**α-arrestin 1 (ARRDC1) and β-arrestins cooperate to mediate Notch degradation in mammals**

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**Summary**

Notch signaling is a conserved signaling pathway implicated in embryogenesis and adult tissue maintenance. Notch signaling strength is strictly regulated, notably by maintaining a controlled pool of functional receptor at the cell surface. Mammalian non-activated Notch receptor is internalized, ubiquitylated by the Itch E3 ubiquitin ligase and degraded in the lysosomes. Here, we show that β-arrestins are necessary for Itch–Notch interaction and for Itch-driven ubiquitylation and degradation of Notch. Interestingly, β-arrestins do not directly bind Itch but heterodimerize with a member of another subfamily of arrestins called ARRDC1 or α-arrestin 1, which harbors PPxY motifs that allow direct interaction with Itch. Cells transfected with ARRDC1 mutated in PPxY motifs show reduced Itch-mediated Notch ubiquitylation and impaired lysosomal degradation of Notch, as observed in β-arrestin⁻/⁻ or Itch⁻/⁻ cells. Our data show for the first time that ARRDC1 and β-arrestins heterodimerize and cooperate in the same complex to promote non-activated Notch receptor degradation, thus acting as negative regulators of Notch signaling.

**Key words:** Arrestin, Endocytosis, Itch, Notch, Ubiquitin

**Introduction**

Notch signaling controls a variety of developmental processes as well as adult tissue and organ maintenance. It relies on the interaction between Notch ligand and receptor, which are transmembrane proteins present on the surface of adjacent cells. This binding triggers a succession of events leading to proteolytic cleavage of Notch receptor, which results in the production of a cytosolic fragment of the receptor. The latter migrates to the nucleus, where it acts as a transcriptional cofactor to regulate target genes transcription (Bray, 2006).

A number of components and post-translational modifications have been implicated in regulating the activity of Notch receptor, either positively or negatively (Brou, 2009). Among those, some affect Notch degradation in a ligand-independent manner, thus regulating the quantity of receptor present at the cell surface and therefore the strength of the Notch response. In mammals, non-activated Notch receptor is constitutively internalized and ubiquitylated by Itch/AIP4 E3 ubiquitin ligase in order to be processed for lysosomal degradation (Chastagner et al., 2008). Moreover, the deubiquitylating complex USP12/UAFL1 is recruited by Itch to Notch and allows Notch deubiquitylation, probably before its entry into the intraluminal vesicles of the multivesicular bodies (MVBs) (Moretti et al., 2012).

These data suggest that Notch degradation in the lysosomes is a regulated and ordered process, where several proteins are necessary to recruit E3 ubiquitin ligases and deubiquitylating enzymes. The first evidence of arrestin involvement in Notch signaling was made in *Drosophila* (Mukherjee et al., 2005) by the identification in a two-hybrid screen of Kurtz (krz), the single *Drosophila* homolog of mammalian non-visual β-arrestins, as an interacting partner of Deltex, a known modulator of Notch activity. Loss of krz function results in an accumulation of Notch receptor, resulting in a partial gain-of-function effect on Notch signaling. On the basis of these genetic data in *Drosophila* and the absence of direct interaction between Itch and Notch in mammals (Chastagner et al., 2008), we asked whether arrestins could have a role in Notch regulation in mammals.

In mammals, β-arrestins (ARRBs) were originally shown to desensitize activated G-protein-coupled receptors (GPCR) and to promote receptor endocytosis. In addition, there is a growing list of examples showing that β-arrestins might also serve as multifunctional adaptors and scaffolds that mediate endocytosis of various types of receptors, have signaling functions or allow E3 ubiquitin ligase recruitment (Kovacs et al., 2009). Among these E3 ligases, Mdm2 and Deltex belong to the RING (a really interesting new gene) family, whereas Nedd4 and Itch are HECT (homologous to E6AP carboxyl terminus) family members. Because β-arrestins lack any canonical PPxY motif known to interact with WW domains of HECT family members (Ingham et al., 2004), the molecular basis of the interaction between β-arrestins and HECT E3 ubiquitin ligases remains unknown. By contrast, members of the related ARRD/α-arrestin family (arrestin domain-containing proteins, ARRD1–ARRD5 and TXNIP in mammals) (Alvarez, 2008; Becuwe et al., 2012a; Masutani et al., 2012) exhibit PPxY motifs in their C-terminal tail (except ARRD5). Until now, ARRDs have been shown to serve, similar to β-arrestins, as adaptors for the recruitment of specific cargo to E3 ubiquitin ligases and endocytosis in yeast (Lin et al., 2008), and also, in a few cases, in mammals (Nabhan...
et al., 2012; Nabhan et al., 2010). Whereas heterodimerization of β-arrestins in cells has been demonstrated (Storez et al., 2005; Xiao et al., 2007), heterodimerization of α- and β-arrestins giving rise to functional complexes has been theoretically proposed (Polo and Di Fiore, 2008), but not yet proven. Here, we show that a complex is formed containing α-arrestins and β-arrestins to recruit the E3 ubiquitin ligase Itch on Notch, which leads to Notch ubiquitylation and lysosomal degradation.

Results

β-arrestins are necessary for Itch–Notch interaction

Previous studies in mammals have demonstrated that non-activated Notch-1 receptor (hereafter Notch-1 receptor is named as Notch) is ubiquitylated by the E3 ubiquitin ligase Itch, even if no direct interaction has been detected (Chastagner et al., 2008). To determine whether β-arrestins promote Itch recruitment to Notch, we performed a coimmunoprecipitation (Co-IP) assay after transfecting HEK 293T cells with expression vectors encoding β-arrestin 1 (β-arr1), Notch (Myc-tagged Notch full-length, FL) together with FLAG-tagged Itch dominant negative (DN) or two other E3 ubiquitin ligases of the HECT family (Nedd4 DN–FLAG and Nedd4.2 DN–FLAG). The E3 ligases were mutated in their active site (indicated as DN) to try to stabilize the transient interactions with their substrates. We pulled down the E3 ubiquitin ligases and analyzed the immunoprecipitates and the whole-cell extracts (WCEs) by western blot using an antibody against the intracellular part of Notch (Notch IC) allowing us to detect the membrane-anchored subunit of mature Notch heterodimer (designated as NFL). Notch weakly co-immunoprecipitated with Itch DN (Fig. 1A, lane 13) and overexpression of β-arrestin 1 increased this interaction more than twofold (Fig. 1A, lane 10 compared with lane 13). By contrast, the interaction between Nedd4/4.2 and Notch FL remained weak even in the presence of overexpressed β-arrestin 1 (Fig. 1A,B lanes 11, 14, and lanes 12, 15), as confirmed by the corresponding densitometry analysis (Fig. 1B). As shown in Fig. 1A (lane 13) and 1C (lane 2) the Itch-DN–Notch complex was established at a basal level even in the absence of overexpressed β-arrestin 1. The presence of endogenous β-arrestins (detected in the WCEs in Fig. 1C) was sufficient to allow some binding of Notch to Itch. To demonstrate this, we knocked down the endogenous β-arrestin 1 and β-arrestin 2 as described previously (Coureuil et al., 2010). The efficiency of siRNA targeting β-arrestins (siβ-arr) was confirmed by western blot (Fig. 1C). β-arrestin silencing almost completely abolished the Itch-DN–Notch-FL interaction compared with control siGFP (31% of signal left in lane 4 versus 2, as quantified in Fig. 1D). These results suggest that β-arrestins are necessary for the formation of the Itch–Notch-FL complex in mammalian cells.

