

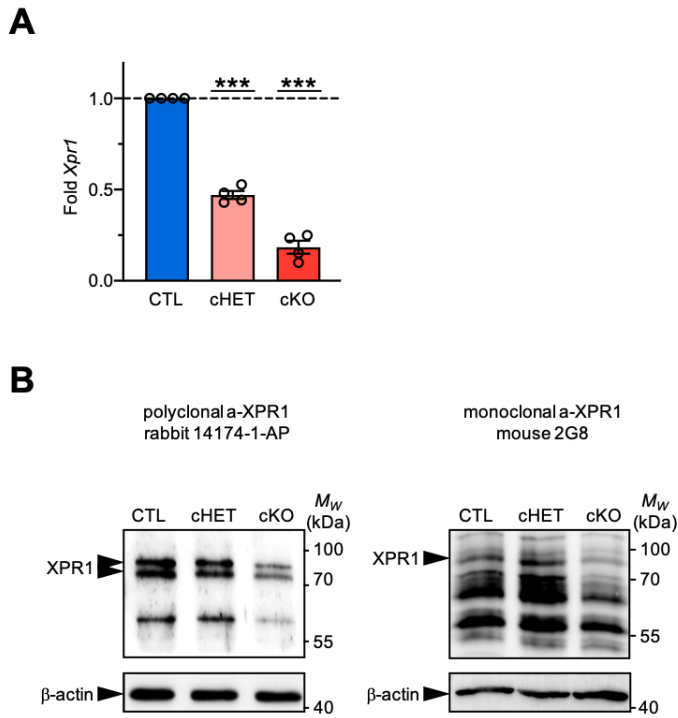
Supplementary Data

XPR1 prevents T-cell hyperactivation through interaction with KIDINS220 for endocytic regulation of CD28 costimulation

Marion Mengel, Benita Kröger, Mandy Malle, Maike Frye, Thomas Renné, Timur A. Yorgan, Björn-Philipp Diercks, Reiner K. Mailer

Supplementary data include Supplementary Figures (fig. S1-12).

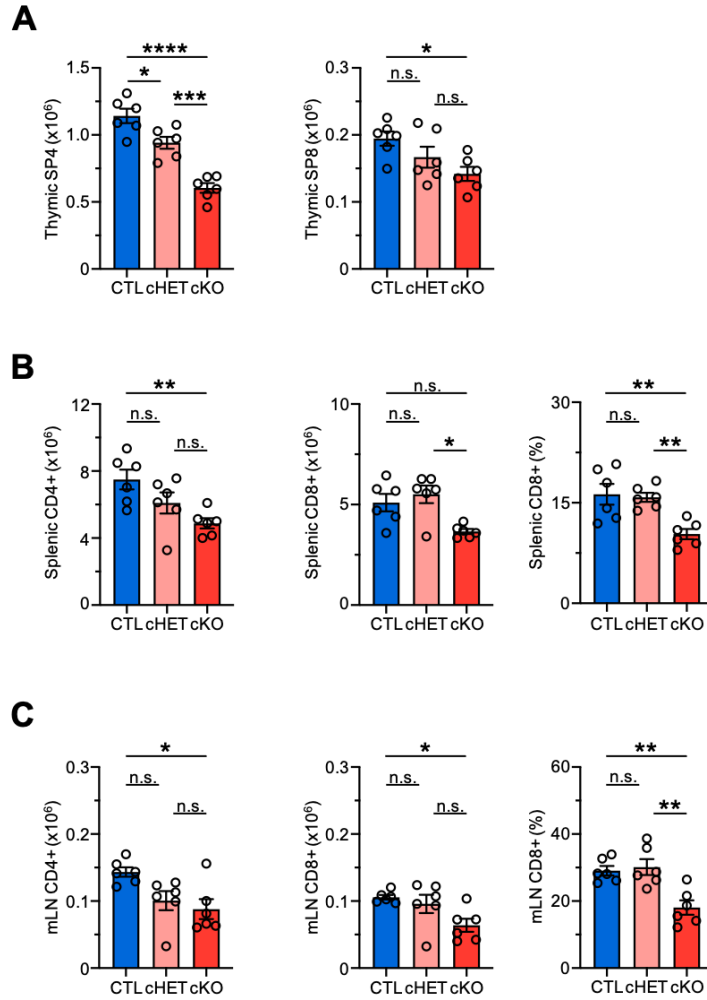
Figure S1



Supplementary Figure 1. Genetic deletion of *Xpr1* reduces XPR1 expression in CD4⁺ T cells from conditional knockout mice.

(A) Real-time PCR of CD4⁺ T-cell transcripts from XPR1 cKO, cHET and CTL mice; $n=4$. Values are expressed as mean $\pm$ SEM; statistical significance is indicated as *** $p < 0.001$. One-sample t test was performed for statistical analysis. **(B)** Immunoblots of CD4⁺ T-cell lysates from XPR1 cKO, cHET and CTL mice probed for XPR1 expression with primary antibodies from two different vendors as indicated; $n=2$.

