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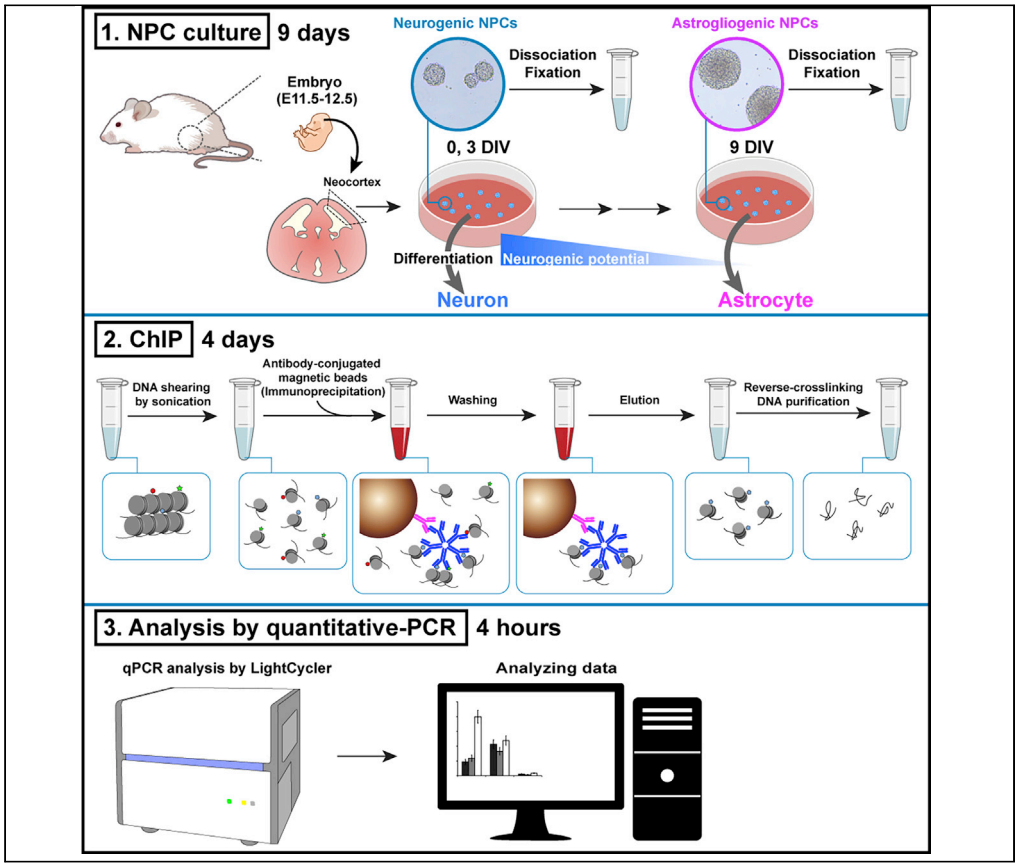
Analysis of histone modifications in mouse neocortical neural progenitor-stem cells at various developmental stages



Dynamic changes in histone modifications mediated by Polycomb group proteins can be indicative of the transition of gene repression mode during development. Here, we present methods for the isolation of mouse neocortical neural progenitor-stem cells (NPCs) and their culture, followed by chromatin immunoprecipitation-qPCR techniques to examine changes in histone H2A ubiquitination patterns at various developmental stages. This protocol can be applied for both in vitro NPCs and NPCs directly isolated from mouse neocortices.

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Highlights

In vitro culture system for obtaining neocortical neurogenic and astrogliogenic NPCs

Analysis for histone modification profiles in mouse neocortical NPCs by ChIP-qPCR

This ChIP protocol is optimized to yield a high signal-to-noise ratio for H2Aub analysis

Applicable for both in vitro NPCs and NPCs directly isolated from mouse neocortices

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Analysis of histone modifications in mouse neocortical neural progenitor-stem cells at various developmental stages

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SUMMARY

Dynamic changes in histone modifications mediated by Polycomb group proteins can be indicative of the transition of gene repression mode during development. Here, we present methods for the isolation of mouse neocortical neural progenitor-stem cells (NPCs) and their culture, followed by chromatin immunoprecipitation quantitative PCR (ChIP-qPCR) techniques to examine changes in histone H2A ubiquitination patterns at various developmental stages. This protocol can be applied for both in vitro NPCs and NPCs directly isolated from mouse neocortices.

For complete details on the use and execution of this protocol, please refer to (Tsuboi et al., 2018).

BEFORE YOU BEGIN

All developmental genes are maintained in a poised state for future transcriptional activation in pluripotent stem cells, whereas a subset of genes becomes persistently repressed after fate restriction during commitment to a specific lineage. PcG proteins mediate both temporary and persistent repression of the developmental genes during development. PcG comprises two main multisubunit complexes referred to as Polycomb repressive complex (PRC) 1 and PRC2. PRC2 mediates trimethylation of histone 3 at lysine-27 (H3K27me3), which is recognized by chromobox proteins (CBX) -containing PRC1 (Cao et al., 2002; Entrevan et al., 2016; Min et al., 2003; Wang et al., 2004b; Yu et al., 2019). PRC1 contains the E3 ubiquitin ligases Ring1A and Ring1B, which catalyze H2A ubiquitination (H2Aub) at K118 and K119 (de Napoles et al., 2004; Elderkin et al., 2007; McGinty et al., 2014; Wang et al., 2004a). H2Aub deposition is enriched at developmental gene loci in mouse embryonic stem cells (ESCs) (Blackledge et al., 2020; Endoh et al., 2012; Tamburri et al., 2020) and dynamically regulated at a subset of neurogenic gene loci during neuronal fate restriction in NPCs (Tsuboi et al., 2018). For instance, the level of H2Aub significantly increases at neurogenic genes such as *Neurog1*, *Fezf2*, *Tcfap2* and, *Lef1* when neurogenic potential of NPCs becomes restricted at the onset of the astrogliogenic phase (Tsuboi et al., 2018). By optimization of some conditions including antibody concentration, this protocol enables us to efficiently perform conventional chromatin immunoprecipitation (ChIP) analysis of H2Aub at K118 and K119 from neocortical NPCs. This ChIP protocol can be applied to both *in vitro* NPC cultures and NPCs directly isolated from mouse neocortices (Tsuboi et al., 2018). The following protocol is for the former, but the same is applicable to the latter after isolation of NPCs according to the method described in (Kishi and Gotoh, 2021).

Prepare solutions for NPC culture

Timing: 2–3 h

1. Prepare solutions listed below (see detailed recipes in the "Materials and equipment" section). Make sure that the volume of all solutions is sufficient for culture of mouse neocortical NPCs. The approximate volume required for 1.0×10^6 of NPCs is indicated.
   a. DMEM-F12 containing 1% penicillin-streptomycin (100 mL, 4°C)
   b. Trypsin solution (20 mL, −20°C)
   c. DNase I solution (500 μL, −20°C)
   d. Ovomucoid solution (200 μL, −20°C)
   e. Artificial cerebrospinal fluid (aCSF) (20 mL, 4°C)
   f. Low Ca^{2+}-aCSF (lc-aCSF) (4°C)
   g. Phosphate-buffered saline (PBS) (Room temperature; i.e., 18°C–24°C)
   h. Fibroblast growth factor 2 (FGF2) solution (50 μL, −80°C)
   i. Epidermal growth factor (EGF) solution (50 μL, −80°C)

   Note: Prepare all the solutions excluding PBS aseptically under laminar flow bench. Ovomucoid, aCSF and lc-aCSF solutions should be filtrated with a 0.22 μm filter (Millipore, SLGSR33SB for Ovomucoid and SCGPS05RE for aCSF and lc-aCSF). PBS is dissolved and then should be autoclaved.

   ⚠ CRITICAL: Dissolve the reagents with ice-cold PBS for preparing FGF2 and EGF solutions. Aliquot 100 μl of each solution into 0.6 ml tubes and flash-freeze them rapidly with liquid nitrogen and store it at −80°C.

2. Before beginning dissection of the mouse neocortex, trypsin solution, DNase I solution, and ovomucoid solution (stored at −20°C), FGF2 and EGF solution (stored at −80°C) should be thawed on ice.
3. Incubate trypsin solution and aCSF in a water bath at 37°C for ~10 min before beginning dissociation of the dissected neocortex.

Prepare solutions for ChIP

Timing: 2–3 h

4. Prepare solutions listed below (Tables in Materials and Equipment). Make sure that the volume of all solutions is sufficient for ChIP and that the solutions a, d–h have been adjusted to the indicated temperature before use. The approximate volume required for 1.0×10^6 of NPCs is indicated.
   a. PBS containing 0.5% bovine serum albumin (BSA) (500 μL, 4°C)
   b. 11% formaldehyde solution (150 μL)
   c. 2.5 M glycine (100 μL)
   d. Swelling buffer (1.5 mL, 4°C)
   e. Cell lysis buffer (10 mL, 4°C)
   f. Wash buffer A (2 mL, 4°C)
   g. Wash buffer B (2 mL, 4°C)
   h. TE buffer (2 mL, 4°C)
   i. Elution buffer (0.5 mL)
   j. Proteinase K solution (20 μL)
   k. 3 M sodium acetate, pH 5.2 (50 μL)
   l. 80% ethanol (1 mL)

   Note: Adjust pH of 3 M sodium acetate with acetic acid while stirring.

