

Mutations in *MRAP*, encoding a new interacting partner of the ACTH receptor, cause familial glucocorticoid deficiency type 2

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Familial glucocorticoid deficiency (FGD), or hereditary unresponsiveness to adrenocorticotropin (ACTH; OMIM 202200), is an autosomal recessive disorder resulting from resistance to the action of ACTH on the adrenal cortex, which stimulates glucocorticoid production. Affected individuals are deficient in cortisol and, if untreated, are likely to succumb to hypoglycemia or overwhelming infection in infancy or childhood^{1–3}. Mutations of the ACTH receptor (melanocortin 2 receptor, MC2R) account for ~25% of cases of FGD^{4–7}. FGD without mutations of MC2R is called FGD type 2. Using SNP array genotyping, we mapped a locus involved in FGD type 2 to chromosome 21q22.1. We identified mutations in a gene encoding a 19-kDa single-transmembrane domain protein⁸, now known as melanocortin 2 receptor accessory protein (MRAP). We show that MRAP interacts with MC2R and may have a role in the trafficking of MC2R from the endoplasmic reticulum to the cell surface.

Several consanguineous families including one or more members with FGD who have no mutations in *MC2R* have been identified. Their disease does not segregate with either *MC2R* or a second locus at 8q12.1–21.2, which was identified to be involved with FGD in a single family⁹. In one such family, we carried out a whole-genome scan by microarray analysis of SNPs using the GeneChip Human Mapping 10K Array from Affymetrix with genomic DNA from the parents, affected children and unaffected siblings¹⁰. Data analysis identified a single candidate region in family 1 at 21q22.1 extending over a 2.75-Mbp region between rs723469 and rs743337, which gave a maximum lod score of 2.64 (Fig. 1a). Genotyping of microsatellite markers *D21S223* and *D21S63* confirmed homozygosity in this region and of markers *D21S1909* and *D21S1898* reduced the size of the region

to 2.2 Mbp in this family and in another consanguineous family (family 2) with a single affected individual.

Thirty known or predicted genes have been localized to this interval, but none is a candidate for involvement with FGD on the basis of its probable function (Fig. 1b and Supplementary Table 1 online). In view of the highly adrenal-specific nature of the disease, we reasoned that the expression of the underlying gene would probably be restricted to a few tissues, including the adrenal cortex. Therefore, we designed intron-spanning RT-PCR primer pairs for all 30 genes and amplified them from cDNA from human adrenal cortex, liver and brain. Most genes were expressed in two or more of these sites (Supplementary Fig. 1 online), but one was expressed only in adrenal cortex and not in liver or brain (Fig. 1c). This gene was known as chromosome 21 open reading frame 61 (*C21orf61*) and described to encode fat tissue-specific low molecular weight protein⁸; it has now been renamed melanocortin 2 receptor accessory protein (*MRAP*).

MRAP consists of six exons; either exon 5 or exon 6 is alternatively spliced to encode the isoforms *MRAP-α* or *MRAP-β*⁸ (Fig. 1d). We screened the entire exon sequence and the relevant flanking intronic regions by DNA sequencing in the two families. Sequence analysis identified the homozygous mutation IVS3ds+1G→T in the proband and affected siblings in family 1 and the mutation IVS3ds+1G→C in the proband of family 2. We screened the probands from another 104 families with confirmed FGD and no mutations in *MC2R* for mutations in *MRAP*. We identified one unrelated individual carrying the mutation IVS3ds+1G→C mutation, one individual carrying the mutation IVS3ds+1G→A, six individuals from five families carrying the mutation IVS3ds+1delG, four individuals from three families carrying the mutation IVS3ds+3insT, nine individuals from eight families carrying the missense mutation M1I and one individual carrying the nonsense mutation V44X (the only mutation located in exon 4; Fig. 2). These homozygous changes cosegregated

