

Allosteric Modulation of the Calcium-Sensing Receptor Rectifies Signaling Abnormalities Associated with G-protein alpha-11 Mutations causing Hypercalcemic and Hypocalcemic Disorders

Valerie N. Babinsky^{†1}, Fadil M. Hannan^{†§1}, Caroline M. Gorvin^{†1}, Sarah A. Howles[‡], M. Andrew Nesbit^{†¶}, Nigel Rust^{||}, Aylin C. Hanyaloglu^{§§}, Jianxin Hu^{**}, Allen M. Spiegel^{††}, Rajesh V. Thakker^{‡2}

From the [†]Radcliffe Department of Medicine, University of Oxford, Oxford, OX3 7LJ, UK, [§]Department of Musculoskeletal Biology, University of Liverpool, Liverpool, L69 3GA, UK, [¶]Biomedical Sciences Research Institute, Ulster University, Coleraine, BT52 1SA, UK, ^{||}Sir William Dunn School of Pathology, University of Oxford, Oxford, OX1 3RE, UK, ^{§§}Institute of Reproductive Biology and Development, Department of Surgery and Cancer, Imperial College London, London, W12 0NN, UK, ^{**}Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, 20892, ^{††}Albert Einstein College of Medicine, Bronx, New York 10461

Running title: *Allosteric CaSR modulation for Ga₁₁ mutations*

¹These authors contributed equally to this work

²To whom correspondence should be addressed: Prof Rajesh V. Thakker, Academic Endocrine Unit, Radcliffe Department of Medicine, Oxford Centre for Diabetes, Endocrinology and Metabolism (OCDEM), Churchill Hospital, Oxford OX3 7LJ, United Kingdom. Tel no: 01865 857501. Fax no: 01865 875502. E-mail: rajesh.thakker@ndm.ox.ac.uk

Key words: calcium, drug action, G protein, genetic disease, parathyroid hormone, *GN11*, familial hypocalciuric hypercalcemia, autosomal dominant hypocalcemia, uveal melanoma

ABSTRACT

Germline loss- and gain-of-function mutations of G-protein alpha-11 (*Ga₁₁*), which couples the calcium-sensing receptor (CaSR) to intracellular calcium (Ca^{2+}_i) signaling, lead to familial hypocalciuric hypercalcemia type 2 (FHH2) and autosomal dominant hypocalcemia type 2 (ADH2), respectively, whereas somatic *Ga₁₁* mutations mediate uveal melanoma development by constitutively upregulating MAPK signaling. Cinacalcet and NPS-2143 are allosteric CaSR activators and inactivators, respectively, that ameliorate signaling disturbances associated with CaSR mutations, but their potential to modulate abnormalities of the downstream *Ga₁₁* protein is unknown. This study investigated whether cinacalcet and NPS-2143 may rectify Ca^{2+}_i alterations associated with FHH2- and ADH2-causing *Ga₁₁* mutations, and evaluated the influence of germline gain-of-function *Ga₁₁* mutations on MAPK signaling by measuring ERK phosphorylation, and assessed the effect of NPS-2143 on a uveal melanoma *Ga₁₁* mutant. WT and mutant *Ga₁₁* proteins causing FHH2, ADH2 or uveal melanoma were

transfected in CaSR-expressing HEK293 cells, and Ca^{2+}_i and ERK1/2 phosphorylation responses measured by flow-cytometry and Alphascreen immunoassay following exposure to extracellular Ca^{2+} (Ca^{2+}_o) and allosteric modulators. Cinacalcet and NPS-2143 rectified the Ca^{2+}_i responses of FHH2- and ADH2-associated *Ga₁₁* loss- and gain-of-function mutations, respectively. ADH2-causing *Ga₁₁* mutations were demonstrated not to be constitutively activating and induced ERK phosphorylation following Ca^{2+}_o stimulation only. The increased ERK phosphorylation associated with ADH2 and uveal melanoma mutants was rectified by NPS-2143. These findings demonstrate that CaSR-targeted compounds can rectify signaling disturbances caused by germline and somatic *Ga₁₁* mutations, which respectively lead to calcium disorders and tumorigenesis; and that ADH2-causing *Ga₁₁* mutations induce non-constitutive alterations in MAPK signaling.

Guanine nucleotide-binding protein (G-protein) alpha-11 (*Ga₁₁*) is a major intracellular signaling

partner of the cell-surface G-protein-coupled calcium (Ca^{2+})-sensing receptor (CaSR), which plays a pivotal role in the parathyroid and renal regulation of extracellular calcium (Ca^{2+}_o) concentrations (1,2). $\text{G}\alpha_{11}$ belongs to the $\text{G}_{q/11}$ class of G-proteins that enhance phospholipase C activity (3), thereby leading to an accumulation of inositol 1,4,5-trisphosphate and rapid increase in intracellular Ca^{2+} (Ca^{2+}_i) concentrations (2,4). These signal transduction events allow the CaSR to respond to small fluctuations in the prevailing Ca^{2+}_o concentration ($[\text{Ca}^{2+}]_o$) by inducing alterations in parathyroid hormone (PTH) secretion and urinary Ca^{2+} excretion (5).

The identification of germline heterozygous loss- and gain-of-function mutations of $\text{G}\alpha_{11}$, which is encoded by the *GN11* gene on chromosome 19p13.3, that lead to forms of familial hypocalciuric hypercalcemia (FHH) or autosomal dominant hypocalcemia (ADH), respectively, has demonstrated the importance of this G-protein subunit in Ca^{2+}_o homeostasis (1,6,7). FHH is a genetically heterogeneous disorder that is inherited as an autosomal dominant condition, which is characterised by lifelong elevations of serum Ca^{2+} concentrations in association with normal or mildly raised serum PTH levels and low urinary Ca^{2+} excretion (8). FHH is considered to represent a benign disorder, however some patients may develop symptomatic hypercalcemia, pancreatitis or chondrocalcinosis (8). FHH type 1 (FHH1, OMIM #145980) is caused by loss-of-function mutations of the *CASR* gene (9), and FHH type 2 (FHH2, OMIM #145981) is caused by loss-of-function $\text{G}\alpha_{11}$ mutations, which comprise a Leu135Gln missense substitution and in-frame isoleucine deletion at codon 199 or 200 (Ile199/200del) that impair CaSR signal transduction and were identified in two unrelated probands and families (1). ADH is also genetically heterogeneous and caused by germline gain-of-function mutations of the *CASR* and *GN11* genes, which lead to ADH types 1 (ADH1, OMIM #601198) and 2 (ADH2, OMIM #615361), respectively (1,6-10). Approximately 50% of ADH patients develop hypocalcemic symptoms such as paraesthesia, carpo-pedal spasms and seizures, and >35% of patients harbor ectopic calcifications within the kidneys or basal ganglia (1,6,7,10). In contrast to germline gain-of-function $\text{G}\alpha_{11}$ mutations, which affect Ca^{2+}_o

homeostasis, somatic gain-of-function $\text{G}\alpha_{11}$ mutations have been reported to lead to uveal melanoma, which is a primary intraocular tumor, by inducing constitutive up-regulation of proliferative signaling involving ERK, which is a component of the MAPK signaling pathway (11).

Compounds that selectively bind to the CaSR and allosterically modulate the function of this family C G-protein-coupled-receptor (GPCR) represent a potential targeted therapy for patients with symptomatic forms of FHH and ADH. Indeed, cinacalcet, which is a licenced CaSR positive allosteric modulator, has been used effectively in FHH1 patients to manage symptomatic hypercalcemia and recurrent pancreatitis (12,13). Furthermore, negative allosteric CaSR modulators, known as calcilytics, have been demonstrated to ameliorate hypocalcemia in mouse models of ADH1 (14,15). The objective of this study was to undertake *in vitro* studies to determine whether allosteric modulators targeted to the CaSR may also rectify the loss- and gain-of-function associated with FHH2- and ADH2-causing germline $\text{G}\alpha_{11}$ mutations, respectively, and the upregulation of ERK phosphorylation caused by a uveal melanoma-associated somatic $\text{G}\alpha_{11}$ mutation. In addition, this study evaluated whether germline ADH2-causing gain-of-function $\text{G}\alpha_{11}$ mutations may constitutively activate MAPK signaling and thus pose a risk for the development of uveal melanomas.

EXPERIMENTAL PROCEDURES

Cell culture and transfection - Functional studies of mutant $\text{G}\alpha_{11}$ proteins were performed in HEK293 cells that stably expressed the CaSR (HEK-CaSR) (1,16,17). HEK293 cells endogenously express $\text{G}\alpha_{11}$, and co-expression of mutant $\text{G}\alpha_{11}$ proteins approximately represented the heterozygous state in FHH2 and ADH2 patients (1). The HEK-CaSR cell line was cultured in high-glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% geneticin, as described (1,16,17). A high level of CaSR expression in these cells was confirmed by Western blot analysis using a mouse monoclonal antibody to human CaSR (ADD; Abcam, ab19347, 1:1,000) (1,16). WT and mutant *GN11*-pBI-CMV2 constructs were transiently transfected into HEK-CaSR cells using Lipofectamine 2000

(1,16,17). The bidirectional pBI-CMV2 cloning vector was used as it facilitated the co-expression of $G\alpha_{11}$ and GFP (1,16,18). Expression of WT and mutant $G\alpha_{11}$ proteins were determined by Western blot analysis using a mouse monoclonal anti- $G\alpha_{11}$ antibody (SantaCruz, sc-390382, 1:750), and the membrane was re-probed with a polyclonal rabbit anti- α -tubulin antibody (Abcam, ab15246, 1:1000) as a loading control. Successful transfection was also confirmed by visualising GFP fluorescence using an Eclipse E400 fluorescence microscope with a Y-FL Epifluorescence attachment and a triband 4,6-diamidino-2-phenylindole-FITC-Rhodamine filter, and images captured using a DXM1200C digital camera and NIS-Elements software (Nikon) (1,16,17).

