CHAPTER 6b

Coregulatory Protein Recruitment by Thyroid Hormone Receptors in Neuronal Cells

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Abstract

Thyroid hormone (TH) binding to its nuclear TH receptors (TRs) alters coregulatory protein recruitment of the TRs to regulate gene transcription. Considering the crucial role of TH in brain development and function, we aimed to identify the coregulatory proteins that are involved in neuronal TR activity by elucidating the interactomes for wild-type TRα1 and TRβ1 in a human-derived neuronal cell line (SH-SY5Y). TR-interacting proteins were purified from nuclear extracts of SH-SY5Y cells stably expressing epitope-tagged TRα1 and TRβ1 (FH-TRα1 and -TRβ1) by tandem-affinity purification and analyzed by LC-MS/MS. One hundred one proteins were co-purified with TRs, including the known TR-interacting proteins, such as retinoid X receptors (RXRs) (regardless of T3, the presence or absence of T3), NCoR1/SMRT/HDAC3 repressor complex (in the absence of T3), and SRCs and the Mediator complex (in the presence of T3). Several chromatin remodeling complexes that have not previously been described as coregulators of TRs were identified, for instance the nucleosome remodeling deacetylase (NuRD) and the Spt-Ada-Gcn5-Acetyltransferase (SAGA) complexes. Most of the proteins were shared between the two TR isoforms. However, we identified nine proteins that only associated with FH-TRα1 and two proteins with FH-TRβ1. These findings suggest that TRs not only interact with classical coregulatory proteins in SH-SY5Y cells but also with several potential novel binding partners. In addition, we identified a subset of distinct nuclear proteins that seem to interact with TRs in an isoform-specific manner.

Introduction

Thyroid hormone (TH) is indispensable for proper neurodevelopment (1). TH regulates gene transcription by binding of the bioactive TH form, triiodothyronine (T3), to thyroid hormone receptors (TRs). Impaired action of TH in the brain is associated with intellectual disability and psychomotor retardation in many conditions, including prolonged untreated congenital hypothyroidism patients (2,3), impaired TH transport to the brain in monocarboxylase transporter 8 (MCT8) deficiency (due to a mutation in MCT8) (4,5), and impaired function of TRα due to a mutation in this receptor leading to resistance to TH alpha (RTHα) (6,7).

Three TR isoforms, TRα1, β1, and β2 that have extensive structural similarity are capable of binding to T3. TRα1 is encoded by *THRA* gene on chromosome 17 whereas TRβ1 and TRβ2 are encoded by *THRB* gene on chromosome 3 (8). It has been shown that TRα1 is the most abundantly expressed in the brain and plays a crucial role in brain development (9-13). TRβ1 is also expressed in many regions of the brain but the distribution is more restricted compared to TRα1 (9,11). TRβ2 is predominantly expressed in the hypothalamus, pituitary, retina, and cochlea (11,14,15). These differential expression patterns suggests that TR isoforms could play different functional roles during development.

TRs mainly form heterodimers with retinoid X receptors (RXRs) on thyroid hormone response elements (TREs) and interact with many nuclear coregulatory proteins to regulate local chromatin structure and gene transcription. In the absence of T3, TRs recruit corepressor proteins, including nuclear receptor corepressor 1 (NCoR1), silencing mediator for retinoid or thyroid hormone receptors (SMRT), and histone deacetylases (HDACs), to modify the histone core of nucleosomes and create a closed chromatin conformation. Binding of T3 induces conformational changes in TRs, which favor recruitment of coactivator proteins, including steroid receptor coactivators (SRCs) and histone acetyltransferases (HATs), to create an open chromatin configuration and accessibility of target genes for general transcription factors (GTFs) and the RNA polymerase II complex (8,16). In addition to the classical TR coregulators, other nuclear proteins have been described that can have a direct or indirect interaction with TRs (17,18) and are involved in transcriptional regulation.

There is evidence indicating that the coregulatory protein recruitment by nuclear receptors is tissue-dependent, which may lead to diverging receptor functions in the different tissues (14,17,19,20). In addition, some studies showed that TRs recruit a subset of coregulatory proteins in an isoform-specific manner (17,18), adding to the complexity of transcriptional gene regulation by TRs. In order to gain more insight into the transcriptional gene regulation by TRs in the brain, we identified the coregulatory proteins that are involved in neuronal TR activity by interactome analysis for wild-type (WT) TRα1 and TRβ1 in a human-derived neuronal cell line (SH-SY5Y). We found that TRs interact with several nuclear proteins, some of which have not previously been described as TR interacting partners. In general, both

TR isoforms shared the same coregulatory proteins; however, a subset of proteins seemed to interact in an isoform-specific manner.

Materials and Methods

Plasmid constructs

Lentiviral constructs containing N-terminal FLAG and Hemagglutinin (HA) double-epitope tagged human WT TRα1 and TRβ1 and a bicistronic messenger RNA that allows the simultaneous expression of TRs, puromycin resistance marker, and green fluorescent protein (GFP) (pLentiFH-TRα1 and pLentiFH-TRβ1) were created as previously described (Chapter 6a). An empty vector construct (pLentiMCS) expressing only the puromycin resistance marker and GFP was also used to generate an empty vector control cell line. The packaging vectors, pMD2.G and psPAX2 (Chapter 6a), were used to produce TR containing lentiviruses as described below.

Stable expression of TRs in SH-SY5Y cells

FH-TRα1, -TRβ1 and empty vector (MCS) were stably expressed in SH-SY5Y human-derived neuroblastoma cells using lentiviral transduction as previously described (Chapter 6a). In brief, lentiviruses containing pLentiFH-TRα1, pLentiFH-TRβ1, and pLentiMCS were produced in HEK293FT cells by co-transfecting 4 µg of pLenti-CMV-FH-TRα1, -TRβ1, or -MCS constructs with 4 µg of psPAX2 and pMD2.G plasmids using Xtreme Gene 9 transfection reagent (Roche Diagnostics, Almere, NL). SH-SY5Y cells were transduced with lentiviruses for 48 hours in 6-well plates (at 25% confluency) and subsequently selected with 2 µg/mL of puromycin. Puromycin-resistant SH-SY5Y cells were expanded in growth medium (DMEM/F12 supplemented with 9%FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 nM Na_2SeO_3) and 2 µg/mL puromycin for subsequent experiments. SH-SY5Y cells stably expressing FH-TRα1, -TRβ1 or -MCS were designated as FH-TRα1, FH-TRβ1, or MCS cells, respectively.

Luciferase assays

To determine T3-induced transcriptional activity of stably expressed receptors, FH-TRα1, FH-TRβ1, and MCS cells were cultured in 24-well plates. At 80% confluency, cells were transfected for 24 hours with 200 ng pdV-L1 luciferase-renilla reporter construct (21) in TH depleted medium (DMEM/F12 supplemented with 9% charcoal-stripped FBS) using Xtreme Gene 9 transfection reagent (Roche Diagnostics, Almere, NL). After that, cells were stimulated with 0-10,000 nM T3 for 24 hours in DMEM/F12 medium supplemented with 0.1% bovine serum albumin (BSA). Luciferase and renilla activities were determined using the Dual Glo Luciferase kit (Promega, Leiden, NL) as previously described (7). The ratio between luciferase

and renilla was calculated to adjust for transfection efficiency. The results were shown as mean ± SEM of three independent experiments performed in triplicate. Dose-response curves were created by GraphPad Prism version 5.0 (GraphPad, La Jolla, CA).

