Nfix* promotes survival of immature hematopoietic cells via regulation of *c-Mpl

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Abstract

Hematopoietic stem and progenitor cells (HSPC) are necessary for life-long blood production and replenishment of the hematopoietic system during stress. We recently reported that nuclear factor I/X (*Nfix*) promotes HSPC survival post-transplant. Here, we report that ectopic expression of *Nfix* in primary mouse HSPCs extends their *ex vivo* culture from about 20 days to 40 days. HSPCs overexpressing *Nfix* display hypersensitivity to supportive cytokines and reduced apoptosis when subjected to cytokine deprivation relative to controls. Ectopic *Nfix* resulted in elevated levels of *c-Mpl* transcripts and cell surface protein on primary murine HSPCs as well as increased phosphorylation of STAT5, which is known to be activated down-stream of c-MPL. Blocking c-MPL signaling by removal of thrombopoietin or addition of a c-MPL neutralizing antibody negated the anti-apoptotic effect of *Nfix* overexpression on cultured HSPCs. Further, NFIX was capable of binding to and transcriptionally activating a proximal *c-Mpl* promoter fragment. In sum, these data suggest that NFIX-mediated up-regulation of *c-Mpl* transcription can protect primitive hematopoietic cells from stress *ex vivo*.

Introduction

Hematopoietic stem and progenitor cells (HSPCs) are necessary for replenishing the blood system during native hematopoiesis and times of stress, such as during a hematopoietic stem cell transplant (HSCT), which is employed routinely in the clinic to treat hematologic disease. Transplant-induced stress exerted on HSPCs has been well documented, resulting in reduced stem cell pools and decreased self-renewal ability [1-3], but regulation of their ability to overcome this stress and successfully replenish hematopoiesis is not well understood. Cell-extrinsic and cell-intrinsic regulators of HSCT have been implicated in HSPC self-renewal and mobilization and homing [4-6]. Better understanding the mechanisms that allow HSPC engraftment post-transplant will facilitate efforts to improve transplantation protocols and clinical outcomes.

Recently, our lab completed a functional screen that identified 17 novel regulators of murine HSCT, including the nuclear factor I (NFI) family member, *Nfix* [7]. NFI family members function as transcriptional activators and repressors [8,9]. Although *Nfix*$^{-/-}$ mice display no overt hematopoietic phenotypes during native hematopoiesis, shRNA-mediated knock-down or genetic deletion of *Nfix* in HSPCs results in a profound loss of competitive *in vivo* repopulating potential, a loss of niche retention post-transplant and increased apoptosis [10]. NFIX and NFIA, a related family member, have also been implicated in regulating hematopoietic lineage fate decisions, with ectopic expression of *NFIA* or *Nfix* promoting HSPC commitment to erythropoiesis or myelopoiesis and depletion promoting granulopoiesis or lymphopoiesis, respectively [11,12].

Although *Nfix* is clearly required by HSPCs during HSCT, little is known about how NFIX regulates HSPCs at the molecular and cellular level. Here we report that *Nfix* can promote *ex vivo* growth, cytokine hypersensitivity, and survival of primitive hematopoietic populations *ex vivo*. We further demonstrate that these effects are in part mediated via up-regulation of the thrombopoietin (TPO) receptor, *c-Mpl*, thus revealing NFIX as a novel transcriptional regulator of *c-Mpl* and illuminating one molecular pathway targeted by NFIX in HSPC.

Materials and Methods

Complete materials and methods can be found in the Supplemental Data.

Results and Discussion

We have previously shown that *Nfix* is critical to HSPC survival post-transplantation [10]. To further interrogate the role of *Nfix* in HSPC biology, we ectopically expressed *Nfix* in Lineage$^-$ Sca-1$^+$c-Kit$^+$ (LSK) cells cultured under serum-free conditions (Supporting Information Fig. S1A). During culture, cells were assessed for growth rate, retention of vector+ (NFIX+) cells, and persistence of an LSK phenotype (Fig. 1A). *Nfix* was over-expressed 20-fold in NFIX+ cells, while other NFI genes remained unperturbed (Fig. 1B). Remarkably, ectopic *Nfix* expression prolonged hematopoietic cell cultures two-fold, allowing cells to persist up to 40 days *ex vivo* (Fig. 1C). However, the relative growth of control and NFIX+ cultures did not significantly diverge until control cells began to display culture exhaustion (p = 0.036) (Fig. 1C). During this extended time, a steady selection for NFIX+ cells was apparent (Fig. 1D). These data suggest that *Nfix* can promote the extended cell culture of hematopoietic progenitors.

 By seven days of culture, the majority of cells in both control and NFIX+ cultures had lost the LSK cell surface phenotype (Fig. 1E), with immunophenotypic LSK cells being almost completely lost from culture by day 14 (Supporting Information Fig. S1B, S1C). However, *Nfix* overexpression appeared to accelerate the loss of this phenotype, evident by the appearance of a Sca-1$^-$c-Kit$^-$ population in NFIX+ cells and reduced overall levels of cell surface c-Kit, relative to control (Fig. 1Ei, 1Eii, Supporting Information Fig. S1D). These data suggest that *Nfix* might promote LSK cell differentiation during *ex vivo* culture. At day seven of culture, control and NFIX+ cells displayed a similar blast-like morphology, with NFIX+ cells retaining this morphology through day 30 of culture (Supporting Information Fig. S2). However, LSK cells overexpressing *Nfix* displayed a loss of *in vivo* competitive hematopoietic repopulating potential,

a myeloid bias in peripheral blood production and a loss of colony-forming unit (CFU) potential

compared to control cells by seven days of culture, with a significant loss in CFU potential by 21

days in culture (p = 0.023) (Supporting Information Fig. S3A-D and S4). A majority of expanded

control and NFIX+ cells were negative for all major lineage markers (excepting CD8) and

expressed c-Kit and CD71, which is a marker of proliferating progenitors (Supporting

Information Fig. S5A-B). High CD71 expression can also be indicative of erythroid progenitors,

and while NFIX+ cells show a significantly higher percentage of a CD71^{hi} population compared

to controls (p = 0.017), this population represents only a small portion (15-25%) of cells

throughout the entirety of the culture (Supporting Information Fig. S5C). Together, these data

suggest that *Nfix* promotes differentiation of LSK cells towards a heterogeneous immature

progenitor population that ultimately lacks CFU potential, suggesting arrested differentiation

potential.

As *Nfix*-deficient HSPCs display elevated apoptosis post-transplant [10], we next tested if

ectopic *Nfix* protects primitive hematopoietic cells from apoptosis during *ex vivo* culture.

Towards this, NFIX+ HSPCs were cultured under normal or reduced cytokine conditions and

monitored for growth rate, NFIX+ cell selection, cell cycle, and apoptosis (Fig. 2A). Control

cells cultured in reduced cytokines displayed a significantly lower growth rate by day 13 (p =

0.048) and an attenuated culture lifespan relative to cells maintained at normal cytokine levels

(Fig. 2B). Remarkably, reduced cytokine levels had no effect on the extended culture of NFIX+

cells (Fig. 2B). NFIX+ cells cultured under reduced cytokines were selected for at a significantly

accelerated rate compared to NFIX+ cells cultured under normal cytokine levels (p = 0.041)

(Fig. 2C). There were no significant differences in cell cycle status between control and NFIX+

cells regardless of cytokine levels (Fig. 2D), suggesting that the reduced growth rate of cytokine-deprived control cells was not due to a reduction in cycling. However, control cells displayed a significant increase in apoptosis (p = 0.032) when cultured in reduced cytokines (Fig. 2E). In contrast, the apoptotic status of NFIX+ cells was unaffected by reduced cytokines (Fig. 2E), even in immunophenotypic HSPCs (Supporting Information Fig. S6). These data reveal that *Nfix* promotes primitive hematopoietic cell survival *ex vivo*.

