6-21-2016

CIS is a potent checkpoint in NK cell-mediated tumor immunity

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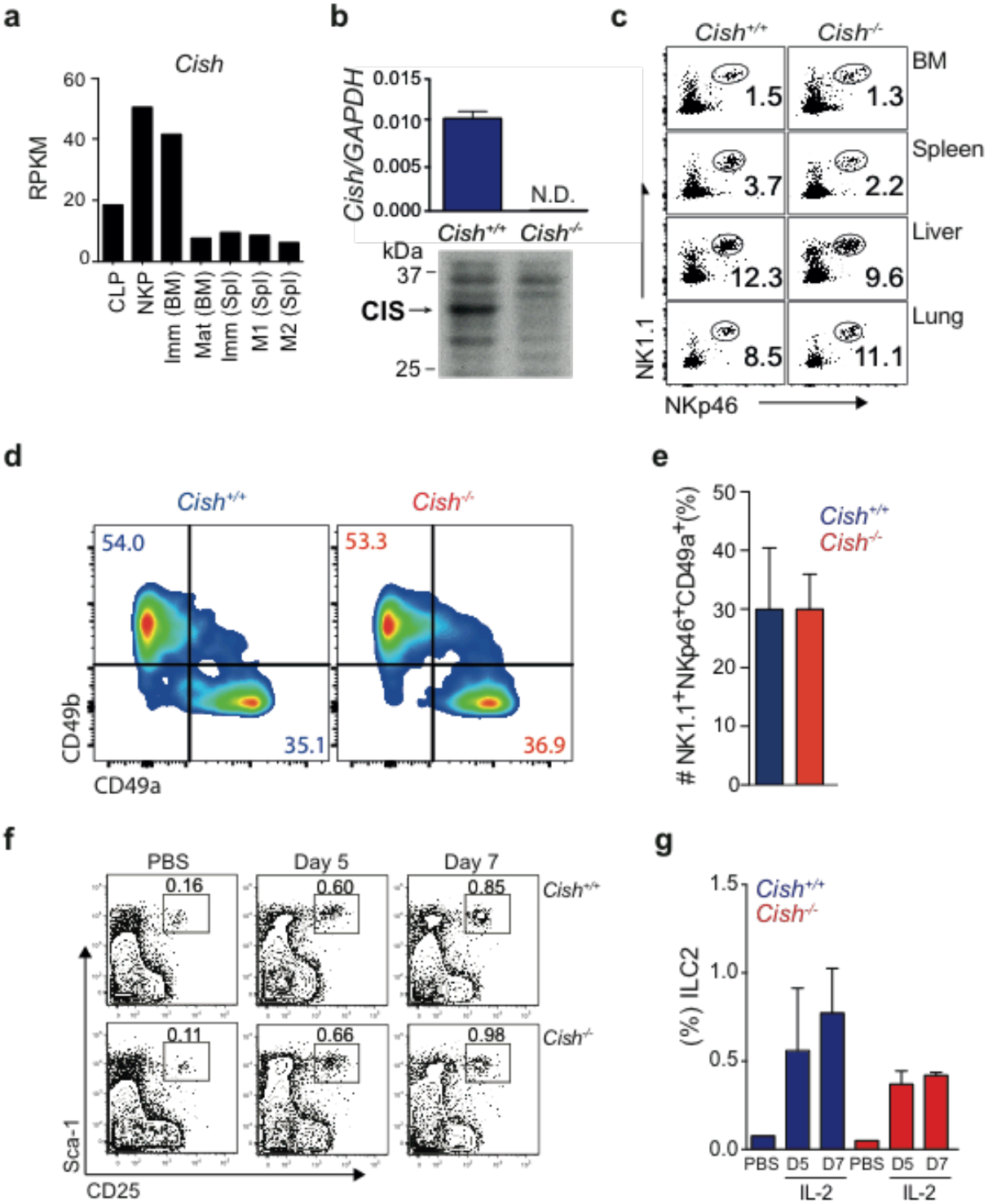
Delconte et al, Supplementary Figure 1



