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Author(s)	Bandara, Thusitha A. M. K.; Otsuka, Kai; Matsubara, Shin et al.
Citation	Biochemical and biophysical research communications, 534, 1007-1012 <a href="https://doi.org/10.1016/j.bbrc.2020.10.049">https://doi.org/10.1016/j.bbrc.2020.10.049</a>
Issue Date	2021-01-01
Doc URL	<a href="https://hdl.handle.net/2115/83746">https://hdl.handle.net/2115/83746</a>
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Type	journal article
File Information	Biochem. Biophys. Res. Commun.534_1007-1012.pdf



## **A dual enhancer-silencer element, DES-K16, in mouse spermatocyte-derived GC-2spd(ts) cells**

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## Abstract

The multifunctionality of genome is suggested at some loci in different species but not well understood. Here we identified a DES-K16 region in an intron of the *Kctd16* gene as the chromatin highly marked with epigenetic modifications of both enhancers (H3K4me1 and H3K27ac) and silencers (H3K27me3) in mouse spermatocytes. *In vitro* reporter gene assay demonstrated that DES-K16 exhibited significant enhancer activity in spermatocyte-derived GC-2spd(ts) and hepatic tumor-derived Hepa1-6 cells, and a deletion of this sequence in GC-2spd(ts) cells resulted in a decrease and increase of *Yipf5* and *Kctd16* expression, respectively. This was consistent with increased and decreased expression of *Yipf5* and *Kctd16*, respectively, in primary spermatocytes during testis development. While known dual enhancer-silencers exert each activity in different tissues, our data suggest that DES-K16 functions as both enhancer and silencer in a single cell type, GC-2spd(ts) cells. This is the first report on a dual enhancer-silencer element which activates and suppresses gene expression in a single cell type.

**Keywords:** Dual enhancer-silencer, Multifunctional genome, Spermatocyte, Histone modification, GC-2spd(ts) cell, ChIP-sequencing

## 1. Introduction

The genome consists of different types of cis-regulatory elements such as transcriptional enhancers and silencers [1,2]. Transcriptional enhancers play a major role in activating gene expression and can be located upstream or downstream of transcriptional start sites of target genes or within gene bodies [2]. A silencer is a cis-regulatory element which negatively regulates the transcription of its target gene. Since its discovery in 1985, various silencers have been identified in different species [3–7], but compared to enhancers, detailed studies about silencers are limited.

Epigenomic analyses have revealed the association of epigenetic marks with cis-regulatory elements. Histone H3 lysine 4 monomethylation (H3K4me1) has been identified as an important and enriched mark at active and primed enhancers, and possibly finetunes, rather than tightly controls, the activity and function of enhancers [8–10]. H3 lysine 27 acetylation (H3K27ac) distinguishes active enhancers from inactive/poised enhancer elements that contain H3K4me1 alone [8,11]. H3K27 trimethylation (H3K27me3) was recently reported to be a mark of silencers when it coincides with DNase I hypersensitivity [12]. Thus, epigenetic marks are useful for exploration of the functional genomic regions.

In addition, the multifunctionality of genome and its association with epigenetic marks have been revealed by recent studies. A dual promoter-enhancer or epromoter is associated with H3K4me1, H3K27ac, and histone H3 lysine 4 trimethylation (H3K4me3), a typical mark of promoter, and possesses both promoter and enhancer activity to control the transcription of two or more genes at a time [13–20]. A dual enhancer-silencer element possesses both enhancer and silencer activity [21,22], and a recent study with *Drosophila* indicated its association with H3K4me1, H3K27ac, and H3K27me3 [23]. However, while dual promoter-enhancers mostly function as both enhancer and promoter in a single cell type, enhancer and silencer activity of a dual enhancer-silencer is exerted in different tissues [21–24]. This raises a question whether a dual enhancer-silencer element can function as both enhancer and silencer in a

single cell type or not.

In this study, we identified a novel gene regulatory genomic element in mouse primary spermatocytes, DESK16, in an intron of the *Kctd16* gene. The present data demonstrate that DES-K16 exhibits unprecedented dual enhancer-silencer activity in a spermatocyte-derived GC-2spd(ts) cell line. To the best of our knowledge, this is the first demonstration of the simultaneous enhancer and silencer activity in a single cell type.

