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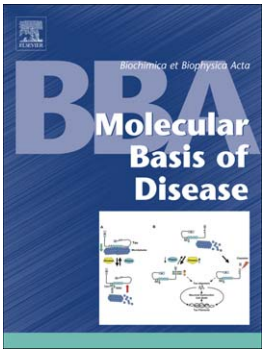

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Untranslated regions of thyroid hormone receptor beta 1 mRNA are impaired in human clear cell renal cell carcinoma

Running title: TRβ1 mRNA UTR in kidney cancer.

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Abstract

Thyroid hormone receptor β1 (TRβ1) is a hormone-dependent transcription factor activated by 3,5,3'-L-triiodothyronine (T3). TRβ1 functions as a tumor suppressor and disturbances of the *THRB* gene are frequent findings in cancer. Translational control mediated by untranslated regions (UTRs) regulates cell proliferation, metabolism and responses to cellular stress, processes that are involved in carcinogenesis. We hypothesized that reduced TRβ1 expression in clear cell renal cell cancer (ccRCC) results from regulatory effects of TRβ1 5' and 3'UTRs on protein translation. We determined TRβ1 expression and alternative splicing of TRβ1 5' and 3'UTRs in ccRCC and control tissue together with expression of the type 1 deiodinase enzyme (coded by *DIO1*, a TRβ1 target gene). Tissue concentrations of T3 (which are generated in part by D1) and expression of miRNA-204 (an mRNA inhibitor for which a putative interaction site was identified in the TRβ1 3'UTR) were also determined. TRβ1 mRNA and protein levels were reduced by 70% and 91% in ccRCC and accompanied by absent D1 protein, a 58% reduction in tissue T3 concentration and 2-fold increase in miRNA-204. Structural analysis of TRβ1 UTR variants indicated that reduced TRβ1 expression may be maintained in ccRCC by posttranscriptional mechanisms involving 5'UTRs and miRNA-204. The tumor suppressor activity of TRβ1 indicates that reduced TRβ1 expression and tissue hypothyroidism in ccRCC tumors is likely to be involved in the process of carcinogenesis or in maintaining a proliferative advantage to malignant cells.

Key words: translational control in cancer, untranslated regions, miRNA, thyroid hormone receptor beta 1, thyroid hormone, renal cancer, ccRCC.

1. Introduction

The thyroid hormones, T3 (3,5,3'-L-triiodothyronine) and T4 (thyroxine), regulate key cellular processes including differentiation, proliferation, apoptosis and metabolism. Thyroid hormone action is mediated via the thyroid hormone receptors TRα1 and β1, which are expressed widely and act as T3-inducible transcription factors [1]. TRs bind specific thyroid hormone response elements (TREs) in T3 responsive genes and regulate expression of target genes including oncogenes [2] and tumor suppressors [3]. Human *THRB* gene contains 10 exons (Fig.1A) with an open reading frame extending from exon 3 to exon 10. Previous studies indicate that TRβ1 expression is perturbed in tumors, including clear cell renal cell cancer (ccRCC) [4]. Several mechanisms have been proposed to contribute to reduced TRβ1 expression in cancer cells. Examples include hypermethylation of the TRβ1 promoter and loss of heterozygosity (LOH) at the *THRB* locus [5] and mutations found in TRβ1 transcripts [6]. Although all these mechanisms may lead to reduced transcript expression, aberrant TRβ1 mRNA expression correlates poorly with protein expression in ccRCC [4], suggesting that TRβ1 translation in human renal carcinomas may involve post-transcriptional regulation.

The final concentration of expressed protein depends on its translation efficiency and stability. Since initiation of translation is a rate-limiting step [7], factors influencing this process limit the efficiency of protein synthesis. Translation initiation events are controlled by secondary structures of UnTranslated Regions (UTRs) within mRNAs. The UTRs contain cis-acting sequences recognized by trans-acting factors including translation initiation and elongation factors and microRNAs [8]. Alternative splicing of the TRβ1 mRNA 5'UTR results in expression of multiple 5'UTR variants [9, 10]. Expression of these variants is tissue-specific, and previous studies indicated they differentially regulate the efficiency of protein translation [10]**.** Similarly, cis-acting elements in the 3'UTR of TRβ1 may influence the efficiency of protein translation [11].

It is well-known that UTRs can be involved in carcinogenesis. For instance, mammary gland BRCA1 suppressor is characterized by a short, efficiently translated 5'UTR variant. In some breast cancers the BRCA1 transcripts posses a longer structured UTR containing upstream open reading frames (uORFs) resulting in reduced BRCA1 protein synthesis [12]. Moreover, tumor cells expressing a shorter, readily translated variant of the retinoic acid receptor RARβ2 5'UTR exhibited greater sensitivity to retinoic acid in comparison to cells expressing a longer 5'UTR variant less efficiently translated [13,14].

Although the protein coding region of TRβ1 has been extensively investigated in various tumor cell types, there is little information regarding UTR-dependent control of the efficiency of TRβ1 translation. In these studies we hypothesized that aberrant TRβ1 protein expression in ccRCC results from reduced levels of TRβ1 mRNA, alternative splicing of 5' and 3'UTR TRβ1 mRNA and altered expression of miRNAs that regulate TRβ1 translation. To address these possibilities we characterized TRβ1 mRNA and protein expression and alternative splicing of TRβ1 UTRs in ccRCC and control tissue as well as the levels of the type 1 deiodinase enzyme (D1, a TRβ1 target gene) and the tissue concentrations of T3 and T4, which are regulated by activity of the D1 enzyme. Finally, we investigated the possible role of mRNA UTRs in the control of TRβ1 protein translation.

2. Materials and Methods

2.1. Tissue samples were obtained from nephrectomies performed on patients with clear cell renal cell cancer (n=39) or non-neoplastic abnormalities (nephrolithiasis, hydronephrosis, injury, n=10) under permission of the Ethical Committee of Human Studies (The Medical Centre of Postgraduate Education). Samples were divided into three groups: cancer tissue (n=39, T) and two control tissues: paired normal tissue from the opposite pole of the malignant kidney with no histological evidence of tumor (n=39, C) and tissue from kidney removed for non-neoplastic disease (n=10, N). The aim of using controls "N" was to clearly separate cancer-specific molecular changes from those resulting from non-cancerous pathologies. Clear cell renal cell cancer was diagnosed according to WHO criteria [15].

2.2. Plasmids and transient transfection assays: TRβ1 5'UTR variants A-G pGL3 constructs were described previously [10] pGL3-F1 was constructed using HindIII/NcoI cloning of PCR amplified F1 variant (primers p1d1F and pex3R-PscI, Supplementary Data Table-3).

Caki-2 cells (clear cell renal cell cancer, American Type Culture Collection, Manassas, VA) were seeded at $5x10^5$ cells per well and after 24 hours were transfected with 100 ng pRL-TK and 1 µg of pGL-5'UTR vectors, using 1 µg/ul PEI (Linear Polyethylenimine, Cat. No. 23966-2, Polysciences Inc., Warrington, PA) and 150 mM NaCl in FBS-free McCoy's medium (Gibco/Invitrogen, Carlsbad, Ca). Five hours after transfection, the medium was replaced with medium plus 10% FBS (Sigma-Aldrich, Saint Louis, MO) and penicillin-streptomycin solution (Sigma-Aldrich, Saint Louis, MO). Cells were grown for 24 hrs at 37°C in 5% CO_2 and harvested for dual-luciferase assay (Promega, Madison, WI) using a Synergy2 luminometer (BioTek, Winooski, VT). All transfections and luciferase assays were performed in triplicate.

For transfection with miRNA precursors, Caki-2 cells were plated at 5×10^5 cells per 12-well dish and transfected 24 h later using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Ca) following manufacturer's protocol. Each transfection reaction contained 37.5 nM of precursor miRNA-204 (PremiR™ miRNA Precursor Molecule, Ambion, Foster City, CA). As control, transfection with scrambled microRNA (Negative microRNA Control, Ambion, Foster City, CA) was performed. Cells were harvested 48 h after transfection for total RNA isolation for real-time RT-PCR and 72 h after transfection for protein isolation.

