



Published in final edited form as:

Sci Signal. 2024 February 13; 17(823): eadd9139. doi:10.1126/scisignal.add9139.

GPCR kinases differentially modulate biased signaling downstream of CXCR3 depending on their subcellular localization

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Abstract

Some G protein–coupled receptors (GPCRs) demonstrate biased signaling, such that ligands of the same receptor exclusively or preferentially activate certain downstream signaling pathways over others. This phenomenon may result from ligand-specific receptor phosphorylation by GPCR kinases (GRKs). GPCR signaling can also exhibit location bias because GPCRs traffic to and signal from subcellular compartments in addition to the plasma membrane. Here, we investigated whether GRKs contributed to location bias in GPCR signaling. GRKs translocated to endosomes following stimulation of the chemokine receptor CXCR3 or other GPCRs in cultured cells. GRK2, GRK3, GRK5, and GRK6 showed distinct patterns of recruitment to the plasma membrane and to endosomes, depending on the identity of the biased ligand used to activate CXCR3. Analysis of engineered forms of GRKs that localized to either the plasma membrane or endosomes demonstrated that biased CXCR3 ligands elicited different signaling profiles that depended upon the subcellular location of the GRK. Each GRK exerted a distinct effect on the regulation of CXCR3 engagement of β -arrestin, internalization, and activation of the downstream effector kinase ERK. Our work highlights a role for GRKs in location-biased GPCR signaling

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Methodology, J.G., D.S.E., C.H., O.M., S.R.

Investigation, J.G., D.S.E., C.H., I.C., U.P., A.C.

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Writing – Reviewing & Editing, J.G., D.S.E., C.H., N.B., C.C.H., U.P., A.C., and S.R.

Visualization, J.G., D.S.E., C.H. and S.R.

Supervision and Funding Acquisition, S.R.

COMPETING INTERESTS

The authors declare that they have no competing interests.

and demonstrates the complex interactions between ligands, GRKs, and cellular location that contribute to biased signaling.

INTRODUCTION

G protein–coupled receptors (GPCRs) represent the largest and most versatile class of receptors in humans and can elicit cellular responses to various stimuli, including photons, odorants, peptides, lipids, and small molecules (1). Due to the extensive involvement of GPCRs in normal physiology and pathophysiology, approximately 35% of all U. S. Food and Drug Administration–approved drugs target one or more of the 800 human GPCRs (2, 3). GPCRs engage an array of signaling effectors to actuate a cellular response, such as G proteins, GPCR kinases (GRKs), and β -arrestins (4). Ligand binding to plasma membrane–embedded GPCRs promotes a conformational change in the receptor that induces the activation of G proteins and subsequent G protein–dependent signaling (5, 6). The GRKs and other kinases then phosphorylate the intracellular surface of the receptor, promoting the binding of β -arrestins, which can desensitize receptor coupling to G proteins, induce receptor internalization into clathrin-coated pits, and act as scaffolds for other signaling effectors, including mitogen activated protein kinases (MAPKs) and E3 ubiquitin ligases (7, 8). Some ligand:GPCR complexes preferentially activate distinct signaling pathways over others, a phenomenon referred to as biased signaling (9–11).

One proposed mechanism for biased signaling, the phosphorylation barcode hypothesis, posits that unique ligand-induced GPCR phosphorylation patterns promote selective engagement with specific signaling effectors, thereby producing functionally selective outputs (12–14). Many studies demonstrate the critical role the phosphorylation barcode plays in driving specific β -arrestin recruitment and conformational signatures, ultimately promoting unique downstream signaling (15–18). Despite the critical function of GRKs in receptor phosphorylation, the role of the GRKs in promoting biased signaling has not been widely studied. The seven identified mammalian GRKs (1–7) are divided into three subfamilies according to sequence and structure (19), wherein GRKs 1 and 7 comprise the GRK1 subfamily, GRKs 2 and 3 the GRK2 subfamily, and GRKs 4, 5, and 6 the GRK4 subfamily (20, 21). GRKs 2, 3, 5, and 6 are ubiquitously expressed in human tissues (22), and it has been demonstrated that each of these GRKs performs a unique signaling role at GPCRs (23). Although no clear consensus target sequence has been identified for specific GRKs (24), GRKs have a strong preference for nearby phosphorylated residues, and GPCR phosphorylation may ultimately be determined by other serine, threonine, and tyrosine kinases that initially phosphorylate the receptor and subsequent phosphorylation by different GRKs (25). GRKs 2 and 3 are localized to the cytoplasm, and their ligand-induced engagement with a GPCR depends upon interaction of the GRK pleckstrin homology (PH) domain with free $G\beta\gamma$ subunit (26), whereas GRKs 5 and 6 are constitutively present on the plasma membrane (27, 28). GPCRs demonstrate heterogeneity in the putative phosphorylation patterns present on intracellular loop 3 (ICL3) and at the C-terminus (29), and it has been difficult to experimentally delineate the specific contributions of individual GRKs to the generation of unique GPCR phosphorylation patterns and subsequent receptor signaling (30). Certain residues on numerous GPCRs are specifically targeted by individual

GRKs, but other sites are phosphorylated by multiple GRKs (13, 31, 32). Differences in the relative amounts of GRKs in various tissues has also been proposed as a means of attaining GRK-specific functionality. For example, GRK5 and GRK6 are present in similar amounts in B- and T-cells, yet GRK5 is the predominant family member present in cardiac tissue (24). Although distinct structural elements, phosphosite targets, and tissue-specific differences in abundance have helped understand GRK specificity, further studies are needed to fully resolve this complex system.

It has been demonstrated that GPCRs can signal from numerous subcellular locations beyond the plasma membrane, including endosomes, mitochondria, the Golgi apparatus, and the endoplasmic reticulum (33–44). We previously demonstrated that biased ligands of the chemokine receptor CXCR3 induce distinct patterns of G protein activation and β -arrestin recruitment at endosomes compared to the plasma membrane, and CXCR3 internalization contributes to the overall biased cellular output of this receptor (45). Given the critical role of receptor phosphorylation in biased GPCR signaling and previous evidence that GRKs can exist in specific subcellular compartments (46–50), it is likely that the GRKs play a substantial role in generating functionally selective responses in different cellular locations. Yet, to our knowledge, few experiments have assessed the specific effects of GRK engagement with GPCRs in endosomes on biased signaling.

Here, we show that biased CXCR3 agonists differentially promoted GRK engagement with CXCR3 at the plasma membrane. We also provide evidence demonstrating that some ligands promoted GRK translocation to endosomes in a pattern entirely distinct from that observed at the plasma membrane. We further show that individual GRKs exhibited distinct effects on CXCR3 signaling depending on the biased ligands used to activate the receptor. We demonstrated that the association of GRKs 2 and 3 with CXCR3 was largely dependent upon the activation of G proteins, but some ligands enabled G protein-independent recruitment. Each GRK demonstrated unique roles in regulating the receptor's ability to engage β -arrestins, become internalized, and activate extracellular signal-regulated kinase (ERK). Using engineered forms of GRKs that localize to specific subcellular locations, we showed that GRK identity and subcellular localization modulated GPCR signaling depending on the ligand used to activate the receptor. Lastly, we demonstrated that GRK recruitment to endosomes was not unique to CXCR3 but was observed for a panel of other therapeutically relevant, well-studied GPCRs, suggesting that location-specific GRK activity occurs across the GPCR superfamily. These findings suggest that subcellular engagement of GRKs with GPCRs may facilitate location-specific signaling responses and that the role of individual GRKs is differentially directed by specific ligands. These data demonstrate a complex interaction between GRK family members, ligands, and cellular locations in driving a GPCR's overall biased signaling outputs.

RESULTS

Biased ligands elicit different GRK recruitment patterns to the plasma membrane and to CXCR3

CXCR3 is a chemokine receptor with endogenous ligands that exhibit biased signaling in various forms, such as in their differential formation of $G\alpha i:\beta$ -arrestin complexes and

markedly different abilities to induce G protein- or β -arrestin-mediated signaling (51–54). CXCR3 is primarily found on activated CD8⁺ T cells and natural killer cells, directs cellular functions like chemotaxis and T cell polarization, and is implicated in inflammatory conditions such as cancer, atherosclerosis, autoimmunity, and allergic contact dermatitis (11). We first determined if activation of CXCR3 using biased ligands promoted distinct patterns of GRK recruitment to the plasma membrane in HEK293 cells. Using a previously validated NanoLuc binary technology (nanoBiT) complementation assay based on the generation of active luciferase by the association of small BiT (SmBiT) and large BiT (LgBiT) subunits with one another (55), we monitored the interaction between the plasma membrane marker CD8 α -SmBiT and GRK2-, GRK3-, GRK5-, or GRK6-LgBiT following stimulation of untagged CXCR3 (Fig. 1A). Using confocal microscopy in HEK293T cells, we demonstrated colocalization of CD8 α -mCherry and CXCR3-mCerulean, consistent with CD8 α and CXCR3 residing in the same subdomains within the plasma membrane (fig. S1). We used three endogenous, biased CXCR3 agonists, CXCL9, CXCL10, and CXCL11, and two synthetic CXCR3 agonists, VUF10661 and VUF11418, for the nanoBiT complementation experiments (56). Previous publications measuring relative amounts of G protein signaling and β -arrestin recruitment demonstrate that CXCL11 is relatively β -arrestin-biased, whereas CXCL10, VUF10661, and VUF11418 are relatively G protein-biased. CXCL9 acts as a β -arrestin-biased partial agonist (52, 57).

After 5 minutes, the recruitment patterns of GRK2 and GRK3 were similar to each other, with differences in efficacy and potency between the biased ligands (Fig. 1B). Specifically, CXCL11 and VUF10661 were the most efficacious agonists followed by CXCL10 and VUF11418. CXCL9 evoked little to no detectable recruitment of GRK2 or GRK3 at physiologic concentrations of the chemokine. GRK2 and GRK3 are known to localize differently from GRK5 and GRK6 in the cell at baseline, and we performed confocal microscopy using YFP-tagged GRKs and CXCR3-mCerulean to confirm cytosolic localization of GRK2 and GRK3 and constitutive membrane localization of GRK5 and GRK6 (58–60) (fig. S2). Thus, ligand-induced recruitment to the plasma membrane was not necessarily an illustrative readout of GRK5 and GRK6 activity, as evinced by the lack of detectable additional recruitment of these GRKs over baseline by physiological chemokine concentrations in our assay (Fig. 1B).