We previously observed by global gene expression analyses [10] that *Nfix* knockdown in HSPC reduced expression of multiple genes implicated in HSPC survival and maintenance including *c-Mpl*, a known regulator of HSC maintenance in the bone marrow niche that has been shown to affect apoptosis via multiple downstream signaling cascades [13-16]. c-MPL is the receptor for TPO, which is added as a supplement to our *ex vivo* serum-free cultures of HSPCs. To further explore possible regulation of *c-Mpl* levels by NFIX, we assessed the expression of *c-Mpl* in NFIX+ cells after seven days of *ex vivo* culture by qRT-PCR and flow cytometry (Fig. 3A, 3B). We found that *c-Mpl* transcripts increased two-fold in NFIX+ cells (p = 0.028) (Fig. 3A). We also observed a two-fold increase in c-MPL cell surface antigen on NFIX+ cells relative to control via flow cytometry (p = 0.042) (Fig. 3Bi, 3Bii). Also, from the number of additional HSPC genes previously observed to be perturbed by loss of *Nfix* [10], *Erg* was significantly upregulated (p = 0.022) (Supporting Information Fig. S7). TPO/c-MPL signaling is classically involved in megakaryopoiesis and platelet production [17-19]. Thus, as expected, NFIX+ cells also displayed a substantial increase in the cell-surface antigen CD41 (Fig. 3C), a known marker of megakaryocytes [20]. Since our data suggested that *Nfix* was driving HSPC towards an immature progenitor population (Supporting Information Fig. S2-S5), we further interrogated our

cultures for CFU-Megs. NFIX+ cells appeared to generate more CFU-Megs than control cells after seven days of culture (p = 0.052), but the absolute frequency of CFU-Megs in NFIX+ cultures was minute (0.016), revealing that megakaryocytic progenitors with colony forming potential are rare in NFIX+ cultures (Supporting Information Fig. S8A-B). This is consistent with the observed low percentage of immunophenotypic megakaryocyte progenitors (c-Kit$^+$Sca-1$^-$CD127$^-$CD9$^+$CD32/CD16^{lo}CD41$^+$) (Supporting Information Fig. S8C) [21]. Indeed, by day 30 almost no CFU-Megs were present in NFIX+ cultures (Supporting Information Fig. S8A).

TPO/c-MPL can activate JAK/STAT, PI3K/AKT, and MAPK/ERK downstream signaling pathways [22]. To determine if NFIX-mediated up-regulation of *c-Mpl* also increased TPO/c-MPL signaling, we examined the phosphorylation status of STAT5, AKT, and ERK1/2 via flow cytometry. NFIX+ cells displayed significant enhancement of STAT5 phosphorylation compared to control cells (p = 0.018), while AKT trended towards enhanced phosphorylation after prolonged TPO treatment (Fig. 3D, Supporting Information Fig. 9A). Also, NFIX+ cells showed no difference in phosphorylation of ERK1/2 compared to control cells (Fig. 3D, Supporting Information Fig. 9A). This suggests that the anti-apoptotic effects displayed by NFIX+ HSPC may be mediated through the STAT5 signaling pathway. Indeed, expression of *Bcl-X$_L$*, an anti-apoptotic factor induced by STAT5 [23], was also significantly upregulated in NFIX+ cells by two weeks of culture compared to controls (p = 0.0038) (Supporting Information Fig. S9B). In sum, these data reveal that up-regulation of *Nfix* induces both *c-Mpl* expression and signaling downstream of c-MPL in primitive hematopoietic cells.

Examination of the *c-Mpl* locus revealed palindromic NFI binding sites within the *c-Mpl* promoter (Figure 3Eii). Promoter analysis by TRANSFAC revealed full NFI consensus sites 101 and 127 nucleotides upstream of the *c-Mpl* transcription start site (TTS, +1) (Figure 3Eii). NFI members are known to bind both full and half NFI consensus sites [24]. Two half sites were identified 18 and 189 nucleotides upstream of the *c-Mpl* TSS (Figure 3Eii). To assess NFIX transcriptional activity against these putative NFI binding sites in the *c-Mpl* proximal promoter, a *c-Mpl* 243 bp genomic fragment 5' of the *c-Mpl* promoter containing the four identified putative NFI binding sites was sub-cloned into the pGL4.14 promoterless luciferase vector. Transient transfection of this vector into K562 cells yielded nearly three-fold higher levels of promoter activity when co-transfected with MND-NFIX relative to co-transfection with MND-Control (Figure 3Eii). This enriched activity was diminished when the half NFI site (-189) furthest from the TSS was removed and was significantly reduced by the additional removal of the two full NFI sites (-127 and -101) (p = 0.0056) (Figure 3Eii). Further, chromatin immunoprecipitation (ChIP) was used to show direct NFIX binding to the *c-Mpl* proximal promoter in the HPC5 bone marrow derived cell line. Primers were designed and validated to amplify the promoter region containing two full NFI consensus sites. In Figure 3Ei, a near 9-fold enrichment is observed in samples where a FLAG-tagged NFIX is present compared to controls. These data suggest that NFIX may directly activate *c-Mpl* promoter activity in a hematopoietic cell line.

To determine if the anti-apoptotic effects of ectopic *Nfix* in primitive hematopoietic cells depends on enhanced TPO/c-MPL signaling, we cultured NFIX+ HSPC in reduced cytokines while also either removing TPO or blocking ligand binding to c-MPL via a neutralizing antibody (AMM2) [13] for 72 hours. Although removal of TPO and neutralization of c-MPL led to

reduced cell expansion in both control and NFIX+ cultures, NFIX+ cultures were significantly more sensitive to the loss of c-MPL stimulation after TPO removal or the addition of AMM2 (p = 0.0021 and 0.033, respectively) (Fig. 4A). The selection for NFIX+ cells under reduced cytokines was also lost when TPO/c-MPL signaling was blocked by TPO removal or the addition of AMM2 (p = 0.0054 and 0.0019, respectively) (Fig. 4B), suggesting an enhanced reliance on TPO/c-MPL signaling for expansion of NFIX+ cells. NFIX+ cells display an accelerated loss of the LSK immuno-phenotype (Fig. 1E), possibly due to enhanced differentiation towards a downstream progenitor (Supporting Information Fig. S3-S5). This loss of immuno-phenotype was mostly due to down-regulation of c-Kit cell surface expression (Fig. 1Ei). When NFIX+ cells were cultured in the absence of TPO or the presence of AMM2, c-Kit was no longer rapidly down-regulated relative to control (Fig. 4C). Finally, while apoptosis was relatively unaffected by a loss of c-MPL signaling in control cells, NFIX+ cells displayed a significant increase in apoptosis after TPO removal (p = 0.045) or addition of AMM2 (p = 0.0098) (Fig. 4D). These data reveal that NFIX-mediated up-regulation of c-MPL, and subsequent downstream signaling, functionally contributes to *Nfix*-induced protection from apoptosis and accelerated differentiation in primitive hematopoietic cells *ex vivo*.

Conclusion

In this study we have utilized *ex vivo* culture of HSPCs to further interrogate the molecular regulation of HSPCs by *Nfix*, which is required for their *in vivo* repopulation potential [10]. Primitive hematopoietic cells overexpressing *Nfix* persist in culture significantly longer than control cells, even when severely deprived of cytokines. We show that this persistence is due to enhanced survival that is mediated, in part, by up-regulation of the TPO receptor, *c-Mpl*, and correlates with our previous finding that loss of *Nfix* is detrimental to HSPC survival post-transplant [10]. *Nfix* appears to promote differentiation of cultured LSK cells towards a heterogeneous mixture of immature progenitors that lack transplantation and CFU potential (Supporting Information Fig. S2-S5, S8), likely indicative of a differentiation block. It is also possible that *Nfix* expression selects for a cell in these cultures that depends on c-MPL signaling for survival. However, the enhanced survival of NFIX+ cells can also be observed in immunophenotypic HSPCs (Supporting Information Fig. S6), demonstrating that this phenomenon is not confined to a particular population.

We further demonstrate that NFIX may function as a transcriptional regulator of *c-Mpl*. Indeed, NFIX was capable of activating a promoter containing multiple NFI consensus binding sites located upstream of *the c-Mpl* promoter. We also show NFIX-FLAG directly associated with the proximal promoter. NFIX may also regulate downstream effectors of the TPO/c-MPL signaling pathway, as *Stat5a* is significantly upregulated in NFIX+ cells compared to controls (p = 0.0012, Supporting Information Fig. 9C). However, this effect may be indirect as there are no NFI consensus binding sites proximal to the *Stat5a* promoter (data not shown). NFI proteins favor binding in the proximal promoter region.

c-Mpl is a well-known regulator of HSPC function, as it is required for the maintenance of adult quiescent HSCs and protection from DNA-damage induced apoptosis *in vivo* [13-15]. Given that *Nfix* is required for HSPC survival during transplant hematopoiesis [10], our data further implicate *Nfix* as a novel regulator of this important HSPC regulatory axis. Further work will be required to determine if *Nfix*-mediated regulation of HSPC responsiveness to TPO contributes to loss of HSPC survival and niche retention following transplant *in vivo*.