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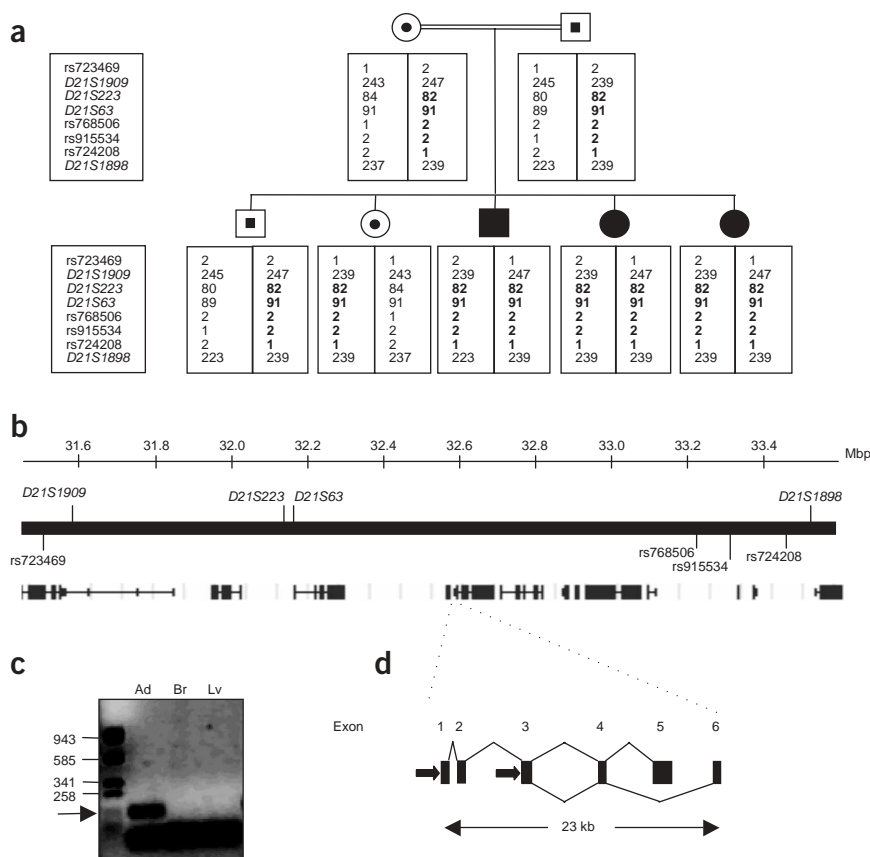


Figure 1 Identification of *MRAP*. **(a)** The pedigree of family 1. Affected individuals are indicated by filled symbols. The haplotype at the identified locus, combining both SNP markers (rs numbers) and microsatellites (D21S numbers; shown beneath each symbol), shows the region of homozygosity. The founder disease haplotype is shown in bold. **(b)** Map of the minimal region on chromosome 21q22 showing positions of SNP and microsatellite markers used in the analysis and the locations of known and predicted genes in the region. **(c)** RT-PCR analysis of *MRAP* after agarose gel electrophoresis showing the presence of a band of the predicted size in cDNA from human adrenal (Ad; arrows) but no signal from human brain (Br) or liver (Lv). Size markers (bp) are shown on left side of gel. **(d)** Diagram of *MRAP* showing the six exons that extend over a distance of 23 kb and the two major splicing variations that encode *MRAP-α* and *MRAP-β*.

with the disease in all affected families but were not found in 120 control chromosomes.

Several of these mutations disrupt or substitute essential residues at the intron 3 donor splice site and are likely to result in a transcript with a foreshortened open reading frame encoding a prematurely terminated translation product. Attempts to confirm this consequence in peripheral blood leukocyte mRNA from affected individuals have been unsuccessful owing to low abundance of *MRAP* mRNA in this tissue. Insertion of the IVS3ds+1G→C, IVS3ds+1G→T and IVSds+3insT mutated exon and flanking intronic sequences into the intron of a well-characterized splicing reporter derived from the adenovirus major late first and second leader exons^{11,12} showed that these mutations resulted in exon skipping in an *in vitro* splicing assay, in contrast to the wild-type exon or exon resulting in the MII mutant (**Supplementary Fig. 2** online).