Coupled *in vitro* transcription and translation assay: *In vitro* protein synthesis was performed from pKS derived plasmids: UTR_A+Luc, TRβ1 ORF+Luc, UTR_A+ TRβ1 ORF+Luc and Control plasmid. Control plasmid was constructed by cloning LUC gene downstream of the T7 promoter in pKS vector [10] and served as a basis for construction of other plasmids used in the study: 1) UTR_A+Luc (THRB UTR-A cloned upstream of luciferase in pKS vector), TRβ1 ORF+Luc (TRβ1 ORF cloned upstream of luciferase in pKS vector), UTR_A+ TRβ1 ORF +Luc (5'UTR A inserted upstream of TRβ1ORF and luciferase reporter gene). Reaction was conducted in RTS 100 Wheat Germ CRCF system (Roche Diagnostics, Mannheim, Germany) in conditions recommended by the manufacturer, using 2 μg of each plasmid.

2.3. RNA and miRNA isolation and reverse transcription: RNAs were isolated from ~100 mg of frozen tissue using GeneMATRIX Universal RNA Purification Kit (EURx, Gdansk, Poland) or mirPremier microRNA Isolation Kit (Sigma-Aldrich, St. Louis, MO). Reverse transcription was performed using RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and 200 ng total RNA with Random Hexamer primers, or 30 ng miRNA and specific stem-loop primers with 5'-overhangs (Supplementary Data, Table-1).

2.4. Real-time PCR:

Quantitative real-time PCR of UTRs was performed using QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) under conditions: 95°C, 5 min; 50 cycles: 95°C, 10 s, 60°C, 30 s; melting curve analysis: 135 cycles: 50°C; 0.3°C increase in each cycle. Ct data were

acquired after reaching the threshold in real-time module, usually between 18 and 36 cycle; cycle efficiency was corrected using iQ5 thermocycler software (Bio-Rad, Hercules, CA). Quantitation of transcripts was determined using serial dilutions of a known number of DNA amplicons and normalized to expression of endogenous *ACTB1*.

Semi-quantitative real-time PCR of *DIO1* was performed using SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) under conditions: 95°C for 10 min; 3 cycles: 95°C, 30 s; 61°C, 30 s; 72°C, 30 s; 50 cycles: 95°C, 20 s; 57°C, 30 s; 72°C, 30 s, 68°C, 15 s (acquisition); melting curve analysis 120 cycles: 58°C; 0.3°C increase in each cycle. Results were normalized to expression of *ACTB1.*

Real-time PCR **quantitation of miRNAs** was performed using QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany) and universal primer UniAmpHindIII with sequence homology to overhangs of primers used in reverse transcription and miRNA-specific primers. The conditions used were: initial denaturation: 95°C, 5 min., 32 cycles: denaturation 95°C, 11 s, annealing 52°C, 11 s, and extension 65°C, 30 s; followed by melting curve analysis: 81 cycles: 55°C with 0.5°C temperature increase in each cycle. For normalization of miRNA expression, U6 snRNA [16] was used as an internal control. Relative miRNA expression was calculated using the $2^{-\Delta Ct}$ method [53].

Primer sequences (Biote21, Krakow, Poland) for all real-time PCR reactions are shown in Supplementary Data, Tables 1-2.

2.5. **Identification of *THRB* UTR splice variants:**

5'UTR variants were amplified using Perpetual OptiTaq Polymerase (EURx, Gdansk, Poland), 8μM of primers (Supplementary Data, Table-3) and conditions: 95°C for 10 min, 5 cycles (95°C, 30 s; 62°C, 45 s; 72°C, 45 s), 50 cycles (95°C, 30 s; 58°C, 30 s; 72°C, 90 s); final extension 30min, 61°C. Sequencing was performed with BigDye Terminator v3.1 (Applied Biosystems, Foster City, CA).

Newly described exons were identified using PCR primers designed basing on the *THRB* genomic sequence (ref.seq.: NC_000003.10) and the most probable donor and acceptor splice sites, predicted using NNSPLICE0.9 [20].

2.6. Protein isolation:

TRβ1: tissue samples were homogenized (buffer: 150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH 8.0, protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO), and 0.5 mM PMSF), incubated for 2 hours at 4°C with shaking and centrifuged at 12,000 rpm for 20 min at 4°C. Caki-2 cells were harvested 72 h after transfection with precursor miRNA-204 (pre-miR-204) or scrambled microRNA, washed once in PBS, and lysed in RIPA buffer (150mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl at pH 8, 2 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and 1mM PMSF.

D1: samples were homogenized (buffer: 0.1 phosphate buffer pH 7.0, 1 mM EDTA, 10 mM DTT, 250 mM sucrose, protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 1 mM PMSF), and centrifuged at 15,000 g for 30 min, at 4°C. The supernatant was centrifuged at 75,000 g for 1,5 h at 4°C. The microsome fraction pellet resuspended in homogenization buffer.

Protein extracts were stored at -70°C.

2.7. Western blotting:

40 µg of protein was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked overnight (8°C, 5% non-fat milk in TBS-T buffer: 10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 7.6), washed three times in TBS-T for 10 min, and incubated overnight at 8°C with primary antibody diluted 1:1000 in TBS-T buffer with 5% non-fat milk (anti- TRβ1 antibody: cat. no. ab2744, Abcam plc, Cambridge, UK; anti-D1 serum [54], a kind gift of T.J. Visser). After washing 3 times for 10 min, membranes were incubated for 1 h at RT with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10000, DakoCytomation, Glostrup, Denmark) (TRβ1) or goat anti-rabbit secondary antibody (1:10000, DakoCytomation, Glostrup, Denmark) (D1), and washed 3 times for 10 min. Proteins were detected using Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL). Subsequently, membranes were stripped and incubated with anti-β-actin antibody diluted 1:10000 (cat. no. ab6276, Abcam plc, Cambridge, UK) in TBS-T buffer for 1h, washed three times and used for detection. Levels of TRβ1 and Dio proteins were normalized to β-actin expression.

2.8. Thyroid hormone levels in tissue samples: Intracellular T4 and T3 concentrations were measured by radioimmunoassay following tissue extraction as described [17].

2.9. Bioinformatic analysis:

5′UTR secondary structures were predicted using RNAstructure 5.0 [18]. Splice site predictions were determined using NetGene2 Server [19], and NNSPLICE 0.9 [20].

Identification of miRNA target sites in the 3′UTR-1 was performed using: RNAhybrid [21], PicTar [22], TargetScan 5.1 [23] and miRanda [24].

Detailed descriptions of bioinformatic analyses are provided in Supplementary Data.

2.10. Statistical analysis:

The Shapiro-Wilk test was used to determine normality of data distribution. Normally distributed data were analyzed by paired t-test and non-parametric data by Wilcoxon matched pairs test. Data from luciferase assays were analyzed by ANOVA followed by Dunnett's multiple comparison test. $p<0.05$ was considered statistically significant.

3. Results

3.1. Identification of new UTR splice variants

The previously described TRβ1 5'UTR variants A-G and variant IVS4A encoding truncated protein [10] were expressed in benign and malignant kidney tissue. Moreover, four new TRβ1 5'UTR variants (A2, A3, F1, F2) were identified (Fig.1B). Two variants, F1 and F2, contained a previously unknown exon located between exons 1d and 1e, designated here as exon 1d1 (Fig.1B). The new variants A2 and A3 were similar in structure to the previously described variant A but contained an additional exon between exons 2a and 3. Variant A2 additionally contained exon 2b and variant A3 additionally contained exon 2c. Finally, a new variant of IVS4A containing a fragment of intron 4 was also identified and named IVS4B. Variant IVS4B, in contrast to IVS4A, contained exon 1c and open reading frame (ORF) that predicts expression of a 28 amino acid protein with a stop codon located in intron 4.