Acknowledgements

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Figure 1. *Nfix* induces longevity in HSPC *ex vivo* culture. (A) Experimental schematic. 48 hours post-transduction, LSK cells transduced with MND-Control or MND-*Nfix* were re-plated in 96-well non-tissue culture treated plates in serum-free expansion medium (SFEM) supplemented with mSCF, mTPO, mIGF-2, and hFGF-a (STIF). Every 48-72 hours of culture, cells were counted, assessed for GFP+ cells, and passaged 1:4. Cells were also periodically assessed for LSK immuno-phenotype. (B) Relative expression of NFI-family genes in NFIX+ cells compared to control cells, quantified by qRT-PCR (n = 3). *Tbp* was used as a housekeeping gene. (C) Relative growth of control and NFIX+ cells during *ex vivo* culture (n = 4). Dotted line indicates the divergence in relative growth between control and NFIX+ cells. (D) GFP percentage of control and NFIX+ cells during *ex vivo* culture, assessed by flow cytometry (n = 4). (E) Percentage of control and NFIX+ cells with an LSK immuno-phenotype at day seven of *ex vivo* culture, depicted as a (i) representative dot plot and (ii) bar plot (n = 6). All values represent mean ± standard deviation. NS denotes not significant.

Figure 2. HSPCs overexpressing *Nfix* can withstand cytokine deprivation and display reduced apoptosis during *ex vivo* culture. (A) Experimental schematic. 48 hours post-transduction, LSK cells transduced with MND-Control or MND-*Nfix* were re-plated in 96-well non-tissue culture treated plates in serum-free expansion medium (SFEM) supplemented with either normal (100%) or reduced (25%) levels of STIF cytokines. Every 48-72 hours of culture, cells were counted, assessed for GFP+ cells, and passaged 1:4. Cells were also assessed by flow cytometry for apoptosis via Annexin V and cell cycle via DAPI at day seven of culture. (B) Relative growth of control and NFIX+ cells during *ex vivo* culture (n = 4). Dotted line indicates the divergence in relative growth between control 100% and control 25% cells. (C) GFP

percentage of control and NFIX+ cells during *ex vivo* culture, assessed by flow cytometry (n =

4). Dotted line indicates significant selection of NFIX+ cells under 25% cytokines. (D) Cell

cycle analysis of GFP+ control or NFIX+ cells at day seven of *ex vivo* culture (n = 3). (E)

Percentage of GFP+ apoptotic cells within control or NFIX+ cell cultures at day seven of *ex vivo*

culture (n = 6). All values represent mean ± standard deviation. NS denotes not significant.

Figure 3. NFIX up-regulates *c-Mpl* expression and downstream signaling in HSPC during

***ex vivo* culture.** (A) Expression of (i) *c-Mpl* in LSK cells transduced with MND-*Nfix* relative to

controls at day seven of culture, quantified by qRT-PCR (n = 3). *Tbp* was used as a

housekeeping gene. (B) Percentage of c-MPL+ cells at four days of *ex vivo* culture for control

and NFIX+ HSPCs, depicted as (i) representative dot plots and (ii) bar plots (n = 3). (C) Relative

level of CD41 cell surface expression in NFIX+ cells compared to controls after four days of *ex*

vivo culture, measured by flow cytometry as gMFI, depicted as (i) representative fluorescence

histogram and (ii) bar plot (n = 3). (D) Relative levels of STAT5, AKT, and ERK1/2

phosphorylation in NFIX+ cells compared to controls after four days of *ex vivo* culture,

measured by flow cytometry as geometric mean fluorescence intensity (gMFI). Depicted as (i)

representative fluorescence histograms and (ii) bar plots (n = 3). (E) (i) Quantitative ChIP

analysis of *c-Mpl* proximal promoter in HPC5 cells. Data are presented as a percentage of total

input chromatin (n = 3). (ii) *Left*, Schematic representation of the *c-Mpl* promoter with half and

full NFI consensus sites cloned into luciferase reporter backbone pGL4.14. *Right*, Results

showing luciferase activity normalized to Renilla luminescence and relative to MND-control

samples (n = 3-5). Values represent mean ± standard error. NS denotes not significant.

Figure 4. The anti-apoptotic effect of *Nfix* in HSPC depends on c-MPL signaling. (A) Relative number of cells in control and NFIX+ cultures (i) with or without TPO and (ii) with or without AMM2 72 hours after replating (TPO, n = 3; AMM2, n = 6). Bar indicates significant difference in the extent of cell loss after 72 hours of culture between control and NFIX+ cells. (B) Percentage of GFP+ cells in control and NFIX+ cultures (i) with or without TPO and (ii) with or without AMM2 72 hours after replating (TPO, n = 3; AMM2, n = 6). (C) Representative fluorescence histograms of GFP+ control and NFIX+ cultures to illustrate shift in c-KIT intensity 72 hours after removal of mTPO or addition of AMM2 (TPO, n = 3; AMM2, n = 6). (D) Relative levels of apoptosis in GFP+ compartment of control and NFIX+ cultures (i) with or without TPO and (ii) with or without AMM2 72 hours after replating (TPO, n = 3; AMM2, n = 6). All values represent mean ± standard deviation. NS denotes not significant.

Supplemental Figure Legends

Supplemental Figure 1. NFIX overexpression promotes accelerated differentiation of LSK cells during *ex vivo* culture. (A) FACS plots depicting the sorting schematic for freshly isolated LSK cells. (B) LSK immunophenotype of control and NFIX+ cells at day 0, 7, and 14 of culture depicted a representative dot plot from three independent experiments. (C) LSK immunophenotype of control and NFIX+ cells at day 14 of culture depicted as bar plot (n = 3). (D) One-way FACS histogram depicting a reduction in c-KIT+ cells among the lineage negative population of control and NFIX+ cells after seven days of *ex vivo* culture. All values represent mean ± standard deviation. NS denotes not significant.

Supplemental Figure 2. HSPCs overexpressing *Nfix* display an immature blast-like morphology similar to control cells. Romanowsky stain of fresh bone marrow (BM) LSK cells, culture day seven (D7) GFP+ MND-Control, D7 GFP+ NFIX+ cells, and day thirty (D30) GFP+ NFIX+ cells. Representative images from two independent experiments are shown. Scale bars represent 50μm.

Supplemental Figure 3. HSPCs overexpressing *Nfix* fail to repopulate the bone marrow of irradiated recipients and display a myeloid bias in lineage distribution. (A) Schematic displaying competitive transplantation assay to assess hematopoietic repopulation potential of HSPC. CD45.2 "test" LSK cells were harvested from bone marrow and transduced with either MND-control or MND-*Nfix* lentiviral vectors. CD45.1 "competitor" LSK cells were mock transduced. 24 hours post-transduction, 5000 test and 5000 competitor cells were harvested and

transplanted into irradiated recipients. (B) Percentage of CD45.2 "test" cells in the peripheral

blood of transplanted recipients over a 16 week period. (C) Percentage of GFP+ cells within

CD45.2 "test" cells in the peripheral blood of transplanted recipients over a 16 week period. (D)

Percentage of T-, B-, and myeloid cells within CD45.2 "test" cells in the peripheral blood of

transplanted recipients over a 16 week period.

Supplemental Figure 4. HSPC overexpressing *Nfix* display reduced CFU potential. (A)

Frequency of colony-forming units among GFP+ control and NFIX+ cells cultured for seven

days *ex vivo* (n = 3). (B) Frequency of colony-forming units among GFP+ control and NFIX+

cells cultured for 21 days *ex vivo* (n = 3). The frequency of colony forming units refers to the

number of colonies scored divided by the total number of cells plated in methylcelluose. All

values represent mean ± standard deviation. NS denotes not significant.

