Cellular Signalling 22 (2010) 900–913

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

KLHL12-mediated ubiquitination of the dopamine D4 receptor does not target the receptor for degradation

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ARTICLE INFO

Article history:
Received 8 October 2009
Received in revised form 15 January 2010
Accepted 16 January 2010
Available online 25 January 2010

Keywords:
GPCR
Dopamine
D4 receptor
KLHL12
Ubiquitination
Degradation
β-arrestin2

ABSTRACT

In previous studies, we identified KLHL12 as a novel interaction partner of the dopamine D4 receptor that functions as an adaptor in a Cullin3-based E3 ubiquitin ligase complex to target the receptor for ubiquitination. In this study, we show that KLHL12 promotes poly-ubiquitination of the receptor by performing ubiquitination assays in eukaryotic cells. Furthermore, we demonstrate that KLHL12 not only interacts with both immature, ER-associated and mature, plasma membrane-associated D4 receptors, but also promotes ubiquitination of both receptor subpools. Unexpectedly, however, KLHL12-mediated receptor ubiquitination does not promote proteasomal degradation of newly synthesized receptors through the ER-associated degradation pathway or lysosomal degradation of mature receptors. Moreover, our data reveal that D4 receptors do not undergo agonist-promoted ubiquitination or degradation, in contrast to many other G-protein-coupled receptors (GPCRs) indicating that ubiquitination of GPCRs does not defaultly lead to receptor degradation. Interestingly, KLHL12 does also interact with β-arrestin2 but this has no effect on the ubiquitination or localization of β-arrestin2 nor on the internalization of the D4 receptor.

1. Introduction

Dopamine is a neurotransmitter that plays an important role in various processes in the mammalian brain, such as reward, motivation, learning, memory, movement, and endocrine regulation, by binding to specific receptors on target cells [1,2]. Dopamine receptors belong to the GPCR (G-protein-coupled receptor) superfamily, and can be subdivided into D1-like (D1, D5) receptors that activate adenylyl cyclase through coupling with Gs-proteins, and D2-like (D2, D3, D4) receptors that inhibit adenylyl cyclase via Gi/o-proteins. The D4 receptor contains an important polymorphism in its third intracellular loop, consisting of a variable number of tandem repeats (VNTR), resulting in different possible receptor variants (from D4.2 to D4.11) [3–5]. Interestingly, 7-repeat alleles have been associated with ADHD, demonstrating the importance of this unique polymorphism [6].

Ubiquitination is a post-translational modification that involves the covalent attachment of ubiquitin (76 aa polypeptide) on lysine residues of substrate proteins: first, the ubiquitin-activating enzyme (E1) activates ubiquitin, which is then transferred to the ubiquitin-conjugating enzyme (E2). Finally, ubiquitin is attached to the substrate by (E3) ubiquitin ligases, which are multiprotein complexes that provide specificity towards the substrate [7]. Attachment of a single ubiquitin moiety to a single residue of the substrate results in mono-ubiquitination. When several lysine residues undergo mono-ubiquitination, this results in multi-ubiquitination. Moreover, ubiquitin itself contains seven lysine (K) residues, which can also serve as acceptor sites for additional ubiquitin moieties, resulting in poly-ubiquitin chains. Although all these ubiquitin residues were demonstrated to be able to form poly-ubiquitin chains, K48- and K63-linked chains are the most abundant [8,9]. These different types of ubiquitination can result in different possible outcomes for ubiquitinated substrates. The most frequent type, K48-linked poly-ubiquitination, targets substrate proteins for proteasomal degradation [10,11]. Besides proteasomal degradation, the second major degradative pathway in the cell is based on lysosomes. In contrast to K48-linked poly-ubiquitination, K63-linked poly-ubiquitination does not target substrates for proteasomal degradation, but rather plays a role in processes such as endocytic trafficking, inflammation, protein

Abbreviations: GPCR, G-protein-coupled receptor; BTB, broad-complex, tramtrack, and Bric à Brac; HEK293, Human Embryonic Kidney 293; CHO, Chinese hamster ovary; IP, immunoprecipitation; IB, immunoblotting; E3, ubiquitin-conjugating enzyme; HA, hemagglutinin; siRNA, small interfering RNA; ER, endoplasmic reticulum; ERAD, ER-associated degradation; PM, plasma membrane; BFA, brefeldin A; wt, wild-type; Cul3, Cullin 3; Ub, ubiquitin; MW, molecular weight.

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doi:10.1016/j.cellsig.2010.01.014

translation, activation of certain protein kinases and DNA repair [12,13]. Similarly, mono-ubiquitination does not lead to proteasomal degradation, but functions in membrane trafficking, regulation of the endocytic machinery and lysosomal degradation, viral budding, and DNA repair [14–17]. In all these different cellular processes, ubiquitin serves as a recognition site for specific and selective recruitment of different ubiquitin-binding domains (UBDs). For example, K48-linked poly-ubiquitin chains have a more dense conformation, whereas K63-linked poly-ubiquitin chains are more extended, and hence, both can be recognized by a different specific subset of UBDs [18–20].

Ubiquitin also plays a role in GPCR regulation. A major type of GPCR ubiquitination is independent of agonist stimulation. For some GPCRs, ubiquitin-dependent proteasomal degradation leads to basal turnover of cell surface receptors [21,22]. Additionally, proteasomal degradation through the ER-associated degradation (ERAD) pathway [23] is involved in the regulation of many GPCRs, such as the dopamine D4 receptor [24]. This quality control mechanism promotes newly synthesized but misfolded proteins to undergo ubiquitination in the ER, targeting them for proteasomal degradation [25].

Another major type of GPCR ubiquitination occurs upon agonist induction and can play a role in receptor internalization [26–28]. For the prototypic β2-adrenergic receptor, and for other GPCRs (CXCR4, V2 vasopressin receptor), it was demonstrated that agonist-dependent receptor ubiquitination is not required for receptor internalization, but rather functions to target internalized receptors to lysosomes for degradation [29–31]. However, exceptions exist; the β1-adrenergic receptor, for example, is resistant to agonist-induced ubiquitination and agonist-promoted degradation [32]. Another exception is the delta opioid receptor (δOR), which undergoes agonist-induced lysosomal degradation but does not seem to require ubiquitination for this process [33,34]. A hallmark for GPCR internalization is often the recruitment of β-arrestins [35,36], and agonist-stimulated ubiquitination of GPCRs and of β-arrestins can play a role in receptor internalization/degradation [29,37,38]. Recently, we have shown that the D4 receptor is resistant to agonist-induced receptor phosphorylation, β-arrestin1/2-recruitment, degradation and internalization [39]. Here we show that receptor stimulation neither induces ubiquitination of the receptor nor of β-arrestin2.

In our previous study, we identified the BTB-Kelch protein KLHL12 as a novel D4 receptor-interacting partner that specifically binds to the VNTR polymorphic region of this receptor [40]. Furthermore, we demonstrated that KLHL12 functions as an adaptor in a Cullin3-based E3 ubiquitin ligase complex that specifically promotes ubiquitination of the D4 receptor. In this study, we wanted to characterize and unravel the function of KLHL12-mediated receptor ubiquitination. We provide evidence that KLHL12 interacts with and promotes ubiquitination of both immature, ER-associated and mature, plasma membrane-associated D4 receptors. However, KLHL12-mediated receptor ubiquitination does not lead to receptor degradation, which is highly remarkable and most interesting in view of the current literature assigning a role for GPCR ubiquitination in targeting receptors for degradation.

We further demonstrate that KLHL12 can interact with β-arrestin2, although this has no effect on ubiquitination or localization of β-arrestin2 or on D4 receptor internalization, upon agonist induction.

2. Materials and methods

2.1. Plasmids and antibodies

Plasmids encoding the HA D4.2 receptor, FLAG D4.4 receptor, HA D2 receptor, Etag KLHL12, and FLAG Ub, were described before [39,40]. The ubiquitin constructs coding for wild-type (wt) HA Ub, the single mutants HA K29R, HA K48R and HA K63R, and the triple mutant FLAG Ub K29,48,63R were kind gifts from Dr. Dikič (Goethe Universität, Frankfurt, Germany), and the wt cmyc Ub was obtained from Dr. Kopito (Stanford University, Stanford, CA). β-arrestin2 GFP, HA β2-adrenergic receptor and FLAG β-arrestin2 were kind gifts from Dr. R. Lefkowitz (Duke University, Durham, NC).

The D4.2 receptor contains four lysine (K) residues in its primary structure, namely at sites 229 (in the third intracellular loop before the 'variable number of tandem repeat' polymorphism), 304, 311 (both in the third intracellular after the polymorphism) and 381 (in the C-terminal tail). The HA D4.2 receptor construct was used as a template for the application of single-nucleotide mutations (encoding arginine instead of lysine) using the QuikChange® Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA). The quadruple mutant D4.2 K229,304,311,381R is further denoted as 4 K➔R.

Primary antibodies used were mouse monoclonal anti-HA (clone 16B12; Covance Research Products, Berkeley, CA), mouse monoclonal anti-Etag (Amersham Biosciences, Roosendaal, The Netherlands), rabbit anti-Etag (GenScript), rabbit anti-c-myc (Sigma), rabbit anti-HA (GeneTex), horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-Etag (Amersham Biosciences), mouse monoclonal anti-ubiquitin clone P4D1 (Cell Signaling, Danvers, MA), mouse monoclonal anti-ubiquitin clone FK1 (Biomol, Plymouth Meeting, PA), mouse monoclonal anti-poly-ubiquitin (K63-linkage specific) clone HWA4C4 (Biomol), mouse monoclonal anti-FLAG M2 (Sigma, St. Louis, MO) (immunoprecipitation), HRP-conjugated mouse monoclonal anti-FLAG M2 (Sigma) (immunodetection in HEK293T cells), mouse monoclonal anti-FLAG M1 (Sigma) (immunodetection in stably transfected CHO FLAG D4.4 cells), mouse monoclonal anti-actin (Sigma), and rabbit polyclonal anti-β-catenin (Abcam, Cambridge, MA). Secondary antibodies used were HRP-conjugated anti-mouse and anti-rabbit IgG (Cell Signaling) and Alexa-350-conjugated and Alexa-594-conjugated anti-mouse and anti-rabbit IgG (Invitrogen).

2.2. Chemical products

Brefeldin A (BFA), the lysosomal inhibitor chloroquine, and the agonists dopamine and quinpirole were purchased from Sigma; the proteasomal inhibitor MG-132 and the D4 receptor antagonist L745-870 were from Calbiochem (Merck; Darmstadt, Germany) and Tocris (Bristol, UK), respectively.

2.3. Cell culture and transfection

HEK293T 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). HEK293T cells were transfected using the Polyethylenimine (PEI) method. Therefore, cells were grown in 10 cm dishes until subconfluency. The medium was refreshed with 9 ml DMEM, supplemented with 2% fetal calf serum, 1 h before transfection. A mixture of 475 μl serum-free medium and 25 μl (1 μg/μl) PEI was added dropwise to a solution of 500 μl serum-free medium containing 10 μg DNA. Upon mixing thoroughly and incubation for 10 min at room temperature, the DNA/PEI mixture was added dropwise to the cells. 6 h later, the medium was refreshed with DMEM, supplemented with 10% fetal calf serum. CHO FLAG D4.4 cells were cultured in αMEM (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% CO_2). CHO FLAG D4.4 cells were transfected using Lipofectamine™ (Invitrogen) (4.5 μl/μg DNA), according to the manufacturer's guidelines. The amount of plasmid DNA used for transfection is indicated for each experiment.

2.4. Immunoblot analysis and co-immunoprecipitation assays

After transfection, cells were washed twice with ice-cold phosphate-buffered saline, harvested and the cell pellet was frozen at

−70 °C for at least 1 h before lysis, unless indicated differently. Cell lysates were subjected to immunoblot analysis, or to immunoprecipitation (IP) followed by immunoblotting (IB), as described before [40]. Upon immunoblotting, antibodies were stripped from the membrane by incubation in stripbuffer (2% SDS, 62.5 mM Tris–HCl pH 6.7, 0.7% freshly added β-mercaptoethanol) for 30 min at 65 °C. Following washing and blocking in blocking buffer (Tris-buffered saline, containing 0.1% Tween-20 and 5% non-fat dry milk), the membranes were incubated with a second pair of primary and secondary antibodies to detect a second protein.

