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Efficient isolation of specific genomic regions and identification of associated proteins by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR

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ABSTRACT

Isolation of specific genomic regions retaining molecular interactions is necessary for their biochemical analysis. Here, we established a novel method, engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP), for purification of specific genomic regions retaining molecular interactions. We showed that enChIP using the CRISPR system efficiently isolates specific genomic regions. In this form of enChIP, specific genomic regions are immunoprecipitated with antibody against a tag(s), which is fused to a catalytically inactive form of Cas9 (dCas9), which is co-expressed with a guide RNA (gRNA) and recognizes endogenous DNA sequence in the genomic regions of interest. enChIP–mass spectrometry (enChIP–MS) targeting endogenous loci identified associated proteins. enChIP using the CRISPR system would be a convenient and useful tool for dissecting chromatin structure of genomic regions of interest.

1. Introduction

Function of eukaryotic genome is mediated by molecular complexes in the context of chromatin [1]. Elucidation of molecular mechanisms of genome functions requires identification of components mediating the genome function. However, biochemical nature of chromatin domains is poorly understood, mainly because methods for biochemical and molecular biological analysis of chromatin structure are limited [2–9].

To purify specific genomic regions retaining molecular interactions *in vivo*, we recently developed the insertional chromatin immunoprecipitation (iChIP) technology to identify molecules interacting with a given genomic region of interest *in vivo* [9,10]. Basically, iChIP consists of locus tagging by insertion of the recognition sequences of an exogenous DNA-binding molecule such as LexA and affinity purification of the tagged DNA-binding protein. iChIP has been combined with downstream analyses such as mass spectrometry (MS) (iChIP-MS) to identify proteins and RT-PCR (iChIP-RT-PCR) to identify RNA [10,11]. Although iChIP is a powerful technique to purify specific genomic regions, it requires insertion of the recognition sequences of an exogenous DNA-binding molecule by gene targeting or other methods.

Recent development of engineered DNA-binding molecules has enabled us to design and generate molecules binding to a given DNA sequence. Such engineered DNA-binding molecules include zinc-finger proteins [12], transcription activator-like (TAL) proteins [13], and the CRISPR (clustered regularly interspersed short palindromic repeats) system consisting of a catalytically inactive form of Cas9 endonuclease (dCas9) and small guide RNA (gRNA) [14]. These molecules have opened a way to tag a specific genomic locus without inserting exogenous DNA sequences.

Among others, the CRISPR system provides a flexible and inexpensive way to target specific genomic regions [15]. The CRISPR system is acquired immunity machinery in bacteria and archaea to confer resistance to foreign DNA elements [16] and uses gRNA which base-pairs with the target DNA sequence to cleave foreign DNA elements [17]. RNA-guided cleavage of foreign DNA elements requires only a single gene encoding the Cas9 protein and a synthetic gRNA [18–21]. The CRISPR system was successfully used for RNA-guided genome editing [22–28]. Recently, dCas9 and gRNA were used to specifically repress expression of target genes [14].

Here, we established a novel method, engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP), for purification of specific genomic regions. In enChIP, specific genomic regions are immunoprecipitated with antibody against a tag(s), which is fused to an engineered DNA-binding molecule recognizing an endogenous DNA sequence in the genomic regions of interest. For this purpose, we used dCas9, which binds to a specific DNA sequence when a gRNA is co-expressed [14]. enChIP mediated by the CRISPR system efficiently enriched the targeted endogenous

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loci. In addition, enChIP–mass spectrometry (enChIP–MS) identified proteins associated with those loci. enChIPenChIP using the CRISPR system would be a convenient and useful tool for dissecting chromatin structure of genomic regions of interest.

2. Materials and methods

2.1. Cell culture

293T cells were maintained in DMEM (Wako) supplemented with 10% fetal calf serum (FCS).

