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Enhanced Membrane Binding of Oncogenic G Protein α Q209L Confers Resistance to Inhibitor YM-254890

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Heterotrimeric G proteins couple activated G protein-coupled receptors (GPCRs) to intracellular signaling pathways. They can also function independently of GPCR activation upon acquiring mutations that prevent GTPase activity and result in constitutive signaling, as occurs with the α Q209L mutation in uveal melanoma. YM-254890 (YM) can inhibit signaling by both GPCR-activated WT α q and GPCR-independent α Q209L. Although YM inhibits WT α q by binding to α q-GDP and preventing GDP/GTP exchange, the mechanism of YM inhibition of cellular α Q209L remains to be fully understood. Here, we show that YM promotes a sub-cellular redistribution of α Q209L from the plasma membrane (PM) to the cytoplasm. To test if this loss of PM localization could contribute to the mechanism of inhibition of α Q209L by YM, we developed and examined N-terminal mutants of α Q209L, termed PM-restricted α Q209L, in which the addition of membrane-binding motifs enhanced PM localization and prevented YM-promoted redistribution. Treatment of cells with YM failed to inhibit signaling by these PM-restricted α Q209L. Additionally, pull-down experiments demonstrated that YM promotes similar conformational changes in both α Q209L and PM-restricted α Q209L, resulting in increased binding to $\beta\gamma$ and decreased binding to regulator RGS2, and effectors p63RhoGEF-DH/PH and phospholipase C- β . GPCR-dependent signaling by PM-restricted WT α q is strongly inhibited by YM, demonstrating that resistance to YM inhibition by membrane-binding mutants is specific to constitutively active α Q209L. Together, these results indicate that changes in membrane binding impact the ability of YM to inhibit α Q209L and suggest that YM contributes to inhibition of α Q209L by promoting its relocalization.

Heterotrimeric G proteins, comprised of an α , β , and γ subunit, act as molecular switches to regulate cell signaling pathways (1–3). Heterotrimeric G proteins couple to G protein-coupled receptors (GPCRs), which upon activation act as guanine-nucleotide exchange factors (GEFs) to

induce conformational changes that promote GDP release from the α subunit in exchange for GTP (2, 3). Binding of GTP to the α subunit results in dissociation of the α subunit from the tightly associated $\beta\gamma$ subunit and subsequent binding of α and $\beta\gamma$ to effector proteins. α subunits are grouped into four families (α s, α i, α q, and α 12/13) that mediate various pathways within the cell (4). The G α q family, which is comprised of α q, α 11, α 14, and α 15/16, classically activate phospholipase C β (PLC β) (5, 6). Active PLC β hydrolyzes phosphatidylinositol 4,5-bisphosphate resulting in production of inositol 1,4,5-trisphosphate and diacylglycerol (DAG) (7).

α q signaling is essential for physiological processes; however, activating mutations in α subunits can result in dysregulation of signaling and disease. α q and α 11, which share 90% identity at the amino acid level and signal similarly, are mutated mutually exclusively in over 90% of uveal melanoma cases resulting in their constitutive activity (8–10). α subunits are comprised of a helical domain and a GTPase domain. The helical domain and GTPase domain form a crevice where the guanine nucleotide binds (11), and the GTPase domain contains three critical switch regions (Sw I, Sw II, and Sw III) that undergo conformational changes allowing for GTP binding. The switch regions are also important for intrinsic GTP hydrolysis activity, which is accelerated by GTPase-activating proteins (GAPs). α q/11 mutations in uveal melanoma most frequently involve a missense mutation at glutamine 209 to leucine or less commonly proline. The mutation at glutamine 209 located in Sw II greatly diminishes the ability of α q or α 11 to hydrolyze GTP rendering them constitutively active (12). The implications of α q mutations in disease warrants an urgent need to better understand the biology of α q under physiological and disease conditions.

Two major signaling pathways that are activated by constitutively active α q to promote cell growth and proliferation are the mitogen-activated protein kinase (MAPK) pathway and the nuclear translocation of the transcription coactivators Yes-associated protein 1 (YAP) and paralog transcriptional coactivator with PDZ-binding motif (TAZ). MAPK pathway activation occurs *via* increased DAG

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production upon α_q activation of the classical effector PLC β . DAG then serves a dual role in membrane recruitment of the RasGEF RasGRP3 and protein kinase C isoforms δ/ϵ (PKC δ/ϵ), which phosphorylate and further activate RasGRP3 (13, 14). The resulting activation of Ras then stimulates the well-known MAPK cascade. On the other hand, activated α_q initiates the YAP/TAZ pathway by directly binding to and activating the RhoGEF Trio (15). Activation of Rho then leads to focal adhesion kinase (FAK)-dependent disruption of the cytoplasmic retention of YAP/TAZ, thereby promoting translocation of YAP/TAZ into the nucleus (16). Both the MAPK pathway and translocation of YAP into the nucleus promote transcription of cell growth and proliferative genes, resulting in uveal melanoma progression (13–16).

A number of studies show the promise of YM-254890 (YM) and FR900359 (FR) as inhibitors of WT and constitutively active $\alpha_q/11$ (17–21). YM and FR are naturally occurring cyclic depsipeptides isolated from *Chromobacterium* and *Ardisia crenata*, respectively. Both compounds, having highly similar structures (22), are thought to have similar mechanisms of action of preventing the release of GDP from α_q and thereby preventing activation by inhibiting the exchange of GDP for GTP (19, 21, 23). Although it is clear that FR/YM can effectively inhibit constitutive α_q Q209L signaling, α_q Q209L is thought to exist predominantly in the GTP-bound form due to the lack of GTP hydrolysis activity, presenting a paradox as to how FR/YM can trap α_q Q209L in the GDP-bound form (24). Thus, the mechanisms of how constitutively active α_q Q209L is regulated in cells and how FR/YM inhibit α_q Q209L remain to be fully understood.

Importantly, membrane localization is critical for signaling by both WT and constitutively active α_q . α_q gains affinity for the plasma membrane (PM) through interaction with $\beta\gamma$ (25, 26), palmitoylation at cysteines 9 and 10 (27), and an N-terminal polybasic motif (28). PM localization is particularly important for interaction with the GPCR and effector proteins such as PLC β . Furthermore, mutational disruption of these membrane-targeting mechanisms of WT and constitutively active α_q results in cytoplasmic localization and attenuation of signaling, demonstrating that PM localization is critical for α_q signaling (25–29). Moreover, α subunits can traffic reversibly between the PM and intracellular organelles, highlighting the importance of subcellular localization for G protein signaling function (29, 30). Understanding further how localization regulates signaling by constitutively active α_q is critical for gaining new insight into ways to inhibit dysregulated α_q signaling.

In our studies, we provide evidence that YM treatment results in the redistribution of α_q Q209L from the PM to the cytoplasm. Additionally, we generated PM-restricted α_q Q209L mutants that displayed resistance to inhibition of signaling by YM. These studies suggest the importance of YM-induced translocation of α_q Q209L into the cytoplasm as an additional way in which YM inhibits constitutively active α_q .

Results

YM promotes the redistribution of α_q Q209L from the PM to cytoplasm

While studying the effect of YM-254890 (YM) on α_q -dependent signaling, we observed surprisingly that YM promoted a change in localization of α_q Q209L and to a lesser extent α_q WT. Immunofluorescence microscopy of HEK 293 cells stably expressing tetracycline-inducible α_q Q209L showed that treatment of cells with 1 μ M YM for 1 h promoted a redistribution of α_q Q209L from a PM localization to an intracellular localization (Fig. 1A). Scoring α_q Q209L localization in individual cells as PM localized, PM and cytoplasmic, or cytoplasmic showed that in dimethyl sulfoxide (DMSO) vehicle-treated cells, α_q Q209L displayed a predominant PM localization in 63% of cells, colocalizing at the PM with GRK5, a protein shown to strongly localize at the PM when expressed in HEK 293 cells (31), and a distribution to both the PM and cytoplasm in 25% of cells (Fig. 1, A and B). However, after 1 h of YM treatment, only 5% of cells showed strong PM localization of α_q Q209L; instead, α_q Q209L was not detected at the PM and was localized in the cytoplasm in 84% of cells. Redistribution of α_q Q209L was also observed after 24 h of YM treatment, but more PM localization remained compared to 1 h YM treatment (Fig. 1, A and B). Although α_q Q209L displays a decrease in PM localization after YM treatment, GRK5 remains strongly localized at the PM, indicating that the observed redistribution of α_q Q209L is not simply due to a general disruption in PM localization of peripheral membrane-bound proteins. In HEK 293 cells stably expressing tetracycline-inducible α_q WT, a small but consistent shift-off of the PM for α_q WT was observed after YM treatment (Fig. 1, A and B), but only α_q Q209L displayed a dramatic redistribution. Translocation of α_q Q209L in response to YM was also observed in HeLa cells (Fig. S1).

To begin to address whether the observed YM-promoted redistribution of α_q Q209L is related to the ability of YM to inhibit constitutive signaling by α_q Q209L, we examined a time course of YM inhibition. α_q Q209L activates the MAPK pathway through its canonical signaling pathway mediated by PLC β . Immunoblotting of cell lysates with a phospho-ERK (pERK)-specific antibody was used as a read out of MAPK signaling. In cells expressing α_q Q209L, a significant decrease in pERK levels was detected after 1 h of YM treatment. pERK continued to decrease at 2 to 3 h and remained inhibited up to 16 h after YM treatment (Fig. 1C). These experiments together show that the YM-promoted redistribution of α_q Q209L appears to precede YM-induced inhibition of signaling, raising the question of whether YM-promoted loss of PM localization of α_q Q209L plays a role in the ability of YM to inhibit constitutive signaling by α_q Q209L.

