Published in final edited form as:
Trends Biochem Sci. 2011 September ; 36(9): 457–469. doi:10.1016/j.tibs.2011.06.003.

Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling

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Abstract

β-arrestins, originally discovered to desensitize activated seven transmembrane receptors (7TMRs; also known as G protein-coupled receptors [GPCRs]), are now well established mediators of receptor endocytosis, ubiquitylation and G protein-independent signaling. Recent global analyses of β-arrestin interactions and β-arrestin-dependent phosphorylation events have uncovered several previously unanticipated roles of β-arrestins in a range of cellular signaling events. These findings strongly suggest that the functional roles of β-arrestins are much broader than currently understood. Biophysical studies aimed at understanding multiple active conformations of the 7TMRs and the β-arrestins have begun to unravel the mechanistic basis for the diverse functional capabilities of β-arrestins in cellular signaling.

The seven transmembrane receptor superfamily

Seven transmembrane receptors (7TMRs), also referred to as G protein-coupled receptors (GPCRs), are integral membrane proteins which constitute the largest class of cell surface receptors in the human genome with about 800 different members [1]. These receptors bind to a diverse array of ligands which include hormones, peptides, neurotransmitters, and lipids [2]. Upon activation, they initiate a range of intracellular signaling pathways to elicit appropriate cellular responses [3]. As 7TMR signaling critically regulates a wide range of physiological and pathophysiological processes, these receptors are one of the most important drug targets [4]. Thus, studies on 7TMR signaling comprise one of the most attractive areas of modern biology not only from the perspective of basic research, but also in terms of potential therapeutic implications and translational opportunities. Activation of G protein-dependent 7TMR signaling involves binding of an agonist that induces conformational changes in the receptor which in turn promote its interaction with the heterotrimeric G proteins ($G_{\alpha\beta\gamma}$) (Figure 1a). This is followed by guanine nucleotide exchange (GTP for GDP) on the G_{α} subunit, activation and dissociation of the heterotrimeric G proteins [5]. Dissociated G proteins subsequently interact with and activate a variety of downstream effectors such as enzymes (e.g. adenylate cyclase and phospholipases) and ion channels to generate second messengers (e.g. cAMP) and initiate appropriate signaling responses [6]. As one activated receptor can sequentially couple to multiple G proteins, cells

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have devised desensitization mechanisms to turn off G protein-dependent signaling in order to avoid the deleterious effects of sustained signaling [8].

β-arrestins and G Protein-coupled receptor kinases (GRKs): a universal desensitization mechanism

7TMR desensitization is essentially a two step process (Figure 1b). In the first step, agonist-occupied receptors are phosphorylated by GRKs, primarily in the carboxyl terminus of the receptors, but also in the intracellular loops [7]. GRKs are a family (GRK1-GRK7) of serine/threonine kinases [8]. Whereas GRK1 and GRK7 expression is confined to the retina, GRK4 also has limited cellular distribution with highest expression in the testes [9]. The other GRKs are widely expressed and phosphorylate non-rhodopsin 7TMRs. GRK2 and 3 contain a pleckstrin homology domain, and their membrane targeting is aided primarily by interactions with $G_{\beta\gamma}$; by contrast, GRK5 and 6 are constitutively localized to the membrane by virtue of various modifications such as palmitoylation [10]. Although four GRKs (i.e. GRK2,3,5 and 6) are primarily responsible for the phosphorylation of most 7TMRs, a consensus GRK target sequence motif has not been firmly established. The primary sequences of 7TMRs are only poorly conserved, therefore GRKs probably recognize certain structural determinants in the activated receptors. Activated 7TMRs are also phosphorylated by second messenger-dependent protein kinases such as protein kinase A (PKA) and protein kinase C (PKC) which also contribute to desensitization [11]. In addition, some studies have shown that other kinases such as the casein kinase II [12] and ribosomal S6 kinase 2 [13] can phosphorylate specific 7TMRs; however, these events appear to be restricted to specific receptor systems and their contributions to receptor desensitization remains to be fully elucidated.

The second step of desensitization involves recruitment of the multifunctional adaptor proteins called arrestins to the activated and phosphorylated receptors. Recruitment of β-arrestin sterically hinders further G protein coupling of the receptor, thereby leading to desensitization [14]. There are four members in the arrestin family, arrestin 1 (also called S antigen or visual arrestin), arrestin 2 (β-arrestin 1), arrestin 3 (β-arrestin 2) and arrestin 4 (also called X arrestin or cone arrestin) [15]. Whereas visual arrestin and cone arrestin are localized to retinal rods and cones and interact exclusively with rhodopsin, the two β-arrestin isoforms are ubiquitously expressed. The crystal structures of all four arrestins have been determined, and they essentially consist of N- and C-domains built almost entirely from antiparallel β sheets [16,17,18]. It is interesting to note that there is high sequence and structural homology among the members of the arrestin family; still, they are capable of strikingly differential functional capabilities. Much like the GRKs, only the two β-arrestin isoforms appear to be sufficient to regulate a large repertoire of 7TMRs, suggesting that these proteins recognize certain common structural domains in the agonist-activated receptors.

Over the past decade, several novel functional aspects of β-arrestins in regulating 7TMR signaling have been discovered. The previously unanticipated capability of β-arrestins to act as signal transducers and mediate G protein independent 7TMR signaling has led to the notion of biased agonism. Moreover, global proteomics approaches have begun to uncover an ever expanding range of β-arrestin interaction partners as well as novel signaling networks which are potentially regulated by β-arrestins in a phosphorylation/dephosphorylation-dependent fashion. The following sections discuss the most recent discoveries in these areas and attempt to provide recent information about the underlying biophysical basis of the multifaceted signaling via 7TMRs.

Expanding horizons: novel connections and novel regulatory roles of β-arrestins for 7TMRs

Following the discovery of the role of β-arrestins in 7TMR desensitization, additional studies found that β-arrestins also interact with and serve as adaptors for a number of key components of the endocytotic machinery to promote receptor internalization (Figure 1c) [19]. These proteins include clathrin heavy chain [20], β2 adaptin of the adaptin proteins AP2 complex [21], the small G protein ADP-ribosylation factor 6 (ARF6) [22] and the *N*-ethylmaleimide-sensitive fusion protein [23]. β-arrestin-dependent clathrin-mediated internalization of 7TMRs appears to be a general mechanism for the majority of 7TMRs; however, there are some examples where, depending on the cell type or the receptor system, β-arrestins are involved either to a lesser extent or not at all [24]. Based on the stability of interaction between the internalizing receptor and the β-arrestins, two classes of receptors have been delineated. For class A receptors, such as the β2-adrenergic receptor (β2AR), the interaction between the receptor and β-arrestins is transient; by contrast, for class B receptors such as the angiotensin receptor subtype 1a (AT1aR), the interaction is significantly more stable [25]. In addition, the stability of receptor–β-arrestin interaction also determines whether the receptors undergo rapid recycling (class A) or slow recycling/endosomal sorting (class B) [26].

Another set of related observations which have broadened the functional span of β-arrestins is an appreciation of their capability to act as E3 ubiquitin ligase adaptors and mediate ubiquitylation of 7TMRs (Figure 1c) [27]. The very first indication of an involvement of β-arrestins in ubiquitylation was reported in the context of the human β2AR: it was proposed that β-arrestin 2 can act as an adaptor for an E3 ubiquitin ligase to promote β2AR ubiquitylation [28]. Subsequent work found that β-arrestin 2 indeed acts as an adaptor for the E3 ubiquitin ligase neural precursor cell expressed, developmentally down-regulated 4 (NEDD4) to promote β2AR ubiquitylation and degradation [29]. Similarly, β-arrestin 1 scaffolds another E3 ubiquitin ligase, AIP4, in response to the activation of chemokine (C-X-C motif) receptor 4 (CXCR4); however, this interaction appears to be required for late endosomal sorting events and degradation instead of ubiquitylation of CXCR4 per se [30]. Because most β-arrestin-dependent functions appear to be conserved across the majority of 7TMRs, the role of β-arrestins in the ubiquitylation, endosomal/lysosomal sorting and degradation of the receptors probably will also be conserved among the members of 7TMR superfamily.

