nature immunology

SUPPLEMENTARY INFORMATION

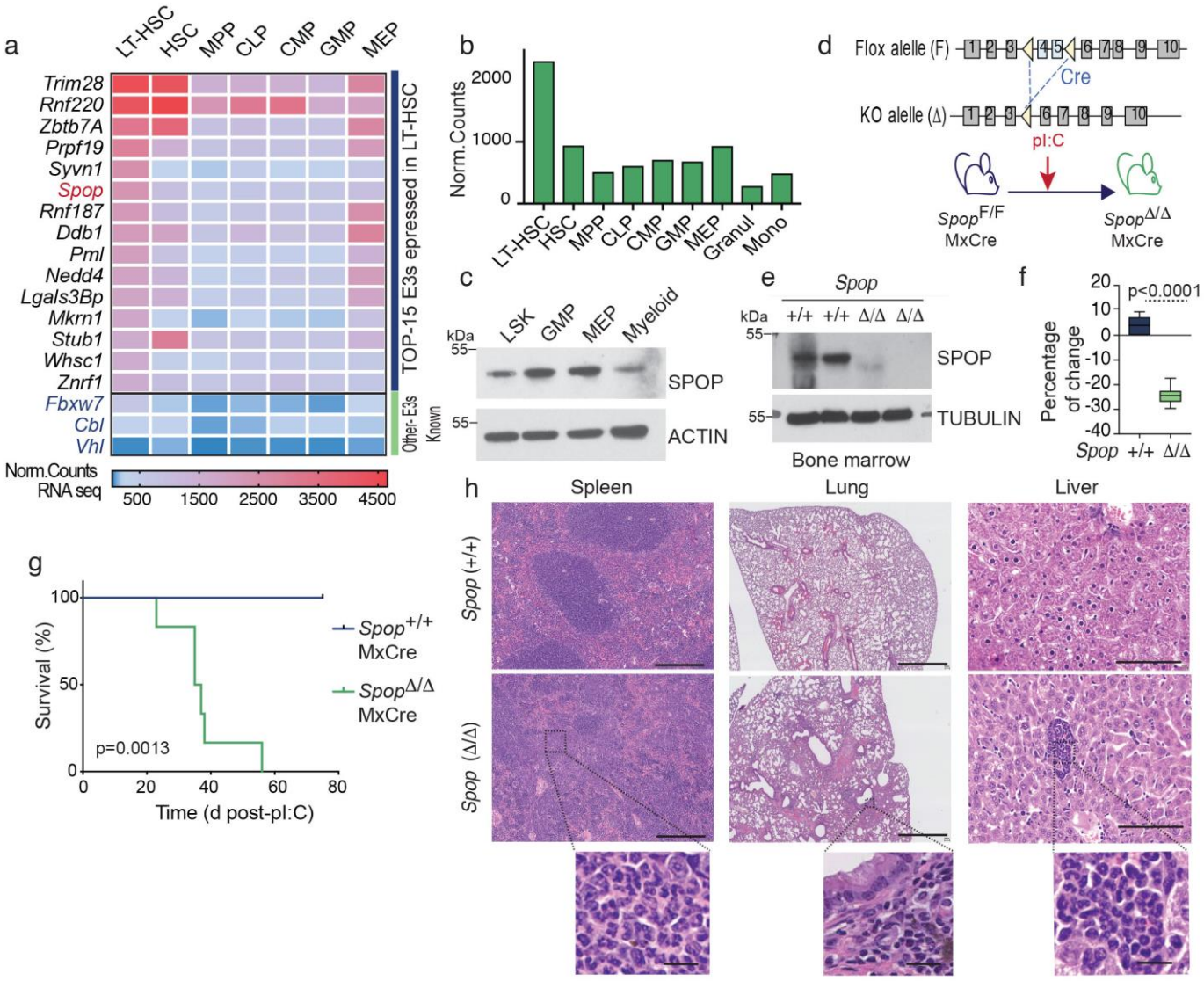
https://doi.org/10.1038/s41590-019-0454-6

In the format provided by the authors and unedited.

The E3 ubiquitin ligase SPOP controls resolution of systemic inflammation by triggering MYD88 degradation

Maria Guillamot[1,2,10]*, Dahmane Ouazia[3,10], Igor Dolgalev[1,2,4], Stephen T. Yeung[5], Nikos Kourtis[1,2], Yuling Dai[1,2], Kate Corrigan[1,2], Luna Zea-Redondo[1,2], Anita Saraf[6], Laurence Florens[6], Michael P. Washburn[6,7], Anastasia N. Tikhonova[1,2], Marina Malumbres[1,2], Yixiao Gong[1,2], Aristotelis Tsirigos[1,2,4], Christopher Park[1,2], Christopher Barbieri[8,9], Kamal M. Khanna[5], Luca Busino[3,10,11]* and Iannis Aifantis[1,2,10,11]*

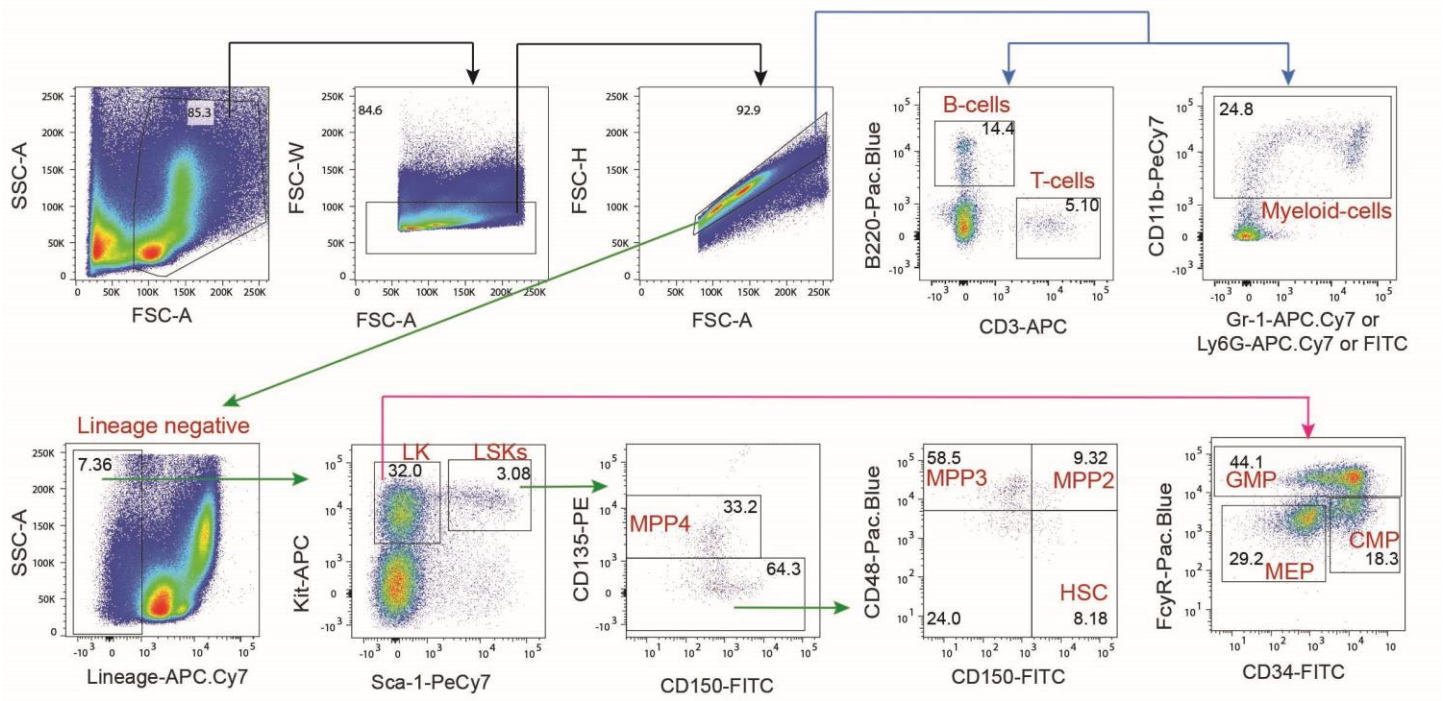
[1]Department of Pathology, NYU School of Medicine, New York, NY, USA. [2]Laura and Isaac Perlmutter Cancer Center, NYU School of Medicine, New York, NY, USA. [3]Department of Cancer Biology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA. [4]Applied Bioinformatics Laboratories, Office of Science & Research, NYU School of Medicine, New York, NY, USA. [5]Department of Microbiology, NYU School of Medicine, New York, NY, USA. [6]The Stowers Institute of Medical Research, Kansas City, MO, USA. [7]Department of Pathology and Laboratory Medicine, The University of Kansas Medical Center, Kansas City, KS, USA. [8]Sandra and Edward Meyer Cancer Center, Weill Cornell Medicine, New York, NY, USA. [9]Department of Urology, Weill Cornell Medicine, New York, NY, USA. [10]These authors contributed equally: Maria Guillamot, Dahmane Ouazia. [11]These authors jointly supervised this work: Luca Busino and Iannis Aifantis. *e-mail: maria.guillamot-ruano@nyumc.org; businol@upenn.edu; iannis.aifantis@nyumc.org



