Published in final edited form as:

Sci Signal. ; 11(546): . doi:10.1126/scisignal.aao6852.

Targeting nucleotide exchange to inhibit constitutively active G protein alpha-subunits in cancer

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Abstract

Constitutively active G protein α -subunits cause cancer, cholera, Sturge-Weber Syndrome, and other disorders. Therapeutic intervention by targeted inhibition of constitutively active $G\alpha$ subunits in these disorders has yet to be achieved. Here we show that constitutively active $G\alpha_q$ in uveal melanoma (UM) cells can be targeted by the cyclic depsipeptide FR900359 (FR). FR allosterically inhibits GDP/GTP exchange to trap constitutively active $G\alpha_q$ as inactive GDP-bound $G\alpha\beta\gamma$ heterotrimers. Allosteric inhibition of other $G\alpha$ subunits can be achieved by introduction of an FR binding site. In UM cells driven by constitutively active $G\alpha_q$, FR inhibits second messenger signaling, arrests proliferation, reinstates melanocytic differentiation or triggers apoptosis. FR has no effect on BRAF-driven UM cells. FR promotes UM cell differentiation by re-activating polycomb repressive complex 2 (PRC2)-mediated gene silencing, a heretofore unrecognized effector system of constitutively active $G\alpha_q$ in UM. Constitutively active $G\alpha_q$ and PRC2 therefore provide therapeutic targets for UM. The development of FR analogs specific for other $G\alpha$ subunit subtypes may provide novel therapeutic approaches for diseases driven by constitutively active $G\alpha$ subunits or multiple G protein-coupled receptors where targeting a single receptor is ineffective.

One-sentence Summary:

The cyclic depsipeptide FR900359 targets nucleotide exchange to trap constitutively active mutant $G\alpha_q$ in the inactive GDP-bound state and uncover novel pathways and therapeutic opportunities in uveal melanoma and other diseases.

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Author contributions: MDO and KJB planned the experiments. MDO, CMM, SW, KMK, SMK, TDT and KJB performed experiments. TJB and KJB purified FR. MDO, TJB, PKR, JAC, and KJB analyzed data and provided comments. MDO, CMM, KMK, SMK, TDT, PKR, JAC and KJB wrote the paper.

Competing Interests: The authors have no competing interests.

Data and Materials Availability: The RNA-Seq data used in this manuscript are available at NCBI's GEO (GSE103761).

Introduction

Heterotrimeric G proteins transduce signals from hundreds of cell-surface G protein-coupled receptors (GPCRs) to intracellular signaling networks that regulate diverse biological processes. By undergoing GPCR-stimulated GDP/GTP exchange followed by GTP hydrolysis, G protein α -subunits cycle between inactive GDP- and active GTP-bound states to determine the duration, magnitude and specificity of biological responses (1). In cholera, certain cancers (2), Sturge-Weber syndrome (3) and other disorders, this cycle is disrupted by mutant or covalently modified $G\alpha$ subunits that, by failing to hydrolyze GTP, are constitutively active.

Constitutively active mutant forms $G\alpha_q$ or its close relative $G\alpha_{11}$ are the oncogenic drivers in nearly 90% of uveal melanoma (UM) patients (4–6). UM is the most common cancer of the eye, and the eye is the second most common site of melanoma. Regardless of primary tumor treatment, nearly half of UM patients develop metastatic disease (7) with mean survival less than one year (8). Therapies to treat primary tumors and treat or prevent metastatic disease are needed. Inhibitors of individual signaling pathways downstream of $G\alpha_q/11$ are being studied in UM clinical trials, but all have failed thus far (9). Thus, therapeutic approaches that directly target constitutively active $G\alpha_q/11$ may be required to inhibit all necessary downstream oncogenic signaling networks.

Constitutively active $G\alpha$ subunits have yet to be targeted pharmacologically in disease due to challenges analogous to those of inhibiting oncogenic Ras (10–12). GTP hydrolysis defects would be extremely difficult to correct pharmacologically, and the high affinity of $G\alpha$ subunits for GTP or GDP precludes the generation of effective competitive inhibitors of guanine nucleotide binding.

However, other evidence led us to consider that constitutively active $G\alpha_q$ can be targeted in UM by pharmacologically inhibiting GDP/GTP exchange. Although nucleotide exchange by soluble $G\alpha_q$ is very slow in vitro (13), it is enhanced strikingly by lipid membranes (14, 15) and Ric-8a (16, 17), a non-receptor guanine nucleotide exchange factor (GEF) and folding chaperone. Nucleotide exchange, therefore, may occur at appreciable rates in cells; however, constitutively active $G\alpha_q$ still would exist predominantly in the active GTP-bound state because average GTP:GDP ratios in human cells are ~8:1 (18) and GTP dissociates ~10-fold slower than GDP (13). Nevertheless, we reasoned that this equilibrium might be driven toward the GDP-bound state by inhibiting GDP dissociation, which would cause constitutively active $G\alpha_q$ to assemble into inactive GDP-bound $G\alpha\beta\gamma$ heterotrimers and thereby attenuate all downstream oncogenic signaling networks.

Here we report that constitutively active $G\alpha_q$ can be targeted pharmacologically in UM cells by FR900359 (FR), a naturally occurring, cyclic depsipeptide that has been shown previously to inhibit wild type $G\alpha_q$ by interfering allosterically with GDP dissociation (19–21). We show that FR can trap constitutively active $G\alpha_q$ in the GDP-bound state and inhibit downstream signaling in UM cells. We also show that constitutively active $G\alpha_q$ drives oncogenesis by a novel mechanism that antagonizes epigenetic silencing. Our results suggest

that targeting nucleotide exchange is a novel, general strategy for inhibiting Gα subunits in cancer and other diseases.

Results

FR traps mutant constitutively active Gαq in the GDP-bound state

To determine whether constitutively active Gαq undergoes appreciable GDP/GTP exchange in cells, we investigated whether Gαq(Q209L), a common oncogenic GTPase-defective mutant in UM, could be trapped in the GDP-bound state by FR. The GDP- and GTP-bound states of Gαq(Q209L) were assessed by detecting interaction with Gβγ subunits, which bind preferentially to GDP-loaded Gα subunits (1), or with RGS2, which binds GTP- but not GDP-loaded Gαq (22). To detect protein-protein interactions, we employed split-luciferase complementation assays (23) using click beetle green (CBG) luciferase to study activation state-dependent interaction between Gα subunits and cognate binding partners in living cells(24), or co-purification with tandem affinity-tagged (FLAG and StrepII (FS)) wild type or constitutively active Gαq.