## 2. Materials and Methods

### 2.1. Animals

C57Bl/6 mice were maintained on 14 hr light/10 hr dark cycles at 25°C and given food and water ad libitum. The experimental procedures in this study were approved by the Institutional Animal Use and Care Committee at Hokkaido University.

### 2.2. ChIP-sequencing data analysis

ChIP-sequencing (ChIP-seq) data were collected and analyzed as previously described [15].

### 2.3. Reporter Constructs

The thymidine kinase (TK) promoter was obtained from a pEBMulti-Neo vector (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) by *NheI* and *BglIII* restriction digestion, blunted by T4 DNA polymerase (Takara, Kusatsu, Japan), and inserted into a pGL3-Basic vector (Promega, Madison, WI, USA) at the *SmaI* site. This construct was named pGL-TK. The DES-K16 sequence in an intron of the *Kctd16* gene was amplified by PCR using KOD FX Neo (Toyobo, Osaka, Japan) with genome DNA

isolated from the mouse liver and a primer pair listed in Supplemental Table 1. The 341-bp PCR product was inserted into pGL-TK at the blunted *Bam*HI site. All constructs were subjected to the DNA sequencing analysis.

#### 2.4. *Cell culture, transfection, and reporter gene assay*

GC-2spd(ts) and Hepa1-6 cells were cultured in Dulbecco's Modified Eagle Medium (Fujifilm Wako) containing 10% fetal bovine serum supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 292 µg/ml L-glutamine (Invitrogen, Carlsbad, CA, USA). The cells were transfected with GeneJuice transfection reagent (Merck, Darmstadt, Germany), and reporter gene assay was performed two days later, as previously described [15,25]. The pRL-CMV vector (Promega) was co-transfected to normalize the transfection efficiency.

#### 2.5. *Genome editing by the CRISPR/Cas9 system*

Guide RNA (gRNA) sequences were designed using the CRISPRdirect web tool (<https://crispr.dbcls.jp/>) [26]. Complementary oligonucleotides (Supplemental Table.1) were annealed and ligated into a *Bbs*I-digested pX330 vector [27], and the completed construct was confirmed by DNA sequencing. The constructs were transfected into GC-2spd(ts) cells together with pKO-Select-Puro (Lexicon Genetics, The Woodlands, TX, USA), and the transfected cells were selected with 1-µg/µl puromycin for 7 days. The surviving cells were used for DNA and RNA isolation. The DNA was used for PCR with a primer pair listed in Supplemental Table 1 to see whether the DES-K16 sequence was deleted. The RNA was used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

#### 2.6. *qRT-PCR analysis*

Total RNA was isolated from the cells selected as above or mouse testes using ISOGEN II

(Nippongene, Tokyo, Japan) according to the manufacturer's instructions. After treatment with TurboDNase (Thermo Fisher Scientific, Waltham, MA, USA), cDNAs were synthesized using the oligo(dT) primer and Superscript III (Thermo Fisher). Quantitative PCR (qPCR) reactions were performed using Power SYBR Green Master mix (Thermo Fisher) in the 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Primers used for this qPCR are listed in Supplemental Table 1. Expression levels were normalized to that of the housekeeping *Gapdh* gene. For *Kctd16* gene, a transcript variant, *Kctd16-202*, which encodes a full-length protein, was detected.

### 2.7. Statistical analysis

The results were expressed as means  $\pm$  standard deviation (S.D.). Dunnett's test and Tukey HSD test were performed by the R software, and Student's t test was performed using Microsoft Excel statistical analysis functions (Microsoft Corp., Redmond, WA, USA). One-way analysis of variance (ANOVA) was done by F test (Microsoft Corp.).  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1. Discovery of DES-K16 with H3K4me1, H3K27ac, and H3K27me3 marks in mouse spermatocytes

To find novel cis-regulatory elements during spermatogenesis, we analyzed publicly available ChIP-seq data indicating H3K4me1, H3K27ac, and H3K27me3 patterns (GEO accession numbers: GSM1202711, GSM1202714, and GSM1674011) in mouse primary spermatocytes [28,29]. We detected a high peak for all of the three histone marks overlapping with an intron region of the *Kctd16* gene on chromosome 18 (Fig. 1). This approximately 350-bp region contained a 100-bp high peak and 250-bp small but clear peaks and was named 'DES-K16'. Because a recent report indicated that a genome region

with the three marks is a dual enhancer-silencer element in *Drosophila* [23], we further analyzed DES-K16 for its transcriptional activity.

