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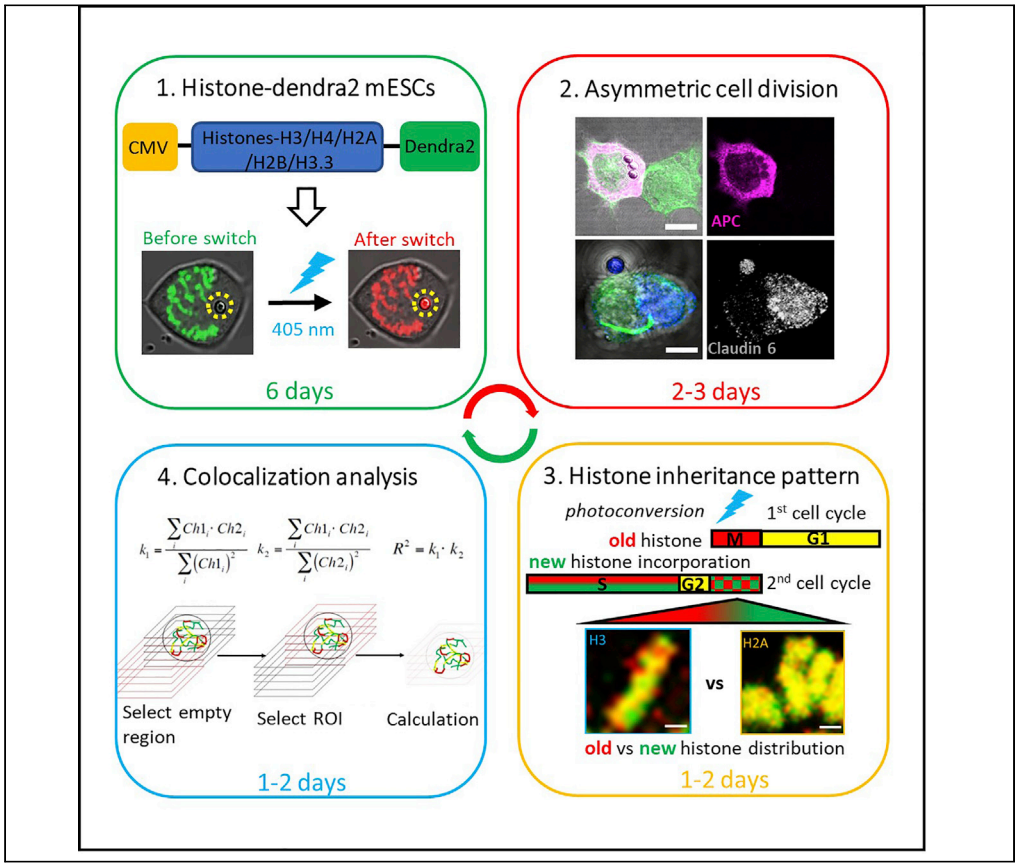
Protocol

Protocol for Establishing Mouse Embryonic Stem Cells to Study Histone Inheritance Pattern at Single-Cell Resolution



Asymmetric histone inheritance can regulate cell-fate determination in *Drosophila* male germline stem cells. However, it remains elusive how this mechanism may be used in mammalian system. Recently, we show mouse embryonic stem cells (mESCs) with Wnt3a beads display non-overlapping H3/H4 patterns. Here, we present a detailed protocol for tracking histone inheritance in asymmetrically dividing mESCs at single-cell resolution. This protocol will establish a new system to study histone inheritance in cultured mammalian cells and could be applied to other parallel systems.

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HIGHLIGHTS

Induction of asymmetric division of mESCs with Wnt3a-coated beads in cell culture

Study of histone inheritance in asymmetrically dividing mESCs at single-cell level

Optimized three-dimensional measurement for colocalization between old and new histones

Compatibility with other Wnt3a-responsive cultured cells or in tissues *in vivo*

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Protocol for Establishing Mouse Embryonic Stem Cells to Study Histone Inheritance Pattern at Single-Cell Resolution

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SUMMARY

Asymmetric histone inheritance can regulate cell-fate determination in *Drosophila* male germline stem cells. However, it remains elusive how this mechanism may be used in mammalian system. Recently, we show mouse embryonic stem cells (mESCs) with Wnt3a beads display non-overlapping H3/H4 patterns. Here, we present a detailed protocol for tracking histone inheritance in asymmetrically dividing mESCs at single-cell resolution. This protocol will establish a new system to study histone inheritance in cultured mammalian cells and could be applied to other parallel systems.

For complete details on the use and execution of this protocol, please refer to Tran et al. (2012), Habib et al. (2013), Lowndes et al. (2017), and Ma et al. (2020).

BEFORE YOU BEGIN

To perform this experiment, you need to prepare all the stock solutions for the culture media of mouse embryonic stem cells (mESCs) culture and mouse epiblast stem cell (mEpiSC) induction, as well as related biochemical assays.

Preparation for Culturing mESCs

Timing: 4–5 days

1. Preparation of Media and Buffers: Refer to "Materials and Equipment" for more details.

2. Thawing the E14TG2a mESCs:
   a) Coat the 24 well plates with 250 μL of 0.2% Gelatin/well and leave in the incubator for 12–16 h.

   Note: Gelatin coating can also be prepared 2 h before you start to thaw the cells.
   b) Prewarm the mESCs culture medium at 20°C–25°C for 15 min.
   c) Thaw a cryovial of mESCs (containing 5 × 10^5 cells) in 37°C water bath with gently rocking (1–2 min), and then transfer to 4 mL prewarmed medium in 15 mL conical centrifuge tube.

   Note: Slow addition is critical for maintaining high viability during the defreezing process, so we recommend to add the prewarmed medium in dropwise method.
   d) Centrifuge the cell suspension at 1,200 × *g* for 5 min at 20°C–25°C, aspirate supernatant, and resuspend the cell pellet in 1 mL fresh mESCs medium.

e) Aspirate the pre-coated gelatin solution, suspend the cells with an appropriate density (500 μL culture medium with 0.5–1 × 10^5 cells per well for 24 well plate).

3. mESCs are cultured at 37°C with 5% CO_2 on gelatin-coated plates. Normally the culture medium needs to be replaced every 24 h, and passaged every 2–3 days.

Note: The routine mycoplasma testing was mainly through DNA staining (Hoechst 33342) or PCR amplifying bacterial DNA. This step is essential to maintain batch-to-batch consistency of mESCs, which is critical for the following differentiation and asymmetric cell division (ACD) induction assay.

Passage for Culturing mESCs

Timing: ~0.5 h

4. Preparation of cell digestion buffer of 0.05% Trypsin according to the "Preparation of Media and Buffers."
5. Wash the culturing mESCs with prewarmed 1× PBS (without Ca^{2+} and Mg^{2+}) for twice, gently aspirate the medium.

Note: It is critical to wash the culturing mESCs with 1× PBS (without Ca^{2+} and Mg^{2+}). First, the FBS in the remanent culture medium should be washed away because it will compromise the digestion activity of trypsin. Second, the PBS buffer should be without Ca^{2+} and Mg^{2+}, because EDTA of cell digestion buffer can compromise the digestion activity of trypsin through chelating Ca^{2+} and Mg^{2+}.

6. Add 100 μL of 0.05% Trypsin to each well/24 well plate, then transfer to 37°C incubator for digesting 1–2 min.
7. Use brightfield microscopy to check the mESCs colonies until they become loosen, then quickly add 200 μL fresh culture medium to stop the trypsin digestion.
8. Pipette the cell suspension gently to physically dissociate the cell colonies or aggregates.
9. Centrifuge the cell suspension at 1,200 × *g* for 5 min at 20°C–25°C, aspirate supernatant, then passage the cell suspension at a 1:10 ratio to new wells.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-APC (used at 1:150)	Santa Cruz	Cat# sc-7930; RRID: AB_2242804
Goat polyclonal anti-Claudin 6 (used at 1:150)	Santa Cruz	Cat# sc-17669; RRID: AB_2083279
Rabbit polyclonal anti-Sox2 (used at 1:200)	Active Motif	Cat# Active Motif 39823; RRID: AB_2793356
Rat monoclonal anti-Nanog (used at 1:200)	Active Motif	Cat# Active Motif 61627; RRID: AB_2793709
Rabbit monoclonal anti-Oct4 (used at 1:200)	Abcam	Cat# ab181557; RRID: AB_2687916
Rabbit polyclonal anti-Rex1 (used at 1:200)	Thermo Fisher	Cat# PA5-27567; RRID: AB_2545043
Mouse monoclonal anti-SSEA-1 (used at 1:50)	Thermo Fisher	Cat# MC-480; RRID: AB_528475

