Haematopoietic stem cells (HSCs) require the right composition of microRNAs (miR) for proper life-long balanced blood regeneration. Here we show a regulatory circuit that prevents excessive HSC self-renewal by upregulation of miR-193b upon self-renewal promoting thrombopoietin (TPO)-MPL-STAT5 signalling. In turn, miR-193b restricts cytokine signalling, by targeting the receptor tyrosine kinase c-KIT. We generated a miR-193b knockout mouse model to unravel the physiological function of miR-193b in haematopoiesis. MiR-193b−/− mice show a selective gradual enrichment of functional HSCs, which are fully competent in multilineage blood reconstitution upon transplantation. The absence of miR-193b causes an accelerated expansion of HSCs, without altering cell cycle or survival, but by decelerating differentiation. Conversely, ectopic miR-193b expression restricts long-term repopulating HSC expansion and blood reconstitution. MiR-193b-deficient haematopoietic stem and progenitor cells exhibit increased basal and cytokine-induced STAT5 and AKT signalling. This STAT5-induced microRNA provides a negative feedback for excessive signalling to restrict uncontrolled HSC expansion.
MicroRNAs (miRs) are small non-coding RNAs, which regulate gene expression by either degrading mRNAs or by inhibiting protein translation. They simultaneously target various miRNAs and thereby fine-tune entire gene expression networks. The necessity of miRs for normal long-term repopulating haematopoietic stem cell (LT-HSC) function became apparent by the haematopoietic-specific deletion of Dicer, an essential nuclease for the generation of mature miRs, leading to the loss of their life-long self-renewal ability. MiR-125a and miR-125b support HSC maintenance, function and renewal, and their upregulation may even drive leukemogenesis. Conversely, miR-126 impedes cell-cycle progression in LT-HSCs, and downregulation of miR-126 causes LT-HSC expansion. Various miRs act at different haematopoietic developmental stages and lineages, and altered miR expression may play a pivotal role in leukaemia onset and progression.

We aimed to identify miRs induced by the self-renewal-promoting cytokine thrombopoietin (TPO)-mediated signalling in LT-HSCs. In particular, we assessed miRs downstream of TPO-induced activation of signal transducer and activator of transcription (STAT) 5A/B. The STAT5A/B signalling pathway is activated by cytokine receptors, such as myeloproliferative leukemia (MPL), c-KIT and the receptors of interleukin 3, granulocyte–macrophage colony-stimulating factor and erythropoietin. LT-HSCs require STAT5A/B activity for self-renewal and maintenance. Constitutively active STAT5A/B promotes marked LT-HSC expansion and the subsequent development of a myeloproliferative syndrome. Therefore, STAT5A/B signalling needs fine-tuning for normal LT-HSC self-renewal.

In this study, we identify miR-193b as a regulatory feedback molecule restricting excessive HSC self-renewal upon the activation of the self-renewal-promoting TPO-MPL-STAT5 signalling. To execute this function, miR-193b restricts cytokine signalling by targeting the tyrosine kinase c-KIT. In turn, miR-193b-deficient haematopoietic stem and progenitor cells (HSPCs) from miR-193b knockout mice exhibited increased basal and cytokine-induced STAT5 and AKT signalling, thereby promoting the consecutive expansion of HSCs. This STAT5-regulated miR balances cytokine signalling via the STAT5 and AKT pathways, providing a negative feedback for excessive signalling to restrict uncontrolled HSC expansion.

Results

In vivo expansion of LT-HSCs in the absence of miR-193b.

To identify miRs that are extrinsically regulated by the self-renewal-promoting signalling axis comprising TPO, its receptor MPL and the transcription factors STAT5A/B, we compared miR expression patterns in LT-HSCs of STAT5A/B-deficient and wild-type (WT) control mice that were stimulated with TPO or kept unstimulated, by quantitative PCR (qPCR; Fig. 1a). The differential miR pattern revealed five miRs that were >2-fold upregulated by TPO only in the presence of STAT5A/B: miR-193b, miR-132, miR-125a, miR-331-5p and miR-669a (Fig. 1a and Supplementary Data 1). We focused on the function of the intergenic miR-193b in haematopoiesis, because miR-193b is selectively expressed in LT-HSCs and to a lesser extend in multipotent progenitors (MPPs), but not in lineage-committed progenitors and mature blood cells, as shown by us (Supplementary Fig. 1a) and others. Furthermore, haematopoietic stress induced by the cytokine storm 10 days after 5-fluorouracil (5-FU) treatment upregulated miR-193b expression in LT-HSCs (about 2.5-fold in comparison to steady-state). Although the induction of miR-193b expression was even more pronounced in lineage-committed progenitors and mature blood cells than in LT-HSCs caused by 5-FU treatment, the expression level in these committed cells was still 1,000 times lower than in LT-HSCs (Supplementary Fig. 1b). Recently, we demonstrated that STAT5A/B binds to the miR-193b promoter in the murine mammary gland. Here we could show that STAT5A/B is required for the cytokine-induced miR-193b transcription in LT-HSCs.

