Mediator subunit MED1 is a T3-dependent and T3-independent coactivator on the thyrotropinβ gene promoter.

松井，啓治

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Mediator subunit MED1 is a T3-dependent and T3-independent coactivator on the thyrotropin β gene promoter

(MED1 は T3 依存性および非依存性の両経路を介在して

TSHβ プロモーターを活性化する)

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神戸大学大学院保健学研究科保健学専攻

松井　啓治

Mediator subunit MED1 is a T3-dependent and T3-independent coactivator on the thyrotropin β gene promoter

ABSTRACT

The MED1 subunit of the Mediator transcriptional coregulator complex is a nuclear receptor-specific coactivator. A negative feedback mechanism of thyroid-stimulating hormone (TSH, or thyrotropin) expression in the thyrotroph in the presence of triiodothyronine (T3) is employed by liganded thyroid hormone receptor β (TRβ) on the TSHβ gene promoter, where conventional histone-modifying coactivetors act as corepressors. We now provide evidence that MED1 is a ligand-dependent positive cofactor on this promoter. TSHβ gene transcription was attenuated in MED1 mutant mice in which the nuclear receptor-binding ability of MED1 was specifically disrupted. MED1 stimulated GATA2- and Pit1-mediated TSHβ gene promoter activity in a ligand-independent manner in cultured cells. MED1 also stimulated transcription from the TSHβ gene promoter in a T3-dependent manner. The transcription was further enhanced when the T3-dependent corepressors SRC1, SRC2, and HDAC2 were downregulated. Hence, MED1 is a T3-dependent and -independent coactivator on the TSHβ gene promoter.

Keywords: Mediator, MED1, negative TR-responsive element, *TSHB* promoter, TRβ

Introduction

In the anterior pituitary thyrotroph, a well-known negative feedback mechanism regulates thyroid stimulating hormone (TSH, or thyrotropin) production in response to triiodothyronine (T3) concentration. The mechanism involves a negative (n) TR-responsive element (TRE) (nTRE) in the TSHβ gene promoter. There is a switch in cofactor function on nTRE, wherein the histone-modifying cofactors (SRC1 and SRC2) that act as coactivators on conventional TREs act as corepressors, and the histone deacetylase (NCoR) that acts as a corepressor on conventional TREs acts as a coactivator [1-3; reviewed in 4]. In addition, HDAC2 acts as a ligand-dependent corepressor on the TSHβ gene promoter [5]. Besides the nTRE in the TSHβ gene promoter, there appears to be a mechanism for T3-dependent transcriptional repression through the action of thyroid hormone receptor β (TRβ) that is not associated with DNA but with GATA2 [6].

We have previously shown through *Med1* knockout mouse studies that MED1 is a positive cofactor on the TSHβ gene promoter in the pituitary thyrotroph. *Med1*$^{-/-}$ mice are lethal, and *Med1*$^{+/-}$ mice suffer from pituitary hypothyroidism because of reduced TSHβ gene transcription [7]. Although the mechanism underlying this mouse phenotype is not known, previous reports suggest a ligand-independent mechanism on the TSHβ gene promoter, in which MED1 interacts with GATA2 and Pit1 and coactivates GATA2- and Pit1-initiated transactivation [8]. On the other hand, the T3-dependent, TRβ-mediated function of MED1 on the TSHβ gene promoter (or nTRE) has not been described.

We have developed mutant mice in which the nuclear receptor-binding ability of MED1 was specifically disrupted. We knocked in cDNA encoding mutant MED1 with two LxxLL nuclear receptor recognition motifs modified to LxxAA (MED1(LX) KI mice) [9]. In this study, we show that TSHβ gene transcription in these mice is reduced, indicating that MED1 is a T3-dependent coactivator on the TSHβ gene promoter. This phenotype was confirmed through cell biological analyses.

Materials and methods

Mice

MED1(LX) KI mice [9], backcrossed at least 10 times with C57BL6, were used for experiments. All animal experiments were performed according to National Institutes of Health (NIH) guidelines in the Rockefeller University Laboratory Animal Research Center or the institutional guidelines of the Animal Research Center, Kobe University, Japan.

Cell culture

Stable lines of *Med1*$^{+/+}$ *p53*$^{-/-}$ and *Med1*$^{-/-}$ *p53*$^{-/-}$ mouse embryonic fibroblasts (MEFs), established from embryonic day 10.0 (E10.0) embryos derived from a single crossing of *Med1*$^{+/-}$ *p53*$^{+/-}$ male and *Med1*$^{+/-}$ *p53*$^{+/-}$ female mice on a C57BL6 background, have been described [10]. *Med1*$^{+/+}$ and *Med1*$^{-/-}$ MEFs (with *p53*$^{-/-}$) and CV-1 cells, distributed by RIKEN BRC through the National Bio-Resource of the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS at 37°C.

