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Epidermal growth factor receptor association with β1-adrenergic receptor is mediated via its juxtamembrane domain

Viren Patwa[1], **Shuchi Guo**[1,*], **Rhonda L. Carter**[1], **Lindsay Kraus**[2], **Jeanette Einspahr**[1], **David Teplitsky**[1], **Abdelkarim Sabri**[2], **Douglas G. Tilley**[1]

[1]Center for Translational Medicine, Temple University, Philadelphia, PA, USA, 19140

[2]Cardiovascular Research Center, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, USA, 19140

Abstract

β1-adrenergic receptor (β1AR)-mediated transactivation of epidermal growth factor receptor (EGFR) engages downstream signaling events that impact numerous cellular processes including growth and survival. While association of these receptors has been shown to occur basally and be important for relaying transactivation-specific intracellular events, the mechanism by which they do so is unclear and elucidation of which would aid in understanding the consequence of disrupting their interaction. Using fluorescence resonance energy transfer (FRET) and immunoprecipitation (IP) analyses, we evaluated the impact of C-terminal truncations of EGFR on its ability to associate with β1AR. While loss of the last 230 amino acid C-terminal phosphotyrosine-rich domain did not disrupt the ability of EGFR to associate with β1AR, truncation of the entire intracellular domain of EGFR resulted in almost complete loss of its interaction with β1AR, suggesting that either the kinase domain or juxtamembrane domain (JMD) may be required for this association. Treatment with the EGFR antagonist gefitinib did not prevent β1AR-EGFR association, however, treatment with a palmitoylated peptide encoding the first 20 amino acids of the JMD domain (JMD-A) disrupted β1AR-EGFR association over time and

Corresponding Author: Douglas G. Tilley, PhD, FAHA, Room 945A MERB, Center for Translational Medicine, Lewis Katz School of Medicine, Temple University, 3500 N. Broad St., Philadelphia, PA 19140, Tel.: 215-707-2791, Fax: 215-707-9890, douglas.tilley@temple.edu.

*Present address: Center for Cardiovascular Research, Washington University School of Medicine

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prevented β1AR-mediated ERK1/2 phosphorylation, both in general and specifically in association with EGFR. Conversely, neither a mutated JMD-A peptide nor a palmitoylated peptide fragment consisting of the subsequent 18 amino acids of the JMD domain (JMD-B) were capable of doing so. Altogether, the proximal region of the JMD of EGFR is responsible for its association with β1AR, and its disruption prevents β1AR-mediated transactivation, thus providing a new tool to study the functional consequences of disrupting β1AR-EGFR downstream signaling.

Keywords

β1-Adrenergic Receptor; epidermal growth factor receptor; juxtamembrane domain; extracellular-regulated kinase

1. Introduction

The β1-adrenergic receptor (β1AR) has long been recognized as an important regulator of cardiac contractility in response to elevated catecholamines via $G\alpha_s$ protein-mediated signaling[1]. Beyond these effects, however, β1AR engages additional signaling pathways including the transactivation of epidermal growth factor receptor (EGFR) to exert effects on processes such as cardiomyocyte growth and survival, manipulation of which may offer potential therapeutic advantages. In particular, we and others have shown that while β1AR-mediated EGFR transactivation may be initially beneficial via the acute activation of pro-survival signaling pathways, over time it may give way to chronic deleterious pro-hypertrophic signaling[2–4]. Since cardiac hypertrophy precedes maladaptive cardiac remodeling and heart failure, but is not actually required to preserve contractile function[5, 6], mechanisms to disrupt its development and/or progression could be advantageous.

Currently, pharmacologic study of the impact of EGFR transactivation on cellular effects is reliant upon tyrosine kinase domain inhibitors, such as gefitinib, chemical compounds with similar structures to ATP that allow them to compete for the ATP-binding domain of EGFR, thereby preventing subsequent phosphorylation of the tyrosine-rich C-terminal domains and activation of signaling scaffolds. However, due to the similarity of ATP-binding sites across the kinome, EGFR inhibitors have varying selectivity profiles that can impact study interpretation[7]. Thus, we have sought to identify alternate methods for modulating β1AR-mediated EGFR transactivation, both for mechanistic study and the development of potential novel therapeutic strategies.

Previously, we demonstrated that in addition to transactivation, β1AR associates with EGFR in an endogenous receptor complex[8]. While the interaction of the receptors following catecholamine stimulation was shown to be dependent upon the recruitment of and continued association with β-arrestin1/2, the mechanism by which they associate in the absence of ligand has not been well-characterized. From the standpoint of the β1AR, mutation of all the putative C-terminal G protein-coupled receptor kinase (GRK) phosphorylation sites decreased its association with EGFR[8], however the reciprocal mechanism(s) by which EGFR associates with β1AR are totally unknown. Since disruption of this primed association may prevent EGFR transactivation and provide a mechanism to

study its effects on cellular functions, we have aimed to characterize the structural components necessary for EGFR association with β1AR.

EGFR contains several domains essential to its interactions with other proteins. In particular, intracellular domains include the kinase domain and the C-terminal tail containing numerous tyrosine residues that become autophosphorylated upon ligand stimulation[9–11]. Phosphorylation of these residues provides several points of regulation and scaffolding sites for the assembly of protein complexes and downstream signaling events. In addition, the juxtamembrane domain (JMD) of EGFR has been recognized to be an important regulator of receptor activity, targeting and interactions with other proteins[12, 13]. Indeed, small peptide disruptors of the JMD have been shown to be effective at regulating EGFR activity in cancer cells[14–18]. Thus, in our current study we have employed various truncated and mutated EGFR constructs as well as JMD-derived palmitoylated peptides to define the required regions responsible for β1AR-EGFR association and the impact of such disruption on EGFR transactivation.

2. Materials and Methods

2.1. Cell culture and infection/transfection.

U2OS cells were cultured in Minimum Essential Medium (MEM, Cellgro Corning) supplemented with 10% fetal bovine serum (Gemini Bioproducts) and 1% penicillin/streptomycin/amphotericin B (Gemini Bio-products). Cells were grown in a humidified atmosphere of 5% CO_2 at 37°C. U2OS cells were seeded at a density of 4.5×10^6 cells in 100 mm dishes (for immunoprecipitation (IP) experiments) or at a density of 4×10^4 cells/well in 96 well plates (for FRET experiments). U2OS cells were infected with adenoviruses encoding HA-β1AR, Flag-β1AR-mCFP, Flag-WT-EGFR-mYFP, Flag-EGFR-CT230-mYFP (each constructed by Vector Biolabs, Malvern, PA) or EGFR-CD533[19] at multiplicities of infection (MOIs) as indicated for 24 hr, after which the cells were used for experiments outlined below.

