CD44 proteolysis increases CREB phosphorylation and sustains proliferation of thyroid cancer cells

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Abstract

CD44 is a marker of cancer stem-like cells and epithelial-mesenchyme transition that is overexpressed in many cancer types, including thyroid carcinoma. At extracellular and intramembranous domains, CD44 undergoes sequential metalloprotease- and γ-secretase-mediated proteolytic cleavage, releasing the intracellular protein fragment CD44-ICD which translocates to the nucleus and activates gene transcription. Here we show that CD44-ICD binds to the transcription factor CREB, increasing S133 phosphorylation and CREB-mediated gene transcription. CD44-ICD enhanced CREB recruitment to the cyclin D1 promoter, promoting cyclin D1 transcription and cell proliferation. Thyroid carcinoma cells harboring activated RET/PTC, RAS or BRAF oncogenes exhibited CD44 cleavage and CD44-ICD accumulation. Chemical blockade of RET/PTC, BRAF, metalloprotease or γ-secretase were each sufficient to blunt CD44 processing. Further, thyroid cancer cell proliferation was obstructed by RNAi-mediated knockdown of CD44 or inhibition of γ-secretase and adoptive CD44-ICD overexpression rescued cell proliferation. Together, these findings reveal a CD44-CREB signaling pathway that is needed to sustain cancer cell proliferation, potentially offering new molecular targets for therapeutic intervention in thyroid carcinoma.
Introduction

CD44 is a glycosylated transmembrane glycoprotein that is implicated in tumor growth and metastasis (1-3). CD44 undergoes intramembrane proteolysis by metalloprotease, that cleaves the extracellular juxtamembrane stem domain, and, then, by γ-secretase that cleaves the transmembrane domain (4-7). The first cleavage results in the shedding of the ectodomain (ecto-CD44) and the release of the membrane-bound CD44 C-terminal fragment (CD44-CTF). The second cleavage causes the release of the CD44 intracellular domain fragment (CD44-ICD) (5, 6). Shedding of ecto-CD44 regulates cell-extracellular matrix interaction (8), while CD44-ICD translocates to the nucleus and activates gene transcription (7).

Papillary thyroid carcinoma (PTC) often features RET/PTC or BRAF oncoproteins, both of which signal through the ERK (extracellular signal regulated kinase) pathway (9-11). RET/PTC result from the fusion of the tyrosine kinase domain of the RET receptor with the N-terminus of heterologous proteins (9). RET tyrosine 1062 (Y1062) plays an important role in RET and RET/PTC signaling, acting as the binding site for several proteins, thus leading to ERK and PI3K (phosphoinositide 3-kinase)/AKT signaling (12-16).

CD44 cleavage produces CD44-ICD in cells transformed by oncogenic RET point mutants (17). CD44 is overexpressed in PTC and in cell lines harboring RET/PTC or BRAF oncogenes (18, 19). Here, we show that CD44-ICD triggers activation of the CREB transcription factor; in thyroid cancer cells, RET/PTC-BRAF signaling cascade uses such a CD44-ICD-CREB axis to sustain cell proliferation.

Materials and Methods

Cell lines. —TPC-1 and BCPAP cell lines were obtained, respectively, in 1990 from M. Nagao (National Cancer Center Research Institute, Tokyo, Japan) and in 1994 from N. Fabien
The anaplastic thyroid carcinoma cell lines ACT-1 (NRAS Q61K), HTH7 (NRAS Q61R), HTH74 (negative for HRAS, KRAS and NRAS mutations) and C643 (HRAS G13R) were obtained in 2005 from N. Onoda (Osaka University of Medicine, Japan) (ACT-1) and from N.E. Heldin (University Hospital, Uppsala, Sweden) (HTH7, HTH74 and C643). These cell lines were DNA profiled by short tandem repeat analysis in 2009 (20) and shown to be unique and identical to those reported in Schweppe et al., 2008 (21). In brief, 15 STR loci were tested by using the Applied Biosystems AmpFLSTR Identifiler kit (ABI no. 4322288, Forest City, CA); DNA profiles were compared manually to the American Type Culture Collection database and to the DNA profiles reported by Schweppe et al. 2008 (21).

