Substrate-specific regulation of mTORC1 activity by G protein-coupled receptors

Samuel J. Atkinson, William Vincent Ritchie, Kyle Thompson, Dawn Thompson, James N. Hislop*, and Riko Hatakeyama*

Institute of Medical Sciences, School of Medicine, Medical Sciences & Nutrition, University of Aberdeen

*Corresponding authors;

riko.hatakeyama@abdn.ac.uk

ORCID 0000-0002-3065-6639

Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

james.hislop@abdn.ac.uk

ORCID 0000-0002-4829-7531

Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

Running title

Substrate-specific GPCR-mTORC1 signaling

Keywords

mammalian/mechanistic Target of Rapamycin Complex 1 (mTORC1); G protein-coupled receptor (GPCR)

Abbreviations

mTORC1: mammalian/mechanistic Target of Rapamycin Complex 1

GPCR: G protein-coupled receptor

M5R: muscarinic acetylcholine receptor M5

FPR2: formyl peptide receptor 2

β2AR: beta-2 adrenergic receptor

DREADD: designer receptor exclusively activated by designer drugs

CNO: Clozapine N-oxide

PKC: protein kinase C

PKA: protein kinase A

Abstract

The mammalian/mechanistic Target of Rapamycin Complex 1 (mTORC1) kinase controls cell growth in response to various external stimuli. mTORC1 has a myriad of substrates, and the activation of distinct downstream pathways has important physiological outcomes. Emerging evidence suggests that mTORC1 can respond to upstream signals in a nuanced manner, differentially regulating individual substrates and downstream biological processes. The nature of signals that determine the signaling selectivity of mTORC1 is not fully understood. Here, we studied mTORC1 regulation by G protein-coupled receptors (GPCRs). We found that muscarinic acetylcholine receptor M5, formyl peptide receptor 2, and beta-2 adrenergic receptor, which are coupled to distinct effector G proteins, all trigger substrate-specific activation of mTORC1. Remarkably, phosphorylation of the TFEB transcription factor, a non-canonical mTORC1 substrate that controls lysosome biogenesis, responded to GPCRs differently, sometimes even oppositely, compared to canonical mTORC1 substrates such as S6K1 and 4EBP1. This study highlights the need to re-evaluate the effects of GPCRs on mTORC1 by parallelly monitoring individual substrates, an important consideration to be made when assessing GPCR ligands as therapeutic tools to manipulate the mTORC1 pathway.

Introduction

Cells adapt to the changing environment by fine-tuning various biological processes. At the core of the cellular adaptation mechanism is the mammalian/mechanistic Target of Rapamycin Complex 1 (mTORC1) kinase, a master regulator of cell growth and metabolism [1][2]. mTORC1 is activated by pro-growth signals such as nutrients (e.g., amino acids) and growth factors (e.g., insulin). mTORC1, in turn, activates pro-growth cellular processes such as anabolic reactions (e.g. protein synthesis) while repressing catabolic reactions (e.g. protein degradation via autophagy), through phosphorylating its substrate proteins. mTORC1 activation by amino acids and by insulin/growth factors is mediated by Rag and Rheb small GTPases, respectively. mTORC1 significantly impacts human health and diseases such as cancer, diabetes, and neurodegeneration, as well as aging. To design therapeutic approaches against these conditions, a deeper mechanistic understanding of mTORC1 regulation is imperative.

An emerging theme in the mTORC1 research field is mTORC1's differential signaling to individual substrates. Depending on upstream stimuli, the phosphorylation pattern of several "non-canonical" mTORC1 substrates including the transcription factor EB (TFEB), a regulator of lysosome biogenesis, responds differently to that of canonical mTORC1 substrates such as the ribosomal protein S6 kinase 1 (S6K1) and the eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), regulators of global protein synthesis [3][4][5]. Unlike phosphorylation of canonical substrates that requires activation of both Rag and Rheb small GTPases, TFEB phosphorylation is thought to only respond to Rag GTPases. Two models have been proposed to explain the distinct behavior of TFEB, which are not mutually exclusive. One model attributes it to the unique substrate recruitment mechanism, in which Rag GTPases physically interact with TFEB (but not S6K1 or 4EBP1) and present it to mTORC1 [3][6]. Another model assigns these substrates to spatially distinct mTORC1 pools; the cytosolic mTORC1 pool phosphorylates S6K1 and 4EBP1, while the lysosomal mTORC1 pool phosphorylates TFEB [5]. In either case, a comprehensive view is missing on how various external cues, that are typically sensed by cell-surface receptors, differentially feed into individual mTORC1 substrates.

Many cell-surface receptors signal to mTORC1. Among them, G protein-coupled receptors (GPCRs) constitute the largest family, being encoded by more than 800 genes in the human genome [7][8]. GPCRs sense diverse signals ranging from chemicals such as growth factors and odorants to physical parameters such as light. Characterizing any GPCR-mTORC1 signaling therefore has wide-reaching implications for cellular adaptation mechanisms operating in diverse contexts across the human body. Moreover, GPCRs are highly "druggable" proteins, as more than 30% of FDA-approved drugs target GPCRs [9]. To utilize these drugs effectively and safely (as well as repurpose for other diseases), we need to understand how GPCRs signal to downstream pathways such as the mTORC1 pathway.

For many years GPCR signaling was considered to be mediated exclusively via activation of heterotrimeric G-proteins, with distinct Gα subtypes triggering distinct downstream events. G_q stimulates phospholipase C; G_s and G_i stimulates and inhibits, respectively, cyclic AMP production; $G_{12/13}$ activates Rho small GTPases [10][11]. This original paradigm has since been challenged, with the identification of G protein-independent signaling cascades, often proposed to be mediated by β-arrestin proteins [12]. More recently, the GPCR field has seen another paradigm shift; both G-protein dependent and -independent signaling can occur from intracellular compartments, particularly endosomes, previously considered to be refractory for signaling [13]. However, the role of intracellular signaling is widely unknown.

Substantial efforts have been made to delineate how GPCRs impact mTORC1 signaling (reviewed in [14]), with some GPCRs activating, while others inhibiting, mTORC1. A largely overlooked aspect,

however, is the substrate specificity of GPCR-mTORC1 signaling, since most past studies monitored phosphorylation of only canonical mTORC1 substrates such as S6K1 and/or 4EBP1. In this study, we parallelly examined the response of S6K1, 4EBP1, and the non-canonical mTORC1 substrate TFEB to GPCR stimulation. We found that TFEB responds to GPCR stimulation differently to S6K1 and 4EBP1, allowing simultaneous activation of protein synthesis and lysosome biogenesis. Our results revealed substrate/downstream specificity as a new dimension of the GPCR-mTORC1 signaling.

