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The orphan nuclear receptor Nurr1 restricts the proliferation of haematopoietic stem cells

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Successful haematopoiesis requires long-term retention of haematopoietic stem cells (HSCs) in a quiescent state. The transcriptional regulation of stem cell quiescence, especially by factors with specific functions in HSCs, is only beginning to be understood. Here, we demonstrate that *Nurr1*, a nuclear receptor transcription factor, has such a regulatory role. Overexpression of *Nurr1* drives early haematopoietic progenitors into quiescence. When stem cells overexpressing *Nurr1* are transplanted into lethally irradiated mice, they localize to the bone marrow, but do not contribute to regeneration of the blood system. Furthermore, the loss of only one allele of *Nurr1* is sufficient to induce HSCs to enter the cell cycle and proliferate. Molecular analysis revealed an association between *Nurr1* overexpression and upregulation of the cell-cycle inhibitor p18 (also known as INK4C), suggesting a mechanism by which *Nurr1* could regulate HSC quiescence. Our findings provide critical insight into the transcriptional control mechanisms that determine whether HSCs remain dormant or enter the cell cycle and begin to proliferate.

Life-long haematopoiesis is sustained by the capacity of haematopoietic stem cells (HSCs) to self-renew as well as to replenish mature blood cells. Most HSCs reside in a quiescent or dormant state[1], a property critical to maintain blood homeostasis. Mutations in a number of genes important for HSC quiescence lead to the loss of haematopoietic repopulating ability[2–4]. This strong correlation between HSC quiescence and regenerative capacity seems counterintuitive, but suggests that most HSC cell divisions (at least under stress) are symmetric, leading uniformly toward differentiation, rather than allowing HSC regeneration. Nevertheless, HSCs are clearly able to self-renew, as demonstrated by single-cell and secondary transplantation experiments[5]. Identifying the activities of genes that regulate self-renewal and differentiation is crucial to realizing the potential of HSCs in regenerative medicine.

The majority of genes known to have roles in HSC quiescence are those with essential functions in cell-cycle regulation (for example, p21, p27, D-type cyclins, RB and p53)[4,6–10] or in transcriptional regulation of haematopoiesis (for example, Runx1, Egr1 and Gfi1)[2,11,12]. To identify genes with a specific role in preservation of quiescence, we analysed genes that were differentially expressed between dormant and proliferating HSCs[13], and uniquely expressed in HSCs[14]. *Nurr1* (also known as *Nr4a2*) was identified as a prime candidate. This ligand-independent nuclear receptor-type transcription factor is paralogous to *Nur77* and *Nor1* (also known as *Nr4a1* and *Nr4a3*, respectively)[15–18]. Although combined loss of *Nor1* and *Nur77* results in an acute myeloid leukaemia (AML)-like phenotype in mice[19], *Nurr1* has not been implicated in haematopoiesis, although it is known to function in terminal differentiation of dopaminergic neurons[20,21], and regulation of apoptosis[22,23]. Thus, the properties of *Nurr1* were sufficiently relevant to warrant its further investigation for a role in HSC quiescence.

Quiescent HSCs proliferate in a synchronized manner after a single injection of the chemotherapeutic agent 5-flurouracil (5FU)[1,13]. Microarray analysis of quiescent versus 5FU-stimulated HSCs identified approximately 800 genes that were activated in quiescent cells. One of these, *Nurr1,* was markedly downregulated in proliferating HSCs (Fig. 1a, b), as well as in differentiated haematopoietic cells (Fig. 1c, d). To examine whether *Nurr1* expression in haematopoietic cells is functionally associated with quiescence, we forced its expression in 32D cells, an IL-3-dependent myeloid progenitor line, by transducing cells with a retrovirus carrying a vector containing *Nurr1* upstream of *IRES-GFP.* This allowed us to follow the transduced cells through expression of green fluorescent protein (GFP; Fig. 1e). An identical vector carrying only *GFP* was used as a control. *Nurr1* overexpression led to a marked block in proliferation that inhibited expansion of the transduced 32D cells, whereas cells transduced with control vector readily proliferated (Fig. 1f). To ensure that the difference in proliferation was not the result of apoptosis, we performed annexin-V staining. This demonstrated that there was no increase in cell death associated with *Nurr1* overexpression (Fig. 1g); the proportion of apoptotic/dead cells in the *Nurr1*-transduced group was significantly lower than in the control group ($P = 0.004$), consistent with a pro-survival role of Nurr1 (ref. 22). Overexpression of *Nurr1* in 32D cells also led to a decrease in cells positive for the proliferative marker Ki67 (Supplementary Information, Fig. 1a), supporting an inhibitory effect of Nurr1 on proliferation. The

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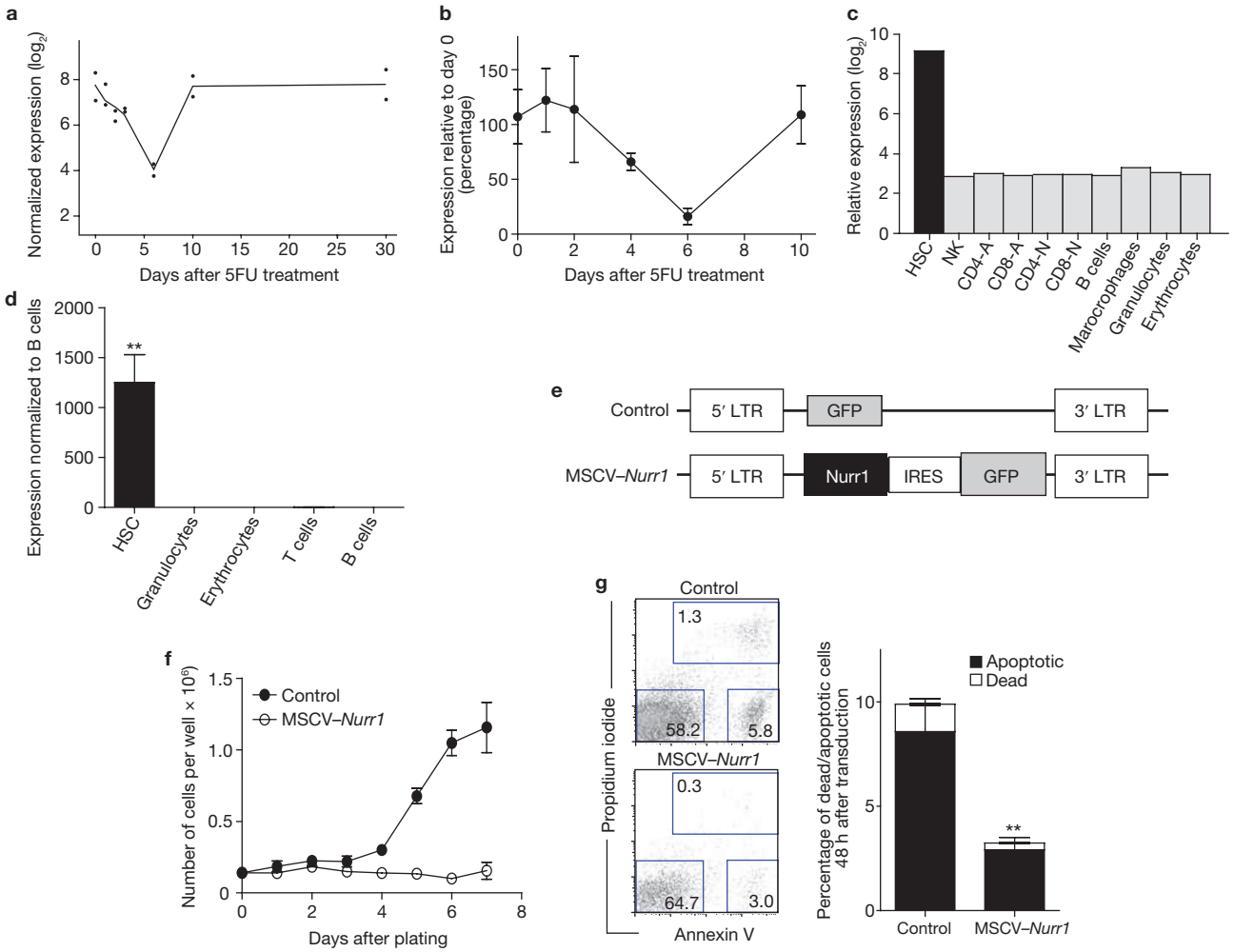

