SUPPLEMENTARY INFORMATION

Megakaryocytes regulate hematopoietic stem cell quiescence via Cxcl4 secretion

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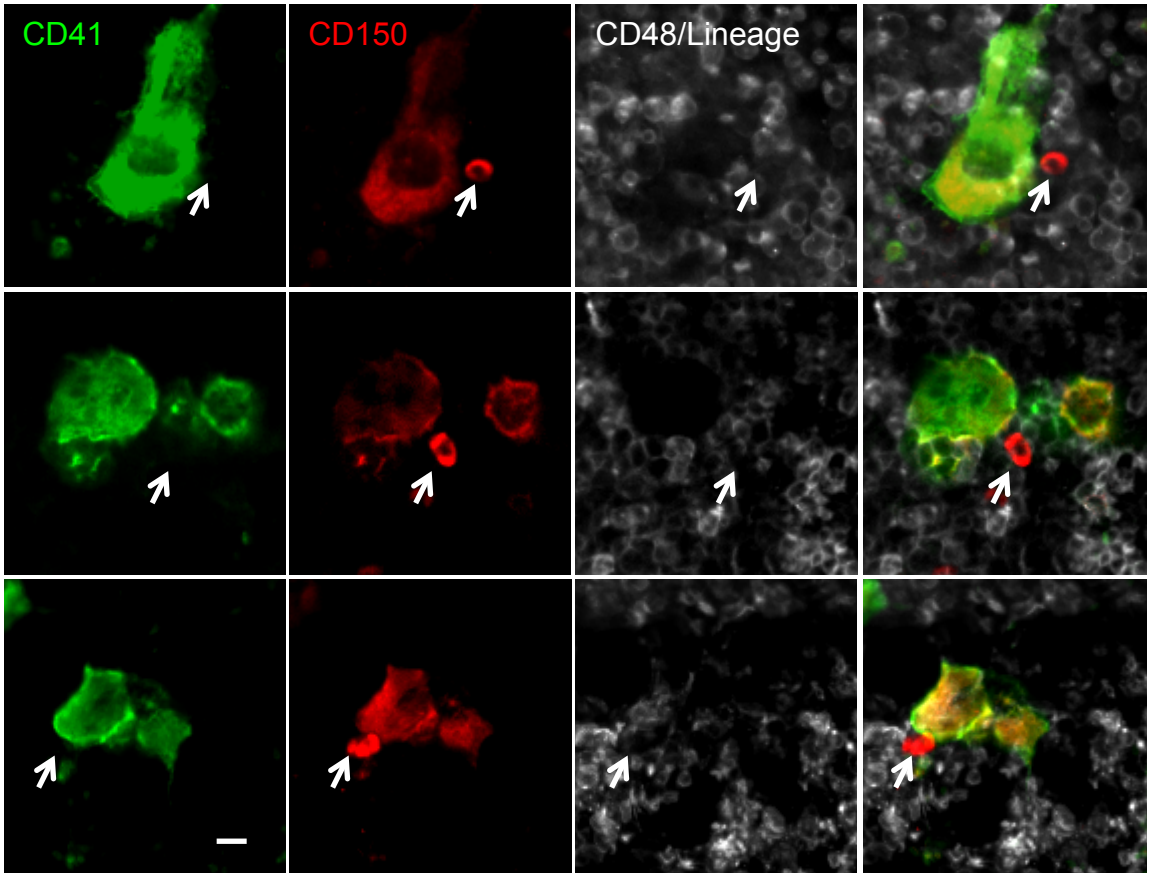
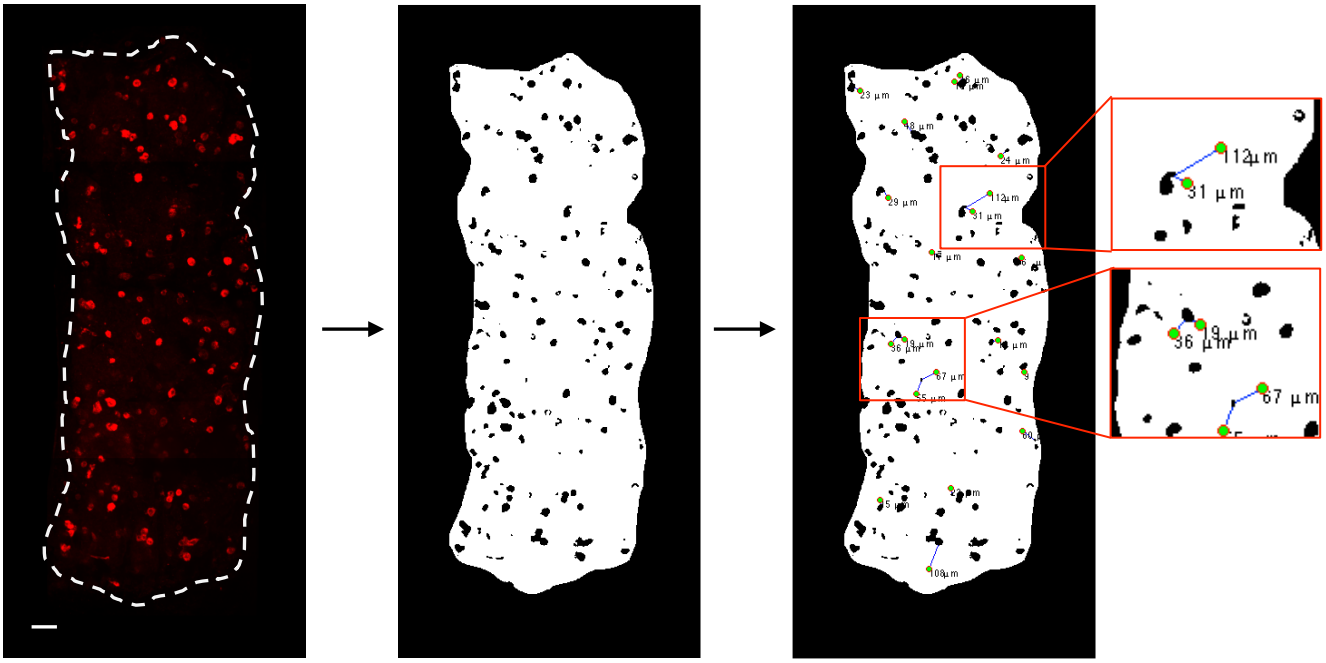
SUPPLEMENTARY FIGURES