Results

M5R regulates mTORC1 in a substrate-specific manner

We examined how phosphorylation of various mTORC1 substrates —S6K1, 4EBP1, and TFEB— respond to GPCR stimulation. To gain broader insight, we decided to cover three model GPCRs, the muscarinic acetylcholine receptor M5 (M5R), the formyl peptide receptor 2 (FPR2), and the beta-2 adrenergic receptor (β2AR), each of which is selectively coupled to a distinct Gα protein, namely G_q, G_i, or G_S, respectively.

As G_q has been previously implicated in mTORC1 signaling [14], we investigated a G_q-coupled GPCR with M5R as a model. To enable selective stimulation of M5R, we took advantage of the previously described mutations that make muscarinic receptors into 'designer receptor exclusively activated by designer drugs (DREADD)' receptors and generated a HEK293 cell line stably expressing an M5R DREADD [15]. The M5R-DREADD cells responded to the synthetic ligand clozapine N-oxide (CNO) in a dose-dependent manner as expected, triggering phosphorylation of its downstream MAP kinase ERK1/2 (Figure 1A and 1B, showing the activation kinetics and dose response curve, respectively).

We then examined phosphorylation of mTORC1 substrates. We serum-starved M5R-DREADD cells to bring the mTORC1 activity to a basal level before stimulation with CNO. CNO treatment triggered phosphorylation of S6K1, as probed by the phospho-specific antibody (Figure 1C and 1D for a short and long time-course, respectively, panel 1). Phosphorylation of S6K1 coincided with that of 4EBP1, which was detected as its upper band shift (Figure 1C and 1D, panel 3). Strikingly, however, phosphorylation of TFEB, as probed by the phospho-specific antibody, was rather downregulated by CNO treatment after 16 minutes (Figure 1C and 1D, panel 4). These results suggest that M5R activates mTORC1 towards S6K1 and 4EBP1, while inhibiting it towards TFEB.

We noticed the slow kinetics of mTORC1 substrate phosphorylation by M5R, which did not peak until 32 minutes in the case of S6K1 (Figure 1C and 1D, panel 1). This was in stark contrast to the kinetics of ERK1/2 phosphorylation that peaked at, or even before, 4 minutes of CNO treatment (Figure 1C, panel 6). This observation raises the intriguing possibility that mTORC1 is activated only after M5R is internalized (see later).

Another notable observation was that M5R failed to stimulate S6K1 and 4EBP1 phosphorylation in amino acid-starved cells (Figure 1E, lanes 2 and 6; compare with serum-starved cells in lanes 3 and 7). This was not due to the failure of M5R activation, because ERK1/2 phosphorylation was similarly triggered by CNO in serum- and amino acid-starved cells (Figure 1E lanes 6 and 7, panel 6). A possible explanation is that M5R can activate the Rheb branch but not the Rag branch, the latter of which requires amino acids.

Distinct effects of M5R stimulation on protein synthesis and lysosome biogenesis

134 Having observed the opposite effects of M5R activation on phosphorylation of canonical substrates
135 (S6K1 and 4EBP1) and that of TFEB, we then examined further downstream cellular processes.
136 Phosphorylation of S6K1 and 4EBP1 by mTORC1 promotes global protein synthesis [1][2]. *De novo*
137 protein synthesis can be monitored by the established puromycin incorporation assay. As shown in
138 Figure 2A, serum starvation caused decreased incorporation of puromycin, suggestive of slowed
139 protein synthesis. Subsequent M5R stimulation by CNO triggered significantly increased puromycin
140 incorporation. Puromycin incorporation was further enhanced 2 hours after washing out CNO,
141 suggesting a prolonged effect of M5R activation. These results suggest the positive effect of M5R
142 signaling on cellular protein synthesis activity, consistent with increased phosphorylation of S6K1 and
143 4EBP1.

144 Since mTORC1 inhibits lysosome biogenesis through phosphorylating TFEB [16], dephosphorylation
145 of TFEB upon M5R activation was predicted to boost lysosome biogenesis. We tested this possibility
146 by quantifying the fluorescent signal of the lysosome-staining dye Lysotracker. As shown in Figure 2B,
147 serum starvation increased the Lysotracker signal, suggestive of accelerated lysosome biogenesis.
148 This trend was, unlike the case for puromycin incorporation, not reversed by subsequent M5R
149 stimulation by CNO. The effect of M5R stimulation on lysosome biogenesis was rather positive, if any
150 (although this positive effect was not statistically significant).

151 Together, M5R activation promoted protein synthesis without suppressing lysosome biogenesis. This
152 observation was consistent with the positive effect of M5R stimulation on phosphorylation of S6K1
153 and 4EBP1, and the negative effect on that of TFEB (Figure 2C). We conclude that M5R regulates
154 mTORC1 in a substrate/downstream-specific manner to achieve this outcome.

155

156 **mTORC1 regulation by FPR2 and β2AR**

157 We then asked whether substrate-specific regulation of mTORC1 is unique to M5R/G_q-coupled
158 GPCRs. We studied a G_i-coupled GPCR using FPR2 as a model, expressed stably in a previously
159 generated HEK293 cell line [17]. FPR2-expressing cells were serum starved and treated with the
160 peptide ligand WKYMVm, a potent FPR2 agonist. As shown in Figure 3A, while WKYMVm triggered
161 phosphorylation of S6K1 and 4EBP1 in 16 minutes (panels 1 and 3), it did not affect phosphorylation
162 of TFEB (panel 4) as strongly as M5R.

163 We then chose β2AR as a model G_S-coupled GPCR. We treated wild-type HEK293 cells, which
164 naturally express β2AR, with the ligand isoprenaline. This triggered phosphorylation of S6K1 in 64
165 minutes (Figure 3B, panel 1), but did not cause a clear band-shift of 4EBP1 (panel 3). Phosphorylation
166 of TFEB was enhanced, and this effect was observed in 16 minutes (panel 4), earlier than S6K1's
167 response.

168 To summarize, M5R, FPR2, and β2AR all activated mTORC1 towards S6K1. M5R and FPR2 also
169 activated the mTORC1-4EBP1 branch. The mTORC1-TFEB axis behaved very differently from those
170 canonical substrates; while M5R inhibited mTORC1 towards TFEB, β2AR activated it. FPR2 did not
171 strongly affect this signaling. Thus, TFEB showed diverse responses to three GPCRs even though all of
172 them activate the mTORC1-S6K1 axis, further highlighting a substrate-specific nature of mTORC1
173 regulation by GPCRs.