Human primary culture of thyroid cells (P5) were obtained from F. Curcio (University Udine, Italy) in 2003 and passaged in our laboratory, as described (22), for fewer than 2 months after resuscitation. They were tested in 2010 by proliferation rate and expression of thyroid differentiation markers (TG, TPO and TSHR). Nthy-ori 3-1 (hereafter referred to as NTHY), a human thyroid follicular epithelial cell line immortalized by the SV40 large T gene, was obtained from European Collection of Cell Cultures (ECACC) (Wiltshire, UK) in 2010. They were tested by ECACC for identity verification by DNA profiling of short tandem repeat sequences and were passaged in our laboratory for fewer than 3 months after resuscitation. PC Cl 3 (hereafter referred to "PC") is a differentiated thyroid follicular cell line derived from 18-month-old Fischer rats in our laboratory in 1987 (23). They were kept in culture for fewer than 3 months after resuscitation and tested in 2010 by proliferation rate, dependence on six hormones for growth and expression of thyroid differentiation markers (TG, TPO and TSHR). HEK293T cells were purchased in 2006 from the American Type Culture Collection and tested by ATCC for identity by DNA profiling of short tandem repeat sequences. HEK293T were passaged in our laboratory for fewer than 3 months after resuscitation. The PC ICD and
BCPAP ICD cell lines were obtained by a stable transfection with the CD44-ICD construct by using the Fugene HD reagent from Roche Diagnostics (Mannheim, Germany). A pool of several cell clones was isolated by G418 selection. Transient transfections were carried out with the Fugene HD reagent. All these cell lines were grown in standard conditions as detailed in Supplemental Methods.

**Plasmids.** — The RET/PTC constructs used in this study were cloned in pBABE or pcDNA3(Myc-His) (Invitrogen, Groningen, The Netherlands) and described elsewhere (14). RET/PTC3(K-) is a kinase-dead mutant, carrying the substitution of the catalytic lysine (residue 758 in full-length RET) with a methionine. In RET/PTC3(4YF) mutant the 4 major autophosphorylation sites (Y826, Y1015, Y1029, Y1062 in full-length RET) are mutated to phenylalanine; Y1062 has been added back in RET/PTC3(3YF). These mutants were generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA, USA). BRAFV600E and HRAS(V12) plasmids are described elsewhere (14). The full-length rat CD44 was cloned into pDEST47 vector and tagged at the C-ter with GFP (17); CD44-ICD was cloned in pDEST47 (GFP-tagged), pDEST40 (V5-tagged), and pCDNA 3.1 (myc-tagged) (17).

**Antibodies, compounds and proteins.** — The antibody against the cytosolic portion of CD44 (CD44cyto) and anti-RET are described elsewhere (14, 17). A list of commercial antibodies, compounds and recombinant proteins used in this study is provided in Supplemental Methods.

**Cell growth and staining.** — For growth curves, cells were seeded in triplicate and counted at the indicated time points. Thyroid cancer cells were maintained in DMEM 2.5% fetal bovine
serum (FBS) and PC cells in medium supplemented with 5% calf serum and without TSH. DNA synthesis was measured by the 5'-bromo-3'-deoxyuridine (BrdU) Labeling and Detection Kit from Boehringer Mannheim (Germany), as indicated by manufacturer (Supplemental Methods).

**ELISA assay.** — Extracellular shedding of the soluble ectodomain of human standard CD44 (CD44st) was measured using the Instant ELISA (Bender MedSystems, Vienna, Austria) according to the manufacturer’s instructions. Conditioned media from cell cultures were analyzed in triplicate at 450 nM with an microplate reader (Model 550, Bio-Rad).

**Protein studies.** — Immunoblotting and immunoprecipitation experiments were performed according to standard procedures. For nuclear extraction, cells were lysed by shearing with 15 passages through a 26-gauge needle mounted in a 1 ml syringe. Nuclei were recovered by centrifugation at 3,000 x g for 10 m (Supplemental Methods).

**Pull down assay.** — The GST-CD44-ICD vector codes for a chimeric protein with CD44-ICD fused to the glutathione S-transferase (GST). It was generated by the Invitrogen Gateway technique using as donor the pDEST47-CD44-ICD vector and as recipient the pDEST15 vector. GST-CD44-ICD was purified from pDEST15-CD44-ICD transformed bacterial lysates using glutathione-Sepharose beads. Recombinant proteins containing the different domains of CREB fused to GST are described elsewhere (24). Pull down assays were performed by standard procedures (Supplemental Methods).

**Reporter assay.** — All the Firefly luciferase reporters were kindly provided by J.S. Gutkind
Twenty-four hours after seeding, the cells were transiently transfected in triplicate with reporters together with pRL-null, a plasmid expressing the enzyme Renilla luciferase, used as an internal control (Promega Corporation, Madison, WI, USA). Luciferase assays were performed according to standard procedures (Supplemental Methods).

Chromatin immunoprecipitation (ChIP). — Chromatin was extracted from CD44-ICD or empty vector transfected HEK293T cells and ChIP was performed with the chromatin immunoprecipitation assay kit (Upstate Biotechnology Inc.) according to manufacturer’s instructions, as described in Supplemental Methods.

