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Oscar Urtatiz, Amanda Haage, Guy Tanentzapf, Catherine D. Van Raamsdonk

Institutions: University of British Columbia

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Crosstalk with keratinocytes causes GNAQ oncogene specificity in melanoma

Oscar Urtatiz¹, Amanda Haage², Guy Tanentzapf², and Catherine D. Van Raamsdonk¹

¹Department of Medical Genetics
Life Sciences Institute
University of British Columbia
2350 Health Sciences Mall
Vancouver, British Columbia, V6T 1Z3
Canada

² Department of Cellular and Physiological Sciences
Life Sciences Institute
University of British Columbia
2350 Health Sciences Mall
Vancouver, British Columbia, V6T 1Z3
Canada

***Corresponding author:**

Catherine D. Van Raamsdonk
604-827-4224
cvr@mail.ubc.ca

RUNNING TITLE: Epidermal crosstalk causes GNAQ oncogene specificity

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31 **ABSTRACT**

32 Different melanoma subtypes exhibit specific and non-overlapping sets of oncogene and tumor
33 suppressor mutations, despite a common cell of origin in melanocytes. For example, activation of
34 the $G\alpha_{q/11}$ signaling pathway is a characteristic initiating event in primary melanomas that arise
35 in the dermis, uveal tract or central nervous system. It is rare in melanomas arising in the
36 epidermis. Here, we present evidence that in the mouse, crosstalk with the epidermal
37 microenvironment actively impairs the survival of melanocytes expressing the $GNAQ^{Q209L}$
38 oncogene, providing a new model for oncogene specificity in cancer. The presence of epidermal
39 cells inhibited cell division and fragmented dendrites of melanocytes expressing $GNAQ^{Q209L}$ in
40 culture, while they promoted the growth of normal melanocytes. Differential gene expression
41 analysis of FACS sorted epidermal melanocytes showed that cells expressing $GNAQ^{Q209L}$ exhibit
42 an oxidative stress and apoptosis signature previously linked to vitiligo. Furthermore, $PLCB4$,
43 the direct downstream effector of $G\alpha_{q/11}$ signaling, is frequently mutated in cutaneous melanoma
44 alongside P53 and NF1. Our results suggest that a deficiency of $PLCB4$ promotes cutaneous
45 melanomagenesis by reducing $GNAQ$ driven signaling. Hence, our studies reveal the flip side of
46 the $GNAQ/PLCB4$ signaling pathway, which was hitherto unsuspected. In the future,
47 understanding how epidermal crosstalk restrains the $GNAQ^{Q209L}$ oncogene could suggest novel
48 melanoma therapies.

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53 INTRODUCTION

54 *GNAQ* and *GNAI1* encode heterotrimeric G protein alpha subunits that are best known for
55 signaling through phospholipase C-beta (PLCB) to release intracellular calcium stores¹. The
56 somatic substitution of glutamine 209 or arginine 183 in either *GNAQ* or *GNAI1* generates
57 constitutive activity of the G protein and is a characteristic early event in uveal melanoma,
58 occurring in up to 90% of cases². These mutations are also frequent in blue nevi and primary
59 melanomas of the central nervous system²⁻⁴. Activation of the pathway in uveal melanoma drives
60 cell proliferation and stimulates the mitogen activated protein kinase ('MAPK') pathway through
61 RasGRP3⁵. G $\alpha_{q/11}$ activation also activates the Hippo pathway through nuclear localization of
62 YAP1 via a Trio-Rho/Rac signaling circuit⁶. On the other hand, Q209 and R183 mutations in
63 either *GNAQ* or *GNAI1* are rare in melanocytic neoplasms arising in the epithelium, which are
64 more likely to carry mutations in the MAPK pathway components, *BRAF*, *NRAS* and *NFI*^{3, 7, 8}.
65 The underlying mechanism for the restriction of *GNAQ* and *GNAI1* mutations to non-epithelial
66 melanomas is unknown.

67
68 There are several possible, non-exclusive explanations for this phenomenon. First, an over-
69 representation of mutations in specific genes in certain neoplasms could be related to exposure to
70 different mutagens in different parts of the body. Concerning melanoma, the skin areas exposed
71 to UV radiation via sunlight accumulate many somatic mutations; characteristically, these are C
72 to T transitions⁹. Most UV radiation does not penetrate very deeply into the body and some areas
73 of the body are rarely exposed, which could generate a varying spectrum of mutations¹⁰.

74
75 Secondly, melanocytes arise from neural crest cells all along the anterior-posterior axis. The
76 molecular pathways for melanocyte cell fate determination along this axis are poorly
77 understood¹¹. However, there are demonstrated differences in the potential for melanomagenesis
78 of melanocytes arising at different anterior-posterior positions in the mouse embryo¹². It is
79 possible that the developmental origin of a melanocyte could influence its migration during
80 embryogenesis, its differentiation potential and program long-term gene expression patterns.
81 Therefore, some melanocytes might only respond to specific driver mutations due to intrinsic
82 differences generated during development.

83
84 Lastly, different microenvironments surround melanocytes located in different parts of the body,
85 which could impact the effects of mutations. Direct cell-cell contact or paracrine signaling
86 produced by the tissue-specific microenvironment might allow the transformation of cells only
87 with certain mutational events, forcing the selection of specific driver mutations in melanoma¹³.
88 Epidermal melanocytes interact mainly with keratinocytes, while internal melanocytes interact
89 with various mesodermal stromas. Therefore, crosstalk between melanocytes and their cellular
90 neighbors might prevent transformation or proliferation driven by specific signaling pathways.

91
92 In this paper, we investigated these possibilities. We forced the expression of oncogenic
93 GNAQ^{Q209L} in all melanocytes in mice beginning in adulthood using *Tyr-creER*. This drove the
94 loss of melanocytes from the interfollicular (IFE) epidermis of the tail, while dramatically
95 increasing melanocyte growth in the dermis. This observation demonstrated that GNAQ^{Q209L}
96 expression was not simply neutral for epidermal melanocytes; it was deleterious. We established
97 primary cultures of normal or GNAQ^{Q209L} expressing melanocytes sorted from the mouse tail
98 epidermis and used time lapse microscopy to quantify melanocyte cell dynamics in the presence
99 of either fibroblasts or keratinocytes. We discovered that paracrine signaling from the epidermis
100 reversibly switches GNAQ^{Q209L} from an oncogene to an inhibitor of melanocyte survival and
101 proliferation. Differential expression analysis of melanocytes sorted from the tail epidermis
102 showed that the melanocytes expressing GNAQ^{Q209L} exhibited alterations in gene expression
103 related to cell adhesion, axon extension, oxidative stress and apoptosis. Since GNAQ^{Q209L}
104 signaling was deleterious to epidermal melanocytes, we wondered whether loss of function
105 mutations in *PLCB4* might promote melanomagenesis. We examined 470 cases in the Cancer
106 Genome Atlas (TCGA) skin cutaneous melanoma (SKCM) dataset and found that 21% had a
107 mutation in *PLCB4*. *PLCB4* mutations were enriched in cases with *TP53* and *NF1* mutations. We
108 describe the relationship between mutation load and the combinations of certain gain and loss of
109 function drivers in the dataset.

110

111 RESULTS

112

113 **Forced GNAQ^{Q209L} reduces melanocytes in mouse tail inter-follicular epidermis (IFE)**

114 We previously generated a conditional GNAQ^{Q209L} allele wherein oncogenic human GNAQ^{Q209L}
115 is expressed from the *Rosa26* locus in mice following the removal of a loxP-flanked stop cassette
116 ("*R26-fs-GNAQ^{Q209L}*")¹⁴. We previously showed that forcing GNAQ^{Q209L} expression in
117 melanocytes beginning during embryogenesis using the *Mitf-cre* transgene reduced the number
118 of melanocytes in the inter-follicular epidermis (IFE)^{14, 15}. To test whether GNAQ^{Q209L} affected
119 melanocyte establishment or melanocyte maintenance, we used the tamoxifen (TM) inducible
120 melanocyte-specific Cre transgene, *Tyrosinase (Tyr)-creERT²* with *R26-fs-GNAQ^{Q209L}* to induce
121 GNAQ^{Q209L} expression in melanocytes beginning in adulthood. We also included the *Rosa26-*
122 *LoxP-Stop-LoxP-LacZ* reporter to label cells expressing GNAQ^{Q209L} with LacZ^{16, 17}. TM was
123 administered once per day for 5 days at 4 weeks of age. One week following the last dose of TM,
124 half of the mice were euthanized to collect and stain tail epidermal sheets with X-gal to
125 determine the average number of melanocytes (LacZ-positive cells) per scale. At this time point,
126 *R26-fs-GNAQ^{Q209L}/R26-fs-LacZ; Tyr-creERT²/+* (hereafter referred to as "GNAQ-lacZ") mice
127 and *+/R26-fs-LacZ; Tyr-creERT²/+* (hereafter referred to as "WT-LacZ") mice showed no
128 significant difference in melanocyte numbers (p=0.18, **Figures 1A, B**). However, 8 weeks
129 following the last dose of TM, GNAQ-LacZ mice had on average 50% fewer melanocytes per
130 scale than WT-LacZ mice (p=0.045, **Figures 1A, B**). While scales in WT-LacZ mice were
131 pigmented evenly, many scales in GNAQ-LacZ mice exhibited partial loss of melanin 8 weeks
132 after TM (p=0.048, **Figure 1C, D**). These results demonstrate that GNAQ^{Q209L} signaling inhibits
133 melanocyte maintenance in the IFE.

134

135 **The effects of GNAQ^{Q209L} signaling in melanocytes are reversible**

136 To isolate melanocytes from the epidermis for analysis *in vitro*, we used *Mitf-cre* with *Rosa26-*
137 *LoxP-Stop-LoxP-tdTomato* and *Rosa26-fs-GNAQ^{Q209L}* to label the melanocyte lineage with a
138 robust and intense fluorescent tdTomato signal. To confirm appropriate labeling, we first
139 compared sections of the tail skin of *+/R26-fs-tdTomato; Mitf-cre/+* ("WT") and *R26-fs-*
140 *GNAQ^{Q209L}/R26-fs-tdTomato; Mitf-cre/+* ("GNAQ^{Q209L}") mice at 4 weeks of age (**Figure 1E-F**).

141 Sections of WT tail skin exhibited tdTomato-positive cells in the IFE, located as expected on the
142 basal membrane, as well as some tdTomato-positive cells in the dermis. In contrast, sections of
143 GNAQ^{Q209L} tail skin contained fewer tdTomato-positive cells in the IFE while exhibiting an
144 abnormally extensive tdTomato signal in the dermis.

145
146 Next, we isolated tdTomato-positive melanocytes from the IFE of WT and GNAQ^{Q209L} mice by
147 Fluorescent Activated Cell Sorting (FACS) to study the melanocytes in *in vitro* settings. To
148 obtain a single cell suspension for FACS, we first split the tail skin IFE from the underlying
149 dermis. Then, we dissociated the scales within the IFE with trypsin to obtain melanocytes and
150 keratinocytes as dispersed single cells. As expected, fewer tdTomato-positive cells were sorted
151 from GNAQ^{Q209L} mice (0.61%) compared to WT mice (0.99%) (p=0.012, **Figure 2A,B**).
152 Previous studies have estimated that melanocytes account for ~1.5% of the total cells in the
153 IFE¹⁸.

154
155 We then seeded the sorted IFE melanocytes on fibronectin-coated plates to study baseline
156 survival. The GNAQ^{Q209L} expressing melanocytes survived better than the WT melanocytes
157 (p=3.3 x 10⁻⁵ for genotype, 2 way ANOVA analysis, **Figure 2C**). Hence, the growth-inhibiting
158 effects of GNAQ^{Q209L} expression in IFE melanocytes are reversible. When removed from the
159 epidermis and grown on fibronectin, IFE melanocyte survival was increased by GNAQ^{Q209L}
160 expression.

161
162 To compare the effects of GNAQ^{Q209L} with another well studied melanoma oncogene, we used
163 the same methods to obtain Braf^{V600E} expressing melanocytes from the mouse tail IFE. In mice,
164 the expression of Braf^{V600E} leads to melanocytic over-growth in both the tail dermis and
165 epidermis¹⁹. We crossed the conditional *Braf*^{V600E} allele, which expresses *Braf*^{V600E} from the
166 endogenous *Braf* locus²⁰ to *R26-fs-tdTomato* and *Mitf-cre*. Then, we used FACS to isolate
167 epidermal melanocytes from the tail IFE of 4-week old mice (+/*R26-fs-tdTomato*; *Mitf-cre*/+;
168 *Braf*^{CA}/+, hereafter referred to as "Braf^{V600E}"). We found that Braf^{V600E} expression increased the
169 survival of sorted IFE melanocytes plated on fibronectin, even more so than GNAQ^{Q209L} (**Figure**

170 **2C).** Melanocytes expressing $\text{Braf}^{\text{V600E}}$ could be maintained in culture for at least 8 days (**Figure**
171 **2G).**

172
173 We observed a striking difference in cell morphology of $\text{GNAQ}^{\text{Q209L}}$ melanocytes compared to
174 WT melanocytes grown *in vitro*. $\text{GNAQ}^{\text{Q209L}}$ melanocytes exhibited a more dendritic
175 morphology with several protrusions per cell, while WT melanocytes appeared mostly spindle-
176 shaped (**Figure 2D**). To study the cellular dynamics of the melanocytes, we analyzed time lapse
177 microscopy for 20 hours (**Figure 2E, F**). No migration, formation of new protrusions or cell
178 divisions were observed in the melanocytes of either the WT or $\text{GNAQ}^{\text{Q209L}}$ cultures plated on
179 fibronectin alone. However, in WT cultures, some melanocytes were observed to adopt a round
180 shape, after which the cells were immediately lost from view (presumed cell death, example
181 shown in the circled cell in **Figure 2E**).

182
183 As another test of $\text{GNAQ}^{\text{Q209L}}$ reversibility, we isolated mouse embryonic fibroblasts (MEFs)
184 and plated them onto fibronectin-coated plates to simulate a dermis-like microenvironment. Then
185 we added FACS sorted IFE melanocytes from either WT or $\text{GNAQ}^{\text{Q209L}}$ mouse tails. The growth
186 of both WT and $\text{GNAQ}^{\text{Q209L}}$ expressing melanocytes was stimulated by MEF co-culture, as there
187 was an increase in cell number above the original number plated, unlike on fibronectin alone
188 (**Figure 2H**). In this situation, we did not detect a difference in survival between WT and
189 $\text{GNAQ}^{\text{Q209L}}$ melanocytes ($p=0.48$, 2 way ANOVA). When grown with MEFs, $\text{GNAQ}^{\text{Q209L}}$
190 melanocytes grew much larger and irregularly shaped than WT melanocytes (**Figure 2I**), similar
191 to their appearance in the dermis of tail sections (**Figure 1F**).

192
193 To summarize, our findings suggest that the attrition of $\text{GNAQ}^{\text{Q209L}}$ expressing IFE melanocytes
194 from the mouse tail epidermis is not due to an inherent characteristic in IFE melanocytes because
195 the phenotype is reversible by switching the microenvironment.

196
197

198 **The IFE impairs the survival and proliferation of GNAQ^{Q209L} melanocytes *in vitro***

199 Keratinocytes are known to affect melanocyte proliferation and survival in the IFE in a normal
200 context, so we suspected that interactions between keratinocytes and melanocytes in the IFE
201 could be modifying the outcome of GNAQ^{Q209L} signaling. To test this, we dissociated tail IFE
202 into single cells and plated the suspension onto fibronectin-coated plates. Co-culturing with
203 dispersed IFE provided a significant boost to WT melanocyte survival compared to fibronectin
204 coating alone (p=0.0071 for culture condition, 2 way ANOVA, **Figure 3A**). In contrast, the co-
205 culture of IFE with GNAQ^{Q209L} expressing melanocytes caused a significant reduction in
206 melanocyte survival throughout the five days of the experiment (p=0.0053 for culture condition,
207 2 way ANOVA, **Figure 3B**).

