Supplementary methods

Oligodendrocyte and neuron differentiation

Oligodendrocyte differentiation of NSC was achieved as described in (46). In brief, cells were plated on polyornithin/laminin-coated glass cover slips and first cultured for 4 days in DMEM/F12 supplemented with N2, 10 ng/ml FGF2 and PDGF each (ProSpec, Israel), 10 µM forskolin (Biaffin, Germany), 2mM L-glutamine (L-Gln), 100U/ml penicillin and 100µg/ml streptomycin (P/S). Differentiation was induced by a 4-day- replacement of FGF2, PDGF and forskolin with 30 ng/ml 3,3,5-tri-iodothyronine (Sigma Aldrich) and 200 µM ascorbic acid (Sigma Aldrich). This protocol also produces a side population of GFAP-positive astrocytes.

Neuron differentiation of NSC was achieved as described in (24). In brief, cells were first grown for 3 days in D1 medium (Euromed-N (Euroclone), supplemented with 1x B27 and 0.5x N2 supplement (Invitrogen), 10ng/ml FGF2 (ProSpec, Israel), L-Gln and P/S. Cell were then plated on laminin (Santa Cruz)-coated glass cover slips in medium A (1:3 DMEM/F12 and Neurobasal), supplemented with 1.5x B27 and 0.5x N2 and10 ng/ml FGF2 and 20ng/ml BDNF (ProSpec, Israel), L-Gln and P/S). After 3 days, medium A was switched to medium B (similar to A, but with 6.7 ng/ml FGF2 and 30ng/ml BDNF).

Immunofluorescence microscopy

Cells cultured on glass cover slips were fixed in 4% paraformaldehyde (PFA, 10 minutes at room temperature (RT)) and permeabilized with 0.2% Triton X100 in PBS (10 minutes at RT). For DDR studies, cells were fixed in methanol/acetone (1:1, 3 minutes at RT) without further permeabilization. Samples were then blocked with 0.5% BSA and 0.2% gelatin in PBS, then probed with appropriate primary antibodies and Alexa-fluor 488-, 568- and 647-labeled secondary antibodies (Invitrogen). Primary antibodies used were: RIP (DSHB, Iowa, USA), Tuj1 (#MAB1637 Millipore), ATM-pS1981 (#200-301-400, Rockland), pS/TQ (phospho-(Ser/Thr) ATM/ATR Substrate Antibody #2851, Cell Signaling), 53BP1 (#NB100-304, Novus Biologicals) and γH2AX (clone JBW301, # 05-636, Millipore) DNA was counterstained with DAPI (Sigma Aldrich). Wide field images were obtained with a Olympus AX70 upright microscope and processed with ImageJ software.

Gene expression analysis

Total RNA was extracted with Trizol reagent (Invitrogen) and precipitated with isopropanol and ethanol. 1 µg of total RNA was used for retrotranscription using VILO® reverse transcription kit (Invitrogen). cDNA was analyzed by quantitative RT-PCR amplification on a Light Cycler 480 system using SYBR Green I assay (Roche). CT-values were obtained by calculation of the second derivative. Primers were designed with Roche UniversalProbe Library software against *Mus musculus* ATM:

FP: TGCAGATTTATATCCATCATCCAC;

RP: TTTCATGGATTCATAAGCACCTT

Supplementary figure legends

Fig. S 1 ES-cell derived NSC are tripotential and can be stimulated to differentiate into neurons, oligodendrocytes and astrocytes

A Confocal microscopy analysis of immunofluorescence staining of NSC and oligodendrocytes cultures derived as in (46). The protocol also yields a side population of astrocytes, positive for GFAP. Oligodendrocyte marker used was RIP, only the nuclear signal is specific. Please note that astrocytes as well as NSC lack any clear nuclear RIP staining.

B Confocal microscopy analysis of immunofluorescence staining of NSC and neuron cultures derived as in (24). Neuron marker used was Tuj1 (also known as β-Tubulin III).

Fig. S 2 NSC exit cell cycle upon exposure to BrdU

A Representative analysis of DNA content and cell cycle phase distribution in control and BrdU-treated cells using propidium iodide and flow cytometry. Quantifications were perfomed using ModFit software.

B Quantitative RT-PCR analysis of expression of the proliferation specific gene Ki67 in BrdU treated NSC, normalized against ctrl cells. β2-microglobulin was used as housekeeping gene. Error bars show SD.

Fig. S 3 Neither thymidine nor cytidine have any detectable effect on stem cell qualities of NSC

A Wide-field microscopy analysis of immunofluorescence staining of NSC cultured for 3 days in medium supplemented with H_2O, BrdU, thymidine or cytidine. Impact on stem cell differentiation was studied using specific antibodies for the presence of stem cell marker Nestin and astrocyte differentiation marker GFAP.

B Quantitative analysis of Nestin and GFAP positive cells shown representatively in (A). Note that while BrdU (3.3µM) exposed NSC lose Nestin expression and become GFAP positive, this does not occur for NSC treated with thymidine (10µM) or cytidine (10µM), despite the three fold higher concentration used.

Fig. S 4 Treatment of NSC with BrdU leads to cell cycle arrest and, unlike in 5-aza-treated cells, to low levels of cell death.

A Flow cytometrical analysis of 5-aza treated NSC for apoptosis-associated DNA fragmentation (Sub-G1). As comparison, BrdU Sub-G1 data from Fig. 1E is shown here again. Cells were scored for DNA content of less than 2N, as detected with propidium iodide, measured on log10 scale. Experiments were done in triplicate, error bars show SD.

