Investigating the role of KLHL12 and β-arrestin2 in dopamine D4 receptor signaling

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<tr>
<td>7TM</td>
<td>Seven transmembrane</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
</tr>
<tr>
<td>AC</td>
<td>Adenyl cyclase</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention-deficit hyperactivity disorder</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase 1</td>
</tr>
<tr>
<td>BACK</td>
<td>BTB and C-terminal Kelch</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Bidi</td>
<td>Aqua bidest</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>BTB</td>
<td>Broad complex, Tramtrack, and Bric à Brac</td>
</tr>
<tr>
<td>C3IP1</td>
<td>Cullin3 interacting protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>coIP</td>
<td>Coimmunoprecipitation</td>
</tr>
<tr>
<td>D4R</td>
<td>Domaine D4 receptor</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>Dopamine and cAMP regulated phosphoprotein of 32 kD</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DRIPs</td>
<td>Dopamine receptor interacting proteins</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiolumthreitol</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitinating enzymes</td>
</tr>
<tr>
<td>EC</td>
<td>Extracellular</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmatic reticulum associated degradation</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide-exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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GIRK  G protein-coupled inwardly rectifying potassium channel
GPCR  G protein-coupled receptor
GRK  GPCR kinase
GTP  Guanosine triphosphate
HA  Hemagglutinin
HECT  Homologous to the E6-AP carboxyl terminus
HEK  Human embryonic kidney
IC  Intracellular
IC3  Intracellular loop 3
IF  Immunofluorescence
JNK  c-Jun terminal kinase
K  Lysine
kan  kanamycine
KLHL12  Kelch-like protein 12
LB  Lysogeny broth
L-DOPA  L-3,4-dihydroxyphenylalanine
LEGEST  Lab for Eukaryotic Gene Expression & Signal Transduction
MAPK  Mitogen-activated protein kinase
MEK  Mitogen-activated protein kinase
MKK4  Mitogen-Activated Protein Kinase Kinase 4
NE buffer  Nuclear extract buffer
NEM  N-ethylmaleimide
ON  Overnight
PEI  Polyethyleneimine
PKA  Protein kinase A
PM  Plasma membrane
PTM  Post-translational modification
R  Arginine
Raf  Rapidly accelerated fibrosarcoma
Rb  Retinoblastoma
RING  Really interesting new gene
RIPA  Radio-immunoprecipitation assay
Rluc  Renilla luciferase
vi
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>Roc1</td>
<td>RING of cullins 1</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulphate – polyacrylamide gelelectrophoresis</td>
</tr>
<tr>
<td>SFM</td>
<td>Serum free medium</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factors</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable number of tandem repeats</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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Figure 1.1: Structure of a family 1 GPCR. These receptors contain seven transmembrane helices that span the cell membrane and are connected by altering intracellular and extracellular loops. Posttranslational modifications are indicated: the palmitoylated cysteine at the C terminus, the disulfide bridge connecting the first and second extracellular loop and the glycosylation tree near the N terminus. The conserved DRY motif in the second intracellular loop is also indicated (red circles, white letters).

Figure 1.2: Activation of effectors by G proteins associated with GPCRs. 1) Binding of the ligand induces a conformational change of the receptor. 2) The activated receptor binds the Gα subunit. 3) The active receptor causes dissociation of GDP from Gα. 4) Binding of GTP to Gα, resulting in association of Gα with the effector. 5) Ligand dissociates from the receptor, Gα binds to effector. 6) Hydrolysis of GTP to GDP and coupled dissociation of Gα from effector, reassociation to Gβγ. Source: (Lodish et al, 2008).

Figure 1.3: Ternary complex models of GPCRs. Left, extended ternary complex model (ETC). This model takes different receptor conformations into account together with the possibility of constitutive GPCR activity. The active receptor (R*) can bind to the G protein (G) in the presence or absence of the ligand (A). Right, cubic ternary complex model (CTC). Both the active (R*) receptor as the inactive receptor (R*) can interact with the G protein in this model. Source: Devi, 2005.

Figure 1.4: GPCR MAPK signaling mechanisms. (a) Heterotrimeric G protein dependent activation of the ERK module, resulting in translocation of ERK to the nucleus. (b) β-arrestin-dependent activation of the ERK module, resulting in cytosolic retention of ERK. However signaling can also be dependent on both mechanisms. Source: (Lefkowitz & Whalen, 2004).

Figure 1.5: Desensitization and internalization of GPCRs. Desensitization and internalization is dependent on GRKs, β-arrestins and clathrin-coated pits. After activation of the receptor with the ligand, both the α and βγ subunits provoke a cellular response (arrow a and b). However, within seconds after activation GRKs are recruited and phosphorylate the receptor. Subsequently β-arrestins are recruited and block activation of the G protein and promote receptor endocytosis via clathrin-coated pits. After internalization, some GPCRs can still signal from the endosomal membrane (arrow c). Source: (Hanyaloglu & von Zastrow, 2008).

Figure 1.6: Influence of ubiquitination in GPCR internalization and trafficking. (a) Activation of GPCR by agonist induces recruitment of GRKs and binding of β-arrestin to the phosphorylated receptor. (b) Following activation, the receptor and β-arrestin are polyubiquitinated. (c) The GPCR is internalized to endosomes (d) and is recycled back to the membrane or (e) is degraded in lysosomes. Ubiquitination of the receptor is not necessary for internalization, but for endocytic sorting to lysosomes. β-arrestin ubiquitination, however, is necessary for internalization. Source: (Wojcikiewicz, 2004).

Figure 1.7: Ubiquitination mechanism. In the first ATP-driven step ubiquitin is conjugated to an E1 conjugating enzyme. In the second step ubiquitin is transferred from E1 to an E2 conjugating enzyme and finally ubiquitin is transferred to the substrate by an E3 ligase. Two types of E3 ligases exist: the RING class and the HECT class. RING E3s functions as adaptors,
while HECT E3s have catalytic activity. Ub can be removed by deubiquitinating enzymes and is recycled. Source: (Kerscher et al, 2006).

**Figure 1.8:** Schematic representation of the D₄ receptor with interaction partners and signaling aspects. PTM of the D₄ receptor are shown: glycosylation, phosphorylation and ubiquitination. The D₄ receptor couples to inhibiting Gᵢ proteins that block the conversion of ATP to cAMP through inhibiting AC. cAMP normally activates PKA, which can activate downstream effectors that translocate to the nucleus and activate transcription factors (TF). The IC3 loop of the D₄ receptor is interacting with KLHL12. β-arrestin2 binds the receptor near two constitutively phosphorylated residues and can also interact with KLHL12. MAPK activation of ERK1/2 can occur through G proteins and possibly via β-arrestin2.

**Figure 1.9:** Schematic illustration of KLHL12. KLHL12 consists of an N terminal BTB domain and a C-terminal Kelch domain (with six Kelch repeats), connected by a central BACK domain. Source: (Stogios & Prive, 2004)

**Figure 1.10:** Cullin3-based based E3 ligase complex. KLHL12 functions as an adaptor to connect the D₄ receptor to the Cullin3-based ubiquitination machinery. KLHL12 binds via its BTB domain to Cullin3 and via its Kelch domain to the D₄ receptor. Cullin3 forms a complex with Roc1, which recruits the E2 enzyme that is conjugated with ubiquitin.

**Figure 3.1:** Visualization of DNA bands on preparative agarose gel. Restriction enzyme digest of Etag-KLHL12 pGFP²-N3 resulted in two fragments (4724bp and 1766bp). The digest of the pEYFP-N3 vector resulted in a fragment of 4715bp. Fragment sizes were determined with the lambda DNA/PstI marker (indicated with arrows).

**Figure 3.2:** Agarose gel from AvrII and Nhel control restriction enzyme digest of pKLHL12-YFP. Agarose gel from the control restriction digest with AvrII and Nhel. M represents the length mark, λ/PstI.

**Figure 3.3:** Agarose gel from NotI control restriction enzyme digest of pKLHL12-YFP. Agarose gel from the restriction digest with NotI. M represents the length mark, λ/PstI.

**Figure 3.4:** Interaction of KLHL12-YFP with the HA D₄₀R or HA D₄₂R. Upper IP blots, IP was done with anti-HA16B12, blots were incubated with anti-GFP mouse to detect KLHL12-YFP (~90kDa) and with anti-HA rat to detect the receptor (mature HA D₄₂R ~52kDa, immature HA D₄₂R ~46kDa). Down, lysate blots were checked for protein expression of KLHL12-YFP.

**Figure 3.5:** Immunofluorescence pictures of HEK293T cells transfected with pKLHL12-YFP. In the upper left panel, the green channel shows the fluorescent signal from KLHL12-YFP. In the upper right panel, the red signal also originates from KLHL12-YFP (antibody staining was used, see text). In the lower left panel, DAPI stains the nuclei of the cells blue. In the lower right panel, a merged picture in all three channels is presented. A clear colocalization between the red and green channel can be noticed.

**Figure 3.6:** Graph plotting fluorescence versus concentration KLHL12-YFP. With increasing concentrations of KLHL12-YFP, the fluorescent signal rises. Values for 2µg and 4µg KLHL12-YFP were excluded (transfection error). The 0µg KLHL12-YFP corresponds to transfection with pcDNA3 alone. a.u., arbitrary units. Error bars represent SEM.

**Figure 3.7:** BRET principle. A GPCR is tagged with the donor enzyme, Rluc, while the GPCR-interacting protein is coupled to YFP. After addition of coelenterazine, it is oxidized by Rluc,
releasing CO₂ and light (480nm). If both proteins are not interacting, no resonant energy transfer will occur (a). If they are interacting, resonant energy transfer occurs and will lead to the emission of light by YFP (530nm). Source: (Poyner & Wheatley, 2010). ........................................ 25

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**Figure 3.10: Interaction of KLHL12 and β-arrestin2 with the different polymorphic variants of the D₄R.** Lysates of HEK293T cells transiently transfected with pHA D₄,R and pCmyc-β-arrestin2 or pEtag-KLHL12, were subjected to IP with anti-HA antibody. Co-immunoprecipitation of β-arrestin2 and KLHL12 was checked after IP (IP blots) using respectively anti-Cmyc and anti-Etag antibodies. In the lysate, β-arrestin2 gives two bands: one at 40kDa and one at 55kDa (lysate blot, lane 2-5), in IP only the band of 55kDa is present (IP blots, lane 2-5). KLHL12 has a size of 63kDa in lysate (lysate blot, lane 6-10) and IP (IP blot, lane 8-10). Receptor expression was checked in lysate (not shown) and IP (IP blots) with anti-HA (mature D₄,R ~52kDa, immature D₄,R ~46kDa). This experiment was done in fourfold. .................................................. 28

**Figure 3.11: Non-specific interaction of tagged β-arrestin2/β-arrestin1 constructs with beads.** Left, in IP there is no signal from Cmyc-β-arrestin2. Center, in IP there is a weak signal for both Flag-β-arrestin2 and β-arrestin1. Right, in IP there is a clear signal coming from GFP-β-arrestin2 and β-arrestin2-GFP2. .......................................................................................... 29

**Figure 3.12: Interaction of β-arrestin2 with the different polymorphic variants of the D₄R.** Lysates of HEK293T cells transiently transfected with pHA D₄,R and pFlag-β-arrestin2, were subjected to IP with anti-Flag antibody. Expression of HA D₄,R (lysate blot, anti-HA, lane 3-8) and Flag-β-arrestin2 (lysate blot, anti-Flag, lane 2, 4-8) was checked in lysate. Co-immunoprecipitation of the receptor was checked after IP (IP blots, lane 4-8) using anti-HA antibody. .......................................................................................... 29

**Figure 3.13: Interaction of the β-arrestin2 deletion mutants with the D₄₂,R.** Lysates of HEK293T cells transiently transfected with pHA D₄₂,R and pFlag-β-arrestin2 WT or its deletion mutants pFlag-β-arrestin2 CT, pFlag-β-arrestin2 NT, were subjected to IP with anti-Flag antibody. In the lysate, Flag-β-arrestin2 gives two bands: one at 40kDa and one at 55kDa (lysate blot, lane 2 and 6), in IP only the band of 55kDa is present (IP blots, lane 2 and 6). Flag-β-arrestin2 CT (IP and lysate blots, lane 3 and 7) and Flag-β-arrestin2 NT (IP and lysate blots, lane 4 and 8) give bands of respectively 35kDa and 20kDa. Receptor expression was checked in lysate (not shown) and IP (IP blots) with anti-HA. Co-immunoprecipitation of the D₄₂,R was checked in IP (IP blots, lane 6-8) using anti-HA antibody. The mature D₄₂,R and immature D₄₂,R can be found respectively at ~52kDa and ~46kDa.................................................. 30

**Figure 3.14: Competition BRET assay between the D₄,R and β-arrestin2/KLHL12.** A constant amount of D₄,R-Rluc and β-arrestin2-GFP² were used, together with an increasing amount of KLHL12. Ratios are expressed as mean ± SEM from three experiments (n=3). One way
analysis of variance (ANOVA) was used for statistical evaluation. Post-hoc tests were used to address significant differences between groups. ***p<0.001. Source: (Skieterska, Unpublished results).

**Figure 3.15: Competition colP between D4R and β-arrestin2/KLHL12.** Lysates of HEK293T cells transiently transfected with constant amounts of pHA D4R and pCmyc-β-arrestin2, and with increasing amounts of pEtag-KLHL12 (0.6µg, 1.5µg, 3µg, 4.5µg, 6µg, 9µg), were subjected to IP with anti-HA antibody. Expression of Etag-KLHL12 (lysate blot, anti-Etag, lane 4, 6-11) and Cmyc-β-arrestin2 (lysate blot, anti-Cmyc, lane 2, 5-11) were checked in lysate. Receptor levels were monitored with anti-HA (IP blot, anti-HA, 3, 5-11). Co-immunoprecipitation of Cmyc-β-arrestin2 was confirmed in IP (IP blots, anti-Cmyc, lane 2, 5-11) using anti-Cmyc antibody.

**Figure 3.16: Double colP ubiquitination assay.** Lysates of HEK293T cells transiently transfected with HA D4.2R, FlagUb and Cmyc-β-arrestin2 and/or KLHL12 were subjected to IP with anti-HA antibody. Expression of Etag-KLHL12 (lysate blot, anti-Etag, lane 5, 7), Cmyc-β-arrestin2 (lysate blot, anti-Cmyc, lane 6, 7) and Flag-Ub (lysate blot, anti-Flag, lane 3-7) were checked in lysate. Receptor levels were examined (IP blot, anti-HA, 2, 4-7) and can be considered equally strong. The ubiquitination pattern of the D4R was monitored by immunoblotting with anti-Flag antibody (IP blot, anti-Flag, 4-7). The experiment was done in threefold.

**Figure 3.17: Quantification of the ubiquitination signal of the D4R.** Upper, the ubiquitination signal of the D4R is compared between setups. Lower, the normalized ubiquitination signal of the D4R is compared between setups. Quantification was performed using ImageJ. a.u., arbitrary units.

**Figure 3.18: Theoretical saturation binding plot.** Graphical representation of the saturation binding plot. From the specific binding curve, the $B_{max}$ (total number of receptors) and $K_D$ (concentration of radioligand at half-maximal binding of the receptors) can be determined. Source: (Vauquelin & Von Mentzer, 2007).

**Figure 3.19: Saturation binding plots of the D4.2R with pcDNA3 or KLHL12.** In both graphs total, non-specific and specific binding are shown. Top, saturation curve of D4.2R with pcDNA3. Bottom, saturation curve of D4.2R with KLHL12. Non-linear regression is performed and the resulting $B_{max}$ and $K_D$ values with their standard errors are listed in the corresponding tables.

**Figure 3.20: Scatchard plots of the D4.2R with pcDNA3 or KLHL12.** Top, Scatchard plot of D4.2R with pcDNA3. Bottom, Scatchard plot of D4.2R with KLHL12. Linear regression was performed and the resulting $B_{max}$ (X-intercept) and $K_D$ (-1/slope) values are listed.

**Figure 3.21: Theoretical competition binding plot.** The percentage bound radioligand is plotted against the concentration competitor (here: dopamine). From this graph the $IC_{50}$ can be determined, the concentration of dopamine at half-maximal binding of the radioligand. From the $IC_{50}$ and the $K_D$ from the saturation experiment, the $K_i$ of the competitor can be calculated. Source: (Vauquelin & Von Mentzer, 2007).

**Figure 3.22: Competition binding plot from the D4.2R with pcDNA3 or KLHL12.** The $IC_{50}$ values from the setup with the D4.2R and pcDNA3, and the setup with D4.2R and KLHL12 are given.
Figure 3.23: EIA cAMP assay. Upper left, plates are precoated with mouse monoclonal anti-rabbit IgG and blocked with a certain mix proteins. Upper right, the wells are incubated with cAMP Tracer, antiserum specific for cAMP and with standard or sample. Lower left, the wells are washed to remove unbound reagents. Lower right, Ellman’s Reagent, the substrate for the acetylcholinesterase, is added. A yellow solution is formed of which the absorbance at 412nm can be measured using a spectrophotometer. Source: (Website_Cayman). 

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Summary

In this thesis, the interaction between the D₄ receptor (D₄R) variants, KLHL12 and β-arrestin2 was characterized. To this end, bioluminescence resonance energy transfer (BRET) was introduced as a novel technique in the lab. This technique required the cloning of the acceptor fluorophore, KLHL12-YFP. The correctness of the KLHL12-YFP construct was confirmed via sequencing and the expression was checked via three different tests. First, expression was confirmed on western blot. Via immunofluorescence experiments it was shown that the green fluorescent signal specifically originated from KLHL12-YFP. Finally, it was shown that when cells were transfected with increasing amounts of pKLHL12-YFP, the fluorescent signal was increased. Together, these three tests confirmed the expression of KLHL12-YFP in different experimental setups. Furthermore, it was shown on western blot that KLHL12-YFP specifically interacts with the D₄₂R and not with the D₄₀R, indicating that the YFP-tag did not influence the interaction properties of KLHL12. With this construct in hand, saturation BRET experiments were performed. This experiment aimed to determine the optimal ratio of donor and acceptor plasmid. However, the obtained saturation curves impeded us from determining this optimal ratio. The main problem was the occurrence of negative BRET ratios. Possible explanations for these negative BRET ratios are the high autofluorescence of the HEK293T cells or the suboptimal filter settings that were used.

Next, co-immunoprecipitation experiments were performed to study the interaction of β-arrestin2 with the D₄R and the role of KLHL12 on this interaction. It was shown that the polymorphic region in the third intracellular loop of the D₄R is not crucial for the binding of β-arrestin2 to the D₄R. Furthermore, by using β-arrestin2 deletion mutants, it was revealed that both the N domain as the C domain are able to interact separately with the D₄R. It was also shown that KLHL12 is able to displace β-arrestin2 from the receptor, confirming results previously obtained by LEGEST. Next, it was revealed that β-arrestin2 can influence the ubiquitination signal of the D₄R. When KLHL12 and β-arrestin2 are present together, the increase in the ubiquitination signal was lower compared to the increase in D₄R ubiquitination with KLHL12 alone.

The functional implications of D₄R ubiquitination and the influence of KLHL12 and β-arrestin2 in this process, were also addressed in this thesis. Via saturation binding experiments, it was shown that overexpression of KLHL12 does not decrease the D₄R expression, because similar B_{max} values were obtained as in the control experiment. Competition binding experiments showed that the affinity of dopamine for the receptor remained unchanged upon overexpression of KLHL12. Through cAMP assays with the D₄₂R and overexpression of KLHL12, it was revealed that a decrease in cAMP levels was obtained at higher concentrations of PD-168077 (D₄R specific agonist), compared to the control setup with pcDNA3. This indicated that KLHL12 could have an influence on cAMP levels. Finally, MAPK phosphorylation assays with the D₄₀R, D₄₂R and D₄₂,2₄KR were performed. From these experiments it was concluded that KLHL12 does not have an influence on MAPK activation, while β-arrestin2 significantly decreased the MAPK activity for each receptor variant.

