Supplementary Appendix

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

Supplement to: Van Raamsdonk CD, Griewank KG, Crosby MB, et al. Mutations in *GNA11* in uveal melanoma. N Engl J Med 2010;363:2191-9. DOI: 10.1056/NEJMoa1000584.

Supplementary Appendix

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Supplementary Methods

Tissues

The archival, paraffin-embedded biopsies were retrieved from the archives of the Department of Ophthalmology, and the Dermatopathology Section of the Departments of Dermatology and Pathology of the University of California, San Francisco, the Department of Pathology of the Memorial Sloan Kettering Cancer Center, and the University of Graz, Austria under the approval of the institutional review boards at the participating institutions.

DNA

DNA from melanoma and nevi samples were obtained from archival paraffin-embedded biopsies. For each sample, DNA was extracted from sections of 5-20 μm thickness from which tumor-bearing tissue had been manually microdissected.

Sequencing

We successfully obtained sequencing data from *GNAQ* and *GNA11* exons 5 for 713 melanocytic neoplasms and from *GNAQ* and *GNA11* exons 4 for 453 of these samples. The exonic regions of *GNAQ* and *GNA11* (NCBI Human Genome Build 36.1) were broken into amplicons of 350 bp or less, and specific primers were designed using Primer 3, to cover the exonic regions plus at least 50 bp of intronic sequences on both sides of intron-exon junctions[1]. M13 tails were added to facilitate Sanger sequencing.

The PCR reaction conditions were 0.25 mM each dNTPs, 0.4X BSA (New England Biolabs), 1 U Hotstar Taq (Qiagen), 1X Hotstar Taq buffer (Qiagen), and 0.5 uM each primer: 5'-cgctgtgtcctttcaggatg-3' and 5'-ccacctcgttgtccgact-3' to examine *GNA11* Q209, 5'-gtgctgtgtccctgtcctg-3' and 5'-ggcaaatgagcctctcagtg-3' to examine *GNA11* R183, and 5'-tggtgtgatggtgtcactgacattctcat-3' and 5'-agctgggaaataggtttcatggactcagt-3' for *GNAQ* R183. Other primer sequences are available on request. PCR consisted of 35 cycles of 95 ^{o}C (30 seconds), 58^{o}C (1 minute), and 72 ^{o}C (1 minute) after initial denaturation at 94 ^{o}C for 15 minutes. PCR

reaction products were purified using QIAquick PCR Purification kit (Qiagen) and then used as templates for sequencing reactions using Big Dye v3.1 (Applied Biosystems); sequencing was performed in both directions. Dye terminators were removed using the CleanSEQ kit (Agencourt Biosciences), and subsequent products were run on the ABI PRISM 3730xl (Applied Biosystems, Foster City, CA). Samples identified with mutations in both sequencing directions were replicated at least twice. For samples with mutations, DNA was sequenced from the adjacent normal tissue to determine whether the mutations were somatically acquired.

We also sequenced all seven coding exons of *GNAQ* and *GNA11* in 97 uveal melanomas and 45 blue nevi. PCR reactions were carried out in 384 well plates, in a Duncan DT-24 water bath thermal cycler, with 10 ng of whole genome amplified DNA (GenomePlex, Sigma) as template, using a touchdown PCR protocol with HotStart Fast Taq (Kapa Biosystems, Cape Town, South Africa). The touchdown PCR method consisted of : 1 cycle of 95°C for 5 min; 3 cycles of 95°C for 30 sec, 64°C for 30 sec, 72°C for 60 sec; 3 cycles of 95°C for 30 sec, 62°C for 30 sec, 72°C for 60 sec; 3 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec; 37 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 60 sec; 1 cycle of 70°C for 5 min. Templates were purified using AMPure (Agencourt Biosciences, Beverly, MA). The purified PCR reactions were split into two, and sequenced bidirectionally with M13 forward and reverse primers. The sequencing reactions were carried out as above.

Mutation detection.