Interestingly, the ability of β-arrestins to mediate internalization and ubiquitylation are not limited to 7TMRs (Box1). β-arrestins are critical for the internalization or ubiquitylation (or both) of the type III transforming growth factor β (TGF-β) receptor [31], the insulin-like growth factor I (IGF1) receptor [32], voltage-dependent calcium channels [33,34], transient receptor potential channel vanilloid type (TRPV4) [35], the Na(+)/H(+) exchanger 1 (NHE1) [36], the neuronal Na(+)/H(+) exchanger NHE5 [37], the androgen receptor [38] and the vascular endothelial (VE) cadherin [39]. Moreover, kurtz, the single nonvisual arrestin expressed in *Drosophila*, is critically involved in deltex-mediated Notch ubiquitylation and degradation [40]. Furthermore, yeast arrestin related trafficking proteins (ARTs), which are distantly related to the mammalian arrestins, but which share the arrestin fold, are involved in internalization and ubiquitylation of plasma membrane transporters [41,42,43]. These findings not only highlight an evolutionarily conserved principle, but also suggest that some of the paradigms originally discovered for 7TMRs, and anticipated to be relevant solely for 7TMRs, might be applicable to other integral membrane proteins. Indeed, it is tempting to speculate that β-arrestins might be global mediators of the ubiquitylation and internalization of membrane proteins.

Box 1

Most recent developments

1. **β-arrestins as E3 ligase adaptors.** One of the latest additions to the ever growing list of proteins for which β-arrestins can act as adaptors, are the E3 ubiquitin ligases. β-arrestins can scaffold MDM2 [28], AIP4 [30] and NEDD4 [29] not only in the context of 7TMRs, but also other non-7TMR membrane proteins.
2. **β-arrestins as regulators of non-7TMR membrane proteins.** Several lines of evidence suggest critical roles of β-arrestins in regulating ion channels and transporters, primarily mediating their internalization and ubiquitylation [33–37]. This activity significantly broadens the repertoire of membrane proteins which are influenced directly or indirectly by β-arrestins.
3. **Arrestin-related trafficking adaptors in yeast (ARTs**). Evolutionarily related to mammalian arrestins and containing structurally conserved features with mammalian arrestins, ARTs can mimic the scaffolding function of β-arrestins for E3 ubiquitin ligases in order to maintain appropriate levels of membrane transporters in *Saccharomyces cerevisiae* [41–43].
4. **Novel ways to induce bias.** In addition to biased ligands, AT1aR also exhibits β-arrestin-biased signaling when mechanical or osmotic stress is applied to cells expressing the receptor. Such stresses do not appear to induce any detectable G protein activation downstream of AT1aR [98]. The bacterium causing cerebrospinal meningitis, *Neisseria meningitides*, selectively engages a β-arrestin-dependent signaling pathway downstream of β2AR in order to open intercellular junctions through cytoskeletal rearrangements which ultimately allows bacterial adhesion to endothelial cells and subsequent colonization [99].

Roles of β-arrestin and GRKs in 7TMR signaling: changing the paradigm

A somewhat surprising, but also one of the most interesting, finding in the area of 7TMRs was the observation that β-arrestins can scaffold the tyrosine kinase c-Src to agonist-activated β2AR, eventually leading to activation of extracellular signal-regulated kinase (ERK1/2) [44] (Figure 1c). This finding not only provided the first hint that β-arrestins could function as signaling molecules, but it has subsequently led to the discovery of the new field of G protein-independent, β-arrestin dependent signaling via 7TMRs. Since this first report, a large body of data has not only established β-arrestins as *bona fide* signal transducers, but also suggested that β-arrestin-dependent signaling is very diverse [45]. For example, β-arrestins can interact with other members of the c-Src family such as Hck, Fgr and Yes and bring them into close proximity of the activated receptor [46]. Moreover, β-arrestins are able to scaffold various other key players of the mitogen-activated protein kinase (MAPK) signaling pathways including ERK1/2, p38 and c-Jun N-terminal kinase-3 (JNK3) [44,47,48]. In addition, β-arrestins are reported to scaffold AKT, PI3 kinase and phosphodiesterase 4 (PDE4) in the context of specific receptors both *in vitro* and *in vivo* [49,50,51,52,53,54,55,56]. The range of signaling capabilities of β-arrestins have been reviewed in detail elsewhere [57]; however, there are at least two important conclusions that can be drawn from the research in this area over the past decade: first, that β-arrestins act as G protein-independent signaling molecules downstream of 7TMRs and second, that the signaling capacities of β-arrestins are much more diverse than originally conceived.

Zooming in: the concept of biased agonism

The finding that β-arrestins can mediate G protein-independent cellular signaling downstream of 7TMRs subsequently led to the discovery that the two signaling arms are pharmacologically separable [58][70]. In other words, it is possible to identify or design ligands which can selectively trigger either G protein-dependent or β-arrestin-dependent signaling. Such ligands, which can selectively trigger one or the other signaling arm, are termed 'biased agonists' and this phenomenon of selective activation is termed 'biased agonism'. This discovery changed the widely held notion of the linear spectrum of efficacy of 7TMRs and uncovered multidimensional signaling modes of these receptors. For example, stimulation of the angiotensin II type 1a receptor (AT1aR) by SII (Sar^1, Ile^4, Ile^8-angiotensin II), a modified version of angiotensin II, leads exclusively to β-arrestin-dependent signaling without any detectable G protein activation making SII a β-arrestin-biased ligand for the AT1aR. Ligands biased for either G protein or β-arrestins have been described for a number of different receptors (reviewed in [59]). Interestingly, for some receptors, one of these two signaling pathways can translate into beneficial physiological effects whereas the other appears to mediate the undesirable outcomes. Therefore, depending on the receptor system, it might be possible to develop biased agonists for one of these two pathways into novel and therapeutically more beneficial drugs. For example, the vitamin niacin, a GPR109A agonist which in high doses is used to treat dyslipidemia, appears to exert its triglyceride lowering effect via a G protein-dependent pathway whereas its side effect of cutaneous flushing depends on β-arrestin 1 [60]. In fact, an agonist of GPR109A which selectively triggers G protein signaling without engaging β-arrestin 1 has been recently described [61]. Such a ligand might maintain the beneficial lipid lowering effects without eliciting the flushing response and therefore might be superior to niacin. Another example for where a β-arrestin-biased ligand might be therapeutically preferred is for AT1aR. TRV120027, a β-arrestin-biased agonist of AT1aR, improves cardiac performance in animal models in addition to reducing the mean arterial pressure like classical unbiased antagonists [62]. These examples highlight the potential for therapeutic improvement by targeting specific signaling arms of 7TMRs and represent an attractive translation of basic 7TMR research.

For the majority of the $G_{\alpha s}$- and $G_{\alpha q}$- coupled receptors, the G protein-dependent and the β-arrestin-dependent activation of ERK MAP kinases appears to be mostly independent of each other, i.e. inhibition of a G protein does not significantly attenuate β-arrestin-dependent ERK activation and vice versa. However, an interesting finding, that remains incompletely understood, is that for $G_{\alpha i}$/$G_{\alpha o}$ coupled receptors (such as the chemokine receptor CXCR7 and the niacin receptor GPR109A), the G protein and the β-arrestin dependent signaling to ERK appear to be interdependent [63] [60]. For these receptors, inhibition of either $G_{\alpha i}$ using pertussis toxin or β-arrestins by siRNA significantly abolishes ERK activation. These findings indicate that at least for ERK activation downstream of $G_{\alpha i}$ coupled receptors, certain key components of both the G protein-dependent and the β-arrestin-dependent signaling pathways are required.