YM does not disrupt the PM localization or signaling of Src- α_q Q209L or Lyn- α_q Q209L

If redistribution from the PM plays a role in YM inhibition of α_q Q209L signaling, we reasoned that a mutant of α_q Q209L

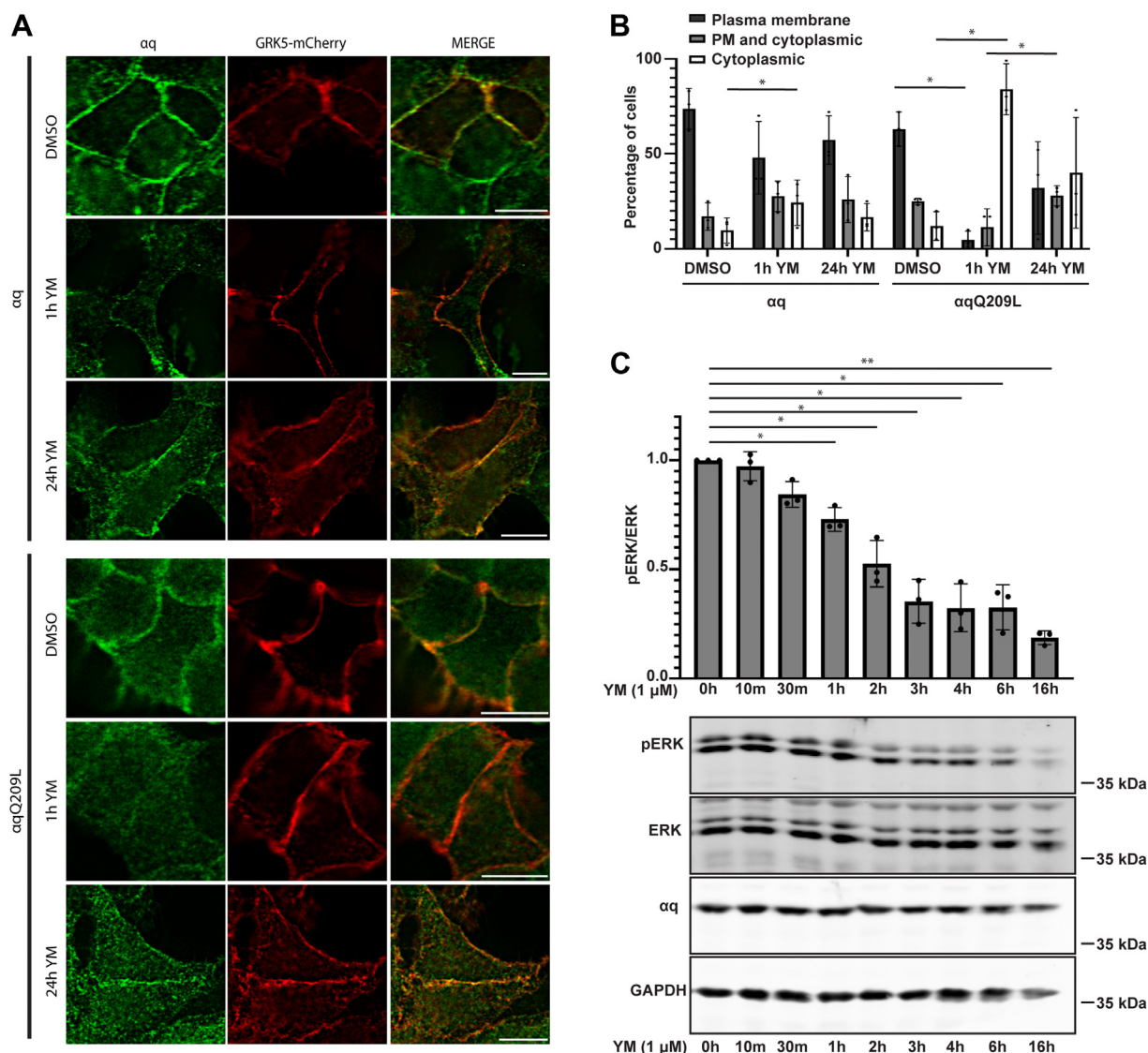


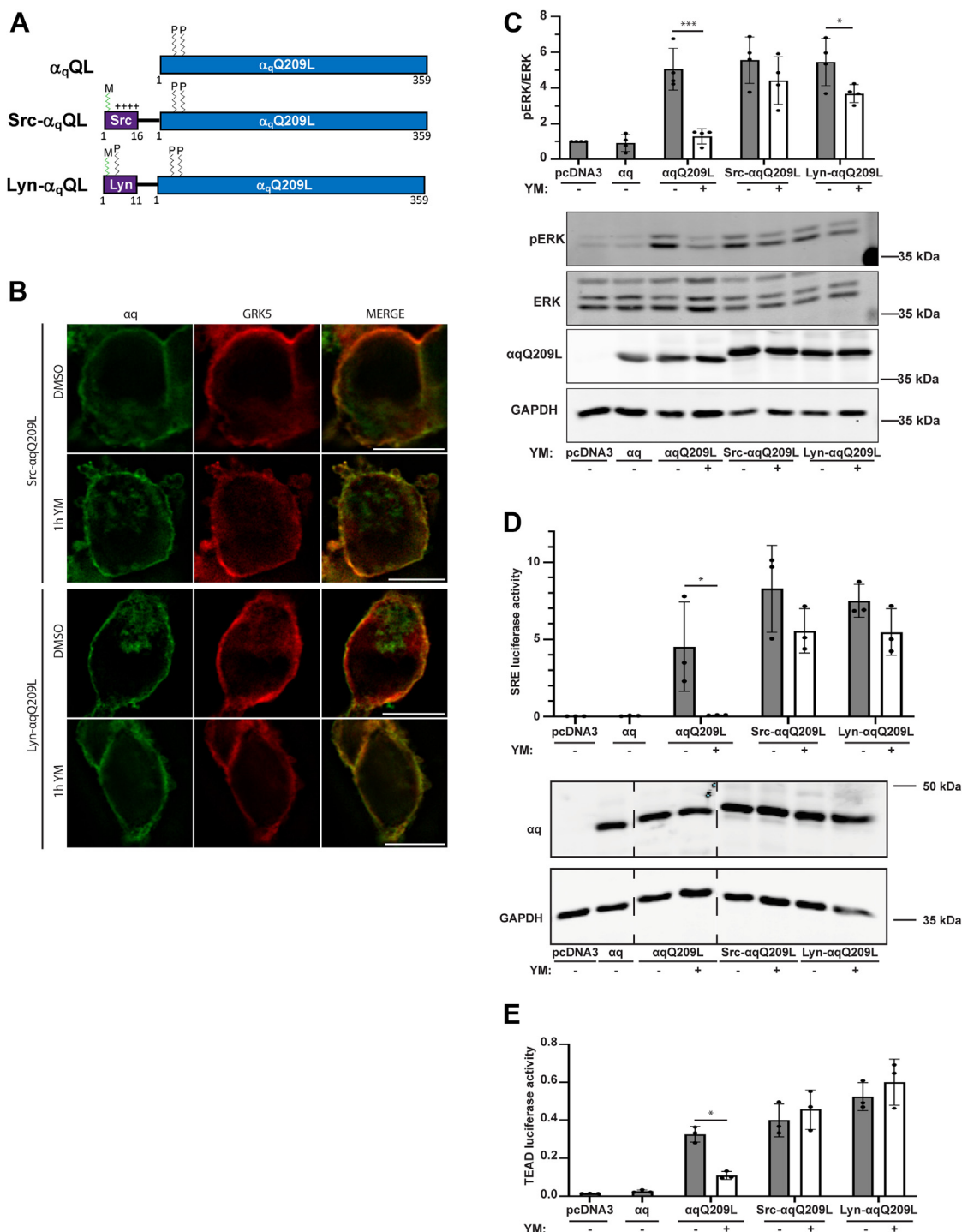
Figure 1. YM promotes dissociation of α_q Q209L from the PM. A, Flp-In HEK 293 cells were transfected with GRK5-mCherry and treated with tetracycline to induce expression of α_q and α_q Q209L. The tetracycline-induced α_q - and α_q Q209L-Flp-In HEK 293 cells were treated with DMSO or 1 μ M YM for 1 h or overnight. α_q , α_q Q209L, and GRK5 were visualized by immunofluorescence microscopy, as described under [Experimental procedures](#). The scale bars represent 10 μ M. B, the localization of α_q - or α_q Q209L in 100 tetracycline-induced Flp-In HEK 293 cells in each of $n = 3$ experiments were scored as either PM localized with little to no observable staining in the cytoplasm, PM, and cytoplasmic localization in which individual cells displayed varying degrees of a partial PM stain and observable cytoplasmic localization of α_q or cytoplasmic in which α_q was distributed throughout the cytoplasm but had no observable PM localization. Statistical significance comparing experimental conditions was determined as described under [Experimental procedures](#). Results are shown as mean \pm SD ($n = 3$; experiments $*p < 0.05$, two-way ANOVA, Fisher's Least Significant Differences test). C, expression of α_q Q209L was tetracycline induced in Flp-In HEK 293 cells, and cells were treated with 1 μ M YM for the indicated times. Cell lysates were immunoblotted using antibodies for the indicated proteins. pERK and ERK signal intensities were quantified and normalized to pERK/ERK signal intensity in DMSO treatment. Results are shown as mean \pm SD ($n = 3$; $*p < 0.05$; $**p < 0.01$, one-way ANOVA, Dunnett's multiple comparisons test). DMSO, dimethyl sulfoxide; PM, plasma membrane.

that remains at the PM upon YM treatment would continue to signal in the presence of YM. To test this idea, we initially constructed mutants of α_q Q209L in which additional PM-targeting motifs were added to the N terminus to augment the ability of reversible palmitoylation at cysteines 9 and 10 to maintain PM localization. Amino acids 1 to 16 of Src or amino acids 1 to 11 of Lyn were fused to the N terminus of α_q Q209L to introduce a myristoylation plus polybasic motif and a myristoylation plus palmitoylation motif, respectively (Fig. 2A). The addition of a similar N-terminal sequence of Src has been shown to strongly localize α_s at the PM and prevent activation-induced subcellular redistribution of α_s

(32). Immunofluorescence microscopy showed that Src- α_q Q209L or Lyn- α_q Q209L displayed strong PM localization and colocalization with GRK5, when expressed in HEK 293 q/11 KO cells in which α_q and α_{11} were knocked out using CRISPR (Fig. 2B). Importantly, Src- α_q Q209L and Lyn- α_q Q209L remained at the PM after YM treatment (Fig. 2B), thus demonstrating that the addition of the Src or Lyn motif is sufficient to prevent YM-promoted redistribution of α_q Q209L.

