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**T3-mediated expression of PGC-1 α via a far upstream located thyroid hormone
response element**

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Running Title: T3 regulation of PGC-1 α

Abstract

30

Thyroid hormone (T3) has a profound influence on normal development, differentiation and metabolism. T3 induces complex gene expression patterns raises the question of how these expression patterns might be regulated. Since the transcriptional coactivator PGC-1 α (peroxisome proliferators-activated receptor γ coactivator-1 α) induces very similar cellular energy metabolic pathways, we investigated the molecular mechanism of T3 regulation of PGC-1 α . PGC-1 α is rapidly regulated by T3, both in vivo and in cell culture. Transient transfection experiments demonstrated binding of the thyroid hormone receptor (TR) to a response element located at -4 kb upstream of the transcriptional start site within the PGC-1 α gene. Introducing of a single copy of the -4 kb TRE in a heterologous promoter context is sufficient to maintain T3 responsiveness. Chromatin immunoprecipitation analysis revealed increased histone acetylation upon stimulation of T3. Finally, TR binds the -4 kb TRE in electrophoretic mobility shift assays, identifying PGC-1 α as a direct target of TR action. Since T3 directly regulates PGC-1 α and PGC-1 α coactivates liganded TR, we suggest an autoregulatory feed-forward loop of PGC-1 α activation upon T3 treatment.

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Keywords: thyroid hormone, metabolism, transcription factor, nuclear receptor, coactivator.

Introduction

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Thyroid hormone (T3) triggers mitochondrial biogenesis, adaptive thermogenesis and hepatic gluconeogenesis (Yen, 2001). Thyroid hormone regulates gene expression via binding to thyroid hormone receptors (TRs), which belong to the family of nuclear receptors. TRs can bind to thyroid hormone response elements (TREs) either as a homodimer or as a

55 heterodimer preferentially with retinoid X receptor (RXR). The TR interacts with co-factors, which mediate T3 signalling to the basal transcriptional machinery and activates or represses gene transcription (Zhang and Lazar, 2000). On positively T3-regulated genes unliganded TR suppresses basal gene transcription by interacting with corepressors such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT).

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Corepressors are associated with histone deacetylases (HDACs), which modify chromatin into a compact and transcriptionally silent structure. Binding of T3 to the receptor (liganded TR) leads to conformational changes of the TR, which releases the corepressor and recruits the coactivators such as steroid receptor coactivator 1 (SRC-1) or peroxisome proliferators-activated receptor γ coactivator-1 α (PGC-1 α). Coactivators possess or recruit proteins with

65 histone acetyltransferase activity (HAT), which remodels chromatin into an open structure allowing for transcription (Rosenfeld et al., 2006). In contrast to positively T3-regulated genes the mechanism of negatively T3-regulated gene transcription is less clear (Lazar, 2003).

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Beside positively and negatively regulated target genes, gene expression analysis indicated different induction kinetics (Miller et al., 2001; Weitzel et al., 2001b; Flores-Morales et al., 2002; Weitzel et al., 2003a). While some target genes were rapidly induced within 6 hours after the administration of T3 ('early' induction), a second subset of T3-target genes were delayed regulated after a lag period of 12-24 hours ('late' induction). Whereas numerous TREs have been indentified in early-induced gene promoters (Zilz et al., 1990; Weitzel et al.,

75 2001a), we failed to detect TREs in late-induced gene promoters (Weitzel et al., 2003a).

80

These later genes might be regulated in a different manner, probably via an 'intermediate factor'. As a candidate factor for such an intermediate factor we suggested PGC-1 α (Weitzel et al., 2003b). PGC-1 α regulates adaptive thermogenesis in brown adipose tissue, hepatic gluconeogenesis and mitochondrial biogenesis in skeletal muscle and heart (among others) all of which resembles those effects which are concurrently regulated by thyroid hormone as well; reviewed in (Finck and Kelly, 2006; Handschin and Spiegelman, 2006). PGC-1 α itself is regulated by several environmental and endocrine signals, including T3 (Weitzel et al.,

2001b; Irrcher et al., 2003; Weitzel et al., 2003a). Furthermore, PGC-1 α binds and coactivates a great variety of transcription factors implicated in the control of cellular energy metabolic pathways (e.g. TRs, ERRs and NRFs) probably serving as bridging factor for inducing late T3-regulated genes.

