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

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## ABSTRACT

The molecular machinery that regulates the balance between self-renewal and differentiation properties of hematopoietic stem cells (HSC) has yet to be characterized in detail. Here we found that the tetratricopeptide repeat domain 7 A (Ttc7a) protein, a putative scaffold protein expressed by HSC, acts as an intrinsic regulator of the proliferative response and the self-renewal potential of murine HSC *in vivo*. Loss of Ttc7a consistently enhanced the competitive repopulating ability of HSC and their intrinsic capacity to replenish the hematopoietic system after serial cell transplantations, relative to wildtype cells. Ttc7a-deficient HSC exhibit a different transcriptomic profile for a set of genes controlling the cellular response to stress, which was associated with increased proliferation in response to chemically induced stress *in vitro* and myeloablative stress *in vivo*. Our results therefore revealed a previously unrecognized role of Ttc7a as a critical regulator of HSC stemness. This role is related, at least in part, to regulation of the endoplasmic reticulum stress response.

## Introduction

In flaky skin (*fsn*) mice, the spontaneous insertion of early transposon into the gene for tetratricopeptide repeat domain 7 A (*Ttc7a*) is known to impair Ttc7a protein expression.[1,2] Consequently, *fsn* mice develop a proliferative lymphoid and myeloid disorder, with hyperplasia of the spleen and lymph nodes, elevated monocyte, granulocyte and lymphoid cell counts,[3-6] and severe anemia.[7] Moreover, *fsn* mice have a reduced lifespan and changes in the skin (epidermal hyperplasia and inflammation)[8,9] and the intestinal tract (gastric papillomas).[10] The marked phenotypic alterations in *fsn* mice suggest that Ttc7a protein has one or more major regulatory roles in the hematopoietic system, and, potentially, in other tissues of epithelial origin.

Ttc7a is a putative scaffolding protein as it contains nine tetratricopeptide repeats (TPR) domains that are predicted to interact with proteins containing their own TPR or other motifs.[11] These TPR-containing proteins are involved in a variety of biological processes, including cell cycle control, protein trafficking, secretion and protein quality control. Indeed, TPR-containing proteins have been shown to bind chaperones such as Hsp90 and Hsp70, controlling their activity.[12-14] Thus, Ttc7a is likely to be involved in a broad range of protein complexes and hence functions. *In vitro* studies have shown that the loss of Ttc7a causes inappropriate activation of RhoA-dependent effectors and thus disrupts cytoskeletal dynamics.[15,16]

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Furthermore, TTC7A reportedly interacts with EFR3 homolog B and phosphatidylinositol 4-kinase alpha, which is known to catalyze the production of phosphatidylinositol 4-phosphate at the plasma membrane in yeast and human cells.[17,18] This observation emphasizes the conservation, at least in part, of the functions of Ttc7a during evolution. However, data on TTC7A's biological function(s) are still scarce.

Inadequate proliferation of peripheral hematopoietic lineages has been reported in several modified murine models; this impairment is ultimately associated with the exhaustion of the hematopoietic stem cell (HSC) pool.[19] Indeed, the production of blood cells requires HSC to leave their quiescent state and differentiate into functional progeny. An excessive requirement for hematopoietic cell production biases HSC function toward differentiation, at the expense of self-renewal.[20] Various intrinsic and extrinsic factors influence HSC fate, i.e. quiescence or proliferation. Endoplasmic reticulum (ER) stress has recently been highlighted as an important regulator of HSC function.[21] This stress is triggered by various stimuli and leads to the accumulation of unfolded proteins in the lumen of the ER, and induction of the unfolded protein response (UPR). The chaperone BIP (Hspa5/GRP78) is the main inducer of the UPR.[22] This response results in enhanced expression of chaperone proteins (heat shock proteins, Hsp), phosphodiesterase (Pdi), and other proteins such as calreticulin that, together with BIP, boost protein folding capacities. Depending on the intensity of the ER stress, UPR activation can lead to apoptosis or survival.[23]

In the present study, we found that Ttc7a regulates murine HSC self-renewal and hematopoietic reconstitution potential and controls the sensitivity of these cells to stress. Loss of Ttc7a consistently enhanced HSC stemness, since Ttc7a-deficient HSC displayed a greater proliferation capacity than control counterparts in response to ER stress *in vitro*, and after myeloablative stress *in vivo*. Hence, our results reveal a new role for Ttc7a as a regulator of self-renewal and response to stress in HSC.

## 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. All mice were maintained in specific pathogen-free conditions and handled according to national and institutional guidelines.

### Repopulations assays

Bone marrow (BM) cells were transferred into CD45.1$^+$ control recipient mice upon irradiation and then 30,000 Lin$^-$ Sca1$^+$ cKit$^+$ (LSK) donor cells were injected into the irradiated recipient mice. For serial transplantations, recipients were reconstituted with $10^7$ BM cells. To perform competitive repopulation assays, 1,000 LSK cells were injected with 2 x $10^6$ unfractionated CD45.1$^+$ BM cells. Twelve weeks after transfer, mice were treated with a single dose of 5-fluorouracil (5-FU, 150 mg/kg).

### Flow cytometry and isolation of hematopoietic stem cells

Splenocytes and peripheral blood cells were incubated with conjugated antibodies and viability exclusion dyes. The antibodies used are listed in *Online Supplementary Table S2*. Stained cells were quantified using a Gallios flow cytometer (Beckman Coulter), and analyzed with FlowJo software (Treestar). HSC and LSK cells were isolated by depleting Lin$^+$ cells using the Lineage Cell Depletion Kit according to the manufacturer's protocol (Miltenyi Biotec), stained with a Lin$^-$ antibody cocktail, and antibodies against CD117, Sca-1, CD150 and CD48, and sorted with FACS Aria™ (BD Biosciences).

### Cell culture

Lin$^-$ cells were cultured in StemSpan medium (StemCell Technologies) supplemented with 5% fetal bovine serum, 1% penicillin/streptomycin, recombinant human thrombopoietin (100 μg/mL), recombinant murine stem cell factor (100 μg/mL) and recombinant murine FLT3 ligand (100 μg/mL). Tunicamycin (Cayman Chemical) was added (0.6 or 1.2 μg/mL) for 24 or 48 h.

### RNA-sequencing

RNA was extracted using the ZR-RNA MicroPrep™ isolation kit (Proteinegene). cDNA libraries were generated using the Ovation SoLo RNA-seq system (NuGEN). The libraries were controlled with a High Sensitivity DNA Analysis Kit and Bioanalyzer (Agilent). NextSeq 500 (Illumina) was used for sequencing. FASTQ files were mapped to the ENSEMBL MM38 reference using Hisat2 and counts were produced with feature Counts. Read count normalization and group comparisons were performed by DESeq2, edgeR, and LimmaVoom. Heatmaps were made with R and imaged by Java Treeview software. Differentially expressed genes were examined with gene set enrichment analysis (GSEA) for functional enrichment in gene ontology (GO) terms using normalized expression values of LimmaVoom.

### Western blot

Lin$^-$ cells were cultured for 3 days and HSC were sorted directly into 10% trichloroacetic acid. Proteins were extracted and solubilized as previously described.[24]

### Statistical analysis

Data were analyzed with GraphPad Prism 6 software. Statistical analyses were performed using two-tailed Student t-test. Differences were considered to be statistically significant when $P<0.05$ (indicated as $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$).

### Data availability

The data are available at the Sequence Read Analysis (SRA) database under accession number SRA139913.

## Results

### Ttc7a is required for the maintenance of immune homeostasis

It has previously been shown that adult Ttc7a-deficient (*fsn*) mice (aged 8 to 10 weeks) develop an imbalance in hematopoiesis, characterized by leukocytosis and anemia.[7] To gain insight into the change over time in the *fsn* mice's pathology, we analyzed the different hematopoietic lineages in the blood and the spleen at 3, 6 and 12 weeks of age. *Fsn* mice had a considerably higher circulating leukocyte count than control littermates (*ctrl*) at all time points (Figure 1A). The spleen was much larger in *fsn* mice than in *ctrl* mice, twice as large at 3 weeks and ten times larger at 12 weeks (Figure 1B). The splenic architecture in *fsn* mice became increasingly disorganized, with an age-related expansion of red and white pulp (Figure 1C).