Figure S2

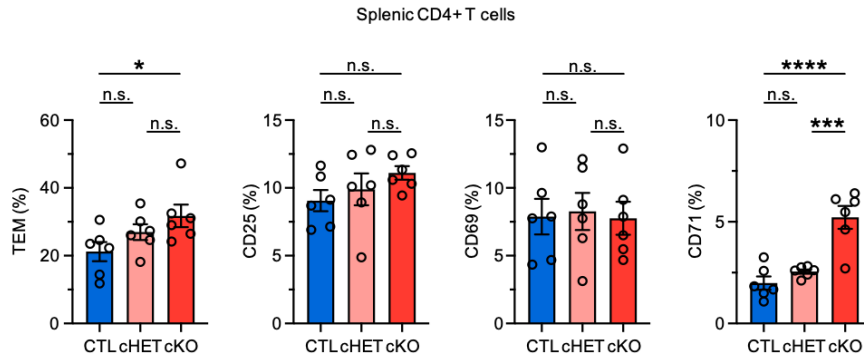


Supplementary Figure 2. XPR1 deficiency decreases development of thymocytes and peripheral T-cell populations.

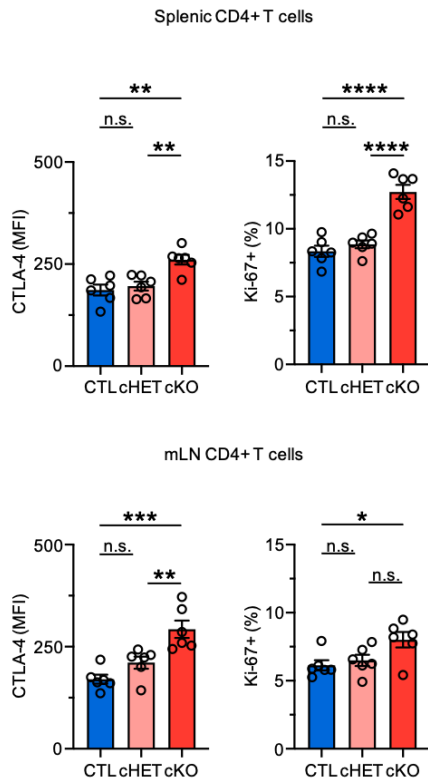
(A) Absolute numbers of SP4 (left) and SP8 (right) thymocytes from XPR1 cKO, cHET and CTL mice. (B) Absolute numbers of CD4+ (left) and CD8+ (middle) T cells, and population size of CD8+ T cells (right) in spleen from XPR1 cKO, cHET and CTL mice. (C) Absolute numbers of CD4+ (left) and CD8+ (middle) T cells, and population size of CD8+ T cells (right) in mesenteric lymph nodes (mLN) from XPR1 cKO, cHET and CTL mice. Values are expressed as mean $\pm$ SEM; statistical significance is indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$; not significant (n.s.). One-way ANOVA followed by Tukey multiple comparisons test was performed for statistical analysis, except for absolute numbers of splenic CD8+ T cells (B) and mLN CD4+ T cells (C) that were analyzed by Kruskal-Wallis ANOVA followed by Dunn multiple comparisons test; $n=6$.

Figure S3

A



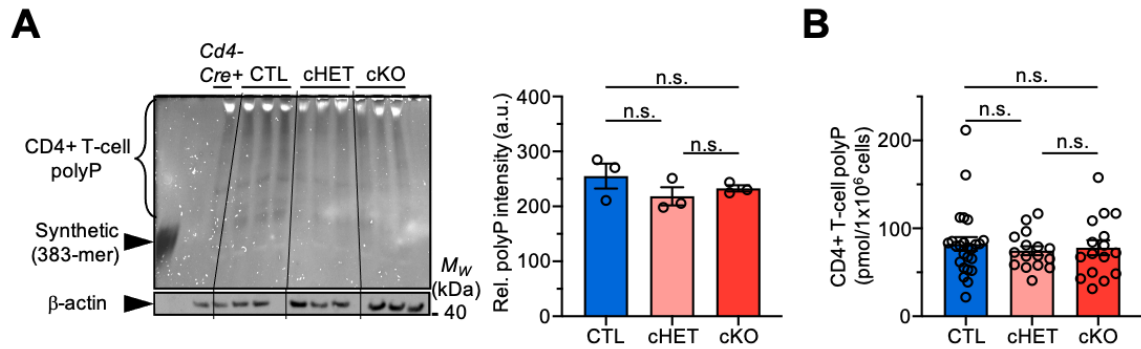
B



Supplementary Figure 3. Lack of both *Xpr1* alleles induces expression of T-cell activation markers.

(A) Population size of effector memory T cells (TEM, CD62L-CD44+) and expression of CD25, CD69 and CD71 among CD4+ T cells in spleen from XPR1 cKO, cHET and CTL mice. (B) Expression level of CTLA-4 and Ki-67 of splenic (upper panel) and mLN (lower panel) CD4+ T cells from XPR1 cKO, cHET and CTL mice. Values are expressed as mean $\pm$ SEM; statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; not significant (n.s.). One-way ANOVA followed by Tukey multiple comparisons test was performed for statistical analysis; $n=6$.

