Chapter 18

cgChIP: A Cell Type- and Gene-Specific Method for Chromatin Analysis

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Abstract

Hox and other homeobox-containing genes encode critical transcriptional regulators of animal development. Although these genes are well known for their roles in the body axis and appendage development, little is known regarding the mechanisms by which these factors influence chromatin landscapes. Chromatin structure can have a profound influence on gene expression during animal body formation. However, when applied to developing embryos, conventional chromatin analysis of genes and *cis*-regulatory modules (CRMs) typically lacks the required cell type-specific resolution due to the heterogeneous nature of animal bodies. Here we present a strategy to analyze both the composition and conformation of in vivo-tagged CRM sequences in a cell type-specific manner, using as a system *Drosophila* embryos. We term this method cgChIP (*c*ell- and *g*ene-specific Chromatin Immunoprecipitation) by which we access and analyze regulatory chromatin in specific cell types. cgChIP is an in vivo method designed to analyze genetic elements derived from limited cell populations. cgChIP can be used for both the analysis of chromatin structure (e.g., long-distance interactions between DNA elements) and the composition of histones and histone modifications and the occupancy of transcription factors and chromatin modifiers. This method was applied to the Hox target gene *Distalless* (*Dll*), which encodes for a homeodomain-containing transcription factor critical for the formation of appendages in *Drosophila*. However, cgChIP can be applied in diverse animal models to better dissect CRM-dependent gene regulation and body formation in developing animals.

Key words *Hox*, Chromatin, Histone modifications, DNA looping, *Cis*-regulatory modules (CRMs), Enhancer-promoter communication, Gene transcription, *LacI/lacO*

1 Introduction

Many developmentally regulated genes are active in a minority of the cells in a multicellular organism. Transcriptional regulation of these genes is typically governed by multiple CRMs that control only a subset of a gene's overall expression pattern. Each CRM can be differentially active with respect to cell type, developmental time, and cellular environment. Furthermore, the activity of each CRM often must be communicated over long distances to their cognate promoter

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Yacine Graba and René Rezsohazy (eds.), *Hox Genes: Methods and Protocols*, Methods in Molecular Biology, vol. 1196,
DOI 10.1007/978-1-4939-1242-1_18,

in order to impact gene transcription. Current approaches to investigating the molecular events controlling gene regulation lack the necessary resolution to assess such dynamic and cell-type-specific events. For example, standard chromatin immunoprecipitation (ChIP) studies, when applied to developing embryos, provide an average of signals, corresponding to all cell types in which the factor of interest is expressed at the time of chromatin preparation. One major obstacle arises from technical difficulties associated with the ability to isolate specific cell types from a tissue that then can be used for chromatin and gene expression studies [1]. Consequently, research on animal models often lacks cell type resolution and thus cannot reveal biologically relevant differences if they exist [2]. Methods based on the ground-breaking approach capturing chromosome conformation (3C), including 4C, 5C, Hi-C, and ChIA-PET, have provided major insights into the analysis of the 3-dimensional interactions that exist between chromosomal elements [3–8]. However, when applied to intact organisms, such as a developing embryo, these methods cannot reveal cell-specific interactions. Furthermore, only ChIA-PET and the ChIP-loop assay [9] have the potential to combine transcription factor binding and/or histone modifications with chromatin conformation in the same experiment.

The above considerations prompted us to develop a method that combines cell specificity with the analysis of chromatin conformation, transcription factor occupancy, and histone modifications at specific loci. We call this method cgChIP (for *c*ell- and *gene-specific* ChIP). We reasoned that expression of a DNA-binding protein used in conjunction with its cognate-binding site would provide a molecular tag that could be used to immunoprecipitate target chromatin. We chose the bacterial DNA-binding protein LacI and its operator sequence *lacO* because LacI binds *lacO* with high specificity and affinity even in nucleosomal DNA [10]. In addition, the *lacI/lacO* system has previously been used in vivo in *Drosophila* and *C. elegans* for other purposes without adverse affects on development [11, 12]. In cgChIP, regulatory elements (CRMs and promoters) are tagged with *lacO*-binding sites and tissue-specific expression of an epitope-tagged LacI allows the immunoprecipitation of the lac*O*-tagged elements from specific cell types [2]. These elements can be analyzed directly (e.g., for looping) or re-precipitated (e.g., double immunoprecipitation) to analyze chromatin conformation and composition, respectively. cgChIP shares some features with other methods, such as iChIP [13], which depend on the tagging of DNA elements and partial purification of the desired chromatin fragment. However, an important aspect of cgChIP is the cell-type-specific expression of LacI, which allows the immunoprecipitation of the tagged DNA only from those cell types (Fig. 1a–d). Although the methods and examples outlined here have been optimized for *Drosophila*, the approach can readily be adapted to any system in which transgenes can be generated.

