

Characterization of a novel thyrotropin-releasing hormone receptor, TRHR3, in chickens

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ABSTRACT The physiological roles of thyrotropin-releasing hormone (TRH) are proposed to be mediated by TRH receptors (TRHR), which have been divided into 3 subtypes, namely, TRHR1, TRHR2, and TRHR3, in vertebrates. Although 2 TRH receptors (TRHR1 and TRHR3) have been predicted to exist in birds, it remains unclear whether TRHR3 is a functional TRH receptor similar to TRHR1. Here, we reported the functionality and tissue expression of TRHR3 in chickens. The cloned chicken TRHR3 (cTRHR3) encodes a receptor of 387 amino acids, which shares high-amino-acid identities (63–80%) to TRHR3 of parrots, lizards, *Xenopus tropicalis*, and tilapia and comparatively lower sequence identities to chicken TRHR1 or mouse TRHR2. Using cell-based luciferase reporter assays and Western blot, we demonstrated that similar to chicken TRHR1 (cTRHR1), cTRHR3 expressed in HEK 293 cells can be

potently activated by TRH and that its activation stimulates multiple signaling pathways, indicating both TRH receptors are functional. Quantitative real-time PCR revealed that *cTRHR1* and *cTRHR3* are widely, but differentially, expressed in chicken tissues, and their expression is likely controlled by promoters located upstream of exon 1, which display strong promoter activities in cultured DF-1 cells. *cTRHR1* is highly expressed in the anterior pituitary and testes, while *cTRHR3* is highly expressed in the muscle, testes, fat, pituitary, spinal cord, and many brain regions (including hypothalamus). These findings indicate that TRH actions are likely mediated by 2 TRH receptors in chickens. In conclusion, our data provide the first piece of evidence that both cTRHR3 and cTRHR1 are functional TRH receptors, which helps to elucidate the physiological roles of TRH in birds.

Key words: chicken, TRH, TRHR3, TRHR1, pituitary

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INTRODUCTION

It is generally believed that thyrotropin-releasing hormone (TRH), also known as thyrotropin-releasing factor, is a releasing hormone secreted by the hypothalamus which constitutes an essential part of the hypothalamic–pituitary–thyroid axis in vertebrates. TRH is mainly produced by neurons in the paraventricular nucleus and transported via the hypophysial portal circulation to the pituitary gland (Taylor et al., 1990). The precursor of TRH is a long polypeptide which often contains multiple repeats of Gln-His-Pro-Gly. These repeats are likely cleaved to form the mature form of TRH

(Richter et al., 1984; Lechan et al., 1986), which is a tripeptide (pyro-Glu-His-Pro-NH₂) that shows a remarkable structural conservation in most vertebrates including mammals, birds, amphibians, reptiles, and fish. TRH was originally named after its ability to promote the release of thyroid-stimulating hormone (TSH) in mammalian pituitaries (Bøler et al., 1969; Burgus et al., 1970). Nevertheless, this function has also been found to be mediated by other hormones in vertebrates, including corticotropin-releasing hormone in nonmammalian vertebrates and glucagon-like peptide (GCGL) in chickens (De Groef et al., 2003; Huang et al., 2014). Furthermore, multiple effects of TRH were found in the central nervous system (CNS) and peripheral tissues in various species. For instance, TRH exerts an antidepressant function in the mammalian CNS (Marangell, 1997). TRH was reported to have a prolactin (PRL)-stimulating effect in cultured rat pituitary cells and chicken pituitary cells (Tashjian et al., 1971; Harvey et al., 1978). It has also been established as a potent growth hormone (GH)-releasing factor in

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vertebrates. As an example, in chickens, TRH stimulates GH release both *in vivo* and *in vitro* (Harvey, 1990).

The physiological roles of TRH are reported to be mediated by thyrotropin-releasing hormone receptor (TRHR). In vertebrates, 3 subtypes of TRHR have been identified or predicted, namely, TRHR1, TRHR2, and TRHR3. All of them belong to G protein-coupled receptor family. Among the three, TRHR1 was the first isolated one, being discovered in mice back in 1990 (Straub et al., 1990). This receptor is widely present across various vertebrate groups and has been reported to mediate the function of TRH in the anterior pituitary of mice (Rabeler et al., 2004). TRHR2 was first identified in rats in 1998 (Itadani et al., 1998). It shares 51% sequence identity with both mouse and rat TRHR1. Unlike TRHR1, TRHR2 has so far been found in frogs, teleosts, and a few mammalian species including rats, mice, and horses (Heuer et al., 2000; O'Dowd et al., 2000). In comparison, studies on TRHR3 are scarce. This receptor has been predicted to exist in a small number of species across different animal kingdoms but is hypothesized to be absent in humans and rodents. Although TRHR3 of *Xenopus laevis* has been functionally analyzed, the binding affinity of *Xenopus* TRHR3 to TRH is considerably lower than that of the other 2 TRH receptors, casting doubt on whether TRHR3 is a functional TRH receptor (Lu et al., 2003).

In birds, 2 TRH receptors (TRHR1 and TRHR3) were predicted from the genome. Similar to mammalian TRHR1, chicken TRHR1 has been established to be a functional receptor coupled to Gq proteins and its activation can stimulate intracellular calcium concentration (Sun et al., 1998). Nevertheless, the tissue expression and functionality of TRHR3 in birds remains in the dark. Hence, in this study, we cloned *TRHR3* from chickens and characterized its tissue expression and signal transduction, aiming to reveal the functional similarity and differences between cTRHR3 and cTRHR1. These findings not only help to unveil the influence of TRH and its receptors on the physiological functions and traits of chickens, such as pituitary hormone (GH, PRL, TSH) secretion and growth, but also provide clues to explore the functional change of TRHR3 during vertebrate evolution.

METHODS AND MATERIALS

Chemicals, Primers, Peptides, and Antibodies

Chicken TRH used in experiments was synthesized by GL Biochem (Shanghai, China). All primers were synthesized by Beijing Genome Institute (China), and primer sequences are shown in Supplementary Table 1. ERK1/2 and pERK1/2 antibodies were purchased from Cell Signaling Technology (Beverly, MA). All chemicals including H89, MDL-12330A, and 2-aminoethoxydiphenyl borate (2-APB) were obtained from Sigma-Aldrich (St. Louis, MO).

Total RNA Extraction

Adult chickens (aged 6 mo) of both sexes (Lohmann layer) and ducks were purchased from local commercial companies. Six adult chickens (3 males and 3 females) were killed, and different tissues (including the telencephalon, midbrain, cerebellum, hindbrain, hypothalamus, spinal cord, anterior pituitary, heart, duodenum, kidneys, liver, lung, ovary, testes, spleen, pancreas, crop, proventriculus, gizzard, jejunum, ileum, cecum, colon, subcutaneous fat, back skin, and breast muscle) were collected and stored at -80°C before use. In addition, anterior pituitary glands collected from 6 adult male chickens were carefully segregated into the caudal lobe (Ca) and cephalic lobe (Ce) and subjected to RNA extraction. Total RNA was extracted using RNeasy (Molecular Research Center, Cincinnati, OH) in accordance with the manufacturer's instruction. The animal experiment protocol was approved by the Animal Ethics Committee of Sichuan University (Chengdu, China).

Reverse Transcription and Quantitative Real-Time PCR

Two micrograms of total RNA and 0.5 pg of oligodeoxythymide were mixed in a total volume of 5 μL , incubated at 70°C for 10 min and cooled at 4°C for 2 min. The first strand buffer, 0.5 mmol each deoxynucleotide triphosphate, and 100 U Moloney murine leukemia virus reverse transcriptase (Takara) were then added into the reaction mix in a total volume of 10 μL . Reverse transcription (RT) was performed at 42°C for 90 min. In accordance with our previously established method (Cai et al., 2015), quantitative real-time RT-PCR was performed on the CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA) to examine the mRNA levels of target genes in chicken tissues.

Cloning of the cDNAs Encoding Chicken TRHR1 and TRHR3 and Duck TRHR1

In accordance with the cDNA sequence of *cTRHR1* (NM_204930) and predicted cDNA sequence of *dTRHR1* (XM_005022942) and *cTRHR3* (XM_004947049) in GenBank, gene-specific primers were designed to amplify the 5'-cDNA and 3'-cDNA ends of *cTRHR1* and *cTRHR3* from adult chicken or duck brain (or pituitary) using SMART-RACE cDNA amplification Kit (Clontech, Palo Alto, CA). The amplified PCR products were cloned into pTA2 vector (TOYOBO, Japan) and sequenced. Finally, the open reading frames were obtained by assembly of resultant sequences.