RNA silencing. — The small inhibitor duplex RNAs (siRNA) were from Dharmacon (Lafayette, CO, USA) and were ON-target plus SMARTpool siCD44 human #L-009999-00, siCREB human: #L-003619-00-0005, and siCREB rat: #L-092995-00-0010. The siCONTROL Non-targeting Pool (#D-001206-13-05) was used as a negative control. Cells were transfected with 100 nM siRNAs using Dharmafect reagent. The day before transfection, cells were plated in 35-mm dishes at 40% of confluence in DMEM supplemented with 10% FBS and without antibiotics. The Sh29merRNA constructs against human CD44 were from OriGene Technologies (Rockville, MD, USA) (TR314080 IDs: TI356313, TI356314, TI356316). shRNA pRS plasmid TR20003 was used as a negative control. Transfection was performed in 100-mm dishes using Fugene HD reagent (Roche, Mannheim, Germany) with 4 μg shRNA construct. Cells were harvested 48 h after transfection.

In vitro PP2A dephosphorylation assay. — Recombinant HIS-CREB was in vitro
phosphorylated by the catalytic subunit of PKA in a buffer containing 1 mM ATP, 10 mM MgCl₂, 50 mM KCl, 10 mM Hepes and 10% glycerol for 1h at 30°C. Phosphorylated CREB was incubated in a phosphatase buffer (40 mM Tris HCl, 34 mM MgCl₂, 4 mM EDTA, 2 mM DTT, 0.05 mg/ml BSA) for 1 h at 37°C with 10 μU of PP2A catalytic subunit. GST-CD44-ICD, or the GST backbone alone, were added to the samples. The reaction was terminated by adding SDS gel loading dye and samples were run on 10% SDS-polyacrylamide gel. Phospho-CREB signal was detected with anti-phospho(Ser/Thr) PKA substrate antibody and the amount of CREB in each reaction was estimated with anti-HIS antibody.

**Statistical analysis.**— The two-tailed unpaired Student’s t test (normal distributions and equal variances) was used for statistical analysis. Differences were significant when $P < 0.05$.

Statistical analysis was performed using the Graph Pad InStat software program, version 3.06.3 (San Diego, CA).

**Results**

**CD44-ICD stimulates CRE-mediated transcription.**—We analyzed the capability of a GFP-tagged CD44-ICD construct to trans-activate a panel of promoter elements, including AP-1 (activating protein-1), SRF (serum response factor), TCF (ternary complex factor), Gli (Glioma-associated oncogene homolog), NFkB (nuclear factor kappa B) and CRE (cAMP-responsive element) reporters (25-28). CD44-ICD strongly (about 10 fold, $P < 0.01$) activated the CRE reporter in HEK293T cells, but not the other promoters (Fig. 1A). This was a specific feature of the cleaved CD44-ICD, because full length GFP-tagged CD44 did not significantly ($P > 0.05$) stimulate the CRE reporter (Fig. 1B).
**CD44-ICD sustains CREB/p300-mediated CCND1 expression.** — CRE elements bind the CREB (cAMP-responsive element binding protein) family of transcription factors (29, 30). Cyclin D1 (CCND1) is a prototypic CREB transcriptional target, containing a CRE element upstream of the mRNA start site (at -58 bp) (25, 31). We studied the effect of CD44-ICD on a CCND1-luciferase reporter. HEK293T cells were kept without serum for 24 h after the transfection to reduce the growth factor-dependent CCND1 promoter activity. CD44-ICD stimulated (approximately 12 fold, P < 0.01) the CCND1 promoter (Fig. 1C, left).

We silenced endogenous CREB by transiently transfecting CREB siRNA (siCREB) in CD44-ICD-transfected HEK293T cells; CREB knock-down (about 70% of reduction) was verified by immunoblot (Fig. 1C, right). CREB silencing, but not a scrambled control, obstructed CD44-ICD-mediated activation of CCND1-luciferase (Fig. 1C, left, P < 0.01).

The co-activator CBP/p300 binds to active S133-phosphorylated CREB and triggers CREB-mediated gene transcription (32). Previous data demonstrated that CD44-ICD stimulates p300-mediated transactivation (33). Thus, we co-transfected CD44-ICD with p300 or a p300-dominant negative construct (LYRR) (34). CD44-ICD upregulated (about 3 fold) endogenous CCND1 expression; the p300-dominant negative mutant blocked CD44-ICD-induced CCND1 expression, whereas wt p300 slightly increased it (Fig. 1D).

Phosphorylated CREB is recruited to CRE sites in DNA (35). We performed a chromatin immunoprecipitation (ChIP) to measure CREB binding to the CRE element of the CCND1 promoter. CD44-ICD expression increased the binding of CREB to the CCND1 promoter by about 5 fold (Fig. 1E).