Plasmids

Mouse (m) GATA2 and human (h) Pit1 cDNAs were kindly provided by M. Yamamoto and Y. Okimura, respectively. These cDNAs (alone or fused with VP16) were inserted into pcDNA3.1(+) (Invitrogen). Gal4-fused truncated hMED1 cDNA was subcloned in pCDM8 (Invitrogen). hMED1, hMED1(LX mutant), and siRNA-resistant hMED1 cDNAs in pIRESneo (Clontech) were described previously [11]. siRNA-resistant hMED1(1-530) in pIRESneo was likewise prepared by site-directed mutagenesis. hTRβ cDNA was prepared by RT-PCR and cloned in pcDNA3.1(+).

For luciferase reporters, the human *TSHB* promoter (−128 to +40) was amplified from genomic DNA using KOD FX (Toyobo, Japan) and cloned into the firefly luciferase reporter plasmid pGL4.10 to create *TSHB*-LUC. The reporter containing five Gal4 binding sites (5×Gal4-LUC) has been described [10].

Luciferase reporter assay and mammalian two-hybrid assay

For luciferase reporter assays, cells (2 × 104) in 24-well plates were transfected with pcDNA3.1-mGATA2 (80 ng), pcDNA3.1-hPit1 (40 ng), pIRESneo-hMED1 (120 ng), pIRESneo-mutant hMED1 (120 ng), and/or pcDNA3.1-hTRβ (30 ng), without or with 10−7 M 3,3′,5-triiodo-L-thyronine (T3) (Sigma), together with *TSHB*-LUC (160 ng) and the *Renilla* control luciferase vector (5 ng) using Lipofectamine2000 (Invitrogen). In some experiments, 5 nM siRNA (Silencer® Select Pre-designed siRNA; Applied

Biosystems) was cotransfected. After 48 h, reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega) and normalized to the activity of the control *Renilla* luciferase [10].

Mammalian two-hybrid assays were similarly performed by transfection with Gal4-fused expression vector (10 ng), VP16-fused expression vector (200 ng), and 5×Gal4-LUC (100 ng), together with the *Renilla* control luciferase vector (5 ng).

Quantification of mRNA

For northern blot analysis, mRNA (10 μg) from five pooled male pituitaries was used [7]. The probes used were a 0.4-kb fragment of mouse TSHβ cDNA, a 0.6-kb fragment of mouse growth hormone cDNA, a 0.5-kb fragment of mouse TSHα cDNA, a 0.3-kb fragment of mouse FSHβ cDNA, a 0.3-kb fragment of mouse LHβ cDNA, and a 0.5-kb fragment of human actin cDNA. Signal intensities were quantified with a STORM840 image analyzer (Molecular Dynamics).

For quantitative PCR (qPCR), total RNA (1 μg) was used to prepare cDNA with the ReverTra Ace qPCR RT kit (Toyobo, Japan). The expression of various genes was identified by qPCR (StepOnePlus™ Real-Time PCR system; Applied Biosystems). Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference marker. The sequences of the primers and the PCR conditions used for amplification are available upon request.

Western blot analysis and measurement of serum hormone levels

For western blot analysis, total cell lysates were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with polyclonal antibodies against MED1 (M255; Santa Cruz Biotechnology), SRC1 (M341; Santa Cruz Biotechnology), SRC2 (F2; Santa Cruz Biotechnology), HDAC2 (AB394; Sigma), and β-actin (BioLegend). Serum levels of free T3 and free thyroxine (T4) were measured by solid phase 125I radioimmunoassay using Coat-a-Count Free T3 and Coat-a-Count Free T4 (Diagnostic Products Corporation, CA).

Statistical analysis

All assays were performed in triplicate in at least three separate experiments. The significance of the differences between independent means was assessed by one-way ANOVA or Student's t-test. We considered a P value of <0.05 statistically significant.

Results

Dysfunction of the pituitary-thyroid axis in MED1(LX) KI mice

We previously reported that *Med1*$^{+/-}$ mice exhibit hypothyroidism because of the reduced level of TSHβ mRNA in the pituitary [7]. To determine if the mechanism of hypothyroidism in the heterozygous knockouts is related to the function of TRβ or another activator(s), we analyzed the pituitary-thyroid axis of MED1(LX) KI mice. The free T4 serum levels were slightly but significantly lower in MED1(LX) homozygous KI males than in wild-type or heterozygous KI male siblings, but the free T3 serum levels were comparable. In contrast, both the free T3 and free T4 serum levels were comparable in MED1(LX) heterozygous KI and wild-type male siblings (Fig. 1A). These findings indicate a reduced thyroid hormone reservoir (but essentially a euthyroid condition) specifically in MED1(LX) homozygous KI mice.