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rabbit polyclonal anti-Histone H3 (used at 1:4,000)	Abcam	Cat# ab1791; RRID: AB_302613
Rabbit polyclonal anti-Histone H4 (used at 1:4,000)	Abcam	Cat# ab10158; RRID: AB_296888
Rabbit monoclonal anti-Histone H2A (used at 1:3,000)	Abcam	Cat# ab18975; RRID: AB_444713
Mouse monoclonal anti-Histone H2B (used at 1:3,000)	Abcam	Cat# ab52484; RRID: AB_1139809
Rabbit monoclonal anti-Histone H3.3 (used at 1:4,000)	Abcam	Cat# ab176840; RRID: AB_2715502
Rabbit monoclonal anti-GAPDH (used at 1:3,000)	Abcam	Cat# ab181602; RRID: AB_2630358
Alexa Fluor-conjugated series (used at 1:600)	Thermo Fisher	N/A
Goat anti-Rabbit IgG(H+L) HRP	Sungene	Cat# LK2001L
Goat anti-Mouse IgG(H+L) HRP	Sungene	Cat# LK2003L
Chemicals and Recombinant Proteins		
Dulbecco's modified Eagle's solution	Thermo Fisher	Cat# 10829018
DMEM (1×) [+] 4.5 g/L D-Glucose, L-Glutamine (LS/L cells)	Gibco	Cat# 41966-029
Neurobasal Medium	Thermo Fisher	Cat# 21103049
DMEM/F-12	Thermo Fisher	Cat# 11320033
PD032501	Axon Medchem	Cat# Axon 1408
CHIR99021	Axon Medchem	Cat# Axon 1386
Activin A, Recombinant Human Protein	Thermo Fisher	Cat# PHC9564
bFGF, Recombinant Human Protein	Thermo Fisher	Cat# 13256029
Recombinant Mouse Wnt3a protein	R&D Systems	Cat# 1324-WN-002
Fetal Bovine Serum, heat inactivated (FBS)	Gibco	Cat# 10082-147
Fetal Bovine Serum (LS/L cells)	Sigma-Aldrich	Cat# F7524
MEM non-essential amino acids	Thermo Fisher	Cat# 11140050
Sodium Pyruvate	Thermo Fisher	Cat# 11360070
Recombinant mouse LIF	Millipore	Cat# ESG1107
β-mercaptoethanol	Thermo Fisher	Cat# 21985023
GlutaMAX	Thermo Fisher	Cat# 35050061
Trypsin-EDTA (0.25%)	Gibco	Cat# 25200056
2% Gelatin Solution	Merck	Cat# G1393-20Ml
7.5% BSA	Thermo Fisher	Cat# 15260037
N2-Supplement	Thermo Fisher	Cat# 17502048
B27-Supplement	Thermo Fisher	Cat# 17504044
Fibronectin human plasma	Merck	Cat# F2006-1MG
Normal Donkey Serum	Merck	Cat# D9663
Lipofectamine 2000	Invitrogen	Cat# 12566014
Nocodazole	Sigma	Cat# M1404
RNase A	TIANGEN	Cat# RT405-02
Dimethyl sulfoxide (DMSO)	Merck	Cat# D2650
Vectashield mounting medium	Vector	Cat# H1200

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
RIPA Lysis and Extraction buffer	Thermo Fisher	Cat# 89901
Protease Inhibitor Cocktail	Beyotime	Cat# P1005
Phosphatase inhibitor cocktail A	Beyotime	Cat# P1081
Penicillin-Streptomycin	Thermo Fisher	Cat# 15140122
Penicillin-streptomycin (LS/L cells)	Sigma-Aldrich	Cat# P4458
Experimental Models: Cell Lines		
E14TG2a embryonic stem cells	Hooper et al., 1987	N/A
Histone-Dendra2 transgenic mESC lines	Ma et al., 2020	N/A
Software and Algorithms		
MATLAB	MathWorks	https://www.mathworks.com/products/matlab.html
FlowJo	FlowJo LLC	https://www.flowjo.com/
ImageJ	NIH	https://imagej.nih.gov/ij/
Huygens	Scientific Volume Imaging	The Netherlands, http://svi.nl
GraphPad Prism 5.0	GraphPad	http://www.graphpad.com/scientific-software/prism/
Mendeley data-additional supplemental data including figures, tables, and movie	Ma et al., 2020	https://data.mendeley.com/datasets/pc8z47jzm5/draft?a=7e80bf22-2723-423b-8bea-45e629c5ec8
Other		
Dendra2-H3.3-N-14 vector	Addgene	Plasmid# 57725
Dendra2-H3-N-14 vector	Ma et al., 2020	Plasmid# 157814
Dendra2-H4-N-14 vector	Ma et al., 2020	Plasmid# 157815
Dendra2-H2A-N-14 vector	Ma et al., 2020	Plasmid# 157816
Dendra2-H2B-N-14 vector	Ma et al., 2020	Plasmid# 157817
HindIII-HF	NEB	Cat# R3104S
BamH I	NEB	Cat# R0136S
Taq 2X Master Mix	NEB	Cat# B0270L
T4 ligase buffer	NEB	Cat# B0202S
T4 ligase	NEB	Cat# M0202S
DH5-alpha competent *E. coli*	NEB	Cat# C2987I
QIAGEN Plasmid Mini Kit	QIAGEN	Cat# 12123
QiaQuick gel extraction kit	QIAGEN	Cat# 28704
GenElute Mammalian Genomic DNA Miniprep Kit	Merck	Cat# G1N10-1KT
Alkaline Phosphatase Detection Kit	Millipore	Cat# SCR004
Immobilon Western chemiluminescence kit	Millipore	Cat# P90720
Pierce BCA Protein Assay Kit	Thermo Fisher	Cat# 23225
Immun-Blot PVDF Membrane	Bio-Rad	Cat# 1620177
Fluorodish	WPI, Inc.	Cat# FD3510-100
Falcon® 35 mm TC-treated Easy-Grip Style Cell Culture Dish	Corning	Cat# 353001
Corning® Costar® TC-Treated 24 Well Plates	Corning	Cat# CLS3527
Freezing Container	Thermo Scientific Nalgene	Cat# C1562

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
NaCl	Sigma-Aldrich	Cat# S7653-1KG
CHAPS (detergent)	Thermo Fischer	Cat# 28299
M-270 Dynabeads	Invitrogen	Cat# 14305D
Dual-Light Luciferase and β-Galactosidase Reporter Gene Assay System Kit	Thermo Fisher Scientific	Cat# T1005
Oligonucleotides		
mH3 F: CTCAAGCTTCCGCGGCGCCACCA TGGCTCGTACTAAGCAGAC	IDT	N/A
mH3 R: GGTGGATCCCCACTGCCTGCGCTT CCGGACCCAGCCCGCTCGCCG CGGATAC	IDT	N/A
mH4F: CTCAAGCTTCCGCGGCGCCACCAT GTCTGGCAGAGGAAAGG	IDT	N/A
mH4R: GGTGGATCCCCACTGCCTGCG CTTCCGGACCCGCCTCCGAAGCC GTAGAGG	IDT	N/A
mH2aF: CTCAAGCTTCCGCGGCGCCACCA TGTCTGGACGCGGCAAGCA	IDT	N/A
mH2a R: GGTGGATCCCCACTGCCTGCGC TTCCGGACCCCTTCCCCTTGGCCT TGTGGT	IDT	N/A
mH2b F: CTCAAGCTTCCGCGGCGCCACC ATGCCAAGCCTTCTAAGTC	IDT	N/A
mH2b R: GGTGGATCCCCACTGCCTGCGC TTCCGGACCCCTTGGAGCTGG TGTACTTGG	IDT	N/A

MATERIALS AND EQUIPMENT

Stock Solutions

- Wnt3a Stock Solution

Reagent	Final Concentration	Amount
Wnt3a	40 μg/mL	2 μg
PBS 1×	N/A	50 μL
BSA	0.1% (w/v)	0.05 μg
Total	**N/A**	**50 μL**

N/A – not applicable

⚠ CRITICAL: Soluble Wnt3a proteins should be stored at 4°C and be tested periodically (we test it every one month and track the activity), using reporter cell lines such as LS/L assay, please refer to (Lowndes et al., 2017) for details.