It should be noted that a signaling bias has been demonstrated at various additional levels, adding further ramifications to 7TMR signaling. For example, certain receptors are able to couple to more than one type of G protein, and specific ligands which promote preferential coupling to one or the other G protein have recently been described. Interestingly, a recent report showed that for β2AR, two stereoisomers of the same ligand fenoterol, exhibit bias by preferentially coupling to either G_s or G_i [64]. It is also possible that certain ligands will preferentially recruit one of the two β-arrestin isoforms to the receptors, thus adding an additional level of bias. Moreover, different ligands can engage different sets of GRKs leading to differential signaling outcomes, as observed for the chemokine receptor, CCR7

[65]. In this case, CCL19 leads to receptor phosphorylation by GRK2/3 and GRK 6, CCL21 promotes only GRK6-mediated phosphorylation; these two ligands, in turn, display differential patterns of receptor desensitization, β-arrestin recruitment and β-arrestin redistribution [65]. One can also imagine that other signaling pathways, in addition to G protein and β-arrestin signaling, can add even further levels of potential bias. For example, stimulation of AT1aR leads to tyrosine phosphorylation and activation of JAK2 (Janus kinase 2), a cytosolic protein typically implicated in cytokine receptor signaling. Activation of JAK2 subsequently results in the activation of its downstream substrate, the transcription factors, signal transducer and activator of transcription (STAT) 1 and 2 [66]. Furthermore, not all cells express all the effectors (e.g. G proteins, GRKs, β-arrestins, etc.) at similar levels and depending on the cell/tissue type and the levels of effectors, ligands might also display bias at one or the other level of receptor regulation [67].

Going global: 'Omics' on β-arrestins

As the list of signaling pathways that β-arrestins can regulate in response to activation of 7TMRs has grown, so too has the application of global approaches to obtain a more comprehensive view of β-arrestin-dependent signaling and regulatory events in cells. Notably, two mass spectrometry (MS)-based systems biology approaches have aimed to provide novel insights into β-arrestin-dependent signaling in an unbiased fashion. These approaches essentially utilize recent advances of MS to first, identify novel interaction partners of β-arrestins upon activation of AT1aR and second, to obtain an extensive list of proteins downstream of AT1aR which might be regulated by β-arrestins via phosphorylation or dephosphorylation.

The β-arrestin interactome

One approach aimed to obtain a comprehensive list of β-arrestin binding partners. HEK-293 cells overexpressing the AT1aR together with either β-arrestin 1 or 2 were stimulated with either vehicle or angiotensin II followed by immunoprecipitation using the FLAG tag at the C-terminus of β-arrestins followed by identification of associated proteins by MS [68]. A total of 337 non-redundant proteins were identified as β-arrestin binding proteins: 173 were found to interact with β-arrestin 1 and 266 were found to interact with β-arrestin 2. Interestingly, a significant number of proteins were found in complex with β-arrestins under basal conditions. However, both the number of proteins and the coverage of interacting proteins changed significantly upon angiotensin stimulation, consistent with receptor activation-dependent changes in the β-arrestin profile. Although some of the identified proteins could represent indirect interactions (e.g. β-arrestin-dependent scaffolding of a signaling complex wherein not all the components directly bind β-arrestins), the reliability score (their likelihood of being in complex with β-arrestins) is high for two reasons. First, a number of proteins which have been reported to interact with β-arrestins such as protein phosphatase 2A (PP2A), clathrin, AP2 and phosphodiesterase 4D, cAMP-specific (PDE4D) were present in this list. Second, secondary testing of several 'hits' by Western blotting essentially reflected a pattern consistent with the MS data. Moreover, since the publication of this study, a number of interactions (e.g. Kif 3A and TRPV4) have been independently confirmed. The results from this study highlight at least two key points. First, β-arrestin interacting proteins are widely distributed across subcellular compartments, with a majority in the cytoplasm and nucleus, but also in nucleolus, plasma membrane, mitochondria, ER and Golgi (Figure 2a). Second, β-arrestin interacting proteins fall into the functional categories of signal transduction, cellular organization, nucleic acid binding, metabolic enzymes, chaperones and ion channels, thus indicating the diverse nature of processes in which β-arrestins are potentially involved (Figure 2b). These proteins include kinases (e.g. ERK, AKT), phosphatases (e.g. PP2A, slingshot), nuclear proteins (e.g. histone deacetylases [HDACs]), non-7TMR membrane proteins (e.g. TRPV4), proteins involved in transcription

(e.g. STAT1) and translation (e.g. elongation factor eIF-4B). This diverse array of interacting proteins highlights the widely distributed roles of β-arrestins in cellular signaling. Even under basal (i.e. non-stimulated) conditions, β-arrestins appear to be in complex with a number of different proteins, thus suggesting that even without receptor activation, they can scaffold a number of proteins which might be critical to sequestering these proteins from their sites of action in order to exert negative regulation of certain pathways.

Although this study provided the first extensive screen to identify novel β-arrestin interaction partners, there are several ways in which this approach could be utilized to yield even more interesting and novel information. First, not all 7TMRs necessarily converge on the same functional outputs. Thus it would not be surprising if, upon activation by different 7TMRs, the β-arrestin interactome shows somewhat non-overlapping profiles with that obtained with the AT1aR. Such a finding would highlight the roles of β-arrestin-dependent regulation of signaling pathways in the context of a specific receptor system. It would also be interesting to explore how the β-arrestin interactome changes in response to a biased ligand. Although significant overlap might be expected between proteins that interact with β-arrestins in response to an unbiased versus biased ligand, there might also be specific sets of proteins which interact or lose interaction with β-arrestins in one of these two conditions. Indeed, because β-arrestin dependent-processes appear to have distinct temporal patterns, it would not be surprising if the β-arrestin interactome changes at different time points during signaling events. For example, the desensitization process occurs within seconds of receptor activation, but β-arrestin-dependent signaling appears to have a somewhat slower onset. Thus, one might expect to identify proteins involved in receptor desensitization and endocytosis such as clathrin or AP2 at early time points and increased signaling proteins such as ERK or Src at later time points. Moreover, it would be very interesting, and probably physiologically more relevant, to perform similar screens using primary cells or even whole tissues.

Cellular signaling maps: phosphoproteomics

Almost every signaling pathway in the cell involves phosphorylation and/or dephosphorylation of key proteins [69]. These events are critical for activating and inactivating key enzymes including kinases and phosphatases, promoting protein–protein interactions, downregulating membrane proteins and promoting the ubiquitylation and degradation of proteins [70,71]. Therefore, obtaining an exhaustive list of proteins whose phosphorylation status changes in a β-arrestin-dependent manner upon activation of a 7TMR is of the utmost importance to gain key insights into β-arrestin signaling pathways. Two parallel but independent approaches have in fact very recently addressed this issue [71,72]. Both of these approaches use the cutting edge technology of quantitative phosphoproteomics to observe changes in phosphorylation levels in an unbiased fashion upon activation of AT1aR. One study used a β-arrestin-biased ligand of the AT1aR namely SII to selectively activate β-arrestin-dependent signaling pathways and used the stable isotope labeling of amino acids in cell culture (SILAC) strategy in conjunction with MS to identify changes in phosphorylation [64]. This study identified 171 proteins (222 different phosphopeptides) which showed increased phosphorylation and 53 proteins (66 different phosphopeptides) which showed decreased phosphorylation upon SII stimulation. Using the data obtained from the β-arrestin interactomics screen [61], the authors utilized multiple bioinformatics tools to construct a kinase network involved in β-arrestin dependent signaling downstream of the AT1aR. By contrast, a different study used both the unbiased ligand angiotensin II and the biased ligand SII to obtain a comparative pattern of G protein-dependent and β-arrestin-dependent signaling upon activation of AT1aR [65]. This approach also utilized SILAC and identified a total of 1183 upregulated phosphorylation sites on 527 phosphoproteins; 36% of these phosphosites (i.e. 427 phosphosites) overlapped in the SII

and angiotensin phosphoproteomes. Interestingly, a smaller set of sites (a total of 58) was found to be regulated exclusively by SII, and not by angiotensin, thus indicating that a significant number of proteins downstream of AT1aR are exclusively phosphorylated in a β-arrestin-dependent manner without any involvement of G protein-dependent signaling.

