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**Mutations of  $\beta$ -arrestin 2 that limit self-association also interfere with interactions with the  $\beta_2$ -adrenoceptor and the ERK1/2 MAP kinases:  
Implications for  $\beta_2$ -adrenoceptor-signalling via the ERK1/2 MAP kinases**

Tian-Rui Xu<sup>1,2</sup>, George S. Baillie<sup>2</sup>, Narinder Bhari<sup>1,2</sup>, Thomas M. Houslay<sup>2,6</sup>, Andrew M. Pitt<sup>1</sup>, David R. Adams<sup>4</sup>, Walter Kolch<sup>1,3</sup>, Miles D. Houslay<sup>2</sup> and Graeme Milligan<sup>2,5</sup>

<sup>1</sup> Sir Henry Wellcome Functional Genomics Facility, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow Glasgow G12 8QQ, Scotland, U.K.

<sup>2</sup> Molecular Pharmacology Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ Scotland, U.K.

<sup>3</sup> Beatson Institute for Cancer Research, Garscube Estate, Glasgow G61 1BD Scotland, U.K.

<sup>4</sup> Department of Chemistry, Heriot-Watt University, Riccarton Campus, Edinburgh, EH14 4AS, Scotland, U.K.

<sup>5</sup> To whom correspondence should be addressed

G. Milligan, Davidson Building, University of Glasgow, Glasgow G12 8QQ Scotland, U.K. Tel + 44 141 330 5557 FAX + 44 141 330 4620

e-mail: [g.milligan@bio.gla.ac.uk](mailto:g.milligan@bio.gla.ac.uk).

<sup>6</sup> Current address: AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leics. LE11 5RH

**Short title:**  $\beta$ -arrestin 2 self association and scaffolding

**Key words:**  $\beta$ -arrestin, scaffolding,  $\beta_2$ -adrenoceptor, ERK MAP kinase, peptide array

**<sup>6</sup>Abbreviations**

DTSP, dithiobis(succinimidyl propionate); FRET, Fluorescence resonance energy transfer; GPCR, G protein-coupled receptor; IP6, inositol 1,2,3,4,5,6-hexakisphosphate.

## SYNOPSIS

Fluorescence resonance energy transfer (FRET) and co-immunoprecipitation studies confirmed the capacity of  $\beta$ -arrestin 2 to self-associate. Amino acids potentially involved in direct protein-protein interaction were identified via combinations of spot-immobilized peptide arrays and mapping of surface exposure. Among potential key amino acids Lys<sup>285</sup>, Arg<sup>286</sup> and Lys<sup>295</sup> are part of a continuous surface epitope located in the polar core between the N- and C-terminal domains. Introduction of Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala mutations into  $\beta$ -arrestin 2-eCFP and  $\beta$ -arrestin 2-eYFP constructs substantially reduced FRET, whilst introduction of a Lys<sup>295</sup>Ala mutation had a more limited effect. Neither of these mutants was able to promote  $\beta_2$ -adrenoceptor-mediated phosphorylation of the ERK1/2 MAP kinases. Both  $\beta$ -arrestin 2 mutants displayed limited capacity to co-immunoprecipitate ERK1/2 and further spot-immobilized peptide arrays indicated each of Lys<sup>285</sup>, Arg<sup>286</sup> and particularly Lys<sup>295</sup> to be important for this interaction. Direct interactions between  $\beta$ -arrestin 2 and the  $\beta_2$ -adrenoceptor were also compromised by both Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala and Lys<sup>295</sup>Ala mutations of  $\beta$ -arrestin 2. These were not non-specific effects linked to improper folding of  $\beta$ -arrestin 2 as limited proteolysis was unable to distinguish the Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala or Lys<sup>295</sup>Ala mutants from wild type  $\beta$ -arrestin 2, whilst interaction of  $\beta$ -arrestin 2 with JNK3 was unaffected by the Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala or Lys<sup>295</sup>Ala mutations. These data suggest that amino acids important for self-association of  $\beta$ -arrestin 2 also play an important role in interaction with both the  $\beta_2$ -adrenoceptor and the ERK1/2 MAP kinases. Regulation of  $\beta$ -arrestin 2 self-association may therefore control  $\beta$ -arrestin 2-mediated  $\beta_2$ -adrenoceptor-ERK1/2 MAP kinase signalling.

## INTRODUCTION

$\beta$ -arrestin 1 and 2 are ubiquitously expressed members of the arrestin protein family. Although long appreciated to play key roles in the desensitization of function of G protein-coupled receptors (GPCRs)<sup>6</sup>, recent studies have begun to demonstrate a much wider ranging set of roles for the  $\beta$ -arrestins, including interactions with receptors outside of the GPCR family and the generation of distinct, G protein-independent signals [1-3]. Many of the recently described functions of  $\beta$ -arrestins, including their ability to regulate activation of members of the MAP kinase families [4-7], reflect the capacity of  $\beta$ -arrestins to act as scaffolds for a substantial number of cellular polypeptides. Indeed, proteomic analysis of  $\beta$ -arrestin-interacting proteins has indicated upwards of 200 distinct polypeptides residing in distinct cellular compartments and with wide ranging functions in signal transduction, cellular organization and nucleic acid binding [8]. These include polypeptides involved in the production and destruction of the secondary messengers cAMP [9] and diacylglycerol [10]. Many of these interactions have been uncovered via combinations of yeast-2 hybrid screens and immunoprecipitation/pull down studies, and sites of interaction have been mapped by mixtures of protein truncation and mutagenesis techniques. Recently, however, the application of peptide array-based techniques has allowed detailed mapping of potential sites of interactions between  $\beta$ -arrestin 2 and protein-interaction partners such as the cAMP specific phosphodiesterase PDE4D5 [11-12]. Such screens rapidly focus attention on key regions, and even individual amino acids, that may contribute to or define protein-protein interaction surfaces.

Protein self-association is an extremely common theme in biology [13] and recent studies have indicated that  $\beta$ -arrestin 1 and  $\beta$ -arrestin 2 are able to both homo- and hetero-dimerize [14-15].  $\beta$ -arrestins have long been known to interact with phosphoinositides [16] and recent studies have indicated that, at least in part, binding of inositol 1,2,3,4,5,6-hexakisphosphate (IP6) to positively charged regions in both the N and C-terminal domains contributes to  $\beta$ -arrestin 1 and 2 dimerization [14]. In that study the 2.9A atomic level structure of a  $\beta$ -arrestin 1/IP6 complex indicated the existence of two IP6 binding sites per protein monomer. Isothermal titration calorimetry and <sup>3</sup>H-IP6 binding assays confirmed two-site binding with a low affinity IP6-binding site in the N-domain and a high affinity site in the C-domain of the arrestin. IP6, therefore, appears to act as a bridge between  $\beta$ -arrestin monomers to generate dimers and, potentially, as has also been observed for members of the GPCR superfamily [17] that interact with arrestins, to generate higher-order  $\beta$ -arrestin structures and arrays. Structural models of  $\beta$ -arrestin- $\beta$ -arrestin interactions [14] also predict that direct protein-protein contacts are likely to contribute to the stability and effectiveness of  $\beta$ -arrestin dimerization/oligomerization.

To identify regions and specific amino acids important in  $\beta$ -arrestin 2 self-association that are distinct from the IP6 binding domains we have employed combinations of spot-immobilized peptide arrays with subsequent supporting mutagenesis and combinations of fluorescence resonance energy transfer (FRET) and co-immunoprecipitation studies. Mutation of positively charged residues in a surface exposed, polar core region between the N and C-terminal IP6 binding domains, which was identified via peptide array studies, limited the effectiveness of  $\beta$ -arrestin 2 self-association. Such mutants of  $\beta$ -arrestin 2 also interacted poorly with both the prototypic

$\beta$ -arrestin-interacting GPCR, the  $\beta_2$ -adrenoceptor, and with the ERK1/2 MAP kinases. This resulted in an inability of the modified forms of  $\beta$ -arrestin 2 to support  $\beta_2$ -adrenoceptor-mediated,  $\beta$ -arrestin 2-dependent ERK1/2 MAP kinase phosphorylation. These studies indicate that sites other than the IP6 binding domains are important for  $\beta$ -arrestin 2 self-association, that the quaternary structure of  $\beta$ -arrestin 2 plays a key role in  $\beta_2$ -adrenoceptor and ERK MAP kinase interactions and suggest that regulation of  $\beta$ -arrestin 2 self-association may, therefore, control  $\beta$ -arrestin 2-mediated  $\beta_2$ -adrenoceptor-ERK1/2 MAP kinase signalling.

## EXPERIMENTAL

### *Antibodies and radiochemicals*

Monoclonal anti-VSV-G antibody and a polyclonal anti-FLAG antiserum were from Sigma. The polyclonal anti- $\beta_2$ -adrenoceptor antibody (H-73) and one of the  $\beta$ -arrestin antibodies used were from Santa Cruz (Calne, Wiltshire, U.K.). Monoclonal p44/42 MAPK (ERK1/2) and phospho-p44/42 MAPK (phospho-ERK1/2) antibodies were from Cell Signalling Technology (Hitchin, Hertfordshire, U.K.). A second anti- $\beta$ -arrestin antibody was the kind gift of Dr R. J. Lefkowitz (Howard Hughes Medical Institute, Duke University, Durham, NC, U.S.A.). Lipofectamine 2000 transfection reagent was from Invitrogen. Protease inhibitor cocktail tablets were from Roche. [ $^3$ H]CGP-12177 (44 Ci mmol $^{-1}$ ) was purchased from GE Healthcare.

