Stem Cell Reports

Supplemental Information

Embryonic Stem Cells License a High Level of Dormant Origins to Protect the Genome against Replication Stress

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Ge_Fig S1

A

Mcm5 siRNA

SC | 60pmol | 30pmol | 15pmol

mean = 74 | mean = 68 | mean = 70 | mean = 75

EdU incorporation

DNA content (arbitrary units)

100 μM HU

mean = 43 | mean = 32 | mean = 36 | mean = 36

EdU incorporation (arbitrary value)

SC

Mcm5 siRNA

SC 60 30 15

pmol *Mcm5* siRNA

+100 μM HU

B

SC 25.93 ± 0.97 kb

siRNA 26.10 ± 0.88 kb

P = 0.6993

SC +HU 17.22 ± 0.61 kb

siRNA +HU 21.09 ± 0.78 kb

P = 0.0001

frequency

mean intra-cluster fork spacing (kb)

C

W1 W2 C1 C2

MCM2

MCM3

MCM4

MCM5

MCM7

ACTIN

H3

chromatin

W3 W4 C3 C4 W3 W4 C3 C4

MCM2

ACTIN

chromatin total

D

W3

W4

C3

C4

growth increase (in fold)

24 48 72 h

E

EdU incorporation (arbitrary value)

W1 W2 W3 W4 C1 C2 C3 C4

F

W3 W4 C3 C4

OCT4

SOX2

50μm

G

nonstained CCE W1 W2

W3 W4 C1 C2

C3 C4

SSEA-1

H

