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Published in:
Basic and Clinical Pharmacology and Toxicology

Link to article, DOI:
10.1111/bcpt.13935

Publication date:
2023

Document Version
Publisher's PDF, also known as Version of record

Received: 20 April 2023 | Revised: 18 August 2023 | Accepted: 21 August 2023

DOI: 10.1111/bcpt.13935

ORIGINAL ARTICLE

BCPT Basic & Clinical Pharmacology & Toxicology

Re-routing GPR56 signalling using $G\alpha_{12/13}$ G protein chimeras

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Funding information
Kirsten and Freddy Johansens Foundation, Grant/Award Number: 2017-112697; Novo Nordisk Foundation, Grant/Award Numbers: NNF22OC0080625, NNF20OC0062899; Independent Research Fund Denmark, Grant/Award Number: 1054-00086B

Abstract

Adhesion G protein-coupled receptors (aGPCRs) constitute the second largest subclass of the GPCR superfamily. Although canonical GPCRs are explored pharmacologically as drug targets, no clinically approved drugs target the aGPCR family so far. The aGPCR GPR56/ADGRG1 stands out as an especially promising target, given its direct link to the monogenetic disease bilateral frontoparietal polymicrogyria and implications in cancers. Key to understanding GPCR pharmacology has been mapping out intracellular signalling activity. Detection of GPCR signalling in the $G\alpha_s/G\alpha_i/G\alpha_q$ G protein pathways is feasible with second messenger detection systems. However, in the case of $G\alpha_{12/13}$-coupled receptors, like GPR56, signalling detection is more challenging due to the lack of direct second messenger generation. To overcome this challenge, we engineered a $G\alpha_q$ chimera to translate $G\alpha_{12/13}$ signalling. We show the ability of the chimeric $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ to translate basal $G\alpha_{12/13}$ signalling of GPR56 to a $G\alpha_q$ readout in transcription factor luciferase reporter systems and show that the established peptide ligands (P7 and P19) function to enhance this signal. We further demonstrate the ability to directly influence the generation of second messengers in inositol-3-phosphate assays. In the future, these chimeric G proteins could facilitate basic functional studies, drug screenings and deorphanization of other aGPCRs.

KEYWORDS
drug discovery and development, G protein chimeras, G protein-coupled 7TM receptors, outcome measures, Type II: adhesion GPCRs

1 | INTRODUCTION

With 33 members, the adhesion G protein-coupled receptors (aGPCRs) constitute the second-largest subclass (class B2) of endogenous GPCRs, only proceeded by class A.[1] aGPCRs share structural features with the other GPCR families, including the seven transmembrane spanning (7TM) helix core. Approximately 35% of all approved drugs target GPCRs, typically the binding pocket of the 7TM core, however despite growing interest in also exploiting aGPCRs in a similar approach,[2–4] currently no drugs have been approved for this subclass. GPR56/ADGRG1 is especially intriguing, as it is one of few aGPCRs with a known causative role in a

monogenetic human disease, namely, bilateral frontoparietal polymicrogyria (BFPP), an autosomal recessive disorder affecting brain development.[5-8] Various GPR56 mutations that result in impaired receptor functionality, including sub-cellular trafficking, protein expression and auto-proteolysis have been associated with BFPP pathology.[6,7,9] Moreover, GPR56 is strongly associated with cancer and has been proposed as a novel immune checkpoint, rendering it an attractive therapeutic target.[10–12] Dependent on the cancer type, GPR56 was described to be tumour-suppressive or tumour-promoting.[11] Although the molecular mechanisms can be distinct in different cancers, GPR56 activation, for example by collagen-III in breast cancer metastasis or by progastrin in colorectal cancer was described to induce tumour-promoting effects.[13,14] On the contrary, transglutaminase-2 (TG-2) binding to GPR56 stimulated receptor endocytosis and TG-2 degradation, antagonizing melanoma cell metastasis.[15] Based on the distinct functions of GPR56 in cancer, the administration of both agonistic and antagonistic ligands targeting receptor interactions or receptor signalling capabilities could be therapeutically relevant, dependent on the cancer type. GPR56 contains an aGPCR proteolytic site (GPS) in its extracellular GPCR autoproteolysis-inducing (GAIN) domain, at which the receptor is autoproteolytically cleaved into two fragments: the N terminal fragment (NTF) and the C terminal fragment (CTF) that stay non-covalently associated.[7,16] The obligated cleaved GPR56-CTF displays a considerably higher constitutive activity compared to full-length (FL) GPR56, as it lacks negative regulation by the NTF,[17,18] and may resemble the activated receptor construct.[16,19]

GPCRs can couple to the α subunit of the heterotrimeric G protein from the four different subfamilies $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$ and $G\alpha_{12/13}$, leading to the activation of distinct signalling pathways.[20] Stimulation of the G protein subunits $G\alpha_s$, $G\alpha_{i/o}$ and $G\alpha_{q/11}$ all influence the generation of second messengers (cAMP for $G\alpha_s$ and $G\alpha_{i/o}$; IP_3 and Ca^{2+} for $G\alpha_{q/11}$), enabling a robust detection of GPCR activity immediately downstream of the receptor due to the strong and rapid signal amplification for $G\alpha_s$ and $G\alpha_q$, respectively and inhibition for $G\alpha_{i/o}$.[21] Activation of these second messengers can be detected using cAMP bioluminescence resonance energy transfer-based CAMYEL (cAMP sensor using YFP-Epac-Rluc) sensors or protein/dye-based Ca^{2+} sensors,[22] such as GCaMP or Fura-2 which enable the detection of rapidly increasing signals that gets highly amplified.[23]

Although $G\alpha_{i/o}$ signalling can be monitored using cAMP-dependent pathway readouts, advances have been made to redirect $G\alpha_{i/o}$ to $G\alpha_{q/11}$ signalling, enabling a more refined activity assessment with an increased dynamic range of detection.[24] Research on the receptor-G protein interaction has shown the C-terminal amino acids and linker of the Gα subunits to be critical for coupling specificity.[25–27] These findings prompted the creation of chimeric $G\alpha_{qi}$ proteins, in which the last six C-terminal amino acids of $G\alpha_q$, determining receptor specificity, are substituted with those corresponding to $G\alpha_i$. Chimeric $G\alpha_{qi}$ proteins thereby effectively translate $G\alpha_i$ signalling into a more robustly detectable $G\alpha_q$ readout due to the rapid and strong generation of the second messengers IP_3 and Ca^{2+}.[24,28,29] Subsequent successful large-scale screenings and chimera generations allowed for testing a panel of chimeras across different GPCRs.[30]

