

1           **Reversing the Thyroid Hormone Mediated Repression of a HSV-1 Promoter through**  
2                           **Computationally Guided Site Directed Mutagenesis**

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14   **Key words:** Herpesvirus, Thyroid Hormone, Differentiation, Transcription Factor, Histone Modification

15  
16   **Abbreviation:** HSV-1, Herpes Simplex Virus Type-1; T<sub>3</sub>, Thyroid Hormone; qPCR, quantitative  
17   Polymerase Chain Reaction; TRβ1, Thyroid Hormone Receptor Beta 1; TRE, Thyroid Hormone Receptor  
18   Element; EMSA, Electro Mobility Shift Assay; ChIP, Chromatin Immuno-Precipitation; Sp1, specificity  
19   protein 1; RXR, Retinoid X Receptor; H3K9me3, histone H3 lysine 9 tri-methylated; VZV, Varicella  
20   Zoster Virus; TK, thymidine kinase; DLuc, Dual Luciferase

21  
22   DISCLOSURE STATEMENT: The authors have nothing to disclose.

## Thyroid hormone mediated viral gene repression

### 25 **Abstract**

26 Thyroid hormones (TH or T3) and their DNA binding nuclear receptors (TRs), direct  
27 transcriptional regulation in different ways depending on the host cell environment and specific promoter  
28 characteristics of TH sensitive genes. This study sought to elucidate the impact on repression of nucleotide  
29 sequence/orientation of TR binding sites, TR elements, (TREs) within TH sensitive promoters.  
30 Computational analysis of the HSV-1 thymidine kinase (TK) gene TRE bound by TR and RXR revealed a  
31 single TRE point mutation sufficient to reverse the TRE orientation. In vitro experiments corroborated that  
32 the TRE point mutation exhibited distinct impacts on promoter activity, sufficient to reverse the TH  
33 dependent negative regulation in neuro-endocrine differentiated cells. EMSA and ChIP experiments  
34 suggest that this point mutation altered the promoter's regulatory mechanism through discrete changes in  
35 transcription factor Sp1 and TR occupancy and altered enrichment of repressive chromatin, histone-3-  
36 lysine-9-trimethyl (H3K9Me3). Incites relating to this negative TRE (nTRE) mechanism impacts the  
37 understanding of other nTREs and TRE mutations associated with TH and herpes diseases.

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51 **Introduction**

52 Thyroid hormones (TH or T<sub>3</sub>) and their nuclear receptor family members Thyroid Hormone  
53 Receptors (TRs) have the ability to increase and decrease the rate of transcription of target genes (Lazar,  
54 1993). These target genes generally contain a thyroid response element, TRE, arrangements of single or  
55 multiple DNA hexamers recognized and bound by the DNA binding domain (DBD) of TR (Yen, 2001). A  
56 host of criteria such as T<sub>3</sub> binding to TRs, TR isoforms binding to target gene promoter regions as  
57 monomers, homo or hetero dimers, and the number of, arrangement and sequence of TREs determine the  
58 type of regulation (Yen, 2001). These criteria and their outcomes are extensively studied but still poorly  
59 understood for most thyroid hormone sensitive genes, which T<sub>3</sub> decreased the target gene expression.

60 It is generally understood that certain TRE arrangements known as direct repeat fours, DR4, consensus  
61 AGGT(C/G)A nnnn AGGT(C/G)A, are bound by a TR and retinoid X receptor (RXR) heterodimer which  
62 recruit corepressor complexes to the target genes promoter region which modify the bound histones to  
63 repress transcription. When T<sub>3</sub> as a ligand binds to the receptor a conformational change causes the  
64 corepressors to leave and be replaced by coactivators that modify the histones to attract the transcription  
65 machinery. Alternatively, several TREs with arrangements known as palindromes, often found on genes  
66 related to the feedback inhibition of T<sub>3</sub> synthesis, regulate transcription in an opposite manner (Chatterjee  
67 et al., 1989). For these negative TREs (nTRE) the un-ligand TR somehow activated gene expression and  
68 then conferred repression upon binding T<sub>3</sub> however this type of regulation is not well described possibly  
69 due to the conflicting circumstances for different genes that contain similar nTREs. There is argument over  
70 whether TR/DNA binding is maintained and what cofactors are involved because there has been evidence  
71 to support many different hypotheses depending on the system/cell line and gene being studied.

72 Our previous studies suggested that T<sub>3</sub> participated in the HSV-1 regulation by repressing viral  
73 replication and gene expression (Bedadala et al., 2010; Figliozzi et al., 2014; Hsia et al., 2010). The HSV-  
74 1 Thymidine Kinase, TK, has been shown to play an intriguing role during reactivation (Kosz-Vnenchak et  
75 al., 1993; Nichol et al., 1996; Tal-Singer et al., 1997; Valyi-Nagy et al., 1994; Wilcox et al., 1992) and is  
76 one of the thyroid hormone sensitive genes that contains a palindromic nTRE which has seen some debate

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77 and intrigue for several decades (Maia et al., 1996; Park et al., 1993). The TK nTRE is in a context of  
78 palindrome with 6 nucleotides spacing (Pal6) (Hsia et al., 2010). In undifferentiated non-neuronal cells  
79 there is no regulation (Figliozzi et al., 2014; Hsia et al., 2010). However, it appears to result in TK being  
80 transcriptionally repressed by T<sub>3</sub> in neuronal cells, evidenced by HSV-1 infection and in transfection  
81 reporter assay experiments (Hsia et al., 2010).

82 We have set out to better understand the precise TK nTRE nucleotide and TR/RXR residue  
83 interactions that define this protein-DNA binding, using web-based molecular biology applications to steer  
84 our benchtop TK nTRE site directed mutagenesis experiments. Performing a point mutation on a  
85 computationally identified nucleotide within the TRE, we generated a mutant that exhibited reversal of T<sub>3</sub>  
86 sensitivity measured by dual luciferase assays. The luciferase reporter system has been used by several labs  
87 to evaluate nTREs in a variety of promoters including TSH $\alpha$ , TSH $\beta$  and HSV-1 TK (Lalli and Sassone-  
88 Corsi, 1995; Latif et al., 2016; Shibusawa et al., 2003). Electro-mobility shift assays (EMSA) were used to  
89 determine whether TR may exhibit differential binding to the wild type and mutant promoters. Chromatin  
90 Immuno-Precipitation (ChIP) Assays were used to determine what effects on the histone modification and  
91 SP-1 transcription factor binding that the mutations caused.