- Wnt3a vehicle buffer

Reagent	Final Concentration	Amount
CHAPS (detergent)	1%	15 g
PBS 10×	10%	150 mL
NaCl	0.5 M	43.83 g
ddH_2O	N/A	Up to 1.5 L
Total	**N/A**	**1.5 L**

N/A– not applicable

Note: Enough ddH_2O for a final volume of 1.5 L (~1.2 L) and adjust to pH 7.5.

- PD03259010 Stock Solution

Reagent	Final Concentration	Amount
PD0325901	2.5 mM	5 mg
DMSO	N/A	4.148 mL
Total	**N/A**	**4.148 mL**

N/A – not applicable

Use the 2.5 mM stock to further dilute 1:2,500 to obtain 1 μM working solution in the mESCs culture medium.

Note: Prepare 20 μL aliquots of the stock solutions and store at −20°C for up to 1 year. Avoid repeated freeze-thaw cycles.

- CHIR99021 Stock Solution

Reagent	Final Concentration	Amount
CHIR99021	3 mM	2 mg
DMSO	N/A	1.433 mL
Total	**N/A**	**1.433 mL**

N/A – not applicable

Use the 3 mM stock to further dilute 1:1,000 to obtain 3 μM working solution in the mESCs culture medium.

Note: Prepare 50 μL aliquots of the stock solutions and store at −20°C for up to 1 year. Avoid repeated freeze-thaw cycles.

- Activin A Stock Solution

Reagent	Final Concentration	Amount
Activin A	20 μg/mL	10 μg
PBS 1×	N/A	0.5 mL
BSA	0.1% (w/v)	0.5 mg
Total	**N/A**	**0.5 mL**

N/A – not applicable

Use the 20 μg/mL stock to further dilute 1:1,000 to obtain 20 ng/mL working solution in the mEpiSCs medium. Avoid repeated freeze-thaw cycles.

Note: Prepare 50 μL aliquots of the stock solutions and store at −20°C for up to 1 year. Avoid repeated freeze-thaw cycles.

- bFGF Stock Solution

Reagent	Final Concentration	Amount
BFGF	12 μg/mL	10 μg
PBS 1×	N/A	0.83 mL
BSA	0.1% (w/v)	0.83 mg
Total	**N/A**	**0.83 mL**

N/A – not applicable

Use the 12 μg/mL stock to further dilute 1:1,000 to obtain 12 ng/mL working solution in the mEpiSCs medium. Avoid repeated freeze-thaw cycles.

Note: Prepare 50 μL aliquots of the stock solutions and store at −20°C for up to 1 year.

- Gelatin Stock Solution

Reagent	Stock Concentration	Final Concentration	Amount
Gelatin	2%	0.2%	5 mL
PBS 1×	N/A	N/A	45 mL
Total	**N/A**	**N/A**	**50 mL**

Use 2% Gelatin stock to further dilute 1:10 to obtain 0.2% working solution.

Note: The surface coverage with 100–200 μg gelatin/cm^2. The working solution can be stored at 4°C for up to 1 month.

- Fibronectin Stock Solution

Reagent	Stock Concentration	Final Concentration	Amount
Fibronectin	1 mg/mL	40 μg/mL	1 mg
ddH_2O	N/A	N/A	1 mL
Total	**N/A**	**N/A**	**1 mL**

N/A – not applicable

Use the 1 mg/mL stock to further dilute 1:25 to obtain 40 ug/mL working solution.

⚠ CRITICAL: The surface coverage with 1–5 μg fibronectin/cm^2. Prepare 10 μL aliquots of the stock solutions (for five fluorodishes) and store at −80°C for up to 1 year.

Preparation of Media and Buffers

- LS/L medium

Reagent	Final Concentration	Amount
DMEM (1×) [+] 4.5 g/L D-Glucose, L-Glutamine	N/A	445 mL
FBS	10%	50 mL
Pen-Strep	1%	5 mL
Total	**N/A**	**500 mL**

Note: Filter the medium using a 0.22 μm filter, then store it in 4°C for up to 1 month.

Prewarm the medium at 20°C–25°C for at least 30 min before use.

- mESCs culture complete medium with LIF and 2i

Reagent	Final Concentration	Amount
KO-DMEM	N/A	40.9 mL
ES qualified FBS	15%	7.5 mL
Sodium Pyruvate	10 μM	0.5 mL
MEM-NEAA	1×	0.5 mL
β-mercaptoethanol	50 μM	0.05 mL
Pen-Strep	1×	0.5 mL
LIF	1,000 U/mL	0.05 mL
CHIR99021	3 μM	0.05 mL
PD0325901	1 μM	0.02 mL
Total	**N/A**	**50 mL**

Note: Filter the medium using a 0.22 μm filter, then store it in 4°C for up to 1 month.

Prewarm the medium at 20°C–25°C for at least 30 min before use.

- N2B27 Basic Medium

Reagent	Final Concentration	Amount
DMEM/F12	N/A	24.09 mL
Neurobasal medium	N/A	24.09 mL
N2 Supplement	0.5%	0.25 mL
B27 Supplement	1%	0.5 mL
BSA 7.5% Solution	0.033%	0.0165 mL
β-mercaptoethanol	10 μM	0.05 mL
Gluta-MAX	1×	0.5 mL
Pen-Strep	1×	0.5 mL
Total	**N/A**	**50 mL**

Note: Filter the basic medium using a 0.22 μm filter, then store it in 4°C for up to 1 month. Add other reagents according to different purpose. Prewarm the medium at 20°C–25°C for at least 30 min before use.

- Cell digestion buffer

Reagent	Final Concentration	Amount
0.25% Trypsin	0.05%	10 mL
PBS 1×	N/A	40 mL
Total	**N/A**	**50 mL**

N/A – not applicable

Note: Store the 0.05% Trypsin in 4°C for up to 1 month. Prewarm the solution at 20°C–25°C for at least 30 min before use.

- Fixation buffer (4% Paraformaldehyde-PFA)

Reagent	Final Concentration	Amount
Paraformaldehyde	4%	40 g
ddH_2O	N/A	1 L
Total	**N/A**	**1 L**

N/A – not applicable

Note: Stir and heat the solution at 60°C, then slowly adding 1 N NaOH dropwise until the solution becoming clear. Finally aliquot and store at −20°C or stored at 4°C for up to 1 month.

- Permeabilization buffer (PBS+0.2% Triton X-100)

Reagent	Final Concentration	Amount
Triton X-100	0.2%	100 μL
PBS 1×	N/A	50 mL
Total	**N/A**	**50 mL**

N/A – not applicable

Note: Prepare the working solution and filter with 0.22 μm filter, then store at 20°C–25°C for up to 1 month.

- Blocking buffer

Reagent	Final Concentration	Amount
Triton X-100	0.2%	200 μL
PBS 1×	N/A	100 mL
Normal donkey serum	10%	10 mL
Total	**N/A**	**100 mL**

N/A – not applicable

Note: Prepare the working solution and filter with 0.22 μm filter. Then prepare 1 mL aliquots and store them at −20°C for up to 1 year.

- Tris-Buffered Saline (TBS) 10× Stock Solution

Reagent	Final Concentration	Amount
Tris Base	0.2 M	12 g
NaCl	1.5 M	44 g
ddH_2O	N/A	500 mL
Total	**N/A**	**500 mL**

N/A – not applicable

Note: Adjust pH to 7.6 with 12 N HCl. The stock solution can be stored at 4°C for up to 3 months. Prepare the 1× working solution and put it at 20°C–25°C for use.

- RIPA Lysis and Extraction buffer

Reagent	Final Concentration	Amount
Tris-HCl	25 mM	0.39 g
NaCl	150 mM	0.88 g
NP-40	1%	1 g
SDS	0.1%	0.1 g
Sodium deoxycholate	1%	1 g
ddH_2O	N/A	100 mL
Total	**N/A**	**100 mL**

N/A – not applicable

Note: Adjust pH to 7.6 with 12 N HCl. Store this solution at 4°C for up to 1 year.

STEP-BY-STEP METHOD DETAILS

Preparation for Histone-dendra2 Plasmids

Timing: ~3 days

1. Digest the vector of Dendra2-H3.3-N-14 with Hind III and BamH I, set up the following reaction system:

Reagents	Amount
Dendra2-H3.3-N-14	3–5 μg
Hind III	1 μL
BamH I	1 μL
NEB CutSmart Buffer	3 μL
ddH_2O	up to 30 μL
Total	**30 μL**

2. Incubate the above mixture for 3 h at 37°C.
3. Run an 1% agarose gel at 100 V for about 0.5 h.
4. Harvest the backbone of Dendra2-H3.3-N-14 (4,667 bp band) with gel extraction kit.

