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COMMENTARY

# α-Arrestins – new players in Notch and GPCR signaling pathways in mammals

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## ABSTRACT

For many years, β-arrestins have been known to be involved in G-protein-coupled receptor (GPCR) desensitization. However, β-arrestins belong to a family of proteins that act as multifunctional scaffolding proteins, in particular during trafficking of transmembrane receptors. The arrestin family comprises visual arrestins, β-arrestins and α-arrestins. In mammals, the functions of the α-arrestins are beginning to be elucidated, and they are described as versatile adaptors that link GPCRs or the Notch receptor to E3 ubiquitin ligases and endocytic factors. These α-arrestins can act in sequence, complementarily or cooperatively with β-arrestins in trafficking and ubiquitylation events. This Commentary will summarize the recent advances in our understanding of the functions and properties of these α-arrestin proteins in comparison to β-arrestins, and will highlight a new hypothesis linking their functional complementarity to their physical interactions. α- and β-arrestins could form transient and versatile heterodimers that form a bridge between cargo and E3 ubiquitin ligases, thus allowing trafficking to proceed.


**KEY WORDS: E3 ubiquitin ligase, GPCR, Notch, α-Arrestin, β-Arrestin**

## Introduction

In 1977, Wacker and colleagues described S-antigen, a protein found in retinal rods and involved in uveitis, an inflammation of the vascular pigmented middle layer of the eye (the uvea). Later, Kuhn et al. discovered a 48-kDa protein involved in the termination of the signal from light-activated phosphorylated rhodopsin, a photoreceptor of the G-protein-coupled receptor (GPCR) family (Wilden et al., 1986; Kühn et al., 1984; Zuckerman and Cheasty, 1986). Kuhn named this protein 'arrestin' for its ability to arrest rhodopsin-mediated signaling. Shortly after, biochemical, immunological, functional and pathological tests demonstrated that S-antigen and the arrestin protein were exactly the same molecule (Pfister et al., 1984). In 1990, Lohse and colleagues identified another arrestin protein that could specifically switch off β-adrenergic signaling and not rhodopsin; for this reason, this new protein was called 'β-arrestin' (Lohse et al., 1990). The arrestin-mediated termination of GPCR signaling is thus a common mechanism for arresting rhodopsin and β-adrenergic receptor stimulation (and that of other GPCRs), and is called 'receptor desensitization' (Lohse, 1992; Benovic et al., 1989; Shenoy and Lefkowitz, 2011).

Beyond this historical role, β-arrestins are involved in a variety of processes (that we will not detail here; for a review, see Kovacs et al., 2009), including some that are linked to ubiquitylation and to the trafficking of cargo (Fig. 1). Ubiquitylation of cell surface receptors (Box 1) in response to environmental cues is described either as an internalization, trafficking or sorting signal, depending on the cargo. It is now well accepted that this post-translational modification increases the affinity of the endocytic machinery factors for the cargo, resulting in the rapid assembly and disassembly of macromolecular complexes and thus affecting trafficking. Among these complexes, the clathrin-coated pits allow endocytic vesicles to pinch off the plasma membrane, the endosomal sorting complexes required for transport (ESCRT) complex (Henne et al., 2011) delivers cargo into the luminal vesicles of the multivesicular bodies (MVBs), and retromers control the trafficking of endosomes to the Golgi. Among the many possible trafficking signals, the recruitment of adaptor proteins that bind to the cytoplasmic tail of a given transmembrane receptor at a given time determines the fate of the receptor and, eventually, the effects on the signaling cascade. Arrestins are able to fulfill such adaptor functions.

Recently, the arrestin family has been extended from visual and β-arrestins to include a new class of arrestins called α-arrestins. This Commentary will emphasize the role of these α-arrestins as scaffolding molecules in receptor signaling, in particular, GPCR and Notch receptor signaling, and discuss the possible functional and physical interactions of α- and β-arrestins.

## The arrestin family

The phylogenetic analysis of arrestins has been revisited in the past few years, uncovering a number of proteins that, in addition to the visual and β-arrestins, create the arrestin clan. These proteins all harbor an arrestin domain and all originate from a unique ancestral arrestin. The clan comprises two families: the Vps26-related proteins (encoded by three genes in mammals, constituting part of the retromer complexes) and the arrestin family (Alvarez, 2008). Within the arrestin family, the new protein members are called α-arrestins, or arrestin-domain-containing proteins (ARRDCs) in mammals (Fig. 2A). In yeast, they are also named arrestin-like yeast proteins (ALY) or arrestin-related trafficking adaptors (ARTs).

α-Arrestins were first studied in fungi and yeast (see, for example, Andoh et al., 2002), even before being recognized as members of the arrestin clan (Boase and Kelly, 2004; Herranz et al., 2005). *Saccharomyces cerevisiae* has 11 α-arrestins but neither visual nor β-arrestins (Becuwe et al., 2012a); therefore, α-arrestins are considered the ancestral factors of the arrestin family. Yeast α-arrestins are thought to serve as adaptors for the

