SUPPLEMENTARY APPENDIX

Ttc7a regulates hematopoietic stem cell functions while controlling the stress-induced response

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Supplementary Methods

Mice. Heterozygous Balb/cByJ *fsn* (CByJ.A-Ttc7fsn/J) mice and Balb/cByJ CD45.1 (CByJ.SJL(B6)-Ptprca/J) mice were obtained from the Jackson Laboratory. Heterozygous $Ttc7a^{fsn/+}$ mice were bred to generate homozygous $Ttc7a^{fsn/fsn}$ mice and their respective control littermates. Genotyping of Ttc7a gene was performed by PCR using the following primers: Ttc7a_F: 5'-CTCTTTCCATGGCTTCCTTG-3', Ttc7awt_R: 5'-GAAGAGCAGCGCTAGCAAGT-3', Ttc7afsn_R: 5'-TAGAAAGGTGCACGGGTGTG-3'.

Repopulations assays. For bone marrow transplantation experiments, $CD45.1^+$ ctrl recipient mice were subjected to a single dose of 8 Gray total body irradiation with a X-ray electric generator RS2000 (RAD SOURCE), and cells were transferred intravenously in the tail vein. After transplantation, peripheral blood was sampled every two weeks to monitor cell engraftment. Blood counts were determined by ScilVet Abc+ analysis. Bone marrow reconstitution with LSK donors was performed by injecting 30 000 cells into irradiated recipient mice. For serial transplantations, primary recipients were reconstituted with 10^7 cells as described above. Bone marrow cells of primary recipients were pulled and 10^7 cells were transferred to secondary recipients. To perform competitive repopulation assays, 1 000 LSK cells were injected concomitantly with 2 x 10^6 unfractionated $CD45.1^+$ bone marrow cells.

For 5-FU (5-fluorouracil) treatment, control recipients were reconstituted with 10^7 control or Ttc7a-deficient bone marrow cells. 12 weeks after transfer, mice were treated with a single dose of 5-FU (150 mg/kg intra peritoneally). Peripheral blood was sampled every 3 days to monitor blood cell counts.

Flow cytometry and isolation of HSCs. Cell suspensions were prepared by mechanical disruption of mouse bone marrow and spleen in PBS. For FACS analysis of spleen and peripheral blood, red blood cells were lysed with RBC lysis buffer (Biolegend, San Diego, CA, US). For erythroid differentiation studies, FACS analyses were performed without RBC lysis. Dead cells were stained with LIVE/DEAD™ Fixable Aqua Stain (ThermoFischer Scientific, Watham, MA, US), 20 minutes at 4°C. Cells were then incubated with conjugated antibodies at the appropriated concentration for 20 minutes at 4°C in darkness. For Annexin V staining, cells were incubated 15 minutes at room temperature with Annexin V (Sony Biotech, San Jose, CA, US)) and 7-AAD (BD

Pharmingen) in Annexin V binding buffer (Biolegend). The antibodies used are listed in Table S2. Stained cells were quantified using a Gallios (Beckman Coulter), and FlowJo software (Treestar) was used to generate flow cytometry plots and histograms. LSKs were isolated by depleting Lin^+ cells using the Lineage Cell Depletion Kit according to the manufacturer's protocol (Miltenyi Biotec). The enriched cells were stained with Lin^- antibody cocktail, and antibodies against CD117 (c-Kit), Sca-1 (Ly6A/E), CD150 (Slamf1) and CD48 (Slamf2), and sorted with FACS Aria™ (BD Biosciences). For BrdU incorporation assay, control and Ttc7a-deficient mice received a single i.p. injection of BrdU. 24 hours later bone marrow cells were harvested and analyzed for BrdU incorporation using the BrdU Flow kit according to manufacturer's instructions (BD Biosciences), associated to viability staining and surface staining to define BM subsets. For cell cycle analysis, Lin^- cells were stained with Ki67 antibody and Hoechst 33342 with Intracellular Fixation and Permeabilization Buffer set according to manufacturer's protocol (Ebiosciences). For aggresome detection, Lin^- cells were stained using the Aggresome detection kit according to the manufacturer's protocol (Abcam, Cambridge, UK).

Cell culture. Bone marrow cells were harvested and lineage depletion was performed using Lineage Cell Depletion Kit according to the manufacturer's protocol (Miltenyi Biotec). 250,000 Lin^- cells were plated at 10^6/mL in StemSpan medium (StemCell Technologies) supplemented with 5% FBS, 1% penicillin/streptomycin, recombinant human TPO (100 µg/mL), recombinant mouse SCF (100 µg/mL) and recombinant mouse FLT3 ligand (100 µg/mL). Tunicamycin (Cayman Chemical) was dissolved in DMSO and added (0,6 or 1,2 µg/mL) for 24 or 48 hours. Cells were counted with Countess™ II Automated Cell Counter (ThermoFisher Scientific).