W1 W2 C1 C2 W1 W2 C1 C2

400μM HU – – – – + + + +

p-CHK1

p-P53

MCM2

ACTIN

total lysate

W3 W4 C3 C4 W3 W4 C3 C4

400μM HU – – – – + + + +

p-CHK1

p-H2AX

MCM2

ACTIN

total lysate

I

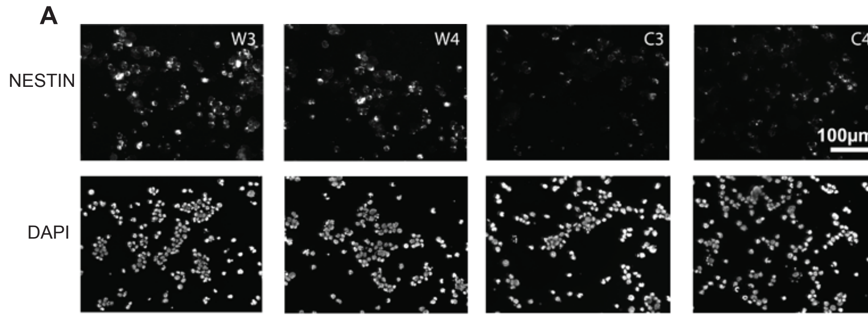
untreated

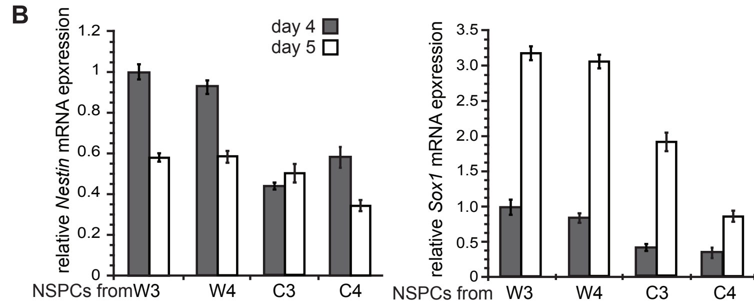
500 μM HU

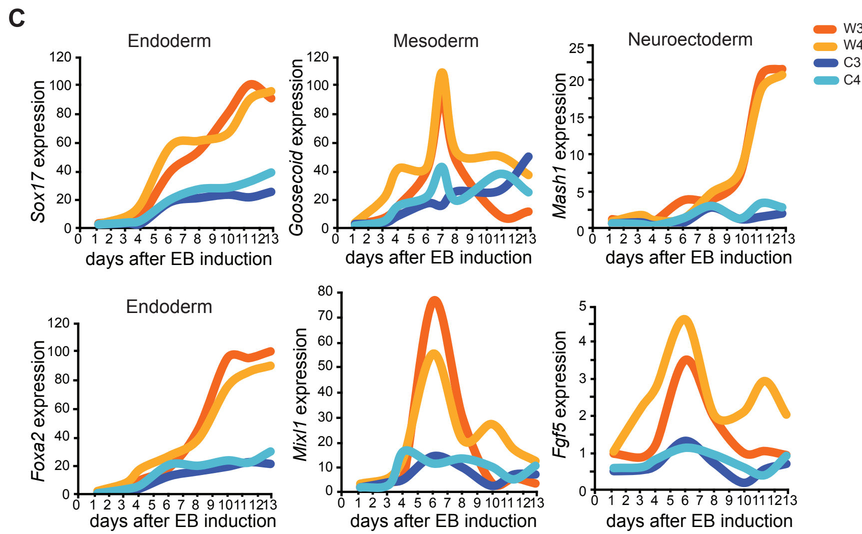
percentage of TUNEL postive cells

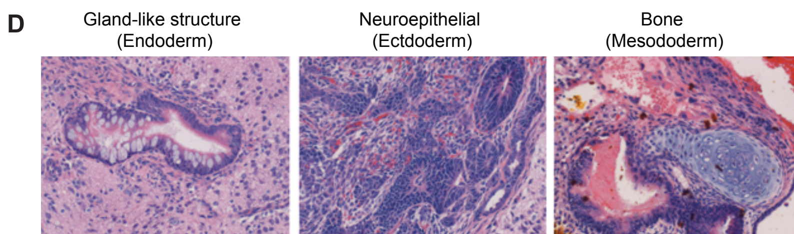
W3 W4 C3 C4

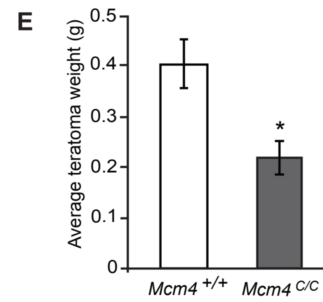
Ge_Fig S2



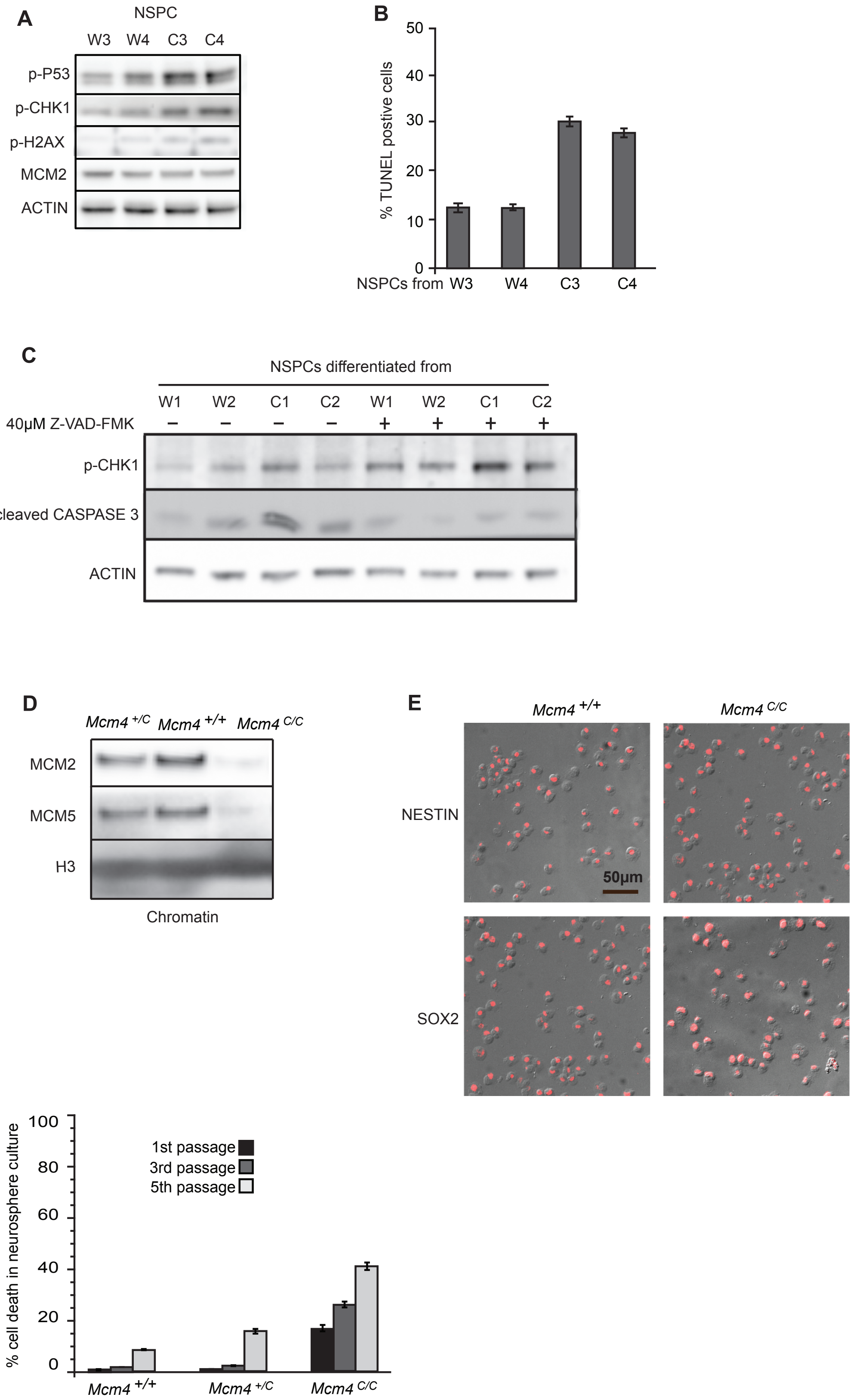


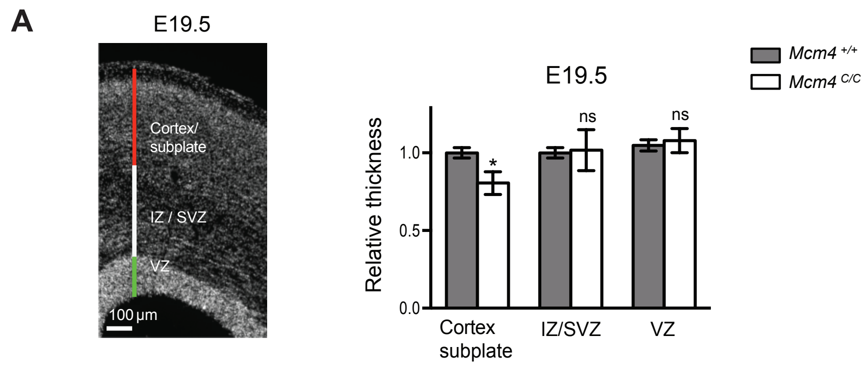


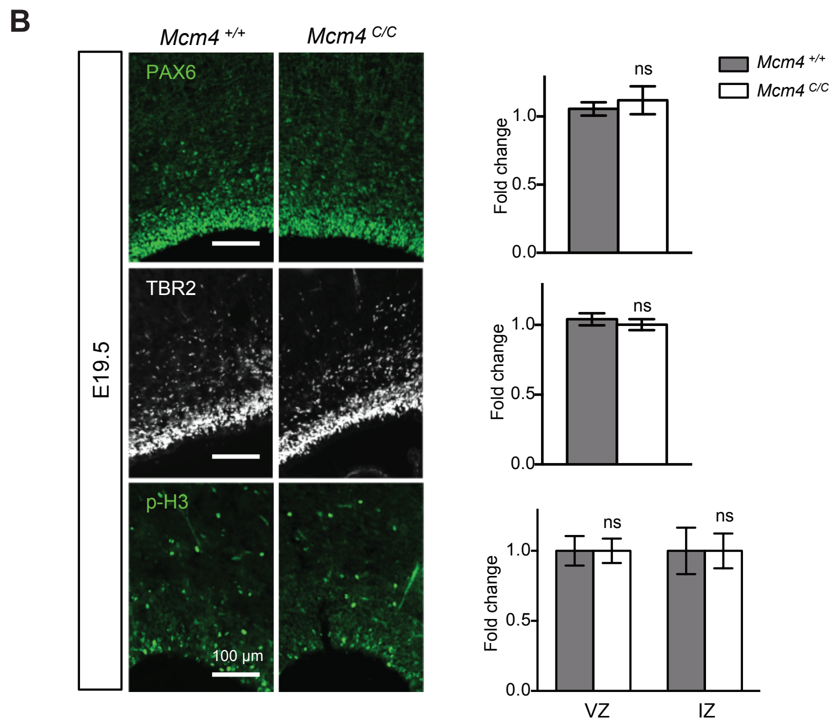




Ge_Fig S3







C

	Expected	Observed
Mcm4 $^{+/+}$	25%	40% (36/89)
Mcm4 $^{Chaos/+}$	50%	49% (44/89)
Mcm4 $^{Chaos/Chaos}$	25%	10% (9/89)

Supplemental Figure Legends

Figure S1. DO reduction does not affect ESC self-renewal, related to Figure 2.

(A) CCE ESCs were transfected with a serial dilution of the *Mcm5* or scrambled siRNA. Overall rate of DNA replication was assayed in cells after EdU pulse followed by FACS