Taken together, these results demonstrate that CD44-ICD stimulates CREB recruitment to CCND1 promoter and CREB/p300-mediated transcription of CCND1.
CD44-ICD increases CREB phosphorylation on serine 133. — Nuclear extracts were prepared from HEK293T cells transfected with CD44-ICD and phosphoS133 (CREB principal activation site) levels were measured by immunoblot. As a positive control, we treated cells with the adenylate cyclase activator forskolin (FSK). Besides CREB, the phospho-CREB antibody recognizes phosphorylated CREB family members CREM (30 kDa) and ATF-1 (38 kDa). Expression of CD44-ICD induced a robust increase of pS133 CREB as well as of phosphorylated ATF-1 (Fig. 2A, left).

Activated pS133 CREB binds p300 and this results in a transcriptionally active complex. Thus, we analyzed CREB-p300 interaction by immunoprecipitating CREB from nuclear extracts of HEK293T cells co-transfected with CD44-ICD and p300, and staining the immunoblot with p300 antibody. CD44-ICD, as well as FSK treatment, increased (about 5 fold) the CREB-p300 interaction (Fig. 2A, right).

These results demonstrate that CD44-ICD expression increases levels of S133-phosphorylated CREB and its binding to p300.

CD44-ICD forms a protein complex with CREB. — We transfected HEK293T cells with V5-tagged CD44-ICD and stained anti-CREB immunoprecipitates with V5 antibody. CD44-ICD formed a protein complex with CREB (Fig. 2B). To verify if the interaction between CREB and CD44-ICD was direct or mediated by other proteins, we performed a pull-down assay using recombinant CREB and GST-CD44-ICD proteins. Figure 2C shows that CREB and CD44-ICD readily interacted in vitro.

We used different domains of CREB expressed as recombinant proteins fused to GST (24) to pull-down myc-tagged CD44-ICD expressed in HEK293T cells. As shown in Figure
2D, CD44-ICD bound two contiguous domains of CREB: Q2 (aa 160-283), the constitutive glutamine-rich activation domain, and bZIP (aa 284-341), the DNA binding/dimerization domain. CD44-ICD did not bind the isolated bZIP construct (aa 1-283) of CREB, that contains Q2 but not bZIP. It is possible that, in the absence of bZIP, the presence of the amino-terminal part of the protein interferes with the interaction between Q2 and CD44-ICD. The fact that CD44-ICD binds to a CREB domain different to that binding to p300 (KID domain) (29) is consistent with the possibility that a complex of three proteins, CREB, p300 and CD44-ICD is formed.

**CD44-ICD reduces the rate of CREB de-phosphorylation.** — Various serine/threonine kinases, i.e. PKA, RSK and MSK, are able to phosphorylate CREB on S133 and to stimulate CREB binding to DNA (29, 35, 36). We evaluated the effects of the blockage of these kinases. To inhibit PKA, we either treated cells for 2 h with the H89 compound, a potent selective inhibitor of PKA, or transfected cells with a plasmid encoding the PKA-specific peptide inhibitor (PKi) (37). RSK and MSK are activated by MEK (36); thus, to inhibit RSK and MSK, we treated cells for 2 h with the MEK-inhibitor U0126. Nuclear extracts were analyzed by Western blot with anti pS133 CREB and normalized with anti-CREB. The inhibition of both PKA and MEK efficiently blocked CD44-ICD-induced CREB phosphorylation (Supplemental informations, Fig. S1).

These results prompted us to hypothesize that, in the presence of CD44-ICD, the increase of CREB phosphorylation levels occurs due to a reduced rate of CREB de-phosphorylation rather than to activation of a specific CREB kinase. To evaluate this possibility, we stimulated serum-starved HEK293T cells for 40 min with the cAMP-analog N6-benzoyl-cAMP to induce S133 CREB phosphorylation, and then measured CREB binding...
to CD44-ICD. GST-CD44-ICD pulled-down a larger amount of CREB upon induction of CREB phosphorylation (Fig. 3A), indicating that CD44-ICD, although able to bind de-phosphorylated CREB, binds preferentially to pS133 CREB.

Then, we induced CREB phosphorylation with N6-benzoyl-cAMP in the presence or not of GFP-tagged CD44-ICD and chased pS133 CREB de-phosphorylation by immunoblot. Figure 3B shows that the pS133 CREB half-life was increased by CD44-ICD; CREB pS133 levels did not decrease up to 12 h in cells expressing CD44-ICD, while the half-life of pS133 CREB was about 8 h in mock-transfected cells (Fig. 3B).

