Chromatin assembly and transcriptional cross-talk in *Xenopus laevis* oocyte and egg extracts

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ABSTRACT Chromatin, primarily a complex of DNA and histone proteins, is the physiological form of the genome. Chromatin is generally repressive for transcription and other information transactions that occur on DNA. A wealth of post-translational modifications on canonical histones and histone variants encode regulatory information to recruit or repel effector proteins on chromatin, promoting and further repressing transcription and thereby form the basis of epigenetic information. During metazoan oogenesis, large quantities of histone proteins are synthesized and stored in preparation for the rapid early cell cycles of development and to elicit maternal control of chromatin assembly pathways. Oocyte and egg cell-free extracts of the frog *Xenopus laevis* are a compelling model system for the study of chromatin assembly and transcription, precisely because they exist in an extreme state primed for rapid chromatin assembly or for transcriptional activity. We show that chromatin assembly rates are slower in the *X. laevis* oocyte than in egg extracts, while conversely, only oocyte extracts transcribe template plasmids. We demonstrate that rapid chromatin assembly in egg extracts represses RNA Polymerase II dependent transcription, while pre-binding of TATA-Binding Protein (TBP) to a template plasmid promotes transcription. Our experimental evidence presented here supports a model in which chromatin assembly and transcription are in competition and that the onset of zygotic genomic activation may be in part due to stable transcriptional complex assembly.

KEY WORDS: *histone, RNA polymerase II, TATA-binding protein, transcriptional complex*

Introduction

Chromatin is the complex of DNA and histones that is the biological form of the eukaryotic genome (Shechter and Allis, 2007). Chromatin is generally repressive for transcription of RNA from the genome. During early metazoan development, chromatin assembly and transcriptional activation and repression are simultaneously acting to establish the embryonic epigenome and transcriptome, building a program essential for development and life of the organism. Chromatin and transcription are interrelated, especially during these important regulatory transitions that occur during early metazoan embryogenesis. The organization of DNA into nucleosomes is critical as it not only allows the long stretch of DNA to be housed inside the nucleus but as it also serves as the fundamental regulatory center for gene expression (Bannister and Kouzarides, 2011, Das and Tyler, 2013).

Early development in animals is primarily controlled by maternal RNAs and proteins, including histones and histone chaperones that form chromatin. New zygotic control over the maternal program occurs at various stages depending on the species at a time point variously called the mid-blastula transition (MBT), maternal-to-zygotic transition (MZT), and zygotic genome activation (O'Farrell *et al.*, 2004, Schier, 2007, Tadros and Lipshitz, 2009). The MBT is typically the period of dramatic cell cycle changes as well as the beginning of differentiation. Since new zygotic transcription occurs during this time, a reasonable hypothesis is that the dramatic chromatin and transcriptional changes are critical for subsequent differentiation. We previously showed that substantial and elaborate changes in abundance of different core and linker histones and discrete classes of histone modifications occur during vertebrate MBT (Nicklay *et al.*, 2009, Shechter *et al.*, 2009a, Shechter *et al.*, 2009b, Wang *et al.*, 2014). Others have shown differences in chromatin assembly ability and rates in different oocyte and embryo contexts (Almouzni *et al.*, 1990, Almouzni *et al.*, 1991, Dimitrov *et al.*, 1993, Hair *et al.*, 1998, Prioleau *et al.*, 1995, Prioleau *et*
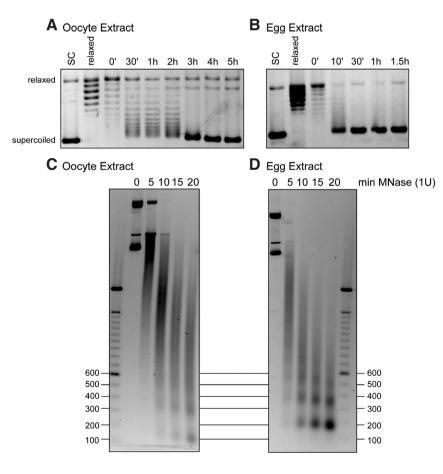

Abbreviations used in this paper: TBP, TATA-binding protein.