Furthermore, histological assessment of splenic sections revealed extramedullary hematopoiesis as evidenced by elevated counts of megakaryocytes (Figure 1C) and of hematopoietic stem and progenitor cells (HSPC) (*Online Supplementary Figure S1*). Relative to *ctrl* mice, the absolute splenic T-cell count in *fsn* mice was slightly lower at 3 weeks of age but higher at 6 and 12 weeks of age (Figure 1D). A large proportion of Ttc7a-deficient T lymphocytes had an effector memory phenotype ($CD44^+$ $CD62L^-$) (Figure 1E). Splenic B-cell counts were slightly elevated, and B cells presented the impaired maturation phenotype previously described in *fsn* mice[6] (Figure 1F). The lymphoid alterations were accompanied by massive myeloproliferation, with an increase over time in the numbers of splenic granulocytes (both neutrophils and eosinophils) and resident and inflammatory monocytes (Figure 1G, H). Thus, Ttc7a-deficient mice displayed a number of persistent hematopoietic alterations (i.e., leukocytosis, T-lymphocyte activation and anemia) at a very early age, whereas other manifestations appeared later in life and/or were exacerbated with age (i.e., myeloproliferation and elevated T-cell counts).

Since all the peripheral hematopoietic lineages were affected in *fsn* mice, we next looked at whether the HSPC compartment was also altered. BM cellularity in *fsn* mice, in contrast to *ctrl* mice, increased between 3 and 12 weeks of age (Figure 2A). The LSK stem cell population was slightly higher in *fsn* mice than in *ctrl* mice at all the time

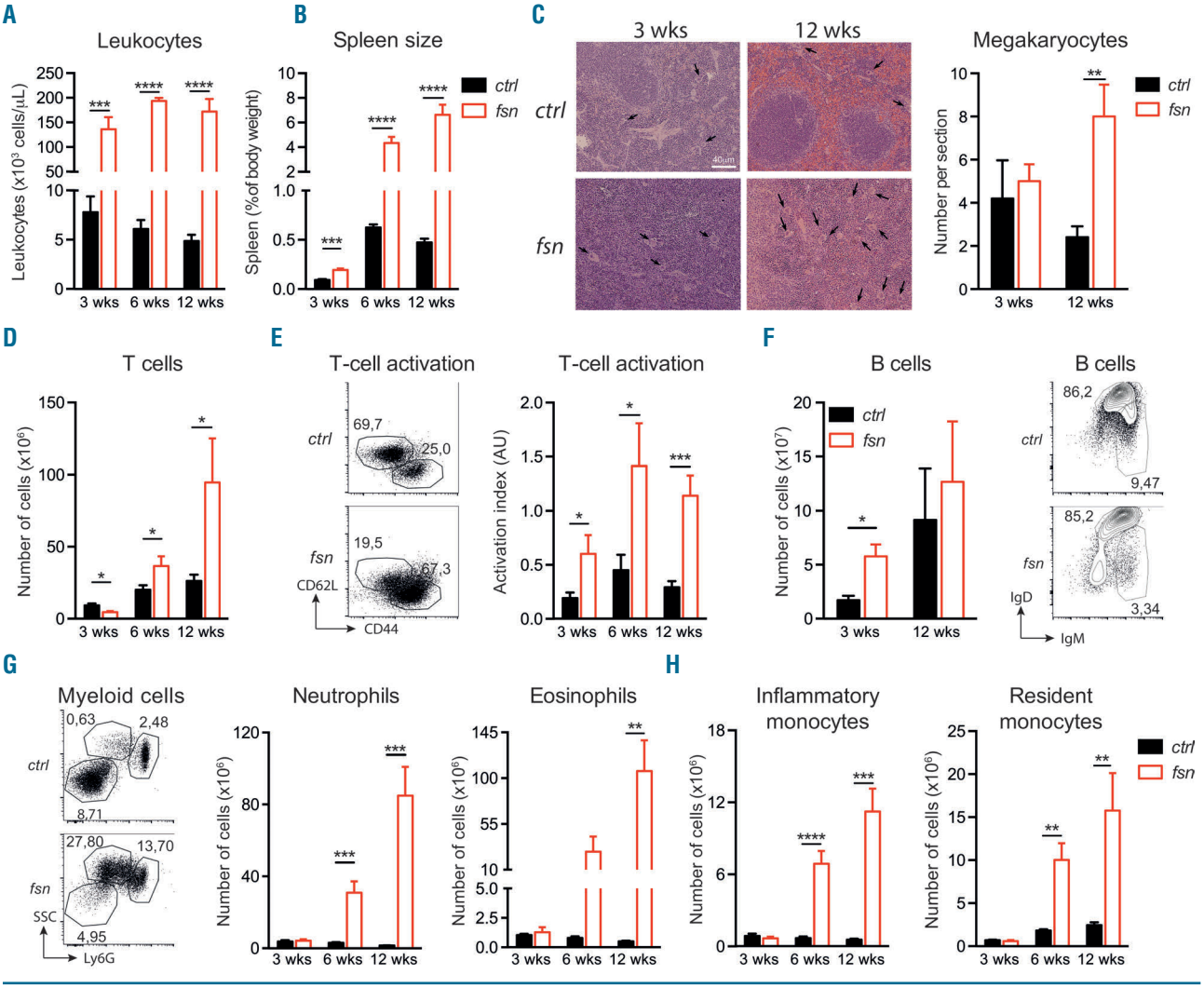


**Figure 1. Ttc7a-deficiency perturbs homeostasis of all immune populations.** Control littermates (*ctrl* – black bars) and Ttc7a-deficient (*fsn* – red bars) mice were analyzed at 3, 6 and 12 weeks of age (mean ± standard error of mean) **P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001 (two-tailed *t*-test). (A) White blood cell count (n≥6). (B) Spleen size determined as percent of body weight (n≥6). (C) Histological sections of spleen stained with hematoxylin and eosin showing megakaryocytes (black arrow) (left panel) and quantification of spleen megakaryocytes (right panel). (D) Total number of T cells in the spleen (n≥7). (E) Flow cytometry representative of T-cell activation (according to CD44 and CD62L expression) at 12 weeks (left panel), and activation index of T cells [ratio of effector memory T cells ($CD44^+$ $CD62L^-$) to naïve T cells ($CD44^-$ $CD62L^+$)] (right panel) of *fsn* and *ctrl* mice (n≥7). Numbers adjacent to the outlined areas indicate percent cells in the parent gate (mean). (F) Total number of B cells in the spleen (n=4) (left panel) and flow cytometry representative of B-cell maturation (according to IgM and IgD expression) (right panel). (G) Representative flow cytometry at 12 weeks (left panel) and total number of neutrophils ($CD11b^+$ $Ly6G^{hi}$) and eosinophils ($CD11b^+$ $Ly6G^{int}$ $SSC^{hi}$). (H) Total number of inflammatory ($CD11b^+$ $Ly6G^-$ $Ly6C^+$) and resident monocytes ($CD11b^+$ $Ly6G^-$ $Ly6C^-$) (n≥6). Numbers adjacent to the outlined areas indicate percent cells among leukocytes in the spleen (mean).

points analyzed (*Online Supplementary Figure S2A*). The proportion of HSC (Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^+$ CD48$^-$)[25] was decreased in *fsn* mice, whereas the proportion of more mature hematopoietic progenitor cells (HPC-1: Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^-$ CD48$^+$) was increased, compared to the proportions in *ctrl* mice (Figure 2B and *Online Supplementary Figure S2B*). At 12 weeks of age, the HSC progenitor count was significantly lower in *fsn* mice than in *ctrl* mice, while the numbers of multipotent progenitors (MPP: Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^-$ CD48$^+$) were unchanged and those of HPC-2 (Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^+$ CD48$^+$) and HPC-1 were slightly higher (Figure 2C). Within the committed progenitor compartment, the numbers of common myeloid progenitors (CMP: Lin$^-$ Sca1$^-$ cKit$^+$ CD34$^+$ CD16/32$^{low}$) and granulocyte-monocyte progenitors (GMP: Lin$^-$ Sca1$^-$ cKit$^+$ CD34$^+$ CD16/32$^+$) were lower than *ctrl* values at 3 weeks of age, although the differences disappeared with time (Figure 2D, E). There were no significant differences in *fsn vs. ctrl* values in the numbers of common lymphoid progenitors (CLP: Lin$^-$ Sca1$^-$ cKit$^{int}$) or megakaryocyte-erythrocyte progenitors (MEP: Lin$^-$ Sca1$^-$ cKit$^+$ CD34$^-$ CD16/32$^-$) (Figure 2D, E). We confirmed previous reports that the profound anemia observed in *fsn* mice (*Online Supplementary Figure S3A*) is peripheral in nature and does not result from a decreased number of early erythroid progenitors but rather from a defect in the last step of erythropoiesis (*Online Supplementary Figure S3B, C*).[7] Erythropoiesis and enucleation processes have been shown to involve chromatin compaction[26] and actin cytoskeleton dynamics.[27] Interestingly, we previously showed that Ttc7a plays a role in actin dynamics[15,16] as well as in chromatin compaction and genomic stability.[28] Hence, it is tempting to speculate that altered actin dynamics and chromatin organization, as a consequence of Ttc7a-deficiency, contribute to defective erythrocyte generation in *fsn* mice. A high splenic erythroblast count