Contrarily to the other G protein pathways, $G\alpha_{12/13}$ signalling is more challenging to detect because of the lack of an immediate effect on second messenger generation.[31] $G\alpha_{12/13}$-mediated signalling regulates Rho GEF family proteins, which triggers the activation of Rho family GTPases mediating downstream signalling responses.[31] Transcription factors activated further downstream in $G\alpha_{12/13}$ signalling, including serum response element (SRE) and myocyte enhancer factor 2, have been exploited as pathway activation readouts.[32,33] SRE-based systems have previously been used to investigate GPR56 which is signalling mainly through the $G\alpha_{12/13}$ signalling pathways,[34] amongst others by Stoveken et al. who identified an antagonist (dihydromunduletone) and a partial agonist (3-a-acetoxydihydrodeoxygedunin) in an SRE transcription factor-based screening.[35,36] These compounds were used in follow-up studies investigating GPR56 biology, highlighting the potential use of the discovery of novel compounds acting on this receptor.[35,37,38] While SRE-based systems offer a simple tool for screening for $G\alpha_{12/13}$-mediated receptor activity, they have the disadvantage of a lower signalling amplification compared to the highly amplifying second messenger-based assay systems. Additionally, second messengers are generated from precursor molecules that are abundant in the cell. Thus, their generation is not rate-limiting and can be performed in a shorter period.[39]

To overcome the limitations of SRE-based assay systems for GPR56, and inspired by previous work, we generated two novel chimeric G proteins by engineering the $G\alpha_q$ subunit to include the receptor binding motif of $G\alpha_{12}$ and $G\alpha_{13}$, respectively, as well as a myristoylation site (myr) to further enhance the membrane anchoring.[24,30] With these chimeras ($G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$), GPR56 signalling can be re-routed from $G\alpha_{12/13}$ to $G\alpha_q$ signalling, allowing a more robust signal amplification. Thus, the co-transfection with the chimeric G proteins could offer a benefit in enhancing a subtle receptor activity and providing a more direct and upstream signalling readout, since GPCR signalling is shown to involve crosstalk downstream of second messengers between the different G protein signalling pathways.[40,41]

Because luciferase-based transcription factor assays have been used to screen GPR56 in previous studies,[35,36] we applied them in our initial assessments of the new chimeras. We show the ability of $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ to translate basal and peptide-induced GPR56 $G\alpha_{12/13}$ activation to $G\alpha_q$ signalling readouts in nuclear factor of activated T cells (NFAT) transcription factor luciferase assay,[42] and further to direct second messenger generation in an IP_3 accumulation assay.

A $G\alpha_{12/13}$-to-$G\alpha_q$ readout assay could advance the understanding of signalling and physiological functions for $G\alpha_{12/13}$ aGPCRs, and potentially improve pharmaceutical screenings, and as such our approach adds another option to the toolbox for testing both GPR56 signalling and other adhesion GPCRs known to signal in the $G\alpha_{12/13}$ pathway.

2 | MATERIALS AND METHODS

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.[43]

2.1 | Cell culture and transfection

HEK293T cells (ATCC #CRL-3216, HEKT), HEK293 parental cells (ATCC #CRL-1573, WT) and HEK293 G protein KO cells[44] (KO) that lack $G\alpha_s$, $G\alpha_{olf}$, $G\alpha_z$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$—but not $G\alpha_{i/o}$—were used in this study. All cells were cultured in DMEM (1X) + GlutaMAX™-I (Gibco®), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich®) and 1% Penicillin + Streptomycin (P/S; Substrate department, University of Copenhagen) at 5% CO2, 95% humidity, at 37°C. Transfection of all cells was performed by lipofection using Lipofectamine 2000® (Invitrogen, Carlsbad, CA) as described previously.[45]

2.2 | Reagents

Human wild-type C-terminally Myc and FLAG-tagged FL GPR56 cDNA was cloned into the expression vector pCMV6. Generation of the N-terminal deletion mutant GPR56-CTF (Δ2-382) was performed by GenScript Europe (Rijswijk, Netherlands). The mutant GPR56-CTF GPR56-A386M was generated using restriction cloning. GPR56-A386M cDNA was amplified by PCR using the primers 5′-CATCATGGTACCATGGTGCTGATGGTCTC CTCGGTG-3′ and 5′-ATATAAAGCGGCCGCGTACG CGTGATG-3′ containing a KpnI or NotI restriction site, respectively. After digestion, cDNA was ligated into pCMV6. The chimeric G protein $G\alpha_{\Delta 6qi4myr}$ was kindly provided by Evi Kostenis. Mutations of $G\alpha_{\Delta 6qi4myr}$ were carried out by GenScript (Rijswijk, Netherlands). For the generation of $G\alpha_{\Delta 6q12myr}$, the last four C-terminal amino acids of $G\alpha_{\Delta 6qi4myr}$ (CGLF) were substituted with the last four C-terminal amino acids corresponding to $G\alpha_{12}$ (IMLQ). Similarly, for the generation of $G\alpha_{\Delta 6q13myr}$, the last four C-terminal amino acids of $G\alpha_{\Delta 6qi4myr}$ (CGLF) were substituted with the last four C-terminal amino acids corresponding to $G\alpha_{13}$ (LMLQ). All mutations were verified by sequencing performed by Eurofins Danmark (Galten, Denmark). Luciferase reporter vectors pGL3-NFAT-Luc, pGL3-SRE-Luc and pFA-CRE/pFR-Luc were purchased from Stratagene (Amsterdam, Netherlands).

GPR56 peptide ligands were synthesized in the laboratory of Katrine Qvortrup (Technical University of Denmark) according to the sequences of P7 (TYFAVLM-NH_2) and P19 (TYFAVLMQLSPALVPAELL-NH_2), previously published by Stoveken et al. and Wilde et al.[35,46,47] Peptides were dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 10 mM.

2.3 | Luciferase transcription factor reporter assays

For all luciferase assays, cells were seeded in poly-D-Lysine-coated (Sigma-Aldrich) white 96-well plates, $3.5*10^4$ per well (Greiner bio-one), incubated overnight and transfected the next day using lipofection.[45] For transfection, gene doses (0–5 ng plasmid DNA) of the receptors (GPR56-FL/GPR56-CTF) were transfected with 50 ng of the respective luciferase reporter and with/without 15 ng G protein chimera per well. For endothelin A (ETA), 0/30 ng of plasmid DNA were transfected with/without 15 ng G protein chimera per well. Five hours post-transfection the medium was replaced with growth medium and supplemented with 10% heat-inactivated FBS and 1% P/S for NFAT and cAMP response element (CRE) assays or serum-free growth medium with 1% P/S for SRE assays and incubated for 18 h. For ligand stimulation experiments of GPR56, 100 μL growth medium containing 20 μM of the respective peptide ligand was incubated for 2 h. After 2 h, 50 μL growth medium containing 20 μM peptide ligand was added on top of the growth medium and incubated for 4 h, as described in previous studies.[35] For ligand stimulation experiments of ETA, 100 μL growth medium containing 100 nM of endothelin-1 (ET-1) was incubated for 6 h. The final DMSO concentrations did not exceed 1% of the total medium volume. The culture medium was aspirated 18 h post-transfection and each well was washed with 100 μL phosphate-buffered saline

(PBS). Subsequently, 100 μL of a 1:1 Steadylite plus substrate (PerkinElmer®)-PBS solution was added and cells were incubated in the dark for 30 min at room temperature. Luminescence was read with an EnVision Multilabel reader (model 2104, PerkinElmer).