In analyzing the kinetic data of GRK recruitment to the plasma membrane in response to 100nM CXCL9, CXCL10, or CXCL11 and 10 μ M VUF10661 or VUF11418, we observed significant differences across ligands. All ligands, except CXCL9, elicited rapid recruitment of GRK2 and GRK3 to the plasma membrane upon receptor activation (Fig. 1C). However, at 30 minutes, all ligands except VUF11418, show a marked decrease in luminescence. Given that most ligands induced maximum GRK recruitment to the plasma membrane at 5 minutes, we elected to show the concentration-response data at this time point (Figs. 1B). These data demonstrate that the ligands not only promoted different magnitudes of GRK2 and GRK3 translocation but also produced unique kinetic signatures for the interaction of individual GRKs with the plasma membrane. Differential GRK recruitment magnitude and kinetics may contribute to the generation of specific phosphorylation barcodes and biased signaling.

When the same experiment was performed using CXCR3-SmBiT to assess GRK recruitment to the receptor, we observed similar concentration-response curves to those seen for plasma membrane recruitment for all GRKs at 5 minutes after stimulation (Fig. 1, D and E). Unexpectedly, when analyzing GRK recruitment over time, we observed an increase in the luminescence signal at 30 minutes for all four GRKs in response to the synthetic ligands but not to the endogenous chemokines (Fig. 1F). For GRK2 and GRK3, following an initial robust phase of GRK recruitment induced by the synthetic ligands, there was a slight decrease in luminescence at 10 minutes, followed by an increase in luminescence at 30 minutes (Fig. 1F). CXCL10 and CXCL11 elicited sustained interaction of GRKs with the receptor even at 30 minutes (Fig. 1F), whereas the luminescence signal returned to baseline at this time when measuring GRK recruitment to the plasma membrane (Fig. 1C). By contrast, GRK5 and GRK6 showed little initial detectable increase in luminescence signal following stimulation with VUF10661 and VUF11418 but demonstrated a very slow and late increase after 10 minutes (Figs. 1F).

GRK recruitment to endosomes differs from recruitment to the plasma membrane

Based on the unexpected kinetic tracings of GRK recruitment to CXCR3 compared to the plasma membrane, we hypothesized this data may reflect GRKs interacting with the receptor at subcellular locations in addition to or other than the plasma membrane. To evaluate the potential recruitment of the GRKs to endosomes, we repeated the NanoBiT complementation assays using the GRK-LgBiT constructs, wild-type (WT) CXCR3, and the previously validated early endosome marker 2x-Fyve tagged with SmBiT (61, 62) (Fig. 2A). We observed that GRK2, GRK3, and GRK5 were recruited to endosomes, with VUF10661 exhibiting the most robust recruitment of these GRKs (Fig. 2B). We also observed detectable recruitment of GRK3 to endosomes using CXCL11, VUF11418, and CXCL10. The overall pattern of ligand-induced GRK recruitment to endosomes substantially differed from the recruitment pattern observed at the plasma membrane. GRK recruitment to endosomes was significantly slower, consistent with previously reported kinetic data on GPCR internalization (63, 64).

To support these findings using confocal microscopy, we transfected HEK293 cells with the GRK-YFP and CXCR3-mCerulean and stimulated cells with agonist for 45 minutes (figs. S3, A and B; S4, A and B; S5, A to E). The 45-minute time point was chosen because this is when maximal CXCR3 internalization was observed using confocal microscopy; however, CXCR3 and GRKs were detected in intracellular puncta as early as 15 minutes following agonist stimulation, consistent with our luminescence data (fig. S6, A and B). These data are consistent with many other reports that demonstrate GPCR localization to early endosomes is dynamic and can occur within a few minutes following agonist stimulation but may last for up to 90 minutes (63, 65–67). Although all ligands promoted receptor internalization, we only observed the formation of intracellular puncta that were both YFP⁺ and mCerulean⁺, as supported by a line scan analysis, suggestive of colocalized receptor and GRK in endosomes, for some ligand:GRK combinations, consistent with our luminescence data (figs. S3, A and B; S4, A and B; S5, A to E). Of note, we observed colocalization of GRK3-YFP and CXCR3-mCerulean following treatment with CXCL11 but not with CXCL10 nor CXCL9 (fig. S3B).

In contrast, following treatment with VUF10661, we observed robust colocalization of CXCR3-mCerulean with GRK2-YFP, GRK3-YFP, and to a lesser extent GRK5-YFP. To more rigorously demonstrate colocalization of GRKs and CXCR3 in endosomes, we repeated the confocal experiment with co-transfection of the endosomal marker EEA1-dsRed following VUF10661 treatment (Fig. 2, C to F). Consistent with our previous confocal experiment, we observed colocalization of GRK2-YFP, GRK3-YFP, and GRK5-YFP with CXCR3-mCerulean and EEA1-dsRed. These findings suggest that ligands may direct specific activities of the individual GRKs through selective trafficking of certain GRKs to endosomes.

GRK2 and GRK3 recruitment to CXCR3 largely depends on G protein activation for some, but not all ligands

A primary feature distinguishing the GRK2 and GRK4 subfamilies is that GRK2 and GRK3 recruitment to a GPCR rely on interactions of their PH domain with free G $\beta\gamma$ following heterotrimeric G protein dissociation (68, 69). Although studies have shown that mutations in the PH domain of GRK2 abolish its ability to interact with free G $\beta\gamma$ and phosphatidylinositol 4,5-bisphosphate (70), previous work also suggests G protein-independent mechanisms of GRK recruitment and functionality (71). Seminal work using purified GRK1, GRK2, GRK5, and the GPCRs β_2 adrenergic receptor (β_2 AR) and rhodopsin demonstrated the ability of GRKs to phosphorylate activated receptors in the absence of G proteins (72–76). Additionally, this work showed that GRK2, but not GRK5, kinase activity is greatly augmented in the presence of G $\beta\gamma$ (73, 76). Another study demonstrated a role for G protein-independent GRK2 recruitment to the dopamine D2 receptor (D2R) in facilitating β -arrestin engagement in cells (77). However, whether biased ligands of CXCR3 can elicit distinct GRK recruitment mechanisms is unclear.

To evaluate the necessity of G protein activation in GRK2 and GRK3 recruitment to CXCR3, we assessed GRK recruitment to this predominantly G α_i -coupled receptor (78) in the absence of G α_i using G6 cells, a previously validated HEK293 cell line devoid of all G proteins except G α_i /o family members (79). Specifically, these cells lack *GNAS*, *GNAL*, *GNA11*, *GNA12*, *GNA13*, and *GNAQ*. Using these cells, we performed the NanoBiT complementation assay using GRK2- or GRK3-LgBiT and CXCR3-SmBiT with and without the addition of pertussis toxin (PTX) to inhibit G α_i /o activation (80, 81) (Fig. 3, A and B). The kinetic tracings for GRK recruitment to CXCR3 in G6 HEK293 cells (fig. S7, A and B) were overall similar to those observed in WT HEK293 cells (Fig. 1F). Unexpectedly, we were able to detect GRK2 and GRK3 recruitment after CXCL9 stimulation in the G6 cells, a process we did not observe in WT HEK293 cells. We found that PTX treatment significantly reduced GRK2 and GRK3 recruitment, although there was incomplete inhibition of GRK2 recruitment induced by the endogenous chemokines (Fig. 3A) and GRK3 recruitment by all ligands (Fig. 3B). It is possible that these findings are due to incomplete inhibition of G α_i /o activation with PTX. However, we observed near complete inhibition of GRK2 recruitment with VUF10661 and VUF11418 but relatively little change with CXCL9, suggesting that PTX treatment completely inhibited G α_i /o activation in these assays (Fig. 3A). These data validate previous reports suggesting that recruitment of GRK2

family proteins is largely, but not completely, dependent on G protein activation, with some contribution of G protein-independent mechanisms for GRK2 and GRK3 recruitment.

To further corroborate these findings, we repeated these experiments in HEK293 cells devoid of all G α proteins (G7 cells), specifically *GNAS*, *GNAL*, *GNAQ*, *GNA11*, *GNA12*, *GNA13*, *GNAI1*, *GNAI2*, *GNAI3*, *GNAO1*, *GNAZ*, *GNAT1*, and *GNAT2* (82). Upon rescued expression of G α i1, we observed a significant increase in GRK2 recruitment with CXCL11 and VUF10661, and in GRK3 recruitment with VUF10661 (fig. S8, A to D). All ligands except CXCL9 demonstrated an increase, albeit not to statistically significant extents, in GRK2 or GRK3 recruitment following rescue of G α i1. Even in the absence of G α i activity, VUF10661 maintained a partial ability to recruit GRK2, whereas CXCL10, CXCL11, VUF10661, and VUF11418 all partially recruited GRK3. Although G protein-dependent mechanisms are responsible for the majority of GRK2 and GRK3 recruitment to CXCR3 in response to stimulation by different ligands, these findings demonstrate an additional aspect of GRK specificity, whereby different ligands can sometimes elicit G protein-independent mechanisms to promote GRK2/3 recruitment.

GRK subcellular localization differentially affects β -arrestin 2 recruitment and CXCR3 internalization

Given that biased CXCR3 agonists promoted differential GRK recruitment across subcellular locations, we next determined if the cellular localization of a GRK could influence signaling. We therefore generated location-restricted GRK constructs to localize GRKs to either the plasma membrane or endosomes. We used a membrane targeting sequence from Lyn kinase to create plasma membrane-tethered forms (Mem-GRK) of GRK2 and GRK3, and a 2x-Fyve early endosome tag to develop endosome-tethered forms (Endo-GRK) of GRK2, GRK3, GRK5, and GRK6. The constructs were validated through confocal microscopy (Fig. 4, A and B) and evaluated further using co-expression with the membrane marker CD8 α and the endosomal marker EEA1 to confirm proper localization (fig. S9, A and B). Endo-GRK5 and Endo-GRK6 demonstrated both plasma membrane and endosome localization upon addition of the 2x-Fyve tag given their baseline localization to the plasma membrane.

Because the GRK N-terminus is involved in recognition of and docking to active GPCRs, it is possible that the N-terminal placement of the localization tags on the GRKs could interfere with their function (83, 84). To determine if the location-specific GRK constructs could interact with CXCR3, we performed Bioluminescence Resonance Energy Transfer (BRET) assays using CXCR3 tagged with Renilla Luciferase II (RLucII) and YFP-tagged GRK constructs. Endo-GRK2 and Mem-GRK3 interacted with the receptor following treatment with VUF10661, providing evidence that these tags did not significantly disrupt GRK:GPCR interactions (figs. S9, C and D). We additionally observed that the Mem-GRK and Endo-GRK constructs had higher basal BRET values as compared to the WT-GRK constructs, demonstrating that the location-specific GRK constructs were in close proximity to both plasma membrane and endosomal CXCR3, even in the absence of ligand stimulation. Given these higher basal BRET values, likely representing increased constitutive receptor

association, we expected and observed a smaller ligand-induced effect using Mem-GRK-YFP and Endo-GRK-YFP as compared to WT-GRK-YFP (figs. S9, C and D).