Tandem-affinity purification

The FH-TRs and TR-interacting proteins were purified from SH-SY5Y cells as previously described (Chapter 6a). Briefly, the FH-TRα1, FH-TRβ1, and MCS cells were cultured until near confluency. Then, the cells were starved of TH for 24 hours in TH depleted medium and incubated for 4 hours with 0 (vehicle) or 100 nM T3 in DMEM/F12 supplemented with 0.1% BSA. After that, the cells were harvested, and nuclear proteins were extracted as previously described (22) (Chapter 6a). Nuclear extracts were incubated overnight with anti-Flag M2 affinity gel (#A2220, Sigma Aldrich) at 4°C to purify the TRs. The bound proteins were eluted with 200 µg/mL Flag peptide (#F3290, Sigma Aldrich) and subsequently incubated for 4 hours with EZview red anti-HA affinity gel (#E6779, Sigma Aldrich) at 4°C for the second purification. The proteins were eluted from HA-resin with 400 µg/mL HA peptide. The purified products (final HA eluates) of two independent tandem-affinity purifications per conditions were subjected for analysis by LC-MS/MS.

Immunoblotting

The expression of TRs in nuclear extracts prepared from FH-TRα1, FH-TRβ1, and MCS cells and FLAG and HA eluates from two tandem-affinity purification were verified by immunoblotting as previous described (21,22) using 1:1000 dilution HA-Tag (C29F4) Rabbit mAb (#3724, Cell Signaling Technology, Leiden, NL).

Proteomic analysis (Orbitrap LC-MS/MS)

Isolated proteins in the purified products were identified by Orbitrap LC-MS/MS as previously described (Chapter 6a). The peptide sequences identified by LC-MS/MS were mapped onto the reference amino acid sequences to determine individual proteins using Scaffold Viewer version 4.8.9 (Proteomic Software Inc., Oregon, USA). Proteins with at least two unique peptides assigned to them with 1% false detection rate (FDR) protein and peptide threshold were accepted as true identifications. Only proteins that were present in the two replicates of at least one TR and T3 conditions but absent in MCS control eluates were selected for further analysis. Individual proteins were clustered and presented as an expressing heat map using the Heatmapper online software (http://www.heatmapper.ca) (23). Pathway enrichment analysis was performed by DAVID functional annotation chart (DAVID Bioinformatics Resources 6.8, NIAID/NIH: https://david.ncifcrf.gov/) using default setting (count 2, ease 0.1). The gene-term enrichment was analyzed based on molecular function Gene Ontology terms. Statistical significance was considered when p-values of modified Fisher's exact test (EASE score) with Benjamini post-test < 0.05. The known protein-protein interactions (from curated databased and experimentally determined) between individual

proteins were analyzed by STRING database version 11.0 (http://string-db.org) with minimum required interaction scores set at medium confidence (0.400).

Results

The expression and transcriptional activation of FH-TRα1 and FH-TRβ1 in SH-SY5Y cells were evaluated before using the cells in TR purification. Both TR isoforms were detected with equal intensity on immunoblots of nuclear extracts (NEs) from FH-TRα1 and FH-TRβ1 expressing cells, showing similar expression levels of the TR isoforms in the cells (Figure 1A). FH-TRα1 and -TRβ1 cells also showed T3-dependent transactivation of the reporter gene in luciferase assays (Figure 1B), confirming that the stably expressed TRs have normal transcriptional activity, and the FH-tag did not interfere with receptor activity.

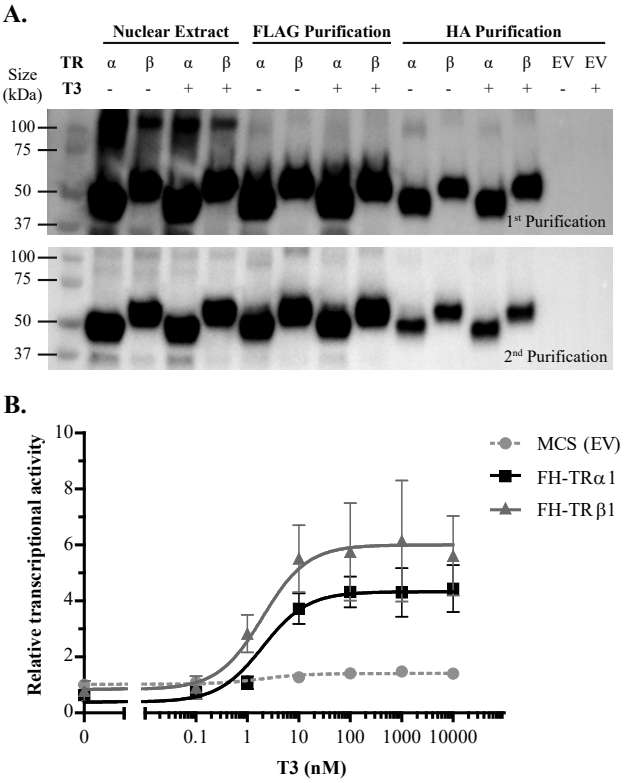


Figure 1. (A) Immunoblots show an equal amount of FH-TRα1 and FH-TRβ1 in nuclear extracts and purification products from SH-SY5Y cells of two tandem-affinity TR purifications (TR, thyroid hormone receptor; EV, empty vector control). (B) The T3-induced transcriptional activity of FH-TRα1, FH-TRβ1, and MCS (EV) confirms a normal function of stably expressing TRs in SH-SY5Y cells (data are presented as mean ± SEM of three independent experiments performed in triplicate).

To identify the nuclear coregulatory proteins that interact with TRs in SH-SY5Y cells, we performed two independent tandem-affinity TR purification using NEs of the FH-TRα1, FH-TRβ1, and MCS cells after 4 hours stimulation with 0 or 100 nM T3. TRs and associated proteins were sequentially co-immunoprecipitated using anti-FLAG and anti-HA resins. To estimate the recovery of the FH-TRs, proportional amounts of NEs and eluates from both FLAG- and HA-purification steps were analyzed by immunoblotting with HA antibodies. The amount of FH-TRα1 and -TRβ1 in the final (HA) eluates after tandem-affinity purification was slightly less than in the NE input but equal between conditions (Figure 1A), ensuring similar amounts of complexes in final eluates. These eluates were then subjected to LC-MS/MS analysis to identify distinct nuclear proteins associated with FH-TRs.

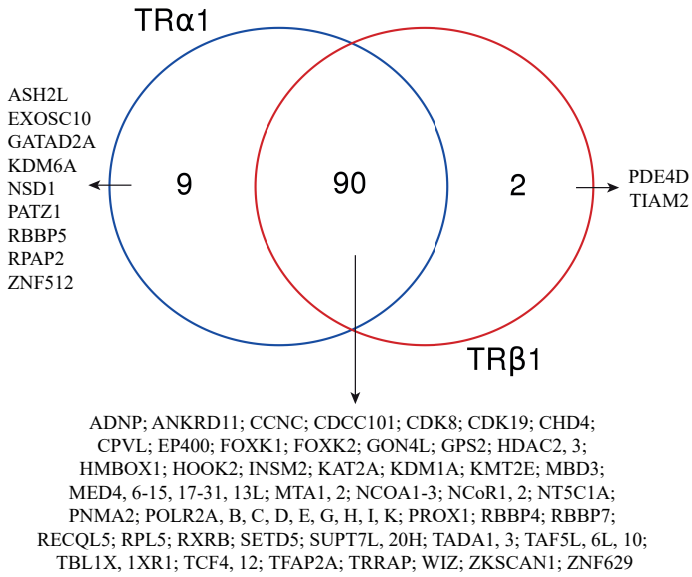


Figure 2. Venn diagram showing the number of distinct nuclear proteins that co-purified with FH-TRs. Most of the proteins are associated with both TR isoforms, indicating that TR action in SH-SY5Y cells is mainly regulated by common coregulatory proteins. However, eleven proteins were found to be exclusively associated with only one TR isoform (nine proteins for TRα1 and two proteins for TRβ1), suggesting an isoform-specific coregulatory protein recruitment.

