**ORIGINAL ARTICLE**

*Ric-8A* gene deletion or phorbol ester suppresses tumorigenesis in a mouse model of \( GNAQ^{Q209L} \)-driven melanoma

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The heterotrimeric G protein alpha subunit oncogenes *GNAQ* or *GNA11* carry Q209X or R183X activating mutations and are present with \(~90\%\) frequency in human uveal melanomas. Forced expression of *GNAQ/11*\(^{Q209L}\) in melanocytes is sufficient to drive metastatic melanoma in immune-compromised mice. No known drugs directly target these oncogenic G proteins. Ric-8A is the molecular chaperone that selectively folds Gaq/11 subunits. Targeting Ric-8A serves as a rational, yet unexplored approach to reduce the functional abundance of oncogenic Gaq/11 in order to blunt cancer signaling. Here, using mouse melanocyte cell graft tumorigenesis models, we determined that *Ric-8A* genetic ablation attenuated the abundance and melanoma-driving potential of Gaq-Q209L. A new conditional *Ric-8A*\(^{Flx/Flox}\;Rosa-CreER\(^{1\;\text{ER}}\) mouse strain was derived and used as a tissue source to culture an immortalized, tamoxifen-inducible *Ric-8A-* knockout melanocyte cell line that required 12-O-tetradecanoylphorbol-13-acetate (TPA, phorbol ester) for growth. The cell line failed to grow tumors when grafted into immune-compromised mice regardless of *Ric-8A* expression. Stable expression of human *GNAQ*\(^{Q209L}\), but not *GNAQ*\(^{WT}\) in the cell line promoted TPA-independent cell proliferation, and upon cell grafting in mice, the initiation and robust growth of darkly-pigmented melanoma tumors. Deletion of *Ric-8A* in *GNAQ*\(^{Q209L}\) cells restored TPA-dependent growth, reduced Gaq-Q209L below detectable levels and completely mitigated tumorigenesis from primary or secondary cell line grafts. Interestingly, TPA treatment of cultured *GNAQ*\(^{Q209L}\) cells or host animals grafted with *GNAQ*\(^{Q209L}\) cells also sharply reduced Gaq-Q209L abundance and tumorigenic capacity. Finally, tumorigenesis initiated from *GNAQ*\(^{Q209L}\) cell grafts, followed by host mouse systemic tamoxifen treatment to delete *Ric-8A* in the grafted cells completely abrogated *GNAQ*\(^{Q209L}\)-driven tumor progression unless a stable human *Ric-8A* transgene was used to rescue the floxed *Ric-8A* alleles. Our work defines two new rational targets that may be developed as potential uveal melanoma therapies through reduction of Gaq/11-Q209L oncoprotein abundance: (1) *Ric-8A* inhibition and (2) phorbol ester treatment.

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**INTRODUCTION**

Uveal melanomas arise from melanocytes contained in the uveal tract of the ocular cavity. Owing to limited responses to systemic chemotherapies, metastatic forms of the cancer have a poor prognosis with a median survival rate of 12 months, accounting for \(~5\%\) of all lethal melanoma cases.\(^1\) Metastases occur in \(~90\%\) of uveal melanoma patients, predominantly to the liver, and to a lesser extent, the lung and bones.\(^2\) The genetic alterations in uveal melanoma are distinct from cutaneous melanoma, which commonly carry driver mutations in *BRAF* or *NRAS*.\(^3\;6\) Uveal melanomas are predominantly \((\geq 85\%)\) driven by mutations in *GNAQ* or *GNA11*, genes that encode the partially redundant heterotrimeric G protein alpha subunits, Gaq and Gai1.\(^3\;7\;10\) The point mutations are restricted to residues Q209 and R183, which are critical for intrinsic GTP hydrolysis (GTPase) activity of the G proteins, resulting in persistently active GTP-bound Gai subunits, and therefore constitutively-active signaling.\(^3\;7\;9\) Q209 mutations are more prevalent than R183 mutations in uveal melanoma because Gaq/11-Q209X proteins are stronger activators of downstream signaling.\(^8\;11\) Gaq/11-Q209X has poorer GTP hydrolytic activity in comparison with Gaq/11-R183X and is insensitive to regulators of G protein signaling (RGS)-stimulated GTP hydrolysis.\(^12\;14\) Interestingly, *GNAQ* or *GNA11* activating mutations induce dermal hyperpigmentation, and are frequently found in cutaneous benign blue nevi and a small subset of melanomas, indicating that overactive Gaq/11 signaling may also be important during priming events of dermal melanocyte neoplasms.\(^6\;8\;9\;15\;16\) Studies using genetic or xenograft mouse models demonstrated that melanocyte-specific *GNAQ* or *GNA11* Q209L expression promoted invasive and metastatic melanoma.\(^8\;16\;17\) Moreover, massive metastatic cutaneous melanomas were induced when the Gq/11-coupled GPCRs, mGluR1 or mGluR5 were ectopically expressed from mouse melanocyte-specific promoters.\(^19\;20\) Both mGluRs 1 and 5 have reasonably high basal ability to activate Gq/11 in the absence of agonist.\(^22\;23\) The emerging evidence is quite convincing that aberrant stimulation of Gaq/11 signaling pathways by hyperactive GPCRs or oncogenic *GNAQ/11* mutations, contributes to the development of various melanocyte neoplasms including cellular transformation and uveal melanoma.\(^9\)