**Supplemental Figure 5. *Nfix*-overexpressing cells display no major lineage markers and an

immature progenitor immuno-phenotype.** (A) Percentage of lineage^{+} cells among GFP+

control and NFIX+ cells as one (n = 3), three (n = 2), and four (n = 3) weeks in *ex vivo* culture.

(B) Percentage of c-Kit^{+} CD71^{+} cells among GFP+ control and NFIX+ cells at various time-

points during *ex vivo* culture. (C) Representative FACS plot depicting the percentage of CD71^{hi}

cells in GFP+ control and NFIX+ cultures at day seven and day 30 of *ex vivo* culture. All values

represent mean ± standard deviation. Note: all comparisons in (A) are not significant.

Supplemental Figure 6. LSK cells overexpressing *Nfix* display reduced apoptosis under cytokine deprivation during *ex vivo* culture. Percentage of GFP+ apoptotic cells within control or NFIX+ LSK cells at day seven of *ex vivo* culture (n = 3). All values represent mean ± standard deviation. NS denotes not significant.

Supplemental Figure 7. *Nfix* overexpression affects the expression of other known regulators of HSPC biology. Relative expression of several regulators of HSPC biology in NFIX+ cells compared to control cells known to be down-regulated upon shRNA-induced *Nfix* knockdown [10] at day seven of *ex vivo* culture (n = 3). All values represent mean ± standard deviation. NS denotes not significant.

Supplemental Figure 8. HSPCs overexpressing *Nfix* are not enriched for megakaryocyte progenitors or CFU-Megs. (A) Frequency of GFP+ CFU-Megs from control day seven, NFIX+ day seven, and NFIX+ day 30 *ex vivo* cells (n = 2). (B) Representative images of CFU-Megs from control day seven, NFIX+ day seven, and NFIX+ day 30 *ex vivo* cells (n = 2). (C) Percentage of megakaryocyte progenitors (c-Kit$^+$Sca-1$^-$CD127$^-$CD9$^+$CD32/CD16^{lo}CD41$^+$) among GFP+ control and GFP+ NFIX+ cells (n = 4). All values represent mean ± standard deviation. Scale bars represent 50μm. MND-Control D7 = 100X magnification; MND-*Nfix* D7 = 100X magnification; MND-*Nfix* D30 = 200X magnification.

Supplemental Figure 9. NFIX+ cells display enhanced TPO/c-MPL signaling sensitivity to mTPO exposure. (A) Relative phosphorylation status of STAT5, AKT, and ERK1/2 in NFIX+ cells during a time-course of mTPO exposure following cytokine starvation, as measured by phosphoflow (STAT5, ERK1/2: n = 4; AKT: n = 3). gMFI: Geometric mean fluorescence intensity. (B) Relative expression of *Bcl-xL* in NFIX+ cells compared to control cells at different time points during *ex vivo* culture, quantified by qRT-PCR (n = 3). *Tbp* was used as a housekeeping gene. (C) Relative expression of *Stat5a* and *Stat5b* in NFIX+ cells compared to control cells at day seven of *ex vivo* culture, quantified by qRT-PCR (n = 6). *Tbp* was used as a housekeeping gene. All values represent mean ± standard deviation. NS denotes not significant.

Graphical Abstract Legend

Hematopoietic stem and progenitor cells (HSPC) are necessary for lifelong blood production and replenishment of the hematopoietic system during stress. Here, we show that nuclear factor I/X (*Nfix*) is capable of protecting HSPC from stress-induced apoptosis during *ex vivo* culture. This protection relies on proper thrombopoietin/c-MPL signaling, as NFIX directly regulates *c-Mpl* expression.

Materials and Methods

Mice

C57BL/6J and C57BL/6.SJL-PtprcaPep3b/BoyJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free facility. All animal experiments were carried out according to procedures approved by the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee.

Vector construction

Mouse *Nfix* cDNA was purchased from GE Healthcare Dharmacon Inc. (Lafayette, Colorado) (Accession: BC003766; Clone ID: 3491917). *Nfix* was cloned into the Gateway entry vector pDONR221 (Thermo Fisher Scientific, Waltham, MA) by BP clonase reaction, followed by transfer into pCCL-MNDU3-Gateway-PGK-GFP by LR clonase reaction to produce pCCL-MNDU3-*Nfix*-PGK-GFP (MND-*Nfix*). pCCL-MNDU3-Gateway-PGK-GFP was prepared by transferring the Gateway cassette from pRFA (Thermo Fisher Scientific) to pCCL-MNDU3-PGK-GFP downstream of the MNDU3 promoter. pCCL-MNDU3-PGK-GFP was used as a control vector (MND-Control).

Lentivirus production

A four plasmid system (transfer vector (i.e. *Nfix*), Gag/Pol, Rev/Tat, and vesicular stomatitis virus glycoprotein (VSVG) envelope plasmid) was used to produce VSVG-pseudotyped lentivirus. Briefly, plasmids were co-transfected into 293T cells using TransIT 293 (Mirus, Madison, WI) and viral supernatant was collected 48 hours post-transfection. 293T cells were maintained in DMEM (GE Healthcare Life Sciences, Logan, UT) supplemented with 10% FCS (Omega Scientific, Tarzana, CA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from 70-200,000 cells after 4-7 days in *ex vivo* culture using the Qiagen RNeasy Micro Kit (Qiagen, Valencia, CA), followed by reverse transcription using the High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific). qRT-PCR was performed on an ABI StepOnePlus thermal cycler using SYBR Green (Thermo Fisher Scientific). *Tbp* was used as a housekeeping gene, and changes in gene expression between test and control samples were calculated using the ΔΔCt method. Primer sequences can be found in Supplemental Table 1.

Fluorescence-activated cell sorting

Bone marrow was harvested from the femurs, tibias, pelvic bones, and spines of mice by crushing. c-KIT$^+$ cells were enriched by staining the bone marrow with anti-cKIT microbeads (Miltenyi Biotec, San Diego, CA), followed by magnetic separation on an autoMACS Pro

Separator (Miltenyi Biotec). Following separation, cells were stained with the following antibodies: c-KIT-APC (2B8) (eBioscience, Inc., San Diego, CA) and SCA-1-FITC (E13-161.7) (BD Biosciences, San Jose, CA). The c-KIT$^+$SCA-1$^+$ fraction was sorted on a FACSAriaIII (BD Biosciences). 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) was used to exclude dead cells.

Lentiviral transduction

96-well non-tissue culture (NTC) treated plates (Thermo Fisher Scientific) were coated with Retronectin (Takara Bio USA, Inc., Mountain View, CA) according to the manufacturer's instructions. After coating, lentivirus was spin loaded onto the plates for one hour at 1000 g at room temperature at 2.5 x 10^6 virus/cm^2. Wells were then washed once with PBS (Thermo Fisher Scientific) and 15,000 sorted LSK cells resuspended in 200 μL of serum-free expansion media (SFEM) (STEMCELL Technologies Canada, Inc., Vancouver, BC) were added to each well. SFEM was supplemented with 10 ng/mL murine stem cell factor (mSCF), 20 ng/mL murine thrombopoietin (mTPO), human fibroblast growth factor acidic (hFGF-a) (Peprotech, Rocky Hill, NJ), 20 ng/mL murine insulin-like growth factor 2 (mIGF-2) (R&D Systems, Inc., Minneapolis, MN), and 5 ug/mL protamine sulfate (Sigma-Aldrich Corp., St. Louis, MO). This cytokine combination will hereafter be referred to as "STIF."

HSPC ex vivo culture

Following 24-48 hours of lentiviral transduction, cells were washed of any residual viral particles with PBS/2% FCS. After washing, 15,000 cells resuspended in 200 μL SFEM supplemented

with STIF and 10 µg/mL heparin (Sigma-Aldrich) were added to each well of a 96-well NTC plate. Cells were collected and passaged 1:4 into new media every 48-72 hours. 50 µL of cells not used for passaging were simultaneously assessed for relative growth and GFP% via flow cytometry analysis using BD LSRFortessa (BD Biosciences) and data analysis using FlowJo (FlowJo, LLC, Ashland, OR). To determine relative cell number, the 50 µL fraction was collected for 30 seconds at medium speed on the same instrument for every time point assessed, and the number of live cells collected was recorded. DAPI was used to exclude dead cells. This value was then entered into the following equation:

relative growth value = ((live cell # x 4)/previous live cell #) x previous relative growth value

In this equation, "4" corresponds to the dilution factor of the previous passage. For cytokine deprivation experiments, the same procedure was followed with the exception that 25% of the normal concentrations of STIF cytokines were used.

