UNIVERSITY OF CALIFORNIA
RIVERSIDE

Molecular Mechanisms of Beta-Arrestin-1 Dependent Regulation of LIMK and Cofilin

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Biochemistry and Molecular Biology

by

Kyu Joon Lee

March 2016

Dissertation Committee:
Dr. Kathryn A. DeFea, Chairperson
Dr. Emma Wilson
Dr. Iryna Ethell

The Dissertation of Kyu Joon Lee is approved:

Committee Chairperson

University of California, Riverside

ACKNOWLEGEMENT

I would like to express the deepest appreciation to my Principal investigator and mentor Dr. Kathryn DeFea. This dissertation would not have been possible without help of Dr. DeFea in so many ways. She provided me with excellent projects, guidance, coworkers, and experiences that I will carry with me long after I have left her laboratory. Also, I would like to thank to my current lab members Mike, Monica, and Rashid for their encouragement and support throughout my school life.

I would like to extend my appreciation to my committee members. Dr. Emma Wilson and Iryna Ethell for your support and precious time.

Special thanks to chair of biochemistry department, Dr. Richard Debus who was more than generous with his expertise and precious time.

Finally and most importantly, special thanks and love to my grandparents, Tae-Won Lee and Jae-Chun Song, my parents, Young-Ja Choi and Sang-Myung Lee. Their faith and support makes this process a very fulfilling experience and one that I will never forget

Dedication

To

My father,

Lee, Sang-Myung

ABSTRACT OF THE DISSERTATION

Molecular Mechanisms of Beta-Arrestin-1 Dependent Regulation of LIMK and Cofilin

by

Kyu Joon Lee

Doctor of Philosophy, Graduate Program in Biochemistry and Molecular Biology
University of California, Riverside, March 2016
Dr. Kathryn A. DeFea, Chairperson

Beta-arrestins are adaptor proteins that can scaffold a number of signaling proteins to promote localized activity within the cell. Downstream of some GPCRs, β-arrestins can promote activation of the actin filament severing protein, cofilin, through two mechanisms: one involving inhibition of LIM Kinase (LIMK) which negatively regulates cofilin activity through phosphorylation on serine 3. The mechanism by which β-arrestin-1 regulates LIMK activity has not been elucidated; however, it has been shown to be important for cell migration downstream of protease-activate-receptor-2 (PAR-2), dendritic spine formation and opioid receptor function. Here my work demonstrate that β-arrestin-1 directly binds both cofilin and LIMK, and inhibits LIMK activity directly and investigate the mechanism by which inhibition of kinase activity occurs. Using serial truncations and site-directed mutagenesis, I identify crucial residues for cofilin and LIMK interaction within amino acids 1-99 of β-arrestin-1 and show that charged residues at 50 and 51 are crucial for binding to LIMK and R51 is required for LIMK inhibition, PAR2 stimulated cofilin dephosphorylation and cell

migration. Additionally, our work reveals that amino acids 1-99 aminos of β-arrestin-1 bind both cofilin and LIMK with a higher apparent affinity than the full length and blocks PAR2-stimulated cofilin dephosphorylaton in HEK293 cells, suggesting it functions as a selective dominant negative β-arrestin-1, inhibiting specifically the cofilin pathway. Thus, residues in the N-terminus of β-arrestin-1 are involved in LIMK inhibition and cofilin activation and this, in turn, is important for cell migration downstream of PAR-2.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE

Introduction

1. Background

1.1 Introduction to G-protein-coupled receptors and β -arrestins

Seven transmembrane G-Protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane receptors in eukaryotes, responding to a diverse set of extracellular stimuli, including hormones, neurotransmitters, peptides, photons and proteases (Bockaert and Pin 1999). GPCRs can associate with heterotrimeric G-proteins and share a common mechanism upon receptor activation. Upon ligand binding, GPCRs undergo conformational changes catalyzing the release of GDP and the binding of GTP on the G_{α} subunits of associated heterotrimeric G proteins. Thus, they can be said to act as guanine exchange factors (GEFs) for the G_{α} subunits. Upon GTP binding, G_{α} subunit dissociates from the $G_{\beta\gamma}$ subunits. Both can associate with downstream effectors to transmit various aspects of cellular response. More recently, several studies have shown that GPCRs are able to signal in a G-protein independent manner, most often mediated through a family of proteins known as β-arrestins (Shenoy and Lefkowtiz 2003; Ma and Pei 2006; Zoudilova et al. 2007; Reiter et al. 2011) (Fig 1.1). The two highly homologous β-arrestins (β-arrestin-1 and 2) were first identified for their ability to uncouple the β2-adrenergic receptor from $G_{\alpha s}$ and facilitate clathrin-mediated receptor endocytosis (Daaka et al.1998). Shortly afterward, they were demonstrated to regulate desensitization of the majority of GPCRs. Nearly a decade ago, paradigm-shifting studies revealed they are not merely signal terminators, but pleiotropic scaffolding proteins capable of localized activation and inhibition of signaling molecules (Defea et al. 2000, Kohout et al. 2001 Shenoy et al 2004).

Some of the first studies demonstrated GPCR signaling through β–arrestins were performed on protease-activated-receptor-2 (PAR-2, discussed below), demonstrating sequestration and activation of ERK1/2 and its upstream regulators, resulting in phosphorylation of non-nuclear substrates (Defea et al. 2000). PAR-2 is activated by proteolytic cleavage at the sequence $R^{34}\downarrow S^{35}$LIGKV/RL (human/mouse) in the N-terminus, to expose the tethered ligand that binds to and activates the receptor (Vu, Hung et al 1991; Hollenberg and Compton 2002). Peptides corresponding to the six amino acid tethered ligand and chemically modified peptidomimetics such as 2-furoyl-LIGRL-ornithine-NH2 (2fAP), can activate PAR-2 without proteolytic cleavage (Bernatowicz, Klimas et al 1996; McGuire, Saifeddine et al 2004; Ishiwata et al. 2005). PAR-2 is widely expressed, in fibroblast, leukocytes, epithelial cells, endothelial cells and neurons and is thus involved in a variety of cellular responses, including proliferation, actin reorganization, chemotaxis, ion transport and production of cytokines and prostaglandins (Macfarlane et al 2001). PAR-2 can couple to $G_{\alpha q/11}$, $G_{\alpha 12/13}$ and $G_{\alpha i}$ but is most commonly associated with $G_{\alpha q/11}$ activation, leading to Ca^{2+} mobilization and PKC activation. PAR-2 can also couple to β-arrestins for the purpose of G-protein uncoupling and receptor internalization as well as scaffolding of signaling complexes, including the MAPK cascade, PI3K, AMPK and the cofilin cascade (Zoudilova, Min et al.2010; DeFea, Vaughn et al. 2000; DeFea, Zalevsky et al. 2000; Ge, Ly et al. 2003; Ge, Shenoy et al. 2004; Stalheim, Ding et al. 2005; Wang and DeFea 2006; Wang, Kumar et al. 2007; Zoudilova, Kumar et al. 2007). Of particular interest here, is the role of PAR-2 in promoting chemotaxis through activation of cofilin.

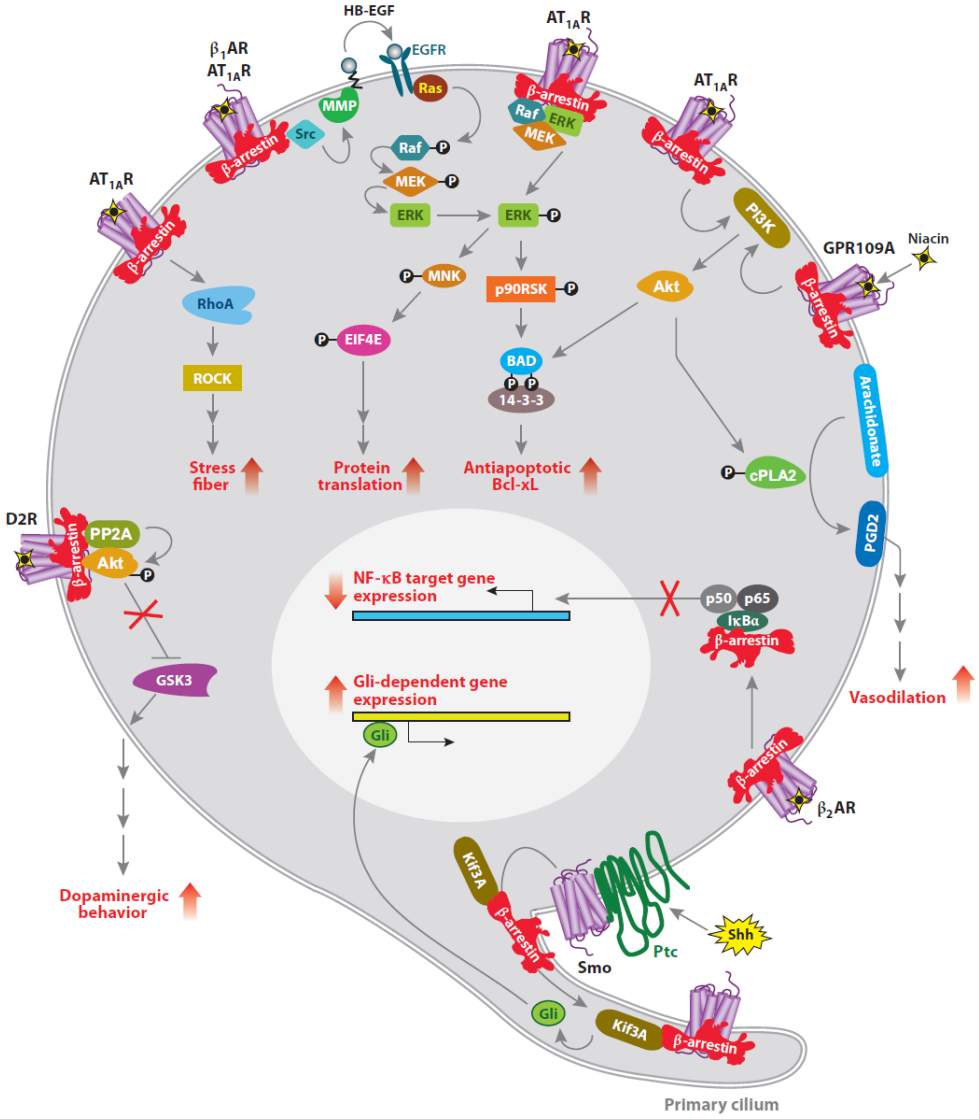


Figure 1.1 Pluridimensionality of β-arrestin-dependent signaling at seven-transmembrane receptors (7TMRs). Some of the best-characterized β-arrestin-induced signaling mechanisms are schematically represented. They include RhoA-dependent stress fiber formation; inhibition of nuclear factor κB (NF-κB)-targeted gene expression through IκB stabilization; protein phosphatase 2A(PP2A)-mediated dephosphorylation of Akt, which leads to the activation of glycogen synthase kinase 3 (GSK3) and dopaminergic behavior; extracellular signal-regulated kinase (ERK)-dependent induction of protein translation and antiapoptosis; PI3K-mediated phospholipase A2 (PLA2) induction and increased vasodilation through GPR109A activation; and Kif3A-dependent relocalization and activation of the protein Smoothened (Smo) in the primary cilium. Courtesy of Reiter et al., 2012, Annual review of Pharmacology and Toxicolgy

1.2 Scaffolding functions of β-arrestins

More than 100 β-arrestins binding proteins have now been reported (Xiao et al. 2007) and studies suggest that β-arrestins can regulate a many of these proteins, independent of, or even in opposition to, the G-protein mediated signal. These effects can be mediated through direct effects of β-arrestins on enzymatic activities, and by bringing components of a signaling cascade in proximity to each other, facilitating sequential activation. Among the signaling cascades regulated by β-arrestin are the Jnk and ERK1/2 cascades, the cofilin cascade (including upstream regulators LIMK and Chronophin), PI3K, Src, AMPK (including upstream activator CAMKK and AMPK) and NFkB. It is noteworthy that many of the proteins regulated by β-arrestins are kinases. The first identified and one of the most extensively studied β-arrestin targets are the MAPKs, ERK1 and 2 (ERK1/2). Several β-arrestin/ERK1/2, complexes have been identified: One set of complexes contains ERK1/2, MEK1 (dual specific kinase which activates ERK1/2), and Raf (MAPKK kinase which activates MEK1) and is formed downstream of a number of GPCRs resulting in prolonged ERK1/2 activation in the membrane (DeFea JCB2000, Luttrell PNAS 2000, + several other refs). Downstream of PAR2, this complex forms independent of G-protein activation and accumulates at the leading edge of migrating cells and facilitates chemotaxis. Another group of complexes contains ERK1/2, Raf and src but only transiently associates with MEK. These complexes facilitate nuclear translocation of the activated ERK which is important for gene transcription and proliferation of cells (Luttrell Science 2000, DeFea, PNAS 2000, + several others). Finally, other MAPK family members, in the first example described above, association of Raf with β-arrestins is required for its activation (Coffa, Breitman et al. 2011).

Another kinase regulated by β-arrestin binding is LIMK, a protein important in the regulation of the actin cytoskeleton (discussed in more detail in section 1.3). LIMK structure consists of a constitutively active C-terminal kinase domain and two N-terminal LIM domain with adjacent PDZ and proline/serine rich regions. The N-terminal domains inhibit the C-terminal kinase domain (auto-inhibition), as evidenced by the fact that mutation and deletion of LIM domain leads to increasing LIMK activity and cleavage of LIMK at Asp 240 a truncated LIMK (aa 240-647) that is constitutively active (Hiraoka, Okano et al. 1996; Tomiyoshi, Horita et al. 2004).

Both Raf and LIMK are auto-inhibited kinases where intramolecular interactions between N- and C-termini suppress the constitutive activity of the C-terminal kinase domain and both are activated by a conformational change that relieves the inhibition (Rebecca and Olson 2007). However, β-arrestins associate with LIMK leading to inhibition of LIMK, while β-arrestins associate with Raf leading activation of Raf. The work described here focuses on the mechanism by which β-arrestin-1 inhibit LIMK, information which may be extrapolated to other kinases similar regulated by β-arrestins.

While not all of the binding sites for the 100+ β-arrestins binding partners have been identified, studies on the crystal structures of β-arrestins provide valuable information about the mechanism of scaffolding and regulation of these downstream signaling moleules by β-arrestins (Han, Gurevish et al. 2001; Vishnivetskiy, Hosey et al. 2004; Zhan, Gimenez et al 2010). Structural studies of β-arrestins suggest it consists of two concave domains of antiparallel β-sheets that can rotate on a central hinge region. Usually, receptors bind to concave sides of β-arrestins, and many other proteins that mediate downstream functions bind to the convex side of β-arrestins (Fig 1.2). This

structural information, combined with receptor-specific β-arrestin scaffolds, suggest that β-arrestins can adopt multiple conformations exposing different sets of binding regions, depending on the receptor to which they are recruited.

Some generalizations can be made from the crystal structure of β-arrestin-1 that help us predict many β-arrestin interactions, and interpret the significance of certain binding sites (Fig 1.3). The sequence between residues 357-382 is the "disordered region", containing the clathrin binding domain, which is exposed upon activation of GPCRs. Binding to most GPCRs appears to induce a conformational change in β – arrestin-1, resulting in closer apposition of the two lobes and exposure of the clathrin binding domain. The polar core, which is common element in all β-arrestin structures, is important for stabilizing the arrestins in their basal conformations (Vishnivetskiy et al. 1999). The residues 46-86 appear to be important for binding to most GPCRs. There are other potential binding sites for receptors, including residues 164-172 and 44-66. However, these sequences do not form a contiguous surface, suggesting that β-arrestin-1 may have more than one conformation for receptor binding (Pulvermuller et al. 2000; Vishniveskiy et al. 2004; Hanson and Gurevish 2006). The receptor-bound β-arrestin adopts a conformation that allows C-tail to move away from its original position, thus exposing potential binding regions for downstream molecules. Because GPCRs differ in terms of which potential binding partners are recruited to β-arrestin upon activation, there is likely to be a multitude of receptor-specific β-arrestin conformations. This allows for recruitment of a subset of potential interacting proteins downstream of any given receptor, thus giving some degree of signaling specificity despite the use of β-arrestins as common effectors.

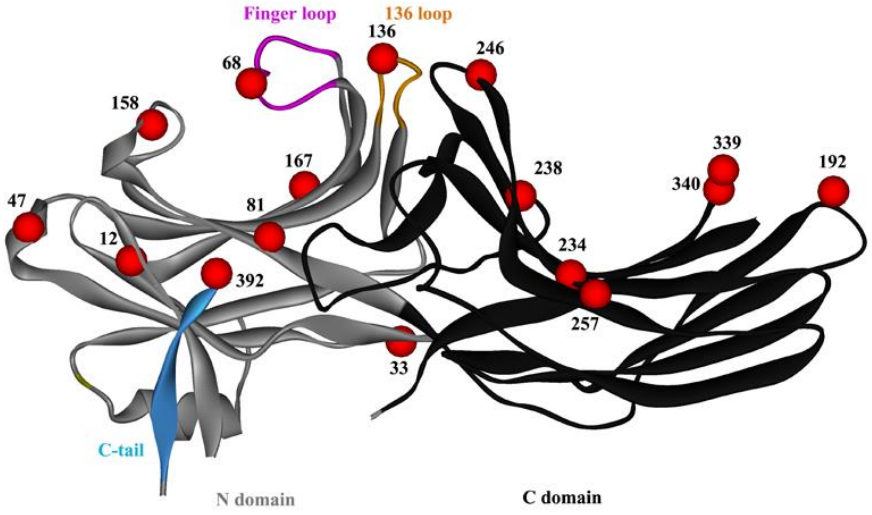


Figure 1.2 Crystal structure of arrestin-2 in the basal conformation (PDB: 1G4M) with the residues studied by DEER spectroscopy shown as red C± CPK models. The backbone structure of the N domain is shown in gray and the backbone structure of the C domain is shown in black. The major structural features of arrestin-2 are indicated.

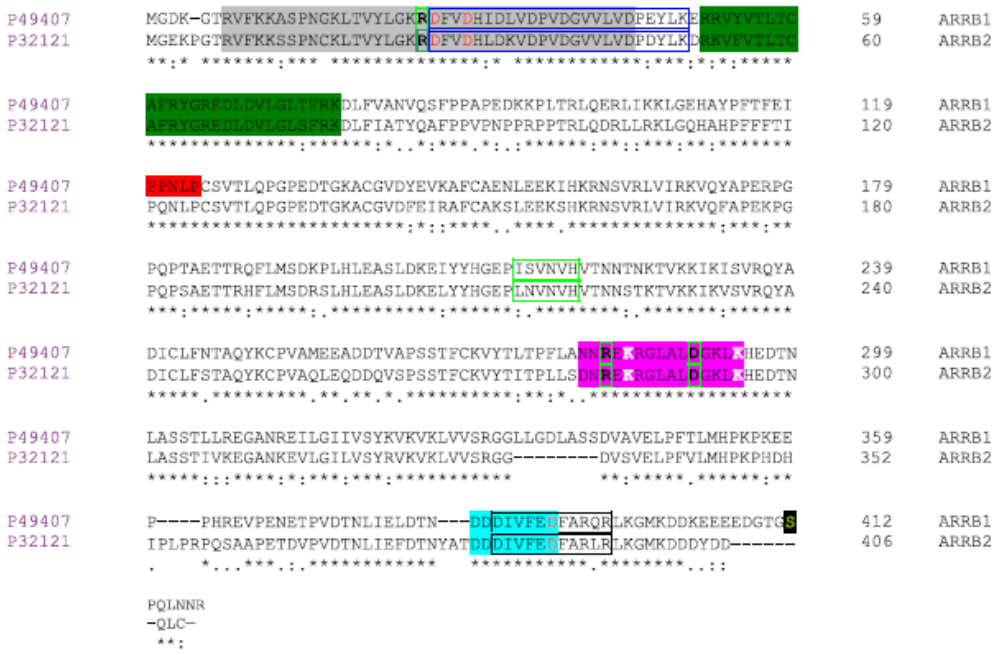


Figure 1.3 Alignment of putative interaction domains on β-arrestin-1 and 2. The sequence of β-arrestin-1 and 2 are aligned and domains that have been shown to mediate interactions with various downstream targets are shown. Gray shading indicates Ask1 binding sites and blue boxes indicate MEK1 binding sites, with red letters indicating specific amino acids that are required for binding to both. Dark green shading indicates MKK4 binding sites. Red shading indicates poly-proline stretches important for Src SH3 domain interactions. Green boxes indicate PDE4D5 binding sites. Pink shading indicates ERK2 binding sites with essential lysines highlighted in white. Light blue shading indicates an Akt binding motif. Black box indicates clathrin binding domain. Blackshading indicates site for C-terminal serine phosphorylation on β-arrestin-1. Courtesy of Defea KA., 2011, Cellular Signalling.