Identification of the Promoter Regions of Chicken TRHR1 and TRHR3

To map the promoter regions of chicken *TRHR1* and *TRHR3*, we designed gene-specific primers and

amplified their 5'-flanking regions (near exon 1) with high-fidelity *Taq* DNA polymerase (TOYOBO, Japan). The PCR products were cloned into pGL3-Basic vector (Promega, Madison, WI), and their sequences verified. Then, a series of promoter-luciferase reporter constructs for *cTRHR1* (−2097/+127Luc, −933/+127Luc, −371/+127Luc, −137/+127Luc) and *cTRHR3* (−2003/+23Luc, −1000/+23Luc, −509/+23Luc, −180/+23Luc) were prepared. In this experiment, the transcription start site on exon 1 of each gene determined by rapid amplification of 5' cDNA ends (5'-RACE) was designated as “+1,” and the first nucleotide upstream of the transcription start site was designated as “−1.” Finally, the promoter activities of these constructs were examined in cultured DF-1 cells by the Dual-Luciferase Reporter Assay (Promega, Madison, MI), as described in our previous study (Wang et al., 2010).

Functional Characterization of Chicken TRHR1 and TRHR3

The open reading frames of *TRHRs* were amplified using gene-specific primers based on their cDNA sequences obtained from the adult chicken brain or pituitary using high-fidelity *Tag* DNA polymerase (TOYOBO, Japan). Then, the PCR products were cloned into the pcDNA3.1(+) expression vector (Invitrogen) and sequenced. In accordance with our previously established methods (Wang et al., 2007; Wang et al., 2012; Mo et al., 2015; Mo et al., 2017), the function of each receptor was examined in HEK 293 cells using the pGL3-NFAT-RE-luciferase reporter system, pGL4-SRE-luciferase reporter system, and pGL3-CRE-luciferase reporter system.

Western Blot

As described in our previous studies (He et al., 2016; Mo et al., 2017), HEK 293 cells transfected with *cTRHR1* or *cTRHR3* expression plasmid were cultured on a 24-well plate at 37°C and then treated by TRH (10 nM) for 10 min. Then, cells were lysed and used for Western blot detection of phosphorylated ERK1/2 (pERK1/2) and ERK1/2.

Data Analysis

The mRNA level of the target gene was first normalized by that of β -*actin* and then shown as a fold difference compared to a selected tissue. The change in luciferase activity of HEK 293 cells expressing TRHR was expressed as a relative fold increase in response to peptide treatment. Data were analyzed by one-way ANOVA followed by Dunnett's test. Dose-response curves were constructed using a nonlinear regression model, and the corresponding half maximal effective concentration (EC₅₀) values were assessed using GraphPad Prism, version 7 (GraphPad, San Diego, CA). The construction of the Maximum Likelihood Tree

phylogenetic tree was carried out using MEGA, version 7 (MEGA software, Sudhir Kumar, Arizona State University, Phoenix, AZ). All experiments were repeated at least twice.

RESULTS

Cloning of the Full-Length cDNA of Chicken TRHR3

Based on the predicted partial cDNA sequence of chicken *TRHR3* (XM_004947049), we cloned the full-length cDNAs of *cTRHR3* from adult chicken brain tissue by RACE-PCR. The cloned *cTRHR3* cDNA is 3,150 bp in length (accession no. MK138989) and predicted to encode a receptor of 387 amino acids (Figure 1A). Comparison of *cTRHR3* cDNA sequence with the chicken genome database (http://www.ensembl.org/Gallus_gallus) shows that *cTRHR3* consists of 2 exons. The first exon of *cTRHR3* consists of a 774-bp coding sequence (CDS) fragment and a 939-bp 5'-untranslated region (5'-UTR), and the second exon consists of a 390-bp CDS fragment and a 1047-bp 3'-UTR region.

To compare the structural and functional difference between *cTRHR3* and *cTRHR1*, we also cloned the chicken TRHR1 of 395 amino acids from pituitary. *cTRHR1* is composed of 3 exons, and its exon 3 contains a very short CDS region of 6 bp and a long 3'-UTR region of 3,274 bp (Figure 1B).

Sequence alignment revealed that *cTRHR3* shows high-amino-acid-sequence identities of 80.3%, 72.9%, 66.5%, and 62.6% with TRHR3 of parrots, lizards, *Xenopus tropicalis*, and tilapia, respectively. Chicken TRHR1 shows high-amino-acid-sequence identity of 81% with human TRHR1 and a much lower identity of 56.2% with chicken TRHR3 (Figure 2, Supplementary Table 2). Both *cTRHR1* and *cTRHR3* possess a G protein-coupling ERY motif located near the third transmembrane domain and a conserved NP (XX)Y sequence located in the seventh transmembrane domain. In addition, both TRH receptors have the same four-amino-acid residues (Tyr¹⁰⁸, Asn¹¹², Tyr²⁷⁷, and Arg³⁰¹) that were proposed to directly interact with TRH (Sun et al., 1998).

To study the evolutionary relationship between *TRHR1*, *TRHR2*, and *TRHR3* in vertebrates, phylogenetic analysis was performed using the maximum likelihood method of MEGA software. As shown in Supplementary Figure 1, TRHR family forms 3 clusters, TRHR1, TRHR2, and TRHR3. Among them, *TRHR1* and *TRHR2* have closer evolutionary relationships, while the *TRHR3* is located on a more distant branch. *TRHR1* exists in all vertebrate groups; *TRHR2* is found in mice, horses, frogs, reptiles, and teleosts (e.g., zebrafish); *TRHR3* is present in birds, frogs, fish, and a few mammalian species (e.g., dogs, pigs, and cattle).

A

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1   AGAGCATGGGGCTGGGCAGGAGGTTTTTTTGTCTGCACTGGGACTGTAGCACCCATGCTGGCAATGTGCCTTTGAAGCCCCAGACTGGAA 90
91  TCTCTCAGCAATGGAAATCCTTCACATTTTCAAATGGGTGTCTATGCTGGGATTAATGGCGTGGCTCTGCTTCAAATGCAATGTAAT 180
181 CTAATTGAAAAGTGGGCTTTTCTCCTTGTCTCTTTCCCTCCACTCCACAGGTTAGGTATGATGAGCACTCTTTCTGCAATCACGGTTGCT 270
271 CTTTCTATCTGAAAATACATTTTCTGAGGCTGCAGAGACATCAGGAGGAGACAGAGCTGACAGCAGAGTCAAGGCTGCTCTGGATTA 360
361 CCTGTCTGGGAGGAAACACATCTCAGCCCCGGGATGGATACAGACAGCCCGGCTGGCCAGCAGAGCTCCCATCATGGGGTGGGTAAGA 450
451 CTTTCCCAGTGGAGTGACACCACTTCTTTGAGATAGGGTGTGTTGACAGACTGATGGGAGGAGGTTGAAGACCCCCAGGAAAGCA 540
541 CTGCTCTGCCCCAGCCCAAGGGAATGGGGAAGCATCATCTGGTCCCATCTCTGAGATGAGGAGGAAAGACAGGACCTCAGCTGGG 630
631 CTCCTTCTCCACCTCTCTCTGTTCTCTCTCTCTCTCTCTCTGGTCCAGCTGTGAACCAGGACCCAAACAAGTGTAGGTGAGGAGCACATG 720
721 CATGAGGGGGTCTGGGGGATTTCTGGGGTCCCCAGGGCTGAGAAGGAGGTTGCAGGGATTTGGGGTTTCTGGATGCTCCCCGCCCTCC 810
811 TAGCAGTGGTTCAGCGAGGGGGGAGAGGGGGTTCAGGAGAGGTTGGGTGGAGAAGAAAGGGGAGGGGAAACGGTTGGGAAGGA 900
901 GGGGAGATCACCGTGTCTGTAGAAATCTGTGCAGCCATCGAGAATGGTTTCAGCCAGCCCCGGAGCCAGCTGGGCAACCAACATTTG 990

