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Supplementary Information for

Early-life midazolam exposure persistently changes chromatin accessibility to impair adult hippocampal neurogenesis and cognition.

Correspondence to:
Taito Matsuda,
Department of Stem Cell Biology and Medicine,
Graduate School of Medical Sciences, Kyushu University,
3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Phone: +81-92-642-6196; E-mail: tmatsuda@scb.med.kyushu-u.ac.jp

Kinichi Nakashima,
Department of Stem Cell Biology and Medicine,
Graduate School of Medical Sciences, Kyushu University,
3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.
Phone: +81-92-642-6195; E-mail: kin1@scb.med.kyushu-u.ac.jp

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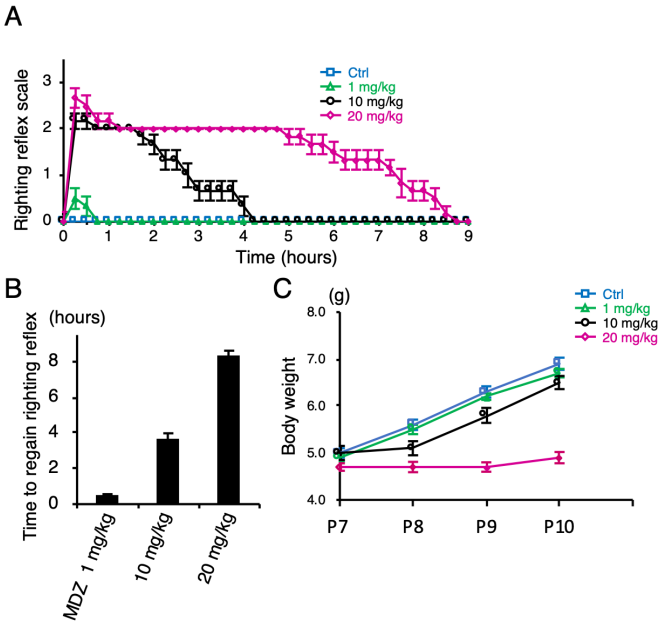

Fig. S1: Sedation measurement after MDZ exposure.

(*A*) Duration of sedation after MDZ treatment ($n = 6$ animals each). (*B*) Bar graph showing time to recovery of righting reflex ($n = 6$ animals each). MDZ treatment at 10 mg/kg is required for sedation without loss of body weight. (*C*) Body weight change after MDZ administration ($n = 6$ animals each). MDZ treatment at 20 mg/kg abolishes body weight gain.

