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Characterization of the interaction between the dopamine D_4 receptor, KLHL12 and β-arrestins

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ABSTRACT

Dopamine receptors are G protein-coupled receptors involved in regulation of cognition, learning, movement and endocrine signaling. The action of G protein-coupled receptors is highly regulated by multifunctional proteins, such as β-arrestins which can control receptor desensitization, ubiquitination and signaling. Previously, we have reported that β-arrestin 2 interacts with KLHL12, a BTB-Kelch protein which functions as an adaptor in a Cullin3-based E3 ligase complex and promotes ubiquitination of the dopamine D_4 receptor.

Here, we have investigated the molecular basis of the interaction between KLHL12 and β-arrestins and questioned its functional relevance. Our data demonstrate that β-arrestin 1 and β-arrestin 2 bind constitutively to the most common dopamine D_4 receptor polymorphic variants and to KLHL12 and that all three proteins can interact within a single macromolecular complex. Surprisingly, stimulation of the receptor has no influence on the association between these proteins or their cellular distribution.

We found that Cullin3 also interacts with both β-arrestins but has no influence on their ubiquitination. Knockout of one of the two β-arrestins hampers neither interaction between the dopamine D_4 receptor and KLHL12, nor ubiquitination of the receptor.

Finally, our results indicate that p44/42 MAPK phosphorylation, the signaling pathway which is often regulated by β-arrestins is not influenced by KLHL12, but seems to be exclusively mediated by $G\alpha_i$ protein upon dopamine D_4 receptor stimulation.

1. Introduction

1.1. Dopamine D_4 receptor

Dopamine receptors (DR) belong to the superfamily of G protein-coupled receptors (GPCR), the largest family of membrane receptors and the most common drug targets. Dopaminergic signaling controls many basic processes such as movement, cognition, learning, reward and endocrine regulation. Aberrant DR signaling has been implicated in several neurological and psychiatric disorders including schizophrenia, Parkinson's disease, Huntington's disease and attention deficit hyperactivity disorder (ADHD). Five different dopamine receptors have been identified which are divided into two subfamilies: the D_1-like dopamine receptors (D_1R and D_5R), which signal through $G\alpha_s$ proteins and the D_2-like dopamine receptors (D_2R, D_3R, and D_4R), which signal through $G\alpha_{i/o}$ proteins [1–3].

The dopamine D_4 receptor (D_4R) has gained increasing interest due to its high affinity for the atypical antipsychotic clozapine, and its potential in antipsychotic medication [4]. Moreover, this receptor possesses a remarkable VNTR (variable number of tandem repeats) polymorphism

Abbreviations: β_2AR, β_2-adrenergic receptor; ADHD, attention deficit hyperactivity disorder; BTB, bric à brac 1, tramtrack, and broad-complex; co-IP, co-immunoprecipitation; CXCR4, C-X-C chemokine receptor type 4; D_1R-D_4R, dopamine D_1-D_4 receptor; DMEM, Dulbecco's modified Eagle's medium; DUB, deubiquitinating enzyme; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; IB, immunoblotting; IC3, third intracellular loop; JNK3, *c*-Jun-*N*-terminal kinase 3; MAPK, mitogen-activated protein kinase; NEM, *N*-ethylmaleimide; PBS, phosphate-buffered saline; PEI, polyethylenimine; PKA, protein kinase A; PKC, protein kinase C; PTX, pertussis toxin; RIPA, radioimmunoprecipitation buffer; USP, ubiquitin-specific protease; V_2R, vasopressin V_2 receptor; VNTR, variable number of tandem repeats.

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in its third intracellular loop (IC3) where a 16 amino acid sequence is repeated two to eleven times [4,5]. The precise role of this polymorphism is not clear, but the seven repeat allele ($D_{4.7}R$), which has been subject to positive selection during recent evolution, is associated with a predisposition to develop ADHD [6].

1.2. Importance of β-arrestins in GPCR regulation

GPCR function is highly regulated in multiple ways by β-arrestin proteins. There are four members of the arrestin family: arrestin 1 (visual arrestin), arrestin 2 (β-arrestin 1), arrestin 3 (β-arrestin 2) and arrestin 4 (cone arrestin). The visual and cone arrestin are localized to retina rods and cones and interact mainly with rhodopsin, in contrast, to the two β-arrestin isoforms which are ubiquitously expressed. All four arrestins share high sequence and structural homology, consisting out of an *N*-terminal and *C*-terminal domain built almost completely from antiparallel β sheets, but still they preserve highly different functional capabilities [7].

The initially discovered function of β-arrestins was regulation of GPCR desensitization which leads to termination of the signal from the activated receptor. In the classical desensitization mechanism the agonist-occupied receptors are first phosphorylated by G protein-coupled receptor kinases (GRKs) or second messenger-dependent protein kinases, e.g. protein kinase A (PKA) and protein kinase C (PKC), mainly in the *C*-terminus but also in the intracellular loops. In the second step, multifunctional adaptor proteins, β-arrestins, are recruited to the activated and phosphorylated receptors. Binding of β-arrestins inhibit further G protein coupling to the receptor thus leading to desensitization. Subsequently, the receptor is internalized as a consequence of recruitment by β-arrestins key components of the endocytic machinery, including clathrin, adaptin and dynamin. Internalized receptors can be either recycled back to the plasma membrane (resensitization) or degraded (down-regulation) [7–10].

Besides their function in receptor desensitization and trafficking, β-arrestins are also able to induce signaling by scaffolding signaling molecules such as p44/42 mitogen-activated protein kinase (p44/42 MAPK), *c*-Jun-*N*-terminal kinase 3 (JNK3), non-receptor tyrosine kinase *c*-Src, p38 and AKT [11–13]. The discovery of β-arrestin-mediated signaling brought a new appreciation of biased agonism. Biased agonism is best explained as a model in which different GPCR active conformations are either able to promote the full range of receptor activities or only a restricted subset of them. The balanced ligands stabilize the conformations that allow for signaling to all downstream pathways, whereas biased ligands stabilize those conformations that promote only a part of the signaling effects [14–19]. Currently, the bias agonism concept and the possibility to create ligands, which exclusively activate β-arrestin-mediated or G protein-mediated signaling are gaining increasing interest due to their possible therapeutic applications. As an example the discovery of β-arrestin–biased D_2R ligands can be mentioned. Those ligands seem to display potent antipsychotic-like activity without inducing motoric side effects which is desired in the therapy [20]. Recently, also a G-protein–biased D_2R ligand was identified which will be helpful in the elucidation of physiological effects mediated by G protein- or β-arrestin-mediated signaling pathways and their involvement in the therapeutic effects of various pharmaceutical agents [21].

An additional level of GPCR regulation by β-arrestins is achieved by their ability to control GPCR ubiquitination. Several studies have demonstrated that β-arrestins can interact with E3 ubiquitin ligases and mediate ubiquitination of GPCRs, other cell surface receptors and non-receptor proteins [22,23]. The initial study performed by Shenoy and coworkers [24] has shown that stimulation of the β_2-adrenergic receptor (β_2AR) leads to receptor ubiquitination and degradation. The presence of β-arrestin 2 and the ubiquitin ligase MDM2 was important for this process, although MDM2 turned out to ubiquitinate β-arrestin 2 and not the receptor. Later work has revealed that β-arrestin 2 also interacts with another ubiquitin ligase, neural precursor cell expressed developmentally downregulated protein 4 (Nedd4), which is responsible for the ubiquitination and lysosomal degradation of β_2AR [25].

The adaptor role of β-arrestins for ubiquitination of the μ-opioid and V_2 vasopressin (V_2R) receptors was also reported, but the identity of the E3 ligase is not known yet [26,27]. On the contrary, there are also examples such as the *C*-X-C chemokine receptor type 4 (CXCR4) which undergo β-arrestin-independent ubiquitination [28].

In addition to ubiquitin ligases, β-arrestins can also interact with deubiquitinating enzymes (DUBs), including ubiquitin-specific proteases (USPs). USP33 was identified as a β-arrestin-interacting protein in a yeast two-hybrid screen [29] and functions as an endogenous inhibitor of β-arrestin-dependent signaling initiated by β_2AR. USP33 and USP20 were also shown to regulate post-endocytic fate of internalized β_2AR.

1.3. KLHL12 a BTB-Kelch protein

KLHL12 is a member of the KLHL protein family. There are 42 members of this family encoded in the human genome and all of them share structural similarity. They all consist of an *N*-terminal BTB domain (bric à brac 1, tramtrack, and broad-complex), a central BACK domain and 5–6 Kelch motifs at the *C*-terminus. KLHL proteins are known to be involved in the process of ubiquitination and in most cases they serve as adaptors for Cullin3-based E3 ligases [30]. As such, they can also bind to multiple substrates for ubiquitination. For example KEAP1 (KLHL19) has at least three different substrates: NRF2, IKKβ and BCL-2 [31]. Besides to D_4R, KLHL12 was described to bind [32] to Dishevelled [33] and SEC31, the COPII vesicle component [34] and in all cases KLHL12 functions as an adaptor for the Cullin3-based E3 ligase and targets proteins for ubiquitination. Polyubiquitination of Dishevelled leads to its degradation which antagonizes the Wnt-β-catenin pathway [33]. In case of SEC31, the KLHL12-Cullin3 E3 ligase complex promotes monoubiquitination which regulates formation of the large COPII vesicles important for the export of collagen from cells [34].

In our previous studies we have shown that KLHL12 interacts with the D_4R and functions as an adaptor in the Cullin3-based E3 ligase complex and in this way enhances ubiquitination of the receptor. The increased ubiquitination of the D_4R, however, does not promote its degradation [32,35]. Moreover, it was also reported that stimulation of the D_4R does not lead to β-arrestin recruitment and receptor internalization [36].

In the current study we show that both β-arrestins bind constitutively with all common D_4R variants as well as with KLHL12 and that these three proteins can co-exist in one complex. However, stimulation of the receptor does neither influence association between these proteins, nor their cellular distribution. Interestingly, the E3 ligase complex member Cullin3 also co-precipitates with both β-arrestins but has no influence on their ubiquitination.

As β-arrestins can function as scaffolds for E3 ligases, the importance of β-arrestins for the interaction between D_4R and KLHL12 and ubiquitination of D_4R was verified. Additionally, we have also examined if KLHL12 has an influence on p44/42 MAPK phosphorylation, the signaling pathway which is often mediated by β-arrestins, but no significant effect was observed.