Figure 1 *Nurr1* is highly expressed in quiescent HSCs (Hoechst 33342 side-population cells that are also c-Kit$^+$, Sca1$^+$ and Lin$^-$; SP-KSL) and its overexpression in 32D cells results in a proliferative block. (**a**) *Nurr1* expression in HSCs on the indicated days after injection with 5FU, as determined by microarray data extracted from ref. 13. Data was normalized to expression profile of *Nurr1* from quiescent HSCs. (**b**) Verification of microarray data shown in **a** by real-time PCR measuring *Nurr1* levels in HSCs on the indicated days after injection with 5FU. Data were normalized relative to expression levels at day 0. Data are means ± s.e.m. from three experiments; $P = 0.02$ between day 0 and day 6. (**c**) Relative expression of *Nurr1* in HSCs and the indicated differentiated cells, as assessed by microarray data extracted from ref. 14. (**d**) *Nurr1* levels in the indicated cells, as assessed by real-time PCR. Expression levels are normalized with respect to B cells (data are means ± s.e.m. of 3 experiments; asterisks indicate $P = 0.003$). (**e**) Schematic representation of the vectors used for expression of *Nurr1* in 32D cells. Both vectors were cloned into MSCV for subsequent infection of cells. The control vector only expresses GFP. MSCV–*Nurr1* expresses Nurr1 upstream of *IRES–GFP*. IRES, internal ribosome entry site; LTR, long terminal repeats. (**f**) Proliferation of 32D cells transduced with the indicated vectors, as represented in **e**. Cells were transduced, cultured for 48 h and GFP-positive cells were identified by flow cytometry, before culture in 96-well plates. Viability was assessed by treatment of the cells with trypan blue on the indicated days. Data are means ± s.e.m.; $n = 3$; The curves were fitted using non-linear curve fitting, and Fisher's exact test indicated they were significantly different ($P < 0.0001$). (**g**) Left: representative flow-cytometry analysis of cells cultured as in **f** and treated with propidium iodide and anti-annexin V to assess cell death and apoptosis. Percentages of cells that are dead (top), apoptotic (bottom right), or alive (bottom left), are indicated. Right: quantification of dead and apoptotic cells from flow cytometric analyses. Data are means ± s.d. from 3 experiments; asterisks indicate $P = 0.004$.

Nurr1-associated decrease in proliferation was not associated with increased differentiation of 32D cells (data not shown).

Although supporting the hypothesis that Nurr1 is a regulator of cell proliferation, these data do not demonstrate an effect on HSC cycling. Thus, we isolated bone marrow from 5FU-treated mice (to permit efficient transduction), magnetically enriched for progenitors using the Sca-1 stem cell marker, and transduced them with either of the vectors shown in Figure 1e (Fig. 2a). After 2 days of culture to allow *GFP* expression, the GFP and Sca-1 double-positive cells were sorted for colony–forming assays, scored on day 14 of growth. The colony-forming ability of *Nurr1*-transduced cells was significantly reduced when compared with control cells (4% versus 20% of total wells of a 96-well plate, $P = 0.0002$; Fig. 2b), suggesting that Nurr1 impedes proliferation of HSCs. The large decrease in colony number seen with *Nurr1* overexpression could not be attributed to cytotoxicity, as the proportion of viable cells transduced with control and *Nurr1*-containing vectors were identical at the time of plating (data not shown). Annexin-V staining approximately 20 h after transduction indicated that the proportion of apoptotic cells was approximately 5% in both the control cells and in cells overexpressing *Nurr1* (Fig. 2c). Culture of more highly enriched progenitors for up to 96 h also resulted in no significant difference in cell viability (Supplementary Information, Fig. 2a), suggesting that *Nurr1* overexpression is not toxic to HSCs.

