SUPPLEMENTAL MATERIALS AND METHODS

cCPE.GST Protein Expression

Expression of the carboxy-terminal fragment of CPE linked to an N-terminal GST fusion protein was performed as previously reported (*1*). The expression of GST fusion protein was performed using *E. coli* BL21 (DE3) (New England Biolabs, cat no C2527). Purification was performed using a 1-ml settled Sepharose 4B agarose column, eluted using glutathione (GE Healthcare). Fractions were analysed and quantified by 280 nm spectroscopy and SDS-PAGE (Supplemental Figure 1), and the fractions containing cCPE.GST combined. A similar process was used to produce GST protein, used as a negative control agent. Since glutathione negatively influences the conjugation reactions described below, it was removed using size exclusion chromatography (Zeba desalting column, Thermo Scientific, 7 kDa MW cut-off), followed by dialysis against sodium bicarbonate buffer (0.1 M, pH 8.4), at 4°C for 15 hours (slide-a-lyser G2 cassette, Pierce, 10 kDa MW cut-off), and finally re-concentrated using an ultrafiltration filter device (Amicon, 3 kDa MW size cut-off). Protein purity was confirmed by SDS-PAGE gel followed by staining using Coomassie blue (Supplemental Figure 1). Although the primary sequence of Claudin-4 receptor would indicate a molecular weight of 22 kDa, it has been reported that the native weight in various cell-lines can be between 16-18 kDa, presumably due to differing post-translational processing (*2*).

Radiolabelling Procedure

Purified cCPE.GST or GST was dissolved at 1 g/l in 0.1 M $NaHCO_3$, pH 8.4, and reacted for 1 h at room temperature (RT) with a four-fold molar excess of the bifunctional metal ion chelator 2-(4-isothiocyanatobenzyl)-diethylenetriaminepentaacetic acid (*p*-SCN-Bn-DTPA; Macrocyclics), freshly dissolved at 2 g/l in in dry dimethylsulfoxide (Sigma-Aldrich). Excess chelator was removed by purification on a Sephadex G25 mini-column (Sigma-Aldrich) eluted with 100 mM sodium citrate, pH 6.0, and microfiltration using a 3 kDa cut-off Amicon spin filter (Millipore). DTPA-conjugation

efficiency was determined using a previously reported assay (*3*). Purified DTPA-conjugated ligands were radiolabeled by incubation of 20 μg ligand, dissolved at 1 g/l in 100 mM sodium citrate, pH 6.0, with 20 MBq of $^{111}InCl_3$ (Perkin Elmer) for 1 h at room temperature. Radiolabelling yield was measured by instant thin layer silica gel chromatography developed in 100 mM sodium citrate pH 6.0, and G25 sephadex SEC minicolumn eluted with PBS.

Balb/neuT **Mouse Model**

Balb/neuT mice express mutated rat *neuT* under *mmtv* promoter control, resulting in the formation of DCIS-like deposits and *neuT*-positive breast cancer in mammary fat pads of female mice. NeuT, a variant of the rat neu HER2-homolog, contains a point mutation in the transmembrane region and is highly tumorigenic compared to wild-type (WT) neu or human HER2. When *balb/neuT* mice reach 21-28 days of age, the neuT protein is over-expressed in mammary glands and areas of atypical hyperplasia start to form. These aplastic sites progress to *in situ* carcinomas at about day 60 and to invasive cancers by day 120-150. Neoplastic change occurs, albeit asynchronously, in all mammary glands so that by about 120 days one or more tumors are palpable and by about day 230 (33 weeks) all 10 mammary glands contain palpable tumors. The model's main advantage is its close resemblance of tumor progression and histopathological presentation to HER2-overexpressing breast cancer in human subjects. A more detailed description may be found here (*4*-*6*).