### *Cell culture and transfection*

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.292 g/liter L-glutamine and 10% (v/v) newborn calf serum at 37 °C in a 5% CO $_2$  humidified atmosphere. A HEK293 cell line stably expressing the human  $\beta_2$ -adrenoceptor has been described previously [18] and was maintained as above but with the addition of 800  $\mu$ g/ml G418. Cells were grown to 50–70% confluence before transient transfection. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

### *Plasmid construction and site-direct mutagenesis*

Bovine  $\beta$ -arrestin 2 was used as a template to generate  $\beta$ -arrestin-eCFP and  $\beta$ -arrestin-eYFP via overlapping PCR. The first set of primers were ACT ATG ACG ACC AGT TCT GT GTG AGC AAG GGC GAG GAG CT (forward) and AG CTC CTC GCC CTT GCT CAC ACA GAA CTG GTC GTC ATA GT (reverse). The overlapping PCR products were amplified by the second set of primers with KpnI and ApaI sites and inserted into pCDNA3, the primers were CGAT GGT AC CCC ATG GGG GAG AAA CCC GGG ACC AGG GT (forward with KpnI site (underlined)) and TGCT GGG CCC TTA CTT GTA CAG CTC GTC CAT GCC GAG AG (reverse with ApaI site (underlined)). eCFP- $\beta$ -arrestin-2 was a kind gift of Dr. Juan F. Lopez-Gimenez (University of Glasgow). VSV-G- $\beta$ -arrestin-2 was generated by PCR and inserted into the KpnI-ApaI site of pCDNA3. The primers were CGAT GGT ACC CC GCC ACC ATG TAC ACC GAT ATA GAG ATG AAC CGC CTT GGA AAG GGG GAG AAA CCC GGG ACC AGG GTC (forward with VSV-G sequence and KpnI site (underlined)) and TGAT GGG CCC TCA ACA GAA CTG GTC GTC ATA GTC CTC GT (reverse with ApaI site (underlined)). FLAG- $\beta$ -arrestin-2 was generated by PCR and also inserted

into the KpnI-ApaI site of pCDNA3. The primers were CGAT GGT ACC CC ATG GAC TAC AAG GAC GAC GAT GAT AAG GGG GAG AAA CCC GGG ACC AGG GT (forward with FLAG sequence and KpnI site (underlined)) and TGAT GGG CCC TCA ACA GAA CTG GTC GTC ATA GTC CTC GT (reverse with ApaI site (underlined)). Site-directed mutagenesis was performed using the QuikChange® II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala double mutation of  $\beta$ -arrestin-2 was introduced using the primers TCA GCA ACA ACC GGG AGG CGG CCG GCC TCG CTC TGG AT (forward) and ATC CAG AGC GAG GCC GGC CGC CTC CCG GTT GTT GCT GA (reverse). The primers for the Lys<sup>295</sup>Ala mutation of  $\beta$ -arrestin-2 were CTC TGG ATGGG AAG CTC GCG CAC GAG GAC ACC AAC C (forward) and GGT TGG TGT CCT CGT GCG CGA GCT TCC CAT CCA GAG (reverse).

### *Live cell FRET imaging*

HEK293 cells grown on poly-D-lysine-treated glass coverslips were transiently transfected with combinations of eCFP- and eYFP-tagged forms of  $\beta$ -arrestin 2. 18-24 hrs after transfection, cells were transferred to a microscope chamber containing HEPES-buffered saline solution: (130 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 20 mM HEPES, 10 mM D-glucose, pH 7.4). eCFP and eYFP expressing cells were excited using an Optoscan monochromator (Cairn Research, Faversham, Kent, UK) which was set to 500/5 and 430/12 nm for the sequential excitation of eYFP and eCFP respectively. A dual dichroic mirror (86002v2bs; Chroma Inc., Rockingham, VT) was used to reflect the excitation light through a Nikon x40, (NA=1.3), oil-immersion Fluor lens. The resultant eCFP and eYFP fluorescence emission signals were detected using a high-speed filterwheel device (Prior Instruments) containing the following band pass emitters: HQ470/30 m for eCFP and HQ535/30 m for eYFP. Bleedthrough coefficients were measured from cells expressing either eCFP or eYFP alone and were quantified by dividing the amount of fluorescence detected in the FRET channel (e.g. FRET<sub>eCFP-eYFP</sub>) by the fluorescence detected from each fluorescent protein, at its own filter channel (e.g. eCFP or eYFP). Corrected FRET (FRET<sub>c</sub>) was calculated using a pixel-by-pixel methodology using the equation  $FRET_c = FRET - (\text{coefficient } B \times \text{CFP}) - (\text{coefficient } A \times \text{YFP})$ , where eCFP, eYFP, and FRET values correspond to background corrected images obtained through the eCFP, eYFP, and FRET channels. *B* and *A* correspond to the values obtained for the eCFP (donor) and eYFP (acceptor) bleedthrough coefficients, respectively. To correct the FRET levels for the varying amounts of donor (eCFP) and acceptor (eYFP), normalized FRET (FRET<sub>N</sub>) was calculated using the equation  $FRET_N = FRET_c / \text{eCFP}$ , where FRET<sub>c</sub> and eCFP are equal to the fluorescence values obtained from single cells. For further details of the FRET procedures see [17, 19].

### *Molecular models*

The crystal structure (PDB: 1ZSH) of bovine arrestin-2 ( $\beta$ -arrestin 1) with bound IP6 was used to locate basic residues from array peptide-57 implicated in arrestin self-association. Rendered surfaces were generated using PyMol (DeLano Scientific LLC; www.pymol.org).

### ***Peptide arrays and alanine scans***

A  $\beta$ -arrestin-2 peptide library was produced by automatic SPOT synthesis as described [20-21]. It was synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membranes using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry with the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany). Alanine scanning peptide libraries were constructed by taking the residues in positive spots and sequentially changing each residue to alanine (or if an alanine was the natural amino acid at that position, to aspartate). The interaction of spotted peptides with purified, recombinant GST and GST- $\beta$ -arrestin-2 fusion proteins was determined by overlaying the cellulose membranes with 10  $\mu$ g/ml recombinant protein. Bound recombinant proteins were then detected following wash steps with rabbit anti-GST and anti  $\beta$ -arrestin antisera, and detection was performed with a secondary anti-rabbit horseradish peroxidase-coupled antibody. Similar studies explored interactions of the  $\beta$ -arrestin-2 peptide library with ERK2 (Millipore 14-198). The relative intensity of interaction of the recombinant proteins with the arrayed peptides was estimated by densitometry.

### ***Co-immunoprecipitation studies***

Cells were harvested 24 h after transfection and resuspended in immunoprecipitation buffer (150 mM NaCl, 0.01 mM NaPO<sub>4</sub>, 2 mM EDTA, 0.5% Triton X-100, and 5% glycerol plus protease inhibitor cocktail tablets). The cell pellet was lysed on a rotating wheel for 30 min at 4°C. Samples were then centrifuged for 15 min at 20,000g at 4°C, and the supernatant transferred to a fresh tube with Protein G/A beads (Sigma) to pre-clear the samples. After incubation on a rotating wheel for 1 h at 4°C, the samples were re-centrifuged at 20,000g at 4°C for 1 min, and the protein concentration of the supernatant was determined. Samples containing equal amounts of protein were incubated for 2 h with 30  $\mu$ l anti-VSV-G agarose beads (Sigma) at 4°C on a rotating wheel. Samples were then washed three times with immunoprecipitation buffer. After addition of 2 x SDS loading buffer and heating to 90°C for 5 min, both immunoprecipitated samples and cell lysate controls were resolved by SDS-PAGE and subsequently immunoblotted to detect proteins of interest.

### ***Limited proteolysis studies***

HEK293 cells were transfected with wild type  $\beta$ -arrestin-2 or its mutants, 24 h after transfection, cells were lysed in lysis buffer (150 mM NaCl, 0.01 mM NaPO<sub>4</sub>, 2 mM EDTA, 0.5% Triton X-100, and 5% glycerol plus protease inhibitor cocktail tablets, pH 8.0) on a rotating wheel for 30 min at 4°C. Samples were then centrifuged for 15 min at 20,000g at 4°C. 60 $\mu$ g protein from the supernatant fraction was incubated with 0.6  $\mu$ g sequencing grade trypsin (Promega) at 37°C varying times indicated. Digestion was terminated by addition of 2 X SDS loading buffer and heating at 95°C for 5 min. Samples were then resolved by SDS-PAGE and subsequently immunoblotted with a  $\beta$ -arrestin-2 antibody (Santa Cruz).

### ***Cross-linking studies***

HEK293 cells stably expressing the  $\beta_2$ -adrenoceptor [18] were transfected transiently with VSV-G tagged forms of wild type and mutated  $\beta$ -arrestin-2. Cells were then treated

with or without isoprenaline. Subsequently, the membrane-permeable and reversible cross-linker dithiobis(succinimidyl propionate) (DTSP) was added at a final concentration of 2 mM. The cells were then incubated under gentle agitation at room temperature for 30 min and washed twice with 50 mM Tris/HCl, pH 7.4, in PBS to neutralize unreacted DTSP. The cells were then lysed and immunoprecipitated as above.

### ***Receptor internalisation***

$\beta_2$ -adrenoceptor internalisation studies were performed as in [18]. Briefly, cells were co-transfected with pCDNA3-  $\beta_2$ -adrenoceptor plus  $\beta$ -arrestin 2 or its mutants in 24 well plates. 24 h after transfection cells were treated with 10  $\mu$ M isoprenaline or vehicle for 30 min. The cells were then washed twice with ice-cold phosphate-buffered saline (KCl 2.7 mM, NaCl 137 mM,  $\text{KH}_2\text{PO}_4$  1.5 mM,  $\text{Na}_2\text{HPO}_4$  8 mM, pH 7.4) followed by addition of 200  $\mu$ l of binding mix ( $[^3\text{H}]\text{CGP-12177}$  (10 nM) in Krebs-Ringer-HEPES buffer (NaCl 130 mM, KCl 5 mM,  $\text{MgSO}_4$  1.2 mM,  $\text{CaCl}_2$  1.2 mM, HEPES 20 mM,  $\text{Na}_2\text{PO}_4$  1.2 mM, glucose 10, 0.1% BSA; pH 7.4), and incubated at 4°C for 90 min. Non-specific binding was assessed by the presence of 10  $\mu$ M propranolol. All experiments were terminated by removal of the binding medium and washing of the cells with ice-cold PBS. 0.5 ml of 0.5 mM EDTA in PBS was added to detach cells from the plate and this volume plus a further 0.5 ml wash of the wells of the microtitre plate were counted.

### ***ERK1/2 MAP kinase phosphorylation assays***

HEK293 cells stably expressing the  $\beta_2$ -adrenoceptor were grown in 6-well plates and transiently transfected with various forms of  $\beta$ -arrestin-2. The cells were rendered quiescent by serum starvation for 12 h prior to stimulation for the indicated times with or without isoprenaline. Cells were then placed on ice and solubilized directly in 500  $\mu$ l Laemmli sample buffer. The samples were then sonicated for 30 secs, heated for 15 min at 95°C, and micro-centrifuged for 5 min before fractionation of the proteins on SDS-PAGE. ERK1/2 MAP kinase phosphorylation was detected by protein immunoblotting using a phospho-ERK1/2-specific antibody (Cell Signalling Technology). The nitrocellulose membranes were subsequently stripped of immunoglobulins and re-probed using an anti-ERK1/2 antibody to assess the equivalence of protein loading.

