THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 25, pp. 15549–15558, June 19, 2015

Astrocyte Elevated Gene-1 (AEG-1) Contributes to Non-thyroidal Illness Syndrome (NTIS) Associated with Hepatocellular Carcinoma (HCC)*

Received for publication, March 6, 2015, and in revised form, April 23, 2015 Published, JBC Papers in Press, May 5, 2015, DOI 10.1074/jbc.M115.649707

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Background: Astrocyte elevated gene-1 (AEG-1) inhibits retinoid X receptor (RXR) function and is overexpressed in human hepatocellular carcinoma (HCC), which is associated with non-thyroidal illness syndrome (NTIS).
Results: AEG-1 inhibits thyroid hormone (T_3) function by down-regulating type I 5′-deiodinase (DIO1) thus contributing to NTIS.
Conclusion: A novel role of AEG-1 is identified regulating cancer-associated NTIS.
Significance: AEG-1 inhibition might alleviate cancer-associated debilitating disorders.

Non-thyroidal illness syndrome (NTIS), characterized by low serum 3,5,3′-triiodothyronine (T_3) with normal L-thyroxine (T_4) levels, is associated with malignancy. Decreased activity of type I 5′-deiodinase (DIO1), which converts T_4 to T_3, contributes to NTIS. T_3 binds to thyroid hormone receptor, which heterodimerizes with retinoid X receptor (RXR) and regulates transcription of target genes, such as DIO1. NF-κB activation by inflammatory cytokines inhibits DIO1 expression. The oncogene astrocyte elevated gene-1 (AEG-1) inhibits RXR-dependent transcription and activates NF-κB. Here, we interrogated the role of AEG-1 in NTIS in the context of hepatocellular carcinoma (HCC). T_3-mediated gene regulation was analyzed in human HCC cells, with overexpression or knockdown of AEG-1, and primary hepatocytes from AEG-1 transgenic (Alb/AEG-1) and AEG-1 knock-out (AEG-1KO) mice. Serum T_3 and T_4 levels were checked in Alb/AEG-1 mice and human HCC patients. AEG-1 and DIO1 levels in human HCC samples were analyzed by immunohistochemistry. AEG-1 inhibited T_3-mediated gene regulation in human HCC cells and mouse hepatocytes. AEG-1 overexpression repressed and AEG-1 knockdown induced DIO1 expression. An inverse correlation was observed between AEG-1 and DIO1 levels in human HCC patients. Low T_3 with normal T_4 was observed in the sera of HCC patients and Alb/AEG-1 mice. Inhibition of co-activator recruitment to RXR and activation of NF-κB were identified to play a role in AEG-1-mediated down-regulation of DIO1. AEG-1 thus might play a role in NTIS associated with HCC and other cancers.

Thyroid hormone is an essential hormone required for development, metabolism, and energy utilization (1). The thyroid gland secretes 3,5,3′,5′-tetraiodo-L-thyronine (thyroxine or T_4),[4] an inactive form, which is converted into functionally active 3,5,3′-triiodothyronine (T_3) in peripheral tissues, such as liver and kidney. This conversion is primarily carried out by type I 5′-deiodinase (DIO1) enzyme (2). T_3 functions as a ligand for thyroid hormone receptor (TR) that heterodimerizes with retinoid X receptor (RXR) (3). Liganded TR/RXR heterodimer binds to target gene promoter and regulates transcription. DIO1 itself is a target of T_3 and contains a thyroid hormone-response element (TRE) in its promoter (4). Non-thyroidal illness syndrome (NTIS), also known as low T_3 syndrome or euthyroid sick syndrome, is characterized by low serum T_3 level with normal serum T_4 levels and is associated with a variety of conditions, including malignancy (5). As a result, patients develop hypothyroidism. One cause of this condition is decreased conversion of T_4 to T_3 due to decreased DIO1 activity in the liver (5).

Astrocyte elevated gene-1 (AEG-1), also known as metadherin and LYRIC, is an oncogene that is overexpressed in a diverse array of cancers and plays a key role in regulating carcinogenesis (6). AEG-1 plays an important role in regulating hepatocellular carcinoma (HCC) (7). AEG-1 overexpression at both mRNA and protein levels has been identified in a high percentage (>90%) of HCC patients, and a significant percentage of patients harbored genomic amplification of the AEG-1 locus in chromosome 8q22 (7). A progressive increase in the

* This work was supported, in whole or in part, by National Institutes of Health Grants R01 CA138540 and R21 CA183954 from NCI (to D. S.) and National Institutes of Health Grant R01 CA134721 (to P. B. F.). This work was also supported by the James S. McDonnell Foundation. The authors declare that they have no conflicts of interest with the contents of this article.
[1] Supported by National Institutes of Health Grant T32DK007150 from NIDDK.
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[4] The abbreviations used are: T_4, L-thyroxine; NTIS, non-thyroidal illness syndrome; T_3, 3,5,3′-triiodothyronine; DIO1, type I 5′-deiodinase; TR, thyroid hormone receptor; TRE, thyroid hormone response element; RXR, retinoid x receptor; HCC, hepatocellular carcinoma.

levels of AEG-1 was observed in HCC patients that directly correlated with the stages of the disease, and the AEG-1 level correlated with a higher recurrence and poor overall survival (7, 8). Overexpression of AEG-1 in the poorly aggressive HCC cell line HepG3, which expresses a low level of AEG-1, significantly increases *in vitro* proliferation, invasion, anchorage-independent growth and chemoresistance, and *in vivo* tumorigenesis, angiogenesis, and metastasis in nude mice (7, 9–11). These observations were further corroborated in a transgenic mouse with hepatocyte-specific overexpression of AEG-1 (Alb/AEG-1) that develops highly aggressive metastatic HCC in a diethylnitrosamine-induced HCC model (12, 13). Conversely, knockdown of AEG-1 in highly aggressive QGY-7703 cells, expressing high levels of AEG-1, significantly abrogates *in vivo* tumorigenesis (7, 10). As a corollary, an AEG-1 knockout (AEG-1KO) mouse shows profound resistance to diethylnitrosamine/phenobarbital-induced hepatocarcinogenesis and metastasis (14).

AEG-1 exerts its function by modulating a variety of signal transduction pathways and altering global gene expression profiles (15). Two particular aspects of AEG-1 function, pertinent to this study, are highlighted here. AEG-1 interacts with RXR and inhibits its function (16). RXR is a ligand-dependent transcription factor that heterodimerizes with one-third of the 48 human nuclear receptor superfamily members, including TR, and regulates gene transcription (17). In the absence of ligand, RXR heterodimers interact with co-repressors that maintain histones in a deacetylated state and inhibit transcription. Upon ligand binding, there is a conformational change so that the co-repressors are replaced by co-activators inducing histone acetylation and transcriptional activation. The co-activators harbor a unique "L*XX*LL" motif through which they interact with the transcription factors (18). Interestingly, AEG-1 also harbors an L*XX*LL motif, and a yeast two-hybrid assay using the region of AEG-1 harboring the L*XX*LL motif identified RXR as its interacting partner (16). We documented that in the nucleus interaction of AEG-1 with RXR blocks co-activator recruitment thereby abrogating retinoic acid-mediated gene transcription (16). Additionally, interaction of cytoplasmic AEG-1 with RXR blocks RXR nuclear translocation and also induces RXR phosphorylation and hence inactivation (16). Thus, AEG-1 interferes with RXR function by multiple ways.