To unravel the function of miR-193b in haematopoiesis, we generated miR-193b knock-out mice, which were viable without visible abnormalities. First we investigated the steady-state haematopoiesis of 2- to 3-month-old miR-193b−/− mice. Compared with WT mice, no significant differences (according to t-tests) were observed in the mature blood cell lineages in peripheral blood, bone marrow (BM) or spleen of miR-193b−/− mice (Supplementary Fig. 2a-c). The percentage and number of defined BM progenitor cells were also unchanged (Fig. 1b and Supplementary Fig. 2d,e). However, miR-193b−/− mice over 6 months of age displayed an unexpected increase in LT-HSCs in the LSK (Lineage− Sca1− c-KIT−) compartment (Fig. 1b), whereas total LSK cell numbers were not altered (Supplementary Fig. 2e). The accumulation of LT-HSCs increased with age, as 1-year-old mice showed a 1:1 ratio of LT-HSCs and MPPs (Fig. 1b). Yet, we only determined the LT-HSC frequency by their well-established marker phenotype, but we needed to confirm their true identity by their long-term blood reconstitution ability. To corroborate that miR-193b−/− LT-HSCs were fully functional, we performed a competitive transplantation of LT-HSCs from 1-year-old miR-193b-deficient or WT mice into recipients and then monitored donor blood reconstitution (Fig. 1c). The miR-193b-deficient LT-HSCs reconstituted equally well as WT LT-HSCs (Fig. 1d) and exhibited normal production of T, B and myeloid cells (Supplementary Fig. 2g). Strikingly, when we analysed the distribution of LT-HSC and progenitor cells in primary recipient BM, we determined a more than twofold increase in phenotypic LT-HSC numbers in the absence of miR-193b in comparison to the WT controls (Fig. 1e). Although donor cell engraftment in the BM was only slightly enhanced in the absence of miR-193b (Supplementary Fig. 2f), overall BM donor cellularity was markedly increased, thereby suggesting that miR-193b−/− LT-HSCs self-renew extensively after transplantation stress to repopulate the recipient (Supplementary Fig. 2h). We further challenged the self-renewal ability of miR-193b-deficient LT-HSCs by transplanting unfractionated BM cells from primary recipients into secondary recipient mice. Again, both the WT and knockout group reconstituted the secondary recipients almost equally well, which clearly indicated that miR-193b-deficient LT-HSCs were fully functional (Fig. 1f). When we gated for LT-HSCs in the BM of secondary recipients, we again measured a consistent increase in LT-HSC numbers in those recipients that received miR-193b−/− cells (Fig. 1g). Assessing the donor-derived HSPC distribution in primary and secondary recipient BM, we observed a consistent increase in miR-193b−/− LT-HSCs and subsequent increase in LSK cell numbers (Fig. 1h,i). Although no difference was found in the primary recipients of either miR-193b−/− or miR-193b+/+ LT-HSCs (Fig. 1h), secondary recipient BM accumulated miR-193b−/− LT-HSCs and LSK cells (Fig. 1i). This proves that the self-renewal of LT-HSCs leading to an enlarged compartment of competent LT-HSCs is intrinsically promoted in the absence of miR-193b. Of note, the recipients that received the miR-193b−/− donor cells displayed a slightly lower donor cell chimerism in the BM (Supplementary Fig. 2f,h), indicating that although they received more LT-HSCs from primary recipients and again showed an enhanced self-renewal and expansion of LT-HSCs, the ability of these LT-HSCs to produce the same output on mature cells seemed altered. Whether this is due to a delay in differentiation of LT-HSCs or an altered clonal fitness remains to be clarified.
More miR-193b-deficient LT-HSCs are in active cell cycle. Most LT-HSCs are quiescent (G0 phase) in homeostasis. The expansion of the LT-HSC population may indicate that an increased proportion of LT-HSCs are actively cycling in the absence of miR-193b. Therefore, we examined the cell-cycle phases in 2- to 3-month-old mice (Fig. 2a). Indeed, there were a significant higher proportion of LT-HSCs and MPPs in cell-cycle (Ki67${}^{+}$) in miR-193b$^{-/-}$ mice (Fig. 2a). The continuous observation of individual LT-HSCs by time-lapse microscopy-based cell tracking allowed us to determine the time of entry into the first division once cells were exposed to in vitro culture. The miR-193b$^{-/-}$ LT-HSCs displayed an earlier time point of division compared with WT controls under minimal (stem cell factor (SCF) only) and self-renewal-promoting (TPO and SCF)
cytokine conditions (Fig. 2b). Next, we functionally confirmed these findings by repetitively treating mice with 5-FU, which eliminates proliferating cells. Quiescent LT-HSCs only become susceptible to 5-FU during haematopoietic stress, driving them into cycle. We injected 5-FU into miR-193b−/− and miR-193b+/− mice once a week, until the mice suffered from fatal haematopoietic failure (Fig. 2c). Although most of the WT mice died after the third round of 5-FU injections, all miR-193b-deficient mice died already after two subsequent injections, most likely because the hyperproliferative LT-HSCs were quickly extinguished (Fig. 2c). These results show at the phenotypic, molecular and functional level that the absence of miR-193b leads to an intrinsically controlled expansion of fully functional LT-HSCs, as they exhibit reduced quiescence and increased self-renewal.