Together, these results provide additional insight into the roles of ubiquitination, KLHL12 and β-arrestin2 in D₄R signaling. More research will be required in order to unravel the complete spectrum of D₄R signaling properties.
Samenvatting

In deze thesis werd de interactie tussen de D₄ receptor (D₄R) varianten, KLHL12 en β-arrestin2 gekarakteriseerd. Hiervoor werd BRET als een nieuwe techniek geïntroduceerd in het labo. Deze techniek vereiste het kloneren van de acceptor fluorofoor, KLHL12-YFP. De juistheid van dit construct werd bevestigd via sequencerig en de expressie ervan werd getest via drie verschillende experimenten. Eerst werd de expressie bevestigd op western blot. Via immunofluorescentie werd gedemonstreerd dat het groen fluorescent signaal specifiek afkomstig was van KLHL12-YFP. Finaal werd aangetoond dat cellen getransfecteerd met toenemende hoeveelheden KLHL12-YFP, een toename in fluorescent signaal vertonen. Deze drie experimenten bevestigden de expressie van KLHL12-YFP in verschillende experimentele condities. Verder, werd via western blot aangetoond dat KLHL12-YFP specifiek interageert met de D₄.2R en niet met de D₄.0R, wat aantoont dat de YFP-tag de interactie eigenschappen van KLHL12 niet beïnvloedt. Met behulp van dit coonstruct werden saturatie BRET experimenten uitgevoerd. Dit experiment had als doel de optimale ratio van donor en acceptor plasmiden te bepalen. Echter, de verkregen saturatie curves lieten niet toe om deze optimale ratio af te leiden. Mogelijke verklaringen voor deze negatieve BRET ratio’s zijn de hoge autofluorescentie van de HEK293T cellen of de suboptimale filterinstellingen. Vervolgens werden co-immunoprecipitatie experimenten uitgevoerd om de interactie tussen β-arrestin2 en de D₄R te bestuderen evenals de invloed van KLHL12 op deze interactie. Er werd aangetoond dat de polymorfe regio in de derde intracellulaire lus van de D₄R niet cruciaal is voor de binding van β-arrestin2 met de receptor. Bovendien werd met β-arrestin2 deletie mutanten bewezen dat zowel het N domein als het C domein afzonderlijk kunnen interageren met de D₄R. Er werd ook aangetoond dat KLHL12 in staat is β-arrestin2 te verdringen van de receptor, wat eerdere resultaten van LEGEST bevestigd. Vervolgens werd aangetoond dat β-arrestin2 het ubiquitinatie signaal van de D₄R kan bevorderen. Wanneer KLHL12 en β-arrestin2 samen aanwezig waren, was de toename in het D₄R ubiqutinatie signaal lager dan de toename in D₄R ubiquitinatie door KLHL12 alleen. De functionele implicaties van D₄R ubiquitinatie en de invloed van KLHL12 en β-arrestin2 in dit proces, werden ook behandeld in deze thesis. Via saturatie bindingsexperimenten werd aangetoond dat overexpressie van KLHL12 de D₄R expressie niet doet afnemen, omdat vergelijkbare B_max waarden werden verkregen als in het controle experiment. Competitie bindingsexperimenten toonden aan dat de affiniteit van dopamine voor de receptor onveranderd blijft wanneer KLHL12 wordt overgeëxpressé. Via cAMP experimenten met de D₄.2R en overexpressie van KLHL12, werd aangetoond dat een afname in de cAMP niveaus pas werd bekomen bij hogere concentraties PD-168077 (D4R specifieke agonist), in vergelijking met het controle experiment. Dit betekent dat KLHL12 een invloed kan hebben op de cAMP niveaus. Finaal werden MAPK fosforylatie experimenten met de D₄.0R, de D₄.2R en de D₄.24KR uitgevoerd. Uit deze experimenten werd besloten dat KLHL12 geen invloed heeft op de MAPK activatie, terwijl β-arrestin2 de MAPK activiteit significant verlaagt voor elke D₄R variant. Deze resultaten verschaffen extra inzicht in de invloed van ubiquitinatie, KLHL12 en β-arrestin2 in D₄R signalisatie. Meer onderzoek is vereist om het complete spectrum aan signalisatie eigenschappen van de D₄R op te helderen.
Chapter 1: Introduction

1.1. G protein-coupled receptors

G protein-coupled receptors (GPCRs) are the largest plasma membrane receptor family and comprise at least 1000 members. GPCRs are important drug targets since almost 50% of all marketed drugs are ligands for these receptors. They represent about 3% of the human genome (Vauquelin & Von Mentzer, 2007).

1.1.1. Structure and organisation

Structurally GPCRs consist of seven transmembrane-spanning α-helices, connected by three intracellular and extracellular loops (Figure 1.1). Therefore GPCRs are also called seven transmembrane (7TM) receptors. The amino terminus is located on the extracellular side and the carboxy terminus on the intracellular side. The ligand docking site is located extracellular and consists of a cavity formed by the helices (Pangalos & Davies, 2002; Vauquelin & Von Mentzer, 2007).

![Figure 1.1: Structure of a family 1 GPCR](image)

These receptors contain seven transmembrane helices that span the cell membrane and are connected by altering intracellular and extracellular loops. Posttranslational modifications are indicated: the palmitoylated cysteine at the C terminus, the disulfide bridge connecting the first and second extracellular loop and the glycosylation tree near the N terminus. The conserved DRY motif in the second intracellular loop is also indicated (red circles, white letters).

GPCRs can undergo several posttranslational modifications including N-glycosylation, palmitoylation, phosphorylation, and ubiquitination (Vauquelin & Von Mentzer, 2007). At the amino terminus, potential glycosylation sites are found. Phosphorylation sites are located at the C-terminus and at the third intracellular loops and play an important role in the regulation of receptor activity. Ubiquitination of GPCRs is also involved in receptor regulation and will be discussed later in the introduction. The majority of the receptors also contain a conserved C-terminal cysteine which is palmitoylated, causing the formation of an extra intracellular loop. Often, GPCRs also contain disulfide bonds between conserved cysteine residues located at the extracellular loops (Figure 1.1).

The GPCR superfamily has been divided into three subfamilies: the rhodopsin-like family (family 1), the secretin receptor family (family 2) and family 3 receptors, including γ-aminobutyric acid (GABA) and glutamate receptors. Family 3 receptors have typically very large extracellular domains. The rhodopsin-like receptor family compromises 90% of all GPCRs. Family 1 receptors contain a conserved DRY motif (Figure 1.1) and do not have large extracellular domains, in contrast to the family 2 receptors (Pangalos & Davies, 2002; Van Craenenbroeck, 2011-2012). Family 2 receptors have a large N-terminus that contains
several cysteine residues which form a disulfide bond network. Although family 1 and 2 receptors are morphologically similar, they share no sequence homology (Vauquelin & Von Mentzer, 2007).

1.1.2. Signaling via G-proteins

GPCRs respond to a diverse set of signals, including amines, amino acids, ions, lipids, peptides, large proteins, light and odorants. The interaction between the ligand and the receptor, induces a conformational change in the receptor which is sensed by a G protein. This G protein forms the central element of signal transduction between the receptor and the effectors (e.g. enzymes or ion channels) (Vauquelin & Von Mentzer, 2007). It are heterotrimeric proteins, consisting of an α, β and γ subunit. Moreover, G proteins are not integral membrane proteins, they are anchored to the membrane via the α and γ subunit through covalent attached lipids (Lodish et al, 2008; Vauquelin & Von Mentzer, 2007). The α subunit has two functions: it binds guanosine diphosphate (GDP) and guanosine triphosphate (GTP) and has GTPase activity. The β and γ subunits are always tightly linked as a heterodimeric Gβγ and also plays a role in signaling.

In the inactive state, when no ligand is bound to the receptor, the α subunit is bound to GDP and forms a complex with βγ dimer (Figure 1.2). Upon activation, the receptor undergoes a conformational change and the cytosolic domains are now accessible for G protein binding. Especially the C terminal region and the third intracellular loop (sometimes also the second intracellular loop) are important for the interaction with the G protein.

![Figure 1.2: Activation of effectors by G proteins associated with GPCRs.](image)

1) Binding of the ligand induces a conformational change of the receptor. 2) The activated receptor binds the Gα subunit. 3) The active receptor causes dissociation of GDP from Gα. 4) Binding of GTP to Gα, resulting in association of Gα with the effector. 5) Ligand dissociates from the receptor, Gα binds to effector. 6) Hydrolysis of GTP to GDP and coupled dissociation of Gα from effector, reassociation to Gβγ. Source: (Lodish et al, 2008).

The activated receptor causes a conformational change in the G protein, triggering the release of GDP from Gα. The ligand-receptor complex thus acts as a guanine nucleotide-
exchange factor (GEF) for Gα. Next, GTP quickly replaces GDP and causes dissociation of Gα from Gβγ and the receptor. The membrane anchored Gα-GTP then interacts with the effector protein (e.g. adenylyl cyclase), resulting in an activation or inhibition of the effector. Also the Gβγ subunit can interact with effectors and play a role in the signal transduction. Next, the GTPase activity of the α subunit will induce hydrolysis of GTP to GDP. The effector increases the rate of hydrolysis and thus acts as a GAP (GTPase activating enzyme). The hydrolysis dissociates Gα from the effector and reassociates Gα with Gβγ (Lodish et al, 2008). However, G proteins are stable molecules with long half-lives and the described regulatory mechanism does not explain the rapid termination of G protein signaling (De Vries et al, 2000). Rapid turnover of G proteins is accomplished by the regulator of G protein signaling (RGS) proteins. These RGS proteins negatively regulate G proteins by acting as a GAP. The GTPase activity of the RGS proteins accelerates GTP hydrolysis and returns the Gα protein quickly to its GDP-bound inactive state, followed by reassembly of the trimeric Gαβγ complex (De Vries et al, 2000).

Several receptor activation models have been defined to capture the effect of agonist activation on G protein binding to the receptor. In the original ternary complex model (TCM), only an agonist-bound activated receptor can form a complex with a G protein, which results in its activation (Devi, 2005). Next, novel features were included in the model such as the existence of different receptor conformations and the possibility of constitutive GPCR activity. This indicated that GPCRs could couple to and activate G proteins in the absence of an agonist (Devi, 2005). In the extended ternary complex model (ETC), active and inactive receptor configurations are taken into account, together with the notion that only the activated receptor can interact with the G protein in the presence or absence of the ligand. The more complete but more complex model, is the cubic ternary complex model (CTC) in which both the inactive as the active receptor can interact with the G protein. (Devi, 2005; Kenakin, 2004).

**Figure 1.3: Ternary complex models of GPCRs.** Left, extended ternary complex model (ETC). This model takes different receptor conformations into account together with the possibility of constitutive GPCR activity. The active receptor (R*) can bind to the G protein (G) in the presence or absence of the ligand (A). Right, cubic ternary complex model (CTC). Both the active (R*) receptor as the inactive receptor (R*) can interact with the G protein in this model. Source: Devi, 2005.

G proteins are divided in four families, based on the amino acid sequence and the function of the Gα subunit. The Gi subfamily consists of inhibitory Gα subunits and negatively regulates adenylyl cyclase (AC). The Gs subfamily on the other hand consists of stimulatory Gα proteins and stimulates AC activation. Once activated, AC catalyses the production of cyclic adenosine monophosphate (cAMP) and cAMP activates protein kinases (e.g. protein kinase A, PKA). The other two subfamilies are the Gq and G12 subfamily (Pangalos & Davies, 2002; Van Craenenbroeck, 2011-2012).
1.1.3. Signaling via β-arrestins

Next to signaling via heterotrimeric G proteins, it has been shown that GPCRs can also signal via G protein independent mechanisms. In this context, β-arrestin proteins play an important role.

The arrestin family is a small gene family that consists of four members (Lefkowitz & Whalen, 2004). All members interact with GPCRs after receptor activation by GRKs. Arrestin1 and arrestin4 are found only in retinal cones and rods, where they regulate color opsins and rhodopsin. On the other hand, β-arrestin1 and β-arrestin2 (also known as arrestin2 and arrestin3) are expressed ubiquitously in all cells and tissues (Lefkowitz & Whalen, 2004). X-ray structures of the inactive state show that arrestin is an elongated molecule with a central polar core flanked by two domains, the N and C domain, which are connected by a linker region (the C tail) (Han et al, 2001; Hirsch et al, 1999). Next to their well-known roles in desensitization and endocytosis (see further), β-arrestins can also act as adaptors or scaffolds for different signaling proteins (Lefkowitz & Shenoy, 2005; Lefkowitz & Whalen, 2004).

A well-studied β-arrestin-mediated signaling system is that leading to mitogen-activated protein kinase (MAPK) activation. MAP kinases are family of conserved serine/threonine kinases that are involved in kinase cascades consisting of MAPKKKs (e.g. Raf, a MAP kinase kinase kinase), MAPKKs (e.g. MEK, a MAP kinase kinase) and the MAPKs (e.g. ERK1/2, a MAP kinase) (DeWire et al, 2007; Lefkowitz & Shenoy, 2005). There are three families of MAPK including extracellular signal-regulated kinase 1/2 (ERK1/2, also known as p44/p42 MAPK), c-Jun terminal kinases (JNK1, JNK2, JNK3) and p38 kinases (isoforms α, β, γ, δ). The activation of ERK1/2 can be considered as a prototypical MAPK signaling process. ERK1/2, a MAPK, is phosphorylated by MEK, a MAPKK. MEK itself is phosphorylated by Raf, a MAPKKK. This mechanism holds true for all MAPK family members; a MAPKKK activates a MAPKK, which activates a MAPK (DeWire et al, 2007). Classically, activated MAPK translocate to the nucleus where they activate transcription factors which regulate cellular processes such as cell division, differentiation and apoptosis. In the context of GPCRs this process is mediated through G proteins (Figure 1.4) (Lefkowitz & Whalen, 2004). However MAP kinases can also activate various cytosolic substrates. The conundrum that appears here is how the cell is able to organize the different MAPK pathways with specificity, efficiency and reproducibility (Lefkowitz & Shenoy, 2005). The answer lies in the fact that scaffolding molecules are used to ensure that the appropriate kinases are recruited in a specific MAPK module. β-arrestin2 acts as a scaffolding molecule and binds to the components of the ERK-activating module (Raf, MEK, ERK) and the JNK module (ASK1, MKK4, JNK3). However receptor-β-arrestin2 complexes are not only associated with the activation of ERK, but also with its cytosolic retention, thereby inhibiting phosphorylation of nuclear transcription factors (Figure 1.4). Cytosolic ERK can phosphorylate cytosolic, plasma membrane and cytoskeletal proteins or can activate other kinases which can translocate to the nucleus and regulate transcription (Lefkowitz & Shenoy, 2005; Lefkowitz & Whalen, 2004).
1.4. Regulation of GPCRs

There are also mechanisms necessary to terminate receptor activity and signaling cascades in order to prevent overstimulation and to allow renewed responsiveness towards new stimuli. These mechanisms include desensitization, internalization, endocytic receptor trafficking and downregulation.

GRKs are a seven-membered family of kinases, which show significant sequence homology. All GRKs have a similar functional organization: a central catalytic domain, an amino terminal domain that is important for substrate recognition and a carboxy terminal domain that mediates the targeting of the kinase to the plasma membrane (Vauquelin & Von Mentzer, 2007). These kinases preferentially phosphorylate receptor which are activated by agonists. They phosphorylate the receptor at serine/threonine residues located in the third intracellular loop and the carboxy terminus. Phosphorylation of the receptor alone has no influence on G protein coupling. However by phosphorylating the receptor, GRKs stabilize the conformation of the receptor in such a way that interaction with β-arrestins is promoted. Thus the affinity of β-arrestin for the receptor is greatly increased by receptor phosphorylation. Mostly, β-arrestins interact only with GPCRs after phosphorylation of the receptor but β-arrestin binding to the receptor can also occur in absence of GRK-dependent phosphorylation. The cell makes use of this mechanism to terminate G protein-dependent signaling after activation of GPCRs. After agonist-promoted activation of the GPCR, GRKs are rapidly recruited and phosphorylate the GPCR. This leads to subsequent recruitment of β-arrestins, which sterically inhibit the binding of the G protein to the receptor and thus terminate G protein-mediated signaling. This process is called desensitization and occurs within seconds after agonist activation of the receptor (Figure 1.5) (Hanyaloglu & von Zastrow, 2008). However one can discriminate between homologous and heterologous desensitization. One speaks of homologous desensitization when an agonist only has an effect on the activity of its own receptor. This form of desensitization is thus mediated by
β-arrestins. The term heterologous desensitization is used when unrelated drugs affect the activity of the receptor (increase or decrease in activity) (Vauquelin & Von Mentzer, 2007).

β-arrestins can also play a role in signaling (see 1.1.3) and internalization of the receptors via endocytosis. β-arrestins can act as adaptors targeting GPCRs to clathrin-coated pits (Hanyaloglu & von Zastrow, 2008; Marchese et al, 2008). After binding to GPCRs, β-arrestins undergo a conformational change whereby their C terminal domain is exposed. This domain can bind clathrin and the β2 subunit of the AP-2 (adapter protein complex-2), proteins of the endocytic machinery (Figure 1.5). This endocytic process is dependent on dynamin, a GTPase that helps in constricting the clathrin vesicles (with the GPCR) from the plasma membrane. Internalization is a process that occurs over a period of minutes after agonist-mediated activation. The internalized receptors can undergo different fates: either resensitization (recycling of the receptor to the membrane) or downregulation (receptor degradation) occurs.

**Figure 1.5: Desensitization and internalization of GPCRs.** Desensitization and internalization is dependent on GRKs, β-arrestins and clathrin-coated pits. After activation of the receptor with the ligand, both the α and βγ subunits provoke a cellular response (arrow a and b). However, within seconds after activation GRKs are recruited and phosphorylate the receptor. Subsequently β-arrestins are recruited and block activation of the G protein and promote receptor endocytosis via clathrin-coated pits. After internalization, some GPCRs can still signal from the endosomal membrane (arrow c). Source: (Hanyaloglu & von Zastrow, 2008)

Sorting between receptor recycling and degradation is often called endocytic receptor trafficking and is dependent on the stability of the receptor-β-arrestin complex and the ubiquitination status of both the receptor and β-arrestin (Hanyaloglu & von Zastrow, 2008; Shenoy & Lefkowitz, 2004; Wojcikiewicz, 2004). It has been shown that receptor ubiquitination is not necessary for receptor internalization, but rather targets internalized receptors to lysosomes for degradation. However β-arrestin ubiquitination, which is an agonist-driven process (ubiquitination occurs after receptor binding), is necessary for intracellular trafficking (Figure 1.6). The reason is that polyubiquitination of β-arrestin modulates the stability and the links with the endocytic proteins. In this context two classes of receptors exist: class A and class B receptors (Shenoy & Lefkowitz, 2004; Wojcikiewicz, 2004). Class A receptors only provoke transient ubiquitination of β-arrestin (β-arrestin2 > β-arrestin1) and rapidly dissociate from it upon internalization. These receptors rapidly recycle back to the membrane. Class B receptor, however, induce more stable ubiquitination of β-
arrestin and remain bound to it upon internalization. These receptor-β-arrestin complexes remain for prolonged period in the endocytic vesicles before being recycled back to the membrane or degraded in lysosomes.

![Diagram of GPCR internalization and trafficking](image)

**Figure 1.6: Influence of ubiquitination in GPCR internalization and trafficking.** (a) Activation of GPCR by agonist induces recruitment of GRKs and binding of β-arrestin to the phosphorylated receptor. (b) Following activation, the receptor and β-arrestin are polyubiquitinated. (c) The GPCR is internalized to endosomes (d) and is recycled back to the membrane or (e) is degraded in lysosomes. Ubiquitination of the receptor is not necessary for internalization, but for endocytic sorting to lysosomes. β-arrestin ubiquitination, however, is necessary for internalization. Source: (Wojcikiewicz, 2004)

Downregulation, as previously mentioned, refers to degradation of the receptor after persistent contact with agonists. This process is often seen in pathological circumstances, e.g. tumors which continuously secrete hormones or neurotransmitters. However, also from therapeutic points of view downregulation is important, because it can be the mechanism after tolerance (Vauquelin & Von Mentzer, 2007).

### 1.1.5. Ubiquitination of GPCRs

Most of the proteins in a cell undergo post-translational modifications (PTM) (Komander, 2009). One of these PTM is ubiquitination, in which a substrate protein is modified with a small polypeptide of 76 amino acids (AA), called ubiquitin and which is ubiquitously expressed in every eukaryotic cell. Ubiquitin is attached covalently via the carboxyl group of its C terminal glycine, to the ε-amino group of a lysine residue that is present in the substrate. The result is the formation of an amide (isopeptide) bond between ubiquitin and the substrate (Kerscher et al, 2006; Komander, 2009). This reaction is performed by a three-step enzymatic cascade (Figure 1.7). In a first ATP-driven step, ubiquitin is activated by an E1 ubiquitin-activating enzyme, resulting in the formation of a thioester linkage. In the second step, ubiquitin is transferred from E1 to an E2 ubiquitin-conjugating enzyme. Finally, ubiquitin is covalently attached to its substrate by an E3 ubiquitin-ligase. Two E1 enzymes, 37 E2 enzymes and more than 600 E3 enzymes are coded in the human genome. Moreover, ubiquitinated proteins are recognized by more than 20 different UBDs (ubiquitin binding domains). Important to note is that ubiquitination is a reversible process and that about 85 deubiquitinating enzymes (DUB) are known today (Komander, 2009). Furthermore, there are two types of E3 enzymes: the really interesting new gene (RING) class and the homologous to the E6-AP carboxyl terminus (HECT) class (Kerscher et al, 2006). RING E3s function as a
kind of adaptor that simultaneously binds the E2 enzyme conjugated with ubiquitin and the substrate. In this way transfer of ubiquitin to the substrate is facilitated. HECT E3s function via a different mechanism: ubiquitin is first transferred to E3 and then to the substrate (Figure 1.7). In contrast to RING E3s, HECT E3s thus have a catalytic function. An example of a RING type E3 ligase, is the BTB-Cullin3-Roc1 complex (see further).