To evaluate the signaling consequences of these location-specific forms of GRK, we used two previously validated CRISPR/Cas9-edited HEK293 cell lines, one devoid of GRK2 and GRK3 (GRK2/3) and the other devoid of GRK5 and GRK6 (GRK5/6) (85, 86), rather than siRNA or pharmacologic strategies, to ensure complete elimination of endogenous GRK kinase activity. We performed immunoblotting on WT, GRK2/3, and GRK5/6 HEK293 cells to determine the amount of each GRK to transfect based on WT expression amounts (fig. S10, A to D). We then used BRET to monitor ligand-induced recruitment of fluorescently tagged β -arrestin 2 (β -arrestin 2-mKO) recruitment to CXCR3-RLucII in either GRK2/3 or GRK5/6 cells transfected with individual WT GRK constructs (Fig. 4C). For CXCL10, CXCL11, and VUF11418 treatment, we observed that expression of WT-GRK2 decreased β -arrestin 2 recruitment, whereas VUF10661 stimulation had no significant effect on WT-GRK2-mediated β -arrestin 2 recruitment (Fig. 4D). Further, expression of WT-GRK3 enhanced β -arrestin 2 recruitment for VUF11418 treatment but had no significant effect for other ligand conditions (Fig. 4E). By contrast, WT-GRK5 and WT-GRK6 had minimal effect on β -arrestin 2 recruitment for all but one ligand condition (figs. S11, A and B). These findings suggest that the WT GRKs have distinct effects on CXCR3 engagement with β -arrestin 2. These effects were conserved across ligands for some GRKs but agonist-dependent for others, indicative of the ability of different ligands to confer unique properties to GRK family members.

Furthermore, the effects of the location-specific GRKs from WT-GRK were ligand-specific. With CXCL11, both Mem-GRK2 and Endo-GRK2 partially reduced the WT effect on β -arrestin 2 recruitment (Fig. 4D). However, upon VUF11418 stimulation, Mem-GRK2, but not Endo-GRK2, altered the WT phenotype. A different profile was observed for GRK3. Although β -arrestin 2 recruitment increased following expression of WT-GRK3 with VUF11418 treatment, this effect was reduced when GRK3 was localized to the plasma membrane and lost when it was bound to endosomes (Fig. 4E). Although WT-GRK3 had no detectable effect on β -arrestin 2 recruitment for CXCL10, CXCL11, or VUF10661, Mem-GRK3 decreased β -arrestin 2 recruitment for all three ligands. Whereas β -arrestin 2 recruitment was susceptible to alterations in GRK2 and GRK3 cellular localization, GRK5 and GRK6 demonstrated little effect (fig. S11, A and B). These data suggest a role of location-dependent activities of the GRKs on β -arrestin 2 recruitment, where the relative direction and magnitude of change is GRK-, ligand-, and location-dependent.

Given the role of β -arrestins in promoting receptor internalization, we next assessed if individual GRKs had distinct effects on CXCR3 trafficking to endosomes. We expressed individual GRKs in GRK2/3 or GRK5/6 cells transfected with CXCR3-RLuc2 and 2x-Fyve-mVenus to assess CXCR3 proximity to early endosomes following ligand stimulation (Fig. 4F). Whereas WT-GRK2 significantly decreased β -arrestin 2 engagement of CXCR3 for CXCL10, CXCL11 and VUF11418 stimulation, expression of WT-GRK2 unexpectedly elicited minimal changes to CXCR3 internalization across most ligands (fig. S11C). Likewise, although we observed a robust increase in β -arrestin 2 recruitment by WT-GRK3 upon VUF11418 stimulation, there was no effect on CXCR3 internalization (fig. S11D). By

contrast, even though WT-GRK6 showed little activity in affecting β -arrestin 2, expression of WT-GRK6 enhanced CXCR3 internalization across all ligands except CXCL9 (Fig. 4G). Expression of WT-GRK5 also showed a minimal effect on CXCR3 internalization for all ligands apart from VUF10661 (fig. S11E). These findings are indicative of specific roles for distinct GRKs in driving different GPCR signaling events. Whereas our data suggest a prominent function of GRK2 and GRK3 in regulating β -arrestin 2 recruitment, we found a substantial role for GRK6 in mediating receptor internalization.

Subcellular localization modulates the distinct effects of GRKs on cytosolic and nuclear ERK1/2 activity in a ligand-dependent fashion

We next investigated the activation of the kinases ERK1 and ERK2 (ERK1/2) as a marker of mitogen-activated protein kinase (MAPK) signaling, a prototypical GPCR downstream signaling pathway that can be activated by both G proteins and β -arrestins (87, 88). We transfected a previously developed BRET-based biosensor that detects ERK kinase activity in the nucleus or cytoplasm (45, 89) into the GRK2/3 or GRK5/6 cell lines. We did not observe any differences in basal ERK activity following expression of any of the GRK constructs (fig. S12, A to D).

WT-GRK2 had little effect on cytosolic ERK activity, with minor decreases in ERK activity with VUF10661 (Fig. 5A). However, WT-GRK2 significantly inhibited nuclear ERK activity across all ligands (Fig. 5B). In contrast, WT-GRK3 was inhibitory to both cytosolic and nuclear ERK activation for most ligands tested (Fig. 5, C and D). WT-GRK5 had an entirely different profile, wherein it diminished cytosolic ERK activity upon CXCL10 and VUF11418 stimulation but had no significant effect on nuclear ERK activity with these ligands (Fig. 5, E and F). CXCL11 also demonstrated reduced cytosolic ERK activity with WT-GRK5 but enhanced nuclear ERK activation. These findings support distinct roles of individual GRKs in regulating the activation of cytosolic and nuclear pools of ERK. Consistent with our previous assays examining upstream signaling events, activities of GRK subtypes were specific for each ligand.

When using the location-specific forms of GRK, Endo-GRK2 inhibited nuclear ERK activity similarly to WT-GRK2, except with CXCL9 and VUF10661 (Fig. 5B). For most ligands, WT-GRK3 and Mem-GRK3 significantly reduced both nuclear and cytosolic ERK activation, whereas Endo-GRK3 had little effect on ERK activity as compared to empty vector (Fig. 5, C and D). Further, although WT-GRK5 and Endo-GRK5 decreased cytosolic ERK activation following treatment with CXCL10, CXCL11, and VUF11418, we observed a substantial increase in nuclear ERK activation with CXCL11 treatment (Fig. 5, E and F). Endo-GRK6 demonstrated little effect in modulating nuclear or cytosolic ERK activity as compared to empty vector, whereas WT-GRK6 increased ERK activation in both the nucleus and cytosol for some ligands (Fig. 5, G and H). These data demonstrated that altering GRK localization had differential effects on the activation of nuclear and cytoplasmic ERK. By modulating the ligand, the GRK, and the subcellular location of the GRK, it was possible to generate numerous and highly specific patterns of ERK activity, highlighting the complex interaction between GRKs and a GPCR activated by a single ligand.

Other GPCRs recruit GRKs to endosomes

Given our finding that biased CXCR3 agonists promoted differential GRK recruitment to the plasma membrane and endosomes, we wondered if this phenomenon occurred at other GPCRs. We selected an array of GPCRs, including β_2 AR, μ -opioid receptor (MOR), angiotensin II type I receptor (AT₁R), V₂ vasopressin receptor (V₂R), and atypical chemokine receptor 3 (ACKR3). We then probed GRK2, GRK3, GRK5, and GRK6 recruitment to the plasma membrane and endosomes following ligand stimulation using the Nano-BiT complementation assay. Cells were transfected with WT receptor, GRK-LgBiT, and either CD8-SmBiT for the plasma membrane or 2xFyve-SmBiT for the endosome.

We found that these receptors differed in their ability to induce GRK recruitment to the plasma membrane. For example, stimulation of the V₂R produced the most robust recruitment of GRK2 and GRK3 to the plasma membrane, whereas stimulation of the β_2 AR induced the greatest recruitment of GRK5 and GRK6 to the plasma membrane (Fig. 6A). Although GRK5 and GRK6 are constitutively membrane-localized, this apparent increase may represent a change in the membrane distribution of these GRKs because CD8 α can be found in specific membrane microdomains (90, 91). We observed substantial recruitment of the GRKs to endosomes for many of the receptors tested, with distinct patterns when compared to plasma membrane recruitment (Fig. 6, A and B). GRK2 and GRK3 were robustly recruited to endosomes upon activation of the AT₁R (Fig. 6B). The β_2 AR, MOR, and V₂R all demonstrated the ability to recruit GRK2 and/or GRK3 to endosomes, albeit to different extents and with distinct kinetic tracings. GRK5 and GRK6 similarly demonstrated recruitment to endosomes across many receptors in a pattern that was different than that observed at the plasma membrane. (Fig. 6, A and B). We further validated these findings with the AT₁R using confocal microscopy. Following treatment with angiotensin II (AngII), we observed colocalization of GRK3-YFP and GRK6-YFP with AT₁R-mKO in endosomes (Fig. 6, C and D). These data demonstrated that GRK trafficking to endosomes is a conserved signaling mechanism for many GPCRs and that distinct patterns of GRK engagement with GPCRs at the plasma membrane and endosomes may facilitate signaling specificity and promote biased responses.

DISCUSSION

Nearly all GPCRs are known to interact with G proteins, GRKs, and β -arrestins, but these interactions have primarily been studied at the plasma membrane. Published studies demonstrate that G proteins and β -arrestins can signal from endosomes (92–94). Here, we provide evidence that GRKs can traffic to endosomes following agonist stimulation of multiple GPCRs (Fig. 6E). Additionally, using both endogenous and synthetic CXCR3 agonists, we showed that the pattern of GRK recruitment to the plasma membrane differed substantially from that observed at endosomes. For example, whereas CXCL11 promoted robust GRK2 recruitment to CXCR3 at the plasma membrane, it did not produce any detectable recruitment of GRK2 at endosomes. Similarly, stimulation of the V₂R promoted more GRK2 and GRK3 recruitment to the plasma membrane than did stimulation of the AT₁R, but this pattern was reversed in endosomes. It is likely that the subcellular localization of the GRKs contributes to the ability of these GPCRs to initiate spatially

distinct signaling patterns. Elucidation of the unique roles of individual GRK subtypes on overall GPCR signaling has received appreciable attention, in large measure due to the potential contribution of single GRKs to a wide range of pathophysiological processes. The dysregulation of GRK2 has been linked to the progression of mitochondrial lesions in Alzheimer's disease, cardiovascular dysfunction, pathological angiogenesis, and chronic inflammation, among others (95–99).

