SUPPLEMENTAL DATA

Identification of Serine-875 as an Inhibitory Phosphorylation Site in the Calcium-Sensing Receptor

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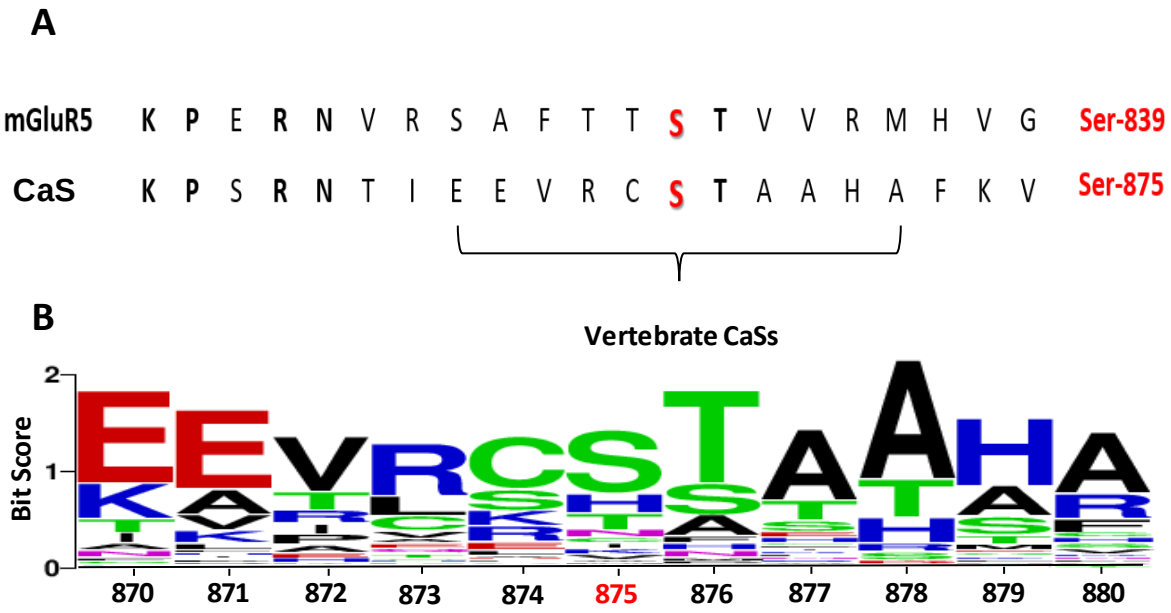
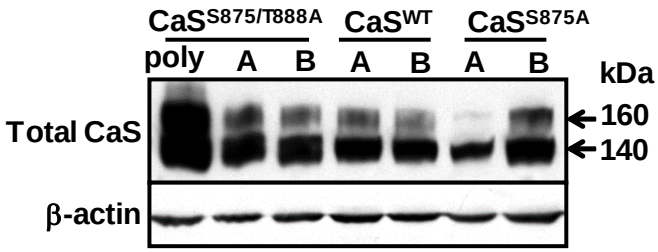
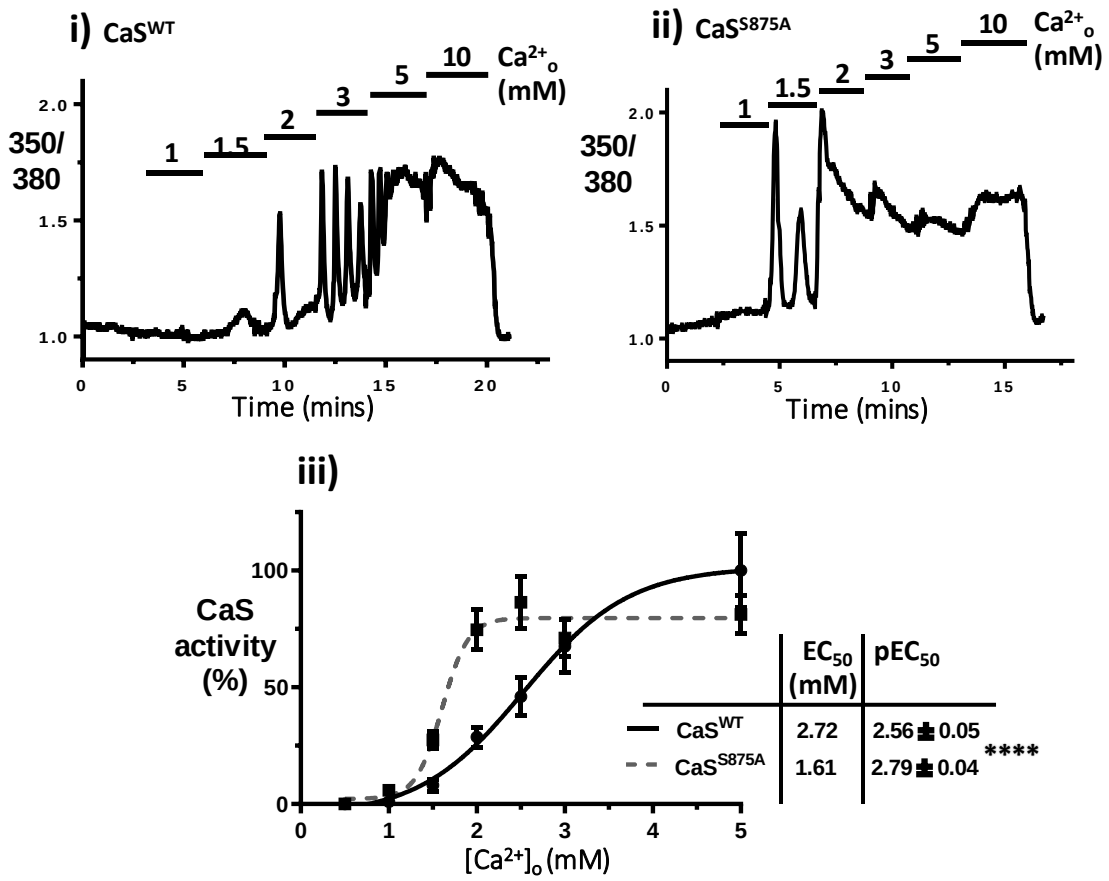