Figure 1.7: Ubiquitination mechanism. In the first ATP-driven step ubiquitin is conjugated to an E1 conjugating enzyme. In the second step ubiquitin is transferred from E1 to an E2 conjugating enzyme and finally ubiquitin is transferred to the substrate by an E3 ligase. Two types of E3 ligases exist: the RING class and the HECT class. RING E3s functions as adaptors, while HECT E3s have catalytic activity. Ub can be removed by deubiquitinating enzymes and is recycled. Source: (Kerscher et al, 2006)

In general, three different ubiquitination layouts exist: mono-ubiquitination, multi-mono-ubiquitination and polyubiquitination (Komander, 2009). Ubiquitin itself contains seven lysine residues (position 7, 11, 27, 29, 33, 48 and 63), which can act as acceptors for other ubiquitin molecules and give rise to ubiquitin polymers (polyubiquitination). Depending on the specific lysine used, polyubiquitin chains can be called, for example, K48 when lysine 48 is used to form the ubiquitin polymer. Next to these homotypic polyubiquitin chains, also heterotypic polyubiquitin chains exist. The latter are either mixed linkages (more than one type of lysine residue is involved) or branched linkages. However, when a single ubiquitin molecule is attached to only one substrate lysine, one refers to mono-ubiquitination, while when attached to several substrate lysines, one speaks of multi-mono-ubiquitination. These different ubiquitination layouts are involved in different cellular processes. Multi-mono-ubiquitination of cell surface receptors, for example, triggers their internalization and subsequent degradation in lysosomes or their recycling to the plasma membrane. Mono-ubiquitination, on the other hand, is involved in the DNA damage response. Likewise, the different polyubiquitin chains have different functions. For example, K48-linked polyubiquitination targets the proteins to the proteasome for degradation and K63-linked polyubiquitination plays a role in cell-surface receptor internalization, DNA repair, inflammation and activation of several protein kinases. Also, different polyubiquitin chains
adapt different folds: K48 chains adapt a rather compact fold, while K63 chains show a more elongated, open conformation (Komander, 2009).

Ubiquitination of GPCRs plays an important role in their regulation. Two major mechanisms are known so far: agonist-dependent and agonist-independent ubiquitination (Shenoy, 2007). In absence of a ligand, some GPCRs are ubiquitinated, targeting them for proteasomal degradation. This leads to a basal turnover of membrane-associated receptors. Agonist-independent ubiquitination can also occur via the endoplasmatic reticulum (ER) associated receptor (ERAD) pathway. This is a quality control mechanism that promotes ubiquitination of misfolded proteins (here GPCRs) in the ER, which targets them for proteasomal degradation. The second is the agonist-dependent ubiquitination pathway. Studies in prototypic β2-adrenergic receptors show that agonist-induced ubiquitination of GPCRs is not required for internalization but targets the receptors for lysosomal degradation (Shenoy et al, 2001). GPCR internalization, on the other hand, requires the recruitment and ubiquitination of β-arrestins, following agonist-stimulation (Shenoy, 2007). Thus, both pathways eventually lead to GPCR degradation. However ubiquitination of GPCRs not necessarily leads to receptor degradation; this will be illustrated further (section 1.2.3) for the dopamine D₄ receptor.

1.2. Dopamine receptors

1.2.1. Dopamine

Dopamine is a hormone and neurotransmitter in the brain and is released by the hypothalamus. Chemically, it is a catecholamine, and is present in exceptionally high concentrations in the basal ganglia. Dopamine regulates several basic functions in the brain such as motor activity, behavior and cognition, attention, learning and memory, motivation and reward, sleep, mood, sexual behavior (D₄R_Database; Iversen & Iversen, 2007). Its hormonal function lies in the inhibition of prolactin release. Dysfunction of dopaminergic signaling has been associated with different neurological disorders, such as Parkinson’s disease, schizophrenia, attention-deficit hyperactivity disorder (ADHD) and addiction (Rondou et al, 2010a). In Parkinson’s disease dopamine can be administered as medication, but under the form of its precursor molecule L-3,4-dihydroxyphenylalanine (L-DOPA), since dopamine itself cannot cross the blood-brain barrier (BBB).

Dopaminergic neurons, which are neurons that primarily use dopamine as a neurotransmitter, represent only a small fraction of the neuronal population in the central nervous system (CNS), but through their enormous branching network, they influence large areas of the brain. Dopamine is not considered simply as an excitatory of inhibitory neurotransmitter, but as a neuromodulator that changes the response of target neurons to other neurotransmitters. Thus, dopamine is not involved in fast synaptic transmission, but rather modulates it by inducing slow-acting effects through signaling pathways (Rondou et al, 2010a).

1.2.2. The dopamine D₄ receptor and its remarkable polymorphism

Dopamine exerts its effects through binding to dopamine receptors. Generally, dopamine receptors are divided into two subfamilies: the D₁-like receptors (D₁ and D₅ receptors) and the D₂-like receptors (D₂, D₃ and D₄ receptors) (Neve, 2010; Pangalos & Davies, 2002). In contrast to D₁-like receptor genes which only consist of exons, the D₂-like receptor exons are
separated by introns allowing the formation of different receptor variants. Another remarkable difference between D₁ and D₂-like receptors is that D₂-like receptors have a long third intracellular loop, while D₁-like receptors have a short third intracellular loop (IC3).

The dopamine D₄ receptor (D₄R) gene is located at chromosome 11p15.5 and consists of four exons. The D₄R gene is about 3400bp in size and is highly polymorphic with several remarkable polymorphisms (Neve, 2010). However, the most extensive polymorphism is located in exon 3, in a region that codes for the third intracellular loop and is a 48bp tandem repeat. This polymorphism consists of a 2-11 repeats (variable number of tandem repeats (VNTR)), giving rise to several polymorphic receptor variants, denoted as D₄.x, where x is the number of repeats (Neve, 2010). The 48bp VNTR has been associated with neuropsychiatric diseases such as ADHD, schizophrenia and with alterations in personality, especially with novelty seeking.

The allele frequencies of the polymorphic variants are quite divergent. The D₄.4 allele is the most frequent (global frequency 64%), followed by the D₄.7 allele (21%) and the D₄.2 allele (8%). However these allele frequencies can differ in different populations (Chang et al, 1996). Furthermore, the D₄.7 allele is much younger than the most frequent D₄.4 allele, however its frequency has increased by positive selection. Despite the numerous negative findings, the association between the D₄.7 allele and ADHD is now well established in literature (Li et al, 2006). Also novelty seeking has been associated with the D₄.7R allele (Ebstein et al, 1996).

Figure 1.8: Schematic representation of the D₄ receptor with interaction partners and signaling aspects. PTM of the D₄ receptor are shown: glycosylation, phosphorylation and ubiquitination. The D₄ receptor couples to inhibiting G proteins that block the conversion of ATP to cAMP through inhibiting AC. cAMP normally activates PKA, which can activate downstream effectors that translocate to the nucleus and activate transcription factors (TF). The IC3 loop of the D₄ receptor is interacting with KLHL12. β-arrestin2 binds the receptor near two constitutively phosphorylated residues and can also interact with KLHL12. MAPK activation of ERK1/2 can occur through G proteins and possibly via β-arrestin2.

The D₂-like receptor family, and thus also the D₄R, inhibits adenylyl cyclase by binding Gi proteins (Figure 1.8). Studies have shown that the D₄.7 receptor is two to three times less
potent in coupling to AC than the D4.2 and D4.4 receptors, what leads to a difference in cAMP levels for these polymorphic variants (Asghari et al, 1995). Levels of cAMP, an important second messenger molecule, can influence downstream effectors such as PKA and dopamine and cAMP regulated phosphoprotein of 32 kD (DARPP-32). DARPP-32 is an important intermediate in different signaling pathways and forms a link to various effectors and transcription factors (Greengard et al, 1999).

The pharmacological profiles of the polymorphic variants, on the other hand, were shown not to be significantly different (Asghari et al, 1994). Furthermore, experiments reveal an interaction between the D4 receptor and the G protein-coupled inwardly rectifying potassium channel (GIRK), a potassium channel of which the opening is followed by a reduced firing of neurons. Studies in *Xenopus* showed that dopamine stimulation of D4.2, D4.4 and D4.7 modulates GIRK currents with a different potency (Wedemeyer et al, 2007). Finally, it can be noted that in CHO cells no difference was observed in the activation or duration of MAPK signaling when comparing the D4.2, D4.4 and D4.7 receptor (Oak et al, 2001).

### 1.2.3. Dopamine D4 receptor regulation

Agonist stimulation of GPCRs normally results in signal attenuation to prevent overstimulation of the receptor and to allow the receptor to respond to new stimuli. As discussed above, GPCRs therefore undergo rounds of phosphorylation and β-arrestin recruitment (desensitization), followed by internalization and finally degradation of the receptor. However, it has been shown that the dopamine D4 receptor does not answer to these classical signal attenuation pathways.

The D4R does not undergo phosphorylation, internalization, downregulation nor β-arrestin recruitment upon agonist stimulation (Spooren et al, 2010). In vivo phosphorylation assays have shown that the D4R exhibits a basal level of phosphorylation but that this level is not increased upon stimulation of the receptor. Although the interaction between β-arrestin2 and the D4R has been confirmed via coimmunoprecipitation (coIP) experiments, the recruitment of β-arrestin2 to the receptor is not increased upon agonist stimulation. Together these data suggest that the D4R is not strongly regulated through desensitization, which was also discussed in the review of (Oak et al, 2000).

Furthermore, constitutive phosphorylation of two serine residues is demonstrated, which are located in the third intracellular loop of the receptor, just upstream of the polymorphic region (Spooren et al, 2010). The normal β-arrestin binding mechanism involves the β-arrestin phosphate sensor, which binds to phosphorylated residues, and an activation sensor, which binds to regions of the receptor that undergo activation-induced conformational changes (Spooren et al, 2010). These authors proposed that β-arrestin will transiently bind to these constitutively phosphorylated residues (via its phosphate sensor) or will bind to constitutive active regions of the receptor. This does not exclude a role for β-arrestin in the internalization of the D4 receptor. Furthermore, the location of these constitutively phosphorylated serines coincides partially with the SH3 domains of the receptor in IC3 (not with SH3 domains in repeat region). In the lab of Van Tol, it was shown by Oldenhof et al, 1998 that these SH3 domains play an important role in receptor internalization. By using SH3 domain-deletion mutants of the D4 receptor, they revealed constitutive internalization of the receptor, indicating a major role for these SH3 domains in internalization.
Finally the importance of dopamine receptor interacting proteins (DRIPs) can be stressed, because they act on different levels of receptor activity (e.g. β-arrestin in termination of signaling) (Rondou et al, 2010a). In this context LEGEST performed a Y2H (yeast two-hybrid) screening to identify DRIPs that specifically interact with the D₄ receptor. They used the IC3 region of the D4.4 receptor as a bait to screen a human brain cDNA library. This lead to the identification of Kelch-like protein 12 (KLHL12) (Rondou et al, 2008).

1.2.4. KLHL12
KLHL12, also known as Cullin3 interacting protein (C3IP1), is a broad complex, Tramtrack, and Bric à Brac-Kelch protein (BTB-Kelch), that specifically interacts with the D₄ receptor. KLHL12 consists of a N-terminal BTB domain, connected to a central BTB and C-terminal Kelch (BACK) domain and a C-terminal Kelch domain consisting of six Kelch repeats (Figure 1.9) (Angers et al, 2006; Stogios & Prive, 2004).

Recently, it has been shown that BTB proteins represent a new class of adaptors in Cullin3-based E3 ligase complexes, that consist of Cullin3 and RING of cullins 1 (Roc1) (Furukawa et al, 2003). Roc1 functions as a carrier of the E2 ubiquitin-conjugating enzyme. The function of the BTB protein in this complex is to confer substrate specificity to the E3 ligase complex. For the interaction with the substrate protein, the BTB-containing protein makes use of its other protein interaction domain (e.g. Kelch domain). Since KLHL12 is known as BTB-Kelch protein, LEGEST wanted to investigate whether KLHL12 can function as an adaptor in a Cul3-based E3 ligase complex, to target the D₄ receptor for ubiquitination.

In a first step the region of the receptor was identified that is responsible for KLHL12 binding. By using mutant receptors such as the D₄.0R (which does not contain the repeat region) and the D₄.ΔΔR (in which regions were deleted that directly flank the repeat region), it was shown that KLHL12 is directly interacting with the polymorphic region in the IC3 loop (Rondou et al, 2008). Next, the region of KLHL12 was determined that is responsible for D₄R binding. Also here mutants were used: KLHL12ΔKelch (does not contain the Kelch domain) and KLHL12ΔBTB (does not contain the BTB domain). With these mutants, it was shown that the Kelch domain of KLHL12 is responsible for the interaction with the D₄ receptor. Furthermore, several coIP studies were carried out to test the hypothesis of KLHL12 as an adaptor for the Cul3-based E3 ligase complex. It was demonstrated that KLHL12 and Cullin3 interact with each other (confirming an earlier study of (Angers et al, 2006)), but that the BTB domain of KLHL12 was not strictly necessary for its interaction with Cullin3 (Rondou et al, 2008). As a control, direct interaction between the D₄ receptor and Cullin3 was ruled out to emphasize the importance of KLHL12 as an adaptor. Also, coIP of Roc1 and KLHL12 was demonstrated, suggesting the possibility of Roc1 and KLHL12 being in the same complex with Cullin3 as a mutual interaction partner. A direct interaction between these two protein could not be ruled out. Nevertheless, these data strongly support the hypothesis that the D₄ receptor can be targeted to the Cul3-based E3 ligase complex through its adaptor protein, KLHL12 (Figure 1.10) (Rondou et al, 2008). Sequential immunoprecipitation assays confirmed specific ubiquitination of the D₄ receptor, excluding the contribution of other ubiquitinated
proteins. Upon overexpression of KLHL12, the ubiquitination signal of the receptor is strongly increased. These data were the first to show ubiquitination of dopamine receptors and the first to identify a protein that specifically interacts with the IC3 polymorphism of the D₄ receptor.

![Diagram of Cullin3-based based E3 ligase complex](image)

**Figure 1.10: Cullin3-based based E3 ligase complex.** KLHL12 functions as an adaptor to connect the D₄ receptor to the Cullin3-based ubiquitination machinery. KLHL12 binds via its BTB domain to Cullin3 and via its Kelch domain to the D₄ receptor. Cullin3 forms a complex with Roc1, which recruits the E2 enzyme that is conjugated with ubiquitin.

In a further study it was shown that KLHL12 is able to promote ubiquitination of as well plasma membrane associated receptors (PM receptor) as endoplasmatic reticulum associated receptors (ER receptors) and that agonist treatment of the receptor does influence the ubiquitination levels of the receptor. This means that the D₄ receptor does not undergo agonist-mediated ubiquitination (Rondou et al, 2010b). Next, the ubiquitination pattern of the D₄ receptor was investigated, since different ubiquitination patterns (mono, multi-mono, poly) are possible (see section 1.1.5). To this end, triple lysine (K) ubiquitin mutants (K29R, K48R and K63R), in which three lysines were mutated to arginines (R), were used and compared to WT (wild type) ubiquitin to study the strength of the receptor ubiquitination signal. When the triple mutant was used, receptor ubiquitination is no longer increased upon KLHL12 coexpression, contrary to when WT ubiquitin was used. Also the smeary pattern, indicative for polyubiquitination, was no longer increased upon KLHL12 coexpression. Furthermore, commercially available antibodies recognizing only polyubiquitination or all types of ubiquitination (mono, multi, poly) confirmed polyubiquitination of the D₄R, promoted by KLHL12 (Rondou et al, 2010b).

Different types of polyubiquitination can have different functions, thus it is important to investigate the consequences of D₄ polyubiquitination. Therefore, single ubiquitin mutants (K29R, K48R and K63R) were used. However, receptor ubiquitination levels were unaffected upon coexpression of each single mutant compared to WT ubiquitin. Remarkably, KLHL12 is still able to promote ubiquitination of the D₄R in the presence of the single ubiquitin mutants (Rondou et al, 2010b). A possible explanation is that KLHL12 is able to promote different types of receptor ubiquitination (e.g. K63 together with K29). This could explain why the
effect of the single mutants is invisible compared to the more significant effect of the triple mutant because of the combined inhibition effect of the different types of polyubiquitination (Rondou et al, 2010b). As mentioned, ubiquitination of GPCRs often targets them for degradation. LEGEST showed that KLHL12 does not promote receptor degradation; neither proteasomal degradation nor lysosomal degradation. Furthermore, ubiquitination of the D₄ receptor did not result in enhanced internalization.

Because of the importance of β-arrestin2 in receptor regulation, it was investigated if KLHL12 can interact with β-arrestin2 and if ubiquitination of the receptor has an influence on internalization. It was shown that β-arrestin2 can interact with KLHL12 in absence of the receptor, and that it is able to bind both the ubiquitinated receptor and the 4KR D₄ receptor (in which all four IC lysines are mutated to arginines) (Rondou et al, 2010b). Also, coexpression of KLHL12 did not increase the interaction between β-arrestin2 and the D₄R, nor did it change the ubiquitination pattern and recruitment of β-arrestin2. A lot of research will be necessary to unravel the functional consequences of the interaction between β-arrestin2 and KLHL12.

D₄ receptor ubiquitination and the interaction of KLHL12 with the D₄R IC3 polymorphic region was first described by LEGEST (Rondou et al, 2008; Rondou et al, 2010b), so the expertise of this lab will be very instructive to study the functional consequences of D₄R ubiquitination and the influence of β-arrestin2 and KLHL12 in this process.
Chapter 2: Aims

2.1. Characterization of the interaction between the polymorphic dopamine D₄ receptor variants and KLHL12 and/or β-arrestin2

KLHL12 is a BTB-Kelch protein that is specifically interacting with the polymorphic region in the third intracellular loop (IC3) of the dopamine D₄ receptor (Rondou et al, 2008). KLHL12 also interacts with β-arrestin2, a multifunctional adaptor protein that is important in GPCR regulation and signaling. LEGEST also demonstrated that KLHL12 is interacting with the different polymorphic variants of the D₄R, except the D₄.₀R (receptor without the polymorphic region in IC3) (Rondou et al, 2008). However, the affinity of KLHL12 for the different polymorphic variants is not necessarily the same and cannot be quantified accurately via coIPs. Therefore, we were planning to use bioluminescence resonance energy transfer (BRET) to quantitatively measure these interactions. Before starting with BRET, the construct KLHL12- Yellow fluorescent protein (YFP) needed to be cloned. The expression of this new construct will be thoroughly tested on the Victor³ plate reader, via western blot (WB) analysis and immunofluorescence. Because BRET still needs to be optimized, saturation BRET experiments will be done to try to determine the optimal ratio of receptor-Rluc/KLHL12-YFP for future experiments.

We also want to know if there is competition between KLHL12 and β-arrestin2 for the same binding region of the receptor. We will test this by performing coIP studies between β-arrestin2 and the different polymorphic variants of the D₄R (D₄.₀R, D₄.₂R, D₄.₄R, D₄.₇R and D₄.₂ΔΔR). LEGEST also demonstrated that the C terminal domain of β-arrestin2 is responsible for the interaction with KLHL12 (Skieterska, Unpublished results). However, it is not known yet which domain of β-arrestin2 is responsible for the binding with the D₄ receptor: the C terminal or N terminal domain or both. By using deletion mutants of β-arrestin2, we will try to elucidate the involved domain(s) by coIP studies.

It has been shown that overexpression of KLHL12 increases the ubiquitination signal of the D₄ receptor (Rondou et al, 2010b). On the other hand, we also want to know if β-arrestin2 plays a role in the ubiquitination of the receptor. Therefore, sequential (double) coIPs will be done. In this way, we can study the ubiquitination pattern of the receptor alone, without the influence of possible ubiquitinated proteins that also interact with the receptor and that could contribute to the ubiquitination signal.

2.2. Functional consequences of D₄ receptor ubiquitination

Next, the functional consequences of D₄ receptor ubiquitination and the influence of KLHL12 and β-arrestin2 in this process are studied. First, ligand binding studies will be performed on the D₄.₂R in the presence or absence of KLHL12. Therefore, we will first determine the maximal number of receptors (B_max) by doing saturation binding assays. Then, the affinity of dopamine for the receptor will be studied by performing competition binding assays. It will be interesting to see whether KLHL12 can influence the affinity of dopamine for the receptor.

Then, the influence of D₄R ubiquitination on cAMP levels will be studied. As mentioned, the D₄R interacts with inhibiting G proteins that block the conversion of ATP to cAMP by adenylyl cyclase. PD-168077, which is a D₄R specific agonist, will be used to activate the D₄R. Then,
cells will be treated with forskolin, an activator of adenylyl cyclase, to start from a reference level of cAMP. In this way, lowering of cAMP levels can be studied more accurately. The cAMP assay will be performed by using a commercial kit from Cayman.