Supplementary Figure 1

Spop is highly expressed in LT-HSC.

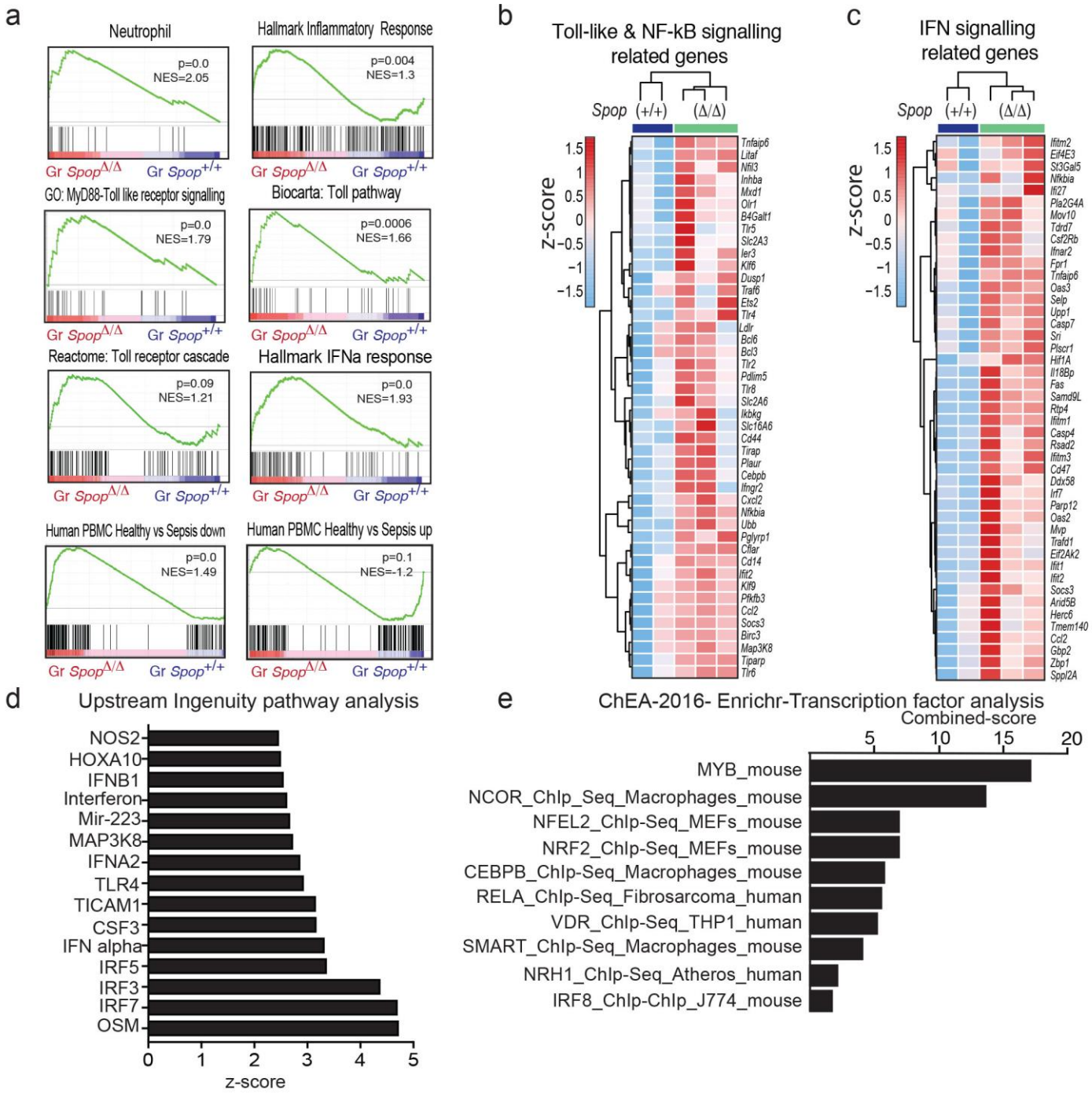
a, Top 15 highest expressed ubiquitin ligase genes in LT-HSC. Heatmap represents the normalized counts relative to 10M reads per gene in the indicated populations. The data was collected from Lara-Astiaso., 2016 [29] **b,** *Spop* expression profile on different hematopoietic cells from the same dataset. **c,** Immunoblot showing Spop protein levels in the indicated sorted cells. Data are representative from two independent experiments **d,** Schematic diagram of *Spop* conditional allele. **e,** Immunoblot analysis of Spop protein levels of total bone marrow cells from wild-type ($Spop^{+/+}$MxCre) and KO ($Spop^{\Delta/\Delta}$MxCre) mice following a single Poly(I:C) injection. Data are representative of three independent experiments. **f,** Percentage of mouse weight-change on d15 after Poly (I:C) injection ($Spop^{+/+}$MxCre: n=9; $Spop^{\Delta/\Delta}$MxCre: n=10). Data represent minimum, first quartile, mean, third quartile and maximum. Statistical analysis: unpaired t-test, two-tailed). **g,** Kaplan-Meier analysis of survival of $Spop^{+/+}$MxCre and $Spop^{\Delta/\Delta}$MxCre hematopoietic chimeras after donor hematopoietic reconstitution and one injection of Poly(I:C) (n=5 wild-type, n=6 *Spop* KO. Statistical analysis: Mantel-Cox test). **h,** Hematoxylin & Eosin stained sections of spleen, lung and liver of wild-type ($Spop^{+/+}$MxCre) and KO ($Spop^{\Delta/\Delta}$MxCre) hematopoietic chimeric mice on d21 post Poly (I:C) injection. Scale bars indicate 100 um (Spleen and liver), 1000 um (lung) and 10 um magnifications. Data are representative from 3 independent experiments(pI:C: Poly (I:C). LT-HSC: $Lin^-c\text{-}Kit^+Sca\text{-}1^+Flk2^-CD34^-$. ST-HSC: $Lin^-c\text{-}Kit^+Sca\text{-}1^+Flk2^-CD34^+$. MPP: $Lin^-c\text{-}Kit^+Sca\text{-}1^+Flk2^+CD34^+$. CMP: Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^+$ $FcgRII^{low}CD34^+$. GMP: $Lin^-c\text{-}Kit^+Sca\text{-}1^+FcgRII^{high}CD34^+$. MEP: $Lin^-c\text{-}Kit^+Sca\text{-}1^+FcgRII^-CD34^-$. CLPs: $Lin^-Flk2^+Il7R^+$).