AEG-1 is fundamentally required for activation of the NF-κB pathway, and AEG-1 regulates NF-κB at multiple levels. AEG-1 directly interacts with the p65 subunit of NF-κB and CREB-binding protein and thus functions as a bridging factor between NF-κB and basal transcriptional machinery promoting NF-κB-induced transcription (19, 20). AEG-1, anchored on endoplasmic reticulum membrane, associates with upstream ubiquitinated activators of NF-κB, such as RIP1 and TRAF2, facilitating their accumulation and subsequent NF-κB activation by diverse stimuli (21). Indeed, lipopolysaccharide (LPS)-induced NF-κB activation is markedly abrogated in AEG-1KO hepatocytes and macrophages when compared with wild type (WT) (14). Studies have shown that NF-κB activation by inflammatory cytokines inhibits DIO1 expression and contributes to NTIS (22).

In this study, we document that inhibition of T_3-mediated gene transcription and activation of NF-κB by AEG-1 results in abrogation of DIO1 expression in human HCC cells and mouse hepatocytes. We also show a decrease in serum T_3 levels in human HCC patients and Alb/AEG-1 mice. AEG-1 is overexpressed in a diverse array of cancers, and this overexpression might contribute to NTIS associated with cancers.

Materials and Methods

Alb/AEG-1 and AEG-1KO Mice—Generation and characterization of a hepatocyte-specific AEG-1 transgenic mouse (Alb/AEG-1) and an AEG-1 knock-out (AEG-1KO) mouse have been described previously (12–14). All animal studies were approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University and were conducted in accordance with the Animal Welfare Act, the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

Patient Samples—Serum samples were collected from hepatitis C virus-HCC patients ($n = 23$) and 14 healthy volunteers. These studies are approved by the Institutional Review Board of the University of Virginia.

Tissue Culture—Primary mouse hepatocytes were isolated and cultured as described (12, 13, 16). HepG3, QGY-7703, and HEK-293 cells were cultured as described (7, 16). Generation of Hep-pc-4 (control clone), Hep-AEG-1-14 (a COOH-terminal HA-tagged AEG-1 overexpressing clone), Hep-siCon (control siRNA expressing clone), and Hep-AEG-1si (expressing AEG-1 shRNA) in HepG3 background has been described before (7, 11).

Plasmids—TRE luciferase reporter plasmids (pGL3.TRE-luc) and expression constructs for TRβ, SMRT, and SRC-1 were kind gifts from Dr. Yoshitaka Hayashi, Nagoya University, Japan. NF-κB luciferase reporter plasmid (NF-κB-luc) has been described before (20). Expression constructs for full-length AEG-1, L*XX*LL mutant construct (L*XX*LLmut), and AEG-1shRNA have been described before (7, 11, 16). Myc-tagged RXRα expression plasmid was obtained from Origene.

Transient Transfection and Luciferase Reporter Assays—Transfections of human HCC cells using Lipofectamine and primary hepatocytes using Promofectin were performed with firefly luciferase reporter plasmid (pGL3.TRE-luc or NF-κB-luc) and *Renilla* luciferase reporter plasmid with or without other expression plasmids as described (13, 16). After 48 h, cells were treated with T_3 (100 nM) for 24 h. Luciferase assays were measured using Dual-Luciferase reporter assay kit (Promega) following the manufacturer's protocol, and firefly luciferase activity was normalized by *Renilla* luciferase activity. Each experiment was performed three times to calculate means and standard errors.

Total RNA Extraction, cDNA Preparation, and Real Time PCR—Total RNA was extracted using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany). cDNA preparation was done using the ABI cDNA synthesis kit. Real time-polymerase chain reaction (RT-PCR) was performed using an ABI ViiA7 fast real time PCR system and TaqMan gene expression assays according to

the manufacturer's protocol (Applied Biosystems, Foster City, CA). The expression level of each gene was determined by the $\Delta\Delta C_T$ method and was normalized by the level of GAPDH. The level of untreated control for each gene was considered as 1 to determine the fold difference.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assays were performed using a kit from Active Motif (Carlsbad, CA) according to the manufacturer's instructions and as described (16, 20). Cells were treated or not with T_3 (100 nM) for 3 h before fixing in formaldehyde. An equal amount of sheared chromatin was immunoprecipitated using antibodies against RXRα (Santa Cruz Biotechnology; sc-553), TRβ1 (Santa Cruz Biotechnology; sc-398007), and SRC-1 (Cell Signaling; catalog no. 2191). The eluted DNA along with the input was subjected to PCR for *DIO1* gene promoter using primers sense, 5′-AACCACCAGC-TAGAAGCCATG-3′, and antisense 5′-TGCCACCACGCC-TGGCTAATT-3′, that flanks a consensus DR4 sequence AGGTCATCTGAGGTCA.

In Vitro Translation and Electrophoretic Mobility Shift Assay (EMSA)—AEG-1, TRβ, and RXRα proteins were generated by *in vitro* translation using TnT® Quick Coupled Transcription/Translation system (Promega). EMSA using these proteins was performed with a radiolabeled DR4 TRE probe (5′-GTAGGT-CATCTGAGGTCAGG-3′) as described (23, 24). The antibodies used for supershift analysis were anti-RXRα, anti-TRβ1, and anti-AEG-1 (chicken, in-house).

Immunohistochemistry and Western Blotting—Immunohistochemistry was performed in human HCC tissue microarrays containing 40 primary HCC, 10 metastatic HCC, and 9 normal adjacent liver samples (Imgenex; IMH-360) (7). The primary antibodies used were as follows: DIO1 (ProteinTech; rabbit polyclonal; 1:100) and AEG-1 (chicken polyclonal; 1:500). The signals were developed by avidin-biotin-peroxidase complexes with a diaminobenzidine substrate solution (Vector Laboratories). Western blotting was performed as described using AEG-1 (chicken polyclonal, 1:3000), DIO1 (ProteinTech, rabbit polyclonal, 1:1000), and EF1α (Millipore, mouse monoclonal, 1:1000) antibodies (7).

Enzyme-linked Immunosorbent Assay (ELISA)—Total T_3 was measured using the total T_3 ELISA kit (Abnova) in human serum samples and the T_3 ELISA kit (MyBiosource) in mouse serum samples following the manufacturer's instructions. Total T_4 in mouse sera was measured by the T_4 ELISA kit (Calbiotech).