B Quantitative RT-PCR analysis of S-phase associated Ki67 gene expression in NSC, treated with BrdU or 5-aza-dC, normalized against untreated NSC. β2-microglobulin was used as housekeeping gene.

Fig. S 5 Impact on methylation by CldU and IdU and on DNMT gene expression by BrdU

A Flow cytometrical analysis of NSC for the relative changes in global DNA methylation upon treatment with BrdU-related reagents CldU and IdU. Global DNA methylation at CpG islands was detected with antibody against 5-methyl-2'-deoxycytidin (5me-C). Cy5-coupled secondary antibody signal was measured on log10 scale. Gates were set to discriminate "medium" methylated cells, while the population shifts out of this gate were classified as "hypo-"or "hyper-methylated". 2nd AB: primary 5me-C antibody was omitted, thus showing that cells in the hypomethylated gate may lack DNA methylation completely.

B Quantitative RT-PCR analysis of expression of the 3 major DNA methyltransferases (DNMT) in BrdU-treated NSC on day 3 and day 7, normalized against untreated NSC. β2-microglobulin was used as housekeeping gene, error bars show SD.

Fig. S 6 NSC exposed to BrdU do not activate the canonical DNA damage reponse (DDR)

A Wide-field microscopy analysis of immunofluorescence staining of control and BrdU-treated NSC. Specific antibodies for canonical DDR markers were used to detect the assembly of ATM-pS1981 and its kinase activity on pS/TQ epitopes (top panel) or focal assembly of 53BP1 and γH2AX at DNA double strand break sites (bottom panel). No increase in signal as compared to control NSC was detectable.

B Similarly, 53BP1 foci assembly was still undetectable after 3 days of BrdU exposure. X-ray irradiated NSC, used as positive control, show 53BP1 foci assembly.

C Quantitative RT-PCR expression analysis of the key DDR gene ATM in BrdU treated NSC, normalized against untreated NSC. β2-microglobulin was used as housekeeping gene.

Fig. S 7 Forebrain NSC express comparably high endogenous levels of GFAP

A Western blot analysis of forebrain NSC for the kinetics of astrocyte marker GFAP upon BrdU treatment. Membrane was normalized for vinculin. Note a slight increase of GFAP protein in BrdU exposed forebrain NSC at later time points.

B Average cDNA quantities of Nestin and GFAP relative to the cDNA of the housekeeping gene B2M in forebrain NSC and ES-derived NSC, as assessed by qRT-PCR analysis. Note that while Nestin mRNA expression levels are similar in both cell types, the endogenous expression of GFAP is comparably high in the NSC from adult forebrain.

Supplementary reference

46. Glaser, T., Pollard, S.M., Smith, A. and Brustle, O. (2007) Tripotential differentiation of adherently expandable neural stem (NS) cells. *PLoS ONE*, **2**, e298.

