Cyba-deficient mice display an increase in hematopoietic stem cells and an overproduction of immunoglobulins

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 doi:10.3324/haematol.2019.233064

Received: July 19, 2019.

Accepted: January 2, 2020.

Pre-published: January 9, 2020.

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Supplementary Materials & methods

Reagents

TRI reagent and PMA (P8139) were from Sigma-Aldrich Spain. Antibodies against STAT5, Vinculin, ERK, phospho-ERK (Tyr 204), p-AKT (Ser 473) and AKT were from Santa Cruz Biotechnology, CA, USA. β-CATENIN antibody was from Abcam (Madrid, Spain); phospho-STAT5 (Tyr 694) antibody was from BD-Biosciences (Madrid, Spain); STAT3 antibody was from Cell Signaling Technology (Danvers, MA, USA); p22phox antibody was from OriGene (Rockville, MD, USA). Antibodies for the detection of murine CD135 (Flt3), CD34, CD16/32, and CD127 (IL-7R±) were from eBioscience, Barcelona, Spain. Mouse IL-3, mouse SCF, mouse TPO, mouse Flt3-Ligand, the Lineage Cell Depletion Kit for mice, FcR Blocking reagent and murine flow cytometry antibodies (CD3e, CD4, CD8a, CD11b, CD19, CD43, CD45R (B220), CD117 (c-Kit), anti-Gr1, anti-Sca-1, anti-Ter-119, anti-IgM) were from Miltenyi Biotec, Madrid, Spain. SuperScript II reverse transcriptase and RNase OUT ribonuclease inhibitor were from Invitrogen, Thermo Fisher Scientific (Madrid, Spain). GoTaqR qPCR master mix was from Promega (Madrid, Spain). Methylcellulose semisolid medium (MethoCult #3234) was from STEMCELL Technologies SARL (Grenoble, France). RNASpin Mini Kit was from GE Healthcare (Barcelona, Spain).

Quantitative RT-PCR

RNA was extracted with RNAspin Mini kit, and cDNA was generated with SuperScript II Reverse Transcriptase. qPCRs were carried out using GoTaq^{R} qPCR Master Mix in a StepOne RealTime PCR System (Applied Biosystems). Analysis of data was performed

by the comparative C_t method ($\Delta\Delta C_t$), using β-actin as endogenous control. Oligonucleotides used for qPCR were as follows:

Gene	Direction	Sequence	Amplicon
b-Actin	Sense	CAGCCTTCCTTCTTGGGTAT	100 pb
b-Actin	Anti-sense	TGGCATAGAGGTCTTTACGG	
Nox1	Sense	CCACTGGCTCTCAGTTTTGT	122 pb
Nox1	Anti-sense	TCCCAAGAATTTCTCTCGTG	
Cybb	Sense	ATGCAGGAAAGGAACAATGC	90 pb
Cybb	Anti-sense	GTGCACAGCAAAGTGATTGG	
Nox3	Sense	GGTCCTGTGGTCTTGTATGC	142 pb
Nox3	Anti-sense	CAGGTGCCATCTTGAAGTCT	
Nox4	Sense	TGGGCCTAGGATTGTGTTTA	127 pb
Nox4	Anti-sense	CTGCTAGGGACCTTCTGTGA	
Duox1	Sense	CATCATGGGGTTCCACTTAG	131 pb
Duox1	Anti-sense	TGTGCTCCATGAGGTTGTTA	
Duox2	Sense	CTCCAAGGACGAGTTCTTCA	106 pb
Duox2	Anti-sense	CTCCCGGAACATAGACTCAA	
Itgam	Sense	CCCATCTTTCCTGCTAATTCTGA	198 pb
Itgam	Anti-sense	ACTCTCATCACTGGTGACAATC	
Ccr1	Sense	CTCTGGAAACACAGACTCACT	164 pb
Ccr1	Anti-sense	AGCAGTCTTTTGGCATGGAG	
Lrg1	Sense	GCATCAAGGAAGCCTCCAG	199 pb
Lrg1	Anti-sense	GGAGAATTCCACCGACAGATG	
Lilrb4	Sense	CTCAGAAACCAAGGACCAGT	217 pb
Lilrb4	Anti-sense	TGGGTTCCAACTGTTCAGC	
Pirb	Sense	TCACAGTCTCAGGACCCATC	114 pb
Pirb	Anti-sense	GGATGAAGGCCACAGACAC	
Stat5a	Sense	AGCACCTTCAGATCAACCAA	290 pb
Stat5a	Anti-sense	GGTCTTCTGGTGCTTCTCAG	
Stat5b	Sense	TACAACAGCATGTCCGTGTC	126 pb
Stat5b	Anti-sense	CCAGTGAGGCTTGAGATGTT	

Production of *Cyba* knockout mice from targeted embryonic stem cells

Cyba^{tm1e} Wtsi Mutant ES Cell Clones (JM8A3.N1) with exon1 knockout targeted mutation were acquired from EUCOMM consortium, and grown according to EUCOMM instructions. ES cells were microinjected into blastocysts from C57BL/6J females, and implanted into pseudopregnant females. The selected founder (F0) was chosen among several chimeras according to its gender (male) and grade of chimerism (80-90% agouti).