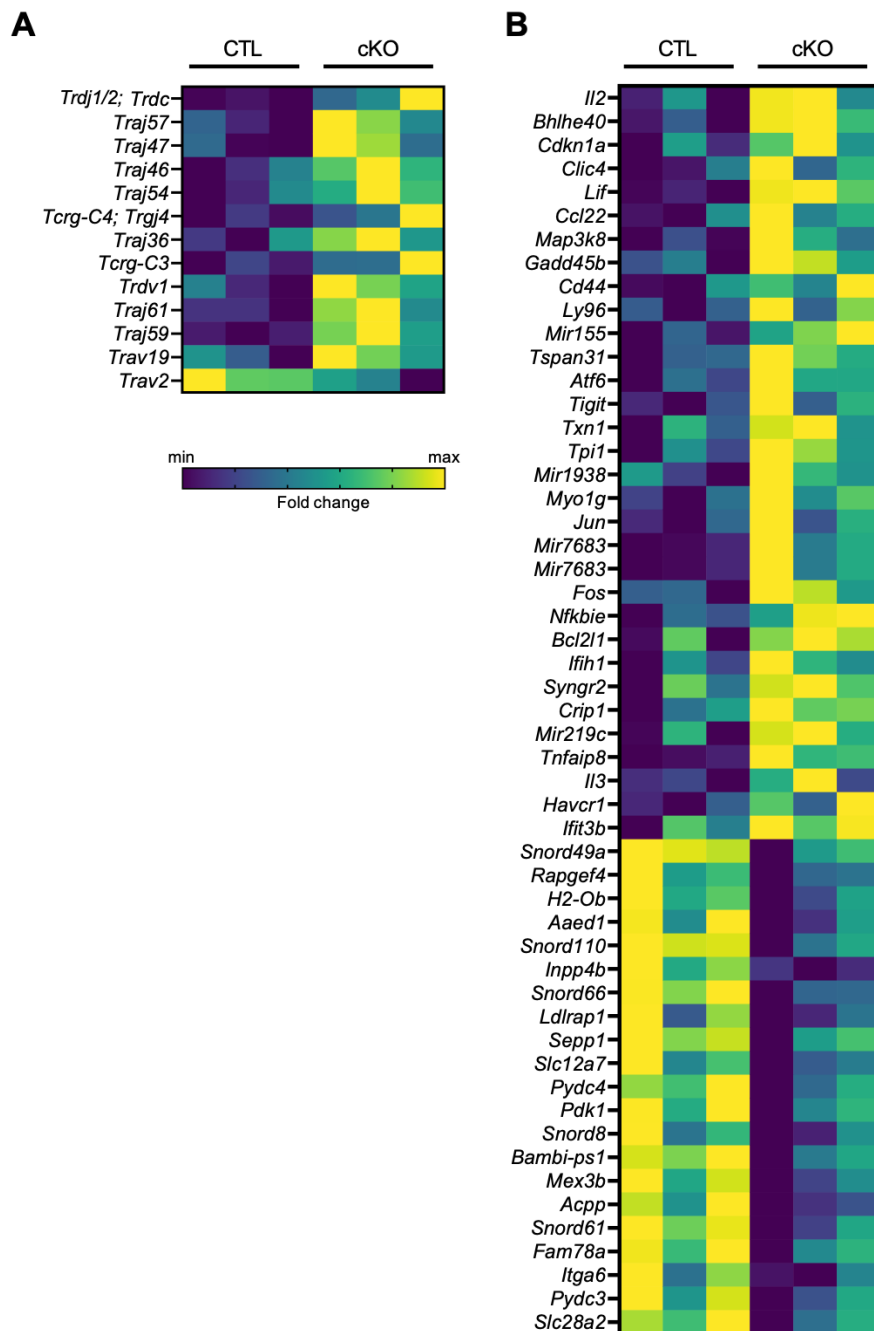
Figure S4



Supplementary Figure 4. Polyphosphate levels are unchanged in XPR1-deficient CD4+ T cells.

(A) Negative DAPI staining of purified polyphosphate (polyP) from CD4+ T cells (1×10^6 per lane) isolated from XPR1 cKO, cHET and CTL mice. Densitometric analysis normalized to β-actin loading control is shown, $n=3$. (B) Quantification of polyP purified from 1×10^6 CD4+ T cells isolated from XPR1 cKO, cHET and CTL mice by malachite green assay; $n=25$ (CTL), $n=16$ (cHET) and $n=16$ (cKO), respectively. Values are expressed as mean $\pm$ SEM; not significant (n.s.). One-way ANOVA followed by Tukey multiple comparisons test was performed for statistical analysis.