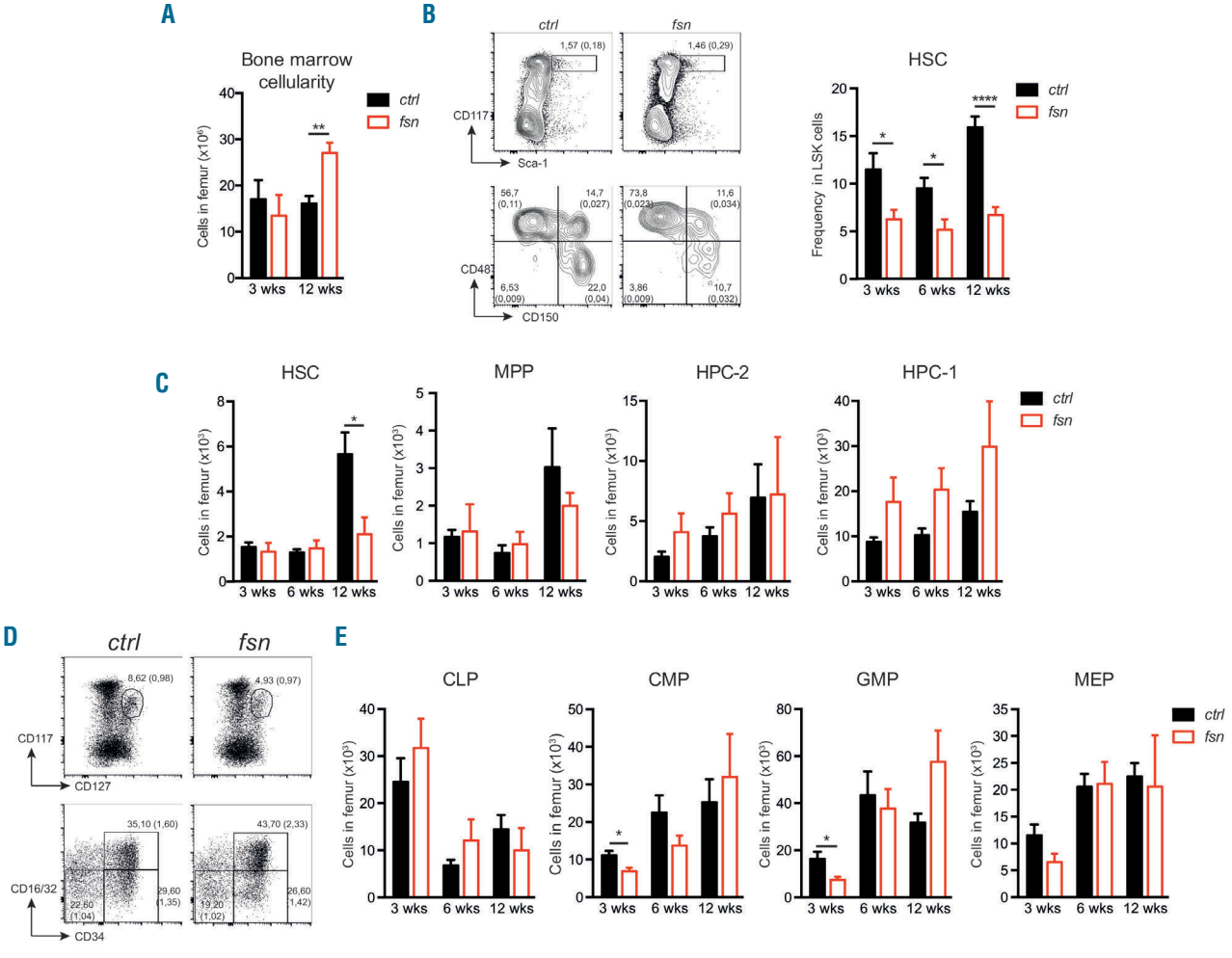


**Figure 2. Ttc7a-deficiency alters the hematopoietic stem and progenitor cell compartment.** The hematopoietic stem and progenitor cell compartment was analyzed in the bone marrow (BM) of 3-, 6-, and 12-week old control (*ctrl* - black bars) and Ttc7a-deficient (*fsn* - red bars) mice (mean ± standard error of mean) *$P$<0.05; **$P$<0.01; ****$P$<0.0001 (two-tailed *t*-test). (A) Quantification of total femoral BM cells (n≥6). (B) Representative flow cytometry at 12 weeks (left panel) and percentage of hematopoietic stem cells (HSC: Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^+$ CD48$^-$) among LSK (Lin$^-$ Sca1$^+$ cKit$^+$) cells (right panel) (n≥7). (C) Quantification of LSK cell populations, HSC, multipotent progenitors (MPP: Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^-$ CD48$^+$), HPC-2 (Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^+$ CD48$^+$) and HPC-1 (Lin$^-$ Sca1$^+$ cKit$^+$ CD150$^-$ CD48$^+$). (D) Representative flow cytometry at 12 weeks and (E) quantification of common lymphoid progenitors (CLP: Lin$^-$ Sca1$^-$ cKit$^{int}$ CD127$^+$), common myeloid progenitors (CMP: Lin$^-$ Sca1$^-$ cKit$^+$ CD34$^+$ CD16/32$^-$), granulocyte-monocyte progenitors (GMP: Lin$^-$ Sca1$^-$ cKit$^+$ CD34+ CD16/32$^+$) and megakaryocyte-erythroid progenitors (MEP: Lin$^-$ Sca1$^-$ cKit$^+$ CD34$^-$ CD16/32$^-$) (n≥7). (B-D) Numbers adjacent to outlined areas indicate percent cells in the parent gate (mean). Numbers in parentheses indicate percentage among leukocytes in the BM (mean).

suggested the presence of stress erythropoiesis as a possible attempt to compensate for the peripheral anemia (*Online Supplementary Figure S3B*).

Thus, our present results show that the absence of Ttc7a in *fsn* mice is associated with deregulation of the homeostatic balance between hematopoietic lineages, from the HSC stage onwards, and a tendency of all leukocyte subsets to expand over time.

## Ttc7a has an intrinsic role in the fate of progenitor cells

Since Ttc7a is broadly expressed, it was not possible to distinguish the respective involvements of hematopoietic factors (i.e., HSC) and non-hematopoietic factors (e.g., BM niches and the thymic epithelium) in the generation of the *fsn* phenotype. In a previous study we found that the skin barrier is impaired in *fsn* mice;[9] this defect may enhance antigen sensitization and thus induce immune system activation. Therefore, in order to determine the Ttc7a-deficient hematopoietic cells' intrinsic contribution to *fsn*-associated hematologic manifestations, we generated chimeric mice by reconstituting lethally irradiated control recipients with LSK cells purified from either *ctrl* or *fsn* mice. Hereafter, these chimeric mice are respectively referred to as Ctrl$^{ctrl}$ and Ctrl$^{fsn}$. Three-week old mice were chosen as donors so that we could use a similar LSK graft inoculum in both control and *fsn* samples, and thus minimize the potential immune consequences caused by the altered *fsn* skin barrier. We monitored the hematologic reconstitution over time by collecting blood samples from the recipient mice every 2 weeks. As observed in native *fsn* mice, white blood cell counts were higher in Ctrl$^{fsn}$ mice than in Ctrl$^{ctrl}$ mice, and a difference was observed as early as 4 weeks after transplantation (Figure 3A). The Ctrl$^{fsn}$ mice were also anemic (Figure 3B) and developed splenomegaly (Figure 3C), although the latter was less pronounced than in native *fsn* mice (Figure 1B). The total body weight of Ctrl$^{ctrl}$ and Ctrl$^{fsn}$ mice was not different. The distribution of the splenic myeloid, T- and B-cell populations was the same as in *ctrl* mice (Figure 3D). As observed in *fsn* mice, BM cellularity was higher in Ctrl$^{fsn}$ mice than in Ctrl$^{ctrl}$ mice (Figure 3E), whereas the LSK counts were slightly increased (Figure 3F). The distribution of the HSC, MPP, HPC-2 and HPC-1 populations was similar in Ctrl$^{fsn}$ and Ctrl$^{ctrl}$ mice, suggesting that the low HSC count observed in 12-week old native *fsn* mice (Figure 2C) is primarily caused by external (i.e., non-hematopoietic) factors. Taken as a whole, these data suggest that Ttc7a has an intrinsic role in hematopoietic cells; the absence of Ttc7a in hematopoietic progenitors results in the over-proliferation of the various cell lineages as seen in native *fsn* mice.