F0 was crossed with albino mice B6(Cg)-*Tyr*$^{c-2J}$/J and germ line transmission was corroborated by coat color and genotyping the offspring (F1) as previously reported [1]. To be maintained on a stable background, F1 heterozygous mutants were backcrossed to C57BL/6J strain.

Production of *Cyba* CRISPR/Cas9-edited mice

Two complementary oligos were designed for *Cyba*-sgRNA, targeting exon 1 with the web tool of the Spanish National Biotechnology Centre (CNB)-CSIC (http://bioinfogp.cnb.csic.es/tools/breakingcas/): 5´-caccgAGACGCCAGCGCCTGTTCGT-3'; 5'- aaacACGAACAGGCGCTGGCGTCTc-3'). These oligos were annealed and cloned into a pX458 vector (Addgene plasmid # 48138), which contains the coding sequence of Cas9 nuclease. The *Cyba*-sgRNA sequence and Cas9 nuclease ORF were PCR-amplified from px458-based vector with primers carrying the T7 RNA polymerase promoter at the 5´ ends. After column purification (Roche) the resulting PCRs were used as a template for T7 RNA polymerase transcription *in vitro* (MEGAshortscript T7 Transcription Kit, Thermo Fisher). 5´ capping (mMESSAGE mMACHINE T7 Transcription Kit, Thermo Fisher), and 3´ poly(A) tailing (Poly(A) Tailing Kit, Thermo Fisher) were added for Cas9 mRNA. Transcription products were purified with RNeasy Mini Kit (Qiagen) and eluted in nuclease-free EmbryoMax microinjection buffer (Millipore). One-cell-staged embryos from superovulated C57BL/6J females were harvested and microinjected with 20 ng/µl of *Cyba*-sgRNA and 20 ng/µl of Cas9 mRNA into the cytoplasm and pronucleus. Microinjected embryos were implanted in pseudopregnant females. The selected founder was chosen among several knockout animals due to its frameshift mutation arising from an indel (+111 bp insertion/ 5 bp deletion), easily detected by PCR (Supplementary Figure

2). The selected founder was crossed with wild-type C57BL/6J to eliminate possible unwanted off-targets and to generate pure homozygotes.

Haematopoietic lineages analysis

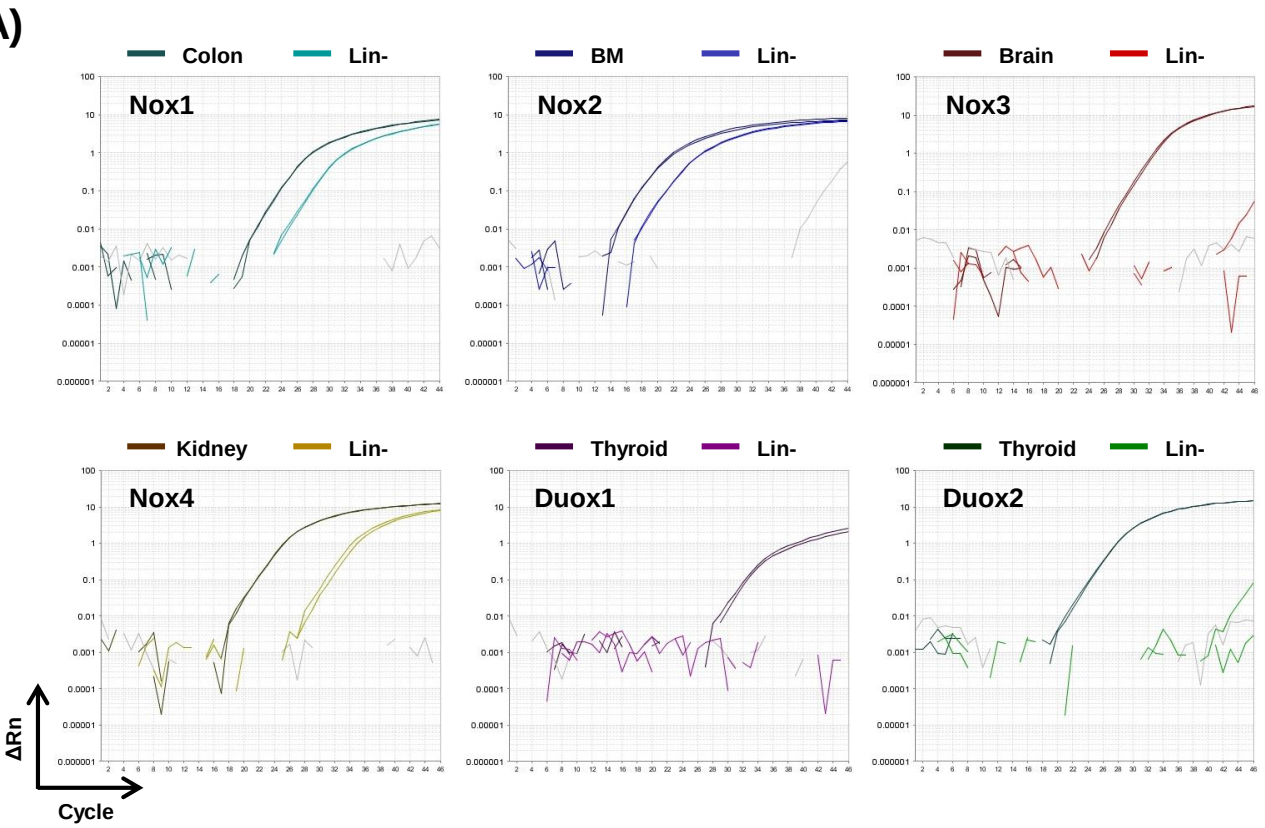
The lineage-specific markers used were as follows: anti-Ter119 for erythrocytes; anti-Gr1 and CD11b for granulocytes and macrophages; CD19 and B220 for B-cells, and CD3 for T-cells. B-cell maturation was analysed in BM: ProB ($B220^+$ $CD43^+$ IgM^-), PreB ($B220^+$ $CD43^-$ IgM^-) and Immature B-cells ($B220^+$ $CD43^-$ IgM^+). BM Lin^- cell subpopulations were identified as follows: LSKs (Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$), LT-HSCs (Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ $CD34^-$ $Flt3^-$), ST-HSCs (Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ $CD34^+$ $Flt3^-$), LMPPs (Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ $CD34^+$ $Flt3^+$), MEPs (Lin^- $Sca\text{-}1^-$ $c\text{-}kit^+$ $CD34^-$ $CD16/32^-$), CMPs (Lin^- $Sca\text{-}1^-$ $c\text{-}kit^+$ $CD34^+$ $CD16/32^-$), GMPs (Lin^- $Sca\text{-}1^-$ $c\text{-}kit^+$ $CD34^+$ $CD16/32^+$), CLPs (Lin^- $Sca\text{-}1^{low}$ $c\text{-}kit^{low}$ IL-7Rα$^-$).