Little is known of the functional role of *MRAP*. It was originally identified as a protein that was upregulated on differentiation of mouse 3T3-L1 cells from fibroblasts into adipocytes⁸. This is notable because *Mc2r* is also upregulated in this cell line during differentiation^{13,14} and is known to be ACTH-responsive in the differentiated state¹⁵. Alternative splicing of human *MRAP* results in two transcripts: *MRAP-α*, derived from exons 1–5 and encoding a protein of 172 residues and predicted molecular weight of 19 kDa, and *MRAP-β*, derived from exons 1–4 and 6 and encoding a protein of 102 residues and predicted molecular weight of 14.1 kDa. These isoforms share a highly conserved N-terminal and predicted transmembrane domain between residues 38 and 58 but are thereafter highly divergent (**Supplementary Fig. 3** online). There is no secretory signal sequence. *MRAP* is similar to another gene (*C6orf117*) that encodes a small single transmembrane-domain protein that is more

than 36% identical to *MRAP* in the N-terminal and transmembrane domains but differs at the C terminus. Membrane prediction tools (TMPRED and TMHMM) suggest that the N terminus of these proteins probably lies on the intracellular cytoplasmic surface of the membrane.

Using RT-PCR, we examined *MRAP-α* and *MRAP-β* mRNA expression in multiple human tissues. Expression was most readily detectable in adrenal cortex, testis, breast, thyroid, lymph node, ovary and fat (**Fig. 3**). *Mrp* is also expressed by mouse Y1 and Y6 cells (data not shown).

It has been very difficult to obtain transfection-mediated functional expression of heterologous *MC2R*. This can only be achieved in a limited number of cell types, notably Y6 and OS3 cells, which are of adrenocortical origin^{16,17}. For example, when mouse *Mc2r*-GFP is expressed in CHO-K1 cells, the protein is retained within the cell and is not processed to the plasma membrane. In Y6 cells, however, it is localized to the plasma membrane and forms a functional signal

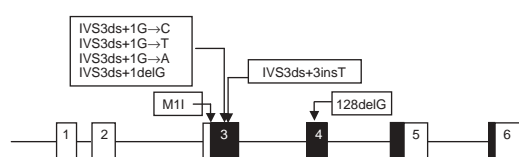


Figure 2 Mutations of *MRAP* in individuals with FGD type 2. Gene structure indicating the locations of mutations identified in 26 affected individuals from 21 families. The location of the single open reading frame encoding *MRAP* is shown by the filled boxes. Exons 5 and 6 are alternatively spliced to generate the *MRAP-α* and *MRAP-β* isoforms, respectively.

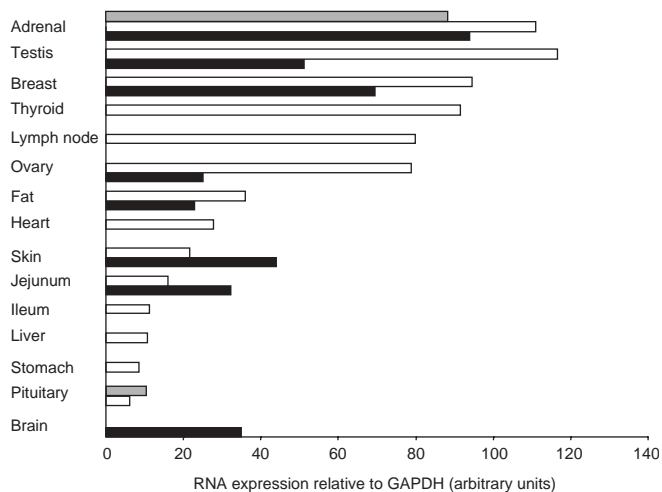


Figure 3 Relative expression of MRAP- α (white bars), MRAP- β (black bars) and *MC2R* (gray bars) in human tissues analyzed by RT-PCR. Minimal expression was identified in other tissues examined, including bladder, colon, duodenum, fallopian tube, gall bladder, kidney, lung, lymphocyte, esophagus, pancreas, placenta, prostate, rectum and thymus (data not shown).

transducing receptor¹⁸ (Fig. 4a). This failure of processing has led to the suggestion that this receptor may require an accessory factor or factors to facilitate correct folding of the receptor, its translocation across the endoplasmic reticulum membrane or its trafficking to the cell surface.