2.5. Ubiquitination assays in HEK293T cells

For regular IP of Etag KLHL12, HA D4.2 or FLAG D4.4 receptors, 2 μg of anti-Etag, anti-HA (16B12), or anti-FLAG M2 antibody, respectively, was used and IP was performed as usually, but N-ethylmaleimide (NEM; final concentration of 10 mM) was additionally added to the RIPA lysis buffer. Double (sequential) immunoprecipitation assays were performed as described before [40]. Briefly, a first, regular IP was performed as described above. Upon three wash steps, the 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 rest of the eluates, containing denatured proteins, were 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 proteins (D4 receptor or β-arrestin2). For the specific IP of cell surface-expressed HA D4.2 receptors (membrane IP), 10 cm dishes with cells were incubated with 5 ml serum-free DMEM containing 2 μg of anti-HA (16B12) antibody for 2 h to allow binding of the antibody to HA-tagged receptors. Then, the medium was removed and the cells were washed twice with ice-cold phosphate-buffered saline, harvested, centrifuged for 5 min (4 °C, 900 rpm) and finally lysed in 500 μl RIPA buffer (with NEM). Upon incubation for at least 2 h (4 °C) under continuous rotation, the lysates were cleared by centrifugation for 15 min (4 °C, 3000 rpm). Finally, washed immobilized protein A-beads (20 μl; Pierce) were added to the samples, followed by overnight incubation at 4 °C under continuous rotation. Final steps of washing the beads and elution of the proteins were performed as described above.

2.6. cAMP tests

cAMP was measured using the LANCE cAMP 384 kit (PerkinElmer Life Sciences, Boston, MA). Cells were stimulated for 30 min according to manufacturer's instructions with 10 μM forskolin, 10 μM quinpirole or combinations thereof. cAMP measurements were performed according to manufacturer's instructions.

2.7. Immunofluorescence microscopy

HEK293 cells were seeded in wells with coverslips and transfected using the calcium phosphate method in a 5:5:1 ratio for the expression plasmids for Etag KLHL12:HA receptor:β-arrestin2 GFP. Immunocytochemistry was performed as described previously [39].

3. Results

3.1. KLHL12 interacts with both immature, ER-associated and mature, plasma membrane-associated D4 receptors

In our previous study, we demonstrated that KLHL12 specifically interacts with the D4 receptor in HEK293T cells. In the lysate of these cells, transiently transfected with tagged D4 receptor constructs, two different species of overexpressed D4 receptors can be detected; the upper band (with a molecular weight of ~52–54 kDa for the HA-tagged D4.2 receptor) represents mature, fully processed receptor (e.g. plasma membrane (PM)-associated), whereas the lower band (molecular weight ~46–48 kDa for HA D4.2) represents immature, endoplasmic reticulum (ER)-retained receptor [24,40]. Finally, the receptor-specific high molecular weight smear probably represents D4 receptor species that are modified with distinct post-translational modifications (glycosylation, ubiquitination), receptor oligomers, or higher order aggregates (see further Fig. 2A; blot of lysates). Although we could clearly demonstrate specific co-purification of KLHL12 upon immunoprecipitation of D4 receptors, this assay setup was inconclusive regarding whether KLHL12 interacts with mature or immature receptors [40]. In order to elucidate this matter, we performed alternative (reverse) co-immunoprecipitation studies in HEK293T cells, transiently transfected with HA D4.2 and Etag KLHL12 constructs (Fig. 1). We could clearly detect co-purification of immature receptor (~47 kDa; further depicted as ER-receptor), but also a smaller fraction of mature receptor (~53 kDa; further depicted as PM-receptor) upon specific immunoprecipitation of KLHL12 (lane 3). These observations strongly suggest that KLHL12 is able to interact with both immature and mature D4 receptors, at the ER and at the plasma membrane (or endosomes), respectively.

3.2. KLHL12 promotes ubiquitination of both ER- and plasma membrane-associated D4 receptors

The observation that KLHL12 could interact with both ER- and plasma membrane-associated D4 receptors, raised the question whether KLHL12 could also target both receptor subpools for ubiquitination by the Cul3-based E3 ubiquitin ligase complex.

To test whether ER-associated receptors show basal ubiquitination, and whether KLHL12 could promote ubiquitination of these receptors, ubiquitination assays were performed upon prolonged treatment of the cells with brefeldin A (BFA). BFA is a fungal metabolite which disrupts the structure and function of the Golgi-apparatus. Thereby, BFA impedes protein transport from the ER to the Golgi-complex and thus prevents the transport of newly synthesized membrane receptors towards the plasma membrane, though it has no significant effect on protein synthesis or endocytosis [41]. As the half-life of mature D4 receptors is relatively long (estimated at ~4–8 h; see further [24]), prolonged treatment of D4

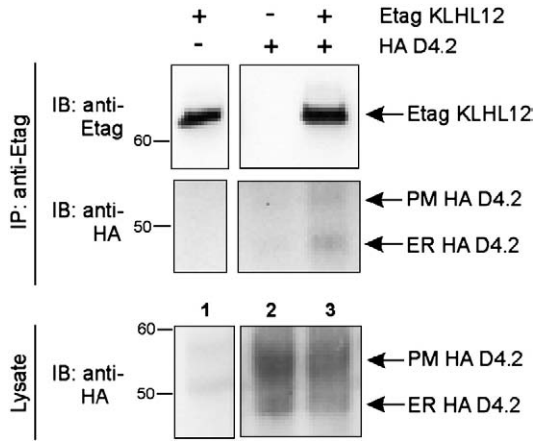

Fig. 1. KLHL12 interacts with both mature, plasma membrane-expressed and immature, ER-associated D4 receptors in HEK293T cells. HEK293T cells were transiently transfected with plasmids encoding HA D4.2 receptor (5.5 μg) and/or Etag KLHL12 (5.5 μg), as indicated. 48 h post-transfection, cells were lysed and 5% of the lysates were subjected to SDS-PAGE and subsequent immunoblotting (IB) with anti-HA to confirm expression of both mature (PM) and immature (ER) HA D4.2 receptors. The rest of the lysates were subjected to immunoprecipitation (IP) with anti-Etag. The D4-KLHL12 association was examined through subsequent IB with anti-HA. IP of KLHL12 was confirmed upon IB with anti-Etag.

receptor-expressing cells with BFA is required to sufficiently decrease the mature, plasma membrane-associated D4 receptor subpool in these cells. In our experiments, HEK293T cells, transiently transfected with the HA D4.2 receptor construct, were treated for 7 or 22 h with BFA prior to cell lysis (Fig. 2A). It was observed that treatment with BFA for 7 h resulted in a significant decrease of mature receptor levels and an increase in ER-associated receptor levels (compare lane 3 to 2 in the *bottom panel* of Fig. 2A). Prolonged treatment with BFA for 22 h resulted in an almost complete loss of mature receptors, whereas the levels of ER-associated receptors remained high (lane 4). Consequently, immunoprecipitation with anti-HA in these BFA-treated cells resulted in selective purification of ER-associated receptors (*upper panel* of Fig. 2A). Subsequent probing for ubiquitin signals demonstrated that ubiquitination levels remain significant upon treatment of the cells with BFA (*middle panel* of Fig. 2A). These observations therefore strongly suggest that ER-associated D4 receptors undergo basal ubiquitination.

To test whether KLHL12 promotes ubiquitination of these ER-associated D4 receptors, similar ubiquitination assays where performed in HEK293T, transiently co-transfected with HA D4.2, Etag KLHL12, and FLAG Ub (the latter to enhance ubiquitin signals) (Fig. 2B). Immunoblotting with anti-HA confirmed that almost exclusively immature, ER-associated D4 receptors were purified and subsequent probing for ubiquitin signals revealed that co-expression of KLHL12 significantly promoted ubiquitination of these ER-associated D4 receptor (compare lane 4 to 3). These observations indicate that KLHL12 promotes ubiquitination of ER-associated D4 receptors.

Similarly, we wanted to explore whether mature, cell surface-expressed D4 receptors undergo basal ubiquitination, and whether KLHL12 could also promote ubiquitination of this receptor subpool. To specifically purify the cell surface-expressed D4 receptor subpool, HEK293T cells, transiently transfected with HA-tagged D4 receptor constructs, were pre-incubated with anti-HA antibody targeting the N-terminal HA-tag of the D4 receptor. Subsequent lysis of the cells and immunoprecipitation selectively purified cell surface-expressed (PM) HA D4.2 receptors (~53 kDa), as demonstrated in Fig. 3 (*left part*). Although only minor basal ubiquitination of the PM-associated receptor was observed (lane 3), a significant increase was visible upon co-expression of KLHL12 (lane 4). Notably, pre-treatment of the cells with dopamine did not alter this pattern (lanes 5 and 6), which will also be discussed later. To exclude the possibility that the observed ubiquitination signals represent ubiquitinated proteins associated

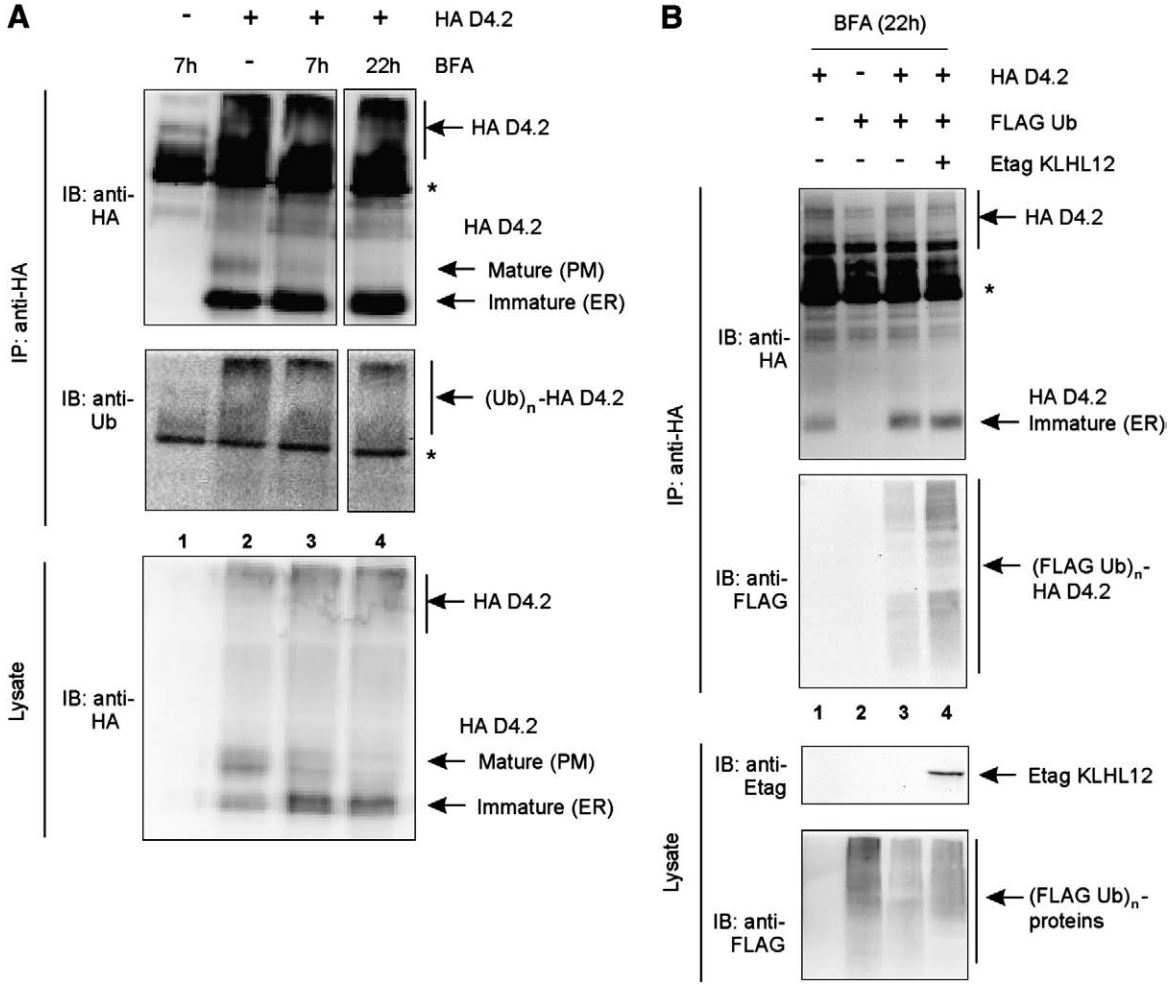

Fig. 2. KLHL12 promotes ubiquitination of ER-associated D4 receptors. (A) HEK293T cells were transiently transfected with either vehicle (pcDNA3; lane 1), or HA D4.2 receptor (5.5 μg). 24 h post-transfection, cells were incubated with a medium containing 5 μg/ml BFA for 7 or 22 h, or left untreated. 5% of the lysates were used to visualize the levels of the mature (PM) and immature (ER) HA D4.2 receptors. The rest of the lysates were subjected to IP with anti-HA. IP of the ER- and/or PM-associated receptors was confirmed upon IB with anti-HA. Finally, ubiquitination of D4 receptors was demonstrated upon IB with anti-ubiquitin (clone P4D1). (B) HEK293T cells, seeded in 10 cm dishes, were transiently transfected as indicated (4 μg/plasmid). 24 h post-transfection, cells were trypsinized and seeded in 6 cm dishes (10^6 cells/dish). Another 24 h later, cells were incubated with a medium containing 5 μg/ml BFA for 22 h. Finally, the cells were harvested, lysed, and 5% of the lysates were used for IB to confirm expression of Etag KLHL12 and $(FLAG\ Ub)_n$-proteins, respectively. The rest of the lysates were subjected to IP with anti-HA. Subsequent IB with anti-HA confirmed specific purification of immature, ER-associated D4.2 receptors in BFA-treated samples. Finally, IB with anti-FLAG revealed ubiquitination of the ER-associated D4 receptors. * Signal representing association of two heavy chains (2×50 kDa) of the anti-HA antibody.