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Fig. 1. *Xenopus laevis* early embryology and extract functionality. (A) *Stereomicrographs of* Xenopus laevis *stage IV oocytes, eggs (stage 1), and Stage 2 through Stage 10 embryos as indicated. Timing of germinal vesicle breakdown (GVBD) and the onset of transcriptional repression is indicated.* **(B)** *Cell-free oocyte extract is prepared from a pool of dissected oocytes (stages II-VI, primarily the later stages as the early stage oocytes are lost), while cell-free egg extract is prepared from laid eggs. The table shows various activities present in the extracts (+, modest activity; +++++, high activity; X, no activity; check, activity).*

al., 1994, Wolffe *et al.,* 1993). A still unanswered question is the direct relationship between the distinct chromatin states and the transcriptional competence of that chromatin.

Xenopus frogs are commonly used vertebrate model systems for studying development, cell cycle, cancer, chromatin, and many other biological phenomena. In particular, studies in *Xenopus* cell free extracts and embryos have established robust biological pathways regulating chromatin assembly and transcription, however many questions about how maternal chromatin modifications and deposition machinery function to control gene expression remain. In this issue (Shechter, *co-submitted*), we provide a short review and overview of the literature on chromatin and transcription in early development and the significance of the cross-talk between these biological events. Here, we present new experiments testing how chromatin assembly and transcription are coincident and correlated with each other.

Results and Discussion

Utility of Xenopus extracts for studying transcription

Xenopus oocyte and egg extracts are prepared from dissected ovaries, prior to germinal vesicle breakdown and arrested in meiosis I, or laid eggs that have entered the somatic cell cycle (Fig. 1). Importantly, there is no active transcription in extracts from *Xenopus* eggs through the mid-blastula transition around developmental stage 8 (Fig. 1A) (Maller *et al.,* 2001, Newport and Kirschner, 1982), yet any DNA can be duplicated in the extract (Fig. 1B). We have used multiple reporter plasmids in the

Fig. 2. Plasmid chromatin assembly in oocyte and egg extracts. (A) *pG5ML plasmid was relaxed by Topoisomerase I and incubated in oocyte extract for up to 5 h as indicated. Reactions were stopped at indicated time, deproteinized and the DNA was run on an agarose gel and stained with EtBr. Supercoiled and relaxed positions are indicated.* **(B)** *pG5ML plasmid was relaxed by Topoisomerase I and incubated in egg extract for up to 1.5 h as indicated. Reactions were stopped at indicated time, deproteinized and the DNA was run on an agarose gel and stained with EtBr. Supercoiled and relaxed positions are indicated.* **(C)** *Plasmid DNA was incubated in oocyte extract for 2.5 h and reactions were treated with 1U of micrococcal nuclease (MNase) for 0, 5, 10, 15, or 20 min. Reactions were deproteinized and the DNA was run on an agarose gel and stained with EtBr. Marker positions are indicated in basepair length. Nucleosomes are 146bp to 200bp fragments.* **(D)** *Plasmid DNA was incubated in egg extract for 2.5 h and reactions were treated with 1U of micrococcal nuclease (MNase) for 0, 5, 10, 15, or 20 min. Reactions were deproteinized and the DNA was run on an agarose gel and stained with EtBr. Marker positions are indicated in basepair length. Nucleosomes are 146bp to 200bp fragments.*

assay presented here as any plasmid with a strong viral promoter (for RNA Polymerase II) will induce transcription in these conditions (Toyoda and Wolffe, 1992). We mainly used the plasmid pG5ML (An *et al.*, 2004, An and Roeder, 2004) and the commercial pC1-CMV-EYFP plasmid.

Chromatin assembly in oocyte and egg extracts

To clearly establish the difference in chromatin assembly and assembly rates, we first prepared *Xenopus laevis* oocyte and egg extracts as we had previously described (Fig. 1B) (Banaszynski *et al.*, 2010). We incubated oocyte and egg extract with supercoiled plasmid DNA as well as Topoisomerase I relaxed plasmid DNA. Products were stopped in SDS-containing solution and DNA was phenol-chloroform isolated at time points up to 5 h (oocyte extract) or 1.5 h (egg extract) and run on an agarose gel in the absence of ethidium bromide. Stained gels are shown in Figs. 2 A,B, with increased supercoiled DNA indicative of chromatin assembly. The rate of assembly was substantially faster in egg extract compared to oocyte extract.