2.2. JMD peptides.

JMD peptides were synthesized by Genscript USA, Inc., with palmitoylation at the N-terminus and amidation at the C-terminus. The sequence for JMD-A corresponds to amino acids 645–664 of EGFR (RRRHIVRKRTLRRLLQEREL) and JMD-B to amino acids 665–682 of EGFR (VEPLTPSGEAPNQALLRI)[12]. JMD-A-5A contains a sequence of 5 amino acid substitutions within JMD-A to alanines (RRRHIVRKRTAAAAAQEREL) [15]. All peptides were dissolved in DMSO at a stock concentration of 1 mM.

2.3. Cell treatment.

Infected U2OS cells were cultured for 24 hr in a humidified atmosphere of 5% CO_2 at 37°C, after which the growth media was replaced by serum-free media for 3 hr. For experiments with pharmacologic treatments, cells were treated with ISO (0 to 10μM) for 5 min, or gefitinib (Gef, 1 μM) versus vehicle (0.1% DMSO) for up to 30 min, prior to processing for IP. In a subset of experiments, prior to ISO simulation cells were treated with 10 μM JMD

peptides versus vehicle (1% DMSO) for 15 min. For FRET experiments, cells were treated with JMD peptides or DMSO for up to 1 hr.

2.4. Fluorescence resonance energy transfer (FRET) detection.

To measure the affinities of association between β1AR and EGFR constructs, U2OS cells seeded in 96-well plates were initially infected with adenoviruses encoding Flag-EGFR-mYFP versus Flag-EGFR-CT230-mYFP and Flag-β1AR-mCFP at increasing MOIs. 24 h post-infection, the cells were rinsed and media replaced with imaging buffer (HBSS buffer (Cellgro), 0.2% bovine serum albumin, 20 mM HEPES) for 3 hr, after which cyan fluorescent protein (CFPex/CFPem), yellow fluorescent protein (YFPex/em) and FRET (CFPex/YFPem) readings were attained using a M1000 multimode plate reader (Tecan). Quantification of FRET (corrected FRET (cFRET) = FRET − (CFP*CFP bleedthrough) − (YFP*YFP bleedthrough) were expressed as a percentage of total CFP emission (%FRET = cFRET/[cFRET + CFP]). Plate reader fluorescence bleedthrough values were: 40% (CFP) and 2% (YFP). Nonlinear regression one-site binding analysis was used to calculate maximal % FRET ($FRET_{max}$) and affinity ($10/K_{FRET}$) values for mCFP/mYFP association (Graphpad Prism 8.4.3). In subsequent stimulation experiments, cells were infected with Ad-Flag-EGFR-mYFP and Ad-Flag-β1AR-mCFP at MOIs indicated to achieve a baseline FRET value of ~10% for evaluation of pharmacologic disruption of β1AR-EGFR association with gefitinib (1 μM), JMD peptides (10 μM) or vehicle (1% DMSO). Following treatment, fluorescence values were attained every 5 min for up to 1 hr.

2.5. Immunoprecipitation.

Infected U2OS cells were washed three times with ice cold PBS followed by cell lysis. Cells were scraped using lysis buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 10% glycerol, 1 mM EDTA, 1% Nonidet P-40, 10 mM NaF (chemicals attained from Fisher Scientific, Pittsburgh, PA), 1× HALT protease inhibitor cocktail (78437; Thermo Scientific, Rockford, IL), and phosphatase inhibitor cocktail set IV (524628; Calbiochem). Cell lysates were incubated on a rotor at 4°C for 2 hr. Following incubation, lysates were centrifuged at 20,000 x g for 20 min at 4°C. The supernatant was collected and protein quantity estimated via use of the Pierce 660 nM Protein Assay Reagent (Thermo Scientific). 50 μg protein/sample was separated for total lysate and 500 μg protein/sample was separated for IP. Total lysates were diluted using 6X SDS buffer, followed by heating for 5 min at 70°C and were stored at −20°C. IP lysates were incubated overnight with anti-HA or anti-FLAG M2 agarose beads at 4°C on a rotor, following which the samples were spun at 8000 x g for 1 min and then washed with PBS, repeating this step three times. Finally, the agarose beads were diluted with 2X SDS buffer and proteins were eluted by incubating the beads with 2X SDS at room temperature for 30 min with intermittent vortexing at a low speed. The eluted samples were transferred to new microtubes, centrifuged at 5000 x g for 1 min and heated at 70°C for 5 min. These samples were either stored at −20°C or resolved immediately using polyacrylamide gels.

2.6. Immunoblotting.

Equal amounts of IP eluates and total lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Odyssey Blocking

Buffer (LI-COR Biosciences; Lincoln, NE) was used to prevent non-specific binding. Immunoblotting was performed overnight at 4°C with diluted antibodies against Flag or HA (1:10,000 or 1:1000, respectively; Sigma; St. Louis, MO), rabbit or mouse anti-phosphorylated ERK1/2, rabbit or mouse anti-total ERK1/2 and rabbit anti-GAPDH (1:1000 each; Cell Signaling), or mouse anti-EGFR clone LA22 (1:500, EMD Millipore). After washing with TBS-T, membranes were incubated at room temperature for 60 min with the appropriate diluted secondary antibody (IRDye680 Donkey anti-rabbit IgG (H + L) at 1:15,000; IRDye800CW Goat anti-mouse IgG (H + L) at 1:15,000; LI-COR Biosciences). Bound antibody was detected using the LI-COR Biosciences Odyssey System (LI-COR Biosciences). Levels of phosphorylated ERK1/2 were normalized to levels of total ERK1/2 in total lysates and to Flag-EGFR in the IP eluates.

