CREB3L2-PPARγ Fusion Mutation Identifies a Thyroid Signaling Pathway Regulated by Intramembrane Proteolysis

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Abstract

The discovery of gene fusion mutations, particularly in leukemia, has consistently identified new cancer pathways and led to molecular diagnostic assays and molecular-targeted chemotherapies for cancer patients. Here, we report our discovery of a novel CREB3L2-PPARγ fusion mutation in thyroid carcinoma with t(3;7)(p25;q34), showing that a family of somatic PPARγ fusion mutations exist in thyroid cancer. The CREB3L2-PPARγ fusion encodes a CREB3L2-PPARγ fusion protein that is composed of the transactivation domain of CREB3L2 and all functional domains of PPARγ1. CREB3L2-PPARγ was detected in <3% of thyroid follicular carcinomas. Engineered overexpression of CREB3L2-PPARγ induced proliferation by 40% to 45% in primary human thyroid cells, consistent with a dominant oncogenic mechanism. Wild-type CREB3L2 was expressed in the thyroid as a bZIP transcription factor with a transmembrane domain that has flanking S1P and S2P proteolytic cleavage sites. Native CREB3L2 was cleaved to nuclear CREB3L2 by regulated intramembrane proteolysis in normal thyroid cells that expressed the S1P and S2P proteases. Nuclear CREB3L2-stimulated transcription 8-fold from the EVX1 cyclic AMP (cAMP) response element in the absence of cAMP, whereas CREB3L2-PPARγ inhibited transcription 6-fold from EVX1 in the same experiments. CREB3L2-PPARγ also inhibited 4-fold the expression of thyroglobulin, a native cAMP-responsive gene, in primary thyroid cells treated with thyroid-stimulating hormone. Our findings identify a novel CREB3L2-PPARγ gene fusion mutation in thyroid carcinoma and reveal a thyroid signaling pathway that is regulated by intramembrane proteolysis and disrupted in cancer. [Cancer Res 2008;68(17):7156–64]

Introduction

Gene fusion mutations underlie a significant subset of human cancers. Scientific investigations of gene fusions, particularly in leukemia, have led to (a) the identification of biological pathways that are deregulated in many cancer types; (b) the development of specific molecular diagnostic assays that identify and monitor cancer in patients; and (c) the implementation of effective molecular-targeted chemotherapies that have relatively few side effects. The discovery of fusion mutations is therefore important, particularly in carcinoma, the most common cancer group in which few gene fusions have been identified (1). The recent discoveries of ERG (2) and ALK (3) gene fusions in prostate and lung carcinoma, respectively, increase the prospect that new diagnostic and therapeutic strategies based on gene fusions will be applicable to common epithelial cancers.

Families of gene fusions tend to characterize specific cancer types. For example, the RUNX1-ETO and CBFβ-SMMHC gene fusions underlie acute myeloid leukemia and deregulate the RUNX/CBF transcription factor complex by distinct molecular mechanisms (4). In a similar fashion, the PML-RARα, PLZF-RARα, NPM-RARα, NuMA-RARα, and Stats5b-RARα gene fusions underlie acute promyelocytic leukemia. The PML-RARα gene fusion is present in >95% of acute promyelocytic leukemia patients, whereas the other RARα fusions are observed at low incidence. Even so, the low incidence gene fusions have revealed molecular mechanisms that were unapparent from investigation of PML-RARα alone (5, 6) and have identified biological pathways that are important in both acute promyelocytic leukemia and other cancer types.

PPARγ is a lipid-binding nuclear receptor that has been implicated in physiologic processes, including adipogenesis and obesity (7, 8), insulin sensitivity and diabetes (9, 10), and inflammation and atherosclerosis (11, 12). The activities of PPARγ have been studied most thoroughly in fat, the development of which requires PPARγ to induce differentiation and regulate the expression of fat-specific genes (7, 8, 13). Mutations of PPARγ in colon (14) and thyroid (15) carcinoma tissues. Such mutations seem to impair ligand-mediated transcription by PPARγ (14, 15), which induces differentiation and inhibits proliferation in many, but not all, cancer cell lines (16–20). Even so, the interpretation of these experiments is not straightforward because data are conflicting as to whether PPARγ suppresses or promotes tumorigenesis in mouse tumor models (21–24). Thus, fundamental mechanisms of PPARγ in cancer remain to be elucidated.

Regulated intramembrane proteolysis is a physiologic process that cleaves transcription factors from cell membranes to activate gene expression (25). For example, SREBP1 and SREBP2 (26), Notch, and activating transcription factor-6 undergo intramembrane proteolysis in cholesterol, fatty acid, protein folding, and intracellular signaling pathways. Cyclic AMP (cAMP) response element binding protein 3 (CREB3) and CREB4 are bZIP transcription factors with transmembrane domains that are thought to be cleaved by intramembrane proteolysis in response to endoplasmic reticulum stress (27, 28). CREB3-like 2 (CREB3L2)
a CREB3-related protein that has domains fused to FUS in human fibromyxoid sarcoma with c(7;16) (29, 30). An oncogenic role for the FUS-CREB3L2 fusion protein is predicted but is not yet determined. Here, we report our discovery of a novel CREB3L2-PPARγ; gene fusion mutation in thyroid follicular carcinoma. Our experiments show that the encoded CREB3L2-PPARγ; fusion protein stimulates proliferation and inhibits cAMP-responsive transcription in normal thyroid cells treated with thyroid-stimulating hormone (TSH). Our discovery of CREB3L2-PPARγ identifies a thyroid signaling pathway that is regulated by intramembrane proteolysis and disrupted in thyroid cancer.