Results indicated that FR is able to trap constitutively active Gαq in the GDP-bound state. Split-luciferase complementation between Gβ1γ2 and wild type or constitutively active Gαq was increased by FR (Fig. 1, A and B; EC₅₀ 3 nM and 9 nM, respectively). FR also increased association between Gβγ and affinity-tagged wild type or constitutively active Gαq (19- and 9-fold, respectively; n=3; p<0.05) as indicated by co-purification (Fig. 1C). Conversely, FR drove constitutively active Gαq out of the active GTP-bound state, as indicated by inhibition of split-luciferase complementation between constitutively active Gαq and RGS2 (Fig. 1D; IC₅₀ 0.4 nM). FR was selective for Gαq, as revealed by its lack of effect on interaction between wild type or constitutively active Gα13(Q226L) and Gβ1γ2 (Fig. 1A), or between constitutively active Gα13 and the RGS domain of LARG (Fig. 1D). Thus, FR drives constitutively active Gαq from its active GTP-bound state into inactive GDP-bound Gαβγ complexes.

As a further test of the ability of FR to inhibit constitutively active Gαq, we measured downstream signaling. FR inhibited induction of a transcriptional reporter driven by constitutively active Gαq (Fig. 1E; IC₅₀ 1 nM), but had no effect on expression of the reporter when driven by constitutively active Gα13 (Fig. 1E). Crystallographic and mutagenesis studies of wild type Gαq identified amino acid residues (Arg⁶⁰, Val¹⁸⁴, Ile¹⁹⁰) that are important for inhibition by YM-254890 (YM) (19), an inhibitor nearly identical to FR. We found that single amino-acid substitutions at any of these sites (R60K, V184S, I190N) in constitutively active Gαq were sufficient to blunt the inhibitory potency of FR (Fig. 1E; IC₅₀ 30 to 70 nM), demonstrating that FR targets constitutively active and wild type Gαq by using the same binding site.

FR inhibits Gαi1 bearing an engineered FR binding site

FR inhibits receptor-evoked signaling by wild type Gαq and its close relatives Gα11 and Gα14, but not other Gα subunits (20). We therefore determined whether an FR-insensitive Gα subunit could be converted into FR-sensitive form by introducing an FR binding site.

This might be possible because: all Gα subunits release GDP by a common allosteric mechanism (25); structural elements of the allosteric relay include the FR binding site (19, 25); and FR-insensitive Gα subunits contain a similar but diverged form of the FR binding site.

To test this hypothesis, we engineered an FR binding site into Gαi1 by changing eight diverged amino acids to match their counterparts in the FR binding site of Gαq, producing “Gi/q” α-subunits (illustrated in Fig. 2A). Gαi1 was chosen because it is insensitive to FR (20) and its function is studied readily in biochemical and cell-based assays. As a control, a Gαi/q(R54K) mutant also was made, corresponding to a Gαq mutant that is less sensitive to FR (19). We found that FR inhibited the rate of nucleotide exchange by Gαi/q in vitro ($IC_{50}=4.6\mu M$), as indicated by the binding kinetics of a fluorescent, non-hydrolyzable GTP analog (BODIPY-GTP γ S; Fig. 2 B and fig. S1A), which is rate-limited by GDP release. As expected, FR was >10-fold less potent toward Gαi/q(R54K) ($IC_{50}=76\mu M$; Figs 2B and S1A). Similarly, we found that FR inhibited Gαi/q signaling in cells. FR attenuated the ability of Gαi/q activated by cannabinoid type 1 receptors to inhibit forskolin-induced cAMP accumulation ($IC_{50}=25$ nM; Fig. 2C and fig. S2, C and D). FR was ~30-fold less potent ($IC_{50}\sim 800$ nM; Fig. 2C and fig. S2, C and D) toward Gαi/q(R54K). Thus, FR targets Gαi/q and Gαq by utilizing the same binding site. This finding suggests that FR-like molecules could be created to target the analogous, but distinct, binding sites of other Gα subtypes, providing a general approach to discover novel chemical probes of Gα function and potential therapeutics for various diseases.

FR targets constitutively active Gα subunits by inhibiting nucleotide exchange

In principle, FR could target constitutively active Gα subunits by inhibiting nucleotide exchange or restoring GTP hydrolysis. We tested both hypotheses by using Gαi/q, because Gαq was unsuitable due to its unusual nucleotide binding properties that confound measuring GTP hydrolysis in vitro. We found that constitutively active Gαi/q(Q204L) (equivalent to Gαq(Q209L)) exhibited a severe defect in the catalytic rate of GTP hydrolysis that was not corrected by FR (Fig. 2D), whereas wild type Gαi/q hydrolyzed GTP effectively (Fig. 2D). In contrast, FR effectively inhibited nucleotide exchange by constitutively active Gαi/q (Fig. 2E; $IC_{50}=2.7\mu M$). Thus, FR targets constitutively active Gα subunits by inhibiting nucleotide exchange rather than restoring GTP hydrolysis.

FR inhibits signaling by constitutively active Gαq in UM cells

To determine whether FR inhibits signal transduction by constitutively active Gαq in UM cells, we analyzed two UM cell lines (Mel202 and 92.1) driven by constitutively active Gαq(Q209L). A third UM cell line (OCM-1A) driven by constitutively active BRAF(V600E) served as a negative control. Signaling by Gαq-stimulated phospholipase Cβ was quantified based on abundance of inositol monophosphate (IP1), a metabolically stable product of inositol 1,4,5-trisphosphate (IP3) produced by cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2). In the absence of FR, IP1 was >50-fold more abundant in Gαq(Q209L)-driven Mel202 and 92.1 cells relative to BRAF(V600E)-driven OCM-1A cells (Fig. 3A). FR reduced IP1 abundance in Mel202 and 92.1 cells >50-fold (Fig. 3B), but had

only modest effect (~2-fold) on OCM-1A cells (Fig. 3B). Thus, FR strikingly inhibited second messenger production driven by constitutively active Gαq in UM cells.