analysis. Cells were treated with HU for 4 hs before the analysis. Mean EdU incorporation is quantified and plotted. Note that 15pmol *Mcm5* siRNA does not change the rate of DNA replication, but make cells hypersensitive to HU. (**B**) Distribution of the mean intra-cluster fork spacing in CCE cells transfected with scrambled siRNA (SC) or 15pmol *Mcm5* siRNA analyzed by DNA fiber assay. Cells were treated with 100μM HU for 4 hs before the analysis. Overall average fork spacing ± standard error of the mean (SEM) is calculated. (**C-I**) Comparison between the *Mcm4*$^{+/+}$ (W1-W4) and *Mcm4*$^{C/C}$ (C1-C4) ESCs. (**C**) Immunoblot assaying chromatin-bound and total MCM protein level. (**D**) Cell proliferation rate analyzed over 72hs. (**E**) Overall rate of DNA replication assayed by EdU pulse and FACS analysis. (**F**) Immunofluorescence of OCT4 and SOX2 in ESCs. The fluorescent images are overlaid with the DIC images. (**G**) SSEA-1 expression assayed by FACS with CCE cells as a positive control. (**H**) Immunoblot analysis on the total lysate of cells after treatment with 400μM HU for 4 hs. (**I**) TUNEL assay of cells after treatment of HU for 48 hs. Error bars in Figure S1A, D, E and I indicate SEM from three independent experiments.

Figure S2. Dormant origin reduction impairs ESC differentiation, related to Figure 2.

(A) Immunofluorescence of NESTIN on day 4 after induced NSPC differentiation from the ESCs. (**B**) qRT-PCR of *Nestin* and *Sox1* expression during NSPC differentiation from ESCs (day 4 and day 5 post-induction). (**C**) qRT-PCR of the markers representing three germ

lineages during day 1 to day 13 of EB differentiation from the ESCs. (**D**) Representative images of endoderm, mesoderm and ectoderm tissues of teratomas generated from both the *Mcm4*$^{+/+}$ and *Mcm4*$^{C/C}$ ESCs. (**E**) The average weight of teratomas from four *Mcm4*$^{+/+}$ and four *Mcm4*$^{C/C}$ ESC lines. Two-tailed *t*-test: p = 0.0194. Error bars in Figure S2B and E indicate SEM from three independent experiments.