2.7. Statistical analysis.

GraphPad Prism 8.4.3 software (GraphPad Software Inc., San Diego, CA) was used for all statistical analysis. Data are presented as the mean ± standard error of the mean (SEM). Two-tailed unpaired t tests were used for comparison between two groups and ordinary one-way ANOVA with Tukey's multiple comparisons test was used for comparison across multiple groups at a single time point. Comparison between groups over time was performed using two-way repeated measures ANOVA with Dunnett's multiple comparisons test. Differences were considered significant at $P < 0.05$, as indicated in figure legends.

3. Results

3.1. β1AR-EGFR association is independent of the phosphotyrosine-rich C-terminus of EGFR

To establish a system in which we could test the determining factors of β1AR-EGFR association reproducibly in vitro, we initially infected U2OS cells with adenoviruses encoding Flag-β1AR-mCFP and increasing amounts of Flag-WT-EGFR-mYFP and measured the association of the receptors via FRET analysis. As we have previously performed[8], we calculated the maximal %FRET (F_{max}) and K_{FRET} (as a measure of relative affinity) achieved between β1AR and EGFR via nonlinear regression analysis (Fig. 1A). Using these values for comparison, we next assessed the FRET parameters attained between β1AR-mCFP and a truncated form of EGFR lacking the last 230 amino acid C-terminal phosphotyrosine-rich domain (Flag-EGFR-CT230-mYFP, Fig. 1B). A similar association curve between β1AR-mCFP and EGFR-CT230-mYFP was attained (Fig. 1C) as had been for β1AR-mCFP and WT-EGFR-mYFP, with comparable F_{max} values (Fig. 1D), and the affinity of β1AR-mCFP for EGFR-CT230-mYFP versus WT-EGFR-mYFP was not significantly different (Fig. 1E). Similarly, via HA-agarose IP analysis, the ability of the Flag-tagged WT versus CT230 EGFR constructs to associate with HA-tagged β1AR was not different (Fig. 2A). Thus, the phosphotyrosine-rich C-terminal domain of EGFR does not play a role in regulating the association between β1AR and EGFR.

3.2. Deletion of the entire C-terminus of EGFR disrupts its association with β1AR in a kinase-independent manner

To next determine whether loss of the entire C-terminus of EGFR would alter its association with β1AR, we infected cells with adenoviruses encoding HA-β1AR and either Flag-WT-EGFR-mYFP or a truncated form of EGFR lacking almost the entire intracellular domain (EGFR-CD533[19], Fig. 2B) and performed IP analysis (Fig. 2C). Compared with either WT-EGFR or EGFR-CT230, β1AR association with EGFR-CD533 was dramatically reduced (Fig. 2D). Since loss of the phosphotyrosine-rich C-terminal domain of EGFR had no effect on its ability to associate with β1AR, these results suggest that either the kinase domain or JMD may be required for this association. To ascertain whether kinase activity is required to maintain β1AR-EGFR association, we examined the impact of the EGFR antagonist gefitinib (Gef) on receptor association (Fig. 3A). Via IP analysis (Fig. 3B), we observed that although Gef treatment reduced ERK1/2 phosphorylation in total lysates and more specifically in the EGFR IP complex (Fig. 3C), confirming inhibition of EGFR, there was no significant decrease in β1AR-EGFR association over time (Fig. 3D). Additionally, we assessed receptor association via FRET analysis. Based on the affinity between EGFR and β1AR (Fig. 1), we infected cells with MOIs of the adenoviruses encoding each receptor to achieve ~half-maximal baseline FRET, such that either increases or decreases in receptor association could be detected. However, Gef treatment did not alter β1AR-EGFR FRET (Fig. 3E), revealing that inhibition of EGFR kinase activity does not impact its ability to associate with β1AR, which may instead be dependent on a structural element proximal to the kinase domain.

3.3. The juxtamembrane domain of EGFR is required for β1AR association

The JMD of EGFR has been recognized to be an important regulator of its activity, targeting and association with other proteins through semi-distinct motifs termed JMD-A, consisting of the first 20 amino acids of the JMD domain, and JMD-B, consisting of the subsequent 18 amino acids of the JMD domain[12, 13]. Therefore we undertook to investigate whether palmitoylated peptides encoding JMD-A or JMD-B could impact β1AR-EGFR association (Fig. 4A). Indeed, treatment with palmitoylated JMD-A disrupted β1AR-EGFR association over time, however, JMD-B was incapable of doing so (Fig. 4B). Sequential alanine mutation of amino acids 655–659 (LRRLL) within JMD-A (JMD-A-5A), which was previously shown to relay effects on EGFR-protein interactions[15], was also incapable of disrupting β1AR-EGFR association. Overall, treatment of the cells specifically with JMD-A resulted in a significant decrease in β1AR-EGFR association compared with all other treatments (Fig. 4C).

3.4. β1AR-mediated EGFR transactivation is attenuated by palmitoylated JMD-A

We next sought to determine whether JMD-A-mediated disruption of β1AR-EGFR association would impact EGFR transactivation in response to β1AR stimulation. To explore this at the level of the receptor complex itself, we initially assessed the association of phosphorylated ERK1/2 with EGFR in response to ISO stimulation at increasing concentrations in U2OS cells expressing HA-β1AR and Flag-WT-EGFR-mYFP (Fig. 5A). ERK1/2 phosphorylation increased in a concentration-dependent manner to a similar extent

in both the total lysates (input, with an EC_{50} of ~28 nM) and Flag-IP eluates (with an EC_{50} of ~15 nM), representing the EGFR-associated activated ERK1/2 (Fig. 5B). Next, we pretreated U2OS cells expressing β1AR and EGFR as above with the JMD peptides, followed by stimulation with ISO at a sub-maximal concentration of 100 nM (Fig. 5C). Similar to its effect on β1AR-EGFR association, pretreatment with JMD-A attenuated the P-ERK1/2 response to β1AR stimulation, both in the total lysates and IP eluates, whereas JMD-A-5A and JMD-B were unable to do so (Fig. 5D). Altogether, these data indicate that the JMD-A region of EGFR is required for its association with β1AR and that its disruption is sufficient to prevent β1AR-mediated EGFR transactivation.