**MiR-193b controls HSPC expansion by altering differentiation.**

We then assessed the immediate consequences of the absence of miR-193b on the function and fate of LT-HSCs. First, we measured the expansion of LT-HSCs in liquid culture (Fig. 3a) and found that LT-HSCs from miR-193b−/− mice gave rise to a twofold increase in cell numbers over a 7-day-period compared with WT LT-HSCs (Fig. 3a). Conversely, lentiviral expression of miR-193b in LT-HSCs led to a dramatic reduction of miR-193b-expressing progeny over time, whereas the percentages of control vector transduced cells or of cells lentivirally expressing the unrelated miRs-132/212 remained largely constant (Fig. 3b,c, and Supplementary Figs 3a and 4a). Of note, ectopic expression of miR-193b in GMPs did not influence their expansion, and the percentage of transduced cells did not change over time (Supplementary Fig. 4b), indicating a distinct function of miR-193b in LT-HSCs. The increased expansion of LT-HSCs and their progeny leading to elevated levels of mature blood cells may be explained by increased proliferation, reduced apoptosis or delayed differentiation. To dissect these various cell fates at a single-cell resolution, we monitored the individual LT-HSCs and their progeny of miR-193b-deficient and WT mice using video-microscopy-based cell tracking (Fig. 3d and Supplementary Fig. 5). Interestingly, the absence of miR-193b had no influence on cell-cycle duration in dividing LT-HSCs and their progeny for many generations (Fig. 3e). Moreover, there was no decrease of cell death events in the absence of miR-193b (Fig. 3f). To verify that the absence of miR-193b does not influence the cell-cycle duration of HSPCs, we determined HSPC proliferation in vivo by pulsing miR-193b−/− and miR-193b+/+ mice with 5-bromodeoxyuridine (BrdU) for 4 h. Accordingly, there was no difference in the cell-cycle distribution in LT-HSCs, MPPs, GMPs or MEPs, which further confirmed that the absence of miR-193b does not shorten the cell cycle of HSPCs (Fig. 3g). These results show that the absence of miR-193b increases the number of LT-HSCs in active cell cycle (Fig. 2), but does not alter the cell-cycle progression of proliferating LT-HSCs or their progeny during steady-state haematopoesis (Fig. 3).
Increased cytokine signalling in miR-193b^{−/−} LT-HSCs. Next, we aimed to assess the molecular mechanism by which miR-193b restrains HSPC expansion and LT-HSC self-renewal. We performed RNA sequencing of LSKs from miR-193b^{−/−} and miR-193b^{+/+} mice to elucidate differences in the gene expression profile. Using a regulation threshold of 1.5-fold (N = 3 independent experiments), we identified 41 upregulated and 117 downregulated genes in the absence of miR-193b (Supplementary Fig. 6 and Supplementary Data 2). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of these regulated genes using DAVID18 and Ingenuity Pathway Analysis suggested the involvement of altered cell signalling (Fig. 4a and Supplementary Data 3 and 4). This result guided us to investigate major signalling pathways that are altered in miR-193b-deficient LT-HSCs compared with WT counterparts (Fig. 4d). Next, we determined the kinetics of STAT5 and AKT signalling in temporal relation to miR-193b expression in LT-HSCs upon cytokine stimulation, and investigated changes in STAT5 and AKT signalling in the absence of miR-193b (Fig. 4e). After a rapid induction already at 5 min of stimulation, the levels of pSTAT5 and pAKT further increased to a maximum at 20 min in LT-HSCs from miR-193b^{+/+} and miR-193b^{−/−} mice. pSTAT5 and pAKT levels remained constant for 60 min before declining again at 120 min. Intriguingly, miR-193b^{−/−} LT-HSCs showed an overshooting activation of pSTAT5 at 20 min, that did not decline as much as the pSTAT5 levels in miR-193b^{+/+} LT-HSCs at 120 min (Fig. 4e). MiR-193b^{−/−} LT-HSCs showed a higher induction of pAKT at 20 min, which remained above the pAKT levels of miR-193b^{+/+} LT-HSCs until 120 min (Fig. 4e). The expression of miR-193b was instantly induced by cytokine stimulation and showed the strongest expression at 60 min after stimulation before it already declined at 120 min. Importantly, already 20 min after stimulation there was a twofold increase in miR-193b expression in comparison to unstimulated LT-HSCs (Fig. 4e).