There are no current drugs that directly target oncogenic Gaq/11 proteins, although encouraging developmental efforts are underway.\(^24\;25\) Existing small-molecule Gaq inhibitors exhibit efficacy to inhibit wild-type Gaq or Gq, and some related G proteins, but fail to attenuate Gaq-Q209L, or provide limited inhibition of Gaq-R183C-dependent signaling in cultured cells.\(^26\;28\) These inhibitors may ultimately prove useful to block pathogenic, hyperactive GPCR signaling, but the inability to inhibit oncogenic Gaq/11 directly has prompted us to explore an alternative means to block oncogenic Ga subunits by targeting...
the highly-substrate-specific molecular chaperones, Ric-8A or Ric-8B that act collectively to maintain the proper abundances of all heterotrimeric G protein α subunits. Studies using cell-free protein translation/folding systems demonstrated that Ric-8A directly participates in the biosynthetic folding of oncogenic Gaq-Q209L and Gαq/i/13. When these G proteins are produced in the absence Ric-8A, they are mis-folded and rapidly degraded, accounting for massive ~90-95% reductions in membrane-associated G protein levels.

Here, we conducted a proof-of-concept investigation demonstrating that genetic ablation of Ric-8A blocked GNAQQ209L-driven melanocyte transformation and melanoma pathogenesis using cell graft mouse tumor models. A new C57Bl6J mouse with floxed Ric-8A alleles was derived for the work that permitted conditional Ric-8A deletion. Primary melanocytes were cultured from this strain and used to create immortalized murine melanocyte cell lines that stably expressed human GNAQWT or oncogenic GNAQQ209L. The GNAQQ209L but not the GNAQWT melanocyte cell line exhibited phorbol ester-independent proliferation in culture; a feature associated with melanocyte transformation, and one that was abolished by Ric-8A deletion. When the GNAQQ209L melanocyte cell line was grafted into immune-compromised mice, pigmented melanoma tumors grew robustly. No tumors formed from GNAQWT melanocyte cell line grafts. Deletion of Ric-8A in culture before GNAQQ209L cell grafting completely abrogated tumor growth. Ric-8AFlox/Flox, GNAQQ209L murine melanoma cell lines were cultured ex vivo from primary tumor explants and secondary tumor formation from these cells was also blocked by in vitro Ric-8A deletion. GNAQQ209L melanocyte cell grafts were then permitted to initiate tumorigenesis, followed by host mouse tamoxifen treatment to delete floxed Ric-8A in the grafted melanocytes. Systemic tamoxifen treatment specifically abrogated GNAQQ209L-driven tumorigenesis from Ric-8AFlox/Flox melanocytes.

We also made an unexpected observation that culture of GNAQQ209L melanocytes in the presence of phorbol ester led to a dramatic decrease in Gaq-Q209L oncoprotein levels. Accordingly, phorbol ester-pre-cultured GNAQQ209L melanocytes completely failed to form melanoma tumors when grafted into mice, and systemic phorbol ester treatment of host mice grafted with GNAQQ209L melanoma cells suppressed tumor initiation and progression. In sum, our study has identified Ric-8A inhibition and phorbol ester (over)stimulation of protein kinase C (PKC) as two new rationale means to attenuate Gαq-Q209L oncoprotein levels. Successful future development of therapeutics against these new targets could provide wanted therapies for GNAQ/11-driven uveal melanoma, and perhaps additional oncogenic G protein-influenced cancers.

Figure 1. Deletion of Ric-8A in murine melanocytes confer a modest cell proliferation advantage, but does not confer TPA- and/or CTX-independent growth. (a) Bright-field images of untreated or 4OHT-treated immortalized Ric-8AFlox/Flox, Rosa-CreER+/- melanocyte cell lines grown in the presence of CTX, or TPA, or both for 4 days. (b) Quantitative western blot analyses of Ric-8A, Gα11/12, Gαq/11, Gα13, Gox, Gvo and Gβ1-4 levels in Cre or GFP (control) lentivirus, or 4OHT-treated and untreated cultured melanocyte cell lines. Relative GAPDH or α-tubulin levels are shown as normalization controls. (c) Cell proliferation analyses of untransduced, and Cre or GFP (control) lentivirus-infected Ric-8AFlox/Flox, Rosa-CreER+/- melanocytes. Error bars are the mean ± s.e.m. of experiments performed in triplicate.
RESULTS

Generation of a conditional Ric-8A mouse

Germline deletion of mouse Ric-8A causes embryonic lethality due to severe gastrulation defects.29,34 We created a C57Bl/6 J mouse strain with potential for conditional Ric-8A knockout using Ric-8A gene-targeted embryonic stem cell lines available from the Knockout Mouse Project (KOMP, #CSD70793) (Supplementary Figure S1). Ric-8A mice with two copies of floxed exon 5 were viable, reproductive and had no obvious defects; findings consistent to those obtained with a conditional Ric-8A mouse produced by a distinct gene targeting strategy.35 Mouse embryonic fibroblasts (MEFs) were cultured from our Ric-8A Flox/Flox mice and infected with a Cre-NLS-expressing lentivirus. PCR analysis of MEF genomic DNA revealed efficient Cre-mediated deletion of Ric-8A exon 5 (Supplementary Figure S2). Immunoblot analyses of MEF lysates demonstrated efficient Cre-mediated