The structure of TRβ1 3'UTR was less complex (Fig.1C). The previously described TRβ1 mRNA contains a 3'UTR sequence designated here as 3'UTR-1. Sequencing revealed that 3'UTR-2 lacks a 123 nucleotide region of exon 10 that is located between nucleotides 2272-2394. This region contains a putative binding site for miRNA-204, located between nucleotides 2313-2319. The sequences of the new UTR variants with splice consensus sites are shown in Supplementary Data.

3.2. Reduced expression of TRβ1 mRNA and protein in ccRCC

Levels of TRβ1 mRNA were determined in ccRCC and control tissues by real-time PCR using primers which amplified the 5'-end of the TRβ1 coding sequence. TRβ1 mRNA expression was reduced by 70% in ccRCC tumor tissue (T) compared with paired control tissue (C). There was no significant difference in TRβ1 mRNA expression between control samples (C) and (N) (Fig.2A). Accordingly, TRβ1 protein expression was reduced by 91% or was undetectable in ccRCC tissue compared with paired control samples (Fig.2B). This 20% difference in reduction of TRβ1 protein compared to mRNA in tumors versus paired controls may result from aberrant post-transcriptional regulation of TRβ1 protein expression in

ccRCC. Thus, we analyzed expression of the TRβ1 5'UTR variants and investigated expression of miRNAs predicted to interact with the TRβ1 3'UTR.

3.3. Expression of TRβ1 UTRs in ccRCC

Expression of 5'UTR variants A, F, F1, as well as 3'UTR-1, 3'UTR-2 and the coding sequence of TRβ1 were analyzed by real-time PCR. Variants, B-E, F2, G, A2 and A3 were either expressed at very low levels or were undetectable in most samples and accurate quantification of their expression was not possible (data not shown). Expression of 5'UTR variants A and F were reduced in tumors by 75% and 62%, respectively, compared to (C) control samples (Fig.3.A.). 5'UTR variant A was expressed at the highest level in ccRCCs and their controls (1.8-7.4 transcripts per 10^3 copies of *ACTB1*), whereas variant F was expressed in all samples at very low levels (3-8 transcripts per 10^{13} copies of *ACTB1*). Expression of newly identified variant F1 (0.05-172 transcripts per 10^6 copies of *ACTB*) was higher than expression of variant F but lower than variant A. The expression profile of variant F1 was complex, however, and two distinct groups of paired samples were defined. In one group (n=8) there was a very high level of F1 expressed in controls compared to ccRCCs (HL, in Fig.3.B.), whereas in a second group, containing most of the tested pairs (n=30) F1 expression was much lower (LL, in Fig.3.B.) and decreased by 90% in paired tumor tissue. In the HL group, expression of F1 in ccRCC tissue was almost undetectable. There was a significantly ($p < 0.0001$) higher level of expression of F1 in N noncancerous samples from the LL group compared to C normal control tissue (Fig.3.B.). Expression of the variants IVS4A and IVS4B was reduced by 99% in tumour (T) compared to paired control (C) tissue although levels were much higher in samples from non-malignant diseased kidneys (N) (Supplementary Data, Fig.1S).

Expression of the 3'UTR-1 and 3'UTR-2 variants was decreased by 79% and 86%, respectively, in tumors, compared to control samples (Fig.3.C). 3'UTR-1 (2.3-11 transcripts per 10^3 copies of *ACTB1*) was expressed at a similar level to 5'UTR variant A, whereas 3'UTR-2 expression was 1000-fold lower (0.8-5.7 transcripts per 10^6 copies of *ACTB1*).

The levels of expression of the TRβ1 transcript containing the coding sequence (ORF) of TRβ1 protein (2.0-7.7 transcripts per 10^3 copies of *ACTBI*) were similar to levels of the 5'UTR A and 3'UTR-1 variants (Fig.2 and 3). Abundances of particular UTR variants relative to the most highly expressed variant A are shown in Fig. 2S.

3.4. The expression of miRNA-204 regulating TRβ1 expression in ccRCC

Bioinformatic analysis of the TRβ1 mRNA 3'UTR identified multiple potential miRNA binding sites, 14 of which were selected for further analysis (Supplementary Data, Table-4) based on their identification by at least two of four independent bioinformatic approaches. In preliminary studies we determined the relative expression of 14 candidate miRNAs in paired control and ccRCC tissue from two kidneys. These studies revealed differential expression of seven miRNAs: miRNA-1, miRNA-211, miRNA-204, miRNA-206, miRNA-154, miRNA-335 and miRNA-512-3p. Subsequent analysis in 39 paired samples revealed that expression of miRNA-204 was increased by 1.2-2.0 fold in ccRCC compared to paired control tissue (Fig.4), whereas expression of the six other candidate miRNAs did not differ significantly between ccRCC and control tissue (data not shown). The predicted miRNA binding site for miRNA-204 was located in the 61bp region of exon 10 that was present in 3'UTR-1 but absent from 3'UTR-2 (Fig. 1C and Fig. 4A).

In order to test whether miR-204 is a true TRβ1 regulator, Caki-2 cells were transfected with miR-204 precursor and scrambled control. Subsequent real-time PCR and Western blot analysis of TRβ1, as well as real-time PCR of TRβ1 dependent gene, *DIO1* were performed. Transfection with miR-204 precursor resulted in a large (three magnitudes of order) induction of miR-204 expression (Fig. 3S. in Supplementary Data) which was followed by ~42% decrease of TRβ1 mRNA expression and ~35% decrease of *DIO1* mRNA expression when compared with scrambled control. Moreover, the protein expression of TRβ1 is also almost twice lower in pre-miR-204 transfected cells in comparison with scrambled control (Fig. 4).

3.5. TRβ1 5'UTR variants influence protein translation efficiency in ccRCC cells

To analyze the effect of the TRβ1 5'UTR variants A-G and new variant F1 on translation efficiency, ccRCC derived Caki-2 cells were transfected with 5'UTR-pGL3 constructs and luciferase activity was determined. In ccRCC Caki-2 cells, inclusion of 5'UTR variant A (folding free energy ΔG=-69.0 kcal/mol), resulted in the highest degree of luciferase activity when compared to other 5'UTR variants (Supplementary Data, Fig.4S.A). By contrast, variant F1 (ΔG=-124.4 kcal/mol) exerted the strongest inhibitory effect on luciferase expression, decreasing activity by more than 98% comparing to activity of the control vector lacking a TRβ1 5'UTR.

To check whether the presence of TRβ1 ORF influences the regulatory effect of UTR on translational efficiency, we performed an *in vitro* experiment using pKS-derived plasmids (Fig. 4S.B). To do this, luciferase transcription and translation rates from construct UTR-A+ TRβ1ORF+Luc were tested in *in vitro* transcription/translation assay together with the following constructs: UTR-A+Luc; TRβ1ORF+Luc; Control. These assays revealed that: 1) there were no differences between transcriptional efficiency of control and none of the constructs; 2) translational efficiency of TRβ1ORF+Luc did not statistically significantly differ when compared with control; 3) translational efficiency of UTR-A+Luc was 22% lower when compared to control ($p < 0.01$); 4) translational efficiency of UTR-A+ TRβ1ORF+Luc was 28% lower when compared to control ($p < 0.01$); 5) there was no statistically significant difference between translational efficiency of UTR-A+Luc and UTR-A+ TRβ1ORF+Luc. Thus, we concluded that TRβ1ORF did not influence the regulatory effect of 5'UTR A on translational efficiency.

The prediction of secondary structures formed by the 5'UTRs revealed that the variants A2, A3, F1, and F2 fold into structures of different free energies. Variant F2 formed the most stable secondary structure (ΔG=-171.0 kcal/mol) and variant A3 was the least stable (ΔG=-86.6 kcal/mol) (Supplementary Data, Table-5 and Fig. 5S-9S).

