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ChIA-PET analysis of transcriptional chromatin interactions

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ABSTRACT

Long-range chromatin contacts between specific DNA regulatory elements play a pivotal role in gene expression regulation, and a global characterization of these interactions in the 3-dimensional (3D) chromatin structure is imperative in understanding signaling networks and cell states. Chromatin Interaction Analysis using Paired-End Tag sequencing (ChIA-PET) is a method which converts functional chromatin structure into millions of short tag sequences. Combining Chromatin Immunoprecipitation (ChIP), proximity ligation and high-throughput sequencing, ChIA-PET provides a global and unbiased interrogation of higher-order chromatin structures associated with specific protein factors. Here, we describe the detailed procedures of the ChIA-PET methodology, unraveling transcription-associated chromatin contacts in a model human cell line.

1. Introduction

The human genome sequencing project revealed that only 1.9% of the entire genome is protein-encoding [1]. The precise control of gene expression at the transcription level involves interaction of multiple *trans*-acting factors with transcriptional regulatory elements (TREs) distributed throughout the vast regions of non-coding DNA. Such TREs include core/proximal promoters, distal enhancers, silencers, insulators and locus control regions (LCRs) (for review, see [2]). Intense international efforts have been directed towards mapping and characterizing the chromatin structure of these elements, through a variety of technologies including ChIP coupled with microarrays (ChIP-chip) or Paired-End Tag sequencing (ChIP-PET) for mapping transcription factor (TF) binding sites [3]. More recently, the ability to directly sequence ChIP-enriched DNA fragments by next generation sequencing (ChIP-seq) [5–7] has greatly advanced our ability to identify TF binding sites with higher resolution and deeper coverage. These studies suggest that TREs are pervasive in the human genome, and a considerable number of TREs are distal regulatory elements, confirming previous observations that TREs can reside far from target genes and in large domains of gene-poor regions [4]. Great interests were raised to understand the potential roles of individual remote TREs through possible long-range chromatin interactions to their target genes [8,9]. In the past decade, development of chromosome conformation capture (3C) technology and its subsequent variants have tremendously improved our understanding of long-range DNA interactions and genome conformation (for review, see [10]). These methods rely on the principle that DNA fragments in close contact within the 3D nuclear space can be ligated and quantified through PCR, microarray or sequencing. The Hi–C method [11] represents a significant improvement over earlier 3C-variants, incorporating selective purification and paired-end sequencing of ligation junctions for whole-genome mapping of DNA contacts. Collectively, these strategies have yielded new insights into the spatial relationships between TREs and provided early insights into folding principles of chromatin.

To address the functional relationships between specific subsets of interacting DNA loci, we have developed ChIA-PET, a global, high throughput and unbiased method for *de novo* detection of interacting DNA segments associated with DNA- or chromatin-binding proteins [12]. This method incorporates ChIP [13], the original nuclear proximity ligation concept [14], Paired-End-Tag (PET) strategy [15] and next-generation sequencing technologies [5,6,16]. Subsequent reference genome mapping of the sequencing reads will reveal protein binding site information, defined by ChIP enrichment, as well as interactions between these binding loci. The feasibility of this strategy was first demonstrated on oestrogen receptor α (ERα) in human breast adenocarcinoma cells [12], revealing complex networks of ERα-bound intra-chromosomal long-range interactions associated with gene transcription. In principle, ChIA-PET can be applied to any factor amenable to ChIP enrichment, opening up a myriad number of possibilities in identifying the associated chromatin interactions which together constitute the heart of genome organization.

Abbreviations: ChIP, chromatin immunoprecipitation; ChIA-PET, chromatin interaction analysis with paired-end tag sequencing; RNAPII, RNA polymerase II.

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The ERα-associated chromatin interactome represented a promising first step towards the complete annotation of all TREs. To obtain a global view of the transcriptional chromatin architecture, ChIA-PET can be applied to components of the general transcription machinery. In eukaryotic systems, basal transcription of a majority of cellular genes requires assembly of a preinitiation complex comprising RNA polymerase II (RNAPII) and several accessory factors (for review, see [17]) on core promoters. We have mapped transcriptional chromatin interactions by constructing RNAPII ChIA-PET libraries using 5 human cell lines and uncovered wide-spread promoter-centered interactions which further aggregated into multigene clusters [18], providing a topological framework for cotranscription which supports the postulated "transcription factory" model [19]. Importantly, this study also comprehensively characterized long-range enhancer-to-promoter interactions and illustrated the potential of ChIA-PET in connecting disease-associated non-coding elements to their target genes, providing a molecular basis for understanding complex genetic disorders.

Global transcriptional landscapes are shaped into higher-order structures by chromatin-organizing factors, for example the CCCTC-binding factor (CTCF) [20]. Recognized as the 'master weaver' of chromatin architecture, CTCF mediates long range chromosomal contacts between a number of developmentally-important genomic loci (for review, see [21]). ChIA-PET experiments in mouse embryonic stem cells revealed that CTCF-mediated interactions occur throughout the genome and demarcate chromatin into distinct domains with unique epigenetic signatures and transcriptional patterns [22]. In addition to the well-accepted enhancer-blocking model at certain loci [23], CTCF is also possible to facilitate enhancer-bridging to distant promoters for transcription activation.

Using ChIA-PET, it is also possible to study specific TREs marked by certain chromatin signatures. Keji Zhao and colleagues demonstrated that enhancer-promoter interactions associated with the active enhancer mark H3K4me2 form complex interaction networks which correlate with target gene expression [24]. This approach can be applied to other histone modifications, such as acetylation [25], phosphorylation [26] and ubiquitination [27] to study their individual and collective contributions to 3D chromatin function.

Collectively, these studies highlight the contribution of ChIA-PET towards linking genome structures with functions, and demonstrated the potential of this approach to be applied to other interesting and important biological systems. Here, we describe the detailed methodology for the three main parts of a ChIA-PET experiment: ChIP sample preparation, ChIA-PET library construction and tag sequencing with the Illumina sequencing platform. This protocol describes the ChIA-PET methodology applied to RNAPII in K562 myelogenous leukemia cells [18], revealing long-range chromatin interactions between promoters and their corresponding regulatory regions.

2. Description of methods

The entire ChIA-PET methodology comprises of many parts. For clarity, we describe the detailed procedures in the following 4 sections, namely ChIP preparation (Section 2.1), ChIA-PET library construction (Section 2.2), DNA sequencing (Section 2.3), and data mapping analysis (Section 2.4).

2.1. ChIP preparation

2.1.1. Overview

A successful ChIA-PET experiment largely depends on both ChIP DNA quantity and quality. However, due to inherent variations of cell types, relative abundance of targeted epitopes, and particular efficacy of antibodies, specific parameters of the ChIA-PET procedure could be variable. The parameters described in this protocol are based on the experiments of RNAPII in K562 (ATCC #CCL-243) cells as references. Empirical optimizations of key parameters are recommended for different cells, protein targets and antibodies. ChIP materials are temperature-sensitive and all samples and buffers should be kept on ice.

