# Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Howles SA, Hannan FM, Thakker RV, et al. Cinacalcet for symptomatic hypercalcemia caused by *AP2S1* mutations. N Engl J Med 2016;374:1396-8. DOI: 10.1056/NEJMc1511646

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## CASE REPORTS

Proband 1, a 34-year old woman had a 12-year history of fatigue, recurrent headaches and persistent generalised aches. She was hypercalcemic (serum ionized calcium concentrations 1.53-1.72 mmol/liter) in association with normal serum PTH concentrations and suppressed urinary calcium excretion. Her three children were hypercalcemic, but both her parents were normocalcemic. Surgical neck exploration was undertaken and an enlarged non-adenomatous parathyroid gland excised. Post-operatively, serum ionized calcium concentrations decreased from 1.53 to 1.41 mmol/liter, but then increased over a 2-month period to values that remained at >1.50 mmol/liter. Proband 2, a 22-year old man presented with fatigue and generalised rib pains. He was hypercalcemic with borderline elevation of serum PTH and suppressed urinary calcium excretion. His father and one of his three siblings were also hypercalcemic. Proband 3, a 52-year old woman had a ~20 year history of recurrent headaches, abdominal pain, vomiting, lethargy, and musculoskeletal pain. She was hypercalcemic with normal serum PTH concentrations and suppressed urinary calcium excretion. Her serum calcium concentrations, adjusted for albumin, ranged from 3.00-3.20 mmol/liter, and treatment with a pamidronate infusion, temporarily reduced the serum calcium concentration to 2.80 mmol/liter, but 4 months later, the serum calcium had increased to >3.10 mmol/liter, and she underwent a parathyroidectomy with excision of an enlarged parathyroid gland, which failed to rectify her hypercalcemia. None of the probands were taking medications that are associated with hypercalcemia. The probands provided informed consent for the studies and for the use of cinacalcet as an experimental therapy.

**Mutational Analysis**

DNA sequence analysis of the *CASR*, *GNA11* and *AP2S1* genes demonstrated probands 1, 2, and 3 to harbor heterozygous *AP2S1* mutations comprising CGC>TGC (Arg15Cys), CGC>CAC (Arg15His) and CGC>CTC (Arg15Leu), respectively, consistent with a diagnosis of familial hypocalciuric hypercalcemia type 3 (FHH3). Sequence abnormalities involving the *CASR* and *GNA11* genes in these three hypercalcemic probands were not detected.

## METHODS

### Measurement of intracellular calcium responses

Previously reported HEK293 cell lines that stably express the calcium-sensing receptor (CaSR) (HEK-CaSR), [1,2] were cultured in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 400 μg/ml geneticin. CaSR expression was confirmed by Western blot analysis using a mouse monoclonal antibody to human CaSR (ADD; Abcam).[1,2] An *AP2S1* expression construct was generated by subcloning the full-length non-mutant and mutant *AP2S1* coding regions from the previously reported pBI-CMV2-GFP vector (Clontech)[1,2] into a bi-directional cloning vector, pBI-CMV4 (Clontech), which allows co-expression of adaptor protein-2 sigma (AP2-sigma) and a red-fluorescent protein (RFP) reporter gene at equivalent levels to aid selection of transfected cells.[3,4] Use of RFP minimized overlap between the emission spectra of this reporter gene and the indo-1-AM $Ca^{2+}$-binding fluorophore, which is further described below. Expression of RFP was used as a surrogate for AP2-sigma expression.[4] Western blot analysis of cellular protein extract was undertaken using a rabbit polyclonal antibody to RFP (Thermo Scientific, PA1-986, 1:500). The membrane was re-probed with mouse anti-GAPDH antibody (Abcam, ab8245) as a loading control. Successful transfection was also confirmed by visualising RFP fluorescence using an Eclipse E400 fluorescence microscope with an epifluorescence filter, and images were captured using a DXM1200C digital camera and NIS Elements software.[1,2] The non-mutant and mutant *AP2S1* constructs were transiently transfected into HEK-CaSR cells using Lipofectamine 2000 (Invitrogen). HEK293 cells were utilised, as suitable parathyroid and renal thick ascending limb cells are not available, and HEK293 cells represent an established model for assessing the effects of mutant AP2-sigma proteins on CaSR signal transduction.[1,5]

