Molecular Cell, Volume *63*

Supplemental Information

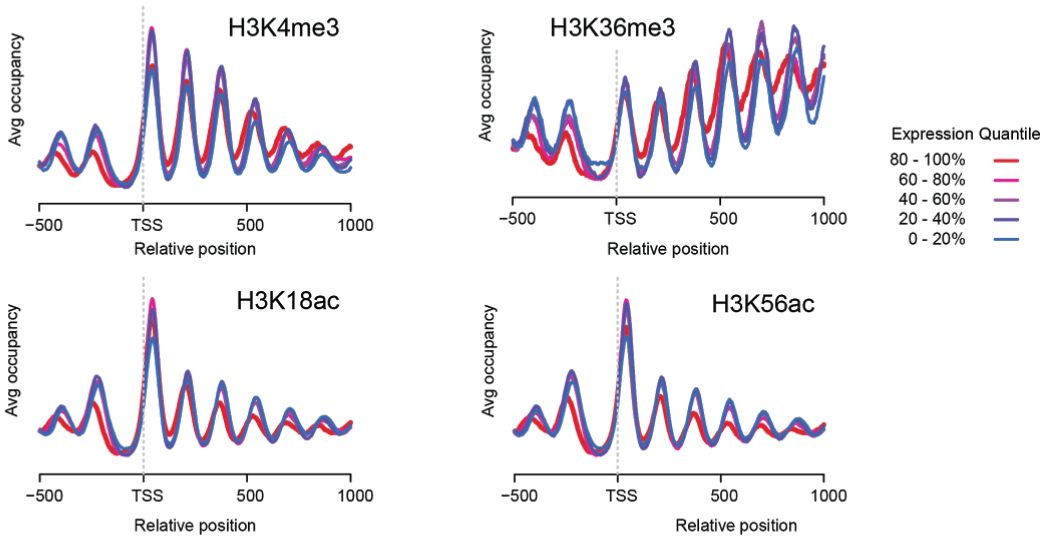
Elucidating Combinatorial Chromatin States at Single-Nucleosome Resolution