          M E N G S A S P G A T L G N Q T L
991  GGCAGGATGCCCCGACGCCCTGGAGCTCCAGGTGGTCAACCTCTGCTGGTGTCTCGTCTGCGGGTGGGCATAGCAGGCAATGCC 1080
    G R M P R Q P L E L Q V V T I L L V L L V C G V G I A G N A
1081 ATGGTGGTGTGGTGGTGGCGCACCAAGCACATGGTGACCCACCACCACTGCTACTGGTGGAGCCTGGCTGTGGCCGATCTGCTGGTA 1170
    M V V L V L V L R T K H M V T P T N C Y L V L V S L A D L L V
1171 CTGCTGGTGGTGGGCTGCCAACATCTCCGAAGTGGTGGCTTCTGGGTGTACGGCTACGGCCGGCTGCCCTGCATCACCTACCTGCGAG 1260
    L L V A G L P N I S E V V A S W V Y G Y A G C L C I T Y L Q
1261 TACCTGGGCATCAACATCTCCGCTGGTCCATCACCAGCTCCACAGTGGVAGGCGCTACATTGCAATCTGCCATGCCATCAGGGCACAGCT 1350
    Y L G I N I S A W S I T A F T V E R Y I A I C H A I R A Q L
1351 CTGTGCACCGTGGCCCGCGCAAGCGCATCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 1440
    L C C T V A R A K R I I S S L W I F T S V Y C L V W F F L V D
1441 ACGTCCAGGTACGTTCTCAGATGGGGAACAAGTCAAGTCCAGTCCGGTACCCGCTCTCCAGAAGCCTTTACATGCCATTTATTTCTTGGAT 1530
    T S Q V T T F S D G E Q V V S C G Y R V S R S L Y M P I Y F L D
1531 TTTGCTGTCTTCTACGTATCCCCGTGGGTCTTTCAGCCGTCCTCTATGGCCCTATTGCACGCATTCTATACATGAGCCCCCTGCTGCT 1620
    F A V F Y V I P L G L A A V L L Y G L I A R I L Y M S P L P A
1621 GCCCTCAGCATGCCCTGGGCTCAGCACATCGGGGTGGCTCCCTCCAGCAGTCTGCCAGGGCAGCAGGGGGACCCCTGAGCTCCCGCAAG 1710
    A P Q H A L G S A H R G V S L Q Q S C Q G S R G T L S S R K
1711 CAGGTGACCAAGATGCTGGCTGCTGGTGGTCTCTCTCGCCCTTCTGTGGCTGCCGTACCCGACACTGGTGGTGGTGAATCTCTCTGCTG 1800
    Q V T K M L A V V V L F A L L W L P Y R T L V V V N S F V
1801 GACCCCTCCGTACCTCAACATTTGGTTCCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT 1890
    D P P Y L N I W F L L F C R M C I Y L N S A I N P I I Y S L
1891 ATGTCCAGAGATTCAGAGCTGCCTCAGGAAGCTCTGCAAGTCCAGTCCGGATGGAAGAGCAGCAGGAGTCCACACCACCCAGCAGCTCCCGGT 1980
    M S Q R F R A A F R K L C R C G W K S T E V P T P R S V P V
1981 CTCTATAGCAGAGCAAGGATGGCTCTCAGGCAAGCTCCGAGCAGCCACCCAGGATGACCTGAACGGTCCCCCTGCACCTGTGGAGA 2070
    L Y S R R A K D G S C Q G S S E H G T H Q D D L N G Q D P P A P V R
2071 AGCAGGGCTGCCATGCACACCCGGGACCTGACACAACCGCAGGCAGGGGAGCTGTGCGGCTGTGTGCACCACGTTGGAAAGTGCCAA 2160
    S R A A M H T P G P

2161 CAGAAATGCTTGGGCATTGCAACAAGCTTAAGTTAATACCTCTCCAGGGCATTAAACAGAACAGATTCTTGGAGGCAAAAATAATTTGCT 2250
2251 TTTCTTAATGCTAATTTATTTCTTGGCTGTGAAGCGTAGGGCAGGTCATGTTTCAAGTCTCTAAAGAAATCTGTAGTTAAATATAGC 2340
2341 AGTGCAACGCCATTCTGTGTATGTGCAAGTGCAGCTGCTTGTGAGTCAAGCCAGAGCATGGGAAGGAAATGCAAGGAAGAAAGGGCAG 2430
2431 TCAAGGAAAACCTGTCCACAGAGCAGATTTCTAGAGGTTGAGCTTCCCTACCCAAATGTAGGAGGCTTCTGACATGAGCTGCTCTCTCC 2520
2521 AGGGTCTGTGGAAATAGCAGGACAATGTGGCACTTTGTCTCTCAATCCAGGAAGAGATGAATGCACCTCTGTGTGTATTTCCT 2610
2611 TCCACCAACACCAAGGGACCCTCTGCTCTCCGATGGAGACTCGTGCATTACAGTGGCCGAGCTGGGTGTCATGAACCTCACCCCG 2700
2701 TGCTTCTGTGCCATTGCTGTGCCAACACAGCCCAAGGACACTTGCACCAGGCTCACAAGCAACTCTCTGGACTGGGAGTAAATGTAAGAG 2790
2791 ATCACACAGGGGGCAGATGCCAAGGCTCCTGCCAAGCAACCAAGAGGAGCCACAGTAACCTTGAAGTGAAGTGAAGTGAAGTGAAGTGAAG 2880
2881 GCAGCCAGATCAGCTCAGCATCCACCACTGCTTCACACAAACCAGAGCATTTAATCAGAACTTTACCTCTGCTTGGAAAAGGCTCTGT 2970
2971 GTGACATTTCAAGTGGACACAATCTTAAAAACCTTTTTTGTGTATGGAAATAACTGCCTTGGCAAGAGACACTCATTCATCCATGCA 3060
3061 GGAAGGTTTCAAGGTTTATTTTGTTCATGTGGTGTGGCTTTGCGTGTGTGATTATTGTATTGTAATTATAATCATCTATTCCGG 3150
    
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B

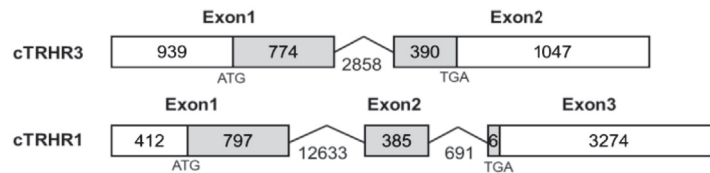


Figure 1. (A) The full-length cDNA and amino acid sequences of chicken *TRHR3*. (B) Exon organization of chicken *TRHR1* and *TRHR3* genes. Numbers in the boxes indicate the size (bp) of the coding region (shaded) and noncoding region. Numbers under the broken lines indicate the size (bp) of the introns.

Identification of *cTRHR1* and *cTRHR3* Promoter Regions

In this study, based on the cloned *cTRHR3* and *cTRHR1* 5'-UTR sequences, the potential promoter regions near or upstream exon 1 of the 2 receptor genes were cloned, and their promoter activity were detected *in vitro* using DF-1 cells.

As shown in **Figure 3A**, the promoter vector (−2003/+23Luc) containing the 5' flanking sequence of the *cTRHR3* gene exhibited strong promoter activity in DF-1 cells. Promoter deletion experiments mapped that the core promoter region of *cTRHR3* gene may be located in the −509/+23 region because the fragment near exon 1 (−180/+23Luc) did not show any promoter

activity. Similar to *cTRHR3*, the promoter vector (−2097/+127Luc) containing the 5' flanking sequence of the *cTRHR1* gene exhibited strong promoter activity in DF-1 cells, indicating that this region likely corresponds to the promoter region for this gene. After the deletion experiment, it was preliminarily determined that the core promoter of the *cTRHR1* gene is likely located in the −137/+127 region. Using online transcription factor-binding site prediction tools such as JASPAR (jaspar.genereg.net), multiple common transcription factor-binding sites were identified in the promoter region of *cTRHR1* (−509/+23) and *cTRHR3* (−371/+127) genes. As shown in **Figure 3B**, the binding sites for many transcription factors such as Sp1, AP4, SRY, and MZF1 were predicted to exist within the

