

A coregulator shift, rather than the canonical switch, underlies thyroid hormone action in the liver

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Thyroid hormones (THs) are powerful regulators of metabolism with major effects on body weight, cholesterol, and liver fat that have been exploited pharmacologically for many years. Activation of gene expression by TH action is canonically ascribed to a hormone-dependent “switch” from corepressor to activator binding to thyroid hormone receptors (TRs), while the mechanism of TH-dependent repression is controversial. To address this, we generated a mouse line in which endogenous TR β 1 was epitope-tagged to allow precise chromatin immunoprecipitation at the low physiological levels of TR and defined high-confidence binding sites where TRs functioned at enhancers regulated in the same direction as the nearest gene in a TR β -dependent manner. Remarkably, although positive and negative regulation by THs have been ascribed to different mechanisms, TR binding was highly enriched at canonical DR4 motifs irrespective of the transcriptional direction of the enhancer. The canonical NCoR1/HDAC3 corepressor complex was reduced but not completely dismissed by TH and, surprisingly, similar effects were seen at enhancers associated with negatively as well as positively regulated genes. Conversely, coactivator CBP was found at all TH-regulated enhancers, with transcriptional activity correlating with the ratio of CBP to NCoR rather than their presence or absence. These results demonstrate that, in contrast to the canonical “all or none” coregulator switch model, THs regulate gene expression by orchestrating a shift in the relative binding of corepressors and coactivators.

[*Keywords:* ligand regulation; negative regulation; nuclear receptor coregulators; nuclear receptors; thyroid receptor]

Supplemental material is available for this article.

Received October 12, 2020; revised version accepted January 12, 2021.

Thyroid hormones (THs) have been known to be powerful regulators of metabolism for >100 yr (Tata 2013; Mullur et al. 2014). In addition to its levels being dysregulated in millions of people with thyroid disease, the hormone or related compounds have long been used pharmacologically to treat metabolic diseases (Kowalik et al. 2018). THs were once a preferred treatment for obesity, until side effects were deemed too harmful. More recently, TH analogs have demonstrated potential for treating fatty liver and nonalcoholic steatohepatitis (NASH) (Sinha et al. 2019). However, >30 yr after the cloning of TH receptors (TRs) as members of the nuclear receptor (NR) superfamily, the mechanism of TH action remains enigmatic.

TH executes its function on gene-network regulation by activating TH receptors (TRs) on chromatin (Sap et al.

1986; Weinberger et al. 1986; Glass et al. 1987; Murray et al. 1988; Hodin et al. 1989). Three functional TRs are encoded from two genes: TR α 1 is encoded from the *Thra* gene, and TR β 1 and TR β 2 are splice variants of *Thrb* (Lazar 2003). The three proteins are homologous and their separate in vivo function is mostly explained by specificity in tissue expression rather than sequence differences (Forrest et al. 1990, 1991; Strait et al. 1990; Bradley et al. 1992). Liver is one of the most important targets of TH where TR β 1 is the predominant isoform (Weiss et al. 1998). While TH action in the liver includes carbohydrate, fatty acids, and cholesterol homeostasis, thyroid dysfunction can cause impairment of liver physiological function

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Article published online ahead of print. Article and publication date are online at <http://www.genesdev.org/cgi/doi/10.1101/gad.345686.120>.

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and, consequently, systemic dysregulation of the whole body metabolism (Sinha et al. 2019). Correlation was found between hypothyroidism and nonalcoholic fatty liver disease (NAFLD) (Chung et al. 2012; Mantovani et al. 2018). Moreover, TH treatment can decrease hepatosteatosis and inflammation and restore mitochondrial function in NAFLD (Sinha et al. 2012; Bruinstroop et al. 2018).

In contrast to steroid hormone receptors, TRs are not anchored to heat shock proteins in the cytoplasm (Carson-Jurica et al. 1990; Dalman et al. 1990). Rather, TRs are located in the cell nucleus and bind to DNA even in the absence of TH (Lazar et al. 1991; Brent et al. 1992; Forman et al. 1992; Ribeiro et al. 1992; Wahlström et al. 1992; Kim et al. 1996). TRs bind DNA with the highest affinity as a heterodimer with retinoid X receptor, favoring sites with a direct repeat of the sequence AGGTCA separated by 4 base pairs (bp) (DR4) (Yu et al. 1991; Kliewer et al. 1992; Wahlström et al. 1992).

While bound to chromatin TRs show the ability to activate or repress the expression of target genes (Lazar 2003; Shibusawa et al. 2003; Yen et al. 2003). For genes activated by TH, current dogma posits that TH binding acts as a switch between repressed and activated states. Unliganded TRs recruit the corepressor complex, mainly NCoR1 and SMRT (Chen and Evans 1995; Hörlein et al. 1995; Ishizuka and Lazar 2003; Choi et al. 2008), while TH binding releases the corepressors and leads to recruitment of coactivators that increase gene transcription (Chakravarti et al. 1996; Jeyakumar et al. 1997; Glass and Rosenfeld 2000). Recent studies suggest that this model is oversimplified and may only pertain to a subset of TH-regulated genes in mouse liver (Præsthholm et al. 2020).

TH-dependent negative regulation is even less well understood, yet it is critical to physiology as repression of pituitary *Tshb* gene expression by TH is the critical physiological regulator of TH levels (Hoskins 1949; Gurr and Kourides 1983; Carr et al. 1985; Chin et al. 1985). Many other genes are repressed by TH in a variety of tissues, including the liver (Lompré et al. 1984; Thompson et al. 1987; Caubín et al. 1994; Feng et al. 1994; Iglesias et al. 1996; Dupré et al. 2004). Several potential mechanisms of TH-mediated repression have been postulated (Lazar 2003). One model, involving direct TR binding to a so-called negative TRE (nTRE) has been proposed to explain regulation of the *Tshb* (Wondisford et al. 1989; Carr et al. 1992; Shibusawa et al. 2003) and other genes (Chin et al. 1998; Kim et al. 2005; Nygård et al. 2006; Gillis et al. 2018). A competing model, known as transrepression, suggests that TR binding to nTREs is not via direct DNA binding but, rather, via tethering to another DNA-binding transcription factor such as the AP1 complex (Desbois et al. 1991; Zhang et al. 1991). The tethering mechanism has also been proposed for other NRs (Jonat et al. 1990; Yang-Yen et al. 1990; Schüle et al. 1991; Joseph et al. 2003).