Ronen Sadeh, Roee Launer-Wachs, Hava Wandel, Ayelet Rahat, and Nir Friedman

Contents

Supplemental Figures and Table

A



B

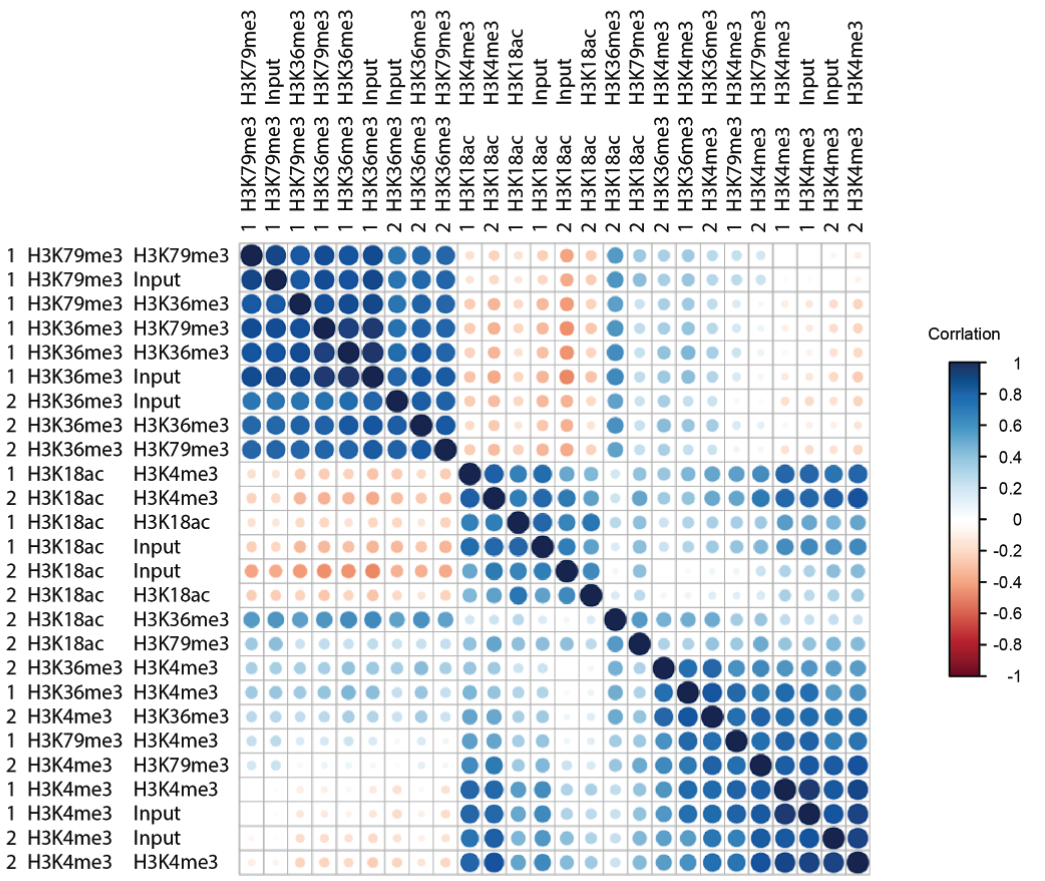


Figure S1: combinatorial-iChIP recapitulates standard ChIP-seq results and is reproducible. Related to Figure 1.

A Metagene profiles of the individual combinatorial-iChIP samples (“input”). Each group of genes (sorted by expression quantiles) are averaged in TSS-aligned manner. **B** Correlation plot of nucleosome coverage of different combinatorial-iChIP tracks from WT strain. Each name consists of batch number (1 - histone mutants, 2 - SET2/RPD3 KOs), first IP, and second IP (“input” first IP without second step). Correlation is denoted by color and by magnitude of the circle. Correlation was computed between coverage counts over nucleosomes (see Methods).