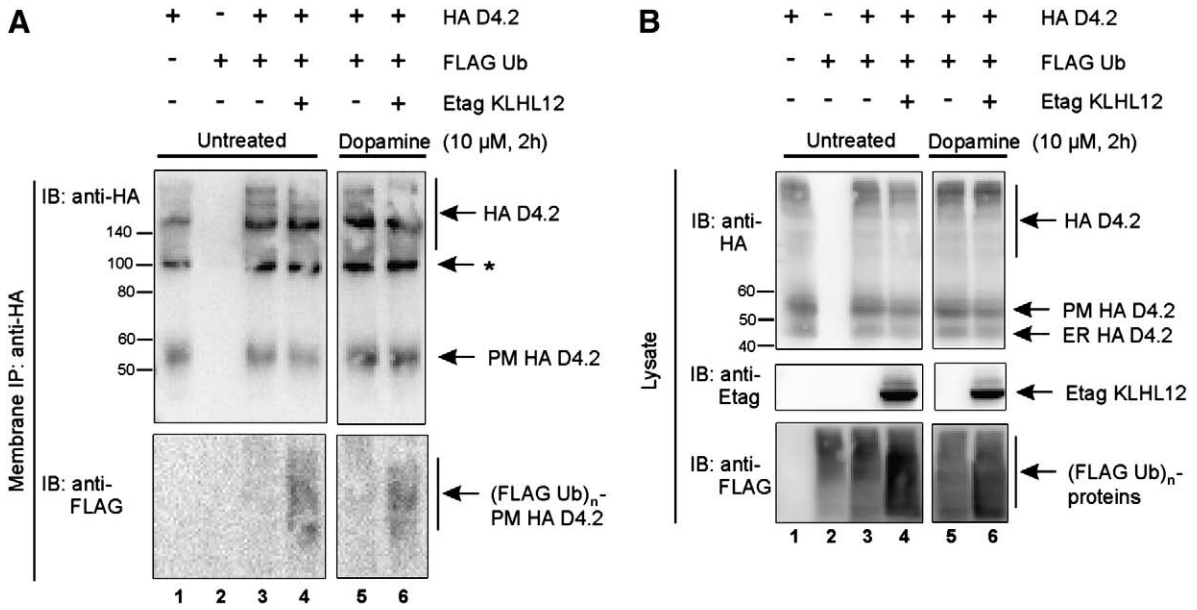

Fig. 3. KLHL12 promotes ubiquitination of plasma membrane-associated D4 receptors. HEK293T cells, seeded in 10 cm dishes, were transiently transfected as indicated (4 μg/plasmid). 48 h post-transfection, cells were incubated for 2 h with 10 μM dopamine (samples 5 and 6) or left untreated (1–4). Then, the medium on all plates was removed and the cells were subsequently incubated for 2 h with serum-free medium containing anti-HA antibody (2 μg/5 ml in each plate). Finally, the medium was removed and cells were washed, harvested, and incubated with lysis buffer for 2 h. 5% of the lysates were used for IB to visualize the HA D4.2 receptor, Etag KLHL12, and $(FLAG\ Ub)_n$-proteins, respectively. All lanes originate from the same gel. To the rest of the lysates, beads were added and the samples were rotated overnight for immunoprecipitation. IP of plasma membrane (PM)-associated HA D4.2 receptors was confirmed upon IB with anti-HA. Finally, ubiquitination of the PM-D4 receptor was revealed upon IB with anti-FLAG. All lanes originate from the same gel. The results shown are representative for three independent experiments. * Association of two heavy chains (each 50 kDa) of the anti-HA antibody.

with the PM-receptor, rather than the receptor itself, additional sequential immunoprecipitation studies were performed, as described before (see Section 2.5 and [40]). These experiments confirmed that the signals represent ubiquitinated PM-associated D4 receptors (data not shown). Although the levels of ubiquitination that can be detected in these experiments are rather low, the results suggest that PM-associated receptors undergo basal ubiquitination levels, and that KLHL12 is able to promote ubiquitination of these receptors.

To summarize, our data demonstrate that KLHL12 can promote ubiquitination of both immature, ER- and mature, PM-associated D4 receptors, which is in accordance with the observation that KLHL12 is able to interact with both receptor subpools.

3.3. Agonists or antagonists do not influence basal or KLHL12-mediated ubiquitination levels of D4 receptors

As for many other GPCRs, agonist-promoted ubiquitination of the β2-adrenergic receptor (the prototype GPCR) is required for targeting internalized receptors to the lysosomes [29]. To investigate whether activation of the D4 receptor enhances basal or KLHL12-mediated receptor ubiquitination, HEK293T cells, transiently transfected with HA D4.2, FLAG Ub and/or Etag KLHL12 constructs, were treated with 10 μM dopamine for different time periods, followed by a ubiquitination assay (Fig. 4A). However, agonist treatment does not seem to promote ubiquitination of the receptor, neither upon short dopamine stimulation (5 or 10 min), nor upon longer incubation times (1, 3 or 6 h). Co-expression of Etag KLHL12 resulted again in higher ubiquitination levels of the D4 receptor in unstimulated cells (compare lane 8 to lane 2 in Fig. 4A), but this remained further unaffected by agonist treatment. Noteworthy, cAMP measurements confirmed that overexpressed D4 receptors are responsive to agonist treatment in the HEK293T cell line (Supplemental Fig. 2A).

The effect of dopamine stimulation was also investigated upon specific immunoprecipitation of cell surface-expressed D4 receptors. Also in this case, dopamine neither promoted basal ubiquitination of the PM-associated receptors (Fig. 3; compare lane 5 to lane 3), nor KLHL12-mediated receptor ubiquitination (compare lane 6 to lane 4).

In parallel, we tested the effect of a D4 receptor-specific antagonist, L745-870 [42]. Similarly, HEK293T cells, transiently transfected with HA D4.2 receptor, FLAG Ub, and/or Etag KLHL12 constructs, were treated with the antagonist for different time periods, followed by a ubiquitination assay (Fig. 4B). Receptor ubiquitination levels remained virtually unaffected upon treatment with antagonist (lanes 3 to 7). Similarly, antagonist treatment did not significantly influence receptor ubiquitination levels in cells co-expressing KLHL12 (lanes 10–14). These data indicate that also antagonists do not influence ubiquitination of the D4 receptor.

Together, the observations suggest that the D4 receptor, in contrast to many other GPCRs, does not undergo agonist-promoted ubiquitination.

3.4. KLHL12 promotes poly-ubiquitination of the D4 receptor

As mono-, multi- and several possible types of poly-ubiquitination generally serve distinct cellular functions, we wanted to investigate which type(s) of ubiquitination is (are) associated with the D4 receptor. More specifically, we were interested to learn which type of receptor ubiquitination is promoted upon assembling the Roc1–Cul3–KLHL12 E3 ubiquitin ligase complex, in which KLHL12 serves as a substrate adaptor to recruit the D4 receptor for ubiquitination [40].

To investigate this, ubiquitination assays were performed upon co-transfection of a mutant FLAG-tagged ubiquitin construct encoding a ubiquitin molecule that carries three lysine (K)➔arginine (R) amino acid substitutions at sites 29, 48 and 63, respectively (further referred to as Ub K29,48,63R). Upon incorporation, such a mutant ubiquitin moiety does not allow further K29-, K48- or K63-linked chain extension on the substrate proteins. We compared the effect of this mutant Ub to wt Ub on basal and KLHL12-mediated ubiquitination of D4 receptors in HEK293T cells (Fig. 5A). Upon immunoprecipitation of the receptor, we could clearly detect basal receptor ubiquitination as a high molecular weight smear in lane 3 (*second panel*), which is strongly promoted by KLHL12 co-expression (lane 4). Upon co-transfection of Ub K29,48,63R, basal levels of D4 receptor ubiquitination could still be detected (lane 6). However, the ability of KLHL12 to