3.6. Reduced expression of the TRβ1 target gene, *DIO1*, in ccRCC

The promoter of type 1 iodothyronine deiodinase (*DIO1*) was shown to be bound *in vitro* by both thyroid hormone receptors: TRα and TRβ [25]. It was also suggested, however, that in the kidney *DIO1* expression is solely activated by TRβ1 [26]. In addition, our unpublished results of experiments performed on the material used in this study show lack of changes between the expression of TRα1 in ccRCC tisues in comparison with control samples. Therefore we assumed that *DIO1* expression would be a good marker of changes of TRβ1 level in kidney samples. To determine whether decreased expression of TRβ1 mRNA and protein observed in ccRCC was accompanied by changes in T3-target gene expression, we analyzed expression of *DIO1* in eleven paired samples of ccRCC and normal kidney tissue. In accordance with the reduced TRβ1 expression in ccRCC, *DIO1* mRNA was decreased by 92% in tumor tissue compared to normal (Fig.5A) and D1 protein was undetectable in tumors but detectable in samples of paired normal tissue (Fig.5B).

3.7. Tissue T3 concentrations are reduced in ccRCC compared with normal kidney

TRβ1 regulates target gene expression in response to T3, whereas T4 does not bind and activate the receptor at physiological concentrations. T4, is a pro-hormone that is converted to T3 by the type 1 and 2 deiodinase enzymes, of which *DIO1* gene is regulated directly by T3 whereas *DIO2* is not [27]. Thus, the level of deiodinase activity determines the intracellular level of T3 available to bind and activate TRs. In order to determine whether reduced expression of *DIO1* correlated with reduced tissue T3 concentration, the levels of T4 and T3 were determined in 11 paired ccRCC and normal kidney tissue samples. The level of T4 did not differ between normal and ccRCC tissue, whereas the concentration of T3 was reduced by 58% in ccRCC tissue (Fig.5C).

4. Discussion

These studies demonstrate reduced TRβ1 mRNA and protein expression in human ccRCC (Fig.2). We found discordance in the magnitude of the change in TRβ1 mRNA level (70% reduction in ccRCC) compared to protein (91% reduction). These results confirm our previous findings achieved by different mRNA analysis method (Northern blot) and different antibodies as well as Western blot protocol and in different tissue samples [28]. These repeatedly obtained, coherent results suggest that TRβ1 expression is subject to posttranscriptional regulation in ccRCC. Reduced TRβ1 expression in ccRCC was accompanied by absent D1 protein and a 58% reduction in tissue T3 concentration (Fig.5). The current findings are consistent with previous findings of abnormal TRβ1 expression [4] and reduced D1 activity [29] in renal cancer. Taken together, the data provide powerful evidence of tissue hypothyroidism and impaired T3 action in ccRCC that is maintained by reduced expression of TRβ1 and D1 (Fig.6).

Previous studies have shown that TRβ1 mRNA 5'UTR undergoes complex alternative splicing, and eight 5'UTR variants that regulate efficiency of TRβ1 protein translation in a tissue-specific manner were identified [10]. In the current studies we show that these variants are expressed in normal kidney and ccRCC, and also identify 4 additional 5'UTR variants, a new 5' variant that is predicted to encode a truncated TRβ1 protein and a new 3'UTR variant that lacks a 123 nucleotide region containing putative miRNA binding sites (Fig.1).

Detailed analysis demonstrated that 5'UTR variant A and 3'UTR-1 are expressed at similar levels to each other and to mRNA containing the TRβ1 protein ORF, indicating the major TRβ1 coding mRNA expressed in kidney and ccRCC contains 5'UTR A and 3'UTR-1. Expression of all TRβ1 UTRs was reduced in ccRCC by between 62% and more than 90% (Fig.3). Differences in the ratios of the various TRβ1 transcripts in healthy tissue compared to tumor suggests that alternative splicing of TRβ1 UTRs is perturbed in ccRCC, as shown previously for *DIO1* in ccRCC [30]. Accordingly, aberrant alternative splicing of other genes has been reported to contribute to carcinogenesis in ccRCC [31-33]. Thus, the loss of

transcripts F1 and IVS4B that were undetectable in ccRCC tissue, and the absence of D1 protein may represent new markers of tumor progression in ccRCC. Interestingly, ccRCC-specific loss of D1 protein is in agreement with our previous paper, concerning disturbed alternative splicing of *DIO1* [30]. In that paper we suggested that alternative splicing of *DIO1* in ccRCC is "shifted" and leads to production of splice variants containing premature termination codons (PTC) that may be degraded by nonsense-mediated mRNA decay mechanism (NMD). Thus, ccRCC-specific decrease of D1 protein would result not only from lowered transcription rate but also from inhibition of translation due to NMD of PTC containing splice variants.

The discordance between changes in levels of TRβ1 mRNA and protein in ccRCC is suggestive of altered posttranscriptional regulation of TRβ1 that may be mediated by alternative splicing of the 5' and 3'UTRs. The major TRβ1 transcript expressed in ccRCC contained 5'UTR variant A and 3'UTR-1. Analysis of the effects of 5'UTR variants on protein expression in Caki-2 renal cancer cells indicated that variant A permitted the highest level of protein expression (Supplementary Data, Fig.4S) and was consistent with previous findings in JEG-3 choriocarcinoma cells [10]. The least efficiently translated 5'UTR variant was F1 (Fig.4S.). As this variant revealed also the largest decrease of expression it could suggest an increase in TRβ1 protein expression, rather than the decrease that we actually observed. It must be noted however that both in control and tumor tissues the expression of F1 was three orders of magnitude lower than expression of the most efficiently translated variant A. Thus, the predicted the impact of F1 on the final level of TRβ1 protein is possibly rather low.

A small number of mRNAs possess long 5'UTRs of complex secondary structure that contain upstream ORFs which inhibit protein translation. However, if such 5'UTRs also contain internal ribosome entry sites (IRES) and conditions favor IRES-dependent translation these sequences can allow efficient protein expression, thereby demonstrating that 5'UTR sequence and secondary structure mediate posttranscriptional regulation of gene expression [34]. Secondary structure analysis of 5'UTR variant A revealed complex mRNA folding, the

presence of four upstream AUG translation initiation sites and two putative IRES sequences (Supplementary Data, Fig.5S), indicating features that may provide a mechanism whereby variant A regulates TRβ1 protein expression. The role of other minor TRβ1 5'UTR variants in determining the total level of TRβ1 protein expressed in ccRCC, however, remains to be elucidated. For example, although variant D was expressed at very low levels in ccRCC, previous studies have shown it permits efficient protein expression in kidney-derived COS-7 cells [10]. In this context, evidence supports a role for two possible mechanisms of translation initiation: by non-classical, hypoxia-induced, IRES-dependent (cap-independent) translation or by cap-dependent protein synthesis in conditions where increased expression of the translation factor eIF4E disproportionately increases translation from pools of mRNAs containing 5'UTRs with complex secondary structure [35]. Although the functional significance of alternative splicing of the TRβ1 5'UTR in ccRCC is currently unknown, aberrant expression of alternative 5'UTRs has been shown to contribute to carcinogenesis mediated by tumor suppressors [12;36], and oncogenes [37]. In the light of the complex secondary structures of low copy number TRβ1 5'UTRs and evidence for selective translation of structured mRNAs in aggressive metastatic cancer or oxygen deprived tumors [35] it is likely that the 5'UTR of TRβ1 plays an important role in controlling levels of receptor protein in ccRCC and this may influence tumor progression.

In addition to the role of the 5'UTR, alternative splicing of the 3'UTR is likely to be important. Analysis of the main TRβ1 3'UTR variant (3'UTR-1) identified miRNA-204 as a candidate regulator of TRβ1 expression. A binding site for miRNA-204 was identified in TRβ1 3'UTR-1 but not in the minor 3'UTR-2 splice variant and miRNA-204 expression was found to be increased 2-fold in ccRCC (Fig.4). Moreover, as we showed, overexpression of miR-204 in Caki-2 cells leads to downregulation of TRβ1 mRNA and protein. These findings are consistent with studies indicating that miRNA-204 acts as an inhibitor of mRNA expression [38].