FR inhibits UM tumor cell proliferation and survival

During the preceding experiments, we also observed that FR treatment decreased viability of Gαq(Q209L)-driven UM tumor cells. Quantification confirmed that FR inhibited proliferation of Gαq(Q209L)-driven Mel202 and 92.1 UM cells (Fig. 4, A and B; EC₅₀ 6 nM and 2 nM, respectively), with no effect on proliferation of BRAF(V600E)-driven OCM-1A cells even at high concentration (10 μM). Flow cytometry revealed that FR induced cell cycle inhibition (decreased fraction of S- or G2/M-phase cells) and apoptosis (increased fraction of sub-G1/G0 cells) in Mel202 and 92.1 cells (Fig. 4, C and D), but had no effect on OCM-1A cells (Fig. 4, C and D). FR therefore inhibited proliferation and survival of UM cells in which constitutively active Gαq is the oncogenic driver.

FR promotes melanocytic re-differentiation of UM cell lines

We observed that FR treatment caused Gαq(Q209L)-driven Mel202 and 92.1 cells to undergo morphological changes indicative of re-differentiation. FR-treated Mel202 and 92.1 cells lost spindle morphology, became flatter, and produced multiple projections (Fig. 5A), when compared with vehicle-treated cells or FR-treated OCM-1A cells. Furthermore, FR increased melanocytic pigmentation in Mel202 and 92.1 cells as compared to vehicle-treated cells or FR-treated OCM-1A cells (Fig. 5B). Similarly, FR increased expression of two pigmentation enzymes (tyrosinase (TYR) and dopachrome tautomerase (DCT)) and a melanosome structural protein (PMEL) in Mel202 and 92.1 cells but not in OCM-1A cells (Fig. 5C). FR increased the proportion of DCT-positive 92.1 and Mel202 cells (3.5- and 1.6-fold, respectively), TYR-positive Mel202 cells (1.8 fold), and PMEL-positive 92.1 and Mel202 cells (6.8- and 3.9 fold, respectively) ($p < 0.01$, Fisher's exact test, for all comparisons). Thus, FR antagonized the de-differentiation process driven by constitutively active Gαq in UM cells.

FR alters expression of genes regulated by constitutively active Gαq in UM cells

To explore how FR regulates phenotypes of UM cells driven by constitutively active Gαq, we analyzed global gene expression by RNA-Seq. Although FR caused an apoptotic response in UM cells, it did not strikingly increase expression of pro-apoptotic genes or decrease expression of survival genes. Instead, FR modestly decreased expression of two pro-apoptotic BCL2 family members (*BBC3/PUMA* by 3.5-fold ($q < 0.01$; FDR) and *PMAIP1/NOXA* by 2.5-fold ($q < 0.01$; FDR); fig. S2). Other BCL2 family members showed insignificant changes in expression ($q < 0.01$; FDR, fold change > 2), and broader examination of apoptosis-related genes showed only small effects (fig. S2). These results are consistent with evidence that intrinsic and extrinsic apoptotic pathways function independently of gene transcription (26, 27).

Cell-cycle genes also were relatively unaffected by FR, as indicated by gene set enrichment analysis and direct comparison of the RNA-Seq data (fig. S2 and supplemental data file 1). The cyclin-dependent kinase inhibitor p21^{CIP1} (*CDKN1A*, down 3.0 fold, $q < 0.01$; FDR) showed reduced expression, and several cell cycle genes showed upward trends lacking

statistical significance ($q > 0.01$; FDR, fold change < 2) (fig. S2). Targets of E2F, a transcription factor positively regulated by cyclin-dependent kinases, were positively enriched (supplemental data file 1). These results suggest that the anti-proliferative effects of FR are not mediated primarily by short-term changes in cell expression of cycle genes.

FR restores UM cell differentiation by promoting polycomb-mediated gene repression

In contrast to the results obtained for apoptosis and cell cycle genes, large changes in expression were observed for differentiation and developmental genes in FR-treated Gαq(Q209L)-driven 92.1 cells. Results of a multi-dimensional gene expression analysis comparing relative patterns of expression of all genes across all samples showed that a large number of gene expression changes were associated specifically with FR treatment (Fig. 6A). Most strikingly, a distinct gene cluster showed dramatic reduction in expression (4- to ~160-fold; Fig. 6B; circled, and supplemental data file 3). Among genes in this cluster, 38% are associated with cell differentiation and development (Fig. 6C and supplemental data file 4), as revealed by Gene Ontology analysis. More important, 42% of genes in this cluster are known targets of the polycomb repressive complex 2 (PRC2) during differentiation from embryonic stem cells (Fig. 6D and supplemental data file 5), as indicated by gene set enrichment analysis. These results were confirmed by quantitative RT-PCR analysis of FR-treated 92.1 cells (fig. S3). In contrast, expression of PRC2-regulated genes in BRAF(V600E)-driven OCM-1A cells was unaffected by FR (fig. S3), demonstrating specificity of FR for developmental and differentiation genes targeted by constitutively active Gαq in UM cells.

The RNA-Seq analyses suggested a novel mechanism for Gαq-induced oncogenesis in UM, in which constitutively active Gαq antagonizes PRC2-mediated gene repression, thereby reactivating genes associated with stemness and driving de-differentiation of UM cells into a more stem-like phenotype. FR treatment inhibits constitutively active Gαq, relieves blockade of PRC2-mediated repression, re-silences these genes, and returns UM cells to a melanocytic state. Consistent with this hypothesis, we found that inhibiting the catalytic subunits of PRC2 complexes (Ezh1/2) with GSK503 maintained 92.1 cells in an undifferentiated state and blocked the ability of FR to re-differentiate these cells as indicated by morphology (Fig. 6E) and pigmentation (Fig. 6F). The effectiveness of GSK503 at inhibiting histone H3(K²⁷) methylation was confirmed by immunoblotting histones isolated from control and FR-treated 92.1 cells (Fig. 6G).

Discussion

GDP/GTP exchange can be exploited as an Achilles heel of constitutively active G protein α-subunits

The most important discovery provided by our studies is that GDP/GTP exchange is an underappreciated vulnerability of constitutively active G protein α-subunits, and one that can be exploited pharmacologically in UM and other diseases. Although Gα subunits undergo GDP/GTP exchange slowly in vitro, we discovered that nucleotide exchange occurs in cells at rates sufficient for constitutively active Gαq to be trapped in the inactive GDP-bound state by treatment with FR, an allosteric inhibitor of GDP release. When trapped by

FR, GDP-bound constitutively active Gαq assembles into Gαβγ heterotrimers, further suppressing GDP release and stabilizing the inactive state. Because FR-bound Gq heterotrimers are refractory to activation by GPCRs (20), signaling networks downstream of constitutively active Gαq are attenuated.