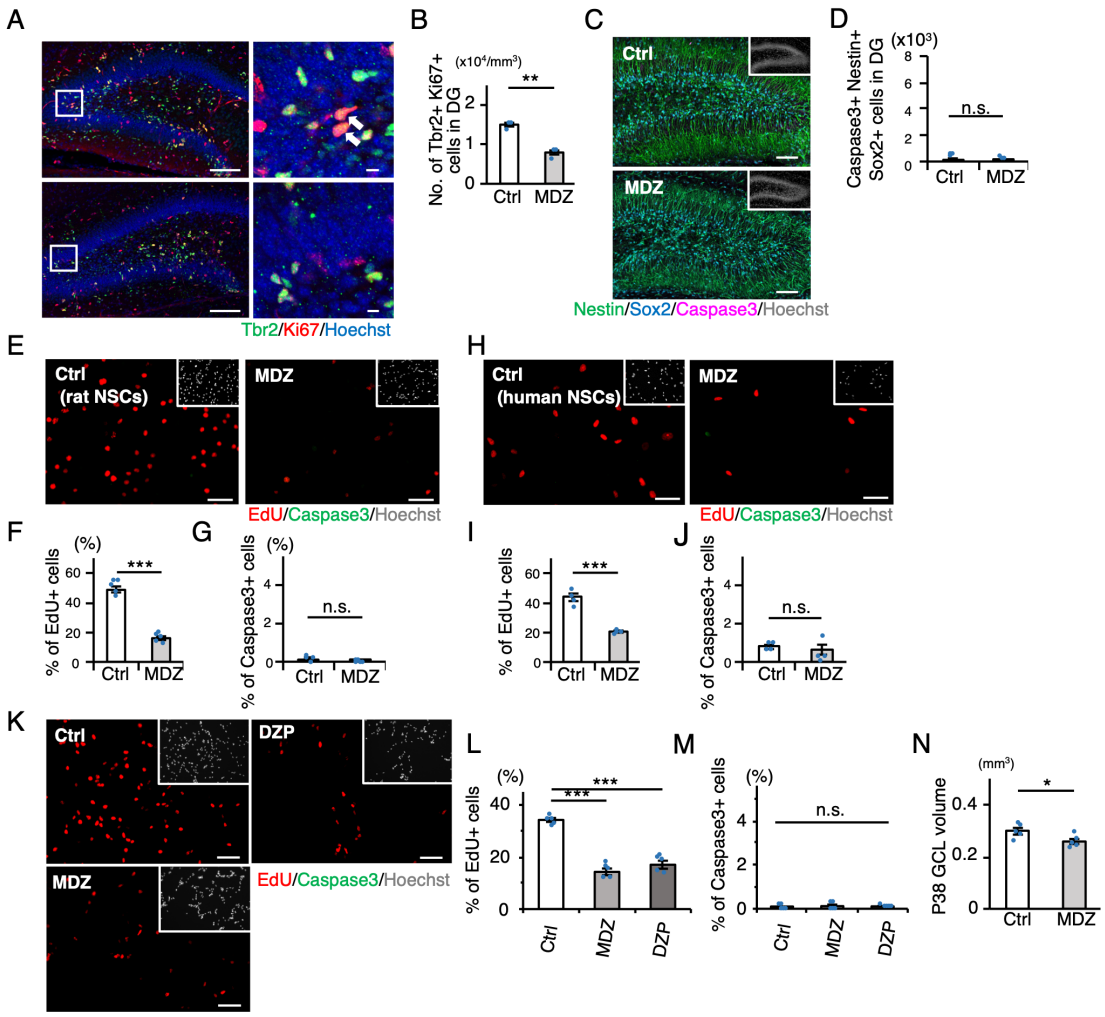

Fig. S2: MDZ suppresses proliferation of rodent and human NSCs without inducing cell death.

(*A*) Representative tile scan confocal images of immunostaining of Tbr2 (green), Ki67 (red), and Hoechst (blue) at P10. The areas outlined by a white rectangle are enlarged to the right. Arrows indicate Tbr2$^+$ Ki67$^+$ proliferating intermediate progenitor cells. Scale bars, 100 μm (left) and 20 μm (right). (*B*) Quantification of the number of Tbr2$^+$ Ki67$^+$ intermediate progenitor cells in the DG of Ctrl and MDZ mice at P10 ($n = 3$ animals each). $^{**}P < 0.01$ by *t* test. (*C*) Representative tile scan confocal images of immunostaining of Nestin (green),

Sox2 (cyan), active Caspase 3 (magenta) and Hoechst (gray; insets). Scale bars, 100 μm. (*D*) Quantification of the number of Caspase 3^+ $Nestin^+$ $Sox2^+$ NSCs in the DG of Ctrl and MDZ mice at P10 (n = 3 animals each). (*E*) Representative images of EdU (red), active Caspase 3 (green) and Hoechst (gray; insets) staining in rat NSCs treated with MDZ (30 μM). Scale bars, 50 μm. (*F* and *G*) Quantification of EdU^+ (*F*) or Caspase 3^+ (*G*) cells in (*E*) (n = 5 animals each). (*H*) Representative images of EdU (red), active Caspase 3 (green) and Hoechst (gray; insets) staining in human NSCs treated with MDZ (30 μM). Scale bars, 50 μm. (*I* and *J*) Quantification of EdU^+ (*I*) or Caspase 3^+ (*J*) cells in (*H*) (n = 4 animals each). (*K*) Representative images of EdU (red), active Caspase 3 (green) and Hoechst (gray; insets) staining in mouse NSCs treated with MDZ (30 μM) or diazepam (DZP, 50 μM) for 24 hours. (*L* and *M*) Quantification of EdU^+ (*L*) or Caspase 3^+ (*M*) cells in (*K*) (n = 5 animals each). (*N*) Quantification of volume of GCL (mm^3) of Ctrl and MDZ mice at P38 (n = 6 animals per group). ***$P < 0.001$ by ANOVA with Tukey post-hoc tests (L and M) and *$P < 0.05$ and *$P < 0.01$ by t test (B, F, I and N). n.s., not significant.

