**Name of Journal: *World Journal of Biological Chemistry***

**Manuscript NO: 41750**

**Manuscript Type: MINIREVIEWS**

**Arrestin-mediated signaling: Is there a controversy?**

Gurevich VV *et al*. Arrestin-mediated signaling

Vsevolod V Gurevich, Eugenia V Gurevich

**Vsevolod V Gurevich, Eugenia V Gurevich,** Department of Pharmacology, Vanderbilt University, Nashville, TN 37232, United States

**ORCID number:** Vsevolod V Gurevich (0000-0002-3950-5351); Eugenia V Gurevich (0000-0002-0563-8295).

**Author contributions:** Gurevich VV and Gurevich EV wrote the manuscript.

**Supported by** National Institutes of Health RO1 grants, No. EY011500; National Institutes of Health R35 grants, No. GM122491; and Cornelius Vanderbilt Endowed Chair (Vanderbilt University), No. NS065868 (to Gurevich VV) and No. DA030103 (to Gurevich EV).

**Conflict-of-interest statement:** The authors declare no conflict of interest.

**Open-Access:** This is an open-access article that was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: <http://creativecommons.org/licenses/by-nc/4.0/>

**Manuscript source:** Invited manuscript

**Correspondence to:** **Vsevolod V Gurevich, PhD, Professor,** Department of Pharmacology, Vanderbilt University, 2200 Pierce Ave, PRB Rm 417D, Nashville, TN 37232, United States. [vsevolod.gurevich@vanderbilt.edu](mailto:vsevolod.gurevich@vanderbilt.edu)

**Telephone:** +1-615-3227070

**Fax:** +1-615-3436532

**Received:** August 28, 2018

**Peer-review started:** August 28, 2018

**First decision:** September 11, 2018

**Revised:** October 20, 2018

**Accepted:** November 2, 2018

**Article in press:**

**Published online:**

**Abstract**

The activation of the mitogen-activated protein (MAP) kinases extracellular signal–regulated kinase (ERK)1/2 was traditionally used as a readout of signaling of G protein-coupled receptors (GPCRs) *via* arrestins, as opposed to conventional GPCR signaling *via* G proteins. Several recent studies using HEK293 cells where all G proteins were genetically ablated or inactivated, or both non-visual arrestins were knocked out, demonstrated that ERK1/2 phosphorylation requires G protein activity, but does not necessarily require the presence of non-visual arrestins. This appears to contradict the prevailing paradigm. Here we discuss these results along with the recent data on gene edited cells and arrestin-mediated signaling. We suggest that there is no real controversy. G proteins might be involved in the activation of the upstream-most MAP3Ks, although *in vivo* most MAP3K activation is independent of heterotrimeric G proteins, being initiated by receptor tyrosine kinases and/or integrins. As far as MAP kinases are concerned, the best-established role of arrestins is scaffolding of the three-tiered cascades (MAP3K-MAP2K-MAPK). Thus, it seems likely that arrestins, GPCR-bound and free, facilitate the propagation of signals in these cascades, whereas signal initiation *via* MAP3K activation may be independent of arrestins. Different MAP3Ks are activated by various inputs, some of which are mediated by G proteins, particularly in cell culture, where we artificially prevent signaling by receptor tyrosine kinases and integrins, thereby favoring GPCR-induced signaling. Thus, there is no reason to change the paradigm: arrestins and G proteins play distinct non-overlapping roles in cell signaling.

**Key words:** G protein-coupled receptors; Arrestin; G protein; Signaling; Extracellular signal–regulated kinase 1/2; c-Jun N-terminal kinase 3

**© The Author(s) 2018.** Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Both arrestins and G proteins play important roles in G protein-coupled receptor (GPCR) signaling, including GPCR-initiated activation of mitogen-activated protein (MAP) kinases extracellular signal–regulated kinase (ERK)1/2 and c-Jun N-terminal kinase 3 (JNK3). Their roles do not overlap. G proteins participate in signal initiation, by activating MAP3Ks. Arrestins, free and GPCR-bound, function as scaffolds of the three-tiered MAP kinase cascades, facilitating signal transduction. Cells express other scaffolds, so that no MAPK cascade relies solely on arrestins. Different experimental paradigms highlight the role of G proteins or arrestins in this process, and neither can be discounted based on available evidence.

Gurevich VV, Gurevich EV. Arrestin-mediated signaling: Is there a controversy? *World J Biol Chem* 2018; In press

**INTRODUCTION**

G-protein-coupled receptors (GPCRs) respond to hormones, neurotransmitters, light, odorants, taste molecules, extracellular calcium, extracellular protease activity, cell adhesion, and a variety of other stimuli[1]. All members of the GPCR super-family (which includes hundreds of receptors encoded by different genes in animals) share a common transmembrane domain consisting of seven α-helices, which are connected by intra- and extra-cellular loops of variable lengths[2]. Their extracellular N-termini and intracellular C-termini also differ widely in size and structure[2]. Upon activation by an appropriate input most GPCRs serve as guanyl nucleotide exchange factors of heterotrimeric G proteins, facilitating the release of guanosine diphosphate (GDP) bound to their inactive α-subunits and its exchange for guanosine triphosphate (GTP), which is a lot more abundant in cells. Activated G proteins then dissociate from the receptors, their α- and βγ-subunits separate and activate or inhibit various effectors. Active GPCRs can sequentially activate several molecules of G proteins, providing signal amplification at this level. Active GPCRs are also specifically phosphorylated by G protein-coupled receptor kinases (GRKs)[3], of which most mammals have seven. Nocturnal rodents only have six, as they are missing GRK7, specialized GRK expressed in cone photoreceptors, which function in relatively bright light.

**ARRESTIN-MEDIATED GPCR DESENSITIZATION**

The first arrestin family member (current systematic name arrestin-1) was discovered in the visual system as the protein that specifically binds active phosphorylated rhodopsin and suppresses its signaling[4]. Thus, desensitization, *i.e.*, the suppression of G protein-dependent signal transduction, was the first arrestin function discovered. Subsequently the first[5] and then the second non-visual arrestin[6-8] were cloned. The demonstration that the first non-visual arrestin preferentially desensitized phosphorylated β2-adrenergic receptors (β2AR) (which gave it the original name, β-arrestin; systematic name arrestin-2), whereas visual arrestin-1 preferentially desensitized phosphorylated rhodopsin[9], suggested the idea that all arrestins desensitize cognate GPCRs *via* specific binding to their active phosphorylated state[10]. Thus, the field came to believe that the model of two-step desensitization, phosphorylation of active GPCRs by specific GRKs, reviewed in[3], followed by arrestin binding to the active phosphorylated receptor, applies to all GPCRs[10-12]. In this paradigm, the role of arrestins is to stop GPCR signaling *via* G proteins. This remains the best characterized biological function of all arrestin proteins[11]. Subsequent findings that receptor-associated non-visual arrestins directly bind clathrin[13] and clathrin adaptor, adaptor protein 2 (AP2)[14], the key components of the coated pit, and that the binding to both is enhanced by arrestin-receptor interactions[15], suggested that arrestins participate in the next step of desensitization, *i.e.* receptor removal from the plasma membrane *via* internalization.