Materials and Methods

Tissues and cells. Thyroid tumors were diagnosed according to WHO criteria (31), with the exception that Hurthle cell tumors were diagnosed separately from, and not as a variant of, thyroid follicular tumors. Some thyroid tumors have been described in previous reports (15, 32). Banked tissues were obtained from thyroidectomy specimens and frozen immediately at −80°C or in liquid nitrogen. Tissue fragments were examined by frozen and/or permanent sections to confirm the proportion of tumor cells. Informed consent was obtained from all patients and the study was approved by the Institutional Review Boards of the University of Chicago, Emory University, and the Ethics Committee of the Karolinska Hospital.

Primary human thyroid cells. Cultures of primary thyroid cells were established from normal thyroid tissues that were removed by surgery for thyroid tumors (33). The thyroid tissues were dissected by an experienced thyroid pathologist (T.G.K.), disaggregated, and digested with collagenase and dispase (Roche) for up to 3 h. Intact thyroid follicles were maintained for 3 to 5 d in serum-free RPMI medium (Invitrogen-Life Technologies, Inc.) containing glutamine, penicillin-streptomycin, and primocin (Sigma). Thyroid cell monolayers of 85% to 90% purity grew from the follicles after the addition of medium with 10% FCS. Primary thyroid cultures from ≈40 different patients have been investigated.

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) was done with yeast artificial chromosomes (YAC) 75337 and 92238 that flank the PPARY; gene, a BAC 377219 that contains the CREB3L2 gene, and BACs 22G20, 351B12, 29B3, 83I11, and 691P5 that flank the CREB3L2 gene, as described previously (15, 34). Briefly, touch preparations from frozen thyroid carcinoma tissues were prepared on glass slides, fixed in either 70% ethanol or methanol/acytic acid (3:1), and air-dried. Slides were treated with pepsin (50 µg/ml in 0.1 N HCl) and postfixed in 10% phosphate buffered formalin (Sigma). FISH probes were labeled with digoxigenin- or biotin-conjugated nucleotides by random priming (Invitrogen), mixed with human Cot-1 DNA (Invitrogen), denatured, and hybridized for 16 to 48 h at 37°C. Probes were visualized with fluorescent avidin (Vector) or anti-digoxigenin (Roche) using a Zeiss Axiosplan2e microscope fitted with Zeiss Neofluar and ApoFluar objectives, a megapixel avidin (Vector) or anti-digoxigenin (Roche) using a Zeiss Axioplan2ie.

Rapid amplification of mRNA ends, reverse transcription-PCR, and mRNA in situ hybridization. cDNA was transcribed from total RNA using oligo dT primers and SMART rapid amplification of cDNA ends (RACE) oligonucleotides, as described (SMART RACE kit, Clontech). CREB3L2-PPARγ; was identified originally by SMART reverse transcription using the PPARY; reverse primer (5’-ACAGGGCCTACCTTGTGGC-3’, 319–341, NM_138712) and universal forward primers. Amplifications were done with Advantage II (Clontech) or PFX (Invitrogen) Taq polymerase. Primers for cloning of the full-length CREB3L2-PPARγ; cDNA were CREB3L2 forward (5’-ATGGAGGCTGCTGAGAACGCGG-3’, 353–373, AJ549092, AJ549387) and PPARγ; reverse (5’-GAGAGTCCTGAGCCACTGCC-3’, 1901–1921, NM_138712) using Advantage II or HotStar Taq DNA polymerase (Qiagen). Quantitative reverse transcription-PCR (RT-PCR) for CREB3L2 was done as previously described (35). Primers for the amplification of SIP were 5’-TACCACACCTCCGGTATCC-3’ (1882–1902, D42053) and 5’-CCCGTACCCAGACAGACG-3’ (2303–2323, D42053). Primers for the amplification of SIP were 5’-TGATGCGTACTCTTCTCCT-3’ (413–433, AF019612) and 5’-AGTGATGAGCAACCCAAACTCT-3’ (876–865, AF019612). Automated nucleotide sequencing was done using Big Dye Terminators (Perkin-Elmer). mRNA in situ hybridization was done as described (36) on normal and thyroid tissue sections. In brief, 5-µm sections were cut from frozen thyroid tissues, hybridized with 32P end-labeled synthetic oligonucleotides, and washed under stringent conditions. Expression was determined by autoradiography after exposure in photographic emulsion. The antisense probe for CREB3L2-PPARγ; was 5’-CTTGGTACACATGTTGATTGC-3’; 297–340, AY222643) was designed to span the CREB3L2-PPARγ; fusion breakpoint. An 18s RNA antisense probe (5’-CCCTGGGAGGCACTCCCC-AAGGTGGTCTTTTGGTGACAC-3’, 441–480, X03205) was used as a positive control and a 17α-hydroxylase sense probe (5’-CCCTGGAAGGAGCATTCCCC-AAGGTGGTCTTTTGGTGACAC-3’, 8290–8329, M63871) was used as a negative control.