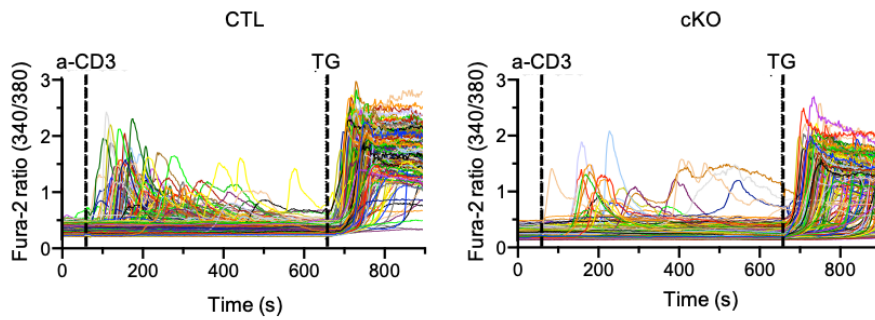
Figure S5



Supplementary Figure 5. Altered TCR repertoire and gene profile of XPR1-deficient CD4⁺ T cells.

Heatmap of (A) TCR-related and (B) TCR-unrelated genes identified as DEGs by transcriptome microarray analysis; $n=3$.

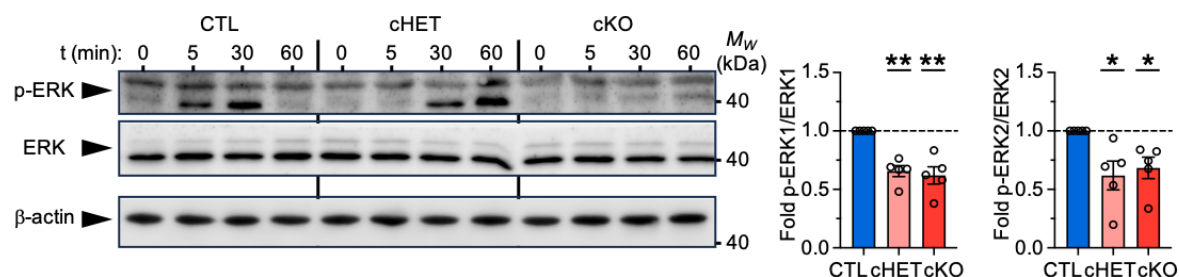
Figure S6



Supplementary Figure 6. Diminished Ca^{2+} mobilization in individual traces of XPR1-deficient CD4^+ T cells.

Live cell imaging of naïve CD4^+ T cells from CTL (left) and XPR1 cKO mice (right) stimulated with 10 $\mu\text{g/ml}$ anti-CD3 (a-CD3) after 1 min, and 1.67 μM thapsigargin (TG) after 11 min measurement. Individual traces ($n=342$ and $n=229$ for CTL and XPR1 cKO T cells, respectively) from one out of three independent time-lapse microscopy experiments are shown.