### 3.2. *Enhancer activity of DES-K16 by in vitro reporter gene assay*

We first assessed transcriptional regulatory activity of DES-K16 by *in vitro* reporter gene assay. As a general promoter, the TK promoter was placed prior to the luciferase gene, and DES-K16 was conjugated with the downstream in both directions. The constructs were transfected into two cell lines, GC2-spd(ts) and Hepa1-6. The GC-2spd(ts) cells were derived from mouse primary spermatocytes, and the Hepa1-6 cells originated from a mouse hepatic tumor but are known to express testicular germ cell-specific genes [30].

The luciferase activity was significantly increased in both cell lines when the DES-K16 sequence was present. In GC-2spd(ts) cells, the activity was dependent on the direction and significantly increased only by a forward direction of DES-K16 (Fig. 2A). On the other hand, both directions of DES-K16 significantly enhanced TK promoter activity in Hepa1-6 cells (Fig. 2B). These results indicated that DES-K16 possessed enhancer activity.

### 3.3. *Effect of a deletion of DES-K16 by the CRISPR/Cas9 system*

Next, we deleted the DES-K16 sequence in GC-2spd(ts) cells by genome editing using the CRISPR/Cas9 system. We transfected the vectors containing gRNAs for 5' and 3' regions of DES-K16 and selected the successfully transfected cells (Fig. 3A). As a control, the vector containing no gRNAs was transfected. We isolated genome DNAs from the selected cells to examine whether the sequence was deleted. As shown in a result of genomic PCR, a specific band was detected at a lower position in the deleted cells than in the control cells (Fig. 3B), and DNA sequencing of the PCR product confirmed the deletion of a 358-bp sequence encompassing DES-K16 (Supplemental Fig. 1).

Results of qRT-PCR demonstrated that the *Yipf5* gene, located at 40-kb upstream of the *Kctd16* gene in the antisense direction, was significantly downregulated by the deletion (Fig. 3C), revealing that DES-K16 functioned as an enhancer of *Yipf5* gene in GC-2spd(ts) cells. Interestingly, the qRT-PCR result also showed significant upregulation of the *Kctd16* gene in the DES-K16-deleted cells (Fig. 3D). The *Kctd16* gene was not expressed in the control GC-2spd(ts) cells (Fig. 3D), but the deletion led to expression of this gene. These results suggested that DES-K16 was a silencer of the *Kctd16* gene in GC-2spd(ts) cells. Expression of *Prelid2* and *Sh3rf2* genes, located downstream of the *Kctd16* gene, was not affected by the deletion (Supplemental Fig. 2), and *Pabpc2* gene, located upstream of DES-K16, was not expressed in either control or deleted cells. Collectively, these results indicated that DES-K16 specifically functioned as a “dual enhancer-silencer”: an enhancer of *Yipf5* and a silencer of *Kctd16* in GC2-spd(ts) cells.

#### 3.4. Expression patterns of *Yipf5* and *Kctd16* genes during mouse testis development

To examine the possibility that DES-K16 is also a dual enhancer-silencer in mouse spermatocytes, we investigated expression of *Yipf5* and *Kctd16* genes in mouse testes at various postnatal days. *Yipf5* gene expression was gradually increased from 7 days postpartum (dpp) to 28 dpp, which coincided with the onset and progression of meiosis (Fig. 4A). This is in good agreement with enhancer activity of DES-K16 in spermatocytes. In contrast, *Kctd16* expression was significantly decreased from 7 dpp to 14 dpp and thereafter (Fig. 4B), suggesting that this gene was suppressed in spermatocytes, in agreement with silencer activity of DES-K16.

## 4. Discussion

Here, we identified the DES-K16 region based on histone modification patterns in mouse spermatocytes and assessed its transcriptional activity in cultured cells. DES-K16 showed significant enhancer activity in two cell types by reporter gene assay, and a deletion resulted in both increase and decrease in expression of linked genes in GC-2spd(ts) cells. These data suggest that DES-K16 functions as a dual enhancer-silencer in this cell type. Hitherto known dual enhancer-silencer elements act as either silencer or enhancer depending on the cell specificity or the cellular condition [21–23]. In contrast, the present study demonstrates that DES-K16 functions as both enhancer and silencer in one cell type, GC-2spd(ts) cells, and that DES-K16 is a novel type of multifunctional genome element.