These results underline that the cytokine-induced activation of STAT5 leads to the rapid upregulation of the miR-193b as a STAT5-dependent miR, and the time shift between maximum pSTAT5 (at 20 min) and maximum miR-193b expression (at 60 min) would be expected from a transcribed target gene of STAT5. However, in the absence of miR-193b there is an
Figure 4 | The absence of miR-193b increases basal and cytokine-stimulated signalling in LT-HSCs. (a) Functional annotation analysis using DAVID (KEGG pathways) of the upregulated and downregulated genes in sorted LSK cells derived from miR-193b 

overshooting pSTAT5 and pAKT activation signal, which would have been dampened in the presence of the already increased miR-193b levels at 20 min. Furthermore, the activation of STAT5 and AKT persisted longer in the absence of miR-193b, which suggests a negative regulation of the signalling kinetics by the miR-193b.

MiR-193b targets c-KIT and thereby modulates signalling. The increased signalling output may be caused by kinase hyper-activation. Therefore, we applied PamGene array technology to quantitatively compare the activity of hundreds of tyrosine and serine/threonine kinases in the BM cells of miR-193b 

As the c-Kit mRNA contains a predicted conserved miR-193b target sequence (Fig. 5b), and c-Kit activity was elevated in the absence of miR-193b, we predicted that miR-193b can modulate c-Kit expression in HSPCs. Indeed, there was a 30% reduction of c-Kit mRNA expression in LT-HSCs ectopically expressing miR-193b (Fig. 5c). More importantly, we determined
a 40% reduction of c-KIT surface expression on HSPCs that expressed miR-193b in comparison to control vector transduced cells, measured by FACS (Fig. 5d). Because LT-HSCs with diminished c-KIT function are severely impaired in recipient repopulation\(^9,20\), we assessed the blood reconstitution of miR-193b-expressing LT-HSCs after transplantation. As hypothesized, no donor cell reconstitution was detected in mice transplanted with LT-HSCs ectopically expressing miR-193b (Fig. 5e). Reduced cell expansion and lack of blood reconstitution following transplantation with LT-HSCs overexpressing miR-193b resembles the phenotype of LT-HSCs harbouring dysfunctional c-KIT\(^9,20\). Next, we assessed the c-KIT protein expression in miR-193b\(^{-/-}\) BM cells and showed via FACS that miR-193b-deficient cells expressed 30% more c-KIT protein in comparison to their respective miR-193b\(^{+/+}\) counterparts (Fig. 6a), indicating that the absence of miR-193b leads to higher c-KIT protein levels.

To consolidate that the observed phenotype in miR-193b-expressing LT-HSCs was at least partly due to the reduced c-KIT expression, we lentivirally expressed both c-KIT lacking the miR-193b target site (Supplementary Fig. 3b) and miR-193b in LT-HSCs (Fig. 6b). We then assessed the expansion of double-transduced cells in culture. Although cells expressing miR-193b and a control vector nearly disappeared over the course 7 days,
miR-193b-expressing cells co-transduced with c-KIT expanded in culture, thereby rescuing the miR-193b-mediated effect (Fig. 6c). These results show that STAT5-activated miR-193b regulates c-KIT expression, probably among other important factors, and thereby subsequently influences signalling networks to control the fate of LT-HSCs.

