Reversing thyroid-hormone-mediated repression of a HSV-1 promoter via computationally guided mutagenesis

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ABSTRACT
Thyroid hormones (THs) and their DNA-binding nuclear receptors (TRs) direct transcriptional regulation in diverse ways depending on the host cell environment and specific promoter characteristics of TH-sensitive genes. This study sought to elucidate the impact on transcriptional repression of nucleotide sequence or orientation within TR binding sites – the TH response elements (TREs) of TH-sensitive promoters – to better understand ligand-dependent transcriptional repression of wild-type promoters. Computational analysis of the HSV-1 thymidine kinase (TK) gene TRE bound by TR and retinoid X receptor (RXR) revealed a single TRE point mutation sufficient to reverse the TRE orientation. In vitro experiments showed that the TRE point mutation had distinct impacts on promoter activity, sufficient to reverse the TH-dependent negative regulation in neuroendocrine differentiated cells. This point mutation altered the promoter’s regulatory mechanism by discrete changes in transcription factor TR occupancy and altered enrichment of the repressive chromatin modification histone-3-lysine-9-trimethylated (H3K9Me3). Insights relating to this negative TRE (nTRE) mechanism aids our understanding of other nTREs and TRE mutations associated with TH and herpes diseases.

KEY WORDS: Herpesvirus, Thyroid hormone, Differentiation, Transcription factor, Histone modification

INTRODUCTION
Thyroid hormones [THs; thyroxine (T4) and triiodothyronine (T3)] and their nuclear receptor family members, thyroid hormone receptors (TRs), have the ability to increase or decrease the rate of transcription of target genes (Lazar, 1993). These target genes generally contain TH response elements (TREs), arrangements of single or multiple DNA hexamers recognized and bound by the DNA binding domain of TRs (Yen, 2001). A host of criteria such as T3 binding to TRs, TR isoform binding to target gene promoter regions as monomers, homo- or hetero-dimers, and the number, arrangement and sequence of TREs impact the type of regulation (Yen, 2001). These criteria and their outcomes are extensively studied but still poorly understood for many TH-sensitive genes, especially those downregulated by T3.

It is generally understood that certain TRE arrangements known as direct repeat fours (DR4) with consensus sequence AGGT(C/G) XXXXAGGT(C/G)A, are bound by a TR and retinoid X receptor (RXR) heterodimer, which recruit co-repressor complexes to the target gene promoter region and modify the bound histones to repress transcription. When T3 as a ligand binds to the receptor, a conformational change causes the co-repressors to be replaced by coactivators that modify the histones to attract the transcription machinery. Alternatively, several TREs with arrangements known as palindromes, often found on genes related to the feedback inhibition of T3 synthesis, can regulate transcription in an opposite manner. Specifically, the thyroid-stimulating hormone α-subunit (TSHα) gene contains a palindrome depicted in Fig. 1A (Chatterjee et al., 1989). For these negative (n)TREs, the non-ligand-bound TR somehow activates gene expression and then confers repression upon binding T3; however, this type of regulation is not well described, possibly because of the conflicting circumstances for different genes that contain similar nTREs. There is argument over whether TR–DNA binding is maintained and what cofactors are involved because there is evidence to support many different hypotheses depending on the system or cell line and gene being studied.

Our previous studies suggested that T3 participates in herpes simplex virus-1 (HSV-1) regulation by repressing viral replication and gene expression (Bedalala et al., 2010; Figliozzi et al., 2014; Hsia et al., 2010). This observation synergizes with the concept that stress triggers both herpes reactivation and thyroid hormone fluctuations. The HSV-1 thymidine kinase (TK), while considered non-essential for lytic replication, has been shown to play a significant role during reactivation from neuronal cells where phosphorylated nucleotides are scarce (Kosz-Vnenchak et al., 1993; Nichol et al., 1996; Tal-Singer et al., 1997; Valyi-Nagy et al., 1992). Additionally, TK is one of the TH-sensitive genes that contains a palindromic nTRE which have been the subject of debate and intrigue for several decades (Maia et al., 1996; Park et al., 1993). The wild-type TK nTRE with a palindromic sequence comprising 6-nucleotide spacing (Pal6) is depicted in Fig. 1B (Hsia et al., 2010). In undifferentiated non-neuronal cells there is no TK regulation (Figliozzi et al., 2014; Hsia et al., 2010). However, TK is transcriptionally repressed by T3 in neuronal cells as evidenced by HSV-1 infection and in transfection reporter assay experiments (Hsia et al., 2010). Under the context of quiescent HSV-1 infections and specifically the HSV-1 TK gene, the role of the repressive histone modification H3K9Me3 is well documented and observed in our experiments (Kwiatkowski et al., 2009; Liang et al., 2013).