## Loss of Ttc7a enhances the reconstitution potential of hematopoietic stem cells

Next, we sought to determine the impact of Ttc7a loss on the reconstitution potential of HSC in a controlled *in vivo* environment. Using lethally irradiated congenic recipients, we transferred equal numbers of LSK cells purified from 3-week old *ctrl* (LSK$^{ctrl}$)- or *fsn* (LSK$^{fsn}$)-(CD45.2$^{+}$) mice together with competitor wildtype-(CD45.1$^{+}$) BM cells (i.e., Ctrl-LSK$^{ctrl}$ or Ctrl-LSK$^{fsn}$). We then assessed the

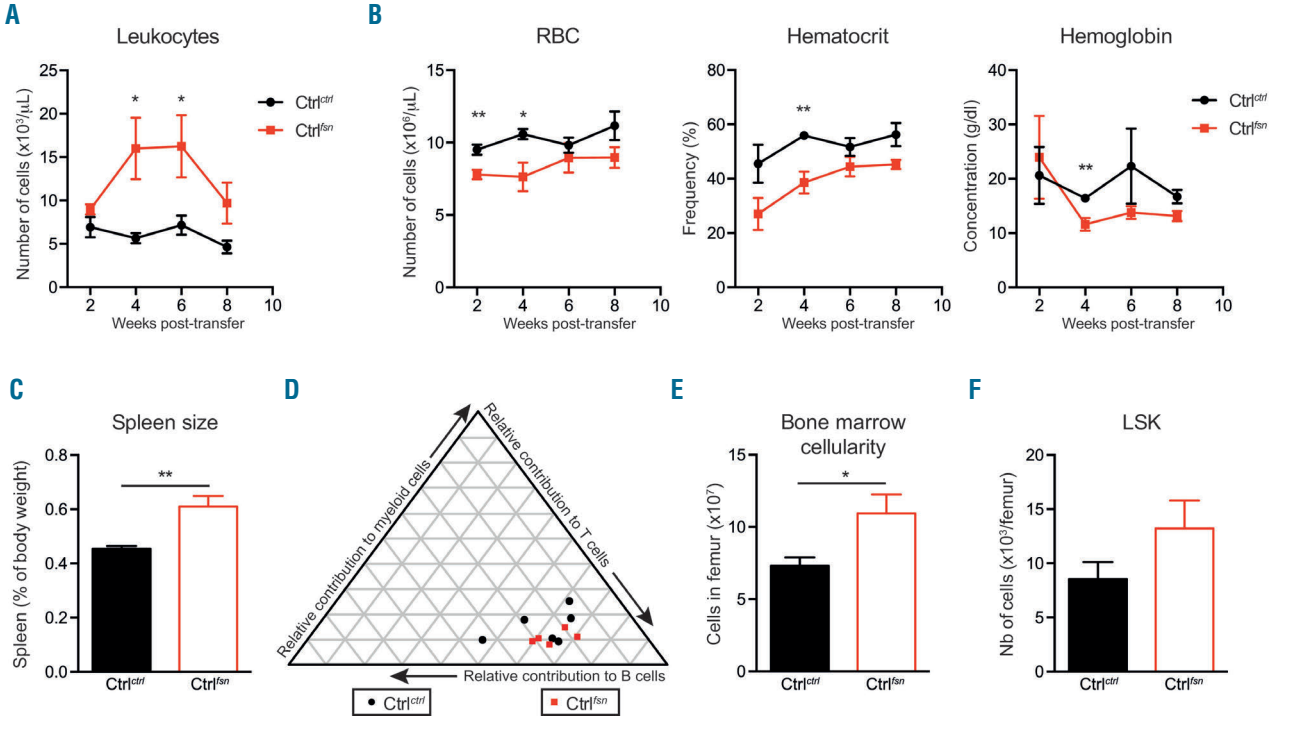


**Figure 3. Transferred Ttc7a-deficient LSK cells reproduce fsn manifestations in a control environment.** Control irradiated mice were transferred with LSK cells purified from 3-week old *ctrl* (Ctrl$^{ctrl}$ – black bars) or Ttc7a-deficient mice (Ctrl$^{fsn}$ – red bars). (mean ± standard error of mean) **P*<0.05; ***P*<0.01 (two-tailed *t*-test). (A, B) Monitoring of the number of leukocytes (A) and red blood cells, hematocrit and hemoglobin (B) during hematopoietic reconstitution. (C) Spleen size determined as percent of body weight 10 weeks after bone marrow (BM) transfer. (D) Relative contribution of myeloid compared to T cells and B cells in the spleen. (E, F) BM cellularity (E) and absolute number of LSK cells (F) 10 weeks after BM transfer. RBC: red blood cells.

respective contributions of cells originating from *ctrl* or *fsn* LSK donors during hematopoietic reconstitution. As early as 2 weeks after transfer, the proportion of LSK$^{fsn}$ donor-derived leukocytes in the recipients' blood was higher than that of LSK$^{ctrl}$. These differences persisted 14 weeks after transfer (Figure 4A). The proportions of cells originating from donor LSK in the recipients' organs, particularly the thymus and BM, were higher in Ctrl-LSK$^{fsn}$ mice than in Ctrl-LSK$^{ctrl}$ mice (Figure 4B). In the spleen, the reconstitution advantage of LSK$^{fsn}$ donor-derived cells led to the expansion of neutrophil, eosinophil and monocyte lineages and to a lesser extent, T-cell lineages (Figure 4C). To further evaluate the effect of Ttc7a loss on long-term reconstitution, total BM cells from primary recipient mice were transplanted into secondary recipients. The competitive advantage of Ttc7a-deficient LSK donor cells with regards to reconstitution was maintained and even enhanced upon secondary and tertiary transplantation (Figure 4D). Thus, our results show that a defect in Ttc7a improves the competitive fitness of HSC following transplantation.

## Loss of Ttc7a increases the long-term self-renewal potential of hematopoietic stem cells

In view of the elevated proliferative capacity of Ttc7a-deficient hematopoietic cells, we next sought to assess the properties of HSC that could modify their reconstitution potential (i.e., quiescence and self-renewal capacity). To evaluate the impact of Ttc7a loss on the quiescence of reconstituting HSC, we measured bromodeoxyuridine (BrdU) incorporation in control and Ttc7a-deficient HSPC before and after BM transplantation. Upon 24 h of BrdU treatment, we observed similar percentages of BrdU-positive (BrdU$^{+}$) HSC, HPC-1 and HPC-2 cells purified from control and *fsn* mice (*Online Supplementary Figure S4A*). Similar results were obtained when comparing cell cycle progression in control and Ttc7a-deficient HSC upon transplantation of irradiated recipients (*Online Supplementary Figure S4B, C*). Altogether, these results suggest that the increased repopulation capacity of *fsn* HSC was not caused by a disturbed quiescent state.