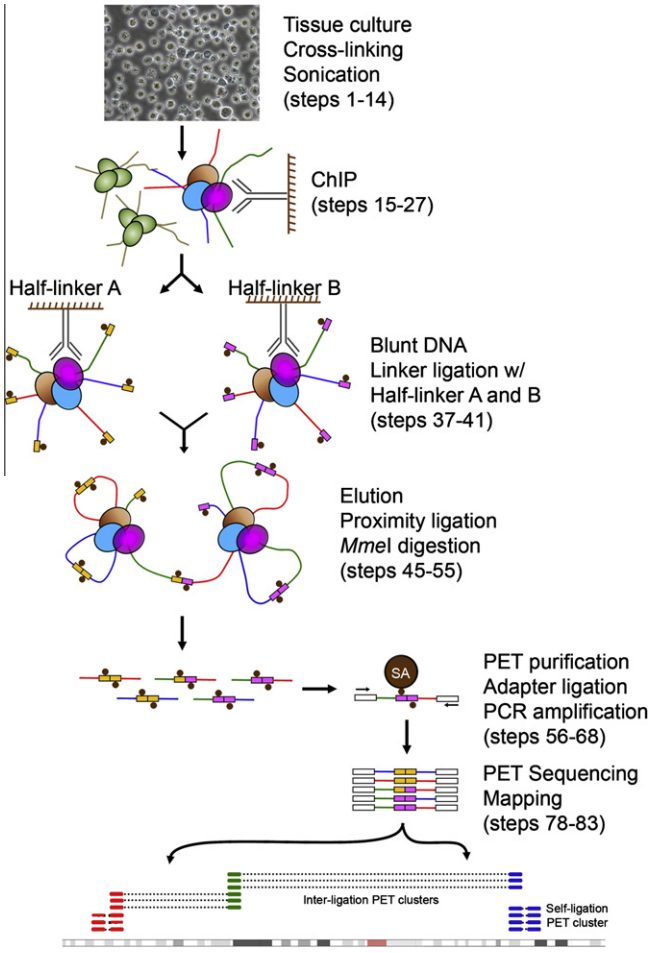

Fig. 1. Schematic veiw of the ChIA-PET methodology SA: streptavidin-coupled magnetic beads.

2.1.2. Step-by-step protocol

2.1.2.1. In vivo dual crosslinking. Covalent fixation of protein-DNA and protein–protein complexes is achieved by a sequential two-step process using crosslinking reagents of varying spacer arm lengths. A combination of crosslinking reagents can increase immunoprecipitation efficiency for transcription factors [28,29], and we have found that dual crosslinking using ethylene glycol bis (succinimidyl succinate) (EGS) followed by formaldehyde increases ChIA-PET library quality. EGS contains a reactive *N*-hydroxysuccinimide ester moiety which hydrolyzes readily. To minimize loss of crosslinking activity, EGS is dissolved in DMSO immediately before use. Crosslinking efficiency is time-dependent and we suggest a range of 30–45 min for EGS crosslinking, followed by 10–20 min of formaldehyde crosslinking. Excessive crosslinking may mask epitopes, reduce antigen accessibility and decrease sonication efficiency. To preserve chromatin complex integrity, supplement phosphate-buffered saline (PBS) from step (5) and subsequent lysis buffers with protease inhibitors (Com-

plete, EDTA-free, Roche Cat # 11873580001). Crosslinking can be performed on both adherent [30] and suspension cells as described.

(1) Transfer 2×10^7 K562 cells into a 50-ml conical tube. We recommend at least 5 tubes (1×10^8 cells) for ChIA-PET library construction. Centrifuge at 800 × rcf for 5 min at room temperature (RT) (22 °C). Discard growth medium and wash twice with PBS.
(2) Discard PBS and add freshly prepared 1.5 mM EGS (Pierce Cat # 21565). Incubate for 45 min at RT with rotation on an Intelli-Mixer (Palico Biotech Cat # RM-2L) (F1, 12 rpm).
(3) Add 37% formaldehyde (Merck Cat # 344198) drop-wise with gentle mixing to a final concentration of 1%. Incubate for 20 min at RT with rotation (F1, 12 rpm).
(4) Add 2 M glycine (Bio-Rad Cat # 161-0718) to a final concentration of 200 mM to quench crosslinking reaction. Incubate for 10 min at RT with rotation (F1, 12 rpm).
(5) Centrifuge at 800× rcf for 5 min at 4 °C and discard supernatant. Wash twice with ice-cold PBS. Proceed with cell lysis or store cell pellet at −80 °C for up to several months. Thaw on ice before proceeding.

2.1.2.2. Lysis and sonication. Crosslinked cells are lysed to remove the bulk of cytosolic proteins to improve ChIP enrichment of chromatin-bound nuclear proteins. Nuclear lysis is then performed to release crosslinked chromatin, increasing chromatin yield and allowing less vigorous sonication conditions. Sonication produces smaller fragments compared to restriction enzyme digestion, reducing the likelihood of non-specific ligation products during self-circularization. In addition, sonication avoids incomplete digestion products, which may contribute up to 30% of a library [31]. Chromatin should be sonicated to between 200 and 600 bp, which can be confirmed on a 1% agarose gel after reverse crosslinking using 20 mg/ml proteinase K (Fermentas Cat # E00491) (step (14), Fig. 2A). The sonication conditions described here work with most cell lines, but further optimization may be required with different equipment and buffer choice.

All buffers used from this point should be ice-cold unless stated otherwise.

(6) Thoroughly resuspend cell pellet in 10 ml Cell Lysis buffer (50 mM Hepes-KOH, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% Na-deoxycholate; 0.1% SDS). Rotate for 15 min at 4 °C (F1, 12 rpm). Centrifuge at 1200× rcf for 10 min at 4 °C. Discard supernatant and repeat cell lysis. To assess lysis efficiency, pipette 5 µl lysate on a glass slide, add a coverslip and observe on a light microscope with non-lysed cells as control.
(7) Resuspend nuclei pellet with 10 ml Nuclear Lysis buffer (50 mM Hepes-KOH, pH 7.5; 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% Na-deoxycholate; 1% SDS). Rotate for 15 min at 4 °C (F1, 12 rpm).
(8) Transfer nuclear lysate into a high speed centrifuge tube (Nalgene Cat # 3119-0050). Centrifuge at 38,000× rcf for 30 min at 4 °C and discard supernatant.
(9) Wash crosslinked chromatin pellet with 10 ml Cell Lysis buffer. Rotate for 15 min at 4 °C (F1, 12 rpm). Centrifuge at 38,000× rcf for 30 min at 4 °C and discard supernatant. Repeat wash.