We set out to better understand the precise TK nTRE nucleotide and TR–RXR residue interactions that define this protein–DNA binding, using web-based molecular biology applications to steer our benchtop TK nTRE site-directed mutagenesis experiments. Performing a point mutation on a computationally identified nucleotide (Fig. 1C bold, underlined red nucleotide) within the TRE, we generated a mutant that surprisingly exhibited reversal of T3 sensitivity measured by dual luciferase assays. The luciferase reporter system has been used by several labs to evaluate nTREs in a
variety of promoters including TSHα, TSHβ and HSV-1 TK (Lalli and Sassone-Corsi, 1995; Latif et al., 2016; Shibusawa et al., 2003). Electro-mobility shift assays (EMSA) were used to determine whether TRs exhibit differential binding to the wild-type and mutant promoters. Chromatin immunoprecipitation (ChIP) assays were used to determine what effects the mutations caused on the histone modification. Consistent with our previous studies, differentiated LNCaP cells were used as a host for this study. Upon androgen deprivation, LNCaP cells halt proliferation and differentiate into a physiological and morphological neuron-like state. Our studies highlighted how these T3-treated differentiated cells are resistant to HSV-1 lytic infection and possess the ability to enter a reversible TH-dependent semi-quiescent state of infection (Figliozzi et al., 2014).

RESULTS

Computer analyses of putative binding of TR to alternative TRES

Swiss PDB viewer (SPV) was used to generate two pdb files from 2NLL (which contains the crystallography structure of the RXR and TR heterodimer bound to a traditional TRE sequence), each with either the DNA binding domain of TRβ or RXRα bound to its half-site. The generated pdb files were used as inputs for PiDNA. PiDNA randomly mutated the half-sites to yield hexamers with altered RXR or TR occupancy based on increased energy release due to binding and position frequency matrix analyses. PiDNA identified 5′-AGGTGA-3′ (TRE-1a) on the positive strand and 5′-AGGCCA-3′ (TRE-2) located at the reverse strand of the HSV-1 TK promoter as the best half-sites for protein binding. The orientation of these half-sites for TRE-1 and TRE-2 was suggested as palindromic TRES with a six nucleotide in between (Pal 6). (C) HSV-1 mutant TK gene TATA box, TRE half-sites and transcription start site with the point mutation on TRE-1a to 5′-AGGTGC-3′ and TRE-1b to 5′-GGGCA-3′ on the reverse strand. This shift would allow TRE-1β and TRE-2 to form a direct repeat separated by three nucleotides (DR 3).

Site-directed mutagenesis

The HSV-1 TK promoter contains a pair of Pal6 TRES, which reside between the TATA box (47933–47937) and the transcription initiation site (47911) based on the HSV-1 complete genome sequence (GenBank accession number X14112). This was found previously to cause negative regulation by T3 in neural cells, but different cell lines generated the opposite results, even in several cell types of neural origin (Figliozzi et al., 2014; Hsia et al., 2010; Maia et al., 1996; Park et al., 1993). To address the importance of the TRE sequence in the T3-mediated downregulation, a point mutation was introduced followed by reporter assays to study the regulatory effects. The TRE adjacent to TATA box was named TRE-1 and the other one was called TRE-2. The mutation was introduced to the end of TRE-1 from 5′-AGGTGA-3′ to 5′-AGGGGAG-3′. The original TK TRE was named wtPal6. The resulting plasmid mtTRE-1 was confirmed by sequencing (data not shown).

The TK promoter and its mutant are not regulated by T3 in undifferentiated LNCaP cells

These reporter plasmids were first tested by dual luciferase (DLuc) assay after transfection of undifferentiated LNCaP cells treated with and without T3. DLuc assays showed that T3 treatment did not affect the promoter activity of any plasmid (Fig. 3A), indicating that there is no T3-mediated regulation in the undifferentiated cellular environment.
TK single nucleotide mutant reporter plasmids show distinct T3-mediated regulation in differentiated LNCaP cells.