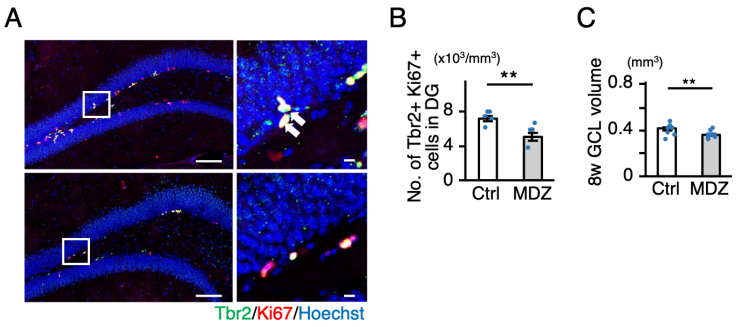

Fig. S3: MDZ suppresses proliferation of progenitor cells in the adult DG.

(*A*) Representative tile scan confocal images of immunostaining of Tbr2 (green), Ki67 (red), and Hoechst (blue) at 8w. The areas outlined by a white rectangle are enlarged to the right. Arrows indicate $Tbr2^+$ $Ki67^+$ proliferating intermediate progenitor cells. Scale bars, 100 μm (left) and 20 μm (right). (*B*) Quantification of the number of $Tbr2^+$ $Ki67^+$ intermediate progenitor cells in the DG of Ctrl and MDZ mice at 8w ($n = 3$ animals each). $^{**}P < 0.01$ by *t* test. (*C*) Quantification of volume of GCL (mm^3) of Ctrl and MDZ mice at 8w ($n = 6$ animals per group). $^{*}P < 0.01$ by *t* test.

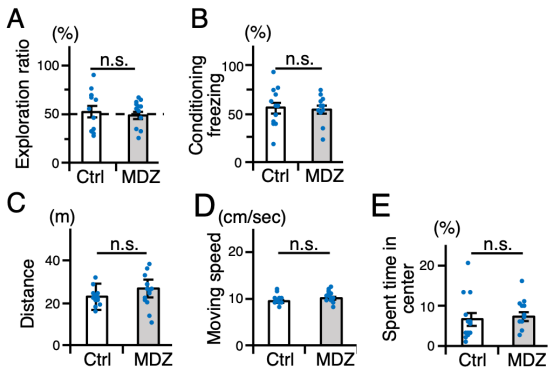

Fig. S4: Behavioral analysis of MDZ-exposed mice.

(*A*) Exploration ratio in Ctrl (n = 12) and MDZ (n = 12) mice during familiarization. Ctrl and MDZ-treated mice showed comparable levels of exploration of the test object. (*B*) Ctrl (n = 13) and MDZ (n = 14) mice showed an equivalent freezing response in the conditioning phase before the testing phase. (*C–E*) Bar graphs showing the distance traveled throughout the open field (*C*), moving speed (*D*), and the percentage of time spent in the center (*E*). MDZ treated mice exhibited no difference in locomotive activity or anxiety level compared to Ctrl mice. n.s., not significant.

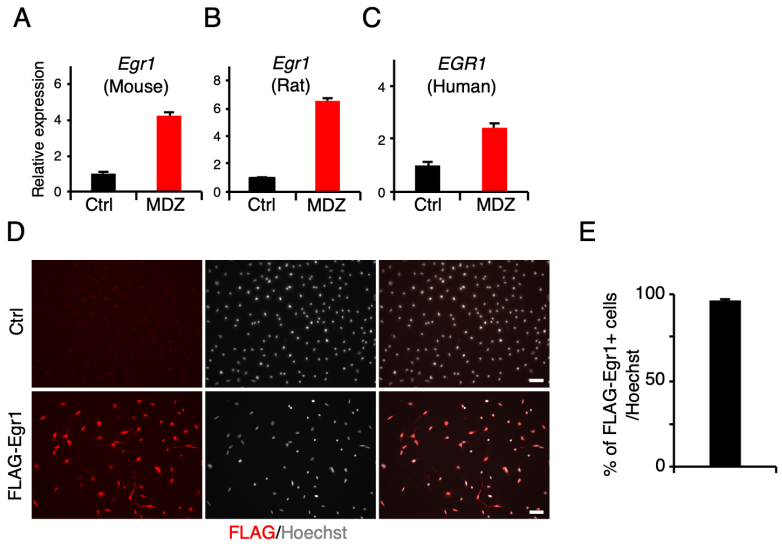

Fig. S5: Egr1 expression in cultured mouse NSCs.