The effects of cinacalcet on HEK-CaSR cells transfected with non-mutant and mutant AP2-sigma proteins, were assessed by an assay that measured the alterations in intracellular calcium concentrations ($[Ca^{2+}]_i$) in response to changes in extracellular calcium concentrations ($[Ca^{2+}]_o$), as previously reported.[1,2,4,5] Briefly, 48 hours following transfection, the cells were harvested, washed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) (Invitrogen) and loaded with 1 μg/ml indo-1-acetoxymethylester (Indo-1-AM) (Molecular Probes) for 1 hour at 37°C. After removal of free dye, the cells were resuspended in calcium- and magnesium-free HBSS that contained 0, 10, 20 or 30 nM of cinacalcet, and maintained at 37°C for 1 hour prior to being stimulated by the addition of $Ca^{2+}_o$ to increase the $[Ca^{2+}]_o$ in a stepwise manner from 0-10 mM, with the cells being exposed to each $[Ca^{2+}]_o$ for 2 min. Cells were analysed by flow cytometry (Beckman Coulter MoFlo XDP equipped with JDSUY Xcyte UV Laser and a Coherent Sapphire 488 Laser) and live, single cells selected on the basis of morphology using forward scatter and side scatter readings. The data on the $[Ca^{2+}]_i$ responses to alterations in $[Ca^{2+}]_o$ were collected from all cells that expressed RFP, by simultaneous flow cytometry fluorescence measurements of RFP (using a 580/30 Bandpass filter and 550 LP Dichroic mirror), $Ca_i^{2+}$-bound Indo-1AM (at 410 nm), and free Indo-1AM (i.e. not bound to $Ca_i^{2+}$) (at 485 nm), as described.[1,2,4,5] To identify cells that were successfully transfected with the RFP-expressing vector, live, single cells were gated by measurement of pulse-width and forward/side scatter and also by measurement of RFP fluorescence. The baseline indo-1AM fluorescence ratio was measured for 2 min, the fluorescence ratio vs time recorded, and data collected for 2 min at each $[Ca^{2+}]_o$. Cytomation summit software was used to determine the peak mean fluorescence ratio of the transient response after each individual stimulus expressed as a normalized response, as previously reported.[1,2,4,5] Nonlinear regression of concentration-response curves was performed with GraphPad Prism (GraphPad, San Diego, CA) using the normalized response

at each of 9 different $[Ca^{2+}]_o$ for each separate experiment (N=8-17) for the determination of the $EC_{50}$ (i.e., $[Ca^{2+}]_o$ required for 50% of the maximal response).

**Measurement of serum response element luciferase reporter activity**

Activation of serum response element (SRE), which represents a downstream target of the extracellular signal-regulated kinase 1/2 (ERK1/2)/MAP kinase pathway and has been previously used to functionally characterize CaSR signal transduction,[6] was assessed using a luciferase reporter assay, as described.[7] Luciferase assays were performed in HEK293 cell-lines stably expressing a plasmid containing either full-length nonmutant (Arg15) or mutant (Cys15, His15 or Leu15) *AP2S1* cDNA. Cells were cultured in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum and 400μg/ml geneticin. Cells were transiently co-transfected in 24-well plates with 200ng of the pGL4.33-Serum Response Element (SRE) reporter gene construct (Promega), 10ng pRL (renilla) control vector, and 200ng of a nonmutant *CASR* vector, using Lipofectamine 2000 (Invitrogen). Thirty-six hours post-transfection cells were incubated in serum-free media overnight. For each cell-line, four biological replicates were treated with 0.5mM $[Ca^{2+}]_o$ and four biological replicates treated with 10mM $[Ca^{2+}]_o$ in the presence of either 0 or 10 nM cinacalcet, and incubated at 37°C for 6 hours. Cells were lysed and assayed for luciferase activity using a Turner Biosystems luminometer and the Dual Luciferase Reporter assay system (Promega), as described.[7] Firefly luciferase activity was adjusted for Renilla luciferase activity (Firefly/Renilla ratio), and the luciferase activity at each $[Ca^{2+}]_o$ was expressed as a fold-change of the luciferase activity at basal (0.5mM) $[Ca^{2+}]_o$, and normalized to that of untreated nonmutant AP2-sigma-expressing cells.