Figure 2. Phorbol ester-independent growth of cultured melanocyte cell lines is conferred by oncogenic GNAQQ209L and reversed by induced Ric-8A knockout. (a, b) Cultured Ric-8A Flox/Flox; Rosa-CreER+/- melanocyte cell lines stably expressing IRES-GFP, GNAQ WT-IRES-GFP or GNAQ Q209L-IRES-GFP were treated with solvent or 4OHT to induce Ric-8A knockout, followed by growth in culture medium ± TPA for 7 days as indicated. Cells were stained with DAPI and counted using Image J, and the data were reported as fold cell growth. Error bars are the mean ± s.e.m. of experiments performed in triplicate. Student’s t-tests were used to denote significant differences. (c) Quantitative immunoblot analyses of relative Ric-8A, Gαq/11 and Gαq-Q209L levels in membrane fractions prepared from melanocyte cell lines treated with solvent or 4OHT, ± TPA as indicated. Relative α- or γ-tubulin levels were measured as loading controls. (d) Semi-quantitative genomic DNA PCR analysis of the stably integrated human GNAQQ209L transgene in the GNAQ Q209L-IRES-GFP mouse melanocyte cell line after culture for 11 days ± continuous TPA treatment. (e) Semi-quantitative RT–PCR analyses of human GNAQ Q209L transcript levels from cDNA prepared from the GNAQ Q209L-IRES-GFP melanocyte cell line after 11 days culture ± continuous TPA treatment. (f) Quantitative western blots of relative Gαq-Q209L and Gαq/11 levels in crude membrane fractions prepared from the GNAQ Q209L melanocyte cell line cultured in the presence of 200 nM TPA for the indicated time course. (g) Time course of MG132 proteasome inhibition-mediated recovery of Gαq-Q209L levels in the GNAQ Q209L-melanocyte cell line cultured continuously in the presence or absence of TPA. Quantitative western blots show relative Gαq-Q209L, Gαq/11 and Ric-8A levels over the 20-h MG132 time course.
Generation of a mouse melanocyte cell line with potential to conditionally delete Ric-8A

To investigate the effect of Ric-8A deletion on Ga subunit abundances in melanocytes and melanocyte transformation induced by oncogenic GaQ-Q209L, we first created and characterized an immortalized melanocyte cell line with inducible Ric-8A knockout potential. Primary melanocytes were isolated from Ric-8Aα/βγΔ mouse embryonic fibroblasts (MEFs) and immortalized by serial passaging in medium containing 12-O-tetradecanoylphorbol-13-acetate (TPA, a phorbol ester) and chola toxin (CTX). The Ric-8AαβγΔ/Δ melanocyte cell line exhibited TPA- and CTX-dependent growth, a shared characteristic with the non-tumorigenic mouse melanocyte cell line, Melan-a (Figure 1a).36 Ric-8A is produced in melanocytes, as are tested examples from all four heterotrimeric G protein α subunit classes (Gaα/GaB, Gaα/GaG, Gaα/GaH, and Gaα/GaA). Ric-8A deletion in the melanocyte cell line mediated by Cre-NLS lentiviral infection or by 4-hydroxynamoxfen (4OHT) activation of Cre recombinase, decreased Ric-8A abundance and caused concomitant decreases in the levels of G protein subunits folded by Ric-8A (Figure 1b). Gas levels remained unchanged because this subunit is folded by Ric-8B.29,32,38

Ric-8A deletion had no effect on the requirements of TPA or CTX for melanocyte cell line growth, but did modestly enhance the cell proliferation rate (Figures 1a and c). Enhanced proliferation has been observed for other Ric-8A-null cell types.29,39 We hypothesize that Ric-8A deletion and the consequent decreases in G protein α subunit abundances (Gaα/i/13 classes) release a modest cell proliferation damper conferred normally by homeostatic G protein signaling.

Ric-8A deletion suppresses phorbol ester-independent growth of GaαQ209L-transformed melanocytes

Cultured melanocytes, Melan-a cells and our Ric-8AαβγΔ/Δ melanocyte cell line require continuous signaling for stable transgene expression (Supplementary Figure S3). The combined action of phorbol-13-acetate (TPA, a phorbol ester) and cholera toxin (CTX) for growth of the GaαQ209L melanocyte cell line was substantially less than that in the TPA-untreated cell line. These results suggest that phorbol esters may present a viable means to reduce GaαQ209L oncprotein levels and attendant cancer-driving signaling. For experimental considerations, TPA exclusion from melanocyte culture medium is required to maintain GaαQ209L oncprotein levels. TPA inclusion is required for growth of all other melanocyte cell lines that lacked the GaαQ209L oncogene.