Cytospin Preparation

Cytospins were prepared and stained using cytopads with caps (Fisher) in a 7120 Aerospray Hematology Slide Stainer/Centrifuge (Wescor, Logan, UT). Briefly, 75,000 GFP+ control or GFP+ NFIX+ cells in 100ul were centrifuged for three minutes at 1000 rpm. After letting the slides air dry, slides were stained using the Romanowsky staining method: eosin (Wescor), Thiazin (azure B, methylene blue) (Wescor) and light eosin rinse (Wescor). Anhydrous methanol (Wescor) was used for fixation.

Bone Marrow Transplantation

CD45.2 "test" LSK cells were collected and transduced with lentivirus as described above. Twenty-four hours post-transduction, 5000 test cells were washed with PBS and transplanted with 5000 mock-transduced CD45.1 LSK cells into lethally irradiated CD45.1/CD45.2 recipients. For lethal irradiation, CD45.1/CD45.2 mice received two doses of 5.5 Gy administered three hours apart.

TPO removal and AMM2 treatment

Ex vivo HSPCs were transduced with lentivirus and plated as described above. After 72 hours in culture, 15,000 cells were collected, washed with PBS, and replated in 200ul SFEM supplemented with 25% STIF ± TPO or ± 2 µg/mL of the c-MPL neutralizing antibody AMM2 [13] (Takara Bio USA). After another 72 hours in culture, cells were collected and counted via hemacytometer, as well as analyzed for GFP%, c-KIT%, and apoptosis via flow cytometry on a BD LSRFortessa.

CFU assays

Control and NFIX+ cells were sorted for GFP+ cells as described above and plated in methylcellulose M3434 (STEMCELL Technologies). Colonies were scored and counted 10-12 days after plating. For identification of CFU-Megs, sorted cells were plated in MegaCult-C medium with collagen (STEMCELL Technologies), along with 50ng/ml TPO, 20ng/ml IL-6, and

10ng/ml IL-3. Colonies were stained and counted 6-8 days after plating, according to manufacturer's instructions (STEMCELL Technologies).

Phosphoflow

Cells were transduced with lentivirus and plated as described above. After four to seven days in culture, 15,000-40,000 cells were collected, washed with PBS, and replated in 200ul SFEM without cytokines for two hours. After the incubation period, cells were collected and treated with 20 ng/mL mTPO for 5, 10, 25, 60, or 120 minutes at 37 ºC, followed by fixation in 1.6% formaldehyde (Avantor Performance Materials, Center Valley, PA) for 10 minutes at room temperature. Fixed cells were then pelleted and resuspended in ice cold methanol (Thermo Fisher Scientific), followed by 30 minutes incubation on ice or storage at -20 ºC for later analysis. After permeabilization, cells were washed with PBS/2% FCS and stained with fluorescent conjugated antibodies for phosphorylation of STAT5, ERK1/2, or AKT for analysis by flow cytometry.

Luciferase Reporter Activity Assay

Constructs for luciferase reporter assays were designed by using primers listed in Supplemental Table 2. HindIII and XhoI restriction sites were included during primer design (bold, lowercase in Table 2). Regions of the *c-Mpl* promoter were amplified via polymerase chain reaction (PCR) using these primers and fragments were purified with Wizard SV Gel and PCR Clean-up system (Promega, Madison, WI). Purified fragments and promoterless luciferase vector pGL4.14 (Promega) were incubated with restriction enzymes HindIII-High Fidelity and XhoI (New England Biolabs, Ipswich, MA) and purified. Each fragment of the *c-Mpl* promoter and digested pGL4.14 backbone were ligated together with T4 DNA ligase overnight at 16°C. Ligation

reactions were transformed into *Escherichia coli* TOP10 One Shot competent cells (Invitrogen) and plated onto LB agar plates supplemented with 100ug/ml Ampicillin. Resulting colonies were sequenced using the RVprimer3 sequencing primer. The MND-control or MND-NFIX constructs (2.5 ug) were co-transfected with pGL4.70 (hRluc) (0.125 ug) and one of the luciferase constructs described earlier (0.875 ug) into 10^6 K562 cells using nucleofector kit V (Amaxa; Lonza Group, Basel, Switzerland) according to the manufacturer's protocol. K562 cells were maintained in DMEM (GE Healthcare Life Sciences) supplemented with 10% FCS (Omega Scientific). 24 hours post-transfection, cells were lysed with passive lysis buffer and tested for reporter activity using the Dual-Luciferase Reporter Activity Assay Stop-and-Glo Kit (Promega) and a BioTek Synergy H1 Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) according to manufacturer's instructions.

Chromatin Immunoprecipitation

HPC5 cells were maintained in IMDM (Hyclone) media supplemented with 5% FCS, 0.15 mM 1-thioglycerol (MTG) (Sigma, M6145), 10 ng/mL human interleukin-6 (hIL-6) (Pepro Tech Inc.), and 100 ng/mL mSCF. The cells were then infected with lentivirus carrying MND-Nfix-FLAG or MND-control constructs. 48 hours after infection, cells were sorted for GFP+ cells. GFP+ HPC5 cells were expanded to 10^7 in culture for no more than one week. 10^7 GFP+ HPC5 cells were crosslinked with 1% formaldehyde and stored at -80°C. After crosslinking, cells were sheared and diluted. At this time, 10% of the total lysate volume was reserved as the total input sample. Each sample received 1μg of an anti-FLAG antibody (CST, clone: D6W5B) or rabbit

IgG antibody (CST, 2729). Samples were incubated overnight at 4°C with gentle rocking. Next, Protein G Dynabeads (Fisher) were added to samples and incubated for two hours at 4°C with gentle rocking. Samples were washed with a series of buffers and then immunoprecipitated chromatin and total input chromatin were eluted. Chromatin were de-crosslinked and RNA and protein were digested by overnight incubations with RNase and Proteinase K in a sodium chloride rich buffer. DNA from ChIP samples and total input samples was extracted using phenol/chloroform. Total input samples were diluted ten times before qPCR. The following primers were designed to encompass NFI consensus binding sites in the *Mus musculus c-Mpl* proximal promoter [Forward: CCCATTCCCCCTCCTCTGG] and [Reverse: CCTGTCAGATACAGCCCCAC]. Primers used for ChIP-qPCR were validated with serial dilutions of HPC5 genomic DNA. Total input samples were first adjusted to represent 100% of the total chromatin present in samples. Finally, percent input was calculated as follows, *% Input = 100 × 2*$^{(adjusted\ input\ Ct\ -\ ChIP\ Ct)}$.

Flow cytometry

All flow cytometry analysis was performed on a BD LSRFortessa and all data was analyzed by FlowJo. For determination of Lineage^{-}SCA-1^{+}c-KIT^{+} immuno-phenotype of *ex vivo* cells, the following antibodies were used: {CD3 (145-2C11), CD4 (GK1.5), CD19 (6D5), GR-1 (Rb6-8C5), TER-119 (TER-119) (BioLegend, San Diego, CA), CD8 (53-6.7), B220 (RA3-6B2) (BD Biosciences)}-PerCP; SCA-1-PerCP-Cy5.5 (E13-161.7) (BioLegend); c-KIT-APC-eFluor780 (2B8) (eBioscience). For determination of c-KIT% of *ex vivo* cells in the TPO and AMM2 experiments, c-KIT-PE-Cy7 (2B8) (BioLegend) was used. For flow cytometry analysis of cell

cycle, cells were collected, washed with PBS/2% FCS, then fixed and permeabilized followed by DAPI staining. For flow cytometry analysis of apoptosis, cells were collected, washed with PBS/2% FCS and resuspended in Annexin Binding Buffer (BD Biosciences). Cells were then stained with DAPI and Annexin V-APC (BD Bioscences). For phosphoflow, the following antibodies were used: STAT5(pY694) (47); ERK1/2(pT202/pY204) (20A); AKT(pS473) (M89-61) (BD Biosciences). For peripheral blood analysis of recipients, blood was collected from the retro orbital plexus in heparinized capillary tubes and lysed in red blood cell lysis buffer (Sigma-Aldrich Corp.). Cells were then stained with CD45.1-APC (A20), CD45.2-v500 (104) (eBioscience), {B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (M1/70)}-PerCP-Cy5.5, {B220 (RA3-6B2), CD4 (GK1.5), CD8 (53-6.7)}-PE-Cy7 (BioLegend). For staining of megakaryocyte progenitors, cells were stained with c-KIT-APC-eFluor780 (2B8) (eBioscience), SCA-1-PE (E13-161.7) (BioLegend), CD127-PE-Cy7 (A7R34) (Tonbo Biosciences, San Diego, CA), CD9-A647 (KMC8) (BD Biosciences), CD32/CD16-A700 (93) (eBioscience), and CD41-PerCP-e710 (eBioMWReg30) (eBioscience).

