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The Diverse Roles of Arrestin Scaffolds in G Protein–Coupled Receptor Signaling

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Abstract—The visual/ β -arrestins, a small family of proteins originally described for their role in the desensitization and intracellular trafficking of G protein-coupled receptors (GPCRs), have emerged as key regulators of multiple signaling pathways. Evolutionarily related to a larger group of regulatory scaffolds that share a common arrestin fold, the visual/ β -arrestins acquired the capacity to detect and bind activated GPCRs on the plasma membrane, which enables them to control GPCR

desensitization, internalization, and intracellular trafficking. By acting as scaffolds that bind key pathway intermediates, visual/ β -arrestins both influence the tonic level of pathway activity in cells and, in some cases, serve as ligand-regulated scaffolds for GPCR-mediated signaling. Growing evidence supports the physiologic and pathophysiologic roles of arrestins and underscores their potential as therapeutic targets. Circumventing arrestin-dependent GPCR desensitization may alleviate

ABBREVIATIONS: AKT, protein kinase B; AP-2, adapter protein-2; ARDC, arrestin domain-containing protein; ASK, apoptosis signal regulating kinase; BRET, bioluminescence resonance energy transfer; CML, chronic myelogenous leukemia; cPLA2, cytosolic phospholipase A2; CXCR, CXC chemokine receptor; Dsh, dishevelled; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FIAsh, fluorescent aresinical hairpin; FRET, fluorescence resonance energy transfer; Fz, Frizzled; GAP, GTPase-activating protein; GDS, guanine nucleotide dissociation stimulator; GEF, guanine nucleotide exchange factor; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; GSK3 β , glycogen synthase kinase 3 β ; ICL, intracellular loop; IL, interleukin; iNOS, inducible nitric oxide synthase; IP6, inositol hexakisphosphate; JNK, c-Jun N-terminal kinase; LEF, lymphoid enhancer factor; LH, luteinizing hormone; LPA, lysophosphatidic acid; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK/ERK kinase; mGluR, metabotropic glutamate receptor; MKK, MAPK kinase; MOR, μ opioid receptor; NES, nuclear export sequence; NF κ B, nuclear factor κ B; NHE, Na⁺/H⁺ exchanger; NLS, nuclear localization sequence; NSF, N-ethylmaleimide-sensitive fusion protein; PAR, protease-activated receptor; PcG, polycomb group; PDB, Protein Data Bank; PDE, phosphodiesterase; PDK1, 3-phosphoinositide dependent protein kinase-1; PDZ, postsynaptic density protein of 95 kDa, disc large, zona occludens-1; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP, protein phosphatase; PPAR, peroxisome proliferator-activated receptor; PTEN, phosphatase and tensin deleted on chromosome 10; PTH, parathyroid hormone; RXR, retinoic acid receptor; SH, Src homology; Shh, Sonic hedgehog; SHP, Src homology region 2 domain-containing phosphatase; SII, [Sar1,Ile4,Ile8]-angiotensin II; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; SpoOM, sporulation stage 0, protein M; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor; TM, transmembrane domain; TNF- α , tumor necrosis factor- α ; TP, thromboxane prostanoid; TRAF, TNF receptor-associated factor; TXNIP, thioredoxin-interacting protein; USP33, ubiquitin-specific protease 33; VPS, vacuolar protein sort.

the problem of tachyphylaxis to drugs that target GPCRs, and find application in the management of chronic pain, asthma, and psychiatric illness. As signaling scaffolds, arrestins are also central regulators of pathways controlling cell growth, migration, and survival, suggesting that manipulating their scaffolding functions may be beneficial in inflammatory diseases, fibrosis, and cancer. In this review we examine the structure–function

relationships that enable arrestins to perform their diverse roles, addressing arrestin structure at the molecular level, the relationship between arrestin conformation and function, and sites of interaction between arrestins, GPCRs, and nonreceptor-binding partners. We conclude with a discussion of arrestins as therapeutic targets and the settings in which manipulating arrestin function might be of clinical benefit.

I. Introduction

The complexity of coordinated cell signaling has necessitated the evolution of scaffold proteins whose role is to control the activity of cellular processes driven by receptors, enzymes, and channels. Scaffolds, proteins or protein domains that themselves lack intrinsic catalytic activity, perform three basic functions: to increase the efficiency of information transfer between successive enzymes in a signaling cascade; to enhance fidelity by dampening crosstalk between parallel cascades; and to target effectors to specific subcellular locations. The true arrestins, consisting of two retinal isoforms, visual arrestin (arrestin1) and cone arrestin (arrestin4), and two nonvisual arrestins, β -arrestin1 (arrestin2) and β -arrestin2 (arrestin3), belong to a superfamily of structurally and functionally related scaffolding proteins that trace their origins to prokaryotes and occur in all eukaryotes except plants (Ferguson, 2001; Alvarez, 2008). Depending on the type of cell and its metabolic state, arrestin family proteins can be found distributed diffusely in the cytosol, bound to the cytoskeleton, concentrated at the centrosome, coating internalizing endosomes, and inside the nucleus. These many pools of arrestin are integral to the control of cell metabolism, division, motility, and cross-talk and are adapted to provide diverse but highly specific signal integration.

What distinguishes the visual/ β -arrestins from other arrestin-like proteins is their capacity to interact with activated heptahelical G protein–coupled receptors (GPCRs). Upon ligand binding, G protein–coupled receptor kinases (GRKs) phosphorylate agonist-occupied receptors on serine or threonine residues within the C terminus or third intracellular loop, creating high-affinity arrestin binding sites. There are seven known GRKs, of which GRK1 and 7, like visual and cone arrestin, are confined to visual sensory tissue, whereas GRK2, 3, 5, and 6, along with β -arrestin1 and 2, are widely expressed (Stoffel et al., 1997). Arrestin binding stabilizes a high agonist affinity state of the receptor, similar to the complex existing between agonist, receptor, and heterotrimeric G protein in the absence of GTP (De Lean et al., 1980; Gurevich et al., 1997). The traditional view is that once bound to arrestin, GRK-phosphorylated GPCRs on the plasma membrane are precluded from G protein coupling, leading to homologous desensitization, the process whereby G protein signaling by agonist-occupied receptors is selectively

dampened (Ferguson, 2001). β -Arrestin1 and 2 further attenuate G protein signaling by linking receptors to the clathrin-dependent endocytic machinery. The β -arrestin C terminus directly binds clathrin heavy chain and the β 2 adaptin subunit of the adapter protein-2 (AP-2) complex (Goodman et al., 1996; Krupnick et al., 1997; Laporte et al., 1999, 2000), causing β -arrestin-bound receptors to cluster in clathrin-coated pits. This β -arrestin-dependent endocytosis, or sequestration, removes receptors from the cell surface, rendering it less responsive to subsequent stimuli. Once inside, the stability of the GPCR– β -arrestin complex determines whether receptors resensitize and recycle to the cell surface or are degraded, with receptors that form transient receptor– β -arrestin complexes undergoing rapid resensitization and recycling back to the plasma membrane, whereas receptors that form more stable complexes either recycle slowly or are targeted for degradation (Oakley et al., 2000, 2001).

The capacity to recognize and bind activated GPCRs is what places the scaffolding functions of visual/ β -arrestins under the control of environmental cues delivered in the form of extracellular hormones. As a result, they can play both silent scaffolding roles, binding and sequestering key signaling pathway intermediates away from potential regulators, and stimulus-dependent scaffolding roles in the positive and negative regulation of GPCR signaling (Luttrell and Gesty-Palmer, 2010; Breitman et al., 2012; Lin and Defea, 2013). Besides clathrin and AP-2, the list of proteins that have been reported to bind arrestins includes Src family tyrosine kinases (Luttrell et al., 1999; Barlic et al., 2000; DeFea et al., 2000a), components of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) and c-Jun N-terminal kinase (JNK)3 mitogen-activated protein (MAP) kinase cascades (DeFea et al., 2000b; McDonald et al., 2000; Luttrell et al., 2001), the Ser/Thr protein phosphatase (PP)2A (Beaulieu et al., 2005), E3 ubiquitin ligases and deubiquitinases (Shenoy et al., 2001, 2008, 2009), second-messenger degrading cAMP phosphodiesterases (PDE) (Perry et al., 2002) and diacylglycerol kinase (Nelson et al., 2007), elements of the nuclear factor κ B (NF κ B) signaling pathway (Wetherow et al., 2004), and regulators of small GTPase activity (Claing et al., 2001; Bhattacharya et al., 2002). It is the interaction with this diverse set of partners that positions arrestins as

critical regulators of GPCR signal transduction and permits them to integrate GPCR-mediated signals with other inputs. In this review, we examine the structure–function relationships that enable arrestins to perform their diverse roles, addressing arrestin structure at the molecular level, the relationship between arrestin conformation and function, and sites of interaction between arrestins, GPCRs, and nonreceptor-binding partners. We conclude with a discussion of arrestins as therapeutic targets, and the settings in which manipulating arrestin function might be of clinical benefit.

II. Arrestin Structure and Function

A. The Taxonomy of Arrestins

In mammals, the extended family of arrestin-like proteins is composed of at least 12 proteins that employ a conserved protein fold to coordinate the temporal and spatial aspects of multiple processes, particularly those related to endosome trafficking, vesicle sorting, and signaling (de Mendoza et al., 2014). Various synonyms for visual/ β -arrestins appear in the literature: Visual arrestin (Gene Symbol: SAG) is also called arrestin-1, S-antigen, 48 kDa protein, or rod arrestin; β -arrestin1 (Gene Symbol: ARRB1) is also called β -arrestin or arrestin-2; β -arrestin2 (Gene Symbol: ARRB2) is also called arrestin-3 and hTHY-ARRX; and cone arrestin (Gene Symbol: ARR3) is also called arrestin-4, arrestin-C, or X-arrestin. The tree that gave rise to arrestins traces its roots to prokaryotes (Alvarez, 2008). The progenitor arrestin-like proteins in *Archaea* are known as the sporulation stage 0, protein M (SpoOM) family, a group of proteins that function as integrators of lipid/nutrient internalization and whose loss or overexpression blocks sporulation (Han et al., 1998; Birko et al., 2009). Eukaryotic arrestins appear to arise from the SpoOM lineage, later diverging into two major families: the α -arrestins, consisting of the arrestin domain-containing proteins (ARRDCs), thioredoxin-interacting protein (TXNIP), and vacuolar protein sort (VPS) 26, which coordinate enzyme activity largely for membrane trafficking; and the visual/ β -arrestins, which possess the ability to interact with GPCRs (Aubry and Klein, 2013).

The eukaryotic α -arrestins appear to have evolved from a single progenitor. The function of the five mammalian ARRDCs is not well understood. ARRDC1–4, along with TXNIP, are known to interact with several E3 ubiquitin ligases and promote the ubiquitination of associated cargo proteins. For example, ARRDC3 binds activated β -4 integrin and controls its ubiquitination, endocytosis, and degradation (Becuwe et al., 2012). ARRDC3 has been reported to recruit the E3 ligase NEDD4 to regulate ubiquitination and endocytosis of the β 2-adrenergic receptor, although other authors attribute this function to the visual/ β -arrestin, β -arrestin2

(Nabhan et al., 2010; Han et al., 2013). ARRDC1–4 and TXNIP also interact with subunits of the endosomal sorting complexes required for transport machinery. Consistent with a general role in vesicle trafficking, ARRDC4 and TXNIP inhibit glucose uptake, suggesting a role in endosomal trafficking of glucose transporters (Patwari et al., 2009). TXNIP also controls cellular redox state by binding and inhibiting thioredoxin (World et al., 2011; Spindel et al., 2014; Yoshihara et al., 2014). The VPS proteins are critical components of the retromer complex that sorts and traffics endosomes to the Golgi to recycle cell surface receptors and other proteins (Haft et al., 2000; Seaman, 2004). One of the three VPS proteins, VPS26, is an arrestin-fold protein (Shi et al., 2006). VPS26 engages postsynaptic density protein of 95 kDa, disc large, zona occludens-1 (PDZ) domain-containing proteins like the sorting nexin, SNX27, to regulate intracellular protein trafficking. The VPS26–SNX27 interaction promotes cooperative binding between the SNX27 PDZ domain and PDZ-binding motifs on cargo proteins (Gallon et al., 2014).

The oldest visual/ β -arrestin in our current genetic record appears in cnidarians. Cnidarians developed ocelli in the absence of a nervous system, most likely to sense the diurnal cycle for feeding and simple navigation (Nordstrom et al., 2003). GPCRs and visual/ β -arrestins evolved very early and in tandem in the earliest eukaryotes (de Mendoza et al., 2014). Given that α -arrestins and visual/ β -arrestins share roles in vesicle trafficking, one might speculate that the shared functions originated first, with the visual/ β -arrestin offshoot gaining the added property of GPCR recognition. The GPCR-regulated arrestins diverged from the rest of the clade very early in arrestin evolution. The visual arrestins appear to have evolved first and given rise to the β -arrestins. Visual arrestin has very high affinity and specificity for the rhodopsin GPCR, whereas cone arrestin has lower affinity and selectivity for rhodopsin and behaves much more like the β -arrestins. This has been postulated as an intrinsic mechanism to speed up rhodopsin recycling to increase photosensitivity and rapid adaptation to changing light conditions (Sutton et al., 2005).

The total sequence drift between the visual and β -arrestins is relatively small, whereas the greatest divergence is between α - and β -arrestins and the entire ARRDC clade (Fig. 1A) (Aubry et al., 2009). Although the visual and β -arrestins form a tight family of GPCR-interacting proteins, the α -arrestins and VPS26 proteins have more divergent sequences and functions from each other. Analysis of the arrestin identity matrix shows the β -arrestins, α -arrestins, and VPS26 proteins as three distinct families (Fig. 1B). However, ARRDP1 and ARRDP5 are quite distinct from all the other arrestins, having a maximum of 20% similarity to any other arrestin. It appears that all the arrestins should

Fig. 1. Evolutionary relationship of proteins sharing the common arrestin fold. (A) Radial phylogram showing the relation of all mammalian arrestins to the archaea arrestin-like molecule SpoOM. It also shows the relation to other mammalian arrestin family members, including the VPS and TXNIP proteins, which suggest that arrestins evolved from a mechanism to regulate environmental sensing toward more complicated signaling. NCBI BLAST was queried using the human β -arrestin2 full-length sequence. Psi-BLAST was used to search the RefSeq database. The sequence analysis was performed using Fitch phylogenetic tree analysis implemented in BioEdit 7.2.5 and visualized using Dendroscope 3.2.4. This graph was inspired from (Alvarez, 2008; Aubry et al., 2009). (B) Sequence identity matrix indicating the overall similarity of human arrestins. The analysis was performed using BLOSUM62 implemented in BioEdit 7.2.5. Cells were colored as a heat map using EXCEL according to percent similarity, with green being most similar and red being divergent. (C) Rectangular phylogram showing the relation of mammalian β -arrestin2 isoforms across several species. It demonstrates an expansion of diversity in humans and mice that may be indicative of the need to regulate complex hormone signaling and olfaction. The sequence analysis was performed using Fitch phylogenetic tree analysis implemented in BioEdit 7.2.5 and visualized using Dendroscope 3.2.4. (D) Structural comparison of β -arrestin, VPS26, and TXNIP showing different rotations between the amino and carboxy arrestin domains. VPS26 β -baskets are nearly symmetrical and on the same plane, and β -arrestins show a compaction of the amino-terminal domain and a slight rotation between domains, whereas TXNIP has an almost 180° degree inversion of the relative position of the β -baskets. Structural PDB files (2WTR, 4P2A, and 4LL4) were superposed using only the amino-terminal domain to highlight the relative differences in the carboxy domain. Gray half circles were added to show the relative positions of the concave portion of the two β sandwiches per molecule. Analysis and visualization were performed using MOE 2014.09.

have the capacity to translocate, although translocation is not required for all of their known functions. The β -arrestins may be the only members of the clade whose functions are dependent upon translocation between multiple cellular compartments.

Illustrating the degree to which β -arrestins have evolved as specialized GPCR regulators, a close look at the β -arrestin2 phylogeny in mammals shows a distinct speciation and branching of β -arrestin2 between rodents and humans (Fig. 1C). Compared with 1783 full-length genes encoding GPCRs in mice,

there are only 799 in humans, with the bulk of the additional 1000 murine genes encoding olfactory receptors. It is thus tempting to speculate that there was evolutionary pressure on β -arrestins to provide for more nuanced regulation of environmental sensing GPCRs in rodents (Gloriam et al., 2007). The analysis supports the conclusion that human olfaction is very distinct from rodents and that GPCR and β -arrestin evolution are tightly linked and tied to the environmental necessities of each species (Haitina et al., 2009).

B. Arrestin Structure

In a general sense, all arrestins are semi-bisymmetric soluble proteins that link plasma membrane-initiated signaling events to intracellular responses. Like most reversible signaling interactions, the affinities are relatively weak, allowing for more dynamic temporal scaffolding. A substantial body of X-ray crystallographic structural data is available for the visual/ β -arrestins (Table 1), whereas the α -arrestins are represented by TXNIP and VPS26. The conserved arrestin fold that defines the family consists of a semi-symmetric fold of 20 β strands condensing into two β strand sandwich structures, forming two baskets connected by a short hinge domain (Fig. 1D) (Vishnivetskiy et al., 2002; Aubry et al., 2009).

A number of domains important to arrestin function have been characterized, some universal, others unique to visual/ β -arrestins. There are three major recognized differences between the α - and visual/ β -arrestins. Visual/ β -arrestins possess an N-terminal helix (R⁹⁹LQERLI¹⁰⁵ in bovine β -arrestin2) that has not appeared in α -arrestin structures and whose sequence is unique to visual/ β -arrestins (Sutton et al., 2005) (Fig. 2). The C terminus of arrestins exhibits a switch from the α -arrestin PPPxYS motif to the β -arrestin DDIVFE motif. Note that arrestin1 and VPS26 diverge from the rest of their respective cohort in this region. Finally, the relative rotations of the two baskets are unique, implying different activation dynamics between α - and visual/ β -arrestins (Fig. 1D). In contrast, the polar core is conserved between α - and visual/ β -arrestins, suggesting that interaction with GPCRs or GPCR-like motifs is central in propagating structural changes. TXNIP has highest sequence identity with the α -arrestins but structurally is most closely related to VPS26 proteins, with the greatest similarity in the N domain. Like visual/ β -arrestins, TXNIP has 20 β sheets divided into N and C domain concave baskets and a polar core. However, the N- and C-terminal domains of TXNIP are rotated away from each other (Polekhina et al., 2013; Hwang et al., 2014). Superposing a β -arrestin1 structure [Protein Data Bank (PDB) file: 2WTR] with TXNIP (PDB file: 4LL4), there is a 15.2 Å root mean square deviation, whereas superposing a VPS26 (PDB file: 2FAU) structure with full-length TXNIP (PDB file: 4LL4) gives a 19.1 Å root mean square deviation. Additionally, although in visual/ β -arrestins the inside of both of the baskets has a positive charge, in TXNIP the N domain is negative, whereas the C domain is positive.

Proceeding from the N to C terminus of visual/ β -arrestins, conserved structural elements include the N-terminal arm, Motif II, the phosphate sensor, the hinge domain, the gate loop/polar core, and the C-terminal arm that contains several protein-protein interaction motifs (Figs. 2 and 3, A–C).

1. N-Terminal Arm. Residues in the N terminus (V¹¹I¹²F¹³ of bovine visual arrestin) are involved in stabilizing arrestin conformation, whereas the nearby residues K¹⁴ and K¹⁵ have been implicated in phosphate binding (Vishnivetskiy et al., 2000). Alanine substitution of residues 11–13 permits visual arrestin to bind to light-activated rhodopsin independent of receptor phosphorylation state, indicating a role in stabilizing the inactive conformation. Conversely, substitution of residues 14 and 15 dramatically impairs visual arrestin binding to phosphorylated light-activated rhodopsin, suggesting that the two lysines help direct receptor-attached phosphates toward the phosphate sensor and participate in phosphate binding in the active state.

2. Finger Loop/Motif II. The next characterized subdomain is motif II, located in the finger loop, the uppermost loop (visual arrestin residues 68–79) on the N-terminal side of the hinge domain (Granzin et al., 1998). The conformational flexibility of motif II has been shown to be important for high-affinity receptor binding (Dinculescu et al., 2002; Vishnivetskiy et al., 2004). Spin-labeling and fluorescence quenching data indicate that in the unbound state motif II makes close contact with the arrestin N domain, but, when bound to phosphorylated light-activated rhodopsin, it is extended and buried in the rhodopsin-arrestin interface (Hanson et al., 2006a; Sommer et al., 2007). The crystal structure of p44 arrestin (Arr1-370A; PDB file: 3UGX), a naturally occurring splice variant with a truncation of the inhibitory C terminus that can terminate phototransduction by binding to nonphosphorylated light-activated rhodopsin (Schroder et al., 2002), indicates that that rearrangement of motif II is a major change associated with constitutive arrestin activity (Granzin et al., 2012). Similarly, a crystal structure of light-activated rhodopsin bound to a peptide analog of the finger loop of visual arrestin (PDB file: 4PXF) shows that the finger loop engages a binding crevice on the surface of the activated receptor in much the same manner as the Gat C terminus (Szczeppek et al., 2014). Engaging the receptor stabilizes the finger loop structure in the form of a reverse turn.