Interestingly, in GC-2spd(ts) cells, DES-K16 enhanced expression of the *Yipf5* gene which was upregulated in spermatocytes during testis development and silenced expression of the *Kctd16* gene which was downregulated in spermatocytes (Figs. 3 and 4). These results showed that dual enhancer-silencer activity of DES-K16 is consistent with expression patterns of its target genes in the mouse testis. Combined with the presence of epigenetic marks of both enhancer and silencer (H3K4me1, H3K27ac, and H3K27me3) in spermatocytes (Fig. 1), the present gene expression analyses verified that DES-K16 is presumed to function as a dual enhancer-silencer element not only in GC-2spd(ts) cells but also in spermatocytes during meiosis.

In general, enhancers and silencers bind to distinct transcription factors for their activity [31,32], and some transcription factors such as GATA1 regulate both enhancer and silencer functions [2,32]. By searching for potential binding sites, the DES-K16 sequence was found to be potentially bound by several such transcription factors that were expressed in spermatocytes (Supplemental Fig. 3). Some of these transcription factors may bind to DES-K16 and lead to incorporated enhancer and silencer activity in the same cellular environment.

The *Kctd16* gene was reported to interact with GABA<sub>B</sub> receptors and control the signaling pathway in the brain [33], and the *Yipf5* gene plays a major role in innate immune responses in mammalian cells

[34]. Although no specific functions of these two genes have been identified in the testis, their expression changes during testis development suggest some roles in spermatogenesis. A significant decrement in expression of *Kctd16* from 7 dpp to 14 dpp (Fig. 4A) raises the possibility that *Kctd16* has a role in spermatogonia, and the increase of *Yipf5* expression (Fig. 4B) implies its role during meiosis. Consequently, DES-K16 may contribute to the regulation of spermatogenesis through its multifunctional gene regulatory activity.

In conclusion, we identified a novel type of dual enhancer-silencer element, DES-K16, which functioned as both enhancer and silencer in a single cell type, GC-2spd(ts) cells. DES-K16 was marked with H3K4me1, H3K27ac, and H3K27me3 in spermatocytes, and it enhanced *Yipf5* expression and silenced *Kctd16* expression in GC-2spd(ts) cells. The present study paves the way for understanding the multifunctionality of genome and the gene regulation during spermatogenesis.

## **Funding**

This work was supported by KAKENHI 15H04317 and 20H03285 from Japan Society for the Promotion of Science and Suhara Memorial foundation.

## **Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Figure legends

**Fig. 1. Identification of a dual enhancer-silencer element, DES-K16, in mouse spermatocytes.** Five protein-coding genes are depicted by blue boxes with the name and length. The position of DES-K16 is shown by a red line within the *Kctd16* gene. Below the gene structure, three transcript variants of *Kctd16* are enlarged and drawn by indicating the exon-intron structure, in which DES-K16 is pointed by red arrowheads. The ChIP-seq data for three histone modification patterns in spermatocytes are shown at the bottom. The epigenetic marks are histone H3 lysine 4 monomethylation (H3K4me1), histone H3 lysine 27 acetylation (H3K27ac), and histone H3 lysine 27 trimethylation (H3K27me3). DES-K16 is a region containing a high peak of all the three histone modifications.

**Fig. 2. *In vitro* reporter gene assay for transcriptional activity of DES-K16.** The reporter gene constructs were generated as indicated at the left side of the graph. Each construct was transfected into GC-2spd(ts) (A) and Hepa1-6 cells (B), and luciferase activity was measured two days later. The activity of the empty vector was set to 1.0 in each experiment, and the values from other constructs were calculated. Data are presented as means  $\pm$  S.D. from five or six independent experiments, and statistical significance was assessed by Dunnett's test to compare the construct not containing DES-K16 with those containing it. \*\*\* $P < 0.001$ .