208
209 Observation using time lapse microscopy showed that both WT and GNAQ^{Q209L} melanocytes
210 exhibited different behavior when cultured with IFE compared to culture on fibronectin alone,
211 with both WT and GNAQ^{Q209L} melanocytes migrating and extending and retracting protrusions
212 (**Figure 3C**, example **Videos 1 and 2**). GNAQ^{Q209L} expressing melanocytes exhibited longer and
213 more numerous protrusions (p=0.0096 and p=0.00041, respectively, **Figure 3E, 3F**). Perhaps
214 related to the unusually long protrusion length, breakage and fragmentation of dendrites was
215 observed in 45% of the GNAQ^{Q209L} melanocytes tracked by time lapse microscopy for 20 hours
216 (**Figure 3H, 3I**). In comparison, this phenomenon was observed in just 5% of WT melanocytes
217 (p=0.021). The average cell area of GNAQ^{Q209L} melanocytes was 2.7-fold greater than WT
218 melanocytes (p=2.6x10⁻¹³, **Figure 3G**) and the cells were less circular (p=5.0x10⁻⁸, **Figure 3D**).
219 These changes in cell morphology and dynamics did not seem to affect cell migration (**Figure**
220 **4A**). We found no difference in the total length traveled or the straightness of path in GNAQ^{Q209L}
221 versus WT melanocytes cultured with IFE (p=0.86 and p=0.18, **Figure 4B, 4C**).

222
223 We also monitored for cell division events using time lapse microscopy. During the 20 hours of
224 observation, 16% of WT melanocytes co-cultured with IFE underwent a cell division event
225 (**Figure 4D**, example shown in **Figure 4E, video 1**). WT melanocytes smoothly progressed from
226 cleavage furrow formation to the separation of daughter cells and on average this took about 50
227 minutes (**Figure 4H**). In contrast, only one of the 99 tracked GNAQ^{Q209L} melanocytes divided

228 (p=0.0040, **Figure 4D**). In this cell, furrow formation was not as clear and the process took 150
229 minutes (**Figure 4F**). We used the same methods to examine the effects of the $\text{Braf}^{\text{V600E}}$
230 oncogene on proliferation (**Figure 4G**). During 20 hours of observation, 32% of $\text{Braf}^{\text{V600E}}$
231 melanocytes underwent a cell division event (**Figure 4D**). Cell division took the least time in
232 $\text{Braf}^{\text{V600E}}$ expressing melanocytes (**Figure 4H**).

233
234 To determine whether the reduced survival of $\text{GNAQ}^{\text{Q209L}}$ expressing melanocytes co-cultured
235 with IFE was due to direct contact inhibition or a paracrine mechanism, we used a transwell
236 assay in which we seeded FACS collected IFE melanocytes on a permeable membrane with
237 dissociated IFE from the same animal plated in the well underneath. If direct cell-cell contact
238 was necessary for the inhibitory effect of the IFE, then we would expect increased survival of
239 $\text{GNAQ}^{\text{Q209L}}$ melanocytes in this culture system compared to simple co-culture. We found that it
240 made no difference whether the IFE was directly in contact with the $\text{GNAQ}^{\text{Q209L}}$ expressing
241 melanocytes or present in the same dish, separated by a membrane (**Figure 5B**). In WT
242 melanocytes, there was no significant difference in survival for the first three days, after which
243 the transwell melanocytes developed an advantage (**Figure 5A**). We conclude that paracrine
244 signaling from the microenvironment helps switch $\text{GNAQ}^{\text{Q209L}}$ from an oncogene to an inhibitor
245 of cell survival.

246
247 Altogether, the cell culture experiments revealed that IFE co-culture stimulates survival and cell
248 division of WT melanocytes, but inhibits these processes in $\text{GNAQ}^{\text{Q209L}}$ melanocytes.
249 Furthermore, the IFE stimulated protrusion activity in both WT and $\text{GNAQ}^{\text{Q209L}}$ cells, with the
250 $\text{GNAQ}^{\text{Q209L}}$ expressing cell hyper-responding. Since both WT and $\text{GNAQ}^{\text{Q209L}}$ melanocytes
251 were static when plated on fibronectin alone, these experiments show that the IFE plays a vital
252 role in melanocyte regulation. We conclude that crosstalk between melanocytes and the IFE
253 reversibly switches GNAQ signaling from promoting to inhibiting melanocyte growth and
254 survival.

255
256
257

258 **Gene expression analysis: Regulation of cell adhesion and pseudopod dynamics**

259 To identify the pathways that change in response to GNAQ^{Q209L} expression in IFE melanocytes,
260 we performed RNA sequencing (RNA-seq) immediately after sorting WT and GNAQ^{Q209L}
261 melanocytes from tail IFE (n=6 mice and n=3 libraries per genotype). There were 14,461 genes
262 with an average FPKM > 0.1 in WT and/or GNAQ^{Q209L} melanocytes (**Supplementary Table 1**).
263 Seven genes previously shown to be directly involved in pigment production and melanosome
264 biology were on the list of the top 20 most highly expressed genes in WT melanocytes,
265 validating our melanocyte isolation protocol (**Supplementary Table 2**). These genes were *Pmel*,
266 *Dct*, *Tyrp1*, *Mlana*, *Cd63*, *Slc24a5*, and *Gpnmb*. We analyzed differential gene expression using
267 DEseq2. 1,745 genes were found to be differentially expressed (DE), of which 729 genes were
268 down-regulated and 1016 genes were up-regulated in GNAQ^{Q209L} melanocytes (q-value < 0.05)
269 (**Supplementary Tables 3 and 4**).

270
271 We first used the DAVID Functional Annotation Tool (6.8) to understand the biological
272 significance of the DE genes in GNAQ^{Q209L} melanocytes. We analysed the combined list of up
273 and down-regulated DE genes with a log₂ fold change ("LFC") of > 2.0 or < -2.0. Using Gene
274 Ontology (GO) analysis and KEGG pathway analysis, there were two dominant signatures. One
275 overlapping set of genes supported the terms: cell adhesion, focal adhesion, extracellular matrix-
276 receptor interactions and extracellular matrix structural constituents (**Figure 6A, Supplementary**
277 **Table 5**). Overlapping genes supported a second signature for the terms: axons, axon guidance
278 and nervous system development (**Supplementary Table 6**).

279
280 For changes related to cellular adhesion, almost all the identified genes were up-regulated in
281 expression. This included types *IV*, *V*, and *XXVII collagens*, *Fibronectin 1 (Fn1)*, *Integrin alpha*
282 *1*, *Integrin beta 3*, *Amigo1*, *Mcam*, *Cadherin 6*, *Cercam* and *Esam*. Examining the complete list
283 of DE genes (with no cut-off), we also found that two Rho GEFs that interact with Gα_{q/11}, *Trio*
284 and *Kalirin (Kalrn)*, were up-regulated (LFC 0.5 and 1.8). RhoGEFs catalyze GDP to GTP
285 exchange on small Rho guanine nucleotide-binding proteins, regulating the actin cytoskeleton.
286 We also noticed that several genes that play an essential role in lamellipodia formation²¹
287 including *RhoB*, *RhoC*, *Cdc42*, and *Rock* were all up-regulated by LFC > 1.0. Increased adhesion

288 and cell-ECM interactions could underlie the changes in cell shape and increased cellular area in
289 GNAQ^{Q209L} melanocytes co-cultured with IFE (**Figure 3**).

290
291 To examine the actin cytoskeleton in GNAQ^{Q209L} and WT melanocytes, we measured the overall
292 level of actin alignment, termed cell fibrousness, using an *in vitro* quantitative approach
293 previously described by Haage *et al*²². First, WT and GNAQ^{Q209L} tdTomato-positive
294 melanocytes co-cultured with IFE were stained for f-actin using phalloidin (**Figure 6B**). Then,
295 confocal z-projections of individual melanocytes were obtained and processed using
296 quantitative image analysis to determine cell fibrousness. GNAQ^{Q209L} expressing melanocytes
297 exhibited less cell fibrousness than WT melanocytes ($p=1.8 \times 10^{-12}$, **Figure 6C**). Hence,
298 GNAQ^{Q209L} expressing melanocytes have less organized actin cytoskeletons.

299
300 Melanocytes are known to share signaling pathways with neurons²³. The DE genes related to
301 axons (identified by pathway analysis in **Supplementary Table 6**) are diverse in function;
302 however, the number of *Semaphorin* genes on the list stands out, suggesting that these signaling
303 molecules might be important. While the autocrine effects of Semaphorins on melanocytes has
304 not been addressed before, it is known that keratinocytes express semaphorins to regulate
305 melanocytes²⁴. Semaphorin signaling controls the formation of cellular extensions such as axons
306 and dendrites in both neurons and melanocytes²⁵. Semaphorins bind to two main classes of
307 receptors, plexins and neuropilins. Some semaphorins can also bind the Integrin beta 3
308 receptor²⁴. Scott *et al.* showed that Sema7a binding to Integrin beta 3 induced melanocyte
309 spreading and dendricity, while Sema7a binding to plexin C1 inhibited dendrite formation²⁴.
310 Another study found that the absence of Sema3A leads to defasciculation and abnormal
311 extension of certain cranial nerves²⁶.

312
313 Among the complete list of DE genes, the semaphorins that were up-regulated were *Sema3c*,
314 *Sema3d*, *Sema3g*, *Sema4c* and *Sema4f*, while *Sema3a*, *Sema3b*, *Sema4b*, *Sema5a*, *Sema6d* and
315 *Sema7a* were down-regulated. *Sema4f* was the most highly up-regulated *Sema* family member
316 (LFC 4.4), followed by *Sema3g* (LFC 2.8). In GNAQ^{Q209L} melanocytes, the expression of
317 *Integrin beta 3* was also up-regulated (LFC 3.6), while *Plexin C1* was down-regulated (LFC -

318 0.7). These expression changes in *Sema3A*, *Sema7A*, *Integrin beta 3* and *Plexin C1* are consistent
319 with the abnormally long dendrites and increased cellular area in GNAQ^{Q209L} expressing
320 melanocytes²⁴.

321
322 To summarize, the RNAseq data suggests that the impact of GNAQ^{Q209L} expression on IFE
323 melanocyte morphology is mediated through increased cell adhesion, a disorganized actin
324 cytoskeleton and the promotion of dendrite extensions possibly stimulated by changes in
325 semaphorin signaling.

326

327 **Gene expression analysis: Cellular stress and apoptosis**

328 Using the RNAseq data, Gene set enrichment analysis (GSEA) revealed that GNAQ^{Q209L} IFE
329 melanocytes express a pattern of genes related to cellular stress and apoptosis. GSEA showed
330 that GNAQ^{Q209L} melanocytes are enriched in the hallmarks for "apoptosis", "p53 pathway", and
331 "hypoxia" (**Figure 6D**). Among the top-ranked DE genes was *Stanniocalcin (Stc1)* which
332 encodes a glycoprotein hormone involved in calcium/phosphate homeostasis. *Stc1* was up-
333 regulated by a LFC of 7.1 in GNAQ^{Q209L} melanocytes. Significant up-regulation of *Stc1* has been
334 reported in tumors under hypoxic or oxidative stress²⁷⁻³¹. Furthermore, *Stc1* expression down-
335 regulates pro-survival ERK1/2 signaling and reduces the survival of MEFs under conditions of
336 oxidative stress²⁸. *Cdkn2a* and *Ccnd1* were up-regulated in GNAQ^{Q209L} expressing melanocytes
337 by a LFC of 5 and 1.7, respectively, similar to the intracellular response to oxidative stress
338 previously described in melanocytes in the disease, vitiligo, which results in the loss of
339 melanocytes from the epidermis³²⁻³⁴. Moreover, the *S100A8*, *S100A9* and *BNIP3* genes, involved
340 in cell death and autophagy via ROS-mediated crosstalk between mitochondria and lysosomes,
341 were all up-regulated in GNAQ^{Q209L} melanocytes by a LFC of 0.7 to 1.7³⁵.

342

343 In terms of apoptosis, *S100b*, a negative regulator of p53 in melanocytes, was down-regulated by
344 (LFC -2.6), while *Annexin V (Anxa5)*, a marker of early apoptosis, was up-regulated (LFC 0.6).
345 We also found up-regulation of the pro-apoptotic *Bok* (LFC 1.3), an effector of mitochondrial
346 outer membrane permeabilization (MOMP) that triggers membrane permeabilization and
347 apoptosis in the absence of Bax and Bak³⁶. *Cystatin C (Cst3)*, a major inhibitor of cathepsins³⁷,

348 was up-regulated (LFC 2.3) in GNAQ^{Q209L} melanocytes. In response to DNA damage, activated
349 p53 can induce *Cystatin C* expression by binding to regulatory sequences in the first *Cst3* intron.
350 Hence, we hypothesized that cellular stress was stimulating increased apoptosis of GNAQ^{Q209L}-
351 expressing melanocytes. To assess the amount of cell death *in vivo*, we dissociated IFE from
352 mouse tails and stained the cells for Annexin-V, which labels cells in early apoptosis. Using
353 FACS, we quantified the percentage of tdTomato and Annexin-V double-positive cells (**Figure**
354 **6E**). There was a greater percentage of Annexin-V positive melanocytes from GNAQ^{Q209L} tail
355 epidermis (4.2% versus 1.2%). This supported the GSEA data that suggests that the loss of
356 GNAQ^{Q209L} expressing melanocytes from the IFE involves cellular stress and apoptosis.

357

358 **Low frequency of *GNAQ* and *GNAI1* oncogenic mutations in human cutaneous melanoma**

359 Our results suggested that oncogenic mutations in *GNAQ* and its closely related homolog,
360 *GNAI1*, are rarely found in cutaneous melanoma because the epidermal microenvironment
361 causes G $\alpha_{q/11}$ signaling to inhibit melanocytes. To determine the frequency of these mutations,
362 we searched the COSMIC database to identify all cutaneous melanoma cases with a *GNAQ* or
363 *GNAI1* oncogenic mutation at either Q209 or R183. 2753 and 2295 samples meeting our
364 inclusion criteria (see legend of **Supplementary Tables 7, 8**) had *GNAQ* or *GNAI1* mutation
365 status reported, respectively. A total of 23 cases carried an oncogenic mutation in either *GNAQ*
366 (n=11) or *GNAI1* (n=12). Five of these cases were actually specified as malignant blue nevus or
367 uveal melanoma in their original publications and so were not cutaneous melanomas. Eight other
368 cases carried one or more mutations in genes also found in uveal^{38, 39} or mucosal^{40, 41} melanoma,
369 but not in cutaneous melanoma: *BAP1*, *SF3B1* or *EIF1AX* and hence were similarly suspect as
370 misclassified. One case was from a cell line and in another case, the frequency of an odd
371 *GNAQ*^{Q209H} mutation in the tumor was less than 5%. This left eight cases that we think could
372 have arisen in the epidermis, for an incidence of 0.15% (4/2753) for *GNAQ* and 0.17% (4/2295)
373 for *GNAI1*. Details on two of these cases can be found in **Supplementary Table 9**.

374

375 **Mutations in *PLCB4* in human cutaneous melanoma**

376 *Phospholipase C beta 4 (PLCB4)* encodes a protein that binds to G α_q and G α_{11} and serves as the
377 primary effector of signaling to downstream components for q class heterotrimeric G proteins.

378 *PLCB4* was linked to $G\alpha_{q/11}$ signaling in melanoma when a recurrent gain-of-function
379 substitution (*PLCB4*^{D630Y}) was found to be mutually exclusive with *GNAQ* and *GNAI1* mutations
380 in uveal and CNS melanomas^{42, 43}. If $G\alpha_{q/11}$ signaling inhibits melanocyte growth in the
381 epidermis, we wondered whether loss of function mutations in *PLCB4* might promote
382 melanomagenesis. In 2012, Wei *et al.* reported that *PLCB4* was one of the most frequently
383 mutated genes in a study of 52 cutaneous melanomas (freq = 15%)⁴⁴. However, in the TCGA-
384 SKCM study published in 2015, *PLCB4* was not identified as a Significantly Mutated Gene
385 (SMG) by the MutSig and InVEx algorithms used to analyze the sequencing data⁴⁵. These
386 particular bioinformatics methods, while effective, left out some known melanoma driver genes
387 identified by other studies⁴⁶. Hayward *et al.* subsequently reported that *PLCB4* was targeted by
388 loss-of-function gene fusion events in their cutaneous melanoma set⁴⁰.

