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# An enChIP system for the analysis of bacterial genome functions

Toshitsugu Fujita<sup>1,2</sup>, Miyuki Yuno<sup>2</sup> and Hodaka Fujii<sup>1,2\*</sup> 

## Abstract

**Objective:** The engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) technology enables purification of specific genomic regions interacting with their associated molecules. In enChIP, the locus to be purified is first tagged with engineered DNA-binding molecules. An example of such engineered DNA-binding molecules to tag the locus of interest is the clustered regularly interspaced short palindromic repeats (CRISPR) system, consisting of a catalytically-inactive form of Cas9 (dCas9) and guide RNA (gRNA). Subsequently, the tagged locus is subjected to affinity purification for identification of interacting molecules. In our previous studies, we developed enChIP systems for analysis of mammalian genome functions. Here, we developed an enChIP system to analyze bacterial genome functions.

**Results:** We generated a plasmid inducibly expressing *Streptococcus pyogenes* dCas9 fused to a 3xFLAG-tag (3xFLAG-dCas9) in bacteria. Inducible expression of 3xFLAG-dCas9 in *Escherichia coli* was confirmed by immunoblot analysis. We were able to purify specific genomic regions of *E. coli* preserving their molecular interactions. The system is potentially useful for analysis of interactions between specific genomic regions and their associated molecules in bacterial cells to understand genome functions such as transcription, DNA repair, and DNA recombination.

**Keywords:** enChIP, dCas9, ChIP, Chromatin immunoprecipitation, CRISPR, Bacteria

## Introduction

To understand the regulatory mechanisms underlying genome functions such as transcription, it is essential to identify the molecules associated with a genomic region of interest in vivo. The engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) technology we developed recently, enables specific isolation of genomic regions of interest interacting with their associated molecules [1, 2]. Examples of such engineered DNA-binding molecules to tag the locus of interest are transcription activator-like proteins [3] and the clustered regularly interspaced short palindromic repeats (CRISPR) system [4, 5] consisting of a catalytically-inactive form of Cas9 (dCas9) and guide RNA (gRNA). Subsequently, the tagged locus is affinity-purified to identify

interacting molecules. A locus of interest can be tagged in the cell by expressing engineered DNA-binding molecules [1, 2, 6–9] (in-cell enChIP) or in vitro using recombinant or synthetic engineered DNA-binding molecules [10, 11] (in vitro enChIP). After purification of the locus of interest, mass spectrometry (MS), RNA sequencing, and next-generation sequencing (NGS) can be used to identify proteins [1, 2, 6], RNAs [7], and genomic regions [9, 11] binding to the locus in a non-biased manner.