The plasmids were later transfected into differentiated LNCaP cells with or without T3 treatment. Fig. 3B shows that T3 treatment caused a 10-fold reduction in wtPal6 promoter activity, consistent with our previous finding that T3 mediated a negative regulation in differentiated cellular background. The TSH\(\alpha\) promoter behaved similarly to the wild-type TK promoter. In contrast, mtTRE-1 activity showed the opposite regulatory profile with a 4-fold increase in activity upon T3 treatment (Fig. 3B). In differentiated LNCaP cells in the presence of T3 the wt promoter activity was at least 70-fold weaker than mtTRE-1 (Fig. 3B). Together, these observations demonstrated that single nucleotide changes within the TREs can disrupt the normal T3-mediated repression of the HSV-1 TK promoter only in differentiated cells. The co-transfection of a dominant-negative TR overexpression vector and the luciferase reporter plasmids caused the T3-mediated differences in luciferase activities to be eliminated with no statistical significance (Fig. 3C).

Differential wtTRE and mtTRE oligomer binding by LNCaP cell proteins with TR participation

Proteins purified from undifferentiated or differentiated LNCaP cells were incubated with either the wtTRE (wtPal6) or mtTRE-1 oligomer, with and without T3 and the anti-TR antibody. The solutions were subjected to the EMSA protocol and protein–DNA complexes were visualized (Fig. 4). No complexes were detected from the three control lanes (Fig. 4, lanes 1). Weak signals from the undifferentiated LNCaP cells were visible with no apparent influence from T3 (Fig. 4A, lanes 2 and 3) and the anti-TR antibody caused the signal to become undetectable (Fig. 4A, lanes 4 and 5). Saturated signals from the wtTRE oligomer and the differentiated LNCaP protein again have no apparent T3 effect (Fig. 4B, lanes 2 and 3). Addition of an anti-TR antibody reduced the signal substantially whereas T3 appears to enhance the signal (Fig. 4B, lanes 4 and 5) compared with the use of a control anti-RXR antibody (data not shown), which independently of T3, exhibited signals statistically similar in strength to the no-antibody lanes (Fig. 4B, lanes 2 and 3). Saturated DNA–protein complex signals were detected between the mtTRE oligomer and differentiated LNCaP protein regardless of T3 or antibody treatment (Fig. 4C).

In the presence of T3, H3K9me3 is enriched at the wtTRE promoter but is reduced at the mtTRE-1 promoter

ChIP was performed to investigate the impact of a single nucleotide change in the TREs on the recruitment of chromatin to the promoter. In this experiment we used an antibody against H3K9me3, which is a repressive histone previously reported to be associated with the TK promoter along with T3 (Figliozzi et al., 2014). We showed that in the wt promoter, the tri-methylated histone interaction was increased 4.3-fold upon T3 addition (Fig. 5), in agreement with our hypothesis that T3 mediates repression in differentiated cells. In contrast, the opposite effect was shown for mtTRE-1, where H3K9me3 recruitment decreased by ~80% in the presence of T3 (Fig. 5). This result indicates that a single nucleotide mutation in the TK promoter can shift its histone profile in the same T3 conditions.
DISCUSSION

Seemingly minute differences in the TRE sequence in promoters would direct chromatin recruitment and determine the type of T3-mediated regulation. However, the mechanisms are poorly understood. Putative TREs were identified within a wide variety of promoters and characterized as bona fide TREs by molecular analyses. Several TREs have been found in viral promoters including HSV-1 TK (Bedadala et al., 2010; Desai-Yajnik and Samuels, 1993; Desvergne and Favez, 1997; Hsia et al., 2011, 2003, 2001; Hsia and Shi, 2002; Zuo et al., 1997). For decades, the HSV-1 TK promoter has been used as a transcriptional control and studied for its T3 sensitivity (Maia et al., 1996). Bioinformatics analyses have demonstrated a pair of non-traditional palindromic TK TREs located between the TATA box and the transcription initiation site, in thyroid-stimulating hormone alpha (TSH\(\alpha\)) TREs, one of the most well-characterized negative TREs (Carr et al., 1992; Hollenberg et al., 1995; Jacobs and Kühn, 1992; Kohn et al., 1992; Rentoumis et al., 1990). It is thus hypothesized that TK TREs would be bound by TR–T3 to confer negative regulation in differentiated cells with a neural phenotype but not non-neural cells (Hsia et al., 2010; Park et al., 1993). Nonetheless, it is quite complicated. First, it was shown that TR–T3 exhibited no regulation of TK transcription on most of the non-neural cells (Hsia et al., 2011; Maia et al., 1996) but generated good negative regulation in differentiated mouse
neuroblastoma cells N2a (Hsia et al., 2010). These observations supported the hypotheses nicely. However, further studies indicated that there was no T3-mediated regulation in rat pituitary GH4C1 cells (Park et al., 1993), a popular model used to investigate molecular mechanisms of TRH receptor function, signal transduction, for electrophysiological studies on plasma membrane calcium channels and to study intracellular calcium homeostasis in pituitary cells. Nevertheless, it appeared that TR–T3 caused downregulation of gene expression in the human JEG-3choriocarcinoma cell line, a placental epithelial-trophoblast-like cell line, which can be seen in the top and free probe can be seen at the bottom of the membrane. Triplet runs of lanes 2–5 were performed and analyzed using pixel densitometry using Bio-Rad image lab software and ANOVA with Holm–Sidak post hoc to determine statistical significance. Lane 5 (B) had a 15% statistically significant increase in pixel density over lane 4 (B) with P<0.0005.