In the past few years, as genome-wide approaches have been used to interrogate gene expression and enhancer activities, another model for negative regulation by NRs has gained wider acceptance. In this model, known as

“quelching” or “coactivator redistribution,” NRs compete for limiting amounts of nuclear transcriptional cofactors without binding near the gene that is negatively regulated by a ligand (Schmidt et al. 2016). This concept was first introduced based on studies in yeast and then extended to NRs in mammalian cell lines (Meyer et al. 1989, 1992). The best evidence for this model in a chromatin context is based on studies of estrogen receptor (ER) (Carroll et al. 2006; Guertin et al. 2014) and PPAR γ . (Step et al. 2014; Nelson et al. 2018).

TRs are not highly expressed in tissues, and existing antibodies are suboptimal, which has hampered previous genome-wide studies relating endogenous TR binding to function. Here we describe a new mouse model with an epitope tag in frame with the first methionine of the TR β 1 protein, and use it to detect endogenous TR β 1 bound to chromatin at physiological expression levels. Up-regulation as well as down-regulation of liver gene expression was highly correlated with TR β 1 binding sites (TRBSs) on chromatin. TRBSs near both positively and negatively regulated genes demonstrated epigenomic characteristics of activating and repressive enhancers, respectively. While TH binding reduced NCoR/HDAC3 recruitment at activated enhancers, the corepressor complex was also found and destabilized at sites near down-regulated genes. In contrast, binding of the coactivator CBP was reduced specifically at repressed enhancers. Thus, unlike other NRs, ligand-dependent repression by TH involves direct TR binding through the canonical TR-binding motif. Furthermore, rather than the all or none canonical switch model, it is the direction of shift of coregulator association with TR β enhancers that determines enhancer activity and transcriptional outcomes.

Results

An in vivo mouse model to interrogate TR β function at physiological levels

TR β is expressed at low levels in liver, estimated to be ~4000–8000 receptors per rat liver nucleus (Oppenheimer et al. 1974; Samuels et al. 1974). To gain insight into the function of TR β at physiological levels, we used CRISPR to insert a triple HA epitope tag in frame with the first methionine of the *Thrb* gene in C57BL/6J mice (Fig. 1A). Liver expression of *Thrb* and the TH target gene *Dio1* was indistinguishable from that of WT mice (Supplemental Fig. S1A). Endogenous TR β was readily detected immunohistologically, demonstrating nearly exclusive nuclear localization in liver (Supplemental Fig. S1B). HA-TR β was readily detected by chromatin immunoprecipitation (ChIP) of liver using anti-HA antibody, with little or no background in livers from wild type (WT) mice (Supplemental Fig. S1C). Mice were rendered hypothyroid by treatment with anti-thyroid drug propylthiouracil (PTU) for 3 wk, leading to low serum levels of thyroid hormones T3 and T4 and high levels of thyroid stimulating hormone (TSH), which were reversed by treatment with a combination of T3 and T4 (Supplemental Fig. S1D). The systemic hypothyroidism and reversal with T3/T4 were confirmed

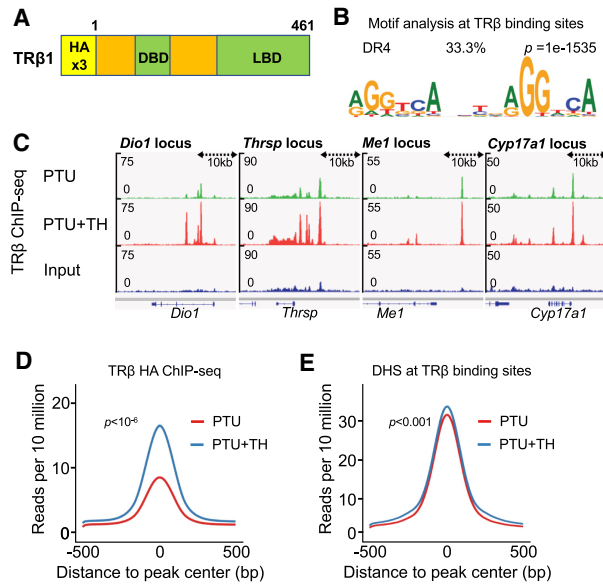


Figure 1. TR β binding sites in hypothyroid and hyperthyroid mice with endogenous epitope-tagged TR β 1. (A) Schematic representation of the HA-TR β protein. (B) Top hit from Homer de novo motif search at all TR β binding sites identified the DR4 motif. (C) Examples of TR β ChIP tracks for *Dio1*, *Thrsp*, *Me1*, and *Cyp17a1* showing nearby TRBSs. (D) Average density profiles in reads per 10 million of TR β ChIP-seq data ($n=3$ biological replicates) at all TRBS in PTU- or PTU + TH-treated mice. (E) Average density profiles in reads per 10 million of DNase-seq data ($n=3$ biological replicates) at all TRBS in PTU- or PTU + TH-treated mice.

by analysis of canonical TH-responsive *Dio1* and *Thrsp* genes (Supplemental Fig. S1E).

HA-ChIP followed by deep sequencing (ChIP-seq) was performed on livers from PTU-treated (hypothyroid) livers as well as livers from PTU-treated mice given T3/T4. This analysis revealed 6500 TR β binding sites (TRBS) using stringent cutoffs of P -value > 0.001 , RPKM > 1 compared with input and were fourfold more than WT mice ChIP. De novo motif analysis of TRBS showed enrichment for the known functional TRBS, which is a direct motif of the AGGTCA sequence spaced by four bases, called the DR4 motif (Fig. 1B). In addition to the DR4, the DR1 motif was also enriched in the TR β cistrome, albeit with a lower P value (Supplemental Fig. S1F). TR β bound in the region of many canonical TH-activated genes including *Dio1*, *Thrsp*, *Me1*, and *Cyp17a1* in both hypothyroid and hyperthyroid conditions (Fig. 1C).