RNA-sequencing. HSCs ($CD150^+$ $CD48^-$ LSK) were directly sorted into RNA Lysis Buffer, and RNAs were extracted using the ZR RNA MicroPrep™ isolation kit (Zymo Research) according to the manufacturer's protocol. cDNA libraries were generated by using Ovation SoLo RNA-seq system (NuGEN). Control of libraries was performed with the High Sensitivity DNA Analysis Kit using the Bioanalyzer (Agilent). NextSeq 500 (Illumina) was used for sequencing with paired-ended sequencing length of 2x75 bases. FASTQ files were mapped to the ENSEMBL MM38 reference using Hisat2 and counts were produced with feature Counts. Read count normalizations and groups comparisons

were performed by three independent and complementary methods – DESeq2, edgeR, LimmaVoom – and the results were compared and merged. The results were filtered at p value ≤0,05 and folds 1,2. Heatmaps were made with the R package ctc: Cluster and tree Conversion, using normalized expression values of LimmaVoom and imaged by Java Treeview software. Differentially expressed genes were examined with GSEA for functional enrichment in GO terms using normalized expression values of LimmaVoom.

Western blot. Lin- cells were cultured for 3 days and HSCs were sorted by FACS directly into trichloroacetic acid (TCA) and adjusted to a final concentration of 10% TCA. Extraction and solubilization of proteins was performed as previously described [24]. Proteins were separated on a 4-12% Bis-tris gel (Invitrogen) and transferred to a PVDF membrane (Millipore). Bip, HSP70 and Gapdh proteins were revealed by western blot using specific antibodies (Cell Signaling Technology, Millipore, Burlington, MA, US and Abcam, Cambridge, UK).

Supplementary Figure legends:

Figure S1 – Total number of HSPCs in the spleen of *ctrl* (black bars) and *fsn* (red bars) mice at 3, 6 and 12 wees of age (n≥6) (mean ± SEM). *P<0,05; **P<0,01; ***P<0,001 (two-tailed *t*-test).

Figure S2 - HSPC compartment was analysed in the bone marrow (BM) of 3, 6 and 12 weeks old ctrl (black bars) and Ttc7a-deficient (*fsn* - red bars) mice (mean ± SEM) ****P<0,0001 (two-tailed *t*-test). (A) Total number of LSK cells (n≥7). (B) Frequency of MPP, HPC-2 and HPC-1 among LSK cells (n≥6).

Figure S3 – (A) Red blood cell count, hemoglobin and hematocrit of control littermates (ctrl – black bars) and Ttc7a-deficient (*fsn* – red bars) mice at 3, 6 and 12 weeks of age (mean ± SEM) (n≥6). (B) Total number of erythroblasts in bone marrow (left panel) and spleen (right panel) of 12 weeks old *ctrl* and *fsn* mice. (C) Flow cytometry representative of erythroid differentiation (according to CD71 expression and forward scatter) at 12 weeks in *fsn* and *ctrl* mice. Numbers adjacent of outlined areas indicate percent cells in parent gate (mean).*P<0,05; **P<0,01; ***P<0,001; ****P<0,0001 (two-tailed *t*-test).

Figure S4 – (A) BM cells were collected from c*trl* (black dots) and *fsn* (red dots) mice at 3 and 12 weeks of age, 24 hours after a single injection of BrdU. Percentage of BrdU positive cells was evaluated in HSC, HPC-2 and HPC-1 by flow cytometry. (B-C) BM cells were collected from Ctrl^{ctrl} (black dots) and Ctrl^{fsn} (red dots) mice 3 weeks after BM transfer and Ki67/Hoeschst 33342 staining was performed. (B) Percentage of HSCs in each cell cycle phase was evaluated. (C) Flow cytometry representative of Ki67/Hoechst 33342 staining of HSCs.

Figure S5 – (A-B) Lethally irradiated mice were serially transplanted with 12 weeks old *ctrl* (black bars and lines) or Ttc7a-deficient (*fsn* – red bars and lines) donor BM cells. These data are representative of 2 independent experiments with at least 3 mice per each round of transplantation (A) Percent survival of recipient mice across the 7 transplantation cycles. (B) Survival of Ctrl^{ctrl} and Ctrl^{fsn} mice during the 6th round and Ctrl^{fsn} mice during the 7th over time (n=5 for control- and n=11 for Ttc7a-reconstituted mice). (C) Relative

contribution of myeloid compared to T and B lymphoid cells in the spleen of irradiated recipients serially transplanted with 3 weeks old *ctrl* (black dots) or *fsn* (red dots) donor BM cells.

Figure S6 - RNA sequencing was performed on 3 weeks old control (*ctrl*) LT-HSCs transcripts. Expression of Ttc7a mRNA transcript was compared to Sca-1, cKit, CD150 and CD48 mRNA transcripts.