**GPCR-DEPENDENT ARRESTIN SIGNALING**

The arrestin-mediated cellular signaling was first discovered upon GPCR stimulation, and therefore was assumed to be strictly receptor-dependent. The binding of non-visual arrestins to their cognate receptors was shown to facilitate the activation of protein kinases proto-oncogene tyrosine-protein kinase Src (c-Src)[16], c-Jun N-terminal kinase 3 (JNK3)[17], then extracellular signal–regulated kinase (ERK)1/2[18]. As JNKs and ERKs are mitogen-activated protein kinases (MAPKs) activated *via* the three-tiered kinase cascade (in general terms, MAP3K, MAP2K, and MAPK[19,20]), the latter two cases suggested that receptor-bound arrestins scaffold the three-kinase modules, thereby facilitating signal transduction in them. Initial studies detected direct arrestin binding to both MAP3Ks, RAF proto-oncogene serine/threonine-protein kinase (cRaf) (a.k.a. Raf1) and apoptosis signal-regulating kinase 1 (ASK1), and corresponding MAPKs, ERK1/2 and JNK3, but not to the MAP2Ks of these cascades, MEK1 or MKK4/7[17,18]. However, subsequently arrestin interactions with MEK1[21], as well as with MKK4 and MKK7[22,23] were documented. Thus, the idea of scaffolding of MAP kinase cascades by arrestin bound to a GPCR received further experimental support. The binding of arrestins to ERK1/2 is barely detectable in the absence of activated GPCR[24], and both arrestin binding to ERK1/2 and arrestin-dependent ERK1/2 activation are greatly facilitated by GPCR stimulation[18]. Therefore, arrestin-dependent ERK1/2 activation following GPCR stimulation in the experimental conditions excluding other inputs (see below) became a readout of choice for arrestin-mediated signaling. It has been shown that GPCRs that form stable complexes with arrestins tend to increase ERK1/2 activity in the cytosol, presumably *via* retaining ERK1/2 activated by the GPCR-bound arrestin scaffold in that compartment, whereas GPCRs that form transient complexes with arrestins induce mitogenic response due to the translocation of active ERK1/2 to the nucleus, where it acts on its nuclear substrates[25]. Moreover, using siRNA knockdown ERK1/2 activation by angiotensin II type 1A receptor *via* G proteins (likely Gq/11) was found to be transient, peaking at 2 min and then rapidly declining, whereas arrestin-mediated activation of ERK1/2 was shown to peak later and last much longer[26]. Even though ERK1/2 can be activated *via* a variety of pathways in the cell[27], it became widely accepted that the late phase (10-30 min after the stimulus) of ERK activation reflects GPCR signaling *via* arrestins[28,29]. However, it has been shown that G protein-mediated ERK1/2 activation can also have a late phase (see as the first report of this phenomenon[30], reviewed in[31]). As the late phase of ERK1/2 activation was subsequently shown to be mediated by G proteins in several other studies involving different GPCRs, the time course of ERK1/2 activation cannot be regarded as an indication of it being G protein- or arrestin-dependent.

The molecular mechanism of arrestin-mediated connection between GPCRs and proteins containing Src homology 3 (SH3) domains was recently extensively investigated using biophysical methods[32]. The data suggest that arrestin “unwinds” the auto-inhibited conformation of c-Src, thereby directly activating this kinase. Free arrestin-2 was found to have detectable effect, but an increase in Src activity upon binding to the arrestin-2 engaged by an appropriately phosphorylated GPCR, or, to a lesser extent, receptor-derived phosphopeptide, was much greater[32]. The authors detected notable differences between conformational changes in arrestin-2 induced by differentially phosphorylated peptides[32], which is consistent with the barcode hypothesis positing that GPCRs phosphorylated at different sites by different GRKs have differential effects on arrestin conformation, which is translated into the activation of distinct branches of arrestin-mediated signaling[33,34].

**GPCR-INDEPENDENT ARRESTIN SIGNALING**

Interestingly, whereas both non-visual arrestin-2 and -3 (a.k.a. β-arrestin1 and 2) appeared to facilitate the activation of c-Src and ERK1/2, only one subtype, arrestin-3, facilitated the activation of JNK3[24,35,36], as well as at least some isoforms of ubiquitously expressed JNK1 and JNK2[37]. Early studies revealed that arrestin-3 can facilitate JNK3 activation even in receptor-independent manner, when the upstream-most kinase, MAP3K ASK1, is overexpressed[24,35]. This finding was confirmed by documenting that arrestin-3 mutant incapable of GPCR binding (which has a deletion in the inter-domain hinge region, precluding domain movement[38,39] necessary for the binding to GPCRs[40-42]) promotes JNK3 activation as effectively as wild type (WT) arrestin-3[43]. It was also shown that replacement of certain residues in the arrestin-3 with their homologues from closely related arrestin-2[6,7] impedes its ability to activate JNK3, rendering it arrestin-2-like[36]. Systematic comparison of the effects of β2AR ligands acting *via* endogenous receptor on the activation of ERK1/2 and JNK3 in the same cells expressing various forms of arrestin-3 proved beyond reasonable doubt receptor-independence of this arrestin-3 function: while the levels of active phosphorylated ERK1/2 reflected the functional state of the receptor, the levels of active phospho-JNK3 did not depend on it, reflecting only the nature of arrestin-3 mutant expressed[43]. Arrestin-3-mediated scaffolding of the two modules of the JNK3-activating cascade, MKK4-JNK3 and MKK7-JNK3, was demonstrated using purified proteins *in vitro* in the absence of any GPCRs, confirming yet again that receptors are not necessary for this arrestin-3 function[22,23]. Recent structure of the arrestin-3 trimer crystallized in the presence of a fairly abundant intracellular small molecule, inositol-hexakisphosphate (IP6)[44] revealed that all three protomers in the trimer are in the “active” (receptor-bound-like) conformation. It was similar to the conformation of arrestin-1 in complex with rhodopsin[41,42], as well as the conformations of constitutively active arrestin-1 splice variant p44[45] and C-terminally truncated arrestin-2 in complex with the phosphopeptide derived from the angiotensin receptor C-terminus[40], thereby suggesting a molecular mechanism of receptor-independent activation of arrestin-3. These data suggest that at least one of non-visual arrestins, arrestin-3, can assume “active” (GPCR bound-like) conformation without the help of GPCRs[44]. Curiously, the molecular mechanism of arrestin-3 activation in this case appears to resemble the mechanism of activation of all arrestins by GPCRs: the phosphates of IP6 engage the same positively charged side chains in arrestin as receptor-attached phosphates[46]. It is also noteworthy that GPCRs might activate arrestins catalytically, *i.e.*, that arrestins can maintain active conformation after dissociation from GPCRs[47]. Thus, multiple mechanisms can generate “active” arrestins in the cytoplasm that are not bound to GPCRs.

The propensity of arrestin-3 mutants to form trimers in the presence of IP6 appeared to correlate with their ability to facilitate JNK3 activation in cells[44]. However, no IP6 was used in the experiments where MKK4-JNK3 and MKK7-JNK3 modules were reconstituted *in vitro* from purified proteins with arrestin-3[22,23], suggesting that this subtype can assume active (at least in terms of the ability to facilitate signaling in the JNK3 activation cascade) conformation spontaneously, without the help of IP6. Indeed, structural data[48] and molecular dynamics simulations[49] indicate that arrestin-3 is more flexible than other arrestin subtypes. A short arrestin-3-derived peptide comprising the first 25 residues was found to facilitate JNK3 activation both *in vitro* and in cells[50]. This peptide is unlikely to trimerize, as it does not contain most of the inter-protomer interfaces observed in the crystal trimer[44]. It was expressed as a fusion with well-folded proteins (MBP in *E. coli* and YFP in mammalian cells), which suggests that it simply needs to have loose conformation to function as a scaffold[50]. Interestingly, the arrestin-3 N-terminus, containing this peptide, does not appear to be particularly loose in the crystal trimer[44], so that the detailed molecular mechanism of arrestin-3-mediated scaffolding of the ASK1-MKK4/7-JNK3 cascade still remains to be elucidated.