1.3 Regulation of actin assembly by β -arrestins

Shortly after the ability of β-arrestins to sequester signaling molecules in specific cellular microdomains was established, it was demonstrated that they are indispensable for chemotaxis downstream a number of receptors. While desensitization of chemotactic receptors as they reach high concentrations of chemoatractant is likely to be one role of β-arrestins in this process, localized actin assembly at the leading edge is also important. The actin filament severing protein, cofilin, was identified as a β-arrestin binding partner in a proteomics screen and as a functional target of β-arrestin signaling to the actin cytoskeleton in our laboratory downstream of PAR-2 (Zoudilova et al. 2007). Cofilin plays a critical role in chemotaxis because it can rapidly reorganize the cell's cytoskeleton allowing it to migrate in the direction of a chemoattractant.

For chemotaxis to occur, cells must reorganize their cytoskeleton and form a protrusion in the direction of the chemoattractant. This process involves disassembly of existing actin filaments and formation of new ones, a process that must be tightly regulated. The core of this process is actin, a polar molecule ATP binding occurs and a pointed end. Actin polymerization and depolymerization occurs at both ends, but the fast growing barbed end dominates assembly kinetics and disassembly occurs primarily at the pointed end. In a resting cell filaments are usually capped at both barbed and pointed ends, limiting both polymerization and depolymerization until this balance is altered by signaling events. For actin polymerization to occur efficiently, the cell needs a high concentration of exposed barbed ends. There are several ways to accomplish this but a frequent early event in chemotaxis is the activation of the actin filament severing protein, cofilin. Active cofilin binds to filamentous actin (F-actin), destabilizing and severing actin filament into smaller F-actin seeds with free barbed ends to which G-

actin monomers rapidly add, allowing for rapid reorganization and providing a driving force for directed cell migration (Fig 1.4).

Cofilin is the only known substrate for LIM Kinase (LIMK), which negatively regulates it by phosphorylating it on serine 3. Cofilin is activated by dephosphorylation on serine 3, by the phosphatases Slingshot (SSH) and chronophin (CIN). Our laboratory has previously shown that β-arrestin-2 scaffolds CIN with cofilin to promote its dephosphorylation, while β-arrestin-1 appears to inhibit LIMK activity, but the mechanism by which β-arrestin-1 regulates LIMK has never been elucidated (Fig 1.5).

The LIM-kinase protein family consists of two members, LIM kinase 1 (LIMK1) and LIM kinase 2 (LIMK2). LIMK1 and LIMK2 have 50% overall identity, with 70% identity in kinase domains. The majority of published studies have focused on LIMK1 and this is the isoform used in these studies. LIMK is widely expressed in both embryo and adult, with highest expression in the brain, kidney, lung, stomach and testis. Because of its crucial role in actin reorganization, defects in LIMK are associated with multiple diseases. One of example is Williams-Beuroen syndrome (WBS), in which a microdeletion of *limk1* gene on chromosome 7 implicated in WBS. Deletion of *limk1* gene in the brain leads to abnormalities in synaptic structure and dendritic spine development most likely due to aberrant regulation of actin cytoskeleton (Hoogenraad et al. 2004; Meng et al. 2002). Dysregulation of LIMK is also implicated in cancer and inflammation; however, the role is more complicated as either too much or too little LIMK activity can disrupt cell migration. Simply put, tight regulation of cofilin activity is required for cell migration and LIMK is crucial to the regulation of cofilin in migratory cells.

LIMK activity is regulated by several upstream signaling pathways, the most well-characterized of which involve activation of Rho GTPases. Phosphorylation of LIMK at Thr^{508} within the catalytic loop by Rho-activated kinases, ROCK and PAK1, results in a conformational change that increases its activity. Binding of hsp90 to LIMK promotes homodimerization which then facilitates trans-autophosphorylation within the kinase domain that further stabilizes the active conformation (Li R, et al. 2006). We know that β-arrestins directly interact with the kinase domain and inhibit the ability of LIMK to phosphorylate cofilin and MBP. Our published data demonstrate that β-arrestins inhibit PAR_2-stimulated LIMK activity but not phosphorylation at the active site, suggesting that β-arrestins do not block activation by upstream kinases (Zoudilova et al. 2007). β-arrestins also bind to both cofilin and hsp90 (Xiao K et al. 2007; Ge, Shenoy et al.2004). Likely mechanisms by which β-arrestins inhibit LIMK activity are: that they compete with cofilin for binding to the catalytic domain; that they block hsp90 binding and trans-autophosphorylation, and/or that they block ATP binding. Because β-arrestins also bind cofilin, we cannot rule out the possibility that they act as a "substrate sponge" sequestering cofilin away from LIMK.

My studies are aimed at understanding the mechanism by which β -arrestins inhibit LIMK activity, thus enhancing cofilin activation. Because the initial characterization of β-arrestin-dependent regulation of cofilin activity was done downstream of PAR-2, I use PAR-2 to investigate the significance of disrupting cofilin or LIMK interactions with β -arrestin-1.

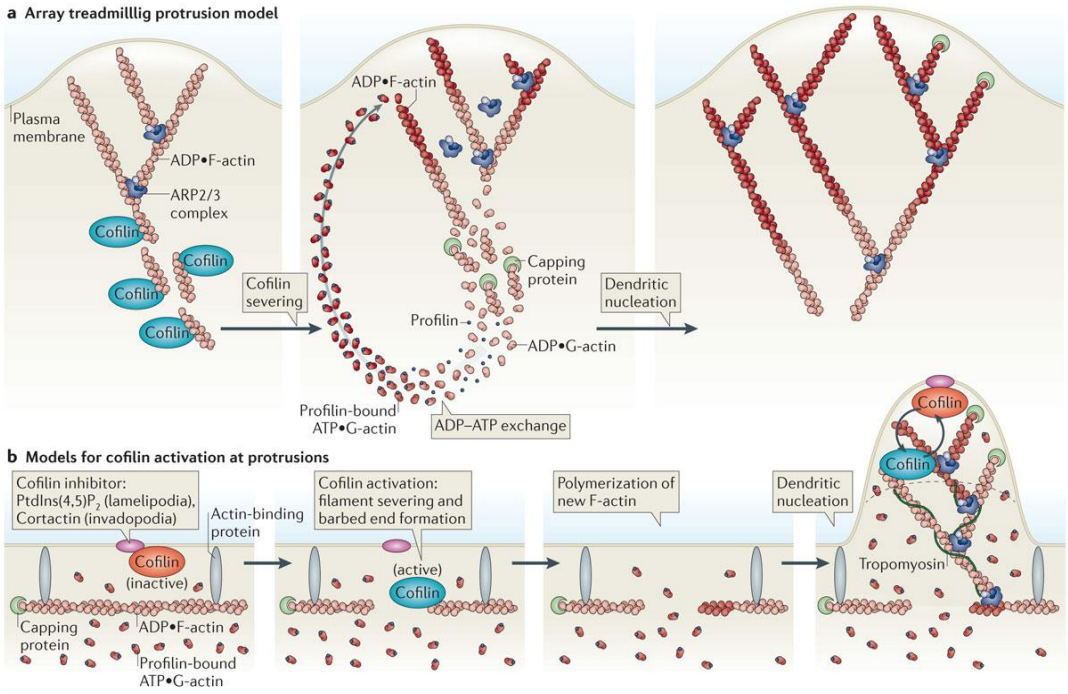


Figure 1.4 Models of cofilin function (a) Array treadmilling protrusion model. Cofilin severs and depolymerizes actin filaments at the base of the lamellipodium, thereby supplying G-actin monomers for steady-state actin polymerization in conditions of G-actin depletion. Dendritic nucleation is mediated by the actin-related protein 2/3 (ARP2/3) complex. (b) Models for cofilin activation at protrusions. Release of cofilin from its inhibitor (phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) at lamellipodia or cortactin at invadopodia) at the plasma membrane increases severing of actin filaments, generating free barbed ends that define the sites of dendritic nucleation by the ARP2/3 complex. G-actin monomers are supplied from an abundant pre-existing G-actin pool. The ARP2/3 complex mediates dendritic nucleation. Tropomyosin limits cofilin action, as it inhibits binding of cofilin to F-actin. This confines cofilin severing to the tip of protrusions (dotted black line), where cofilin continues its cycles of activation and deactivation due to the local cofilin activity cycle. Courtesy of Bravo-Cordero et al., 2013, Nature Reviews Molecular Cell Biology

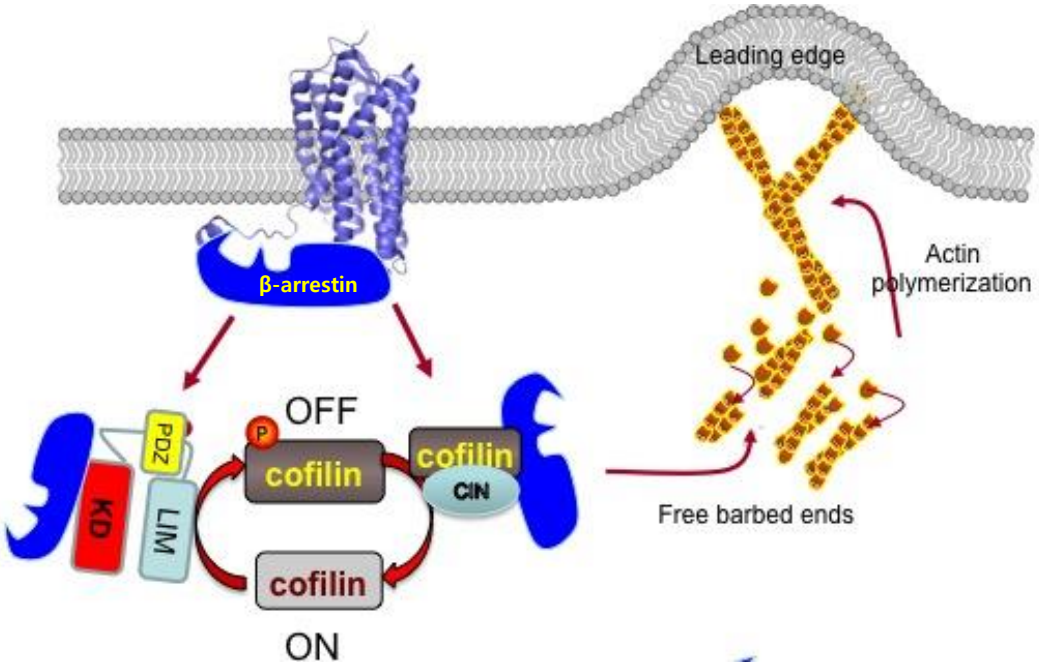


Figure 1.5 Model for β -arrestin regulation of cofilin. Receptor promotes recruitment of β-arrestins, which results in the formation of two complexes that facilitate cofilin dephosphorylation and activation: one containing β-arrestins, the phosphatase CIN and cofilin and the other containing LIMK and β-arrestins.

CHAPTER TWO

Basic residues in the N-terminus of β-arrestin-1 regulate cofilin and LIMK binding

2.1 Introduction

β-arrestin-dependent signals play important roles in chemotaxis and actin reorganization by spatially and temporally regulating cofilin activity. Previously, our lab demonstrated that protease-activated-receptor-2 (PAR-2) promotes dephosphorylation and activation of cofilin, both of which are inhibited by siRNA knockdown or genetic deletion of β-arrestins. These results were verified with pyrene actin assays that showed a PAR-2-induced actin filament severing activity that could be immuno-depleted with cofilin antibody or abolished with β-arrestin siRNA knockdown. Cofilin activation is independent of classical $G_{\alpha q}$ signaling, as demonstrated by the fact that it was not blocked by siRNA knockdown of $G_{\alpha q}$, or pretreatment with a blocking peptide of $G_{\alpha q,}$ a PLC β inhibitor or the intracellular Ca2+ chelating agent, BAPTA-AM. In fact, inhibition of $G_{\alpha q}$ signaling increased PAR-2 stimulated cofilin dephosphorylation, suggesting the two pathways may work not only independently but in opposition to each other. Our studies suggest that β-arrestin-dependent cofilin activation involves the formation of two scaffolding complexes. One complex contains β-arrestin1 and 2, with cofilin and CIN and appears to localize cofilin activity to the leading edge and facilitate dephosphorylation. The scaffolding complex containing β-arrestins-1 and 2, CIN and cofilin was identified in cultured breast cancer cells and primary bone marrow leukocytes and had an apparent Stoke's radius of 5nm. A role for CIN was established by transfection of a dominant negative mutant, which abolished PAR-2-stimulated cofilin dephosphorylation, as well as PAR-2 stimulated actin barbed end formation. Another complex contains β-arrestin1 and 2 with LIMK; this complex is associated with inhibition of LIMK activity which maintains a pool of active cofilin necessary for driving cell migration. Thus by inhibiting LIMK and

facilitating interaction of cofilin with CIN, β-arrestins promotes dephosphorylation and activation of cofilin.

While β-arrestin-2-dependent scaffolding of CIN with cofilin has been well studied, the role of β-arrestin-1-dependent LIMK inhibition is less clear. A role for inhibition of LIMK was first observed in a breast cancer cell line (MDA MB-468) that expresses high levels of endogenous β-arrestins and favors β-arrestin-dependent signaling downstream of PAR-2. In these cells, PAR-2 activation decreased LIMK activity to below baseline levels, and when β-arrestin-1 was knocked down with siRNA, a PAR2-stimulated increase in LIMK activity was unmasked. The increase in LIMK activity was inhibited Ca2+ chelation suggesting once again that PAR2 elicits opposing signals through $G_{\alpha q}$ and β-arrestin pathways. However, detailed mechanisms by which β-arrestins regulate LIMK activity and how this contributes to cofilin activation and migration upon PAR-2 activation had not been elucidated. The studies described in this chapter identify the sites on β-arrestin-1 that mediate binding to LIMK and cofilin, and the effect of disrupting these sites on PAR-2 stimulated signaling to the actin cytoskeleton.

2.2 Results

2.2.1 β-arrestin-1 directly interacts with LIMK and cofilin

Previous studies demonstrated that cofilin can be co-immunoprecipated with β-arrestin-1 and 2 in cultured cells (Zoudilova et al 2007), and flag-tagged β-arrestin-1 and 2, isolated from HEK293 cells, directly bind cofilin (Pontrello et al 2012). Size exclusion chromatography revealed two complexes formed upon PAR-2 activation: one containing LIMK and β-arrestin-1 (Fig 2.1) and another containing cofilin, CIN and both β-arrestin- 2 (with some β-arrestin-1 found in this complex as well). In those same studies, association of cofilin with CIN was dependent upon β-arrestin-2, and unpublished studies (Figurexxx, courtesy of A. Lin) revealed that LIMK could be co-immunoprecipitated with b-arrestin-1 upon PAR2 activation. To further dissect the roles of these molecular interactions in the regulation of cofilin activity, I used sandwich immunoassays to assess directing binding of cofilin and LIMK to β-arrestin-1. Recombinant his-LIMK (kinase domain only; KD-LIMK)) or recombinant cofilin was captured on 96-well plates, and increasing concentrations of either GST or GST-β-arrestin1 were added. I observed concentration dependent binding of β-arrestin-1 to both LIMK and cofilin, with relative EC50s of 22.05nM for LIMK binding and 626.4nM for cofilin binding (Figure 2.2 and 2.3).

Next, I examined whether PAR-2 stimulated direct binding of β-arrestin-1 to LIMK or cofilin in live cells using Bioluminescence Resonance Energy Transfer (BRET) assays in which luciferase-tagged β-arrestin-1 was co-transfected with YFP-tagged LIMK or YFP-tagged cofilin and cells were treated with 2fAP to activate PAR-2. Net BRET signal is the BRET measured ratio of emission at 535nm (YFP) and 435nm (luciferase) minus background (BRET signal in cells transfected with β-arrestin-

1-luciferase alone). Because transfer only efficiently occurs when the two tags are less than 10 nm apart, an increase in net BRET upon PAR-2 activation effectively reflects a direct interaction between the two proteins. Net bret was .0167 for cofilin/β-arrestin-1and .013 for LIMK/β-arrestin-1 in untreated cells and these values increased to .0223 and .097 in 2fAP treated cells, respectively (Fig 2.4 and 2.5. Together these data demonstrate direct binding of both LIMK and cofilin to β-arrestin-1 upon PAR-2 activation in live cells, although β-arrestin-1 may bind preferentially to LIMK over cofilin.

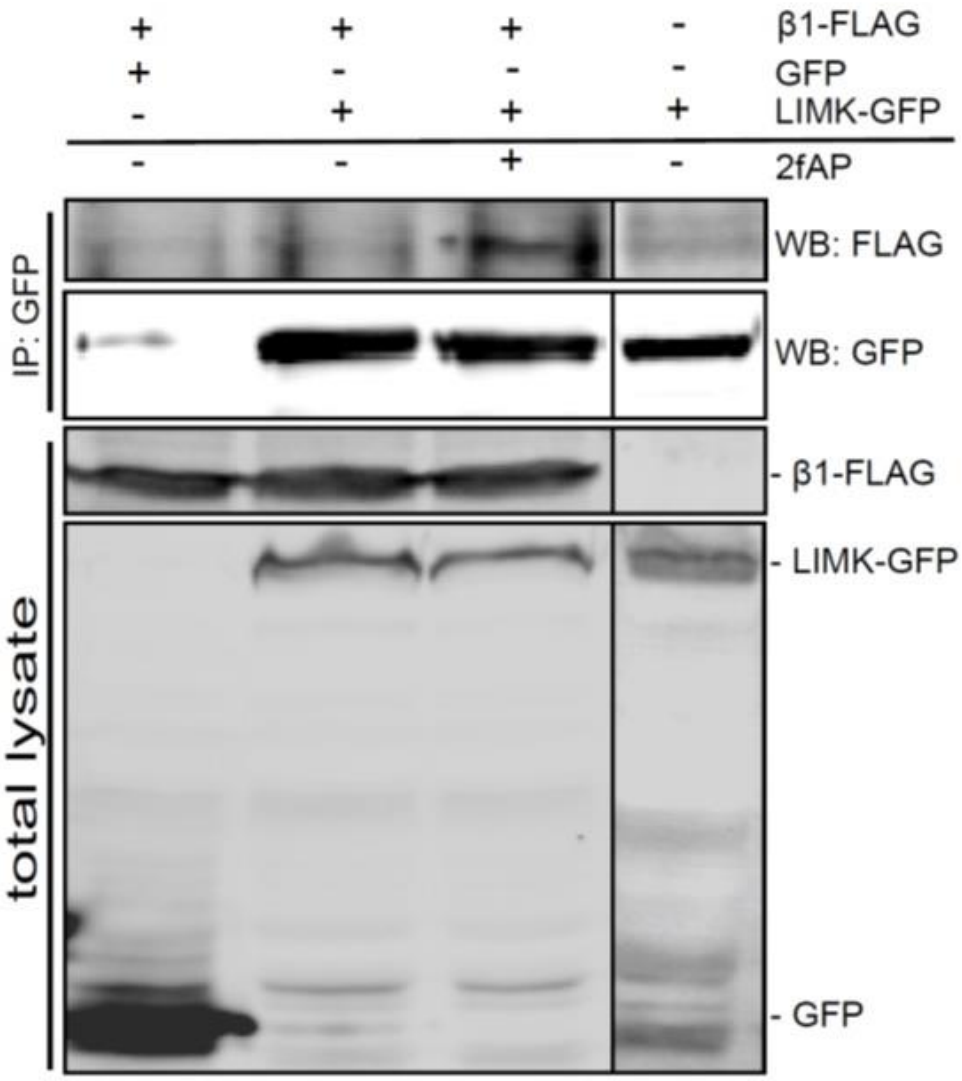


Figure 2.1. PAR2-stimulates LIMK interaction with β-arrestin-1. Coimmunoprecipitation of LIMK and β-arrestin-1. HEK293 cells, expressing β-arrestin-1-flag and either LIMK-GFP or GFP (negative control) or with LIMK-GFP alone, were treated with or without 2fAP for 5 minutes and LIMK-GFP immunoprecipitated with anti-GFP, followed by SDS-PAGE and western blotting with either anti-GFP or anti-flag.