Fig. 4. Electro-mobility shift assays. Labeled wtTRE oligo was incubated with undifferentiated (A) or differentiated (B) LNCaP cell protein extract and various anti-TRβ and T3 treatments, followed by gel electrophoresis. (C) Differentiated LNCaP protein extract and mtTRE oligo. Lane 1, no protein; lanes 2–5 were incubated with cell protein extract; lanes 4 and 5 with anti-TRβ antibody; lanes 3 and 5 were treated with T3. Protein–oligo complex can be seen at the top and free probe can be seen at the bottom of the membrane. Triplet runs of lanes 2–5 were performed and analyzed using pixel densitometry using Bio-Rad image lab software and ANOVA with Holm–Sidak post hoc to determine statistical significance. Lane 5 (B) had a 15% statistically significant increase in pixel density over lane 4 (B) with P<0.0005.

Fig. 5. Enrichment of repressive chromatin H3K9me3 at the HSV-1 TK TREs. ChIP assay measuring the enrichment of H3K9me3, a repressive chromatin protein marker, to HSV-1 TK TREs from differentiated LNCaP cells transfected with wtTRE and mtTRE1 treated with and without T3 presented as percentage input with IgG background subtracted. T3 treatment of wtTRE resulted in a 4.5-fold increase in comparison to no T3 treatment. The mtTRE1, however, showed an opposite effect where T3 treatment caused a reduction. As a control, immunoprecipitated chromatin was subjected to qPCR analysis for a region 1.5 kb downstream of the TK promoter. The differences between wtTRE TK promoter and mtTRE TK promoter treated or not treated with T3 are statistically significant (P<0.05 by ANOVA and Holm-Sidak post hoc analysis). Results are mean±s.d. of n=3 replicate experiments.
cell line with little evidence of differentiation (Maia et al., 1996). Furthermore, HSV-1 TK was negatively regulated by T3 in differentiated but not true neural LNCaP cells (Figliozzi et al., 2014). Together, these results suggested that T3-mediated regulation on a promoter with palindromic TREs may be controlled by multiple factors such as cell origin, host regulatory protein recruitment, TRE sequence composition/context, nuclear receptor subtypes, dimerization preferences, differentiation status and chromatin context.

Downregulation of gene expression by T3 and TR is not as well characterized as upregulation, but is of equal importance. A number of hypotheses have been suggested to account for such gene downregulation, such as suppression of Sp1 stimulation (Xu et al., 1993), cAMP response element binding (CREB) competition (Mendez-Pertuz et al., 2003), recruitment of the chromatin insulator protein CTCF (Burke et al., 2002), downstream binding to the TATA box and direct interaction of TFIID (Crone et al., 1990), hetero- to homodimer conformational alteration (Bendik and Pfahl, 1995), interaction of GATA2 or TRAPP2 dissociation (Matsushita et al., 2007), TR and Sp1 competition in the first exon (Villa et al., 2004), ligand-mediated recruitment of histone deacetylase (HDAC) complex (Sasaki et al., 1999), interaction of TR with TATA binding protein (TBP) and HDAC (Sánchez-Pacheco and Aranda, 2003), and conversion of a co-repressor SMRT to coactivator by TR (Berghagen et al., 2002). It is being debated whether T3-mediated downregulation requires TR–DNA binding (Madison et al., 1993; Shibusawa et al., 2003; Tagami et al., 1999). In this study, another layer of complication – differentiation – was added to the sophisticated regulation. It is known that T3 induces differentiation in several different tissues of many distinct species (Nygård et al., 2003). Studies suggested that T3 induces withdrawal from the cell cycle through S-phase inhibition of genes such as E2F1, S-phase-specific DNA polymerase α, thymidine kinase and dihydrofolate reductase (Nygård et al., 2003). For instance, ligand TR triggers differentiation by suppressing expression of E2F1, a key transcription factor that controls G1- to S-phase transition (Nygård et al., 2003).