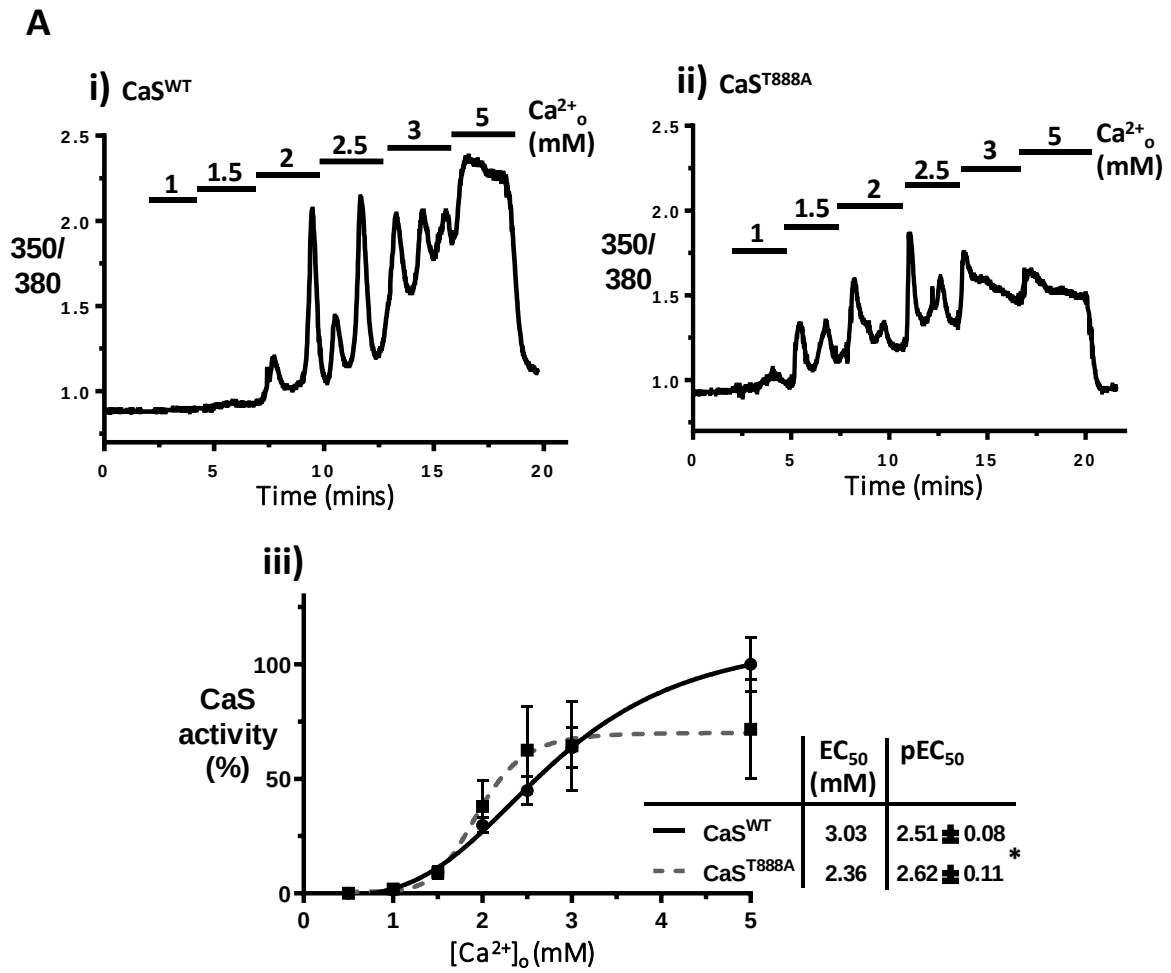
Supplementary Figure 1: Sequence alignment of the CaS and mGluR5 intracellular domains. A) Sequence alignment of the juxtamembrane regions of the CaS and mGluR5 intracellular domains indicates that the putative PKC phosphorylation site Ser-839 in mGluR5 aligns with Ser-875 in CaS (serine residues shown in red). **B)** Weblogo multiple sequence alignment of Ser-875 phosphorylation site. The multiple sequence alignment of the vertebrate CaSs obtained from (Herberger and Loretz, 2013) is showing amino acid residues surrounding Ser-875 phosphorylation site at positions −5 to +5. The calculated bit scores indicate an overall high score for the respective amino acids reflecting their conservation and stabilisation among different species.