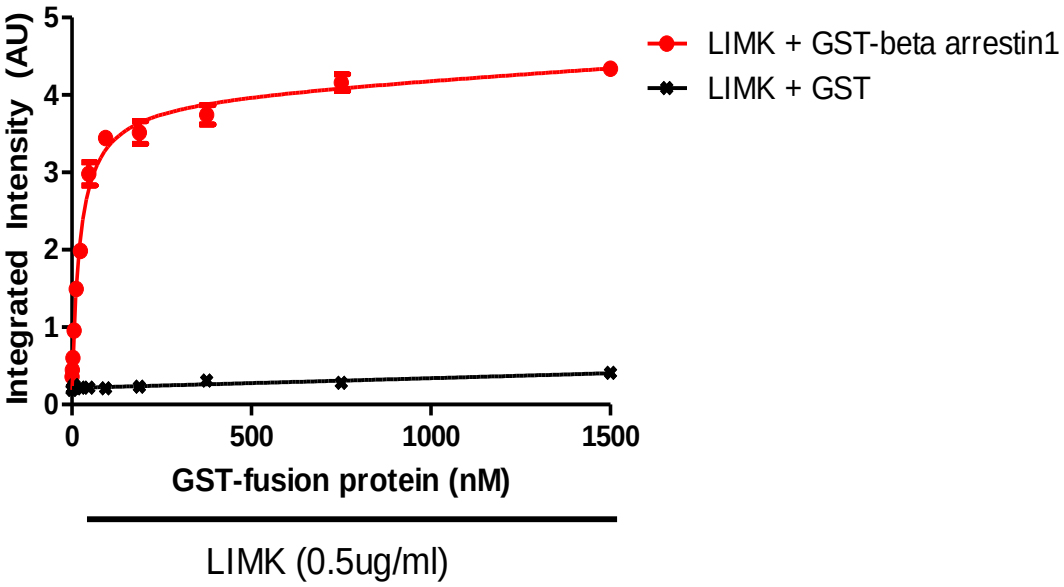


Figure 2.2 β-arrestin1 interacts with LIMK Direct binding of β-arrestin-1 to LIMK-kinase domain (KD) using sandwich immunoassays. Fixed amounts of LIMK-KD was captured on 96 well plates, and increasing concentrations of GST-β-arrestin1 or GST alone were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=2). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

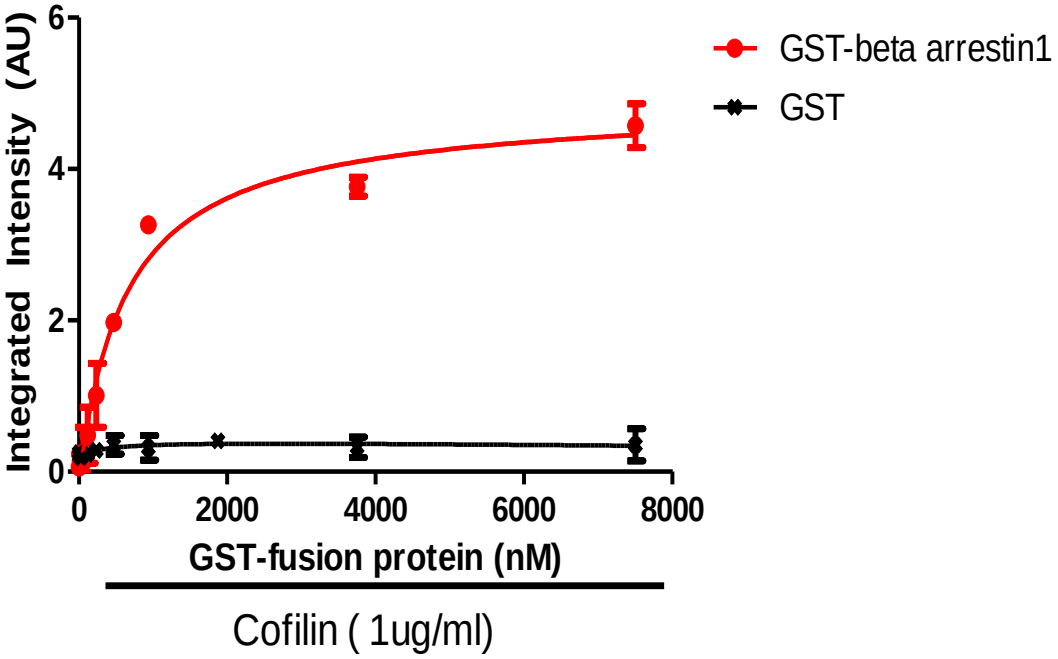


Figure 2.3 β-arrestin1 interacts with cofilin. Direct binding of β-arrestin-1 to cofilin using sandwich immunoassays. Fixed amounts of cofilin was captured on 96 well plates, and increasing concentrations of GST-β-arrestin1 or GST alone were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=2). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

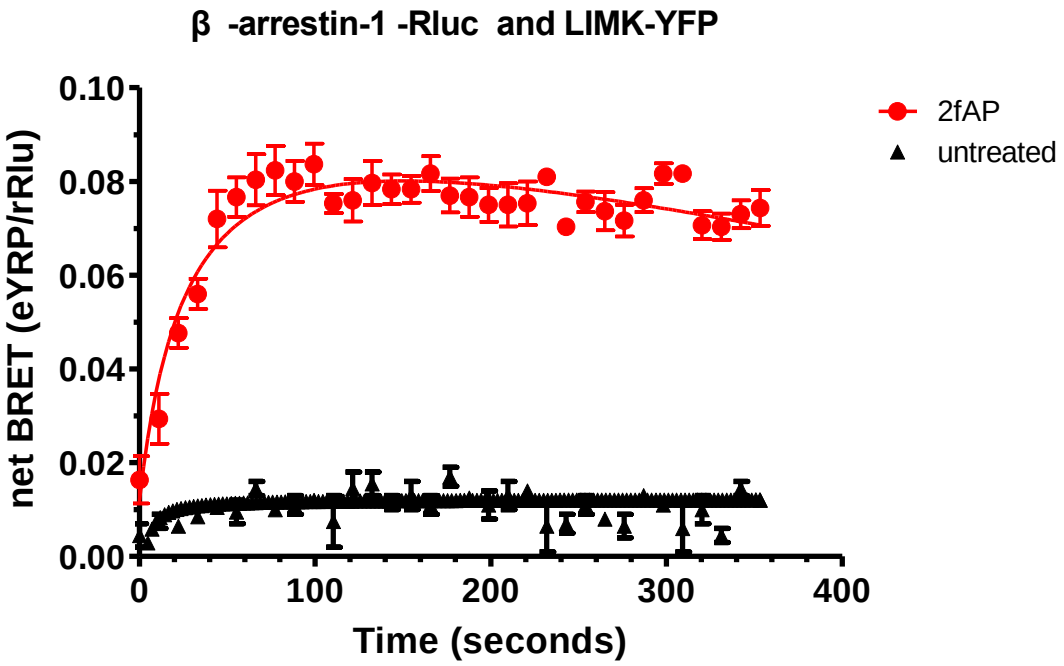


Figure 2.4 PAR-2 activation stimulates β-arrestin1 interaction with LIMK. Bioluminescence Resonance Energy Transfer (BRET) between β-arrestin1-Rluc and LIMK-YFP was determined in real time. Net Bret values in the presence and absence of 2fAP were determined and expressed as eYFP (535nm)/rLuc (488nm) (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

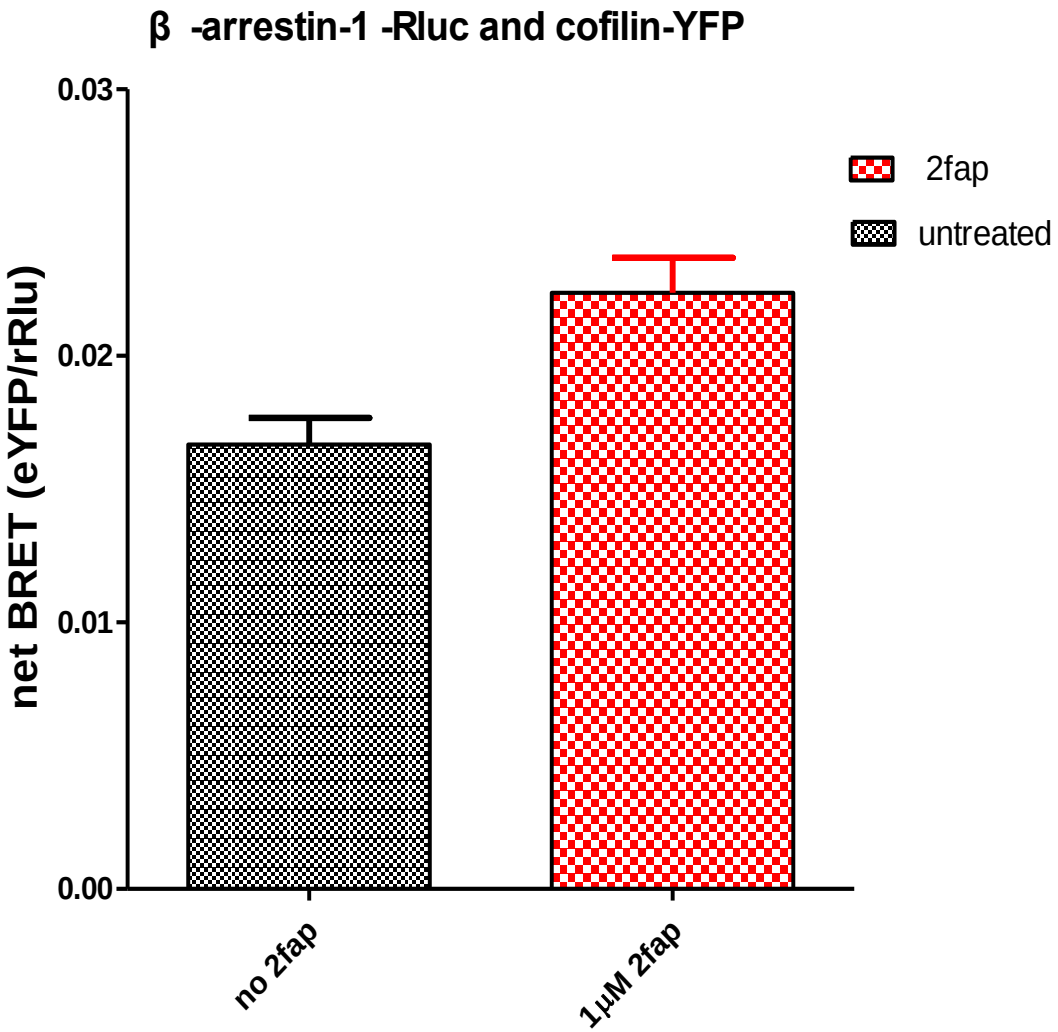


Figure 2.5 PAR-2 activation stimulates β-arrestin1 interaction with cofilin. Bioluminescence Resonance Energy Transfer (BRET) between β-arrestin1-Rluc and Cofilin-YFP was determined in real time. Net Bret values in the presence and absence of 2fAP were determined and expressed as eYFP (535nm)/rLuc (488nm) (n=3). The bar graph were analyzed and graphed by *GraphPad Prism***.**

2.2.2 β-arrestin-1 directly inhibits LIMK phosphorylation of cofilin

Previous studies showed that, upon PAR-2 activation, β-arrestin-1 appears to antagonize LIMK activity in live cells, likely contributing to the overall dephosphorylation of cofilin. Because, β-arrestin-1 does not block LIMK activation by upstream kinases such as ROCK or PAK (Zoudilova et al 2007), I proposed that it directly inhibits LIMK activity and that this is dependent upon association of LIMK with β-arrestin-1. To test this, purified recombinant cofilin was incubated with recombinant LIMK-kinase domain (LIMK-KD; amino acids 285-629) in the presence of increasing concentrations of either GST or GST-β-arrestin-1 for 1 hour after which reactions were terminated by addition of Laemmli sample buffer and samples were analyzed by SDS-PAGE followed by Western blotting with anti-phospho cofilin (Fig 2.6). Phosphorylation of cofilin by LIMK was reduced by 47% by addition of GST- β-arrestin-1 but not GST alone. This result suggests that β-arrestin-1 is able to directly inhibit LIMK activity, which might involve blocking ATP binding. To determine whether β-arrestin-1 prevented ATP binding to LIMK, I incubated KD-LIMK with ATP-agarose beads and either GST or GST-β-arrestin-1. (Fig 2.7). KD-LIMK was incubated with ATP-agarose beads in the presence of increasing amount of GST-β-arrestin-1 or GST for 4hr and samples were analyzed by SDS-PAGE followed by Western blotting with anti-His. The result showing that β-arrestin-1 did not significantly affect ATP binding to KD-LIMK. Another possible mechanism by which β-arrestin-1 inhibits LIMK activity involves β-arrestin-1 acting as a substrate sponge, sequestering cofilin away from LIMK. We performed sandwich competition immunoassays to verify whether β-arrestin-1 is able to inhibit cofilin binding to LIMK. When KD-LIMK was first captured on microplates and then bound to GST-cofilin, adding increasing

concentrations of β-arrestin-1 did not compete cofilin off of KD-LIMK (Fig 2.8). However, in reciprocal experiment, when β-arrestin-1 was prebound to LIMK, addition of GST-cofilin resulted in a 40% loss of bound β-arrestin-1 (Fig 2.9). These results suggest that cofilin can compete with β-arrestin-1 for binding to LIMK. Thus, it is likely that cofilin has a higher binding affinity for LIMK than β-arrestin-1 and the mechanism by which b-arrestin-1 inhibits cofilin phosphorylation by LIMK involves an allosteric mechanism.

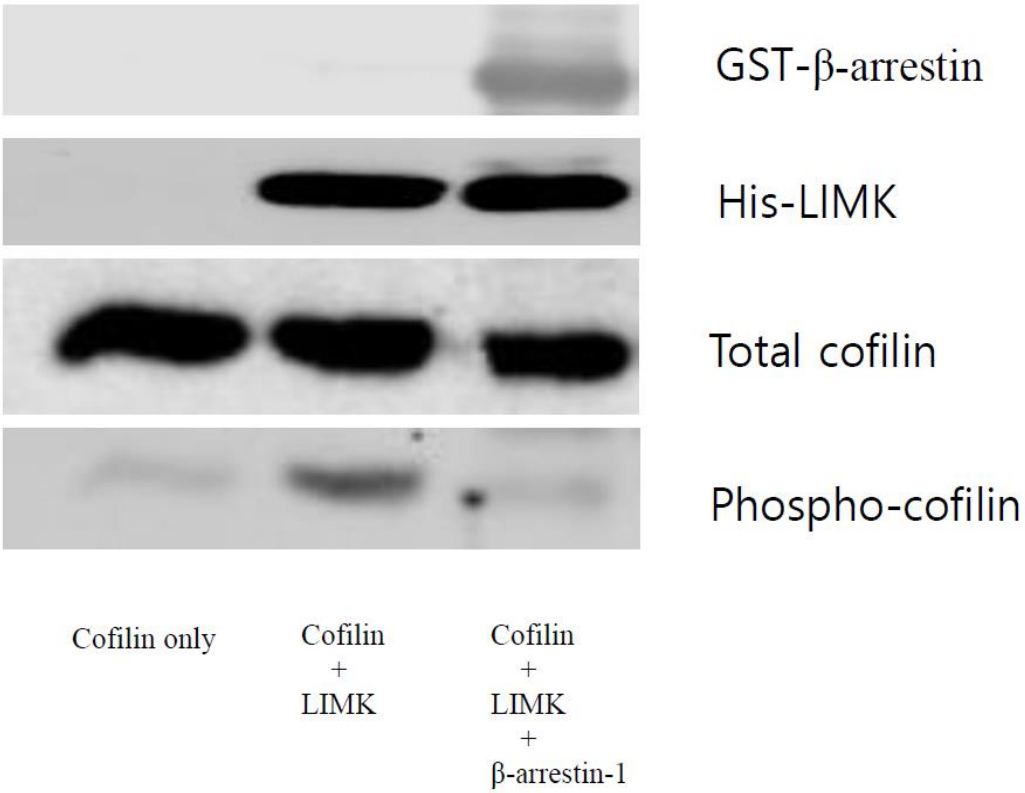


Figure 2.6 β-arrestin-1 directly inhibits LIMK activity. 1μM of cofilin was incubated with KD-LIMK in the presence of ATP and 3μM GST-β-arrestin1 or GST (negative control). Reactions were stopped and phospho-cofilin level was determined by Western blotting by using total and phospho-cofilin antibodie

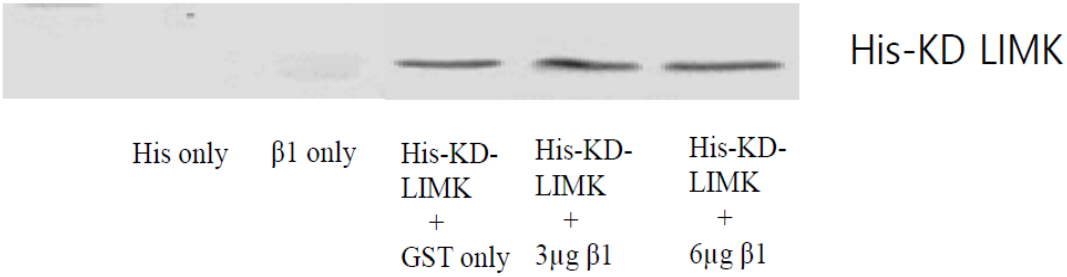


Figure 2.7 β-arrestin-1 does not inhibit ATP binding to LIMK. His-KD-LIMK was immobilized on ATP cross-linked agarose beads and incubated with GST-β-arrestin-1 or GST. The beads were washed and bound proteins were analyzed by Western blotting using anti-His antibodies.