Fig. S1

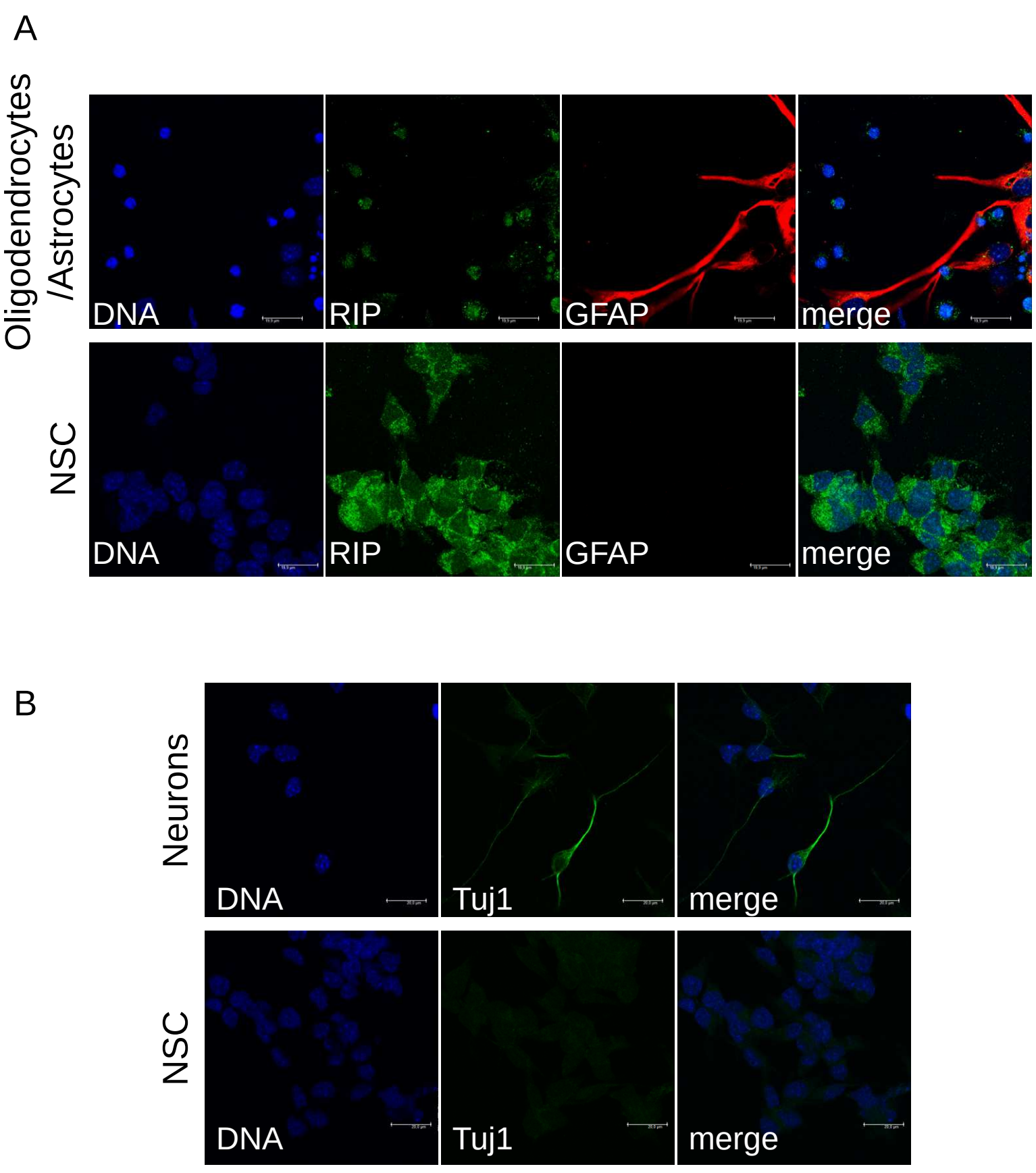
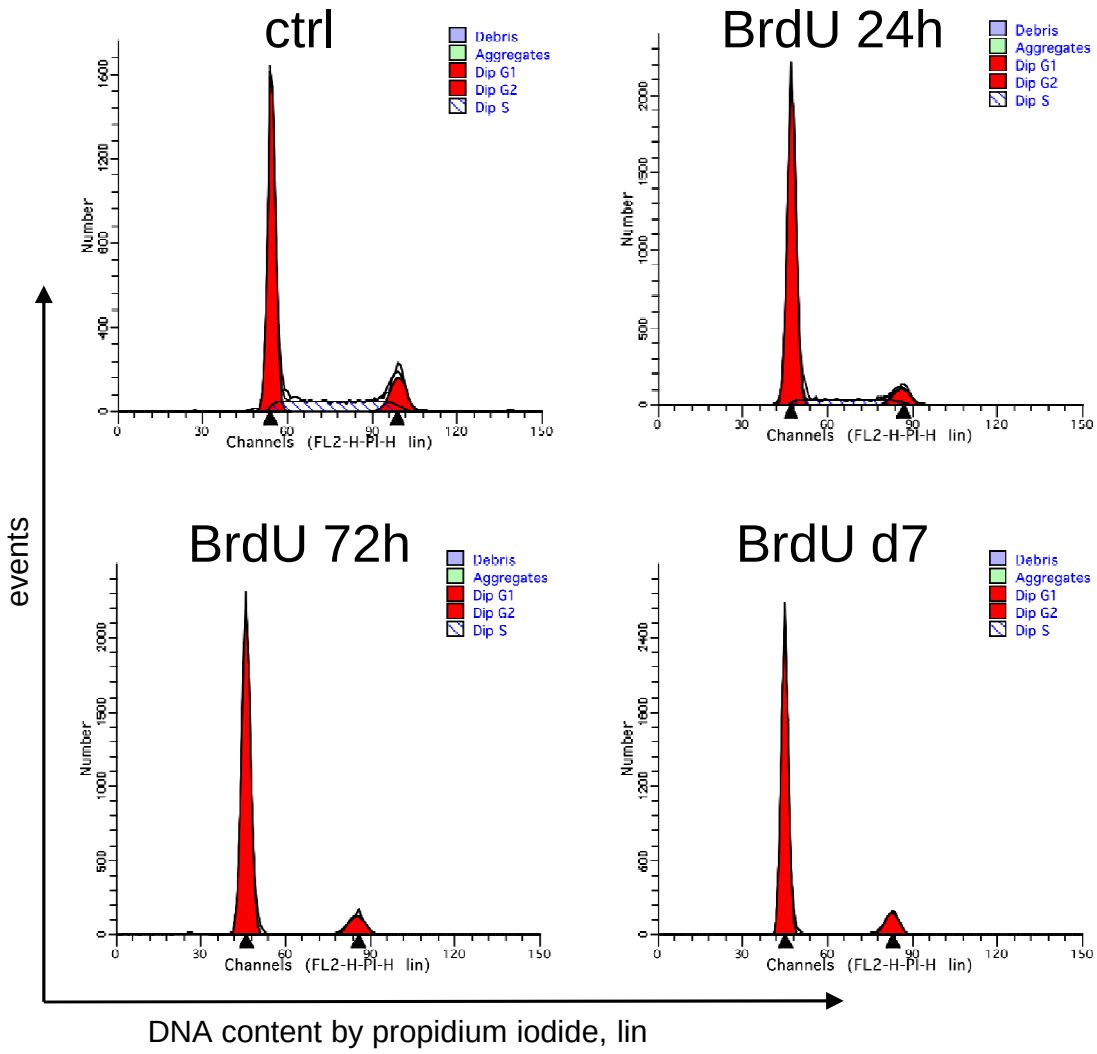

A
Oligodendrocytes /Astrocytes
DNA
RIP
GFAP
merge
NSC
DNA
RIP
GFAP
merge
B
Neurons
DNA
Tuj1
merge
NSC
DNA
Tuj1
merge

Fig. S2

A


ctrl
BrdU 24h
BrdU 72h
BrdU d7
Debris
Aggregates
Dip G1
Dip G2
Dip S
Number
Channels (FL2-H-PI-H lin)
events
DNA content by propidium iodide, lin