(*A –C*) Relative expression of *Egr1* after MDZ treatment (30 μM) in cultured mouse (*A*), rat (*B*), and human (*C*) NSCs. (*D*) Representative images of FLAG-tagged Egr1 (red) and Hoechst at 24 h after the induction of Egr1 expression in cultured mouse NSCs. (*E*) Bar graph showing the percentage of FLAG-tagged $Egr1^+$ cells among $Hoechst^+$ cells.

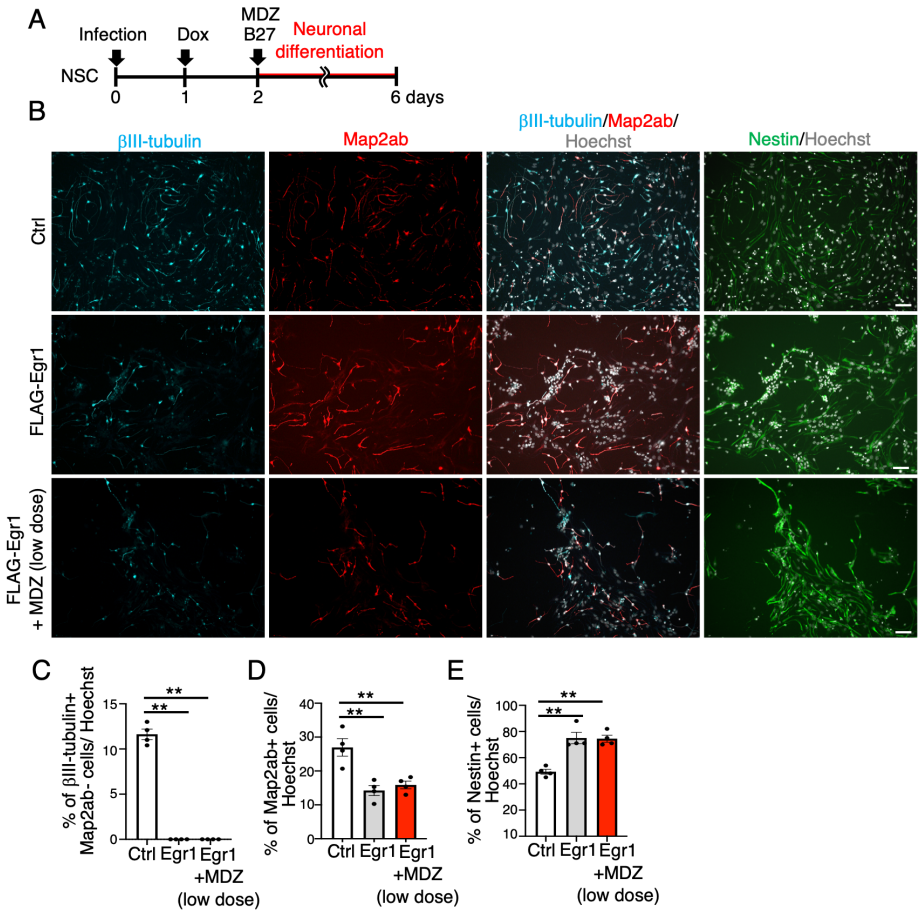

Fig. S6: Egr1 represses neuronal differentiation of NSCs.

(*A*) Experimental scheme for investigating the neuronal differentiation of Egr1-transduced NSCs. (*B*) Representative images of immunostaining of βIII-tubulin (cyan), Map2ab (red), Nestin (green), and Hoechst (gray) at 5 d after doxycycline-induced Egr1 expression. (*C* and *D*) Quantification of βIII-tubulin$^+$ (*C*), Map2ab$^+$ (*D*) and Nestin$^+$ (*E*) cells in (*B*) (n = 4). **P< 0.01 by ANOVA with Tukey post-hoc tests.

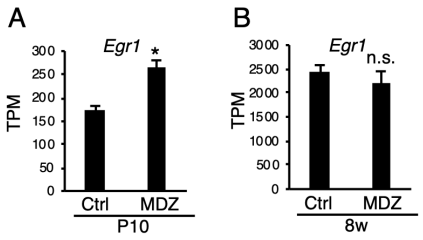

Fig. S7: Transient up-regulation of *Egr1* after MDZ exposure.

(*A* and *B*) Bar graphs showing the expression levels of *Egr1* in Nestin-GFP^{+} cells in the DG at P10 (*A*) and 8w (*B*) ($n = 3$). *$q < 0.05$. n.s., not significant.