## **RESULTS**

$\beta$ -arrestin 2 has been reported to self-associate [14-15]. To examine the basis of such interactions we set out to gain insight into amino acid residues that are important for this interaction. The aim was to generate self-association compromised  $\beta$ -arrestin 2 point mutants and determine how interfering with self-association might affect function. We chose two complementary strategies, FRET and classical co-immunoprecipitation experiments to detect self-association. For FRET experiments bovine  $\beta$ -arrestin 2 was tagged at the C-terminus with enhanced yellow fluorescent protein (eYFP) and with enhanced cyan fluorescent protein (eCFP) at either the N- or C-terminus. Following transfection of either individual forms or pairs of constructs into HEK293 cells, imaging of eCFP and eYFP auto-fluorescence demonstrated each of the constructs to be predominantly cytoplasmic and excluded from the nucleus (Figure 1A and data not shown). Measurements of eCFP to eYFP FRET demonstrated a significant signal that is consistent with the self-association of  $\beta$ -arrestin 2. The FRET signal obtained was



substantially greater when the fluorescent protein tags were each located at the C-terminus of  $\beta$ -arrestin 2 (Figure 1B) than when C-terminally-tagged eCFP- $\beta$ -arrestin 2 and N-terminally-tagged  $\beta$ -arrestin 2-eYFP were co-expressed (Figure 1B). Although other interpretations are possible, this observation suggests that  $\beta$ -arrestin 2 self-association might be sterically constrained to favour conformations where the C-termini are juxtaposed.

Spot-immobilized peptide array analysis has proved to be a powerful novel technology to gain insight into sites that putatively underpin protein-protein interaction. Indeed, we have recently exploited this technique to identify sites that define the interaction of the PDE4D5 isoform with the signalling scaffold proteins,  $\beta$ -arrestin and RACK1 [11-12] and between PDE4B1 and the scaffold protein, DISC1 whose gene disruption is implicated in schizophrenia [22]. 25-mer peptides covering the entire primary sequence of  $\beta$ -arrestin 2 were spot synthesized on cellulose membranes with a 5 amino acid moving frameshift. These immobilized peptide arrays were probed with recombinantly expressed GST- $\beta$ -arrestin 2 or, as a control, with GST alone. Subsequent to washing, the peptide arrays were exposed to either an anti- $\beta$ -arrestin antiserum or an anti-GST antiserum and interactions between GST- $\beta$ -arrestin 2 and the target peptides monitored immunologically. Neither the anti-GST nor  $\beta$ -arrestin antiserum identified interactions on the peptide array probed with GST alone (Figure 2 and not shown). However, both the anti-GST and anti- $\beta$ -arrestin antisera identified specific spots, a number of which were non-contiguous, on the peptide arrays that had been overlaid with GST- $\beta$ -arrestin 2 (Figure 2 and data not shown). A number of the spots identified by the two antisera were the same and these represent regions potentially involved in  $\beta$ -arrestin 2 self-association. This included spot 57 (amino acids 281-305) (Figure 2).

The peptide array represents linear 25-amino acid segments of  $\beta$ -arrestin 2 that are not necessarily surface exposed in the mature, folded protein. However, regions involved in self-association must necessarily be at the surface of the protein. We, therefore, overlaid the residues of  $\beta$ -arrestin 2 identified via the peptide array on the surface of  $\beta$ -arrestin 1 (PDB: 1ZSH) (Figure 3) in order to define regions identified by the peptide array studies that are surface-exposed and, hence, most likely to be available for protein interactions. These included residues within the sequence encompassing amino acids 281 to 305 of  $\beta$ -arrestin 2, but not a number of the other peptides suggested by the array interaction studies (not shown). We subsequently narrowed the search for key interacting amino acids within amino acids 281 to 305 of  $\beta$ -arrestin 2 by generating further spot-immobilized peptide arrays that incorporated an alanine scan throughout this sequence (Figure 4). For residues 289 and 302 that are naturally alanine, these were replaced by aspartate. Three clear and distinct sets of patterns were observed in the alanine scan of this region. Compared to the wild type sequence, certain alterations e.g. Asn<sup>282</sup>Ala, Leu<sup>288</sup>Ala, Leu<sup>290</sup>Ala and Leu<sup>294</sup>Ala had little or no effect on the capture of GST- $\beta$ -arrestin 2, whilst certain alterations, e.g. Asp<sup>281</sup>Ala, Glu<sup>284</sup>Ala and Asp<sup>291</sup>Ala increased the interaction. Most importantly, changes of Arg<sup>283</sup>Ala, Lys<sup>285</sup>Ala, Arg<sup>286</sup>Ala, Lys<sup>293</sup>Ala and Lys<sup>295</sup>Ala all decreased or even ablated interaction with the  $\beta$ -arrestin 2 probe (Figure 4). The point mutations that substantially decreased these interactions were then overlaid onto the surface of the  $\beta$ -arrestin 1 structure (Figure 3). Of these Arg<sup>283</sup>, Lys<sup>285</sup>, Arg<sup>286</sup> and Lys<sup>295</sup> were clearly surface exposed and Lys<sup>285</sup>, Arg<sup>286</sup> and Lys<sup>295</sup> formed a

contiguous ribbon (Figure 3) in a region between the N- and C-terminal IP6 binding domains that have recently been suggested to play an important role in  $\beta$ -arrestin self-association [14]. It should be noted that while a role of IP6 for  $\beta$ -arrestin 2 self-association has been demonstrated [14], this is likely to provide a priming/facilitating role that is substantiated by direct amino acid-mediated interactions. No IP6 was employed in the peptide array analyses suggesting that these experiments identify core  $\beta$ -arrestin 2 self-association surfaces.

To explore the roles of Lys<sup>285</sup>, Arg<sup>286</sup>, and Lys<sup>295</sup> in  $\beta$ -arrestin 2 self-association in more detail, both Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 and Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 mutants were C-terminally tagged with either eCFP or eYFP. Following transfection into HEK293 cells, eCFP to eYFP FRET was again measured. Compared to the FRET signal obtained when wild type forms of  $\beta$ -arrestin 2-eCFP and -eYFP were co-expressed, co-expression of Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2-eCFP with wild type  $\beta$ -arrestin 2-eYFP resulted in a lower normalized FRET signal and this was further reduced following co-expression of Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2-eCFP with Lys<sup>285</sup>Ala, Arg<sup>286</sup>Ala  $\beta$ -arrestin 2-eYFP (Figure 5). The differences were statistically significant. The co-expression of Lys<sup>295</sup>Ala  $\beta$ -arrestin 2-eCFP with either wild type  $\beta$ -arrestin 2-eYFP or Lys<sup>295</sup>Ala  $\beta$ -arrestin 2-eYFP resulted in a trend towards reduced FRET signal (Figure 5) but this was not statistically significant. To further explore the importance of Lys<sup>285</sup>, Arg<sup>286</sup>, and Lys<sup>295</sup> in the self-association of  $\beta$ -arrestin 2, wild type and mutated forms of  $\beta$ -arrestin 2 were N-terminally epitope-tagged with either the FLAG or VSV-G sequences. Following co-transfection of wild type FLAG- and VSV-G- $\beta$ -arrestin 2 into HEK293 cells, immunoprecipitation with an anti-VSV-G antiserum resulted in effective co-immunoprecipitation of FLAG- $\beta$ -arrestin 2 (Figure 6). Interestingly, in the SDS-PAGE conditions employed, approximately 50% of the co-immunoprecipitated FLAG- $\beta$ -arrestin 2 migrated with  $M_r$  of some 45kDa, consistent with the anticipated size of the monomeric protein. However, most of the rest of the FLAG- $\beta$ -arrestin 2 migrated with  $M_r$  close to 90kDa, consistent with the maintained presence of an SDS-resistant dimer (Figure 6). There was also some evidence for yet higher level organization of  $\beta$ -arrestin 2, as FLAG-immunoreactive material of higher apparent  $M_r$  was also detected in the co-immunoprecipitated samples (Figure 6). Co-transfection of FLAG- and VSV-G-Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 into HEK293 cells demonstrated that the mutations were without effect on construct expression level. However, co-immunoprecipitation of  $\beta$ -arrestin 2 was substantially decreased for the various mutant forms (Figure 6). Similar reductions in, but not abolition of, co-immunoprecipitation was observed with tagged forms of Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 (Figure 6) and this was not further affected by combination of the Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala and Lys<sup>295</sup>Ala mutations (Figure 6). It was clearly possible that alterations in the observed FRET and co-immunoprecipitation studies reflected alterations in folding of the  $\beta$ -arrestin 2 mutants. Although it is impossible to exclude potential folding and maturation effects without detailed analysis, we employed limited proteolysis to detect any gross perturbation of  $\beta$ -arrestin 2 structure associated with the mutations studied. We were unable to detect differences in the rate or pattern of limited tryptic fragmentation (data not shown).

$\beta$ -arrestin 2 is known to interact with many GPCRs in both a constitutive and receptor agonist-dependent manner. The  $\beta_2$ -adrenoceptor has been perhaps the most

extensively studied. To explore functional consequences of the  $\beta$ -arrestin 2 self-association interface mutations HEK293 cells stably expressing the  $\beta_2$ -adrenoceptor [18] were transfected transiently with wild type VSV-G  $\beta$ -arrestin 2. Following treatment of the cells with the cell-permeant cross-linker dithiobis(succinimidyl propionate) (DTSP), VSV-G immunoprecipitation allowed co-immunoprecipitation of the  $\beta_2$ -adrenoceptor (Figure 7A). As anticipated from the known role of  $\beta$ -arrestin 2 in interactions with agonist-activated GPCRs, the amount of  $\beta_2$ -adrenoceptor co-immunoprecipitated in such experiments was increased substantially when cells were first exposed to the  $\beta$ -adrenoceptor agonist isoprenaline (10  $\mu$ M, 10 min) (Figure 7A). By contrast, when the  $\beta_2$ -adrenoceptor expressing HEK293 cells were transfected with either VSV-G Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 or VSV-G Lys<sup>295</sup>Ala  $\beta$ -arrestin 2, the amount of  $\beta_2$ -adrenoceptor that could be co-immunoprecipitated with the VSV-G tagged  $\beta$ -arrestin 2 was reduced substantially, whether the studies were performed with or without pre-exposure to isoprenaline (Figure 7A). Despite the reduced interaction of the  $\beta$ -arrestin 2 mutants with the  $\beta_2$ -adrenoceptor this was insufficient to limit their ability to enhance isoprenaline-mediated internalization of the  $\beta_2$ -adrenoceptor from the surface of HEK293 cells when the wild type and mutated forms of  $\beta$ -arrestin 2 were transfected transiently into these cells (Figure 7B).