Design primers for qPCR

Timing: 30–60 min

5. Obtain genomic DNA sequences for the target gene loci from the mouse genomic DNA database with the UCSC genome browser (Mouse GRCm39/mm39: https://genome.ucsc.edu/). ChIP-seq databases for Ring1B and H3K27me3 in the developing mouse neocortices (Albert et al., 2017; Bonev et al., 2017) are informative to identify the sites of H2Aub deposition.
6. Design quantitative polymerase chain reaction (qPCR) primer sets with the use of LightCycler Probe Design Software 2.0 (Roche). Design primers as follows; Tm value of primer, 60.0°C; primer concentration, 0.5 μM; primer size, 19~ bp; amplicon size, 80–200 bp. Confirm that designed primer sets show no non-specific binding with the use of the BLAST tool.

Alternatives: Other qPCR design tools can be adopted for design of qPCR primer sets.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-H2Aub (E6C5) (1:62.5)	Merck Millipore	Cat#05–678; RRID: AB_309899
Rabbit anti-mouse IgMμ (1:125)	Merck Millipore	Cat#12–488; RRID: AB_390193
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium (DMEM)–F12 (1:1, v/v)	Thermo Fisher Scientific	Cat#1130-032
Penicillin-streptomycin	Thermo Fisher Scientific	Cat#15140122
B-27	Thermo Fisher Scientific	Cat#17504-44
Human FGF2	Thermo Fisher Scientific	Cat#PHG0023
Human EGF	Thermo Fisher Scientific	Cat#PHG0314
Neuron Dissociation Solutions	FUJIFILM Wako Pure Chemical Corporation	Cat#291-78001
Trypsin (2.5%), no phenol red	Thermo Fisher Scientific	Cat#15090046
Ovomucoid (trypsin inhibitor from chicken egg white Type II-O)	Sigma	Cat#T9253
Bovine serum albumin solution 7.5% in DPBS	Sigma	Cat#A8412
DNase I	Roche	Cat#11284932001
PBS	NISSUI	Cat#05913
0.4w/v% Trypan blue solution	FUJIFILM Wako Pure Chemical Corporation	Cat#207-17081
Bovine serum albumin lyophilized powder, ≥96%	Sigma	Cat#A2153
Pierce™ 16% formaldehyde (w/v), methanol-free	Thermo Fisher Scientific	Cat#28908
Aprotinin from bovine lung	Sigma	Cat#A6279
Hepes	Nacalai	Cat#17546-05
Nonidet P-40	Sigma	Cat#21-3277
Dithiothreitol (DTT)	FUJIFILM Wako Pure Chemical Corporation	Cat# 042-29222
EDTA	Nacalai	Cat#15105-35
Triton X-100	Nacalai	Cat#12967-45
Sodium dodecyl sulfate (SDS)	FUJIFILM Wako Pure Chemical Corporation	Cat#194-13985
Lithium chloride (LiCl)	Nacalai	Cat#20645-92
Proteinase K	Nacalai	Cat#29442-14
Protein A/G beads	Pierce	Cat#88803
Dynabeads protein A	Thermo Fisher Scientific	Cat#10002D
Phenol-chloroform-isoamyl alcohol	Nacalai	Cat#25970-56
Sodium acetate trihydrate	FUJIFILM Wako Pure Chemical Corporation	Cat#198-01055

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Acetic acid	FUJIFILM Wako Pure Chemical Corporation	Cat#017-00256
Ethanol	FUJIFILM Wako Pure Chemical Corporation	Cat#054-07225
Glycogen solution	FUJIFILM Wako Pure Chemical Corporation	Cat#076-06621
Critical commercial assays		
THUNDERBIRD® SYBR® qPCR Mix	Toyobo	Cat#QPS-201
Neural Tissue Dissociation Kit	Sumitomo Bakelite	Cat#MB-X9901
Experimental models: Organisms/strains		
Mouse: Jcl:ICR	CLEA Japan	MGI Cat# 5652875; RRID:MGI:5652875
Oligonucleotides		
**Neurog1* mRNA F: 5′-ATCACCACTCTCTGACCC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Neurog1* mRNA R: 5′-GAGGAAGAAAGTATT GATGTTGCCTTA-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Fezf2* mRNA F: 5′-CTCTACTGACAGCAAACCCA-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Fezf2* mRNA R: 5′-CTTTGCACACAAACGGTCT-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Lef1* mRNA F: 5′-CCGGTGGCTCTTTATTGT-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Lef1* mRNA R: 5′-CAAGAGGCTCCCTTTGC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Tcfap2c* mRNA F: 5′-TTGTCTCATTTCAGCCTGATAAC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Tcfap2c* mRNA R: 5′-CGATTAGAGCCTCCT TGATATAATTC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Gfap* mRNA F: 5′-TCCCTAGAGCGGCAAAT-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Gfap* mRNA R: 5′-TCCTCCTTGAGGCTTTGG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Gapdh* mRNA F: 5′-TGGGTGTGAACCACGAG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
**Gapdh* mRNA R: 5′-AAGTTGTCATGGATGACCTT-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Fezf2* F: 5′-TTCCTTGGGTGATTCACAAC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Fezf2* R: 5′-CTTTGAGGTAGCGCGTC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Lef1* F: 5′-GGCAGCTCTTTGCTTTGAC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Lef1* R: 5′-CCCGATCCCTCTTTGTTC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Tcfap2c* F: 5′-CTTCCCATTAAGGCGTTCG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Tcfap2c* R: 5′-GCCGTGGGTGTGTAGAG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Cdkn2d* F: 5′-CGGAGACCCAGGACAG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Cdkn2d* R: 5′-CCAGGATTCCGGTGC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Gapdh* F: 5′-TGCAGTCCGTATTTATAGGAACC-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Gapdh* R: 5′-CTTGAGCTAGGACTGGATAAGCA-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Cdkn1a* F: 5′-CACCTCTAAGGCCAGCTA-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Cdkn1a* R: 5′-AGCAATGTCAAGAGTCGG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Cdkn1c* F: 5′-CTGGCTGATTGGTGATGG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Cdkn1c* R: 5′-GACTGAGAGCAAGCGAACA-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Dlk1* F: 5′-ACGTTCTAAGGTGCAACT-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
***Dlk1* R: 5′-GGAAATTAGGGACTACGACTG-3′	(Tsuboi et al., 2018)	https://doi.org/10.1016/j.devcel.2018.11.018
Software and algorithms		
LightCycler Probe Design Software 2.0	Roche	Cat#04342054001
LightCycler 480 Software, version 1.5.1	Roche	https://lifescience.roche.com/global_en/products/lightcycler14301-480-software-version-15.html
Other		
100-mm Plastic culture dish	VIOLAMO	Cat#VTC-D100
Millex-GS Syringe Filter Unit, 0.22 μm	Merck Millipore	Cat#SLGSR33SB
Steritop Threaded Bottle Top Filter	Merck Millipore	Cat#SCGPS05RE
Cell counting chamber according to Neubauer	Hirschmann	Cat#8100104
Inverted microscope Eclipse Ts2-FL	Nikon	N/A
10-mL Disposable pipette	VIOLAMO	Cat#4733
Heat block	Astec	Cat#BI-516S

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vortex mixer	LMS	Cat#VTX-3000L
Tube rotator	TAITEC	Cat#AT-5
27G Injection needle	Terumo Corporation	Cat#NN-2719S
Aspirator	AS ONE	Cat#1-689-04
LightCycler 480 System II	Roche	N/A
DynaMag-2 Magnet	Thermo Fisher Scientific	Cat#12321D
Prelubricated microcentrifuge tubes (1.7 mL)	Costar	Cat#3207
Cool rack for 96-well plate	AS ONE	Cat#IO-2
Sapphire PCR microplate, 96 Well	Greiner Bio-One	Cat#669285
LightCycler® 480 Sealing Foil	Roche	Cat#04729757001
Handy Sonic UR-20P	Tomy	Cat#UR-20P

* Primers for qPCR of mRNA expression analysis
** Primers for ChIP-qPCR analysis

MATERIALS AND EQUIPMENT

Buffers for neocortical NPC culture

DMEM-F12 containing 1% penicillin-streptomycin		
Reagent	Final concentration	Amount
DMEM-F12		495 mL
Penicillin-streptomycin	1% (v/v)	5 mL
Total		**500 mL**

Store at 4°C, and do not store more than 3 months.

