

1 Identification of the DNA binding element of ZNFO, an oocyte-specific zinc finger
2 transcription factor in cattle

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Abstract

The maternal effect genes are essential components of oocyte competence, which orchestrate the early developmental events before zygotic genome activation (ZGA). The Krüppel-associated box (KRAB) domain-containing zinc finger proteins (KRAB-ZFPs) constitute the largest transcription factor family in mammals. As a novel maternal effect gene, *ZNFO* was identified previously in our laboratory. The gene codes for a KRAB-ZFP specifically expressed in bovine oocytes and early embryos and gene silencing experiments have demonstrated that *ZNFO* is required for early embryonic development in cattle. In the present study, we identified a consensus sequence, ATATCCTGTTTAAACCCC, as the DNA binding element of *ZNFO* (ZNFOBE) using a library of random oligonucleotides by cyclic amplification of sequence target (CAST) analysis. Sequence-specific binding of *ZNFO* to the DNA binding element was confirmed by an electrophoretic mobility shift assay (EMSA), and the key nucleotides in the ZNFOBE that are required for specific binding by *ZNFO* were further determined by a competitive EMSA using mutant competitors. Through a luciferase-based reporter assay, it was confirmed that the interaction between *ZNFO* and ZNFOBE is required for the repressive function of *ZNFO*. These results provide an essential step towards the identification of *ZNFO* regulated genes that play important roles during early embryonic development.

Key words: zinc finger, DNA binding, maternal effect gene, cattle

1. Introduction

Oocyte developmental competence or oocyte intrinsic quality describes the capability of oocytes to resume meiosis, cleave and develop to blastocyst stage after fertilization, and further establish implantation and sustain fetal growth and development until birth (Picton et al., 1998; Krisher, 2004; Sirard et al., 2006). Numerous studies have demonstrated that early embryonic mortality is the major cause of pregnancy loss in both human and domestic animals (Ginther et al., 1985; Wilcox et al., 1988; Sartori et al., 2009; Reese et al., 2020) primarily due to poor oocyte quality. Mammalian mature oocytes and early embryos before zygotic genome activation (ZGA) are transcriptionally quiescent and the early development events are highly dependent on maternal factors or maternal effect genes. As essential components of intrinsic oocyte quality, a growing number of maternal effect genes have been identified in model species such as mice (Li et al., 2010; Zhang and Smith, 2015). They are involved in orchestrating different biological processes during early stages including degradation of maternal transcripts and proteins, epigenetic reprogramming and zygotic genome activation (Li et al., 2010; Kim and Lee, 2014).

Zygotic genome activation (ZGA) occurs in two phases: minor ZGA and major ZGA. The minor ZGA, which occurs at the zygotic stage in mice (Li et al., 2021) and the 2-cell stage in cattle (Memili and First, 2000; Vassena et al., 2011), results in low level of transcription and is essential for the major ZGA (Halstead et al., 2020). The major ZGA occurs at the 2-cell stage in mice (Qiu et al., 2003) and 8-cell stage in cattle (Memili and First, 2000; Vassena et al., 2011). The timing difference in ZGA between mice and cattle suggests potential species differences in mechanisms and mediators of the maternal-to-zygotic transition (Telford et al., 1990). To date, our knowledge of the contribution of maternal factors to the maternal-to-zygotic transition during early embryogenesis in cattle is limited. Although a number of maternal effect genes have been

recently identified in bovine species such as JY-1 (Bettegowda et al., 2007), KPNA7 (Tejomurtula et al., 2009), NOBOX (Tripurani et al., 2011) and ZNFO (Hand et al., 2017), our understanding of the mechanisms of action of these genes in controlling the key events during early bovine embryogenesis is far less complete.