4. Discussion

EGFR has long been recognized to act as a signaling node for many neurohormone GPCRs[9], impacting numerous processes in various cell types, including cardiomyocyte hypertrophy and survival following acute cardiac injury or during the development of heart failure[3, 20–22]. In particular, we and others have studied β1AR-mediated EGFR transactivation for years, revealing that while transactivation may be initially beneficial via the acute activation of pro-survival signaling pathways, over time it may give way to chronic deleterious pro-hypertrophic signaling. While the use of neurohormone antagonists, such as β-blockers, can prevent EGFR transactivation, they can also reduce cardiac inotropic support, which becomes increasingly important to maintain during the progression of heart failure. Similarly, EGFR inhibitors can block β1AR-mediated EGFR transactivation, but have various non-selective kinase inhibitor profiles that may produce off-target effects[7]. Thus, to understand the impact of β1AR-EGFR association at baseline and during transactivation, molecular tools to disrupt this receptor complex would be of great benefit. We have previously shown that β1AR-EGFR association occurs basally, at endogenous levels of expression and is sensitive to mutation of the C-terminal putative GRK phosphorylation sites of β1AR[8], however there lacked insight into how these receptors associate from the perspective of EGFR itself.

EGFR is comprised of several structural elements that impact its interactions with other proteins, including intracellular domains incorporating the kinase domain and the C-terminal tail, which contain numerous tyrosine residues that become autophosphorylated upon ligand stimulation[9–11]. Phosphorylation of these residues provides several points of regulation and scaffolding sites for the assembly of protein complexes and downstream signaling events, which could regulate its association with β1AR. However, using FRET and IP assays we determined that truncation of the C-terminal 230 amino acid phospho-tyrosine domain did not alter EGFR association with β1AR. Conversely, truncation of almost the entire intracellular domain of EGFR resulted in the loss of a majority of its interaction with β1AR, suggesting that either the kinase domain or JMD may be required for this association. However, treatment with the EGFR antagonist gefitinib, a direct tyrosine kinase inhibitor, did not prevent β1AR-EGFR association. Conversely, treatment with a palmitoylated peptide encoding the first 20 amino acids of the JMD domain (JMD-A) disrupted β1AR-EGFR association over time, though a palmitoylated peptide fragment consisting of the subsequent 18 amino acids of the JMD domain (JMD-B) was incapable of doing so. Therefore, the proximal JMD sequence of EGFR specifically relays its ability to associate with β1AR.

The JMD of EGFR has been recognized to be an important regulator of receptor activity, targeting and interactions with other proteins[12, 13]. JMD-A has been shown to regulate the transition of EGFR dimers toward a stabilized active state and the amino acid 655–659 LRRLL sequence in particular to be essential in this process[15]. Indeed, alanine mutation of the LRRLL sequence of JMD-A into JMD-A-5A in our study attenuated the ability of the peptide to prevent β1AR association. While this could suggest that the activation status of EGFR is an important factor in mediating the association between the receptors, again, use of the kinase domain inhibitor gefitinib was unable to alter β1AR-EGFR association. Thus, JMD-A more likely relays a scaffolding function that mediates interaction between the receptors at baseline, in the absence of EGFR activity. For instance, JMD-A is known to associate with other proteins including calmodulin, ADP ribosylation factor nucleotide-binding site opener (ARNO) and sequestosome 1 (SQSTM1)[13, 18, 23]. Thus, while we have narrowed down the region of EGFR responsible for relaying its association with β1AR, future work will aim to define additional protein constituents that may act to scaffold the receptor complex together.

Notably, the predominant β1AR-coupled G protein, $G\alpha_s$, has previously been shown to associate with EGFR in a JMD-dependent manner that could be disrupted with a peptide encoding a majority of JMD-A[24], suggesting that the receptors exist in a G protein-dependent complex at baseline. This is intriguing since β1AR activation would induce $G\alpha_s$ protein dissociation from the complex to allow JMD-A dimer formation and EGFR activation, concurrent with β-arrestin1/2 recruitment. We previously demonstrated that while β-arrestins were not required for basal association of β1AR and EGFR, their recruitment to the receptor complex was essential for not only EGFR transactivation, but the continued association between β1AR and EGFR after catecholamine stimulation[8], which presumably occurs via distinct structural elements of EGFR than the JMD. Thus, β1AR-EGFR association may be regulated both before and after EGFR transactivation via the coordinated actions of G protein- and β-arrestin-dependent mechanisms, respectively. Further, EGFR has been shown to associate with other GPCRs[9], thus it is possible that disruption of the JMD could more broadly impact the ability of EGFR to be engaged as a signaling node by additional receptor systems.

Targeting the JMD has been studied with more frequency in recent years as a means by which to disrupt EGFR in cancer cells. Peptides engineered from the JMD-A sequence in particular have been shown effective at reducing EGFR activity and cancer cell survival and decreasing tumor burden, while having minimal effects on survival of cells with lower levels of endogenous EGFR expression[14–18]. A recent study identified a small molecule, EE-02, that inhibited EGFR association with EGFR pathway substrate 8 (Eps8), ultimately reducing viability specifically in cancer cells, while noncancerous cells retained normal survival responses[25]. Molecular modeling of EE-02 predicted its binding to the JMD-B region of EGFR, thus we don’t expect that it would be effective for preventing β1AR-EGFR association, however it does establish an important proof-of-concept for small molecule-mediated, rather than peptide-mediated, disruption of a β1AR-EGFR complex for future development.

5. Conclusion

In summary, we have demonstrated that the JMD of EGFR is essential to its interaction with β1AR, disruption of which dissociates the receptor complex and prevents EGFR transactivation. Although the JMD has been shown to regulate EGFR activity[12, 13], EGFR kinase activity and C-terminal phosphorylation sites are dispensable for β1AR association, thus scaffolding of JMD with other proteins likely mediates receptor complex assembly. Refined tools targeting the JMD or associated proteins may therefore provide a means by which to explore the impact of loss of the β1AR-EGFR receptor complex on cellular signaling and outcomes such as growth and survival.