Two prior studies evaluated TRBS in mouse liver: Ramadoss et al. (2014) performed ChIP-seq on biotinylated TR β 1 that has been overexpressed in mouse liver, while Grøntved et al. (2015) used an antibody to endogenous TRs that detects both TR α and β with relatively low specificity and sensitivity. There were numerous differences between the conclusions of the studies, likely related to the detection methods and overexpression model. For example, many more TRBSs were identified in the overexpression model. Moreover, Grøntved et al. (2015) detected little or no TR β binding at TH-inducible genes

in hypothyroid livers (e.g., black arrow in Supplemental Fig. S2A), while Ramadoss et al. (2014) reported TR β binding at the sites that was nearly the same in hypothyroid and hyperthyroid livers (Supplemental Fig. S2A). Our results with endogenous HA-TR β revealed binding that was equally or more robust with fewer nonspecific peaks (e.g., red arrow in Supplemental Fig. S2A) in both hypothyroid and TH-treated livers (Supplemental Fig. S2A).

We next performed global comparison of the TR β cistromes using a single bioinformatics pipeline. As expected, the overexpression model of Ramadoss et al. (2014) had by far the most binding sites. The DR4 motif was enriched in all three studies, yet the HA-TR β cistrome showed stronger enrichment, and sites common to all three studies and common to the HA and Grøntved et al. (2015) were the highest (10-fold and 15-fold, respectively) (Supplemental Fig. S2B). Focusing on these common sites, we noted that the effect of TH on TR β binding was also notably different across studies. Ramadoss et al. (2014) observed a very small overall increase with TH treatment, while Grøntved et al. (2015) noted an increase with TH with binding strength that was generally quite low (Supplemental Fig. S2C). In contrast, the present studies using an antibody that was highly specific for endogenous tagged TR β demonstrate robust binding that was markedly stimulated by TH (Supplemental Fig. S2C). A similar result was obtained when all of the HA-TR β binding sites were included in the analysis (Fig. 1D). Interestingly, integrating the TR β binding sites with published DNase-seq data generated from hypothyroid and hyperthyroid liver mice (Grøntved et al. 2015) revealed no significant difference between the openness of chromatin at these sites in the presence or absence of TH (Fig. 1E).

TH alters chromatin structure at TR binding sites near TH-regulated genes

We next sought to understand the hepatic regulatory network and function of TR β by integrating the TR β cistromes with TH-dependent gene expression. Similar numbers of genes were up-regulated (777) and down-regulated (867) by TH (Fig. 2A). Each TRBS was mapped to the nearest gene then partitioned based on the effect of TH on the nearest gene relative to PTU-only treatment: down-regulated, no change in expression, and up-regulated (differential expression [DE]: $|FC| > 1.5$, adjusted FDR < 0.05). Remarkably, although we showed earlier that TH did not change overall chromatin accessibility on a genome-wide scale, TH increased chromatin accessibility at TRBSs near up-regulated genes (Fig. 2B, red dots). Conversely, chromatin accessibility decreased upon TH treatment at TRBSs near down-regulated genes (Fig. 2B, blue dots). These findings suggest that TH binding to TR β can alter chromatin structure in a manner that is associated with the outcome of gene regulation, with chromatin opening correlating with activation and closing being characteristic of ligand-induced repression. Since TR β binding was globally increased by TH, we considered whether there might be a difference at sites associated

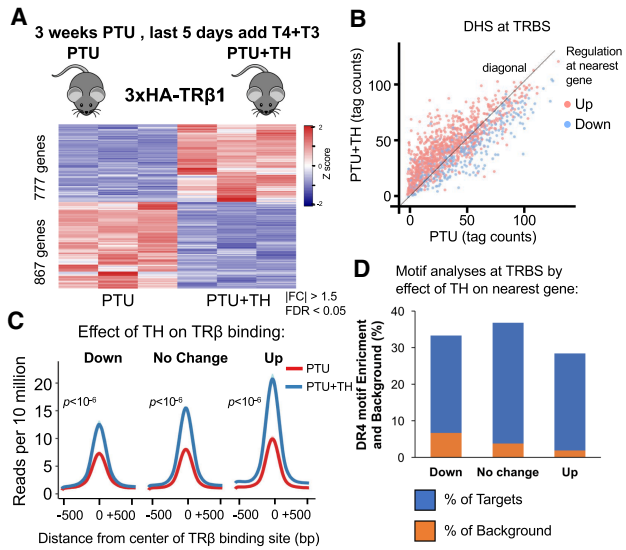


Figure 2. TR β binding sites near genes that are induced or repressed by TH. (A) Heat map showing TH-dependent differentially regulated genes. $n = 3$ biological replicates, DE cutoff: $|FC| > 1.5$, two-sided Benjamini–Hochberg-adjusted FDR < 0.05 as determined by edgeR likelihood ratio test. (B) Scatter plot of DNase-seq data showing read counts centered to TRBSs from PTU-treated mice against PTU + TH-treated mice ($n = 2$ biological replicates). (Red) Up-regulated, (blue) down-regulated. DE cutoff taken from A. (C) Average density profiles in reads per 10 million of TR β ChIP-seq data ($n = 3$ biological replicates) at TRBSs in PTU- or PTU + TH treated mice. TRBSs were categorized by the effect of TH on the nearest gene annotated to them. (D) DR4 motif enrichment (percentage) depicted as blue bars for each TRBSs group, categorized by the effect of TH on the nearest gene annotated to them. The orange bars overlay showing the (percentage) background level (Homer used for de novo motif search).

with gene activation versus those associated with TH-dependent repression. However, despite the differences in chromatin accessibility, TH increased TR β binding approximately twofold regardless of the relationship of the binding site to TH-dependent changes in chromatin structure and gene regulation (Fig. 2C).

TR is known to bind preferentially in vitro as a heterodimer with RXR to DNA containing two directly repeated hexameric sequences related to AGGTCA spaced by 4 bp, which is referred to as a DR4 motif (Yu et al. 1991; Kliwer et al. 1992; Marks et al. 1992). While the DR4 motif has been shown to mediate TR binding and TH activation of gene expression (Umesono et al. 1991; Mader et al. 1993; Shulemovich et al. 1995), its role in TH-dependent repression is debated (Lazar 2003). Interestingly, de novo motif analysis revealed that the DR4 motif was enriched at TRBSs regardless of the relationship of the binding site to TH regulation of the nearest gene (Fig. 2D). These results suggest that TR β binds directly to DR4 sites near both positively and negatively regulated genes. TH induces TR β binding nearly equally at sites, which are distinguishable by the direction of chromatin accessibility induced by TH.