It has been suggested that interactions with  $\beta$ -arrestin 2 can be an important element in the ability of many GPCRs, including the  $\beta_2$ -adrenoceptor, to elicit the phosphorylation and activation of the MAP kinases ERK1 and ERK2. Even without introduction of VSV-G  $\beta$ -arrestin 2, addition of isoprenaline to the  $\beta_2$ -adrenoceptor expressing HEK293 cells resulted in a rapid enhancement of ERK1/2 phosphorylation (Figure 8). Transfection of wild type VSV-G  $\beta$ -arrestin 2 resulted in a marked enhancement of ERK1/2 phosphorylation in response to isoprenaline (Figure 8). By contrast, introduction of either VSV-G Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 or VSV-G Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 was unable to increase isoprenaline-mediated ERK1/2 phosphorylation (Figure 8). Because the effects of the Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala and Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 mutations on the ability of isoprenaline to promote ERK1/2 phosphorylation were virtually complete whilst interaction of these mutated forms with the  $\beta_2$ -adrenoceptor was only partially compromised we explored the contribution of this region of  $\beta$ -arrestin 2 to direct interactions with ERK2. Spot-immobilized peptide arrays, akin to those employed above to define sites of  $\beta$ -arrestin 2 self-association, were overlaid with recombinant GST-ERK2. Following washing the arrays were probed with anti-ERK1/2 antibody. Peptides 54 and particularly 55 (amino acids 271-295) of the  $\beta$ -arrestin 2 sequence were identified (Figure 9). A peptide array incorporating an alanine scan through this segment of  $\beta$ -arrestin 2 identified a number of amino acids, including both Lys<sup>285</sup> and Arg<sup>286</sup>, where alteration to Ala reduced interaction with GST-ERK2 (Figure 9). Furthermore, replacement of Lys<sup>295</sup> by Ala in such arrays virtually eliminated detectable interactions with ERK2 (Figure 9). Following transfection of HEK293 cells with VSV-G tagged  $\beta$ -arrestin 2, immunoprecipitation of cell lysates with anti-VSV-G caused the co-immunoprecipitation of ERK1/2 (Figure 10). Transfections with either VSV-G-Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 or VSV-G-Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 resulted in similar levels of expression of these forms of  $\beta$ -arrestin 2 as wild type but following anti-

VSV-G immunoprecipitation, substantially lower levels of ERK1/2 were co-immunoprecipitated (Figure 10). This suggests an important role of this surface exposed region of  $\beta$ -arrestin 2 in interactions with ERK1 and ERK2. Loss of interaction with ERK1 and ERK2 was selective. Neither VSV-G-Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 nor VSV-G-Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 displayed reduced ability to interact with JNK3 in co-immunoprecipitation studies (Figure 11).

## DISCUSSION

Dimerization or multimerization is one of the most common themes in biology [13] and can involve either two or more copies of the same polypeptide (homodimerization/multimerization), or interactions between different polypeptides with varying degrees of relatedness (hetero-dimerization/multimerization). Recent studies utilizing FRET have demonstrated the capacity of  $\beta$ -arrestin 1 to both self-associate and to interact with the closely related and also ubiquitously expressed arrestin,  $\beta$ -arrestin 2 [15]. The FRET studies we report herein confirm the capacity of  $\beta$ -arrestin 2 to self-associate and, although indirect, suggest that  $\beta$ -arrestin 2 self-association might be sterically constrained to favour conformations where the C-termini are juxtaposed. We also demonstrate that mutations of positively charged amino acids at the surface limit  $\beta$ -arrestin-2 self association and interfere with interactions with the ERK1/2 MAP kinases and with the  $\beta_2$ -adrenoceptor. As these same elements of  $\beta$ -arrestin-2 are important for each of these interactions this may explain why cytoplasmic  $\beta$ -arrestin-2 is not in a conformation to constitutively activate ERK1/2. Indeed, ERK1/2 would likely be shielded from interactions with  $\beta$ -arrestin-2 until the arrestin was in monomeric state. Although interactions between  $\beta$ -arrestin-2 and ERK1/2 have previously been reported [1] until now the site of this interaction on  $\beta$ -arrestin-2 has not been mapped. Equally, the mutated forms of  $\beta$ -arrestin-2 studied herein appear to interact relatively poorly with the  $\beta_2$ -adrenoceptor. However, it remains to be established if this is a general feature of  $\beta$ -arrestin-2-GPCR interactions or that the specific mutations we have explored here are relevant only to interactions with the  $\beta_2$ -adrenoceptor. Importantly, the mutations introduced did not modulate interaction with all proteins that are well-known to be scaffolded by  $\beta$ -arrestin-2. For example, interactions with JNK3 were unaltered, suggesting, as did the lack of variation in limited proteolysis studies, that the mutations generated in  $\beta$ -arrestin-2 did not simply result in a generalized unfolding or denaturation. Given that the JNK3 interaction has previously been mapped to the RRS motif of  $\beta$ -arrestin-2, between amino acids 195-202 [1, 5], the lack of effect of the Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala and Lys<sup>295</sup>Ala mutants on interactions with JNK3 are not unexpected and are consistent with no overall disruption of this scaffold.

Although many proteins that are central to signal transduction processes are known to have the capacity to self-associate and/or to interact with other polypeptides, in many cases little is known about the key amino acids involved in the interaction interface(s). Such information is, however, of utmost importance to inform the rational design of mutants that may limit protein-protein interaction and hence permit an assessment of the functional importance of these interactions. Recently, spot-immobilized peptide arrays have provided a valuable approach to focus attention on regions of proteins that may contribute to such interactions [11-12, 20-23]. Importantly, we have

shown via co-immunoprecipitation studies that effective  $\beta$ -arrestin 2 self-association is important for interaction with the  $\beta_2$ -adrenoceptor and, therefore, potentially with other GPCRs. Moreover, in recent studies Boularan et al. [24] have shown that interfering with the oligomerization of  $\beta$ -arrestin 2 by mutation of IP6 binding sites limits effective interactions of the arrestin with the protooncogene Mdm2, a previously characterized  $\beta$ -arrestin 2 binding partner [25-27]. Given the vast array of  $\beta$ -arrestin-interacting partners identified via proteomic approaches [8] it is evident that deciphering the importance of  $\beta$ -arrestin 2 monomers versus oligomers in scaffolding protein complexes, and the identity of  $\beta$ -arrestin sub-populations characterised by specific cohorts of partners in a particular complex, will be a substantial undertaking. Furthermore, although the traditional role of  $\beta$ -arrestins in agonist-induced GPCR desensitisation and internalisation has been supplemented in recent times by an appreciation of their role in the initiation of distinct, G protein-independent signalling cascades [1-4], the contribution of the  $\beta$ -arrestin monomer/dimer/oligomer equilibrium in defining these distinct processes remains to be established. The results provided herein indicate that  $\beta$ -arrestin 2 self-association may be critically important for transmitting signals from the  $\beta_2$ -adrenoceptor to the ERK1/2 MAP kinases and, in conjunction with the recent data of Boularan et al. [24], demonstrate that the effects on downstream effectors may be limited to specific cohorts of scaffolded proteins, thereby instilling pathway selectivity.

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Stage 2(a) POST-PRINT



## Figure legends

### Figure 1

#### FRET studies indicate that $\beta$ -arrestin-2 is able to self-associate

- A.  $\beta$ -arrestin-2 was C-terminally tagged with eCFP (upper panels) or eYFP (lower panels), expressed transiently in HEK293 cells and auto-fluorescence corresponding to eCFP and eYFP imaged. No eCFP to eYFP FRET signal was detected when either construct was expressed alone.
- B. HEK293 cells were transiently transfected to co-express eCFP and  $\beta$ -arrestin-2-eYFP, eCFP- $\beta$ -arrestin-2 and  $\beta$ -arrestin-2-eYFP or  $\beta$ -arrestin-2-eCFP and  $\beta$ -arrestin-2-eYFP. eCFP to eYFP FRET was then measured as described in Experimental. In the absence of energy transfer the normalized FRET data has a value of 1. Values of greater than 1 reflect the occurrence of FRET (17). Because eCFP and eYFP have no inherent mutual affinity, eCFP plus  $\beta$ -arrestin-2-eYFP acts as negative control.

Data are means  $\pm$  S.E.M. of three independent experiments. Greater than co-expression of eCFP and  $\beta$ -arrestin-2-eYFP, \*  $p < 0.05$ , \*\*  $p < 0.01$ .

### Figure 2

#### Detection of potential $\beta$ -arrestin-2 self-association elements using spot-immobilized peptide arrays

25-mer peptides covering the entire primary sequence of  $\beta$ -arrestin 2 and with a 5 amino acid moving frameshift were spot synthesized on cellulose membranes. Such membranes were overlaid with recombinant GST or GST- $\beta$ -arrestin 2 (each at 10  $\mu$ g/ml), expressed in and purified from *E coli*. Following washing, rabbit anti-GST or anti- $\beta$ -arrestin antisera were added and their interactions with the peptide arrays subsequently detected via binding of a horseradish peroxidase-coupled, secondary anti-rabbit antibody. Peptides spots 53-58 (amino acids 261-310) are displayed. Peptide 57 (amino acids 281-305) was identified by both the anti-GST and anti- $\beta$ -arrestin antisera. The identified region lies within the C-terminal domain of  $\beta$ -arrestin 2.

### Figure 3

#### A model of $\beta$ -arrestin-2 highlighting surface residues potentially involved in self-association

Surface rendition of bovine  $\beta$ -arrestin 1 basal conformation (PDB: 1ZSH), mapping the location of surface-exposed basic residues in the array peptide-57 sequence (amino acids 281-305) that are implicated in  $\beta$ -arrestin 2 self-association (see Figure 4). The high and low affinity phosphoinositide binding sites in the N- and C-domain [14] are shown occupied by IP6 (stick).

### Figure 4

#### An alanine scanning peptide array of residues 281-305 of $\beta$ -arrestin-2 identifies a number of key amino acids involved in self-association

Alanine scanning, 25-mer peptides based on the sequence of amino acids 281-305 of  $\beta$ -arrestin 2 (spot 57 from Figure 2) were synthesized on a cellulose membrane. **Cont** corresponds to the wild type sequence, whilst in the remaining spots the wild type amino

acid(s) noted was altered to alanine (or in the cases where alanine was the wild type amino acid it was converted to aspartate). In a number of cases multiple amino acids were converted in concert to alanine. The membrane was overlaid with GST- $\beta$ -arrestin 2 (10  $\mu$ g/ml). Following washing, rabbit anti- $\beta$ -arrestin was added and interactions with the peptide array detected via binding of a horseradish peroxidase-coupled, secondary anti-rabbit antibody. Densitometric quantification of the array is shown in the lower panel.

### Figure 5

#### **FRET studies suggest altered self-association of Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala $\beta$ -arrestin 2 whilst Lys<sup>295</sup>Ala $\beta$ -arrestin 2 is less affected**

- A. HEK293 cells were transiently transfected to co-express eCFP and  $\beta$ -arrestin-2-eYFP,  $\beta$ -arrestin-2-eCFP and  $\beta$ -arrestin-2-eYFP, Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala (KR285/6AA)  $\beta$ -arrestin-2-eCFP and  $\beta$ -arrestin-2-eYFP or Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin-2-eCFP and Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin-2-eYFP. eCFP to eYFP FRET was subsequently measured as in Figure 1. (M) represents the relevant mutant.
- B. HEK293 cells were transiently transfected as in A except Lys<sup>295</sup>Ala (K295A)  $\beta$ -arrestin 2 replaced Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin.