Figure S3. Dormant origin reduction impairs the differentiation of neural stem/progenitor cells (NSPCs), related to Figure 3.

(**A**) Immunoblot of NSPCs at 96 hs after induced differentiation from the *Mcm4*$^{+/+}$ (W3, W4) and *Mcm4*$^{C/C}$ ESCs (C3, C4). (**B**) TUNEL assay of NSPCs at 96 hs after induced differentiation from ESCs. Error bars represent SEM from three experiments. (**C**) Immunoblot showing the effect of CASPASE inhibitor Z-VAD-FMK on the levels of phospho-CHK1 and cleaved CASPASE 3 in NSPCs differentiated from the *Mcm4*$^{+/+}$ (W1, W2) and *Mcm4*$^{C/C}$ ESCs (C1, C2). 40μM Z-VAD-FMK was added at 48 hs after induced differentiation, and NSPCs were analysed at 96 hs. **(D**) Immunoblot of chromatin bound MCMs in the NSPCs from the embryo brains. (**E**) Immunofluorescence of NESTIN and SOX2 in the neurospheres generated from the NSPCs of embryo brains. The fluorescent images are overlaid with the DIC images. (**F**) Percentage of dead cells measured by trypan blue exclusion in the clonogenic neurosphere culture. Error bars in Figure S3B and F indicate SEM from three independent experiments.

Figure S4. The *Mcm4*$^{Chaos3/Chaos3}$ mutant displays thinner cortical layers, without neurogenesis alteration, at late embryonic stage E19.5, related to Figure 4.

(A) DAPI staining and measurement of the successive cortical zones. Note that outer layers (cortex and subplate) are thinner in the *Mcm4*$^{C/C}$ mutants, without alteration of the VZ and IZ/SVZ layers. **(B**) Immunolabeling of VZ/SVZ cells (PAX6^{+}), intermediate progenitor cells (TBR2^{+}) and dividing cells (p-H3^{+}) on coronal sections of the cortex. At late-stage neurogenesis, MCM4 partial loss-of-function does not affect the number of ventricular stem cells, intermediate progenitors and dividing cortical cells. lv: lateral ventricles. Error bars represent SEM of three independent experiments totaling 4 *Mcm4*$^{+/+}$ and 7 *Mcm4*$^{C/C}$ embryos. Two-tailed *t*-test: non-significant (ns), $p < 0.05$ (*). **(C)** Expected and observed frequencies of wild-type, heterozygote and homozygote embryos after self-crossing of the *Mcm4*$^{+/C}$ mice.

Supplemental Experimental Procedures

ES cell derivation and culture

Feeder-free CCE mouse ESCs were grown in standard ES cell culture medium consisting of high glucose DMEM supplemented with 15% ES cell-qualified fetal bovine serum, 2 mM L-Glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM Monothioglycerol and 1000 U/ml LIF at 37°C, 5% CO_2. *Mcm4*$^{chaos3/chaos3}$ and wide-type mouse ESC lines were derived from the blastocysts generated by crossing *MCM4*$^{chaos3/+}$ heterozygous mice using previously described procedures (Bryja et al. 2006). They were maintained on mouse embryonic fibroblast (MEF) feeder layer in standard ES cell culture medium. Before experiments, feeders were removed and ES cells were grown on plates coated with 0.1% gelatin.

Mice

129/Sv mice were purchased from Taconic. *Mcm4*^{Chaos3} mice (C57BL/6J and C3HeB/FeJ mixed background) were maintained as heterozygotes. All experiments involving mice were approved by the Institutional Animal Care and Use Committee (IACUC).

Neural stem/progenitor cell culture

NSPCs were isolated and cultured as previously described (Saxe et al. 2007). Briefly, NSPCs were isolated from the forebrain of E13.5 mice. They were dissociated into single cells and cultured as neuropsheres by plating 50,000 cells/ml in DMEM/F12 media supplemented with B27, 1,000U/mL penicillin-streptomycin, 20ng/ml bFGF, 50ng/ml EGF and 2ug/ml heparin. For clonal neurosphere assays, dissociated single cells were re-plated at 1,000 cells/ml in 6 well plates in neurobasal medium with B27, 2mM L-glutamine, 1,000U/ml penicillin-streptomycin, 20ng/ml bFGF, 2ng/ml EGF, and 2ug/ml heparin. Neurosphere culture on day 6 was dissociated into single cells and plate at 1,000 cells/ml to initiate a new round of neurosphere growth.