Fig. 1. β-arrestins are necessary for Itch–Notch FL complex formation. (A) Co-immunoprecipitation of β-arrestin 1 and Notch FL with the catalytically inactive E3 proteins Itch DN, Nedd4 DN and Nedd4.2 DN (represented by black, dark grey and grey squares respectively). (C) Co-immunoprecipitation of Notch FL with Itch DN in the presence of siRNA targeting β-arrestins. In A and C, HEK 293T cells were transfected as indicated above the lanes. The immunoprecipitations (IPs) were performed with anti-FLAG antibody coupled to agarose beads followed, when indicated, by a FLAG peptide elution (2 mg/ml). IPs and whole-cell extracts (WCEs, 5% of the total lysates) were resolved on SDS-PAGE and analyzed by western blot using the antibodies recognizing the proteins indicated on the left. NFL corresponds to the membrane-anchored fragment of the mature Notch heterodimer. The molecular masses (kDa) are represented on the right. eIF3a was used as a loading control. (B,D) The amounts of Notch signal in lanes 10–15 in A, and lanes 2 and 4 in C were quantified by densitometry analysis and normalized to total NFL in WCE.
β-arrestins are required for Itch-mediated Notch ubiquitylation and degradation, acting as negative regulators of Notch signaling

To determine whether the function of β-arrestins in the Notch pathway is related to the Itch-mediated Notch ubiquitylation, we monitored Notch ubiquitylation when endogenous β-arrestins were knocked down or not. We transfected HEK 293T cells with vectors encoding Itch, Notch and 6×His-tagged ubiquitin and we purified the ubiquitylated proteins on Nickel-charged beads in denaturing conditions. Ubiquitylated Notch products were specifically detected when the cells were transfected with His–Ub (a monoubiquitylated Notch indicated by an asterisk and a smear corresponding to the polyubiquitylated forms, indicated by NFLub, compare lane 2 with lane 1 in Fig. 2A). A low amount of ubiquitylated Notch was detected without overexpressed Itch, in contrast to what was observed when the cells were transfected...
with Itch-expressing plasmid and a control siRNA (NT) (Fig. 2A, lane 2 compared with lane 3). Silencing the endogenous β-arrestins significantly decreased Itch-mediated Notch ubiquitylation (Fig. 2A, lane 4), suggesting that β-arrestins are not only important for the formation of the complex, but are also required for the proper function of Itch in the Notch pathway.

To further assess the role of β-arrestins in Notch trafficking and degradation, we used mouse embryonic fibroblasts (MEFs) either WT or knocked out for both β-arrestins to avoid possible redundancy. We infected these cells with a retroviral vector encoding Notch, harboring a HA tag in its extracellular part and we selected stable clones expressing comparable amount of Notch, either WT or β-arrestin−/− cells (C2). The amount of cell surface Notch was quantified by flow cytometry and was found to be higher in WT cells compared with C2 (supplementary material Fig. S1A). Living cells were labeled with an anti-HA antibody to recognize Notch molecules present at the cell surface, then extensively washed and incubated at 37°C for various period of time to allow Notch endocytosis and trafficking. At time point 0 (no incubation at 37°C) HA–Notch was localized to the plasma membrane in both cell lines, as exemplified in Fig. 2B panels A and B, where the upper slice of each field was chosen to easily visualize the staining. After 30 and 60 minutes of incubation at 37°C, Notch was mostly detected in intracellular vesicles, showing that the receptor was internalized in WT as well as in β-arrestin−/− cells with comparable kinetics (Fig. 2B, panels C–F). At 90 and 180 minutes, almost no Notch signal was detectable in WT cells (G,J), whereas it was still clearly visible in C2 cells (H,J). Quantification of spot numbers revealed that they are seven times more frequent at time 180 minutes in C2 cells compared with WT cells (Fig. 2C). Thus, Notch degradation was delayed in the absence of β-arrestins with a kinetics similar to that described in Itch−/− cells (Chastagner et al., 2008). As shown in Fig. 2B, panels K,L, β-arrestin expression could complement the defect of β-arrestin−/− cells, because Notch degradation was restored after 180 minutes of incubation as quantified in Fig. 2D. In light of this result, we decided to assess whether β-arrestins, as previously demonstrated for Itch, were necessary to target non-activated Notch receptor towards lysosomes for degradation. We performed a receptor-uptake experiment in WT, C2 and Itch−/− cells (3F4) in the presence of leupeptin, an inhibitor of lysosomal proteases to impair Notch degradation and visualize it in late endosomes and lysosomes. In leupeptin-treated WT cells, Notch colocalized with LAMP1, a late endosome/lysosomal marker, after 3 hours of internalization (Fig. 2E, panels A–C and see quantification in supplementary material Fig. S1B). As expected, in 3F4 cells, Notch signal was much less associated with the lysosomal marker (Fig. 2E, panels G–I) (Chastagner et al., 2008). Similarly, in C2 cells, the percentage of colocalization between Notch and LAMP1 showed a significant decrease (Fig. 2E, panels D–F) compared with WT cells (supplementary material Fig. S1B), suggesting that in β-arrestin−/− cells as in Itch−/− cells, Notch did not reach the lysosomes for degradation and rather remained trapped in endocytic vesicles.

We next assessed the role of β-arrestins in Notch activation. We transfected WT and C2 cells with a Notch reporter vector, an internal Notch-insensitive control vector and an expression vector encoding β-arrestin 1. WT and C2 cells were then co-cultured with cells expressing Delta-like 1 (a Notch ligand) for 18 hours. As a reference, we performed the co-culture assay in the presence of DAPT, a potent inhibitor of gamma-secretase activity and involved in Notch trafficking.

Together, these results show that, in the absence of β-arrestins, Itch-mediated Notch ubiquitylation and lysosomal localization are strongly perturbed. Moreover, β-arrestin complementation restores Notch degradation and significantly affects Notch activation, confirming that β-arrestins are negative factors involved in Notch trafficking.

