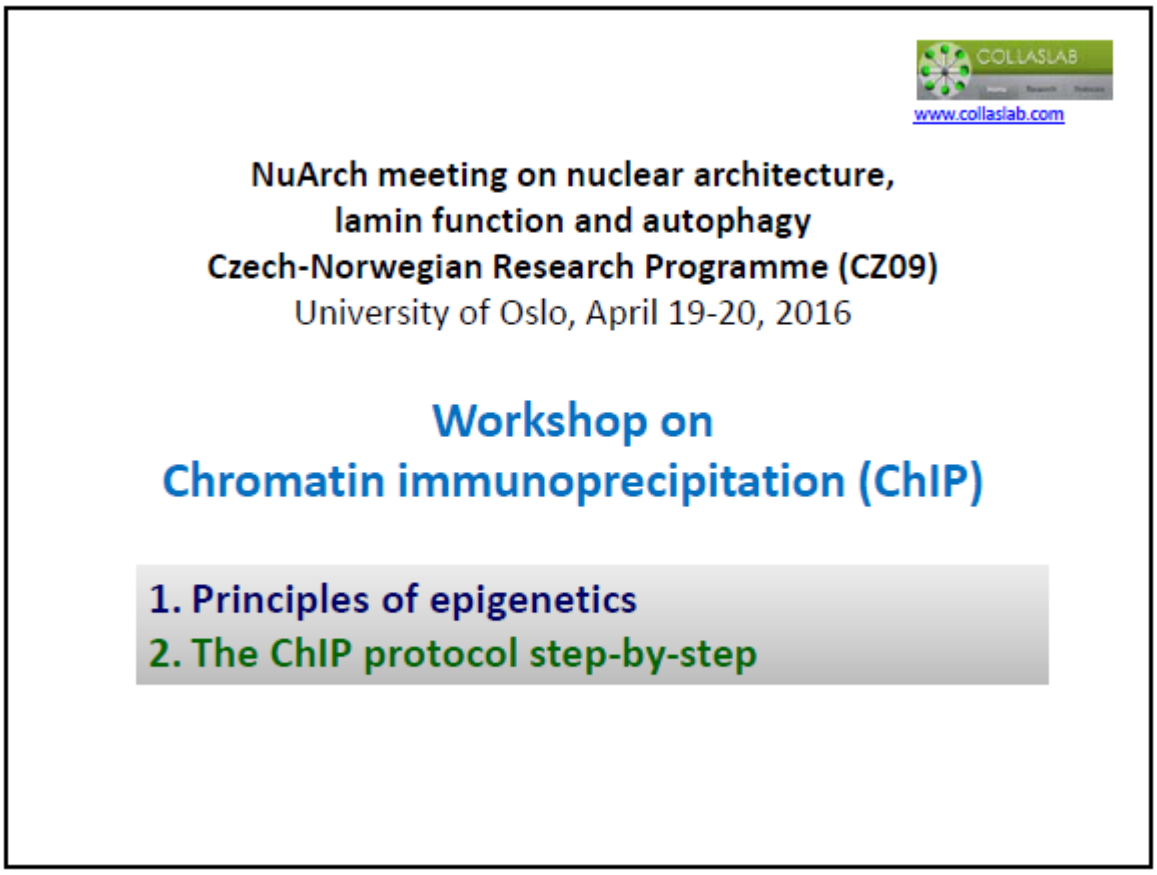
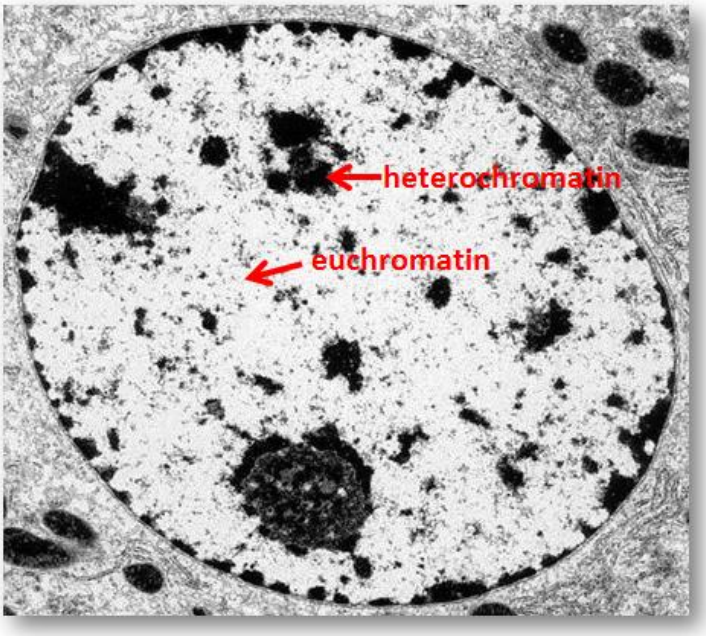
**NuArch meeting on nuclear architecture,
lamin function and autophagy
Czech-Norwegian Research Programme (CZ09)**
University of Oslo, April 19-20, 2016

Workshop on Chromatin immunoprecipitation (ChIP)