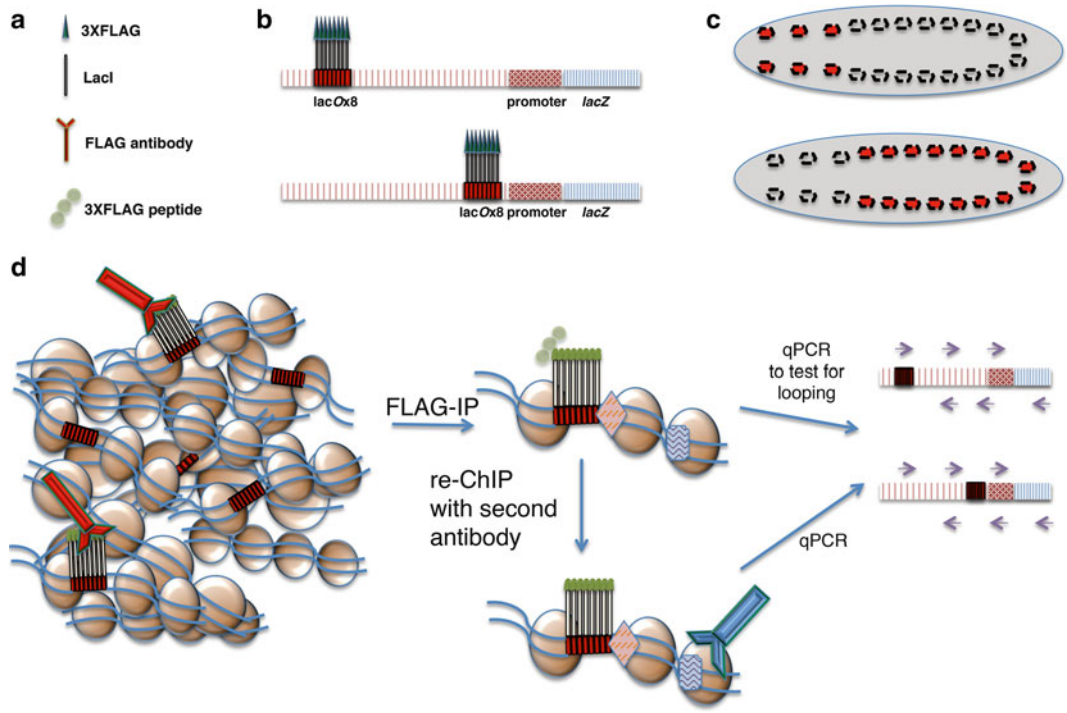

Fig. 1 A graphical synopsis of the cgChIP method. (**a**) Overview of the schematics. (**b**) Schematic representation of 3× FLAG::LacI chimeras bound on lac*O*-tagged CRM. The insertion of the lac*O* repeats can be either proximal (*bottom*) or distal (*upper*) from the transcription start site of the transgene. (**c**) Graphical representation of *Drosophila* embryos (viewed ventrally) expressing 3× FLAG::LacI (*red*) across thoracic (*upper*) or abdominal (*bottom*) tissues. (**d**) Embryonic lac*O*-tagged chromatin can be isolated by anti-FLAG precipitation and used as template either directly for cg looping experiments or re-precipitated and analyzed in double-cgChIP experiments. Both approaches are finally analyzed by semi- and quantitative PCR

2 Materials

2.1 CRMs of Interest

The identification and functional characterization of a regulatory element (CRM or promoter) is an essential prerequisite for carrying out cgChIP. CRMs integrate temporal and spatial information by binding groups of transcription factors [14]. Proximal or distal CRMs can regulate gene transcription in several manners, including controlling assembly of transcriptional pre-initiation complexes and regulating release of stalled RNA polymerase at a gene's promoter. For many developmentally regulated genes such as the *Hox* genes and their targets, in vivo *lacZ*- or GFP-based reporter gene studies typically provide the initial characterization of regulatory elements. In addition to classical analyses (http://redfly.ccr.buffalo.edu/), a large screen recently characterized more than 6,000 potential regulatory elements in *Drosophila* at several stages and in different tissues, providing a large number of novel CRMs [15–17]. In addition to these resources, histone modifications,

DNase I sensitivity, and formaldehyde-assisted identification of regulatory elements (FAIRE) can be used to identify candidate CRMs [18, 19]. Thus, technical and methodological advances have improved the capacity to identify regulatory elements in the genome which then can be functionally characterized by cgChIP.