While our study involved constitutively active Gαq in UM, we anticipate that constitutively active forms of Gα subunit subtypes that drive other types of cancer also may be vulnerable to allosteric inhibitors of GDP release. Constitutively active Gα11 in UM (12) and Gα14 in vascular tumors (28) should be susceptible because wild type forms of these Gα subunits are sensitive to FR (20). Although other subtypes of Gα subunits are insensitive to FR, all Gα subunits possess a diverged but related form of the allosteric regulatory site in Gαq that binds FR. This site includes conserved features of linker 1, which stabilizes the GDP-bound state by interacting with helix 1, helix A, and helix F as part of the universal mechanism that regulates GDP release. Indeed, as predicted by this hypothesis, we found that engineering an FR binding site into an FR-insensitive Gα subunit was sufficient to confer FR sensitivity. Thus, we speculate that a collection of FR-like inhibitors, each of which selectively targets the diverged allosteric regulatory site of certain Gα subunits, may provide a novel approach toward therapeutic development in cancers associated with other mutant constitutively active Gα subunits (2), cholera, and Sturge-Weber Syndrome. In addition, this approach may be efficacious for diseases that are driven by multiple GPCRs, in which blocking a single receptor is ineffective.

Uveal melanoma cells are addicted to constitutively active Gαq

Another important discovery emerging from our study is that constitutively active Gαq has unexpectedly diverse functional roles as an oncogenic driver in UM. Instead of affecting a single cell biological process, FR inhibits proliferation, triggers apoptosis and drives melanocytic re-differentiation of UM cells. Our findings help to explain why previous studies using MEK, AKT, and PKC inhibitors to target individual signaling pathways downstream of Gαq/11 failed in clinical trials of UM (9). By inhibiting constitutively active Gαq and consequently attenuating all downstream signaling networks, FR or related inhibitors may impair the growth and survival of cancer cells in UM primary tumors, and they may also decrease the probability or extent of metastasis because melanocytic differentiation correlates inversely with metastatic potential in UM (29, 30). For primary tumors, FR may slow conversion from the indolent state to aggressive Class 2 tumors (31), or it may impair the growth or spread of metastatic lesions, including those that are clinically undetectable. Tumor-targeted or focal delivery of FR may be required because systemic administration might cause unacceptable side effects by inhibiting Gαq/11 in normal tissues. Nevertheless, the striking FR-sensitivity of UM tumor cells driven by constitutively active Gαq suggests that clinical investigation of this or related inhibitors could be considered.

Constitutively active Gαq antagonizes gene silencing by the polycomb repressive complex 2

We found a striking and unanticipated consequence of inhibiting constitutively active Gαq in UM – the repression of gene sets that control differentiation and development. Many of these repressed genes are involved in embryonic stem cell lineage specification and

differentiation, and are targets of epigenetic silencing by the polycomb repressive complex 2 (PRC2), which acts through histone H3(Lys²⁷) trimethylation (32–34). These repressed genes include *ADRA2A* (alpha-adrenergic receptor-2A) and *HAND2* (heart and neural crest derivatives expressed-2). The *ADRA2A* gene promoter was identified in independent screens for PRC2 subunit binding and for histone H3(Lys²⁷) trimethylation (32–34), and *ADRA2A* has been linked to cancer progression and severity (35). The *HAND2* gene promoter is also targeted by PRC2 binding and histone H3(Lys²⁷) trimethylation (32–34), especially in migrating cranial neural crest cells, where *HAND2* expression distinguishes neural crest cell lineages during facial development (36).

Together, our findings reveal a novel mechanism in which signaling by constitutively active Gα_q in UM cells antagonizes PRC2-mediated gene silencing, thereby maintaining UM cells in a less differentiated state similar to pre-melanocytic cranial neural crest cells (36). This finding, coupled with prior studies of BAP1 (30, 37), indicates that a temporal hierarchy of epigenetic regulation drives tumorigenesis and progression in UM. Early in tumorigenesis, mutations that constitutively activate Gα_q are acquired, which inhibits PRC2-mediated repression. Subsequent loss of BAP1, a histone H2A(Lys¹¹⁹) deubiquitinase that antagonizes repression by polycomb repressive complex 1 (PRC1), then leads to metastasis.

Materials and Methods

FR900359

FR900359 was purified from *A. crenata* according to published methods (20). The structure of purified FR900359 relative to a commercially available equivalent (UBO-QIC; University of Bonn (Germany)) was established by NMR.

Biochemical assays

Split luciferase assays were performed as described (24). The N-terminal portion of click beetle green luciferase (*CBGN*) was inserted into the αB-αC loop within the helical domain of wild type (WT) and constitutively active (c.626A>T; Q209L) mutant forms of *GNAQ* (Gα_q) and *GNAI3* (Gα₁₃) (Q226L). Insertion of foreign proteins at this site preserves Gα subunit function (38). The C-terminal region of click beetle green luciferase (*CBGC*) was fused to the N-termini of: *GNB1* (Gβ1), which was co-transfected with untagged *GNG2* (Gγ2); *RGS2*; and the RGS domain of *ARHGEF12* (LARG), which interacts with Gα₁₃ only in the active GTP-bound state (39). HEK293 cells transiently transfected with various combinations of fusion constructs generating tagged proteins were treated 18 hours with vehicle (DMSO) or FR and luciferase assays were performed to measure reconstituted luciferase activity.

Assays of transcriptional reporters driven by Gα subunits were performed as described (40). Gα-driven firefly luciferase activity was normalized to co-transfected Renilla luciferase expressed from a constitutive promoter.

Experiments used to measure agonist-evoked inhibition of forskolin-induced cAMP formation in Neuro2A cells were performed in a 96-well plate format as described in (24) with slight modifications. Cells were transfected with a cAMP FRET reporter and pertussis

toxin (PTX)-resistant forms of G*ai*1, G*ai*/q or G*ai*/q(R54K). After cells were treated 16 hours with PTX (100 ng/ml) to inactivate endogenously expressed Gi, FRET was used to measure inhibition of forskolin-induced cAMP formation by a CB1 cannabinoid receptor agonist (WIN 55,212-2; WIN). A Synergy H4 hybrid plate reader (Biotek) was used to measure FRET every 57 seconds by exciting the FRET donor (420/20nm bandpass filter), and simultaneously detecting donor and acceptor emissions (480/20nm and 540/20nm bandpass filters, respectively). FRET measurements of changes in intracellular [cAMP], were expressed ratiometrically as:

$$\frac{\Delta R}{R_o} = \frac{FRET\ ratio - baseline\ FRET\ ratio}{baseline\ FRET\ ratio}$$

where FRET ratio = (480nm emission/540nm emission) at a given time point, and the baseline FRET ratio = average (480nm emission/540nm emission) prior to FSK stimulation. Independent experiments were performed in triplicate. Effects of FR on agonist-evoked adenylyl cyclase inhibition was quantified as the percentage of R/R_o relative to vehicle control. FR concentration-response curves were generated by a three-parameter fit.