**Fig 3. Effect of a deletion of DES-K16 by genome editing in GC-2spd(ts) cells.** (A) The exon-intron structure of *Kctd16* gene is indicated as in Fig. 1. The region amplified by PCR for genotyping is enlarged and the positions of primers (P1 and P2) and gRNA targets are shown. (B) A representative result of PCR for genotyping. GC-2spd(ts) cells were transfected with the CRISPR/Cas9 vectors containing and not containing gRNA sequences, and DNAs were isolated after selection. PCR was done with primers shown in (A), and the products were analyzed by electrophoresis and ethidium bromide staining. The band size confirmed by DNA sequencing is indicated. (C) qRT-PCR for *Yipf5* gene expression in GC-2spd(ts) cells edited by the CRISPR/Cas9 system. Total RNAs were isolated from the cells selected in (B) and qRT-PCR was performed for *Yipf5* gene. *Gapdh* was used as an internal control. Data are presented as means  $\pm$  S.D. from four or eight independent experiments, and statistical significance was assessed by Student's t test. \*\* $P < 0.01$ . (D) qRT-PCR for *Kctd16* gene expression in GC-2spd(ts) cells edited by the CRISPR/Cas9 system. Total RNAs were isolated and qRT-PCR was performed as in (C). In the control sample, *Kctd16* expression was not detected. Data are presented as means  $\pm$  S.D. from eight independent experiments, and statistical significance was assessed by Student's t test. \*\*\* $P < 0.001$ .

**Fig 4. *Yipf5* and *Kctd16* expression during mouse testis development.** Expression patterns of *Kctd16* (A) and *Yipf5* (B) genes in postnatal testes by qRT-PCR. Total RNAs were isolated from whole testes at the indicated ages, and qRT-PCR was performed. *Gapdh* was amplified as an internal control and used to calculate the relative expression level. Data are presented as means  $\pm$  S.D. from six (A) and eight (B) independent experiments, respectively, and statistical significance was analyzed by one-way ANOVA followed by the Tukey post hoc test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