Statistical analysis

Statistical significance was determined using two-sample/one sample Student's t-tests or exact Wilcoxon rank sum tests, depending on the normality of the data as determined by the Shapiro-Wilk test. In Fig. 4A, a linear regression model was used to examine the reduction in cell number in controls compared with NFIX+ cells. p-values < 0.05 were considered statistically significant in all analyses.

Supplemental Tables

Supplemental Table 1. qRT-PCR primer sequences

Designed sequences:

Target	Forward Primer	Reverse Primer	Amplicon Size (bp)

Nfix	AGGCTGACAAGGTGTGGC	CACTGGGGCGACTTGTAGAG	103
Nfia	GAGTCCAGGAGCAATGAGG	CCATTTCATCCTCCACAGAC	86
Nfic	CCGGCATGAGAAGGACTCTAC	TTCTTCACCGGGGATGAGATG	187
c-Mpl	CTGGTCCTTCCCTGTGACT	GCGGTTCCTCCTCTTCACAT	206
Bcl-xL	GACAAGGAGATGCAGGTATTGG	TCCCGTAGAGATCCACAAAAGT	124
Erg	CTAAGACAGAGATGACCGCA	GTGGTCATATTGGGAGGCG	274
Gata3	CTCGGCCATTCGTACATGGAA	GGATACCTCTGCACCGTAGC	134
Hlf	CCGTCTCCGAACTGTATGC	AGAACTTCCGTTTGCGAGG	187
Mecom	ACATGGGAGAGCAGAGATCAG	TGATCATAGCAGCCAGCG	151
Robo4	TGTGTTGCTCCTGAGGCTG	TCTGTTCACCCACTACGGTC	203
Tek	GATTTTGGATTGTCCCGAGGTCAAG	CACCAATATCTGGGCAAATGATGG	306
Tie1	AGGAGGTGTATGTGAAGAAGAC	CCTCCAAGGCTCACTATCTC	142
Tbp	GAAGAACAATCCAGACTAGCAGCA	CCTTATAGGGAACTTCACATCACAG	129

Sequences obtained from Qiagen:

Target	Qiagen Order Number	Amplicon Size (bp)
Hemgn	QT00106099	127
Mycn	QT00252196	111

Gata2	QT00160524	95
Stat5a	QT00164367	87
Stat5b	QT00126126	99

Supplemental Table 2. Primer sequences for luciferase constructs		
Sites Included	**Forward (5'-3')**	**Reverse (5'-3')**
-189,-127,-101,-18	GGGG**ctcgag**AATATATACCTCTGTGTCCCTGCC	GGG**aagctt**CACTGTGTGCCTGCCTTA
-127,-101,-18	GGGG**ctcgag**ATATATACCTCTGTGTCCCT	GGG**aagctt**CACTGTGTGCCTGCCTTA
-18	GGGG**ctcgag**GGACGTGGGGCTGTATCTGA	GGG**aagctt**CACTGTGTGCCTGCCTTA
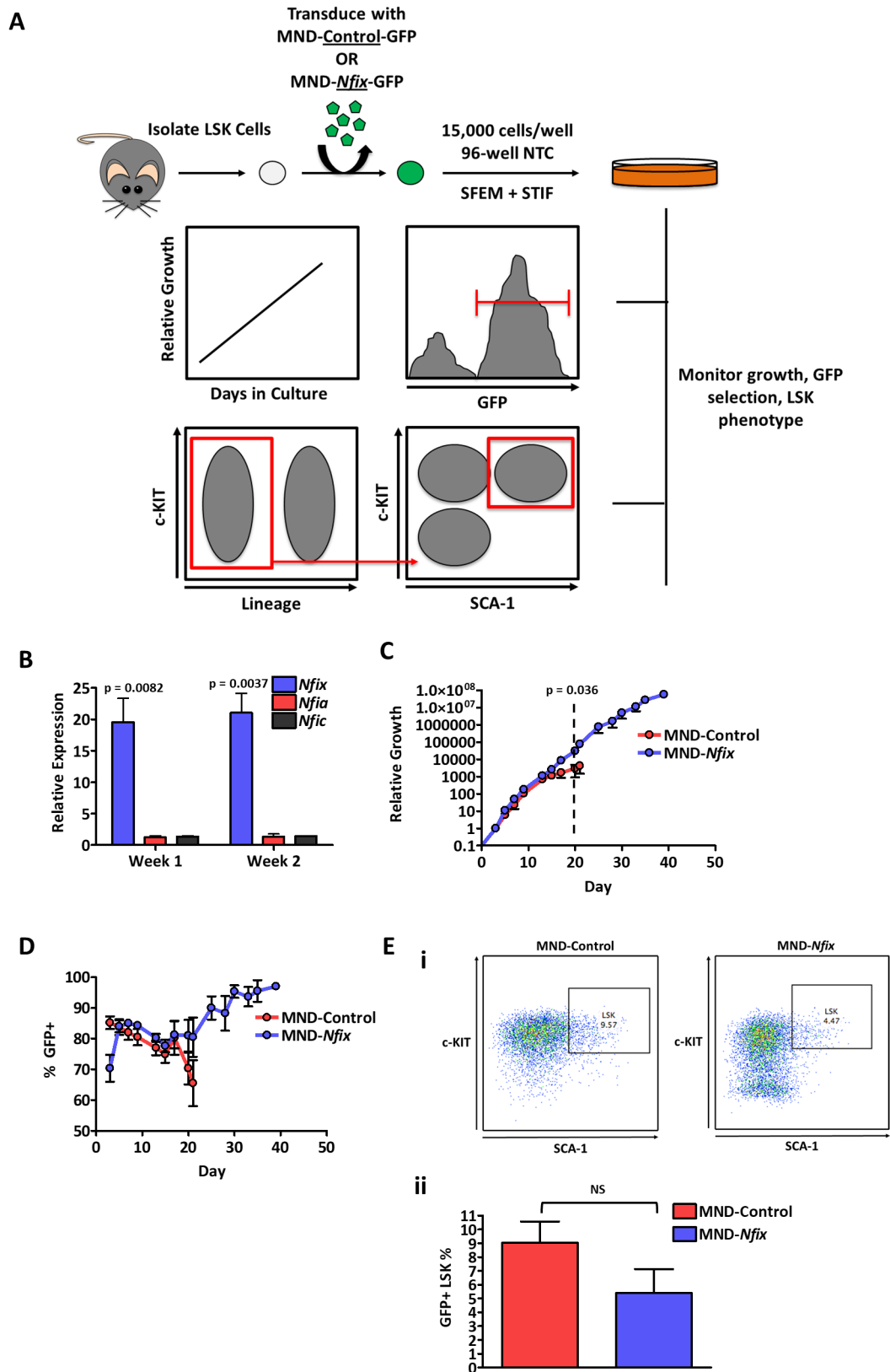
Figure 1
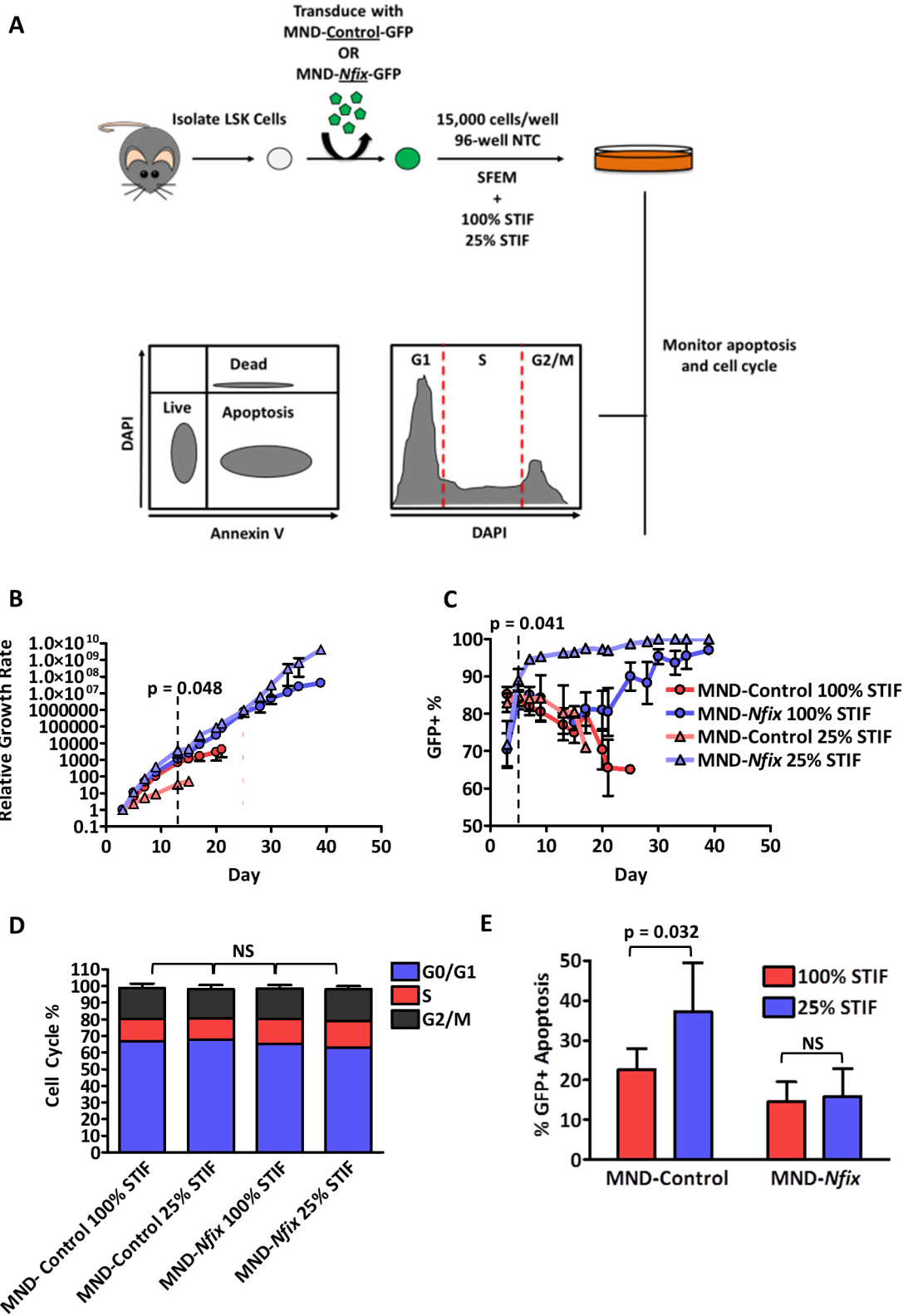
Figure 2