Trypsin			
Reagent	Final concentration	Stock concentration	Amount
Trypsin (2.5%), no phenol red	0.0625% (v/v)	2.5%	1.25 mL
BSA	0.01875% (v/v)	7.5%	0.125 mL
lc-aCSF			Up to 50 mL
Total			**50 mL**

Store at –20°C, and do not store more than 6 months.

Ovomucoid		
Reagent	Final concentration	Amount
Ovomucoid	70 mg/mL	1 g
aCSF		14.3 mL
Total		**14.3 mL**

Store at –20°C, and do not store more than 6 months.

DNase I		
Reagent	Final concentration	Amount
DNase I	5 mg/mL	100 mg
lc-aCSF		20 mL
Total		**20 mL**

Store at –20°C, and do not store more than 6 months.

aCSF		
Reagent	Final concentration	Amount
NaCl	124 mM	7.25 g
KCl	5 mM	0.37 g
$MgCl_2/6H_2O$	1.3 mM	0.26 g
$CaCl_2/2H_2O$	2 mM	0.29 g
$NaHCO_3$	26 mM	2.18 g
D-Glucose	10 mM	1.80 g
ddH_2O		Up to 1 L
Total		**1** L
Store at 4°C, and do not store more than 3 months.		

lc-aCSF		
Reagent	Final concentration	Amount
NaCl	124 mM	7.25 g
KCl	5 mM	0.37 g
$MgCl_2/6H_2O$	3.2 mM	0.65 g
$CaCl_2/2H_2O$	0.1 mM	0.015 g
$NaHCO_3$	26 mM	2.18 g
D-Glucose	10 mM	1.80 g
ddH_2O		Up to 1 Ll
Total		**1** Ll
Store at 4°C, and do not store more than 3 months.		

PBS	
Reagent	Amount
PBS	4.8 g
ddH_2O	500 mL
Total	**500 mL**
Store at 18°C–24°C, and do not store more than 1 year.	

FGF2 solution		
Reagent	Final concentration	Amount
FGF2	20 μg/mL	25 μg
PBS		1.25 mL
Total		**1.25 mL**
Store at –80°C, and do not store more than 1 year.		

EGF solution		
Reagent	Final concentration	Amount
EGF	20 μg/mL	100 μg
PBS		5 mL
Total		**5 mL**
Store at –80°C, and do not store more than 1 year.		

DMEM-F12 medium		
Reagent	Final concentration	Amount
DMEM-F12		24.45 mL
B-27	2% (v/v)	0.5 mL
FGF2 solution	20 ng/mL	0.025 mL
EGF solution	20 ng/mL	0.025 mL
Total		25 mL
Please prepare the required volume of fresh medium just before use.		

Buffers for H2Aub ChIP

PBS containing 0.5% bovine serum albumin (BSA)		
Reagent	Final concentration	Amount
Bovine serum albumin lyophilized powder	0.5% (w/v)	0.05 g
PBS		10 mL
Total		**10 mL**
Store at 4°C, and do not store more than 1 month.		

11% formaldehyde solution		
Reagent	Final concentration	Amount
16% formaldehyde	11% (v/v)	6.875 mL
PBS		3.125 mL
Total		**10 mL**
Please prepare the required volume of fresh solution just before use.		

2.5 M Glycine		
Reagent	Final concentration	Amount
Glycine	2.5 M	0.94 g
ddH_2O		5 mL
Total		**5 mL**
Store at –20°C, and do not store more than 6 months.		

Swelling buffer			
Reagent	Final concentration	Stock concentration	Amount
Hepes (pH 7.9)	20 mM	1 M	0.1 mL
$MgCl_2$	1.5 mM	1 M	0.0075 mL
KCl	10 mM	1 M	0.05 mL
NP-40	0.1% (v/v)	10% (v/v)	0.05 mL
Aprotinin[a]	0.2% (v/v)		0.01 mL
DTT[a]	1 mM	1 M	0.005 mL
ddH_2O			Up to 5 mL
Total			**5 mL**
Store at 18°C–24°C, and do not store more than 6 months. [a]Add fresh before use			

Cell lysis buffer			
Reagent	Final concentration	Stock concentration	Amount
Tris (pH 8.0)	10 mM	1 M	0.5 mL
NaCl	140 mM	5 M	1.4 mL
EDTA	1 mM	0.5 M	0.1 mL
Triton X-100	1% (v/v)	10% (v/v)	5 mL
Sodium deoxycholate	0.1% (w/v)		0.05 g
SDS	0.1% (w/v)	10% (w/v)	0.5 mL
ddH_2O			Up to 50 mL
Total			**50 mL**
Store at 4°C, and do not store more than 6 months.			

Wash buffer A			
Reagent	Final concentration	Stock concentration	Amount
Tris (pH 8.0)	10 mM	1 M	0.5 mL
NaCl	500 mM	5 M	5 mL
EDTA	1 mM	0.5 M	0.1 mL
Triton X-100	1% (v/v)	10% (v/v)	5 mL
Sodium deoxycholate	0.1% (w/v)		0.05 g
SDS	0.1% (w/v)	10% (w/v)	0.5 mL
ddH_2O			Up to 50 mL
Total			**50 mL**
Store at 4°C, and do not store more than 6 months.			

Wash buffer B			
Reagent	Final concentration	Stock concentration	Amount
Tris (pH 8.0)	10 mM	1 M	0.5 mL
EDTA	1 mM	0.5 M	0.1 mL
LiCl	250 mM	5 M	2.5 mL
NP-40	0.5% (v/v)	10% (v/v)	2.5 mL
Sodium deoxycholate	0.5% (w/v)		0.25 g
ddH_2O			Up to 50 mL
Total			**50 mL**
Store at 4°C, and do not store more than 6 months.			

TE buffer			
Reagent	Final concentration	Stock concentration	Amount
Tris (pH 8.0)	10 mM	1 M	0.5 mL
EDTA	1 mM	0.5 M	0.1 mL
ddH_2O			Up to 50 mL
Total			**50 mL**
Store at 18°C–24°C, and do not store more than 6 months.			

Elution buffer			
Reagent	Final concentration	Stock concentration	Amount
Tris (pH 8.0)	10 mM	1 M	0.5 mL
EDTA	5 mM	0.5 M	0.5 mL
NaCl	300 mM	5 M	3 mL
SDS	0.5% (w/v)	10% (w/v)	2.5 mL
ddH_2O			Up to 50 mL
Total			**50 mL**
Store at 18°C–24°C, and do not store more than 6 months.			

Proteinase K solution		
Reagent	Final concentration	Amount
Proteinase K	10 mg/mL	100 mg
ddH_2O		Up to 10 mL
Total		**10 mL**
Store at –20°C, and do not store more than 1 year.		

3M sodium acetate (Adjust to pH 5.2)		
Reagent	Final concentration	Amount
Sodium acetate trihydrate	3 M	40.8 g
ddH_2O		Up to 100 mL
Total		**100 mL**
Store at 18°C–24°C, and do not store more than 1 year.		

80% Ethanol		
Reagent	Final concentration	Amount
Ethanol	80% (v/v)	40 mL
ddH_2O		Up to 50 mL
Total		**50 mL**
Store at –20°C, and do not store more than 1 year.		

Equipment

- Handy Sonic UR-20P (Tomy)

  Alternatives: This protocol was developed with the use of a Handy Sonic UR-20P device to shear chromatin DNA. Picoruptor (diagenode, P-150504) can also be used for this purpose.

- LightCycler 480 System II (Roche) + THUNDERBIRD® SYBR® qPCR Mix (Toyobo)

  Alternatives: This protocol is based on a qPCR procedure performed with a LightCycler 480 System II (Roche). Any other qPCR machine can also be used.

  Alternatives: This protocol describes a qPCR procedure performed with THUNDERBIRD® SYBR® qPCR Mix in a reaction volume of 16 µl. SYBR Premix Ex Taq (Takara), LightCycler 480 SYBR Green I Master (Roche), and KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) are potential alternatives.

- DynaMag-2 magnet

  Alternatives: Any other magnetic stand for 1.5-ml tubes can also be used.

STEP-BY-STEP METHOD DETAILS

Isolation and *in vitro* culture of neocortical (E11-12) NPCs

Cross-linking of the chromatin—Days 1–10

Timing: 9 days

Neocortical NPCs can be propagated in suspension culture with FGF2 and EGF, during which they form cell aggregates known as neurospheres. The cells cultured for 0 or 3 days in vitro (DIV)

correspond to the neurogenic phase of development, whereas those cultured for 9 DIV correspond to the astrogliogenic phase (Hirabayashi and Gotoh, 2005; Qian et al., 2000).

Day 1

1. Kill pregnant Institute of Cancer Research (ICR) mice by cervical dislocation at embryonic day (E) 11 to 12, with E1 being defined as 12 h after detection of the vaginal plug.

   Note: Approximately 5×10^5 NPCs can be obtained from a single mouse embryo at E11-12.

   Note: C57BL/6 mice also can serve as a source of NPCs for culture.

   Note: The procedure for sacrifice of mice should be in accordance with the institutional animal care and use committee.