Supplementary Figure 1



Supplementary Figure 1. Phenotypic HSCs are frequently located immediately adjacent to Mk. Representative whole-mount images of the mouse BM stained with anti-Lineage (anti-Mac-1, anti-Gr-1, anti-Ter119, anti-B220, anti-CD3e), anti-CD48, anti-CD41 and anti-CD150 antibodies showing HSCs located adjacent to Mk. Arrows denote Lin^{-} $CD48^{-}$ $CD41^{-}$ $CD150^{+}$ phenotypic HSCs. Mk are distinguished by their size and CD41 expression. Scale bar: 10 μm.

Supplementary Figure 2

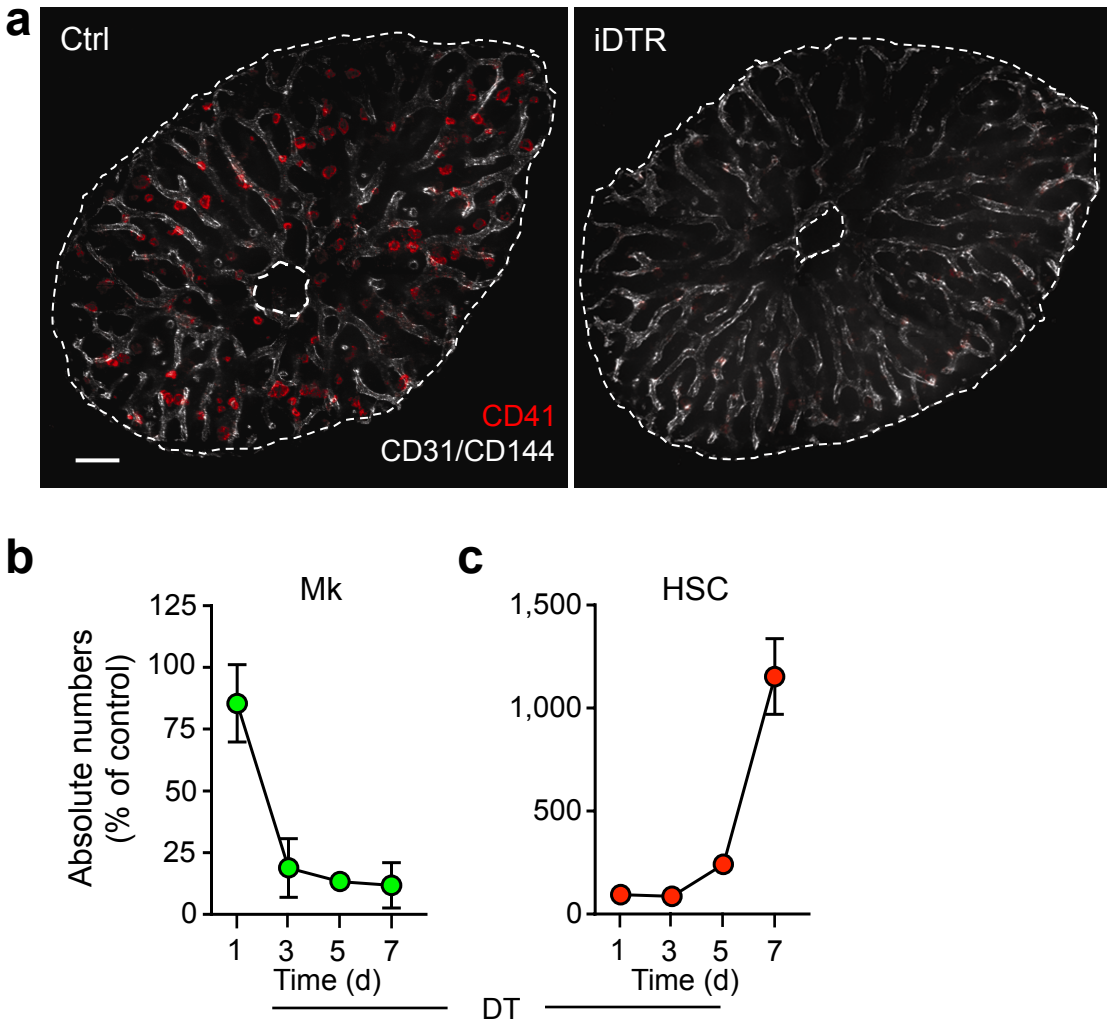


Define binary map of Mk and boundaries and apply median filtering.

Random placement of “HSC dots” within boundaries and subsequent distance measurements.