Figure 3

Figure 4

A

SSC-A / FSC-A

C-Kit / Sca-1 — LSK

DAPI — Viable / FSC-A

FSC-A / SSC-W — Singlets

B

C-Kit / Sca-1 — (L)SK — Day 0

MND-Control   MND-*Nfix*

LSK 6.10   LSK 4.59   Day 7

LSK 1.97   0.99   Day 14

C-Kit / Sca-1

C

Culture Day 14

NS

GFP+ LSK %

MND-Control
MND-*Nfix*

D

Culture Day 7
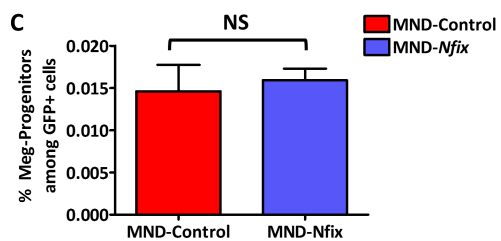
Count

c-KIT

MND-Control
MND-*Nfix*

Bone Marrow LSK cells

MND-Control D7

MND-*Nfix* D7

MND-*Nfix* D30

50 µm

Supplemental Figure 2

A

Transduce with
MND-Control-GFP
OR
MND-*Nfix*-GFP

CD45.2

Isolate LSK cells

Mock Transduce

Pool 1:1

Transplant into irradiated recipients

CD45.1

CD45.1/CD45.2

B

MND-Control
MND-*Nfix*

% Chimerism

Week

C

MND-Control
MND-*Nfix*

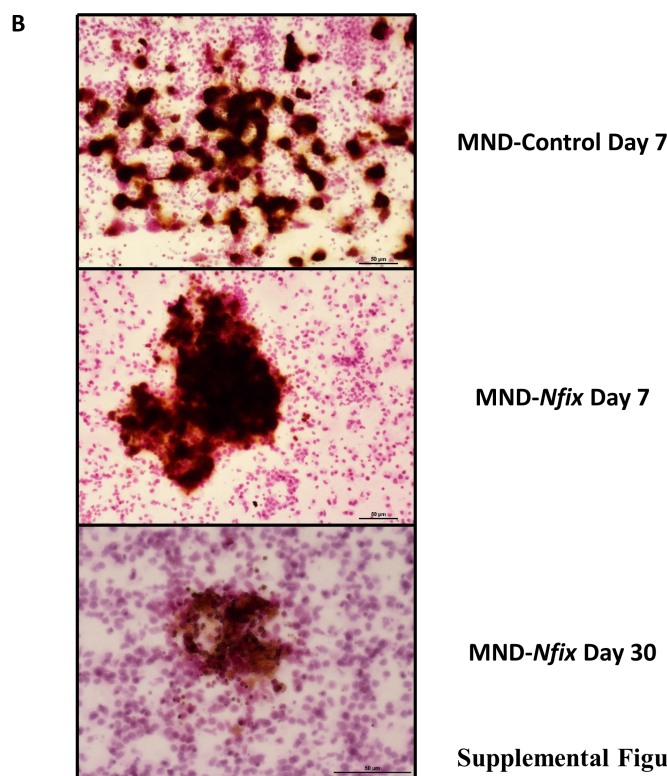% GFP+ Test

Week

D

T-Cell
B-Cell
Myeloid

% Lineage

