**Chromatin Immunoprecipitation from *Drosophila* heads**

**Experimental Conditions for ChIP-Seq Samples**

For the ChIP-seq experiments, about 200 fresh-frozen fly heads were used per assay. There were three bioreplicates per species and condition. The two species of Drosophila heads used were Drosophila *melanogaster* and Drosophila *simulans*. They were divided by sex and then per treatment (Ethanol-treated and No-Ethanol-treatment) for a total of 24 samples as depicted below:

**Protocol**

*Preparing Homogenate*

Fresh-frozen fly heads were transferred with a 5 ml serological pipette into a Pink Eppendorf RNA Lysis Kit tube (Next Advance, Cat. # PINKE1-RNA) using 1 ml NEB Buffer (10 mM HEPES pH 8.0, 0.1 mM EGTA pH 8.0, 0.5 mM EDTA pH 8.0, 10 mM NaCl, 0.5 % v/v Tergitol NP-10, 1 mM DTT, 1X cOmplete Protease Inhibitor Cocktail (Roche)). The samples were then centrifuged at 10,000 rpm (Eppendorf 5418) for 5 min to spin heads down and 800 µl NEB Buffer was carefully removed without bringing up any heads. The samples with the remaining 200 µl NEB Buffer were homogenized with the Bullet Blender Storm (Next Advance, Cat. # BBY24M) at speed 10 for 5 min (1 min intervals; rest sample on ice) in a cold room. The homogenate was transferred to a fresh tube and the total volume increased to 1 ml with NEB Buffer. Afterwards, the samples were removed from ice to reach room temperature (RT) for 5 min.

*Crosslinking Proteins to DNA*

Formaldehyde was added to the samples to a final concentration of 1% (27 μl of 37% formaldehyde), and the tubes rotated for 10 min at room temperature (RT). To quench the formaldehyde, glycine solution was added to a final concentration of 125 mM (51 μl of 2.5 M glycine). The sample tubes were rotated at RT for 5 min and then kept on ice moving forward. Following centrifugation at 10,000 rpm (Eppendorf 5415R) for 5 min at 4°C, the supernatant was removed, and the pellet washed twice with 1 ml ice-cold PBS before resuspension in 200 μl 0.3% SDS-RIPA buffer (1% v/v NP-40, 0.5% w/v Sodium Deoxycholate, 0.3% w/v SDS, 1X Protease Inhibitor Cocktail in 1X PBS). The samples were transferred into 1.5 ml Bioruptor Plus TPX microtubes (Diagenode, Cat. # C30010010-50) and sonicated with BioRuptor (Diagenode, Cat. # UCD-200) in High Power setting for 15 min (5 min intervals; 30 sec ON, 30 sec OFF). After centrifugation at 13,200 rpm for 15 min at 4°C, the supernatant was transferred to a fresh tube followed by the addition of 800 µl 0.05% SDS-RIPA buffer (1% v/v NP-40, 0.5% w/v Sodium Deoxycholate, 0.05% w/v SDS, 1X Protease Inhibitor Cocktail in 1X PBS). Each sample was split into two 450 μl (for immunoprecipitation) and one 60 μl (for Input) aliquots and all samples except the Input aliquot were stored at -80°C.

*Verifying Sheared Input DNA*

To the Input tube (60 μl aliquot), 2 μl Proteinase K (5PRIME, Cat. # 2900405) was added to start the DNA purification overnight in an Eppendorf 1.5 ml Thermomixer R (65°C; 1,400 rpm; 5 min every hr). The DNA was purified with Monarch® PCR & DNA Cleanup Kit (5 µg) (New England BioLabs, Cat. # T1030L) and eluted in 20 μl 1X TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0). The samples were analyzed by Thermo Scientific NanoDrop 1000 Spectrophotometer for quantity and by Agilent 2200 TapeStation System to verify that the chromatin was sheared to a fragment size of 200-400 bp, using High Sensitivity ScreenTape D1000 (Agilent, Cat. # 5067-5584).

*Immunoprecipitation*

2 μl of H3K4me3 antibody (EMD Millipore, Cat. # 04-745) or H3K27me2me3 antibody (Active Motif, Cat. # 39535) was added to 450 μl aliquot, and the tube rotated at 4oC overnight. 1 ml PBS/BSA (100 mg in 20 ml 1X PBS) and 20 μl Protein A/G Magnetic Beads (ThermoFisher Scientific, Cat. # 88803) were added to a fresh tube and the beads washed twice on a magnetic stand with 1 ml PBS/BSA. To recover antibody-bound chromatin complexes, the antibody-bound chromatin sample was incubated with the pre-washed protein A/G magnetic beads for 2 hr at 4oC. The sample was then placed on a magnetic stand and the supernatant was discarded. 1 ml of LiCl Wash Buffer (100 mM Tris pH 7.5, 1 mM EDTA pH 8.0, 500 mM LiCl, 1% v/v NP-40, 1% w/v Sodium Deoxycholate, 1X Protease Inhibitor Cocktail) was added to wash the beads for 3 min at RT and the sample was resuspended and transferred into a fresh tube. The beads were washed with LiCl Wash Buffer for a total of 5 times and once with 1 ml 1X TE buffer, incubating on a rotator at RT for 3 min for each wash.

For the first elution, the beads were resuspended in 75 μl fresh IP Elution Buffer (1% w/v SDS, 100 mM NaHCO3; prepare fresh for each experiment)and placed on a thermomixer for 2 hr (65°C; 1,400 rpm; 5 min every hr). Afterwards, the tubes were placed on a magnetic stand and the supernatant containing the immunoprecipitated DNA was collected and stored at 4 oC overnight. For the second elution, 75 μl IP Elution Buffer was added to the beads and the tubes incubated in the thermomixer overnight as before. The next day, the first elution was warmed for 5 min to 65°C before combining with the second elution. Then, the DNA was purified using Monarch® PCR & DNA Cleanup Kit (5 µg) and eluted in 20 μl 1X TE Buffer. For quality control, 1:2 dilution (1X TE Buffer) with 5 μl DNA was prepared for all samples, both input and immunoprecipitated samples. 1 μl per 10 μl ChIP-qPCR analysis were done. The remaining 15 μl of the undiluted sample was used for library preparation.

**Library Preparation**

96 libraries were prepared using the following kits: NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (NEB #E7645S/L) and NEBNext® Multiplex Oligos for Illumina® (NEB #E6609S/L). 35 μl 1X TE was added to 15 μl samples (50 μl total) for library preparation following the manufacturer’s protocol. The libraries were prepared in sets of 8 with WTB16 iSLK DNA as a positive control and water as a negative control. For the adaptor ligation step, a 1:25 adaptor dilution was used. The cleanup of adaptor-ligated DNA was performed without size selection. The PCR amplification of adaptor-ligated DNA was run in 8 cycles.

The 72 ChIP-seq libraries were sequenced by Illumina paired-end sequencing multiplexed in one lane (without the controls).

**References**

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