ZNFO is a new member of the Krüppel-associated box (KRAB) domain-containing zinc finger protein (KRAB-ZFP) family identified previously in our laboratory (Hand et al., 2017). As a maternal factor, *ZNFO* transcript is highly abundant in germinal vesicle (GV), MII-stage oocytes, and early stage embryos but barely detectable in morula and blastocyst stage embryos (Hand et al., 2017). RNAi experiments demonstrated that embryonic development to 8- to 16-cell stage and blastocyst stage was significantly reduced in ZNFO siRNA-injected embryos indicating that ZNFO is required for early embryonic development in cattle (Hand et al., 2017). However, the regulatory mechanism of ZNFO remains elusive. In the present study, we performed cyclic amplification of sequence target (CAST) assay followed by Sanger sequencing to identify the DNA binding element of ZNFO (ZNFOBE). Electrophoretic mobility shift assay (EMSA) combined with a binding competition assay were performed to confirm the specificity of ZNFO binding to the identified DNA element. Furthermore, a luciferase reporter assay was performed to demonstrate that the binding of ZNFO to ZNFOBE represses transcription.

2. Materials and Methods

2.1. Plasmid construction

The sequence encoding ZNF domain (amino acid residues: 412-714) of ZNFO was amplified by PCR from a *ZNFO* expression construct used in a previous study (Hand et al., 2017) using the High-Fidelity DNA polymerase (Thermo Scientific) and cloned into pH6HTN

His₆HaloTag® T7 Vector (Promega) to generate the ZNFO-ZNF expression construct (pH6HTNHis₆Halo-ZNFO-ZNF). For the luciferase reporter assay, 3 copies of the ZNFO binding element (3xZNFOBE) were cloned into the pGL3-promoter vector (Promega) to generate the reporter construct (3xZNFOBE-pGL3). A mutant reporter construct containing 3 copies of the mutated ZNFO binding element (3xZNFOBEmut) was also generated (3xZNFOBEmut-pGL3). The ZNFO open reading frame (ORF) fused with a N-terminal 3xFLAG tag was cloned into pcDNA3.1A vector (Invitrogen) for overexpression in the mammalian cells, which is named pcDNA3.1A-ZNFO. The PCR primers are listed in Supplementary Table 1. All constructs were verified by Sanger sequencing.

2.2 Expression and purification of recombinant proteins

The construct pH6HTNHis₆Halo-ZNFO-ZNF or pH6HTNHis₆Halo vector was transformed into single step (KRX) competent cells (Promega) to induce the expression of His₆Halo-ZNFO-ZNF or His₆Halo fusion proteins, respectively according to the manufacturer's instructions. Briefly, a fresh colony from each transformation was inoculated into LB medium containing 100 µg/ml Ampicillin and 0.4% of glucose followed by culturing at 37 °C overnight. The overnight culture was further diluted in fresh LB containing 100 µg/ml Ampicillin, 0.05% glucose and 0.1% rhamnose (1:100) and the protein expression was induced at 23 °C. The fusion proteins were purified using HaloLink resin (Promega) or Dynabeads™ His-Tag Magnetic beads (Thermo Fisher Scientific).

2.3 Cyclic amplification of sequence target (CAST) analysis

A library of single-stranded oligonucleotides containing a 20 nt random core sequence flanked on each side by 21 nucleotides (Supplementary Table 1) was generated by Integrated DNA Technologies (IDT). Double-stranded oligonucleotides were prepared by incubating the

single-stranded library with the reverse primer (Supplementary Table 1) in a PCR buffer (Promega) containing 10 μ M of each dNTPs, and 5 units of Taq polymerase and amplified in a thermocycler. The double-stranded random oligonucleotides library was purified using the QIAquick nucleotide removal kit (Qiagen).

To perform the binding reaction, the double-stranded DNA library was incubated with 160 ng of purified His₆Halo-ZNFO-ZNF protein immobilized on HaloLink resin in a binding buffer containing 25 mM Tris (pH = 7.5), 100 mM KCl, 1 mM MgCl₂, 5 mM Dithiothreitol (DTT), 5% glycerol, 100 μ M ZnCl₂, 0.1% Tween-20, 100 μ g/ml poly (dI-dC) and 1 mg/ml bovine serum albumin (BSA) for 60 minutes at room temperature with rotation. Unbound oligonucleotides were washed away with cold wash buffer, comprised of binding buffer without poly (dI-dC). Bound DNAs were used for PCR amplification to generate a library for the next round of CAST analysis. To generate a purified library for ZNFO, a total of 6 rounds of CAST were performed. The final PCR products were purified using the QIAquick nucleotide removal kit (Qiagen) followed by cloning into pGEM-T easy vector (Promega) for Sanger sequencing. The sequences were trimmed and aligned using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>), an online multiple sequence alignment software.