Statistical Analysis—Data were represented as the mean ± S.E. and analyzed for statistical significance using one-way analysis of variance followed by Newman-Keuls test as a post hoc test. A p value of <0.05 was considered as significant. To assess the strength of association between AEG-1 and DIO1, an ordinal logistic regression was conducted. Pearson's χ^2 goodness of fit test with six degrees of freedom was performed.

Results

TR/RXR-mediated Promoter Activities Are Inhibited by AEG-1—For our studies, we used the following clones in the human HCC cell line HepG3 background: Hep-pc-4 expressing empty vector; Hep-AEG-1-14 overexpressing AEG-1; Hep-siCon expressing control siRNA; and Hep-AEG-1si expressing AEG-1 siRNA. These clones have been employed in multiple previous studies and have been thoroughly characterized (7, 9–11, 16). Additionally, we used primary mouse hepatocytes isolated from a transgenic mouse with hepatocyte-specific overexpression of AEG-1 (Alb/AEG-1) and an AEG-1 knockout (AEG-1KO) mouse (12–14). We used these cells because the liver is a major target organ of thyroid hormone (1). The AEG-1 level in these cells is shown in Fig. 1*A*. Luciferase reporter activity of thyroid hormone-response element (TRE) was measured in these cells. Upon T_3 treatment, TRE luciferase (TRE-luc) activity was induced in Hep-pc-4 and Hep-siCon cells (Fig. 1*B*). This induction was significantly inhibited in Hep-AEG-1-14 cells and increased in Hep-AEG-1si cells (Fig. 1*B*). Transfection of TRβ markedly augmented TRE-luc activity in all cells (Fig. 1*C*). However, compared with Hep-pc-4 cells, Hep-AEG-1-14 cells showed significantly decreased TRE-luc activity, although Hep-AEG-1si cells showed a significant increase *versus* Hep-siCon cells (Fig. 1*C*). Additionally, T_3-induced TRE-luc activity was significantly decreased in Alb/AEG-1 hepatocytes and significantly elevated in AEG-1KO hepatocytes, with or without TRβ co-transfection, when compared with their corresponding WT counterparts (Fig. 1, *D* and *E*).

We corroborated the findings in stable clones by transient transfection of AEG-1 or AEG-1 siRNA in HepG3 and QGY-7703 cells. Endogenous AEG-1 levels in QGY-7703 cells are markedly higher than in HepG3 cells (7). As a corollary, T_3-mediated induction of TRE-luc activity was significantly lower in QGY-7703 cells *versus* HepG3 cells when TRβ was co-transfected (Fig. 1*F*). AEG-1 overexpression suppressed and AEG-1 knockdown by siRNA augmented T_3-induced TRE-luc activity in both the cells (Fig. 1*F*). These findings were corroborated in HEK-293 cells with transient overexpression of AEG-1 (Fig. 1*G*).

We documented that AEG-1 interacts with RXR via its L*XX*LL motif (16). We checked whether this interaction was necessary for regulation of TRE-luc activity by AEG-1. HepG3 and QGY-7703 cells were transfected with TRβ and with either full-length AEG-1 or an L*XX*LL mutant construct (L*XX*LL-mut), which do not interact with RXR (16), and TRE-luc activity was measured. The L*XX*LLmut construct not only lost its ability to inhibit TRE-luc activity, as opposed to full-length AEG-1, it augmented TRE-luc activity when compared with empty vector indicating that L*XX*LLmut might function as a dominant negative inhibitor of endogenous AEG-1 (Fig. 1*H*). Taken together, AEG-1 up-regulation led to a decrease in TRE-luc activities that were increased upon inhibition of AEG-1.

AEG-1 Abrogates Expression of DIO1—The expression of TR/RXR-regulated gene DIO1 was next analyzed. At a basal level, DIO1 was strongly inhibited in Hep-AEG-1-14 cells and Alb/AEG-1 hepatocytes in comparison with their respective controls (Fig. 2*A*). Knockdown of AEG-1 did not significantly affect the basal expression of DIO1 (Fig. 2*A*). Upon treatment with T_3, DIO1 mRNA was induced in all the cells (Fig. 2, *B–D*). However, the induction level was markedly less in Hep-AEG-1-14 cells and Alb/AEG-1 hepatocytes when compared with Hep-pc-4 cells and WT hepatocytes, respectively, in the presence or absence of TRβ (Fig. 2, *B* and *C*). As a corollary, upon T_3