The aim of the following study was to investigate the molecular mechanism of T3-mediated activation of PGC-1 α . Our data indicate that PGC-1 α is regulated via a far upstream located TRE within the PGC-1 α gene. PGC-1 α is a direct target of TR and is rapidly regulated by T3. Given that T3 activates PGC-1 α and PGC-1 α coactivates liganded TR, our data indicate an autoregulatory feed-forward loop upon stimulation by T3.

Material and Methods

Animals and treatments

Adult male Wistar rats (Charles River Deutschland GmbH, Sulzfeld, Germany) were housed in the animal care facility of the University Hospital Hamburg-Eppendorf according to the UKE animal care guidelines. Hypothyroidism was provoked by an intraperitoneal injection of Na¹³¹I (Amersham Biosciences, Freiburg, Germany) (250 μ Ci/100 g body weight) and hyperthyroidism was provoked by i.p. injection of T3 (50 μ g/ 100 g body weight) (Weitzel et al., 2001b; Weitzel et al., 2003a). Zero and 6 hours after thyroid hormone injection, rats were killed by decapitation and livers were isolated for RNA preparation. Total RNA was prepared using RNA-Clean (Hybaid-AGS, Heidelberg, Germany) according to the recommendations of the manufacturer and were subject to real-time PCR using the LC-DNA Master SYBR Green kit in a LightCycler (Roche Applied Science, Mannheim, Germany) as described (Weitzel et al., 2001b; Weitzel et al., 2003a; Wulf et al., 2007).

Isolation and characterisation of DNA sequences

The TRE-containing promoter fragment B(-316/+109) of rat mGPDH in pGL2-basic has been described previously (Weitzel et al., 2001a) and was further subcloned into pRL-MA (Promega, Mannheim, Germany). The promoter fragment from -4483 to +78 relative to the transcriptional start site of mouse PGC-1 α in pGL3-basic was a kind gift of James Rhee (Boston, USA). Further deletion fragments thereof were amplified by PCR and subcloned into pGL3-basic or pRL-MA vectors. Point mutations were introduced using the QuickChange

site-directed mutagenesis system (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's recommendations. A sequence portion from -4025 to -4010 (5'-
 120 tgcacttgcttagct-3') was designed as double stranded oligonucleotides and ligated upstream of the thymidine kinase (TK) promoter into pRL-MA, generating the construct TRE-TK-Luc. All clones were confirmed by sequencing. In silico analysis to predict nuclear receptor binding sites were performed using NUBIScan (Podvinec et al., 2002) and MatInspector (Genomatix, München, Germany).

125 *Transient Transfection analyses*

Human hepatocarcinoma HepG2 were cultured under standard conditions in Dulbecco's modified Eagle's medium plus Glutamax (Invitrogen, Karlsruhe, Germany) and 10% fetal calf serum (Biochrom, Berlin, Germany) as described (Weitzel et al., 2001a; Weitzel et al., 2003a). Rat pituitary GC cells (a gift of Danielle Gourdji, Paris, France) were cultured in a
 130 1:1 mixture of Dulbecco's modified Eagle's and Ham's F12 medium plus 10% fetal calf serum (final concentration of T3: ~ 180 pM). Cells were treated with 100 nM T3 for various time points before cells were harvested and RNA was prepared. Transient transfection experiments and mammalian one-hybrid assays were performed using a calcium phosphate technique described previously.

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Electrophoretic mobility shift assay (EMSA)

In vitro-translated chicken TR α 1 was synthesized by a transcription/translation-coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. One μ l of in vitro-translated TR was incubated with 40 fmol of radioactively labelled, double-stranded
 140 oligonucleotide probe (-4029 to -4006 of the PGC-1 α gene, 5'-ctgctgcacttgcttagctgtag-3') in the presence of 1 μ g poly (dA-dT) x (dA-dT) as described previously (Weitzel et al., 2001a). For competition experiments a 100-fold molar excess of double-stranded oligonucleotides 5' mut (5'-ctgcgaattcttgcttagctgtag-3'), 3' mut (5'-ctgctgcactttgcgaattcgtag-3') or TRE-mGPDH (5'-accgtggccccgctgaccggtg-3') were added to the binding reaction. Shifted DNA bands
 145 were separated on 5% polyacrylamide gels and visualized by autoradiography on XAR films (Kodak, Rochester, NY).