B

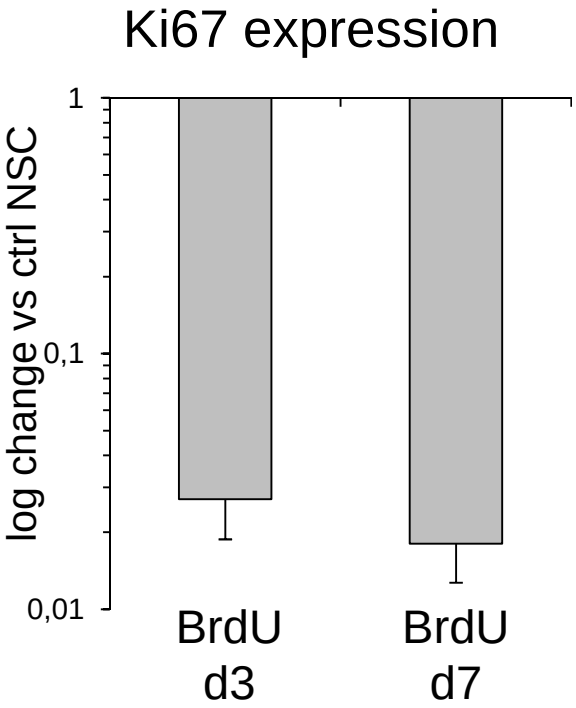
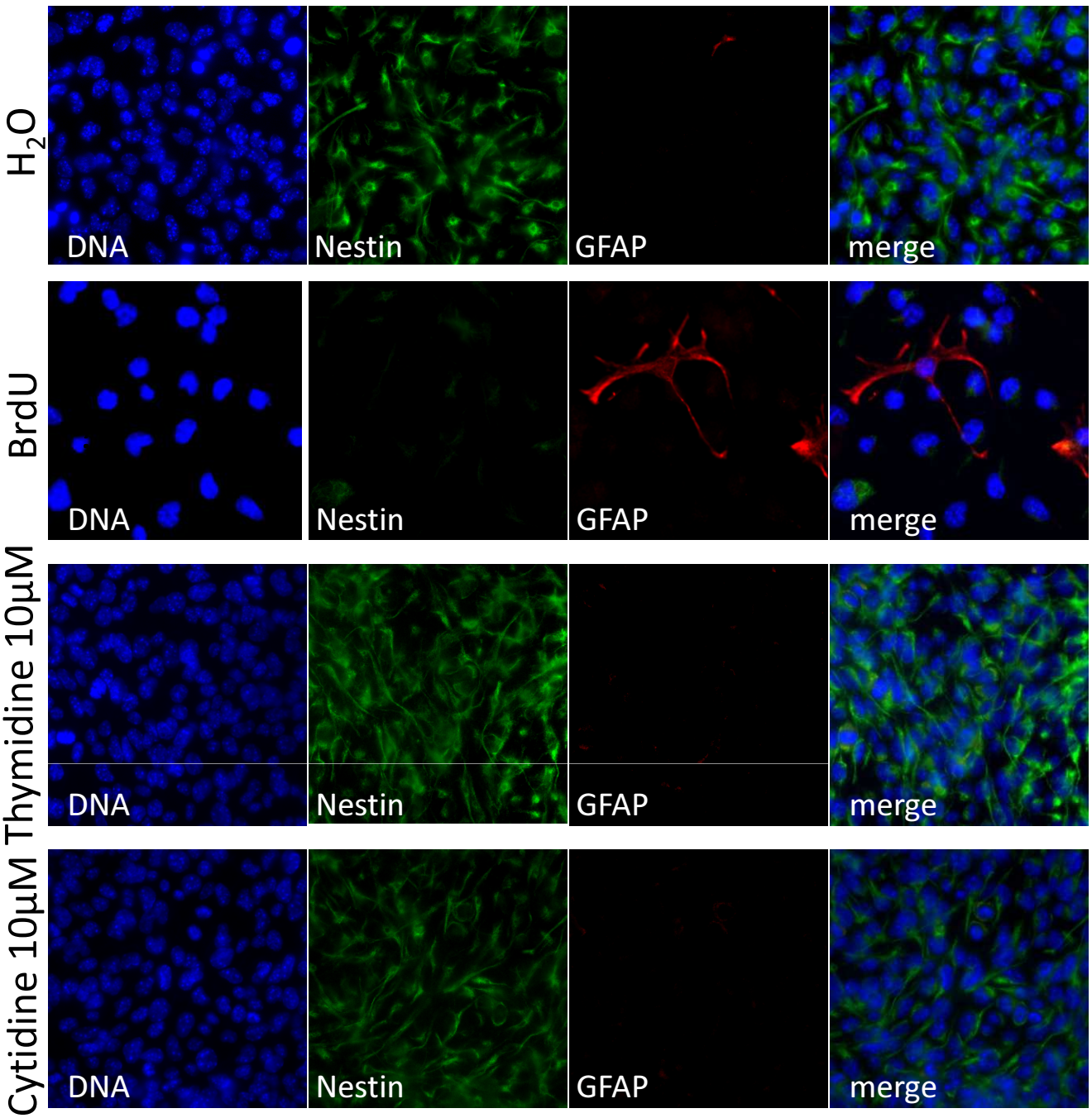