To determine if these PM-restricted α_q Q209L mutants are sensitive to inhibition by YM, we assayed pERK levels $-/+$ YM treatment by immunoblot analysis. Expression of α_q Q209L,

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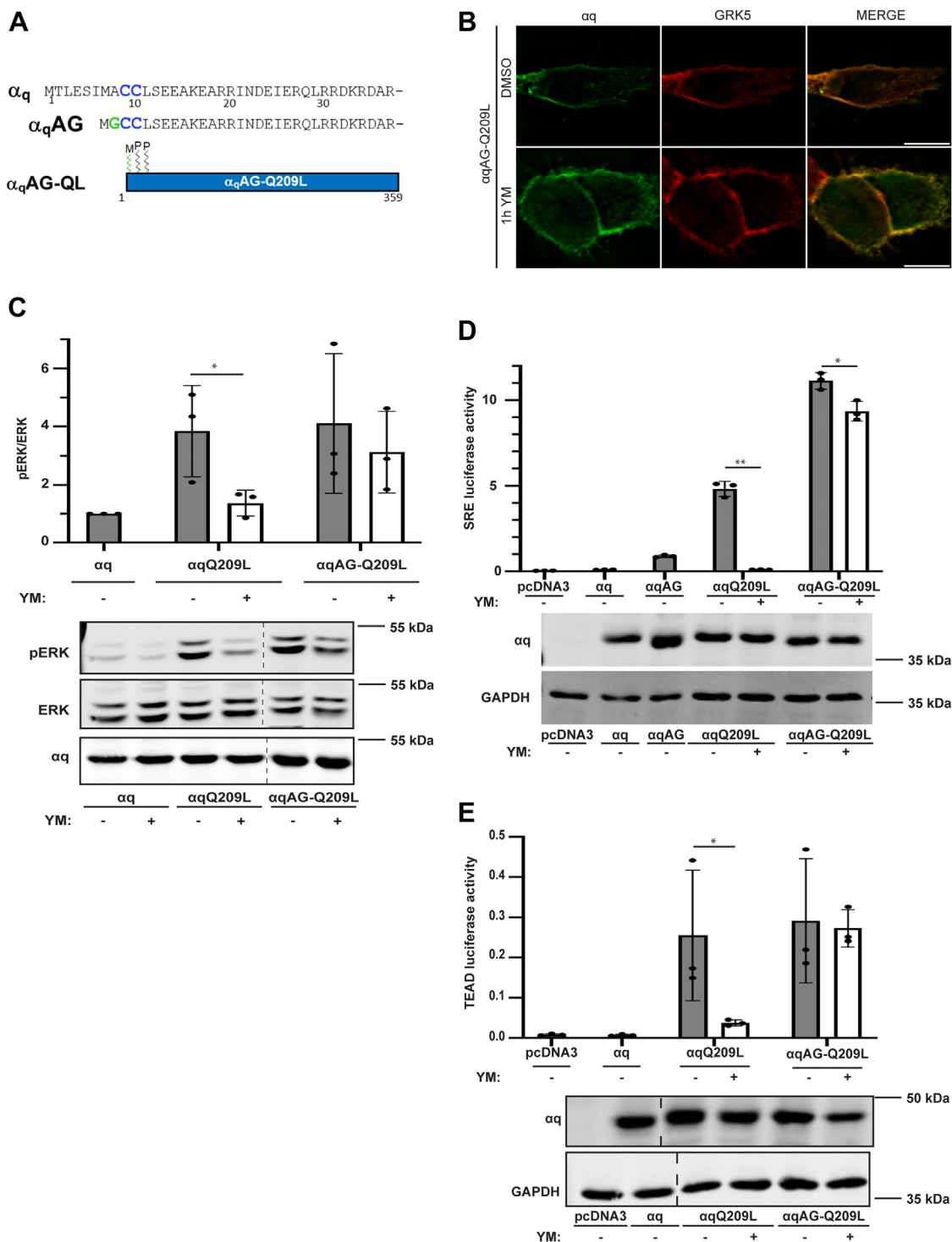


Figure 3. α_q -AGQ209L remains at the PM upon YM treatment and is resistant to YM inhibition. *A*, schematic of α_q , α_q AG, and α_q AGQ209L. *B*, α_q AG-Q209L was cotransfected with GRK5-mCherry in HEK 293 q/11 K/O cells, and cells were treated with DMSO or 1 μ M YM for 1 h. α_q AG-Q209L and GRK5-mCherry were visualized by immunofluorescence microscopy. The scale bars represent 10 μ m. *C*, HEK 293 q/11 K/O cells were transfected with α_q -pcDNA3, α_q Q209L-pcDNA3, α_q AG-Q209L, or pcDNA3 vector alone and treated with DMSO or 1 μ M YM overnight. Cell lysates were immunoblotted using antibodies for the indicated proteins. pERK and ERK signal intensities were quantified and normalized to pERK/ERK signal intensity in DMSO treatment in α_q -transfected cells. *D* and *E*, HEK 293 q/11 K/O cells were transfected with α_q -pcDNA3, α_q AG-pcDNA3, α_q Q209L-pcDNA3, α_q AG-Q209L-pcDNA3, or pcDNA3 alone, along with *renilla* luciferase and either pSRE luciferase (*D*) or 8xGTIIC (TEAD) luciferase (*E*). Cell lysates were prepared, and luciferase assays were performed and quantitated as described under [Experimental procedures](#). Cell lysates were immunoblotted using antibodies for the indicated proteins. In (*C*–*E*) results are shown as mean \pm SD ($n = 3$; * $p < 0.05$; ** $p < 0.01$, two-way ANOVA, Šidák's multiple comparisons test). DMSO, dimethyl sulfoxide; PM, plasma membrane.

Src- α_q Q209L, or Lyn- α_q Q209L in HEK 293 q/11 KO cells resulted in similar constitutive activation of pERK, approximately 5-fold higher than in vector transfected or α_q WT

expressing cells (Fig. 2C). While YM treatment abolished α_q Q209L activation of pERK, Src- α_q Q209L- and Lyn- α_q Q209L-dependent activation of pERK was strongly resistant

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to inhibition by YM. These results provide the first evidence that signaling by PM-restricted forms of α_q Q209L is not effectively inhibited by YM.

To examine further this insensitivity to YM of PM-restricted α_q Q209L, we utilized two transcriptional reporter assays. The serum response element (SRE) luciferase reporter assay detects α_q -dependent signaling *via* both MAPK- and Rho-dependent pathways, and the TEAD luciferase reporter assay detects α_q -dependent signaling *via* Rho-dependent translocation into the nucleus of the transcription coactivator YAP. Consistent with the pERK assay (Fig. 2C), expression of α_q Q209L, Src- α_q Q209L, or Lyn- α_q Q209L in HEK 293 q/11 KO cells resulted in similar constitutive activation of SRE-luciferase and TEAD-luciferase signals (Fig. 2, D and E). However, and again consistent with the pERK assays, YM treatment resulted in little or no inhibition of signaling by Src- α_q Q209L or Lyn- α_q Q209L, even though α_q Q209L signaling was abolished after YM treatment. Taken together, these results demonstrate that the addition of N-terminal PM-targeting motifs to α_q Q209L does not affect constitutive signaling but does confer resistance to YM.

Myristoylated α_q AG-Q209L remains localized to the PM and is resistant to YM

To further determine if signaling by PM-restricted α_q Q209L fails to be inhibited by YM, we took advantage of a previously described N-terminal, membrane-binding mutant of α_q . In this AG mutant, the first methionine codon is mutated so that α_q utilizes the methionine codon at position 7 as the initiating ATG, and the alanine at position 8, now position 2 in the AG mutant, is changed to a glycine codon to introduce a new site for myristoylation (25). Thus, the α_q AG-Q209L mutant is singly myristoylated and dually palmitoylated, in contrast to only dual palmitoylation for α_q Q209L, thereby providing an additional lipid modification to increase PM localization (Fig. 3A). Consequently, immunofluorescence microscopy showed that α_q AG-Q209L localized at the PM, colocalizing with GRK5 (Fig. 3B), and α_q AG-Q209L remained at the PM after YM treatment (Fig. 3B). Thus, the simple introduction of a site for N-terminal myristoylation is sufficient to prevent YM-promoted redistribution of α_q AG-Q209L, similar to that observed for the more complex Src- α_q Q209L or Lyn- α_q Q209L mutants (Fig. 2, A and B).

To determine if α_q AG-Q209L signaling, like Src- α_q Q209L and Lyn- α_q Q209L signaling, was also insensitive to YM inhibition, we first examined pERK levels stimulated by α_q AG-Q209L and the effect of YM treatment. Expression of α_q AG-Q209L in HEK 293 q/11 KO cells resulted in strong constitutive activation of the MAPK pathway, as revealed by pERK immunoblotting, similar to α_q Q209L (Fig. 3C). However, the constitutive pERK activation by α_q AG-Q209L was resistant to YM treatment. Upon YM treatment, α_q Q209L signaling to pERK was significantly reduced but α_q AG-Q209L retained its pERK signaling. We also used SRE and TEAD luciferase reporter assays to further confirm α_q AG-Q209L's lack of inhibition of by YM (Fig. 3, D and E). Expression of

either α_q Q209L or α_q AG-Q209L in HEK 293 q/11 KO cells resulted in strong constitutive activation in both luciferase transcriptional reporter assays. However, overnight YM treatment failed to inhibit SRE-dependent and TEAD-dependent luciferase activity in cells expressing α_q AG-Q209L, even though YM treatment completely blocked signaling by α_q Q209L. Collectively, our results demonstrate that enhanced membrane binding, displayed by three different PM-restricted α_q Q209L mutants, prevents relocalization and inhibition by YM, supporting the idea that YM-induced subcellular redistribution of α_q Q209L is important for inhibition of signaling by YM.