Chromatin Immunoprecipitation (ChIP)

For ChIP analyses, 1 μ g of luciferase reporter, 0.1 μ g TR and 0.1 μ g RXR expression
 150 plasmids were mixed and transfected into HepG2 cells using 4 μ l Metafectene (Biontex,

München, Germany) per 9.6 cm² dish according to the instructions of the manufacturer and stimulated with 100 nM T3 for 24 hours. Transfection efficiencies were ~ 11%. Cells were fixed with 1% formaldehyde for 4 min at 37°C, washed with PBS (136 mM NaCl, 50 mM Na₂HPO₄, pH 7.4) containing 1 mM protease inhibitor AEBSF (Roche Applied Bioscience) and resolved in Lysis buffer (Upstate, Hamburg, Germany). DNA was sheared by ultra sound for 30 sec in a Sonorex ultrasonic bath (Bandelin, Berlin, Germany), centrifuged and the supernatant was diluted in ChIP dilution buffer. Twenty µl of this material was taken away as input control and to the remaining portion a 1: 500 dilution of anti-acetylated histone H3 antibodies (Upstate) or irrelevant antibodies was added and incubated overnight at 4°C. Sixty µl of salmon sperm DNA/protein A agarose slurry was added and incubated at 4°C for 1 hour, centrifuged, washed several times according to the instructions of the manufacturer and finally suspended in elution buffer (1% SDS, 100 mM NaHCO₃). Histone-DNA cross links were reversed by adding NaCl to a final concentration of 200 mM and incubation at 65°C for 4 hours. Immunoprecipitated DNA was purified by phenol/chloroform extraction according to standard procedures, and finally resolved in 20 µl water. PCR amplification reactions were performed using the forward primer WL328 (5'-ctaagcggtacttactgag-3') and the reverse primer WL339 (5'-ctttatgttttggcgtcttc-3'). DNA amplification products (~ 300 bp in length) were radioactively labelled due to the incorporation of radioactively labelled nucleotides, separated on 5% polyacrylamide gels and visualized by autoradiography on XAR films (Kodak).

Results

We previously described PGC-1 α as a target of thyroid hormone action (Weitzel et al., 2001b; Weitzel et al., 2003a). After administration of T3 to hypothyroid rats, endogenous steady state levels of PGC-1 α mRNA rise 13-fold in liver compared to untreated control animals (Figure 1A). This activation is accomplished by increased PGC-1 α protein concentrations. A similar induction has also been detected in rat pituitary GC cells (Figure 1B) which mimic thyroid hormone-mediated hepatic gene expression patterns (Miller et al., 2001; Weitzel et al., 2001b; Weitzel et al., 2003a; Wulf et al., 2007). Since this activation occurs fast (within 4 to 6 hours, Figure 1B) we suggested a direct mechanism of activation by thyroid hormone, probably via a thyroid hormone response element.

185 To analyze whether PGC-1 α is directly regulated via a TRE, we investigated a fragment
comprising sequences from -4483 to +78 relative to the transcriptional start site of the PGC-
1 α gene. Transient transfection experiment of a luciferase reporter construct of this 4.5 kb
190 fragment together with a fusion protein of TR with the viral activation domain VP16 (TR-
VP16) demonstrated an activation of gene expression in a mammalian one-hybrid assay
(Figure 2). As positive control for this assay we used an mGPDH promoter-luciferase reporter
construct which contains a well characterized TRE (Weitzel et al., 2001a) (not shown),
whereas as negative control we used a VP16-only construct without a binding site for a TRE.
Similar results have been observed using the 4.5 kb reporter by cotransfection of TR and
stimulation with T3 in ‘classical’ transfection experiments (supplementary figure 1).