2.4 | IP_3 accumulation assay

For all IP_3 accumulation assays, cells were seeded in poly-D-Lysine-coated (Sigma-Aldrich) clear 96-well plates, $3.5*10^4$ per well (Greiner bio-one), incubated overnight and transfected the next day using lipofection.[45] For transfection, gene doses (0–5 ng plasmid DNA) of the receptors (GPR56- FL/GPR56-CTF) were transfected with/without 15 ng G protein chimera per well. For ETA, 0/30 ng of plasmid DNA were transfected with/without 15 ng G protein chimera per well. The IP_3 accumulation assays were performed as described previously.[48] Briefly, the culture medium was aspirated 24 h post-transfection and replaced with 100 μL growth medium containing 0.5 μL of 1 mCi/mL myo-[2-^{3}H(N)]-inositol (NET114A005MC, PerkinElmer) and incubated overnight at 5% CO2, 95% humidity, at 37°C. After 24 h incubation, the medium was aspirated and wells were washed twice with Hanks' Balanced Salt Solution (HBSS, Gibco). For GPR56/chimera co-expression experiments, 100 μL HBSS assay buffer containing 10 mM LiCl was added to each well and incubated for 90 min. For ligand stimulation experiments of ETA, 100 μL assay buffer containing 100 nM of the ET-1 was incubated for 90 min. The final DMSO concentrations did not exceed 1% of the total medium volume. After the incubation, plates were put on ice, assay medium was aspirated and cells were lysed using 40 μL of 10 mM formic acid for 30–60 min. Hereafter, 35 μL of the lysate were transferred to a new white 96-well plate and 60 μL of 12.5 mg/mL SPA-YSi (PerkinElmer) bead solution was added. Plates were sealed and shaken thoroughly for 30 min. Afterwards, plates were spun down at 1500 rpm for 5 min and radioactivity was measured using MicroBeta2® (2450-0060, PerkinElmer) with an 8 h delay.

2.5 | Quantification and statistical analysis

For all signalling assays, three to seven independent experiments were performed each in triplicates. Data are depicted as the mean of the replicates ± standard error of means (SEM). All data analysis was performed using GraphPad Prism version 9.4.1 using *t*-test or ANOVA. p values < 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Generation of $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ chimeras

The two chimeric G proteins, $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$, were generated on the basis of the chimeric G protein $G\alpha_{\Delta 6qi4myr}$. $G\alpha_{\Delta 6qi4myr}$ is a $G\alpha_i$-to-$G\alpha_q$ G protein chimera, which has the last six $G\alpha_q$ residues deleted and the binding-determining $G\alpha_i$ residues (CGLF) inserted, harbours a myristoylation (G201-R208; sequence GGQRSERR),[49] and has been widely used in redirecting G protein signalling.[24,28,29] For the $G\alpha_{12}$-to-$G\alpha_q$ signalling re-directing $G\alpha_{\Delta 6q12myr}$, the last four C-terminal amino acids of $G\alpha_{\Delta 6qi4myr}$ (CGLF) were substituted with the corresponding four C-terminal amino acids of $G\alpha_{12}$ (IMLQ) (Figure 1A). Accordingly, for $G\alpha_{13}$ the last four C-terminal amino acids of $G\alpha_{\Delta 6qi4myr}$ (CGLF) were substituted with the corresponding four C-terminal amino acids of $G\alpha_{13}$ (LMLQ) to obtain the chimeric G protein $G\alpha_{\Delta 6q13myr}$ (Figure 1A).[24,30] These C-terminal residues of the Gα subunits have previously been reported to be significant for receptor specificity and G protein activation.[25,26,50–52] The expected outcome of the substitutions was a redirection of the canonical $G\alpha_{12/13}$ signalling to $G\alpha_q$ signalling for $G\alpha_{12/13}$-coupled GPCRs (Figure 1B).

3.2 | GPR56-CTF, but not GPR56-FL activates NFAT signalling pathway

We first assessed the signalling of FL human GPR56 (GPR56-FL) and GPR56-CTF (Δ2-382), a receptor construct truncated at the GPS-cleavage site and thus lacking the entire NTF.[46] For a handful of receptors, it has previously been established that truncating aGPCRs at the GPS site led to enhanced signalling activity[17,53] and that the peptide stretches directly following the GPS site towards the TM1 region can act as a tethered agonist to activate the receptor,[19] thus the CTF construct may resemble an activated receptor construct.[16,19]

To test that both FL and CTF constructs were functional, we assessed the signalling in HEK293T cells using a panel of transcription factor luciferase assays as done previously.[17,53] We used NFAT which is reported to read out mainly in $G\alpha_q$, SRE which is reported to read out mainly in $G\alpha_{12/13}$, and CRE which is reported to readout mainly in $G\alpha_{s/i/q}$, (Figure 1C–E).[48]

While no significant basal NFAT activity was observed for GPR56-FL, increasing concentrations of GPR56-CTF receptor cDNA resulted in signalling activity in NFAT, reaching a maximum of 1.8-fold over the cellular baseline (Figure 1C). In SRE, expression of FL and

