124; Cytoskeleton, Inc., Denver, CO, USA), according to the manufacturer's instructions. Briefly, cell were lysed in ice-cold lysis buffer with a protease-inhibitor cocktail, and then centrifuged at 10000 × g at 4°C for 1 min. The supernatants were harvested and protein concentrations were measured using the Precision Red Advanced Protein Assay Reagent and were finally equalised with ice-cold lysis buffer to 1.0 mg/ml. Equalised protein extractions were transferred to a Rho-GTP-binding protein pre-coated plate. The plate was placed on an orbital microplate shaker at 0.72 × g for 30 min at 4°C, and then incubated with monoclonal mouse anti-human anti-RhoA primary antibody (cat. no. GL01A; 1:250; Cytoskeleton, Inc.), followed by a polyclonal goat anti-mouse horseradish-conjugated secondary antibody (cat. no. GL02; 1:62.5; Cytoskeleton, Inc.), on an orbital microplate shaker (SSM1; Bibby Scientific Limited Group) at 0.72 × g at room temperature, for 45 min each. The plate was then incubated with the HRP detection reagent at 37°C for 15 min. Subsequent to the addition of HRP stop buffer, absorbance was read at 490 nm using a microplate reader.

Statistical analysis

All experiments were independently repeated at least three times unless otherwise indicated. All data were shown as mean ± SEM. Statistical significance was analysed by either Mann-Whitney or Kruskal-Wallis test and Dunn’s multiple comparison test.