Given the similarities in phenotype resulting from mutations in *MC2R* or *MRAP* and the coexpression of the two genes in the adrenal (Fig. 3) and differentiating adipocyte, we wanted to test the hypothesis that MRAP could form a complex with *MC2R* and act as such an accessory factor. We investigated the subcellular localization of MRAP using CHO cells transfected with mouse *Mrap- α* with a FLAG epitope tag at the C terminus⁸. Immunocytochemical analysis of live nonpermeabilized cells using the FLAG antibody showed that the C terminus of the protein was present on the extracellular face of the plasma membrane (Fig. 4a), confirming the predicted topology. When the cells were permeabilized, we also detected an intracellular pool of *Mrap* (Fig. 4a), which colocalized with markers for the endoplasmic reticulum (Supplementary Fig. 4 online). Expression of *Mrap-FLAG* and mouse *Mc2r-GFP* together resulted in a shift in the localization of both proteins. The amount of *Mrap* present on the plasma membrane decreased, and the amount of intracellular *Mrap* that colocalized with *Mc2r* at the endoplasmic reticulum increased (Fig. 4b). Furthermore, in some cells, *Mc2r* colocalized with *Mrap* at the plasma membrane (Fig. 4c).

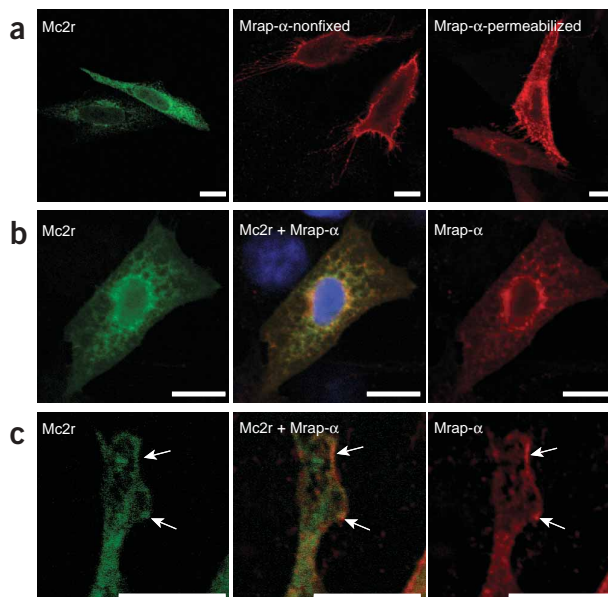
Figure 4 Colocalization of *Mrap* with *Mc2r*. (a) Subcellular localization of *Mc2r-GFP* and *Mrap-FLAG* in CHO cells. CHO cells were transfected with *Mc2r* and *Mrap*. GFP fluorescence was visualized, and antibody to FLAG was used to detect *Mrap-FLAG* in live nonpermeabilized cells (center) or fixed and permeabilized cells (right). (b) Coexpression of *Mc2r* and *Mrap* leads to a redistribution of both proteins. *Mc2r* and *Mrap* colocalized in a reticular pattern within the cell consistent with endoplasmic reticulum staining, which corresponded with less *Mrap* being present on the plasma membrane. Nuclei are counterstained blue with DAPI in the merge panel (center). (c) In some cells, *Mc2r* colocalized with *Mrap* at the plasma membrane (arrows). Scale bars, 10 μ m.

Because *Mrap* and *Mc2r* colocalized, we tested whether they form a complex by reciprocal coimmunoprecipitation. *Mrap-FLAG* migrated as three bands of ~ 15 kDa, ~ 22 kDa and ~ 40 kDa when resolved by SDS-PAGE (Fig. 5a). The smallest species corresponds to the predicted molecular weight of mouse *Mrap-FLAG*; the other bands may result from post-translational modification or oligomerization. Immunoprecipitation of *Mc2r-GFP* using an antibody to GFP specifically coprecipitated all three species of *Mrap-FLAG* when the two proteins were coexpressed (Fig. 5a). In the reciprocal experiment, *Mc2r-GFP* was specifically coprecipitated with *Mrap-FLAG* using an antibody to the FLAG tag, as determined by GFP fluorescence (Fig. 5b).