We then looked at whether the long-term self-renewal ability of HSC was altered in this context. We therefore performed serial BM transplants from irradiated mice having received whole BM from 3-week old *ctrl* or *fsn* mice. Unexpectedly, Ttc7a-deficient cells successfully sustained BM reconstitution longer than *ctrl* cells did. The *ctrl* HSC sustained six rounds of transplantation (Figure 5A) but all the Ctrl$^{ctrl}$ mice died during the seventh round of BM transfer (Figure 5A, B). In contrast, most Ctrl$^{fsn}$ mice survived 6 weeks after the seventh round and were able to undergo

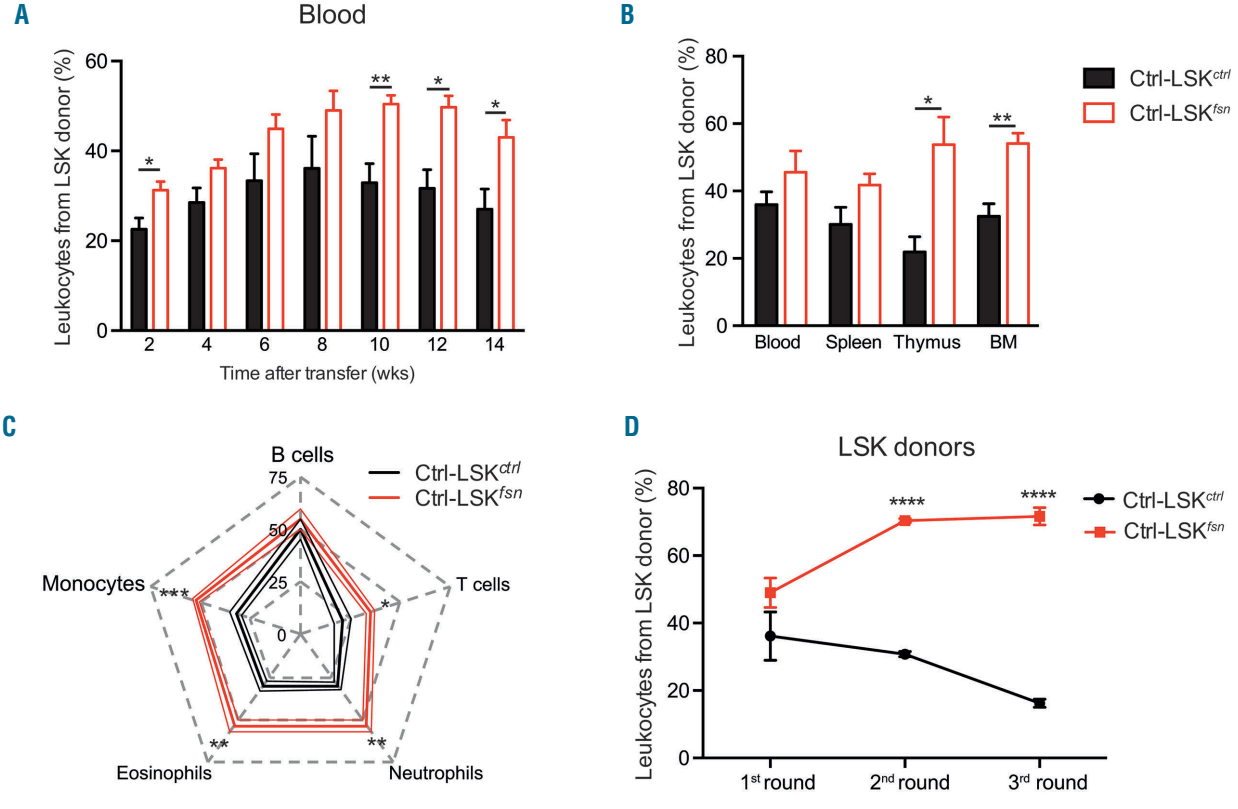


**Figure 4. Ttc7a-deficient hematopoietic stem cells have a higher repopulation capacity.** (A-C) Lethally irradiated CD45.1 mice were reconstituted with a mix of control whole bone marrow (BM) and sorted LSK cells purified from *ctrl* (Ctrl-LSK$^{ctrl}$ – black bars and lines) or Ttc7a-deficient mice (Ctrl-LSK$^{fsn}$ – red bars and lines) (mean ± standard error of mean) *$P$<0.05; **$P$<0.01; ***$P$<0.001; ****$P$<0.0001 (two-tailed $t$-test). These data are representative of three independent experiments. Proportion of LSK donor-derived leukocytes in the blood over time (A), in lymphoid organs (15 weeks after transfer) (B) and in the spleen for the different types of leukocytes (C) (n=8). (D) BM cells from first and then second round recipient mice of each group were pulled and transplanted to secondary and tertiary *ctrl* CD45.1 recipients, respectively. The proportions of LSK donor-derived leukocytes of Ctrl-LSK$^{ctrl}$ and Ctrl-LSK$^{fsn}$ mice were determined in each round (n=14 for control and n=16 for Ttc7a-reconstituted mice).

an additional round of transplantation before dying after the eighth round (Figure 5A, B). Similar results were obtained in experiments with 12-week old donors (*Online Supplementary Figure S5A, B*). We next determined the ability of *ctrl* and Ttc7a-deficient BM cells from 3-week old mice to properly reconstitute hematopoiesis over the different rounds of transplantation. During the first five rounds, the same phenotype was always observed. Ctrl$^{fsn}$ mice displayed anemia (Figure 5C), together with an elevated leukocyte count up until the third round (Figure 5D). The spleen was larger in Ctrl$^{fsn}$ mice than in Ctrl$^{ctrl}$ mice until the fifth round (Figure 5E). However, differences between Ctrl$^{fsn}$ and Ctrl$^{ctrl}$ mice were no longer observed in the sixth round; this was probably caused by exhausted donor cells that failed to properly reconstitute recipient mice at the end of the reconstitution process (Figure 5C-E). Interestingly, the distribution of splenic leukocyte subsets in the Ctrl$^{fsn}$ mice was progressively biased toward myeloid populations at the expense of B cells, and to a lesser extent, T cells, as notably observed for the fifth round (Figure 5F). In Ctrl$^{fsn}$ mice, this bias became detectable in the third round and persisted until the seventh round (*Online Supplementary Figure S5C*). In summary, these data show that Ttc7a-deficient HSC have a greater ability to self-renew and to induce myeloid cell expansion.

## Ttc7a-deficiency perturbs the transcriptomic profile of the endoplasmic reticulum stress response in hematopoietic stem cells

In order to gain further mechanistic insight into the Ttc7a-related regulation of HSC homeostasis, we carried out a transcriptomic analysis of HSC isolated from Ttc7a-deficient and control BM (from 4-week old mice). A two-way hierarchical clustering analysis of differentially expressed genes ($P$ value ≤0.05, fold change ≥1.2) revealed a clear-cut separation between Ttc7a-deficient and control HSC samples (Figure 6A). We found that 1,103 genes were significantly upregulated and 928 significantly downregulated in Ttc7a-deficient HSC relative to the expression levels in control HSC (Figure 6B). Among the differentially