3. Phosphate Sensor. Because visual/ β -arrestin function depends on its ability to detect a phosphorylated GPCR on the plasma membrane, the location of the phosphate sensor has been a major area of interest (Palczewski et al., 1991b; Gurevich and Benovic, 1993, 1997; Vishnivetskiy et al., 2011; Gurevich and Gurevich, 2013; Gimenez et al., 2014a). Although all visual/ β -arrestins can bind to unphosphorylated GPCR C-terminal peptides, different arrestin-GPCR pairs vary in their affinities for nascent versus phosphorylated receptor. Visual arrestin binding to rhodopsin is highly dependent on phosphorylation, leading to a rapid dissociation rate when bound to unphosphorylated light-activated rhodopsin, whereas β -arrestin binding to GPCRs is less phosphorylation-dependent, relying on

TABLE 1
Exemplary arrestin PDBs and structural form

PDB File	Structural Form	Arrestin State	References
α-Arrestins			
4GEJ/4GEI	TXNIP	N-terminal domain	Polekhina et al., 2013
4LL4/4LL1/4GFX	TXNIP-TRX complex	Bimolecular complex	Hwang et al., 2014
2FAU	VPS26	Monomer	Shi et al., 2006
4P2A	VPS26A-SNX27 PDZ domain	Bimolecular complex	Gallon et al., 2014
Visual/β-arrestins			
1AYR	Bovine rod arrestin1	Homotetramer	Granzin et al., 1998
1CF1	Bovine rod arrestin1	Monomer	Hirsch et al., 1999
3UGX/3UGU	Bovine p44 arrestin1	Monomer	Granzin et al., 2012
4J2Q	Bovine p44 arrestin1	Active conformation	Kim et al., 2013
1SUJ	<i>Ambystoma tigrinum</i> cone arrestin	Monomer	Sutton et al., 2005
1VQX/1NZS	Bovine arrestin1-rhodopsin C terminus	Monomer	Kisselev et al., 2004a,b
4PXF	Bovine arrestin1(67–77) –retinal-free rhodopsin	Bimolecular complex	Szczepek et al., 2014
4ZWJ	T4 lysozyme-rhodopsin–arrestin1 chimera	Monomer	Kang et al., 2015
1G4M	Bovine β -arrestin1	C-terminal truncation	Han et al., 2001
2WTR	Bovine β -arrestin1	Dimer	N/A
1JSY	Bovine β -arrestin2	Monomer	Milano et al., 2002
3P2D	Bovine β -arrestin2	Monomer	Zhan et al., 2011a
1ZSH	Bovine β -arrestin1–inositol hexakisphosphate	Bimolecular complex	Milano et al., 2006
3GC3	Bovine β -arrestin1(1–385)-clathrin	Bimolecular complex	Kang et al., 2009
4JQ1	Rat β -arrestin1-V2 receptor phosphopeptide-Fab	Trimolecular complex	Shukla et al., 2013
2IV8	Human β -arrestin2(317–410)-AP2 β -appendage	Bimolecular complex	Schmid et al., 2006

contact with nonphosphorylated receptor elements to add stability to the interaction (Vishnivetskiy et al., 2011). The primary site of phosphorylated receptor interaction is on the positively charged concave surface of the two arrestin β -baskets. Phosphate-binding residues have been localized to three segments of visual arrestin, including residues 68–78, 170–182, and 330–348 (Mokarzel-Falcon et al., 2008). Interestingly, the two β -baskets are not symmetrical, and the majority of direct receptor affinity appears to lie in the N-terminal basket. The phosphate sensor, which is integral to controlling the polar core, is designed to be a charge-operated trigger, with R¹⁷⁵ and other amino acids within the 158–185 region driving arrestin activation through propagating structural rearrangements (Gurevich and Benovic, 1993; Kieselbach, et al., 1994; Vishnivetskiy et al., 2000; Han et al., 2001; Nobles et al., 2011). Mutations that destabilize the polar core by disrupting the phosphate sensor, e.g., R¹⁶⁹E and R¹⁷⁵D/E, or removal of the auto-inhibitory C terminus, e.g., D³⁸³STOP, generate constitutively active arrestins that lose the ability to recognize receptor phosphorylation while retaining selectivity for the ligand-activated receptor conformation (Gurevich and Benovic, 1997; Kovoor et al., 1999).

4. Hinge Domain. The arrestin hinge domain is formed by the conjunction of N- and C-terminal loops from each half of the protein that come together to create an area of regulated flexibility. The hinge region is defined primarily as the loops from motifs I and IV (Figs. 2 and 3, A–C) (Vishnivetskiy et al., 2002). The entire midsection of the arrestin molecule, which contains the polar core and the hinge, is composed of three loops from the N-terminal half and two loops from the C-terminal half of the upper surface, along with a series of crossing peptide sequences from the bottom face of

the protein. This interface between the N and C domain is involved in interactions with the arrestin N-terminal helix, GPCR domains, and microtubules.

5. Gate Loop and Polar Core. The polar core is essential to the activation of arrestin. In visual arrestin, rearrangement of the polar core is controlled primarily by the gate loop (D²⁹⁶-N³⁰⁵), also known as the lariat loop (residues 282–309) (Han et al., 2001; Kim et al., 2013). The gate loop is so named because of its ability to stabilize the polar core using charge pair atoms from aspartic acid that interact with arginine in the polar core. The arrestin C terminus (residues 384–391) interacts with both the N terminus and areas on the C-terminal side of the hinge domain, stabilizing the inactive arrestin molecule to prevent structural rearrangements and blocking effector and specific hydrophobic binding sites (Gurevich and Benovic, 1993). Receptor binding displaces the arrestin C terminus, destabilizing the core and exposing new receptor interaction sites (Kim et al., 2013). A salient difference between visual arrestin and the nonvisual β -arrestins is that the interdomain hydrogen bonds that stabilize the polar core are weakened in β -arrestins, such that the hydrogen bond networks in β -arrestin1 and 2 resemble that seen in the preactivated p44 arrestin1 structure (Granzin et al., 2012; Kim et al., 2013). This suggests the nonvisual arrestins may exist in a partially preactivated state, accounting for their lower dependence upon receptor phosphorylation compared with visual arrestin (Gurevich et al., 1995).

6. C-Terminal Arm. The major role of the arrestin C-terminal arm is to work with the gate loop to stabilize the inactive or closed conformation of arrestin (Shukla et al., 2013). Additionally, the very C-terminal region is the area of highest divergence among the arrestin superfamily, indicating that this region has adapted to

Fig. 2. Amino acid sequence alignment of arrestins indicating extensions, insertions, deletions, and functional domains among the arrestin clade. Domains depicted include the N- and C-terminal arms, Motif I-IV, NES, three NLS, SH3 domain, four PPxY motifs, clathrin binding domain (CBD), and the middle, C, and Lariat loops. The analysis was performed using ClustalW multiple alignment analysis implemented in BioEdit 7.2.5. Amino acids are colored according to their chemical properties, and conserved consensus residues are colored filled.

their divergent functions. The α -arrestins ARRD1 and ARRD3 possess a PPxY motif within the C terminus that confers binding to WW proteins like the E3 ubiquitin ligase NEDD4 (Nabhan et al., 2010; Puca

et al., 2013). In the nonvisual arrestins, the C terminus contains clathrin and AP-2-binding motifs that are essential for supporting GPCR endocytosis. Residues 89–100 of the clathrin heavy chain bind C-terminal

Fig. 3. Visual/ β -arrestin topologic structural analysis showing the overall tertiary fold of arrestins, the charge distribution surface, and the major functional and interaction domains. (A) Ribbon diagram indicating arrestin folding from N terminus (blue) to C terminus (red). (B) Surface diagram indicating the positive (red) and negative (blue) charge regions. (C) Functional domain diagram showing areas of functional importance from X-ray and mutagenesis studies. Domains are colored such that red regions are involved in receptor binding, green regions are involved in oligomerization, blue are important in arrestin activation, and yellow regions interact with microtubules. Analysis and visualization were performed using MOE 2014.0. (D) Comparison of multiple X-ray crystal structures of β -arrestins shows plasticity and signaling diversity. The images show large conformational rearrangements localized to the outer loops, and the hinge domain proximal to the N-terminal domain. Note the disordered and unresolved loops present in the bottom of each image corresponding to the beginning of the C-terminal arm containing the CBD, Motif V, and RxR motifs. Topologic flexible regions of arrestin from PDB files 2WTR, 3GD1, 3GC3, 1AYR.A, and 1AYR.B. The two images are rotated 180° to each other to show the amino or carboxy domains. Structures were aligned and superposed using all carbon α atoms. Each chain has a unique color. Analysis and visualization were performed using MOE 2014.09.

residues 373–377 in β -arrestin2, which comprise a conserved LIEFE/LD motif shared by the β -arrestins (Goodman et al., 1996; Krupnick et al., 1997). This same region of β -arrestin2 contains an RxR motif that binds the β 2-adaptin subunit of the AP-2 complex and promotes GPCR clustering in clathrin-coated pits (Laporte et al., 2000; Kim and Benovic, 2002; Schmid et al., 2006).

C. Arrestin Activation

Binding of a visual/ β -arrestin to the phosphorylated intracellular domains of an activated GPCR induces conformational rearrangements that enable it to perform its extracellular ligand-regulated scaffolding functions. Both static structural data from X-ray crystallography and dynamic data obtained using spin labels and resonance energy transfer probes offer insights into how arrestins are activated and how different receptors are able to initiate different arrestin functions.

Arrestin activation commences upon interaction with receptor-attached phosphates, a step that displaces the arrestin C terminus and destabilizes the polar core, priming the arrestin for a conformational rearrangement that enables high-affinity receptor binding (Schleicher et al., 1989; Schroder et al., 2002; Kirchberg et al., 2011; Gimenez et al., 2012a). The

open activated conformation forms when the finger loop/motif II is stabilized into a fold that leaves the polar core exposed and allows interaction with receptor loops (Gurevich and Benovic, 1993). This is associated with rearrangements in motifs I, III, and IV, leading to dissociation of the hinge salt bridges and protein flexing (Granzin et al., 1998). Analysis of conformational shifts in β -arrestin1 and 2 binding to rhodopsin in vitro, using double-electron electron resonance spectroscopy, confirms release of the C-terminal tail and movement of the finger loop toward the predicted location of the receptor (Zhuo et al., 2014). The crystal structure of a preactivated C-terminally truncated variant of visual arrestin (Arr1-370A), which was activated during crystallization by incubation with retinal-free rhodopsin (PDB file: 4JQ2), shows a dramatic 21° twist between the N- and C-terminal domains and local changes in loop conformation and hydrogen-bonding networks, compared with the structure of unactivated full-length visual arrestin (Kim et al., 2013). Similar findings were reported for a structure of β -arrestin1 cocrystallized in the presence of a Fab fragment and a phosphopeptide corresponding to the last 29 amino acids of the V2 vasopressin receptor (PDB file: 4JQI), where a 20° rotation between the N- and C-terminal domains is present compared with the structure of inactive β -arrestin1 (Shukla et al., 2013). Finally, the crystal

structure of a fusion protein composed of T4 lysozyme, mutationally stabilized rhodopsin, and a preactivated visual arrestin mutant (PDB file: 4ZWJ) demonstrates that arrestin activation involves a 20° rotation between the N and C domains that opens a cleft in the arrestin surface to accommodate a short helix in the rhodopsin second intracellular loop (Kang et al., 2015). Figure 3D depicts an overlay of basal and activated arrestin structures, illustrating the major areas of conformational flexibility. This two-step model wherein arrestin first engages the phosphorylated GPCR C terminus, prompting a conformational rearrangement that enables it to dock with the heptahelical receptor core, has been visualized using single-particle negative-stain electron microscopy (Shukla et al., 2014).

The dynamics of arrestin recruitment and the corresponding conformational shifts in the arrestin molecule have been probed using resonance energy transfer. Intermolecular bioluminescence resonance energy transfer (BRET) between *Renilla* luciferase–tagged β -arrestin2 and yellow fluorescent protein–tagged GPCRs indicates that half-maximal association occurs within 1–2.5 minutes (at 25°C) for arrestin recruitment to the receptor upon agonist stimulation (Charest and Bouvier, 2003; Charest et al., 2005). Measured by intermolecular fluorescence resonance energy transfer (FRET), the process appears even faster, taking only seconds to occur (Nuber et al., 2016). Recruitment is followed closely in time by a conformational shift (Charest et al., 2005). Consistent with the model that arrestins undergo structural rearrangement after the phosphate sensor is triggered by phosphorylated receptor domains, the intramolecular shifts in β -arrestin2 conformation upon binding the β 2-adrenergic receptor are slower than the initial binding (τ 1.2 seconds for recruitment versus 2.2 seconds for conformational change) (Nuber et al., 2016).

The dynamic conformational shifts observed in β -arrestin2 upon receptor stimulation vary, suggesting that information about ligand and receptor is encoded within the activated arrestin structure. When intramolecular fluorescent arsenical hairpin (FAsH) FRET or BRET is used to report on arrestin conformational changes from multiple vantage points within the β -arrestin2 molecule, it is apparent that different GPCRs impose characteristic arrestin conformational signatures (Lee et al., 2016; Nuber et al., 2016). Interestingly, the β -arrestin2 FAsH BRET signal at some positions relates to the avidity of GPCR–arrestin binding and at others correlates with arrestin engagement of downstream signals (Lee et al., 2016). Even different ligands binding the same GPCR influence arrestin conformation and function. When measured by intramolecular BRET between the β -arrestin2 N and C terminus (Charest et al., 2005), conventional GPCR agonists produce similar increases in BRET signal, whereas biased agonists that recruit arrestin without

activating heterotrimeric G proteins trigger modest shifts in the opposite direction (Shukla et al., 2008). For a panel of biased angiotensin AT_{1A} receptor peptides, the β -arrestin2 FAsH BRET signal detected from discrete positions within the C-terminal domain correlates with the avidity of GPCR–arrestin binding measured by fluorescence recovery after photobleaching (Zimmerman et al., 2012; Lee et al., 2016). Although the factors, beyond ligand-induced changes in receptor structure, that dictate arrestin conformation are incompletely understood, reports that different GRKs specify arrestin signaling versus desensitization (Kim et al., 2005; Ren et al., 2005; Nobles et al., 2011; Zimmerman et al., 2012) suggest that the pattern of receptor phosphorylation may imprint a phosphorylation code that regulates arrestin function by influencing its activated conformation (Tobin et al., 2008; Liggett, 2011).

Once bound, the lifetime of the GPCR–arrestin complex is determined by several factors, including receptor structure (Oakley et al., 2000, 2001), ligand off-rate (Krasel et al., 2005), and post-translational modifications such as reversible arrestin ubiquitination (Shenoy et al., 2001, 2007, (2009; Shenoy and Lefkowitz, 2003, 2005) and phosphorylation (Lin et al., 1997, 1999; Khoury et al., 2014). Most GPCRs fall into one of two classes based on their selectivity for the two β -arrestin isoforms and the longevity of the receptor–arrestin interaction (Oakley et al., 2000). One, termed class A, exhibits higher affinity for β -arrestin2 than β -arrestin1 and forms transient receptor–arrestin complexes that dissociate soon after the receptor internalizes. These receptors are rapidly resensitized and recycled back to the plasma membrane. The other, class B group exhibits equivalent affinities for β -arrestin1 and β -arrestin2 and forms long-lasting receptor–arrestin complexes that remain intact as the receptor undergoes endosomal sorting. These receptors tend to be sequestered in endosomes and either recycle slowly or are degraded. Intriguingly, the conformational shift in β -arrestin2 induced by binding to the class A β 2-adrenergic receptor persists for as much as 5 seconds after the receptor–arrestin complex itself dissociates (Nuber et al., 2016), suggesting that whereas class B GPCRs may tie up arrestins in stoichiometric signal-some complexes that distribute to endosomes, class A receptors might be able to act upon arrestins catalytically, much in the same manner as heterotrimeric G proteins.

D. The Arrestin–GPCR Interface

Two facets of the visual/ β -arrestin–GPCR interaction that are critical to its function are the ability to interdict GPCR coupling to heterotrimeric G proteins, which is the physical basis for homologous GPCR desensitization, and the ability to recognize a multitude of activated GPCRs despite variations in their

transmembrane domain orientation and intracellular loop (ICL) structure.

The former is accomplished through a shared GPCR interface. G protein activation involves binding of the $G\alpha$ subunit C terminus within a cytoplasmic crevice in the GPCR transmembrane bundle that opens upon receptor activation (Cherezov et al., 2007; Rasmussen et al., 2007, 2011a,b). The finger loop/motif II of all four visual/ β -arrestins contains a consensus sequence, (E/D)x(I/L)xxxGL, that is shared with the C termini of $G\alpha i/t$ family heterotrimeric G proteins, and the crystal structure of a peptide analog of the finger loop of visual arrestin in complex with rhodopsin (PDB file: 4PXF) shows that G proteins and arrestins use a common interface (Szczeppek et al., 2014). The T4 lysozyme–rhodopsin–visual arrestin chimera structure (PDB file: 4ZWJ) representing the active GPCR–arrestin complex identifies four principal rhodopsin–arrestin interface patches involving mostly the N-terminal arrestin basket (Kang et al., 2015). When fully engaged, the visual arrestin finger loop (residues 70–78) is stabilized as a short α -helix that interacts with the C terminus of transmembrane domain (TM)7, the N terminus of helix 8, and ICL1 of rhodopsin, whereas the adjacent arrestin β -strand (residues 79–86) makes contact with residues from TM5, TM6, and ICL3. Upon activation, the arrestin middle and C-loops move apart to accommodate rhodopsin ICL2, such that another interface patch is formed by the middle loop of the N domain (around residue 140) and the C-loop of the C domain (around residue 251) that interact with ICL2, and the arrestin back loop (residues 319–320) that contacts the C terminus of TM5. The N terminus of ICL2 contains the conserved DRY motif that is critical for regulating GPCR activation state, G protein coupling, and intracellular localization (Rovati et al., 2007; Kim et al., 2008c; Stambouli et al., 2014). Although not visualized in the crystal structure, additional contacts between the visual arrestin N-terminal β -strand (residues 11–19) and the C-terminal tail of rhodopsin can be modeled (Kang et al., 2015). Whereas the structure of the unphosphorylated C terminus of GPCRs appears to be flexible, the nuclear magnetic resonance spectroscopy structure of a fully phosphorylated rhodopsin C terminus bound to visual arrestin shows a distinct structure imposed by very clear charge coupling between the negative receptor phosphates and the positively charged outer loop of the arrestin N domain basket (Kisselev et al., 2004a,b).

The GPCR selectivity of visual/ β -arrestins varies. Visual arrestin, which is expressed only in visual sensory tissue and exists primarily to desensitize rhodopsin, is highly dependent upon receptor phosphorylation and exhibits the largest differences in affinity between rhodopsin and other GPCRs (Gurevich et al., 1995). The ubiquitously distributed β -arrestin1 and 2, in contrast, which must regulate hundreds of different

GPCRs, have a weaker hydrogen-bonding network within the polar core that makes them less dependent upon receptor phosphorylation and more easily activated by diverse receptor structures (Gurevich et al., 1995). β -Arrestin2 is the least selective member of the visual/ β -arrestins in terms of GPCR interaction, and this is correlated with increased flexibility within the C-terminal basket (Zhan et al., 2011a). This area, which in most arrestins forms a stabilized β sheet, has less defined secondary structure in β -arrestin2, probably reflecting greater flexibility to accommodate differences in GPCR structure.

Given that a few discrete contact patches form the GPCR–arrestin interface, it is not surprising that mutating a small number of residues can change the receptor selectivity of arrestins. Studies performed using visual arrestin/ β -arrestin1 chimeras and alanine substitution mutagenesis have identified a handful of such receptor discriminator residues. Alanine substitution of the two highly conserved phosphate-sensing Lys residues in the visual arrestin N terminus ($K^{14,15}A$) reduces binding to active phosphorylated rhodopsin (Vishnivetskiy et al., 2000; Gimenez et al., 2012a), reflecting the dependence of visual arrestin–rhodopsin binding on receptor phosphorylation. In contrast, the analogous mutation in β -arrestin2 ($K^{11,12}A$) produces receptor-specific effects. For example, [$K^{11,12}A$]- β -arrestin2 is markedly impaired in binding to neuropeptide Y2 receptor, but not the closely related Y1 receptor (Gimenez et al., 2014a). Substitution of as few as 10 nonconserved residues located in two regions on the exposed surface of arrestins (N domain residues 49–90 and C domain residues 237–268) markedly impairs the ability of visual arrestin, β -arrestin1, or β -arrestin2 to bind most GPCRs (Vishnivetskiy et al., 2011). Combining these two sets of mutations in β -arrestin2 eliminates both predocking and agonist-induced recruitment to both neuropeptide Y1 and Y2 receptors (Gimenez et al., 2014a).

Even point mutations in the C-terminal central loop of β -arrestin2 (residues 230–260) dramatically affect GPCR selectivity. For example, the $Y^{239}T$ mutation enhances β -arrestin2 interaction with β 2-adrenergic receptor at the cost of affinity for M2 muscarinic, D1 dopamine, and D2 dopamine receptors. The $D^{260}K/Q^{262}P$ mutant has the opposite effect, virtually eliminating β 2-adrenergic receptor binding, while preserving M2, D1, and D2 receptor binding, and the $Q^{256}Y$ mutant selectively reduces affinity for the D2 receptor. The $Y^{239}T/Q^{256}Y$ mutation preserves D1 receptor binding while reducing affinity for the β 2-adrenergic and M2 receptors and eliminating D2 receptor binding (Gimenez et al., 2012b). For the Y1 and Y2 receptors, a $Y^{238}T$ point mutation introduces several-fold selectivity for the Y1 over Y2 receptor, suggesting that arrestins can be customized to preferentially recognize specific GPCR targets (Gimenez et al., 2014a,b).

When fully engaged, arrestin and heterotrimeric G protein binding should be mutually exclusive. Yet some GPCRs, e.g., the type 1 parathyroid hormone (PTH), thyroid-stimulating hormone, type 1 sphingosine-1-phosphate, and V2 vasopressin receptors, are able to mediate prolonged G protein–dependent signaling from within endosomal compartments even after undergoing arrestin-dependent internalization (Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009; Feinstein et al., 2013; Vilardaga et al., 2014). One proposal to account for this seeming paradox, supported by biophysical and single-particle negative-stain electron microscopy data, is that some GPCRs can assemble signaling “megaplexes,” composed of receptor, G protein, and arrestin (Thomsen et al., 2016). In this setting, the arrestin appears to linger in its initial binding mode, engaging the receptor C-tail, but failing to envelop the receptor intracellular domains, which would deny G protein access to the receptor. The resulting complex continues to generate G protein–mediated signals while nonetheless undergoing arrestin-dependent redistribution into internalized vesicles.