REFERENCES FOR SUPPLEMENTARY METHODS

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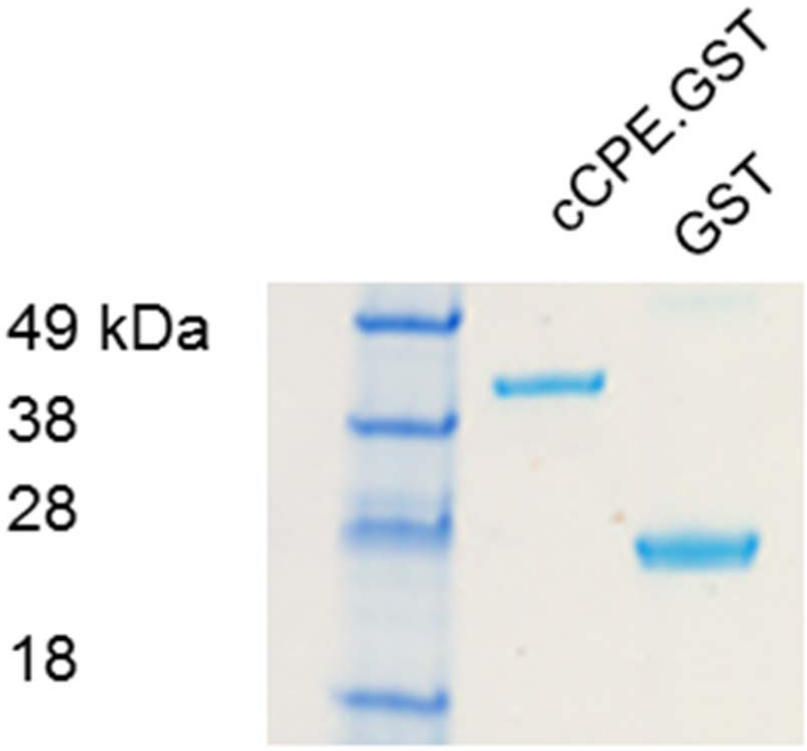
2. Sjo A, Magnusson KE, Peterson KH. Protein kinase C activation has distinct effects on the localization, phosphorylation and detergent solubility of the claudin protein family in tight and leaky epithelial cells. *J Membr Biol.* 2010;236:181-189.

3. Hnatowich DJ, Layne WW, Childs RL. The preparation and labeling of DTPA-coupled albumin. *Int J Appl Radiat Isot.* 1982;33:327-332.

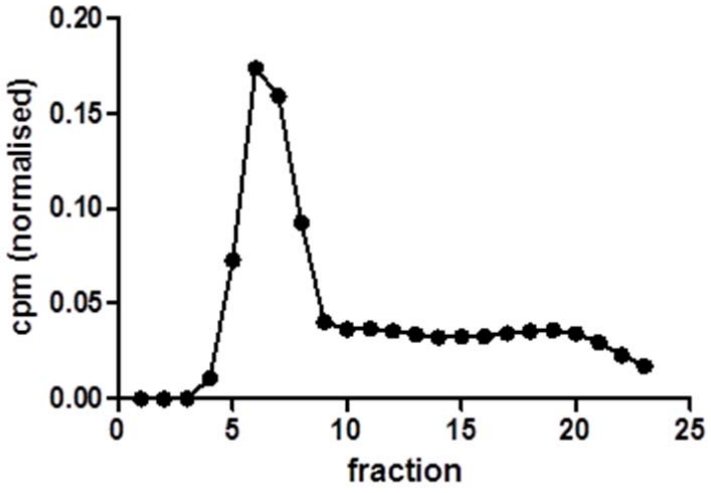
4. Quaglino E, Mastini C, Forni G, Cavallo F. ErbB2 transgenic mice: a tool for investigation of the immune prevention and treatment of mammary carcinomas. *Curr Protoc Immunol.* 2008;Chapter 20:Unit 20 29 21-20 29-10.

5. Di Carlo E, Diodoro MG, Boggio K, et al. Analysis of mammary carcinoma onset and progression in HER-2/neu oncogene transgenic mice reveals a lobular origin. *Lab Invest.* 1999;79:1261-1269.