2.2 CRMs and Promoters Tagged with lacO-Binding Sites and Fusion to lacZ

An ~300 bp long cassette consisting of 8 sequential repeats of the binding site for the bacterial repressor LacI (*lacO*) is inserted adjacent to the 5′ or the 3′ edge of the CRM of interest. The endogenous gene's promoter or a minimal promoter sequence (e.g., *hsp43*)—in cases where the endogenous promoter is not part of the CRM—and coding sequences for a nuclear version of β-galactosidase (β-Gal, encoded by *lacZ*) are fused downstream of the *lacO*-tagged element (Fig. 1b). Thus, β-Gal expression can be used as a reporter in order to monitor the ability of the cloned CRM to recapitulate the endogenous expression pattern, and to ensure that the *lacO*-binding sites do not interfere with CRM activity. Transgenic flies with these tagged reporter genes are generated using standard methods. Ideally, the tagged transgenes should generate the same expression pattern regardless of where the *lacO* sites are inserted, or where the transgene is integrated in the genome (Fig. 2a). In case of incorrect or leaky expression the *lacO*-tagged constructs should be redesigned, either by moving the location of the *lacO* or by decreasing the number of *lacO* repeats. Indeed, in pilot experiments, as few as 2 *lacO* repeats were sufficient for successful immunoprecipitation of tagged DNA (DJM and RSM, unpublished results).

cgChIP can also be used to monitor interactions between a distally located CRM and its promoter. In the specific example we have characterized, the ~12 kb region 5′ of the *Dll* promoter, which contains several CRMs, was cloned upstream of the β-Gal-coding sequence [2]. Constructs were generated in which either distal CRMs were tagged or the promoter was tagged (Fig. 1b). In both cases, expression of β-Gal was unaffected and closely matched the endogenous *Dll* expression pattern (Fig. 2a).

2.3 Fly Lines for Cell-Type-Specific Expression of Epitope-Tagged LacI

In addition to being objects for study, well-characterized CRMs serve as drivers for the cell-type-specific expression of LacI. This can be done directly (by fusing the CRM to LacI-coding sequences) or indirectly by, for example, the Gal4/UAS or the LexA/LexO system [11, 20–22]. The large availability of GAL4 "enhancer trap" fly lines serves as a standard source for such genetic tools. Further, the use of repressors (e.g., Gal80) and other intersectional methods (e.g., splitGal4) can be used to fine-tune expression patterns [23]. For cgChIP, these methods are used to drive the expression of an epitope-tagged LacI protein. In our experience, three tandem copies of the "FLAG" tag (3× FLAG) worked well, because of its small size and the availability of high-affinity commercially