Studies involving siRNA knockdown of endogenous $G\alpha_{11}$ were undertaken in HEK293 cells that stably expressed WT or mutant $G\alpha_{11}$ proteins (HEK- $G\alpha_{11}$). The HEK- $G\alpha_{11}$ cells were generated using HEK293 T-Rex-Flp-in stable cell-lines (Life Technologies), as reported (19). WT and mutant *GNAI1* constructs were cloned into the pcDNA5/FLP recombination target (FRT) expression vector (Life Technologies), and silent mutations introduced to render the constructs resistant to *GNAI1*-targeted siRNA, thereby allowing investigation of the mutant $G\alpha_{11}$ protein in the absence of endogenous WT $G\alpha_{11}$. *GNAI1* constructs were transiently transfected into T-Rex-Flp-in cells, and those cells expressing the $G\alpha_{11}$ protein selected by culturing cells in media containing Hygromycin (Gibco). The presence of the $G\alpha_{11}$ protein, and its resistance to siRNA was confirmed by Western blot analysis. Forty-eight hours prior to measuring Ca^{2+}_i responses, HEK- $G\alpha_{11}$ cells were transiently transfected with the reported pEGFP-CaSR construct (9) and three different commercially available *GNAI1*-targeted siRNA constructs (Trilencer-27 siRNA kit, catalog number SR301839, Origene) or a commercially available scrambled siRNA (Trilencer-27 universal scrambled negative control siRNA duplex, catalog number SR30004, Origene), and successful transfection confirmed by fluorescence microscopy, as described for pBI-CMV2-expressing HEK-CaSR cells (1,16,17).

Measurement of Ca^{2+}_i responses - The effect of allosteric CaSR modulators on HEK-CaSR cells expressing WT or mutant $G\alpha_{11}$ proteins was assessed by a flow cytometry-based

Ca^{2+}_i assay, as reported (1,16,17). In brief, 48 hours after transfection, the cells were harvested, washed in Ca^{2+} - and magnesium (Mg^{2+})-free Hank's balanced salt solution (HBSS) (Invitrogen) and loaded with 1 μ g/ml indo-1-acetoxymethylester (Indo-1-AM) (Molecular Probes) for 1 hour at 37 °C (1,16,17). Transfected HEK-CaSR cells were incubated with either a 20% aqueous solution of 2-hydroxypropyl- β -cyclodextrin (Sigma) (vehicle), or positive or negative CaSR allosteric modulators, known as cinacalcet or NPS-2143, respectively, at concentrations ranging from 10-40 nM for 1 hour (15). Flow cytometry was performed with a Beckman Coulter MoFlo XDP equipped with JDSUY Xcyte UV Laser and a Coherent Sapphire 488 Laser using a 550LP dichroic mirror and 580/30 bandpass filter (17). Single cells were isolated and stimulated by sequentially adding Ca^{2+} to the Ca^{2+} - and Mg^{2+} -free HBSS to increase the $[Ca^{2+}]_o$ in a stepwise manner from 0-15 mM. The range of $[Ca^{2+}]_o$ used to activate CaSR signaling in HEK293 cells was not representative of physiological levels of serum ionized calcium, which are homeostatically maintained between 1.1-1.3 mM (20), but use of these Ca^{2+}_o concentrations *in vitro* allowed a comprehensive assessment of CaSR signaling responses, which included threshold responses (1-1.5 mM Ca^{2+}_o), half-maximal (EC_{50}) responses (2-4 mM Ca^{2+}_o) and near-maximal responses (>10 mM), as reported (21). The baseline fluorescence ratio was measured for 2 min, the fluorescence ratio compared to the time was recorded and data were collected for 2 min at each $[Ca^{2+}]_o$, as described (1,16,17). Cytomation Summit software was used to determine the peak mean fluorescence ratio of the transient response after each individual stimulus, which was expressed as a percentage normalized response (1,16,17). Concentration-response curves were generated using a 4-parameter non-linear regression curve-fit model (GraphPad Prism) to calculate the half-maximal (EC_{50}) and area under the curve (AUC) mean \pm SEM responses for each separate experiment (17).

Measurement of ERK phosphorylation - HEK-CaSR cells, transfected with WT or mutant $G\alpha_{11}$ proteins for 24 hours, were seeded in 48-well plates and cultured overnight in high glucose DMEM containing 10% FBS, prior to being incubated for 4 hours with serum-free DMEM

containing 0.5 mM Ca^{2+} , 25 mM HEPES buffer with or without cinacalcet or NPS-2143 at 10-500 nM concentrations. Cells were stimulated for 4 min with pre-warmed serum-free DMEM that contained Ca^{2+} concentrations ranging from 0.5-10 mM, as reported (22), and lysed in Surefire lysis buffer. Alphascreen Surefire ERK phosphorylation assays were performed on whole cell lysates, as reported (23), and the fluorescence signal measured using a PHERAStar FS microplate reader (BMG Labtech) (23). ERK phosphorylation responses measured at each $[\text{Ca}^{2+}]_o$ were normalized to the mean responses of WT expressing cells and expressed as a fold-change of responses obtained at basal (0.5 mM) $[\text{Ca}^{2+}]_o$.

Statistical analysis - The Ca^{2+}_i and ERK phosphorylation responses of cells expressing WT or mutant $\text{G}\alpha_{11}$ proteins were compared from a minimum of four experiments using the F-test and Mann-Whitney U test, respectively (1). All analyses were undertaken using GraphPad Prism (GraphPad), and are presented as mean \pm SEM. A value of $p < 0.05$ was considered significant for all analyses.

RESULTS

Effect of cinacalcet on the Ca^{2+}_i responses of FHH2-associated $\text{G}\alpha_{11}$ mutations - The FHH2-associated Leu135Gln and Ile199/200del $\text{G}\alpha_{11}$ mutations have been reported to impair the sensitivity of CaSR-expressing cells to Ca^{2+}_o (1), and we hypothesized that cinacalcet-mediated allosteric activation of the CaSR would ameliorate the loss-of-function associated with germline mutations of $\text{G}\alpha_{11}$, thereby rectifying the signal transduction abnormalities in cells expressing these FHH2-associated mutant $\text{G}\alpha_{11}$ proteins. To investigate this hypothesis, WT or mutant *GN11*-pBI-CMV2 constructs were transiently transfected in HEK-CaSR cells and the effect of cinacalcet on the responses of Ca^{2+}_i concentrations ($[\text{Ca}^{2+}]_i$) to alterations in $[\text{Ca}^{2+}]_o$ was assessed. Expression of the CaSR and $\text{G}\alpha_{11}$ was confirmed by fluorescence microscopy and/or Western blot analysis of whole-cell lysates (Fig. 1A and B). CaSR expression, which was normalised by comparison to α -tubulin expression, did not differ between cells transfected with WT or FHH2-associated mutant *GN11*-pBI-CMV2 vectors when compared to cells transfected with empty vector, whereas the expression of $\text{G}\alpha_{11}$ was greater in cells transfected with WT or mutant

constructs (Fig. 1B). HEK-CaSR cells transiently transfected with WT or mutant $\text{G}\alpha_{11}$ proteins were exposed to varying $[\text{Ca}^{2+}]_o$, and measurement of Ca^{2+}_i responses by flow cytometry revealed the FHH2-associated Gln135 and del199/200 $\text{G}\alpha_{11}$ mutants to result in a rightward shift of the concentration-response curves (Fig. 1C) with a significant reduction in AUC values and increases in EC_{50} values (Gln135 = 3.54 ± 0.07 mM, del199/200 = 3.49 ± 0.04 mM) compared to WT $\text{G}\alpha_{11}$ (2.67 ± 0.03 mM; $p < 0.0001$) (Fig. 1D-E), as reported (1). A dose-titration of cinacalcet in cells expressing the Gln135 $\text{G}\alpha_{11}$ mutant revealed this calcimimetic to act in a dose-dependent manner, with 10 and 20 nM drug concentrations significantly ($p < 0.0001$) reducing the Gln135 mutant EC_{50} values to 2.75 ± 0.03 and 2.61 ± 0.09 mM, respectively (Fig. 1E). Indeed, 10 nM of cinacalcet induced a leftward shift of the mutant concentration-response curve, so that this was indistinguishable from that of WT-expressing cells (Fig. 1F). The addition of 10 and 20 nM cinacalcet lowered the EC_{50} values of cells expressing the del199/200 $\text{G}\alpha_{11}$ mutant (Fig. 1E). However, despite the del199/200 mutant having an almost identical EC_{50} value to the Gln135 $\text{G}\alpha_{11}$ mutant protein, these cinacalcet doses were insufficient to rectify the loss-of-function associated with the del199/200 $\text{G}\alpha_{11}$ mutant (Fig. 1E). Subsequently, when cinacalcet was added at a 40 nM concentration to cells expressing the del199/200 mutant, this lowered the EC_{50} value to 2.68 ± 0.04 mM (Fig. 1E), so that the del199/200 mutant concentration-response curve overlapped with that of the WT $\text{G}\alpha_{11}$ protein (Fig. 1G).