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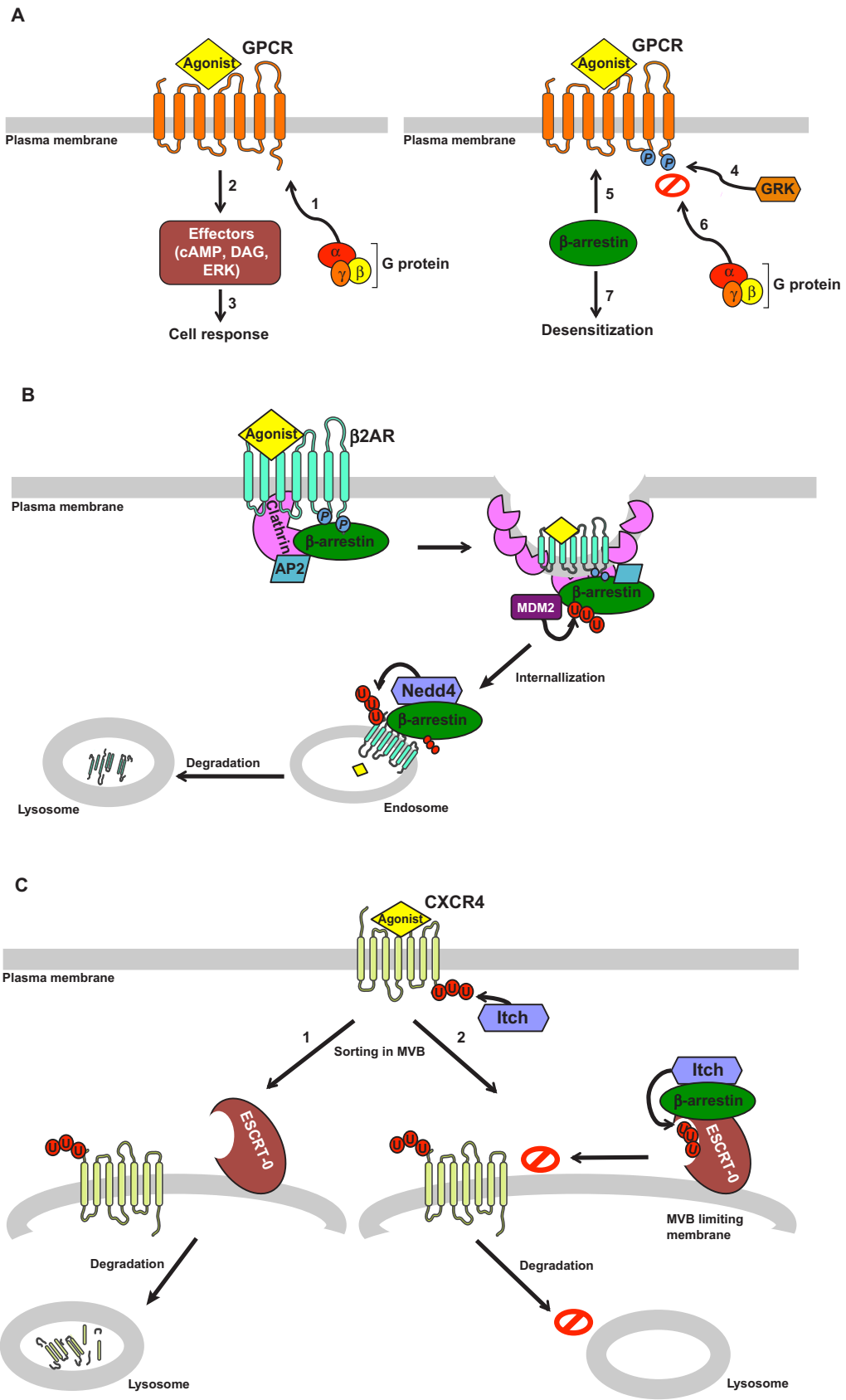


**Fig. 1. Multifaceted functions of β-arrestins in receptor trafficking.** (A) β-arrestins and GPCR desensitization. Following agonist binding, G proteins are recruited to the receptor (1) and activate downstream effectors, such as cAMP, diacylglycerol (DAG) and ERK (2), promoting multiple cellular events (3). On prolonged stimulation, the agonist-dependent GPCR phosphorylation by G-protein-coupled receptor kinases (GRK) (4) facilitates binding of β-arrestin to the receptor (5), impairing the recruitment of other G proteins (6), thereby arresting receptor signaling (7). (B) β-arrestin and β2-adrenergic receptor (β2AR) degradation. Following agonist binding, β-arrestin is recruited to the phosphorylated receptor, where it binds clathrin and AP2, thereby promoting association of β2AR with clathrin-coated pits and its internalization. β-arrestin also serves as an adaptor for E3 ubiquitin ligases; consequently, β-arrestin and β2AR are ubiquitylated by MDM2 and Nedd4 ubiquitin ligases, respectively. These modifications direct receptor trafficking and lysosomal degradation. (C) β-arrestin and CXCR4 sorting to MVBs. On agonist binding, CXCR4 is ubiquitylated by the E3 ubiquitin ligase Itch at the plasma membrane, and is then endocytosed and recognized by the ESCRT-0 complex before its lysosomal degradation (1). β-arrestin is not necessary for receptor ubiquitylation, but controls receptor sorting by acting as an adaptor for Itch to promote ESCRT-0 ubiquitylation (2). This modification affects the ability of ESCRT-0 to recognize the ubiquitylated cargo (indicated by a red crossed circle) and thus prevents the lysosomal degradation of CXCR4. U, ubiquitin; *P*, phosphorylated residues.



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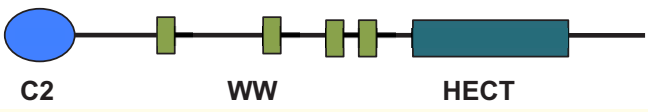
### Box 1. The HECT family of E3 ubiquitin ligases

Ubiquitylation of a substrate involves the covalent attachment of ubiquitin, a protein of 76 amino acids, or of a chain of polymerized ubiquitin moieties, to a lysine residue in the substrate through an isopeptidic bond. This modification is the result of the action of three enzymes: an E1 (activating), an E2 (conjugating) and an E3 (ubiquitin ligase). The E3 ligases (E3s, of which there are more than 600 in the mammalian genome) are specific to a limited number of substrates. They belong to two main families, defined by their active domain: the really interesting new gene (RING) and the homologous to E6-AP C-terminus (HECT) families. The HECT catalytic domain contains a cysteine residue that acts as an acceptor of ubiquitin from the E2 enzymes. Once accepted by the E3, ubiquitin is transferred to a specific substrate.