Supplementary Figure 1

Analysis of NK, ILC1 and ILC2 in *Cish*-deficient mice

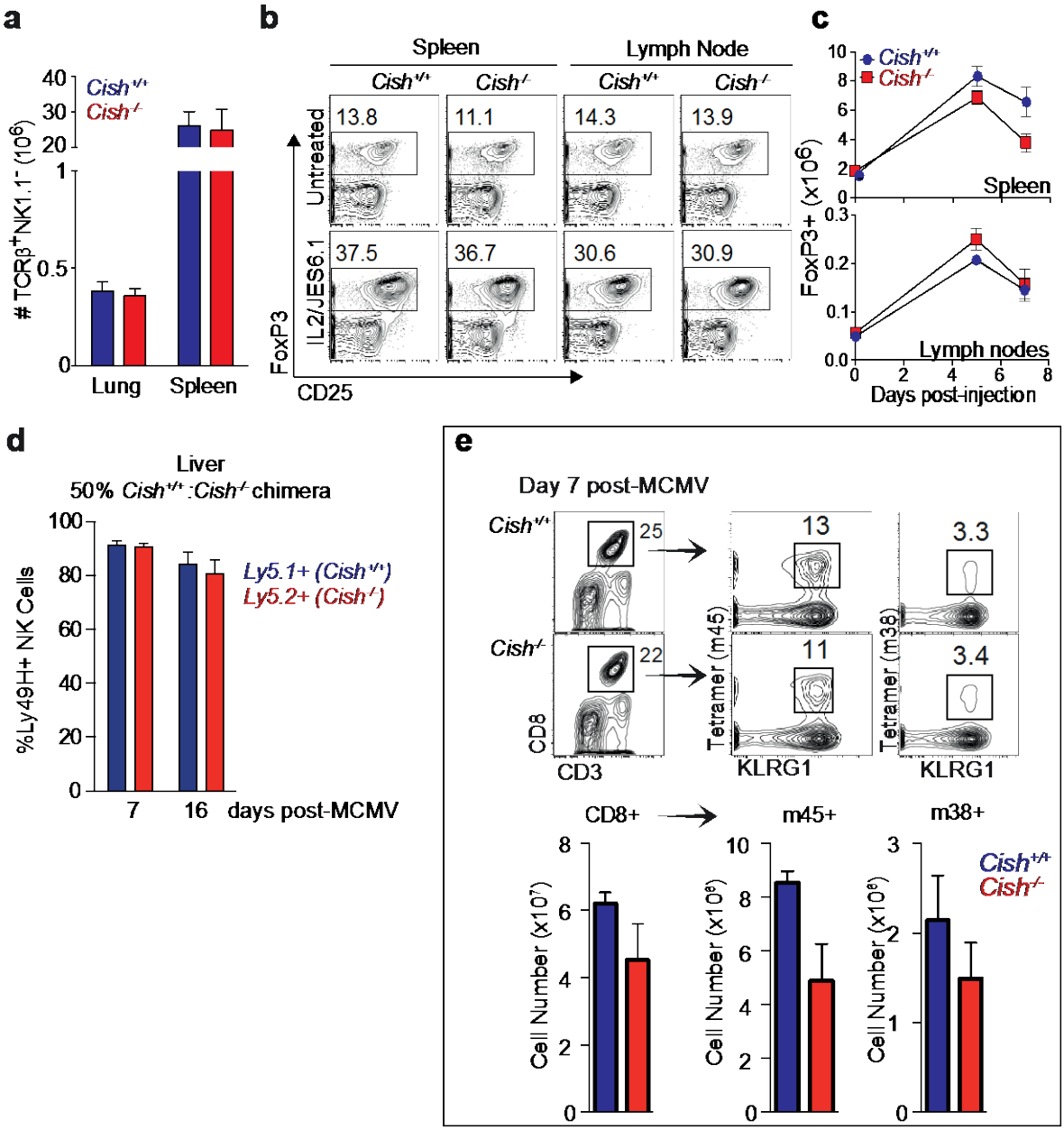
(a) *Cish* expression in NK cell subsets from C57BL/6 mice. Shown as reads per kilobase of exon per million reads (RPKM). Subset definition and RNAseq data have been described previously[1-3]. **(b)** *Cish*$^{+/+}$ or *Cish*$^{-/-}$ NK cells were cultured in IL-15, lysed and *Cish* mRNA analyzed by Q-PCR. Data were normalized to expression of GAPDH mRNA (upper panel). N.D.: not detected. *Cish*$^{+/+}$ or *Cish*$^{-/-}$ NK cells were cultured in IL-15 and the proteasomal inhibitor MG132 for 4 h prior to cell lysis and CIS protein detected in whole cell lysates by Western blotting (lower panel). **(c) NK cells** (NK1.1^{+}NKp46^{+}TCR-β^{-}) were analyzed in the indicated organs from *Cish*$^{+/+}$ and *Cish*$^{-/-}$ mice by flow cytometry. **(d) ILC1** (NK1.1^{+}NKp46^{+}TCR-β^{-}CD49a^{+}CD49b^{-}) in the liver of *Cish*$^{+/+}$ and *Cish*$^{-/-}$ were analyzed by flow cytometry and **(e)** quantified (vertical axis: % of ILC1). **(f) ILC2** *Cish*$^{+/+}$ and *Cish*$^{-/-}$ mice were treated with PBS or IL-2 complexed with anti-IL-2 antibodies (IL-2-JES6.1; expansion of CD25+ cells) every 2 days and were sacrificed after 5 or 7 days (D5, D7). Representative flow cytometry plots of ILC2 in the bone marrow gated on CD3/19/NK1.1/B220/Gr1 negative cells. **(g)** Frequency of ILC2 in the bone marrow following IL-2-JES6.1 treatment. **(b, e, g)** Mean ± s.e.m. n=3 biological replicates.