Several interesting conclusions can be drawn from these studies. Similar to β-arrestin interacting proteins, the functional and cellular distribution of the proteins phosphorylated or dephosphorylated in a β-arrestin-dependent manner are quite diverse. These proteins are widely distributed amongst the plasma membrane, cytoplasm and nucleus (Figure 2c). Moreover, functional analysis reveals that these proteins fall into several different categories including signal transduction, cellular organization, nucleic acid binding and metabolic enzymes (Figure 2d). Interestingly, a large number of kinases were identified in these screens, including several MAPKs, cell division control 42 (CDC42), ribosomal S6 kinase, AMP-activated kinase (AMPK) and non-receptor tyrosine kinases. Although some of these kinases were previously reported to be regulated by β-arrestins, these studies identified several proteins (e.g. AMPK) whose β-arrestin-dependent phosphorylation was previously unknown. The enrichment of kinases in the phosphoproteome is especially noteworthy given that most kinases are activated upon phosphorylation or dephosphorylation; therefore, this finding provides important clues regarding the specific roles of β-arrestins in various signaling pathways. Moreover, there was a significant overlap between the angiotensin- and SII-induced phosphoproteomes; however, several dozen proteins were phosphorylated only in response to SII stimulation, thus suggesting the existence of β-arrestin signaling pathways distinct from G protein-dependent ones. Bioinformatics tools, used to obtain a system wide view, generated a β-arrestin-dependent kinase network which highlights potential roles of β-arrestin in several previously unanticipated pathways such as those involved in cell cycle and DNA damage repair [64]. These studies also identified consensus motifs within several kinases that are enriched among the phosphoproteins and therefore provide a better understanding of kinases which might be critically involved in β-arrestin signaling. The large sets of data generated from these proteomic studies can be utilized to build β-arrestin-dependent signaling networks which should be able to provide hints at novel levels of regulatory nodes in 7TMR signaling (Figure 3). It is important to note here that both phosphoproteomics studies were performed using cells expressing the AT1aR. As a number of 7TMR signaling mechanisms are conserved across different receptor systems, a great number of phosphorylation events described in these studies are likely to exist for other receptors as well. However, at the same time, similar studies with other receptors might reveal novel and non-overlapping regulatory networks which can be receptor type specific.

Despite the fact that the signaling function of β-arrestins was discovered only about ten years ago, there has been remarkable progress in the field since then. Not only have several unanticipated connections been identified, but the physiological importance of β-arrestin signaling has begun to be deciphered from studies carried out in animal models [73]. This body of work suggests that β-arrestin-dependent regulatory events are most likely as diverse and equally physiologically important as those mediated by G proteins. Methodological developments in the area of MS and systems biology have not only made it possible to generate global views of β-arrestin-dependent signaling, but also hold significant promise for uncovering novel regulatory networks in a relatively quick fashion. Indeed, these global analyses of β-arrestin-mediated events serve as robust hypothesis-generating tools. For example, the interaction of β-arrestins with kinesin family member 3A (KIF3A), first uncovered by the interactomics approach, led to the discovery of a novel mechanism of smoothened translocation to the primary cilium [74]. Similarly, analysis of TRPV4, which was identified as an interaction partner in the β-arrestin interactome, led to the discovery of a novel mechanism of downregulating TRPV4 through β-arrestin-mediated ubiquitylation [75]. Similarly, the phosphoproteomic analysis identified cytoskeletal reorganization as one

of the enriched signaling pathways downstream of AT1aR; a number of key players in this pathway (e.g. cofilin, slingshot) undergo change in their phosphorylation status upon SII stimulation. This finding prompted a subsequent biochemical analysis which not only confirmed the findings of phosphoproteomics, but also revealed that the roles of β-arrestins in regulating AT1aR-mediated cytoskeletal reorganization are analogous to the roles of β-arrestins reported earlier for protease activated receptor 2 (PAR2) [76]. In the case of AT1aR, β-arrestins scaffold slingshot 1 to mediate dephosphorylation of cofilin, however, for PAR2, β-arrestins mediate cofilin dephosphorylation via scaffolding chronofin. Most likely, this systems biology approach will guide the evolution of the field and will help to uncover a range of novel regulatory networks which would otherwise take much longer to elucidate by conventional iterative processes.

Functional diversity of β-arrestins and the issue of specificity

As mentioned earlier, there are about 800 genes encoding 7TMRs in the human genome. Although the basic architecture of seven transmembrane bundles is highly conserved, there is relatively little sequence similarity among the primary sequences of the receptors. Despite this, the central paradigms of G protein- and β-arrestin-dependent signaling holds true for most receptors examined to date. This obviously raises the question of how the specificity of signaling is determined and tightly controlled. Mammalian cells express 16 G_{α} subunits, 5 G_{β} subunits and 12 G_{y} subunits which can yield sufficient combinatorial permutations to account for the large number of receptors. Conversely, there are only four GRK subtypes which are ubiquitous in cells and only two isoforms of β-arrestins which are responsible for desensitization, internalization and signaling via a large repertoire of the 7TMRs. Because the GRKs are kinases, their activity depends on the presence of serine and threonines in an appropriate context. Concerted regulation of β-arrestin-dependent events, by contrast, demands several levels of regulatory circuits.

A clue to the functional multiplicity of β-arrestins comes from their intracellular localization patterns. Whereas β-arrestin 1 is localized primarily in the cytoplasm, it contains a nuclear localization signal and it is known to translocate to the nucleus in response to specific stimuli [77]. It is possible that the nuclear pool of β-arrestin 1 is involved in range of processes which are distinct from those regulated by the cytoplasmic pool of β-arrestin 1, thus extending its functional coverage. By contrast, β-arrestin 2 harbors a nuclear export signal [78] which facilitates its nuclear exclusion, possibly ensuring that the two isoforms can simultaneously be involved in non-overlapping signaling events. Obviously, β-arrestins cannot bind all 337 proteins present in the interactome at the same time; instead, they most likely interact with distinct sets of proteins at different time points of signaling, leading to different functional outputs. Although, mutually exclusive interactions of β-arrestins with their binding partners have not been documented directly, there are reports which suggest at least partially overlapping binding interfaces for different proteins on β-arrestins. For example, c-Src, Mdm2 and PDE4 appear to share an interaction interface at the N-domain of β-arrestins and therefore, it would seem likely that their binding to β-arrestins would be mutually exclusive.

Another level of specificity may arise from β-arrestin oligomerization [79,80,81,82]. There are several lines of evidence which suggest that β-arrestins can form both homo- and heterodimers, at least when overexpressed in cells. This was first documented by bioluminescence resonance energy transfer (BRET) and co-immunoprecipitation studies and recently, the homodimerization interface for β-arrestin 2 was mapped by spot peptide array analysis. Interestingly, co-expression of β-arrestin 1 and 2 in HEK cells prevents nuclear localization of β-arrestin 1 which in turn might affect β-arrestin 1 dependent signaling events. One can also envisage the possibility that β-arrestin dimers present a unique and

different interface for interactions with distinct sets of proteins than that presented by β-arrestin monomers. It was recently reported that interaction of Mdm2 requires β-arrestin 2 oligomerization as impairing β-arrestin 2 oligomerization via mutation of IP6 binding sites reduces its interaction with Mdm2. However, the interactions of this oligomerization impaired mutant with other binding partners such as clathrin, AP2 and ERK2 are not affected. Furthermore, it was recently shown that mutations in β-arrestin 2 which reduced homodimerization, also inhibited β2AR dependent ERK activation without affecting receptor internalization. Another important difference in the functional specificity of β-arrestins is observed depending on the extent and site of ubiquitylation. For example, non-overlapping putative ubiquitylation sites on β-arrestin 2 appear to govern its interaction with different receptors and subsequent endocytosis and signaling [83]. Thus, depending on the context of stimulation (e.g. receptor, ligand etc.), a differential pattern of ubiquitylation might arise to fine-tune the downstream response. It is tempting to speculate that similar types of regulation can also result from other post-translational modifications to β-arrestins, e.g. phosphorylation and sumoylation. All of these potential mechanisms to govern the functional specificity of β-arrestins ultimately rely on the ability of β-arrestins to adopt multiple conformations in response to specific stimulation.