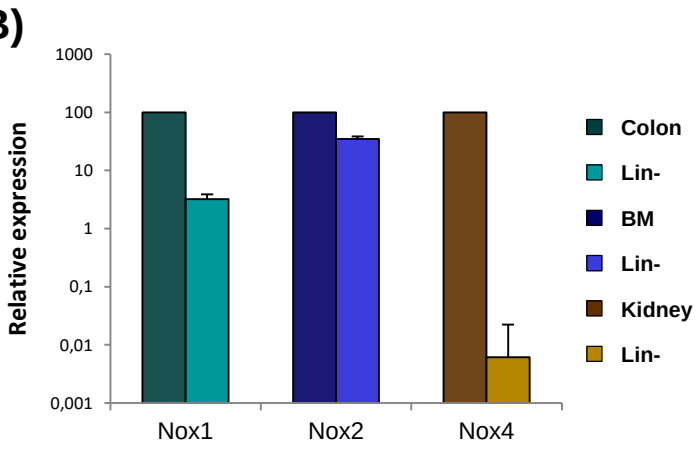
Serum immunoglobulin levels

The levels of IgA, IgG and IgM in serum were measured with commercial ELISA kits (IgA Mouse Uncoated ELISA Kit with Plates, IgG (Total) Mouse Uncoated ELISA Kit with Plates and IgM Mouse Uncoated ELISA Kit with Plates, from Invitrogen, Thermo Fisher Scientific) following the manufacturer instructions.