Ki67 expression
log change vs ctrl NSC
1
0,1
0,01
BrdU d3
BrdU d7

Fig. S3

A


H2O
BrdU
Thymidine 10µM
Cytidine 10µM
DNA
Nestin
GFAP
merge

B

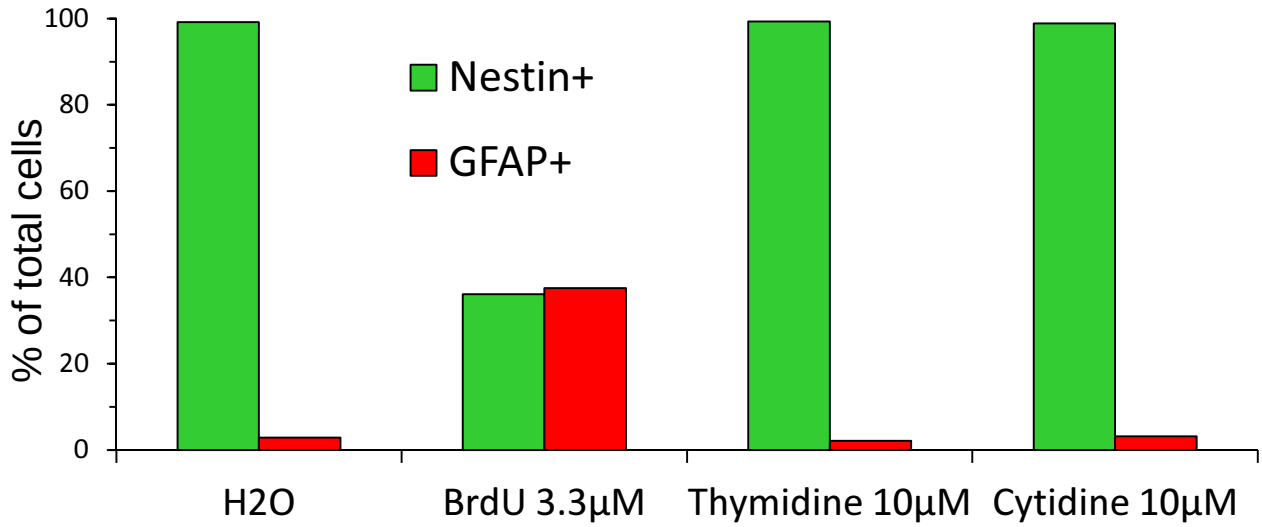
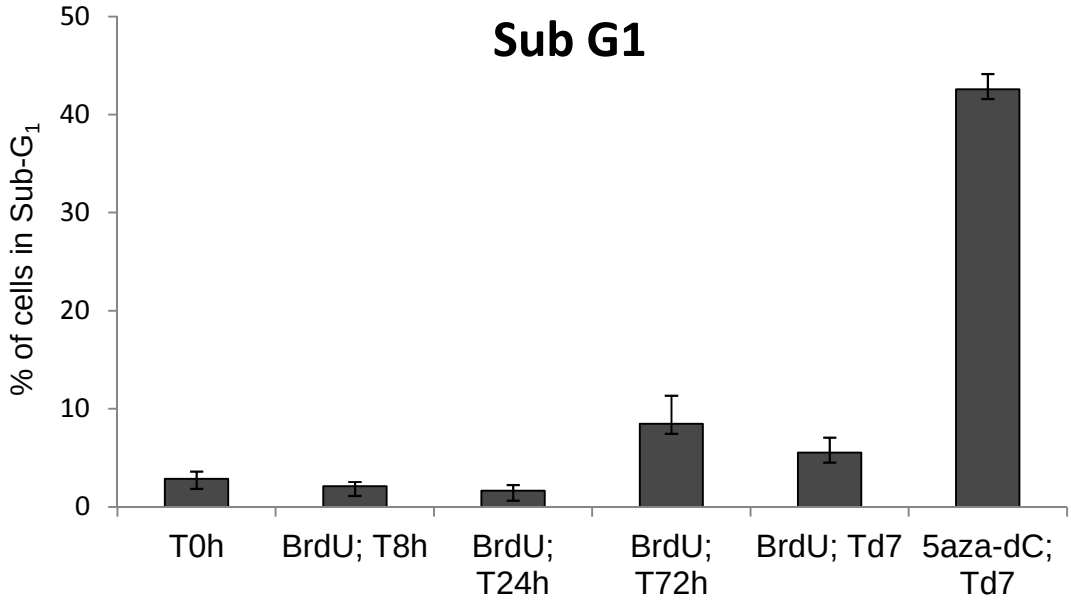

100
80
60
40
20
0
% of total cells
Nestin+
GFAP+
H2O
BrdU 3.3µM
Thymidine 10µM
Cytidine 10µM

Fig. S4

A


Sub G1
% of cells in Sub-G1
50
40
30
20
10
0
T0h
BrdU; T8h
BrdU; T24h
BrdU; T72h
BrdU; Td7
5aza-dC; Td7