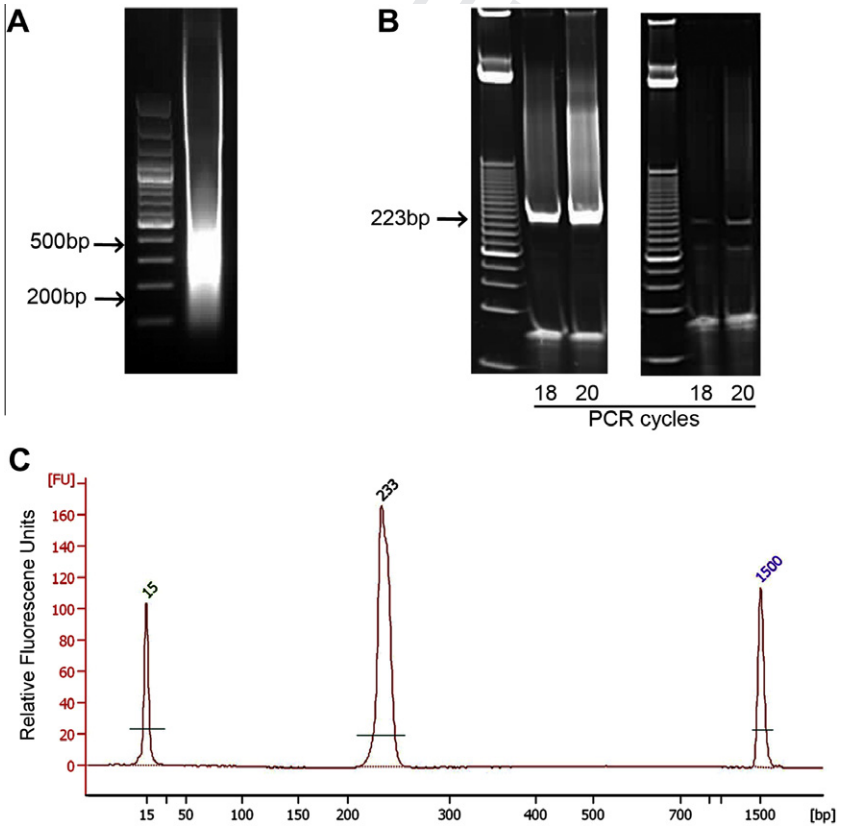

Fig. 2. Experimental results of ChIA-PET library construction. (A) Sonicated chromatin DNA resolved by agarose gel electrophoresis. (B) ChIA-PET templates amplified using PCR and resolved by polyacrylamide gel electrophoresis. Left: Successful library amplification depicted by a prominent 223-bp band. Right: An unsuccessful ChIA-PET library represented by a weak 223-bp band. Excess un-ligated adapters and amplified self-ligated adapters appear as a ~40-bp and ~150-bp band respectively. (C) Bioanalyzer electropherogram of a RNAPII ChIA-PET library from 10^8 K562 cells amplified with 18 PCR cycles. The resulting ChIA-PET library concentration is 68.1 nM. Note that the bioanalyzer reports a higher-than-expected fragment size (233-bp instead of expected 223-bp).

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(10) Proceed with sonication or store chromatin pellet at −80 °C for up to 3 months. Thaw on ice before proceeding.
(11) Transfer pellet into a Polystyrene Round Bottom Test Tube (BD Biosciences Cat # 352057). Resuspend pellet in 1 ml Cell Lysis buffer and remove any bubbles to maximize sonication efficiency.
(12) Sonicate chromatin-DNA suspension with a Digital Sonifier Cell Disruptor (Branson Cat # 450D-0101063591) in an ice-water bath. Sonicate at 35% for 5 cycles, each 30 s ON and 30 s OFF. Transfer chromatin to a new 1.5-ml tube.
(13) Centrifuge at 16,000× rcf for 30 min at 4 °C and transfer supernatant to a new 1.5-ml tube.
(14) Aliquot 10 μl sonicated chromatin to check fragmentation efficiency by gel electrophoresis.

2.1.2.3. Chromatin wash.

(15) Wash 50 μl Protein G magnetic beads (Invitrogen Cat # 100-04D) thrice with 1 ml Beads Wash buffer (PBS; 0.1% Triton X-100). This and future washes involving Protein G magnetic beads should be performed as described in step (16).
(16) Reclaim beads using a Magnetic Particle Concentrator (MPC) (Invitrogen Cat # 123-21D) and discard supernatant. Wash beads with 1 ml Beads Wash buffer and rotate for 5 min at 4 °C (F1, 12 rpm). Centrifuge at 100× rcf for 1 min at 4 °C.
(17) Add 1 ml sonicated chromatin from step (13) to washed magnetic beads. Rotate overnight (~16 h) at 4 °C (F1, 12 rpm).

2.1.2.4. Coating of antibody to beads. When performing ChIP for the first time or with new antibodies, validate antibody specificity by Western Blot, immunohistochemistry and/or immunoprecipitation. For antibodies with known binding characteristics, ChIP-quantitative PCR (ChIP-qPCR) using appropriate positive and negative controls should be performed. We recommend the following amounts of RNAPII antibody (Covance Cat # MMS-126R) for these cells: 12.5 μg/10^8 (K562), 15 μg/10^8 (HCT116), 5 μg/10^8 (hESC3) and 35 μg/10^8 (MCF7).

(18) Wash 50 μl Protein G magnetic beads thrice with 1 ml Beads Wash buffer.
(19) Resuspend beads in 200 μl Beads Wash buffer and add 2.5 μg (12.5 μg/10^8 K562 cells) RNAPII antibody (Covance Cat # MMS-126R). Rotate overnight (~16 h) at 4 °C (F1, 12 rpm).
(20) Wash antibody-coated beads twice with 1 ml Beads Wash buffer. Keep tube on ice after the second wash.

2.1.2.5. Chromatin immunoprecipitation.

(21) Place tube from step (17) in MPC and save 10 μl precleared chromatin as input DNA for subsequent ChIP enrichment analysis. Store input DNA at 4 °C.
(22) Discard Beads Wash buffer from antibody-coated beads from step (20) and combine with pre-cleared chromatin (supernatant) from step (21). Rotate overnight (~16 h) at 4 °C (F1, 12 rpm).
(23) Wash ChIP-enriched beads thrice with 1 ml Cell Lysis buffer.
(24) Wash once with 1 ml High Salt buffer (50 mM Hepes-KOH, pH 7.5; 350 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% Na-deoxycholate; 0.1% SDS).
(25) Wash once with 1 ml ChIP Wash buffer (10 mM Tris–HCl, pH 8.0; 250 mM LiCl; 1 mM EDTA; 0.5% Nonidet P-40 or Igepal CA-630; 0.5% Na-deoxycholate).
(26) Wash once with 1 ml TE buffer (10 mM Tris–HCl, pH 8; 1 mM EDTA).
(27) Resuspend beads in 1 ml TE buffer and store at 4 °C.

2.1.2.6. Elution and crosslink reversal for quantitation. A portion of ChIP DNA is eluted for quantitation and enrichment check. Once sufficient ChIP material has been enriched, samples can be combined for ChIA-PET library construction. We recommend ChIP DNA storage durations of <1 month to minimize loss of chromatin complex integrity.

(28) Use 10% beads by issue from step (27) for quantitation and enrichment check.
(29) Place tube in MPC to collect beads and remove supernatant.
(30) Add 200 μl ChIP Elution buffer (50 mM Tris–HCl, pH 7.5; 10 mM EDTA; 1% SDS; RT) and rotate for 30 min at 37 °C (F1, 12 rpm).
(31) Add 190 μl ChIP Elution buffer to 10 μl of input DNA from step (21).
(32) Add 2 μl proteinase K to ChIP and input DNA, mix and incubate for 2 h at 50 °C.
(33) Add an equivalent volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 7.9) and separate phases with 2-ml MaXtract High Density (Qiagen Cat # 129056) according to manufacturer's instructions.
(34) Transfer upper aqueous phase of each sample into a new tube containing 20 μl 3 M sodium acetate, 1 μl 15 mg/ml Glycoblue (Ambion Cat # AM9740) and 200 μl isopropanol. Vortex to mix and incubate for 20 min at −80 °C.
(35) Centrifuge at 16,000× rcf for 30 min at 4 °C to pellet DNA. Wash DNA pellets with 1 ml 70% ethanol, dry in a SpeedVac (Tomy Cat # MV-100) and resuspend each pellet in 20 μl TE buffer.
(36) Measure DNA concentration with a Quant-IT PicoGreen DS DNA assay (Invitrogen Cat # P11495).