Figure S7 – (A) Proliferation index (calculated as the ratio between the number of cells at 48 hours and 24 hours) of MPP, HPC-2 and HPC-1 after Lin^- cells were sorted from *ctrl* (black bars) and Ttc7a-deficient (*fsn* – red bars) mice and cultured for two days with or without tunicamycin (TM). (B) Representative histograms of protein aggregation level of *ctrl* (black line) and *fsn* (red line) HSCs after 3 days *in vitro* expansion. Representative from 3 independent experiments (n=3). (C) Normalized expression of ER stress receptors and effectors in *ctrl* and Ttc7a-deficient HSCs (LimmaVoom analysis). (D) Annexin V staining of HSCs, HPC-1 and HPC-2 after Lin^- cells were sorted from *ctrl* (black bars) and Ttc7a-deficient (*fsn* – red bars) mice and cultured for two days with or without tunicamycin (TM). *p<0,05 (two-tailed *t*-test). (E) Protein expression of Hsp70 in *ctrl* and *fsn* HSCs after 3 days *in vitro* expansion (representative of two independent experiments).

Table S1 - List of DEGs related to ER stress (p≤0,05; 0,833≥fold change≥1,200)

Sondes	*Gene*	*RatiosMoys_Fsn_vs_WT*	*Delta*	*pval Limmavoom*	*pval edgeR*	*pval DEseq2*
ENSMUSG00000090877	Hspa1b	0.08802294	53.81062	1,54969E-19	1,6719E-15	3,18277E-09
ENSMUSG00000029657	Hsph1	0.40476508	320.17351	2,23955E-27	1,44893E-13	2,15459E-08
ENSMUSG00000026864	Hspa5	0.53204545	890.45856	4,07427E-32	1,71998E-08	4,20833E-08
ENSMUSG00000015656	Hspa8	0.59971221	1105.28708	3,09075E-28	5,30123E-06	1,60832E-07
ENSMUSG00000032575	Manf	0.60433485	82.48086	2,91807E-06	0,000735798	0,002012401
ENSMUSG00000032553	Srprb	0.60691657	8.599529	0,004331196	0,03258362	NS
ENSMUSG00000028410	Dnaja1	0.61410102	353.97319	2,45433E-12	2,57138E-05	5,39726E-05
ENSMUSG00000005374	Tbl2	0.64061516	47.77602	0,001385277	0,01300467	0,02770783
ENSMUSG00000004460	Dnajb11	0.64292208	64.18646	0,00020224	0,004711647	0,01021892
ENSMUSG00000005078	Jkamp	0.65123690	10.030755	0,009853081	0,03230204	NS
ENSMUSG00000005483	Dnajb1	0.65521199	125.18250	5,97843E-06	0,001307077	0,001630244
ENSMUSG00000002835	Chaf1a	0.69009974	39.26364	0,004823073	0,03352666	0,04348155
ENSMUSG00000025198	Erlin1	0.69583222	58.06665	0,000898242	0,01672315	0,02648279
ENSMUSG00000070426	Rnf121	0.70942962	6.9890015	0,01416127	NS	NS
ENSMUSG00000057789	Bak1	0.71608208	12.957568	0,005523162	0,0408372	NS
ENSMUSG00000031770	Herpud1	0.71698985	87.60374	0,000628255	0,01306436	0,02067868
ENSMUSG00000035890	Rnf126	0.72067420	9.6012957	0,02039035	NS	NS
ENSMUSG00000053317	Sec61b	0.72271861	8.6464277	0,02394618	NS	NS
ENSMUSG00000032115	Hyou1	0.72542206	111.90740	0,000261459	0,01184542	0,009999353
ENSMUSG00000029992	Gfpt1	0.73010342	128.74029	0,000181333	0,01169538	0,009300651
ENSMUSG00000025823	Pdia4	0.73578803	99.75082	0,001245015	0,01889195	0,01937855
ENSMUSG00000027274	Mkks	0.73859129	10.1872651	0,01692847	NS	NS
ENSMUSG00000024807	Syvn1	0.74925435	12.5888520	0,01491	NS	NS
ENSMUSG00000022136	Dnajc3	0.75897388	107.92095	0,001613378	0,02941396	0,0224176
ENSMUSG00000036752	Tubb4b	0.76208405	128.46661	0,00137721	0,02961917	0,02992061
ENSMUSG00000021270	Hsp90aa1	0.76412886	487.61517	3,71512E-09	0,01869743	0,01748928
ENSMUSG00000020571	Pdia6	0.76530663	115.60622	0,001585131	0,03259344	0,02569314
ENSMUSG00000022403	St13	0.76694814	183.61440	0,000159801	0,02485634	0,01006615
ENSMUSG00000020048	Hsp90b1	0.77268789	333.65080	3,62403E-06	0,02545554	0,01035048
ENSMUSG00000038991	Txndc5	0.78533220	21.6381701	0,01223921	NS	NS
ENSMUSG00000057177	Gsk3a	0.79325964	9.3428609	0,04547464	NS	NS
ENSMUSG00000020964	Sel1l	0.80117569	39.8227204	0,01028297	NS	NS
ENSMUSG00000003814	Calr	0.80683350	280.52363	5,73728E-05	NS	0,03557301
ENSMUSG00000014905	Dnajb9	0.81077498	13.6103914	0,04756119	NS	NS
ENSMUSG00000027248	Pdia3	0.82023420	266.61918	0,000844391	NS	0,03250441
ENSMUSG00000025980	Hspd1	0.83155570	39.3474128	0,005265961	NS	NS
ENSMUSG00000027006	Dnajc10	1.20432485	36.3353195	0,004916254	NS	NS
ENSMUSG00000030102	Itpr1	1.23316873	37.0042251	0,003676613	NS	NS
ENSMUSG00000057329	Bcl2	1.24012846	12.5516511	0,03456831	NS	NS
ENSMUSG00000041417	Pik3r1	1.30733252	390.20651	7,967E-08	0,0122928	0,02680347
ENSMUSG00000033538	Casp4	1.32151410	14.8300797	0,01902025	NS	NS
ENSMUSG00000093904	Tomm20	1.34528949	74.42460	0,001110166	0,01932788	0,02751819
ENSMUSG00000020303	Stc2	1.52415772	5.5675880	0,02826457	NS	NS
ENSMUSG00000036052	Dnajb5	1.99140600	4.632706	0,03248627	0,04135138	NS