B

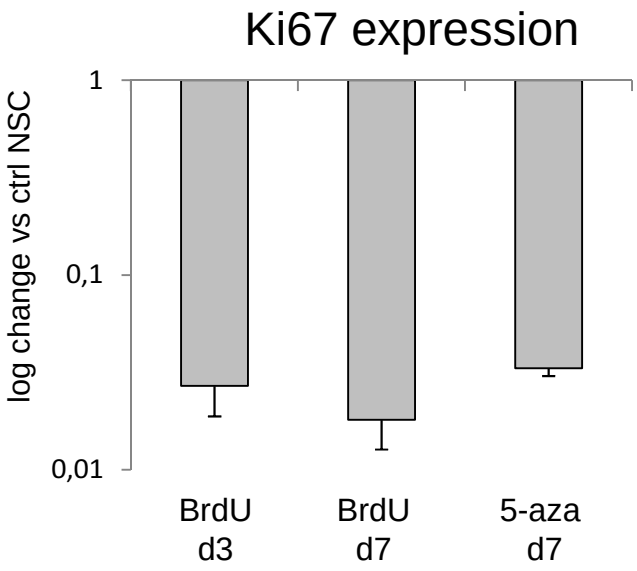
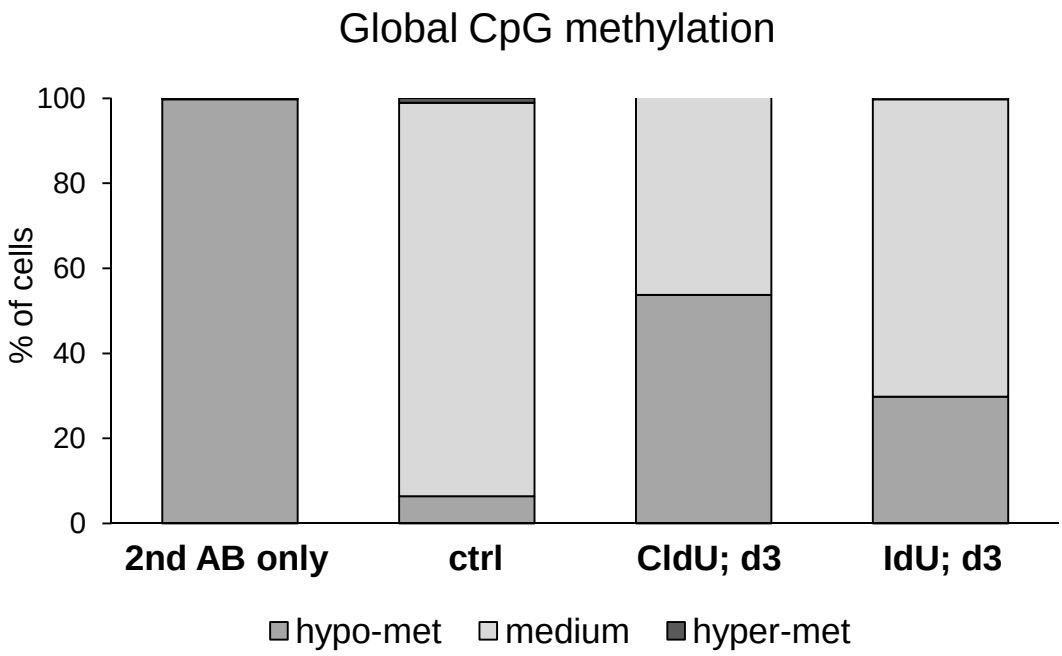

Ki67 expression
log change vs ctrl NSC
1
0,1
0,01
BrdU d3
BrdU d7
5-aza d7

Fig. S5

A


Global CpG methylation
100
80
60
40
20
0
% of cells
2nd AB only
ctrl
CldU; d3
IdU; d3
hypo-met
medium
hyper-met