**Discussion**

Two prominent signalling pathways guiding LT-HSC self-renewal and proliferation, STAT5A/B and PI3K/AKT, are hyperactivated in the absence of miR-193b leading to a more active LT-HSC population that expands over time (Supplementary Fig. 7). Constitutive active STAT5 signalling induces LT-HSC expansion and finally results in a myeloproliferative disease in mice12,13. Furthermore, many leukemicogenic alterations (for example, mutated c-KIT, Flt3-ITD and BCR-ABL) require chronically high STAT5 activation for disease development22–24. PI3K/AKT signalling promotes cell proliferation and survival25, and hyperactivation of this pathway results in LT-HSC expansion and exhaustion, and in the development of leukaemia26. Furthermore, SCF is essential for the survival and proliferation of all HSPCs, and it transmits its signal after binding to c-KIT leading to STAT5, PI3K/AKT and ERK signalling21. Activating c-KIT mutations, which are common in acute leukaemias, cause SCF-independent constitutive, dysregulated signalling and eventual uncontrolled expansion of leukaemic blasts27. Conversely, in mouse models, hypomorphic c-KIT mutations compromise LT-HSCs, which can easily be outcompeted by WT LT-HSCs in transplantsations without harsh conditioning28. STAT5 and AKT signalling need to be in tight balance26, and are controlled by negative regulators such as suppressor of cytokine signaling (SOCS) and phosphatase and tensin homolog (PTEN)10,29–32. PTEN-deficient LT-HSCs display increased PI3K/AKT signalling and a hyperproliferative phenotype with long-term exhaustion9,30,32. In this study, we found that miR-193b is an important negative regulator of basal and cytokine-stimulated signalling and hyperactivation in LT-HSCs. Therefore, as miR-193b expression is triggered by STAT5 signalling, this axis represents a classical negative feedback mechanism. Strikingly, although miR-193b is a key player in the cell-cycle length in proliferating HSCs, it often regulates the decision of active cell-cycle entry versus quiescence.

It is not surprising that miR-193b is downregulated in leukaemia, and other cancer entities33–36. Although we did not observe increased cancer incidence in 1-year-old miR-193b−/− mice, it would be intriguing to test whether the absence of miR-193b cooperates with known oncogenes. Especially, the initiation of pre-cancerous (stem) cells at an early disease stage might be supported by enhanced self-renewal in the absence of the tumour-suppressing miR-193b, as STAT5 activation plays a key role in establishing pre-cancerous clonal dominance in stem cells37,38. We recently reported the expansion of mammary epithelial stem cells in the absence of miR-193b, which suggests a general miR-193b function in restricting adult stem cell proliferation. Further evaluation is warranted to determine whether miR-193b downregulation is an early event in tumourigenesis.

**Methods**

**Mice.** Male and female C57BL/6, B6.SJ-Ly-5.1/J, B6129S6-Stat5a/Stat5btm2Mam/Mmjax x B6.Gt(RecIfLV151St)/Ifaj and B6.129S6-Stat5a/Stat5btm2Mam/Mmjax x B6.Gt(RecIfLV151St)/Ifaj were purchased from Jackson Laboratory or bred in our animal facility. Male miR-193b-deficient (referred to as miR-193b−/−) and corresponding WT littermate control mice (referred to as miR-193b+/+) were used in this study. The mice were 8–14 weeks of age unless stated otherwise. All mice were bred and maintained under specific pathogen-free conditions. Experiments were performed in accordance with German animal welfare legislation and approved by the relevant authorities (Regierungspräsidium Darmstadt).

**FACS analysis and sorting of HSPCs.** BM cells isolated from femurs, tibias, coxae and sternum were either crushed or flushed (excluding sternum) followed by a depletion of lineage marker-positive cells (EasySep Biotin Selection Kit, Stemcell Technologies) using the following biotin-labelled antibodies (CD3, CD45R, CD19, CD11b, CD41, Ter119 and Gr1). Alternatively, BM mononuclear cells were depleted of lineage marker-positive cells (EasySep Biotin Selection Kit, Stemcell Technologies) using the following biotin-labelled antibodies (CD3, CD45R, CD19, CD11b, CD41, Ter119 and Gr1). The cells were stained with fluorochrome-labelled antibodies and sorted using a FACS Aria I and DIVA 7.0 software (BD) or FlowJo software (FlowJo).

**MiR expression array by quantitative RT-PCR.** Conditional deletion of STAT5A/B was induced in 6-week-old mice (STAT5A−/− x MxI:Cre and STAT5B−/−, 16–20 mice per group and experiment) via Poly(I:C) injections as previously described34. LT-HSCs were isolated from 12- to 16-week-old mice via FACS sorting. 5,000–10,000 viable LT-HSCs were starved for 5 h and subsequently stimulated with 100 ng ml−1 TPO (Peprotech) for 2 h. The RNA was isolated by
using a miRNeasy mini kit (Qiagen). DNA was synthesized from total RNA using a miRNA gene-specific RT-primer pool according to the MicroRNA Megaplex Assay protocol (Megaplex Pool A. Applied Biosystems). The preamplification reaction was performed according to the manufacturer’s protocol. The TaqMan Array MicroRNA Rodent Cards were analysed using the 384-well TaqMan Low Density protocol (Megaplex Pools—Applied Biosystems). The preamplification reaction using a miRNeasy mini kit (Qiagen). cDNA was synthesized from total RNA using (MOI) pRRL.PPT.SFFV.eGFP.wPRE (Schambach 2006) was used to construct the protocol. Reverse transcribed samples from KL and Lin (Life Technologies) the reverse transcription was performed with the TaqMan dx.doi.org/10.6084/m9.figshare.1554878.