2. Remove the uterus and immerse it in ice-cold DMEM-F12 containing 1% penicillin-streptomycin in a 100-mm plastic culture dish.

   Note: After isolation of embryos from the uterus, all manipulations are performed in ice-cold DMEM-F12 containing 1% penicillin-streptomycin unless indicated otherwise.

3. Remove embryos one by one from the embryonic sacs and transfer then to another 100-mm dish filled with ice-cold medium.
4. Hold down the head of each embryo with forceps and cut off the telencephalon with a scalpel.
5. Pull and tease apart the skin from the telencephalon and separate the telencephalon from the brainstem.
6. Remove the vessel membrane (meninges) from the telencephalon.
7. Isolate the neocortex from the ventral telencephalon.
   a. Collect the dissected neocortices into a 1.7-mL prelubricated microcentrifuge tube containing 500 μL of DMEM-F12 containing 1% penicillin-streptomycin.
   b. Maintain the tube on ice until the neocortices settle to the bottom of the tube and then remove the medium.
8. Digest the neocortical tissue with Neuron Dissociation Solutions (FUJIFILM Wako Pure Chemical Corporation) consisting of Enzyme solution, Dispersion solution and Isolation solution.
   a. Add 300 μL of Enzyme solution to the tissue and incubate for 20 min at 37°C.
   b. Centrifuge the digest at 160 × *g* for 2 min at room temperature and remove the resulting supernatant.
   c. Add 300 μL of Dispersion solution to the pellet and triturate gently with the use of a 1-mL micropipette.

   ⚠ CRITICAL: Take care during trituration not to damage the cell suspension by pipetting too vigorously.
   d. Add 300 μL of Isolation solution as a layer below the cell suspension.

   Note: The specific gravity of Isolation solution is greater than that of Dispersion solution. Therefore, by gently adding Isolation solution to the bottom of the tube, these solutions become separated into two layers— Dispersion solution in the top layer and Isolation solution in the bottom layer. Be careful not to disturb the interface between the layered solutions. If the solutions become mixed, it will not be possible to remove the papain.
   e. Centrifuge the tube at 160 × *g* for 5 min at room temperature and then remove the supernatant.

9. Prepare the DMEM-F12 supplemented with B-27 (final B-27 concentration of 2%), recombinant human FGF2 (final FGF2 concentration of 20 ng/mL), and recombinant human EGF (final EGF concentration of 20 ng/mL).

Note: Prewarm the DMEM-F12 (without any supplements) to 37°C in a water bath for at least 20 min before starting dissociation of neocortices.

10. Gently suspend the cell pellet in 4 mL of DMEM-F12 supplemented with B-27, FGF2, and EGF.
11. Count the number of isolated cells with the use of a cell counting chamber (Neubauer haemocytometry, Electron Microscopy Sciences)

Note: When counting the number of cells, it is recommended to mix 10 μl of cell suspension with an equal volume of Trypan blue solution and evaluate the cell viability under a microscope. The percentage of cells stained with Trypan blue should be less than 2–3%.

12. Collect 0.5×10^6 to 1.0×10^6 dissociated cells into a 1.7-mL prelubricated microcentrifuge tube as neurogenic NPCs.
13. Add 0.1 volume of 11% formaldehyde solution to the cell suspension (final formaldehyde concentration of 1%) and rotate the tube for 10 min on a tube rotator.

Note: The formaldehyde solution should be added dropwise to the cell suspension in order to avoid a localized high formaldehyde concentration.

14. Add 2.5 M glycine to the cell suspension to achieve a final concentration of 125 mM.
15. Centrifuge the mixture at 2,700 × *g* for 5 min at 4°C.
16. Remove the resulting supernatant and then freeze the pellet in liquid nitrogen and store it at –80°C.

Pause point: The pellet can be stored at –80°C for at least several months.

17. The remaining dissociated cells are cultured at a density of 3.0×10^5 cells/mL in DMEM-F12 medium supplemented with B-27, FGF2, and EGF, resulting in neurosphere formation.

Note: Culture the dissociated cells in a dish at least 60 mm in diameter (e.g., 100-mm dish, 3.0×10^6 cells in 10 mL medium; 60-mm dish, 1.2×10^6 cells in 4 mL medium). Use of a smaller culture dish can result in aggregation of neurospheres, with such large aggregates being difficult to dissociate completely.

Note: Do not move the culture dish after its transfer to the incubator. Even a slight movement can also result in the aggregation of neurospheres.

Day 4

18. After culture for 3 DIV, transfer the neurospheres to a 15-mL Falcon tube. Centrifuge the tube at 200 × *g* for 2 min at room temperature and remove the supernatant.

⚠ CRITICAL: Remove as much of the supernatant as possible after centrifugation. Residual medium in the tube can inhibit the enzymatic activity of trypsin.

19. Add 100 μL of DNase I solution into 4 mL of trypsin solution (final DNase I concentration of 0.1 mg/mL).
20. Add 4 mL of trypsin solution containing DNase I to the neurosphere pellet.

⚠ CRITICAL: Take care not to prewarm trypsin at 37°C (in a water bath) for >10 min. Incubation for longer can result in self-digestion of trypsin and a consequent reduction in dissociation efficiency.

Note: DNase I solution should be added to the trypsin solution immediately before the addition of trypsin to the neurosphere pellet. Otherwise, trypsin digests the DNase I and reduces the efficiency of genomic DNA digestion.

21. Incubate the mixture for 10 min at 37°C.

Note: Gently mix the suspension every 2–3 min with the use of a 10-mL disposable pipette in order to promote dissociation of the neurospheres.

22. Add 40 μL of ovomucoid solution into 4 mL of aCSF (final ovomucoid concentration of 0.7 mg/mL).
23. Add 4 mL of aCSF containing ovomucoid to the cell suspension from step 21.
24. Centrifuge the suspension at 200 × *g* for 2 min at room temperature and then remove the supernatant.
25. Resuspend the cell pellet in 5 mL of DMEM-F12 medium supplemented with B-27, FGF2, and EGF and then mechanically dissociate the NPCs with the use of a 10-mL disposable pipette.

⚠ CRITICAL: Lightly press the tip of the pipette against the bottom of the tube and pipette the cell suspension at least 10 times. This step is critical for complete dissociation of the cells. Be careful not to press the tip of the pipette too hard against the bottom of the tube, however, as this can result in cell damage or death.

26. Determine the cell number with a cell counting chamber and set aside 0.5 × 10^6 to 1.0 × 10^6 of the cells for ChIP analysis of NPCs in the neurogenic phase at 3 DIV.
27. Culture the remaining dissociated cells at a density of 3.0 × 10^5 cells/mL in DMEM-F12 medium supplemented with B-27, FGF2, and EGF.
28. Repeat steps 18–27 after successive 3-day intervals to obtain NPCs at 6 or 9 DIV.

Day 10

29. Transfer 0.5 × 10^6 to 1.0 × 10^6 dissociated cells after culture for 9 DIV into a 1.7-mL prelubricated microcentrifuge tube for analysis as astrogliogenic NPCs.
30. Add 0.1 volume of 11% formaldehyde solution to the cell suspension (final formaldehyde concentration of 1%) from steps 26 or 29 and rotate the tube for 10 min on a tube rotator.
31. Add 2.5 M glycine to the cell suspension to achieve a final concentration of 125 mM.
32. Centrifuge the mixture at 2,700 × *g* for 5 min at 4°C.
33. Remove the supernatant and then freeze the pellet in liquid nitrogen and store it at –80°C.

Pause point: The pellet can be stored at –80°C for at least one year.

Preparation of antibody-conjugated magnetic beads—Days 11 and 12

Timing: 1 day

Antibodies that bind the protein of interest are linked to magnetic beads. The antibody to H2Aub from Merck-Millipore (#05–678) is mouse immunoglobulin M (IgM) and shows low affinity for both protein A and G beads. The antibody to the μ light chain of mouse IgM (Merck-Millipore, #12–488) shows high affinity for the beads, and is therefore used to link the antibody to H2Aub and the protein A beads. The specificity of this H2Aub antibody has been confirmed by the reduction

of signals in Ring1A/B double knockout NPCs compared to contorl NPCs in Western blotting and ChIP-qPCR analyses (Tsuboi et al., 2018).

Note: The following H2Aub ChIP protocol is optimized from one previously described (Endoh *et al.*, 2012; Stock et al., 2007).

Day 1

34. Resuspend protein A magnetic beads (Dynabeads) by vortex-mixing for >30 s.

Alternatives: Protein A/G beads (Pierce) can be used as an alternative to protein A beads (Dynabeads).

35. Transfer a volume of beads corresponding to 20 μL (600 μg) of beads per ChIP reaction and 20 μL of beads per sample (for preclearing) to a 1.7-mL prelubricated microcentrifuge tube.
36. Place the tube on the magnet (DynaMag-2) to separate the beads from the solution and then remove the supernatant by aspiration.