Data are means  $\pm$  S.E.M. of three independent experiments. \*\* Less than co-expression of wild type of  $\beta$ -arrestin-eCFP and  $\beta$ -arrestin-2-eYFP,  $p < 0.01$ .

### Figure 6

#### **Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala $\beta$ -arrestin 2 and Lys<sup>295</sup>Ala $\beta$ -arrestin 2 display markedly reduced capacities to co-immunoprecipitate**

A. N-terminally FLAG- and VSV-G tagged forms of  $\beta$ -arrestin 2, Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 or Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 were co-expressed in HEK293 cells. Immunoblotting of cell lysates with anti-FLAG or VSV-G antibodies confirmed expression and demonstrated that the mutations did not alter cellular expression levels. Immunoprecipitation with anti-VSV-G resulted in each case in co-immunoprecipitation of anti-FLAG reactivity but the extent of co-immunoprecipitation was substantially lower for each mutant  $\beta$ -arrestin 2 than for wild type  $\beta$ -arrestin 2. Expression of FLAG- $\beta$ -arrestin 2 in the absence of VSV-G- $\beta$ -arrestin 2 resulted in a lack of immunoprecipitation of anti-FLAG reactivity by anti-VSV-G. WT = wild type. **B.** Densitometric quantification of FLAG- $\beta$ -arrestin 2 immunoprecipitated with VSV-G- $\beta$ -arrestin 2 (arbitrary units) Data are means  $\pm$  SEM from 3-6 independent experiments. \*\*,  $p < 0.01$ .

### Figure 7

#### **Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala $\beta$ -arrestin 2 and Lys<sup>295</sup>Ala $\beta$ -arrestin 2 interact poorly with the $\beta_2$ -adrenoceptor but do not limit internalization of the receptor**

VSV-G tagged forms of wild type  $\beta$ -arrestin 2, Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2, or Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 were expressed transiently in HEK293 cells stably expressing the  $\beta_2$ -adrenoceptor (18). **A.** Blotting of cell lysates with VSV-G demonstrated that each form of  $\beta$ -arrestin 2 was expressed equally. Anti-VSV-G immunoprecipitations were performed with and without treatment of the cells with isoprenaline (10  $\mu$ M, 10 min) and following cross-linking of samples with DTSP. The relative amounts of  $\beta_2$ -adrenoceptor

in the immunoprecipitated samples were assessed by blotting with an anti- $\beta_2$ -adrenoceptor antiserum. Data are representative of three independent experiments. **B.** The ability of isoprenaline (10  $\mu$ M, 30 min) to cause internalization of the  $\beta_2$ -adrenoceptor was measured based on the loss of specific binding of the cell impermeant  $\beta$ -adrenoceptor antagonist [ $^3$ H]CGP-12177 (18). Data are means  $\pm$  S.E.M. n = 3.

### Figure 8

#### **Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala $\beta$ -arrestin 2 and Lys<sup>295</sup>Ala $\beta$ -arrestin 2 are unable to enhance $\beta_2$ -adrenoceptor-mediated ERK1/2 MAP kinase phosphorylation**

VSV-G tagged forms of wild type  $\beta$ -arrestin 2, Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2, or Lys<sup>295</sup>Ala $\beta$ -arrestin 2 were expressed transiently in HEK293 cells stably expressing the  $\beta_2$ -adrenoceptor as in Figure 7. Cells were subsequently challenged with isoprenaline (10  $\mu$ M, 5 min). Cell lysates were resolved by SDS-PAGE and the presence of phospho-ERK1/2 (upper panel) and total ERK1/2 amounts (lower panel) probed by immunoblotting with appropriate antibodies. Data are representative of three independent experiments.

### Figure 9

#### **Detection of potential ERK2 interaction sites in $\beta$ -arrestin-2 using spot-immobilized peptide arrays**

Upper panels: 25-mer peptides covering the entire primary sequence of  $\beta$ -arrestin 2 and with a 5 amino acid moving frameshift were spot synthesized on cellulose membranes as in Figure 2 and Such membranes were probed for interactions with recombinant GST-ERK2. Spots 51-59 are displayed. Lower panels: Based on the strong reactivity of spot 55 with GST-ERK2 a peptide array comprising an Ala scan of peptide 55 was generated as in Figure 4 and re-probed with ERK2. The entire array is shown and relative interaction intensity quantitated by densitometry.

### Figure 10

#### **Interactions between $\beta$ -arrestin-2 and ERK1/2: Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala $\beta$ -arrestin 2 and Lys<sup>295</sup>Ala $\beta$ -arrestin interact poorly**

HEK293 cells were transfected with or without VSV-G- $\beta$ -arrestin-2 or with Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 or Lys<sup>295</sup>Ala  $\beta$ -arrestin. **A.** Cell lysates were immunoblotted to detect the VSV-G tag or to detect ERK1/2. Samples were immunoprecipitated with anti-VSV-G and after resolution by SDS-PAGE, immunoblotted to detect ERK1/2. The amount of ERK1/2 was substantially lower in the immunoprecipitates of Lys<sup>285</sup>Ala, Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 and Lys<sup>295</sup>Ala  $\beta$ -arrestin. **B.** Densitometric quantification of ERK1/2 immunoprecipitated with VSV-G  $\beta$ -arrestin 2 (arbitrary units). Data are means  $\pm$  SEM from 3 independent experiments. \*, p<0.05. \*\*, p<0.01.

### Figure 11

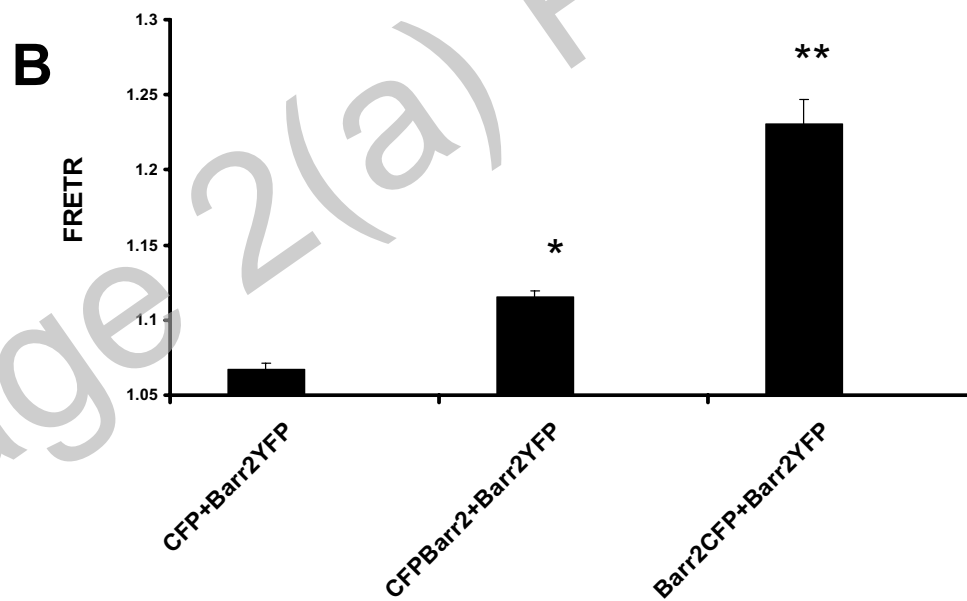
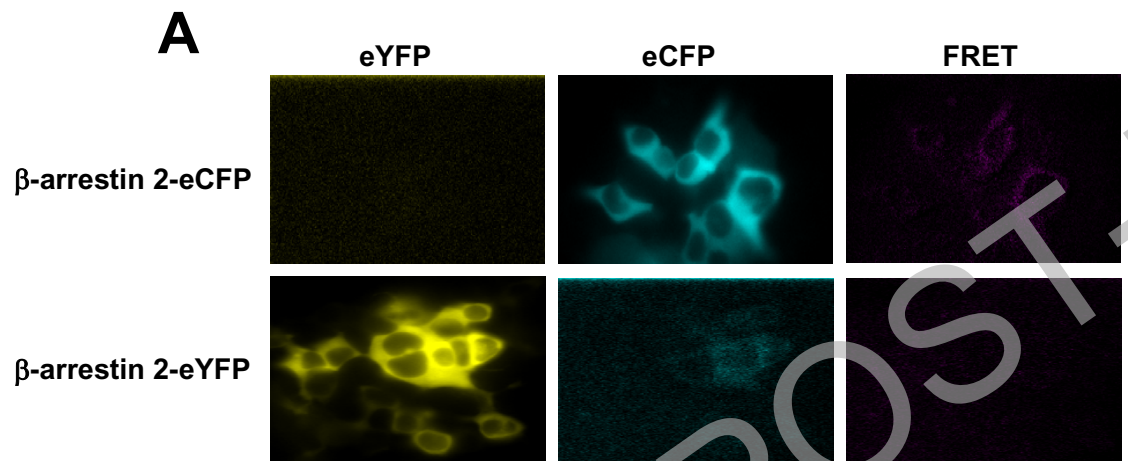
#### **Both Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala $\beta$ -arrestin 2 and Lys<sup>295</sup>Ala $\beta$ -arrestin 2 interact with JNK3 as well as wild type $\beta$ -arrestin 2**

HEK 293 cells were transfected with (+) or without (-) GST-JNK3 and either VSV-G-tagged forms of wild type (wt), Lys<sup>285</sup>Ala:Arg<sup>286</sup>Ala  $\beta$ -arrestin 2 (KR285/6AA) or

Lys<sup>295</sup>Ala  $\beta$ -arrestin 2 (K295A). SDS-PAGE resolved cell lysates were probed to detect expression of JNK3 or following immunoprecipitation with anti-VSV-G samples were immunoblotted to detect JNK3.