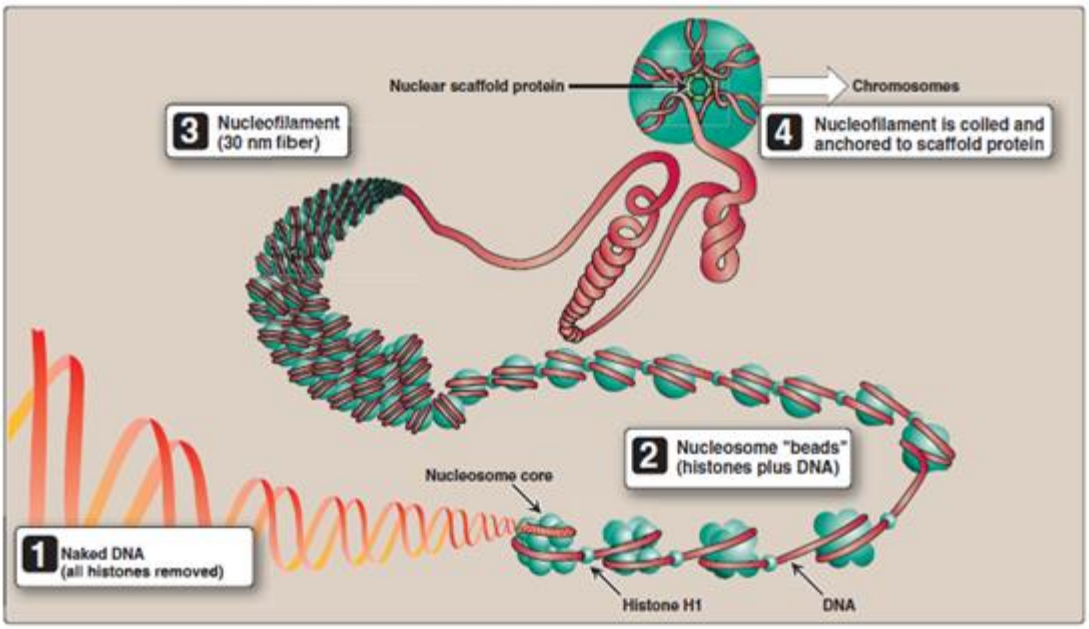


Prepared by Torunn Rønningen, Anja Oldenburg, Philippe Collas

Various degrees of chromatin packaging in the eukaryotic nucleus



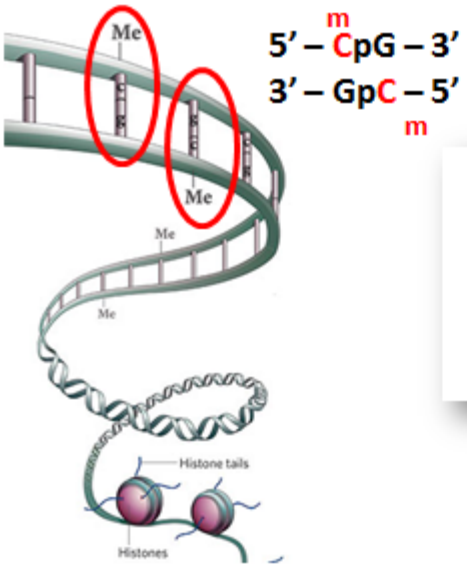
Structural organization of eukaryotic chromatin



Epigenetics:
Heritable modifications of DNA or chromatin (histones) that affect gene expression without changing the DNA sequence

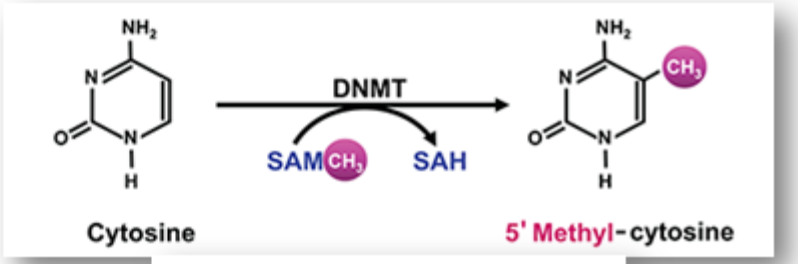
Two main components of epigenetic regulation:
DNA methylation
Post-translational modifications of histones

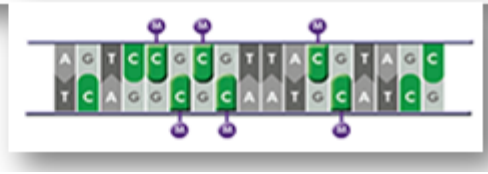
DNA methylation occurs **on cytosines** in CG dinucleotides