Mutations were detected using an automated detection pipeline at the MSKCC Bioinformatics Core. Bi-directional reads and mapping tables (to link read names to sample identifiers, gene names, read direction, and amplicon) were subjected to a QC filter which excludes reads that have an average phred score of < 10 for bases 100-200. Passing reads were assembled against the reference sequences for each gene, containing all coding and UTR exons including 5Kb upstream and downstream of the gene, using command line Consed 16.0[2]. Assemblies were passed on to Polyphred 6.02b[3] which generated a list of putative candidate mutations, and to Polyscan 3.0[4] which generated a second list of putative mutations. The lists were merged

together into a combined report, and the putative mutation calls were normalized to '+' genomic coordinates and annotated using the Genomic Mutation Consequence Calculator[5]. The resulting list of annotated putative mutations was loaded into a Postgres database along with select assembly details for each mutation call (assembly position, coverage, and methods supporting mutation call). All putative mutations detected by the automated pipeline were repeated by a second PCR and sequencing reaction, in parallel with amplification and sequencing of matched normal tissue DNA. All traces for mutation calls were manually reviewed.

Comparative genomic hybridization

Samples were labeled with a Bioprime Array CGH Genomic Labeling Kit according to the manufacturer's instructions (Invitrogen, Carlsberg, CA). Briefly, 500 ng test DNA and reference DNA (Promega, Madison, WI) were labeled with dCTP-Cy5 and dCTP-Cy3, respectively (GE Healthcare, Piscataway, NJ). Genome-wide analysis of DNA copy number changes was performed using oligonucleotide arrays according to the manufacturer's protocol version 6.0 (Agilent, Santa Clara, CA). Slides were scanned with Agilent's microarray scanner G2505B and analyzed using Agilent DNA Analytics software 4.0.76.

Plasmids

For the allograft tumor formation experiments, cDNA of $GNA11^{Q209L}$ was obtained from UMR cDNA Resource Center, epitope-tagged with an N-terminal Myc-tag and cloned into Wzl retroviral expression vectors. $GNA11^{wt}$ and $GNA11^{R183C}$ versions were engineered from $GNA11^{Q209L}$ using the Stratagene Lightning Mutagenesis Kit. The *GNAQ* and *GNA11* constructs used in Western blots were obtained from the Missouri S&T cDNA resource center. They are internally Glu-Glu tagged, with altered residues at position 171-176 from AYLPTQ (Gαq) or GYLPTQ (Gα11) to EYMPTE[6], and were cloned into Wzl-retrovirus vectors. All constructs were sequenced for confirmation.

Transduction

Viral supernatants were generated using appropriate packaging cell lines and transfected with 10 μg plasmid and lipofectamine 2000. Media were changed 16 hr after transfection and the virus was harvested 40 to 56 hr later. Melan-a cells were transduced and positively selected with blasticidin.

Cell culture

Melan-a cells[7] (a gift from Dr. Dorothy C Bennett, St. George University, London, UK), were cultured in glutamine-containing RPMI media supplemented with 10% FCS and 200 nM TPA.

Western blot analysis

Cells were washed twice with ice-cold PBS and lysed in 50mM Tris–HCl pH7.8, 1% NP-40, 10% glycerol, 150mM NaCl, 1% sodium deoxycholate, 1% sodium dodecyl sulphate, supplemented with protease inhibitor, phosphatase inhibitor and EDTA (Pierce Biotechnologies). The protein content of the lysates was determined by the BCA Protein Assay Reagent (Pierce Biotechnologies). 15μg of protein were separated by SDS–PAGE and transferred to Immobilon-P membrane (Millipore). Primary antibodies were pERK (E-4, Santa Cruz Biotechnology) and β-actin (Sigma). Secondary antibodies were labeled with horseradish peroxidase.

Tumorigenicity study

Melan-a cells were transduced with the respective *GNA11* expression constructs or a β-galactosidase control vector, and selected with 5 μg/ml blasticidin over two weeks. Selected cells were expanded, trypsinized, washed in PBS, and re-suspended in DMEM at 10 million cells per milliliter. Four month old female NOD/SCID/interleukin 2 receptor γ^{null} were injected with 1 million cells subcutaneously in both flanks. Mice were palpated weekly for the development of tumors and tumor sizes were determined using calipers.

Statistical Analysis

Association of mutation status with location, cell type and initial treatment and comparison of different mutation types were performed using two sided Fisher's exact tests. Both trichotomized (*GNAQ*, *GNA11* or neither) and dichotomized (mutant or not) mutation status were used. For 2 by 2 association tests, p-values were obtained from hyper-geometric distribution while 2 by 3 and larger cases were tested using Mehta and Patel's algorithm[8]. Survival analyses were performed using log-rank tests.