One hundred and one different proteins were present in the two replicates of at least one TR (FH-TRα1 or -TRβ1) and T3 (0 or 100 nM) condition, but absent in MCS control eluates (Figure 2, Supplementary Table S1). Our strategy was validated by the identification of known TR-interacting proteins, such as RXRβ, the NCoR1/SMRT/HDAC3 corepressor complex, SRC coactivators, the Mediator complex, and the RNA polymerase II complex with a high percent coverage. Approximately 90% of the proteins overlapped between FH-TRα1

and FH-TRβ1, indicating that both TR isoforms mainly interact with the same coregulatory proteins to regulate gene transcription. However, nine proteins (8.9%) were only associated with FH-TRα1 (such as ASH2L, KDM6A (as known as UTX), and RBBP5) and two (2.0%) only with FH-TRβ1 (PDE4D and TIAM2) (Figure 2), suggesting that TRs could also be partly regulated by an isoform-specific subset of coregulatory proteins.

All identified proteins were then categorized by heat map analysis into two clusters (Figure 3). Cluster 1 consists of proteins that are predominantly identified in the absence of T3 (vehicle enriched group), and cluster 2 consists of proteins that are mainly identified in the presence of T3 (T3-enriched group). The molecular functions of proteins in these two clusters were explored by gene ontology (GO) enrichment analysis. The results showed that the proteins in the cluster 1 or vehicle enriched group (total N = 21) were significantly associated with transcriptional gene repression GO terms, such as transcription corepressor activity (N = 7, p-value <0.001) and histone deacetylase activity (N = 3, p-value <0.05) (Figure 4A). The proteins in the cluster 2 or T3-enriched group (total N = 80) were significantly associated with transcriptional gene activation GO terms, for instance, RNA polymerase II transcriptional cofactor activity (N = 22, p-value <0.01), transcriptional coactivator activity (N = 24, p-value <0.01), and histone acetyltransferase activity (N = 11, p-value <0.01) (Figure 4B).

Each cluster was subsequently analyzed for known interactions based on experimental data using the STRING online database (Figure 5). The analysis showed that the NCoR/SMRT complex was enriched in the absence of T3 for both isoforms. Apart from the direct TR-binding proteins NCoR1 and NCoR2/SMRT, the profiles included other members of the complex, such as HDAC3, transducin beta like 1 X-linked (TBL1X), transducing beta like 1 X-linked receptor 1 (TBL1XR1), and G-protein pathway suppressor 2 (GPS2). Many other proteins in this cluster have never been described to be associated with TRs, including the Forkhead box (FOX) transcription factor K1 and K2 (FOXK1 and FOXK2) proteins that are known to be part of Wnt/β-catenin signaling complex (Supplementary Table S2), ankyrin repeat domain-containing protein 11 (ANKRD11), and activity-dependent neuroprotector homeobox protein (ADNP).

The majority (77%) of the identified proteins co-purified with TRs in the presence of T3 (Figure 5B). The nuclear receptor coactivator (NCoA, also known as SRC) isoforms 1, 2, and 3, the Mediator complex, and RNA polymerase II complex that are known to associate with liganded TRs were all present in the T3-enriched cluster (Figure 5B, Supplementary Table S2). We also identified proteins belonging to chromatin remodeling complexes, including the nucleosome remodeling deacetylase (NuRD), the Spt-Ada-Gcn5-Acetyltransferase (SAGA), and the MLL/SET methyltransferase complexes (Figure 5B, Supplementary Table S2), all of which have not previously been reported to interact with TRs. The T3-enriched also contained other proteins which are unknown to interact with TRs and could not be categorized into any known complex, such as the transcription factors Prospero homeobox protein 1 (PROX1) and transcription factor 4 and 12 (TCF4 and TCF12).

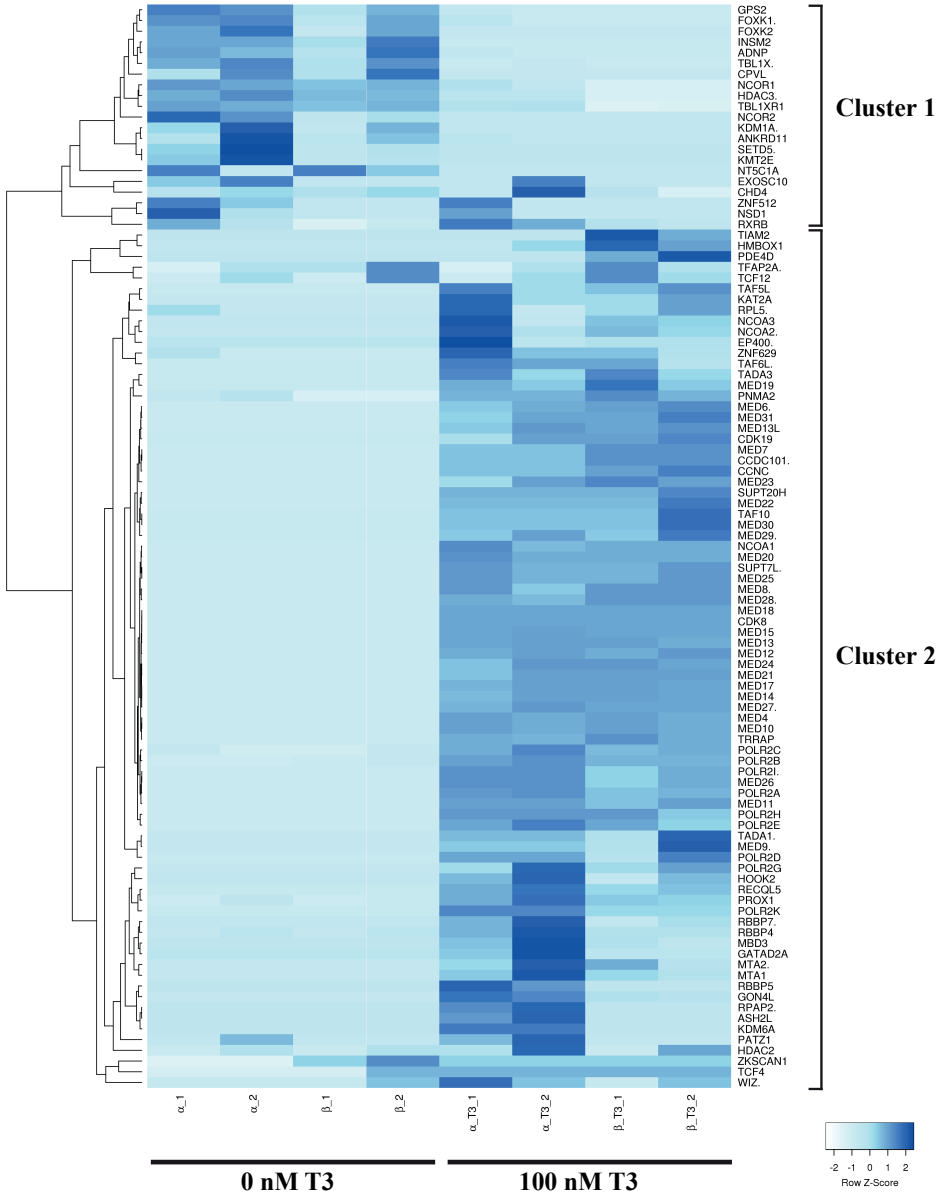


Figure 3. Heat map showing the profile of distinct nuclear proteins co-purified with FH-TRα1 and FH-TRβ1 after stimulating with 0 or 100 nM T3. The color intensity correlates with the Z-score of exclusive unique peptides identified by LC-MS/MS (white, low or undetectable level; blue, high level). According to hierarchical clustering (demonstrated by dendrogram on the left), the proteins are divided into two main clusters. The cluster 1 consists of proteins that are predominantly identified in the absence of T3 (vehicle enriched group), and the cluster 2 consists of proteins that are mainly identified in the presence of T3 (T3-enriched group). (Proteins are clustered by average linkage clustering and Pearson distance measurement methods using Heatmapper online software.)