B

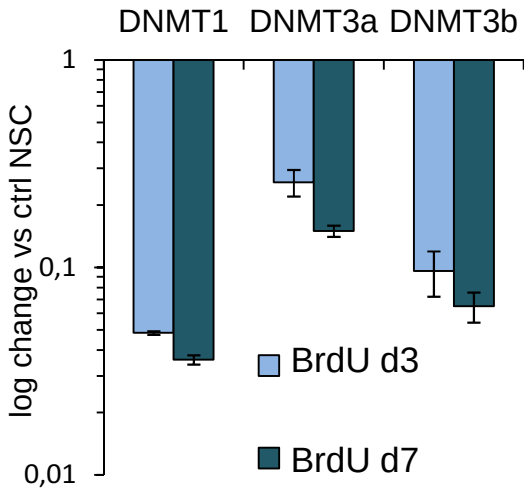
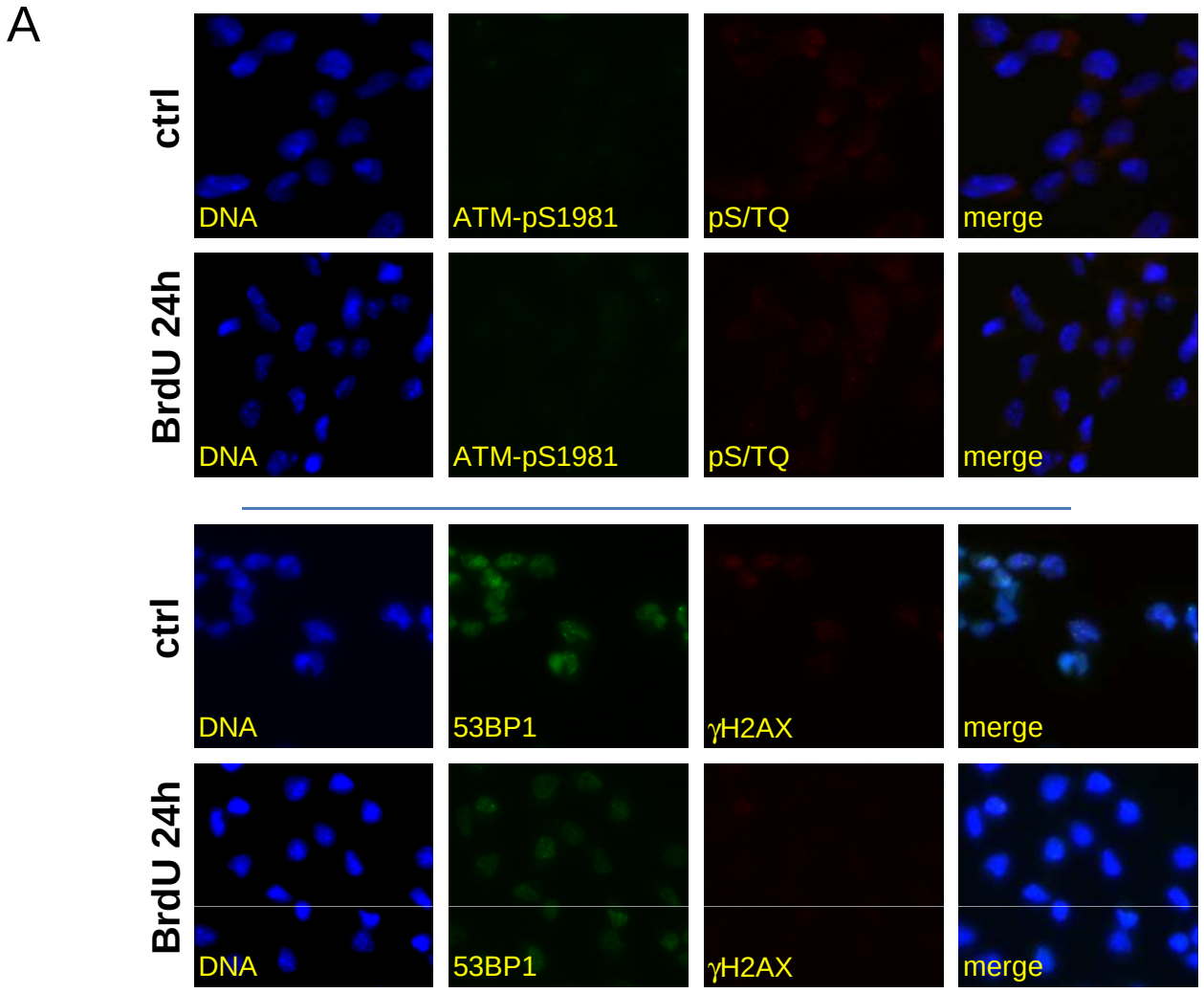
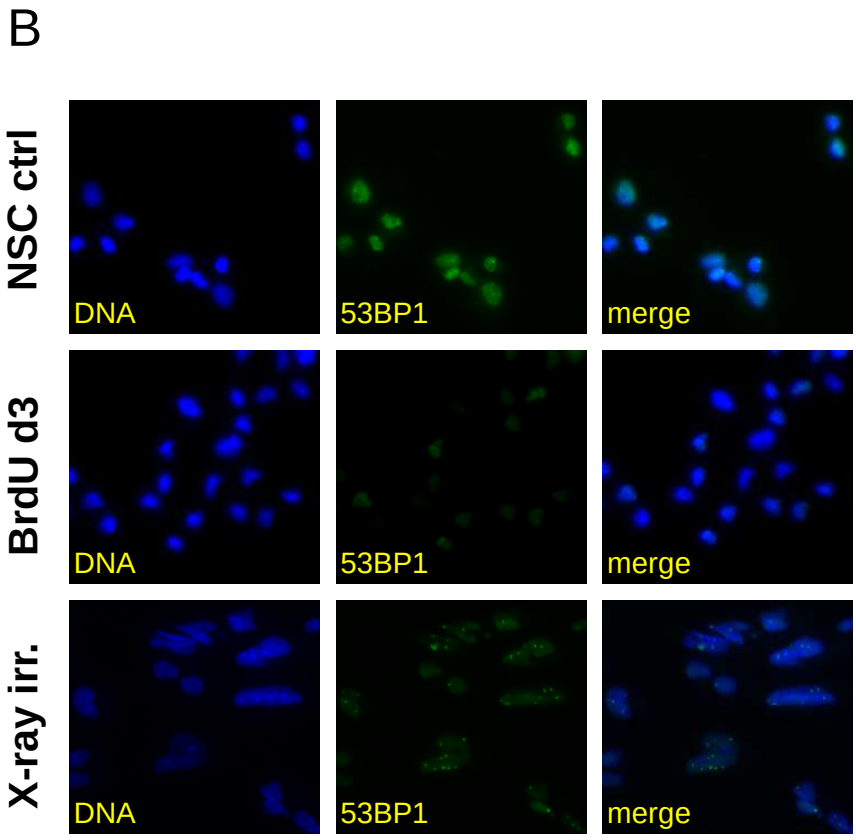

