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Exon capture analysis of G protein-coupled receptors identifies activating mutations in *GRM3* in melanoma

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

AUTHOR CONTRIBUTIONS

T.D.P., I.C.-N., X.W., E.H.M. and Y.S. designed the study. K.S.-H., M.A.D., J.E.G. and S.A.R. collected and analyzed the melanoma samples. X.W., I.C.-N., J.K.T., J.G., P.F.C. and J.C.L. analyzed the genetic data. T.D.P., A.M., J.J. and V.W. produced and analyzed the functional data. All authors contributed to the final version of the paper.

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Abstract

G protein-coupled receptors (GPCRs), the largest human gene family, are important regulators of signaling pathways. However, knowledge of their genetic alterations is limited. In this study, we used exon capture and massively parallel sequencing methods to analyze the mutational status of 734 GPCRs in melanoma. This investigation revealed that one family member, *GRM3*, was frequently mutated and that one of its mutations clustered within one position. Biochemical analysis of *GRM3* alterations revealed that mutant *GRM3* selectively regulated the phosphorylation of MEK, leading to increased anchorage-independent growth and migration. Melanoma cells expressing mutant *GRM3* had reduced cell growth and cellular migration after short hairpin RNA-mediated knockdown of *GRM3* or treatment with a selective MEK inhibitor, AZD-6244, which is currently being used in phase 2 clinical trials. Our study yields the most comprehensive map of genetic alterations in the GPCR gene family.

Melanoma is a common form of skin cancer and, despite years of research, metastatic disease is often fatal¹. Although some targeted treatment has been effective², the results of these treatments are not long lasting, showing a need for new clinically relevant targets. GPCRs, which are activated by ligands, are integral in regulating various signaling pathways^{3,4}. The importance these molecules play in human diseases is evident by the fact that 50–60% of the US Food and Drug Administration–approved therapeutics target GPCRs⁵. As GPCRs regulate pathways that affect cell growth, their genetic analysis in cancer is warranted.

To identify somatic mutations in GPCRs, we performed a GPCR-targeted mutational analysis in tumor DNA derived from 11 melanoma samples. For each sample, we performed DNA capture using molecular inversion probes of 7,059 regions corresponding to 2,400 GPCR exons encoding 734 GPCR genes⁶. We then analyzed DNA samples enriched for GPCR exons by massively parallel sequencing using the Illumina GAII platform. We identified 755 potential non-synonymous mutations when comparing the molecular inversion probe results to the known HapMap sequence. To determine which alterations were somatic, we examined the genomic DNA derived from matched normal tissue. From these alterations, we confirmed 106 to be somatic in 94 different genes (Supplementary Table 1). Eleven of these genes harbored at least two somatic mutations (Supplementary Table 2). The coding sequence of these 11 genes was analyzed for somatic mutations in a total of 80 melanoma samples, as described previously⁷. Supplementary Figure 1 depicts the stages described above.

From the ~3.4 Mb of sequencing information obtained, we identified 115 non-synonymous, somatic mutations in 42 of the 80 tumors (Table 1). The number of C>T mutations was significantly greater than the number of other substitutions, resulting in a high prevalence of C:G>T:A transitions ($P < 1 \times 10^{-6}$) (Supplementary Fig. 2), consistent with melanoma mutation signatures⁸. We found a total of seven nonsense mutations and two splice site alterations, potentially resulting in aberrant or truncated proteins for five of the genes. We identified recurring alterations in *CHRM3*, *RXFP1*, *OR8B8* and *OR1J2*, which harbored p.Pro421Leu/p.Pro421Ser, p.Ser269Phe, p.Ser74Phe and p.Asp109Asn, respectively, in two individuals. Using SIFT (sorting intolerant from tolerant)⁹, we determined a computational estimation of the effects of the mutations (Supplementary Table 3). A description of these genes and the likely nature of their mutations is given in the Supplementary Note.

GRM3 and *GPR98* were the most frequently mutated genes in our screen. *GRM3* had a 16.3% mutation rate, with 18 non-synonymous mutations in 13 of 80 tumors. *GPR98* had a total of 42 non-synonymous mutations in 22 of 80 tumors (27.5%). As *GRM3* belongs to the metabotropic family and was previously shown to be linked to tumorigenesis¹⁰, it was

evaluated genetically in an additional panel consisting of 57 melanoma specimens¹¹. In this screen, we discovered 11 non-synonymous alterations in nine tumors affecting 15.7% of the individuals analyzed and yielding a non-synonymous to synonymous ratio of 29:7, which is significantly higher than the non-synonymous to synonymous ratio of 2:1 predicted for nonselected passenger mutations ($P < 0.05$). This investigation allowed the identification of a mutational hotspot in *GRM3*; we found p.Glu870Lys in four different individuals with melanoma (one individual from the original panel and three from the second panel) (Supplementary Table 4 and Supplementary Fig. 3). The likelihood for the occurrence of four identical mutations is approximately 1.8×10^{-12} , suggesting that the *GRM3* hotspot mutation is functionally important. Clinical information for all tumors harboring GPCR mutations is given in Supplementary Table 5.

We focused on *GRM3* (the group II metabotropic glutamate receptor-3 gene, or *mGluR3*), as two genetic observations suggested that the mutations in this gene may be functionally important for melanoma tumorigenesis: (i) *GRM3* was one of the most highly mutated genes in the screen, and (ii) it contained a mini-hotspot (p.Glu870Lys).