2.1.2.7. Validate enrichment by ChIP-qPCR. A high ChIP enrichment ensures that transient or weak interactions which occur in a small subset of cells can be adequately captured and detected by sequencing. Conversely, poor enrichment leads to libraries yielding few binding and interaction events, requiring higher sequencing depth to detect interactions. ChIP enrichment can be verified with primers specific for known binding sites and normalized with background binding by control primers. By comparing normalized qPCR data between ChIP and input DNA, fold enrichment can be calculated. Primer pairs should span 100–200 bp for adequate binding site coverage. Using this protocol we routinely obtain 100–300 ng RNAPII ChIP DNA from 10^8 K562, HCT116, MCF7 and hESC3 cells with more than 200 × ChIP enrichment using the qPCR primers listed in Fig. 3A.

2.2. ChIA-PET library construction

2.2.1. Overview

This ChIA-PET protocol converts ChIP-enriched chromatin complexes into interacting PET DNA fragments which can be sequenced by the Illumina Genome Analyzer IIx or Hiseq2000 platform (Fig. 1). Chromatin complexes tethered on magnetic beads are first end-polished and ligated (linker ligation) with biotinylated half-linker oligonucleotides bearing a recognition site (TCCAAC) for the type IIS restriction enzyme *Mme*I. Following linker end-phosphorylation, chromatin complexes are eluted, and the tethered DNA fragments with half-linkers are further ligated(proximity ligation) under dilute conditions, forming a com-

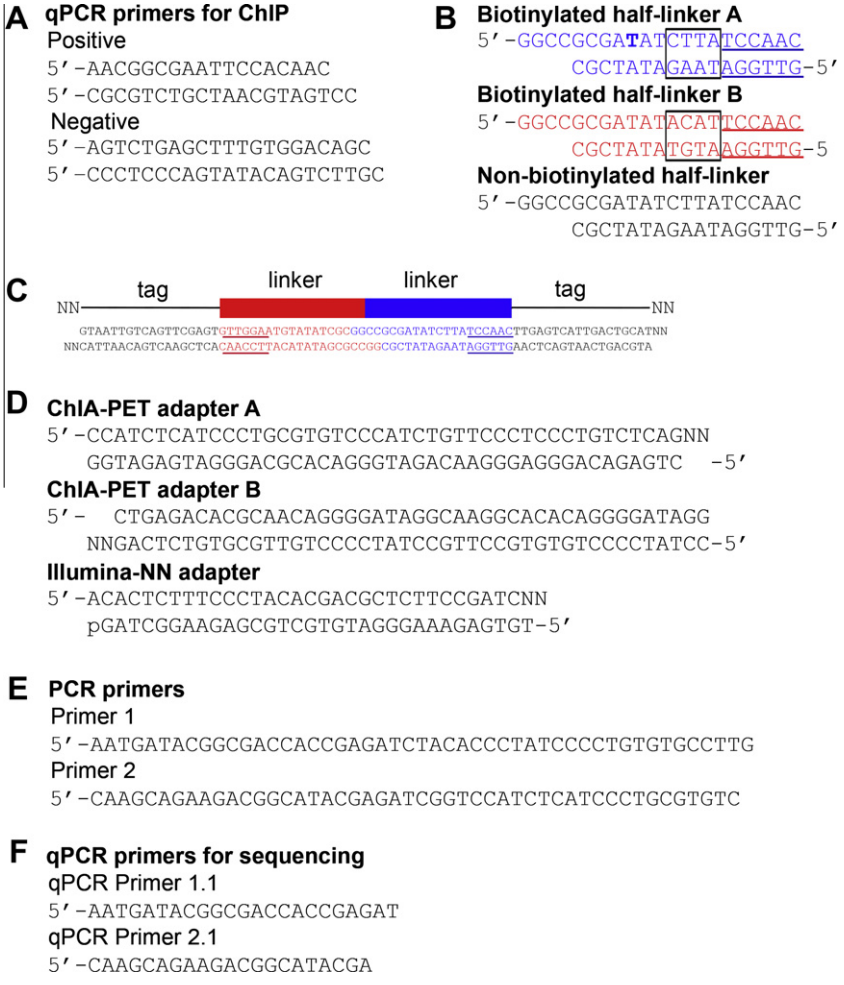

Fig. 3. Oligonucleotide sequences used in ChIA-PET (A) qPCR primers to assess RNAPII ChIP-enrichment in K562 cells. (B) Half-linker sequences. The internal T (in bold) is biotin-modified. Linker barcodes are boxed and *Mme*I recognition sequence is underlined. (C) Schematic representation of the tag-linker-tag construct formed by proximity ligation and *Mme*I digestion. (D) In-house designed ChIA-PET adapters. (E) PCR primers for ChIA-PET amplification. (F) qPCR Primers for ChIA-PET one-point qPCR quantitation prior to sequencing.

plete linker with flanking *Mme*I restriction sites (Fig. 3C). During this ligation, we expect the tethered DNA fragments within chromatin complex are ligated to each other. However, inevitably, DNA fragments tethered in different complexes could also be ligated under such conditions. To assess such non-specific ligation rate, we designed specific nucleotide barcodes in the two different half-linkers (A and B), which are added in separate linker ligation aliquots and then mixed together for proximity ligation (Fig 1). The resulting full linker barcode composition with the mixed linker barcodes of A and B in the ligation products is an indication of non-specific ligation. Restriction digestion of the purified DNA by MmeI allows the extraction of tag-linker-tag constructs (ChIA-PETs). Through isolation by streptavidin-coupled magnetic beads, high-throughput sequencing and reference genome mapping, ChIA-PETs can reveal the relationships between any pair of DNA loci associated with specific protein factors and connected through proximity ligation. Such interacting loci may be further validated using 3C-based methods and/or fluorescence in situ hybridization in combination with protein knock-down or knock-out to establish functional dependence between protein binding and chromatin interaction. As extensive manipulation is required for library construction, we recommend at least 100 ng RNAPII ChIP DNA as starting material, and multiple aliquots of ChIP DNA may be pooled as a single ChIA-PET library. Note that the efficiency of subsequent processing steps can only be assessed through a diagnostic gel run at step (69). The following processing steps have been carefully optimized and in our hands generate a positive gel band in more than 80% of all ChIA-PET libraries.

2.2.2. Step-by-step protocol

2.2.2.1. End-blunt ChIP DNA. Sonication shears chromatin DNA and produce variable 5′- and/or 3′-overhangs incompatible with half-linker ligation. These overhangs can be enzymatically filled in (5′-overhang) or removed (3′-overhang) through the polymerase and exonuclease activities of T4 DNA polymerase.

All incubations involving magnetic beads are performed on an Intelli-Mixer (F8, 30 rpm, U-50, u = 60). Program F8 repeatedly rotates the tube 180° and shakes a few seconds. This combination of rotation and shaking ensures thorough mixing and suspending of beads throughout the entire incubation period.