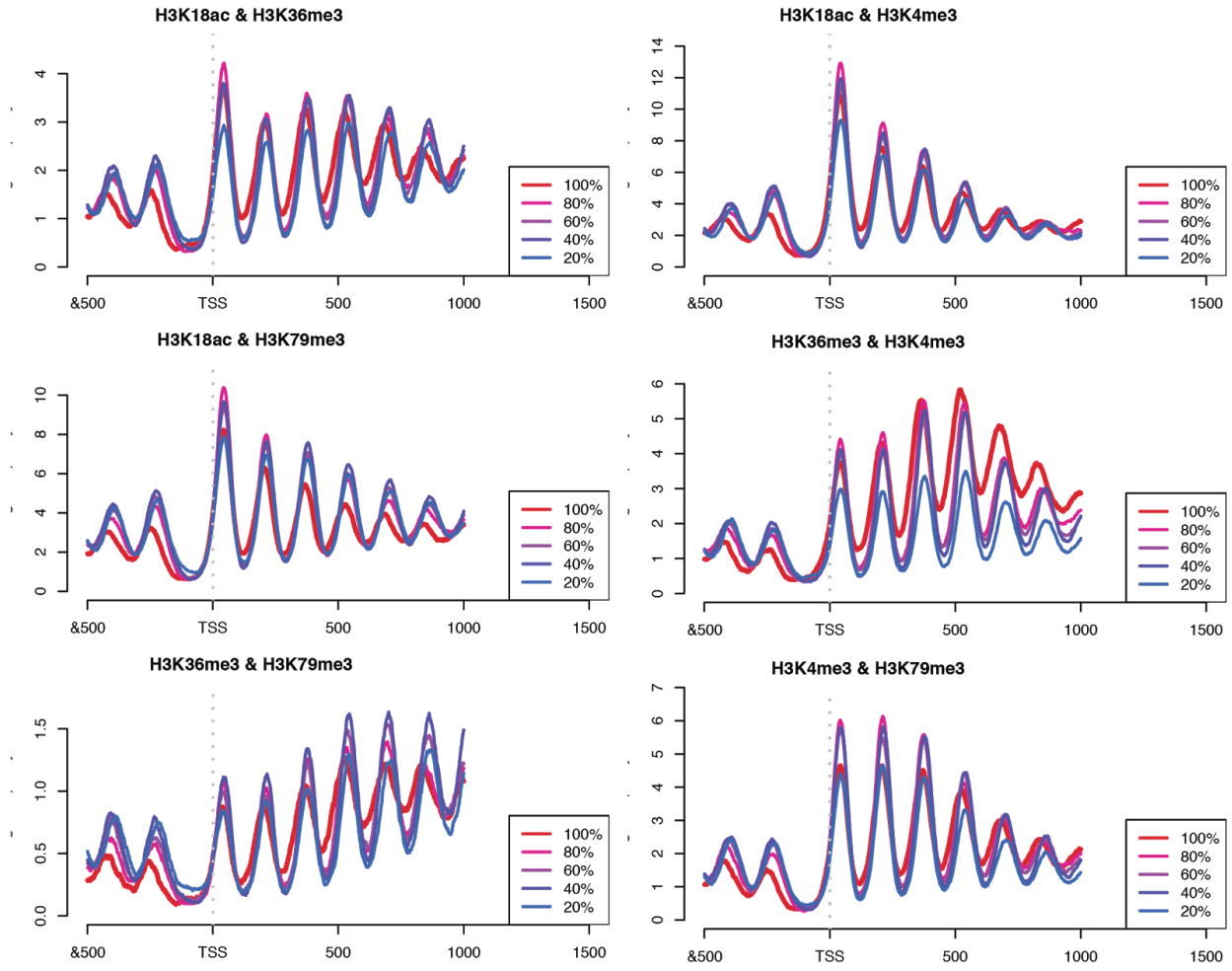


Figure S2: combinatorial-iChIP genic patterns. Related to Figure 1.

Metagene profiles of combinatorial-iChIP. Each group of genes (sorted by expression quantiles) are averaged in TSS-aligned manner.