Phorbol ester- or Ric-8A deletion-induced GaαQ209L abundance reduction suppresses GaαQ209L-driven melanoma tumorigenesis

The GaαQ209L, GaαWT and the GFP (Ric-8AαβγΔ/Δ; Rosa-CreER+/−) melanocyte cell lines (5 × 10⁵ cells each) were grafted subcutaneously into the rear flanks of immune-compromised NSG mice. GaαQ209L cells pre-cultured in the absence of TPA formed tumors that could first be measured beneath the skin after an ~28-day latency. The tumors continued to grow for an additional ~30 days until the mice were killed and the heavily melanin-pigmented tumors were excised and weighed. The GaαQ209L tumors were roughly 0.6–2.0 cm in diameter and often oblong and/or multi-lobed. No tumors were formed from GaαWT or GFP cell grafts (Figure 3a).

The GaαQ209L and control GFP melanocyte cell lines were then treated with or without 4OHT in culture to delete Ric-8A before grafting into NSG mice (left flanks, solvent-treated cells and right flanks, 4OHT-treated cells). The GFP-only melanocyte cell line had no tumorigenic capacity regardless of Ric-8A expression or deletion (Figures 3a and d, bottom panel). Ric-8A deletion substantially attenuated GaαQ209L-driven tumor progression (Figure 3b) and measured tumor weights at the completion of the experiments (Figures 3c and d). This indicates that the modest in vitro melanocyte proliferation advantage conferred by Ric-8A knockout (Figure 1c) was negated by loss of GaαQ209L oncprotein folding capacity during in vivo tumor growth.

GaαQ209L cells pre-cultured in the presence of TPA also had dramatically reduced GaαQ209L levels (Figure 2c) and accordingly, failed to grow melanoma tumors when grafted into mice (Figures 3e and f). PKC overstimulus by the combined action of GaαQ209L-stimulated diacylglycerol (DAG) production and the exogenous DAG mimetic, TPA may induce a melanocyte cytotoxicity that is overcome by GaαQ209L protein downregulation. This raises the enticing possibility that phorbol esters may be used therapeutically to induce cancer cell toxicity in GaαWT-driven uveal melanomas.
Ric-8A deletion suppresses secondary tumor progression of grafted, ex vivo cultured GNAQ\textsuperscript{Q209L} melanoma cell lines

Excised Ric-8A\textsuperscript{Flox/Flox}, Rosa-CreER\textsuperscript{1/2}−/−; Tg (GNAQ\textsuperscript{Q209L}) tumors from Figure 3a were cultured ex vivo in standard melanocyte culture medium lacking TPA to derive two independent GNAQ\textsuperscript{Q209L} melanoma cell lines, with which to determine the effect of Ric-8A deletion on secondary tumor progression. The melanoma cell lines exhibited TPA-independent growth and acquired TPA dependence after Ric-8A deletion, both characteristics of the parental GNAQ\textsuperscript{Q209L} melanocyte cell line used to generate the primary tumors (Figures 4a and b). The GNAQ\textsuperscript{Q209L} melanoma cell lines retained melanin pigmentation but had adopted a morphology that was more spindle-shaped or epithelial-like in comparison with the parental melanocyte cell line (Figure 4b). Both tumor cell lines retained the capacity to induce Ric-8A deletion and deplete endogenous G proteins and Gaq-Q209L (Figure 4c). The melanoma cell lines were pre-treated with 4OHT ex vivo to induce Ric-8A knockout before secondary graft experiments (Figure 4d). Ric-8A knockout dramatically blunted Gaq-Q209L-driven secondary melanoma tumor progression. One tumor cell line exhibited accelerated tumorigenic onset and progression whereas the second cell line exhibited kinetics similar to, or slightly delayed in comparison with the parental GNAQ\textsuperscript{Q209L} melanocyte cell line.

Host animal tamoxifen treatment induces Ric-8A deletion in grafted tumor cells and blunts GNAQ\textsuperscript{Q209L} tumorigenesis

The Ric-8A\textsuperscript{Flox/Flox}, Rosa-CreER\textsuperscript{1/2}−/−; Tg (GNAQ\textsuperscript{Q209L}) melanocyte cell line was transduced with a human Ric-8A cDNA or control lentivirus and selected for stable expression of the transgene. 4OHT treatment of both melanocyte cell lines in culture induced deletion of the floxed, mouse Ric-8A alleles, which resulted in greatly reduced Gaq-Q209L and Gaq/11 levels in the cell line lacking the Ric-8A transgene. Gaq-Q209L remained at normal levels in the counterpart Ric-8A transgene expressing cell line, despite 4OHT treatment (Figure 5a).