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Highlights

- β1AR and EGFR associate in a receptor complex
- EGFR kinase activity and distal C-terminus are dispensable for association with β1AR
- Disruption of the juxtamembrane domain (JMD) dissociates the β1AR-EGFR complex
- JMD disruption prevents β1AR-mediated ERK1/2 phosphorylation in association with EGFR

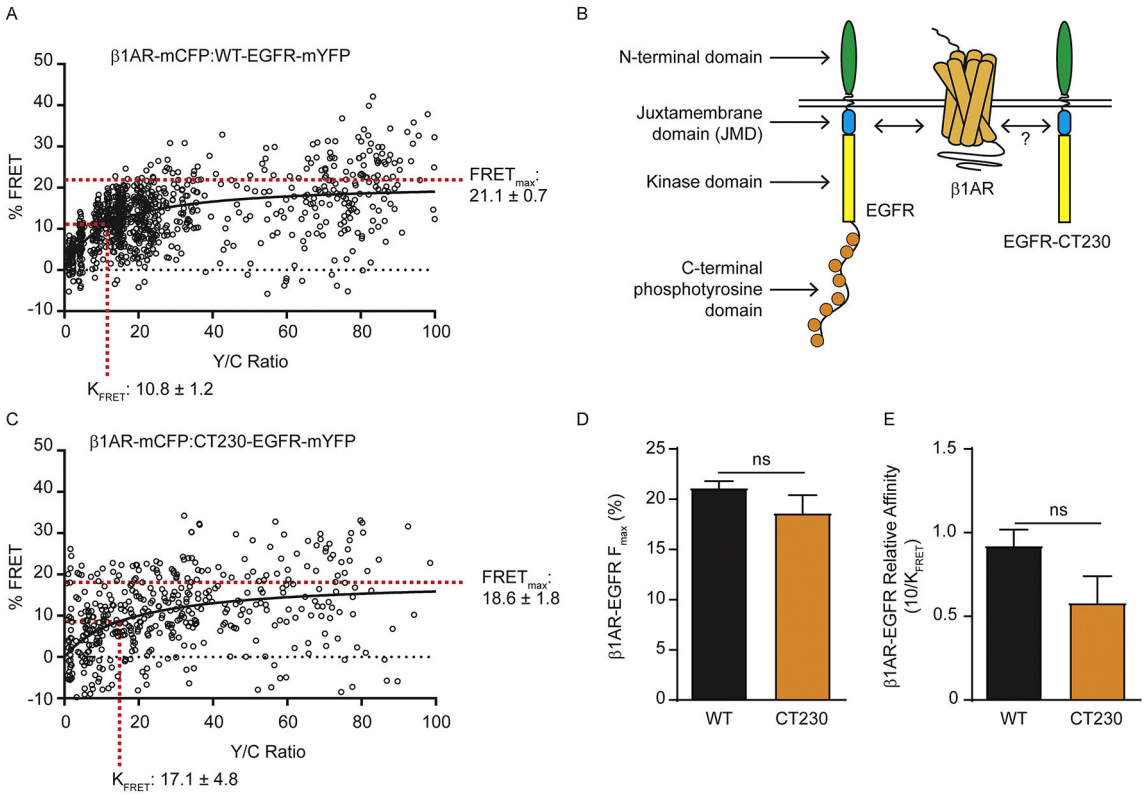

Figure 1: β1AR-EGFR association is independent of the phosphotyrosine-rich C-terminus of EGFR.

A) U2OS cells were infected with increasing MOIs (0.03–30) of adenoviruses encoding Flag-WT-EGFR-mYFP and Flag-β1AR-mCFP. %FRET values attained between β1AR and EGFR were plotted against the increasing mYFP/mCFP ratio and nonlinear regression analysis performed to calculate the maximal FRET values (F_{max}) and Y/C ratio at which half the F_{max} was achieved (K_{FRET}). B) Schematic comparing WT-EGFR versus EGFRCT230. C) U2OS cells were infected with increasing MOIs (0.03–30) of adenoviruses encoding Flag-EGFR-CT230-mYFP and Flag-β1AR-mCFP. %FRET values attained between β1AR and EGFR were plotted against the increasing mYFP/mCFP ratio and nonlinear regression analysis performed to calculate the maximal FRET values (F_{max}) and Y/C ratio at which half the F_{max} was achieved (K_{FRET}). No significant differences were detected between WT and CT230 EGFR for either F_{max} (D) or relative affinity (E) for association with β1AR. NS=not significant, two-tailed unpaired t-test, n=894 from 12 independent experiments (WT) and n=456 from 6 independent experiments (CT230).