Note: Complete separation of the beads from the solution can be facilitated by swirling the beads gently until the solution becomes clear.

Note: When removing the supernatant by aspiration, attach a 27G injection needle to the tip of the aspirator. This will reduce the suction force and the risk of aspirating the magnetic beads by accident.

37. Wash the beads once with 250 μL of PBS containing 0.5% BSA by vortex-mixing or gentle tapping.
38. Place the tube on the magnet to separate the beads from the solution and again remove the supernatant.
39. Add 250 μL of PBS containing 0.5% BSA to the tube containing the beads. Subsequent nonspecific binding of protein to the magnetic beads is minimized by this beads-blocking step.

Note: This step is for blocking beads with BSA, and a volume of 250 μl is required for the solution to be stirred by the rotator.

40. Incubate the tube on a tube rotator at approximately 5 rotations per minute for 1 h at 4°C (in a cold room).
41. Place the tube on the magnet to separate the beads from the solution and remove the supernatant.
42. Add a volume of PBS containing 0.5% BSA corresponding to that of the beads transferred to the tube in step 35 (for example, if 100 μL of beads were used initially, then add 100 μL of PBS containing 0.5% BSA to the tube).
43. Transfer beads for ChIP reaction into a new 1.7-mL prelubricated microcentrifuge tube and proceed to step 44. Keep rotating the tube containing beads used for preclearing at 4°C (in a cold room).

Note: It is frequently observed that magnetic beads precipitate into the bottom of the tube after the rotation. Gently resuspend the beads-containing solution by pipetting.

44. Add PBS containing 0.5% BSA to the tube containing beads up to 250 μL and then add 2 μg of antibodies to mouse IgMμ per ChIP reaction to the tube containing the blocked magnetic beads.

Note: Given that the optimal amounts of antibodies vary from lot to lot, it is recommended that ChIP be performed in advance with various amounts of antibody by using 9 DIV NPC

culture in order to determine the optimal amount of antibody that yields a high signal-to-noise ratio [e.g., signal value for the *HoxA1* locus (positive control) in NPCs at 9 DIV should be >10 times than that for the *Gapdh* locus (negative control)].

45. Incubate the tube on a tube rotator for >2 h at 4°C (cold room).
46. Place the tube on the magnet to separate the beads from the solution and then remove the supernatant.
47. Wash the anti-IgMμ–conjugated beads five times with 500 μL of Cell lysis buffer.

⚠ CRITICAL: Washing of the beads at least five times at this stage is important to reduce nonspecific binding of chromatin.

Note: When washing the beads, place the tube on the magnet to separate the beads from the solution and then remove the supernatant, add the washing buffer, and resuspend the beads by gentle rotation of the magnetic stand.

48. Resuspend the anti-IgMμ–conjugated beads in 250 μL of Cell lysis buffer.
49. Add 4 μg of antibodies to H2Aub per ChIP reaction to the beads.

Note: This step is for binding of anti-H2Aub antibody to the anti-IgMμ–conjugated beads, and a volume of 250 μl is required for the solution to be stirred by the rotator.

Note: It is recommended to determine the optimal amount of anti-H2Aub antibody that yields a high signal-to-noise ratio by using 9 DIV NPC culture, given that the efficiency of immunoprecipitation varies depending on the antibody lot [e.g., signal value for the *HoxA1* locus (positive control) in NPCs at 9 DIV should be >10 times than that for the *Gapdh* locus (negative control)].

50. Incubate the tube on a tube rotator overnight (10–16 h) at 4°C (cold room).

Day 2

51. Place the tube on the magnet to separate the beads from the solution and then remove the supernatant.

Note: Complete separation of the beads from the solution can be facilitated by swirling the beads gently until the solution becomes clear.

Note: When removing the supernatant by aspiration, attach a 27G injection needle to the tip of the aspirator. This will reduce the suction force and the risk of aspirating the magnetic beads by accident.

52. Wash the beads three times with 500 μL of Cell lysis buffer.

Note: When washing the beads, place the tube on the magnet to separate the beads from the solution and then remove the supernatant, add the washing buffer, and resuspend the beads by gentle rotation of the magnetic stand.

53. Resuspend the antibody–conjugated beads in 20 μL of Cell lysis buffer per ChIP reaction.

Note: Given that the protein A beads tend to form pellets under the influence of gravity, pipette the bead suspension gently and thoroughly before transfer to tubes containing chromatin.

Lysis of the cell pellet and shearing of chromatin—Day 12

Timing: 3–4 h

NPCs are lysed to extract chromatin from the nucleus. Chromatin is then fragmented by ultrasonic treatment, which is necessary to increase the resolution of ChIP.

54. Resuspend the cell pellet with 1 mL of swelling buffer per sample and incubate for 20 min on ice.
55. Centrifuge the tube at 5,800 × *g* for 5 min at 4°C.
56. Remove the resulting supernatant and resuspend the pellet in 150 μL of Cell lysis buffer.
57. Shear the chromatin with the use of a Hand Sonic UR-20P device (Tomy). The power setting should be "3" for 8 cycles of ON for 10 s and OFF for 20 s and then "4" for 2 cycles of ON for 10 s and OFF for 20 s.

⚠ CRITICAL: The sonication setting is essential for the appropriate shearing of chromatin. The power of sonication can vary depending on the equipment. After shearing of chromatin, it is recommended that its length be 200–1000 base pairs. It is also recommended to run the samples on an agarose gel electrophoresis and confirm that the size of chromatin is appropriate. If shearing is insufficient, increase the power setting step by step.

Note: While sonicating the cell suspension, place the tube on ice in order to prevent an increase in temperature. Be careful not to cause bubbling of the suspension. If bubbling occurs, centrifuge the tube at 20,380 × *g* for 5 min at 4°C. This operation will remove bubbling that have been generated.

58. Centrifuge the tube at 20,380 × *g* for 15 min at 4°C. Cell debris such as insoluble membranes should be removed by this step.
59. Transfer all supernatant (~150 μL) to a new 1.7-mL prelubricated microcentrifuge tube.
60. Add 100 μL of Cell lysis buffer and 20 μL of blocked Dynabeads for preclearing from step 43 to the tube containing the sheared chromatin. Incubate the mixture on a tube rotator for 1 h at 4°C (cold room). Subsequent nonspecific binding of protein to the magnetic beads is minimized by this preclearing step.
61. Place the tube on the magnet to separate the beads from the solution and then transfer 100 μL of the supernatant to a new 1.7-mL prelubricated microcentrifuge tube for ChIP as well as 50 μL to another new tube for determination of input (the amount of chromatin DNA used for the immunoprecipitation). Store the latter tube at –20°C until reversal of cross-linking.
62. Add 150 μL of Cell lysis buffer to the tube containing the supernatant for ChIP reaction.

Immunoprecipitation, washing, elution, and reversal of cross-linking—Days 12–14

Timing: 2 days

In the following steps, H2Aub is specifically captured by antibodies conjugated to magnetic beads. Nonspecific binding of nonmodified histone and nonbound DNA are largely eliminated by washing of the beads with various buffers. Immunoprecipitated chromatin is then eluted from the magnetic beads, and histone-DNA cross-linking is reversed to release DNA fragments.

Day 1

63. Add 20 μL of anti-H2Aub–conjugated magnetic beads per ChIP to the chromatin extract from step 62.
64. Incubate the tube on a tube rotator overnight (10–16 h) at 4°C (cold room).

Day 2

65. Place the tube on the magnet to separate the beads from the solution and then remove the supernatant by aspiration.

    Note: Complete separation of the beads from the solution can be facilitated by swirling the beads gently until the solution becomes clear.

    Note: When removing the supernatant by aspiration, attach a 27G injection needle to the tip of the aspirator. This will reduce the suction force and the risk of aspirating the magnetic beads by accident.

66. Wash the beads six times with 500 μL of Cell lysis buffer.
67. Wash the beads twice with 500 μL of Wash buffer A.
68. Wash the beads twice with 500 μL of Wash buffer B.
69. Wash the beads twice with 500 μL of TE buffer.

    Note: After the addition of each wash buffer, separate the beads on the magnetic stand, remove the supernatant, and resuspend the beads by agitation.

70. Resuspend the beads in 200 μL of Elution buffer and proceed to step 71. At the same time, thaw the tube containing the chromatin solution for determination of total input (from step 61), add 150 μL of Elution buffer and proceed to step 72.
71. Incubate the former tube at 65°C for 15 min.

    Note: For efficient elution of chromatin from the magnetic beads, tap and agitate the tube gently every 2–3 min during the incubation.

72. Add 5 μL of proteinase K solution to a final enzyme concentration of 0.25 mg/mL.
73. Incubate overnight (10–16 h) at 37°C.

Day 3

74. Transfer the tubes to a 65°C incubator for reversal of cross-linking and incubate for at least 6 h.

    Pause point: The chromatin can be stored at –20°C for at least several days at this point.