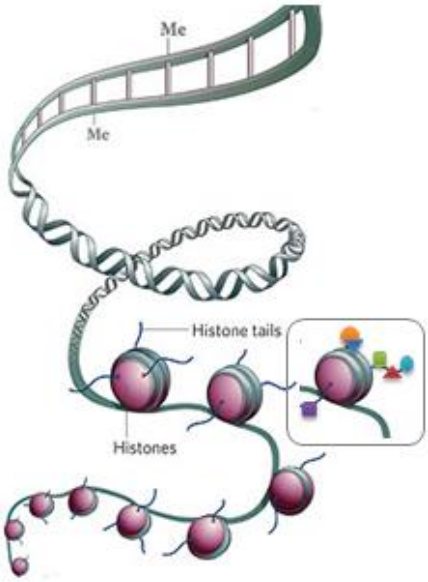
5′ – C(m)pG – 3′
3′ – GpC(m) – 5′

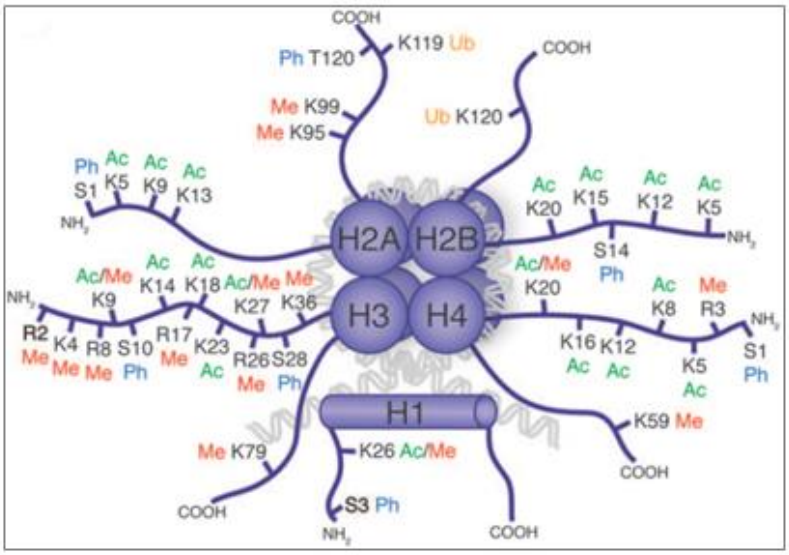
DNA methylation reaction



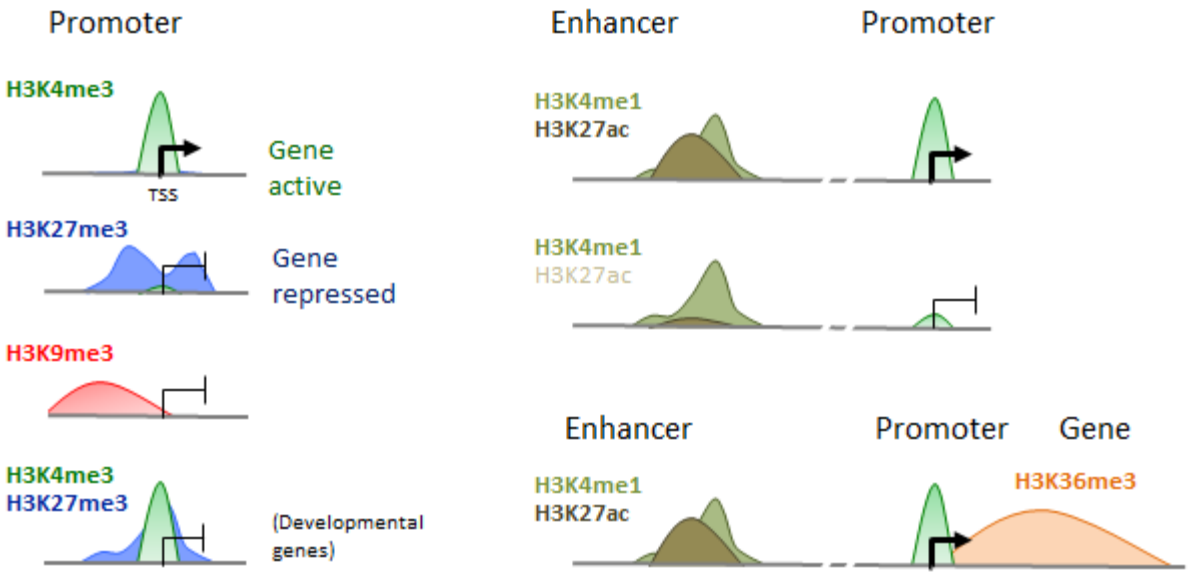


The N-terminal tails of histones are post-translationally modified





Histone modifications mark specific regions in the genome and correlate with a gene expression status



How to analyze histone modifications? Chromatin immunoprecipitation (ChIP)