ARRDC1/α-arrestin 1 interacts with β-arrestins and Itch

β-arrestins lack canonical PPxY motifs, which are known to interact with WW domains of HECT proteins (Ingham et al., 2004). We thus wondered whether β-arrestins could directly interact with Itch. We performed a GST pull-down assay, where

Fig. 2. β-arrestins are required for Itch-mediated Notch ubiquitylation and degradation, acting as negative regulators of Notch signaling.

(A) Effect of siRNA targeting β-arrestins on Notch FL ubiquitylation. HEK 293T cells were transfected as indicated above the lanes. The nickelformase-purified ubiquitylated products and WCEs (5% of the total lysates) were resolved on SDS-PAGE and analyzed by western blotting using anti-Notch IC. White lines indicate that intervening lanes have been spliced out.

Notch activation was defined as 100% in the presence of Dll1-α-arrestin

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reporter activation in WT (top) and C2 (bottom) cells. Cells were transfected with csl-firefly luciferase (Notch reporter) and TK-Remilla luciferase (internal control) together with β-arrestin 1 expression vector (100 ng), when indicated. Notch activation was defined as 100% in the presence of Dll1-expressing cells without β-arrestin 1 overexpression (lane 1, in each cell line). Error bars correspond to s.e.m. P values are calculated with Student’s t-test (*P<0.05; n.s., P>0.05 compared with the activated control, lane 1). Scale bars: 10 μm.
in vitro translated [35S]methionine-labeled β-arrestin 1 was incubated with either purified GST–Iitch or GST alone adsorbed on glutathione-agarose beads. As shown in Fig. 3A, no interaction was detected between β-arrestin 1 and Iitch (lane 6), which is inconsistent with a direct binding between the two native proteins. We thus asked whether another member of the arrestin family could interact with Iitch. α-arrestins/ARRDCs (arrestin-domain containing proteins) have been classified after phylogenetic analysis as members of the large family of proteins that includes the well-characterized β-arrestins (Alvarez, 2008). Among the six mammalian α-arrestins, ARRDC1–ARRDC4 harbor conserved PPxY motifs (Alvarez, 2008; Rauch and Martin-Serrano, 2011). We first tested by coimmunoprecipitation from transiently transfected cells, the interaction of ARRDC1–ARRDC4 with Iitch. We found that only ARRDC1 and ARRDC3 were able to coimmunoprecipitate with Iitch-DN (supplementary material Fig. S3A). For technical reasons we used ARRDC1 in all our subsequent experiments.

We performed in vitro translations and a GST pull-down assay with HA–ARRDC1 and HA–ARRDC1-ΔPY (which lacks the PPxY-containing C-terminal part, as schematized in Fig. 3B) in parallel with β-arrestin 1. In contrast to β-arrestin 1, ARRDC1 directly bound Iitch, whereas GST–Iitch interaction with the mutant ARRDC1-ΔPY was almost undetectable (Fig. 3A, lanes 2 and 4). Therefore the PPxY-containing domain of ARRDC1 is necessary to directly bind Iitch. A PY* mutant (which contains a double point mutation in the PPxY motifs; Fig. 3B) was not able to coimmunoprecipitate with Iitch DN from transfected cells, in contrast to WT ARRDC1, confirming that the PPxY motifs of ARRDC1 indeed account for Iitch interaction (supplementary material Fig. S3B).

We next asked whether β-arrestins could heterodimerize with ARRDC1 and so cooperate as adaptors for Iitch recruitment. In transfected HEK 293T cells, β-arrestin 1 co-immunoprecipitated with WT ARRDC1 constructs (Fig. 3C, compare lanes 5 and 7 with lanes 2 and 4). This interaction does not depend on PPxY motifs because the binding was also detectable with PY* and ΔPY mutants (lanes 3, 6 and 8), suggesting that ARRDC1 and β-arrestin 1 interact through the arrestin domain. Therefore, β-arrestin 1 is not able to directly interact with Iitch, but it can heterodimerize with ARRDC1, a direct Iitch interactor. In addition, in vitro pull-down experiments and coimmunoprecipitations from transfected cells showed that ARRDC1 is able to directly interact with β-arrestin 1 and β-arrestin 2 (supplementary material Fig. S3C,D). In light of these results, we hypothesized that ARRDC1 could form a complex with β-arrestin 1 and β-arrestin 2 that is able to recruit Iitch to Notch.

**ARRDC1 cooperates with β-arrestins on Notch signaling**

To determine whether ARRDC1 plays a role in Notch regulation we first decided to assess whether it could influence β-arrestin–Notch interaction. We pulled down β-arrestin 1 (β-ar1–GFP) in transfected HEK 293T and we observed that Notch coimmunoprecipitated with β-arrestin 1 (Fig. 4A, compare lane 7 with lane 2). When ARRDC1 WT was overexpressed, β-arrestin–Notch complexes were less abundant (Fig. 4A, lane 5). Overexpression of ARRDC1 could increase the quantity of the degradative complexes formed with endogenous Iitch and.

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**Fig. 3. ARRDC1 interacts with Iitch and β-arrestin 1.** (A) GST pull down with Iitch and ARRDC1. HA-tagged ARRDC1 and HA ARRDC1 ΔPY proteins were in vitro-translated in the presence of [35S]methionine. The translated products were incubated with purified GST–Iitch fusion protein (lanes 2,4,6) or control GST (lanes 1,3,5) adsorbed to glutathione-agarose beads and then resolved on SDS-PAGE. The input lanes (7–9) show the in vitro-translated products before incubation with beads (10% of the total). (B) Schematic drawing of human ARRDC1 constructions used: ARRDC1 wild type (WT), double point mutation mutant in PPxY motifs (PY*), HA-tagged ARRDC1 wild type (HA-WT) and HA-tagged ARRDC1 truncation mutant (HA-ΔPY) showing N- and C-terminal arrestin-like domains, as well as PPxY motifs in the C-terminus, where indicated. All the constructions are compared with β-arrestin 1 and β-arrestin 2, which contain an arrestin domain, but lack a PPxY motif. The numbers indicate the amino acids. (C) Coimmunoprecipitation of ARRDC1 WT/PY* and HA-WT/HA-ΔPY with β-arrestin 1. HEK 293T cells were transfected as indicated above the lanes. IP was performed with the anti-FLAG antibody coupled to agarose beads and followed by FLAG peptide elution (2 mg/ml). IPs and WCEs (5% of the total lysates) were analyzed by western blot using the antibodies recognizing the proteins indicated on the left. White lines indicate that intervening lanes have been spliced out. elf3a was used as a loading control. The molecular sizes (kDa) are represented on the right.
therefore further promote Notch degradation. This hypothesis was corroborated by the fact that ARRDC1 PY*, which is not able to bind Itch, stabilized \(\alpha\)-arrestin-Notch complexes (lane 6), probably avoiding the whole Itch-containing complex assembly and therefore Notch degradation. In all cases, the levels of Notch were comparable in the WCEs, reflecting the fact that a small percentage of whole cellular Notch is undergoing degradative trafficking at a given time. Together, these results suggest that complexes containing Notch and \(\alpha\)–\(\beta\) heterodimeric arrestins are formed and that they are able to target Notch for degradation. To further prove and localize the interaction of endogenous ARRDC1 with Notch, we detected Notch–ARRDC1 complexes by using \emph{in situ} PLA technology (Fig. 4B, panels G,M). Notch- and ARRDC1-containing spots were twice as numerous when cells were transfected with ARRDC1 PY* (see quantification on Fig. 4C), confirming the stabilization of the complexes when Itch cannot be recruited.