				TMD1										TMD2																			
Chicken	TRHR3	1	MENGSASP-GATL----	GNQTLG-RMPRQPLELQVVT	ILLVLLVCGVGIAGNAMVVL	VVLRTRKHMVPTNC	YLVSLAVADLLVLL																										
Parrot	TRHR3	1A.S-GS.E.....II.....V.V.....C.D.....I.....																											
Lizard	TRHR3	1	...Q.WDFF.TP.GNSST...L	LDK..QL..V.....F.....I.....I.....I.....																											
Xenopus	TRHR3	1	...QTL.IGMQSINATGM.E.V.-	S.QIA.V.....S.....I.....I.....M.....I.....I.....																											
Tilapia	TRHR3	1	...NTTTFQDVSR-----	VVN.T-E..LN..E.I..	F.TM.I.....I.....																											
Mouse	TRHR2	1	...DGP.NVSLVHG-----	DTTLGLP.YK..SV.....T.....V.....TSRD.H.....L.....I.....																										
Chicken	TRHR1	1	...TGDEQNH.GL-----	LSSQEFVTA.Y.....I.....L.....V.....I.....R.....R.....M.V																										
Human	TRHR1	1	...ETV.ELNQ-----	QLQPRAVVA.Y.....II.....L.....V.....I.....M.....R.....M.V																										
				TMD3										TMD4																			
Chicken	TRHR3	80	VAGLPNISE-VVASW	VGYAGCLCITYLOYLGINISA	AWSITAF	VERYIAICHAIRAQLLCTVARAKR	ISSLWIFTSVYCVLWFW																										
Parrot	TRHR3	83K.....S.....L.....M.....																										
Lizard	TRHR3	86	A.....I.....C.....S.....K.....ACV.F.A.....M.....																										
Xenopus	TRHR3	85	A.....V.....C.....S.....K.....F.....I.....S.....K.....AFV.F.....M.....																										
Tilapia	TRHR3	80	A.....D.....F.....I.....T.....V.....S.....C.....I.....S.....K.....F.....S.....AGV.....L.....TM.....																									
Mouse	TRHR2	74	A.....V.....DSL.GH.....I.....H.....G.....F.....V.....S.....C.....L.....I.....PM.....TV.....R.....AGI.GV.....L.....L.....																									
Chicken	TRHR1	79	A.....T.....SLYK.....V.....A.....S.....F.....I.....P.....K.....F.....F.....S.....K.....IFV.S.A.....L.....																									
Human	TRHR1	77	A.....TDSIYG.....V.....A.....S.....C.....I.....P.....K.....F.....F.....S.....K.....IFV.A.....L.....ML.....																									
				TMD5										TMD6										TMD7									
Chicken	TRHR3	164	FLV	DTSQVTFSDGEQVSCGYRVSRLYMP	TYFLDFAVFYIPLGLAAVLYGL	IARILYMSPLPAAPQH-ALGSAHR-----	GGSG																										
Parrot	TRHR3	167T.....A.....I.....V.....F.....N.....TSL.SCP.M.Q-----	S.S																										
Lizard	TRHR3	170I.....AV.....MK.....N.....T.....V.....T.....F.....N.....S.....GGRP.K.SSI-----	STSS																									
Xenopus	TRHR3	169I.TE.K.A.V.N.....N.....T.....T.....I.....V.....T.....F.....N.....SN.DLSRM.SKHYG--	KPYN																										
Tilapia	TRHR3	164DET.VYTN.VV.T.....TL.....I.V.T.....F.....SHLNDR.G.GSVH-----	Q.H																									
Mouse	TRHR2	159LN--VR.NQRLE.....K.....G.....L.....L.....FIA.LGTL.....F.G.....FO.....SQEAWQKERO.HGQ-----	SEG																									
Chicken	TRHR1	164L.LNIAVYK.TTV.....K.....Y.S.....MM.GI.....L.MV.T.....FLN.I.SD.KENSNTWKNDMAQQNKTVN																										
Human	TRHR1	162L.LNIS.YK.AIVI.....KI.NY.S.....LM.G.....V.MI.T.....F.....FLN.I.SD.KENSNTWKNDSTHQTNLN																										
Chicken	TRHR3	242	LQSCQGRSGLTSSRRKQVTKM	LAVVVVLFALLWLPYRTLTVV	VNSFVDP	PYLNIFLLFCRMCYILNSAINPIIYSLMS	QRFRAAF																										
Parrot	TRHR3	246	..KL..R.N.AA.....M.....M.....L.....K.....																										
Lizard	TRHR3	253	MKL..R.NKSA.....I.....M.....M.....V.....L.....NV.....K.....																										
Xenopus	TRHR3	252	IKL.GK.NKN.A.....I.....M.....M.....V.....V.....L.....N.....K.....																										
Tilapia	TRHR3	243	SSTTNKANK.AV.A.....I.....I.....M.....I.....H.T.....T.....N.....K.....V.....																										
Mouse	TRHR2	235	TPGN.SR.KSSM.....A.R.....I.....L.....V.....T.....LL.LAR.F.DP.V.....T.V.T.....V.....K.....																									
Chicken	TRHR1	249	SKMTNKSFNS.IA.R.....F.....M.....LSS.FQEN.....I.....V.....N.....K.....																									
Human	TRHR1	247	VNT.NRCFNS.V.....I.....M.....LSS.FQEN.....I.....V.....N.....K.....																									
Chicken	TRHR3	327	RKLCRCGWKSTEVPTPRSPVPLYSRAKDGSGSSEHG-THQDD	LNGPPAPVRSRAAMHTFGP-----																													
Parrot	TRHR3	331	...K.EA.A.N.VSYHN..Y..	VT..HFHD..N.N-.E.E.SIL.V.AKK-----																													
Lizard	TRHR3	338	K...K.ES.R..ST.QF.A.F..	TM..Y.HD...DI.EHE...Y.SVA.K----																													
Xenopus	TRHR3	337	KN..K.EQ.R..KAAKYN...Y..	VM..S.HE.PD.DV.V.E...F..																													
Tilapia	TRHR3	328	K...K.P.G--HREAEN..MY..	VM.NSTHECN.PV-.E.EEVS																													
Mouse	TRHR2	320	L...W.RAAGSQRAACAPTSN.A.	QET.L.TEKMQLGSRVSGPAA.ASLRCQ																													
Chicken	TRHR1	334	...N.HL.RDKK.ANY..ALN.NVI.	ESDHF...IEDITVTNT-YLSSAKT.IGDTCL																													
Human	TRHR1	332	...N.KQ.P..K.ANY..ALN..VI.	ESDHF.T.LDDITVTT-T-YLS.TKV.FDDTCL																													

Figure 2. Amino acid alignment of chicken TRHR3 (MK138989) with parrot TRHR3 (XP_005146871.1), lizard TRHR3 (XP_003220685.1), *Xenopus tropicalis* TRHR3 (XP_002933212.1), tilapia TRHR3 (XP_003439144.1), mouse TRHR2 (NP_573465.1), chicken TRHR1 (NP_990261.1), and human TRHR1 (NP_003292). The seven transmembrane domains (TMD1-7) are shaded. The ERY motif is boxed, and the 4 conserved amino acid residues (Tyr¹⁰⁸, Asn¹¹², Tyr²⁷⁷, Arg³⁰¹) associated with ligand binding are marked with black arrows. Note: dots indicate the amino acids identical to chicken TRHR3, and dashes denote the gaps in the sequence.

core promoter region of *cTRHR3*, while the binding sites for STAT3, CEBP α , SRY, and Sp1 were predicted to exist within the core promoter region of *cTRHR1*.

Functional Analysis of *cTRHR3* and *cTRHR1*

In mammals, it was reported that TRHR can couple to G_{q/11} protein upon ligand activation and cause changes in intracellular calcium levels and activate the MAPK/ERK1/2 signaling pathway (Sun et al., 1998; Kanasaki et al., 1999). In this study, the signaling pathways coupled to the newly cloned receptor TRHR3 in chickens were first investigated using the pGL3-NFAT-RE-luciferase reporter system, pGL4-SRE-luciferase reporter system, and pGL3-CRE-luciferase reporter system. In addition, chicken TRHR1 and duck TRHR1 (dTRHR1, MN561700) were examined in parallel to compare their functional similarity and difference with *cTRHR3*.