2. Materials and Methods

2.1. Plasmids and antibodies

HA $D_{4.0}R$, HA $D_{4.2}R$, HA $D_{4.4}R$, HA $D_{4.7}R$, HA $D_{4.2\ \Delta\Delta}R$, HA Cullin3, Etag KLHL12, Etag KLHL12ΔKelch (aa 1–280), Etag KLHL12ΔBTB (aa 118–568), Flag KLHL12 were described before [32]. The coding sequence of BTB domain of KLHL12 was amplified from the pOTB7-KLHL12 Vector (I.M.A.G.E. Clone ID: IRAL p962K062) by PCR using primers anchored with *NotI* and *XhoI* recognition sequences. The *NotI-XhoI* fragment was cloned into the *NotI-XhoI* sites of the Etag pCAGGS/A20 vector to

generate Etag BTB (aa 1–134). Details concerning primers used and cloning strategy are available upon request. The Etag Kelch (aa 277–568) construct was obtained by digesting Etag KLHL12 with *AvrII*, treating with Klenow fragment and next digesting with *XhoI* and *DraI*. This sequence was later cloned to the Etag pCAGGS/A20 vector treated with *NotI*, Klenow fragment and *XhoI*, respectively. The integrity of all constructed KLHL12 vectors was confirmed by sequence analysis.

Flag β-arrestin 1, Flag β-arrestin 2, β-arrestin1-GFP, β-arrestin2-GFP were described before [36]. Flag β-arrestin 2 NT encoding the *N*-terminal domain of β-arrestin 2 (aa 1–320) and Flag β-arrestin 2 CT encoding the *C*-terminal domain of β-arrestin 2 (aa 284–409), were kind gifts from Prof. Dr. R. Lefkowitz (Duke University, Durham, NC).

Primary antibodies used were mouse monoclonal anti-HA (clone16B12; Covance Research Products), rabbit anti-Etag (Abcam), rabbit anti-HA (GeneTex), rat anti-HA (Roche), mouse monoclonal anti-Flag M2 (Sigma), rabbit anti-phospho-p44/42 MAPK (Cell Signaling), mouse anti-p44/42 MAPK (Cell Signaling), mouse anti-tubulin (Sigma).

Secondary antibodies used were goat anti-rabbit IRDye680 LT, goat anti-mouse IRDye680 LT, goat anti-mouse IRDye680 RD (used for in-cell-western), goat anti-rabbit IRDye800, goat anti-mouse IRDye800, goat anti-rat IRDye680 LT and were all purchased from LI-COR Biosciences. Secondary antibodies used for immunofluorescence were: Alexa Fluor 350 donkey anti-mouse, Alexa Fluor 594 donkey anti-rabbit (Invitrogen).

2.2. Cell culture and transfection

HEK293T cells and MEF cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a controlled environment (37 °C, 98% humidity, 5% CO_2). For co-immunoprecipitation experiments HEK293T cells were transfected using the Polyethylenimine (PEI) method as described before [35]. A total amount of 10 μg of DNA was used for transfection of cells in one 10-cm dish.

For immunofluorescence experiments HEK293T cells were transfected using calcium phosphate method [35].

HEK293S cells stably expressing HA $D_{4.2}R$ or HA $D_{4.0}R$ were grown in DMEM/F12 (Gibco, Invitrogen) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 0.5 mg/ml G418 (Geneticin, Gibco) in a controlled environment (37 °C, 98% humidity, 5% CO2). The PEI method was also used for transient transfection.

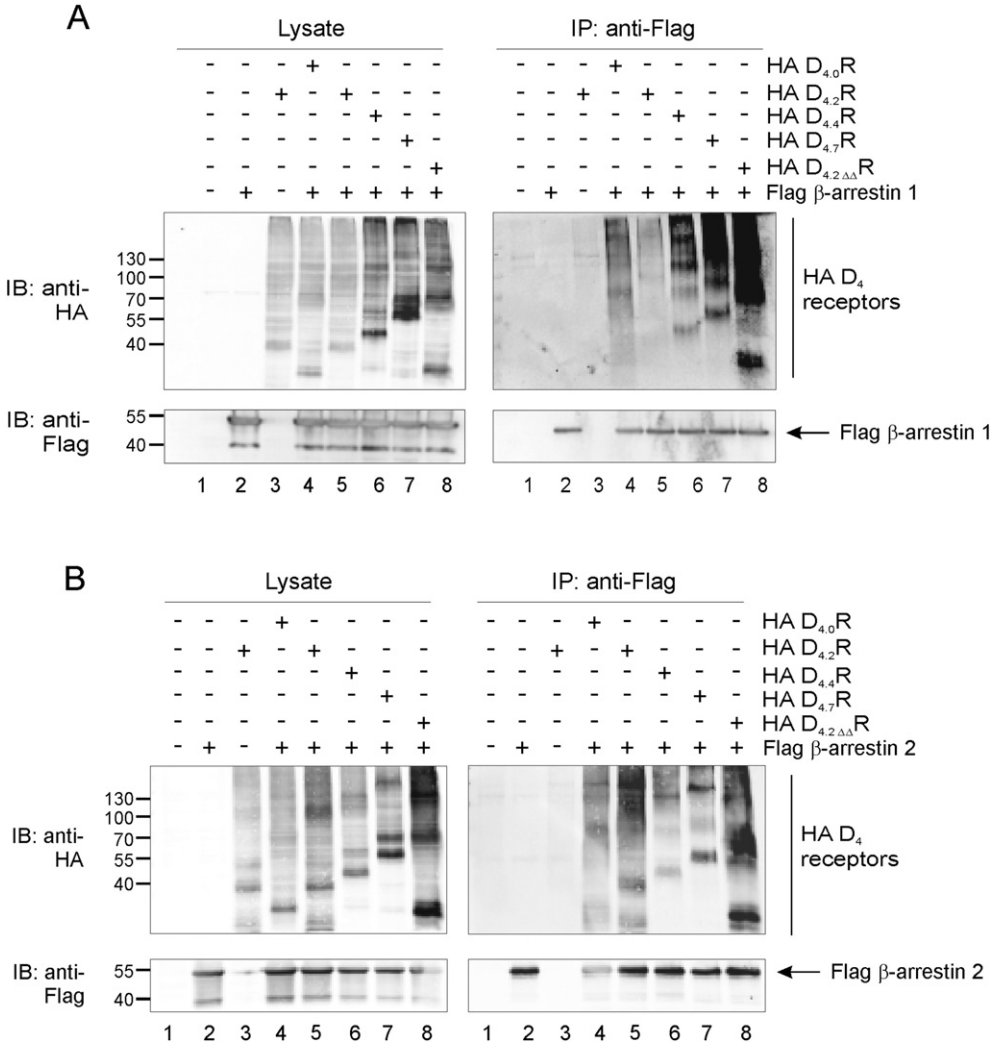

Fig. 1. β-arrestin 1 and β-arrestin 2 interact with all common D_4R polymorphic variants HEK293T cells were transiently transfected as indicated. 48 h post-transfection, cells were harvested and lysed. Part of the lysate was used for IB to verify expression of HA-tagged receptors and Flag β-arrestin 1 (A) or Flag β-arrestin 2 (B). The remainder of the lysate was subjected to IP with anti-Flag. Specific purification of both β-arrestins after IP was confirmed upon IB with anti-Flag. Interaction of D_4R variants with β-arrestins was verified by IB with anti-HA. Data shown are representative of four independent experiments.

MEF cells were transfected using Lipofectamine® 2000 (Invitrogen) (3μl/μg DNA) according to the manufacturer's instruction with 25 μg of total DNA used for transfection of cells in one 10-cm dish.

2.3. Co-immunoprecipitation (co-IP)

Forty eight hours after transfection cells were washed twice with cold phosphate-buffered saline (PBS), harvested and the cell pellet was frozen at −70 °C for at least 1 h before lysis. Cell lysates were subjected to immunoblot analysis, or to immunoprecipitation (IP) followed by immunoblotting (IB), as described before [37].

2.4. Ubiquitination assay – sequential double immunoprecipitation

Ubiquitination assay was described before [38]. Briefly, cells were lysed in radioimmunoprecipitation buffer (RIPA) supplemented additionally with the inhibitor of deubiquitinating enzymes, *N*-ethylmaleimide (NEM). For the first IP, 2 μg of anti-HA (16B12) antibody or 10 μl of HA-beads (Sigma) were used and samples were incubated overnight with antibody and agarose beads. Next, three wash steps with the lysis buffer were performed and proteins bound to the beads (receptor and interacting partners) were eluted under denaturing conditions, and a quarter of the eluate was used to confirm the first IP. The remainder of the eluate, containing denatured proteins, was diluted with lysis buffer and subjected to a second IP to remove receptor-interacting proteins from the first IP and specifically isolate the protein of interest. Finally, the eluates from the second IP were subjected to immunoblotting for the detection of ubiquitinated receptor.

2.5. Single-molecule pull-down assay (SIMPull)

a) Lysate preparation

HEK293T cells were grown in 12-well plates and transfected using the PEI method, first with Flag-$D_{4.4}$R (300 ng) and Venus-KLHL12 (600 ng). 24 h later, the transfection complex was removed and cells were further transfected overnight with mCherry-βarrestin 2 (300 ng).

Cells sequentially transfected with Venus-KLHL12 and mCherry-βarrestin 2, but without Flag-$D_{4.4}$R, served as negative control. Next, cells were lysed with 200 μl/well of lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 2 mM EDTA, 1% NP40, 1.5 mM MgCl2, with protease inhibitors). Lysates were clarified by centrifugation (13,000 g, 15 min, 4 °C).

b) Single-molecule pull-down

SIMPull slides containing chambers were prepared according to the protocol described in [39]. Chambers were first washed twice with T50 buffer (10 mM Tris/HCl pH 7.8; 50 mM NaCl). Next, NeutrAvidin (Thermo) was added and slides were incubated for 5 min. After washing, biotinylated anti-Flag antibody (~ 10 nM) was added (10 min incubation at RT). Subsequently, undiluted cell lysates were added, followed by 20 min incubation at RT. Unbound antibodies and sample were removed from the chamber by washing with T50 buffer twice between successive additions. Proteins immobilized on the slides were visualized by a prism type total internal reflection fluorescence (TIRF) microscope equipped with excitation laser 488nm (Venus) and 561nm (mCherry), and DV2 dichroic 565dcxr dual-view emission filters (520/30nm and 630/50nm). In all cases, mCherry fluorescence was collected first, followed by Venus fluorescence at the same position. Mean spot counts per image and standard deviations were calculated from images taken from 5 to 10 different regions. Single-molecule colocalization between Venus and mCherry was calculated based on pictures from two different channels, the Venus channel (ex. 488 nm, em. 520/30 nm) and the mCherry channel (ex. 561 nm, em. 630/50 nm), made at exactly the same imaging area using a method described previously [40]. The mCherry and Venus molecules within a 2-pixel (~300 nm) distance were considered as colocalized. The number of molecules where colocalization occurred divided by the total number of mCherry molecules was calculated as overlap percentage.