Mechanistic basis of biased signaling

The fact that G protein-dependent and β-arrestin-dependent pathways signaling are pharmacologically separable, i.e. that it is possible to design ligands which can selectively trigger one or the other pathway, argues for the existence of multiple active receptor conformations. In this conceptual framework, one could envisage that an unbiased ligand induces a conformation which is capable of coupling to both arms of downstream signaling. However, a distinct conformation of the receptor is induced when it is occupied by a biased ligand (Figure 4a). Obviously, these receptor conformations probably overlap significantly given that the conformational space of the 7TMR bundle is restricted. Still, the ligand binding pocket, upon binding to a biased ligand, must display a set of interactions which are significantly different and non-overlapping with those induced upon binding of an unbiased ligand. These differences in the ligand-binding pocket must in turn lead to corresponding structural rearrangements of the transmembrane helices as well as the intracellular face of the receptor. Because the intracellular surface of the receptor is the major interface for receptor–effector coupling, these distinct structural changes at the intracellular surface of the receptor should govern whether the receptor couples to a G protein or β-arrestin, and therefore which of these signaling arms undergoes activation. Although direct evidence for distinct conformations of the receptor in response to unbiased and biased ligands is lacking, some findings do suggest that functionally different ligands can induce significantly different receptor conformations [84,85]. For example, fluorescence spectroscopy has been used to monitor conformational changes in purified and reconstituted β2AR labeled with an environmentally sensitive fluorophore, monobromobimane at Cys^{271} in the 3rd intracellular loop of the receptor. Bimane fluorescence quenching experiments using this receptor suggest that not only do full and partial agonists alter critical activation switches in the β2AR to different degrees but also, partial agonists with different efficacies are capable of affecting these switches to different extents [84]. More recently, an intramolecular fluorescence resonance energy transfer (FRET)-based β2AR biosensor was utilized to monitor conformational changes of the receptor in live cells. A systematic analysis of FRET changes in response to ligand stimulation revealed qualitative and kinetic differences in the conformations induced by two endogenous agonists of the receptor, epinephrine and norepinephrine [85]. Even stronger evidence for the existence of multiple active conformations of 7TMRs comes from the observation that certain receptor mutants, e.g. $\beta2AR^{TYY}$ [86] and $AT1R^{AAY}$ [87], do not exhibit any detectable activation of classical heterotrimeric G protein signaling even upon stimulation by their endogenous unbiased

agonists. However, these mutant receptors robustly recruit β-arrestins and activate β-arrestin-dependent signaling. These findings clearly indicate the inability of these mutant receptors to adopt a conformation that readily couples to their cognate G proteins and argue that a specific conformation exists that selectively couples to β-arrestins (Figure 4b). The structural aspects of these multiple active conformations of the 7TMRs are very poorly understood at present and will require thorough biophysical and crystallographic characterization. Interestingly, a crystal structure of the β2AR bound to a synthetic full agonist (i.e. unbiased ligand) in an active conformation was determined very recently. This structure shows significant outward movement of TM 5 and 6 and inward movement of TM 3 and 7 towards the central core of the 7TM bundle in comparison to the previously determined crystal structure of the β2AR in an inactive conformation [88]. It will be interesting to see how these features change when crystal structures of the β2AR in alternative signaling conformations are determined.

The bar code hypothesis

Conformational differences at the receptor level must be recognized by immediate downstream effectors such as GRKs and β-arrestins in order to initiate specific sets of functional responses. For a number of different receptors, depletion of either GRK 2/3 or GRK 5/6 exert clearly distinguishable effects on receptor regulation and signaling [89,90]. GRK2/3 appears to be responsible for bulk phosphorylation of the receptor and their depletion negatively affects receptor internalization and β-arrestin recruitment but not β-arrestin mediated signaling. Conversely, depletion of GRK5/6 appears to significantly reduce β-arrestin signaling to ERK 1/2 but has relatively lesser effects on internalization and β-arrestin recruitment. These observations provide indirect evidence for the idea that different sets of GRKs phosphorylate distinct non-overlapping sites on the receptors which in turn constitute a phosphorylation ‘bar code’ on the receptor. Thus it is tempting to speculate that the conformation of the receptor induced by an unbiased ligand will engage specific GRKs which will differ, partially or completely, from those engaged by receptor conformations induced by a biased ligand. Such phosphorylation bar codes could significantly affect the topology and charge of the intracellular face of the receptor in a specific fashion, in turn governing the conformation adopted by the recruited β-arrestins. The distinct conformations adopted by β-arrestins in response to differential phosphorylation bar codes on the receptor might then direct β-arrestins to engage in specific functions. Although differential receptor phosphorylation by different GRKs upon stimulation by unbiased versus biased ligands remains to be directly demonstrated, emerging data points to the existence of differential receptor phosphorylation in response to full versus partial agonists [67] and some preference for phosphorylation of specific sites by specific GRKs [91].

In vitro studies of the interaction of isolated non-rhodopsin receptors with β-arrestins have been technically very challenging. Therefore, a phosphorylated peptide corresponding to the C-terminus of the vasopressin receptor has been used as a mimic for receptor–β-arrestin interaction [92,93]. Binding of these phosphopeptides induces a conformational change in β-arrestins similar to that induced in visual arrestin upon binding of a rhodopsin phosphopeptide [94]. Limited proteolysis and MS-based analyses reveal that the conformational change induced by the phosphopeptide leads to the release of the C-terminus of arrestins which in turn exposes its binding site for clathrin. Such a model is compatible with the activation mechanism of β-arrestins upon recruitment to phosphorylated 7TMRs and with the ability of β-arrestins to promote receptor internalization. Further support for a conformational change in β-arrestin 2 upon binding to the activated receptor is provided by experiments with a BRET-based biosensor in live cells [95,96]. The idea of a phosphorylation bar code on the receptor takes the issue of β-arrestin conformational change

a step further, and suggests that multiple distinct active conformations of β-arrestins are induced corresponding to the phosphorylation pattern of the receptor. In fact, strong evidence for such a possibility comes from a study that used a previously described BRET based β-arrestin biosensor in live cells [96]. Structural changes in β-arrestin cause rearrangement of the two ends of the molecule, thus altering BRET efficiency in such a way as to indicate conformational changes. In three different receptor systems, receptor activation by an unbiased ligand increases the BRET signal, thus indicating β-arrestin recruitment to the receptor and a conformational change. Strikingly, when the receptor is stimulated by a β-arrestin-biased agonist, there is a decrease in BRET signal suggesting that β-arrestin adopts a different conformation upon recruitment to the receptor. It is tempting to speculate that an increase in BRET signal corresponds to a conformation of β-arrestin which is capable of both receptor desensitization and signaling, whereas a decrease in BRET signal reflects a conformation which is selectively engaged in signaling. In fact, the use of β-arrestin-biased receptor mutants strongly supports this hypothesis. A deceased BRET signal is observed when β-arrestin-biased mutants of the β2AR and the AT1aR are stimulated by unbiased ligands. Even an unbiased ligand can only place the receptor in a conformation that is selectively engaged in β-arrestin signaling, therefore, recruited β-arrestin probably will adopt a signaling conformation which in this case is reflected by a decrease in BRET signal. These findings establish the notion that β-arrestins can not only adopt multiple active conformations, but also that these conformations are coupled to distinct functional outcomes. An interesting, but very challenging, goal is to map the structural details of these distinct conformational changes either by indirect biophysical tools or X-ray crystallography (Box 2). Moreover, structural determination of β-arrestins in complex with activated receptors, preferably occupied by different ligands, will help to delineate the interaction interface as well the critical determinants responsible for inducing functionally distinct conformations in β-arrestins.