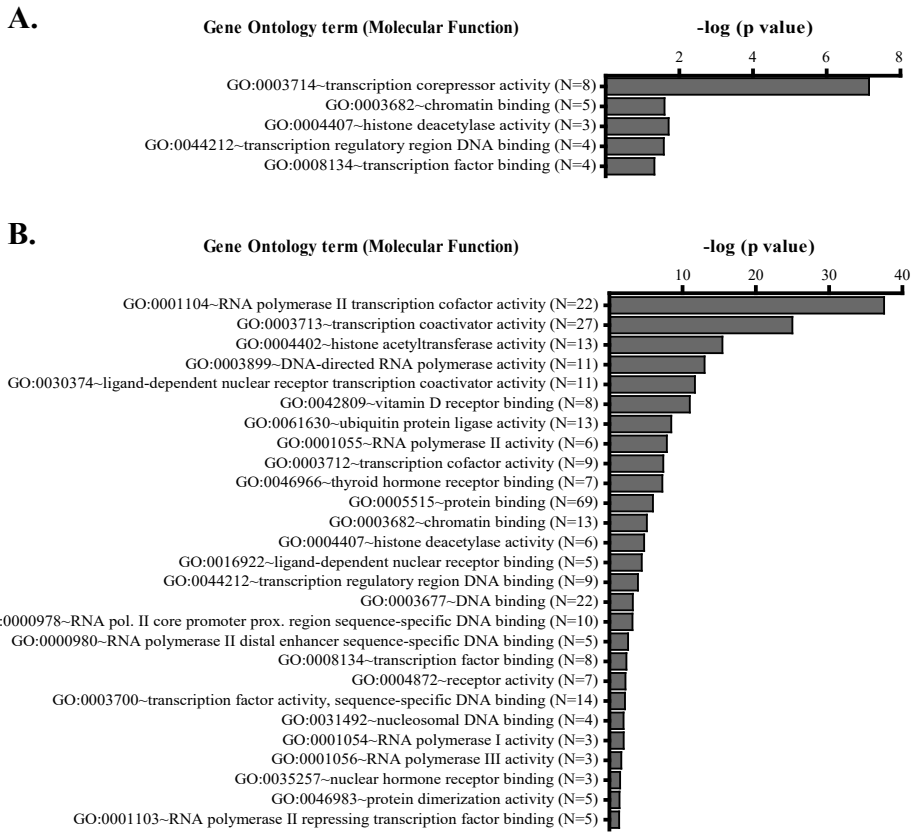
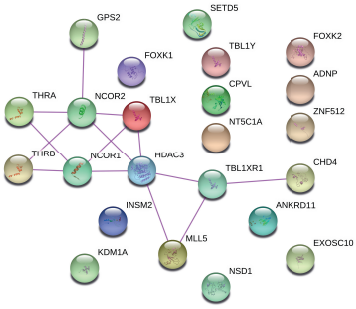


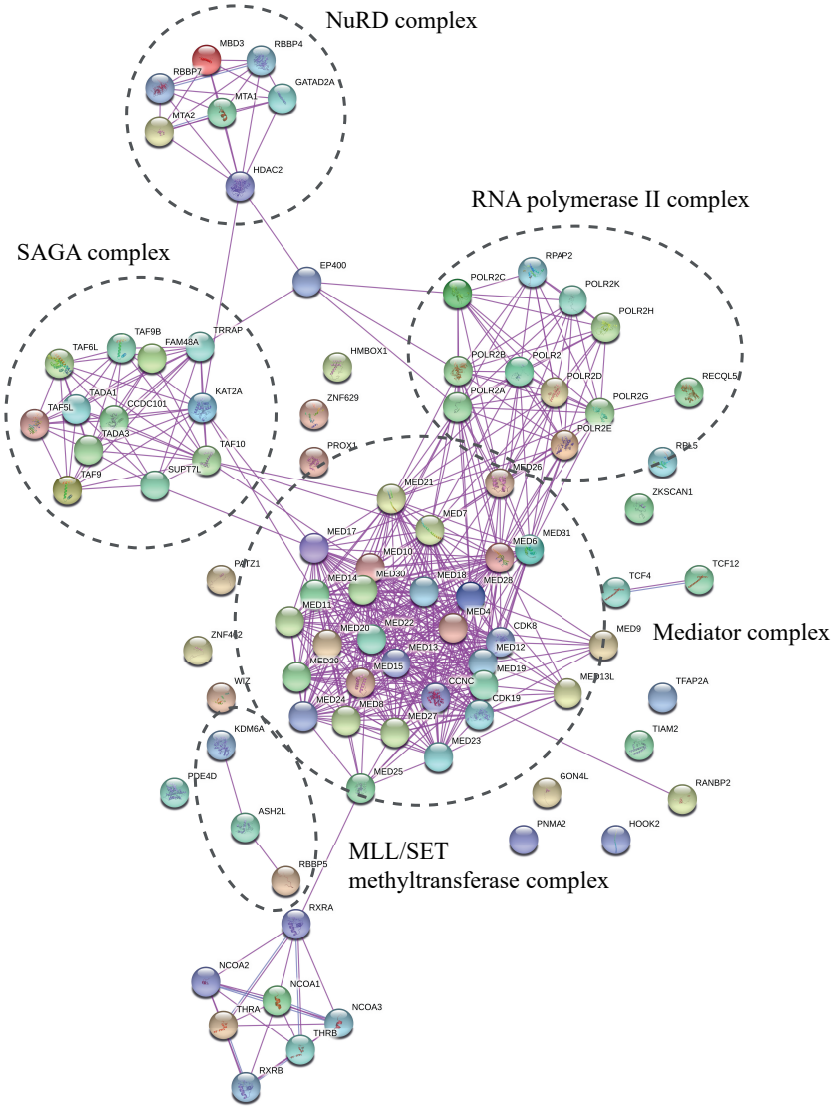
Figure 4. Gene Ontology term (molecular function) enrichment analysis. (A) The proteins found in the absence of T3 (cluster 1) are significantly related to transcriptional corepressor activity and histone deacetylase activity whereas (B) the proteins found in the presence of T3 (cluster 2) are related to transcriptional coactivator activity, histone acetyltransferase activity, and RNA polymerase II activity. Data are presented as –log (p value) of modified Fisher's exact test (EASE score) with Benjamini post-test from DAVID functional annotation chart [DAVID Bioinformatics Resources 6.8, NIAID/NIH]. (N = number of proteins associated to GO term)

Figure 5 (right page). Protein-protein interaction (PPI) network demonstrating known interactions (pink line) between identified proteins in the (A) absence and (B) presence of T3. Known TR-interacting proteins, such as the NCoR1, NCoR2 (SMRT), HDAC3, TBL1X, and TBL1XR1, in the absence of T3, and the SRC 1-3 coactivators, the Mediator complex, and RNA polymerase II complex, in the presence of T3, were identified, validating our strategy and suggesting a role for these proteins in neuronal TR activity. Strikingly, many other identified proteins are members of protein complexes that are involved in chromatin remodeling but have never been described as TR coregulators, for instance, ADNP, ANKRD11, FOXK1, and FOXK2, all of which were co-purified with TRs in the absence of T3, and the nucleosome remodeling deacetylase (NuRD), the Spt-Ada-Gcn5-Acetyltransferase (SAGA) complexes, and the MLL/SET methyltransferase complex, all of which were co-purified with TRs in the presence of T3. (Data are analyzed by STRING version 11.0)

A.