92

### 93 **Experimental Procedures**

#### 94 *Computational analyses of protein-DNA binding*

95 The protein data bank (pdb) file 2NLL which depicts the crystallography structure of the RXR and  
96 TR heterodimer bound to a traditional TRE sequence 5'-CAG GTC ATT TCA GGT CAG-3' was  
97 manipulated using the Swiss PDB Viewer, SPV, (<http://spdbv.vital-it.ch/>) (Johansson et al., 2012) to obtain  
98 2 separate pdb files for each protein monomer bound to a 5'-AGGTCA-3' hexamer TRE half-site. The  
99 interaction of TREs and nuclear receptors was computationally analyzed by the web based application  
100 PiDNA (<http://dna.bime.ntu.edu.tw/pidn>) (Lin and Chen, 2013). Each monomer-hexamer pdb was used as  
101 a template in the PiDNA web application to computationally predict the binding affinities of the monomers  
102 to the wild type hexamers and randomly generated single base pair mutant hexamers. A variety of criteria

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103 such as energy release index and position frequency matrix analyses. Hydrogen bond interactions between  
104 the DNA binding domain residues and hexamer nucleotides were measured using Python Molecule Viewer  
105 software (<http://mgltools.scripps.edu/documentation/tutorial/python-molecular-viewer>), Swiss PDB  
106 viewer, and PDBSum ([http://www.ebi.ac.uk/thornton-srv/databases/cgi-  
107 bin/pdbsum/GetPage.pl?pdbcode=index.html](http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html)) (Johansson et al., 2012). PDBSum calculates all hydrogen  
108 bonds between the DNA and the Protein in the pdb files. Hydrogen bonds were predicted using the  
109 HBPLUS (<http://www.ebi.ac.uk/thornton-srv/software/HBPLUS/>) default parameters were satisfied. The  
110 default parameters H-A distance is < 2.7, D-A distance is < 3.35, and the D-H-A angle is > 90. Schematic  
111 diagrams of protein-nucleic acid interactions were created by NUCPLOT ([http://www.ebi.ac.uk/thornton-  
112 srv/software/NUCPLOT/](http://www.ebi.ac.uk/thornton-srv/software/NUCPLOT/)) program (Luscombe et al., 1997; Luscombe et al., 1998).

113

### 114 *Construction of TRE mutant plasmid*

115 The wild-type TK reporter plasmid pGL4.74-hRluc/TK was purchased from Promega (Cat #  
116 E6921). The single nucleotide mutation was introduced into the promoter region using Phusion Site-  
117 Directed Mutagenesis Kit from Thermo Fisher Scientific (Cat#: F-541) as described by the manufacturer.  
118 In short, template plasmid pGL4.74 was used in a PCR reaction using mutagenesis primers with 5'  
119 phosphorylation modifications. The PCR reaction resulted in amplified linear point mutated plasmid in its  
120 entirety followed by a ligation to cyclize the linear PCR product to form circular plasmid containing single  
121 point mutation. The sequence and size of the generated plasmid were confirmed by gel electrophoresis and  
122 sequencing. The computational results discussed in a later section and depicted in Fig. 1 led to the  
123 development of the forward, 5'-tcgcatattaaggtgccgcgtgtggc-3', and reverse, 5'-agtggacctgggaccgcgcc-3',  
124 primer used for mutagenesis and strategy depicted in Fig. 2.

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### 127 *Cells*

128 The human prostate cancer cell line, LNCaP, was obtained from ATCC (Cat#: CRL-1740) and  
129 grown in RPMI-1640 supplemented with 10% FBS. Vero cells (ATCC Cat#: CCL-81) were grown in  
130 DMEM supplemented with 10% FBS. Cells were grown and maintained at 37 °C and 5% CO<sub>2</sub> in a cell  
131 culture incubator.

132

### 133 *Neuroendocrine differentiation (NED)*

134 The differentiation was achieved by removing androgen from the culture condition. In short,  
135 proliferating LNCaP cells were cultured in phenol red-free RPMI 1640 supplemented with 10% charcoal  
136 dextran treated FBS. The cells were seeded onto culture dishes at  $4.0 \times 10^3$  cells per cm<sup>2</sup> of culture dish  
137 growth area. These conditions were maintained for at least 5 days before being treated further. A detailed  
138 protocol was reported previously (Figliozzi et al., 2014).

139

### 140 *Transfection*

141 Lipofectamine 3000 (Cat#: L3000, Life Technologies) was used for transfection of LNCaP  
142 cells. Detailed protocol was provided by the manufacturer.

143

### 144 *Reporter assays*

145 Luciferase activity was measured by a luminometer using the Dual-Luciferase reporter assay  
146 system (Cat#: PR-E1910, Promega). The cell lysate was collected for the luciferase assay after 48 h of  
147 transfection essentially described by the manufacturer. Luminescence was measured over a 10-s interval  
148 with a 2-s delay on the Synergy HTX multi-mode plate reader (Cat#: S1L, Biotek). The renilla luciferase  
149 activities were normalized against the internal firefly luciferase control driven by HSV-1 VP16 promoter

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150 to correct for transfection efficiency. The results were presented as the percent induction of the reporter  
151 plasmid in the presence or absence of T<sub>3</sub> (100 nM).

152

### 153 *Antibodies*

154 Detailed in Table 1. Anti-TRβ1 antibody was obtained from Thermo-Fisher (Cat#: MA1-215).  
155 Anti-RXRα (Cat#: ab41934) and anti-histone H3 tri-methylated K9 (Cat#: ab8898) was procured from  
156 Abcam Biotech. Anti-SP1 antibody was purchased from Santa Cruz Biotechnology (Cat#: SC-59).

157

### 158 *Electro-mobility Shift Assays (EMSA) and protein extraction*

159 EMSA was performed using the LightShift™ Chemiluminescent EMSA Kit (Cat#20148, Fisher  
160 Scientific). The manufactures protocol was followed using 3'-biotin modified oligos corresponding to the  
161 wild type and TRE1 mutant sequences. The oligos were annealed and incubated with protein extracts from  
162 undifferentiated or differentiated LNCaP cells pretreated or without pretreatment of T<sub>3</sub> in binding buffer  
163 for 30 min. The binding reaction mixtures were loaded onto 6% DNA Retardation Gel (Cat#: EC6365BOX,  
164 Invitrogen) and ran at 100V. The samples were transferred onto positively charged nylon membranes  
165 overnight using the capillary method. The membranes were blocked, washed, and exposed per the  
166 manufacturer. The membranes were sealed in blotting envelopes and photographed in a Bio-Rad ChemiDoc  
167 Imaging system. The sequences of the oligos are TK-Pal6-TRE-for: 5'-TAT TAA GGT GAC GCG TGT  
168 GGC CTC GAA CAC CG-3' [BioTEG-Q] and TK-TRE1-mut1; 5'-TAT TAA GGT GCC GCG TGT GGC  
169 CTC GAA CAC CG-3' [BioTEG-Q]. Their complementary sequences were synthesized and annealed using  
170 protocol described previously (Bedadala et al., 2010).