2.6. Immunofluorescence microscopy

HEK293 cells were seeded in wells with coverslips and transfected using the calcium phosphate method in a 5:5:1 ratio of the expression plasmids for Flag KLHL12:HA receptor:β-arrestin1/2-GFP. Immunocytochemistry was performed as described previously [36].

2.7. Detection of p44/42 mitogen-activated protein kinase (MAPK) phosphorylation by in cell western

HEK293S cells stably expressing HA $D_{4.2}$R or HA $D_{4.0}$R were grown and transiently transfected on 10-cm dish using PEI method. 6 h post-transfection cells were reseeded to poly-*D*-lysine coated 96-well plates. 24 h later, medium on the cells was replaced with serum free medium with or without 100 ng/ml of pertussis toxin (PTX). After 18 h of incubation, cells were treated with 100 nM PD 168077 for 1 to 90 min at 37 °C. Incubation was stopped by removing the culture medium, followed by addition of fixing solution (3.7% formaldehyde in PBS) for 20 min at room temperature (RT). Next, cells were permeabilized by washing 4 times for 5 min with Triton washing solution (0.1% Triton X-100 in PBS). Subsequently, cells were blocked with a blocking buffer (LI-COR Biosciences) for 90 min and finally, cells were incubated overnight at 4 °C with two primary antibodies: rabbit anti-phospho-p44/42 MAPK and mouse anti-p44/42 MAPK diluted in the blocking buffer. The next

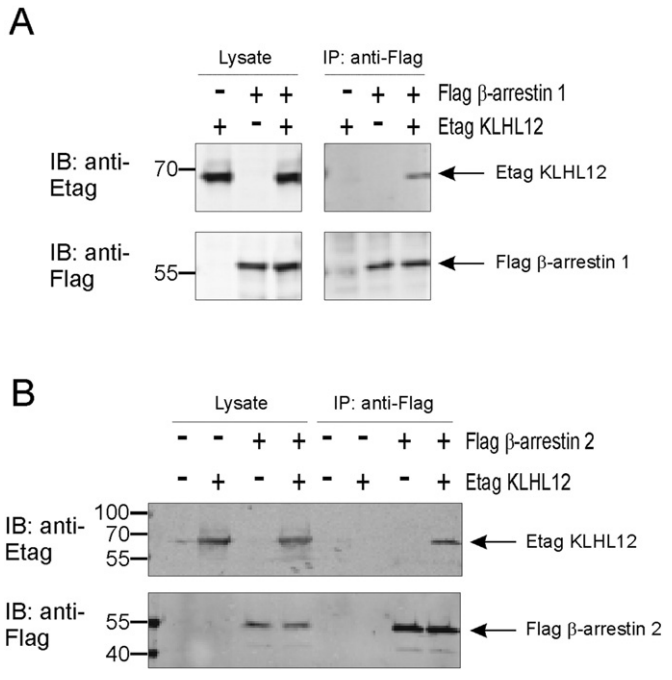

Fig. 2. KLHL12 interacts with β-arrestin 1 and β-arrestin 2 HEK293T cells transiently transfected as indicated were lysed 48 h post-transfection. 5% of the lysate was used for IB to verify expression of Etag KLHL12 and Flag β-arrestin 1 (A) or Flag β-arrestin 2 (B). The remainder of the lysate was used for IP with anti-Flag. Specific purification of β-arrestins was confirmed upon IB with anti-Flag, whereas co-immunoprecipitation of KLHL12 was revealed upon IB with anti-Etag. The results shown are representative of three independent experiments.

day, the plate was washed 4 times for 5 min at RT with Tween washing solution (0.1% Tween-20 in PBS) and incubated with fluorescently labeled secondary antibodies: goat anti-rabbit IRDye800 and goat anti-mouse IRDye680 RD for 1 h at RT. After final washing with Tween washing solution, the fluorescent signal was detected with the Odyssey Infrared Imaging system. In the analysis, background values of the secondary antibody are subtracted and the phospho-p44/42 MAPK signal is normalized against the total p44/42 MAPK signal.

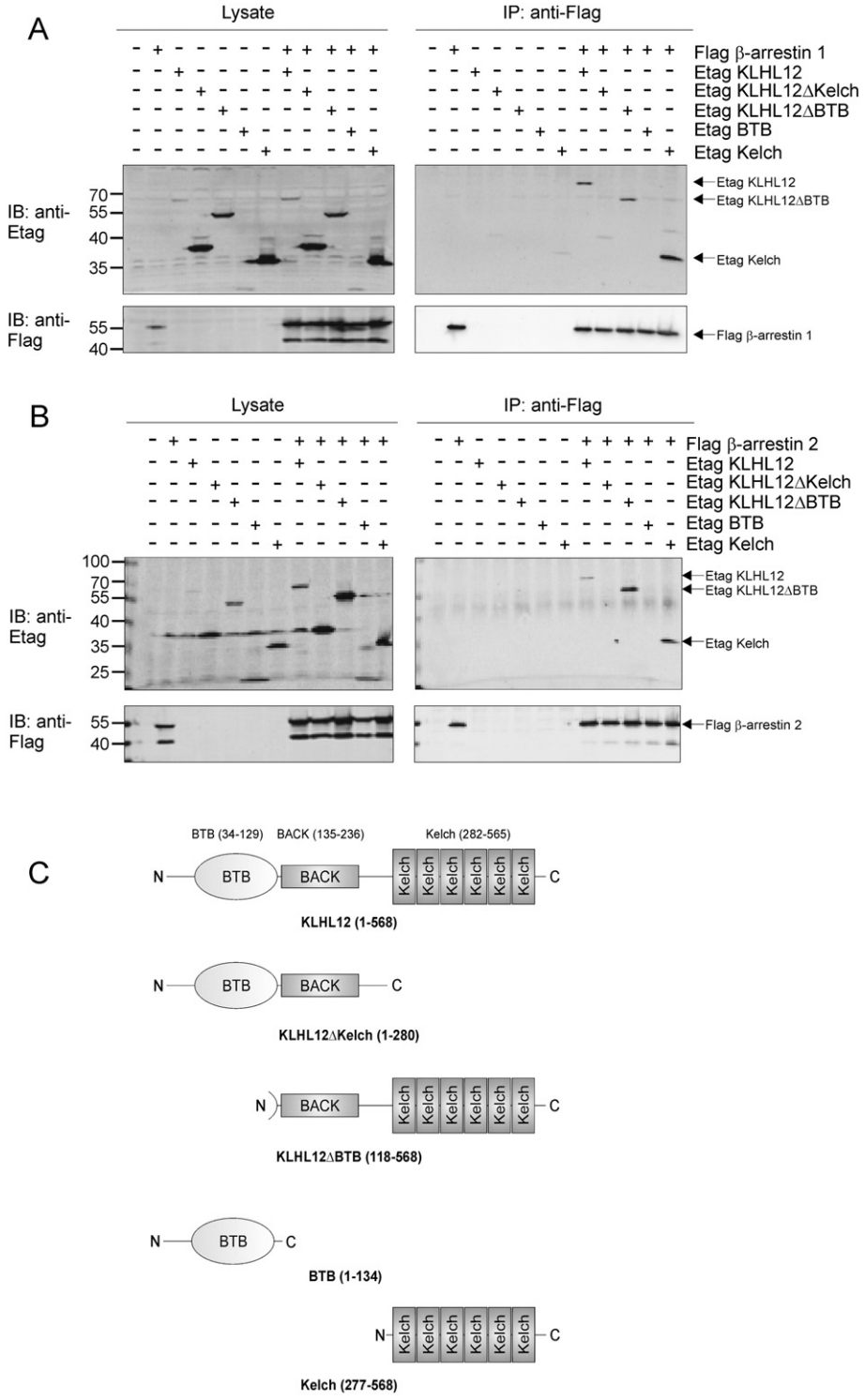

Fig. 3. Kelch repeats of KLHL12 are involved in the interaction with β-arrestins HEK 293T cells were transiently transfected as indicated. 48 h post-transfection, cells were harvested and lysed. Part of the lysate was used for IB to verify expression of full length Etag KLHL12, KLHL12 deletion mutants and Flag β-arrestin 1 (A) or Flag β-arrestin 2 (B). The remainder of the lysate was subjected to IP with anti-Flag. Specific purification of both β-arrestins after IP was confirmed upon IB with anti-Flag. Interaction of KLHL12 WT or deletion mutants with β-arrestins was verified by IB with anti-Etag. The results shown are representative of three (β-arrestin 2) and two (β-arrestin 1) independent experiments. C) Schematic illustration of the wild type KLHL12 protein and deletion mutants used in this study. The depicted sequences were fused to an *N*-terminal Etag.