SETD5
GPS2
FOXK2
TBL1Y
FOXK1
ADNP
CPVL
THRA
NCOR2
TBL1X
ZNF512
NT5C1A
THRB
NCOR1
HDAC3
CHD4
TBL1XR1
INSM2
ANKRD11
MLL5
EXOSC10
KDM1A
NSD1

B.


NuRD complex
MBD3
RBBP4
RBBP7
GATAD2A
MTA1
MTA2
HDAC2
RNA polymerase II complex
SAGA complex
EP400
RPAP2
POLR2C
POLR2K
POLR2H
TAF6L
TAF9B
FAM48A
TRRAP
HMBOX1
POLR2B
POLR2
POLR2D
RECQL5
TADA1
KAT2A
POLR2A
POLR2G
TAF5L
CCDC101
ZNF629
POLR2E
TADA3
TAF10
PROX1
RPA5
TAF9
SUPT7L
MED26
MED21
ZKSCAN1
MED7
MED17
MED31
MED10
MED6
PATZ1
MED30
MED14
MED18
MED28
TCF4
TCF12
MED11
MED4
MED22
CDK8
ZNF462
MED20
MED9
Mediator complex
MED13
MED12
MED15
MED19
CCNC
MED13L
WIZ
TFAP2A
MED24
MED8
CDK19
MED27
KDM6A
TIAM2
MED23
PDE4D
MED25
6ON4L
RANBP2
ASH2L
PNMA2
HOOK2
RBBP5
MLL/SET
methyltransferase complex
RXRA
NCOA2
NCOA1
NCOA3
THRA
THRB
RXRB

Discussion

In this study, we identified known and potentially novel binding partners for TRs in a human neuronal cell model. The majority of identified proteins associated with both TRα1 and TRβ1, suggesting that these two TR isoforms interact with common coregulatory proteins to regulate gene transcription. However, a subset of distinct nuclear proteins seems to interact with TRs in an isoform-specific manner.

We successfully identified a large number of TR associated proteins in this study. The yield of our study is higher than previous reports (17,18). Although the tandem-affinity TR purification technique we used was adopted from Fozzatti et al. (17), we performed direct LC-MS/MS analysis of the eluates rather than separated proteins by SDS-PAGE gel and subjected only proteins that are visualized on gel to LC-MS/MS analysis, which may lead to a higher yield. Therefore, we suggest that the methods we used is an optimal and effective technique for the interactome analysis of TRs. We also replicated the purifications for each condition and found some previously reported TR specific proteins in our controls. We, therefore, suggest to replicate the purifications as well, in order to increase the reliability of the results.

The nuclear proteins associated with TRs were categorized by heat map and hierarchical clustering into two groups. A vehicle enriched group is a group of proteins identified in the absence of T3, and a T3-enriched group is a group of proteins identified in the presence of T3. Gene ontology enrichment analyses confirmed this separation since the proteins from the two groups were significantly enriched with transcriptional gene repression and activation terms, respectively.

In the vehicle enriched group, we identified two well-known TR corepressors, NCoR1 and SMRT (NCoR2), showing the effectiveness of our approach. We also identified other members of the NCoR/SMRT complex that do not directly interact with TRs, namely HDAC3, TBL1X, TBL1XR1, and GPS2 (24-27), which shows that our method preserves protein complexes and can find distant binding partners for TRs. HDAC3 is responsible for remodeling of the chromatin by deacetylating histone tails, which promotes transcriptional repression of the TRs (24,27). TBL1X and its homolog TBL1XR1 stabilize the NCoR/SMRT complexes on the chromatin and promote efficient histone deacetylation by HDAC3 (26). GPS2 associates with the NCoR/SMRT complex to regulate gene repression of estrogen receptor (ER) α (28) and peroxisome proliferator-activated receptor (PPAR) γ (29). Although Guo et al. showed that the transcriptional activity of TRα1 is not affected in embryonic fibroblast cells derived from GPS2 knock-out mice (29), this does not necessarily rule out a role for GPS2 in neuronal T3-regulated gene expression.

Another protein that was identified in the absence of T3 is MLL5. This protein has been grouped into the lysine N-methyltransferase (KMTs) family because of its structural homology; however, it lacks intrinsic histone methyltransferase (HMT) activity (30). An orthologue protein, SETD5, was also found in the absence of T3 but with a lower peptide count than MLL5.

Osipovich et al. showed that SETD5 associates with NCoR repressor complexes (NCoR1, TBL1X, and HDAC3) and regulates histone deacetylation in HEK 293T cells (31). Evidence in yeast shows that Set3 (orthologue of human MLL5) interacts with Snt1 and Sif2 (i.e. Hif2) proteins (orthologues of human SMRT and TBL1X, respectively) to regulate gene transcription (32,33). Recently, MLL5 was found to interact with SMRT and also TBL1X and TBL1XR1 in nuclear extracts of HeLa cells (34)(www.nursa.org/10.1621/datasets.01002), indicating the role of this protein in corepressor complex. Therefore, the identification of MLL5 and SETD5 in our profiles suggests that they may play a role in transcriptional gene repression by TRs in neuronal cells as well.

Two closely related Forkhead box (FOX) transcription factors, FOXK1 and FOXK2, were also identified in the absence of T3 and have previously been shown to regulate gene repression. FOXK1/2, in combination with Sin3A-HDAC transcriptional repression complexes (Sin3A, SIN3 transcription regulator family member A), inhibit expression of cell autophagy genes in muscle cells and fibroblast (35). FOXK1 also interacts with Ten-eleven translocation 1 (TET1), a tumor suppressor protein, in breast cancer cell line and repress vascular endothelial growth factor VEGF gene expression (36). Wang et al. reported that FOXK1 and 2 positively regulate Wnt/β-catenin pathway by promoting the nuclear localization of phosphorylated Dishevelled (DVL) protein which helps to stabilize β-catenin/T-cell factor (TCF) transcriptional complex at the promoter region of Wnt target genes (37). Although the interaction between TRs and FOXK1/2 is unknown, both physical and functional interactions between TRs and Wnt/β-catenin signaling have been established (38-40).

In the T3-enriched group, we found many know TR-coactivator proteins, including SRC 1, 2, and 3, the RNA polymerase II complex, and the Mediator complex, together with both TR isoforms. In addition, several proteins belonging to the NuRD complex were co-purified with both TR isoforms in the presence of T3. Although originally identified as a gene repressing complex, subsequent studies have shown that the NuRD complex is involved in both transcriptional repression (41-43) and activation (44) by forming direct interactions with many nuclear receptors (45-47). The identification of the NuRD complex in the presence of T3 was similar to our previous TRα1 interactomes (Chapter 6a), suggesting that this complex may take part in T3-induced transcriptional activation. However, we and others (17) identified CHD4, one of the core components of the NuRD complex, also with TRs in the absence of T3. Ostapcuk et al. showed that apart from the NuRD complex, CHD4 can also form a complex with ADNP and HP1β and γ (heterochromatin protein 1 β and γ), called ChAHP, to represses gene transcription, which seems to be essential for neuronal cells differentiation (48). Since we and others (17) identified both CHD4 and ADNP with TRs in the absence of T3, TRs may interact with the ChAHP complex to facilitate T3-dependent gene repression as well.