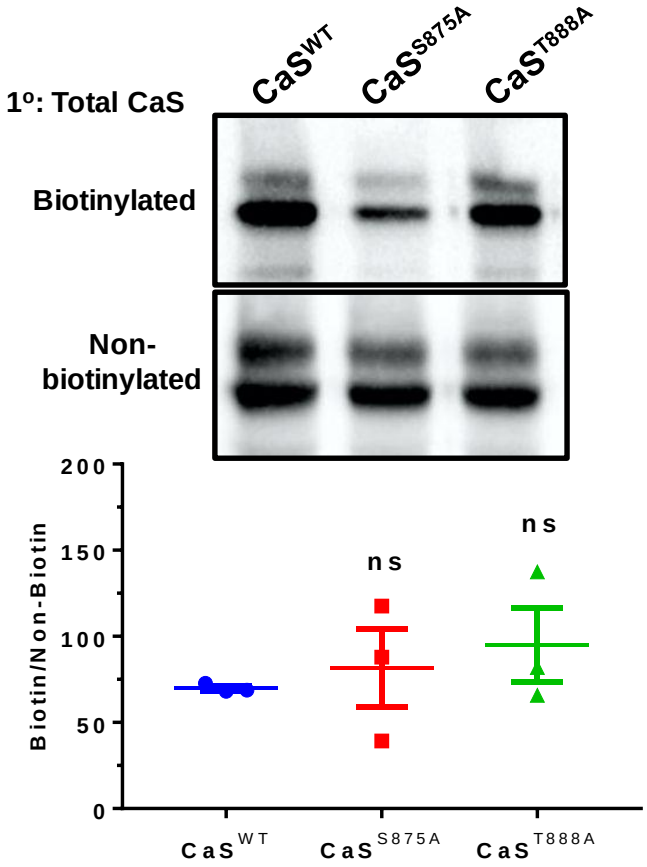
Supplementary Figure 2: Western blot analysis of CaS in HEK-293 cells stably transfected with either wild-type or mutant CaS cDNAs. Wild-type HEK-293 cells were transfected with linearised vectors encoding CaS^{WT}, CaS^{S875A} and $CaS^{S875A/T888A}$ then selected with 400 μg/ml hygromycin for two weeks. Single clones from the stable polyclonal pool were then expanded for an additional three weeks under hygromycin maintenance dose (200 μg/ml). Representative western blots show robust CaS expression in lysed cells from either the initial stable polyclone (poly) or from two individual monoclones (A & B). Clonal cell lines that expressed mutant receptors at levels close to the CaS^{WT}-expressing cells were selected for subsequent studies (clone A, CaS^{WT}; clone B, CaS^{S875A}; clone A, $CaS^{S875A/T888A}$). The CaS immunoreactivity (ADD monoclonal anti-CaS antibody) included the distinctive immature 140-kDa and mature 160-kDa CaS proteins. β-actin was included as a loading control.