Accumulation of IP1 in UM cells was measured using the IP-One kit (CiSbio, Inc; catalog number 62IPAPB) according to the supplier's instructions. 10,000 Mel 202 cells, 20,000 92.1 cells and 20,000 OCM 1A cells were seeded into white-bottom tissue culture grade 384-well plates. Following an overnight incubation, cells were treated with FR or DMSO and returned to the incubator. The next day, stimulation buffer was added for 1 hour, after which IP1-d2 and Ab-Cryp were added, and the cells were incubated at room temperature for 60 minutes. Plates were read in a Synergy H4 hybrid plate reader. Standard curves were generated using reagents supplied with the kit.

Co-purification of tandem affinity-tagged G*aq* and endogenous G*βγ* was assayed as follows. A gBlock Gene Fragment (Integrated DNA Technologies) encoding a glycine/serine linker (GGGSSGGG) followed by a FLAG-StrepII- StrepII (FS) tag and another glycine/serine linker (GGGSSGGG) was inserted between sequences encoding amino acid residues 124 and 125 of mouse G*aq* (wild type and Q209L mutant), cloned into pcDNA3.1+, and verified by DNA sequencing. HEK293 cells were plated in 10cm dishes and transfected the next day with the indicated plasmids. After 24 hours, transfection media was removed and replaced with fresh media containing 1 FR (1 μ M) or vehicle (DMSO). 24 hours after treatment, media was removed and cells were washed with Dulbecco's PBS and processed for tandem affinity purification (TAP). TAP was performed as described previously (24) with the following modifications. All subsequent steps were performed on ice or at 4 °C. HEK293 cells were lysed in (50 mM Tris (pH 8.0), 5 mM EDTA, 100 mM NaCl, 0.5% (v/v) IGEPAL CA-630, 1 mM MgCl₂, complete protease inhibitor mix (Roche, cat. 11697498001), with or without 1 μ M FR), by sonication on ice for 2 minutes (30 seconds on, 30 seconds off, 60% A), rotated end-over-end for 30 minutes, and cleared by ultracentrifugation at 100,000*g* for 15 minutes. Cleared lysates were incubated with StrepTactin resin (IBA cat# 2-1206-010, lot# 1206-0350) overnight with end-over-end rotation and washed three times in batch with a 10× column volume of wash buffer (50 mM Tris (pH 8.0), 5 mM EDTA, 100 mM NaCl, 0.5% (v/v) IGEPAL CA-630, 1 mM MgCl₂,

complete protease inhibitor mix, $\pm 1 \mu\text{M}$ FR). Protein complexes were eluted two consecutive times by incubating with a 5-fold column volume of elution buffer (desthiobiotin buffer E (IBA cat# 2-1000-025, lot# 1000-5170), 0.1% (v/v) IGEPAL CA-630, 1 mM magnesium chloride, Complete protease inhibitor mixture, $\pm 1 \mu\text{M}$ FR) for 30 minutes in batch. StrepTactin elution fractions were combined and incubated with anti-FLAG M2-agarose (Sigma cat# A2220, lot# SLBW1929) in batch for 2 hours and washed three times in batch with a 10-fold column volume of wash buffer. Protein complexes were eluted from FLAG-agarose by incubating with a 4-fold column volume of FLAG elution buffer (200 $\mu\text{g}/\text{ml}$ 3xFLAG peptide, Sigma cat# F4799, lot# SLBM1190V in wash buffer) for 30 minutes in batch. Lysates and FLAG eluates were resolved on 12% SDS-PAGE gels and transferred to Immobilon(P^{SQ}) PVDF membranes (Milipore, cat# ISEQ00010). Membranes were blocked with 5% w/v milk in TBST (25 mM Tris pH 7.2, NaCl 150 mM, 2.7 mM KCl, 0.1% v/v Tween 20) and incubated with primary antibodies. Lysate primary antibody mixture was ANTI-FLAG® M2 Sigma cat# F1804, lot# SLBN5629V (1:50,000) and G β H-1 Santa Cruz cat# sc-166123, lot# G2414 (1:500). TAP eluates were probed with a primary antibody mixture consisting of anti-FLAG® M2 Sigma cat# F1804, lot# SLBN5629V (1:50,000) and anti-G β 1 H-1 Santa Cruz cat# sc-166123, lot G2414 (1:500). Membranes were washed with TBST at least three times and incubated with goat anti-mouse IgG coupled to IRDye 800CW (LI-COR, cat#926-32210, lot#C70712-15). Following incubation, membranes were washed at least three times with TBST and signals were detected using an Odyssey model 9120 imaging system (LI-COR).

Nucleotide exchange by purified His-tagged G α subunits was assayed as follows. Plasmid pet14B-6xHIS-G α i1 was a generous gift from Dr. Maurine Linder, Cornell University, New York. A pet14B-6xHIS-G α i/q plasmid was generated by cloning a custom synthesized gBlocks gene fragment (Integrated DNA Technologies) containing mutations encoding eight amino acid substitutions (V50I, K54R, Y69F, V72L, K180P, V185I, T187Y, and H188P) in 6xHIS-G α i1. Site directed mutagenesis of this plasmid was used to generate pet14B-6xHIS-G α i/q(R54K). Recombinant His-tagged G α subunits were expressed and purified from *E. coli* according to published methods (41). To detect nucleotide exchange, fluorescence of the GTP analog BODIPY-GTP γ S (ThermoFisher cat.G22183) was recorded with a Synergy H4 hybrid plate reader in the absence or presence of recombinant G α subunits (42). The indicated G α subunits (1 μM) were incubated in a black wall clear bottom 96 well-plate (Costar, cat.3603) with vehicle (DMSO) or FR at the indicated concentrations for 20 minutes at 25°C in reaction buffer (50mM Tris-HCl pH 8.0, 1mM EDTA, 10mM MgCl₂). BODIPY-GTP γ S then was added to the reaction mixture at a final concentration of 25nM to initiate nucleotide exchange. The intensity of fluorescence emission was recorded at 30°C every 10 seconds for 30 minutes with excitation and emission wavelengths of 485nm and 528nm, respectively, and the monochromator set to a bandwidth of 9nm. Specific fluorescence was normalized to background fluorescence as follows:

$$\frac{\Delta F}{F_0} = \frac{(\text{fluorescence from BODIPY-GTP}\gamma\text{S-bound G}\alpha) - (\text{fluorescence from BODIPY-GTP}\gamma\text{S alone})}{\text{fluorescence from BODIPY-GTP}\gamma\text{S alone}}$$

Graphpad Prism was used to fit fluorescence (F/F_0) curves to a pseudo first-order rate equation and obtain k_{obs} values relative to vehicle controls. FR concentration-response curves were generated by a three-parameter fit of normalized k_{obs} values.