To further assess the role of ARRDC1 in Notch trafficking and degradation, we used WT and C2 MEFs transiently transfected with either ARRDC1 (WT or HA-WT) or the mutated forms (PY* or DPY) to perform a receptor-uptake experiment on living cells. Expression of each ARRDC1 construct was confirmed by western blotting (supplementary material Fig. S6A). Notch was mostly detected in intracellular vesicles at early time points, with a comparable kinetics in all cases (supplementary material Fig. S4), meaning that overexpression of any ARRDC1 construct had no effect on the early steps of Notch endocytosis. As shown in Fig. 5A and quantified in Fig. 5B, the presence of ARRDC1 WT affected neither Notch degradation observed in WT cells (panels E,I compared with A,C) nor the delayed degradation observed in C2 (panels F,J compared with B,D). This shows that ARRDC1 did not complement the trafficking defect of \(\beta\)-arrestin \(2/2\) cells, in contrast to what was observed when \(\beta\)-arrestin 1 was transfected into C2 cells (Fig. 2B, panels K,L). Therefore, these factors are not interchangeable. We observed Notch-positive spots remaining in WT cells transfected with ARRDC1 PY* or

Fig. 4. ARRDC1 effects on Notch signaling pathway. (A) Co-immunoprecipitation of Notch and ARRDC1/PY* with \(\beta\)-arrestin 1. Cells were transfected as indicated above the lanes. IPs and WCE were analyzed by western blot using the indicated antibodies. (B) Detection of Notch–ARRDC1 interaction using \emph{in situ} PLA technology. Living cells were transfected, permeabilized and labeled with anti-Notch (NIC) and anti-ARRDC1 antibodies, as indicated on the left. After addition of secondary antibodies conjugated with oligonucleotides, ligation and amplification with fluorescently labeled nucleotides were performed. Red fluorescent spots indicate that ARRDC1 and Notch are closely located (interaction). DAPI staining is in blue. (C) Quantification of Notch–ARRDC1 PLA spots. Means ± s.e.m. of a minimum of 20 cells are shown. Data are reported as average of spots per cell. ****\(P<0.00001\). (D) Effect of ARRDC1 WT and PY* on Notch FL ubiquitylation. Cells were transfected as indicated above the lanes. The ubiquitylated products and WCEs were obtained and analyzed as in Fig. 2A. In A and B, white lines indicate that intervening lanes have been spliced out, \(\alpha\)-tubulin was used as loading control and the molecular sizes (kDa) are shown on the right. Scale bar: 10 \(\mu\)m.
ΔPY (Fig. 5A, panels G and K), as was the case in all C2 conditions (Fig. 5A, panels B,D,F,H,J,L and quantification Fig. 5B). Thus ARRDC1 PY* or ΔPY overexpression converted WT cells into cells that behaved like β arrestin−/− cells. To further assess whether this effect was due to an impairment of Notch lysosomal degradation, we looked whether

Fig. 5. See next page for legend.
the remaining Notch-positive vesicles (visible after 3 hours of incubation in Fig. 5A) colocalized with LAMP1. As expected, Notch signal colocalized with LAMP1 in the presence of leupeptin either in WT cells transfected with control vector (Fig. 5C, panels A–C), or in C2 cells transfected with β-arrestin 1 expression vector (Fig. 5C, panels M–O). In WT cells expressing ARRDC1 PY* or ΔPY (Fig. 5C, panels D–F and G–I, respectively), Notch colabeling with LAMP1 was barely detected, as in C2 cells (panels J–L). These findings strongly suggest that the overexpression of mutated ARRDC1 (PY* or ΔPY) in WT cells impairs Notch targeting to lysosomal degradation, as observed in β-arrestin–/– cells. The vesicles where Notch was trapped in C2 cells or in ARRDC1-PY*-transfected WT cells, after 180 minutes of internalization were partially colocalized with the early endosome maker EEA1 in both conditions (supplementary material Fig. S5), suggesting that ARRDC1 and β-arrestins are required after early endocytosis for Notch degradation.

We finally monitored the role of endogenous arrestins in Notch signaling by co-culture assay. We transfected human-Notch-expressing cells (U2OS-FL) with Notch reporter, a control vector (as described in Fig. 2D) and different siRNAs: NT (nontargeting) or targeting ARRDC1, β-arrestin, CSL, USP12, respectively, as indicated in Fig. 5D. The efficiency of both siRNAs targeting β-arrestin and ARRDC1 was quantified by qRT-PCR (see supplementary material Fig. S6B). After co-culturing Delta-like-1-expressing cells with U2OS-FL cells transfected with siNT, Notch activation was significantly induced compared with that in the DAPT-treated co-cultures (33.3 fold, see Fig. 5D). As expected, knockdown of CSL, the key DNA-binding subunit of the Notch-containing transcriptional complex (Bray, 2006), led to a significant reduction of Notch activation. By contrast, silencing of USP12, a negative regulator of Notch signaling (Moretti et al., 2012), significantly increased Notch activity (233% over siNT). Strikingly, knockdown of either β-arrestins or ARRDC1 also induced Notch reporter activity (161% and 127%, respectively) showing that ARRDC1 and β-arrestins are negative regulators of Notch signaling. Moreover, the simultaneous ablation of both ARRDC1 and β-arrestins further increased the effect of single knockdown (184% for the combination vs 127% and 161% for siARRDC1 and siβ-arrestin respectively) suggesting that indeed ARRDC1 and β-arrestins cooperate as negative regulators of Notch signaling.

Taken together, these data show that ARRDC1 is involved in Itch-mediated Notch ubiquitylation and lysosomal degradation at the same step, but not redundantly, with β-arrestins. Moreover, ARRDC1 in combination with β-arrestins acts as a negative regulator of Notch signaling in accordance with ARRDC1 and β-arrestins being members of the same complex.