We next investigated ACTH-responsiveness of cells transfected with *Mc2r* in the presence of *Mrap*. Although CHO cells showed no responses in these experiments, the human SK-N-SH cell line showed both a strong enhancement of cell surface expression of *Mc2r-GFP* in the presence of *Mrap* (Fig. 6a) and a substantial cAMP response to ACTH stimulation (Fig. 6b). These cells also showed some expression of a melanocortin receptor other than *MC2R*, as evidenced by the small cAMP response to NDP-MSH and ACTH in mock-transfected cells. Transfection of *Mrap* alone did not enhance this response, implying that it is specific for *Mc2r*. These findings support the idea that *Mrap* is an essential cofactor for *Mc2r* expression.

MRAP joins a group of small single-transmembrane domain proteins that include the RAMP proteins, which support expression of the calcitonin and calcitonin-like receptors^{19,20}, and the recently described RAP-1 and RAP-2 proteins, which support olfactory receptor trafficking and expression²¹. No homology exists between these GPCR accessory proteins, although functional parallels do exist between them.

In conclusion, we show that a potentially lethal disorder caused by mutations in *MC2R* can also result from mutations in *MRAP*. Our data indicate that MRAP and *MC2R* interact and that both are colocalized in the endoplasmic reticulum and plasma membrane. MRAP is required for *MC2R* expression in certain cell types, supporting the idea that MRAP has a role in the processing, trafficking or function of *MC2R*. This is a new cause of receptor insensitivity that may have clinically important parallels in other receptor systems.



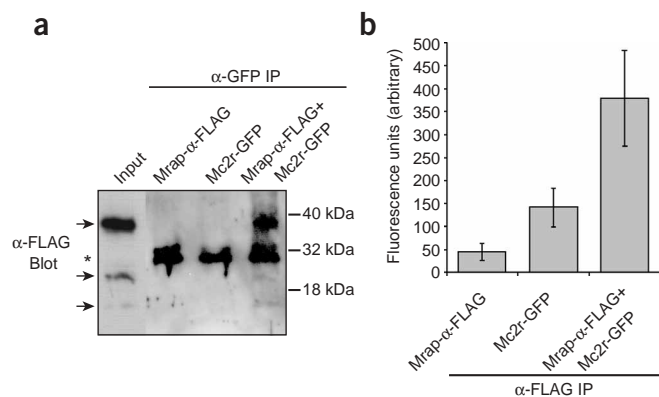


Figure 5 Mrap and Mc2r form a complex. **(a)** Mrap-FLAG coprecipitates with Mc2r-GFP. Western blot with antibody to FLAG showing the migration of Mrap-FLAG as three species on SDS-PAGE (Input, arrow on left). Mrap-FLAG specifically coprecipitated with an antibody to GFP (α -GFP) only in the presence of Mc2r-GFP. The immunoglobulin light chain was detected as a nonspecific band (asterisk). Molecular weight marker positions are indicated on the right. **(b)** Mc2r-GFP coprecipitated with Mrap-FLAG. Mrap-FLAG precipitated with antibody to FLAG (α -FLAG) in the presence and absence of Mc2r-GFP. GFP fluorescence was measured using a spectrofluorimeter. Error bars represent s.d. of three experiments. IP, immunoprecipitation.

METHODS

Affected individuals and families. Affected individuals were clinically diagnosed with FGD on the basis of clinical history and biochemical measurements. We isolated genomic DNA from blood samples from affected individuals and family members after obtaining informed consent from them and/or their parents. This study was approved by the East London and City Health Authority Ethics Committee. In all cases, the sequence of *MC2R* had been determined to be normal.