**Fig 1.**

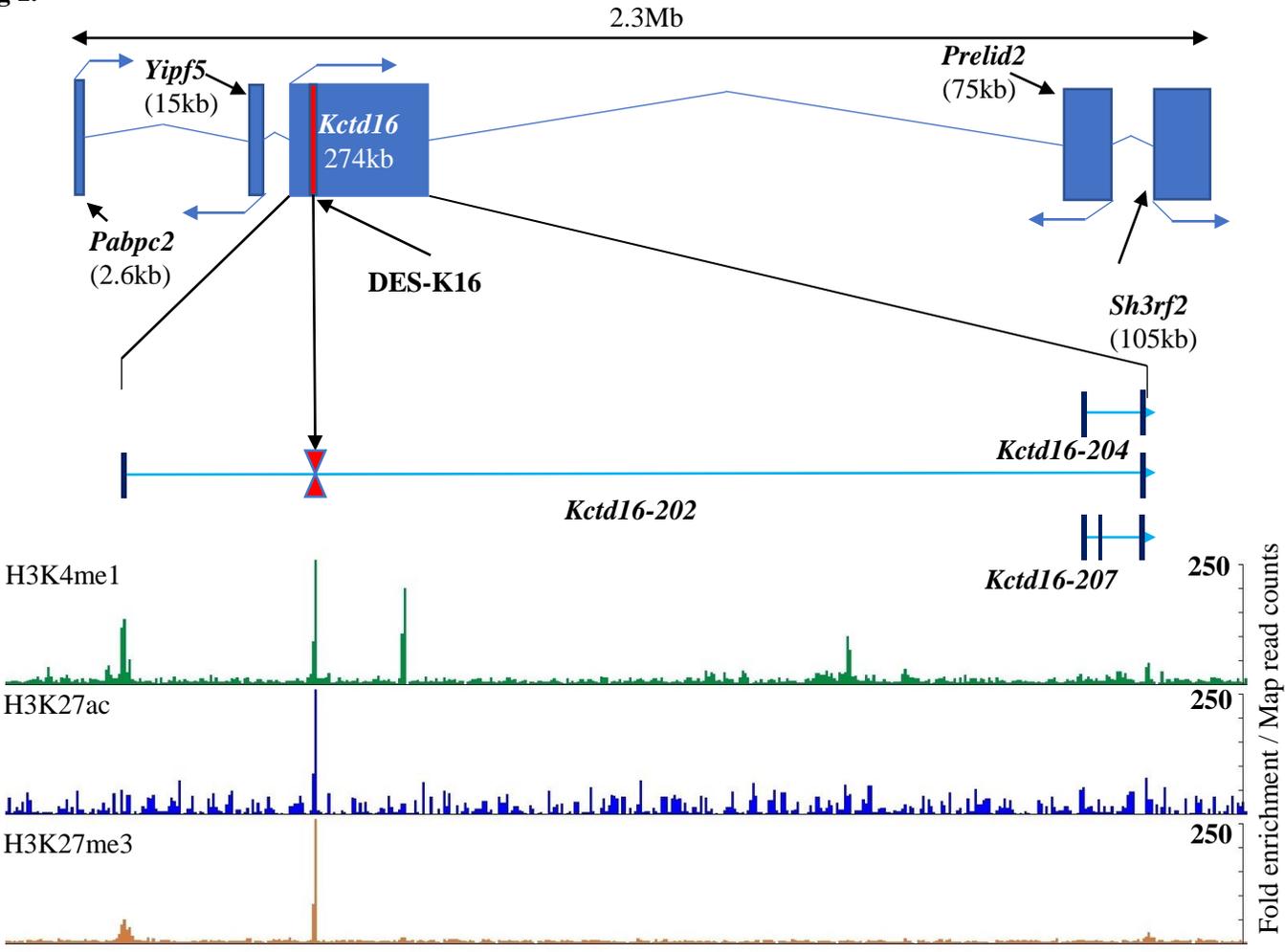
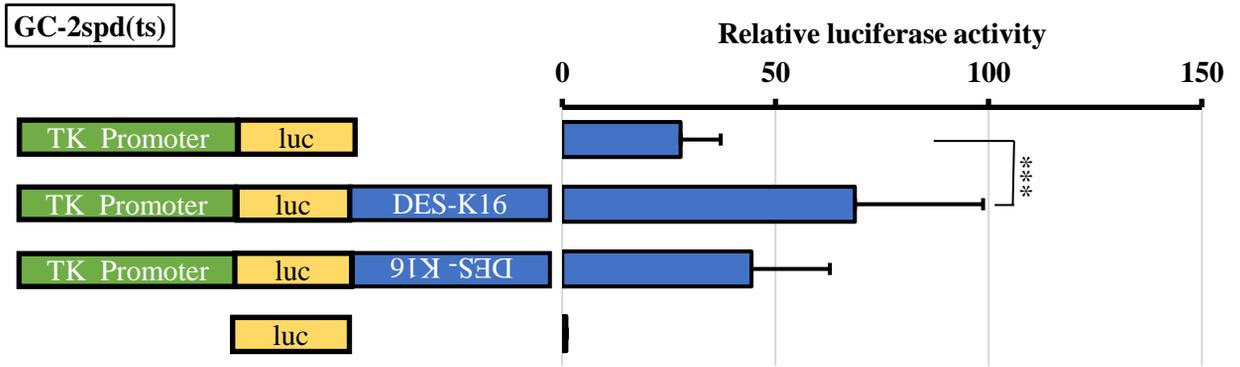


Fig 2.

A



B

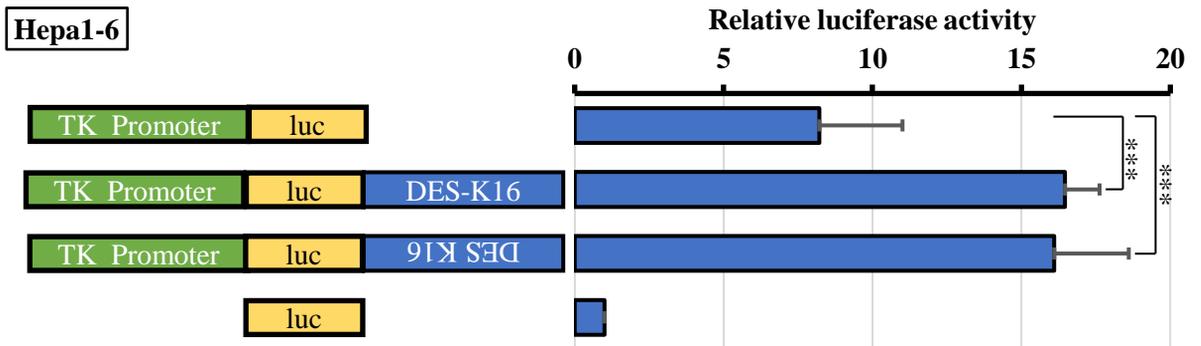


Fig 3.