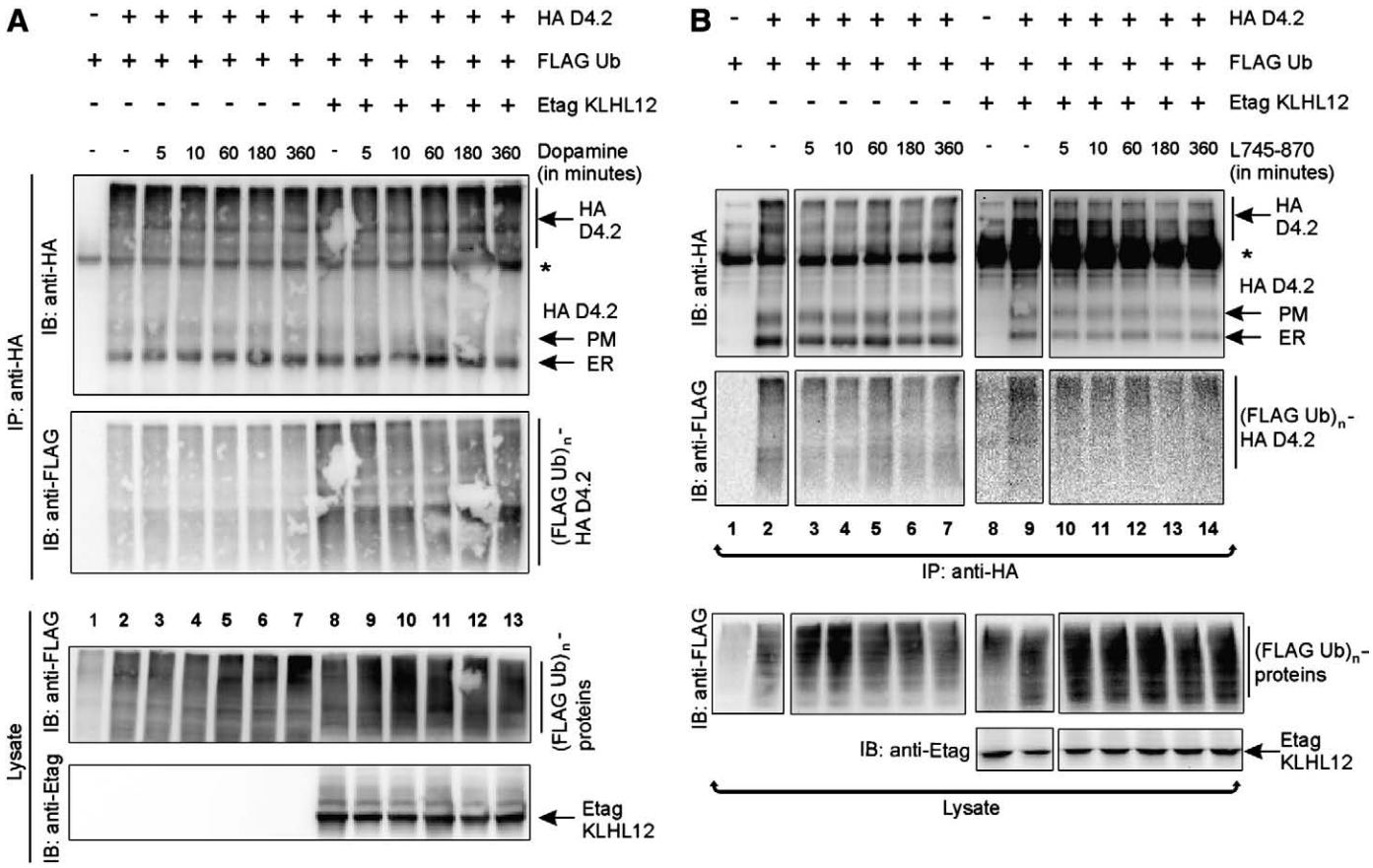

Fig. 4. Agonists/antagonists do not influence ubiquitination levels of the D4 receptor. (A) HEK293T cells were transiently transfected as indicated (4 μg/plasmid). 48 h post-transfection, all dishes were refreshed with serum-free medium. Then, dopamine was added (to a final concentration of 10 μM) to the samples at different time points, starting with the longest time period (360 min) and ending with the shortest (5 min). Finally, the cells from all samples were harvested at the same time and lysed. 5% of the lysates were used for IB to visualize Etag KLHL12, and (FLAG Ub)$_n$-proteins, respectively. The rest of the lysates were subjected to IP with anti-HA. IP of the receptor was confirmed upon IB with anti-HA, whereas receptor ubiquitination was observed upon subsequent IB with anti-FLAG. The results shown are representative for three independent experiments. (B) HEK293T cells were transiently transfected as indicated. Cells were treated with antagonist, applying the same protocol as in (A). 5% of the lysates were used for IB to visualize Etag KLHL12, and (FLAG Ub)$_n$-proteins, respectively. Samples 1–7 originate from the same gel, whereas samples 8–14 originate from a second gel. The rest of the lysates were subjected to IP with anti-HA. IP and receptor ubiquitination were detected as in (A). Again, samples 1–7 originate from the same gel, whereas samples 8–14 originate from a second gel. The results shown are representative for two independent experiments. * Association of two heavy chains (each 50 kDa) of the anti-HA antibody.

promote D4 receptor ubiquitination is almost completely blocked upon co-transfection of Ub K29,48,63R instead of wt Ub (compare lane 7 to lane 6, and lane 7 to lane 4, respectively). Although these data do not exclude mono- or multi-ubiquitination of the D4 receptor, the observations strongly suggest that KLHL12 promotes poly-ubiquitination of the D4 receptor via K29-, K48-, or K63-linked chain formation. The results presented in Fig. 5A were confirmed upon sequential immunoprecipitation of the receptor under the same conditions, ensuring that the observed signals merely reflect ubiquitination of the receptor (data not shown).

The Ub K29,48,63R mutant has been used before to demonstrate that receptor tyrosine kinases (RTKs) undergo multi-ubiquitination [43]. In our experimental approach, we wanted to further substantiate that the Ub K29,48,63R mutant is useful to demonstrate inhibition of poly-ubiquitination of substrate proteins. Therefore, we compared its effect to that of wt Ub on the ubiquitination levels of KLHL12, a protein that has been described to undergo poly-ubiquitination and subsequent proteasomal degradation [40,44] (Fig. 5B). Upon co-expressing Etag KLHL12 and wt or mutant FLAG Ub in HEK293T cells, poly-ubiquitination of KLHL12 is strongly reduced with co-expression of Ub K29,48,63R, compared to wt ubiquitin (compare lane 3 to lane 2 in the *middle panel*). This is in accordance with the general idea that most proteins undergo K48-linked poly-ubiquitination prior to proteasomal degradation. These observations confirm the usefulness of this Ub construct to reveal poly-ubiquitination of certain substrate proteins.

Further evidence for (KLHL12-mediated) poly-ubiquitination of the D4 receptor was obtained from studies with commercially available antibodies, which show specificity towards poly-ubiquitinated proteins (Fig. 5C). Again, we could clearly detect both basal ubiquitination of the receptor (lane 3 in the *middle part* of Fig. 5C) and increased KLHL12-mediated ubiquitination (lane 6) upon immunoprecipitation of the receptor and immunoblotting with anti-FLAG. Another volume of the same IP eluates was then loaded on a second gel (*upper part* of Fig. 5C), and now, the first immunoblotting was performed with the anti-Ub (clone FK1) antibody, which does not recognize mono- or multi-ubiquitinated substrate proteins but instead shows specificity towards poly-ubiquitinated substrates. This revealed specific signals for the D4 receptor (lanes 1 and 3 in the *first blot*) which were significantly higher in cells co-expressing KLHL12 (lanes 4 and 6; originating from the same gel). Next, the blot was stripped and reprobed with the anti-Ub (clone P4D1) antibody that recognizes all types of ubiquitination (mono-, multi, and poly-ubiquitination) on substrate proteins (*second blot*). Again, the specific ubiquitination signal for the D4 receptor (lanes 1 and 3) was increased upon KLHL12 co-expression (lanes 4 and 6). Finally, the same blot was reprobed with anti-HA antibody to confirm specific immunoprecipitation of the D4 receptor (*third blot*). These data provide further evidence that the D4 receptor is indeed poly-ubiquitinated, which is further promoted by KLHL12.

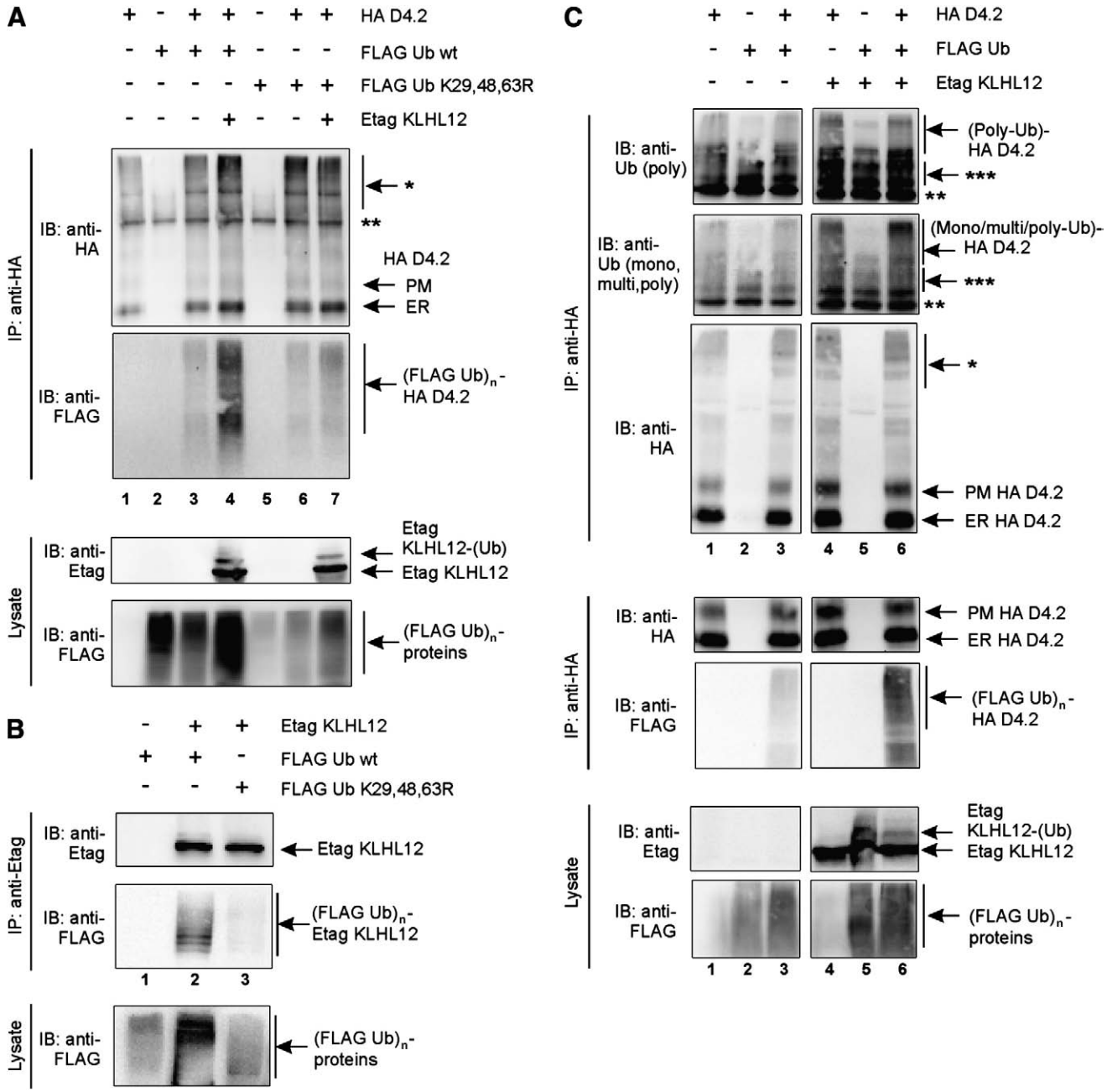

Fig. 5. KLHL12 promotes poly-ubiquitination of the D4 receptor. (A) HEK293T cells were transiently transfected as indicated (4 μg/plasmid). 48 h post-transfection, cells were lysed and 5% of the lysates were used for IB to visualize Etag KLHL12 and (wt/mutant FLAG Ub)$_n$-proteins, respectively. The rest of the lysates were subjected to IP with anti-HA. IP of the receptor was confirmed upon IB with anti-HA, whereas subsequent IB with anti-FLAG revealed ubiquitination levels of the receptor. The results shown are representative for three independent experiments. (B) HEK293T cells were transiently transfected as indicated (4 μg/plasmid). 48 h post-transfection, cells were harvested and lysed. Proteins, modified with wt or mutant FLAG Ub, were visualized upon SDS-PAGE of 5% of the cell lysate, whereas the rest of the lysates were subjected to IP with anti-Etag. IP of KLHL12 was confirmed upon IB with anti-Etag, whereas subsequent IB with anti-FLAG demonstrated KLHL12 ubiquitination. The results shown are representative for four independent experiments. (C) HEK293T cells were transiently transfected as indicated (4 μg/plasmid). 48 h post-transfection, cells were harvested and lysed. 5% of the lysates were used for IB to visualize Etag KLHL12 (unmodified and ubiquitinated species) and (FLAG Ub)$_n$-proteins, respectively (*bottom part*). The rest of the lysates were subjected to IP with anti-HA. Half of the IP eluates were applied in a first SDS-PAGE (upper part). IB with anti-Ub (clone FK1) revealed specific poly-ubiquitination of the D4 receptor (first blot). Upon stripping, the blot was reprobed with anti-Ub (clone P4D1) to detect total (mono/multi/poly) ubiquitination of the receptor (second blot). Finally, the blot was stripped again and reprobed with anti-HA to confirm IP of the HA D4.2 receptor (third blot). All lanes originate from the same gel. The other halves of the same IP eluates were applied in a second SDS-PAGE (*middle part*). IP of the receptor was confirmed with anti-HA, whereas ubiquitination with FLAG Ub moieties was revealed upon IB with anti-FLAG. Again, all lanes originate from the same gel. * High MW HA D4.2 receptor-specific signals. ** Association of two heavy chains of the anti-HA antibody (100 kDa). *** Combination of specific and non-specific signals from the anti-Ub antibodies (clone FK1 and P4D1, respectively).