(37) Pool the ChIP-enriched beads from step (27) to the equivalent of at least 1×10^8 cells. Wash ChIP-enriched beads from step (27) with 1 ml ice-cold TE buffer. Mix by flicking and centrifuge at 100× rcf for 1 min at 4 °C. Place tube in MPC and remove wash buffer. Perform all subsequent washes involving ChIP-enriched beads as described in this step.

(38) Add the following mix in the indicated order. For multiple samples, a master mix can be prepared.

Water	616.0 μl
T4 DNA polymerase buffer (Promega Cat # M831A)	70.0 μl
dNTP mix, each 10 mM (Fermentas Cat # R0191)	7.0 μl
T4 DNA polymerase (Promega Cat # M4215)	7.0 μl
Total	700.0 μl

(39) Incubate for 40 min at 37 °C with rotation (F8, 30 rpm).

2.2.2.2. Ligate biotinylated half-linkers to ChIP DNA. Each half-linker oligonucleotide contains a 5′ cohesive end (GGCC) to facilitate proximity ligation between each other. Ideally, all proximity ligation products are derived from DNA fragments within individual chromatin complexes. However, ligations can occur randomly between different complexes and do not represent true *in vivo* interactions. To assess the frequencies of such random inter-complex ligations, two half-linkers are designed with specific nucleotide barcodes A and B (Fig. 3B). A and B half-linkers are ligated to two separate aliquots of the same ChIP preparation, which are subsequently combined for proximity ligation. PETs derived from chimeric ligations can thus be identified by their A and B linker composition. In principle, additional linker barcodes can be designed for each biological replicate, allowing multiple samples to be processed through a single library construction step. To facilitate PET extraction for sequencing, a *Mme*I restriction digestion site is included in each half-linker. In addition, linkers are biotin-modified to enable purification with streptavidin-coated magnetic beads. This biotin moiety is attached to the internal C6 of the 9th base (T) from the 5′ end through a 15-atom triethylene glycol spacer to minimize steric hindrance to proximity ligation. Linkers are purchased single-stranded and annealed in-house (Appendix A). Annealed linkers should be thawed on ice to prevent denaturation.

(40) Wash beads thrice with 1 ml ChIA-PET Wash buffer (10 mM Tris–HCl, pH 7.5; 1 mM EDTA; 500 mM NaCl). Leave beads in buffer after the final wash.
(41) Aliquot ChIP sample into two equal portions labeled A and B. Discard wash buffer and add the respective reaction mix into each tube in the indicated order. Mix by inverting tube immediately to minimize self-ligation of half-linkers. Incubate overnight (~16 h) at 16 °C with rotation (F8, 30 rpm).

Water	553.5 μl
200 ng/μl Biotinylated half-linker (A or B)	3.5 μl
T4 DNA ligase buffer (Invitrogen Cat # 46300018)	140.0 μl
T4 DNA ligase (Fermentas Cat # EL0013)	3.0 μl
Total	700.0 μl

2.2.2.3. Add phosphate groups to 5′ ends.
(42) Wash beads thrice with 1 ml ChIA-PET Wash buffer. At the third wash, combine beads from tubes A and B using the MPC.
(43) Discard wash buffer and add the following mix in the indicated order.

Water	616.0 μl
T4 DNA ligase buffer (NEB Cat # B0202S)	70.0 μl
T4 DNA polynucleotide kinase (NEB Cat # M0201L)	14.0 μl
Total	700.0 μl

(44) Incubate for 50 min at 37 °C with rotation (F8, 30 rpm).

2.2.2.4. Elute chromatin-DNA complex from beads. The elution of chromatin-complexes from beads allows circularization to be performed under extremely dilute conditions to minimize inter-complex "chimeric" ligations which do not represent true *in vivo* interactions. Subsequent to elution, excess SDS is sequestered with TritonX-100 to prevent denaturation of T4 DNA ligase during proximity ligation.

(45) Reclaim beads with MPC and discard supernatant. Add 200 μl ChIA-PET Elution buffer (10 mM Tris–HCl, pH 8; 1 mM EDTA; 1% SDS; RT). Incubate for 30 min at RT with rotation (F8, 30 rpm).
(46) Reclaim beads with MPC and transfer eluate into a new 1.5-ml tube. Wash beads with 900 μl buffer EB (10 mM Tris–HCl, pH 8.5) and combine the two elutions.
(47) Add 90 μl 20% Triton X-100 and incubate for 1 h at 37 °C.

2.2.2.5. Proximity ligation of DNA fragments with linkers. The circularization conditions described here are optimized for minimal ligations between non-interacting DNA fragments from different chromatin complexes.

(48) Add the following mix in a 50-ml conical tube in the indicated order.

Water	7800.0 μl
T4 DNA ligase buffer (NEB Cat # B0202S)	1000.0 μl
T4 DNA ligase (Fermentas Cat # EL0013)	33.00 μl
Total	8833.00 μl

(49) Add sample from step (47), invert to mix and incubate 20–24 h at 16 °C.

2.2.2.6. Reverse crosslink and DNA purification. Proteins in the samples are enzymatically digested to release crosslinked DNA, which are purified by phenol–chloroform extraction and isopropanol precipitation.

(50) Add 100 μl proteinase K, mix and incubate for 2 h at 50 °C.
(51) Add an equivalent volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 7.9) and separate phases with 50-ml MaXtract High Density (Qiagen Cat # 129073) according to manufacturer's instructions.
(52) Transfer upper aqueous phase of each sample (~10 ml) into a high speed centrifuge tube (Nalgene Cat # 3119-0050) containing 1 ml 3 M sodium acetate, 5 μl glycoblue and 10 ml isopropanol. Invert to mix and incubate for 1 h at −80 °C.
(53) Thaw for 10 min and centrifuge at 38,000× rcf for 30 min at 4 °C. Decant supernatant and transfer DNA pellet to a new 1.5-ml tube. Centrifuge at 16,000× rcf for 1 min at 4 °C and wash pellet with 1 ml 70% ethanol. Dry pellet in a SpeedVac and resuspend in 34 μl buffer EB.

*2.2.2.7. Digest with MmeI to release ChIA-PET. Mme*I exhibits low enzymatic turnover and excess *Mme*I blocks cleavage through substrate methylation [32]. A 1:1 ratio of *Mme*I molecules to restric-

tion sites is required for optimal digestion. To achieve stoichiometric *Mme*I concentration, we have included double-stranded non-biotinylated linkers (Fig. 3B) containing the *Mme*I restriction site in the digestion reaction. S-adenosylmethionine (SAM), required for *Mme*I activity, is extremely unstable and should be freshly diluted from stock solutions before every use.

(54) Add the following mix in the indicated order.

DNA sample	34.0 μl
NEBuffer 4 (NEB Cat # B7004S)	5.0 μl
500 μM SAM (32 mM stock: NEB Cat # B9003S)	5.0 μl
200 ng/μl Non-biotinylated half-linker	5.0 μl
*Mme*I (NEB Cat # R0637L)	1.0 μl
Total	50.0 μl

(55) Incubate for 2 h at 37 °C.