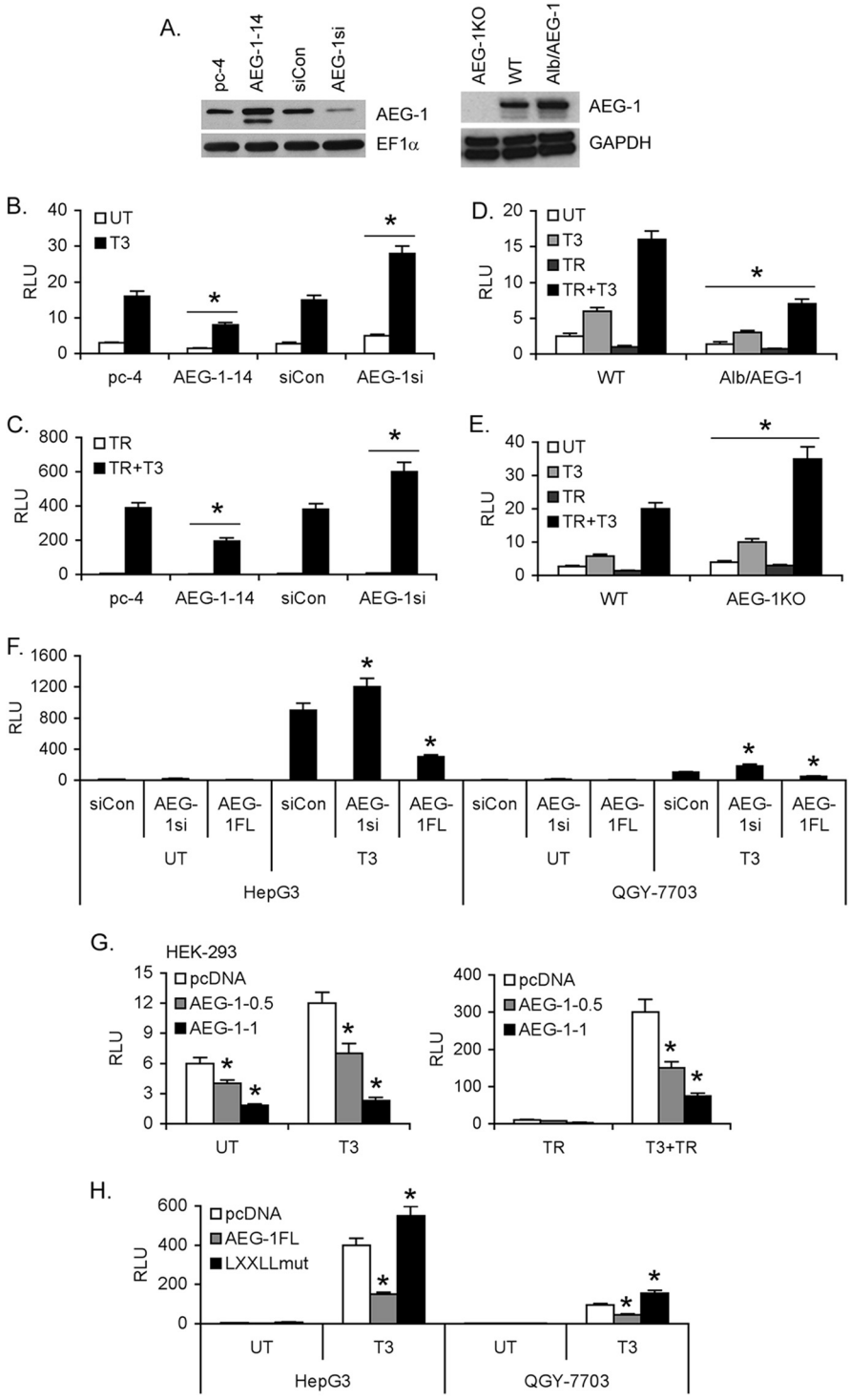


FIGURE 1. **AEG-1 abrogates TRE-luc activities.** *A,* AEG-1 level was detected in the indicated cells by Western blot. *B* and *C,* Hep-pc-4, Hep-AEG-1-14, Hep-siCon, and Hep-AEG-1si cells were transfected with a TRE luciferase reporter plasmid (TRE-luc) with (*B*) or without TRβ (TRβ) (*C*) and treated with T_3 (100 nM), and luciferase activity was measured. *D* and *E*, TRE-luc activity was measured in Alb/AEG-1 (*D*) and AEG-1KO (*E*) hepatocytes. *F,* TRE-luc activity was measured in HepG3 and QGY-7703 cells transiently transfected with TRβ along with plasmids expressing control shRNA (*siCon*), AEG-1 shRNA (*AEG-1si*), or full-length AEG-1 (*AEG-1FL*). *G,* TRE-luc reporter plasmid was co-transfected with pcDNA3.1, AEG-1, and human (h)TRβ (TRβ) expression constructs into HEK-293 cells, which were harvested for luciferase assays 48 h post-transfection. T_3 treatment (100 nM) was performed during the last 24 h. Two different concentrations of AEG-1 (0.5 and 1 μg) were transfected to see the dose-dependent effect of AEG-1. *H,* TRE-luc activity was measured in HepG3 and QGY-7703 cells transiently transfected with TRβ along with plasmids expressing empty vector (pcDNA), full-length AEG-1 (AEG-1FL), or AEG-1 with mutation in the L*XX*LL motif. Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$ *versus* corresponding controls. *UT,* untreated. *RLU,* relative luciferase unit.

treatment and TRβ co-transfection, a significant up-regulation of DIO1 expression was observed in Hep-AEG-1si cells and AEG-1KO hepatocytes in comparison with their respective controls (Fig. 2, *B* and *D*). The expression of additional T_3-regulated genes, namely malic enzyme 1 (ME1), nuclear receptor co-activator 4 (NCOA4), and fatty acid-binding protein-1, liver

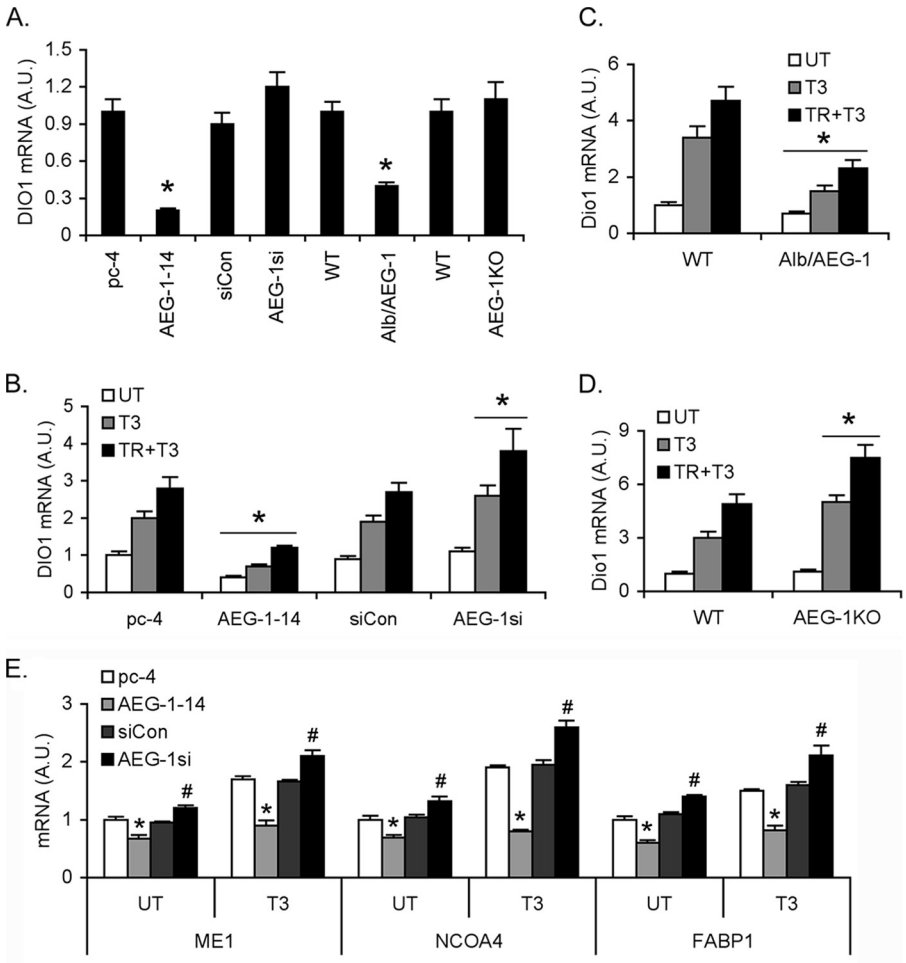
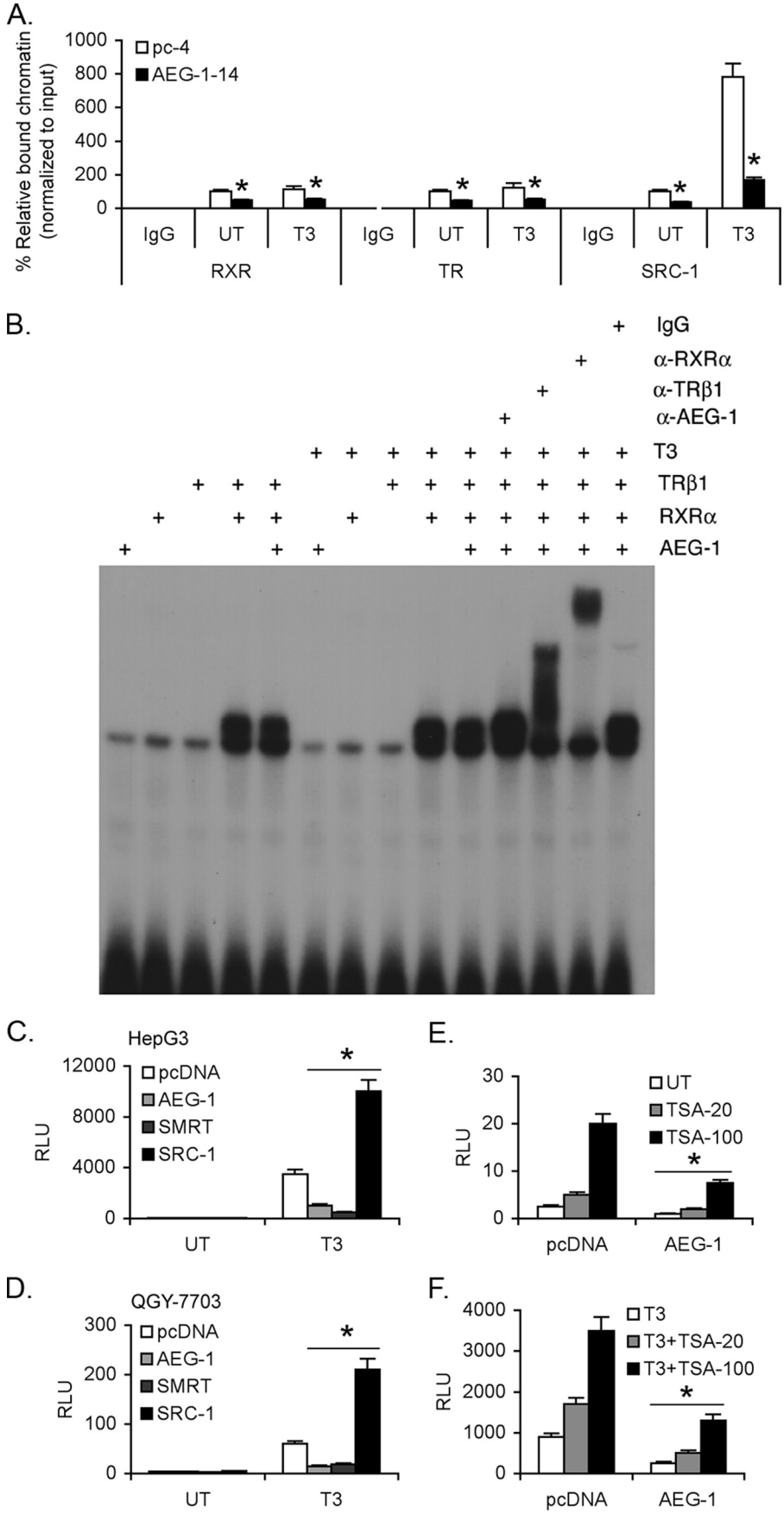