MND-Control   MND-Nfix

Supplemental Figure 3

Supplemental Figure 4

A

B

p = 0.017   p = 0.36

Control Day 7   **Nfix Day 7**   **Nfix Day 30**

5.88% ± 0.055   15.02% ± 1.21   26.04% ± 9.20
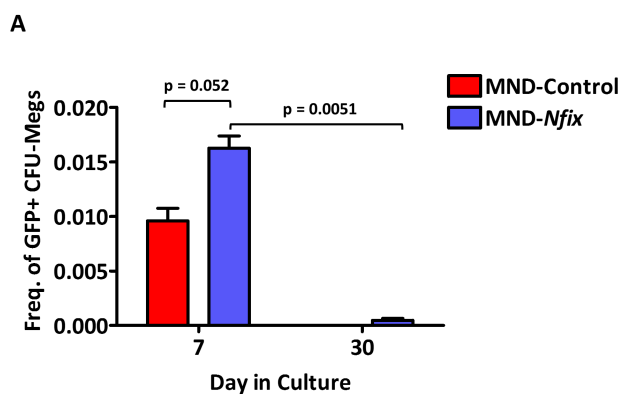
FSC-A
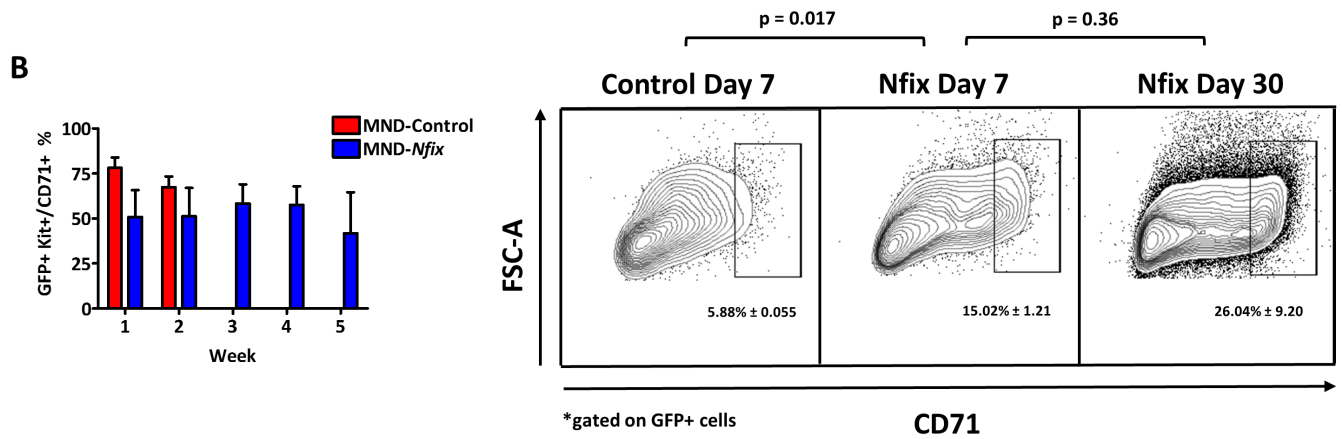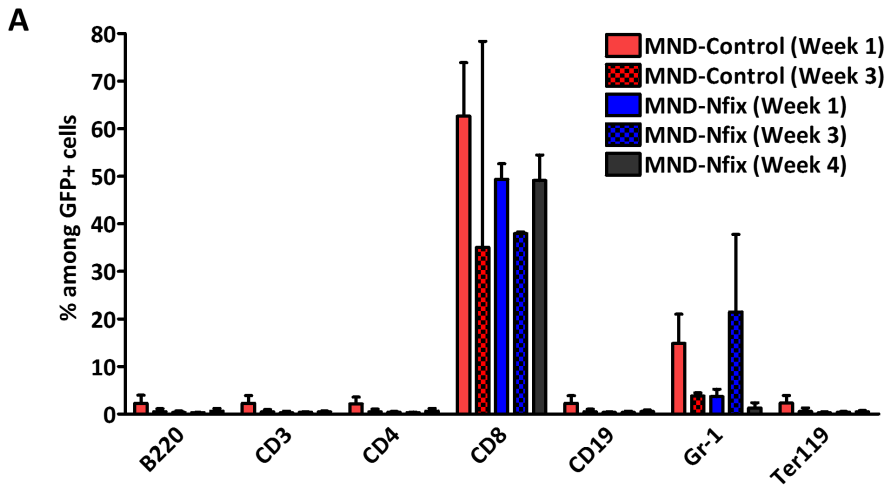
*gated on GFP+ cells   **CD71**
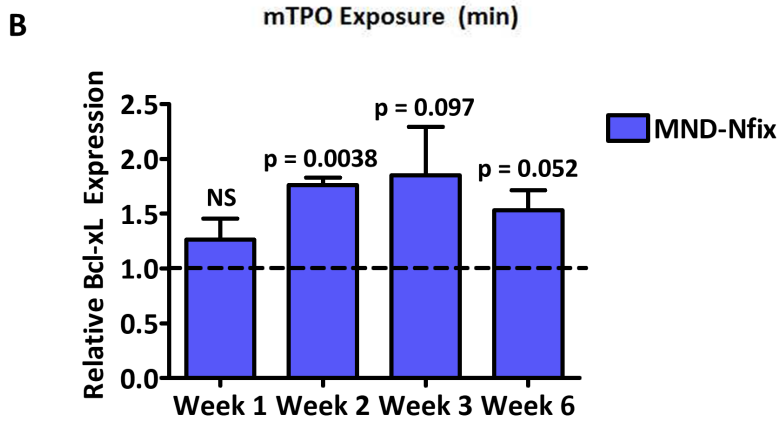
Supplemental Figure 5
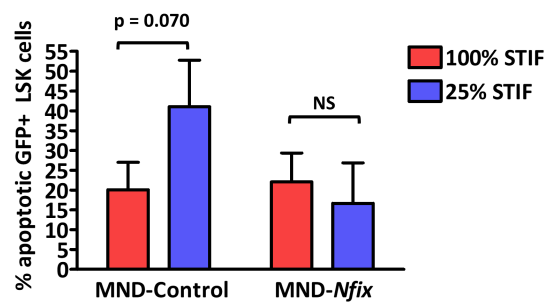
Supplemental Figure 6

Supplemental Figure 7

A

p = 0.052

p = 0.0051

■ MND-Control
■ MND-*Nfix*

Freq. of GFP+ CFU-Megs

Day in Culture

C

NS

■ MND-Control
■ MND-*Nfix*

% Meg-Progenitors among GFP+ cells

MND-Control   MND-Nfix

B

MND-Control Day 7

MND-*Nfix* Day 7

MND-*Nfix* Day 30
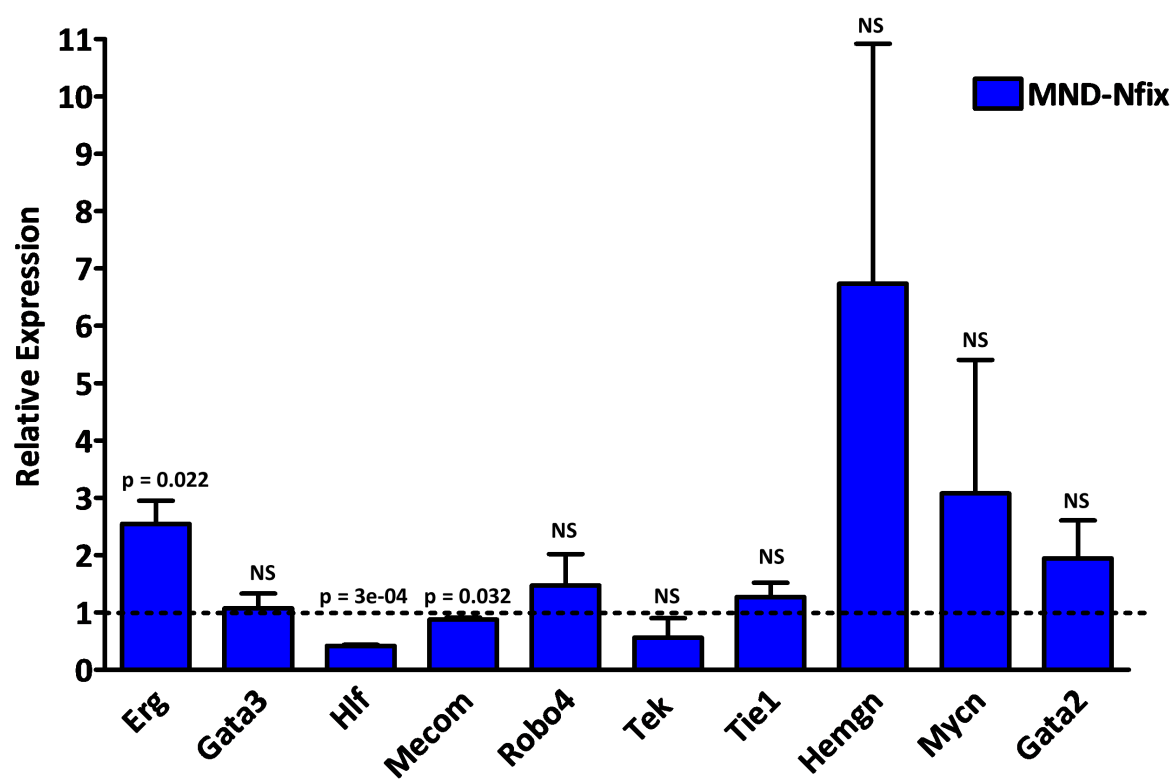
Supplemental Figure 8

Supplemental Figure 9