75. Add 200 μL of phenol–chloroform–isoamyl alcohol to each tube and shake vigorously.
76. Centrifuge at 20,380 × *g* for 15 min at room temperature.
77. Collect the resulting aqueous upper phase (~200 μL) and transfer it to a new 1.7-mL prelubricated microcentrifuge tube.
78. Add 2.5 volumes of ethanol, 0.1 volume of 3 M sodium acetate, and 1 μL of glycogen (final glycogen concentration of 0.1 mg/mL) and apply the tube to a vortex mixer.
79. Incubate at –20°C for 30 min.
80. Centrifuge at 20,380 × *g* for 15 min at room temperature.
81. Remove the resulting supernatant and wash the DNA pellet with 300 μL of 80% (v/v) ethanol.
82. Allow the DNA pellet to dry in air and then dissolve it in 50 μL of ddH_2O.

Quantification of binding by qPCR—Day 15

Timing: 4 h

The amount of H2Aub at target genes is quantified by qPCR. The relative binding frequency is estimated by calculating the percentage of total input that is recovered in the immunoprecipitate.

Note: Neurogenic gene loci such as *Neurog1*, *Fezf2*, *Lef1*, and *Tcfap2c* and housekeeping gene loci such as *Actb* and *Gapdh* can be used as positive and negative controls, respectively, to verify the fidelity of the H2Aub ChIP assay. The binding ratio of the signals at target gene loci relative to those at negative genes loci can then be calculated. However, if the background is very low, it can be difficult to quantify. If there is a lot of variation in the background signal, the enrichment over background will also have a lot of variation. To confirm that neuronal differentiation potential is indeed restricted in NPC cultures at 9 DIV, the level of H2Aub at neurogenic gene loci should be examined.

Note: This qPCR protocol was designed for a 16 μL reaction mixture in the 96-well format and for the use of THUNDERBIRD® SYBR® qPCR Mix (Toyobo) and a LightCycler 480 System II (Roche). The precise procedure will differ depending on the reagents and machine used, but the general design principles should be the same as described here.

Reaction mixture for a single 16 μL qPCR reaction with THUNDERBIRD® SYBR® qPCR Mix

Reagent	Volume
THUNDERBIRD® SYBR® qPCR Mix	8 μL
ddH_2O	4.4 μL
Forward + reverse primer mix (10 μM each)	1.6 μL
DNA template	2 μL

⚠ CRITICAL: When designing primers for qPCR, ensure that the amplicons are not too large (optimal size of ~100 bp).

83. Prepare a stock solution of both forward and reverse primers in ddH_2O at a final concentration of 10 μM.
84. Combine 4.4 μL of ddH_2O, 8 μL of THUNDERBIRD® SYBR® qPCR Mix, and 1.6 μL of primer stock solution per reaction for 1.1 times the desired number of reactions as a premixture. Mix well by pipetting or tapping.

Note: Assay samples at least in duplicate and ensure that there is no substantial difference between the duplicate values.

Note: When performing qPCR, always generate a standard curve for the level of PCR amplification by the use of each primer pair on each plate with the use of purified genomic DNA of the input sample and with the same premixture as used for the immunoprecipitated samples. We only used the range of PCR amplification cycles for each primer pair that shows the level of amplification greater than 1.5 at each cycle.

85. Cool a 96-well plate on a plate cooler and add 14 μL of the premixture followed by 2 μL of the purified DNA to each well.

Note: Keep the plate cold during preparation of the reaction mixture with the use of a plate cooler so as to avoid evaporation.

Note: When adding DNA to the wells, be careful not to lose track of those to which it has been added. Use of a printed plate layout, on which the wells to which the DNA has been added can be checked off, is recommended.

86. Seal the plate with a seal compatible with qPCR.

Note: Before transferring the plate to the qPCR machine, wipe the surface of the seal to remove dirty spots such as hand grease. Given that the qPCR instrument measures fluorescence through the seal, any material on the seal that is in the light path can affect the fluorescence intensity.

87. Vortex the plate briefly and centrifuge at 100 × *g* for a few seconds at room temperature.
88. Perform the qPCR reaction with an appropriate program (see examples below).

Example of qPCR reaction program for THUNDERBIRD® SYBR® qPCR Mix

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	30 s	1
Denaturation	95°C	5 s	40
Annealing + Extension	60°C	30 s	

Example of melting curve analysis

Step	Target temperature	Incubation time	Temperature transition rate (°C/s)	Acquisition mode
1	95°C	15 s	20	NONE
2	65°C	15 s	20	NONE
3	95°C	0 s	0.20	CONT

Note: Given that the precise qPCR program will differ depending on the reagents used, check the protocol recommended by the product manufacturer.

Note: A melting curve analysis at the end of the program is essential to confirm that only a single specific product was formed. If two or more peaks are obtained in the melting curve analysis, the reaction should be performed again or the qPCR primer set should be redesigned.

89. Calculate the relative concentration of the target DNA for all reactions for each primer pair with the use of the corresponding standard curve (Roche software will do this automatically).

Note: We recommend use of the second derivative max method for calculation of the Cp (Ct) value.

90. Export the data to the ''.txt'' format and calculate the average of relative concentration of DNA for each sample from two or more replicates.

Q, the average value of relative concentration of each sample; *q*, the value of relative concentration of input sample; *q'*, the value of relative concentration of immunoprecipitate with H2Aub antibody, *n*: the number of qPCR replicates

$$Q_{total} = \frac{(q_1 + q_2 + \cdots + q_n)}{n},\ Q_{H2Aub} = \frac{(q'_1 + q'_2 + \cdots + q'_n)}{n}$$

91. Given that the total input sample was diluted, correct the relative concentration value of each reaction by multiplying by the dilution factor.

I, corrected value of qPCR result of input sample; *D*, dilution factor (e.g., If the equal volume of ddH2O is added to the input sample, the dilution factor is calculated as ''2''.)

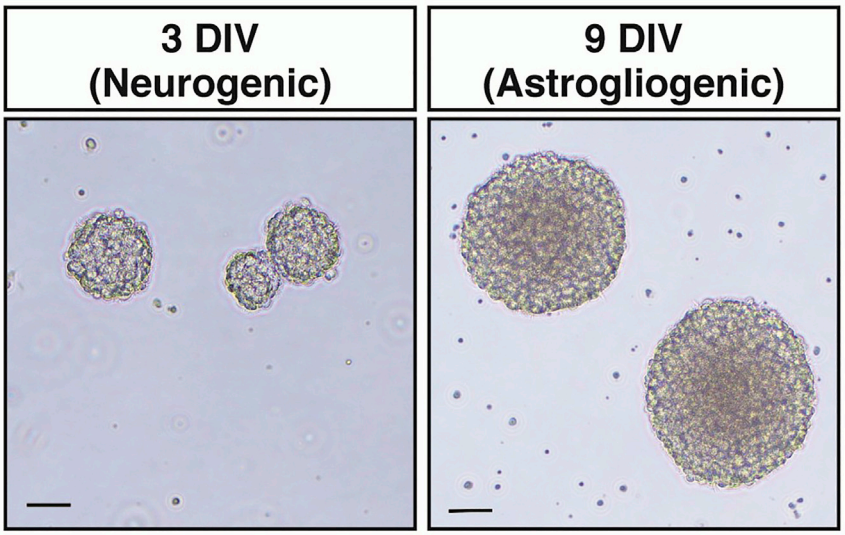

Figure 1. Mouse neocortical NPCs cultured as neurospheres for ChIP-qPCR analysis

NPCs isolated from the mouse neocortex at E11.5 were cultured in the presence of FGF2 and EGF for 3 or 9 DIV and then imaged by phase-contrast microscopy. Scale bars, 50 μm.

$$I_{total} = Q_{total} \times D$$

Note: If ChIP is performed with several antibodies in addition to anti-H2Aub antibody, the input sample will be used for qPCR reaction many times. In this case, it is recommended the input samples be diluted to 2–5 times with ddH2O.

92. Calculate the percent of input by dividing the value for each immunoprecipitate by that for its corresponding input.

r, The ratio of the volume of input samples to that of immunoprecipitated samples

$$[\%\ of\ input] = \frac{(Q_{H2Aub} \div r)}{I_{total}} \times 100$$

Note: Q_{H2Aub} must be divided by "*r*" when *r*-fold amount of the solution to input sample is used for immunoprecipitation of H2Aub antibody at step 61.

EXPECTED OUTCOMES

Progressive neuronal fate restriction of neocortical NPC cultures

Neocortical cells isolated from mouse embryos at E11.5 and cultured in suspension with FGF2 and EGF for 3 or 9 DIV form spherical aggregates known as neurospheres (Figure 1). Under these culture conditions, NPCs undergo a progressive neuronal fate restriction. Reverse transcription (RT) and qPCR analysis (Kishi and Gotoh, 2021) should show that the expression of neurogenic genes *Neurog1*, *Fezf2*, *Lef1* and *Tcfap2c* is down-regulated, whereas that of the astrogliogenic gene for glial fibrillary acidic protein (*Gfap*) is up-regulated, at 9 DIV compared with 0 or 3 DIV (Tsuboi et al., 2018) (Figure 2).