III. Visual/ β -Arrestins as Scaffolds

A. The Arrestin Interactome

Beyond their traditional roles in GPCR desensitization/internalization, arrestins have been implicated in the control of multiple signaling processes. Early efforts to identify arrestin-binding partners using yeast two-hybrid or proteomic approaches uncovered myriad potential interactions. For example, one proteomics-based screen reported that 337 distinct proteins coprecipitated with epitope-tagged β -arrestin1 or 2 under varying conditions (Xiao et al., 2007). Although it is unlikely that so many proteins directly engage arrestins, it seems clear that arrestins do bind elements of several intracellular signaling cascades, and in many cases contribute to their positive or negative regulation (Luttrell and Gesty-Palmer, 2010). Table 2 summarizes many of the reported arrestin-dependent GPCR signaling pathways that have a degree of experimental validation. Viewed as a whole, arrestin signaling appears to encompass a fairly discrete set of functions, linking GPCRs to nonreceptor tyrosine kinases, MAP kinases (MAPKs), lipid kinases, protein phosphatases, ubiquitin ligases and deubiquitinating enzymes, enzymes involved in second-messenger degradation, and regulators of Ras-family small GTPases. Many of these putative effectors are not known to be regulated by heterotrimeric G proteins, suggesting that arrestin–effector pathways function in parallel with G protein–effector pathways to add dimensions to GPCR signaling.

1. *Clathrin-Dependent Endocytic Machinery.* The β -arrestins, but not the visual arrestins, mediate clathrin-dependent sequestration of GPCRs (Ferguson,

2001). This is accomplished through regulated interaction of the β -arrestin C terminus with elements of the clathrin-coated pit following arrestin activation. As noted, arrestin activation initially involves interaction between the phosphate sensor and receptor-attached phosphates, leading to displacement of the C terminus. Once exposed, a conserved LIEFE/LD motif in the distal C terminus binds residues 89–100 of the clathrin heavy chain (Goodman et al., 1996; Krupnick et al., 1997). Mutating L³⁷³, I³⁷⁴, and F³⁷⁶ of β -arrestin2 to alanine disrupts clathrin binding and markedly impairs GPCR sequestration (Goodman et al., 1997). Nearby the clathrin-binding motif is an RxR motif that binds the β 2-adaptin subunit of the AP-2 complex (Laporte et al., 2000; Kim and Benovic, 2002; Schmid et al., 2006). Mutating the β -arrestin2 RxR motif does not impair recruitment to the plasma membrane, but prevents β 2-adrenergic receptors from clustering in clathrin-coated pits.

2. *Tubulin and Microtubules.* All of the visual/ β -arrestins bind microtubules, with β -arrestin1 and 2 showing the stronger interaction (Nair et al., 2004; Hanson et al., 2006b, 2007a). The site of interaction involves the concave sides of both the N and C domain baskets and substantially overlaps the receptor binding sites, but the affinity for microtubules is much lower than for activated GPCRs, allowing receptors to out-compete microtubules for arrestin binding. Interestingly, deletion mutations within the hinge region that reduce receptor binding enhance binding to microtubules, suggesting distinct microtubule-bound and receptor-bound conformations (Vishnivetskiy et al., 2002; Hanson et al., 2007a).

3. *Phosphoinositides.* Several species of phosphoinositide bind to visual/ β -arrestins, of which the abundant cytosolic phosphoinositide, inositol hexakisphosphate (IP6), has the highest affinity (Palczewski et al., 1991a; Gaidarov et al., 1999). Two independent IP6 binding sites have been identified in β -arrestin1 both by mutational analysis and X-ray crystallography, a low-affinity site in the N domain involving residues K¹⁵⁷ K¹⁶⁰ R¹⁶¹, and a high-affinity site in the C domain involving residues K²³² R²³⁶ K²⁵⁰ K³²⁴ K³²⁶ (Gaidarov et al., 1999; Milano et al., 2006). IP6 binding promotes β -arrestin1 self-association and is involved in receptor clustering in clathrin-coated pits and their subsequent internalization. Similarly, *Drosophila* visual arrestin contains a C-terminal domain IP6 binding site that when mutated interferes with arrestin trafficking in photoreceptor cells and light adaptation (Lee et al., 2003). In contrast, visual arrestin binding to IP6 involves principally the N domain residues K¹⁶³ K¹⁶⁶ K¹⁶⁷. Unlike β -arrestin1, IP6 inhibits both self-association and its recruitment to light-activated rhodopsin (Hanson et al., 2006a; Zhuang et al., 2010). Solution NMR studies indicate that IP6 binding leads to release of the visual arrestin C-tail, presumably

TABLE 2
The nonreceptor arrestin interactome

Effector	Arrestin	Reported Function	References
Clathrin heavy chain β 2-adaptin subunit of AP-2	β -Arrestin1 β -Arrestin2	Clathrin-dependent GPCR endocytosis	Goodman et al., 1996 Krupnick et al., 1997 Laporte et al., 1999 Laporte et al., 2000 Hanson et al., 2006b Hanson et al., 2007a
Tubulin; microtubules	Arrestin1 β -Arrestin1 β -Arrestin2 Arrestin4	Sequestration of arrestin Attenuation of MAPK activity Enhanced ubiquitination of cytoskeletal proteins	Hanson et al., 2007a
Ca ²⁺ -calmodulin	Arrestin1 β -Arrestin1 β -Arrestin2 Arrestin4	Cytosolic sequestration of Ca ²⁺ -calmodulin	Wu et al., 2006
Inositol hexakisphosphate	Arrestin1 β -Arrestin1 β -arrestin2 Arrestin4	Arrestin oligomerization Rhodopsin binding Receptor endocytosis	Palczewski et al., 1991a Gaidarov et al., 1999 Milano et al., 2006 Hanson et al., 2008
Src family tyrosine kinases c-Src; c-Yes; c-Hck; c-Fgr; c-Fyn	Arrestin1 β -Arrestin1 β -Arrestin2	Arrestin nuclear translocation ERK1/2 activation Dynamin 1 phosphorylation Exocytosis/Granule release Phosphorylation/Destabilization of GRK2 FAK phosphorylation EGF receptor transactivation Phosphorylation of β 2 adaptin subunit of AP-2	Luttrell et al., 1999; DeFea et al., 2000a Miller et al., 2000 Barlic et al., 2000; Imamura et al., 2001 Penela et al., 2001 Galet and Ascoli, 2008 Noma et al., 2007 Fessart et al., 2007; Zimmerman et al., 2009
c-Raf1-MEK1/2-ERK1/2	β -Arrestin1 β -Arrestin2	Activation of cytosolic ERK1/2 Receptor internalization and trafficking p90RSK phosphorylation Actin cytoskeletal reorganization/chemotaxis ERK1/2-dependent transcription Mnk1/eIF4E phosphorylation/protein translation	DeFea et al., 2000b; Luttrell et al., 2001 Lin et al., 1999; Khoury et al., 2014 Seta et al., 2002 Ge et al., 2003 Gesty-Palmer et al., 2005 DeWire et al., 2008
ASK1-MKK4-JNK3	β -Arrestin2	Activation of cytosolic JNK3 Sequestration of JNK outside the nucleus	McDonald et al., 2000; Song et al., 2006 Breitman et al., 2012
ASK1-MKK3/7-p38 MAPK	β -Arrestin1 β -Arrestin2	Scaffolding/Activation of p38 MAPK Inhibition of p38 MAPK	Sun et al., 2002; Yang et al., 2012 Zhao et al., 2004
I κ B α -I κ B kinase α/β	β -Arrestin1 β -Arrestin2	Attenuation of NF κ B signaling	Gao et al., 2004; Witherow et al., 2004
Casein kinase II cAMP phosphodiesterases PDE4D3; PDE4D5	β -Arrestin2 β -Arrestin1 β -Arrestin2	Activation of CK2 Attenuation of cAMP signaling	Kendall et al., 2011 Perry et al., 2002; Baillie et al., 2007
Diacylglycerol kinases	β -Arrestin1 β -Arrestin2	Attenuation of PKC signaling	Nelson et al., 2007
PI 4-phosphate 5-kinase I α	β -Arrestin1 β -Arrestin2	Control of clathrin-dependent GPCR internalization	Nelson et al., 2008
Phosphatidylinositol 3-kinase PTEN	β -Arrestin1 β -Arrestin1	Localized inhibition of PI3K Inhibition of AKT signaling and cell proliferation	Wang and Defea, 2006 Lima-Fernandes et al., 2011
PP2A-Akt-GSK3 β	β -Arrestin2 β -Arrestin2	Increased cell migration Inactivation of Akt/GSK3 β Activation of β -catenin signaling Activation of Akt	Beaulieu et al., 2005 Beaulieu et al., 2008 Kendall et al., 2011 Walters et al., 2009
Phospholipase A2 Nitric oxide synthases	β -Arrestin1 β -Arrestin1 β -Arrestin2	Vasodilation and cutaneous flushing Suppression of stress-induced iNOS transcription Post-translational activation of iNOS eNOS-dependent S-nitrosylation of β -arrestin2	Tan et al., 2015 Kuhr et al., 2010 Ozawa et al., 2008
Cofilin; chronophin; LIM kinase Filamin A	β -Arrestin2 β -Arrestin1 β -Arrestin2	Actin cytoskeletal reorganization/chemotaxis Membrane ruffling	Zoudilova et al., 2007, 2010 Scott et al., 2006
SHP-1; SHP-2 E3 ubiquitin ligases Mdm2; parkin; Nedd4; AIP4; TRAF6	β -Arrestin2 Arrestin1 β -Arrestin1 β -Arrestin2	Inhibition of NK cell cytotoxicity Ubiquitination of β -arrestin2 Stabilization of GPCR-arrestin complex Increased p53-mediated apoptosis Inhibition of Toll-like receptor signaling Stabilization of GPCR-arrestin-ERK1/2 signalsome	Yu et al., 2008 Shenoy et al., 2001 Shenoy and Lefkowitz, 2003 Wang et al., 2003 Wang et al., 2006 Shenoy et al., 2007
Ubiquitin-specific protease 33	β -Arrestin2	GPCR ubiquitination and downregulation Deubiquitination of β -arrestin2 Control of GPCR internalization	Bhandari et al., 2007; Shenoy, et al., 2008 Shenoy et al., 2009
Na ⁺ /H ⁺ exchanger1	β -Arrestin1	Ubiquitination of NHE1 by Nedd4 Negative regulation of NHE1 activity	Simonin and Fuster, 2010
Ral-GDS	β -Arrestin1 β -Arrestin2	Cytoskeletal reorganization/granule exocytosis	Bhattacharya et al., 2002

(continued)

TABLE 2—Continued

Effector	Arrestin	Reported Function	References
ARF-GAP21	β -Arrestin1	RhoA activation	
ARF6-ARNO	β -Arrestin1	Membrane ruffling	
	β -Arrestin2	GPCR endocytosis	Claing et al., 2001; Houndolo et al., 2005
N-ethylmaleimide-sensitive factor	β -Arrestin1	Control of GPCR internalization	McDonald et al., 1999
Dishevelled 2	β -Arrestin1	Frizzled endocytosis and Wnt signaling	Chen et al., 2004
Kif3A kinesin motor protein	β -Arrestin1	Targeting and internalization of Smoothed	Chen et al., 2004
	β -Arrestin2	Gli-dependent transcription	Kovacs et al., 2008
Histone acetyltransferase p300	β -Arrestin1	Transcription of p27 and c-Fos	Kang et al., 2005
Enhancer of zeste homolog 2	β -Arrestin1	Promote histone H4 acetylation of BCR/ABL	Qin et al., 2014
		Stimulate chronic myelogenous leukemia progression	
YY1 transcription factor	β -Arrestin1	Repression of cdx4-hox transcription	Yue et al., 2009
PPAR γ	β -Arrestin1	Repression of PPAR γ -RXR α transcription	Zhuang et al., 2011
STAT1-TC45	β -Arrestin1	Dephosphorylation/Inactivation of STAT1	Mo et al., 2008

BCR, B cell receptor; FAK, focal adhesion kinase; eNOS, endothelial nitric oxide synthase.

by mimicking the multiphosphorylated rhodopsin C terminus.

4. Ca^{2+} -Calmodulin. All four visual/ β -arrestins also bind Ca^{2+} -liganded calmodulin with micromolar affinity (Wu et al., 2006). The binding site is on the concave side of the C domain basket, again overlapping the receptor- and microtubule-binding surface, such that Ca^{2+} -calmodulin can only interact with free cytosolic arrestin.

5. Src Family Nonreceptor Tyrosine Kinases. Several Src family tyrosine kinases have been reported to bind visual/ β -arrestins, including c-Src, c-Fgr, c-Fyn, c-Hck, and c-Yes (Luttrell et al., 1999; Barlic et al., 2000; DeFea et al., 2000a; Galet and Ascoli, 2008). As with many non-GPCR arrestin-binding partners, the sites of interaction have not been mapped with any degree of precision, and appear to involve several elements of both proteins. The N domain of β -arrestin1 is proline rich and contains three PxxP motifs that interact with the Src homology (SH)3 domain of c-Src (Luttrell et al., 1999), but additional contacts with the c-Src SH1 (catalytic) domain are also involved (Miller et al., 2000). Additionally, visual arrestin binding to c-Src appears to involve the SH2 domain (Ghalayini et al., 2002). The arrestin–Src interaction appears to be constitutive, and it is not known whether arrestin-dependent recruitment of Src to GPCRs results in its activation, but immunostaining for the activated (Y^{530} dephosphorylated) form of c-Src indicates that c-Src in the GPCR–arrestin complex is active (Luttrell et al., 1999).

6. Mitogen-Activated Protein Kinases. One of the better understood arrestin-signaling functions is scaffolding of MAPK cascades. The ubiquitous MAPKs play critical roles in cell cycle regulation/proliferation and survival/apoptotic signaling by controlling phosphorylation of nuclear transcription factors, e.g., Elk1 and c-Jun, as well as diverse regulatory functions mediated through phosphorylation of cytosolic substrates (Davis, 2000; Kyriakis and Avruch, 2001; Pearson et al., 2001). Each MAPK module consists of three kinases: MAPK kinase kinase, MAPK kinase, and MAPK, which must

phosphorylate one another in succession, and arrestins play a traditional scaffold protein role, binding the component kinases to regulate the efficiency, fidelity, and compartmentalization of signaling (Luttrell and Miller, 2013). Significantly, although arrestins appear to engage all three major MAPK modules, ERK1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK, the direction of regulation, positive or negative, varies. This most likely reflects the differing functions of MAPK modules and a consistent role of arrestins to favor activation of proliferative/survival pathways and suppression of apoptotic signals (Gurevich and Gurevich, 2013).

ERK1/2 activity is required for G0–G1 cell cycle transition and the passage of cells through mitosis or meiosis (Pearson et al., 2001). All three kinases of the ERK1/2 MAPK module, cRaf1–MAPK/ERK kinase (MEK) 1/2–ERK1/2, bind β -arrestin1/2 individually and collectively (Luttrell, et al., 2001; Meng et al., 2009). In vitro, all three kinases can interact with either the isolated N- or C-terminal domains (Song et al., 2009a), suggesting a broad interaction surface involving the cytoplasmic surface of both domains, although the single R³⁰⁷A point mutation in β -arrestin1 is sufficient to reduce c-Raf1 binding and prevent arrestin scaffolding of the cascade (Coffa et al., 2011a). The ERK1/2 cascade is unique among the MAPK modules engaged by arrestins in that receptor activation regulates its assembly (Luttrell et al., 2001; Coffa et al., 2011b). Active ERK2 exhibits the highest affinity for receptor-bound arrestin, with virtually no binding to free cytosolic protein. C-Raf1 also shows a preference for the active arrestin conformation, whereas MEK1 binds equally to active and inactive arrestins. Interestingly, a constitutively inactive arrestin mutant that mimics the microtubule-bound conformation binds ERK1/2 and c-Raf1 better than the inactive cytosolic form, leading to recruitment of inactive ERK1/2 to microtubules, where it is sequestered away from membrane-generated activating signals (Hanson et al., 2007a; Coffa et al., 2011b). Thus, arrestin scaffolds perform the dual roles of

dampening basal ERK1/2 pathway activity, while specifically promoting the assembly of GPCR-associated signalsome complexes.

JNK family MAPKs play a major role in cellular stress responses and are involved in the normal physiologic processes of cell proliferation, apoptosis, differentiation, and migration (Davis, 2000; Kyriakis and Avruch, 2001). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 expression is largely confined to neuronal tissues. As with the ERK1/2 MAPK module, arrestins bind the component kinases of the neuronal JNK3 cascade, apoptosis signal regulating kinase (ASK) 1-MAPK kinase (MKK) 4/7-JNK3 (McDonald et al., 2000), but the consequences for JNK pathway regulation are much different. Similar to the ERK1/2 cascade, all four visual/ β -arrestins can bind ASK1-MKK4/7-JNK3 in vitro (Song et al., 2009a). Both the N and C domains contribute to the JNK3-binding surface, although a 25-amino-acid stretch within the N domain exhibits the highest affinity for JNK3 α 2 (Zhan et al., 2014). Phosphorylation of JNK3 by both MKK4 and MKK7 is required for full activation. In vitro, β -arrestin2 interacts directly with MKK7 to phosphorylate JNK3 α 2 (Zhan et al., 2013). JNK3 binding causes the arrestin to lose affinity for MKK7 and gain affinity for MKK4, indicating that the pathway components actually regulate one another's interaction with the scaffold. β -Arrestin2-dependent activation of MKK4-JNK3 has been reconstituted using purified proteins in vitro, and the efficiency of JNK3 activation demonstrates the biphasic dependence on arrestin concentration that is characteristic of a true scaffold protein (Zhan et al., 2011b, 2013). Interestingly, although all arrestins bind JNK3, only β -arrestin2 is able to efficiently scaffold pathway activation. This was initially attributed to the presence of a consensus MAPK-docking motif, RRSLHL, in the β -arrestin2 C-terminal domain that conferred JNK3 binding and scaffolding activity when substituted for the corresponding sequence in β -arrestin1 (Miller et al., 2001). However, this motif is unique to rat β -arrestin2, whereas the capacity to activate JNK3 is shared with other mammalian β -arrestin2 proteins. The two β -arrestin isoforms exhibit similar affinities for ASK1, MKK4, and JNK3, but apparently only β -arrestin2 is able to orient the kinases so as to support efficient phosphorylation (Seo et al., 2011). Bovine β -arrestin2 can also bind the ubiquitous JNK isoforms, JNK1 and JNK2, and support their activation by ASK1 and MKK4 or MKK7 in cells, suggesting the possibility that the activity of all three JNK isoforms can be modulated by arrestins (Kook et al., 2013).

The p38 MAPKs comprise the other major family of MAPKs. Like the JNKs, they are activated primarily in response to cell stress and proinflammatory signals (Kyriakis and Avruch, 2001, 2012) and are controlled via a modular ASK1-MKK3/6-p38MAPK phosphorylation

cascade. Although the molecular mechanisms of activation have not been characterized in any detail, several studies have reported arrestin-dependent regulation of the p38 MAPK cascade (Luttrell and Miller, 2013).

7. Regulators of Nuclear Factor κ B Signaling. β -Arrestin1 and 2 both bind the NF κ B inhibitor, I κ B α , and attenuate basal NF κ B signaling (Gao et al., 2004). In the cytoplasm, NF κ B dimers are bound to I κ B. In response to inflammatory signals, I κ B is phosphorylated by I κ B kinase, causing it to undergo proteosomal degradation. Once dissociated from I κ B, NF κ B translocates to the nucleus, where it promotes transcription of proinflammatory genes (Karin and Ben-Neriah, 2000). The N-terminal domain of β -arrestin2, within residues 1–60, binds to the C-terminal 40-amino-acid residues of I κ B α , preventing its phosphorylation and subsequent degradation in response to stress signals like UV irradiation or activation of Toll-like receptor (TLR) 4 by bacterial lipopolysaccharide (Luan et al., 2005; Fan et al., 2007). Stimulation of β 2-adrenergic receptors enhances the β -arrestin2–I κ B α interaction, enhancing the stabilization of I κ B α and inhibiting NF κ B-dependent transcription. The I κ B kinases, I κ B kinase α and β , also coprecipitate with β -arrestins, and down-regulation of β -arrestin1, but not β -arrestin2, enhances basal NF κ B transcriptional activity in HeLa cells (Witherow et al., 2004). In addition, interaction between β -arrestin2 and the non-GPCR type III transforming growth factor- β receptor negatively regulates NF κ B transcriptional activity in breast cancer cells (You et al., 2009).

In some circumstances, however, β -arrestin1 can function as an enhancer of GPCR-stimulated NF κ B transcription. β -arrestin1 has a bipartite nuclear localization sequence (NLS) located between residues 157–161 and 169–170 in the N domain that allows it to bind importin β 1 and engage the nuclear import machinery (Hoepfner et al., 2012). Introducing a K¹⁵⁷A mutation blocks importin binding and prevents bradykinin receptor-mediated nuclear translocation of β -arrestin1. Inside the nucleus, β -arrestin1 forms a complex with the p65/RelA subunit of NF κ B. Arrestin binding facilitates p65/RelA acetylation by acetyltransferase CREB binding protein and phosphorylation by nuclear MSK1, two post-translational modifications that stabilize its DNA binding. As a result, cells expressing the K¹⁵⁷A β -arrestin1 mutant exhibit less p65/RelA promoter binding and a decrease in bradykinin-stimulated transcription of NF κ B targets like IL-1 β .