endogenous miR-193b levels under steady-state and under stress, LT-HSCs, MPPs, mice (CD45.1) together with 2.5 (100 LT-HSCs/mouse) into sub-lethally irradiated (2.5 Gy) 6- to 8-week-old NSG mice (CD45.1) were transplanted into secondary sublethally irradiated NSG recipients (CD45.1). The third-generation self-inactivating lentiviral vector pRRL.PPT.SFFV.eBFP.wPRE (both co-expressing eBFP2). A MOI 50 was used for each vector. The cells were isolated and reverse transcribed using the Cells-to-Ct-Kit (Life Technologies) according to the manufacturer’s protocol. To quantify the ability of miR-193b to target c-Kit mRNA expression, FACS-sorted LT-HSCs and MPPs were lentivirally transduced for eGFP (enhanced green fluorescent protein) was replaced with eBFP2. The genomic region of miR-193b was amplified from spleenocytes of C57Bl/6 mice using the forward primer 5'-GAGCCCTGACGATAGGTTGCTGG TTGTTT-3` and reverse primer 5'-TAAAGGTACCATTAAAAATATCTACG GAGGCTTTCCGGGATG-3`. The PCR product harboured the sequence from 13449305 to 13449984 of chromosome 11 from spleenocytes of C57Bl/6 mice (Supplementary Fig. 3a). The ectopic expression level of mature miR-193b and miR-132 was confirmed via qPCR using an ABI TaqMan microRNA Assay ID002467 and ID000457 (Life Technologies).

To generate a c-KIT rescue vector, the egFP ORF of the pRRL.PPT.SFFV.eBFP.wPRE vector was replaced by the IRES-VENUS-hilmportin subunit α (AA2-67) and a multiple cloning site (MCS) was inserted downstream of the SFV promoter sequence (pRRL.PPT.SFFV.IRES.VENUSsm.wPRE). The murine c-KIT ORF was amplified from the plasmid PENTRIA-ckt (a gift from Christian Brandts) with the forward (5'-CTTACTAGTGACGCGGAGG) and the reverse (5'-GAATACGGTGTCCTCCA CGACATTCTC-3`) primer pair and then cloned into the MCS of pRRL.PPT.SFFV.MCS.IRES.VENUSsm.wPRE (Supplementary Fig. 3b).

Kie67/7-AAD staining for cell cycle and quiescence. Lineage-depleted BM cells were stained for CD117, Cat, CD150, CD48, CD16/32, CD34 and Staphyvcin. The cells were assessed for Ki67 expression and DNA content (7-AAD) according to the manufacturer’s instructions and measured via flow cytometry using an LSR Fortessa, BD.

Time-lapse imaging and cell tracking. Microscopy and tracking of LT-HSCs and their progeny was performed using a self-written computer programme (TIT) as previously described14,37 until the fate of all progeny in the third cell generation was determined. The generation time of an individual cell was defined as the time span from cytokinesis of its mother cell division to its own division. Dead cells were easily depicted by their shrunk, non-refracting and immobile appearance. Cell tracking was carried out by sacrificing the current animals on days 7 to 8 of the transduction assay. The health status of the animals was monitored daily.

Peripheral blood cell counts. Peripheral blood cell counts were determined from tail vein blood using a Scilvet animal blood cell counter (Scil Animal Care Company).

In vitro SFU treatment. SFU (Medac) was intraperitoneally injected into miR-193b−/- and miR-193b+/- mice (150 mg kg−1) once a week. The weight of the animals was monitored daily.

In vitro cell proliferation assay. FACS-sorted LT-HSCs (100 cells per well) were cultured for 7 days in SFEM supplemented with 100 ng ml−1 murine SCF and TPO, 20 ng ml−1 murine IL3 and IL6 (Peprotech) and 5 U ml−1 human EPO (Promokine). Viable cells were assessed using the Vivialight Plus Cell Proliferation and Cytotoxicity BioAssay Kit (Lonza) at days 3, 5 and 7 according to the manufacturer’s instructions. Luminescence was measured using a Mithras LB940 luminometer (Berthold Technologies). For ectopic miR-193b expression experiments, FACS-sorted LT-HSCs (100 cells per well in 96-well format) were lentivirally transduced (MOI = 100) and cultured for up to 9 days in SFEM supplemented with 100 ng ml−1 SCF and TPO. Viable cells were counted with Trypan blue exclusion. The percentage of transduced cells (eBFP+) was analysed using FACS (BD Cantoll). Ectopic expression of the unrelated miRs-132/212 in LT-HSCs using the same lentiviral expression strategy served as an additional control. FACS-sorted LT-HSCs per well were lentivirally transduced (MOI = 20) and cultured for up to 7 days in SFEM supplemented with 100 ng ml−1 SCF and 20 ng ml−1 murine IL3 and IL6 (Peprotech). The percentage of transduced cells (eBFP+) was analysed via FACS.