HECT E3s can be divided into three groups: the neural-precursor-cell-expressed, developmentally downregulated (Nedd4) family, the HECT domain and RCC1-like domain-containing protein (HERC) family and other HECTs (Rotin and Kumar, 2009). In mammals, the Nedd4 family comprises nine members: Nedd4, Nedd4-2, Itch (also known as AIP4 in human), WWP1, WWP2, SMURF1, SMURF2, Nedl1 and Nedl2; Rsp5, however, is the only known member of this family in *Saccharomyces cerevisiae*. The E3s of this family are generally involved in endocytosis and trafficking of plasma membrane proteins (Shearwin-Whyatt et al., 2006).

In addition to the HECT domain (see figure; dark green rectangle), located in the C-terminus of the protein, these E3s harbor an N-terminal C2 domain (blue ellipse) that binds membrane phospholipids, targeting the E3s to intracellular compartments and to the plasma membrane (Dunn et al., 2004), and two to four WW domains (light green rectangles) that recognize and bind their substrates. In particular, WW domains bind predominately proline-rich motifs, including PPXY (amino-acid single letter code, in which x is any amino acid) (Staub et al., 1996), PPLP (Bedford et al., 1997), PR (Bedford et al., 1998; Bedford et al., 2000) and phosphoserine/phosphothreonine (pS/pT) residues that precede a proline residue (Lu et al., 1999).




E3 ubiquitin ligase Rsp5 [the unique neural-precursor-cell-expressed developmentally downregulated gene 4 (Nedd4) homolog in yeast] (Box 1) in the endocytosis of plasma membrane transporters, such as the arginine transporter Can1 and the methionine transporter Mup1 (both of which involve the yeast α-arrestin Art1/Cvs7/Ldb19), and the lysine transporter Lyp1 (involving the α-arrestin Art2/Ecm21) (Lin et al., 2008; MacGurn et al., 2011). After a specific stimulus, an α-arrestin is recruited to the plasma membrane where it binds to Rsp5, which ubiquitylates the transporter, inducing its internalization and its degradation in a vacuole (lysosome) (Lin et al., 2008). Another α-arrestin, Rod1 (also known as Art4), however, is directly targeted by glucose signaling, which allows it to be ubiquitylated by Rsp5, thereby promoting sugar transporter endocytosis (Becuwe et al., 2012b).

As well as in yeast, arrestins are found in the earliest eukaryotes. *Caenorhabditis elegans*, for example, has multiple arrestin-related proteins but also has one β-arrestin; fish and amphibians have a rod arrestin, a cone arrestin, one β-arrestin and multiple α-arrestins; and flies have two sensory arrestins, a single β-arrestin (called Kurtz in *Drosophila melanogaster*) and multiple α-arrestins. There are ten proteins that belong to the mammalian arrestin family (Fig. 2A): two visual and two very closely related non-visual β-arrestins (89% similarity), and six α-arrestins. In spite of the divergence in the primary amino acid sequences of α- and β-arrestins (11–15% identity and 60–68% similarity between α- and β-arrestins, and between α-arrestins) (Fig. 2A), they are predicted to exhibit the same structural features (Polekhina et al., 2013). The crystal structures of visual S-arrestin and of β-arrestin 1 were solved some years ago (Granzin et al., 1998; Han et al., 2001, Hirsch et al., 1999; Milano et al., 2002), and looks like a saddle, consisting of two related β-sheet-rich subdomains, termed arrestin C- and N-domains, joined by a set of buried salt bridges, with a less-organized C-terminal tail (Fig. 2B). A polar core of charged residues in the N-domain acts as a phosphate sensor: when β-arrestin binds a phosphorylated GPCR receptor, its conformation changes from an inactive to an active state, in which the C-terminal tail is released from the N-domain and can then bind proteins involved in the endocytosis machinery, such as clathrin and AP2 (Kim et al., 2012; Shukla et al., 2013).

For the α-arrestins, the crystal structure of the N-terminal domain of human thioredoxin-interacting protein (TXNIP) has only been recently reported, so the comparison between α- and β-arrestins is based on structural predictions from these data (Polekhina et al., 2013) (Fig. 2B). Nevertheless, two structural differences distinguish α-arrestins from β-arrestins and visual arrestins: first, α-arrestins probably do not encode the particular α-helix in the N-terminal domain that participates in keeping β-arrestins in the inactive conformation (Sutton et al., 2005; Alvarez, 2008); and second, α-arrestins harbor PPXY motifs in their C-terminal tail that bind proteins with WW domains, such as the E3 ubiquitin ligases of the HECT family (Fig. 2; Box 1). These structural similarities and differences between α- and β-arrestins provide the core foundation of functional similarities, differences and complementarities that we will highlight here.

## Arrestins and GPCRs

Among the multitude of GPCRs (about 1000 in the human genome), which transmit extracellular signals into cells, the prototypic adenylyl-cyclase-coupled β2-adrenergic receptor (β2AR, also known as ADRB2) for catecholamines is by far the most studied since the 1980s. β-arrestins are required for activated GPCR internalization and ubiquitylation (Fig. 1A,B), and have also been shown to regulate the ability of the ESCRT-0 complex to recognize ubiquitylated cargo, thereby affecting the sorting of GPCRs into MVBs (Fig. 1C) (Malik and Marchese, 2010).

Because the role of β-arrestins in β2AR internalization and ubiquitylation (Fig. 1) has been well documented (Shenoy et al., 2001; Shenoy et al., 2008), it has been surprising to discover that α-arrestins also have a role in the endocytosis of activated GPCRs. ARRDC3 was the first α-arrestin shown to be required for the regulation of β2AR after prolonged stimulation of β2AR (Nabhan et al., 2010). Depletion of ARRDC3 abolishes the interaction between β2AR and Nedd4, affecting receptor ubiquitylation and decreasing β2AR degradation (Nabhan et al., 2010). In addition, ARRDC3 associates with β2AR following agonist stimulation, as shown by co-immunoprecipitation, leading to the proposal that ARRDC3 mediates the association between Nedd4 and β2AR.