171 Protein Extracts were prepared from undifferentiated or differentiated LNCaP cells grown in T75s  
172 treated overnight with or without T<sub>3</sub>. Protein was isolated using RIPA buffer (Thermo Scientific cat# 89900)  
173 based on the protocol of the manufacturer. Briefly, the cells were washed twice with cold DPBS and  
174 exposed to cold RIPA buffer with Halt protease inhibitor cocktail (Thermo Sci cat# 78410) for 5 min on

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175 ice with gentle rocking. The lysate was scraped and collected followed by centrifuging at  $14,000 \times g$  for 15  
176 minutes to pellet the cell debris. The supernatant was used for EMSA assays.

177

### 178 *Chromatin Immuno-precipitation (ChIP)*

179 The ChIP was performed using ChromaFlash High-Sensitivity ChIP Kit from Epigentek,  
180 Farmingdale, NY (Cat#: P-2027-48). The protocol was described per the manufacturer. In short, test  
181 antibodies were first bound to Assay Strip Wells as well as Anti-RNA polymerase II (positive control) and  
182 non-immune IgG (negative control). The cells were subjected to cross-linking by adding media containing  
183 formaldehyde to a final concentration of 1% with incubation at room temperature (20–25 °C) for 10 min  
184 on a rocking platform (50–100 rpm). Glycine (1.25 M) was added to the cross-link solution (1:10) to stop  
185 the cross-linking. After appropriate mixing and ice-cold PBS washing and centrifuging, Working Lysis  
186 Buffer was added to re-suspend the cell pellet and incubate on ice for 10min. After carefully removing the  
187 supernatant, ChIP Buffer CB was added to re-suspend the chromatin pellet. Shear chromatin using Water  
188 Bath Sonication (EpiSonic 1100 Station, Cat No. EQC-1100, Epigentek). The program was set up at 20  
189 cycles of shearing under cooling condition with 15 s On and 30 s Off, each at 170–190W. ChIP samples  
190 were centrifuged at 12,000 rpm at 4 °C for 10min after shearing and transferred supernatant to a new vial.  
191 Next set up the reactions by adding the ChIP samples to the wells that are bound with test antibodies,  
192 positive control, or negative control. The reaction incubation condition was 4°C overnight. ChIP samples  
193 then were washed per the protocol and subjected to reverse cross-linking at 60 °C for 45 min. Finally, the  
194 DNA samples were purified by spin column for quantitative PCR (qPCR).

195

### 196 *ChIP-qPCR*

197 Quantitative analyses of ChIP and gene expression were performed by qPCR using myiQ SYBR  
198 green super-mix and iScript One-Step RT-PCR kits (BIO-RAD). Experiments were performed in triplicate  
199 with one set of primers per reaction. The ChIP primer sequences for TK TRE are 5'-ATG GCT TCG TAC  
200 CCC TGC CAT-3' and 5'-GGT ATC GCG CGG CCG GGT A-3'. The qPCR reactions were carried out at



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201 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  
202 were calculated using Percent Input method with pre-immune antibody background subtracted.

203

### 204 **Results:**

#### 205 *Computer analyses of putative binding of TR to alternative TRE*

206 Swiss PDB Viewer (SPV) was used to generate two pdb files from 2NLL, each with either the  
207 DBD of TR $\beta$  or RXR $\alpha$  bound to its half-site. The generated pdb files were used as inputs for PiDNA.  
208 PiDNA randomly mutated the half-sites to yield hexamers with altered RXR or TR occupancy based on  
209 increased energy release due to binding and position frequency matrix analyses. PiDNA identified 5'-  
210 AGGTGA-3' (TRE-1a) on the positive strand of the wt HSV-1 TK promoter (Fig. 1A) and 5'-AGGCCA-  
211 3' (TRE-2) on the reverse strand as the optimal half-sites for protein binding (data not shown). The  
212 orientation of these half-sites suggests a palindromic TRE with a six-base pair spacer (Pal 6) as suggested  
213 by us and others (Figliozzi et al., 2014; Hsia et al., 2010; Maia et al., 1996; Park et al., 1993). Interestingly,  
214 PiDNA also suggested an alternative binding to 5'-GCGTCA-3' (TRE-1b) located at the reverse strand  
215 (Fig. 1A). This half-site exhibited the same binding strength in comparison to TRE-1a (data not shown).

216 Computational observations revealed that a TRE-1a 6A/C mutation on the positive strand to  
217 become 5'-AAGTGC-3' would create a new TRE-1b' with half-site sequence of 5'-GCGGCA-3' on the  
218 reverse strand identified by PiDNA as preferentially bound by RXR in comparison to TRE-1a' (Fig. 1B)  
219 (Rastinejad et al., 2013; Rastinejad et al., 1995). Thus, TRE-1b' and TRE-2 would create a pair of direct  
220 TRE repeat with three nucleotide spacing, likely to behave as positive TRE conferring positive regulation.  
221 PDBSum analysis of TR $\beta$  and RXR $\alpha$  hydrogen bonding further suggests the likelihood of a direct repeat  
222 on the mutant promoter. The mutant TRE1b' would gain one more hydrogen bond from Arg160 from the  
223 RXR $\alpha$  (Fig. 1B) to strengthen the interaction which is absent from the wild type.

224

225

226 *Site-directed mutagenesis*

227 HSV-1 TK promoter contains a pair of TREs exhibiting palindrome repeats with six nucleotides  
228 separating each other (Pal6 TREs). These Pal6 TREs reside between the TATA box (47933–47937) and  
229 the transcription initiation site (47911) (Fig. 2) based on the HSV-1 complete genome sequence accession  
230 number X14112. This context was discussed previously to produce negative regulation by T<sub>3</sub> in neural cells  
231 but different cells generating different opposite results even in several cells with neural origin (Figliozzi et  
232 al., 2014; Hsia et al., 2010; Maia et al., 1996; Park et al., 1993). To address the importance of TRE sequence  
233 in the T<sub>3</sub> -mediated negative regulation, a point mutation was introduced followed by reporter assays to  
234 study the regulatory effects. For easy discussion, the TRE adjacent to TATA box was named TRE1 and the  
235 other one was called TRE2 (Fig. 2). The mutation was introduced to the end of TRE1 from 5'-AGGTGA-  
236 3' to 5'-AGGTGC-3' (Fig. 2, mtTRE1). The original TK TRE was named wtPal6. The resulting plasmid  
237 mtTRE1 was confirmed by sequencing (data not shown).