To confirm that the supercoiled DNA product represented *bona fide* nucleosome assembly, we subjected the 2.5 h time point of the oocyte and egg extract chromatin assembly reactions to micrococcal nuclease (MNase) digestion. MNase specifically cleaves chromatin between nucleosomes and reveals the size and regularity of assembled chromatin by producing a ladder of 146-200bp DNA molecules, the typical size of nucleosome fragments. As shown in Fig. 2C, oocyte extract assembled chromatin digested by 1 unit of MNase resulted in a smear of DNA and a few regularly spaced but smaller fragments. This suggests either poor and incomplete assembly or irregular assembly. In contrast, chromatin assembled on a plasmid in egg extract exhibited a clear ladder of nucleosomal sized DNA fragments, indicating robust assembly (Fig. 2D).

Our conclusions from these initial experiments are that egg extract is more efficient at assembling chromatin than is oocyte extract. This is likely due to differences in regulation of histone chaperones, as we previously observed for Nucleoplasmin (Onikubo *et al.*, 2015), differences in post-translational modifications of core and linker histones (Nicklay *et al.*, 2009, Shechter *et al.*, 2009b), or some other modulation of remodeling factors, such as ISWI (Kikyo *et al.*, 2000). As with most work from *Xenopus* extracts due to the heterogeneity of the population of frogs, while there were batch-to-batch variations, egg extract was routinely more efficient than oocyte extract at assembling chromatin (*data not shown*).

The time course of chromatin assembly in egg extract revealed apparently complete supercoiling by 1 h. To further probe chromatin assembly rate and density, we probed assembled plasmid DNA in egg extract at 1 and 5 h with micrococcal nuclease. As shown in Fig. 3A, chromatin assembled at 1 h showed a repeating nucleosomal ladder, consistent with our experiment in Fig. 2D. However, chromatin assembled for 5 h was largely resistant to micrococcal nuclease, consistent with a large assembly of proteins on the chromatin, perhaps indicative of the artificial nature of this experimental setup. In embryos, cell divisions occur at 90 min after fertilization and then roughly every 45 min, so chromatin assembly would not persist for 5 h. Alternatively, there are large quantities of linker histones and HMG proteins in the egg and robust assembly

of these proteins onto chromatin may preclude MNase digestion.

Hair *et al.*, (1998) showed that titration of plasmid DNA into egg extract revealed a limited ability to supercoil DNA above 25 ng DNA per μl of egg extract, likely due to titration of histones from the extract. This observation of limiting histones in eggs was recently shown to be responsible for setting the DNA-to-cytoplasm ratio
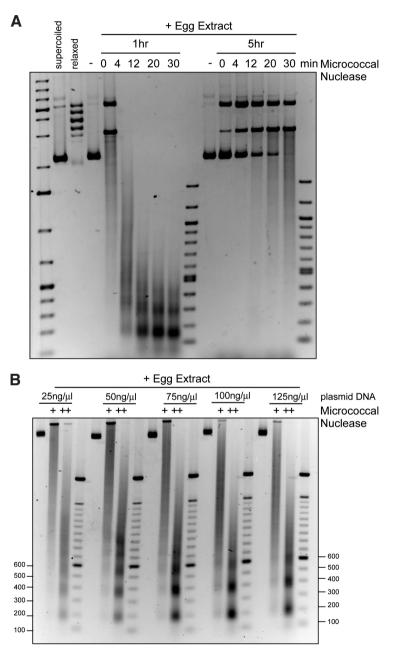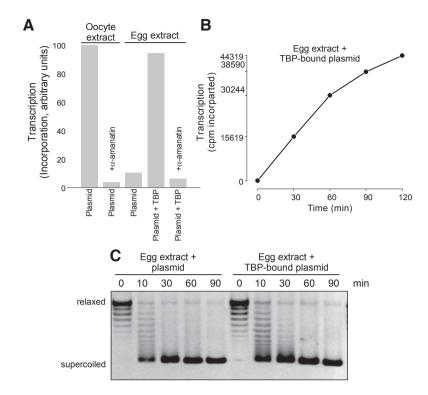

Fig. 3. Plasmid chromatin assembly in egg extract is concentration and time dependent. (A) *pG5ML plasmid was relaxed by Topoisomerase I and incubated in egg extract for 1 or 5 h, as indicated. Reactions were treated with 1U of MNase for 0, 4, 12, 20, or 30 min and the DNA was run on an agarose gel and stained with EtBr. Nucleosomal sized fragments were readily apparent in the 1 h incubation but not the 5 h incubation.* **(B)** *pG5ML plasmid was relaxed by Topoisomerase I and incubated in egg extract for 1 h at 25, 50, 75, 100, or 125 ng DNA / μl of egg extract, as indicated. Reactions were treated with 1 or 2 units of MNase and DNA was run on an agarose gel and stained with EtBr.*