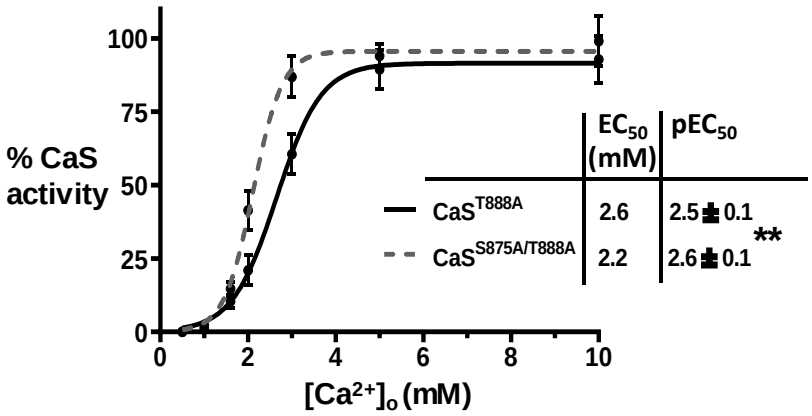
Supplementary Figure 3: Effect of CaS^{S875A} mutation on Ca^{2+}_i mobilisation following stable transfection into HEK-293 cells. Fura-2-loaded HEK-293 cells stably expressing either CaS^{WT} (i) or CaS^{S875A} (ii) were stimulated with increasing Ca^{2+}_o concentrations (0.5–10 mM) and Ca^{2+}_i assayed. Representative Ca^{2+}_i traces are shown (i and ii) with the resulting concentration-effect curves shown in panel iii. ***P<0.001 for pEC_{50}s of CaS^{S875A} vs CaS^{WT} by unpaired t-test; n ≥ 12 coverslips from three independent experiments.



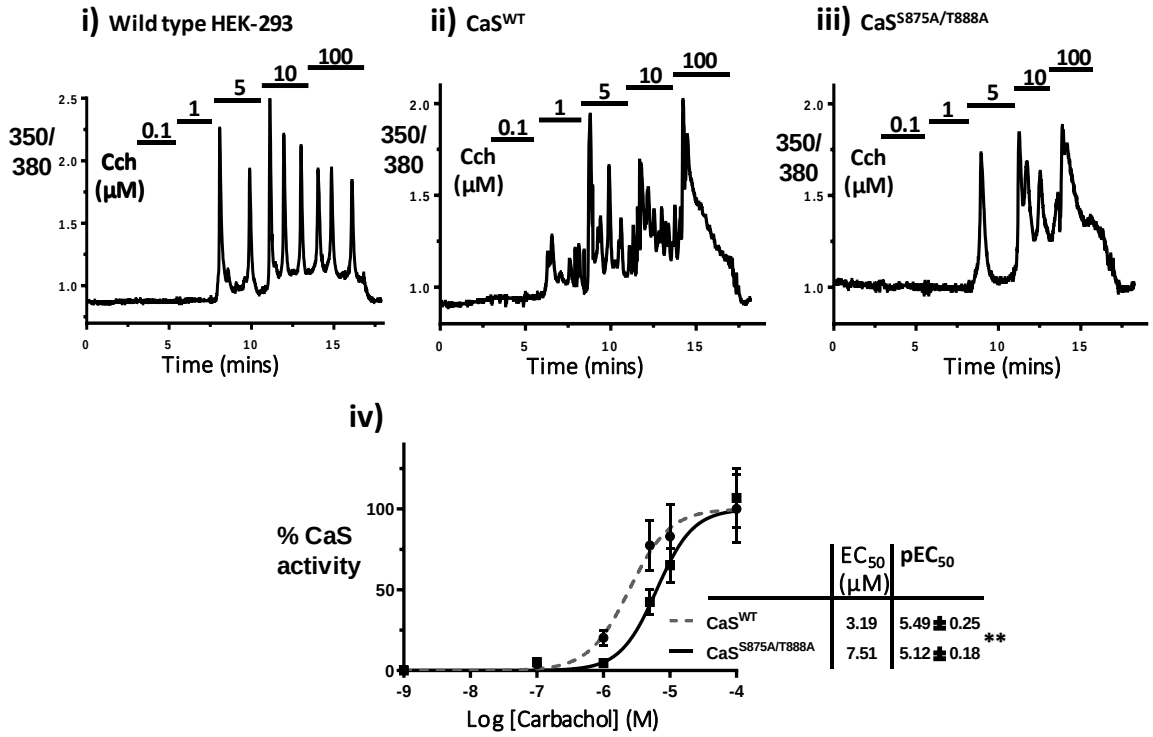
Supplementary Figure 4: CaS^{T888A} exhibits enhanced Ca^{2+}_i mobilisation. Fura-2-loaded HEK-293 cells stably expressing either CaS^{WT} (i) or CaS^{T888A} (ii) were stimulated with increasing Ca^{2+}_o concentrations (0.5–5 mM) and Ca^{2+}_i assayed. Representative Ca^{2+}_i traces are shown (i and ii) with the resulting concentration-effect curves shown in panel iii. *P<0.05 for pEC_{50}s of CaS^{T888A} vs CaS^{WT} by unpaired t-test; n ≥ 6 coverslips from three independent experiments.