TH-dependent gene expression requires TR β

We next studied *Thrb* knockout mice (Forrest et al. 1996; Amma et al. 2001) to determine the TH-regulated genes that require TR β . *Thrb*^{-/-} and *Thrb*^{+/+} mice were made hypothyroid by treatment with methimazole (MMI) for 4 wk in parallel with MMI-treated groups that were made hyperthyroid by additional treatment with T3 for the fourth week (MMI + T3), followed by RNA-seq analysis of the liver. Since this hypothyroid/hyperthyroid protocol was different from that used for the HA-TR β mice (3 wk PTU, with T4 + T3 given i.p. during the last 5 d), we compared the RNA-seq results from the control group with that of the HA-TR β mice (performed at different institutions) (Supplemental Fig. S3A) and noted a strong correlation in TH-dependent gene expression (Supplemental Fig. S3B), which was even higher when differentially expressed genes were tested (Supplemental Fig. S3C). Additionally, $>75\%$ of both up-regulated and down-regulated genes in the HA-TR β /PTU model (DE: $|FC| > 1.5$, adjusted FDR < 0.05) overlapped with the control of the methimazole model (Fig. 3A). Focusing on the genes that were TH-regulated in both models, genetic deletion of TR β markedly reduced TH-responsiveness of both up-regulated (Fig. 3B) and down-regulated genes (Fig. 3C), consistent with TR β being the dominant TR subtype in the liver (Weiss et al. 1998). This was observed in both models, validating with high confidence this TH/TR-dependent transcriptome in liver.

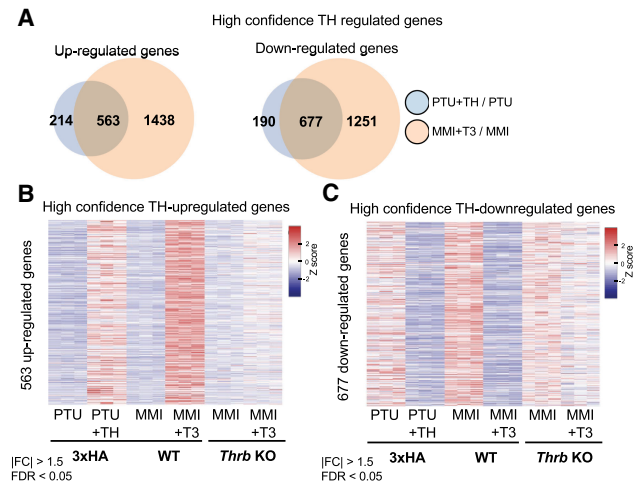


Figure 3. Defining high-confidence TH-regulated genes that require TR β in liver. (A) Venn diagrams displaying overlap of differentially expressed genes identified in PTU + TH/PTU and MMI + T3/MMI experiments. Overlapped up-regulated genes on the left and down-regulated genes on the right. (B) Heat map showing relative expression of overlapped TH-dependent up-regulated genes between the two treatment systems and the treated *Thrb*^{-/-} mice. (C) Heat map showing relative expression of overlapped TH-dependent down-regulated genes between the two treatment systems and the treated *Thrb*^{-/-} mice. For all experiments, $n = 3$ biological replicates, DE cutoff: $|FC| > 1.5$, two-sided Benjamini–Hochberg-adjusted FDR < 0.05 as determined by edgeR likelihood ratio test.

TR β binding sites near TH/TR-dependent genes are functional enhancers that depend on ligand receptor interaction

A large number of these TH/TR-dependent genes were highly associated with nearby TR β binding sites and generated a greater overlap of differentially regulated genes between the two models (Supplemental Fig. S4). The effect of TR β deletion on the magnitude of the TH-dependent change in gene expression (either up-regulation or down-regulation of expression) was >1.5-fold in >84% of the 505 TH-regulated, TR β -dependent genes with proximal TRBSs (Fig. 4A). Moreover, one-third of these functional binding sites are near genes that are repressed by TH (Fig. 4B). For TRBS near both up-regulated and down-regulated genes, the most enriched DNA sequence motif was the DR4 (Fig. 4C), which thus did not discriminate between functional transcriptional outcome. Chromatin accessibility increased with TH at TRBS near high-confidence up-regulated genes and decreased near repressed genes (Fig. 4D), similar to what was observed for the cistrome near all differentially expressed genes.

Functional binding sites, commonly known as enhancers, are characterized by modification of histone H3 by acetylation at lysine 27 (H3K27Ac) that is related to the regulation of the gene whose transcription is being controlled. H3K27Ac was markedly increased by TH at TRBSs near TR-dependent up-regulated genes (Fig. 4E). In contrast, H3K27Ac went down in a TH-dependent manner at TRBSs near down-regulated genes (Fig. 4E). These results show that TRBSs near up-regulated as well as down-regulated genes have the characteristics of regulated enhancers. Moreover, the finding that TH reduces enhancer activity at TRBSs where TR is required for negative regulation strongly suggests that repression is mediated by liganded receptor binding directly at these sites. This is of great interest because the mechanism of TH-dependent down-regulation is controversial (Lazar 2003), and ligand-dependent negative regulation by other NRs including PPAR γ and the estrogen receptor has been shown to occur without direct binding to the target genes (Carroll et al. 2006; Step et al. 2014).

To better understand the role of TH binding to TR β at TRBSs near negatively regulated genes, we integrated data from a mouse model in which a frameshift mutation in the ligand binding domain of TR β (TR β -PV) results in its inability to bind TH and to activate or to repress genes in a ligand-dependent manner (Kaneshige et al. 2000). A recent study showed blunted H3K27Ac at TR β -PV binding sites after TH treatment (Præsthholm et al. 2020), and we interrogated those ChIP-seq data with a focus on the TRBSs at high-confidence TR/TH-requiring genes as defined herein. Analysis of TH-induced genes at TRBS where H3K27Ac is normally stimulated by TH showed that these sites lost their ability to gain acetylation in the TR β -PV livers, consistent with the recent study (Fig. 4F; Præsthholm et al. 2020). Importantly, performing the same analysis on TRBSs near negatively regulated genes revealed that TH-dependent effects on H3K27Ac were lost as for positive regulated TRBSs (Fig. 4F). Furthermore,

