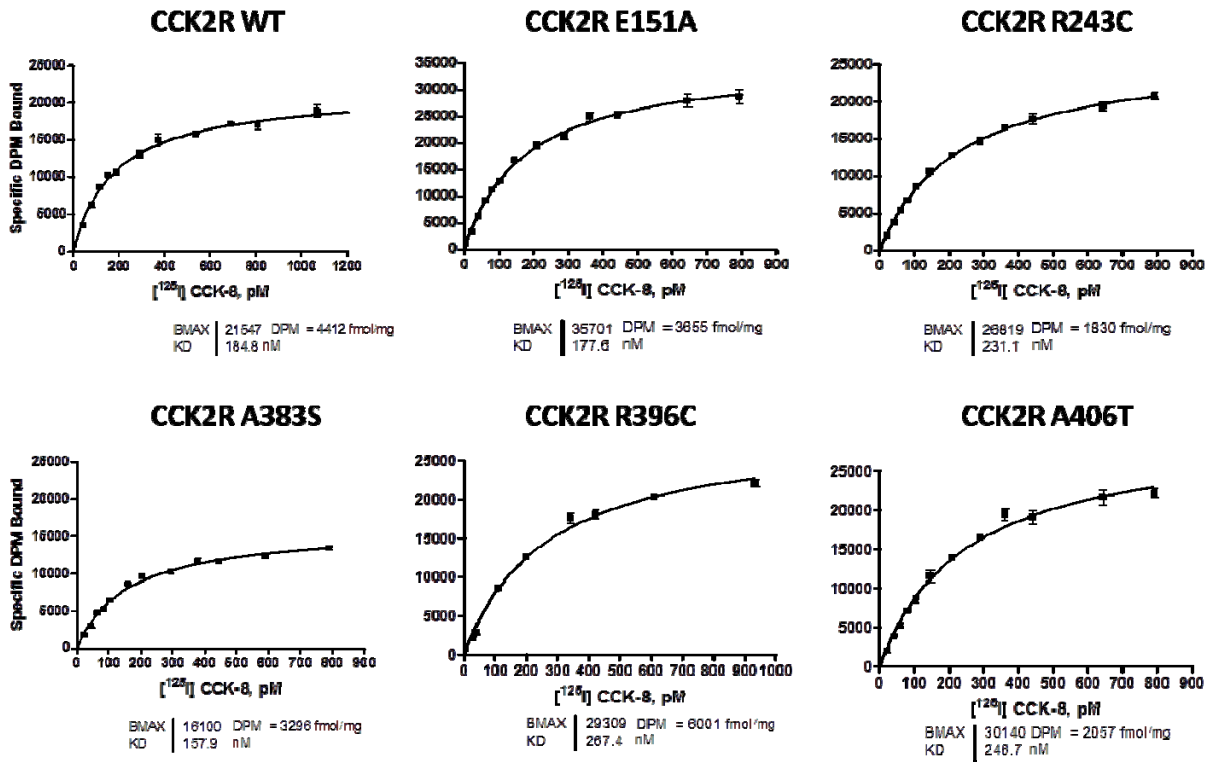
Supplementary Information

Supplementary Table S1. Somatic mutations in CCK2R found in cancers		
Protein Change	**Cancer Subtype**	**References**
*E151A	Genetically engineered mutation	(1-5)
R243C	Colon cancer	-
G267V	NSCLC (squamous)	(6)
R278H	NSCLC (adenocarcinoma)	(6)
V287F	Colon cancer	(7)
#i4sv	Colorectal and pancreatic cancer	(8-10)
A383S	Gastric cancer	-
R395H	Colon cancer	-
R396C	Colon cancer	-
C405F	NSCLC (adenocarcinoma)	(6)
A406T	Colon cancer	-
A406V	Gastric cancer	-
A431D	NSCLC (adenocarcinoma)	(6)

Supplementary Figure 1



Supplementary Table S2. Functional activity of CCK2R variants					
Variant	**IP-1**	**Ca^{2+} flux#**	**cAMP**	**pERK**	**pAkt**
WT	10	20	3	27	8
E151A	4	140	2	30	-
R243C	3	50	1	30	29
A383S	7	30	5	15	3
R396C	9	120	1	27	18
A406T	10	20	1	27	20

Supplementary Table S1. Somatic mutations in CCK2R found in cancers. #All mutations are in the coding region and are missense excluding i4sv which is an aberrantly spliced transcript. *E151A is a synthetic constitutively active published mutation that promotes tumorigenesis.

Supplementary Figure 1. Saturation binding experiments to determine receptor expression level (Bmax) of HEK293 CCK2R stable clones. Data were analyzed using Prism 4.03 and the nonlinear regression (curve fit) equation “one site binding hyperbola” to determine Kd and Bmax.

Supplementary Table S2. Functional activity of CCK2R variants in second messenger and downstream pathway activation assays. Agonist potency of HEK293 stable clones at CCK2R WT, CCK2R E151A and CCK2R variants (R243C, A383S, R396C and A406T) in the following assays: IP-1, Ca^{2+} flux, cAMP, pERK and pAkt. #All values displayed are the EC_{50} at each receptor expressed in nM, excluding Ca^{2+} flux which is pM. All experiments conducted with CCK-8 except Ca^{2+} flux where gastrin was used.