174

175 **mTORC1 regulation towards S6K1 and 4EBP1 involves kinases downstream of Gα**

176 We next addressed the mechanism of GPCR-mTORC1 signaling. We specifically examined the
177 contribution of kinases activated downstream of Gα proteins. Because G_q activates protein kinase C
178 (PKC) via phospholipase C [10][11], we examined the effect of the PKC inhibitor GF109203x on the
179 M5R-mTORC1 signaling. As shown in Figure 4A, CNO-triggered phosphorylation of S6K1 and 4EBP1 in
180 M5R-DREADD cells was blocked by GF109203x (lanes 4 and 5), suggesting that these responses
181 require PKC activity.

182 G_i, which is coupled to FPR2, activates MEK kinase [10][11]. The MEK inhibitor PD 98059 blocked
183 WKYMVm-triggered phosphorylation of S6K1 and 4EBP1 in FPR2-expressing cells (Figure 4B, lanes 4
184 and 5), suggesting the role of MEK in this signaling process. MEK inhibition did not affect TFEB
185 phosphorylation.

186 G_S, which is coupled to β2AR, activates protein kinase A (PKA) via cAMP production [10][11].
187 Interestingly, the PKA inhibitor PKI rather enhanced isoprenaline-triggered phosphorylation of S6K1
188 (Figure 4C, lanes 4 and 5), suggesting the negative contribution of PKA. This is consistent with the
189 inhibitory phosphorylation of the mTORC1 subunit Raptor by PKA previously reported [18]. Our
190 results thus suggest that β2AR sends both positive and negative signals to the mTORC1-S6K1 axis, the
191 latter of which is mediated by PKA. Which signal dominates may depend on the context and/or cell
192 types. The contribution of β1AR, which is also expressed in HEK293 cells at a low level, should also be
193 taken into account. PKA inhibition, on the other hand, did not enhance TFEB phosphorylation.

194 To confirm that β2AR signals to mTORC1 mainly via G_S rather than its alternate effector β-arrestin, we
195 examined the effect of carvedilol, which primarily stimulates the β2AR-β-arrestin signaling [19].
196 Carvedilol did not detectably affect phosphorylation of S6K1, 4EBP1, and TFEB (Figure 4D),
197 suggesting that β-arrestin does not mediate the β2AR-mTORC1 signaling. Overall, our observations
198 suggest that M5R, FPR2, and β2AR regulate the mTORC1-S6K1 axis via standard second messenger-
199 dependent protein kinases downstream of Gα proteins. These kinases however do not similarly
200 regulate the mTORC1-TFEB axis.

201

202 **mTORC1 activation by GPCRs involves endocytosis**

203 As GPCRs signal from both the plasma membrane and endosomes, we asked where GPCRs signal to
204 mTORC1. The remarkably slow kinetics of mTORC1's response to GPCRs led us to hypothesize that
205 GPCRs only signal to mTORC1 after being internalized. We addressed this possibility using the
206 dynamin inhibitor Dyngo-4a that blocks endocytosis [20]. As shown in Figures 5A to 5C (lanes 4 and
207 5), Dyngo-4a significantly reduced phosphorylation of S6K1 and 4EBP1 triggered by M5R, FPR2, and
208 β2AR. These observations favor the post-internalization signaling model, although we do not exclude
209 the possibility that this effect was caused by general endocytosis defects or off-target effects of
210 Dyngo-4a.

211 We noticed that Dyngo-4a treatment causes a reduction in the total TFEB level. Because this effect
212 towards TFEB was observed even without GPCR ligands, it should be attributed to a general
213 endocytosis defect or off-target effects. Our results thus did not inform the location where GPCRs
214 signal to the mTORC1-TFEB axis.

215 Together, the most straightforward interpretation of our data is that three GPCRs signal to the
216 mTORC1-S6K1/4EBP1 axis through Gα after being internalized. This signaling mechanism is substrate-
217 selective, as it does not work for the mTORC1-TFEB axis in the same way.

218

Discussion

This study revealed the substrate/downstream-specific nature of mTORC1 regulation by GPCRs, adding a new layer of complexity to this signaling. In particular, the behavior of TFEB was very different from that of canonical mTORC1 substrates, S6K1 and 4EBP1. A lesson to be drawn from this is that we need to monitor multiple mTORC1 substrates including TFEB when assessing the effects of GPCRs, a viewpoint that has largely been missing in past studies [14].

Mechanistically, the unique behavior of TFEB upon GPCR stimulation can be partly explained if GPCRs signal to mTORC1 through the Rheb branch, which TFEB is uniquely deaf to. This possibility is consistent with our observation that M5R stimulation overrides FBS starvation but not amino acid starvation (Figure 1E). It is indeed known that the MEK-ERK1/2 kinase cascade, which acts downstream of many GPCRs including the ones examined here, activates Rheb by inhibiting its negative regulator TSC complex [21]. Another factor could be the location of phosphorylation events. Given that TFEB is phosphorylated at lysosomes rather than the cytoplasm as proposed for S6K1 and 4EBP1 [5], GPCRs may exert substrate-specific effects by modulating mTORC1 localization.

The non-canonical behavior of TFEB observed in this and other studies means the regulatory decoupling of a catabolic process (lysosome biogenesis) from an anabolic process (protein synthesis), even though both are governed by mTORC1. A similar decoupling is observed in the budding yeast model, in which protein synthesis and autophagy are regulated by spatially distinct TORC1 pools [22]. This may look counterintuitive from the traditional view in the TORC1 field that activation of anabolism or catabolism should coincide with inhibition of the other. Such coupling would make sense in the context of nutrient response, where cells adapt their bulk mass to nutrient availability. However, the situation is different when cells respond to other stimuli such as growth factors or stresses. Cells would need to change themselves qualitatively by remodeling the proteome, not necessarily changing their total mass. For that, they would need to synthesize proteins that are needed in the new environment, and simultaneously degrade proteins not needed anymore. The latter catabolic process and subsequent recycling of resources (e.g., amino acids) fuel the former anabolic processes. In such a situation, it would be beneficial to decouple anabolism and catabolism.