Using biased ligands, we observed that G protein activation was a critical mechanism underlying the biased recruitment of GRKs to CXCR3 at the plasma membrane. These findings are consistent with previous reports that have demonstrated that GRK2 and GRK3 are recruited to GPCRs through their PH domain recognizing free G $\beta\gamma$ following G protein activation (26, 68, 74). However, using two different CRISPR/Cas9 GRK knockout cell lines, we found that GRK2 and GRK3 could be recruited to CXCR3 independently of G protein activation. Furthermore, the relative amount of G protein-independent GRK recruitment observed was dependent on the ligand, the specific GRK, and the cell type. It is possible that these observations are a consequence of using genetically modified HEK293 cells, but other work has demonstrated a similar phenomenon for the D2R, which can recruit GRK2 in the absence of G protein activation (77). We build upon this previous work, because our data suggest that a single GPCR may use both G protein-dependent and -independent mechanisms to recruit GRKs to the plasma membrane, depending on the specific ligand that activates the receptor.

This observation of G protein-independent GRK recruitment raises the question of whether this phenomenon has physiologic relevance. Although our data demonstrated that GRK recruitment robustly increased in the presence of cognate G proteins, we were still able to detect GRK recruitment using cells devoid of G proteins. Changes in the abundances of GRKs and G proteins are associated with some disease states. For example, GRK2 is present in almost three-fold higher amounts in the failing heart compared to the healthy heart, leading to enhanced activity in adrenergic receptor desensitization (100). Similarly, there are reports of decreased and increased heterotrimeric G proteins in disease states like asthma, myocardial infarction, and breast cancer (101). It is possible that G protein-independent mechanisms of GRK recruitment are present in both physiologic and pathophysiological states, depending on the specific context of the cellular system. Further work is warranted to better understand these findings.

We explored the contributions of GRK subcellular localization to biased GPCR signaling by developing location-specific forms of GRKs and characterizing CXCR3 signaling using GRK knockout cell lines. We demonstrated that modifications to GRK cellular localization could alter the receptor's ability to recruit β -arrestin 2, internalize, and activate ERK, depending on the specific ligand and GRK. Thus, GRK subtype functionality appears to be not only driven by distinctions between the proteins themselves, but also by unique properties conferred to each GRK by different GPCR ligands. We observed that expression of GRK2 and GRK3 in GRK2/3 cells led to decreased β -arrestin 2 recruitment. These findings were unexpected considering canonical GPCR signaling suggests receptor phosphorylation by GRKs leads to increased β -arrestin recruitment. It is possible that GRK2 and GRK3 phosphorylate sites on CXCR3 that are inhibitory to β -arrestin recruitment or

that β -arrestin recruitment to CXCR3 is primarily mediated through a GRK-independent process. Similarly, GRK5 and GRK6 (which are still present in GRK2/3 cells) may promote arrestin recruitment for some agonist conditions, and GRK2 and GRK3 may compete with these kinases for the receptor. There is evidence that different GRKs can serve unique functions at a single GPCR (102, 103), and the specific role of GRKs 2, 3, 5 and 6 at CXCR3 similarly seem to be nonredundant.

We also observed an increase in CXCR3 internalization following expression of GRK6 in GRK5/6 cells at most ligands (Fig. 4G). However, we did not observe any change in β -arrestin 2 recruitment under similar transfection conditions (fig. S11B). There are many possible explanations for these findings. Although β -arrestins are known to play a role in GPCR internalization through interactions with AP-2 and clathrin, there are β -arrestin-independent mechanisms of internalization as well (104–106). It is therefore likely that GPCR internalization is not always directly correlated with β -arrestin recruitment. GRKs could regulate GPCR internalization through mechanisms independent of β -arrestin recruitment, possibly by modulating β -arrestin conformation or receptor interactions with other effector proteins (107, 108). Previous work has demonstrated that different ligands can cause the same receptor to differentially engage the GRKs. For example, at the GPCR CCR7, the ligand CCL19 leads to GRK3- and GRK6-dependent receptor phosphorylation, whereas the ligand CCL21 only activates GRK6 (103). Similarly, at the AT1R, knockdown of GRK2 and GRK3 lead to decreased receptor phosphorylation, β -arrestin recruitment, and endocytosis, whereas knockdown of GRK5 and GRK6 abolish β -arrestin-mediated ERK activation (109). Additionally, we did not investigate the role of β -arrestin 1 in this system, and it is possible that the GRKs may have different effects on β -arrestin 1 recruitment and functionality as compared to β -arrestin 2, as others have recently reported (86, 107). Further insight is needed into understanding how the GRKs modulate receptor internalization and signaling through β -arrestin-dependent, and possibly β -arrestin-independent, mechanisms.

Our data demonstrated that the GRK location and ligand identify both impacted the ability of a GPCR to modulate ERK activity. We also did not expect to find that the regulation of ERK activation also depended on which subcellular pool of ERK was being investigated (cytosolic versus nuclear ERK). There is burgeoning evidence demonstrating that many GPCR second messengers or effector proteins including cAMP (110), ERK (45, 111), JNK (112), and G proteins (113) exhibit location-specific effects. However, the physiologic importance and molecular determinants of this phenomenon are incompletely understood. It will be important to determine the functional consequences of location-specific GPCR signaling and how GRKs modulate and contribute to this activity.

Receptor internalization and recycling were initially considered a mechanism of signal attenuation and re-sensitization (114, 115). Specifically, β -arrestins sterically inhibit GPCRs to prevent further G protein activation while simultaneously promoting receptor internalization to prevent further ligand binding. However, subsequent studies have demonstrated that many GPCRs can continue to signal through both G proteins and β -arrestins from endosomes. Previous work on the β_2 AR showed that endosomal cAMP production is required to generate a complete transcriptional response (39). Subsequent work has shown that signaling from endosomes also impacts the global phosphoproteome

(38). Many studies support the paradigm of location bias, and it is now appreciated that endocytosis does not solely serve as a mechanism to attenuate GPCR signaling; rather, it also can be a critical component to achieving maximal GPCR signaling (36, 44, 116–118). Additionally, prior work demonstrates the therapeutic potential of pharmacologically targeting signaling from endosomes by the neurokinin 1 receptor, MOR, and other GPCRs (119–122). Recent studies have observed that a specific ligand:GPCR complex can activate signaling pathways from endosomes that are distinct from those activated at the plasma membrane (37, 39, 118). It is likely that signaling from endosomes and other subcellular locations is one mechanism by which a GPCR can generate diverse signaling outputs in response to biased ligands. We previously demonstrated that the biased agonists of CXCR3 demonstrate different relative amounts of G protein and β -arrestin signaling at the plasma membrane and endosome, providing evidence that the degree of biased agonism observed for a GPCR depends on where the signaling output is measured (45).

Notably, our data demonstrate that changes in a given readout of CXCR3 signaling were not necessarily predictive of the alterations for other signaling events canonically viewed as sequential. As mentioned above, although differential GRK6 localization had little effect on the ability of CXCR3 to engage β -arrestin 2, GRK6 increased CXCR3 internalization across all ligands. These results are consistent with GRKs demonstrating function beyond receptor phosphorylation and increasing the affinity of β -arrestin for a GPCR. Other studies demonstrate that the GRKs can modulate β -arrestin conformation, which is tightly associated with its ability to scaffold other signaling effectors (86, 108, 123, 124). Additionally, GRKs are known to phosphorylate other non-GPCR receptors, such as receptor tyrosine kinases and toll-like receptors, transcription factors, and more (23). GRKs are also known to have kinase-independent functionality. For example, a reduction in GRK2 leads to impairment in zebrafish development, but a kinase-dead mutant form of GRK2 restores normal development (125). This led to the discovery that GRK2 interacts with the cyclin B1 regulator patched homolog 1 (PTCH1) in manner that is independent of its kinase activity, and the interaction is necessary for normal embryogenesis (125). Similar work has demonstrated that GRK5 can inhibit nuclear factor κ B (NF- κ B) signaling by promoting the nuclear accumulation of the NF- κ B inhibitor I κ B α independently of its catalytic activity (126).

Biased agonism at GPCRs is traditionally characterized by a ligand's relative ability to activate G proteins and recruit β -arrestins. However, we now appreciate that there are many other dimensions of biased agonism including location bias, receptor bias, kinetic bias, and more (4). In this manuscript, we determined that VUF10661 was the only CXCR3 ligand to appreciably recruit GRKs to endosomes, even though all chemokines except CXCL9 could recruit GRKs to the plasma membrane. Although previous studies demonstrate that VUF10661 and VUF11418 have similar biased signaling profiles regarding G protein activation and β -arrestin recruitment (127), these ligands differed substantially in their abilities to recruit GRKs to the endosome. Notably, we did not observe a relationship between G protein activation, β -arrestin recruitment, or GRK recruitment to the plasma membrane and a ligand's ability to recruit GRKs to the endosome. Together, our findings suggest another functionally distinct dimension of biased GPCR signaling, one that incorporates ligand and location bias.

Our findings highlight the importance of the GRKs in initiating biased responses at GPCRs and demonstrate the complexity of downstream signaling that can be achieved through a single GPCR. These data support the phosphorylation barcode hypothesis by demonstrating that differential GPCR engagement with the GRKs can generate multiple agonist-specific, signaling pathways. This complex signaling exists at different compartments within the cell. Together, our data demonstrate the therapeutic promise and simultaneous complexity of drugging GPCRs and GPCR effectors given the vast diversity of signaling that can be achieved within this receptor family. The molecular determinants that promote GRK translocation to endosomes are still unclear at this time, and the complex interconnection between ligand and GRK subtype bias has not been fully resolved. It will be important to determine the mechanisms by which individual GRKs modulate signaling at different locations. Deconvoluting the structural elements that exist between a GPCR and GRK are critical to understanding the biochemical basis for biased signaling observed across different cellular compartments.

MATERIALS AND METHODS

Bacterial Strains

XL-10 Gold ultracompetent *E. coli* (Agilent) were used to express all constructs used in this manuscript.