2.4. Electrophoretic mobility shift assay (EMSA)

A sense and an antisense oligonucleotide labeled with IRDye 700 (Supplementary Table 1) were obtained from IDT. The EMSA probe was generated by annealing of the two oligonucleotides. To perform the near-infrared fluorescent EMSA, 20 pmole of probe was incubated with 160 ng of purified His₆Halo-ZNFO-ZNF protein in a binding buffer containing 10 mM Tris-HCl (pH = 7.5), 2.5 mM DTT, 0.25% tween-20, 2.5% glycerol, 50 ng/ μ l polydI-dG, 1 μ g/ μ l BSA, 0.05% NP-40, 50 μ M KCl, and 1 mM ZnCl₂ at 23 °C for 20 minutes. For the

binding competition assay, purified protein was incubated with cold competitors (Supplementary Table 1) for 10 minutes before the addition of the probe. The mixtures were subjected to electrophoresis on a 5% protean TBE precast gel (Bio-rad) in 0.5 × TBE buffer. The gel was analyzed on the Odyssey imaging system (Li-COR).

2.5. Cell culture and luciferase reporter assay

HEK293 cells (2.5×10^4 /well) were plated in a 96-well plate. For each transfection, 10 ng of reporter construct (3xZNFOBE-pGL3 or 3xZNFOBEmut-pGL3), 40ng of ZNFO expression construct (pcDNA 3.1A-ZNFO), and 1 ng of pRL-TK renilla luciferase control vector were co-transfected into the HEK293 cells using X-tremeGENE 9 (Roche) according to the manufacturer's instructions. The luciferase activities were determined 48 hours after transfection using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. The luminescence was measured by the Synergy H1 microplate reader (BioTek). All experiments were repeated in triplicate.

2.6. Statistical analysis

The relative luciferase activities were analyzed in JMP using Student's t-test. Data were obtained from a representative of three independent experiments, and each group represents the mean $\pm$ SEM. $P < 0.05$ indicates significant difference.

3. Results

3.1. Determination of the DNA sequence recognized by ZNFO

To identify the sequences recognized by ZNFO, we performed the CAST assay. The ZNF domain of ZNFO was fused with a His₆Halo tag, generating a His₆Halo-ZNFO-ZNF fusion protein. The recombinant protein was induced, purified and immobilized on the beads. Through

6 rounds of CAST reactions, a purified ZNFO binding element library was generated from the original random oligonucleotide library (Fig. 1A). The final library was cloned into the pGEM-T easy vector, and individual clones (55) were selected for Sanger sequencing. The selected sequences were aligned using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) and an 18-nt consensus sequence, ATATCCTGTTTAAACCCC, was identified as the ZNFOBE (Fig. 1B). Within this sequence, TATCCTG appears to be specific and therefore can be considered as the core element. However, the nucleotides at positions 9, 10, 11 and 15 are not specific.

3.2. Confirmation of the interaction between ZNFO and ZNFOBE

To demonstrate the interaction between ZNFO and the identified ZNFOBE, we performed a near-infrared fluorescent EMSA using an IRDye700 labeled ZNFOBE probe designed based on the sequences of clone 4 and 5 (ATATCCTGCTTAAACCCC). The purified ZNFO-ZNF protein was incubated with the IRDye700 labeled probe. As shown in Fig. 2, the addition of purified ZNFO-ZNF protein causes a shift of the ZNFOBE probe (lane 2). To further elucidate the specificity of the interaction, a competitor (identical to the ZNFOBE probe sequence but unlabeled) was included in the reaction. The bound complex was competed out by 10-fold and 100-fold molar excess of unlabeled cold competitor (lane 3 and 4).