Figure S7

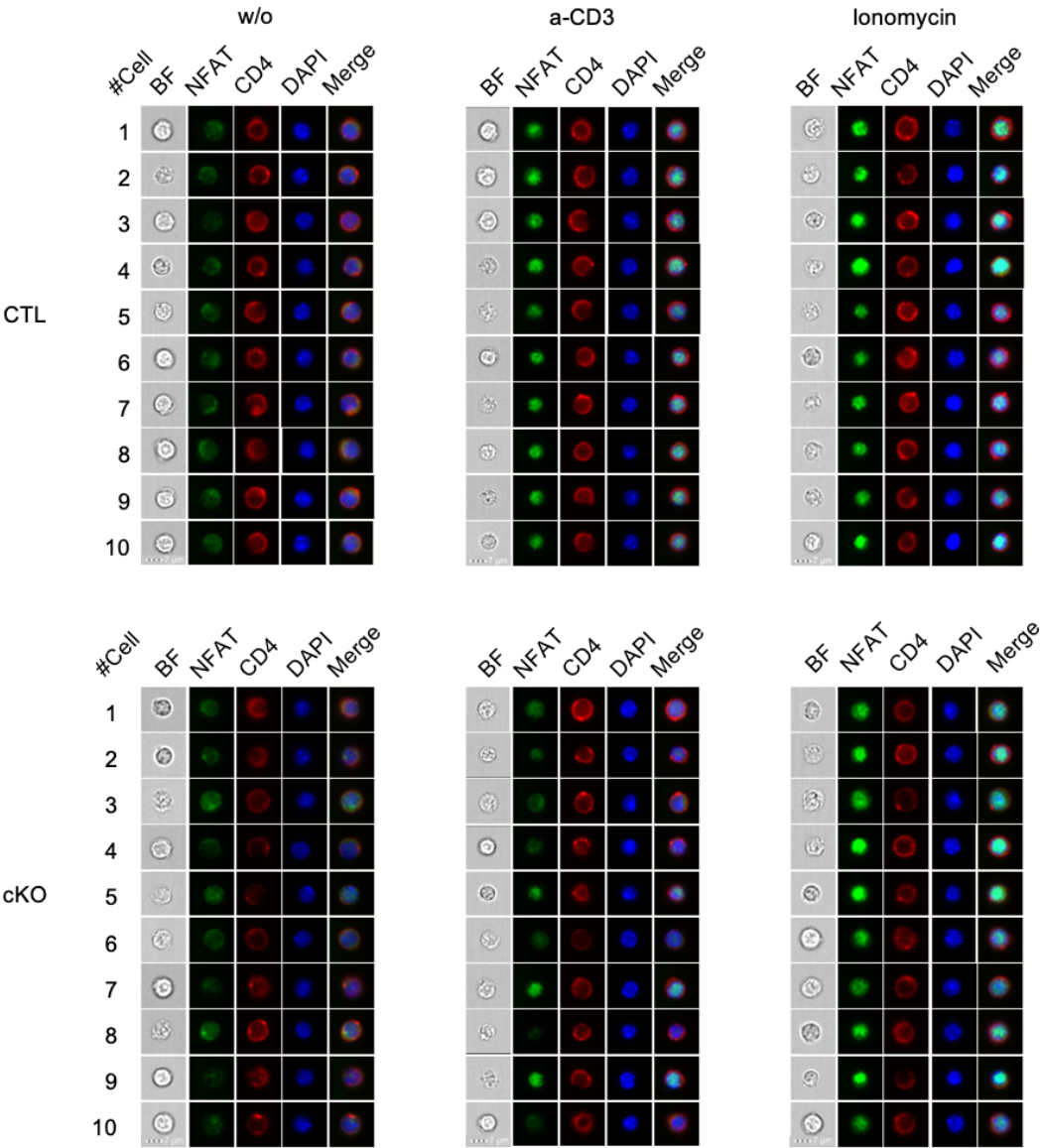


Supplementary Figure 7. Decreased phosphorylation of both ERK isoforms upon stimulation of naïve XPR1-deficient CD4⁺ T cells.

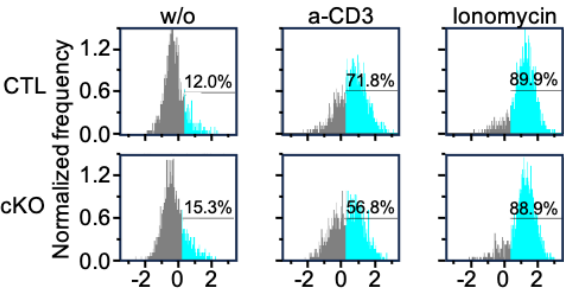
Phosphorylation of ERK (p-ERK) in naïve CD4⁺ T cells from XPR1 cKO, cHET and CTL mice after stimulation with 5 μ g/ml anti-CD3 for indicated time. Normalized p-ERK1/ERK1 and p-ERK2/ERK2 ratios after 5 min stimulation were analyzed in relation to CTL values (set to 1); $n=5$. Values are expressed as mean $\pm$ SEM; statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$. One-sample t test was performed for statistical analysis.

Figure S8

A



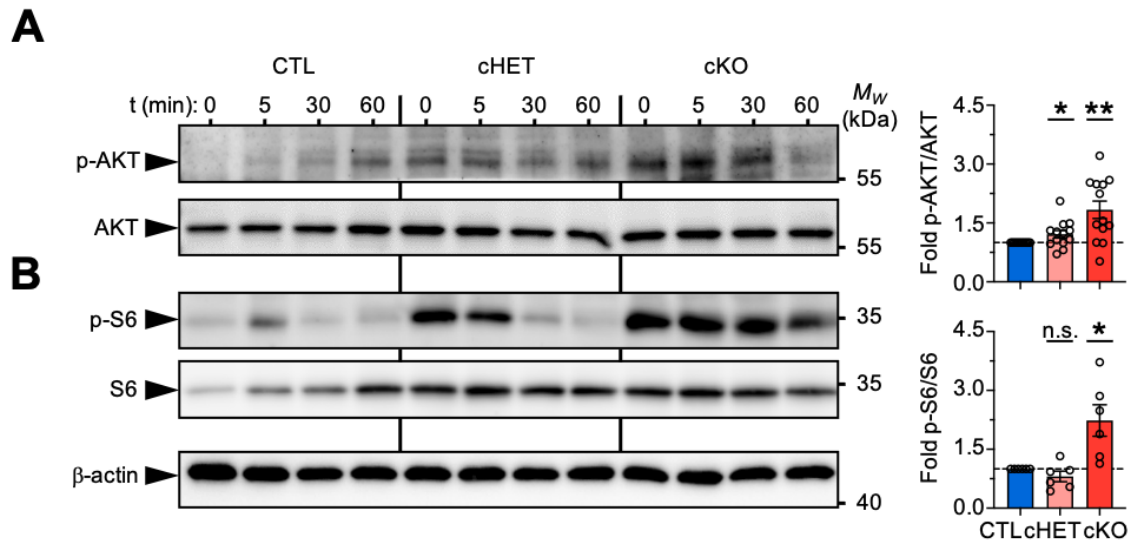
B



Supplementary Figure 8. Attenuated nuclear translocation of NFAT in XPR1-deficient CD4⁺ T cells upon anti-CD3 and ionomycin treatment.

(A) NFAT nuclear translocation in response to 10 min stimulation without (w/o) or with 5 µg/ml anti-CD3 (a-CD3) or treatment with 1 µg/ml ionomycin measured by imaging flow cytometry of CD4⁺ T cells from CTL (upper panel) and XPR1 cKO mice (lower panel) stained for CD4 and intracellular NFAT/DAPI. Ten representative T cells each with bright field (BF), single fluorescence and merged overlay images are shown. (B) Analysis of colocalization gates (cyan) assessed by similarity score analyses (IDEAS v6.2) to quantify overlapping NFAT and DAPI fluorescence signals from one out of five independent experiments is shown.