Finally, the MAPK activation of different D₄R variants, including D₄.0R, D₄.2R and D₄.24KR, will be studied in the presence of KLHL12 or β-arrestin2 or both. In this way, we will know if ubiquitination of the D₄R and the presence of one of these proteins, influences the levels of ERK1/2 phosphorylation.
Contributions of third parties

- Figure 3.11: Kamila Skieterska (transfection and coIP)
- Figure 3.12: Kamila Skieterska (transfection and coIP)
- Figure 3.15: Kamila Skieterska (competition BRET)
- Figure 3.20: Béatrice Lintermans (transfection)
- Figure 3.22: Béatrice Lintermans (transfection)
- Figure 3.26: Kamila Skieterska (repeating the assay)
Chapter 3: Results

The results from both the interaction and the functional experiments will be presented in this chapter. We will start with the cloning strategy of the pKLHL12-YFP vector and proceed with the discussion of the saturation BRET experiments. Then, the different co-immunoprecipitation experiments will be discussed and finally the results from the functional assays will be presented.

3.1. Cloning of pKLHL12-YFP

A successful cloning experiment starts with a good strategy. We started from two constructs: the pEYFP-N3 vector, which is our destination vector, and the Etag-KLHL12 pGFP2-N3 vector, which contains the Etag-KLHL12 sequence. The elements of both vectors are identical, except for the YFP/GFP sequence. Our goal was to insert the Etag-KLHL12 sequence into the pEYFP-N3 vector, which would result in the Etag-KLHL12-pEYFP-N3 vector. The resulting vector could then be used for BRET experiments.

3.1.1. Cloning procedure

The first step in our cloning procedure consists of a restriction enzyme digest to generate the insert (Etag-KLHL12) and vector (pEYFP-N3) fragments. The enzymes Nhel and BglII were used to cut both vectors; the expected fragment sizes were calculated using Clonemanager and are presented in the table below (Table 3.1). Via double digest finder (Website_neb), the optimal buffer and reaction conditions were determined (NEBuffer 2, 37°C, with addition of BSA). The restriction enzyme digest was done overnight.

Table 3.1: Expected fragment sizes Nhel and BglII double digest. Both vectors sizes are listed, together with their vector fragment sizes after being cut with Nhel and BglII. The fragments indicated in bold, were used in subsequent experiments.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Full size vector (bp)</th>
<th>Vector fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etag-KLHL12 pGFP2-N3</td>
<td>6606</td>
<td>4724</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1766</td>
</tr>
<tr>
<td></td>
<td></td>
<td>116</td>
</tr>
<tr>
<td>pEYFP-N3</td>
<td>4733</td>
<td>4715</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18</td>
</tr>
</tbody>
</table>

The resulting fragments were then separated according to size using (preparative) agarose gel electrophoresis. Only the borders of the gel lanes were used for visualization with EtBr, to avoid introduction of mutations in the desired DNA fragments (Figure 3.1). The restriction digest of the Etag-KLHL12 pGFP2-N3 vector gave us two fragments (4724bp and 1766bp) instead of three (Table 3.1), because the third fragment was so small that it already ran off the gel. The digest of the pEYFP-N3 vector gave us one fragment (4715bp) instead of two (Table 3.1), again because of the small size of the second fragment (18bp).

Next, the desired fragments (1766bp insert, 4715bp vector) were cut out from the gel and DNA was eluted from the gel fragments. A small fraction of the eluted DNA was again loaded on an agarose gel to check the correctness of our fragments (data not shown). This gave us enough proof to proceed with these fragments.
Figure 3.1: Visualization of DNA bands on preparative agarose gel. Restriction enzyme digest of Etag-KHL12 pGFP-N3 resulted in two fragments (4724bp and 1766bp). The digest of the pEYFP-N3 vector resulted in a fragment of 4715bp. Fragment sizes were determined with the lambda DNA/PstI marker (indicated with arrows).

Then, a ligation reaction was performed between the insert and the vector fragments. We used a 4:1 molar excess of insert (Etag-KHL12) compared to vector (pEYFP-N3) and performed the ligation reaction using T4 DNA ligase in the presence of ATP and spermidine, a molecule that increases the activity of the T4 DNA ligase enzyme. The ligation was done overnight at 16°C.

The new construct (pKLHL12-YFP) was then transformed into competent bacteria (MC1061 cells) using the heat shock method. Bidi was included as a negative control and the pEYFP-N3 vector as a positive control. Because pKLHL12-YFP encodes the kanamycine resistance gene (kan^R) as a selection marker, the transformed bacteria were plated on LB agar plates supplemented with kanamycine. Bacteria growing on these plates (LB + kan) have taken up the plasmid of interest. The plates were incubated overnight at 37°C, to allow for bacterial growth. The next day, the plates were checked for colony formation. About fifteen colonies were counted and were transferred to separate culture flasks containing LB and kanamycine. This bacterial minipreparation allowed the amplification of our new construct in bacterial cells. Plasmids were then isolated from the bacteria using the Birnboim method (Birnboim & Doly, 1979).

Next, the correctness of the isolated plasmids was checked with a restriction enzyme (RE) digest. In a first control experiment, AvrII and Nhel were used for the RE digest. A negative control (bidi) and three positive controls (pEYFP-N3 vector cut with AvrII, Nhel or both) were also included. The expected fragment sizes were calculated using Clonemanager (Table 3.2). The digested fragments were loaded on an agarose gel. The results are given in Figure 3.2.

Table 3.2: Expected fragment sizes upon restriction enzyme digest of vector. The new construct, pKLHL12-YFP, was cut with AvrII and Nhel in the first control experiment and with NotI in the second control experiment.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Avr II + Nhe I: fragments (bp)</th>
<th>Not I: fragments (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etag-KHL12 pEYFP-N3 (6481bp)</td>
<td>2852 1730 1015 884</td>
<td>3972 2509</td>
</tr>
</tbody>
</table>
The negative control did not give a band, as was expected. The expected fragment sizes of the positive controls, shown in Figure 3.2, match the band fragment sizes. With the samples from the clones, lane 5-11, four fragments were expected. However, only two bands were clearly visible (2852bp, 1730bp) because the gel was run too long to see the 1015bp and 884bp bands. This first control restriction enzyme digest gave us an indication that our construct was correct.

To confirm, a second restriction enzyme digest was carried out using the NotI enzyme. Expected fragment sizes are shown in Table 3.2. The results from the agarose gel is presented in Figure 3.3. This time two bands (3972bp and 2509bp) were detected for all the clones except number four; probably this sample was not digested properly. This additional control gave us an extra indication that the construct was properly cloned.

Figure 3.2: Agarose gel from AvrII and Nhel control restriction enzyme digest of pKLHL12-YFP. Agarose gel from the control restriction digest with AvrII and Nhel. M represents the length mark, λ/PstI.

Figure 3.3: Agarose gel from NotI control restriction enzyme digest of pKLHL12-YFP. Agarose gel from the restriction digest with NotI. M represents the length mark, λ/PstI.
Next, the bacterial minipreparation was repeated with one clone, followed by a bacterial maxipreparation in big culture bottles with a mixture of LB and kanamycine. Plasmid DNA was now isolated using a commercial kit (QIAGEN Plasmid midi kit). The DNA concentration was measured using the Nanodrop spectrophotometer; the concentration was 4.2µg/µL. The 260/280 ratio, which is an indication of the purity of the DNA, was 1.86, thus approximating the ideal value of 1.8 (lower values indicate the presence of impurities like organic solvents). Finally, KLHL12-YFP was sequenced, which confirmed the correctness of the construct.

3.1.2. Expression tests

To test the expression and the fluorescence of the pKLHL12-YFP, three types of tests were performed: a Western blot, an immunofluorescence experiment and an expression test on the Victor plate reader.

**Western blot**

HEK293T cells were transiently transfected with pKLHL12-YFP and pHA D₄.₀R or pHA D₄.₂R. Because KLHL12 does not interact with the D₄.₀R, co-transfection of these plasmids was used as a negative control. This control is introduced to check whether the tag (YFP) influences the interaction with the receptors. Additional controls were added: cells transfected with the receptor alone or with pKLHL12-YFP alone.

Cells were lysed and co-immunoprecipitation was carried out using anti-HA 16B12 antibody, thus immunoprecipitating the D₄.R. Proteins were separated via SDS-PAGE, then western blotting was performed. To detect the proteins of interest, KLHL12-YFP and the D₄.xR, blots were incubated with respectively anti-GFP mouse and anti-HA 16B12. Fluorescent antibodies were used to visualize the proteins on the Odyssey (infrared scanner). The results of this WB experiment are presented in Figure 3.4.

![Figure 3.4: Interaction of KLHL12-YFP with the HA D₄.₀R or HA D₄.₂R.](image)

Lane 4-6 in the lysate blots show that the KLHL12-YFP fusion protein is well expressed. Moreover, KLHL12-YFP seems to interact specifically with the D₄.₂R and not with the D₄.₀R (IP blots anti-GFP, lane 5 compared to 6). This proves that the YFP-tag, which is attached to KLHL12, is not influencing interaction properties of KLHL12. Also, D₄.₀R and D₄.₂R expression was confirmed in the lysate (not shown) and IP (IP blot, anti-HA lane 2,3 and 5,6).
Immunofluorescence

HEK293T cells in 6-well plates on coverslips, were transiently transfected either with pKLHL12-YFP, pcDNA3 (negative control) or pEYFP-N3 vector (positive control). 48 hours post-transfection, cells were fixed with formaldehyde, permeabilized and incubated with anti-GFP mouse (primary antibody) and anti-mouse alexa fluor 594 (secondary antibody). Nuclei were stained with DAPI.

Normally, the fluorescence coming from the KLHL12-YFP construct should be already visible without prior incubation with antibodies. Suppose, however, that no fluorescence was detected from the protein as such (e.g. due to improper folding of YFP caused by fusion to KLHL12), but when using antibodies a YFP signal was detected. This means that by using antibodies, it can be proven that the received signal specifically originates from the construct. If a fluorescent signal can be directly seen under the microscope (without using antibodies), then incubation with the antibodies will show colocalization of KLHL12-YFP in the green and red channel. This antibody incubation step can thus be seen as an additional control. The results from the immunofluorescence experiment are shown in Figure 3.5. These pictures show that KLHL12-YFP is well expressed, both in the green and red channel. In the lower right panel, a clear colocalization of the green and red signal can be seen, an additional confirmation of the specific expression of KLHL12-YFP.

![Immunofluorescence pictures of HEK293T cells transfected with pKLHL12-YFP.](image)

Figure 3.5: Immunofluorescence pictures of HEK293T cells transfected with pKLHL12-YFP. In the upper left panel, the green channel shows the fluorescent signal from KLHL12-YFP. In the upper right panel, the red signal also originates from KLHL12-YFP (antibody staining was used, see text). In the lower left panel, DAPI stains the nuclei of the cells blue. In the lower right panel, a merged picture in all three channels is presented. A clear colocalization between the red and green channel can be noticed.
Expression test on Victor

HEK293T cells seeded on 6-well plates, were transiently transfected with increasing amounts of pKLHL12-YFP. Transfection with pcDNA3 was taken as a negative control, and the pEYFP-N3 vector as a positive control. Then, 48 hours post-transfection cells were collected in HBSS buffer. The protein concentration of each sample was determined via BCA analysis (see section 5.4.6) and samples were diluted to equal concentration. The fluorescent signal (coming from the KLHL12-YFP) was then measured using the Victor plate reader. The resulting graph is presented in Figure 3.6. The positive control was approximately 300 times more expressed than KLHL12-YFP (not shown). The fluorescent signal increases with increasing amounts of KLHL12-YFP, as expected. The fluorescence value at 0µg KLHL12-YFP corresponds to transfection with pcDNA3 alone (negative control) and is not zero due to autofluorescence from the cells.

Figure 3.6: Graph plotting fluorescence versus concentration KLHL12-YFP. With increasing concentrations of KLHL12-YFP, the fluorescent signal rises. Values for 2µg and 4µg KLHL12-YFP were excluded (transfection error). The 0µg KLHL12-YFP corresponds to transfection with pcDNA3 alone. a.u., arbitrary units. Error bars represent SEM.

Together, these three expression tests prove that our new construct, pKLHL12-YFP, is well expressed and that fluorescence is detectable in different experimental set-ups. This construct will be used for BRET experiments.

3.2. BRET

Via sequential immunoprecipitations, it has been shown that KLHL12 can have a different affinity for different D₄.xR polymorphic variants. Upon overexpression of KLHL12, the ubiquitination signal of the D₄.2R was less increased compared to the D₄.3R and D₄.4R ubiquitination levels (Rondou, 2009). Classical co-immunoprecipitation studies, however, only provide us with semi-quantitative information. In order to quantify these differences in affinity more reliable, BRET will be used.

3.2.1. BRET principles

BRET is a useful tool for studying protein-protein interactions. We will use this technique to study the interaction of KLHL12 with D₄.xR. Other applications are e.g. the dimerization of GPCRs. This technology involves the fusion of donor and acceptor molecules to the proteins of interest (Poyner & Wheatley, 2010). Thus, the D₄.xR will be tagged with the donor enzyme,
Renilla luciferase (Rluc), and the intracellular protein will be tagged with an acceptor fluorophore, YFP. The D4.2-Rluc and D4.7-Rluc constructs were already available in the lab and the KLHL12-YFP construct was newly cloned (section 3.1). The BRET process occurs via the oxidation of the coelenterazine substrate to coelenteramide, by the donor enzyme Rluc; a reaction in which CO₂ is produced and light is emitted. If the donor protein is sufficiently close (i.e. interacts) to the acceptor fluorophore, resonant energy transfer will occur and the YFP protein will be excited (Figure 3.7). Consequently, the fluorophore will emit light from a longer wavelength. The energy emitted by both processes can be measured and compared using a dual-filter luminometer. An increased level of energy emitted by the YFP protein compared with the emission of Rluc at the same time point, is indicative of an interaction between the proteins coupled to Rluc and YFP. The physical distance that BRET can measure is ≤10nm, a range suggestive for protein-protein interactions.

Figure 3.7: BRET principle. A GPCR is tagged with the donor enzyme, Rluc, while the GPCR-interacting protein is coupled to YFP. After addition of coelenterazine, it is oxidized by Rluc, releasing CO₂ and light (480nm). If both proteins are not interacting, no resonant energy transfer will occur (a). If they are interacting, resonant energy transfer occurs and will lead to the emission of light by YFP (530nm). Source: (Poyner & Wheatley, 2010).

3.2.2. Saturation BRET

The determination of the optimal ratio of donor and acceptor tagged proteins is of utmost importance for obtaining significant BRET signals, as well as for obtaining conclusive results. In this context, the overall expression of the constructs, as well as the relative expression levels of donor and acceptor, are important. In general, the ratio of acceptor to donor should be higher than one, typical ratios are 2:1 or 4:1 (Poyner & Wheatley, 2010). The optimum ratio depends, however, on the GPCR-interacting protein pair under investigation.

Saturation BRET experiments were performed to determine the optimal ratio between the donor D4.2-Rluc (or D4.7-Rluc) and the acceptor KLHL12-YFP, important for future BRET experiments. HEK293T cells seeded on 6-well plates were used and were transiently transfected with donor, acceptor and pcDNA3 to equal the amount of DNA in every well. The principle of this experiment is to keep the concentration D4.2-Rluc constant, while
increasing the amount of KLHL12-YFP. An appropriate background control in the analysis of the BRET ratio is given by transfection with the receptor alone. In literature, one can find a broad spectrum of total amounts of DNA that can be used per well and a large range of acceptor/donor ratios. Therefore, different transfection setups were tried, using total DNA amounts ranging from 6µg to 2µg (Issad & Jockers, 2006). An example is given in Table 3.3.

Table 3.3: BRET transfection ratios. The amount of donor was kept constant at 0.2µg. The amount of acceptor was increased starting from 0.1µg and going to 1.8µg. To equal the amount of DNA in every well, pcDNA3 was added. The ratio of acceptor to donor is also given.

<table>
<thead>
<tr>
<th>W0</th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
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<tbody>
<tr>
<td>Donor (µg)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
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<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Acceptor (µg)</td>
<td>/</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
</tr>
<tr>
<td>pcDNA3 (µg)</td>
<td>1.8</td>
<td>1.7</td>
<td>1.6</td>
<td>1.4</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>0.8</td>
<td>0.7</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Ratio (acceptor/donor)</td>
<td>/</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>3.5</td>
<td>4.0</td>
<td>5.0</td>
<td>5.5</td>
<td>6.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

From this type of BRET experiment, a saturation curve can be obtained, by plotting the BRET ratio (see equation) against the ratio of the background corrected fluorescence to the luminescence ((YFP-YFP)/Rluc).

\[
BRET\ _{ratio} = \frac{\text{long wavelength emission (donor - acceptor pair)}}{\text{short wavelength emission (donor - acceptor pair)}} - \frac{\text{long wavelength emission (donor only)}}{\text{short wavelength emission (donor only)}}
\]

Theoretically, if a specific interaction occurs between the donor and acceptor proteins, a hyperbolic curve should be obtained (Figure 3.8). Saturation (BRETmax) is reached when all the receptors (D4.2R-Rluc) are specifically interacting with their interacting proteins (KLHL12-YFP). However, when the measured BRET signal results from random collisions between these proteins, a linear curve is obtained (non-specific interaction). The optimal ratio of acceptor to donor can be interpolated from the saturation curve using the half-maximal BRET ratio, the BRET50.

![Figure 3.8: Theoretical BRET saturation curve.](image)

From Figure 3.9, the saturation curve is presented for the experiment where a constant amount of donor 0.2µg and increasing pKLHL12-YFP amounts were used (Table 3.3). From Figure 3.9, however, one cannot determine the BRET50 accurately because the curve does not converge.
completely to the maximal BRET value (saturation). The main problem in these experiments is that a lot of negative BRET ratios were obtained that need to be excluded from the data set, which impedes obtaining a good saturation curve. One reason for the negative BRET ratios is that the background BRET signal (measured in the sample with donor only) is often higher than for the samples were KLHL12-YFP was included. This means that the HEK293T cells show considerable autofluorescence. To circumvent this problem, other cell lines can be tested for their autofluorescence characteristics e.g. HEK293 cells. Another possible reason for the negative BRET values, is the suboptimal settings of the filters of the Victor plate reader. Rluc emission is measured at 460nm, while the optimum emission wavelength of Rluc is at 480nm. It still has to be ruled out whether these suboptimal settings can play a role.

**Figure 3.9: Saturation BRET curve.** The BRET ratio is plotted against the ratio of fluorescence minus background (YFP-YFP) to luminescence (Rluc). The optimal ratio, BRET50, cannot be determined accurately from this graph.

### 3.3. Interaction of β-arrestin2 and KLHL12 with the D₄R polymorphic variants

It has been shown that KLHL12 is specifically interacting with the D₄R polymorphic repeat region (Rondou et al, 2008). KLHL12 can also interact with β-arrestin2, even in absence of the D₄R (Rondou et al, 2010b). In this work it was investigated whether β-arrestin2 can interact with the same binding of the D₄R as KLHL12.

HEK293T cells were transiently transfected with the different polymorphic variants of the D₄R (pHA D₄.0R, pHA D₄.2R, pHA D₄.4R, pHA D₄.7R) and with pCmyc-β-arrestin2 or pEtag-KLHL12. Lysates of these cells were subjected to immunoprecipitation with anti-HA antibody. The resulting immunoblots are presented in Figure 3.10. Co-immunoprecipitation of β-arrestin2 and KLHL12 was checked in IP, using respectively anti-Cmyc and anti-Etag antibodies. Receptor expression was confirmed in lysate (not shown) and IP (IP blots, anti-HA); a typical stairway pattern is observed due to the increasing size of the D₄,R variants (from D₄.0R, D₄.2R, D₄.4R to D₄.7R). KLHL12, as expected, is interacting with all the receptor polymorphic variants except the D₄.0R (IP blots, anti-Etag, lane 8-10). β-arrestin2, however, interacts with all the receptor variants: D₄.0R, D₄.2R, D₄.4R, D₄.7R (IP blots, anti-Cmyc, lane 2-5) and also D₄.2ΔΔR (not shown in Figure 3.10). Because β-arrestin2 is interacting with the D₄.0R, which does not contain the polymorphic repeat region, it can be concluded that the polymorphic repeat region is not crucial for the interaction of β-arrestin2 with the D₄R.
Figure 3.10: Interaction of KLHL12 and β-arrestin2 with the different polymorphic variants of the D₄R. Lysates of HEK293T cells transiently transfected with pHA D₄R and pCmyc-β-arrestin2 or pEtag-KLHL12, were subjected to IP with anti-HA antibody. Co-immunoprecipitation of β-arrestin2 and KLHL12 was checked after IP (IP blots) using respectively anti-Cmyc and anti-Etag antibodies. In the lysate, β-arrestin2 gives two bands: one at 40kDa and one at 55kDa (lysate blot, lane 2-5), in IP only the band of 55kDa is present (IP blots, lane 2-5). KLHL12 has a size of 63kDa in lysate (lysate blot, lane 6-10) and IP (IP blot, lane 8-10). Receptor expression was checked in lysate (not shown) and IP (IP blots) with anti-HA (mature D₄R ~52kDa, immature D₄R ~46kDa). This experiment was done in fourfold.