The SAGA complex is one of the known coactivator complex which consists of a core structural module, a histone deubiquitinase module (DUB), a histone acetyltransferase module (HAT), and an activator-biding module (49,50). In this study, we co-purified many

nuclear proteins that belong to the SAGA complex with the TRs in the presence of T3, including members of core structural module (TAF5L, TAF6L, TAF10, SUPT7L, and SUPT20H), HAT module (KAT2A, TADA3, and CCDC101), and activator-binding module (TRRAP). A recent study showed that SAGA is recruited to upstream activating sequences (UASs) of most RNA polymerase II-transcribed genes (51,52), indicating that SAGA may act as a cofactor to modify chromatin structure and recruit the preinitiation complex (PIC) for transcriptional activation. To our knowledge, this is the first study showing an interaction between the SAGA complex and TRs.

Although the majority of identified proteins were associated with both TR isoforms, we identified a subset of nuclear proteins interacts with TRs in an isoform-specific manner, which is in line with previous reports (17,18). The confidence of these hits being isoform-specific is strengthened by the fact that they were explicitly identified with certain TR isoform in two independent purifications. Interestingly, we identified that lysine-specific demethylase 6A (KDM6A or UTX), set1/Ash2 histone methyltransferase complex subunit ASH2 (ASH2L), and retinoblastoma-binding protein 5 (RBBP5) are specific for TRα1. KDM6A removes repressive histone marks (H3K27me), and ASH2L and RBBP5, in combination with MLL1-4 (KMT2A-D) and WDR5 proteins, subsequently add active histone marks (H3K4me) to establish transcriptionally permissive chromatin for many key developmental genes such as HOX genes (53-56). The importance of these proteins in neurodevelopment was postulated because mutations in genes encoding KDM6A and MLL4 lead to a similar phenotype, including growth retardation, intellectual disability, and characteristic facial features (long palpebral fissures and ectropion of the lateral 1/3 of lower eyelids), as known as Kabuki syndrome (55,57,58). However, further studies are needed to explore the specificity for TRα1 and the role in transcriptional regulation of these proteins.

In summary, we identified known and potential novel binding partners of TRs in SH-SY5Y cells. In addition, a subset of distinct nuclear proteins seems to interact with TRs in an isoform-specific manner. These findings enable us to gain more understanding of the transcriptional gene regulation by TRs in the brain. However, additional experiments are needed to confirm the interaction between these proteins and TRs and to elucidate a functional relevance of these proteins in TR actions.

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Author Disclosure Statement

The authors have nothing to disclose.

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Supplementary Table S1. List of proteins identified by LC-MS/MS.

Protein	0 nM T3												100 nM T3											
	TRα1						TRβ1						TRα1						TRβ1					
	1st			2nd			1st			2nd			1st			2nd			1st			2nd		
	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob
ADNP	9	11.7	100.0	7	10.7	100.0	3	4.3	100.0	12	18.4	100.0	1	1.1	12.3	0	0	0	0	0	0	0	0	0
ANKRD11	3	2.8	100.0	17	11.4	100.0	1	0.4	99.7	7	4.3	100.0	1	0.3	8.6	0	0	0	0	0	0	0	0	0
ASH2L	0	0	0	0	0	0	0	0	0	0	0	0	2	3.8	99.9	3	6.4	100.0	0	0	0	0	0	0
CCDC101	0	0	0	0	0	0	0	0	0	0	0	0	2	8.9	100.0	2	8.2	100.0	3	13.3	100.0	3	10.6	100.0
CCNC	0	0	0	0	0	0	0	0	0	0	0	0	3	9.9	100.0	3	8.5	100.0	4	14.5	100.0	5	20.1	100.0
CDK19	0	0	0	0	0	0	0	0	0	0	0	0	4	16.7	100.0	8	31.5	100.0	8	26.7	100.0	10	36.5	100.0
CDK8	0	0	0	0	0	0	0	0	0	0	0	0	8	19.4	100.0	8	23.5	100.0	8	22.4	100.0	8	25.2	100.0
CHD4	4	2.8	100.0	6	5.7	100.0	5	4.9	100.0	6	4.4	100.0	3	3.5	100.0	12	11.5	100.0	4	4.1	100.0	1	1.15	98.7
CPVL	2	4.2	100.0	6	13.9	100.0	2	3.8	100.0	7	16.8	100.0	0	0	0	0	0	0	0	0	0	0	0	0
EP400	0	0	0	0	0	0	0	0	0	0	0	0	8	4.0	100.0	0	0	0	1	1.0	16.8	1	0.5	39.8
EXOSC10	1	1.1	27.1	2	3.3	100.0	0	0	0	0	0	0	0	0	0	2	3.3	99.9	0	0	0	0	0	0
FOXK1	9	20.9	100.0	10	22.0	100.0	2	3.7	100.0	8	18.0	100.0	2	4.1	99.8	0	0	0	0	0	0	0	0	0
FOXK2	4	7.0	100.0	6	14.8	100.0	0	0	0	4	14.1	100.0	0	0	0	0	0	0	0	0	0	0	0	0
GATAD2A	0	0	0	0	0	0	0	0	0	0	0	0	1	3.8	99.4	3	7.6	100.0	0	0	0	0	0	0
GON4L	0	0	0	0	0	0	0	0	0	0	0	0	20	13.8	100.0	18	11.9	100.0	5	3.4	100.0	3	2.2	100.0
GPS2	9	41.9	100.0	8	42.5	100.0	3	15.9	100.0	6	24.5	100.0	1	2.5	87.4	0	0	0	0	0	0	0	0	0
HDAC2	0	0	0	1	3.3	87.6	0	0	0	1	9.0	92.70	1	1.6	87.4	3	11.1	100.0	0	0	0	2	5.5	99.9
HDAC3	17	48.4	100.0	21	66.4	100.0	15	42.8	100.0	15	44.6	100.0	7	25.9	100.0	7	27.6	100.0	0	0	0	0	0	0
HMBOX1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4.5	100.0	3	10.2	100.0	2	8.6	100.0
HOOK2	0	0	0	0	0	0	0	0	0	0	0	0	1	1.0	38.7	2	3.3	93.1	0	0	0	1	1.0	39.3
INSM2	13	38.9	100.0	13	40.6	100.0	7	19.8	100.0	18	57.2	100.0	0	0	0	0	0	0	0	0	0	0	0	0