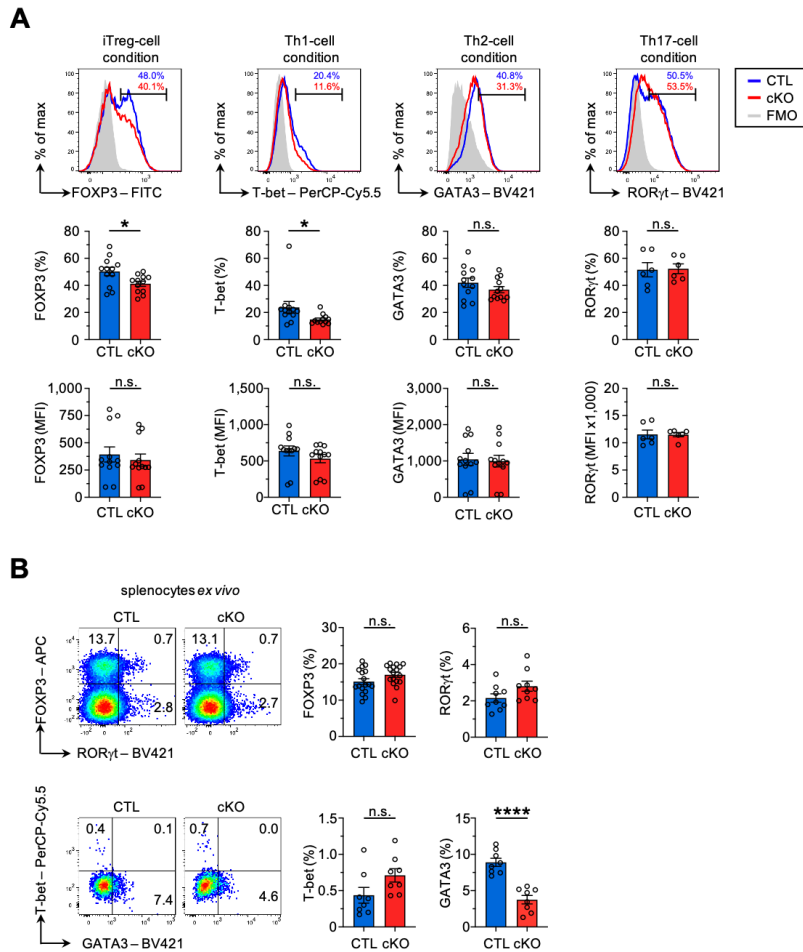
Figure S9



Supplementary Figure 9. Enhanced phosphorylation of AKT and S6 upon stimulation of naïve XPR1-deficient CD4⁺ T cells.

Phosphorylation of (A) AKT (p-AKT) and (B) S6 (p-S6) in naïve CD4⁺ T cells from XPR1 cKO, cHET and CTL mice after stimulation with 5 μ g/ml anti-CD3 for indicated time. Normalized p-AKT/AKT ratios ($n=13$) and p-S6/S6 ratios ($n=6$) after 5 min stimulation were analyzed in relation to CTL values (set to 1). Values are expressed as mean $\pm$ SEM; statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$; not significant (n.s.). One-sample t test was performed for statistical analysis.