195 Consecutive 5’ deletion of the 4.5 kb sequences (-2500, -1200, -200 fragments, Figure 2) lost
the ability to activate TR-mediated gene expression, indicating a TRE within the 2 kb most
upstream located sequence portion. In silico analyses indicated the sequence 5’-
TGCACTttgcTTAGCT-3’ at position -4025 with the highest score matching a DR+4 thyroid
hormone response element. This sequences was excised and integrated upstream of a TK
200 promoter-driven luciferase reporter. As demonstrated in Figure 2, a single copy of this
element was sufficient to activate TR-mediated gene expression (construct TRE-TK-Luc).
Thus, the TRE-like sequence at -4 kb is functionally active in cell experiments even in a
heterologous promoter context.

205 To investigate the molecular consequences of thyroid hormone-mediated gene expression we
performed chromatin immunoprecipitations. The 4.5 kb PGC-1 α promoter reporter construct
together with TR was transfected into HepG2 cells and stimulated with T3. Chromatin was
fixed and immunoprecipitated with an antibody directed against acetylated histone H3, a
marker for transcriptionally active chromatin. Immunoprecipitated DNA was amplified by
210 PCR reaction. As shown in Figure 3, withdraw of T3 leads to under-acetylated histone
occupancy of DNA. In contrast, administration of T3 leads to higher acetylated histone
occupancy of target sequences. These data indicate that thyroid hormone-mediated activation
of luciferase reporter activity correlates with a higher acetylated histone occupancy, indicating
an open, transcriptionally active chromatin structure upon T3 stimulation.

215 To finally test whether TR is able to physically bind the -4 kb TRE sequence, we performed
electrophoretic mobility shift assays. As demonstrated in Figure 4, an in vitro-translated
thyroid hormone receptor α 1 is able to bind the -4 kb TRE sequence (Figure 4, arrow). The

interaction could be competed with a 100-fold molar excess of unlabelled fragment (wt) or
220 with a control TRE from the mGPDH promoter (TRE-mGPDH). In contrast, TR-DNA
binding could not be blocked by an excess of mutated TRE variants bearing mutations either
within the 5' or 3' nuclear receptor half sites (5' mut and 3' mut, respectively; for sequences
see Material and Methods). These data indicate that TR is able to bind the -4 kb TRE in
EMSA experiments.

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Discussion

Gene expression analysis in vivo and cell culture indicated remarkable differences in thyroid
230 hormone-mediated induction kinetics. Whereas ~ 50% of T3-induced genes are rapidly
regulated within 6 hours, another ~ 50% of T3 target genes are regulated after a lag period of
12-24 hours within 48 hours. Surprisingly, these expression patterns are very similar in vivo
(Weitzel et al., 2001b; Weitzel et al., 2003a; Helbing et al., 2003), primary cell culture
(Moeller et al., 2005) and permanent cell lines (Miller et al., 2001; Shih et al., 2004),
235 suggesting common regulation mechanisms. In particular rat pituitary GC cells have been
shown to be an ideal model system to investigate thyroid hormone-mediated gene expression
patterns (Miller et al., 2001; Wulf et al., 2007). We hypothesized that different regulation
mechanisms might be responsible for the different induction kinetics. In line with this
hypothesis, we suggested that early regulated genes are direct targets of TR. Indeed thyroid
240 hormone response elements have been described in the regulatory elements of Spot 14 and
mGPDH (among others), both of which are regulated rapidly by T3 (Zilz et al., 1990; Weitzel
et al., 2001a). In contrast delayed regulated target genes might be indirectly regulated via an
intermediate factor. Indeed, we failed to identify TREs in several late induced gene promoters
(Weitzel et al., 2003a); however, we could not completely rule out the existence of TREs in
245 far distally located sequences which are difficult to address experimentally. Based on a
candidate approach, we suggested PGC-1 α to be an intermediate factor of T3 action (Weitzel
et al., 2003b). PGC-1 α has a profound influence on development, differentiation and
metabolism. In particular, activation of PGC-1 α in muscle induces the full program of
mitochondrial biogenesis including an increase in mitochondrial mass and oxygen
250 consumption capacity, whereas a decrease of PGC-1 α leads to a reduced mitochondrial mass
and oxygen consumption rates. Furthermore, PGC-1 α activation in liver induces
gluconeogenesis whereas blunted PGC-1 α expression leads to reduced expression levels of

gluconeogenic key enzymes; reviewed in (Finck and Kelly, 2006; Handschin and Spiegelman, 2006). These and other effects resemble those effects which are regulated by thyroid hormone.