It can be remarked that the control setup in which cells were transfected with pCmyc-β-arrestin2 alone, gave an non-specific band in IP (IP blots, anti-Cmyc, lane 1). However, β-arrestin2 should not give rise to any bands in IP because no receptor was present. Still, in each of the four performed experiments, a non-specific band was obtained in IP from this β-arrestin2 control. A possible explanation is that β-arrestin2 non-specifically interacts with the beads (protein A beads, used in IP). To investigate this further, a test experiment was performed. Different tagged β-arrestin2 and β-arrestin1 constructs (Flag-β-arrestin2/1, Cmyc-β-arrestin2, GFP-β-arrestin2/1) were used in a precipitation reaction where only beads were added to the cell lysate (without antibody). If the beads and β-arrestin2 are non-specifically interacting with each other, then in IP a signal should be obtained from β-arrestin2. The resulting blots are shown in (Figure 3.11). A non-specific interaction between the beads and β-arrestin2 could be shown for the GFP-tagged β-arrestin2 and Flag-tagged β-arrestin1 and 2 constructs (IP blots, right and center). For Cmyc-β-arrestin2, no non-specific interaction could be detected in this experiment. In general this test shows that a non-specific interaction between β-arrestin2 and the beads can occur and that the tag attached to β-arrestin2 can have an influence on the strength of this non-specific interaction.
Figure 3.11: Non-specific interaction of tagged β-arrestin2/β-arrestin1 constructs with beads. Left, in IP there is no signal from Cmyc-β-arrestin2. Center, in IP there is a weak signal for both Flag-β-arrestin2 and β-arrestin1. Right, in IP there is a clear signal coming from GFP-β-arrestin2 and β-arrestin2-GFP2.

A possible way to circumvent this problem is to use an anti-Flag antibody to do the immunoprecipitation reaction (Figure 3.12). In the setup with β-arrestin2 alone, a band is now expected in IP (IP blot, anti-Flag, lane 2); a band which is now specific. The other β-arrestin2 bands in IP (IP blot, anti-Flag, lane 4-8) are now more reliable. The overall conclusions of this experiment remain valid when IP is done with anti-Flag antibody.

Figure 3.12: Interaction of β-arrestin2 with the different polymorphic variants of the D4R. Lysates of HEK293T cells transiently transfected with pH4 D4.R and pFlag-β-arrestin2, were subjected to IP with anti-Flag antibody. Expression of HA D4.R (lysate blot, anti-HA, lane 3-8) and Flag-β-arrestin2 (lysate blot, anti-Flag, lane 2, 4-8) was checked in lysate. Co-immunoprecipitation of the receptor was checked after IP (IP blots, lane 4-8) using anti-HA antibody.

3.4. Interaction of β-arrestin2 deletion mutants with the D4.R

Previous experiments from LEGEST have shown that KLHL12 and β-arrestin2 can interact with each other in the absence of the receptor (Rondou et al, 2010b). β-arrestin2 comprises a C domain and a N domain, which are connected through a linker region. KLHL12 consists of
a central BACK domain, which is linked to a BTB domain and to six Kelch repeats (Figure 1.9). The Kelch repeats of KLHL12 are important for the interaction with the D4R but also for the interaction with β-arrestin2. On the other hand, β-arrestin2 itself is interacting with KLHL12 via its C domain (Skieterska, Unpublished results). Because it is not known yet which domain(s) of β-arrestin2 are important for D4R binding, this topic is investigated in this section.

HEK293T cells were transiently transfected with the pHA D4.2R and pFlag-β-arrestin2 WT or the deletion mutants of β-arrestin2 (pFlag-β-arrestin2 NT, pFlag-β-arrestin2 CT). β-arrestin2 consists of 409 amino acids (AA). Flag-β-arrestin2 NT only contains the N domain (AA 1-320), while Flag-β-arrestin2 CT only contains the C domain (AA 284-409). Cells were lysed and subjected to IP with anti-Flag antibody, thus immunoprecipitating β-arrestin2. The resulting immunoblots from one representative experiment are presented in (Figure 3.13). These results were confirmed in a second experiment.

Expression of the different β-arrestin2 constructs was checked in lysate and IP (lane 2-4 and 6-8). Co-immunoprecipitation of the D4.2R was checked in IP using anti-HA antibody (IP blots, lane 6-8). Lane 6 (IP blots, anti-HA) shows that Flag-β-arrestin2 WT is, as expected, interacting with the D4.2R. Remarkably, both Flag-β-arrestin2 CT and Flag-β-arrestin2 NT can interact individually with the D4 receptor (IP blots, anti-HA, lane 7 and 8). This means that the C domain and N domain can interact individually with the D4R in vitro.
3.5. Competition between KLHL12 and β-arrestin2 for interaction with the D4.0R and D4.2R

As previously mentioned, it was shown that KLHL12 and β-arrestin2 can interact with each other, even in the absence of the D4R. Earlier studies also revealed that coexpression of KLHL12 together with β-arrestin2 and the D4R, does not increase the interaction between the receptor and β-arrestin2 nor does it change the cellular localization of β-arrestin2 (Rondou et al, 2010b). By performing competition BRET experiments (Figure 3.14), LEGEST showed that the constitutive interaction between β-arrestin2 and the D4.2R (and also the D4.7R) was decreased upon overexpression of KLHL12 (Skieterska, Unpublished results). This indicated that KLHL12 was displacing β-arrestin2 from the receptor.

![D4.2R - β-arrestin2/KLHL12 competition assay](image)

**Figure 3.14:** Competition BRET assay between the D4.2R and β-arrestin2/KLHL12. A constant amount of D4.2R-Rluc and β-arrestin2-GFP were used, together with an increasing amount of KLHL12. Ratios are expressed as mean ± SEM from three experiments (n=3). One way analysis of variance (ANOVA) was used for statistical evaluation. Post-hoc tests were used to address significant differences between groups. ***p<0.001. Source: (Skieterska, Unpublished results).

To confirm these results, a competition co-IP was performed in which the amounts of D4.2R and β-arrestin2 were kept constant and the amount of KLHL12 was increased. This experiment, however, did not give conclusive results (not shown). As a control experiment, the competition co-IP was repeated with the D4.0R, which does not interact with KLHL12. Therefore, HEK293T cells were transiently transfected with a constant amount of pHA D4.0R and pCmyc-β-arrestin2 and increasing amounts of pEtag-KLHL12. Cell were lysed and IP was performed using anti-HA antibody. The results from this experiment are presented in Figure 3.15. In the lysate, the expression of the receptor was confirmed (not shown). Also the expression of Cmyc-β-arrestin2 (lysate blot, anti-Cmyc, lane 2, 5-11) and Etag-KLHL12 (lysate blot, anti-Etag, lane 4, 6-11) was checked. Co-immunoprecipitation of Cmyc-β-arrestin2 was confirmed upon IB with anti-Cmyc (IP blot, anti-Cmyc, lane 2, 5-11). The receptor levels after IP were checked by incubating with anti-HA (IP blot, anti-HA, lane 3, 5-11). KLHL12 was not present in the IP fraction, as expected (not shown). It must be remarked that with cells transfected with pCmyc-β-arrestin2 alone, again a non-specific band is obtained in IP (see also section 3.3).
Figure 3.15: Competition coIP between D4.0R and β-arrestin2/KLHL12. Lysates of HEK293T cells transiently transfected with constant amounts of pHA D4.0R and pCmyc-β-arrestin2, and with increasing amounts of pEtag-KLHL12 (0.6µg, 1.5µg, 3µg, 4.5µg, 6µg, 9µg), were subjected to IP with anti-HA antibody. Expression of Etag-KLHL12 (lysate blot, anti-Etag, lane 4, 6-11) and Cmyc-β-arrestin2 (lysate blot, anti-Cmyc, lane 2, 5-11) were checked in lysate. Receptor levels were monitored with anti-HA (IP blot, anti-HA, 3, 5-11).

When no KLHL12 is present, Cmyc-β-arrestin2 is interacting with the D4.0R. However, if the amount of transfected pEtag-KLHL12 was increased from 0.6µg to 9µg (IP blot, anti-Cmyc, lane 6-11), a decrease in the amount of Cmyc-β-arrestin2 could be observed. From this experiment, it is hypothesized that KLHL12 can displace Cmyc-β-arrestin2 from the receptor. It must be remarked that this experiment was done in twofold, but that these conclusions are derived from only one experiment because the second was not conclusive. Nevertheless, these results are presented because they are in accordance with the BRET competition assay performed by K. Skieterska (see higher).

However, two configurations remain possible. Either, cytoplasmatic KLHL12 is responsible for displacing β-arrestin2 from the receptor or the receptor-bound pool of KLHL12 is able to fulfill this function. To rule out one of both configurations, a membrane competition coIP using the D4.2R was performed. This experiment, however, did confirm nor rejection our working hypothesis.

3.6. Influence of β-arrestin2 and KLHL12 on D4R ubiquitination

KLHL12 overexpression has been shown to increase the ubiquitination signal of the D4R. KLHL12 acts as an adaptor that targets the Cullin3-based E3 ligase complex to the D4R for ubiquitination (Rondou et al, 2010b). In this work it is investigated whether β-arrestin2, which is an adaptor protein for many signaling molecules (DeWire et al, 2007), can influence the ubiquitination signal of the D4R. It will be also investigated whether KLHL12 and β-arrestin2 can work synergistically to alter the ubiquitination signal of the D4R.

HEK293T cells were transiently transfected with the pHA D4.2R, pFlag-Ub and pEtag-KLHL12 and/or pCmyc-β-arrestin2. Cell were lysed and subjected to two sequential IPs with anti-HA, immunoprecipitating the receptor. This second IP is performed to remove receptor interacting proteins (e.g. ubiquitinated proteins) that could contribute in a non-specific way to the ubiquitination signal of the D4R. The resulting immunoblots are presented in Figure 3.16.
Figure 3.16: Double coIP ubiquitination assay. Lysates of HEK293T cells transiently transfected with HA D₄R, FlagUb and Cmyc-β-arrestin2 and/or KLHL12 were subjected to IP with anti-HA antibody. Expression of Etag-KLHL12 (lysate blot, anti-Etag, lane 5, 7), Cmyc-β-arrestin2 (lysate blot, anti-Cmyc, lane 6, 7) and Flag-Ub (lysate blot, anti-Flag, lane 3-7) were checked in lysate. Receptor levels were examined (IP blot, anti-HA, 2, 4-7) and can be considered equally strong. The ubiquitination pattern of the D₄R was monitored by immunoblotting with anti-Flag antibody (IP blot, anti-Flag, 4-7). The experiment was done in threefold.

In the lysate, expression of Etag-KLHL12 (lysate blot, anti-Etag, lane 5, 7), Cmyc-β-arrestin2 (lysate blot, anti-Cmyc, lane 6-7), Flag-Ub (lysate blot, anti-Flag, lane 3-7) and the D₄R receptor (not shown) was confirmed. Coinmunoprecipitation of KLHL12 and β-arrestin2 in IP1 was confirmed upon incubation with respectively anti-Etag and anti-Cmyc (not shown). In IP2, KLHL12, β-arrestin2 and other (ubiquitinated) receptor interacting proteins are no longer co-immunoprecipitated with the receptor. The D₄R expression levels in IP2 are checked (IP blot, anti-HA, lane 2, 4-7). These receptor levels are however not equal: e.g. in lane 5 (IP blot, anti-HA) there is less receptor compared to lane 4, in lane 6 and 7 there is more receptor than in lane 4. Ubiquitination of the D₄R was checked by performing immunoblotting with anti-Flag antibody, specifically recognizing the Flag-Ub. The ubiquitination levels were quantified to assist the interpretation of the blots (Figure 3.17). In the left graph, the absolute ubiquitination level is given and in the right graph, the ubiquitination signals were normalized to the receptor expression levels in IP2 (because these are not equal in the different setups).

In Figure 3.16, it is shown that the presence of Flag-Ub together with the receptor already slightly increases the ubiquitination of the receptor (IP blot, anti-Flag, lane 4). When β-arrestin2 and KLHL12 were cotransfected (IP blot, anti-Flag, lane 7), the D₄R ubiquitination signal was 1.5 times higher (Figure 3.17, left, 5-7) than when KLHL12 was present alone (IP blot, anti-Flag, lane 5). When the D₄R ubiquitination signal of β-arrestin2 alone (IP blot, anti-Flag, lane 6) is compared to D₄R signal with Flag-Ub alone (IP blot, anti-Flag, lane 4), the signal was found to be 1.8 times stronger in the combined setup (Figure 3.17, left, 4-6). However, the receptor expression levels were not constant in every setup. Therefore, it is more accurate to look at the normalized ubiquitination signals. In Figure 3.17 it is shown that when KLHL12 and β-arrestin2 are present together, that the increase in the ubiquitination of the D₄R was lower (0.8 times) compared to the increase in D₄R ubiquitination with KLHL12.
alone. This suggests that KLHL12 is less ubiquitinating the D₄R in the presence of β-arrestin2. From Figure 3.17 it can also be derived that the increase in D₄R ubiquitination, when KLHL12 and β-arrestin2 are present together, is lower (1.45 times) than the increase in D₄R ubiquitination with β-arrestin2.

![Ub signal and Normalized Ub signal](image)

**Figure 3.17: Quantification of the ubiquitination signal of the D₄R.** Upper, the ubiquitination signal of the D₄R is compared between setups. Lower, the normalized ubiquitination signal of the D₄R is compared between setups. Quantification was performed using ImageJ. a.u., arbitrary units.

### 3.7. Ligand binding experiments

In this section, we will investigate whether the presence of KLHL12 has an influence on the affinity of dopamine for the D₄.2R. In a first step, the maximal number of receptors (Bₘₐₓ) needs to be determined with saturation binding experiments. In a second step, competition binding experiments will be done to determine the affinity of dopamine for the D₄.2R in the presence or absence of KLHL12.

#### 3.7.1. Saturation binding experiments

HEK293S HA D₄.2R cells were transiently transfected with pcDNA3 or KLHL12. Membrane preparations were made, which were incubated with increasing concentrations of [³H]spiperone (D₂-like antagonist) and with binding buffer, to determine total binding, or with haloperidol (also a D₂-like antagonist), to determine non-specific binding. The specific binding of the radioligand can be determined by subtracting the non-specific binding from the total binding. The samples were done in duplicate.

The interaction of a radioligand with the receptor can be generally described as a reversible reaction (1), with an equilibrium dissociation constant K_D (2) (Vauquelin & Von Mentzer, 2007).

\[
L + R \rightleftharpoons L - R \quad (1)
\]

\[
K_D = \frac{[L][R]}{[L-R]} \quad (2)
\]

The relationship between the concentration of bound receptor [L-R] and the concentration of free radioligand [L], is described by (3).

\[
[R - L] = R_{tot} \left( \frac{1 + K_D}{[L]} \right) \quad (3)
\]
Substituting [L-R] with B (total bound receptor) and [Rtot] with \( B_{\text{max}} \) (total number of receptors), gives rise to (4) (Vauquelin & Von Mentzer, 2007).

\[
B = \frac{B_{\text{max}}}{1 + \frac{K_D}{[L]}}
\]  

(4)

Equation (4) can be graphically represented as a saturation binding plot that describes the amount of bound \([^{3}\text{H}]\text{spiperone} \) (fmol bound \([^{3}\text{H}]\text{spiperone}/\text{mg protein}\) in function of the concentration of \([^{3}\text{H}]\text{spiperone} \) (nM) (Figure 3.18). Initially, B increases linearly with [L]. However, when L is further increased, the curve will reach saturation (Vauquelin & Von Mentzer, 2007). The corresponding B value is the \( B_{\text{max}} \), the total number of receptors. In this graph (Figure 3.18), \( K_D \) corresponds to the concentration of radioligand at half-maximal binding (\( B_{\text{max}}/2 \)) of the receptors and describes the affinity of spiperone for the receptor (Vauquelin & Von Mentzer, 2007). A low \( K_D \) means a high affinity, while a high \( K_D \) means a low affinity.

![Graphical representation of the saturation binding plot.](image)

**Figure 3.18: Theoretical saturation binding plot.** Graphical representation of the saturation binding plot. From the specific binding curve, the \( B_{\text{max}} \) (total number of receptors) and \( K_D \) (concentration of radioligand at half-maximal binding of the receptors) can be determined. Source: (Vauquelin & Von Mentzer, 2007).

The results of our saturation binding experiments are plotted in Figure 3.19. The \( K_D \) and \( B_{\text{max}} \) values can be obtained by non-linear regression analysis of the data points. For the D4.2R with pcDNA3 we have more receptors (i.e. 8428) compared to the D4.2R with KLHL12 (i.e. 6984). This would indicate a decrease in the expression of the receptor in the presence of KLHL12. However, a substantial error is introduced when we extrapolate the \( B_{\text{max}} \) and \( K_D \) values from these graphs, because the specific binding curve has not reached saturation.
Figure 3.19: Saturation binding plots of the $D_{4.2}R$ with pcDNA3 or KLHL12. In both graphs total, non-specific and specific binding are shown. Top, saturation curve of $D_{4.2}R$ with pcDNA3. Bottom, saturation curve of $D_{4.2}R$ with KLHL12. Non-linear regression is performed and the resulting $B_{\text{max}}$ and $K_D$ values with their standard errors are listed in the corresponding tables.

Because equation (4) describes a non-linear relationship and because $B_{\text{max}}$ cannot be determined experimentally (this would require an infinite concentration of radioligand, $L=\infty$), $K_D$ and $B_{\text{max}}$ cannot be easily and correctly determined from the saturation plot. Therefore, (4) can be mathematically transformed to linear equation (5).

$$
\frac{B}{[L]} = \frac{B}{K_D} + \frac{B_{\text{max}}}{K_D}
$$

The corresponding plot is called a ‘Scatchard plot’. This plot shows the concentration of bound over free [$^3$H]spiperone in function of the amount of bound spiperone (fmol spiperone/mg protein). From this plot, $K_D$ can be calculated as the negative reciprocal of the slope ($K_D= -1/$slope) and $B_{\text{max}}$ as the intercept with the x-axis. The advantage of this Scatchard analysis is that the error on the interpolation of $B_{\text{max}}$ is smaller compared to the non-linear regression error of the saturation curve. The resulting Scatchard plots with the
extrapolated $B_{\text{max}}$ and $K_D$ values are shown in Figure 3.20. When the $B_{\text{max}}$ values are compared between both setups, the number of receptors is approximately the same. This suggests that the presence of KLHL12 does not result in breakdown of the receptor (in contrast to the results of the saturation curve analysis) and thus that KLHL12 does not significantly influences receptor expression. The $K_D$ values can be used to determine the $K_i$ (section 3.7.2).

![Figure 3.20: Scatchard plots of the D$_{4.2}$R with pcDNA3 or KLHL12. Top, Scatchard plot of D$_{4.2}$R with pcDNA3. Bottom, Scatchard plot of D$_{4.2}$R with KLHL12. Linear regression was performed and the resulting $B_{\text{max}}$ (X-intercept) and $K_D$ (-1/slope) values are listed.](image)

### 3.7.2. Competition binding experiments

HEK293S HA D$_{4.2}$R cells were transiently transfected with pcDNA3 or KLHL12. Membrane preparations were made, which were incubated with increasing amounts of dopamine (agonist) and constant amounts of $[^3]$H]spiperone and binding buffer. Samples were done in sextuplet. To determine the contribution of non-specific binding, a few membrane samples were incubated with $[^3]$H]spiperone and haloperidol (instead of binding buffer).

In general $[^3]$H]spiperone and dopamine will compete for the same site of the receptor. The interaction between the radioligand (L) or dopamine (competitor, I) with the receptor (R) can again be described by reversible reactions (6).

$$L + R \rightleftharpoons L - R$$

$$I + R \rightleftharpoons I - R$$

The equilibrium dissociation constants for these interactions are: $K_D$ for the radioligand-receptor interaction and $K_i$ for the competitor-receptor interaction. To obtain a competition binding plot, the bound radioligand in percentage is plotted against the dopamine concentration (logarithmic scale) (Figure 3.21).

When dopamine has decreased the radioligand binding with 50%, the corresponding concentration of dopamine, denoted as the IC$_{50}$, is equal to $K_i (1 + [L]/K_D)$. This means that the $K_i$ of dopamine can be calculated from its IC$_{50}$ value and from the $K_D$ obtained from the saturation binding experiment (7).

$$K_i = IC_{50}/(1 + [L]/K_D)$$
Figure 3.21: Theoretical competition binding plot. The percentage bound radioligand is plotted against the concentration competitor (here: dopamine). From this graph the IC$_{50}$ can be determined, the concentration of dopamine at half-maximal binding of the radioligand. From the IC$_{50}$ and the K$_D$ from the saturation experiment, the K$_i$ of the competitor can be calculated. Source: (Vauquelin & Von Mentzer, 2007).