3.3. Determination of the key nucleotides in ZNFOBE

It appears that the sequence in the middle of the consensus sequence is not specific (Fig. 1B). To determine the regions that are required for specific binding by ZNFO, we performed a competitive EMSA using mutant competitors. A series of oligonucleotides with base substitutions in different regions of the ZNFOBE were synthesized and used as cold competitors (Fig. 3A and Supplementary Table 1). As expected, the intensity of the shifted band was reduced by adding 200-fold molar excess of wild-type competitor (Fig. 3B, lane 4 and Fig. 3C, lane 4). In

contrast, the mutant competitors with base substitutions at positions 1 to 8 (mutant 1) and positions 13 to 18 (mutant 3) failed to compete for binding with ZNFO (Fig. 3B, lanes 5, 6, 9 and 10), indicating that both regions of the consensus sequence are indispensable for the interaction between ZNFO and ZNFOBE. The mutant competitor with base substitutions at positions 9 to 12 (mutant 2) was able to compete for binding with ZNFO when 200-fold molar excess of competitor was used (Fig. 3B, lane 8), indicating the middle region (position 9 to 12) of the consensus sequence is not essential for specific binding to ZNFO. Further analysis revealed that the mutant competitors with base substitutions at positions 15 to 18 (mutant 4), positions 15 to 16 (mutant 6) and positions 17 to 18 (mutant 7) did not compete for binding with ZNFO (Fig. 3C, lanes 5, 6, 9, 10, 11 and 12), indicating that nucleotides at these positions are required for specific binding to ZNFO. Interestingly, the mutant competitor with base substitutions at positions 13 to 14 (mutant 5) could partially compete for binding of ZNFO to the ZNFOBE probe as evidenced by slightly decreased intensity of the shifted band (Fig. 3C, lane 8).

3.4. Transcriptional regulation of ZNFOBE.

To determine the regulatory function of ZNFOBE, we cloned 3xZNFOBE into the pGL3 vector, which contains a SV40 promoter located upstream of the luciferase gene (Fig. 4A). The 3xZNFOBE-pGL3 construct was co-transfected into HEK293 cells with the ZNFO expression construct (pcDNA 3.1A-ZNFO). Overexpression of ZNFO decreased the SV40 promoter-driven luciferase activity (Fig. 4B), indicating that the interaction of ZNFO and ZNFOBE has a repressive effect. Furthermore, the mutation of ZNFOBE in the reporter construct rescued the repressive effect of ZNFO overexpression (Fig. 4C).

4. Discussion

As a zinc finger transcription factor, ZNFO needs to bind to its specific DNA elements to regulate transcriptional activity of downstream genes. In this study, we identified ATATCCTGTTTAAACCCC as the ZNFO binding element and TATCCTG therein as the core element. Sequence-specific binding of ZNFO to the DNA binding element was confirmed by EMSA and competition assays. Furthermore, the luciferase-based reporter assay demonstrated that the ZNFOBE is essential for the repressive function of ZNFO.

The tandem two cysteine and two histidine (C2H2) zinc finger proteins (ZFPs) represent the largest transcription factor family in mice and human (Emerson and Thomas, 2009), while the KRAB-ZFPs are the largest subtype (Urrutia, 2003; Emerson and Thomas, 2009). The zinc finger motifs of KRAB-ZFPs serve as modular DNA binding units. Each finger may interact with three successive nucleotides on the forward strand (Pavletich Nikola and Pabo Carl, 1991) and one nucleotide in the reverse strand (Fairall et al., 1993). The KRAB domain interacts with KAP1 co-repressor, which serves as a platform to recruit repressive heterochromatin-inducing macromolecular complex. Even though a growing number of KRAB-ZFPs have been identified and characterized to have essential functions in various cellular, physiological and even pathological process including cell proliferation, differentiation, apoptosis and cancer (Lupo et al., 2013), the target elements of mammalian KRAB-ZFPs are largely unknown. In general, transcription factors, such as Nobox (Choi and Rajkovic, 2006) and Figla (Liang et al., 1997) bind to short sequences. The KRAB-ZFPs are expected to have longer target elements (Emerson and Thomas, 2009), since they can contain up to 36 zinc finger motifs. However, not each individual fingers from KRAB-ZFPs is involved in DNA binding (Hoffmann et al., 2003; Johnson David et al., 2007). The identified ZNFOBE in the present study has 18 nucleotides,

which is relatively longer than the general binding elements for transcription factors. However, ZNFO is expected to capture a 27-nucleotide element, since it has 9 zinc finger motifs. Apparently, ZNFO may have some fingers that are not involved in DNA binding.