FIGURE 2. **AEG-1 suppresses T_3-induced gene expression.** *A*, basal DIO1 mRNA expression analysis in the indicated cells. *B–D*, DIO1 mRNA expression analysis upon transfection of TRβ and treatment of T_3 (100 nM) in AEG-1-overexpressing and knockdown clones of HepG3 cells (*B*), Alb/AEG-1 (*C*), and AEG-1KO hepatocytes (*D*). Data represent mean ± S.E. of three independent experiments. *A–D*, *, $p < 0.01$ *versus* corresponding controls. *E*, analysis of mRNA expression of the indicated genes in the indicated cells upon treatment with T_3 (100 nM). Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$ between Hep-pc-4 and Hep-AEG-1-14 cells; #, $p < 0.01$ between Hep-siCon and Hep-AEG-1si cells. *A.U.*, arbitrary unit; *UT*, untreated.

(FABP1), was checked in AEG-1-overexpressing and knockdown cell lines in the presence or absence of T_3. Both basal and T_3-induced levels of these genes were significantly decreased in Hep-AEG-1-14 cells and increased in Hep-AEG-1si cells when compared with their corresponding controls, further confirming a regulatory role of AEG-1 in T_3-mediated gene expression (Fig. 2*E*).

AEG-1 Impairs Co-activator Recruitment to TR/RXR—We checked the effect of AEG-1 on TR/RXR DNA binding and co-activator recruitment on the *DIO1* promoter. Chromatin immunoprecipitation (ChIP) assays were performed using antibodies against RXRα, TRβ1, and SRC-1 and lysates from Hep-pc-4 and Hep-AEG-1-14 cells. TR/RXR recruitment was significantly inhibited in Hep-AEG-1-14 cells when compared with Hep-pc-4 cells in the presence or absence of T_3 (Fig. 3*A*). Upon T_3 treatment, a robust recruitment of SRC-1 on the *DIO1* promoter was observed in Hep-pc-4 cells, which was markedly abrogated in Hep-AEG-1-14 cells. These studies indicate that AEG-1 interferes with ligand-dependent co-activator recruitment. We previously documented that cytoplasmic AEG-1 entraps RXR and prevents its nuclear translocation. This decreased availability of RXR in the nucleus might explain decreased TR/RXR binding to the *DIO1* promoter upon AEG-1 overexpression. To confirm this hypothesis and to further check whether AEG-1 interferes with the DNA binding property of TR/RXR, an electrophoretic mobility shift assay (EMSA) was performed in a cell-free system using *in vitro* translated TRβ1, RXRα, and AEG-1 and a DR4 T_3 response element as a probe. TR/RXR heterodimer efficiently bound to DNA in the absence or presence of T_3, which was not affected by addition of AEG-1 (Fig. 3*B*). Supershift of the band was observed with TRβ1 and RXRα antibodies (Fig. 3*B*). The authenticity of the *in vitro* translated AEG-1 was confirmed by Western blot analysis (data not shown). These findings suggest that AEG-1 does not interfere with DNA binding of TR/RXR and exerts its effect predominantly by inhibiting co-activator recruitment.

To define a possible correlation between AEG-1 expression and histone acetylation/deacetylation, we measured TRE-luc activity in HepG3 and QGY-7703 cells with co-expression of a co-repressor Silencing mediator for retinoid/thyroid-hormone receptors (SMRT) or a co-activator, SRC-1. TRE-luc activity was inhibited upon overexpression of AEG-1 (Fig. 3, *C* and *D*).