The supreme druggability of GPCRs, and their tissue/context-specific expression patterns, make GPCR ligands attractive therapeutic tools to manipulate mTORC1 pathway to treat cancer, diabetes, neurodegeneration, and aging. The substrate/downstream specificity revealed in this study further enhances the potential of GPCR ligands, as it theoretically enables selective manipulation of desired downstream functions of mTORC1. Such a targeted approach should be more effective and safer than pan-mTORC1 manipulation. This potential warrants a comprehensive investigation of the mTORC1 substrate specificity for a wider range of GPCRs.

Materials and methods

Antibodies and reagents

All antibodies were purchased from Cell Signaling technologies except anti-GAPDH (Proteintech 60004-1-Ig). Anti-S6K (2708), anti-P-S6K (9205), anti-4EBP1 (9644), anti-TFEB (4240), anti-P-TFEB (8793) and anti-P-MAPK (4370) were used at 1:1000. Anti-GAPDH was used at 1:30,000. Unless otherwise stated, common reagents were purchased from ThermoFisher Scientific.

Cell culture

All cell lines (HEK293 and derivatives) were cultured in DMEM high glucose (Merck D5671) supplemented with 1 mM sodium pyruvate (ThermoFisher Scientific 11360-039), 1% glutamine (Merck G7513) and 10% FBS (Biosera 10011500) at 37°C in an atmosphere of 5% CO_2. Cell lines were regularly tested for the presence of mycoplasma contamination. FPR2 stably expressing cells were previously generated as described in [17]. DMEM without amino acids (VWR USBID9800-13) was supplemented with glucose to match DMEM high glucose above, 1 mM sodium pyruvate and 10% dialysed FBS (lacking amino acids). The same FBS batch for all experiments was dialysed against PBS twice overnight at 4°C using a 3500 Da cutoff Snakeskin™ dialysis membrane (ThermoFisher Scientific 88242).

Muscarinic Acetylcholine Receptor M5 DREADD stable cell line

HEK293 cells were from the EACCC. The M5 DREADD receptor was generated based on the M3R DREADD [23]. Briefly, M5R cDNA was obtained from cDNA.org and subcloned into the Flag pcDNA3.1+ zeo [17]. Site-directed mutagenesis (QuickChange, Agilent) was then used to introduce the conserved mutations in the 3rd and 5th transmembrane domain (Y111C and A201G) to confer sensitivity to CNO. Stables were generated by selection with zeocin and identified by anti-Flag immunofluorescence determined by microscopy.

GPCR stimulation

All cell lines were washed with PBS then starved of amino acids or FBS for 2 hours before treatment with the GPCR antagonists at 1 µM: CNO (Tocris/bio-techne 6329), Isoprenaline/Carvedilol (Sigma/Merck I6504; Sigma/Merck C3993) or WKYMVm (Tocris/BioTechne 1800) in M5R-DREADD, WT or FPR2-expressing HEK293 cells respectively for the indicated timepoints. Dyngo®-4a (Abcam ab120689) was added to culture media 20 minutes before stimulation at 10 µM. MEK inhibitor PD 98059 (Abcam ab120234) was added for 1.5 hours before stimulation at 50 µM. PKC inhibitor GF109203x (Sigma/Merck B6292) and PKA inhibitor PKI (Tocris/BioTechne 2546) were added for 1.5 hours before stimulation at 1 µM.

Global translation assay

Cells were stimulated as above, and puromycin (ThermoFisher Scientific 227420500). was added 15 minutes before lysis at 5 ug ul^{-1} as per the SUNSET assay protocol [24].

Western blotting

Cells were washed with PBS before being lysed directly in high SDS lysis buffer (3% SDS, 60 mM sucrose, 65 mM Tris-HCl pH 6.8), denatured at 95°C for 5 minutes, homogenised by sonication and cleared by centrifugation at 18,000 x g for 30 minutes. Lysates were quantified by a linearised bradford assay (Abcam ab119216) using 595nm/450nm ratio.

294 25 µg of proteins were separated in SDS-PAGE gels before being transferred to nitrocellulose
295 membranes (VWR 10600002). After ponceau staining, membranes were blocked with 5% milk/TBST
296 (for anti-TFEB, P-TFEB, GAPDH, P-MAPK antibodies) or 5% BSA/TBST (for anti-S6k, PS6k, 4EBP1
297 antibodies) and probed with antibodies overnight at 4°C in 5% BSA/TBST (for anti-S6k, PS6k, 4EBP1,
298 TFEB, P-TFEB, P-MAPK antibodies) or 5% Milk/TBST (for anti-GAPDH antibody). Membranes were
299 visualised after secondary antibody incubation (Cell Signaling Technologies 7074 and 7076) and
300 washing with TBST using ECL substrate (ThermoFisher Scientific 12994780). Membranes were
301 imaged in a Gbox gel/blot imaging system (Syngene) and densitometry analysis for was performed
302 using ImageJ [25].

303 **Flow cytometry**

304 After stimulation, cells were treated with 20 nM LysoTracker™ Red DND-99 (ThermoFisher Scientific
305 L7528) before being placed on ice detached, resuspended in cold FACS buffer (PBS 3% FBS, 25 mM
306 HEPES, 5 mM EDTA) and analysed on an Attune NxT flow cytometer (ThermoFisher Scientific),
307 collecting at least 10,000 single cell events. Data was analysed using https://floreada.io.

308 **Statistical analysis**

309 All statistical analyses and graphs were produced in GraphPad Prism. Technical replicates (n) were
310 averaged for each biological replicates (N). Control and experimental condition's biological repeats
311 were compared using unpaired student's t tests. $P<0.05$ was considered significant and $P>0.05$ non-
312 significant.

Acknowledgments

We thank Kei Sakamoto (University of Copenhagen) and Fiona Murray (University of Aberdeen) for helpful suggestions; Colin Ferguson and the Flow Cytometry Core Facility (University of Aberdeen) for technical support. This work was funded by BBSRC (BB/V016334/1 to RH), British Heart Foundation (PG/19/30/34327 to DT), and the University of Aberdeen. The authors declare no conflict of interest.

Author contributions

Conceptualization: SJA, DT, JH, and RH; Methodology: SJA, WR, KT, and JH; Investigation: SJA, WR, KT, and JH; Formal Analysis: SJA and JH; Validation: SJA, WR, and JH; Supervision: DT, JH, and RH; Funding acquisition: DT, JH and RH; Visualization: SJA, JH, and RH; Writing – original draft: SJA and RH; Writing – review & editing: SJA, DT, JH, and RH.

References

1. Shimobayashi, M.; Hall, M.N. Making New Contacts: The MTOR Network in Metabolism and Signalling Crosstalk. *Nat Rev Mol Cell Biol* **2014**, *15*, 155–162, doi:10.1038/nrm3757.