The global epigenetic reprogramming is an essential process, which ensures that the embryonic genome is activated properly. After fertilization, the zygote undergoes a global demethylation followed by a *de novo* methylation establishment (Kohli and Zhang, 2013), which is required for the cell lineage differentiation. During pre-implantation stage, the imprinted control regions are protected from the global demethylation process. It has been demonstrated that KRAB-ZFPs are highly involved in the process. For example, Zfp57, a KRAB-ZFP, is highly involved in imprinting maintenance (Lau et al., 2016). In mouse, Zfp57 binds to the methylated hexanucleotide motif, TGCCGC (Quenneville et al., 2011), and deficiency of both zygotic and maternal Zfp57 fails to maintain DNA methylation at multiple differentially methylated regions and results in embryonic death (Li et al., 2008). The corepressor KAP1 is indispensable for Zfp57 to maintain the genomic imprints (Zuo et al., 2012). The activation of endogenous retroelements is another event that occurs during ZGA (Peaston et al., 2004; Jiang et al., 2018) due to the global epigenetic reprogramming during the early developmental stage. The endogenous retroelements are considered parasite DNA and are silenced by KRAB-ZFPs to avoid them agitating the integrity of host genome (Wolf et al., 2015). As a new member of the KRAB-ZFP family, ZNFO may also be involved in the maintenance of imprinting and silencing of endogenous retroelements during early embryonic development. Since ZNFO is known to interact with KAP1 with high affinity and the expression of ZNFO is restricted to female gamete and pre-implantation embryos (Hand et al., 2017), it is likely that, together with KAP1, ZNFO may regulate genes specifically or predominantly expressed in oocyte and early embryos.

Identification of ZNFOBE in this study would help identify putative ZNFO target genes in future studies based on analysis of differentially expressed genes in early embryos following ZNFO knockdown.

In conclusion, the present study identified a consensus sequence, ATATCCTGTTTAAACCCC, as the binding element of ZNFO. The interaction between ZNFO and ZNFOBE is required for the repressive function of ZNFO. The identification of ZNFOBE is essential for future studies to investigate the molecular mechanism, in which ZNFO contributes to early embryonic development.

Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

Mingxiang Zhang: Conceptualization, Investigation, Formal analysis, Writing - Original Draft. Heather L. Chaney and Jaelyn Z. Current: Investigation. Jianbo Yao: Supervision, Writing- Reviewing and Editing, Funding acquisition.

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Figure legends

Fig. 1. Determination of the consensus sequence recognized by ZNFO. (A) Progressive enrichment for the ZNFO binding elements by CAST assay. The original random oligonucleotide library was incubated with His₆Halo-ZNFO-ZNF fusion protein or His₆Halo protein immobilized on beads to pull down the potential binding elements. The selected oligonucleotides were subjected to PCR amplification followed by next round of incubation to further purify the library. A total of 6 rounds of CAST reactions were performed. The products of each round were subjected to electrophoresis on 1% agarose gel. (B) Alignment of individual sequences obtained by Sanger sequencing of cloned PCR products after 6 rounds of CAST reactions. The consensus sequence recognized by ZNFO (ZNFOBE) was generated using MEME suite (<http://meme-suite.org>).

Fig. 2. Confirmation of the interaction between ZNFO and ZNFOBE using EMSA. Purified ZNFO-ZNF protein was incubated with IRDye 700 labeled ZNFOBE probe (ATATCCTGCTTAAACCCC). A 10-fold or 100-fold molar excess of cold wild-type competitor, which is identical to the probe but not labeled, was included in the reaction to further confirm the specificity of the interaction. The shifted bands represent the ZNFO-ZNF/probe complex.