YM insensitivity of PM-restricted α_q AG-Q209L is not due to expression level or YM concentration

To address concerns that the aforementioned demonstrations of resistance to YM are artefacts of high levels of α_q AG-Q209L expression or inappropriate concentrations of YM, we performed key control experiments. First, we assessed TEAD-dependent transcription using decreasing amounts of expression of α_q Q209L and α_q AG-Q209L. α_q Q209L displayed a strong and significant decrease in TEAD luciferase activity upon YM treatment at both high and low expression levels (Fig. 4A). However, α_q AG-Q209L signaling remained resistant to YM at all expression levels, indicating that lack of inhibition by YM observed for this PM-restricted mutant was not due to high levels of expression. Second, we analyzed YM concentration curve. Using concentrations from 10 nM to 5 μ M, we observed the expected YM concentration-dependent inhibition of α_q Q209L signaling with an approximate IC₅₀ of 9 nM, as measured here by TEAD luciferase activity. However, α_q AG-Q209L signaling remained resistant to YM inhibition at all concentrations, including 5 μ M YM (Fig. 4B). Additionally, we performed TEAD luciferase assays in HEK 293 cells, rather than the HEK 293 q/11 KO cells that lack endogenous α_q and α_{11} (Fig. 4C). Signaling by expressed α_q AG-Q209L in HEK 293 cells remained resistant to YM inhibition, indicating that this failure to be inhibited by YM occurs for PM-restricted α_q AG-Q209L regardless of the presence or absence of endogenous WT α_q and α_{11} . Taken together, these results demonstrate that insensitivity to YM of PM-restricted α_q AG-Q209L is maintained at various expression levels of α_q AG-Q209L, various concentrations of YM, and in HEK 293 parental cells.

YM affects α_q Q209L and PM-restricted α_q AG-Q209L similarly in regulating binding to regulators and effectors

We next tested whether YM was binding to the PM-restricted mutants by assaying the ability of added YM to affect the interaction of α_q AG-Q209L with several binding partners. Previous work showed that addition of YM to cells expressing α_q Q209L promoted increased interaction with G β y and decreased interaction with regulator of G protein signaling 2 (RGS2), consistent with a proposed mechanism of YM binding to α_q Q209L and shifting it into an inactive form (19). Here, we expressed α_q Q209L and α_q AG-Q209L, as well as the

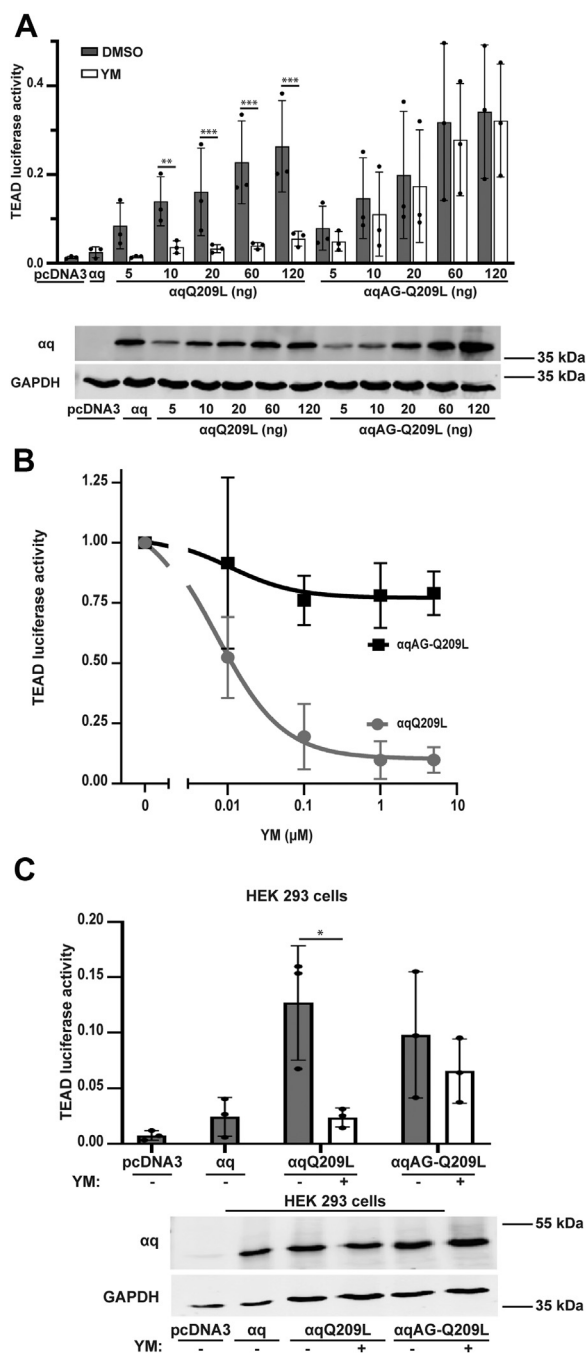


Figure 4. The resistance of PM-restricted α_q Q209L mutants to YM occurs independently of protein expression level, YM concentration, or presence of endogenous α_q /11. A, HEK 293 q/11 K/O cells were transfected with *renilla* luciferase, 8xGTIIc luciferase, and increasing amounts of α_q Q209L or α_q AG-Q209L, as indicated. Cells were treated with 1 μ M YM overnight. Cell lysates were prepared, and luciferase assays were performed and quantitated as described. Cell lysates were also immunoblotted using antibodies for the indicated proteins. B, HEK 293 q/11 K/O cells were transfected with *renilla* luciferase, 8xGTIIc luciferase, and α_q Q209L or α_q AG-Q209L. Cells were treated with increasing concentrations of YM overnight. Lysates were prepared, and luciferase assays were performed and quantitated. C, α_q Q209L or α_q AG-Q209L were cotransfected with *renilla* luciferase and 8xGTIIc luciferase in HEK 293 cells then treated with DMSO or YM overnight. Lysates were prepared, and luciferase assays were performed and quantitated. Cell lysates were also immunoblotted using antibodies for the indicated proteins. In (A and C) results are shown as mean \pm SD ($n = 3$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, two-way ANOVA, Sidak's multiple comparisons test). DMSO, dimethyl sulfoxide.

WT forms of α_q and α_q AG, in HEK 293 cells stably expressing poly-His-tagged β_1 . Previous work has demonstrated that G β_1 associates with α_q Q209L similarly to its association with WT α_q ; the mechanism of such strong interaction of α_q Q209L with G β_1 remains to be fully understood (26, 33). To observe the association of expressed α_q subunits with G β_1 , we used nickel nitrilotriacetic acid (Ni-NTA) magnetic agarose beads to pull down poly-His- β_1 from cell lysates and then immunoblotted for α_q . These β_1 pull-down assays showed that YM treatment promoted a similar increased interaction of both α_q Q209L and α_q AG-Q209L with G β_1 upon YM treatment (Fig. 5A). Additionally, both WT α_q and α_q AG showed an expected increase in association with G β_1 upon YM treatment.

We also interrogated the ability of α_q AG-Q209L to interact with RGS2 and the effect of YM. RGS2 binds to GTP-bound forms of α_q and acts as a GTPase-activating protein (GAP) to stimulate the hydrolysis of GTP. Although the Q209L mutation greatly reduces α_q 's intrinsic and RGS2-stimulated GTPase activity, α_q Q209L is still able to associate with RGS2 (19). However, GDP-bound α_q displays a poor affinity for RGS2, and therefore, reduced association of Q209L mutants with RGS2 in response to YM treatment would be consistent with YM binding and inducing an inactive conformation. To test this, we performed a GST-RGS2 pull-down assay, in which purified GST-RGS2, immobilized on GSH sepharose beads, was incubated with cell lysates containing the expressed α_q subunits (Fig. 5B). The pull-down results showed that both α_q Q209L and α_q AG-Q209L displayed a significant and similar reduction in association with RGS2 after treating the cells with YM.

Similarly, binding to key effector proteins was examined. GST pull downs were performed with the extended PH/DH domain of p63RhoGEF, a direct effector of α_q that stimulates activation of RhoA and downstream signaling (34). In addition, coimmunoprecipitation experiments were performed after coexpression of PLC β -3 with α_q Q209L or α_q AG-Q209L (35). In both assays, α_q Q209L and α_q AG-Q209L were coisolated with the protein domain or effector protein at a similar level (Fig. 5, C and D). Consistent with the GST-RGS2 pull downs (Fig. 5B), treatment with YM reduced the level of α_q Q209L or α_q AG-Q209L that interacted with GST-p63RhoGEF-DH/PHext or PLC β -3. Pull-down and immunoprecipitation experiments provide an important approach for monitoring changes in protein-protein interactions in response to YM; however, we note that protein-protein interactions detected in a cell lysate may not fully reflect the situation in the intact cell. Collectively, these results indicate that although YM fails to inhibit signaling mediated by PM-restricted α_q AG-Q209L, YM is able to bind to and promote an inactive conformation of α_q AG-Q209L.