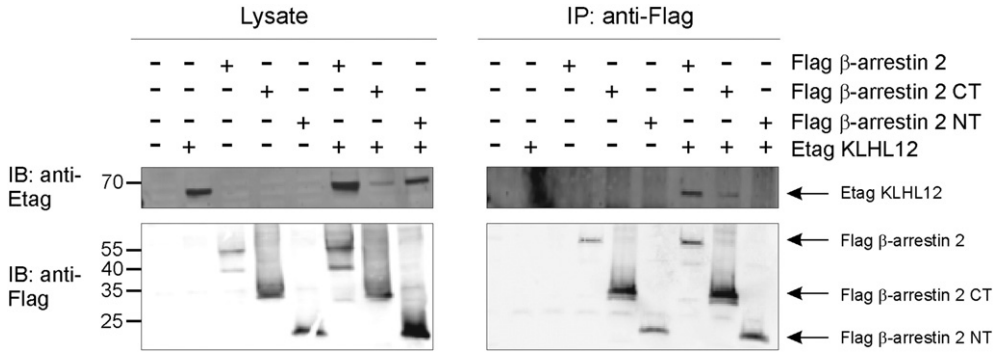

Fig. 4. KLHL12 interacts with the *C*-terminal domain of β-arrestin 2 48 h post-transfection HEK293T cells were harvested and lysed. Part of the lysate was used for IB to verify expression of Etag KLHL12 and Flag-tagged full length β-arrestin 2, β-arrestin 2 *C*- (CT) or *N*-terminal (NT) deletion mutants. The remainder of the lysate was subjected to IP with anti-Flag. Specific purification of β-arrestins after IP was confirmed upon IB with anti-Flag. Interaction of KLHL12 with β-arrestin 2 WT and deletion mutants was verified by IB with anti-Etag. Data shown are representative of three independent experiments.

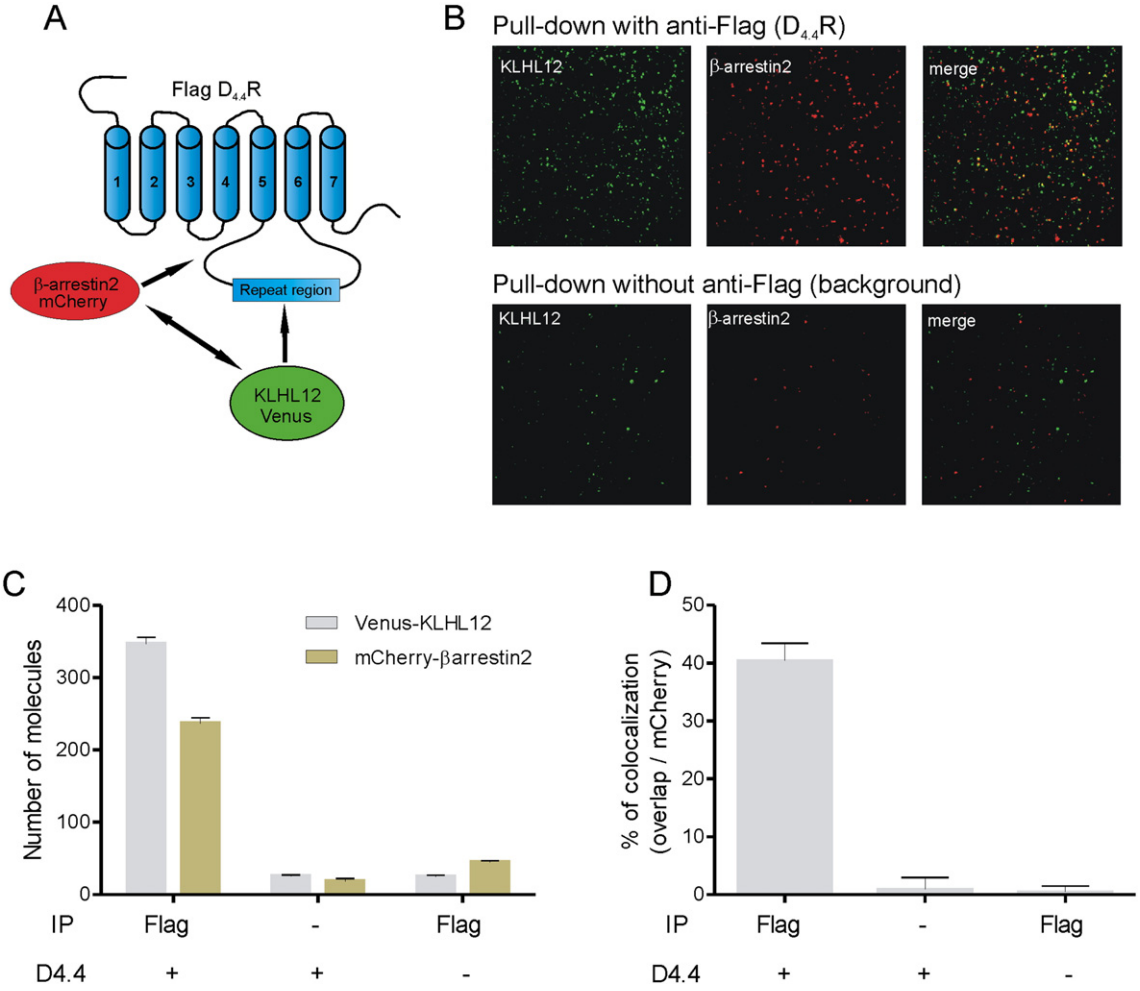

Fig. 5. D_4R, KLHL12 and β-arrestin 2 form a trimeric complex HEK293T cells expressing Flag $D_{4.4}R$, KLHL12-Venus and β-arrestin 2-mCherry (specific pull-down) or KLHL12-Venus and β-arrestin 2-mCherry (background) were lysed and the lysates were applied to the microscope slide containing chambers coated with biotinylated anti-Flag antibody or without coating (background). Slides were imaged with the single-molecule prism-type TIRF microscope to verify co-precipitation of β-arrestin 2-mCherry and KLHL12-Venus. A) Schematic representation of the protein complex investigated in this study. B) Example of single triple-complex image. The upper row shows specific pull-down of KLHL12-Venus (green) and β-arrestin 2-mCherry (red) where lysate was applied to a chamber coated with biotinylated anti-Flag antibody. The lower row shows non-specific binding (background) of KLHL12-Venus and β-arrestin 2-mCherry to the slide which was not coated with the antibody. In the merged picture the colocalization between the two molecules is visible as yellow puncta. C) Mean spot counts per image and standard deviations were calculated from images taken from 5 to 10 different regions of the imaging surface. D) Single-molecule colocalization between Venus and mCherry was performed using a method described previously [40], the mCherry and Venus molecules within a 2-pixel (~300 nm) distance were considered as colocalized. The number of molecules where colocalization occurred divided by the total number of mCherry molecules was calculated as overlap percentage. The results shown are representative of three independent experiments.

3. Results and Discussion

3.1. β-arrestin 1 and β-arrestin 2 constitutively interact with all common D_4R variants

In our previous study [36] it was shown that the $D_{4.2}R$ interacts with β-arrestin 2 and that stimulation of the receptor with its agonist, dopamine, has no influence on the association between D_4R and β-arrestin 2.

As mentioned before, the D_4R has an important polymorphism in its IC3 where a 16 amino acid sequence is repeated two to eleven times giving rise to different polymorphic variants. Although many variants exist, the $D_{4.4}R$ (64%), $D_{4.7}R$ (21%), and $D_{4.2}R$ (8%) are the most frequent in the human population [5]. Therefore, the binding of β-arrestin 1 and β-arrestin 2 to all these polymorphic variants was verified by co-IP. Additionally, we have tested possible interaction with $D_{4.0}R$, an artificial receptor variant from which the polymorphic repeats were removed. The results presented in Fig. 1 clearly show that both β-arrestins interact with all tested receptor variants even without stimulation with an agonist. Co-immunoprecipitation of β-arrestins with $D_{4.0}R$, which does not contain the polymorphic repeats in the IC3, suggests that the polymorphism-containing domain is not required for the interaction between D_4R and β-arrestins. Binding of β-arrestins often requires phosphorylation of the intracellular part of the receptor [19,41,42]. We have identified before [36], by mass spectrometry, two serines: Ser^{239} and Ser^{245}, which are phosphorylated in the D_4R and their phosphorylation does not depend on agonist stimulation of the receptor. To verify if these two serines play a role in β-arrestin binding, the interaction between both β-arrestins and the $D_{4.2\ \Delta\Delta}R$ mutant was tested. In this mutant the regions: aa 233–253 (just before the polymorphic repeats) and aa 283–307 (just after the polymorphic repeats) are deleted. Although in the $D_{4.2\ \Delta\Delta}R$ both serines are missing the receptor can still interact with both β-arrestins suggesting that phosphorylation of these two residues is not crucial for the interaction between the receptor and β-arrestins. It is worth to mention that previously it was already reported that GRK2 can constitutively regulate D_2R expression and signaling independently of receptor phosphorylation [43]. The suppression of receptor expression was correlated with constitutive association of GRK2 with the receptor complex which did not depend on the phosphorylation of the receptor and was not enhanced by agonist stimulation.