238 *TK promoter and its mutant were not regulated by T<sub>3</sub> in undifferentiated LNCaP cells*

239 These reporter plasmids were first tested by dual luciferase (DLuc) assay after transfection of  
240 undifferentiated LNCaP cells treated with and without T<sub>3</sub>. DLuc assays showed that the T<sub>3</sub> treatment did  
241 not affect the promoter activity of any plasmid (Fig. 3A), indicating that there is no T<sub>3</sub> -mediated regulation  
242 in undifferentiated cellular environment.

243

244 *TK single nucleotide mutant reporter plasmids exhibited distinct T<sub>3</sub> -mediated regulation in*  
245 *differentiated LNCaP cells*

246 The plasmids were later transfected into differentiated LNCaP cells with or without T<sub>3</sub> treatment.  
247 Fig. 3B shows T<sub>3</sub> treatment caused a 10-fold reduction in wtPal6 promoter activity, consistent with our  
248 previous finding that T<sub>3</sub> mediated a negative regulation in differentiated cellular background. In contrast  
249 mtTRE1 activity showed the opposite regulatory profile with a 4-fold increase upon T<sub>3</sub> treatment (Fig. 3B,

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250 mtTRE1 panel). In differentiated LNCaP, under T<sub>3</sub> the wt promoter activity was at least 70-fold weaker  
251 than mtTRE1 (Fig. 3B). Together these observations demonstrated that single nucleotide changes within  
252 the TREs can disrupt the normal T<sub>3</sub> -mediated repression of HSV-1 TK promoter only in differentiated  
253 cells.

254

255 *Addition of T<sub>3</sub> to differentiated cells without prior treatment of T<sub>3</sub> reduced its capacity of TR to*  
256 *bind wt TREs*

257 The binding of TR to the TREs under the influence of hormone was investigated by EMSA. Wild  
258 type promoter oligo and protein extract from differentiated cells not pretreated with T<sub>3</sub> were first tested.  
259 Shifted bands were detected where the addition of T<sub>3</sub> slightly reduced the band intensity (compare Fig. 4A,  
260 lane 2 and 3). The decreased interaction by T<sub>3</sub> was more significant if longer electrophoresis was allowed  
261 (Fig. 4C, lane 2 and 3). These observations demonstrated that proteins from the extract interacted with the  
262 TRE oligo and T<sub>3</sub> reduced the binding. To address if TR contributed to the binding to the TRE, antibodies  
263 against TR or RXR were introduced in the EMSA. The results showed that the retardation bands were  
264 completely abolished upon the addition of anti-TR Ab and only slightly affected upon the addition of anti-  
265 RXR Ab (Fig. 4A, lane 4-5 and 6-7, respectively), supporting that TR played a critical role in this  
266 interaction, perhaps by direct binding to the TK-TREs with RXR functioning as a partner.

267

268 *TK-TREs were not bound by TR from undifferentiated cells*

269 Protein extract from undifferentiated LNCaP cells of equivalent total protein concentrations used  
270 in differentiated extract experiments in Fig. 4A were used to compare the TRE/TR interaction. Surprisingly  
271 the strong interaction between wtTRE and TR from differentiated cells (Fig. 4B, lane 8) was not observed  
272 (Fig. 4B, lane 1-7) even though TR is detectable at this dilution (data not shown). The mutant TRE oligo  
273 yielded similar results of no interaction (Fig. 4B, lane 9-15). Previous study indicated that TR $\beta$  was

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274 expressed in undifferentiated LNCaP cells (Figliozzi et al., 2014). This results suggested that differentiated  
275 status played critical roles in TR binding to TK TREs.

276

277 *Interaction of T<sub>3</sub> to TR of differentiated cells pretreated with T<sub>3</sub> increased its binding capacity to*  
278 *wt TREs*

279 To mimic the physiological condition, we pretreated differentiated LNCaP cells with T<sub>3</sub> for 48  
280 hours and wash the cells with PBS followed by protein extraction (prior T<sub>3</sub> pretreatment). It was noted that  
281 T<sub>3</sub>-pretreated protein extracts caused the band intensity to increase upon T<sub>3</sub> addition (compare Fig. 4C,  
282 lane 4 and 5). Upon addition of anti-TR Ab, the lane lacking T<sub>3</sub> binding treatment nearly disappeared (Fig.  
283 4C, lane 6) whereas its T<sub>3</sub> treated counterpart band was still clearly visible with reduced intensity (Fig. 4C,  
284 lane 7), suggesting that ligand TR, if pretreated with hormone, exhibited a stronger binding capacity to  
285 TREs. The addition of anti-RXR Ab had little effect on these bands (Fig. 4C, Lane 8 and 9).

286

287 *mtTRE1 demonstrated different pattern of binding in comparison to wt TREs*

288 TRE mutants were investigated for their binding by TR. It was shown that mtTRE1 band intensity  
289 decreased to very low level in the presence of T<sub>3</sub> (compare Fig. 4D, lane 2 and 3), similar to the pattern of  
290 no prior T<sub>3</sub> treatment (compare Fig. 4A, lane 2 and 3). Anti-TR Ab experiments again suggested that this  
291 interaction required TR (Fig. 4D, lane 4 and 5). Unlike the wtTRE, the addition of anti-RXR Ab also  
292 reduced the band signal, which suggested that RXR was also involved in DNA binding on the mtTRE (Fig.  
293 4C, Lane 6 and 7). Together these observations suggested that the mutation disrupted the interaction of  
294 nuclear hormone receptors to the wild-type TRE thus altering the negative regulation.

295

296 *Repressive chromatin H3K9me3 enriched at wt promoter in the presence of T<sub>3</sub> was released in*  
297 *mtTRE1 promoter*

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298           ChIP was performed to investigate the impact of single nucleotide change of the TREs on the  
299 chromatin recruitment to the promoter. In this experiment using an antibody against H3K9me3, a repressive  
300 histone previously reported to be associated with TK promoter with T<sub>3</sub> (Figliozzi et al., 2014), we showed  
301 that in the wt promoter the tri-methylated histone interaction was increased 4.3-fold upon T<sub>3</sub> addition (Fig.  
302 5A), in agreement with our hypothesis that T<sub>3</sub> mediated repression in differentiated cells. In contrast the  
303 opposite effect was shown for the mtTRE1, where H3K9me3 recruitment decreased by as many as 5.5-fold  
304 in the presence of T<sub>3</sub> (Fig. 5A). This result indicated that one nucleotide mutation shifted the histone profile  
305 under the same condition.