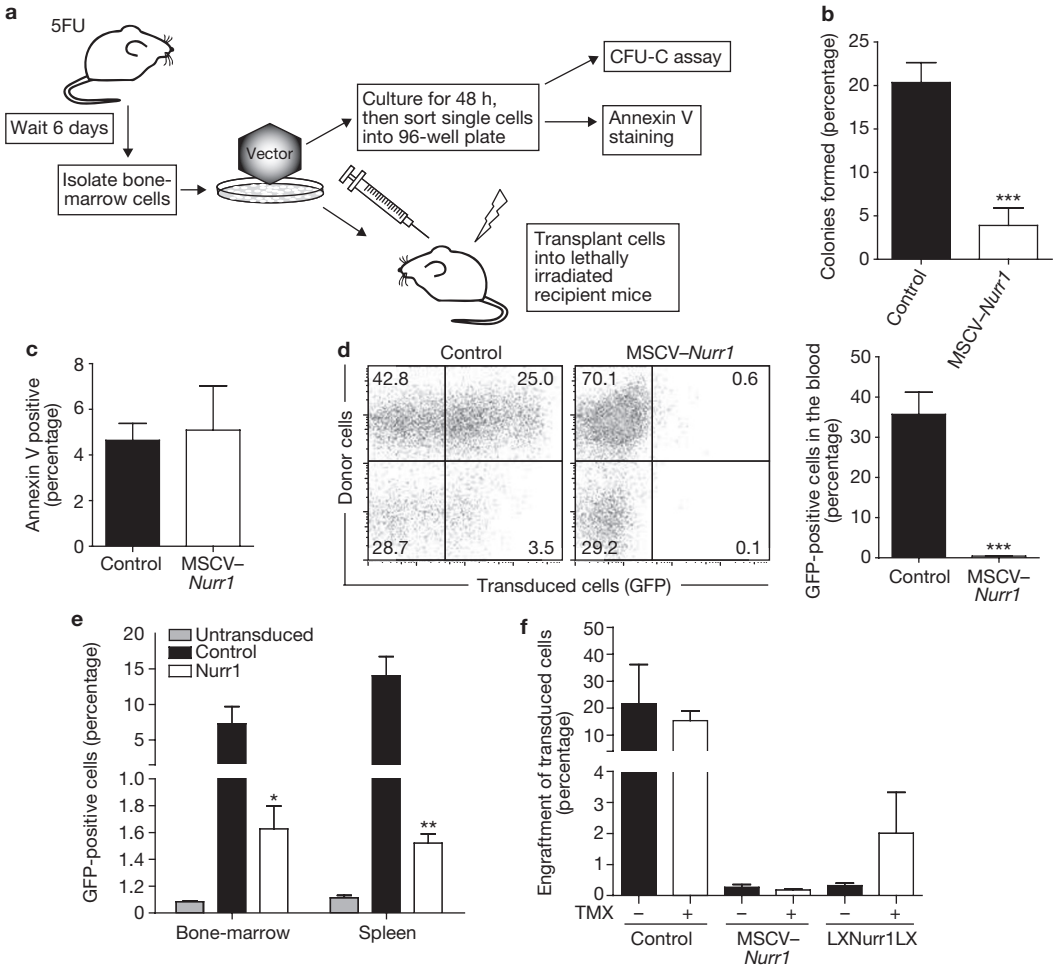

Figure 2 Overexpression of *Nurr1* in bone-marrow cells reversibly blocks proliferation. (**a**) Schematic representation of overexpression experiments in bone-marrow cells using control and *Nurr1* vectors shown in Fig. 1e. (**b**) CFU-C assay on cells cultured as indicated in **a** ($n = 4$; asterisks indicate $P < 0.0002$). Data are colonies formed as a percentage of the total wells plated. (**c**) Cells were treated as indicated in **a**, before treating with antibodies against annexin V and analysing staining by flow cytometry. Data are means ± s.e.m. of five experiments. (**d**) Lethally irradiated mice were transplanted with bone-marrow cells transduced with control or MSCV–*Nurr1* vectors, as indicated in **a**. Left: representative flow-cytometric analysis of peripheral blood 4 weeks after transplantation. Numbers indicate percentages of cells in each indicated quadrant. Right: quantification of GFP-positive cells from the flow cytometric analyses. Control; $n = 7$, *Nurr1* $n = 9$; asterisks indicate $P < 0.0001$. (**e**) Quantification of GFP-positive cells in the bone marrow and spleen of lethally irradiated mice, transplanted with bone-marrow cells that were untransfected, or transfected with control or MSCV–*Nurr1* vector. GFP-positive cells were identified by flow cytometric analysis 4 weeks after transplantation. Data are means ± s.e.m. of three experiments. Asterisk indicates $P = 0.03$ and double asterisks indicate $P < 0.005$. (**f**) Lethally irradiated mice were transplanted with bone-marrow cells transduced with control, MSCV–*Nurr1* or the LXNurr1LX vector. The LXNurr1LX vector contains a *loxP*-flanked *Nurr1* gene (schematic representation of vector is shown in Supplementary Information, Fig. 3), with Cre-mediated excision of the gene inducible by treatment of transduced cells with tamoxifen. Engraftment of transduced cells was assessed by flow cytometry, 8 weeks after the initial transplant (4 weeks after tamoxifen induction). Data are means ± s.e.m. Control, $n = 4$; *Nurr1*, $n = 4$; LXNurr1LX, $n = 6$; $P = 0.3$ for the comparison of TMX treated and non-treated LXNurr1LX.

The *in vitro* data suggested that transplanted *Nurr1*-overexpressing cells would not be able to contribute to the replenishment of blood lineages *in vivo*. To test this, we transplanted haematopoietic progenitors transduced with control or *Nurr1*-containing vectors into lethally irradiated mice within approximately 4 h of transduction, thereby avoiding any effect that *Nurr1* overexpression might have on homing (because the protein is not expressed immediately after transduction), and analysed the peripheral blood of recipients 4 weeks after transplantation for the presence of donor-derived transduced cells, using flow cytometry. Control-transduced cells were found to contribute to about 36% of the peripheral blood, whereas the contribution of *Nurr1*-transduced cells was only 0.43% (Fig. 2d). Similar levels of non-transduced donor-derived cells were present between infusions of cells transduced with control vectors and vectors containing *Nurr1*, indicating successful transplantation. Flow-cytometric analysis performed 12 months post-transplantation revealed a persistent lack of contribution to peripheral blood from *Nurr1*-transduced donor cells, ruling out delayed engraftment (Supplementary Information, Fig. 2b). With so few *Nurr1*-overexpressing progeny, we cannot exclude a simultaneous impact of Nurr1 on proliferation and differentiation.

To explain the negligible contribution of *Nurr1*-overexpressing donor cells to peripheral blood reconstitution, we hypothesized that the transduced HSCs home to bone marrow, where they enter the cell cycle at very low levels, if at all. To verify their presence, the bone marrow and spleen were examined after transplantation. Non-transduced transplanted cells functioned as a background autofluorescence control. Four weeks

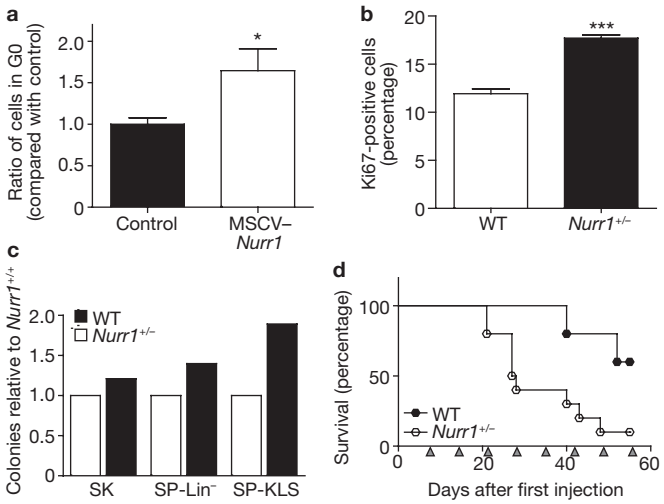

Figure 3 *Nurr1* overexpression leads to reduced cell-cycle proliferation. (**a**) Quantification of cells in G_0. Sca-1-enriched bone-marrow cells were transduced with the indicated vectors . After transduction GFP-positive and Sca-1-positive cells were sorted by flow cytometry, and stained with Hoescht 33342 and pyronin Y to enable quantification of cells in G_0 and G_1 phases by flow cytometry. Data are means ± s.e.m. Control $n = 5$, *Nurr1* $n = 7$. Asterisk indicates $P = 0.05$. (**b**) Quantification of Ki67-positive cells by flow cytometry. Haematopoietic progenitors (c-Kit$^+$ Sca-1$^+$ Lin$^-$; KSL) were isolated from adult wild-type (WT) and *Nurr*$^{+/-}$ heterozygous mice by flow cytometry and Ki67-positive cells were then quantified by flow cytometry. Data are means ± s.e.m. of three experiments. Asterisks indicate $P = 0.0007$. (**c**) Colony forming assay of cells isolated from the bone marrow of wild-type and *Nurr1*$^{+/-}$ mice. Bone-marrow cells were sorted by flow cytometry into HSCs (SK; Sca-1$^+$c-kit$^+$), side population Lin$^-$ (SP-Lin$^-$) and side population c-Kit$^+$ Sca-1$^+$ Lin$^-$ (SP-KSL) before the assay. (**d**) Survival curves of *Nurr1*$^{+/+}$ mice ($n = 5$), compared with *Nurr1*$^{+/-}$ mice ($n = 10$) mice, over 55 days with weekly injections of 5FU (indicated by arrowheads). The survival curves were significantly different ($P = 0.022$), based on Gehan-Breslow-Wilcoxon test.

post-transplantation, *Nurr1*-transduced GFP-positive cells were present in spleen and bone marrow (Fig. 2e). Although these populations were small (< 1% of all cells in the tissues), they were significantly higher than the autofluorescent background levels of about 0.1%. The low proportion of *Nurr1*-overexpressing cells in the bone marrow seems similar to the proportion of transduced cells after initial transplantation. This suggests that control cells were able to proliferate and generate progeny, whereas the *Nurr1*-overexpressing cells remained dormant. As a single HSC can reconstitute a lethally irradiated mouse[24,25], we suggest that a sufficient number of transduced HSCs were present in the bone marrow to contribute to the reconstitution of peripheral blood; their inability to do so supports the hypothesis that Nurr1 is a regulator of cell proliferation.