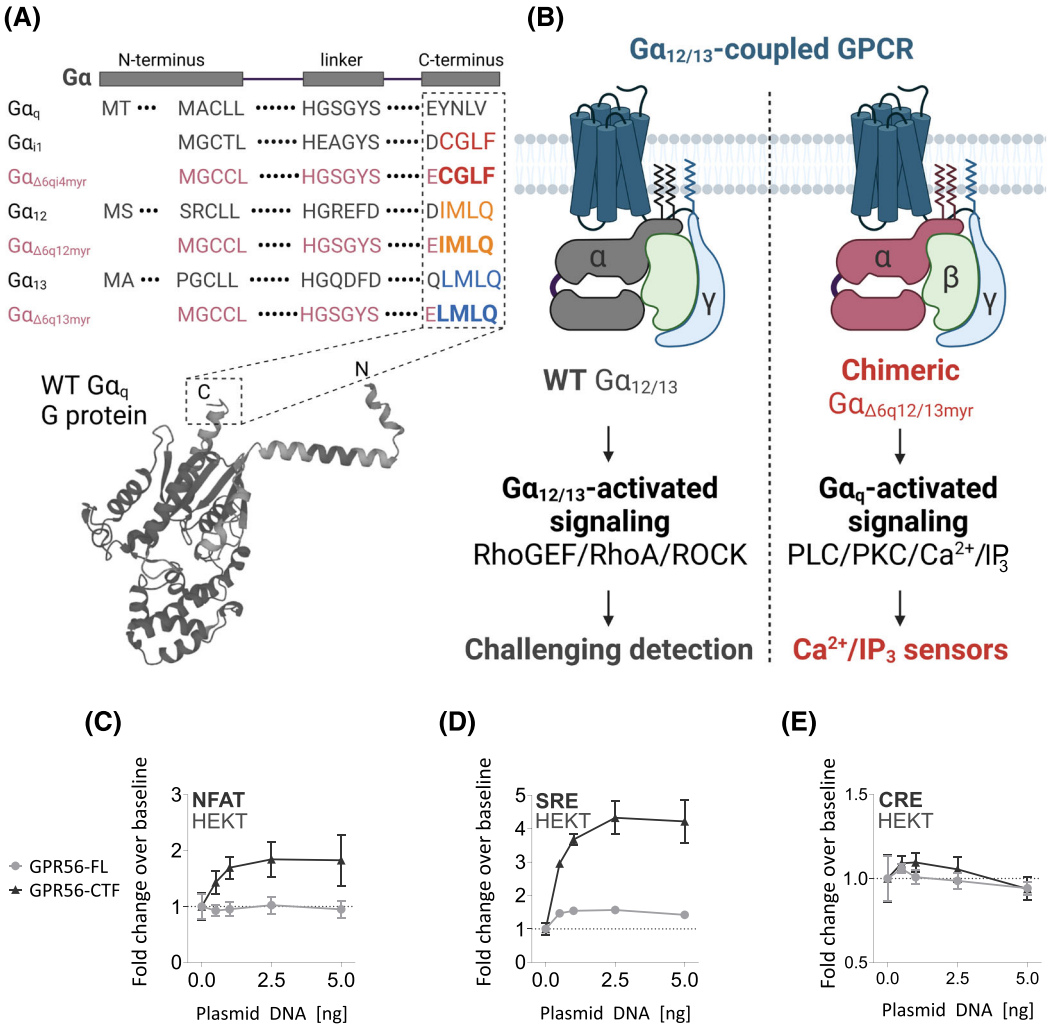

FIGURE 1 Generation of chimeric G proteins $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ for redirecting GPR56 signalling. (A) Sequence alignment of the N-terminal, linker, and C-terminal amino acid sequences of wild-type (black) and mutant (red) Gα subunits. Gaps (⋯) were introduced for optimal sequence alignment. $G\alpha_{\Delta 6qi4myr}$, $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ denote mutant $G\alpha_q$ constructs in which the four C-terminal amino acids are replaced with the corresponding $G\alpha_i$ ($G\alpha_{\Delta 6qi4myr}$), $G\alpha_{12}$ ($G\alpha_{\Delta 6q12myr}$) or $G\alpha_{13}$ ($G\alpha_{\Delta 6q13myr}$) sequence, respectively, with substituted residues being highlighted bold. The single-letter amino acid code is used. Structure of $G\alpha_q$ from AlphaFold with denoted N and C-terminus. (B) Cartoon outlining the principle of the $G\alpha_{12/13}$-to-$G\alpha_q$ signalling redirection of a $G\alpha_{12/13}$-coupled G protein-coupled receptor (GPCR) using the chimeric G proteins. Image created with BioRender.com. (C–E) Gene dose-dependent basal signalling of GPR56-full-length (GPR56-FL) and GPR56-C terminal fragment (GPR56-CTF) in (C) nuclear factor of activated T cells (NFAT), (D) serum response element (SRE) and (E) cAMP response element (CRE) luciferase reporter assays in HEK293T cells (HEKT). Data in (C–E) represent means normalized as fold change over baseline (empty vector) ± standard error of means (SEM) of five to six independent experiments, each performed in triplicates.

CTF constructs resulted in increased signalling activity with GPR56-FL reaching a maximum of 1.5-fold and GPR56-CTF reaching a maximum of 4-fold over the cellular baseline (Figure 1D). As expected, no significant signalling activity in CRE was observed for GPR56-FL or GPR56-CTF (Figure 1E), showing that both FL and CTF constructs are functional and suitable for testing the G protein chimeras.

3.3 | $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ redirect GPR56 signalling through $G\alpha_{12/13}$ to a $G\alpha_q$ readout

The ability of $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ to redirect signalling was first verified using a HEK293 knockout (KO) cell line lacking $G\alpha_s$, $G\alpha_{olf}$, $G\alpha_z$, $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{12}$ and $G\alpha_{13}$—but not $G\alpha_{i/o}$—using the class A ETA

receptor (Figure 2A–D).[44,54] We chose ETA as a control because it displayed a strong ligand-dependent $G\alpha_{12/13}$ signalling activation in previous studies using a similar approach.[55] In the KO cell line, lacking the relevant Gα subunits, we observed robust ligand-dependent ETA signalling in both SRE transcription and IP_3 accumulation readouts (Figure 2B,D) only in conditions with $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ co-transfection, establishing that both chimeras were able to redirect signalling.

Having established that $G\alpha_{\Delta 6q12myr}$ or $G\alpha_{\Delta 6q13myr}$ functioned to generate a $G\alpha_q$ rerouting signalling response, we next tested the signalling redirection for GPR56-FL and GPR56-CTF in HEK293T cells using a