Assays of GTP hydrolysis by His-tagged Gi/q α -subunits were performed as follows. Reactions contained wild type G*α*i/q (1.5 μ M) or constitutively active G*α*i/q(Q204L) (3 μ M) in reaction buffer (50mM Tris-HCl, 5mM MgCl₂, 1mM EDTA, 0.5mM DTT, pH 8.0). Reactions performed in triplicate were started by adding reaction buffer containing γ ³²P-GTP (final concentration of 100nM and specific activity of 100Ci/mmol). G*α* subunits were present at >10-fold molar excess over GTP to assess the catalytic rather than steady state rate of GTP hydrolysis. As indicated, vehicle (DMSO) or FR (25 μ M final concentration) was added. Aliquots were removed at intervals, quenched by addition of 1N formic acid and spotted on polyethyleneimine (PEI)-cellulose thin layer plates (Sigma-Aldrich cat. Z122882). Plates were developed in 0.5M LiCl₂ and 0.5M formic acid, dried, wrapped in plastic, and exposed 1 hour to a phosphor storage screen (GE Healthcare Life Sciences) that was visualized on a Typhoon FLA 9500 scanner (GE Healthcare Life Sciences) at a resolution of 100 μ m and photomultiplier voltage of 750V. Scans were analyzed using Image Studio Lite to quantify the amount of labeled GTP and orthophosphate in each lane. GTP hydrolysis was calculated by dividing the magnitude of the orthophosphate signal by the sum of the orthophosphate and GTP signals. Data were fit using Graphpad Prism to a pseudo first-order rate equation.

Uveal melanoma cell culture assays

Cells were cultured at 37°C in 5% CO₂. Human uveal melanoma (UM) cell lines 92.1, Mel202, and OCM-1A were derived by Drs. Martine Jager (Laboratory of Ophthalmology, Leiden University), Bruce Ksander (Schepens Eye Institute, Massachusetts Eye and Ear Infirmary) and June Kan-Mitchell (Biological Sciences, University of Texas at El Paso). UM cell lines were grown in RPMI 1640 medium (Life Technologies, Carlsbad, CA) supplemented with 10% FBS and antibiotics. Cell viability was measured using a water-soluble tetrazolium salt, WTS-8 (Bimake, Houston, TX), following the manufacturer's protocol. Flow cytometry for analysis of cell proliferation and apoptosis was performed at the Siteman Cancer Center Flow Cytometry Core on a FACScan analyzer (BD Biosciences, San Diego CA, USA) using a standard propidium iodide staining protocol as described previously (43).

Immunofluorescence staining of UM cell lines was carried out by adding an equal volume of 2 $\times$ fixative (PBS with 4% paraformaldehyde and 0.4% glutaraldehyde) to UM cells in RPMI growth medium. After 15 minutes at 37°C, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 minutes, washed with PBS, and blocked with 2% fish gelatin (Sigma-Aldrich) in PBS. Primary and secondary antibodies were diluted in 2% fish gelatin in PBS. Primary antibodies included mouse monoclonal anti-pre-melanosomal protein (One World Lab, San Diego, CA), rabbit polyclonal anti-tyrosinase (One World Lab), rabbit polyclonal anti-dopachrome tautomerase (One World Lab), rabbit polyclonal anti-S100 (DakoCytomation, Denmark), and mouse monoclonal anti-BrdU (Life Technologies). Secondary antibodies were Alexa-fluor conjugates (Life Technologies), and the mounting agent was ProLong

Gold (Life Technologies). Cell morphology was assessed by phase contrast imaging with an inverted microscope (Olympus IX72) using a 10× objective.

Immunoblotting of UM cell lines was performed by lysing cells in radioimmunoprecipitation assay buffer (150 mM sodium chloride, 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0) with 1× complete protease inhibitor mix (Roche, cat.11697498001). Cleared lysates were analyzed by immunoblotting as described above for HEK293 cells.

Histones were isolated from UM cell lines by using the Active Motif histone purification mini kit (cat.40026, Active Motif, Carlsbad, CA). Lysates were resolved on 15% SDS-PAGE gels and transferred to Immobilon(P) PVDF membrane (Millipore, cat.IPVH00010). Membranes were blocked with 5% w/v milk in TBST (25 mM Tris pH 7.2, NaCl 150 mM, 2.7 mM KCl, 0.1% v/v Tween 20) and incubated with primary antibodies. Membranes were washed with TBST at least three times and incubated with IRDye 680-coupled goat anti-rabbit and IRDye 800 or goat anti-mouse antibodies (LI-COR, Lincoln, NE). Following incubation, membranes were washed at least three times with TBST and signals were detected using Odyssey model 9120 imaging system (LI-COR). Other primary antibodies used for immunoblotting were: anti-EE (Covance, cat. MMS-115P, lot E12BF00285), anti-Actin C4 (Millipore, cat. MAB1501), anti-Histone H3 (clone A3S, cat.05-928, Millipore) and Anti-histone H3-trimethyl-K27 (cat.6002, Abcam).