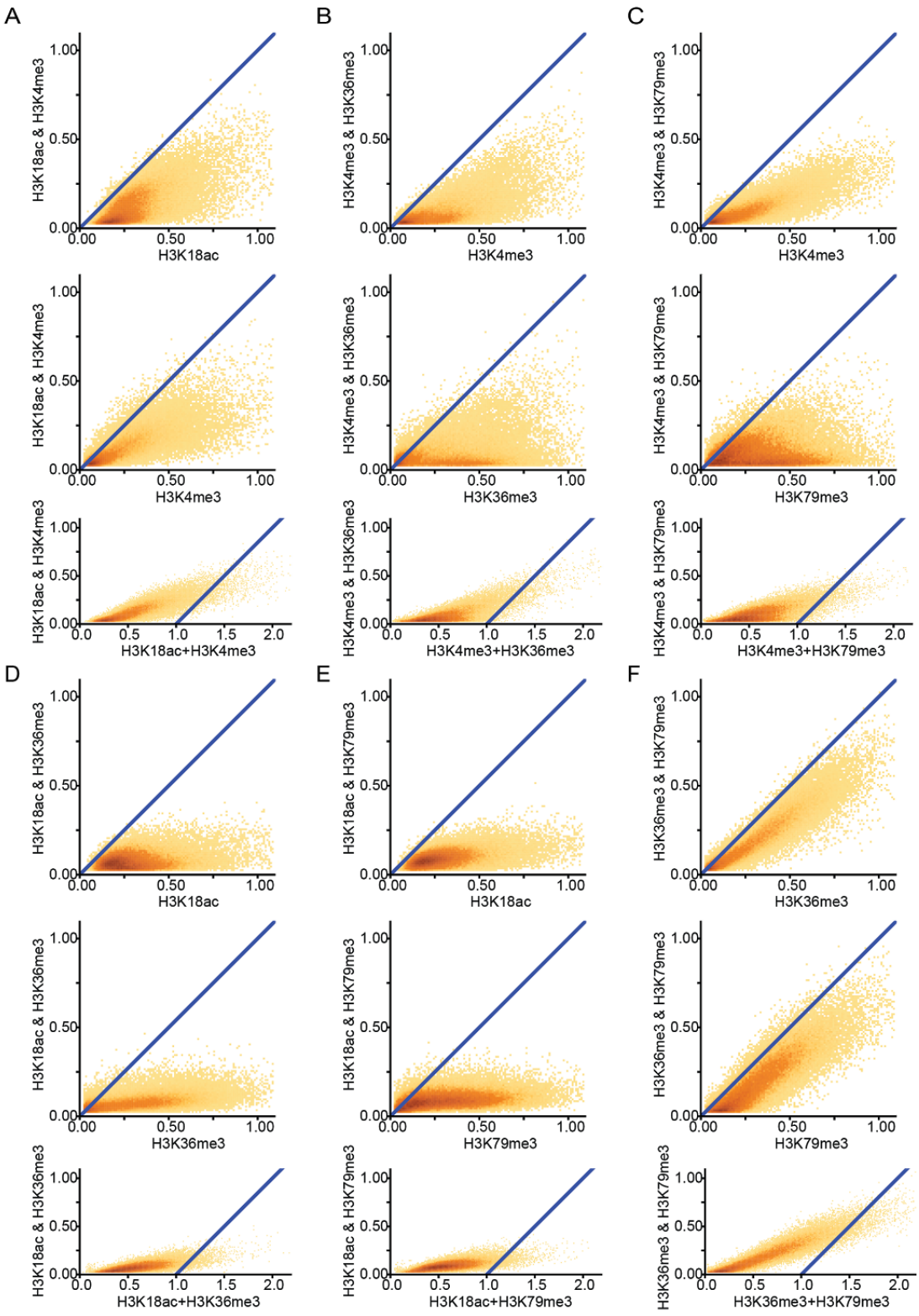


Figure S3: Quantitative combinatorial-iChIP patterns. Related to Figure 3

A-F Panels corresponding to Figure 2B-D for each pair of marks

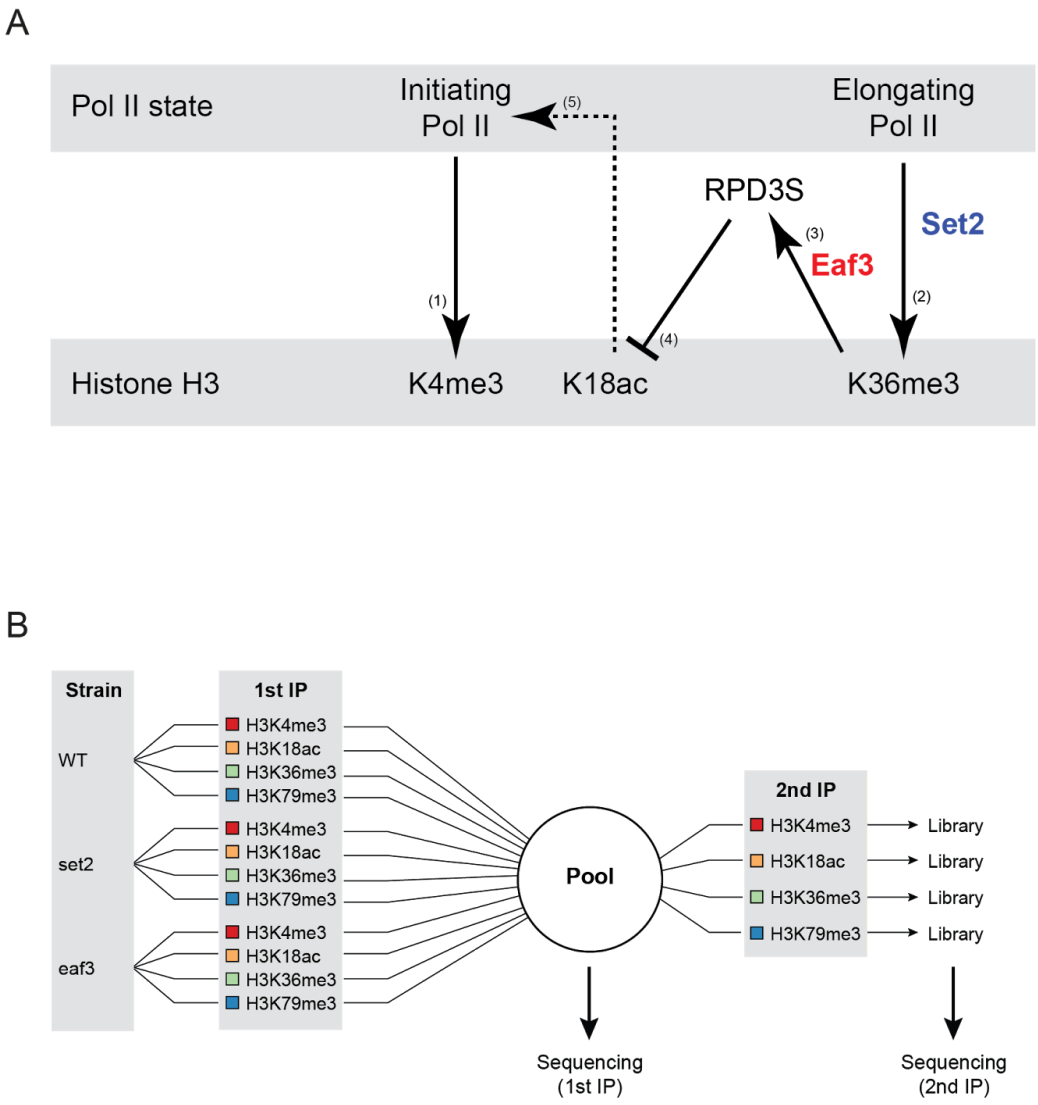


Figure S4: The Set2-RPD3 pathway. Related to Figure 5.

A Summary of the positive and negative relations between Pol II states, RPD3S recruitment, Histone H3 modifications, and turnover (Rando and Winston 2012). Solid lines represent established connections: (1) H3K4me3 is deposited by Set1 recruited to initiating Pol II; (2) H3K36me3 is deposited by Set2 recruited to elongating Pol II (Li et al. 2003); (3) RPD3S is recruited to active gene bodies by RNA Pol II and likely gets activated by binding of its Eaf3 subunit to H3K36me3 (Drouin et al. 2010; Govind et al. 2010); (4) RPD3S deacetylates H3 tail lysines; (5) Deacetylated H3 nucleosomes repress transcriptional initiation. **B** Outline of combinatorial-iChIP experiment to probe this pathway.

Table S1: Unique read counts for each sample. Related to Figure 2.

For each sample, listed the number of reads and the number of unique reads in two size ranges 50-220bp and 220-1000bp.

Detailed Combinatorial-iChIP Protocol

Yeast growth

- Yeast cells were grown in YPD media at 30ºC with constant shaking to OD 0.6-0.8.

Cells fixation

- Add formaldehyde (1% final concentration) directly to cells and rotate 15 minutes at RT.
- Add glycine (0.125M final concentration from 2.5M stock) to the fixed cells and rotate 5 minutes at RT.
- Pellet cells by centrifugation (4000g, 5 minutes, 4ºC).
- Resuspend the cells pellet in 1ml cold ddH2O supplemented with a EDTA-free protease inhibitors cocktail (Roche).
- Pellet cells by centrifugation (4000g, 5 minutes, 4ºC).

Note: It is possible at this point to flash freeze the cell pellet and store at -80ºC.