A prerequisite of an intermediate factor would be that its gene expression is rapidly regulated by T3. For PGC-1 α , this has been demonstrated in rat liver in vivo and GC cells, respectively. Endogenous transcript levels rise within 4 – 6 hours after administration of T3 (Figure 1). A second prerequisite would be that PGC-1 α is directly regulated by a TRE. As demonstrated in Figure 2, TR activates PGC-1 α gene expression via a TRE which is located at -4 kb upstream of the transcriptional start site. Integration of a single copy of this TRE sequence is sufficient to activate a luciferase reporter in transfection experiments (Figure 2; construct TRE-TK-Luc). Thyroid hormone-mediated gene expression via its nuclear receptor is accomplished by alterations in histone acetylation rates in the direction toward a higher acetylated ‘open’ chromatin (Figure 3). Finally, TR is able to bind the TRE sequence at -4 kb physically in EMSA experiments. These data indicate that the PGC-1 α gene is a direct target of TR via binding to a response element at -4 kb. Of note, this TRE is not identical to other described nuclear receptor binding sites within the PGC-1 α gene, in particular a PPRE site at -2 kb and a ERR binding site at -600 bp (Hondares et al., 2006; Laganriere et al., 2004) which are not used by TR (Figure 2).

Since thyroid hormone regulates PGC-1 α gene expression via thyroid hormone receptor binding (see above) and PGC-1 α coactivates TR (Puigserver et al., 1998; Knutti et al., 2001; Zhang et al., 2004; Sadana et al., 2007) a T3-mediated activation of PGC-1 α leads to an autoregulatory feed-forward loop. Interestingly, a feed-forward mechanism has also been described for PPAR-, ERR- and MEF2-mediated activation of PGC-1 α (Hondares et al., 2006; Laganriere et al., 2004; Handschin et al., 2003). However; this raises the question of how this activating signal might be switched off. One possibility would be the RNA processing capacity of PGC-1 α . PGC-1 α has been shown to regulate the splicing ratio of the TR α gene toward the TR α 2 variant which has dominant negative properties (Thijssen-Timmer et al., 2006). Thus, a decreased TR α 1 concentration relative to TR α 2 concentration would lead to dampened thyroid hormone-mediated stimulation. Another mechanism might be interference between the thyroid hormone and the cAMP signal transduction pathway. cAMP activates phosphorylation and transcriptional activity of transcription factors such as CREB which binds to CRE sites within target genes. A CRE site has been described within the PGC-

1 α gene (Herzig et al., 2001) and we and others could detect interference between the thyroid hormone and the cAMP signal pathway (Irrcher et al., 2007) (and not shown) which has also been described for PPAR and cAMP-mediated regulation of the PGC-1 α gene (Hondares et al., 2006). Furthermore, an antagonism of the thyroid hormone and the cAMP signal pathway via protein-protein interaction between CREB and TR has been described (Mendez-Pertuz et al., 2003). Finally, the presence of CRE sites in several thyroid hormone target genes (e.g. PEPCCK, a key gluconeogenic gene) further supports this idea.

295 What might be the role of PGC-1 α in thyroid hormone-mediated gene expression? Previous data from our laboratory indicated that complex thyroid hormone-mediated gene expression patterns are not completely depending on PGC-1 α activation (Wulf et al., 2007). Furthermore, recent data by Holloszy and coworkers indicated that PGC-1 α activation appears to be rather a cause than a reason of exercise-induced mitochondrial biogenesis (Wright et al., 2007). This observation would also fit the phenotype of PGC-1 α knockout mice which show relative subtle alterations in mitochondrial biogenesis and architecture (Lin et al., 2004; Leone et al., 2005; Arany et al., 2005). However, isolated hepatocytes from PGC-1 α knockout mice clearly demonstrated reduced mitochondrial performance suggesting a role of PGC-1 α in fine tuning of mitochondrial biogenesis (Lin et al., 2004; Leone et al., 2005). Our data indicate that PGC-1 α is a direct target of thyroid hormone via its nuclear receptor. Since PGC-1 α coactivates transcription factors such as TR, NRF-1, NRF-2 and ERRs, all of which are involved in regulating mitochondrial biogenesis, a thyroid hormone-mediated activation of PGC-1 α might help this coactivator to exert its function in adaptation to nutritive, endocrine and other environmental signals.