1 **Delconte, R. B. *et al.* The Helix-Loop-Helix Protein ID2 Governs NK Cell Fate by Tuning Their Sensitivity to Interleukin-15. *Immunity* 44, 103-115, doi:10.1016/j.immuni.2015.12.007 (2016).**

2 **Revilla, I. D. R. *et al.* The B-cell identity factor Pax5 regulates distinct transcriptional programmes in early and late B lymphopoiesis. *EMBO J* 31, 3130-3146, doi:10.1038/emboj.2012.155 (2012).**

3 **Holmes, M. L. *et al.* Peripheral natural killer cell maturation depends on the transcription factor Aiolos. *EMBO J* 33, 2721-2734, doi:10.15252/embj.201487900 (2014).**

Delconte et al. Supplementary Figure 2

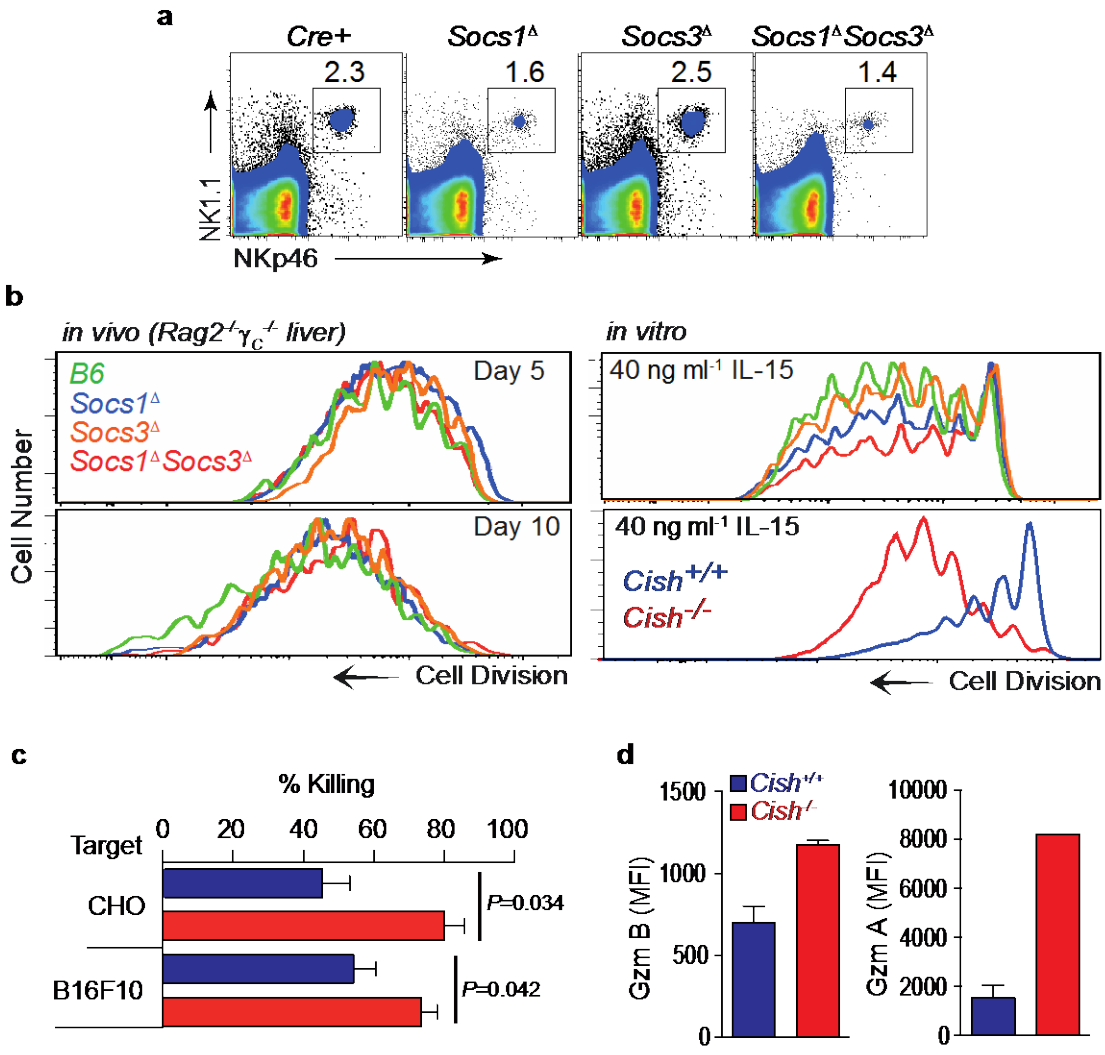


Supplementary Figure 2

Analysis of T cells, regulatory T cells and MCMV responses in *Cish*-deficient mice