8. Casein Kinase II. Casein kinase II is a ubiquitously expressed, constitutively active Ser/Thr protein kinase that performs diverse functions related to cell survival and tumorigenesis. The catalytic subunit of casein kinase II was identified in a proteomic screen of β -arrestin2-binding proteins as well as a phosphoproteomic screen of angiotensin AT_{1A} receptor-mediated phosphorylation following stimulation with

the arrestin pathway-selective biased angiotensin analog, Sar¹Ile⁴Ile⁸-AngII (Xiao et al., 2010; Kendall et al., 2011).

9. *cAMP Phosphodiesterases*. β -Arrestins1 and 2 interact with all five type 4D isoforms of cAMP phosphodiesterase, PDE4D1–5 (Perry et al., 2002). The common catalytic subunit of PDE4D5 interacts with β -arrestin2 via interactions with the cytoplasmic surface of both the N domain, near residues 18–26, and C domains, near residues 215–220 and 286–291 (Baillie et al., 2007). The main function of arrestin-scaffolded PDE4 appears to be to enhance the negative regulation of G protein signaling by accelerating second-messenger degradation.

10. *Diacylglycerol Kinases*. Both β -arrestins associate with the α , β , γ , δ , ϵ , ζ , or ι isoforms of diacylglycerol kinase, via interaction between the β -arrestin C-domain and Cys-rich domains in diacylglycerol kinase (Nelson et al., 2007). Diacylglycerol kinases phosphorylate the phospholipase C (PLC) β -generated second messenger, diacylglycerol, to produce phosphatidic acid. Arrestin-dependent recruitment of diacylglycerol kinase, which inhibits protein kinase C (PKC) by converting diacylglycerol produced by phospholipase C β to phosphatidic acid, dampens M1 muscarinic receptor-mediated PKC activity.

11. *Phosphatidylinositol 4-Phosphate 5-Kinase*. The phosphatidylinositol 4,5-bisphosphate (PIP₂)-producing enzyme, phosphatidylinositol 4-phosphate 5-kinase I α , binds β -arrestin2 via both N and C domain interactions. Arrestin binding to phosphatidylinositol 4-phosphate 5-kinase I α is increased by β 2-adrenergic receptor stimulation, resulting in its recruitment to activated internalizing receptors (Nelson et al., 2008). β -Arrestin-bound phosphatidylinositol 4-phosphate 5-kinase I α generates PIP₂ on the inner leaflet of the clathrin-coated pit, promoting polymerization of clathrin and AP-2 and assembly of the clathrin coat. Hence, its recruitment facilitates GPCR endocytosis. Consistent with this, a β -arrestin2 mutant deficient in PIP₂ binding fails to recruit phosphatidylinositol 4-phosphate 5-kinase I α or support β 2-adrenergic receptor internalization (Nelson et al., 2008).

12. *Phosphatidylinositol 3-Kinase*. The lipid kinase, phosphatidylinositol 3-kinase (PI3K), is an upstream regulator of protein kinase B (AKT) signaling. PI3K activity is necessary to recruit the constitutively active Ser/Thr kinase 3-phosphoinositide-dependent protein kinase-1 (PDK1) to the plasma membrane, where it phosphorylates AKT Thr³⁰⁸, leading to AKT activation. During protease-activated receptor (PAR)2-mediated chemotaxis, PAR2 receptors stimulate PI3K activity through a G_{q/11}-Ca²⁺-dependent pathway, whereas β -arrestin1 binds directly to the catalytic p110 α subunit of PI3K and inhibits its activity (Wang and DeFea, 2006). It has been proposed that arrestin-dependent targeting of PI3K to PAR2 receptors in pseudopodia modulates chemotaxis by locally inhibiting PI3K activity.

13. *Phosphatase and Tensin Deleted on Chromosome 10*. The tumor suppressor, phosphatase and tensin deleted on chromosome 10 (PTEN), regulates AKT-dependent proliferative and survival signaling via both lipid phosphatase-dependent and -independent mechanisms. β -Arrestin1 and 2 directly bind the C-terminal C2 domain of PTEN (Lima-Fernandes et al., 2011). GPCR-dependent activation of RhoA/Rho kinase signaling promotes the arrestin–PTEN association, and arrestin binding recruits PTEN to the plasma membrane and activates its lipid phosphatase activity, leading to negative regulation of AKT signaling and inhibition of cell proliferation. At the same time, β -arrestins inhibit the lipid phosphatase-independent antimigratory effects of PTEN, promoting increased cell migration.

14. *Protein Phosphatase 2A–Akt–Glycogen Synthase Kinase 3 β* . The Ser/Thr phosphatase PP2A holoenzyme is composed of regulatory A and B subunits that target the catalytic C subunit to specific intracellular locations, thereby restricting its otherwise promiscuous activity to selected targets. The PP2A catalytic subunit was identified as a β -arrestin2-interacting protein in a proteomic screen (Xiao et al., 2010), and a native β -arrestin2–PP2A–AKT–glycogen synthase kinase 3 β (GSK3 β) complex has been purified from the striatum of mice (Beaulieu et al., 2005), suggesting that β -arrestins may serve an analogous function. In vitro, β -arrestin2, but not β -arrestin1, interacts directly with the B and C subunits of PP2A, AKT, and GSK3 β . In vivo, increasing synaptic dopamine release with amphetamine promotes PP2A–AKT association in wild-type, but not β -arrestin2 knockout mice, suggesting that arrestins mediate assembly of the complex upon D2 receptor stimulation. Within the complex PP2A dephosphorylates AKT Thr³⁰⁸, keeping its kinase activity suppressed. Because AKT phosphorylation of GSK3 β inhibits its catalytic activity, the net result is increased GSK3 β signaling (Beaulieu et al., 2008).

The same complex, under other circumstances, may promote AKT signaling. Angiotensin AT_{1A} receptor-mediated, G protein-independent phosphorylation of the PP2A inhibitor, I2PP2A, transiently inhibits β -arrestin2-bound PP2A, resulting in increased AKT activity and phosphorylation-dependent inhibition of GSK3 β (Kendall et al., 2011). Stimulation of PAR1 receptors also reportedly promotes rapid AKT activation through an unknown β -arrestin1-dependent mechanism (Goel et al., 2002). PP2A is also known to promote ERK1/2 activation by acting on c-Raf1 Ser²⁵⁹, an inhibitory site that must be dephosphorylated for Raf activation (Abraham et al., 2000). Because PP2A both positively regulates c-Raf (Abraham et al., 2000; Adams et al., 2005) and negatively regulates ERK1/2 (Silverstein et al., 2002; Zhou et al., 2002), its presence in GPCR–arrestin complex may modulate arrestin-dependent ERK1/2 regulation.

15. Phospholipase A2. The nicotinic acid receptor, GPR109A, recruits cytosolic phospholipase A2 (cPLA2), stimulating the release of arachidonate, the precursor of prostaglandin D2 that is the vasodilator responsible for the cutaneous flushing response seen after niacin administration. In vitro, β -arrestin1 binds active cPLA2 and recruits it to GPR109A (Walters et al., 2009). In β -arrestin2 null mice, the free fatty acid-lowering effects of niacin are preserved, indicating that they are mediated via G protein signaling, but the cutaneous flushing response is diminished, suggesting that arrestin-dependent recruitment of cPLA2 mediates the response.

16. Nitric Oxide Synthases. β -Arrestin2 reportedly binds and is S-nitrosylated by endothelial nitric oxide synthase (Ozawa et al., 2008). S-nitrosylation of β -arrestin2 following β 2 adrenergic receptor activation promotes dissociation of endothelial nitric oxide synthase from the complex and facilitates arrestin binding to clathrin and AP-2, thereby accelerating receptor internalization. Although regulated transcription is the primary mechanism of inducible nitric oxide synthase (iNOS) activation, some data suggest that GPCRs can also regulate post-translational iNOS activity via an arrestin-dependent mechanism (Kuhr et al., 2010). Stimulation of bradykinin B1 receptors in lung microvascular endothelial cells promotes the association of β -arrestin2 and iNOS and increased nitric oxide production. Conversely, the ability of β -arrestin1 to dampen NF κ B signaling and repress p65/RelA transcription reportedly inhibits endoplasmic reticulum stress-induced upregulation of iNOS and moderates the inflammatory response (Tan et al., 2015).

17. Cofilin–Chronophin–LIM Kinase. GPCR-driven chemotaxis involves formation of a dominant pseudopodium at the leading edge of the cell that protrudes forward driven by F-actin polymerization and actin–myosin contraction forces. Upon activation of PAR2 receptors, β -arrestin1 assembles a complex containing the actin filament-severing protein, cofilin, Lin11, Isl-1, and Mec-3 (LIM) kinase, and the cofilin-specific phosphatase, chronophin, that is localized to membrane protrusions. Complex assembly promotes dephosphorylation and activation of cofilin, leading to localized generation of the free barbed ends on actin filaments that permit filament extension (Zoudilova et al., 2007, 2010). Regions of both the N and C domains of β -arrestin1 and 2 also interact with C-terminal repeat sequences within the actin-bundling protein, filamin A. Assembly of an angiotensin AT_{1A} receptor– β -arrestin–ERK1/2–Filamin A complex is involved in the formation of membrane ruffles in Hep2 cells (Scott et al., 2006).

18. Phosphotyrosine Phosphatases. The SH2 domain-containing protein tyrosine phosphatase (SHP)-1 modulates AKT activation by the ghrelin receptor, GHSR1 α . In adipocytes, ghrelin activates AKT by both an early pertussis toxin-sensitive Gi/o-mediated pathway and a slower arrestin-dependent pathway (Lodeiro

et al., 2011). GHSR1 α activation leads to c-Src activation, tyrosine phosphorylation of the p85 regulatory subunit of PI3K, PDK1 phosphorylation, and PDK1-dependent activation of AKT. SHP-1 localizes to a receptor-associated arrestin–scaffold complex, where it attenuates ghrelin-induced c-Src and AKT activation. A β -arrestin2-dependent mechanism also appears to negatively regulate the activity of natural killer cells, a key component of the innate immune response. β -Arrestin2 mediates recruitment of SHP-1 and SHP-2 to KIR2DL1, an inhibitory receptor of natural killer cells (Yu et al., 2008).

19. E3 Ubiquitin Ligases. Visual/ β -arrestins interact with at least five different E3 ubiquitin ligases: Mdm2, parkin, Nedd4, AIP4, and tumor necrosis factor (TNF) receptor-associated factor (TRAF)6 (Shenoy et al., 2001, 2008; Wang et al., 2006; Bhandari et al., 2007; Ahmed et al., 2011). All four visual/ β -arrestins bind Mdm2 in cells. Unlike JNK3, which binds epitopes located in both the N and C domains of arrestin1, Mdm2 binding involves primarily the N domain (Song et al., 2007). In cells, Mdm2 binds with highest affinity to inactive forms of visual arrestin and β -arrestin1 and 2, suggesting that arrestin is preloaded with Mdm2 in the cytosol (Song et al., 2006). Mdm2-mediated ubiquitination of arrestins is nonetheless stimulated by receptor binding, suggesting that the conformational shifts that occur upon receptor binding promote both arrestin ubiquitination and dissociation of Mdm2 from the complex (Shenoy and Lefkowitz, 2005). Parkin likewise exhibits higher affinity for the cytosolic and microtubule-bound arrestin conformations than for the mutationally activated conformation (Ahmed et al., 2011). The arrestin–parkin interaction is complex, however, as it promotes Mdm2 binding while at the same time attenuating stimulus-dependent arrestin ubiquitination.

Whereas Mdm2 catalyzes arrestin ubiquitination, other arrestin-bound E3 ligases mediate stimulus-dependent ubiquitination of the receptor. The β 2 adrenergic receptor is ubiquitinated by the E3 ligase Nedd4, which is recruited by β -arrestin2, possibly in collaboration with ARDC3 (Nabhan et al., 2010; Han et al., 2013). Nedd4 promotes β 2 adrenergic receptor downregulation by accelerating its proteosomal degradation (Shenoy et al., 2008). The CXC chemokine receptor (CXCR)4 is ubiquitinated by AIP4, which binds to the amino-terminal half of β -arrestin1 (Bhandari et al., 2007). β -Arrestin binding to another E3 ligase, TRAF6, negatively regulates TLR–interleukin (IL)-1 signaling (Wang et al., 2006). TRAF6 is normally recruited to TLR/IL-1 receptors, where it facilitates I κ B kinase and NF κ B activation. Binding of TRAF6 to β -arrestin1 and 2 in response to lipopolysaccharide or IL-1 stimulation prevents TRAF6 oligomerization and autoubiquitination, inhibiting lipopolysaccharide and IL-1 signaling.

20. Deubiquitinases. The deubiquitinase, ubiquitin-specific protease 33 (USP33), binds both β -arrestin1 and 2 in vitro and in cells (Shenoy et al., 2009). Whereas Mdm2 catalyzes β -arrestin2 ubiquitination upon activation of β 2 adrenergic receptors, USP33 catalyzes the reverse reaction. Analysis of the kinetics of β -arrestin2 ubiquitination/deubiquitination by BRET shows that ubiquitination is detectable within 2 minutes of stimulation of either β 2 adrenergic or vasopressin V2 receptors, but within 5 minutes the β 2 receptor-bound arrestin is deubiquitinated, whereas V2 receptor-bound arrestin remains stably ubiquitinated beyond 10 minutes (Perroy et al., 2004).

21. Na^+/H^+ Exchanger Type 1. The Na^+/H^+ exchanger regulatory factor binds to PDZ domain-binding motifs located at the very C terminus of several GPCRs, including the β 2 adrenergic and type 1 PTH receptors, to regulate the activity of Na^+/H^+ exchanger (NHE) type 3 controlling cell volume and pH (Hall et al., 1998; Mahon et al., 2002). The C terminus of the ubiquitous NHE1 isoform binds β -arrestin1, allowing it to be ubiquitinated by arrestin-bound Nedd4 (Simonin and Fuster, 2010). Ubiquitylation of NHE1 leads to its proteosomal degradation, and cells lacking β -arrestin1 or Nedd4 exhibit increased plasma membrane NHE1 levels and greatly enhanced Na^+/H^+ transport activity.

22. Regulators of Small GTPases. There are over 150 small GTPases in humans, traditionally classified into five families: Ras/Ral/Rap (>30 members), Rho/Rac/Cdc42 (>20 members), Rab (>60 members), Arf (6 members), and Ran (1 member) (Takai et al., 2001). Although only one of these small GTPases has been shown to bind directly to visual/ β -arrestins, arrestins have been implicated in the GPCR-dependent regulation of several family members, typically through scaffolding guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors that regulate their activity. In so doing, visual/ β -arrestins contribute to the regulation of cell proliferation/survival, cell migration, intracellular vesicle trafficking, and cytoskeletal remodeling (Claing, 2013).

The modulation of Ras activity by β -arrestins appears to result primarily from scaffolding of Src family tyrosine kinases that function upstream of Ras (Luttrell et al., 1999) and the ERK1/2 MAPK cascade that is a major downstream Ras effector pathway (Luttrell et al., 2001). In response to β 1 adrenergic receptor activation, β -arrestin-dependent recruitment of c-Src reportedly promotes transactivation of epidermal growth factor (EGF) receptors, which in turn activate Ras by recruiting the Shc-Grb2-Sos complex (Noma et al., 2007). Conversely, data obtained using BRET biosensors suggest that in some systems the dominant role of arrestins is to dampen G protein-mediated Ras activation signals (Balla et al., 2011). In

the case of Ral-GTPases, β -arrestin1 binds directly to Ral-guanine nucleotide dissociation stimulator (GDS), a GEF for the Ras-like Ral GTPases (Bhattacharya et al., 2002). Ral-GDS binding to cytosolic β -arrestin sequesters it in the cytosol, thereby maintaining Ral in the inactive state. Recruitment of β -arrestin to activated GPCRs on the plasma membrane allows Ral-GDS to dissociate from the arrestin and activate RalA.

Although GPCR-dependent activation of Rho is mediated primarily through $\text{G}\alpha_{12/13}$ proteins (Buhl et al., 1995), RhoA activation by the angiotensin $\text{AT}_{1\text{A}}$ receptor is inhibited by knockdown of β -arrestin1 and can be stimulated by an arrestin pathway-biased angiotensin analog (Barnes et al., 2005). β -Arrestin1 is involved in RhoA-mediated actin stress fiber formation and membrane blebbing. One mechanism by which arrestin-dependent RhoA regulation may occur is through direct binding of ARFGAP21, a RhoA GAP (Anthony et al., 2011). Stimulation of angiotensin $\text{AT}_{1\text{A}}$ receptors promotes the β -arrestin1-ARFGAP21 interaction, occluding its GAP domain and leading to increased RhoA activity and membrane ruffling. Notably, RhoA activation potentiates the interaction between β -arrestin1 and PTEN, whereas knockdown of β -arrestin1/2 inhibits RhoA-dependent PTEN activation by the lysophosphatidic acid (LPA) receptor, suggesting bidirectional crosstalk between arrestin and RhoA signaling (Lima-Fernandes et al., 2011). β -Arrestins have been reported to play both positive and negative roles in Rac1 signaling. Arrestins inhibit the NADPH oxidase-dependent oxidative cell burst produced by IL-8 and protect against cell death, while at the same time mediating Rac1 activation by Wnt-5A (Zhao et al., 2004; Bryja, et al., 2008). In HEK293 cells, β 2 adrenergic receptor-dependent activation of Rac1 is blocked by knockdown of β -arrestin1, as is Rac1-dependent activation of NADPH oxidase and p38 MAPK (Gong et al., 2008). β -Arrestins negatively regulate PAR2 receptor-mediated Cdc42 activation through an undefined mechanism (Wang et al., 2007). Similarly, in breast and ovarian cancer cell lines, activation of Cdc42 by the type III transforming growth factor- β receptor, a non-GPCR tumor suppressor, alters actin cytoskeletal rearrangement and reduces random cell migration (Finger et al., 2008; Mythreye and Blobe, 2009).

Rab family GTPases control most aspects of vesicular trafficking, and Rab4, Rab5, Rab7, and Rab11 are involved in GPCR endocytosis, recycling, and lysosomal targeting (Seachrist and Ferguson, 2003). Although the stability of the GPCR-arrestin complex has a profound impact on intracellular trafficking, there are no data to indicate that arrestins directly bind either Rabs or their GEFs and GAPs. In contrast, ARF6, a small GTPase involved in sequestration of many GPCRs, binds directly to the C-terminal domain of β -arrestin1 and 2 in

the GDP-bound state, where it is activated by ARNO, a constitutively arrestin-associated ARF-GEF (Claing et al., 2001; Houndolo, et al., 2005; Macia et al., 2012). Activated ARF6 mediates the recruitment of clathrin and AP-2 to the GPCR–arrestin complex, nucleating the assembly of endocytic vesicles (Paleotti et al., 2005; Poupart et al., 2007). The association between ARNO and β -arrestin also facilitates GPCR binding and desensitization, as shown for the luteinizing hormone (LH) receptor (Mukherjee et al., 2000).

23. N-Ethylmaleimide–Sensitive Fusion Protein. N-ethylmaleimide–sensitive fusion protein (NSF) is a homohexameric ATPase that regulates the disassembly of soluble NSF attachment protein (SNAP) receptor (SNARE) complexes that facilitate the membrane fusion events necessary for vesicle transport (Rizo and Südhof, 2002). Full-length β -arrestin1 preferentially binds to the N-terminal SNAP/SNARE binding domain of NSF when ATP-bound (McDonald et al., 1999). Overexpression of NSF facilitates β 2 adrenergic receptor endocytosis, suggesting that arrestin-dependent NSF recruitment contributes to clathrin-dependent GPCR internalization. Similarly, visual arrestin binds to NSF and stimulates its activity, enhancing neurotransmitter secretion in rod photoreceptors (Huang et al., 2010).

24. Wnt Signaling Pathway—Dishevelled. Wnts are secreted glycoproteins involved in embryologic patterning and development. They bind to seven-membrane-spanning receptors called Frizzleds, which cluster within the GPCR superfamily but do not signal via heterotrimeric G proteins. Frizzleds (Fz) recruit cytosolic proteins called Dishevelleds (Dsh), and the Fz–Dsh complex mediates the endocytosis and degradation of Wnt protein, a key step in establishing morphogen gradients during development (Dubois et al., 2001). During canonical Wnt signaling, Wnts bind to Fz, activating Dsh, and preventing Axin/GSK3 β -mediated phosphorylation and degradation of β -catenin. The resulting nuclear accumulation of β -catenin leads to activation of T cell–specific factor/lymphoid enhancer factor (LEF) transcription factors. In the alternative noncanonical Wnt/planar cell polarity signaling pathway, Wnts signal through Fz to the small GTPases Rho and Rac to promote rearrangement of the actin cytoskeleton. Another noncanonical Wnt pathway promotes increases in intracellular Ca²⁺ to negatively regulate the canonical Wnt/ β -catenin pathway. β -Arrestin1 binds phosphorylated Dsh1 and Dsh2 and enhances LEF-mediated transcription (Chen et al., 2001). β -Arrestin2 interacts with both Axin and Dsh after Wnt3A stimulation (Bryja et al., 2007), suggesting both β -arrestins are involved in negatively regulating GSK3 β activity and promoting canonical Wnt signaling. During noncanonical wnt5A signaling, β -arrestin2 binds phosphorylated Dsh2, and, in a heterologous expression system, Wnt5A-stimulated endocytosis of

Fz4 is dependent upon both β -arrestin1 and Dvl2 (Chen et al., 2003).