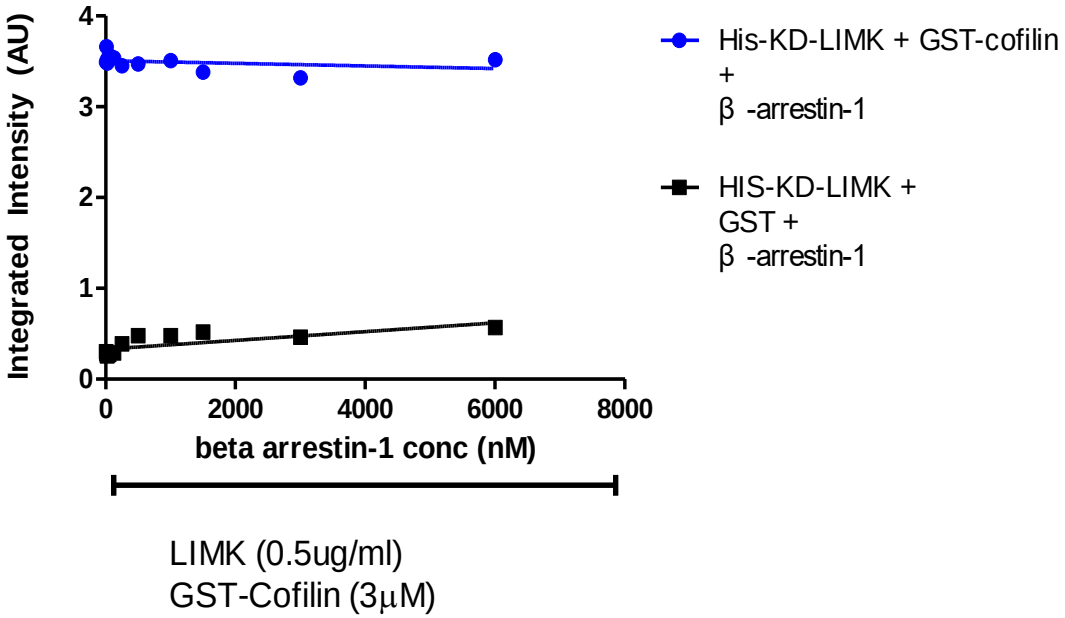


Figure 2.8 β-arrestin-1 is unable to inhibit cofilin binding to LIMK. 3μM GST-cofilin was incubated with 96 well plates coated with LIMK-KD. Increasing concentration of untagged β-arrestin1 was then added. Bound GST-fusion protein was visualized with IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

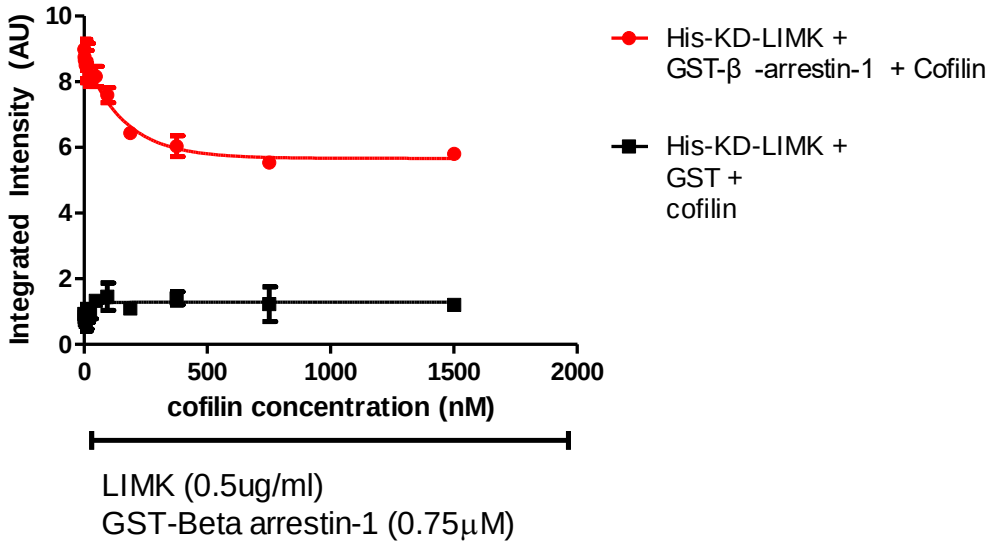


Figure 2.9 cofilin inhibits β-arrestin-1 binding to LIMK. 0.75μM β-arrestin-1 was incubated with 96 well plates coated with LIMK-KD. Increasing concentration of untagged cofilin was then added. Bound GST-fusion protein was visualized with IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

2.2.3 N-terminus of β-arrestin-1 contains important binding sites for LIMK and cofilin

The above results confirm direct interaction between LIMK, cofilin, and β-arrestin-1 (Fig 2.1 ~ Fig 2.5). To narrow down the regions of β-arrestin-1 important for binding LIMK and cofilin, β-arrestin-1 truncation mutants consisting of the following residues were constructed: amino acids 1-163 (β1 1-163), amino acids 1-99 (β11-99), carboxyl terminal residues amino acids 164~418 (β1164-418), and amino acids 184-418 (β1 184-418) were generated. Sandwich immunoassays were repeated and show that while both N and C-terminal truncations (β1 1-163 and β1 164-418), bound to LIMK and cofilin, 184-419 showed no significant binding and 1-99 appeared to bind both with higher apparent affinity than full length β-arrestin-1 (Fig 2.10 and 2.11). These results suggest that β-arrestin-1 may have multiple binding sites for cofilin and LIMK and the N-terminal amino acids may contain a high affinity binding sites. Furthermore, we observed that β11-99 has much higher binding affinities to LIMK and cofilin than full-length β-arrestin-1 as indicated by 45 fold lower EC50 value for LIMK binding and more than 20000 fold lower for cofilin binding. These results suggest that amino acids 1-99 may either be sufficient for LIMK and cofilin binding or it may represent a dominant negative fragment (if it can bind tightly to either or both proteins and prevent their interaction with endogenous β-arrestin-1). To test this we transfected the 1-99 truncation into HEK 293T, treated cells with or without 2fAP and analyzed cofilin dephosphorylation by Western blotting with anti-pcofilin (Fig 2.12). As expected, activation of PAR-2 with 2fAP in promoted cofilin desphosphorylation (to 45% of control values) and expression of flag-1-99 blocked this. These results suggest that 1-99 truncation mutant is able to bind to LIMK and cofilin with higher binding

affinity than full length β-arrestin-1, but lacks the ability to promote cofilin dephosphrylation after PAR-2 activation.. If this mutant is unable to block other β-arrestin-1-dependent signaling events, such as ERK1/2 activation, it can be viewed as selective dominant negative mutant.

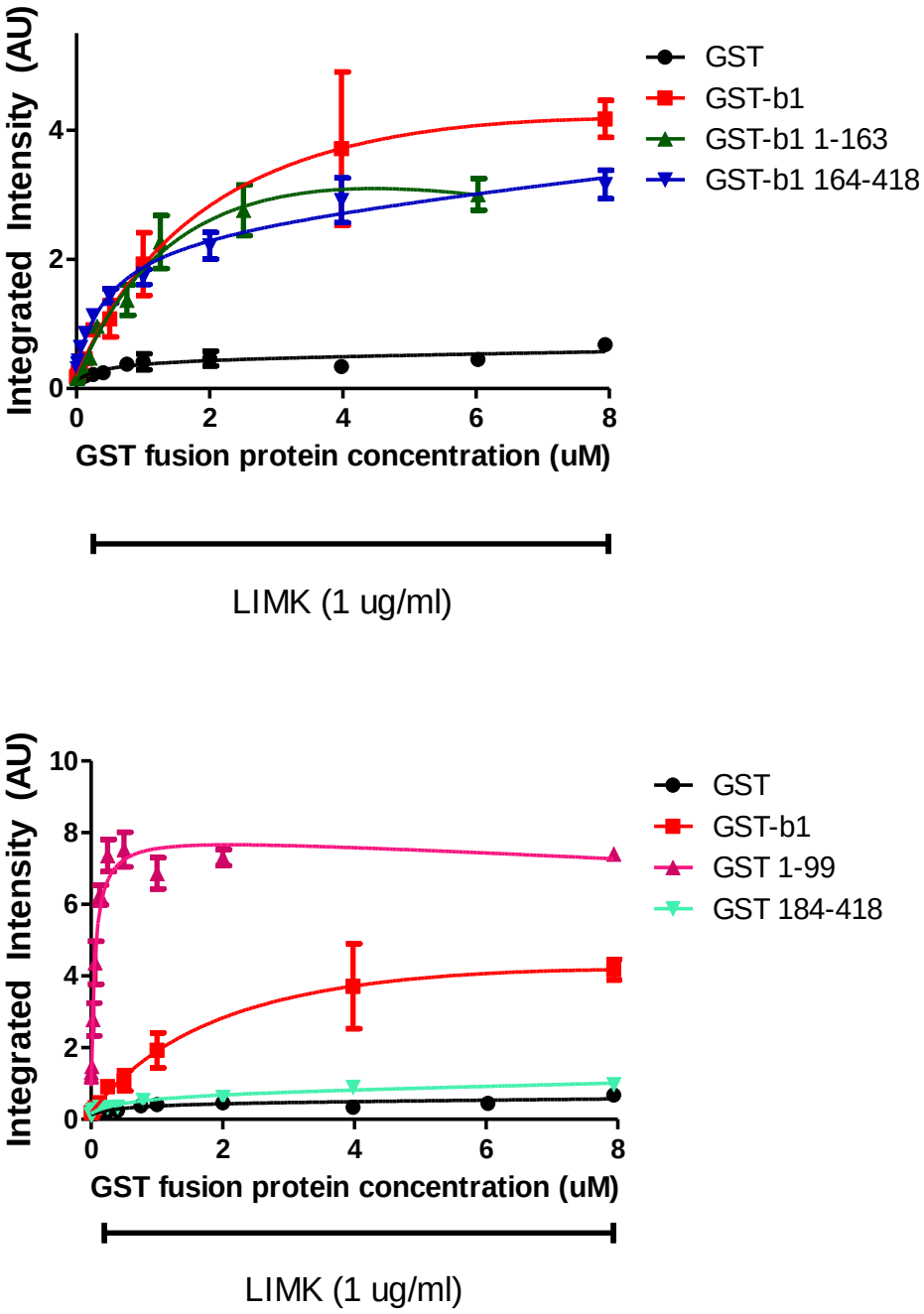


Figure 2.10 The N-terminus (amino acids 1-99) of β-arrestin1 binds with high affinity to LIMK. Fixed amounts of KD-LIMK was captured on 96 well plates, and increasing concentration of GST-β-arrestin1 or GST- β-arrestin1-trucation mutants were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

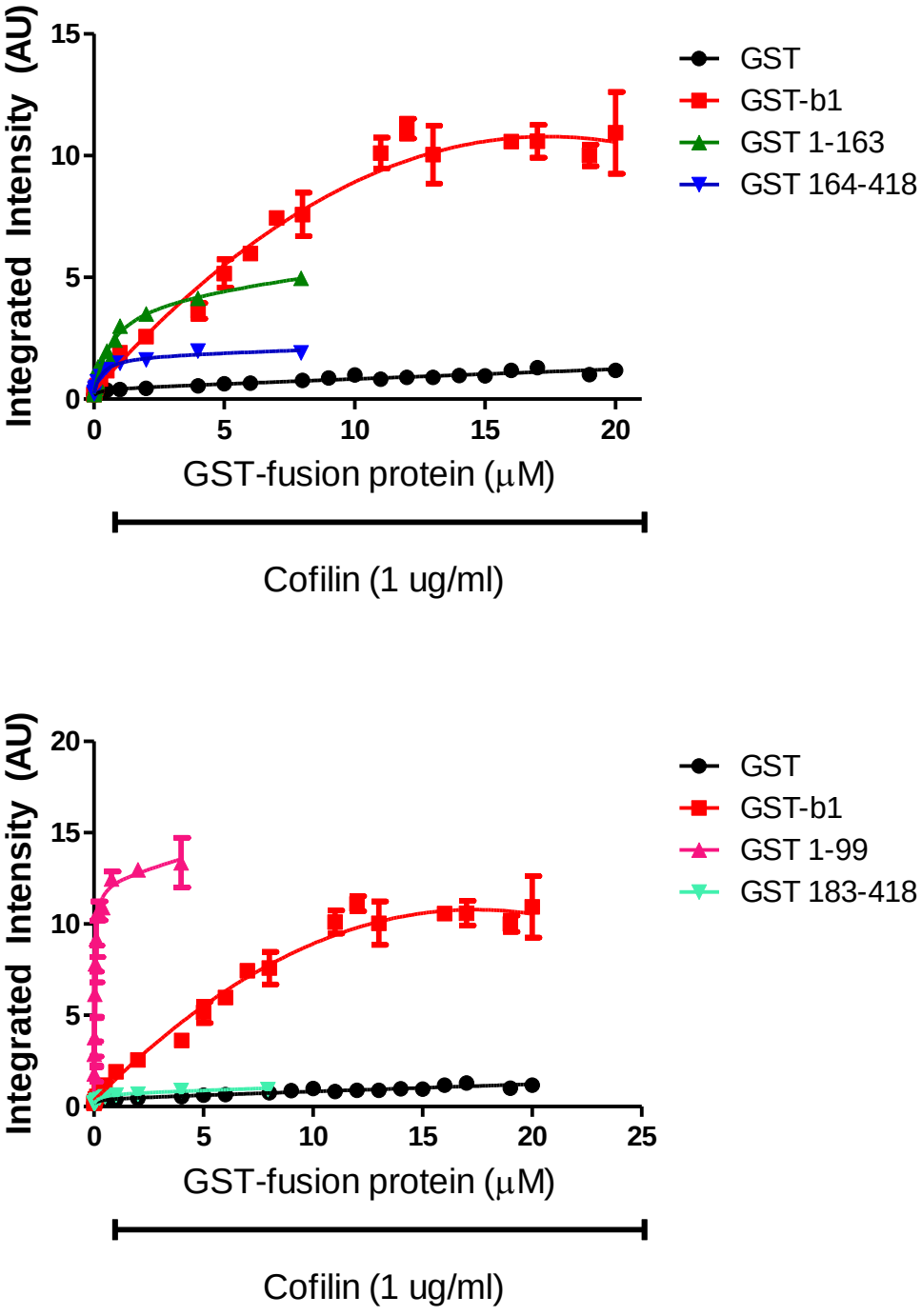


Figure 2.11 The N-terminus (amino acids 1-99) of β-arrestin1 binds with high affinity to cofilin. Fixed amounts of untagged cofilin was captured on 96 well plates, and increasing concentration of GST-β-arrestin1 or GST- β-arrestin1-trucation mutants were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

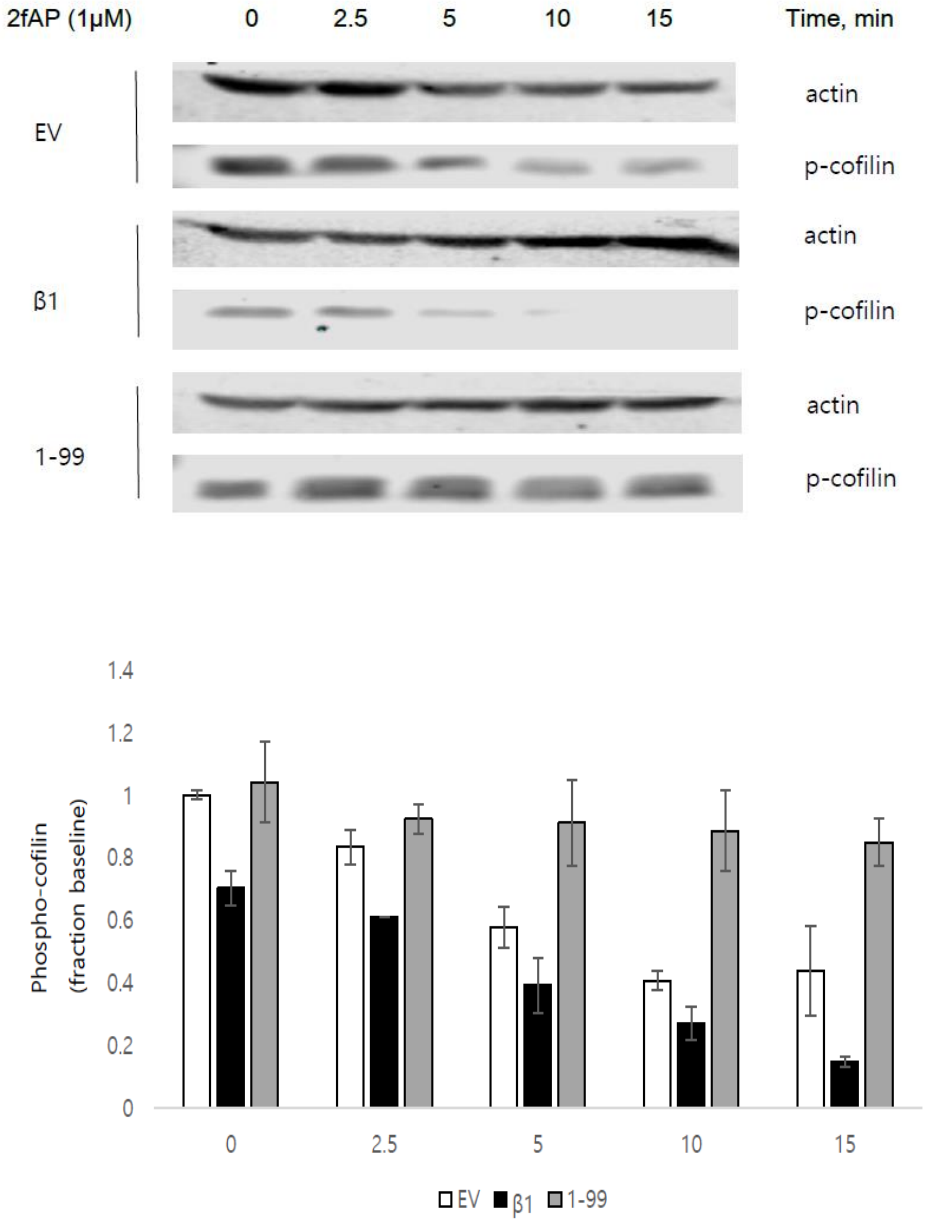


Figure 2.12 Inhibition of cofilin dephosphorylation by 1-99 β-arrestin-1. HEK293 cells were transfected with vector only, full-length β-arrestin-1-flag or 1-99- β-arrestin-1 and treated with 2fAP for 0-15 minutes. Representative Western blot with phospho-cofilin, total cofilin and actin levels after PAR2 treatment (upper panel). Cofilin activation (dephosphorylation) is shown in a bar graph as normalized phosphor-cofilin levels (fraction of baseline) (lower panel).

2.2.4 Residues Arg 51 in β-arrestin-1 is important for interaction with LIMK and cofilin

To narrow down specific amino acids involved we used spot peptide arrays, in which recombinant LIMK or cofilin are incubated with nitrocellulose filters on which 25 amino encompassing the entire sequence of β-arrestin-1, overlapping by 5 amino acids are spotted. These experiments identified peptides corresponding to amino acids 46~115 and 145~160 as LIMK binding regions and amino acids 46~70 and 115~130 cofilin binding regions (Fig 2.13). Given that the 1-99 truncation mutant had a high affinity for both LIMK and cofilin (Fig 2.10 and 2.11), we hypothesized that N-terminal peptides identified in the spot peptide assays contained major binding sites for LIMK and cofilin. Using alanine scanning mutagenesis of peptides 46-70, we identified a charged sequence (ERR) between residues 50~52 that appeared to be important for interaction with cofilin (Figure 2.14). Superimposing this sequence onto the structure of β-arrestin-1, residues 50-52 are located at a flexible region (cyan) of the N-terminal lobe (Figure 2.15). Structural studies suggest that, in the "inactive state", R51 interacts with N162 and Y47 via hydrogen bonding (Figure 2.15 lower image) and R52 interacts with Y54. If these intramolecular interactions are necessary to expose a specific binding domain, mutation may disrupt hydrogen bonding such that the binding site is not accessible. To further investigate the role of amino acids 50-52 of β-arrestin-1, we generated Glu 50 to Ala (E50A), Arg 51 to Ala (R51A), and Arg 52 to Ala (R52A) β-arrestin-1 mutants, and determined whether E50A, R51A, and R52A were able to bind cofilin and LIMK using sandwich immunoassays. While no significant decrease in binding to cofilin was observed for any of the mutants (Fig 2.16), E50A and R51A have

lower binding affinity to LIMK than wild type β-arrestin-1 (Fig 2.17). R52A had a slightly reduced affinity for β-arrestin-1.

To determine whether disrupting binding also prevented the ability of β-arrestin-1 to inhibit LIMK, we performed vitro kinase assays with increasing amounts of WT β-arrestin-1 or the point mutants in presence of purified cofilin. While E50A, and R52A were able to inhibit LIMK activity to nearly the same extent as wild type, R51A mutant lost ability to inhibit LIMK activity (Fig 2.18).

Taken together, these results suggest that R51 is required for LIMK binding and inhibition, which should translate into reduced PAR-2 stimulated cofilin dephosphorylation. We first established that R51A was recruited to PAR-2 upon activation to the same extent as WT β-arrestin-1 by using BRET assay. HEK293T cells were transfected with luciferase-tagged β-arrestin-1 or luciferase-tagged R51A and YFP-tagged PAR-2. Net BRET values were monitored over a period of 20 min after the 2fAP addition (Fig 2.19). As the result shows, R51A recruits to PAR-2 to the same extent as wild type β-arrestin-1. Next, we determined whether R51A is unable to promote cofilin dephosphorylation. Expression of wild type β-arrestin-1 decreases baseline phospho-cofilin levels as well as PAR2-stimulated dephosphorylation, but this is not observed with expression of the R51 mutant (Fig 2.20). Finally, the functional significance of R51 was confirmed by examining PAR-2 stimulated cell migration in HEK 293T cells expressing wild type β-arrestin-1 compared with R51A mutant. Wild type β-arrestin-1 or R51A overexpressed HEK29T cells were seeded onto Transwell filters and treated with or without the PAR-2 peptide agonist, 2fAP, for 3hr. Non-migraotory cells were removed from the top of the membrane, and cells that had migrated to the filter underside were stained and counted. In cells expressing wild type

β-arrestin-1, PAR-2 promoted a 8 fold increase in migration but only a 3.6 fold increase in cells expressing R51A, which was essentially same as empty vector expressing cells(Fig 2.21). Taken together, these results suggest disrupting binding of β-arrestin-1 to LIMK by mutating a crucial residue disrupts PAR2-stimulated cofilin activation and cell migration. Thus inhibition of LIMK by β-arrestin-1 likely represents a major mechanism by which β-arrestin-1 facilitates cell migration.