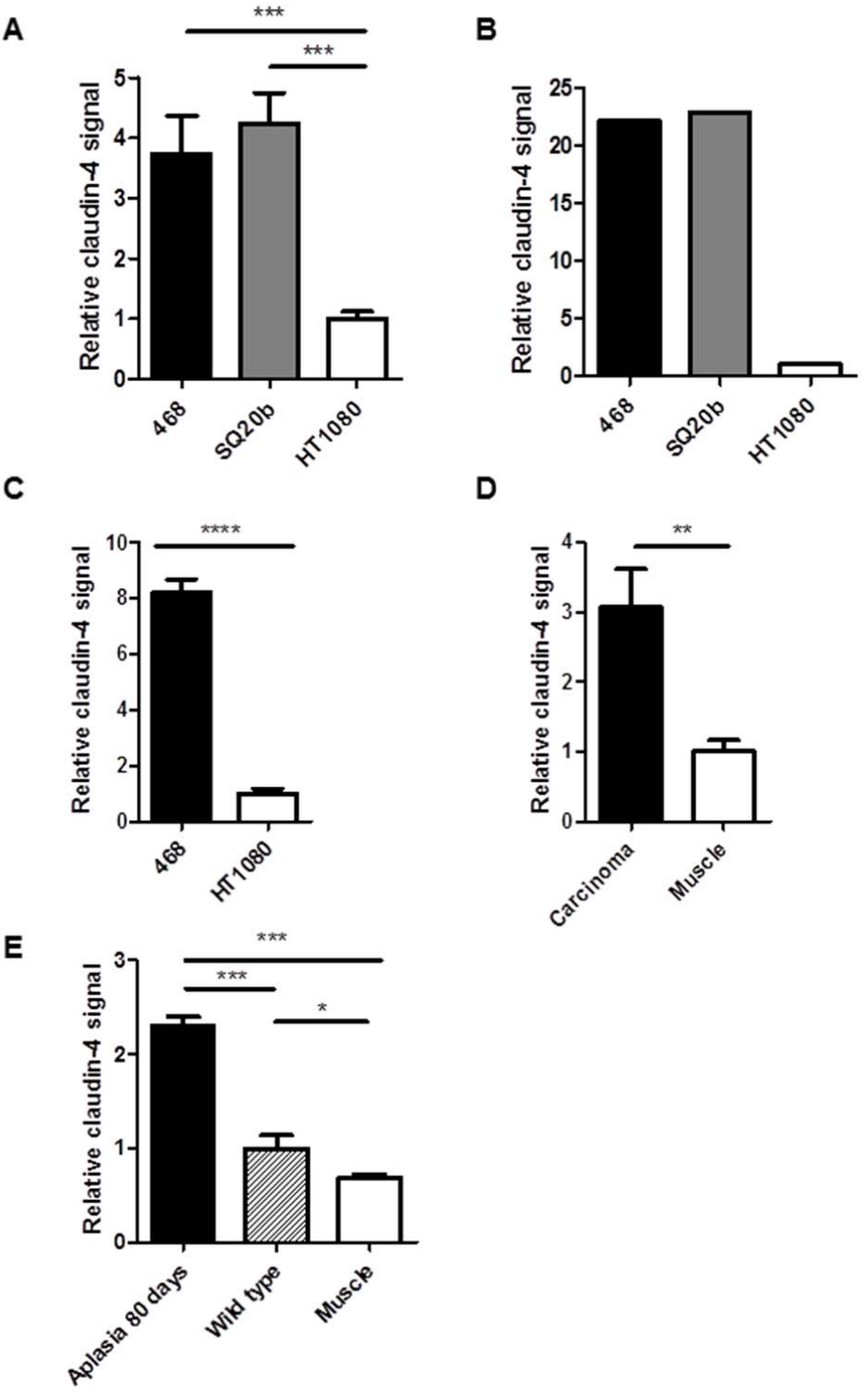
6. Calogero RA, Cordero F, Forni G, Cavallo F. Inflammation and breast cancer. Inflammatory component of mammary carcinogenesis in ErbB2 transgenic mice. *Breast Cancer Res.* 2007;9:211.



Supplemental Figure 1. SDS-PAGE gel chromatography showing purified cCPE.GST or GST, stained using Coomassie blue dye.