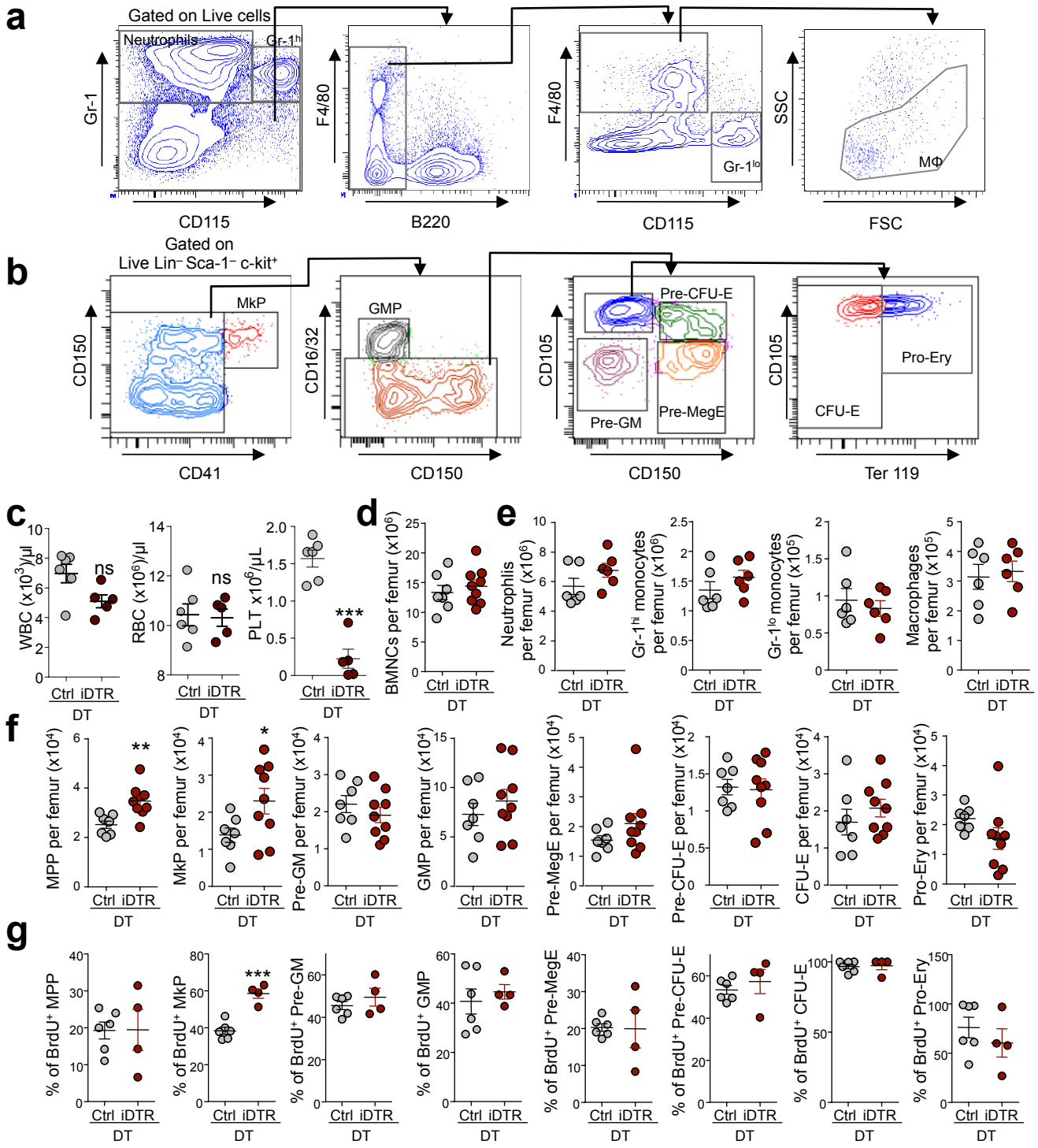
Supplementary Figure 2. Computational simulation of randomly distributed HSCs. To establish the null-model, binary spatial maps of Mk (marked by staining with anti-CD41 antibody) were defined from the images of sternal BM segments. To define a random distribution in which HSCs are not preferentially localized near any marrow structures, we randomly placed HSCs on the unoccupied regions of the spatial maps and measured the Euclidean distance of each HSC to the nearest Mk. For statistical testing of preferential HSC localization *in situ,* their observed distances to a nearby Mk are compared to those obtained by random placement of HSCs *in silica.* Scale bar: 100 µm.