389
390 We re-examined the data concerning *PLCB4* in the TCGA-SKCM dataset, which currently
391 consists of 470 cases (a list of these cases with description can be found in **Supplementary**
392 **Table 9**). Most cases (n=420) had a primary diagnosis of 'Malignant melanoma, not otherwise
393 specified (NOS)'. 50 cases were variously described as 'Epithelioid cell melanoma', 'Nodular
394 melanoma', 'Amelanotic melanoma', 'Lentigo maligna melanoma', 'Acral lentiginous melanoma,
395 malignant', 'Superficial spreading melanoma', 'Spindle cell melanoma, NOS', or 'Desmoplastic
396 melanoma, malignant'. Excluding synonymous alterations, small somatic mutations in the exons,
397 introns, UTRs or splice sites of *PLCB4* were found in 97 TCGA-SKCM cases (21% overall). Of
398 the affected cases, 72 carried missense mutations. Other types of mutations (stop gained, splice
399 site, intronic, 5'UTR, 3'UTR and frameshift) occurred along with a missense mutation in 17
400 additional cases and without one in 8. The most frequent recurrent mutations were S670L,
401 G876E and E1104K, which together were found in 12% of the 97 *PLCB4* affected cases. No case
402 carried a mutation affecting the D630 residue previously identified as a gain-of-function
403 alteration in uveal and CNS melanomas^{42, 43}.

404
405 We compared *PLCB4* to three of the most frequently mutated tumor suppressor SMGs in
406 melanoma: *PTEN*, *TP53* and *NF1*, considering small somatic mutations within each gene,
407 excluding synonymous alterations (all mutations are listed in **Supplementary Table 9**). The

408 *NRAS*^{Q61} and *BRAF*^{V600} oncogenic mutations occur in an exact mutually exclusive pattern in the
409 TCGA-SKCM dataset (116 and 205 cases, respectively). Therefore, we began by comparing the
410 frequency of *PLCB4*, *PTEN*, *TP53* and *NF1* mutations in these subsets. *PLCB4* was mutant in
411 24% of the *NRAS*^{Q61} mutant cases, 17% of the *BRAF*^{V600} mutant cases and 23% of the remaining
412 cases (hereafter referred to as 'other'). *TP53* and *NF1* were also less frequent in *BRAF*^{V600} mutant
413 cases and more frequent in the *NRAS*^{Q61} and other cases (**Figure 7A**). *NF1* mutations were
414 highly enriched in the 'other' subset, as expected from the literature^{8, 40}. *PTEN* mutations, in
415 contrast, were more likely to be found in the *BRAF*^{V600} mutant subset compared to the *NRAS*^{Q61}
416 mutant subset or other cases. We calculated the average total number of all gene associated small
417 somatic mutations in each of the three subsets and found that the 'other' subset had 1.7-fold
418 greater mutation load than the *NRAS*^{Q61} mutant subset. Hence, *TP53* and *PLCB4* mutations in the
419 *NRAS*^{Q61} subset could be enriched when considering the overall mutation burden. Lastly, 32% of
420 the *PLCB4* mutant cases had more than one mutation in *PLCB4* (*i.e.* potential biallelic mutation),
421 compared to 38% for *NF1*, 14% for *TP53* and 4% for *PTEN* (**Figure 7B**).

422
423 We then identified all cases that had a mutation in more than one of these four genes of interest
424 (*PTEN*, *NF1*, *PLCB4* and *TP53*) and counted the number of times a double hit for any of the two
425 gene combinations was found (**Figure 7C**). In agreement with the frequencies in Figure 8A,
426 *PTEN* mutations were less often found with other members of this group, particularly *NF1*.
427 *PLCB4* mutations were most often seen with *TP53* and *NF1*, which were found less frequently
428 with each other than with *PLCB4*.

429
430 Finally, we grouped all of the cases into bins based upon the total number of small somatic
431 mutations in all genes. This was done to examine the relationship between the overall mutation
432 load with the frequency of mutation in the genes of interest (*BRAF*^{V600}, *NRAS*^{Q61}, *PTEN*, *NF1*,
433 *PLCB4* and *TP53*) (**Figure 7D**). We calculated the mutation rate of *BRAF*^{V600}, *NRAS*^{Q61}, *PTEN*,
434 *NF1*, *PLCB4* and *TP53* in each bin by dividing the total number of small somatic mutations (not
435 including synonymous) found within each gene by the summed total number of mutations in all
436 genes in each bin. The mutation rate of *BRAF*^{V600} and *NRAS*^{Q61} was highest in the bin with the
437 smallest mutation load. As mutation load increased, the mutation rate of *BRAF*^{V600} and *NRAS*^{Q61}

438 consistently decreased. *PTEN* also generally followed this pattern. In the 2000 to 3000 mutation
439 load/case range, the mutation rate of *BRAF*^{V600}, *NRAS*^{Q61} and *PTEN* was low, while *NF1* and
440 *PLCB4* reached their maximum. The rate of *NF1* and *PLCB4* mutations declined in the 3000+
441 mutation load/case bin. *TP53* showed no particular pattern across the bins.

442

443 We then organized all of the current TCGA-SKCM cases into **Figure 8**, reflecting on how the
444 overall mutation load relates to mutations in these six genes of interest. At the lowest mutation
445 load (average of 35 total mutations per case), we note that there were five cases of *GNAQ* or
446 *GNAI1* oncogenic mutations plus a mutation in either *BAP1* or *SF3B1*. We suspect that these
447 cases could be tumors from uveal or mucosal melanomas, although they were not described as
448 such. Next, there were 74 cases with an average of 431 mutations per case, with no mutations in
449 any of the genes of interest described here. Cases with just *BRAF*^{V600} or *NRAS*^{Q61} closely
450 followed, with an average of 453 and 587 mutations per case, respectively. As the mutation load
451 continued to increase, hits in multiple genes of interest accumulated. First, there were cases with
452 just one tumor suppressor mutation plus *BRAF*^{V600} or *NRAS*^{Q61} (at 900 mutations/case). The
453 tumor suppressor mutation was more likely to be *PTEN* in the *BRAF*^{V600} mutant subset. Next,
454 there were combinations of two or more tumor suppressor mutations along with either *BRAF*^{V600}
455 or *NRAS*^{Q61} (at 1543 mutations/case). Cases with only tumor suppressor mutations made up the
456 last group, which had an average of 2543 mutations per case. Half of these cases had just one
457 mutant tumor suppressor, while the remaining half had more than one mutated (**Figure 8**).

458

459 These findings are consistent with previous studies. For example, Mar *et al* found that
460 *BRAF/NRAS* WT cutaneous melanomas have a higher average mutation rate than *BRAF* or *NRAS*
461 mutant melanomas⁴⁷. The mutations in the *BRAF/NRAS* WT tumors were spread over various
462 signaling pathways and included *NF1*, *KIT* and *NOTCH1*⁴⁷. Palmieri *et al* also summarized a
463 number of gene mutations that vary in frequency between *BRAF/NRAS* WT vs. mutant
464 melanomas⁴⁸.

465

466 The overall pattern is that gain of function drivers are more frequently found when mutation load
467 is lower and loss of function drivers predominate when mutation load is higher. Perhaps when

468 mutagen exposure is low, a stronger acting *BRAF* or *NRAS* mutation can drive the melanoma
469 because an inactivating hit to the same allele is less likely to occur. However, when mutagen
470 exposure is high, it alternatively allows for the accumulation of loss of function mutations in
471 multiple genes, which has a positive synergistic effect when they occur in tumor suppressors.

472

473 Our finding that $G\alpha_{q/11}$ signaling unexpectedly restrains melanocyte growth in the epidermis
474 provides an explanation for the frequent occurrence of *PLCB4* mutations in cutaneous
475 melanoma.

476

477 **DISCUSSION**

478 Oncogenic *GNAQ* and *GNAI1* mutations are observed at a high frequency in human melanocytic
479 neoplasms in non-epithelial tissues. Still, they are rare in anatomical locations with an epithelial
480 component, such as the inter-follicular epidermis or conjunctiva of the eye. One theoretical
481 explanation for this skewed frequency is that some unknown mechanism mutates *GNAQ* and
482 *GNAI1* only in non-epithelial locations. We found that this is not the case. The forced expression
483 of oncogenic *GNAQ*^{Q209L} in mature melanocytes in mice did not result in cutaneous melanoma,
484 but instead had the opposite effect: the gradual loss of melanocytes from the IFE. Others have
485 hypothesized that subtle geographic variances in the embryonic origin of epithelial versus non-
486 epithelial melanocytes could explain the restriction of certain driver mutations to specific
487 melanoma subtypes^{49, 50}. Alternatively, since epidermal melanocytes interact with keratinocytes,
488 whereas non-epidermal melanocytes interact with the mesodermal stromas, it was possible that
489 direct cell contact or paracrine signaling produced by the tissue-specific microenvironment might
490 interfere with the oncogenic signaling pathway¹³. Here, we have presented evidence that the IFE
491 microenvironment drives the loss of melanocytes expressing the *GNAQ*^{Q209L} oncogene,
492 providing a new model for oncogene specificity.

493

494 **Melanocytes taken from the IFE and cultured *in vitro* can switch phenotypes**

495 Previous studies have shown that stable transfection of *GNAQ*^{Q209L} induced anchorage-
496 independent growth in soft agar of hTERT/CDK4^{R24C}/p53^{DD} mouse melanocytes in a TPA-
497 independent manner, a feature associated with cellular transformation^{3, 51}. In addition,
498 *GNAQ*^{Q209L} signaling produced an abnormal cell morphology with irregular contours. A similar
499 observation was described in *GNAQ*^{Q209L}-transformed human melanocytes and melanocytes of a
500 *Gnaq*^{Q209L} zebrafish model⁵². Our study complements these previous results to show that
501 melanocytes directly taken from the mouse IFE and grown in primary culture without any other
502 cues survive better if they express *GNAQ*^{Q209L}. We also found that *Braf*^{V600E} had a stronger effect
503 than *GNAQ*^{Q209L} in this same situation, although the promoters driving the expression of these
504 two oncogenes were not the same. In addition, both WT and *GNAQ*^{Q209L} expressing melanocytes
505 survived better in culture when grown on MEFs. Therefore, the attrition of *GNAQ*^{Q209L}
506 expressing IFE melanocytes from the mouse tail epidermis is not due to an inherent characteristic

507 in IFE melanocytes because the phenotype is reversible by changing the microenvironment. This
508 is exciting because it suggests that there might be a way to shut the GNAQ^{Q209L} oncogene off
509 through some external cues.

510

511 **The IFE microenvironment inhibits melanocytes expressing GNAQ^{Q209L}**

512 It is well known that keratinocytes, which make up the vast majority of the epidermis, secrete
513 various growth factors and cytokines in a paracrine manner that regulate growth, survival,
514 adhesion, migration, and differentiation of melanocytes⁵³. In WT melanocytes, we found that the
515 presence of dissociated IFE in co-cultures provided a significant boost. In contrast, co-culturing
516 GNAQ^{Q209L} expressing melanocytes with IFE reduced their survival capacity and inhibited cell
517 division. Moreover, the effects of the IFE on melanocyte survival did not require direct cell-cell
518 contact and could be replicated in a transwell culture system. Our studies of IFE melanocytes
519 suggests that GNAQ^{Q209L} expression causes increased cell adhesion, a disorganized actin
520 cytoskeleton and the promotion of long dendrite extensions, which frequently break, possibly
521 related to changes in semaphorin signaling in pathways shared with neurons. We conclude that
522 the microenvironment surrounding melanocytes is the main factor controlling whether
523 GNAQ^{Q209L} signaling is oncogenic or inhibits growth. IFE melanocytes have the innate capacity
524 to be transformed by GNAQ^{Q209L}, but whether or not they are permitted to do so is dictated by
525 the microenvironment in which they reside.

526

527 **A cellular stress response signature in GNAQ^{Q209L} IFE melanocytes related to vitiligo**

528 Reactive oxygen species (ROS) are highly active radicals produced during multiple cellular
529 processes that, upon accumulation, can damage most biological macromolecules⁵⁴. ROS
530 overproduction in melanocytes by exogenous and endogenous stimuli has been implicated in
531 vitiligo, a common skin depigmentation disorder caused by a loss of melanocytes from the
532 epidermis. ROS accumulation can lead to membrane peroxidation, decreased mitochondrial
533 membrane potential, and apoptosis of melanocytes in vitiligo epidermis³². Interestingly,
534 GNAQ^{Q209L} IFE melanocytes shared a similar intracellular response to oxidative stress as
535 observed in vitiligo melanocytes, including activation of p53-dependent signaling and Akt
536 signaling, and induction of both *CDKN2A* (*p16*) and cyclin D1 (*CCND1*) expression³²⁻³⁴.

537 Disruption in these cellular processes might lead to metabolic disturbances, cell cycle arrest,
538 senescence and cell death in melanocytes³². Previous studies have shown that melanocytes have
539 an increased susceptibility to oxidative stress compared with keratinocytes or fibroblasts and that
540 *CDKN2A* (*p16*) plays a crucial role in regulating oxidative stress independently of Rb-tumor-
541 suppressor function⁵⁵. Furthermore, oncogene activation in melanocytes generates oxidative
542 stress and increased ROS and p16 expression has been implicated in oncogene-induced
543 melanocyte senescence⁵⁶. Thus, up-regulation of *Cdkn2a* (p16) may reflect a systemic oxidative
544 stress experienced by *GNAQ*^{Q209L} melanocytes in an epithelial context.

545
546 In support of this idea, Cystatin C (*Cst3*), a potent inhibitor of cysteine proteinases and some
547 lysosomal caspases involved in apoptotic-related processes³⁷, was up-regulated in *GNAQ*^{Q209L}
548 melanocytes. Increased *Cst3* expression was correlated with oxidative stress-induced apoptosis
549 in cultured neurons⁵⁷, and with the induction of intracellular ROS from mitochondria by
550 depletion of glutathione in dendritic cells⁵⁸. Moreover, *S100A8/A9* and *BNIP3* genes involved in
551 cell death and autophagy via ROS-mediated crosstalk between mitochondria and lysosomes were
552 also upregulated³⁵. Similarly, we found up-regulation of the pro-apoptotic BOK, an effector of
553 mitochondrial outer membrane permeabilization (MOMP) that triggers membrane
554 permeabilization and apoptosis in the absence of BAX and BAK³⁶.

555
556 In addition, among the top-ranked DE genes was *Stanniocalcin* (*Stc1*), a glycoprotein hormone
557 involved in calcium/phosphate homeostasis. Significant up-regulation of *STC1* was reported in
558 tumors under hypoxic or oxidative stress²⁷⁻³¹. Furthermore, *Stc1* expression down-regulates pro-
559 survival Erk1/2 signaling and reduces survival of MEFs under conditions of oxidative stress²⁸.
560 The slow loss of melanocytes from the IFE in *GNAQ*^{Q209L} expressing mice is consistent with
561 damage induced by ROS. This represents a novel and intriguing potential consequence of
562 *GNAQ*^{Q209L} signaling induced by the interfollicular epidermal microenvironment.