3.5. The D4 receptor could undergo distinct types of ubiquitination, whereas KLHL12 undergoes K48-linked poly-ubiquitination

Our experiments with the mutant Ub K29,48,63R strongly suggest that KLHL12 promotes poly-ubiquitination of the D4 receptor via K29-, K48-, or K63-linked chains (Fig. 5A). As distinct types of poly-ubiquitination serve several distinct functions in the cell, it was of interest to determine which of these three types of poly-ubiquitin chains is conjugated to the D4 receptor. In order to unravel this matter, we used single mutant ubiquitin constructs, each encoding a ubiquitin molecule with only one amino acid substitution (K➔R), K29R, K48R or K63R. As these mutants were HA-tagged, a FLAG-tagged version of the

D4.4 receptor variant was used in the following ubiquitination assays. The effect of each individual ubiquitin mutant was compared to the effect of wt HA Ub on the basal or KLHL12-mediated ubiquitination levels of the D4 receptor, by performing a double sequential immunoprecipitation of the FLAG D4.4 receptor in HEK293T cells (Fig. 6A). Although expression of the different subpools of the D4.4 receptor was confirmed in the cell lysates, only the higher molecular weight fractions of the D4.4 receptor could be detected upon sequential IP with anti-FLAG. Interestingly, the receptor ubiquitination levels remained virtually unaffected upon co-expression of each of the single ubiquitin mutants, respectively HA K29R (second blot; lane 4), HA K48R (lane 6), and HA K63R (lane 8), compared to wt Ub (lane 2). These observations might indicate that the D4 receptor is able to undergo distinct types of ubiquitination, such as mono-, multi-, or poly-ubiquitination via ubiquitin chains other than K29-linked, K48-linked, or K63-linked. Ubiquitination of FLAG D4.4 receptors is again strongly promoted by KLHL12 with co-expression of wt Ub (compare lane 3 to lane 2). Remarkably, KLHL12 is also able to promote receptor ubiquitination in the presence of each individual Ub mutant, although this effect is slightly reduced in the presence of Ub K48R (compare lanes 7 and 3 to lanes 6 and 2, respectively). These results could indicate that KLHL12 does not promote K29-linked or K63-linked poly-ubiquitination of the D4 receptor, and could slightly promote K48-linked receptor poly-ubiquitination. Alternatively, these observations could also reflect the possibility that KLHL12 promotes receptor mono-, multi-, or poly-ubiquitination via chain formation other than K29-linked, K48-linked, or K63-linked. However, as demonstrated in Fig. 5A, co-expression of the triple mutant Ub K29,48,63R almost completely blocks the ability of KLHL12 to promote D4 receptor ubiquitination, suggesting that KLHL12 promotes receptor poly-ubiquitination via K29-linked, K48-linked and/or K63-linked ubiquitin chains. All the above observations (Figs. 5 and 6) could also reflect the possibility that KLHL12 can promote distinct types of receptor poly-ubiquitination. This might explain why the effects of the single Ub mutants are invisible (for K29R and K63R) or only partial (for K48R), whereas the effect of the triple mutant is more significant, due to the combined effect of inhibition of several types of poly-ubiquitin chain formation.

As a positive control, we wanted to demonstrate that the individual Ub mutants can be useful to elucidate the preferred type of poly-ubiquitination of certain substrate proteins. Therefore, the three mutants, HA Ub K29R, HA Ub K48R, and HA Ub K63R, were used to study the ubiquitination pattern of KLHL12, a protein that has been described to undergo proteasomal degradation, most likely upon K48-linked poly-ubiquitination [40,44] (Fig. 6B). Basal ubiquitination of KLHL12 was visible upon co-expression of wt HA Ub (lane 2 in the second blot). Co-expression of the mutants Ub K29R (lane 3) or Ub K63R (lane 5) did not significantly affect this ubiquitination pattern. These observations suggest that KLHL12 does not undergo K29-linked or K63-linked poly-ubiquitination. However, co-expression of the mutant Ub K48R resulted in a significant stronger signal for the mono-ubiquitinated form of KLHL12 (~71–72 kDa, versus ~63 kDa for unmodified Etag KLHL12) upon immunoblotting with anti-HA, as visible in lane 4 (second blot). This band was also observed upon immunoblotting with anti-Etag (lane 4 in the first blot), confirming that this signal represents mono-ubiquitinated KLHL12. These observations are in accordance with the idea that, upon attachment to the substrate protein KLHL12, HA Ub K48R blocks poly-ubiquitination via K48-linked chains. As mono-ubiquitinated proteins cannot be recognized by the proteasome, they escape degradation and thus accumulate in the cell. Notably, the high molecular weight smeary pattern, representing KLHL12 molecules conjugated with multiple Ub moieties,

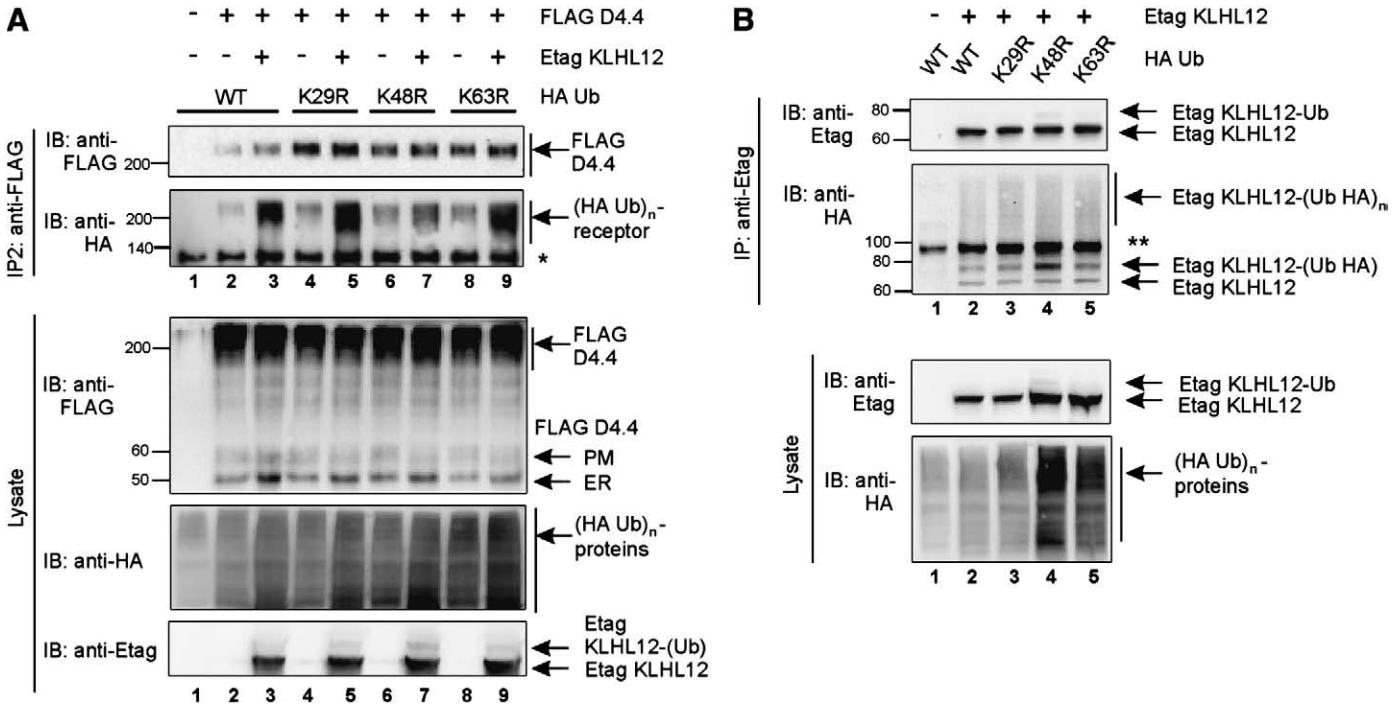

Fig. 6. The use of single mutant ubiquitin constructs K29R, K48R and K63R does not allow elucidation of the preferred type of D4 receptor poly-ubiquitination, but in contrast clearly demonstrates K48-linked poly-ubiquitination of KLHL12. (A) HEK293T cells were transiently transfected as indicated (4 μg/plasmid). 48 h post-transfection, cells were harvested and lysed. 5% of the lysates used for IB to visualize FLAG D4.4 receptors, $(HA\ Ub)_n$-proteins, and Etag KLHL12, respectively (*bottom part*). The rest of the lysates were subjected to a sequential double IP with anti-FLAG. Specific purification of the receptor after the second IP was confirmed upon IB with anti-FLAG, whereas receptor ubiquitination was revealed upon IB with anti-HA (upper part). (B) HEK293T cells were transiently transfected as indicated (4 μg/plasmid). 48 h post-transfection, cells were lysed and 5% of the lysates were used for IB to visualize Etag KLHL12 and $(HA\ Ub)_n$-proteins, respectively (*bottom part*). The rest of the lysates were subjected to IP with anti-Etag. IP of KLHL12 was confirmed upon IB with anti-Etag. Following, KLHL12 ubiquitination was revealed upon subsequent IB with anti-HA (second blot from the top); the signal at ~63 kDa, representing unmodified Etag KLHL12, results from the binding of HRP-coupled anti-mouse antibody in the second immunoblotting to residual primary anti-Etag antibody from the first immunoblotting, due to incomplete stripping of the membrane. * and ** Associations of two heavy chains (100 kDa) of the anti-HA or anti-Etag antibody, respectively.

could still be observed with co-expression of the mutant Ub K48R. The latter observation suggests that K48-linked poly-ubiquitin chain formation on KLHL12 was still possible in these cells, probably through the incorporation of wt endogenous ubiquitin molecules. This can also explain our observations that relative strong poly-ubiquitination signals for the D4 receptor could still be detected in the presence of these single ubiquitin mutants (Fig. 6A). Nevertheless, no specific mono-ubiquitinated receptor species could be detected, in contrast to KLHL12 (Fig. 6B).

Together, these data demonstrate that the use of single ubiquitin mutant constructs helps to elucidate the preferred type of poly-ubiquitination for some substrates, as demonstrated for KLHL12 that undergoes K48-linked poly-ubiquitination (Fig. 6B), whereas they yield inconclusive results about the preferred type of poly-ubiquitination of other substrate proteins, such as D4 receptors (Fig. 6A). However, when combined, the data from Figs. 5A, C and 6A suggest that KLHL12 could promote distinct types of D4 receptor ubiquitination.

3.6. KLHL12 does not promote D4 receptor degradation

In most cases, ubiquitination of substrate proteins targets them for subsequent degradation. Also GPCRs can be targeted for ubiquitin-dependent degradation via two major pathways. Agonist-dependent ubiquitination of membrane receptors often marks them for degradation in the lysosomes, whereas the ERAD pathway targets misfolded proteins for proteasomal degradation following ubiquitination. Albeit we clearly demonstrated that KLHL12 promotes ubiquitination of the D4 receptor, we could not observe enhanced degradation of the receptor in the presence of KLHL12. In contrast, somewhat elevated levels of receptor could be observed in some, but not all experiments, for reasons that are unknown so far. Although in most experiments, HEK293T cells were transfected with equal amounts of D4 receptor and KLHL12, the ratio of expression levels of the two proteins could still be important, as lower KLHL12 to D4 receptor ratios could imply competition of KLHL12 for interaction with other proteins, whereas higher ratios might target more D4 receptors for ubiquitination and degradation. Therefore, various distinct ratios of Etag KLHL12 versus HA D4.2 constructs were transfected in HEK293T cells (Fig. 7A), varying from 0.1, 0.25, 0.5, 1, 1.5, to 2, respectively (lanes 4 to 9). The expression levels of the D4 receptor are definitely not decreased upon gradually increasing KLHL12 levels. Hence, KLHL12 is not observed to promote degradation of D4 receptors at any ratio. As this experiment is representative for multiple independent experiments, we conclude that overall, KLHL12 does not promote D4 receptor degradation. These results are also in accordance with our previous findings that small amounts of KLHL12 (low KLHL12/D4 ratio) are already able to strongly promote receptor ubiquitination, but are not observed to promote receptor degradation (data not shown).