Apart from TRβ1, another TR isoform is present in the kidney, TRα1, which has been also shown to bind the promoter of *DIO1* gene *in vitro* [25]. It was suggested, however, that

DIO1 expression in the kidney is solely activated by TRβ1 [26]. In addition, our unpublished results of experiments performed on the material used in this study show that expression of TRα1 does not change in ccRCC when compared control samples. Finally, miR-204-mediated downregulation of TRβ1 in Caki-2 cells is accompanied by decrease of *DIO1* mRNA expression, what additionally confirms the linkage between changes in TRβ1 and *DIO1* expression in the kidney.

The biological significance of our findings comes from emerging evidence demonstrating that TRβ1 functions as a tumor suppressor [39-41]. Its role is complex, however, as the T3-dependent activity of TRβ1 may have a biphasic effect on cell growth and proliferation. Knockout mice lacking TRβ1 show delayed hepatocyte proliferation and enhanced apoptosis in response to partial hepatectomy [42] and tumor cell growth is retarded in hypothyroid mice [39]. On the other hand, the phenotype of tumors induced in hypothyroid hosts is more mesenchymal, and their invasiveness and metastatic behaviour are enhanced [39]. These findings are in line with previous reports documenting reduced tissue T3 in human gliomas [43] and demonstrating an association between the low T3 syndrome and renal cancer [44], although decreased concentrations of T3 have also been shown to reduce proliferation of Caki-2 cells *in vitro* [45]. Interestingly, variations in T3 and TRs level may directly initiate changes in activity of other target genes with direct effect on proliferation including those engaged in the regulation of cell cycle progression. An example of such a gene is E2F1, a transcription factor controlling G1 to S phase transition whose expression is negatively regulated by TRs [46]. As we showed, the expression of E2F1 is increased in renal cancer tissues, and this is in agreement with disturbed function of TRs [47]. E2F1 is known to regulate cellular proliferation [48] therefore its disturbed expression may contribute to ccRCC carcinogenesis. Moreover, Liu et al., [49] showed that CD74 induced tumorigenesis of ccRCC is mediated by PI3K/AKT pathway and it is known that this pathway is regulated by TRβ1/T3 [50]. Thus, it is highly probable that disturbances of TRβ1 and T3 observed in this study may initiate wide net of changes in multiple target genes, causing direct effect on proliferation.

In summary, we demonstrate that reduced expression of TRβ1 in ccRCC is likely to be maintained by posttranscriptional mechanisms mediated by alternative splicing of the TRβ1 mRNA 5' and 3'UTRs along with increased expression of miRNA-204. Reduced expression of TRβ1 results in tissue T3 deficiency mediated by inhibition of *D1* expression. The recently identified tumor suppressor activity of TRβ1 [39-41] indicates that reduced TRβ1 expression and tissue hypothyroidism in ccRCC tumors may be involved in the process of carcinogenesis itself or in maintaining a proliferative advantage to the malignant cells.

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Conflict of Interests: None declared.

- TRβ1 mRNA and protein levels are significantly reduced in renal cancer
- Loss of D1 and reduced level of intracellular T3 are found in kidney tumors
- TRβ1 decrease in renal cancer is caused by miRNA-204 upregulation
- 5'UTR and 3'UTR are involved in posttranscriptional TRβ1 regulation in renal cancer
- Renal cancer is characterized by tissue hypothyroidism

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Figure Legends

Fig.1. Human *THRB* gene.

A. *THRB* gene (Ref.seq.:NC_000003.11: 378609bp region of chromosome 3 from base 24133709 to 24512317), TRβ1 mRNA (NM_000461.3) and protein (NP_000452.2). Exons are numbered 1-10 and sized above. Amino acid positions below the protein demonstrate boundaries of the DNA and ligand binding domains (DBD and LBD) in TRβ1 protein.

B. Previously published (A-IVS4A) [10] and newly identified (A2-IVS4B) splice variants of TRβ1 mRNA 5'UTR. Exons are labeled 1a-1e, 2a-2c and 3.

C. Previously published (3'UTR-1) and newly identified (3'UTR-2) splice variant of TRβ1 3'UTR that lacks the 61bp region shown, which contains a potential binding site for miRNA-204 (bold underlined). Primers are indicated by arrows.

Fig.2. Expression of TRβ1 mRNA and protein in ccRCC

A. Expression of TRβ1 mRNA containing the TRβ1 protein ORF. T: ccRCC tissue, C: paired normal control tissue from ccRCC kidneys, N: non-neoplastic kidney. The data are means ± S.E. (n=10 for N, n=39 for C, n=39 for T).

B. Expression of TRβ1 protein. Upper panel: Western blot showing expression of TRβ1 (52kDa protein) and β-actin (42kDa protein) in four representative C and T paired samples. Lower panel: mean TRβ1 expression in paired samples C and T normalized to β-actin (n=11 for C, n=11 for T). The data are means ± S.E. Statistical analysis was performed using paired t-test to compare C and T. * $p < 0.05$.

Fig.3. Expression of TRβ1 5′ and 3′UTR variants

Expression of 5′UTR variants A and F (Panel A), F1 (Panel B) and 3′UTR variants (Panel C) in paired tumor (T) and control tissue (C) and in non-neoplastic kidney (N). Analysis of F1 expression revealed two groups of samples: one with low levels (LL) of F1 (74% of samples) and one with high levels (HL) of F1 in control tissues C and N. The data are means ± S.E. in HL samples and as median values with 95%CI in LL samples as data were not normally

distributed in this group. Statistical analysis was performed using paired t-test for variants A, F, F1 in HL samples, 3'UTR-1, and 3'UTR-2 or Wilcoxon paired test for F1 in LL samples. Variant A: n=10 for N, n=37 for C, n=37 for T; variant F: n=10 for N, n=35 for C, n=35 for T; F1 LL samples: n=30 for C, n=30 for T; F1 HL samples: n=10 for N, n=8 for C, n=8 for T; variant 3'UTR-1: n=10 for N, n=32 for C, n=32 for T, variant 3'UTR-2: n=30 for C, n=30 for T. *** $p < 0.001$. ** $p < 0.01$

Fig.4. Levels of miRNA-204 in tissues and its regulatory effect on TRβ1 expression. A. Positions of potential miRNA binding sites in TRβ1 3'UTR identified by TargetScan and RNAHybrid. miRNAs binding to 3'UTRs requires only partial complementarity to the target. Nucleotides of perfect complementarity are shown as seed match.

B. Increased miRNA204 expression in ccRCC tumor tissue (T). Data are means ± S.E. (n=35 for T, n=35 for C, n=10 for N). Statistical analysis was performed using paired t-test to compare C and T samples. * $p< 0.05$. **C.** miR-204 mediated downregulation of TRβ1 and its target gene D1 transcript in Caki-2 cell line. Semi-quantitative real-time PCR assay for TRβ1 and D1 in the Caki-2 cell line transfected with pre-miR-204 or scrambled microRNA (negative control). Data are expressed as mean values +/- SEM. Statistical analysis was performed using paired t-test to compare cells transfected with pre-miR-204 and with scrambled microRNA. *** $p < 0.0001$.

Western blotting for TRβ1 and β-actin (loading control) in the Caki-2 cell line transfected with pre-miR-204 or scrambled pre-miR-204 (negative control). Cells were harvested 72 h after transfection; the relative density of bands was quantified by densitometry.

Fig.5. Expression of *DIO1* and tissue concentrations of thyroid hormones in ccRCC

A. Expression of *DIO1* mRNA in tissue samples. n=33 for T, n =33 for C, n=10 for N. Data are means +/- S.E, *** $p<0.001$. Statistical analysis was performed using a paired t- test.

B. Western blot showing expression of D1 (28kDa protein) and β-actin (42kDa protein) in four representative C and T paired samples.

C. Tissue concentrations of T4 and T3, in paired control (C) and ccRCC (T) samples. Data are shown as mean ± S.E. (n=12 for T, n=12 for C). Statistical analysis was performed using paired t-test. ***p<0.001.