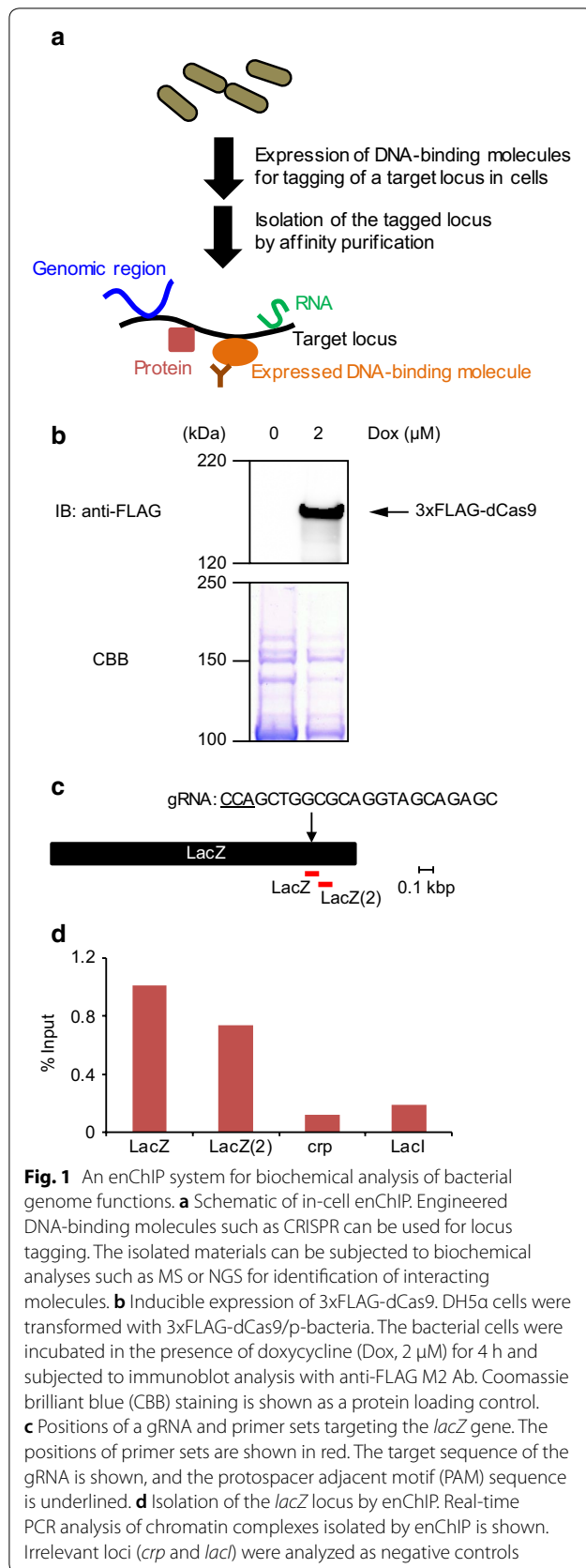
Previously, we developed enChIP systems for analysis of mammalian genome functions. Here, we report development of an in-cell enChIP system for bacterial cells (Fig. 1a). The system consists of plasmids expressing *Streptococcus pyogenes* dCas9 fused to a 3xFLAG-tag (3xFLAG-dCas9) and gRNA in bacteria. The developed enChIP system isolated target genomic regions from *Escherichia coli*. The system might enable identification of molecules associated with a specific genomic region in bacteria, and thus help to elucidate their genome functions.

\*Correspondence: hodaka@hirosaki-u.ac.jp

<sup>1</sup> Department of Biochemistry and Genome Biology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

Full list of author information is available at the end of the article





## Main text

### Materials and methods

#### Plasmids

To construct the doxycycline (Dox)-inducible 3xFLAG-dCas9 expression plasmid 3xFLAG-dCas9/p-bacteria (Addgene #64325), pdCas9-bacteria (Addgene #44249) [12] was digested with *Bgl*II and *Bst*Z17I and treated with bacterial alkaline phosphatase (*E. coli* C75) (2120A, Takara-Bio). The double-strand DNA (dsDNA) containing the coding sequence of the 3xFLAG-tag and the N-terminal portion of dCas9 (agatctaaagaggagaagatct-ATGGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGCTCATGGATAAGAAATACTCAATAGGCTTAGCTATCGGCACAAATAGCGTTCGGATGGGCGGTGATCACTGATGAATATAAGGTTCCGTCTAAAAGTTCAAGGTTCTGGGAAATACAGACCGCCACAGTATCAAAAAAATCTTATAGGGGCTCTTTTATTTGACAGTGGAGAGACAGCGGAAGCGACTCGTCTCAAACGGACAGCTCGTAGAAGGTATAC) (underlined: 3xFLAG-tag; italic: dCas9) was synthesized (Invitrogen) and digested with *Bgl*II and *Bst*Z17I. The cleaved pdCas9-bacteria and the synthetic DNA fragment were purified by agarose gel electrophoresis and ligated.