The competition binding plot from our experiments is shown in Figure 3.22. From this graph we can conclude that KLHL12 does not influence the affinity of dopamine for the receptor, because the competition curves overlap almost completely. This can also be derived from the IC$_{50}$ values from both setups, because they are almost identical (Figure 3.22).

Figure 3.22: Competition binding plot from the D$_{4.2}$R with pcDNA3 or KLHL12. The IC$_{50}$ values from the setup with the D$_{4.2}$R and pcDNA3, and the setup with D$_{4.2}$R and KLHL12 are given.

3.8. cAMP assay

The D$_4$R binds to inhibitory G$_i$ proteins which negatively regulate adenylyl cyclase. The inactivity of AC impedes the formation of cAMP from ATP (Rondou et al, 2010a). Thus, we will investigate whether the interaction of KLHL12 with the D$_4$R can have an influence on the concentration of cAMP in the cell.
3.8.1. Principle of assay

cAMP is important in signaling because it links membrane receptors with their ligands to activation of downstream effectors. The concentration of cAMP in the cell is a function of the ratio of the rate of synthesis by AC and of the rate of breakdown by phosphodiesterases (Website_Cayman). Cayman’s cAMP assay is a competitive enzyme immunoassay (EIA). The experiment is based on the competition between free cAMP and a cAMP-acetylcholinesterase (AChE) conjugate, called cAMP Tracer, for a limited number of cAMP-specific antibody binding sites. This cAMP-specific antibody binds to an IgG antibody that is coated on the bottom of a 96-well plate (Figure 3.23). In the cAMP assay, the amount of cAMP Tracer is kept constant and the concentration of cAMP is varied. Therefore, the amount of cAMP Tracer that binds this cAMP-specific antibody will be inversely proportional to the concentration of cAMP that is present in the well (Website_Cayman). After washing the plate, Ellman’s reagent is added as a substrate for the acetylcholinesterase enzyme of the cAMP Tracer. This enzymatic reaction generates a yellow colored product, of which the absorbance can be measured at 412nm using a spectrophotometer. The absorbance will be directly proportional to the amount of cAMP Tracer and inversely proportional to the amount of free cAMP (Website_Cayman).

**Figure 3.23: EIA cAMP assay.** Upper left, plates are precoated with mouse monoclonal anti-rabbit IgG and blocked with a certain mix proteins. Upper right, the wells are incubated with cAMP Tracer, antiserum specific for cAMP and with standard or sample. Lower left, the wells are washed to remove unbound reagents. Lower right, Ellman’s Reagent, the substrate for the acetylcholinesterase, is added. A yellow solution is formed of which the absorbance at 412nm can be measured using a spectrophotometer. Source: (Website_Cayman).

3.8.2. Influence of KLHL12 on cAMP levels

HEK293T cells were transiently transfected with pHA D₄R and pcDNA3 or pEtag-KLHL12. The cells were pretreated with EBSS buffer and then incubated with different concentrations of PD-168077, a D₄R specific agonist (1nM, 10nM, 100nM, 1µM, 10µM). Forskolin, an activator of AC, was then added to increase the cAMP concentration in the cell to a significant reference level. In this way, the lowering of the cAMP levels by activation of the D₄R receptor with PD-168077 could be monitored better.
Following the conclusions from previous optimizations of the assay, samples were diluted 1/100, 1/150 and 1/200 (each time in duplicate). Standards (with known cAMP concentrations) were included in the assay to set up a standard curve. Different controls were included that are important for the data analysis: the blank (Bln), the non-specific binding control (NSB), the maximum binding ($B_0$) and the total activity control (TA). The Bln corrects for the background absorbance of the Ellman’s reagent and this value should be subtracted from all other absorbance values. NSB corrects for non-specific binding of the cAMP Tracer to the wells. $B_0$ is the maximum amount of tracer that the antibody can bind (in absence of free cAMP). Thus, the absorbance of every standard and sample is corrected with the Bln and the NSB and divided by the $B_0$ to compare the sample absorbance to the maximal absorbance. This corrected absorbance is described as $B/B_0$. In the standard curve the logit($B/B_0$) is plotted versus the cAMP concentration (pmol/mL); linear (y) and log (x) axes are used (Figure 3.24).

**Figure 3.24: Standard curve cAMP assay.** The logit($B/B_0$) is plotted versus the concentration cAMP (pg/mol). The equation of the standard curve and the $R^2$ are also mentioned.

Using the standard curve equation, the concentrations of the samples (pmol/mL) are determined according to their logit($B/B_0$) values. In both setups, HA D$_{4.2}$R with pcDNA3 and HA D$_{4.2}$R with KLHL12, the concentrations of cAMP (pmol/mL) are plotted against the concentrations of PD-168077. The corresponding graphs are presented in Figure 3.25. As mentioned previously, three dilutions of the samples, each done in twofold. The corresponding cAMP concentrations of the samples (three dilutions per sample) were merged in one graph per receptor setup (Figure 3.25). The error bars represent the SEM (standard error of the mean). Significant differences in cAMP concentration are calculated with a one-sample Student t-test (*$p<0.05$, **$p<0.01$) assuming equal variances. The cAMP levels of the samples are statistically compared to the 0nM sample, in which only forskolin is present. This sample (with forskolin alone and no PD) allows to start from a reference level of cAMP. In this way, a decrease in cAMP levels upon agonist stimulation, can be studied more accurately. For the setup with D$_{4.2}$R and pcDNA3 (Figure 3.25, left), it is shown that when the concentration PD-186077 is increased, the cAMP levels decrease significantly starting from 10nM PD-168077. At higher concentrations of PD-168077, the cAMP levels stay
constant, but are still significantly different from the reference sample (0nM PD). However, in the presence of KLHL12 a significant decrease in cAMP concentration was obtained at a higher concentration PD-168077, 100nM (Figure 3.25), compared to the setup with D$_{4.2}$R and pcDNA3. This can indicate that KLHL12 has an influence on the cAMP levels in the cell.

![Figure 3.25: Influence of PD stimulation on cAMP levels. cAMP levels (pmol/mL) are plotted against the concentration of PD. FK corresponds to treatment with forskolin alone (no PD). Upper left, transfection with D$_{4.2}$R alone, to see differences in cAMP levels within set-up. Upper right, transfection with D$_{4.2}$R and KLHL12, to see differences in expression within set-up. Down, combined graph of both set-ups to differences in cAMP levels between set-ups. Error bars represent the SEM (n=6). Significant differences are indicated: * p<0.05, **p<0.01, ***p<0.001.](image)

It must be remarked that this experiment was performed twice, but that the results are derived from one experiment. The results of one of the experiments were excluded because most measured values were outside of the range of the standard curve. The assay should be repeated twice more to be conclusive.

### 3.9. MAPK-phosphorylation assay

β-arrestin2 can act as a scaffolding protein that binds to different components of the ERK-activation cascade: Raf, MEK, ERK1/2 (DeWire et al, 2007; Lefkowitz & Whalen, 2004). Earlier western blot experiments performed by LEGEST, studied the influence of KLHL12 on MAPK activation. It was shown that KLHL12-mediated ubiquitination did not significantly influence MAPK activation and this for different D$_{4}$R variants (Rondou, 2009; Skieterska, Unpublished results). However, this procedure involved western blot experiments and the handling of a lot of samples. Therefore, the experiment was optimized to an in cell Western assay, in which cell lysis, SDS-PAGE and immunoblotting steps are eliminated. In this work it was investigated for the D$_{4.0}$R, the D$_{4.2}$R and the D$_{4.2KR}$R whether KLHL12 alone, β-arrestin2 alone or KLHL12 and β-arrestin2 could influence the MAPK activation.

For this experiment, HEK293S cells stably transfected with either HA D$_{4.0}$R, HA D$_{4.2}$R or HA D$_{4.2KR}$R, were used. Four experimental setups were designed to transiently transfect the stable cells: 1) pcDNA3, 2) pEtag-KLHL12, 3) pFlag-β-arrestin2, 4) pEtag-KLHL12 and pFlag-β-arrestin2. The different receptor variants, each in four different setups (1-4), were stimulated with quinpirole (10µM) for different time points (0’, 1’, 5’, 10’, 20’, 30’, 45’, 60’, 75’, 90’, 120’). After fixing, permeabilizing and blocking the cells, they were incubated with two primary antibodies: an anti-phospho MAPK p42/44 antibody and an anti-total ERK antibody. The first antibody recognizes phosphorylated ERK1/2 (p42/p44), while the latter recognizes non-phosphorylated and phosphorylated ERK1/2. The incubation with the anti-total ERK antibody was done to normalize the levels of phosphorylated ERK1/2 to the total population of ERK1/2. The MAPK activation values are corrected by subtracting the
background values. In our graphs, the MAPK activation is presented as fold basal. This means that the (normalized) MAPK values are divided by (normalized) MAPK values at 0’.

The results are given in Figure 3.26. For all studied receptor variants, the maximal MAPK activation is obtained 5’ after stimulation with quinpirole (Figure 3.26). When KLHL12 was present alone, no significant influence on MAPK activation could be detected, confirming the earlier studies of LEGEST. In the setup where β-arrestin2 was present alone, a statistically significant decrease in MAPK activation was obtained for D_{4.0}R, D_{4.2}R and D_{4.24KR}R when compared to transfection with pcDNA3 alone. This indicates that β-arrestin2 can negatively influence MAPK activation. When the MAPK values of the setup with KLHL12 and β-arrestin2 are compared to the setup with pcDNA3 alone, no consistent trend is observed for the three receptor variants under investigation.
Figure 3.26: MAPK activation of different D₄R variants transfected with KLHL12 and/or β-arrestin2. The MAPK activation fold basal values are plotted against the time points after quinpirole (10µM) stimulation. Top, graph for D₄.0R. Center, graph for D₄.2R. Bottom, graph for D₄.2KRR. The influence of Etag-KLHL12 and/or Flag-β-arrestin2 on MAPK activation is studied. The MAPK values are averages from two experiments. Error bars represent SEM.
Chapter 4: Discussion

4.1. Discussion

In previous studies, it has been shown that KLHL12 is a BTB-Kelch protein that specifically interacts with the polymorphic region in the third intracellular loop of the D₄R (Rondou et al, 2008). KLHL12 also acts as an adaptor protein for the Cullin3-based E3 ligase complex that targets the D₄R for ubiquitination (Rondou et al, 2010b). Sequential co-immunoprecipitation studies between the different polymorphic D₄R variants (D₄.2R, D₄.4R and D₄.7R) and KLHL12 revealed that the ubiquitination signal of the D₄.7R was less increased upon overexpression of KLHL12, compared to the D₄.2R and D₄.4R ubiquitination signals (Rondou, 2009). This could indicate that KLHL12 has a different affinity for the different polymorphic receptor variants. However, affinities cannot be determined via co-immunoprecipitation experiments. Therefore, BRET was introduced into the lab to study these differences in affinity. As the necessary donor (D₄.2R-Rluc, D₄.4R-Rluc) constructs were already available, only the acceptor plasmid KLHL12-YFP needed to be cloned.

In this thesis the cloning procedure of KLHL12-YFP was presented. The expression of the construct was confirmed via western blot, immunofluorescence and an expression test on the Victor plate reader. Via a Western blot test, the expression of KLHL12-YFP was shown. Using fluorescence microscopy, a green fluorescent signal was picked up upon transfection of cells with KLHL12-YFP. Moreover, after staining with an YFP-recognizing antibody, a nice colocalization of the green and red signal was observed, indicating that the green fluorescence specifically originated from KLHL12-YFP (Figure 3.5). Finally, the expression of KLHL12 was tested on the Victor plate reader by using cells that were transfected with increasing amounts of KLHL12-YFP. A clear increase in the fluorescent signal was obtained upon increasing the amount of pKLHL12-YFP (Figure 3.6). Finally, the correctness of the KLHL12-YFP construct was confirmed via sequencing. Together, these three successful expression tests and the sequencing results gave us enough proof to assume that KLHL12-YFP is well expressed under different experimental setups. Next, a western blot experiment was performed, in which it was shown that KLHL12-YFP specifically interacts with the D₄.2R and not with the D₄.0R (Figure 3.4). This confirmed that the YFP-tag was not influencing the interaction properties of KLHL12.

Because BRET was newly introduced in the lab, it required optimization. An important parameter that needed optimization was the ratio of the amount of donor plasmid (D₄R-Rluc) over acceptor plasmid (KLHL12-YFP). Therefore, saturation BRET experiments with constant amounts of donor and increasing amounts of acceptor were performed. However, we did not succeed in obtaining a saturation plot that would enable us to find the optimal donor over acceptor ratio (Figure 3.9). The main problem was the frequent occurrence of negative BRET ratios that needed to be excluded from the data set. These negative BRET ratios originated primarily from the high background fluorescence of the cells that were transfected with donor alone. This means that the cells showed significant autofluorescence. Therefore, different cell lines (e.g. HEK293 cells) can be tested for their autofluorescence. One could also argue that another reason for the negative BRET ratios, is the low expression levels of the acceptor plasmid. However, the expression of the KLHL12-YFP construct was thoroughly tested and cannot be the reason of our problem. Another possible reason for the negative BRET ratios is the suboptimal settings at which the Rluc emission was measured.
The emission filter could be set maximally at 460 nm, but the optimal emission occurs at 485 nm. It still needs to be ruled out experimentally whether these suboptimal measuring settings can have an influence on the results.

In previous experiments, it was revealed that KLHL12 was not interacting with the D4R, the artificial receptor were the polymorphic repeat region is removed. This was an extra confirmation of the Y2H study performed by (Rondou et al, 2008), in which KLHL12 was shown to be an interaction partner of the polymorphic region of the D3R. In this study it was investigated whether β-arrestin2, a known interactor of the D3R, was interacting with the same binding region of the receptor that binds with KLHL12 (i.e. the polymorphic repeat region in the third intracellular loop). Different receptor variants, namely D4R, D2R, D4, D3R and D3ΔΔR, were tested for their interaction with β-arrestin2. These co-immunoprecipitation studies revealed that β-arrestin2 is interacting with all the tested receptors, including the D4R (Figure 3.10, Figure 3.12). From these results it can be concluded that the polymorphic IC3 repeat region is not crucial for the binding of β-arrestin2 to the D3R.

When studying the protein domains involved in the interaction cycle between the D3R, β-arrestin2 and KLHL12, it became clear that some links were still missing. It was already known that KLHL12 is interacting via its Kelch domain with the D4R and with β-arrestin2 and that KLHL12 binds the repeat region in the third intracellular loop of the D3R (Rondou et al, 2008; Skieterska, Unpublished results). From β-arrestin2, it was only known that the C domain is important for its interaction with KLHL12. In studies with prototypical β2-adrenergic receptors and β-arrestin2 deletion mutants, it was shown that the β-arrestin2 mutant that only contained the N domain could still bind to the receptor, while the C domain mutant (only contained the C domain) could no longer bind the receptor (Orsini & Benovic, 1998). However, it was not yet revealed which domain of β-arrestin2 was binding to the D3R. Therefore, in this work it was investigated whether the C domain, the N domain, or both domains of β-arrestin2 were responsible for the interaction with the D3R. By using deletion mutants of β-arrestin2, in which either the C domain or the N domain was deleted, it was shown that both domains can bind individually to the receptor (Figure 3.13). However, it should be remarked that these conclusions are derived from in vitro experiments and that in vivo these β-arrestin2 mutants do not occur.

It can be of interest to investigate whether overexpression of KLHL12 in this deletion mutant experiment, could change the outcome of the experiment. It can be, that in the presence of KLHL12 the C domain mutant can no longer bind to the receptor, while the N domain mutant still can. This would introduce a bias towards the N domain of β-arrestin2, as is seen for the β2-adrenergic receptor. This experiment would rule out whether KLHL12 can have an influence on the binding properties of β-arrestin2 or not.

Another point of interest is to identify which region of the receptor is involved in β-arrestin2 binding. From classical β2-adrenergic receptor studies it is known that β-arrestin2 is preferentially recruited to phosphorylated receptor sites upon agonist stimulation (Vauquelin & Von Mentzer, 2007). However, the D3R does not respond to agonist-induced β-arrestin2 recruitment (Spooren et al, 2010), but rather interacts in a constitutive way with β-arrestin2. It has been suggested that two constitutive phosphorylated serine in the third intracellular loop of the D3R and other constitutive active regions are involved in the interaction with β-arrestin2 (Gurevich et al, 2008; Spooren et al, 2010). Also the DRF motif,
located in the second intracellular loop, could play a role in β-arrestin2 binding. Important to note is that the SH3-domains of the receptor coincide with the two constitutive phosphorylated serines and that these SH3-domains have been linked to internalization (Oldenhof et al, 1998; Rondou et al, 2010a). SH3-domains are present along the entire IC3 loop of the D₄R. It was also shown that upon deletion of the entire third intracellular loop, an interaction (although weakened) between the D₄R and Grb2 (SH3 adaptor protein) could still occur (Oldenhof et al, 1998). Not only does this mean that the IC3 loop is important for the interaction with Grb2, but also that probably multiple binding sites are possible. In this work, it was shown that β-arrestin2, which also binds to SH3 domains, can still interact with receptor variants in which the repeat region or the repeat flanking regions are removed. Also, because of its general role in internalization, it would be interesting to investigate whether β-arrestin2 can still bind to receptor mutants in which both the third intracellular loop is removed and in which the DRF motif is mutated to DRY.

Former studies revealed that coexpression of KLHL12 together with β-arrestin2 and the D₄R does not increase the interaction between the receptor and β-arrestin2 nor does it change the cellular localization of β-arrestin2 (Rondou et al, 2010b). Moreover, by performing competition BRET experiments, LEGEST showed that the constitutive interaction between β-arrestin2 and the D₄R and (also the D₄R) was decreased upon overexpression of KLHL12 (Skieterska, Unpublished results). This meant that KLHL12 was removing β-arrestin2 to a certain extent from the receptor. To confirm these results, a competition co-immunoprecipitation was performed in which the amounts of D₄R and β-arrestin2 were kept constant and the amount of KLHL12 was increased. This experiment, however, did not give conclusive results. As a control experiment, the competition co-IP was repeated but this time with the D₄R, which does not interact with KLHL12. This experiment showed that KLHL12 can displace β-arrestin2 from the receptor and that KLHL12 does not necessarily need to interact with the D₄R to perform this function. This experiment was done in twofold, but the results were derived from only one representative experiment. Together these results indicate that two possible interaction configurations remain possible when the KLHL12 levels are increased. One option is that KLHL12 is displacing β-arrestin2 from the receptor when KLHL12 is attached to the receptor, resulting in the formation of a trimeric complex. Another option is that a subpool of KLHL12 in the cytoplasm is responsible for displacing β-arrestin2 from the receptor, while another subpool of KLHL12 interacts with the receptor. In the latter configuration, β-arrestin2 would no longer be localized at the receptor, while in the former it would still be indirectly attached to the receptor via KLHL12. Both the competition BRET and the competition coIPs do not exclude one of the two configurations. Therefore, a competition membrane IP was performed to check whether β-arrestin2 was still present at the membrane. Unfortunately, these experiments were also not conclusive. Another possible approach to distinguish between the configurations, would be to perform a yeast-three-hybrid screen or to use split-GFP constructs (one GFP-domain attached to KLHL12 and another to β-arrestin2) in competition BRET experiments.

From previous studies it is known that KLHL12 acts as an adaptor protein for the Cullin3-based E3 ligase complex that targets the D₄R for ubiquitination (Rondou et al, 2008). It has also been shown that the ubiquitination of the D₄R does not target the receptor for degradation (Rondou et al, 2010b). In this context, it was investigated whether β-arrestin2, a multifunctional scaffolding protein for many signaling molecules (DeWire et al, 2007), can
influence the ubiquitination signal of the D_4R. In the sequential co-immunoprecipitation studies, it was shown that β-arrestin2 could increase the D_4R ubiquitination signal 1.8 times compared to transfection with pFlag-Ub alone (Figure 3.16, Figure 3.17). When β-arrestin2 and KLHL12 were cotransfected, the ubiquitination signal was 1.5 times higher than when KLHL12 was present alone (Figure 3.17). However, it can be remarked that the receptor expression levels were not constant in every setup. Therefore the ubiquitination signals were normalized to the receptor expression levels (Figure 3.17). In Figure 3.17 it is shown that when KLHL12 and β-arrestin2 were present together, the increase in the ubiquitination of the D_4R was lower (0.8 times) compared to the increase in D_4R ubiquitination with KLHL12 alone. This suggests that the ubiquitinating effect of KLHL12 on the D_4R is lowered in the presence of β-arrestin2. From Figure 3.17 it can also be derived that the increase in D_4R ubiquitination, when KLHL12 and β-arrestin2 are present together, is also lower (1.5 times) than the increase in D_4R ubiquitination with β-arrestin2 alone. Together these results suggest that a certain pool of KLHL12 is no longer involved in D_4R ubiquitination when β-arrestin2 is present and vice versa that a certain pool of β-arrestin2 is no longer involved in D_4R ubiquitination when KLHL12 is present. These results can support our (previously mentioned) hypothesis that KLHL12 is able to displace β-arrestin2 from the receptor. Because KLHL12 binds to β-arrestin2 and Cullin3, which itself binds to Roc1, it could be investigated whether β-arrestin2 interacts with Cullin3 or Roc1. This would be a possible explanation for the increased ubiquitination of the D_4R in the presence of β-arrestin2 (1.4 times), compared to the setup with Flag-Ub alone.