25. Hedgehog Signaling Pathway—Smoothed. The Hedgehog signaling pathway regulates cell fate determination during embryologic patterning. Smoothed, a non-G protein–coupled seven-membrane-spanning receptor, is constitutively suppressed by binding to Patched, a 12-membrane-spanning coreceptor that binds the extracellular glycoprotein, Sonic hedgehog (Shh). Shh binding to Patched relieves its inhibition of Smoothed, which in turn activates Gli family transcription factors by dissociating them from their negative regulator, Su(fu). β -Arrestin2 binds activated Smoothed in a GRK2-dependent manner and promotes its internalization (Chen et al., 2004). Arrestins also promote the association of Smoothed with the kinesin motor protein, Kif3A, causing β -arrestin, Smoothed, and Kif3A to colocalize in primary cilia (Kovacs et al., 2008). In NIH3T3 cells, downregulation of β -arrestin1 or 2 causes mislocalization of Smoothed and disrupts activation of Gli1.

26. Nuclear Proteins and Transcription Factors. By virtue of its NLS, β -arrestin1 is able to participate in protein–protein interactions within the nucleus that modify the activity of several transcription factors. Besides binding nuclear p65/RelA and enhancing NF κ B signaling by the bradykinin receptor, activation of δ -opioid receptors causes β -arrestin1 to move into the nucleus, where it interacts with the p27 and c-Fos promoters and stimulates transcription by recruiting histone acetyltransferase p300 and enhancing local histone H4 acetylation (Kang et al., 2005). Nuclear β -arrestin1 also binds enhancer of zeste homolog 2, a polycomb group (PcG) protein involved in gene silencing (Qin et al., 2014). Knockdown of β -arrestin1 results in reduced histone H4 acetylation of many genes in K562 chronic myelogenous leukemia (CML) cells, notably including the B cell receptor/Abelson murine leukemia viral oncogene homolog 1 (ABL) fusion oncogene, leading to reduced B cell receptor/ABL expression. Importantly, depletion of β -arrestin1 slows proliferation of K562 and primary CML cells and increases survival of CML mice. In zebrafish, β -arrestin1 binds the PcG recruiter, YY1, a ubiquitously expressed transcription factor essential for embryonic development (Yue et al., 2009). β -Arrestin1 binding sequesters YY1, relieving PcG-mediated repression of Cdx4-hox transcription. Without β -arrestin1, hox gene expression is downregulated, leading to developmental defects and failed hematopoiesis.

Within the nucleus, β -arrestin1 interacts directly with the peroxisome proliferator–activated receptor (PPAR) γ via a short region in the C-terminal domain between M²⁵⁵ and A²⁶³ (Zhuang et al., 2011). β -Arrestin competes with the 9-cis retinoic acid receptor (RXR) α , inhibiting PPAR γ –RXR α -dependent transcription and promoting PPAR γ nuclear receptor corepressor

function. As a result, loss of β -arrestin1 promotes, and overexpression of β -arrestin1 inhibits, adipogenesis, macrophage infiltration, and diet-induced obesity, and improves glucose tolerance and systemic insulin sensitivity in vivo. The β -arrestin1 C-terminal domain also directly interacts with signal transducers and activators of transcription (STAT)1 and its regulatory phosphatase TC45 in the cell nucleus following STAT1 activation by interferon- γ (Mo et al., 2008). By acting as a scaffold for STAT1 dephosphorylation by the nuclear phosphatase TC45, β -arrestin1 negatively regulates interferon- γ signaling and cellular antiviral responses.

In contrast to β -arrestin1, β -arrestin2 contains a classic leucine-rich nuclear export sequence (NES) located between amino acid residues 390–400 of the C-terminal domain (Scott et al., 2002). As a result, β -arrestin2 is actively excluded from the nucleus. Interestingly, β -arrestin2 actively accumulates in the nucleus when the NES is mutated to the corresponding residues of β -arrestin1 or when nuclear export is pharmacologically inhibited by leptomycin B, suggesting that it may also engage in nucleocytoplasmic shuttling.

B. Functional Pools of Arrestin

The visual/ β -arrestins possess three properties essential to their scaffolding function: the flexibility to bind multiple cargo proteins; the capacity to exist in different intracellular pools wherein they adopt different conformations; and the ability to recognize activated GPCRs. Whereas many arrestin cargo proteins appear to be constitutively associated, others exhibit a distinct preference for the cytosolic, microtubule-bound, or GPCR-bound arrestin. Thus, arrestin binding can constrain signaling proteins to one cellular compartment until an external GPCR-mediated stimulus prompts a conformational change that causes them to release some cargos and associate with others (Fig. 4).

1. Cytosolic Arrestin. In the absence of an acute stimulus, most of the visual/ β -arrestin pool resides either in the cytosol in an inactive conformation or bound to microtubules. Several cargos have been shown to have higher affinity for inactive arrestin than for either the microtubule- or GPCR-bound conformations. The binding site for Ca^{2+} -calmodulin overlaps the GPCR and microtubule-binding surfaces such that it can only interact with free arrestin protein (Wu et al., 2006). Binding to cytosolic arrestins probably serves to buffer intracellular Ca^{2+} -calmodulin and to maintain an equilibrium between cytosolic and microtubule-bound arrestin in the absence of higher-affinity GPCR docking sites. JNK3 also exhibits higher affinity for the cytosolic form of β -arrestin2 (Song et al., 2006; Breitman et al., 2012). Because the NES of β -arrestin2 keeps it out of the nucleus, arrestin-bound JNK3 is maintained in the cytosol away from its nuclear transcription factor

targets. In the case of β -arrestin1, which freely enters the nucleus in its monomeric form, IP6 binding promotes arrestin self-association and hinders spontaneous nuclear translocation (Milano et al., 2006; Song et al., 2006, 2007). Another cargo that prefers inactive arrestin is Mdm2 (Song et al., 2006). In this case, the differential affinity probably permits dynamic regulation, in that Mdm2 preferentially ubiquitinates GPCR-bound arrestin, a step that stabilizes the GPCR–arrestin complex (Shenoy and Lefkowitz, 2005). Once the arrestin is in the ubiquitinated GPCR-bound conformation, the drop in affinity may allow Mdm2 to dissociate, allowing other cargos to take its place in the receptor-associated complex.

2. Microtubule-Bound Arrestin. Like Ca^{2+} -calmodulin, the microtubule binding sites on arrestins overlap the GPCR interacting sites such that microtubule and GPCR binding are mutually exclusive (Nair et al., 2004; Hanson et al., 2006b). Also like Ca^{2+} -calmodulin, the affinity of arrestins for microtubules is much lower than for activated GPCRs. Thus, competition between abundant low-affinity microtubule binding sites and a smaller number of ligand-induced high-affinity GPCR binding sites probably allows arrestin to sequester itself, and certain cargos, in a microtubule-associated pool under basal conditions. All visual/ β -arrestins can bind microtubules, although β -arrestins have somewhat higher affinity (Hanson et al., 2007a). Notably, deletions in the hinge region of β -arrestin1 that restrict its flexibility enhance microtubule binding compared with either the wild-type protein or preactivated mutants that show enhanced receptor binding. This suggests the GPCR- and microtubule-bound conformations both differ from that of free cytosolic arrestin. Because the binding of some arrestin cargos is sensitive to arrestin conformation, it is thus likely that cytosolic, microtubule-bound, and GPCR-bound arrestins carry different cargos. For example, ERK1/2 has significant affinity only for the GPCR- and microtubule-bound arrestin conformations, but is activated only upon recruitment to the receptor. Sequestration of inactive ERK1/2 within the microtubule-bound arrestin pool effectively dampens basal ERK1/2 activity, thereby enhancing the signal-to-noise ratio upon receptor activation (Hanson et al., 2007a; Coffa et al., 2011b). Conversely, Mdm2 loses affinity for the GPCR-bound arrestin conformation, such that overexpressing arrestins markedly increases ubiquitination of microtubule-associated substrates (Hanson, et al., 2007a). These conformational effects are cargo-specific, in that other arrestin cargos, e.g., JNK3 and PP2A, are not preferentially targeted to microtubules.

The capacity of β -arrestins to bind both microtubules and clathrin enables them to regulate focal adhesion dynamics (Hanson et al., 2007a; Cleghorn et al., 2015). β -Arrestin1/2 null murine fibroblasts exhibit increased cell spreading and adhesion, reduced motility, and

Fig. 4. Dynamic regulation of functionally discrete arrestin pools. Visual/ β -arrestins exist in equilibrium between a large intracellular pool, where they are either freely cytosolic or associated with low-affinity microtubule binding sites, and a small pool bound with high affinity to activated GPCRs. Cytosolic, microtubule-bound, and GPCR-bound arrestins adopt different conformations, such that some cargos preferentially associate with free arrestins, e.g., Ca^{2+} -calmodulin and components of the ASK1/MKK4/JNK3 cascade, others prefer microtubule-bound arrestin, e.g., Mdm2, whereas still others preferentially associate with GPCR-bound arrestin, e.g., Raf-MEK-ERK1/2. Upon ligand (H) binding, GRK-phosphorylated GPCRs recruit β -arrestins from the cytosolic and microtubule-bound pools to the plasma membrane, where they can engage clathrin and AP2, leading to receptor endocytosis. Assembly of multiprotein signaling complexes on the GPCR-arrestin scaffold leads to spatially constrained pools of activated cargo, e.g., ERK1/2. Although β -arrestin2 (β Arr2) is excluded from the cell nucleus by its NES, β -arrestin1 is in equilibrium between cytosolic and nuclear pools. IP6 binding promotes β -arrestin1 self-association, which, like microtubule binding, sequesters it from the nucleus and restrains interactions with transcriptional regulatory proteins, e.g., histone acetyltransferase p300.

reduced focal adhesion turnover. Reintroduction of wild-type β -arrestin1 and 2, or mutants defective in GPCR binding, can each restore focal adhesion dynamics, whereas mutants defective in clathrin binding cannot. Because other arrestin cargos, e.g., c-Src, ERK1/2, and JNK, are known regulators of focal adhesion assembly, it is likely that β -arrestin1 and 2 regulate cell adhesion by bringing clathrin and possibly other effectors to microtubules in a GPCR-independent manner.

3. GPCR-Bound Arrestin. The unique capacity of visual/ β -arrestins to respond to extracellular stimuli derives from their ability to recognize and bind agonist-occupied GRK phosphorylated GPCRs on the plasma membrane. Heptahelical GPCRs function by detecting the presence of extracellular ligands that, upon interacting with the receptor, promote conformational

rearrangements, which are in turn transmitted across the plasma membrane to affect the conformation and activity of intracellular effectors, such as heterotrimeric G proteins and arrestins (Kenakin, 2012; Manglik and Kobilka, 2014). The conformational shifts occurring in visual/ β -arrestins not only permit tight binding to receptors, but also affect their affinity for some cargos. Destabilization of the arrestin polar core upon interaction with receptor-attached phosphates exposes the LIEF and RxR motifs in the C terminus of β -arrestin1/2, permitting them to engage clathrin and AP-2, steps that are essential for clathrin-dependent endocytosis of GPCRs (Goodman et al., 1997; Laporte et al., 2000). The binding of some signaling cargos is likewise affected. cRaf-1 and activated ERK1/2 have highest affinity for the receptor-bound arrestin conformation, allowing the GPCR-arrestin complex to nucleate

assembly of a signalsome that not only activates ERK1/2, but also keeps it spatially constrained (Luttrell et al., 2001; Coffa et al., 2011b). Receptor-bound arrestins also direct assembly of the cofilin–chronophin–LIM kinase complex necessary for actin cytoskeletal rearrangement and chemotactic cell migration (Zoudilova et al., 2007, 2010). Other cargos, for example Ral-GDS (Bhattacharya et al., 2002) and Mdm2 (Song et al., 2006), lose affinity for GPCR-bound arrestin, such that they are released upon receptor binding, freeing them to engage other membrane-associated substrates and making room for new signalsome components. This potential for dynamic regulation of GPCR-bound arrestin signalsomes is highlighted by biophysical studies of the effects of receptor binding on arrestin conformation, which indicate that differences in GPCR intracellular domain structure stabilize different β -arrestin2 conformations (Lee et al., 2016; Nuber et al., 2016). To the extent that the affinity of at least some arrestin cargos is sensitive to arrestin conformation, this implies that GPCRs may specify which arrestin effectors can bind.

The stability of the GPCR–arrestin complex also impacts the kinetics of arrestin-dependent signaling. For example, GPCRs that form stable arrestin complexes, like the angiotensin AT_{1A} and vasopressin V₂ receptors (Oakley et al., 2000), remain bound to activated ERK1/2, leading both to prolonged ERK1/2 activation and targeting of the kinase to endosomes (DeFea et al., 2000b; Luttrell et al., 2001; Ahn et al., 2004; Jafri et al., 2006). These constraints have profound effects on ERK1/2 function. Whereas ERK1/2 activated by classic growth factors or G protein-dependent GPCR signaling is able to translocate to the nucleus and elicit a transcriptional response, ERK1/2 bound to β -arrestin scaffolds is retained in the cytosol and silent in Elk-1 reporter assays (Tohgo et al., 2002; Lee et al., 2008). Arrestin-bound ERK1/2 performs other functions, for example, regulating arrestin–clathrin interaction during GPCR endocytosis (Lin et al., 1999), modulating actin cytoskeletal reorganization during chemotaxis (Ge et al., 2003), and activating MNK1, p90RSK, and p70S6K signaling to stimulate protein translation (DeWire et al., 2008; Kendall et al., 2014). Thus, by compartmentalizing signaling, arrestin scaffolding can change the functional consequences of pathway activation, even when the pathway is subject to convergent regulation by multiple mechanisms.

4. Nuclear Arrestin. With the exception of β -arrestin2, all visual/ β -arrestins are able to enter the nucleus. The presence of a NES in β -arrestin2 (Scott et al., 2002) largely confines it to cytosolic, microtubule-bound, and GPCR-bound pools, and favors cytosolic sequestration of cargos that regulate nuclear substrates, e.g., JNK3 (Song et al., 2006). In contrast, β -arrestin1, by virtue of its intrinsic NLS, appears capable of regulating events within the nucleus. At concentrations present in the

cytosol, IP6 promotes β -arrestin1 self-assembly and retards its nuclear translocation (Milano et al., 2006; Hanson et al., 2007a). Because the low-affinity IP6 interaction must be displaced for GPCR binding to occur, receptor activation might be expected to disassemble β -arrestin1 oligomers and promote nuclear signaling through engagement of components of the NK κ B/p65/RelA pathway (Hoeppner et al., 2012), STAT1/TC45 (Mo et al., 2008), p300 histone acetyltransferase (Kang et al., 2005), and other regulators of transcription. The role of IP6 in regulating nuclear arrestin signaling is likely to be complex, however, because IP6 inhibits, rather than enhances, self-association of visual arrestin (Hanson et al., 2007a).

C. Arrestins as GPCR-Activated Scaffolds

Nearly all GPCRs function as GEFs for heterotrimeric G proteins. Agonist binding stabilizes receptor conformations that enable it to catalyze GTP for GDP exchange on heterotrimeric G protein G α subunits, leading to dissociation of GTP-bound G α and G $\beta\gamma$ subunits, which in turn regulate the activity of enzymatic effectors, such as adenylate cyclases, PLC isoforms, and ion channels, and generate small-molecule second messengers that control the activity of key enzymes involved in intermediary metabolism. What then are the principal roles of arrestin scaffolds in cells? For the most part, arrestin-mediated signals appear to coordinate a few basic biologic processes, some related to modulation of G protein signaling and others accomplished by conferring upon GPCRs the ability to regulate noncanonical GPCR signaling pathways (Fig. 5).

1. Negative Regulation of Heterotrimeric G Protein Signaling. The most conserved arrestin function is the negative regulation of G protein signaling through direct steric hindrance of the GPCR–G protein interaction, and in the case of β -arrestins mediation of clathrin-dependent receptor sequestration (Ferguson, 2001). Due to common sites of interaction, GPCR binding to arrestins and G proteins is mutually exclusive (Kang et al., 2015). Even the formation of megaplexes, in which β -arrestins adopt a partially engaged binding pose that permits simultaneous binding of arrestin and G protein (Thomsen et al., 2016), appears to support receptor endocytosis, removing receptors from the plasma membrane even while permitting ongoing G protein signaling (Calebiro et al., 2009; Ferrandon et al., 2009; Mullershausen et al., 2009; Feinstein et al., 2013; Vilardaga et al., 2014).

Besides serving as adaptors linking GPCRs to clathrin and AP-2, β -arrestins also carry cargos that either promote second-messenger degradation or modulate the endocytic process. Gs-coupled β 2 adrenergic receptors form a complex with β -arrestin2 and PDE4D3/5, leading to accelerated cAMP degradation (Perry et al., 2002). Whereas wild-type β -arrestin2 is able to rescue arrestin-dependent inhibition of β 2-adrenergic

Fig. 5. Diverse cellular functions of arrestin scaffolds. By associating with different cargos in different subcellular locations, visual/ β -arrestins regulate multiple signaling networks. In quiescent cells, free cytosolic and microtubule-bound arrestins dampen basal pathway activity by sequestering signaling pathway intermediates away from their site of activation/action. Free arrestins can buffer cytosolic Ca²⁺-CaM concentration, suppress NFκB signaling by sequestering IκB kinases, and tonically inhibit β-catenin signaling by promoting GSK3β-dependent β-catenin phosphorylation and degradation. They also keep proapoptotic JNK kinases away from their nuclear substrates, whereas, in the case of β-arrestin2, they promote activation of cytosolic JNK. Microtubule-bound arrestins sequester inactive ERK1/2 away from the plasma membrane, dampening basal pathway activity, while directing Mdm2 toward cytoskeletal substrates. In some settings, this has the effect of increasing proapoptotic p53 signaling by preventing p53 ubiquitination and degradation. Microtubule-bound arrestins also regulate cell adhesion by binding to regulators of focal adhesions such as Src, ERK1/2, and JNK. Once recruited to plasma membrane-bound GPCRs, arrestins promote GPCR desensitization, support clathrin-dependent endocytosis, and accelerate second-messenger degradation by recruiting cAMP phosphodiesterases and diacylglycerol kinase. At the membrane they also stimulate cell proliferation by promoting Src-dependent transactivation of EGF receptor tyrosine kinases (RTKs) and promote cell survival by activating AKT. Through their interactions with numerous regulators of actin cytoskeletal dynamics, arrestin stimulate membrane ruffling, cell migration, and chemotaxis. Several β-arrestin cargos, e.g., ERK1/2, continue to signal from endosomal GPCR-arrestin signalsome complexes, where they regulate aspects of GPCR trafficking and recycling and preferentially phosphorylate cytosolic ERK substrates, leading to increased protein translation. β-Arrestins also stimulate canonical Wnt signaling by engaging Dsh and inhibiting GSK3β to stabilize β-catenin, and promote hedgehog signaling by internalizing and targeting smoothened to primary cilia. Within the nucleus, β-arrestin1 interacts with a number of transcription factors to either increase or tonically inhibit transcription.

receptor-stimulated protein kinase A (PKA) activation in β-arrestin1/2 null fibroblasts, R²⁶A and R²⁸⁶A mutants of β-arrestin2 that lack PDE4D5 binding but retain the ability to bind activated GPCRs are much less effective terminators of cAMP-PKA signaling (Baillie et al., 2007). In an analogous manner, arrestin-dependent recruitment of diacylglycerol kinase, which inhibits PKC by converting diacylglycerol produced by PLCβ to phosphatidic acid, dampens Gq/11-mediated signaling by the M1 muscarinic receptor (Nelson et al., 2007). It remains unclear whether or how specificity is achieved in arrestin-dependent targeting of PDE4D3/5 and diacylglycerol kinase, e.g., whether activation of adenylyl cyclase or PLC generates a coregulatory

signal that directs these second-messenger degrading enzymes to the appropriate receptor. The original reports suggest that their interaction with β-arrestins is constitutive; however, the effect of arrestin conformation on binding of these cargos has not been determined.

The reversible ubiquitination of arrestin3 regulates the stability of the GPCR-arrestin complex and thereby the kinetics of receptor desensitization, internalization, and intracellular trafficking. Ubiquitination of β-arrestin2 by Mdm2 stabilizes the receptor-arrestin interaction, allowing the receptor to undergo endocytosis, after which USP33 removes the ubiquitin moiety, promoting arrestin dissociation (Shenoy and Lefkowitz,

2003; Shenoy et al., 2009). This allows class A GPCRs, like the $\beta 2$ adrenergic receptor, to recycle to the plasma membrane. In contrast, β -arrestin recruited to class B receptors, like the angiotensin AT_{1A} receptor, remains ubiquitinated, stabilizing the receptor–arrestin complex and favoring slow recycling or receptor degradation. Hence, a lysine-less β -arrestin2 mutant is unable to remain associated with the $\beta 2$ adrenergic receptor (Shenoy et al., 2007); expression of a β -arrestin2–ubiquitin chimera converts the $\beta 2$ adrenergic receptor from class A to class B trafficking (Shenoy and Lefkowitz, 2003); and expression of a β -arrestin2 K^{11/12}R mutant that cannot be ubiquitinated upon binding to the angiotensin AT_{1A} receptor promotes class A trafficking (Shenoy and Lefkowitz 2005). By stabilizing the receptor–arrestin interaction, ubiquitination of β -arrestin2 also favors ERK1/2 activation in GPCR-based signalsomes (Shenoy et al., 2007). As mentioned, ubiquitination of the receptor itself by other β -arrestin-bound ubiquitin ligases, e.g., Nedd4 and AIP4, influences GPCR fate by accelerating their degradation in proteosomes (Bhandari et al., 2007; Shenoy et al., 2008).