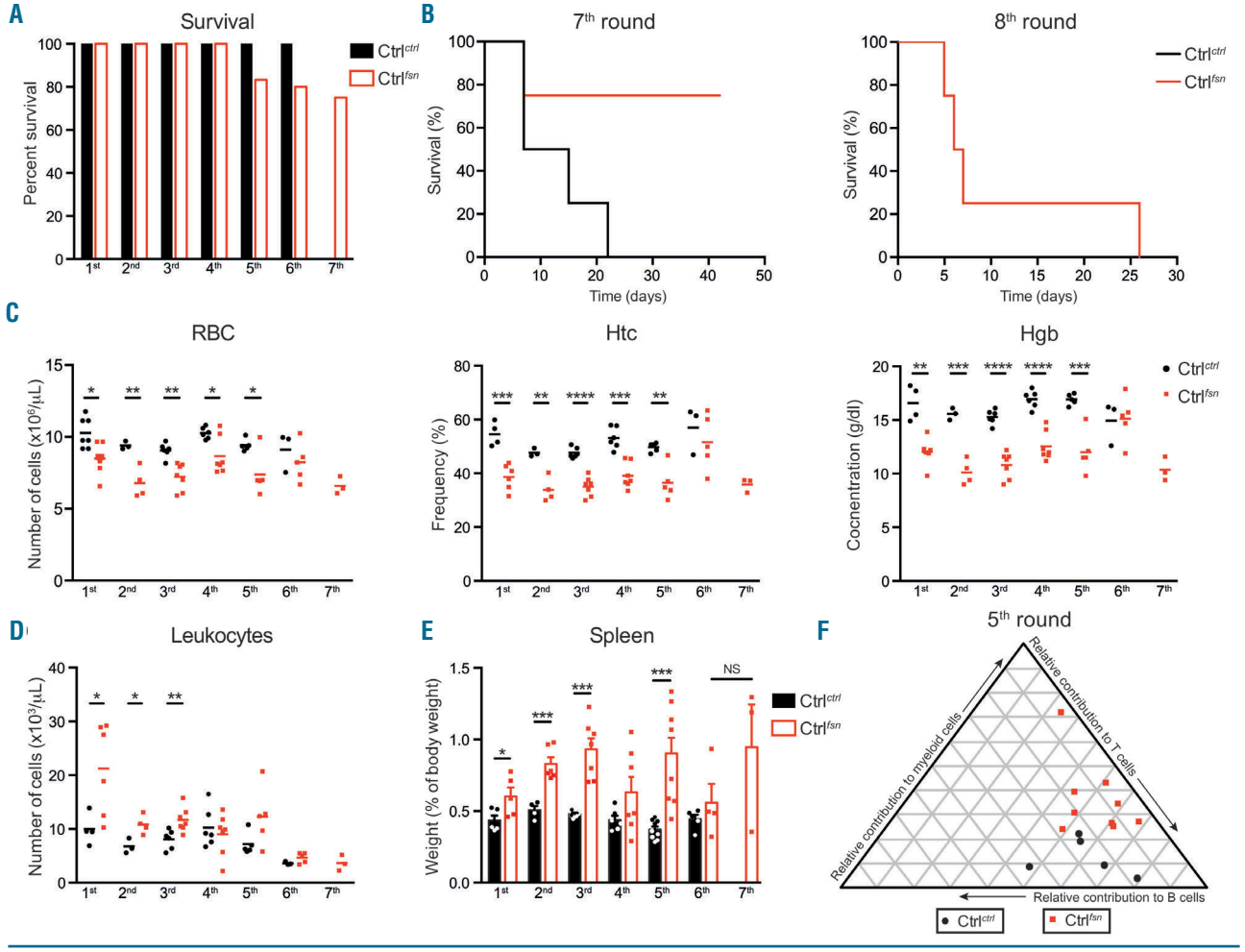


**Figure 5. Ttc7a-deficiency promotes self-renewal ability of hematopoietic stem cells.** Lethally irradiated mice were serially transplanted with 3-week old *ctrl* (Ctrl$^{ctrl}$ – black bars, dots and lines) or Ttc7a-deficient (Ctrl$^{fsn}$ – red bars, dots and lines) donor bone marrow (BM) cells (mean ± standard error of mean) *$P$<0.05; **$P$<0.01; ***$P$<0.001; ****$P$<0.0001 (two-tailed $t$-test). These data are representative of four independent experiments with at least four mice in each round of transplantation. (A) Percent survival of recipient mice across the seven transplantation cycles. (B) Survival of Ctrl$^{ctrl}$ and Ctrl$^{fsn}$ mice during the seventh round and Ctrl$^{fsn}$ mice during the eighth over time (n=4 for control- and n=10 for Ttc7a-reconstituted mice). (C-F) Red blood cell count, hematocrit, and hemoglobin level (C), leukocytes count (D) and spleen weight (E) across the transplantation cycles. (F) Relative contribution of myeloid compared to T- and B-lymphoid cells in the spleen of mice transplanted with BM cells that had undergone five transplantation cycles. RBC: red blood cells; Htc: hematocrit; Hgb: hemoglobin.

expressed genes, the most statistically significant differences were observed in the group of downregulated genes (Figure 6B). To determine the functional profile of the differentially expressed genes, we performed GSEA for genes with a fold change ≥1.5. GO analysis of the identified gene signature revealed a significant enrichment of genes in three main categories. Two categories are related to chromatin organization/modification and DNA damage repair; this observation fits with our recent finding that Ttc7a is a chromatin-binding nuclear factor involved in chromatin compaction and nuclear organization[28] (Figure 6C, D). Another category corresponds to genes involved

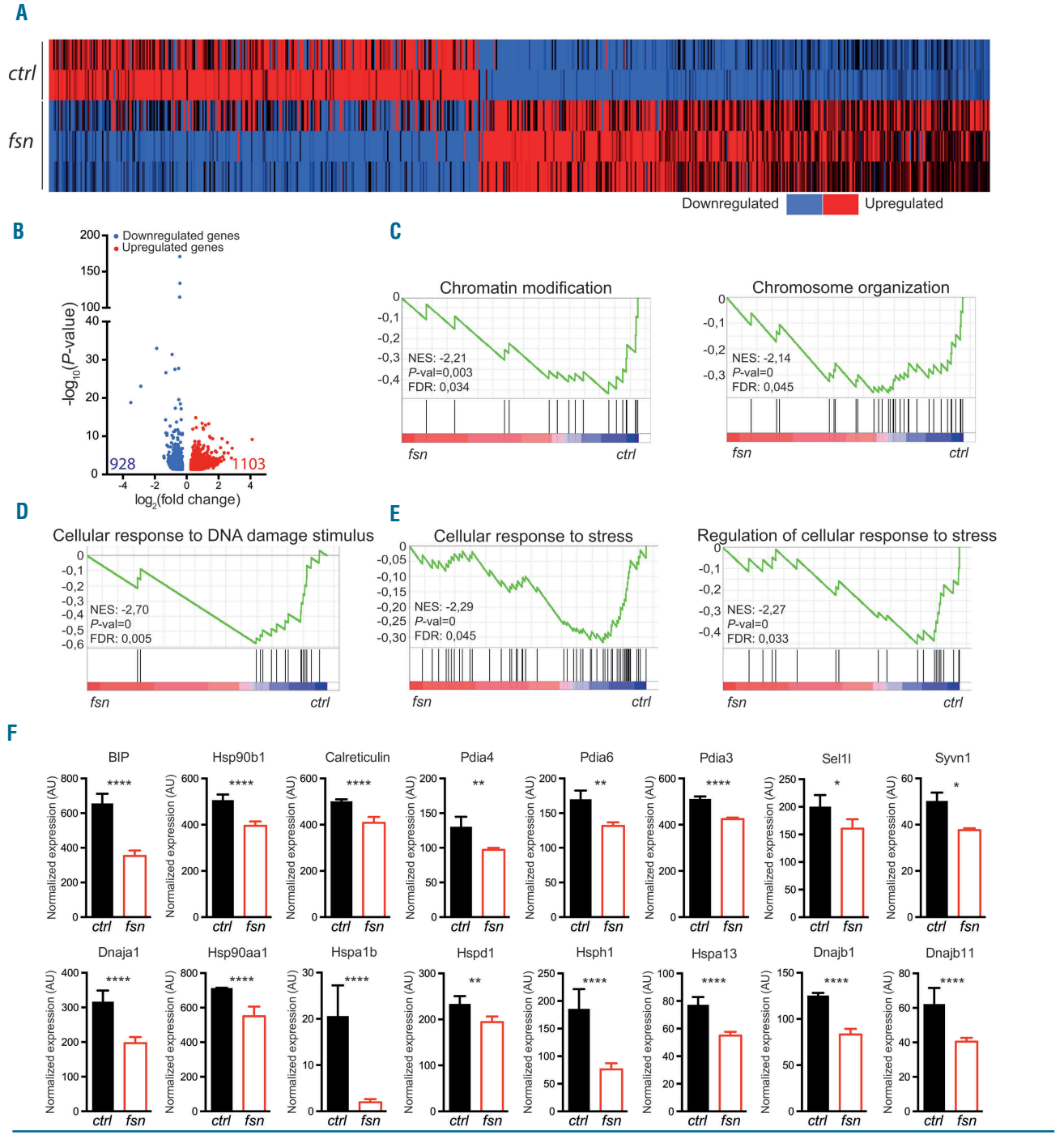


**Figure 6. Ttc7a deficiency results in reduced expression of endoplasmic reticulum stress response genes in hematopoietic stem cells.** RNA sequencing was performed on 3-week old control (*ctrl* – black bars) and Ttc7a-deficient (*fsn* – red bars) hematopoietic stem cell (HSC) transcripts. (A) Heatmap of relative expression of differentially expressed genes (fold change ≥1.2) in Ttc7a-deficient HSC compared to control. (B) Volcano plot of differentially expressed genes (fold change ≥1.2) in Ttc7a-deficient HSC compared to control HSC showing the adjusted *P*-value ($-\log_{10}$) *vs.* fold change ($\log_2$). Upregulated and downregulated genes are shown in red and blue, respectively. Total numbers in each group are indicated in red and blue, respectively. (C-E) Enriched gene sets in Ttc7a-deficient HSC compared to control HSC, as determined by gene set enrichment analysis of differentially expressed genes (fold change ≥1.5). (F) Normalized expression of endoplasmic reticulum stress response genes downregulated in Ttc7a-deficient HSC (fold change ≥1,2) **P*<0.05; ***P*<0.01; *****P*<0.0001 (LimmaVoom analysis). NES: normalized enrichment score; FDR: false discovery rate; AU: arbitrary units.