Effect of NPS-2143 on the Ca^{2+}_i responses of ADH2-associated $\text{G}\alpha_{11}$ mutations - We previously reported the germline Arg181Gln and Phe341Leu $\text{G}\alpha_{11}$ mutations to enhance the sensitivity of CaSR-expressing cells to Ca^{2+}_o (1), thereby giving rise to the hypocalcemic disorder of ADH2. To determine whether allosteric inhibition of the CaSR can rectify the gain-of-function associated with ADH2-causing $\text{G}\alpha_{11}$ mutations, WT or ADH2-associated mutant *GN11*-pBI-CMV2 vectors were transiently transfected into HEK-CaSR cells, and the responses of $[\text{Ca}^{2+}]_i$ to alterations in $[\text{Ca}^{2+}]_o$ assayed. Expression of the CaSR and $\text{G}\alpha_{11}$ was demonstrated by fluorescence microscopy and/or Western blot analysis (Fig. 2A

and B). Western blot analysis confirmed an increase in the expression of $G\alpha_{11}$ in cells transfected with WT or ADH2-associated mutant proteins, when compared to cells transfected with empty vector alone (Fig. 2B). An assessment of the Ca^{2+}_i responses of HEK-CaSR cells transiently transfected with WT or ADH2-associated mutant $G\alpha_{11}$ proteins following stimulation with Ca^{2+}_o , demonstrated cells expressing the Gln181 or Leu341 mutants to induce a leftward shift of the concentration-response curves (Fig. 2C) with a significant increase in AUC values and reduction in EC_{50} values of 2.38 ± 0.08 and 2.29 ± 0.07 mM, respectively, compared to 2.57 ± 0.03 mM for WT expressing cells ($p < 0.0001$) (Fig. 2D and E), as previously reported (1). The addition of NPS-2143 to cells expressing the Gln181 $G\alpha_{11}$ mutant revealed a 10 nM concentration of this calcilytic compound to normalize the mutant EC_{50} value to 2.57 ± 0.07 mM (Fig. 2E), so that the concentration-response curve resembled that of WT $G\alpha_{11}$ (Fig. 2F), whereas 20 nM NPS-2143 significantly increased the mutant EC_{50} value to 2.72 ± 0.12 mM when compared to WT expressing cells (Fig. 2E). In contrast to these studies involving the Gln181 $G\alpha_{11}$ mutant protein, the addition of 20 nM NPS 2143 to cells expressing the Leu341 $G\alpha_{11}$ mutant did not significantly alter the EC_{50} . (Fig. 2E). Indeed, NPS-2143 at a concentration of 30 nM was required to decrease the Leu341 mutant EC_{50} value to 2.66 ± 0.09 mM and rectify the shift in the mutant concentration-response curve (Fig. 2E and G).

Effect of CaSR allosteric modulators on the Ca^{2+}_i responses in absence of endogenously expressed WT $G\alpha_{11}$ protein - To determine whether CaSR-targeted drugs rectify the Ca^{2+}_i responses of FHH2- and ADH2-mutant expressing cells by directly influencing mutant $G\alpha_{11}$ -signaling or by indirect effects on WT $G\alpha_{11}$ protein that is endogenously expressed in HEK293 cells, siRNA knockdown of endogenous WT $G\alpha_{11}$ was undertaken in HEK- $G\alpha_{11}$ cells stably expressing WT, FHH2-associated Gln135, or ADH2-associated Gln181 mutant $G\alpha_{11}$ proteins. Western blot analysis demonstrated that siRNA with a scrambled sequence did not alter endogenous WT $G\alpha_{11}$ expression in untransfected HEK293 cells (Fig. 3A). In contrast, *GN11*-targeted siRNA reduced endogenous WT $G\alpha_{11}$ expression in

untransfected HEK293 cells (Fig. 3A), and decreased the level of transiently expressed WT $G\alpha_{11}$ in HEK293 cells (Fig. 3B), but did not affect the levels of stably expressed WT or mutant $G\alpha_{11}$ proteins in HEK- $G\alpha_{11}$ cells (Fig. 3B), which contained constructs with silent mutations that had rendered them resistant to *GN11*-targeted siRNA. CaSR constructs were transiently transfected into HEK- $G\alpha_{11}$ cells, and CaSR expression confirmed by fluorescence microscopy (Fig. 3C). The effects of cinacalcet or NPS-2143 on the Ca^{2+}_i responses of the FHH2- and ADH2-associated $G\alpha_{11}$ mutants were assessed following knockdown of endogenous WT $G\alpha_{11}$ using *GN11*-targeted siRNAs (Fig. 3D-G). These studies revealed that: 10 nM of cinacalcet could rectify the rightward shift in the concentration-response curve and lower the significantly raised EC_{50} of the FHH2-associated Gln135 $G\alpha_{11}$ mutant from a value of 3.85 ± 0.12 mM to values of 3.23 ± 0.1 mM and 3.17 ± 0.08 mM, respectively, in the presence of *GN11*-targeted or scrambled siRNA (Fig. 3D and E), so that these values were not significantly different from HEK- $G\alpha_{11}$ cells stably expressing WT $G\alpha_{11}$ ($EC_{50} = 3.33 \pm 0.06$ mM); and that 10 nM of NPS-2143 could normalize the leftward shift of the concentration-response curve and increased the EC_{50} of the ADH2-associated Gln181 $G\alpha_{11}$ mutant from a value of 2.70 ± 0.07 mM to values of 3.26 ± 0.06 mM and 3.11 ± 0.08 mM, respectively, in the presence of *GN11*-targeted or scrambled siRNA (Fig. 3F and G), so that these values were not significantly different from WT-expressing HEK- $G\alpha_{11}$ cells. Thus, these results show that CaSR-targeted drugs can influence the signaling responses of downstream mutant $G\alpha_{11}$ proteins.

Effect of ADH2-associated $G\alpha_{11}$ mutants on MAPK signaling - To investigate whether the germline Arg181Gln and Phe341Leu ADH2-associated mutant $G\alpha_{11}$ proteins may lead to constitutive upregulation of MAPK signaling, WT and mutant *GN11*-pBI-CMV2 vectors were transiently transfected into HEK-CaSR cells and fold-change ERK phosphorylation (phospho-ERK) responses assessed following exposure to varying $[Ca^{2+}]_o$. The effects of the ADH2-associated mutants on phospho-ERK responses were compared to the uveal melanoma-associated Gln209Leu $G\alpha_{11}$ mutation (11). Following stimulation with Ca^{2+}_o , the germline Gln181 and

Leu341 mutants were revealed to have significantly ($p < 0.001$) increased maximal phospho-ERK fold-change responses (Gln181 = 18.1 ± 1.1 , Leu341 = 18.3 ± 0.9) compared to WT $G\alpha_{11}$ (14.7 ± 0.3), consistent with a gain-of-function (Fig. 4A). However, in the absence of Ca^{2+}_o stimulation, the basal phospho-ERK responses of the ADH2 mutants were demonstrated to not differ from WT $G\alpha_{11}$ (Fig. 4A and B), and thus these mutants are not constitutively activating. In contrast, the tumor-associated somatic Gln209Leu $G\alpha_{11}$ mutation led to both significantly ($p < 0.0001$) increased basal and maximal phospho-ERK fold-change responses when compared to the ADH2 mutants or WT $G\alpha_{11}$, consistent with a constitutive upregulation of MAPK signaling (Fig. 4A and B). The effect of NPS-2143 on the phospho-ERK responses of HEK-CaSR cells expressing the ADH2-associated Gln181 or Leu341 mutants, or the uveal melanoma-associated Leu209 mutant, was also assessed. NPS-2143 was added at 10 and 30 nM concentrations to cells expressing the Gln181 and Leu341 mutants, respectively, as these doses had rectified the Ca^{2+}_i responses of the $G\alpha_{11}$ mutants (Fig. 2F and G). The addition of 10 and 30 nM NPS-2143 significantly lowered the maximal fold-change responses of the Gln181 and Leu341 mutants to 14.0 ± 0.5 and 14.9 ± 0.4 , respectively, so that these values did not differ from the phospho-ERK responses of cells expressing WT $G\alpha_{11}$ (Fig. 4C and D). However, cells expressing the uveal melanoma-associated Leu209 mutant required NPS-2143 at a higher dose of 500 nM to successfully rectify increases in phospho-ERK responses (Fig. 4E).

DISCUSSION

Our studies demonstrate that cinacalcet and NPS-2143, which are allosteric CaSR activators and inactivators, respectively, can successfully rectify the loss-of-function associated with FHH2-causing $G\alpha_{11}$ mutations and the gain-of-function associated with $G\alpha_{11}$ mutations that lead to ADH2 or uveal melanomas (1,11). Cinacalcet and NPS-2143 are allosteric modulators that are predicted to bind to the CaSR transmembrane domain (24) and influence receptor activity by altering its conformational status. These compounds have been reported to rectify the activity of FHH1- and ADH1-

associated mutant CaSR proteins *in vitro* (15,25-27). However, the ability of these agents to normalise CaSR sensitivity in the presence of an abnormality downstream of the CaSR remained unknown. The *in vitro* findings of our study indicate allosteric modulation at the level of the receptor can rectify such loss- and gain-of-function associated with mutations of the intracellular $G\alpha_{11}$ protein. Indeed, these studies demonstrate that pharmacological GPCR modulation may directly overcome abnormalities affecting the downstream effector G-protein rather than by indirect effects on endogenously expressed WT G-proteins.

However, the $G\alpha_{11}$ mutations showed differences in their responsiveness to allosteric CaSR modulators. For example, our study of the FHH2 mutants revealed that a 4-fold increase in the cinacalcet dose was required to normalise the loss-of-function associated with Ile199/200del compared to the Leu135Gln mutation, despite both mutations having similar EC_{50} values. Similarly, a 3-fold increase in the NPS-2143 dosage was required to rectify the gain-of-function due to the ADH2-associated Phe341Leu mutation when compared with the gain-of-function Arg181Gln mutation, despite both mutations having similar EC_{50} values. Thus, the Ile199/200del and Phe341Leu mutations showed diminished sensitivity to cinacalcet and NPS-2143, respectively, and these differences in the sensitivities of the mutants to CaSR-targeted drugs may be explained by a reported crystallography study, which showed residues homologous to Ile199 and Phe341, in the related Gas protein to be located at the interface between GPCR and $G\alpha$ -subunit (28). Thus, $G\alpha_{11}$ mutations located at the GPCR- $G\alpha$ interface may potentially influence the efficacy of CaSR allosteric modulators.

Cells expressing loss- and gain-of-function $G\alpha_{11}$ mutants responded to nanomolar concentrations of cinacalcet (10-40 nM, which is equivalent to 3.6-14.3 ng/mL) and NPS-2143 (10-30 nM, which is equivalent to 4.4-13.3 ng/mL), respectively. However, previous *in vitro* studies of CaSR mutations leading to FHH and ADH have indicated that micromolar concentrations of these drugs may be required to rectify associated signal transduction abnormalities (25-27), and *in vivo* studies in WT rats have reported that the plasma drug concentrations of cinacalcet and NPS-2143

required to alter PTH secretion are ≥ 20 ng/mL and >100 ng/mL, respectively (29,30). The responsiveness of $G\alpha_{11}$ mutants to low doses of CaSR-targeted drugs may be explained by the finding that these mutants induced only minor disturbances of CaSR signal transduction. Indeed, the FHH2 and ADH2 mutants were associated with up to a 30% shift in the EC_{50} values of HEK-CaSR cells used in this study, whereas CaSR mutations leading to FHH1 and ADH1 generally cause a $>50\%$ shift in the EC_{50} value (9,21,31,32). However, it remains to be established whether such low concentrations of calcimimetic and calcilytic drugs will be able to rectify *in vivo* the alterations in mineral homeostasis in FHH2 and ADH2 patients.