Supplementary Figure 3



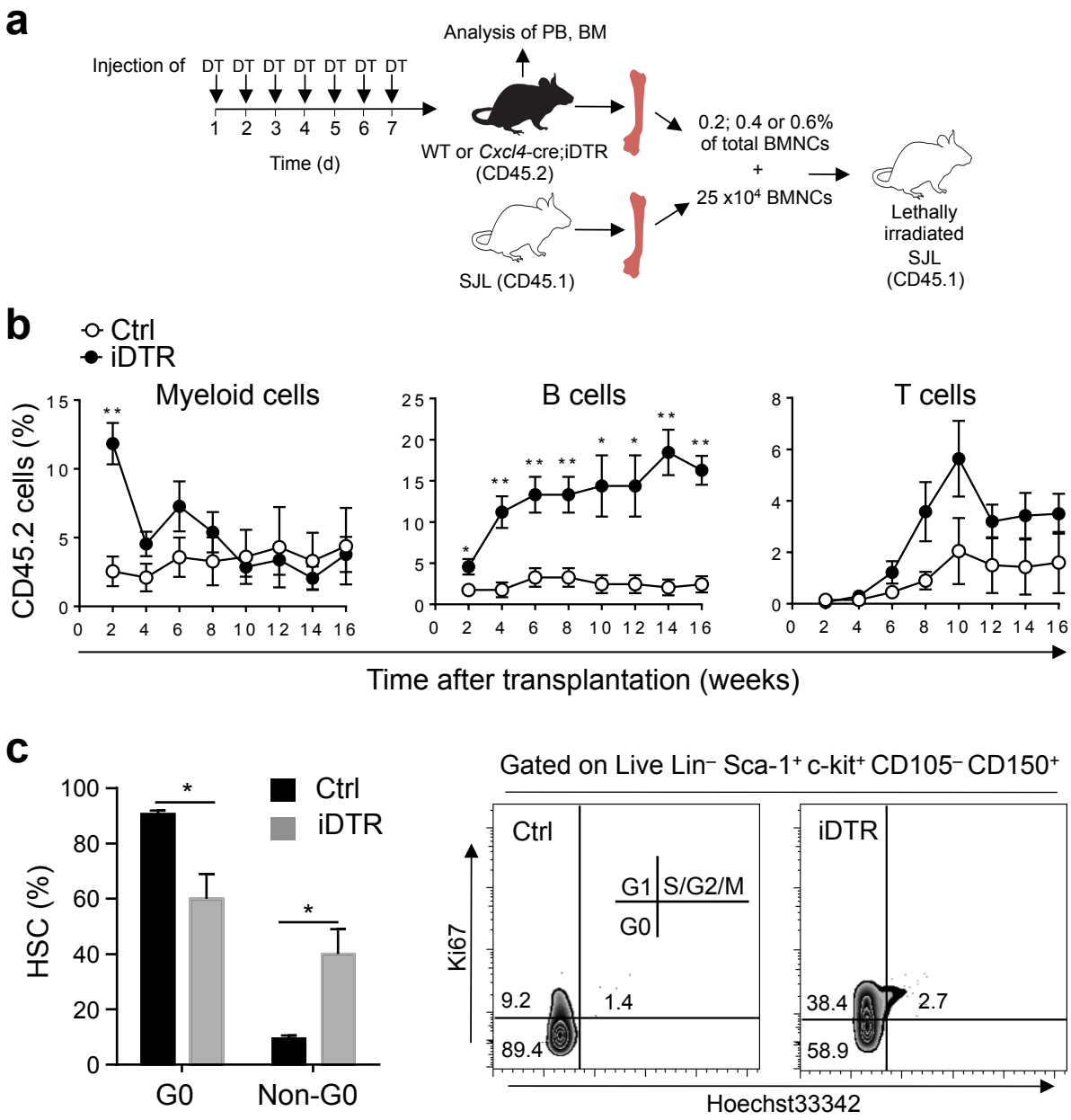
Supplementary Figure 3. *Cxcl4*-cre;iDTR mice show specific Mk depletion and expansion of phenotypic HSCs after treatment with diphtheria toxin (DT). (**a**) Representative whole-mount images of transverse-shaved femoral BM (cross sections) of control (left) and *Cxcl4*-cre;iDTR mice (right) stained with anti-CD41, anti-CD31 and anti-CD144 antibodies after 7 days of DT treatment. (**b,c**) Time course analysis of Mk (**b,** measured by immunofluorescence) and HSC (**c,** measured by FACS analysis) numbers in *Cxcl4*-cre;iDTR mice treated with DT (days 1, 3, 5 and 7 after DT injection). Data was normalized to control mice. For Mk analysis, *n* = 4 control and *n* = 5 *Cxcl4*-cre;iDTR male mice for day 1, *n* = 3 female mice per group for day 3, *n* = 3 male mice per group for day 5 and *n* = 4 male mice per group for day 7 time points where used. For HSC analysis *n* = 3 male mice per group and time point, except for day 7 (*n* = 5), and the day 3 time point where female mice were used. Error bars indicate SEM. Scale bar: 200 µm.

Supplementary Figure 4



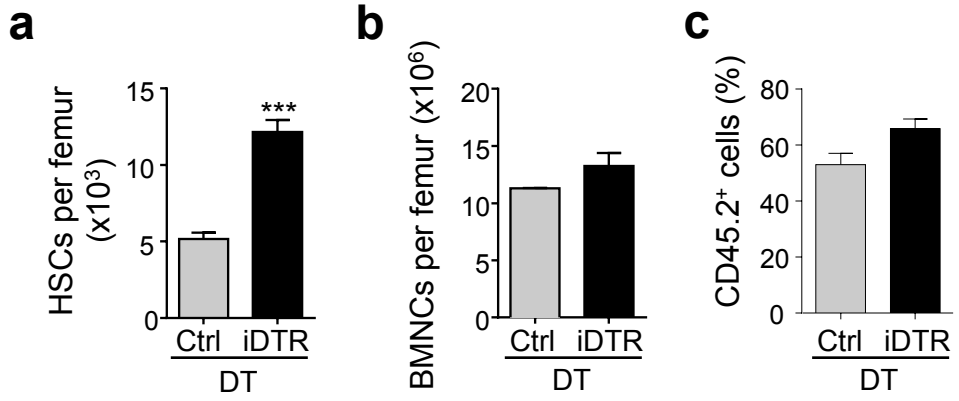
Supplementary Figure 4. Depletion of Mk does not impair hematopoiesis. (**a**,**b**) Gating strategies for the analyses of BM mononuclear phagocytes (**a**) and HSC and progenitor populations (**b**). (**c**,**d**) WBC, RBC and PLT counts in the peripheral blood (**c**) and BMNCs per femur (**d**) of control and *Cxcl4*-cre;iDTR mice after 7 days of DT treatment. (**e**,**f**) Number of neutrophils, Gr-1^{hi} and Gr-1^{lo} monocytes and macrophages (**e**) and progenitor populations (MPP, MkP, Pre-GM, GMP, Pre-MegE, Pre-CFU-E, CFU-E and Pro-Ery) (**f**) in the BM of control and *Cxcl4*-cre;iDTR mice after 7 days of DT treatment. (**g**) Percentage of proliferating cells in the progenitor fractions listed in (**f**) as determined by BrdU incorporation. *n* = 4-9 mice per group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's t-test).