Protein	0 nM T3												100 nM T3											
	TRα1						TRβ1						TRα1						TRβ1					
	1st			2nd			1st			2nd			1st			2nd			1st			2nd		
	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob
KAT2A	0	0	0	0	0	0	0	0	0	0	0	0	3	4.7	100.0	1	1.3	75.7	1	2.3	97.7	2	3.1	99.9
KDM1A	3	4.5	100.0	10	17.0	100.0	0	0	0	5	8.6	100.0	0	0	0	0	0	0	0	0	0	0	0	0
KDM6A	0	0	0	0	0	0	0	0	0	0	0	0	3	3.9	100.0	3	4.1	100.0	0	0	0	0	0	0
KMT2E	3	2.9	100.0	9	6.9	100.0	0	0	0	1	1.3	40.7	0	0	0	0	0	0	0	0	0	0	0	0
MBD3	0	0	0	0	0	0	0	0	0	0	0	0	2	8.9	99.8	5	19.9	100.0	1	5.5	67.1	0	0	0
MED10	0	0	0	0	0	0	0	0	0	0	0	0	7	60.0	100.0	6	57.8	100.0	7	59.3	100.0	6	57.8	100.0
MED11	0	0	0	0	0	0	0	0	0	0	0	0	4	55.6	100.0	4	55.6	100.0	3	49.6	100.0	4	55.6	100.0
MED12	0	0	0	0	0	0	0	0	0	0	0	0	53	33.6	100.0	58	32.7	100.0	52	32.2	100.0	61	39.2	100.0
MED13	0	0	0	0	0	0	0	0	0	0	0	0	49	30.6	100.0	52	31.7	100.0	51	32.0	100.0	46	29.5	100.0
MED13L	0	0	0	0	0	0	0	0	0	0	0	0	25	20.5	100.0	39	31.4	100.0	35	27.9	100.0	40	31.9	100.0
MED14	0	0	0	0	0	0	0	0	0	0	0	0	34	31.7	100.0	43	37.6	100.0	42	36.1	100.0	41	37.9	100.0
MED15	0	0	0	0	0	0	0	0	0	0	0	0	12	23.0	100.0	13	23.9	100.0	12	23.0	100.0	12	21.1	100.0
MED17	0	0	0	0	0	0	0	0	0	0	0	0	24	43.8	100.0	28	50.8	100.0	28	51.2	100.0	27	45.3	100.0
MED18	0	0	0	0	0	0	0	0	0	0	0	0	3	15.4	100.0	3	15.4	100.0	3	15.4	100.0	3	15.4	100.0
MED19	0	0	0	0	0	0	0	0	0	0	0	0	4	32.4	100.0	3	21.7	100.0	6	42.2	100.0	3	21.7	100.0
MED20	0	0	0	0	0	0	0	0	0	0	0	0	6	31.1	100.0	5	26.9	100.0	5	29.2	100.0	5	29.2	100.0
MED21	0	0	0	0	0	0	0	0	0	0	0	0	3	39.6	100.0	4	53.5	100.0	4	53.5	100.0	4	53.5	100.0
MED22	0	0	0	0	0	0	0	0	0	0	0	0	3	12.0	100.0	3	14.0	100.0	3	18.5	100.0	5	23.0	100.0
MED23	0	0	0	0	0	0	0	0	0	0	0	0	11	10.8	100.0	20	17.6	100.0	24	24.7	100.0	20	17.4	100.0
MED24	0	0	0	0	0	0	0	0	0	0	0	0	17	23.2	100.0	23	28.9	100.0	23	29.5	100.0	21	26.8	100.0
MED25	0	0	0	0	0	0	0	0	0	0	0	0	6	19.0	100.0	5	17.4	100.0	5	13.4	100.0	6	20.2	100.0
MED26	0	0	0	0	0	0	0	0	0	0	0	0	13	29.7	100.0	13	36.5	100.0	8	19.7	100.0	11	25.2	100.0

Protein	0 nM T3												100 nM T3											
	TRα1						TRβ1						TRα1						TRβ1					
	1st			2nd			1st			2nd			1st			2nd			1st			2nd		
	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob
MED27	0	0	0	0	0	0	0	0	0	0	0	0	9	28.6	100.0	11	39.5	100.0	10	27.7	100.0	10	30.5	100.0
MED28	0	0	0	0	0	0	0	0	0	0	0	0	7	47.8	100.0	6	41.0	100.0	8	46.1	100.0	8	48.9	100.0
MED29	0	0	0	0	0	0	0	0	0	0	0	0	2	13.0	100.0	3	19.0	100.0	2	13.0	100.0	4	34.0	100.0
MED30	0	0	0	0	0	0	0	0	0	0	0	0	1	5.1	96.2	1	5.1	96.2	1	5.1	96.4	2	11.8	100.0
MED31	0	0	0	0	0	0	0	0	0	0	0	0	2	24.4	100.0	3	38.9	100.0	3	31.3	100.0	4	40.5	100.0
MED4	0	0	0	0	0	0	0	0	0	0	0	0	10	43.0	100.0	9	39.6	100.0	10	43.3	100.0	9	39.6	100.0
MED6	0	0	0	0	0	0	0	0	0	0	0	0	8	30.1	100.0	10	37.8	100.0	11	40.7	100.0	13	45.1	100.0
MED7	0	0	0	0	0	0	0	0	0	0	0	0	2	15.0	100.0	2	15.0	100.0	3	24.5	100.0	3	24.5	100.0
MED8	0	0	0	0	0	0	0	0	0	0	0	0	6	29.9	100.0	4	20.9	100.0	6	28.4	100.0	6	32.5	100.0
MED9	0	0	0	0	0	0	0	0	0	0	0	0	2	17.8	100.0	2	12.3	99.9	1	12.3	96.4	5	40.4	100.0
MTA1	0	0	0	0	0	0	0	0	0	0	0	0	6	15.1	100.0	15	29.4	100.0	5	14.4	100.0	3	10.9	100.0
MTA2	0	0	0	0	0	0	0	0	0	0	0	0	3	8.1	100.0	9	21.7	100.0	5	11.8	100.0	1	2.4	69.3
NCOA1	0	0	0	0	0	0	0	0	0	0	0	0	36	34.4	100.0	25	26.2	100.0	29	32.8	100.0	29	31.9	100.0
NCOA2	0	0	0	0	0	0	0	0	0	0	0	0	12	12.5	100.0	3	2.7	100.0	6	5.5	100.0	4	4.8	100.0
NCOA3	0	0	0	0	0	0	0	0	0	0	0	0	12	12.6	100.0	0	0	0	5	5.4	100.0	4	4.5	100.0
NCOR1	105	52.8	100.0	96	50.5	100.0	81	43.6	100.0	86	45.3	100.0	54	32.6	100.0	29	17.3	100.0	2	1.4	100.0	1	0.7	95.6
NCOR2	35	22.2	100.0	26	17.3	100.0	2	3.0	98.0	10	7.1	100.0	1	2.9	71.1	0	0	0	0	0	0	0	0	0
NSD1	5	2.5	100.0	1	0.5	53.8	0	0	0	0	0	0	3	1.8	100.0	0	0	0	0	0	0	0	0	0
NT5C1A	2	7.3	98.6	0	0	0	2	9.2	100.0	1	4.6	96.8	0	0	0	0	0	0	0	0	0	0	0	0
PATZ1	0	0	0	1	1.9	96.8	0	0	0	0	0	0	1	1.9	96.2	2	2.9	99.9	0	0	0	0	0	0
PDE4D	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3.2	96.4	2	5.9	100.0
PNMA2	2	14.0	100.0	3	16.5	100.0	0	0	0	0	0	0	6	22.3	100.0	6	23.4	100.0	8	27.5	100.0	6	23.4	100.0