To construct vectors for expression of gRNAs, pgRNA-bacteria (Addgene #44251) [12] was digested with *Spe*I and *Hind*III and treated with bacterial alkaline phosphatase (*E. coli* C75). The dsDNA for targeting the *lacZ* gene (ACTAGTGCTCTGCTACCTGCGCCAGCGTTT TAGAGCTAGAAATAGCAAGTTAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTTGAAGCTT) (underlined: the target sequences) was synthesized (Invitrogen) and digested with *Spe*I and *Hind*III. The cleaved pgRNA-bacteria and the synthetic DNA fragment were purified by agarose gel electrophoresis and ligated. For construction of gRNAs targeting the promoter region of the *rpoH* gene, oligodeoxyribonucleotides (Table 1) were synthesized, annealed, and phosphorylated. Combination of oligodeoxyribonucleotides was; *E. coli* gRNA common: 27816 + 27817; *rpoH* p 158–180: 27818 + 27819; *rpoH* p 184–206: 27820 + 27821. The annealed dsDNAs were subjected to ligation reactions with the *Spe*I and *Hind*III-digested pgRNA-bacteria plasmid.

#### *E. coli* strains

DH5α (9057, Takara-Bio) was transformed with 3xFLAG-dCas9/p-bacteria alone or together with gRNA expression plasmids, and transformed bacteria were selected with chloramphenicol (Cam) (25 μg/ml) for 3xFLAG-dCas9/p-bacteria alone or a combination of Cam (25 μg/ml) and ampicillin (Amp) (50 μg/ml)

**Table 1 Oligodeoxyribonucleotides used in this study**

Number	Name	Sequence (5' → 3')	Experiments
27816	<i>E. coli</i> gRNA common S	agttaaataaggctagctccgttatcaacttgaaaagtgccaccgagtcg-gtgcctttttga	Construction of plasmids targeting the <i>rpoH</i> gene promoter
27817	<i>E. coli</i> gRNA common A	agcttcaaaaaagaccgactcggtgccacttttcaagtgataacg-gactagcct	Construction of plasmids targeting the <i>rpoH</i> gene promoter
27818	<i>rpoH</i> p 158–180 S	ctagtggtatactcttccctgcaagtttagagctgaaatagca	Construction of the plasmid targeting the <i>rpoH</i> gene promoter 158–180
27819	<i>rpoH</i> p 158–180 A	tattttaactgctatttctagctctaaaactgcagggaaagagtataaca	Construction of the plasmid targeting the <i>rpoH</i> gene promoter 158–180
27820	<i>rpoH</i> p 184–206 S	ctagtcggggtctcttccctgctagtttagagctgaaatagca	Construction of the plasmid targeting the <i>rpoH</i> gene promoter 184–206
27821	<i>rpoH</i> p 184–206 A	tattttaactgctatttctagctctaaaactgcagggaaagagaccccgca	Construction of the plasmid targeting the <i>rpoH</i> gene promoter 184–206
27792	LacZ- <i>E. coli</i> -F	gcgattaccgttgatgttgaagt	Real-time PCR in Figs. 1d and 2d (LacZ)
27793	LacZ- <i>E. coli</i> -R	agtaaggcggtcggtgatgtttt	Real-time PCR in Figs. 1d and 2d (LacZ)
27794	LacZ- <i>E. coli</i> -F2	aaaactatcccgaccgcttact	Real-time PCR in Fig. 1d (LacZ(2))
27795	LacZ- <i>E. coli</i> -R2	gggaagacgtacggggtatacat	Real-time PCR in Fig. 1d (LacZ(2))
27796	<i>crp</i> - <i>E. coli</i> -F	tcacttcagagaaagtgggcaac	Real-time PCR in Fig. 1d ( <i>crp</i> )
27797	<i>crp</i> - <i>E. coli</i> -R	gtcatagcgtctggtttgttgc	Real-time PCR in Fig. 1d ( <i>crp</i> )
27798	LacI- <i>E. coli</i> -F	cgtcagtgggctgacattaact	Real-time PCR in Fig. 1d (LacI)
27799	LacI- <i>E. coli</i> -R	atcaagaataaacgccgaacat	Real-time PCR in Fig. 1d (LacI)
27902	<i>rpoH</i> - <i>E. coli</i> -F	aagcttgattgaactgtggat	Real-time PCR in Fig. 2d ( <i>rpoH</i> -prom)
27903	<i>rpoH</i> - <i>E. coli</i> -R	tatctctggcgcttcagtggtga	Real-time PCR in Fig. 2d ( <i>rpoH</i> -prom)
27896	<i>rpoH</i> -coding-F	tacgtctgcgtaactggcggtat	Real-time PCR in Fig. 2d ( <i>rpoH</i> -cod)
27897	<i>rpoH</i> -coding-R	accatttcgactcatcctggtt	Real-time PCR in Fig. 2d ( <i>rpoH</i> -cod)

for 3xFLAG-dCas9/p-bacteria plus gRNA expression plasmid.