Supplementary Figure 2

Gating strategy.

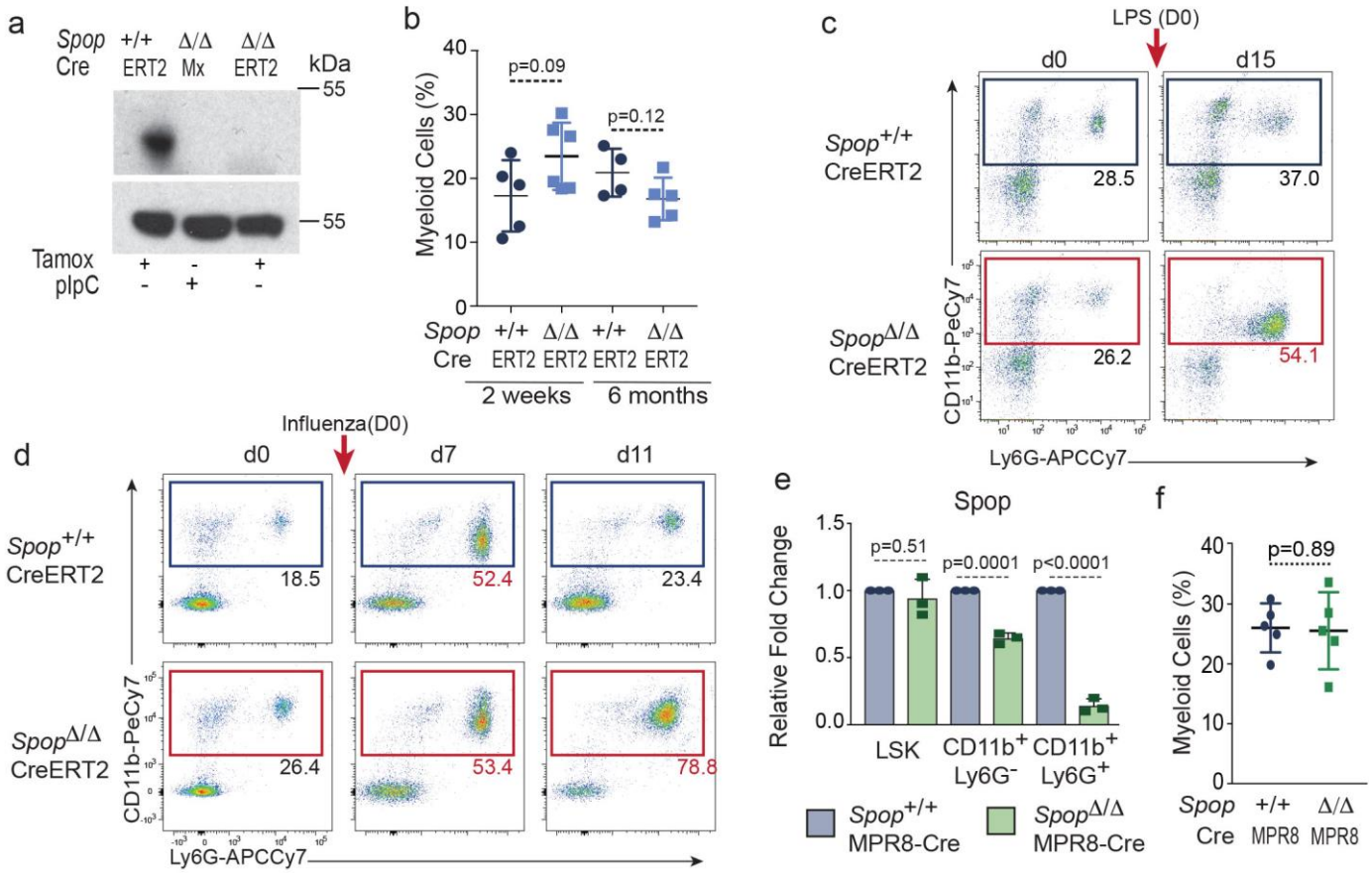
Representative image of the Flow Cytometry gating strategy to identify B-, T- and myeloid cells in peripheral blood and bone marrow of the mice and HSC and progenitors in the bone marrow of the mice.



Supplementary Figure 3

Spop$^{\Delta}$*MxCre* neutrophils display upregulation of inflammatory response gene programs.

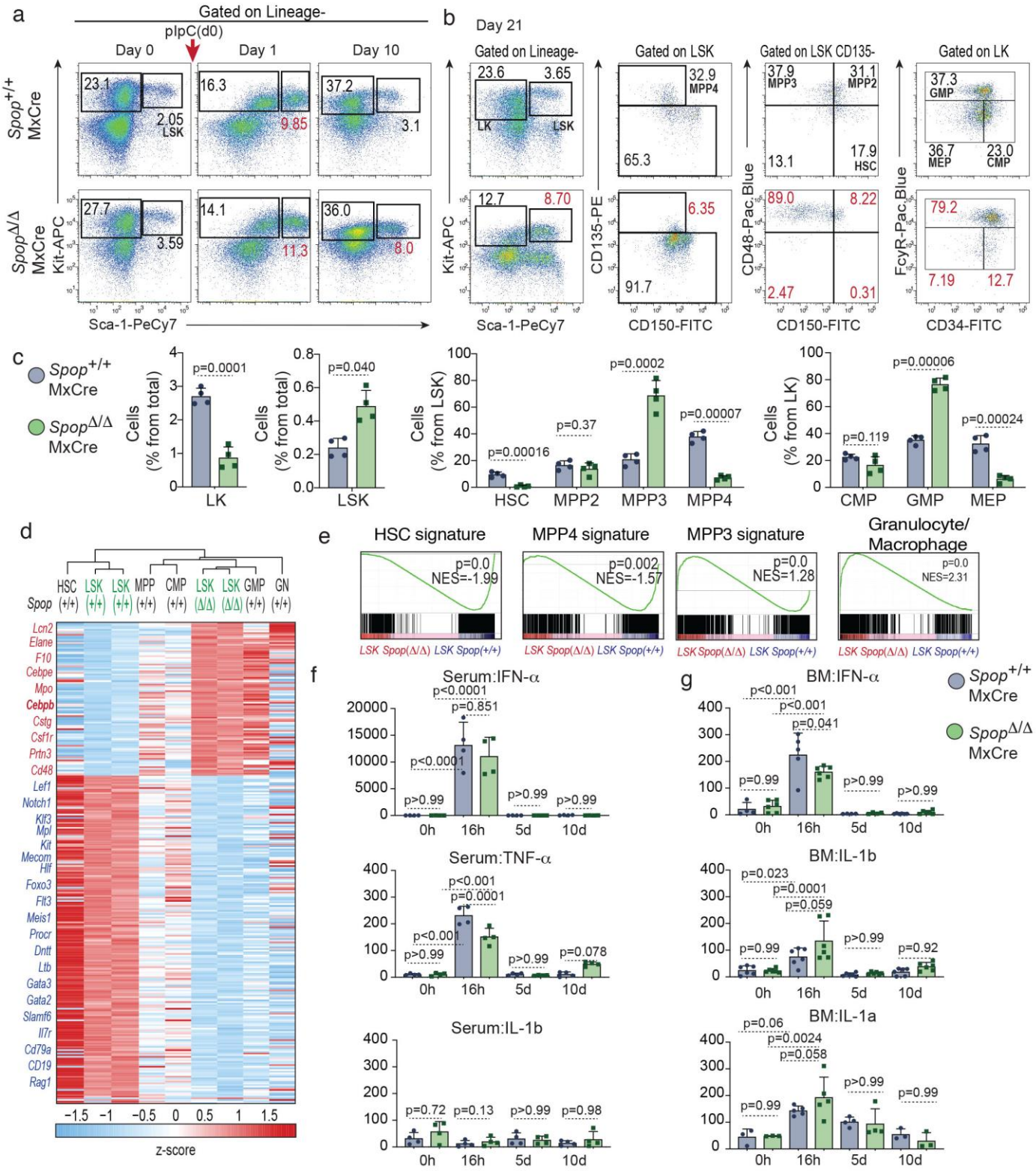
a, GSEA enrichment plots of differentially expressed genes in *Spop* KO *Spop*$^{\Delta/\Delta}$MxCre neutrophils (n=3 mice) compare to control *Spop*$^{+/+}$MxCre (n=2 mice). Statistical significance determined by GSEA Nominal p-value **b,** Heatmap showing the relative expression of selected Toll-like and NF-kB signaling genes identified to be upregulated in *Spop* KO compare to controls. **c,** Heatmap showing the relative expression of selected interferon response genes identified to be upregulated in *Spop* KO compare to controls. **d,** Ingenuity upstream analysis of differentially expressed genes in *Spop*$^{\Delta}$*MxCre* neutrophils compare to control. **e,** ENRICHR transcription factor analysis of the identified differential upregulated genes in *Spop* KO neutrophils compare to control. (NES: normalized enrichment score).