Protein	0 nM T3												100 nM T3											
	TRα1						TRβ1						TRα1						TRβ1					
	1st			2nd			1st			2nd			1st			2nd			1st			2nd		
	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob
POLR2A	0	0	0	0	0	0	0	0	0	0	0	0	34	18.8	100.0	37	21.8	100.0	25	17.3	100.0	28	18.7	100.0
POLR2B	1	1.6	76.6	0	0	0	2	2.4	99.5	4	4.9	100.0	25	25.3	100.0	28	25.0	100.0	21	19.9	100.0	21	17.9	100.0
POLR2C	2	12.0	100.0	0	0	0	0	0	0	2	13.5	100.0	10	52.7	100.0	13	69.5	100.0	9	46.9	100.0	10	56.7	100.0
POLR2D	0	0	0	0	0	0	0	0	0	0	0	0	3	28.2	100.0	3	34.5	100.0	1	11.3	98.7	4	40.8	100.0
POLR2E	0	0	0	0	0	0	0	0	0	0	0	0	3	17.1	100.0	4	21.4	100.0	3	17.1	100.0	2	9.1	97.3
POLR2G	0	0	0	0	0	0	0	0	0	0	0	0	1	12.8	78.0	3	23.8	100.0	1	12.8	96.4	2	17.4	99.9
POLR2H	0	0	0	0	0	0	0	0	0	0	0	0	3	34.7	100.0	3	34.7	100.0	3	34.7	100.0	2	16.7	99.5
POLR2I	0	0	0	0	0	0	0	0	0	0	0	0	5	56.8	100.0	5	56.8	100.0	3	40.8	100.0	4	46.4	100.0
POLR2K	0	0	0	0	0	0	0	0	0	0	0	0	2	13.8	94.3	2	13.8	93.7	1	12.1	89.8	1	12.1	39.3
PROX1	0	0	0	5	8.3	100.0	0	0	0	2	3.8	99.9	18	34.6	100.0	28	48.6	100.0	14	28.4	100.0	13	22.4	100.0
RBBP4	0	0	0	1	3.8	96.8	0	0	0	1	3.8	81.4	5	29.9	100.0	10	61.2	100.0	2	14.1	100.0	2	12.0	100.0
RBBP5	0	0	0	0	0	0	0	0	0	0	0	0	3	7.6	100.0	2	5.8	100.0	0	0	0	0	0	0
RBBP7	0	0	0	0	0	0	0	0	0	0	0	0	2	12.2	85.8	4	30.4	100.0	0	0	0	1	5.7	99.8
RECQL5	0	0	0	0	0	0	0	0	0	0	0	0	12	14.0	100.0	18	24.8	100.0	8	11.6	100.0	10	16.0	100.0
RPAP2	0	0	0	0	0	0	0	0	0	0	0	0	3	8.0	100.0	4	6.7	100.0	0	0	0	0	0	0
RPL5	1	3.4	93.6	0	0	0	0	0	0	0	0	0	3	18.5	100.0	0	0	0	1	11.8	96.4	2	16.5	100.0
RXRB	14	41.3	100.0	8	33.8	100.0	2	6.6	100.0	5	22.7	100.0	18	52.7	100.0	14	49.9	100.0	9	38.3	100.0	7	28.9	100.0
SETD5	2	2.8	100.0	7	9.3	100.0	0	0	0	1	1.8	49.7	0	0	0	0	0	0	0	0	0	0	0	0
SUPT20H	0	0	0	0	0	0	0	0	0	0	0	0	5	10.8	100.0	5	10.4	100.0	5	8.6	100.0	7	17.3	100.0
SUPT7L	0	0	0	0	0	0	0	0	0	0	0	0	5	14.3	100.0	4	11.8	100.0	4	12.3	100.0	5	14.3	100.0
TADA1	0	0	0	0	0	0	0	0	0	0	0	0	2	8.4	100.0	2	8.7	90.2	1	3.9	73.4	4	17.3	100.0
TADA3	0	0	0	0	0	0	0	0	0	0	0	0	4	11.1	100.0	2	7.6	100.0	4	14.6	100.0	2	7.6	100.0

Protein	0 nM T3												100 nM T3											
	TRα1						TRβ1						TRα1						TRβ1					
	1st			2nd			1st			2nd			1st			2nd			1st			2nd		
	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob	UPC	% Cov	% Prob
TAF10	0	0	0	0	0	0	0	0	0	0	0	0	1	11.5	96.2	1	11.5	65.8	1	11.5	85.5	2	17.9	100.0
TAF5L	0	0	0	0	0	0	0	0	0	0	0	0	7	13.6	100.0	3	6.1	100.0	4	8.0	100.0	6	12.1	100.0
TAF6L	0	0	0	0	0	0	0	0	0	0	0	0	4	10.5	100.0	3	7.2	100.0	3	7.2	100.0	1	2.4	98.3
TBL1X	10	39.3	100.0	13	54.2	100.0	5	32.1	100.0	12	56.5	100.0	0	0	0	1	19.9	100.0	0	0	0	0	0	0
TBL1XR1	27	74.7	100.0	25	80.2	99.7	22	65.8	100.0	24	80.2	100.0	16	59.5	100.0	17	60.7	100.0	3	14.6	100.0	4	16.9	100.0
TCF12	0	0	0	1	4.11	95.4	0	0	0	2	5.0	99.9	0	0	0	1	2.8	96.2	2	6.5	100.0	1	2.8	96.3
TCF4	0	0	0	0	0	0	0	0	0	1	2.4	96.8	1	3.8	99.8	1	2.4	96.2	1	3.8	99.8	1	2.4	96.3
TFAP2A	0	0	0	1	4.6	96.8	1	4.6	97.9	2	6.6	100.0	0	0	0	1	4.6	96.2	2	6.6	100.0	1	4.6	96.3
THRA	25	60.6	100.0	25	61.2	100.0	2	11.2	100.0	1	8.37	99.7	27	59.2	100.0	20	54.7	100.0	1	6.7	99.7	1	8.4	99.9
THRB	1	6.9	100.0	1	6.9	100.0	26	59.9	100.0	32	66.6	100.0	2	10.8	100.0	2	8.5	100.0	29	63.6	100.0	28	65.9	100.0
TIAM2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1.2	92.1	1	0.8	28.6
TRRAP	0	0	0	0	0	0	0	0	0	0	0	0	17	5.5	100.0	16	6.3	100.0	20	7.2	100.0	17	6.8	100.0
WIZ	0	0	0	0	0	0	0	0	0	1	1.2	29.1	2	2.2	99.6	1	1.0	9.5	0	0	0	1	1.2	99.0
ZKSCAN1	0	0	0	0	0	0	2	3.7	99.5	3	7.1	100.0	2	5.3	100.0	2	4.6	98.9	2	3.7	99.9	2	5.0	100.0
ZNF512	2	5.5	99.8	1	2.3	32.3	0	0	0	0	0	0	2	4.9	100.0	0	0	0	0	0	0	0	0	0
ZNF629	1	1.5	97.5	0	0	0	0	0	0	0	0	0	4	6.0	100.0	2	3.0	97.3	2	2.7	100.0	1	1.5	89.5

UPC, exclusive unique peptide count; **%Cov**, percent coverage; **%Prob**, percent probability

Supplementary Table S2. Identified TR-interacting proteins are associated in large protein complexes.

Protein complex	Identified proteins	Associated condition
NCoR/SMRT corepressor	NCoR1; SMRT (NCoR2); HDAC3; TBL1X; TBL1XR1; GPS2	Unliganded TRα and β
Wnt/β-catenin signalling	FOXK1; FOXK2	Unliganded TRα and β
RNA polymerase II	POLR2A, B, C, D, E, G, H, I, K; RECQL5; RPAP2	Liganded TRα and β
Mediators	CCNC; CKD8, 19; MED 4, 6-15, 17-31, 13L	Liganded TRα and β
MLL/SET methyltransferase	ASH2L; KDM6A; RBBP5	Liganded TRα only
NuRD (Nucleosome Remodeling Deacetylase)	GATAD2A*; MBD3; MTA1, 2; CHD4**; RBBP4, 7	Liganded TRα and β
SAGA (Spt-Ada-Gcn5-Acetyltransferase)	CCDC101 (SGF29); KAT2A (GCN5); SUPT20H; SUPT7L; TADA3; TAF5L, 6L, 10; TRRAP	Liganded TRα and β

*TRα1 only; **predominant with unliganded TRs