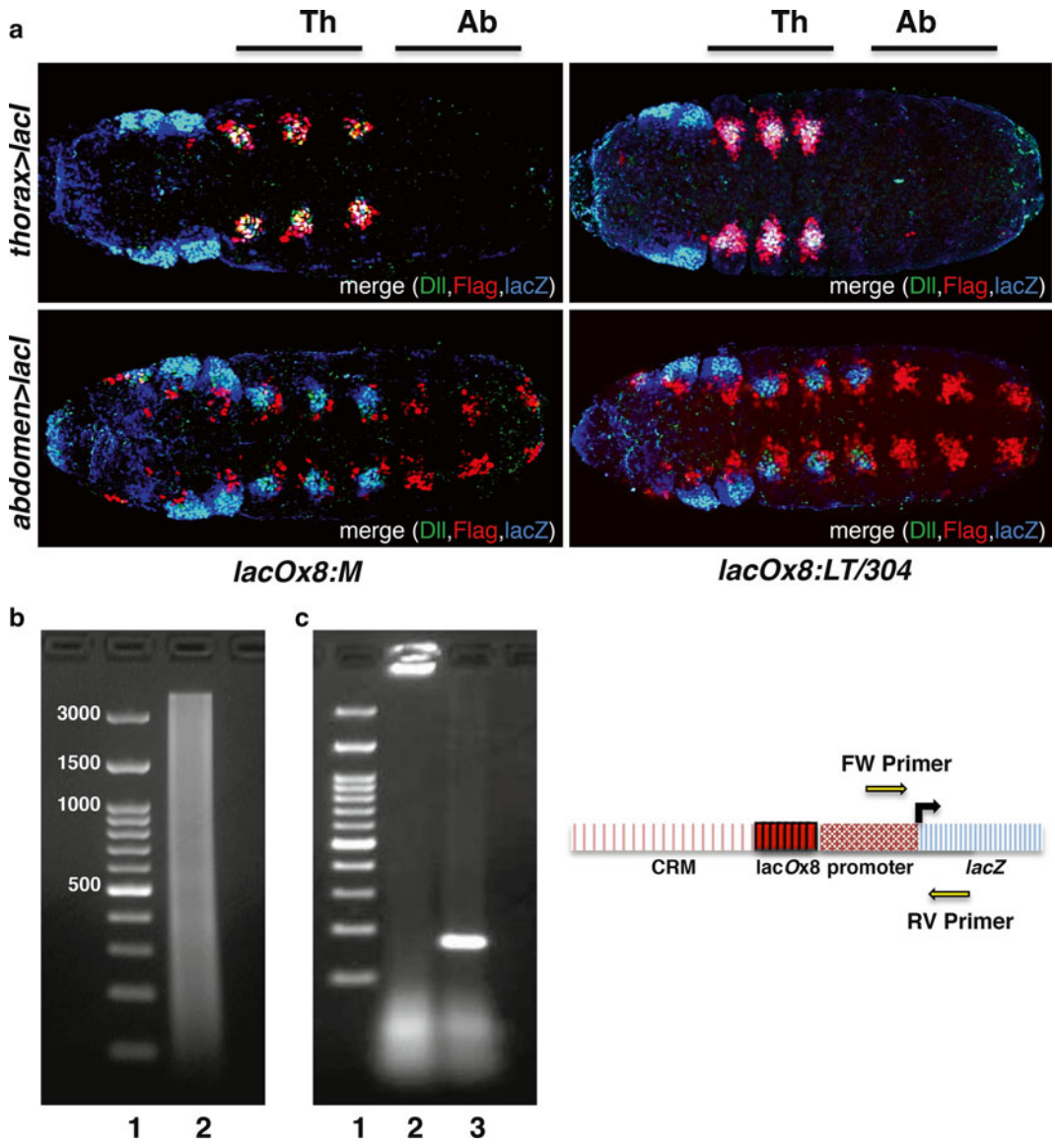

Fig. 2 Control steps required for an accurate chromatin preparation used for cgChIP assays. (**a**) Antibody-staining reactions depicting spatiotemporally accurate expression of 3× FLAG::LacI, endogenous gene, and the reporter gene of study. (**b**) Agarose gel electrophoresis of sheared isolated DNA derived from total embryonic chromatin shows a robust distribution ranged between 300 and 3,000 bp molecular weight (lane 1: 100 bp DNA marker; lane 2: sheared isolated DNA). (**c**) Control PCR for evaluation of chromatin cross-linking efficiency. Equal amounts of total chromatin and extracted DNA are amplified in PCR reactions with a set of primers hybridized at the promoter (forward) and the *lacZ* (reverse) sequences, respectively. The total chromatin-containing PCR reaction fails to amplify the above region (chromatin is detected inside the well). In sharp contrast, a robust/specific amplification signal is produced when extracted DNA from the same chromatin preparation is used as a template. The above quality control step ensures for the absence of non-cross-linked material inside the embryonic chromatin preparation (lane 1: 100 bp DNA marker; lane 2: PCR reaction with total chromatin; lane 3: PCR reaction with extracted DNA)

available anti-FLAG antibodies and peptides (Figs. 1a and 2a). Importantly, the version of LacI used in these experiments is missing its tetramerization domain, thus preventing LacI dimer-dimer interactions when expressed in vivo [24].

Ideally, 3× FLAG::LacI should be expressed in a pattern as precise as possible to analyze CRM structure and function in specific cell types. For *Dll*, we generated two 3× FLAG::LacI transgenes (referred as "*flag-lacI*" in the genotypes) to compare chromatin organization in cells where *Dll* is differentially regulated in the early embryo. To purify chromatin from *Dll*-expressing cells of the thoracic limb primordia, we used the early *Dll* enhancer, *Dll304*, to drive Gal4 and indirectly activate a *UAS-flag-lacI* transgene (Figs. 1c and 2a). To purify chromatin from the analogous population of *Dll* non-expressing cells in the abdomen, we used a derivative of *Dll304* that lacks the Hox repressor element (DME^{ACT}) to drive Gal4 and indirectly activate the *UAS-flag-lacI* transgene [2, 25]. In addition, we used *Dll304* and *DMX* (a thoracic specific derivative of the *Dll304* enhancer) to directly drive the expression of the Gal4 inhibitor, Gal80, thereby blocking Gal4 activity in the thoracic segments [2, 25]. This combination of transgenes (*DMX-Gal80*, *304-Gal80*; DME^{ACT}*-Gal4*,*UAS-flag-lacI*) resulted in the expression of 3× FLAG::LacI in cells in the abdominal segments that have the potential to express *Dll* but normally do not due to Hox-mediated repression (Fig. 2a) [2, 25]. We note that in this genotype, 3× FLAG::LacI is also expressed in several nonabdominal cells, but these do not express *Dll* (Fig. 2a) [2].

2.4 Fly Culture Components

1. Commercial or homemade cages can be used to host a large population of adults. To provide sufficient circulation, homemade cages should have multiple openings covered with netting.
2. Agar-apple juice plates for egg collections (*see* **Note 1**).
3. Yeast paste (*see* **Note 1**).
4. Cell strainers: 70 μm cell strainer.
5. Paintbrush.
6. Wide-mesh metal strainer.