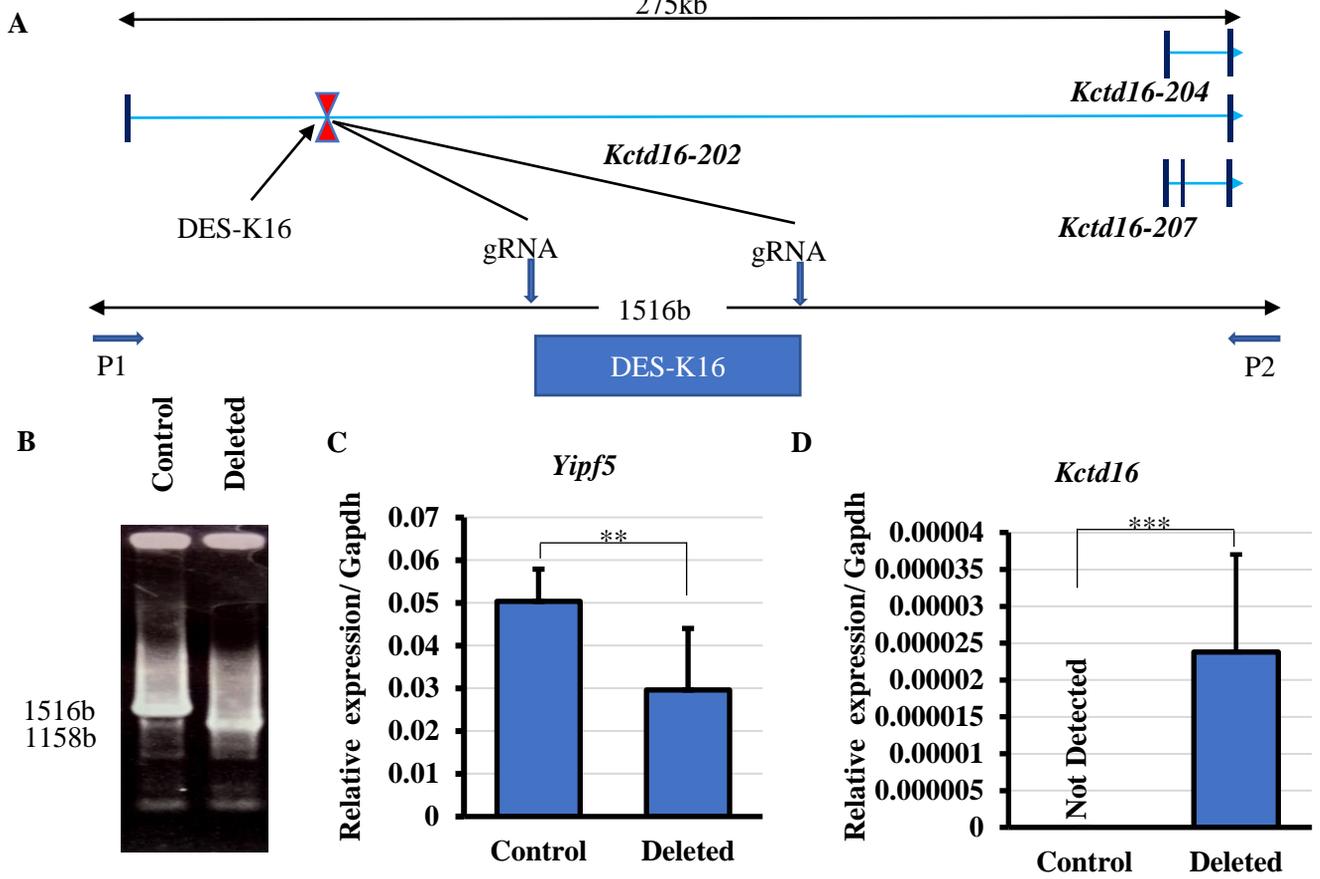


Fig 4.