(a) T cells ($NK1.1^-NKp46^-TCR\text{-}\beta^+$) were analyzed in the indicated organs from $Cish^{+/+}$ and $Cish^{-/-}$ mice by flow cytometry. **(b) Regulatory T cells (Tregs)** Expression of FoxP3 and CD25 on $CD4^+$ cells from spleen and lymph nodes of $Cish^{+/+}$ and $Cish^{-/-}$ mice before and 5 days after IL-2-JES6-1 treatment. Representative flow cytometry plots are shown. **(c)** Expansion and contraction of Tregs in the spleen and lymph nodes following IL-2-JES6-1 complex treatment (Mean ± S.E.M., n=1-2 mice per group). **(d)** 50:50 bone marrow chimeras ($5x10^6$ $Ly5.1^+$ $Cish^{+/+}$ and $Ly5.2^+$ $Cish^{-/-}$ cells) were injected intraperitoneally with $5x10^3$ plaque forming units (PFU) of salivary gland-propagated virus stock of MCMV-K181-Perth and $Ly49H^+$ NK cells frequency monitored by flow cytometry. (Mean ± S.E.M., n=6 mice). **(e)** Intact $Cish^{+/+}$ and $Cish^{-/-}$ *mice* were injected intraperitoneally with $5x10^3$ plaque forming units (PFU) of salivary gland-propagated virus stock of MCMV-K181-Perth. On day 7 spleens were analyzed for $MCMV^+$ $CD8^+$ T cell responses by flow cytometry. Numbers of indicated $tetramer^+$ $CD8^+$ T cells are shown (mean ± s.e.m., n=5 mice

Delconte et al. Supplementary Figure 3

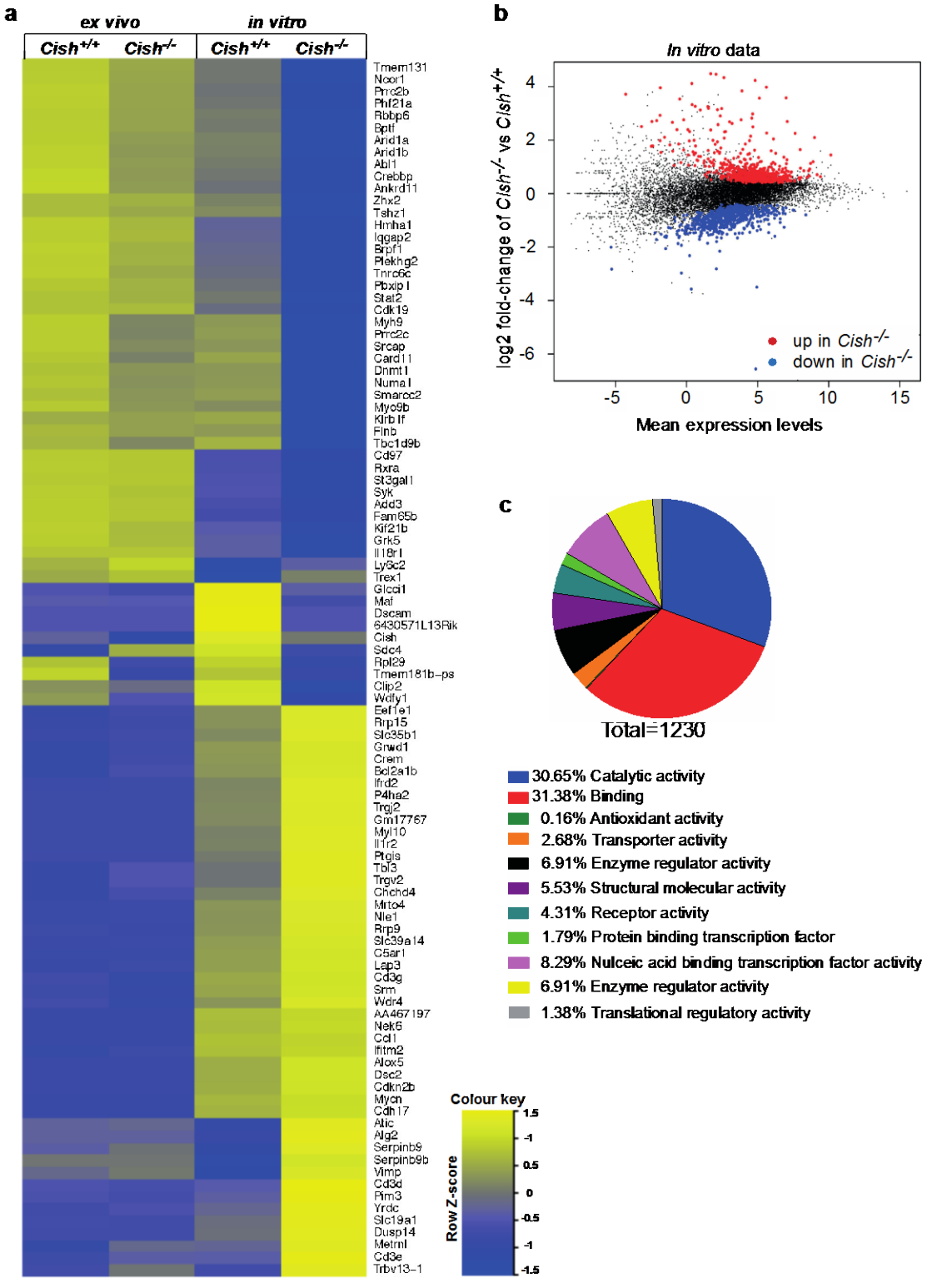


Supplementary Figure 3

Loss of *Socs1* and/or *Socs3* does not alter IL-15 responses in NK cells.