2. Liu, G.Y.; Sabatini, D.M. MTOR at the Nexus of Nutrition, Growth, Ageing and Disease. *Nat Rev Mol Cell Biol* **2020**, *21*, 183–203, doi:10.1038/s41580-019-0199-y.

3. Napolitano, G.; Di Malta, C.; Esposito, A.; de Araujo, M.E.G.; Pece, S.; Bertalot, G.; Matarese, M.; Benedetti, V.; Zampelli, A.; Stasyk, T.; et al. A Substrate-Specific MTORC1 Pathway Underlies Birt–Hogg–Dubé Syndrome. *Nature* **2020**, *585*, 597–602, doi:10.1038/s41586-020-2444-0.

4. Hasegawa, J.; Tokuda, E.; Yao, Y.; Sasaki, T.; Inoki, K.; Weisman, L.S. PP2A-Dependent TFEB Activation Is Blocked by PIKfyve-Induced MTORC1 Activity. *Mol Biol Cell* **2022**, *33*, doi:10.1091/mbc.E21-06-0309.

5. Fernandes, S.A.; Angelidaki, D.-D.; Nüchel, J.; Pan, J.; Gollwitzer, P.; Elkis, Y.; Artoni, F.; Wilhelm, S.; Kovacevic-5 Sarmiento, M.; Demetriades, C. Spatially and Functionally Distinct MTORC1 Entities Orchestrate the Cellular Response to Amino Acid Availability. *bioRxiv* **2023**, doi:10.1101/2023.10.03.559930.

6. Cui, Z.; Napolitano, G.; de Araujo, M.E.G.; Esposito, A.; Monfregola, J.; Huber, L.A.; Ballabio, A.; Hurley, J.H. Structure of the Lysosomal MTORC1–TFEB–Rag–Ragulator Megacomplex. *Nature* **2023**, *614*, 572–579, doi:10.1038/s41586-022-05652-7.

7. Pierce, K.L.; Premont, R.T.; Lefkowitz, R.J. Seven-Transmembrane Receptors. *Nat Rev Mol Cell Biol* 2002, *3*, 639–650.

8. Rosenbaum, D.M.; Rasmussen, S.G.F.; Kobilka, B.K. The Structure and Function of G-Protein-Coupled Receptors. *Nature* **2009**, *459*, 356–363, doi:10.1038/nature08144.

9. Hauser, A.S.; Attwood, M.M.; Rask-Andersen, M.; Schiöth, H.B.; Gloriam, D.E. Trends in GPCR Drug Discovery: New Agents, Targets and Indications. *Nat Rev Drug Discov* **2017**, *16*, 829–842, doi:10.1038/nrd.2017.178.

10. Milligan, G.; Kostenis, E. Heterotrimeric G-Proteins: A Short History. *Br J Pharmacol* **2006**, *147*, doi:10.1038/sj.bjp.0706405.

11. Goldsmith, Z.G.; Dhanasekaran, D.N. G Protein Regulation of MAPK Networks. *Oncogene* **2007**, *26*, 3122–3142, doi:10.1038/sj.onc.1210407.

12. Scheerer, P.; Sommer, M.E. Structural Mechanism of Arrestin Activation. *Curr Opin Struct Biol* **2017**, *45*, 160–169, doi:10.1016/j.sbi.2017.05.001.

13. Irannejad, R.; Von Zastrow, M. GPCR Signaling along the Endocytic Pathway. *Curr Opin Cell Biol* 2014, *27*, 109–116.

14. Melick, C.H.; Lama-Sherpa, T.D.; Curukovic, A.; Jewell, J.L. G-Protein Coupled Receptor Signaling and Mammalian Target of Rapamycin Complex 1 Regulation. *Mol Pharmacol* **2022**, *101*, 181–190, doi:10.1124/molpharm.121.000302.

15. Urban, D.J.; Roth, B.L. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs): Chemogenetic Tools with Therapeutic Utility. *Annu Rev Pharmacol Toxicol* 2015, *55*, 399–417.

16. Puertollano, R.; Ferguson, S.M.; Brugarolas, J.; Ballabio, A. The Complex Relationship between TFEB Transcription Factor Phosphorylation and Subcellular Localization . *EMBO J* **2018**, *37*, doi:10.15252/embj.201798804.

17. Thompson, D.; McArthur, S.; Hislop, J.N.; Flower, R.J.; Perretti, M. Identification of a Novel Recycling Sequence in the C-Tail of FPR2/ALX Receptor: Association with Cell Protection from Apoptosis. *Journal of Biological Chemistry* **2014**, *289*, 36166–36178, doi:10.1074/jbc.M114.612630.

18. Jewell, J.L.; Fu, V.; Hong, A.W.; Yu, F.-X.; Meng, D.; Melick, C.H.; Wang, H.; Lam, W.-L.M.; Yuan, H.-X.; Taylor, S.S.; et al. GPCR Signaling Inhibits MTORC1 via PKA Phosphorylation of Raptor., doi:10.7554/eLife.43038.001.

19. Ippolito, M.; Benovic, J.L. Biased Agonism at β-Adrenergic Receptors. *Cell Signal* **2021**, *80*, 109905, doi:10.1016/j.cellsig.2020.109905.

20. Mccluskey, A.; Daniel, J.A.; Hadzic, G.; Chau, N.; Clayton, E.L.; Mariana, A.; Whiting, A.; Gorgani, N.N.; Lloyd, J.; Quan, A.; et al. Building a Better Dynasore: The Dyngo Compounds Potently Inhibit Dynamin and Endocytosis. *Traffic* **2013**, *14*, 1272–1289, doi:10.1111/tra.12119.

21. Ma, L.; Chen, Z.; Erdjument-Bromage, H.; Tempst, P.; Pandolfi, P.P. Phosphorylation and Functional Inactivation of TSC2 by Erk: Implications for Tuberous Sclerosis and Cancer Pathogenesis. *Cell* **2005**, *121*, 179–193, doi:10.1016/j.cell.2005.02.031.