Signaling by GPCR-activated α_q AG is sensitive to YM

We next wanted to determine if GPCR-dependent signaling by WT PM-restricted α_q is also refractory to inhibition by YM. To test this, we transfected α_q or α_q AG into HEK 293 q/11

Localization-dependent inhibition of α_q Q209L by YM-254890

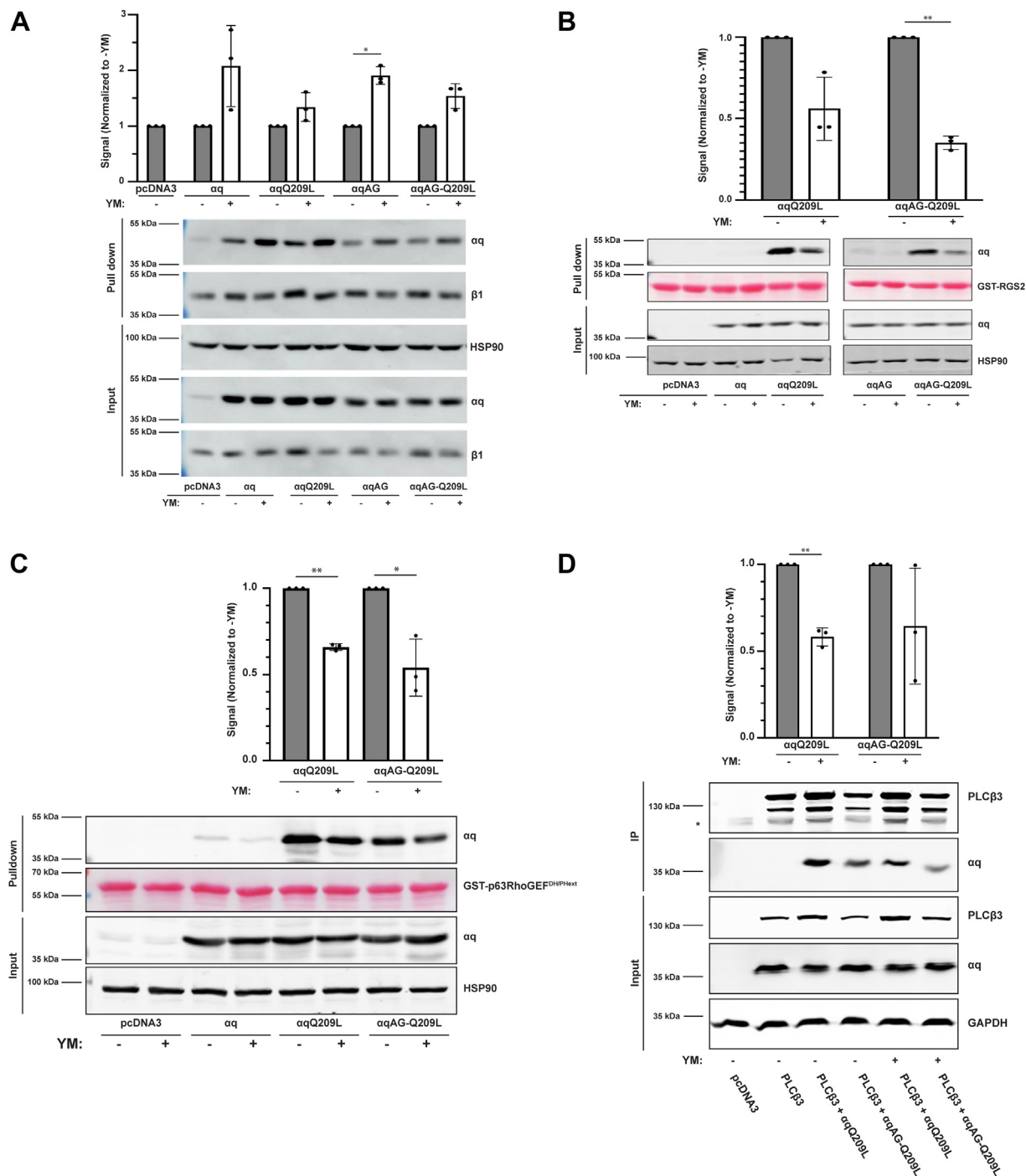


Figure 5. PM-restricted α_q Q209L displays YM-induced conformational changes. A, HEK 293 cells stably expressing His-Myc- β 1 γ 2 were transfected with α_q -pcDNA3, α_q AG-pcDNA3, α_q Q209L-pcDNA3, α_q AG-Q209L-pcDNA3, or pcDNA3 vector alone then treated with 1 μ M YM overnight. A β γ pull-down assay was done after the overnight YM treatment. Pull-down and whole cell lysates were immunoblotted using antibodies for the indicated proteins. α_q signal intensities in the pull down were quantified and normalized to their respective signal intensities in untreated (-YM) cells to quantify the increase in association with β 1 γ 2 after YM treatment. B, HEK 293 q/11 K/O cells were transfected with α_q -pcDNA3, α_q AG-pcDNA3, α_q Q209L-pcDNA3, α_q AG-Q209L-pcDNA3, or pcDNA3 vector alone then treated with 1 μ M YM overnight. A GST-RGS2 pull-down assay was done after the overnight YM treatment. Pull-down and whole cell lysates were immunoblotted using antibodies for the indicated proteins, and GST-RGS2 in the pull down was visualized with Ponceau S staining. α_q Q209L and α_q AG-Q209L signal intensities in the pull-down were quantified and normalized to their respective signal intensities in untreated cells to quantify the decrease in association with RGS2 after YM treatment. C, HEK 293 q/11 K/O cells were transfected and treated with 1 μ M YM as in B, and a GST-p63RhoGEF-DH/PHext pull-down assay was performed. Proteins were visualized and quantitation performed as in (B). D, HEK 293 q/11 K/O cells were transfected with α_q Q209L-pcDNA3, α_q AG-Q209L-pcDNA3, or pcDNA3 together with pcDNA3.1-FLAG-PLC β -3 where indicated. FLAG-PLC β -3 was immunoprecipitated from cell lysates as described under Experimental Procedures. Immunoprecipitates and cell lysates were immunoblotted using antibodies for the indicated proteins. The asterisk in the PLC β -3 IP panel indicates a nonspecific band. Immunoprecipitated α_q Q209L and α_q AG-Q209L signal intensities were divided by the corresponding PLC β -3 signal and then normalized to the respective signal intensities in the untreated sample. Results are shown as mean \pm SD (n = 3; *p < 0.05, **p < 0.01, Student's t test). PM, plasma membrane.

KO cells and then activated the endogenous muscarinic acetylcholine m3 receptor (m3AChR) with 100 μ M carbachol in the presence or absence of pretreatment with YM for 2 h prior to carbachol treatment. Both α_q and α_q AG were able to activate the MAPK pathway within 5 min of carbachol treatment as assessed by immunoblotting for pERK (Fig. 6A). Importantly, treatment with YM abolished carbachol stimulation of the MAPK pathway for both α_q or α_q AG, indicating that signaling by GPCR-activated α_q AG, in contrast to

constitutive signaling by α_q AG-Q209L, is fully inhibited by YM. To further assess the sensitivity of GPCR-activated α_q AG to YM, we used the SRE luciferase reporter and TEAD luciferase reporter assays. Overnight treatment with carbachol resulted in robust stimulation of SRE luciferase and TEAD luciferase activity *via* either α_q and α_q AG (Fig. 6B), and similar to the results with pERK (Fig. 6A), both α_q - and α_q AG-mediated signaling was completely blocked by the addition of YM. Taken together, our results show that the surprising

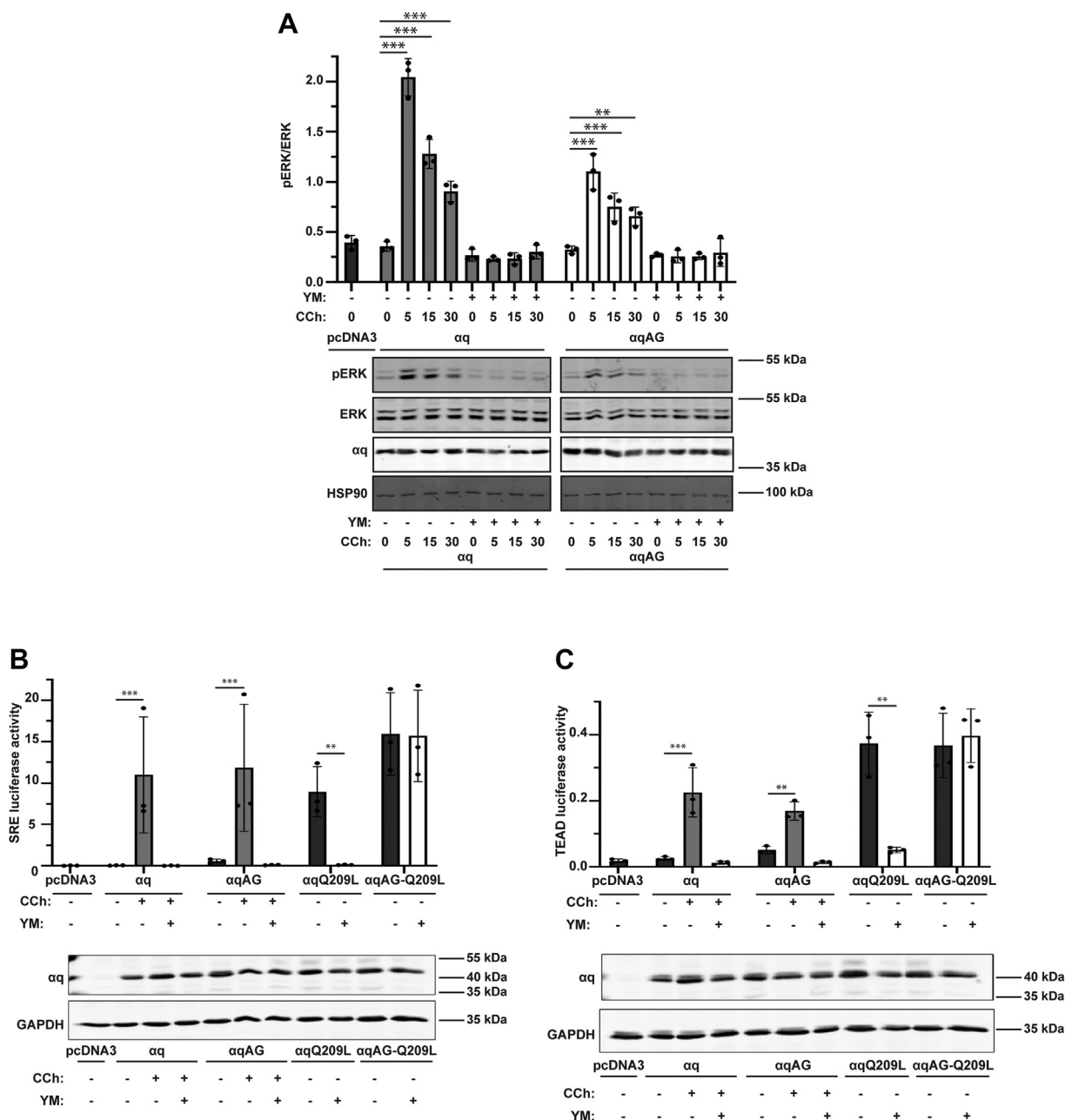


Figure 6. GPCR-activated α_q AG is sensitive to YM. A, HEK 293 q/11 K/O cells were transfected with α_q or α_q AG, then treated with 100 μ M carbachol for 5, 15, or 30 min after 2 h pretreatment with 1 μ M YM or DMSO vehicle control. Cell lysates were immunoblotted using antibodies for the indicated proteins and pERK/ERK signal intensities were quantified. Results are shown as mean \pm SD ($n = 3$; $**p < 0.01$; $***p < 0.001$, two-way ANOVA, Dunnett's multiple comparisons test). B and C, HEK 293 q/11 K/O cells were transfected with α_q -pcDNA3, α_q Q209L-pcDNA3, α_q AG-Q209L, or pcDNA3 alone, along with muscarinic acetylcholine m3 receptor, renilla luciferase, and either pSRE luciferase (B) or 8xGT1C (TEAD) luciferase (C). Cells were then treated with 100 μ M carbachol overnight after a 1 h pretreatment with 1 μ M YM or DMSO vehicle control overnight. Luciferase assays were performed and quantitated as described previously. Cell lysates were immunoblotted using antibodies for the indicated proteins. Results are shown as mean \pm SD ($n = 3$; $**p < 0.01$; $***p < 0.001$, two-way ANOVA, Sidák's multiple comparisons test). DMSO, dimethyl sulfoxide; GPCR, G protein-coupled receptor.