#### Confirmation of inducible expression of 3xFLAG-dCas9

DH5 $\alpha$  transformed with 3xFLAG-dCas9/p-bacteria was cultured in 2 ml of LB media containing Cam (25  $\mu$ g/ml) at 37 °C overnight with shaking. One hundred microliters of the culture liquid was mixed with 900  $\mu$ l of LB media containing Cam (25  $\mu$ g/ml) and incubated for 1 h with shaking. Subsequently, Dox (2  $\mu$ M) was added to the culture media for induction of expression of 3xFLAG-dCas9. After incubation with shaking for 4 h, 400  $\mu$ l of the culture liquid was centrifuged at 5000 rpm for 1 min, and the bacterial pellets were suspended in 100  $\mu$ l of 4 $\times$  SDS buffer. After boiling at 100 °C for 5 min, 10  $\mu$ l of the sample was subjected to SDS-PAGE with a 5–20% gradient gel followed by immunoblot analysis with anti-FLAG M2 antibody (Ab) (F1804, Sigma-Aldrich).

#### enChIP-real-time PCR

DH5 $\alpha$  cells transformed with 3xFLAG-dCas9/p-bacteria alone or together with a gRNA expression plasmid were cultured in 5 ml of LB media containing Cam

(25  $\mu$ g/ml) (3xFLAG-dCas9/p-bacteria) or Cam (25  $\mu$ g/ml) plus Amp (50  $\mu$ g/ml) (3xFLAG-dCas9/p-bacteria and a gRNA expression plasmid) overnight at 37 °C with shaking. The bacterial culture was added to 100 ml of LB media containing the same antibiotics and cultured at 37 °C with shaking. When OD<sub>600</sub> of the culture media reached 0.5, Dox (2  $\mu$ M) was added for induction of 3xFLAG-dCas9 expression. After incubation at 37 °C for 4.5 h with shaking, the bacterial cells were fixed with 1% formaldehyde at 37 °C for 5 min and neutralized with glycine at room temperature for 10 min. After centrifugation, the cell pellets were suspended in 800  $\mu$ l of modified lysis buffer 3 [10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 0.1% sodium deoxycholate, 0.1% SDS, and 1 $\times$  protease inhibitors (Complete-Mini, EDTA-free, 11836170001, Roche)], and DNA was fragmented by sonication using Ultrasonic disruptor UD-201 (TOMY SEIKO) with conditions: Output, 3; Duty, 100% (continuous); Time, Free; 6 cycles of sonication for 10 s and cooling on ice for 20 s (the average length of chromatin fragments was 1 kbp). The sonicated chromatin was subjected to enChIP-real-time PCR analysis as described previously [1, 2]. Primers used in the analysis are shown in Table 1.