Using the pGL3-NFAT-RE-luciferase reporter assay, we demonstrated that chicken TRHR3 expressed in HEK 293 cells can be activated by cTRH potently with an EC₅₀ value of 1.23 nM, clearly indicating that

cTRHR3 is a functional receptor for TRH and its activation can elevate intracellular Ca²⁺ concentration (Table 1). Furthermore, 2-aminoethoxydiphenyl borate (an inhibitor of inositol triphosphate receptor) could inhibit this induced luciferase activity, supporting that similar to chicken TRHR1, TRHR3 is likely coupled to G_q protein and its activation can induce intracellular calcium mobilization. Using the pGL4-SRE-luciferase reporter assay and Western blot, we also elucidated that TRHR3 activation can activate downstream MAPK/ERK signaling cascade. In contrast, TRHR3 activation cannot stimulate cAMP/PKA signaling pathway at any concentration tested, as monitored by the pGL3-CRE-luciferase reporter system (Figure 4, Table 1).

Similar to TRHR3, TRHR1 activation can stimulate intracellular Ca²⁺ mobilization and MAPK/ERK signaling pathways (Table 1), as monitored by the 2 respective cell-based luciferase reporter systems and Western blot. Interestingly, unlike *cTRHR3*, *cTRHR1* activation can stimulate adenylyl cyclase/cAMP/PKA signaling pathway in the pGL3-CRE-luciferase reporter assay, and this stimulatory effect could be inhibited by either an adenylyl cyclase inhibitor (MDL-12330A) or a PKA inhibitor (H89) (Figure 5). To further elucidate the functions of bird TRHR1, we

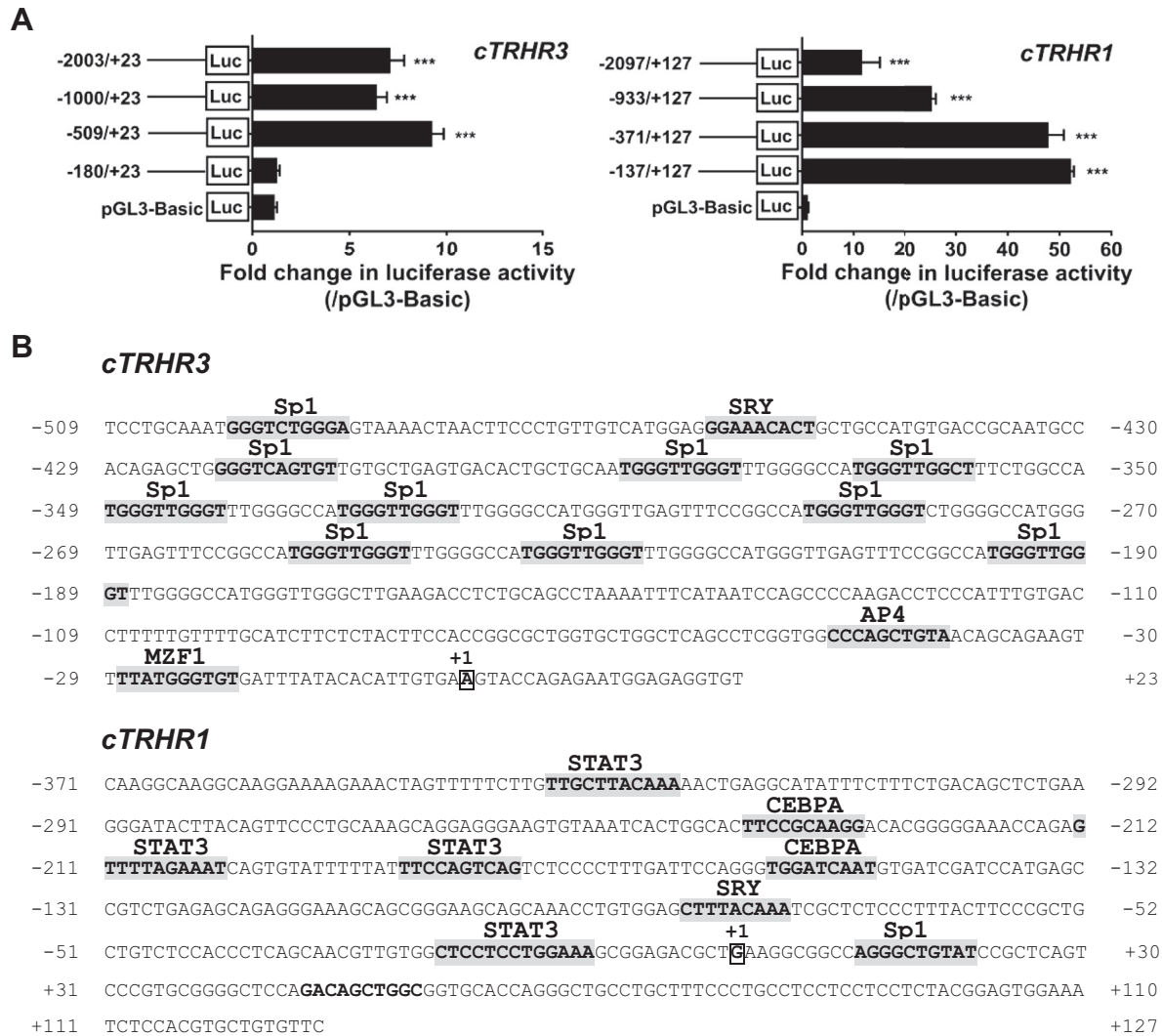


Figure 3. (A) Detection of promoter activities of the 5'-flanking region of chicken *TRHR1* and *TRHR3* in cultured DF-1 cells. Various stretches of the 5'-flanking regions of *TRHR1* and *TRHR3* were cloned into pGL3-Basic vector for the generation of multiple promoter-luciferase constructs. The promoter-luciferase vector and pRL-TK vector were cotransfected into DF-1 cells, and the promoter activities were detected by the dual-luciferase reporter system. All data represent the mean \pm SEM of 4 replicates ($N = 4$). *** $P < 0.001$ vs. pGL3-Basic vector. The transcriptional start site (A/G, boxed) identified by rapid amplification of 5' cDNA ends (5'-RACE) was designated as '+1'. (B) Predicted transcription factor binding sites in the promoter regions of *cTRHR1* and *cTRHR3*. The corresponding binding sites are shaded. Sp1, specificity protein 1; SRY, sex-determining region; AP4, activating enhancer-binding protein 4; MZF1, myeloid zinc finger 1; STAT3, signal transducer and activator of transcription 3; CEBPA, CCAAT/enhancer-binding protein alpha.

cloned and performed the same test with duck *TRHR1*. The results showed that d*TRHR1* can activate multiple signaling pathways including calcium mobilization, MAPK/ERK pathways, and cAMP/PKA pathways (Table 1) similar to *cTRHR1* (Supplementary Figure 2), supporting that d*TRHR1* is a functional receptor as well. For easy reference, a schematic figure is included to show the similarities and differences of the downstream signaling pathways of *cTRHR1* and *cTRHR3* when activated by *cTRH* (Figure 6).

Tissue Expression of *cTRH*, *cTRHR1*, and *cTRHR3*

To investigate the tissue expression of *TRH* and its receptors *cTRHR1* and *cTRHR3* in chickens, quantitative real-time PCR was used to detect their mRNA

expression in adult chickens. As shown in Figure 7, within the CNS, *cTRH* is highly expressed in the hypothalamus, moderately expressed in the hind-brain, and weakly expressed in other remaining brain regions. In peripheral tissues, only the testes showed a high expression level of *TRH*. *cTRHR1* has the highest expression level in the anterior pituitary and moderate expression levels in other brain regions. In peripheral tissues, besides moderate expression level in the fat, muscle, skin, testes, and intestine, only weak expression of *cTRHR1* was detected in other remaining peripheral tissues. Unlike *cTRHR1*, *cTRHR3* has a relatively higher expression level in the muscle, pituitary, skin, spinal cord, midbrain, cerebellum, hindbrain, and hypothalamus, while *cTRHR3* is moderately or weakly expressed in other remaining tissues.