Supplementary Figure 5



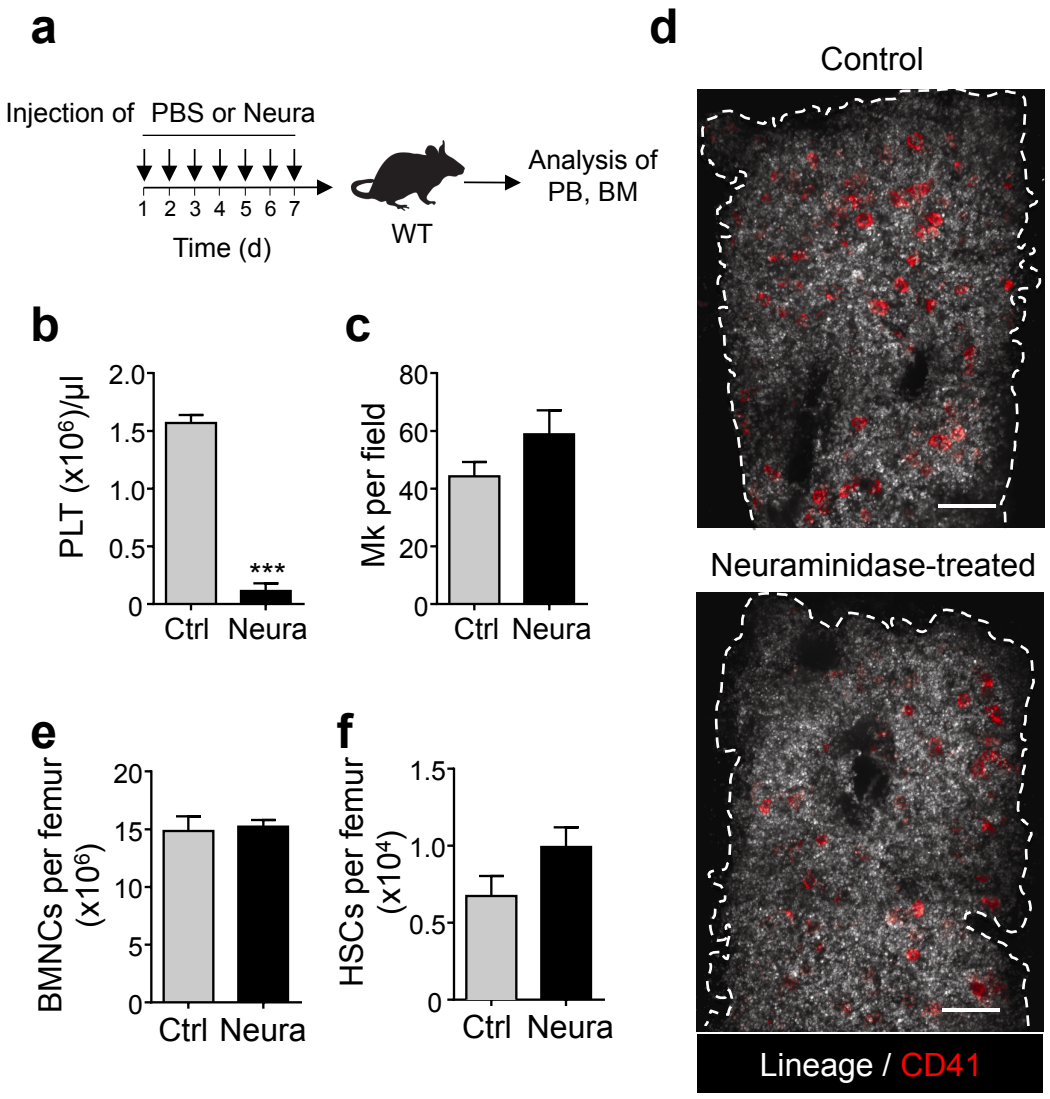
Supplementary Figure 5. Tri-lineage engraftment and cell cycle analysis of HSCs from the BM of control and Mk-depleted mice. (**a**) Experimental design to determine the effect of Mk depletion on HSCs. *Cxcl4*-cre;iDTR mice were injected daily with 250 ng DT for one week. Peripheral blood (PB) and BM were harvested on day 7, analyzed and 0.2%, 0.4% or 0.6% of total BM nucleated cells (BMNCs) obtained from one femur were transplanted together with 2.5 x 10^5 CD45.1$^+$ competitor cells into lethally irradiated SJL recipient mice. (**b**) Quantification of tri-lineage (myeloid, B-cell and T-cell) engraftment in the mice analyzed in **Fig. 2f**. (**c**) Cell cycle analysis by FACS using anti-Ki67 and Hoechst 33342 staining of HSCs from control and *Cxcl4*-cre;iDTR mice after 7 days of DT treatment. *n* = 3 male mice per group. **P* < 0.05, ***P* < 0.01 (Student's t-test).