in cellular response to stress (Figure 6E). Our transcriptomic analysis also highlighted high expression levels of Ttc7a in HSC (*Online Supplementary Figure S6*). A growing body of evidence suggests that ER stress regulates the function of the HSC pool.[21] In particular, a recent study highlighted a link between ER stress perturbation in HSC and an elevated reconstitution capacity following BM transplantation.[29] Accordingly, we found that several effectors of the ER stress response were significantly downregulated in Ttc7a-deficient HSC, including the UPR master regulator Bip (Hspa5/GRP78), calreticulin, Pdia3, Pdia4, Pdia6, several Hsp, as well as Sel1l and Syvn1 which have been shown to regulate an ER-associated protein degradation (ERAD) pathway[30,31] (Figure 6F and *Online Supplementary Table S1*). Overall, our data suggest that Ttc7a loss affects the cellular response to ER stress in HSC.

## Ttc7a controls the response to stress in hematopoietic stem cells

ER stress is mainly triggered in response to altered protein homeostasis leading to pro-apoptotic or pro-survival responses. Notably, Ttc7a-deficient HSC had reduced lev-

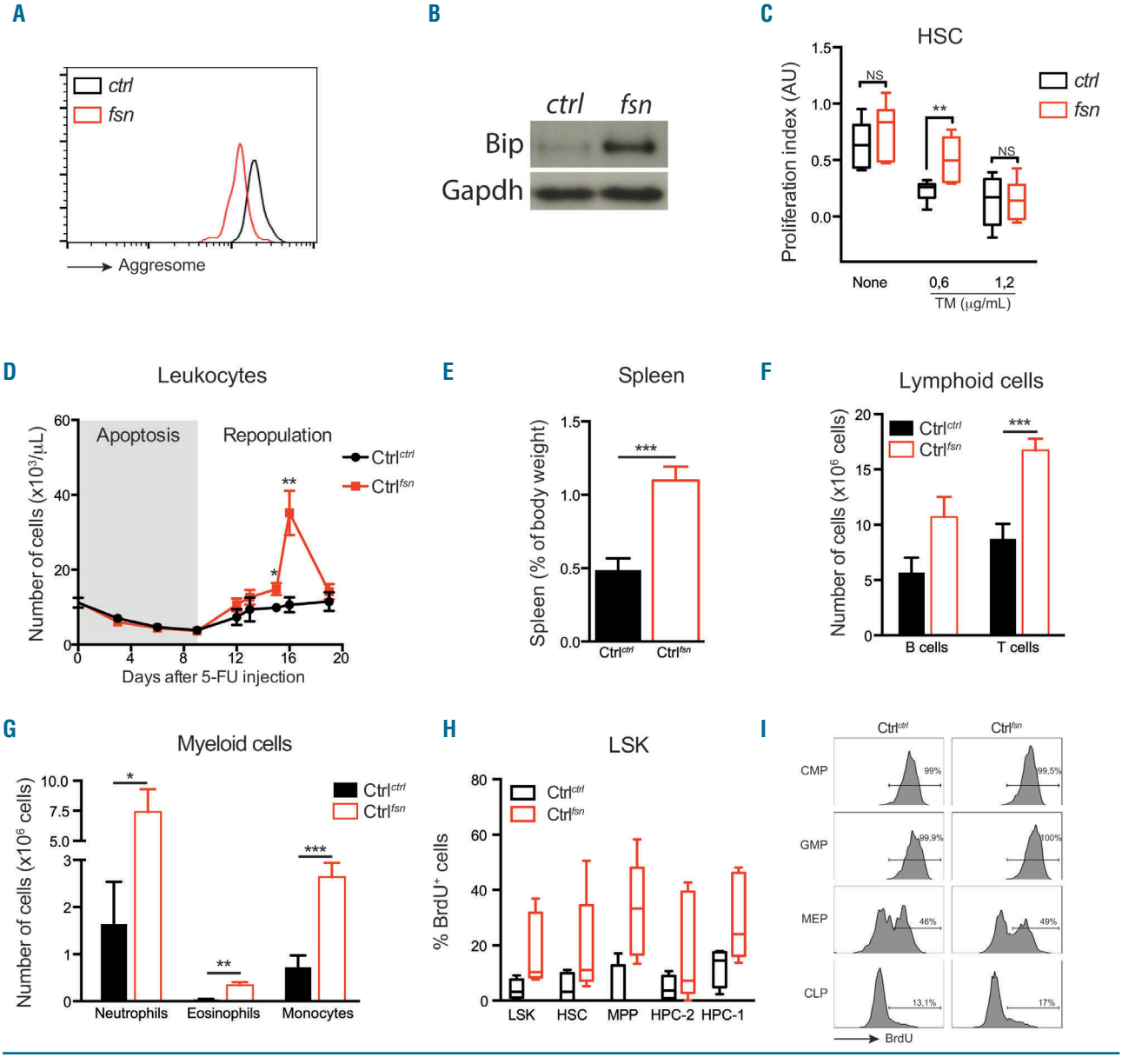


**Figure 7. Ttc7a controls the response to stress in hematopoietic stem cells.** (A) Representative histograms of protein aggregation level of 3-week old *ctrl* (black line) and *fsn* (red line) hematopoietic stem cells (HSC). (B) Protein expression of Bip in *ctrl* and *fsn* HSC after 3 days of *in vitro* expansion. (C) Proliferation index (calculated as the ratio between the number of cells at 48 h and 24 h) of HSC after Lin⁻ cells were sorted from *ctrl* (black bars) and Ttc7a-deficient (*fsn* – red bars) mice and cultured for 2 days with or without tunicamycin. $^{**}P<0.01$ (two-tailed *t*-test). (D-I) Ctrl$^{ctrl}$ (black line and bars) and Ctrl$^{fsn}$ (red line and bars) mice were analyzed after they had received a single intraperitoneal injection of 150 mg/Kg of 5-fluorouracil (5-FU), 12 weeks after bone marrow transfer. White blood cell count over time (D). Spleen size (E) and absolute number of lymphoid (F) and myeloid (G) cells in the spleen 15 days after 5-FU injection (n=9). Percentage bromodeoxyuridine (BrdU) incorporation in LSK subpopulations (H) and representative flow cytometry histograms of BrdU incorporation in LK (Lin⁻ Kit⁺) populations (I) 7 days after 5-FU injection. (n=10 for control- and n=12 for Ttc7a-reconstituted mice) $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ (two-tailed t-test). AU: arbitrary units; LSK: Lin⁻ Sca1⁺ cKit⁺ cells; MPP: multipotent progenitors; HPC: hematopoietic progenitor cells; CMP: common myeloid progenitors; GMP: granulocyte-monocyte progenitors; MEP: megakaryocyte-erythroid progenitors; CLP: common lymphoid progenitors.