Two recent studies have compared the respective functions of β-arrestin 2 (also known as ARRB2) and ARRDC3 in β2AR

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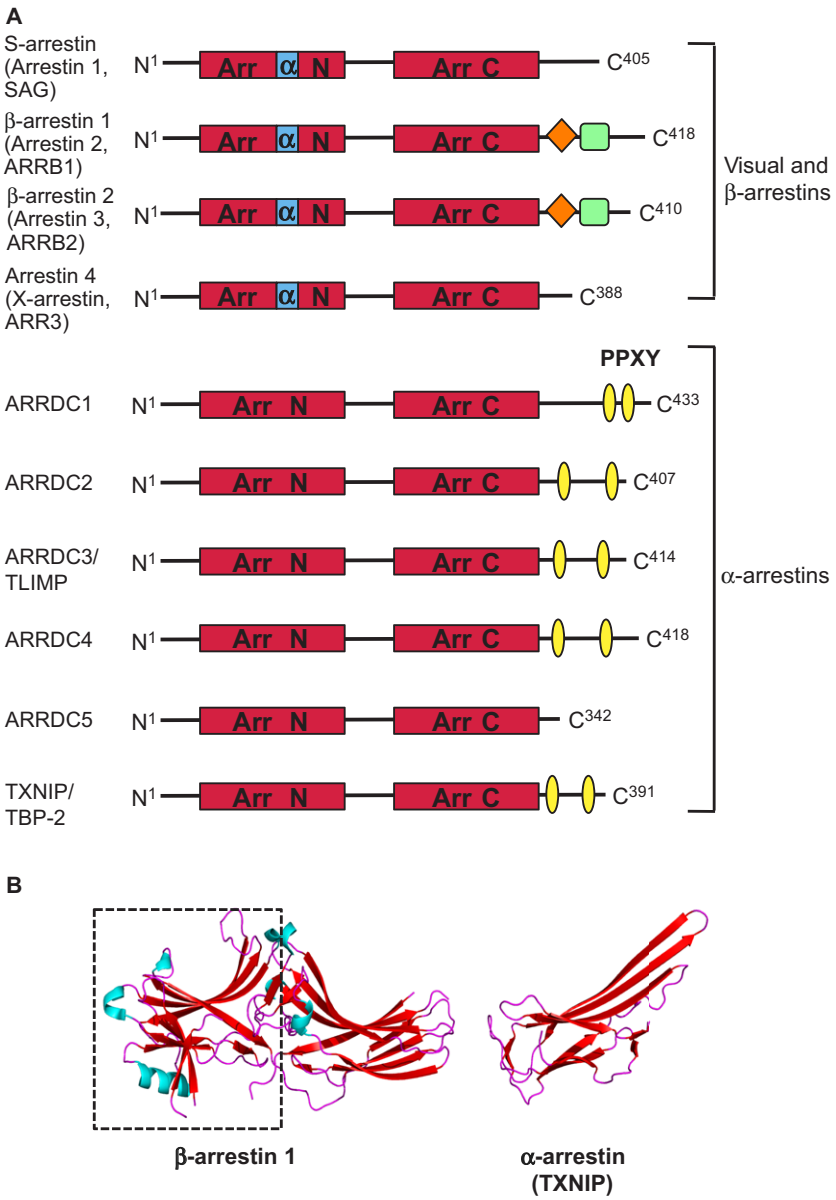


**Fig. 2. Mammalian arrestin family members.** (A) Schematic representation of the domain organization. The arrestin domain comprises an N-terminal domain (Arr N) and a C-terminal domain (Arr C) (both shown in red). The C-terminal tail of β-arrestins contains binding sites for clathrin and AP2 (orange rhombus and light green round rectangle, respectively). α-Arrestins lack the short α-helix inside the arrestin N-terminal domain (blue) and contain PPXY motifs (yellow) in their C-terminal tails (except ARRDC5), accounting for the interaction with WW domains of HECT E3 ubiquitin ligases. The numbers indicate the amino acids. Primary amino acid identity is ~70% between β-arrestins, and only 11–15% between β- and α-arrestins. (B) Comparison of the Arr N-terminal domains of the β-arrestin 1 (PDB ID 1G4M) and the α-arrestin TXNIP (PDB ID 4GEI). The whole structure of β-arrestin is shown, with the Arr N-terminal domain framed by a dotted line. The structural features (in particular, the α-helix) that are unique to β-arrestins are highlighted in blue. The structures were compared using PyMol, v1.7.

trafficking, but with conflicting conclusions. First, Shea and colleagues show that ARRDC3, ARRDC4 and β-arrestins work together to promote Nedd4 recruitment to the activated vasopressin receptor and β2AR (Shea et al., 2012) (Fig. 3A). In their paper, a kinetic study of the interaction of α-arrestins with both receptors shows their recruitment at two main time points: shortly after the addition of ligand (1 minute), and then after 15–30 minutes of ligand treatment. Accordingly, recruitment of Nedd4 family members and ubiquitylation of their receptors is detected ~5 minutes after ligand stimulation. Shea and colleagues observed that α- and β-arrestins partially colocalize in endocytic vesicles after 30 minutes of agonist addition. Notably, they also observed co-immunoprecipitation of overexpressed ARRDC3 with β-arrestins, regardless of whether the cells were treated with agonist. From these data, the authors propose that α- and β-arrestins are recruited at the same time as receptor trafficking and work cooperatively to eventually sort the activated receptors into MVBs (Shea et al., 2012) (Fig. 3A). By contrast, Han and colleagues propose that β-arrestins and ARRDCs are required at two different steps of receptor endocytosis (Han et al., 2013) (Fig. 3B). They show that β-arrestins are required for clathrin-mediated internalization of β2AR, after a short exposure to the agonist, and recruit the E3 ubiquitin ligases. ARRDC2, ARRDC3 and ARRDC4, although already present at the plasma membrane, are rather secondary adaptors that promote the sorting of the Nedd4–β2AR complex to endosomes positive for hepatocyte growth-factor-regulated tyrosine kinase substrate (Hrs, also known as HGS, a key component of the ESCRT-0 complex) after prolonged agonist treatment (Han et al., 2013), consistent with the fact that, in contrast to β-arrestins, α-arrestins have no conserved clathrin-interacting motifs (Alvarez, 2008). In this study, however, the possibility of heterodimerization between the two subfamilies of proteins was not addressed (Han et al., 2013).