Box 2

Areas requiring significant progress in the coming years

1. **Structural characterization of β-arrestin signaling complexes.** With the crystal structures of all four isoforms of β-arrestins available, the next step will be to trap the complexes of β-arrestins with their interacting partners. Such structures should shed light on the interaction interface, functional plasticity and the multiple conformations of these multifunctional molecules.
2. **Structural characterization of receptor-β-arrestin signaling complexes.** Although the phosphopeptides corresponding to the C-terminus of receptors have been used as a surrogate to study receptor–β-arrestin interactions, a complete understanding of these interactions will require co-crystallization of receptors with β-arrestins. Such experiments should provide direct information on the interaction interface as well as activation-dependent conformational changes in β-arrestins which govern receptor-mediated β-arrestin-dependent signaling and regulatory events in cells.

Concluding remarks and future perspectives

As systems biology approaches become more routine with advances at technological and bioinformatics levels, the number of large data sets describing global views of the functional capabilities of β-arrestins in the context of either specific receptors or specific stimuli should accumulate rapidly. This will hopefully result in construction of system wide signaling networks and uncover many new avenues for future investigation. However, the direct demonstration and functional analysis of these networks in physiological settings will

remain challenging. Developing a better understanding of the physiological consequences of G protein-dependent and β-arrestin-dependent signaling for any given receptor will be extremely important (Box 3). Such studies, utilizing knock out animal models, will be critical to subsequent design of appropriate biased ligands as potential drugs. The structural characterization of 7TMRs in multiple signaling conformations and in complex with their effectors such as GRKs and β-arrestins needs to be carried out in order to provide in-depth molecular details of biased signaling. Structures of β-arrestins in complex with their own interaction partners will be critical to understanding the broad functional capabilities of these molecules. Such structures will not only provide molecular details of the interactions yielding distinct functional outcomes but should also help in the design of better tools to modulate these complicated networks for therapeutic purposes.

Box 3

Hottest areas of research

1. **Physiological implications of β-arrestin-dependent signaling using animal models.** Studies with β-arrestin knockout mice have provided several key insights into the physiological implications of β-arrestin-dependent signaling. As new signaling paradigms emerge from global screens, *in vivo* studies in animal models will be crucial for gaining a better understanding of the finer ramifications of these signaling events in order to design tools for therapeutic manipulations.
2. **Developing biased ligands as novel therapeutics.** Biased ligands for several receptors present a unique opportunity to explore the possibilities of designing a novel class of drugs targeting 7TMRs. TRV120027, the first β-arrestin biased agonist that targets AT1aR has just entered in phase II clinical trials for congestive heart failure.
3. **Systems biology approaches to decipher β-arrestin-dependent signaling networks.** Global screens using proteomics-based approaches clearly highlight the way forward to uncover β-arrestin-dependent signaling networks in cells. Systematic validation of novel networks *in vivo*, however, will remain challenging.

A major theme that has emerged in the last decade is that β-arrestins, in addition to their classical roles in 7TMR desensitization, are also critically involved in 7TMR endocytosis, ubiquitylation and signaling events. Moreover, it is now well established that β-arrestin-dependent signaling is extremely diverse and regulates an ever-growing list of signaling pathways. Furthermore, the regulatory effects of β-arrestins clearly go beyond the realm of 7TMRs and involve several other classes of membrane proteins such as ion channels, transporters, receptor tyrosine kinases and cytokine receptors. These findings imply that the regulatory roles of β-arrestins span a wider spectrum of physiological and pathological processes than currently appreciated.

Acknowledgments

We thank Drs. Seungkirl Ahn, Jeffrey Kovacs and Tanya Daigle for critical reading of the manuscript. R.J.L. is an investigator with the Howard Hughes Medical Institute (HHMI). We acknowledge grant support from the National Institutes of Health (HL16037 and HL70631). We thank Donna Addison and Quivetta Lennon for excellent administrative assistance.

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Fig. 1A

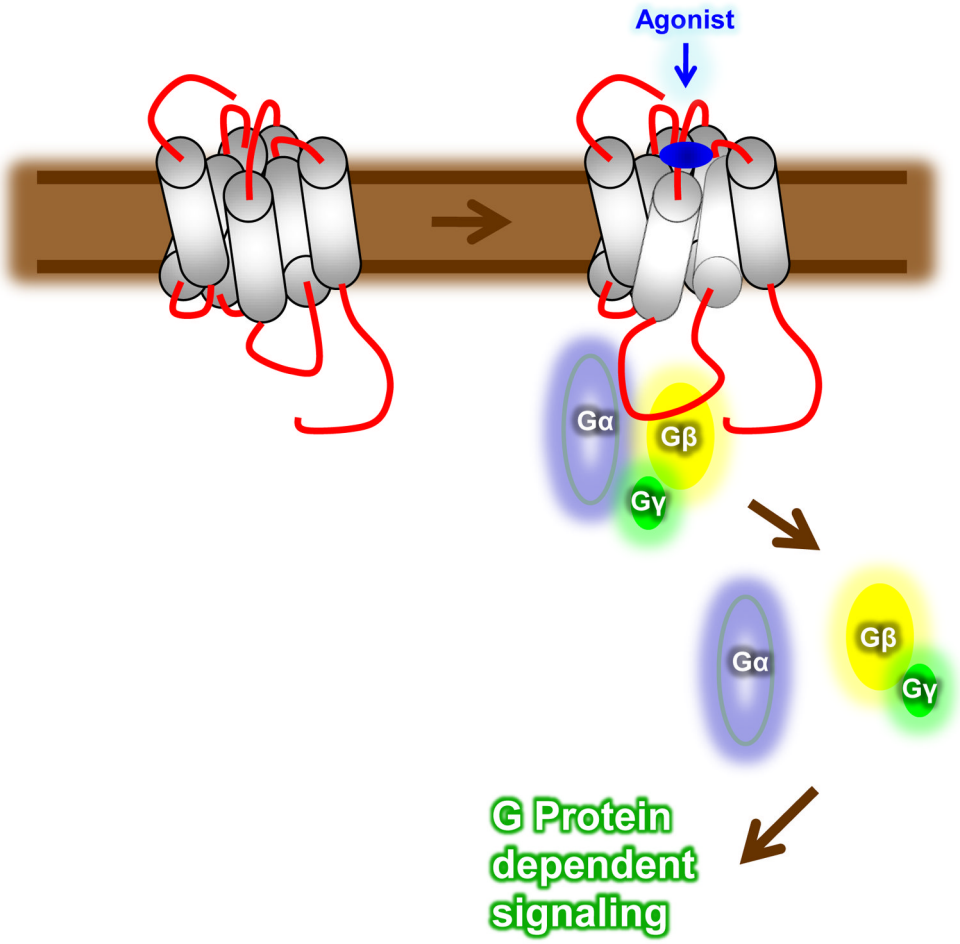
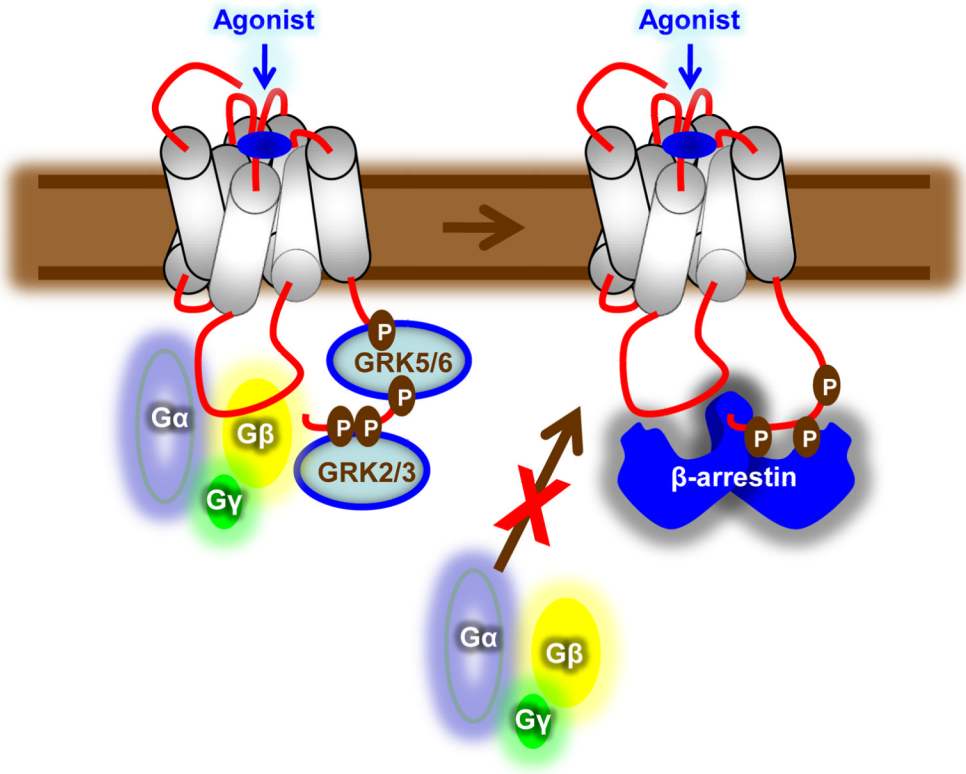