Supplementary Figures

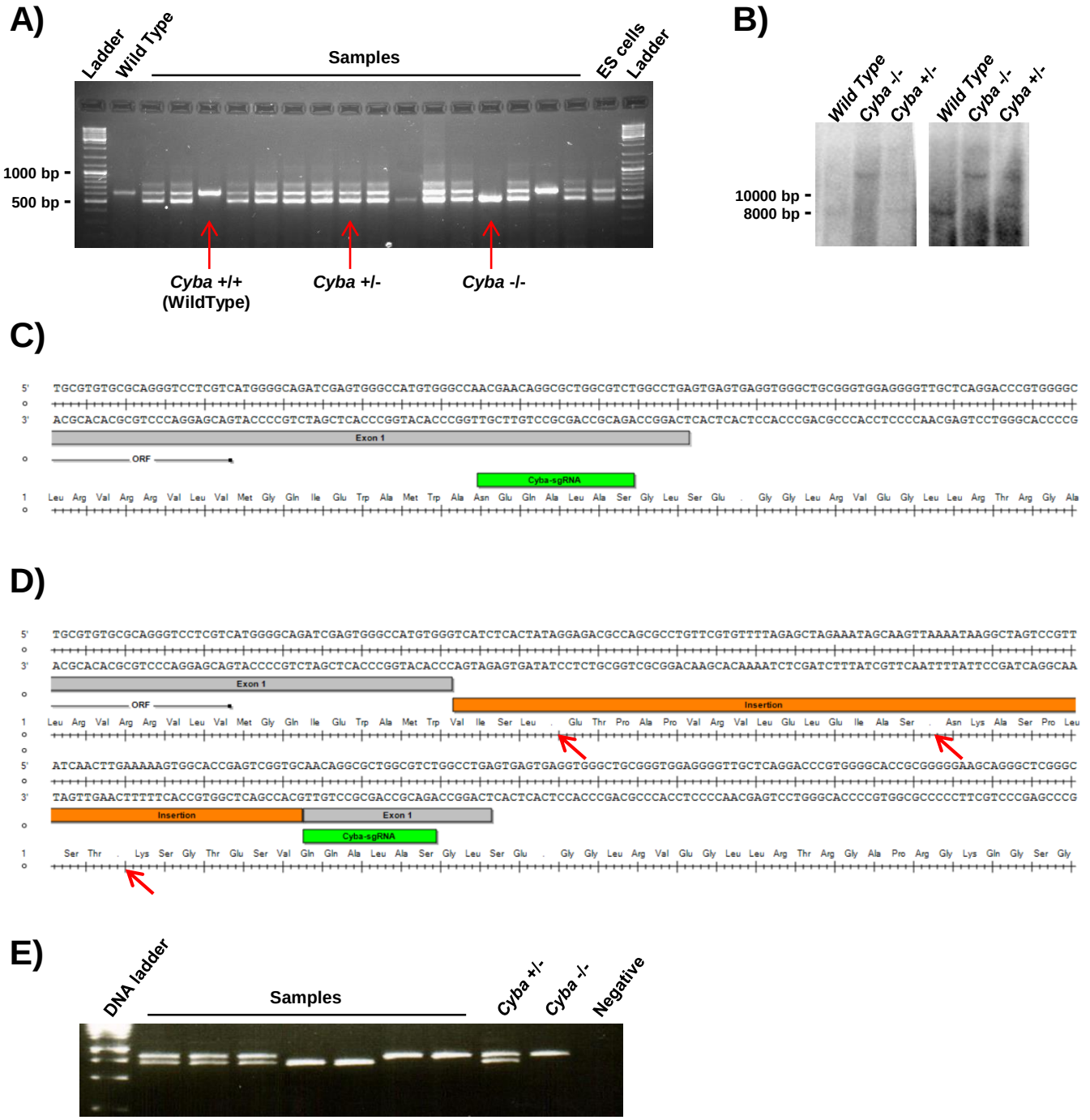
Supplementary Figure 1





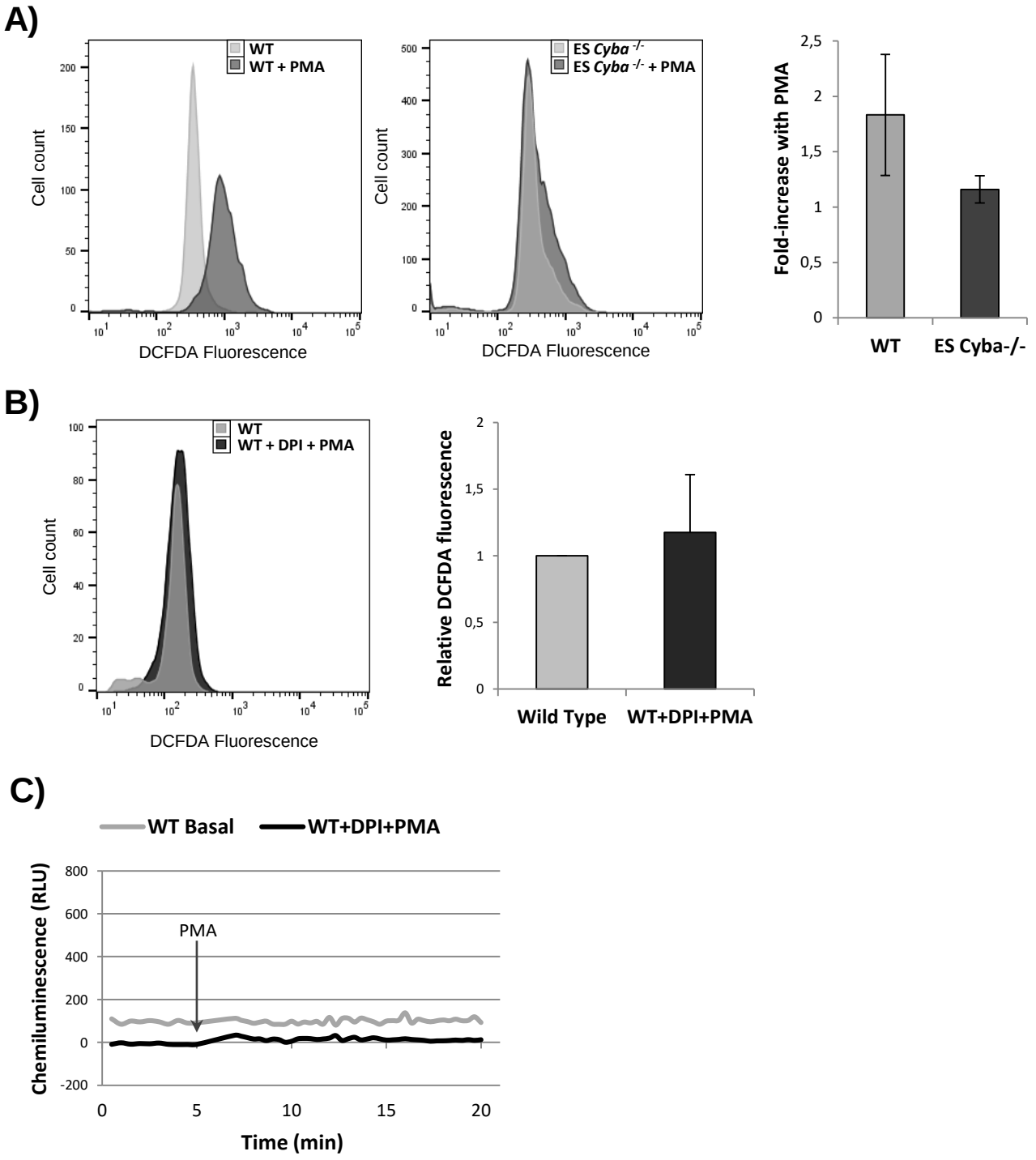
Supplementary Figure 1. Lin$^-$ cells express Nox1, Nox2 and Nox4. The expression of NADPH oxidase family members was analyzed by qRT-PCR (n=2). A) Amplification plots of each NADPH oxidase in mouse Lin$^-$ cells (light color), and in control tissues (dark color) in which they are highly expressed. Grey lines correspond to negative reaction controls. B) Histogram displaying the relative amount of NADPH oxidases expressed in mouse Lin$^-$ cells with respect to their control tissues.