Supplementary Figure 6



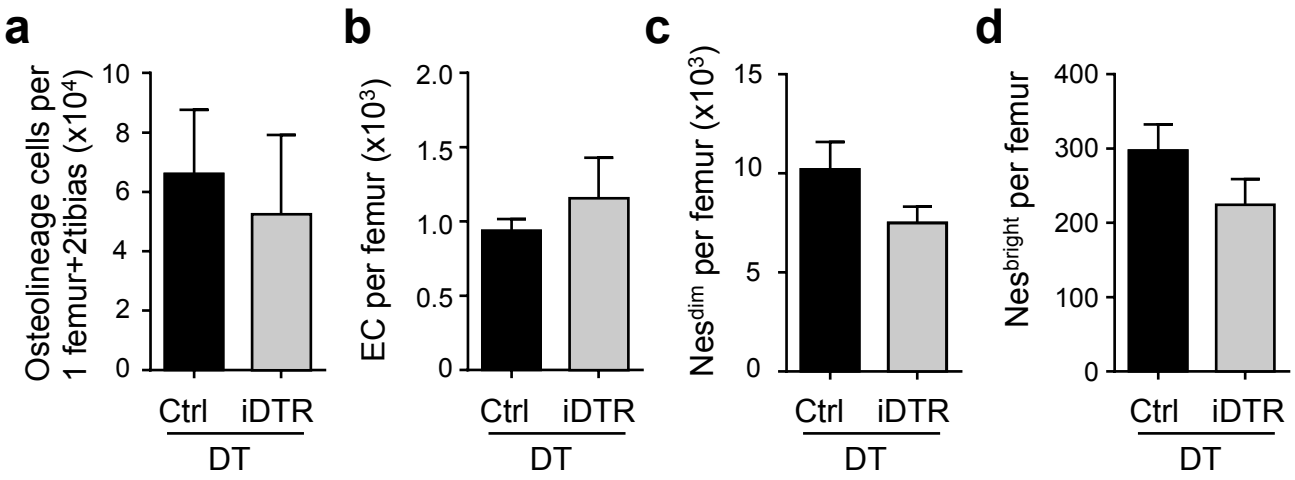
Supplementary Figure 6. Long-term depletion of Mk. (**a**,**b**) Number of HSCs (**a**) and BMNCs (**b**) per femur in control (*n* = 4) and *Cxcl4*-cre;iDTR (*n* = 3) mice treated with DT (250 ng, daily) over a period of 6 weeks. (**c**) Percentage of CD45.2+ cells in the blood of lethally irradiated CD45.1^{+} female mice transplanted with 2.5 x 10^5 BM cells from a CD45.1^{+} female mouse together with 3% of total BM cells purified from the mice analyzed in (**a**,**b**). Analyses were performed 16 weeks after transplantation. *n* = 3 (control group) and *n* = 5 (*Cxcl4*-cre;iDTR) female recipient mice. ***$P < 0.001$ (Student's t-test).

Supplementary Figure 7



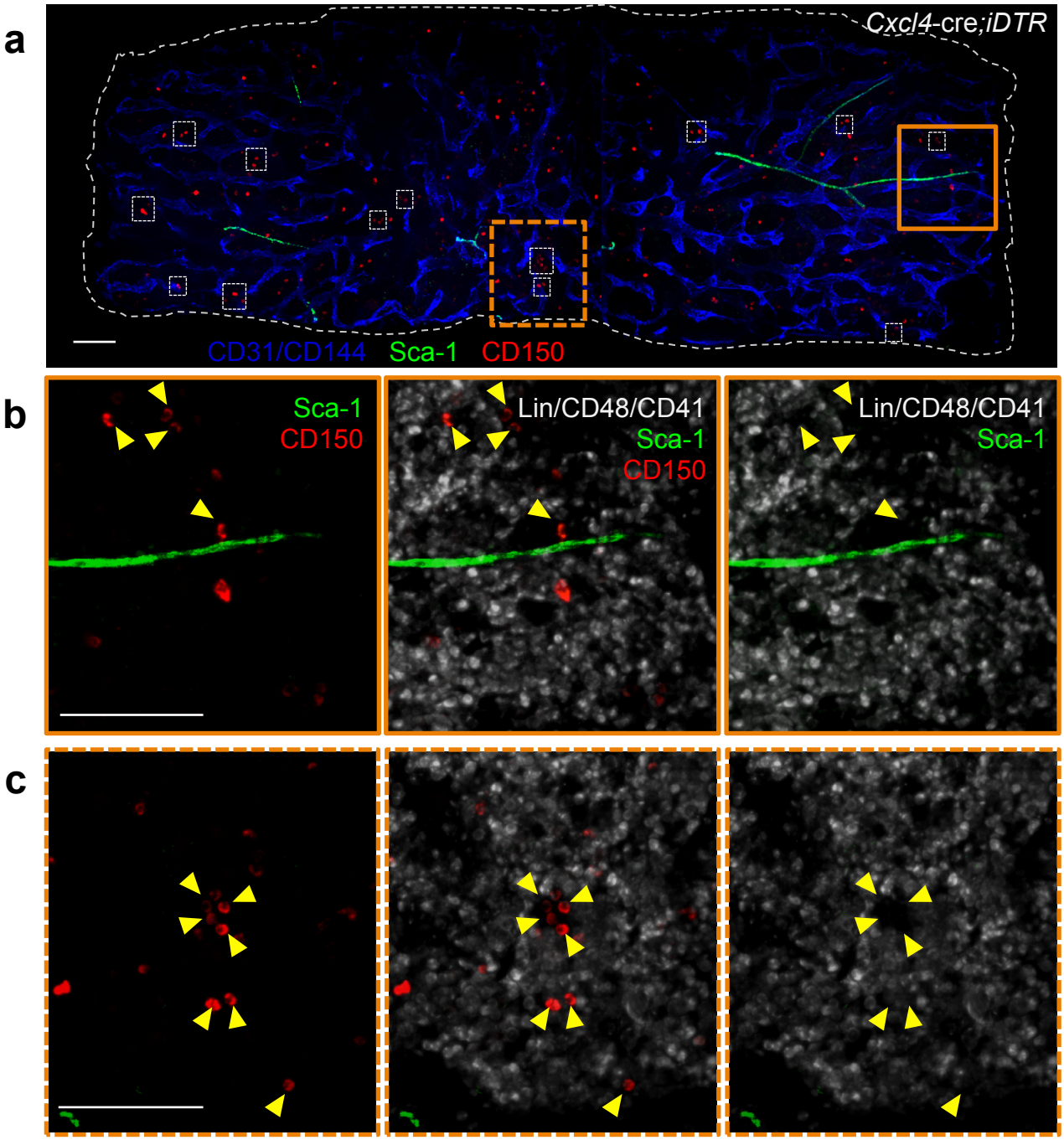
Supplementary Figure 7. Selective depletion of platelets does not significantly affect HSCs in the BM. (**a**) Experimental design to assess the effect of selective platelet depletion by neuraminidase (Neura) on HSCs *in vivo*. C57BL/6 mice were injected daily with either PBS or neuraminidase. Peripheral blood (PB) and BM were harvested on day 7. (**b**) Platelet (PLT) counts in the PB of mice treated with either PBS or neuraminidase. (**c,d**) Quantification of Mk per BM field (**c**) after 7 days of treatment with PBS or neuraminidase and representative whole-mount images of sternal BM stained with anti-CD41 and anti-Lineage (anti-Mac-1, anti-Gr-1, anti-Ter119, anti-B220, anti-CD3e) antibodies (**d**). (**e,f**) BMNCs (**e**) and HSCs (**f**) per femur after treatment with PBS or neuraminidase. $n = 4$ female mice per group. $^{***}P < 0.001$ (Student's t-test). Scale bar: 100 µm.