(a) *Socs3*$^{+/+}$*ERT2*$^{Cre/+}$ (Cre+), *Socs1*$^{-/-}$*Ifnγ*$^{-/-}$ (Socs1$^{\Delta}$), *Socs3*$^{fl/fl}$*ERT2*$^{Cre/+}$ (Socs3$^{\Delta}$), and *Socs1*$^{-/-}$*Ifnγ*$^{-/-}$*Socs3*$^{fl/fl}$*ERT2*$^{Cre/+}$ (Socs1$^{\Delta}$Socs3$^{\Delta}$) mice were treated with 4-hydroxytamoxifen (4-OHT; to induce *Socs3* deletion) by oral gavage and splenic NK cells (TCR-β^{-}NK1.1^{+}NKp46^{+}) analysed 14 days later by flow cytometry. Plots and values (%) are representative of 3 mice analysed for each genotype. **(b)** Splenic NK cells from mice in (a) were FACS sorted and cultured in IL-15 (50 ng ml^{-1}) for 7 days before being CFSE labelled and either i.v. transferred into alymphoid *Rag2*$^{-/-}\gamma_C{}^{-/-}$ recipients or cultured in IL-15 (50 ng ml^{-1}) *in vitro*. Five and ten days post-transfer, recipient livers were analysed for donor NK cells by flow cytometry. *In vitro* cultures were analysed on day 5. *Cish*$^{+/+}$ and *Cish*$^{-/-}$ NK cell cultures serve as a reference for differential proliferation (lower right panel). **(c) Enhanced effector function in *Cish*$^{-/-}$ NK cells.** *Cish*$^{+/+}$ and *Cish*$^{-/-}$ NK cells were cultured for 7 days prior to co-culture with CHO or B16F10 target cells at a ratio of 1:1. Target cell killing at 5 h was determined by relative changes in electrical impedance using the xCELLigence system. *Cish*$^{+/+}$ and *Cish*$^{-/-}$ NK cells achieved maximal killing at 9:1 effector:target ratios (defined as 100% killing). **(d)** *Cish*$^{+/+}$ and *Cish*$^{-/-}$ mice where injected with RMA-m157 cells i.p and peritoneal NK cells analysed 18 h later for intracellular granzyme-B production by flow cytometry. Mean±SD of two experiments. n = 2 mice. MFI: Mean Fluorescence Index.