Fold change
≥1,500
≥1,200
≤0,833
≤0,666
≤0,500

Table S2 – Antibodies used for flow cytometry staining and cell sorting

Antibody	*Conjugated*	*Clone*	*Dilution*	*Manufacturer*
CD45.2	PB	104	1/200	Sony Biotech
CD45.1	PE	A20	1/100	BD Biosciences
TCRβ	APCCy7	H57-597	1/200	Sony Biotech
CD4	PECy7	RM4-5	1/200	Sony Biotech
CD8	PECy5	53-6.7	1/200	BD Biosciences
CD44	APC	IM7	1/200	Sony Biotech
CD62L	FITC	MEL-14	1/200	Sony Biotech
CD19	PECy7	1D3	1/200	BD Biosciences
IgM	APC	RMM-1	1/200	Sony Biotech
CD11b	APCCy7	M1/70	1/200	Sony Biotech
CD11b	AF488	M1/70	1/200	Sony Biotech
CD115	PECy7	AF598	1/200	eBiosciences
Ly6C	FITC	AL-21	1/200	BD Biosciences
Ly6G	PerCPCy5.5	1A9	1/200	Sony Biotech
Sca-1	PE	D7	1/200	Sony Biotech
CD117	APC	ACK2	1/200	eBiosciences
CD117	AF700	ACK2	1/200	eBiosciences
CD150	PECy5	TC15-12F12.2	1/200	Biolegend
CD48	APCCy7	HM48-1	1/200	Sony Biotech
CD34	FITC	RAM34	1/200	BD Biosciences
CD127	APCeF780	A7R34	1/200	eBiosciences
TER-119	PE	TER-119	1/200	BD Biosciences
CD71	FITC	C2	1/200	BD Biosciences
CD16/32	PECy7	93	1/200	Sony Biotech
LIN (CD3, Ly6G/Ly6C; CD11b; B220; TER-1119)	PB	17A2; RB6-8C5; M1/70; RA3-6B2; Ter-119	1/10 (1/20 for cultured cells)	Biolegend
CD3ε	APC	145-2C11	1/200	BD Biosciences
CD11b	APC	HL3	1/200	BD Biosciences
CD19	APC	6D5	1/200	Sony Biotech
CD19	APCCy7	1D3	1/200	BD Biosciences