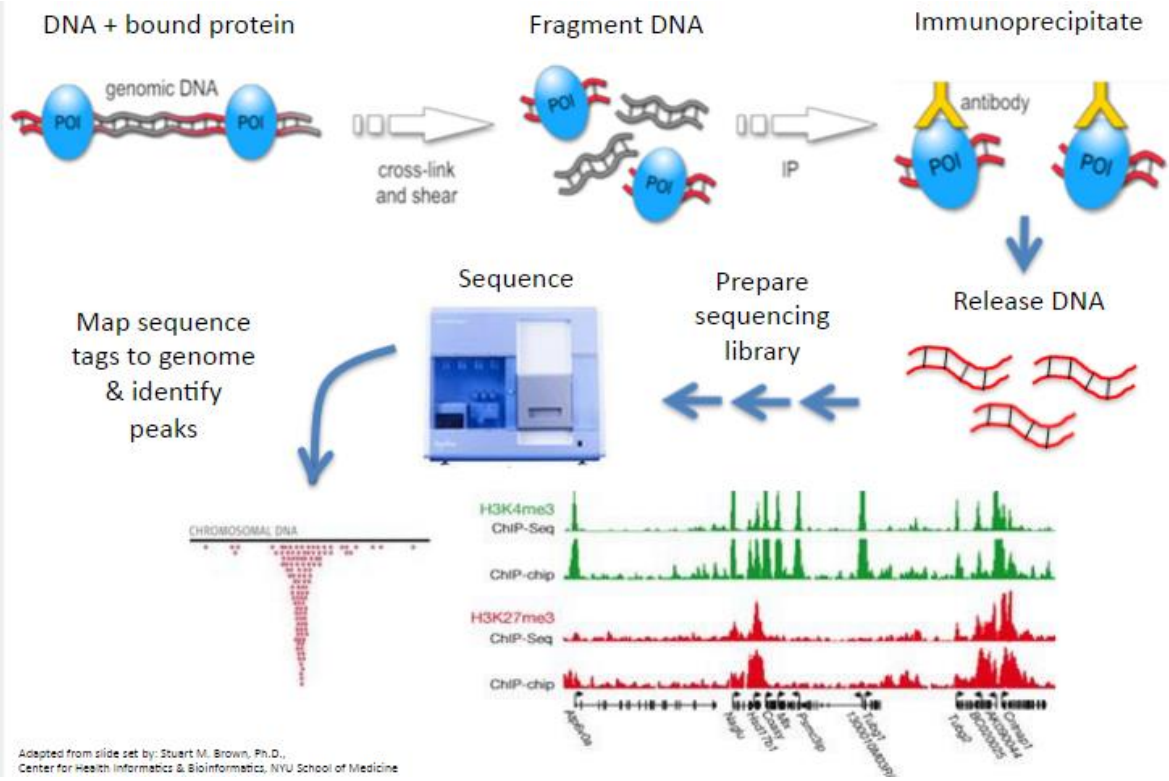


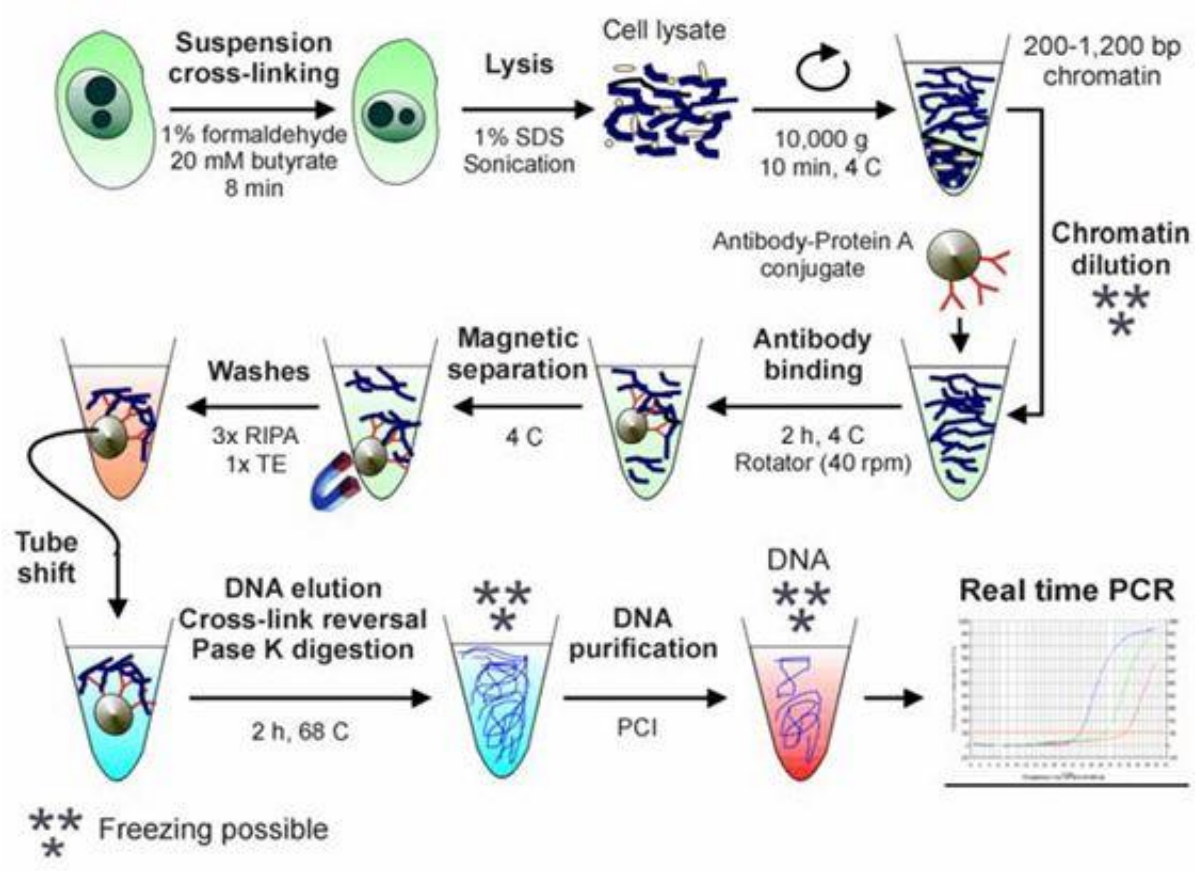
Fig. 1. Overview of the ChIP procedure

Objective of the ChIP assay:
Determine DNA sequences associated with a protein of interest (POI)* in the genome

- Single gene or region level: ChIP-qPCR
- Genome-wide: ChIP-seq

- *Transcription factor, histone, RNA polymerase, chromatin remodeling enzyme, DNA repair enzymes, other chromatin-bound factor

ChIP Protocol



The CollasLab histone ChIP protocol

Cell harvesting

1. Harvest cells according to your lab protocol for the cell type of interest
2. Count cells and sediment them at 300 g for 10 min at room temp
3. Resuspend up to $2x10^6$ cells in 500 µl PBS containing 20 mM Na-butyrate (optional); see notes
4. Transfer into a 1.5-ml tube

Notes:

- If you have > $2x10^6$ cells, and up to $10x10^6$ cells, resuspend them in 1 ml PBS in Step 3.
- Na-butyrate (NaBu) is an HDAC inhibitor and prevents deacetylation of proteins.

DNA-protein crosslinking with formaldehyde

5. In a fume hood, and for 500 µl PBS containing cells, add 14 µl formaldehyde from a 36.5% stock (Sigma)
6. Incubate for 10 min at room temp. Note: proteins other than histones may need longer incubation time.
7. Add 57 µl of 1.25 M glycine stock made in PBS (this gives 0.125 M final concentration)
8. Incubate for 5 min at room temp

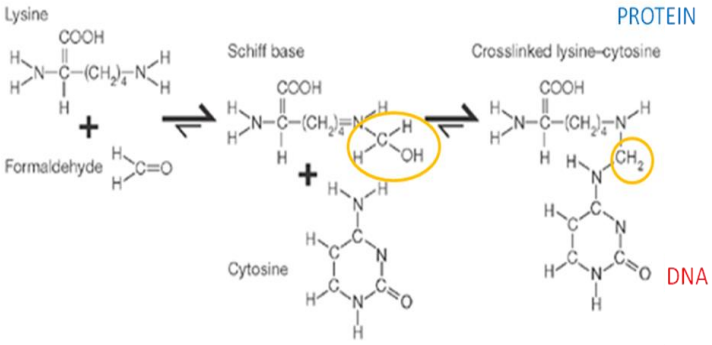


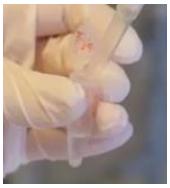
Figure: Formaldehyde cross-links amino or nitro groups within 2 Å of each other.

Washing of crosslinked cells (on ice)

9. Centrifuge formaldehyde cross-linked cells at 470 g for 10 min at 4°C (the swing out rotor in the cold room). Place the formaldehyde waste into a separate flask in the fume-hood.
10. Resuspend the cell pellet in 500 µl ice-cold PBS containing 20 mM Na butyrate (optional)
11. Centrifuge at 470 g for 10 min at 4°C
12. Repeat steps 10 and 11 once

Cell lysis (on ice)

13. Resuspend the $2x10^6$ cell pellet in 180 µl lysis buffer w/ Na butyrate (optional), protease inhibitor cocktail (Pi) and PMSF on ice.
14. Vortex and allow lysis to occur for 5 min on ice. Pipette if necessary.



Notes:

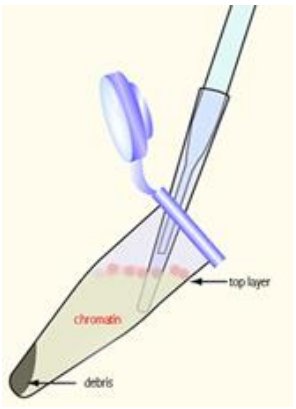
- If less than $2x10^6$cells, resuspend them in 120 µl lysis buffer w/ Na butyrate (optional), Pi and PMSF on ice.
- Na butyrate, Pi and PMSF should be added to the lysis buffer just before use; see page 6. Half -life of PMSF is 30 min.
- This tube should not be kept on ice because SDS might precipitate.
- Time of cell lysis might depend on cell type. Check lysis under a phase contrast microscope after putting 2 µl of sample between a glass slide and a coverslip.

Chromatin fragmentation by sonication

15. Fragment chromatin by sonication using a Bioruptor (Diagenode; **Figure**) with the following settings: 30 sec ON/OFF at HIGH power for 3 or 4 times 10 min

Notes:

- Remember to turn on the water pump before starting sonication to allow cooling
- The tubes should be balanced in the Bioruptor wheel (as you would in a centrifuge)
- Time of sonication should be optimized to get chromatin fragment sizes of 200-500 bp
- Efficiency of sonication depends on cell type and number, cross-liking conditions, sample volume and number of tubes in the Bioruptor wheel



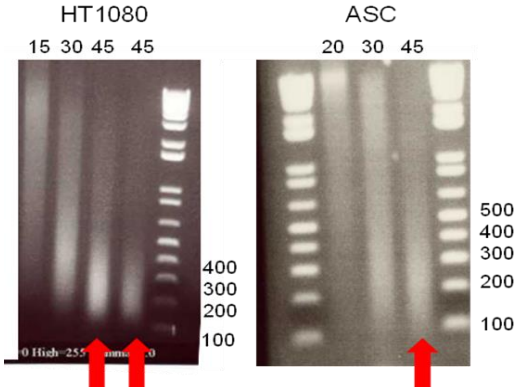
16. Centrifuge at 10000 g for 10 min at 4°C using a swing-out rotor
17. Transfer the supernatant ('chromatin') into a new tube; take care to avoid the top layer and the bottom fraction (debris) at the bottom of the tube (**Figure**). The chromatin must be kept on ice or frozen.
18. Transfer 10 µl of chromatin to a new tube for fragment size analysis on gel

Analysis of chromatin fragment size

It is important to assess the length of chromatin fragments (200-500 bp) before using chromatin for ChIP in order to ensure you have fragments that are long enough for PCR analysis, and short enough to cover only the genomic region of interest (resolution).