Finally, we performed an in vitro CREB de-phosphorylation assay. Recombinant HIS-tagged CREB was phosphorylated in vitro by PKA and then incubated with PP2A phosphatase in the presence or the absence of GST-CD44-ICD. As shown in Fig. 3C, GST-CD44-ICD, but not the GST backbone, protected phosphoCREB from PP2A-mediated de-phosphorylation.

These findings demonstrate that CD44-ICD reduces the rate of CREB de-phosphorylation on S133.

**Expression of CD44-ICD-CREB complex in human thyroid carcinoma cell lines.** —We selected thyroid cancer cells as a model whereby to study effects of CD44-ICD. Initially, we looked for CD44-ICD, in two papillary thyroid carcinoma (PTC) cell lines, TPC-1 and BCPAP, that feature the RET/PTC1 rearrangement and the BRAFV600E mutation, respectively; as a control, we used P5, a normal thyroid primary cell culture. Both PTC cell lines, but not non transformed thyrocytes, expressed full-length CD44 as well as CD44-ICD (Supplemental informations, Fig. S2A). Moreover, pS133 CREB as well as phosphoATF-1
levels were detected in BCPAP and TPC-1 while they were only barely detectable in P5 cells (Supplemental informations, Fig. S2A).

GST-CD44-ICD, but not the GST backbone, was able to pull-down pS133CREB and phosphoATF-1 in both BCPAP and TPC-1 cell lysates (Supplemental informations, Fig. S2B, left). Finally, in thyroid cancer cells, the pCREB-CD44-ICD interaction was shown by immunoprecipitating pS133-phosphorylated CREB and staining the blot with anti-CD44cyto antibody (Supplemental informations, Fig. S2B, right).

These findings demonstrate that thyroid cancer cells express CD44-ICD and that, in these cells, CD44-ICD exists in a complex with CREB.

**RET/PTC and BRAFV600E oncogenes induce CD44 cleavage.**—We treated TPC-1 and BCPAP cells for 48 h with BB94, a broad-spectrum metalloprotease inhibitor; moreover, we treated the RET/PTC1 positive TPC-1 cell line with ZD6474, a RET kinase inhibitor, and the BRAFV600E-positive BCPAP cell line with U0126, a MEK inhibitor. EctoCD44 (sCD44st, soluble standard CD44) shedding into the cell culture media was measured by ELISA.

EctoCD44 release was detected in TPC-1 and BCPAP conditioned media (Supplemental informations, Fig. S3A, left); treatment with BB94, ZD6474 or U0126 blocked ectoCD44 shedding (P < 0.01), indicating that RET and BRAF signaling stimulates CD44 cleavage (Supplemental informations, Fig. S3A, left). In TPC-1 cells, treatment with BB94, ZD6474 and U0126 downregulated CD44-ICD (Supplemental informations, Fig. S3A, middle). CD44 down-regulation by siRNA proved that the protein band with a relative molecular weight of about 10 kDa was indeed CD44-ICD (Supplemental informations, Fig. S3A, right). Finally, γ-secretase blockade by COMP X or DAPT decreased the amount of CD44-ICD, while it increased the amount of a polypeptide of ∼31 kDa, which corresponds to the
molecular weight of CD44-CTF (Supplemental informations, Fig. S3A, middle). This effect was consistent with a block of transmembrane conversion of CD44-CTF to CD44-ICD. In contrast, CD44-CTF did not accumulate upon RET/PTC1 or MEK inhibition, which suggests that the RET/PTC1 and MEK block acts upstream CD44-CTF generation, possibly at the level of metalloprotease-mediated CD44 cleavage (Supplemental informations, Fig. S3A, middle).

We co-expressed a GFP-tagged full-length CD44 with the two most prevalent RET/PTC oncogenes, RET/PTC1 and RET/PTC3. RET/PTC oncogenes stimulated the generation of CD44-ICD and CD44-CTF (Supplemental informations, Fig. S3B). That the band migrating above CD44-ICD corresponded to CD44-CTF was suggested by the fact that when we used the metalloprotease inhibitor BB94 in cells co-expressing CD44-GFP and RET/PTC1, this band was strongly reduced (Supplemental informations, Fig. S4A). Instead, when we used a CD44 mutant lacking γ-secretase cleavage site (Δ287-290), this band was not affected while CD44-ICD band was strongly attenuated (Supplemental informations, Fig. S4B). RET/PTC-mediated CD44 cleavage depended on RET/PTC kinase activity and on the integrity of tyrosine 1062, because CD44-ICD did not accumulate when kinase-dead (K-) and (Y1062F) RET/PTC1 mutants were expressed (Supplemental informations, Fig. S3C, left). CD44-ICD did not accumulate when the RET/PTC3 4YF mutant (in which the 4 major RET signaling tyrosines are mutated to phenylalanine) was expressed (Supplemental informations, Fig. S3C, right), but CD44 cleavage was rescued when Y1062 was added back (3YF) (Supplemental informations, Fig. S3C, right). We treated RET/PTC1-transfected HEK293T cells with U0126. Treatment with U0126 blocked CD44-ICD generation (Supplemental informations, Fig. S3D). Transient expression of the myc-tagged constitutively active forms of RAS (RASV12) and BRAF (BRAFV600E) induced CD44-ICD formation (Supplemental
informations, Fig. S3D), which further supports the concept that the ERK pathway plays an important role in CD44 cleavage. Accordingly, also human thyroid carcinoma cell lines featuring mutant RAS alleles upregulated both CD44 and CD44-ICD with respect to non-transformed thyrocytes (Supplemental informations, Fig. S5).