2.5 Chromatin Preparation Buffers and Solutions

1. Embryo wash buffer (EWB): 1× PBS, 0.01 % Triton X-100.
2. *n*-Heptane.
3. Fixing solution (FS): 1× PBS, 3.7 % formaldehyde.
4. Quenching solution (QS): 1× PBS, 0.125 M glycine.
5. Buffer A (BA): 0.25 % Triton X-100, 10 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM HEPES pH 7.9.
6. Buffer B (BB): 0.2 M NaCl, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM HEPES pH 7.9.

7. Sonication buffer (SB): 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM HEPES pH 7.9.
8. Glycerol-containing storage buffer (GSB): 10 % Glycerol, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 10 mM HEPES pH 7.9.

2.6 Immunoprecipitation Components, Solutions, and Apparatus

1. Magnetic beads: Protein G magnetic beads (Invitrogen).
2. Magnetic particle concentrator: MPC-S (Invitrogen) or an equivalent model like DynaMag™-2 (Invitrogen).
3. FLAG antibody: M2 (Sigma-Aldrich).
4. 3× FLAG peptide: Lyophilized powder (Sigma-Aldrich).
5. LacI antibody (Rockland antibodies and assays).
6. Reaction buffer (1× RIPA): 0.5 % Triton X-100, 140 mM NaCl, 0.07 % Sodium deoxycholate (deoxycholic acid), 10 mM HEPES pH 7.9.
7. Wash RIPA buffer (1× WRP): 0.5 % Triton X-100, 140 mM NaCl, 0.14 % Sodium deoxycholate (deoxycholic acid), 0.2 % SDS, 10 mM HEPES pH 7.9.
8. Elution buffer (EB): 200 μg/ml 3× FLAG peptide, 140 mM NaCl, 0.2 % SDS, 10 mM HEPES pH 7.9.
9. Phenol.
10. Chloroform.
11. Proteinase K solution: 0.25 mg/ml Proteinase K, 0.4 % SDS.
12. Precipitation mix: 0.2 mg/ml Glycogen, 0.3 M sodium acetate pH 5.2, 3 volumes ice-cold ethanol.

2.7 Semiquantitative and Real-Time PCR Components, Solutions, and Apparatus

1. Transgene-specific amplicon: In cases where *lacZ* is used as a readout, a reverse primer hybridizing within *lacZ* but close to the transcriptional start site can be combined with a forward primer complementary to the promoter sequence (Fig. 2c). This combination restricts the amplification to the transgenic and not the endogenous sequences. Thus the final amplification product derived absolutely from the tagged transgene.
2. Reference amplicon: A pair of primers hybridizing at an endogenous sequence that is not included into the tagged transgene is used as a negative reference.
3. Gene-specific amplicons: If the regulatory region under investigation is large (>10 kb), unique regions should be scanned by amplicons spaced every ~2 kb.
4. *Taq* DNA Polymerase.
5. Polyacrylamide gel electrophoresis system.
6. Alpha ^{32}P-dCTP.
7. Phosphorimager/screens.
8. qPCR application: 2× SYBR Green mix, 96-well plates.

3 Methods

The experimental procedure described below can be completed within 3 days assuming that transgenic fly lines are already available.

3.1 Fly Strain Development and Culture

Large populations must be established to enable the isolation of a sufficient amount of staged embryos (*see* **Note 2**). In our experience with *Dll*, at least six 500 ml population cages were required to collect a sufficient amount of staged embryos in a 2–3-day window. Importantly, in order to avoid any contamination each population should be initiated from a single vial and disposed of once they are no longer productive. In addition, once expanded the population should be retested prior to embryo collection by antibody staining (*see* **Note 3**). The adults should eclose approximately 2 days prior to the first collection in order to achieve efficient egg laying. Moreover, when multiple transgenic fly lines are analyzed in parallel, synchronization of egg laying helps to maximize the amount of embryos collected simultaneously.

3.2 Embryo Collections and Fixation

1. Collect embryos in ddH_2O with the help of a paintbrush from apple juice agar plates and pour them through a wide-mesh metal strainer followed by extensive wash with ddH_2O. Adults, body parts, and debris remain in the strainer and embryos are collected in a glass beaker. Transfer embryos to 70 μm cell strainers and wash with ddH_2O to remove agar and yeast.
2. Place the 70 μm cell strainer with embryos inside a Petri dish filled with bleach and shake for 3 min on a laboratory shaker operating at low speed (<50 rpm).
3. Wash embryos extensively with ddH_2O followed by EWB. This step is required in order to remove the bleach and chorion fragments.
4. Remove most of the moisture with laboratory wipes.
5. At this point a small subset of embryos should be used for immunostaining, to confirm the correct age of the embryos, the expression of the target and reporter genes, and the transcription factor(s) of interest. In addition, an anti-FLAG antibody staining should be performed to monitor the tissue-specific expression of the 3× FLAG::LacI protein (*see* **Note 3**).
6. Dechorionated embryos are placed in a Petri dish filled with heptane. Upon slight shaking, any debris, chorion fragments, and agar should be aspirated. Transfer "pure" embryonic mass with a glass pipette into a 50 ml glass bottle.
7. Balance the volume of heptane at 30 ml and add directly 10 ml of the fixing solution (FS). Close the cap tightly to avoid any leaks of the above harmful solutions.