From these contradictory data, the two models summarized in Fig. 3 can be depicted according to the described arrestin localizations and interactions with the receptor, the endocytic machinery and the E3 ubiquitin ligases. Each hypothesis is based on the same type of experimental approaches (gain and loss of

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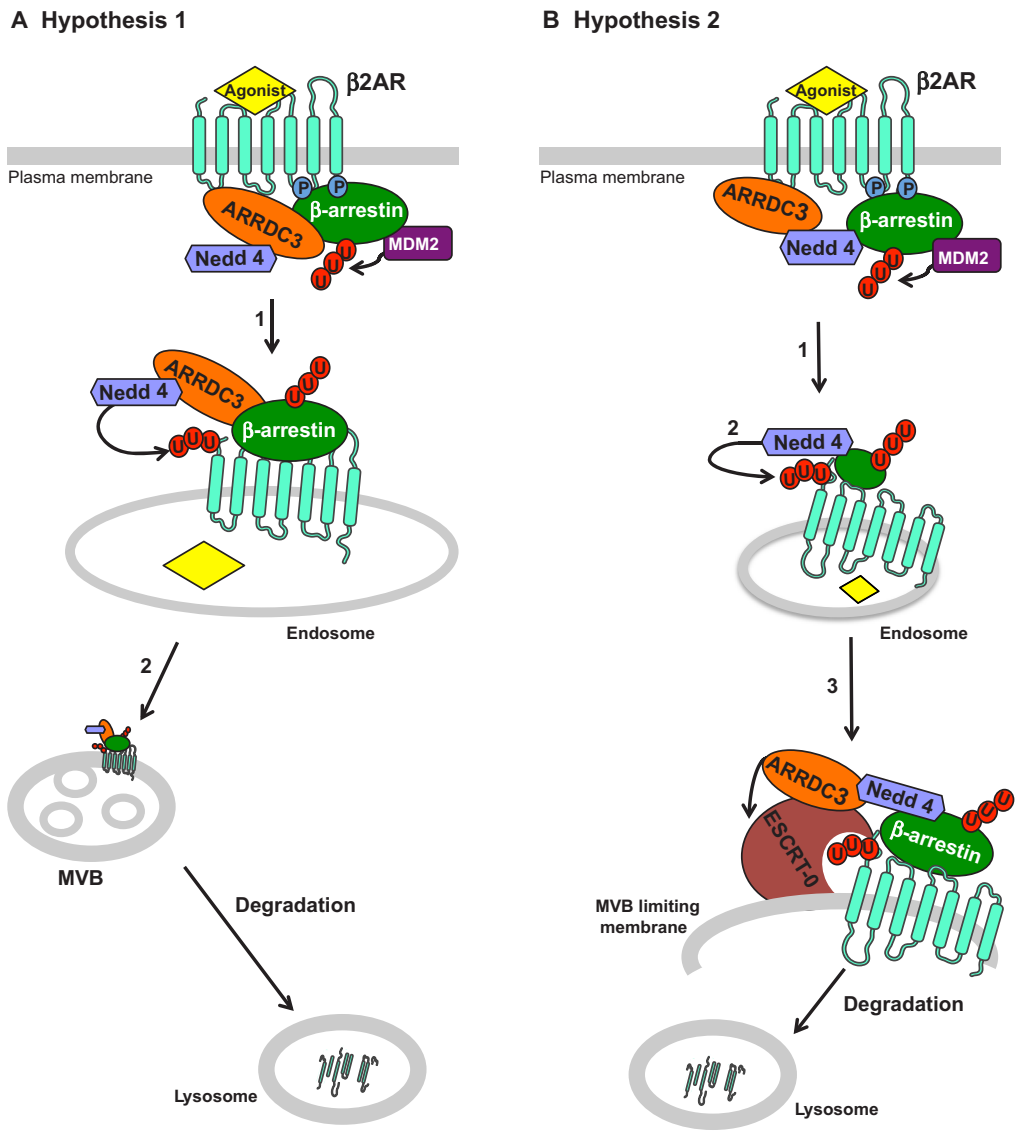


**Fig. 3. Two models for recruitment of ARRDCs (α-arrestins) and β-arrestins in β2AR trafficking.** (A) Hypothesis 1, from Shea and colleagues, states that α- and β-arrestins act coordinately from the plasma membrane in the early stages of receptor endocytosis (1) to promote Nedd4 recruitment to the activated β2AR receptor, β2AR ubiquitylation and subsequent lysosomal degradation (2) (Shea et al., 2012). (B) Hypothesis 2, from Han and colleagues, suggests that and α- and β-arrestins are recruited sequentially during receptor trafficking: β-arrestin is rapidly recruited at the plasma membrane to the activated receptor, promoting internalization of β2AR (1) and recruiting Nedd4 for receptor ubiquitylation (2) (as in Fig. 1C). ARRDC3 (orange oval), which is constitutively activated at the plasma membrane and associated with Hrs (an ESCRT-0 member)-containing vesicles, secondarily recognizes and binds Nedd4–β2AR complexes (3), leading to post-endocytic sorting of internalized, ubiquitylated β2AR (Han et al., 2013). U, ubiquitin.

function), which suffer from technical limitations (i.e. controversial specificity of the siRNAs targeting ARRDC3, overexpression experiments with GFP-tagged proteins for *in vivo* localization and co-immunoprecipitations, and different time courses for agonist treatments) and impair a direct comparison between the results. One can imagine that a unified model will emerge from further studies.

Although all GPCRs share common mechanisms for signal transduction and arrest, whether they all use the same adaptors (in particular α- and β-arrestins), or the same combinations of adaptors, remains to be investigated in each case.