To substantiate the observation that KLHL12 does not promote D4 receptor degradation, we investigated the influence of KLHL12 on the half-life ($t_{1/2}$) of the mature D4 receptor (Fig. 7B). For these purposes, D4 receptor-expressing cells were treated with BFA to block the expression of new, fully mature D4 receptors at the plasma membrane. The existing pool of mature receptors will gradually decrease upon longer BFA treatment, due to intrinsic receptor degradation in the cell. For these experiments, we used CHO cells that were stably transfected with FLAG D4.4 receptors. This cell line has been used before to demonstrate chaperone-effects of agonists in the upregulation of D4 receptor expression levels [24]. It is also noteworthy to mention that the receptors in these cell lines are functional ([24] and our own unpublished data). In contrast to endogenous KLHL12 expression in HEK293T cells [39], it is not known whether this protein is also expressed in CHO cells. However, we were able to demonstrate that overexpressed KLHL12 not only interacts with D4 receptors but also promotes receptor ubiquitination in CHO cells (Supplemental Fig. 1A and B). In untreated cells, the mature, PM-associated receptor subpool is more abundant than the immature, ER-associated receptor subpool (upper part of Fig. 7B, lane 1). Upon BFA treatment for 2 h, already a small reduction in the mature receptor levels is observed (lane 3), which are further decreased upon longer treatments with BFA (lanes 5, 7, 9, 11, 13). From these data, the half-life of the receptor can be estimated to lie between 4 and 8 h. Simultaneously with decreasing mature receptor levels, levels of immature receptors augment upon longer BFA treatment, due to their retention in the ER. However, similar patterns can be observed in KLHL12 co-expressing cells. Although receptor levels might be slightly higher, compared to the untransfected cell upon 2 h treatment (compare lane 4 to lane 3) and 4 h treatment (compare lane 6 to lane 5), the actin levels, which function as internal controls in this experiment, are also elevated. Taken this into account, the general pattern for receptor levels in KLHL12-expressing cells upon BFA treatment is very similar to the pattern in the control cells, with an estimated mature receptor half-life between 4 and 8 h, approximately. The levels of the immature receptors (relative to the actin control levels) are also comparable between the control and KLHL12-expressing cells. In previous studies, it was demonstrated that the agonist quinpirole is able to act as a pharmacological chaperone for the D4 receptor, by stabilizing the correct folding of the receptor at the ER and leading to upregulated receptor levels [24]. In a similar setup as above, we investigated whether the combined treatment with BFA and quinpirole would result in different receptor levels between control and KLHL12-expressing cells (*bottom part* of Fig. 7B). Regarding the mature receptor, the effect of BFA and quinpirole was comparable to the cells that were treated with BFA alone. Similarly, KLHL12 did not affect the stability of the mature receptor in these experiments with quinpirole. These experiments therefore also indicate that mature, plasma membrane-associated D4 receptors do not undergo agonist-promoted downregulation, even not in the presence of their adaptor KLHL12. Regarding the immature receptor, the combined treatment with BFA and quinpirole resulted in dramatically increased ER-associated receptor levels due to the chaperone-effect of quinpirole. However, ER-associated receptor levels (relative to actin control levels) are not significantly different upon KLHL12 co-expression. Together, these data suggest that (1) KLHL12 does not influence the half-life (stability) of the mature D4 receptor (2) the chaperone-effect of quinpirole is not affected by KLHL12, and (3) the mature, PM-associated D4 receptor does not undergo agonist-dependent degradation, even not in the presence of KLHL12.

To further substantiate these findings, we investigated the effect of KLHL12 on D4 receptor expression levels upon proteasomal or lysosomal inhibition. For this, CHO FLAG D4.4 cells, either untransfected or transfected with Etag KLHL12, were treated for 6 h with combinations of BFA, the proteasomal inhibitor MG-132 (M) or its solvent control DMSO (D), and the lysosomal inhibitor chloroquine (Chl), as indicated in Fig. 7C. Treatment of the cells with chloroquine resulted in increased levels of mature receptor (compare lane 5 to lane 1), whereas the mature receptor levels were less decreased upon combined BFA and chloroquine treatment, compared to levels upon BFA treatment alone (compare lane 6 to lane 2). Although these data indicate that the mature receptor indeed undergoes lysosomal degradation, we observed no effect of KLHL12 on this (compare lanes 7 and 8 to lanes 5 and 6). Treatment of the cells with the proteasomal inhibitor MG-132 resulted in increased immature receptor levels (compare lane 9 to lane 13), and the combined incubation with BFA also resulted in stabilization of the immature receptor (compare lane 10 to lane 14). These results indicate that the immature, ER-associated D4 receptor indeed undergoes proteasomal degradation. Again, similar receptor expression levels were observed in KLHL12-expressing cells. Remarkably, in this experiment, a stronger signal for the mature D4 receptor was observed in

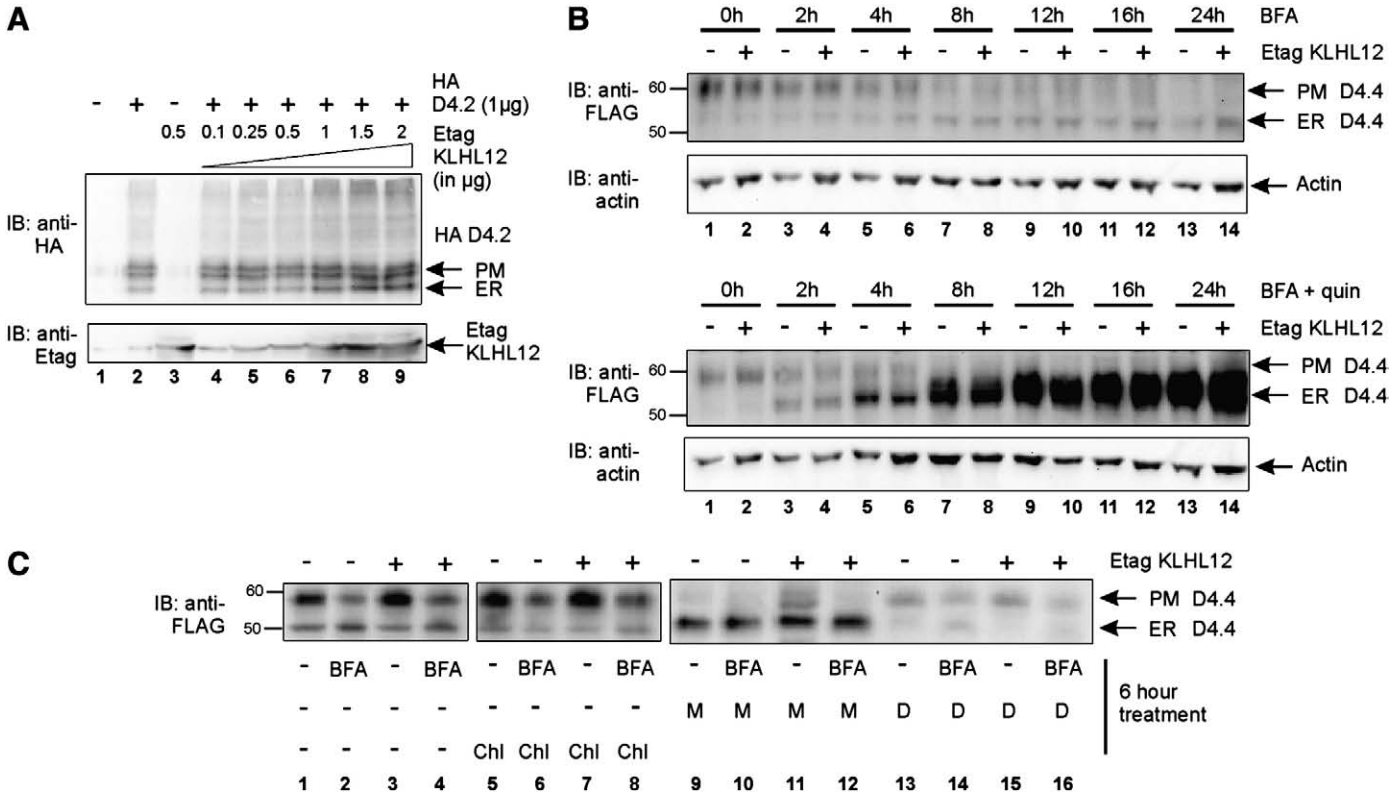

Fig. 7. KLHL12 does not promote D4 receptor degradation. (A) HEK293T cells, seeded in 6-well plates, were transiently transfected with 1 μg of HA D4.2 and increasing amounts of Etag KLHL12 (from 0.1 to 2 μg/sample), as indicated. 48 h post-transfection, cells were harvested and lysed. Parts of the lysates were used for IB with anti-HA to reveal receptor expression levels, followed by IB with anti-Etag to confirm gradually increasing KLHL12 expression levels. The experiment in this picture is representative for multiple independent experiments (B) CHO FLAG D4.4 cells, seeded in 10 cm dishes, were transiently transfected with either vehicle (pcDNA3; 10 μg/plate) or Etag KLHL12 plasmids (10 μg/plate). 24 h post-transfection, the cells were trypsinized and seeded again in 6-well plates, to obtain the same amounts of transfected receptor in each sample. Another 24 h later, the cells were left untreated and collected (1, 2) or treated with either 5 μg/ml BFA alone (*upper panel*), or BFA + 10 μM quinpirole (quin) (*lower panel*). The cells were harvested at different time points, upon incubation periods varying from 2 to 24 h. Upon cell lysis, equal amounts of lysates were used for IB to reveal receptor expression levels. Afterwards, blots were reprobed with anti-actin, as an internal control for cell death and protein levels in the lysates. (C) CHO FLAG D4.4 cells, seeded in 10 cm dishes, were transiently transfected and reseeded in 6-well plates, as in (B). Another 24 h later, all samples were treated for 6 h with different combinations of BFA (5 μg/ml), the lysosomal inhibitor chloroquine (chl; 0.25 mg/ml), and the proteasomal inhibitor MG-132 (M; 20 μM) or its solvent control DMSO (D; 0.2%), as indicated. After 6 h, all cells were harvested and lysed. Equal protein amounts were subjected to SDS-PAGE and subsequent IB with anti-FLAG to reveal receptor expression levels. Samples 1–8 and samples 9–16 originate from two different gels.

KLHL12-expressing cells that had been treated with MG-132, compared to control cells (compare lane 11 to lane 9). Although this might suggest that MG-132 is able to stabilize the mature receptor in the presence of KLHL12, this proteasomal inhibitor was, however, not able to stabilize mature receptors upon combined BFA treatment (compare lane 12 to lane 11). Furthermore, another proteasomal inhibitor, epoxomycin, was tested in parallel in these experiments but did not stabilize the mature receptor in the presence of KLHL12 (data not shown). Altogether, these data demonstrate that KLHL12 does neither promote lysosomal degradation of mature D4 receptors, nor proteasomal degradation of immature D4 receptors.

3.7. KLHL12 interacts with β-arrestin2

Upon continuous agonist stimulation, a number of GPCRs are targeted to the lysosomes via a conserved mechanism requiring covalent addition of ubiquitin to the receptor. Figs. 3 and 4 demonstrate that stimulation of the D4 receptor by agonist does not enhance receptor ubiquitination and we published before that also other hallmarks for receptor internalization are blunted [39]. To explore the role of receptor ubiquitination in the internalization mechanism of the D4 receptor, we performed several experiments with the mutant D4.2 4 K➔R receptors, in which all four intracellular lysines (K) which can be a substrate for ubiquitination, are mutated to arginine (R). First of all, cAMP-ELISA assays in HEK293T cells confirmed that the D4.2 4 K➔R mutant is responsive to agonist stimulation (Supplemental Fig. 2A). Next, immunofluorescence microscopy studies were performed in transiently transfected cells, to compare the subcellular localization pattern of the mutant D4.2 4 K➔R receptor to the wild-type D4.2 receptor. Interestingly, the mutant D4.2 4 K➔R receptor showed a similar expression pattern as the wild-type receptor (Supplemental Fig. 2B). The strong expression of ubiquitination-deficient mutant receptors at the cell surface suggests that receptor ubiquitination is not required to target (newly synthesized) mature receptors to the plasma membrane and that these mutant D4 receptors do not show strong constitutive internalization. The reciprocal experiment in which an enhanced receptor ubiquitination, due to the co-expression of KLHL12, was provoked did neither affect receptor localization (see further, Fig. 10).

We have shown before, by immunofluorescence microscopy and co-immunoprecipitation studies that β-arrestin2 interacts with the D4 receptor [39] and now we have evaluated whether ubiquitination of the receptor affects the interaction with β-arrestin2. To study the influence of enhanced ubiquitination, we overexpressed KLHL12, and to study a decreased ubiquitination, we used the D4 receptor mutant (HA D4.2 4 K➔R). HEK293T cells were transiently transfected as indicated in Fig. 8 and a co-immunoprecipitation experiment was performed. Both receptors (wild-type and 4 K➔R mutant) are able to interact with β-arrestin2 indicating that inhibition of receptor ubiquitination on intracellular lysines does not prevent β-arrestin2 binding. Furthermore, increasing receptor ubiquitination, by overexpression of KLHL12, did neither result in a significant augmented interaction of FLAG β-arrestin2 with the D4 receptor.