Spheroplasting

- Resuspend the cell pellet in 100µl buffer Z.
- Add zymolyase 20T (Seikagaku) at 0.3 - 1 units per 1 ml of original culture (2x10^7 cells/ml). Incubate cells at 30ºC for 20 minutes.
- To test spheroplasting efficiency, remove 1-5µl of the cells into 1% SDS solution and check the cells under the microscope. Estimate spheroplasting efficacy (counted cells/expected # of cells*100). This number should be lower than 5%. If it is higher than 5% continue incubating cells at 30ºC and test again.

MNase digestion

Note: It is important to calibrate your MNase digest by running a titration experiment to achieve the desired nucleosome pattern (see digest evaluation below). This calibration should be done for each batch of enzyme and each strain. We usually aim for ~ 80% mono nucleosomes with no or minimally apparent over digested nucleosomes.

- Pellet spheroplasts (6500 g, 10 minutes), remove the supernatant, and resuspended in NP buffer at final concentration of $0.2x10^7$ cells/µl.
- Prewarm samples to 37ºC for 5 minutes.
- Add 12.5 units/ml of MNase (Worthington) diluted in NP buffer for 20 minutes at 37ºC.
- Remove tubes to ice and add one volume of ice cold MNase stop buffer.

- Keep tubes on ice for 10 minutes, vortexed 3 x 10 seconds (this step increase chromatin yield but can be skipped when using 96 well plates to avoid sample spilling).
- Centrifuged samples (16,000g, 10 minutes, 4ºC - for 96 wells plates we centrifuge 30 minutes at 5000g).
- Remove the supernatant containing the chromatin to fresh tubes.

MNase digest evaluation

- Remove 8µl of MNase digested chromatin to a fresh tube.
- Add 2µl of 0.5ug/µl RNase A and incubate for for 30 minutes at 37ºC.
- Add 40µl of proteinase K solution (10 mM Tris pH 8.0, 5 mM EDTA, 300 mM NaCl, 0.6% SDS) containing 50 units of proteinase K.
- Incubate for 2 hours at 37ºC, and for 12-16 hours at 65ºC.
- Isolate DNA by 2X SPRI beads cleanup, resuspend DNA in 20µl of 10mM Tris pH-8.0, and measure DNA concentration by Qubit.
- Expect total of ~ 50-100 ng of DNA
- Visualize DNA by TapeStation (Agilent) or agarose gel. For nucleosomes ChIP we aim for ~ 75-80% mono nucleosomes.

Chromatin immobilization

Note: The amount of DNA used for ChIP can vary and depends on the abundance of the target and antibody yield and specificity. We find that using chromatin from ~$1x10^7$ cells (~0.5ml of yeast culture at log phase) gives good results for most chromatin marks.

- Remove MNase digested chromatin samples (from ~$1x10^7$ cells) to a fresh 96 well plate and adjust the volume to 80 µl with ice cold RIPA buffer and antibody (for specific details see antibodies section below).
- Incubate the samples with gentle tumbling for 2 hours at 4ºC.
- While samples are incubated wash protein G dynabeads three times in RIPA buffer (20µl beads per sample). Resuspend beads to the original volume with RIPA buffer.
- Centrifuge the samples shortly, add 20µl of protein G to each sample, and incubate the samples with gentle tumbling for an additional hour at 4ºC.
- Magnetize the samples and wash: 6 X RIPA buffer, 3 X RIPA 500 RIPA buffer containing 500 mM NaCl), 3 X LiCl wash buffer. It is possible to use vacuum for these washes.
- **Important**: From this point on DO NOT use vacuum to aspirate the supernatant.
- Wash the beads 3 X 10 mM Tris pH 7.5 supplemented with protease inhibitors.

Chromatin barcoding and release

These steps are a modified version of the iChIP protocol (Lara-Astiaso et al. 2014).

End repair:

- Resuspend the immobilized chromatin in 20 µl of 10 mM Tris pH 7.5.
- Add 40 µl of end repair and mix well by pipetting.
- Incubated for 22 minutes at 12°C followed by 22 minutes at 25°C.
- Magnetize beads and wash once in 150 µl 10 mM Tris pH 8.0 and resuspended the beads in 40 µl of 10 mM Tris pH 8.0.