To determine whether the proliferative block with *Nurr1* overexpression is reversible, we used the Cre/*loxP* system. *Nurr1* flanked by *loxP* sites was cloned into the retroviral vector shown in Figure 1e, and used to transduce cells expressing Cre recombinase under the control of a tamoxifen-inducible promoter (Supplementary Information, Fig. S3). Four weeks after transplantation of the transduced cells, tamoxifen was administered to half the group. As expected, mice transplanted with unfloxed *Nurr1*-overexpressing cells or control vector did not respond to tamoxifen treatment: *Nurr1*-transduced cells (measured by GFP) remained at very low levels, whereas control-vector-transduced cells were abundant. However, in mice transplanted with cells harbouring the floxed *Nurr1* construct, there was an increase in GFP-expressing cells in peripheral blood 8 weeks after tamoxifen treatment, suggesting removal of the Nurr1-proliferative block with *Nurr1* deletion (Fig. 2f). The appearance of transduced cells 8 weeks after tamoxifen treatment supports the hypothesis that Nurr1 functions in HSCs rather than committed progenitors to restore depleted blood cell populations, and suggests that, as well as the dependency on niche occupancy, the timing of excision is also important.

The transplantation data, coupled with the *in vitro* data, indicate that *Nurr1* overexpression restricts HSC proliferation. To directly determine the cell-cycle status of *Nurr1*-overexpressing cells, 48 h after transduction we sorted and stained GFP-expressing Sca-1-positive haematopoietic progenitors with pyronin Y and Hoechst 33342 dye, allowing us to distinguish between cells in G_0 versus G_1. Flow-cytometric analysis revealed an approximate 50% increase of cells in G_0 with *Nurr1* overexpression when compared with the control cells (Fig. 3a), supporting an early block of HSC proliferation by Nurr1 activity.

To verify the physiologic relevance of *Nurr1*, we sought to determine whether the loss of *Nurr1* expression would promote HSC proliferation. Homozygous *Nurr1* knockout mice die shortly after birth due to defects in respiratory function[26]. Haematopoietic progenitors (c-Kit$^+$ Sca-1$^+$ Lin$^-$ or KSL) were therefore selected from adult wild-type versus *Nurr1* heterozygous mice (*Nurr1*$^{+/-}$) mice, and their cell cycle status was compared by Ki67 staining. The results indicate that approximately 18% of the *Nurr1*$^{+/-}$ cells were in cycle compared with 12% of the wild-type cells ($P = 0.0007$; Fig. 3b). Analysis of Sca-1-enriched c-Kit$^+$ bone-marrow cells with pyronin Y and Hoechst 33342 demonstrated that approximately half as many of the cells from *Nurr1*$^{+/-}$ mice, compared with wild-type mice progenitors, were in G_0 (data not shown). As predicted by these results, the colony-forming ability of HSCs from *Nurr1*$^{+/-}$ mice clearly exceeded that of wild-type cells. Indeed, when bone-marrow populations enriched to different purities for HSCs (Sca-1$^+$ c-Kit$^+$; SK versus side population Lin$^-$; SP-Lin$^-$ versus side population c-Kit$^+$ Sca-1$^+$ Lin$^-$; SP-KSL) were plated onto methylcellulose, the colony-forming potential of progenitors from *Nurr1*$^{+/-}$ mice was consistently higher than the control value (Fig. 3c).

If greater numbers of HSCs with reduced levels of *Nurr1* enter the cell cycle, they should be more sensitive to killing by anti-mitotic agents. To test this, we injected *Nurr1*$^{+/-}$ and wild-type mice weekly with 5FU and then compared death rates between the two groups over 55 days. By 60 days after the first injection, only 2 of 5 wild-type mice had died, compared with 9 of 10 *Nurr1*$^{+/-}$ mice ($P = 0.022$, Fig. 3d), supporting our *in vitro* findings. When we examined the HSC population 7 days after 5FU injection, we observed a higher level of cell death in the HSCs from *Nurr1*$^{+/-}$ mice (Supplementary Information, Fig. 4a, b). Thus, the exhaustion of the HSCs from *Nurr1*$^{+/-}$ mice that leads to premature death after repeated 5FU injection is probably because of the higher level of HSC cycling (Fig.3b), but a contribution from higher levels of apoptosis (potentially permitted by *Nurr1* loss) cannot be excluded. Thus, both loss- and gain-of-function studies point to a physiologically important role for Nurr1 in HSC dormancy.

Next, we investigated how Nurr1 might exert its inhibitory effect on HSC proliferation. The expression levels of four cell cycle inhibitors (p18 INK4c, p19 INK4d, p21 C.p1/Waf1, and p27 Kip1) were measured in KSL progenitors from the livers of wild-type, *Nurr1*$^{+/-}$ and *Nurr1*$^{-/-}$ 14.5-day-old embryos, the site of haematopoietic progenitors during this stage of development. The mRNA expression levels of *p18*, *p19*, *p21* and *p27* generally decreased with the loss of *Nurr1* alleles (Fig. 4a). To gain insight