Supplemental Figure 2. Size exclusion chromatogram of ^{111}In-cCPE.GST, using a G25 Sephadex mini-column.


A
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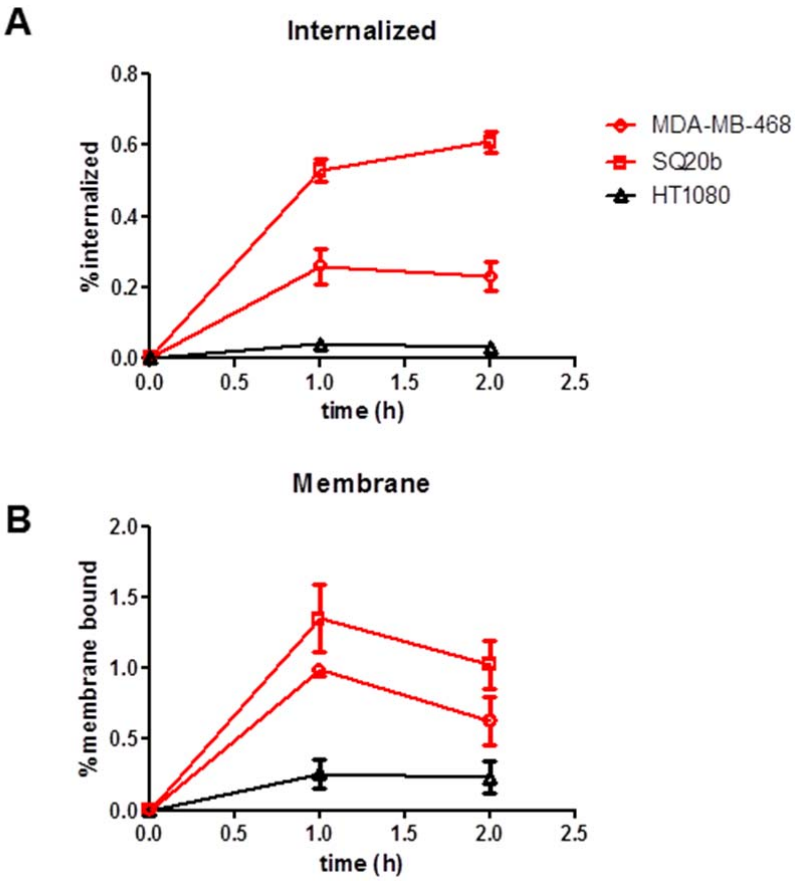
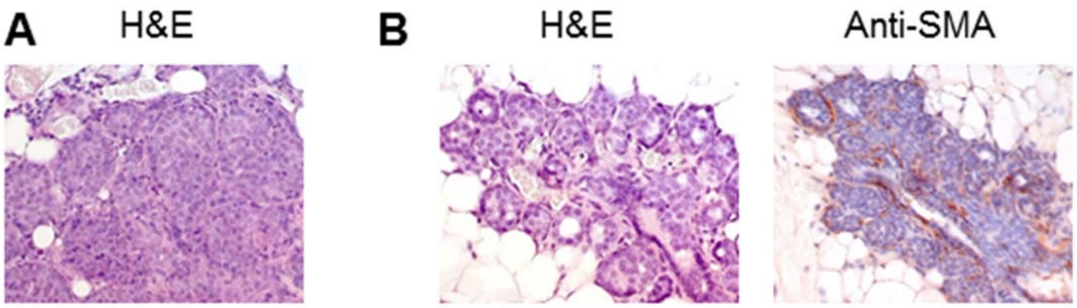
Relative claudin-4 signal
0
1
2
3
4
5
468
SQ20b
HT1080
B
Relative claudin-4 signal
0
5
10
15
20
25
468
SQ20b
HT1080
C
****
Relative claudin-4 signal
0
2
4
6
8
10
468
HT1080
D
**
Relative claudin-4 signal
0
1
2
3
4
Carcinoma
Muscle
E
***
***
*
Relative claudin-4 signal
0
1
2
3
Aplasia 80 days
Wild type
Muscle