Delconte et al. Supplementary Figure 4

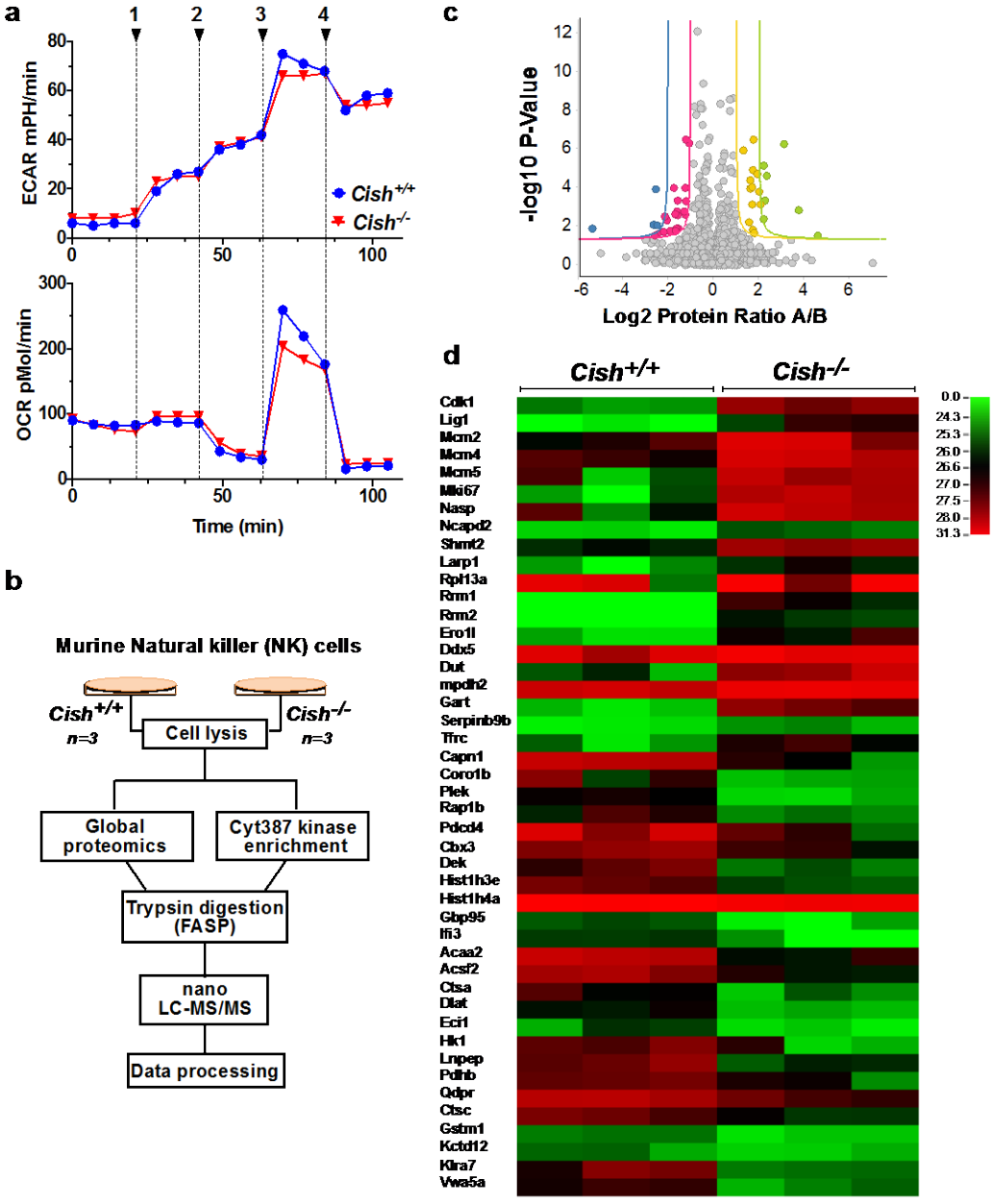


Supplementary Figure 4

Transcriptome profiling of *ex vivo* and *in vitro* cultured *Cish*$^{-/-}$ NK cells

100bp single-ended RNAseq was performed on freshly sorted *ex vivo Cish*$^{+/+}$ and *Cish*$^{-/-}$ NK cells, and on *Cish*$^{+/+}$ and *Cish*$^{-/-}$ NK cells that had been cultured for 7 days in IL-15 (50 ng/ml). **(a)** Relative expression levels (Z-scores) of the top ~100 most differentially expressed genes in *Cish*$^{-/-}$ cells are shown in the heatmap, color-coded according to the legend. Rows are scaled to have a mean of 0 and s.d. of 1. n=2 biological replicates. **(b)** Mean-difference plot of the cultured NK cell data generated in Figure 2, showing Log2-fold change versus mean expression. **(c)** Functional analysis of the 1230 differentially expressed genes observed in IL-15 cultured *Cish*$^{-/-}$ NK cells. Gene ontology was performed using the PANTHER classification system. Major gene networks are shown as a percentage of total differentially expressed genes in *Cish*$^{-/-}$ cells.