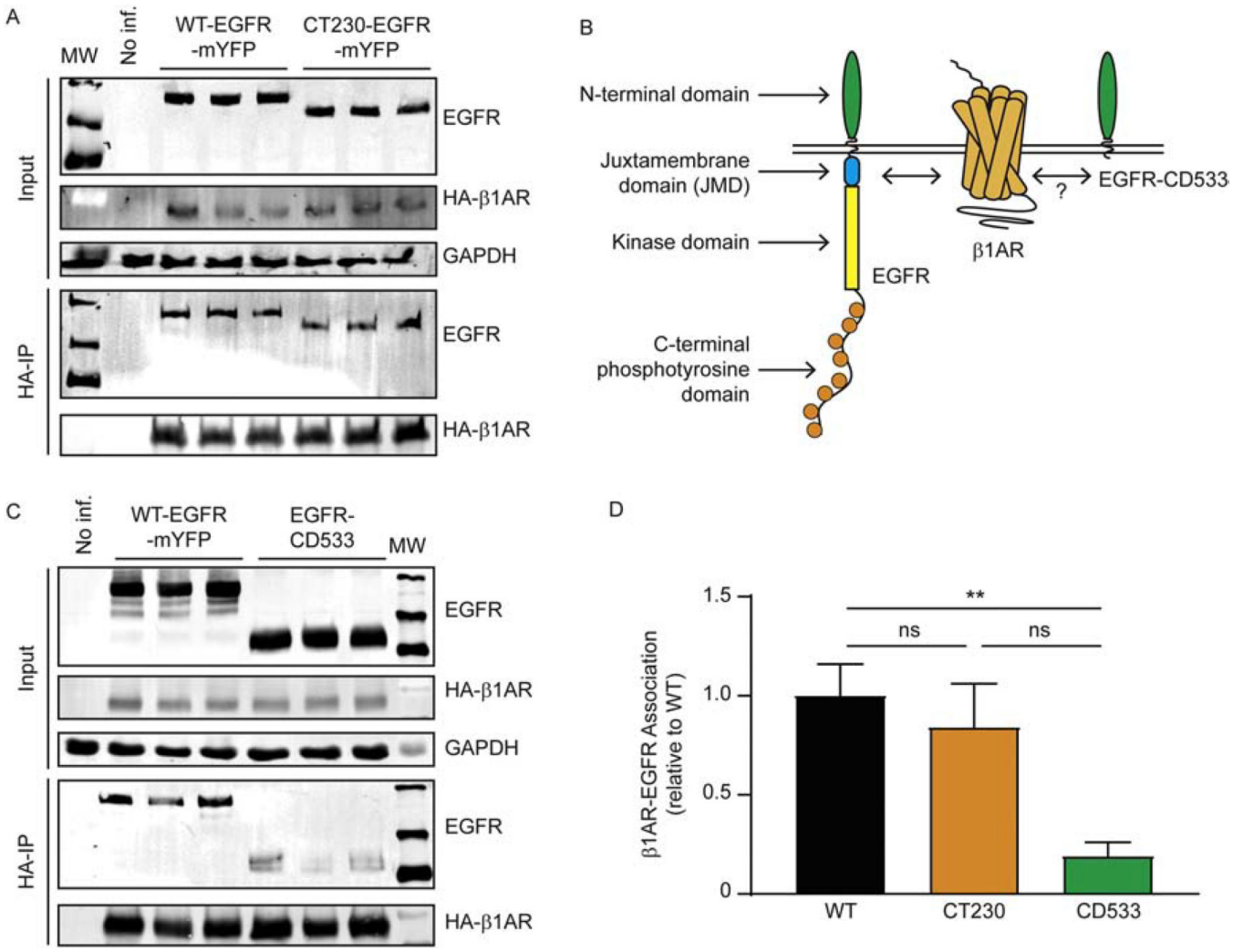

Figure 2: Truncation of the entire C-terminus of EGFR disrupts its association with β1AR.

A) U2OS cells were infected with adenoviruses encoding Flag-WT-EGFR-mYFP (MOI 5) versus Flag-EGFR-CT230-mYFP (MOI 40) in conjunction with HA-β1AR (MOI 10). Immunoprecipitation of HA-β1AR was performed using HA-conjugated agarose beads and its association with each EGFR construct assessed via immunoblotting with anti-EGFR antibody. MW = molecular weight markers, No Inf. = non-infected controls. B) Schematic comparing WT-EGFR versus EGFR-CD533. C) U2OS cells were infected with adenoviruses encoding Flag-WT-EGFR-mYFP (MOI 10) versus EGFR-CD533 (MOI 2.5) in conjunction with HA-β1AR (MOI 10), and immunoprecipitations performed as above. MW = molecular weight markers, No Inf. = non-infected controls. D) Both WT-EGFR and EGFR-CT230 associated to the same extent with β1AR, but EGFR-CD533 had significantly reduced association with association. ns = not significant, **P<0.01, ordinary one-way ANOVA with Tukey's multiple comparisons test, n=9 (WT), n=3 (CT230) and n=6 (CD533).

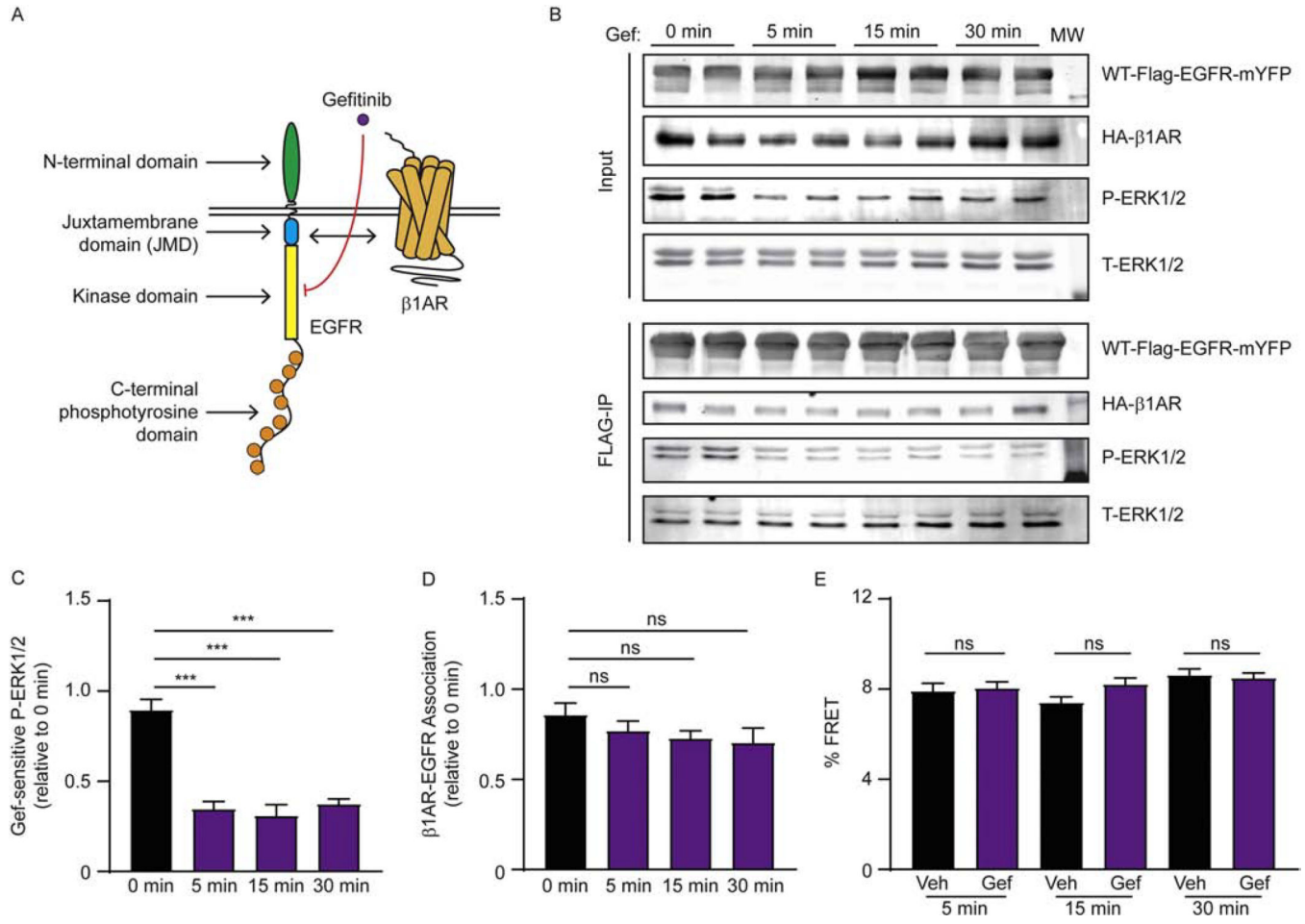

Figure 3: Kinase activity of EGFR is not required for its association with β1AR.