Somatic gain-of-function $G\alpha_{11}$ mutations that induce constitutive MAPK activation have been reported in uveal melanoma and are associated with an increased likelihood of metastases (11). We therefore assessed the effects of germline ADH2-associated Arg181Gln and Phe341Leu gain-of-function $G\alpha_{11}$ mutations on MAPK signaling by measuring phospho-ERK responses. Our studies demonstrated that the ADH2 $G\alpha_{11}$ mutants induced a milder increase in ERK phosphorylation when compared to the uveal melanoma Gln209Leu $G\alpha_{11}$ mutant. Moreover, up-regulation of ERK phosphorylation by the ADH2-associated $G\alpha_{11}$ mutants only occurred in the presence of Ca^{2+}_o stimulation, and therefore these Arg181Gln and Phe341Leu $G\alpha_{11}$ mutants do not harbor constitutive activity. These findings are consistent with a recent report of an ADH2-associated Arg60Leu $G\alpha_{11}$ mutation, which also enhanced MAPK activation in a non-constitutive manner (7). The finding that ADH2-associated mutations are not constitutively activating can be explained by their locations within the GTPase

domain of the $G\alpha$ subunit. Thus, the Gln209 residue, which is mutated in uveal melanomas (11), is required to spatially orientate the terminal phosphate group of $G\alpha$ -bound GTP (33), thereby facilitating its hydrolysis and the conversion of GTP to GDP. Mutations affecting the Gln209 residue have been shown to abolish GTP hydrolysis, thereby leaving the $G\alpha$ subunit in a permanent GTP-bound state of activation (34). In contrast, the Arg181 and Phe341 $G\alpha_{11}$ residues, which are mutated in ADH2, are not located near to the terminal phosphate of GTP, and likely induce more indirect and subtle effects on GTP hydrolysis (1). The ADH2-associated $G\alpha_{11}$ mutations represent the first reports of non-constitutively activating G-protein mutations (1,6,7), and the milder nature of these mutations is consistent with post-natal survival, in contrast to the constitutively activating Gln209Leu mutation, which has been shown to be cytotoxic when expressed at high levels (35), and is likely to be embryonically lethal. The occurrence of non-constitutively activating $G\alpha_{11}$ mutations that are tolerated in humans and heritable, highlights the potential for such germline mutations to affect other G-proteins and be associated with disease-related phenotypes, and this possibility remains to be explored.

In summary, our studies have revealed that germline gain-of-function $G\alpha_{11}$ mutations induce non-constitutive alterations in MAPK signaling, and that CaSR-targeted compounds may rectify signaling disturbances caused by germline and somatic $G\alpha_{11}$ mutations, which are associated with calcium disorders and tumorigenesis, respectively. These findings indicate that allosteric modulation at the level of the receptor may influence signaling disturbances associated with mutations of the downstream G-protein.

Conflicts of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: F.M.H., M.A.N., A.C.H. and R.V.T. designed the experiments. V.N.B., S.A.H. and N.R. performed the Ca^{2+}_i measurement experiments. C.M.G. performed the siRNA knockdown experiments. V.N.B. performed the ERK phosphorylation experiments. J.H. and A.M.S. prepared and supplied the NPS-2143 compound. F.M.H., V.N.B., C.M.G. and R.V.T. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

1. Nesbit, M. A., Hannan, F. M., Howles, S. A., Babinsky, V. N., Head, R. A., Cranston, T., Rust, N., Hobbs, M. R., Heath, H., 3rd, and Thakker, R. V. (2013) Mutations affecting G-protein subunit alpha11 in hypercalcemia and hypocalcemia. *N Engl J Med* **368**, 2476-2486
2. Hofer, A. M., and Brown, E. M. (2003) Extracellular calcium sensing and signalling. *Nat Rev Mol Cell Biol* **4**, 530-538
3. Wu, D. Q., Lee, C. H., Rhee, S. G., and Simon, M. I. (1992) Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells. *J Biol Chem* **267**, 1811-1817
4. Breitwieser, G. E., and Gama, L. (2001) Calcium-sensing receptor activation induces intracellular calcium oscillations. *Am J Physiol Cell Physiol* **280**, C1412-1421
5. Riccardi, D., and Brown, E. M. (2010) Physiology and pathophysiology of the calcium-sensing receptor in the kidney. *Am J Physiol Renal Physiol* **298**, F485-499
6. Mannstadt, M., Harris, M., Bravenboer, B., Chitturi, S., Dreijerink, K. M., Lambright, D. G., Lim, E. T., Daly, M. J., Gabriel, S., and Juppner, H. (2013) Germline mutations affecting Galpha11 in hypoparathyroidism. *N Engl J Med* **368**, 2532-2534
7. Li, D., Opas, E. E., Tuluc, F., Metzger, D. L., Hou, C., Hakonarson, H., and Levine, M. A. (2014) Autosomal dominant hypoparathyroidism caused by germline mutation in GNA11: phenotypic and molecular characterization. *J Clin Endocrinol Metab* **99**, E1774-1783
8. Hannan, F. M., and Thakker, R. V. (2013) Calcium-sensing receptor (CaSR) mutations and disorders of calcium, electrolyte and water metabolism. *Best Pract Res Clin Endocrinol Metab* **27**, 359-371
9. Hannan, F. M., Nesbit, M. A., Zhang, C., Cranston, T., Curley, A. J., Harding, B., Fratter, C., Rust, N., Christie, P. T., Turner, J. J., Lemos, M. C., Bowl, M. R., Bouillon, R., Brain, C., Bridges, N., Burren, C., Connell, J. M., Jung, H., Marks, E., McCredie, D., Mughal, Z., Rodda, C., Tollefsen, S., Brown, E. M., Yang, J. J., and Thakker, R. V. (2012) Identification of 70 calcium-sensing receptor mutations in hyper- and hypo-calcaemic patients: evidence for clustering of extracellular domain mutations at calcium-binding sites. *Hum Mol Genet* **21**, 2768-2778
10. Pearce, S. H., Williamson, C., Kifor, O., Bai, M., Coulthard, M. G., Davies, M., Lewis-Barned, N., McCredie, D., Powell, H., Kendall-Taylor, P., Brown, E. M., and Thakker, R. V. (1996) A familial syndrome of hypocalcemia with hypercalciuria due to mutations in the calcium-sensing receptor. *N Engl J Med* **335**, 1115-1122
11. Van Raamsdonk, C. D., Griewank, K. G., Crosby, M. B., Garrido, M. C., Vemula, S., Wiesner, T., Obenauf, A. C., Wackernagel, W., Green, G., Bouvier, N., Sozen, M. M., Baimukanova, G., Roy, R., Heguy, A., Dolgalev, I., Khanin, R., Busam, K., Speicher, M. R., O'Brien, J., and Bastian, B. C. (2010) Mutations in GNA11 in uveal melanoma. *N Engl J Med* **363**, 2191-2199
12. Festen-Spanjer, B., Haring, C. M., Koster, J. B., and Mudde, A. H. (2008) Correction of hypercalcaemia by cinacalcet in familial hypocalciuric hypercalcaemia. *Clin Endocrinol (Oxf)* **68**, 324-325
13. Gannon, A. W., Monk, H. M., and Levine, M. A. (2014) Cinacalcet monotherapy in neonatal severe hyperparathyroidism: a case study and review. *J Clin Endocrinol Metab* **99**, 7-11
14. Dong, B., Endo, I., Ohnishi, Y., Kondo, T., Hasegawa, T., Amizuka, N., Kiyonari, H., Shioi, G., Abe, M., Fukumoto, S., and Matsumoto, T. (2015) Calcilytic Ameliorates Abnormalities of Mutant Calcium-Sensing Receptor (CaSR) Knock-in Mice Mimicking Autosomal Dominant Hypocalcemia (ADH). *J Bone Miner Res* **30**, 1980-1993
15. Hannan, F. M., Walls, G. V., Babinsky, V. N., Nesbit, M. A., Kallay, E., Hough, T. A., Fraser, W. D., Cox, R. D., Hu, J., Spiegel, A. M., and Thakker, R. V. (2015) The Calcilytic Agent NPS 2143 Rectifies Hypocalcemia in a Mouse Model With an Activating Calcium-Sensing Receptor (CaSR) Mutation: Relevance to Autosomal Dominant Hypocalcemia Type 1 (ADH1). *Endocrinology* **156**, 3114-3121
16. Nesbit, M. A., Hannan, F. M., Howles, S. A., Reed, A. A., Cranston, T., Thakker, C. E., Gregory, L., Rimmer, A. J., Rust, N., Graham, U., Morrison, P. J., Hunter, S. J., Whyte, M. P., McVean, G., Buck,