5. Combine the following reagents to generate each of histone coding sequences for mouse H3, H4, H2A, and H2B. Here is an example with mH3:

Reagents	Amount
Genomic DNA from mESCs	<100 ng
10 μM mH3-Forward primer	0.5 μL
10 μM mH3-Reverse primer	0.5 μL
Taq 2× Master Mix	25 μL
ddH_2O	up to 50 μL
Total	**50 μL**

6. Harvest the ~456 bp PCR band using PCR extraction kit.
7. Do the digestion for mH3 PCR product for 3 h at 37°C with the following reaction system:

Reagents	Amount
mH3 PCR product	1 μg
Hind III	1 μL
BamH I	1 μL
NEB CutSmart Buffer	5 μL
ddH_2O	up to 50 μL
Total	**50 μL**

8. Harvest the ~444 bp PCR band using Gel or PCR extraction kit.
9. Do the ligation with T4 ligase for 2 h at 20°C–25°C with the following reaction system:

Reagents	Amount
Backbone of Dendra2-H3.3-N-14	50 ng
mH3 PCR product	20 ng
T4 ligase	0.5 μL
T4 ligase buffer	1 μL
ddH_2O	up to 10 μL
Total	**10 μL**

10. Mix well the reaction system, then use the following PCR reaction condition:

Step	Cycle	Temperature (°C)	Time	Repeat
1	Initial denaturation	98	3 min	
2	Denaturation	98	30 s	×35
3	Anneal	58	30 s	
4	Elongation	72	45 s	
5	Final Extension	72	5 min	
6	Hold	12	Hold	

11. Select 5 μL of ligation product for DH5-alpha competent *E. coli* (NEB) transformation according to the high efficiency transformation protocol.
12. Then pick up 3–5 single colonies from the plate with sterile inoculating loop and transfer to LB medium with 50 μg/mL of kanamycin. Incubate at 37°C for 12–16 h with continuous shaking.
13. Extract the plasmids with QIAGEN Plasmid Mini Kit, then use Sac II to digest 1 μg of H3-dendra2 plasmid for 3 h at 37°C: the plasmids which can be digested into two bands (4,676 bp +405 bp) are positive, the negative one should be linearized.
14. Send the positive colonies for Sanger sequencing with the primer CMV- Forward (CGCAAATGGGCGGTAGGCGTG).

⚠ CRITICAL: Dendra2 is a photoconvertible protein, which can switch from green to red fluorescence at the protein level. When the photoconversion occurs in mitotic phase, it will enable us to clearly distinguish old versus new histones and then trace their dynamics without concerning the transgene copy number issues.

Preparation for Histone-dendra2-Expressing mESC Lines

Timing: ~1 week

15. Histone-dendra2 mESC lines preparation: The transfection of E14TG2a mESCs was performed using Lipofectamine 2000 according to the manufacturer's instructions.
    a) Dilute 500 ng histone-dendra2 plasmids (for one well of 24 well plate) into 50 μL of Opti MEM and mix gently.
    b) Mix Lipofectamine 2000 gently before use, then dilute 2 μL Lipofectamine 2000 into 50 μL of Opti MEM. Incubate at 20°C–25°C for 5 min.
    c) After incubation, combine the diluted DNA with diluted Lipofectamine 2000 reagent (total volume 100 μL). Mix gently and incubate at 20°C–25°C for 20 min.
    d) Add 100 μL of the histone-dendra2 plasmid-lipid complex to one well of 24 well plate with WT E14 mESCs and incubate for 1–3 days at 37°C prior to checking the green dendra2 fluorescence.

Note: We usually seed the cells at a ratio of 1:5 one day prior the transfection. The cells will form the medium-sized colonies, which normally have active proliferation capacities. This will be beneficial for the incorporation of transgenes.

    e) Passage the transfected mESCs for 2–3 times to remove the transient histone-dendra2 positive cells.
    f) Purify the dendra2 positive cells with manual selection or Flow Cytometry Cell Sorting (FACS, SONY SH800S) by gating the green fluorescence of Dendra2. Before photoconversion, the excitation for dendra2 is 490 nm.

⚠ CRITICAL: The density level of Dendra2 fluorescence will affect the photoconversion efficiency, so we purify and isolate mESCs with medium expression level of Histone-Dendra2 based on the fluorescence signal. The main purpose of this step is to remove those cells with super bright or very weak signal.

Cryopreservation for Histone-dendra2-Expressing mESC Lines

Timing: ~1 day

16. Prepare the cryovials labeled with each histone-dendra2 mESC lines and passage numbers, date, etc.
17. Collect the single cell suspension for each histone-dendra2 mESC lines and check the cell density and viability with hemocytometer.

18. Centrifuge at 1,200 × *g* for 5 min at 20°C–25°C.
19. Remove the supernatant and gently loosen the pellet.
20. Prepare the freezing medium with 10% DMSO + 90% FBS. Then add the freezing medium to the cells with gently rocking.
21. Transfer the cryovials into a freezing container with isopropanol and put into −80°C for about 24 h.
22. Finally transfer the cryovials from the freezing container to liquid nitrogen.

⚠ CRITICAL: Each histone-dendra2 mESC line must be labeled clearly. When culture different histone-dendra2 mESC lines at the same time, try to separate them into different plates in case of the cross contamination.

Pause Point: All the histone-dendra2 tagged mESC lines can be stored in liquid nitrogen for long term preservation.

Note: The density should be at least 10^6 cells/mL. The viability should be higher than 80% for cryopreservation.

Induce Histone 3-dendra2-Expressing mESCs Differentiation toward mEpiSCs

Timing: ~1–5 days

23. First use 0.2% gelatin to coat the 24 well plates, then we usually seed 3,000–5,000 cells/well for the EpiSC differentiation assay.
24. Culture the Histone 3-dendra2 mESCs in complete medium with LIF and 2i for one day, you should see low density of small colonies, each with about 3–5 cells.
25. Then gently change the medium to N2B27 medium with Activin A and bFGF, try not to detach the cell colonies.
26. Culture the Histone 3-dendra2 mESCs in N2B27 medium with Activin A and bFGF for 1 day and then fix the cells with 4% paraformaldehyde (PFA) for 30 min at 20°C–25°C for subsequent ACD detection.

⚠ CRITICAL: Due to different mESCs lines, their abilities to secret Wnt proteins may be different (ten Berge et al., 2011). For example, E14 mESC line usually secrets more Wnt proteins than other mESC lines. Thus, different mESC lines may respond to exogenous Wnt proteins distinctively.

27. If you will perform alkaline phosphatase (AP) assay, continue to culture the Histone 3-dendra2 mESCs in N2B27 medium with Activin A and bFGF for 4 more days, change the medium every day.

⚠ CRITICAL: It is important to first seed the mESCs in complete medium with LIF/2i, which will help the cells attach on the plates. Normally when treating with N2B27 medium with Activin A and bFGF for about one day, the colonies with dome-shaped morphology start to become flatten.

Validation of the mESCs Markers for Histone 3-dendra2-Expressing mESCs

Timing: ~3 days

28. Immunofluorescence staining of mESCs was performed using standard procedures.
    a) Culture the mESCs in fluorodish for about 2 days and then fix them with 4% PFA at 20°C–25°C for 30 min .

b) Wash three times with 1× PBS, each for 5 min, then permeabilize with PBS+0.2% Triton X-100 at 20°C–25°C for 30 min.
c) Block the cells with blocking buffer at 20°C–25°C for 1 h.
d) Use blocking buffer to dilute the primary antibodies and then incubate cells at 4°C for 12–16 h.
e) Wash three times with PBS+0.2% Triton X-100, each for 5 min and use blocking buffer to dilute the secondary antibodies, then incubate cells in the dark at 20°C–25°C for 2 h.
f) Wash three times with PBS+0.2% Triton X-100, each for 5 min, then image using confocal microscopy.

Note: For the immunostaining with nuclear markers, we use permeabilization buffer to treat the samples; while for some membrane markers, like SSEA1, we use 1× PBS with 0.05% Tween-20 instead of 0.2% Triton X-100 in our experiment.