Supplementary Figure 8



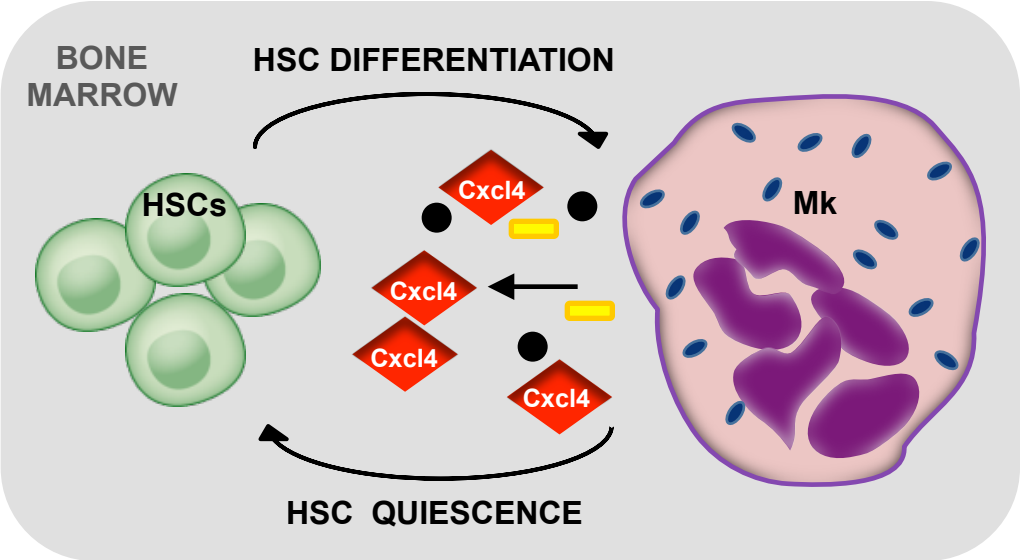
Supplementary Figure 8. Mk depletion does not affect the number of stromal BM and compact bone HSC niche constituents. (**a**) Number of osteolineage (CD45$^-$ Ter119$^-$ CD31$^-$ CD51$^+$ Sca-1$^-$) cells in the compact bone of control and *Cxcl4*-cre;iDTR mice after 7 days of DT treatment. (**b-d**) Number of BM EC (CD45$^-$ Ter119$^-$ CD31$^+$ CD105$^+$ endothelial cells) (**b**), Nes^{dim} (CD45$^-$ Ter119$^-$ CD31$^-$ Nestin-GFP^{low}) (**c**) and Nes^{bright} (CD45$^-$ Ter119$^-$ CD31$^-$ Nestin-GFP^{high}) (**d**) niche cells in control and *Cxcl4*-cre;iDTR;Nestin-GFP mice after 7 days of DT treatment. *n* = 3 male mice per group and cell type, except for EC where *n* = 9 (control) and *n* = 8 (*Cxcl4*-cre;iDTR) male mice were analyzed.

Supplementary Figure 9



Supplementary Figure 9. HSC expansion after Mk depletion occurs distant from arterioles. (**a-c**) Representative whole-mount images of a *Cxcl4*-cre;iDTR sternum compartment after 7 days of DT treatment (**a**) and magnified high power views (**b,c**). Arterioles are identified by $CD31^+$ $CD144^+$ $Sca\text{-}1^+$ expression. Yellow arrowheads denote $CD150^+$ Lineage (Lin)/$CD48/CD41^-$ phenotypic HSCs. (**b,c**) Representative images showing that after Mk depletion, the number of HSCs adjacent or in close proximity to arterioles is not significantly altered (**b**), while HSCs expand significantly distant from arterioles as shown by a representative cluster of 4 HSCs (**c**). White squares mark clusters of 2 or more HSCs. Scale bars: 100 μm.

Supplementary Figure 10



Supplementary Figure 10. Megakaryocytes (Mk) constitute a functional component of the HSC niche in the BM. Schematic representation illustrating how a terminally differentiated HSC progeny, the Mk, regulates HSC quiescence directly through Cxcl4, thereby controlling its own replenishment by a feedback loop.