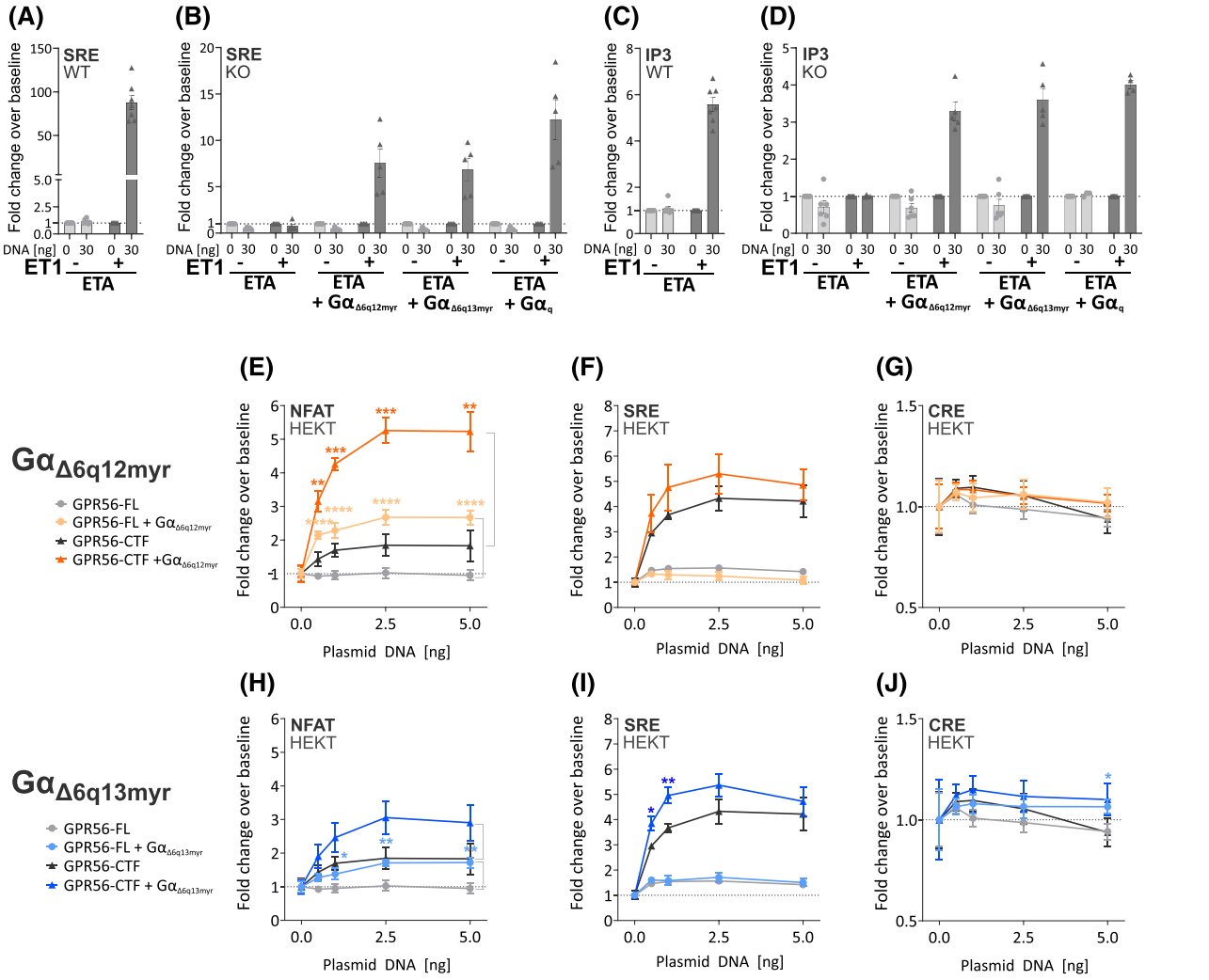

FIGURE 2 Signalling redirection using chimeric G proteins. (A–B) Basal (light grey) and endothelin-1 (ET-1) agonist-induced (dark grey) signalling of endothelin A (ETA) in serum response element (SRE) in (B) HEK293 wild type (WT) cells and (B) HEK293 G protein knockout (KO) cells without/with $G\alpha_{\Delta 6q12myr}$/$G\alpha_{\Delta 6q13myr}$/$G\alpha_q$ as fold change over baseline (empty vector). $n = 7$. (C–D) Basal (light grey) and ET-1-induced (dark grey) effect of ETA on IP_3 turnover in (C) HEK293 WT cells and (D) HEK293 G protein KO cells without/with $G\alpha_{\Delta 6q12myr}$/$G\alpha_{\Delta 6q13myr}$/$G\alpha_q$ as fold change over baseline (empty vector). $n = 4$–6. (E–G) Gene dose-dependent signalling of GPR56-full-length (GPR56-FL) without (grey circles) or with (light orange circles) $G\alpha_{\Delta 6q12myr}$ and of GPR56-C terminal fragment (GPR56-CTF) without (black triangles) or with (dark orange triangles) $G\alpha_{\Delta 6q12myr}$ in (E) nuclear factor of activated T cells (NFAT), (F) SRE and (G) cAMP response element (CRE) luciferase reporter assays in HEK293T cells (HEKT). (H–J) Gene dose-dependent signalling of GPR56-FL without (grey circles) or with (light blue circles) $G\alpha_{\Delta 6q13myr}$ and of GPR56-CTF in without (black triangles) or with (dark blue triangles) $G\alpha_{\Delta 6q13myr}$ in (H) NFAT, (I) SRE and (J) CRE luciferase reporter assays in HEK293T cells (HEKT). (E–J) Depicted basal signalling profiles for GPR56-FL (grey circles) and GPR56-CTF (black triangles) are reprinted data from Figure 1. Data in (A–J) represent means normalized as fold change over baseline (empty vector) ± standard error of means (SEM) of 4–7 independent experiments, each performed in triplicates. Unpaired *t*-tests were performed for comparison of experimental conditions with baseline signalling control (empty vector). (*$p \leq 0.005$; **$p \leq 0.002$; ***$p \leq 0.0001$; ****$p < 0.0001$).

panel of transcription factor luciferase assays (Figure 2E–J). Increasing concentrations of GPR56-FL co-transfected with $G\alpha_{\Delta 6q12myr}$ resulted in a significant increase of NFAT activity, reaching a maximum of 2.8-fold over baseline (Figure 2E). For GPR56-CTF, co-transfection with $G\alpha_{\Delta 6q12myr}$ also resulted in a significant increase in NFAT activity, reaching a maximum of 5.2-fold over baseline (Figure 2E). In SRE (Figure 2F) and CRE (Figure 2G), neither co-transfection of GPR56-FL nor GPR56-CTF with $G\alpha_{\Delta 6q12myr}$ had a significant effect on signalling.

GPR56-FL co-transfection with $G\alpha_{\Delta 6q13myr}$, resulted in a significant increase in NFAT activity with increasing receptor cDNA, reaching a maximum of 1.8-fold over cellular baseline (Figure 2H), whereas co-transfection of GPR56-CTF displayed only a tendency for an increase. While co-transfection of GPR56-FL with $G\alpha_{\Delta 6q13myr}$ had no measurable effect in SRE, a significant signalling increase mediated by the chimeras was observed for increasing receptor amounts of GPR56-CTF with $G\alpha_{\Delta 6q13myr}$ (Figure 2I). No strong signalling-enhancing effect was observed in CRE. However, in the presence of $G\alpha_{\Delta 6q13myr}$, GPR56-FL signalling was kept at a constant level above cellular baseline signalling, while basal GPR56-FL signalling in the absence of the chimeras was decreasing (Figure 2J).