2.2. Plasmids

The plasmid encoding hCas9 D10A was purchased from Addgene (#41816) [22]. To construct dCas9, the H840A mutation was introduced into hCas9 D10A with QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). The coding sequence of dCas9 (D10A/H840A) was cleaved and ligated into the p3XFLAG-CMV-7.1 vector (Sigma-Aldrich) to generate 3xF-dCas9/pCMV-7.1. An expression vector of guide RNA was constructed as previously described [22]. Two oligo nucleotides 5′-TTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGCGGGGGCGCTGGGCTGTCC-3′ and 5′-GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACGGACAGCCCAGCGCCCCCGC-3′ were annealed and extended to make a 100 bp double-stranded DNA fragment using Phusion polymerase (New England Biolabs). The fragment was purified after agarose electrophoresis and subjected to Gibson assembly (New England Biolabs) with the linearized gRNA cloning vector (Addgene #41824) [22] to generate gRNA-hIRF-1 #12, the expression vector of the gRNA targeting the human *IRF-1* locus. The expression vector of a 3xFLAG-tagged LexA DNA-binding domain, 3xFNLDD, was described previously [29].

2.3. enChIP-real-time PCR

3×10^6 of 293T cells in 10-cm tissue culture plates were transfected with 2 μg of 3xF-dCas9/pCMV-7.1 and 2 μg of gRNA cloning vector or gRNA-hIRF-1 #12 with Lipofectamine 2000 (Invitrogen). On the following day, cells were replated into 15-cm tissue culture plates. Two days after transfection, cells were fixed with 1% formaldehyde at 37 °C for 5 min. The chromatin fraction was extracted and fragmented by sonication (the average length of fragments was about 2 kb) as described previously [10]. The sonicated chromatin in Sonication Buffer with 1% Triton X-100 was pre-cleared with 15 μg of normal mouse IgG (Santa Cruz Biotechnology) conjugated to 150 μl of Dynabeads-Protein G (Invitrogen) and subsequently incubated with 15 μg of anti-FLAG M2 Ab (Sigma–Aldrich) conjugated to 150 μl of Dynabeads-Protein G at 4 °C overnight. The Dynabeads were washed twice each with 1 ml of Low Salt Wash Buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), High Salt Wash Buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 2 mM EDTA, 1% TritonX-100, 0.1% SDS), LiCl Wash Buffer (10 mM Tris, pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% IGEPAL-CA630, 0.5% sodium deoxycholate), and TE Buffer (10 mM Tris, pH 8.0, 1 mM EDTA). The Dynabeads were suspended in 285 μl of TE and 12 μl of 5 M NaCl and incubated at 65 °C overnight for reverse crosslink. After RNase A treatment at 37 °C for 1 h, Proteinase K treatment was done at 45 °C for 2 h. Subsequently, DNA was purified by phenol/chloroform treatment and used as template for real-time PCR with SYBR Select PCR system (Applied Biosystems) using the Applied Biosystems 7900HT Fast Real-Time PCR System. PCR cycles were as follows: heating at 50 °C for 2 min followed by 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers used in this experiment are hIRF1-prom-F (27310): 5′-cgcctgcgttcgggagatatac-3′; hIRF1-prom-R1 + 2 (27312): 5′-ctgtcctctcactccgccttgt-3′; hSox2-prom-F (27222): 5′-attggtcgctagaaacccatttatt-3′; and hSox2-prom-R (27223): 5′-ctgccttgacaactcctgatacttt-3′.

2.4. enChIP–MS

For the enChIP–MS analysis, the enChIP procedure was performed as described for enChIP-real-time PCR with 6×10^6 cells for transfection, 30 μg of Abs and 300 μg of Dynabeads-Protein G. TBS Buffer (50 mM Tris, pH 7.5, 150 mM NaCl) with 0.1% IGEPAL CA-630 was used instead of TE in the final step of washing procedure. The immunoprecipitants were eluted by incubating with 200 μl of 3xFLAG peptide (Sigma–Aldrich) (500 μg/ml) in TBS with 0.1% IGEPAL CA-630 at 37 °C for 20 min. The eluted samples were precipitated by isopropanol. The precipitants were suspended in 40 μl of 2× sample buffer, incubated at 100 °C for 30 min for reverse-crosslinking and denaturation of proteins, and subjected to SDS–PAGE. The proteins were visualized by Coumassie Brilliant Blue (CBB) staining. Protein bands were excised and analyzed using a nanoLC–MS/MS system, composed of LTQ Orbitrap Velos (Thermo Fisher Scientific) coupled with nanoLC (Advance, Michrom BioResources) and HTC-PAL autosampler (CTC Analytics) at DNA-chip Development Center for Infectious Diseases (RIMD, Osaka University).