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315

Figure Legends

Figure 1

320 **Thyroid hormone induces PGC-1 α gene expression in rat liver and pituitary GC cells.**
(A) Hypothyroid male rats were treated with T3 for 0 and 6 hours. Livers were prepared, RNA isolated and quantified by real-time PCR. Expression levels were normalized to the expression levels of the housekeeping gene ubiquitin. (B) Rat pituitary GC cells were treated with T3 for 0, 2, 4, 6 and 10 hours. Cells were lysed, RNA isolated and quantified by real-time PCR. Expression levels were normalized to the housekeeping gene Gs α . Data are mean
325 +/- SD of at least three independent experiments performed in duplicates and are expressed as fold activation data relative to un-stimulated expression values.

Figure 2

330 **TR activates gene expression via a distal thyroid hormone response element.** A chimeric protein of TR α 1 with the viral activator domain VP16 (TR-VP16) or the VP16 domain alone (VP16) plus the indicated promoter fragments of PGC-1 α in pGL3 luciferase reporter vectors were transfected into HepG2 cells. Promoter activities were calculated as fold activation rates of the construct by TR-VP16 relative to VP16-only cotransfection +/- S.D. A TRE-like
335 sequence at -4 kb (see text) was integrated upstream of a TK luciferase reporter, given the construct TRE-TK-Luc.

Figure 3

Acetylated histone H3 is associated with the PGC-1 α gene upon T3 stimulation. The
340 promoter fragment of PGC-1 α (-4483/+78-Luc) was transfected into HepG2 cells. An expression vector for TR was cotransfected and stimulated with 100 nM T3 for 24 hours. Chromatin was fixed by formaldehyde and preserved as input control or immunoprecipitated with an anti-acetylated histone H3 antibody (IP: acH3) or irrelevant antibody (IgG). Immunoprecipitated DNA was quantified by PCR and visualised on 5% polyacrylamide gels.
345 A graphic representation is shown in the lower part of the figure.

Figure 4

TR binds to the PGC-1 α gene at the -4 kb TRE. In vitro-translated chicken TR α 1 was incubated with a labelled DNA fragment from -4029 to -4006 of the PGC-1 α gene. For
350 competition experiments, a 100-fold molar excess of wild type fragment (wt), mutated

versions of this DNA fragment or the TRE from the mGPDH promoter (TRE-mGPDH) was added. Mutated DNA sequences contain mutations either within the 5' nuclear receptor half-site (5' mut) or the 3' half-site (3' mut) of the suggested TRE (for sequences, see Material and Methods). Specific protein-DNA complexes are indicated by an arrow.

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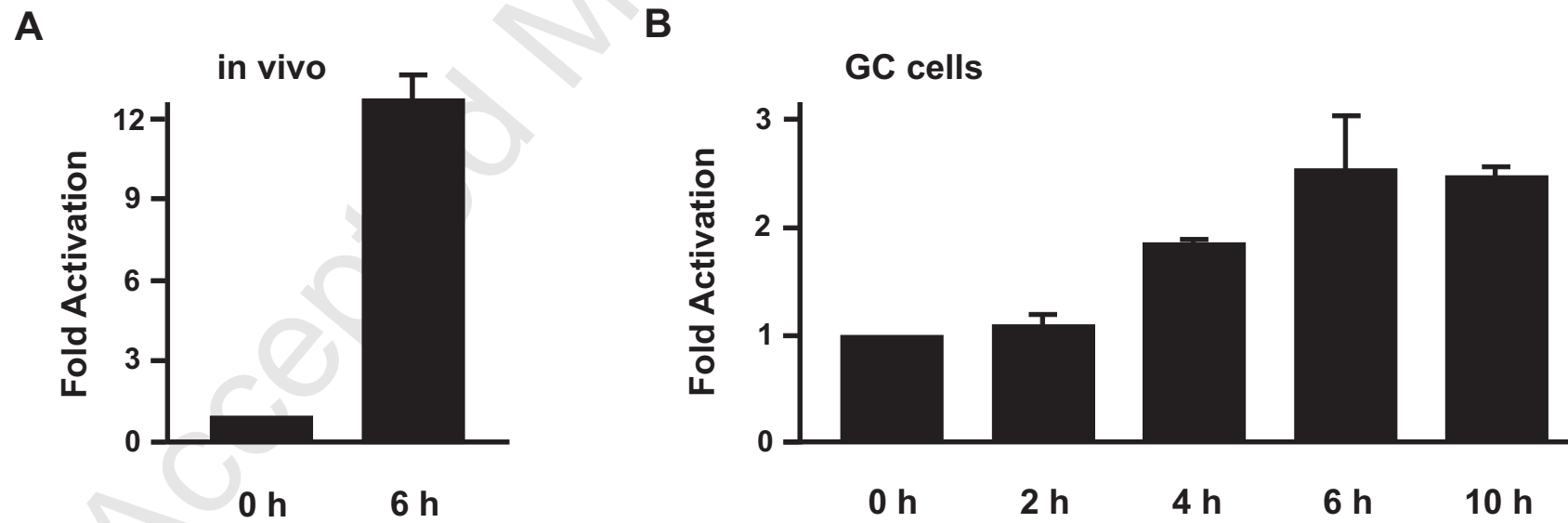