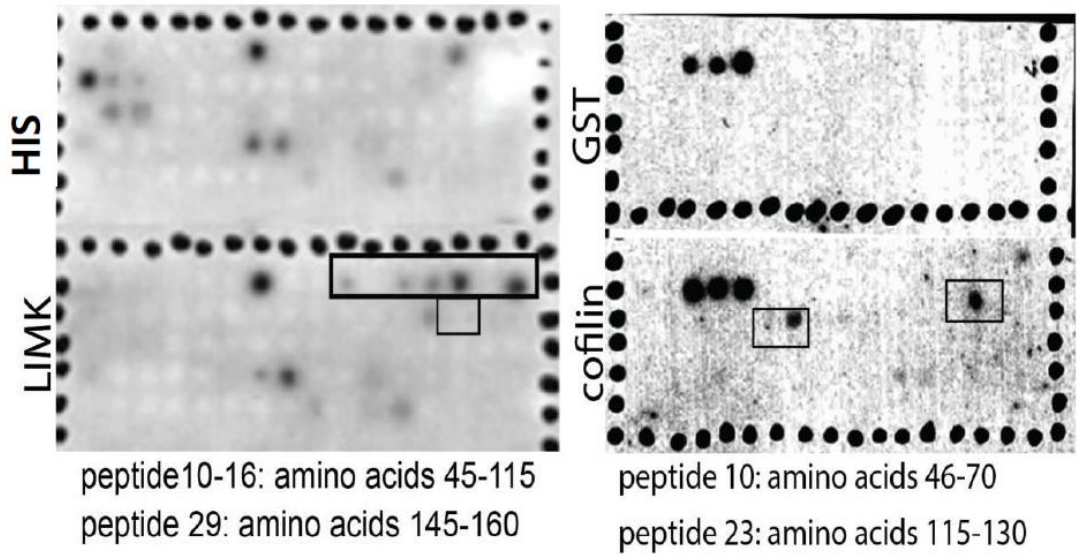


Figure 2.13 Identification of key residues within β-arrestin1 that mediate binding to LIMK and cofilin. Spot peptide arrays in which 25 amino acids encompassing the entire sequence of β-arrestin-1, overlapping by 5 amino acids each, were spotted onto nitrocellulose and incubated with either His-alone (control), His-LIMK **(A),** GST alone (control), or GST-cofilin. The positive spots are indicated by rectangular box.

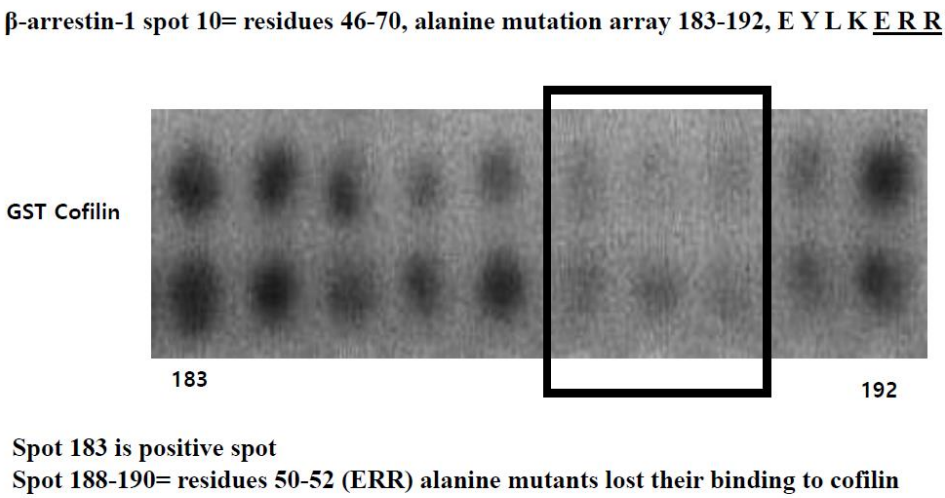


Figure 2.14 50~52 residues within β-arrestin1 that mediate binding to cofilin Alanine scanning mutagenesis of peptide 10 which is identified as positive spot for cofilin was performed, and amino acids from 50 to 52 appear to be essential for binding.

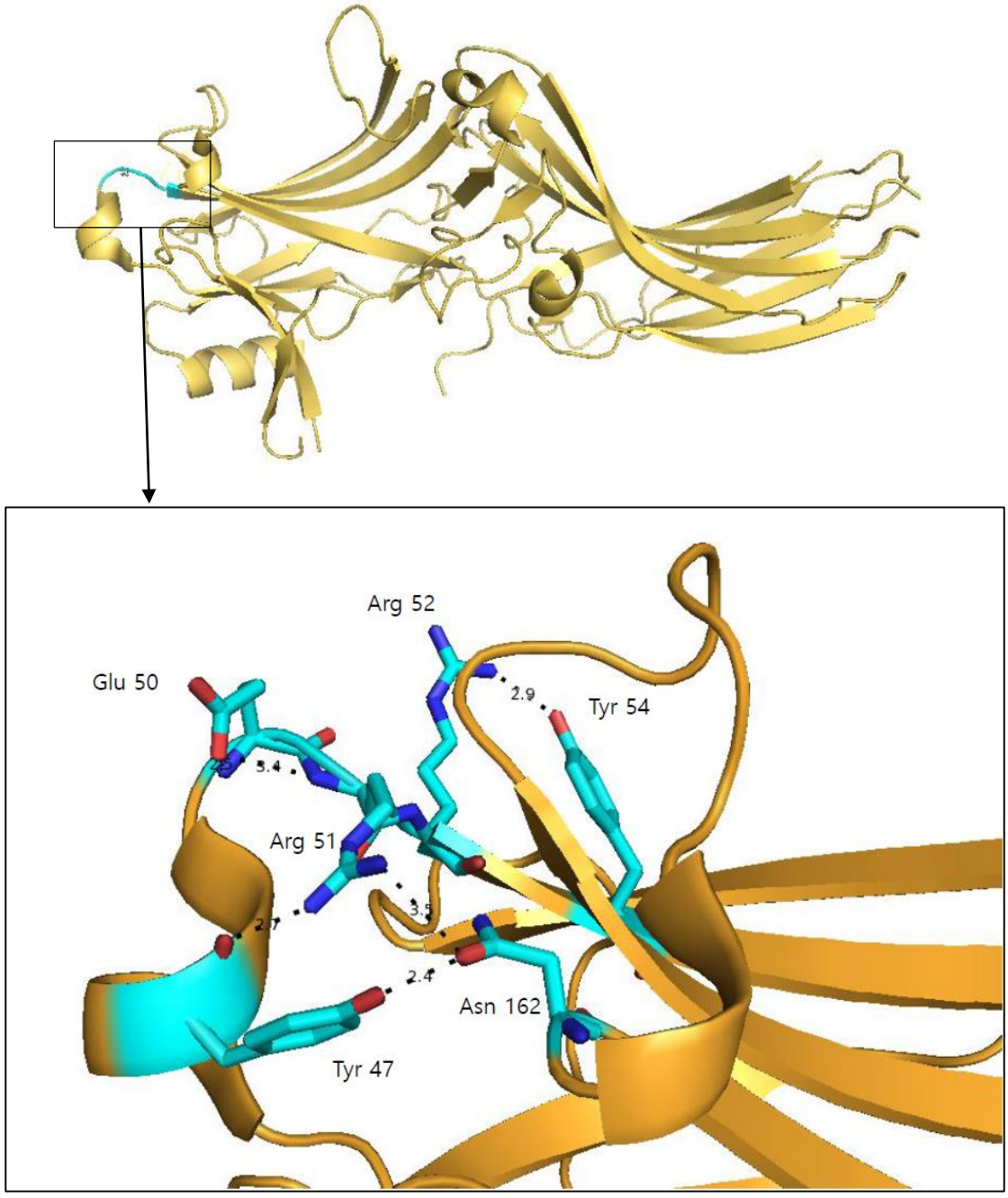


Figure 2.15 The structures of inactive β -arrestin-1. Overall view of β-arrestin-1, with region of interest in box (upper panel). Residues 50-52, 47, 50, 54, and 162 of β-arrestin-1 are highlighted in cyan on the structure of β -arrestin-1 (Protein Data Bank(PDB) 1G4M). R51 interacts with N162 and Y47 via hydrogen bonding (lower panel).

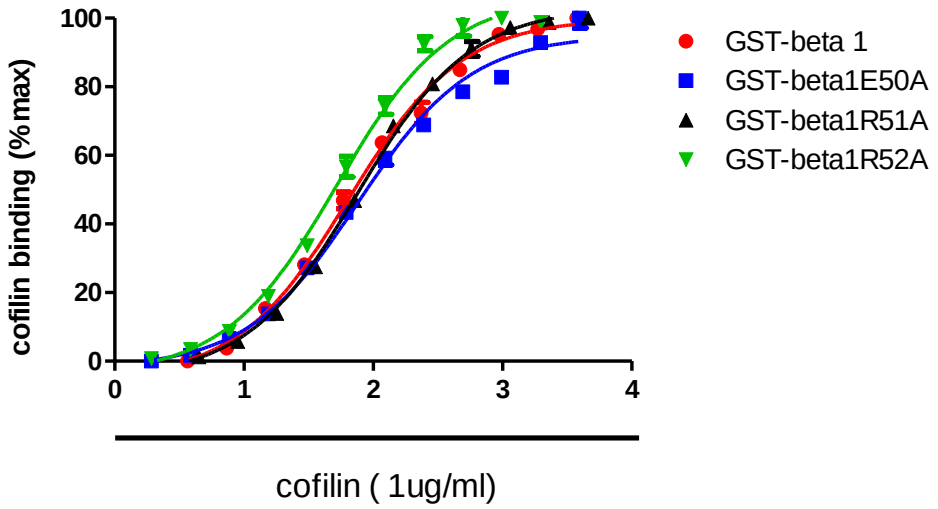


Figure 2.16 The point mutants of β-arrestin-1 are able to bind to cofilin. Fixed amounts of untagged cofilin was captured on 96 well plates, and increasing concentration of GST-β-arrestin1 or GST- β-arrestin-1 point mutants were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

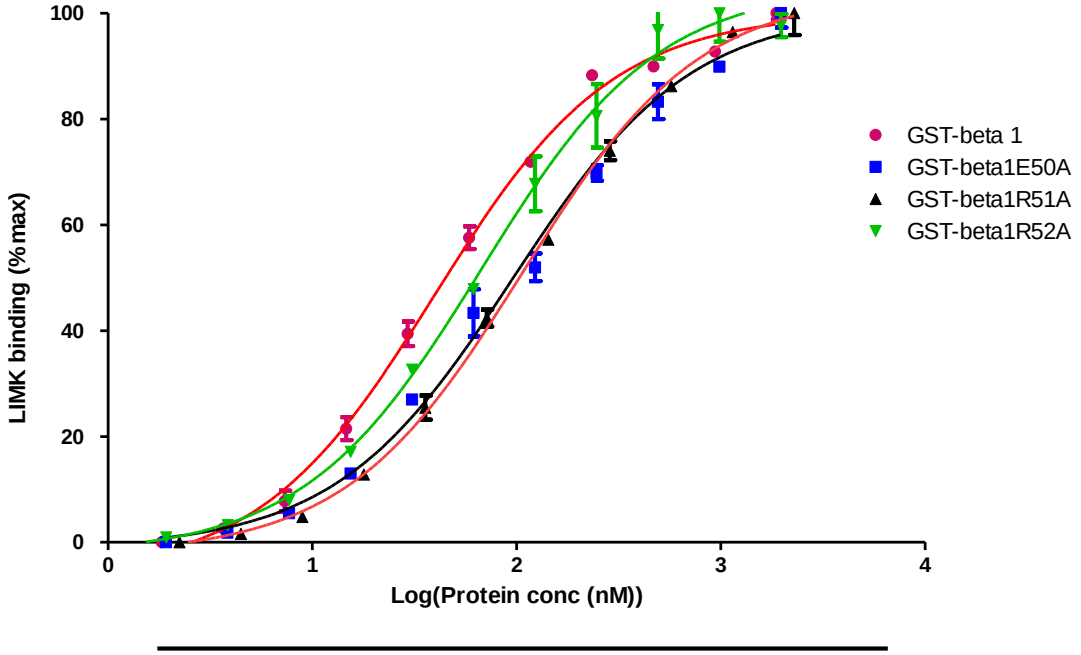
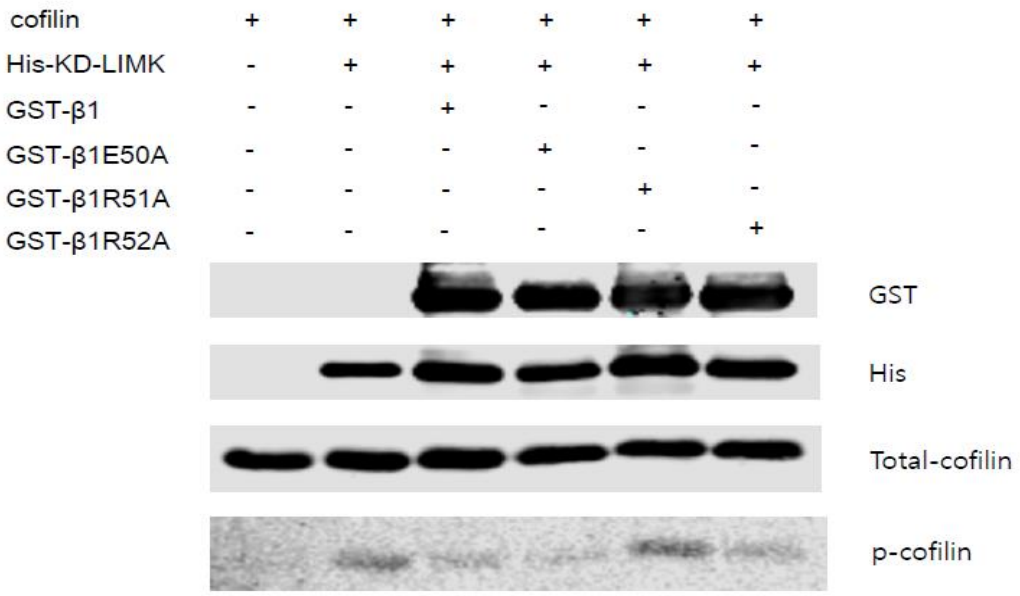


Figure 2.17 E50A and R51A of β-arrestin-1 have lower binding affinity to LIMK. Fixed amounts of untagged cofilin was captured on 96 well plates, and increasing concentration of GST-β-arrestin1 or GST- β-arrestin-1 point mutants were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Pris*



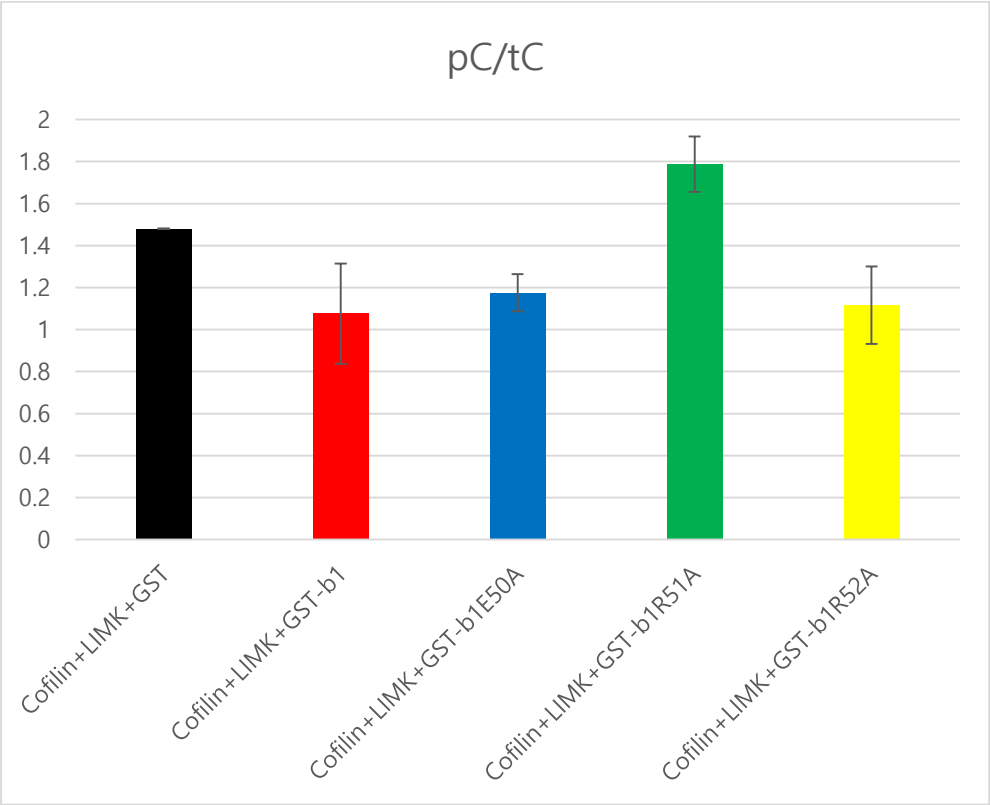


Figure 2.18 R51A mutant of β-arrestin-1 is unable to inhibit LIMK activity. 1μM of cofilin was incubated with KD-LIMK in the presence of ATP and 3μM GST-β-arrestin-1 or point mutants. Reactions were stopped and phospho-cofilin level was determined by Western blotting by using total and phospho-cofilin antibodies

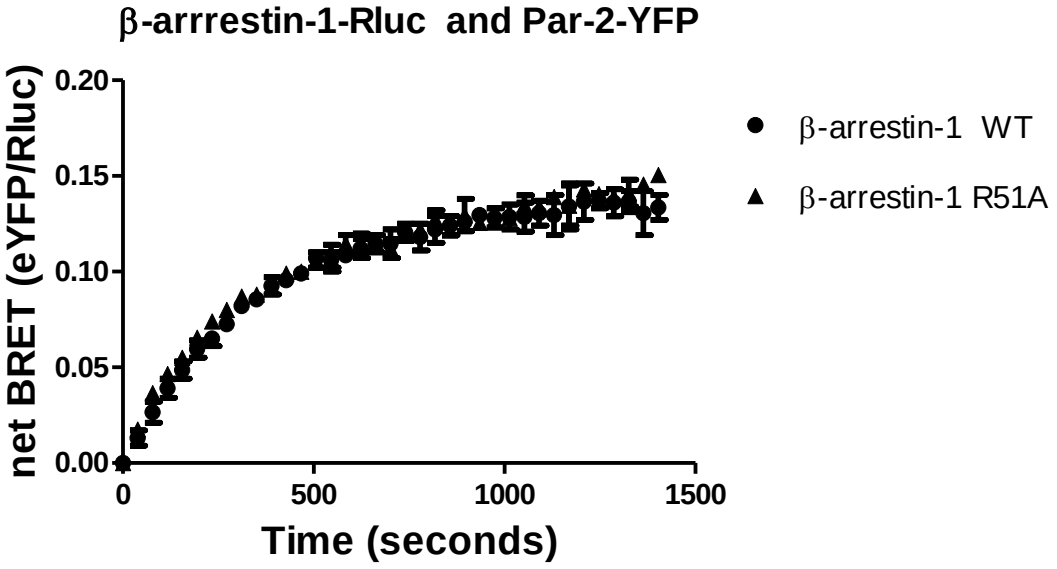


Figure 2.19 The stable and directly binding of WT β-arrestin-1 or R51A mutant to PAR-2. HEK293T cells were transiently co-transfected with PAR-2-YFP and β-arrestin-1 or R51A-Rluc. Net BRET values were monitored over a period of 20 min after the agonist addition.

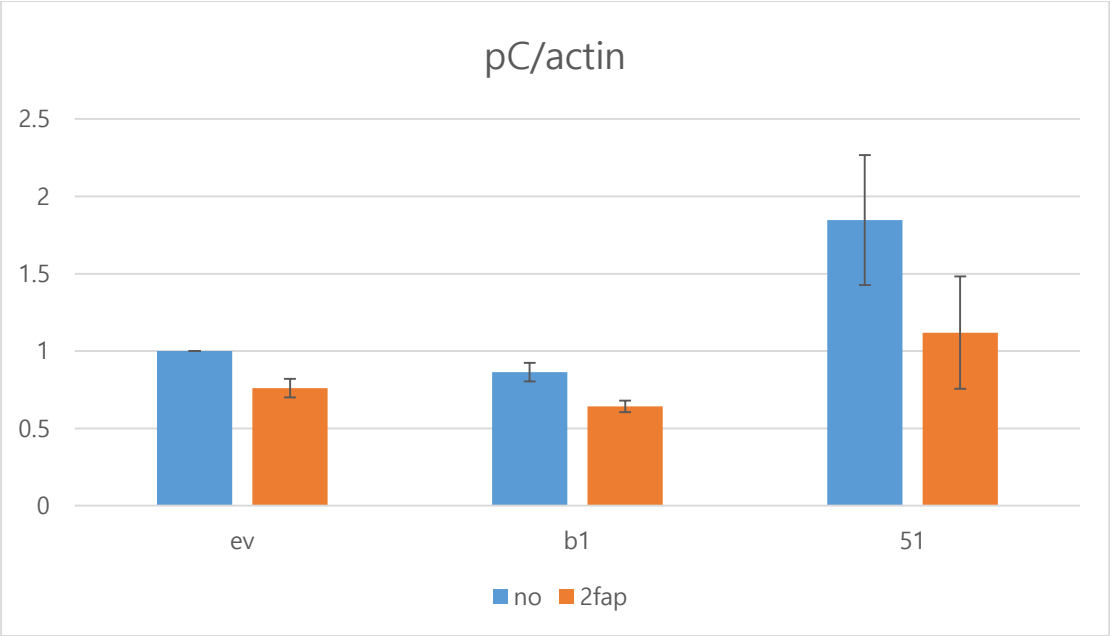


Figure 2.20 R51A mutant is unable to promote cofilin dephosphorylation HEK293 cells were transfected with vector only, full length b-arrestin-1-flag or R51A b-arresin-1 and treated with 2fAP for 15 minutes. Cofilin activation (dephosphorylation) is shown in a bar graph as normalized phosphor-cofilin levels (fraction of baseline).