Figure S1

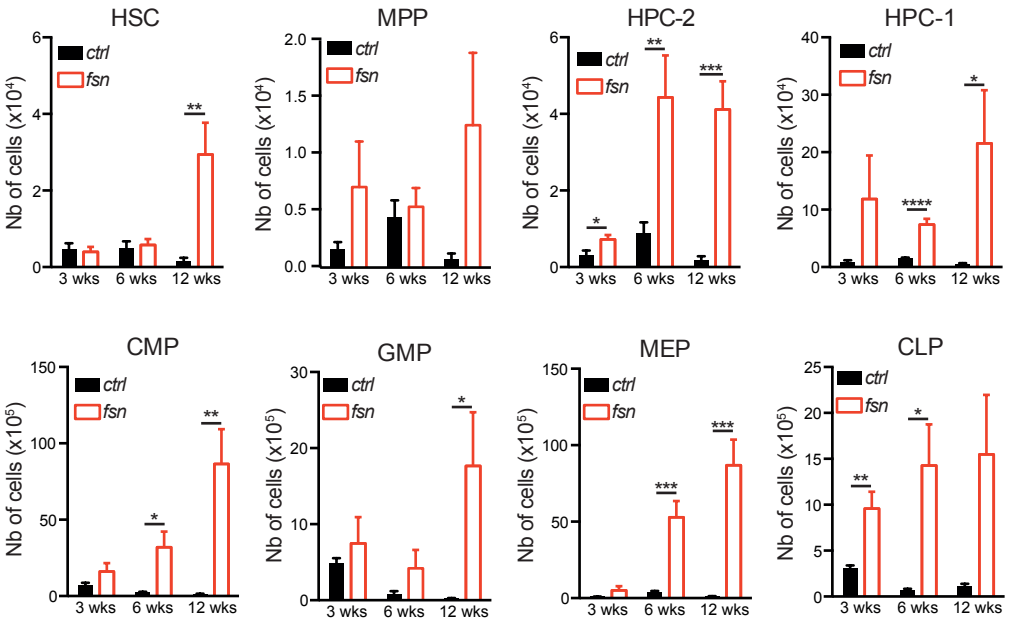

HSC
MPP
HPC-2
HPC-1
CMP
GMP
MEP
CLP
ctrl
fsn
Nb of cells (x10⁴)
Nb of cells (x10⁵)
3 wks
6 wks
12 wks
*
**
***
****

Figure S2

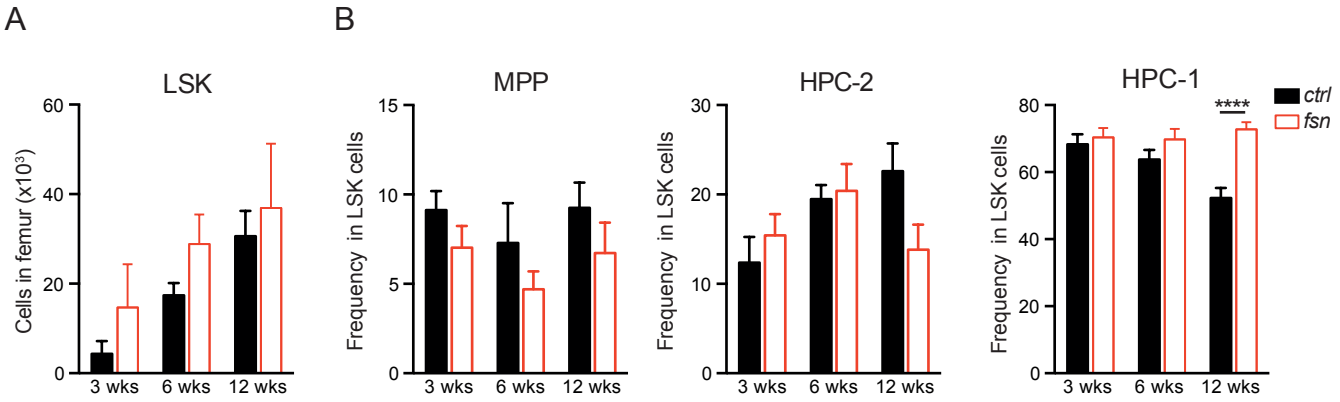

A
B
LSK
MPP
HPC-2
HPC-1
Cells in femur (X10³)
Frequency in LSK cells
3 wks
6 wks
12 wks
****
ctrl
fsn