Arrestin-bound kinases and phosphatases also modulate receptor endocytosis and trafficking. Arrestin-scaffolded c-Src phosphorylates GRK2, providing negative feedback on receptor desensitization by destabilizing GRK2 and promoting its rapid proteosomal degradation (Penela et al., 2001). Arrestin-Src binding promotes phosphorylation of Tyr⁴⁹⁷ of dynamin1, which regulates dynamin self-assembly (Ahn et al., 1999, 2002). The $\beta 2$ adaptin subunit of AP-2 is also subject to regulation by arrestin-dependent Src phosphorylation (Fessart et al., 2005, 2007; Zimmerman et al., 2009). c-Src stabilizes the association of β -arrestin2 and $\beta 2$ adaptin independent of its kinase activity. Src-mediated phosphorylation of $\beta 2$ adaptin Tyr⁷³⁷ in clathrin-coated pits leads to dissociation of AP-2 from the complex, permitting the receptor–arrestin complex to exit the clathrin-coated vesicle.

The fact that active ERK1/2 only has high affinity for receptor-bound arrestins enables class B GPCRs to generate a spatially constrained pool of ERK1/2 that localizes to endosomes (DeFea et al., 2000b; Tohgo et al., 2002, 2003; Ahn et al., 2004; Lee et al., 2008). As a result, arrestin-dependent ERK1/2 catalytic activity appears to be directed toward membrane or cytosolic substrates. ERK1/2 phosphorylates Ser⁴¹² in the C terminus of β -arrestin1, limiting its ability to bind clathrin (Lin et al., 1999). β -arrestin1 in the cytosol is almost stoichiometrically phosphorylated on Ser⁴¹², and dephosphorylation of Ser⁴¹² upon receptor binding promotes receptor internalization and ERK1/2 activation. Rephosphorylation by ERK1/2 in the signalsome complex probably either provides negative feedback regulation of receptor endocytosis or facilitates receptor internalization by promoting dissociation of β -arrestin1 and clathrin, allowing the receptor to exit clathrin-

coated vesicles. In rodent, but not human, β -arrestin2, ERK1/2 phosphorylation of S¹⁷⁸ stabilizes the GPCR–arrestin complex in endosomes and delays recycling (Khoury et al., 2014). Because casein kinase II has been implicated in phosphorylation of Thr³⁸³ of β -arrestin2, which destabilizes the interaction between arrestin and $\beta 2$ adrenergic receptors (Lin et al., 2002), it is possible that arrestin recruitment of casein kinase II plays a role similar to that proposed for ERK1/2-mediated phosphorylation of β -arrestin1 Ser⁴¹² (Lin et al., 1997).

Arrestin-bound PP2A may also contribute to GPCR trafficking and resensitization. β -Arrestin1-bound PP2A reportedly dephosphorylates β -arrestin1 Ser⁴¹², a step that regulates the interaction between arrestin and the clathrin-coated pit (Hupfeld et al., 2005). Dephosphorylation of GRK-phosphorylated receptors, a prerequisite for receptor resensitization, also involves PP2A. A 150-kDa oligomeric form of PP2A catalyzes the dephosphorylation of $\beta 2$ and $\alpha 2$ adrenergic receptors within the acidic microenvironment of endosomes, allowing receptors to recycle to the plasma membrane (Pitcher et al., 1995; Krueger et al., 1997). Arrestin-bound modulators of cytoskeletal dynamics also play a role. Activated ARF6 mediates the recruitment of clathrin and AP-2 to the GPCR–arrestin complex, nucleating the assembly of endocytic vesicles (Paleotti et al., 2005; Poupert et al., 2007). The association between ARNO and β -arrestin also facilitates GPCR binding and desensitization, as shown for the LH receptor (Mukherjee et al., 2000). β -arrestin1 binding to the SNAP/SNARE binding domain of ATP-bound NSF facilitates the membrane fusion events necessary for vesicle transport, and overexpression of NSF facilitates $\beta 2$ adrenergic receptor endocytosis, suggesting that arrestin-dependent NSF recruitment contributes to clathrin-dependent GPCR internalization (McDonald et al., 1999).

2. Cell Proliferation. β -Arrestins interface with several pathways involved in cell cycle progression, leading to context-dependent effects on cell proliferation. Primary calvarial preosteoblasts from β -arrestin2 null mice proliferate faster than wild type, and treatment with an arrestin pathway-selective biased agonist of the PTH receptor, [D-Trp¹², Tyr³⁴]-bPTH(7-34), slows proliferation in a β -arrestin2-dependent manner, suggesting that arrestins restrain osteoblast proliferation (Gesty-Palmer et al., 2013). Such effects are consistent β -arrestin-dependent sequestration of inactive ERK1/2 in a microtubule-bound pool (Hanson et al., 2007a; Coffa et al., 2011b) and cytosolic retention of active ERK1/2 bound to stable GPCR–arrestin complexes (DeFea et al., 2000b; Tohgo et al., 2002; Ahn et al., 2004). Conversely, neointimal hyperplasia following carotid endothelial injury is reduced in β -arrestin2 null mice, where its loss is associated with decreased GPCR-stimulated vascular smooth muscle cell proliferation and migration, consistent with a stimulatory role for

β -arrestin2 signaling in the proliferative response (Kim et al., 2008b). In this system, knockout of β -arrestin1 has the opposite effect, suggesting that β -arrestin1 and 2 play opposing roles.

One mechanism by which GPCRs stimulate cell proliferation is by mediating crosstalk with the EGF receptor family of tyrosine kinase kinases, which in turn promote Ras-dependent activation of mitogenic ERK1/2 signaling. EGF receptors are activated by matrix metalloprotease-dependent shedding of EGF family growth factors, like heparin-binding EGF, which exist as preformed membrane-associated precursors (Carpenter, 2000), and GPCRs possess both G protein-dependent and G protein-independent mechanisms of promoting their release. EGF receptor transactivation is the major mechanism underlying ERK1/2 activation by endogenous LPA receptors in β -arrestin1/2 null murine embryo fibroblasts, clearly indicating that arrestin signaling is not essential for GPCR-EGF receptor crosstalk (Gesty-Palmer et al., 2005). In contrast, in HEK293 cells, β 1 adrenergic receptor-mediated EGF receptor transactivation and ERK1/2 activation are inhibited by downregulating β -arrestin1/2 or GRK5/6, inhibiting Src or matrix metalloprotease activity, or exposing cells to a heparin-binding EGF neutralizing antibody, suggesting that β 1 receptor-mediated EGF receptor transactivation is β -arrestin-dependent (Noma et al., 2007). Consistent with this, a GRK site mutant β 1 receptor that cannot undergo arrestin-dependent desensitization fails to transactivate EGF receptors despite exaggerated G protein signaling. In human coronary smooth muscle cells, an arrestin pathway-selective angiotensin AT_{1A} receptor agonist, [Sar¹,Ile⁴,Ile⁸]-angiotensin II (SII), induces ERK1/2 activation and proliferation by promoting EGF receptor transactivation (Miura et al., 2004), whereas in rat vascular smooth muscle both angiotensin II and SII stimulate Src-dependent EGF receptor phosphorylation on Tyr⁸⁴⁵, an effect that is lost when β -arrestin2 is downregulated by RNA interference (Kim et al., 2009). Similarly, the LH receptor activates c-Fyn in a β -arrestin2-dependent manner (Galet and Ascoli, 2008). Downregulating arrestin expression reduces the rate of LH receptor internalization and inhibits LH-mediated activation of c-Fyn, phosphorylation of the anti-apoptotic focal adhesion kinase, and the release of EGF-like growth factors.

β -Arrestin-bound Src has also been implicated in ERK1/2 activation by the β 2-adrenergic and neurokinin NK1 receptors (Luttrell et al., 1999; DeFea et al., 2000a). In the latter case, substance P-mediated cell survival and proliferation have been attributed to arrestin-Src signaling. Similarly, β -arrestin1-dependent activation of c-Src and EGF receptor appears to contribute to the tumor-promoting effects of prostaglandin EP2 receptors in papilloma formation (Chun et al.,

2009). Another mechanism by which β -arrestin1-Src increases cell proliferation is by activating a retinoblastoma Rb protein-Raf1 pathway that promotes Rb dissociation from E2F-responsive proliferative promoters, leading to increased E2F1 binding, transcription of S-phase genes, and cell cycle progression (Dasgupta et al., 2006). In bladder cancer, malignant transformation is associated with a thromboxane prostanoid (TP) receptor isoform switch (Moussa et al., 2008). Human bladder cancer cells express high levels of both the TP- β receptor isoform and β -arrestin2. The TP- α and TP- β splice variants differ only in the C terminus, with TP- β carrying a longer tail that allows it to engage β -arrestin2 and undergo agonist-dependent internalization (Parent et al., 1999). Expressing TP- β in nontransformed SV-HUV urothelial cells confers agonist-dependent ERK1/2 and focal adhesion kinase phosphorylation and enhances cell proliferation, migration, and invasion in vitro, responses that are lost when β -arrestin2, but not β -arrestin1, is downregulated by RNA interference.

3. Nonproliferative Cell Growth. Some evidence suggests that arrestins mediate GPCR effects on cell growth. In vitro, an angiotensin AT_{1A} receptor mutant with a deletion in ICL2 that inhibits G protein coupling nonetheless activates a Src-Ras-ERK1/2 pathway leading to cytosolic ERK1/2 and p90RSK activation (Seta et al., 2002). In vivo, cardiomyocyte-specific overexpression of this mutant produces more cardiomyocyte hypertrophy and fetal cardiac gene expression than comparable overexpression of the wild-type receptor (Zhai et al., 2005).

Mechanistically, β -arrestin scaffolding of the ERK1/2 cascade allows it to preferentially target cytosolic substrates involved in the control of protein translation, including the ribosomal S6 kinase, p90RSK (Aplin et al., 2007) and the MAPK-interacting kinase, MNK1, a regulator of the ribosomal protein translation initiation complex. β -Arrestin2-dependent ERK1/2 activation by the AT_{1A} receptor increases phosphorylation of MNK1 and eukaryotic translation initiation factor 4E, increasing rates of mRNA translation (DeWire et al., 2008). In addition, arrestin scaffolding of the PP2A-AKT-GSK3 β complex is involved in regulation of mammalian target of rapamycin-dependent protein translation (Kendall et al., 2014). Increased rates of protein translation in response to angiotensin II or the arrestin pathway-selective AngII analog SII involve activation of arrestin-bound pools of both ERK1/2 and AKT, AKT-mediated phosphorylation of mTOR and its downstream effector p70/p85 ribosomal S6 kinase, and ERK1/2 phosphorylation p90 ribosomal S6 kinase.

4. Cell Survival and Apoptosis. Several arrestin signaling complexes modulate cell survival and apoptotic pathways. In vitro, the arrestin pathway-selective PTH analog [D-Trp¹²,Tyr³⁴]-bPTH(7-34) protects wild-type, but not β -arrestin2 null, osteoblasts from a

proapoptotic etoposide challenge (Gesty-Palmer et al., 2013). Likewise, the arrestin-selective AT_{1A} receptor agonist, Sar¹Ile⁴Ile⁸-AngII, is anti-apoptotic in primary vascular smooth muscle (Ahn et al., 2009). Arrestin signaling also protects cardiomyocytes from undergoing apoptosis in vivo. In response to chronic isoproterenol administration, transgenic mice expressing a GRK site mutant β 1 adrenergic receptor that cannot bind arrestins develop more severe dilated cardiomyopathy, with increased left ventricular end-diastolic dimension and greater myocardial apoptosis, than wild-type β 1 receptor transgenic mice (Noma et al., 2007).

Through their scaffolding functions, arrestins are able to affect the balance between activation of proapoptotic MAPK and anti-apoptotic AKT signaling pathways. β -Arrestin-dependent assembly of the PP2A-AKT-GSK3 β complex allows it to function as a positive regulator of PI3K/AKT-dependent survival signaling (Kendall et al., 2011). Following AT_{1A} receptor activation, PP2A activity within the complex is transiently inhibited, relieving the tonic inhibition of AKT by allowing phosphorylation of T³⁰⁸, the activating AKT phosphorylation site, to rise. Arrestin-dependent activation of the ERK1/2 substrate p90RSK (Seta et al., 2002; Aplin et al., 2007) acts in concert with the PI3K-AKT pathway to downregulate phospho-BAD, inducing anti-apoptotic cytoprotective effects in rat vascular smooth muscle (Ahn et al., 2009). Along with these prosurvival effects on AKT, arrestins tonically suppress proapoptotic signaling by the stress-activated JNK and p38 MAPKs. All of the JNK MAPKs can bind to all visual/ β -arrestins, but they exhibit highest affinity for the inactive arrestin conformation, suggesting that the dominant role of arrestins is to repress basal JNK signaling (Song et al., 2007, 2009a). Likewise, in at least some systems, arrestins serve primarily to attenuate G protein-dependent p38 MAPK activation through GPCR desensitization. β -Arrestin1/2 null murine fibroblasts exhibit enhanced activation of ERK1/2, JNK1/2, and p38 MAPK in response to the chemokine CXCR2 receptor agonist, IL-8, whereas β -arrestin expression confers protection from oxidative burst-induced cell death by attenuating JNK/p38MAPK activation (Zhao, et al., 2004). In murine embryo fibroblasts, β -arrestin expression increases resistance to serum deprivation-induced apoptosis by increasing AKT and dampening basal ERK1/2 and P38 MAPK pathway activity (Yang et al., 2012).

Although much evidence suggests that on balance, arrestin scaffolds promote cell survival, arrestins may mediate proapoptotic signals under certain conditions. By sequestering Mdm2, β -arrestin2 restricts its access to other substrates. Mdm2 is a major negative regulator of the p53 tumor suppressor, because ubiquitination of p53 by Mdm2 promotes its proteosomal degradation. Arrestin binding attenuates p53 ubiquitination, increasing p53 abundance, and enhancing p53 signaling.

As a result, overexpressing β -arrestin2 in HEK293 enhances, and downregulating it attenuates, p53-mediated apoptosis (Wang et al., 2003). Similarly, the angiotensin receptor blocker, losartan, has been reported to attenuate neuronal damage in an animal model of cerebral ischemia by inhibiting the assembly of a β -arrestin2-ASK1-MKK4 signaling module and repressing the activation of JNK3, c-jun, and caspase-3, and the release of cytochrome C (Zhang et al., 2012).

Additional evidence that arrestins can trigger apoptotic cell death comes from the study of *Drosophila* phototransduction. Phototransduction in the fly is different from the rhodopsin-transducin-cGMP phosphodiesterase mechanism found in mammals, relying instead on a Gq-coupled pathway to activate light-sensitive Trp channels. Desensitization of *Drosophila* rhodopsin involves both arrestin and the diacylglycerol kinase, RdgA. Loss of function mutation of either protein results in constitutive Trp channel activation and photoreceptor cell necrosis due to excessive G protein signaling (Dolph et al., 1993; Alloway et al., 2000; Kiselev et al., 2000; Raghu et al., 2000). Conversely, enhancing arrestin function appears to promote photoreceptor cell apoptosis. RdgC is a calcium-dependent kinase that normally dissociates the rhodopsin-arrestin complex. Introducing inactivating mutations of RdgC leads to retinal degeneration due to apoptosis of photoreceptor cells (Davidson and Steller, 1998). Complementary mutations that stabilize the rhodopsin-arrestin complex, such as Gq loss of function or deletion of the regulatory arrestin phosphorylation domain, enhance this form of retinal degeneration. The phenotype can be rescued either by expression of a p35 caspase inhibitor or by triple inactivation of G α q, arrestin, and RdgC, suggesting that it is the result of an arrestin-dependent apoptotic signal originating from a stable rhodopsin-arrestin complex (Kiselev et al., 2000). Consistent with this, apoptotic photoreceptor cell death caused by deletion of the eye-specific PLC gene, which likewise promotes assembly of constitutive rhodopsin-arrestin complexes, can be reversed by simultaneous deletion of arrestin or inhibition of rhodopsin endocytosis (Alloway et al., 2000).

5. Cell Migration and Chemotaxis. β -arrestin scaffolds mediate GPCR effects on actin cytoskeletal rearrangement and play important roles in cell migration, chemotaxis, and cancer metastasis. GPCR-stimulated chemotaxis is dependent upon two factors: the ability to sense a chemoattractant gradient and to establish cell polarity through cytoskeletal rearrangement at the leading edge (DeFea, 2013). Arrestin-dependent GPCR desensitization and recycling are critical to the former (Tomhave et al., 1994; Aragay et al., 1998), whereas the coordinated regulation of several processes by β -arrestin scaffolds, including cofilin dephosphorylation, filamin A recruitment, MAPK activation, and regulation of small GTPases, collectively contributes

to the cellular shape changes needed for the latter (Ge et al., 2003, 2004; Barnes et al., 2005; Hunton et al., 2005).

Consistent with the critical role of β -arrestin scaffolds in the spatial control of cytoskeletal dynamics, splenocytes derived from β -arrestin2 null mice exhibit strikingly impaired chemotactic responses to stromal cell–derived factor-1, CXC chemokine ligand 12 (Fong et al., 2002). Downregulation of either β -arrestin1 or 2 in MDA-MB-468 cells inhibits PAR2-stimulated cofilin dephosphorylation and chemotaxis, suggesting that β -arrestin scaffolding of the cofilin–chronophin–LIM kinase complex is necessary for the localized generation of free barbed ends on actin filaments that produce filament extension and generate membrane protrusions (Zoudilova et al., 2007, 2010). Similarly, assembly of a complex containing the angiotensin AT_{1A} receptor, β -arrestin, ERK1/2, and the actin filament–bundling protein, filamin A, is involved in the formation of membrane ruffles in Hep2 cells (Scott et al., 2006).

Arrestin-dependent targeting of MAPKs also plays a role. During PAR2-induced chemotaxis, PAR2–arrestin–ERK1/2 complexes localize to the leading edge of the cell, where ERK1/2 activity is required for actin cytoskeletal reorganization (Ge et al., 2003). In HeLa and HEK293 cells, overexpression/downregulation of β -arrestin2 reciprocally enhances/attenuates activation of both p38 MAPK and ERK1/2 by the chemokine receptor CXCR4, and inhibition of p38MAPK blocks β -arrestin2–dependent chemotaxis, suggesting a role for arrestin scaffolding of p38MAPK in CXCR4-stimulated cell migration (Sun et al., 2002). Arrestin-dependent scaffolding of p38 MAPK has likewise been implicated in control of cell polarization, actin bundle formation, and internalization of platelet-activating factor receptors in polymorphonuclear neutrophils (McLaughlin et al., 2006). Arrestin-dependent regulation of the small GTPase, RalA, through its interaction with Ral-GDS, has been implicated in formyl-Met-Leu-Phe receptor–stimulated membrane ruffling (Bhattacharya et al., 2002), Ral-dependent activation of PLC δ 1 by the angiotensin AT_{1A} receptor (Godin et al., 2010), and LPA receptor–mediated proliferation and migration of breast cancer cells (Li et al., 2009). Regulation of RhoA via the interaction between β -arrestin1 and ARFGAP21 promotes angiotensin AT_{1A} receptor-dependent RhoA activation and membrane ruffling (Anthony et al., 2011).

6. Modulation of the Immune Response. In immune system, β -arrestin scaffolds perform key roles through the negative regulation of G protein–mediated responses, promotion of chemotaxis, regulation of exocytosis and degranulation, and signal dampening through sequestration of pathway components (Jiang et al., 2013).

Within the innate immune system, β -arrestin2 null neutrophils show enhanced CXCR2-mediated Ca²⁺

signaling and superoxide generation, reflecting the loss of β -arrestin–dependent desensitization (Su et al., 2005). β -arrestins regulate macrophage chemotaxis both by desensitizing chemokine CCL2-induced Ca²⁺ signaling and by scaffolding ERK1/2-dependent assembly of the actin cytoskeleton in pseudopodia (Aragay et al., 1998; Ge et al., 2003; Cheung et al., 2009). In polymorphonuclear leukocytes, β -arrestin1–bound c-Hck and c-Fgr regulate IL-8 CXCR1 receptor–stimulated granule exocytosis (Barlic et al., 2000), similar to the reported role of a β -arrestin1–c-Yes complex in the control of endothelin-1–stimulated translocation of exocytic granules containing the glucose transporter GLUT4 (Imamura et al., 2001). Isolated polymorphonuclear leukocytes lacking β -arrestin2 exhibit increased basal and lipopolysaccharide-stimulated release of the inflammatory cytokine TNF- α and IL-6 (Basher et al., 2008), perhaps due to the loss of tonic inhibition of NF κ B transcriptional pathways by β -arrestin2–dependent sequestration of I κ B α and I κ B kinases (Witherow et al., 2004). β -Arrestin2 also negatively regulates the activity of natural killer cells by recruiting SHP-1 and SHP-2 to the inhibitory receptor KIR2DL1 (Yu et al., 2008).

β -Arrestin scaffolds also play important regulatory roles within T and B cells of the adaptive immune system. T cell receptor activation by major histocompatibility complex antigens leads to activation of a cAMP–PKA–Csk pathway in lipid rafts that inhibits proximal T cell signaling (Bjorgo et al., 2010, 2011). Full T cell activation requires the binding of costimulatory molecules to CD28, which uses β -arrestins to recruit the cAMP phosphodiesterase PDE4 into lipid rafts, relieving PKA-dependent phosphorylation of Csk and allowing T cell activation to proceed (Abrahamsen et al., 2004; Baillie and Houslay, 2005; Bjorgo et al., 2010). As in macrophages, β -arrestin2 null CD4⁺ T cells exhibit impaired chemotactic migration (Walker et al., 2003; Raghuwanshi et al., 2008; Walker and DeFea, 2014). By regulating histone H4 acetylation at the Bcl2 locus, β -arrestin1 enhances Bcl2 expression in both naive and activated CD4⁺ T cells, promoting T cell survival and inhibiting apoptosis following cytokine withdrawal (Kang et al., 2005; Shi et al., 2007).

7. Developmental Regulation. Arrestins play important roles in embryological development, perhaps reflecting their interaction with non-GPCR elements of the Shh–Smoothed, Wnt, and Notch signaling pathways (Kovacs et al., 2009). As mentioned, β -arrestins bind Smoothed, the non-G protein–coupled seven-transmembrane receptor component of the Shh signaling pathway, and data from zebrafish suggest that β -arrestin2 functions in the Hedgehog pathway between Smoothed and its downstream transcription factor targets, Su(fu) and Gli1, to promote Shh-dependent transcription (Wilbanks et al., 2004).