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ability of PM-restricted α_q to resist inhibition by YM is unique to the constitutively active Q209L mutants; GPCR-activated signaling by PM-restricted α_q retains sensitivity to YM.

Discussion

The studies presented here suggest a novel mechanism for inhibition of constitutively active α_q Q209L by the depsipeptide YM-254890 (YM). It is well accepted that YM and the very similar FR900359 (FR) inhibit GPCR activation of α_q by locking α_q in the GDP-bound state (17, 21, 23). In addition to inhibiting classical GPCR activation of α_q , recent work has shown that YM can inhibit signaling and cell proliferation driven by α_q Q209L (18–20). However, constitutively active α_q Q209L is assumed to exist in a GTP-bound active state due to its lack of GTPase activity, and thus an understanding of how YM could similarly trap constitutively active α_q Q209L in an inactive state has remained a challenge. Results herein address this challenge by supporting an additional mechanism for YM inhibition of α_q Q209L. The key findings are that (1) YM promotes subcellular redistribution of α_q Q209L off of the PM and (2) YM fails to inhibit signaling by PM-restricted mutants of α_q Q209L. Our results propose a model in which YM binding promotes the dissociation of α_q Q209L from the PM, and the resulting decreased PM localization prevents α_q Q209L signaling.

Our studies have revealed an unexpected subcellular redistribution of α_q Q209L in response to YM treatment. Importantly, previous work on the trafficking of $G\alpha$ subunits has led to a model in which $G\alpha$ can reversibly shuttle between the PM and internal membranes in both constitutive and activation-dependent manners (29, 30, 36–39). Although it is clear that G proteins can traffic to different locations in the cell, a detailed mechanistic understanding remains to be defined. The main insight into a mechanism is that changes in palmitoylation and depalmitoylation of $G\alpha$ can play a key role in their trafficking (29, 40). In this regard, it was reported that α_q fused to a photoconvertible fluorescent protein could rapidly shuttle between the PM and intracellular membranes and also that blocking palmitoylation of α_q by treatment with 2-bromopalmitate or depletion of specific palmitoyl acyltransferases led to loss of PM localization of α_q (29). Thus, an attractive model for our observation of YM-promoted redistribution of α_q Q209L is that binding of YM by α_q Q209L leads to changes in its palmitoylation status. However, in initial studies, we have not been able to demonstrate consistent changes in palmitoylation of α_q or α_q Q209L when cells are treated with YM. Moreover, our studies so far have not been able to conclusively determine the subcellular localization of α_q Q209L upon translocation from the PM. After YM treatment, α_q Q209L appears diffusely localized throughout the cell with no clear colocalization with intracellular organelles. Intriguingly, cellular fractionation experiments show a small shift of α_q Q209L into a soluble, cytosolic fraction after YM treatment, consistent with a potential mechanism of depalmitoylation (Fig. S2). Future studies will be needed to understand the mechanism of this YM-promoted redistribution of

α_q Q209L; it is currently unclear whether YM binding by α_q Q209L stimulates retrograde movement off of the PM or inhibits anterograde trafficking for return to the PM. To our knowledge, results reported herein are the first demonstration of a small molecule binding to a heterotrimeric G protein subunit that affects its subcellular localization. YM is likely to provide an important tool to help unravel trafficking mechanisms of $G\alpha$.

The most compelling findings in our studies were that simply adding additional membrane binding motifs to the N terminus of α_q Q209L were sufficient to prevent YM from inhibiting signaling by these mutant α_q Q209L. Palmitoylation at cysteines 9 and 10 is essential for PM localization and signaling of WT and constitutively active mutants of α_q , and cycles of enzymatic palmitoylation and depalmitoylation can control the subcellular distribution of α_q proteins. We reasoned that appending additional membrane binding motifs to α_q Q209L would result in a stronger attachment to the PM, and indeed N-terminal fusion of a myristoylation + basic sequence from Src, a myristoylation + palmitoylation sequence from Lyn, or the simple AG mutation to introduce a site of myristoylation resulted in PM-restricted mutants of α_q Q209L that were predominantly localized at the PM and remained at the PM upon YM treatment. Signaling by Src- α_q Q209L, Lyn- α_q Q209L, and α_q AG-Q209L failed to be inhibited by YM. Moreover, we demonstrated that insensitivity to YM was maintained at different concentrations of YM and different expression levels of α_q AG-Q209L (Fig. 4, A and B). A previous study reported an IC₅₀ of 12 nM YM for inhibiting signaling by α_q Q209L (41), consistent with the YM concentration curve in our studies (Fig. 4B). Nonetheless, concentrations of up to 5 μ M YM still failed to inhibit α_q AG-Q209L. Increasing expression of α_q Q209L led to increased signaling, and YM fully inhibited signaling by α_q Q209L at all levels of expression; however, YM was unable to inhibit α_q AG-Q209L signaling at any level of expression (Fig. 4A). Taken together, the failure of YM to inhibit the PM-restricted forms of α_q Q209L supports a model in which translocation off of the PM contributes to the mechanism of YM inhibition of α_q Q209L.

The surprising resistance to YM of PM-restricted mutants was specific to the constitutively active α_q Q209L. In our studies, both WT α_q and WT α_q AG efficiently and similarly coupled carbachol-stimulated endogenous m3AChR or expressed m3AChR to activation of ERK and stimulation of luciferase readouts, respectively. However, in these assays, signaling by both WT α_q and α_q AG were able to be completely inhibited by YM. This sensitivity to YM of GPCR-stimulated signaling by α_q AG was in contrast to our demonstrations that signaling by α_q AG-Q209L was insensitive to YM. These results are consistent with the proposal that there are key mechanistic differences in cells regarding how WT α_q versus GTPase-deficient α_q mutants are inhibited by YM.

A key question that we addressed was whether PM-restricted α_q AG-Q209L is capable of binding YM. Importantly, although our experiments showed that signaling by PM-restricted α_q AG-Q209L is insensitive to YM, our studies determined that YM is still able to bind PM-restricted

α_q AG-Q209L and promote conformational changes similar to those that occur upon YM binding by α_q Q209L. Based on a crystal structure of YM bound to WT α_q and biochemical studies with YM or the highly similar FR900359, the accepted mechanism of action is that YM binds to GDP-bound WT α_q and thereby locks it in the inactive state (19, 21, 23). Moreover, cell-based studies have indicated that FR900359 binding can also promote a conformation in α_q Q209L, consistent with it being in an inactive, GDP-bound form (18, 19). Our studies used pull-down experiments to show that α_q Q209L displayed increased association with G $\beta\gamma$ and decreased association with RGS2, p63RhoGEF-DH/PHex, and PLC β after treatment of cells with YM (Fig. 5), showing that YM can promote an inactive conformation of α_q Q209L, consistent with similar reported experiments using FR900359 (19). Critically, α_q AG-Q209L showed the same YM-dependent changes of interaction with these binding partners as did α_q Q209L. Thus, the failure of YM to inhibit signaling by α_q AG-Q209L cannot be ascribed to an inability of α_q AG-Q209L to bind YM.

How then does signaling by PM-restricted α_q Q209L mutants avoid inhibition by YM? Taken together, our results suggest a model in which YM-promoted subcellular redistribution of α_q Q209L contributes to the mechanism of inhibition, in addition to the already recognized potential of YM to shift α_q subunits into an inactive conformation. In this model, YM binding induces translocation of α_q Q209L off of the PM; the redistributed α_q Q209L is then unable to stimulate effector proteins and signaling pathways. This is supported by reports showing that α_q and constitutively active mutants of α_q completely lack signaling function when PM localization is disrupted (25–29). PM-restricted α_q Q209L mutants, on the other hand, remain associated with the PM in the presence of YM and thus continue to signal, even if bound to YM. Furthermore, our results showing that YM promotes a similar conformation in both α_q Q209L and α_q AG-Q209L raises the possibility that YM binding to constitutively active α_q Q209L and the apparent resulting inactivating conformational change, may not be sufficient for inhibition by YM. Because α_q Q209L lacks GTPase activity, it is assumed that a substantial proportion of α_q Q209L exist in the active, GTP-bound form in cells; however, it is also assumed that YM binds to an inactive, GDP-bound form of α_q Q209L, and thus it remains to be fully understood how YM could rapidly lock enough α_q Q209L in a GDP-bound form to fully inhibit signaling. It is worthwhile to note a recent study of constitutively active α sR201C (42). The authors demonstrated that GDP-bound α sR201C can exist in an active conformation capable of activating the effector adenylyl cyclase, contrary to assumptions that α sR201C would be inactive when bound to GDP. Thus, the results of Hu and Shokat raise the possibility that YM binding to α_q Q209L and the resulting shift to a GDP form, may not be sufficient to generate an inactive α_q Q209L; additional mechanisms, such as subcellular redistribution, may be necessary. Further support for our model is our demonstration that GPCR-stimulated signaling *via* WT α_q or WT α_q AG is efficiently inhibited by YM treatment. These WT α_q are likely to be predominantly in the GDP-bound inactive form when YM is added to cells,

allowing YM to rapidly lock them in an inactive form. Thus, additional mechanisms of YM action are likely not required for inhibition of WT α_q , highlighting the uniqueness of constitutively active α_q Q209L.