Cell Lines

Human Embryonic Kidney (HEK293) cells were grown in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO₂. GRK2/3, GRK5/6, G6, and G7 CRISPR/Cas9 KO HEK293 cells were provided by Asuka Inoue, Tohoku University, Japan, and validated as previously described (Table S1) (55, 79, 82).

Generation of Constructs

Construct cloning was performed using conventional techniques such as restriction enzyme/ligation methods. Linkers between the fluorescent proteins or luciferases and the cDNAs for receptors, transducers, or other proteins were flexible and ranged between 2 and 18 amino acids. Fluorescence resonance energy transfer (FRET) based ERK1/2 biosensors previously published (89) were used to generate BRET versions of these sensors by removing the N-terminal mCerulean through restriction digest and inserting a nanoluciferase. We utilized the 2x-Fyve targeting sequence (RKHHCRACG) from the hepatocyte growth factor-regulated tyrosine kinase substrate to target labeled constructs to endosomes. We utilized the membrane targeting sequence (MGCIKSKGKDS) from Lyn kinase to target labeled constructs to the plasma membrane. Location tags were attached to the N-terminus of all constructs, followed by a flexible amino acid linker. CD8 α -smBiT and CD8 α -mCherry were cloned using human CD8 α isoform 1. Human EEa1-dsRed was a gift from the Nina Tsvetanova Laboratory. Bovine GRK2, human GRK3 Isoform 1, human GRK5, and human GRK6 Isoform B were used for all confocal microscopy experiments (GRK-YFP), and all experiments involving expression of either the WT or location-specific mutant GRKs (WT-GRK, Mem-GRK, Endo-GRK). GRK-LgBiT constructs were provided by Asuka Inoue (55).

Specifically, we used human GRK2-LgBiT that is codon optimized, human GRK3-LgBiT that is codon optimized, human GRK5-LgBiT, and human GRK6 Isoform B-LgBiT. Human Gαi1 was used in rescue experiments in G7 cells. Rat β-arrestin 2-mKO was used for recruitment BRET assays. Human CXCR3A, AT1R, V2R isoform 1, B2AR, MOR, and ACKR3 were used in this study.

Cell Culture and Transfection

For luminescence-based assays, HEK293 cells were transiently transfected with an optimized calcium phosphate protocol as previously described unless otherwise indicated (77). In the calcium phosphate transfection method, cell culture media was replaced 30 minutes prior to transfection. Plasmid constructs were suspended in water to a final volume of 90μL. 10μL of 2.5 M calcium chloride was added to the plasmid constructs and mixed. 100μL of 2x HEPES-buffered saline solution (10mM D-Glucose, 40mM HEPES, 10 mM potassium chloride, 270 mM sodium chloride, 1.5 mM disodium hydrogen phosphate dihydrate) was added to the solution, allowed to incubate for two minutes, and subsequently added to the cells. For BRET based assays, luminescence-based assays in G protein KO cell lines, and confocal microscopy, cells were transiently transfected using polyethylenimine (PEI). In the PEI transfection method, cell culture media was replaced 30 minutes prior to transfection. Plasmid constructs were suspended in Opti-MEM (Gibco) to a final volume of 100μL and, in a separate tube, PEI at a concentration of 1 mg/mL was added to Opti-MEM to a final volume of 100μL. For experiments in this manuscript, 3μL of PEI was used per 1μg of plasmid DNA. After 5 minutes, the 100μL PEI solution was added to the 100μL DNA solution, gently mixed, and allowed to incubate at room temperature for 10–15 minutes, after which the mixture was added to the cells.

BRET and Split Luciferase Assays

For all BRET and Split Luciferase assays, HEK293 cells seeded in 6 well plates (~750,000 cells/well) were transiently transfected with the appropriate constructs using the calcium phosphate or PEI method previously described. GRK recruitment was assessed using a NanoBiT complementation assay, where GRK2, GRK3, GRK5, or GRK6 were tagged with a C-terminal LgBiT and transfected with either CXCR3-smBiT (receptor), wild-type CXCR3 and 2x-Fyve-SmBiT (endosome), or wild-type CXCR3 and CD8α-SmBiT (plasma membrane). The role of G protein activation in GRK2/3 recruitment was assessed using PEI transfection of CXCR3-SmBiT and the indicated GRK-LgBiT, with rescue of Gαi1 in ΔG7 GKO cell lines and pertussis toxin (PTX) treatment at a final concentration of 200ng/mL in ΔG6 GKO cells.

To examine role of GRK subcellular localization on effector engagement and downstream signaling, we assessed β-arrestin 2 recruitment and CXCR3 internalization using the wild-type GRKs, membrane-bound GRK2 or 3 constructs, or endosome-bound GRK2, GRK3, GRK5, or GRK6. Rescue of GRK2 or GRK3 was performed in GRK2/3 KO cells while rescue of GRK5 or GRK6 was performed in GRK 5/6 KO cells. For β-arrestin 2 recruitment assays, cells were transfected with the indicated WT- or mutant GRK, CXCR3-RLuc2, and β-arrestin 2-mKO. CXCR3 internalization assays used the indicated WT- or mutant GRK with CXCR3-RLuc2 and a 2x-Fyve-mVenus to assess proximity to the early endosome.

Location-specific BRET-biosensors of ERK activity were transfected using PEI. This ERK biosensor consists of a target ERK substrate that, following phosphorylation by activated phosphorylated ERK, binds to a phosphorylation binding domain, causing a conformational change in the biosensor and subsequent change in BRET efficiency (45, 89).

Twenty-four hours after transfection, cells were washed with phosphate buffered saline (PBS), collected with trypsin, and plated onto clear-bottomed, white-walled, Costar 96-well plates at 50,000 to 100,000 cells/well in BRET medium (clear minimum essential medium (Gibco) supplemented with 2% FBS, 10 mM HEPES, 1x GlutaMax (Gibco), and 1x Antibiotic-Antimycotic (Gibco)).

The following day, media was removed, and cells were incubated at 37°C with 80µL of HBSS supplemented with 20mM HEPES, and 3µM coelenterazine h for all BRET or NanoBiT complementation assays (Cayman Chemical, Ann Arbor, MI and Nanolight Technology, Pinetop, AZ) for 10 to 15 minutes. For all BRET assays, a standard 480nm RLuc emission filter and 530nm (for experiment using mVenus) or custom 542-nm (for experiments using mKO) long pass filter was utilized (Chroma Technology Co., Bellows Falls, VT). Cells were stimulated with either vehicle control (HBSS with 20 mM HEPES) or the indicated concentration of ligand.

For NanoBiT complementation and BRET experiments, three initial reads were taken prior to the addition of ligand to quantify baseline luminescence or BRET signal before adding ligand. The change in luminescence after ligand stimulation was subsequently normalized to vehicle treatment. For BRET experiments, the BRET ratio was calculated by dividing the acceptor signal by the luminescent signal, and a net BRET ratio was calculated by normalizing to vehicle treatment. Plates were read with a BioTek Synergy Neo2 plate reader or Berthold Mithras LB 940 set at 37°C. All readings were performed using a kinetic protocol.

Confocal Microscopy

HEK293 cells were plated on 35 mm glass bottomed dishes (MatTek Corporation, Ashland, MA) and transiently transfected using PEI with the listed constructs. To evaluate the baseline expression of wild-type GRK2-YFP, GRK3-YFP, GRK5-YFP, and GRK6-YFP, or the Lyn-tagged or 2xFyve-tagged indicated GRK-YFP, cells were imaged forty-eight hours after transfection. To assess ligand-induced GRK localization, forty-eight hours after transfection, the cells were washed once with PBS and then serum starved for one hour. The cells were subsequently treated with a control of serum free media or the listed chemokine at 100nM or listed VUF compound at 10µM for the indicated time at 37°C. Cells were then imaged with a Zeiss CSU-X1 spinning disk confocal microscope using the corresponding lasers for YFP (480nm excitation), mCerulean (433nm excitation), dsRed (561nm excitation). Images were analyzed using ImageJ (NIH, Bethesda, MD) and false colors were applied for clarity.

Immunoblotting

Immunoblotting was performed as described previously (51). GRK2/3 and GRK5/6 cells seeded in 6 well plates were transiently transfected with increasing amounts of GRK using the calcium phosphate transfection method. 24 hours after transfection, cells

were serum starved in minimum essential medium supplemented with 0.01% bovine serum albumin (BSA) and 1% penicillin/streptomycin for 16 hours. The cells were then washed with ice cold PBS and lysed in ice cold RIPA buffer supplemented with protease inhibitors (cOmplete EDTA free (Sigma)). The samples were rotated at 4°C for forty-five minutes and cleared of insoluble debris by centrifugation at 17,000g at 4°C for 15 minutes, after which the supernatant was collected. Protein was resolved on SDS-10% polyacrylamide gels, transferred to nitrocellulose membranes, and immunoblotted with the indicated primary antibody overnight at 4°C. GRK2 (Santa Cruz, #sc-13143), GRK3 (Cell Signaling Technology, #80362), GRK5 (Santa Cruz, #sc-518005), and GRK6 (Cell Signaling Technology, #5878) antibodies were used to compare rescued GRK expression level in GRK2/3 and GRK5/6 cells to GRK expression levels WT HEK293 cells (128). Immunoblots were normalized using an alpha-tubulin antibody (Sigma, #T5168). Horseradish peroxidase-conjugated anti-rabbit-IgG or anti-mouse-IgG were used as secondary antibodies. The nitrocellulose membranes were imaged by SuperSignal enhanced chemiluminescent substrate (Thermo Fisher) using a ChemiDoc MP Imaging System (Bio-Rad). To determine the amount of GRK to rescue to match the expression levels in wild-type HEK293 cells, we performed a linear regression analysis of the tubulin normalized signal intensity versus the amount of GRK transfected. We then used these derived equations to solve for the amount of GRK plasmid needed to generate a western blot signal comparable to that seen in the wild-type HEK293 cells.