19. Add 1 µl RNase (from a 500 ng/µl stock) to 10 µl chromatin (final: 50 ng/µl)
20. Incubate at 37°C for 20 min
21. Add 190 µl elution buffer containing 1% SDS; if you started with more than 10 µl chromatin to a total volume of 200 µl.
22. Add 5 µl Proteinase K (from a 2 mg/ml stock)
23. Incubate at 68°C, 500 rpm for 2 h on Thermomixer (**Figure**)
24. Purify DNA; see p. 7 DNA purification, starting from step 2. Adjust volumes to 200 ul.
25. Dissolve DNA in 10 µl MQ H_2O.
26. Analyze DNA in a 1.5% agarose gel with suitable size markers to determine fragment size

Figure: Assessment of chromatin fragmentation after sonication (time indicated in min). Two cell types examined here: HT1080 and adipose stem cells (ASC). **Red arrows** example of appropriately sonicated chromatin.



Bioruptor sonication
- 45 min of:
- High power
- 30 sec on /30 sec off

Chromatin fragments
- 150-400 bp
- Main fraction 200 bp

Chromatin measurement and dilution prior to ChIP

27. Determine the amount of chromatin on a Nanodrop spectrophotometer at A_{260}.
28. Dilute the chromatin to 2 U/ µl with RIPA w/ NaBu (optional), protease inhibitor cocktail (Pi) and PMSF on ice.

Note:
- Remember that you need 100 µl of input chromatin later in the procedure.

Coupling of antibody to magnetic Dynabeads

Note:
- Use Dynabeads 'Protein A' or 'Protein G' depending on the animal origin of your ChIP antibody

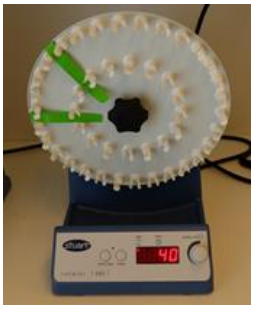
29. Vortex the bottle of Dynabeads stock for 1 min before taking out beads
30. Calculate the amount of beads you need based on the number of ChIPs you will do. In a 1.5 ml tube, place 10 µl of beads per ChIP to be done, and add 10 % of beads extra; use several tubes if necessary, given that the total volume in step 34 cannot exceed 1.4 ml
31. Add 2.5 volumes of RIPA buffer and vortex
32. Incubate on magnet for 1 min and remove the buffer (**Figure**).
    Note: to avoid beads trapped in the lid, snap-spin the tube in a mini-centrifuge (**Figure**) before putting the tube on the magnet.
33. Repeat steps 31 and 32
34. Resuspend the beads in 10 x volume of RIPA buffer
35. To each 0.2 ml tube of an 8-tube strip pre-labeled with right antibody, add 100 µl Dynabeads (from step 34) and 2.5 µg antibody
36. Incubate the tubes at 40 rpm on a rotator in the cold room for at least 2 h or overnight (**Figure**)

Notes:

- Different antibodies might have different concentrations
- Ensure the beads are mixed well in the solution throughout the procedure as they sink fast

Magnetic rack for 200 µl tubes

Minifuge



Rotator

Immunoprecipitation

37. Snap-spin the tubes in a mini-centrifuge to bring down what is trapped in the lid (**Figure**)
38. Place tubes on a magnet on ice for 1 min
39. Remove the supernatant while the tubes are on the magnet; throw away the supernatant. Remember to change pipette tips between tubes as these may contain different antibodies
40. Add 100 µl of chromatin at 2 U/µl (from step 28) and incubate for 2 h at 40 rpm on a rotator in the cold room

Washing of the immunoprecipitated material (on ice)

41. Snap-spin the tubes in a micro-centrifuge to bring down any solution trapped in the lid
42. Place the tubes on a magnet on ice for 2 min
43. Remove the supernatant while tubes are on the magnet; throw away the supernatant, being careful to avoid the Dynabeads
44. Add 100 µl RIPA buffer and incubate tubes for 4 min at 40 rpm on a rotator in cold room
45. Repeat step 41 to 43 twice
46. Add 100 µl of TE buffer (pH 8.0) and incubate tubes for 4 min at 40 rpm on a rotator in the cold room
47. Transfer the content of the tubes to new tubes, place on magnet for 1 min and remove TE

Elution and crosslink reversal

48. Add 150 µl elution buffer (see ChIP Buffers) containing 1% SDS and 50 ng/µl Proteinase K
49. To 100 µl of INPUT chromatin, add 200 µl of elution buffer containing 1% SDS and 50 ng/µl Proteinase K
50. Incubate ChIP DNA and Input chromatin tubes at 68^{o}C for 2 h at 1300 rpm on a Thermomixer
51. Snap-spin ChIP DNA tubes and place on magnet for 1 min; transfer buffer into 1.5 ml tubes
52. To the Dynabeads on magnet, add another 150 µl of elution buffer and incubate at 68^{o}C for 5 min at 1300 rpm on a Thermomixer
53. Snap-spin the tubes and place tubes on magnet for 1 min; transfer the buffer into the 1.5 ml tubes of step 51 above (total is 300 µl per tube).

Note:

- If ChIP DNA is to be used for 'whole genome' amplification, microarray analysis or sequencing, RNase treatment is necessary for DNA purification; see separate protocol.
- At this stage, you have collected ChIP material containing DNA ready to be purified.