Figure 1

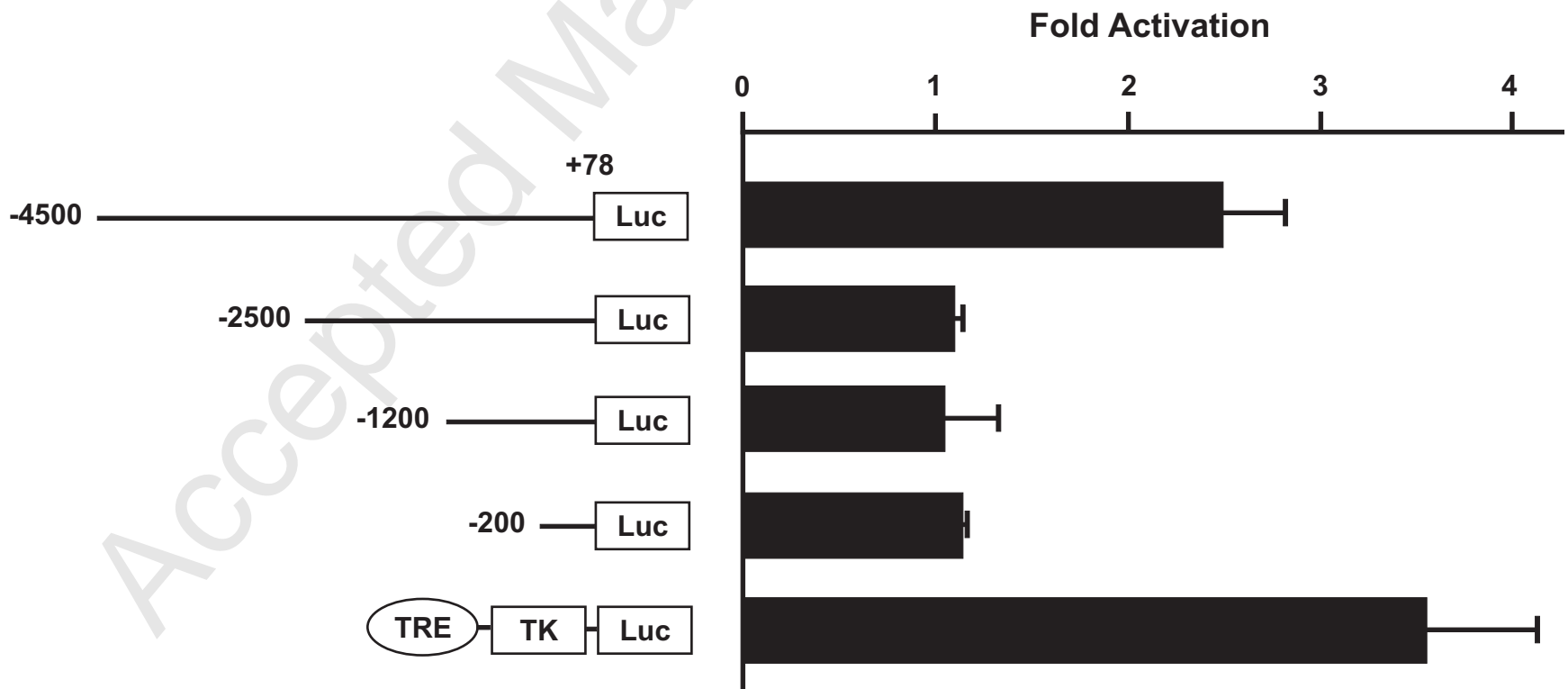


Figure 2

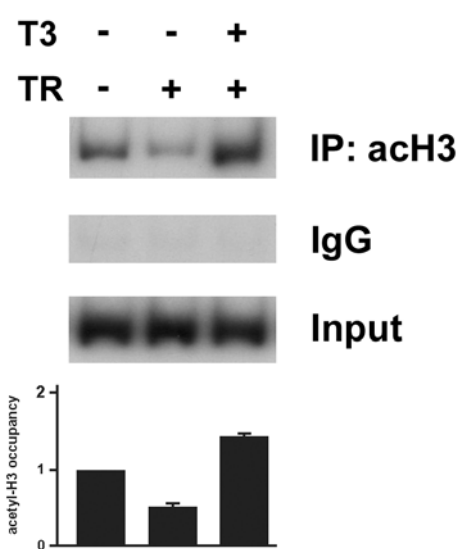