Supplementary Figure 4

Granulocytic specific Spop deletion does not promote neutrophilia.

a, Immunoblot analysis of Spop protein levels of bone marrow cKit+ cells of wild type, *Spop*$^{\Delta/\Delta}$MxCre and *Spop*$^{\Delta/\Delta}$CreERT2 mice after Poly(I:C) or Tamoxifen treatment. **b,** Percentage of myeloid (CD11b^{+}Ly6G^{+}) cells in the peripheral blood of (CD11b^{+}Ly6G^{+}) cells in the peripheral blood of Spop KO and control hematopoietic chimeras on the indicated times after Tamoxifen treatment. Data represent mean±s.d. and dots represent different mice. Statistical analysis: unpaired t-test (two-tailed) **c,** Representative flow cytometry analysis plots of the proportion of myeloid (CD11b^{+}Ly6G^{+}) cells in the peripheral blood of Spop KO and control hematopoietic chimeras on d15 after a sub-lethal LPS injection. **d,** Representative flow cytometry analysis plots of the proportion of myeloid (CD11b^{+}Ly6G^{+}) cells in the peripheral blood of Spop KO (and control hematopoietic chimeric mice on the indicated days after intranasal influenza inoculation. **e,** Spop mRNA relative expression levels in the indicated sorted bone marrow cells. Data represent mean±s.d. (n=3 mice per genotype). The results were first standardized for Gapdh expression levels and then each Spop$^{\Delta/\Delta}$ sample was expressed as a fraction of the expression detected in the correlated control population from the control littermate. **g,** Percentage of myeloid (CD11b^{+}Ly6G^{+}) cells in the peripheral blood of the indicated mice on d10 following pIpC-challenge (n=5 per genotype). Data represent mean±s.d. and dots represent different mice. Statistical analysis: unpaired t-test (two-tailed). **a-f,** Data representative of 3 independent experiments.

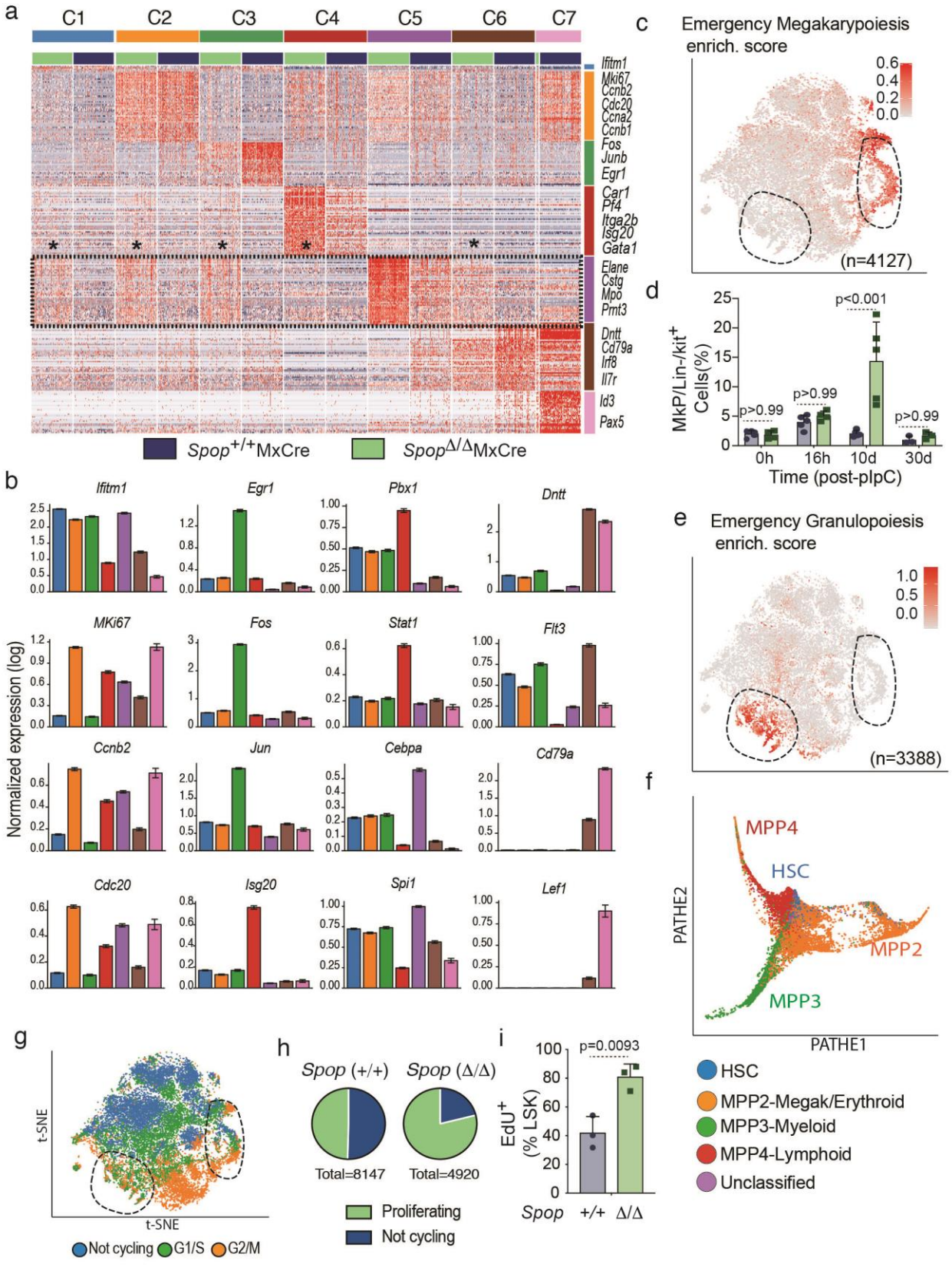


Supplementary Figure 5

Conditional deletion of *Spop* leads to HSPC expansion and myeloid skewing.