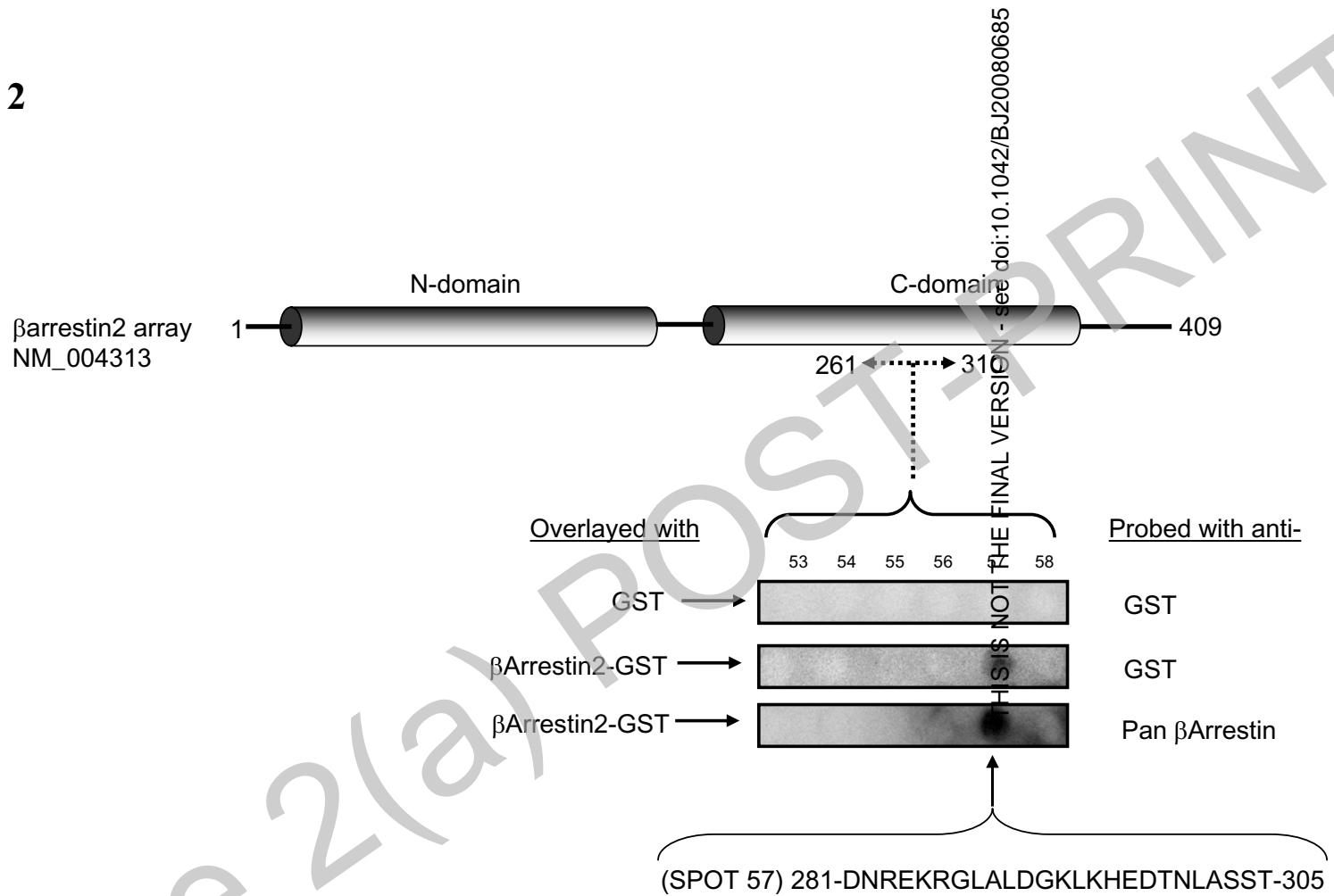
Stage 2(a) POST-PRINT

**Figure 1**

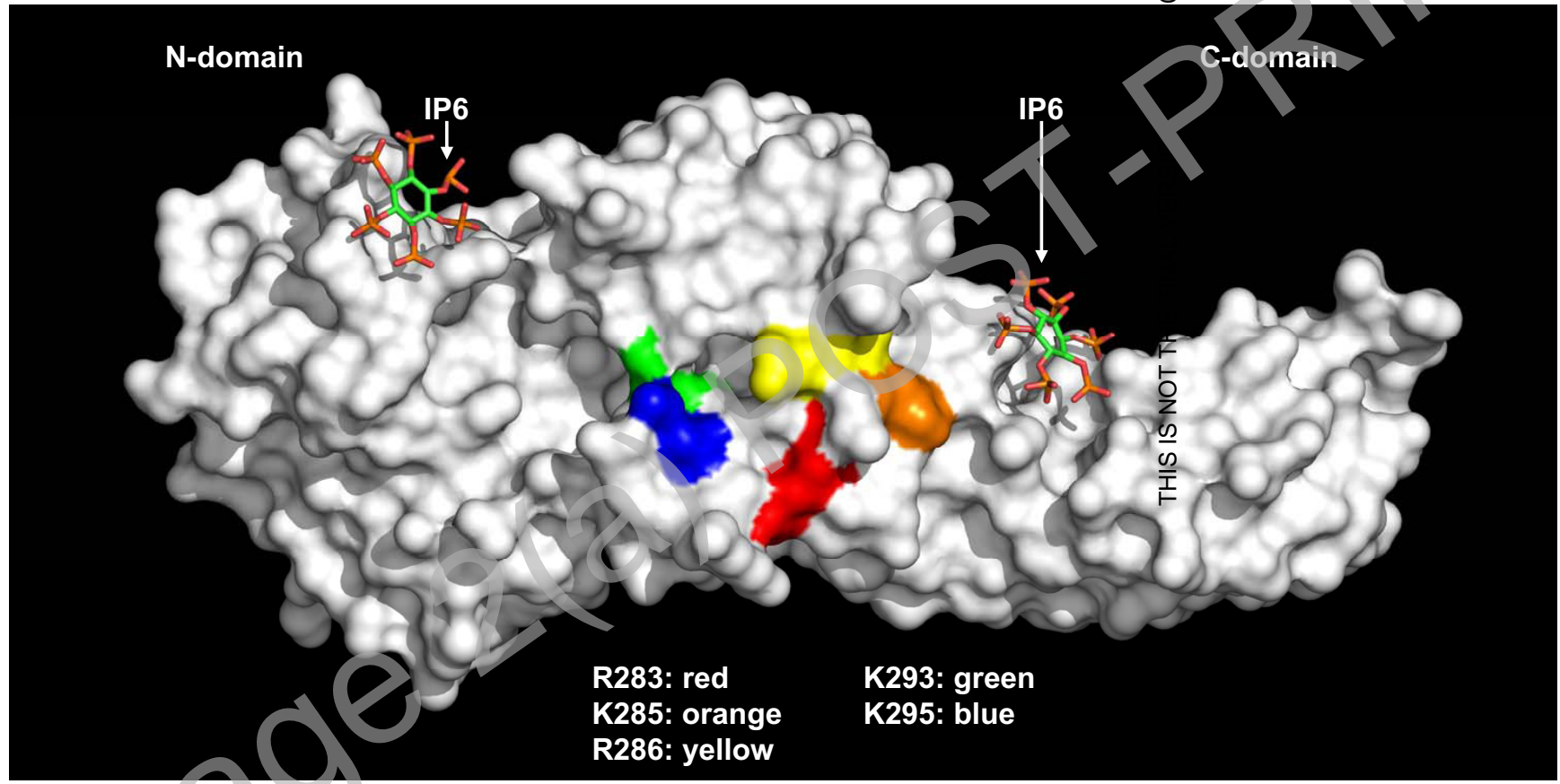


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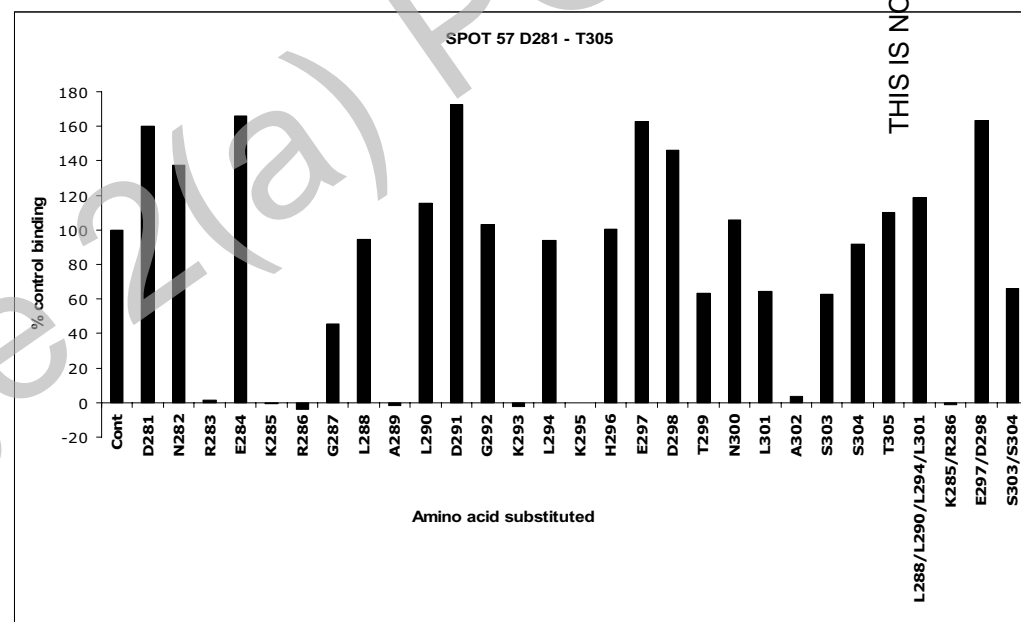
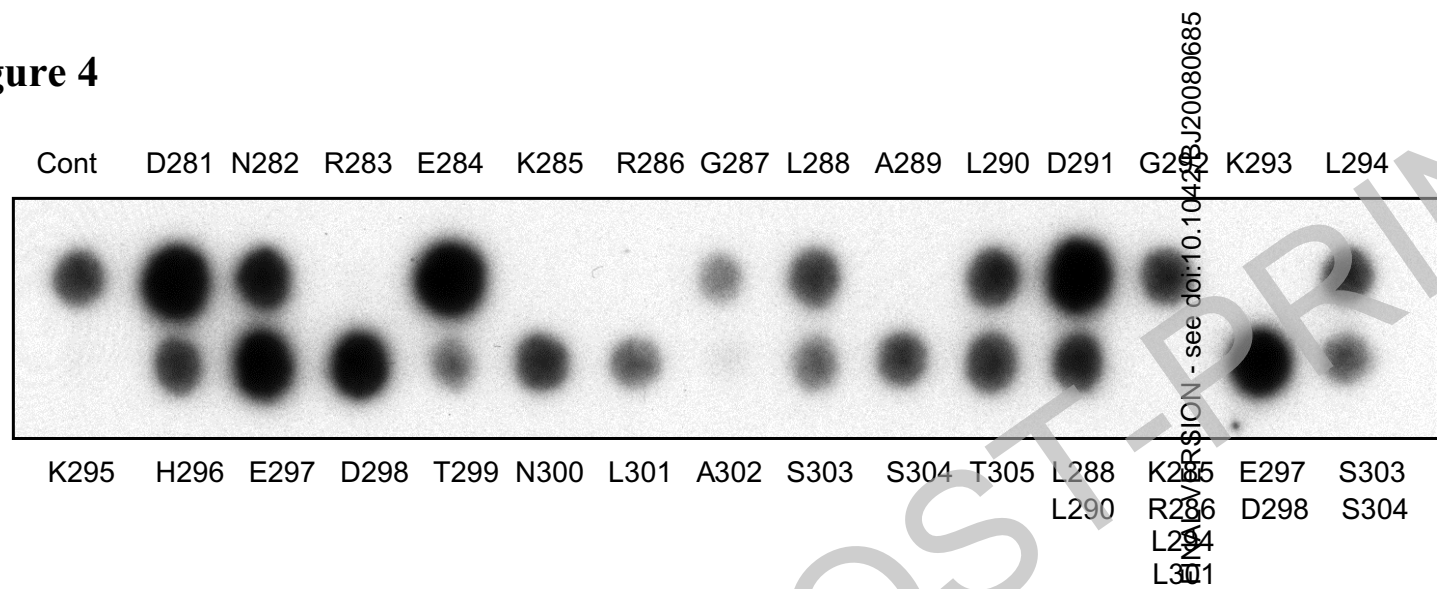
**Figure 2**