In summary, the two chimeras increased the NFAT activity of both GPR56-FL and GPR56-CTF. In both cases, $G\alpha_{\Delta 6q12myr}$ elicited a greater increase over control than $G\alpha_{\Delta 6q13myr}$. Signalling in SRE was not altered for GPR56-FL, while it was significantly enhanced for GPR56-CTF with $G\alpha_{\Delta 6q13myr}$, demonstrating the feasibility of redirecting GPR56-FL and CTF $G\alpha_{12/13}$ activity to $G\alpha_q$ signalling using chimeric G proteins.

Next, we examined the ability of peptide ligands to further enhance the $G\alpha_{\Delta 6q12myr}$/$G\alpha_{\Delta 6q13myr}$ signalling redirection in NFAT using the HEK293 G protein KO cells. Previous studies established the synthetic GPR56-activating peptide ligands P7 (TYFAVLM-NH_2), mimicking the GPR56 tethered agonist (Figure 3A),[35,46,47] and P19 (TYFAVLMQLSPALVPAELL-NH_2) which originally was developed for activating GPR114/ADGRG5, but shares the conserved tethered agonist sequence with GPR56[47] and displayed greater GPR56-CTF receptor activation compared to P7.[35]

For GPR56-FL, stimulation with 20 μM P7 and P19 in the presence of $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ resulted in a receptor-dependent increase over the vehicle-treated baseline (Figure 3B). For GPR56-CTF, we observe a similar trend for P19 treatment with $G\alpha_{\Delta 6q12myr}$ (Figure 3C). In the controls without receptor co-transfection treatment with 20 μM P7 or P19 did not result in an increase for $G\alpha_{\Delta 6q12myr}$, while P19 showed a slight increase in the

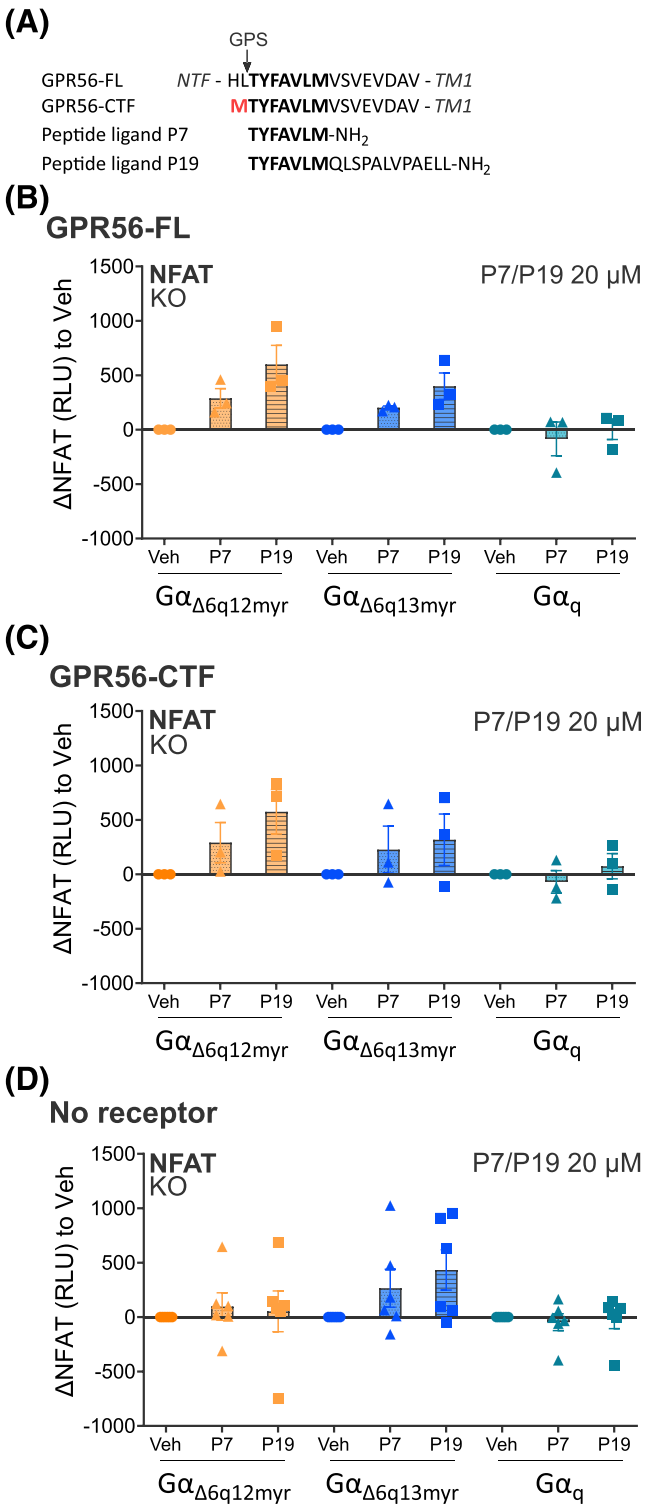

FIGURE 3 Legend on next page.

presence of $G\alpha_{\Delta 6q13myr}$ (Figure 3D). Ratio paired *t*-tests were performed on the raw NFAT data (data not shown) and showed a significant signal increase upon peptide stimulation for GPR56-FL with both $G\alpha_{\Delta 6q12myr}$ with P7 ($p = 0.0246$), or P19 ($p = 0.0133$), and $G\alpha_{\Delta 6q13myr}$ with

FIGURE 3 Peptide-activated signalling redirection. (A) Schematic of the tethered agonist region (bold black) of GPR56-full-length (GPR56-FL) and GPR56-C terminal fragment (GPR56-CTF) in comparison with the synthetic peptide ligands P7 and P19. Displayed is the amino acid sequence between N terminal fragment (NTF) and transmembrane helix 1 (TM1). The arrow indicates the GPCR proteolytic site (GPS) of GPR56-FL and the artificially inserted methionine for GPR56-CTF is displayed in red. (B) Bar charts represent the difference (Δ) of nuclear factor of activated T cells (NFAT) signalling activation of GPR56-FL compared to vehicle (Veh; circles) for 20 μM P7 (triangles) or 20 μM P19-treated (squares) conditions in the presence of $G\alpha_{\Delta 6q12myr}$ (orange), $G\alpha_{\Delta 6q13myr}$ (blue) or $G\alpha_q$ (cyan). (C) Bar charts represent the difference (Δ) of NFAT signalling activation of GPR56-CTF compared to vehicle (Veh; circles) for 20 μM P7 (triangles) or 20 μM P19-treated (squares) conditions in the presence of $G\alpha_{\Delta 6q12myr}$ (orange), $G\alpha_{\Delta 6q13myr}$ (blue) or $G\alpha_q$ (cyan). (D) Bar charts represent the difference (Δ) of NFAT signalling activation of the no receptor control compared to vehicle (Veh; circles) for 20 μM P7 (triangles) or 20 μM P19-treated (squares) conditions in the presence of $G\alpha_{\Delta 6q12myr}$ (orange), $G\alpha_{\Delta 6q13myr}$ (blue) or $G\alpha_q$ (cyan). Data in (B–D) represent means normalized as difference (Δ) to baseline (respective vehicle treatment) ± standard error of means (SEM) of three independent experiments, each performed in triplicates.