a, Representative flow cytometry analysis plot of Lineage negative bone marrow cells at the indicated days after Poly (I:C) injection. **b,**

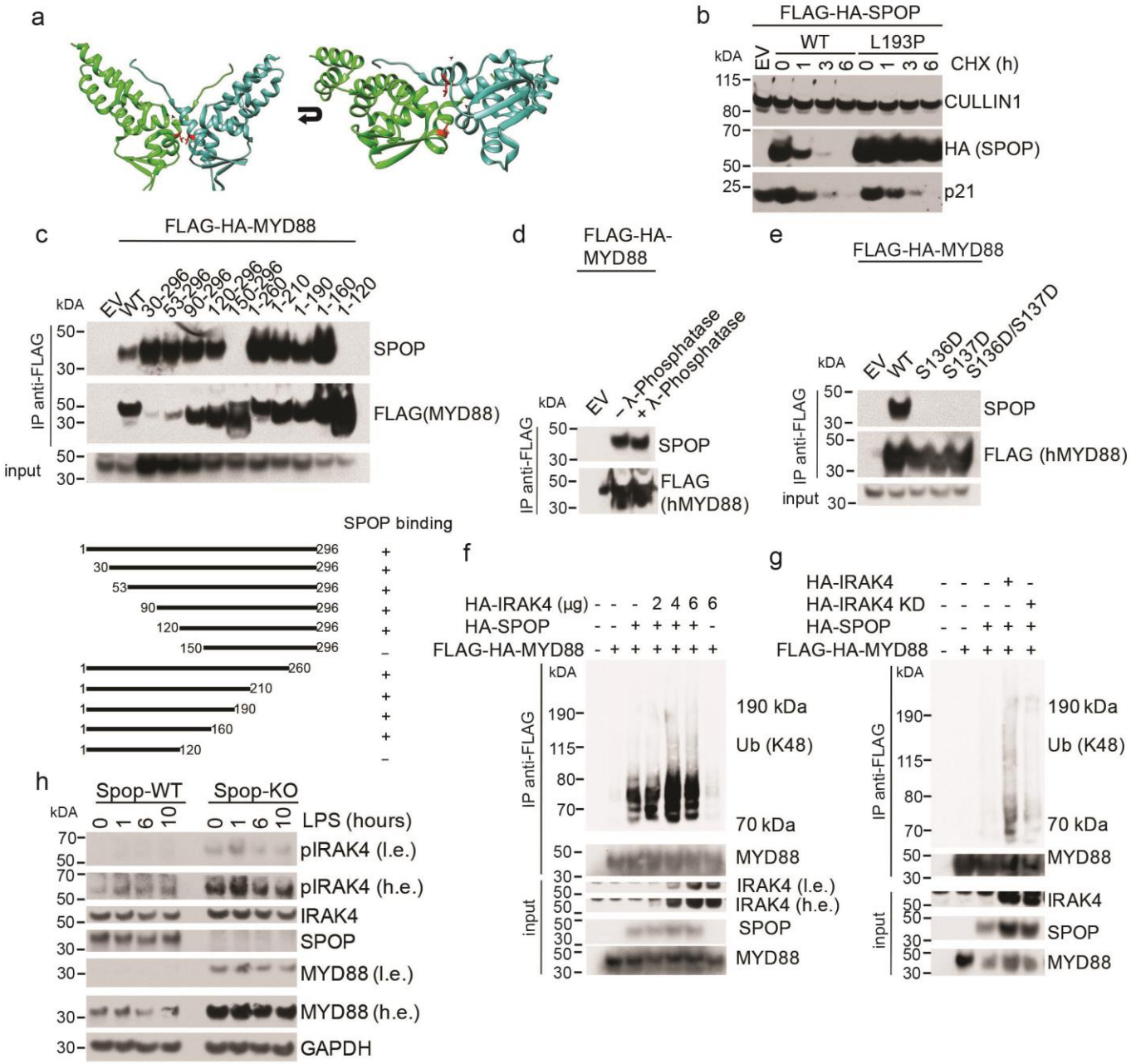
Representative flow cytometry analysis plots of the proportion of HSPC populations, including LSK (Lineage$^-$Sca-1$^+$Kit$^+$), LK (Lineage$^-$ Sca-1$^-$Kit$^+$), HSC (LSK CD135$^-$, CD48$^-$ CD150$^+$), MPP2 (LSK CD135$^-$ CD48$^+$ CD150$^+$), MPP3 (LSK CD135$^-$ CD48$^+$ CD150$^-$), CMP (LK FcyR^{low} CD34$^+$), GMP (LK FcyR^{high} CD34$^+$), MEP (LK FcyR^{low} CD34$^+$). **c,** Percentage of LSK and LK populations in the total bone marrow, percentage of HSC, MPP2, MPP3 and MPP4 in LSKs and percentage of CMP, GMP and MEP in LKs (n=4 mice per genotype. Data represent mean±s.d and dots represent different mice. Statistical analysis: unpaired t-Student, two-tailed). **a-d** Data are representative from 3 independent experiment. **d,** Heatmap showing the relative expression of the selected GSEA-analyzed leading edge genes in *Spop*$^{\Delta/\Delta}$MxCre and control sorted LSKs (n=2 mice per genotype) together with control HSC, MPP, CMP, GMP and GN (Granulocyte) wild-type populations from *Lara-Astiaso et al., 2016*. **e,** GSEA enrichment plots for HSC, MPP3, MPP4 and granulocytic and macrophage precursor signatures[45] in *Spop*$^{\Delta/\Delta}$MxCre and control sorted LSKs (n=2 per genotype). Statistical analysis: Normalized Enrichment Score (NES) and GSEA Nominal p-value. **f,** Cytokine levels in the serum of the indicated hematopoietic chimeras following one pI:C injection (n=4 per genotype and condition). **g,** Cytokine levels in the bone marrow fluids of the indicated hematopoietic chimeras following one pI:C injection. **f,g** Data represent mean±s.d and dots represent different mice. Data are representative of two independent experiments.(h=hours, d=days)



Supplementary Figure 6

Defined gene signature per HSPC clusters.

