Appendix C

General Protocol for Chromatin Immunoprecipitations[1]

C1 Isolation and preparation of chromatin

Cells were plated on 15 cm diameter dishes (30 milliliters each dish) and grown under the conditions described for each experiment. When the cells were ready to be harvested, media was emptied and replaced with 20 milliliters 1% formaldehyde (Ted Pella) in the cells' normal growth media without FBS. Cells were fixed for ten minutes, rinsed with cold phosphate buffered saline, rinsed with a stop-fix solution containing glycine (Active Motif), rinsed again with cold phosphate buffered saline, then scraped using a razor in ice-cold phosphate buffered saline containing PMSF and protease inhibitors. Pooled cells were centrifuged for 10 minutes at 720 RCF, 4°C. Cell pellets were suspended in 1 milliliter cold lysis buffer (Active Motif) supplemented with PMSF and protease inhibitors and left on ice for 30 minutes. Cells were dounce-homogenized with 10 strokes to aid in nuclei release. Cells were transferred to 14 milliliter conical tube and centrifuge for 10 minutes at RCF 2400, 4°C to pellet the nuclei. Pellets were resuspended in 1.4 milliliters shearing buffer (Active Motif) supplemented with protease inhibitors, and sheared four times each using a digital Branson Sonifier under the following settings: 25% power, 30 seconds. Samples were kept on ice between sonications. Sheared DNA samples were centrifuged for 12 minutes at 10,000 to 15,000 rpm in a bench-top micro-centrifuge at 4°C. Supernatants containing the sheared

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[1] * *This protocol is based on several sources including the Active Motif Chip-it Kit and D. Herman et al.*

Herman, D., Janssen, K., Burnett, R., Soragni, E., Perlman, S. L., & Gottesfeld, J. M. (2006) *Nat Chem Biol.* **2**, 551-558.

chromatin were stored at -80°C if necessary, or carried on to the next step. For initial experiments, sonication efficiency was checked using agarose gel electrophoresis.

C2 Immunoprecipitations and analysis of enrichment

To pre-clear chromatin samples, 650 microliters of each chromatin sample was added to 60 microliters of a 1:1 mixture of Protein A Agarose/Salmon Sperm DNA and Protein G Agarose/Salmon Sperm DNA (Upstate) and incubated at 4°C on a gentle inverting rotor for 1.5 hours. Beads were twice pelleted gently at 3000 rpm for 30 seconds and supernatants then divided into aliquots of 300 microliters each. To one aliquot of each chromatin sample was added up to 10 micrograms of appropriate antibody (the antibody immunoprecipitated sample); to the other aliquot was added no antibody (the mock-immunoprecipitated control sample). Both samples were incubated overnight at 4°C on a gentle inverting rotor. Subsequently, 100 microliters of a 1:1 mixture of Protein A Agarose/Salmon Sperm DNA and Protein G Agarose/Salmon Sperm DNA (Upstate) was added to the antibody and mock- immunoprecipitated samples at 4°C on a gentle inverting rotor for 2 hours. Immunoprecipitated complexes were pelleted gently at 3000 rpm for 30 seconds. Pelleted beads were then washed with low salt buffer (1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris pH 8) for 5 minutes at 4°C on a gentle inverting rotor. Beads were pelleted again, and the low salt wash was repeated two more times. Beads were then washed with high salt buffer (1% Triton X-100, 0.1% SDS, 500 mM NaCl, 2 mM EDTA pH 8, 20 mM Tris pH 8) for 5 minutes at 4°C on a gentle inverting rotor. Beads were pelleted, and DNA eluted with 150 microliters of elution buffer (1% SDS, 100 mM $NaHCO_3$) for 10 minutes at 65°C. Beads

were eluted again as described and elution fractions pooled to 300 microliters. Samples elutions were treated with 5 microliters of 20 milligrams/milliliters Proteinase K for 45 minutes at 37°C. Cross-links were reversed by incubation overnight at 65°C. Phenol-chloroform was used to extract DNA, and the DNA was then ethanol precipitated, dried, and resuspended in RNase/DNase free water. PCRs using primers flanking regions of interest were performed on an ABI 7300 instrument using SYBR Green PCR Master Mix reagents (Applied Biosystems) to measure amplifications. Antibody immunoprecipitated samples were compared to mock-immunoprecipitated samples to obtain fold-enrichments.