**Discussion**

**Arrestins are new factors involved in mammalian Notch degradation**

It has been shown in various systems that full-length Notch receptor has a limited half-life at the cell surface and is constantly internalized and degraded through a lysosomal pathway (Chastagner et al., 2008; Sakata et al., 2004; Vaccari et al., 2008; Wilkin et al., 2008). This mechanism is a way of maintaining a functional receptor, and of eventually regulating Notch signal strength by acting on the receptor level at the cell surface. Notch trafficking towards the lysosomes involves ubiquitylation by the E3 ubiquitin ligase Itch/AIP4 in mammals (Chastagner et al., 2008) and Su(dx) or Nedd4 in *Drosophila* (Sakata et al., 2004; Wilkin et al., 2004). However, whereas *Drosophila* Notch contains a canonical PPxY motif that could account for direct binding of the receptor to the E3 ligase, this is not the case for mammalian Notch molecules (except Notch 3). Therefore, scaffolding proteins are probably necessary, at least in mammals, to form and stabilize complexes that allow Notch ubiquitylation and degradation. Our work shows that Notch degradation in the absence of ligand is regulated by β-arrestins, together with the β-arrestin ARRDC1. Biochemical data demonstrate that β-arrestins are necessary for Notch–Itch interaction, although they cannot bind Itch directly. Moreover, ARRDC1, which contains PPxY motifs, is able to interact with Notch and directly with Itch, and with β-arrestin 1 and β-arrestin 2. In addition, complexes containing Notch and β-arrestin are stabilized in the presence of mutant ARRDC1 constructs that are unable to recruit Itch. These data strongly suggest that Itch is recruited to Notch by the ARRDC1 subunit of β-arrestin–ARRDC1 heterodimers. This hypothesis is corroborated by functional data, because we show that Notch ubiquitylation and lysosomal degradation both depend on the presence of both β-arrestins and β-arrestins. Interestingly, in β-arrestin-knockout cells, ARRDC1 overexpression cannot restore Notch degradation, in contrast to overexpression of β-arrestin 1. Conversely, a mutant form of ARRDC1, affected in its ability to recruit Itch, can convert WT cells to cells that behave like β-arrestin-deficient cells, because Notch degradation is blocked at the same step in both conditions. In addition, ARRDC1 and β-arrestins act as negative regulators of Notch signaling, as shown in a co-culture assay, allowing us to monitor the involvement of these endogenous proteins on Notch activation. Taken together, our observations substantiate the idea that β-arrestins and β-arrestins are not redundant, but instead work together in complexes recruiting Itch to Notch.

**How and when are arrestins heterodimers recruited to Notch?**

β-arrestins and β-arrestins are cytosolic proteins that were described as being recruited to the plasma membrane upon cargo activation or upon environmental signals in yeast (Polo and
Di Fiore, 2008). However, recent data localized these proteins to the plasma membrane (Lin et al., 2008), along the endocytic pathway (Becuwe et al., 2012b; O’Donnell et al., 2010; Patwari et al., 2009), or even in the nucleus (Boulan et al., 2007; Hara et al., 2011) during cargo recognition. Similar to β-arrestins, ARRDCs have been described to interact with clathrin and clathrin adaptors, suggesting that they could act on early endocytosis (Becuwe et al., 2012a; O’Donnell et al., 2010). However, our results suggest that this is not the case for Notch trafficking, because the formation of early endocytic vesicles occurs similarly in WT, β-arrestin 2/2 or PY*-ARRDC1-overexpressing cells, as well as in Itch 2/2 cells. In these conditions we observed an increased number of Notch-containing vesicles that colocalized with EEA1, but not a complete overlap of staining. Therefore recruitment of arrestins and Itch to Notch probably takes place after early endocytosis. This result reflects the fact that arrestins are multifunctional molecules that fulfil complex and diverse functions, depending on the substrates. For example, β-arrestin1 can interact with STAM-1, a component of ESCRT-0 to control CXCR4 downregulation (Malik and Marchese, 2010). Recent data on retroviral and vesicle budding mechanisms (Nabhan et al., 2012; Rauch and Martin-Serrano, 2011) show that ARRDC1 can directly interact with HECT ubiquitin ligases, including Nedd4 and Itch, and also with ALIX and Tsg101, components of the ESCRT pathway. In this case, the proposed model is that ARRDCs could provide a physical link between HECT ubiquitin ligases and ESCRT machinery, via interactions with ALIX and Tsg101 through their C-terminus, and with uncharacterized factors through their arrestin domains. Similarly, during Notch trafficking, arrestin heterodimers (bound through their arrestin domain) could be recruited to Notch when the receptor becomes associated with specific factors of the endocytic machinery in sorting endosomes. This scaffold could recruit Itch (through the C-terminus of ARRDC1) and target Notch for subsequent degradation (see model in Fig. 6).

Comparison with the Drosophila model
The first description of the involvement of arrestins in Notch signaling was made in Drosophila (Mukherjee et al., 2005). The Drosophila genome encodes a single β-arrestin, Kurtz (krz), that is involved in development and survival; however, many of the classical functions of β-arrestins are conserved in the fly. In addition to Deltex (dx) and krz, shrub has been recently identified as affecting subcellular localization of Notch (Hori et al., 2011). shrub encodes the Drosophila homologue of vps32, a core component of the ESCRT III complex. Whereas Drosophila shrub antagonizes dx, it enhances the downregulating activity of krz on Notch (Hori et al., 2011). The proposed model in Drosophila is that Krz and the E3 ubiquitin ligase Deltex could form a complex with Notch and regulate Notch degradation via an endosomal-lysosomal pathway. In addition to the identification of Krz by two-hybrid screen using Deltex as bait, this model is mainly based on genetic interaction experiments using the adult wing phenotype as a readout of Notch signaling activation. Our data complete and extend these results in mammals, even though some discrepancies might exist between both systems. It is of note that although Drosophila Notch does contain a PPxY motif, Krz is still necessary for Notch degradation, suggesting that Drosophila β-arrestin could actually stabilize degradation complexes in the same way as we show here for mammalian β-arrestins. Regarding α-arrestins, there are at least 13 members in the fly ARRDC family (Alvarez, 2008). Further genetic studies

**Fig. 6. Model of Notch degradative complex.** After early endocytosis, non-activated Notch receptor (orange heterodimer) reaches the sorting endosome (1) where it is recognized, possibly together with other factors of the endocytic machinery (not shown), by arrestins. β-arrestin 1 or β-arrestin 2 (in green) forms a heterodimer with α-arrestin 1 (ARRDC1) through the arrestin domain (split into N and C domains). The dotted lines indicate interaction. PPxY motifs of α-arrestin account for Itch direct interaction, therefore Notch can be ubiquitylated (2) and subsequently targeted to lysosomes (3) for degradation.
are needed to see whether some of these genes can have a role in Notch signalling; however, compensatory effects could complicate the analysis.