563

564 ***PLCB4* mutations in cutaneous melanoma**

565 *PLCB4* encodes a protein that binds to $G\alpha_q$ and $G\alpha_{11}$ and serves as the primary effector of
566 signaling to downstream components in the pathway. If $G\alpha_{q/11}$ signaling inhibits melanocyte

567 growth in the epidermis, then we wondered whether loss of function mutations in *PLCB4* might
568 promote melanomagenesis. Like Wei *et al.*⁴⁴, we found that somatic *PLCB4* mutations are
569 frequent in cutaneous melanomas. Excluding synonymous alterations, small somatic mutations in
570 *PLCB4* are present in 97 of the 470 TCGA-SKCM cases (21%). Of the affected cases, most carry
571 a missense mutation, with S670L, G876E and E1104K as the most frequent recurrent
572 substitutions. In addition, 32% of *PLCB4* mutant cases have more than one mutation in the gene
573 (potential biallelic mutation), which was similar to *NF1* at 38% of cases. The pattern of *PLCB4*
574 mutation across the set was not random; instead, *PLCB4* mutations were less frequent in
575 *BRAF*^{V600} mutant cases and more frequent in cases with a higher overall mutation load, alongside
576 *NF1* and *TP53*. Our finding that *GNAQ*^{Q209L} signaling restrains melanocyte growth in the
577 epidermis provides an explanation for the frequent occurrence of *PLCB4* mutations in cutaneous
578 melanoma. It will be of interest to study the relationship between *PLCB4* loss and frequency and
579 location of metastases, since metastasis involves melanoma cells leaving the epidermal
580 microenvironment and interacting with mesodermal fibroblasts and stromas.

581
582 In conclusion, we have shown that the *GNAQ*^{Q209L} oncogene switches from promoting to
583 inhibiting melanocyte growth due to paracrine crosstalk with the IFE microenvironment. Our
584 studies revealed evidence for multiple potential mechanisms, including oxidative stress,
585 apoptosis, inhibition of cell division, changes to cell adhesion and cell fragmentation. Hence, the
586 $G\alpha_{q/11}$ signaling pathway is not only important for uveal melanoma; it also has implications for
587 both vitiligo and cutaneous melanoma. The critical paracrine signal(s) remain to be determined
588 through further biochemical and cell culture studies. It is also possible that a better understanding
589 of the ability of *GNAQ*^{Q209L} to switch from acting as an oncogene to an inhibitor of melanocyte
590 growth could lead to new therapies for uveal melanoma, which lacks effective treatment options
591 for metastatic disease.

592

593 **METHODS**

594 **Mice**

595 Animal research was conducted under the approval of the UBC Animal Care Committee
596 (Protocols A18-0080 and A19-0148, C.D.V.R.). DNA from ear notches was isolated using
597 DNeasy columns (Qiagen) and amplified using PCR. *Mitf-cre* (Tg(Mitf-cre)^{7114Gsb}),
598 *Tyrosinase-creERT²* (Tg(Tyr-cre/ERT2)^{13Bos/J}), *Rosa26-LoxP-Stop-LoxP-GNAQ^{Q209L}*
599 (Gt(ROSA)26Sor^{tm1(GNAQ*)Cvr}), *Rosa26-LoxP-Stop-LoxP-tdTomato* (Gt(ROSA)26Sor^{tm14(CAG-}
600 ^{tdTomato)Hze}), *Rosa26-LoxP-Stop-LoxP-LacZ* (Gt(Rosa)26Sor^{tm1Sor/J}), and *Braf^{CA}* (*Braf^{tm1Mmcm}*)
601 mice were genotyped as previously described^{14-17, 20, 59}. All strains were backcrossed to the
602 C3HeB/FeJ genetic background for at least 3 generations.

603

604 **LacZ staining**

605 We crossed *R26-fs-GNAQ^{Q209L}/+*; *+/+* mice to *R26-fs-LacZ/+*; *Tyr-creERT²/+* mice and
606 genotyped the resulting progeny. At 4 weeks of age, two groups of mice were tamoxifen-treated
607 for 5 consecutive days: *R26-fs-GNAQ^{Q209L}/R26-fs-LacZ*; *Tyr-creER/+* (GNAQ-LacZ) mice and
608 *+/R26-fs-LacZ* mice; *Tyr-creER/+* (WT-LacZ) mice. Tamoxifen treatment consisted of one daily
609 intraperitoneal injection of 1 mg tamoxifen (Sigma T5648) alongside a topical treatment for the
610 tail skin (tails were dipped for 5 seconds in 25 mg/ml 4-hydroxytamoxifen (Sigma H6278) in
611 DMSO). Mice were harvested either 1 week or 8 weeks after tamoxifen treatment. At each
612 experimental endpoint, a piece of tail skin of 1.5 cm in length was incubated in X-gal solution
613 for 48 hours as previously described^{60, 61}. The tail dermis and epidermis were split using sodium
614 bromide and fixed in 4% PFA. The number of LacZ-positive cells was counted in three rows of
615 epidermal scales per sample.

616

617 **Histochemistry**

618 For H&E staining, mouse tail skin samples were fixed in 10% buffered formalin overnight at
619 room temperature with gentle shaking, dehydrated, cleared, and embedded in paraffin. 5 μm
620 sections were taken for H&E staining using standard methods and imaged using a DMI 6000B
621 microscope (Leica). To examine tdTomato expression in sections, samples were fixed in 10%
622 buffered formalin overnight at 4°C, taken through a sucrose gradient, embedded in O.C.T., and

623 sectioned at 10 μ m. After washing in PBS for 30 minutes, sections were counter stained with
624 DAPI and imaged using a Histotech III slide scanner.

625

626 **Isolation of inter-follicular melanocytes from mouse tail skin**

627 We crossed *R26-fs-GNAQ^{Q209L}/+* mice to *+/R26-fs-tdTomato; Mitf-cre/+* mice and identified
628 tdTomato expressing progeny by genotyping. At four weeks of age, the tails were waxed to
629 remove hair follicles. The next day, tail skin from *GNAQ^{Q209L}* and WT tdTomato-positive mice
630 was harvested, and the IFE was split from the dermis by gentle dispase treatment. The IFE was
631 then incubated with trypsin into single cells, using forceps to scrape cells from the scales to
632 accelerate the process. (A detailed description of this method will be concurrently submitted to
633 *Bio Protocol*). Cells were centrifuged and the pellet was resuspended in suspension buffer (HBSS
634 + 10% FBS + EDTA 0.1mM EDTA) prior to FACS sorting.

635

636 **FACS**

637 Dissociated cells were first analyzed for viability based on forward and side scatter plots and
638 using eBioscience™ Fixable Viability Dye. Next, filters for forward scatter (height versus width)
639 and side scatter (height versus width) were used to select single cells. Lastly, viability dye
640 fluorescence (y-axis) was plotted against tdTomato (x-axis) and gates were set to capture only
641 viable tdTomato positive cells. Cells from mice lacking *Mitf-cre* (*+/R26-fs-tdTomato; +/+*) were
642 used as negative controls to define the threshold for tdTomato-positive cells. Sorted cells were
643 collected in 15ul of HBSS+FBS 10% +EDTA 0.1 mM solution and 1 ul of RiboLock RNase
644 inhibitor (Thermo Scientific, EO0381). For Annexin V staining, we used the Annexin V Fluos
645 staining kit according to the manufacturer's directions (Sigma Aldrich, 11858777001).

646

647 **Primary cell culture**

648 Cells were plated into 96-well plates previously coated with 0.1 mg/ml fibronectin in Dulbecco's
649 Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum, 1% penicillin-
650 streptomycin (15140122 Thermo-Fisher), 2 mM L-Glutamine, and 1 mM Sodium Pyruvate. For
651 cultures of sorted melanocytes only, cells were plated at 6000 to 8000 cells per well. For co-
652 culture with MEFs, 6000 to 8000 FACS sorted melanocytes were plated into 96-well plates

653 previously seeded with MEFs that had formed a confluent monolayer. For direct co-culture
654 experiments with IFE, the IFE was dissociated and plated at 100,000 cells per well (*i.e.* with no
655 FACS sorting for the melanocytes first). For transwell co-culture with IFE, 6000 to 8000 FACS
656 sorted melanocytes were plated in the well suspended above 100,000 dissociated IFE cells plated
657 below. All cultures were incubated at 37°C in 5% CO₂. Media was changed every third day by
658 exchanging 1/3 of the existing volume. Images were taken at 5x, 10x, and 20x magnification.
659 tdTomato positive cells in the 5x field of view were counted as live melanocytes.

660

661 **Live cell imaging**

662 Sorted tdTomato positive melanocytes or IFE cells containing tdTomato positive melanocytes
663 were plated on 0.1 mg/ml fibronectin-coated coverslips within a 35mm cell culture dish. Cells
664 were incubated overnight before imaging in a live-cell imaging culture chamber. Cells were
665 imaged at 37°C (5% CO₂) every 5 minutes for 20 hours at 10X magnification. Cell movements
666 were determined using the MTrackJ software on ImageJ and analysis was performed using
667 Chemotaxis and Migration Tool (Ibidi).

668

669 **Automated image analysis**

670 Custom Matlab scripts were utilized to analyze cell morphology and actin fiber orientation in
671 fixed cells as described in Haage *et al*⁶² and posted to GitHub. Cell contours were obtained
672 automatically by processing confocal z-projections of cells stained for F-actin (Phalloidin) in
673 MatLab. Images were first blurred with a Gaussian filter, and then an edge detection algorithm
674 was applied to identify cell borders. The resultant binary images were refined through successive
675 dilations and erosions to yield the final cell contour. These contours were used to measure cell
676 area, and circularity ($4\pi \cdot \text{area} / \text{perimeter}^2$), protrusions. The number and length of protrusions
677 were quantified automatically in MatLab. First, cell contours were identified as outlined above.
678 Next, we obtained the contour coordinates of the convex hull of the binary image representing
679 the cell area. At each point along the cell contour, we computed the minimum distance between
680 the convex hull and the actual cell contour. Based on these distances, minima corresponding to
681 protrusions could be identified. To be counted as protrusions, minima had to be at least 10 pixels

682 apart along the contour and of height greater than 5 pixels. Based on the coordinates of adjacent
683 peaks, the width, height, and aspect ratio of protrusions could be computed.

684

685 **Actin fiber organization**

686 First, we identified cell contours as described above. Next, the cell was subdivided into 32x32
687 pixel windows overlapping by 50%. We then computed the two-dimensional Fourier transform
688 of each window. If a window contains no fibers, the Fourier transform will be a central, diffuse
689 point of bright pixels. However, if a window contains aligned fibers, the Fourier transform will
690 consist of an elongated accumulation of bright pixels at a 90 degree angle to the original fibers.
691 Based on the aspect ratio and orientation of the Fourier transform, we determined fibrousness
692 and fiber orientation in a given window. The data for individual windows could then be
693 compared across the entire cell to estimate the cell fibrousness, defined here as the percentage of
694 cell area (% of windows) with aspect ratio greater than a cut-off value.

695

696 **RNA-seq data and bioinformatics analysis**

697 Total RNA from sorted cells was extracted using Trizol (Life Technologies) following the
698 manufacturer's protocol. For each library preparation, sorted melanocytes from two mice of the
699 same litter were pooled to increase the number of cells for RNA extraction. We generated 3 Wt
700 libraries and 3 GNAQ libraries from a total of 12 mice. Sample quality control was performed
701 using the Agilent 2100 Bioanalyzer. Qualifying samples (RNA Integrity Number ≥ 9) were then
702 prepped following the standard protocol for the NEB next Ultra ii Stranded mRNA (New
703 England Biolabs). Sequencing was performed on the Illumina NextSeq 500 with Paired End
704 42bp \times 42bp reads. Sequencing data was demultiplexed using Illumina's bcl2fastq2. De-
705 multiplexed read sequences were then aligned to the *Mus Musculus* mm10 reference sequence
706 using STAR aligner⁶³. The fastq sequences have been deposited at the Sequencing Read Archive
707 (SRA) of the NCBI under BioProjectID PRJNA736153.

708

709 [Private link to PRJNA736153 sequences:

710 <https://dataview.ncbi.nlm.nih.gov/object/PRJNA736153?reviewer=no8fe0hmv2ukgvoplvlu0kbu>
711 bu]

712 Assembly and differential expression analysis were performed using Cufflinks⁶⁴ through
713 bioinformatics apps available on Illumina Sequence Hub. Gene Ontology and KEGG Pathways
714 analysis was performed using DAVID Bioinformatics Resources 6.8^{65, 66}. Gene Set Enrichment
715 Analysis (GSEA) was performed using the JAVA GSEA 2.0 program⁶⁷. The gene sets used for
716 analysis were the Broad Molecular Signatures Database gene sets H (Hallmark gene sets).

717

718 **Statistical analysis**

719 Sample-size estimation: Target sample sizes were not explicitly computed during the study
720 design phase because the standard deviations between wildtype and GNAQ^{Q209L} epidermal
721 melanocytes were unknown. However, in our past experience of studying skin pigmentation
722 phenotypes on inbred genetic backgrounds, 4 mice of each genotype is usually sufficient to
723 detect statistically significant differences. For primary cell culture experiments, we planned to
724 perform each experiment in 3 complete and independent runs, from FACS collection to cell
725 culture. For RNAseq, we planned to generate 3 wildtype libraries and 3 GNAQ^{Q209L} libraries,
726 based on previously published studies.

727

728 Replicates: All replicates were biological replicates, *i.e.* each sample or independently derived
729 cell culture was established from a different mouse. In some experiments, one biological
730 replicate contained melanocytes collected from two mice of the same age and genotype, which
731 were pooled together to form one sample. When pooling occurred, it was done at the FACS step.
732 The number of replicates (number of mice and number of melanocytes derived from the mice)
733 can be found in Supplementary Table 10, organized by figure number.

734

735 Statistical reporting: The statistical tests that were used in each figure are described and shown in
736 Supplementary Tables 10 and 11. Raw data was plotted in graphs. Except for the survival graphs,
737 the mean for each group and the standard error of the mean is shown. Exact p values, t values,
738 degrees of freedom and number of replicates are detailed in Supplementary Tables 10 and 11.
739 Statistical analysis was performed using GraphPad Prism software. For RNAseq, the magnitude
740 and significance of differential gene expression and multiple test corrections were determined
741 using the Cufflinks suite via the Illumina Sequence Hub. Gene Ontology and KEGG Pathways

742 analysis was performed using DAVID Bioinformatics Resources 6. Gene Set Enrichment
743 Analysis (GSEA) was performed using the JAVA GSEA 2.0 program.

744

745 Group allocation: Samples were allocated into experimental groups based on genotype (WT or
746 GNAQ) or culture condition. WT and GNAQ mice/melanocytes that were compared to each
747 other were produced from the same set of breeder parents. Masking was used whenever possible
748 (melanocytes/skin samples with an obvious difference in pigmentation are not maskable).

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761

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767

768 **AUTHOR CONTRIBUTIONS**

769

770 O.U. and C.D.V.R conceived the project and designed the experiments. O.U. and A.H.
771 performed experiments. O.U., A.H., G.T. and C.D.V.R. performed data analyses. O.U. and
772 C.D.V.R wrote the manuscript.