Control and Ric-8A transgene cell lines were grafted into the left and right flanks of NSG mice, respectively. At days 6 through 26 post-subcutaneous grafts, the mice were intraperitoneally (i.p.) injected with tamoxifen q.a.d. to induce floxed Ric-8A deletion in the grafted cells. Solvent injections were performed similarly for control, Ric-8A\textsuperscript{Flox/Flox} grafted mice (that is, no Ric-8A transgene). In vivo tamoxifen treatment effectively ablated tumorigenesis driven by GNAQ\textsuperscript{Q209L} in the Ric-8A\textsuperscript{Flox/Flox} background, but not in the background in which the floxed Ric-8A alleles were rescued by expression of the Ric-8A cDNA transgene (Figures 5b and c). These results clearly show that loss of tumor cell Ric-8A expression and

Figure 3. Attenuation of melanocyte Gaq-Q209L levels by Ric-8A deletion or phorbol ester treatment blocks GNAQ\textsuperscript{Q209L}-driven melanoma tumor progression in engrafted mice. (a) Tumor growth kinetics of subcutaneously grafted Ric-8A\textsuperscript{Flox/Flox}, Rosa-CreER\textsuperscript{1/2}−/− melanocyte cell lines that stably expressed GFP, GNAQ\textsuperscript{WT} or GNAQ\textsuperscript{Q209L}. Images of GNAQ\textsuperscript{Q209L} tumors excised at the termination of the experiment are shown alongside each individual growth rate curve. (b) Tumor progression kinetics of GFP-expressing or GNAQ\textsuperscript{Q209L}-transformed Ric-8A\textsuperscript{Flox/Flox}, Rosa-CreER\textsuperscript{1/2}−/− melanocyte cell lines that were untreated or 4OHT treated to induce Ric-8A deletion before subcutaneous engraftment. (c) Average weights of Ric-8A\textsuperscript{Flox/Flox} or Ric-8A\textsuperscript{KO/KO} GNAQ\textsuperscript{Q209L}–driven melanoma tumors at the experimental end point of 59 days. (d) Representative images of isolated GNAQ\textsuperscript{Q209L}–driven tumors and the mouse injection sites of the GFP melanocyte cell line that showed no signs of tumor growth at day 59 post injection. Error bars are the mean ± s.e.m. of 3–4 independent experiments. (e) Tumor growth kinetics of subcutaneously grafted Ric-8A\textsuperscript{Flox/Flox}, Rosa-CreER\textsuperscript{1/2}−/−; Tg (GNAQ\textsuperscript{Q209L}–IRES-GFP) melanocyte cell line pre-cultured in the presence or absence of TPA. (f) Representative images of excised tumors obtained from grafted GNAQ\textsuperscript{Q209L} cells that had been pre-cultured without TPA and a representative subcutaneous injection site of grafted GNAQ\textsuperscript{Q209L} cells that had been pre-cultured in the presence of TPA at the termination of the experiment (day 55). Data are the mean ± s.e.m. (n = 4).
not whole animal tamoxifen treatment per se accounts for the block of GNAQQ209L-driven melanoma tumorigenesis.

Host animal phorbol ester treatment suppresses melanoma tumorigenesis of grafted GNAQQ209L melanocytes
A Ric-8A Flox/Flox, Rosa-CreER12/12; Tg (GNAQ Q209L) melanoma cell line was generated from an excised primary tumor in Figure 4 and continuously cultured in the absence of TPA. This cell line was treated acutely with or without TPA for 48 h and then for an additional 24 h in the presence of MG132 ± TPA (Figure 6a). MG132 treatment provided a substantial boost in Gαq-209L abundance, as shown for the parental GNAQ Q209L melanocyte cell line in Figure 2g. The acute TPA treatment markedly reduced Gαq-Q209L levels, as well as the ability of MG132 to provide recovery, demonstrating that the established melanoma cell line exhibits in vitro responsiveness to phorbol ester-mediated Gaq-Q209L oncoprotein level reduction (Figure 6a). Finally, the prospective ability of phorbol ester to suppress Gaq-Q209L-driven tumorigenesis in vivo was measured following subcutaneous grafting of GNAQ Q209L cells and systemic treatment of the host animal with TPA as outlined in the schedule of Figure 6b. TPA-treated host animals had markedly delayed tumor onset and a modestly reduced progression rate in comparison with vehicle-treated animals. Overall, these results collectively demonstrate two future possibilities to ameliorate GNAQ/11-induced uveal melanoma; reduction of Gαq/11-Q209L driver oncoprotein levels through Ric-8A inhibition or phorbol ester treatment.

DISCUSSION
The present study provides a genetic demonstration that melanocyte deletion of the molecular chaperone Ric-8A suppressed tumorigenesis mediated by the uveal melanoma oncogenic driver G protein, Gaq-Q209L. The means of inhibition was to deplete cellular oncoprotein levels below a threshold
required to manifest hyperactive, cancer-driving signaling. Our intent with this work is to provide proof-of-concept that a properly developed Ric-8A inhibition strategy may ultimately be used to block onco-G protein-driven cancers. Therapeutic Ric-8A inhibition could be extended to GPCR-driven diseases where depletion of endogenous G proteins might be more efficacious in comparison with antagonism of select GPCR(s). Blunting the abundance of client oncoproteins through inhibition of the chaperones that fold them is an active strategy in cancer therapeutic development, with Hsp90 inhibitors providing a prominent precedent. Blocking the highly specialized chaperones Ric-8A or B, that to date are known to only fold subsets of G protein subunits, represents an untapped target against onco-G-protein-driven cancers. Uveal melanoma is perhaps the clearest example in which the oncoprotein driver is a G protein, Gnaq/Q209L, which is folded by Ric-8A.