Cell Cycle Analysis of Histone-dendra2 mESCs

Timing: ~5 days

29. For cell cycle analysis, we culture the Histone-dendra2 mESCs in Falcon® 35 mm TC-treated Easy-Grip Style Cell Culture dishes, use WT mESCs and WT mEpiSCs as controls.
    a) To get a homogenous population of WT mEpiSC, we treat the WT mESCs with Activin A and bFGF on next day and then culture for 5 days.
    b) Then we use 0.05% Trypsin to digest each type of mESCs/mEpiSCs to single cell suspensions. Harvest the cells at the density of 10^5–10^7 cells/mL to 15 mL centrifuge tubes and wash in 1× PBS once and then fix them in 1 mL of cold 70% ethanol/tube at 4°C for 30 min.
    c) Wash the cells in 1× PBS twice. Spin at 1,200 × *g* in the centrifuge and discard the supernatant.
    d) Add 2 μL 20 mg/mL RNase A stock (100×) to 50 μL 1× PBS with cells/tube and incubate at 20°C–25°C for 5 min.
    e) Then add 50 μL propidium iodide-PI (1 mg/mL stock solution) to 450 μL RNase A/1× PBS/cell suspension, incubate for 10 min in the dark.
    f) Measure the cell cycle of mESCs carrying different histone-dendra2 transgenes with BD FACS Verse machine, with WT mESCs and WT mEpiSCs as controls.
    g) The exported data were analyzed with FlowJo software (Dean and Jett, 1974). Specifically, to launch the cell cycle platform, select the target samples with gated population, where you have gated out the dead cells and debris. Then choose "cell cycle analysis" from the "Workspace" menu. With the same setting and parameters, FlowJo can generate the parallel results for cell cycle data of different target samples.

⚠ CRITICAL: To acquire good FACS result, make sure the cells are in good condition. First try to reduce the dead cells and cell debris because their autofluorescence will interfere the cell sorting; Second try to digest to the single cells without clumping, then use 40 μm strainers to filter the cell suspensions because those cell clusters will clog the instrument.

Detection of the Histone Transgenes Expression Level

Timing: ~1 days

30. For the histone transgene expression analysis, we culture the Histone-dendra2 mESCs in Falcon® 35 mm TC-treated Easy-Grip Style Cell Culture dishes, use WT mESCs as control.
    a) The cells are lysed in RIPA Lysis and Extraction buffer with 1× Protease Inhibitor Cocktail and 1× Phosphatase inhibitor cocktail A.

Note: Normally one 35 mm dish of cells need 1 mL RIPA buffer for complete lysis. We recommend to first pipette the RIPA buffer to detach the cells and then collect the suspension to 1.5 mL centrifuge tube. Finally use vortex mixer for homogenization.

b) The total protein levels in mESCs with or without histone-dendra2 are quantified using the Pierce BCA Protein Assay Kit.

c) Use 10 μg of protein for 10% SDS-polyacrylamide gel electrophoresis, run the gel with constant voltage (100 V) for approximately 1 h until the dye front runs to the bottom of the gel.

d) Followed by transferring the protein to Immun-Blot PVDF Membrane.

e) After blocking in 3% bovine serum albumin in 1× TBS with 0.05% Tween-20 (TBST), the membranes are incubated with Rabbit polyclonal anti-Histone H3, Rabbit polyclonal anti-Histone H4, Rabbit monoclonal anti-Histone H2A, Mouse monoclonal anti-Histone H2B, Rabbit monoclonal anti-Histone H3.3 and Rabbit monoclonal anti-GAPDH antibody in 4°C for 12–16 h. The agitation is recommended in this step.

f) The secondary antibodies are Goat anti-Rabbit IgG (H+L) HRP and Goat anti-Mouse IgG (H+L) HRP with dilution ratio of 1:3,000.

g) The blots are processed with Immobilon Western chemiluminescence kit.

h) The images are visualized using the Image Quant LAS 4000 Gel Imager.

AP Expression Detection of WT mESCs and WT mEpiSCs

Timing: ~1 h

31. AP expression detection, we culture the WT mESCs and WT mEpiSCs in Falcon® 35 mm TC-treated Easy-Grip Style Cell Culture dishes.
    a) Maintain the mESCs in complete medium with LIF and 2i or mEpiSCs in N2B27 medium with bFGF and Activin A for 5 days prior to analyzing AP activity.
    b) Then fix the cells with 4% PFA in 1× PBS for 1–2 min.
    c) Aspirate the fixative and rinse with 1× Rinse buffer twice.
    d) Prepare the AP staining solution by mixing Fast Red Violet with Naphthol AS-BI phosphate solution and water in a 2:1:1 ratio.
    e) Incubate the cells in dark at 20°C–25°C for 30 min. Aspirate the staining solution and rinse with 1× Rinse buffer twice.
    f) Cover the cells with 1× PBS and then image the colonies with AP activity using brightfield microscopy.

Induce Asymmetric Division of Histone-dendra2-Expressing mESCs with Wnt3a-Coated Beads

Timing: 4–6 days

This step introduces the methods for inducing ACD of histone-dendra2-expressing mESCs with Wnt3a beads. Because the activity of Wnt3a beads are critical for the ACD induction efficiency, here we will emphasize the assessment of biological activity of Wnt3a beads with LS/L cells.

32. Preparation of active and inactive Wnt3a coated beads.
    a) Mouse Wnt3a proteins were produced in *Drosophila* S2 cells and purified as described in Willert et al. (2003).
    b) Active Wnt3a-coated beads were prepared as described in Lowndes et al. (2017). Step 1 - beads activation: Activate the carboxylic acid groups of magnetic beads with 50 mg/mL carbodiimide and N-hydroxyl succinimide in 25 mM cold MES buffer. After activation, wash the beads for three times with 25 mM MES buffer (pH 5). Step 2 - Wnt3a protein immobilization: Reconstitute the Wnt3a protein at 40 μg/mL in sterile PBS. Then dilute the 5 μL protein solution with 20 μL 25 mM cold MES buffer (pH 5), incubate at 20°C–25°C with gentle rocking

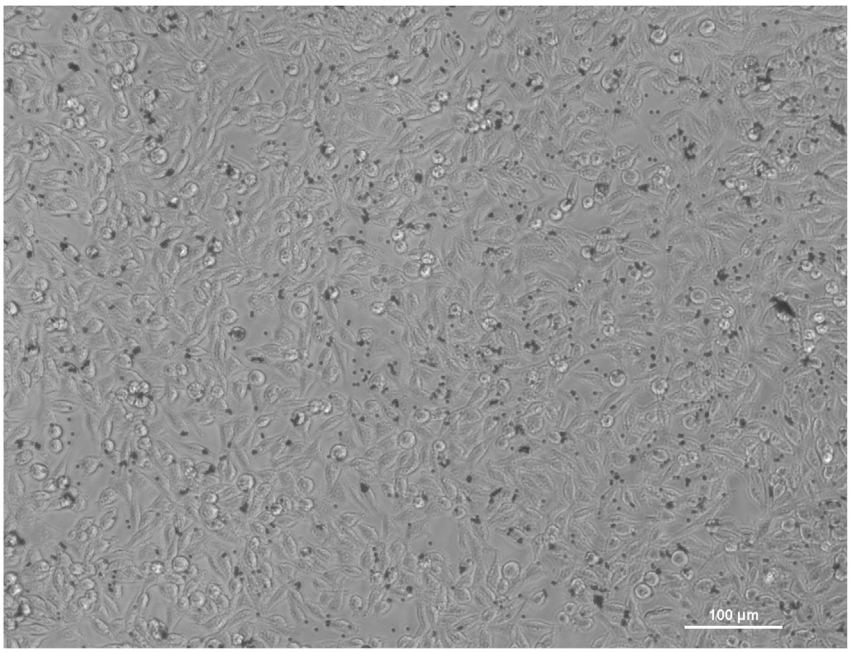

Figure 1. Phase-Contrast Image of LS/L Cells Incubated after 16 h with Active Wnt3a-Coated Beads (Dark Spot)
Objective 10×, 0.25 numerical aperture (NA). Scale bar, 100 µm.

for 1 h. Step 3 - The active Wnt3a beads were washed three times with MES pH 5 and additional three times with PBS, then store in PBS/ 1% BSA buffer at 4°C.

⚠ CRITICAL: For each wash, gently rocking the beads for completely washing away the unconjugated Wnt3a protein. Because of the gravity of magnetic beads, we recommend to flick the incubation tube every 5 min to better mix the beads and Wnt3a protein solution.

c) Preparation of inactive Wnt3a coated bead requires active Wnt3a beads which were incubated in Dithiothreitol (DTT) at 37°C for 30 min. DTT treatment denatures the Wnt protein and loses its activity. Finally store the inactive Wnt3a coated beads in PBS/1% BSA buffer at 4°C.