A) Schematic depicting antagonism of WT-EGFR via gefitinib-mediated inhibition of the kinase domain. B) U2OS cells were infected with adenoviruses encoding Flag-WT-EGFR-mYFP (MOI 5) in conjunction with HA-β1AR (MOI 10), treated with vehicle (0.1% DMSO) or gefitinib (Gef, 1 μM) for indicated timepoints and underwent HA-agarose IP and immunoblotting analysis. Quantified changes in P-ERK1/2 (D) and β1AR association with EGFR (D) in the IP eluates in response to Gef treatment (fold changes compared to non-stimulated control at time 0). IP blots were probed with rabbit anti-P-ERK1/2 and mouse anti-T-ERK1/2, while input blots were probed with mouse anti-P-ERK1/2 and rabbit anti-T-ERK1/2. MW = molecular weight markers, ns = not significant, ***P<0.001 ordinary one-way ANOVA with Tukey's multiple comparisons test, n=6 per condition from 3 independent experiments. E) U2OS cells were infected with adenoviruses encoding Flag-WT-EGFR-mYFP (MOI 10) and Flag-β1AR-mCFP (MOI 3) to produce ~½ maximal FRET were subsequently treated with vehicle (Veh, 0.1% DMSO) or gefitinib (Gef, 1 μM) for indicated timepoints. FRET between β1AR and EGFR was unaltered in the presence of Gef. ns = not significant, two-tailed unpaired t-test, n=24 per condition from 2 independent experiments.

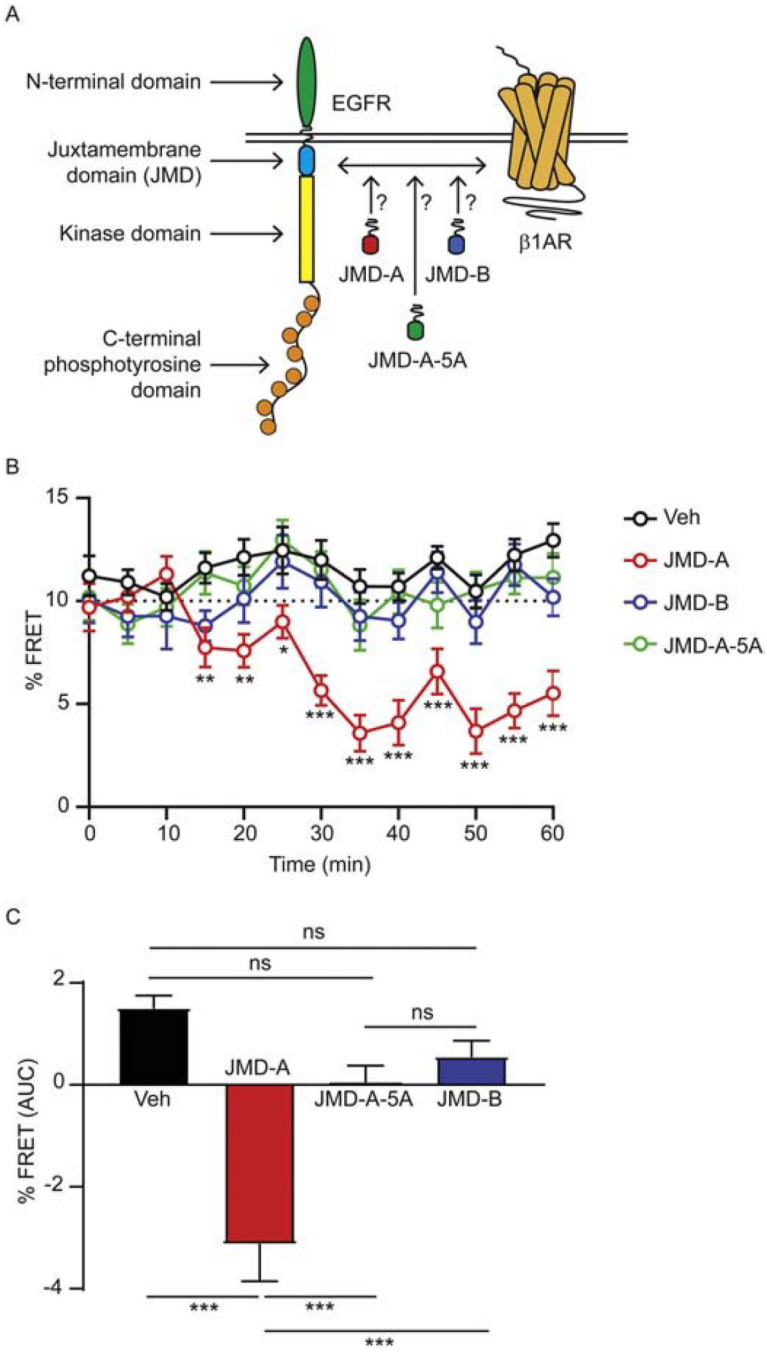

Figure 4: Targeting the juxtamembrane domain of EGFR disrupts its association with β1AR.