Cell growth assays and electroporation. CREB3L2-PPARγ, PAX8-PPARγ, and wild-type PPARY1 were expressed from the mammalian expression vectors pcDNA3.1 or pcDNA3.2 (Invitrogen). Electroporation was done in primary thyroid cells using the Amaxa system (Amaxa). Growth experiments were repeated in three to six separate experiments with different isolates of primary thyroid cells. Cell numbers were determined in duplicate or triplicate using a Z2 particle/cell counter (Beckman-Coulter). Tryptophan and rosiglitazone PPARγ agonists were obtained from Cayman or Biomol. Tunicamycin and thapsigargin were purchased from Sigma-Aldridge.

Transfection and transcription assays. Transfection assays were done with the EVXI CRE reporter (37) in the dual luciferase system (Promega). Transfections were done for 24 h with FuGene as described by the manufacturer (Roche). In some experiments, troglitazone or rosiglitazone was added at 24 to 36 h in medium with charcoal-stripped FCS (Hyclone). Luciferase activities were determined in duplicate or triplicate as the mean ± SE.

Immunoblotting and immunoreagents. Total protein was extracted from tissues or cells with lysis buffers containing nonionic (NP40 or Triton X-100) and/or ionic (SDS and sodium deoxycholate) detergents (U.S. Biochemical or Sigma) and complete protease inhibitors (Roche). Total cellular protein (20–50 µg) was mixed with SDS sample buffer containing 50 mM/L DT or 2% to 2.5% 2-mercaptoethanol, heated to 95°C for 5 min, and fractionated by SDS-PAGE. Proteins were blotted onto nitrocellulose (Protran) or polyvinylidene difluoride membranes. Membranes were incubated sequentially with 5% to 10% nonfat dry milk, primary antibody or antisera, and secondary rabbit anti-mouse or donkey anti-rabbit horseradish peroxidase-conjugates (Santa Cruz or Amersham). Detection was carried out with enhanced chemiluminescence (Amersham). Kaleido-scope (Bio-Rad) and Magicmark (Invitrogen) markers were used to determine relative molecular weights. Protein levels were quantified on X-ray films from immunoblots using Image) software.6 Values were normalized to levels of control (β-actin in the same lanes. Immunoreagents included the E8 PPARγ monoclonal antibody (Santa Cruz), the H7 PPARγ antisera (Santa Cruz), a β-actin antibody (Santa Cruz), a calreticulin antibody (Abcam), and a thyroglobulin antisera (M0781; DAKO). The CREB3L2 antisera was created by New England Peptide, Inc., against a synthetic peptide, Ac-LQWDRKLSELSEPGDC-amide, from the NH2 terminus of wild-type CREB3L2 (residues 12–27, NP_919047). The specificity of the CREB3L2 antisera was verified by ELISA (New England Peptide), immunoblotting, and peptide blocking experiments (Supplementary Fig. S1).

Thyroid tissue microarrays. FISH was done as described above on thyroid tissue microarrays that were constructed from formalin-fixed, paraffin-embedded human thyroid tissues selected from 103 patients by two experienced endocrine pathologists (T.G.K. and M.T.). The tissue

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6 http://rshbinfo.nih.gov/ji/
microarrays contained 127 benign thyroid tissues, including 18 follicular adenomas, 10 Hurthle cell adenomas, 7 multinodular goiters, 3 cases of Graves’ disease, 6 cases of Hashimoto’s thyroiditis, and 83 normal thyroid sections. A minimum of two tissue cylinders with a diameter of 1 mm were arrayed using an automated tissue microarrayer (ATA-27, Beecher Instruments). The recipient blocks were cut into 4-μm-thick sections on Surgipath silane-coated positive-charged slides.

Statistical analyses. Data from cell growth, proliferation, and transcription assays were calculated as the mean of duplicate or triplicate measurements ± SD. P values were determined using the Student t test for continuous variables. P < 0.05 was considered to be statistically significant.

Results

Discovery of the CREB3L2-PPARγ fusion mutation. We physically mapped the breakpoints of the chromosomal rearrangement t(3;7)(p25q34) that we identified previously in a thyroid follicular carcinoma (32). Dual-color FISH with YAC probes that flank the PPARγ gene at 3p25 (932f3 and 753f7) showed split red and green signals that are indicative of PPARγ rearrangements (15, 34) in interphase nuclei of thyroid carcinoma with t(3;7) (Fig. 1A). Single-color FISH with a bacterial artificial chromosome (BAC) probe that contains the CREB3L2 gene at 7q34 (377b19) showed one intact and two split red signals in the thyroid carcinoma cells (Fig. 1B), consistent with simultaneous rearrangement of CREB3L2. Dual-color FISH with BAC probes that flank CREB3L2 confirmed rearrangement of CREB3L2 (Fig. 1C). These experiments showed that both CREB3L2 and PPARγ are rearranged in thyroid carcinoma with t(3;7).