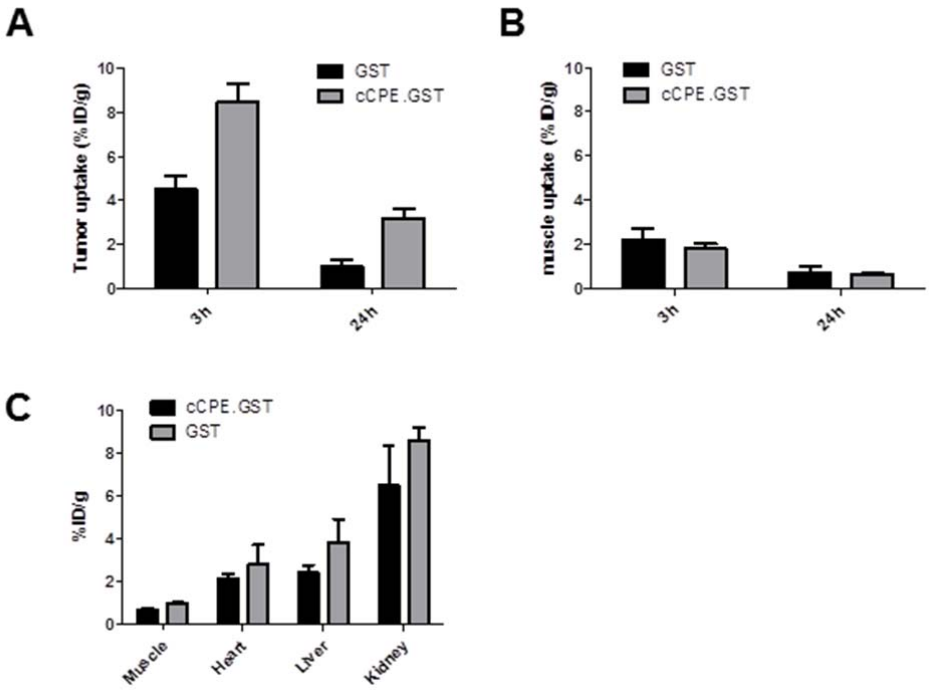
Supplemental Figure 3. (A) MDA-MB-468, SQ20b, and HT1080 cells were stained for claudin-4. Immunofluorescence results were quantified using densitometry. **(B)** Western blot demonstrating presence or absence of claudin-4 in whole cell lysates obtained from MDA-MB-468, SQ20b, and HT1080 cells. Results were quantified using densitometry. **(C)** Sections obtained from MDA-MB-468 or HT1080 xenograft tumors were stained using anti-claudin-4 antibodies. IHC results were quantified using densitometry. **(D)** Sections obtained from tumors harvested from a 139 day old *balb/neuT* mouse were stained using anti-claudin-4 antibodies. Immunofluorescence results were quantified using densitometry. (**E**) Sections obtained from aplastic lesion or muscle harvested from an 80 day old *balb/neuT* mouse, or ductal tissue obtained from a wild-type *balb/c* mouse were stained using anti-claudin-4 antibodies. Scale bar: 20 μm. IHC results were quantified using densitometry.



Supplemental Figure 4. Cells were exposed for various times at 37°C to ^{111}In-labelled cCPE.GST and the extent of membrane-binding and internalised ^{111}In was determined. (**A**) Percentage of internalized ^{111}In-cCPE.GST in cells, 1 h after exposure at 37°C. (**B**) Percentage of membrane-bound ^{111}In-cCPE.GST in cells, 1 h after exposure at 37°C.



Supplemental Figure 5. (**A**) H&E staining confirmed the overt adenocarcinoma stage of tumors harvested from *balb/neuT* mice aged 139 days. (**B**) H&E staining confirmed the aplastic stage of tumors harvested from balb/neuT mice aged 80 days.



Supplemental Figure 6. (**A-C**) VOI analysis of balb/neuT mice bearing overt tumors 3 or 24 hours post injection of ^{111}In-cCPE.GST or ^{111}In-GST. Each group contained at least three animals.