Thyroid hormone production in the thyroid is stimulated by pituitary TSH, but subject to a strict feedback mechanism via T3-bound TRβ. In order to determine the organ responsible for the low thyroid hormone reservoir, TSH production in the pituitary was analyzed by assessing the mRNA levels of the two TSH subunits in the pituitary. In multiple northern blot analyses, TSHβ mRNA levels were significantly lower in MED1(LX) homozygous KI males, whereas TSHα mRNA levels were comparable (Fig. 1B). By contrast, the mRNA levels of other pituitary hormones that use the common α subunit, including follicle stimulating hormone (FSH) β and luteinizing hormone (LH) β, were normal (Fig. 1B). Taken together, these findings indicate that the observed low thyroid hormone reservoirs in MED1(LX) homozygous KI mice are due to the reduced level of TSHβ mRNA in the pituitary and that the T3-dependent interaction of MED1 with TRβ has a crucial role in the normal transcriptional control of TSHβ in the pituitary.

MED1 is a positive cofactor for GATA2 and Pit1 on the TSHB promoter

GATA2 and Pit1 bind to the TSHβ gene promoter and activate transcription [12]. The involvement of MED1 in this process may underlie the pituitary hypothyroidism observed in *Med1*$^{+/-}$ mice. We analyzed the role of MED1 in TSHβ gene promoter activation using a series of luciferase reporter assays in cell culture. In CV-1 cells, in agreement with previous studies, TSHβ gene promoter activity was upregulated by GATA2 or Pit1, and the reporter activity was further enhanced when GATA2 and Pit1 were added together (Fig. 2A). In *Med1*$^{-/-}$ MEFs, enhanced reporter activity via

GATA2 and Pit1 was observed in the absence of MED1. When MED1 was re-introduced into these cells, the reporter activity was further enhanced (Fig. 2B). These data indicate that MED1 is a positive cofactor for GATA2 and/or Pit1, but that transcription can also occur in the absence of MED1.

In GST pull-down assays, GATA2 and Pit1 interacted with MED1 at its N-terminus [8]. We also assessed the intracellular interactions by mammalian two-hybrid assays using *Med1*$^{-/-}$ MEFs. Both GATA2 and Pit1 specifically interacted with MED1(1–530), the smallest fragment of MED1 that integrates into the Mediator complex [13] (Fig. 2C, D). Thus, MED1(1–530) appears to be the interface between GATA2/Pit1 and MED1.

Similar assays using *Med1*$^{+/+}$ MEFs and the TSHB promoter-reporter were used to confirm the role of MED1 in GATA2/Pit1 regulation of TSHβ gene transcription. When MED1 was knocked down in the presence of GATA2 and Pit1 in these cells, reporter activity was attenuated. However, when siRNA-resistant full-length MED1 or MED1(1–530) was reintroduced, reporter activity was recovered (Fig. 2E–G). These observations indicate that MED1 is a coactivator for GATA2 and Pit1 on the TSHβ gene promoter.

MED1 is a positive cofactor for liganded TRβ on the TSHβ gene promoter

Because TSHβ gene transcription was attenuated in MED1(LX) KI mice, we asked if MED1 enhanced T3-dependent transcription on the TSHβ gene promoter, as it does on promoters that possess a conventional TRE. To this end, we first performed promoter assays using *Med1*$^{-/-}$ MEFs in the presence of TRβ, but without exogenous GATA2 and Pit1, in a setting where T3 did not change the promoter activity (Fig. 3A). When wild-type MED1 was added, the T3-independent reporter activity was upregulated 2.3-fold. In the presence of MED1 and T3, the reporter activity was further upregulated 4.3-fold (Fig. 3A). However, ligand-dependent activation was not observed when LX-mutant MED1 or MED1(1–530) was introduced (Fig. 3A). These results indicate that T3-dependent transactivation on the TSHβ gene promoter is dependent on the MED1 LxxLL-mediated interaction between MED1 and liganded TRβ.

We next asked if T3-dependent transactivation occurs concomitant with elevated levels of GATA2 and Pit1. In the setting of exogenously added GATA2, Pit1, and TRβ, but in the absence of MED1, reporter activity was mildly attenuated (0.8-fold) when T3 was added (Fig. 3D). However, when MED1 was added, the reporter activity was enhanced

(1.4-fold) in a T3-dependent matter. SRC1 and SRC2 act cooperatively as T3-dependent corepressors on the TSHβ gene promoter [2]. HDAC2 is also recruited to liganded TRβ on the TSHβ gene promoter and is involved in transcriptional repression [5]. To exclude the interference of these negative cofactors, we reduced the endogenous levels of the cofactors by cotransfection with their respective siRNAs and tested reporter activity. When SRC1 and SRC2 and/or HDAC2 were knocked down, the T3-dependent transactivation was significantly enhanced (up to 2-fold) compared to the reporter activity in the absence of the siRNAs for these cofactors (Fig. 3B–D). These results clearly indicate that MED1 is a T3-dependent positive cofactor on the TSHβ gene promoter.