Rescue experiment with ectopic c-KIT. Freshly sorted LT-HSCs (100 cells per well) were double transduced with four different combinations of either a vector coding for murine c-KIT (without 3'-untranslated region and miR-193b target site, Supplementary Fig. 3b) or a corresponding empty vector (both co-expressing eBFP2). The MOI 50 was used for each vector. The cells were cultured for up to 9 days in SFEM (Stemcell Technologies) supplemented with 100 ng ml−1 murine SCF and TPO. Reporter fluorescence was measured over time via FACS (BD Canto II). The viability of double transduced cells was calculated by dividing the absolute number of double transduced cells at day 9 by the initial absolute number of double transduced cells at day 3.

QPCR of miR-193b basal, stress and cytokine stimulation. To assess the endogenous miR-193b levels under steady-state and under stress, LT-HSCs, MPPs, KL and Lin cells were FACS-sorted 10 days after injection of 150 mg kg−1 5-FU in four individual 12-week-old miR-193b−/- mice. As a reference-control in two experiments, FACS-sorted samples from KL and Lin cells were isolated and reverse transcribed using the Cells-to-Ct-Kit (Life Technologies) according to the manufacturer’s protocol. The pre-amplification for 14 cycles was performed according to the manual of TaqMan-PreAmp-Master-Mix (Life Technologies) using TaqMan assays for c-Kit (ID: MM00452121) and Gapdh (ID: MM999999999_g1), before qPCR was performed according to the manufacturer’s instructions and measured via flow cytometry using an LSR Fortessa, BD.

Competitive repopulation assay. FACS-sorted LT-HSCs from 12-month-old miR-193b−/- or miR-193b+/+ mice (CD45.2) were transplanted intravenously (100 LT-HSCs/mouse) sub-lethally irradiated NSG mice (CD45.1) together with 2 × 10^7 BM competitor recipient cells (CD45.1). For the miR-193b overexpression transplantation, 350 FACS-sorted LT-HSCs from 3-month-old C57BL/6 mice (CD45.2) were lentivirally transduced 24 h prior transplantation and injected into the tail vein of lethally irradiated B6.SL−/−PtpceraPepch/Boy (CD45.1) recipients (6- to 8-week-old) together with 2 × 10^5 BM competitor recipient cells (CD45.1). Transduction efficiency of the transplanted LT-HSCs was determined via FACS of a remaining cell aliquot after 3 days in culture. Multilineage reconstitution was measured every 4 to 6 weeks post transplantation in PB. Briefly, red blood cells were lysed with PharmLysis Buffer (BD), and the cells were washed with antibodies against CD45.1, CD45.2, CD11b, B220, Ter119 and CD11b/Ly6/6 and a dead/live cell exclusion (Fixable Viability Dye, Ebioscience). Lentivirally transduced haematopoietic cells were detected by their enhanced blue fluorescent protein (eBFp) expression via FACS. The primary recipients were killed 16–24 weeks after transplantation, and 1 × 10^6 BM cells per mouse were transplanted into secondary sublethally irradiated NSG recipient mice (2.5 Gy) or lethally irradiated B6.SL−/−PtpceraPepch/Boy mice. For BM reconstitution analyses (primary and secondary recipients), follicl gradient-enriched BM cells (Histoplate 1083, Sigma) were stained with antibodies against CD45.1, CD45.2, CD3, B220, Ter119 and CD11b/Grl and a dead/live cell exclusion (Fixable Viability Dye, Ebioscience), as well as for CD117, Sc1, CD150, CD48, CD16/32, CD34, Lineage markers, and investigated via FACS.

Vector construction. The third-generation self-inactivating lentivector rPRL.PPT.SFFV.eGFP.wPRE (Schambach 2006) was used to construct the miR-193b and the miR-132/212 expression vectors. The open reading frame (ORF) of eGFP.