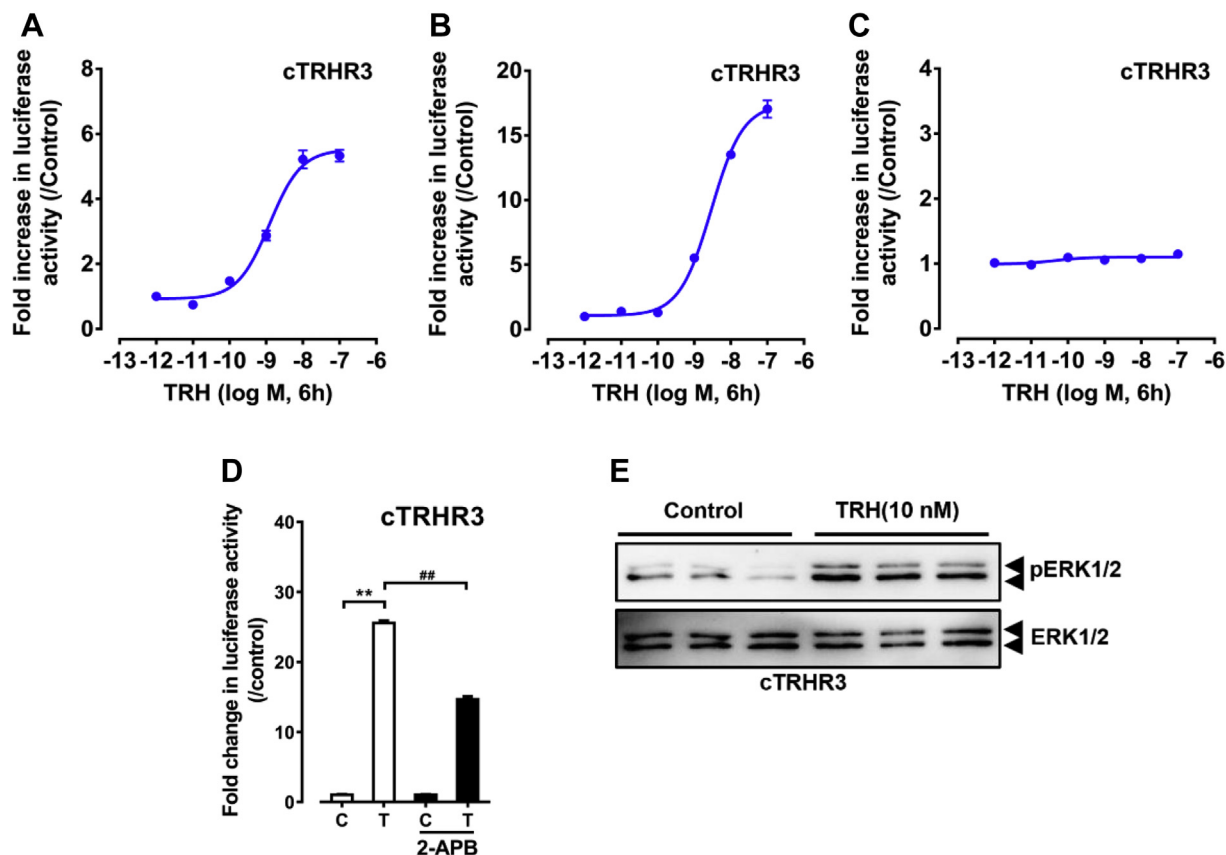


Figure 4. (A–C) Effects of chicken thyrotropin-releasing hormone (TRH) on activating chicken TRHR3, as monitored by the (A) pGL3-NFAT-RE-luciferase reporter system, (B) pGL4-SRE-luciferase reporter system, and (C) pGL3-CRE-luciferase reporter system. All data represent the mean \pm SEM of 3 replicates ($N = 3$). (D) Effects of 2-aminoethoxydiphenyl borate (2-APB) on TRH-induced luciferase activities of HEK 293 cells expressing chicken TRHR3, as monitored by the pGL3-NFAT-RE-luciferase reporter system. 2-APB (100 μ M) was added 0.5 h before TRH treatment. T represents TRH treatment (10 nM, 6 h), and C represents control group without TRH treatment. All data represent the mean \pm SEM of 3 replicates ($N = 3$). ** $P < 0.01$ vs. control; ## $P < 0.01$ between 2 treatment groups. (E) Western blot detection of ERK1/2 phosphorylation (pERK1/2) and total ERK1/2 in HEK 293 cells expressing cTRHR3 treated by cTRH (10 nM) and without TRH (control) for 10 min.

DISCUSSION

In this study, the full-length cDNA sequence of *TRHR3* was cloned from chickens. Cell-based luciferase reporter assays revealed that cTRHR3 is a novel functional receptor of TRH, and it has a signaling property similar, but not identical, to that of cTRHR1 or duck TRHR1. Quantitative real-time PCR assays showed that both cTRHR3 and cTRHR1 are widely distributed among chicken tissues but that their tissue expression is differentially regulated, probably controlled by their promoters mapped upstream of their respective exon 1. To our knowledge, our study represents the first to

characterize the functionality and tissue expression of TRHR3 in avian species.

Structure of cTRHR1 and cTRHR3 and Phylogenetics of Vertebrate TRHR Family

Although 2 *TRHRs* (*TRHR1* and *TRHR3*) have been predicted from avian genome, the full-length cDNAs of *TRHR3* has not been cloned in any avian species. Here, we cloned *cTRHR3* from chickens. Sequence comparison reveals that both cTRHRs show considerable conservation in structure, for instance, completely retaining the 4 amino acid residues that were proposed

Table 1. EC₅₀ values of cTRH in activating different signaling pathways in HEK 293 cells transfected with chicken TRHR1 and TRHR3 and duck TRHR1.

EC ₅₀ values (nM)			
Receptors	Calcium signaling pathway	MAPK/ERK signaling pathway	cAMP/PKA signaling pathway
cTRHR3	1.23	2.91	-
cTRHR1	0.22	4.50	0.30
dTRHR1	0.13	2.14	0.55

- means that cTRHR3 may not couple to this signaling pathway.

Abbreviations: cTRH, chicken thyrotropin-releasing hormone; EC₅₀, half maximal effective concentration.