For this work, a new C57Bl/6 J transgenic mouse strain with inducible Ric-8A knockout potential was derived and used as a source to create immortalized melanocyte cell lines from neonatal dermal explants. The parental melanocyte cell line was made to stably express human Gnaq WT or Gnaq Q209L. Induced Ric-8A deletion in the cultured melanocyte cell lines effectively reduced Gnaq Q209L protein levels and had no obvious cytotoxic effects. In fact, Ric-8A deletion imparted a modest in vitro growth advantage to cultured melanocytes and melanoma cell lines. This effect has been observed for other Ric-8A-null cell types and suggests that
the net effect of endogenous G protein signaling is to provide a cell proliferation brake. However, this in vitro growth enhancement provided no tumorigenic propensity when Ric-8A-null cells lacking a driver oncogene were grafted into immune-compromised mice. Ric-8A deletion completely blocked melanoma tumorigenesis of the grafted GNAQα-Q209L melanocyte cell line and secondarily-derived GNAQα-Q209L melanoma cell lines. A potential complication of any approach to genetically disrupt or inhibit Ric-8A is the reduction in endogenous G protein levels that will occur. On the other hand, if Ric-8A inhibition could be directed to tumor cells, the effect on endogenous G proteins may provide added therapeutic efficacy of tumor inhibition. G protein signaling imparts important advantages within the tumor microenvironment including pro-migratory properties of cancer cells, secretion of angiogenic factors that promote tumor vascularization, responsiveness to host growth factors and usurpation of the immune system. Ric-8A deletion/inhibition-mediated reduction of endogenous G proteins would potentially mitigate all of these processes. An additional observation made with Ric-8A null cells may be specifically relevant to a potential uveal melanoma therapy; Gq/11-Q209L-mediated uveal melanoma progression involves constitutive stimulation of Rho/Rac guanine nucleotide exchange factors (GEFs) that regulate actin polymerization, which is thought to contribute the signal that activates YAP as the main signaling driver of the cancer. Ric-8A null mice embryonic stem cells and Ric-8A shRNAi-treated MEFs have substantial deficiencies in polymerized actin levels (F-actin) that may be attributable to reductions in the G proteins (Gα12/13/11) primarily responsible for stimulating Rho-signaling pathways. So, in addition to inhibited Ric-8A resulting in reduced Gq/11-Q209L oncoprotein levels, there could be additional attenuation of the YAP pathway at the level of reduced actin polymerization.

Our findings help clarify a current controversy regarding Ric-8 cell activity. Ric-8A was initially characterized as a GEF that stimulates Gα subunit guanine nucleotide exchange in vitro. Evidence from many organismal systems showed that Ric-8 orthologs are required to maintain proper G protein abundances. Ric-8 acts as a chaperone during biosynthesis to facilitate Gα subunit protein folding. G proteins produced in the absence of Ric-8 are mis-folded and rapidly degraded. It is not clear whether GEF and chaperoning activities are one and the same, or whether Ric-8 is a multi-functional protein that acts during biosynthetic G protein folding and later facilitates Ga GTP binding to evoke signaling outputs. The fact that Ric-8A deletion suppresses the oncogenic action of Gqα-Q209L indicates that Ric-8A chaperoning activity is either the authentic cellular activity or is more penetrant than GEF activity. The Gq/11-Q209L oncoprotein has greatly impaired GTP hydrolysis activity that renders it constitutively active, therefore bypassing the need of a GEF for sustained signaling output.

A key remaining question is to understand why GNAQα-Q209L drives the majority of uveal melanomas, yet the oncogene is far less prevalent in cutaneous melanomas. Deciphering this difference will provide useful insight toward the development of melanoma therapies. We hypothesize that uveal melanocytes possess a survival privilege stemming from an innate ability to tolerate a higher ‘dose’ of Gq/11 signaling, or as others have suggested, ocular melanocytes are compartmentally shielded from attack by the host immune system. Cutaneous melanocytes may be more sensitive to Gq/11 signaling and have a capacity to downregulate GNAQα-Q209L transcript levels or Gq/11-Q209L protein levels, or otherwise enter into an apoptotic cell death program. Constitutively-active GNAQ/11 mutations are in fact highly prevalent in benign melanocytic neoplasms and can cause skin hyperpigmentation. These pre-cancerous dermal lesions could require a lower threshold of Gq/11 signaling to develop, yet the higher level necessary to drive cellular transformation may not be well tolerated by dermal melanocytes. There are many clear examples in which modestly elevated Gq/11 signaling induces proliferation in specific tissues or cultured cell lines, but chronic Gq/11-coupled GPCR agonist treatment(s) or constitutively-active mutant overexpression induces apoptosis.