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In vitro differentiation. FACS-sorted LT-HSCs (100 cells per well) were cultured from mIr-193b−/− and mIr-193b+/− mice were cultured in SFEM (Stemcell Technologies) supplemented with 100 ng/ml SCF and 10 ng/ml IL-6 and LIF and with 100 ng/ml 3% FCS and 1% Pen/Strep for 120 min with the aforementioned cytokine cocktail. The cells were then fixed with Fix Buffer I after centrifugation. To assess basal signalling activity, the BM cells were transduced of NIH3T3 cells with various concentrations of virus supernatant and Virus production. Visceral Stat3-Stat5−/− mice were infected using a split genome approach via calcium-phosphate-mediated transient transfection of human embryonic kidney HEK293T producer cells as recently described26. After 48 h, supernatant was collected, filtered (45 µm) and enriched via ultracentrifugation (50,000 g, 2 h). Viral titres were determined via transduction of NIH3T3 cells with various concentrations of virus supernant and FACS analysis.

RNA sequencing. A total 10,000 LSK cells (CD117+ Sca1− Lineage−) from four mIr-193b−/− and mIr-193b+/− mice were sequenced. The quality and concentration of the libraries were determined using an Agilent 2,100 Bioanalyzer and RiboGreen fluorescence on QuBit (Life Technologies). The libraries were sequenced using a HiSeq2000 system (illumina).

RNA-seq statistical analysis was performed using Partek genomics suite 6.6 software.

DAVID Bioinformatics Resources functional cluster analysis. The list of significantly differentially regulated genes (1.5-fold upregulated or downregulated in mIr-193b−/− LSK, P < 0.05) was submitted as a list with official gene symbols for DAVID analysis and aligned with murine genetic background. The count threshold and the EASE value were set to 2 and 0.1, respectively. Functional annotation was performed via KEGG pathway analysis.

Ingenuity pathway analysis. To obtain information about the mIr-193b-dependent biological processes from the RNAseq data, gene expression data were analysed using Ingenuity Pathways Analysis with default settings according to the manufacturer’s instructions (IPA) v5.0 (Ingenuity Systems Inc). This tool provides information about diseases, molecular function and biological process categories, as well as biological pathways related to the genes obtained from the RNAseq analysis (Supplementary Data 2). In addition, IPA maps each gene within a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. Gene networks are generated algorithmically based on their connectivity in terms of expression, activation, transcription and/or inhibition. We used IPA to identify canonical pathways that are affected by mIr-193b and upstream regulators predicted to be responsible for the observed mRNA expression changes.

Phosphoflow cytometry. To assess basal signalling activity, the BM cells were flushed in ice-cold PBS and directly fixed in Fix Buffer I after centrifugation. To examine cytokine activation, FACS-sorted LT-HSCs were starved for 1 h in SFEM at 37 °C/5% CO2. Next, the cells were either left untreated (control) or stimulated for 20 min with 10 ng/ml SCF and TPO, 20 ng/ml IL-6 and IL-3 and 5 U/ml human EPO (PromoKine). Cells were analysed via FACS with antibodies against CD117 and CD16/32 and Streptavidin. The cells were analysed using an Agilent 2,100 Bioanalyzer and RiboGreen fluorescence on QuBit (Life Technologies). The quality and concentration of the libraries were determined using a MinElute columns kit (Qiagen) and sequenced. The quality and concentration of the libraries were determined using a MinElute columns kit (Qiagen) and sequenced. The quality and concentration of the libraries were determined using a MinElute columns kit (Qiagen) and sequenced.

Endogenous c-KIT expression determined by FACS. Total c-KIT expression was determined after fixation and permeabilization of BM cells in BD Cytofix Fixation Buffer and Perm Buffer III. Next, the cells were stained with anti-c-KIT-PE-Cy7 or the respective isotype control and analysed by FACS.

Statistics. Statistical analysis was performed with GraphPadPrism software (version 6.0, STATCON). Significant statistical determination was carried out via t-test (two-tailed, unpaired and equal variances) unless otherwise mentioned (see Figure legends). The significance level for all tests was set to p = 0.05; *P value < 0.05; **P value < 0.01 and ***P value < 0.001.

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Author contributions

N.H. and Y.F. designed and performed the experiments, and analysed and interpreted the data. S.W. M.R., F.B.T. and C.W. performed the experiments, H.B. and K.I. performed and analysed the PAMGene data. T.S. developed the cell tracking software. H.S., T.O. and L.H. advised the study and commented on the manuscript. L.H. provided miR-193b knock-out mice and supported experiments at the NIH. M.A.R. conducted and supervised the study, designed and interpreted experiments, and wrote the manuscript together with N.H.

Additional information

Accession codes: The RNA-seq data have been deposited in the BioProject database under accession code PRJNA267041.

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