8. Place the bottle at horizontal position on a shaker that operates at 200–250 rpm for 30 min at room temperature (*see* **Note 4**).
9. Transfer embryos into a Falcon tube and spin down for 5 min at 3,000 × *g*.
10. Aspirate fixing and heptane solutions.
11. Quench fixing reaction by washing with 50 ml quenching solution (QS).
12. Gently help embryos settle to the bottom of the tube with a glass pipette.
13. Aspirate quenching solution and transfer embryos into a new Falcon tube. Repeat **steps 11–13**.
14. Wash embryos three times with 50 ml of EWB for 3 min on ice. In between washes, spin down for 2 min at 3,000 × *g* and aspirate.
15. Dry embryos with the help of a glass pipette. Put the pipette tightly at the bottom of the Falcon tube and remove any remaining EWB without disrupting the pellet.
16. Place embryos at −80 °C for at least 4 h to pulverize. An overnight incubation at −80 °C is more effective for that aspect.

3.3 Chromatin Isolation for cgChIP

1. Incubate pulverized embryonic mass twice with 15 ml of buffer A (BA) for 10 min at room temperature. Place the Falcon tube at a semi-angled position on a laboratory rocker operating at medium speed. Spin down embryonic mass for 2 min at 1,700 × *g* and discard supernatant.
2. Incubate twice with 15 ml of buffer B (BB) for 15 min at 4 °C. Place the Falcon tube at a semi-angled position on a laboratory rocker operating at medium speed. Spin down embryonic mass for 2 min at 1,700 × *g* and discard supernatant.
3. Add 6 ml of sonication buffer (SB) and about 2 g of glass beads directly to the pellet. At this stage the structure of the embryonic mass is changed. The solid/compact structure of the pellet becomes loose and the embryos are no longer settled at the bottom of the tube. The above morphological change is a sign for the correct collection, dechorionation, fixing, and quenching procedures as described above.
4. Place the samples on ice and proceed with sonication. The sonicator's probe should be placed in the middle of the liquid at a semi-angle (~15° angle) in order to achieve efficient mixing of the sample during sonication. The sonication should be performed at maximum power for seven 40-s rounds with 2-min intermediate incubations on ice (*see* **Note 5**).
5. Isolate sheared chromatin by performing two rounds of centrifugation. Initially spin down at ~1500 × *g* in a microcentrifuge

for 10 min. The pellet includes the glass beads and the majority of the cellular debris, and should be discarded. Transfer the supernatant into new 1.5 ml tubes and spin for 20 min at ~13300 × *g* in a microcentrifuge at 4 °C (*see* **Note 6**). Transfer the chromatin-containing supernatant into new tubes and add an equal volume of GSB.

6. Store chromatin at −80 °C (*see* **Note 7**).

3.4 Titration and Quality Control of Chromatin Preparation

1. Calculate chromatin concentration (μg/μl) with a Nanodrop Spectrophotometer and digest 5 μg with proteinase K for 2 h at 55 °C. Reverse formaldehyde cross-linking by overnight incubation at 65 °C.
2. Isolate sonicated DNA by phenol/chloroform extraction and ethanol/glycogen-based precipitation. Resuspend precipitated DNA pellets in 100 μl of ddH_2O.
3. Analyze (~500 ng) sonicated DNA by agarose gel electrophoresis to determine the distribution of fragment sizes. The majority of the sonicated DNA should be more than 300 bp and less than 3,000 bp in order to ensure that shearing was effective but *lacO* sequences were not separated from the CRMs and promoter-reporter sequences (Fig. 2b).
4. The presence of non-cross-linked material in the chromatin sample can interfere with immunoprecipitation results. Therefore, it is important to evaluate the efficiency of chromatin cross-linking for each chromatin preparation by PCR (Fig. 2c). Amplify 10–50 ng of cross-linked chromatin and an equivalent amount of reverse-cross-linked/purified DNA side by side for 25 rounds of PCR. In our experiments with *Dll*, we used primers that specifically recognized the *lacO*-tagged transgene (*see* Subheading 2.7, **step 1**). Analyze PCR products by agarose gel electrophoresis. Chromatin samples that produce amplification products without prior proteinase K digestion and reversal of cross-linking should be discarded.