Specific enrichment of H2Aub at PRC1 target genes

A successful H2Aub ChIP assay should reveal clear enrichment of the modified histone at PRC1 target gene loci relative to non–PRC1 target gene loci and intergenic regions. Obtained signal values (% of input) for H2Aub vary depending on immunoprecipitation efficiency (which is affected by culture conditions, antibody titer, fixation conditions, and the extent of DNA shearing, among other factors). Comparison of the signal values for PRC1 target gene loci with those for non–PRC1

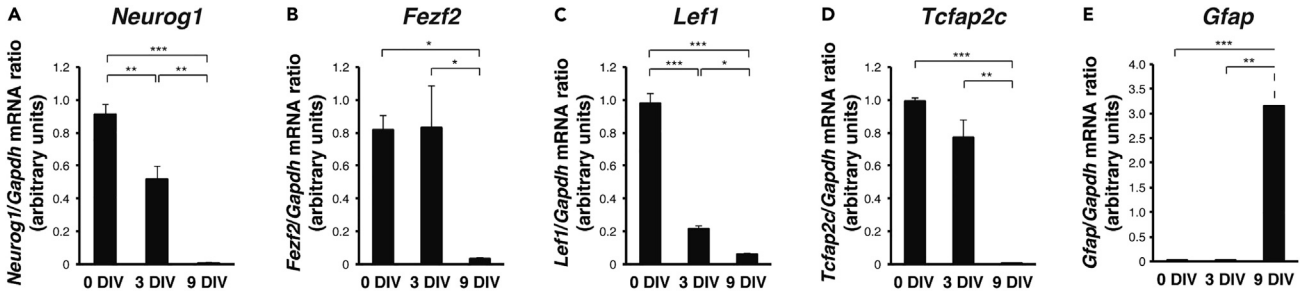

Figure 2. Confirmation of the neurogenic to astrogliogenic fate switch in mouse neocortical NPCs by RT-qPCR analysis

NPCs isolated from the mouse neocortex at E11.5 were cultured with FGF2 and EGF for 0, 3, or 9 DIV, after which the amounts of *Neurog1* (A), *Fezf2* (B), *Lef1* (C), *Tcfap2c* (D), and *Gfap* (E) mRNAs were measured by RT-qPCR analysis (Tsuboi et al., 2018). Data are means ± SEM from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Tukey-Kramer's test).

target gene loci provides an indication of whether H2Aub ChIP was successful or not (for example, signal values for the *Fezf2* locus in NPCs at 9 DIV should be ~10 times those for the *Gapdh* locus). An example of H2Aub levels at four PRC1 targets (*Fezf2*, *Lef1*, *Tcfap2c* and *Cdkn2d*) and a negative control gene (*Gapdh* and *Cdkn1a*) in NPCs at 9 DIV is shown in Figure 3.

Increase in H2Aub at neurogenic gene loci during development

Successful NPC culture and H2Aub ChIP should show an increase in the level of H2Aub specifically at neurogenic gene loci during neuronal fate restriction. Such an increase should not be observed at negative control loci (such as *Gapdh* and *Cdkn1a*) or other PRC1 target gene loci (such as *Cdkn2d*, *Cdkn1c* and *Dlk1*). An example of developmental changes in the amount of H2Aub at neurogenic gene loci (*Fezf2*, *Lef1* and *Tcfap2c*) as well as at other PRC1 targets (*Cdkn2d*, *Cdkn1c* and *Dlk1*) and non–PRC1 targets (*Gapdh* and *Cdkn1a*) gene loci in NPCs is shown in Figure 4.

LIMITATIONS

This protocol reveals changes in histone modification patterns in cortical NPCs at various developmental time points with a highly favorable signal-to-noise ratio. However, there are several technical limitations to the protocol.

In addition to H2Aub, we tried to apply this protocol to ChIP-qPCR analysis of trimethylated (H3K27me3) or acetylated (H3K27ac) lysine-27 of histone H3 mediated by PRC2 and histone acetyltransferase complexes, respectively (Tsuboi et al., 2018). However, in those cases, the signal-to-noise ratios were not as high as that for H2Aub. Optimization of the protocol may thus be required for ChIP-qPCR analysis of certain other histone modifications.

We optimized the ratio between the number of cells and the amount of antibodies to H2Aub and were able to minimize both in the described protocol. However, use of fewer than 1×10^5 cells per immunoprecipitation may result in a loss of signal intensity and a consequent poor signal-to-noise ratio.

TROUBLESHOOTING

Problem 1

Neurospheres are not well dissociated by the mechanical dissociation with a disposable pipette at step 25.

Potential solution

There are several potential causes of this problem. First, the residual medium in the tube at step 18 may inhibit the enzymatic activity of trypsin. Therefore, remove as much of the supernatant as possible after centrifugation. Second, large aggregates formed by neurospheres are difficult to be dissociated. Increase the number of times of pipetting with disposable pipette during the incubation of trypsin at step 21, or in the mechanical dissociation at step 25.

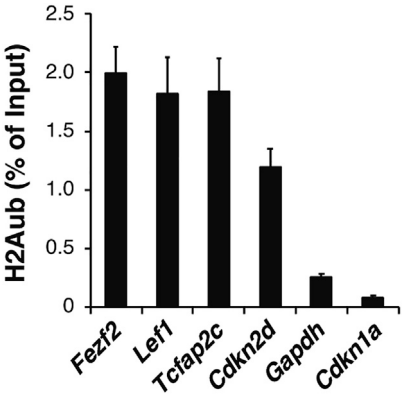

Figure 3. Confirmation of the quality of H2Aub ChIP

NPCs isolated from the mouse neocortex at E11.5 were cultured with FGF2 and EGF for 9 DIV, after which ChIP-qPCR analysis of H2Aub was performed with primers specific for *Fezf2*, *Lef1*, *Tcfap2c*, *Cdkn2d*, *Gapdh* and *Cdkn1a* loci. Data are means ± SEM from three independent experiments.

Problem 2

Neocortical NPCs do not form neurospheres in suspension culture at step 18 and at step 28.

Potential solution

There are several potential causes of this problem. First, it may be a result of the dissociation of previously formed neurospheres with trypsin. NPCs are sensitive to mechanical dissociation with a disposable pipette. If the pipette is pushed too forcefully to the bottom of the 15-mL tube, the NPCs may experience membrane damage. Conversely, if the pipette is not pushed with enough force, the cells will not dissociate sufficiently. Appropriate mechanical force should be determined by checking for membrane-damaged cells by staining with trypan blue as well as for remaining small cell aggregates in the cell counting chamber when counting cell number. Second, variability between lots of B-27, FGF2, and EGF can affect the growth or survival rate of NPCs, which should be examined for two or three lots by quantification of cell number at each dissociation step.

Problem 3

NPCs do not manifest the neurogenic to astrogliogenic fate switch during *in vitro* culture at steps 29 and 92.

Potential solution

Several growth factor or cytokine signaling pathways—including those triggered by EGF, FGF, leukemia inhibitory factor (LIF), and bone morphogenetic protein (BMP)—as well as cell-cell signal transduction (such as that mediated by Notch-Delta) have been implicated in the fate switch of neocortical NPCs (Hirabayashi and Gotoh, 2010; Miller and Gauthier, 2007). Dissociation of NPCs into single cells and expansion of neurospheres from single-cell clones are thought to be important for the proper fate switch of NPC cultures (Coles-Takabe et al., 2008; Jessberger et al., 2007; Singec et al., 2006). It should therefore be examined whether the neocortical tissue and neurospheres are dissociated into single cells after each dissociation step (at steps 8c and 25).

Problem 4

No obvious cell pellet of NPCs is observed after centrifugation at steps 15 and 32.

Potential solution

Even if prelubricated microcentrifuge tubes are used, it occasionally occurs that dissociated NPCs stick to the wall of tubes, resulting in no obvious cell pellet at the bottom of the tube. In this case, coat the inner surface of prelubricated microcentrifuge tubes with PBS containing 0.5% BSA before centrifugation to ameliorate this problem.

Problem 5

Significant amplification is observed in the negative control sample (only ddH2O is added) in the qPCR reaction at step 92.