Knockdown of β -arrestin2 blocks expression of several Shh-regulated genes and phenocopies developmental defects observed in Smoothed loss-of-function mutants, while restoring β -arrestin2 or constitutively activating the Hedgehog pathway downstream of Smoothed rescues the defect. Interestingly, β -arrestin1 has no effect on the expression of Shh-regulated genes in zebrafish, but nonetheless plays a key role in embryologic development (Yue et al., 2009). Zebrafish embryos lacking β -arrestin1 fail to undergo hematopoiesis and exhibit severe posterior defects resulting from downregulation of *cdx4*, a homeobox transcription factor that specifies the hematopoietic lineage by modulating *hox* gene expression. Hematopoiesis can be rescued either by reintroducing β -arrestin1 or injecting *cdx4*, *hoxa9a*, or *hoxb4a* mRNA. The mechanism appears to involve sequestration of the polycomb group (PcG) recruiter YY1 by nuclear β -arrestin1, which relieves PcG-mediated repression of *cdx4*-*hox* pathway.

The binding of β -arrestins to Dsh in the Wnt-Fz signaling pathway is involved in the regulation of canonical Wnt signaling in *Xenopus*, where knockdown of β -arrestin2 reduces β -catenin signaling and blocks Wnt8- or Dsh-induced axis duplication (Bryja et al., 2007). β -Arrestin2 is also required for convergent extension during *Xenopus* axis elongation, a process mediated by the noncanonical Wnt/planar cell polarity pathway (Kim et al., 2008a). During convergent extension, β -arrestin2 and the Ryk receptor tyrosine kinase cooperatively mediate endocytosis of Fz7 and Dsh after Wnt11 stimulation, a process involving activation of Rac1 (Bryja et al., 2008). Without this, β -arrestin2-deficient mesoderm fails to polarize and intercalate with wild-type mesoderm at the embryonic midline (Kim et al., 2008a).

During *Drosophila* development, the single nonvisual arrestin homolog, Kurtz, regulates the functions of Notch, a single-transmembrane-spanning receptor involved in the process of lateral inhibition (Chastagner et al., 2008). Notch ligands are transmembrane proteins that initiate juxtacrine signals resulting in proteolysis of Notch and translocation of the free Notch intracellular domain to the nucleus where it acts as a transcriptional regulator (Kopan and Ilagan, 2009). The single Notch receptor in flies is regulated via a complex with Kurtz, wherein Kurtz promotes ubiquitination of Notch by the E3 ligase, Deltex, in much the same manner that β -arrestins regulate the ubiquitination of several mammalian GPCRs (Mukherjee et al., 2005).

8. Central Nervous System Function, Learning, and Behavior. G protein-coupled metabotropic neurotransmitter receptors are critical to central nervous system function, and arrestin-dependent scaffolding and GPCR desensitization have been shown to be important in diverse central nervous system processes. The original phenotype described in β -arrestin2

null mice was impaired μ opioid receptor (MOR) desensitization leading to prolonged analgesic effects of morphine, indicating that arrestin-dependent desensitization was the principal determinant of opiate duration of action (Bohn et al., 1999, 2000). Subsequent work indicated that some nonanalgesic effects of opiates, e.g., MOR-mediated constipation, respiratory suppression and physical dependence (Raehal et al., 2005, 2011; Raehal and Bohn, 2011), and κ opioid receptor-mediated dysphoria (Bruchas et al., 2006; Redila and Chavkin, 2008), were diminished in the absence of β -arrestin2, suggesting that β -arrestin2 signaling may underlie these responses.

Dopaminergic neurotransmission in the central nervous system regulates behavioral responses such as locomotor activity and neural reward mechanisms, and several lines of evidence suggest that arrestin-signaling complexes regulate dopamine-dependent behaviors. In striatum, the β -arrestin2-PP2A-AKT-GSK3 β complex modulates D2 dopamine receptor-mediated behaviors by tonic repression of β -catenin signaling. GSK3 β phosphorylates β -catenin, accelerating its degradation. Thus, striatal extracts from β -arrestin2 null mice have higher levels of β -catenin, resulting from the loss of signalsome-mediated GSK3 β activation (Beaulieu et al., 2005, 2008). Mice lacking β -arrestin2 exhibit a reduced locomotor hyperactivity response when striatal dopamine signaling is increased either by administration of the drug apomorphine or by dopamine transporter knockout. Inhibiting PP2A or GSK3 β produces similar effects in wild-type mice. Interestingly, at therapeutic concentrations, lithium disrupts the β -arrestin2-PP2A-AKT complex, leading to GSK3 β inhibition, the mechanism by which it exerts its mood-stabilizing effects (Beaulieu et al., 2008).

β -Arrestin signaling has also been implicated in processes related to learning and memory. In mice, deletion of β -arrestin2, but not β -arrestin1, results in deficits in plasticity mediated selectively by group I metabotropic glutamate receptors (mGluRs) in CA3 and CA1 pyramidal neurons (Eng et al., 2016). mGluR1 modulation of intrinsic conductances as well as non-mGluR-mediated long-term potentiation is preserved in β -arrestin 2 null mice, indicating that the arrestin dependency is specific to a subset of mGluR-mediated responses. Experiments performed using pharmacological inhibitors implicate the β -arrestin2 cargos c-Src and ERK1/2 in the effects, suggesting that arrestin scaffolds are involved in regulating mGluR changes in synaptic strength.

D. Silent Scaffolds and Tonic Effects on Pathway Activity

A fourth property of visual/ β -arrestins that contributes to their effectiveness as scaffolds is simply that they are relatively abundant in relation to the GPCRs they desensitize and catalytically active signaling

proteins they carry as cargos. The ability to shuttle between abundant low-affinity binding sites on microtubules and smaller numbers of high-affinity binding sites on activated GPCR permits them to sequester key pathway intermediates away from their site of activation and dampen basal pathway activity until called to respond to an extracellular stimulus.

The dichotomous effects of β -arrestins on ERK1/2 and JNK pathway activation underscore the point that dampening basal signaling pathway may be as important to arrestin function as their ability to support pathway activation. As discussed, ERK1/2 has significant affinity for only the microtubule-bound and receptor-bound arrestin conformations, whereas cRaf-1 and activated ERK1/2 have highest affinity for the receptor-bound arrestin conformation (Luttrell et al., 2001; Coffa et al., 2011b). The result is that inactive ERK1/2 can be sequestered by a microtubule-bound arrestin pool until called to a receptor, where cRaf-1 activation by plasma membrane-delimited effectors, e.g., Ras, initiates the ERK1/2 activation cascade (Jafri et al., 2006). In contrast, activation of the ASK1–MKK4/7–JNK3 cascade does not appear to be a GPCR-regulated process. Although the original study reported that stimulation of angiotensin AT_{1A} receptors activated JNK3 and caused it to colocalize with β -arrestin2 in endosomal vesicles (McDonald et al., 2000), later work performed using the β 2 adrenergic receptor found no evidence of receptor-mediated JNK3 activation under conditions where ERK1/2 was being robustly activated via the β -arrestin2 pathway (Breitman et al., 2012). In fact, JNK3 affinity is highest for inactive arrestin mutants that do not bind GPCRs, further supporting the concept that whereas β -arrestin-dependent ERK1/2 activation is receptor-dependent, JNK3 activation is not (Song et al., 2006; Breitman et al., 2012). Instead, the principal effect of arrestins on JNK appears to be to dampen pathway activity. Although all four visual/ β -arrestins bind JNK3 comparably and redirect it from the nucleus, where it spontaneously localizes, to the cytoplasm (Song et al., 2007), only β -arrestin2 scaffolds JNK3 activation. In the other arrestin isoforms, the properties of JNK3 binding and activation are dissociated (Song et al., 2009a; Zhan et al., 2011b). Even though JNK3 associated with cytosolic β -arrestin2 is active, the β -arrestin2 NES keeps JNK3 out of the nucleus. Thus, whereas arrestins target ERK1/2 to specific substrates through GPCR-dependent activation and tethering to GPCR–arrestin complexes, arrestin binding appears to keep JNK3 away from both GPCRs and its nuclear transcription factor targets (Lin and Defea, 2013).

Similar signal-dampening effects of arrestin binding have been reported for other arrestin effectors. Downregulating β -arrestin1 in HeLa cells increases NF- κ B activation by TNF- α consistent with the hypothesis that arrestins tonically inhibit NF- κ B signaling by

protecting I κ B α from degradation (Witherow et al., 2004). In HEK-293 cells, downregulating arrestin expression attenuates TLR4-mediated ERK1/2 activation while at the same time enhancing NF κ B reporter activity, indicating that arrestins exert opposing effects on the ERK1/2 and NF κ B pathways (Fan et al., 2007). Another example is the binding of nuclear β -arrestin1 to PPAR γ , which competes for RXR α binding and attenuates PPAR γ -RXR α -dependent transcription (Zhuang et al., 2011).

E. Self-Association of Arrestins

Although GPCR-bound arrestins appear to be monomeric, three of the four visual/ β -arrestins are capable of self-association in solution (Chen et al., 2014), with only cone arrestin being a constitutive monomer. Despite this shared property, the structure, regulation, and physiologic significance of arrestin multimers differ between visual arrestin and the β -arrestins.

Visual arrestin forms tetramers both in crystal form and in solution; however, the crystallized and solution tetramers differ in organization (Granzin et al., 1998; Hirsch et al., 1999; Hanson et al., 2007b; Kim et al., 2011). In solution structure, visual arrestin tetramers adopt a circular conformation in which all self-association interfaces are engaged, explaining why self-association stops at tetramers. As importantly, in the solution tetramer all visual arrestin elements involved in rhodopsin binding are either engaged or shielded by other protomers, which is why only monomeric visual arrestin can bind rhodopsin (Hanson et al., 2007b; Beyriere et al., 2015; Kang et al., 2015). After rhodopsin, visual arrestin is the most abundant protein in the rod outer segment, and a delicate balance between spontaneous visual arrestin self-association and light-dependent translocation works together to keep a constant supply of active rhodopsin-binding visual arrestin monomer in the outer segment (Gurevich et al., 2011). In this system, the tetrameric visual arrestin complex appears to function as a storage form, whose role is to protect photoreceptor cells from potentially toxic effects of excess free monomeric visual arrestin (Schubert et al., 1999; Hanson et al., 2007b; Kim et al., 2011). To maintain light sensitivity at the single photon level, rods must contain high enough levels of visual arrestin to quench signaling by all expressed rhodopsin. Loss of visual arrestin or defective rhodopsin phosphorylation resulting from deletion of the rhodopsin kinase, GRK1, led to photoreceptor death, presumably due to excessive signaling (Song et al., 2013). Paradoxically, introducing an enhanced phosphorylation-independent visual arrestin mutant that is impaired in self-association into a GRK1/visual arrestin null background, which would be expected to rescue the defect, produces biphasic effects. When expressed at 50% of wild-type levels, the expected rescue is observed, but at higher levels of expression

the mutant itself is toxic. The difference appears to relate to its inability to form inactive cytosolic multimers. At physiologic levels of expression, monomeric wild-type visual arrestin comprises only about 1.5% of the total arrestin complement of dark-adapted rods, with the balance sequestered in dimeric or tetrameric form. Expressing the mutant at 240% of wild-type levels increases free visual arrestin about threefold over physiologic levels, causing accelerated photoreceptor degeneration (Huang et al., 2010; Moaven et al., 2013; Song et al., 2013). Thus, in the retina, visual arrestin self-association appears to provide a mechanism for maintaining arrestin levels high enough to meet physiologic needs while keeping monomeric arrestin levels low.

The β -arrestins likewise readily form homo- and hetero-oligomers at physiologic levels of expression (Storez et al., 2005; Milano et al., 2006). As noted, the abundant cellular metabolite, IP6, which inhibits self-association of visual arrestin, greatly enhances β -arrestin1 self-association (Hanson et al., 2008). In the presence of IP6, β -arrestin2 forms dimers wherein IP6 connects the C domains of two adjacent molecules, whereas β -arrestin1 forms infinite chains with IP6 connecting the concave sides of the N and C domains of adjacent protomers (Chen et al., 2014). As both IP6 binding sites on β -arrestin1/2 overlap the receptor binding sites, it is likely that oligomeric β -arrestins represent a storage form, as in the case of visual arrestin (Milano et al., 2006). Because β -arrestin1 mutants with impaired IP6 binding show increased nuclear localization, oligomerization may also serve to keep constitutively bound arrestin cargos, like JNK3, out of the nucleus. The formation of β -arrestin1/2 hetero-oligomers may have a similar function, in that the NES of the β -arrestin2 protomer may help sequester β -arrestin1 in the cytosol (Storez et al., 2005). Interestingly, a β -arrestin2 mutant that does not bind IP6 was also found to lack affinity for Mdm2 and could not suppress Mdm2-dependent degradation of p53, suggesting that Mdm2 sequestration is a specific function of dimeric β -arrestins (Boularan et al., 2007). Another report found that K²⁸⁵A/R²⁸⁶A mutations in the polar core of β -arrestin1 that reduced formation of homodimers also impaired β 2-adrenergic receptor binding, ERK1/2 binding, and β 2-adrenergic receptor-dependent ERK1/2 activation (Xu et al., 2008). Whether this indicates β -arrestin1 self-association is involved in controlling arrestin-dependent scaffolding of the ERK1/2 complex, or that common residues are involved in both β -arrestin oligomerization and scaffolding, remains to be determined.

IV. Visual/ β -Arrestins as Therapeutic Targets

A. Orthosteric and Allosteric Modulation of GPCR Signaling

Because they are involved in the coordination and control of nearly every physiologic process, it is not

surprising that GPCRs are the most commonly exploited drug targets by a wide margin (Lappano and Maggiolini, 2011). The visual/ β -arrestins, in turn, by virtue of their unique combination of desensitizing/scaffolding functions, are key determinants of the balance between G protein and non-G protein signals stemming from GPCR activation. As the cellular responses mediated by these two distinct signaling systems differ, manipulating them independently, i.e., changing the balance between G protein and arrestin-dependent processes, might offer distinct advantages over conventional agonist or antagonist approaches (Luttrell, 2013).

GPCRs, which were originally envisioned as simple detectors of hormones in the extracellular environment (Ahlquist, 1948), then as binary switches whose proportional distribution in the off and on states is determined by the intrinsic efficacy and concentration of agonist ligands (Samama, et al., 1993), have come to be viewed more broadly as components of an allosterically regulated signal transduction machinery whose function is to bind molecules, i.e., extracellular ligands, at one location, and change shape to affect the binding and conformation of other molecules, i.e., intracellular effectors, at another location (Kenakin, 2012). Embedded within this general allosteric model of GPCR function are the concepts of pluridimensional efficacy, i.e., that GPCRs signal by engaging multiple G protein and non-G protein effectors (Galandrin et al., 2007), and functional selectivity, i.e., that ligand-specific signaling patterns can emerge because of differences in the efficiency with which ligands stabilize the structural conformations that couple the receptor to each of its possible downstream effectors (Kenakin and Christopoulos, 2013).

Numerous examples of ligand bias have been described, both favoring G protein coupling over GRK phosphorylation and arrestin recruitment, and favoring arrestin coupling over G protein signaling, and the potential of biased ligands as therapeutics has been the subject of multiple reviews (Luttrell and Kenakin, 2011; Whalen et al., 2011; Kenakin and Christopoulos, 2013; Kenakin, 2015). It is less clear at present whether more nuanced form of bias is attainable, e.g., dissociating arrestin-dependent desensitization from arrestin-dependent signaling. Because some GPCRs appear able to engage arrestins in a manner that permits endocytosis without precluding heterotrimeric G protein coupling (Thomsen et al., 2016), it is possible that ligand structure might be able to drive both receptor internalization and prolonged G protein signaling by preferentially stabilizing different arrestin-docking poses.

The main attraction of ligand bias as a means to modulate arrestin function is tissue selectivity, a critical consideration for a target as ubiquitous and multifunctional as the β -arrestins. Because the ligand–GPCR complex, not just the GPCR, determines how the cell

will respond, biased ligands can in effect create new receptors whose signaling properties differ from that of the same receptor bound to its native ligand. Although the structure of the ligand–GPCR complex will determine which effectors are engaged, the tissue distribution/expression of the receptor will determine where that activation occurs (Luttrell, 2014). Another intriguing property of such ligands is that they can activate the same effector in different ways. Biophysical measurements of the conformational signature of β -arrestin2, induced by different angiotensin AT_{1A} receptor agonist peptides, show that structurally distinct ligands can produce different arrestin conformations, leading to differences in arrestin–receptor avidity and the efficiency of arrestin-dependent ERK1/2 activation (Zimmerman et al., 2012; Lee et al., 2016). Similar findings have been reported for G proteins, where human and salmon calcitonin activating the calcitonin receptor produce distinct G α s conformations that differ in their rate of GTP exchange and efficiency with which they activate adenylyl cyclase (Furness et al., 2016).

Modulation of arrestin function through allosteric sites on GPCRs is conceptually similar. Allosteric modulators are molecules that bind GPCR domains that are topographically distinct from the orthosteric site, leading to an increase or decrease in the ability of the orthosteric ligand to interact with the receptor and/or modulate its ability to stabilize active receptor conformations (Kenakin and Miller, 2010). Allosteric modulators have several theoretical advantages over orthosteric ligands, including improved subtype selectivity for closely related GPCRs with conserved ligand-binding pockets (Lazareno et al., 1998; Ellis and Seidenberg, 2000) and low risk of overdose, as their effects are saturable. Because the activity of an allosteric modulator is dependent upon the presence of the endogenous ligand, its effects are tied to endogenous patterns of ligand release and less likely to produce tachyphylaxis (Kenakin, 2009; Wang et al., 2009). Additionally, allosteric modulators may possess intrinsic agonism, i.e., allo-agonism, and can bias the stimulus leading to signaling pathway-selective effects, e.g., favoring G protein or arrestin coupling (Sachpatzidis et al., 2003; Zhang et al., 2005).

B. Inhibiting or Ablating Arrestins

In some settings, it may be desirable to inhibit arrestin function, either to enhance G protein signaling by reducing GPCR desensitization, or to block potentially deleterious arrestin-mediated signaling. One strategy that has been exploited is to target GRKs. Interestingly, of the four widely expressed extraretinal GRKs, GRK2/3 and GRK5/6 have been shown to perform different functions with respect to GPCR desensitization and signaling (Kim et al., 2005; Ren et al., 2005; Noma et al., 2007; Zimmerman et al., 2012). Whereas downregulating GRK2/3 expression inhibits

β -arrestin-dependent desensitization and internalization of the angiotensin AT_{1A} and vasopressin V2 receptors, downregulation of GRK5/6 disproportionately reduces β -arrestin-dependent ERK1/2 activation, suggesting that isoform-selective GRK inhibitors might offer a means to modulate arrestin function. In congestive heart failure, GRK2 is upregulated as a consequence of chronic activation of the sympathetic nervous system, leading to chronic desensitization and downregulation of cardiac β 1 adrenergic receptors and a marked reduction of myocardial inotropic reserve (Cannavo et al., 2013; Woodall et al., 2014; Sato et al., 2015). In several animal models of heart failure, genetic deletion of GRK2, or its inhibition through transgenic or adenoviral expression, a G $\beta\gamma$ subunit sequesterant peptide has been shown to improve functional and morphologic cardiac parameters, including enhanced responsiveness to adrenergic stimuli due to the alleviation of arrestin-dependent tachyphylaxis (Rockman et al., 1998; Harding et al., 2001; Raake et al., 2013). In addition, small-molecule inhibitors of G $\beta\gamma$ subunits (Casey et al., 2010; Piao et al., 2012) or GRK2 itself (Thal et al., 2012) have shown beneficial effects in rodent heart failure models, and newer GRK isoform-specific inhibitors are in development (Homan et al., 2015).

Direct inhibitors of visual/ β -arrestins have not been described to date, although modulation of arrestin function through binding small molecules has been demonstrated in the case of IP6, which interacts with discrete binding sites in the N and C domains (Milano et al., 2006; Hanson et al., 2008). By promoting self-association, IP6 binding sequesters β -arrestins in an inactive cytosolic pool, away from the plasma membrane and nucleus, and changing its affinity for some cargos (Boularan et al., 2007; Xu et al., 2008). Although its highly charged nature and relatively low affinity make IP6 unsuitable as an arrestin inhibitor, its effects illustrate the feasibility of small molecules targeting arrestins.

Rather than small molecules, experimental approaches to inhibiting arrestins have involved genetic ablation or downregulation by RNA interference. β -Arrestin downregulation predictably results in decreased GPCR internalization, prolonged G protein-mediated signaling, and loss of arrestin-dependent responses, including arrestin-dependent chemotaxis (Ahn et al., 2003; Hunton et al., 2005). In vivo, β -arrestin2 downregulation by intrathecal administration of small-interfering RNA in rats has been shown to potentiate the antinociceptive effects of opiates and attenuate opiate tolerance (Yang et al., 2011), consistent with observations made in β -arrestin2 knockout mice (Bohn et al., 1999, 2000). Arrestin-specific aptamers, oligonucleotides whose secondary and tertiary structures enable selective binding to the surface of protein targets and blockade of protein–protein

interactions, have also been developed (Kotula et al., 2014). Administration of a β -arrestin2-specific aptamer to leukemic cells has been shown to impair β -arrestin-dependent signaling and inhibit tumor progression in CML models and samples obtained from human patients. Conjugating the arrestin-specific aptamer to another aptamer that is tumor cell specific enhanced its effectiveness.