3. Results

3.1. Scheme of enChIP

In order to isolate specific genomic regions retaining molecular interactions and identify associated molecules, we established a novel method, enChIP. The scheme of this system is as follows (Fig. 1):

(i) A DNA-binding molecule/complex (DB) is generated to recognize DNA sequence in a genomic region of interest (Fig. 1). This can be achieved by utilizing technologies such as engineered zinc-finger proteins [12], TAL proteins [13], and the CRISPR system consisting of dCas9 together with gRNA [14]. The engineered DB is fused with a tag(s) and the nuclear localization signal (NLS) (Fig. 1A), and expressed into the cell to be analyzed.

(ii) The resultant cell is stimulated and crosslinked with formaldehyde or other crosslinkers, if necessary. This process crosslinks proteins, RNA, DNA and other molecules interacting with the genomic region of interest.

(iii) The cell is lysed, and DNA is fragmented by sonication or digested with nucleases such as restriction enzymes.

(iv) The complexes including the engineered DB are subjected to affinity purification such as immunoprecipitation.

(v) The isolated complexes retain molecules interacting with the genomic region of interest. Reverse crosslinking and subsequent purification of DNA, RNA, proteins, or other molecules allow identification and characterization of these molecules (Fig. 1B).

3.2. Isolation of endogenous loci by enChIP

To show the feasibility of this system, dCas9 was fused with 3xFLAG tag and NLS (3xF-dCas9) (Fig. 1A), and transiently expressed into a human embryonic kidney-derived 293T cells together with the negative control empty gRNA cloning vector or gRNA-hIRF-1 #12 targeting the human *IRF-1* locus. Expression of 3xF-dCas9 was confirmed by immunoblot analysis (Fig. 2A). Cells were crosslinked with formaldehyde, and crosslinked

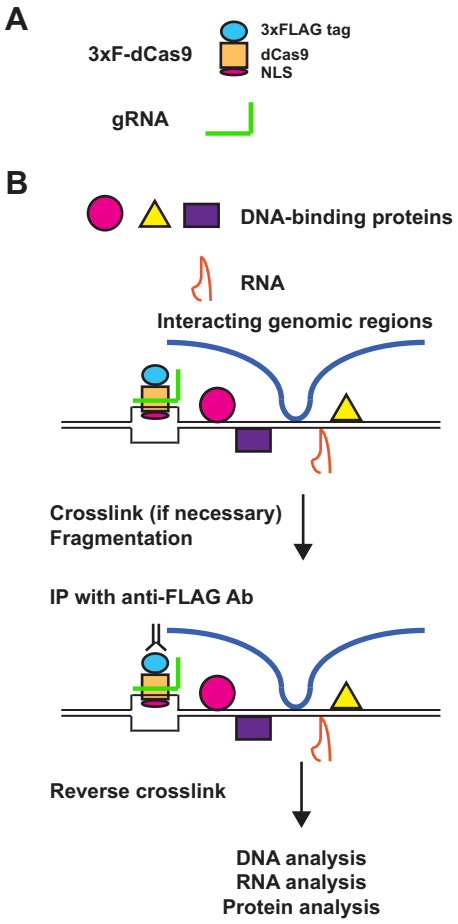

Fig. 1. Scheme of engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using the CRISPR system consisting of dCas9 and gRNA. (A) The system is composed of a fusion protein, 3xF-dCas9, consisting of 3xFLAG-tag, dCas9, and the nuclear localization signal (NLS) of SV40 T-antigen, and a guide RNA (gRNA). (B) The 3xF-dCas9 and gRNA is expressed in appropriate cells. The cells are crosslinked, if necessary, lysed, and fragmented by sonication or other methods. The complexes are immunoprecipitated with anti-FLAG Ab, and crosslink is reversed when crosslinkers are used. Molecules (DNA, RNA, proteins, and others) associated with the target genomic region are isolated and characterized.