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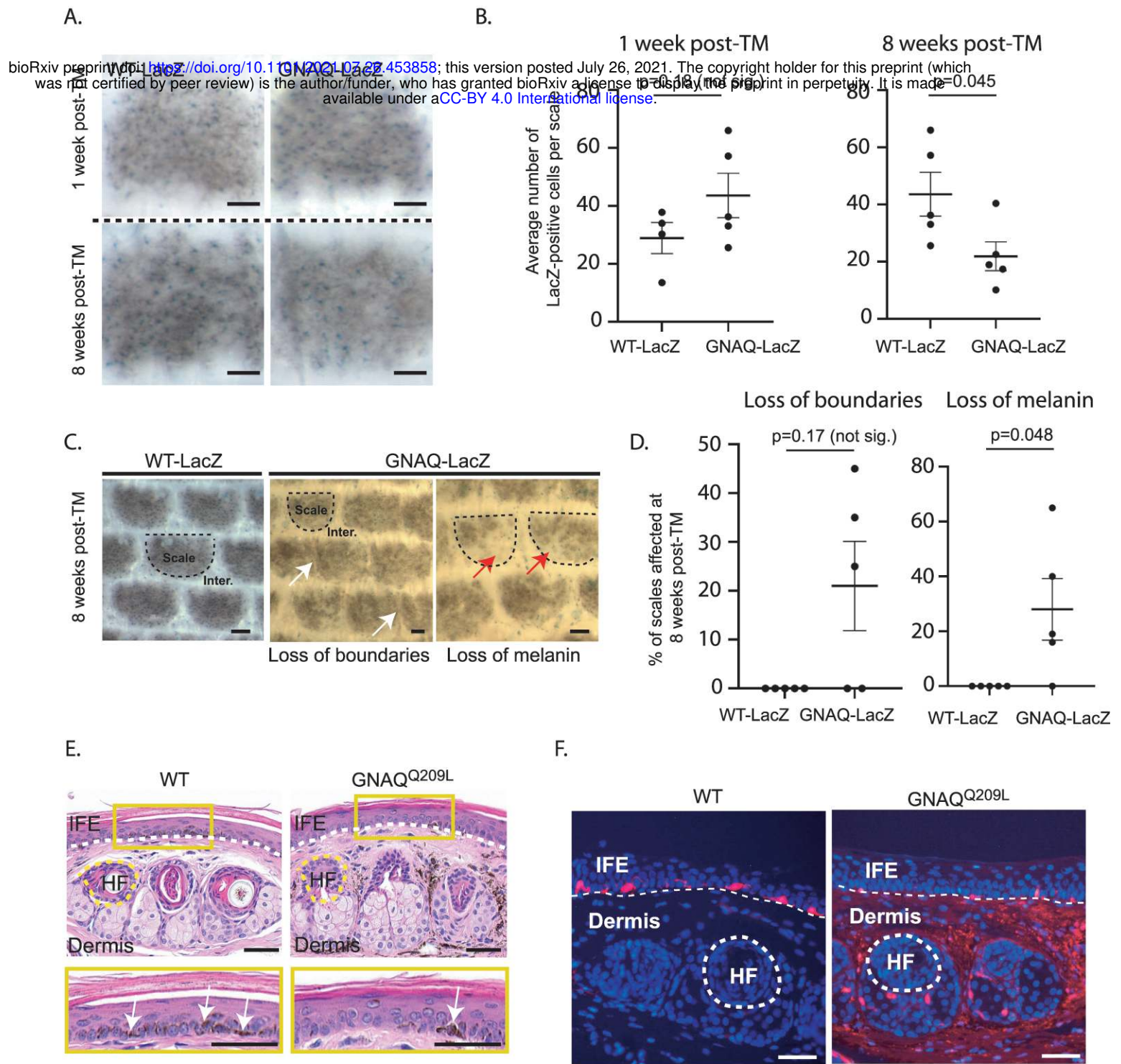


Figure 1. Forced GNAQ-Q209L signaling reduces the number of melanocytes in the IFE

A) Representative example of LacZ-positive cells within scales of WT-LacZ and GNAQ-LacZ tails at 1 week or 8 weeks post-tamoxifen (TM) treatment. **B)** Quantification of the average number of LacZ-positive cells per scale at 1 week and 8 weeks post-TM treatment. (Each point represents avg for 1 mouse; mean +/- SEM, Unpaired t-test). **C)** Representative examples of X-gal stained whole mount epidermal tail sheets in WT-LacZ and GNAQ-LacZ mice at 8 weeks post-TM, showing loss of boundaries in scale pigmentation (white arrows) or loss of melanin (red arrows) in GNAQ-LacZ mice. Example scales are outlined in dashed line for reference. **D)** Percentage of epidermal scales exhibiting loss of boundaries or loss of melanin in 5 WT-LacZ and 5 GNAQ-LacZ mice at 8 weeks post-TM. (Each point represents % for 1 mouse; Kolmogorov-Smirnov test, mean +/- SEM). **E)** H&E stained cross-sections of tail skin in WT and GNAQ-Q209L mice. The yellow box below shows a magnified area of the inter-follicular epidermis (IFE). Less melanin was observed in the IFE of GNAQ-Q209L skin (white arrows point to examples). Dashed lines indicate the boundaries between the IFE, dermis, and an example hair follicle (HF). **F)** tdTomato expression (red) in cross sections of tail skin of WT and GNAQ-Q209L mice at 4 weeks of age showing a reduced number of melanocytes (tdTomato+ cells) in the IFE of GNAQ-Q209L mice and an abnormal expansion of melanocytes in the dermis. Sections are counterstained with DAPI (blue). Dashed lines indicate the boundaries between the IFE, dermis and an example hair follicle (HF). In A and C, scale bars represent 100 μ m, while in E and F, scale bars represent 50 μ m.

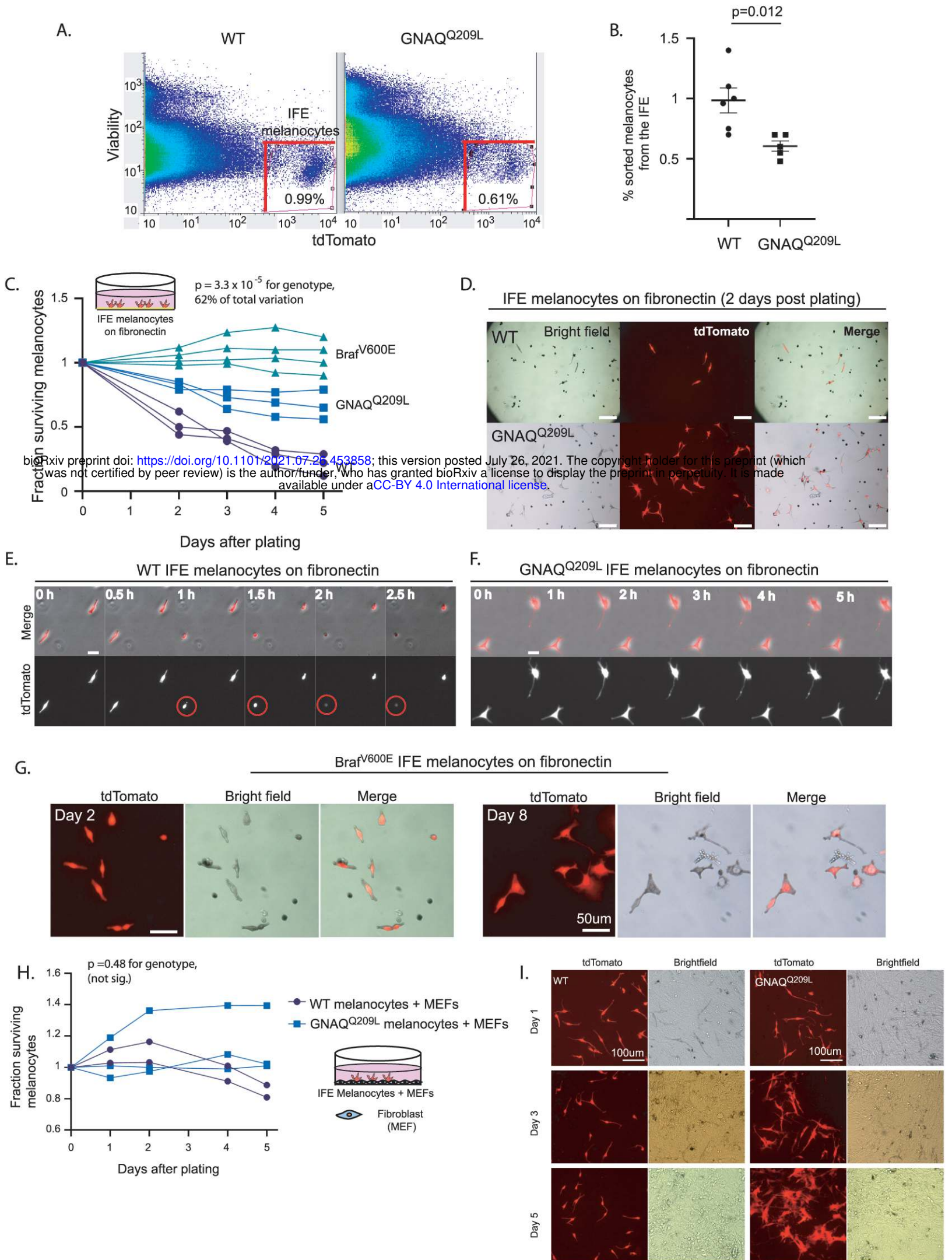


Figure 2. FACS sorted GNAQ-Q209L and BRAFV-600E IFE melanocytes cultured on fibronectin have increased survival compared to WT

A) Representative examples of FACS dot plots of single-cell suspension from the IFE epidermis of WT and GNAQ-Q209L mice. **B)** Average percentage of tdTomato+ cells sorted from the IFE of WT and GNAQ-Q209L mice, with a significantly smaller percentage in GNAQ-Q209L. (Each point represents % from 1 mouse, mean \pm SEM. Unpaired t test). **C)** Fraction of surviving WT, GNAQ-Q209L, and BraF-V600E FACS sorted melanocytes plated on fibronectin (each line represents an independently derived primary culture, 2 way Anova). **D)** Representative images of melanocyte morphology 2 days post-plating on fibronectin-coated wells, showing increased dendrite formation in GNAQ-Q209L melanocytes. **E)** Time-lapse microscopy of two WT melanocytes plated on fibronectin. The circled cell adopted a round shape shortly before being lost from view. **F)** Time-lapse microscopy of two GNAQ-Q209L melanocytes showing a dendritic cell morphology that remained stable over time. **G)** Representative images of BRAF-V600E melanocytes at 2 and 8 days post-plating on fibronectin. Scale bars represent 100 μ m in D, 20 μ m in E and F, and 50 μ m in G. **H)** Fraction of surviving WT and GNAQ-Q209L IFE melanocytes co-cultured with mouse embryonic fibroblasts (MEFs), showing initial growth above the baseline for both genotypes before loss began (each line represents an independently derived primary culture, 2 way Anova). **I)** Representative images of FACS sorted WT and GNAQ-Q209L IFE melanocytes co-cultured with MEFs. While the WT and GNAQ-Q209L IFE melanocytes had a similar spindle cell morphology at day 1, the GNAQ-Q209L melanocytes progressively developed large and abnormal shapes.

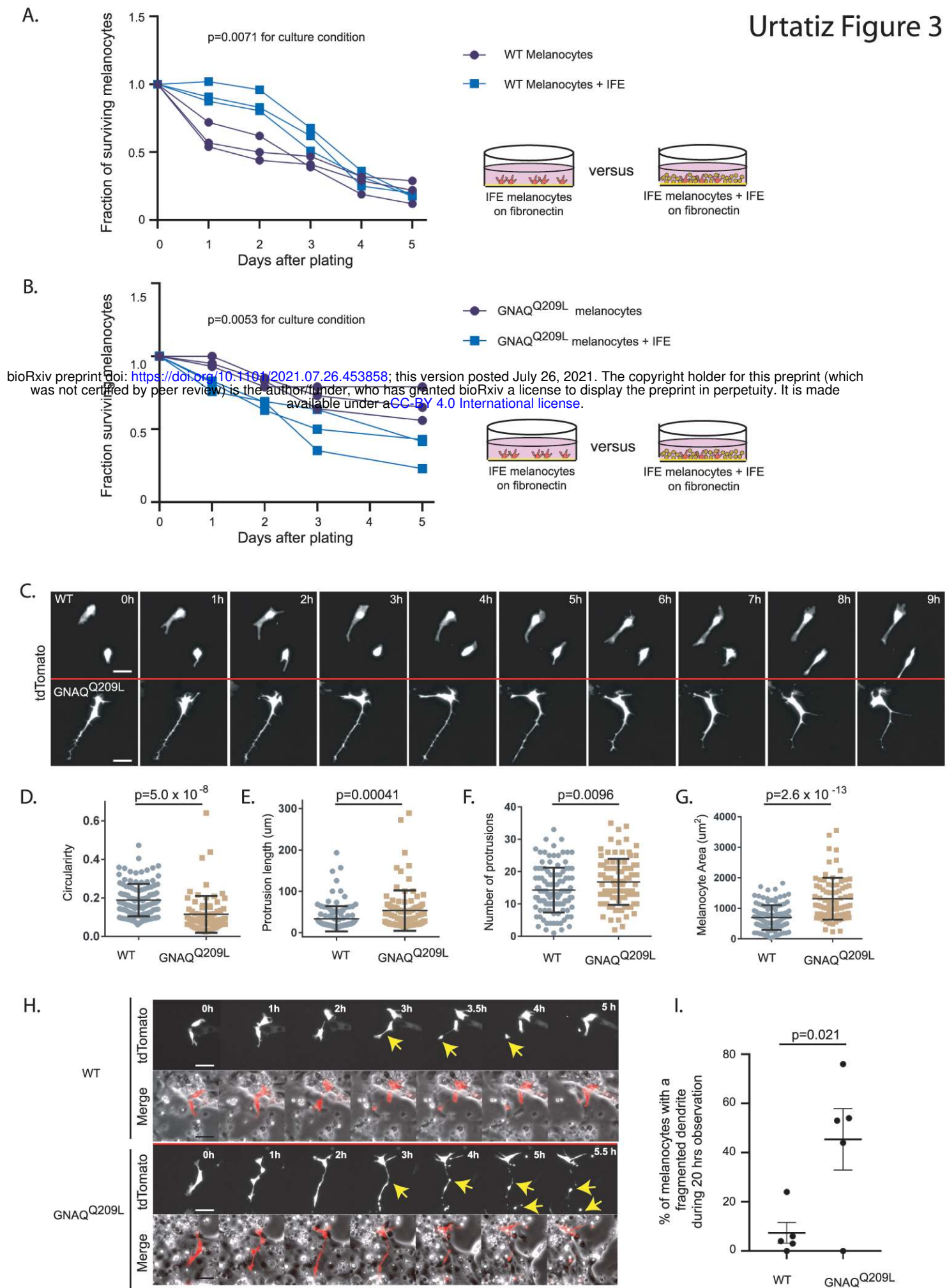


Figure 3. The IFE impairs survival and alters pseudopod dynamics in GNAQ-Q209L melanocytes

A) Survival of unsorted WT melanocytes plated with its IFE, compared to sorted IFE WT melanocytes plated onto fibronectin. The presence of IFE increased the survival of WT melanocytes. (Each line represents one independently derived culture, 2 way Anova). **B)** Survival of unsorted GNAQ-Q209L melanocytes plated with its IFE, compared to sorted IFE GNAQ-Q209L melanocytes plated onto fibronectin. The presence of IFE decreased the survival of GNAQ-Q209L melanocytes. (Each line represents one independently derived culture, 2 way Anova). **C)** Time-lapse images show representative WT and GNAQ-Q209L melanocytes co-cultured with IFE between 0 and 8 hours. The GNAQ-Q209L cell exhibits abnormally long dendrites and a less circular (more polygonal) cell body shape. **D-G)** Quantification of circularity (D), protrusion length (E), number of protrusions (F), and melanocyte area (G), in WT and GNAQ-Q209L melanocytes co-cultured with IFE. (Each point represents the measurement of one cell, mean \pm SEM; Unpaired t test). **H)** Time-lapse microscopy showing a representative example of dendrite fragmentation in WT and GNAQ-Q209L melanocytes. Arrows indicate dendrite breakage points and subsequent fragments that form up into balls. **I)** Quantification of the percent of cells experiencing dendrite fragmentation in WT and GNAQ-Q209L melanocytes cultured with IFE. (Each point represents the measurement from one culture, mean \pm SEM; Unpaired t test). Scale bars represent 40 μ m in C and H.