2.2.2.8. Immobilize ChIA-PETs on magnetic beads.

(56) Wash 50 μl M-280 Streptavidin magnetic beads (Invitrogen Cat # 11206D) twice with 150 μl 2× B&W buffer (10 mM Tris–HCl, pH 7.5; 1 mM EDTA; 2 M NaCl).
(57) Resuspend beads in 50 μl 2× B&W buffer and combine with digestion mix from step (55). Incubate for 45 min at RT with rotation (F8, 30 rpm).

2.2.2.9. Ligate ChIA-PET adapters. This procedure ligates in-house-designed adapters (Fig. 3D) to ChIA-PET constructs, which are subsequently amplified for sequencing with the Genome Analyzer IIx or HiSeq2000 platform. Adapters are purchased single-stranded and annealed before use (Appendix A). Thaw all annealed adapters on ice to prevent denaturation.

(58) Wash beads thrice with 150 μl of 1× B&W buffer (5 mM Tris–HCl, pH 7.5; 0.5 mM EDTA; 1 M NaCl).
(59) Reclaim beads using MPC and discard supernatant.
(60) Add the following mix in the indicated order.

Water	36.0 μl
T4 DNA ligase buffer (Fermentas Cat # B69)	5.0 μl
200 ng/μl ChIA-PET Adapter A	4.0 μl
200 ng/μl ChIA-PET Adapter B	4.0 μl
T4 DNA ligase (Fermentas Cat # EL0013)	1.0 μl
Total	50.0 μl

(61) Resuspend beads by pipetting and incubate overnight at 16 °C with rotation (F8, 30 rpm).

2.2.2.10. Nick translation. The extensive manipulation of ChIP DNA, involving heat exposure and mechanical shearing through pipetting or mixing, may introduce nicks in ChIA-PETs. In addition, nicks are formed during adapter ligation as adapters are non-phosphorylated to prevent self-concatenation. Nick translation is performed to prevent these nicks from affecting downstream PCR and sequencing efficiency.

(62) Wash beads thrice with 150 μl of 1× B&W buffer.
(63) Reclaim beads using MPC and discard supernatant.
(64) Add the following mix in the indicated order.

Water	38.5 μl
NEBuffer 2 (NEB Cat # B7002S)	5.0 μl
dNTP mix, each 10 mM (Fermentas Cat # R0191)	2.5 μl
Escherchia coli DNA polymerase I (NEB Cat # M0209L)	4.0 μl
Total	50.0 μl

(65) Resuspend beads by pipetting and incubate for 2 h at RT with rotation (F8, 30 rpm).

2.2.2.11. PCR amplification and purification of ChIA-PETs. A successful ChIA-PET experiment should yield an intense and well-defined 223-bp band on a diagnostic gel run after PCR amplification (Fig. 2B, left), while a faint band is observed when PCR or library construction conditions are sub-optimal (Fig. 2B, right). To generate sufficient ChIA-PET DNA for sequencing, vary PCR amplification from 16 to 20 cycles (step (68)). To minimize amplification errors and loss of library complexity, use as few PCR cycles as possible with high-fidelity DNA polymerases. The PCR primers in Fig. 3E generate libraries compatible with the Illumina sequencing platform. Amplified libraries are size selected and purified to remove excess adapters and non-specific amplification products. During size selection, excise the 223-bp band on a Dark Reader Transilluminator (Clare Chemical Research Cat # DR46B) with SYBR Green I (Invitrogen Cat # S-7585) staining to prevent DNA damage by UV light exposure and intercalating DNA dyes.

Accurate library quantitation is important for cluster generation during sequencing and use of the Agilent Bioanalyzer is recommended. A single sharp electrophoregram peak against a flat baseline should be observed (Fig. 2C). Note that the Bioanalyzer has up to 10% sizing inaccuracy and may report larger-than-expected fragment sizes.

(66) Wash beads twice with 150 μl 1× B&W buffer and resuspend in 50 μl buffer EB.
(67) Prepare the following PCR mix for each cycling condition to be tested. Store remaining beads at −20 °C for up to 6 months.

Water	21.0 μl
Beads suspension	2.0 μl
10 μM PCR primer	11.0 μl
10 μM PCR primer	21.0 μl
Phusion master mix (Finnzymes Cat # F-531L)	25.0 μl
Total	50.0 μl

(68) Run PCR program:
Step 1: 98 °C, 30 s
Step 2: 98 °C, 10 s
Step 3: 65 °C, 30 s
Step 4: 72 °C, 30 s
Step 5: Repeat steps (2–4) for 15–19 times
Step 6: 72 °C, 5 min
Step 7: Hold at 4 °C
(69) Resolve 25 μl PCR product in a 6% TBE PAGE gel (Invitrogen Cat # EC6263BOX) at 200 V for 35 min. Assess intensity of the 223-bp band and amplify the remaining library from step (67) through a large-scale PCR (24 reactions) with the established PCR cycling conditions.
(70) Combine all PCR products by isopropanol precipitation and resolve in a 6% TBE gel at 200 V for 35 min.

(71) Excise the 223-bp band on a Dark Reader Transilluminator with SYBR Green I staining.
(72) Transfer excised gel slices to a 0.6-ml tube with bottom pierced using a 21-G needle. Place each pierced tube inside a 1.5-ml screw-cap tube and shred gel slices by centrifuging at 16,000× rcf for 5 min at 4 °C.
(73) Add 200 μl TE buffer to each 1.5-ml screw-cap tube and ensure shredded gel is fully immersed in buffer. Incubate for 1 h at −80 °C, followed by overnight incubation (~16 h) at 37 °C.
(74) Transfer shredded gel together with buffer into the filter cup of a 2.0-ml Spin-X tube filter (Costar Cat # 8160). Centrifuge at 16,000× rcf for 5 min at 4 °C. Transfer eluate into a new 1.5-ml tube.
(75) Rinse each screw-cap tube with 200 μl TE buffer and transfer rinsing buffer to the tube filter from step (74). Centrifuge at 16,000× rcf for 5 min at 4 °C.
(76) Purify and combine DNA by isopropanol precipitation. Resuspend DNA in 15 μl TE buffer.
(77) Run 1 μl ChIA-PET library using a Bioanalyzer DNA 1000 assay for quantitation and sizing.

2.3. ChIA-PET library sequencing

2.3.1. Overview

ChIA-PETs can be sequenced using the Genome Analyzer IIx [33] and more recently the HiSeq2000, generating ~15 million and ~70 million pass filtered reads per lane respectively. A sequencing depth of at least 20 million unique reads is required for a high-quality library. Using the HiSeq2000 it is possible to perform sample multiplexing by incorporating a unique identifier barcode for each sample within the half-linkers. This protocol describes ChIA-PET sequencing using the Illumina platforms but in principle most other platforms can be adopted, depending on the choice of sequencing adapters and amplification primers.

2.3.2. Step-by-step protocol

2.3.2.1. Quantitate ChIA-PET library via qPCR. A library concentration of 2.5 pM is required for sequencing on the Genome Analyzer IIx or HiSeq2000. Accurate quantitation of library molecules is critical to sequencing yield, quality and reproducibility. ChIA-PET libraries are first quantified electrophoretically using a Bioanalyzer but this method is not specific for all amplifiable molecules for sequencing. qPCR is a robust method for library quantitation to ensure uniform cluster densities [34]. Perform a qPCR (Illumina qPCR Quantification Protocol Guide, available from http://www.illumina.com/support/documentation.ilmn) using the qPCR primers in Fig. 3F with appropriate controls and dilutions. Alternatively a one-point qPCR routinely performed in our lab as described can provide accurate and consistent quantitation. The qPCR cycling conditions described here are suitable with the Roche LightCycler® 480 system, and use of a different system or reagents may require additional optimization.