CXCR3 Ligands

Recombinant Human CXCL9, CXCL10, and CXCL11 (PeproTech) were diluted according to the manufacturer's specifications, and aliquots were stored at -80°C until needed for use. VUF10661 (Sigma-Aldrich) and VUF11418 (Aobius) were reconstituted in dimethyl sulfoxide (DMSO) and stored at -20°C in a desiccator cabinet.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed in Excel (Microsoft, Redmond, WA) and graphed in Prism 9.0 (GraphPad, San Diego, CA). Dose-response curves were fitted to a log agonist versus stimulus with three parameters (span, baseline, and EC50), with the minimum baseline corrected to zero using Prism 9.0. Statistical tests were performed using a one or two-way ANOVA followed by Tukey's multiple comparison's test when comparing treatment conditions. When comparing ligands or treatment conditions in concentration-response assays or time-response assays, a two-way ANOVA of ligand and concentration or ligand and AUC, respectively, was conducted. For experiments using mutant GRKs, a two-way ANOVA was performed followed by Tukey's multiple comparison's test when comparing transfection conditions within a ligand. If a significant interaction effect was observed ($P < 0.05$), then comparative two-way ANOVAs between individual experimental conditions were performed. Further details of statistical analysis and replicates are included in the figure legends. Lines represent the mean, and error bars signify the SEM, unless otherwise noted. Experiments were not randomized, and investigators were not blinded to treatment conditions. Critical plate-based experiments were independently replicated by at least two different investigators when feasible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank Asuka Inoue (Tohoku University, Japan), Robert J. Lefkowitz (Duke University, USA), Christopher Cole Honeycutt (National Institutes of Health, USA), and Noelia Boldizar (National Institutes of Health, USA) for reagents, guidance, and thoughtful feedback throughout this work; N. Nazo for laboratory assistance. Graphical figures were created with BioRender.

FUNDING

Duke Cardiovascular Research Center Undergraduate Research Fellowship (C.H.)

Duke University Deans Summer Research Fellowship (C.H.)

National Institutes of Health grant T32GM007171 (D.S.E.)

Duke Medical Scientist Training Program (D.S.E.)

American Heart Association Predoctoral Fellowship 20PRE35120592 (D.S.E.)

National Institutes of Health grant 1R01GM122798 (S.R.)

Burroughs Wellcome Career Award for Medical Scientists (S.R.)

DATA AND MATERIALS AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. All plasmids generated in this study will be distributed upon reasonable request and completion of a materials transfer agreement with Duke University. All data reported in this paper will be shared by the lead contact upon request.

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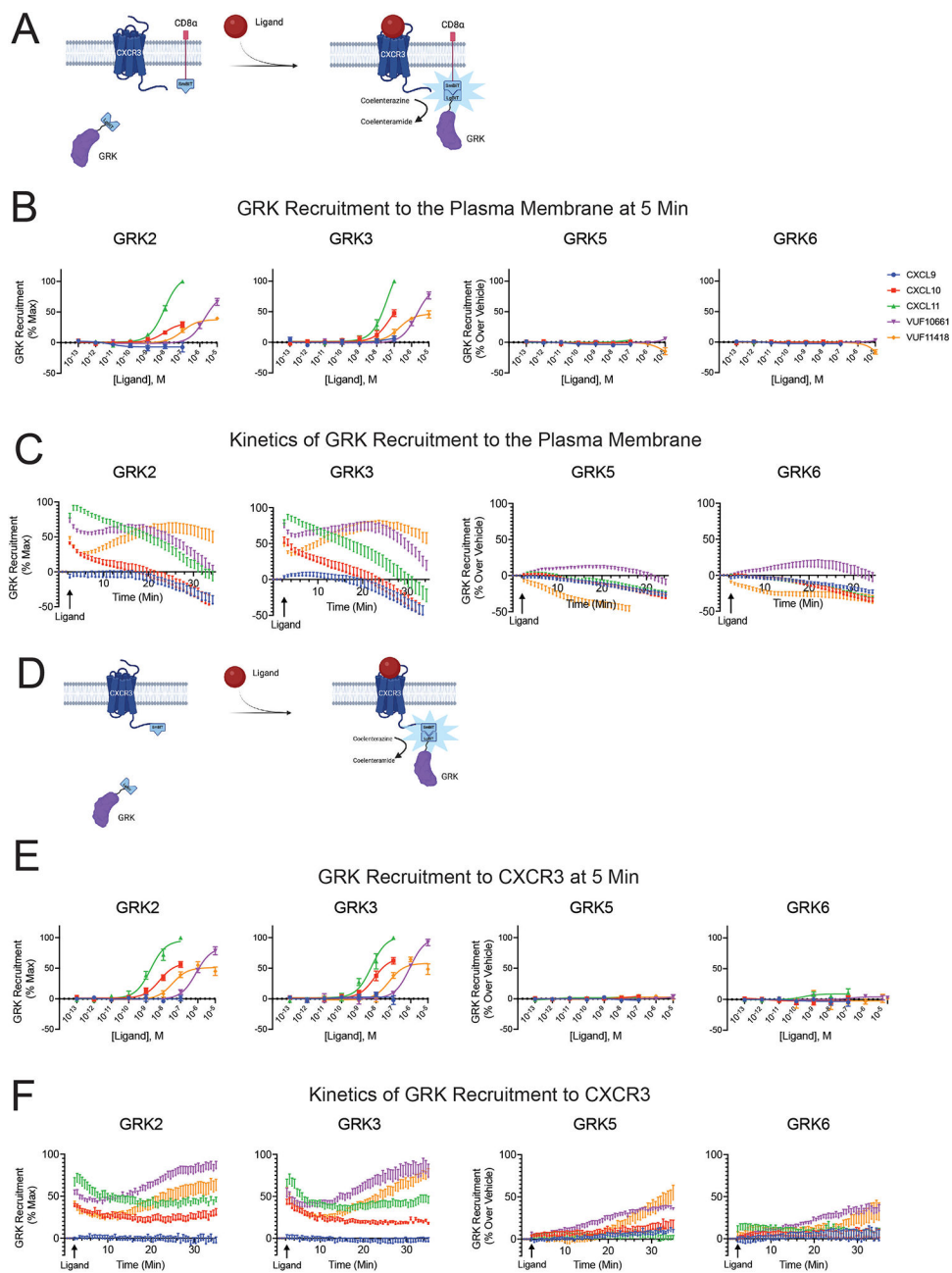


Fig. 1. GRK recruitment to the plasma membrane and to CXCR3.

(A) Schematic representation of a NanoBiT complementation assay detecting GRK recruitment to the plasma membrane using GRK-LgBiT and CD8 α -SmBiT in HEK293 cells stimulated with CXCR3 agonists. Upon recruitment of GRK to the plasma membrane, the smBiT and LgBiT generate a functional luminescent signal as a readout of GRK.

(B) Agonist concentration-response at five minutes and (C) kinetic time-course of GRK2, GRK3, GRK5, and GRK6 recruitment to the plasma membrane in response to the indicated ligands. (D) Schematic representation of a NanoBiT complementation assay detecting GRK recruitment to CXCR3 using GRK-LgBiT and CXCR3-SmBiT in HEK293 cells stimulated with CXCR3 agonists. (E) Agonist concentration-response at five minutes and (F) kinetic

time-course of GRK2, GRK3, GRK5, and GRK6 recruitment to CXCR3. All experiments were performed following stimulation with 100nM CXCL9, CXCL10, or CXCL11 or 10 μ M VUF10661 or VUF11418. Data shown are the mean \pm SEM of n=5 independent experiments. Concentration-response curves and kinetic data for GRK2 and GRK3 are normalized to maximum observed signal across all ligands. Concentration-response curves and kinetic data for GRK5 and GRK6 are shown as luminescence change over vehicle for each ligand. GRK2 and GRK3 both demonstrate maximum recruitment patterns of CXCL11 > VUF10661 > (CXCL10=VUF11418) > CXCL9 as assessed using one-way ANOVAs and Tukey's post hoc testing on the luminescence values at maximum dose.

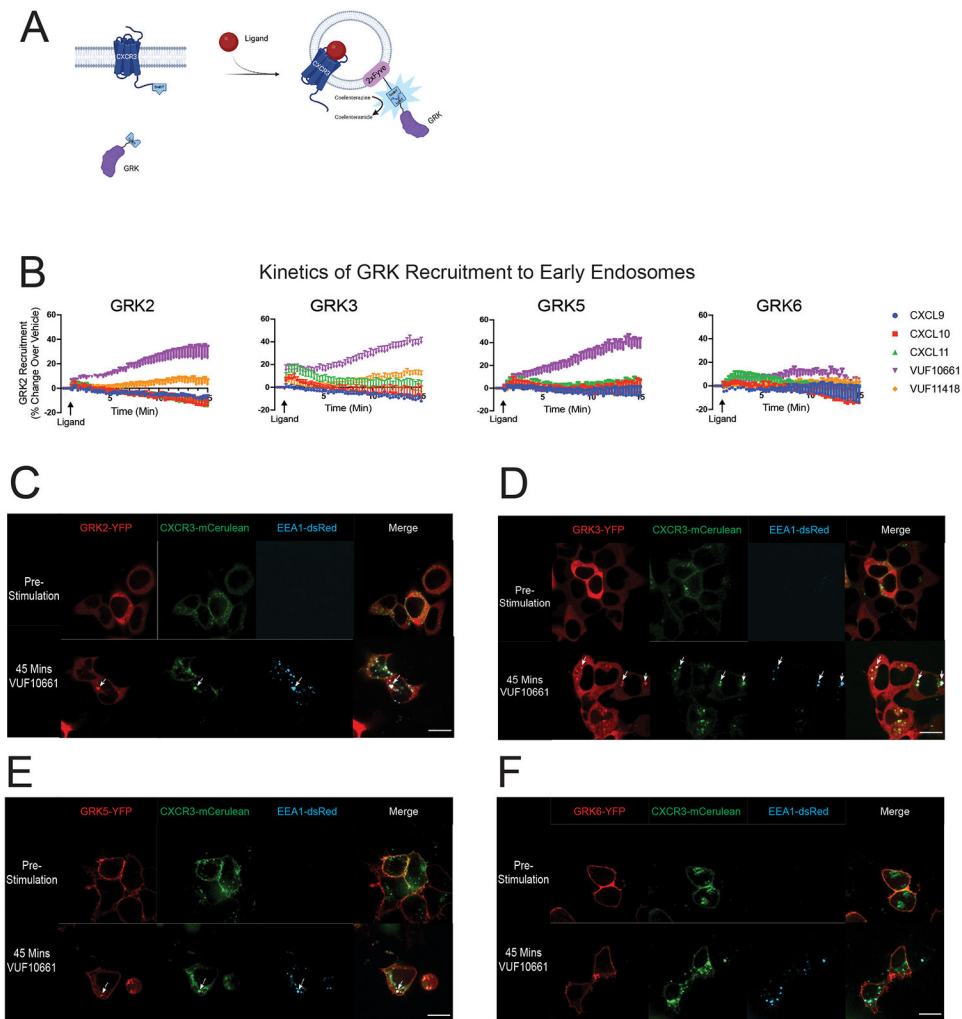


Fig. 2. CXCR3-mediated GRK recruitment to endosomes.