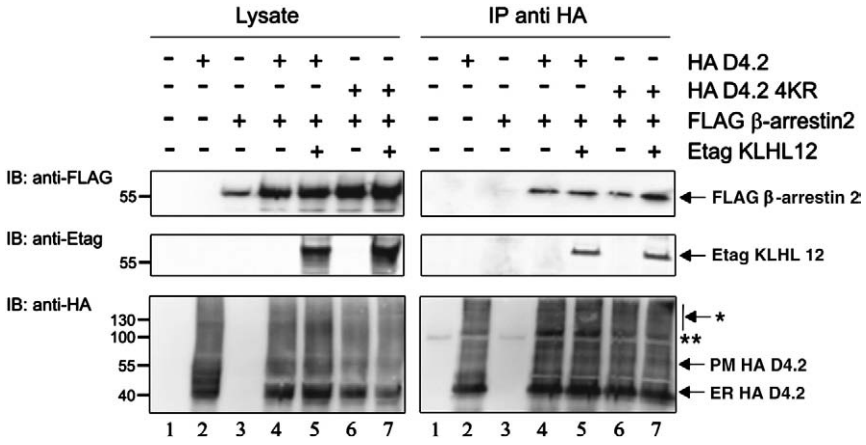

Fig. 8. Ubiquitination of the D4 receptor does not influence its interaction with β-arrestin2. HEK 293 T cells, seeded in 10 cm dishes, were transiently transfected as indicated (4 μg/plasmid/dish) 48 h post-transfection, cells were lysed and 5% of the lysates were used for IB to visualize FLAG β-arrestin2, Etag KLHL12 and HA D4.2 wt/4KR mutant, respectively. The rest of the lysates were subjected to immunoprecipitation (IP) with anti-HA. Interaction between HA D4.2 DR and FLAG β-arrestin2 or Etag KLHL12 was shown after IP by IB with anti-FLAG-HRP and anti-Etag, respectively. Specific purification of the receptor after IP was confirmed upon IB with anti-HA. * High MW HA D4.2 receptor-specific signals. ** Associations of two heavy chains of the anti-HA antibody (100 kDa).

We also investigated if KLHL12 was able to induce ubiquitination of β-arrestin2. A sequential immunoprecipitation in which β-arrestin2 was isolated did not show an increased ubiquitination of this protein. Stimulation of the D4 receptor did neither have an effect on the ubiquitination of β-arrestin2 in the absence or presence of KLHL12. This co-immunoprecipitation assay also shows that KLHL12 can interact with β-arrestin2 in the absence of the D4 receptor (Fig. 9, first immunoprecipitation, second blot, lanes 8 and 9). This was also confirmed in a co-immunoprecipitation assay with only overexpressed FLAG β-arrestin2 and Etag KLHL12 (data not shown).

Before, we have visualized β-arrestin2 upon D4 receptor stimulation and could not show an influence of agonist treatment on β-arrestin1/2 recruitment [39]. We have now investigated whether KLHL12 could promote translocation of β-arrestin2 and could have a subsequent effect on D4 receptor internalization. Immunofluorescence microscopy studies in HEK293T cells overexpressing β-arrestin2 GFP, Etag KLHL12 and HA D4 receptor do not show D4 receptor internalization upon dopamine treatment nor a relocalization of β-arrestin2 GFP (Fig. 10). These results are comparable with the data we obtained from cells overexpressing only β-arrestin2 GFP and HA D4 receptor [39]. As a positive control we have used HA D2 receptor (data not shown) and HA β2-adrenergic receptor.

From these data we can conclude that KLHL12 interacts with β-arrestin2, but has no effect on ubiquitination nor on localization of β-arrestin2.

4. Discussion

We previously demonstrated that KLHL12 functions as an adaptor in a Cul3-based E3 ubiquitin ligase complex, and specifically promotes

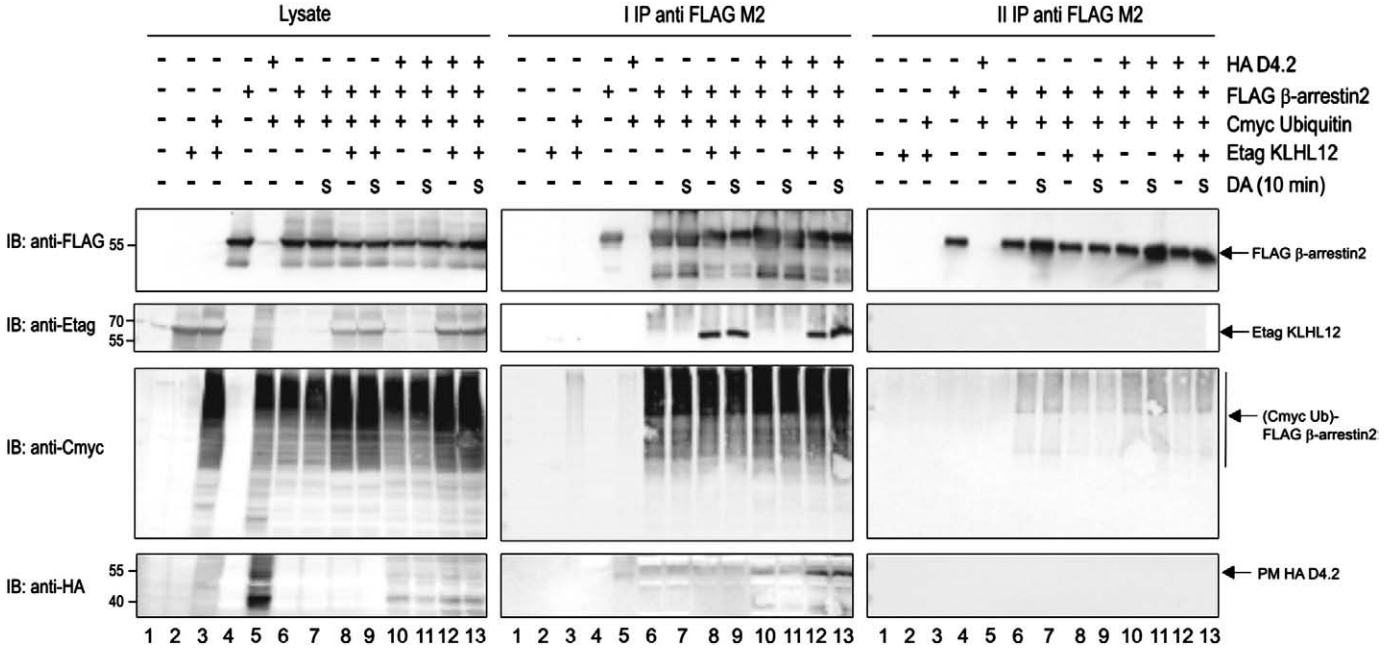

Fig. 9. Ubiquitination of β-arrestin2 is not influenced by KLHL12. HEK 293T cells, seeded in 10 cm dishes, were transiently transfected as indicated (3 μg/plasmid/dish). 48 h post-transfection, cells were incubated for 10 min with 10 μM dopamine (DA) or left untreated. Then cells were washed, harvested and lysed. 5% of the lysates were used for IB to visualize FLAG β-arrestin, Etag KLHL12, $(C\text{-myc Ub})_n$-proteins, and HA D4.2 receptor. The rest of the lysates were subjected to a sequential double IP with anti-Flag M2. After the first IP interaction of HA D4.2 and Etag KLHL12 with FLAG β-arrestin was shown by IB with anti-HA and anti-Etag, respectively. Specific purification of FLAG β-arrestin2 after the first and second IP was confirmed upon IB with anti-FLAG-HRP, whereas FLAG β-arrestin2 ubiquitination was revealed after second IP upon IB with anti-c-myc.

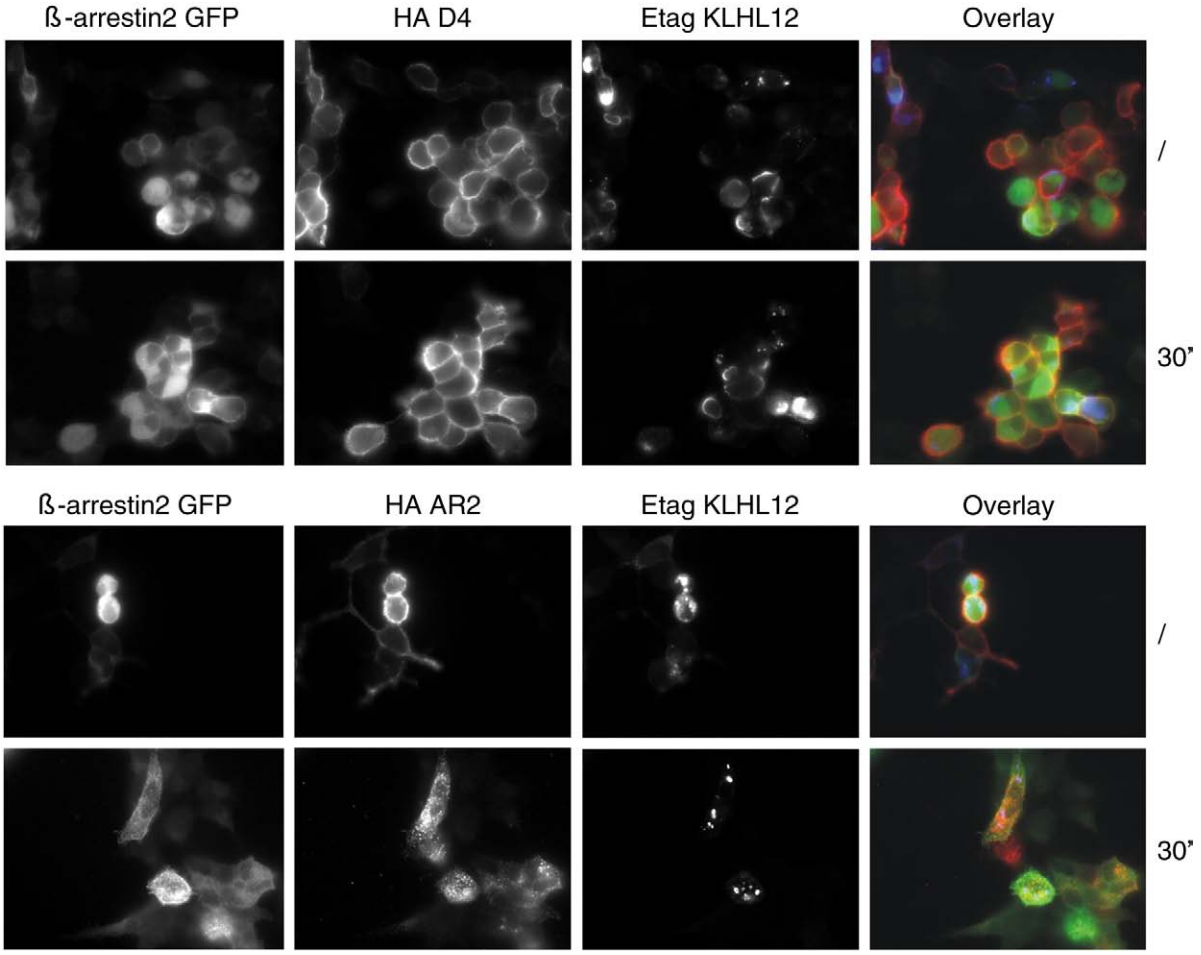

Fig. 10. Recruitment of β-arrestin2 after stimulation of HA D4 or HA β2-adrenergic receptor in the presence of KLHL12. HEK293T cells were transfected with pHA D4.2 or pHA β2-adrenergic receptor (HA AR2) as a positive control, pEtag KLHL12 and pβ-arrestin2 GFP in a 5:5:1 ratio. Membrane receptors were labelled with antibody as described before and subsequently cells were induced with dopamine (DA,10 μM) for HA D4.2 and isoproterenol (iso, 10 μM) for HA β2-adrenergic receptor (HA AR2) for the indicated time periods. Receptors are visualised in red, KLHL12 in blue and β-arrestin2 in green in the overlay picture.

ubiquitination of the D4 receptor [40]. In the present study, we characterized the KLHL12-mediated ubiquitination of the D4 receptor and questioned its functional relevance. First of all, co-immunoprecipitation studies (Fig. 1) indicated that KLHL12 is able to interact with both mature D4 receptors at the plasma membrane, and immature D4 receptors at the ER. Ubiquitination assays in HEK293T cells not only revealed that ER-associated, immature receptors show rather strong basal ubiquitination levels (Fig. 2A), but also that their ubiquitination is strongly promoted by KLHL12 (Fig. 2B). Alternatively, specific isolation of mature, cell surface-expressed D4 receptors, and subsequent ubiquitination assays not only revealed that these receptors undergo basal ubiquitination, but also that KLHL12 significantly promotes mature receptor ubiquitination (Fig. 3). Although basal D4 receptor ubiquitination levels in HEK293T cells could result from endogenously expressed KLHL12, we also observed basal D4 receptor ubiquitination in CHO cells (see Supplemental Fig. 1B) in which it is not known whether KLHL12 is expressed. Furthermore, we previously demonstrated basal ubiquitination of a D4 receptor mutant (D4.0) that does not interact with KLHL12 in HEK293T cells [40], indicating that other adaptors besides KLHL12 could also exist for the D4 receptor or that alternative or redundant systems could regulate D4 receptor ubiquitination.