HSV-1 TK is not essential during lytic infection (Coen et al., 1986; Knipe and Howley, 2013); however, it plays a critical role in the drug action of acyclovir and in viral reactivation (Kosz-Vnenchak et al., 1993; Nichol et al., 1996; Tal-Singer et al., 1997; Valyi-Nagy et al., 1994; Wilcox et al., 1992). In resting cells such as neurons, dNTPs are absent and viral replication requires TK action to provide the necessary dNTPs (Knipe and Howley, 2013). In contrast to lytic infection, viral replication is not essential for α and β gene expression; TK is suggested to promote replication followed by efficient α and β gene expression in neurons during reactivation to complete the life cycle (Nichol et al., 1996; Tal-Singer et al., 1994). In addition, a TK null mutant showed decreased viral gene expression upon reactivation (Kosz-Vnenchak et al., 1993) and in vivo reactivation studies revealed that TK was one of the first genes to be expressed (Pesola et al., 2005; Tal-Singer et al., 1994).

Several limitations to this study exist and will be addressed briefly here. The study of HSV-1 transcriptional regulation in its natural host would be ideal to elucidate related mechanisms of transcriptional regulation by T3. However, the morality and host would be ideal to elucidate related mechanisms of here. The study of HSV-1 transcriptional regulation in its natural genes to be expressed (Pesola et al., 2005; Tal-Singer et al., 1994). Reactivation studies revealed that TK was one of the first expression upon reactivation (Kosz-Vnenchak et al., 1993) and to complete the life cycle (Nichol et al., 1996; Tal-Singer et al., 1994).

In conclusion, the unique negative TREs of HSV-1 TK were characterized in this report by site-directed mutagenesis. T3 was not sufficient to control TK promoter activity in undifferentiated conditions but conferred repression when cells were differentiated. A point mutation at TRE1 close to the TATA box changed the T3-mediated downregulation into a significant upregulation. In vitro EMSA signals suggested that protein from undifferentiated cells formed weak interactions with the wtTRE. Considering that TRβ was expressed in undifferentiated LNCaP cells (Figliozzi et al., 2014) and the anti-TR antibody could abolish the signal, the role of TR in this interaction can be suggested (Fig. 4A). In comparison, protein from differentiated cells incubated with the mtTRE displayed a more robust signal (Fig. 4B, lanes 2 and 3) indicating an enhanced DNA–protein interaction, suggesting that differentiated cell protein exists in a biochemical context more supportive of these interactions. Together, these results suggest that differentiation status plays a critical role in TR binding to TK TREs. The anti-TR antibody reduced but did not abolish the signal (Fig. 4B, lane 4), suggesting that TR plays a dynamic role in the DNA binding protein complex. Under these conditions, T3 appears to strengthen the complex signal (Fig. 4B, lane 5), suggesting that ligand binding enhances DNA–protein interactions. In contrast, mtTRE and differentiated cell protein resulted in DNA–protein complex signals that were not disrupted by the anti-TR antibody or affected by T3 treatment (Fig. 4C), suggesting that this mtTRE is capable of DNA–protein interaction but with a reduced TR role in binding, while the luciferase assay and previous experiments suggest that T3 and TR impart regulatory activation of the mtTRE promoter. Ligand TR under differentiation appeared to suppress TK transcription by creating a repressive chromatin environment. Understanding of this complex regulation may have implications for the control of HSV-1 infection and reactivation.

**MATERIALS AND METHODS**

**Computational analyses of protein–DNA binding**

The protein databank (pdb) file 2NLL, which depicts the crystallography structure of the RXR and TR heterodimer bound to a traditional TRE sequence 5′-CAG GTC ATT TCA GGT CAG-3′, was manipulated using the Swiss PDB Viewer SPV (http://spdbv.vital-it.ch/) (Johansson et al., 2012) to obtain two separate pdb files for each protein monomer bound to a 5′-AGGTCA-3′ hexamer TRE half-site. The interaction of TREs and nuclear receptors was computationally analyzed by the web-based application PiDNA (http://dna.biome.rtu.edu.tw/pidna) (Lin and Chen, 2013). Each monomer–hexamer pdb was used as a template in the PiDNA web application to computationally predict the binding affinities of the monomers to the wild-type hexamers and randomly generated single base-pair mutant hexamers using a variety of criteria such as energy release index and position frequency matrix analyses. Hydrogen bond interactions between the DNA-binding domain residues and hexamer nucleotides were measured using Python molecule viewer software (http://mgltools.scripps.edu/documentation/tutorial/python-molecular-viewer), Swiss PDB viewer and PDBeSum (http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pf?pdbcode=...
using computational results depicted in Fig. 2 led to the development of the forw-