(78) Dilute library to 10 pM based on Bioanalyzer concentration from step (77).
(79) Set up the following reaction mix in triplicates.

Water	3.8 μl
Standard/ChIA-PET DNA	1.0 μl
qPCR primer 1 (10 μM)	0.1 μl
qPCR primer 2 (10 μM)	0.1 μl
LightCycler480 DNA SYBR Green I Master Mix (Roche Cat # 03752186 001)	5.0 μl
Total	10.0 μl

(80) Run qPCR program:

Initial denaturation		Ramp rate
95 °C	5 min	4.8 °C/s
Amplification (30 cycles)		
95 °C	10 s	4.8 °C/s
60 °C	1 min	2.5 °C/s
72 °C	30 s	4.8 °C/s
Melting curve		
95 °C	5 s	4.8 °C/s
65 °C	1 min	2.5 °C/s
95 °C	5 Acquisitions per °C	
Cooling		
40 °C	10 s	2.0 °C/s

2.3.2.2. Generate clusters and basic data pipeline. Reduced flowcell loading is necessary to ensure correct base-calling of linkers which contain barcode sequences. The amount of library DNA loaded depends on the version of the sequencing data analysis software (Illumina Sequencing Control Studio) (SCS). In general, this amount ranges from one-third to half the maximum capacity of each tile for SCS version 2.9.

(81) Load DNA samples (2.5 pM) onto an Illumina flowcell.
(82) Perform cluster generation with the Illumina cBot Cluster Generation System according to onscreen instructions. Store remaining DNA at −20 °C.
(83) Proceed with library sequencing on the Genome Analyzer IIx or HiSeq2000 using the sequencing primers in Fig. 3F.

2.4. DNA sequence data analysis

2.4.1. Overview

The Genome Analyzer IIx and HiSeq2000 generate image files which are automatically processed by Real Time Analysis (RTA) and Off Line Basecaller (OLB) incorporated in the standard Illumina analysis pipeline. RTA converts raw image files to intensity files and performs base-calling, while OLB converts binary base-call files to text format (qseq.txt). Tag sequences are aligned to a reference genome using Batman [35], a Burrows-Wheeler-transform-based method (downloadable with software package, ChIA-PET Tool, Version 4.1) and processed with the ChIA-PET Tool [36].

2.4.2. Step-by-step protocol

2.4.2.1. Filtering of linker sequences from raw reads. Nonredundant PETs are classified as homo-dimeric A–A (TAAG/TAAG) or B–B (ATGT/ATGT), or hetero-dimeric A–B (TAAG/ATGT) based on their linker sequence compositions. The linker proportion in each group is subsequent used to evaluate noise levels (Section 2.4.2). Linker sequences are then trimmed from PETs.

2.4.2.2. Evaluation of proximity ligation noise in ChIA-PET data. Proximity ligation of DNA fragments tethered in chromatin complexes could introduce significant non-specific ligation products (ligations between different chromatin complexes instead of within complex). The nucleotide barcodes incorporated in the linker sequences provide a measurement of such non-specific ligation with hetero-dimer barcodes (A–B) in a full linker sequence. The proportion of hetero-dimeric A–B PETs is calculated to quantify non-specific ligation noises. In general, non-specific noise ranges from 10% to 30% depending on each experiment. For this particular experiment of RNAPII ChIA-PET in K562 cells, the hetero-dimer rate is 11%. The