A.
% Relative bound chromatin (normalized to input)
pc-4
AEG-1-14
IgG
UT
T3
RXR
TR
SRC-1
B.
IgG
α-RXRα
α-TRβ1
α-AEG-1
T3
TRβ1
RXRα
AEG-1
C.
HepG3
pcDNA
AEG-1
SMRT
SRC-1
RLU
D.
QGY-7703
E.
UT
TSA-20
TSA-100
pcDNA
AEG-1
F.
T3
T3+TSA-20
T3+TSA-100

The magnitude of this inhibition was similar to the inhibition upon overexpression of SMRT (Fig. 3, *C* and *D*). SRC-1 overexpression profoundly elevated TRE-luc activity in both cells so that the ligand-induced TRE-luc activity in these cells exceeded that in cells transfected with empty pcDNA plasmid (Fig. 3, *C* and *D*). Histone deacetylase inhibitor, trichostatin A, markedly induced basal and T_3-induced TRE-luc activity, with TR overexpression, in HepG3 cells in a dose-dependent manner and provided partial rescue from AEG-1-mediated inhibition of TRE-luc activity (Fig. 3, *E* and *F*). Co-immunoprecipitation analysis did not detect any interaction between AEG-1 with SRC-1 or SMRT (data not shown). Collectively, these findings suggest that overexpressed AEG-1 blocks recruitment of co-activators to TR/RXR maintaining histones in a deacetylated state.

AEG-1 Overexpression Leads to Hypothyroidism by Down-regulating DIO1—The observation that AEG-1 significantly down-regulated DIO1 prompted us to analyze the consequence of this finding because DIO1 mediates peripheral conversion of prohormone thyroxin (T_4) to its active form T_3. DIO1 mRNA and protein levels were markedly down-regulated in the livers of Alb/AEG-1 mice in comparison with WT mice (Fig. 4, *A* and *B*). DIO1 expression was significantly less in human HCC patients compared with normal liver, and a negative correlation was observed between DIO1 and AEG-1 levels with stages of the disease in human HCC patients (Fig. 4, *C* and *D*, and Table 1). These observations were confirmed by analyzing publicly available databases in Oncomine. In four independent studies, a significant increase in AEG-1 mRNA and a corresponding decrease in DIO1 mRNA was detected in livers of human HCC patients *versus* normal individuals (Table 2). As a corollary, a significant decrease in serum T_3 level was observed in human HCC patients *versus* normal individuals (Fig. 4*E*) as well as in Alb/AEG-1 mice *versus* WT mice (Fig. 4*F*). Serum T_4 levels and TRβ (Thrb) mRNA levels in the liver were similar between Alb/AEG-1 and WT mice (Fig. 4, *G* and *H*).

Activation of NF-κB by AEG-1 Contributes to Inhibition of DIO1 Expression—Previous studies have documented that TNFα-mediated activation of NF-κB interferes with DIO1 expression (22). Because AEG-1 induces NF-κB activity, we measured TRE-luc activity after TNFα and T_3 treatment in AEG-1-overexpressing and knockdown cells. TNFα significantly inhibited T_3-induced TRE-luc activity in Hep-pc-4 and Hep-siCon cells (1.84- and 1.76-fold, respectively, when TR is co-expressed) (Fig. 5*A*). The magnitude of this inhibition was significantly more in Hep-AEG-1-14 cells (3.33-fold when TR is co-expressed). Although TNFα inhibited TRE-luc activity in Hep-AEG-1si cells, the magnitude of this inhibition was significantly less than that in Hep-siCon cells (1.32-fold when TR is co-expressed) (Fig. 5*A*). A similar finding was observed in AEG-1KO hepatocytes (Fig. 5*B*). DIO1 mRNA expression profile corroborated with TRE activity in AEG-1-overexpressing and knockdown cells (Fig. 5*C*). Although TNFα inhibited T_3-induced DIO1 mRNA expression, the magnitude of this inhibition was significantly augmented upon AEG-1 overexpression and significantly blunted upon knockdown of AEG-1 when compared with the controls (Fig. 5*C*). Basal and TNFα-induced NF-κB luciferase reporter activity was significantly increased upon overexpression of either full-length AEG-1 or L*XX*LLmut construct indicating that inhibition of RXR interaction does not interfere with NF-κB activation by AEG-1 (Fig. 5*D*). To demonstrate the role of NF-κB in AEG-1-induced inhibition of DIO1 expression, Hep-pc-4 and Hep-AEG-1-14 cells were treated with TNFα along with the NF-κB inhibitor BMS-345541. In Hep-pc-4 cells, BMS-345541 treatment completely rescued TNFα-induced inhibition of DIO1 expression (Fig. 5*E*). In Hep-AEG-1-14 cells, BMS-345541 treatment partially rescued both basal and TNFα-induced inhibition of DIO1 expression (Fig. 5*E*). Thus, both RXR inhibition and NF-κB activation might mediate AEG-1-induced inhibition of DIO1 expression and decreased serum T_3 levels (Fig. 5*F*).

Discussion

Several mechanisms underlie the development of NTIS. There might be inhibition of the central hypothalamopituitary axis with decreased secretion of thyrotropin-releasing hormone and thyroid-stimulating hormone (5). Serum thyroxine-binding globulin might be decreased, and there might be decreased tissue uptake of T_4/T_3 (5). Decreased activity of DIO1 in liver and kidneys is one of the most common causes of NTIS (5). In this study we describe significant inhibition of ligand-dependent TR/RXR activity and downstream DIO1 gene expression in human HCC cells and AEG-1 transgenic hepatocytes and reversal of these observations upon AEG-1 knockdown. In addition to DIO1, DIO2 in skeletal muscle also contributes to T_4 and T_3 conversion. Studies using knock-out mouse models have shown that DIO2 might compensate for DIO1, and DIO1/DIO2 double knock-out mice maintain plasma T_3 levels (25, 26). A possible explanation of this unexpected finding is increased production of T_3 by thyroid gland itself driven by increased thyroid-stimulating hormone production in these animals. In livers of Alb/AEG-1 mice, we observed profound inhibition in DIO1 levels (Fig. 5*A*). Inhibition of co-activator recruitment to the TR/RXR complex by AEG-1 abrogates T_3-induced DIO1 expression. This decrease in DIO1 inhibits conversion of T_4 to T_3 in the liver. The low amount of T_3 that is generated is further inhibited to exert its transcription regulation function by AEG-1 thereby further decreasing the level of DIO1. This situation is accentuated by the ability of AEG-1 to activate NF-κB resulting in marked inhi-

FIGURE 3. **AEG-1 interferes with the recruitment of co-activators to *DIO1* promoter and histone acetylation rescues TRE-luc activity from AEG-1-mediated inhibition.** *A,* ChIP assay was performed using anti-RXRα, anti-TRβ1, and anti-SRC-1 antibodies in Hep-pc-4 and Hep-AEG1–14 cells, treated or not with T_3, and PCR primers amplifying the promoter region of *DIO1*. Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$ *versus* corresponding controls. *B,* AEG-1, RXRα, and TRβ proteins were *in vitro* translated and were used in EMSA in the absence or presence of 100 nM T_3 and ^{32}P-labeled DR4 TRE probe. The reaction mixture was incubated with anti-RXRα, anti-TRβ1, or anti-AEG-1 antibody for supershift analysis. *C* and *D,* assessment of TRE-luc activity in HepG3 (*C*) and QGY-7703 (*D*) cells upon transfection with TRβ and pcDNA3.1, AEG-1, SMRT, or SRC-1 expression plasmids in the absence or presence of 100 nM T_3. *E* and *F,* TRE-luc activity was measured in HepG3 cells after co-transfection with TRβ and pcDNA3.1 or AEG-1 expression construct and treatment with trichostatin A (*TSA*) (20 and 100 nM) alone (*E*) and with T_3 (100 nM) (*F*). Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$ *versus* corresponding controls. *RLU,* relative luciferase unit; *UT*, untreated.