306

### 307           *Transcription factor Sp1 occupancy was reversed by TRE sequence changes*

308           Recruitment of transcription factor was studied by ChIP Assays using antibodies against Sp1. It  
309 was revealed that Sp1 recruitment to the wt promoter increased 4-fold without T<sub>3</sub>, whereas decreased  
310 approximately 3-fold in the mtTRE1 promoter (Fig. 5B). There are two Sp1 binding sites, -111 and -59 bp,  
311 respectively (Coen et al., 1986) and they did not overlap with TK TREs. This observation suggested that  
312 mutant TREs may alter chromatin configuration therefore influence the interaction of transcription factor  
313 to its binding sites.

314

### 315   **Discussion:**

316           Seemingly minute TRE sequence differences in promoters would direct chromatin recruitment and  
317 determine the type of T<sub>3</sub> -mediated regulation. However, the mechanisms are poorly understood. Putative  
318 TREs were identified within a wide variety of promoters and characterized as *bona fide* TREs by molecular  
319 analyses. Several TREs were depicted in viral promoters including HSV-1 TK (Bedadala et al., 2010;  
320 Desai-Yajnik and Samuels, 1993; Desvergne and Favez, 1997; Hsia et al., 2011; Hsia and Shi, 2002; Hsia  
321 et al., 2003; Hsia et al., 2001; Zuo et al., 1997). For decades the HSV-1 TK promoter has been used as a  
322 transcriptional control and studied for its T<sub>3</sub> sensitivity (Maia et al., 1996). Bioinformatics demonstrated a  
323 pair of nontraditional palindromic TK TREs located between TATA box and the transcription initiation

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324 site, in a context like the thyroid stimulating hormone alpha (TSH $\alpha$ ) TREs, one of the most well  
325 characterized negative TREs (Carr et al., 1992; Hollenberg et al., 1995; Jacobs and Kuhn, 1992; Kohn et  
326 al., 1992; Rentoumis et al., 1990). It is thus hypothesized that TK TREs would be bound by TR/T<sub>3</sub> to confer  
327 negative regulation in differentiated cells with neural phenotype but not non-neural cells (Hsia et al., 2010;  
328 Park et al., 1993). Nonetheless it is quite complicated. First it was shown that TR/T<sub>3</sub> exhibited no regulation  
329 of TK transcription on most of the non-neural cells (Hsia et al., 2011; Maia et al., 1996) but generated good  
330 negative regulation in differentiated mouse neuroblastoma cells N2a (Hsia et al., 2010). These observations  
331 supported the hypotheses nicely. However, more studies indicated that there was no T<sub>3</sub> -mediated regulation  
332 in rat pituitary GH4C1 cells (Park et al., 1993), a popular model to investigate molecular mechanisms of  
333 TRH receptor function, signal transduction, electrophysiological studies on plasma membrane calcium  
334 channels, and intracellular calcium homeostasis in pituitary cells. Nevertheless, it appeared that TR/T<sub>3</sub>  
335 delivered negative regulation in human choriocarcinoma cell line JEG-3, a placental epithelial-trophoblast-  
336 like cells had little evidence of differentiation (Maia et al., 1996). Furthermore, HSV-1 TK was negatively  
337 regulated by T<sub>3</sub> in a differentiated but not a true neural cells LNCaP cells (Figliozzi et al., 2014). Together  
338 these statements suggested that T<sub>3</sub> -mediated regulation on a promoter with palindromic TREs may be  
339 controlled by multiple factors such as cell origin, host regulatory protein recruitment, TRE sequence  
340 composition/context, nuclear receptor subtypes, dimerization preferences, differentiation status, and  
341 chromatin context.

342 The negative regulation of T<sub>3</sub> and TR is not as well-characterized as the positive regulation but is  
343 of equal importance. A number of hypotheses have been suggested such as suppression of Sp1 stimulation  
344 (Xu et al., 1993), cAMP response element binding (CREB) competition (Mendez-Pertuz et al., 2003),  
345 recruitment of chromatin insulator protein CTCF (Burke et al., 2002), the downstream binding to TATA  
346 box and direct interaction of TFIID (Crone et al., 1990), hetero- to homodimer conformational alteration  
347 (Bendik and Pfahl, 1995), interaction of GATA2 and TRAP220 dissociation (Matsushita et al., 2007),  
348 TR/Sp1 competition in the first exon (Villa et al., 2004), ligand-mediated recruitment of histone  
349 deacetylases (HDAC) complex (Sasaki et al., 1999), interaction of TR/TATA binding protein (TBP) and

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350 HDAC (Sanchez-Pacheco and Aranda, 2003), and conversion of corepressor SMRT to coactivator by TR  
351 (Berghagen et al., 2002). It is being debated that if  $T_3$  -mediated negative regulation required TR-DNA  
352 binding (Madison et al., 1993; Shibusawa et al., 2003; Tagami et al., 1999). In this study, another layer of  
353 complication, differentiation, was added to the sophisticated regulation. It is known that  $T_3$  induces  
354 differentiation in several different tissues of many different species (Nygard et al., 2003). Studies suggested  
355 that  $T_3$  induced withdrawal from the cell cycle through S-phase genes inhibition such as E2F1, S-phase-  
356 specific DNA polymerase alpha, thymidine kinase, and dihydrofolate reductase genes. For instance,  
357 ligand TR triggered differentiation by suppressing E2F-1 gene expression, a key transcription factor that  
358 controls G1- to S-phase transition (Nygard et al., 2003). It is surprising to learn that differentiated cells with  
359 prior treatment of  $T_3$  exhibited stronger TR binding capacity to wtTRE in the presence of  $T_3$ . The exact  
360 mechanisms are not understood. It is likely that prior exposure to  $T_3$  changed the gene expression profiles  
361 to modulate the TR/TRE interaction. It cannot be ruled out that differentiated and undifferentiated  
362 conditions generated distinct chromatin environments allowing these discrete interactions.

363         The occupancy of transcription factor Sp1 to the TK promoters with different TREs was assessed.  
364 The ChIP results matched the DLuc assays and indicated that Sp1 occupancy promoted active transcription  
365 in the absence of  $T_3$  (compare Fig. 3B and Fig. 5B). It is worth noting that in the presence of  $T_3$ , Sp1  
366 recruitment to mtTRE1 was enhanced by 16-fold in comparison to wtTRE (Fig. 5B). It seemed that Sp1  
367 binding was determined, at least in part, by TR binding (compare Fig. 5B to Fig. 4C and 3D) and the ligand  
368 appeared to play a critical role. Position weight matrix comparison of the wild type and mutant promoter  
369 for Sp1 binding sites did not reveal additional sites upon mutation (data not shown). Two Sp1 binding sites  
370 are not found overlapping the TK TRE but well upstream of the TATA box (-111 bp and -59 bp), therefore  
371 the mutations did not affect the Sp1 binding site but still altered its binding profile (Coen et al., 1986). It is  
372 unclear how the mutation achieved this phenomenon. We therefore speculate that in both cases  $T_3$   
373 modulated histone modification and the resulting chromatin environment at this promoter determined Sp1  
374 occupancy.