We functionally characterized four somatic mutations (resulting in p.Gly561Glu, p.Ser610Leu, p.Glu767Lys and p.Glu870Lys) discovered in *GRM3* based on sequence conservation and their location within particular functional domains. To examine the biological effects of *GRM3* mutations, we established stable pooled clones expressing either a vector control or wild-type or mutant (p.Gly561Glu, p.Ser610Leu, p.Glu767Lys or p.Glu870Lys) *GRM3*. We selected the melanoma cell lines A375 or Mel-STR, as they express wild-type *GRM3*. We saw similar levels of expression of *GRM3* protein in both the A375 and Mel-STR stable clone cell lines, except for in the p.Glu767Lys and p.Glu870Lys mutants, which had reduced protein expression (Supplementary Fig. 4a,b).

To examine the effects of *GRM3* mutations on cell growth, we investigated growth rate on plastic (Fig. 1a). In the presence of media containing 10% serum, all clones grew similarly (Supplementary Fig. 5a,b). However, if we reduced the serum concentration, wild-type clones grew at a lower rate than mutant clones, except for the clone expressing the p.Glu870Lys alteration (Fig. 1a and Supplementary Fig. 5c). We also observed this difference in cell growth when we assessed the cells for anchorage independence, where cells expressing mutant *GRM3* formed a significantly higher number of colonies compared to wild type or empty vector (Fig. 1b; $P < 0.05$ *t*-test).

The C-terminal region of GPCR proteins is important for binding signaling molecules involved in pathways such as the RAS-RAF-MEK pathway^{3,4}. Mutations in or near this region of *GRM3* may therefore affect signal transduction leading to increased cell proliferation, thus providing the biochemical basis for the growth differences described above. As group 2 metabotropic glutamate receptors can be activated by agonists such as DCG-IV^{12–14}, we tested the biochemical effects of *GRM3* alterations in the presence and absence of DCG-IV. We saw striking differences in MEK1/2 phosphorylation. When we stimulated Mel-STR clones with DCG-IV, there was a sevenfold to tenfold increased phosphorylation of MEK1/2 compared to wild-type-*GRM3*-expressing cells (Fig. 1c). We observed similar results in the A375 clones (Fig. 1d). Notably, Mel-STR cells harbor mutant RAS and A375 cells harbor mutant BRAF, both of which are known to activate MEK¹⁵, and so mutant *GRM3* thus allowed further activation of the MEK pathway.

Previous studies reported that activation of the MEK pathway increases cell migration^{16,17}. As *GRM3* variants activate the MEK pathway, we determined whether these variants also affect migration. To test this, we seeded A375 or Mel-STR pooled clones in serum-free medium in the presence or absence of DCG-IV and looked for migration (Fig. 2a,b). Mutant

GRM3 expression increased migration compared to wild-type GRM3 or an empty vector containing cells in the absence of agonist (Fig. 2a,b; $P < 0.05$ *t*-test). Upon stimulation with DCG-IV, vector and wild-type-expressing cells migrated similarly to mutant-GRM3-expressing cells in the absence of stimuli (Fig. 2a,b). Our results suggest that expression of mutant GRM3 increases migration in the absence of growth factors or receptor agonists.

To determine whether these phenotypes occur *in vivo*, A375 pooled clones expressing vector, wild-type or mutant GRM3 were administered to NOD/SCID mice by tail vein injection. Nine weeks after injection, macroscopic assessment of lung colonization showed that the groups injected with cells expressing vector, wild-type or mutant p.Gly561Glu had two to three mice with gross lung tumors. In contrast, most of the mice injected with cells expressing the p.Ser610Leu, p.Glu767Lys or p.Glu870Lys GRM3 alterations had pulmonary macrometastases (Fig. 2c). Microscopic examination allowed for the detection of micrometastases, with no significant difference being seen in their number or size (Supplementary Fig. 6a,b). Thus, expression of mutant forms of GRM3 in melanoma cells affects growth *in vivo* once the lung is colonized.

To assess if melanoma cells with endogenous *GRM3* mutations are dependent on GRM3 signaling for proliferation and migration, we used short hairpin RNA (shRNA) to stably knock down GRM3 protein levels in melanoma cells that harbor either wild-type *GRM3* or endogenous mutant *GRM3*. We confirmed specific targeting of *GRM3* by transient transfection in HEK293T cells and immunoblotting, as well as by quantitative RT-PCR analysis (Fig. 3a–c). The shRNA had little effect on cells harboring wild-type GRM3 but significantly reduced the growth of cells harboring mutant forms of GRM3 (Fig. 3d–h and Supplementary Fig. 7a,b; $P < 0.005$, $P < 0.008$ and $P < 0.004$ for Fig. 3f,g,h, respectively). Depletion of GRM3 by shRNA in mutant-GRM3-expressing cells reduced their ability to migrate significantly compared to wild-type GRM3 cells targeted with GRM3 shRNA (Fig. 3i–m and Supplementary Fig. 7c,d). To determine whether similar results occur *in vivo*, melanoma cells harboring either wild-type or mutant GRM3 targeted with GRM3 or control shRNA were administered into Nu/Nu mice by subcutaneous injection. Nineteen days after injection, depletion of GRM3 had little effect on *in vivo* growth of cells harboring wild-type GRM3. In contrast, GRM3 knockdown significantly reduced the tumor growth induced by cells harboring mutant GRM3 (Fig. 3n–q; $P < 0.0005$ and $P < 0.02$ for Fig. 3o,q, respectively). As the shRNA-mediated phenotypes could be caused by specific or nonspecific effects, we engineered an exogenous, non-targetable wild-type GRM3 construct that harbors silent mutations in the region of GRM3 targeted by shRNA #3 to rescue the effects of knockdown of endogenous GRM3. Melanoma cells harboring the p.Glu573Lys alterations stably expressing either control or GRM3 shRNA #3 construct were transduced either with the lentiviral non-targetable GRM3 construct or with the empty vector as a control. To show that the non-targetable GRM3 is not knocked down in the presence of GRM3 shRNA #3, we transiently transfected HEK293T cells and immunoblotted for FLAG-GRM3 and GAPDH as a loading control (Supplementary Fig. 8a). Importantly, non-targetable GRM3-reconstituted cells showed significantly more migration than cells infected with the control vector (Supplementary Fig. 8b). These results suggest that certain GRM3 mutations are essential for cellular proliferation as well as for cell migration in melanoma cells.