The facilitation of JNK3 phosphorylation by arrestin-3 is not the only receptor-independent function of arrestin proteins documented. It was recently shown that arrestin-2-(1-380) fragment generated by caspase cleavage in the absence of receptor stimulation translocates to the mitochondria, where it assists caspase-cleaved tBid in releasing cytochrome c, thereby promoting apoptotic cell death[51]. Both non-visual arrestins and their receptor binding-deficient mutants affect cell spreading and motility *via* disassembly of focal adhesions and regulation of small GTPases[52,53]. Thus, several signaling functions of arrestins do not appear to be dependent on GPCRs, and, by extension, on G proteins.

**ROLE OF G PROTEINS**

As discussed above, some signaling functions could be performed by free arrestins independently of their interaction with GPCRs and, consequently, of G proteins activated by these receptors. However, arrestin-dependent signaling has long been considered to require arrestin binding to GPCR but at the same time to be G protein-independent serving as an alternative pathway of the GPCR signaling (*e.g.*, see[54]; recently reviewed in[55,56]). Indeed, free arrestins have minimal effect on certain signaling pathways, such as ERK1/2 activation, whereas arrestins bound to agonist-activated phosphorylated GPCRs are able to facilitate signaling in these pathways. Recently the notion of G protein independence of the GPCR-initiated arrestin signaling function has been called into question.

The use of CRISPR-Cas9 gene editing enabled the creation of cells lacking individual G proteins or several G proteins at the same time[57]. The only class of G proteins that could not be eliminated by CRISPR-Cas9 gene editing was Gi/o subtypes, but these G proteins can be inactivated by pertussis toxin[57,58]. Thus, the combination of inactivation of Gi/o proteins by pertussis toxin in cells where all other G protein subtypes were knocked out by CRISPR-Cas9 made possible the construction of cells lacking all G protein-mediated signaling (termed “zero functional G” cells). A comprehensive study was performed in these cells, with numerous GPCRs, including β2AR and angiotensin1 receptor often used to demonstrate arrestin-dependent ERK1/2 activation[58]. The results showed that neither arrestin recruitment to GPCRs nor receptor internalization requires G protein signaling. However, the authors did not detect any arrestin-mediated ERK1/2 activation in “zero functional G” cells using a variety of methods, including label-free dynamic mass redistribution and ERK1/2 phosphorylation in response to receptor stimulation[58]. Interestingly, the authors documented the role of arrestins in ERK1/2 activation by comparing “zero arrestin” cells with parental line, but only when at least some G protein-mediated signaling remained (illustrated by the Supplementary Figure 4 in Grundmann *et al*[58]). The main take-home message of that study was that while GPCR-induced arrestin-mediated signaling exists, it requires G protein action. The results suggested that without G proteins arrestins do not regulate ERK1/2 activation. Thus, the field has to decide whether these data call for yet another paradigm change.

**MAPKS ARE ACTIVATED BY VARIOUS INPUTS**

It is important to note that *in vivo* the main activators of MAPK cascades are not GPCRs. In most cases upstream MAP3Ks are activated by growth factor receptors[19,59], death receptors[60], integrins[61], or various stressors[62]. We should keep in mind that experimental paradigms used to study arrestin-mediated signaling actually exclude non-GPCR inputs. Cultured cells are usually plated on supports that do not activate integrins. In addition, cells where GPCR-induced MAPK activation is studied are routinely serum-starved, *i.e.*, maintained in growth factor-deficient conditions, which prevents MAPK activation *via* growth factor and/or death receptors, likely the prevalent mechanisms *in vivo*. Mammals have 20 different MAP3Ks that integrate signaling inputs[20]. The mechanisms of MAP3Ks activation are usually complex. For example, one of the MAP3Ks of the ERK1/2 cascade, cRaf (a.k.a. Raf1) is activated by active (GTP-liganded) small G proteins of Ras family, which recruit it to the membrane and promote its dimerization. cRaf dimerizes with other members of RAF family and kinase suppressor of Ras (KSR). An element adjacent to the Ras-binding domain, cysteine-rich domain stabilized by zinc, binds phosphatidylserine, facilitating membrane anchoring[63]. Several additional events contribute to cRaf activation[63]: Ras binding facilitates dephosphorylation of the site upstream of the kinase domain that in the inactive state of Raf1 binds 14-3-3 protein. Dimerized cRaf molecules apparently phosphorylate the activation segment, which stabilizes the active form of the kinase. For full activity, the negatively charged N-terminal region and the C-terminal 14-3-3 binding site also need to be phosphorylated. Another example of complex activation mechanism is ASK1, one of the MAP3Ks of JNK1/2/3 cascades. It is activated by oxidative stress, endoplasmic reticulum (ER) stress, calcium influx, or mechanical stress, and inhibited by the interactions with reduced thioredoxin and 14-3-3 protein[62]. Its phosphorylation on three different serines in the N- and C-terminal elements is inhibitory, whereas the phosphorylation of the three threonines in the kinase domain is stimulatory[62]. Thus, in addition to being phosphorylated on threonines, for full activation the three serines in ASK1 must be dephosphorylated, and both thioredoxin and 14-3-3 protein must dissociate[62]. To the best of our knowledge, none of these events is regulated by heterotrimeric G proteins. It is entirely possible that when MAP3Ks are activated *via* GPCR-independent mechanisms by integrins, death or growth factor receptors, or stressors, G proteins are not involved, whereas arrestins might still act as scaffolds bringing the three kinases of MAPK cascades together.

**MAPK ACTIVATION IN DIFFERENT SUBCELLULAR COMPARTMENTS**

Another important aspect of MAP kinase signaling is related to cell compartmentalization. Most MAPKs phosphorylate transcription factors in the nucleus, although practically every MAP kinase has cytoplasmic or even plasma membrane-localized substrates[64]. Naturally, the biological impact of MAPK activity towards nuclear and non-nuclear proteins has very different biological meaning. Free arrestins are soluble cytoplasmic proteins, whereas GPCR-bound arrestins localize even more restrictively, to the plasma membrane and endosomes. Localization of scaffolds determines where active MAPKs are generated, thus directing their signaling towards substrates in a particular cellular compartment. The original studies suggested that ERK1/2 activated *via* arrestin scaffold remains in the cytoplasm[18], where it phosphorylates its non-nuclear substrates, whereas ERK1/2 activated *via* G protein- and growth factor receptor-mediated mechanisms translocates to the nucleus[18]. Indeed, in some cases arrestin-mediated activation of ERK1/2 was shown not to affect transcription[65]. However, a recent study showed that arrestin-2 in adrenocortical zona glomerulosa facilitates aldosterone production by ERK1/2 activation[66], apparently *via* transcription regulation. Similarly, ERK1/2 activation by angiotensin 1A receptor in vascular smooth muscle *via* both Gq and arrestin was shown to involve transactivation of EGF receptor[67]. Thus, direct biological consequences of ERK1/2 activation also cannot be used to distinguish between arrestin-dependent and -independent mechanisms of its activation.