Purification of the ChIP DNA

1. Add 200 µl elution buffer to 300 µl (2x150 µl) supernatant and to the tube containing input chromatin (see Note, Step 28)
2. Add 500 µl (1x volume) phenyl-chloroform isoamylalcohol (pre-mixed at 24:24:1)
3. Invert the tubes several times and centrifuge at 15000 g for 5 min
4. Use a pipette to carefully collect 450 µl of the upper phase and transfer into a new tube
5. Add 450 µl (1x volume) of chloform isoamylalcohol (pre-mixed at 24:1)
6. Invert the tubes and centrifuge at 15000 g for 5 min
7. Carefully collect 400 µl of the upper phase and transfer into a new tube
8. Add 40 µl (0.1x volume) 3 M Na-acetate, 10 µl acrylamide carrier (0.25%) and 1 ml (2.5x volume) ice-cold 96% EtOH
9. Place samples at -80^{o}C for 2 h or overnight to allow the DNA to precipitate
10. Centrifuge at 20000 g for 15 min at 4^{o}C
11. Dry the DNA pellet for 15 min at room temp by leaving the tube open
12. Dissolve the DNA in 100 µl MQ H_2O. Note: this volume may vary depending on the chromatin concentration you started with; if the DNA is for amplification, microarray or sequencing, dissolve in 10 µl MQ H_2O.
13. Leave the tube at room temp to dissolve for several hours to overnight
14. Freeze the DNA at -20^{o}C. DNA can be stored frozen for months.

ChIP Buffers

LYSIS BUFFER	**Stock**	**50 ml**	**1 ml**	**Room temp**
1% SDS	10%	5 ml		
10 mM EDTA (pH 8)	0.5M	1 ml		
50 mM Tris-HCl (pH 8)	1M	2.5 ml		
dH_2O		41.5 ml		
Protease inhibitor cocktail	*100x*		*10 µl*	***Add to 1 ml of***
1 mM PMSF	*100 mM*		*10 µl*	***lysis buffer just***
20 mM Na-butyrate	*1 M*		*20 µl*	***before use***

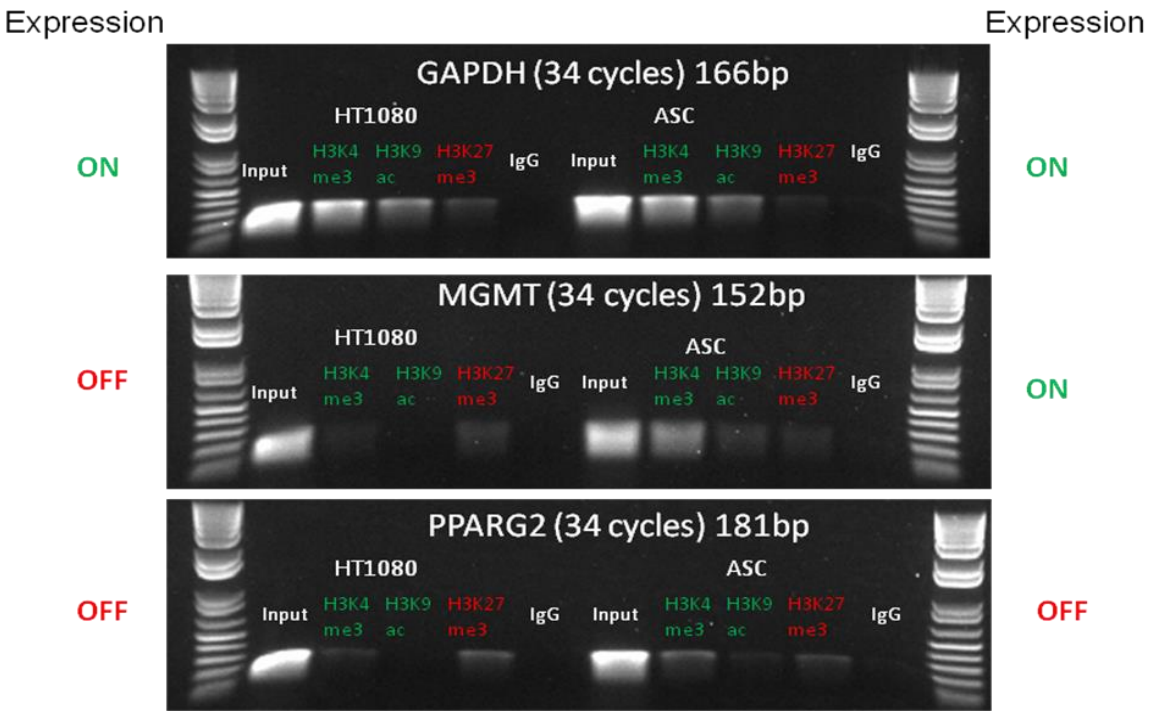
RIPA BUFFER	**Stock**	**100 ml**	**1 ml**	**4°C**
0.1% SDS	10%	1 ml		
0.1% sodium deoxycholate		0.1 g		
1% Triton X-100	100%	1 ml		
1 mM EDTA (pH 8)	0.5 M	200 µl		
0.5 mM EGTA (pH 8)	0.5 M	100 µl		
140 mM NaCl	5 M	2.8 ml		
10 mM Tris-HCl (pH 8)	1 M	1 ml		
dH_2O		93.9 ml		
Protease inhibitor cocktail	*100 x*		*10 µl*	***Add to 1 ml of***
1 mM PMSF	*100 mM*		*10 µl*	***RIPA buffer just***
20 mM Na-butyrate	*1 M*		*20 µl*	***before use***

ELUTION BUFFER	**Stock**	**50 ml**		**4°C**
50 mM NaCl	5 M	500 µl		
20 mM Tris-HCl (pH 7.5)	1 M	1 ml		
5 mM EDTA (pH 8)	0.5 M	500 µl		
dH_2O		42 ml		
1%SDS	*10%*	*5 ml*		***Add just before use***