To evaluate whether inhibition of GRM3 signaling would result in a similar phenotype to depletion of endogenous GRM3, we exposed melanoma cells harboring either wild-type *GRM3* or mutant *GRM3* to AZD-6244 (Selumetinib, ARRY-142886), which is a selective, non-ATP-competitive small molecule inhibitor of MEK1/2 that is being tested in phase 2 clinical trials (see URLs). Exposure of melanoma cells to AZD-6244 inhibited MEK in mutant as well as in wild-type cells (Fig. 4a). However, the relative degree of inhibition was

concentration dependent, resulting in greater inhibition of the mutant cells compared to wild-type cells. This suggested that mutant cells would be more sensitive to growth inhibition by AZD-6244 than wild-type cells. Indeed, exposure of most melanoma cells expressing mutant forms of GRM3 were 2–200-fold more sensitive to AZD-6244 compared to wild-type GRM3 cells (Fig. 4b and Table 2). The GRM3 mutations may be dependent on MEK signaling via BRAF, as evidenced by the genotypes of the investigated cells (Supplementary Table 6). To further investigate the relevance of mutant GRM3 to AZD-6244 sensitivity, we tested whether the sensitivity to MEK inhibition can be altered by modulating GRM3. To do this, we established a stable cell line overexpressing wild-type GRM3 in a mutant GRM3 background (Supplementary Fig. 9a). Exposure of these stable pools to AZD-6244 resulted in reduced cell proliferation, with a fourfold increased resistance in cells overexpressing wild-type GRM3 compared to control infected cells (Supplementary Fig. 9b,c). These results further suggest that AZD-6244 preferentially inhibits the signaling of cells expressing mutant GRM3.

The decreased growth of the mutant cells in the presence of AZD-6244 could have arisen either through alteration of the cell cycle or through increased cell death. To distinguish between these two possibilities, we performed FACS analysis and found that cells harboring mutant GRM3 showed a substantial increase in the levels of subG1-population cells (apoptotic cells) compared to wild-type cells (Fig. 4c). We observed similar results when we performed the experiment on a larger panel of mutant *GRM3* cell lines (Fig. 4d; $P < 0.05$ *t*-test). We confirmed the apoptotic events by protein blot analysis of the cell lysates analyzed by FACS, which showed increased levels of cleaved PARP in mutant cells compared to wild-type cells (Fig. 4e). Thus, melanoma cell lines harboring mutant *GRM3* are markedly more sensitive to MEK1/2 inhibition by AZD-6244, leading to increased cell death.

In this study, we used a systematic approach combining exon capture and massively parallel sequencing to genetically characterize the GPCR gene family, allowing for the identification of *GRM3* somatic mutations in melanoma. The high frequency of mutations found in *GRM3*, the finding of a mutational hotspot, as well as the functional assays, suggest *GRM3* to be a driver in melanoma.

Taken together, our data highlight a model for melanoma pathogenesis in which activation of MEK by GRM3 alterations promotes the proliferation and migration of melanoma cells. Although further investigation into the mechanism of GRM3 activation of the MEK pathway is required, our study suggests that the presence of *GRM3* mutations is expected to indicate subpopulations of individuals whose tumors are dependent on MEK signaling. The prior failure of MEK inhibitors to obtain significant tumor responses in many *BRAF* p.Val600Glu melanomas^{18,19} may have resulted at least in part from the absence of additional mutations that activate the MEK pathway, such as those in *GRM3*. Therefore, targeting MEK signaling in the presence of *GRM3* mutations may have a role in the treatment of melanoma.

ONLINE METHODS

Tumor tissues

Tissue and melanoma cell lines used for the discovery and first validation in this study were described previously²⁰.

URLs. Short Read Archive (SRA), <http://trace.ncbi.nlm.nih.gov/Traces/sra/>; phase 2 clinical trials, <http://clinicaltrials.gov/>; Consed, <http://www.phrap.org/consed/consed.html>; R, <http://www.r-project.org/>; ImageJ, <http://rsbweb.nih.gov/ij/>.

For the validation set of melanomas, optimum cutting temperature– embedded frozen clinical specimens were obtained from the University of Texas MD Anderson Cancer Center under institutional review board–approved protocols. Hematoxylin and eosin–guided dissection and isolation of DNA from the tumor-enriched isolates has been described previously¹¹.

GPCR exon capture and analysis

See the Supplementary Note and Supplementary Table 7.

PCR, sequencing and mutational analysis of melanoma samples

PCR and sequencing was done as previously described^{7,20,21}. The primers used for PCR amplification are listed in Supplementary Table 8. The primary phase mutation screen was analyzed using Consed (see URLs). Variants were called using Polyphred 6.11 and DIPDetector (N. Hansen, unpublished data), an indel detector for improved sensitivity in finding insertions and deletions. Sequence traces of the secondary screen were analyzed using the Mutation Surveyor software package (SoftGenetics).

Construction of wild-type, mutant and non-targetable *GRM3* expression vectors

Human *GRM3* (NM_000840) was cloned by PCR into pCDF1 or pcDNA3.1 as previously described^{7,20} using a clone (cat # MHS1010-9203778) purchased from Open Biosystems and with the primers listed in Supplementary Table 8. The p.Gly561Glu, p.Ser610Leu, p.Glu767Lys and p.Glu870Lys point mutants were made using Phusion PCR for site-directed mutagenesis. Amplification of a non-targetable insert using the primers listed in Supplementary Table 9 was done as previously described^{7,20} and cloned into pCDF1 vector.