DNMT1
DNMT3a
DNMT3b
1
0,1
0,01
log change vs ctrl NSC
BrdU d3
BrdU d7

Fig. S6


A
ctrl
BrdU 24h
DNA
ATM-pS1981
pS/TQ
merge
ctrl
BrdU 24h
DNA
53BP1
γH2AX
merge


B
NSC ctrl
BrdU d3
X-ray irr.
DNA
53BP1
merge

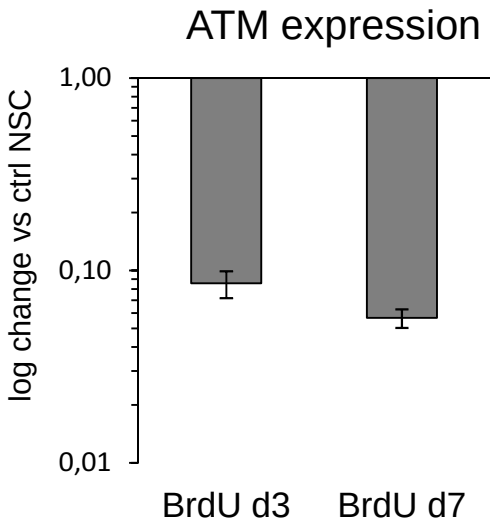
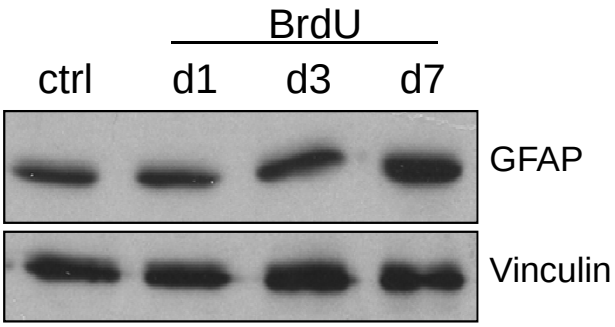
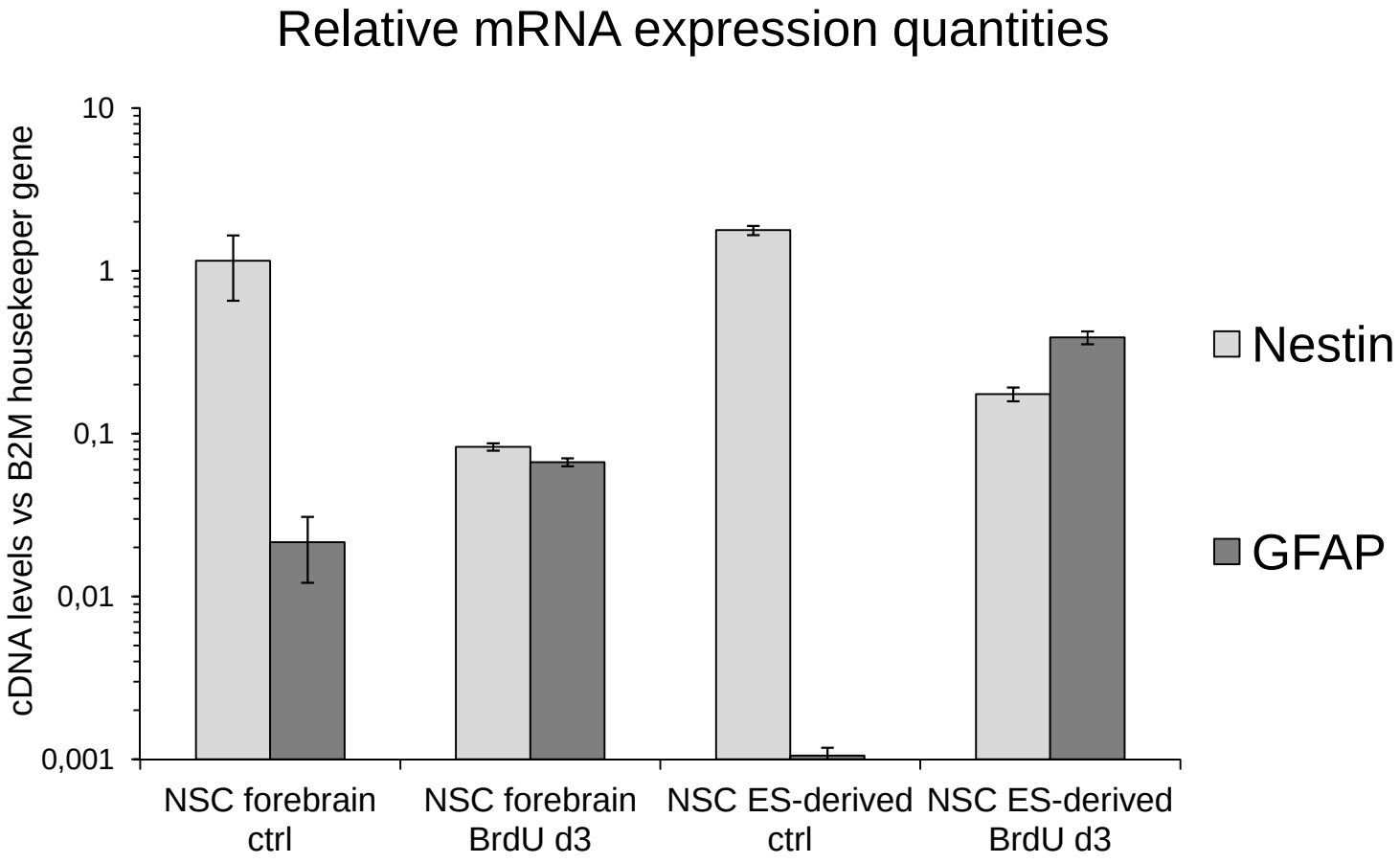

C
ATM expression
log change vs ctrl NSC
1,00
0,10
0,01
BrdU d3
BrdU d7

Fig. S7

A


BrdU
ctrl
d1
d3
d7
GFAP
Vinculin

B


Relative mRNA expression quantities
cDNA levels vs B2M housekeeper gene
10
1
0,1
0,01
0,001
NSC forebrain ctrl
NSC forebrain BrdU d3
NSC ES-derived ctrl
NSC ES-derived BrdU d3
Nestin
GFAP