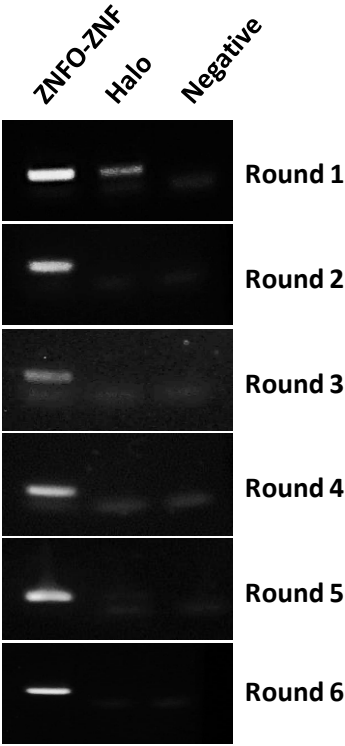
Fig. 3. Determination of the key nucleotide(s) of ZNFOBE by EMSA and competition assays. (A) Sequences of the wild-type and mutant competitors. The mutated nucleotides are highlighted in yellow. (B) and (C) EMSA and competition assays using different mutant competitors. IRDye 700 labeled ZNFOBE probe was incubated with purified ZNFO-ZNF protein.

A 20-fold or 200-fold molar excess of cold wild-type or mutant competitors were included in the reactions. The shifted bands represent the ZNFO-ZNF/probe complex.

Fig. 4. Luciferase reporter assays to determine the transcriptional regulatory function of ZNFOBE. (A) Schematic representation of pGL3-promoter vector, wild type ZNFOBE reporter construct (3xZNFOBE-pGL3) and mutant ZNFOBE reporter construct (3xZNFOBEmut-pGL3). (B) Luciferase reporter assay showing ZNFO regulates the expression of wild type ZNFOBE-driven reporter gene. HEK293 cells were transiently co-transfected with ZNFO expression construct (pcDNA3.1A-ZNFO) or pcDNA3.1A along with ZNFOBE luciferase reporter construct (3xZNFOBE-pGL3) and pRL-TK renilla luciferase reporter plasmid in a 96-well plate (n = 20). Relative luciferase activities were determined by the ratio of firefly to renilla luciferase activity. Data were obtained from a representative of three independent experiments, and each group represents the mean $\pm$ SEM of 20 wells of cells ($P < 0.05$). (C) Luciferase reporter assay showing ZNFO fails to regulate the expression of mutant ZNFOBE-driven reporter gene. HEK293 cells were transiently co-transfected with ZNFO expression construct (pcDNA3.1A-ZNFO) along with wild type ZNFOBE luciferase reporter construct (3xZNFOBE-pGL3) or mutant construct (3xZNFOBEmut-pGL3) and pRL-TK renilla luciferase reporter plasmid in a 96-well plate (n = 18). The relative luciferase activities were determined by the ratio of the firefly to renilla luciferase activity. Data were obtained from a representative of three independent experiments, and each group represents the mean $\pm$ SEM of 18 wells of cells ($P < 0.05$).

Figure 1

A



B

1	CTGAG	TTATCCTGTTTAAAACCC	GTT
2	AACG	ATATCCTGTCCATACCCC	ACAG
3	AAC	ATATCCTGGAAGAATCCC	TTCAG
4	AAC	ATATCCTGCTTAAACCCC	TAACAG
5	AAC	ATATCCTGCTTAAACCCC	TAACAG
6	AACCAA	ATATCCTGTCTAAAACCC	AG
7	AACCG	ATATCCTGGTTAAACCCC	AG
8	AAC	ATATCCTGCTTAAACCCCT	TCCAG