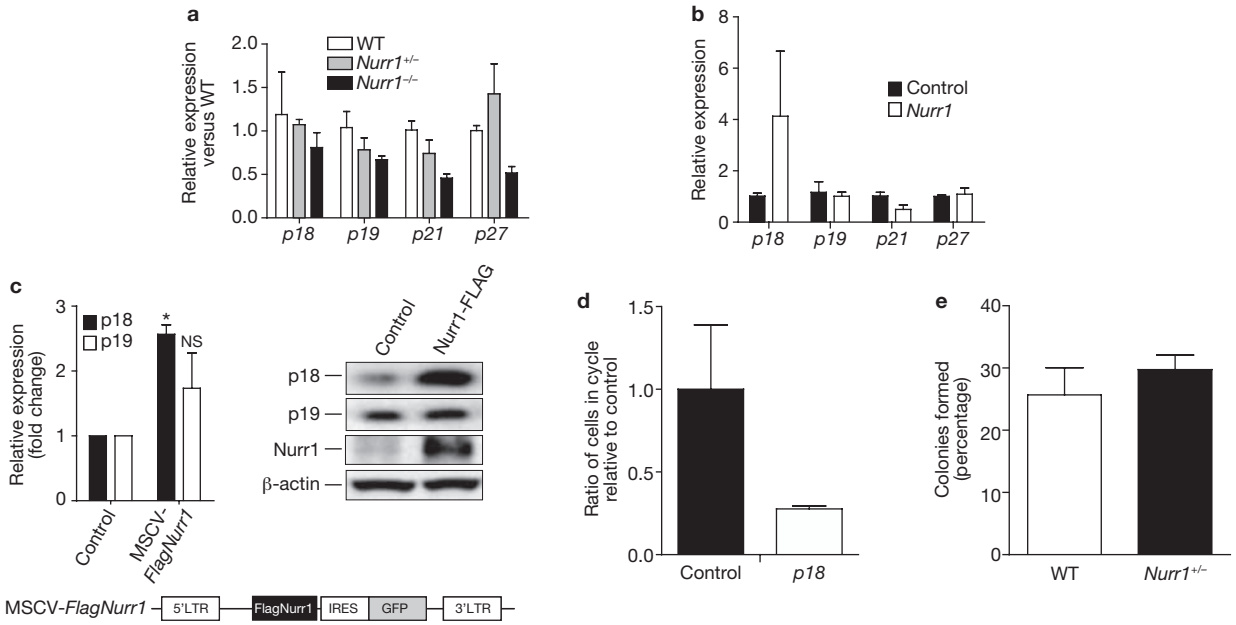

Figure 4 Dose effect of *Nurr1* on expression of cell-cycle inhibitors and rescue of the *Nurr1* phenotype by p18 expression. (**a**) Expression level of the indicated genes in SP-KSL cells isolated from the fetal liver of wild-type, *Nurr1*$^{+/-}$ and *Nurr1*$^{-/-}$ mice at E14.5. SP-KSL were isolated and sorted by flow cytometry and level of mRNA was assessed by real-time PCR. Data are means ± s.e.m. ($n = 3$). (**b**) Expression level of the indicated genes in HSCs (KSL, CD150^{+}) transduced with the indicated vectors (GFP^{+}) was assessed by real-time PCR. Cells were sorted by flow cytometry. Data are means ± s.e.m. ($n = 4$) and expression levels are relative to control. (**c**) Left: fold-change in p18 or p19 protein levels as quantified from the western blot shown to the right, after transfection of 32D cells with empty control vector, or vector containing construct shown in schematic representation at the bottom. Fold-change in expression is calculated relative to cells transfected with control vector. Data are means ± s.e.m. ($n = 3$; asterisk indicates $P = 0.011$; NS, not significant). Right: western blot of lysates from 32D cells transfected with the indicated vectors. Lysates were immunoblotted with antibodies against the indicated proteins. β-actin was used as a loading control. (**d**) KSL HSC progenitor cells were isolated from *Nurr1*$^{+/-}$ mice and transduced with either a control vector (GFP only) or a vector expressing *p18*. Proliferation of the cells was assessed by flow cytometry. Data are means ± s.e.m. and calculated relative to control cells ($n = 3$). (**e**) Colony formation assay of cells isolated from wild-type mice, and *Nurr1*$^{+/-}$ overexpressing *p18* (compare with Fig. 3c). Data are means ± s.e.m. ($n = 3$). Uncropped image of blot is shown in Supplementary Information, Fig. S6.

into the cell cycle phase affected by Nurr1, we sorted *Nurr1*-transduced HSCs for real-time PCR analysis. Interestingly, *p18,* but not *p19*, *p21* or *p27,* was upregulated in *Nurr1*-overexpressing HSCs (Fig. 4b), although there seemed to be a slight increase in *p19* levels in a more heterogeneous progenitor population (Supplementary Information, Fig. 5a). p18 and p19 are inhibitors of the cyclin D–CDK4–CDK6 complex needed for cell-cycle progression through G_1, whereas p21 and p27 inhibit later-appearing cyclin–CDK complexes. This indicates that Nurr1 activates cell-cycle inhibitors specific for progression through G_1, rather than later cell-cycle phases. To confirm upregulation of p18 at the protein level on *Nurr1* overexpression, 32D cells were transduced with a control vector or a vector encoding Flag-tagged *Nurr1*. After 4 days of culture (optimal based on earlier experiments; Fig. 1f), protein levels of p18 and p19 were determined by western blot analysis. The results showed a significant increase ($P = 0.011$) in p18 but not p19 levels with *Nurr1* overexpression (Fig. 4c), suggesting that the inhibitory effects of Nurr1 on HSC self-renewal and differentiation may be mediated by p18. This could be direct or indirect activation of p18, as we observed no Nurr1-binding sequence in the *p18* promoter.

To determine whether the hyper-proliferation phenotype of *Nurr1*$^{+/-}$ mice could be relieved by forcibly expressing p18, we expressed *p18* from a retrovirus in haematopoietic progenitors from *Nurr1*$^{+/-}$ mice. Cell-cycle analysis demonstrated reduced progenitor proliferation when compared with cells transduced with the control vector (Fig. 4d). Furthermore, when *p18* expression was forced in cells from *Nurr1*$^{+/-}$ mice, their proliferative advantage relative to cells from wild-type mice was lost (Fig. 4e), thereby relieving the Nurr1$^{+/-}$ over-proliferation phenotype. Enforced expression of *Nurr1* in *p18*-null cells was not able to cause the proliferation block as it does in wild-type cells (Supplementary Information, Fig. 5b, c), consistent with a role for p18 downstream of Nurr1.

Here, we show that Nurr1 tightly regulates HSC quiescence. Loss of only a single allele of *Nurr1* in HSCs results in their enhanced cycling and sensitivity to the anti-mitotic agent 5FU. By contrast, overexpression of *Nurr1* inhibits proliferation of the haematopoietic progenitor cell line 32D, as well as that of bone-marrow progenitors plated *in vitro*. Remarkably, *Nurr1* overexpression in transplanted HSCs inhibits their proliferation, but this block is released when *Nurr1* is deleted with the Cre recombinase. Finally, consistent with its role in retaining HSCs in G_0, Nurr1 appears to function through upregulation of the early G_1-phase cell-cycle inhibitor p18.

In general, genes known to have a role in HSC dormancy have a broad function in many different tissues. Only a few transcription factors besides *Nurr1* with purported roles in quiescence have expression patterns restricted to haematopoietic progenitors. One of these, the PR-domain-containing transcription factor Evi1, is expressed in HSCs at level approximately 1000-fold higher than in differentiated haematopoietic cells[14] and is involved in leukaemia generation through chromosomal translocation[27]. Necdin is another transcription factor that has much higher expression in HSCs than differentiated haematopoietic cells[14,28], but it appears to have a modest functional role in HSCs[4,28].

Importantly, overexpression of *Necdin* fails to impose quiescence in HSC as does *Nurr1* (J. Berg and M.A.G., unpublished observations).

Although *Nurr1* expression seems to be restricted to haematopoietic progenitors, the paralogues *Nur77* and *Nor1* are expressed in progenitors and myeloid cells. Mice deficient in *Nur77* and *Nor1* died 2–4 weeks after birth with an AML phenotype[19]. Furthermore, leukaemic blasts from AML patients demonstrated downregulation of *NUR77* and *NOR1*, consistent with a role in the proliferation of haematopoietic progenitors[19]. As both Nur77 and Nor1 are expressed in mouse HSCs and bind to the same DNA sequence recognized by Nurr1 (refs 29–31), we suggest that all three may cooperate to maintain HSC dormancy.

To identify mechanisms by which Nurr1 may regulate quiescence, we measured expression of four cell-cycle inhibitors associated with modulation of HSC proliferation[6,7,32–34]. Only p18 was unequivocally upregulated on *Nurr1* overexpression. As p18 is hypothesised to preserve HSC function by limiting cell-cycle entry[34], its regulation by Nurr1 provides a plausible mechanism for the effects of *Nurr1* overexpression or deletion seen in this study. Whether Nurr1 targets the p18 gene directly or indirectly remains to be determined.

Because of the importance of Nurr1 in brain development, its role in this tissue has been extensively studied. Recently, it was shown to interact with co-repressor for element-1-silencing transcription factor (CoREST) to protect neurons from inflammation-induced apoptosis[35]. Nurr1 may have a similar role in HSCs during inflammatory stress[36]. Nurr1 has also been shown to interact with Pitx3, to prevent apoptosis in the brain[37,38]. Although not detected in mature haematopoietic cells, Pitx1 is highly expressed in HSCs[14], suggesting that common mechanisms may regulate parallel processes in HSCs and developing neurons.