In accordance with this hypothesis, when our GNAQα-Q209L cutaneous murine melanocyte or melanoma cell lines were treated in culture with a phorbol ester, GqQ209L protein abundance, but not wild-type Gqα11 abundance was reduced dramatically, an effect on the order of that which occurred when Ric-8A is deleted. The TPA-treated GNAQα-Q209L cell line did not form tumors when grafted, despite efficient production of the GNAQα-Q209L-ires-GFP transcript as visualized via the efficient GFP fluorescent signal (Supplementary Figure S3) and by RT-PCR analyses (Figure 2e). We suspect that overstimulation of the phospholipase Cβ DAGPKC branch of the Gq/11 signaling axis is responsible for a feedback pathway that can reduce Gq/11 oncoprotein levels in dermal melanocytes. TPA is a DAG mimetic that activates PKC. Gq/11 oncoprotein cancer signaling is thought to be primarily driven by the distinct Rho/YAP branch of the Gq/11 signaling axis. When oncoprotein levels are reduced by feedback inhibition through PKC, cancer signaling through Rho/YAP would also be reduced, potentially explaining the great difference in frequency of cutaneous versus uveal melanomas driven by oncogenic GNAQ/11. We are actively investigating the potentially distinct signaling properties of ocular and cutaneous melanocytes to decipher out an explanation of why GNAQα-Q209L-induced oncogenesis is highly biased toward uveal melanocytes.

Our results raise the intriguing prospect that phorbol esters may be an effective way to mitigate uveal melanoma oncogenesis. Phorbol esters directly activate PKC and are commonly thought of as tumor promoters. Yet for GNAQα/Q209L-driven uveal melanoma, super-activation of the Gq/11-Q209L-stimulated PKC pathway could provide two distinct mechanisms of therapy: (1) PKC-activated feedback reduction of Gq/11 oncoprotein abundance or (2) induction of cancer cell apoptosis. PKC activators, including phorbol esters, inhibited growth of various non-uveal melanoma cell lines and tumors through induction of cell-cycle arrest or apoptosis. Wild-type PKC rescues loss-of-function PKC alleles in human tumor cells inhibited tumorigenesis, showing that PKC is a tumor suppressor. Our demonstration in Figure 6b that host animal systemic TPA treatment delayed melanoma tumor onset and progression from a grafted GNAQα-Q209L cell line is highly encouraging. Our ongoing and future work involves active trials to reduce Gq/11-Q209L oncoproteins through Ric-8A inhibition or phorbol ester inhibitory feedback as two new potential strategies to treat GNAQα-Q209L melanoma.

**MATERIALS AND METHODS**

Creation of a conditional Ric-8A knockout mouse

The Ric-8A knockout-first allele targeted mouse embryonic stem cell (ES) lines were purchased from Knockout Mouse Project (KOMP) Repository (Project ID CSD070973) at UC Davis. Microinjection and implantation of Ric-8A-Neo targeted ES cells was carried out by the Gene Targeting and Transgenic Mouse core facility at the University of Rochester Medical Center. Chimeras were mated with C57BL/6J mice (Jackson Lab, Bar Harbor, ME, USA, stock # 000664). One out of four chimeras carried a germline copy of the Ric-8A-Neo allele. Mice harboring the Ric-8A Neo allele were crossed with FLPer mice (Jax Lab stock # 009086) to generate Ric-8A floxed allele progeny. Ric-8A floxed mice that express tamoxifen-inducible-Cre (CreER) recombinase driven by the ubiquitous Rosa promoter were generated upon breeding to R26-Cre-ER T2 mice (Jax Lab stock # 008463). Mice were handled and maintained in accordance with University of Rochester Institutional Animal Care and Use committee.

Cell culture

MEFs were isolated from day E13.5 embryos and cultured in DMEM containing 10% FBS. Spontaneously immortalized melanocyte cell
lines were generated from epidermal cell explants isolated from 3-day old neonates as described. In detail, cell explants were co-cultured in melanocyte medium (RPMI-1640, 10% FBS, 2 mM L-Glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 μg TPA, 200 μM CTX and 200 μM phenylthiourea) with Xβ2 keratinocyte feeder cells (ATCCCL-177, ATCC, Manassas, VA, USA) that had been mitotically-inactivated with mitomycin C. Growth of melanocytes was selectively promoted by TPA and CTX supplementation. After passage 4, Xβ2 feeder cells were eliminated and melanocytes were passaged past the senescence phase until colonies began to grow. Spontaneously immortalized, feeder cell-free, melanocyte cell line growth was confirmed by examination of cells by microscopy for the presence of melanin pigment granules.

**MG132** (in DMSO) at 10 μM final concentration in melanocyte medium was used to inhibit proteasome-mediated degradation in GNAQQ209L; melanocyte or -melanoma cell line culture. The MG132-treated cells were washed with phosphate-buffered saline with protease inhibitor mixture and melanocyte or -melanoma cell line culture. The MG132-treated cells were lysed by nitrogen cavitation using a Parr bomb (Parr Instrument Co., Carlsbad, CA, USA) to recombine the Cre-NLS-IRES-GFP, and created using the Gateway LR Clonase Enzyme kit (Life Technologies, Carlsbad, CA, USA) to recombine the Cre-NLS-IRES-GFP, and inserted 5′ and 3′ flanking regions of the IRES-GFP cassette from pIRES2-GFP (gift of Dr. J. Chen). The resulting cell suspension was washed twice and cultured in fresh melanocyte medium.