Figure S3

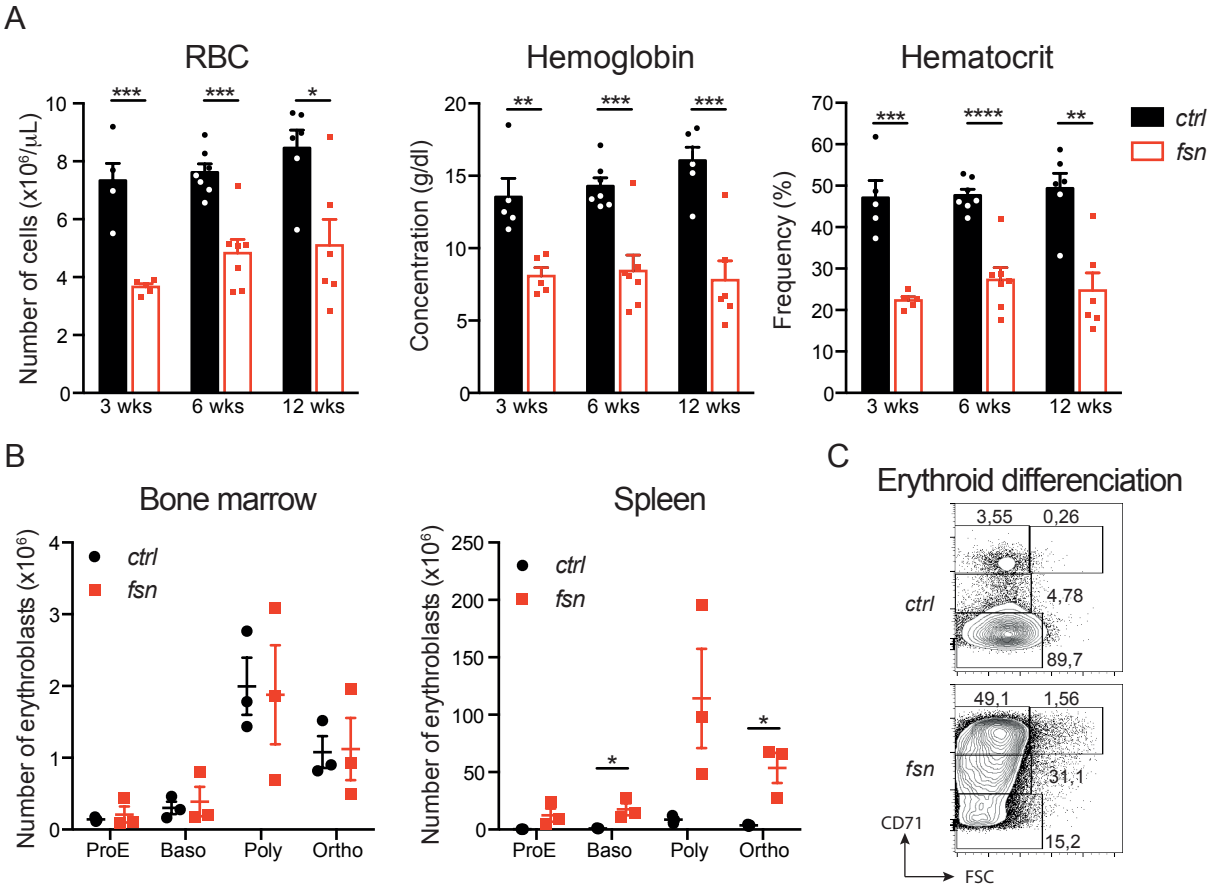

A
RBC
Hemoglobin
Hematocrit
Number of cells (x10⁶/µL)
Concentration (g/dl)
Frequency (%)
3 wks
6 wks
12 wks
ctrl
fsn
B
Bone marrow
Spleen
Number of erythroblasts (x10⁶)
ProE
Baso
Poly
Ortho
C
Erythroid differenciation
3,55
0,26
4,78
89,7
49,1
1,56
31,1
15,2
CD71
FSC

Figure S4

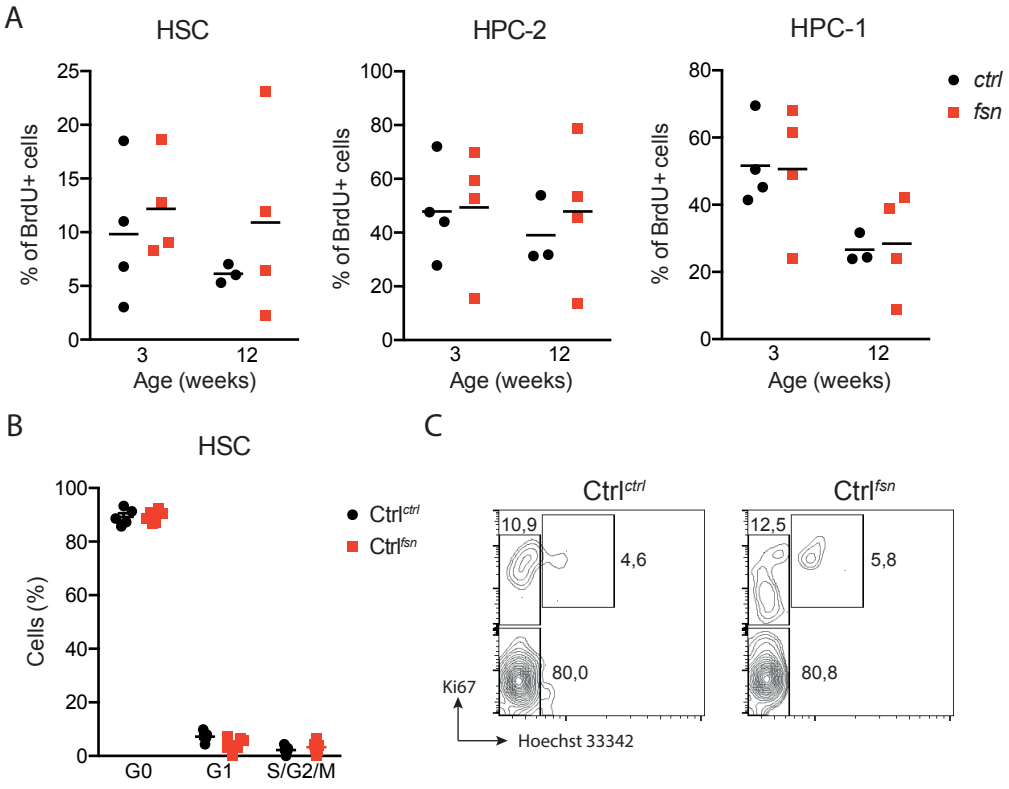

A
HSC
HPC-2
HPC-1
% of BrdU+ cells
Age (weeks)
3
12
ctrl
fsn
B
HSC
Cells (%)
G0
G1
S/G2/M
Ctrl^ctrl
Ctrl^fsn
C
Ctrl^ctrl
Ctrl^fsn
10,9
4,6
80,0
12,5
5,8
80,8
Ki67
Hoechst 33342