SUPPLEMENTARY TABLES

Supplementary Table 1. Probability distributions of HSC distances on maps of sternal BM in relation to arterioles and Mk in wild-type mice (Actual model; relative to Fig. 4d)

Distance to Mk (μm) \ Distance to arterioles (μm)	25	50	75	100	125	150	175	200	225	250	275
25	0.1468	0.0952	0.0913	0.0794	0.1071	0.0516	0.0357	0.0079	0.0119	0.0000	0.0000
50	0.0952	0.0556	0.0159	0.0317	0.0238	0.0000	0.0079	0.0119	0.0000	0.0000	0.0000
75	0.0476	0.0040	0.0119	0.0000	0.0000	0.0000	0.0040	0.0040	0.0000	0.0000	0.0000
100	0.0278	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
125	0.0238	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
150	0.0079	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
175	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Supplementary Table 2. Probability distributions of mean distances from simulations of randomly positioned HSCs on maps of sternal BM in relation to arterioles and Mk (Random 1 model; relative to Fig. 4d)

Distance to Mk (μm) \ Distance to arterioles (μm)	25	50	75	100	125	150	175	200	225	250	275
25	0.0507	0.0611	0.0518	0.0538	0.0342	0.0104	0.0072	0.0052	0.0031	0.0031	0.0000
50	0.0331	0.0559	0.0704	0.0600	0.0528	0.0217	0.0093	0.0083	0.0031	0.0031	0.0000
75	0.0259	0.0383	0.0559	0.0404	0.0248	0.0321	0.0135	0.0072	0.0031	0.0052	0.0000
100	0.0145	0.0124	0.0176	0.0166	0.0083	0.0135	0.0062	0.0093	0.0021	0.0041	0.0000
125	0.0052	0.0083	0.0093	0.0031	0.0052	0.0041	0.0021	0.0010	0.0021	0.0000	0.0000
150	0.0021	0.0031	0.0000	0.0031	0.0000	0.0021	0.0000	0.0000	0.0000	0.0000	0.0000
175	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Supplementary Table 3. Probability distributions of mean distances from simulations of randomly positioned HSCs on maps of sternal BM in relation to arterioles and randomly positioned Mk (Random 2 model; relative to Fig. 4d)

Distance to Mk (μm) \ Distance to arterioles (μm)	25	50	75	100	125	150	175	200	225	250	275
25	0.0408	0.0529	0.0662	0.0540	0.0309	0.0176	0.0099	0.0022	0.0077	0.0066	0.0000
50	0.0419	0.0617	0.0639	0.0485	0.0419	0.0276	0.0143	0.0088	0.0044	0.0088	0.0000
75	0.0287	0.0518	0.0485	0.0320	0.0254	0.0165	0.0132	0.0077	0.0099	0.0066	0.0000
100	0.0110	0.0132	0.0232	0.0232	0.0187	0.0044	0.0011	0.0033	0.0011	0.0000	0.0000
125	0.0066	0.0099	0.0121	0.0033	0.0022	0.0022	0.0000	0.0000	0.0000	0.0011	0.0000
150	0.0022	0.0022	0.0055	0.0011	0.0000	0.0000	0.0000	0.0000	0.0011	0.0000	0.0000
175	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Supplementary Table 4. Probability distributions of HSC distances on maps of sternal BM in relation to arterioles and Mk in *Cxcl4*$^{-/-}$ mice (*Cxcl4*$^{-/-}$ model; relative to Fig. 4d)

Distance to Mk (µm) \ Distance to arterioles (µm)	25	50	75	100	125	150	175	200	225	250	275
25	0.1795	0.0769	0.0855	0.0342	0.0171	0.0171	0.0000	0.0000	0.0085	0.0000	0.0000
50	0.0769	0.0598	0.0342	0.0171	0.0256	0.0085	0.0000	0.0171	0.0000	0.0000	0.0000
75	0.0513	0.0256	0.0598	0.0085	0.0085	0.0085	0.0085	0.0000	0.0000	0.0000	0.0000
100	0.0171	0.0000	0.0342	0.0085	0.0085	0.0000	0.0000	0.0085	0.0000	0.0000	0.0000
125	0.0000	0.0000	0.0000	0.0085	0.0256	0.0085	0.0000	0.0000	0.0000	0.0000	0.0000
150	0.0000	0.0171	0.0000	0.0171	0.0085	0.0085	0.0000	0.0000	0.0000	0.0000	0.0000
175	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Supplementary Table 5. Primers for the amplification of mouse transcripts by real time quantitative PCR.

Mouse primers		***Sequence 5'-3'***
Gapdh	*s*	TGTGTCCGTCGTGGATCTGA
	as	CCTGCTTCACCACCTTCTTGA
Cxcl12	*s*	CGCCAAGGTCGTCGCCG
	as	TTGGCTCTGGCGATGTGGC
Angpt1	*s*	CTCGTCAGACATTCATCATCCAG
	as	CACCTTCTTTAGTGCAAAGGCT
Kitl	*s*	CCCTGAAGACTCGGGCCTA
	as	CAATTACAAGCGAAATGAGAGCC
Ccne1	*s*	GCAGCGAGCAGGAGACAGA
	as	GCTGCTTCCACACCACTGTCTT
Cxcl4	*s*	AGTTTGGTCTTGCTGCTGGT
	as	GGTCTTGACATGAGCGTCG
Tgfb1	*s*	CTCCCGTGGCTTCTAGTGC
	as	GCCTTAGTTTGGACAGGATCTG
Thpo	*s*	CTCTGTCCAGCCCCGTAGC
	as	CCCCAAGAGGAGGCGAAC
Ifna4	*s*	TGATGAGCTACTACTGGTCAGC
	as	GATCTCTTAGCACAAGGATGGC
Igfbp3	*s*	AATGGCCGCGGGTTCTGC
	as	TTCTGGGTGTCTGTGCTTTGAG
Igfbp2	*s*	GGCGCGGGTACCTGTGAAAA
	as	TCTCCTGCTGCTCGTTGTAG
Ifng	*s*	ATGAACGCTACACACTGCATC
	as	CCATCCTTTTGCCAGTTCCTC
Ifnb1	*s*	CAGCTCCAAGAAAGGACGAAC
	as	GGCAGTGTAACTCTTCTGCAT
Igfbp1	*s*	CCGCCACGAGCACCTTGTTCA
	as	TGTTGGGCTGCAGCTAATCTCT
Cdk2	*s*	CCTGCTTATCAATGCAGAGGG
	as	GTGCTGGGTACACACTAGGTG

s- indicates the sense and ***as***- the anti-sense primer.