Cell culture and transient expression

Metastatic melanoma tumor, HEK 293T, A375, Mel-STR cell lines were maintained as previously described^{7,20}. HEK 293T cells were transfected with Lipfectamine2000 reagent (Invitrogen) at a 6:1 ratio with DNA (μl:μg) using 3–5 μg of plasmid DNA.

Immunoprecipitation and protein blotting

Transfected cells or stable pooled clone cell extracts were analyzed by immunoprecipitation or protein blot analysis as previously described^{7,22}.

Pooled stable expression

Production of *GRM3* lentivirus was done as previously described⁷. A375 and Mel-STR cells were seeded at 1.5×10^6 per T75 flask 24 h before infection. Lentivirus for *GRM3* (wild type, p.Gly561Glu, p.Ser610Leu, p.Glu767Lys and p.Glu870Lys) or empty vector control were used to infect cells as previously described⁷. A375 or Mel-STR pooled clones were selected in the presence of 3 μg/ml puromycin or 300 μg/ml G418 for several days, respectively. Stable expression of *GRM3* proteins (wild type and mutants) was determined by SDS-PAGE analysis followed by immunoblotting with anti-FLAG and anti-α-tubulin to show equivalent expression among the pools.

Signaling pathway stimulation

A375 or Mel-STR pooled *GRM3* clones were seeded in 6-well plates at $1-2 \times 10^5$ cells per well the day before transfection. Cells were serum starved for 16 h before stimulation with 2.5 μM DCG-IV (Tocris Biosciences-Ellisville) for 10 min. Cell lysates were generated by direct lysis into 2× SDS sample buffer and then subsequently analyzed by protein blotting using anti-phospho-MEK1/2 (cat # 9154), anti-MEK1/2 (cat # 9126), anti-GAPDH (cat # CB

1001) (Millipore-Billerica) or anti- α -tubulin (cat # CP06) (EMD-Calbiochem-Gibbstown) as a loading control.

Proliferation assays

To examine growth potential, pooled A375 and Mel-STR GRM3 clones were seeded into 96-well plates at 250 cells per well in either 1%, 2.5% or 10% serum-containing medium and incubated for 13–17 days. Samples were analyzed as previously described⁷.

Soft agar assay

Mel-STR pooled GRM3 clones were plated in triplicate at 1,000 cells per well and in top plugs consisting of sterile 0.33% Bacto-Agar (Becton, Dickinson and Company) and 10% FBS (HyClone) in a 24-well plate. The lower plug contained sterile 0.5% Bacto-Agar and 10% FBS. After two weeks, the colonies were photographed and quantitated using ImageJ (NIH software).

Migration assays

Mel-STR or A375 pooled clones were seeded at 30,000 cells per well in serum-free medium or serum-free medium plus 2.5 μ M DCG-IV and incubated for 16–18 h before harvesting. Inserts were analyzed as previously described²⁰.

Lentiviral shRNA

Constructs for stable depletion of GRM3 (cat # RHS4533-NM_000840) were obtained from Open Biosystems and were confirmed to efficiently knockdown GRM3 at the protein level. Lentiviral stocks were prepared as previously described²⁰. Melanoma cell lines (31T, 34T, 36T, 63T and 76T) were infected with shRNA lentiviruses for each condition (vector and two different *GRM3*-specific shRNAs). Selection of stable pooled clones was done in the presence of 3 μ g/ml puromycin containing normal medium for 3–5 days before determining knockdown efficiency. Stably infected pooled clones were tested in functional assays. For the shRNA, inducible system constructs for stable depletion of GRM3 (cat # RHS4740-NM_000840) were obtained from Open Biosystems and were confirmed to efficiently knockdown GRM3 at the message level. Lentiviral stocks were prepared as previously described²⁰. Melanoma cell lines (31T or 36T) were infected with shRNA lentiviruses for each condition (vector and two different *GRM3*-specific shRNAs). Selection and growth were done as described above. Stably infected pooled clones were tested in functional assays. Sequences of stable shRNA knockdown plasmids for GRM3 are listed in Supplementary Table 10. Doxycycline-inducible shRNA knockdown clones were grown in the presence of 0.2 μ g/ml of doxy-cycline for 7 days before any functional assays. The clones were kept in the presence of 0.2 μ g/ml doxycycline throughout the experiment.

To rescue shRNA-mediated knockdown of GRM3 in melanoma cell lines, the non-targetable *GRM3*-expressing lentivirus was made as described above and used to infect the melanoma cell line 63T. After infection, cells were given 96–120 h to recover from infection before being tested in functional assays.

Quantitative real-time PCR

Total RNA was extracted from pooled clones of melanoma cells 36T and 34T stably knocked down for endogenous GRM3 or from the doxycycline-induced clones 31T and 63T targeting GRM3 (in the presence of 0.2 μ g/ml doxycycline for 7 days) following the manufacturer's protocol for the RNeasy Mini Kit (QIAGEN #74101). Total RNA was eluted in 30 μ l diethylpyrocarbonate (DEPC)-treated distilled H₂O. A total of 1 μ g of total RNA was used for single-strand complementary DNA (cDNA) synthesis using a SuperScript III

First Strand kit (Invitrogen #18080-051). cDNA was amplified using the olido dT₂₀ primer supplied in the kit. To test for loss of GRM3 message, we used 2 µl of cDNA in the PCR with either GRM3 primers or GAPDH primers (Supplementary Table 11) mixed with 2× Fast SYBR Green PCR mix at a final volume of 20 µl in triplicate (Applied Biosystems cat # 4355612). qRT-PCR analysis was done using the ABI 7900HT Fast Real-Time PCR system (with a standard program of stage 1: 50 °C for 2 min; stage 2: 95 °C for 10 min; stage 3: 40 cycles of 95 °C for 15 s and 60 °C for 1 min). Results were analyzed using Microsoft Excel.