Figure S5

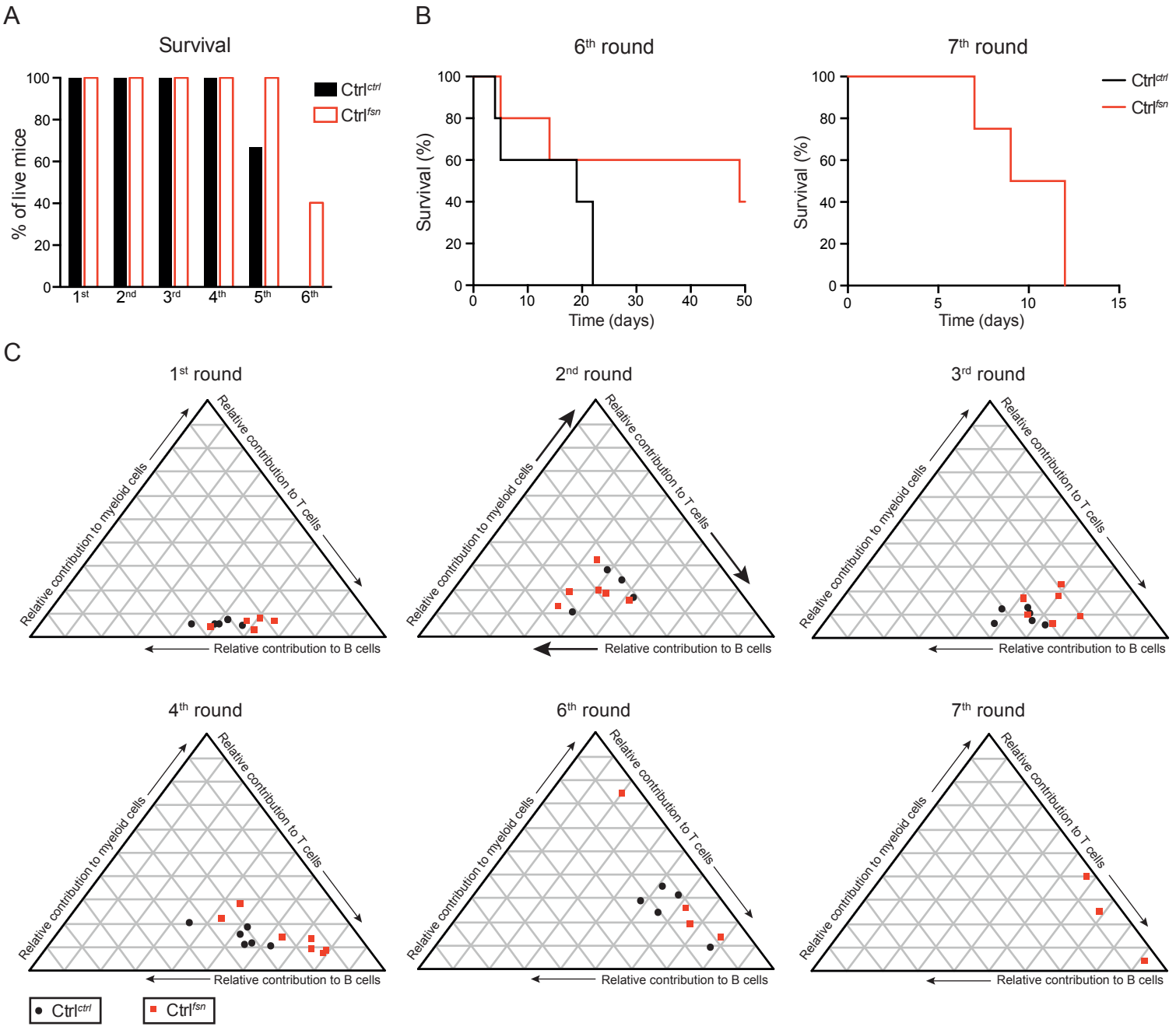

A
Survival
% of live mice
100
80
60
40
20
0
1st
2nd
3rd
4th
5th
6th
Ctrl ctrl
Ctrl fsn
B
6th round
7th round
Survival (%)
Time (days)
Ctrl ctrl
Ctrl fsn
C
1st round
2nd round
3rd round
4th round
6th round
7th round
Relative contribution to myeloid cells
Relative contribution to T cells
Relative contribution to B cells
Ctrl ctrl
Ctrl fsn