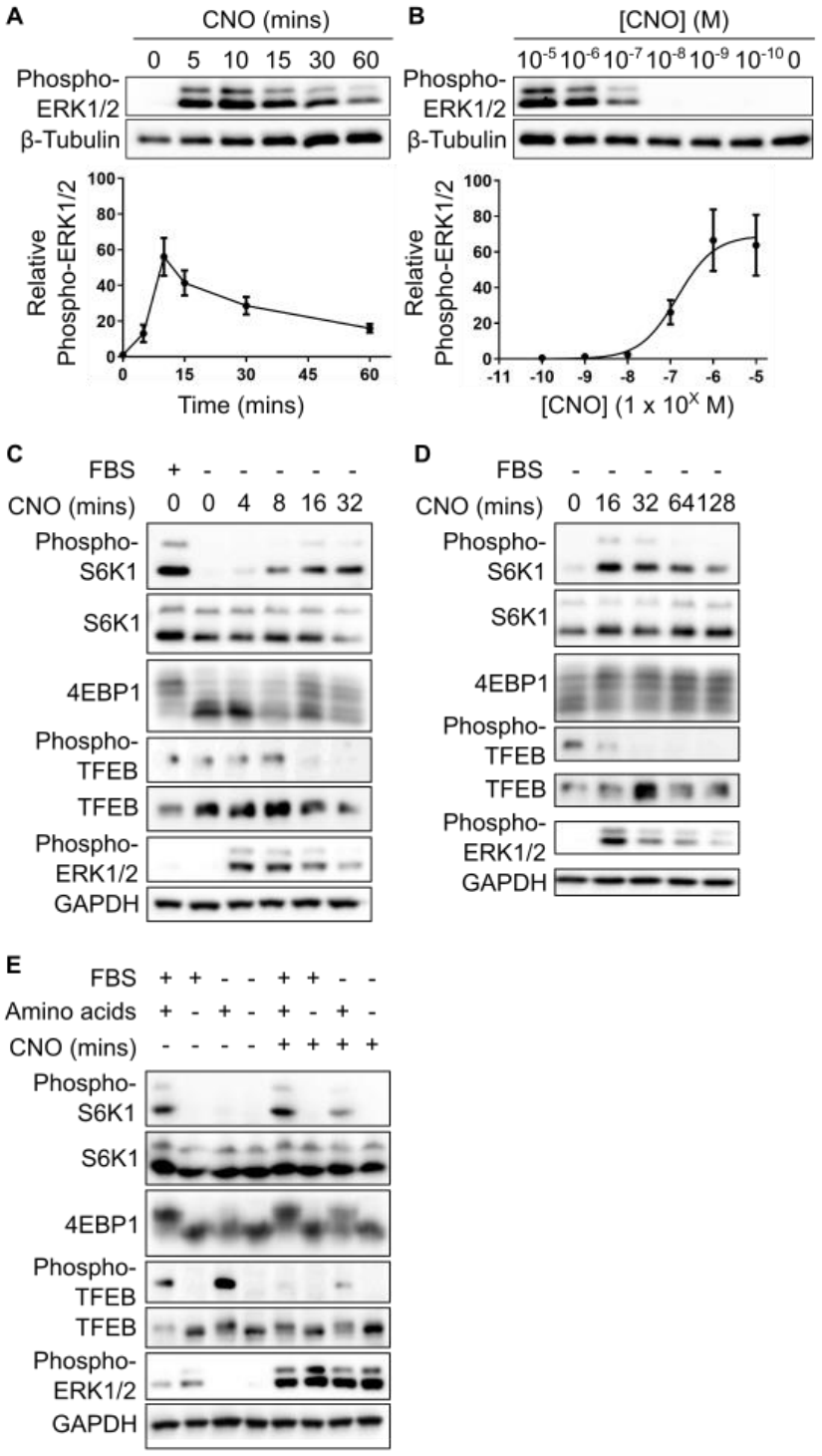
22. Hatakeyama, R.; Péli-Gulli, M.P.; Hu, Z.; Jaquenoud, M.; Garcia Osuna, G.M.; Sardu, A.; Dengjel, J.; De Virgilio, C. Spatially Distinct Pools of TORC1 Balance Protein Homeostasis. *Mol Cell* **2019**, *73*, 325-338.e8, doi:10.1016/j.molcel.2018.10.040.

23. Armbruster, B.N.; Li, X.; Pausch, M.H.; Herlitze, S.; Roth, B.L. Evolving the Lock to Fit the Key to Create a Family of G Protein-Coupled Receptors Potently Activated by an Inert Ligand. **2007**, *104*, 5163–5168, doi:10.1073/pnas.0700293104.

24. Piecyk, M.; Fauvre, J.; Duret, C.; Chaveroux, C.; Ferraro-Peyret, C. SUrface SEnsing of Translation (SUnSET), a Method Based on Western Blot Assessing Protein Synthesis Rates in Vitro. *Bio Protoc* **2024**, *14*, doi:10.21769/BioProtoc.4933.

25. Schindelin, J.; Arganda-Carreras, I.; Frise, E.; Kaynig, V.; Longair, M.; Pietzsch, T.; Preibisch, S.; Rueden, C.; Saalfeld, S.; Schmid, B.; et al. Fiji: An Open-Source Platform for Biological-Image Analysis. *Nat Methods* 2012, *9*, 676–682.