We used rapid amplification of cDNA ends (RACE) to identify 5’coding sequences of PPARγ fused in-frame to 5’untranslated and coding sequences of wild-type PPARγ (exons 1 and 2) fused in-frame to coding and 3’untranslated sequences of PPARγ (exons 1–6). The full-length CREB3L2-PPARγ cDNA predicts a CREB3L2-PPARγ fusion protein that consists of amino acids 1 to 106 of wild-type CREB3L2, a new glutamic acid (E) at position 107, and all 477 amino acids of wild-type PPARγ1. Blue, CREB3L2 sequences; yellow, PPARγ sequences; red, PAX8 sequences; gray, noncoding exons.

Figure 1. Discovery of the CREB3L2-PPARγ fusion mutation. A, dual-color FISH showed that YAC probes that flank the PPARγ gene were split (white arrowheads) in interphase nuclei of thyroid carcinoma with t(3;7), showing rearrangement of PPARγ. One carcinoma cell nucleus is shown. B, single-color FISH showed that a BAC that contains the CREB3L2 gene was also split (white arrowheads) in interphase nuclei of thyroid carcinoma with t(3;7), showing simultaneous rearrangement of CREB3L2. Two carcinoma nuclei are shown. C, dual-color FISH with BAC probes that flank the CREB3L2 gene confirmed rearrangement of CREB3L2 (white arrowheads) in interphase nuclei of paraffin-embedded thyroid carcinoma with t(3;7). D, RACE and RT-PCR generated a CREB3L2-PPARγ cDNA that consisted of 5’untranslated and coding sequences of wild-type CREB3L2 (exons 1 and 2) fused in-frame to coding and 3’untranslated sequences of PPARγ (exons 1–6). The full-length CREB3L2-PPARγ cDNA predicts a CREB3L2-PPARγ fusion protein that consists of amino acids 1 to 106 of wild-type CREB3L2, a new glutamic acid (E) at position 107, and all 477 amino acids of wild-type PPARγ1. Blue, CREB3L2 sequences; yellow, PPARγ sequences; red, PAX8 sequences; gray, noncoding exons.
glutamic acid (E) at position 107, and all 477 wild-type amino acids of wild-type PPARγ (Fig. 1D). The extra glutamic acid and all wild-type PPARγ amino acids are also present in the PAX8-PPARγ fusion protein (Fig. 1D) that we previously discovered (15). FISH showed the CREB3L2-PPARγ gene fusion in 1 of 42 (2.4%) additional paraffin-embedded thyroid follicular carcinomas that we have reported in a previous series (34), whereas no CREB3L2 rearrangements were detected in 127 benign thyroid tissues that included 18 follicular adenomas, 10 Hurthle cell adenomas, 7 multinodular goiters, 3 cases of Graves’ disease, 6 cases of Hashimoto’s thyroiditis, and 83 normal thyroid tissue sections. Thus, CREB3L2-PPARγ is a low-incidence fusion mutation that is present in <3% of thyroid follicular carcinomas.

Expression of CREB3L2-PPARγ. RT-PCR showed CREB3L2-PPARγ mRNA in thyroid follicular carcinoma with t(3;7) but not in normal thyroid tissues (Fig. 2A). The extra glutamic acid and all wild-type PPARγ1 amino acids are also present in the PAX8-PPARγ fusion protein (Fig. 1D) that we previously discovered (15). FISH showed the CREB3L2-PPARγ gene fusion in 1 of 42 (2.4%) additional paraffin-embedded thyroid follicular carcinomas that we have reported in a previous series (34), whereas no CREB3L2 rearrangements were detected in 127 benign thyroid tissues that included 18 follicular adenomas, 10 Hurthle cell adenomas, 7 multinodular goiters, 3 cases of Graves’ disease, 6 cases of Hashimoto’s thyroiditis, and 83 normal thyroid tissue sections. Thus, CREB3L2-PPARγ is a low-incidence fusion mutation that is present in <3% of thyroid follicular carcinomas.

Expression of CREB3L2-PPARγ. RT-PCR showed CREB3L2-PPARγ fusion mRNA in thyroid follicular carcinoma with t(3;7) but not in normal thyroid tissues (Fig. 2A). Expression of CREB3L2-PPARγ mRNA was also observed by in situ hybridization (data not shown). Immunoblots with the E8 PPARγ antibody, which reacts with both PPARγ1 and PPARγ2, showed overexpression of native CREB3L2-PPARγ compared with wild-type PPARγ1 in thyroid carcinoma with t(3;7) (Fig. 2B, left). The CREB3L2-PPARγ fusion protein was not expressed in normal thyroid tissue and comigrated with transfected CREB3L2-PPARγ (67 kDa) and between transfected PPARγ1 (54 kDa) and transfected PAX8-PPARγ (98 kDa) that were expressed in 293T epithelial cells. Nonspecific, nonimmunospecific band. C, immunohistochemistry with the E8 PPARγ antibody showed overexpression of the CREB3L2-PPARγ fusion protein in nuclei of thyroid carcinoma with t(3;7). Immunoreactivity is brown and the nuclear counterstain (Gill’s hematoxylin) is blue.