Consensus **ATATCCTGTTTAAAACCCC**

T GCCGT A T

CAA T

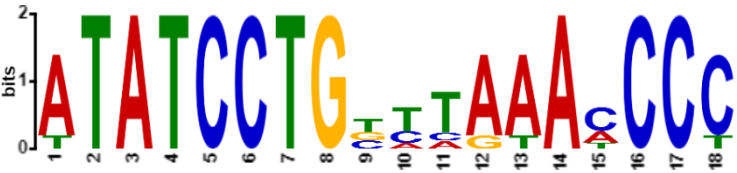


Figure 2

ZNFO-ZNF	-	+	+	+
IRDye 700 labeled probe	+	+	+	+
Cold competitor	-	-	10	100

Probe + ZNFO-ZNF →

Free probe →

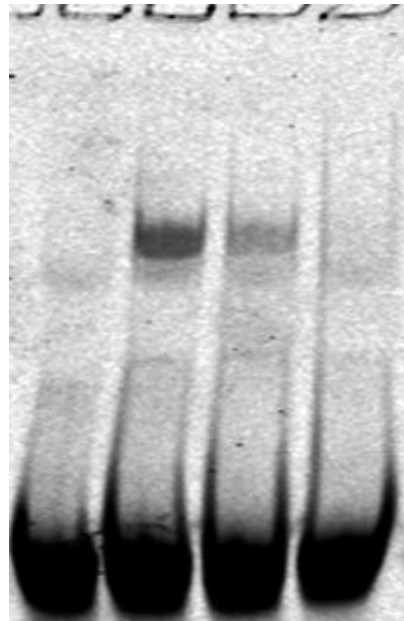


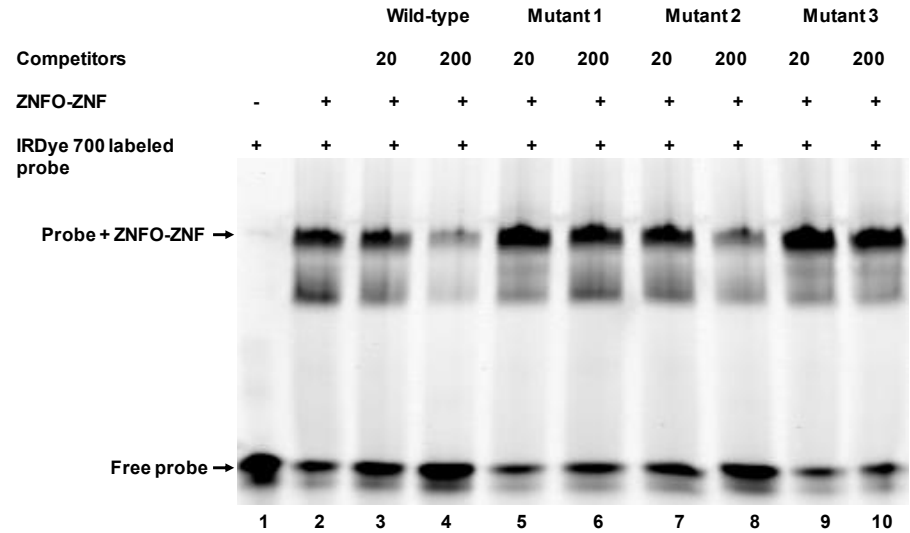
Figure 3

A

Cold competitors

Wild-type ATATCCTGCTTAAACCCC
 Mutant 1 GCGCTTCACTTAAACCCC
 Mutant 2 ATATCCTGTCCGAACCCC
 Mutant 3 ATATCCTGCTTAGGTTTTT
 Mutant 4 ATATCCTGCTTAAATTTT
 Mutant 5 ATATCCTGCTTAGGCCCC
 Mutant 6 ATATCCTGCTTAAATTCC
 Mutant 7 ATATCCTGCTTAAACCTT