a,Heatmap showing the expression levels of the 50 most significant markers per cluster, displaying 100 randomly-selected cells of wild-type (*Spop*$^{+/+}$MxCre) and Spop KO (*Spop*$^{\Delta/\Delta}$MxCre) LSK. * indicate the upregulation of the C5 specific signature in the cells of other clusters. **b,** Normalized expression levels of selected population-specific-markers across clusters (C1=4714, C2=2461, C3=1649, C4=1689, C5=1328, C6=1030, C7=196 cells) Data represent mean±s.d. **c,** Spectral tSNE plot of *Spop* wild-type (8184) and KO (4920) LSK cells showing the Enrichment Score for Emergency Megakaryocyte gene signature. **d,** Percentage of Megakarycyte progenitors (Lineage$^-$, Sca-1$^-$, Kit$^-$, CD150$^+$, CD41$^+$) from total progenitor (Lineage$^-$cKit$^+$Sca-1$^-$) cells from the indicated mice (n=4 per genotype and time-point). **e,** Spectral tSNE plot of *Spop* wild-type (8184) and KO (4920) LSK cells showing the Enrichment Score for Emergency Granulopoietic gene signature. **f,** Cell differentiation trajectory using PHATE visualization of wild-type and Spop KO cells, color code for HSC (n=1099), MPP2 (Megakrycyte/Erythroid biased, n=5111), MPP3 (myeloid biased, n=2414) or MPP4 (lymphoid biased, n=3737) gene signatures. **g,** Spectral tSNE plot of cell cycling color-coded cells. **h,** Frequency of cells expressing cell cycle genes in *Spop*$^{\Delta/\Delta}$MxCre and control LSKs. **i,** Percentage of *Spop*$^{\Delta/\Delta}$MxCre and control cell cycling (EdU$^+$) LSKs on d10 post a Poly (I:C) injection. Data represent mean±s.d. n=3. Statistical analysis: unpaired t-student, two-tailed. **d,i,** Data are representative from 2 independent experiments

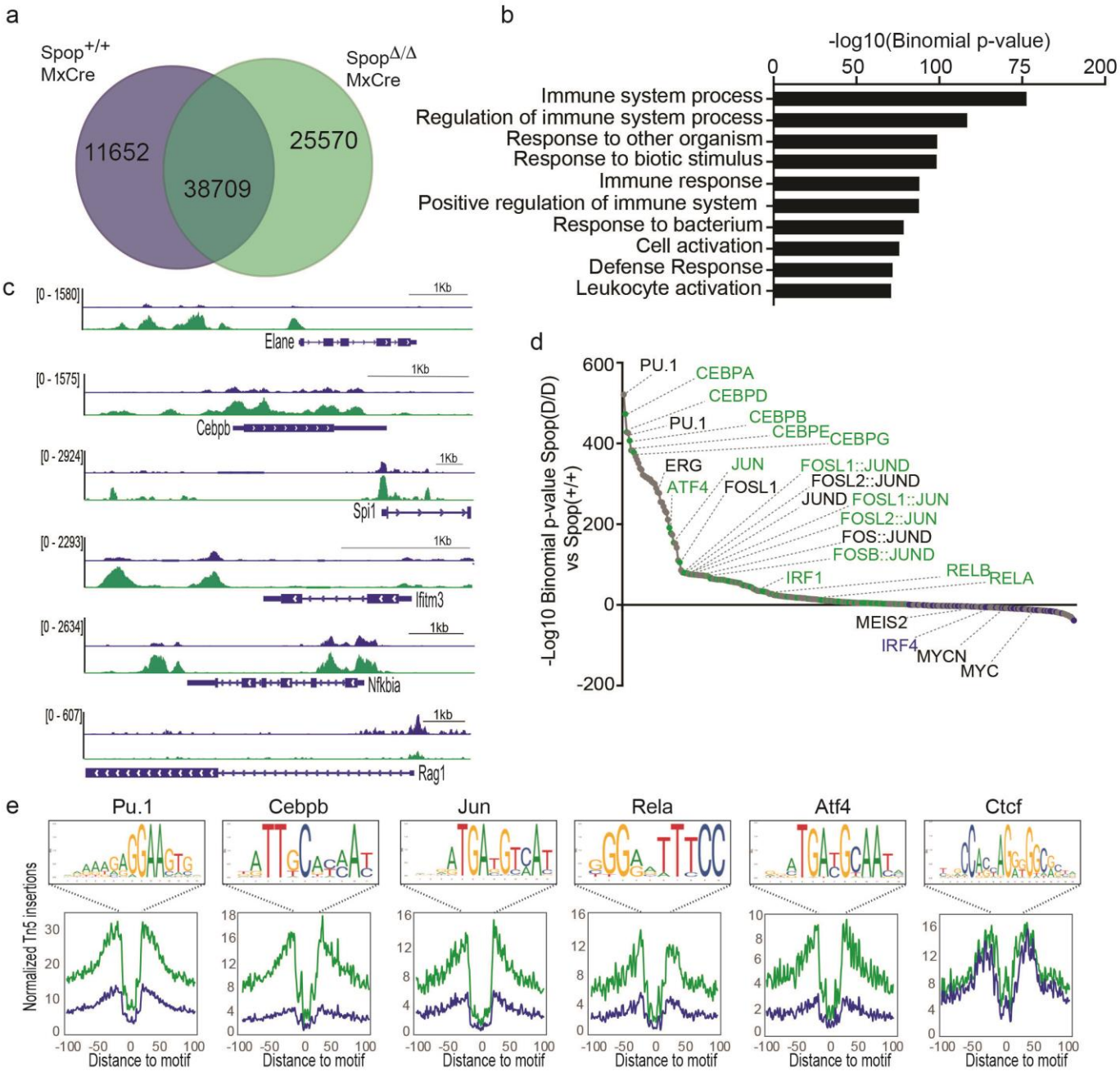


Supplementary Figure 7

SPOP interacts with MYD88 in a phosphorylation independent manner to inhibit IRAK4 signalling.

a, Left, overall view of the SPOP-BTB dimer (pdb access code = 3HTM (Zhuang *et al*., 2009)) with protomers in green and cyan. Right, overall view of dimer interface rotated 90° in x. The L193 residue on each protomer is shown in red. The structural images were obtained using UCSF chimera software. **b,** Immunoblot analysis of whole cell lysates from K562 cells stably expressing HA-tagged SPOP(WT) and SPOP(L193P). Cells were treated with cycloheximide (CHX) for the indicated times. EV, empty vector. **c,** Top, immunoblot analysis of immunoprecipitated WT and truncated forms of FLAG-tagged MYD88 transiently expressed in HEK 293T cells. EV, empty vector. Bottom, diagram showing MYD88 truncations as well as the differential binding to SPOP. **d,** Immunoblot analysis of immunoprecipitated FLAG-tagged MYD88 and of FLAG-tagged CDC25A, both transiently expressed in HEK293T cells. Equal volumes of the immunopurified protein were both treated with the λ-phosphatase reaction buffer with or without the enzyme. EV, empty vector. **e,** Immunoblot analysis of immunoprecipitated FLAG-tagged MYD88(WT) and phosphomimetic mutants (S136D, S137D, S136D/S137D) transiently expressed in HEK 293T cells. EV, empty vector. **f,** *In vivo* ubiquitylation of immunoprecipitated FLAG-tagged MYD88 upon co-expresison with HA-tagged SPOP and increasing amounts of HA-tagged IRAK4 in HEK293T cells. A low (l.e.) and high exposure