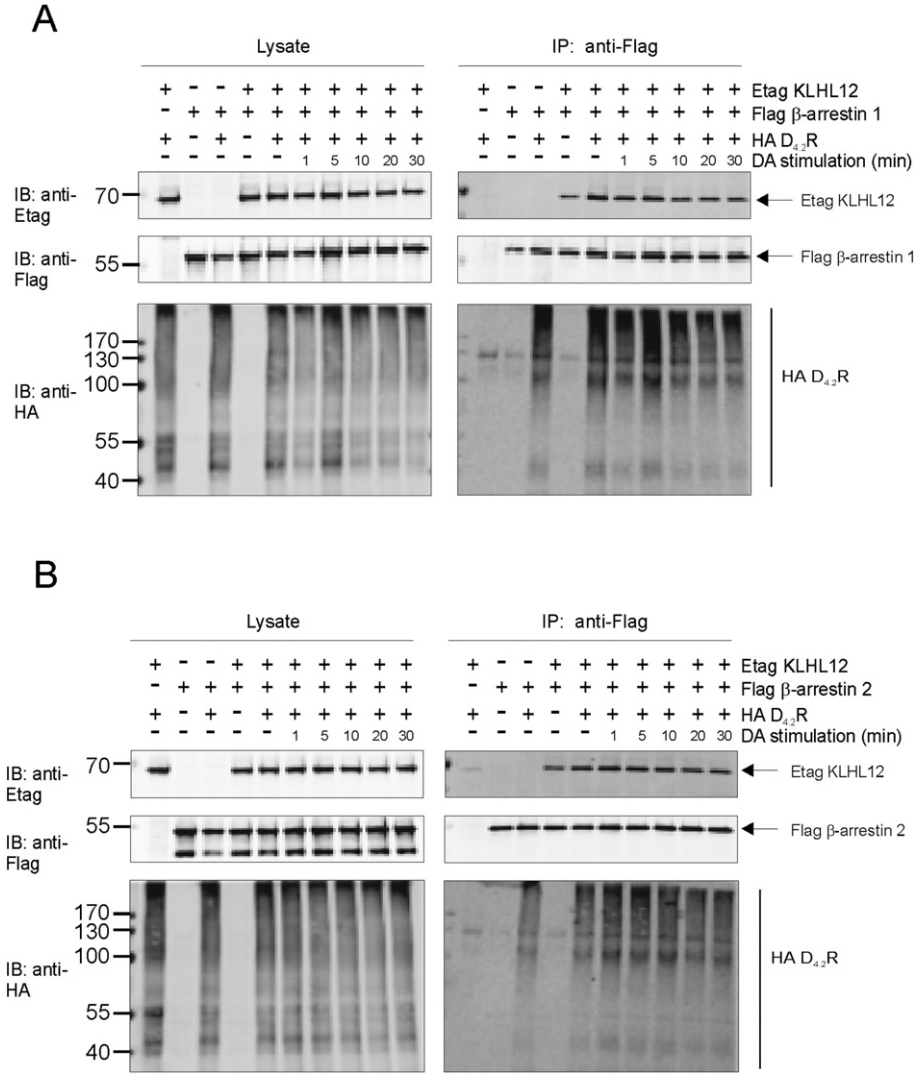

Fig. 6. Dopamine stimulation has no influence on the interaction between β-arrestin 1 or 2 and KLHL12 or D_4R HEK293T cells were transiently transfected as indicated. 48 h post-transfection, cells were treated with 10 μM dopamine (DA) for 1 to 30 min (as indicated) and next, washed twice with cold PBS, harvested and lysed. 5% of the lysate was used for IB to verify expression of Etag KLHL12, HA $D_{4.2}R$ and Flag β-arrestin 1 (A) or Flag β-arrestin 2 (B). The remainder of the lysate was subjected to IP with anti-Flag. Specific purification of both β-arrestins after IP was confirmed upon IB with anti-Flag. Interaction of KLHL12 with β-arrestins was verified by IB with anti-Etag and co-purification of $D_{4.2}R$ was visualized by IB with anti-HA. The results shown are representative of three (β-arrestin 2) and two (β-arrestin 1) independent experiments.

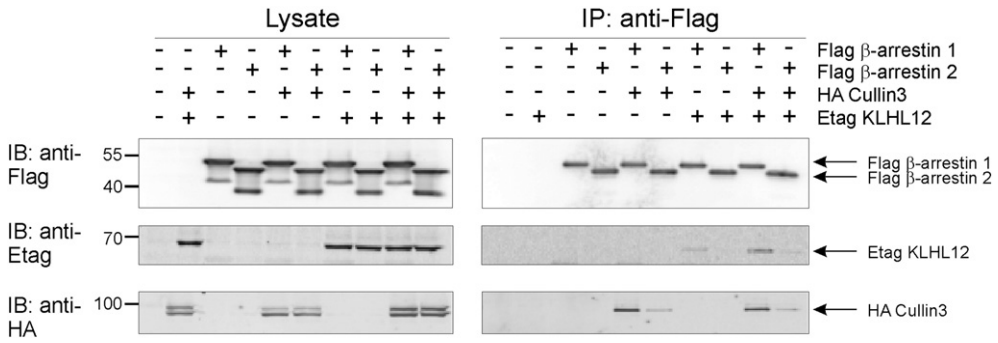

Fig. 7. Cullin3 forms a complex with both β-arrestin 1 and 2 HEK 293T were transiently transfected as indicated. 48 h post-transfection cells were lysed. 5% of the lysate was used to verify expression of Etag KLHL12, HA Cullin3 and Flag-tagged β-arrestin 1 and 2. The remainder of the lysate was subjected to IP with anti-Flag. Specific purification of both β-arrestins after IP was confirmed upon IB with anti-Flag. Interaction of Cullin3 with β-arrestins was verified by IB with anti-HA. Co-purification of KLHL12 was checked by IB with anti-Etag. The results shown are representative of three independent experiments.

3.2. KLHL12 interacts with β-arrestin 1 and β-arrestin 2

KLHL12 is a protein that specifically binds to the polymorphic region in the D_4R and promotes ubiquitination of the receptor [32]. β-arrestins are multifunctional proteins which can function as adaptors for E3 ubiquitin ligases and be involved in regulation of protein ubiquitination [23]. To verify if β-arrestins play a role in ubiquitination of the D_4R, the interaction between KLHL12 and β-arrestin 1 and 2 was verified. Co-IP studies performed in HEK293T cells show a clear interaction between both β-arrestins and KLHL12 (Fig. 2).

To further characterize this novel interaction between KLHL12 and β-arrestins we investigated which regions of KLHL12 are involved in the interaction. Co-IP studies in HEK293T cells using deletion mutants of KLHL12 (Fig. 3C) showed that deletion of the BTB region does not disrupt the interaction with β-arrestin 1 (Fig. 3A) and β-arrestin2 (Fig. 3B), since co-IP of the KLHL12ΔBTB and Kelch mutant with both β-arrestins could be clearly detected. In contrast, by deleting the Kelch domain from KLHL12, interaction with both β-arrestins is completely abolished. These results suggest that the Kelch domain of KLHL12, but not the BTB domain, is sufficient and required for the interaction with β-arrestin 1 and β-arrestin 2. Of interest, it was also demonstrated before that the Kelch repeats are important for the interaction of KLHL12 with D_4R [32].

Similarly, we wanted to pinpoint the region in β-arrestins important for the interaction with KLHL12. β-arrestins have two conserved domains: the *N*-, and *C*-terminal domain. Co-IP studies with β-arrestin 2 *C*- or *N*-terminal deletion mutants revealed that KLHL12 can bind with the *C*-terminal domain of β-arrestin 2 (Fig. 4).

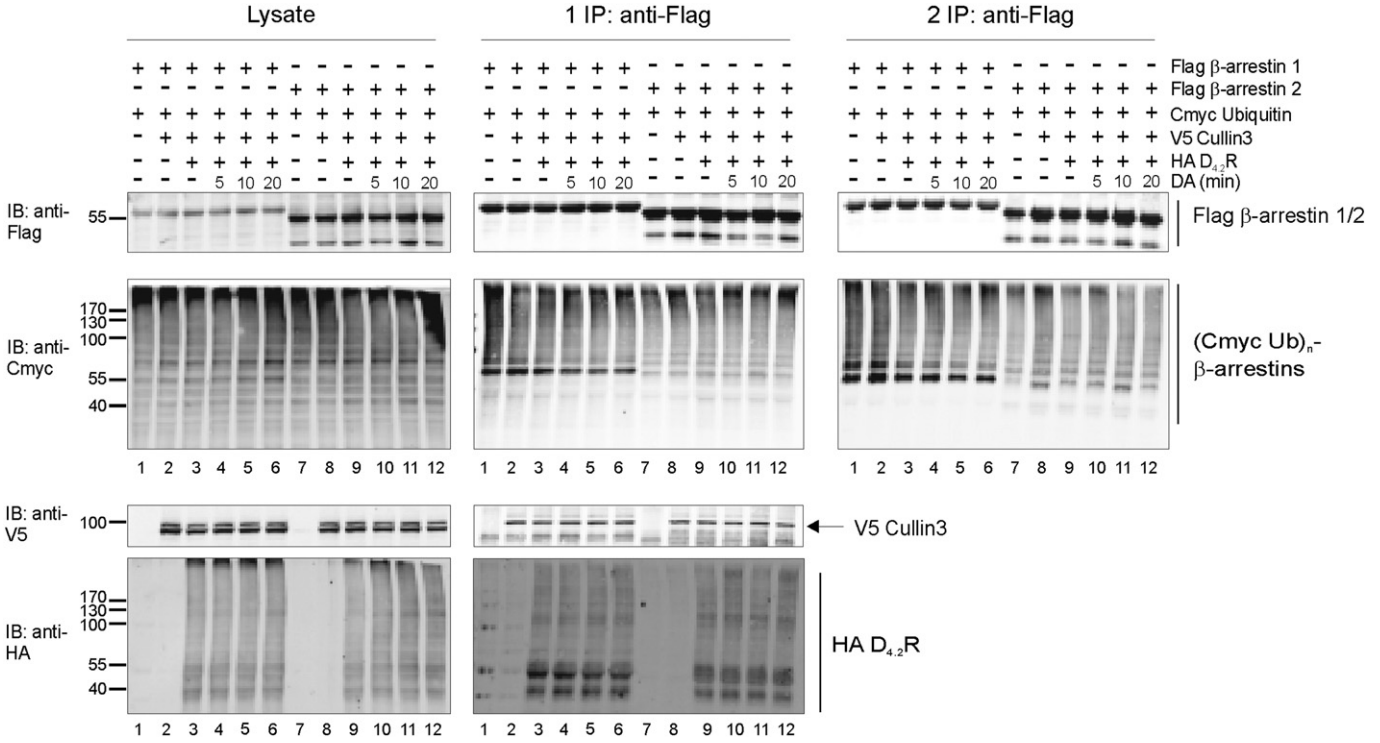

Fig. 8. Dopamine stimulation and Cullin3 overexpression do not influence β-arrestin 1 and 2 ubiquitination HEK293T cells were transfected as indicated. 48 h post-transfection, cells were treated with 10 μM dopamine for 5 to 20 min (as indicated), washed twice with cold PBS, harvested and lysed. 5% of the lysate was used to verify expression of HA $D_{4.2}$R, Flag β-arrestins, V5 Cullin3 and (Cmyc Ub)*n*-proteins. The remainder of the lysate was subjected to double sequential IP with agarose beads coupled to mouse anti-Flag. Specific purification of β-arrestins after first and second IP was verified by IB with anti-Flag DyLight 800, whereas their ubiquitination was revealed upon IB with rabbit anti-Cmyc. Interaction of Cullin3 and $D_{4.2}$R with β-arrestins was examined after the first IP by IB with mouse anti-V5 and rat anti-HA, respectively. The results shown are representative of two independent experiments.

To summarize, these data show that the Kelch repeats of KLHL12 and the C-terminal domain of β-arrestin 2 are important for the interaction between these proteins.