Figure 2. Expression of CREB3L2-PPARγ in thyroid carcinoma tissue. A, RT-PCR with primers that flank the CREB3L2-PPARγ breakpoint showed expression of CREB3L2-PPARγ mRNA in thyroid carcinoma with t(3;7) (FC) but not in normal thyroid tissue. mw, molecular weight markers. B, immunoblots with the E8 PPARγ antibody, which immunoreacts with both PPARγ1 and PPARγ2, showed overexpression of native CREB3L2-PPARγ compared with wild-type PPARγ1 in thyroid carcinoma with t(3;7) (FC; left). The CREB3L2-PPARγ fusion protein was not expressed in normal thyroid tissue and comigrated with transfected CREB3L2-PPARγ (67 kDa) and between transfected PPARγ1 (54 kDa) and transfected PAX8-PPARγ (98 kDa) that were expressed in 293T epithelial cells. Nonspecific, nonimmunospecific band. C, immunohistochemistry with the E8 PPARγ antibody showed overexpression of the CREB3L2-PPARγ fusion protein in nuclei of thyroid carcinoma with t(3;7). Immunoreactivity is brown and the nuclear counterstain (Gill’s hematoxylin) is blue.

Figure 3. CREB3L2-PPARγ stimulates growth and induces proliferation in primary thyroid cells. A, the CREB3L2-PPARγ and PAX8-PPARγ fusion proteins stimulated the growth of primary human thyroid epithelial cells by 25% to 30% compared with wild-type PPARγ or vector controls over 5 d. Similar results were obtained with several isolates of primary cells. Columns, mean from duplicate or triplicate culture dishes; bars, SD. *, P < 0.007. B, the CREB3L2-PPARγ and PAX8-PPARγ fusion proteins also induced thyroid cell proliferation by 42% and 58%, respectively, over vector controls based on the incorporation of BrdUrd during a 5-h incubation period. The incorporation of BrdUrd was determined in duplicate culture dishes on day 3 after electroporation. Columns, mean; bars, SD. *, P < 0.0003.
Immunohistochemistry with the E8 PPARγ antibody showed increased expression of CREB3L2-PPARγ in the nuclei of thyroid carcinoma with t(3;7) relative to normal thyroid tissue on the same slide (Fig. 2C). These experiments show that CREB3L2-PPARγ fusion mRNA and protein are expressed highly in thyroid carcinoma with t(3;7).

The CREB3L2-PPARγ fusion protein induces proliferation in primary thyroid cells. To begin to determine the oncogenic mechanism of CREB3L2-PPARγ, we measured the effects of the CREB3L2-PPARγ fusion protein on the growth of primary human thyroid cells. Cultures of primary thyroid cells were 85% to 90% pure (33) and retained differentiated functions such as the production of thyroperoxidase and thyroglobulin in response to TSH.7 Primary thyroid cells were transduced at 50% to 70% efficiency by electroporation based on a green fluorescent protein construct that was followed by fluorescence microscopy and flow cytometry.7 The CREB3L2-PPARγ and PAX8-PPARγ fusion proteins increased the number of primary thyroid cells by 25% to 30% (P < 0.007) compared with vector controls over 5 d (Fig. 3A). In contrast, wild-type PPARγ, which contains the same PPARγ amino acids as these fusion proteins, had no stimulatory activity (Fig. 3A). The addition of PPARγ agonists troglitazone (1–10 μmol/L) or rosiglitazone (10–1,000 nmol/L; ref. 10) also had little effect in the presence or absence of the fusion proteins.7 Thus, the CREB3L2-PPARγ fusion protein is a potent inducer of thyroid cell growth and seems to be unresponsive to synthetic PPARγ ligands.