P7 ($p = 0.0427$), or P19 ($p = 0.0133$). For GPR56-CTF and the no receptor control, the observed differences were not statistically significant. For all receptors and treatments, no signal activation was observed in any of the conditions co-transfected with $G\alpha_q$, indicating $G\alpha_{\Delta 12/13}$-chimera specificity.

Finally, we sought to establish whether the chimeras directly affect the second messenger generation. We assessed the basal effect of GPR56 co-transfection with $G\alpha_{\Delta 6q12myr}$ in IP_3 accumulation assays in HEK293 G protein KO cells (Figure 4A). Since $G\alpha_{\Delta 6q12myr}$ displayed a greater increase in the transcription factor assays, we decided to focus on this chimera for investigating IP_3 accumulation. Increasing concentrations of GPR56-FL co-transfected with $G\alpha_{\Delta 6q12myr}$ resulted in a significant increase in IP_3-turnover, reaching a maximum of 1.3-fold over baseline (Figure 4B). Additionally, we tested the basal IP_3 accumulation of GPR56-CTF and GPR56-A386M. GPR56-A386M resembles a CTF construct where the first three residues of the tethered agonist (T, Y, F) are deleted, and A386 is substituted for an initiator methionine. This construct shows particularly low constitutive activity as previously described by Stoveken et al.[35,46]

While the absence of exogenous G proteins did not induce an immediate IP_3 response (Figure 4C), co-transfection of GPR56-CTF with $G\alpha_{\Delta 6q12myr}$ resulted in a significant increase in IP_3 accumulation compared to no receptor control and low activity GPR56-A386M (Figure 4D). Co-transfection with $G\alpha_{\Delta 6q13myr}$ or $G\alpha_q$ did not induce any significant IP_3 responses (Figure 4E,F), indicating that for GPR56 the $G\alpha_{\Delta 6q12myr}$ chimera is more suited for detection of IP_3 generation. These results confirm the direct $G\alpha_{12/13}$-to-$G\alpha_q$ signalling redirection of $G\alpha_{\Delta 6q12myr}$, for GPR56 and GPR56-CTF. In the future, experiments are required to explore the more acute effects and kinetics of the ligand-activated receptor signalling on IP_3 turnover in the presence of G protein chimeras.

4 | DISCUSSION

By developing and studying the two novel chimeric G proteins $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$, we here highlight their use in fundamental receptor research for $G\alpha_{12/13}$-coupled adhesion GPCRs. We were focusing on GPR56 which resembles an attractive therapeutic target given its association with cancer and its causative role in the brain-affecting disorder BFPP.[6,11,56] Previous studies evaluating GPR56 signalling have used SRE and NFAT-based luciferase reporters, GTPγS binding assays, Rho-pulldown assays and β-arrestin assays.[16,46,53,57,58] Especially SRE reporter assays have proven useful for the identification of GPR56-targeting small molecules.[35,36] Since luciferase reporter gene assays provide a feasible and scalable tool for GPCR activity detection with documented success in compound discovery, they were used in this study as a first step to evaluate chimeric G protein signalling-redirection. When we applied $G\alpha_{\Delta 6q12myr}$ and $G\alpha_{\Delta 6q13myr}$ to transcription factor luciferase assays with the $G\alpha_{12/13}$-coupled adhesion receptor GPR56-FL and the truncated GPR56-CTF, we were able to demonstrate their $G\alpha_{12/13}$-to-$G\alpha_q$ signalling-redirecting.

$G\alpha_{qs/i/o}$ chimeric G proteins have been used in adhesion GPCR research for a handful of receptors.[47,59–65] However, to date, no screenings for class B2 GPCRs with G protein chimeras have been performed using $G\alpha_{12/13}$-to-$G\alpha_q$ chimeras. We showed the basal signalling redirection for GPR56-FL and GPR56-CTF in NFAT, where $G\alpha_{\Delta 6q12myr}$ displayed a stronger redirection capability than $G\alpha_{\Delta 6q13myr}$. Interestingly for the ETA receptor, both chimeras displayed comparable re-routing abilities in SRE, suggesting that the effect of the chimeric G proteins on signalling redirection could vary across different $G\alpha_{12/13}$-coupled receptors.

While previous studies have evaluated the effect of stimulating GPR56 with peptide agonists predominantly in GTPγS binding assays and SRE-based transcription factor assays,[35,46] we demonstrated their effect using the