Because we observed a direct interaction of human Deltex1 with β-arrestins (supplementary material Fig. S7), one putative model that would integrate all the data would be that Notch internalization could be directed by Deltex (Yamada et al., 2011), which harbors conserved internalization motifs in its C-terminal part. Once non-activated Notch reaches an adequate compartment, arrestins could bind the receptor and recruit Itch to eventually trigger Notch degradation through ESCRT-dependent mechanisms. Alternatively if Notch is activated by its ligand and cleaved by ADAM-10, the γ-secretase complex could recognize it after Deltex-mediated internalization, and the activation process could follow. Further experiments are needed to prove this hypothesis and build a full model.

α–β-arrestins and β-arrestins can heterodimerize and are required together in Notch signalling: is this a general way of working?

Our data show that β-arrestin 1 is able to heterodimerize in vitro and in vivo with ARRD1C1 through the structurally conserved arrestin domain. Do α–β complexes pre-exist in the cell cytoplasm? A strong argument against this possibility comes from a recent census of human soluble complexes (Havugimana et al., 2012), which did not identify arrestin-containing preformed complexes. In addition, a previous proteomic analysis using cells stably expressing β-arrestin 1 or β-arrestin 2 identified more than 300 proteins able to interact with one or both β-arrestins (Xiao et al., 2007). Among them, β-, S- and X-arrestins were detected, suggesting that these proteins are able to heterodimerize in solution, however neither α-arrestins nor E3 ubiquitin ligases are listed in the paper. Therefore α–β complexes could form when recruited to a specific substrate. Our work demonstrates for the first time that α-arrestins and β-arrestins heterodimerize and function coordinately to fine-tune Notch recognition and recruitment of the E3 ubiquitin ligase Itch, eventually targeting Notch for lysosomal degradation. The same type of model could apply in other contexts. In the case of β2AR (β2-adrenergic receptor), challenging results suggest that β-arrestin 2 as well as ARRD3C3, are necessary for Nedd4-dependent ubiquitylation and degradation of the receptor after its activation by isoproterenol (Han et al., 2012; Nabhan et al., 2010; Shea et al., 2012; Shenoy et al., 2008; Shenoy et al., 2001). Therefore, and in accordance with our hypothesis, it is tempting to speculate that β-arrestin 2 and ARRD3C3 (like β-arrestin 1 and ARRD1C1 in our case) could heterodimerize to fulfill this scaffolding function. Of note, Shea and colleagues (Shea et al., 2012) visualized a vesicular colocalization of ARRD3C3/4 with β-arrestin 1 after stimulation of β2AR, as well as a co-immunoprecipitation between α-arrestins and β-arrestins. More generally, various types of arrestin heterodimers could be formed, depending on the cargo and its subcellular localization, and could recruit the specific E3 ubiquitin ligase involved in the process. Given that there are two β-arrestins and a larger number of α-arrestins (six in mammals), the various combinations would thus increase the repertoire of intermediary factors able to specifically recognize defined cargo during their trafficking.

Materials and Methods

Antibodies and peptides

Antibodies for western blots were supplied by Abcam [anti-ARRDC1, anti-ARRDC3 and ARRD4, EEA1, anti-Notch1 (ab49990)], Bethyl (polyclonal anti-eIF3A), Covance (monoclonal anti-HA), Invitrogen (polyclonal anti-GFP), Sigma (monoclonal anti-FLAG M2; polyclonal anti-FLAG; monoclonal anti-α-tubulin, anti-Myc [9E10], BD Transduction Laboratories (monoclonal anti-Itch and anti-β-arrestin) and Novagen (monoclonal anti-S-Tag). Anti-LAMPI was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies for immunofluorescence were supplied by Molecular Probes (Alexa Fluor conjugates). Anti Notch IC antibody has been already described (Logeat et al., 1998). FLAG peptide was provided by GenECUST and used at 2 mg/ml.

Constructions

The following constructs have been already described: murine Mhc-tagged Notch1 full-length (FL), deleted of a C-terminal fragment (Schoeter et al., 1998), human Notch1 retroviral construct (Rand et al., 2000). GST-β-arrestin-1/2 (Hara et al., 2011), GFP-β-arrestin 1 and FLAG-β-arrestin-1/2 (Scott et al., 2002; Storez et al., 2005), β-arrestin 1 and HA-β-arrestin-1 (Bhandari et al., 2007), HA-ARRDC1 (Rauch and Martin-Serrano, 2011), GST–Itch (Angers et al., 2004).

ARRDC1, ARRDC1 PY*/(P PxYs were mutated in PAXys), S-tagged ARRDC2, ARRDC3, ARRDC4 were gifts from Olivier Staub (Department of Pharmacology and Toxicology, University of Lausanne). VSV-tagged DTX was described previously (Chastagner et al., 2006), 6xUBi-His was from M. Treier (EMBL, Heidelberg, Germany). CSL-Luc was a gift from T. Honjo (Kyoto University, Japan) and is referred to as pklu981-R (Minoguchi et al., 1997). HA-tagged ARRDC1 and HA-tagged ARRDC1 ΔPY were generated by inserting PCR products in pcDNA3-HA backbone vector (Ecor1/Xmlol), using as forward primer: 5’-CTAGCGGAACTCGGCGAGTGACAGCTCTCTC-3’ and as reverse primers: 5’-TCATGCATTGGCTAGCTCTCGTGGCTCAG-3’ for HA-ARRDC1 and 5’-TCATGCATTGGCTAGCTCTCGTGGCTCAG-3’ for HA-ARRDC1 ΔPY. All constructs were verified by sequencing.

Cell lines and transfections

Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). OP9-DL1 and U2OS-FL cell lines were described previously (Six et al., 2004; Moretti et al., 2012). Mouse embryo fibroblast (MEF) cells isolated from β-arrestin 1 and β-arrestin 2 double-knockout (DKO) embryos and matched wild-type (WT) embryos were provided by Robert J. Lefkowitz (Duke University, Durham, NC). These cells were retroviral transduced: high titers of recombinant HA-tagged Notch FL viruses were obtained 48 hours after transfection of the Plat-E ecotropic packaging cell line with retroviral expression plasmids. Clonal populations were obtained by limiting dilution. Transfections were performed in HEK 293T with calcium phosphate or jetPRIME™ (Polyplus) according to the manufacturer’s instructions. MEF-FL, OP9 DLL1 and U2OS-FL were transfected with jetPRIME™ (Polyplus) or Fugene HD (Promega).