Genome scan and haplotype analysis. For the whole-genome scan, we used the GeneChip Mapping 10K Array version Xba131 (Affymetrix) in accordance with the manufacturer's guidelines. This array comprised 11,555 SNPs with a mean intermarker distance of 210 kb, equivalent to 0.32 cM. We used nine informative SNP markers for haplotype reconstruction and analysis: rs723470, rs723469, rs2409411, rs768506, rs915534, rs724070, rs724208, rs743337 and rs915574 (dbSNP Home Page). We used PedCheck to detect mendelian errors²². We removed from the data set any genotype data from SNPs showing such errors. We identified nonmendelian errors by MERLIN²³ and deleted them in the individuals in which they occurred. Parametric linkage and haplotype analysis were done by a modified version of GENEHUNTER²⁴, using a sliding window with sets of 50 SNPs. All physical positions are derived from National Center for Biotechnology Information build 34, July 2003. We analyzed dinucleotide repeat markers *D21S265*, *D21S1280*, *D21S2049*, *D21S1694*, *D21S223*, *D21S63*, *D21S1834*, *D21S1413*, *D21S1898*, *D21S2039*, *D21S1987*, *D21S219* and *D21S65* using an ABI 377 automated sequencer.

Candidate gene identification. We identified candidate genes within the region of homozygosity using data available in GenBank and Ensembl databases. We designed RT-PCRs for these genes to be intron-skipping and to target exons common to all known transcripts of each gene. Primer sequences are available on request. We assessed the expression of each gene using cDNA from adrenal cortex, brain and liver (Ambion). PCR reaction mixtures contained 50 ng of cDNA, 1× PCR buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂), 200 μM each dNTP and 1 U *Taq* DNA polymerase (Sigma-Aldrich) in a total volume of 50 μl. After an initial denaturation step of 5 min at 95 °C, PCR cycling was done for 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min.

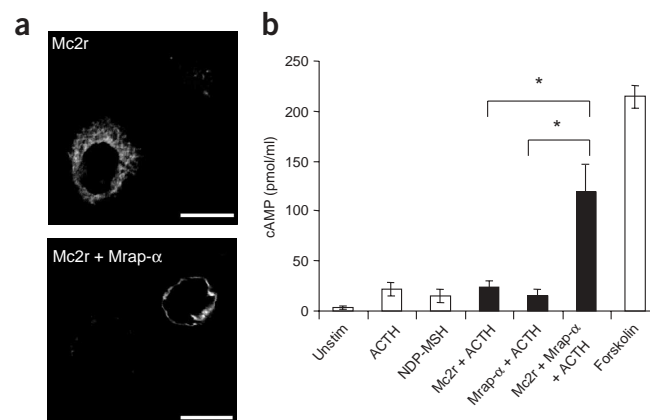


Figure 6 Mrap is required for Mc2r responsiveness in SK-N-SH cells. **(a)** Cells were transfected with Mc2r-GFP alone (top panel) or with Mc2r-GFP and Mrap-FLAG (bottom panel). Confocal imaging shows efficient expression of receptor at the cell surface. **(b)** SK-N-SH cells were either mock-transfected (white bars) or transfected with Mc2r-GFP alone, with Mrap-FLAG alone or with Mc2r-GFP and Mrap-FLAG together (black bars). Cells were then left unstimulated (Unstim) or were stimulated with ACTH (10⁻⁶ M), NDP-MSH (10⁻⁶ M) or forskolin (10⁻⁵ M), and cAMP was assayed. Graph shows the mean ± s.e.m. of three independent experiments, each done in duplicate. **P* < 0.05.