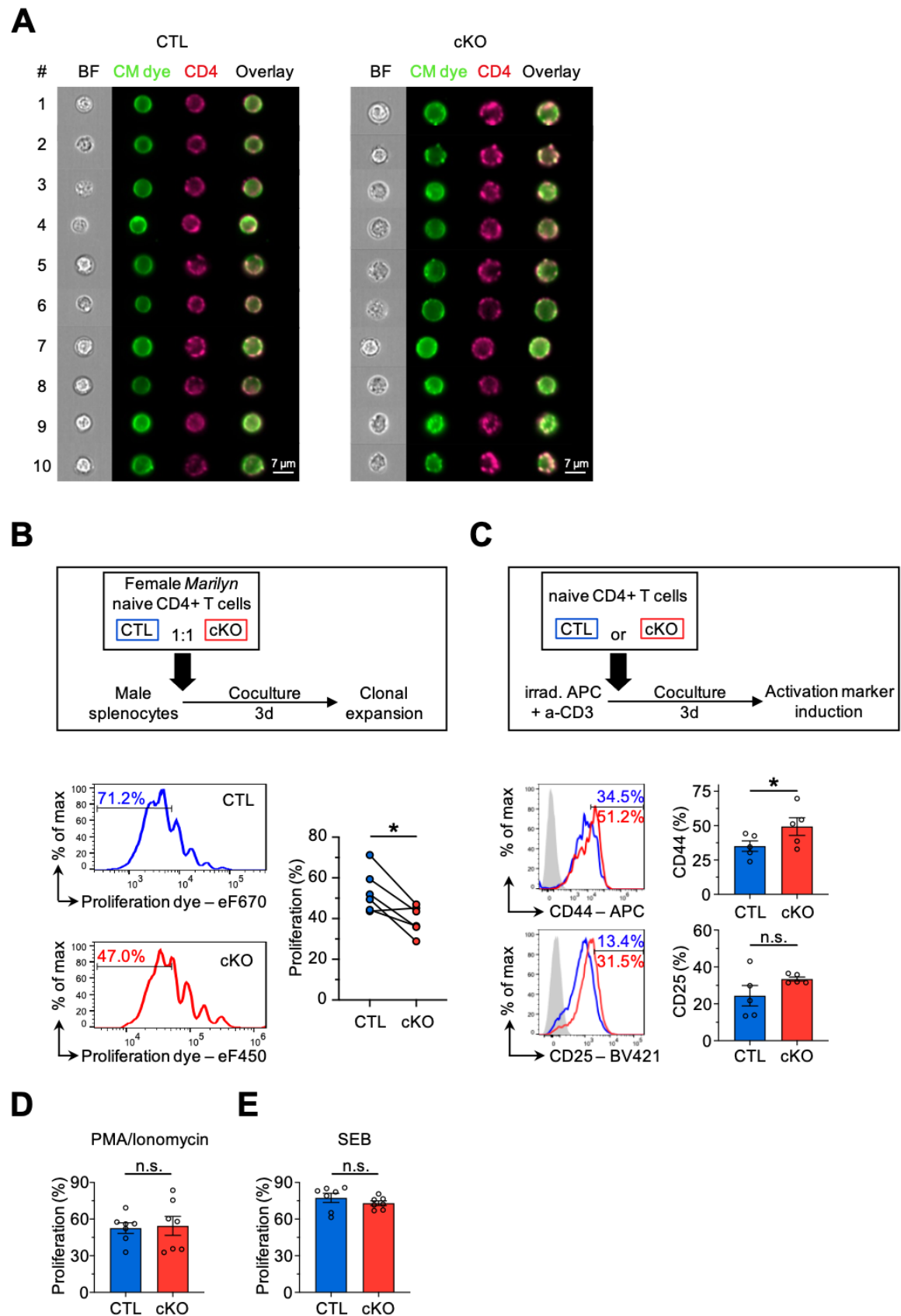
Figure S10



Supplementary Figure 10. Limited impact on transcription factor expression by XPR1 deficiency.

(A) Transcription factor expression of CD4⁺ T cells from XPR1 cKO and CTL mice differentiated *in vitro* under iTreg-skewing (far left, $n=12$), Th1-skewing (middle left, $n=12$) Th2-skewing (middle right, $n=12$) and Th17-skewing (far right, $n=6$) conditions. Representative histograms with fluorescence-minus-one (FMO) controls (upper row), population size (middle row) and expression level analysis (lower row) of lineage-specific transcription factors FOXP3, T-bet, GATA3 and ROR γ t. Values are expressed as mean $\pm$ SEM; statistical significance is indicated as * $p < 0.05$; not significant (n.s.). For statistical analysis of population sizes unpaired Student t test was performed, except for T-bet (%) that was analyzed with Mann-Whitney test. For statistical analysis of expression levels Mann-Whitney test was performed, except for GATA3 (MFI) that was analyzed with unpaired Student t test. (B) Representative flow cytometry plots and quantification of FOXP3 ($n=17$), ROR γ t ($n=9$), T-bet ($n=8$) and GATA3 ($n=8$) expression of splenic CD4⁺ T cells from XPR1 cKO and CTL mice *ex vivo*. Values are expressed as mean $\pm$ SEM; statistical significance is indicated as **** $p < 0.0001$; not significant (n.s.). Unpaired Student t test was performed for statistical analysis.