**Figure 3**

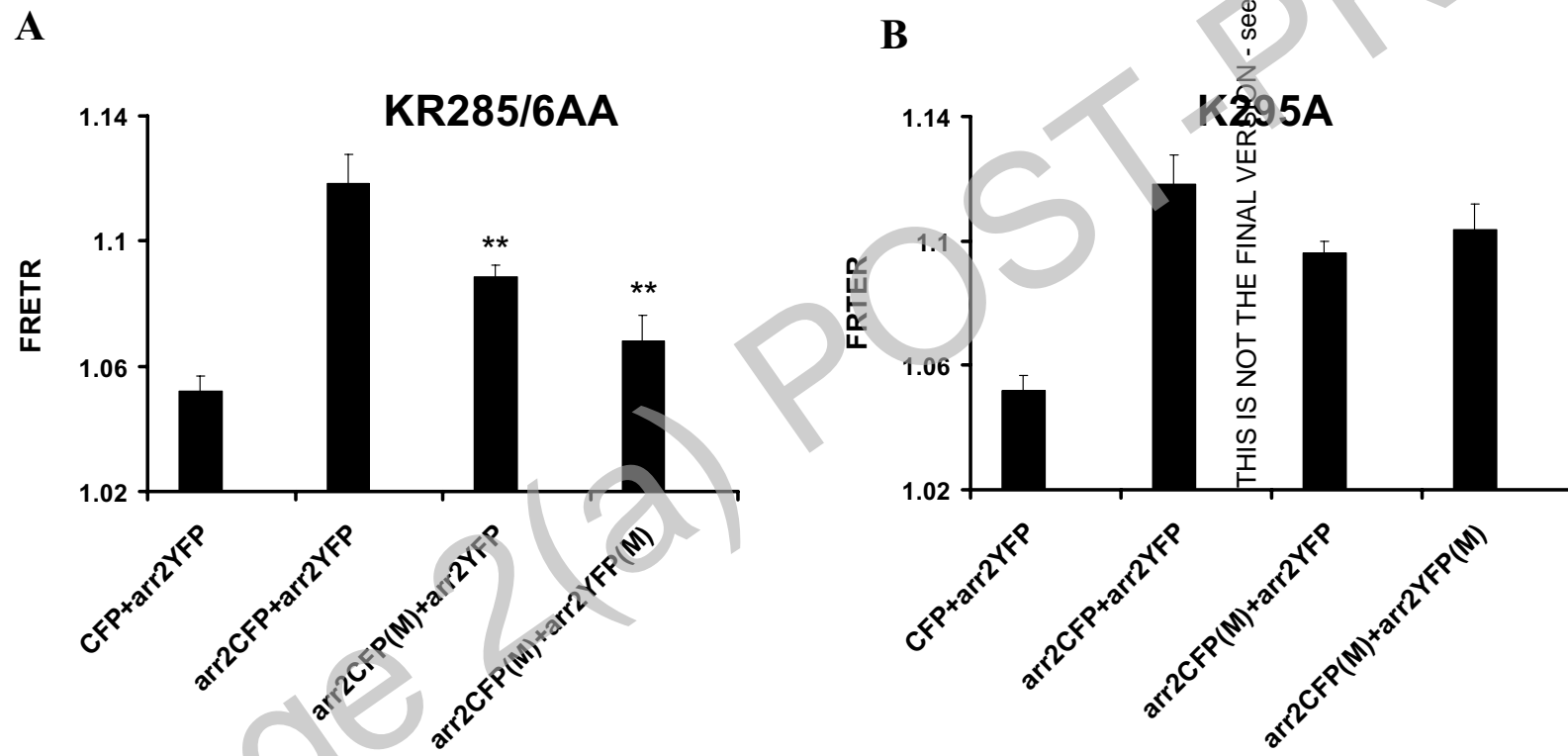


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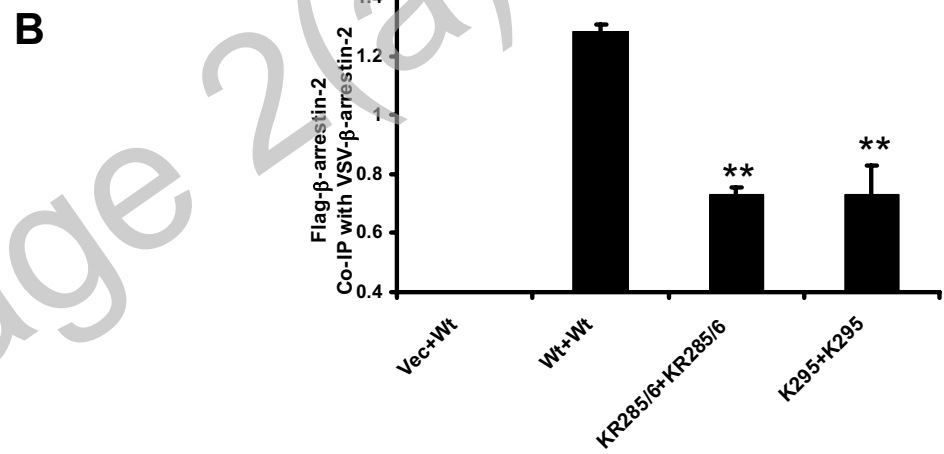
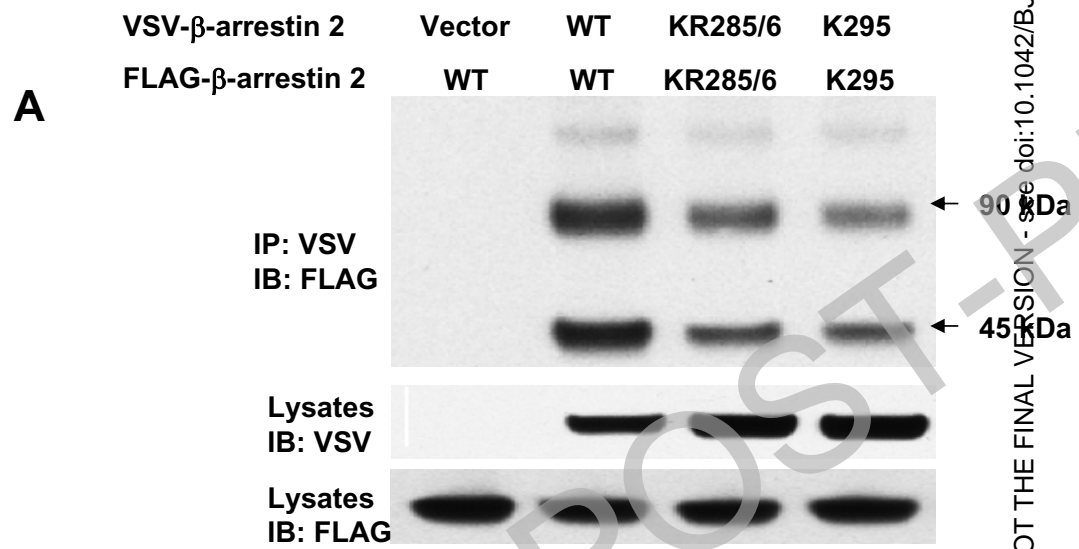
**Figure 4**