**BIASED GPCR SIGNALING**

Recently GPCR ligands that bias the signaling towards G proteins or arrestins have attracted a lot of attention as tools that might help achieving desired therapeutic outcome while minimizing unwanted side effects[55,56]. Ligand-activated GPCRs[68], as well as light-activated prototypical GPCR rhodopsin[69], exist in an equilibrium of multiple conformational states (reviewed in[70]). Thus, distinct subsets of active GPCR conformations might preferentially bind particular signal transducers, such as different G proteins and/or arrestins. The data suggesting that G protein action is required for arrestin-mediated signaling appears to be inconsistent with the concept of arrestin-biased signaling. Indeed, if we envision a ligand with the 100% bias towards arrestin, then it might have to rely on alternative signaling inputs to provide an initial “push”, at least, for some signaling pathways such as the ERK activation, before arrestins could step in. However, these findings do not contradict the idea that GPCR ligands that promote arrestin recruitment to a greater extent than G protein activation can yield signaling outcomes quite different from those generated by unbiased ligands promoting the activation of both G proteins and arrestins.

Indeed, it is likely that a relatively low level of G protein activity is sufficient to provide the initial activation of the MAPK pathways where arrestins play the role of scaffolds or signaling enhancers. Furthermore, practically all G proteins have measurable rate of spontaneous exchange of GDP for GTP[71], *i.e.*, activation. In addition, non-GPCR activators, such as AGS proteins[72], or other proteins containing G protein regulatory (GoLoco) motif[71], were shown to catalyze nucleotide exchange, leading to G protein activation. Thus, a fraction of the G protein pool in the cell is always active. In practical terms, considering that GPCRs and their endogenous ligands were designed by evolution to signal in both directions, it is highly unlikely that 100% effective bias can be achieved by manipulation of ligand structure. For example, it was recently shown that carvedilol, which was traditionally considered to be a “clean” arrestin-biased ligand of β-adrenergic receptors, actually promoted β1-adrenoreceptor coupling to Gi proteins, and this unconventional Gi activation by the receptor that was believed to be strictly Gs-specific is required for observed “arrestin-biased” signaling[73]. In the same vein, recent comprehensive analysis of 65 different ligands of β2AR identified many G protein-biased ones, but none specifically biased towards arrestin recruitment[74], reinforcing the notion that the evolution “designed” GPCRs primarily to activate G proteins. Thus, it appears likely that any synthetic arrestin-biased ligand will have sufficient ability to produce necessary G protein activation, so that the proposed model suggesting the involvement of G proteins in arrestin-mediated signaling does not negate the possibility of exploiting biased signaling for therapeutic purposes. For example, even weak partial agonism towards G proteins combined with a stronger agonism towards arrestins might generate sufficient “push” to activate MAP3Ks and enable arrestin-mediated scaffolding, but not enough G protein signaling to yield the biological effect of a full agonist. Conversely, a strong GPCR agonist biased towards G proteins might produce an effect without activating the arrestin brunch, thereby avoiding arrestin-mediated signaling.

Alternatively, when MAP3Ks are activated *via* G protein-independent mechanisms (which is the most likely scenario *in vivo*), GPCR-bound arrestins might function as signal-enhancing scaffolds, facilitating MAPK activation and other pathways independently of G proteins. Arrestin-mediated scaffolding would restrict the localization of generated active MAPKs to the vicinity of GPCRs, *i.e.*, to plasma membrane and endosomes, thereby directing them to substrates in these locales. Thus, arrestin-biased GPCR agonists would affect cell signaling in a different manner than unbiased ones. These ideas must be explored experimentally, preferably in cells that are meant to be targeted under the conditions where the cell receives all inputs, including stimulation *via* growth factor receptors and integrins.

**COOPERATION OF ARRESTINS AND G PROTEINS**

Conceivably, there might be situations *in vivo* where the bulk of MAP kinase activation depends on GPCRs, similarly to the experimental conditions used to study arrestin-mediated signaling to MAPKs. In these cases, it might appear counter-intuitive that the signaling of arrestins, which suppress G protein coupling to GPCRs, might require G protein activity. However, it is very likely that any GPCR agonist, including those with arrestin bias, also activates G proteins to a certain extent, as arrestin binding to any GPCR is inevitably delayed by the need of receptor phosphorylation by GRKs to increase arrestin affinity[10,11]. There are known examples where particular biological outcomes, such as Rho A activation and stress fiber formation, require simultaneous input from active G proteins and arrestins[75]. Another known mechanism where G proteins and arrestins might cooperatively participate in signaling that requires both types of transducers involves complexes between certain GPCRs and growth factor receptors that create distinct signaling platforms (reviewed in[76]).

**PUTTING PIECES TOGETHER**

Here the focus of the discussion is GPCR-dependent activation of ERK1/2 in the experimental conditions used to study arrestin-mediated signaling, which exclude non-GPCR inputs. The data obtained with gene knockout appear to be less ambiguous than those obtained with siRNA knockdown often used earlier[28,29,54]: the knockdown is never complete and one can never be sure that only the targeted proteins were knocked down. A good example demonstrating problems with knockdown specificity are the two siRNA studies[77,78], where opposite conclusions regarding the role of arrestins and arrestin domain-containing proteins in β2AR trafficking were made based on the data. Thus, strictly speaking, without the demonstration of rescue by the expression of knockdown-resistant exogenous protein substituting for that targeted by siRNA, the results of knockdown cannot be unambiguously interpreted[79,80]. The same applies to knockout: only rescue with knocked out protein proves that the phenotype observed emerged due to the elimination of an intended target. In addition, complete knockout of an important signaling protein has other caveats: the cells might be inadvertently selected for their ability to survive without eliminated protein due to changes in signaling pathways. For example, simultaneous knockout of both non-visual arrestins is embryonic lethal in mice[81], whereas mouse embryonic fibroblasts[81] and HEK293 cells[57,58,82,83] lacking arrestins are viable and can be transfected to generate cells exclusively expressing individual arrestins or particular mutants[52,53,84,85]. Thus, some cells can live without arrestins, whereas others cannot. It is possible (and very hard to check) that “zero functional G” cells also have unanticipated and uncontrolled changes in their signaling pathways. A recent study using three independently generated lines of “zero arrestin” HEK293 cells suggested that these lines are quite different, particularly in terms of signaling: the elimination of arrestins resulted in enhanced, reduced, or unchanged ERK1/2 phosphorylation in response to GPCR activation, as compared to parental cell lines[83]. These data clearly showed that non-visual arrestins do play a role in signaling, at least in GPCR-dependent ERK1/2 activation[83].

So, does arrestin-mediated signaling *via* GPCRs that is G protein-independent, as previously claimed[54], exist? While unambiguous answers require further experimentation, one plausible explanation for the apparent controversy between a large body of data describing arrestin-dependent signaling (reviewed in[55,56]) and recent findings in “zero functional G” and “zero arrestin” cells[57,58,82] can be proposed. An important point that was consistently overlooked in studies of GPCR-dependent arrestin-mediated signaling *via* MAP kinases is the issue of signal initiation. MAP kinase cascades are highly conserved in eukaryotes, from yeast to mammals, and always contain three protein kinases (MAP3K-MAP2K-MAPK) that sequentially activate each other by phosphorylation[86]. The signaling in these cascades is initiated by the activation of the upstream-most MAP3Ks[19]. Yet is was never taken into account that MAP3Ks of the ERK1/2 and JNK3 cascades (cRaf and ASK1, respectively), have to be activated to initiate signaling that eventually leads to the observed phosphorylation of ERK1/2 or JNK3, which usually depends on various protein scaffolds bringing the three kinases of each cascade together. Non-visual arrestins were found to serve as scaffolds but were never shown to facilitate MAP3K activation (reviewed in[12,87-89]). Thus, it is entirely possible that in “real life” the first “push” leading to the activation of MAP3Ks is provided by GPCRs *via* G proteins, or, more likely, by numerous non-GPCR signaling mechanisms, whereas signal propagation is facilitated by scaffolds, including receptor-bound or free arrestins. In case of MAP3K activation by growth factor receptors or integrins arrestin-dependent ERK1/2 activation might appear GPCR-dependent but G protein-independent. In contrast, under experimental conditions so far used to study arrestin-mediated signaling G proteins activated in response to GPCR stimulation might be the only remaining source of MAP3K activation, which would translate into G protein dependence of arrestin signaling to the ERK pathway, as described recently[58]. The need, or lack thereof, of active G proteins for apparently receptor-independent JNK3 activation by arrestin-3 and arrestin-3-derived peptide has never been tested experimentally, although ASK1 activation *via* G protein-independent mechanisms is more likely in this case.