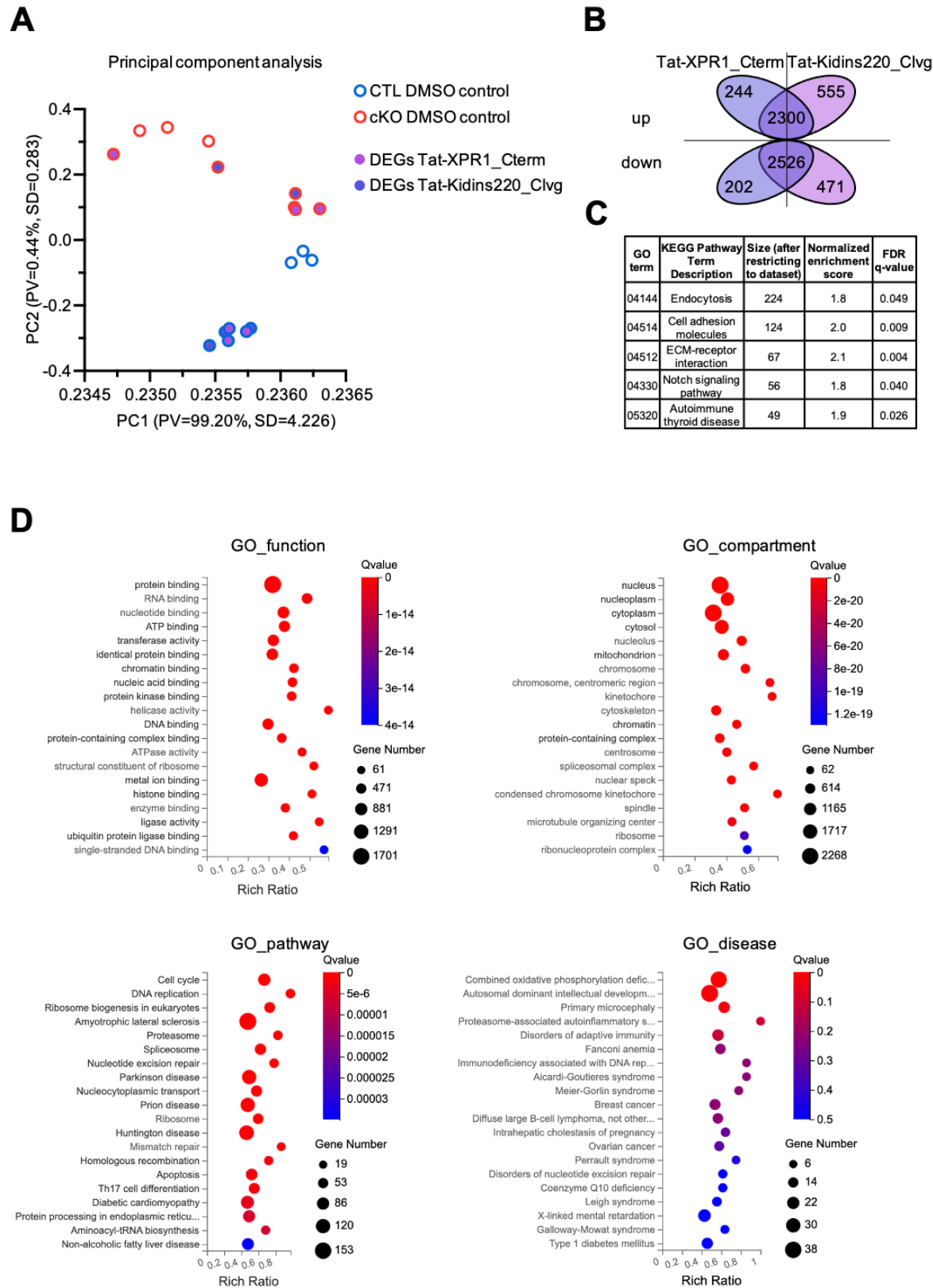
Figure S11



Supplementary Figure 11. Hyperproliferation of XPR1-deficient CD4⁺ T cells depends on impaired costimulation and membrane rearrangement.

(A) Spot formation on naïve CD4⁺ T cells from CTL (left) and XPR1 cKO mice (right) in response to TCR stimulation for 90 min from one out of two independent experiments. Membrane rearrangement measured by imaging flow cytometry of ten representative T cells for each genotype; bright field (BF), single fluorescence analysis for Cellbrite cytoplasmatic membrane (CM) dye and CD4, and merged overlay images are shown. (B) Clonal expansion of 5×10^4 naïve CD4⁺ T cells from female XPR1 cKO and CTL *Marilyn* mice labelled with either eFluor450 or eFluor670 proliferation dye in a 1:1 mixture were cocultured with 1.5×10^6 male splenocytes and 1 $\mu\text{g/ml}$ soluble $\alpha\text{-CD28}$ for 72 h. Representative histograms of proliferating CD4⁺V β 6⁺ T cells and quantification of proliferative responses are shown; $n=6$. (C) Expression levels of CD44 and CD25 in naïve CD4⁺ T cells from CTL and XPR1 cKO mice stimulated by anti-CD3 (5 $\mu\text{g/ml}$) and irradiated APCs (30 Gy, 1:2 ratio) for three days; representative histograms with fluorescence-minus-one (in gray) and quantification is shown; $n=5$. (D and E) Proliferation of CD4⁺ T cells from XPR1 cKO and CTL mice stimulated CD28-independently via PMA/ionomycin (D) or SEB (E) treatment; $n=7$. Values are expressed as mean $\pm$ SEM; statistical significance is indicated as $*p < 0.05$; not significant (n.s.), paired Student t test (B) and unpaired Student t test (C-E) was performed for statistical analysis.

Figure S12



Supplementary Figure 12. Transcriptome and gene ontology pathway analysis induced by XPR1-KIDINS220 association interference.

(A) Principal component analysis (PCA) of samples shown in Figure 5, demonstrating transcriptome similarities for Tat-XPR1-Cterm and Tat-KIDINS220-Clvg treated CD4+ T cells; $n=3$. (B) Venn diagram with detailed mapping of up- and down-regulated DEGs uniquely or shared between protein transduction-mediated changes. (C) Table of top hits for pathway regulation by DEGs that are shared among groups with protein transduction as identified by gene ontology (GO) pathway analysis. (D) Prediction of function, cell compartment, cellular mechanisms and relation for disease of shared DEGs among protein transduction-mediated changes.