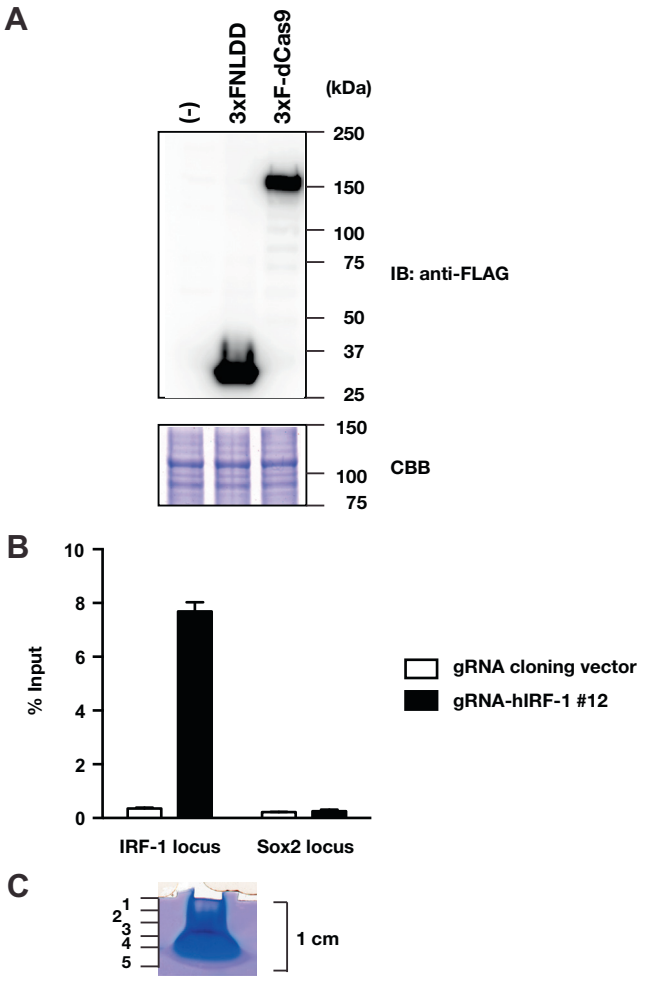

Fig. 2. (A) Expression of 3xF-dCas9. The expression vector of 3xF-dCas9 or the control 3xFNLDD protein was transiently transfected into 293T cells. Nuclear extracts were prepared and subjected to immunoblot (IB) analysis with anti-FLAG Ab. Coumassie Brilliant Blue (CBB) staining is shown as a protein loading control. (B) Isolation of the *IRF-1* locus by enChIP with 3xF-dCas9 and gRNA. Real-time PCR analysis of chromatin complexes isolated by enChIP using 3xF-dCas9 and gRNA. The error bar represents the range of duplicate experiments. (C) Protein complexes purified by enChIP were subjected to SDS–PAGE followed by CBB staining. The gel was cut into five pieces and subjected to mass spectrometry.

chromatin was fragmented by sonication. Subsequently, complexes containing 3xF-dCas9 were immunoprecipitated with anti-FLAG M2 Ab. Real-time PCR showed that the *IRF-1* locus was specifically detected in the immunoprecipitants prepared from 293T cells transfected with gRNA-hIRF-1 #12 but not in those from 293T cells transfected with control gRNA cloning vector. 7.7% of input genomic DNA was immunoprecipitated from cells transfected with gRNA-hIRF-1 #12 (Fig. 2C). In contrast, marginal signals were detected for the irrelevant *Sox2* locus. These results showed that enChIP with 3xF-dCas9 in the presence of specific gRNA can specifically and efficiently isolate target genomic regions.

3.3. Identification of proteins associated with endogenous loci by enChIP–MS

Next, enChIP–MS was used to perform non-biased search for proteins associated with the *IRF-1* locus. 6×10^6 cells were transfected with 3xF-dCas9 and gRNA-hIRF-1 #12. 2 days after transfection, cells were harvested and subjected to enChIP followed by elution with 3xFLAG peptide. Eluate was reverse-crosslinked and subjected to SDS–PAGE. After staining with CBB, proteins were excised for MS analysis (Fig. 2C). We detected many proteins associated with the *IRF-1* locus (Table 1, Supplemental Table 1). The list contained histones as expected. The list also contained various proteins including metabolic enzymes, RNA helicases, and others. These data clearly showed that it is feasible to identify proteins interacting with endogenous genomic regions by enChIP–MS using the CRISPR system.

4. Discussion

In this study, we developed enChIP for purification of specific genomic regions retaining molecular interactions *in vivo* for non-biased identification of binding molecules using the CRISPR system (Fig. 1). We showed that enChIP is able to isolate the endogenous *IRF-1* locus (Fig. 2B). enChIP–MS enabled us to detect known and novel proteins interacting with the *IRF-1* locus (Table 1, Supplemental Table 1). The list of detected proteins included histones, metabolic enzymes, RNA helicases, and others. Many of these proteins were also detected in iChIP-MS (T.F. and H.F., unpublished data), suggesting that enChIP–MS can also identify relevant

Table 1
Examples of proteins detected in enChIP–MS.