**Statistical analysis**

The $Ca^{2+}_{i}$ and SRE responses of cells expressing nonmutant, mutant and cinacalcet-treated mutant AP2-sigma proteins were compared using the F-test and one-way ANOVA, respectively.[1,4,7] All analyses were undertaken using GraphPad Prism (GraphPad), and are presented as mean ± SE. A value of $P<0.05$ was considered significant for all analyses.

**FIGURES**

## Figure S1

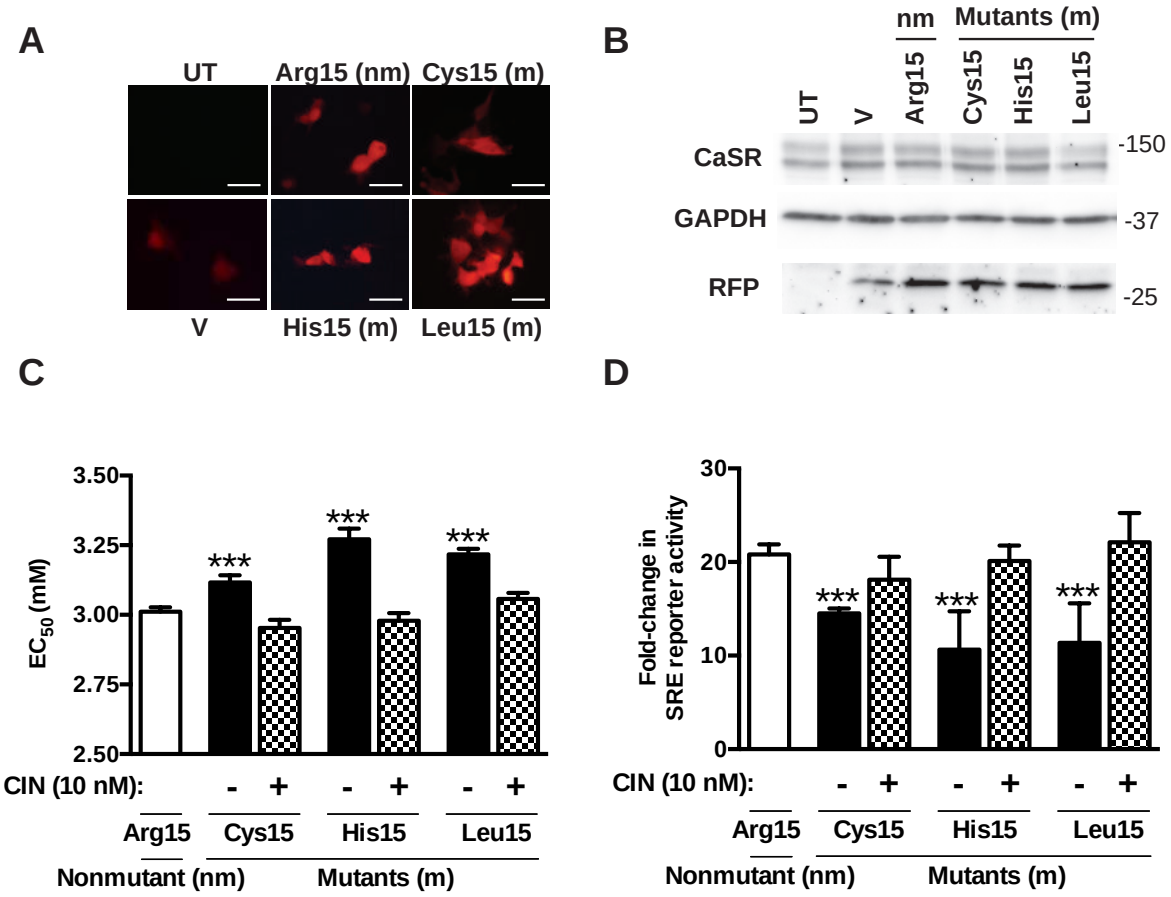


**Figure S1. Use of cinacalcet to rectify impaired calcium-sensing receptor signal transduction associated with *AP2S1* mutations.**

Panel A shows fluorescence microscopy of HEK293 cells expressing CaSR and transfected with vector (V), nonmutant (nm) Arg15 or FHH3-associated mutants (m) Cys15, His15 and Leu15. RFP expression in these cells indicates successful transfection and expression by these constructs. UT, untransfected cells. Bar indicates 20μm. Panel B shows Western blot analysis using anti-CaSR, anti-GAPDH and anti-RFP antibodies. The nonmutant and FHH3-mutant AP2-sigma proteins were expressed at similar levels. Panels C and D show the effects of 10 nM cinacalcet (CIN) on the half-maximal ($EC_{50}$) intracellular calcium responses and serum response element (SRE) luciferase reporter activity, respectively, of HEK293 cells expressing CaSRs, and transfected with nonmutant or mutant *AP2S1* constructs. The FHH3-

associated *AP2S1* mutants, which result in mutant AP2-sigma proteins harboring Cys15, His15 and Leu15, were all associated with significantly increased $EC_{50}$ values and reduced SRE reporter activity, when compared to the nonmutant AP2-sigma protein that has Arg15. Cinacalcet (10 nM) decreased $EC_{50}$ values and increased SRE reporter activity of cells expressing mutant Cys15, His15 or Leu15 AP2-sigma proteins to values that were not significantly different from cells expressing the nonmutant, Arg15, AP2-sigma protein. Results are shown as mean (±SE) of 4-17 assays from 3 or more independent transfections. SRE reporter activity is shown as a fold-change of responses to alterations in extracellular calcium from 0.5 mM (basal) to 10.0 mM (stimulated). ***P<0.001 compared to cells expressing nonmutant *AP2S1*.