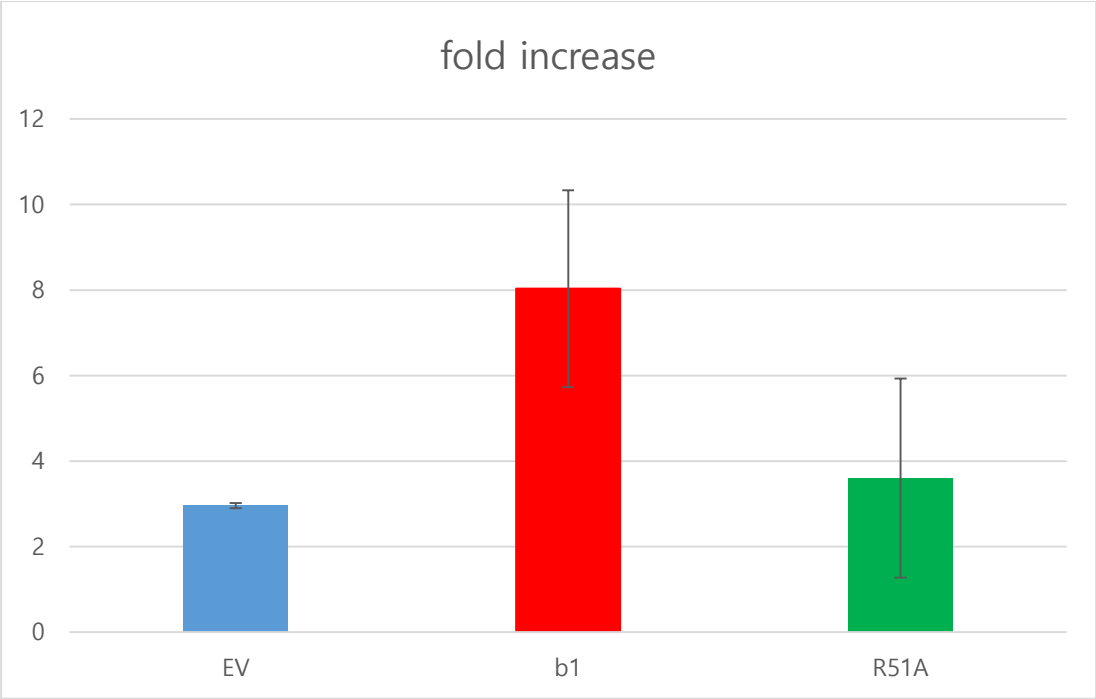


Figure 2.21 R51A inhibits PAR-2 stimulated cell migration Graph showing -fold increase (over non-treated controls) of PAR-2 stimulated cell migration in HEK 293T cells expressing wild type β-arrestin-1, R51A, or vector only.

2.3 Discussion

Our previous published data demonstrate that PAR-2 promotes dephosphorylation and activation of cofilin, which is inhibited by siRNA knockdown of both β-arrestins. The mechanism of β-arrestin-dependent cofilin activation appears to involve both facilitation of cofilin dephosphorylation by the phosphatase Chronophin (aka CIN) and inhibition of LIMK. While the positive effect of β-arrestins on cofilin dephosphorylation appears to involve the formation of a scaffolding complex that brings cofilin in contact with its upstream phosphatase (CIN), the mechanism by which β-arrestins regulate LIMK remains unknown. In this study, we show that β-arrestin-1 directly bind to LIMK and cofilin, and PAR-2 activation stimulates direct interaction between β-arrestin-1 and LIMK or cofilin. Furthermore, we have shown that β-arrestin-1 is capable of inhibiting LIMK activity directly by using a kinase assay. These results suggest that direct binding and inhibition of LIMK by β-arrestin-1 contributes to PAR-2 induced cofilin dephosphorylation. We then examined the molecular mechanisms by which β-arrestin-1 regulate LIMK activities. We tested two possible mechanisms; β-arrestin-1 could potentially act as a "substrate sponge" sequestering cofilin away from LIMK, and β-arrestin-1 could prevent ATP binding to the active site in the catalytic domain of LIMK. However, both hypotheses were proven to be false. Actually, we found that cofilin inhibits β-arrestin-1 binding to LIMK and that β-arrestin-1 didn't inhibit ATP binding of LIMK. One possible explanation for these results could be that we used purified GST- β-arrestin-1, which was not the PAR-2 stimulated activated form of β-arrestin-1. Therefore, it still remains to be determined whether the activated form of β-arrestin-1 sequesters cofilin away from LIMK, or if β-arrestin-1 prevents ATP binding, or both. Also, there are two other hypotheses which need to be elucidated; β-

arrestin-1 competes with cofilin for binding to LIMK, essentially serving as a substrate mimic, and β-arrestins prevent trans-autophosphorylation of LIMK. LIMK assays can be performed using the physiological substrate, cofilin, or myelin basic protein (MBP) as the substrate. MBP is a common substrate for many ser/thr kinases in vitro. Interestingly, sequence comparison of cofilin, myelin basic protein (MBP) and β-arrestin-1, revealed that MBP and β-arrestin-1 both have cofilin phosphorylation site-like motifs (Figure 8). However, the pseudosubstrate site in β-arrestin-1 (ACGV) has a cysteine in place of the serine in cofilin (ASGV), leading us to hypothesize that β-arrestin might bind to the active site as LIMK, in order to mimic the substrate. Since LIMK inhibition is important for PAR-2 stimulated dephosphorylation of cofilin, we examined the ability of a mutant β-arrestin-1 (in which ACGV is deleted) to promote PAR-2 stimulated cofilin dephosphorylation in HEK293T cells (Fig 2.22). As shown in Figure 2.22, PAR 2 promoted cofilin dephosphorylation in cells transfected with wt β-arrestin-1 but not the ACGV deletion mutant. While this is likely due to an inability of this mutant to bind LIMK, it is also possible this deletion mutant could not bind to cofilin because the alanine mutagenesis scanning result indicates V142 is important to cofilin binding. There is another important protein which we have to consider for LIMK regulation: Hsp 90. Hsp 90 regulates LIMK by promoting homodimerization and trans-autophosphorylation of LIMK, leading to the production of stable LIMK dimers. And a previous study showed that β-arrestins are able to bind Hsp 90. Therefore, it is possible that β-arrestins regulate LIMK activity through regulating Hsp 90 and LIMK interaction.

Our lab has shown that both cofilin and LIMK directly bind to β-arrestins, and also identified several important putative binding sites of β-arrestin-1 to LIMK and

cofilin. However, further characterization of binding sites will be required to understand regulatory mechanisms of β-arrestin-1. In this study, we demonstrate for the first time, that R51 of β-arrestin-1 is an important residue for regulating cofilin activity and cell migration. Specifically, we show that, without R51, β-arrestin-1 mediated direct LIMK inhibition is lost, and β-arrestin-1 mediated cofilin dephosphorylation at Ser3 and cell migration are attenuated. Three dimensional structures of inactive β-arrestin-1 shows R51 is located in a flexible region of the N-terminal lobe, and interacts with N162 and Y47 via hydrogen bonding. This result implies that upon PAR-2 activation, β-arrestin-1 undergoes the necessary conformational changes which makes R51 of β-arrestin-1 much more accessible to LIMK, which leads to inhibition of LIMK activity (Fig 2.23).

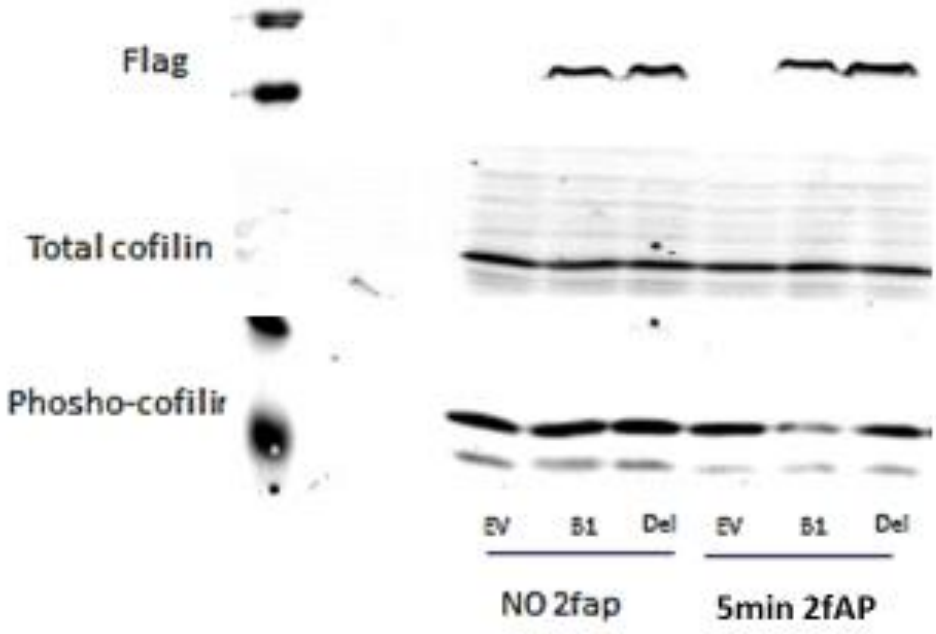


Figure 2.22. Dephosphorylation comparison after PAR-2 activation. HEK 293 cells were transfected with empty vector, β-arrestin-1, or ACGV deletion mutant. HEK293 cells were treated with 2fAP as indicated time after 48h transfection.

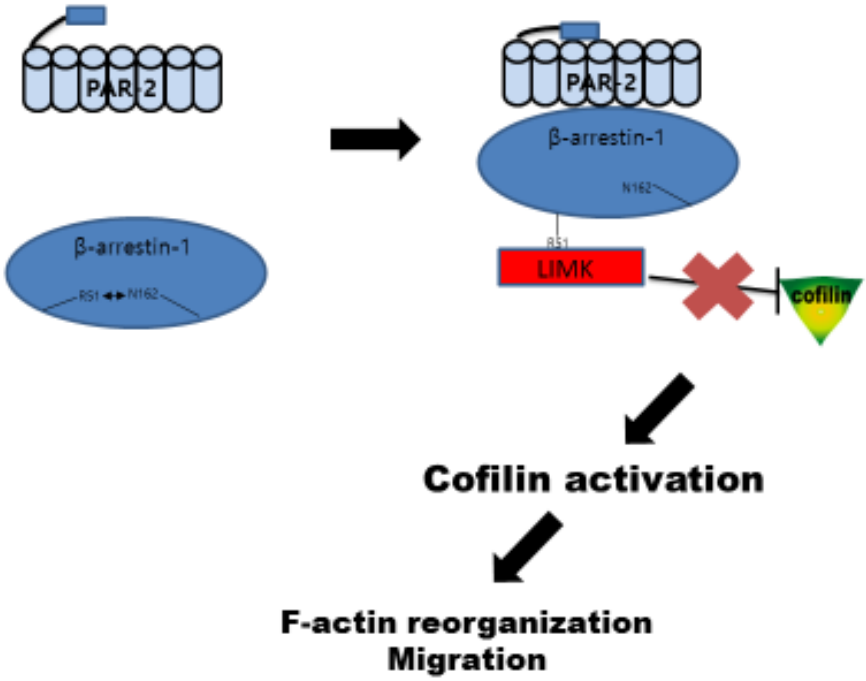


Figure 2.23 Model for regulation of LIMK by β-arrestin-1. upon PAR-2 activation, β-arrestin-1 undergoes the necessary conformational changes which makes R51 of β-arrestin-1 much more accessible to LIMK, which leads to inhibition of LIMK activity

CHAPTER THREE

Define the functional importance of hetero-oligomerization in the regulation of LIMK and cofilin interactions

3.1 Introduction

β-arrestins, once thought to serve only as mediators of G-protein coupled receptor (GPCR) signal termination, are now accepted as essential signaling molecules, governing important cellular outcomes independent of heterotrimeric G-protein coupling. β-arrestin-1 and 2 have very similar amino acid sequences (78% identical), and the two β-arrestins appear to be somewhat functionally redundant. However, functional redundancy of β-arrestins is highly dependent on receptors. For some receptors, one β-arrestin predominantly runs the desensitization and internalization side of the receptor, while the other one scaffolds signaling complex (Kohout et al. 2001; Oakely et al 2000; Paing et al 2002). Over the past years, one interesting question has arisen: what is the functional importance of homo- and hetero oligomerization of β-arrestins in β-arrestins signaling? First, β-arrestins oligomers were considered as an inactive form of β-arrestins. β-arrestin oligomers are able to bind microtubules, but IP_6-mediated β-arrestin-1 oligomers are unable to bind receptors (Schubert el at. 2000;.Milano et al. 2006. Fig 3.1). These results suggest β-arrestin oligomers function as a local store of inactive β-arrestins. Another study showed β-arrestin-2 mutants (K285A/R286A β-arrestin-2 and K295A β-arrestin-2), which were unable to form oligomers, had intact receptor internalization function, while they were unable to promote ERK1/2 phosphorylation (Xu et al 2008, Fig 3.2). However, this study also showed that ERK1/2 binds to a region overlapping the dimer interface. Although it is possible that β-arrestin oligomer is non-functional and only monomeric β-arrestins are capable of binding and activating of ERK1/2, we should consider that β-arrestin oligomer may affect other β-arrestin-mediated signaling complex.

Our lab previously had shown that β-arrestins are required for PAR-2-mediated motility, suggesting they are not redundant, even for receptors that appear to utilize both (Lan et al. 2004). Also, we had shown that β-arrestin-1 inhibits LIMK activity to a greater extent than β-arrestin-2, and cofilin constitutively associated with overexpressed β-arrestin-2 but not β-arrestin-1 (Zoudilova et al 2007). Furthermore, in vivo studies suggest β-arrestin-2 is essential for a number of PAR2-dependent functions, whereas β-arrestin-1 is not. While for overall cofilin dephosphorylation the two β-arrestins appear to be somewhat functionally redundant, they may differ in the mechanisms by which they regulate cofilin. In this chapter, my study will propose the following mechanism: that β-arrestin-1 and 2 work cooperatively to regulate LIMK and cofilin activation.

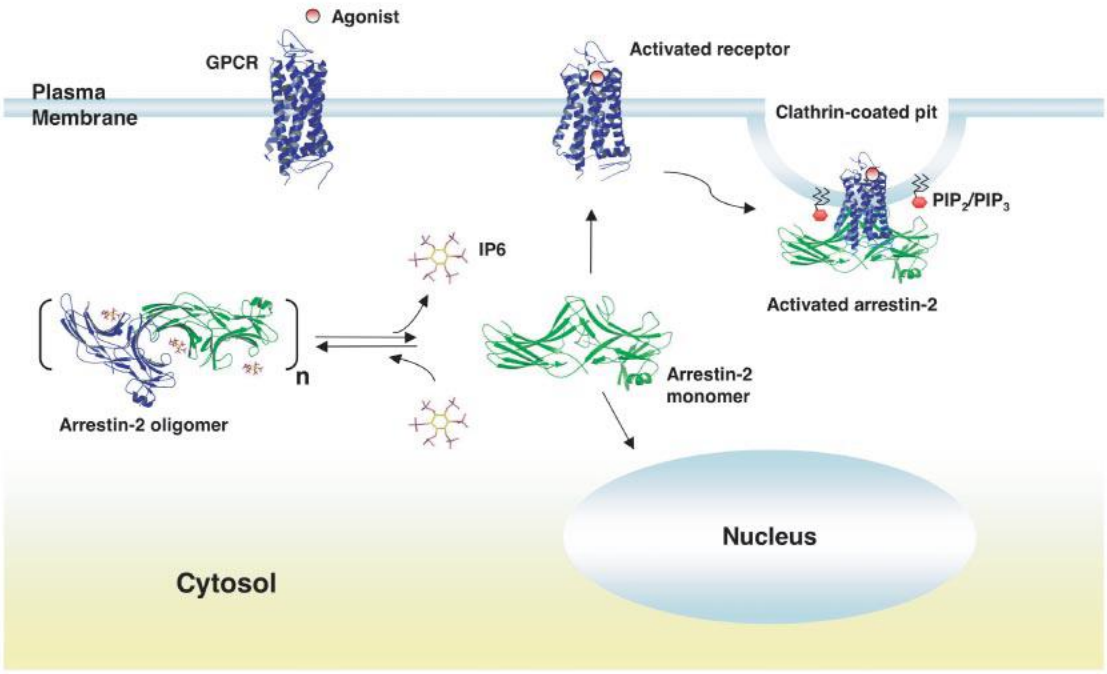


Figure 3.1 Model for IP6-dependent regulation of arrestin oligomerization and function. Arrestin-2 oligomers, unable to bind receptors, function as a local store of inactive arrestin. The combination of arrestin-2 containing a low and high affinity site for IP6 along with the changing level of free IP6 within the cell would regulate the dissociation of arrestin oligomers and the movement of monomeric arrestin to activated receptors. Arrestin-2 oligomers are localized primarily in the cytoplasm enabling the mobilization of arrestin-2 rapidly to the membrane. Arrestin-2 monomers, however, are imported into the nucleus where their interaction with nuclear binding partners might be enhanced. *PIP2*, phosphatidylinositol 4,5-bisphosphate; *PIP3*, phosphatidylinositol 3,4,5-trisphosphate; *GPCR*, G protein-coupled receptor.

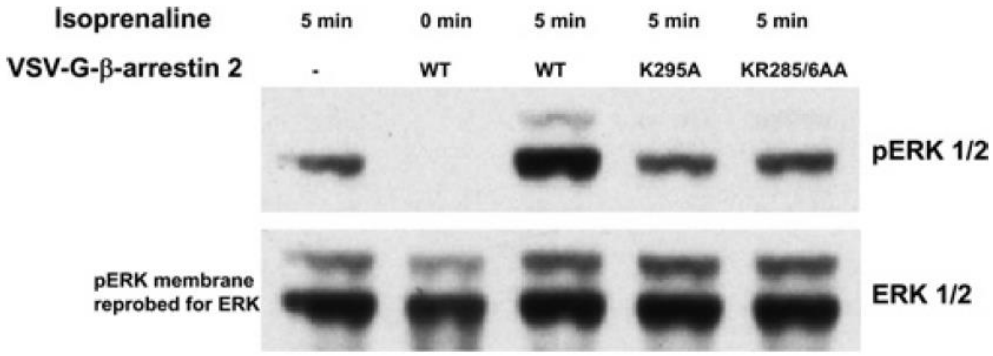


Figure 3.2 K285A/R286A *β*-arrestin 2 and K295A *β*-arrestin 2 are unable to enhance *β*2-adrenoceptor-mediated ERK1/2 MAPK phosphorylation VSV-G-tagged forms of wild-type (WT) *β*-arrestin 2, K285A/R286A (KR285/6AA) *β*-arrestin 2 or K295A *β*-arrestin 2 were expressed transiently in HEK-293 cells stably expressing the *β*2-adrenoceptor Cells were subsequently challenged with isoprenaline (10 μM for 5 min). Cell lysates were resolved by SDS/PAGE and the presence of phospho-ERK1/2 (upper panel; pERK 1/2) and total ERK1/2 amounts (lower panel) was detected by immunoblotting with appropriate antibodies. Results are representative of three independent experiments.

3.2 Results and Discussion

Our lab previously showed that β-arrestin-1 and 2 may differ in the mechanisms by which they regulate cofilin (Zoudilova et al 2007). Sandwich immunoassays, in which recombinant untagged cofilin was captured on 96-well plates, and increasing concentrations of GST or GST- β-arrestin1 were added, demonstrated that β-arrestin-1 and 2 have different affinity to cofilin. On computing the EC50 value, we observed that β-arrestin-2 has higher binding affinity over β-arrestin-1 as indicated by its 70% lower EC50 value (Fig 3.3).