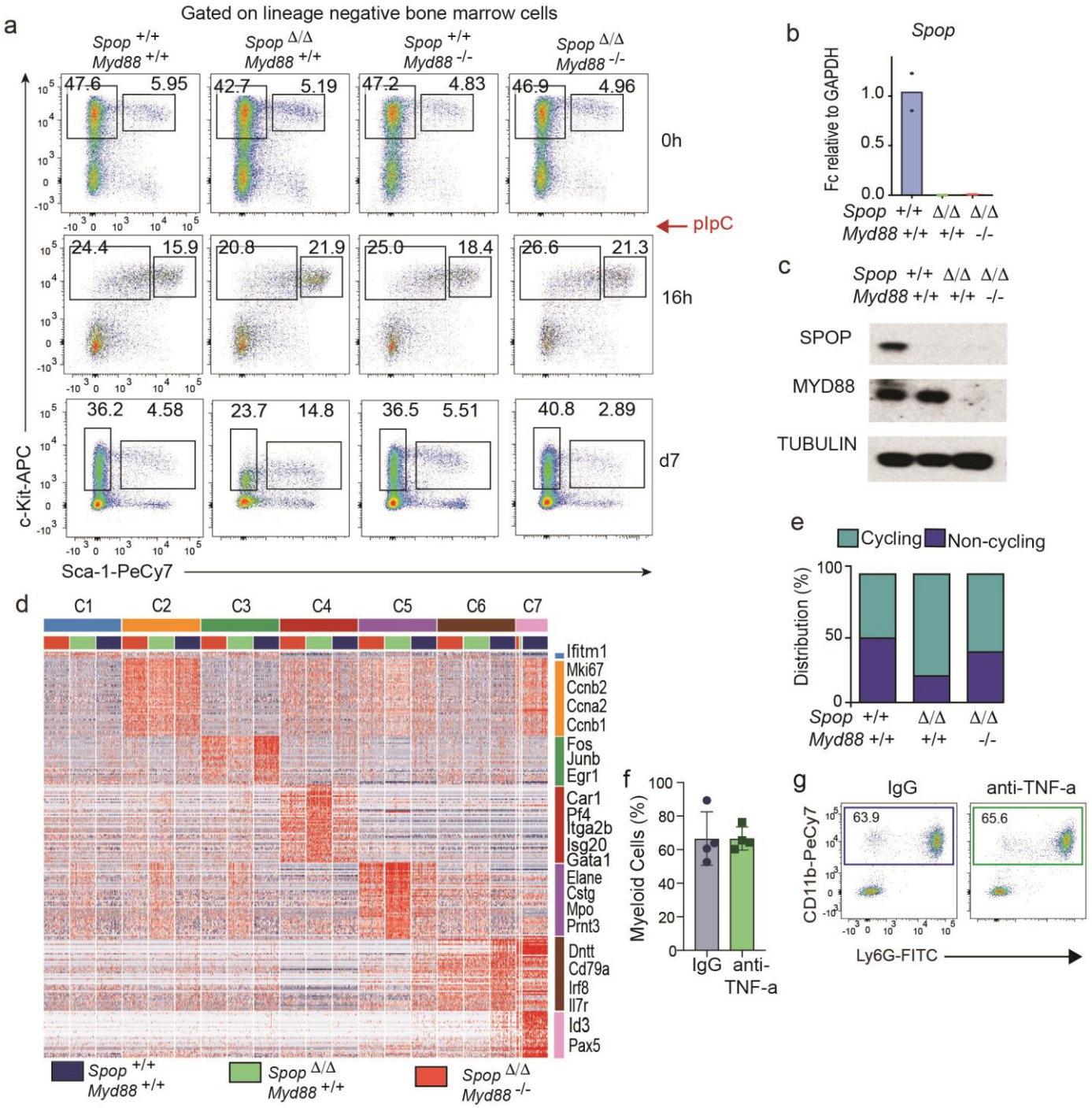
(h.e.) are shown. Protein purification was performed in denaturing conditions. **g,** Immunoblot analysis of immunoprecipitated FLAG-tagged MYD88 co-expressed with HA-tagged SPOP and either HA-tagged IRAK4(WT) or kinase dead (KD) mutant in HEK293T cells. Protein purification was performed in denaturing conditions. **h,** Immunoblot analysis of whole cell lysates of HPC-7 cells *Spop*-WT and *Spop*-KO. Cells were treated with 10 µg/ml lipopolysaccharides (LPS) for the indicated times. A low (l.e.) and high exposure (h.e.) are shown. **a-h** Data are representative from 3 independent experiments



Supplementary Figure 8

Spop loss of function reshapes the open chromatin landscape of the HSPCs.

a, Venn-diagram showing the ATAC-seq signals common and unique for wild-type (*Spop*$^{+/+}$MxCre n=2 mice) and *Spop*$^{\Delta/\Delta}$MxCre (n=2 mice) LSKs (Lineage$^-$cKit$^+$Sca-1$^+$ bone marrow cells), sorted on d10 following a Poly (I:C) injection (FDR<0.05). **b,** GREAT Gene Ontology Biological Function analysis of the 25570 *Spop*$^{\Delta}$*MxCre* differential open chromatin elements. Statistical analysis: GREAT enrichment binomial test from GREAT **c,** Genome Browser plots showing the normalized ATAC-seq profiles at the promoter and distal elements of the indicated genes for *wild-type* (blue) and *Spop*$^{\Delta}$MxCre (green) samples. Data are representative from two mice per genotype. **d,** Ranking of the most enriched transcription factor (TF) motif within *Spop*$^{\Delta}$*MxCre* vs wild-type differential open chromatin elements (cumulative binomial distribution P<10^{-10} .Green=TF motif enriched in the *Spop*$^{(\Delta/\Delta)}$*MxCre* differential open chromatin elements. Blue=TF motif enriched in the *Spop*$^{+/+}$MxCre differential open chromatin elements. Black= TF motif enriched in the *Spop*$^{\Delta/\Delta}$MxCre *Spop*$^{+/+}$MxCre differential open chromatin elements).Statistical analysis: HOMER enrichment binomial test **e,** ATAC-seq Footprint visualization for the indicated TF. Aggregated plot of the Tn5 (transposase) insertions counts per nucleotide.