A

Transcription (Incorporation, arbitrary units)

Oocyte extract   Egg extract

B

Transcription (cpm incorparted)

Egg extract + TBP-bound plasmid

Time (min)

C   Egg extract + plasmid   Egg extract + TBP-bound plasmid

0 10 30 60 90   0 10 30 60 90   min

relaxed

supercoiled

Fig. 4. Transcription in oocyte and egg extracts. (A) *pCMV-EYFP plasmid was incubated in oocyte or egg extracts in the presence of ^{32}P-α-ATP α-amanitin was added or the plasmid pre-incubated with TBP protein, as indicated. The reaction products were stopped and RNA extracted with TRIzol. Precipitated RNA was directly counted in a scintillation counter and resulting transcription radioactivity was scaled to the oocyte plasmid control.* **(B)** *pCMV-EYFP plasmid was incubated with TBP protein and then in egg extract in the presence of ^{32}P-α-ATP and the reactions stopped and RNA extracted at 0, 30, 60, 90, and 120 min after addition. Resulting radioactive incorporation is plotted (cpm) demonstrating transcription over time.* **(C)** *Topoisomerase-I relaxed pCMV-EYFP plasmid or plasmid pre-bound with TBP was incubated in egg extracts and stopped at indicated time. The reactions were deproteinized and the DNA was run on an agarose gel and stained with EtBr. Supercoiled and relaxed positions are indicated.*

in embryos, partly responsible for regulating timing of the MBT (Amodeo *et al.*, 2015). To test the extent of limited histones in micrococcal nuclease digestion, we titrated the concentration of DNA from 25 to 125 ng/μl of egg extract (Fig. 3B). We observed maximal laddering of nucleosomes at 50 ng/μl of egg extract, but clear nucleosomal formation at the highest concentration. This suggests that even though histones are limiting in the egg, nucleosomal formation still occurs but at reduced density.

Transcription in oocyte and egg extracts

To measure transcription in oocyte and egg extracts, we spiked extracts with ^{32}P-α-ATP and a plasmid with a CMV promoter and TATA box (pCMV-EYFP), incubated the extract, isolated total RNA with Trizol, and counted the resulting alcohol precipitated RNA in a scintillation counter. As shown in Fig. 4A, oocyte extract incubated with the plasmid exhibited robust incorporation of the radiolabeled ribonucleotide and this incorporation was blocked upon the addition of α-amanitin, a highly potent and specific RNA Polymerase II inhibitor (Wieland and Faulstich, 1978), consistent with Pol II dependent transcription off of the plasmid. In contrast, the same plasmid incubated in egg extract was not transcribed.

We confirmed the earlier studies that suggested that pre-incubating the plasmid template with TATA binding protein (TBP) stimulated transcription (Almouzni and Wolffe, 1995), which we also demonstrated was blocked by α-amanitin confirming the specific Pol II transcription (Fig. 4A). Plasmid incubated with exogenous TBP at the same time as egg extract was not transcribed (*data not shown*). We then performed a time course of incorporation into RNA on TBP-bound plasmid, showing a linear increase in incorporation (Fig. 4B).

These observations were all consistent with a potential model in which chromatin assembly was competitive with transcriptional complex assembly. Therefore we tested supercoiling on a plasmid

in egg extract or pre-bound with TBP and then incubated in extract and observed a modest reduction in the rate of supercoiling (Fig. 5A), consistent with a reduction in chromatin assembly as a mechanism of promoting transcription. To test this model with a different approach, we incubated *Xenopus* sperm chromatin in oocyte or egg extract and measured transcription (Fig. 5B). Sperm pre-incubated with TBP was transcribed while sperm in the absence of TBP was not transcribed. To measure chromatin assembly on sperm pronuclei, we isolated chromatin at time points after incubation in egg extract and immunoblotted for PCNA as an indication of DNA replication and histone H2A as an indication of histone deposition and chromatin assembly. As shown in Fig. 4E, we observed reduced PCNA and H2A abundance on the TBP-bound sperm chromatin as opposed to the sperm alone, consistent with our hypothesis. To independently confirm that the observed incorporation off of the template was indeed due to RNA Pol II, we isolated chromatin assembled in egg or oocyte extract and immunoblotted for Pol II and Pol II CTD Ser5 phosphorylation (S5ph), an indication of transcriptional initiation. We only observed S5ph in chromatin from oocyte extract, consistent with the observed transcriptional readout (Fig. 5C).