3.5 Cell-Type- and Gene-Specific Looping of Cis-Regulatory Modules

1. Pre-clear 500 μg of chromatin by incubation with 10 μl of magnetic beads in reaction buffer (1× RIPA) for 1 h at 4 °C. Set up pre-clearing reaction at a final volume of 800 μl.
2. Remove the magnetic beads with the help of an MPC-S and divide the reaction in two tubes.
3. Add 2.5 μg of anti-FLAG or IgG antibodies, respectively, and incubate for 4 h at 4 °C by continuous rocking.
4. Transfer the reactions at room temperature and couple antibody to the magnetic beads. Use 2 μl of beads per sample and incubate for 1 h by continuous rocking.
5. Immobilize bead/antibody/chromatin complexes with the help of an MPC-S and aspirate supernatant.

6. Wash beads twice with 1 ml of 1× WRP for 7 min at room temperature by continuous rocking and aspirate with the help of an MPC-S.
7. Dilute bead/antibody/chromatin complexes in 300 µl 1× WRP and transfer into a new tube with the help of a cut filter tip.
8. Wash beads once with 1 ml of 1× WRP at room temperature and mix gently for 5 min without rocking.
9. Aspirate and proceed to proteinase K digest, cross-linking reversal and DNA extraction/precipitation reactions as in Subheading 3.4, **steps 1** and **2** (*see* **Note 8**).
10. Design and perform ^{32}P-based radioactive PCR reactions and analyze the amplification products in 8 % acrylamide gels [2, 26] (*see* **Note 9**).
11. Design and perform SYBR Green-based qPCR in order to quantify the amplification signals (*see* **Note 9**).

3.6 Cell-Type- and Gene-Specific Double Immunoprecipitation

1. Collect and fix 8 g of staged embryos and isolate/titrate total chromatin as in Subheadings 3.1–3.3 in order to perform cell-type- and gene-specific chromatin analysis. An initial collection of 8 g of staged embryos is required in order to probe chromatin with five different antibodies at the final stage of the cgChIP protocol (*see* **Note 2**).
2. Pre-clear total chromatin as in Subheading 3.5, **steps 1** and **2**. Set up multiple pre-clearing reactions at a final volume of 800 µl by using 10 µl of magnetic beads for each 500 µg chromatin sample.
3. Isolate Flag:lacI/lac*O*-tagged chromatin by FLAG immunoprecipitation as in Subheading 3.5, **steps 3–5**. For an initial 8 g collection, a total of eight reactions can be set up.
4. Immobilize bead/antibody/chromatin complexes with the help of an MPC-S and aspirate supernatant.
5. Wash beads twice with 1 ml of 1× WRP for 7 min at room temperature by continuous rocking and aspirate with the help of an MPC-S.
6. Dilute bead/antibody/chromatin complexes in 300 µl 1× WRP and transfer into a new tube with the help of a cut filter tip. Aspirate with the help of an MPC-S.
7. Wash beads once with 1 ml of 1× WRP at room temperature and mix gently for 5 min without rocking. Aspirate with the help of an MPC-S.
8. Dilute Flag:lacI/lac*O*-tagged chromatin complexes in 600 µl of elution buffer (EB) and transfer into a new 0.6 ml tube.
9. Incubate for 4 h at 4 °C by continuous rocking.
10. Pre-clear eluted chromatin as in Subheading 3.5, **steps 1** and **2**.

11. Aliquot a small fraction for an additional control step (*see* Subheading 3.7, **step 2**).
12. Carry out the second round of immunoprecipitation for 12 h at 4 °C in a total volume of 300 μl. Set up the reaction in 0.6 ml tube in order to achieve efficient mixing during rocking. Incubate equal samples of chromatin with 2 μg of antibody against the transcription factor of interest side by side with equivalent amounts of IgG as negative or anti-LacI antibody as positive control, respectively.
13. Transfer the reactions at room temperature and couple antibody to magnetic beads as in Subheading 3.5, **step 4**.
14. Transfer the reactions into 1.5 ml tubes with the help of a cut filter tip.
15. Immobilize bead/antibody/chromatin complexes with the help of an MPC-S and aspirate supernatant.
16. Wash beads twice with 1 ml of 1× WRP for 7 min at room temperature by continuous rocking. Intermediately, aspirate with the help of an MPC-S.
17. Aspirate and proceed to proteinase K digest, cross-linking reversal and DNA extraction/precipitation reactions as in Subheading 3.4, **steps 1** and **2**. Analyze as in Subheading 3.5, **steps 10** and **11** (*see* **Note 9**).