The existing evidence of the role of non-visual arrestins in cell signaling[83] does not actually contradict the idea that G protein activity might be necessary for the arrestin-mediated signaling under conditions where the inputs from growth factor receptors, integrins, and stressors are excluded. Arguably, the situation where GPCRs assume the leading role in the MAPK activation can be encountered only in rather artificial experimental conditions, although we cannot exclude that this situation sometimes exists *in vivo*. The data obtained in “zero functional G” cells do not contradict the notion that arrestin-mediated signaling exists and plays a role in cell biology. Regardless of the potential role of G proteins, signal propagation to MAPKs would still depend on scaffolds, possibly including non-visual arrestins. Experiments where the activity of MAP3Ks and MAP2Ks in each cascade, rather than only the phosphorylation state of downstream MAPKs, such as ERK1/2 and JNK3, is determined in cells expressing non-visual arrestins with or without functional G proteins are necessary to test this hypothesis. It would be instructive to test whether the activation of growth factor receptors, which are the main known activators of MAP3Ks[19], or the activation of integrins (*e.g.*, by plating cultured cells on fibronectin) bypasses the requirement for the G protein activity. If non-visual arrestin scaffolds contribute to MAPK activation under any of these conditions, their function is likely to be G protein-independent.

**CONCLUSION**

Available evidence strongly indicates that non-visual arrestins scaffold three-tiered MAP kinase cascades, facilitating signal propagation. Other signaling functions of arrestins are also well documented. However, arrestins were never implicated in the activation of upstream-most MAP3Ks. Cells have numerous MAP3Ks that are activated by various inputs, including, but not limited to, G protein-mediated GPCR signaling. Thus, arrestins and heterotrimeric G proteins have distinct non-overlapping functions in cell signaling. In MAPK cascades, under experimental conditions that exclude non-GPCR inputs, G proteins might play a role in MAP3K activation, whereas arrestins act as scaffolds facilitating signal transduction.

**REFERENCES**

1 **Bockaert J**, Pin JP. Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J* 1999; **18**: 1723-1729 [PMID: 10202136 DOI: 10.1093/emboj/18.7.1723]

2 **Fredriksson R**, Lagerström MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* 2003; **63**: 1256-1272 [PMID: 12761335 DOI: 10.1124/mol.63.6.1256]

3 **Gurevich EV**, Tesmer JJ, Mushegian A, Gurevich VV. G protein-coupled receptor kinases: more than just kinases and not only for GPCRs. *Pharmacol Ther* 2012; **133**: 40-69 [PMID: 21903131 DOI: 10.1016/j.pharmthera.2011.08.001]

4 **Wilden U**, Hall SW, Kühn H. Phosphodiesterase activation by photoexcited rhodopsin is quenched when rhodopsin is phosphorylated and binds the intrinsic 48-kDa protein of rod outer segments. *Proc Natl Acad Sci U S A* 1986; **83**: 1174-1178 [PMID: 3006038 DOI: 10.1073/pnas.83.5.1174]

5 **Lohse MJ**, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science* 1990; **248**: 1547-1550 [PMID: 2163110 DOI: 10.1126/science.2163110]

6 **Attramadal H**, Arriza JL, Aoki C, Dawson TM, Codina J, Kwatra MM, Snyder SH, Caron MG, Lefkowitz RJ. Beta-arrestin2, a novel member of the arrestin/beta-arrestin gene family. *J Biol Chem* 1992; **267**: 17882-17890 [PMID: 1517224]

7 **Sterne-Marr R**, Gurevich VV, Goldsmith P, Bodine RC, Sanders C, Donoso LA, Benovic JL. Polypeptide variants of beta-arrestin and arrestin3. *J Biol Chem* 1993; **268**: 15640-15648 [PMID: 8340388]

8 **Rapoport B,** Kaufman KD, Chazenbalk GD. Cloning of a member of the arrestin family from a human thyroid cDNA library. *Mol Cell Endocrinol* 1992; **84**: R39-43 [DOI: 10.1016/0303-7207(92)90038-8]

9 **Lohse MJ**, Andexinger S, Pitcher J, Trukawinski S, Codina J, Faure JP, Caron MG, Lefkowitz RJ. Receptor-specific desensitization with purified proteins. Kinase dependence and receptor specificity of beta-arrestin and arrestin in the beta 2-adrenergic receptor and rhodopsin systems. *J Biol Chem* 1992; **267**: 8558-8564 [PMID: 1349018]

10 **Gurevich VV**, Gurevich EV. The molecular acrobatics of arrestin activation. *Trends Pharmacol Sci* 2004; **25**: 105-111 [PMID: 15102497 DOI: 10.1016/j.tips.2003.12.008]

11 **Carman CV,** Benovic JL. G-protein-coupled receptors: turn-ons and turn-offs. *Curr Opin Neurobiol* 1998; **8**: 335-344 [DOI: 10.1016/S0959-4388(98)80058-5]

12 **Gurevich VV**, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol Ther* 2006; **110**: 465-502 [PMID: 16460808 DOI: 10.1016/j.pharmthera.2005.09.008]

13 **Goodman OB Jr**, Krupnick JG, Santini F, Gurevich VV, Penn RB, Gagnon AW, Keen JH, Benovic JL. Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor. *Nature* 1996; **383**: 447-450 [PMID: 8837779 DOI: 10.1038/383447a0]

14 **Laporte SA**, Oakley RH, Zhang J, Holt JA, Ferguson SS, Caron MG, Barak LS. The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc Natl Acad Sci USA* 1999; **96**: 3712-3717 [PMID: 10097102 DOI: 10.1073/pnas.96.7.3712]

15 **Kim YM**, Benovic JL. Differential roles of arrestin-2 interaction with clathrin and adaptor protein 2 in G protein-coupled receptor trafficking. *J Biol Chem* 2002; **277**: 30760-30768 [PMID: 12070169 DOI: 10.1074/jbc.M204528200]

16 **Luttrell LM**, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakatsu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ. Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. *Science* 1999; **283**: 655-661 [PMID: 9924018 DOI: 10.1126/science.283.5402.655]

17 **McDonald PH**, Chow CW, Miller WE, Laporte SA, Field ME, Lin FT, Davis RJ, Lefkowitz RJ. Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* 2000; **290**: 1574-1577 [PMID: 11090355 DOI: 10.1126/science.290.5496.1574]

18 **Luttrell LM**, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL, Lefkowitz RJ. Activation and targeting of extracellular signal-regulated kinases by beta-arrestin scaffolds. *Proc Natl Acad Sci USA* 2001; **98**: 2449-2454 [PMID: 11226259 DOI: 10.1073/pnas.041604898]

19 **Garrington TP,** Johnson GL. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol* 1999; **11**: 211-218 [DOI: 10.1016/S0955-0674(99)80028-3]

20 **Cuevas BD**, Abell AN, Johnson GL. Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene* 2007; **26**: 3159-3171 [PMID: 17496913 DOI: 10.1038/sj.onc.1210409]