**Lentiviral production and infection**

Lentiviruses were prepared from the pLenti-Cre-NLS, pLenti-IRES-GFP and pLenti-GNAQWT or -GNAQQ209L, IRES-GFP donors as follows: For the production of 4×10⁸ HEK293T cells were seeded in 10-cm diameter culture dishes in DMEM containing 10% FBS. After 24 h, cells were transfected with 4 μg of pLenti construct, 2.5 μg of pMDLg/pRRE, 2.5 μg of pRSV-Rev and 2 μg of pSVM/pMD2.g using Lipofectamine 2000 (Thermo Fisher). Lentiviral-containing media overlaying the transfected cells was harvested at 48 h, filtered through a 0.45-μm syringe filter and supplemented with 8 μg/ml of polybrene (Sigma-Aldrich, St. Louis, MO, USA). Immunofluorescent mouse melanocytes were infected with 1:1 ratio of viral medium: fresh melanocyte medium for 48 h. Transduced melanocytes were selected with 200 μg/ml and then 100 μg/ml hygromycin B over 72 h and maintained in melanocyte medium containing 50 μg/ml hygromycin B (Thermo Fisher). The GNAQWT and GFP stable melanocyte cell lines were continuously cultured in the presence of TPA. The GNAQQ209L melanocyte cell line was cultured in the absence of TPA in order to maintain GnaQ-Q209L abundance.

**Genetic ablation of Ric-8A**

Ric-8Afllox/floxMEFs were infected with Cre-NLS-lentivirus for 48 h, selected with 100 μg/ml hygromycin B for an additional 48 h to achieve Ric-8A knockout. Cell lysates were harvested for quantitative western blot analysis and mRNA isolation. Deletion of Ric-8A in cultured Ric-8Afllox/flox, Rosa-CreER+/− melanocyte cell lines was achieved by treating the cells in culture with 500 nM 4OH-T (Sigma-Aldrich) for 5 days or by infection with Cre-NLS-lentivirus for 48 h followed by selection with 2 μg/ml puromycin (Sigma-Aldrich).

**Cell graft melanoma tumorigenesis models**

Eight-week-old female NSG (NOD.Cg-Prkdcscid Ii2rgtm1Wjl/Szjl) mice were obtained from the Jackson Laboratory (Stock #005557). For the subcutaneous cell graft tumor model, live melanocytes were suspended by brief trypsinization, washed extensively in melanocyte medium and counted as a hemacytometer after Trypan blue dye exclusion. NSG host mice were injected subcutaneously in each hind flank with 5×10⁶ cells of the GFP- GNAQWT- or GNAQQ209L-melanocyte cell lines in RPMI base medium, or 1×10⁶ Ric-8Afllox/flox, Rosa-CreER+/− tumor-derived melanocyte cell lines for the secondary tumor growth progression studies. In vivo deletion of Ric-8A in melanocyte cell lines grafted into NSG mice was achieved by treating host animals with 10–15 i.p. injections of 1 mg tamoxifen or vehicle over a course of 20 days. Tamoxifen was dissolved in ethanol at 100 mg/ml followed by 1:10 dilution in sterile corn oil. The TPA treatment of host mice was performed by mixing melanocytes in a suspension of RPMI base medium and 0.2 μM TPA or vehicle before cell grafting. At day 1 post graft, one i.p. injection of 200 μg TPA (in 10% ethanol, 90% corn oil) or vehicle was performed, followed by i.p. injection of 4 μg TPA or vehicle, q.a.d, over days 5–12 post cell grafting. Injection sites were monitored thrice weekly for tumor growth. Tumor size was measured using calipers and volume was calculated using the formula: length × width²/2.6 Mice were killed when measured tumors reached ≥ 1000 mm³ and the tumors were isolated from the adhered tissue.

**Tumor explant cultures**

Subcutaneous GNAQQ209L primary melanoma tumors were excised from host animals, chopped into small pieces and treated with 0.1% w/v Collagenase A (Roche, Indianapolis, IN, USA) for 5–10 min at 37 °C. The resulting cell suspension was washed twice and cultured in fresh melanocyte medium.

**Cell imaging**

Melanocyte cell lines were fixed with 4% paraformaldehyde and stained with DAPI nuclear stain as described. DAPI-stained images of fixed cells were analyzed and imaged using Metamorph (Universal Devices, Sunnyvale, CA, USA) with UPLSAPX10× (n.a. 0.35) objective on an Olympus IX70 microscope and an ORCA-ER digital camera (Hamamatsu, Japan). Phase-contrast images were captured with a Nikon Eclipse TS100 inverted microscope (Nikon, Tokyo, Japan), ×10 objective (n.a. 0.25) using an attached Canon EOS Rebel XSI camera (Canon, Tokyo, Japan).
Immunoblotting
Rabbit polyclonal Ric-8A [1184],29 Gao [521]65 and Gai1/2 [508]66 antisera were described previously. Rabbit polyclonal Goq/11 [C-19] and Gai13 [H-300] antisera were purchased from Santa Cruz (Dallas, TX, USA). Monoclonal α-tubulin and γ-tubulin were from Sigma-Aldrich. The antibody against the Goaq-Q209L oncoprotein was purchased from NewEast Biosciences, King of Prussia, PA, USA [26328].

Statistical analysis
Error bars throughout are the mean ± s.e.m. Two-tailed Student’s t-tests were performed as indicated using GraphPad Prism (La Jolla, CA, USA).

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Supplementary Information accompanies this paper on the Oncogenesis website (http://www.nature.com/oncsis)