Another approach to study the ubiquitination pattern of the D_4R, is to use an in vitro ubiquitination assay (Belz et al, 2002; Beyaert & Carpentier, 2010-2011; Website_Rubicon). Five basic components are necessary for this assay: the E1, E2 and E3 enzymes, ubiquitin (recombinant) and the substrate. The substrate, here the D_4R, can be immunoprecipitated out of cell lysates and coupled to beads. As a source of E1 and E2 enzymes, one can use rabbit reticulocyte lysate or recombinant proteins (Beyaert & Carpentier, 2010-2011). The E3 ligase complex (KLHL12-Cullin3-Roc1) can be purified in vitro, after transfecting cells with the corresponding constructs and immunoprecipitating Cullin3. To study the influence of KLHL12, β-arrestin2 or the combination of both proteins on the ubiquitination of the D_4R, one could add these as recombinant proteins to the reaction mixture. However, these proteins can also be purified in vitro. The different reagents can then be incubated for one hour at 37°C in ubiquitination buffer, in the presence of ATP. Finally, the eluted proteins can be subjected to SDS-PAGE and immunoblotting.

In another part of this work the functional consequences of D_4R ubiquitination and the presence of KLHL12 and/or β-arrestin2 in this process were addressed. As a starting point, the number of receptors was determined in a setup with the D_4_2R and pcDNA3, and in setup with the D_4_2R and KLHL12. Via Scatchard analysis, it was shown that the receptor levels were comparable in both setups (Figure 3.20). This experiment also confirmed that overexpression of KLHL12 does not promote receptor degradation (Rondou et al, 2010b). Next, the affinity of dopamine for the receptor was determined in both setups via competition binding experiments (Figure 3.22). It was shown that the affinity of dopamine is almost unchanged upon overexpression of KLHL12. These results imply that no differences in affinity must be taken into account in further functional studies. To test the influence of ubiquitination, the experiment could also be repeated with the D_4_24KR transfected with
pcDNA3 or pKLHL12. If ubiquitination has no influence on the affinity of dopamine for the receptor, similar results should be obtained as with the D$_{4.2}$R ligand binding experiments. Because of the constitutive interaction between the D$_{4}$R and β-arrestin2, it could be interesting to investigate whether overexpression of β-arrestin2 has an influence on the receptor levels and on the affinity of dopamine for the receptor.

The D$_{4}$R couples to inhibiting G$_{i}$ proteins which negatively regulate AC and thereby the production of cAMP from ATP (Rondou et al, 2010a). It has been shown that the potency to inhibit AC can be different among receptor variants. It was showed by (Asghari et al, 1995) that the D$_{4.7}$R is two to three times less potent in coupling to AC, which influences the cAMP levels. It was not yet known whether KLHL12 could influence the cAMP levels in the cell. As a starting point, the D$_{4.3}$R was used to study the influence of KLHL12 on cAMP production. Due to the inhibiting nature of the associated G proteins, the cells were treated with different concentrations of PD and forskolin (10µM), an activator of AC which brought the concentration cAMP in the cell to a reference level. These experiments revealed that the cAMP levels were decreased significantly at 10nM PD in the absence of KLHL12 (Figure 3.25). However, in the presence of KLHL12 a significant decrease in cAMP concentration was obtained at a higher concentration PD, 100nM (Figure 3.25). This can indicate that KLHL12 has an influence on the cAMP levels in the cell. Four possible outcomes are possible: 1) KLHL12 has no influence, because follow-up experiments cannot confirm this experiment, 2) KLHL12 has an influence and its binding to the receptor is important, 3) KLHL12 has an influence and its binding to receptor as well as ubiquitination play a role, or 4) the cytoplasmatic pool of KLHL12 has influence. Therefore, different follow-up experiments can be suggested. As a negative control, the experiment can be repeated with the D$_{4.0}$R transfected with pcDNA3 or pKLHL12. Also, the D$_{4.2}$R transfected with pKLHL12ΔKelch (in which the Kelch domain is deleted) can be interesting to investigate. This setup could also indicate the importance of receptor binding and in parallel whether a cytoplasmatic pool of KLHL12 could influence the cAMP concentration. To study the role of ubiquitination on cAMP levels, the D$_{4.24Kr}$R with KLHL12 can be used.

It is known that β-arrestin2 can act as a scaffolding protein that is able to bind to the different components of the ERK-activation cascade: Raf, MEK, ERK1/2 (DeWire et al, 2007; Lefkowitz & Whalen, 2004). A consequence of the β-arrestin2-dependent activation of the ERK1/2 pathway, is the cytoplasmatic retention of activated ERK1/2, thereby inhibiting its translocation to the nucleus (Lefkowitz & Whalen, 2004). Previous experiments performed by LEGEST, studied the influence of KLHL12 on MAPK activation. For different receptor variants, KLHL12-mediated ubiquitination did not significantly influence MAPK activation (Rondou, 2009; Skieterska, Unpublished results). In this work it was investigated for the D$_{4.0}$R, the D$_{4.2}$R and the D$_{4.24Kr}$R whether KLHL12 alone, β-arrestin2 alone or KLHL12 and β-arrestin2 could influence the MAPK activation. The maximal MAPK activation was obtained 5 minutes after stimulation with quinpirole (Figure 3.26). For KLHL12 alone, no significant influence of MAPK activation could be detected, confirming the earlier studies of LEGEST. In the setup where β-arrestin2 was present alone, a statistically significant decrease in MAPK activation was obtained for D$_{4.0}$R, D$_{4.2}$R and D$_{4.24Kr}$R when compared to transfection with pcDNA3 alone. This indicates that β-arrestin2 can negatively influence MAPK activation. This finding is in contrast with reported results from prototypic GPCRs, where β-arrestin2 increases MAPK activation (DeWire et al, 2007; Lefkowitz & Whalen, 2004).
values of the setup with KLHL12 and β-arrestin2 are compared to the setup with pcDNA3 alone, no consistent trend is observed for the three receptor variants under investigation. It might be interesting to repeat the experiment for other receptor variants (e.g. the D₄.₄R and the D₄.₇R), focusing on the influence of β-arrestin2 on the MAPK activation.

Because the D₄R is not a prototypical GPCR, a lot of open questions concerning the regulation of the D₄R, the influence of β-arrestin2 and KLHL12 on the signaling properties of the receptor and the role of receptor ubiquitination remain. In this work, it was shown that the repeat region is not crucial for the binding of β-arrestin2 to the receptor and that both the C domain and N domain of β-arrestin2 can interact individually with the D₄R. It was also shown that KLHL12 can displace β-arrestin2 from the receptor and that β-arrestin2 can increase the ubiquitination signal of the D₄R. Furthermore, it was revealed that the affinity of dopamine for the D₄R does not change in the presence of KLHL12. It was also shown that KLHL12 can influence the cAMP levels in the cell, because a decrease in cAMP levels is obtained at higher concentrations of PD. Finally, it was revealed for different D₄R variants that β-arrestin2 decreases the MAPK activity.

4.2. Discussie

In eerdere studies is aangetoond dat KLHL12 een BTB-Kelch proteïne is, dat specifiek interageert met de polymorfe regio in de derde intracellulaire lus van de D₄R (Rondou et al, 2008). KLHL12 fungeert ook als een adapter eiwit voor het Cullin3-gebaseerde E3 ligase complex dat de D₄R voor ubiquitinitatie target (Rondou et al, 2010b). Sequentiële co-immunoprecipitatie studies tussen de verschillende polymorfe D₄R varianten (D₄.₂R, D₄.₄R en D₄.₇R) en KLHL12 toonden aan dat het ubiquitinatiesignaal van de D₄.₇R minder verhoogd was bij overexpressie van KLHL12 in vergelijking met de D₄.₂R en D₄.₄R ubiquitinatiesignalen. Dit zou erop kunnen wijzen dat KLHL12 een verschillende affiniteit heeft voor de verschillende polymorfe receptorvarianten. Affiniteiten kunnen echter niet bepaald worden via co-immunoprecipitatie experimenten. Daarom werd BRET geïntroduceerd in het labo om deze verschillen in affiniteit te bestuderen. Aangezien de nodige donor constructen (D₄.₂R-Rluc, D₄.₇R-Rluc) reeds beschikbaar waren, diende enkel het acceptor plasmide, KLHL12-YFP, gekloneerd te worden.

In deze thesis werd de kloneringsprocedure van KLHL12-YFP gepresenteerd. De expressie van het construct werd bevestigd via western blot, immunofluorescentie en een expressietest op de Victor plaatlezer. Via een testexperiment op western blot werd de expressie van het KLHL12-YFP aangetoond. Met behulp van fluorescentie microscopie werd een groen fluorescent signaal opgepikt na transfectie van cellen met pKLHL12-YFP. Bovendien werd na kleuring met een YFP-herkennend antilichaam, een mooie colokalisatie van het groene en rode signaal waargenomen, wat aangaf dat de groene fluorescentie specifiek afkomstig was van KLHL12-YFP (Figuur 3.5). Tenslotte werd aangetoond dat wanneer cellen getransfecteerd werden met toenemende hoeveelheden KLHL12-YFP, er een duidelijke toename in fluorescent signaal werd verkregen (Figuur 3.6). Tot slot, werd de juistheid van het KLHL12-YFP construct bevestigd via sequenering. Samen gaven deze drie succesvolle expressietesten en de sequeneringsresultaten ons voldoende bewijs om te veronderstellen dat KLHL12-YFP goed wordt geëxpressieerd. Vervolgens werd een western blot experiment uitgevoerd, waarin werd aangetoond dat KLHL12-YFP specifiek interageert
met de $D_{4.2}R$ en niet met de $D_{4.0}R$ (Figuur 3.4). Dit bevestigde dat de YFP-tag de interactie-eigenschappen van KLHL12 niet beïnvloed.

Omdat BRET sinds kort in het labo was geïntroduceerd, vereiste de techniek optimalisatie. Een belangrijke parameter die geoptimaliseerd moest worden, was de verhouding van de hoeveelheid donor plasmide ($D_{4.0}R$-RLuc) op acceptor plasmide (KLHL12-YFP). Hiervoor werden saturatie BRET experimenten uitgevoerd met constante hoeveelheden donor en toenemende hoeveelheden acceptor. Echter, de verkregen saturatie curves lieten ons niet toe om deze optimale verhouding te bepalen (Figuur 3.9). Het grootste probleem was het veelvuldig voorkomen van negatieve BRET ratio's die uitgesloten moesten worden van de dataset. Deze negatieve BRET ratio's waren hoofdzakelijk afkomstig van de hoge achtergrondfluorescentie van de cellen die getransfecteerd waren met uitsluitend donor. Dit betekent dat de cellen een significante autofluorescentie vertoonden. Daarom kunnen verschillende cellijnen (bijvoorbeeld HEK293 cellen) getest worden op hun autofluorescentie. Men zou eveneens de lage expressieve matie van het acceptorplasmide kunnen vooropstellen als verklaring voor de negatieve BRET ratio's. De expressie van het KLHL12-YFP construct werd echter grondig getest en kan daarom niet de reden van ons probleem zijn. Een andere mogelijke reden voor de negatieve BRET ratio's is de suboptimale instelling van de filter waarmee de RLuc emissie werd gemeten. De emissie filter kan maximaal ingesteld worden op 460nm, hoewel de optimale emissie plaatsvindt bij 485nm.

In eerdere experimenten werd aangetoond dat KLHL12 niet interageert met de $D_{4.0}R$, de kunstmatige receptor waarmee de polymorfe repeat regio is verwijderd. Dit was een extra bevestiging van de Y2H studie uitgevoerd door (Rondou et al, 2008), waarin werd aangetoond dat KLHL12 specifiek interageert met de polymorfe regio van de $D_{4}R$. In deze studie werd onderzocht of $\beta$-arrestin2 een gekende interactiepartner van de $D_{4}R$, met dezelfde regio van de receptor interageert als KLHL12. Verschillende receptor varianten, namelijk $D_{4.0}R$, $D_{4.2}R$, $D_{4.4}R$, $D_{4.7}R$ and $D_{4.2\Delta\Delta}R$, werden getest op hun interactie met $\beta$-arrestin2. Deze co-IP studies toonden aan dat $\beta$-arrestin2 interageert met alle geteste receptoren, inclusief de $D_{4.0}R$ (Figuur 3.10, Figuur 3.12). Dit toont aan dat de polymorfe IC3 repeatregio niet cruciaal is voor de binding van $\beta$-arrestin2 aan de $D_{4}R$.

Bij het bestuderen van de proteïne domeinen die betrokken waren bij de interactiecyclus tussen de $D_{4}R$, $\beta$-arrestin2 en KLHL12, werd duidelijk dat een aantal links nog ontbraken. Het was reeds gekend dat KLHL12 via zijn Kelch domein met zowel de $D_{4}R$ als $\beta$-arrestin2 interageert en dat KLHL12 bindt aan de derde intracellulaire lus van de $D_{4}R$ (Rondou et al, 2008; Skieterska, Unpublished results). Van $\beta$-arrestin2 was enkel gekend dat het C domein belangrijk is voor de interactie met KLHL12. In studies met prototypische $\beta_{2}$-adrenerge receptoren en $\beta$-arrestin2 deletiemutanten werd aangetoond dat de $\beta$-arrestin2 mutant die enkel het N domein bevatte, nog steeds kon binden op de receptor, terwijl de C domein mutant (bevat enkel C-domein), niet langer aan de receptor kon binden (Orsini & Benovic, 1998). Het was echter nog niet onthuld welk domein van $\beta$-arrestin2 bond aan de $D_{4}R$. Daarom werd in dit werk onderzocht of respectievelijk het C domein, het N domein of beide domeinen van $\beta$-arrestin2 verantwoordelijk waren voor de interactie van $D_{4}R$. Door gebruik te maken van deletiemutanten van $\beta$-arrestin2, waarin hetzij het C domein of het N domein was verwijderd, werd aangetoond dat beide domeinen afzonderlijk kunnen interageren met de receptor (Figuur 3.13). Er moet echter worden opgemerkt dat deze conclusies afgeleid worden uit in vitro experimenten en dat in vivo deze $\beta$-arrestin2 mutanten niet optreden.

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Het kan interessant zijn om te onderzoeken of overexpressie van KLHL12 in dit deletiemutant experiment, het resultaat van het experiment zou kunnen wijzigen. Het kan zijn dat in de aanwezigheid van KLHL12, het C domein niet meer bindt aan de receptor, terwijl de N domein mutant dit wel kan. Dit zou een bias naar het N domein van β-arrestin2 introduceren, zoals waargenomen kan worden voor de β2-adrenerge receptor. Dit experiment zou aantonen of KLHL12 al dan niet een invloed heeft op de bindingseigenschappen van β-arrestin2.

Een ander belangrijk punt is om te bepalen welke regio van de receptor betrokken is bij de binding van β-arrestin2. Uit klassieke β2-adrenerge receptor studies is bekend dat β-arrestin2 preferentieel wordt gerekruiteerd naar gefosforyleerde receptoren na agonist stimulatie (Vauquelin & Von Mentzer, 2007). De D₄R reageert echter niet op agonist-geïnduceerde β-arrestin2 rekrutering (Spooren et al, 2010), maar interageert eerder op een constitutieve manier met β-arrestin2. Het is gesuggereerd dat twee constitutief gefosforyleerde serines in de derde intracellulaire lus van de D₄R en andere constitutieve actieve regio's betrokken zijn in de interactie met β-arrestin2 (Gurevich et al, 2008; Spooren et al, 2010). Ook het DRY motief, gelegen in de tweede intracellulaire lus, zou een rol kunnen spelen in β-arrestin2 binding. Verder, vallen de SH3-domeinen van de receptor samen met de twee constitutief gefosforyleerde serines en zijn deze SH3-domeinen betrokken zijn in internalisatie (Oldenhof et al, 1998; Rondou et al, 2010a). SH3-domeinen zijn aanwezig langs de gehele IC3 lus van de D₄R. In deze thesis werd aangetoond dat β-arrestin2, dwelke bindt aan SH3-domeinen, nog steeds kan interageren met receptor varianten in dwelke de repeat regio of de repeat flankerende gebieden zijn verwijderd. Omwille van zijn algemene rol in internalisatie, zou het interessant kunnen zijn om te onderzoeken of β-arrestin2 nog steeds kan binden aan receptor mutants waarin zowel de IC3 lus verwijderd is, als het DRY motief gemuteerd is tot DRY.

Eerdere studies toonden aan dat co-expressie van KLHL12 met β-arrestin2 en de D₄R, de interactie tussen de receptor en de β-arrestin2 niet doet toenemen en dat de cellulaire lokalisatie van β-arrestin2 ook niet verandert (Rondou et al., 2010b). Via competitie BRET experimenten toonde LEGEST aan dat de constitutieve interactie tussen β-arrestin2 en D₄₂R (en ook de D₄₇R) afnam na overexpressie van KLHL12 (Skieterska, Unpublished results). Dit betekende dat KLHL12 in staat was om β-arrestin2 van de receptor te verdringen. Om deze resultaten te bevestigen, werd een competitie co-immunoprecipitatie uitgevoerd waarbij de hoeveelheid D₄₂R en β-arrestin2 constant werd gehouden en de hoeveelheid KLHL12 werd verhoogd. Dit experiment gaf echter geen overtuigende resultaten. Als controle experiment werd de competitie co-IP herhaald maar nu met de D₄₀R, dwelke niet reageert met KLHL12. Dit experiment toonde aan dat KLHL12 β-arrestin2 kan verdringen van de receptor en dat KLHL12 niet noodzakelijk hoeft te interageren met de D₄₀R om deze functie uit te oefenen. Dit experiment werd gedaan in tweeëvoud, maar de resultaten zijn afgeleid van slechts een representatief experiment. Samen tonen deze resultaten aan dat twee interactie configuraties mogelijk blijven wanneer de concentratie KLHL12 wordt verhoogd. Een mogelijkheid is dat KLHL12 β-arrestin2 verdringt van de receptor terwijl KLHL12 gebonden is aan de receptor, resulterend in de vorming van trimeer complex. Een andere mogelijkheid is dat een subpool van KLHL12 in het cytoplasma verantwoordelijk is voor het verdringen van β-arrestin2 van de receptor, terwijl een andere subpool van KLHL12 interageert met de receptor. In deze laatste configuratie zou β-arrestin2 niet meer gelokaliseerd zijn ter hoogte
van de receptor; in de eerste configuratie zou β-arrestin2 nog indirect aan de receptor gebonden zijn via KLHL12. Zowel de competitie BRET experimenten als de competitie coIPs sluiten geen van beide configuraties uit. Daarom werd een competitie membraan IP uitgevoerd om te controleren of β-arrestin2 nog steeds aanwezig was aan het membraan. Helaas waren deze experimenten ook niet overtuigend. Een andere mogelijke aanpak om onderscheid te maken tussen beide configuraties, zou een yeast-three-hybrid screening zijn of competitie BRET experimenten met split-GFP constructen (één GFP-domein gehecht aan KLHL12 en het ander aan β-arrestin2).

Uit eerdere studies is gebleken dat KLHL12 fungeert als een adapter eiwit voor het Cullin3-E3 ligase complex dat de D₄R target voor ubiquitinatie (Rondou et al., 2008). Er is ook aangetoond dat ubiquitinatie de D₄R niet target voor afbraak (Rondou et al., 2010b). In deze context werd onderzocht of β-arrestin2, een multifunctioneel scaffold eiwit voor veel signaalmoleculen (DeWire et al, 2007), het ubiquitinatie signaal van de D₄R kan beïnvloeden. In sequentiële co-immunoprecipitatie studies werd aangetoond dat β-arrestin2 het D₄R ubiquitinatie signaal met 1,8 keer verhoogt ten opzichte van transfectie met pFlag-Ub alleen (Figuur 3.16, Figuur 3.17). Wanneer β-arrestin2 en KLHL12 samen getransfecteerd werden, was het ubiquitinatie signaal 1,5 keer hoger dan wanneer KLHL12 alleen aanwezig was (Figuur 3.17). Er kan echter worden opgemerkt dat de receptor expressieniveaus niet constant waren in de verschillende opstellingen. Daarom werd het ubiquitinatiesignaal genormaliseerd naar de receptor expressieniveaus (Figuur 3.17). In Figuur 3.17 wordt aangetoond dat wanneer KLHL12 en β-arrestin2 tegelijk aanwezig waren, de toename in ubiquitinatie van de D₄R (0.8 keer) lager was dan de toename in D₄R ubiquitinatie met KLHL12 alleen. Dit suggereert dat het ubiquitinerend effect van KLHL12 op de D₄R vermindert is in de aanwezigheid van β-arrestin2. Van Figuur 3.17 kan tevens afgeleid worden dat de toename in D₄R ubiquitinatie, wanneer KLHL12 en β-arrestin2 tegelijk aanwezig zijn, tevens (1.5 keer) lager is dan de toename in D₄R ubiquitinatie met β-arrestin2 alleen. Samen suggereren deze resultaten dat een bepaalde groep van KLHL12 niet langer betrokken is bij D₄R ubiquitinatie wanneer β-arrestin2 aanwezig is en dat vice versa een bepaalde groep van β-arrestin2 niet langer betrokken is bij D₄R ubiquitinatie wanneer KLHL12 aanwezig is. Deze resultaten kunnen onze (eerder vermelde) hypothese steunen dat KLHL12 in staat is om β-arrestin2 van de receptor te verdringen. Omdat KLHL12 bindt aan β-arrestin2 en Cullin3, welke zelf aan Roc1 bindt, kan onderzocht worden of β-arrestin2 interageert met Cullin3 of Roc1. Dit zou een mogelijke verklaring zijn voor de toegenomen ubiquitinatie van de D₄R in de aanwezigheid van β-arrestin2 (1.4 keer), in vergelijking met de opstelling met enkel Flag-Ub.