21 **Meng D**, Lynch MJ, Huston E, Beyermann M, Eichhorst J, Adams DR, Klussmann E, Houslay MD, Baillie GS. MEK1 binds directly to betaarrestin1, influencing both its phosphorylation by ERK and the timing of its isoprenaline-stimulated internalization. *J Biol Chem* 2009; **284**: 11425-11435 [PMID: 19153083 DOI: 10.1074/jbc.M806395200]

22 **Zhan X**, Kaoud TS, Dalby KN, Gurevich VV. Nonvisual arrestins function as simple scaffolds assembling the MKK4-JNK3α2 signaling complex. *Biochemistry* 2011; **50**: 10520-10529 [PMID: 22047447 DOI: 10.1021/bi201506g]

23 **Zhan X**, Kaoud TS, Kook S, Dalby KN, Gurevich VV. JNK3 enzyme binding to arrestin-3 differentially affects the recruitment of upstream mitogen-activated protein (MAP) kinase kinases. *J Biol Chem* 2013; **288**: 28535-28547 [PMID: 23960075 DOI: 10.1074/jbc.M113.508085]

24 **Song X**, Coffa S, Fu H, Gurevich VV. How does arrestin assemble MAPKs into a signaling complex? *J Biol Chem* 2009; **284**: 685-695 [PMID: 19001375 DOI: 10.1074/jbc.M806124200]

25 **Tohgo A**, Choy EW, Gesty-Palmer D, Pierce KL, Laporte S, Oakley RH, Caron MG, Lefkowitz RJ, Luttrell LM. The stability of the G protein-coupled receptor-beta-arrestin interaction determines the mechanism and functional consequence of ERK activation. *J Biol Chem* 2003; **278**: 6258-6267 [PMID: 12473660 DOI: 10.1074/jbc.M212231200]

26 **Ahn S**, Shenoy SK, Wei H, Lefkowitz RJ. Differential kinetic and spatial patterns of beta-arrestin and G protein-mediated ERK activation by the angiotensin II receptor. *J Biol Chem* 2004; **279**: 35518-35525 [PMID: 15205453 DOI: 10.1074/jbc.M405878200]

27 **Luttrell LM**. 'Location, location, location': activation and targeting of MAP kinases by G protein-coupled receptors. *J Mol Endocrinol* 2003; **30**: 117-126 [PMID: 12683936 DOI: 10.1677/jme.0.0300117]

28 **Kim J**, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei H, Lefkowitz RJ. Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. *Proc Natl Acad Sci USA* 2005; **102**: 1442-1447 [PMID: 15671181 DOI: 10.1073/pnas.0409532102]

29 **Ren XR**, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. *Proc Natl Acad Sci USA* 2005; **102**: 1448-1453 [PMID: 15671180 DOI: 10.1073/pnas.0409534102]

30 **Luo J**, Busillo JM, Benovic JL. M3 muscarinic acetylcholine receptor-mediated signaling is regulated by distinct mechanisms. *Mol Pharmacol* 2008; **74**: 338-347 [PMID: 18388243 DOI: 10.1124/mol.107.044750]

31 **Gurevich VV**, Gurevich EV. Rich tapestry of G protein-coupled receptor signaling and regulatory mechanisms. *Mol Pharmacol* 2008; **74**: 312-316 [PMID: 18515421 DOI: 10.1124/mol.108.049015]

32 **Yang F,** Xiao P, Qu C-X, et al. Allosteric mechanisms underlie GPCR signaling to SH3-domain proteins through arrestin. *Nat Chem Biol* 2018 [DOI: 10.1038/s41589-018-0115-3]

33 **Tobin AB**, Butcher AJ, Kong KC. Location, location, location...site-specific GPCR phosphorylation offers a mechanism for cell-type-specific signalling. *Trends Pharmacol Sci* 2008; **29**: 413-420 [PMID: 18606460 DOI: 10.1016/j.tips.2008.05.006]

34 **Nobles KN**, Xiao K, Ahn S, Shukla AK, Lam CM, Rajagopal S, Strachan RT, Huang TY, Bressler EA, Hara MR, Shenoy SK, Gygi SP, Lefkowitz RJ. Distinct phosphorylation sites on the β(2)-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci Signal* 2011; **4**: ra51 [PMID: 21868357 DOI: 10.1126/scisignal.2001707]

35 **Miller WE**, McDonald PH, Cai SF, Field ME, Davis RJ, Lefkowitz RJ. Identification of a motif in the carboxyl terminus of beta -arrestin2 responsible for activation of JNK3. *J Biol Chem* 2001; **276**: 27770-27777 [PMID: 11356842 DOI: 10.1074/jbc.M102264200]

36 **Seo J**, Tsakem EL, Breitman M, Gurevich VV. Identification of arrestin-3-specific residues necessary for JNK3 kinase activation. *J Biol Chem* 2011; **286**: 27894-27901 [PMID: 21715332 DOI: 10.1074/jbc.M111.260448]

37 **Kook S**, Zhan X, Kaoud TS, Dalby KN, Gurevich VV, Gurevich EV. Arrestin-3 binds c-Jun N-terminal kinase 1 (JNK1) and JNK2 and facilitates the activation of these ubiquitous JNK isoforms in cells via scaffolding. *J Biol Chem* 2013; **288**: 37332-37342 [PMID: 24257757 DOI: 10.1074/jbc.M113.510412]

38 **Vishnivetskiy SA**, Hirsch JA, Velez MG, Gurevich YV, Gurevich VV. Transition of arrestin into the active receptor-binding state requires an extended interdomain hinge. *J Biol Chem* 2002; **277**: 43961-43967 [PMID: 12215448 DOI: 10.1074/jbc.M206951200]

39 **Hanson SM**, Cleghorn WM, Francis DJ, Vishnivetskiy SA, Raman D, Song X, Nair KS, Slepak VZ, Klug CS, Gurevich VV. Arrestin mobilizes signaling proteins to the cytoskeleton and redirects their activity. *J Mol Biol* 2007; **368**: 375-387 [PMID: 17359998 DOI: 10.1016/j.jmb.2007.02.053]

40 **Shukla AK**, Manglik A, Kruse AC, Xiao K, Reis RI, Tseng WC, Staus DP, Hilger D, Uysal S, Huang LY, Paduch M, Tripathi-Shukla P, Koide A, Koide S, Weis WI, Kossiakoff AA, Kobilka BK, Lefkowitz RJ. Structure of active β-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. *Nature* 2013; **497**: 137-141 [PMID: 23604254 DOI: 10.1038/nature12120]

41 **Kang Y**, Zhou XE, Gao X, He Y, Liu W, Ishchenko A, Barty A, White TA, Yefanov O, Han GW, Xu Q, de Waal PW, Ke J, Tan MH, Zhang C, Moeller A, West GM, Pascal BD, Van Eps N, Caro LN, Vishnivetskiy SA, Lee RJ, Suino-Powell KM, Gu X, Pal K, Ma J, Zhi X, Boutet S, Williams GJ, Messerschmidt M, Gati C, Zatsepin NA, Wang D, James D, Basu S, Roy-Chowdhury S, Conrad CE, Coe J, Liu H, Lisova S, Kupitz C, Grotjohann I, Fromme R, Jiang Y, Tan M, Yang H, Li J, Wang M, Zheng Z, Li D, Howe N, Zhao Y, Standfuss J, Diederichs K, Dong Y, Potter CS, Carragher B, Caffrey M, Jiang H, Chapman HN, Spence JC, Fromme P, Weierstall U, Ernst OP, Katritch V, Gurevich VV, Griffin PR, Hubbell WL, Stevens RC, Cherezov V, Melcher K, Xu HE. Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. *Nature* 2015; **523**: 561-567 [PMID: 26200343 DOI: 10.1038/nature14656]