Our studies provide critical insight into the transcriptional machinery that protects HSCs from excessive proliferation that could lead to stem cell exhaustion. Taken in the context of other transcription factors that regulate HSC proliferation such as Gfi1, Mef1/E1f4, GATA2, and members of the FoxO family[12,39,40], Nurr1 seems to be part of an intrinsic core regulatory circuit that maintains HSC quiescence, much in the way that Nanog, Oct4 or Sox2 control the pluripotency of embryonic stem cells[41]. Understanding how each of these components functions, and the degree of cross-talk among them, will be crucial to manipulating HSC toward desired therapeutic end-points. □

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturecellbiology/

Note: Supplementary Information is available on the Nature Cell Biology website

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AUTHOR CONTRIBUTIONS

This study was developed and designed by O.S., who also performed the experiments and co-wrote the manuscript. G.L.L. and R.M. helped carry out the experiments. O.M.C. provided *Nurr1*$^{-/-}$ mice and discussion. M.A.G. designed experiments and co-wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Retroviral production and transduction of Sca-1$^+$ bone-marrow cells. Vectors containing *Nurr1* and N-terminal Flag-tagged Nurr1 (*Nurr1–Flag*) were subcloned into a murine stem cell virus (MSCV), and retroviral particles were generated by co-transfecting a viral vector containing *Nurr1* with *pCL-Eco* into 293T cells[42]. The virus particles were collected 48 h later and frozen. Virus was titred on 3T3 cells to ensure transduction of experimental cells with equivalent multiplicity of infection (m.o.i.). Mice were treated with 5FU at 150 mg kg^{-1} in intraperitoneal injections (with PBS; American Pharmaceutical Partners) and killed 6 days later. Bone-marrow cells were collected and enriched for Sca-1$^+$ cells using magnetic enrichment (AutoMACS, Miltenyi). The Sca-1-enriched population was then transduced by spin infection for 2 h at 250*g* at room temperature with an appropriate amount of virus[43]. Transduction media contained Stempro 34 (GIBCO), penicillin/streptomycin, L-glutamine (2 mM), nutrient supplement, mSCF (murine stem cell factor; 10 ng ml^{-1}; R&D Systems), mTPO (murine thrombopoietin; 100 ng ml^{-1}; R&D Systems), and polybrene (4 μg ml^{-1}; Sigma). After transduction, the cells were either incubated for 3 h at 37 °C, washed and transplanted into lethally irradiated mice, or cultured further for annexin-V staining or methocult assay.

Proliferation of 32D cells. 32D cells were transduced with viral vectors. After 48 h of culture GFP$^+$ cells were sorted and cultured further following ATCC protocols. The number of viable cells in each well were counted or scored daily by treating cells with trypan blue (1:1 dilution of 0.4% solution; Sigma).

Colony forming assay. After 48 h of culture in transduction media, GFP$^+$ Sca$^+$ double-positive cells were sorted by flow cytometry into 96-well plates containing MethoCult GF M3434 (StemCell Technologies) and incubated at 37 °C, in 5% CO_2. The numbers of colonies were scored 14 days after plating.

Bone-marrow transplantation. Donor bone-marrow cells were isolated from 8 to 12-week-old C57Bl/6 (CD45.2) mice. C57Bl/6 (CD45.1) recipient mice were lethally irradiated with a split dose of 10.5 Gy and then transplanted by retro-orbital intravenous injection. Peripheral blood was analysed at 4, 8, 12 and subsequent weeks to assess engraftment, transduction and lineage distribution, as described in ref. 14. Flow cytometry for cell sorting and analyses were performed using standard procedures[14] on a Becton Dickinson LSRII, FACSAria, or Beckman-Coulter MoFlow

Annexin-V staining. After bone-marrow or 32D cells had undergone transduction (20 h), Sca$^+$ GFP$^+$ double-positive cells were sorted into a carrier population (500,000 B220$^+$ spleen cells), washed with cold PBS, and stained with annexin-V antibody (Becton Dickinson) in Annexin-binding buffer according to the manufacturer's instructions (BD). Cells were analysed with a LSRII flow cytometer (Becton Dickinson).

Pyronin Y/Hoechst staining. At 48 h post-transduction, transduced Sca-1$^+$ cells were sorted into a carrier population (500,000 B220$^+$ spleen cells) and stained with 20 μg ml^{-1} Hoechst dye and 50 μg ml^{-1} Verapamil for 45 min at 37 °C, at which time pyronin Y was added to a final concentration of 1 μg ml^{-1} and the cells incubated for another 15 min. Samples were washed and analysed with a LSRII flow cytometer (Becton Dickinson).

Survival analysis. Mice were injected weekly with a single dose of 5FU (150 mg kg^{-1}) and observed daily for changes in physical health. The times of death were scored and converted to survival plots, with the Gehan-Breslow-Wilcoxon test used to assess statistical significance.

Real-time PCR Analysis. After RNA isolation in lysis buffer (GE Healthcare), reverse transcription was performed with random hexamer primers and the Superscript II kit (Invitrogen). The cDNA was then used in PCR amplifications with either Taqman mastermix, an 18 s ribosomoal RNA probe (VIC-MGB), and a gene-specific probe (FAM-MGB, Applied Biosystems) or Sybrgreen mastermix and primers targeting the gene of interest as well as GAPDH (for normalization). Reactions were run with an AbiPrism 7900HT (Applied Biosystems) for 40 cycles.

Immunoblotting. Cultured 32D cells were transduced with *GFP*-only or Flag-tagged *Nurr1* and *GFP*-expressing MSCV retrovirus. After 96 h of culture, 6×10^6 GFP-positive cells were solubilized in lysis buffer (PBS at pH 7.4, 2% (v/v) IGEPAL (octylphenyl-polyethylene glycol) and Protease Inhibitor Cocktail; Sigma). The lysates were passed 13 times through a 25-gauge needle and centrifuged at maximum speed for 10 min at 4 °C in an Eppendorf microfuge. The clarified lysates were then mixed in even proportions with 2 × SDS (sodium dodecyl sulfate) sample buffer (Bio-Rad) and resolved on 12% (v/v) Ready Tris-HCl gels (Bio-Rad). The gels were immunoblotted with antibodies against: Flag, 1:1000 dilution (Sigma); p18, 1:500 dilution (Invitrogen); p19, 1:100 dilution (Invitrogen); and β-actin, 1:2000 dilution (Santa Cruz Biotechnology) monoclonal primary antibodies, and 1:4000 dilution of anti-mouse HRP-conjugated (Calbiochem) secondary antibody. Immunoblots were developed with the ECL Plus chemiluminescence reagent (Amersham) and visualized with a Storm 860 phosphorimager or Kodak BIOMAX Light Film (Sigma).

Statistics. Descriptive statistics (means and standard deviations) were used to summarize normally distributed data and the two tailed *t*-test was used to compare results. Differences with a P value of < 0.05 were considered statistically significant. The Gehan-Breslow-Wilcoxon test used to assess statistical significance in survival assays.

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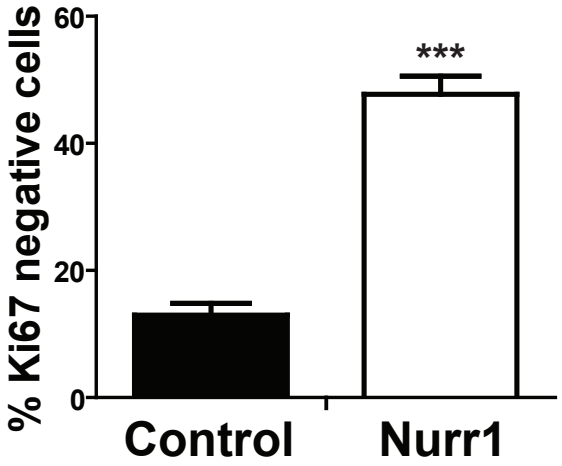

Figure S1 Enforced expression of Nurr1 in 32D cells results in a decrease of cells in cycle. This is demonstrated by an increase of Ki67 negative cells from ~13% quiescent to ~48% with elevated Nurr1 levels (n=6; ***p= 1.27817E-06). Error bars in panel denote sem.