(A) Schematic representation of the NanoBiT complementation assay used to detect GRK recruitment to endosomes using wild-type CXCR3, LgBiT-tagged GRK, and 2xFyve-SmBiT in HEK293 cells stimulated with CXCR3 agonists. Following activation of the receptor and recruitment of GRK to endosomes, the smBiT and LgBiT undergo complementation and generate a functional luminescent signal as a readout of GRK endosomal recruitment. (B) Kinetic data for GRK2, GRK3, GRK5, and GRK6 recruitment to early endosomes upon stimulation with 100nM CXCL9, CXCL10, or CXCL11 or 10 μ M VUF10661 or VUF11418. Kinetic tracings are shown as luminescence change over vehicle. (C to F) Confocal microscopy images of HEK293 cells transfected with CXCR3-mCerulean and EEA1-dsRed plus GRK2-YFP (C), GRK3-YFP (D), GRK5-YFP (E), or GRK6-YFP (F). Images were taken before or 45 minutes after stimulation with 10 μ M of VUF10661. Scale bar, 21 μ m. Plate-based experiments show the mean \pm SEM of n=5 independent experiments. Confocal microscopy images are representative of n=3 independent experiments. Using one-way ANOVA with Tukey's post hoc testing, the time to maximum GRK2 and GRK3 recruitment to the plasma membrane (~2 minutes) was significantly different than the time to maximum recruitment to the endosome (~13 minutes) at a p-value of <0.05.

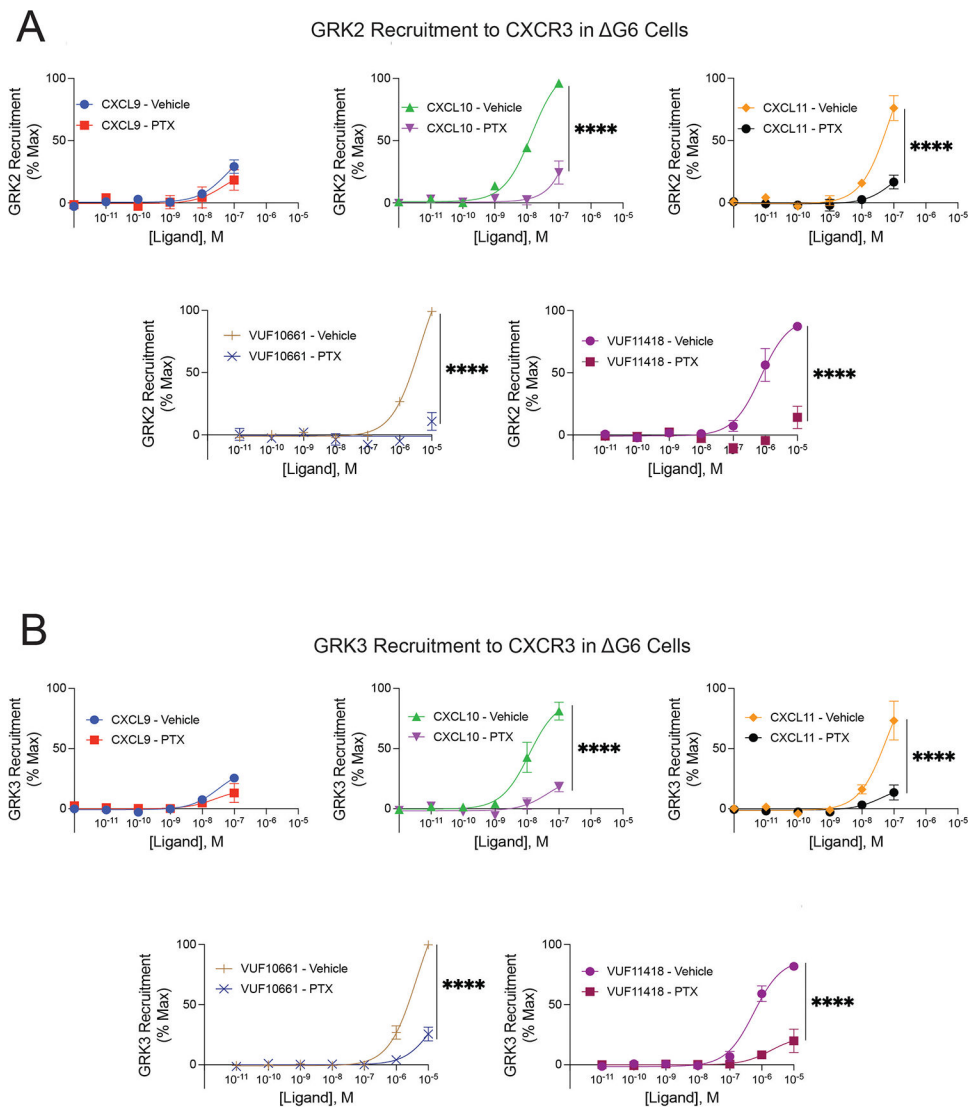


Fig. 3. GRK recruitment to CXCR3 in G protein-deficient cells.

Agonist concentration-response of (A) GRK2 and (B) GRK3 recruitment to CXCR3 with and without pretreatment with 200ng/mL pertussis toxin (PTX) in $\Delta G6$ knockout cells treated with CXCL9, CXCL10, CXCL11, VUF10661 or VUF11418. GRK recruitment was measured using a split luciferase assay involving LgBiT-tagged GRK2 or GRK3 and CXCR3-SmBiT. Data shown are the mean \pm SEM of $n=3$ independent experiments, significance testing by one-way ANOVA with Šidák post hoc testing comparing luminescent signal at maximum dose between treatment conditions (vehicle vs. PTX). Concentration-response curves are normalized to maximum signal observed across all ligands. * $P<0.05$, ** $P<0.005$, *** $P<0.0005$, **** $P<0.0001$.

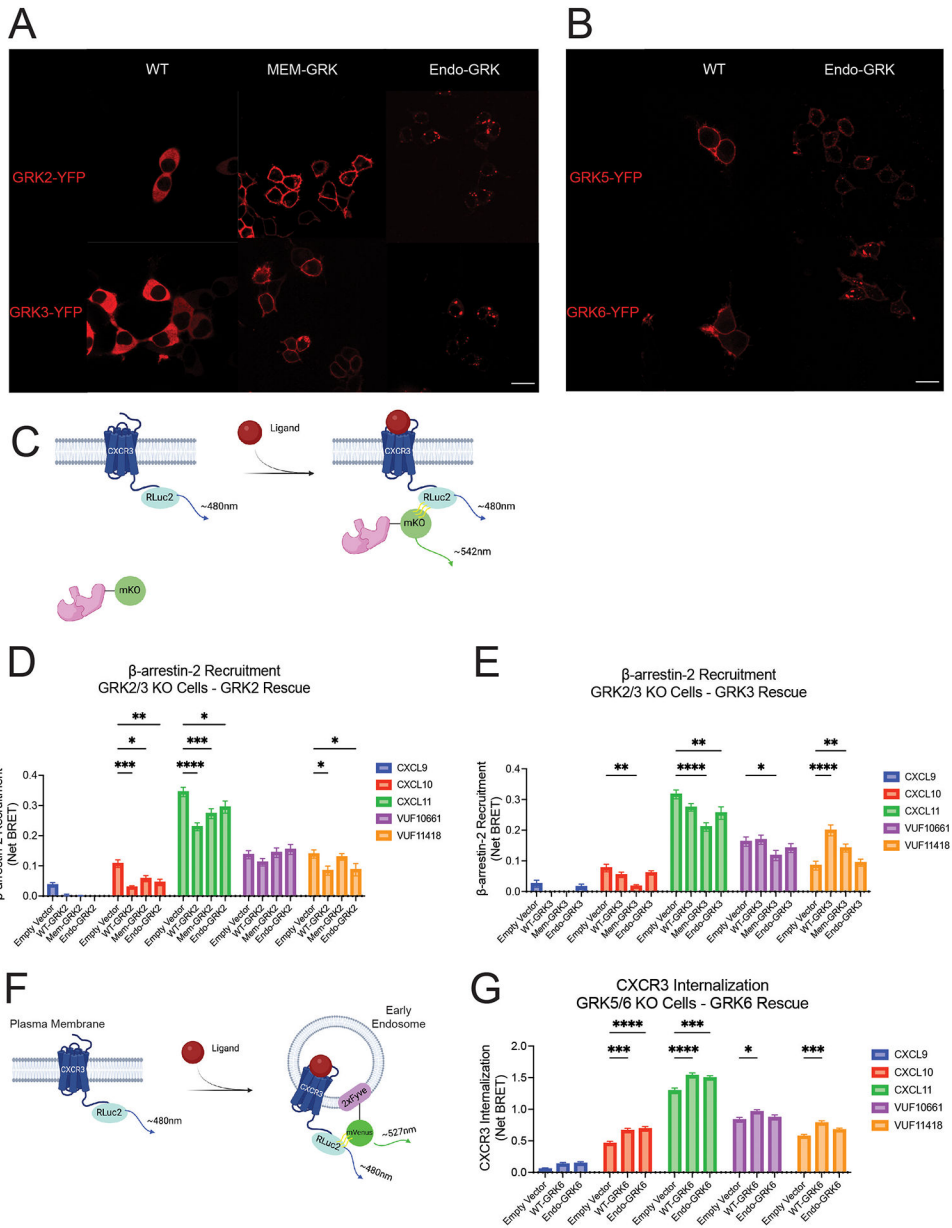


Fig. 4. β -arrestin recruitment and CXCR3 internalization in cells expressing location-specific forms of GRK2, GRK3, or GRK6. (A and B) Confocal microscopy images of HEK293T cells transfected with plasma membrane–localized (Mem) or endosome-localized (Endo) forms of GRK2 or GRK3 (A) and GRK5 or GRK6 (B) tagged with YFP (false red coloring for clarity). Scale bars, 26 μ m. (C) Schematic representation of β -arrestin 2-mKO recruitment to CXCR3-RLuc2 using bioluminescence resonance energy transfer (BRET). (D and E) BRET experiments were performed in GRK2/3 cells transfected with 50ng of the indicated GRK2 constructs (D), 25ng of the indicated GRK3 constructs (E), or empty vector. (F and G) Schematic representation of BRET-based assay (F) to assess CXCR3 internalization in GRK5/6 cells transfected with 50ng of the indicated GRK6 constructs or empty vector (G). Cells were stimulated with 100nM CXCL9, CXCL10, or CXCL11 or 10 μ M VUF10661 or VUF11418

in all experiments. Data shown are the mean AUC \pm SEM of n=5 independent experiments,, significance testing by two-way ANOVA with Tukey post hoc testing conducted between empty vector and other transfections conditions. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001.