33. Assessment of biological activity of Wnt3a coated beads with LS/L assay.
    a) As described in Lowndes et al. (2017), luciferase reporter cells (LS/L cells) was used for testing the biological activity of Wnt3a coated beads. LS/L cells were cultured in LS/L medium. Specifically, per testing condition, 5 × 10^4 LS/L cells (in 100 µL of LS/L medium) were seeded in triplicate, in a 96-well tissue culture plate. Incubate LS/L cells for 4–6 h. When cells have properly attached, add soluble Wnt3a protein or controls ("Vehicle buffer" refers to the buffer used to elute and store Wnt3a protein after its purification from *Drosophila* S2 cells; or LS/L medium refers to LS/L "Cells only") or Wnt3a coated beads (active or inactive) into the corresponding wells. For beads condition in triplicate, dilute 6 µg of beads in 300 µL of LS/L medium and distribute 100 µL of bead mix in each well of the triplicate.
    b) To ensure single-bead suspension, pipet (≥50 times) before adding 100 µL of bead mix or control mix directly to the corresponding wells (final amounts/concentration: beads, 2 µg/well; soluble Wnt3a protein, 50 ng/mL). Incubate LS/L cells with samples or controls at 37°C with 5% CO_2. After 12–18 h, capture representative images of the wells to assess the level of well coverage and bead distribution (see Figure 1).
    c) Luciferase activity was measured as described in Lowndes et al. (2017), with Dual-Light Luciferase & β-Galactosidase Reporter Gene Assay System Kit (contains Buffer A, Buffer B, and

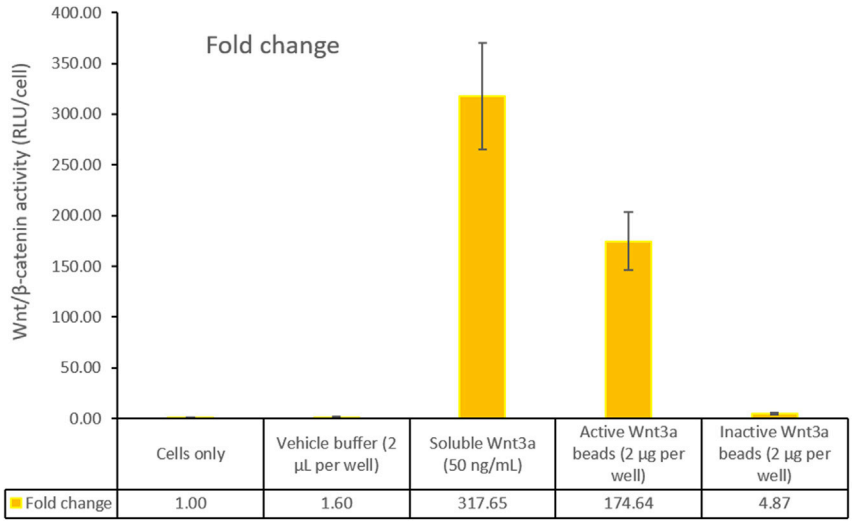

Figure 2. Normalized Results from the LS/L Assay Showing the Fold Change of the Biological Activity of Soluble Wnt3a Protein and Wnt3a Coated Beads (Active or Inactive)

6 µg of beads (active or inactive) were distributed in each well of the triplicate. 6 µL of soluble Wnt3a protein were distributed in the corresponding triplicate (final concentration of soluble Wnt3a protein = 50 ng/mL). 6 µL of LS/L medium or Vehicle buffer were distributed in each triplicate (negative controls: "Cells only" and "Vehicle buffer").

Accelerator-II) and a luminometer. To normalize the results, divide the first luciferase intensity by the β-galactosidase product intensity. To produce the fold change, the average of the triplicate normalized values can be divided by the average of the triplicate control condition (i.e., negative control, in our case "Cells only" containing only LS/L cells and LS/L medium) (see Figure 2).

34. We provide a flow chart for medium switching and inducing asymmetric division of mESCs with Wnt3a-coatd beads, as well as two representative figures showing the validation of ACD in mESCs (see Figures 3, 4, and 5). Please check the detailed information in Habib et al. (2013) and Lowndes et al. (2017).

Synchronization and Photoconversion of Histone-dendra2-Expressing mESCs with Wnt3a-Coated Beads

Timing: ~16.5 h

This step introduces the synchronization of mESCs using microtubule depolymerizing drug-Nocodazole (NZ), which is a critical step to use photoconversion to distinguish old (red fluorescent) versus new (green fluorescent) histones during cell cycle, as newly synthesized histones are mainly incorporated during S phase. Therefore, using the two color to label old versus new histones is only interpretable when we know the precise timing of photoconversion in the context of cell cycle.

35. Mix the cells with Wnt3a-coated beads in a ratio with about 2000 cells per 1.5 µg Wnt3a beads and then seed the mixture to fibronectin-coated fluorodishes.

⚠ CRITICAL: It is important to validate the activity of Wnt3a beads, as described in step 33, before use. Lower biological activity of Wnt3a coated beads can significantly reduce the percentage of ACD.

36. Use 50 ng/mL of NZ to treat mESCs with Wnt3a beads for 16 h, until you see lots of mitotic cells with round morphology.

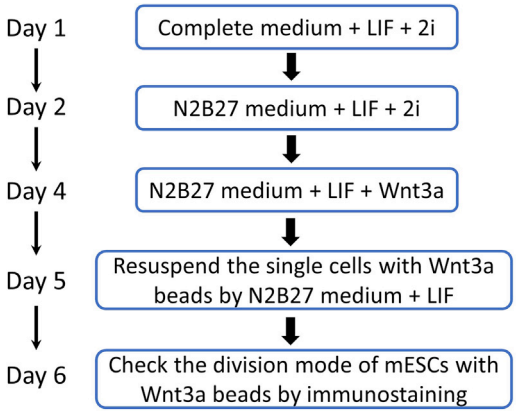

Figure 3. The Flow Chart for Inducing the Asymmetric Division of mESCs with Wnt3a-Coated Beads

Note: Synchronization can disrupt the polarization pattern of components of Wnt/β-catenin pathway in cells contacting Wnt3a bead and they may seem to be diffused. Moreover, avoid overtreating the cells with NZ because you need to release them to proceed until next cell cycle. Moreover, the mitotic cells can easily detach due to the NZ overtreatment. Here we recommend using lower concentration and longer time for NZ treatment. It may need to optimize this condition with different cell types prior to your formal experiments.

37. Wnt3a-induced mESCs expressing Histone-dendra2 are then photoconverted with pulses of 405 nm blue light. This step is empirical, which depends on Dendra2 protein expression level and set up of equipment, here is just an example as a starting point.
    a) Select the 20× objective of LSM 780 microscope for photoconversion.
    b) Then draw the region of interest using the tools in "Regions," and select mitotic cells positive with green Histone-dendra2 signals as targets.
    c) Use the 405 nm laser at 6%–10% power for 30-s pulses for 100–200 iterations to photoconvert Dendra2 protein from green fluorescence to red fluorescence.

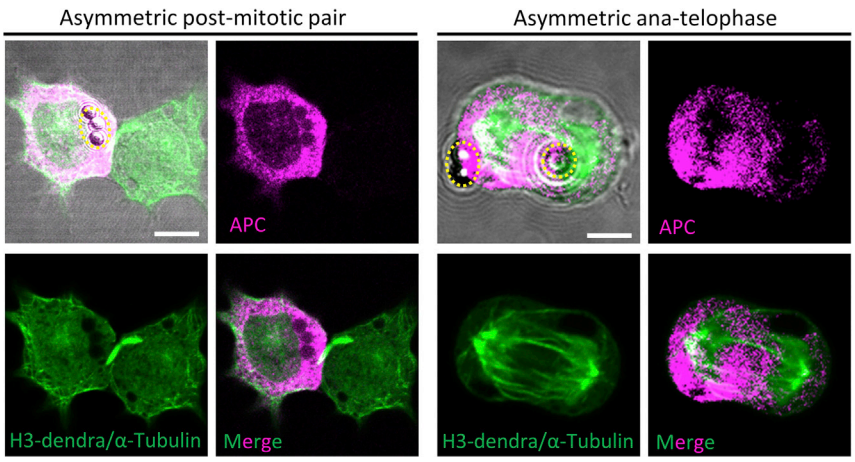

Figure 4. The Localization of Adenomatous Polyposis Coli (APC) in Post-mitotic and Ana-telophase mESCs
Left: A representative immunofluorescence image of the post-mitotic pair of cells with Wnt3a beads. Right: A representative immunofluorescence image of an ana-telophase mESC with Wnt3a beads. Magenta, APC immunostaining signal. Green, H3-dendra2 and α-Tubulin. Yellow dotted circle, Wnt3a-coated beads. Scale bar, 5 μm. Note: APC is a component of the β-catenin destruction complex, and can be detected in proximity to the Wnt3a beads during the asymmetric dividing mESCs.