Supplementary Figure 2



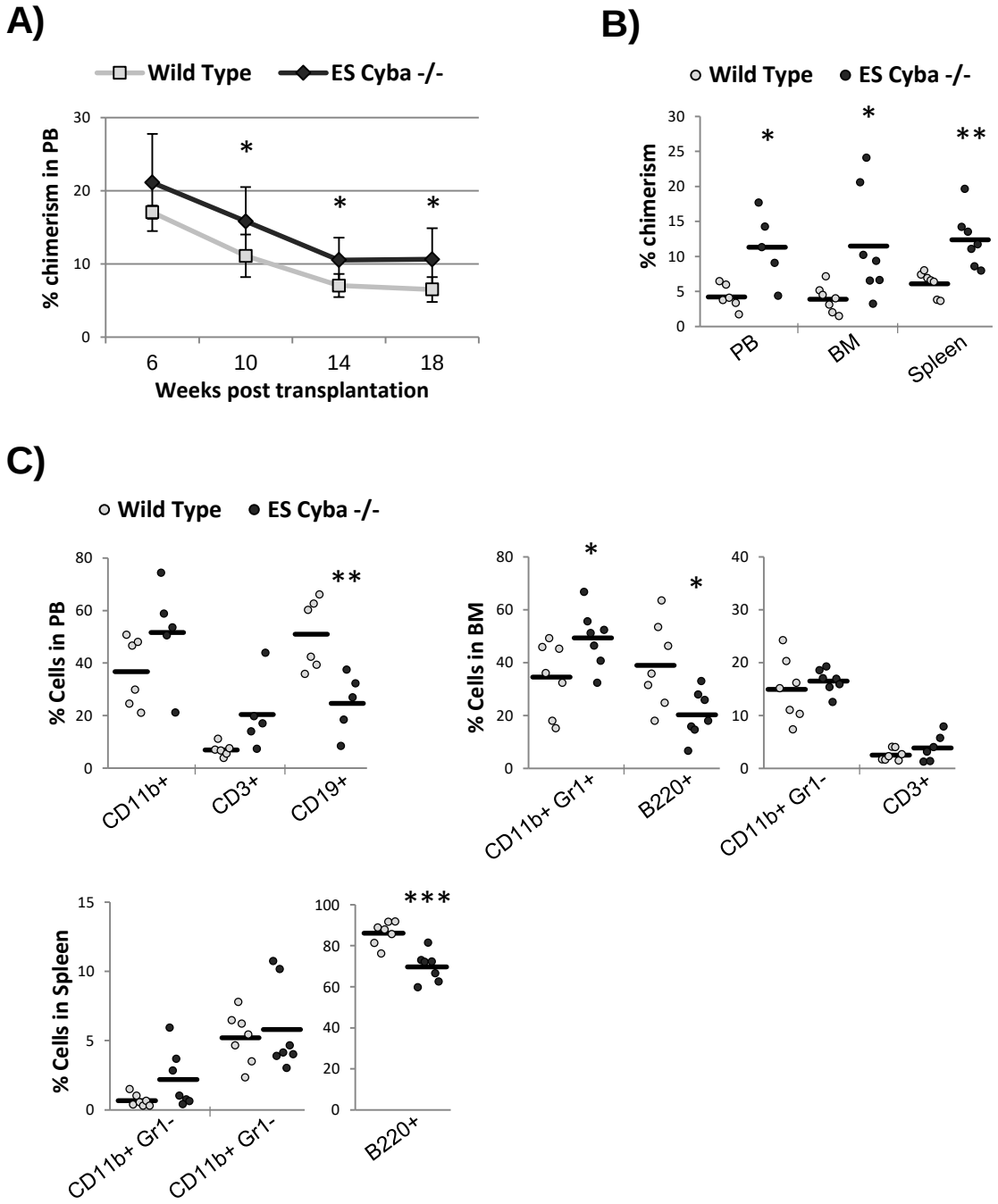
Supplementary Figure 2. Genotyping of *Cyba*$^{-/-}$ mice. A) PCR genotyping of *ES Cyba*$^{-/-}$, *ES Cyba*$^{+/-}$ and wild type mice. **B)** The same as in A) by southern blot analysis. **C**) *Cyba*-sgRNA sequence designed for the generation of the CRISPR/Cas9-edited mice. **D**) Sequencing of the selected mice strain showing the 111 bp insertion within the *Cyba* exon 1. ORF stop codons are indicated by arrows. **E**) PCR genotyping of *CR Cyba*$^{-/-}$, *CR Cyba*$^{+/-}$ and wild type mice.