Implications for the regulation of chromatin assembly and transcription: a race between chromatin assembly and transcription?
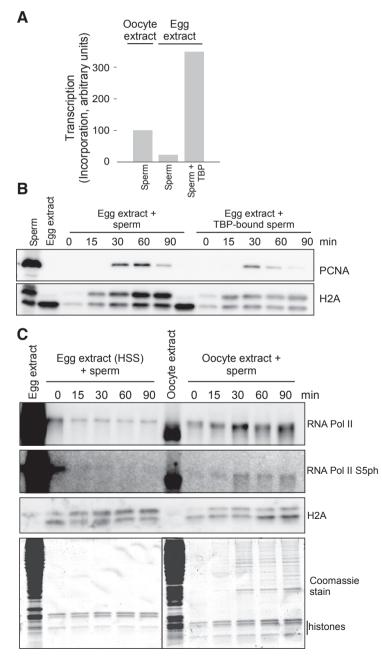
Here we demonstrated that *Xenopus* oocyte and egg extracts are compelling models for the crosstalk between chromatin assembly and transcription as they represent an extreme state not found in somatic cells. Since there are dramatic shifts between transcriptional activity and transcriptional quiescence, concomitant with altered chromatin assembly rates, the developing embryos and as recapitulated in the cell free extracts, these experimental systems can be directly probed for regulatory mechanisms. We confirmed earlier reports that simple pre-incubation of TBP protein to a plasmid containing a TATA-box promoter induced RNA Pol II dependent transcription in egg extracts. Furthermore, we showed that TBP modestly altered the rate of chromatin assembly and histone deposition.

In conclusion, we showed experiments probing the relative roles of chromatin assembly in the regulation of transcription in a cell free system. Since we can add and remove components from

cell free extracts to probe chromatin assembly directly (Onikubo *et al.*, 2015), future studies can directly test how chromatin assembly competes with basal and specific transcriptional output. In particular, studies of transcriptional regulation by abundant histone modifications and modifying activities present in the egg, such as extensive arginine methylation and serine phosphorylation (Wang *et al.*, 2014, Wilczek *et al.*, 2011), may provide novel insights not testable in any other model system. The utility of cell free extracts will continue to be important for new discovery.

Materials and Methods

Chemicals and antibodies
Chemicals and reagents were obtained from Sigma (St. Louis, MO), RPI (Illinois) or Fisher Scientific (Pittsburgh, PA). Antibodies were from Millipore/Upstate.

Animals
Frogs were handled in an ethical manner according to our animal use protocols 20151003, 20121005 and 20110603 approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee (IACUC). The following procedures were performed in strict accordance with the protocol and every effort was made to minimize suffering. *Xenopus* frogs were primed with 50 U PMSG 3-5 days before injection with Human Chorionic Gonadotropin (Sigma) for egg laying or before dissection for oocyte collection. Frogs were anesthetized with 0.2 % MS-222 (Thermo Fisher Scientific, Bridgewater, NJ) pH 7.0 and sacrificed according to IACUC-approved protocols.

Egg and oocyte preparation
Extracts were prepared as described (Banaszynski *et al.*, 2010, Wang *et al.*, 2014). Briefly, High-speed supernatant clarified egg extract (HSS) was prepared from low speed supernatant by spinning in an SW-55 rotor at 55,000 rpm × 45 min. The clarified middle layer was removed and respun for 30 min, and glycerol was added to 5%, aliquoted, and flash frozen. *Xenopus* oocyte extracts were prepared from freshly dissected ovaries by disrupting the follicular layer as described above. The defolliculated oocytes were then washed extensively with 1× MMB containing 200 mm sucrose and 1 mm DTT, and the later staged oocytes settled to the bottom (the less dense stage I and II oocytes were mostly lost during the preparation). The oocytes were settled in 13 × 51-mm Beckman ultracentrifuge tubes, and excess buffer was removed. They were then spin-crushed at 35,000 rpm (150,000 × *g*) for 40 min in an SW-55 rotor. The middle layer was removed with a pipette or a syringe punctured through the side wall and respun for 30 min. The middle layer was again removed, glycerol was added to 5% final, and the extract was aliquoted and flash frozen in liquid nitrogen.