- D., and Thakker, R. V. (2013) Mutations in AP2S1 cause familial hypocalciuric hypercalcemia type 3. *Nat Genet* **45**, 93-97
17. Hannan, F. M., Howles, S. A., Rogers, A., Cranston, T., Gorvin, C. M., Babinsky, V. N., Reed, A. A., Thakker, C. E., Bockenhauer, D., Brown, R. S., Connell, J. M., Cook, J., Darzy, K., Ehtisham, S., Graham, U., Hulse, T., Hunter, S. J., Izatt, L., Kumar, D., McKenna, M. J., McKnight, J. A., Morrison, P. J., Mughal, M. Z., O'Halloran, D., Pearce, S. H., Porteous, M. E., Rahman, M., Richardson, T., Robinson, R., Scheers, I., Siddique, H., Van't Hoff, W. G., Wang, T., Whyte, M. P., Nesbit, M. A., and Thakker, R. V. (2015) Adaptor protein-2 sigma subunit mutations causing familial hypocalciuric hypercalcaemia type 3 (FHH3) demonstrate genotype-phenotype correlations, codon bias and dominant-negative effects. *Hum Mol Genet* **24**, 5079-5092
 18. Fang, Y., Huang, C. C., Kain, S. R., and Li, X. (1999) Use of coexpressed enhanced green fluorescent protein as a marker for identifying transfected cells. *Method Enzymol* **302**, 207-212
 19. Leach, K., Wen, A., Davey, A. E., Sexton, P. M., Conigrave, A. D., and Christopoulos, A. (2012) Identification of molecular phenotypes and biased signaling induced by naturally occurring mutations of the human calcium-sensing receptor. *Endocrinology* **153**, 4304-4316
 20. Brown, E. M. (1991) Extracellular Ca²⁺ sensing, regulation of parathyroid cell function, and role of Ca²⁺ and other ions as extracellular (first) messengers. *Physiol Rev* **71**, 371-411
 21. Pearce, S. H., Bai, M., Quinn, S. J., Kifor, O., Brown, E. M., and Thakker, R. V. (1996) Functional characterization of calcium-sensing receptor mutations expressed in human embryonic kidney cells. *J Clin Invest* **98**, 1860-1866
 22. Davey, A. E., Leach, K., Valant, C., Conigrave, A. D., Sexton, P. M., and Christopoulos, A. (2012) Positive and negative allosteric modulators promote biased signaling at the calcium-sensing receptor. *Endocrinology* **153**, 1232-1241
 23. Newey, P. J., Gorvin, C. M., Cleland, S. J., Willberg, C. B., Bridge, M., Azharuddin, M., Drummond, R. S., van der Merwe, P. A., Klenerman, P., Bountra, C., and Thakker, R. V. (2013) Mutant prolactin receptor and familial hyperprolactinemia. *N Engl J Med* **369**, 2012-2020
 24. Miedlich, S. U., Gama, L., Seuwen, K., Wolf, R. M., and Breitwieser, G. E. (2004) Homology modeling of the transmembrane domain of the human calcium sensing receptor and localization of an allosteric binding site. *J Biol Chem* **279**, 7254-7263
 25. Rus, R., Haag, C., Bumke-Vogt, C., Bahr, V., Mayr, B., Mohlig, M., Schulze, E., Frank-Raue, K., Raue, F., and Schofl, C. (2008) Novel inactivating mutations of the calcium-sensing receptor: the calcimimetic NPS R-568 improves signal transduction of mutant receptors. *J Clin Endocrinol Metab* **93**, 4797-4803
 26. Letz, S., Rus, R., Haag, C., Dorr, H. G., Schnabel, D., Mohlig, M., Schulze, E., Frank-Raue, K., Raue, F., Mayr, B., and Schofl, C. (2010) Novel activating mutations of the calcium-sensing receptor: the calcilytic NPS-2143 mitigates excessive signal transduction of mutant receptors. *J Clin Endocrinol Metab* **95**, E229-233
 27. Leach, K., Wen, A., Cook, A. E., Sexton, P. M., Conigrave, A. D., and Christopoulos, A. (2013) Impact of clinically relevant mutations on the pharmacoregulation and signaling bias of the calcium-sensing receptor by positive and negative allosteric modulators. *Endocrinology* **154**, 1105-1116
 28. Rasmussen, S. G., DeVree, B. T., Zou, Y., Kruse, A. C., Chung, K. Y., Kobilka, T. S., Thian, F. S., Chae, P. S., Pardon, E., Calinski, D., Mathiesen, J. M., Shah, S. T., Lyons, J. A., Caffrey, M., Gellman, S. H., Steyaert, J., Skinotitis, G., Weis, W. I., Sunahara, R. K., and Kobilka, B. K. (2011) Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature* **477**, 549-555
 29. Gowen, M., Stroup, G. B., Dodds, R. A., James, I. E., Votta, B. J., Smith, B. R., Bhatnagar, P. K., Lago, A. M., Callahan, J. F., DelMar, E. G., Miller, M. A., Nemeth, E. F., and Fox, J. (2000) Antagonizing the parathyroid calcium receptor stimulates parathyroid hormone secretion and bone formation in osteopenic rats. *J Clin Invest* **105**, 1595-1604
 30. Nemeth, E. F., Heaton, W. H., Miller, M., Fox, J., Balandrin, M. F., Van Wagenen, B. C., Colloton, M., Karbon, W., Scherrer, J., Shatzen, E., Rishton, G., Scully, S., Qi, M., Harris, R., Lacey, D., and

- Martin, D. (2004) Pharmacodynamics of the type II calcimimetic compound cinacalcet HCl. *J Pharmacol Exp Ther* **308**, 627-635
31. Bai, M., Quinn, S., Trivedi, S., Kifor, O., Pearce, S. H., Pollak, M. R., Krapcho, K., Hebert, S. C., and Brown, E. M. (1996) Expression and characterization of inactivating and activating mutations in the human Ca²⁺-sensing receptor. *J Biol Chem* **271**, 19537-19545
 32. Hu, J., McLarnon, S. J., Mora, S., Jiang, J., Thomas, C., Jacobson, K. A., and Spiegel, A. M. (2005) A region in the seven-transmembrane domain of the human Ca²⁺ receptor critical for response to Ca²⁺. *J Biol Chem* **280**, 5113-5120
 33. Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G., and Sprang, S. R. (1994) Structures of active conformations of Gi alpha 1 and the mechanism of GTP hydrolysis. *Science* **265**, 1405-1412
 34. Landis, C. A., Masters, S. B., Spada, A., Pace, A. M., Bourne, H. R., and Vallar, L. (1989) GTPase inhibiting mutations activate the alpha chain of Gs and stimulate adenylyl cyclase in human pituitary tumours. *Nature* **340**, 692-696
 35. Radhika, V., and Dhanasekaran, N. (2001) Transforming G proteins. *Oncogene* **20**, 1607-1614

FOOTNOTES

This work was supported by the United Kingdom Medical Research Council (MRC) programme grants - G9825289 and G1000467 (to M.A.N., F.M.H., C.M.G. and R.V.T), and National Institute for Health Research (NIHR) Oxford Biomedical Research Centre Programme (to M.A.N. and R.V.T.); European Commission Seventh Framework Programme (FP7-264663) (to V.N.B.); S.A.H. is a Wellcome Trust Clinical Training Fellow; and R.V.T. is a Wellcome Trust Investigator and NIHR Senior Investigator.

The abbreviations used are: ADH, autosomal dominant hypocalcemia; ADH1, ADH type 1; ADH2, ADH type 2; AUC, area under the curve; Ca²⁺, calcium; Ca²⁺_i, intracellular calcium; Ca²⁺_o, extracellular calcium; CaSR, calcium-sensing receptor; EC₅₀, half-maximal effective concentration; FBS, fetal bovine serum; FHH, familial hypocalciuric hypercalcemia; FHH1, FHH type 1; FHH2, FHH type 2; FRT, FLP recombination target; Gα₁₁, G-protein alpha-11; Gas, G-protein alpha-s; GPCR, G-protein-coupled-receptor; HBSS, Hank's balanced salt solution; HEK-CaSR, HEK293-CaSR; HEK293-Gα₁₁, HEK-Gα₁₁; Indo-1-AM, indo-1-acetoxymethylester; m, mutant; Mg²⁺, magnesium; PTH, parathyroid hormone.

FIGURE LEGENDS

FIGURE 1. Effect of cinacalcet on the Ca²⁺_i responses of FHH2-associated Gα₁₁ mutations. *A.* Fluorescence microscopy of HEK293 cells stably expressing CaSR (HEK-CaSR) and transiently transfected with WT or FHH2-associated (Gln135 and del199/200) mutant (m) *GNAl1*-pBI-CMV2-GFP constructs, or with vector only. GFP expression in these cells indicates successful transfection and expression by these constructs. Bar indicates 20µm. *B.* Western blot analysis of whole cell lysates using antibodies to CaSR, α-tubulin and Gα₁₁. Transient transfection of WT or FHH2-associated mutant constructs resulted in over-expression of Gα₁₁ when normalised to α-tubulin expression. *C-D.* Ca²⁺_i response to changes in [Ca²⁺]_o of HEK-CaSR cells transfected with WT or FHH2-associated Gα₁₁ mutants. The Ca²⁺_i responses to changes in [Ca²⁺]_o are expressed as a percentage of the maximum normalized responses and shown as the mean ± SEM of 6-16 assays from 2-4 independent transfections. The FHH2-associated Gα₁₁ mutants (Gln135 and del199/200) led to a rightwards shift of the concentration-response curves (blue) with significantly reduced AUC values when compared with WT Gα₁₁ (black), which harbors Leu and Ile residues at codons 135 and 199/200, respectively. *E.* The FHH2-associated Gln135 and del199/200 mutants (blue bars) are associated with significantly increased EC₅₀ values compared to cells expressing WT Gα₁₁ (open bar). The addition of 10 and 20 nM cinacalcet (Cin) decreased the EC₅₀s of cells expressing Gln135 to values that were not significantly different from WT,

whereas 40 nM cinacalcet was required to rectify the increased EC_{50} value of cells expressing the del199/200 mutant. *F-G*. The addition of cinacalcet at 10 and 40 nM concentrations rectified the rightward shift in the concentration-response curves of the Gln135 and del199/200 mutant $G\alpha_{11}$ proteins, respectively. * $p < 0.05$, *** $p < 0.0001$.