taining a single point mutation. The sequence and size of the generated

reaction resulted in amplified linear point mutated plasmid in its entirety

cells were seeded onto culture dishes at 4.0×10³ cells per cm² of culture dish

free RPMI 1640 supplemented with 10% charcoal

incubator.

2014). Lipofectamine 3000 (cat. #L3000, Life Technologies) was used for

treated further. A detailed protocol was reported previously (Figliozzi et al.,
growth area. These conditions were maintained for at least 5 days before being

Cy3-TAT AGT GCC TGT TGG CTC CTC CAC CGC-3’ (BioTEG-Q) and TK-TRE-mut1-

5’-TAT TAA GGT GCC CCG TGG CTC CAC CGC-3’ (BioTEG-Q). Their complementary

sequences were synthesized and annealed using a protocol described previously (Bedadala et al., 2010).

Protein extracts were prepared from undifferentiated or differentiated LNCaP cells. Protein was isolated using RIPA buffer (Thermo Fisher Scientific, cat. #89900) based on the protocol of the manufacturer. Briefly, the cells were washed twice with cold DPBS and exposed to cold RIPA buffer with Halt protease inhibitor cocktail (Thermo Fisher Scientific, cat. #78410) for 5 min on ice with gentle rocking. The lysate was scraped and collected followed by centrifuging at 14,000 g for 15 min to pellet the cell debris. The supernatant was used for EMSA assays. Triplicate runs were performed and analyzed using pixel densitometry using Bio-Rad image lab software and ANOVA with Holm–Sidak post hoc test to determine statistical significance.

Chromatin immunoprecipitation

ChIP was performed using ChromaFlash High-Sensitivity ChIP Kit from Epigentek, Farmingdale, NY (cat #P-2027-48) following the manufacturer’s protocol. In short, test antibodies were first bound to assay strip wells as well as anti-RNA polymerase II (positive control) and non-immune IgG (negative control). The cells were subjected to cross-linking by adding cell culture medium containing formaldehyde to a final concentration of 1% with incubation at room temperature (20–25°C) for 10 min on a rocking platform (50–100 rpm). Glycine (1.25 M) was added (1:10) to stop the crosslinking. After appropriate mixing and ice-cold PBS washing and centrifuging, lysis buffer was added to resuspend the cell pellet and cells were incubated on ice for 10 min. After carefully removing the supernatant, ChIP buffer was added to resuspend the chromatin pellet. Chromatin was sheared using water bath sonication (EpiSonic 1100 Station, cat. #EQC-1100, Epigentek). The program was set up at 20 cycles of shearing under cooling conditions with 15 s on and 30 s off, each at 170–190 W. Samples were then centrifuged at 12,000 rpm at 4°C for 10 min after shearing and supernatant was transferred to a new vial. ChIP samples were then added to the wells bound with test antibodies, positive control or negative control and incubated at 4°C overnight, washed as per the protocol and subjected to reverse crosslinking at 60°C for 45 min. Finally, the DNA samples were purified using a spin column for quantitative PCR (qPCR).

ChIP-qPCR

Quantitative analyses of ChIP and gene expression were performed by qPCR using myQ SYBR green super-mix and iScript One-Step RT-PCR kits (Bio-Rad). Experiments were performed in triplicate with one set of primers per reaction. The ChIP primer sequences for TK TRE are 5’-AGT GCT TCG TAC CCC TGC CAT-3’ and 5’-GGT ATC CCG CCG GGT A-3’. A negative control primer pair, 5’-AATCGAGCTCAAGTGGAGG-3’ and 5’-ACGACCTACACCGAATCTGGA-3’, against a non-coding region 1.5 kb downstream of the promoter was used to determine specificity of the ChIP assay. The qPCR reactions were carried out at 94°C for 3 min, followed by 30 cycles of 94°C for 15 s, 69°C for 15 s and 72°C for 15 s. The results were calculated using the percentage input method with pre-immune antibody background subtracted.

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Competing interests

The authors declare no competing or financial interests.
Author contributions
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