Tail-vein injection studies in mice

NOD/SCID mice were purchased from Jackson Labs. All mice were housed in a pathogen-free facility and were given autoclaved food and water. A375 pooled clones with empty vector or wild-type GRM3 or mutant GRM3 were grown in T-175 flasks to 70–80% confluency. We resuspended 1×10^6 cells in 100 µl of sterile PBS and injected them intravenously (in the tail) into 7–10-week-old NOD/SCID mice. Mice were monitored biweekly, and lungs were examined and quantified for tumor formation by excision from mice killed at day 60 (9 weeks) after injection.

Subcutaneous injection studies in mice

Nu/Nu mice were purchased from Charles River Labs. All mice were housed in a pathogen-free facility and were given autoclaved food and water. 31T (pLKO.1, shRNA #1 or shRNA #3) or 36T (pLKO.1, shRNA #1 or shRNA #3) cell lines were grown in 4–6 T-175 flasks, each to 90% confluency. We resuspended 2.5×10^6 cells in 200 µl of sterile 1× PBS and matrigel solution (1:1 v/v) (BD Biosciences #354234) and injected them subcutaneously into 6–8-week-old female Nu/Nu mice. Mice were monitored biweekly, and tumor diameters were measured using precision calipers for 17–20 days. For the shRNA inducible system, all mice were given sterile doxycycline food (5 g/mouse/day) and water. 31T (TRIPz-NC, sh639 or sh742) or 63T (TRIPz-NC, sh639 or sh742) cell lines were grown in 4–6 T-175 flasks, each to 90% confluency. We resuspended 2.5×10^6 cells in 200 µl of sterile 1× PBS and matrigel solution (1:1 v/v) (BD Biosciences #354234) and injected subcutaneously into 6–8-week-old female Nu/Nu mice. Mice were monitored biweekly, and tumor diameters were measured using precision calipers for 17–20 days.

Growth inhibition analysis

To test AZD-6244 (cat # S1008) (Selleck Chemicals LLC) inhibition on melanoma cell lines, we seeded 96-well plates at 2,000–5,000 cells per well and incubated them for 24 h before addition of inhibitor at concentrations from 2 nM to 30 µM. Once the inhibitor was added, cells were incubated for 72 h at 37 °C. Cells were then analyzed using CellTiter-Glo according to the manufacturer's protocol (cat# G7571) (Promega). Plates were read on a Luminoskan Ascent (Thermo Scientific) plate reader and analyzed using Microsoft Excel and GraphPad Prism v5.

Flow cytometry analysis

Melanoma cells were seeded into T-25 flasks at densities of 3×10^5 cells per flask in normal complete medium and incubated at 37 °C for 24 h before addition of AZD-6244. AZD-6244 or DMSO was added for 72 h at a concentration of 2 µM. Cells were then analyzed by FACS as previously described⁷.

Statistical analysis

Statistical analyses were performed using the R statistical package (see URLs) and Microsoft Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effects of GRM3 alterations on cell growth and MEK phosphorylation. **(a)** Somatic alterations in GRM3 cause increased proliferation in reduced serum. We seeded A375 pooled GRM3 clones expressing wild-type, p.Gly561Glu, p.Ser610Leu, p.Glu767Lys, p.Glu870Lys or vector alone in 96-well plates in the presence of reduced serum (1% FBS). We harvested the plates and analyzed them by SYBR Green I on a BMG Labtech FluorOptima. Error bars, standard deviation (s.d.). **(b)** Mutant GRM3 shows anchorage-independent growth. We seeded Mel-STR cells in a top plug of agar and allowed them to incubate for 2 weeks before analysis by light microscopy and counting using US National Institutes of Health (NIH) ImageJ software (see URLs). Error bars, s.d. $n = 3$. Students t -tests in all instances showed a $*P < 0.05$, except for in the test of vector versus wild type. **(c)** Mutant GRM3 activates MEK1/2 upon DCG-IV stimulation in Mel-STR and A375 cells. We serum starved Mel-STR pooled GRM3 clones seeded in 6-well dishes for 4 h before the addition of either 2.5 μ M DCG-IV or vehicle for 10 min. After lysis of the cells, we analyzed the lysates on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and immunoblotted them with corresponding antibodies. We generated the ratios shown by ImageJ and Microsoft Excel analysis of phosphorylated protein to total protein blots. **(d)** Mutant GRM3 activates MEK1/2 upon DCG-IV stimulation in A375 cells. We analyzed A375 pooled GRM3 clones as described in **c**.

Figure 2. GRM3 mutations increase migration *in vitro* and *in vivo*

(a) A375 pooled GRM3 clones in the absence of stimulus migrate as well as those stimulated with the group 2 metabotropic agonist DCG-IV. We seeded A375 clones in Boyden chambers in either the absence of stimulus of, or in the presence of, 2.5 μ M DCG-IV and assessed them for their ability to migrate 16 h later. Error bars, s.d. (b) We analyzed Mel-STR pooled GRM3 clones for migration as described in a. We analyzed stained wells using a Zeiss microscope 10 $\times$ lens and counted them with NIH ImageJ software. Error bars, s.d. $n = 3$. (c) We intravenously injected NOD/SCID mice with A375 pooled GRM3 clones expressing the wild-type, p.Gly561Glu, p.Ser610Leu, p.Glu767Lys, p.Glu870Lys or vector alone and examined them after nine weeks. The graph indicates the number of mice that had lung macrometastases ($n = 10$; $P < 0.05$, Fisher's exact test). Shown are representative images of lungs from mice injected with the vector, wild type or mutant-expressing A375 clones.