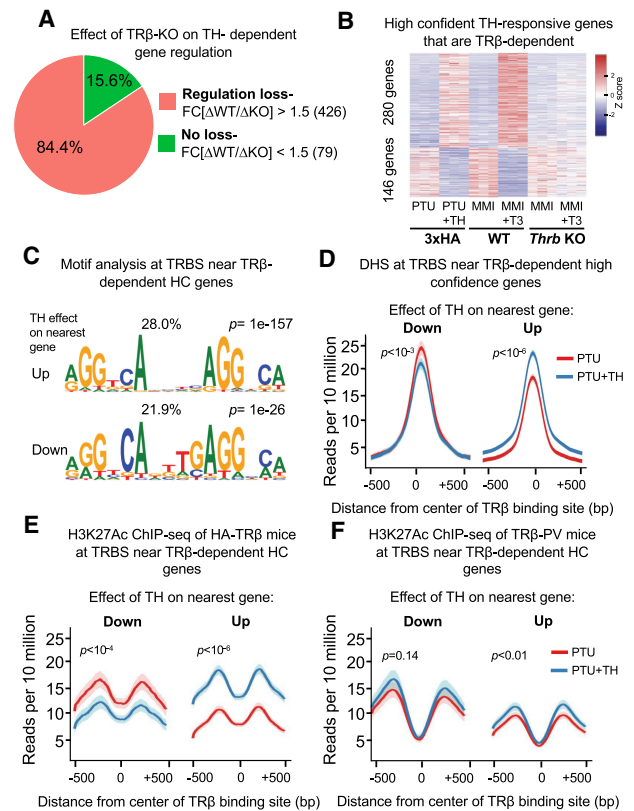


Figure 4. Functional TRBSs demonstrate enhancer characteristics that correlate with nearby gene expression. (A) Fraction of TH-regulated, TR β -dependent genes with proximal TRBSs in which the effect of TR β deletion on gene expression is >1.5-fold. (B) Heat map showing the effect of TR β deletion on relative expression of TH-regulated, TR β -dependent genes with proximal TRBS genes. $n = 3$ biological replicates, DE cutoff: $|FC| > 1.5$, two-sided Benjamini–Hochberg-adjusted FDR < 0.05 as determined by edgeR likelihood ratio test. (C) Homer de novo motif search at TRBS near high-confidence TH-regulated genes that are TR β -dependent showing DR4 motif enrichment near both up-regulated and down-regulated genes. (D) Average density profiles in reads per 10 million of DNase-seq data ($n = 3$ biological replicates) at TRBSs near high-confidence TH up-regulated or down-regulated genes that are TR β -dependent. The difference in reads between PTU and PTU + TH treatments is shown. (E) Average density profiles in reads per 10 million of H3K27Ac ChIP-seq data ($n = 2$ biological replicates) at TRBSs near high-confidence TH up- or down-regulated genes that are TR β -dependent, showing the difference in reads between PTU and PTU + TH treatments. (F) Average density profiles in reads per 10 million of H3K27Ac ChIP-seq data in TR β -PV mice lacking the ability to bind TH ($n = 2$ biological replicates) at TRBSs near high-confidence TH up-regulated or down-regulated genes that are TR β -dependent. The difference in reads between PTU and PTU + TH treatments is shown.

in TR β -PV livers, the acetylation at these TRBSs was higher than at TRBSs near up-regulated genes and were comparable with acetylation levels noted in 3xHA-TR β hypothyroid livers. Together, these results suggest that inability to bind ligand diminished TR β /TH-dependent enhancer function for both direct activation and repression.

TR β canonically activates genes by dismissing the NCoR/HDAC3 corepressor complex while recruiting coactivators such as CBP. Analysis of available ChIP-seq data for NCoR1, HDAC3, and CBP (Præstholm et al. 2020) showed enrichment for expected transcription factors (Supplemental Fig. S5A–C) and binding motifs (Supplemental Fig. S5D–F), and thus we integrated these data with the high-confidence TRBSs. NCoR and HDAC3 binding was reduced by hyperthyroid conditions at TRBSs near TH-induced genes, as expected (Fig. 5A,B). However, TH also reduced NCoR and HDAC3 binding at TRBSs near down-regulated genes (Fig. 5A,B). Thus, TH-dependent transcriptional outcomes were not necessarily correlated with reduced binding of NCoR/HDAC3. A recent study proposed two distinct mechanisms of hyperacetylation at TR-occupied enhancers, distinguished by the presence of HDAC3 in the hypothyroid condition (Præstholm et al. 2020). However, rather than two distinct kinds of TRBS, analysis of the high-confidence TRBS revealed a continuous range of occupancy states (Supplemental Fig. S6A,B), with high correlation between NCoR1 and HDAC3 binding (Supplemental Fig. S6C).

We next considered the binding of coactivator CBP, which has intrinsic histone acetyltransferase (HAT) activity and is recruited as part of a coactivator containing SRC (Li et al. 2000; Sheppard et al. 2001). CBP also showed a continuous range of occupancy rather than bimodal binding (Supplemental Fig. S6D). Interestingly, CBP binding was robust but not changed between hypothyroid and hyperthyroid conditions at positively regulated genes. However, at enhancers near negatively regulated genes, CBP binding was high in the hypothyroid state and reduced by TH (Fig. 5C).

To further interrogate the role of coregulators in positive and negative regulation by TH, we normalized CBP binding to that of NCoR1 or HDAC3 at TRBSs of high-confidence TR/TH-requiring genes between hypothyroid and hyperthyroid conditions. Genome-wide, for up-regulated genes, TH increased the ratio of CBP to NCoR (or HDAC3) binding at nearby enhancers whereas, in contrast, the CBP to NCoR (or HDAC3) ratio was greater at down-regulated TR-bound enhancers under hypothyroid conditions (Fig. 5D,E). This is illustrated at specific TRBSs of genes that are positively regulated (*Dio1*, *Idh3*, and *Gdp2*) and negatively regulated (*Gys2*, *Gsta2*, and *Etnpp1*) by TH (Supplemental Fig. S7).