3.3. D_4R, KLHL12 and β-arrestin 2 form a trimeric complex

In order to verify if D_4R, KLHL12 and β-arrestins form a trimeric complex a single-molecule pull-down (SIMPull) analysis was performed. SIMPull is a new technique which allows studying cellular protein complexes with single-complex resolution. In this method macromolecular complexes are pulled down from cell or tissue extracts directly to the imaging surface of a single-molecule fluorescence microscope [40].

The HEK293T cells transiently expressing Flag $D_{4.4}R$, KLHL12-Venus and β-arrestin 2-mCherry were used. After cell lysis, Flag $D_{4.4}R$ together with its interacting partners was pulled down using biotinylated anti-Flag antibody (Fig. 5). The slide was imaged with the single-molecule TIRF microscope and the number of spots (molecules) representing co-immunoprecipitated β-arrestin 2-mCherry and KLHL12-Venus was calculated (Fig. 5C). Non-specific binding of both fluorescently labeled proteins (β-arrestin 2-mCherry and KLHL12-Venus) was verified by applying lysate from the cells expressing β-arrestin 2-mCherry and KLHL12-Venus alone to the chamber or by applying lysates of cells expressing all three proteins to the chamber which was not coated with the biotinylated anti-Flag antibody. As shown in Fig. 5C a very significant pull-down of KLHL12-Venus and β-arrestin 2-mCherry was obtained compared to the background samples. Next, the percentage of colocalization between the spots representing β-arrestin 2-mCherry and KLHL12-Venus was calculated (Fig. 5D).

Our results show that about 40% of β-arrestin 2-mCherry molecules colocalize with KLHL12-Venus suggesting that D_4R, KLHL12 and β-arrestin 2 can exist in a trimeric complex. Although this percentage of colocalization may seem low, it has to be taken into account that GFP has been reported to have ~75% fluorescently active and ~25% fluorescently inactive molecules among total GFP proteins expressed in cells [40,44]. Assuming that mCherry and Venus have similar fluorescently active ratios as GFP, then the maximal colocalization percentage calculated based on fluorescent observation is 60% rather than 100%.

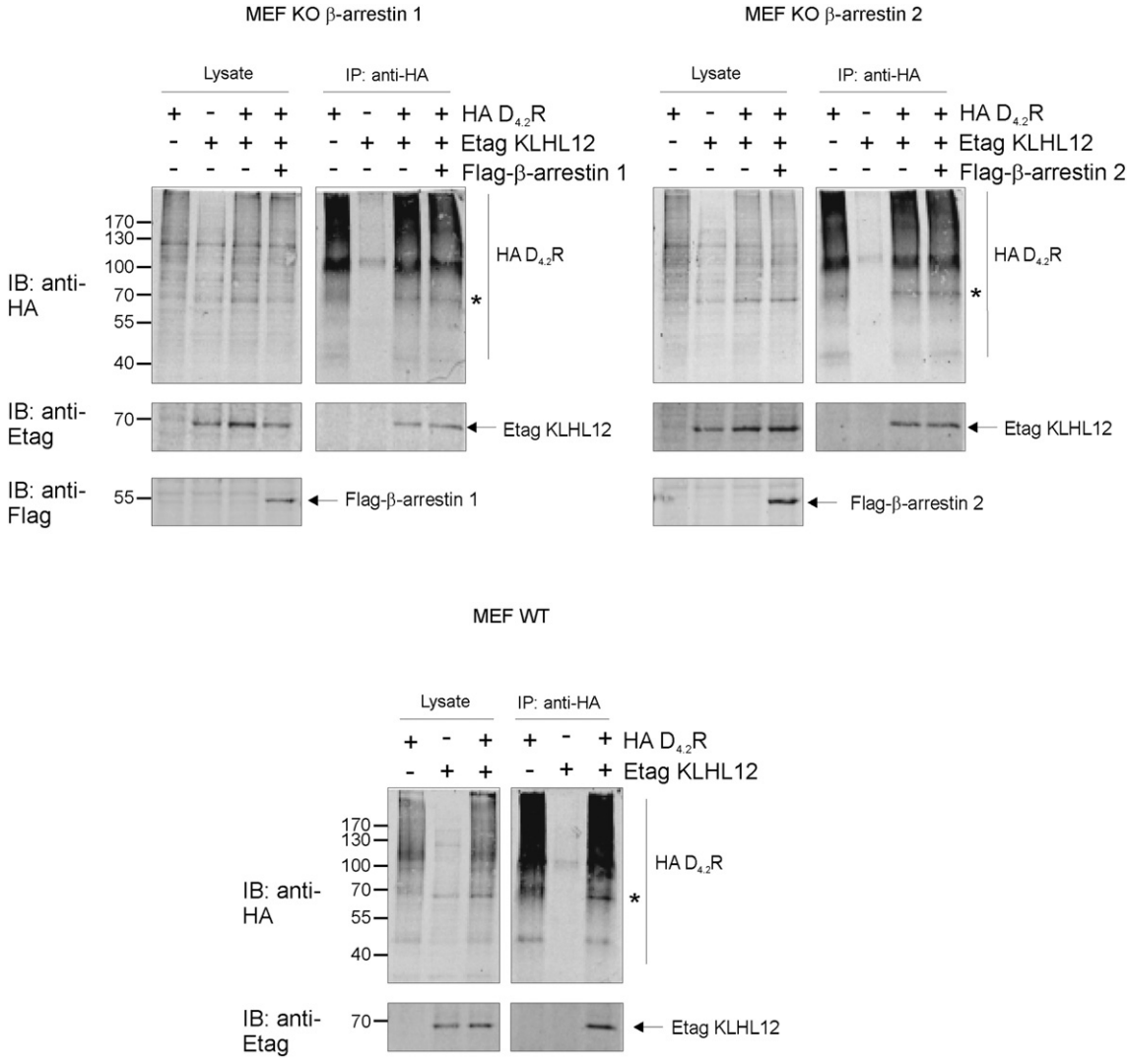

Fig. 9. β-arrestin 1 or β-arrestin 2 are not important for the interaction between D_4R and KLHL12 WT MEF cells and MEF cells with knockout of β-arrestin 1 or β-arrestin 2 were transfected as indicated. 48 h post-transfection cells were harvested and lysed. 5% of the lysate was used to verify expression of HA $D_{4.2}R$, Etag KLHL12 and Flag β-arrestin 1 or Flag β-arrestin 2. The remainder of the lysate was subjected to IP with mouse anti-HA. Specific purification of the receptor after IP was verified by IB with rabbit anti-HA. Interaction of KLHL12 with the receptor was revealed by IB with rabbit anti-Etag. * Indicates Etag KLHL12 visible on the blot as both antibodies used for IB were of rabbit origin. Data shown are representative of three independent experiments.

Therefore, the incomplete colocalization in the assay thus arises mainly from inactive chromophores and unbalanced expression of two proteins in individual cells.

3.4. Dopamine stimulation has no effect on the interaction of KLHL12 with β-arrestin 1/2

β-arrestin 1 and β-arrestin 2, as well as KLHL12, interact constitutively with D_4R [32,36].

The fact that D_4R, KLHL12 and β-arrestin 2 can exist as a trimeric complex encouraged us to further investigate whether KLHL12 and β-arrestins cooperate to regulate D_4R functionality.

β-arrestins are known to regulate ubiquitination of GPCRs through binding with different proteins like E3 ubiquitin ligases or deubiquitinating enzymes and this complicated regulatory mechanism is often initiated by stimulation of the receptor. For example, USP33 is pre-coupled to β_2AR but upon stimulation dissociates from the receptor and associates with β-arrestin 2. Therefore, we were interested if stimulation of the receptor with an agonist has an influence on the interaction between β-arrestins and KLHL12. To answer this question, Flag β-arrestin 1 and Flag β-arrestin 2 were immunoprecipitated from HEK293T cells which were treated with dopamine for various time points. Stimulation of the receptor for 1 to 30 min did not cause any significant changes in the level of co-precipitated Etag KLHL12 (Fig. 6). This observation suggests that stimulation of the receptor does not influence the interaction between KLHL12 and β-arrestins. Moreover, in the same experiment no influence of receptor stimulation on its association with β-arrestins was observed. The latter observation is in agreement with our previous findings in which the resistance of D_4R to agonist induced β-arrestin recruitment, internalization and desensitization was described [36]. In that study we have investigated the interaction between D_4R and β-arrestins using co-immunoprecipitation and also immunofluorescence studies were performed to visualize recruitment of β-arrestins to the plasma membrane and internalization of the receptor. Similar results were also described by Cho and colleagues [45] who investigated β-arrestin recruitment upon dopamine activation of five different dopamine receptors. They observed robust β-arrestin 2 recruitment to the plasma membrane in the cells expressing D_1R, D_2R and D_5R, however, hardly any effect was noticed when D_3R or D_4R were activated. On the other hand several recent studies described recruitment of β-arrestin 2 upon D_4R activation measured using novel assays including the Presto-TANGO assay in which lisurdine was used as D_4R agonist [46]; BRET assay in which additionally GRK 2 was overexpressed to promote receptor phosphorylation and β-arrestin 2 recruitment [47]; and PathHunter assay from DiscoverX which was performed in CHO cells stably expressing D_4R [48]. The contradictory results observed by these groups cannot be easily explained but the use of other cell lines, ligands and overexpression of additional signaling molecules may play an important role.

3.5. Cullin3, member of the E3 ubiquitin ligase complex which ubiquitinates D_4R, interacts also with β-arrestin 1 and β-arrestin 2

KLHL12 functions as an adaptor protein between D_4R and the Cullin3-based E3 ubiquitin ligase and in this way promotes ubiquitination of the receptor. For some GPCRs, e.g. β_2AR [24] ubiquitination of β-arrestins is required for subsequent ubiquitination, internalization and degradation of the receptor. It was shown before by us [35] that overexpression of KLHL12 has no influence on ubiquitination of β-arrestin 2. Now, we were interested if Cullin3 is also present in the complex with KLHL12 and β-arrestins. Fig. 7 shows that Cullin3 is co-immunoprecipitated with β-arrestin 1 and also with β-arrestin 2, although for the latter the signal is significantly weaker. Similarly, more KLHL12 is co-immunoprecipitated with β-arrestin 1 than with β-arrestin 2.