Results

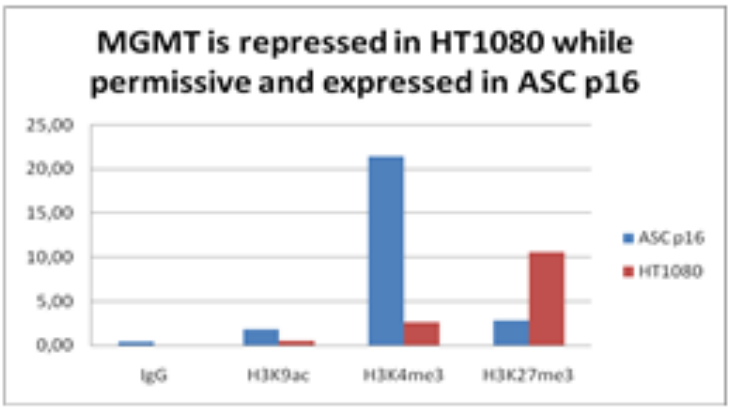
ChIP-endpoint PCR

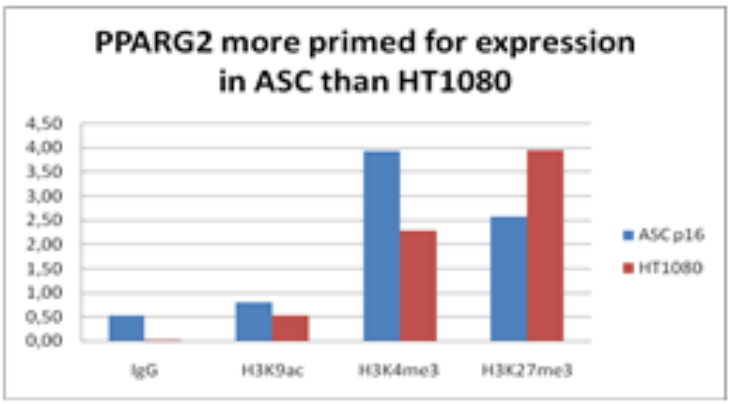
HT1080: cancer cell line; **ASC**: primary adipose stem cells
GAPDH: housekeeping gene, expressed in both cell types
MGMT: tumor suppressor, expressed in ASCs not in HT1080
PPARG2: adipogenic gene "poised" in ASCs, no expressed in HT1080.