Figure 4. Wild-type CREB3L2 is expressed in thyroid tissue and regulated by intramembrane proteolysis. A, Northern blots showed expression of three wild-type CREB3L2 mRNA transcripts, ~7.5, 2.3, and 1.1 kb, in thyroid and other human tissues. B, the 7.5- and 2.3-kb CREB3L2 mRNAs encode a CREB3L2 protein of 520 amino acids that has an NH2-terminal transactivation domain, a bZIP DNA binding domain, and a carboxyl transmembrane (TM) domain (residues 379–395; light red) that is flanked by S2P (LCX, P; residues 385–402; light blue) and S1P (RXXL, residues 427–430; light green) proteolytic cleavage sites. S2P and S1P sites are often cleaved by intramembrane proteolysis to release transcription factor domains to the nucleus to activate gene expression. The transactivation domain of CREB3L2 is fused to PPARγ in the CREB3L2-PPARγ fusion protein. C, full-length CREB3L2-V5 was expressed in 293T epithelial cells that were treated with brefeldin A, an inducer of intramembrane proteolysis. Brefeldin A produced rapid conversion of full-length CREB3L2-V5 to cleaved CREB3L2-V5 beginning at 0.5 h. The expression of control β-actin was constant under these same conditions. Immunoblots with CREB3L2 antiserum and β-actin antibody are shown. D, cleaved CREB3L2-V5 localized to nuclear and total, but not cytoplasmic, fractions of 293T cells that had been treated with brefeldin A. In contrast, full-length CREB3L2-V5 localized to cytoplasmic, nuclear, and total fractions of untreated 293T cells. We attribute CREB3L2-V5 in the nuclear fractions to result from incomplete separation of nuclear and endoplasmic reticulum/membrane proteins, which is dependent on protein solubility. In support of this possibility, calreticulin, a protein marker of the endoplasmic reticulum, was also present in all nuclear fractions. The control transcription factor PPARγ was enriched highly in nuclear fractions. Immunoblots with CREB3L2 antiserum, PPARγ antiserum, and calreticulin antibody are shown.
We also measured the effect of CREB3L2-PPARγ on the incorporation of bromodeoxyuridine (BrdUrd) into nuclear DNA as a measure of thyroid cell proliferation. CREB3L2-PPARγ and PAX8-PPARγ increased the incorporation of BrdUrd by 42% and 58% (*P < 0.0003*), respectively, compared with vector controls on day 3 after electroporation (Fig. 3B). In contrast, the expression of wild-type PPARγ had little effect on the incorporation of BrdUrd (Fig. 3B). Thus, CREB3L2-PPARγ and PAX8-PPARγ each induced marked proliferation in normal thyroid cells, consistent with a dominant transforming mechanism.

**CREB3L2 is a transcription factor regulated by intramembrane proteolysis.** To further understand the mechanism of CREB3L2-PPARγ and PAX8-PPARγ, we investigated whether native CREB3L2 was processed by intramembrane proteolysis in normal thyroid cells. Nucleotide probes for wild-type CREB3L2 hybridized on Northern blots to three main mRNA transcripts, approximately 7.5, 2.3, and 1.1 kb (Fig. 4A). These transcripts corresponded in size to three CREB3L2 sequences in GenBank (NM_194071, AJ549387, BC063666). The 7.5-kb transcript was expressed at highest levels in the thyroid and at lower levels in stomach, adrenal gland, lymph node, and tracheal (salivary gland) tissues (Fig. 4A), showing that the native CREB3L2 promoter is sufficient to drive high expression of CREB3L2-PPARγ in thyroid cells. Both the 7.5- and 2.3-kb transcripts encode a wild-type CREB3L2 protein of 520 amino acids (AJ549387, AJ549092) that contains an NH2-terminal transactivation domain, a middle bZIP DNA binding domain, and a carboxy transmembrane domain (amino acids 379–395) that is flanked by S1P (RXXL, 427–430) and S2P (LCX,P, 385–402) proteolytic cleavage sites (Fig. 4B). The S1P serine protease and S2P zinc metalloprotease cleave membrane-bound transcription factors in a process termed regulated intramembrane proteolysis that depends on metabolic, endoplasmic reticulum, and/or protein folding signals (26). The membrane-bound transcription factors are thereby released to the nucleus to activate gene expression. The CREB3L2-PPARγ fusion protein contains only the transactivation domain of CREB3L2 (Fig. 4B).

We next sought direct experimental evidence that CREB3L2 is regulated by intramembrane proteolysis in epithelial cells. CREB3L2 was tagged with V5 and expressed by transfection in 293T kidney cells that were treated with brefeldin A. Brefeldin A has been shown to induce regulated intramembrane proteolysis at S1P and S2P sites (38) by altering the endoplasmic reticulum-Golgi, endosome-lysosome, and protein secretory pathways (39, 40). Transfected 293T cells expressed full-length CREB3L2-V5 protein (78 kDa) that was lost after 3 hours of treatment with brefeldin A (Fig. 4C). The transfected 293T cells produced a smaller, cleaved CREB3L2-V5 protein (54 kDa) beginning at 0.5 hour of brefeldin A treatment (Fig. 4C). Expression of control β-actin was constant in the same experiment (Fig. 4C). This precursor-product relationship between full-length and cleaved CREB3L2-V5, the rapid kinetics of CREB3L2-V5 cleavage, and the relative molecular weights of full-length and cleaved CREB3L2-V5 are all consistent with the regulated intramembrane proteolysis of CREB3L2 at its S1P and S2P sites.