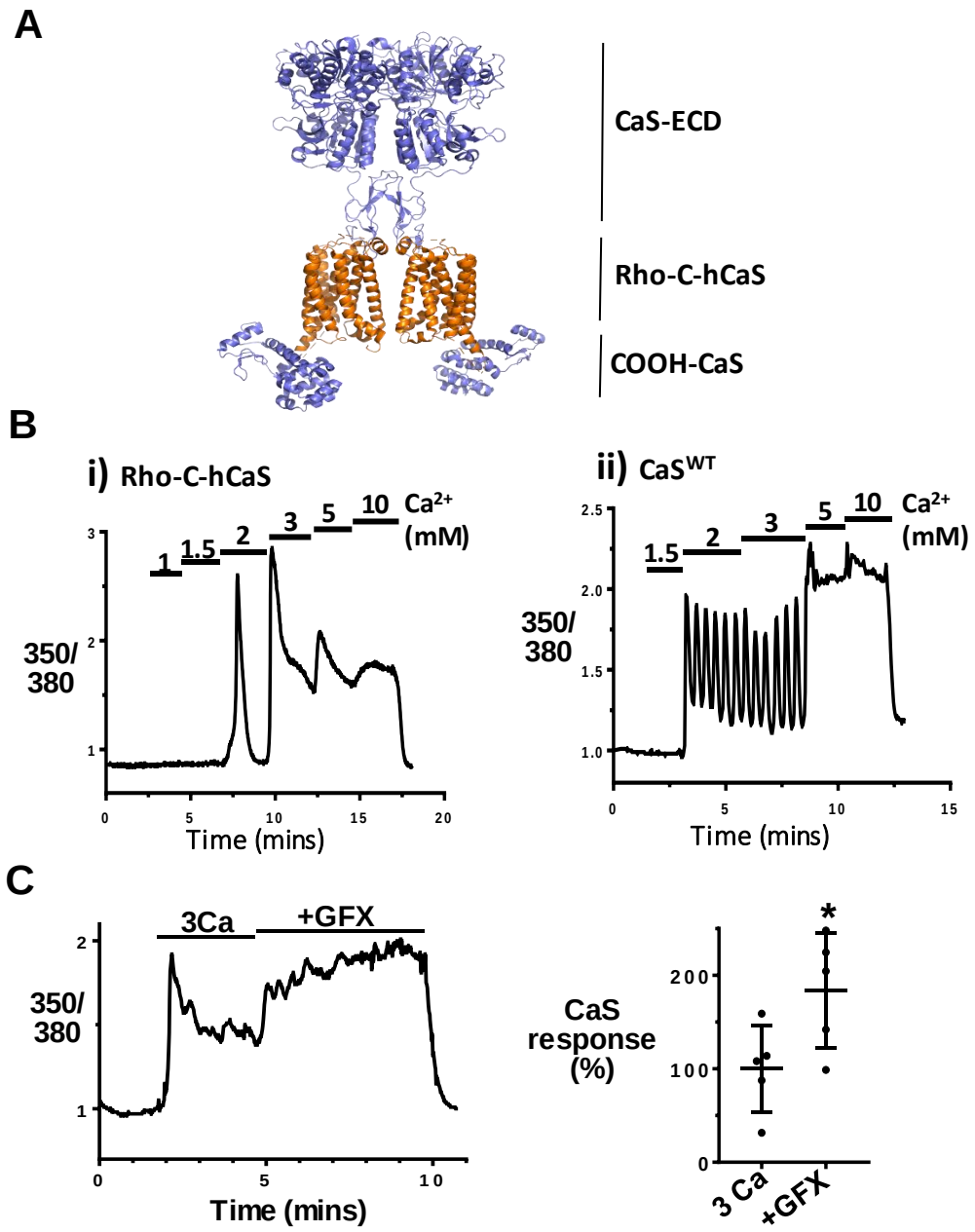
Supplementary Figure 5: Relative membrane localisation of receptor mutants CaS^{S875A} and CaS^{T888A} following transient transfection in HEK-293 cells. HEK-293 cells were transiently transfected with either CaS^{S875A} or CaS^{T888A} and then transferred to 35 mm dishes. Cell surface CaR expression was determined using the Pierce Cell Surface Protein Isolation Kit (Fisher Scientific) according to the manufacturer's instructions. Sulfo-NHS-SS-Biotin-labelled cell surface proteins were collected using NeutrAvidin Agarose beads. There were no gross or statistically significant differences in membrane localization between the 3 receptors (P=0.55 RM-ANOVA). Data are shown graphically as mean ± SEM and include individual data points.