A base addition:

- Add 20 µl of A-Base mix [10 mM Tris pH 8, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, 0.58 mM dATP, 0.75 units Klenow fragment (NEB)] to the beads and mix well by pipetting.
- Incubate samples at 37°C for 30 minutes.
- Magnetize beads and wash once in 150 µl 10 mM Tris pH 8.0 and resuspended the beads in 18 µl of 10 mM Tris pH 8.0.

Adapters ligation:

- Add 5µl of indexed adapters (Blecher-Gonen et al. 2013) to each sample mix well by pipetting. Add 34µl of ligation mix [29 µl of 2X quick ligase buffer (NEB), 5µl quick ligase (NEB)], mix well by pipetting, and incubate at 25°C for 45 minutes.
- Magnetize beads and wash once in 150 µl 10 mM Tris pH 8.0

Chromatin release:
This step releases bound chromatin and inactivates the antibodies used in the first ChIP.

Note: From this point it is important to keep samples at temperature higher than 15°C to prevent SDS precipitation.

- Resuspend beads in 12.5 µl fresh 0.1 M DTT and incubate at RT for 5 minutes.
- Add 12.5 ul of freshly prepared 2X Chromatin Release Buffer, mix well by pipetting, and incubate at t 37°C for 45 minutes.
- Pool all the samples that are going to be ChIPed together during the second ChIP into a 1.5ml tube.
- Magnetize the pooled samples and remove the supernatant into a fresh 1.5 ml tube.
- Centrifuged max speed, 5 minutes, 15-20°C, and remove the supernatant into a 15 ml falcon tube.
- Measure the volume of the pooled sample and dilute by adding 9 volumes of dilution buffer.
- Load the diluted samples on an amicon filter (Millipore UFC905024) containing 12 ml of Amicon buffer. We usually load ~2 ml of diluted sample per Amicon filter.

Note: In case that many samples are pooled from the first ChIP it is possible to leave the concentrated sample in the Amicon filter after sample concentration and reload it with additional 12 ml of Amicon buffer and 2 ml of pool sample. This will allow for a more concentrated chromatin for the second chIP.

- Centrifuge at 2000g, 15-20°C until ~ 0.25ml of concentrated sample is left in the filter (5-10 minutes).
- Pool the concentrated samples together and add 1 volume of equilibration buffer and vortex the samples for 10 seconds.

Note: At this point samples can be flash frozen and stored at -80°C or proceed to the next step.

Second ChIP:

Note: A critical point in this protocol is to use sufficient amount of barcoded chromatin from the first ChIP in the second ChIP step to obtain enough barcoded DNA for efficient library amplification. The amount of barcoded chromatin that should be used is dependent on factors such as antibody yield, modification abundance, and adapter ligation efficiency and should be determined empirically for each experiment. However, we find that pooling ~ 5 samples from the first ChIP gives good results in most cases. This means, for example, that 20 pooled samples from the first ChIP should be sufficient for ~ 4 different antibodies used during the second ChIP.

- Divide the pooled barcoded chromatin into fresh 96 well plate or PCR tubes according to the number of antibodies to be used for the second ChIP step.
- Adjust the volume to 80µl with RIPA buffer and the antibody.
- Chromatin immobilization and washes were done as for the first ChIP.
- Resuspend the beads in 23µl of chromatin elution buffer supplemented with 2µl of 0.5µg/µl RNase A and incubate for for 30 minutes at 37ºC.
- Add 24µl of chromatin elution buffer supplemented with 1 µl of proteinase K (50 units/µl, epicenter).
- Incubate for 2 hours at 37ºC, and for 12-16 hours at 65ºC.
- Isolate DNA by 0.8 X SPRI beads cleanup, resuspend DNA in 25µl of 10mM Tris pH-8.0.

Library amplification:

- Remove 23µl of the eluted chromatin into a fresh PCR tube. Add add 2µl of barcoded amplification primers mix (Different barcode for each antibody used in second ChIP, see primer sequence below) and 25µl of 2 X Kapa hifi hotstart ready mix.
- Run PCR for 12-16 cycles.
- The number of PCR cycles depends on the second ChIP yield. It is recommended to use the lowest number of cycles that yield sufficient library for next generation sequencing to reduce PCR duplicates.