A) Schematic depicting possible disruption of β1AR-EGFR association via palmitoylated peptides comprising JMD-A, JMD-B or a mutated form of JMD-A (JMD-A-5A). B) U2OS cells were infected with adenoviruses encoding Flag-WT-EGFR-mYFP (MOI 3) and Flag-β1AR-mCFP (MOI 2.5) to produce ~½ maximal FRET and were subsequently treated with vehicle (Veh, 1% DMSO) or palmitoylated JMD peptides (10 μM each) and FRET monitored every 5 min for 60 min total. JMD-A reduced β1AR-EGFR FRET over time versus Veh control, whereas neither JMD-A-5A nor JMD-B were able to do so. *P<0.05,

**P<0.01, ***P<0.001 versus Veh at corresponding timepoint, two-way repeated measures ANOVA with Dunnett's multiple comparisons test. C) JMD-A was the only peptide to produce a composite negative change in FRET area under the curve (AUC) over the full timecourse. ns = not significant, ***P<0.001, ordinary one-way ANOVA with Tukey's multiple comparisons test, n=24 from 4 independent experiments (Veh, JMD-A) and n=18 from 3 independent experiments (JMD-A-5A, JMD-B).

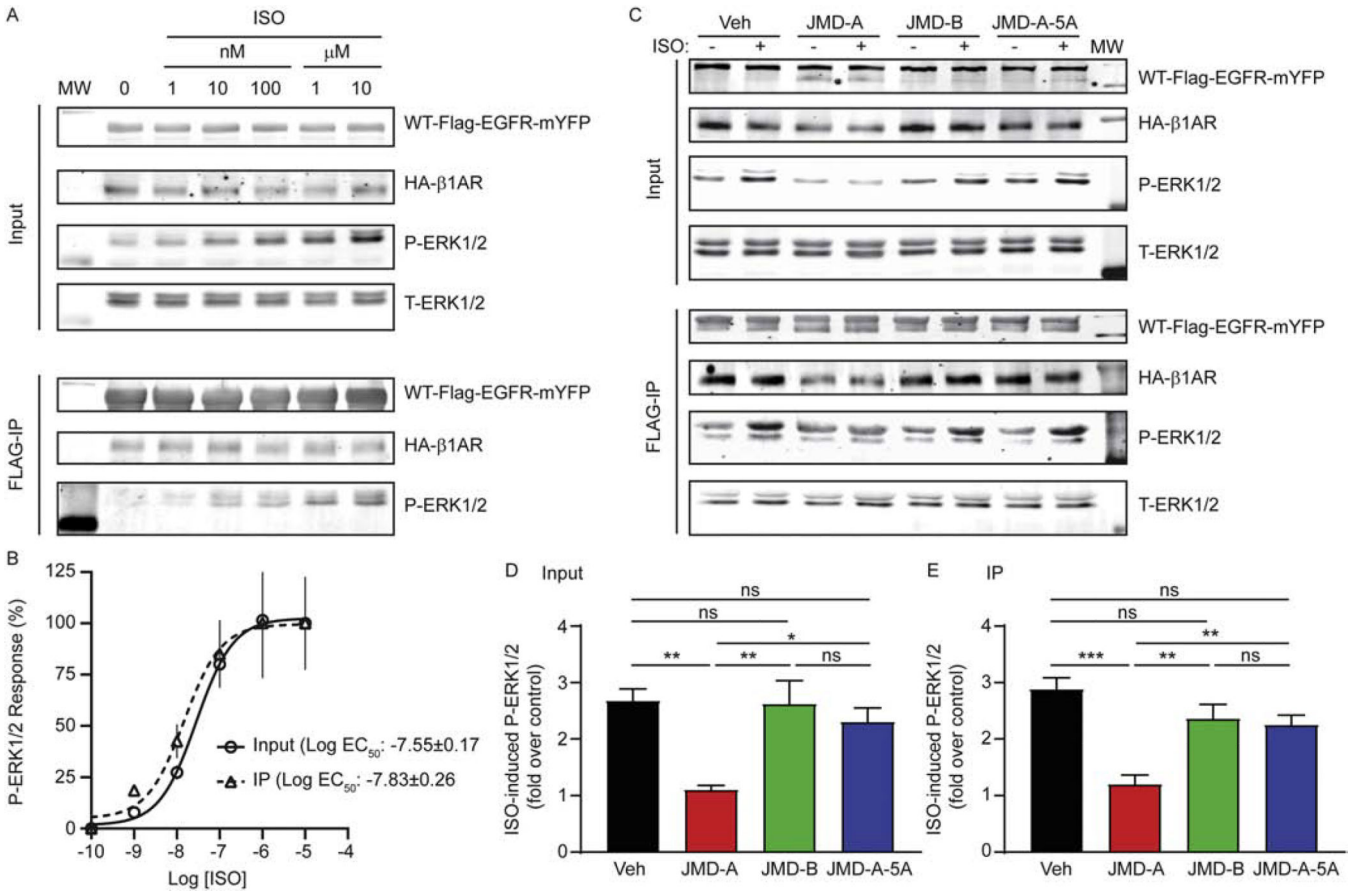

Figure 5: JMD-A impact on β1AR and EGFR proximal signaling responses alone.

A) U2OS cells infected with Flag-WT-EGFR-mYFP (MOI 1) and HA-β1AR (MOI 10) were stimulated with increasing concentrations of ISO for 5 min, followed by immunoprecipitation using Flag-M2 agarose beads. Increasing ISO concentrations acted to increase phosphorylation of ERK1/2 in input (total lysate) as well as Flag-IP lysates. MW = molecular weight markers, n = 8 (Veh), 7 (1 nM), 8 (10 nM), 6 (100 nM), 5 (1 μM), 4 (10 μM). B) Concentration-dependent curves depict similar log EC_{50} values for ERK1/2 phosphorylation in both the input (total lysate) and IP samples. C) Representative immunoblots of U2OS cells infected with Flag-WT-EGFR-mYFP and HA-β1AR as described and pretreated with Vehicle (Veh, 1% DMSO) or JMD peptides (10 μM each) for 15 min followed by ISO stimulation (100 nM) for 5 min and immunoprecipitation using Flag-M2 agarose beads. MW = molecular weight markers. IP blots were probed with rabbit anti-P-ERK1/2 and mouse anti-T-ERK1/2, while input blots were probed with mouse anti-P-ERK1/2 and rabbit anti-T-ERK1/2. Quantified P-ERK1/2 responses to ISO stimulation (fold changes compared to non-stimulated controls within corresponding treatment condition) in the input (D) and IP samples (E). *P<0.05, **P<0.01, ***P<0.001 ordinary one-way ANOVA with Tukey's multiple comparisons test, n=8 per condition.