Figure S6

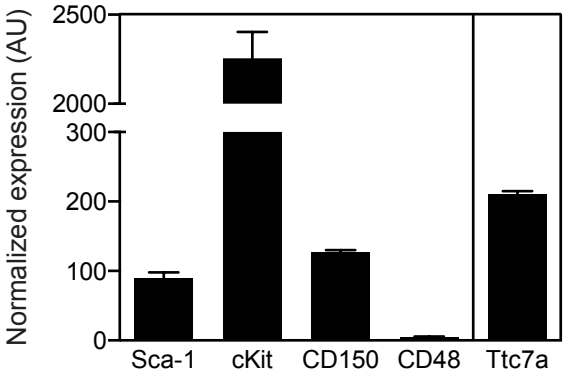
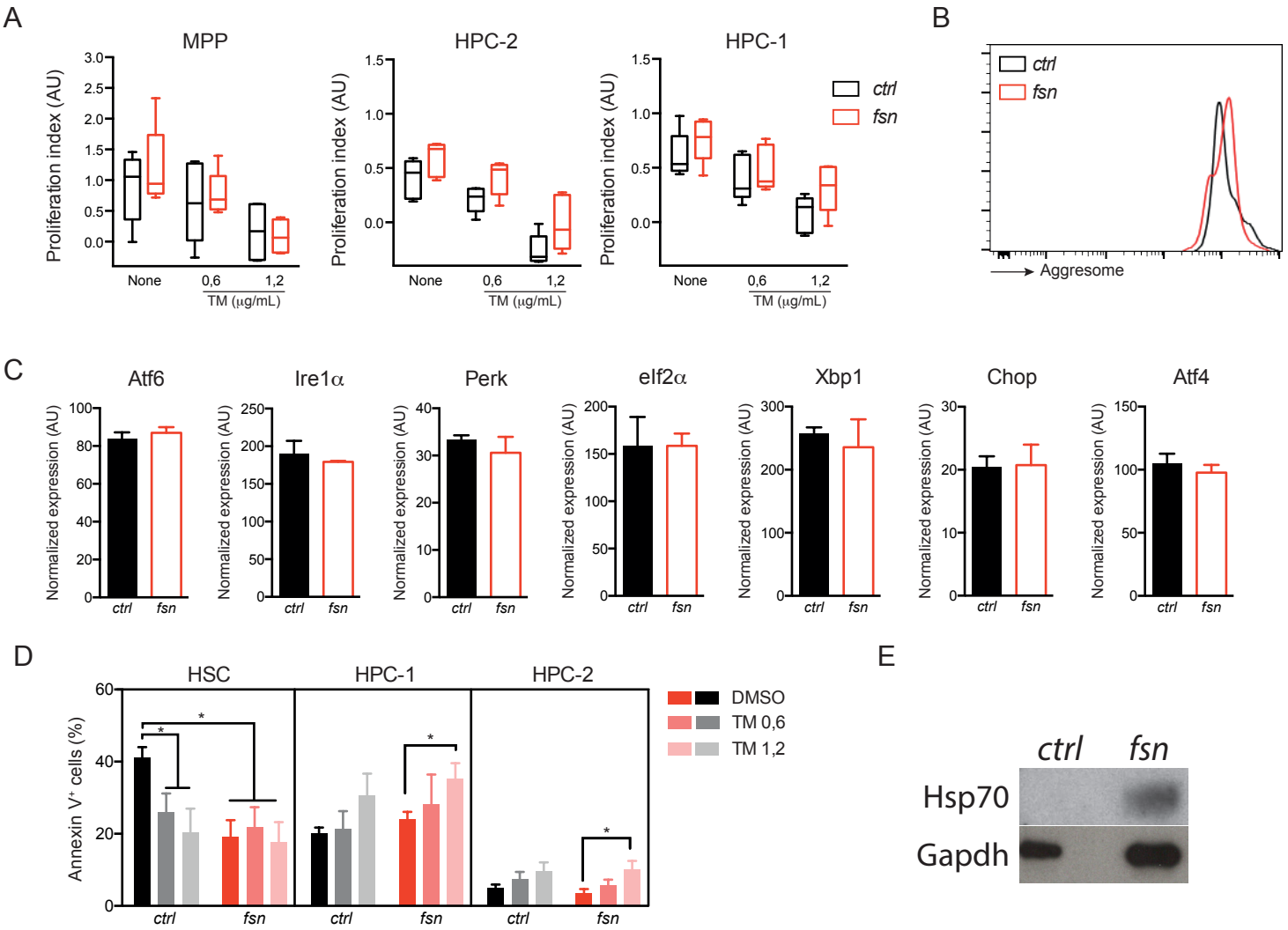

Normalized expression (AU)
2500
2000
300
200
100
0
Sca-1
cKit
CD150
CD48
Ttc7a

Figure S7


A
MPP
HPC-2
HPC-1
Proliferation index (AU)
None
0,6
1,2
TM (µg/mL)
ctrl
fsn
B
Aggresome
C
Atf6
Ire1α
Perk
eIf2α
Xbp1
Chop
Atf4
Normalized expression (AU)
D
HSC
HPC-1
HPC-2
Annexin V+ cells (%)
DMSO
TM 0,6
TM 1,2
E
Hsp70
Gapdh