Protein
Histones: H1, H2A, H2B, H3, H4
Histone-binding protein RBBP7
Metabolic enzymes: pyruvate kinase isoenzymes M1/M2, alpha-enolase, fatty acid synthase, creatinin kinase B, fructose-bisphosphate aldolase A, poly [ADP-ribose] polymerase, peroxiredoxin-1/2, phosphoglycerate kinase 1, L-lactate dehydrogenase B
RNA helicases: DDX1, DDX3X, DDX6, DDX17, DDX21, DDX39B
Elongation factors: 1-α, 1-γ, 1-δ, 2
Tubulins: α-1B, β-5, β-4B
Eukaryotic translation initiation factors
Peptidyl-prolyl cis–trans isomerases: A, FKBP4
Actin and actin-related proteins: actin-5C, actin-related protein 2/3 complex subunit 4, actin-related protein 3B
Nucleolin
Ribosomal proteins
Polyadenylate-binding protein 1
T-complex proteins
Heat shock proteins: HSP7C, HSP70-1A/1B, HSP70-4, HSP90-α, HSP90-β, HS105

All of identified proteins are shown in Supplemental Table 1.

proteins. Thus, enChIP–MS could detect a wide variety of chromatin-interacting proteins, which is beneficial for comprehensive understanding of chromatin biology. In addition, the results showed that enChIP–MS would be useful for non-biased identification of proteins associated with a given genomic locus *in vivo*. It would be interesting future issues to investigate the functions of newly identified chromatin-binding proteins.

We recently developed iChIP, which is a method to biochemically isolate genomic regions of interest [9,11]. iChIP has been successfully used to directly identify proteins, RNA and DNA bound to specific genomic regions *in vivo* [10,30]. One drawback of iChIP is that it is necessary to insert the recognition sequences of an exogenous DNA-binding molecule such as LexA into the genomic regions of interest. In this regard, enChIP does not require insertion of recognition sequences of exogenous DNA-binding proteins, making the procedure much more straightforward. The yield of isolated genomic regions in iChIP was about 15% of input genomic DNA [29]. In this regard, the yield of isolated genomic regions in enChIP using the CRISPR system was 7.7% of input genomic DNA (Fig. 2C). Although the yield for enChIP using the CRISPR system was about half of that for iChIP, considering that enChIP can isolate both alleles in the genome whereas iChIP can isolate only the tagged allele, the expected total amounts of the isolated genomic regions would be comparable between these two approaches for genomic regions in autosomes. Importantly, we could show that enChIP–MS using the CRISPR system successfully identifies proteins associated with the isolated loci.

Recently, proteomics of isolated chromatin (PICh) has been developed as a novel technique to isolate specific genomic regions retaining molecular interaction [8]. PICh utilizes specific biotinylated nucleic acid probes such as locked nucleic acids (LNAs) that hybridize target genomic regions and isolates the regions using streptavidin beads to analyze interacting proteins. It has been reported that proteins associated with telomeres can be identified by PICh. However, it has not been shown if PICh can be used for identification of proteins associated with low copy number loci. In this regard, we could successfully show that endogenous low copy number loci can be purified by enChIP. In addition, we could show that associated proteins can be identified by MS, showing the utility of enChIP. enChIP can be combined with many different downstream analytical methods. For example, combination of enChIP with microarray analysis (enChIP-chip) or RNA-Seq analysis (enChIP-RNA-Seq) would enable us to perform non-biased search for RNA species associated with a given genomic region. enChIP can also be combined with genomic PCR to detect interactions between given loci or next generation sequencing (enChIP-Seq) to perform non-biased search for interactions between genomic regions in a genome-wide scale.

In summary, enChIP is a novel technology to isolate specific genomic regions for non-biased search for interacting molecules. Because it is very easy to generate gRNA targeting specific genomic regions, enChIP using the CRISPR system would be a convenient way to perform enChIP analysis. enChIP might facilitate understanding of molecular mechanisms of a broad range of biological phenomena involving genome functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.013.

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