## Arrestins and Notch signaling

In contrast to other signaling pathways in which activated receptors often have accelerated turnover and degradation, thus allowing signal shutdown, activated Notch receptor is transformed into its own effector – a short-lived transcription factor (Box 2). The strength and duration of the Notch signal thus depends on the quantity and availability of Notch receptor that is capable of being activated at the cell surface. Studies in *Drosophila* and mammals have shown that Notch receptor turnover depends on its internalization and degradation through the lysosomal pathway (Chastagner et al., 2008; Wilkin et al., 2004). In the absence of activation, the Notch receptor is constantly internalized. Following the early endocytosis of the unactivated receptor, which is directed by as yet unknown events or factors, further Notch trafficking requires a ubiquitylation step mediated by the HECT family E3 ubiquitin ligases Nedd4 and Suppressor of Deltex [Su(dx) in *Drosophila*; Itch or AIP4 in mammals] (Chastagner et al., 2008; Wilkin et al., 2004; Sakata et al., 2004) (Box 1), which eventually leads to lysosomal degradation of Notch (Fig. 4).

Studies in *Drosophila* have considered the involvement of arrestins in Notch ubiquitylation and degradation. Using immunocytochemical and biochemical approaches, it has been shown that Notch, Kurtz and the RING family E3 ubiquitin ligase Deltex colocalize in intracellular vesicles, forming a trimeric complex (Mukherjee et al., 2005). It has been proposed a bridge between Kurtz and Notch is formed by Deltex (Mukherjee et al., 2005; Matsuno et al., 1995). In addition, Shrub, the fly homolog of an ESCRT-III component (CHMP4 in mammals), promotes Notch delivery into multivesicular bodies (MVBs) by enhancing Kurtz activity (Hori et al., 2011). Are there α-arrestins involved in

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### Box 2. Key features of Notch signaling

Notch signaling is an evolutionarily conserved signaling pathway implicated in cell fate decisions during the development of multicellular organisms and in adult tissue homeostasis. In mammals, there are four Notch receptors and five Notch ligands (Delta-like 1, 3, 4 and Jagged 1 and 2) (Bray, 2006).

The effects of Notch signaling on an individual cell are highly dependent on signal dose and context, and include proliferation, differentiation and apoptosis. Because of these recurrent roles, mutations in Notch components or abnormal Notch activation are frequently associated with diseases that can begin during development (such as congenital abnormalities) or adulthood [such cerebral autosomal-dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), an arteriopathy associated with frequent strokes], or with cancers (such as T-cell acute lymphoblastic leukemia or breast cancer) (Louvi and Artavanis-Tsakonas, 2012).

Notch receptor is synthesized as a precursor of 300 kDa. During its maturation, Notch is highly glycosylated and cleaved by the furin-like convertase, resulting in the presentation at the cell surface of a heterodimer consisting of the Notch extracellular domain associated with the Notch transmembrane and intracellular domains (Kopan and Ilagan, 2009). Notch signaling relies on the contact of Notch receptor with one of its ligands, which is anchored at the surface of an adjacent cell. This binding triggers the transformation of the receptor into a transcription factor, which acts on various target genes depending on the cellular context. This conversion requires that several modifications are undergone by the receptor, including its trafficking along the endocytic pathway and its ubiquitylation. However, the most prominent events are two proteolytic cleavages: first, an ADAM protease cleaves the activated receptor outside the transmembrane domain, removing most of the extracellular domain; next, γ-secretase cleavage, occurring inside the transmembrane domain of the receptor, releases the Notch intracellular domain (NICD) that translocates to the nucleus and activates a transcriptional switch by cooperating with the DNA-binding protein CSL (named after CBF1, suppressor of hairless and LAG-1) and other co-activators (Musse et al., 2012). Notch signaling is rapidly arrested owing to degradation of the NICD through the proteasome pathway, so the effect of the Notch signal on its target genes is transient. The production of the active form of Notch and the strength of the Notch response depend not only on the efficiency of signal transduction but also on the quantity of Notch receptor at the cell surface. This last parameter, in particular, is specifically regulated by arrestins.

this pathway in *Drosophila*? A genetic study aimed at identifying new regulators of Notch signaling tested, among other factors, the 13 α-arrestins in the *Drosophila* sensory organ lineage (Le Bras et al., 2012). The authors did not observe any Notch-related phenotype in adults caused by gene silencing of single α-arrestins, or of Kurtz, Nedd4 or Su(dx). The authors explain these results by suggesting that these factors might be associated only with wing vein development (Matsuno et al., 1995; Sakata et al., 2004); α-arrestins, therefore, remain possible candidates in this context (Le Bras et al., 2012). Alternatively, redundancy between α-arrestins and/or between E3 ubiquitin ligases could explain the lack of obvious effects.

Beyond observations in *Drosophila*, our recent study has addressed the functions of α- and β-arrestins in Notch signaling in mammals (Puca et al., 2013). In contrast to *Drosophila*, mammalian Notch receptors (with the exception of Notch3) have no PPXY motif and are not able to bind directly to the E3