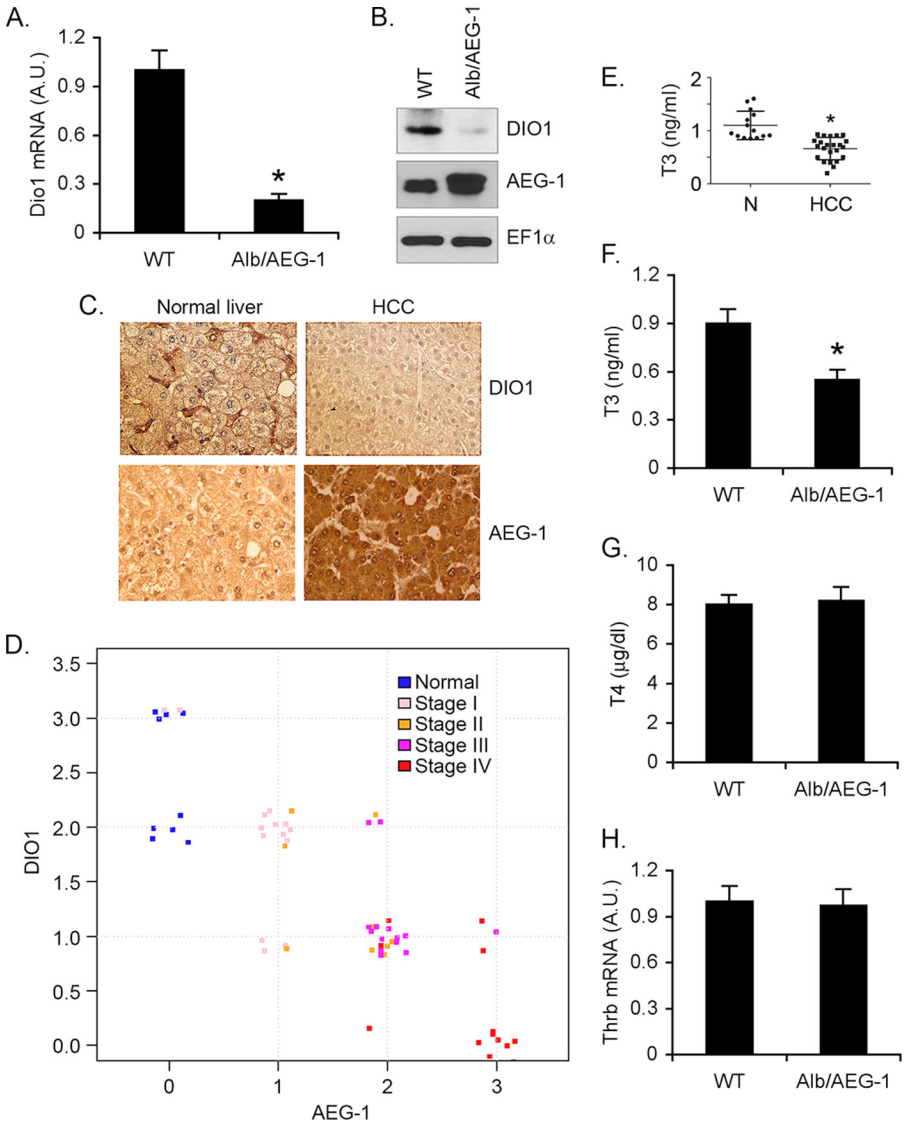


FIGURE 4. **AEG-1 decreases total serum T_3 level.** *A* and *B*, expression analysis of DIO1 in livers of WT and Alb/AEG-1 mice ($n = 5$ for each group) at mRNA level by TaqMan quantitative RT-PCR (*A*) and protein level by Western blotting (*B*). Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$. *C*, immunohistochemical analysis of DIO1 and AEG-1 in normal liver and matched HCC from the same patient. The panels represent data from one patient. A similar finding was observed in other HCC patients. *D*, expression analysis of AEG-1 and DIO1 proteins in normal and HCC samples at different stages. To assess the strength of association between AEG-1 and DIO1, an ordinal logistic regression was conducted. Pearson's χ^2 goodness of fit test with six degrees of freedom was performed. *p* value, 2.354×10^{-14}. *E* and *F*, determination of total T_3 levels in the serum of normal controls ($n = 14$), and hepatitis C virus-HCC patients ($n = 23$) (*E*), and WT and Alb/AEG-1 mice ($n = 5$ per group) (*F*) by ELISA. Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$. *G*, determination of total T_4 levels in the serum of WT and Alb/AEG-1 mice ($n = 5$ per group) by ELISA. *H*, analysis of thyroid hormone receptor β (*Thrb*) mRNA level in the livers of WT and Alb/AEG-1 mice ($n = 5$ per group). *A.U.*, arbitrary unit.

TABLE 1

Cross tabulation of AEG-1 and DIO1 expression

Pearson's χ^2 test was used. The following were used: C_T, $\chi^2 = 84.2027$. Degrees of freedom = 6. *p* value is 2.354×10^{-14}.

	0	1	2	3
0	0.00	0.00	5.00	6.00
1	0.00	4.00	11.00	0.00
2	1.00	18.00	3.00	0.00
3	8.00	3.00	0.00	0.00

bition of DIO1 expression. In these mice, the level of DIO1 in the liver is so low that DIO1 in the kidneys and DIO2 in skeletal muscle may not compensate for low liver DIO1 resulting in a significant decrease in serum T_3 levels. A similar scenario might be true for human HCC patients in whom there is a marked increase in AEG-1 levels with a corresponding decrease in DIO1 levels in the liver. However, additional factors associated with chronic liver disease, such as decreased serum thyroxine-binding globulin or decreased tissue uptake of T_4/T_3, might also play a role in decreased serum T_3 levels in these patients.

Apart from malignancy, other causes of NTIS include sepsis and AIDS (5). AEG-1 might play a role in NTIS in these cases as well. We have documented that AEG-1 is fundamentally required for NF-κB activation (14). NF-κB is a pivotal determinant of inflammation (27). Although 16-month-old WT mice showed signs of aging-associated inflammation, no such changes were observed in AEG-1KO littermates (14). Infiltration of macrophages was observed in aged WT liver and spleen but not in AEG-1KO (14). As a corollary, NF-κB activity is significantly augmented in Alb/AEG-1 hepatocytes (12, 13).