els of protein aggregation compared to control HSC (Figure 7A). In keeping with this, *in vitro*-expanded *fsn* HSC had an elevated level of BIP protein (Figure 7B). These results suggest that Ttc7a loss could modify HSC susceptibility to ER stress. Therefore, to determine the impact of Ttc7a loss in the response of HSC to ER stress, we analyzed the proliferative capacity of Ttc7a-deficient HSC and progenitor cells (i.e., HSC, MPP, HPC-2 and HPC-1) upon chemical induction of ER stress *in vitro*. To do so, lineage-negative cells from 4-week old *fsn* and *ctrl* mice were cultured for 48 h in the presence or absence of tunicamycin, which blocks the synthesis of N-linked glycoproteins, leading to an accumulation of unfolded proteins and the induction of ER stress.[32] As expected, on day 2, tunicamycin treatment reduced the proliferation ability of control cells in a dose-dependent manner (Figure 7C). In contrast, at a low dose of tunicamycin, the proliferative capacity of Ttc7a-deficient HSC was significantly greater than that of control HSC (Figure 7C). These differences were particular to HSC, as Ttc7a-deficient MPP, HPC-2 and HPC-1 subsets had a similar response to tunicamycin as their control counterparts (*Online Supplementary Figure S7A*). The alterations in the ER stress response in Ttc7a-deficient HSC were not due to protein aggregation (Figure 7A and *Online Supplementary Figure S7B*), nor to low expression of the ER stress sensors and effectors (Ire1α, Perk, Atf6, etc.), as no differences were observed in our transcriptomic analysis (*Online Supplementary Figure S7C* and *Online Supplementary Table S1*). Surprisingly, the reduction in cell proliferation of *ctrl* HSC in response to tunicamycin was independent of apoptosis, in contrast to that of other progenitor cells. Apoptosis of Ttc7a-deficient HSC was reduced compared to that of unstimulated *ctrl* cells, and remained unchanged upon tunicamycin treatment (*Online Supplementary Figure S7D*). No differences were observed in other progenitor populations (*Online Supplementary Figure S7D*). Altogether, these data suggest that *ex vivo* purified Ttc7a-deficient HSC had a higher level of ER stress compared to their control counterparts.

Interestingly, we observed that the expression of Hsp70, a chaperone protein associated with broad cellular stresses, was also higher in *fsn* HSC than in controls (*Online Supplementary Figure S7E*). In order to determine whether Ttc7a regulates the cellular response to stress *in vivo*, we monitored the proliferative response of Ttc7a-deficient cells following the induction of stress by 5-FU. The depletion of cycling cells by 5-FU stimulates HSC to replenish peripheral leukocytes,[33,34] inducing a broad stress response in HSC, not limited to ER stress (e.g., oxidative stress, proliferative stress). We injected 5-FU into Ctrl$^{fsn}$ and Ctrl$^{ctrl}$ mice 3 months after BM transplantation and monitored the replenishment of peripheral leukocytes for 19 days. The Ttc7a-deficient and *ctrl* leukocyte counts fell until day 9 post-injection, and then increased. On day 15, Ttc7a-deficient leukocytes were growing significantly more strongly than *ctrl* cells, with a peak on day 16 (Figure 7D). Interestingly, the spleen of Ctrl$^{fsn}$ mice enlarged further after 5-FU treatment (Figure 7E, compared with Figure 3D), with higher lymphoid and myeloid counts (Figure 7F-G). To assess the proliferative response of Ttc7a-deficient HSC following stress injury, we assayed BrdU uptake by LSK subsets between day 6 and day 7 after 5-FU injection. The greater BrdU uptake in Ttc7a-deficient HSC, MPP, HPC-2 and HPC-1 (Figure 7H), suggested that Ttc7a controls the cell cycle progression of HSC under stress conditions. Strikingly, BrdU uptake did not differ in committed progenitors, CLP, CMP, GMP and MEP (Figure 7I). These results suggest that Ttc7a is involved in the regulation of the proliferative response of HSC under stress conditions but not in that of committed progenitor cells.

## Discussion

The present study revealed a previously unrecognized role for Ttc7a in the negative regulation of HSC function. Using murine transplantation models and Ttc7a-deficient HSC, we found that Ttc7a intrinsically regulates the maintenance and proliferation of HSC *in vivo*, and the subsequent homeostasis of downstream cell populations. We also found that *Ttc7a* expression in HSC is closely associated with the transcriptional response to ER stress.

HSC are the only cells capable of self-renewing and differentiating into all mature blood lineages. The quiescence of HSC must be tightly regulated in order to control proliferation, maintain normal homeostasis, and prevent stem cell exhaustion[35,36] Various intrinsic and cell-extrinsic regulatory factors of the HSC cell cycle have been described, such as phosphatase and tensin homologue (Pten) signaling, Wnt signaling and cytokine signaling.[37] Indeed, in mice that lack growth factor independent 1 (Gfi1),[38] Pten, forkhead box proteins 1, 3, 4, or M1[39] or other proteins,[20,36] excessive HSC proliferation is associated with stem cell exhaustion and the loss of self-renewal. In contrast, we found that mice reconstituted with Ttc7a-deficient progenitors exhibit a characteristic phenotype with enhanced HSC function, higher HSC-derived peripheral blood cell counts and no evidence of stem cell exhaustion when compared with Ttc7a-proficient HSC. Indeed, Ttc7a-deficient HSC were able to repopulate the hematopoietic system better in serial transplantation experiments, indicating that the self-renewal of Ttc7a-deficient HSC is not compromised by repeated rounds of proliferation. Although this situation clearly differs from the above-mentioned knock-out mice, a few similar observations have been reported after the deletion of the cyclin-dependent kinase 4 inhibitor C (CDKN2C),[40] the ubiquitin-mediated protein degradation Cbl[41] and Itch,[41] and the transcription factors Hif1a[42] and Egr1.[43] The loss of these proteins enabled the maintenance of HSC, despite an increase in the cells' proliferative capacity. However, the specific mechanisms by which these proteins regulate HSC function remain largely unknown.

Our findings support a role for the ER stress response in the enhanced function of Ttc7a-deficient HSC. Since long-lived HSC are particularly sensitive to stress stimuli, their response must be tightly controlled in order to prevent either a loss of function or the clonal persistence of oncogenic mutations. It has been shown that HSC are enriched in components of the UPR pathway. Upon exposure to acute stress *in vitro*, HSC are more prone to apoptosis, via upregulation of the canonical UPR genes, than related progenitors that have lost their self-renewal capacity.[21,44] Along these lines, the ectopic expression of developmental pluripotency-associated 5 (Dppa5) was associated with enhanced HSC function, via suppression of the ER stress response (by downregulating the expression of ER stress chaperones) and the subsequent apoptotic signals.[29] However, UPR activation can also have an anti-apoptotic outcome in HSC. It has been shown that stimulation of

estrogen receptor α (ERα) confers HSC resistance to proteotoxic stress by activating the Ire1α-Xbp1 branch of the UPR and promoting the cells' reconstituting potential.[24] In Ttc7a deficient HSC, expression levels of ER stress response genes were abnormally low, whereas the level of the Bip chaperone protein was increased. Furthermore, Ttc7a deficient HSC were less sensitive to stress induction than Ttc7a-proficient-HSC both *in vitro* and *in vivo*. A similar phenomenon was observed in mouse liver in which mild chronic ER stress decreases the mRNA level of Bip while maintaining its protein level. This response allows hepatocytes to avoid the overproduction of UPR effectors that could lead to apoptosis.[45] It is, therefore, tempting to speculate that Ttc7a deficiency could be associated with mild chronic stress. In this context, the increased resistance of *fsn* HSC to tunicamycin could be caused by a cellular adaptive response aiming to increase the threshold of ER stress sensitivity, and ensure cell survival. Interestingly, HSC exposed to other sources of persistent cellular stress develop mechanisms of stress resistance resulting in increased self-renewal capacity and reconstitution potential.[46] Knowing that TTC7A stabilizes several interacting proteins, the role of additional components, altered as a consequence of Ttc7a deficiency, cannot be excluded. Ttc7a could represent a pivotal connection between ER stress regulation and the maintenance of HSC functions.

Along with an abnormally proliferative hematopoietic system, fsn mice develop hyperplasia of the epidermis and the gastric epithelium. Notably, stem cells from other tissues can similarly sense ER stress and activate the UPR pathway to control self-renewal and differentiation. This has been shown for the intestinal epithelium in particular, and several lines of evidences support the concept whereby ER stress and UPR activity regulate the differentiation of intestinal stem cells.[47] An attractive hypothesis would be that the other phenotypic manifestations that characterized Ttc7a deficiency might be due to perturbation of the ER stress response.

In summary, our results show that Ttc7a has a critical but previously unrecognized role as a regulator of HSC homeostasis and function through the regulation of the ER stress response.

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