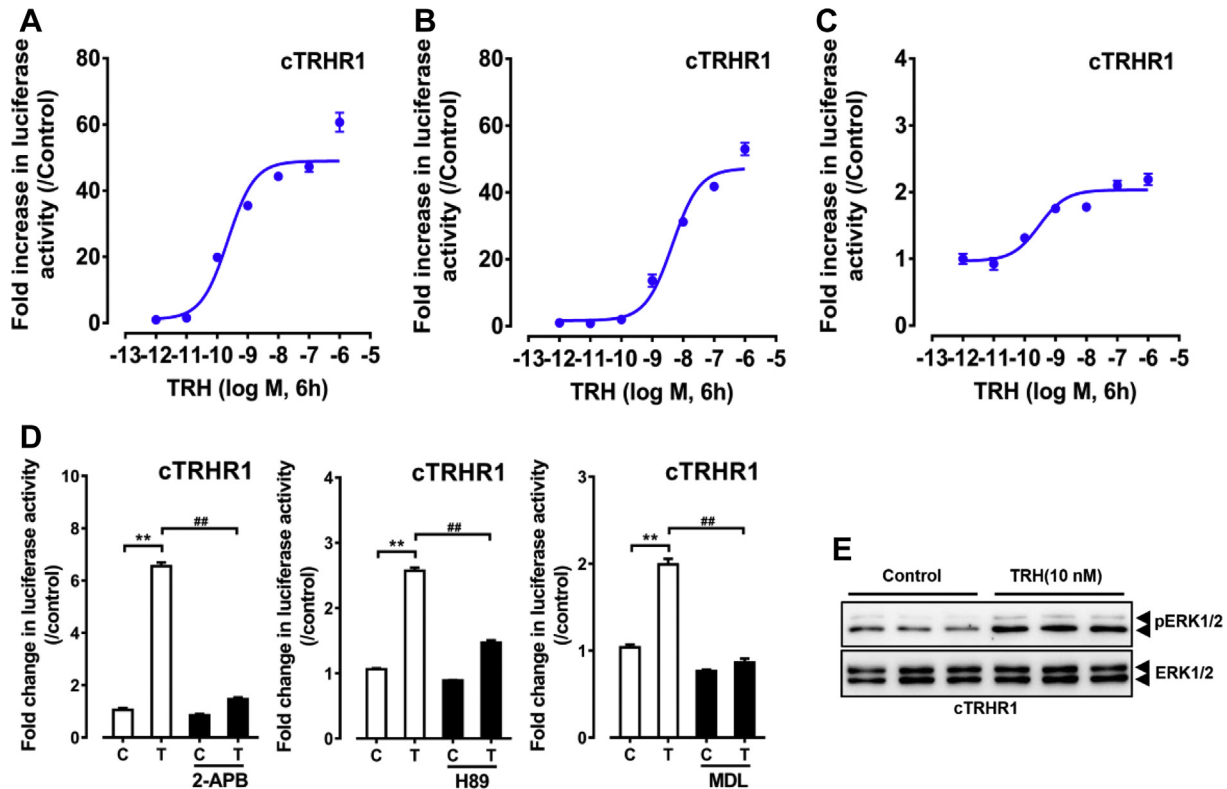


Figure 5. (A–C) Effects of chicken thyrotropin-releasing hormone (TRH) on activating chicken TRHR1, as monitored by the (A) pGL3-NFAT-RE-luciferase reporter system, (B) pGL4-SRE-luciferase reporter system, and (C) pGL3-CRE-luciferase reporter system. All data represent the mean \pm SEM of 3 replicates ($N = 3$). (D) Effects of 2-aminoethoxydiphenyl borate (2-APB), H89, and MDL-12330A (MDL) on TRH-induced luciferase activities of HEK 293 cells expressing chicken TRHR1, as monitored by the pGL3-NFAT-RE-luciferase reporter system and pGL3-CRE-luciferase reporter system. 2-APB (100 μ M), H89 (10 μ M) or MDL (20 μ M) was added 0.5 h before TRH treatment. T represents TRH treatment (10 nM, 6 h), and C represents control group without TRH treatment. All data represent the mean \pm SEM of 3 replicates ($N = 3$). ** $P < 0.01$ vs. control; ### $P < 0.01$ between 2 groups. (E) Western blot detection of ERK1/2 phosphorylation (pERK1/2) and total ERK1/2 in HEK 293 cells expressing cTRHR1 treated by TRH (10 nM) and without TRH (control) for 10 min.

to be important for binding TRH (i.e., Tyr¹⁰⁸, Asn¹¹², Tyr²⁷⁷, Arg³⁰¹). These sites are thought to facilitate ligand binding by altering the conformation of TRHRs to form a more affinitive structure (Sun et al., 1998). TRH was found to initially bind with low affinity to the extracellular region of TRHR, and then the conformation of ligand–receptor complex changes so that TRH binds to the transmembrane region of TRHR with a higher affinity, thus allowing the transduction of downstream signals (Engel and Gershengorn, 2007).

We found that the number of *TRHR* subtypes varies greatly among vertebrate species, despite their generally conserved structure. To date, only *TRHR1* has been identified in the majority of mammalian species including humans, while *TRHR2* has only been found in a few mammals such as rats (Duthie et al., 1993; Itadani et al., 1998). In chickens, only *TRHR1* and *TRHR3* have been identified. By contrast, in lower vertebrates such as amphibians and teleosts, all 3 *TRHR* subtypes have been found in the majority of these species (Harder et al., 2001; Mekuchi et al., 2011). Taken into account the proposed theory on evolutionary history of vertebrate species and our phylogenetic and synteny analyses on *TRHR* subtypes shown in Supplementary Figure 1, we hypothesize that multiple gene loss events might have occurred for

different subtypes of *TRHR* in various species during evolution (Kumar and Hedges, 1998; International Chicken Genome Sequencing Consortium, 2004). *TRHR2* was likely lost during the evolution of birds as well as many mammals, and *TRHR3* might be lost

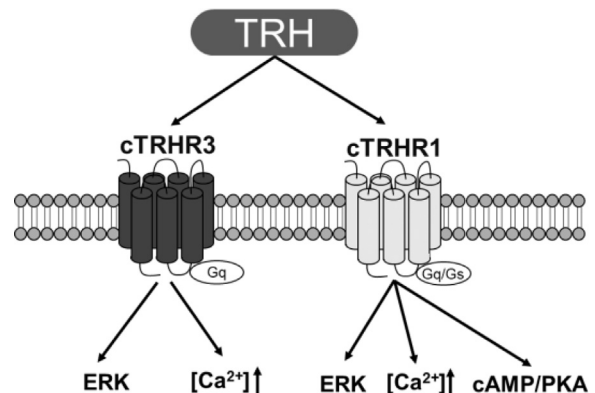


Figure 6. Schematic diagram showing the similarities and differences of the downstream signaling pathways of cTRHR1 and cTRHR3 when activated by thyrotropin-releasing hormone (TRH). Both receptors can couple to Gq protein, and their activation stimulates intracellular calcium mobilization and MAPK/ERK signaling pathway. Unlike cTRHR3, cTRHR1 can also couple to Gs protein and stimulate adenylate cyclase/cAMP/PKA signaling pathway.

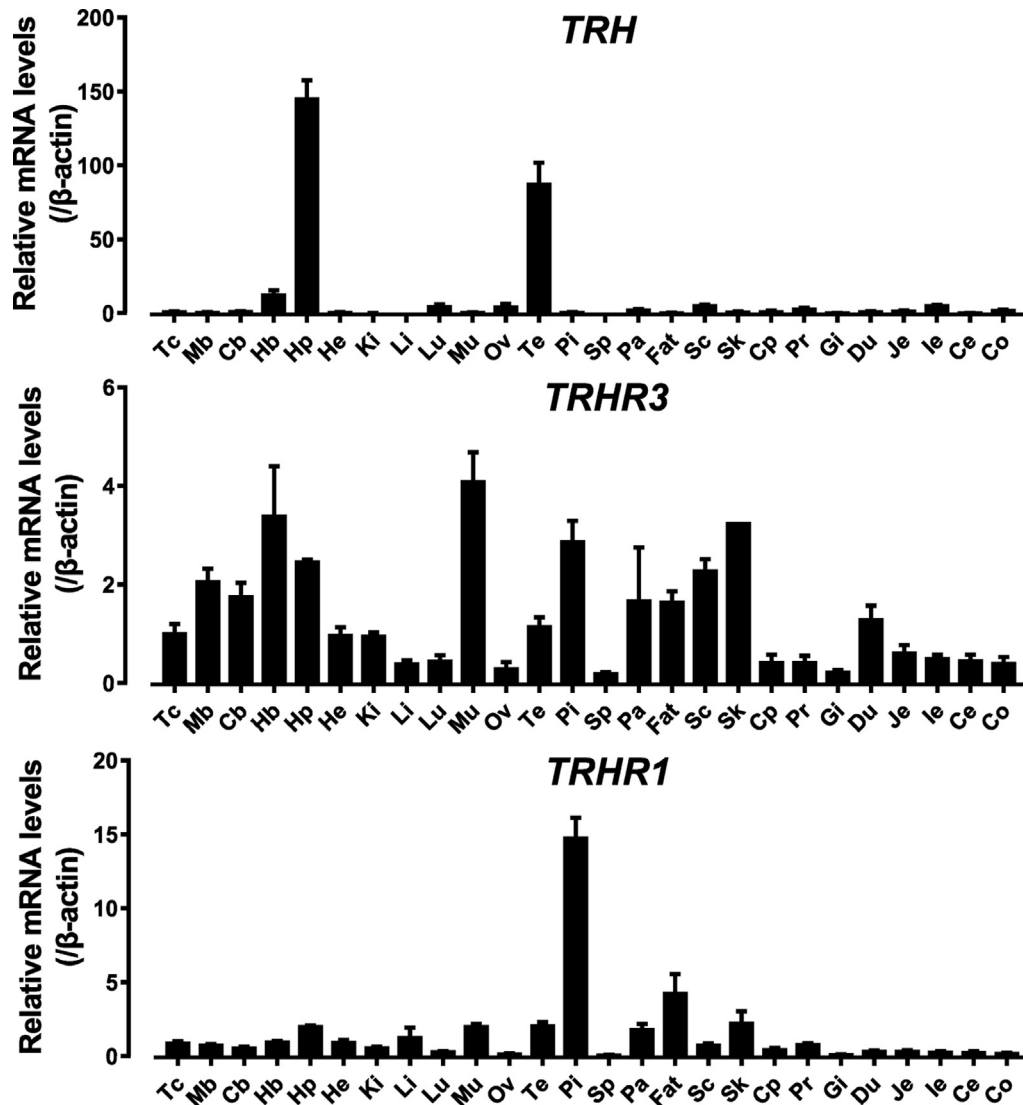


Figure 7. Quantitative real-time PCR assay of *cTRH*, *cTRHR3*, and *cTRHR1* mRNA expression in adult chicken tissues, including the telencephalon (Tc), midbrain (Mb), cerebellum (Cb), hindbrain (Hb), hypothalamus (Hp), heart (He), kidneys (Ki), liver (Li), lung (Lu), muscle (Mu), ovary (Ov), testes (Te), anterior pituitary (Pi), spleen (Sp), pancreas (Pa), subcutaneous fat (Fat), spinal cord (Sc), skin (Sk), duodenum (Du), crop (Cp), proventriculus (Pr), gizzard (Gi), jejunum (Je), ileum (Ie), cecum (Ce), and colon (Co). The mRNA level of each gene was normalized with the mRNA level of β -actin as an internal control and expressed as the fold difference compared with that of telencephalon (Tc). All data represent the mean \pm SEM of 6 individual adult chickens (3 males and 3 females) ($N = 6$).

during the evolution of mammals, eventually giving rise to the current repertoire of *TRHR* subtypes in vertebrates.