FIGURE 2. Effect of NPS-2143 on the Ca^{2+}_i responses of ADH2-associated $G\alpha_{11}$ mutations. *A*. Fluorescence microscopy of HEK293 cells stably expressing CaSR (HEK-CaSR) and transiently transfected with vector, WT or ADH2-associated (Gln181 and Leu341) mutant (m) constructs. GFP expression in these cells indicates successful transfection and expression by these constructs. Bar indicates 20 μ m. *B*. Western blot analysis of whole cell lysates using antibodies to CaSR, α -tubulin and $G\alpha_{11}$. Transient transfection of WT or ADH2-associated mutant constructs resulted in over-expression of $G\alpha_{11}$ when normalised to α -tubulin expression. *C-D*. Ca^{2+}_i response to changes in $[Ca^{2+}]_o$ of HEK-CaSR cells transfected with WT or ADH2-associated $G\alpha_{11}$ mutants. The Ca^{2+}_i responses to changes in $[Ca^{2+}]_o$ are expressed as a percentage of the maximum normalized responses and shown as the mean \pm SEM of 6-16 assays from 2-4 independent transfections. The ADH2-associated $G\alpha_{11}$ mutants (Gln181 and Leu341) led to a leftwards shift of the concentration-response curves (red) with significantly increased AUC values when compared with WT $G\alpha_{11}$ (black), which harbors Arg and Phe residues at codons 181 and 341, respectively. *E*. The ADH2-associated Gln181 and Leu341 mutants (red bars) are associated with significantly reduced EC_{50} values compared to cells expressing WT $G\alpha_{11}$ (open bars). The addition of 10 nM NPS-2143 (2143) increased the EC_{50} value of cells expressing Gln135 so that this was not significantly different from WT, whereas 30 nM of NPS-2143 was required to rectify the reduced EC_{50} value of cells expressing the Leu341 mutant. *F-G*. The addition of NPS-2143 at 10 and 30 nM concentrations rectified the leftward shift in the concentration-response curves of the Gln181 and Leu341 mutant $G\alpha_{11}$ proteins, respectively. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

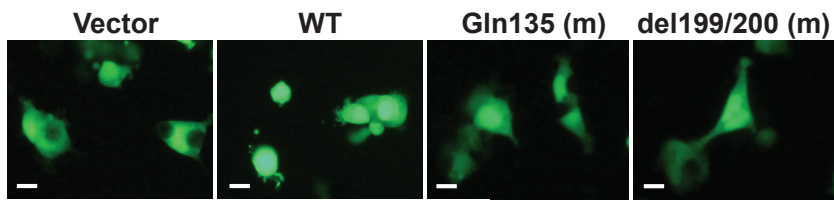
FIGURE 3. Effect of cinacalcet and NPS-2143 on the Ca^{2+}_i responses of FHH2- and ADH2-associated $G\alpha_{11}$ mutations following siRNA knockdown of endogenously expressed WT $G\alpha_{11}$. *A*. Western blot analysis of untransfected HEK293 cells, which express endogenous WT $G\alpha_{11}$ only, and have been treated with either scrambled siRNA, or three different *GN11*-targeted siRNAs (siRNAs 1-3), either alone or all together (siRNAs 1+2+3), and compared to untreated HEK293 cells. All three *GN11*-targeted siRNAs, but not scrambled siRNA, reduced endogenous WT $G\alpha_{11}$ expression. *B*. Western blot analysis showing combined effects of the three *GN11*-targeted siRNAs on $G\alpha_{11}$ protein expression in HEK293 cells transiently expressing WT $G\alpha_{11}$ proteins and in HEK- $G\alpha_{11}$ cells, which stably express WT or mutant $G\alpha_{11}$ proteins. Use of *GN11*-targeted siRNAs reduced $G\alpha_{11}$ expression in HEK293 cells, but not in HEK- $G\alpha_{11}$ cells, which are resistant to *GN11*-targeted siRNA. *C*. Fluorescence microscopy confirming transfection of untreated and siRNA-treated (scrambled or combined siRNAs 1+2+3) HEK- $G\alpha_{11}$ cells with the pEGFP-CaSR construct. Bar indicates 50 μ m. *D-E*. Ca^{2+}_i response to changes in $[Ca^{2+}]_o$ of FHH2-associated mutant Gln135 HEK- $G\alpha_{11}$ cells following siRNA knockdown of endogenous WT $G\alpha_{11}$. The Ca^{2+}_i responses to changes in $[Ca^{2+}]_o$ are expressed as a percentage of the maximum normalized responses and shown as the mean \pm SEM of 4-5 independent transfections (i.e. biological replicates). The FHH2-associated $G\alpha_{11}$ mutant (Gln135) led to a rightward shift of the concentration-response curve (blue), with a significant increase in EC_{50} value compared to WT $G\alpha_{11}$ (black). The addition of 10 nM cinacalcet (Cin) normalized the EC_{50} values of cells in the presence of scrambled (grey) or *GN11*-targeted siRNAs (siRNAs 1+2+3) (red). *F-G*. Ca^{2+}_i response to changes in $[Ca^{2+}]_o$ of ADH2-associated mutant Gln181 HEK- $G\alpha_{11}$ cells following siRNA knockdown of endogenous WT $G\alpha_{11}$. The ADH2-associated $G\alpha_{11}$ mutant (Gln181) led to a leftward shift of the concentration-response curve (blue), with a significant decrease in EC_{50} value compared to WT $G\alpha_{11}$ (black). The addition of 10 nM NPS-2143 (2143) normalized the EC_{50} value of cells in the presence of scrambled (grey) or *GN11*-targeted siRNAs (siRNAs 1+2+3) (red). *** $p < 0.0001$; -, nil; scram, scrambled.

FIGURE 4. Phospho-ERK responses of $G\alpha_{11}$ mutations associated with ADH2 or uveal melanoma.

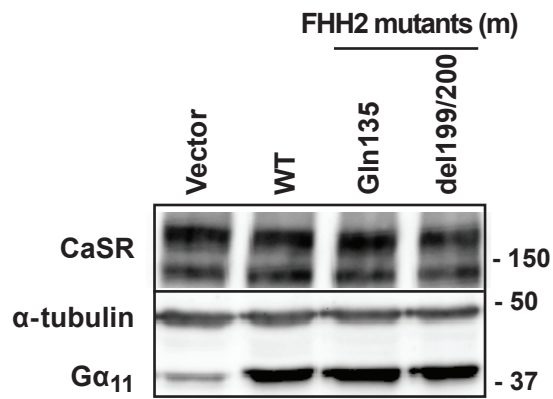
A. The phospho-ERK response to changes in $[Ca^{2+}]_o$ was measured by quantitative immunoassay (Alphascreen) in HEK-CaSR cells transiently transfected with WT or ADH2-associated $G\alpha_{11}$ mutants (Gln181 and Leu341), or the uveal melanoma (UV)-associated Leu209 $G\alpha_{11}$ mutant protein. Phospho-ERK responses at each $[Ca^{2+}]_o$ are expressed as a fold-change of the response of cells stimulated with basal (0.5 mM) $[Ca^{2+}]_o$, and are shown as the mean \pm SEM of 9-24 assays from 3-8 independent transfections. The uveal melanoma-associated Leu209 $G\alpha_{11}$ mutant is associated with significantly increased maximal phospho-ERK fold-change responses compared to WT (Qln209) and the ADH2 mutant $G\alpha_{11}$ proteins. The Gln181 and Leu341 mutants also induce significant increases in maximal phospho-ERK fold-change responses compared to WT $G\alpha_{11}$, which harbors Arg and Phe residues at codons 181 and 341, respectively. *B.* Quantification of the basal phospho-ERK responses shown in *A.* Values are expressed as a percentage of the WT basal phospho-ERK response. The uveal melanoma-associated Leu209 $G\alpha_{11}$ mutant induces a significant phospho-ERK elevation when exposed to basal 0.5 mM $[Ca^{2+}]_o$, whereas the basal phospho-ERK responses of the ADH2-associated $G\alpha_{11}$ mutants are not significantly different compared to WT $G\alpha_{11}$. *C-E.* The addition of NPS-2143 (2143) at 10, 30 and 500 nM concentrations significantly decreased the phospho-ERK responses of the ADH2-associated Gln181 and Leu341 $G\alpha_{11}$ mutants, and the UV-associated Leu209 mutant $G\alpha_{11}$ protein, respectively, to values that were not significantly different from WT $G\alpha_{11}$. *** $p < 0.0001$.