Discussion

We determined a role for MED1 in both T3-dependent and T3-independent transcriptional activation on the promoter of the gene encoding TSHβ. This implies that MED1 is an important positive regulator in the pituitary thyrotroph that maintains the homeostasis of thyroid hormone by a mechanism distinct from the p160 family cofactors.

We propose two mechanisms for the recruitment of MED1 to the TSHβ gene promoter. N-terminal MED1(1–530) mediates recruitment via interaction with GATA2 and/or Pit1 in a T3-independent manner, and the LxxLL nuclear receptor recognition motif of MED1 serves as an interface for the interaction with liganded TRβ in a T3-dependent manner (Fig. 4). The existence of a T3-dependent coactivator on this promoter suggests an interesting model for the transcriptional control of negative feedback in the thyrotroph. While the manner in which HDAC2 is recruited to liganded TRβ has not been demonstrated, the interaction of MED1 and the p160 family of coactivators with the AF2 domain of nuclear receptors is mutually exclusive [14]. Therefore, the interaction of the positive cofactor MED1 and a p160 family negative cofactor with liganded TRβ is competitive. When MED1 interacts with liganded TBβ, LxxLL-mediated recruitment of MED1 to the promoter could enhance the concomitant or subsequent association of MED1(1–530) with GATA2/Pit1, thus further stabilizing the recruitment of Mediator and the formation of the preinitiation complex (PIC) for transcriptional initiation. SRC1/SRC2 associated with liganded TRβ could block Mediator and the functional formation of PIC on the promoter. On the other hand, when

SRC1/SRC2 is downregulated, MED1-led Mediator recruitment becomes dominant and contributes to an elevated TSH level.

The location of nTRE corresponds to the transcription initiation site (Fig. 4), raising the question of whether TRβ stably associates with DNA during promoter melting induced by PIC. At the nTRE located within the Necdin gene promoter, TRβ dissociates from DNA during transcription [15]. In this regard, the hypothesis of an activator switch (TRβ to GATA2/Pit1) during MED1-led Mediator recruitment to this promoter is attractive and should be tested in the future.

In human diseases with mutant TRβ that result in generalized resistance to thyroid hormone and in mouse models lacking TRβ (and/or TRα), TSHβ production is prominently upregulated [16,17]. In the absence of liganded TRβ, the negative cofactor is absent, and GATA2/Pit1-led transcriptional activation predominates in our model (Fig. 4).

In one study, TRβ associated with GATA2 and acted in a similar manner as nTRE-associated TRβ [6]. In this situation, MED1 recruitment to DNA-bound GATA2 occurs in two ways: direct interaction between GATA2 and N-terminal MED1(1–530) and indirect interaction with the LxxLL domain of MED1 and GATA2 mediated by liganded TRβ. The additional possibility of MED1 LxxLL-mediated and TRβ-mediated recruitment of Mediator enhances the likelihood that a functional PIC forms for effective transcriptional initiation.

In conclusion, MED1 acts a positive cofactor in T3-independent and T3-dependent manners on the promoter of gene encoding TSHβ. The multiple modes of MED1 recruitment to the promoter contribute to the fine-tuning of the physiological homeostasis of the pituitary-thyroid axis and might contribute to some pathological conditions.

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Figure legends

Fig. 1. Reduced thyroid hormone reservoir and pituitary TSHβ gene transcription in MED1(LX) KI mice.

(A)Serum levels of free T3 and free T4 were measured in five 12-week-old male mice of each genotype. Values are the mean ± SD of a representative experiment (*$P < 0.05$; **$P < 0.01$).

(B)Northern blot of pooled pituitary total RNA from the same mice. The relative signal intensities of RNA blot analyses were quantified with an image analyzer, and the expression of each mRNA (normalized to the level of actin mRNA) was expressed as the fold increase versus the value in wild-type mice. A decrease in the TSHβ signal was observed in MED1(LX) homozygous knock-in mice, but the signals for the common α subunit and two other β subunits that use the common α subunit (FSHβ and LHβ) did not change. The signals in heterozygous knock-in mice did not change either. These results were reproduced in three independent experiments.

Fig. 2. MED1 is a positive cofactor for GATA2 and Pit1 on the TSHβ gene promoter.