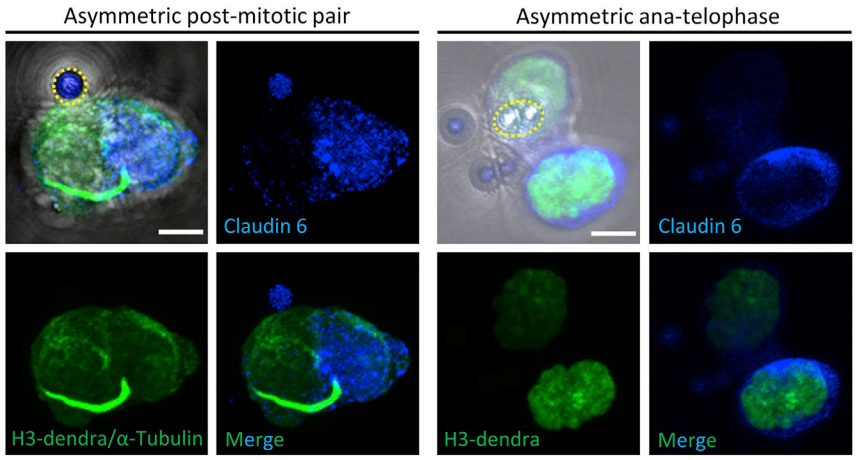

Figure 5. The Localization of Claudin 6 in Post-mitotic and Ana-telophase mESCs

Left: A representative immunofluorescence image of the post-mitotic pair of cells with Wnt3a beads. Right: A representative immunofluorescence image of an ana-telophase mESC with Wnt3a beads. Both images are shown with maximum projection. Blue, Claudin 6. Green, H3-dendra2 and α-Tubulin. Yellow dotted circle, Wnt3a-coated beads. Scale bar, 5 μm. Note: Claudin 6 is a marker for the cell fate of mEpiSCs, and can be detected in distal side of the Wnt3a beads during the asymmetric dividing mESCs.

Note: This photoconversion setting is performed on individual mESCs expressing medium level of histone-dendra2. For your own sample, it is necessary to optimize these settings to acquire a high photoconverting efficiency. The photoconversion efficiency can be calculated by quantifying the total intensity of green dendra2 before photoconversion (DBP) and the total intensity of green dendra2 right after photoconversion (DRAP) using the following equation: Photoconversion efficiency(%) = $\frac{DBP-DRAP}{DBP} \times 100\%$.

38. Release the cell cycle synchronization by gently washing off NZ with prewarmed 1× PBS buffer for three times, and then change with the fresh N2B27+ LIF culture medium.

⚠ CRITICAL: Try to gently aspirate and add 1× PBS buffer or culture medium during the washing to avoid misplacement of Wnt3a beads.

Tracking the Old versus New Histone Distribution in Histone-dendra2-Expressing mESCs

Timing: ~14–16 h

39. Prepare each histone-dendra2 mESCs line, perform steps 34–38, then release the cells to allow them to progress into the second M phase (see Figure 6).

Note: Some cells may be sensitive to NZ treatment and still arrest after washing off NZ. Those cells can be distinguished as they will carry most photoconverted red fluorescent dendra2 but no or very low green fluorescent dendra2, which will not be included in imaging and data analyses.

40. After 14–16 h, fix cells with 4% PFA at 20°C–25°C for 30 min and then image the mitotic cells carrying comparable red and green histone-dendra2 signals with confocal microscopy with 40× or 63× oil immersion objectives, 1.4 NA.

⚠ CRITICAL: To better perform colocalization analysis, we recommend to capture images for the entire nucleus with Z stack interval of 0.5 μm.

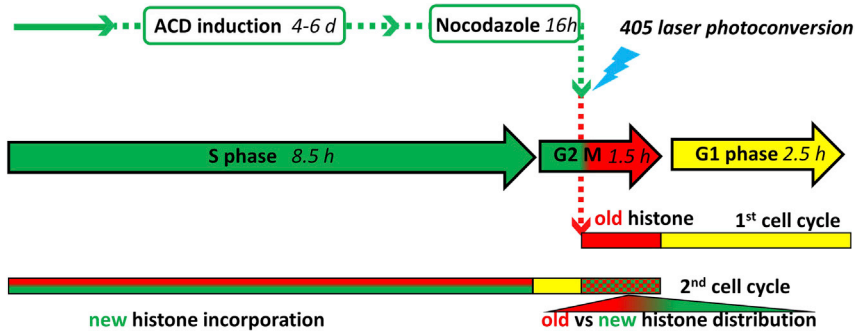

Figure 6. The Regime for Tracking the Distribution of Old versus New Histone in Asymmetric Dividing mESCs

The photoconversion of histone-Dendra2 is performed in the first mitotic phase. Then, new histone will be incorporated in next S phase. The old versus new histone distribution patten can be detected on the second mitotic phase.

EXPECTED OUTCOMES

Using this protocol, we have established an optimized method for tracking different histone distribution patterns in the Wnt3a bead-induced asymmetric dividing mESCs. This method comprises two major parts:

The first part of this protocol is adapted from (Habib et al., 2013; Lowndes et al., 2017) to establish asymmetric divisions of mESCs with Wnt3a-coated beads. Polarized APC or Claudin6 distribution in post-mitotic or anaphase-to-telophase mESCs with active Wnt3a beads should be higher than 50% at least, while control group with inactive Wnt3a beads or other culture conditions for symmetric cell division should show dominantly symmetric cell division mode (see Figures 4 and 5 for the polarized distribution of APC and Claudin 6).

The second part of this protocol is adapted from previous assays of asymmetric histone inheritance pattern in *Drosophila* male germline stem cells (Tran et al., 2012; Xie et al., 2015; Wooten et al., 2019). In this protocol, we use the photoconvertible protein dendra2 to trace old versus new histone distribution pattern in Wnt3a bead-induced mESCs. Similar to results in fly male germline stem cells, histone H3 and H4 show non-overlapping patterns during ACD of mESCs, but H2A, H2B, and H3.3 show overlapping patterns (see Figure 7).

This protocol is suitable for other types of mammalian cells to explore histone inheritance patterns in either cultured cells or tissue context *in vivo*.

QUANTIFICATION AND STATISTICAL ANALYSIS

Measurement of Colocalization of Old versus New Histone in mESCs

To quantify the overlapping degree between old versus new histones, we developed a MATLAB code for optimization of "overlapping coefficient" k (see Figure 8). The k overlap coefficient is defined using the following equation:

$$k_1 = \frac{\sum_i Ch1_i \cdot Ch2_i}{\sum_i (Ch1_i)^2} \quad k_2 = \frac{\sum_i Ch1_i \cdot Ch2_i}{\sum_i (Ch2_i)^2} \quad R^2 = k_1 \cdot k_2$$

Step 1: For each focal plane of the cell, the intensity value of individual voxel for one channel is normalized by percentage of intensity of that channel.

$$\left(\text{e.g. } Ch1i = \frac{\text{intensity value for one voxel in a single focal plane}}{\sum \text{intensity values of all voxels in a single focal plane}}\right).$$

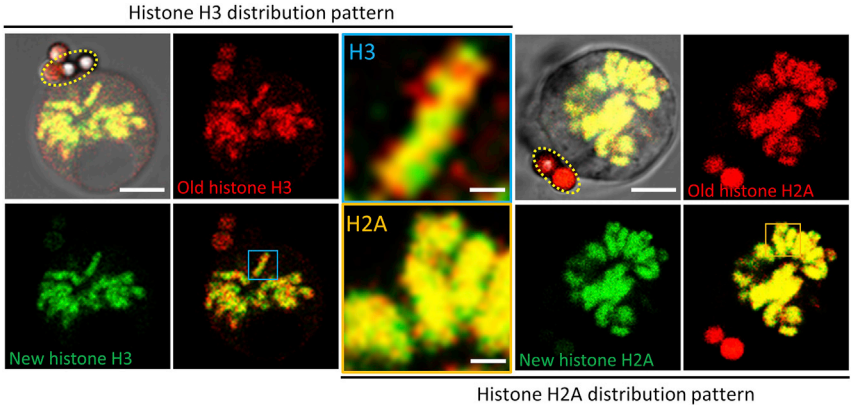

Figure 7. The Histone Distribution Patterns in Mitotic H3-dendra2-Expressing and H2A-dendra2- Expressing mESCs, Respectively

Left: A representative immunofluorescence image of non-overlapping old versus new H3 in a prometaphase mESC with Wnt3a beads. Right: A representative immunofluorescence image of overlapping old versus new H2A in a prometaphase mESC with Wnt3a beads. Red, Old histone. Green, New histone. Yellow dotted circle, Wnt3a-coated beads. Scale bar, 5 μm. Scale bar for insets (outlined by cyan or blue line), 1 μm.