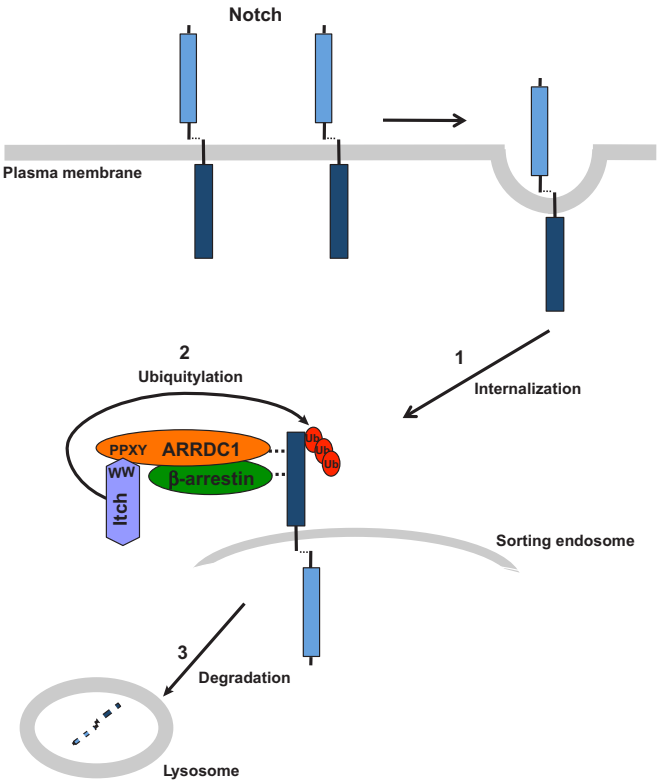


**Fig. 4. Model of Notch degradative complex.** After early endocytosis (1), unactivated Notch receptor (blue heterodimer, with the extracellular subunit in light blue) is recognized by arrestins. β-arrestin (β-arrestin 1 or 2, green oval) forms a heterodimer with α-arrestin 1 (ARRDC1, orange), which recruits Itch. The dotted lines indicate interactions. PPXY motifs of ARRDC1 account for direct interaction with Itch WW domains. Itch mediates Notch ubiquitylation (2) and Notch is eventually degraded in the lysosomes (3). Ub, ubiquitin.

ubiquitin ligase Itch (Chastagner et al., 2008). Our results have shown that ARRDC1 (and possibly ARRDC3) is able to recruit Itch to the unactivated Notch receptor through the PPXY motifs in the α-arrestin C-terminal tail. However, β-arrestins also participate in Itch-mediated Notch ubiquitylation and degradation: Notch ubiquitylation is impaired and Notch does not reach the lysosomes in β-arrestin-null (knockout for both isoforms) cells. A Notch degradation defect in these knockout cells is rescued by adding back β-arrestin 1 (also known as ARRB1), but not ARRDC1, indicating that ARRDC1 and β-arrestin are not redundant, but rather are both required for proper Notch trafficking. Interestingly, α- and β-arrestins are able to associate with each other through the arrestin domain, as shown by glutathione S-transferase (GST) pulldown and co-immunoprecipitation experiments. The first steps of Notch endocytosis occur similarly in wild-type cells and in conditions in which Notch degradation is impaired (i.e. in β-arrestin- or Itch-null cells), or in wild-type cells that overexpress a mutant version of ARRDC1 in which its PPXY motifs are deleted and that is therefore unable to recruit Itch. These results led us to propose the model in Fig. 4, in which an ARRDC1–β-arrestin–Notch complex necessary to recruit Itch is assembled in sorting endosomes. The arrestin heterodimers constitute the platform that links the receptor to the Itch E3 ubiquitin ligase and possibly to the ESCRT machinery.

### Arrestins and endocytic machinery

α- and β-arrestins are mainly membrane-associated (plasma membrane and cytoplasmic vesicular) and diffuse cytoplasmic proteins, respectively. However, in response to different stimuli, arrestins can change their subcellular localization. Following agonist stimulation of the GPCR, β-arrestins are recruited to the plasma membrane where they bind clathrin and the AP2 adaptor complex, thereby promoting GPCR endocytosis (Kim and Benovic, 2002) (Fig. 1B). However, β-arrestins can also participate in receptor trafficking by re-localizing themselves in specific subcellular compartments. In the case of the chemokine receptor CXCR4, β-arrestins are required for the endosomal sorting of the receptor and binding members of the ESCRT-0 machinery, such as Hrs and signal transducing adaptor molecule (STAM), at the limiting membrane of endosomes (Malik and Marchese, 2010).

Similar to β-arrestins, α-arrestins have multiple subcellular localizations. TXNIP interacts with importin-α (one of the transport carriers allowing nuclear import of large proteins) and shuttles into the nucleus where it binds specific substrates (Nishinaka et al., 2004); nevertheless, a fraction of TXNIP is localized at the plasma membrane (Wu et al., 2013). ARRDC3 is found on the inner sides of the plasma membrane, lysosomes and endosomes (Shea et al., 2012; Oka et al., 2006; Han et al., 2013; Nabhan et al., 2010), and ARRDC1 is associated partly with the plasma membrane and partly with cytoplasmic vesicles (Nabhan et al., 2012; Shea et al., 2012). In addition, ARRDCs (primarily ARRDC1 and ARRDC3) show high affinity for proteins of the ESCRT machinery, such as Tsg101 (a ESCRT-I subunit) and Alix [an accessory subunit that bridges ESCRT-I and -III (Henne et al., 2011)], in contrast to β-arrestins, which can only bind ESCRT-0 members (Hrs and STAM). ARRDC1 and Tsg101 can also be recruited to the plasma membrane, confirming previous results in yeast for Rim8 (also known as Art9) and Vps23 (Herrador et al., 2010), and are involved in microvesicle budding, a process similar to the viral budding process (Galindo et al., 2012; Nabhan et al., 2012; Rauch and Martin-Serrano, 2011; Hayashi et al., 2005). These changing and multiple subcellular localizations of arrestin proteins highlight their versatility for different cellular processes and probably specific stimuli.