(A) A luciferase reporter assay in CV-1 cells showed that GATA2 and Pit1 cooperatively enhanced *THSB* promoter activity.

(B) A luciferase reporter assay in *Med*$^{-/-}$ MEFs showed that GATA2 and Pit1 cooperatively enhanced *TSHB* promoter activity in the absence of MED1. The promoter activity was further enhanced in the presence of MED1.

(C, D) Mammalian two-hybrid assays using *Med1*$^{-/-}$ MEFs showed that both Pit1 (C) and GATA2 (D) specifically interacted with the N-terminal fragment MED1(1–530). (E, F) Quantitative RT-PCR (E) and western blot (F) of MED1 2 days after transfection of *Med1*$^{+/+}$ MEFs with MED1 siRNA.

(G) A luciferase reporter assay in *Med1*$^{+/+}$ MEFs showed that MED1(1–530) as well as full-length MED1 enhanced GATA2- and Pit1-mediated transcription. Both siRNA-resistant MED1(1–530) and full-length MED1 rescued the reporter activity attenuated by MED1 siRNA.

The means ± SD from a representative experiment performed in triplicate are shown (A–F). Values were plotted as the fold increase versus the value without GATA2 or Pit1 (A, B), versus the value with Gal-MED1(1–530) and VP16 (C, D), versus the value of control scrambled siRNA (E), or versus the value with GATA2 and Pit1 (F).

Fig. 3. MED1 is a T3-dependent positive cofactor on the TSHβ gene promoter.
(A) In a luciferase reporter assay in *Med1*$^{-/-}$ MEFs, wild-type MED1 enhanced *TSHB* promoter activity in a T3-dependent manner, but MED1(LX-mutant) or MED1(1–530) did not.
(B, C) Quantitative RT-PCR (B) and western blot (C) of SRC1, SRC2, and HDAC2 2 days after transfection of *Med1*$^{-/-}$ MEFs with SRC1/SRC2 and/or HDAC2 siRNA.
(D) In a luciferase reporter assay in *Med1*$^{-/-}$ MEFs, MED1 further enhanced T3-dependent *TSHB* promoter activity when SRC1/SRC2 or HDAC2 expression was suppressed. The reporter activity was tested in the presence of GATA2 and Pit1 with either scrambled siRNA or siRNA for SRC1/SRC2 and/or HDAC2.
The means ± SD from a representative experiment performed in triplicate are shown (A, B, D). The values were plotted as the fold increase versus the value with TRβ and without T3 (A), versus the value of control scrambled siRNA (B), or versus the value with GATA2, Pit1, and scrambled siRNA (D).

Fig. 4. Regulation of the TSHβ gene promoter by MED1 in the presence of T3.
The following specific sites are present on the *TSHB* promoter: Pit1-binding site (−120 to −108) [18], GATA2-binding sites (−107 to −102, −96 to −91) [12,19], TATA box (−29 to −24) [20], and nTRE (−2 to +33) [21-25]. MED1 is recruited to the *TSHB* promoter either through the ligand-independent interaction of N-terminal MED1(1–530) with GATA2 or Pit1 or through the ligand-dependent interaction of the LxxLL motifs of MED1 with the AF2 domain of liganded TRβ. The AF2 domain of liganded TRβ binds to the negative cofactor SRC1/SRC2 or the positive cofactor MED1 in a mutually exclusive, and probably competitive, manner. Liganded TRβ also recruits the negative cofactor HDAC2. LxxLL-mediated recruitment of MED1 to the *THSB* promoter may facilitate the interaction of MED1(1–530) with GATA2 and stabilize Mediator recruitment to the promoter and the subsequent formation of a functional preinitiation complex (PIC) during promoter melting. The involvement of multiple activators and both positive and negative cofactors may fine-tune the ligand-dependent negative feedback mechanism of THSβ production.