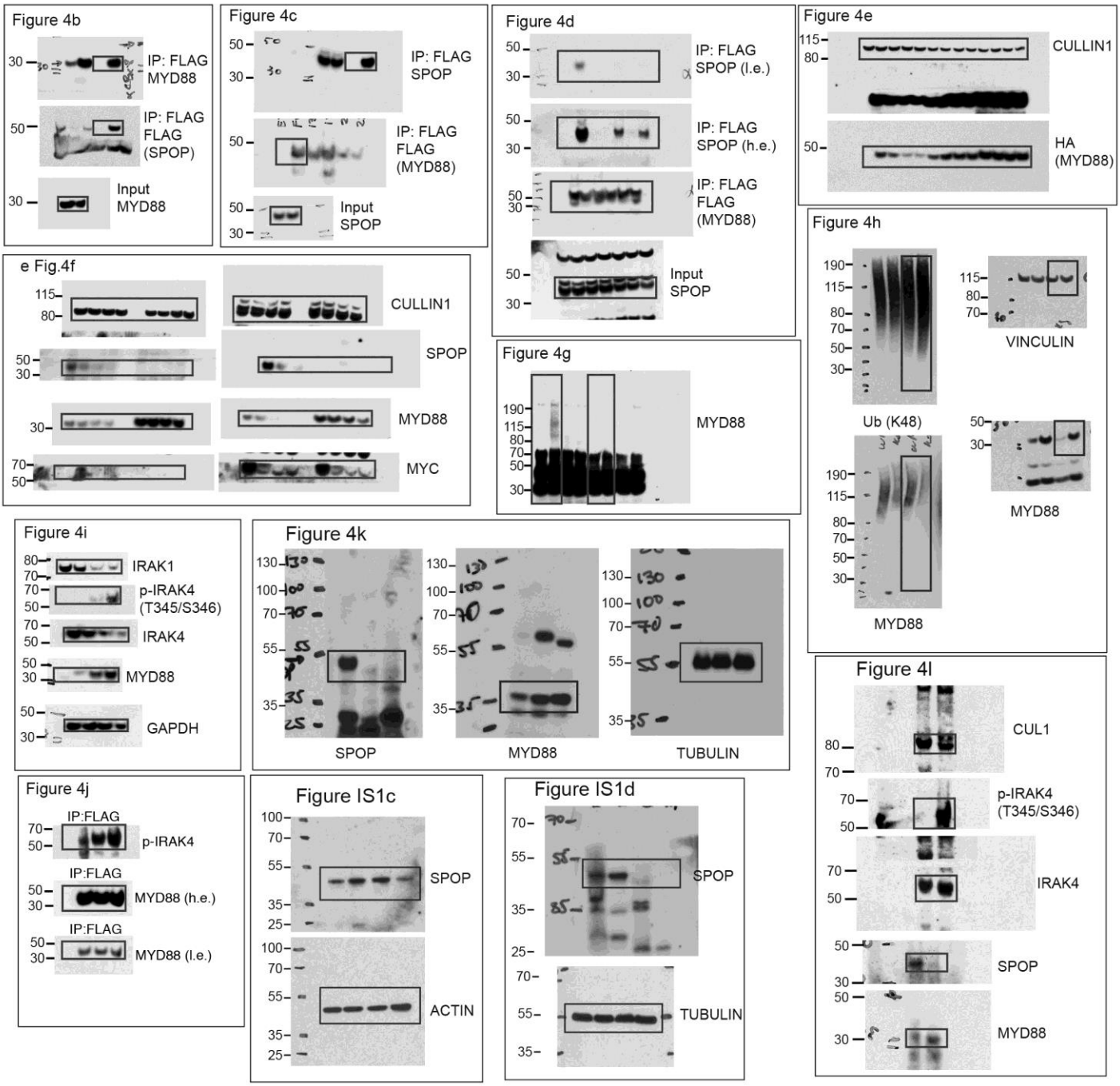


Supplementary Figure 9

Myd88 deficiency restores the steady-state transcriptional program in *Spop* KO LSKs.

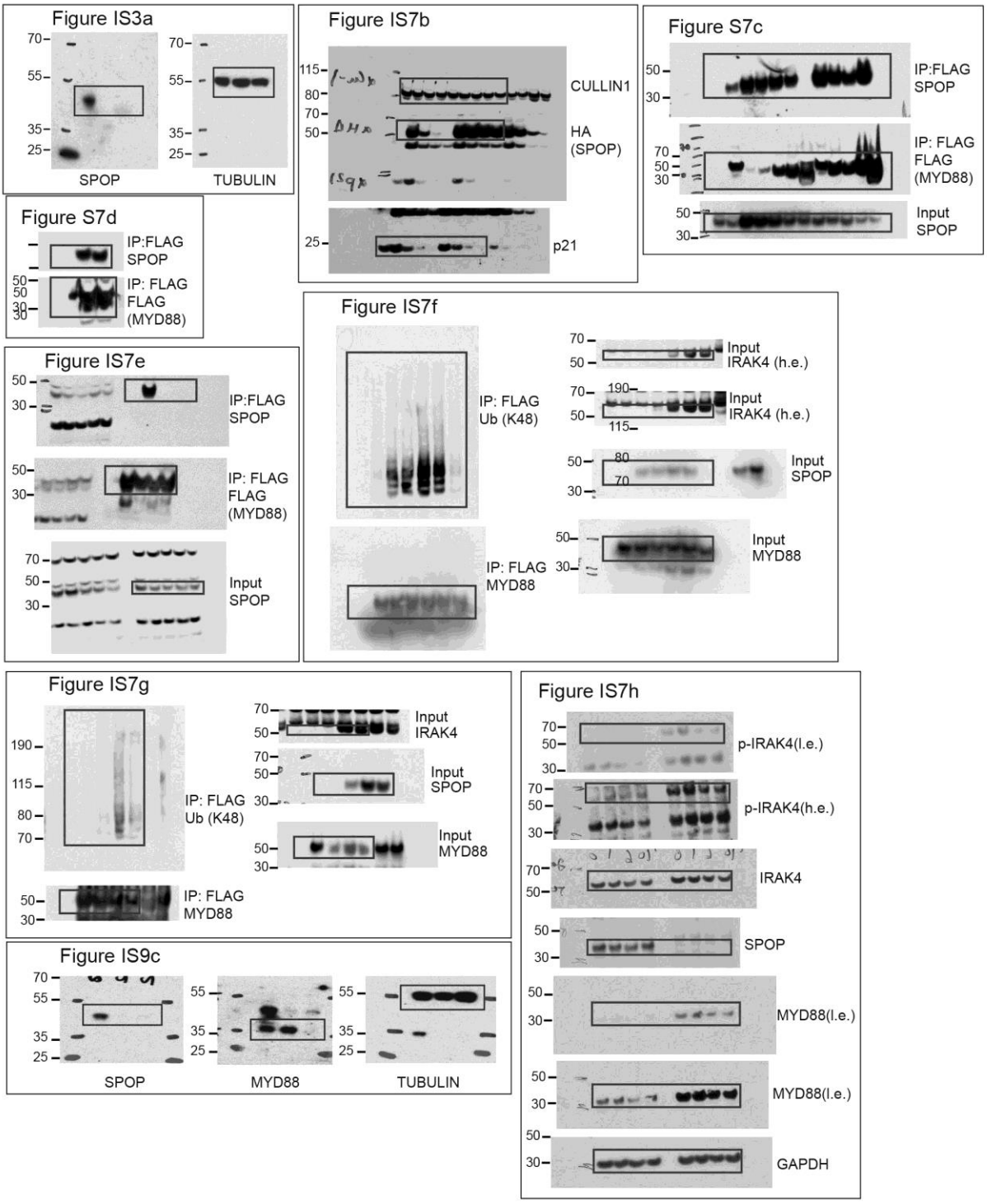
a, Representative flow cytometry analysis plots of the proportion of HSPC in the lineage$^-$ bone marrow from hematopoietic chimeric mice following Poly(I:C) injection including: control (*Spop*$^{+/+}$MxCre *MyD88*$^{+/+}$), Spop KO (*Spop*$^{\Delta/\Delta}$MxCre *MyD88*$^{+/+}$), Myd88 KO (*Spop*$^{+/+}$MxCre *MyD88*$^{-/-}$) and dKO (*Spop*$^{\Delta/\Delta}$*MxCre MyD88*$^{-/-}$. Data are representative from 2 independent experiments. b, *Spop* mRNA expression levels of LSKs on d10 post Poly (I:C) injection (n=2). c, Immunoblot analysis of HSPC cKit$^+$ bone marrow cells of wild-type (*Spop*$^{+/+}$MxCre *MyD88*$^{+/+}$), Spop KO (*Spop*$^{\Delta/\Delta}$MxCre *MyD88*$^{+/+}$) and dKO (*Spop*$^{+/+}$MxCre *MyD88*$^{-/-}$) mice. Data are representative from 3 independent experiments. d, Heatmap showing the expression levels of the 50 most significant markers per cluster, displaying 100 randomly-selected cells. e, Percentage of Cycling cells per genotype. f, Percentage of myeloid Cd11b+Ly6G+ cells in peripheral blood

of *Spop*$^{\Delta/\Delta}$MxCre mice on day 20 following pIpC challenge and antibody treatment. Bar plots represent mean+-s.d, n=4 mice per condition. g, Representative Flow Cytometry Analysis Plots of the proportion of Myeloid (Cd11b+, Ly6G+) cells in the peripheral blood of the pIpC-stimulated mice.



Uncropped gels

Uncropped western blot gels from the indicated figures



Uncropped gels 2

Uncropped western blot gels