Supplementary Figure 6: Effect of CaS$^{S875A/T888A}$ double mutation on Ca^{2+}_i mobilisation following transient transfection into HEK-293 cells. Fura-2-loaded HEK-293 cells were transiently transfected with either CaS^{T888A} or CaS$^{S875A/T888A}$, then stimulated with increasing Ca^{2+}_o concentrations (0.5–10 mM) and their Ca^{2+}_i content assayed. The concentration-effect curves show the significantly enhanced Ca^{2+}_o sensitivity in the CaS$^{S875A/T888A}$ double mutant cells. **P<0.01 pEC_{50} CaS^{T888A} vs CaS$^{S875A/T888A}$ by unpaired t-test; n ≥ 12 coverslips from three independent experiments.



Supplemental Figure 7: CaS$^{S875A/T888A}$ double mutation does not enhance muscarinic receptor responsiveness. Fura-2-loaded HEK-293 cells stably transfected with either CaS^{WT} (ii) or CaS$^{S875A/T888A}$ (iii) were stimulated with increasing concentrations of carbachol (0.1-100 μM; Cch) and their Ca^{2+}_i content assayed. The response to carbachol in wild-type HEK-293 cells is shown in panel i. The Ca^{2+}_o sensitivity of CaS$^{S875A/T888A}$ was not increased but was, in fact, decreased. **P<0.01 pEC_{50} vs CaS^{WT} by unpaired t-test. Results are representative of a minimum of nine coverslips from three independent experiments.



Supplemental Figure 8: $Ca^{2+}{}_i$ mobilisation response for the ECD truncated mutant, Rho-C-hCaS. **A)** Homology model of the full CaS structure. The ECD is adapted from the crystallography data of CaS (Geng et al., 2016, PDB: 5K5S). TMD and ICD are adapted from the crystallography data of mGluR5 (Doré et al., 2014, PDB:4OO9). The portion of the CaS that is Rho-C-hCaS is shown in orange. **B)** Fura-2-loaded HEK cells transiently transfected with Rho-C-hCaS were stimulated with increasing $Ca^{2+}{}_o$ concentrations and then assayed for their $Ca^{2+}{}_i$ content (i). N=8 coverslips from three independent

transfections. The response of CaS^{WT}-expressing cells to the same treatment is shown for comparison in panel ii. **C)** Rho-C-hCaS-transfected HEK cells were exposed to 3 mM Ca^{2+}_o then cotreated with the PKC inhibitor GF109203X (250 nM; GFX). The response to GFX (area-under-the-curve) was normalised to the initial 3 mM Ca^{2+}_o response. *P<0.05 by paired t-test; n = 5 coverslips from two independent transfections.