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375 HSV-1 TK is not essential during lytic infection (Coen et al., 1986; Knipe and Howley, 2013)  
376 however it plays a critical role in the drug action of acyclovir and reactivation (Kosz-Vnenchak et al., 1993;  
377 Nichol et al., 1996; Tal-Singer et al., 1997; Valyi-Nagy et al., 1994; Wilcox et al., 1992). In resting cells  
378 such as neurons dNTPs are absent and requires TK action to provide dNTPs for viral replication (Knipe  
379 and Howley, 2013). In contrast to the lytic infection that viral replication is not essential for  $\alpha$  and  $\beta$  gene  
380 expressions, TK was suggested to promote replication followed by efficient  $\alpha$  and  $\beta$  gene expressions in  
381 neurons during reactivation to complete the life cycle (Nichol et al., 1996; Tal-Singer et al., 1994). In  
382 addition, TK null mutant showed decreased viral gene expression upon reactivation (Kosz-Vnenchak et al.,  
383 1993) and in vivo reactivation studies revealed that TK was among the first genes to be expressed (Pesola  
384 et al., 2005; Tal-Singer et al., 1994).

385 In conclusion, the unique negative TREs of HSV-1 TK were characterized in this report by side-  
386 directed mutagenesis.  $T_3$  was not sufficient to control TK promoter activity in undifferentiated condition  
387 but conferred repression while cells were differentiated. A point mutation at TRE1 close to TATA box  
388 changed the  $T_3$  -mediated negative regulation into positive fashion. In vitro EMSA suggested this wide  
389 spaced TREs would favor TR monomer binding and strong TR interaction to the TREs particularly in the  
390 presence of  $T_3$  during differentiation. Ligand TR under differentiation appeared to suppress TK  
391 transcription by creating a repressive chromatin environment and by reducing the recruitment of the  
392 important transcription factor Sp1 to the promoter. This understanding of this complex regulation may have  
393 implication to control alpha herpes virus infection and reactivation.

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398

### 399 **Conflict of Interest**



## Thyroid hormone mediated viral gene repression

400 The authors declare no conflict of interest.

401

### 402 **Author contributions**

403 RWF and SVH initiated this investigation and designed the study. FC provided the cultured cells. RWF  
404 did all transfection experiments and analyzed the promoter activity. RWF performed the EMSA. FC  
405 designed the primers and performed all the ChIP analyses. RWF performed the computational approach.  
406 SVH wrote the original draft of the manuscript. RWF and FC contributed to portion of the manuscript  
407 writing. All authors analyzed and validated the results and approved the final version of the manuscript.

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565 **Table 1. Antibodies**

<b>Peptide/Protein Target</b>	<b>Name of Antibody</b>	<b>Manufacturer, Catalog No., or Name of Source</b>	<b>Species Raised in Monoclonal or Polyclonal</b>	<b>Dilution Used</b>
THRA from human, mouse, rat, amphibian, avian, chicken, non-human primate, <i>Xenopus laevis</i> NULL	<b>Mouse Anti-Thyroid Hormone Receptor Monoclonal Antibody, Unconjugated, Clone C3</b>	<b>Thermo Fisher, MA1-215</b>	<b>Mouse, monoclonal</b>	<b>1:20</b>
Retinoic Acid Receptor alpha antibody [H1920] - ChIP Grade human, human	Retinoic Acid Receptor alpha antibody [H1920] - ChIP Grade	<b>Abcam, ab41934</b>	<b>Mouse, monoclonal</b>	<b>1:20</b>
Histone H3 (tri methyl K9) - ChIP Grade bovine, canine, chicken/avian, donkey, drosophila, feline, guinea pig, hamster, horse, human, mouse, other, porcine, rabbit, rat, sheep, simian, xenopus, yeast, this antibody reacts with tri methylated k9 within a sequence found in all mammals and a wide range of other species, including arabidopsis, s pombe, n crassa, aspergillus nidulans, d melanogaster, saccharomyces cerevisiae (pubmed 17371840) s ocellaris, c reinhardtii, c elegans, xenopus, chicken, zebrafish and tobacco the antibody will react with any species where the modification is presentreacts with human, mouse, rat and indian muntjac	Rabbit Anti-Histone H3, trimethyl (Lys9) ChIP Grade Polyclonal Antibody, Unconjugated	<b>Abcam, ab8898</b>	<b>Rabbit, polyclonal</b>	<b>1:20</b>
Sp1 (PEP 2) mouse, rat, human, mouse, rat, human	Sp1 (PEP 2) antibody	<b>Santa Cruz, sc-59</b>	<b>Rabbit, polyclonal</b>	<b>1:20</b>

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570 **Figure Legend:**

571 **Fig. 1: Computational analysis to predict binding of nuclear receptor to putative TK TREs**

572 The interactions of protein and DNA were investigated by PiDNA, a web based application,  
573 to interpret the results from the biochemical assays. The high-scored putative TRE half-sites were  
574 further assessed for hydrogen bond interactions using Python Molecule Viewer (PMV) software  
575 and PDBSum. PMV was used to visualize and measure distances between the DNA binding  
576 protein residues and the bases of the DNA TRE half-sites. PDBSum calculates and diagrams  
577 putative hydrogen bonds between the DNA and the Protein in the pdb files.

578 A. Random screening of PiDNA identified 5'-AGGTGA-3' (TRE-1a) on the positive strand and  
579 5'-AGGCCA-3' (TRE-2) located at the reverse strand of the HSV-1 TK promoter as the best  
580 half-sites for protein binding. The orientation of these half-sites for TRE-1 and TRE-2 was  
581 suggested as palindromic TREs with a six nucleotide in between (Pal 6). The point mutation  
582 changed the TRE-1a to 5'-AGGTGC-3' and TRE-1b to 5'-GCGGCA-3' on the reverse  
583 strand. This shift would allow TRE-1b' and TRE-2 to form a direct repeat separated by three  
584 nucleotides (DR 3), presumably to generate positive regulation.

585 B. PDBSum hydrogen bonding analysis of TR $\beta$  and RXR $\alpha$  hydrogen bonding to 5'-AGGTGA-  
586 3' (wt TRE-1a), 5'-AGGTGC-3' (mt TRE-1a'), and 5'-GCGGCA-3' (mt TRE-1b') suggested  
587 strongest binding to TRE-1b' after the mutation due to one more hydrogen bond established  
588 from Arg160 of the RXR $\alpha$ .