Figure 1

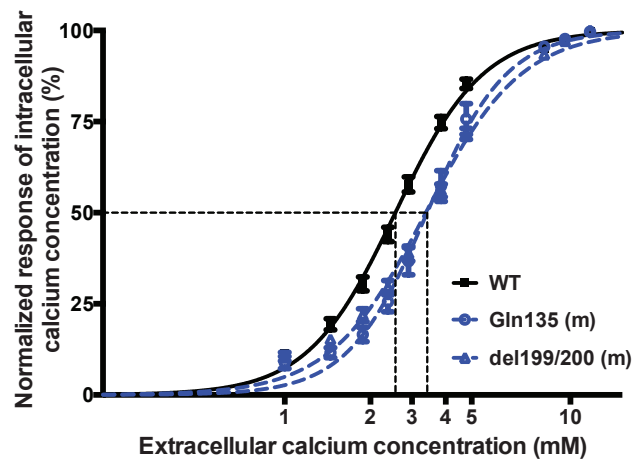
A



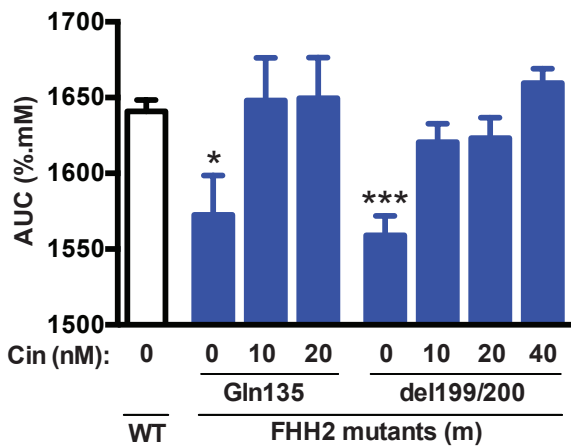
B



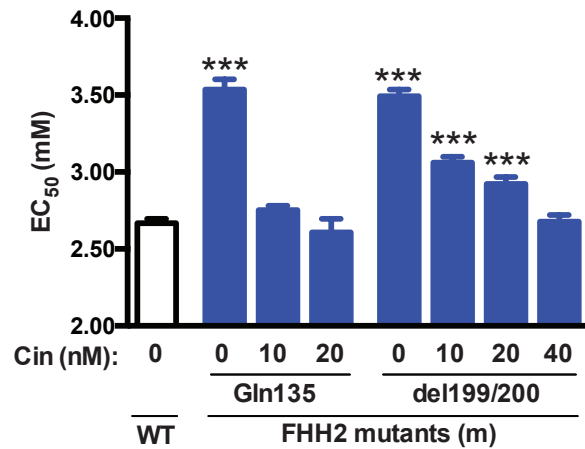
C



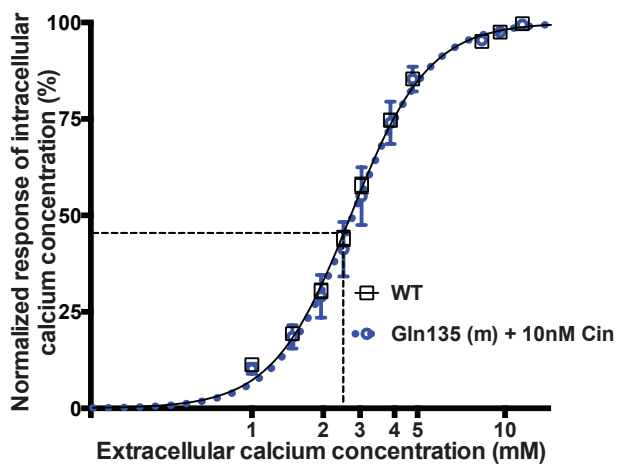
D



E



F



G

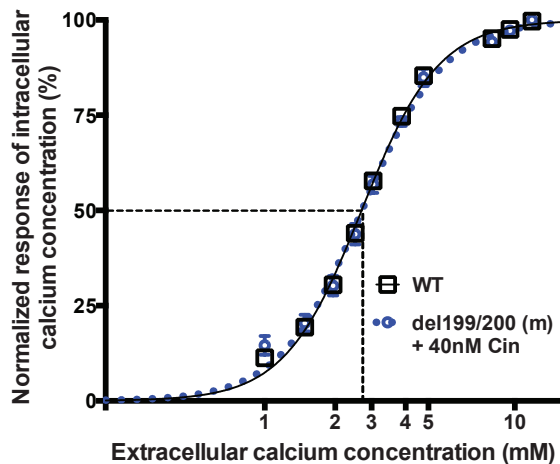


Figure 2

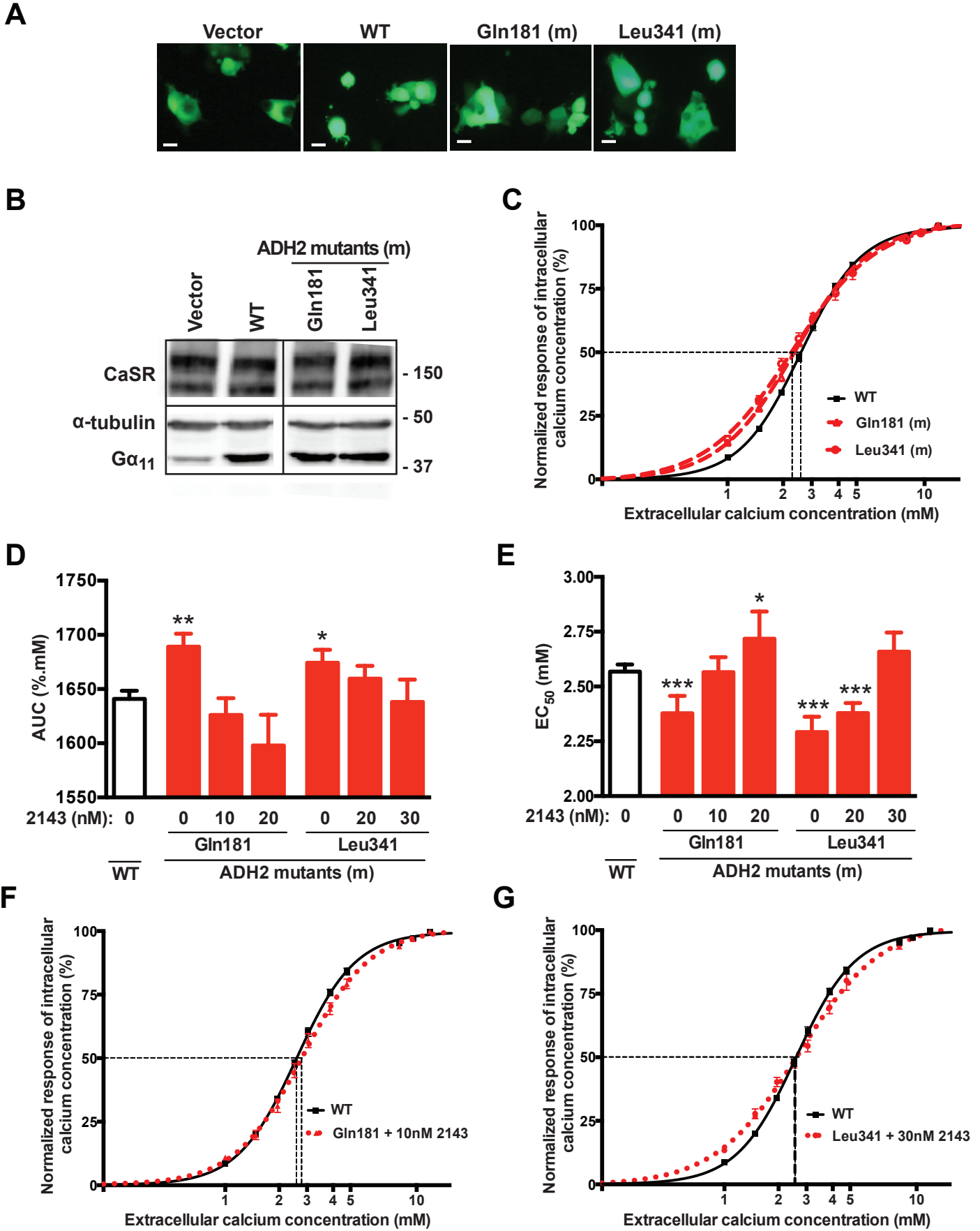
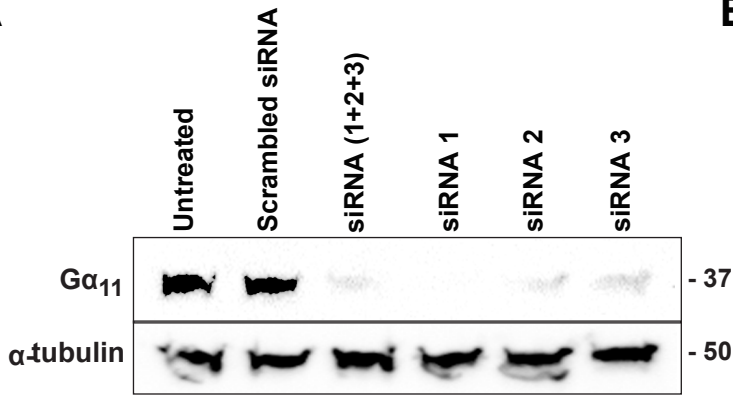
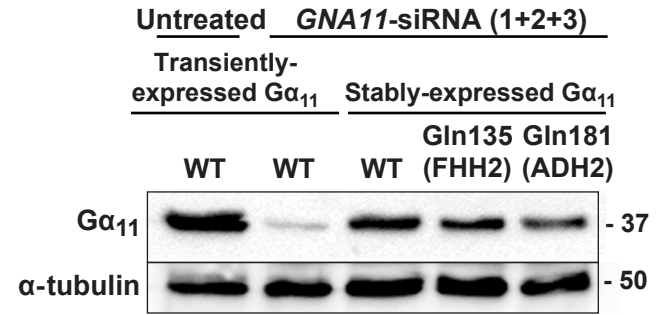