## Results and discussion

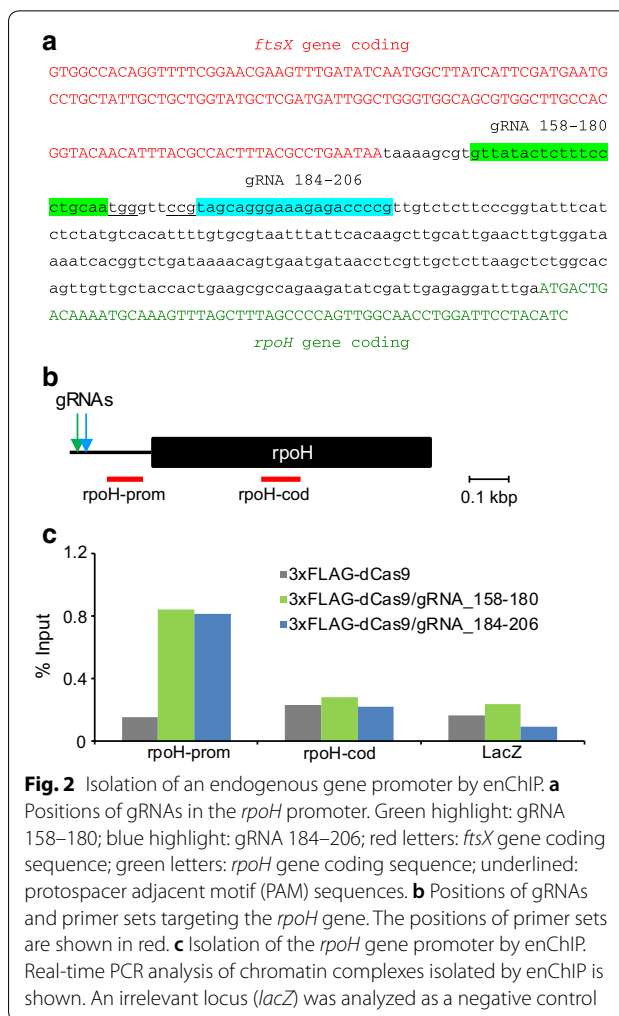
### An enChIP system for the locus-specific biochemical analysis of bacterial genome functions

To analyze the locus-specific functions of the bacterial genome biochemically, we constructed a bacterial expression plasmid (3xFLAG-dCas9/p-bacteria (Addgene #64325)) encoding *S. pyogenes* dCas9 fused with the 3xFLAG-tag. The *E. coli* DH5 $\alpha$  strain was transformed with 3xFLAG-dCas9/p-bacteria, and inducible expression of 3xFLAG-dCas9 by Dox was confirmed by immunoblot analysis with anti-FLAG Ab (Fig. 1b).

Next, for enChIP analysis, we designed a gRNA targeting the coding region of the *lacZ* gene (Fig. 1c). We transformed DH5 $\alpha$  with 3xFLAG-dCas9/p-bacteria together with a gRNA expression plasmid targeting the *lacZ* gene in the DH5 $\alpha$  genome. Four hours after the addition of Dox (2  $\mu$ M), cells were crosslinked with formaldehyde and subjected to sonication for fragmentation of chromatin DNA. Next, chromatin complexes containing 3xFLAG-dCas9/gRNA were immunoprecipitated with anti-FLAG Ab, and DNA was purified. Real-time PCR showed enrichment of the *lacZ* gene (Fig. 1c, d). The percentage of input of negative control genomic regions (the *crp* and *lacI* genes) was markedly lower than that of the *lacZ* gene (Fig. 1d). These results showed that the bacterial enChIP system can specifically and efficiently isolate target genomic regions.

### enChIP analysis of an endogenous gene promoter

Next, we targeted the promoter region of an endogenous gene, *rpoH*, which encodes RNA polymerase sigma (32) factor [13]. This gene is a single operon gene and one of the essential genes of *E. coli* [Profiling of *E. coli* Chromosome (PEC): <https://shigen.lab.nig.ac.jp/ecoli/pec/>]. First, we designed two gRNAs targeting the promoter region of the *rpoH* gene (Fig. 2a). Next, we transformed DH5 $\alpha$  with 3xFLAG-dCas9/p-bacteria alone or together with one of two gRNA expression plasmids targeting the promoter region. After enChIP, real-time PCR showed that the promoter region of the *rpoH* gene was specifically enriched only in the immunoprecipitants prepared from DH5 $\alpha$  cells transformed with both 3xFLAG-dCas9/p-bacteria and either one of two gRNA expression plasmids, but not from those transformed with 3xFLAG-dCas9/p-bacteria alone (Fig. 2b, c). The percentage of input of negative control genomic regions (the coding region of the *rpoH* gene or the *lacZ* gene) was markedly lower than that of the *rpoH* promoter region (Fig. 2b, c). These results confirmed that the bacterial enChIP system can specifically and efficiently isolate endogenous target genomic regions.