Next, it was examined if Cullin3 has an influence on the ubiquitination of β-arrestins. The ubiquitination assay was performed in HEK293T cells

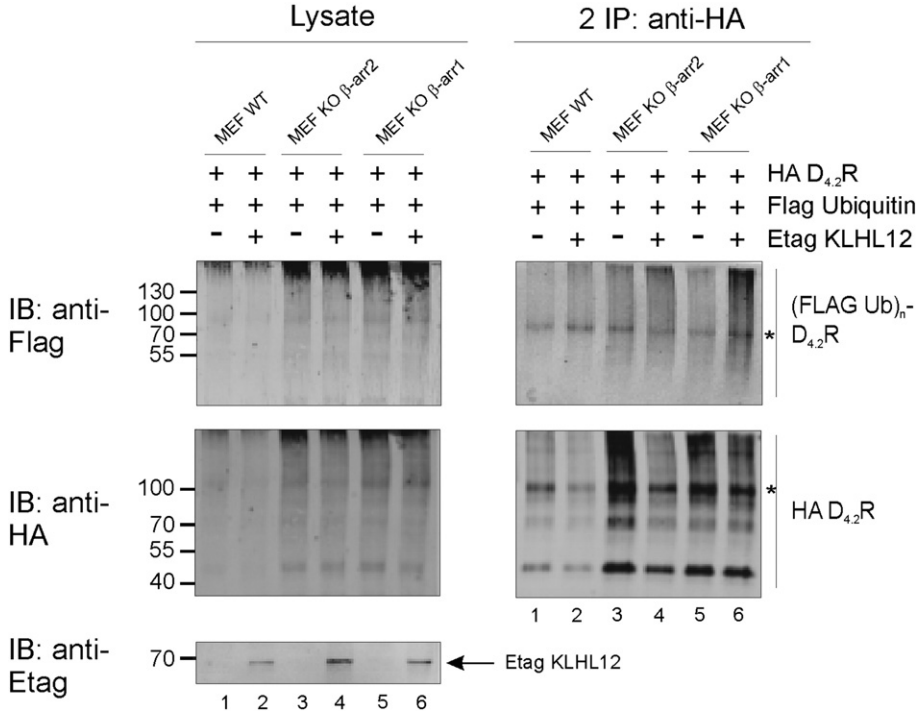

Fig. 10. KLHL12 enhances D_4R ubiquitination in β-arrestin 1 and β-arrestin 2 knockout MEF cells. WT MEF cells and MEF cells with knockout of β-arrestin 1 or β-arrestin 2 were transfected as indicated. 48 h post-transfection cells were harvested and lysed. 5% of the lysate was used to verify expression of HA $D_{4.2}R$, Etag KLHL12 and (Flag Ub)*n*-proteins. The remainder of the lysate was subjected to double sequential IP with agarose beads coupled to mouse anti-HA. Specific purification of the receptor after the second IP was verified by IB with rat anti-HA whereas receptor ubiquitination was revealed upon IB with anti-Flag-HRP. * Association of two heavy chains of mouse anti-HA antibody (each 50 kDa), the primary mouse antibody used for IP is detected by the anti-rat secondary antibody due to cross-reactivity. The results shown are representative of two independent experiments.

to verify β-arrestins ubiquitination status. Additionally, in the same experiment HA D_4R was overexpressed and cells were incubated for different time points with D_4R agonist, dopamine, to test if stimulation of the receptor promotes or inhibits interaction between β-arrestins and Cullin3. The obtained results (Fig. 8) do not reveal any differences in association between both β-arrestins and Cullin3 upon D_4R

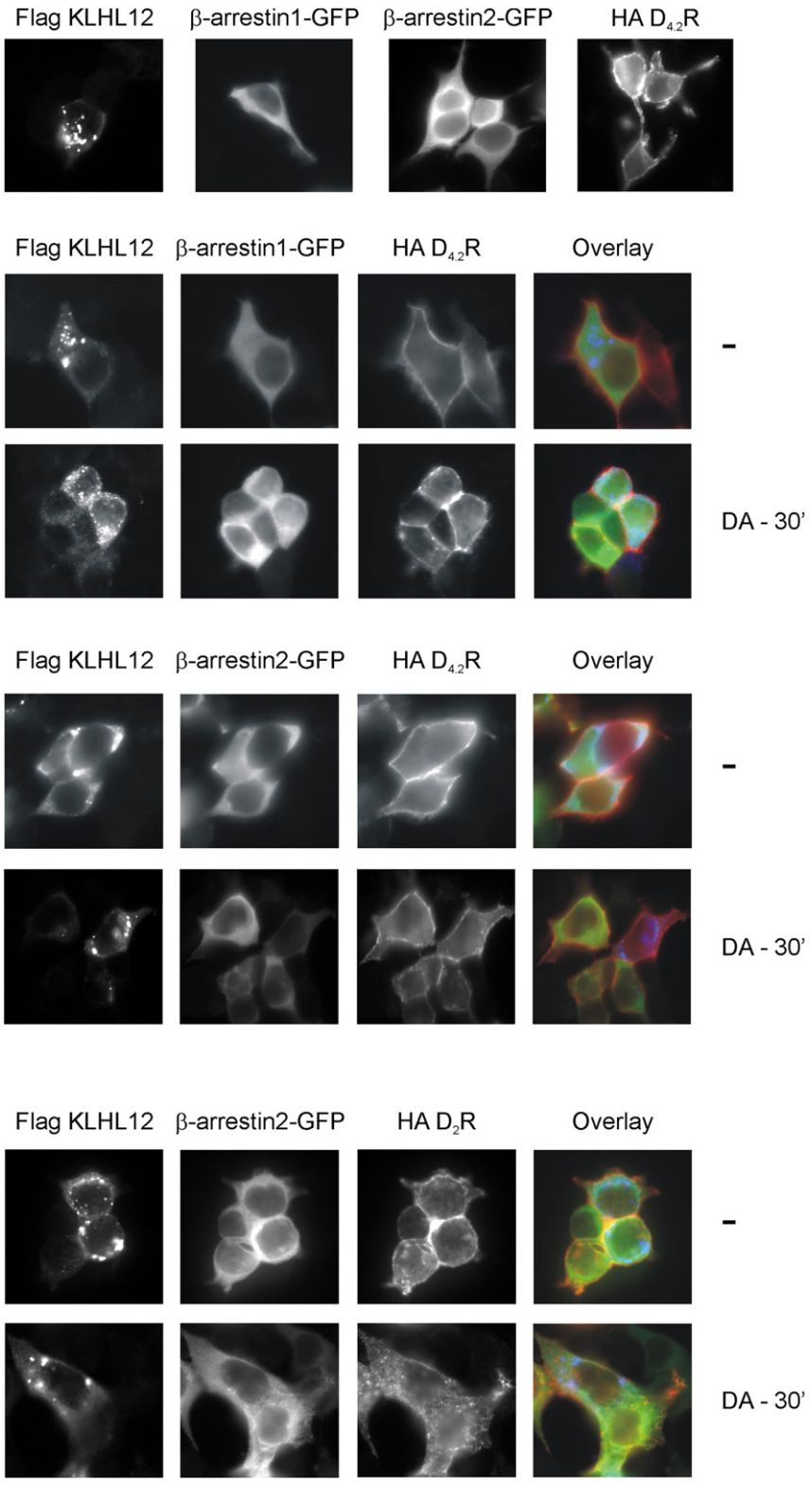

Fig. 11. Stimulation of D_4R with dopamine has no influence on cellular distribution of KLHL12 and β-arrestins HEK293T were seeded in wells with coverslips and transfected with pHA $D_{4.2}R$, or pHA D_2R as a positive control, pFlag KLHL12 and pβ-arrestin1-GFP or pβ-arrestin2-GFP in a 5:5:1 ratio. 48 h post-transfection membrane receptors were labeled with rabbit anti-HA and subsequently, cells were induced with dopamine (DA, 10 μM, 30 min). Next, cells were fixed, permeabilized and Flag KLHL12 was labeled with mouse anti-Flag. Finally, cells were incubated with fluorescent secondary antibodies, anti-mouse Alexa 350 and anti-rabbit Alexa 594. Receptors are visualized in red, KLHL12 in blue and β-arrestins in green in the overlay picture. The results shown are representative of three independent experiments.

stimulation. Moreover, no increase in ubiquitination of β-arrestin 1 and 2 was observed. Taken together, all these data, indicate that activation of D_4R does not promote binding of Cullin3 to β-arrestins, or the ubiquitination of β-arrestins. Overexpression of Cullin3 also does not influence ubiquitination of β-arrestins suggesting that β-arrestins probably do not function as substrates for Cullin3-mediated ubiquitination but rather as adaptor proteins which recruit Cullin3 to other target proteins.

3.6. β-arrestin 1 and β-arrestin 2 are neither required for the interaction between D_4R and KLHL12, nor for KLHL12-mediated ubiquitination of the D_4R

The presence of Cullin3 in the complex further suggests the importance of β-arrestins in ubiquitination of D_4R. To examine this possibility, it was first tested if β-arrestins are necessary for the interaction between D_4R and KLHL12. β-arrestins are ubiquitously expressed proteins and therefore, co-IP experiments were performed in MEF cells with knockout of β-arrestin 1 or β-arrestin 2 described before [49] and the results were compared with those obtained from WT MEF cells (Fig. 9). Etag KLHL12 was clearly co-immunoprecipitated in MEF cells with knockout of β-arrestin 1 or β-arrestin 2 suggesting that β-arrestins do not function as scaffolds between D_4R and KLHL12 and are not critical for the interaction between these two proteins. We cannot exclude the possibility that there is a redundancy between the two β-arrestins, as we were unable to obtain conclusive results from the MEF cells with a double knockout of β-arrestin 1 and β-arrestin 2 due to too low transfection efficiency.

Although β-arrestins seem not to be required for the interaction between D_4R and KLHL12 they can still be involved in the ubiquitination of the receptor. To examine this hypothesis, we have performed a ubiquitination assay in WT MEF cells and MEF with knockout of either β-arrestin 1 or β-arrestin 2 (Fig. 10). In all tested cell lines KLHL12 could still enhance ubiquitination of D_4R which suggests that KLHL12-mediated ubiquitination of D_4R does not depend on the presence or activity of β-arrestins. Of course, as mentioned before, redundancy between the two β-arrestins cannot be ruled out.