We localized full-length and cleaved CREB3L2 proteins by cell fractionation. Cleaved CREB3L2-V5 was present in the nuclear and total, but not cytoplasmic, fractions of 293T cells that were treated with brefeldin A for 3.5 hours (Fig. 4D). In contrast, full-length CREB3L2-V5 was absent from 293T cells that were treated with brefeldin A but was present in cytoplasmic, nuclear, and total fractions of untreated 293T cells (Fig. 4D). We believe that full-length CREB3L2-V5 in the nuclear fraction of untreated 293T cells resulted from incomplete separation of endoplasmic reticulum and nuclear proteins in this fractionation procedure, which is known to be influenced by protein solubility. In support of this possibility, calreticulin, a protein marker of the endoplasmic reticulum (41), was also apparent in all nuclear fractions (Fig. 4D). On the other hand, the nuclear receptor PPARγ that served as a control transcription factor was enriched highly in the nuclear fractions (Fig. 4D). These experiments show that wild-type CREB3L2 is cleaved by intramembrane proteolysis and transported to the nucleus.

We investigated whether native CREB3L2 was processed by regulated intramembrane proteolysis in normal thyroid cells. Full-length native CREB3L2 was synthesized in cultures of primary human thyroid cells, whereas only native cleaved CREB3L2 was produced after treatment of thyroid cells with brefeldin A (Fig. 5A). In addition, RT-PCR showed mRNA transcripts encoding the S1P and S2P proteases in primary thyroid cells and control HepG2 cells (Fig. 5B), supporting the possibility that S1P and S2P cleave CREB3L2 in the thyroid gland. Taken together, these experiments indicate that wild-type full-length CREB3L2 is cleaved by intramembrane proteolysis to nuclear CREB3L2 as...
part of a thyroid signaling pathway that is involved in growth regulation and cancer.

**CREB3L2-PPARγ inhibits cAMP-responsive transcription activities.** To further determine the mechanism of CREB3L2-PPARγ, we measured the abilities of nuclear CREB3L2 and CREB3L2-PPARγ to activate transcription from the EVX1 cAMP response element (CRE). Nuclear CREB3L2 or CREB3L2-PPARγ were transfected with the EVX1 CRE reporter into 293T epithelial cells that are responsive to cAMP (37). Nuclear CREB3L2 stimulated transcription from the EVX1 CRE 8-fold over vector controls ($P = 0.002$) in the absence of cAMP, whereas CREB3L2-PPARγ inhibited transcription from the EVX1 CRE 6-fold ($P = 0.01$) in the same experiments (Fig. 6A). Transcription from the EVX1 CRE was shown to be dose dependent under these conditions (Fig. 6B). Furthermore, overexpression of CREB3L2-PPARγ inhibited 4-fold the expression of native thyroglobulin, a cAMP responsive thyroid gene, in primary thyroid cells that were treated with TSH (Fig. 6C). These experiments show that the CREB3L2-PPARγ fusion protein inhibits transcription from an established CRE and inhibits expression of a native cAMP-responsive gene in normal thyroid cells.

**Discussion**

Our discovery of the CREB3L2-PPARγ fusion mutation shows for the first time that a family of somatic PPARγ fusion mutations exist in thyroid cancer. An important aspect of fusion mutations is that they identify molecular mechanisms that underlie cancer pathogenesis. In fact, our discovery of CREB3L2-PPARγ shows that CREB3L2 is the target of at least two fusion mechanisms in cancer. In thyroid follicular carcinoma, the NH2-terminal transactivation domain of CREB3L2 is fused to PPARγ. In fibromyxoid sarcoma, the bZIP and carboxyl domains of CREB3L2 are fused to FUS (29). Thus, the identification of the CREB3L2-PPARγ fusion underscores the importance of CREB3L2 deregulation in diverse cancer types and adds to a growing list of CREB-related alterations that have recently been identified in human cancer tissues (42, 43).

Our experiments showed that CREB3L2-PPARγ is a low-incidence PPARγ gene fusion that is present in <3% of thyroid follicular carcinomas. The PAX8-PPARγ gene fusion has been reported in 25% to 45% of thyroid follicular carcinomas in pathologically well-defined series that separate follicular from Hurthle cell carcinomas (34, 44, 45). Little is understood about the function of CREB3L2 in cancer, and our experiments provide new insights into CREB3L2 mechanisms by showing that (a) the CREB3L2-PPARγ fusion protein stimulates proliferation in normal human thyroid cells; (b) wild-type CREB3L2 is cleaved by intramembrane proteolysis in a thyroid signaling pathway; (c) nuclear CREB3L2, but not CREB3L2-PPARγ, stimulates transcription from the EVX1 CRE; and (d) the CREB3L2-PPARγ fusion protein inhibits transcription from the EVX1 CRE and also inhibits expression of thyroglobulin in normal thyroid cells that have been treated with TSH, the major thyroid regulator that acts through cAMP.