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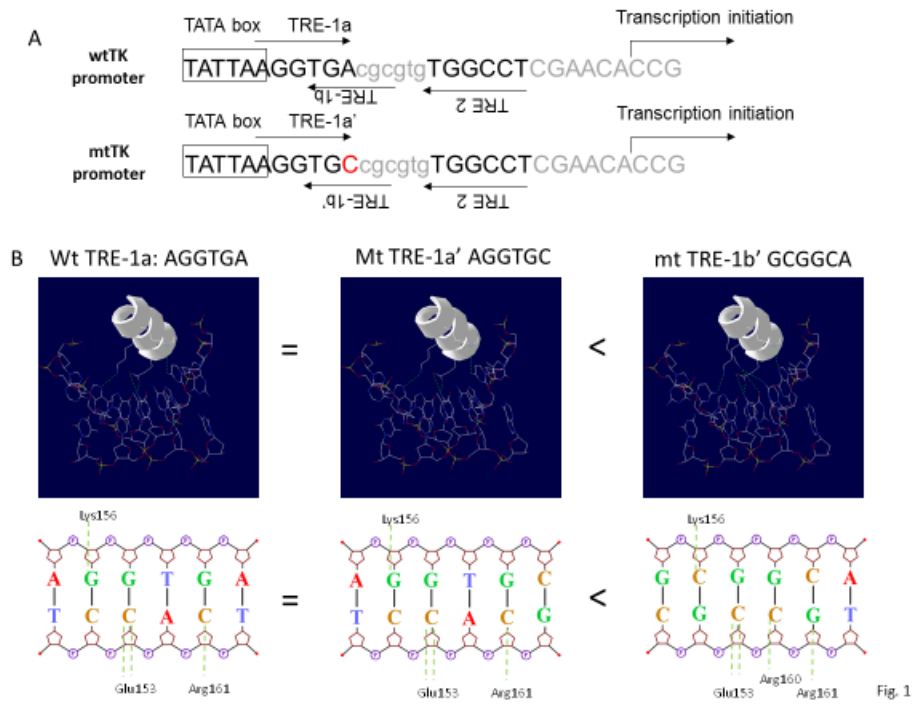
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## Thyroid hormone mediated viral gene repression



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## Thyroid hormone mediated viral gene repression

### 611 **Fig. 2: Site-directed mutagenesis**

- 612 A. HSV-1 TK TREs and sequences of the mutant TREs. A 32-base of HSV-1 TK promoter regions  
613 from TATA box through the transcription initiation site was shown. The TATA box is framed  
614 and bases matching TRE half-site consensus sequences are identified by asterisks (\*). TRE half-  
615 site directions are identified by arrows. TREs are described and classified in reference to the well  
616 described TRE consensus sequence AGGT(G/C)A. The wt HSV-1 TK TRE is described as a  
617 palindrome with 6-nucleotide spacing (Pal6).
- 618 B. Mutagenesis strategy. Specific primers were designed to introduce the mutation into the precise  
619 position using Phusion Site-Directed Mutagenesis system.

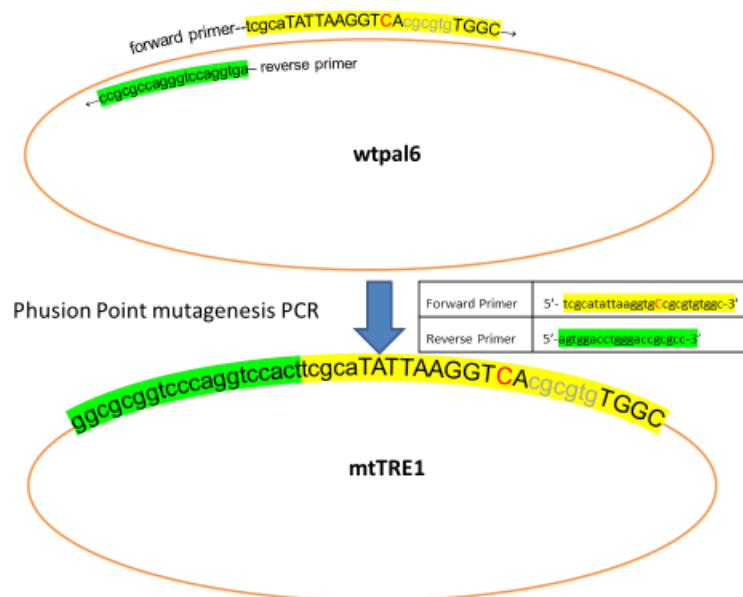


Fig. 2

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Thyroid hormone mediated viral gene repression

626 **Fig. 3: Transfection and DLuc assays**

- 627 A. Transfection of undifferentiated cells. Dual-Luciferase assays of undifferentiated LNCaP cells  
628 transfected with HSV-1 TK TRE luciferase reporter plasmids: wtPal6 and mtTRE treated with  
629 and without T<sub>3</sub>, were performed in triplicate. The effects of T<sub>3</sub> on promoter activity were not  
630 significant and wt promoter generally had stronger activity in comparison to mt counterpart. Two-  
631 way ANOVA with Holm-Sidak post-hoc analysis suggests that the differences in activity is not  
632 significant.
- 633 B. Transfection of differentiated cells. Similar Dual-Luciferase assays of differentiated LNCaP cells  
634 transfected with the reporter plasmids in the presence or absence of T<sub>3</sub>, were done in triplicate. It  
635 appeared that mt plasmids exhibited different T<sub>3</sub> -mediated regulation compared to the wt version.  
636 Two-way ANOVA with Holm-Sidak posthoc analysis suggests that the differences in activity is  
637 statistically significant where \* denotes p<0.05.

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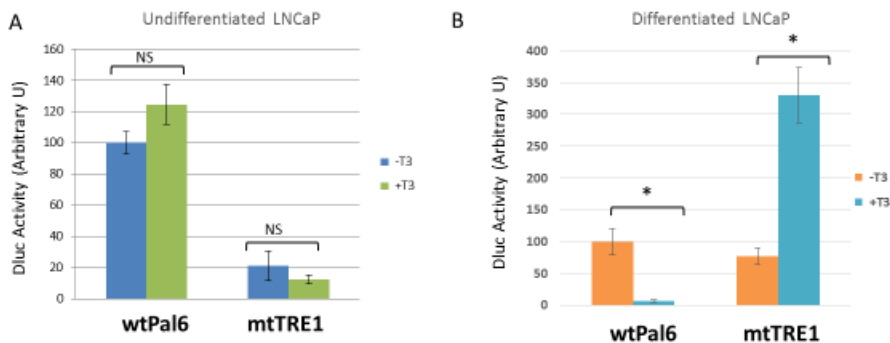


Fig. 3

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Thyroid hormone mediated viral gene repression