These findings demonstrate that RET/PTC and BRAF signaling to ERK stimulates CD44 processing.

**CD44-ICD sustains CREB phosphorylation and CCND1 expression in thyroid cancer cells.**—In BCPAP and TPC1 thyroid cancer cells, CD44 silencing by shCD44, but not the empty vector, attenuated CREB phosphorylation on S133 (Fig. 4A). In shCD44-transfected cells, CREB phosphorylation was rescued by the transfection of a V5-tagged rat CD44-ICD mRNA that is shCD44-resistant because it has several mismatches with human shCD44 (Fig. 4A). BB94 and DAPT, able to block CD44 cleavage, reduced CREB pS133 (Fig. 4B). Downregulation of either CREB or CD44 by siRNA, but not a scrambled control, reduced (about 2 fold) the expression of CCND1 in BCPAP and TPC-1 cell lines (Fig. 4C). These findings demonstrate that CD44-ICD sustains CREB-dependent CCND1 expression in thyroid cancer cells.

**CD44-ICD sustains proliferation of thyroid cells.**—We silenced CD44 by transiently transfecting BCPAP and TPC-1 cells with a human shCD44 plasmid. To exclude off-target effects, cells were co-transfected with the shCD44-resistant rat CD44-ICD construct. CD44 silencing reduced BrdU incorporation (P < 0.05) and this effect was rescued by adoptive expression of rat CD44-ICD (P < 0.05) (Fig. 5A). CD44-ICD was not able to rescue the reduction of BrdU incorporation mediated by CREB silencing (P >0.05), that is consistent
with CREB acting downstream CD44-ICD (Fig. 5A). We treated BCPAP cells with the γ-secretase inhibitor COMP X and, to rescue the effect, we transfected CD44-ICD or the empty vector. As shown in Figure 5B, COMP X inhibited DNA synthesis in BCPAP, and CD44-ICD was able to revert this effect. We stably expressed CD44-ICD in BCPAP and selected one mass population by G418 treatment. While treatment with COMP X reduced the growth rate of parental (P < 0.01) (Fig. 5C, left), it did not change significantly (P > 0.05) the proliferation of BCPAP-ICD cells (Fig. 5C, right).

Non transformed thyroid PC cells require a mixture of 6 hormones (6H), including TSH, for proliferation (23). In hormone-starved PC cells, transiently transfected CD44-ICD stimulated CRE-mediated transcription (about 9 fold) (Fig. 6A) (P < 0.01) and activated the CCND1 promoter (almost 4 fold) (Fig. 6B) (P < 0.01). CREB silencing, but not a scrambled control, obstructed CD44-ICD-mediated activation of CCND1-luciferase (Fig. 6B) (P < 0.01).

A mass population of CD44-ICD transfected PC cells (PC ICD pool) showed increased (about 8 fold) CRE-reporter luciferase activity with respect to empty vector transfected cells (Fig. 6C) (P < 0.01). Finally, we measured the rate of DNA synthesis in the absence of TSH. BrdU incorporation in PC ICD cells was higher (about 10 fold) than in empty vector transfected cells (Fig. 6D) (P < 0.01). Thus, CD44-ICD is sufficient to trigger proliferation of non transformed thyrocytes.

**Discussion**

Here we report a novel functional link between CD44 and the CREB transcription factor. CREB is involved in neoplastic transformation and, being activated via PKA by cAMP, is also involved in the growth of normal thyroid follicular cells (38-41). Our data show that...
CD44-ICD binds CREB and increases pS133 CREB levels. CD44-ICD stimulated CREB-mediated gene transcription and recruitment of CREB to CCND1 gene promoter. We noted that CD44-ICD preferentially binds to phosphorylated CREB and that CD44-ICD expression attenuates the rate of CREB dephosphorylation, thus suggesting that interaction with CD44-ICD impairs CREB de-phosphorylation on S133. Accordingly, CD44-ICD protected pCREB from PP2A-mediated dephosphorylation in vitro.