Fig.6. Mechanisms of aberrant TRβ1 action in ccRCC

Disruption of TRβ1 activity may result from loss of heterozygosity (LOH) at the *THRB* locus [51] and several other mechanisms: **(1a)** reduced TRβ1 expression, **(1b),** decreased intracellular T3 concentrations, **(2)** mutations of TRβ1 (triangles) that inhibit binding of T3, **(3)** DNA [6], or **(4)** altering interactions with coregulators [52]. Mechanisms that contribute to reduced TRβ1 expression include: **(5)** aberrant splicing and expression of TRβ1 UTRs, **(6)** increased expression of miRNAs that interact with the TRβ1 3'UTR and **(7)** increased expression of the TRβ1 target gene *DIO1*, which results in reduced intracellular T3. The arrows accompanying the percentages show the decrease of expression.

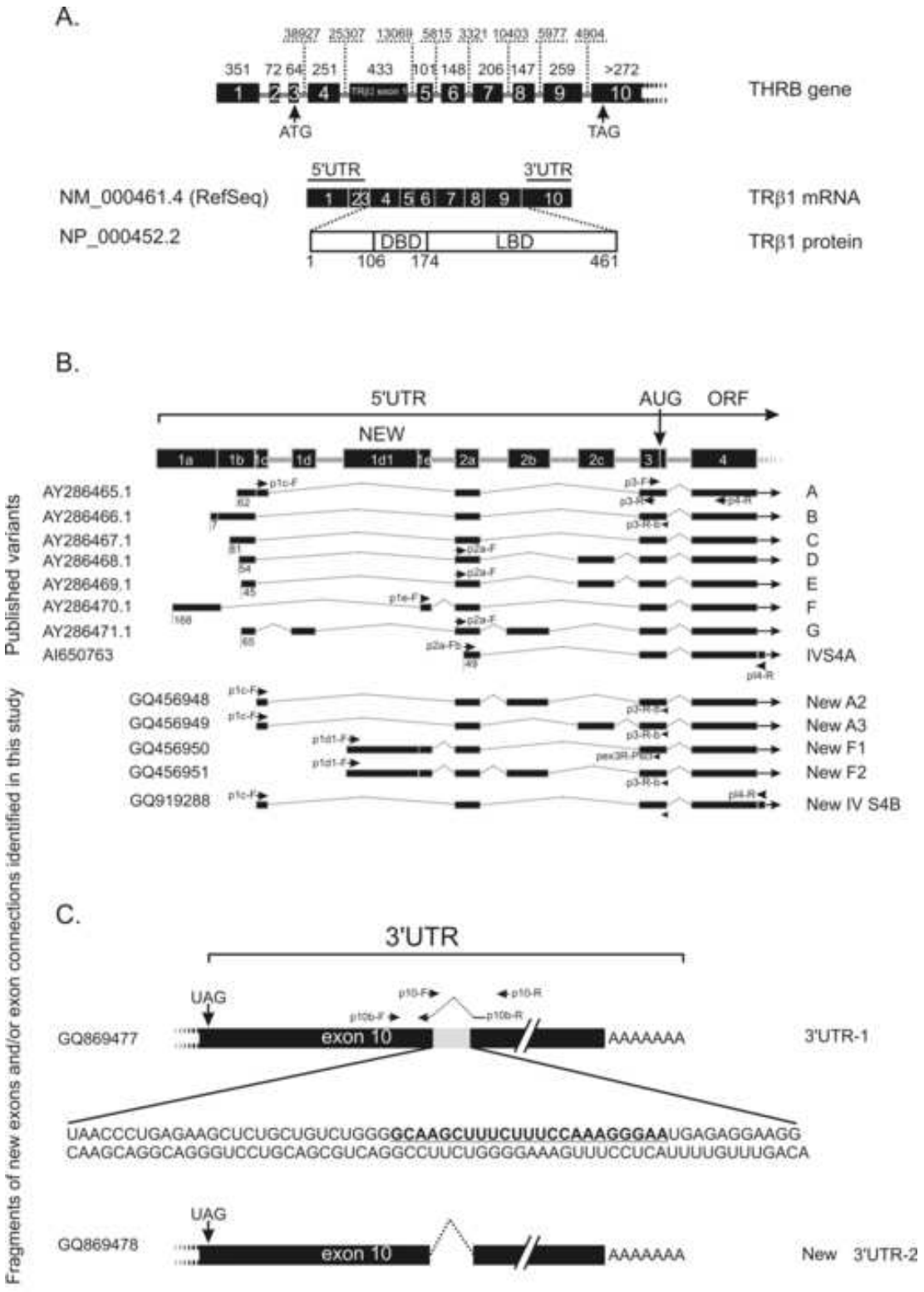

Figure 1

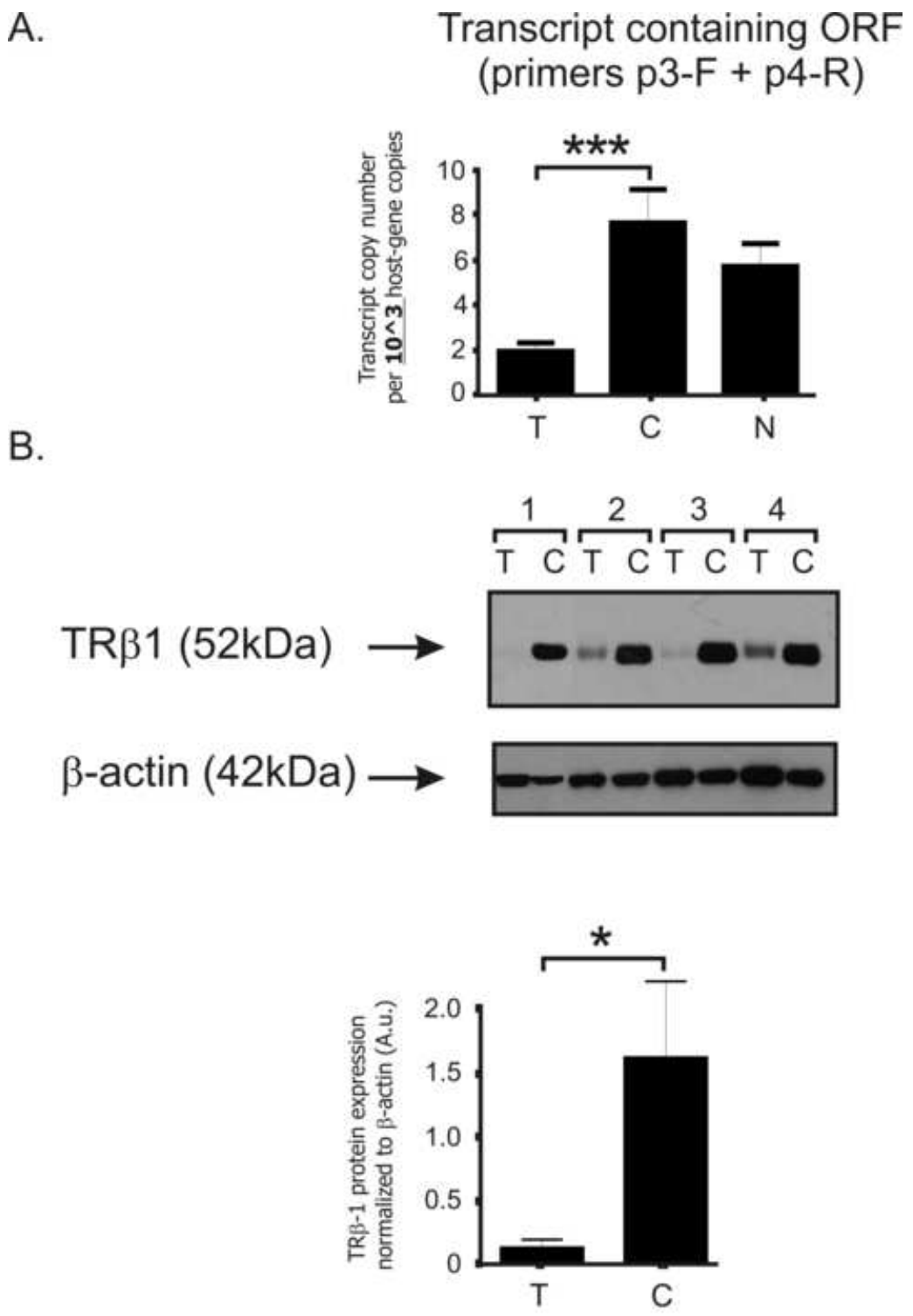

Figure 2