References for the supplementary information

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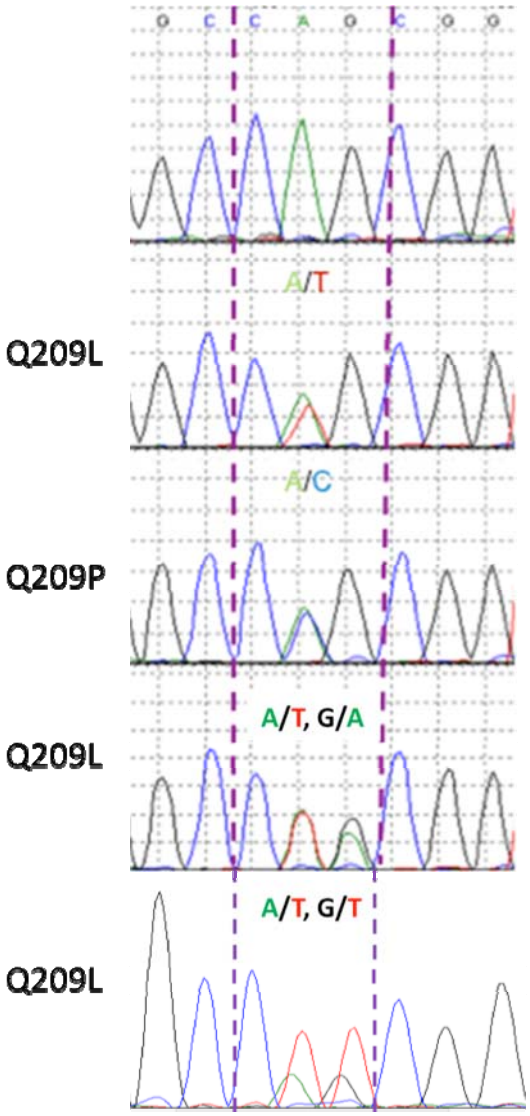
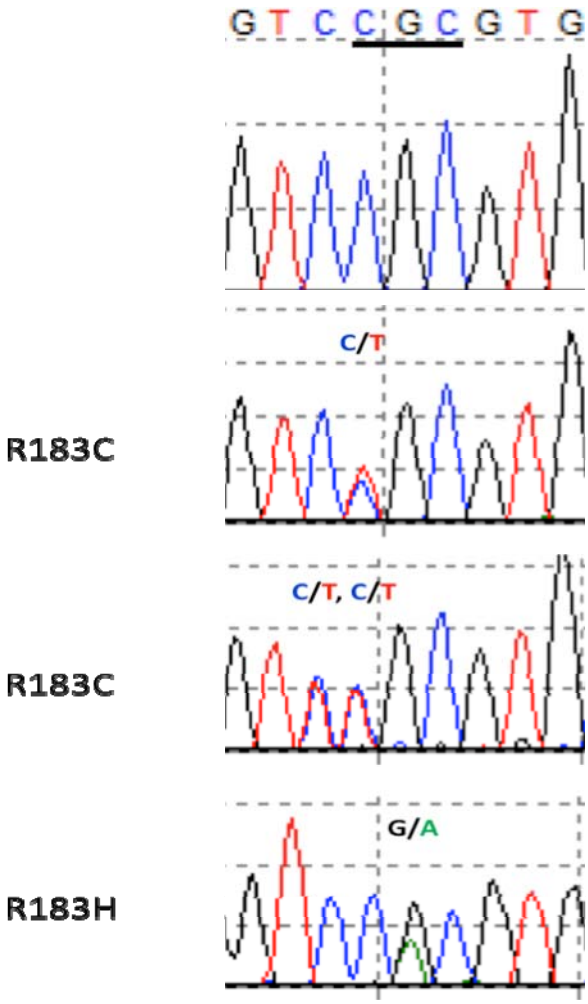
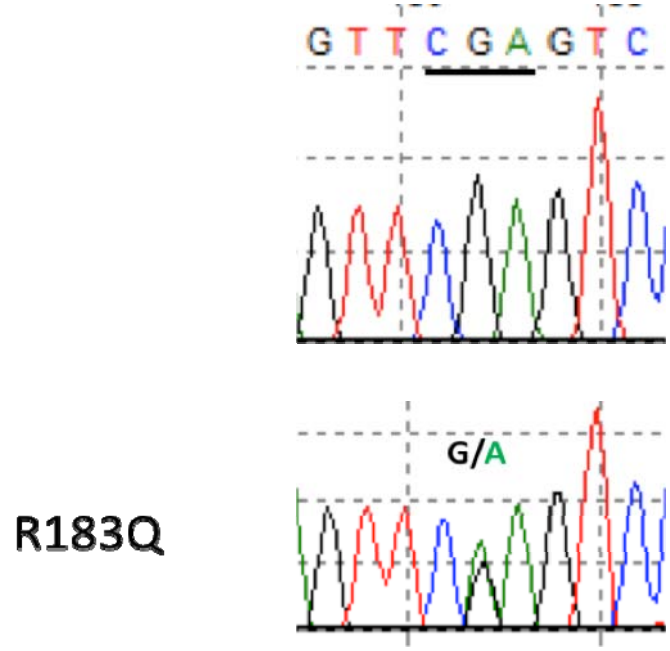