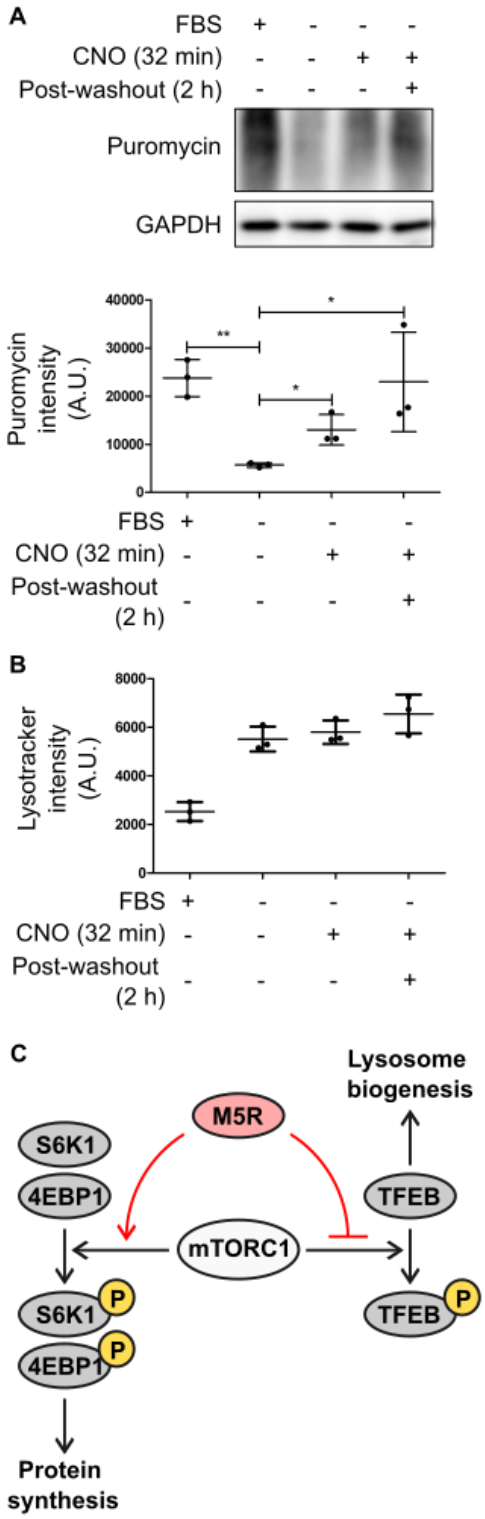
Figure 1


A
CNO (mins)
0 5 10 15 30 60
Phospho-ERK1/2
β-Tubulin
Relative Phospho-ERK1/2
Time (mins)
B
[CNO] (M)
10-5 10-6 10-7 10-8 10-9 10-10 0
Phospho-ERK1/2
β-Tubulin
Relative Phospho-ERK1/2
[CNO] (1 x 10X M)
C
FBS + - - - - -
CNO (mins) 0 0 4 8 16 32
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
D
FBS - - - - -
CNO (mins) 0 16 32 64 128
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
E
FBS + + - - + + - -
Amino acids + - + - + - + -
CNO (mins) - - - - + + + +
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH

397 **Figure 1. Activation of M5R-DREADD by CNO and downstream signalling to mTORC1**.

398 HEK293 cells stably expressing the M5R DREADD were serum starved for 2 hours prior to stimulation
399 with 1 μM CNO for the indicated time (**A**) or with the indicated concentration of CNO for 7 minutes
400 (**B**), before lysis and analysis by SDS-PAGE. Representative anti-phospho-ERK1/2 and anti-tubulin
401 Western blots are presented alongside quantification relative to untreated samples (n=3). The same
402 M5R DREADD cells were seeded overnight and then starved of FBS or given fresh FBS-containing
403 media for 2 hours before being stimulated with 1 μM CNO for up to 32 minutes (**C**) or 128 minutes
404 (**D**) before lysis and separation by SDS-PAGE. (**E**) M5R-DREADD cells were treated as above but with
405 the additional conditions of amino acid and combined amino acid/FBS starvation. Cells were treated
406 with 1 μM CNO for 32 minutes before lysis.

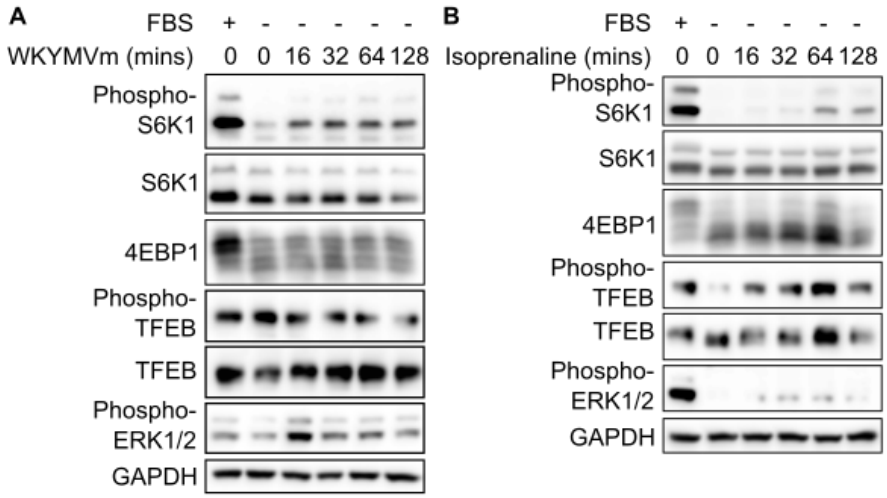
Figure 2


A
FBS + - - -
CNO (32 min) - - + +
Post-washout (2 h) - - - +
Puromycin
GAPDH
Puromycin intensity (A.U.)
40000
30000
20000
10000
0
**
*
*
FBS + - - -
CNO (32 min) - - + +
Post-washout (2 h) - - - +
B
Lysotracker intensity (A.U.)
8000
6000
4000
2000
0
FBS + - - -
CNO (32 min) - - + +
Post-washout (2 h) - - - +
C
Lysosome biogenesis
M5R
S6K1
4EBP1
TFEB
mTORC1
S6K1
P
4EBP1
P
TFEB
P
Protein synthesis

407

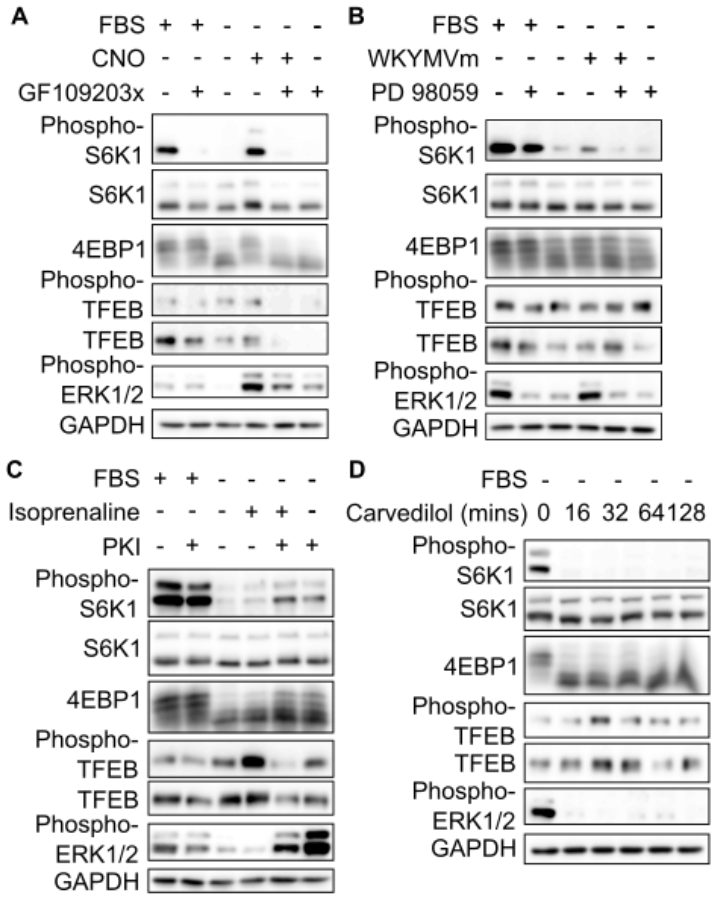
408 **Figure 2. Protein synthesis and lysosome biogenesis regulated by M5R.** HEK293 cells expressing
409 M5R-DREADD were seeded overnight then starved of FBS or given fresh FBS-containing media for 2
410 hours before being stimulated with 1 µM CNO for 32 minutes. After stimulation, CNO was removed
411 and fresh FBS starvation media was added for a further 2 hours in one condition. Puromycin at 5 µg
412 ml^{-1} was added to each condition for 15 minutes before lysis and separation by SDS-PAGE (**A**) (top
413 panel representative western blot, bottom panel – quantitative analysis of N = 3 experiments) or
414 LysoTracker™ Red DND-99 at 20 nM was added before detachment for flow cytometry analysis (**B**).
415 (**C**) A schematic showing the proposed dualistic regulation of downstream mTORC1 substrates by
416 M5R where, in contrast to normally observed signaling (e.g. Figure 1E without CNO lanes),
417 S6K1/4EBP1 phosphorylation is promoted and TFEB phosphorylation is suppressed – activating all
418 three substrates simultaneously. Significance was calculated by unpaired student's t test with *
419 denoting $P<0.05$ and ** $P<0.01$.

