Supplementary Figure Legends

Figure S1—PKA and MEK inhibition blunts CD44-ICD mediated CREB phosphorylation. HEK293T cells were transiently transfected with CD44-ICD or the empty vector; when indicated, cells were also co-transfected with a plasmid encoding the PKA-specific peptide inhibitor (PKi). Cells were treated for 2 h with H89 (1 μM) or with U0126 (10 μM). Nuclear extracts were prepared and analyzed by Western blot with anti pS133 CREB. Blots were normalized with anti-CREB.

Figure S2 — CD44-ICD-CREB complex in human PTC cell lines. (A) Protein lysates (50 μg) extracted from normal (P5) and PTC (BCPAP and TPC-1) cells were analyzed by Western blot with anti-CD44cyto antibody (which recognizes the cytosolic region of CD44); tubulin was used for normalization. Migration of full-length CD44 (CD44-FL, ~89 kDa) and CD44 intracellular domain (CD44-ICD, ~10kDa) is indicated. pS133CREB levels were measured by immunoblot in the same cells. Anti-CREB antibody was used for normalization. (B, left) Recombinant GST-CD44-ICD or the GST backbone were used to pull-down protein lysates from BCPAP or TPC-1 cells. pS133CREB and phosphoATF-1 were detected by pS133 CREB immunostain. (B, right) PhosphoCREB (+) was immunoprecipitated from BCPAP or TPC-1 protein lysates (1 mg) and immunocomplexes stained with anti-CD44cyto or pS133 CREB antibody. Protein A sepharose alone (-) was used as a control. These data are representative of 3 independent experiments.
Figure S3 — The RET/PTC-RAS-BRAF cascade triggers CD44 cleavage. (A, left) ELISA-based measurement of ectoCD44 fragment-shedding in TPC-1 or BCPAP cells; the cells were treated for 48 h in serum free media with 100 μM BB94 (metalloprotease inhibitor), 0.5 μM ZD6474 (RET kinase inhibitor), or 10 μM U0126 (MEK kinase inhibitor), supernatants were collected, and 20 μl used for ectoCD44 measurement. Data are the average of 3 independent experiments ±SD. P values were determined by the two-tailed unpaired Student's t test. (A, middle) Following overnight starvation, protein lysates (100 μg) were extracted from TPC-1 cells after 48 h of treatment with 100 μM BB94, 10 μM COMP X (γ-secretase inhibitor), 10 μM DAPT (γ-secretase inhibitor), 0.5 μM ZD6474, or 10 μM U0126, and immunoblotted with anti-CD44cyto. Migration of CD44-FL, CD44-ICD, and CD44 C-terminal fragment (CD44-CTF, about 31 kDa) is indicated. These data are representative of 3 independent experiments. (A, right) Immunoblot stained with anti-CD44cyto of TPC-1 cells treated with siCD44 or scrambled control (si CTR). Tubulin was used for normalization. (B) Protein lysates (50 μg) were extracted from HEK293T cells transiently transfected with GFP-tagged full-length CD44 with or without RET/PTC1 (PTC1) or RET/PTC3 (PTC3) (−: mock-transfected cell lane) and immunoblotted with anti-GFP antibody. CD44-ICD was transfected as a molecular weight control. Migration of CD44-FL and CD44-ICD is indicated. RET/PTC1 and RET/PTC3 protein expression is shown. (C) Protein lysates (50 μg) were extracted from HEK293T cells transiently cotransfected with GFP-tagged full-length CD44 and the indicated RET/PTC1 or RET/PTC3 mutants and immunoblotted with anti-GFP. RET/PTC1 and RET/PTC3 protein expression is shown. A schematic representation of RET/PTC protein is shown: TK, tyrosine kinase;
the catalytic lysine (K758) and major RET autophosphorylation sites are indicated. (D) Protein lysates (50 μg) were extracted from HEK293T cells transiently co-transfected with GFP-tagged full-length CD44 and the indicated myc-tagged oncogenes and immunoblotted with anti-GFP. Where indicated, cells were treated for 24 h with 10 μM U0126. Oncogenes expression is shown by anti-myc tag immunoblot.

Figure S4 — RET/PTC triggers CD44-ICD and CD44-CTF production. (A) Protein lysates (50 μg) were extracted from HEK293T cells transiently transfected with GFP-tagged full-length CD44 with or without RET/PTC1 (PTC1); cells co-transfected with CD44 and RET/PTC1 were treated or not with 100 μM BB94 (metalloprotease inhibitor) and immunoblotted with anti-GFP antibody. Migration of CD44-FL, CD44-CTF and CD44-ICD is indicated. (B) Protein lysates (50 μg) were extracted from HEK293T cells transiently transfected with GFP-tagged full-length CD44 or with its deletion mutant Δ287-290 (lacking γ-secretase cleavage site) with or without RET/PTC1 (PTC1) and immunoblotted with anti-GFP antibody. RET/PTC1 expression was detected by the myc tag antibody. Migration of CD44-FL, CD44-CTF and CD44-ICD is indicated.

Figure S5 — CD44-ICD expression in human thyroid cancer cell lines. (A) Protein lysates (50 μg) extracted from the indicated cell lines were analyzed by Western blot with anti-CD44cyto antibody; tubulin was used for normalization. Migration of full-length CD44 (CD44-FL, ~89 kDa) and CD44 intracellular domain (CD44-ICD, ~10kDa) is indicated. Blot was normalized with anti-tubulin.