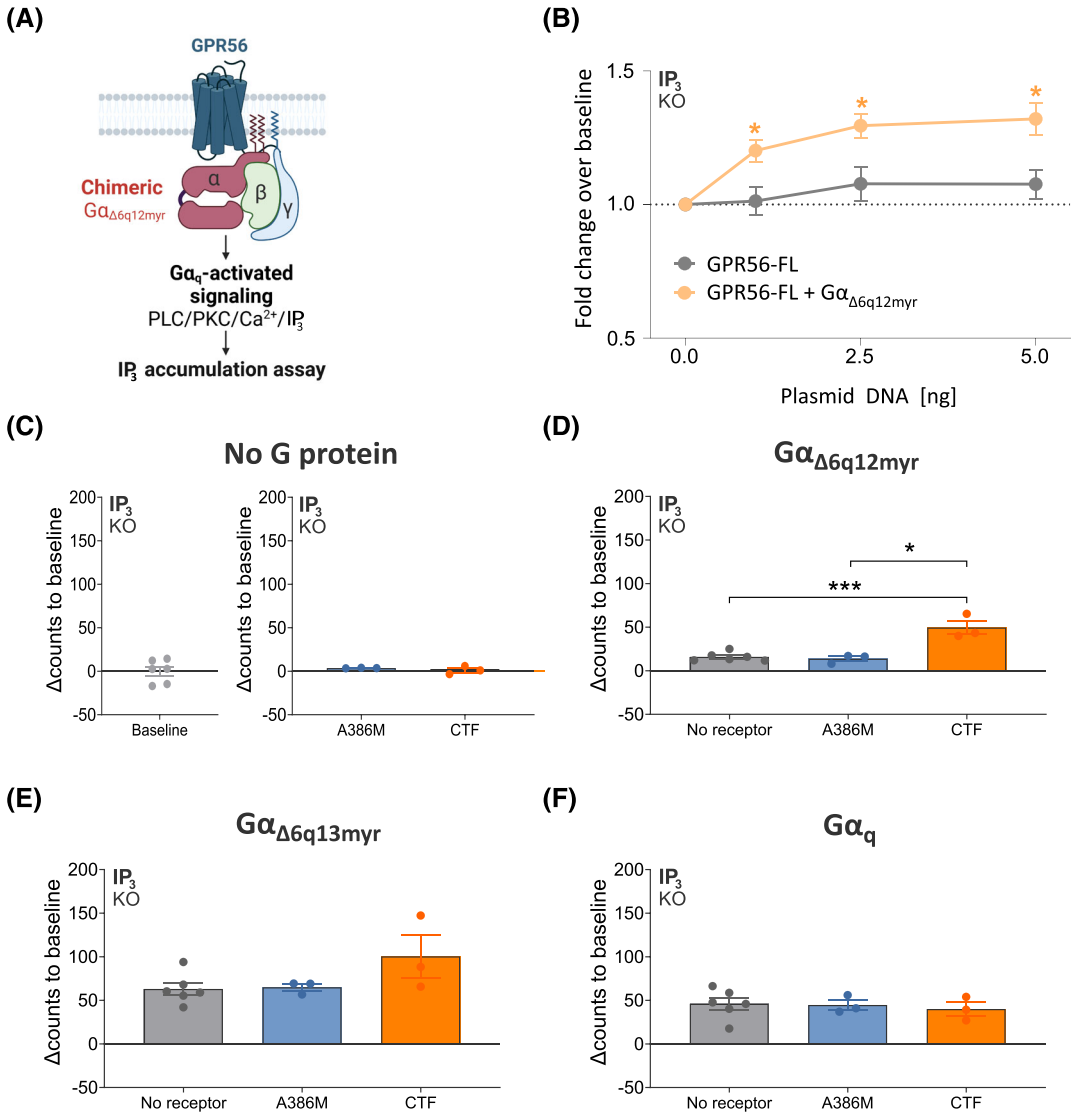

FIGURE 4 Redirection of GPR56 signalling to an IP_3 readout. (A) Schematic principle of GPR56 signalling redirection to IP_3 accumulation assay using $G\alpha_{\Delta 6q12myr}$. (B) Gene dose-dependent IP_3 turnover of GPR56-full-length (GPR56-FL) in the absence (grey circles) or presence (orange circles) of $G\alpha_{\Delta 6q12myr}$ normalized to the no receptor control. Data represent means normalized as fold change over baseline (empty vector) ± standard error of means (SEM) of four independent experiments, each performed in triplicates. (C–F). Bar charts represent the difference (Δ) of counts to (C) baseline (light grey) IP_3 turnover of no receptor (dark grey), GPR56-A386M (A386M; blue) and GPR56-CTF (CTF; orange) for (C) no G protein, (D) $G\alpha_{\Delta 6q12myr}$, (E) $G\alpha_{\Delta 6q13myr}$ and (F) $G\alpha_q$. (C–F) Data represent means normalized difference (Δ) of counts to baseline (empty vector with no G proteins) ± SEM of three independent experiments, each performed in triplicates. (B–F). Unpaired *t*-tests were performed for comparison of experimental conditions. (*$p \leq 0.005$; **$p \leq 0.002$; ***$p \leq 0.001$; ****$p < 0.0001$).

chimeric G proteins in NFAT-based assays. For GPR56-FL with $G\alpha_{\Delta 6q12myr}$, we observe a signalling increase over receptor control that is slightly higher for P19 compared to P7. The stronger activation of GPR56-CTF with P19 is in line with previous work by Stoveken and colleagues that showed a roughly 1.5-fold increase of P19 stimulation over P7 stimulation for the related GPR56 A386M CTF construct.[35] While GPR56-FL was lacking the ability to be activated on membranes using P7 in GTPγS binding assays, other studies have reported signalling activation of GPR56-FL by the P7 peptide in BRET-based assays in HEK293 cells,[66] and activation of Ca^{2+} signalling in MIN6 mouse pancreatic beta cells.[67] Additionally, murine GPR56-FL could be activated by the GPR56/GPR114 peptide ligand P19,[68] and administration of P7 or P19 resulted in GPR56-mediated antidepressant-like effects in mice and upregulated related signalling pathways.[69]

Interestingly, treatment with P19 in $G\alpha_{\Delta 6q13myr}$-transfected cells resulted in a slight signalling activation in

the absence of GPR56. The peptide ligands used in this study resemble the tethered agonist sequence that is highly conserved across the whole aGPCR family, with the amino acid consensus sequence being TXFAVLMXX.[46,70] GPR56 shares the tethered agonist sequence with GPR114 (TYFAVLM) and displays strong sequence homology with GPR128, LPHN1, LPHN3 (all TNFAVLM), LPHN2 (TNFAILM), and CELSR2 (TSFAVLM).[70] Peptide stimulation of aGPCRs was reported to activate receptors in the same subfamily, as well as across subfamilies.[61] While HEK293 aGPCR expression studies found no endogenous expression of GPR56, a slight expression of GPR114 and expression of CELSR2, LPHN2 and LPHN3 was detected.[71] Therefore, we speculate that the P7/P19 peptides could be able to activate related aGPCRs in HEK239 G protein KO cells.

Finally, we show GPR56 signalling redirection for IP_3 second messenger accumulation for GPR56-FL and GPR56-CTF. Here, $G\alpha_{\Delta 6q12myr}$ displayed a more robust signalling redirection, indicating that this chimera is more efficient in the signalling rerouting and could be more feasible for investigating GPR56 signalling.

In luciferase assays the expression of the reporter gene may be affected by various upstream signalling cascades that can result in a high baseline noise. To substantially enhance the signal-to-noise ratio, $G\alpha_q$-re-routing and more upstream and direct readouts could be used, such as IP_3 detection systems or Ca^{2+}-sensors.[23] Our results on IP_3 accumulation with GPR56-FL and GPR56-CTF hint at the feasibility and applicability of using the chimeric G protein $G\alpha_{\Delta 6q12myr}$ to redirect $G\alpha_{12/13}$ signalling to a direct second messenger readout. Further studies using these chimeras should evaluate the effect on IP_3/Ca^{2+} levels and demonstrate the kinetics of GPR56 activation through the chimeric G proteins. They could also explore the combination of G protein chimeras with the available upstream $G\alpha_q$ signalling sensors aiming to develop a technique for screening activation of $G\alpha_{12/13}$-coupled adhesion GPCRs.

ACKNOWLEDGEMENTS

We thank Maibritt Sigvardt Baggesen, Søren Petersen and Rajesh Regmi for excellent technical assistance in conducting the experiments.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicting interests.

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How to cite this article: Faas F, Nørskov A, Holst PJ, et al. Re-routing GPR56 signalling using $G\alpha_{12/13}$ G protein chimeras. *Basic Clin Pharmacol Toxicol*. 2023;133(4):378-389. doi:10.1111/bcpt.13935