Figure 3.

Expression of mutant GRM3 provides cell proliferation and survival signals in melanoma. (a) Our protein blot analysis shows that expressing GRM3 shRNA decreases endogenous GRM3 levels. We analyzed HEK 293T cells co-transfected with shRNA targeting GRM3 and FLAG-GRM3 by immunoblot. We analyzed the lysates in parallel using anti-GAPDH. (b,c) Our quantitative RT-PCR (qRT-PCR) analysis shows that GRM3 shRNA decreases endogenous levels of GRM3. Error bars, s.d. $n = 3$. qRT-PCR analysis of the wild-type GRM3 cell line (34T) (b) and the mutant (p.Gly561Glu) GRM3 cell line (36T) (c) using *GRM3*- or *GAPDH*-specific primers. (d–h) Growth curves of representative melanoma cell lines transduced with shRNA. Error bars, s.d. $n = 4$. (i–m) Stable knockdown of GRM3 in mutant-GRM3-expressing cells causes decreased migration compared to wild-type-expressing cells. We seeded wild-type GRM3 melanoma cell lines stably transduced (i,j) or mutant GRM3 melanoma cell lines stably transduced (k–m) with either empty vector, shRNA #1 or shRNA #3 (GRM3) in Boyden chamber wells in triplicate and incubated them for 16–72 h before analysis. We quantitated the results using a Student's *t*-test. Error bars, s.d. $n = 3$. (n,o) We subcutaneously injected Nu/Nu mice with either 31T or 36T GRM3 clones stably infected with pLKO.1, shRNA #1 or shRNA #3 against endogenous *GRM3* or 31T (wild-type GRM3) or 63T (p.Glu573Lys) GRM3 clones stably infected with the doxycycline-inducible TRIPz NC, sh639 or sh742 shRNA against endogenous *GRM3*.

Graphs show volumetric measurements of 31T (**n**), 36T GRM3 (constitutive knockdown clones) (**o**), 31T (doxycycline-inducible clones) (**p**) or 63T GRM3 (doxycycline-inducible clones) (**q**) tumor-bearing mice. We performed all *in vivo* studies from $n = 6$ mice. Error bars, s.d.

Figure 4.

Melanoma cell lines expressing GRM3 mutants show increased sensitivity to inhibition of MEK by AZD-6244. **(a)** Immunoblot analysis of representative melanoma cell lines harboring either wild-type or mutant GRM3. We treated the cells with the indicated concentration of AZD-6244 and analyzed them for ERK1/2 activation. We treated cells for 1 h with AZD-6244 or vehicle alone as a control. We subjected the lysates to protein blot analysis with anti-ERK1/2 (α -ERK1/2), anti-P-ERK1/2 (α -P-ERK1/2) and anti-GAPDH as a loading controls. **(b)** Representative dose-response curves showing the efficacy of AZD-6244 against GRM3 mutant lines compared to wild-type GRM3 lines. The relative cell numbers after we treated the cells for 72 h with increasing concentrations (0.002–30 μ M) of AZD-6244, as estimated by CellTiter-Glo and plotted as percent survival, as compared to vehicle-treated control, versus log [AZD-6244] concentration in nM (where 1 is 10 nM AZD-6244). We generated fitted lines using four-parameter nonlinear regression. Error bars, s.d. $n = 3$. **(c)** FACS analysis of wild-type (2T) and p.Ser154Phe, p.Asp280Asn, p.Arg352Trp or p.Glu870Lys mutant (68T) cells showing the cell-cycle distribution (propidium iodide staining; x axis) versus cell count (y axis). Shown are representative plots. **(d)** Quantification of FACS-sorted AZD-6244-treated cells. We determined the percentage of apoptotic cells based on the subG1 population for vehicle-treated cells or AZD-6244-treated cells. Error bars, s.d. **(e)** Immunoblot analysis of representative melanoma lines expressing wild-type or mutant GRM3 after AZD-6244 treatment using the indicated antibodies to assess PARP cleavage.

Table 1

Somatic mutations identified in recurrently mutated GPCRs

Gene	Other names	CCDS accession ^a	RefSeq accession ^d	No. of mutations (% tumors affected) ^b	Tumor	Exon	Nucleotide ^c	Amino Acid ^c	Functional domain
<i>GRM3</i>	<i>GLUR3</i>	5600.1	NM_000840	18 (16.3)	85T	1	c.53G>A	p.Gly18Glu	N/A
	<i>GPRC1C</i>				21T	1	c.176G>A	p.Arg59Gln	PBP1_mGluR_groupII
	<i>MGLUR3</i>				76T	1	c.263G>A	p.Gly88Glu	PBP1_mGluR_groupII
	<i>mGlu3</i>				68T	1	c.461C>T	p.Ser154Phe	PBP1_mGluR_groupII
					85T	2	c.575C>T	p.Pro192Leu	PBP1_mGluR_groupII
					68T	2	c.838G>A	p.Asp280Asn	PBP1_mGluR_groupII
					68T	2	c.1054C>T	p.Arg352Trp	PBP1_mGluR_groupII
					29T	3	c.1324-13T>C	Splice site	PBP1_mGluR_groupII
					6T	3	c.1402G>T	p.Val468Leu	PBP1_mGluR_groupII
					98T	3	c.1531G>A	p.Asp511Asn	NCD3G
<i>CHRM3</i>	<i>HM3</i>	1616.1	NM_000740	8 (10.0)	39T	3	c.1549G>A	p.Glu517Lys	NCD3G
					7T	3	c.1642G>A	p.Asp548Asn	NCD3G
					36T	3	c.1682G>A	p.Gly561Glu	N/A
					63T	3	c.1717G>A	p.Glu573Lys	N/A
					76T	3	c.1829C>T	p.Ser610Leu	7TM_3
					32T	3	c.2299G>A	p.Glu767Lys	7TM_3
					22T	4	c.2543G>A	p.Gly848Glu	N/A
					68T	5	c.2608G>A	p.Glu870Lys	N/A
					34T	1	c.74C>T	p.Ser25Phe	N/A
					39T	1	c.94G>A	p.Gly32Arg	N/A
<i>LPHN2</i>	<i>CIRL2</i>	689.1	NM_012302	10 (8.8)	7T	1	c.599G>A	p.Trp200X	7TM_1
	<i>CL2</i>				21T	1	c.1261C>T	p.Pro421Ser	7TM_1
					91T	1	c.1262C>T	p.Pro421Leu	7TM_1
					12T	1	c.1282C>T	p.Pro428Ser	7TM_1
					100T	1	c.1330A>C	p.Asn444His	7TM_1
					19T	1	c.1741T>A	p.Phe581Ile	N/A
					85T	4	c.529C>T	p.Pro177Ser	OLF
					48T	4	c.563C>T	p.Ser188Phe	OLF