Figure

Figure 1

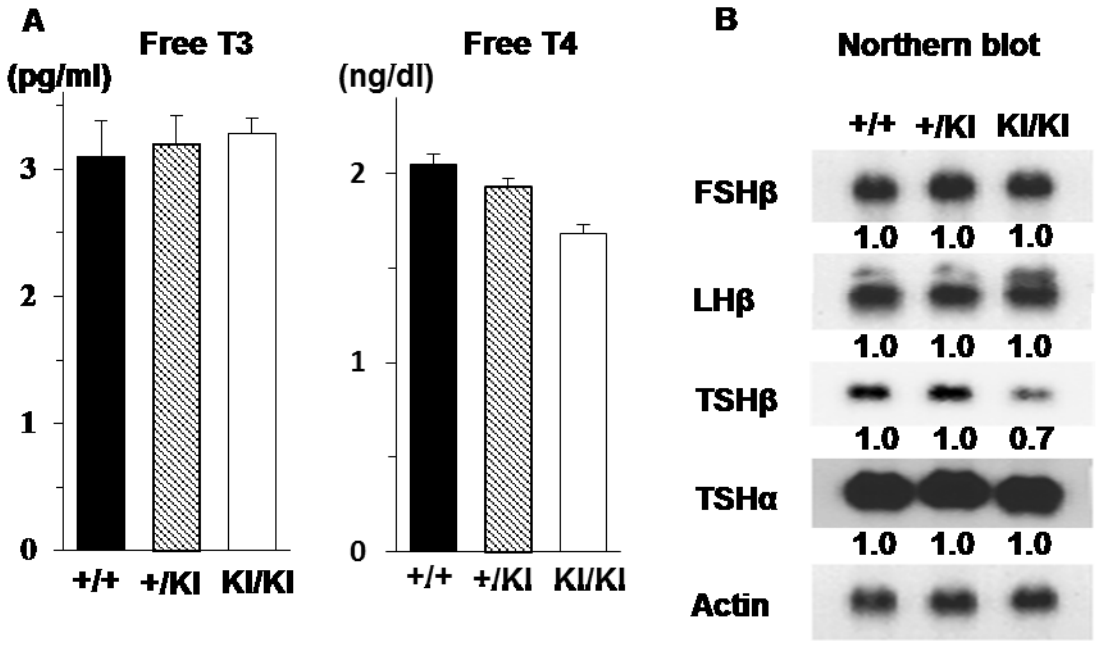


Figure 2

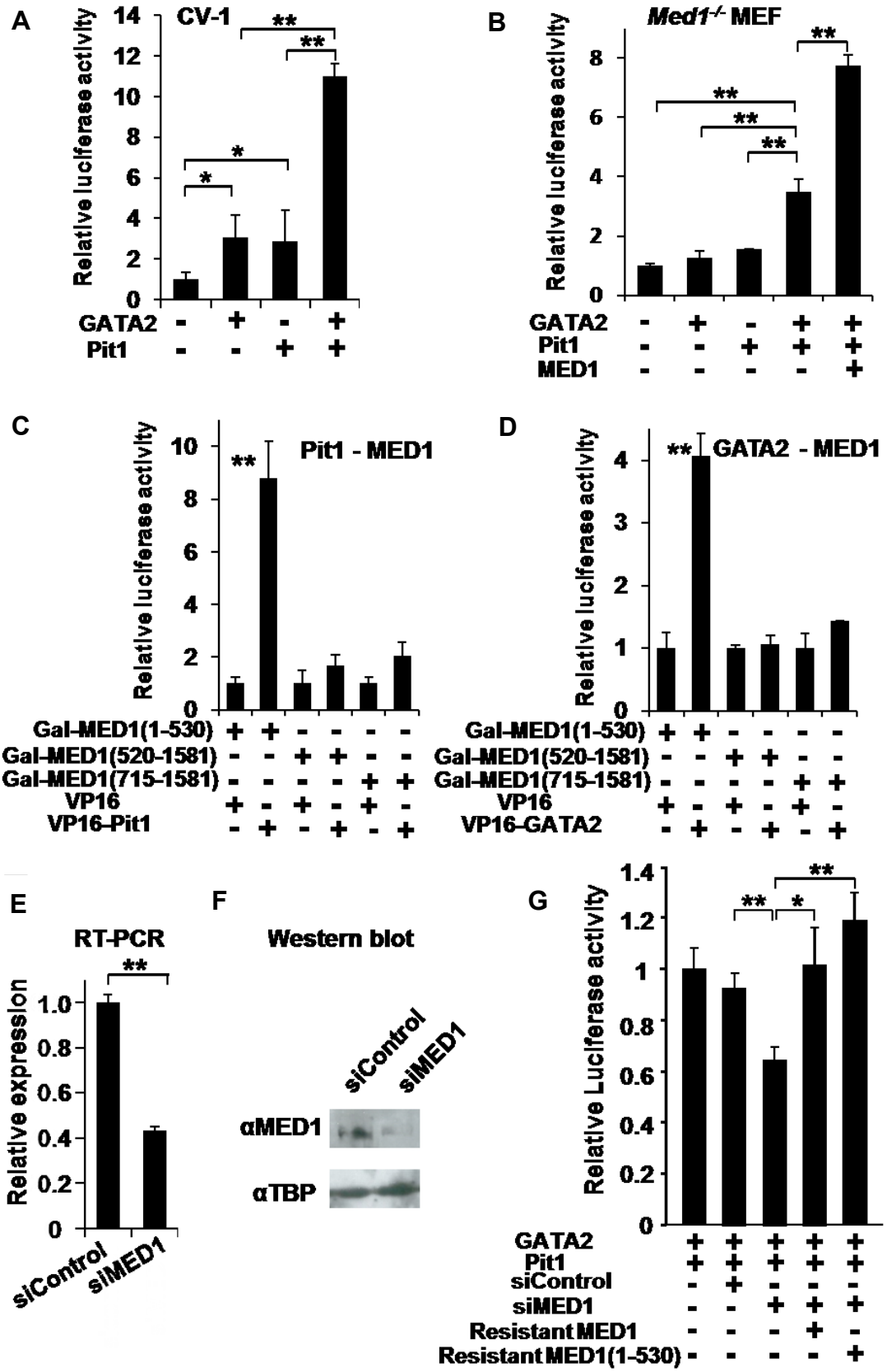

A
CV-1
Relative luciferase activity
GATA2
Pit1
B
Med1-/- MEF
Relative luciferase activity
GATA2
Pit1
MED1
C
Pit1 - MED1
Relative luciferase activity
Gal-MED1(1-530)
Gal-MED1(520-1581)
Gal-MED1(715-1581)
VP16
VP16-Pit1
D
GATA2 - MED1
Relative luciferase activity
Gal-MED1(1-530)
Gal-MED1(520-1581)
Gal-MED1(715-1581)
VP16
VP16-GATA2
E
RT-PCR
Relative expression
siControl
siMED1
F
Western blot
siControl
siMED1
αMED1
αTBP
G
Relative Luciferase activity
GATA2
Pit1
siControl
siMED1
Resistant MED1
Resistant MED1(1-530)