Recalling Chapter 2, β-arrestin1 did not significantly affect ATP binding to KD-LIMK. To demonstrate whether β-arrestin-2 can inhibit LIMK ATP binding, an in vitro ATP binding assay was performed by using ATP cross-linked agarose beads (Fig 3.4). This result suggests that only β-arrestin-2 can inhibit LIMK ATP binding. However, our lab's previous data showed that β-arrestin-1 inhibits LIMK activity to a greater extent than β-arrestin-2. Therefore, it is possible that β-arrestin-1 and 2 have different mechanisms in regulating LIMK activity. The C-terminal amino acid sequence difference between β-arrestin-1 and 2 may account for the LIMK ATP binding inhibition by β-arrestin-2. The amino acid sequences of the two β-arrestins are 78% identical, and most of the sequence differences appear on the C-terminal end. There are several noteworthy differences between β-arrestin-1 and 2 in the C-terminal negatively charged amino acids stretch. (Residues from 402 to 408 for β-arrestin-1 and residues from 402 to 406 for β-arrestin-2.). These differences may possibly be responsible for the LIMK ATP binding inhibition. β-arrestin-1 not only contains 10 more amino acids after the last negatively charged amino acid in the negative stretch, but also contains a lysine residue between negatively charged amino acids in the C-terminal negatively

charged stretch. Presumably, β-arrestin-2 inserts this negatively charged C-terminal end to the ATP binding site of LIMK to mimic ATP, but β-arrestin-1 cannot do this because of a longer C-terminal end after the negatively charged stretch, and the positively charged Lysine residue that may cause electrostatic repulsion.

Several research studies have shown that β-arrestins can make hetero-oligomers. However, the functional importance of hetero-oligomers is largely unknown. To further investigate the role of hetero-oligomers, we verified multimerization of β-arrestins by using the Sandwich immunoassays (Fig 3.5). The Sandwich immunoassays demonstrated direct binding of β-arrestin-1 to β-arrestin-2. Finally, to address whether PAR-2 promotes interaction of β-arrestin-1 with β-arrestin-2, flag-β-arrestin-1 was overexpressed and immunoprecipitated from HEK293T cells after PAR-2 activation with anti-flag, and immunocomplexes were analyzed by SDS-PAGE followed by Western blotting with anti- β-arrestin-2. Overexpressed β-arrestin-1 was associated with endogenous β-arrestin-2 at 5 min of PAR-2 activation (Fig 3.6). These results suggest the possibility that hetero-mulitimer of β-arrestins has a significant role for cofilin regulation, particularly cooperative cofilin regulation. Previous literature identified β-arrestin-1 N-terminal residues, including K157, K160, R161 and C-terminal residues including K232, R236, K250, K324, and K326 that are important to make β-arrestin homo or hetero oligomers (Milano et al. 2006). Using the information above, β-arrestin-1 mutants which cannot make homo or hetero oligomers can be generated and used to provide insights into functional outcomes of oligomerization.

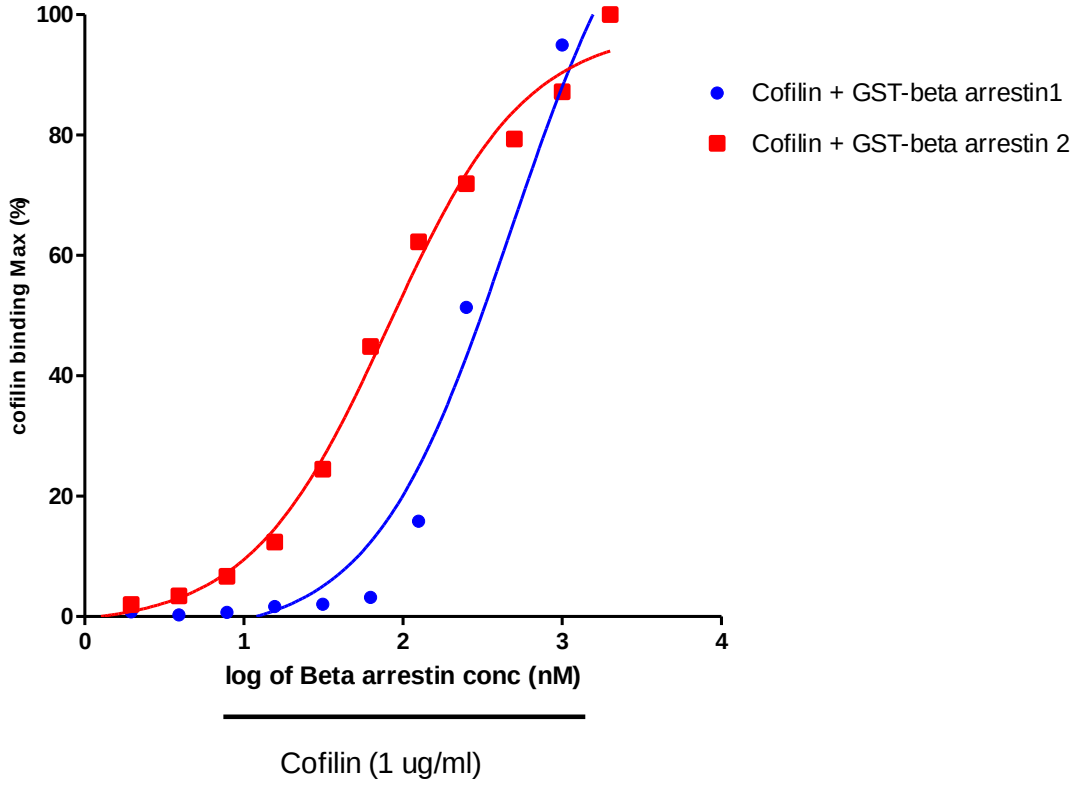


Figure 3.3. β-arrestin-1 and 2 interact with cofilin. Sandwich immunoassay. Fixed amounts of cofilin was captured on 96 well plates, and increasing concentrations of GST-β-arrestin-1 or GST-β-arrestin-2 were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

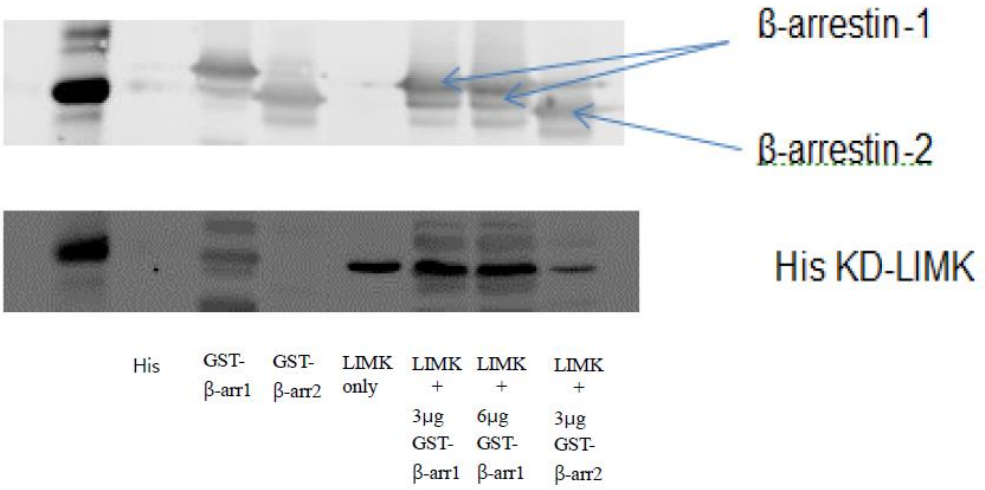


Figure 3.4 β-arrestin-2 inhibit ATP binding to LIMK. His-KD-LIMK was immobilized on ATP cross-linked agarose beads and incubated with GST-β-arrestin-1 or GST- β-arrestin-2. The beads were washed and bound proteins were analyzed by Western blotting using anti-His antibodies.

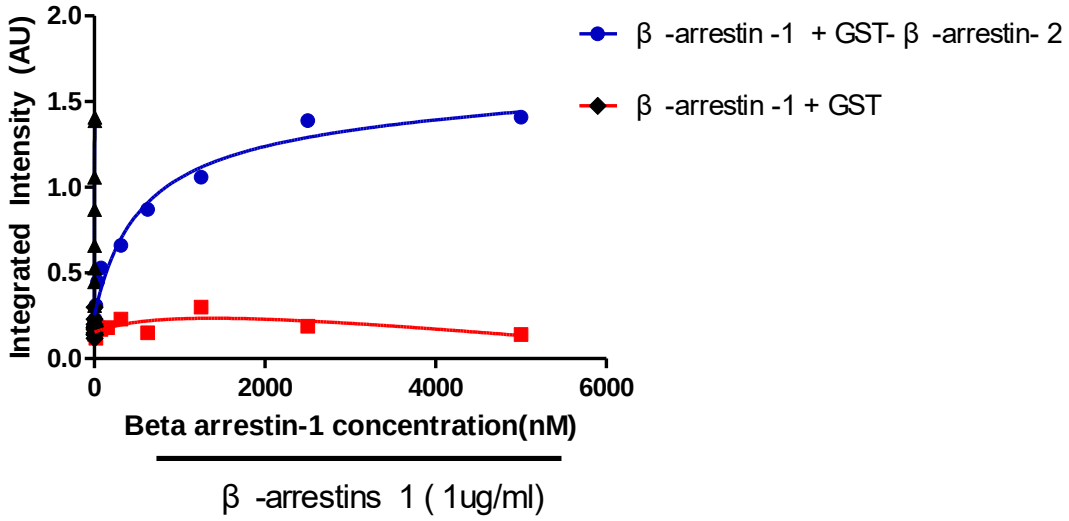


Figure 3.5. β-arrestin-1 and 2 form hetero-oligomers. Sandwich immunoassay. Fixed amounts of β-arrestin-1 was captured on 96 well plates, and increasing concentrations of GST-β-arrestin-2 or GST were added. Bound protein was quantified by using IR-800 conjugated anti-GST, and integrated intensity was obtained by a LI-COR Odyssey system (n=3). The best fit curves were analyzed and graphed by *GraphPad Prism***.**

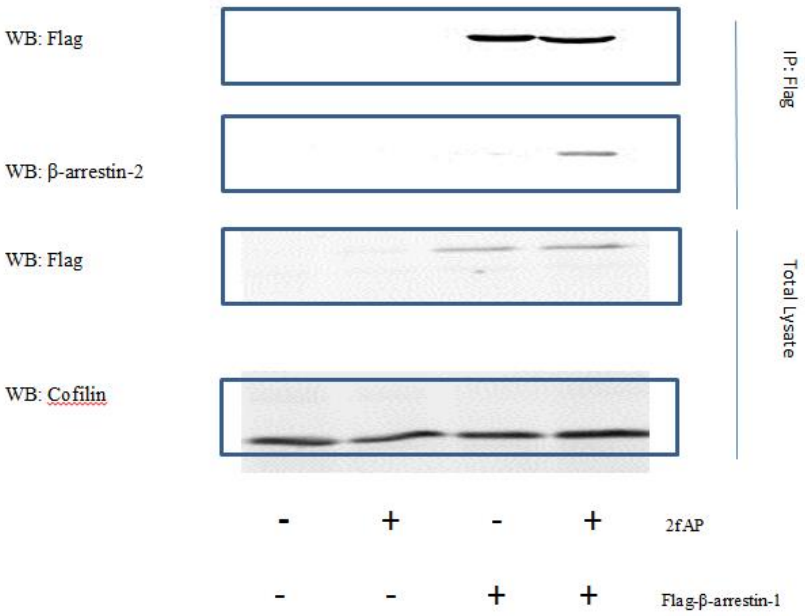


Figure 3.6 PAR-2 stimulates association of β-arrestin-1 with β-arrestin-2. β-arrestin-1 was immunoprecipitated from HEK293T cells after activation of PAR-2 with 2fAP for 5 min and analyzed by SDS-PAGE followed by Western blotting with anti β-arrestin-2

CHAPTER FOUR

Conclusion

Conclusion

My studies reveals several important aspects about how β-arrestins regulate LIMK. First, β-arrestin-1 interacts with LIMK and directly inhibits the kinase activity, which leads to cofilin activation. However, mutation of arginine 51 to alanine (R51A) eliminates the ability of β-arrestin-1 to inhibit LIMK activity and blocks PAR-2-mediated cofilin dephosphorylation. Furthermore, R51A inhibits PAR-2 stimulated migration in HEK293T cell. Thus, this study confirms our previously proposed signaling model; downstream of PAR-2 activation, (1) beta-arrestins form a complex with cofilin and its phosphatase, CIN, which leads cofilin dephosphorylation, (2) β-arrestins further mediate cofilin activation by scaffolding LIMK, which leads to inhibition of LIMK activity (Fig 4.1). Also, we show possibility that β-arrestin-1 and 2 work cooperatively to regulate LIMK and cofilin activation.

Many of the targets of β-arrestin-dependent scaffolding are kinases. In some cases the association leads to activation and in others, inhibition of the target kinase activity. LIMK contain a C-terminal kinase domain that, when expressed alone, is constitutively active and this activity is suppressed by intramolecular interactions between residues in the N and C-terminal. Binding of upstream activators and phosphorylation of key residues relieves the auto-inhibition resulting in activation of the kinases. Mechanisms elucidated here are likely to be extrapolated to other kinases and aid in understanding the full range of GPCR stimulated β-arrestin-dependent activities.

Because β-arrestins are capable of taking on multiple conformations in response to receptor recruitment, the relative affinities of each of the signaling proteins

for individual β-arrestins, their binding on and off rates, and the location of various binding sites on each β-arrestin, are likely to play important roles in determining how association with β-arrestins affects their activities. Their activities in turn will ultimately help determine the cellular consequences of β-arrestin-dependent signaling. By further characterizing the interactions of cofilin and LIMK with each β-arrestin, and assessing the effect of mutating putative sites of interaction on the formation of their cognate scaffolding complexes and downstream signaling events, we can build a model for receptor-specific, β-arrestin-dependent events. This information can eventually be used to identify minimal domains necessary for β-arrestin-dependent signaling, and to assess the physiological consequences of PAR_2/β-arrestin-dependent signaling.

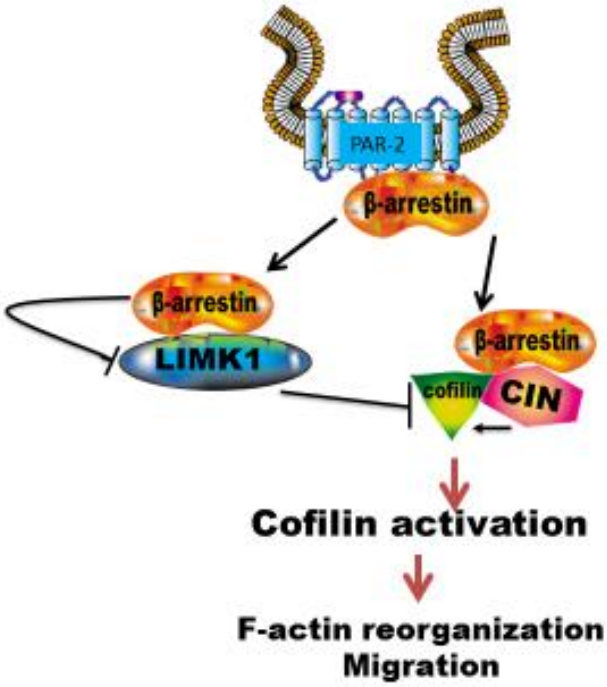


Figure 4.1 the mechanism of the regulation of cofilin activation by β-arrestins. Downstream of PAR-2 activation, β-arrestins form a complex with cofilin and its phosphatase, CIN. Cofilin can be dephosphorylated and activated and leads to actin reorganization. β-arrestins further mediate cofilin activation by scaffolding LIMK, which leads to LIMK inhibition

CHAPTER FIVE

Materials and Methods

Materials and Methods

All chemicals are from Sigma or Fisher Scientific unless stated otherwise. Kinase domain LIMK1 *(*amino acids 285-629, millipore*)* were used for kinase assay and sandwich immunoassay. All restriction enzymes were from New England Biolabs. Glutathione sepharose 4B *(GE Healthcare)* or Glutathione agarose *(BD Biosciences)* were for the GST-tagged protein purification. PAR2 activating peptide 2fAP (2-furoyl-LIGRLOrnithine-NH2) was synthesized by *Tocris Bioscience*. PVDFfl (*Millipore)* was the membrane for western blots.

Antibodies

Antibodies and final dilutions for Western blot (WB), immunoprecipitation (IP) and sandwich immunoassay (SI) were as follows: rabbit anti-phospho (Ser3)-cofilin (*Cell Signaling*, 1:1000 WB), mouse anti-total cofilin (*BD Bioscience*, 1:1000 WB rabbit anti-GST (*Santa Cruz Biotechnology*, 1:1000 WB), rabbit anti-Flag (*Sigma*, 1:000 WB), mouse anti-β-arrestin-1(*BD Biosciences*, 1:1000 WB), mouse anti-6xHis (*BD Biosciences*, 1:1000 WB and SI), IR680-conjugated anti-rabbit secondary (Invitrogen, 1:30000 WB), and IR800-conjugated anti-mouse (invitrogen, 1:30000 WB). IR-dye-conjugated secondary antibodies (*Rockland Biosciences)* 1 μg/ml is used in sandwich immunoassay.

Plasmids

The following plasmids were used in transient expression experiments: Flagtagged β-arrestin-1 plasmids and truncated mutants containing 1 to 163 or 164 to 418 amino acids of β-arrestin-1 were provided from Dr. Robert Lefkowitz (Duke University, Durham, NC). Flag-tagged β-arrestin-1 containing 1 to 99 was excised with HindIII and XhoI and subcloned into pcDNA-hygro. For expression in bacteria, GST-tagged WT β-

arrestin-1 was from Dr. Robert Lefkowitz (Duke University, Durham, NC). GST-tagged truncated β-arrestin-1 containing 1 to 163 and 164 to 418 amino acids were subcloned into pGEX4T-1. GST- tagged E50A, R51A, and R52A were subcloned into pGEX4T-1 and pcDNA 3.1. *Renilla* luciferase-tagged β-arrestin-1 construct, and FLAG tagged β-arrestin-1 and -2 constructs were obtained as gifts from Dr. JoAnn Trejo (University of California San Diego, La Jolla, CA), Dr. Michel Bouvier (University of Montreal), and Dr. Robert Lefkowitz (Duke University Medical Center), respectively.

Cell Culture and Transfection

HEK 293 Cells were grown in 1X DMEM substituted with 10% FCS. Transient transfections were performed on 70-80% confluent cells Bio T (*Bioland*) and experiments were performed between 48 and 72 hours after transfection.

Western Blotting and Immunoprecipitation

60-mm dish (grown for 24 h) were serum starved for 3 h, treated with 100 nM 2fAP for 0–15 min at 37 ℃, lysed in 0.125 ml of lysis buffer (50mM Tris-HCl(pH 8.0), 1% Nonidet P-40, 120 mM NaCl, 1 mM dithiothreitol, 10µg/ml aprotinin, 10mMNaF, 2mM NaVO3, 1mM PMSF and 10µg/ml leupeptin). Cell lysates were subjected to SDS-PAGE (12.5%) followed by Western blotting. Blots were imaged using the LICOR Odyssey imaging system, and LICOR software was used to calculate integrated intensities of bands. For the interaction of transfected Flag-tagged β-arrestin-1 with endogenous β-arrestin-2, HEK293T cells (10cm dish) were transfected with Flag-tagged β-arrestin-1 plasmids by using BioT. 48 h after transfection, cells were serum starved for 3 h, treated with 100 nM 2fAP for 15 min at 37 ℃, lysed in 0.250 ml of lysis buffer. Immunoprecipitation was performed using either anti-flag monoclonal

antibody (M2; Sigma). The amounts of coprecipitated proteins were determined by immunoblotting

Purification ore recombinant proteins

GST-tagged WT β-arrestin-1, its truncations (1-163 and 164-418 amino acids of β-arrestin-1), and point mutants (E50A, R51A, and R52A) were expressed and purified from BL21 *Escherichia coli* cells using glutathione-sepharose 4B (GE Healthcare). Briefly, *E. coli* BL21 (DE3) cells were transformed with GST-tagged fusion proteins, and grown to exponential phase. When OD600 is 0.6, recombinant proteins were induced with 0.5 mM IPTG (isopropyl-1-thio-β-Dgalactopyranoside) for 4 hours at 30℃. Bacterial cells were subsequently lysed by sonication (6 bursts of 10 s at 30% power) in PBS supplemented with 10 μg/ml lysozyme,10 μg/ml DNase 1 and 1% (v/v) Triton X-100. Cleared lysates were incubated with 400μl of glutathione–Sepharose 4B for 1 hour, washed with 20 volumes of binding buffer supplemented with 1 mM ATP, and the bound protein was eluted using increasing concentrations of free reduced glutathione. Elution fractions containing the fusion protein were dialyzed overnight against PBS and stored in 10% (v/v) glycerol at −80℃ until needed. The protein concentration of each elution was determined using the Bradford assay, and the relative purity was determined by SDS/ PAGE (10% gels), followed by staining with Coomassie Brilliant Blue R250. Some breakdown of β-arrestin was observed in each preparation, which was quantified by the Coomassie-stained gels. Removal of the GST moiety from the protein of interest is accomplished through a thrombin cleavage (10U/ml overnight at 4℃) site located between the GST moiety and the recombinant polypeptide. For solution digestions, GST is easily removed by a second round of

chromatography on the glutathione column. Removal of thrombin is facilitated by the use of a benzamidine-agarose column or a gel-filtration step.