Figure 3

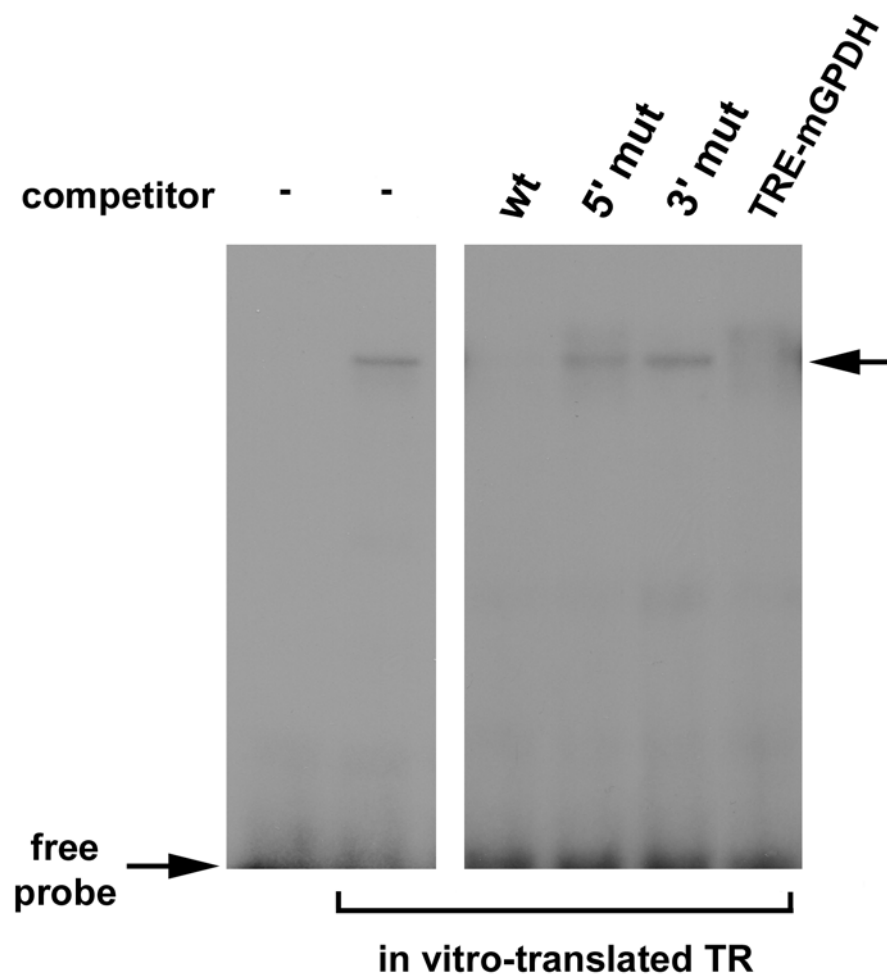


Figure 4