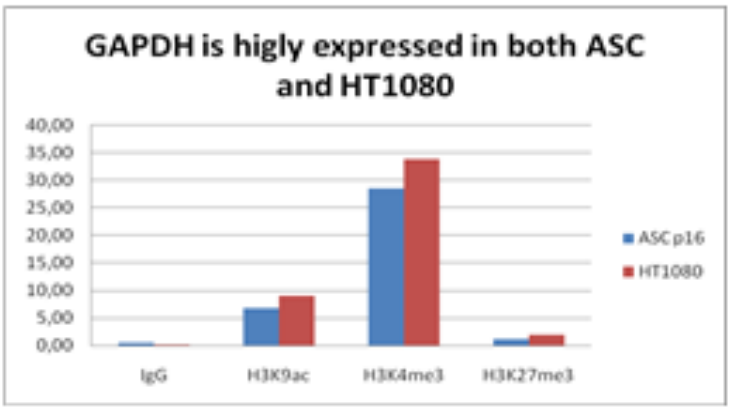


Results

ChIP-qPCR



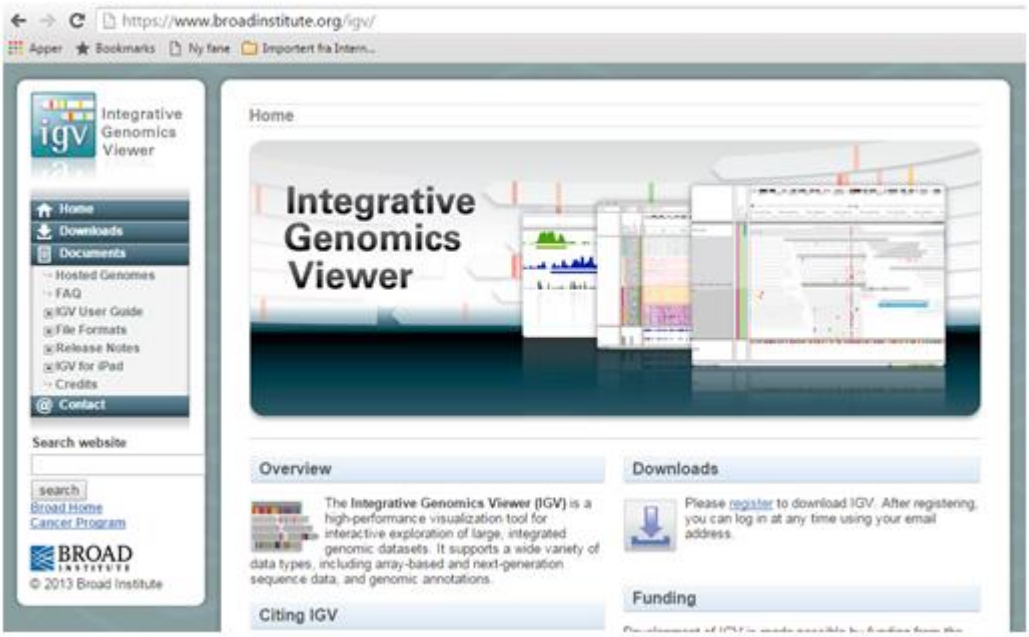




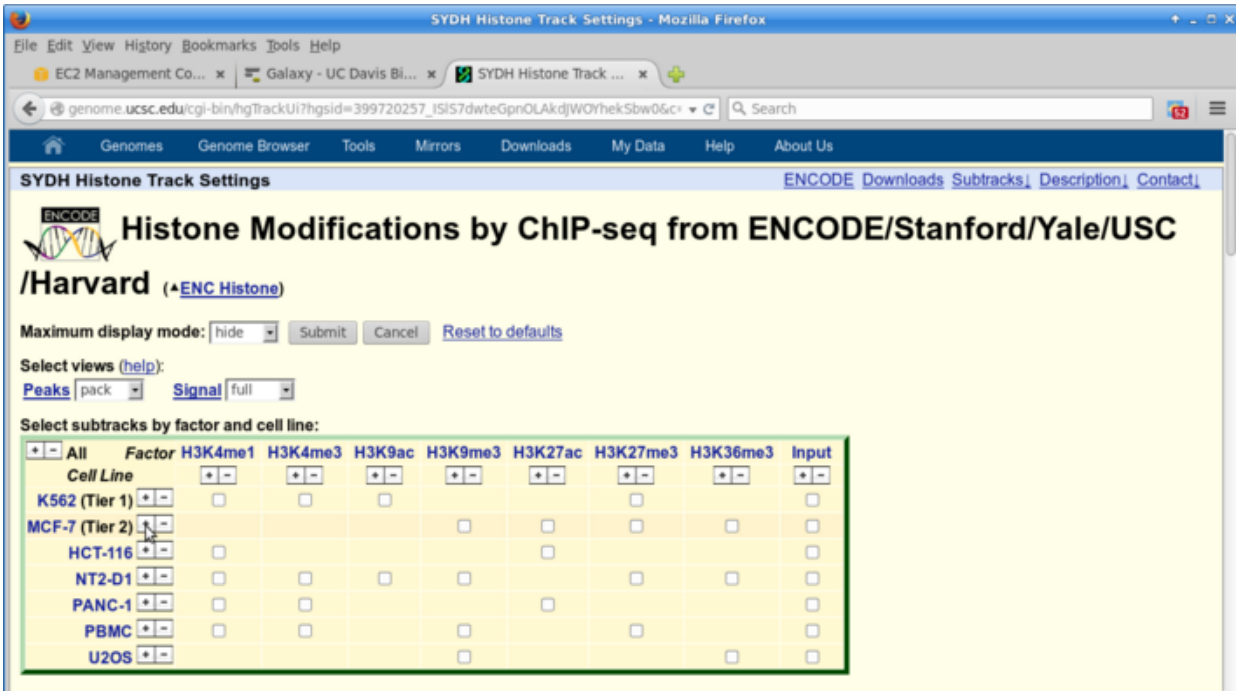
Results

ChIP-seq

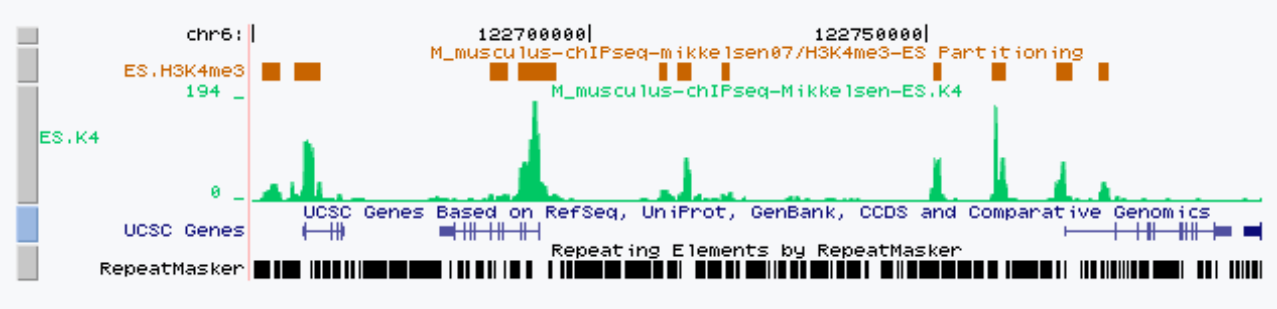
- ❑ Integrated Genomics Viewer (IGV): https://www.broadinstitute.org/igv/



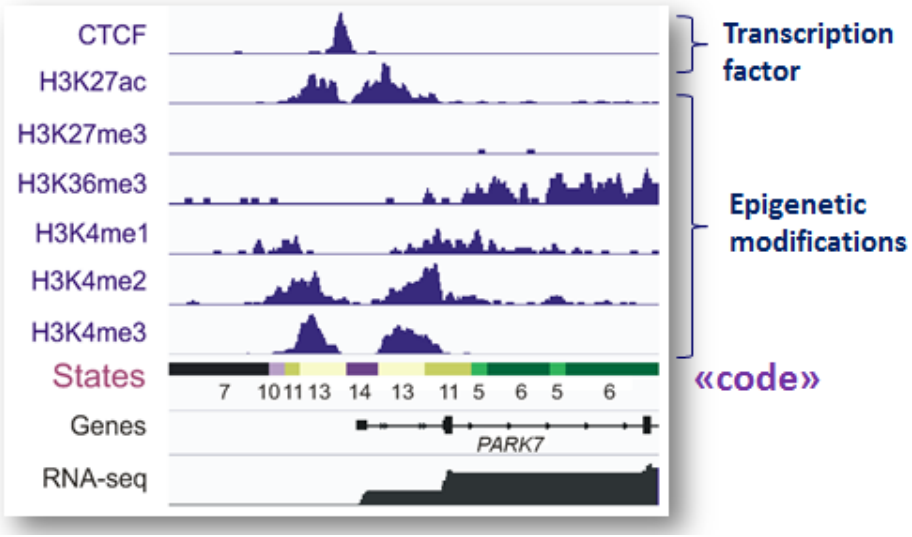
- UCSC Genome Browser: https://genome.ucsc.edu/



Example: H3K4me3 profiling in mouse ES cells

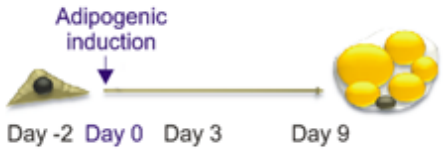


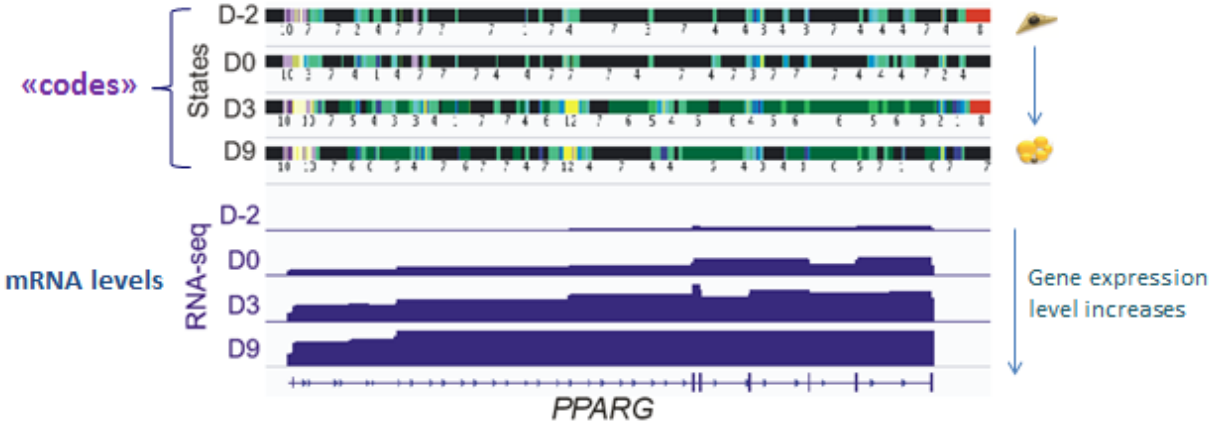
❑ Combinations of epigenetic modifications can be modeled
- ❑ Easier to visualize
- ❑ Easier to analyze at the level of the whole genome



Epigenetic patterns change during cell differentiation

Example: differentiation of adipose stem cells into adipocytes





Three international research programs are mapping epigenetic modifications

ENcyclopedia Of DNA Elements
http://www.genome.gov/encode/

http://www.blueprint-epigenome.eu/

http://fantom.gsc.riken.jp/