Figure 3

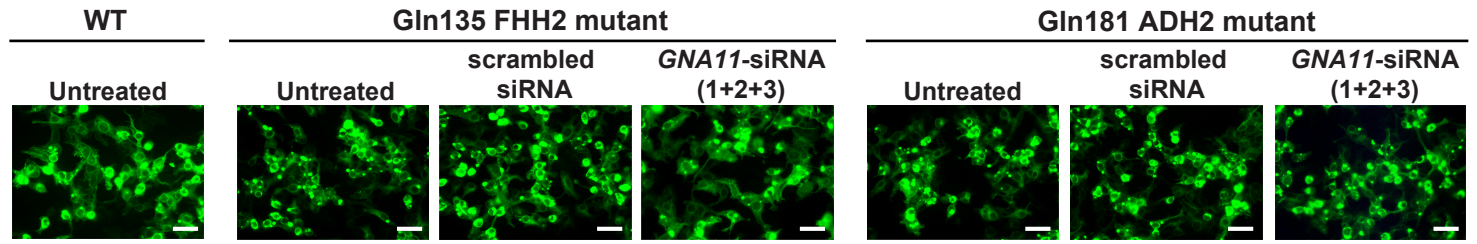
A



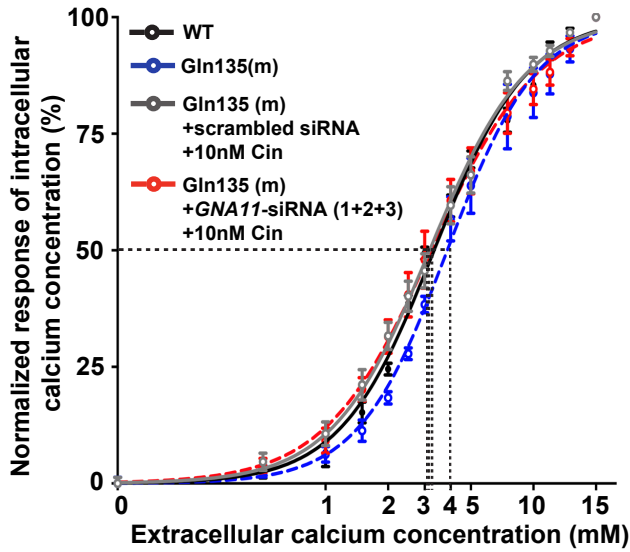
B



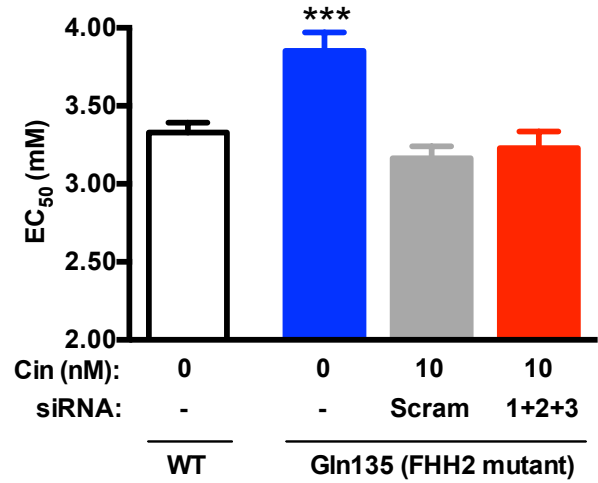
C



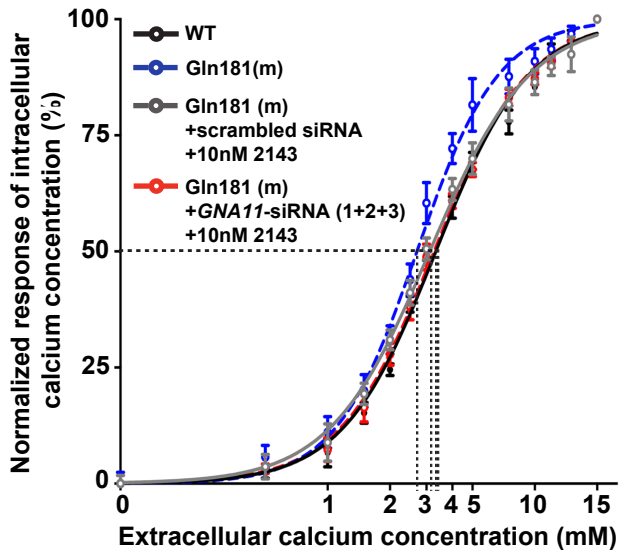
D



E



F



G

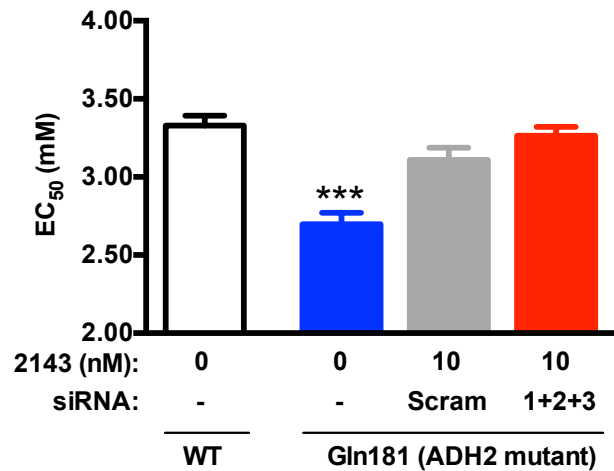
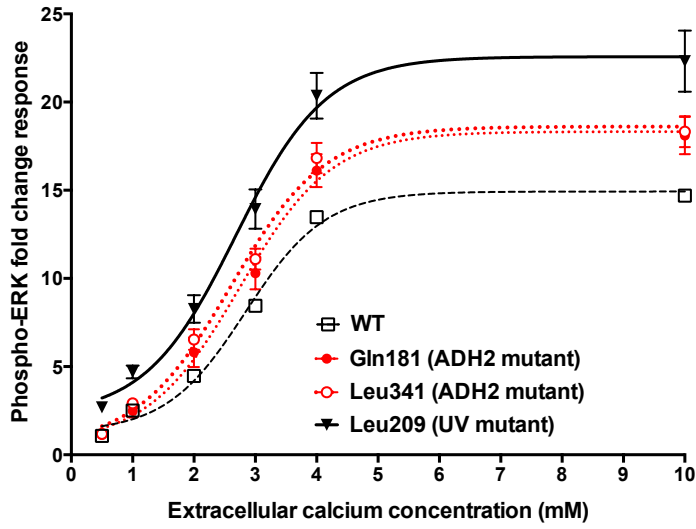
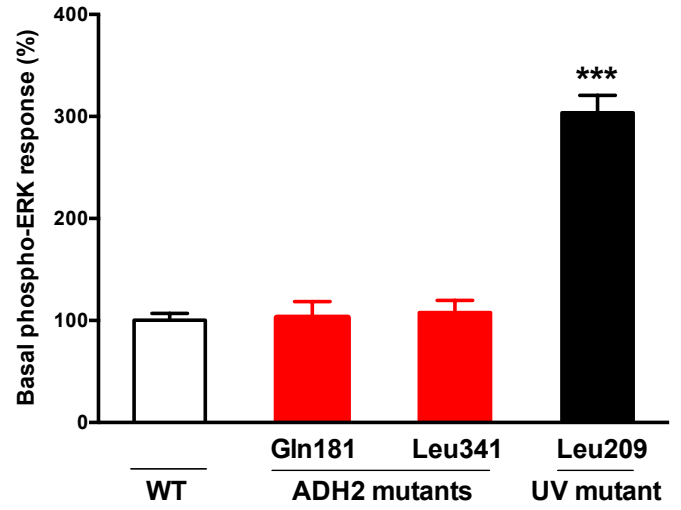


Figure 4

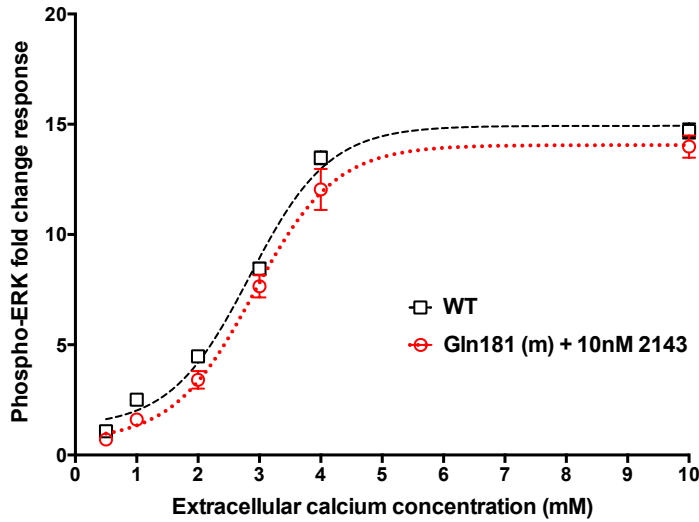
A



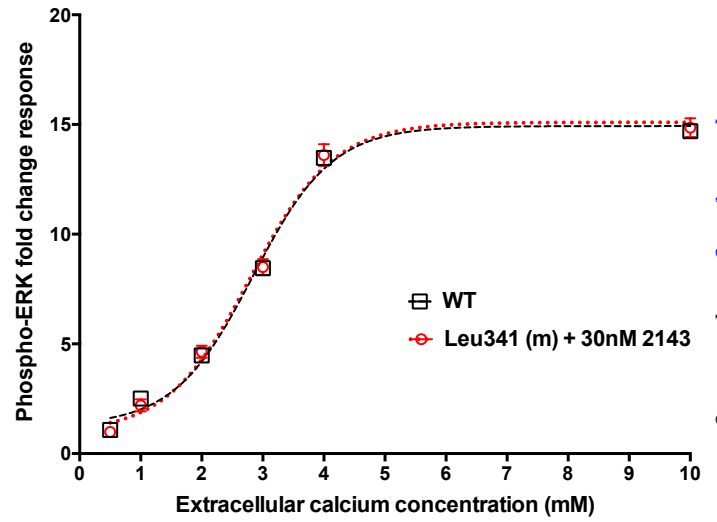
B



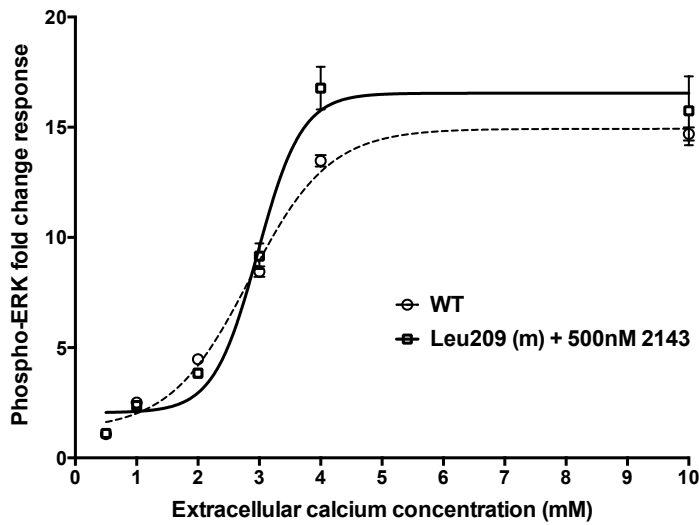
C



D



E



Allosteric Modulation of the Calcium-Sensing Receptor Rectifies Signaling Abnormalities Associated with G-protein alpha-11 Mutations causing Hypercalcemic and Hypocalcemic Disorders

Valerie N. Babinsky, Fadil M. Hannan, Caroline M. Gorvin, Sarah A. Howles, M. Andrew Nesbit, Nigel Rust, Aylin C. Hanyaloglu, Jianxin Hu, Allen M. Spiegel and Rajesh V. Thakker

J. Biol. Chem. published online March 18, 2016

Access the most updated version of this article at doi: [10.1074/jbc.M115.696401](https://doi.org/10.1074/jbc.M115.696401)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at <http://www.jbc.org/content/early/2016/03/18/jbc.M115.696401.full.html#ref-list-1>