Supplementary Materials and Methods

Membrane preparation and radioligand binding assay

HEK membranes were prepared from HEK293 cells CCK2R stable clones (or untransfected cells). Cells were cultured, washed 1x with 10 ml PBS (Ca^{2+}/Mg^{2+} -free), then harvested by triteration in PBS and centrifuged at 100 x g for 5 min. Supernatant was aspirated and dry pellets were frozen in liquid N_2 and stored at -80^{o}C. To prepare membranes, pellets were thawed on ice and homogenized in ice-cold TEE homogenization buffer [25 mM Tris (pH 7.5), 5 mM EDTA, 5 mM EGTA, cOmplete Protease Inhibitors, EDTA-free (Roche)] in a glass dounce homogenizer. Homogenate was centrifuged at 4^{o}C, 250 x g, 10 min and supernatant collected. Pellets were re-homogenized in TEE and centrifuged again. Supernatants were pooled and centrifuged at 4^{o}C, 47,000 x g for 40 min. Supernatants were discarded and membrane pellets were washed 1x in ice-cold TEE by homogenization in glass dounce, then centrifuged again.

Membrane pellets were resuspended in ice-cold binding buffer [50 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 0.2 mg/ml bacitracin, cOmplete Protease Inhibitors, EDTA-free) to a concentration of 1-2 mg protein/ml. Protein concentrations were determined using Pierce BCA protein assay kit. Membrane preparations were aliquoted, snap frozen in liquid N_2, and stored at -80^{o}C.

Saturation binding experiments to determine receptor expression level (Bmax) of HEK293 CCK2R stable clones were performed in 96-well plates. 1-5 µg of membrane protein were incubated in binding buffer plus 0.02% BSA with increasing concentrations of [^{125}I] CCK-8 (20 pM to 800 pM) in the presence or absence of 10 µM JB95008 (CCK2R antagonist). Non-specific binding (determined in the presence of JB95008) was < 10% of total binding. Incubation at RT for 5 h was terminated by harvesting assay wells onto 96-well GF-C filter plates using a Unifilter Harvester (Perkin Elmer) followed by 3-40 ml washes of ice-cold wash buffer (50 mM Tris pH 7.5, 0.2% BSA). Plates were dried and backsealed, and Microscint 20 was added before topsealing and reading plates in a TopCount scintillation counter (Perkin Elmer). Aliquots of radioligand dilutions were counted on a gamma counter to determine dpm added/well. Total binding was always less than 10% of dpm added. Data were analyzed using Prism 4.03 and the nonlinear regression (curve fit) equation “one site binding hyperbola” to determine Kd and Bmax.

IP-1 assay

IP-1 accumulation was measured using the IP-One HTRF kit according to the manufacturer’s instructions. HEK293 CCK2R stable clones (or untransfected cells) were seeded in white, clear-bottom, 96-half-well PDL-coated plates at 40,000 cells/well, and

cultured O/N. To initiate the assay, media was removed and cells were stimulated for 60 min at 37^{o}C, 5% CO_2 with CCK-8 or gastrin in Stimulation Buffer (10 mM Hepes, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4). Dilutions of an IP-1 standard were prepared and added to empty wells for a standard curve. At the end of the stimulation period, d2 conjugated IP-1 and anti-IP-1 antibody labeled with Lumi4-Tb cryptate, both diluted in Lysis Buffer from the kit, were added to cells and standard wells. Plates were incubated at RT for 3-15 h and then read on an EnVision® (Perkin Elmer) multilabel plate reader with excitation at 340 nm and emission at 665 and 620 nm. Assay signal was expressed as the fluorescence ratio (665 nm / 620 nm). A standard curve was constructed plotting fluorescence ratio v. IP-1 concentration. IP-1 concentrations of unknowns were interpolated from the standard curve. EC_{50} values for concentration response curves were determined using Prism 4.03 and the nonlinear regression (curve fit) equation “sigmoidal dose-response (variable slope)”.

cAMP assay

cAMP accumulation was measured using the cAMP dynamic 2 kit according to the manufacturer’s instructions. HEK293 CCK2R stable clones (or untransfected cells) were seeded in white, clear-bottom, 96-half-well PDL-coated plates at 40,000 cells/well, and cultured O/N. To initiate the assay, media was removed from wells and replaced with Stimulation Buffer (DMEM w/o phenol red, 500 μM isobutylmethylxanthine, 0.1% BSA) and incubated at 37^{o}C, 5% CO_2 for 15 min. CCK-8 or gastrin diluted in Stimulation Buffer were added to the wells and plates were incubated at 37^{o}C, 5% CO_2 for 30 min for cAMP accumulation. Dilutions of cAMP standard were made and added to empty

wells for creation of a standard curve. At the end of the stimulation period, d2 conjugated cAMP and anti-cAMP antibody labeled with Eu^{3+} cryptate, both diluted in Lysis Buffer from the kit, were added to cells and standard wells. Plates were incubated at RT for 3-15 h and then read on an EnVision® (Perkin Elmer) multilabel plate reader with excitation at 320 nm and emission at 665 and 620 nm. Assay signal was expressed as the fluorescence ratio (665 nm / 620 nm). A standard curve was constructed plotting fluorescence ratio v. cAMP concentration. cAMP concentrations of unknowns were interpolated from the standard curve. EC_{50} values for concentration response curves were determined using GraphPad Prism and the nonlinear regression (curve fit) equation "sigmoidal dose-response (variable slope)".

SureFire® assays

The combined levels of phosphorylated ERK1/2 (Thr202/Tyr204) or phosphorylated Akt 1/2 (Ser473) were measured using the AlphaScreen® *SureFire*® p-ERK1/2 or p-Akt 1/2 Assay (PerkinElmer), respectively. HEK293 cells were plated and transfected as described above. 48 h post-transfection, cells were starved in serum-free DMEM for 6 h prior to agonist stimulation. The reaction was then terminated by removal of the media followed by lysis using the proprietary lysis buffer supplied with the SureFire® kit. Samples were processed according to the manufacturer's instructions, and samples were read on an EnVision® plate reader (PerkinElmer).

Supplementary References

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