We next investigated potential mechanisms that might dictate the coregulator shifts distinguishing positive and negative enhancers. Epigenetic landscape in silico deletion analysis (LISA) (Qin et al. 2020) did not uncover differences in regulatory candidate transcription factors between up-regulated and down-regulated genes (Supplemental Fig. S8A), and thus we turned our attention to whether three-dimensional chromatin architecture could influence TR β -dependent gene activation or repression. Integration of our data with HiC data from liver tissue (Kim et al. 2018) revealed a small fraction of genes topological-associated domains (TADs) could have a regulatory function, but no difference between up-regulated or down-regulated genes (Supplemental Fig. S8B,C). Of

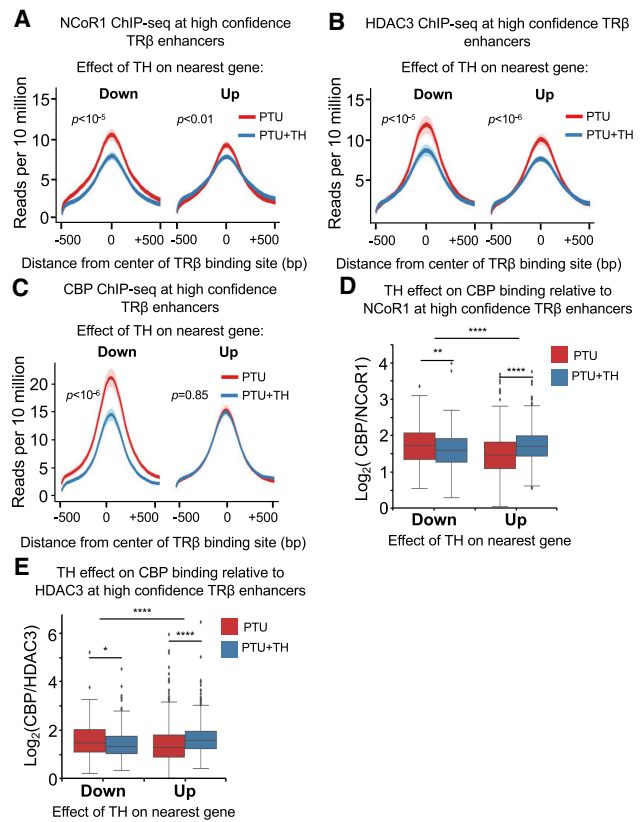


Figure 5. TH action involves a shift between corepressor and coactivator occupancy at enhancers of both positively and negatively regulated genes. (A) Average density profiles in reads per 10 million of NCoR1 ChIP-seq data ($n=2$ biological replicates) at TRBSs near high-confidence TH-regulated and TR β -dependent genes; showing the difference in reads between PTU and PTU + TH treatments. (B) Average density profiles in reads per 10 million of HDAC3 ChIP-seq data ($n=2$ biological replicates) at TRBSs near high-confidence TH-regulated and TR β -dependent genes; showing the difference in reads between PTU and PTU + TH treatments. (C) Average density profiles in reads per 10 million of CBP ChIP-seq data ($n=2$ biological replicates) at TRBSs near high-confidence TH-regulated and TR β -dependent genes; showing the difference in reads between PTU and PTU + TH treatments. (D) Box plot showing the effect of TH on CBP binding normalized to NCoR1. Value at each peak is log ratio of CPB to NCoR1 ChIP-seq signal. The effect is shown separately for active and repressive enhancers. (E) Box plot showing the effect of TH on CBP binding normalized to HDAC3. Value at each peak is log ratio of CPB to HDAC3 ChIP-seq signal. The effect is shown separately for active and repressive enhancers. For D and E, within the same region: paired t -test; different regions: unpaired t test. P -values are shown. (*) $P < 0.05$, (**) $P < 0.01$, (****) $P < 0.0001$.

note, however, positively regulated genes were more frequently positioned in the same TAD than negatively regulated (Supplemental Fig. S8D,E). Also, TRBSs where the CBP/NCoR1 ratio goes up or down with TH had a tendency to cluster in the same TAD (Supplemental Fig. S8F,G), while TAD-TAD interaction did not effect this propensity (Supplemental Fig. S8H,I). These results show that, in some cases, positive regulation by TH was favored by

TADs, whereas negative regulation was less influenced by TAD structures. Together, the results show that transcriptional outcomes of TH binding to TR correlated with the relative recruitment of corepressor or coactivator rather than an absolute switch in their presence at enhancers, with similar principles pertaining to positively and negatively regulated genes.

Discussion

For many years, TH has served as a model for hormone action and TR β as a model representative of the nuclear receptors family (Tata 2013). However, the sparse number of TRs in the cell and the unavailability of high-quality antibodies generated discrepancies of *in vivo* experiments (Ramadoss et al. 2014; Grøntved et al. 2015). Here, we have introduced an HA-TR β -tagged mouse model, where TR β is expressed under its endogenous promoter and can be detected using the highly sensitive antibody, and used the model to better understand the mechanism of TH action and the role of TR β in chromatin.

Our data showed consistency with previous studies demonstrating TR β binding to TRBSs at enhancers near up-regulated genes. These enhancers exhibited higher H3K27ac and chromatin accessibility upon TH treatment. Moreover, *de novo* motif analysis revealed enrichment for the DR4 motif at these sites. TRBSs related to the well-accepted hepatic TH responsive genes *Dio1*, *Thrsp*, *Me1*, and *Cyp17a1* were occupied with TR β in the unliganded state as well as in the liganded state. This is in accordance with early *in vitro* studies (Lazar et al. 1991; Brent et al. 1992; Forman et al. 1992; Ribeiro et al. 1992; Wahlström et al. 1992) and with the ChIP-seq results of Ramadoss et al. (2014), using TR β 1 overexpression in liver, but not with the results of Grøntved et al. (2015), which suggested that TR binding was ligand-dependent and nearly undetectable in the absence of TH. This is probably related to the ChIP-seq detection method, which used a less specific antibody requiring very stringent bioinformatic cutoffs (Grøntved et al. 2015).

Importantly, in this study we show that TH gene repression mediated by TR β in the liver correlates with direct binding to a DR4-like sequence on a genome-wide scale. This is in contrast to ligand-dependent repression by other NRs using tethering or squelching mechanisms (Meyer et al. 1992; Joseph et al. 2003; Carroll et al. 2006; Step et al. 2014). While a previous ChIP-seq study of endogenous TR β did not find TRBSs near TH-dependent repressed genes (Grøntved et al. 2015), a ChIP-seq study on overexpressed TR β in the liver did observe such binding (Ramadoss et al. 2014). However, the overexpression study suggested that TH-facilitated gene repression is associated with reduction in TR β occupancy and thus alluded to an indirect mechanism. Rather, our data show that, in general, TH increases endogenous TR β binding regardless of its effect on the nearest gene. Moreover, the DR4 motif was enriched in these TRBSs, and the causal role of TR β was demonstrated by the abrogation of TH-mediated repression in *Thrb* knockout mice.