3.7. Stimulation of D_4R does not influence the subcellular distribution of KLHL12 and β-arrestins

β-arrestins are common regulators of GPCR internalization and down-regulation. Upon receptor stimulation β-arrestins are recruited to the plasma membrane to form a complex with the receptor and to further assist during its internalization. To examine whether the complex formed between D_4R, β-arrestin 1 or 2 and KLHL12 plays a role in receptor internalization, the cellular distribution of all three proteins with or without stimulation of the receptor was investigated by immunofluorescence studies. HA $D_{4.2}R$, Flag KLHL12 and GFP tagged β-arrestin 1 or 2 were transiently overexpressed in HEK293T cells. 48 h after transfection, the receptor on the plasma membrane was labeled with specific antibodies followed by agonist treatment (10 μM dopamine) for 30 min. Upon cell fixation and permeabilization, Flag KLHL12 was labeled with primary antibody. Finally, the receptor-antibody and KLHL12-antibody complexes were recognized by anti-rabbit-Alexa 594 and anti-mouse-Alexa 350, respectively and visualized together with GFP-tagged β-arrestins by fluorescence microscopy (Fig. 11). As a positive control, D_2R was used. Cell surface expressed D_2R (visible at the plasma membrane in non-stimulated cells) is clearly internalized upon treatment with dopamine (visible as intracellular puncta in bottom panels). This confirms the activity of the agonist and the presence of all required components of the endocytic machinery, necessary to allow a typical agonist-induced response in HEK293T cells. In contrast to D_2R, no significant effect of dopamine stimulation on the distribution of D_4R was observed. We reported before that the D_4R is resistant to agonist-induced receptor internalization and β-arrestin recruitment [36]. Here, we have additionally investigated cellular distribution of KLHL12 and both β-arrestins upon D_4R stimulation but no significant changes in their cell localization were detected. These results are also in agreement with the co-IP studies presented in Fig. 6, showing that dopamine stimulation does not seem to influence β-arrestin 1 and β-arrestin 2 interactions with KLHL12 or D_4R.

The atypical behavior of D_4R upon agonist stimulation can also suggest that the internalization of the D_4R is regulated in another way, independent of β-arrestins. One of the possibilities is endophilin-mediated endocytosis. The interaction between SH3 binding domains in the IC3 of D_4R and endophilin was described recently [50] and the importance of endophilin in the internalization of D_4R was also demonstrated in cells with endophilin knockdown.

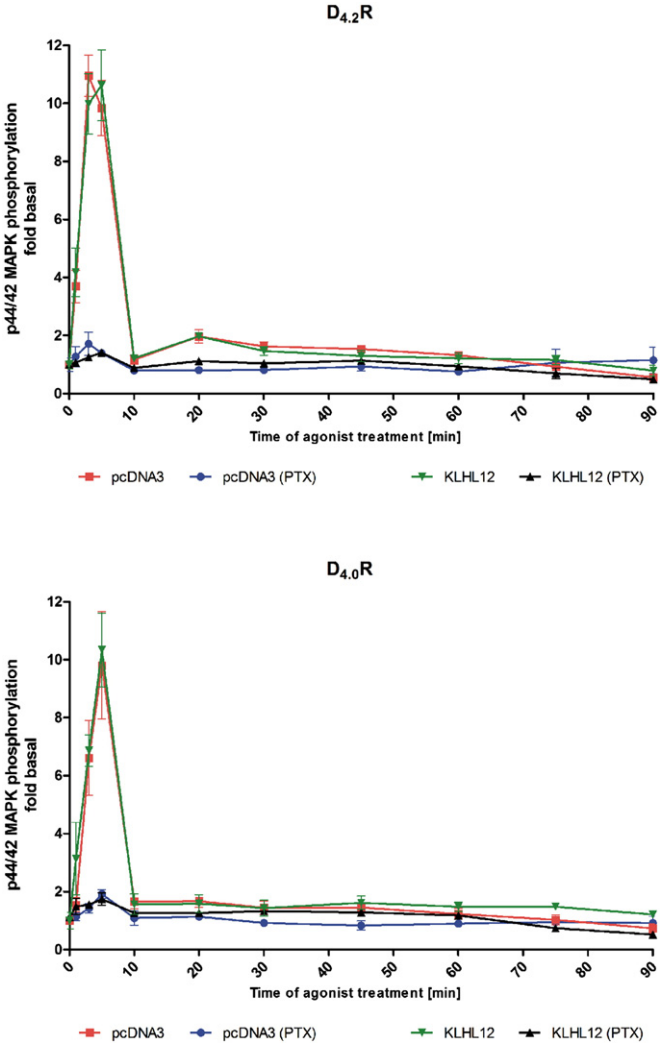

Fig. 12. Pretreatment of cells with pertussis toxin abolishes completely p44/42 MAPK phosphorylation upon D_4R stimulation and KLHL12 does not influence phosphorylation of this kinase significantly HEK293S cells stably expressing HA $D_{4.2}R$ and HA $D_{4.0}R$ were transiently transfected with Etag KLHL12 or empty vector (pcDNA3). After overnight starvation in serum free medium with or without addition of pertussis toxin, cells were incubated with specific D_4R agonist PD 168077 (100 nM) for 1 to 90 min. An in-cell-western assay was performed and phosphorylated p44/42 MAPK was detected with rabbit anti-phospho p44/42 MAPK. Values were normalized against the signal detected with mouse anti-p44/42 MAPK. Graphs show results from three independent experiments performed in triplicate (mean ± SEM).

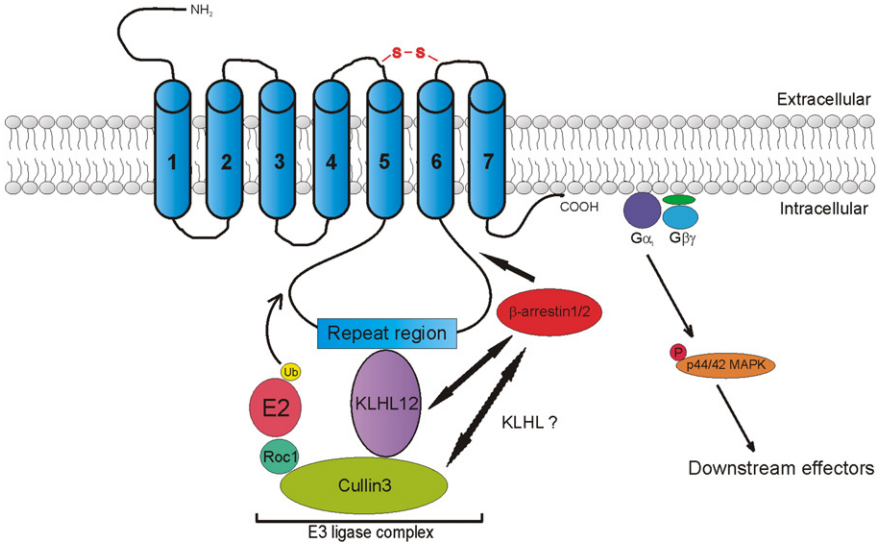

Fig. 13. Schematic representation of the D_4R – KLHL12 - β-arrestin complex KLHL12 interacts through its Kelch repeats with the polymorphic region of D_4R and functions as adaptor protein in a Cullin3-based E3 ligase complex to promote ubiquitination of D_4R. β-arrestin 1 and 2 interact with: D_4R in the region outside the polymorphic repeats; with the Kelch repeats of KLHL12; with Cullin3, but it remains unknown if the KLHL adaptor protein is required for this interaction. Stimulation of D_4R initiates phosphorylation of p44/42 MAPK which is exclusively mediated by $G\alpha_i$ protein.

3.8. p44/42 MAPK phosphorylation upon D_4R stimulation is mediated exclusively by G proteins and KLHL12 does not play a role in this signaling pathway

Signaling of GPCRs is mediated primarily by G proteins and by β-arrestins leading to different outcomes. One of the frequently studied signaling pathways that can be activated by both G proteins and β-arrestins is the p44/42 MAPK pathway. It was reported before that activation of D_4R leads to the phosphorylation of p44/42 MAPK [51]. We wanted to investigate whether this signaling pathway is mediated only by G proteins or if β-arrestins also play a role in this process and whether KLHL12 or ubiquitination of the receptor influence agonist-induced MAPK activation. D_4R is coupled to $G\alpha_i$ proteins which functions can be blocked by pertussis toxin. A series of MAPK phosphorylation assays was performed in HEK293 cells stably expressing HA $D_{4.2}R$ or HA $D_{4.0}R$ which were transiently transfected with Etag KLHL12 or empty vector (pcDNA3). First, cells were pretreated overnight with pertussis toxin or left untreated and next, incubated with D_4R specific agonist, PD 168077 (100 nM), for different time points. β-arrestin-mediated signaling of a GPCR is often slower than G protein-mediated signaling [52] and therefore, incubation with the agonist for up to 90 min was performed. As shown in Fig. 12 for both $D_{4.2}R$ and $D_{4.0}R$ the highest phosphorylation signal is detected after 3–5 min stimulation with the agonist and the phosphorylation is completely abolished in cells in which $G\alpha_i$ proteins are blocked by pertussis toxin. These results suggest that phosphorylation of p44/42 MAPK upon D_4R activation is mediated exclusively by $G\alpha_i$ proteins and that β-arrestins do not seem to be important in this process. Additionally, no significant effect of KLHL12 overexpression was detected in these experiments. Taken together, these observations suggest that the KLHL12-mediated ubiquitination of D_4R or interaction between KLHL12 and β-arrestins do not play a role in the regulation of p44/42 MAPK phosphorylation initiated by D_4R.

4. Conclusions

We have demonstrated before that KLHL12, a BTB-Kelch protein which specifically interacts with the polymorphic repeats in the D_4R and promotes its ubiquitination also binds to β-arrestin 2 [35]. In this study we characterized the interaction between KLHL12, β-arrestins and D_4R (see Fig. 13 for a schematic representation of all interactions described in this study) and questioned its functional relevance.

Our data show that KLHL12 and Cullin3 interact with both β-arrestin 1 and 2, but no influence of these interactions on D_4R ubiquitination, trafficking or p44/42 MAPK phosphorylation was observed. Taking into account that D_4R, KLHL12 and β-arrestin 2 can exist in one protein complex, it is unlikely that these interactions do not play a role in regulation of the D_4R function. Therefore, in future research the possibility of the cooperative action of KLHL12 and β-arrestins in the modulation of other D_4R signaling pathways should be taken into consideration.

Acknowledgements

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