Figure 3


A
FBS + - - - - -
WKYMVm (mins) 0 0 16 32 64 128
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
B
FBS + - - - - -
Isoprenaline (mins) 0 0 16 32 64 128
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH

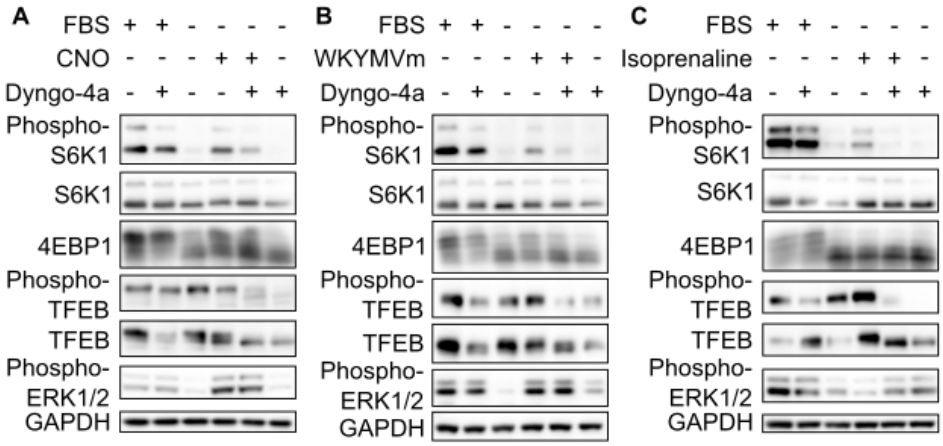
421 **Figure 3. Activation of mTORC1 by alternative classes of GPCRs.** HEK293 cells expressing FPR2 (**A**) or
422 WT HEK293 (**B**) were seeded overnight then starved of FBS or given fresh FBS-containing media for 2
423 hours before being stimulated with 1 µM WKYMVm peptide or 1 µM isoprenaline respectively for up
424 to 128 minutes before being lysed and separation by SDS-PAGE.

Figure 4


A
FBS + + - - - -
CNO - - - + + -
GF109203x - + - - + +
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
B
FBS + + - - - -
WKYMVm - - - + + -
PD 98059 - + - - + +
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
C
FBS + + - - - -
Isoprenaline - - - + + -
PKI - + - - + +
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
D
FBS - - - - -
Carvedilol (mins) 0 16 32 64 128
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH

426 **Figure 4. Activation of mTORC1 by GPCRs occurs through distinct Gα regulated downstream**
427 **pathways.** (**A**) HEK293 cells expressing M5R-DREADD were seeded overnight then starved of FBS or
428 given fresh FBS-containing media for 2 hours before being stimulated with 1 µM CNO for 32 minutes
429 then lysed and separated by SDS-PAGE. A major effector of the G_q coupled M5R, PKC, was inhibited
430 using 1 µM GF109203x for 1.5 hours before CNO treatment. (**B**) HEK293 cells expressing FPR2 were
431 seeded overnight then starved of FBS or given fresh FBS-containing media for 2 hours before being
432 stimulated with 1 µM WKYMVm peptide for 32 minutes then lysed and separated by SDS-PAGE. The
433 MEK inhibitor PD-98059 was used 1.5 hours before WKYMVm treatment. (**C**) WT HEK293 cells were
434 seeded overnight then starved of FBS or given fresh FBS-containing media for 2 hours before being
435 stimulated with 1 µM isoprenaline for 64 minutes then lysed and separated by SDS-PAGE. A major
436 effector of the G_s coupled β2AR, PKA, was inhibited using 1 µM PKI for 1.5 hours before isoprenaline
437 treatment. (**D**) WT HEK293 were seeded overnight then starved of FBS or given fresh FBS-containing
438 media for 2 hours before being stimulated with the β-arrestin biased agonist 1 µM carvedilol for up
439 to 128 minutes before being lysed and separated by SDS-PAGE.

Figure 5


A
FBS + + - - - -
CNO - - - + + -
Dyngo-4a - + - - + +
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
B
FBS + + - - - -
WKYMVm - - - + + -
Dyngo-4a - + - - + +
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH
C
FBS + + - - - -
Isoprenaline - - - + + -
Dyngo-4a - + - - + +
Phospho-S6K1
S6K1
4EBP1
Phospho-TFEB
TFEB
Phospho-ERK1/2
GAPDH

441 **Figure 5. Internalisation of GPCRs is required for mTORC1 activation.** HEK293 cells expressing M5R-
442 DREADD (**A**) or FPR2 (**B**) were seeded overnight then starved of FBS or given fresh FBS containing
443 media for 2 hours before being stimulated with 1 µM CNO or WKYMVm peptide for 32 minutes
444 respectively and were then lysed and western blotted. WT HEK293 cells were treated as above
445 expect stimulated for 64 minutes with 1 µM isoprenaline (**C**). The dynamin inhibitor Dyngo-4a was
446 used 20 minutes before GPCR stimulation to prevent endocytosis of GPCR receptors at 10 µM.