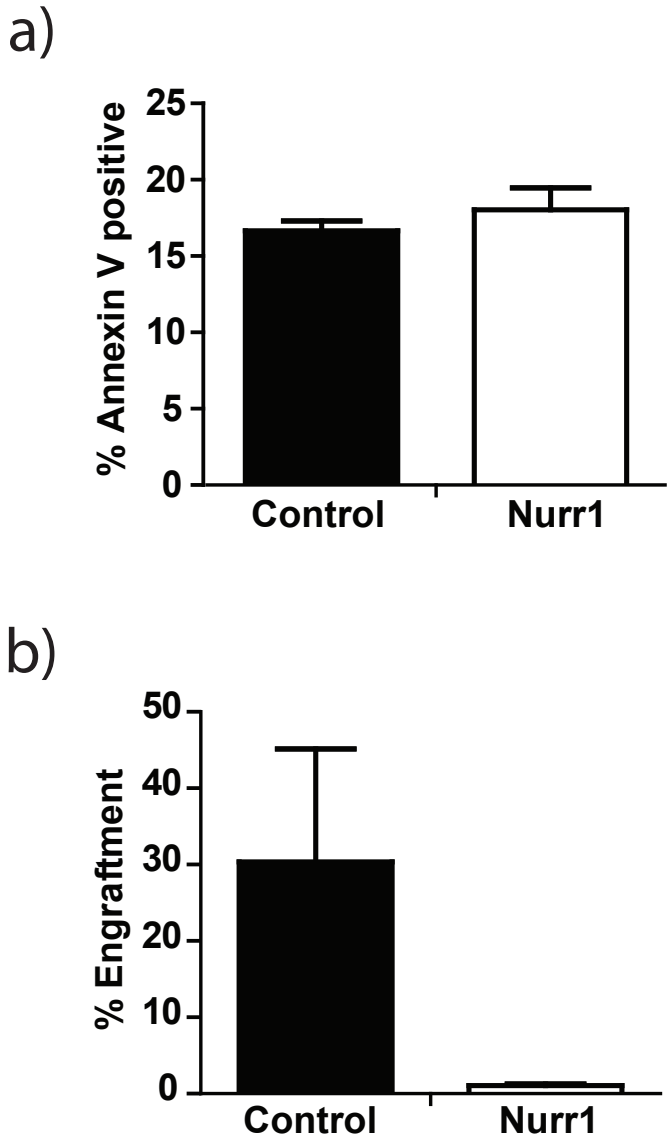

Figure S2 There is no difference in the outcome of the cells with prolonged Nurr1 exposure. (**a**) Annexin V staining of samples as late as 96 hours after transduction with Nurr1 still reveals no significant difference in viability between Control and Nurr1 overexpression (SK CD150+) (n=3). (**b**) Cells overexpressing *Nurr1* show no contribution to peripheral blood as late as 1 year post transplantation (Control n=5; Nurr1 n=7; p=0.0378). Error bars in panel a and b denote sem.

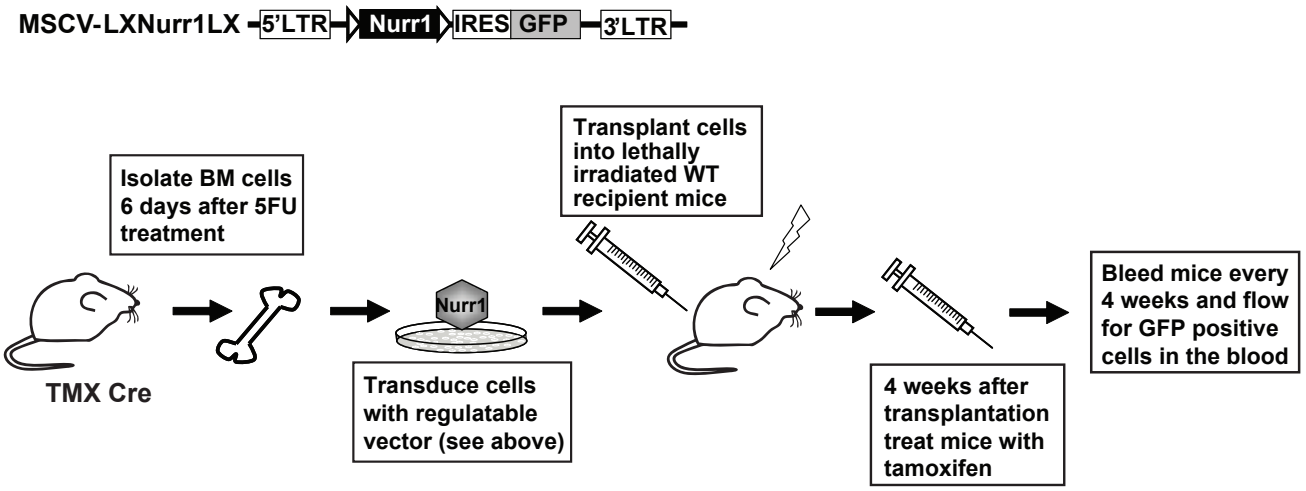

Figure S3 Experimental design and vector used for regulated Nurr1 expression. Triangles flanking the *Nurr1* gene represent the loxp sites. In the presence of Cre recombinase, *Nurr1* should be excised and no longer over-expressed. Donor cells for transplant are isolated from BM of Tamoxifen responsive Cre (TMX Cre) mice. TMX Cre Sca+ cells are isolated and transduced with either control vector containing only GFP, Nurr1-containing vector or the regulatable vector with the Nurr1 gene flanked by loxp sites. Transduced cells are subsequently transplanted into lethally irradiated mice. Four weeks later mice are bled and analyzed for engraftment. Subsequently, half the mice are treated with tamoxifen to induce Cre nuclear translocation and excision of the *Nurr1* gene carried within the retroviral-vector. Changes in contribution of transduced cells to the peripheral blood are followed by monitoring GFP positive cells in peripheral blood in the following weeks.

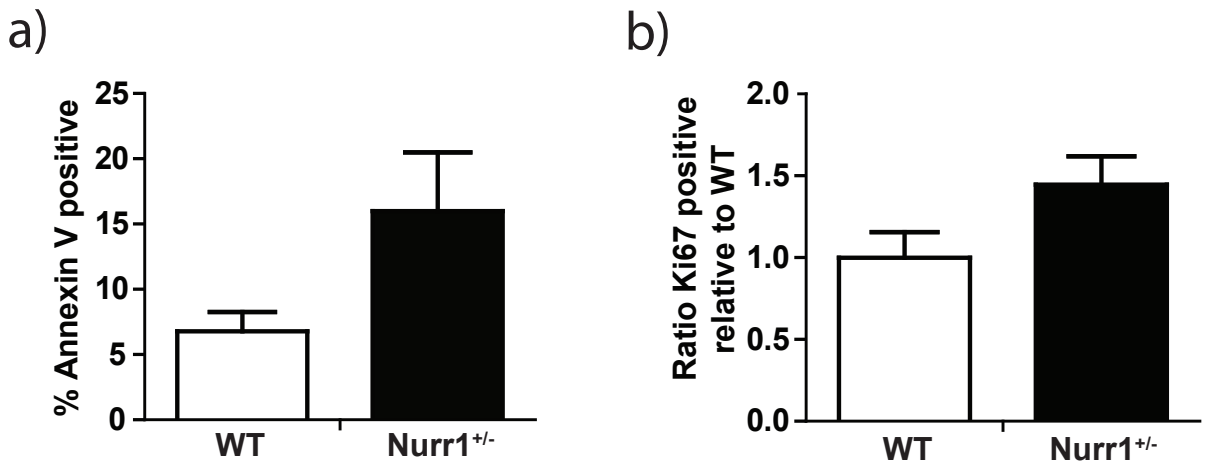

Figure S4 5FU treatment of Nurr1$^{+/-}$ mice results in increased cell death. (**a**) Annexin V staining of HSCs (KSL CD150+) seven days after 5FU injection (corresponding to the day the second 5FU dose would be administered) reveals a higher level of stem/progenitor cell death in the Nurr1$^{+/-}$ mice (n=5) (p=0.09). (**b**) A greater proportion of the Nurr1$^{+/-}$ HSCs are also in cycle, likely also contributing to their increased sensitivity to 5FU treatment (n=5) (p=0.08). Error bars in panels a and b denote sem.