The canonical coregulator switch model describes corepressor binding to TR at functional enhancers, with ligand binding triggering the dismissal of corepressor and recruitment of coactivator (Glass and Rosenfeld 2000). Mutational studies have demonstrated that NCoR and SRC1 both contribute to the magnitude of TH effects but do not provide evidence for an “either/or” mechanism of coregulator binding (Vella et al. 2014). A recent study investigated the switch model by focusing on the extremities of TH-dependent histone acetylation at TRBSs and suggested that the switch does occur but only at a subset of TH-activated genes (Præstholm et al. 2020). Here, however, focusing on functional enhancers in the context of a highly specific TR β 1 cistrome, we found that TH globally reduced without completely dismissing NCoR/HDAC3 binding at regulated enhancers correlating with down-regulated as well as up-regulated genes. Indeed, despite the presence of HDAC3, at down-regulated enhancers we observed elevated H3K27 acetylation in hypothyroid conditions. This is likely because of the abundance of CBP, a powerful HAT, at enhancers associated with TH-repression or other coactivators with redundant activity. It should be recognized that NCoR1 and CBP are not unique but rather representative of TRs corepressors and coactivators, respectively.

Together, these results lead us to suggest a novel view of gene regulation by TH, particularly in its negative transcriptional effects. Rather than a switch, TH functions to shift the ratio of corepressor to coactivator at both positive and negative enhancers that are bound and regulated by TR. On a genome-wide basis, repression of gene expression by TH involves direct binding of TR β to repressive enhancers containing canonical DR4 motifs. The recruitment of activating versus repressive coregulator complexes could be mediated by several layers of regulation. We did not detect major differences in motifs found at TRBSs related to up-regulated versus down-regulation, suggesting that factors in addition to the TRBS must specify whether a given site functions as an activating or repressive enhancer. We did note some influence of three-dimensional architecture, particularly for positively regulated genes, but this could not fully explain the differences between genes that were up-regulated versus down-regulated by TH. Thus, other factors such as response type-specific enhancer-to-enhancer interactions and protein recruitment are likely to explain the direction of regulation by TH at a given TRBS.

Materials and methods

Animals and treatments

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Mice were housed in a temperature-controlled specific pathogen-free facility under 12-h light/dark cycles. Adult male mice of 12–14 wk were used in all experiments. For HA-TR β mice, controls were age-matched WT C57BL/6 mice that were purchased from Jackson Laboratories. *Thrb*^{-/-} mice (Forrest et al. 1996), originally on a mixed C57BL/6 \times 129/Sv background, were back-crossed for multiple generations (more than nine) onto

C57BL/6J; wild-type controls were C57BL/6J mice. For PTU treatment, mice were provided with drinking water containing 1% perchlorate and 0.05% propylthiouracil for 3 wk. In the last 5 d, all animals were intraperitoneally injected with vehicle (0.9% saline in 100- μ L volume) for the hypothyroid group or 40 μ g/100 g T4 with 4 μ g/100 g T3 for the hyperthyroid group. For MMI treatment, mice were provided with 0.05% methimazole and 1% potassium perchlorate in the drinking water for 4 wk, inducing hypothyroidism, adapted from established methods (Ammal et al. 2001). To induce hyperthyroidism, half of the groups were given T3 at 5 μ g/mL in the same drinking water (MMI + T3) during the fourth week of treatment.

Generation of HA epitope-tagged TR β 1 mice

To generate Cas9 mRNA, a plasmid containing Cas9-HA-2NLS was linearized with XbaI (gift from Jorge Henao-Mejia, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA). Approximately 1 μ g of linearized plasmid was incubated with a HiScribeTM T7 quick high-yield RNA synthesis kit (NEB E2050S). RNA was purified using RNeasy mini columns (Qiagen 74106), and the capping reaction used the Vaccinia capping system (NEB M2080S). RNA was purified using RNeasy micro clean-up columns (Qiagen 74004). Capped Cas9 mRNA was then subjected to polyadenylation (NEB M0276S) and purified over an RNeasy micro clean-up column and eluted in RNase-free water. Cas9 mRNA integrity was validated using RNA BioAnalyzer. T7 promoter was added onto the gRNA template targeting the ATG start site by PCR amplification using specific primers (targeting guide sequence: TCATAGGTTAGTAGT CATGC). The T7-sgRNA product was purified using a PCR purification kit (Qiagen) and used as the template for in vitro transcription using the MegaShortScript kit (Life Technologies) following the manufacturer's instructions. Subsequent sgRNA was purified using the MegaClear kit (Life Technologies) and verified by RNA BioAnalyzer before dilution for microinjection. The following ssDNA homology donor (IDT) containing the 3 \times HA tag was resuspended in water and prepared using DNA Clean and Concentrator (Zymo): C*A*C*AGTGTAATCAGGAAAGGACACAAAGATACCTGTCATACTGTTAGGAGTGCCAGCGTAATCTGGAACGTCATAAGGATACGATCCTGCATAGTCCGGGACGTCATAGGGATAGCCCGCATAGTCAGGAACATCGTATGGATACATAGGTTAGTAGTCATGCTGCGTCATCTTCTGTACTGGCATTCCCTAAAA*G*A*G. Microinjection was performed by the Transgenic and Chimeric Mouse Facility at the University of Pennsylvania using C57BL/6J mice from Jackson Laboratories. The microinjection buffer consisted of 1 mM Tris (pH 8.0), 0.1 mM EDTA, 100 ng/ μ L Cas9 mRNA, 50 ng/ μ L sgRNA, and 100 ng/ μ L of ssDNA homology donor. Insertion of epitope tag coding sequence was detected by PCR and confirmed by Sanger sequencing. HA-TR β mice were backcrossed to the C57BL/6J genetic background for at least four to five generations and genotyped using the PCR primers: 5'-TGTCACATCGCAAGTTCCAG-3' and 3'-ATGAGTTCAGGGCATAGGGGTG-5'.