C. Customizing Arrestin Function

Many diseases result from either gain- or loss-of-function mutations that render signaling pathways insensitive to normal regulation, such as constitutive activation of GPCRs (Spiegel, 1996; Schöneberg et al., 2004) or inactivating mutations of GRK1 and visual arrestin (Baylor and Burns, 1998). One strategy for gene therapy rescue of such defects is to introduce compensatory mutations into other pathway components that restore pathway regulation. The structure of visual/ β -arrestins is such that they can be customized in a manner that enhances or removes certain functions without compromising their overall function (Gurevich and Gurevich, 2013). GPCR binding involves the concave surfaces of the N and C domains, where a relatively few residues determine receptor selectivity (Vishnivetskiy et al., 2011; Gimenez et al., 2014a), whereas most other cargo proteins interact with the outer surfaces that remain exposed on the receptor-bound arrestins. Similarly, the clathrin and AP2 binding sites in the C-terminal tail do not overlap with the sites for other binding partners. Hence, mutation of these sites can readily dissociate β -arrestin-dependent desensitization, which only requires recruitment to GPCRs on the plasma membrane, from clathrin-dependent GPCR endocytosis, which requires clathrin and AP2 binding (Goodman et al., 1997; Laporte et al., 2000).

Several customized arrestins have been developed, and one has even been tested in gene therapy rescue experiments (Gurevich et al., 2014). As noted, arrestin activation is triggered by the interaction between phosphate residues on the GPCR C terminus and the arrestin phosphate sensor, and point mutations within the phosphate sensor produce an arrestin molecule that binds activated receptors independent of their phosphorylation state (Gurevich and Benovic, 1995, 1997). One such enhanced arrestin1 mutant has been shown to partially compensate for the loss of GRK1, facilitating the rate of rod recovery and improving rod survival more efficiently than equivalently overexpressed wild-type arrestin-1 (Song et al., 2009b). Similar enhanced phosphorylation-independent versions of all visual/ β -arrestins have been constructed and shown to effectively desensitize several GPCRs without receptor phosphorylation (Kovoor et al., 1999; Celver et al., 2001, 2002), although their utility as therapeutic agents outside the retina would probably also require

mutagenic manipulation of their GPCR selectivity to avoid global attenuation of G protein signaling.

All visual/ β -arrestins except cone arrestin are capable of self-association, and arrestin oligomers most likely represent a cytosolic storage form of the protein, as only monomeric arrestins can bind GPCRs. The capacity to self associate appears most physiologically relevant for visual arrestin, as the equilibrium between tetrameric, monomeric, and rhodopsin-bound pools protects photoreceptor cells from the toxic effects of free visual arrestin monomers (Song et al., 2013). Targeted elimination of two homologous phenylalanine residues in bovine and mouse visual arrestin generates self-association deficient mutants, indicating that the equilibrium between the storage and reactive forms of visual arrestins is a factor that can be manipulated independent of other arrestin functions (Hanson et al., 2008; Kim et al., 2011). Unlike visual arrestin, self-association of β -arrestins is enhanced by IP6 binding, and the behavior of β -arrestin mutants with impaired IP6 binding suggests that self-association plays a regulatory role (Hanson et al., 2008; Chen et al., 2014). By virtue of its NLS, monomeric β -arrestin1 will readily enter the nucleus. In contrast, β -arrestin1 oligomers are predominantly cytosolic, allowing them to sequester nuclear cargos like JNK3 (Milano et al., 2006). Although β -arrestin2 is normally cytosolic due to the presence of a NES, IP6-dependent oligomerization appears to regulate its interaction with Mdm2, such that elimination of IP6-binding residues interferes with its tonic Mdm2-dependent inhibition of p53 signaling and antiproliferative effects (Boularan et al., 2007).

Arrestin's scaffolding functions can also be manipulated. Although GPCR binding is essential for β -arrestin-dependent activation of ERK1/2 (Coffa et al., 2011b), β -arrestin2-mediated facilitation of JNK3 phosphorylation is receptor-independent, suggesting that free cytosolic arrestin performs this function (Song et al., 2006; Zhan et al., 2011b). Although the binding sites of MAPKs remain poorly defined, several mutated forms of β -arrestin that affect MAPK signaling have been created. An R³⁰⁷A mutant of β -arrestin1 has impaired cRaf-1 binding but binds normally to MEK1/2 and ERK1/2 (Coffa et al., 2011a). When expressed in β -arrestin1/2 null fibroblasts, this mutant retains its ability to engage phosphorylated β 2-adrenergic receptors, but fails to reconstitute arrestin-dependent ERK1/2 activation. Thus, it might function as a dominant-negative inhibitor of arrestin-dependent ERK1/2 activation while not impairing receptor desensitization. In the case of JNK3, both wild-type β -arrestin2 and a seven-residue deletion mutant in the hinge region that preferentially binds microtubules (Vishnivetskiy et al., 2002; Hanson et al., 2007a) efficiently scaffold the ASK1-MKK4-JNK3 module, whereas the preactivated β -arrestin2 mutant that exhibits enhanced GPCR binding (Celver et al., 2002) and a receptor-binding deficient

mutant wherein receptor-binding residues were mutated to alanine (Vishnivetskiy et al., 2011; Gimenez et al., 2012b) do not support JNK3 activation (Breitman et al., 2012). The latter mutant is able to bind ASK1, MKK4, and JNK3 efficiently, but, like β -arrestin1, does not promote JNK3 activation and does not recognize GPCRs. Thus, it behaves as a silent scaffold, inhibiting JNK3 activation by sequestering ASK1, MKK4, and JNK3 away from productive scaffolds while not transitioning to the low JNK3 affinity conformation induced by receptor binding, and might serve to suppress proapoptotic signaling in cells. Conversely, a 25-amino-acid miniscaffold derived from β -arrestin2 is sufficient to bind ASK1, MKK4/7, and JNK3 and scaffold JNK3 activation in cells, suggesting that arrestin-derived peptides can be used to manipulate antiproliferative and proapoptotic JNK signaling in cells (Zhan et al., 2016).

D. Potential Therapeutic Applications

Regardless of the approach used to manipulate visual/ β -arrestins, their universality and myriad functions must raise concerns when thinking of them as drug targets. Even functionally selective orthosteric ligands, which derive specificity from the cell/tissue distribution of the cognate GPCR, might produce unexpected on-target effects as a result of unbalanced receptor activation (Appleton and Luttrell, 2013). Still, data from animal models offer several examples in which selective attenuation or enhancement of arrestin function might be beneficial.

1. Potential Benefits of Reducing Arrestin Activity. Inhibiting arrestin function would have two expected consequences: G protein-dependent signaling would be enhanced by the impairment of homologous desensitization, and signals generated by arrestin scaffolds would be reduced. Depending on the setting, both effects can be therapeutically desirable. As noted, β -arrestin2 null mice experience prolonged analgesic effects of morphine due to the loss of MOR desensitization (Bohn et al., 1999, 2000), while at the same time encountering less constipation, respiratory suppression, and physical dependence (Raehal et al., 2005, 2011; Raehal and Bohn, 2011). To capitalize on this clinically in the form of more efficacious and safer narcotic analgesics, it would be desirable to decrease the arrestin-dependent efficacy of opioid receptor ligands (DeWire et al., 2013; Schmid et al., 2013; Zhou et al., 2013). In murine models, a weakly G protein-biased MOR agonist, TRV130, reportedly achieves favorable separation between analgesic efficacy and gastrointestinal and respiratory side effects (DeWire et al., 2013). This compound has entered human clinical trials, where it has proven to be a very potent analgesic, but has yet to show the expected reduction in side effects (Soergel et al., 2014), raising the question of whether biased agonists at MOR can dissociate analgesia from

respiratory depression/constipation, or if a greater degree of bias is necessary to translate into human therapeutic responses (Luttrell et al., 2015). Analogous G protein-biased κ opioid receptor agonists are under development (Schmid et al., 2013; Zhou et al., 2013; White et al., 2014, 2015), aimed at dissociating beneficial Gi/o-mediated antinociceptive effects (Gullapalli and Ramarao, 2002), from the putatively arrestin-mediated undesirable effects of dysphoria, sedation, diuresis, hallucination, and depression (Bruchas et al., 2006; Land et al., 2008; Redila and Chavkin, 2008).

Excessive dopaminergic neurotransmission underlies several cognitive disorders, including schizophrenia and attention deficit hyperactivity disorder. As a result, D2 dopamine receptor antagonists are effective neuroleptics. D2 receptors modulate Gs-cAMP signaling through coupling to Gi/o family G proteins, and engage AKT-GSK3 signaling via arrestin scaffolding of a β -arrestin2-PP2A-AKT-GSK3 β complex (Beaulieu et al., 2005, 2008). Locomotor hyperactivity induced by the dopaminergic drug apomorphine, a D2 receptor agonist, is reduced in β -arrestin2 null mice, implicating arrestin-dependent signaling in D2 receptor-dependent behaviors, and the mood stabilizer lithium acts by destabilizing the β -arrestin2-PP2A-AKT-GSK3 β complex, which enhances AKT-dependent inhibition of GSK3 β (Beaulieu et al., 2008). Strikingly, the clinical efficacy of nearly all mood-stabilizing drugs correlates better with their ability to inhibit β -arrestin2-dependent signaling than with their effects on D2 receptor-G protein signaling (Masri et al., 2008; Gao et al., 2015). Although different classes of antipsychotics vary in D2 receptor-G protein agonist/antagonist efficacy, they share the ability to block β -arrestin2 recruitment to D2 receptors.

β -Arrestin1-dependent scaffolding of cPLA2 underlies the cutaneous flushing side effect of nicotinic acid that limits its utility in the treatment of hyperlipidemia. Activation of GPR109A by niacin lowers serum-free fatty acids by activating G_i/G_o signaling. At the same time, it stimulates cPLA2-dependent production and release of the vasodilator, prostaglandin D₂, producing an increase in cutaneous blood flow and flushing (Kather et al., 1983; Pike, 2005). β -arrestin1-cPLA2 scaffolding underlies the GPR109A-dependent release of arachidonate, the precursor of prostaglandin D₂ (Walters et al., 2009). Although deletion of β -arrestin1 or 2 has no effect on nicotinic acid-induced changes in free fatty acid levels, deletion of β -arrestin1 blocks niacin-stimulated flushing in a murine model, suggesting that a G protein-selective GPR109A agonist would mimic the effects of niacin on lipid metabolism while eliminating flushing. Consistent with this, the GPR109A agonist, MK-0354, which decreases serum-free fatty acids without causing flushing (Semple et al., 2008), does not promote arrestin recruitment in vitro.

One of the mainstays of asthma therapy is the use of inhaled bronchodilators acting as agonists of β_2 adrenergic receptors on bronchial smooth muscle, both as short-acting rescue inhalers and as long-acting preparations. Although long-acting β_2 agonists, when used in combination with anti-inflammatory inhaled corticosteroids, have been shown to improve asthma control overall, the chronic desensitization of β_2 receptors resulting from their use may reduce the effectiveness of rescue inhalers and increase mortality from acute asthma attacks (Oppenheimer and Nelson, 2008). One of the curious phenotypes of β -arrestin2 null mice is that they do not accumulate T helper 2 cells in the airway or develop airway inflammation in an ovalbumin sensitization and inhaled challenge model of allergic asthma (Walker et al., 2003). Given the proposed role of β -arrestin scaffolds in T cell migration (Fong et al., 2002; Sun et al., 2002), it appears likely that β -arrestins exacerbate the asthma phenotype both by desensitizing bronchodilator receptors leading to enhanced bronchospasm and promoting chemokine receptor-mediated airway inflammation. Consistent with this, the bronchodilatory effects of PAR2, which are mediated via prostaglandin E2 production, are preserved in β -arrestin2 null mice, whereas lung eosinophil and CD4⁺ T cell infiltration, and production of IL-4, IL-6, IL-13, and TNF- α , are lost (Nichols et al., 2012). Hence, inhibiting arrestin function in the airway might be expected to augment treatment of allergic asthma (Walker and DeFea, 2014). If β -arrestins perform similar roles with respect to PAR1 and PAR2 regulation of ion transport, mucosal permeability, epithelial cell motility, immune cell recruitment, and nociception in the gut, then inhibiting arrestin function in the gut may have value in the treatment of irritable bowel syndrome and colitis (Ramachandran et al., 2012).

Because of their roles in supporting cell growth, survival, and migration, β -arrestin scaffolds also appear to contribute to a more aggressive cancer phenotype. The finding that β -arrestin2 knockdown reverses TP- β receptor-induced effects on bladder cancer cell proliferation, migration, and invasion of SV-HUV urothelial cells in vitro (Parent et al., 1999) is consistent with data from a murine xenograft model of TP- β -dependent human bladder cancer, where a TP- β receptor antagonist delays tumor growth and prolongs survival (Moussa et al., 2008). Upregulation of LPA1 and LPA2 receptors, β -arrestins 1 and 2, Ral, and Ral-GDS occurs in more advanced stages of human breast cancer, and knockdown of either β -arrestin or Ral expression inhibits LPA-stimulated migration and invasion of MDA-MB-231 breast cancer cells in vitro (Li et al., 2009). Upregulation of endothelin endothelin type A receptors and β -arrestin1 is associated with advanced grades of human ovarian cancer, whereas silencing β -arrestin expression in vitro inhibits endothelin type

A receptor activation of c-Src, ERK1/2, Akt, and EGF receptor and blocks β -catenin-dependent activation of a T cell-specific factor/LEF reporter (Rosano et al., 2009). In a tumor xenograft model, ovarian tumor cells expressing an inhibitory S⁴¹²D mutant of β -arrestin1 mutant develop fewer metastases, suggesting that arrestin signaling contributes to ovarian tumorigenesis and progression. Papilloma formation following exposure to tumor-promoting phorbol esters results from prostaglandin E2 production, which triggers EGF receptor transactivation by a prostaglandin E2 receptor- β -arrestin1-c-Src complex (Chun et al., 2009). Thus, arrestin-dependent signaling may be a therapeutic target in a host of malignancies.

2. Benefits of Promoting Arrestin Activity. Not all in vivo consequences of visual/ β -arrestin signaling are deleterious, however. In several settings, restoration of arrestin-dependent desensitization or selective activation of arrestin-dependent signaling pathways may have uniquely beneficial effects. Visual phototransduction involves a delicate balance between light sensitivity engendered by very high rhodopsin density in the rod outer segment, and rapid signal quenching achieved through GRK1 phosphorylation and visual arrestin-mediated desensitization. Perturbations that result in either excessive signaling or constitutive desensitization are toxic to photoreceptor cells. Oguchi disease, a hereditary form of stationary night blindness, results from inactivating mutations in GRK1 or visual arrestin that impair photoreceptor desensitization and increase signaling (Baylor and Burns, 1998). Conversely, a K²⁹⁶E point mutation in the opsin binding site of rhodopsin that leads to constitutive GRK1 phosphorylation and desensitization in vivo underlies one autosomal dominant form of retinitis pigmentosa (Li et al., 1995). The capacity to customize arrestin function suggests that a strategy of compensatory gene therapy could be a viable means of restoring lost arrestin function (Gurevich et al., 2014). As proof-of-concept, introducing a phosphorylation-independent visual arrestin mutant into GRK1 null rods, which would replace the normal two-step process of GRK phosphorylation followed by visual arrestin binding with a one-step process that bypasses the defect, was able to suppress rhodopsin signaling and enhance photoreceptor survival, functional performance, and photoresponse recovery (Song et al., 2009b).

The roles of β -arrestins in the cardiovascular system are complex, as they appear not only to modulate vasoconstrictor tone through GPCR desensitization, but to mediate effects on vascular smooth muscle proliferation, survival, and migration (Ahn et al., 2009; Kim et al., 2009). In isolated cardiomyocytes, treatment with the arrestin pathway-selective angiotensin AT_{1A} receptor agonist, Sar¹Ile⁴Ile⁸-AngII, stimulates cardiomyocyte proliferation, while generating positive inotropic and lusitropic effects (Rajagopal et al., 2006; Aplin

et al., 2007), whereas in the heart, arrestin-dependent signaling may contribute to cardiomyocyte hypertrophy while promoting cell survival (Zhai et al., 2005). In contrast, β -arrestin1-dependent ERK1/2 signaling in the adrenal gland promotes salt retention by regulating expression of the steroidogenic acute regulatory protein, StAR, the rate-limiting enzyme in aldosterone biosynthesis (Lympopoulos et al., 2009), leading to volume retention and worsening of congestive heart failure (Bathgate-Siryk et al., 2014; Dabul et al., 2015). Because afterload reduction using conventional antagonists of angiotensin AT_{1A} receptor signaling is known to promote beneficial cardiac remodeling and improve survival in congestive heart failure (Michel et al., 2016), the question arises whether, on balance, enhancing or inhibiting β -arrestin signaling would provide the most benefit. Some data suggest that the positive inotropic and lusitropic effects of Sar¹Ile⁴Ile⁸-AngII in vitro translate into improved cardiac contractility in vivo. An analogous arrestin pathway-selective AT_{1A} receptor agonist, TRV120027 [Sar-Arg-Val-Tyr-Ile-His-Pro-(D)-Ala-OH], which also stimulates arrestin-dependent activation of Src, ERK1/2, and endothelial nitric oxide synthase in vitro, similarly improves cardiomyocyte contractility in preclinical animal models (Violin et al., 2010). Administration of TRV120027 to rats reduces mean arterial blood pressure, as do the nonpeptide AT_{1A} receptor antagonists losartan and telmisartan, but, unlike the neutral antagonists, TRV120027 increases cardiac performance and preserves cardiac stroke volume. In a canine rapid-pacing model of heart failure, TRV120027 decreases cardiac afterload while preserving renal function, suggesting that it may have utility in heart failure treatment (Boerrigter et al., 2011, 2012). Similar improvements in cardiac performance were observed upon treatment with a related β -arrestin-biased angiotensin analog, TRV120023, in a murine model of familial dilated cardiomyopathy due to expression of a mutant tropomyosin with reduced myofilament Ca²⁺ sensitivity (Tarigopula et al., 2015). Another arrestin-selective AT_{1A} receptor agonist, TRV027, has been studied in acute heart failure in humans, but failed to demonstrate efficacy in any of the study endpoints in a phase IIb clinical trial (Felker et al., 2015; Greenberg, 2016). Given the complexity of arrestin functions in the cardiovascular system, such a failure to translate in vitro and animal data underscores the challenges of translating arrestin-selective bias into viable human therapeutics.

Osteoporosis is another therapeutic area where selective activation of β -arrestin signaling may confer benefit. During physiologic bone remodeling, PTH stimulates bone-forming osteoblasts, increasing both osteoblast number and activity, while at the same time accelerating bone turnover by causing osteoblasts to secrete soluble factors that increase the number and activity of bone-resorbing osteoclasts (Qin et al., 2004).

Traditionally, the actions of PTH in bone have been attributed to Gs-cAMP signaling (Mohan et al., 2000), yet male mice treated with the β -arrestin-biased PTH analog, [D-Trp¹², Tyr³⁴]-bPTH(7-34), exhibit paradoxical increases in bone formation rate, with greater trabecular bone volume, increased osteoblast number, and accelerated mineral apposition, but no effect on osteoclast number or markers of bone turnover (Gesty-Palmer et al., 2009). Functional transcriptomic analysis of microarray data from treated tissues suggests that the key to this unexpected phenotype lies in the ability of [D-Trp¹², Tyr³⁴]-PTH(7-34) to expand the osteoblast pool through arrestin-dependent cell cycle regulation and anti-apoptotic signaling, while uncoupling the PTH₁ receptor from Gs-cAMP-dependent activation of osteoclasts (Gesty-Palmer et al., 2013; Maudsley et al., 2015).

V. Conclusions

The visual/ β -arrestins, like their evolutionary cousins, the α -arrestins, arose to exploit a conserved arrestin fold to coordinate the temporal and spatial aspects of processes related to endosome trafficking, vesicle sorting, and signaling through their capacity to bind specific cargos and localize them to defined intracellular locations. Beyond the conformational flexibility to bind a wide variety of cargo proteins, visual/ β -arrestins possess additional properties that make them uniquely suited to signal regulation. The first is the capacity to exist in different intracellular pools wherein they adopt conformations that attract and hold different cargos. The second is the ability to recognize activated GPCRs, which enables them to be responsive to extracellular stimuli. The third is that they are sufficiently abundant to sequester key pathway intermediates away from their site of activation until called to the plasma membrane. In cells, the dynamic equilibrium between large pools of inactive arrestin, held in place by low-affinity interactions with IP6, Ca²⁺-calmodulin and microtubules, and a small pool of active arrestin bound to high-affinity GPCR docking sites, serves to keep certain signaling pathways quiet until an extracellular stimulus is received.

The other unique feature of visual/ β -arrestins is their duality of function. Arrestin binding to GRK-phosphorylated GPCRs marks the dividing line between early signaling events mediated via heterotrimeric G proteins that are terminated by arrestin-dependent homologous desensitization and receptor endocytosis, and the initiation of arrestin-dependent signals transmitted through scaffolding of multiprotein GPCR signalsome complexes. Although G protein signaling accounts for the short-term effects on intermediary metabolism transmitted by soluble second messengers, arrestin signaling mediates longer-term effects on processes such as cell proliferation/growth,

survival/apoptosis, and migration/chemotaxis. In effect, G proteins and arrestins perform complementary roles downstream of activated GPCRs, and GRK phosphorylation/arrestin binding is the switch between signaling modes. Given their position and the center of GPCR signaling, manipulating arrestin function may be the key to developing new generations of GPCR-targeted therapeutics. Impairing arrestin activity, whether using G protein-biased orthosteric agonists or allosteric modulators, inhibiting GRKs, or targeting arrestins themselves, will enhance G protein signaling, reduce tachyphylaxis, and block arrestin signaling in settings where it may be deleterious. In contrast, selective activation of arrestin-dependent signaling with arrestin-biased agonists may find application in the converse situation, in which excessive GPCR stimulation underlies a pathophysiologic process.

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