Statistical analyses

All statistical analyses were performed with Graphpad Prism. For split luciferase complementation assays, IP1 assays, and viability assays, cells were plated in triplicate wells and treated in parallel (technical replicates), and each experiment was performed three times on different days (biological replicates). Mean values and standard errors of the means (SEM) were calculated from at least nine replicate values for each condition and t-Tests were performed comparing each condition to controls to determine significance of FR treatment. For guanine nucleotide exchange and GTP hydrolysis assays, experiments were performed in triplicate, and each experiment was performed at least three times on different days with different protein preparations. Mean values and standard errors of the means (SEM) were calculated from at least nine replicate values for each condition and t-Tests were performed comparing each condition to controls to determine significance of FR treatment. For FRET reporter assays, FRET fluorescence signals were quantified relative to vehicle controls from three independent experiments, and mean values and SEM were calculated from the combined data of three experiments. IC₅₀ values and confidence intervals were calculated by the least-square non-linear curve fitting method for normalized response to FR. For histone methylation assays, densitometry was performed on immunoblots for trimethyl-histone H3(Lys²⁷) and compared to DMSO control and normalized to total histone H3 from three independent experiments. t-Tests were performed to determine the significance of response to FR treatment.

Gene expression analysis

92.1 UM cells were treated with 100 nM FR or vehicle (DMSO) in RPMI growth medium and collected after 1 and 3 days of treatment. RNA was isolated using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol and including the optional DNase I treatment step. RNA quality was assessed on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). mRNA was extracted from total RNA using a Dynal mRNA Direct kit, fragmented and reverse transcribed to double stranded cDNA with random primers before addition of adapters for library preparation. Library preparation and HiSeq2500 sequencing were performed by the Washington University Genome Technology Access Center (gtac.wustl.edu). FastQ files were aligned to the transcriptome and the whole-genome with STAR. Biologic replicates were simultaneously analyzed by edgeR and Sailfish analyses of gene-level/exon-level features. Unexpressed genes and exons were removed from the analyses. Unsupervised principal component analysis and volcano plots were generated in Bioconductor using edgeR. Significance Analysis of Microarrays (SAM), Version 4.0 was used to generate a ranked gene list, and a threshold of $q < 10\%$ and fold-change > 2.0 was then used to select the most highly significant genes that showed reduced expression in FR-treated versus vehicle control cells. This list was used as signature gene sets for Gene Ontology (GO) analysis and Gene Set Enrichment Analysis (GSEA) (44). GO groups were assembled by merging the lists of genes from related GO terms that were significantly enriched ($p < 0.01$ using the Kolmogorov-Smirnov statistic) in the signature gene set. Significant gene sets ($p < 0.01$ using the Kolmogorov-Smirnov statistic) from GSEA analyses were combined such that genes associated with multiple related signatures were only counted once and each gene was assigned only to a single combined group based on the signature with the highest enrichment score for that gene. Gene expression changes were validated in all three UM cell lines by qPCR using fast SYBR Green Mastermix (Fisher Scientific) following the manufacturers' protocol. GAPDH was used as an endogenous control. Primer sets used for the assay are listed in data file S6.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We are grateful to Dr. Jerry Y. Niederkorn (UT Southwestern) for providing UM cell lines, and Dr. Robert P. Mecham (Washington University in St. Louis) for supporting this project.

Funding: This study was supported by a multi-PI grant from the Siteman Cancer Center and Pedal the Cause (KJB and MDO) and grants from the National Institute of Health GM044592 and GM124093 (KJB) and GM118171 (JAC).

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Figure 1: FR traps mutant constitutively active $G\alpha$ q in the inactive GDP-bound state.

(A, B, D) Effect of FR on the activity state of $G\alpha$ subunits as determined by interaction with $G\beta\gamma$ or RGS domains in split luciferase complementation assays, measured as reconstitution of click beetle green (CBG) luciferase activity normalized to co-transfected, constitutively expressed Renilla luciferase. A) Effect of FR on interaction of $G\beta 1\gamma 2$ with the indicated wild type (WT) or mutant constitutively active forms of $G\alpha$ q (q) or $G\alpha 13$ (13) (Q209L and Q226L, respectively); mean values $\pm$ SEM of 3 experiments. B) Potency of FR as a driver of interaction between $G\beta 1\gamma 2$ and wild type or mutant constitutively active $G\alpha$ q; mean values

± SEM from 3 experiments. C) Effect of FR treatment of cells on co-purification of endogenous Gβγ with overexpressed, affinity-tagged wild type or constitutively active (Q209L) Gαq. Affinity-tagged Gαq and Gβγ were detected by immunoblotting with FLAG and Gβ antibodies, respectively. Results shown are representative of 3 independent experiments. D) Potency of FR as an inhibitor of interaction between RGS2 and mutant constitutively active Gαq(Q209L), or between the RGS domain of LARG (LARG-RGS) and mutant constitutively active Gα13(Q226L); mean values ± SEM from 3 experiments. E) Potency of FR as inhibitor of signal transduction by constitutively active Gαq or Gα13 detected with an SRE(L) promoter-driven transcriptional reporter; mean values ± SEM from 3 experiments. Constitutively active Gαq(Q209L), constitutively active Gαq bearing the indicated FR binding site mutations, and constitutively Gα13(Q226L) were studied. Concentration-response curves were fit by non-linear regression to derive EC50 or IC50 values, which were compared by t-Test. p<0.05; ** p<0.01 by t-Test, significance confirmed using q<0.05 or q<0.01 by the FDR method of Benjamini and Hochberg.

Figure 2: Inhibition of Gai1 bearing an engineered FR-binding site.

A) Amino acid residues in Gai1 identical to or diverged from the FR binding site in Gaq are indicated in blue and red, respectively. An FR binding site was engineered in Gai1 by introducing the eight indicated amino acid substitutions so as to match the corresponding residues of Gaq, thereby producing a chimeric α subunit termed Gi/q. B) Effect of FR on guanine nucleotide exchange by Gi/q α -subunits in vitro. Nucleotide exchange was assayed by measuring increase in BODIPY-GTP γ S fluorescence upon binding to the indicated purified His-tagged α subunits in the absence or presence of the indicated concentrations of FR. Gi/q(R54K) corresponds to a Gaq mutant that is less sensitive to inhibition by FR. Nucleotide exchange rates (k_{obs}) of Gai/q and Gai/q(R54K) in the absence of FR were 0.24 and 0.12 minute^{-1} , respectively. Mean values $\pm$ SEM from 3 independent experiments are shown. C) Effect of FR on agonist-evoked signaling mediated by Gi/q. Inhibition of forskolin-induced cAMP formation by Gi-coupled cannabinoid receptors was measured in Neuro2A cells transfected with a cAMP FRET reporter and pertussis toxin (PTX)-resistant and EE epitope-tagged forms of the indicated α subunits, and treated with PTX to inactivate endogenous Gi. Inhibition of forskolin-induced cAMP formation by a cannabinoid receptor agonist (WIN 55,212-2; WIN) was measured by FRET. Attenuation of this inhibitory effect by FR was quantified relative to vehicle controls; mean values $\pm$ SEM from 3 experiments are shown. IC_{50} values of FR toward cells expressing Gi/q and Gi/q(R54K) were 4.6 and 76 μM , respectively. D) Failure of FR to correct the GTP hydrolysis defect of Gi/q(Q204L) in vitro. Hydrolysis of $\gamma^{32}\text{P}$ -GTP by the indicated α subunits present at 10-fold molar excess over GTP was measured over time. FR was added at the indicated time point (arrow). Mean values $\pm$ SEM from 3 independent experiments are shown. E) Effect of