Supplementary Figure 3



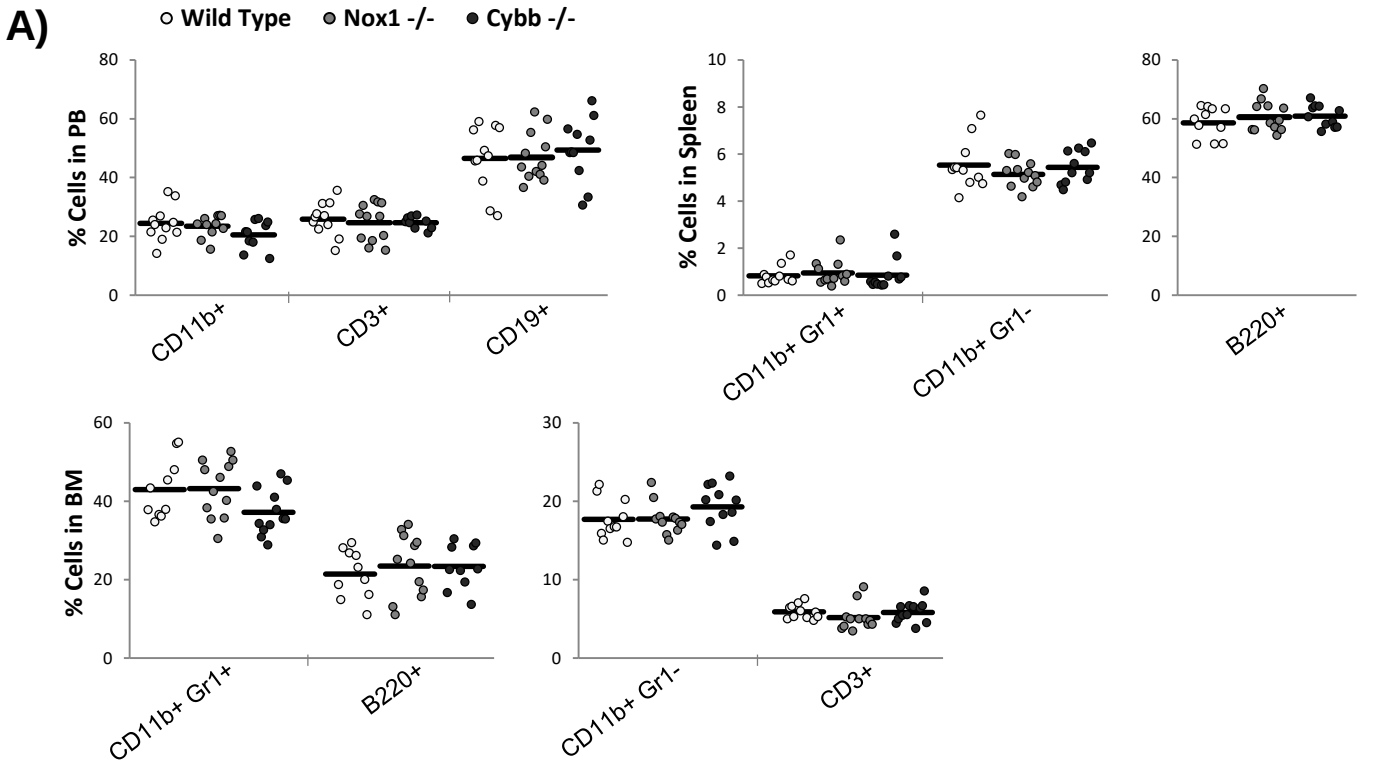
Supplementary Figure 3. The increase in ROS with PMA is NADPH oxidase-dependant. A) Peripheral blood cells from wild type and ES *Cyba*$^{-/-}$ mice were stimulated with 2µM PMA for 30 min. The level of ROS was analysed in the granulocyte population. Flow cytometry experiment and the fold-increase of ROS following incubation with PMA are shown (WT n=2; ES *Cyba*$^{-/-}$ n=3). B) Peripheral blood cells from wild type mice were incubated with 1µM of diphenyleneiodonium (DPI), and then stimulated with 2µM PMA for 30 min. The level of ROS was analysed in the granulocyte population. A representative flow cytometry experiment is shown along with the mean (± SD) fold-increase in ROS (n=4). **C)** 200 000 peripheral blood cells from wild type mice were incubated with 1µM of diphenyleneiodonium (DPI) and with 100 µM luminol, and then stimulated with 2µM PMA. Chemiluminescence was monitored for 15 min at 37°C after addition of PMA (n=1). RLU: Relative luminescence units.