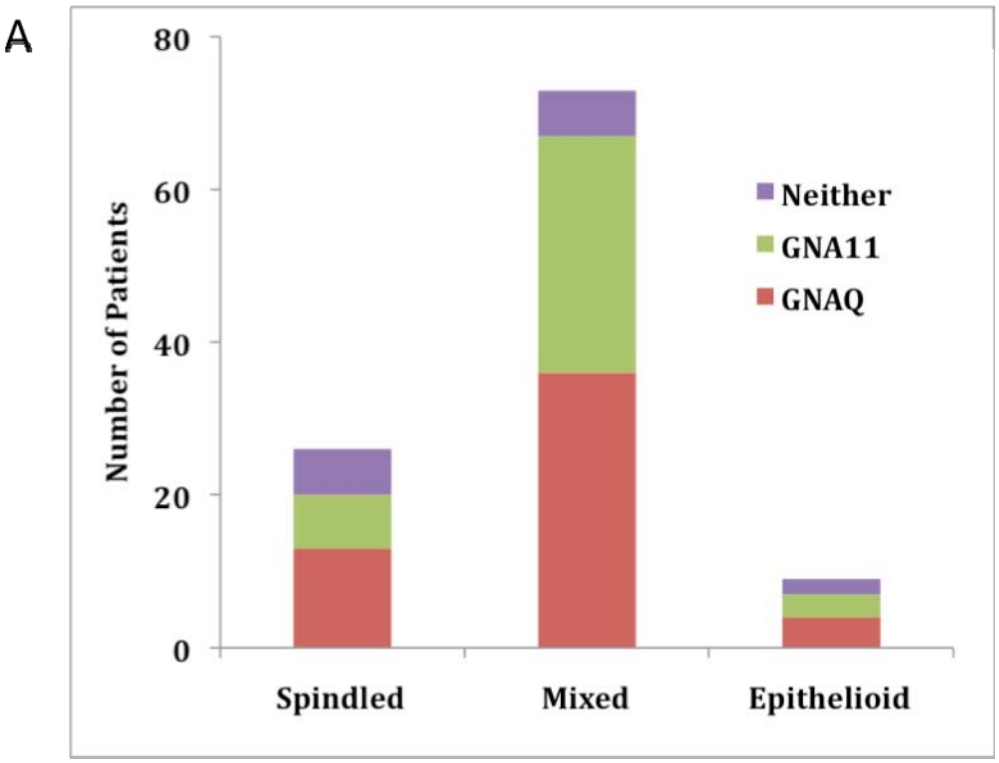
Supplementary Figure 1. Mutation spectrum of *GNA11* at codon 209. The top panel shows the wild type sequence of codon 209 coding for glutamine (between dashed lines). The most common mutation was an adenine to thymine transversion resulting in a substitution to leucine (*GNA11*^{Q209L}) (96% of cases). We saw one case each of CAG to CTA (Q209L), CAG to CTT (Q209L), and CAG to CCG (Q209P).