Antibodies, chemicals and siRNA

Antibodies used were: phospho CHK1 ser345 (2341, Cell Signaling), phospho H2AX ser139 (2577, Cell Signaling), phospho P53 ser15 (9284, Cell Signaling), β-ACTIN (4967, Cell Signaling), H3 (4499, Cell Signaling), MCM2 (BM28, BD), MCM3 (4012, Cell signaling), MCM4 (ab4459, Abcam), MCM5 (ab75975, Abcam), MCM7 (ab2360, Abcam), SSEA-1 (560127, BD), cleaved CASPASE 3 (9661, Cell Signaling), NESTIN (clone 10C2, Millipore), OCT4 (C-10, Santa Cruz), SOX2 (560292, BD), PAX6 (Millipore (AB2237), Phospohistone

H3 (Millipore 06-570), TBR1 (Abcam ab31940), TBR2 (eBioscience 14-4875-82), Hydroxyurea (Sigma), Aphidicolin (Sigma), caffeine (Sigma) or Z-VAD-FMK (Sigma) were added to the medium, incubation time and concentrations of which were indicated in the results section. *Mcm5* siRNA (5'-CGAGCAUUCGGAUUCUGAATT-3') as well as scrambled siRNA (Dharmacon) were transfected into ES cells using a protocol adapted from siRNA transfection in human embryonic stem cells (Ma et al. 2010). Briefly, 60 pmol of siRNAs were mixed with 5 μl of Lipofectamine 2000 (Invitrogen) and then diluted in 100μl of OPTI-MEMI (Invitrogen). Cell pellet (1×10^6 cells) were then incubated in the resulting 100 μl of transfection solution for 15 min before seeded in standard growth medium. Cells were analyzed 72 hs after siRNA transfection.

DNA fiber analysis

DNA fiber analysis was carried out as previously described (Ge et al. 2007). Briefly, ES cells and neural stem/progenitor cells were pulsed with 50μM BrdU for 10min (without HU) or 20min (in presence of 100μM HU) in order to achieve examinable track length after spreading. Cells were then harvested and DNA fiber spread was prepared. For immunodetection of BrdU labeled tracks, acid treated fiber spreads were incubated with mouse anti-BrdU (BD) and Alexa Fluor 555-conjugated rabbit anti-mouse IgG. DNA fibers were examined using Zeiss microscope using 65x lens. Fibers containing at least four consecutive tracks within the range of expected length were selected for analysis, and average track spacing was determined. For each sample, at least 50 measurements were performed giving rise to overall fork spacing ± standard error of the mean (SEM).

Examination of chromatin-bound MCM2-7 complexes

To assay chromatin-bound MCMs by immunoblot, experiments were performed as previously described (Ge et al. 2007). Briefly, cells were incubated with cytoskeleton (CSK) buffer supplemented with 0.2% triton for 10 min on ice. Cells were then centrifuged at 5000g for 5min to obtained pellet containing chromatin-bound proteins. The pellets were suspended in Nupage LDS sample buffer (Invitrogen, supplemented with 5% 2-mercaptoethal) and subject to immunoblot. To assay chromatin bound MCM2 by FACS, cells were incubated with CSK buffer supplemented with 0.2% triton for 10min on ice, centrifuged at 400g for 5min and fixed with 2% paraformaldehyde at 37 ^{o}C for 10min. MCM2 was labeled with primary mouse antibody (347580, BD) and Alexa Fluor 488-conjugated rabbit anti-mouse IgG. DNA was labeled with 20μg/ml 7AAD, and all samples were analysed using a FACSCalibur flow cytometer (BD) and Flowjo software. To quantify chromatin bound MCM2 in G1 phase, the geographical mean of immunolabeled MCM2 was calculated (since the data is collected on a logarithmic scale).