Tissue distribution. We investigated expression of MRAP- α , MRAP- β , MC2R and GAPDH using a panel of cDNAs derived from 30 adult tissues (adrenal, bladder, breast, buccal mucosa, colon, duodenum, fallopian tube, fat, gastric antrum, gastric fundus, gall bladder, ileum, jejunum, kidney, lung, lymph node, lymphocyte, skeletal muscle, myocardium, esophagus, ovary, pancreas, placenta, pituitary, prostate, rectum, skin, testis, thyroid and thymus). We carried out RT-PCR for MRAP- α , MRAP- β , MC2R and GAPDH for each tissue in a reaction mixture containing 50 ng of cDNA, 1× PCR buffer, 200 μM each dNTP, 200 nM each primer and 1 U *Taq* DNA polymerase (Sigma-Aldrich) in a total volume of 50 μl. After an initial denaturation step of 5 min at 95 °C, touchdown PCR cycling was done for 16 cycles of 95 °C for 30 s, *x* °C for 30 s (where *x* began at 65 °C and fell in 1-°C increments per cycle) and 72 °C for 1 min, followed by 25 cycles of 95 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. We carried out gel electrophoresis and scanning densitometry to determine relative expression levels of MRAP- α , MRAP- β and MC2R.

Mutation detection. We carried out PCR using primers directed to intronic sequences in a total volume of 50 μl. Primer sequences are available on request. The reaction mixture contained 100 ng of DNA template, 1× PCR buffer, 200 μM each dNTP, 200 nM each primer and 1 U *Taq* DNA polymerase (Sigma-Aldrich). After an initial denaturation step of 5 min at 95 °C, PCR cycling was done for 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by a final extension step at 72 °C for 5 min. We purified PCR-amplified fragments by spin columns (Qiagen) and sequenced them using the ABI Prism Big Dye Sequencing kit and an ABI 3700 automated DNA sequencer (Applied Biosystems) in accordance with the manufacturer's instructions.

Cell culture maintenance, transfection and confocal microscopy. We monitored cell maintenance, transfections and GFP fluorescence and carried out cell fixation and permeabilization for immunocytochemistry as previously described²³ with the following antibody titers: antibody to FLAG (M2; Sigma-Aldrich), 1:200; Cy3-conjugated donkey secondary antibody (Jackson ImmunoResearch), 1:50. For live cell staining, we incubated live cells with antibody to FLAG and secondary antibody and washed them on ice before fixating and mounting them. Fluorescence was imaged using a Zeiss LSM510 confocal microscope. SK-N-SH cell transient transfections using Fugene (Roche) were ~50% efficient as judged by transfection with pEGFP. We

measured cAMP as previously described^{14,17} after stimulating cells with ACTH (10^{-6} M), NDP-MSH (10^{-6} M) or forskolin (10^{-5} M) for 30 min in the presence of 3-isobutyl-1-methylxanthine (10^{-5} M).

Western blotting and coimmunoprecipitation. We carried out western blotting and coimmunoprecipitation essentially as described^{25,26}. We cotransfected CHO cells with Mc2r-GFP and Mrap-FLAG. Twenty-four hours after transfection, we washed cells twice in phosphate-buffered saline and scraped them into 400 μ l of phosphate-buffered saline with 1% n-dodecyl- β -D-maltoside (Sigma-Aldrich) containing a cocktail of protease inhibitors (buffer A). After a 30-min incubation at 4 °C, we centrifuged cell lysates at 17,500 g for 15 min. We incubated the supernatant overnight at 4 °C with 5 μ g of antibody to FLAG or antibody to GFP (A.v. peptide; Clontech). We added protein G-Sepharose (Amersham Bioscience) in buffer A and incubated it for another 3 h. After five 30-min washes in buffer A, we resuspended Sepharose beads in 100 μ l of phosphate-buffered saline. We monitored Mc2r coprecipitation by using GFP fluorescence measured using a SAFFIRE fluorescence plate reader (Tecan Ltd.), because the protein does not resolve well by SDS-PAGE, and we subtracted the plate background before carrying out statistical analyses. To analyze Mrap-FLAG, we eluted the proteins in SDS-PAGE sample buffer and subjected them to western-blot analyses using antibody to FLAG at 1:1,000. The Mrap-FLAG plasmid was a gift from G. Cooper (University of Auckland, New Zealand).

Accession numbers. Human MRAP, AF454915; human MRAP- α , AY079152; human MRAP- β , AF483549; mouse MRAP- α , AY079153; human *C6orf117*, BC010003.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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