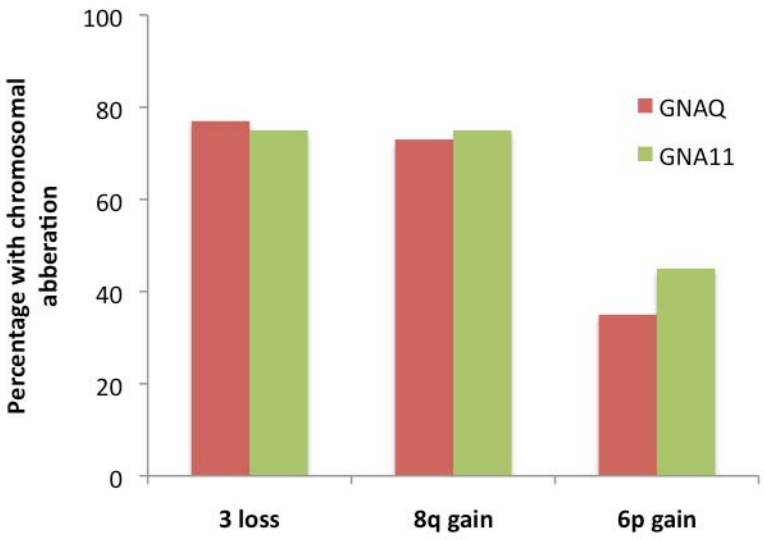
Supplementary Figure 2. Mutation spectrum of *GNA11* at codon 183. The top panel shows the wild type sequence of codon 183 coding for arginine (underlined). Most mutations resulted in a substitution to cysteine (*GNA11*^{R183C}), either caused by a single cytosine to thymine transition (one case) or a double cytosine to thymine transition (three cases), with the latter resulting in a synonymous mutation also affecting codon 182. A single blue nevus had a guanine to adenine transition, causing a substitution to histidine (*GNA11*^{R183H}).



Supplementary Figure 3. Mutation spectrum of *GNAQ* at codon 183. The top panel shows the wild type sequence of codon 183 coding for arginine (underlined). The only type of mutation found was a guanine to adenine transition, resulting in a substitution to glutamine (*GNAQ*^{R183Q}).



Supplementary Figure 4. **Histopathological features and mutation status.** There was a trend towards increased *GNAQ* and *GNA11* mutations in primary uveal melanomas with mixed or epithelioid cell histopathology compared to primaries with pure spindle-cell morphology but this was not statistically significant ($p>0.05$).



Supplementary Figure 5: Chromosomal aberrations and mutation status. *GNAQ* and *GNA11* were not associated with established prognostically relevant chromosomal aberrations in uveal melanoma. All cases for which copy number status was available had either a *GNAQ* or a *GNA11* mutation.

Survival Patient Characteristics	Total	GNAQ	GNA11	Neither	p-value
n	81	43	26	12	n.d.
Female/Male	40/41	25/18	11/15	4/8	n.s.
Median age and range at diagnosis (years)	63 (33-87)	63 (33-87)	63 (41-82)	63 (34-86)	n.s.
Average Length of Follow-up (months)	36 (1-248)	34 (1-248)	38 (1-148)	41 (7-99)	n.s.
Incidence of Metastases	27% (22/81)	26%(11/43)	15%(4/26)	58% (7/12)	n.s.
Incidence of death	22% (18/81)	21% (9/43)	12% (3/26)	50% (6/12)	n.s.
Mean tumor thickness and range (mm)	9.30 (1.2-18)	8.79 (1.2-18)	10.32 (3.2-18)	8.73 (2-15.1)	n.s.
Mean tumor diameter and range (mm)	13.12 (1-25)	12.34 (1-25)	13.96 (4.8-23.2)	14.56 (6.50-24)	n.s.
Initial Treatment					*
Primary Enucleation	44 (54%)	20 (47%)	20 (77%)	4 (33%)	**
Proton Beam	25 (31%)	13 (30%)	5 (19%)	7 (58%)	n.s.
Ru-106 Plaque	4 (5%)	3 (7%)	1 (4%)	0	n.s.
Gamma-Knife	8 (10%)	7 (16%)	0	1 (8%)	n.s.
Transthermal Therapy	1	1	0	0	n.d.

Supplementary Table 1. Demographic and treatment information of the 81 patients included in survival analysis.

* Mutation status was significantly associated with initial treatment (p-value 0.043). Comparing primary enucleation to any other treatment, *GNA11* was more common in tumors treated by primary enucleation (p-value: *GNA11* vs. Neither/*GNAQ* 0.044). The single case treated with transthermal therapy sample was excluded from these analyses.