GST pull-down analysis

GST fusion constructs were transformed into E. coli DG1 cells and expression was induced by IPTG. Approximately equal amounts of glutathione-S transferase (GST) alone or in fusion with Itch, as estimated from a Coomassie-stained gel, were bound to glutathione-Agarose beads (Sigma). In vitro transcription and translation were made according to the manufacturer’s instructions in reticulocyte lysates (Promega), in the presence of [35S]methionine. The products were resolved and immunoblotted.

Ubiquitin-conjugate purification

HEK293T cells were harvested 36 hours after transfection and lysed in 8 M urea, 0.1 M NaH2PO4, 10 mM Tris-HCl (pH 8), 1% Triton X-100 and 20 mM Imidazole at room temperature. His–Ub-conjugated proteins were purified by chelating Sepharose beads (Pharmacia), previously charged with Nickel. Ubiquitinated proteins were washed extensively with the same buffer and then with a Tris-HCl, pH 6.3, buffer. The Ubiquitin-conjugated products were then eluted in Laemmli buffer for western blot analysis.

Cell extracts and immunoprecipitation

HEK293T cells were collected 36 hours after transfection and lysed in 50 mM Tris-HCl (pH 7.9), 5 mM MgCl2, 300 mM NaCl, 1% Triton X-100, supplemented with protease inhibitor cocktail (Roche) and N-ethylmaleimide (NEM), an inhibitor of deubiquitylases (Sigma). The lysates were cleared by centrifugation at 14,000 rpm for 20 minutes at 4°C. Immunoprecipitations were performed with the appropriate antibodies in the same buffer. When indicated, the immunoprecipitates were eluted by FLAG peptide competition (2 mg/ml) for 1 hour at 4°C. Samples were denatured in Laemmli buffer for SDS-PAGE resolution and immunoblotting.
HA antibody-uptake assay
Cells were grown on glass coverslips for 24 hours, and when indicated, transfected (Fugene HD, Promega) with plasmids for the following 24 hours. After 1 hour of incubation in serum-free medium, cells were incubated for 15 minutes with anti-HA, washed again and incubated in serum-free medium at 37°C for various periods of time (0, 30, 60, 90 and 180 minutes). Cells were then quickly washed with cold PBS, fixed with 4% paraformaldehyde for 20 minutes and permeabilized with 0.2% Triton X-100 for 5 minutes before incubation with appropriate primary and secondary antibodies. Cells were mounted in Mowiol (Calbiochem, Merck Biosciences, Darmstadt, Germany) and images acquired with 0.2 μm sections using an Axioplan 2 imaging system with ApoTome (Carl Zeiss MicroImaging, Le Pecq, France). When indicated, cells were treated with Leupeptin (Sigma) at 20 μM.

Co-culture assay
WT, C2 MEFs or U2OS-FL cells were grown in 24-well plates and transiently transfected with reporter genes (Notch reporter: CSL-firefly luciferase; internal control: TK-Renilla luciferase) in triplicate with FuGeneHD transfection reagent (Roche, Mannheim, Germany), then co-cultured with OP9-Dll1 cells for 18 hours. The co-cultured cells were lysed using Passive Lysis buffer (Promega). A fraction of cell lysates was used to measure Firefly and Renilla luciferase activities in a luminometer Centro XS (Berthold). For western blot analysis, culture wells were lysed with 0.1 M Na2HPO4, 10 mM Tris-HCl (pH 8), 1% Triton X-100 and 20 mM Imidazole.

RNA interference
β-arrestin expression was silenced using a validated siRNA (Coureuil et al., 2010). siARRDC1 target sequence was 5′-CACCACUGUCGUCCAUUUAA-3′. siSuperTarget sequence was UAGCAGAUCUCUCCAUAG. ON-TARGET plus smart pool targeting human CSL (L-007772-00-0005), non-targeting pool (D-001810-10-20) or GFP Duplex I (P-002048-01-20) were from Thermo Scientific.

Flow cytometry
Cells were harvested and washed twice with PBS, fixed in 4% paraformaldehyde and labeled with anti-HA followed by incubation with appropriate secondary antibody (Alexa Fluor 488). Finally, the cells were washed and analyzed by using Cyan ADF Flow Cytometer (Beckman Coulter). The data was analyzed using FlowJo (Tree Star) software.

Colocalization images quantification using Icy
The colocalization events were quantified using Icy software (de Chaumont et al., 2012), http://icy.bioimageanalysis.org, composed of (1) efficient fluorescence-labeled spot detection based on wavelet transform and (2) distance object-based colocalization. The colocalization distance was defined as a positive hit when the pixel distance between Notch and LAMP1 centroids was less or equal to 5 pixels. The colocalization distance was defined as a positive hit when the pixel distance between Notch and ARRDC1 centroids is less or equal to 5 pixels (0.5 μm). Percentage of colocalization was defined as a ratio of colocalization value over spot detection number.

Quantification and statistical analysis
Protein bands and images were quantified by densitometry and analyzed with Quantity One Software (BioRAD Laboratories) and ImageJ. Data were analyzed using Student’s t-test. Error bars correspond to s.e.m.

**In situ PLA technology**
The PLA technique was used to detect *in situ* interaction between Notch and ARRDC1 using primary antibodies specific for these proteins (rabbit polyclonal anti-ARRDC1 and mouse anti-Notch, Abcam) coupled to a Duolink II kit (http://www.onlink.com/). This technique allows detection of a single fluorescent spot when Notch and ARRDC1 are closely located (<40 nm) (Söderberg et al., 2006).

**qRT-PCR**
U2OS-FL cells were lysed and RNAs were purified using RNeasy mini kit (Qiagen). A reverse transcription following by quantitative PCR was then performed. CFX96TM real-time PCR detection system and the CFX ManagerTM Software (Bio-Rad) were used for analysis. The following oligonucleotides were used: human β-arrestin 1, 5'-GCCCAAGTGTACAGACAGAG-3' (forward) and 5'-AGGCACAGGATGAAAGACT-3' (reverse); human ARRDC1, 5'-CATGGCC-CACTACACGAC-3' (forward) and 5'-ATAGGGTGATCCTTGAACGAC-3' (reverse); human UBCHB5, 5'-TGAAGAGGAATCCAAAGAATGTA-3' (forward) and 5'-CAAGACGACCTGACGTAACCTG-3' (reverse). Relative expression of β-arrestin 1 and ARRDC1 was normalized using UBCSB5.

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**Author contributions**
L.P. and P.C. performed experiments; L.P. and C.B. designed experiments; L.P., V.M.Y. and C.B. analyzed data; L.P., A.I. and C.B. wrote the paper.