42 **Zhou XE**, He Y, de Waal PW, Gao X, Kang Y, Van Eps N, Yin Y, Pal K, Goswami D, White TA, Barty A, Latorraca NR, Chapman HN, Hubbell WL, Dror RO, Stevens RC, Cherezov V, Gurevich VV, Griffin PR, Ernst OP, Melcher K, Xu HE. Identification of Phosphorylation Codes for Arrestin Recruitment by G Protein-Coupled Receptors. *Cell* 2017; **170**: 457-469.e13 [PMID: 28753425 DOI: 10.1016/j.cell.2017.07.002]

43 **Breitman M**, Kook S, Gimenez LE, Lizama BN, Palazzo MC, Gurevich EV, Gurevich VV. Silent scaffolds: inhibition OF c-Jun N-terminal kinase 3 activity in cell by dominant-negative arrestin-3 mutant. *J Biol Chem* 2012; **287**: 19653-19664 [PMID: 22523077 DOI: 10.1074/jbc.M112.358192]

44 **Chen Q**, Perry NA, Vishnivetskiy SA, Berndt S, Gilbert NC, Zhuo Y, Singh PK, Tholen J, Ohi MD, Gurevich EV, Brautigam CA, Klug CS, Gurevich VV, Iverson TM. Structural basis of arrestin-3 activation and signaling. *Nat Commun* 2017; **8**: 1427 [PMID: 29127291 DOI: 10.1038/s41467-017-01218-8]

45 **Kim YJ**, Hofmann KP, Ernst OP, Scheerer P, Choe HW, Sommer ME. Crystal structure of pre-activated arrestin p44. *Nature* 2013; **497**: 142-146 [PMID: 23604253 DOI: 10.1038/nature12133]

46 **Chen Q**, Iverson TM, Gurevich VV. Structural Basis of Arrestin-Dependent Signal Transduction. *Trends Biochem Sci* 2018; **43**: 412-423 [PMID: 29636212 DOI: 10.1016/j.tibs.2018.03.005]

47 **Eichel K**, Jullié D, Barsi-Rhyne B, Latorraca NR, Masureel M, Sibarita JB, Dror RO, von Zastrow M. Catalytic activation of β-arrestin by GPCRs. *Nature* 2018; **557**: 381-386 [PMID: 29720660 DOI: 10.1038/s41586-018-0079-1]

48 **Zhan X**, Gimenez LE, Gurevich VV, Spiller BW. Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual subtypes. *J Mol Biol* 2011; **406**: 467-478 [PMID: 21215759 DOI: 10.1016/j.jmb.2010.12.034]

49 **Sensoy O**, Moreira IS, Morra G. Understanding the Differential Selectivity of Arrestins toward the Phosphorylation State of the Receptor. *ACS Chem Neurosci* 2016; **7**: 1212-1224 [PMID: 27405242 DOI: 10.1021/acschemneuro.6b00073]

50 **Zhan X**, Stoy H, Kaoud TS, Perry NA, Chen Q, Perez A, Els-Heindl S, Slagis JV, Iverson TM, Beck-Sickinger AG, Gurevich EV, Dalby KN, Gurevich VV. Peptide mini-scaffold facilitates JNK3 activation in cells. *Sci Rep* 2016; **6**: 21025 [PMID: 26868142 DOI: 10.1038/srep21025]

51 **Kook S**, Zhan X, Cleghorn WM, Benovic JL, Gurevich VV, Gurevich EV. Caspase-cleaved arrestin-2 and BID cooperatively facilitate cytochrome C release and cell death. *Cell Death Differ* 2014; **21**: 172-184 [PMID: 24141717 DOI: 10.1038/cdd.2013.143]

52 **Cleghorn WM**, Branch KM, Kook S, Arnette C, Bulus N, Zent R, Kaverina I, Gurevich EV, Weaver AM, Gurevich VV. Arrestins regulate cell spreading and motility via focal adhesion dynamics. *Mol Biol Cell* 2015; **26**: 622-635 [PMID: 25540425 DOI: 10.1091/mbc.E14-02-0740]

53 **Cleghorn WM**, Bulus N, Kook S, Gurevich VV, Zent R, Gurevich EV. Non-visual arrestins regulate the focal adhesion formation via small GTPases RhoA and Rac1 independently of GPCRs. *Cell Signal* 2018; **42**: 259-269 [PMID: 29133163 DOI: 10.1016/j.cellsig.2017.11.003]

54 **Shenoy SK**, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ. beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem*2006; **281**: 1261-1273 [PMID: 16280323 DOI: 10.1074/jbc.M506576200]

55 **Wisler JW**, Rockman HA, Lefkowitz RJ. Biased G Protein-Coupled Receptor Signaling: Changing the Paradigm of Drug Discovery. *Circulation* 2018; **137**: 2315-2317 [PMID: 29844068 DOI: 10.1161/CIRCULATIONAHA.117.028194]

56 **Smith JS**, Lefkowitz RJ, Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. *Nat Rev Drug Discov* 2018; **17**: 243-260 [PMID: 29302067 DOI: 10.1038/nrd.2017.229]

57 **Alvarez-Curto E**, Inoue A, Jenkins L, Raihan SZ, Prihandoko R, Tobin AB, Milligan G. Targeted Elimination of G Proteins and Arrestins Defines Their Specific Contributions to Both Intensity and Duration of G Protein-coupled Receptor Signaling. *J Biol Chem* 2016; **291**: 27147-27159 [PMID: 27852822 DOI: 10.1074/jbc.M116.754887]

58 **Grundmann M**, Merten N, Malfacini D, Inoue A, Preis P, Simon K, Rüttiger N, Ziegler N, Benkel T, Schmitt NK, Ishida S, Müller I, Reher R, Kawakami K, Inoue A, Rick U, Kühl T, Imhof D, Aoki J, König GM, Hoffmann C, Gomeza J, Wess J, Kostenis E. Lack of beta-arrestin signaling in the absence of active G proteins. *Nat Commun* 2018; **9**: 341 [PMID: 29362459 DOI: 10.1038/s41467-017-02661-3]

59 **McKay MM**, Morrison DK. Integrating signals from RTKs to ERK/MAPK. *Oncogene* 2007; **26**: 3113-3121 [PMID: 17496910 DOI: 10.1038/sj.onc.1210394]

60 **Sabio G**, Davis RJ. TNF and MAP kinase signalling pathways. *Semin Immunol* 2014; **26**: 237-245 [PMID: 24647229 DOI: 10.1016/j.smim.2014.02.009]

61 **Stupack DG,** Cheresh DA. Get a ligand, get a life: integrins, signaling and cell survival. *J Cell Sci* 2002; **115**: 3729-3738 [DOI: 10.1242/jcs.00071]

62 **Pleinis JM**, Davis CW, Cantrell CB, Qiu DY, Zhan X. Purification, auto-activation and kinetic characterization of apoptosis signal-regulating kinase I. *Protein Expr Purif* 2017; **132**: 34-43 [PMID: 28082061 DOI: 10.1016/j.pep.2017.01.002]

63 **Lavoie H**, Therrien M. Regulation of RAF protein kinases in ERK signalling. *Nat Rev Mol Cell Biol* 2015; **16**: 281-298 [PMID: 25907612 DOI: 10.1038/nrm3979]

64 . Guan KL. The mitogen activated protein kinase signal transduction pathway: from the cell surface to the nucleus. *Cell Signal* 1994; **6**: 581-589 [DOI: 10.1016/0898-6568(94)90041-8]