- Isolate DNA by 0.8 X SPRI beads cleanup, resuspend in 20µl of 10mM Tris pH-8.0, and measure DNA concentration by Qubit.
- The total amount of amplified DNA can vary between 40-1000 ng of DNA.
- Visualize DNA by TapeStation. A good library should display discrete bands at MW higher in ~140bp than the original MNase digested DNA used for ChIP with no or minimal amount of adapter dimers at 140bp.

Forward amplification primer:
5' - AATGATACGGCGACCACCGAGATCTACAC [8bp barcode] ACACTCTTTCCCTACACGAC
Reverse amplification primer:
5' - CAAGCAGAAGACGGCATACGAGAT

Note: We find that different antibodies have varying chromatin barcoding efficiency that does not always correlate with IP efficiency. This is likely due to the mode of binding of the antibody to the nucleosome and/or the antibody-antigen binding affinity. As a result, we recommend testing the barcoding efficiency for each antibody. If barcoding efficiency is low, it is possible to perform several repeats of the 1st ChIP and pool the samples prior to the 2nd ChIP.

Buffers:

Buffer Z: 1 M sorbitol, 50 mM Tris 7.4, 10 mM β-mercaptoethanol (freshly added).

NP buffer: 10 mM Tris pH 7.4, 1 M sorbitol, 50 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, and 0.075% NP-40, freshly supplemented with 1 mM β-mercaptoethanol, 500 µM spermidine, and EDTA-free protease inhibitor cocktail.

MNase stop buffer: 220 mM NaCl, 0.2% SDS, 0.2% sodium deoxycholate, 10 mM EDTA, 2%,Triton X-100, EDTA-free protease inhibitor cocktail.

RIPA buffer: 10 mM Tris pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, EDTA-free protease inhibitor cocktail.

LiCl wash buffer: 10 mM Tris pH 8.0, 0.25 M LiCl, 0.5% NP-40, 0.5% Sodium Deoxycholate, 1 mM EDTA, EDTA-free protease inhibitor cocktail

End Repair mix: 50 mM Tris pH 7.5, 10 mM MgCl2, 10 mM DTT, 10 mM ATP, 10 mM each dATP , dCTP, dGTP, dTTP, 0.375 units T4 polynucleotide kinase (NEB), 0.01 units T4 polymerase (NEB).

A-Base mix: 10 mM Tris pH 8, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, 0.58 mM dATP, 0.75 units Klenow fragment (NEB).

Chromatin Release Buffer: 500 mM NaCl, 2% Deoxycholate, 2% SDS, 2 mM EDTA, 2X EDTA-free protease inhibitor cocktail.

Dilution buffer: 100 mM Nacl, 10 mM Tris pH 8, 1 mM EDTA, EDTA-free protease inhibitor cocktail

Amicon buffer: 0.1% SDS, 0.1% Sodium Deoxycholate, 10 mM Tris pH 8,, 1 mM EDTA, 140 mM NaCl

Equilibration buffer: 2% Triton X-100, 0.1% SDS, 0.1% Sodium Deoxycholate, 10 mM Tris pH 8, 1 mM EDTA, 140 mM NaCl, 2X EDTA-free protease inhibitor cocktail.

Chromatin elution buffer: 10 mM Tris pH 8.0, 5 mM EDTA, 300 mM NaCl, 0.6% SDS

Antibodies

The following antibodies were used in this study:

*For each antibody we used qPCR to determine the amount of antibody that results in the best yield /to background ratio.

Antigen	Catlog #	µg antibody / ChIP
H3	ab1791 (Abcam)	3
H3K4me3	07-473 (Millipore)	1
H3K18ac	07-354 (Millipore)	3
H3K36me3	ab9050 (Abcam)	3
H3K79me3	ab2621 (Abcam)	1