Gene	Other names	CCDS accession ^d	RefSeq accession ^e	No. of mutations (% tumors affected) ^b	Tumor	Exon	Nucleotide ^c	Amino Acid ^c	Functional domain
<i>LEC1</i>	<i>LPHH1</i>				80T	4	c.1057T>C	p.Tyr353His (LOH)	OLF
					76T	7	c.1609G>A	p.Glu537Lys	N/A
<i>RXFP1</i>	<i>LGR7</i>	43276.1	NM_021634	8 (7.5)	39T	10	c.2225C>T	p.Ser742Leu	N/A
					76T	13	c.2896G>A	p.Glu966Lys (LOH)	7TM_2
					63T	16	c.3116C>T	p.Ser1039Phe	7TM_2
					13T	18	c.3422C>T	p.Ser1141Leu	Latrophilin
					76T	19	c.3618G>A	p.Met1206Ile	Latrophilin
					63T	19	c.3992G>A	p.Ser1331Asn	Latrophilin
					104T	4	c.365C>T	p.Pro122Leu	N/A
					7T	4	c.368C>T	p.Ser123Leu	N/A
					76T	10	c.806C>T	p.Ser269Phe	N/A
					104T	10	c.806C>T	p.Ser269Phe	N/A
<i>GRM8</i>	<i>FLJ41058</i>	5794.1	NM_000845	8 (8.8)	55T	16	c.1405C>T	p.Arg469Cys	7TM_1
					55T	16	c.1525G>A	p.Glu509Lys	7TM_1
					13T	16	c.1636C>T	p.Pro546Ser	7TM_1
					85T	17	c.1837G>A	p.Ala613Thr (LOH)	7TM_1
					76T	1	c.-11C>T	Splice site	N/A
					55T	1	c.143T>A	p.Leu48X	N/A
					34T	3	c.776C>T	p.Pro259Leu	ANF_R
					95T	7	c.1387G>A	p.Glu463Lys	ANF_R
					29T	7	c.1394G>A	p.Gly465Glu	ANF_R
					17T	8	c.1946C>T	p.Ser649Phe	7TM_3
<i>CNR1</i>	<i>RPI-23D17.1</i>	5015.1	NM_016083	6 (6.3)	76T	8	c.2318T>A	p.Val773Asp (LOH)	7TM_3
					85T	10	c.2681C>T	p.Ser894Phe	N/A
					104T	1	c.28G>C	p.Asp10His	N/A
					19T	1	c.145C>T	p.Pro49Ser	N/A
					71T	1	c.659G>A	p.Arg220Lys	7TM_1
					76T	1	c.765G>A	p.Trp255X	7TM_1
					64T	1	c.905A>C	p.His302Pro (LOH)	7TM_1
					64T	1	c.919C>T	p.Arg307Cys (LOH)	7TM_1

Gene	Other names	CCDS accession ^d	RefSeq accession ^e	No. of mutations (% tumors affected) ^b	Tumor	Exon	Nucleotide ^c	Amino Acid ^c	Functional domain
<i>CNR</i>									
<i>OR1J2</i>	<i>RP1J-542K23.4</i>	35121.1	NM_054107	4 (5.0)	85T	1	c.325G>A	p.Asp109Asn (LOH)	7TM_1
	<i>FLJ16828</i>				36T	1	c.325G>A	p.Asp109Asn	7TM_1
	<i>HG152</i>				72T	1	c.467C>T	p.Ser156Phe	7TM_1
	<i>HSA5</i>				23T	1	c.730C>T	p.His244Tyr (LOH)	7TM_1
	<i>OR1J3</i>								
	<i>OR1J5</i>								
	<i>OR9-19</i>								
	<i>OST044</i>								
<i>OR8B8</i>	<i>TPCR85</i>	8446.1	NM_012378	6 (5.0)	55T	1	c.221C>T	p.Ser74Phe (LOH)	7TM_1
					76T	1	c.221C>T	p.Ser74Phe (LOH)	7TM_1
					74T	1	c.361G>A	p.Asp121Asn	7TM_1
					76T	1	c.412C>T	p.Pro138Ser	7TM_1
					1T	1	c.885G>T	p.Lys295Asn	N/A
					1T	1	c.G925T	p.Ala309Ser	N/A
<i>OPN5</i>	<i>NEUROPSIN</i>	4923.1	NM_181744	3 (3.8)	90T	3	c.278C>A	p.Ser93Tyr	7TM_1
	<i>PGR12</i>				85T	4	c.545C>T	p.Ser182Leu	7TM_1
	<i>TMEM13</i>				64T	6	c.1007C>T	p.Thr336Ile	N/A
	<i>OR11-182</i>	31528.1	NM_001002907	2 (2.5)	36T	1	c.409C>A	p.Leu137Met	7TM_1
<i>OR8K1</i>	<i>OR8NIP</i>				13T	1	c.770T>C	p.Phe257Leu	7TM_1
<i>GPR98</i>	<i>DKFZp761P0710</i>	47246.1	NM_032119	43 (27.5)	85T	7	c.853C>T	p.Arg285Cys	N/A
	<i>FEB4</i>				76T	14	c.2570G>A	p.Trp857X	N/A
	<i>KIAA0686</i>				104T	20	c.3925C>T	p.Pro1309Ser	N/A
	<i>MASS1</i>				37T	24	c.5282C>T	p.Ser1761Phe	Calx-beta
	<i>USH2B</i>				76T	26	c.5515C>T	p.His1839Tyr	N/A
	<i>USH2C</i>				1T	28	c.5815G>A	p.Glu1939Lys	Calx-beta
	<i>VLGR1</i>				55T	28	c.6229G>A	p.Glu2077Lys	Calx-beta
	<i>VLGR1b</i>				77T	31	c.6901C>T	p.Gln2301X	N/A
					92T	31	c.6905C>T	p.Thr2302Ile	N/A
					39T	33	c.7330C>T	p.Leu2444Phe (LOH)	N/A
					58T	34	c.8093A>T	p.Asn2698Ile	N/A