## Conclusions

In this study, we developed an enChIP system for analysis of bacterial genomes. This system enables efficient isolation of specific genomic regions from the *E. coli* genome while preserving their chromatin structures, and potentially contributes to the understanding of bacterial genome functions such as transcription and DNA repair.

## Limitations

Further studies might be necessary to assess the utility of this system combined with MS and NGS to identify molecules associated with the target genomic regions in bacteria.

## Abbreviations

enChIP: engineered DNA-binding molecule-mediated chromatin immunoprecipitation; CRISPR: clustered regularly interspaced short palindromic repeats; dCas9: a catalytically-inactive form of Cas9; gRNA: guide RNA; NLS: nuclear

localization signal; MS: mass spectrometry; NGS: next-generation sequencing; Cam: chloramphenicol; Amp: ampicillin; Ab: antibody.

#### Authors' contributions

HF conceived the idea of development of an enChIP system for analysis of bacterial genome functions and constructed expression plasmids. TF and HF designed and performed experiments and wrote the manuscript. MY performed experiments. HF directed and supervised the study. All authors read and approved the final manuscript.

#### Author details

<sup>1</sup> Department of Biochemistry and Genome Biology, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan. <sup>2</sup> Chromatin Biochemistry Research Group, Combined Program on Microbiology and Immunology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan.

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#### Competing interests

TF and H.F. have patents on enChIP (Patent name: Method for isolating specific genomic region using molecule binding specifically to endogenous DNA sequence; Patent number: Japan 5,954,808; Patent application number: WO2014/125668). T.F. and H.F. are founders of Epigeneron, Inc.

#### Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

#### Consent for publication

Not applicable.

#### Ethics approval and consent to participate

Not applicable.

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#### References

- Fujita T, Fujii H. Efficient isolation of specific genomic regions and identification of associated proteins by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR. *Biochem Biophys Res Commun*. 2013;439:132–6.
- Fujita T, Asano Y, Ohtsuka J, Takada Y, Saito K, Ohki R, Fujii H. Identification of telomere-associated molecules by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP). *Sci Rep*. 2013;3:3171.
- Bogdanove AJ, Voytas DF. TAL effectors: customizable proteins for DNA targeting. *Science*. 2011;333:1843–6.
- Harrison MM, Jenkins BV, O'Connor-Giles KM, Wildonger J. A CRISPR view of development. *Genes Dev*. 2014;28:1859–72.
- Wright AV, Nuñez JK, Doudna JA. Biology and applications of CRISPR systems: harnessing nature's toolbox for genome engineering. *Cell*. 2016;164:29–44.
- Fujita T, Fujii H. Identification of proteins interacting with genomic regions of interest *in vivo* using engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP). *Bio Protoc*. 2014;4:e1124.
- Fujita T, Yuno M, Okuzaki D, Ohki R, Fujii H. Identification of non-coding RNAs associated with telomeres using a combination of enChIP and RNA sequencing. *PLoS ONE*. 2015;10:e0123387.
- Fujita T, Yuno M, Fujii H. Allele-specific locus binding and genome editing by CRISPR at the p16INK4a locus. *Sci Rep*. 2016;6:30485.
- Fujita T, Yuno M, Suzuki Y, Sugano S, Fujii H. Identification of physical interactions between genomic regions by enChIP-Seq. *Genes Cells*. 2017;22:506–20.
- Fujita T, Yuno M, Fujii H. Efficient sequence-specific isolation of DNA fragments and chromatin by *in vitro* enChIP technology using recombinant CRISPR ribonucleoproteins. *Genes Cells*. 2016;21:370–7.
- Fujita T, Kitaura F, Yuno M, Suzuki Y, Sugano S, Fujii H. Locus-specific ChIP combined with NGS analysis reveals genomic regulatory regions that physically interact with the Pax5 promoter in a chicken B cell line. *DNA Res*. 2017;24:537–48.
- Qi LS, Larson MH, Gilbert LA, Doudna JA, Weissman JS, Arkin AP, Lim WA. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell*. 2013;152:1173–83.
- Grossman AD, Erikson JW, Gross CA. The htpR gene product of *E. coli* is a sigma factor for heat-shock promoters. *Cell*. 1984;38:383–90.

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