3.7 Internal Controls for Evaluation of cgChIP Results

1. Ideally, cgChIP experiments should include several controls to assess possible contamination of LacI-tagged tissue-specific chromatin with chromatin derived from other cell types. In case of tissue-specific expression of transcription factors (e.g., *Hox* genes) additional controls may include anti-Ubx or anti-AbdA cgChIPs to test the purity in cases where thoracic derived chromatin is analyzed. Conversely, thoracic specific factors (e.g., *Dll*) may be used as a control for cgChIPs where abdominal derived chromatin is analyzed. If any contamination is observed (as evidenced by the presence of a factor that should not be there, for example, Dll in abdominal derived chromatin), the experiment should be aborted and new chromatin preparations should be carried out.
2. Additional controls are performed in order to ensure for the specificity of the immunoprecipitation and the efficiency of both rounds of cgChIP. For one, IgG samples are processed side by side with anti-FLAG immunoprecipitations, to detect any nonspecific enrichment during the first round of precipitation. Second, primers hybridizing across the sequence of the LacZ reporter gene are used in combination with gene-specific primers in order to ensure that transgenic and not endogenous sequences are precipitated and amplified (*see* Subheading 2.7, **step 1**). Third, upon 3× FLAG peptide-based elution of

LacI-tagged tissue-specific derived chromatin and prior to the second round of precipitation, a small fraction of the eluted material is processed as in Subheading 3.4, **steps 1** and **2**, and analyzed by PCR. The precipitated DNA is amplified with transgenic specific and off-target primer pairs in order to evaluate for the efficiency and specificity of FLAG immunoprecipitation. Fourth, at the second round of precipitation, IgG and anti-LacI are used to assess the quality and the efficiency of both rounds of cgChip. A weak LacI:IgG ratio suggests that the experiment was unsuccessful. In our experience, an eight-fold enrichment of LacI compared to IgG is satisfactory when LacI signal recovers at least 1 % of the input used at the second round of precipitation.

4 Notes

1. Apple juice containing agar plates and yeast paste must be freshly prepared and stored at 4 °C. The efficiency of laying is enhanced by pre-warm of the plates and yeast paste for 10–15 min at 25 °C. Avoid changes in apple plate batches and/or recipes during a single experiment. The fly-cage culture system is very sensitive and slight changes in conditions can dramatically reduce the rhythm of egg laying.
2. The exact amount of embryos required depends on the target gene, the embryonic tissue, and the developmental stage of the study. For example, leg primordial cells at stages 11–14 generate less than 200–300 cells from about 6,000 total embryonic cells. For *Dll*, we determined that 8 g of embryos were required to purify a sufficient quantity of chromatin derived from these cells. Preparation of chromatin in this manner provided enough template for five different immunoprecipitations in the second round of cgChIP.
3. Immunostaining of embryos can be performed by standard formaldehyde/heptane fixing and basic incubation steps as follows: (1) Blocking is carried out overnight in PBST with 5 % BSA at 4 °C. (2) Both the primary and the secondary antibody incubations are carried out for 12 h at 4 °C. For β-galactosidase and FLAG expression, commercially available antibodies are used.
4. It is important to place the bottle in horizontal position in order to maximize the interface between fixing solution and heptane where embryos equilibrate during mixing.
5. It is important to follow the electronic display of the sonicator in order to keep the output power at high levels by making slight changes of the Falcon/probe relative position. For example Fisher Sonic Dismembrator Model 100 sonicator has

an electronic display that provides information for the output power. In general the shearing procedure requires to be standardized specifically for each sonicator type.

6. It is important to perform sequential centrifugation. The first (low-speed) round results in separation of chromatin from other cellular debris and glass beads. Chromatin can be trapped inside these particles if a direct high-speed centrifugation step is performed.
7. GSB-diluted chromatin can be stored at −80 °C for at least 6 months.
8. The immunoprecipitated DNA can be stored at −20 °C until further use.
9. It is important to perform radioactive PCRs in order to evaluate the specificity and efficiency of both DNA looping and double-cgChIP readouts. This is especially important for developmental genes that are expressed in small populations of cells. Radioactive PCR products help with the design of amplicons and subsequent qPCRs. In addition, we suggest that interpretation of qPCR results should be based both on the percentage of recovery of input DNA for each antibody or IgG reaction and the fold enrichment of the antibody versus IgG ChIPs. By making both comparisons we reduce the possibility that background signals can be interpreted as true positives due to a high ratio of antibody to IgG ChIPs that may happen in cases where input DNA is poorly recovered.

Acknowledgments

This work was supported by GM058575 and GM054510 awarded to R.S.M. D.J.M. was supported by 5T32DK07328 and M.A. was supported by a long-term EMBO fellowship. We thank D. Arvanitis and S. Tsiftsoglou for critical proofreading.

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