### Arrestin and E3 ubiquitin ligases

E3 ubiquitin ligases of the Nedd4 family regulate many cellular processes and are localized to the plasma membrane or to endosomal membranes because of a domain called C2, which can interact with membrane phospholipids (Ingham et al., 2004). These proteins also harbor WW domains that interact with particular motifs – the PPXY sequences (Staub et al., 1996). These sequences are present on only some substrates of the E3 ubiquitin ligases, raising the question of how these ligases interact with their substrates. ARRDC proteins contain such PPXY motifs in their C-terminal tail and interact with several members of the HECT family of E3 ubiquitin ligases, such as WWP1, WWP2, Nedd4 and Itch, as shown by a two-hybrid screening in yeast (Rauch and Martin-Serrano, 2011). To date, the specific PPXY-dependent interactions of ARRDC1 with WWP1 and WWP2, and of ARRDC3 and ARRDC4 with Nedd4, Itch and WWP1, have been validated by co-immunoprecipitation experiments (Maskos et al., 1998; Nabhan et al., 2012; Shea et al., 2012). ARRDC1, ARRDC3 and ARRDC4 show stronger affinity for E3 ubiquitin ligases than do ARRDC2 and TXNIP, which lack a detectable interaction with WWP2 and Itch (Masutani et al., 2011; Rauch and Martin-Serrano, 2011). By contrast, β-arrestins do not harbor PPXY motifs and are not able to interact directly with Itch (Puca et al., 2013), suggesting that the lack of PPXY motifs affects Itch binding. However, previous studies have described β-arrestins as scaffolding proteins for various E3 ubiquitin ligases, including those of the HECT family. For example, it has been demonstrated that β-arrestin 1 interacts with Nedd4 to promote NHE1 ubiquitylation (Simonin and Fuster, 2010). Moreover, β-arrestins can recruit Nedd4 to β2AR (Shenoy et al., 2007) or interact with Itch to mediate the endosomal sorting of CXCR4 (Bhandari et al., 2007). In summary, β-arrestins can interact with proteins of the HECT family; however, most of the experiments have been performed with lysates from transfected cells under conditions in which intermediary factors necessary for these interactions could be present (Bhandari et al., 2007; Shenoy et al., 2008). The recent discoveries that ARRDC3 and ARRDC1 can, respectively, recruit Nedd4 to the β2AR (Shea et al., 2012), and Itch to Notch (Puca et al., 2013), strongly argue in favor of the possibility that ARRDCs and β-arrestins could work together in recruiting E3 ubiquitin ligases to receptors. In any case, it is possible that a single PPXY E3-docking site is not sufficient to anchor the substrate to the E3 ubiquitin ligase, and several interactions (through scaffolding molecules) could increase the stability of the enzyme–substrate complex.

### Arrestin homo- and hetero-associations

Arrestin domains have the ability to effect self-association or hetero-association between different arrestins. Although crystallization and analytical ultracentrifugation have given contradictory results concerning the ability of β-arrestins to dimerize (Milano et al., 2002), biochemical evidence and both bioluminescence and fluorescence resonance energy transfer experiments have suggested that, at a physiological concentration, β-arrestins can homo- and hetero-dimerize (Storez et al., 2005). The homo- and hetero-association (β1–β2) of β-arrestins has been confirmed by proteomic approaches (Xiao et al., 2007). Several hypotheses have been made for the relevance of such associations. First, the ability to form oligomers might facilitate the interaction of β-arrestins with multiple substrates; alternatively, oligomers could represent an inactive pool of β-arrestins in the cytoplasm, as suggested by the heterodimerization between β-arrestins 1 and 2 that impairs the nuclear translocation of β-arrestin 1 (Storez et al., 2005). Impaired β-arrestin 2 oligomerization affects β2AR-dependent ERK activation without interfering with receptor internalization, suggesting that oligomers could also constitute an interface for the interaction with a distinct set of proteins (Boularan et al., 2007; Xu et al., 2008). However, these studies have not addressed the possible heterodimerization of α- with β-arrestins that has been hypothesized by Alvarez (Alvarez, 2008) and discussed in a review about the involvement of α-arrestins in the trafficking of yeast membrane transporters (Polo and Di Fiore, 2008). This heterodimerization of α- and β-arrestins has been addressed experimentally by Shea and colleagues; they demonstrated that ARRDC3 or ARRDC4 can heterodimerize with β-arrestins upon agonist binding by β2AR, and that this hetero-association is essential to recruit Nedd4 to the activated receptor (Shea et al., 2012). This scenario is very similar to the model that we propose for Notch degradation, in which ARRDC1–β-arrestin heterodimerization, observed in co-immunoprecipitation experiments and GST pulldown assays, is necessary to recruit Itch to unactivated Notch receptor (Puca et al., 2013) (Fig. 4).

Further work is necessary to understand how different complexes containing α-arrestins and β-arrestin 1 or 2 could be formed, as such complexes have not been identified in a recent census of soluble protein complexes present in cytoplasmic extracts generated from cultured cells (Havugimana et al., 2012). The α- and β-arrestin interactions are therefore likely to be transient and dynamically regulated. Nevertheless, the fact that arrestins can form α-arrestin–β-arrestin heterodimers that functionally cooperate brings to light the possibility that multiple arrestin combinations might exist, exhibiting different affinities for specific substrates in defined trafficking steps, thus driving different outcomes.

## Conclusions and perspectives

α-Arrestins expand the arrestin family and the possible roles of these proteins as adaptors linking cargo to E3 ubiquitin ligases and to endocytic factors. Their functions are no longer limited to the GPCRs, but include coupling to other transmembrane proteins, including integrins (Draheim et al., 2010), glucose transporter GLUT1 (Wu et al., 2013) and the Notch receptor (Puca et al., 2013). In the light of this versatility, several questions remain. For example, how do the arrestins recognize their cargo, considering that the surfaces of adaptors or cargo proteins alone are unlikely to account for specificity? Examples of post-translational modifications of cargo exist (by phosphorylation, for instance), as well as post-translational modifications of arrestins (by phosphorylation or ubiquitylation). It is also possible that the arrestin–cargo interaction requires additional intermediary factors. However, the possible cooperation between α- and β-arrestins could make these complexes more adaptable to specific cargo. Taken together, these factors and/or events could eventually provide the time- and space-regulated high-affinity bridge that acts between cargo and arrestins. This coupling would result in the recruitment of E3 ubiquitin ligases acting on the cargo and/or in the recognition of the complex by the endocytic machinery, finally allowing trafficking to proceed.

It will be very interesting to examine whether dimerization between α- and β-arrestins is more widely used than in Notch and β2AR signaling. If that is the case, this could further increase the number of combinations and therefore the repertoire of scaffolding factors allowing specific cargo recruitment. However, α-arrestin–β-arrestin heterodimers could provide a physical basis for the sequential events undergone by cargo along the endocytic pathway. In conclusion, much remains to be discovered to understand the specificity of each member of this fascinating family towards cargo, other interacting proteins, the mechanisms of action and resulting endocytic events.

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### Competing interests

The authors declare no competing interests.

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