An additional model to explain the insensitivity of PM-restricted α_q Q209L mutants is that the addition of N-terminal membrane-binding motifs affects the ability of α_q Q209L to bind key regulatory proteins that may help mediate the inhibitory effect of YM on α_q Q209L. For example, as of yet unidentified proteins may facilitate the inhibitory effect of YM by mediating relocalization of α_q Q209L, but such proteins may not effectively interact with PM-restricted α_q Q209L. Likewise, other proteins may prevent the inhibitory effect of YM by maintaining signaling function of PM-restricted α_q Q209L. In this regard, a recent report argued that the proportion of α_q Q209L already bound to the effector PLC β would be refractory to inhibition by FR, an inhibitor almost identical to YM; because α_q Q209L would not be susceptible to the GTPase accelerating protein (GAP) activity of PLC β , the α_q Q209L-PLC β complex would be longer lived than an α_q -PLC β complex and consequently inaccessible to the inhibitor FR or YM (20). In this model, we can speculate that a PM-restricted α_q Q209L, with tighter membrane binding than α_q Q209L, may exist in a membrane-bound complex with PLC β and other proteins that is more difficult to disrupt and thus less able to be inhibited by YM. However, in contrast to this idea, our immunoprecipitation studies failed to show any increase in association of α_q AG-Q209L with PLC β -3 compared to α_q Q209L association with PLC β -3 (Fig. 5D). Nonetheless, we cannot rule out that in the intact cell α_q AG-Q209L, compared to α_q Q209L, is more strongly associated with PLC β -3 or other interacting proteins. Future studies to identify novel proteins that regulate inhibition of α_q Q209L and structural studies to understand how YM and/or GDP binding affects α_q Q209L are needed to more fully understand YM inhibition of constitutively active α_q mutants.

In summary, our studies show that YM promotes a change in localization of α_q Q209L from the PM to cytoplasm and that restricting α_q Q209L to the PM results in a loss of inhibition by YM, suggesting that this change in localization is important for YM's mechanism of action. The mutation of Q209L in α_q or the highly identical α 11, is the major oncogenic driver mutation in uveal melanoma. A deeper understanding of how the depsipeptide inhibitor YM inhibits α_q Q209L will guide the development of new inhibitors and potentially reveal new therapeutic targets.

Experimental procedures

Reagents, antibodies, and plasmids

YM-254890 (catalog no.: # 257-00631) was purchased from Wako Chemicals USA Inc. DMSO (catalog no.: # BP231-1) was purchased from Fisher. HEK 293 cells and HEK293 α_q /11 K/O cells were cultured in Dulbecco's modified Eagle's medium (Corning, catalog no.: # 10-017-CV) containing 10% fetal bovine serum (Gemini, catalog no.: 900-108) and 1 \times penicillin/streptomycin solution (Sigma, catalog no.: # P4333).

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All other cell culture plates and materials were from GenClone, Costar, or Fisher. Lipofectamine 2000 (catalog no.: # 11668-019), used for all transient transfections, was obtained from Invitrogen. Ponceau S (catalog no.: # P-3504) and carbachol were obtained from Sigma–Aldrich.

The following primary antibodies were used. The GAPDH (catalog no.: # 60004-1-Ig) and α_q (catalog no.: #13927-1-AP) antibodies were purchased from ProteinTech. This α_q antibody (catalog no.: #13927-1-AP) was used for all immunofluorescence microscopy experiments together with a GRK4-6, clone A16/17 (catalog no.: #05-466) antibody, which was obtained from Sigma–Aldrich. An additional α_q antibody (catalog no.: # ab199533) was purchased from Abcam and was used for all immunoblotting experiments. The HSP90 antibody (catalog no.: # sc-7947) was obtained from Santa Cruz Biotechnology. The pERK1/2 (catalog no.: #9101S) and ERK1/2 (catalog no.: #4696S) antibodies were obtained from Cell Signaling Technologies. The anti-myc tag antibody, clone 9E1 (catalog no.: # 05-419) was obtained from Sigma–Aldrich. For immunofluorescence microscopy, the secondary goat anti-rabbit Alexa Fluor 488 (catalog no.: # A-11034) and goat antimouse Alexa Fluor 594 (catalog no.: # A-11032) conjugated secondary antibodies were obtained from Invitrogen. The secondary LI-COR antibodies, IRDye 800CW donkey antimouse IgG (H + L) (catalog no.: # 92532212), and IRDye 680RD goat anti-rabbit IgG (H + L) (catalog no.: #92568071) were purchased from LI-COR and were used to visualize protein on immunoblots. Alternatively for immunoblotting, horseradish peroxidase-conjugated secondary antibodies (catalog no.: # PR-W4011 and PR-W4021) were used from Promega.

HA-tagged α_q , α_q Q209L, α_q AG, and α_q AGQ209L in pcDNA3 were described previously (25–27). Nontagged human α_q (catalog no.: # GNA0Q00000) and α_q Q209L (catalog no.: # GNA0Q000C0) in pcDNA3.1 were received from the complementary DNA Resource Center. Src- α_q Q209L and Lyn- α_q Q209L in pcDNA3 were generated using synthetic DNA (GenScript) and subcloning into HA-tagged α_q Q209L-pcDNA3 and resulted in coding sequences for amino acids 1 to 16 of Src and 1 to 11 of Lyn, respectively, followed by DNA coding for the linker Gly-Thr-Gly-Gly-Ser-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Ser-Gly-Ala and then fusion to Thr2 of α_q Q209L. pmCherry(N1)-GRK5, in which mCherry is fused to the C terminus of GRK5 was described previously (31), and the bacteria expression plasmid for GST-RGS2 has been described (43, 44). A bacteria expression plasmid for GST-p63RhoGEF-DH/PHext and pcDNA3.1-FLAG-PLC β -3 were generously provided by Mikel Garcia-Marcos (Boston University).

Cell lines

HEK 293 cells were received from ATCC. HEK 293 q/11 K/O cells have been described (17). HEK 293 cells stably expressing myc-His-tagged β 1 and γ 2 were described previously (26). The cells were maintained in HEK293 cell media plus 0.5 mg/ml G418. For generation of HEK 293 cells expressing tetracycline-induced α_q and α_q Q209L, HEK

293 cells stably expressing an integrated Flp Recombination Target (FRT) site and Tet repressor (TR) (Flp-In T-REx HEK 293 cells) were received from Diane Merry (Jefferson) and were cultured in HEK 293 media plus 15 μ g/ml blasticidin and 100 μ g/ml zeocin. HA-tagged α_q and α_q Q209L complementary DNA in a pcDNA3 vector backbone were cut using the restriction endonucleases Hind III and Apa I and ligated into the pcDNA5/FRT/TO expression vector, which contained a hybrid human cytomegalovirus (CMV)/TetO₂ promoter for tetracycline-regulated expression of α_q or α_q Q209L, FRT site for Flp recombinase-mediated integration of the vector into the Flp-In T-REx HEK 293 cells, and a hygromycin resistance gene for selection. The HEK 293 cells were transfected with 2 μ g of HA-tagged α_q -pcDNA5/FRT/TO or α_q Q209L-pcDNA5/FRT/TO plus 13 μ g of POGG44 flip recombinase. HEK 293 cells with integrated α_q or α_q Q209L were selected by using HEK 293 media plus 15 μ g/ml blasticidin and 100 μ g/ml hygromycin and single colonies were expanded under blasticidin and hygromycin selection. α_q and α_q Q209L Flp-In T-REx HEK293 cell clones were validated by observation of equal expression across cells by immunofluorescence upon 100 nM tetracycline treatment and nuclear YAP and increased pERK upon tetracycline treatment in α_q Q209L Flp-In T-REx HEK 293 cells.

Immunofluorescence microscopy

Immunofluorescence microscopy experiments involved coexpression of α_q or α_q Q209L and GRK5-mCherry-N1, which localizes at the PM and does not interact with α_q (31, 45). α_q and α_q Q209L Flp-In T-REx HEK 293 cells were initially transfected with GRK5-mCherry-N1 before tetracycline treatment then reseeded onto 6-well plates with coverslips 24 h after transfection. About 100 ng/ml tetracycline was then used to induce expression of α_q and α_q Q209L. The cells were then treated with DMSO or 1 μ M YM in DMSO overnight or for 1 h prior to fixing. To detect localization of Src- α_q Q209L or Lyn- α_q Q209L HEK 293 q/11 K/O cells were seeded onto poly-L-lysine-coated coverslips and transfected with Src- α_q Q209L or Lyn- α_q Q209L plus GRK5-mCherry-N1. Forty-eight hours after transfection, the cells were treated with YM for 1 h then fixed. All cells were fixed with 3.7% formaldehyde in PBS for 15 min, then washed three times with PBS. The cells were then blocked and permeabilized by incubation with 2.5% milk and 1% Triton X-100 in tris-buffered saline (TBS) for 20 min. Coverslips were then incubated with an anti-rabbit α_q antibody and antimouse GRK 4 to 6 antibody in 2.5% milk and 1% Triton X-100 in TBS for 1 h. The cells were then washed five times in 2.5% milk and 1% Triton X-100 in TBS then incubated with goat anti-rabbit Alexa Fluor 488 and goat antimouse Alexa Fluor 594 secondary antibodies in 1% Triton X-100 in TBS for 30 min. After five washes in 1% Triton X-100 in TBS, the cells were rinsed in distilled water and mounted onto glass slides with ProLong Diamond Anti-fade Mountant (Invitrogen, catalog no.: # P36970). Images were acquired on an Olympus IX83 microscope with a 60 \times oil immersion objective and an ORCA Fusion sCMOS camera (Hamamatsu)

controlled by cellSense (Olympus software). Images were subjected to constrained iterative deconvolution to remove background fluorescence. Hundred tetracycline-induced α_q and α_q Q209L Flp-In HEK 293 cells under each treatment were counted and scored as either PM localized with little to no observable staining in the cytoplasm, PM, and cytoplasmic localization in which individual cells displayed varying degrees of a partial PM stain and observable cytoplasmic localization of α_q or cytoplasmic in which α_q was distributed throughout the cytoplasm but had no observable PM localization.