RNA extraction and gene expression measurement

Total RNA was extracted from snap-frozen liver tissues using TRIzol (Invitrogen) followed by an RNeasy mini kit (Qiagen). For RNA-seq, all samples were sequenced separately for three biological replicates. RNA integrity was examined using an Agilent RNA 6000 Nano kit. RNA samples with RNA integrity number >7 (1 μ g) were used for RNA cleanup, library preparation, and sequencing by Novogene. For MMI-treated mice, total RNA was prepared from frozen liver, using TRIzol reagent (Invitrogen

15596-026), then mRNA-enriched from 5- μ g aliquots of total RNA using a Dynabeads mRNA purification kit (Ambion # 61006) and quantified using a Qubit RNA broad-range assay kit (Thermo Fischer Scientific). Double-stranded cDNA was prepared using SuperScript double-stranded cDNA synthesis kit (Invitrogen), and cDNA libraries for sequencing were generated using a ThruPLEX DNA-seq kit (Takara R400428). Multiplex sequencing was performed, yielding single-end 50-base-length reads, at the NIDDK Genomic Core using an Illumina HiSeq 2500 instrument. All RNA-seq experiments were performed with three biological replicates per condition.

RNA-seq analysis

Reads were mapped to the mm9 reference genome using STAR (Dobin et al. 2013), selecting only uniquely mapped reads. Expression was quantified at the gene level with featureCounts (Liao et al. 2014), using Ensembl v.67 gene annotations. Only genes that have at least one read per million in at least two samples are considered in the following analysis. We then used edgeR (McCarthy et al. 2012) to find genes differentially expressed between two conditions. A gene is considered differentially expressed if the corresponding *P*-value (Benjamini-Hochberg-adjusted) is <0.05 and the fold change between conditions is at least 1.5.

For visualization purposes, we use FPKM values computed by edgeR. Heat maps show gene-wise z-scores of FPKM across all shown samples, unless indicated otherwise.

Gene ontology term enrichment in gene sets is computed using the g:Profiler web service (Raudvere et al. 2019). Benjamini-Hochberg-adjusted *P*-value must be <0.05 and enrichment >2 for a term to be considered enriched.

Chromatin immunoprecipitation

HA-TR β ChIP-seq was performed as previously described (Armour et al. 2017). HA magnetic beads (Pierce) were used to perform the immunoprecipitation. For ChIP-seq of HA-TR β , three biological replicates were used for sequencing, and WT mice were used as a negative control. The ChIP-seq library has been performed as previously described (Armour et al. 2017).

ChIP-seq analysis

ChIP-seq experiments on PTU mice and PTU + TH mice were performed independently on three biological replicates ($n=3$), except for H3K27ac ChIP-seq experiments in PTU- and PTU + TH mice for which there were two independent biological replicates ($n=2$) per condition. All sequencing reads of biological replicates were aligned to the mm9 genome using Bowtie2 (v.2.2.6) (Langmead and Salzberg 2012) allowing for one mismatch (-N1). Duplicate reads were removed, and unique reads were extracted using SAMtools (v.1.7) (Li et al. 2009). Tag directories were generated from alignment files using HOMER (v.4.9.1) (Heinz et al. 2010).

Peaks were called using HOMER's findPeaks function with parameter "-style factor" (for transcription factors) or "-style histone" (for H3K27ac) for each individual replicate with a corresponding input directory as background, with default parameters. Each peak file was transposed to a bed format using pos2bed Perl script. The resulting replicate files were then sorted and merged to a single PTU and PTU + TH peak files using BEDOPS (v.2.4.30) (Neph et al. 2012). To find genes nearest to the peaks, we used HOMER's annotatePeaks command using mm9 genome. Tags were counted for each replicate and reported as reads per million (RPM). Annotated peaks were further filtered using R and RStudio (R v.3.6.3), leaving only peaks with reads >1 RPM in

at least two replicates. To further increase the confidence in our peaks, we used annotatePeaks to calculate tag counts for each pooled condition and compared it with WT mice. We filtered out peaks for each pooled condition that had less than fourfold reads compared with WT mice. Peaks from each condition were finally merged to form the set of confident TR β peaks.

Genome browser tracks were generated with HOMER function "makeUCSCfile -bigWig -fsize 1e20." Track visualization was performed using the Integrated Genomics Viewer (IGV) (Thorvaldsdóttir et al. 2013). ChIP-seq signal densities at specific peak regions were obtained with annotatePeaks with a 1-kb window from the peak center, using the option "-size 1000 -hist" (for histograms) with 10-bp bin size. Average density profiles were generated with mean signal in normalized RPM and error bands for biological replicates. De novo motif analyses were performed with the HOMER function findMotifsGenome.pl using parameters "-len 8 to 18 and -p 4."

Density profiles of H3K27ac PTU and PTU+TH aligned, pooled, tag directories around TR β peak files were performed using annotatePeaks function with parameters "-size 1000, -rlog" and plotted using custom Rscript.

Statistical analysis

For analysis of data other than sequencing experiments' output, the following procedures were used: Data are presented as means \pm standard error of the mean. A two-tailed Student's *t*-test was used for comparison between two groups. All of the statistical analyses were performed with GraphPad Prism (not significant [ns], $P < 0.5$ [*], $P < 0.01$ [**], $P < 0.001$ [***], $P < 0.0001$ [****]).

Deep sequencing data

All data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE159648.

Competing interest statement

The authors declare no competing interests.

Acknowledgments

We acknowledge the Functional Genomics Core (J. Schug and K. Kaestner) and the Biomarkers and RIA Core (H. Collins and M. Rickels) of the Penn Diabetes Research Center (DK19525) for next-generation sequencing, and hormone assays, respectively, and the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Genomics Core (H. Smith) for next-generation sequencing at the National Institutes of Health. This work was supported by NIDDK grant DK43806, the Cox Institute for Medical Research (M.A.L.), and the intramural research program at NIDDK (Y.-W.C. and D.F.).

Author contributions: Y.S. and M.A.L. conceived the project. Y.S. performed experiments and interpreted the data. Y.-W.C. performed *Thrb*^{-/-} experiments. Y.G. performed the IF assay. J.R. generated the HA-TR β mice. N.K.N. and K.B. analyzed the data. M.A.L. and D.F. provided supervision. Y.S. wrote the manuscript with input from Y.-W.C., D.F., and M.A.L.

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