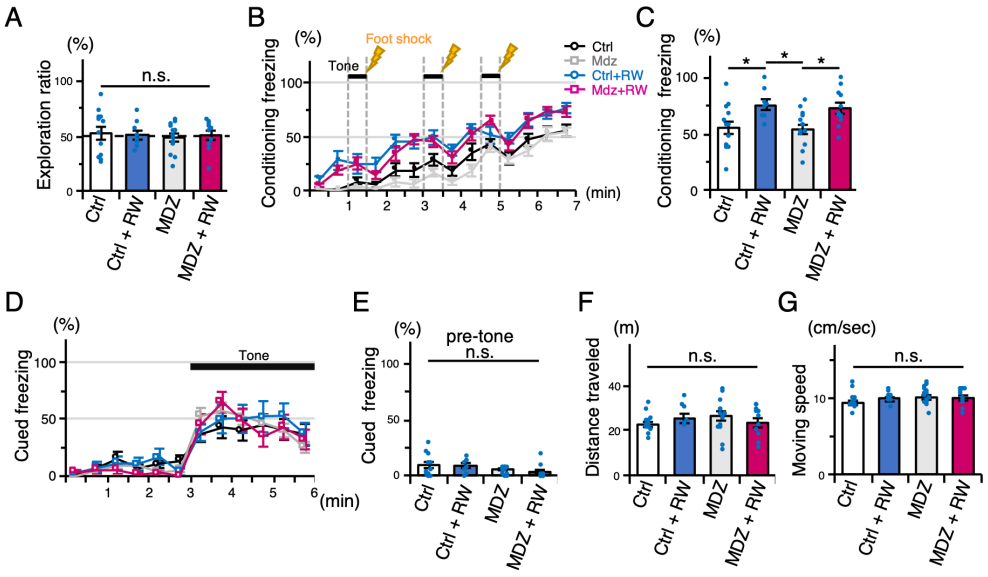

Fig. S8: Behavioral analysis of MDZ-exposed mice after voluntary running.

(*A*) Exploration time in each group during the familiarization phase. All 4 groups showed comparable levels of exploration of the test object (Ctrl (n = 13), Ctrl+RW (n = 8), MDZ (n = 14), and MDZ+RW (n = 11) mice). (*B* and *C*) Time course of freezing response (*B*) and quantification of freezing rate (*C*) in the conditioning phase. *$P < 0.05$ by ANOVA with Tukey post-hoc tests. (*D* and *E*) Time course of freezing response (*D*) and quantification of freezing rate (*E*) prior to the tone in the cued FC test (Ctrl (n = 13), Ctrl+RW (n = 8), MDZ (n = 14), and MDZ+RW (n = 11) mice). (*F* and *G*) Bar graphs showing the distance traveled throughout the open field (*F*) and the moving speed (*G*) (Ctrl (n = 13), Ctrl+RW (n = 8), MDZ (n = 14), and MDZ+RW (n = 11) mice). Mice with or without running exhibited no difference in locomotive activity. n.s., not significant.