Delconte et al. Supplementary Figure 5

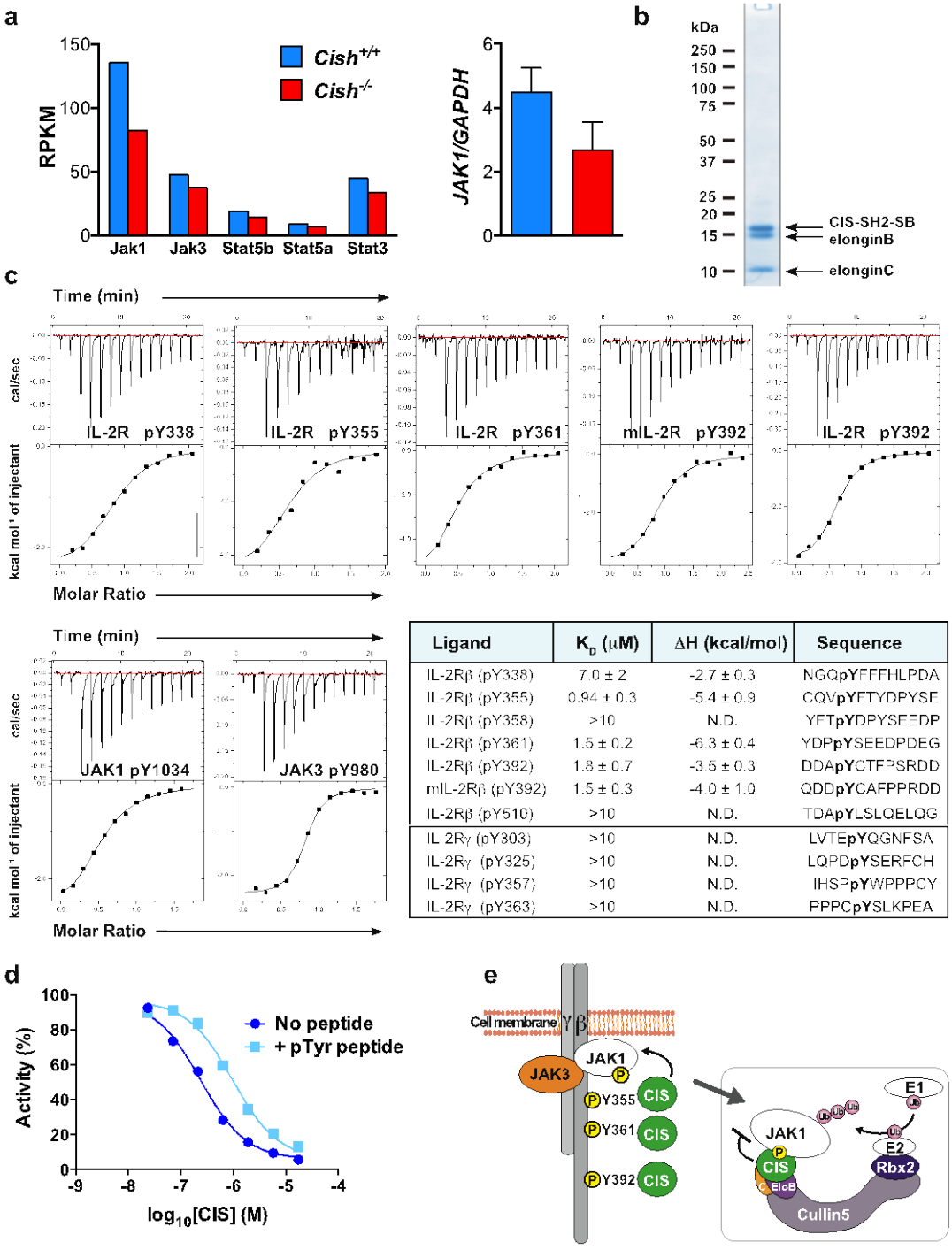


Supplementary Figure 5

Cish$^{-/-}$ NK cells display increased JAK/STAT signaling and normal respiration and glycolysis.

(a) *Cish*$^{-/-}$ NK cell respiration and glycolysis is unperturbed. *Cish*$^{+/+}$ and *Cish*$^{-/-}$ NK cells were cultured in the presence of IL-15 and the extracellular acidification rater (ACR; glycolysis) and oxygen consumption rate (OCR; mitochondrial respiration) measured using the XF Analyzer system. Glucose (1), Oligomycin (2), FCCP and pyruvate (3) and Antimycin A/Rotenone (4) were added at times indicated by the numbered arrows. **(b)** Overview of the proteomic workflow used in this study. Equal numbers of cultured NK cells derived from *Cish*$^{+/+}$ and *Cish*$^{-/-}$ mice were lysed and subjected to kinase enrichment using NHS-CYT-387 beads. Protein eluates from the CYT-387 resin, in addition to a portion of whole cell lysate (WCL; pre-kinase enrichment) were subjected to trypsin digestion and nanoLC-MS/MS. **(c)** Label-free quantification of global protein expression. Volcano plot showing the Log2 protein ratios following the quantitative pipeline analysis (*Cish*$^{+/+}$ vs *Cish*$^{-/-}$) from WCL. The red and yellow lines represent a 2-fold change in protein expression (log2 ratio of 1), while blue and green lines represent a 4-fold change (log2 ratio of 2); dots are colored accordingly and represent individual proteins. Proteins with a -$\log_{10}$ p-value of 1.3 or greater (corresponding to a p-value of ≤ 0.05) were deemed differentially abundant. **(d)** Heat map displaying Log2-transformed summed peptide intensities (non-imputed) for proteins with significantly differential expression in (d). Data from individual biological replicates are shown (n=3). Green to red indicates increasing expression levels. *See also* Extended Data **Table 2**.