FR on guanine nucleotide exchange by constitutively active Gi/q(Q204L) in vitro, as determined by methods described in panel (B). The rate of nucleotide exchange (k_{obs}) by Gi/q(Q204L) in the absence of FR was 0.14 minute^{-1} . Concentration-response curves were fit by non-linear regression to derive IC50 values, which were compared by t-Test. * $p < 0.05$, ** $p < 0.01$ by t-Test, significance confirmed using $q < 0.05$ or $q < 0.01$ by the FDR method of Benjamini and Hochberg.

Figure 3: FR inhibits signaling by constitutively active Gαq in UM cells.

Inhibition of signaling by constitutively active Gαq in UM cell lines was quantified by measuring intracellular inositol 1-phosphate (IP1), a metabolically stable product of inositol 1,4,5-trisphosphate produced by Gαq-stimulated phospholipase Cβ. A) Basal IP1 values in UM cell lines driven by constitutively active Gαq(Q209L) (92.1 and Mel202 cells) and BRAF(V600E) (OCM-1A cells); mean values ± SEM from 3 experiments performed in triplicate. B) Effect of FR on IP1 abundance in 92.1, Mel202 and OCM-1A cells; mean values ± SEM of 4 experiments performed in triplicate. *p<0.01 by t-Test, significance confirmed using q<0.01 by the FDR method of Benjamini and Hochberg.

Figure 4: FR-sensitive growth and viability of UM cells driven by constitutively active $G\alpha_q$.
A) Changes in viability of UM cells treated with FR. Cell viability was quantified using a water-soluble tetrazolium salt assay. The fold-change in cell viability over time is shown for $G\alpha_q(Q209L)$ -driven 92.1 and Mel202 cells, and for BRAF(V600E)-driven OCM-1A cells in response to increasing concentration of FR; mean values $\pm$ SEM from 4 experiments performed in triplicate. B) Potency of FR as an inhibitor of UM cell viability measured as in (A); mean values $\pm$ SEM from 4 experiments performed in triplicate. The indicated UM cell lines were treated for 3 days with the indicated concentrations of FR and analyzed for DNA

content. C) Representative histograms from flow cytometry of the Gα_q(Q209L)-driven cell lines (92.1 and Mel202) and BRAF(V600E)-driven OCM-1A cells. D) Potency of FR as inducer of apoptosis (sub-G1 cells) and inhibitor of cell proliferation (S- and G2/M-phase cells); mean values ± SEM from 4 experiments. *p<0.01 by t-Test, significance corrected for multiple comparisons using the Holm-Sidak method.

Figure 5: FR induces re-differentiation of UM cells driven by constitutively active Gαq.
A) Morphological changes elicited by FR. UM cell lines were treated 3 days with FR and imaged by phase contrast microscopy. Representative fields from one of 3 experiments showing FR caused UM cells driven by constitutively active Gαq (92.1 and Mel202) to lose their characteristic spindle shaped and assume a stellate shape with multiple projections. FR had no effect on BRAF-driven UM cells (OCM-1A). B) Melanocytic differentiation of FR-treated UM cells indicated by pigmentation. UM cell lines were treated 3 days with FR. Cells were pelleted and examined macroscopically; representative images from one of 3

experiments performed in triplicate. C) Induction of melanocytic markers by FR as indicated by immunofluorescence staining of tyrosinase (TYR), dopachrome tautomerase (DCT) and pre-melanosomal protein (PMEL) of Gαq-mutant cell lines (92.1 and Mel202) but not BRAF-driven UM cells (OCM-1A); representative fields from one of 3 experiments. Scale bar = 50 μm.

Figure 6. FR represses expression of differentiation genes by restoring function of the polycomb repressive complex 2.

Gαq-mutant 92.1 UM cells were treated with FR or vehicle, and RNA was collected 1 and 3 days after treatment for RNA-Seq analysis. A) Results of a multi-dimensional gene expression analysis that compares the relative patterns of expression of all genes across all samples and groups genes with similar patterns. The graph shows samples positioned by their relative gene expression values within each pattern. Dimension 1 (x-axis; the most represented pattern) shows separation based on vehicle treatment (red balls) versus FR treatment (blue balls), whereas Dimension 2 (y-axis; the second most represented pattern)

shows separation based on time in culture (indicated by 1d or 3d on balls). B) MA plot (M = log ratio, and A = mean average) comparing gene expression between FR- and vehicle-treated samples identifies a group of significantly reduced genes (circled; fold change > 2; false discovery rate $q < 0.01$) associated with FR treatment. C) Gene ontology analysis of the FR-repressed geneset (circled in B with arrow) shows that most are involved in developmental processes and differentiation. D) FR-repressed genes (circled in B with arrow) identified as targets of the polycomb repressive complex 2 by Geneset enrichment analysis. E) The Ezh1/2 inhibitor GSK503 blocks morphological differentiation elicited by FR. Representative fields are shown from one of 3 experiments of 92.1 UM cells treated 7 days with GSK503 and 3 days with FR and then imaged by phase contrast microscopy. F) GSK503 decreased pigmentation of FR-treated cells, visualized by macroscopic inspection. 92.1 cells were treated 7 days with GSK503 and 3 days with FR and pelleted; representative images from one of 3 experiments. G) PRC2 inhibition by GSK503. Immunoblots of 92.1 cells treated 7 days with GSK503 show reduced histone H3(Lys²⁷) trimethylation. Plot shows relative fraction of trimethyl-histone H3(Lys²⁷) compared to DMSO control and normalized to total histone H3 from densitometry data from 3 independent experiments. * $p < 0.01$ by t-Test, significance confirmed using $q < 0.01$ by the FDR method of Benjamini and Hochberg.