Immunofluoresence

ESCs and neural stem cells were harvested and trypsinized into single cells before the experiments. Cells were than fixed with 2% paraformaldehyde for 10 minutes at room temperature and cytospined onto microscope slides. After permeabilization with 0.2% triton for 10min, cells were stained with primary and secondary antibody as described in the above section.

Tissue collection, immunohistochemistry and data analysis

Pregnant mice (stage of pregnacy E13.5, 15.5 and 19.5) were anaesthetized with

isoflurane inhalation and embryos were removed from the uterus. Embryonic heads (E13.5) and brains (E15.5 and 19.5) were fixed by immersion in 4% paraformaldehyde in PBS, pH7.4 overnight at 4°C. After washing in PBS, samples were soaked overnight at 4°C in 20% sucrose in PBS to cryoprotect the tissues. Brain coronal cryosections (20 μm thick) were sequentially incubated with blocking solution (Dako X0909) for 1 hr at room temperature, primary antibodies diluted in PBS containing 0.1~1% Triton X-100 for overnight at 4°C, and secondary antibodies for 3 hrs at 4°C. Cell nuclei were co-stained by DAPI and slides were mounted with Fluoromount (Southern Biotechnologies Associates). Images were acquired on a Nikon ECLIPSE 80i microscope and ImageJ was used for quantification. For quantification of the cortical and ventral forebrain size, coronal sections were selected at the same anterior-posterior level of the forebrain, just rostral to the anterior emergence of the corpus callosum. In each embryo, three measurements were performed on 4x images, on both the left and the right hemisphere. For quantification of cell number, 10x images were taken. TBR2, p-H3 and cleaved-CASPASE 3 positive cells were counted, and the thickness of PAX6 and TBR1 cell layer was measured.

Super-resolution 3D structured illumination microscopy

Cells were synchronized by incubation in 2.5 mM thymidine for 12 hs followed by 50ng/ml of nocodazole for 7 hs. Cells were then released for 2 hs into G1 phase and chromatin-bound MCMs were extract using methods described in this paper. MCM proteins were labeled with primary antibodies and Alexa Fluor 568-conjugated rabbit anti-mouse or rabbit IgG. To image MCM foci in cells, Delta Vision OMX imaging system (Applied Precision) was used to collect 125nm serial Z sections using a 63× NA 1.4 lense. SoftWorx was used to process raw images for reconstruction to reveal structures in three dimensions.

3D image stacks were then analyzed with Volocity (PerkinElmer) where a threshold-based segmentation was applied to define MCM foci. The same degree of threshold was used across different samples to cover 90% of all visually defined foci in each cell. To calculate cellular foci number, >100 cells pooled from three sets of experiments were used to generate mean foci number and SEM.

DNA synthesis, cell growth and TUNEL assay

To assay the rate of DNA synthesis, cells were labeled with 10 μM EdU for 30 min and stained according to manufacturer's instruction (Click-iT™ EdU Alexa Fluor 647 Flow Cytometry, Invitrogen). DNA was stained with 20 μg/ml 7AAD. All samples were analyzed using a FACSCalibur flow cytometer (BD) and Flowjo software. To quantify EdU incorporation, the geographical mean of incorporated EdU was then calculated (since the data is collected on a logarithmic scale), from which the overall rate of DNA synthesis was derived. Cell growth rate was assayed with the alamarBlue CellViability Reagent (Invitrogen). Absorbance was measured at 570 and 600 nm. Apoptosis of the samples was assayed by ApopTag Fluorescein *in situ* Apoptosis Detection Kit according to the manufacturer's instruction (Millipore).