Thyroid carcinoma overexpresses CD44 and such overexpression is associated to the oncogenic conversion of the ERK signaling pathway (18, 19). Moreover, as in other cell types (42), CD44 was expressed in prospectively identified thyroid cancer stem cells that induce tumors when injected orthotopically into mouse thyroid (43). Thus, it is feasible that CD44 plays a role in thyroid cancer as well as in thyroid cancer stem cells.

Oncogenic RET point mutants induce CD44 cleavage (17). Here, we show that CD44-ICD is expressed in thyroid cancer cell lines harboring RET/PTC or BRAFV600E oncogenes and that RET/PTC and BRAF trigger CD44 cleavage. CD44-ICD is necessary for the proliferation of thyroid cancer cells and sufficient to trigger proliferation of non transformed thyrocytes. These effects are mediated by CREB and by increased rate of CCND1 transcription. The fact that extracellular shedding of ectoCD44 accompanies the generation of CD44-ICD and that RET and BRAF blockade impairs CD44-CTF accumulation indicates that metalloprotease-mediated cleavage is one level at which CD44-ICD generation is stimulated in cells expressing RET/PTC and BRAF. Accordingly, RET/PTC and BRAF up-regulate the transcription of several metalloproteases (44). CD44 cleavage can be triggered by binding to different extracellular ligands, including low molecular weight hyaluronan acid (45). RET, RAS and BRAF oncogenes stimulate the expression of osteopontin, one extracellular CD44 ligand; whether this facilitates CD44 cleavage remains to be determined.
Our findings support a model whereby CD44 cleavage acts as an amplifier of oncogenes signaling to CREB (Fig. 7). According to this model, RET/PTC and BRAF promote CREB phosphorylation via ERK-mediated activation of CREB kinases and stabilize pS133-CREB through CD44-ICD. In turn, the CD44-ICD/CREB axis stimulates CCND1 transcription and thyroid cell proliferation. Should these results be validated in vivo, they may prompt the possibility of pharmacological manipulation of the pathway.
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References


Figure Legends

Figure 1 — (A) Luciferase assays were performed in HEK293T cells to measure effects of a GFP-tagged CD44-ICD (ICD) on the indicated reporters. (B) CRE-LUC reporter assay in HEK293T cells transiently transfected with GFP-tagged full-length CD44 or CD44-ICD (ICD). (C) HEK293T cells were transiently transfected with CycD1(CCND1)-LUC and CREB siRNA or control scrambled siRNA (si CTR). CREB silencing and CD44-ICD (ICD) expression was verified by immunoblot. In (A), (B) and (C) co-transfected Renilla luciferase was used for normalization. Results are reported as fold change with respect to the empty vector (-). Triplicates ± SD are shown. P values were determined by the two-tailed unpaired Student's t test. CD44 or CD44-ICD (ICD) expression was verified by immunoblot with anti-GFP antibody (inset). (D) Immunoblot stained with cyclin D1 antibody of HEK293T cells co-transfected with CD44-ICD-GFP together with p300 or p300LYRR dominant negative mutant. CD44-ICD expression was verified with anti-GFP. Anti-tubulin was used for normalization. (E) HEK293T cells were transiently transfected with CD44-ICD. Mock or anti-CREB immunoprecipitated chromatin was analyzed by semiquantitative PCR with primers spanning the CRE site on the CCND1 promoter. Input DNA levels are shown for normalization. Data in (D) and (E) are representative of three independent experiments.

Figure 2 — (A, left) Nuclear extracts were obtained by HEK293T transiently transfected with CD44-ICD (ICD) and pS133 CREB levels were determined by immunoblot. Forskolin (FSK, 40 μM 30 min) was used as a positive control. Anti-CREB was used for normalization. (A, right) Nuclear extracts were obtained by HEK293T transiently transfected with CD44-ICD (ICD); anti-CREB immunocomplexes (1 mg) were immunoblotted with p300. Lysis buffer
alone was used as a negative control (buffer); FSK was used as a positive control. Anti-CREB and -p300 were used for normalization. (B) HEK293T were transiently transfected with V5-tagged CD44-ICD (ICD) or empty vector (-). Proteins were immunoprecipitated (1 mg) with anti-CREB. CREB antibody incubated with no lysate (buffer) was used as a control. Immunocomplexes were blotted with V5; CREB antibody was used for normalization. (C) CD44-ICD-GST (30 µg) was incubated with 1 µg of recombinant CREB (CREB-REC). Complexes were recovered by glutathione beads and analyzed by immunoblot with anti-CREB. CREB input is reported for normalization. (D, upper) Schematic representation of different portions of CREB used in pull-down assay; (D, bottom) The indicated portions of GST-CREB were used (30 µg) to pull-down myc-tagged CD44-ICD transiently expressed in HEK293T cells (1 mg). GST alone was used as negative control. Data are representative of three independent experiments.