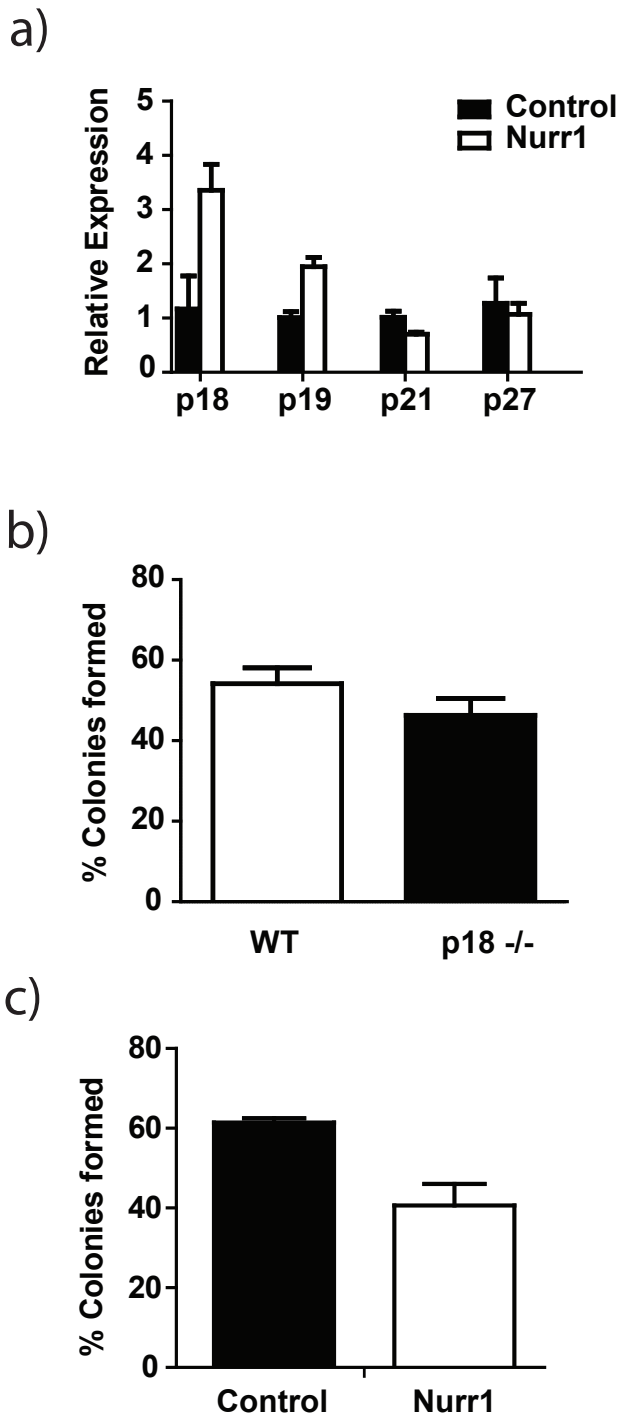

Figure S5 Nurr1 acts primarily via p18 (**a**) Dose effect of *Nurr1* on expression of cell cycle inhibitors *p18*, *p19*, *p21* and *p27* in Sca-1+ cells transduced with control vector and Nr4a2 vector (n=3). Error bars in panels a and b denote sem. (**b**) p18-null KSL CD150+ cells have a similar colony forming ability to WT cells. (**c**) When Nurr1 is over-expressed in p18-null KSL CD150+ cells, colony forming capacity is diminished only slightly relative to control-vector-transduced cells. This is in contrast to the effect when Nurr1 is over-expressed in WT cells (Figure 2b).

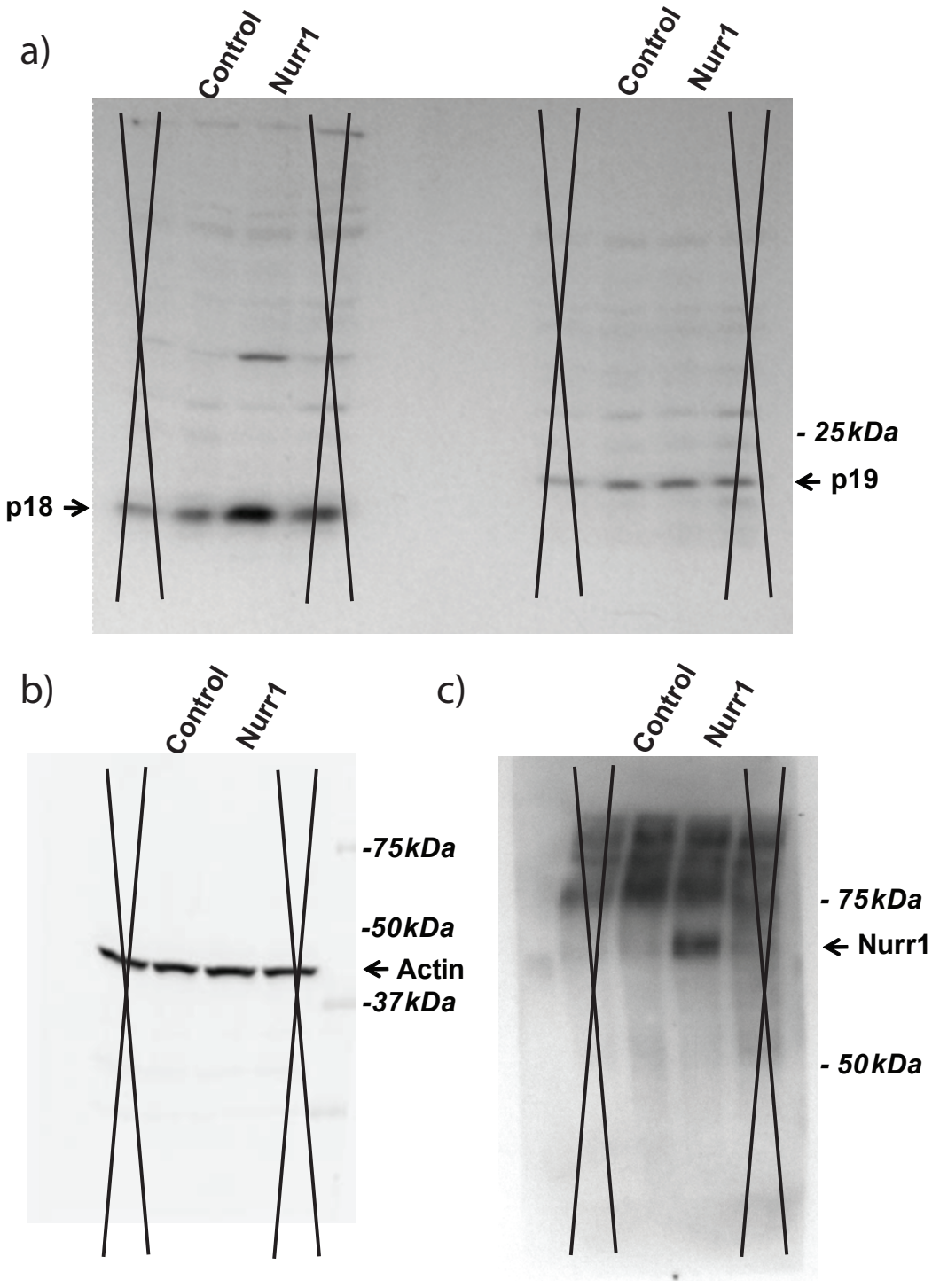

Figure S6 Full scans of blots shown in Figure 4c. Blots stained with (**a**) anti-p18 and anti-p19 (**b**) anti-actin and (**c**) anti-Nurr1.