Fig. 1B



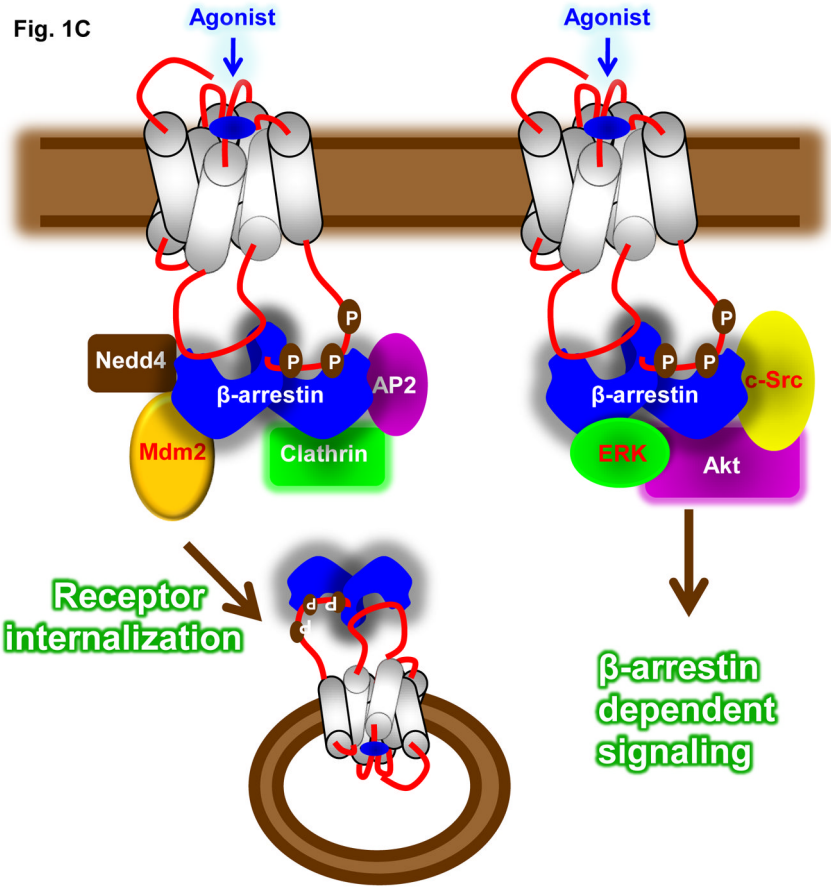


Figure 1.

Multifaceted roles of β-arrestins and GRKs in 7TMR signaling and regulation. **(a)** The classical paradigm of G protein-dependent signaling via 7TMRs where binding of an agonist leads to conformational changes in the receptor. Activated receptor in turn couples to and activates heterotrimeric G proteins. Upon activation, heterotrimeric G proteins dissociate and activated G_{α} and $G_{\beta\gamma}$ lead to generation of downstream signaling. **(b)** GRK-mediated phosphorylation and β-arrestin-mediated desensitization of 7TMRs. Agonist occupied activated receptors are phosphorylated by GRKs at serine/threonines primarily in the C-terminus but also in the intracellular loops. Phosphorylated receptors recruit multifunctional adaptor proteins β-arrestins which sterically hinder further G protein coupling to the receptor and in turn lead to receptor desensitization. **(c)** Novel roles of β-arrestins as endocytosis adaptors, E3 ubiquitin ligase adaptors and the new paradigm of β-arrestin dependent signaling downstream of 7TMRs. Receptor bound β-arrestins also recruit several components of clathrin dependent internalization machinery to the activated receptors and subsequently promote receptor endocytosis via clathrin coated pits. Moreover, β-arrestins also act as adaptors for a number of different E3 ubiquitin ligases to facilitate receptor ubiquitination. Surprisingly, β-arrestins are also capable of scaffolding a number of signaling molecules such as c-Src, Akt and ERK in order to initiate G protein-independent signaling downstream of activated 7TMRs. Please note that the different binding partners of β-arrestins shown in the figure probably do not bind β-arrestins simultaneously.

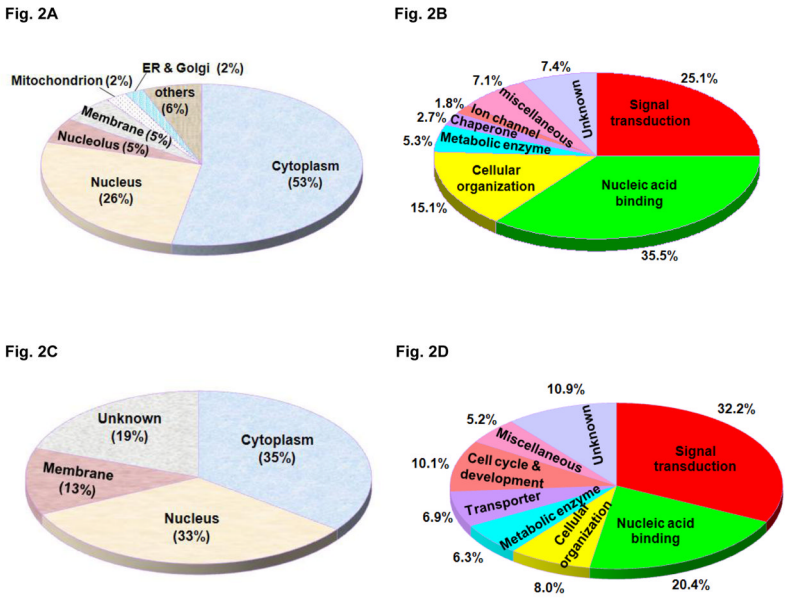


Figure 2.