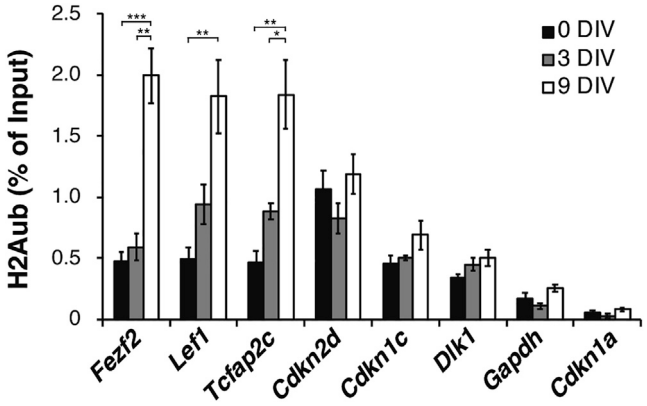

Figure 4. Increase in the level of H2Aub at neurogenic gene loci in astrogliogenic NPCs

NPCs isolated from the mouse neocortex at E11.5 were cultured with FGF2 and EGF for 0, 3, or 9 DIV, after which ChIP-qPCR analysis of H2Aub was performed with primers specific for *Fezf2*, *Lef1*, *Tcfap2c*, *Cdkn2d*, *Cdkn1c*, *Dlk1*, *Gapdh* and *Cdkn1a* loci. Data are means ± SEM from three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Tukey-Kramer's test).

Potential solution

Such amplification can be caused by the contamination with mouse genomic DNA. Therefore, it is recommended to renew the water used for the preparation of qPCR reaction mixtures. Moreover, if possible, it is recommended to use dedicated micropipettes, reagents, and a bench for performing qPCR (and related experiments). If this is difficult, clean the bench and equipments thoroughly before preparing the reagents.

Problem 6

The ratio of the signal intensity for H2Aub at neurogenic gene loci to that at non–PRC1 targets is not sufficiently high at step 92.

Potential solution

The degree of chromatin shearing is likely not appropriate. If chromatin fragments are oversheared and too small (<100 bp), they may not be detected by qPCR primer pairs that flank targeted genomic regions of >100 bp. If background enrichment at negative control regions is high, it is possible that chromatin shearing is insufficient and chromatin fragments are too large. The extent of chromatin shearing should be checked by agarose gel electrophoresis or with the use of a bioanalyzer. It is also possible that the ratio between the number of cells and the antibody amount is not appropriate. If the amount of H2Aub relative to that of anti-H2Aub is too high, the antibody amount will be rate limiting and the signal-to-noise ratio will be adversely affected. Optimization of the ratio of cell number to antibody amount is thus required to obtain a high signal-to-noise ratio for ChIP-qPCR.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Masafumi Tsuboi (tsuboi@chembio.t.u-tokyo.ac.jp).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This study did not generate any unique data sets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.T. and Y.G.; investigation, M.T.; writing—original draft, M.T.; writing—review and editing, Y.G.; funding acquisition, M.T. and Y.G.; supervision, Y.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Albert, M., Kalebic, N., Florio, M., Lakshmanaperumal, N., Haffner, C., Brandl, H., Henry, I., and Huttner, W. (2017). Epigenome profiling and editing of neocortical progenitor cells during development. EMBO J. *36*, 2642–2658. https://doi.org/10.15252/embj.201796764.

Blackledge, N.P., Fursova, N.A., Kelley, J.R., Huseyin, M.K., Feldmann, A., and Klose, R.J. (2020). PRC1 catalytic activity is central to polycomb system function. Mol. Cell *77*, 857–874.e9. https://doi.org/10.1016/j.molcel.2019.12.001.

Bonev, B., Cohen, N., Szabo, Q., Fritsch, L., Papadopoulos, G., Lubling, Y., Xu, X., Lv, X., Hugnot, J., Tanay, A., and Cavalli, G. (2017). Multiscale 3D genome rewiring during mouse neural development. Cell *171*, 557. https://doi.org/10.1016/j.cell.2017.09.043.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039–1043. https://doi.org/10.1126/science.1076997.

Coles-Takabe, B.L., Brain, I., Purpura, K.A., Karpowicz, P., Zandstra, P.W., Morshead, C.M., and van der Kooy, D. (2008). Don't look: growing clonal versus nonclonal neural stem cell colonies. Stem Cells *26*, 2938–2944. https://doi.org/10.1634/stemcells.2008-0558.

de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M., et al. (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev. Cell *7*, 663–676. https://doi.org/10.1016/j.devcel.2004.10.005.

Elderkin, S., Maertens, G.N., Endoh, M., Mallery, D.L., Morrice, N., Koseki, H., Peters, G., Brockdorff, N., and Hiom, K. (2007). A phosphorylated form of Mel-18 targets the Ring1B histone H2A ubiquitin ligase to chromatin. Mol. Cell *28*, 107–120. https://doi.org/10.1016/j.molcel.2007.08.009.

Endoh, M., Endo, T.A., Endoh, T., Isono, K., Sharif, J., Ohara, O., Toyoda, T., Ito, T., Eskeland, R., Bickmore, W.A., et al. (2012). Histone H2A mono-ubiquitination is a crucial step to mediate PRC1-dependent repression of developmental genes to maintain ES cell identity. PLoS Genet. *8*, e1002774. https://doi.org/10.1371/journal.pgen.1002774.

Entrevan, M., Schuettengruber, B., and Cavalli, G. (2016). Regulation of genome architecture and function by polycomb proteins. Trends Cell Biol. *26*, 511–525. https://doi.org/10.1016/j.tcb.2016.04.009.

Hirabayashi, Y., and Gotoh, Y. (2005). Stage-dependent fate determination of neural precursor cells in mouse forebrain. Neurosci. Res. *51*, 331–336. https://doi.org/10.1016/j.neures.2005.01.004.

Hirabayashi, Y., and Gotoh, Y. (2010). Epigenetic control of neural precursor cell fate during development. Nat. Rev. Neurosci. *11*, 377–388. https://doi.org/10.1038/nrn2810.

Jessberger, S., Clemenson, G.D., and Gage, F.H. (2007). Spontaneous fusion and nonclonal growth of adult neural stem cells. Stem Cells *25*, 871–874. https://doi.org/10.1634/stemcells.2006-0620.

Kishi, Y., and Gotoh, Y. (2021). Isolation of genetically manipulated neural progenitors and immature neurons from embryonic mouse neocortex by FACS. STAR Protoc. *2*, 100540. https://doi.org/10.1016/j.xpro.2021.100540.

McGinty, R.K., Henrici, R.C., and Tan, S. (2014). Crystal structure of the PRC1 ubiquitylation module bound to the nucleosome. Nature *514*, 591–596. https://doi.org/10.1038/nature13890.

Miller, F.D., and Gauthier, A.S. (2007). Timing is everything: making neurons versus glia in the developing cortex. Neuron *54*, 357–369. https://doi.org/10.1016/j.neuron.2007.04.019.

Min, J., Zhang, Y., and Xu, R.M. (2003). Structural basis for specific binding of Polycomb chromodomain to histone H3 methylated at Lys 27. Genes Dev. *17*, 1823–1828. https://doi.org/10.1101/gad.269603.

Qian, X., Shen, Q., Goderie, S.K., He, W., Capela, A., Davis, A.A., and Temple, S. (2000). Timing of CNS cell generation: a programmed sequence of neuron and glial cell production from isolated murine cortical stem cells. Neuron *28*, 69–80. https://doi.org/10.1016/s0896-6273(00)00086-6.

Singec, I., Knoth, R., Meyer, R.P., Maciaczyk, J., Volk, B., Nikkhah, G., Frotscher, M., and Snyder, E.Y. (2006). Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. Nat Methods *3*, 801–806. https://doi.org/10.1038/nmeth926.

Stock, J.K., Giadrossi, S., Casanova, M., Brookes, E., Vidal, M., Koseki, H., Brockdorff, N., Fisher, A.G., and Pombo, A. (2007). Ring1-mediated ubiquitination of H2A restrains poised RNA polymerase II at bivalent genes in mouse ES cells. Nat. Cell Biol. *9*, 1428–1435. https://doi.org/10.1038/ncb1663.

Tamburri, S., Lavarone, E., Fernández-Pérez, D., Conway, E., Zanotti, M., Manganaro, D., and Pasini, D. (2020). Histone H2AK119 mono-ubiquitination is essential for polycomb-mediated transcriptional repression. Mol. Cell *77*, 840–856.e5. https://doi.org/10.1016/j.molcel.2019.11.021.

Tsuboi, M., Kishi, Y., Yokozeki, W., Koseki, H., Hirabayashi, Y., and Gotoh, Y. (2018). Ubiquitination-independent repression of PRC1 targets during neuronal fate restriction in the developing mouse neocortex. Dev. Cell *47*, 758. https://doi.org/10.1016/j.devcel.2018.11.018.

Wang, H., Wang, L., Erdjument-Bromage, H., Vidal, M., Tempst, P., Jones, R.S., and Zhang, Y. (2004a). Role of histone H2A ubiquitination in Polycomb silencing. Nature *431*, 873–878. https://doi.org/10.1038/nature02985.

Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004b). Hierarchical recruitment of polycomb group silencing complexes. Mol. Cell *14*, 637–646. https://doi.org/10.1016/j.molcel.2004.05.009.

Yu, J.R., Lee, C.H., Oksuz, O., Stafford, J.M., and Reinberg, D. (2019). PRC2 is high maintenance. Genes Dev. *33*, 903–935. https://doi.org/10.1101/gad.325050.119.