Both *cTRHR3* and *cTRHR1* are Functional Receptors for TRH in Chickens

Although the functionality of *TRHR1* has been reported in a variety of species including mice (Gershengorn and Osman, 1996), chickens (Sun et al., 1998), and *X. laevis* (Bidaud et al., 2002), little is known about the function of its homologous receptor subtype, *TRHR3*. In previous studies, TRH treatment can increase inositol triphosphate levels of COS-1 cells transfected with chicken/mouse *TRHR1* (Sun et al., 1998), and thus, *TRHR1* has been proved to couple to $G_{q/11}$ and it can activate intracellular signaling pathways,

such as activating phospholipase C signaling pathway and inducing intracellular calcium mobilization. In *X. laevis*, when TRH was applied to HEK 293 cells expressing x*TRHR1*, an increase in intracellular Ca^{2+} levels was detected (Bidaud et al., 2002). In our study, we found that similar to mouse *TRHR1*, chicken *TRHR1* is likely coupled to G_q protein and its activation causes intracellular calcium mobilization and activates the MAPK/ERK signaling pathway, as monitored by luciferase reporter assays and Western blot. Interestingly, we also found an increase in pGL3-CRE-luciferase signal in HEK 293 cells transfected with *TRHR1* after TRH treatment, suggesting that *TRHR1* may also act through the G_s protein which activates the cAMP/PKA signaling pathway. Our finding contrasts a previous report in chickens, in which *TRHR1* activation fails to increase the intracellular cAMP levels. This discrepancy is perhaps due to the different experimental approaches

used. Nevertheless, our finding in chickens actually concurs with previous report in mammals, in which TRHR1 was found to be coupled to G_s protein in addition to G_q -phospholipase C and able to stimulate adenylate cyclase in rat GH3 cells and contribute to the function of TRH in stimulation of GH release (Paulssen et al., 1992).

Compared with TRHR1, the studies on TRHR3 have been very limited. In this study, we took the first step in characterizing the function of *cTRHR3* and found that after TRH treatment of HEK 293 cells transfected with *cTRHR3*, an increase in intracellular Ca^{2+} concentration and the activation of the MAPK/ERK signaling pathway were detected. This indicates that chicken TRHR3 is a new functional receptor for TRH. However, this should be noted that *cTRHR3* cannot stimulate cAMP/PKA signaling pathway, which suggests that the functionality of the 2 *cTRHR* receptors is similar but distinct from each other. Our finding, for the first time, indicates that both TRHR1 and TRHR3 can mediate the biological actions of TRH in birds. This contrasts a previous report in *X. laevis*, in which TRHR3 cannot be activated by TRH effectively, although the other 2 TRH receptors (TRHR1 and TRHR2) can (Bidaud et al., 2002; Lu et al., 2003). Interestingly, in bull frogs, TRHR3 is reported to be a functional receptor for TRH and its activation can increase intracellular calcium level (Nakano et al., 2018). Moreover, TRHR3 is the major receptor subtype expressed in the anterior pituitary and found to be colocalized with PRL cells, which may mediate the PRL-stimulating effect of TRH in this species (Lu et al., 2003; Nakano et al., 2018). These findings strongly suggest that TRHR3 may play distinct roles in different vertebrate classes.

Tissue Expression of TRH, TRHR1, and TRHR3 in Chickens

In this study, we found that *cTRH* is widely expressed in chicken tissues examined with the highest expression level noted in the hypothalamus and testes. In amphibians (Lamacz et al., 1989), rodents (Segerson et al., 1987), and birds (Péczy et al., 1988; Vandenberg et al., 2005; Aoki et al., 2007), *TRH* and its precursors are found to be highly expressed in multiple nuclei of the hypothalamus including the supraoptic nucleus, paraventricular nucleus, lateral hypothalamic nucleus, and arcuate nucleus. This highly conserved expression profile of *TRH* across species in the hypothalamic regions hints that TRH plays an important regulatory role therein, for instance, as a major regulator of TSH synthesis and secretion and secretion of other pituitary hormones such as PRL and GH (Scanes, 1974; Harvey et al., 1978; Harvey, 1990). In addition, the high expression of *cTRH* in the testis suggests that as in mammals, *cTRH* may regulate testis function (e.g., testosterone secretion) as an autocrine/paracrine factor in chicken testes (Wilber and Xu, 1998; Geris et al., 2000).

On the other hand, *cTRHR1* and *cTRHR3* were found to be widely, but differentially, expressed in chicken tissues, and their expression is likely controlled by their promoters upstream of respective exon 1, which contains multiple transcription factor-binding sites. *cTRHR1* was detected to be preferentially expressed in the anterior pituitary and weakly or moderately expressed in peripheral tissues such as skin, testis, fat, and muscle. This is consistent with reports in other species, suggesting that *cTRHR1* plays important roles in mediating TRH actions on chicken pituitary, such as involvement in the regulation of TSH, GH, and PRL secretion (Scanes, 1974; Harvey et al., 1978; Harvey, 1990). It was reported that in frogs and humans, TRH acts on the skin to regulate wound healing (Meier et al., 2013). In humans, TRH and TRHR1 are coexpressed in hair follicles to regulate hair growth (Gaspar et al., 2010) and have physiological functions that regulate melanin synthesis (Gaspar et al., 2011). The relatively high expression of *TRHR1* in chicken testes is consistent with the findings in rats (Satoh et al., 1994). In rat testes, TRHR1 expression levels were detected to be approximately 10% of that in the pituitary gland, which were specifically expressed in mesenchymal cells and shown to regulate testosterone secretion (Wilber and Xu, 1998). Both TRH and TRHR1 genes are highly expressed in porcine fat tissue, which may suggest a paracrine or autocrine role of TRH in this tissue (Jiang et al., 2012). A genome-wide association study on lean body mass variation in humans also identified the functional relevance of TRHR1 in muscle metabolism (Liu et al., 2009). The question whether TRHR1 signaling in chicken plays roles similar to that found in mammals awaits further investigation.

Unlike *cTRHR1*, *cTRHR3* is widely expressed in nearly all tissues examined with a relatively high expression level in various brain regions. This finding, together with the wide distribution of *cTRH* in chicken tissues, suggests that TRH-TRHR3 signaling may play important autocrine and paracrine roles in the CNS and peripheral tissues such as in testes. Similarly, in *Oryzias latipes*, *TRHR3* was detected by RT-PCR to be widely expressed in various tissues examined, including the anterior pituitary, brain, testes, ovary, kidneys, spleen, various parts of the gastrointestinal tract, and skin (Mekuchi et al., 2011). In *X. laevis*, *TRHR3* expression was detected in tissues such as the brain, heart, and stomach (Bidaud et al., 2002). These findings fall in line with the hypothesis that TRHR3 signaling plays diverse roles in both the CNS and peripheral tissues in nonmammalian vertebrates. The differential expression of both receptors in various chicken tissues, together with the wide tissue expression of *cTRH*, lead us to hypothesize that TRH plays a wide range of role in chickens and that its action is likely mediated by either TRHR1 or TRHR3, or both receptors in chicken tissues.

In summary, we identified and characterized a new chicken TRH receptor, TRHR3. Our results confirmed that similar to *cTRHR1*, *cTRHR3* is a functional receptor and is able to mediate TRH function through the

intracellular G_q protein-related signaling pathway. Furthermore, we found that both TRH receptors are widely, but differentially, expressed in chicken tissues. These findings, together with the wide expression of TRH in chicken tissues, not only support TRH-TRHR1 signaling plays a role in the regulation of pituitary hormone expression and secretion but also suggest that TRH may play diverse roles in extrapituitary tissues such as the testes, hypothalamus, fat, skin, and spinal cord, via TRHR1 and/or TRHR3 in birds.

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SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.psj.2019.10.062>.

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