Table S1: List of quiescence-associated factors

No.	Gene Name	Reference
1	**Pten**	M. Groszer, et al., PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry. Proc Natl Acad Sci U S A. 103, 111–116 (2006).
2	**Id1**	H. Nam, et al., High levels of Id1 expression define B1 type adult neural stem cells. Cell Stem Cell. 5, 515–526 (2009).
3	**Foxo3**	J. Paik, et al, FoxOs Cooperatively Regulate Diverse Pathways Governing Neural Stem Cell Homeostasis. Cell Stem Cell. 5, 540–553 (2009). ; V. M. Renault, et al., FoxO3 regulates neural stem cell homeostasis. Cell Stem Cell. 5, 527–539 (2009).
4	**Smad4**	H. Mira, et al., Signaling through BMPR-IA regulates quiescence and long-term activity of neural stem cells in the adult hippocampus. Cell Stem Cell. 7, 78–89 (2010).
5	**Bmpr1a**	H. Mira, et al., Signaling through BMPR-IA regulates quiescence and long-term activity of neural stem cells in the adult hippocampus. Cell Stem Cell. 7, 78–89 (2010).
6	**Rest**	Z. Gao, et al., The master negative regulator REST/NRSF controls adult neurogenesis by restraining the neurogenic program in quiescent stem cells. J Neurosci. 31, 9772–9786 (2011).
7	**Vcam1**	E. Kokovay, et al., VCAM1 is essential to maintain the structure of the SVZ niche and acts as an environmental sensor to regulate SVZ lineage progression. Cell Stem Cell. 11, 220–230 (2012).
8	**Nfix**	B. Martynoga, et al., Epigenomic enhancer annotation reveals a key role for NFIX in neural stem cell quiescence. Genes Dev. 27, 1769–1786 (2013).
9	**Cdkn1a**	E. Porlan, et al., Transcriptional repression of Bmp2 by p21Waf1/Cip1 links quiescence to neural stem cell maintenance. Nat Neurosci. 16, 1567–1575 (2013).
10	**Cdkn1c**	S. Furutachi, at al., P57 controls adult neural stem cell quiescence and modulates the pace of lifelong neurogenesis. EMBO J. 32, 970–981 (2013).
11	**Dll1**	D. Kawaguchi, et al., Dll1 maintains quiescence of adult neural stem cells and segregates asymmetrically during mitosis. Nat Commun. 4, 1–12 (2013).
12	**Cdh2**	E. Porlan, et al., MT5-MMP regulates adult neural stem cell functional quiescence through the cleavage of N-cadherin. Nat Cell Biol. 16, 629–638 (2014).
13	**Gabbr1**	C. Giachino, et al., GABA suppresses neurogenesis in the adult hippocampus through GABAB receptors. Development. 141, 83–90 (2014).
14	**Hopx**	D. Li, et al., Hopx distinguishes hippocampal from lateral ventricle neural stem cells. Stem Cell Res. 15, 522–529 (2015).
15	**Sox2**	A. Amador-Arjona, et al., SOX2 primes the epigenetic landscape in neural precursors enabling proper gene activation during hippocampal neurogenesis. Proc Natl Acad Sci U S A. 112, E1936–E1945 (2015).
16	**Pros1**	K. Zelentsova, et al., Protein S regulates neural stem cell quiescence and neurogenesis. Stem Cells. 35, 679–693 (2017).
17	**Mfge8**	Y. Zhou, et al., Autocrine Mfge8 Signaling prevents developmental exhaustion of the adult neural stem cell pool. Cell Stem Cell. 23, 444-452.e4 (2018).
18	**Id4**	R. Zhang, M. et al., Id4 downstream of Notch2 maintains neural stem cell quiescence in the adult hippocampus. Cell Rep. 28, 1485-1498.e6 (2019).
19	**Notch2**	Same as above
20	**Hes5**	Same as above
21	**Hes1**	I. Luque-Molina, et al., The orphan nuclear receptor TLX represses Hes1 expression, thereby affecting NOTCH signaling and lineage progression in the adult SEZ. Stem Cell Reports. 13, 132–146 (2019).; R. Sueda, et al., High Hes1 expression and resultant Ascl1 suppression regulate quiescent vs. active neural stem cells in the adult mouse brain. Genes Dev. 33, 511–523 (2019).
22	**Egr1**	This study

Table S2: Top 10 up-regulated and down-regulated genes at P10.

Up			References associated with stem cell quiescence
Gene	**Fold change**	**q-value**	
Nab2	0.559971366	3.07E-07	-
Gm12504	0.54608964	1.20E-08	-
Meox1	0.517183233	0.000362172	-
Egr3	0.503974661	0.000499342	-
Frs3	0.491933285	3.42E-05	-
Jade2	0.49117208	0.000499342	-
A330093E20Rik	0.483481999	0.000279103	-
Egr1	0.482020383	6.39E-06	I. M. Min, et al., The transcription factor EGR1 controls both the proliferation and localization of hematopoietic stem cells. *Cell Stem Cell*. **2**, 380–391 (2008).
Ier2	0.479184607	5.60E-05	-
Nr4a1	0.476795492	7.73E-05	P. R. Freire, et al., NR4A1 and NR4A3 restrict HSC proliferation via reciprocal regulation of C/EBPα and inflammatory signaling. *Blood*. **131**, 1081–1093 (2018).

Down			References associated with stem cell quiescence
Gene	**Fold change**	**q-value**	
Fmod	-0.55241454	3.09E-05	-
Cfh	-0.5250434	0.000217271	-
Lamc3	-0.50422156	0.000377014	-
Col18a1	-0.46869736	0.000177616	-
Colec12	-0.45339981	0.000780575	-
Ctsk	-0.43126638	0.006331009	-
Filip1l	-0.42877304	0.004962135	-
Aoc3	-0.42165482	0.008230287	-
Rnasel	-0.41906401	0.000420459	-
Il33	-0.41758698	0.003325697	-