**Figure 5**

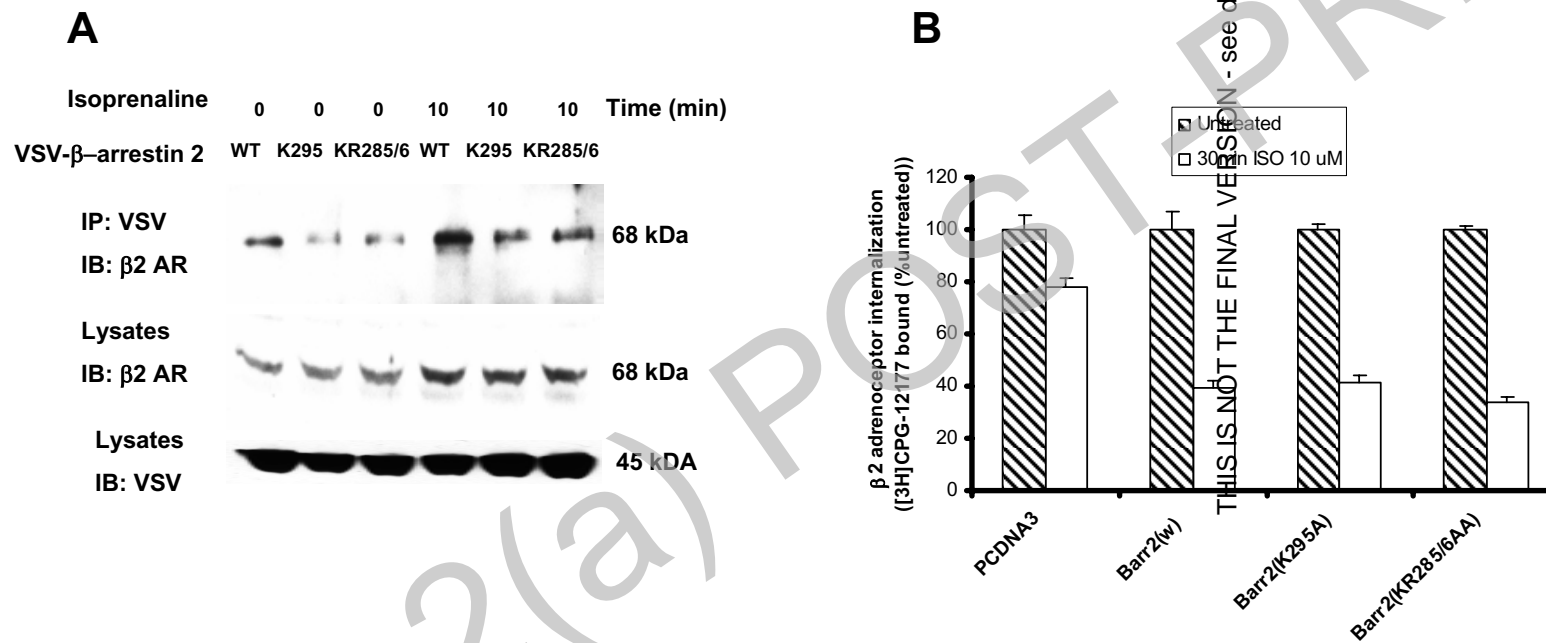


**Figure 6**

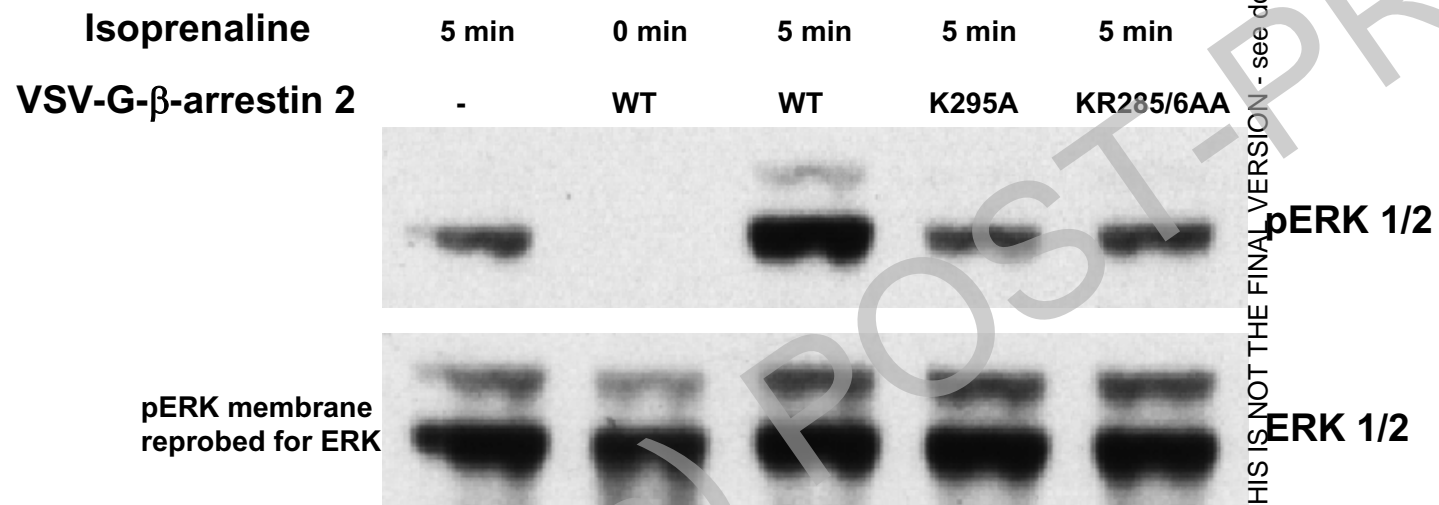


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**Figure 7**

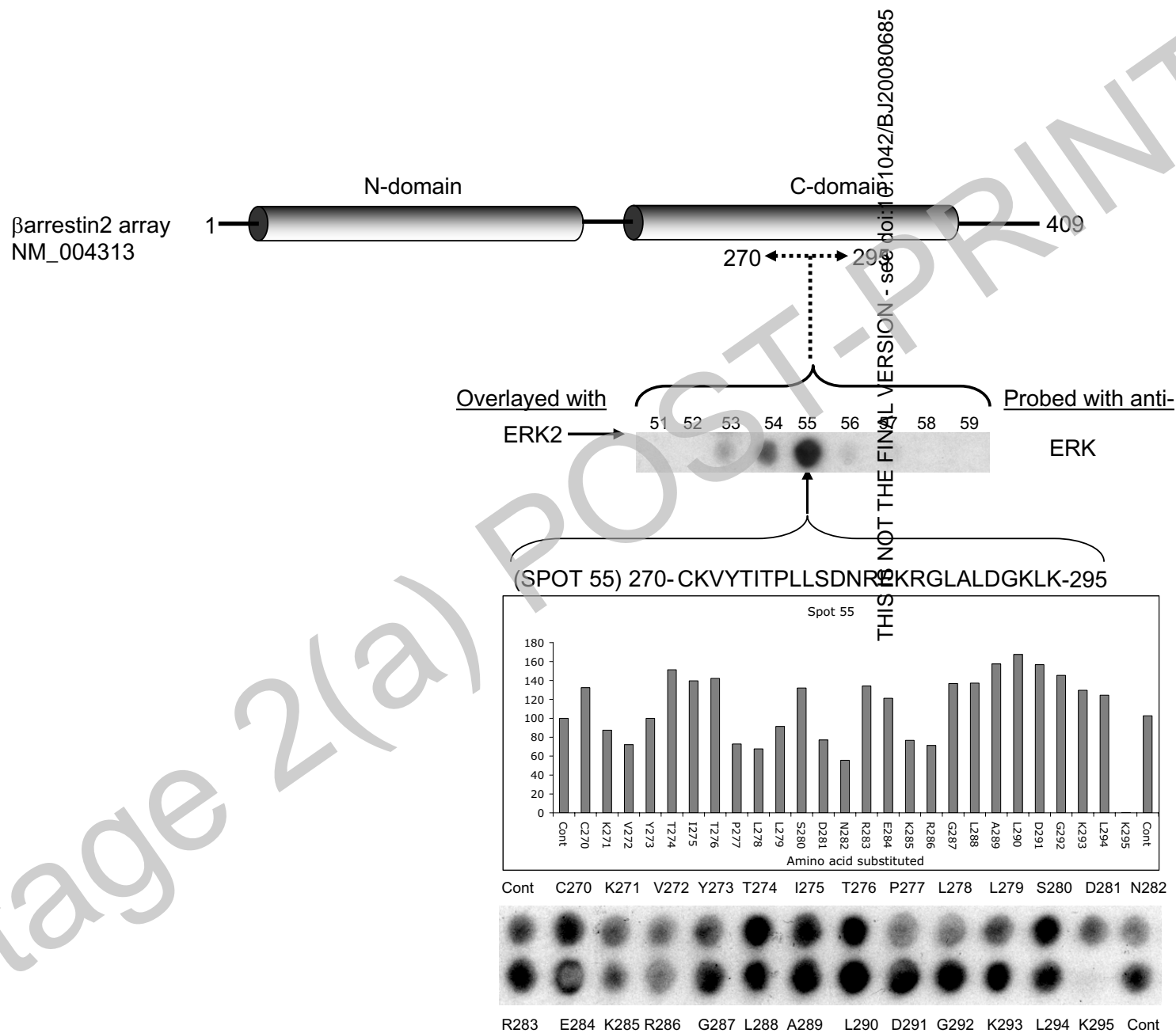


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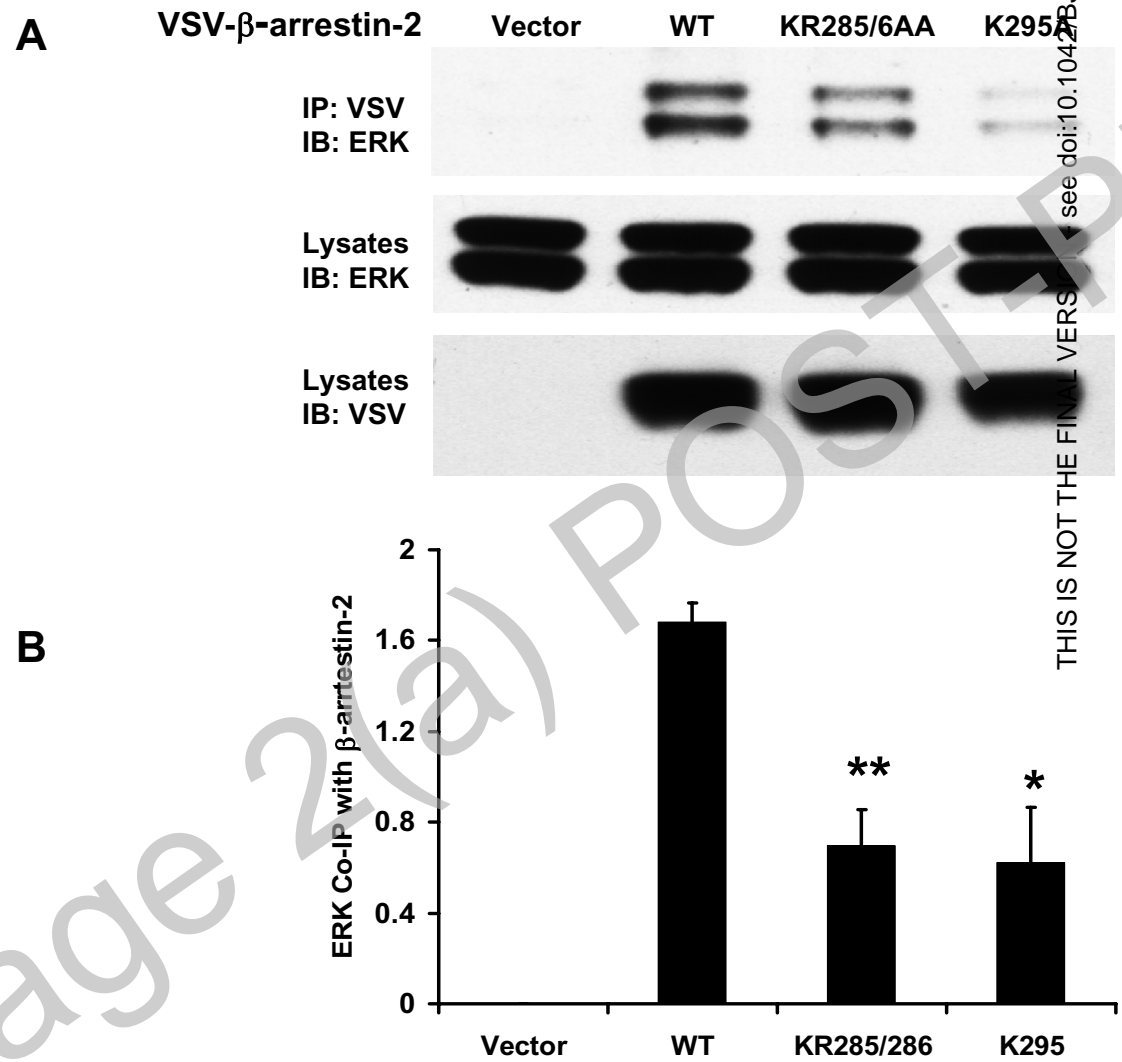
**Figure 8**

Stage 2(a)

Figure 9



**Figure 10**



**Figure 11**