Dual luciferase assay

HEK 293 α_q /11 K/O cells were cotransfected with *Renilla* luciferase and either pSRE-luciferase or the synthetic TEAD reporter 8xGTIIC-luciferase along with the respective α_q construct. 8xGTIIC-luciferase was received from Stefano Piccolo (Addgene plasmid # 34615) and pSRE-luciferase was described previously (46). For experiments using constitutively active mutants, the media was changed to serum-free media plus 1 μ M YM or DMSO vehicle control 2 h after transfection, then lysed approximately 16 h after transfection. For experiments involving GPCR-activated α_q , the cells were treated with 1 μ M YM or DMSO vehicle control 2 h after transfection, treated with 100 μ M carbachol or vehicle control 1 h after DMSO or YM treatment, and then lysed approximately 16 h after transfection. The cells were lysed in 1 \times passive lysis buffer and luciferase activity was detected using the Dual-Luciferase Reporter Assay System kit (Promega, catalog no.: # E1960) according to the manufacturer's directions, using an opaque white 96-well plate and GloMax Explorer luminometer.

$\beta\gamma$ pull-down assay

The $\beta\gamma$ pull-down assay was done as described in (26). Briefly, HEK 293 cells stably expressing $\beta_1\gamma_2$ were transiently transfected with HA-tagged α_q -pcDNA3, α_q Q209L-pcDNA3, α_q AG-pcDNA3, α_q AG-Q209L-pcDNA3, or pcDNA3 alone. Twenty-four hours after transfection, the cells were treated with 1 μ M YM or DMSO vehicle control overnight. The cells were then washed in PBS and lysed in 500 μ l of lysis buffer C (20 mM Hepes pH 7.5, 100 mM NaCl, 0.7% Triton X-100, 5 mM MgCl₂, and 1 mM EDTA supplemented with the protease inhibitors 1 mM PMSE, 2 μ g/ml leupeptin, and 2 μ g/ml aprotinin, and 0.1 \times cOmplete mini protease inhibitor cocktail). The lysates were incubated for 1 h on ice then centrifuged at 13,000 rpm (10,000g) for 3 min to pellet the nuclei and insoluble material. Forty microliters of the supernatant was reserved for the input, and 20 μ l of Ni-NTA magnetic beads (New England Biolabs, catalog no.: #S1423S) were added to a new tube with the remaining supernatant. The tubes were rotated e-o-e for 2 h at 4 °C. The beads were then pelleted by centrifugation for 1 min, then the supernatant was aspirated while the beads were pelleted on a magnetic rack. The beads were washed three times in lysis buffer C to remove nonspecific proteins. Fifty microliters of elution buffer (0.25 M imidazole in lysis buffer C) was added to each sample to elute poly-His β_1 and any associated proteins. SDS-PAGE sample

buffer with 1% β -mercaptoethanol were added to the Ni-NTA beads and the whole cell lysates. The pull down and input were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with an α_q (Abcam, catalog no.: # ab199533) or an anti-myc monoclonal antibody (9E10) to detect β_1 or HSP90 antibody as a loading control. The bands were visualized by chemiluminescence and subjected to densitometry. Each sample was normalized to its respective DMSO treatment.

GST-RGS2 and GST-p63RhoGEF-DH/PHext pull-down assays

The GST-RGS2 and GST-p63RhoGEF-DH/PHext pull-down assays was performed as described in (35, 43). Briefly, GST-RGS2 and GST-p63RhoGEF-DH/PHext was expressed in BL-21 cells and BL-21(DE3) cells, respectively. GST-RGS2 expression was induced with 0.5 mM IPTG for 3 h at 25 °C and GST-p63RhoGEF-DH/PHext was induced with 1 mM IPTG overnight at 18 °C. The GST fusion proteins were then purified using GSH-Sepharose 4B beads (GE Healthcare, catalog no.: # 17-0765-01) as described (35, 44). HEK 293 q/11 K/O cells were transfected with pcDNA3, HA-tagged α_q -pcDNA3 or α_q Q209L-pcDNA3, or α_q AG-Q209L-pcDNA3. Twenty-four hours after transfection, the cells were treated with 1 μ M YM or DMSO vehicle control overnight. The cells were then washed with cold PBS and lysed in 300 μ l of lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 100 nM NaCl, 5 mM MgCl₂, 0.7% Triton X-100, 1 mM PMSF, and 5 μ g/ml leupeptin and aprotinin). The cells were lysed on ice for 1 h, then centrifuged for 3 min at full speed to pellet the nuclei and insoluble material. About 50 μ l of the supernatant was reserved for the input and the remaining supernatant was added to a new tube with 8 μ g of GST-RGS2 or GST-p63RhoGEF-DH/PHext prebound to GSH Sepharose beads and rotated e-o-e at 4 °C for 1 h. After incubation with GST-RGS2 or GST-p63RhoGEF-DH/PHext, the samples were pelleted at 13,000 rpm (10,000g) for 1 min, the flow through was removed and the beads were washed three times in lysis buffer. The proteins were eluted from the beads in 50 μ l of SDS sample buffer plus 1% β -mercaptoethanol and the protein was separated by SDS-PAGE and transferred onto nitrocellulose membrane then probed with an antibody detecting α_q (Abcam) or HSP90 as a loading control. GST-RGS2 and GST-p63RhoGEF-DH/PHext were detected by staining the membrane with Ponceau S. The bands were visualized using LI-COR secondary antibodies and subjected to densitometry. Each sample was normalized to its respective DMSO treatment.

PLC β -3 immunoprecipitation

Experiments were performed similarly to a recent report (35). HEK 293 q/11 KO cells were transiently transfected with FLAG-tagged PLC β -3 and α_q Q209L-pcDNA3 or α_q AG-Q209L-pcDNA3. FLAG-tagged PLC β -3 alone and pcDNA3 alone were transfected as positive and negative controls, respectively. Three hours after transfection, media was changed and respective plates were treated with 1 μ M YM overnight. The cells were washed with cold PBS and lysed in

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500 μ l of lysis buffer C (20 mM Hepes pH 7.5, 100 mM NaCl, 0.7% Triton X-100, 5 mM MgCl₂, and 1 mM EDTA supplemented with 1 mM DTT, 1 mM PMSF, 2 μ g/ml leupeptin, and 2 μ g/ml aprotinin, and 0.1 \times cOmplete mini protease inhibitor cocktail). Lysates were incubated for 30 min on ice and then centrifuged at 13,000 rpm for 10 min. Fifty microliters of the supernatant was reserved for the input, while the remaining supernatant was added to a new tube containing 40 μ l of EZview Red ANTI-FLAG M2 Affinity Gel bead slurry (Sigma—Aldrich, catalog no.: # F2426). Lysates and beads were rotated e-o-e for 2 h at 4 °C. The beads were then pelleted by centrifugation for 1 min. Supernatant was removed, and the beads were washed three times with lysis buffer C. Protein was eluted from the beads in 60 μ l of 1 \times SDS sample buffer plus 1% β -mercaptoethanol. Pull-down and input samples were run on 10% SDS-PAGE gels, transferred onto nitrocellulose membrane, and probed with antibodies detecting PLC β -3 (Sigma—Aldrich ANTI-FLAG M2, catalog no.: # F1804), α_q (ProteinTech, catalog no.: #13927-1-AP), and GAPDH as a loading control. The bands were visualized using LI-COR secondary antibodies and subjected to densitometry. Coimmunoprecipitated α_q was normalized to the amount of PLC β -3 detected in the immunoprecipitate.

pERK/ERK analysis

Protein lysates were run on 10% SDS-PAGE gels then transferred onto nitrocellulose membranes. The membranes were then probed for with a rabbit polyclonal pERK antibody (catalog no.: # 9101S) or a mouse monoclonal ERK antibody (catalog no.: #4696S). A duplicate membrane was probed with an antibody detecting α_q (Abcam) or GAPDH. LI-COR secondary antibodies were used to visualize the protein bands. The pERK and ERK bands were subjected to densitometry and the signal obtained from the pERK bands were divided by the signal detected from ERK. All signals were normalized to 0 h YM treatment to normalize between experiments.

Western blotting and quantification

All protein lysates were run on 10% SDS-PAGE gels and transferred onto nitrocellulose, blocked in 2.5% bovine serum albumin (BSA) or 5% milk in TBS plus 0.05% Tween (TBS-Tween), incubated with primary antibodies overnight in 2.5% BSA or 5% milk in TBS-Tween, washed three times in TBS-Tween, incubated with secondary antibodies in 2.5% BSA or 5% milk in TBS-Tween for 1 h, and washed three times in TBS-Tween. Membranes blotted with LI-COR secondary antibodies were then washed once in PBS then visualized on a LI-COR Odyssey. Membranes blotted with horseradish peroxidase-conjugated secondary antibodies were incubated with SuperSignal West Dura Extended Duration Substrate (catalog no.: # 34075) from Thermo Scientific Dura then imaged on an Amersham Imager 680 (GE Healthcare).

Statistical analysis

All figures were analyzed using GraphPad Prism (GraphPad Software Inc). A two-way ANOVA followed by multiple

comparison's testing was used to calculate significance. Multiple comparison testing was performed using Tukey's test when all groups were compared with all other groups, and a Dunnett's test was used when all groups were compared with a single control group. Šidák's test was performed when DMSO *versus* YM treatments were compared as opposed to Tukey's test, which was used to compare all groups with all other groups. The Fisher's least significant differences test was used for the cell count comparison in [Figures 1B](#) and [S1](#) and the cell fractionation in [Fig. S2](#) to compare all groups without correcting for multiple comparisons. *t* tests were used in [Figure 5](#) to compare to DMSO treatments set at 1. In all experiments, error bars indicate mean \pm SD with significant differences indicated as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Data availability

All data is contained within the paper.

Supporting information—This article contains supporting information.

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Abbreviations—The abbreviations used are: BSA, bovine serum albumin; DAG, diaacylglycerol; DMSO, dimethyl sulfoxide; GPCR, G

protein-coupled receptor; Ni-NTA, nickel nitrilotriacetic acid; PM, plasma membrane.

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