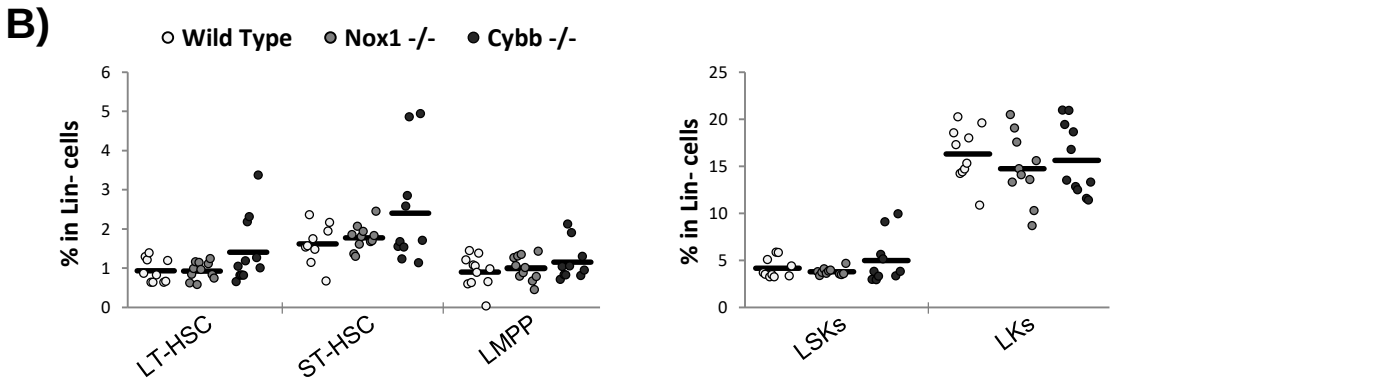
Supplementary Figure 4

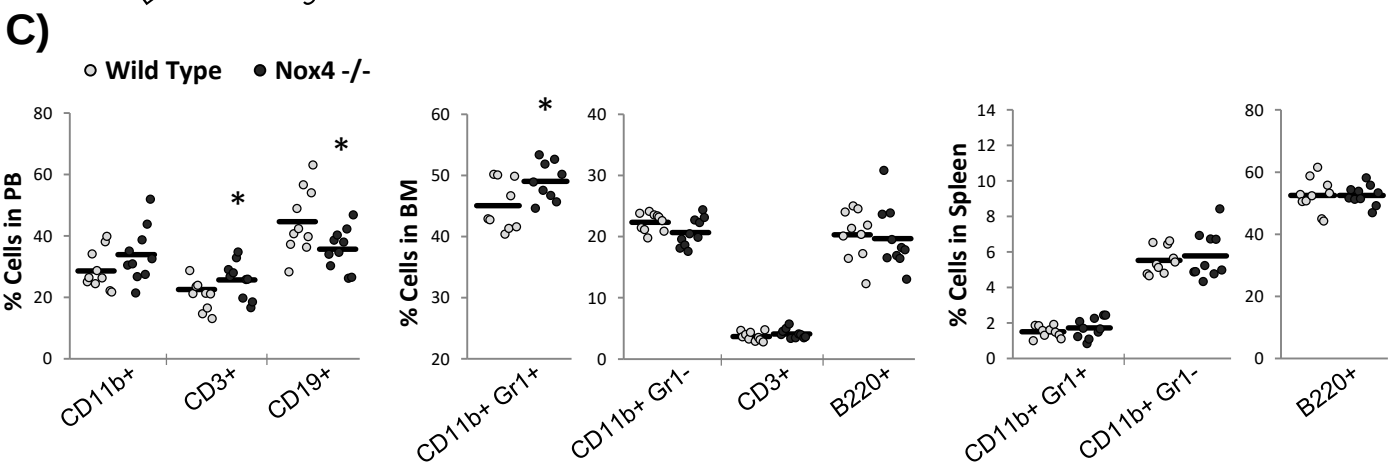


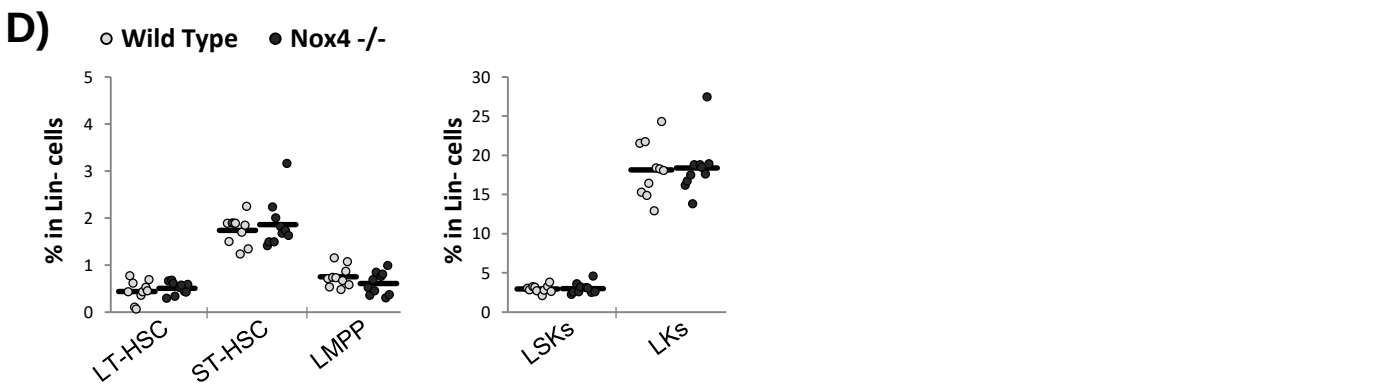
Supplementary Figure 4. *Cyba*$^{-/-}$ cells show a higher haematopoietic reconstitution capacity than wild type control cells in competitive secondary transplant experiments. Secondary transplants were generated from primary recipients of *ES Cyba*$^{-/-}$ and control cells, this time combining their BM cells with wild type competitor cells until a 1:1 ratio of *ES Cyba*$^{-/-}$ and control cells was reached. **A)** Percentage of chimerism in PB in the time following secondary transplant. Graphic show mean ± SD (n=7). **B)** Percentage of chimerism in PB, BM and spleen 20 weeks post-transplant. **C)** Haematopoietic mature lineages in PB, BM and spleen of the transplanted animals.