Gene	Other names	CCDS accession ^a	RefSeq accession ^d	No. of mutations (% tumors affected) ^b		Tumor	Exon	Nucleotide ^c	Amino Acid ^c	Functional domain
CALX				45T	35	c.8201G>A		p.Gly2734Glu		N/A
				76T	37	c.8461G>A		p.Glu2821Lys		N/A
				76T	42	c.9049C>T		p.His3017Tyr		Calx-beta
				4T	42	c.9109C>T		p.Pro3037Ser		N/A
				21T	44	c.9524C>T		p.Thr3175Ile		N/A
				81T	45	c.9679C>T		p.Arg3227 ^d (LOH)		N/A
				17T	46	c.9826G>A		p.Glu3276Lys		N/A
				63T	50	c.10472C>T		p.Ser3491Phe		N/A
				76T	52	c.10789G>A		p.Glu3597Lys		Calx-beta
				85T	52	c.10852G>A		p.Glu3618Lys		Calx-beta
				85T	52	c.10888G>A		p.Gly3630Arg		N/A
				55T	53	c.11087G>A		p.Gly3696Glu		N/A
				17T	53	c.11107C>T		p.Pro3703Ser		N/A
				18T	55	c.11563G>A		p.Glu3855Lys		N/A
				37T	57	c.11869G>A		p.Ala3957Thr		N/A
				44T	61	c.12425G>A		p.Arg4142Gln		N/A
				17T	62	c.12595G>A		p.Gly4199Arg		N/A
				1T	65	c.13151C>T		p.Ser4384Phe		N/A
				55T	69	c.14032G>A		p.Gly4678Arg		N/A
				71T	73	c.14837G>A		p.Gly4946Glu		N/A
			44T	73	c.14846C>T		p.Ser4949Leu		N/A	
			76T	74	c.15106G>A		p.Asp5036Asn (LOH)		N/A	
			21T	74	c.15376G>A		p.Asp5126Asn		N/A	
			76T	74	c.15398T>A		p.Met5133Lys		N/A	
			76T	74	c.15407C>T		p.Ser5136Phe		N/A	
			34T	74	c.15644C>T		p.Ser5215Phe		N/A	
			76T	74	c.15688G>A		p.Glu5230Lys		N/A	
			33T	74	c.15832G>A		p.Gly5278Ser		N/A	
			76T	79	c.17041C>T		p.Gln5681X		N/A	
			74T	78	c.16825G>A		p.Asp5609Asn		N/A	

Gene	Other names	CCDS accession ^a	RefSeq accession ^a	No. of mutations (% tumors affected) ^b		Tumor	Exon	Nucleotide ^c	Amino Acid ^c	Functional domain
						21T	84	c.17965C>T	p.Leu5989Phe	7TM_2

^a Accession numbers for mutated GPCR in the UCSC and GenBank datasets.

^b Number of non-synonymous and splice site mutations observed and percent of tumors affected for each of the 11 genes in the panel of 80 melanoma cancers.

^c Nucleotide and amino acid changes resulting from mutation. 'LOH' refers to cases wherein the wild-type allele was lost and only the mutant allele remained. 'Splice site' refers to a case wherein the alteration affected fifteen bases spanning the exon. 7TM_1, 7 transmembrane receptor; 7TM_2, 7 transmembrane receptor (secretin family); 7TM_3, 7 transmembrane sweet-taste receptor of 3 GPCR; PBP1_mGluR_groupII, receptor family ligand binding region; latrophilin cytoplasmic, C-terminal region; NCD3G, 9 cysteines domain of family 3 GPCR; OLF, olfactomedin-like domain; calx-beta, calx-beta domain; N/A, not available.

Table 2EC₅₀ growth inhibition

Tumor line	EC₅₀ (nM)^a	s.e.m.
68T (p.Ser154Phe, p.Asp280Asn, p.Arg352Trp or p.Glu870Lys)	128	131
63T (p.Glu573Lys)	179	95
29T (splice site)	226	100
36T (p.Gly561Glu)	1075	145
55T (wild type)	13,357	156
76T (p.Gly88Glu or p.Ser610Leu)	15219	205
39T (p.Glu517Lys)	>30,000	>500
71T (wild type)	>30,000	478
49T (wild type)	>30,001	>500
13T (wild type)	>30,000	>500

^aEC₅₀ (half maximal effective concentration) values for cell growth inhibition by AZD-6244 of melanoma cell lines harboring wild-type or mutant GRM3.