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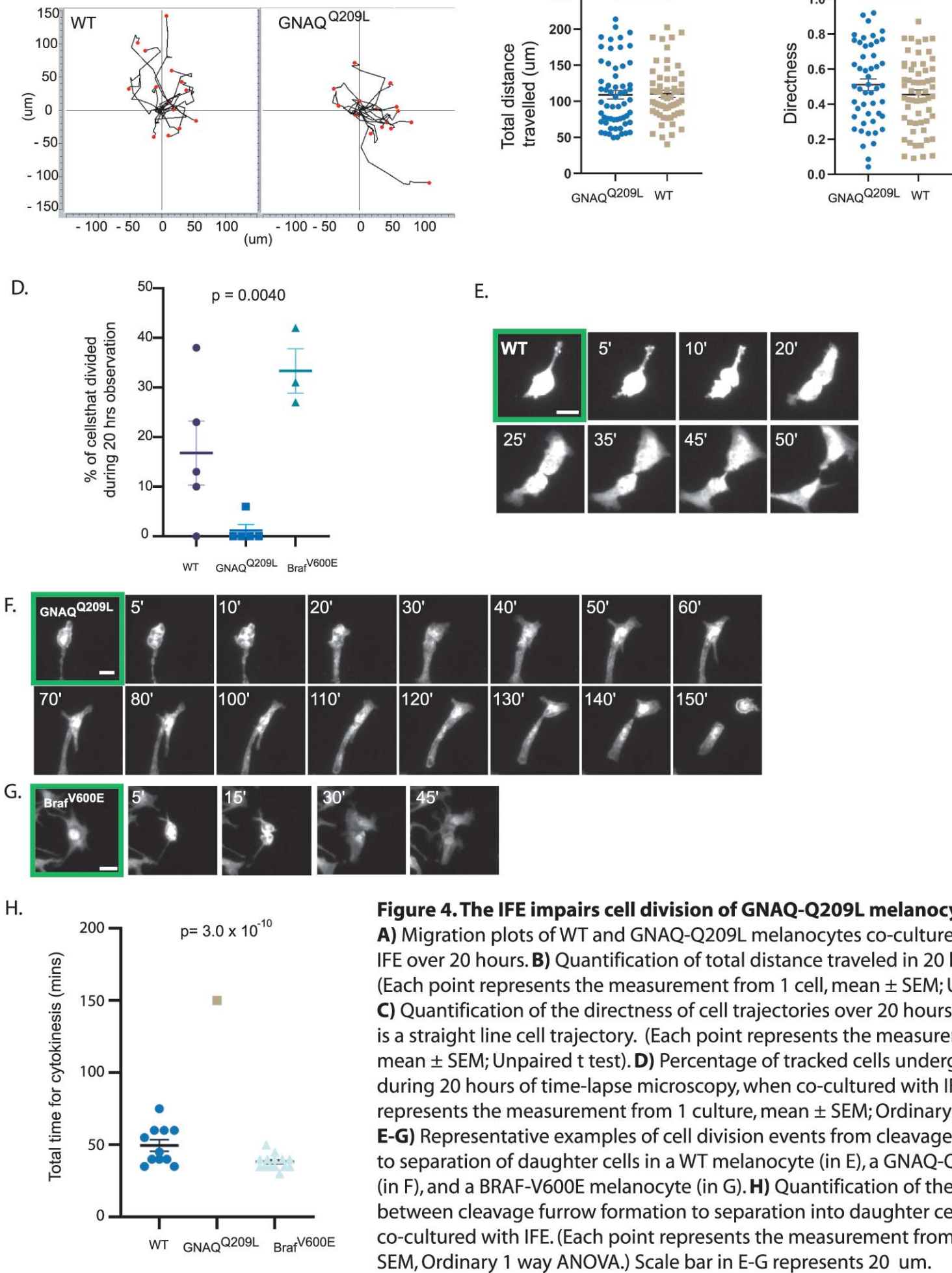


Figure 4. The IFE impairs cell division of GNAQ-Q209L melanocytes

A) Migration plots of WT and GNAQ-Q209L melanocytes co-cultured with IFE over 20 hours. **B)** Quantification of total distance traveled in 20 hours. (Each point represents the measurement from 1 cell, mean \pm SEM; Unpaired t test). **C)** Quantification of the directness of cell trajectories over 20 hours. Directness=1 is a straight line cell trajectory. (Each point represents the measurement from 1 cell, mean \pm SEM; Unpaired t test). **D)** Percentage of tracked cells undergoing division during 20 hours of time-lapse microscopy, when co-cultured with IFE. (Each point represents the measurement from 1 culture, mean \pm SEM; Ordinary 1 way ANOVA). **E-G)** Representative examples of cell division events from cleavage furrow formation to separation of daughter cells in a WT melanocyte (in E), a GNAQ-Q209L melanocyte (in F), and a BRAF-V600E melanocyte (in G). **H)** Quantification of the time taken between cleavage furrow formation to separation into daughter cells in melanocytes co-cultured with IFE. (Each point represents the measurement from 1 cell, mean \pm SEM, Ordinary 1 way ANOVA.) Scale bar in E-G represents 20 μm .

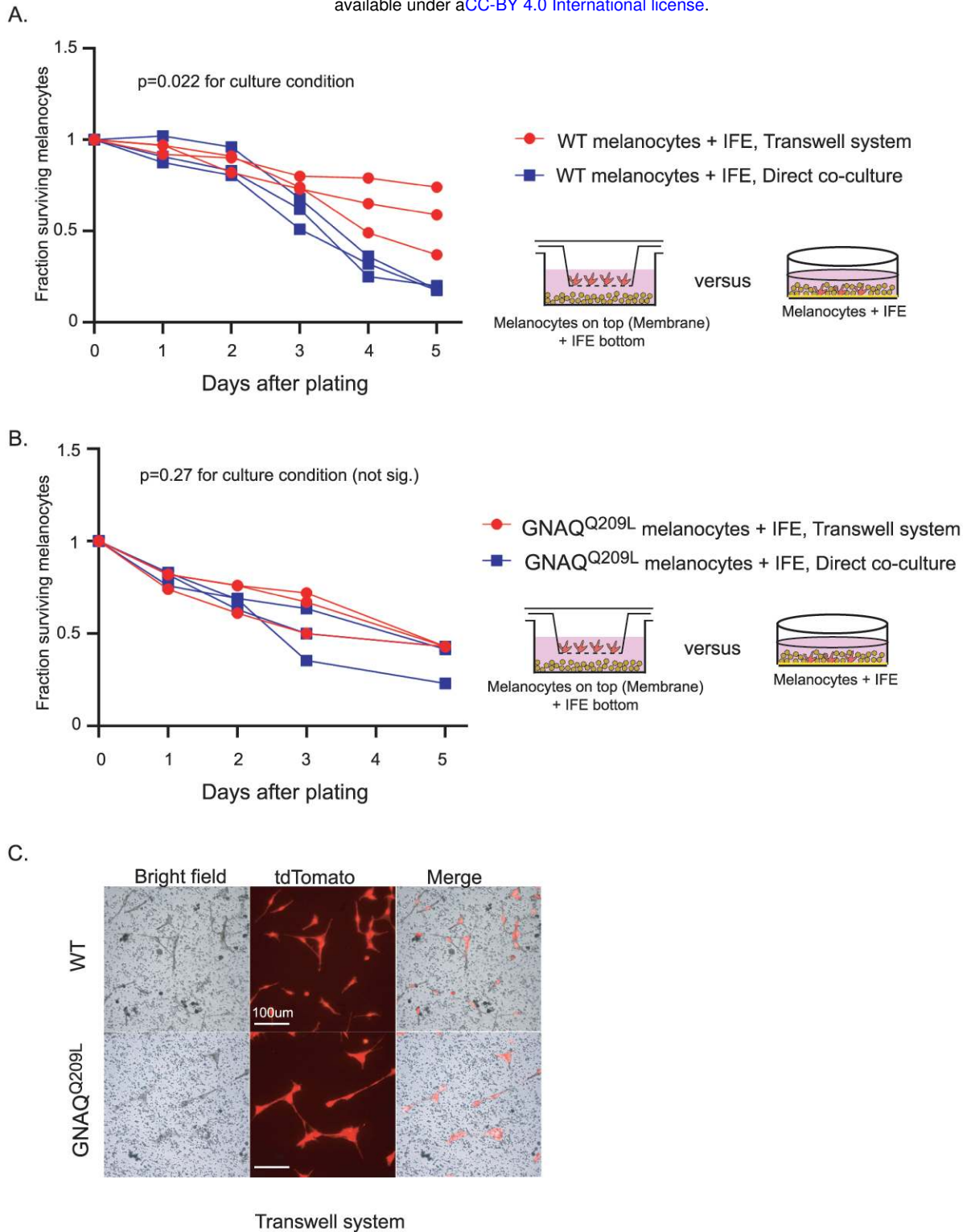
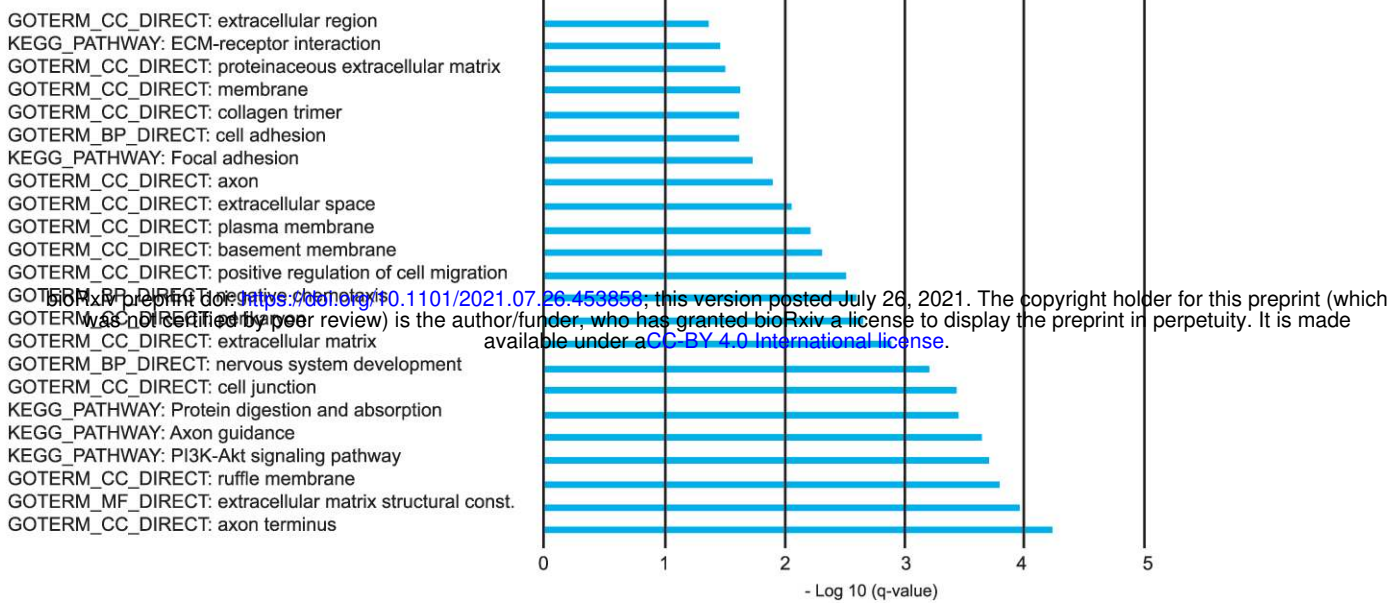


Figure 5. IFE control of melanocytes was maintained in a transwell culture system

A) Comparison of survival for WT melanocytes in direct contact with IFE ("direct co-culture") versus a transwell system where the two populations are separated by a permeable membrane. In WT melanocytes, there was no significant difference in survival for the first three days, after which the transwell melanocytes developed an advantage (Each line represents 1 independent culture, mean \pm SEM; 2 way ANOVA.)

B) Comparison of survival for GNAQ-Q209L melanocytes as in A. There was no significant difference in survival throughout the culture period. (Each line represents 1 independent culture, mean \pm SEM; 2 way ANOVA.)

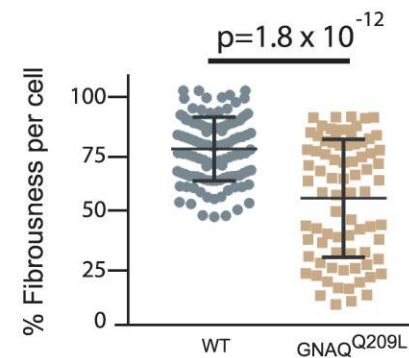
C) Representative images of WT and GNAQ-Q209L melanocytes at day three on the transwell membrane (no direct cell contact with IFE). GNAQ-Q209L melanocytes were larger than WT melanocytes, as in direct co-culture. Scale bar represents 100 μ m in C.

A. GO terms and KEGG pathways enriched in GNAQ^{Q209L}

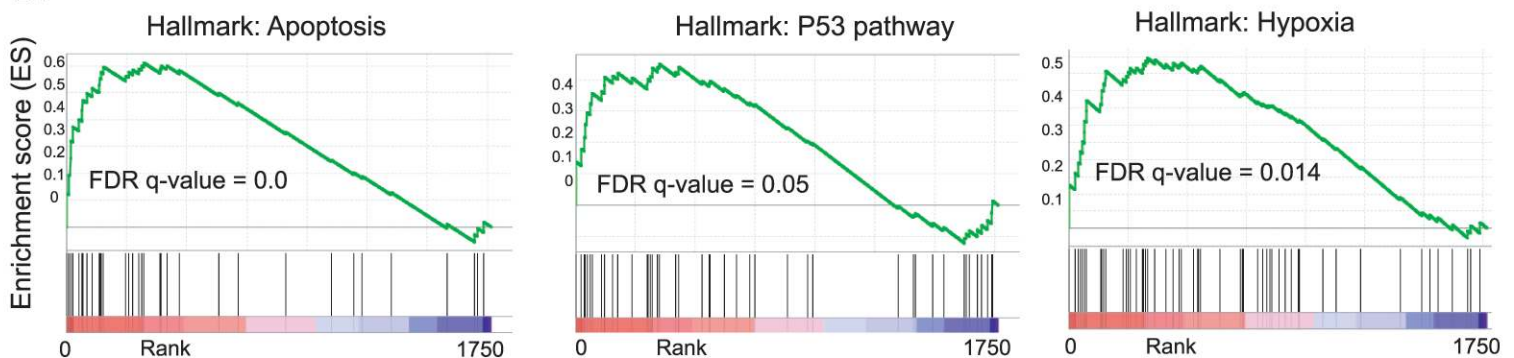
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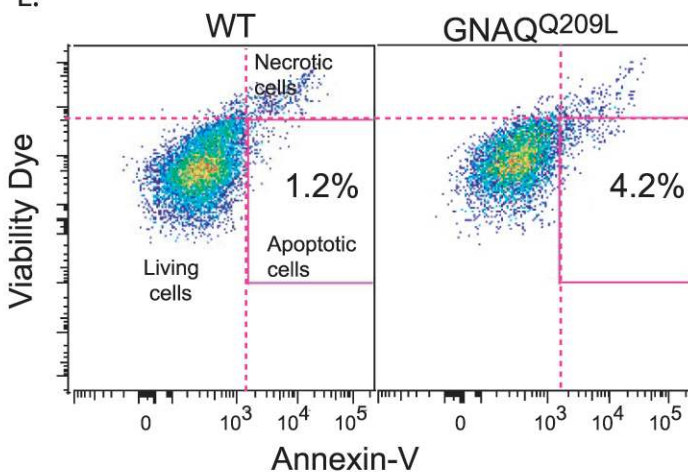


Figure 6. Analysis of GNAQ-Q209L expressing melanocytes in the IFE reveals alterations in the actin cytoskeleton and cell death.

A) Significant terms identified by gene ontology analysis for DE genes (LFC >2 or <-2) in GNAQ-Q209L melanocytes.

B) Representative examples of phalloidin staining for f-actin in WT and GNAQ-Q209L melanocytes co-cultured with IFE. **C)** Quantification of fibrousness as a measure of organization of the actin cytoskeleton. (Each point represents a measurement from 1 cell, mean ± SEM; Unpaired t test).