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**Supplementary material available online at**

**References**


Fig. S1. Quantifications (related to Figure 2). (a) Quantification of cell surface Notch (refers to figure 2b). WT and C2 cells were harvested, resuspended and fixed with 4% PFA before incubation with anti-HA antibody at 4°C, then with Alexa 488-coupled secondary antibody and analyzed using a Cyan ADP flow cytometer (Beckman Coulter). Dead cells and cell debris were excluded by gating the population according to the forward and side light scatters. A minimum of 10,000 total events was analyzed and reported on a % scale. The two peaks in the histogram represent the fluorescence background (only secondary antibody) or HA fluorescence for each cell line (WT in black, C2 in red). Note that WT cells are labeled with higher intensity, therefore the remaining staining in C2 cells in the antibody uptake experiment of figure 2b is not because C2 cells express more Notch at the cell surface, but rather because of lack of degradation. (b) Quantification of Notch-LAMP1 colocalization (relating to figure 2c). Colocalized spots (Notch-LAMP1) in WT, C2 and 3F4 cells were quantified using Icy Program Software (de Chaumont et al., 2012). Means +/- SD of 20 different cells are shown. (*** p<0.0001, compared to control cells (WT). After 3 hours in the presence of leupeptin, Notch positive vesicles are mainly (65%) colocalized with LAMP1 in WT cells, in contrast to C2 (β-arrestins -/-) or 3F4 (Itch-/-) cells. Note that a partial labeling overlap, which is more frequent in C2 and 3F4, is counted as “colocalized” with this software, resulting in an overestimation of the colocalization efficiency in these cells.

Fig. S2. β-arrestin 1 overexpression in WT cells and C2 cells (related to co-culture assay in Figure 2D). WT (a) and C2 (b) cells were transfected and treated in parallel for luciferase assay (figure 2d) and western blot analysis. The WCE were resolved on SDS-PAGE and analyzed by Western blot using the antibodies recognizing the proteins indicated on the left side of the panels. eIF3A was used as a loading control. The molecular weights (kDa) are indicated on the right side of the panels. The figure shows that β-arrestin 1 is overexpressed in both cell lines.
**Fig. S3.** ARRDCs interactions with Itch and β-arrestins (related to Figure 3).

(a) Co-immunoprecipitation of ARRDC1, S-tag ARRDC2, ARRDC3 or ARRDC4 with Itch DN. HEK293T cells were transfected as indicated above the lanes. The IP's were performed with the anti-FLAG antibody coupled to agarose beads followed by FLAG peptide elution (2mg/ml). IP's and WCE (5% of the total) were resolved on SDS-PAGE and analyzed by Western blot using the antibodies recognizing the proteins indicated on the left side of the panels, i.e. for the first and third panels of a: anti-ARRDC1 (lanes 1, 2), anti-S-tag (lanes 3, 4), anti-ARRDC3 (lanes 5, 6) or anti-ARRDC4 (lanes 7, 8). eIF3a and α-tubulin were used as loading controls. The molecular weights (kDa) are represented on the right side of each panel. These experiments show that ARRDC1 and ARRDC3, but not ARRDC2, ARRDC4 or ARRDC1 PY* are able to interact with Itch DN.

(b) Direct interaction of ARRDC1 with β-arrestin 1/2. HA-tagged ARRDC1 was in vitro transcribed and translated in the presence of 35S methionine (lane 4, input), then incubated with purified GST-β-arrestin 1 or 2 fusion proteins (lanes 2, 3) or control GST (lane 1) adsorbed to glutathione-agarose beads. After several washes, the retained material was eluted, resolved on SDS-PAGE and autoradiographed. (d) Co-immunoprecipitation of ARRDC1 with β-arrestins 1 and 2. The protocol was as in (a). The experiments in c and d show that ARRDC1 is able to directly interact with β-arrestins 1 and 2.
Fig. S4. Kinetics of Notch internalization in C2 (β-arrestins -/-) cells transfected or not with ARRDC1 (WT, PY*, HA-WT, HA-ΔPY) (related to Figure 5). Cells were transfected as indicated (CTRL: non-transfected, pcDNA3: empty vector). Living cells were labeled with anti-HA antibody and incubated at 37°C for different time points (time 0’ and 30’ are shown). Then cells were fixed, permeabilized and stained with Alexa 488-coupled secondary antibody and DAPI. The third row shows 4-fold enlargements of regions indicated by arrowheads at 30’ time point. Each field is representative of the experiment and should statistically contain at least one transfected cell, according to the transfection efficiency (30%). Scale bar: 10μm. This figure shows that endocytosis kinetics is similar in control and transfected cells.
Fig. S5. Notch receptor partially colocalizes with EEA1 in WT cells transfected with ARRDC1 PY* and in C2 cells (related to Figure 5). Cells were transfected as indicated on the left side of the panel. Living cells were labeled with an anti-HA antibody (green), incubated for 3h at 37 °C in the presence of leupeptin (20 μM) when indicated (A-C and J-L). After fixation cells were additionally permeabilized and stained with an anti EEA1 antibody (red) and DAPI (blue). Insets are 4-fold enlargements of regions indicated by arrows. Scale bar: 10μm. No colocalization is detected in leupeptin-treated WT cells or C2 cells complemented with β-arrestin 1. In contrast, Notch partially colocalizes with EEA1 in β-arrestins -/- cells or in WT cells transfected with ARRDC1 PY* suggesting that Notch can reach this endocytic compartment without β-arrestins or with a mutated form of ARRDC1.
Fig. S6. Efficiency of transfection of ARRDC1 constructs and siRNAs (related to Figure 5). (a) WT and C2 cells were transfected as indicated on the top of the panel (CTRL: non-transfected, pcDNA3: empty vector). WCE was resolved on SDS-PAGE and analyzed by Western blot using the antibodies recognizing the proteins indicated on the left side of the panel. The arrows indicate the exogenous ARRDC1 proteins. This experiment shows that all the ARRDC1 constructs used are expressed in WT and C2 cells. (b) U2OS- FL cells were transfected with siRNAs targeting β-arrestins, ARRDC1 or non-targeting siRNA (si nt). RNA was extracted 48 hours post transfection. A reverse transcription followed by a quantitative PCR were performed. Data are presented after normalization with a housekeeping gene and are reported in a % scale. This experiment shows the efficiency of siRNAs targeting ARRDC1 and β-arrestin 1.

Fig. S7. DTX directly interacts with β-arrestins and Itch (related to Discussion). DTX-VSV was in vitro transcribed and translated in the presence of 35S methionine (lane 5, input), then incubated with purified GST-Itch, GST-β-arrestin 1 or 2 fusion proteins (lanes 2, 3, 4) or control GST (lane 1) adsorbed to glutathione-agarose beads. After several washes, the retained material was eluted, resolved on SDS-PAGE and autoradiographed. DTX interaction with GST-Itch was used as positive control (Chastagner et al., 2006). The molecular weights (kDa) are represented on the right side of the panel. The experiment shows that DTX is also able to directly interact with β-arrestins 1 and 2.