Figure 3 — (A) HEK293T were treated for 1 h with N6-benzoyl-cAMP (100 µM). Protein lysates (2 mg) were pulled-down with GST-CD44-ICD (ICD-GST) and analysed by immunoblot with the indicated antibodies. Normalization was obtained by CREB blotting, and CREB phosphorylation was determined by phosphoCREB. (B) GFP-tagged CD44-ICD (ICD-GFP) transfected HEK293T cells, or cells transfected with the empty vector (-), were treated with N6-benzoyl-cAMP (100 µM) and harvested at different time points. CREB and ATF-1 phosphorylation was determined by immunoblot. Anti-GFP and anti-CREB were used for normalization. (C) PKA-phosphorylated recombinant HIS-tagged CREB (0.05 µg) was incubated with 10 µU of PP2A catalytic subunit for 1 h at 37°C. Where indicated, GST-CD44-ICD (ICD-GST), or the control GST, was added. CREB phosphorylation was detected on May 30, 2017. © 2012 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from
with an antibody reacting with phospho(Ser/Thr) PKA substrates. Normalization was performed with anti-HIS. Data are representative of three independent experiments.

Figure 4 — (A) BCPAP and TPC-1 were transiently transfected with shCD44 or the empty vector (-) with or without RNAi-refractory V5-tagged rat CD44-ICD (ICD). Silencing was verified by immunoblot with anti-CD44cyto that does not react with rat CD44. Anti-V5 was used to verify the expression of rat CD44. PhosphoCREB and phosphoATF-1 levels were measured by immunoblot; tubulin and CREB were used for normalization. (B) BCPAP and TPC-1 cells were treated with BB94 or DAPT. CREB phosphorylation was measured by immunoblot. Anti-CREB and anti-phosphoMAPK were used for normalization. CD44-ICD levels are shown. (C) Immunoblot stained with cyclin D1 antibody of BCPAP and TPC-1 cells treated with siCD44, siCREB, or scrambled control (si CTR). The efficiency of RNA interference was verified by immunoblot with anti-CD44cyto and -CREB. Data are representative of 3 independent experiments.

Figure 5 — (A) BrdU-positive cells in BCPAP and TPC-1 mock-transfected (-), or transfected with siCREB or shCD44 plasmid with or without shRNA-refractory rat CD44-ICD. (B) BrdU-positive cells in BCPAP treated for 48 h with 10 μM COMP X and transfected with CD44-ICD or empty PCDNA vector. In (A) and (B) at least 100 cells were counted in 5 fields and results are expressed as percentage of BrdU positive cells ± SD. P values were determined by the two-tailed unpaired Student's t test. (C) One mass population of BCPAP transfected with CD44-ICD was selected by G418; growth curves are reported as triplicate determinations ± SD with or without 10 μM COMP X. P values were determined by the two-tailed unpaired Student's t test.
Figure 6 — (A) CRE-LUC assay in PC cells transiently transfected with CD44-ICD. (B) CycD1-LUC assay in PC cells transiently transfected with CREB siRNA or scrambled siRNA (siCTR). In (A) and (B) cells were harvested at 48 h, luciferase assay was performed and results are reported as fold change with respect to the vector (-); co-transfected Renilla luciferase was used for normalization. Triplicates ± SD are shown. P values were determined by the two-tailed unpaired Student's t test. (C, upper) PC cells were stably transfected with GFP-tagged CD44-ICD (PC-ICD pool) or the empty GFP vector and mass populations were isolated by G418 selection. Immunoblot with anti-GFP was performed; equal loading was ascertained by anti-tubulin immunostain. (C, bottom) CRE-LUC assay in PC ICD cell pool. Results are reported as fold change with respect to empty vector transfected cells. Co-transfected Renilla luciferase was used for normalization. (D) Cells were seeded without TSH on glass coverslips, pulsed with BrdU, and BrdU incorporation measured by indirect immunoflorescence. At least 100 cells were counted in 5 different microscopic fields and results are expressed as percentage of BrdU positive cells ± SD. P values were determined by the two-tailed unpaired Student's t test.

Figure 7 — A model of functional interaction between oncogene signaling, CD44 and CREB in thyroid cancer cells.
CD44 proteolysis increases CREB phosphorylation and sustains proliferation of thyroid cancer cells

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