Figure 3

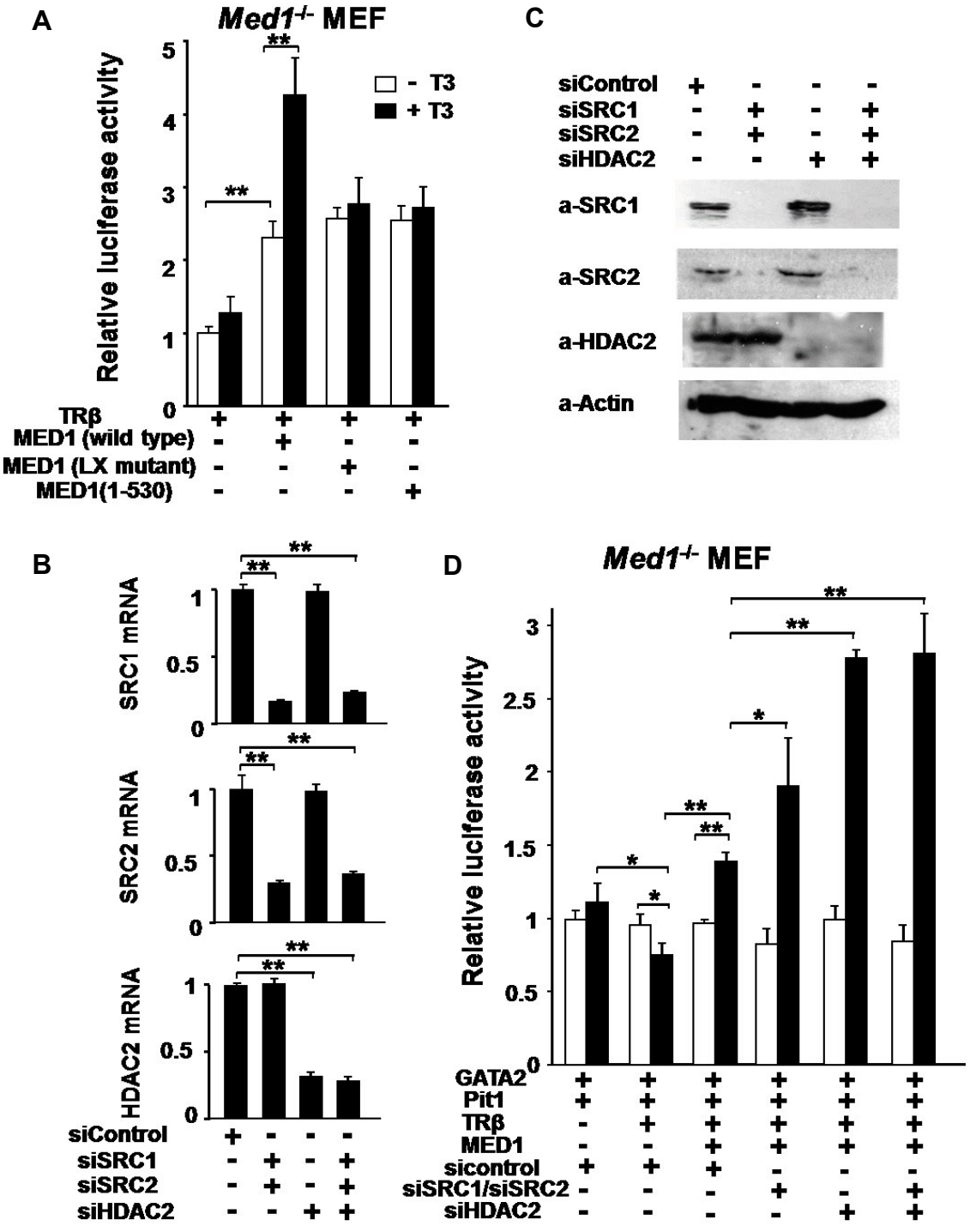
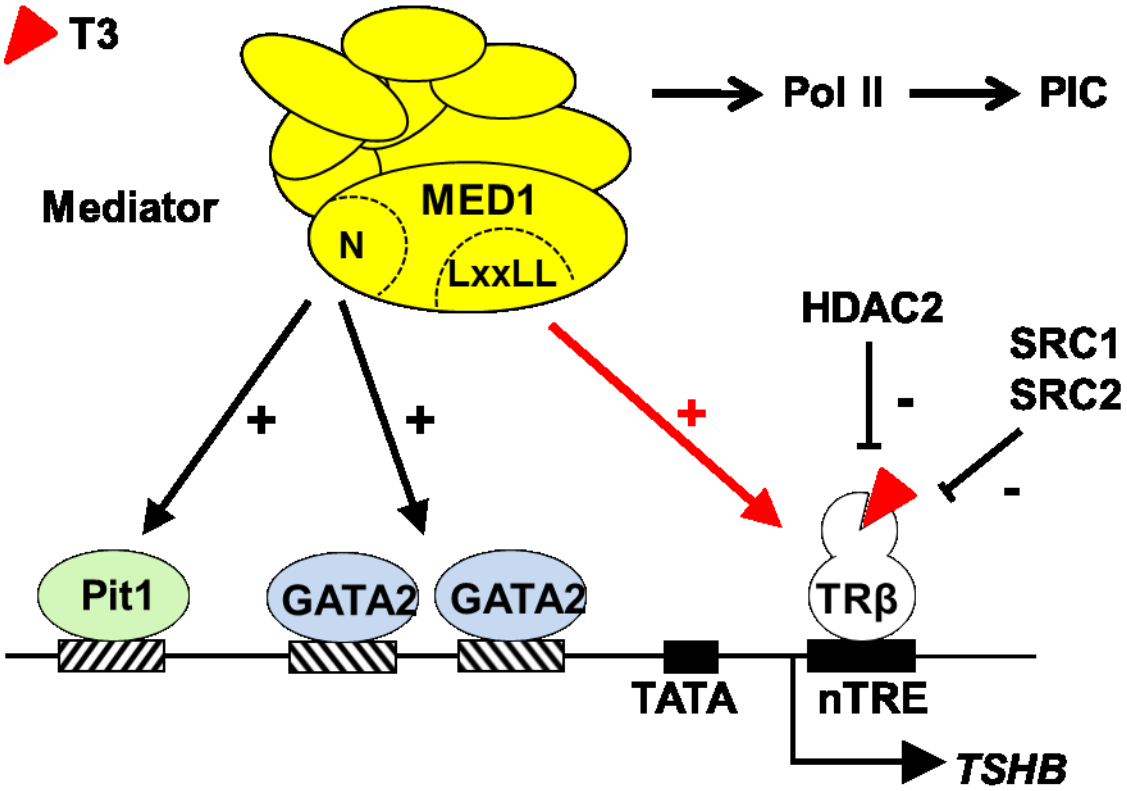

A
Med1-/- MEF
Relative luciferase activity
- T3
+ T3
TRβ
MED1 (wild type)
MED1 (LX mutant)
MED1(1-530)
C
siControl
siSRC1
siSRC2
siHDAC2
a-SRC1
a-SRC2
a-HDAC2
a-Actin
B
SRC1 mRNA
SRC2 mRNA
HDAC2 mRNA
siControl
siSRC1
siSRC2
siHDAC2
D
Med1-/- MEF
Relative luciferase activity
GATA2
Pit1
TRβ
MED1
sicontrol
siSRC1/siSRC2
siHDAC2

Figure 4


T3
Mediator
MED1
N
LxxLL
Pol II
PIC
HDAC2
SRC1
SRC2
+
+
+
-
-
Pit1
GATA2
GATA2
TRβ
TATA
nTRE
TSHB