641 **Fig. 4: EMSA**

- 642 A. The labeled wt TRE oligo was incubated with differentiated protein extract without hormone  
643 pretreatment followed by gel electrophoresis and the image was captured. Lane 1, no protein;  
644 Lane 2-7, incubated with protein extract from differentiated cells; Lane 4-5, with anti-TR $\beta$  Ab;  
645 Lane 6-7, with anti-RXR $\alpha$  Ab. The image came from a 10-well 6% DNA Retardation Gel.
- 646 B. Undifferentiated protein extract was used for comparison. It is shown that TRE-TR interaction  
647 was not seen in undifferentiated extract. Both wtTRE (lane 2-7) and mtTRE1 (lane 10-15) failed  
648 to interact with TR. positive control was wtTRE interacted with differentiated protein extract  
649 without T<sub>3</sub> pretreatment, like Fig. 4A, lane 2. Note that this gel had 15-well so the bands were not  
650 as sharp as the other gels, which were 10-well gels.
- 651 C. The comparison of TREs oligo/TR interaction with or without T<sub>3</sub> pretreatment was analyzed.  
652 Lane 2-3, extract without T<sub>3</sub> pretreatment. Lane 4-7, extract with prior T<sub>3</sub> pretreatment. It  
653 appeared that TRE/TR interaction increased in the presence of T<sub>3</sub> with hormone pretreatment  
654 (compare lane 4 and 5). Anti-TR Ab failed to completely abolish the interaction (lane 6 and 7),  
655 suggesting a robust binding of TR to TRE in T<sub>3</sub>. The free oligos were run out of the gel due to  
656 increased electrophoresis time to improve the resolution.
- 657 D. The interaction of mutant TRE1 to TR with pretreatment was investigated. Lane 1, no extract;  
658 Lane 2 and 4, no T<sub>3</sub>; Lane 3 and 5, with T<sub>3</sub>; Lane 4-5, anti-TR Ab added. The intensity of  
659 mtTRE1 was reduced in the presence of T<sub>3</sub> (compare lane 2 and 3). Anti-TR Ab addition did not  
660 alter the interaction (lanes 4 and 5). Anti-RXR Ab addition altered the interaction (lanes 6 and 7).  
661 The free oligos were not seen because of elongated electrophoresis.

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Thyroid hormone mediated viral gene repression

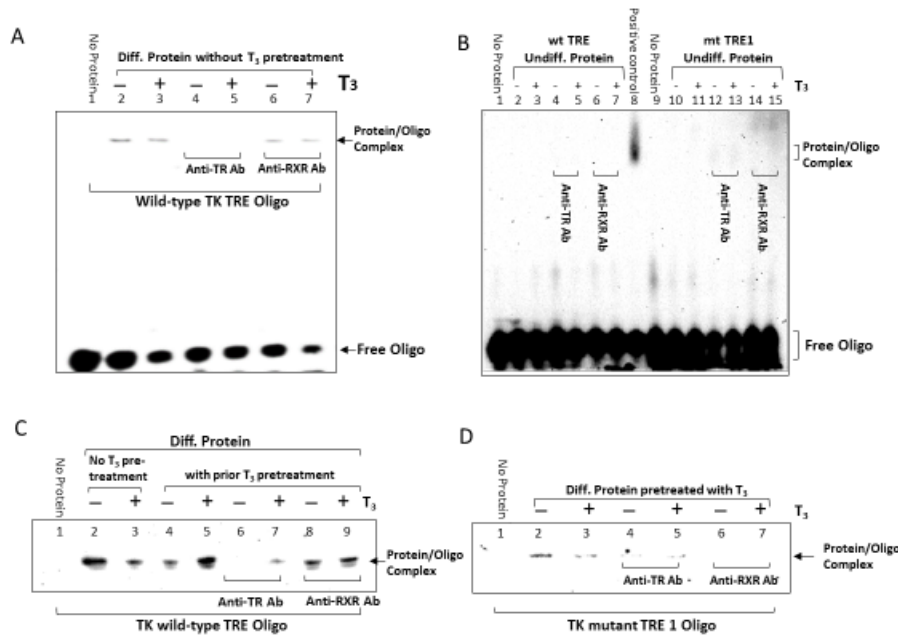


Fig. 4

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Thyroid hormone mediated viral gene repression

682 **Fig. 5a: Enrichment of repressive chromatin H3K9me3 at the HSV-1 TK TREs**

683 ChIP assay measuring the enrichment of H3K9me3, a repressive chromatin protein marker, to  
684 HSV-1 TK TREs from differentiated LNCaP cells transfected with wtTRE and mtTRE1 treated  
685 with and without T<sub>3</sub> presented as percent input with IgG background subtracted. T<sub>3</sub>  
686 treatment of wtTRE resulted in a 4.5-fold increase in comparison to no T<sub>3</sub> treatment. The  
687 mtTRE1, however, showed an opposite effect where T<sub>3</sub> treatment caused a reduction.

688

689 **Fig. 5b: Recruitment of Sp1 to the TK TREs**

690 The occupancy of transcription factor Sp1 to HSV-1 TK TREs under the influence of T<sub>3</sub> was  
691 analyzed by ChIP from differentiated LNCaP cells transfected with wtTRE and mtTRE1. The  
692 results were presented as percent input with IgG background subtracted. For the wtTREs the T<sub>3</sub>  
693 treatment resulted in a 4-fold reduction of Sp1 recruitment comparing to no T<sub>3</sub> treatment. The  
694 mtTRE1 showed a reversed effect that T<sub>3</sub> increased the Sp1 occupancy in comparison to no T<sub>3</sub>.

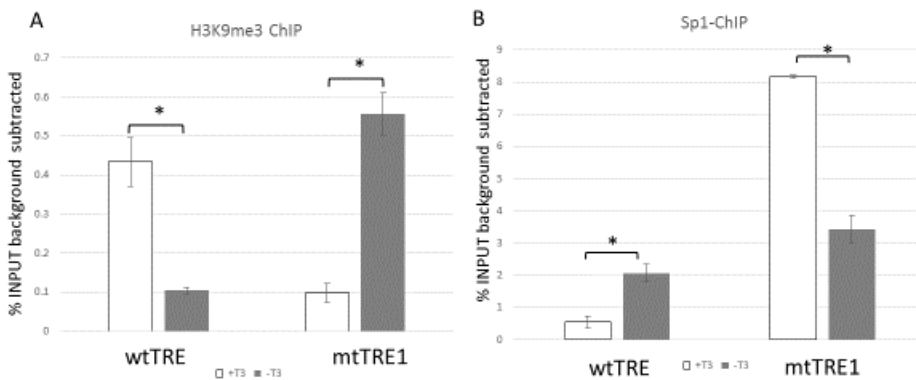


Fig. 5

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