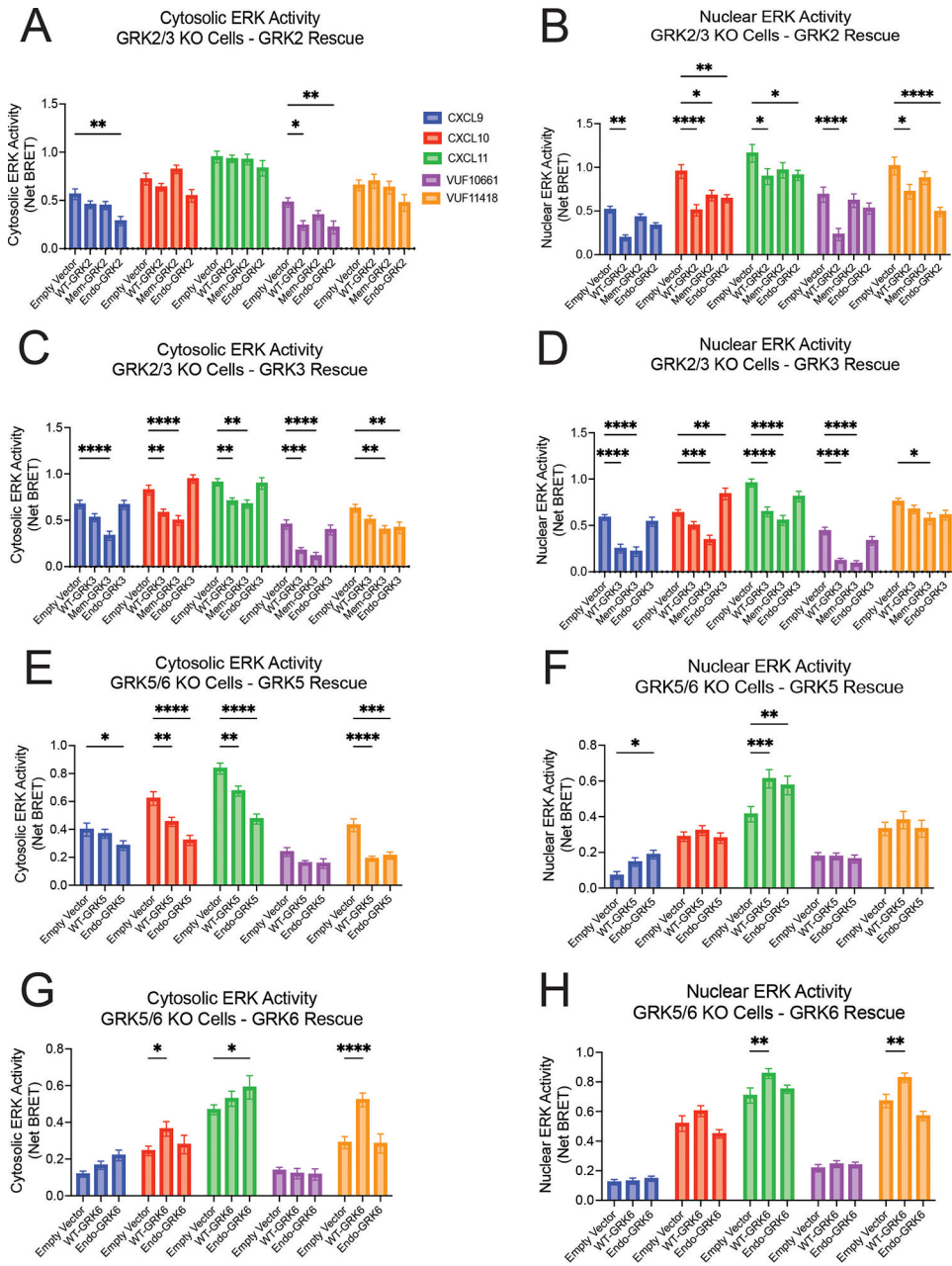


Fig. 5. Cytoplasmic and nuclear ERK activation by GRKs localized to distinct cellular compartments.

(A to D) Cytoplasmic and nuclear ERK activity in GRK2/3 cells expressing the indicated GRK2 (A and B) or GRK3 (C and D) constructs or empty vector. Cytoplasmic and nuclear ERK activity in GRK5/6 cells expressing the indicated GRK5 (E and F) or GRK6 (G and H) constructs or empty vector. 50 ng GRK2 and GRK6 constructs and 25 ng GRK3 constructs were transfected into cells. 100nM CXCL9, CXCL10, or CXCL11 or 10μM VUF10661 or VUF11418 were used in all experiments. Data shown are the mean AUC ± SEM of n=5 independent experiments, significance testing by two-way ANOVA with Tukey post hoc testing conducted between empty vector and other transfections conditions. *P<0.05, **P<0.005, ***P<0.0005, ****P<0.0001.

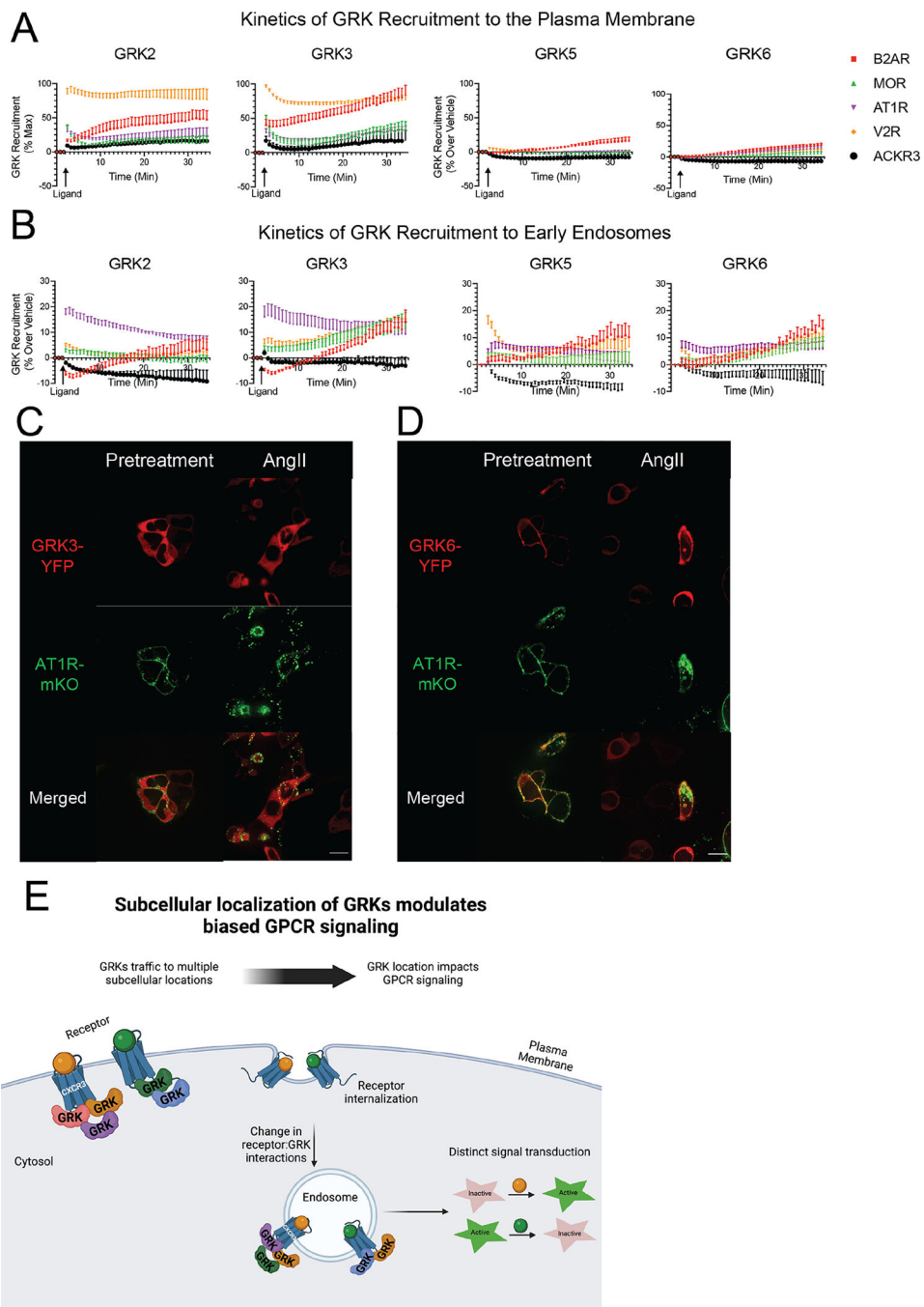


Fig. 6. GRK recruitment to plasma membrane and endosomes at other GPCRs. (A) Recruitment of GRK2, GRK3, GRK5, or GRK6 to the plasma membrane in HEK293 cells upon stimulation of each specified receptor with a single application of the corresponding agonist. (B) Recruitment of GRK2, GRK3, GRK5, and GRK6 to the endosome in HEK293 cells upon stimulation of each specified receptor with a single application of the corresponding agonist. (C and D) Confocal microscopy of mKO-tagged angiotensin II type I receptor (AT1R-mKO) and either GRK3-YFP (C) or GRK6-YFP (D) 45 minutes after addition of vehicle or 10 μ M angiotensin II (AngII). Scale bars, 16 μ m.

The β_2 -adrenergic receptor (B2AR) was stimulated with 10 μ M isoproterenol, the μ -opioid receptor (MOR) was stimulated with 10 μ M DAMGO, the angiotensin II type I receptor (AT1R) was stimulated with 10 μ M angiotensin II, the V₂ vasopressin receptor (V2R) was stimulated with 1 μ M arginine vasopressin, and the atypical chemokine receptor 3 (ACKR3) was stimulated with 10 μ M of WW36. Data shown are the mean \pm SEM of n=5 independent experiments, and are normalized luminescence change over vehicle. Confocal microscopy images are representative of n=3 independent experiments. **(E)** Working model showing how the subcellular location of GRKs influences GPCR signaling induced by biased ligand. Biased ligands (orange and green) activate GPCRs, such as CXCR3, at the plasma membrane and engage distinct GRK subtypes. Following receptor internalization, the GRK subtypes interacting with each receptor can change, demonstrating spatially-dependent GPCR signaling. These unique ligand:receptor:GRK complexes at different cellular locations drive distinct signaling outputs.