TABLE 2
Analysis of Oncomine dataset for AEG-1 and DIO1 mRNA expression in normal liver and HCC samples

Dataset	Liver no.	HCC no.		Gene rank	*p* value	Fold change
Chen liver	76	103	AEG-1/MTDH	In top 9%	4.89E-8	1.571
			DIO1	In top 11%	3.17E-7	−2.093
Wurmbach liver	10	35	AEG-1/MTDH	In top 19%	0.006	1.634
			DIO1	In top 4%	4.62E-5	−2.593
Roessler liver	21	22	AEG-1/MTDH	In top 12%	8.61E-5	2.036
			DIO1	In top 29%	0.013	−2.325
Roessler liver 2	220	225	AEG-1/MTDH	In top 9%	2.59E-36	1.769
			DIO1	In top 16%	1.01E-11	−2.149

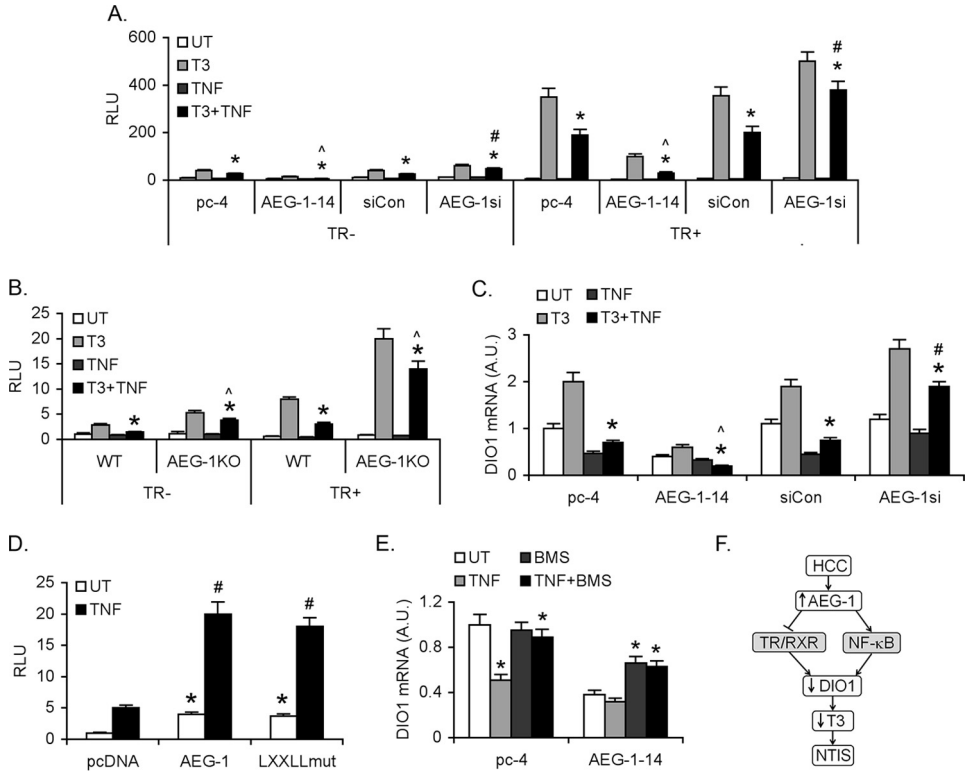


FIGURE 5. **NF-κB activation mediates AEG-1-induced inhibition of DIO1 expression.** *A,* measurement of TRE-luc activity in the indicated cells transfected with or without TRβ followed by treatment with T_3 and TNFα (10 nM) for 16 h. Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$ between T_3 *versus* T_3 + TNF in each group. [caret], $p < 0.01$ between T_3 + TNF groups of Hep-pc-4 and Hep-AEG-1-14. #, $p < 0.01$ between T_3 + TNF groups of Hep-siCon and Hep-AEG-1si. *B,* measurement of TRE-luc activity in WT and AEG-1KO hepatocytes transfected with or without TRβ followed by treatment of T_3 and TNFα (10 nM) for 16 h. Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$ between T_3 *versus* T_3 + TNF in each group. [caret], $p < 0.01$ between T_3 + TNF groups of WT and AEG-1KO. *C,* measurement of DIO1 mRNA levels in the indicated cells treated with T_3 and TNF. *, $p < 0.01$ between T_3 *versus* T_3 + TNF in each group. [caret], $p < 0.01$ between T_3 + TNF groups of Hep-pc-4 and Hep-AEG-1-14. #, $p < 0.01$ between T_3 + TNF groups of Hep-siCon and Hep-AEG-1si. *D,* measurement of NF-κB luciferase reporter activity in HepG3 cells transfected with the indicated plasmids and treated with TNFα for 24 h. Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$ between the untreated (*UT*) groups. #, $p < 0.01$ between the TNFα-treated groups. *E,* Hep-pc-4 and AEG-1-14 cells were treated with TNFα and BMS-345541 (10 μM), and DIO1 mRNA expression was measured. Data represent mean ± S.E. of three independent experiments. *, $p < 0.01$. *F,* schematic showing the mechanism by which AEG-1 mediates NTIS in HCC.

Thus, regulation of NF-κB and inflammation is an important function of AEG-1. In inflammatory states, such as sepsis, inflammatory cytokines play an important role in mediating NTIS. Although the AEG-1KO mouse is resistant to aging-associated inflammation, its susceptibility to septic inflammation has not been studied. It would be intriguing to check the response of the AEG-1KO mouse to sepsis and development of NTIS. AEG-1 was first identified as an HIV-inducible gene in primary astrocytes (28). AEG-1 might contribute to HIV-induced dementia by decreasing the level of glutamate transporters in the astrocytes resulting in accumulation of glutamate in the synapses and leading to excitotoxic neuronal death (29). Whether AEG-1 has any role in AIDS-associated NTIS requires testing.

There is a reciprocal relationship between thyroid hormone function and HCC. An association of long term hypothyroidism with HCC, independent of other major HCC risk factors, has been identified (30). Down-regulation of DIO1 mRNA has been detected in HCC as well as in thyroid and renal cancers (31, 32). TRs have been shown to play a tumor suppressor role, and their mutations or aberrant expression is evident in various cancers, including HCC (30). A recent study documented decreased TRα1 and TRβ1 expression in preneoplastic lesions in rat liver and concluded that local hypothyroidism facilitates the progression of these lesions to HCC (33). By interfering with T_3 function and a decreasing DIO1 level, AEG-1 might create a local hypothyroid state in the liver that might facilitate

HCC progression. Thus, in addition to activation of NF-κB and other pro-tumorigenic signaling pathways, regulation of TR/RXR function might be another mechanism by which AEG-1 contributes to HCC.

In summary, this study unravels a novel function of AEG-1 in regulating T_3 function. AEG-1 contributes to HCC initiation and progression by a variety of mechanisms. Thus, AEG-1 inhibition might be an effective way to counteract this fatal malady for which there is no effective treatment. AEG-1 inhibition will not only eliminate HCC but also ameliorate detrimental effects associated with the disease such as NTIS. Efforts need to be spent to develop clinically relevant AEG-1 inhibitors.

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