The CREB3L2-PPARγ and PAX8-PPARγ fusion proteins each induced the growth of normal human thyroid cells by stimulating cell proliferation. The 42% to 58% induction of proliferation by these PPARγ fusion proteins is remarkable because other potent thyroid oncogenes such as mutant RAS or B-RAF often elicit growth arrest and/or apoptosis in primary cells (46, 47), presumably because they activate mechanisms that protect against cell transformation. In fact, expression of the B-RAF or ELE1-RET (RET-PTC3) mutants, which activate constitutive mitogen-activated protein kinase signaling in thyroid papillary carcinomas, induced cell death or little proliferation, respectively, in normal thyroid cells in our experiments.7 Thus, CREB3L2-PPARγ and PAX8-PPARγ induce proliferation by mechanisms that seem to abrogate or bypass antitumor pathways in a manner different from mutant B-RAF (48) or ELE1-RET (49). Such molecular differences may determine, at least in part, the morphologic and clinical differences that are observed in patients with follicular versus papillary thyroid carcinoma.

CREB3L2 was cleaved by regulated intramembrane proteolysis in an unappreciated thyroid signaling pathway. CREB3L2 contains a transactivation domain, a bZIP DNA binding domain, and a transmembrane domain that has flanking SIP and S2P protease cleavage sites. Intramembrane proteolysis by SIP and S2P controls
the SREBPs, which are key transcriptional regulators of cholesterol metabolism (26), and CREB3, CREB4 and CREB-H, which are thought to be involved in protein folding and stress responses in the endoplasmic reticulum (28, 50, 51). In our study, full-length CREB3L2 exhibited a precursor-product relationship with and rapid conversion to cleaved CREB3L2 after treatment of thyroid cells with brefeldin A, a known inducer of intramembrane proteolysis (38). In addition, full-length CREB3L2 localized predominantly to cytoplasmic fractions, whereas cleaved CREB3L2 localized to nuclear fractions, as expected if CREB3L2 is processed by intramembrane proteolysis. We also showed that the S1P and S2P proteases are expressed in human thyroid cells. Interestingly, treatment of thyroid cells with either thapsigargin or tunicamycin, two inducers of endoplasmic reticulum stress, did not generate cleavage of CREB3L2 in our experiments,7 suggesting that physiologic stimuli other than endoplasmic reticulum stress may control CREB3L2 proteolysis in the thyroid gland. Furthermore, antisera that we have raised against distinct domains of CREB3L2 have localized CREB3L2 to the plasma membrane, in addition to the nucleus and cytoplasm, of thyroid and other epithelial cells.7 These findings suggest that CREB3L2 is part of an unappreciated thyroid signaling pathway that is regulated by intramembrane proteolysis and important in cancer. The data also suggest that CREB3L2 may function in cancer through mechanisms that are different than senescence (52) or apoptosis (53) that have been connected recently to intramembrane proteolysis by S1P and S2P during endoplasmic reticulum stress. The CREB3L2-PPARγ gene fusion provides a natural mutation model with which to determine the contribution of CREB3L2 and regulated intramembrane proteolysis in carcinoma, the most common and clinically significant cancer type. The physiologic signals that control intramembrane proteolysis of CREB3L2 in the thyroid gland remain to be elucidated.

The high expression of CREB3L2-PPARγ in nuclei of thyroid carcinoma cells with (t3/7) suggests a transforming mechanism that involves altered transcription. In fact, our experiments showed that the CREB3L2-PPARγ fusion protein has little ability to stimulate transcription from the EVX1 CRE compared with wild-type nuclear CREB3L2. This finding is consistent with the fact that the CREB3L2 bZIP domain, which facilitates dimerization and DNA binding to CREs, is not retained in the CREB3L2-PPARγ fusion protein. Furthermore, CREB3L2-PPARγ inhibited transcription from the EVX1 CRE and also inhibited expression of thyroglobulin, a cAMP-responsive gene, in normal thyroid cells. These data suggest that CRE-containing genes in thyroid carcinoma with CREB3L2-PPARγ may be down-regulated by (a) disruption of one CREB3L2 allele during CREB3L2-PPARγ formation and (b) functional inhibition of transcription at cAMP responsive genes by the CREB3L2-PPARγ fusion protein. In fact, we have observed by quantitative RT-PCR that expression of CAMP-responsive thyroid genes such as thyroglobulin, thyroperoxidase, and the sodium-iodide transporter is lower in thyroid follicular carcinoma with CREB3L2-PPARγ than in 95% of other thyroid follicular tumors (n = 76), including five follicular carcinomas with the PAX8-PPARγ fusion protein.7 These data support a model in which CREB3L2-PPARγ acts by interfering with CRE-related transcription that is central to the regulation of differentiation and proliferation in the thyroid gland (54). Interestingly, wild-type CREB3L2 mRNA is induced at least 2-fold in normal human thyroid cells by treatment with TSH (55), further connecting the CREB3L2 and TSH pathways. Thus, it will be important to determine the exact molecular interactions between the CREB3L2 and TSH pathways in future studies, although it is likely that CREB3L2-PPARγ has other oncogenic activities as well. In fact, fusion proteins exhibit potent growth activities because they tend to target multiple cell pathways in cancer. Systematic determination of CREB3L2-PPARγ versus PAX8-PPARγ activities should help clarify mechanisms of PPAR-related functions in cancer as well.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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