Step 2: The *k* overlap coefficient for each focal plane, is calculated as the equations described above on normalized intensity values of voxels.

Step 3: The overall *k* coefficient of the whole nucleus is a weighted average of *k* overlap coefficient for each focal plane, based on the green (first) channel.

$$\left(\text{e.g. } R^2_{whole\ \ cell} = \frac{\sum \text{Green intensity values of single focal plane} * R^2_{focal\ \ plane}}{\text{Green intensity values of all focal plane}}\right)$$

⚠ CRITICAL: The algorithm was developed given two premises: (1) Since different channels have variabilities in many aspects and cannot be compared directly, voxels of different channels were normalized respectively in each focal plane. (2) To recapitulate the *k* coefficient in a spherical nucleus (SNR), the second normalization of all focal planes is done on green channel, as it has a better SNR in our imaging setups.

LIMITATIONS

For the current protocol, several improvements could be made to better explore the relationship between non-overlapping pattern for old versus new H3/H4 and distinct cell fates derived from ACD of

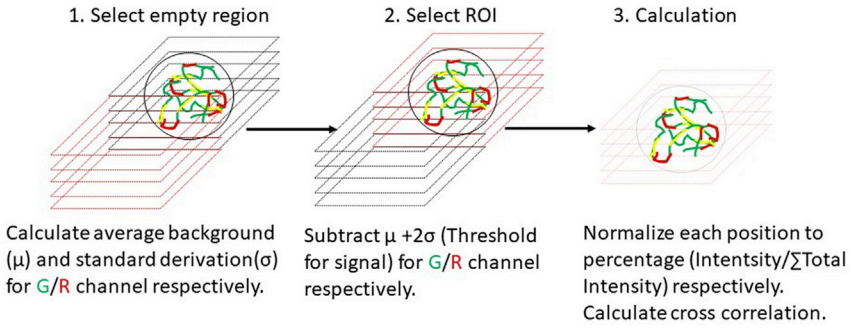

Figure 8. The Quantification Flow with Computational Model of Green and Red Histone Signals on Chromosomal Regions Using Confocal Images

Red, old histone dendra2 signal. Green, new histone dendra2 signal.

mESCs. (1) We used the localization of APC and Claudin6 to indicate ACD in this Wnt3a-induced mESCs system. Because both proteins showed diffused localization during mitosis especially at prophase and prometaphase (see Figure 9), it is difficult to investigate the histone inheritance mode in a single cell undergoing mitosis. (2) We report the similar asymmetric inheritance patterns of old H3 and H4, which is consistent with previous study of H3-H4 splitting events (Xu et al., 2010). Based on those findings, we hypothesized that distinct nucleosome reassembly modes could have regional specificities during DNA replication (Ma et al., 2020. Schematic model-E in Mendeley data): the conservative model could occur at genomic regions that need to be differentially expressed in the two daughter cells derived from Wnt3a-induced asymmetrically dividing mESCs, such as stemness genes, differentiation genes, or Wnt signaling pathway genes. On the other hand, for those genes, which have comparable expressions between the two daughter cells, the dispersive model could be applied to make sure the sister chromatids inherit the identical epigenetic information. Thus, to pinpoint the local H3/H4 asymmetry at specific genomic loci, more *in situ* technologies like CAS-FISH (Deng et al., 2015) or CARGO-FISH (Gu et al., 2018) could show candidate gene loci with the non-overlapping pattern for old and new histone enrichment information.

TROUBLESHOOTING

Problem 1

Low attachment for the mESCs with Wnt3a beads to the dish when seed to the fluorodish.

Potential Solution

First make sure the mESCs are in good condition without spontaneous differentiation when maintaining the cells; Second try to optimize the working concentration and incubation time of fibronectin to coat the surface of fluorodish.

Problem 2

A single mESC carries too few or too many beads.

Potential Solution

Adjust the ratio of single cells and Wnt3a beads: For the 10 mm fluorodish (WPI) we used in this protocol, the surface area is 0.785 cm^2. We usually seed 2000 single cells with ~1.5 μg Wnt3a beads. The beads need to be resuspended ca. 50 times to make sure they are not aggregating before adding them to the cells.

Problem 3

Low ACD induction efficiency of Wnt3a-coated beads.

Potential Solution

If the ACD induction efficiency is quite low, there are several factors you may need to consider: 1. The Wnt3a protein activity. Please make sure the activity of Wnt3a protein is good to maintain the mESCs self-renewal. Please refer to Lowndes et al. (2017) for Wnt3a protein activity, Wnt3a immobilization, and storage. Or you can use the 7× TCF-luciferase reporter to test the Wnt3a activity. 2. Please make sure the immobilization procedures are all performed as indicated in step 32-b, as the concentration and PH value of the reagents will affect the activation of carboxylic acid groups of the magnetic beads. 3. mESCs should be well-maintained at the ground naïve state before any induction procedure, which is essential for Wnt3a-mediated ACD. 4. According to the size of your dishes for confocal imaging, the appropriate ratio of cells to Wnt3a beads is another critical factor to acquire high induction efficiency. The recommended ratio has been mentioned in potential solution of Problem 2.

Problem 4

Low Dendra2 photoconversion efficiency.

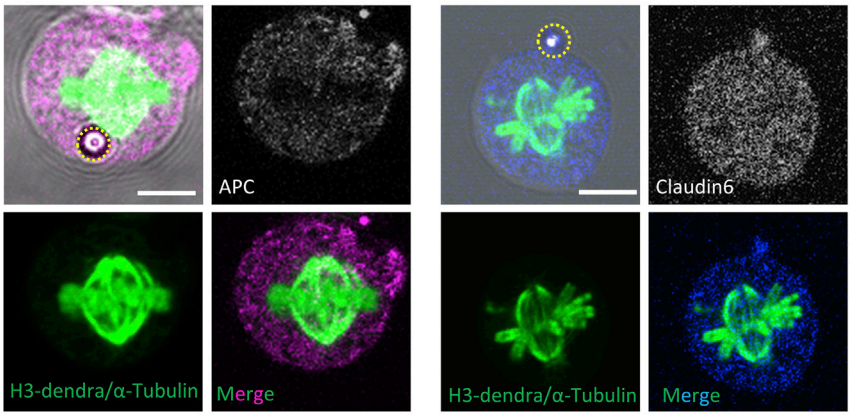

Figure 9. The Diffused Localization of APC and Claudin 6 in Mitotic mESCs

Left: A representative immunofluorescence image of a metaphase mESC with Wnt3a beads. Magenta, APC. Green, H3-dendra2 and α-Tubulin. Right: A representative immunofluorescence image of a prometaphase mESC with Wnt3a beads. Blue, Claudin 6. Green, H3-dendra2 and α-Tubulin. Yellow dotted circle, Wnt3a-coated beads. Scale bar, 5 μm.

Potential Solution

This step is a multifactorial procedure, which might be affected by the thickness of different samples, the expression level of Dendra2 fusion protein, the 405 nm light exposure settings, and the cell tolerance. Thus, we recommend optimizing the settings for Dendra2 photoconversion according to your own samples empirically.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests of reagents can be directed to and fulfilled by the Lead Contact, Xin Chen (xchen32@jhu.edu).

Materials Availability

All Histone-Dendra2 plasmids generated in this study have been deposited to Addgene. Moreover, all Histone-dendra2 mESC lines used in this study are available upon request from the Lead Contact, Dr. Xin Chen, xchen32@jhu.edu.

Data and Code Availability

We have deposited the quantification code used for *k* overlap coefficient measurement onto the Mendeley website and made it accessible to public: https://data.mendeley.com/datasets/pc8z47jzm5/draft?a=7e80bf22-2723-423b-8bea-445e629c5ec8

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AUTHOR CONTRIBUTIONS

Conceptualization, B.M. and X.C.; Methodology, B.M., T.-J.T., S.J.H., and X.C.; Writing – Original Draft, B.M. and X.C.; Funding Acquisition, B.M., S.J.H., and X.C.; Supervision, S.J.H. and X.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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