The global scope of the β-arrestin interactome and β-arrestin dependent phosphorylation events. **(a)** Subcellular and **(b)** functional distribution of β-arrestin interacting proteins with and without stimulation of the AT1aR by angiotensin II [68]. Note that the majority of β-arrestin interaction partners are distributed in the cytoplasm but a significant fraction are nuclear proteins highlighting the potential nuclear roles of β-arrestins. The functional distribution of β-arrestin binding proteins highlights their major roles in cellular signaling, cellular organization and nucleic acid binding. **(c)** Subcellular and **(d)** Functional distribution of the proteins which are phosphorylated upon activation of the AT1aR by a β-arrestin-biased ligand, SII-angiotensin [68]. Note that both the cellular and functional distribution of proteins which are phosphorylated/dephosphorylated upon SII stimulation in a β-arrestin dependent manner mimics the pattern of proteins identified in the interactomics screen.

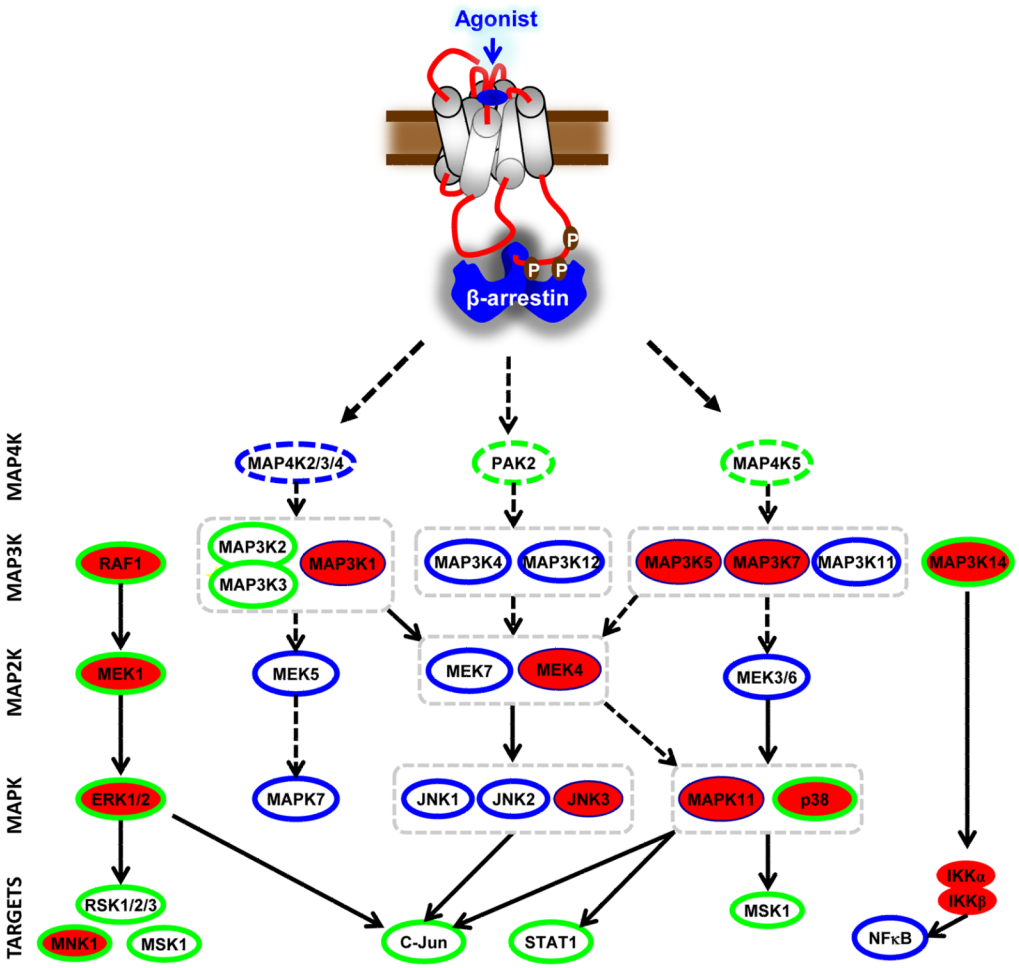


Figure 3.

An example of a β-arrestin dependent MAP kinase signaling network downstream of AT1aR. The proteins highlighted in red interact with β-arrestins, the proteins outlined in green are phosphorylated upon activation of AT1aR by a β-arrestin biased ligand, SII-angiotensin. The protein highlighted in red and outlined in green were present in both, the β-arrestin interactome and the β-arrestin phosphoproteome. The proteins outlined in blue are present in the corresponding MAP kinase pathways but are not identified in the proteomics screen. Please note that this figure highlights a potential β-arrestin dependent MAP kinase signaling network based on interactomics and phosphoproteomics studies and all the MAP kinase modules depicted here have not necessarily been documented to be regulated by β-arrestins. The dotted arrows indicate the pathways which have not yet been confirmed by independent studies to be directly activated by β-arrestins, the solid arrows indicate the pathways which have been studied in detail and which are well established to be modulated by β-arrestins.

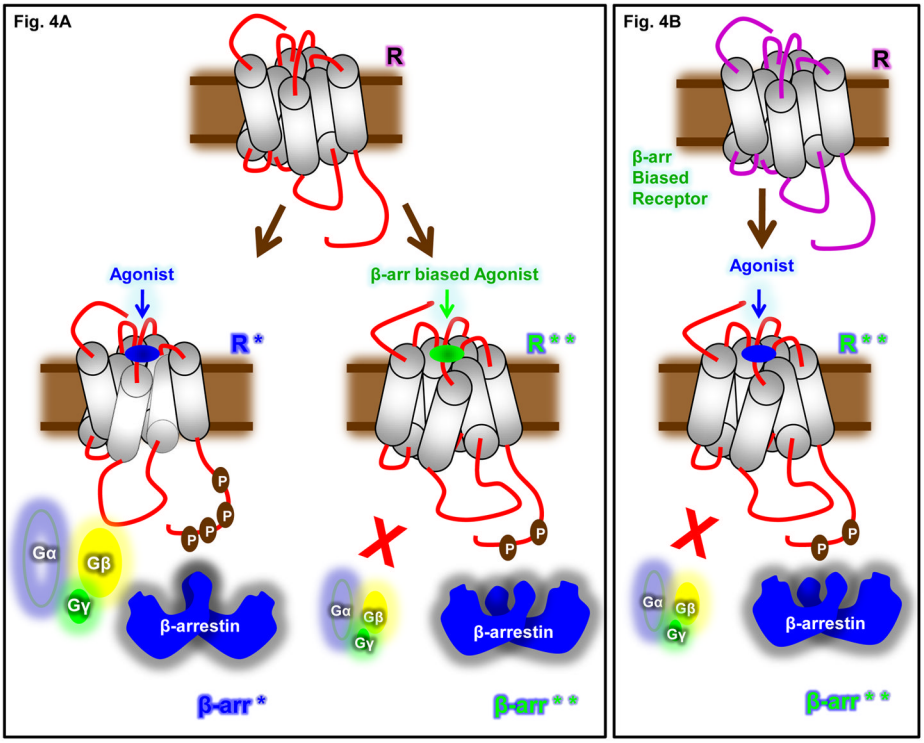


Figure 4.

A schematic representation of a simple conceptual framework to explain the mechanistic basis of β-arrestin biased signaling. **(a)** Binding of an unbiased ligand to the receptor induces an active conformation of the receptor (R*) whereas binding of a β-arrestin-biased ligand promotes a different active conformation of the receptor (R**). The distinct receptor conformations are coupled to corresponding active conformations of β-arrestin (β-arr * and β-arr **) which govern different functional outcomes. **(b)** Binding of an unbiased agonist to a β-arrestin biased receptor also induces a distinct conformation in the receptor which is likely to be similar to that induced by a β-arrestin-biased ligand to the wild-type receptor and a corresponding conformation in β-arrestin. The exact structural details of these multiple active conformations of receptors and β-arrestins remain to be deciphered by crystallography and other biophysical methods.