D) Gene set enrichment plots for apoptosis, P53 pathway, and hypoxia hallmarks enriched in GNAQ-Q209L melanocytes. **E)** Percentage of (tdTomato+/Annexin-V+) cells collected by FACS from 4 week old tail IFE. N=2 WT and 2 GNAQ-Q209L mice, melanocytes pooled.

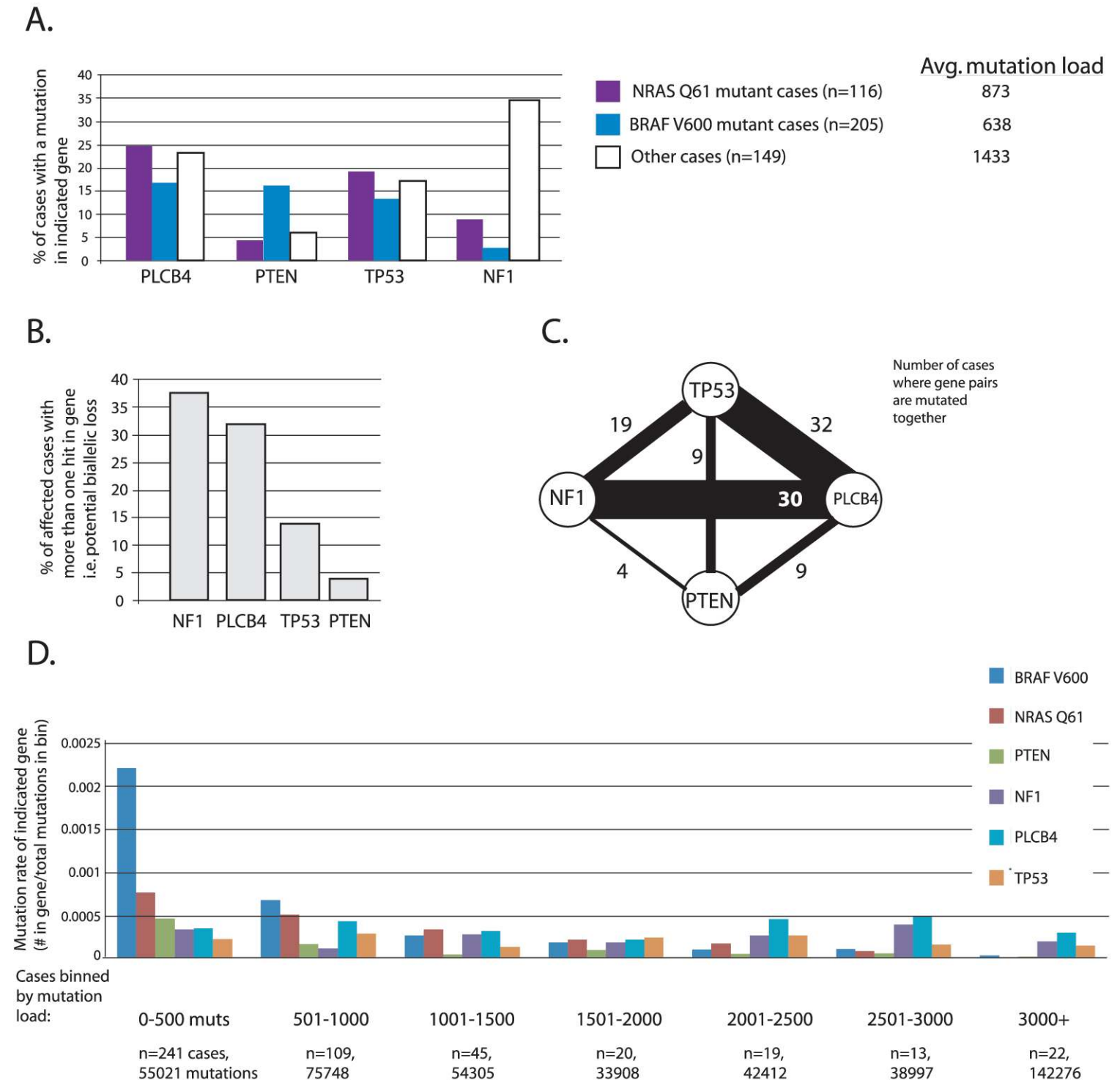


Figure 7. Analysis of PLCB4 mutations in TCGA-SKCM dataset.

A) Percentage of cases with mutations in PLCB4, PTEN, TP53 or NF1, in cases that were either NRAS-Q61 or BRAF-V600 mutant, or did not carry these two mutations ('other'). Shown to the right is the average mutation load for NRAS-Q61, BRAF-V600, or the other cases. **B)** Proportion of cases that had more than one mutation in the indicated gene, out of all cases with a mutation in that gene. **C)** Map illustrating the number of cases with a mutation in more than one of the genes of interest in the study (PLCB4, PTEN, TP53, NF1). PLCB4 mutation was most often found with mutations in TP53 and NF1. **D)** Graph showing the mutation rate of BRAFV600, NRASQ61, PTEN, NF1, PLCB4 or TP53 (excluding synonymous mutations) in cases that were binned by their total number of mutations. The number of cases in each bin and the total number of mutations in those cases is shown below the graph.

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470 cases in TCGA-SKCM project

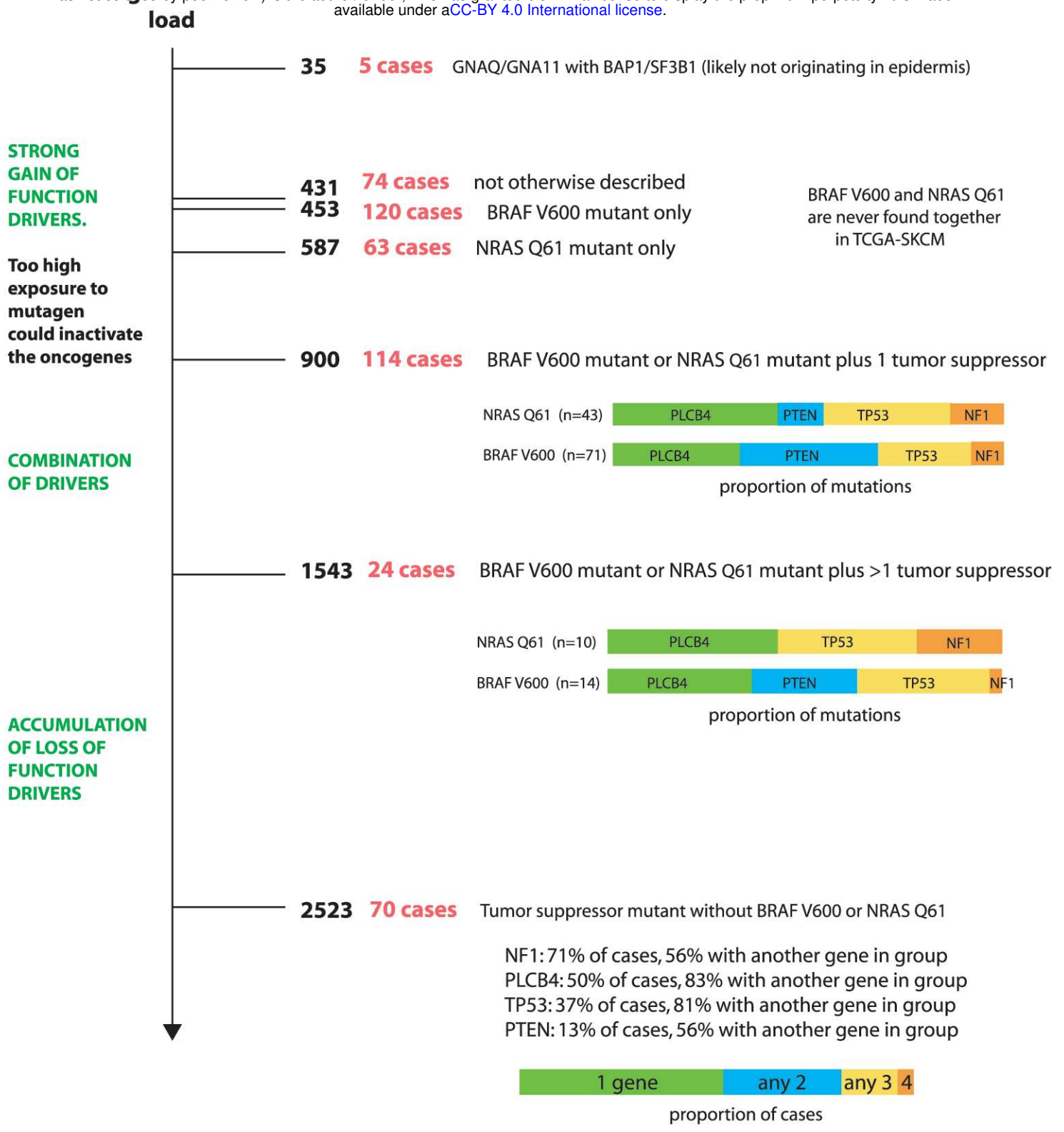


Figure 8. Summary of TCGA-SKCM cases, organized by average mutation load

As mutation load increases, gain of function drivers (BRAF-V600 and NRAS-Q61) become less frequent, while loss of function drivers (NF1, TP53, PTEN) become more frequent, as do mutations in PLCB4, a candidate tumor suppressor. When mutagen exposure is lower, oncogenic BRAF or NRAS mutations could be more likely to drive melanoma because an inactivating hit to the same allele is less likely. However, when mutagen exposure is higher, it instead allows for the accumulation of loss of function mutations in multiple tumor suppressors. At an intermediate mutation load, BRAF-V600 and NRAS-Q61 mutations co-occur with tumor suppressor mutations. BRAF-V600 preferentially associates with PTEN.

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Video 2. GNAQ melanocytes co-cultured with IFE, 625 minute time lapse.

Rank	Gene symbol	Gene name	Directly related to pigment production/melanosome biology?	Reference
1	Pmel	Premelanosome protein	Yes	(Lee et al., 1996)
2	Dct	Dopachrome tautomerase	Yes	(Tsukamoto et al., 1992)
3	Mir5133	MicroRNA 5133		
4	Tyrp1	Tyrosinase-related protein 1	Yes	(Kobayashi et al., 1994)
5	Ptgds	Prostaglandin D2 synthase (brain)		
6	Mlana	Melan-A	Yes	(Du et al., 2003)
7	Eef1a1	Eukaryotic translation elongation factor 1 alpha 1		
8	Vim	Vimentin		
9	Cd63	CD63 antigen	Yes	(van Niel et al., 2011)
10	Krt14	Keratin 14		
11	Lgals1	Lectin, galactose binding, soluble 1		
12	Fth1	Ferritin heavy polypeptide 1		
13	Rplp1	Ribosomal protein, large, P1		
14	Gstp1	Glutathione S-transferase, pi 1		
15	Slc24a5	Solute carrier family 24, member 5	Yes	(Lamason et al., 2005)
16	Gpnmb	Glycoprotein (transmembrane) nmb	Yes	(Loftus et al., 2009)
17	Rps27rt	Ribosomal protein, S27, retrogene		
18	Ftl1	Ferritin light polypeptide 1		
19	Krt10	Keratin 10		
20	Rpl8	Ribosomal protein L8		

Supplementary Table 2. Top 20 most highly expressed genes in mouse WT IFE melanocytes.

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GO_BP: Cell adhesion	Gene	Log2(fold_change)	p_value	q_value
Wisp1	WNT1 inducible signaling pathway protein 1(Wisp1)	-2.12367	5.00E-05	0.000658
Adam12	a disintegrin and metallopeptidase domain 12 (meltrin alpha)(Adam12)	7.1915	5.00E-05	0.000658
Adam23	a disintegrin and metallopeptidase domain 23(Adam23)	3.02523	0.0012	0.011002
Amigo1	adhesion molecule with Ig like domain 1(Amigo1)	2.03507	5.00E-05	0.000658
Aplp1	amyloid beta (A4) precursor-like protein 1(Aplp1)	3.5315	5.00E-05	0.000658
Cdh6	cadherin 6(Cdh6)	3.45939	5.00E-05	0.000658
Cercam	cerebral endothelial cell adhesion molecule(Cercam)	2.9412	5.00E-05	0.000658
Col5a1	collagen, type V, alpha 1(Col5a1)	2.94044	5.00E-05	0.000658
Col14a1	collagen, type XIV, alpha 1(Col14a1)	3.42669	5.00E-05	0.000658
Emilin2	elastin microfibril interfacier 2(Emilin2)	2.94389	0.00015	0.00177
Emb	embigin(Emb)	2.08491	5.00E-05	0.000658
Esam	endothelial cell-specific adhesion molecule(Esam)	6.68228	5.00E-05	0.000658
Fn1	fibronectin 1(Fn1)	3.50797	5.00E-05	0.000658
Itga1	integrin alpha 1(Itga1)	4.6513	0.00015	0.00177
Itgb3	integrin beta 3(Itgb3)	3.62889	5.00E-05	0.000658
Lama1	laminin, alpha 1(Lama1)	-2.1764	5.00E-05	0.000658
Mcam	melanoma cell adhesion molecule(Mcam)	3.70166	5.00E-05	0.000658
Ntm	neurotrimin(Ntm)	5.51819	5.00E-05	0.000658
Pdpn	podoplanin(Pdpn)	-2.08454	5.00E-05	0.000658
Ptpu	protein tyrosine phosphatase, receptor type, U(Ptpu)	2.2332	0.00015	0.00177
Sdk1	sidekick cell adhesion molecule 1(Sdk1)	-2.3421	5.00E-05	0.000658
Thbs2	thrombospondin 2(Thbs2)	3.33993	5.00E-05	0.000658

GO_MP: extracellular matrix structural constituent	Gene	Log2(fold_change)	p_value	q_value
Col4a1	collagen, type IV, alpha 1(Col4a1)	3.50241	5.00E-05	0.000658
Col4a2	collagen, type IV, alpha 2(Col4a2)	4.2194	5.00E-05	0.000658
Col5a1	collagen, type V, alpha 1(Col5a1)	2.94044	5.00E-05	0.000658
Col5a2	collagen, type V, alpha 2(Col5a2)	2.46268	5.00E-05	0.000658
Col27a1	collagen, type XXVII, alpha 1(Col27a1)	3.02033	5.00E-05	0.000658
Lama1	laminin, alpha 1(Lama1)	-2.1764	5.00E-05	0.000658

KEGG pathway: ECM-receptor interactions	Gene	Log2(fold_change)	p_value	q_value
Col4a1	collagen, type IV, alpha 1(Col4a1)	3.50241	5.00E-05	0.000658
Col4a2	collagen, type IV, alpha 2(Col4a2)	4.2194	5.00E-05	0.000658
Col5a1	collagen, type V, alpha 1(Col5a1)	2.94044	5.00E-05	0.000658
Col5a2	collagen, type V, alpha 2(Col5a2)	2.46268	5.00E-05	0.000658
Col27a1	collagen, type XXVII, alpha 1(Col27a1)	3.02033	5.00E-05	0.000658
Fn1	fibronectin 1(Fn1)	3.50797	5.00E-05	0.000658
Itga1	integrin alpha 1(Itga1)	4.6513	0.00015	0.00177
Itgb3	integrin beta 3(Itgb3)	3.62889	5.00E-05	0.000658
Lama1	laminin, alpha 1(Lama1)	-2.1764	5.00E-05	0.000658
Thbs2	thrombospondin 2(Thbs2)	3.33993	5.00E-05	0.000658

KEGG pathway: Focal adhesions	Gene	Log2(fold_change)	p_value	q_value
Col4a1	collagen, type IV, alpha 1(Col4a1)	3.50241	5.00E-05	0.000658
Col4a2	collagen, type IV, alpha 2(Col4a2)	4.2194	5.00E-05	0.000658
Col5a1	collagen, type V, alpha 1(Col5a1)	2.94044	5.00E-05	0.000658
Col5a2	collagen, type V, alpha 2(Col5a2)	2.46268	5.00E-05	0.000658
Col27a1	collagen, type XXVII, alpha 1(Col27a1)	3.02033	5.00E-05	0.000658
Fn1	fibronectin 1(Fn1)	3.50797	5.00E-05	0.000658
Itga1	integrin alpha 1(Itga1)	4.6513	0.00015	0.00177
Itgb3	integrin beta 3(Itgb3)	3.62889	5.00E-05	0.000658
Lama1	laminin, alpha 1(Lama1)	-2.1764	5.00E-05	0.000658
Myl9	myosin, light polypeptide 9, regulatory(Myl9)	2.47356	5.00E-05	0.000658
Pgf	placental growth factor(Pgf)	4.00998	5.00E-05	0.000658
Pdgfa	platelet derived growth factor, alpha(Pdgfa)	2.21579	5.00E-05	0.000658
Thbs2	thrombospondin 2(Thbs2)	3.33993	5.00E-05	0.000658

Supplementary Table 5. Genes supporting pathway analysis terms related to cell adhesion, focal adhesion and the extracellular matrix.