ESC differentiation and quantitative RT-PCR

Neural stem/progenitor cell (NSPC) differentiation was performed as previously described using N2B27 medium (Ying et al. 2003). NSPCs were harvested on day 4 to day 6 after induction of differentiation depending on different experiments. Embryoid body differentiation was achieved by hanging drop method as described previously (Jackson et al. 2010). Teratoma assay was performed by injecting one million ES cells subcutaneously into

six to eight-week-old SCID-beige mice as previously described (Wesselschmidt 2011). Three weeks post-injection, teratomas were harvested, fixed and stained with hematoxylin and eosin for histological analysis. For quantitative RT-PCR analysis of gene expression, total RNA was extracted using RNeasy Plus Kit (Qiagen), reverse transcribed and amplified by lineage specific primers (*Nestin, Sox1, Sox17, Goosecoid, Mash1, Fgf5, Mixl1, Foxa2*) as previously described (Ivanova et al. 2006; Kwon et al. 2014). *Gapdh* was used as internal control for qRT-PCR, and signals in each sample was normalized against it. Primer sequences:

Nestin – For; 5′-CCAGAGCTGGACTGGAACTC-3’, Rev; 5′-ACCTGCCTCTTTTGGTTCCT-3’

*Sox1 – For; 5’-*ATGCACCGCTACGACATGGG-3’, Rev; 5’-GCTCCGACTTGACCAGAGATCC-3’

*Sox17 – For; 5’-*AAGAAACCCTAAACACAAACAGCG-3’,

Rev; 5’TTTGTGGGAAGTGGGATCAAGAC-3’

Goosecoid – For; 5′- AAACGCCGAGAAGTGGAACAAG-3’,

Rev; 5′- AAGGCAGGGTGTGTGCAAGTAG-3’

Mash1 – For; 5′-TAACTCCAAACCACTAACAGGC -3’, Rev; 5′-TGAGGAAAGACATCAACGCAGT-3’

Fgf5 – For; 5′-CTGTATGGACCCACAGGGAGTAAC -3’, Rev; 5′- ATTAAGCTCCTGGGTCGCAAG-3’

Mixl1 – For; 5′-TTGAATTGAACCCTGTTGTCCC-3’, Rev; 5′- GAAACCCGTTCTCCCATCCACC-3’

Foxa2 – For; 5′-GGCACCTTGAGAAAGCAGTC-3’, Rev; 5′-GACATACCGACGCAGCTACA-3’

Gapdh – For; 5′-TCCCACTCTTCCACCTTCGATGC-3’,

Rev; 5′- GGGTCTGGGATGGAAATTGTGAGG-3’

Supplemental References

Bryja, V., S. Bonilla, and E. Arenas. 2006. Derivation of mouse embryonic stem cells. *Nature protocols*. 1:2082-2087.

Ivanova, N., R. Dobrin, R. Lu, I. Kotenko, J. Levorse, C. DeCoste, X. Schafer, Y. Lun, and I.R. Lemischka. 2006. Dissecting self-renewal in stem cells with RNA interference. *Nature*. 442:533-538.

Jackson, M., A.H. Taylor, E.A. Jones, and L.M. Forrester. 2010. The culture of mouse embryonic stem cells and formation of embryoid bodies. *Methods Mol Biol*. 633:1-18.

Kwon YR, Jeong MH, Leem YE, Lee SJ, Kim HJ, Bae GU, Kang JS. 2014. The Shh coreceptor Cdo is required for differentiation of midbrain dopaminergic neurons. *Stem cell research* **13**: 262-274.

Ma, Y., J. Jin, C. Dong, E.C. Cheng, H. Lin, Y. Huang, and C. Qiu. 2010. High-efficiency siRNA-based gene knockdown in human embryonic stem cells. *Rna*. 16:2564-2569.

Saxe, J.P., H. Wu, T.K. Kelly, M.E. Phelps, Y.E. Sun, H.I. Kornblum, and J. Huang. 2007. A phenotypic small-molecule screen identifies an orphan ligand-receptor pair that regulates neural stem cell differentiation. *Chemistry & biology*. 14:1019-1030.

Wesselschmidt, R.L. 2011. The teratoma assay: an in vivo assessment of pluripotency. *Methods Mol Biol*. 767:231-241.

Ying, Q.L., M. Stavridis, D. Griffiths, M. Li, and A. Smith. 2003. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nature biotechnology*. 21:183-186.