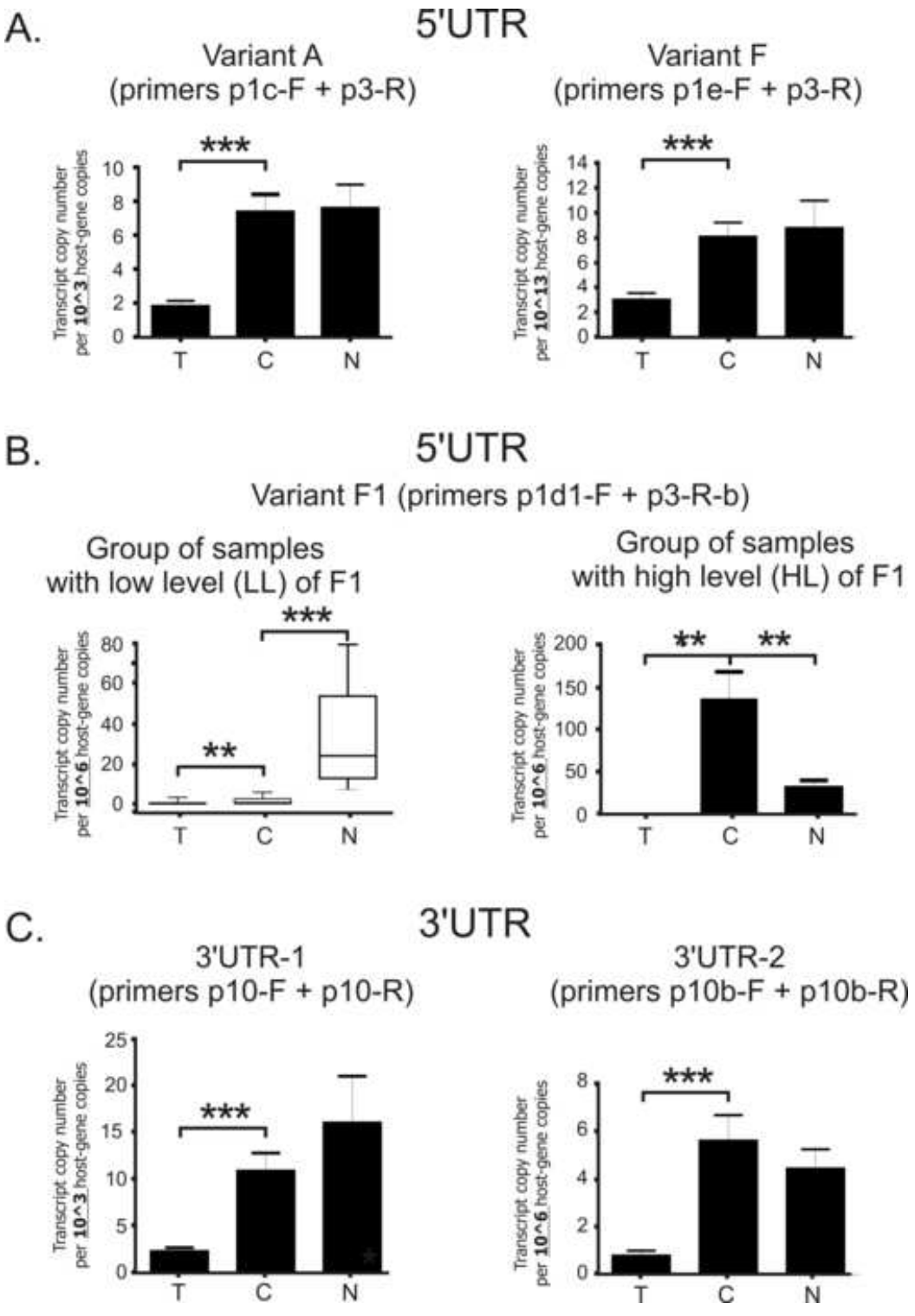

Figure 3

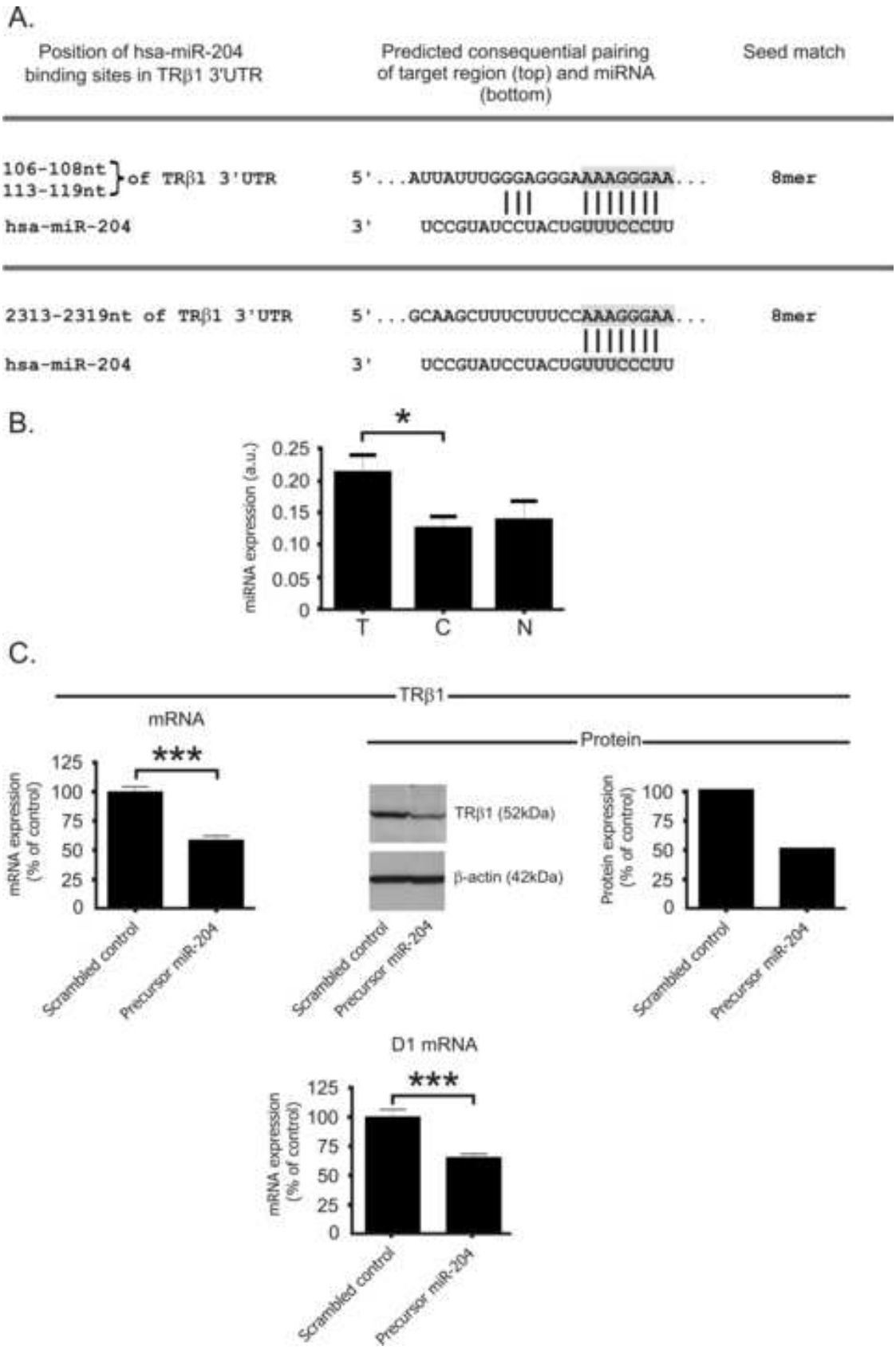

Figure 4

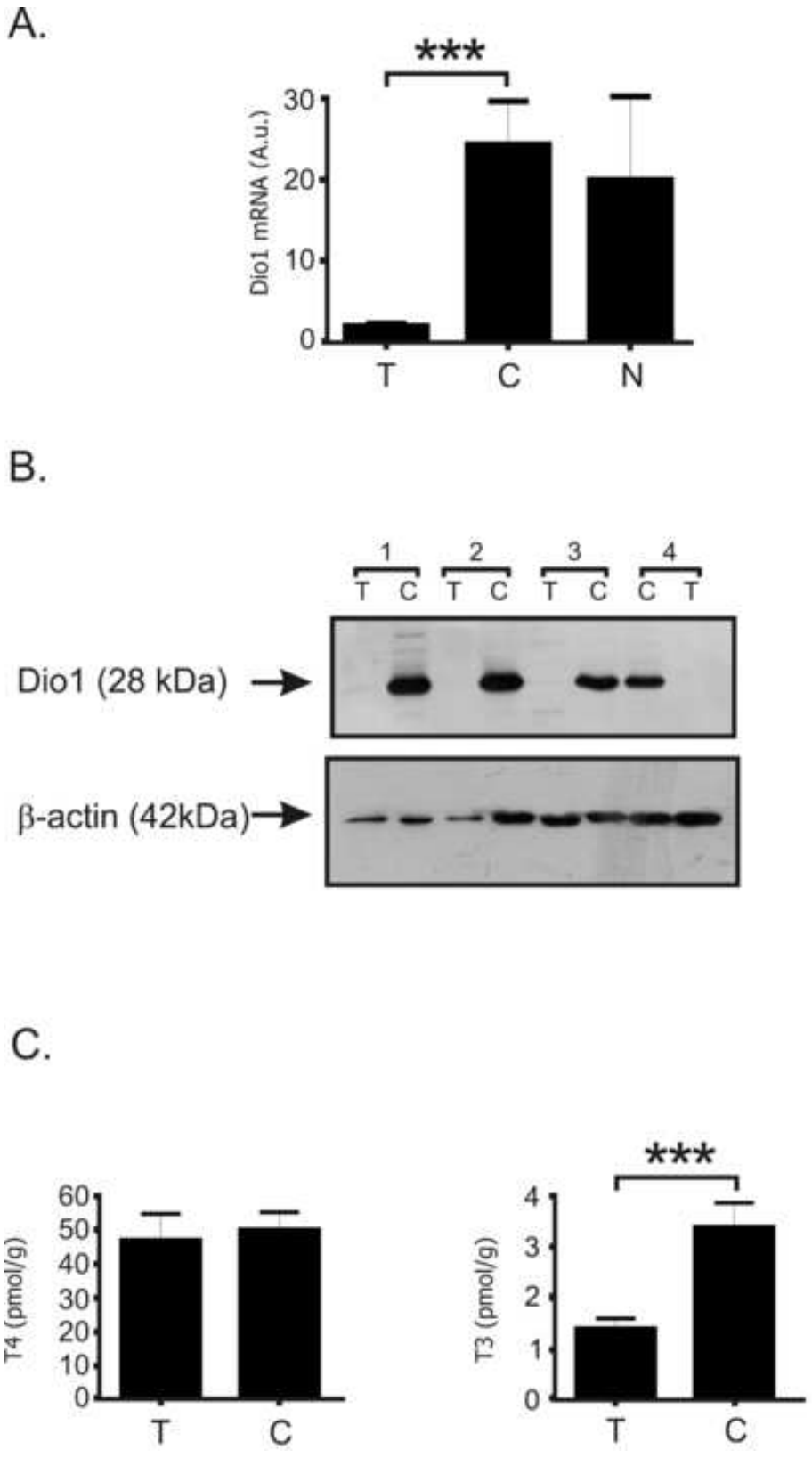

Figure 5

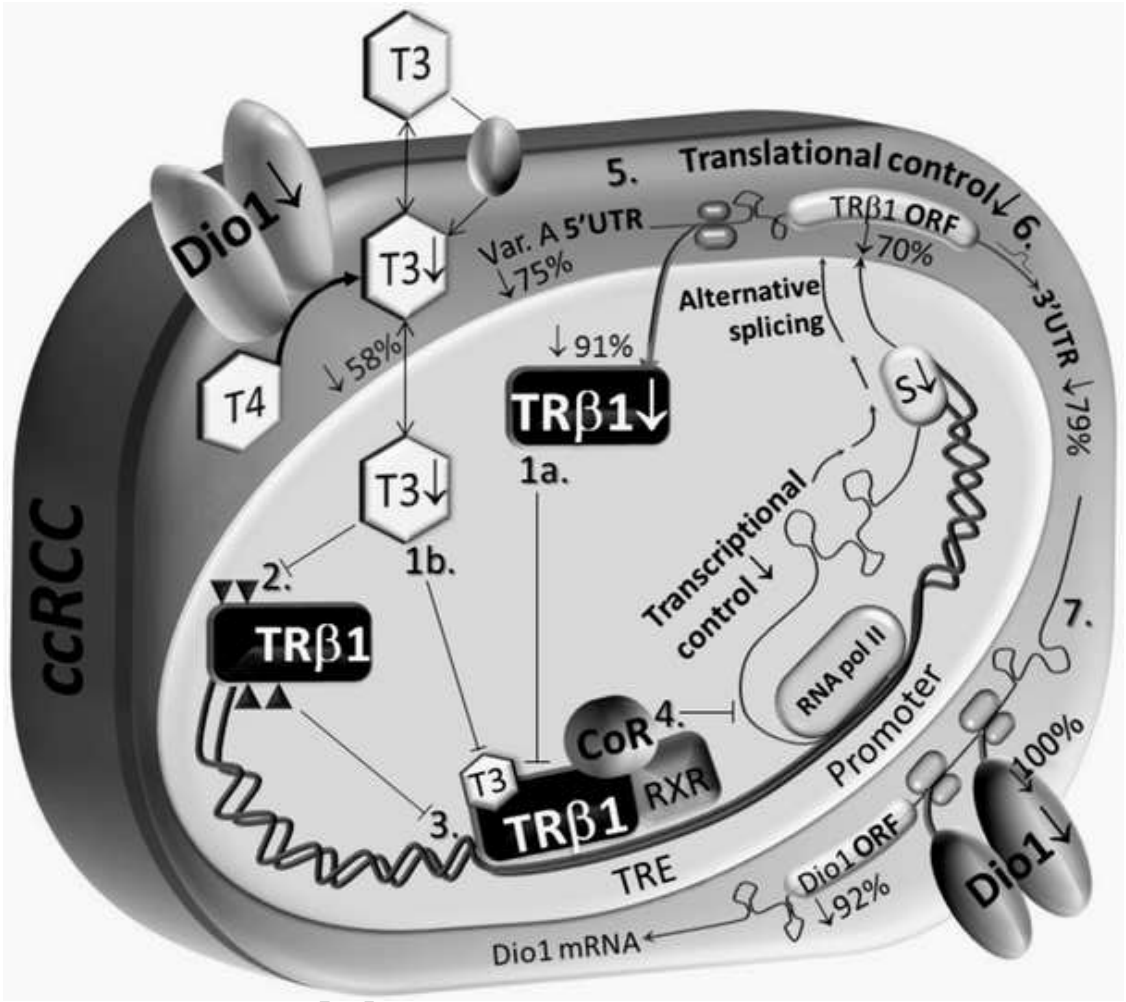
T3
Dio1↓
T3↓
Var. A 5'UTR
↓75%
5. Translational control↓
TRβ1 ORF
↓70%
6.
3'UTR ↓79%
Alternative
splicing
↓58%
↓91%
T4
TRβ1↓
1a.
S↓
T3↓
1b.
Transcriptional
control↓
2.
TRβ1
7.
RNA pol II
CoR
4.
Promoter
T3
RXR
TRβ1
3.
TRE
100%
Dio1↓
Dio1 ORF
↓92%
Dio1 mRNA
ccRCC

Figure 6