B



C

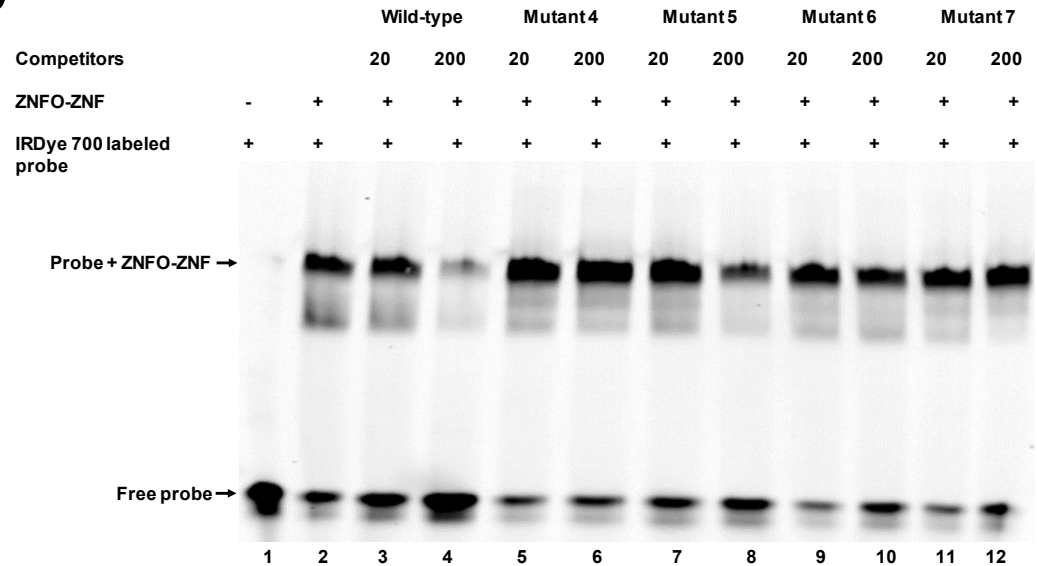
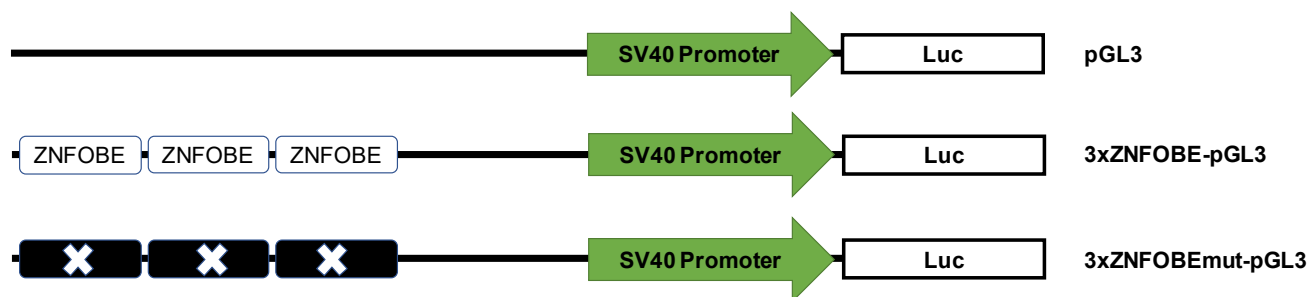
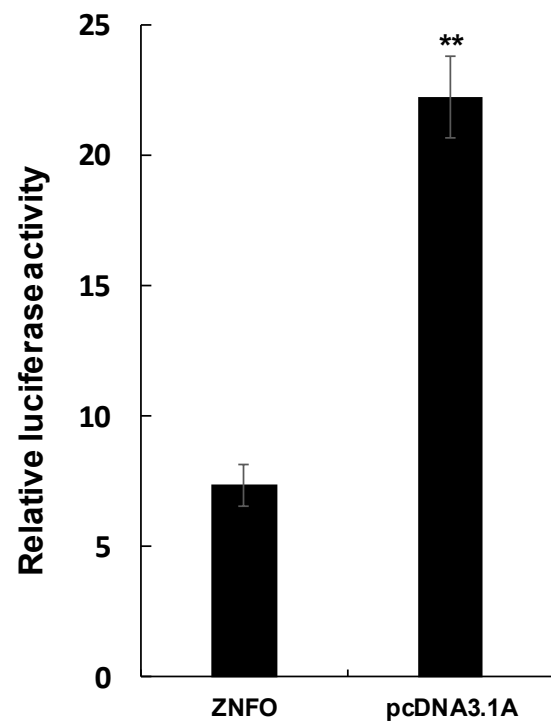


Figure 4

A



B



C