Delconte et al. Supplementary Data Figure 6



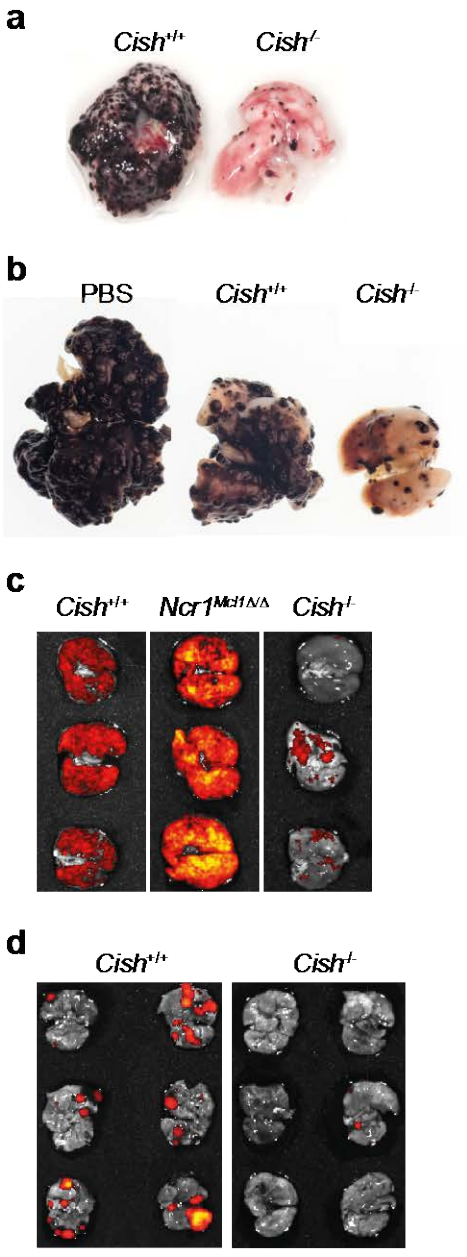
Ligand	K_D (μM)	ΔH (kcal/mol)	Sequence
IL-2Rβ (pY338)	7.0 ± 2	-2.7 ± 0.3	NGQ**pY**FFFHLPDA
IL-2Rβ (pY355)	0.94 ± 0.3	-5.4 ± 0.9	CQV**pY**FTYDPYSE
IL-2Rβ (pY358)	>10	N.D.	YFT**pY**DPYSEEDP
IL-2Rβ (pY361)	1.5 ± 0.2	-6.3 ± 0.4	YDP**pY**SEEDPDEG
IL-2Rβ (pY392)	1.8 ± 0.7	-3.5 ± 0.3	DDA**pY**CTFPSRDD
mIL-2Rβ (pY392)	1.5 ± 0.3	-4.0 ± 1.0	QDD**pY**CAFPPRDD
IL-2Rβ (pY510)	>10	N.D.	TDA**pY**LSLQELQG
IL-2Rγ (pY303)	>10	N.D.	LVTE**pY**QGNFSA
IL-2Rγ (pY325)	>10	N.D.	LQPD**pY**SERFCH
IL-2Rγ (pY357)	>10	N.D.	IHSP**pY**WPPPCY
IL-2Rγ (pY363)	>10	N.D.	PPPC**pY**SLKPEA

Supplementary Figure 6

CIS targets JAK and the IL-2R complex

(a) Cultured NK cells from wild-type and *Cish*$^{-/-}$ mice were lysed, mRNA purified and analyzed by RNAseq. Mean RPKM values for duplicate samples (left panel). JAK1 mRNA levels were analyzed by Q-PCR (right panel). Mean and s.d., n=3. **(b)** 4-12% Coomassie-stained SDS-PAGE gel showing purified hCIS-SH2-SB, elongin B and elongin C complex (CIS-SH2-BC). **(c)** Isothermal calorimetry (ITC) was used to measure the affinity of hCIS-SH2-BC binding to phosphopeptides corresponding to tyrosines within the JAK1/3 kinase domain activation loops and IL-2Rβ and γ cytoplasmic domains. 300 μM phosphopeptides were titrated into a 30 μM solution of the GST-CIS-SH2-BC ternary complex. ITC titration curves and tabular view of some results (inset) showing average and range from two independent experiments. N.D.=Not detectable, p=phosphorylated. The titration curves all fitted well to a single-site model. **(d)** Kinase inhibition assays were performed with the kinase domain (JH1) of JAK1 in the presence of CIS-SH2-BC with and without excess JAK1-Y1034 phosphopeptide as a competitor. The pY1034 peptide partially reduced CIS-mediated inhibition. Data were normalised to no-CIS controls. **(e)** Diagram illustrating the *in vitro* E3 ligase ubiquitination components and proposed model for CIS-mediated inhibition of JAK activity, whereby CIS recruitment to the receptor complex promotes binding to active JAK1 and results in kinase inhibition and proteasomal degradation. eloB: elongin B; eloC: elongin C.

Delconte et al., Supplementary data Figure 7



Supplementary Figure 7

CIS-null mice resist tumor metastasis

(a) Metastatic burden in lungs of *Cish*$^{+/+}$ and *Cish*$^{-/-}$ mice 14 days following i.v injection of B16F10 melanoma cells (as in **Figure 7a)**. **(b)** Metastatic burden in the lungs of NK cell-deficient (*Ncr1*$^{Mcl1\Delta/\Delta}$) mice injected i.v. with B16F10 melanoma cells and *Cish*$^{+/+}$ or *Cish*$^{-/-}$ NK cells or PBS (as in **Figure 7e)**. (**c**) Metastatic burden in the lungs measured by imaging (IVIS; mCherry fluorescence) of *Cish*$^{+/+}$, *Cish*$^{-/-}$ and *Ncr1*$^{Mcl1\Delta/\Delta}$ (NK-null) mice 14 days following i.v injection of E0771-mCherry^{+}luciferase^{+} breast cancer (as in **Figure 8a)**. (**d**) Orthotopic E0771.LMB tumors generated as in **(Figure 8c)** were surgically removed at 400–600 mm^3 and spontaneous lung metastases measured by IVIS (mCherry fluorescence) 14 days later.

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Supplementary Figure 8

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