Supplementary Figure 5



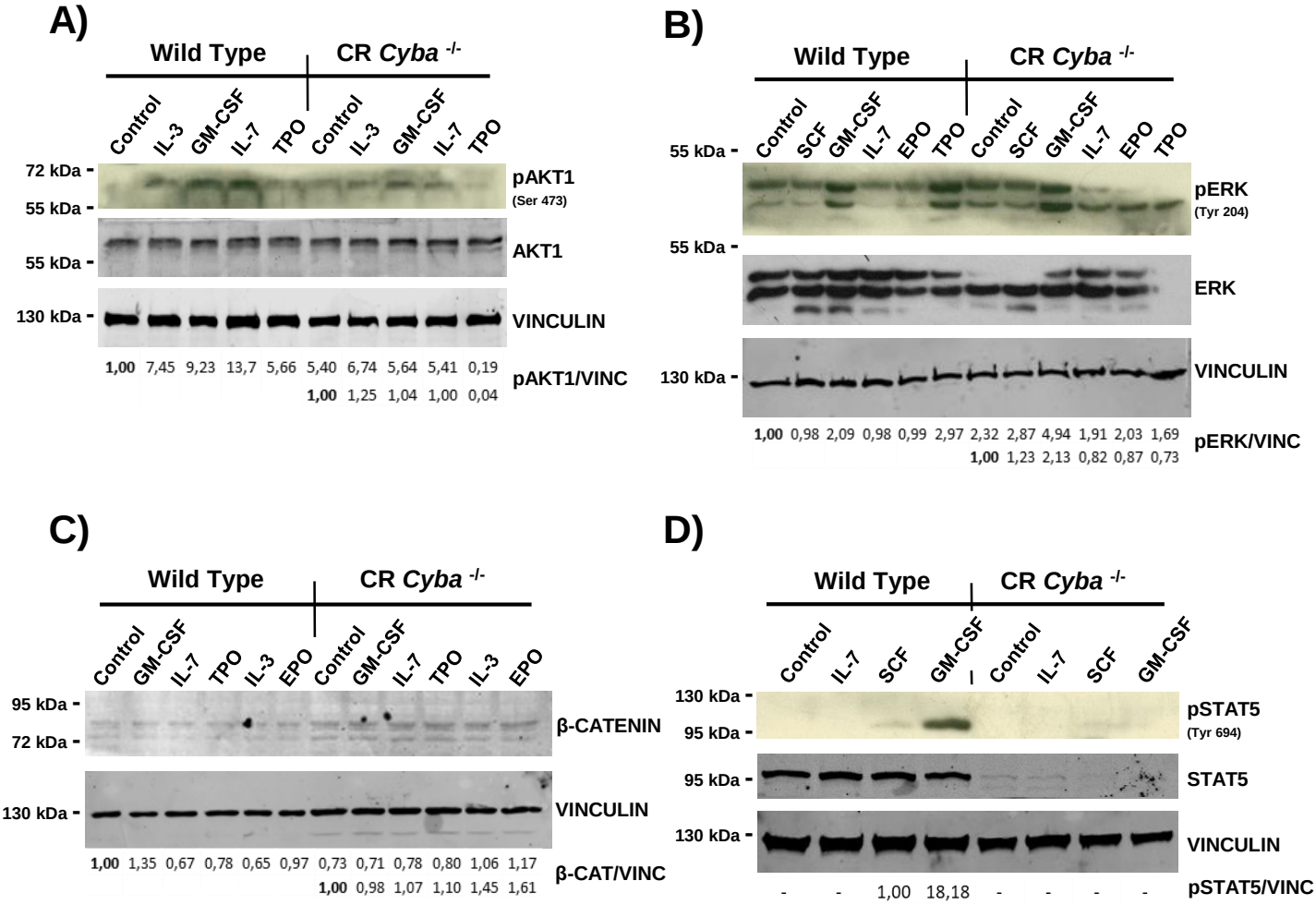






Supplementary Figure 5. Analysis of haematopoietic lineages in the primary transplant experiments performed with NADPH oxidases deficient and control mice. A) Haematopoietic mature lineages in PB, BM and spleen of the transplanted animals coming from *Nox1*$^{-/-}$, *Cybb*$^{-/-}$ and control cells. **B)** HSPCs in the BM of the transplanted animals coming from *Nox1*$^{-/-}$, *Cybb*$^{-/-}$ and control cells. **C)** Haematopoietic mature lineages in PB, BM and spleen of the transplanted animals coming from *Nox4*$^{-/-}$ and control cells. **B)** HSPCs in the BM of the transplanted animals coming from *Nox4*$^{-/-}$ and control cells.

Supplementary Figure 6



Supplementary Figure 6. The deficiency of p22^{phox} hampers the activation of AKT1 and induces downregulation of STAT5 protein. Signaling pathway analysis in BM cells from *CR Cyba*$^{-/-}$ and control mice. BM cells were treated with the indicated cytokines for 30 min and the activation of several signaling pathways was analysed by immunoblotting: **A)** AKT1 (n=4). **B)** ERK1/2 (n=3). **C)** Levels of β-CATENIN (n=3). **D**) STAT5 (n=3).