As many GPCRs show agonist-promoted ubiquitination, we tested the effect of dopamine treatment on the basal and KLHL12-mediated ubiquitination levels of the D4 receptor (Fig. 4A). Ubiquitination assays revealed that dopamine does not influence basal or KLHL12-mediated ubiquitination of the D4 receptor, neither upon short stimulation, nor upon longer D4 receptor activation. Similarly, the D4 receptor-specific antagonist L745-870 did not influence this receptor ubiquitination (Fig. 4B). The data from the experiments shown in Fig. 4 should, however, be evaluated carefully. Indeed, both immature, ER-associated and mature, PM-associated receptors could have contributed to the total ubiquitin signals. However, in case that D4 receptors could hypothetically undergo agonist-induced ubiquitination, only cell surface-expressed receptors are expected to show increased ubiquitination levels upon administration of plasma membrane-impermeable dopamine. In other words, upon agonist treatment, dopamine-independent ubiquitination levels of ER-associated D4 receptors could mask more subtle differences in dopamine-dependent ubiquitination levels of PM-associated receptors. For these reasons, the effect of dopamine treatment was also investigated upon specific immunoprecipitation of PM-associated D4 receptors (Fig. 3). Although basal ubiquitination levels of PM-associated receptors are rather weak, dopamine is not able to promote receptor ubiquitination, even in the presence of KLHL12. Together, these data strongly suggest that the D4 receptor does not undergo agonist-promoted ubiquitination.

As different types of ubiquitination can result in different outcomes for the modified substrate proteins, we investigated which type(s) of ubiquitination the D4 receptor can undergo, and more specifically which type(s) of ubiquitination is (are) promoted by KLHL12. Experiments with Ub K29,48,63R revealed that the basal ubiquitination level of the D4 receptor is not affected upon co-

expression of this mutant (Fig. 5A). This could possibly reflect that the D4 receptor shows basal levels of mono- or multi-ubiquitination, or poly-ubiquitination via chains other than K29-, K48-, or K63-linked. Alternatively, the possibility exists that the triple mutant is not able to sufficiently block basal (K29-, K48-, or K63-linked) poly-ubiquitination of D4 receptors. Indeed, despite the presence of exogenous mutant Ub molecules, the large endogenous pool of wild-type ubiquitin moieties in these cells still allows the formation of normal K29-, K48-, or K63-linked poly-ubiquitin chains on substrates. However, the Ub K29,48,63R mutant strongly inhibits KLHL12-mediated receptor ubiquitination, as opposed to strong KLHL12-mediated ubiquitination in the presence of wt exogenous Ub molecules (Fig. 5A). This observation therefore suggests that KLHL12 promotes poly-ubiquitination of the D4 receptor via K29-, K48-, and/or K63-linked chains. Poly-ubiquitination of the D4 receptor, promoted by KLHL12, was further validated by ubiquitination assays with poly-ubiquitin-specific antibodies (Fig. 5C).

In an attempt to further elucidate the preferred linkage type of D4 receptor poly-ubiquitination, the single Ub mutants K29R, K48R and K63R were used separately in ubiquitination assays (Fig. 6A). Again, no effect was observed on basal D4 receptor ubiquitination levels. Furthermore, the K29R and K63R mutants did not inhibit KLHL12-mediated ubiquitination of the D4 receptor, whereas the Ub K48R mutant showed some inhibition of KLHL12-mediated receptor ubiquitination, indicating the possibility of K48-linked poly-ubiquitination. However, the effect of K48R seems only partial, and not as robust as the observed effect of the triple Ub mutant in several other experiments (Fig. 5A). The possibility that the D4 receptor undergoes combinations of K29-, K48-, and/or K63-linked poly-ubiquitination might explain why the effect of the triple mutant is significantly stronger than the effect of the single ubiquitin mutants. Additionally, as the triple mutant does not completely inhibit basal and KLHL12-mediated ubiquitination of the receptor, mono-, multi-, or poly-ubiquitination via chains other than K29-, K48-, and K63-linked are also not excluded. Altogether, these data suggest that D4 receptors might undergo different types of ubiquitination. Our observations that both plasma membrane-associated and ER-associated D4 receptor subpools undergo basal and KLHL12-mediated ubiquitination might imply different possible functions for ubiquitination, and would thus be in accordance with these data. Moreover, recent studies clearly demonstrate that many types of ubiquitination can be found in a cell; besides the well characterized mono-, multi-, and poly-ubiquitination via K48- or K63-linked chains, there is compelling evidence for 1) atypical homotypic chains (via lysine residues other than K48 or K63), 2) mixed-chains, assembled through several distinct lysines in the ubiquitin monomers, forming bifurcations and 3) heterologous chains, that also integrate ubiquitin-like modifiers [8,45–47]. These recent studies demonstrate that ubiquitination is a versatile modification that is believed to serve several possible functions in the cell. Hence, the possibility of multiple and complex D4 receptor ubiquitination patterns would also explain why the experiments with ubiquitin mutants were not conclusive regarding a preferred linkage type, and might reflect several possible functions for D4 receptor ubiquitination in the cell.

To demonstrate the usefulness of ubiquitin mutants for the determination of the preferred linkage type of substrate proteins, parallel ubiquitination assays were performed with KLHL12, which undergoes proteasomal degradation [40,44]. Whereas no effect on KLHL12 ubiquitination was observed for the single mutants K29R and K63R, the K48R mutant clearly resulted in accumulation of mono-ubiquitinated KLHL12 species, indicating inhibition of further K48-linked poly-ubiquitination and therefore inhibition of subsequent recognition by the proteasome (Fig. 6B). A strong inhibitory effect on KLHL12 poly-ubiquitination was also observed upon application of the mutant Ub K29,48,63R (Fig. 6B). Remarkably, the triple mutant resulted in strongly reduced high molecular weight smearing, characteristic for poly-ubiquitinated species, whereas the single K48R Ub mutant rather induced accumulated levels of mono-ubiquitinated KLHL12 species. These results therefore suggest the possibility that these single ubiquitin mutants cannot sufficiently inhibit poly-ubiquitination of substrates because of the incorporation of endogenous wild-type ubiquitin molecules, while, apparently, the triple Ub mutant is able to inhibit this more robustly. Although the reasons for these different effects are not completely clear, both mutants, however, indicate that they inhibit KLHL12 poly-ubiquitination. Furthermore, these differences also demonstrate that effects observed in experiments with ubiquitin mutants should be interpreted with great care.

Ubiquitination of proteins (including GPCRs) often functions as a signal for degradation. However, KLHL12 does not seem to target the D4 receptor for degradation: 1) Cul3 overexpression or downregulation, alone or in combination with KLHL12 co-expression, did not significantly affect D4 expression levels; 2) downregulation of KLHL12 via siRNA was not observed to increase receptor levels [40]; 3) in addition, variation of the ratio exogenous KLHL12 versus D4 receptor does not promote downregulation or decreased expression levels of the receptor (Fig. 7A). Therefore, upon evaluation of many independent experiments, we conclude that co-expression of exogenous KLHL12 does not result in degradation of exogenous D4 receptor in HEK293T cells. Additional evidence came from the observation that KLHL12 does not influence the half-life of the mature receptor, which was demonstrated in the stable CHO FLAG D4.4 cell line (Fig. 7B). Moreover, these experiments also indicate that mature, PM-associated receptors do not undergo agonist-promoted degradation, as the half-life of the mature receptor upon quinpirole treatment is not observed to be different from the half-life in unstimulated cells (Fig. 7B). Finally, it was demonstrated that KLHL12 does not influence receptor levels upon lysosomal or proteasomal inhibition (Fig. 7C) in the CHO FLAG D4.4 cell line. Although these experiments clearly confirm that mature, plasma membrane D4 receptors undergo lysosomal degradation, and that immature, ER-associated receptors undergo proteasomal degradation (see also [24]), KLHL12 is not observed to affect expression levels of both receptor subpools.

Initially, it was tempting to speculate that KLHL12 might promote ubiquitination and subsequent proteasomal degradation of misfolded, ER-retained D4 receptors through the ERAD pathway, as we clearly demonstrated that 1) KLHL12 interacts with and 2) promotes ubiquitination of immature, ER-associated D4 receptors (Figs. 1 and 2); 3) KLHL12-mediated ubiquitination occurs agonist-independent (Fig. 4); 4) D4 receptors could undergo K48-linked poly-ubiquitination (Figs. 5 and 6), which generally targets proteins for proteasomal degradation. However, this hypothesis can be rejected based upon several lines of evidence: 1) overexpression of KLHL12 does not promote D4 receptor degradation at any ratio (Fig. 7A); 2) downregulation of Cul3, inhibiting KLHL12-mediated D4 ubiquitination, does not result in increased D4 levels [40]; 3) Inhibition of KLHL12-mediated receptor poly-ubiquitination, via co-expression of triple or single ubiquitin mutants, did not result in elevated immature receptor levels (Figs. 5A and 6A). Together, these data indicate that although KLHL12 promotes agonist-independent ubiquitination of ER-associated immature D4 receptors, it does not target these receptors for subsequent proteasomal degradation.

Alternatively, KLHL12 might function as an adaptor in the agonist-dependent ubiquitination of the D4 receptor, which could subsequently target the latter for lysosomal degradation, in analogy with several other GPCRs. Although our data clearly demonstrate that 1) KLHL12 is able to interact with and 2) promotes ubiquitination of cell surface-expressed D4 receptors (Figs. 1 and 3), several lines of evidence fail to substantiate this hypothesis: 1) the D4 receptor is not able to undergo agonist-promoted ubiquitination (Fig. 4A); 2) although cell surface-expressed D4 receptors show minor basal ubiquitination levels, agonists are not able to promote receptor ubiquitination, even in the presence of KLHL12 (Fig. 3); 3) agonist treatment does not result in decreased receptor

expression levels, even upon long stimulation (Figs. 3 and 4A) or 4) does not influence the half-life of the mature D4 receptor, even in the presence of KLHL12 (Fig. 7B). To summarize, we conclude that poly-ubiquitination of the receptor promoted by KLHL12 does not result in receptor degradation, suggesting that KLHL12-mediated receptor ubiquitination might have alternative functions.

Ubiquitination of GPCRs can also function as a signal for internalization and although enhanced ubiquitination of plasma membrane-expressed D4 receptor in cells overexpressing KLHL12 was shown, this did not result in a decreased receptor isolation (Fig. 3) indicating that D4 receptor ubiquitination did not result in enhanced internalization. This was also confirmed by immunofluorescence microscopy (Fig. 10). Besides GPCR ubiquitination, also β-arrestin2 and its ubiquitination can play crucial roles in GPCR internalization [29,35–38]. Co-immunoprecipitation studies (Figs. 8 and 9) did not indicate a cross-talk between D4 receptor ubiquitination and β-arrestin2 recruitment, although they showed a direct interaction between KLHL12 and β-arrestin2 (Fig. 9). It has become evident that β-arrestins act as multifunctional scaffold proteins affecting different signalling pathways [48], therefore it will be challenging to unravel the role of the interaction between KLHL12 and β-arrestin2 in this signalling complex.

Acknowledgments

This work was financially supported partly by FWO (Fonds voor Wetenschappelijk onderzoek) Vlaanderen: Project No. G010909N. Kamila Skieterska is supported by a predoctoral grant from Ghent University; BOF (Bijzonder Onderzoeksfonds). Kathleen Van Craenenbroeck has a post-doctoral FWO fellowship.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellsig.2010.01.014.

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