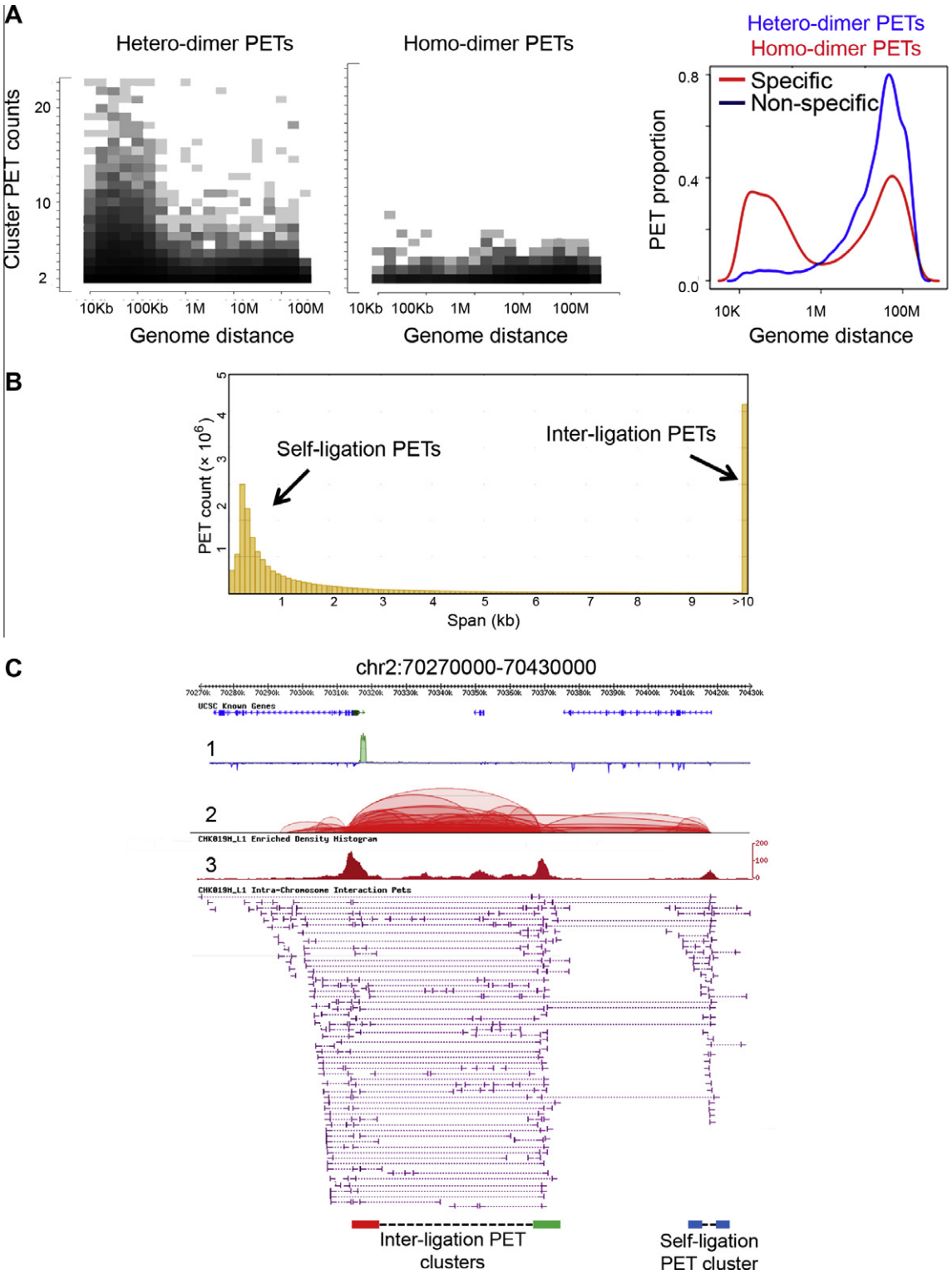

Fig. 4. ChIA-PET sequence mapping analysis. (A) Heatmaps of PET cluster counts versus genomic spans for interactions identified by heterodimeric PETs (left panel) or homodimeric PETs (middle panel). In the right panel, densities of interactions from homodimeric and heterodimeric PETs over genomic distance is shown. (B) Filtered PETs are mapped to the human reference genome and sorted by genomic spans of their interactions. PETs mapped within 3-kb are referred to as self-ligation PETs, while the remaining are inter-ligation PETs, which may map onto the same or different chromosomes. (C) Functional RNAPII loops are revealed through ChIA-PET mapping and clustering. Mapped PETs are clustered to distinguish true interactions from non-specific ones. Data tracks are: (1) RNA-Seq data of K562; (2) visual representation of ChIA-PET detected contacts formed by interaction clusters; (3) RNAPII binding peaks and ChIA-PET data.

vast majority of such hetero-dimer PET data are singletons (no overlap PET, or not recurrent), mapped in different chromosomes or in super-long distance if the paired tags map onto the same chromosome, all of which are characteristics of non-specific noise (Fig. 4A).

2.4.2.3. Mapping tag sequences to reference genomes. After linker trimming, PETs are mapped to the corresponding reference genome and classified into uniquely mapped, multiply mapped or non-mappable PETs. To minimize false positive calls from PCR

clonal amplification, all similarly mapped PETs (within ±1 bp) are merged into one unique PET. To determine the nature of proximity ligation products (i.e. self-ligation, inter-ligation), PETs are classified by the ChIA-PET Tool [36] according to mapping spans which follow a power-law distribution; self-ligation PETs map to the same chromosome within 8 kb apart, while inter-ligation PETs span more than 8 kb and can be further sub-categorized into intra-chromosomal or inter-chromosomal PETs (Fig. 4B).

2.4.2.4. Identification of binding sites and chromatin interactions. The self-ligation PET data is derived from the same DNA fragments circularized during proximity ligation and similar to the ChIP-seq data. Therefore, in our analysis, protein factor binding sites are represented by clusters of overlapping self-ligation PETs, and a false discovery rate can be assigned to individual clusters based on a Monte Carlo simulation, similar to previous approaches in ChIP-PET [37]. Enrichment scores are subsequently assigned to each binding site using similar protocols employed in ChIP-seq analysis [5].

The two mapping tag sites (20 bp each) of an inter-ligation PET represent a possibility of two DNA fragments derived from distant regions that are in close spatial proximity. Since the fragment size by sonication for ChIA-PET analysis is approximate 500 bp, we extend the PET mapping site for 500 bp to establish the digital ChIP fragments under analysis. We also reasoned that if an interaction event between two chromosomal regions is statistically significant, such event should be detected recurrently. Using the overlapping parameter of 500 bp extended from the mapping sites, overlapping inter-ligation PETs are grouped into interaction clusters to define interaction anchor regions. A statistical analysis framework is subsequently applied to take into account the higher probability of random ligation between ChIP-enriched fragments, allowing the false discovery rate of each interaction to be calculated [36]. A large majority ($\geqslant$99.5%) of interaction PETs should overlap with binding sites defined by overlapping self-ligation PETs. To reduce false negatives, only high-confidence PET clusters (FDR < 0.05, PET $\geqslant$ 3) are included in downstream analyses. Using the above experimental and analysis methods, RNAPII ChIA-PET experiments in K562 cells (three independent libraries) generate an average of 38% self-ligation PETs, 29% inter-chromosomal PETs and 21% intra-chromosomal PETs. Of the inter-ligation PETs, 87% consists of singleton PETs, which may represent weak interactions that are indistinguishable from background noise. The processed ChIA-PET data are uploaded onto a mySQL database for organization and visualized with a generic graphical genome browser [38] (Fig. 4C).

3. Concluding remarks

Since its development in 2009, ChIA-PET has proven to be an invaluable adaptation of the original ChIP assay for global mapping of transcription factor binding sites as well as interactions between these sites. Through the interrogation of active chromatin marks [24], components of the transcriptional machinery [12,18] and a key chromatin organizer [22], ChIA-PET has provided an exciting first glimpse into the spectacular intricacies of 3D chromatin organization.

The general transcription machinery is harnessed by general transcriptional cofactors, specific regulatory transcription factors and chromatin-organizing factors to achieve gene- or cell-specific transcription. General transcription cofactors, such as TATA-binding protein (TBP) [39], TBP-associated factors (TAFs) [40–44], Mediator [45] and negative cofactor 1 (NC1) [46] function in combination to fine-tune promoter activities, and an understanding of their associated chromatin interactions will no doubt yield further insights into key principles governing general eukaryotic transcription. A global chromatin contact map associated with "master" regulatory factors can reveal distal TREs involved in specific biological processes and potentially extend known transcription networks in normal and diseased cell states. Some examples of these factors include the homeobox protein NANOG [47], paired box (PAX) proteins [48], HOX proteins [49] and nuclear receptors (for review, see [50]). At a higher organizational level, chromatin fibers are organized by architectural proteins such as topoisomerases [51], cohesin [52] and special AT-rich binding protein (SATB) [53], together with components of the nuclear matrix and transcription or replication factories. By targeting specific factors and analyzing each of their roles in global chromatin interaction, ChIA-PET represents a powerful approach for dissecting the complex intricacies of chromatin structure for functional annotation.

Although the current ChIA-PET methodology is applicable to many cultured cells, the requirement of large amount of cells in the range of 10^8 precludes its application to many interesting biological questions where only small amounts of cell samples are available. Further efforts are certainly needed to streamline the ChIA-PET protocol with a clear goal of reducing the starting material for analysis. Other incremental technical improvements in the ChIA-PET methodology are also expected. The use of protein–protein crosslinking agents in addition to formaldehyde can increase ChIP enrichment of transcriptional cofactors in large multiprotein complexes usually refractory to crosslinking by formaldehyde alone [29,54]. Here, we described a dual crosslinking step using EGS in conjunction with formaldehyde to capture RNAPII-bound chromatin interactions. Crosslinking agents with varying spacer lengths such as disuccinimidylglutarate (DSG) or disuccinimidylsuberate (DSS) represent promising alternatives to enhance detection of ChIP genomic targets and their associated chromatin contacts. Longer PETs map to repetitive regions of the genome with greater accuracy, reducing false positive chromatin interactions. As existing PET strategies rely on restriction digestion for tag extraction, the length of ChIA-PET constructs is constrained by available restriction enzymes. Leveraging on the ability of Illumina platforms to sequence long tags of 200–500 bp, proximity ligation products can be further sonicated, e.g. by adaptive focused acoustics (Covaris), to between 200 and 400 bp and processed with existing ChIA-PET methodologies. Paired-end sequencing using read lengths of 75–100 bp will generate longer tags with increased mapping accuracies.

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