65 **Tohgo A**, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. beta-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J Biol Chem* 2002; **277**: 9429-9436 [PMID: 11777902 DOI: 10.1074/jbc.M106457200]

66 **Lymperopoulos A**, Rengo G, Zincarelli C, Kim J, Soltys S, Koch WJ. An adrenal beta-arrestin 1-mediated signaling pathway underlies angiotensin II-induced aldosterone production in vitro and in vivo. *Proc Natl Acad Sci U S A*2009; **106**: 5825-5830 [PMID: 19289825 DOI: 10.1073/pnas.0811706106]

67 **Kim J**, Ahn S, Rajagopal K, Lefkowitz RJ. Independent beta-arrestin2 and Gq/protein kinase Czeta pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor. *J Biol Chem* 2009; **284**: 11953-11962 [PMID: 19254952 DOI: 10.1074/jbc.M808176200]

68 **Manglik A**, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilka TS, Thian FS, Hubbell WL, Prosser RS, Kobilka BK. Structural Insights into the Dynamic Process of β2-Adrenergic Receptor Signaling. *Cell* 2015; **161**: 1101-1111 [PMID: 25981665 DOI: 10.1016/j.cell.2015.04.043]

69 **Van Eps N**, Caro LN, Morizumi T, Kusnetzow AK, Szczepek M, Hofmann KP, Bayburt TH, Sligar SG, Ernst OP, Hubbell WL. Conformational equilibria of light-activated rhodopsin in nanodiscs. *Proc Natl Acad Sci USA* 2017; **114**: E3268-E3275 [PMID: 28373559 DOI: 10.1073/pnas.1620405114]

70 **Weis WI**, Kobilka BK. The Molecular Basis of G Protein-Coupled Receptor Activation. *Annu Rev Biochem* 2018; **87**: 897-919 [PMID: 29925258 DOI: 10.1146/annurev-biochem-060614-033910]

71 **Natochin M**, Gasimov KG, Artemyev NO. Inhibition of GDP/GTP exchange on G alpha subunits by proteins containing G-protein regulatory motifs. *Biochemistry* 2001; **40**: 5322-5328 [PMID: 11318657 DOI: 10.1021/bi015505w]

72 **Cismowski MJ,** Lanier SM. Activation of heterotrimeric G-proteins independent of a G-protein coupled receptor and the implications for signal processing. *Rev Physiol Biochem Pharmacol* 2005; **155**: 57-80 [DOI: 10.1007/3-540-28217-3\_3]

73 **Wang J**, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, Ahles A, Engelhardt S, Rockman HA. Gαi is required for carvedilol-induced β1 adrenergic receptor β-arrestin biased signaling. *Nat Commun*2017; **8**: 1706 [PMID: 29167435 DOI: 10.1038/s41467-017-01855-z]

74 **Littmann T**, Göttle M, Reinartz MT, Kälble S, Wainer IW, Ozawa T, Seifert R. Recruitment of β-arrestin 1 and 2 to the β2-adrenoceptor: analysis of 65 ligands. *J Pharmacol Exp Ther* 2015; **355**: 183-190 [PMID: 26306764 DOI: 10.1124/jpet.115.227959]

75 **Barnes WG**, Reiter E, Violin JD, Ren XR, Milligan G, Lefkowitz RJ. beta-Arrestin 1 and Galphaq/11 coordinately activate RhoA and stress fiber formation following receptor stimulation. *J Biol Chem* 2005; **280**: 8041-8050 [PMID: 15611106 DOI: 10.1074/jbc.M412924200]

76 **Pyne NJ**, Pyne S. Receptor tyrosine kinase-G-protein-coupled receptor signalling platforms: out of the shadow? *Trends Pharmacol Sci* 2011; **32**: 443-450 [PMID: 21612832 DOI: 10.1016/j.tips.2011.04.002]

77 **Nabhan JF**, Pan H, Lu Q. Arrestin domain-containing protein 3 recruits the NEDD4 E3 ligase to mediate ubiquitination of the beta2-adrenergic receptor. *EMBO Rep* 2010; **11**: 605-611 [PMID: 20559325 DOI: 10.1038/embor.2010.80]

78 **Han SO**, Kommaddi RP, Shenoy SK. Distinct roles for β-arrestin2 and arrestin-domain-containing proteins in β2 adrenergic receptor trafficking. *EMBO Rep* 2013; **14**: 164-171 [PMID: 23208550 DOI: 10.1038/embor.2012.187]

79 **Gurevich VV**, Gurevich EV. Analyzing the roles of multi-functional proteins in cells: The case of arrestins and GRKs. *Crit Rev Biochem Mol Biol* 2015; **50**: 440-452 [PMID: 26453028]

80 **Gurevich EV**, Gurevich VV. Beyond traditional pharmacology: new tools and approaches. *Br J Pharmacol* 2015; **172**: 3229-3241 [PMID: 25572005 DOI: 10.1111/bph.13066]

81 **Kohout TA,** Lin FS, Perry SJ, Conner DA, Lefkowitz RJ. beta-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc Nat Acad Sci USA* 2001; **98**: 1601-1606 [DOI: 10.1073/pnas.041608198]

82 **O'Hayre M,** Eichel K, Avino S, et al. Genetic evidence that β-arrestins are dispensable for the initiation of β2-adrenergic receptor signaling to ERK. *Sci Signal* 2017; **10**: 484 [DOI: 10.1126/scisignal.aal3395]

83 **Luttrell LM,** Wang J, Plouffe B, et al. Manifold roles of beta-arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9. *Sci Signal* 2018; **11**: eaat7650

84 **Coffa S**, Breitman M, Spiller BW, Gurevich VV. A single mutation in arrestin-2 prevents ERK1/2 activation by reducing c-Raf1 binding. *Biochemistry* 2011; **50**: 6951-6958 [PMID: 21732673 DOI: 10.1021/bi200745k]

85 **Prokop S**, Perry NA, Vishnivetskiy SA, Toth AD, Inoue A, Milligan G, Iverson TM, Hunyady L, Gurevich VV. Differential manipulation of arrestin-3 binding to basal and agonist-activated G protein-coupled receptors. *Cell Signal* 2017; **36**: 98-107 [PMID: 28461104 DOI: 10.1016/j.cellsig.2017.04.021]

86 **Tian T**, Harding A. How MAP kinase modules function as robust, yet adaptable, circuits. *Cell Cycle* 2014; **13**: 2379-2390 [PMID: 25483189 DOI: 10.4161/cc.29349]

87 **Gurevich EV**, Gurevich VV. Arrestins: ubiquitous regulators of cellular signaling pathways. *Genome Biol* 2006; **7**: 236 [PMID: 17020596 DOI: 10.1186/gb-2006-7-9-236]

88 **Peterson YK**, Luttrell LM. The Diverse Roles of Arrestin Scaffolds in G Protein-Coupled Receptor Signaling. *Pharmacol Rev* 2017; **69**: 256-297 [PMID: 28626043 DOI: 10.1124/pr.116.013367]

89 **Gurevich VV**, Gurevich EV. Arrestins and G proteins in cellular signaling: The coin has two sides. *Sci Signal* 2018; **11**: eaav1646

**P-Reviewer:** Alcántara-Hernández R, Lymperopoulos A, Rajagopal S

**S-Editor:** Ma RY **L-Editor:** **E-Editor:**

**Specialty type:** Biochemistry and molecular biology

**Country of origin:** United States

**Peer-review report classification**

Grade A (Excellent): A, A

Grade B (Very good): B

Grade C (Good): 0

Grade D (Fair): 0

Grade E (Poor): 0