Sandwich Immunoassay

Appropriate antibodies against the His-tagged-KD-LIMK or purified cofilin were coated to the bottom of the EIA/RIA 96-well plate to capture LIMK or cofilin. The plate was then blocked with 1%BSA in PBS for 1 hour. GST tagged wt β-arrestins or GST tagged β-arrestin-1 mutants were incubated and followed by probing with anti-GST conjugated with IR-800. Wash 3 times by 0.01% Triton X-100 in PBS performed between incubations. Integrated intensity were obtained by the Li-Cor odyssey.

Spot peptide array

Spot peptide array experiments were performed by cooperate laboratory, Dr. G. Baillie, University of Glasgow, Scotland. Peptide libraries were produced by automatic SPOT synthesis and synthesized on continuous cellulose membrane supports on Whatman 50 cellulose membrane using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry with the AutoSpot-RobotASS222 (*Intavis Bioanalytical instruments AG*). The interaction of spotted peptides with GST and GST-fusion proteins was determined by overlaying the cellulose membranes with 10μg/mL recombinant protein. Bound recombinant proteins were detected with specific rabbit antisera and detection was performed with secondary anti-rabbit horseradish-peroxidase-coupled antibody (1:2500 dilution) (*Dianova*) and visualization by ECL.

Bioluminescence Resonance Energy Transfer (BRET)

YFP-tagged protein constructs were transiently co-expressed with β-arrestin-1 - luciferase in HEK293T cells. 48 h post-transfection, the cells were treated with appropriate concentrations of 2fAP and 5 μM coelenterazine. Light emission was

detected (460–500 nm for Rluc and 510–550 nm for YFP) using a TRISTAR LB941 multilabel plate reader from Berthold Technologies. BRET signal was calculated as the ratio of the light emitted by eYFP and the light emitted by luciferase. For a negative control, cells transfected with the β-arrestin-1 -luciferase construct alone were used to determine the background. The ratio observed in β-arrestin-1 -luciferase -only-transfected cells was subtracted from that observed in the presence of YFP-tagged receptors to give the net BRET values

REFERENCE

1. Zoudilova, M., Kumar, P., Ge, L., Wang, P., Bokoch, G. M., & DeFea, K. a. (2007). Beta-arrestin-dependent regulation of the cofilin pathway downstream of protease-activated receptor-2. *The Journal of Biological Chemistry*, *282*(28), 20634–46. doi:10.1074/jbc.M701391200

2. Zoudilova, M., Min, J., Richards, H. L., Carter, D., Huang, T., & DeFea, K. a. (2010). beta-Arrestins scaffold cofilin with chronophin to direct localized actin filament severing and membrane protrusions downstream of protease-activated receptor-2. *The Journal of Biological Chemistry*, *285*(19), 14318–29. doi:10.1074/jbc.M109.055806

3. Milano, S. K., Kim, Y.-M., Stefano, F. P., Benovic, J. L., & Brenner, C. (2006). Nonvisual arrestin oligomerization and cellular localization are regulated by inositol hexakisphosphate binding. *The Journal of Biological Chemistry*, *281*(14), 9812–23. doi:10.1074/jbc.M512703200

4. DeFea, K. a, Zalevsky, J., Thoma, M. S., Déry, O., Mullins, R. D., & Bunnett, N. W. (2000). beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *The Journal of Cell Biology*, *148*(6), 1267–81.

5. Luttrell, L. M., & Lefkowitz, R. J. (2002). The role of β - arrestins in the termination and transduction of G-protein-coupled receptor signals. Journal of Cell Science 115, 455-465

6. Nichols, H. L., Saffeddine, M., Theriot, B. S., Hegde, A., Polley, D., & El-mays, T. (2012). β -Arrestin-2 mediates the proin fl ammatory effects of proteinase-activated receptor-2 in the airway, 2–7. doi:10.1073/pnas.1208881109/-/DCSupplemental.www.pnas.org/cgi/doi/10.1073/pnas.1208881109

7. Ge, L., Ly, Y., Hollenberg, M., & Defea, K. (2003). A [illegible] -Arrestin-dependent Scaffold Is Associated with Prolonged MAPK Activation in Pseudopodia during Protease-activated Receptor-2-induced Chemotaxis *, *278*(36), 34418–34426. doi:10.1074/jbc.M300573200

8. DeWire, S. M., Ahn, S., Lefkowitz, R. J., & Shenoy, S. K. (2007). β-Arrestins and Cell Signaling. *Annual Review of Physiology*, *69*(1), 483–510. doi:10.1146/annurev.physiol.69.022405.154749

9. Shukla, A. K., Manglik, A., Kruse, A. C., Xiao, K., Reis, R. I., Tseng, W.-C., Lefkowitz, R. J. (2013). Structure of active β-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature*, *497*(7447), 137–41.

10. Storez, H., Scott, M. G. H., Issafras, H., Burtey, A., Benmerah, A., Muntaner, O. Marullo, S. (2005). Homo- and hetero-oligomerization of beta-arrestins in living cells. *The Journal of Biological Chemistry*, *280*(48), 40210–5. doi:10.1074/jbc.M508001200

11. Xiao, K., McClatchy, D. B., Shukla, A. K., Zhao, Y., Chen, M., Shenoy, S. K., Lefkowitz, R. J. (2007). Functional specialization of beta-arrestin interactions revealed by proteomic analysis. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(29), 12011–6. doi:10.1073/pnas.0704849104

12. Song, X., Raman, D., Gurevich, E. V, Vishnivetskiy, S. a, & Gurevich, V. V. (2006). Visual and both non-visual arrestins in their "inactive" conformation bind JNK3 and Mdm2 and relocalize them from the nucleus to the cytoplasm. *The Journal of Biological Chemistry*, *281*(30), 21491–9. doi:10.1074/jbc.M603659200

13. Edwards, D. C., & Gill, G. N. (1999). Structural features of LIM kinase that control effects on the actin cytoskeleton. *The Journal of Biological Chemistry*, *274*(16), 11352–61. Retrieved from

14. DeFea, K. a. (2011). Beta-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold? *Cellular Signalling*, *23*(4), 621–9. doi:10.1016/j.cellsig.2010.10.004

15. Min, J., & Defea, K. (2011). ᴺL -Arrestin-Dependent Actin Reorganization : Bringing the Right Players Together at the Leading Edge, *80*(5), 760–768. doi:10.1124/mol.111.072470.

16. Chen, Q., Zhuo, Y., Kim, M., Hanson, S. M., Francis, D. J., Vishnivetskiy, S. A., Gurevich, V. V. (2014). Arrestins - Pharmacology and Therapeutic Potential, *219*, 205–223. doi:10.1007/978-3-642-41199-1

17. Parisis, N., Metodieva, G., & Metodiev, M. V. (2013). Pseudopodial and β-arrestin-interacting proteomes from migrating breast cancer cells upon PAR2 activation. *Journal of Proteomics*, *80C*, 91–106.

18. Song, X., Raman, D., Gurevich, E. V, Vishnivetskiy, S. a, & Gurevich, V. V. (2006). Visual and both non-visual arrestins in their "inactive" conformation bind JNK3 and Mdm2 and relocalize them from the nucleus to the cytoplasm. *The Journal of Biological Chemistry*, *281*(30), 21491–9.

19. Oser, M., & Condeelis, J. (2009). The cofilin activity cycle in lamellipodia and invadopodia. *Journal of Cellular Biochemistry*, *108*(6), 1252–62.

20. Lefkowitz, R. J., & Shenoy, S. K. (2005). Transduction of receptor signals by beta-arrestins. *Science (New York, N.Y.)*, *308*(5721), 512–7.

21. Rajagopal, S., Kim, J., Ahn, S., Craig, S., Lam, C. M., Gerard, N. P., … Lefkowitz, R. J. (2010). Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(2), 628–32. doi:10.1073/pnas.0912852107

22. Coffa, S., Breitman, M., Spiller, B. W., & Gurevich, V. V. (2011). A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf1 binding. *Biochemistry*, *50*(32), 6951–8. doi:10.1021/bi200745k

23. LIM-Kinase (LIMK) which inhibits it by phosphorylating serine 3 and by. (2006), *2590*, 2590.

24. Edwards, D. C., & Gill, G. N. (1999). Structural features of LIM kinase that control effects on the actin cytoskeleton. *The Journal of Biological Chemistry*, *274*(16), 11352–61. Retrieved from

25. Kestler, C., Knobloch, G., Tessmer, I., Jeanclos, E., & Gohla, A. (n.d.). Role of chronophin dimerization Role of chronophin dimerization, (4).

26. Lin, T., Zeng, L., Liu, Y., DeFea, K., Schwartz, M. a, Chien, S., & Shyy, J. Y.-J. (2003). Rho-ROCK-LIMK-cofilin pathway regulates shear stress activation of sterol regulatory element binding proteins. *Circulation Research*, *92*(12), 1296–304.

27. Macfarlane, S. R., Seatter, M. J., Kanke, T., Hunter, G. D., & Plevin, R. (2001). Proteinase-activated receptors. *Pharmacological Reviews*, *53*(2), 245–82. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11356985

28. Shenoy, S. K., Barak, L. S., Xiao, K., Ahn, S., Berthouze, M., Shukla, A. K., Lefkowitz, R. J. (2007). Ubiquitination of beta-arrestin links seven-transmembrane receptor endocytosis and ERK activation. *The Journal of Biological Chemistry*, *282*(40), 29549–62. doi:10.1074/jbc.M700852200

29. Zhan, X., Gimenez, L. E., Gurevich, V. V, & Spiller, B. W. (2011). Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual subtypes. *Journal of Molecular Biology*, *406*(3), 467–78. doi:10.1016/j.jmb.2010.12.034

30. Hu, S., Wang, D., Wu, J., Jin, J., Wei, W., & Sun, W. (2013). Involvement of β-arrestins in cancer progression. *Molecular Biology Reports*, *40*(2), 1065–71. doi:10.1007/s11033-012-2148-0

31. McGuire, J. J., M. Saifeddine, et al. (2004). "2-furoyl-LIGRLO-amide: a potent and selective proteinase activated receptor 2 agonist." J Pharmacol Exp Ther 309(3): 1124-31

32. Bockaert, J. and J.P. Pin (1999), *Molecular tinkering of G protein-coupled receptors: an evolutionary success.* EMBO J, 18(7): p. 1723-9.

33. Shenoy, S. K. and R. J. Lefkowitz (2003). "Multifaceted roles of beta-arrestins in the regulation of sevenmembrane-spanning receptor trafficking and signalling." Biochem J 375(Pt 3): 503-15.

34. Shenoy, S. K. and R. J. Lefkowitz (2005). "Seven-transmembrane receptor signaling through beta-arrestin."Sci STKE 2005(308): cm10.

35. Ma, L. and G. Pei (2006). "beta-arrestin signaling and regulation of transcription." Journal of Cell Science 120: 213-218.

36. Eric Reiter., Seungkirl Ahn., Arun K. Shukla.,Robert J.Lefkowitz (2012). "Molecular Mechanism of β-Arrestin-Biased Agonism at Seven-Transmembrane Receptors". Annu. Rev. Pharmacol. Toxicol. 52:179–97

37. Daaka, Y., et al., Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J Biol Chem, 1998. 273(2): p. 685-8.

38. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) "β-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking" Proc.Natl.Acad.Sci U.S.A 98, 1601-1606

39. Vu, T. K., D. T. Hung, et al. (1991). "Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation." Cell 64(6): 1057-68.

40. Hollenberg, M. D. and S. J. Compton (2002). "International Union of Pharmacology. XXVIII. Proteinaseactivated receptors." Pharmacol Rev 54(2): 203-17.

41. McGuire, M. S., C. E. Klimas, et al. (1996). "Development of potent thrombin receptor antagonist peptides." J Med Chem 39(25): 4879-87.

42. Kanke, T., H. Ishiwata, et al. (2005). "Binding of a highly potent protease-activated receptor-2 (PAR2) activating peptide, [H-3]2-furoyl-LIGRL-NH2, to human PAR2." British Journal of Pharmacology 145(2): 255-263

43. Defea, K. (2008). "Beta-arrestins and heterotrimeric G-proteins: collaborators and competitors in signal transduction." Br J Pharmacol 153 Suppl 1: S298-309.

44. DeFea, K. A. (2011). "Beta-arrestins as regulators of signal termination and transduction: how do they determine what to scaffold?" Cell Signal 23(4): 621-9.

45. DeFea, K. A., Z. D. Vaughn, et al. (2000). "The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex." Proc Natl Acad Sci U S A 97(20): 11086-91.

46. Wang, P. and K. A. DeFea (2006). "Protease-activated receptor-2 simultaneously directs beta-arrestin-1-dependent inhibition and Galphaq-dependent activation of phosphatidylinositol 3-kinase." Biochemistry 45(31): 9374-85

47. Wang, P., Y. Jiang, et al. (2010). "Beta-arrestin inhibits CAMKKbeta-dependent AMPK activation downstream of protease-activated-receptor-2." BMC Biochem 11: 36.

48. Wang, P., P. Kumar, et al. (2007). "Differential regulation of class IA phosphoinositide 3-kinase catalytic subunits p110 alpha and beta by protease-activated receptor 2 and beta-arrestins." Biochem J 408(2): 221-30

49. Luttrell, L. M. (2003). "'Location, location, location': activation and targeting of MAP kinases by G protein-coupled receptors." J Mol Endocrinol 30(2): 117-26.

50. Luttrell, L. M., S. S. Ferguson, et al. (1999). "Beta-arrestin-dependent formation of beta 2 adrenergic receptor-Src protein kinase complexes." science 283(5402): 655-61.

51. Luttrell, L. M., F. L. Roudabush, et al. (2001). "Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds." Proc Natl Acad Sci U S A 98(5): 2449-54.

52. Luttrell, L. M., F. L. Roudabush, et al. (2001). "Activation and targeting of extracellular signal-regulated kinases by β-arrestin scaffolds." Proceedings of the National Academy of Sciences 98(5): 2449-2454.

53. Okano I, Hiraoka J, Otera H, Nunoue K, Ohashi K, Iwashita S, Hirai M, Mizuno K (1995) Identification and characterization of a novel family of serine/threonine kinases containing two Nterminal LIM motifs. J Biol Chem 270:31321–31330

54. Tomiyoshi G, Horita Y, Nishita M, Ohashi K, Mizuno K (2004) Caspase-mediated cleavage and activation of LIM-kinase 1 and its role in apoptotic membrane blebbing. Genes Cells 9:591–600

55. Hiraoka J, Okano I, Higuchi O, Yang N, Mizuno K (1996) Selfassociation of LIM-kinase 1 mediated by the interaction between an N-terminal LIM domain and a C-terminal kinase domain. FEBS Lett 399:117–121

56. Rebecca W. Scott & Michael F. Olson (2007) LIM kinases: function, regulation and association with human disease. J Mol Med (2007) 85:555–568 DOI 10.1007/s00109-007-0165-6

57. Han, M., V. V. Gurevich, et al. (2001). "Crystal Structure of -Arrestin at 1.9 A°: Possible Mechanism of Receptor Binding and Membrane Translocation." structure 9: 869-890

58. Vishnivetskiy, S. A., M. M. Hosey, et al. (2004). "Mapping the arrestin-receptor interface. Structural elements responsible for receptor specificty of arrestin proteins." J. of Biol. Chem. 279(2): 1262-1268.

59. Jose Javier Bravo-Cordero, Marco A. O. Magalhaes, Robert J. Eddy, Louis Hodgson & John Condeelis (2013). Functions of cofilin in cell locomotion and invasion. Nature Reviews Molecular Cell Biology 14, 405–415

60. Hoogenraad CC, Akhmanova A, Galjart N, De Zeeuw CI. (2004) LIMK1 and CLIP-115: linking cytoskeletal defects to Williams syndrome. Bioessays;26(2):141-50.

61. Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron. 35(1):121-33.

62. Li, R., J. Soosairajah, et al. (2006). "Hsp90 increases LIM kinase activity by promoting its homodimerization." The FASEB Journal 20(8): 1218-1220.

63. Shukla, A. K., K. Xiao, et al. (2011). "Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling." Trends in Biochemical Sciences 36(9): 457-469.

64. Xu, T. R., G. S. Baillie, et al. (2008). "Mutations of beta-arrestin 2 that limit self-association also interfere with interactions with the beta2-adrenoceptor and the ERK1/2 MAPKs: implications for beta2-adrenoceptor signalling via the ERK1/2 MAPKs." Biochem J 413(1): 51-60.

65. Zang, M., J. Gong, et al. (2008). "Characterization of Ser338 Phosphorylation for Raf-1 Activation." Journal of Biological Chemistry 283(46): 31429-31437.

66. Van Troys, M., L. Huyck, et al. (2008). "Ins and outs of ADF/cofilin activity and regulation." Eur J Cell Biol 87(8-9): 649-67.

67. Terrillon, S. and M. Bouvier (2004). "Receptor activity-independent recruitment of [beta]arrestin2 reveals specific signalling modes." EMBO J 23(20): 3950-3961.

68. Tohgo, A., E. W. Choy, et al. (2003). "The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation." J Biol Chem 278(8): 6258-67.

69. Takeuchi, T., J. L. Harris, et al. (2000). "Cellular localization of membrane-type serine protease 1 and identification of protease-activated receptor-2 and single-chain urokinase-type plasminogen activator as substrates." J Biol Chem 275(34): 26333-42.

70. Stefansson, K., M. Brattsand, et al. (2008). "Activation of proteinase-activated receptor-2 by human kallikrein-related peptidases." J Invest Dermatol 128(1): 18-25.

71. Rajagopal, K., E. J. Whalen, et al. (2006). "Beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes." Proc Natl Acad Sci U S A 103(44): 16284-9.

72. Rajagopal, S., J. Kim, et al. (2010). "Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7." Proc Natl Acad Sci U S A 107(2): 628-32.

73. Ma, L. and G. Pei (2006). "beta-arrestin signaling and regulation of transcription." Journal of Cell Science 120: 213-218.

74. Yildirim, M., W. G. M. Janssen, et al. (2008). "Estrogen and aging affect synaptic distribution of phosphorylated LIM kinase (pLIMK) in CA1 region of female rate hippocampus." Neuroscience 152: 360-370.

75. Wu, N., S. M. Hanson, et al. (2006). "Arrestin Binding to Calmodulin: A Direct Interaction Between Two Ubiquitous Signaling Proteins." Journal of Molecular Biology 364(5): 955-963.

76. Willoughby, E. A. and M. K. Collins (2005). "Dynamic interaction between the dual specificityphosphatase MKP7 and the JNK3 scaffold protein beta-arrestin 2." J Biol Chem 280(27): 25651-8

77. Gurevich, V.V., E.V. Gurevich, and W.M. Cleghorn, Arrestins as multi-functional signaling adaptors. Handb Exp Pharmacol, 2008(186): p. 15-37.

78. Ge, L., et al., Constitutive protease-activated receptor-2-mediated migration of MDA MB-231 breast cancer cells requires both beta-arrestin-1 and -2. J Biol Chem, 2004. 279(53): p. 55419-24.

79. Luan, B., et al., Deficiency of a beta-arrestin-2 signal complex contributes to insulin resistance. Nature, 2009. 457(7233): p. 1146-9.

80. Pearson, G., et al., Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev, 2001. 22(2): p. 153-83.

81. Laporte, S.A., et al., The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. Proc Natl Acad Sci U S A, 1999.96(7): p. 3712-7.