Plasmid chromatin assembly reactions
25 µl egg or oocyte extract was incubated with 1 µl relaxed-pG5ML vector (625 ng/µl) and 1 µl 50X energy mix at 23°C for the indicated time. Then 5 µl of product was transferred into 200 µl of chromatin assembly stop buffer (10 mM Tris pH 8.0, 20 mM EDTA, 0.5% SDS) with 2 µl of RNase A (10 mg/ml) and incubated at 37°C for 30 min. The sample was digested with 5 µl 20mg/ml Proteinase K (final 50 µg/ml) at 56 °C for 1hr. Plasmid DNA were then purified through Phenol/Chloroform exraction and ethanol precipitated in the presence of NaOAC and 1 µl GlycoBlue (15 mg/ml). Finally, the pellets were dissolved in 12 ul of DNA loading buffer containing RNase A (final 10 ng/ml) and loaded on 0.8 % agarose gel run at 25 V overnight in the cold room. The gel was stained afterwards with ethidium bromide (EtBr).

Micrococcal nuclease digestion of assembled chromatin
After the assembly reaction in extract, 11 µl of the product was transferred into 90 ul of MNase Reaction buffer (10 mM Hepes pH8.0,

Fig. 5. Sperm Chromatin transcription in oocyte and egg extracts.
(A) *Sperm chromatin was incubated in oocyte or egg extracts in the presence of ^{32}P-α-ATP. Sperm was pre-incubated with TBP protein as indicated. The reaction products were stopped and RNA extracted with TRIzol. Precipitated RNA was directly counted in a scintillation counter and resulting transcription radioactivity was scaled to the oocyte sperm control.* **(B)** *Sperm chromatin was incubated in egg extract either without or pre-incubated with TBP protein as indicated. The reaction product chromatin was isolated after 0, 15, 30, 60 or 90 min through a sucrose cushion and pelleted proteins were immunoblotted for PCNA and histone H2A.* **(C)** *Sperm chromatin was incubated in oocyte or egg extracts. The reaction product chromatin was isolated after 0, 15, 30, 60 or 90 min through a sucrose cushion and pelleted proteins were immunoblotted for RNA Pol II, Pol II S5ph, and H2A as indicated. A total Coomassie stained gel of the isolated products is also shown at the bottom.*

50 mM KCl, 5 mM MgCl$_2$, 3 mM CaCl$_2$, 1mM DTT, 0.1 % NP40 and 8% Glycerol) containing 1 unit of MNase and incubated at room temperature for the indicated length of time. 110 ul Stop Buffer was added to stop the reaction. 8 μl RNase A (10 mg/ml) was added to each sample and incubated at 37 °C overnight. 5 μl of Protenase K (20 mg/ml) was added to each sample and incubate at 56 °C for 1 hr. Plasmid DNA was phenol-chloroform extracted and ethanol precipitated as above. The final products were loaded on a 2% agarose gel and run at 100 V for 60 min at room temperature.

Sperm chromatin isolation

2,000 demembranated sperm chromatin per μl were incubated in 80 μl of extract. Samples were flash frozen, suspended in 800 μl of ELB-CIB buffer (10 mM Hepes, pH 7.8, 250 mM sucrose, 2.5 mM MgCl$_2$, 50 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM spermidine, 1 mM spermine, 0.1% Triton X-100, 10 mM sodium butyrate, 1× phosphatase inhibitors, and 1× protease inhibitors) and chromatin isolated via centrifugation at 4000 rpm for 5 min through a 0.3 ml sucrose cushion of ELB-CIB with 0.5 M sucrose underlayered in the tube. The pellet was washed once with ELB-CIB plus 250 mM KCl.

Immunoblotting

Gels were transferred to polyvinylidene difluoride membrane (Millipore) using 1× NuPAGE transfer buffer (Invitrogen) plus 20% methanol and 0.1 % SDS. Membranes were stained using Direct Blue 71 stain to ensure proper transfer; any membranes with inadequate or uneven transfer were discarded. Membranes were blocked in 2% skim milk or 3% BSA and blotted with antibodies. Secondary horseradish peroxidase-coupled antibodies were applied and then visualized using ECL Advance, with images captured using the ImageQuant LAS 4000 digital system (GE, Pittsburgh). Images were incrementally exposed until CCD saturation, and the exposure before saturation was used for analysis. Images were aligned with molecular weight markers by simultaneous capture of the lit membrane and subsequently cropped, and levels were adjusted for contrast in Adobe Photoshop; no exposed bands were eliminated upon adjustment.

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