KEGG pathway: Axon guidance	Gene	Log2 fold change	p value	q value
Srgap3	SLIT-ROBO Rho GTPase activating protein 3(Srgap3)	-2.32519	5.00E-05	0.000658
Ablim2	actin-binding LIM protein 2(Ablim2)	3.03219	5.00E-05	0.000658
Plxna4	plexin A4(Plxna4)	-2.23771	5.00E-05	0.000658
Rgs3	regulator of G-protein signaling 3(Rgs3)	2.27044	5.00E-05	0.000658
Sema4f	sema domain, immunoglobulin domain (Ig), TM domain, and short cytoplasmic domain(Sema4f)	4.38799	5.00E-05	0.000658
Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A(Sema3a)	-2.29021	5.00E-05	0.000658
Sema3d	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D(Sema3d)	2.01686	5.00E-05	0.000658
Sema3g	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G(Sema3g)	2.84317	5.00E-05	0.000658

GO_BP: Nervous system development	Gene	Log2(fold change)	p_value	q_value
Chac1	ChaC, cation transport regulator 1(Chac1)	3.61727	0.00405	0.030694
Amigo1	adhesion molecule with Ig like domain 1(Amigo1)	2.03507	5.00E-05	0.000658
Avil	advillin(Avil)	3.5039	0.0003	0.00327
Cspg5	chondroitin sulfate proteoglycan 5(Cspg5)	3.80311	5.00E-05	0.000658
Dok5	docking protein 5(Dok5)	2.39296	0.0036	0.027827
Mef2c	myocyte enhancer factor 2C(Mef2c)	2.01656	5.00E-05	0.000658
Nes	nestin(Nes)	4.87602	5.00E-05	0.000658
Ndnf	neuron-derived neurotrophic factor(Ndnf)	2.33147	5.00E-05	0.000658
Nr4a2	nuclear receptor subfamily 4, group A, member 2(Nr4a2)	2.06084	5.00E-05	0.000658
Plxna4	plexin A4(Plxna4)	-2.23771	5.00E-05	0.000658
Rgs9	regulator of G-protein signaling 9(Rgs9)	3.33195	5.00E-05	0.000658
Sema4f	sema domain, immunoglobulin domain (Ig), TM domain, and short cytoplasmic domain(Sema4f)	4.38799	5.00E-05	0.000658
Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A(Sema3a)	-2.29021	5.00E-05	0.000658
Sema3d	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D(Sema3d)	2.01686	5.00E-05	0.000658
Serpine2	serine (or cysteine) peptidase inhibitor, clade E, member 2(Serpine2)	5.82485	5.00E-05	0.000658
Ttl7	tubulin tyrosine ligase-like family, member 7(Ttl7)	2.07648	5.00E-05	0.000658

GO_CC: Axon	Gene	Log2(fold change)	p_value	q_value
Avil	advillin(Avil)	3.5039	0.0003	0.00327
Aqp1	aquaporin 1(Aqp1)	5.98438	5.00E-05	0.000658
C4b	complement component 4B (Chido blood group)(C4b)	-2.89878	5.00E-05	0.000658
Cst3	cystatin C(Cst3)	2.26692	5.00E-05	0.000658
Dtna	dystrobrevin alpha(Dtna)	4.21663	5.00E-05	0.000658
Mme	membrane metallo endopeptidase(Mme)	-2.20961	5.00E-05	0.000658
Npy1r	neuropeptide Y receptor Y1(Npy1r)	-3.2591	5.00E-05	0.000658
Ntm	neurotrimin(Ntm)	5.51819	5.00E-05	0.000658
Kcna2	potassium voltage-gated channel, shaker-related subfamily, member 2(Kcna2)	-2.49415	5.00E-05	0.000658
Kcna6	potassium voltage-gated channel, shaker-related, subfamily, member 6(Kcna6)	2.01729	5.00E-05	0.000658
Penk	preproenkephalin(Penk)	5.7203	0.0003	0.00327
Pgr	progesterone receptor(Pgr)	4.05452	5.00E-05	0.000658

Sema3a	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A(Sema3a)	-2.29021	5.00E-05	0.000658
Sphk1	sphingosine kinase 1(Sphk1)	3.34582	5.00E-05	0.000658
Sncg	synuclein, gamma(Sncg)	2.01133	5.00E-05	0.000658
Uchl1	ubiquitin carboxy-terminal hydrolase L1(Uchl1)	2.83131	5.00E-05	0.000658

Supplementary Table 6. Genes supporting pathway analysis terms related to axon guidance, nervous system development, and axon cellular component.

Cosmic Sample ID	PMID	GNAQ mutation	Other key mutations	Scope of study
1175905	19078957	Q209L	N/A	GNAQ only
2543893	26275246: "Malignant melanoma arising in blue nevus"	Q209L	N/A	N/A
2724266	28481359	Q209H (freq =0.04)	N/A	N/A
2719694	28481359	Q209L	BAP1	MSK-Impact screen
2718695	28481359	Q209L	SF3B1 ^{R625C}	MSK-Impact screen
1993392	21726664	Q209L	None	30 gene screen
1993559	21726664	Q209P	None	30 gene screen
2088283-2088289 From one patient	24504448	Q209P	BRAF ^{V600E}	NGS
2380390 (RP-A690)	26091043	Q209P	SF3B1 ^{R625H}	NGS (TCGA-SKCM)
2121734 (ER-A2NF)	26091043	Q209P	SF3B1 ^{R625H}	NGS (TCGA-SKCM)
2380420 (BF-AAP8)	26091043	Q209P	BAP1	NGS (TCGA-SKCM)

Supplementary Table 7. Identification of GNAQ hotspot mutations among human malignant melanomas potentially arising in the epidermis.

The Cosmic database was searched for GNAQ Q209 or R183 missense mutations in tumors with the following criteria: Primary site: Skin and Histology: Malignant melanoma. Must have one of the following terms for Subhistology: Superficial spreading, Nodular, Spitzoid, Lentigo maligna, Acral lentiginous, Amelanotic, Epithelioid, Nevoid, or NS (Not Specified). (Excluded subhistology terms: Benign, Desmoplastic, Mucosal, Blue). Must have one of the following terms for Subsite: ear, lip, elbow, back, upper back, lower back, ankle, trunk, groin, hand, knee, chest, scalp, face, leg, shoulder, arm, breast, neck, flank, extremity, upper arm, forearm, foot, chronically sun exposed, intermittently sun exposed, eye, non chronically sun exposed or NS (Not specified). (Excluded subsite terms: mucosal, axilla, subungual, penis, nipple, vulva). The total number of GNAQ tested samples defined by these terms was 2753. Samples that could have arisen in the epidermis are highlighted in yellow. N/A, non-applicable; NGS, Next generation sequencing.

Cosmic Sample ID	PMID	GNA11 mutation	Other mutations	Scope of study
2544691	26825879	Q209L	None	4 genes screened
1838375	22817889: "uveal melanoma"	Q209L	N/A	N/A
2013581	22842228: "uveal melanoma"	R183C	N/A	N/A
2013602	22842228	R183C	NRAS ^{Q61H}	NGS exome
2013703	22842228: "uveal melanoma"	Q209L	N/A	N/A
2013594	22842228	Q209L	EIF1AX	NGS exome
753596	none	R183C	N/A - Cell line	N/A
2237811	24714776: "most likely represents metastatic uveal melanoma"	Q209L	N/A	N/A
2380396 (RP-A6K9)	26091043	Q209L	BAP1	NGS (TCGA-SKCM)
2339744 (W3-A285)	26091043	R183C	NRAS ^{Q61R}	NGS (TCGA-SKCM)
2121738 (ER-A3ET)	26091043	Q209L	P53 ^{Y220C} , PLCB4 splice	NGS (TCGA-SKCM)
2121737 (ER-A3ES)	26091043	Q209L	SF3B1 ^{R625H}	NGS (TCGA-SKCM)

Supplementary Table 8. Identification of *GNA11* hotspot mutations among human malignant melanomas potentially arising in the epidermis. The Cosmic database was searched for GNA11 Q209 or R183 missense mutations in tumors with the following criteria: Primary site: Skin and Histology: Malignant melanoma. Must have one of the following terms for Subhistology: Superficial spreading, Nodular, Spitzoid, Lentigo maligna, Acral lentiginous, Amelanotic, Epithelioid, Nevoid, or NS (Not Specified). (Excluded subhistology terms: Benign, Desmoplastic, Mucosal, Blue). Must have one of the following terms for Subsite: ear, lip, elbow, back, upper back, lower back, ankle, trunk, groin, hand, knee, chest, scalp, face, leg, shoulder, arm, breast, neck, flank, extremity, upper arm, forearm, foot, chronically sun exposed, intermittently sun exposed, eye, non chronically sun exposed or NS (Not specified). (Excluded subsite terms: mucosal, axilla, subungual, penis, nipple, vulva). The total number of *GNA11* tested samples defined by these terms was 2295. Samples that could have arisen in the epidermis are highlighted in yellow. N/A, non-applicable; NGS, Next generation sequencing.

Supplementary Table 10. Details on statistical tests.

	Comparison of:	Statistical test used:	Exact p value:	Other values:	Degrees of freedom:	Definition of replicates:
1B	Average number of LacZ+ cells per scale in Wt vs GNAQ mice at 1 week of age	Unpaired t-test (two tailed)	0.18	t=1.491	df=7	Experiment performed on 4 Wt mice and 5 GNAQ mice. At least 25 scales were examined per mouse (depending on the number of scales in the row).
1B	Average number of LacZ+ cells per scale in Wt vs GNAQ mice at 8 weeks of age	Unpaired t-test (two tailed)	0.045	t=2.367	df=8	Experiment performed on 5 Wt mice and 5 GNAQ mice. At least 25 scales were examined per mouse (depending on the number of scales in the row).
1D	Percentage of scales affected by loss of boundaries at 8 weeks of age	Kolmogorov-Smirnov test	0.18			Experiment performed on 5 Wt mice and 5 GNAQ mice. At least 25 scales were examined per mouse (depending on the number of scales in the row).

1D	Percentage of scales affected by loss of melanin at 8 weeks of age	Kolmogorov-Smirnov test	0.048			Experiment performed on 5 Wt mice and 5 GNAQ mice. At least 25 scales were examined per mouse (depending on the number of scales in the row).
2B	Percentage of tomato positive cells sorted from Wt vs GNAQ IFE	Unpaired t-test (two tailed)	0.012	t=3.132	df=9	Percentages measured in 6 Wt tails/FACS runs and 5 GNAQ tails/FACS runs
2C	Survival of WT, GNAQ and BRAF IFE melanocytes plated on fibronectin	2 way ANOVA with Tukey's multiple comparisons test	3.3 x 10 ⁻⁵ for genotype 1.6 x 10 ⁻⁵ for time	Geisser-Greenhouse's epsilon = 0.3539		Fraction of survival measured in 10 independent cell cultures derived from 3 or 4 mice of each genotype
2H	Survival of WT vs GNAQ IFE melanocytes plated on MEFs	2 way ANOVA with Tukey's multiple comparisons test	0.48 for genotype 0.31 for time	Geisser-Greenhouse's epsilon = 0.3030		Fraction of survival measured in 3 and 2 independent cell cultures derived from 3 GNAQ mice and 2 WT mice

3A	Survival of WT melanocytes plated with IFE vs WT melanocytes plated on fibronectin	2 way ANOVA	0.0071 for culture condition 1.0 x 10 ⁻⁵ for time	Geisser-Greenhouse's epsilon = 0.3081		Fraction of survival measured in 3 and 3 independent cell cultures derived from 6 Wt mice.
3B	Survival of GNAQ melanocytes plated with IFE vs GNAQ melanocytes plated on fibronectin	2 way ANOVA	0.0053 for culture condition 0.00063 for time	Geisser-Greenhouse's epsilon = 0.3370		Fraction of survival measured in 3 and 3 independent cell cultures derived from 6 GNAQ mice.
3D	Circularity of WT vs GNAQ melanocytes plated with IFE	Unpaired t-test (two tailed)	0.000000050	t=5.675	df=193	Averages calculated from 116 WT cells and 79 GNAQ cells from four independent culture experiments
3E	Protrusion length of WT vs GNAQ melanocytes plated with IFE	Unpaired t-test (two tailed)	0.00041	t=3.593	df=207	Averages calculated from 115 WT cells and 94 GNAQ cells from four independent culture experiments
3F	Number of protrusions in WT vs GNAQ melanocytes plated with IFE	Unpaired t-test (two tailed)	0.0096	t=2.615	df=207	Averages calculated from 115 WT cells and 94 GNAQ cells from four independent culture experiments
3G	Cell area of WT vs GNAQ melanocytes plated with IFE	Unpaired t-test (two tailed)	2.6 x 10 ⁻¹³	t=7.862	df=193	Averages calculated from 116 WT cells and 79 GNAQ cells from four independent culture experiments

3I	Percentage of melanocytes cultured with IFE exhibiting fragmentation of dendrites	Unpaired t-test (two tailed)	0.021	t=2.875	df=8	Percentage calculated from 5 WT cultures and 5 GNAQ cultures (total cells tracked = 187 and 98)
4B	Total distance traveled by WT vs GNAQ cells co-cultured with IFE	Unpaired t-test (two tailed)	0.86	t=0.1827	df=114	Averages calculated from 57 WT cells and 59 GNAQ cells from four independent culture experiments
4C	Directness of cell migration in WT vs GNAQ cells co-cultured with IFE	Unpaired t-test (two tailed)	0.18	t=1.359	df=106	Averages calculated from 59 WT cells and 49 GNAQ cells from four independent culture experiments
4D	Percentage of cells that divided during 20 hours observation, when co-cultured with IFE.	Ordinary 1 way ANOVA with Tukey's multiple comparisons test	0.0040 overall			Averages calculated from 5 WT, 5 GNAQ and 3 BRAF independent cultures. Total cell number tracked: 187 WT cells, 98 GNAQ cells and 121 BRAF cells.
4H	Average time for cytokinesis in cells plated with IFE.	Ordinary 1 way ANOVA, with Tukey's multiple comparisons test	3.0×10^{-10} overall			Average time calculated from 1 GNAQ, 11 WT and 14 BRAF cells that divided in 5, 5 or 3 independent culture experiments.

5A	Survival of WT melanocytes directly co-cultured with IFE vs transwell cultured with IFE	2 way ANOVA	0.022 for culture condition			Fraction of survival calculated from 3 and 3 independent cell cultures for each condition, derived from 6 WT mice.
5B	Survival of GNAQ melanocytes directly co-cultured with IFE vs transwell cultured with IFE	2 way ANOVA	0.27 for culture condition			Fraction of survival calculated from 3 and 3 independent cell cultures for each condition, derived from 6 GNAQ mice.
6C	Cell fibrousness in WT vs GNAQ melanocytes plated with IFE	Unpaired t-test (two tailed)	1.8×10^{-12}	t=7.544	df=193	Averages calculated from 116 WT cells and 79 GNAQ cells from four independent culture experiments