## Figure S2

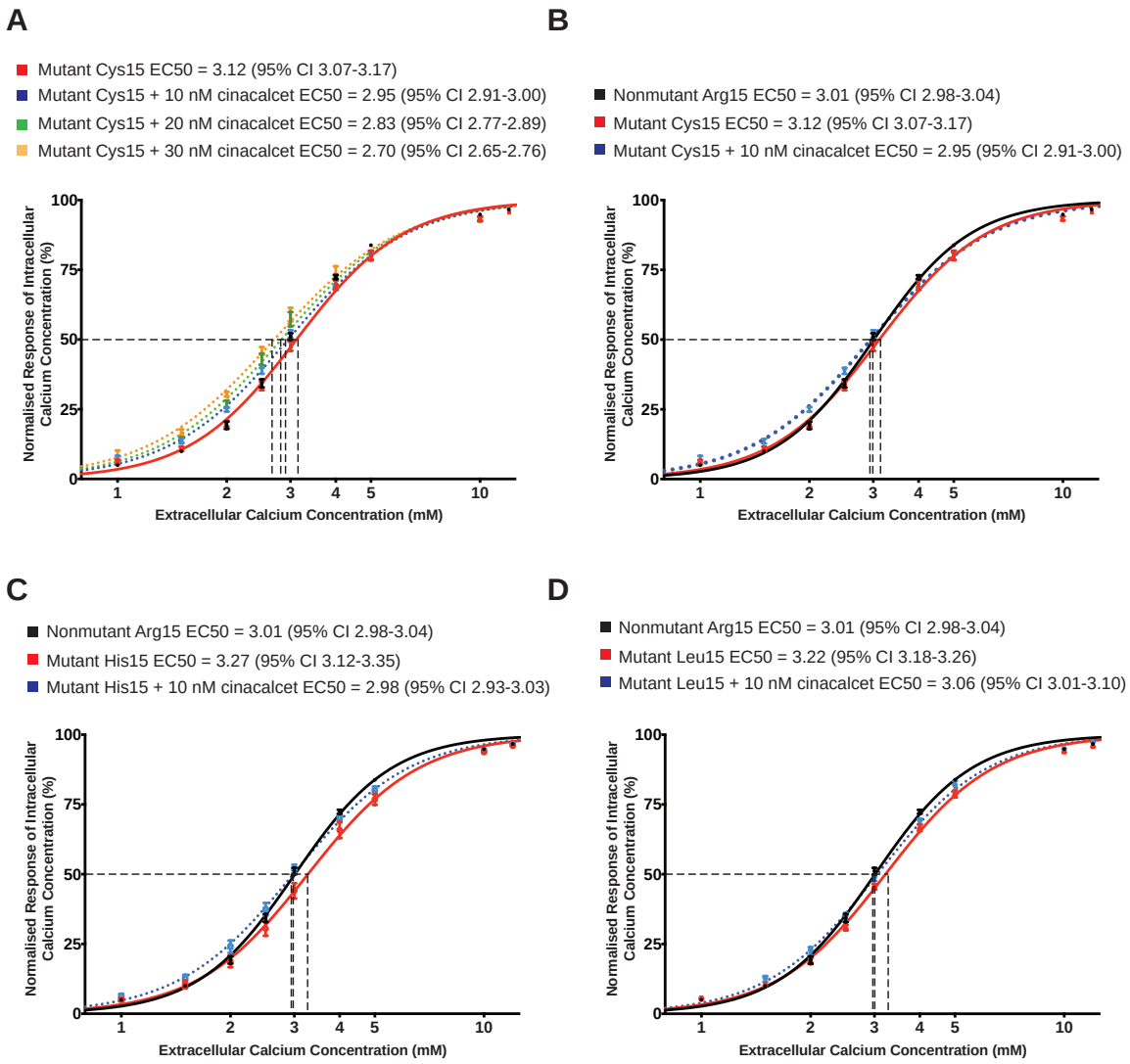


**Figure S2. Effect of cinacalcet on the intracellular calcium responses of HEK-CaSR cells transfected with FHH3-associated *AP2S1* mutants.**

Panel A shows the intracellular calcium ($Ca^{2+}_i$) response to changes in extracellular calcium ($Ca^{2+}_o$) concentrations following a dose-titration of cinacalcet in HEK-CaSR cells transiently transfected with the mutant Cys15 adaptor protein-2 sigma subunit (AP2-sigma) protein. Cinacalcet at concentrations ranging from 10 to 30 nM induced a progressive leftward shift of the mutant Cys15 concentration-response curve. Panels B-D show the $Ca^{2+}_i$ response to changes in $Ca^{2+}_o$ concentrations in HEK-CaSR cells transiently transfected with either nonmutant Arg15 or mutant Cys15, His15 or Leu15 AP2-sigma proteins. The FHH3-associated Cys15, His15 and Leu15 mutants led to a signficant ($P<0.001$) rightward shift of

the concentration-response curves. The addition of 10 nM cinacalcet to cells expressing the Cys15, His15 or Leu15 AP2-sigma proteins rectified the shift in their concentration-response curves. The $Ca^{2+}_i$ responses are expressed as a percentage of the maximum normalized response, and shown as the mean (±SE) of 8-17 assays from 3 independent transfections.

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