Een andere manier om het ubiquitinatie signaal van de D₄R te bestuderen, is door gebruik te maken van een in vitro ubiquitinatie assay (Belz et al., 2002; Beyaert & Carpentier, 2010-2011; Website_Rubicon). Vijf basiscomponenten zijn vereist: de E1, E2 en E3 enzymen, ubiquitine (recombinant) en het substraat. Om de invloed van KLHL12, β-arrestin2 of een combinatie van beide eiwitten op de ubiquitinatie de D₄R te bestuderen, kunnen deze toegevoegd worden als recombinante eiwitten. De verschillende componenten worden gedurende een uur geïncubeerd bij 37°C in ubiquitinatie buffer (met aanwezigheid van ATP). Tenslotte kunnen de geëlueerde eiwitten worden onderworpen aan SDS-PAGE en immunoblotting.
In een ander deel van dit werk, werden de functionele implicaties van D₄R ubiquitinatie en de aanwezigheid van KLHL12 en/of β-arrestin2 in dit proces bestudeerd. Als uitgangspunt werd het aantal receptoren bepaald in de setup met de D₄.2R en pcDNA3, en in de setup met de D₄.2R en KLHL12. Via Scatchard analyse werd aangetoond dat het aantal receptoren vergelijkbaar was in beide setups (Figuur 3.20). Dit experiment bevestigde eveneens dat overexpressie van KLHL12 geen receptor degradatie bevordert (Rondou et al., 2010b).

Vervolgens werd de affiniteit van dopamine voor de receptor bepaald in beide opstellingen via competitie bindingsexperimenten (Figuur 3.22). Er werd aangetoond dat de affiniteit van dopamine vrijwel onveranderd is bij overexpressie van KLHL12. Deze resultaten impliceren dat er in verdere functionele studies (met KLHL12) geen rekening moet worden gehouden met een verschil in affiniteit. Om de invloed van ubiquitinatie te testen, zou het experiment eveneens herhaald kunnen worden met de D₄.24KR en pcDNA3 of pKLHL12. Omwille van de constitutieve interactie tussen D₄R en β-arrestin2, zou het interessant kunnen zijn om te onderzoeken of overexpressie van β-arrestin2 al dan niet een invloed heeft op de receptorniveaus en de affiniteit van dopamine voor de receptor.

De D₄R koppelt met inhiberende Gᵢ proteïnen die AC negatief reguleren en de daarmee gepaard gaande productie van cAMP uit ATP (Rondou et al., 2010a). Het was echter niet bekend of KLHL12 de cAMP niveaus in de cel kan beïnvloeden. Als uitgangspunt werd de D₄.2R gebruikt. Door de inhiberende aard van de geassocieerde G-eiwitten, werden de cellen behandeld met verschillende concentraties PD en forskoline (10 µM), een activator van AC waardoor de concentratie cAMP in de cel gebracht wordt op een referentieniveau. Uit deze experimenten is gebleken dat de cAMP concentratie reeds significant verminderd is bij 10nM PD in afwezigheid van KLHL12 (Figure 3.25). In aanwezigheid van KLHL12 werd een significante afname van de cAMP concentratie pas vastgesteld bij een hogere concentratie PD, 100 nM (Figuur 3.24). Dit kan betekenen dat KLHL12 een invloed heeft op de cAMP niveaus in de cel. Maar in totaal zijn er vier mogelijke resultaten: 1) KLHL12 heeft geen invloed, 2) KLHL12 heeft invloed en de binding aan de receptor is belangrijk, 3) KLHL12 heeft invloed en de binding en ubiquitinatie spelen een rol, of 4) de cytoplasmatische pool van KLHL12 heeft invloed op de cAMP niveaus in de cel. Verschillende vervolgexperimenten kunnen voorgesteld worden. Als negatieve controle kan het experiment herhaald met D₄.0R, getransfecteerd met pcDNA3 of pKLHL12. Ook kan de D₄.2R getransfecteerd met pKLHL12ΔKelch (waarin het Kelch domain is verwijderd) interessant zijn om te onderzoeken. Om de rol van ubiquitinatie op de cAMP niveaus te bestuderen, kan de D₄.24KR met KLHL12 worden gebruikt.

Het is gekend dat β-arrestin2 kan fungeren als een multifunctioneel adaptor eiwit dat de verschillende componenten kan binden van de ERK-activatie cascade: Raf, MEK, ERK1/2 (DeWire et al., 2007; Lefkowitz & Whalen, 2004). Eerdere experimenten uitgevoerd door LEGEST, bestudeerden de invloed van KLHL12 op MAPK activatie. Voor verschillende receptor varianten, bleek KLHL12-afhankelijke ubiquitinatie geen significante invloed te hebben op MAPK activatie (Rondou, 2009; Skieterska, Unpublished results). In dit werk werd onderzocht voor de D₄.0R, de D₄.2R en de D₄.24KR of KLHL12, β-arrestin2 of KLHL12 en β-arrestin2 de MAPK activatie konden beïnvloeden. De maximale MAPK activatie werd telkens verkregen vijf minuten na stimulatie met quinpirole (Figure 3.26). Met KLHL12 alleen kon geen significante invloed op de MAPK activatie worden vastgesteld, wat de eerdere studies van LEGEST bevestigt. In de setup met enkel β-arrestin2 werd een statistisch
significante daling van de MAPK activatie waargenomen voor de D₄₀R, de D₄₂R en D₄₂₄KR in vergelijking met transfectie met pcDNA3 alleen. Dit geeft aan dat β-arrestin2 een negatieve invloed kan hebben op de MAPK activatie. Deze bevinding staat in contrast met de gerapporteerde resultaten van de prototypische GPCR's, waar β arrestin2 toeneemt MAPK activatie (Lefkowitz & Whalen, 2004). Het zou interessant zijn om dit experiment met andere receptor varianten (bijvoorbeeld de D₄₄R en de D₄₇R) te herhalen, met de nadruk op de invloed van β arrestin2 op de MAPK activatie.

De D₄R is geen prototypische GPCR, waardoor er veel open vragen blijven met betrekking tot de regulatie van de D₄R, de invloed van β-arrestin2 en KLHL12 op de signalisatie eigenschappen van de receptor en de rol van receptor ubiquitinatie. In dit werk werd aangetoond dat de repeat regio niet cruciaal is voor de binding van β-arrestin2 aan de receptor en dat zowel het C domein als het N domein van β-arrestin2 individueel kunnen interageren met de D₄R. Er werd tevens aangetoond dat KLHL12 β-arrestin2 kan verdringen van de receptor en dat β-arrestin2 het ubiquitinatiesignaal van de D₄R van verhogen. Bovendien werd aangetoond dat de affiniteit van dopamine voor de D₄R niet verandert in de aanwezigheid van KLHL12. Er werd ook aangetoond dat KLHL12 de cAMP niveaus in de cel kan beïnvloeden, omdat een daling in cAMP niveaus verkregen wordt bij hoge concentraties PD. Tot slot werd aangetoond dat voor verschillende D₄R varianten β-arrestin2 de MAPK activiteit doet afnemen.
Chapter 5: Materials and methods

In this chapter, the used techniques are described together with their principle. For detailed protocols, composition of buffers, primer sequences and suppliers, the reader is referred to the attachments.

5.1. Tissue culture

5.1.1. Cell lines

HEK293T cells are human embryonic kidney cells (HEK) cells that were transformed with parts of the adenovirus 5 genome: E1A and E1B. E1A plays a role in viral genome replication by disrupting the function of the host retinoblastoma (Rb) protein, a key cell cycle protein that normally blocks the transition from the G1 to the S phase; E1B blocks p53-dependent apoptosis. These cells also stably express the SV40 large T-antigen. Therefore, HEK293T cells transfected with plasmids containing a SV40 origin are replicated with a higher copy number (compared to in HEK293 cells), and proteins are thus expressed in higher levels (Van Craenenbroeck, 2010-2011; Website_HEK293). They also express a neomycine resistance gene. Also HEK293S cells were used, stably transfected with different polymorphic D₄R: HA D₄₀R, HA D₄₂R or HA D₄₂₄KR (HA, hemagglutinin).

5.1.2. Subculturing, counting and seeding of adherent cells

Cells are grown at 37°C in a CO₂-incubator (5% CO₂), that is flushed with sterile air. CO₂ is used to keep the cells at constant pH. Therefore, culture flasks may never be completely closed after splitting, to allow buffering of the cells. HEK293T cells are grown in Dulbecco’s modified eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS), L-glutamine and antibiotics (penicillin and streptomycin). HEK293S cells are grown in F12 medium, also supplemented with 10%FCS, L-glutamine and antibiotics. FCS contains a complex mix of nutrients, hormones and growth factors, attachment and spreading factors (e.g. fibronectin), binding proteins for carrying hormones and lipids (e.g. albumin) and protease inhibitors (trypsin neutralization, see further). Antibiotics are added to the medium to inhibit growth of bacteria in the medium. Cells were grown in cell culture flasks (175cm², 75cm²) until confluence.

After reaching confluence, cells are splitted. Therefore, cells need to be detached from the surface of the flask and from each other by adding ethylenediaminetetraacetic acid (EDTA)/trypsin buffer. EDTA is a chelating agent that removes divalent ions and disconnects cells from their substratum into suspension. In combination with trypsin, a protease, this detachment is much faster. Prolonged exposure to trypsin, however, can cause cell damage. Therefore, when cells are sufficiently loose, trypsin is neutralized by adding an equal amount of medium (10% FCS DMEM). Then, a small amount of this cell mixture is transferred to a new recipient with fresh medium, the rest of the cells can be disposed or kept for counting and seeding.

Before seeding cells, one needs to know the number of cells per mL. Counting cells can be done using a Bürker hemocytometer counting chamber and a light microscope. Cells are first stained with Trypan blue (1:10 in PBSA) and loaded on the counting chamber. This enables the discrimination between dead cells, permeable for Trypan blue and colored blue, and living cells.
The number of cells per mL is then determined as:

\[
\text{cells/mL} = \frac{\# \text{ counted living cells} \times 10 \times \text{(dilution factor)} \times 10\,000 \times \text{(counting chamber constant)}}{9 \times \text{(number of squares in counting chamber)}}
\]

Cells are then diluted with fresh medium (10% FCS DMEM) and transferred to the appropriate recipient. 24 hours after seeding, cells are transiently transfected.

### 5.2. Transient transfection

Transient transfection includes a number of methods to transfer the plasmid of interest into the cell. The plasmid is then transported to the nucleus and replicates episomal. The gene of interest will be expressed and the highest concentration of protein will be achieved 48 hours post-transfection; later the plasmid will be diluted in the growing cell population. In our experiments we used two types of chemical transfection methods: calcium phosphate transfection and PEI transfection.

#### 5.2.1. Calcium phosphate transfection

The cell membrane consists of negatively charged polar head groups and hydrophobic tails. To transfer DNA into the cells, the negative charge of DNA needs to be masked or neutralized. In the calcium phosphate method the DNA is first mixed with a calcium chloride ($\text{CaCl}_2$) solution (neutralization of negative charge) and then added to a buffered saline/phosphate solution (2x BS/Hepes). This generates a DNA-calcium phosphate precipitate that is taken up by the cells via endocytosis (Website_BioRad). The relative amounts of the reagents are presented (Table 5.1).

**Table 5.1: Calcium phosphate transfection volumes.** Relative volumes of reagents for different recipients.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>6-well plate</th>
<th>10 cm dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% FCS DMEM (refresh)</td>
<td>1.8mL</td>
<td>9mL</td>
</tr>
<tr>
<td>DNA (1µg/µL)</td>
<td>2µL</td>
<td>10µL</td>
</tr>
<tr>
<td>$\text{CaCl}_2$ (1.25M, pH=7.05)</td>
<td>20µL</td>
<td>100µL</td>
</tr>
<tr>
<td>0.1xTE (pH=7.6)</td>
<td>78µL</td>
<td>390µL</td>
</tr>
<tr>
<td>2xBS/Hepes (pH=7.05)</td>
<td>100µL</td>
<td>500µL</td>
</tr>
</tbody>
</table>

Approximately 24 hours after seeding, medium is refreshed (10% FCS DMEM) and cells are incubated with the transfection mixture. Then cells are placed back at 37°C and medium is refreshed again (10% FCS DMEM) six hours post-transfection. After 48 hours cells can be collected. Remark: if HEK293S cells are transfected, then 10% FCS F12 medium is used.

#### 5.2.2. PEI transfection

Cationic polymers, such as polyethyleneimine (PEI), interact with the negatively charged phosphate groups of DNA and efficiently condense the DNA. This complex is taken up by the cell via endocytosis. Once in the cell, the amine groups of PEI act as a proton sponge, that buffer the acidification of the endosomes. This inhibits the endocytic pathway and promotes rupture, thereby releasing the DNA that can enter the nucleus (Van Craenenbroeck, 2010-2011; Website_BioRad).
Medium is refreshed 24 hours after seeding with 2% FCS DMEM. Next two mixes are prepared: a DNA-serum free medium (SFM) mix and a PEI-SFM mix. Both mixes are combined, vortexed and incubated at room temperature (RT) for 10-15 min. The transfection mixture is added to the cells and 5 hours post-transfection medium is refreshed with 10% FCS DMEM. The relative amounts of the reagents are presented (Table 5.2).

### Table 5.2: PEI transfection volumes. Relative volumes of reagents for a 10 cm dish.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>10 cm dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% FCS DMEM (refresh)</td>
<td>9mL</td>
</tr>
<tr>
<td>DNA-SFM mix</td>
<td>DNA (1µg/µL)</td>
</tr>
<tr>
<td></td>
<td>SFM</td>
</tr>
<tr>
<td>PEI-SFM mix</td>
<td>PEI (1mg/mL stock)</td>
</tr>
<tr>
<td></td>
<td>SFM</td>
</tr>
</tbody>
</table>

### 5.3. Cloning of the construct KLHL12-YFP

For BRET, cloning of the construct KLHL12-YFP was necessary. The different steps in the cloning process are described.

#### 5.3.1. Restriction enzyme digest

We started from two vectors: a green fluorescent protein (GFP) vector that contains Etag-KLHL12 (insert) and the YFP vector (vector). Via clone manager, two restriction enzymes were selected that would cut the areas of interest: BglII and Nhel. Via double digest finder (Website_neb) we got information about the optimal buffer, temperature, addition of BSA, heat inactivation of the enzymes. The relative amounts of insert/vector were calculated as 3/1, which means an excess of insert over vector. The reaction mixtures (Table 5.3) were incubated overnight (ON) at 37°C.

### Table 5.3: Restriction enzyme digest. Reaction mixtures for the generation of the insert and vector.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Insert</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (1µg/µL)</td>
<td>30µL</td>
<td>10µL</td>
</tr>
<tr>
<td>BSA (100x stock)</td>
<td>0.5µL</td>
<td>0.5µL</td>
</tr>
<tr>
<td>NE Buffer 2 (10x stock)</td>
<td>5µL</td>
<td>5µL</td>
</tr>
<tr>
<td>BglII (10U/µL)</td>
<td>3µL</td>
<td>1µL</td>
</tr>
<tr>
<td>Nhel (10U/µL)</td>
<td>3µL</td>
<td>1µL</td>
</tr>
<tr>
<td>Bidi</td>
<td>8.5µL</td>
<td>32.5µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>50µL</td>
<td>50µL</td>
</tr>
</tbody>
</table>

#### 5.3.2. Agarose gel electrophoresis

Agarose gel electrophoresis is a technique used to separate DNA (or RNA) fragments according to their size in an agarose gel. After application of an electric field, negatively charged DNA will migrate from the negative to the positive pole of the electric field.
(Haegeman, 2010-2011). The length of the fragments is then compared to a molecular length marker; we used lambda DNA that was cut with the restriction enzyme PstI. The percentage agarose determines the range of DNA fragments that can be efficiently separated. We used a 1.2% agarose gel, suitable for separating DNA fragments of 0.4 – 6 kb. Gels were run at 120V, 400mA in 1xTBE buffer. Visualization of the DNA was done using an ethidiumbromide solution (EtBr, 10µg/L). EtBr is a planar molecule that intercalates between the bases of DNA and that releases a fluorescent signal after applying UV light (300nm). We used agarose gel electrophoresis for preparative and analytical purposes. Preparative, for the isolation of the vector and insert fragments and analytical to control restriction digests.

5.3.3. Elution of DNA fragments using the Nucleo Spin purification kit

For the elution of DNA from the agarose gel a commercial kit (Nucleo Spin purification kit, MN) was used (Website_Nucleospin_kit). The gel fragments were incubated with NT buffer and heated in the thermoshaker (50°C, 10min). This liquid is transferred to a column and centrifugation is applied to speed up passage of liquid through the column. The DNA is now bound to the column and the column is washed with NT3 buffer. Finally, DNA is eluted from the column using NE buffer.

5.3.4. Ligation reaction

Next, insert and vector DNA fragments, eluted from the agarose gel, are ligated. For the ligation reaction T4 DNA ligase is used, an ATP-dependent enzyme that creates a phosphodiester bond between dsDNA fragments (works on blunt and sticky ends). The ligation requires ATP, Mg\(^{2+}\), present in the T4 DNA ligase reaction buffer, stabilizing additives (spermidine) and an appropriate reaction temperature of 16°C. Spermidine stimulates the activity of the T4 DNA ligase. For the ligation reaction a 4x molar excess of insert is used compared to the amount of vector. The relative amounts of the reaction partners are given (Table 5.4). This reaction gives rise to the new KLHL12-YFP construct.

Table 5.4: Ligation reaction. Relative amounts of the reaction partners in the ligation reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volumes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>4µL</td>
</tr>
<tr>
<td>Insert</td>
<td>1µL</td>
</tr>
<tr>
<td>rATP</td>
<td>2µL</td>
</tr>
<tr>
<td>Spermidine</td>
<td>2µL</td>
</tr>
<tr>
<td>T4 DNA ligase buffer (10x stock)</td>
<td>2µL</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1µL</td>
</tr>
<tr>
<td>Bidi</td>
<td>8µL</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td><strong>20µL</strong></td>
</tr>
</tbody>
</table>

5.3.5. Transformation of competent bacteria

First, competent bacteria (MC1061) are subjected to the heat shock method, a method which enables the DNA to penetrate the bacteria. During the procedure, one needs to work as sterile as possible near the flame. 100ng of DNA is mixed with the competent cells and is
incubated on ice (15min). Then, the heat shock is applied by incubating the reaction mixture at 37°C for 5min. Now cells need to recover and are put on ice again. Next, lysogeny broth (LB) medium is added and the mixture is incubated 1 hour at 37°C; in this phase transformed bacteria will start to express the kanamycine resistance gene (selection marker) that is present in the new vector, pKLHL12-YFP. Then, LB agar plates supplemented with kanamycine (kan) are prepared. Finally, the bacterial culture is plated out on the kan plates to allow individual growth of bacteria. The plates are incubated ON at 37°C. Only transformed bacteria, which have taken up the plasmid with the kan resistance gene, will survive this selection and give rise to separate colonies. Appropriate positive (pEYFP-N3) and negative (Bidi) controls were included.

5.3.6. **Bacterial minipreparation**

After overnight incubation, the kan plates are checked for colonies. The colonies are labeled with a number. Then, a mixture of LB with kanamycine (100x stock) is prepared and added to culture flasks (3mL/flask). Next, individual colonies are picked up and transferred to the culture flasks with LB and kan. Bacteria are grown at 37°C during the day with application of rotation. Finally, 1.5mL of bacteria is transferred to eppendorfs and the bacterial pellet is frozen. The kan plates with the colonies are kept at 4°C.

5.3.7. **Plasmid DNA isolation via Birnboim**

The bacterial pellet is dissolved in TEG buffer and a mix of SDS and NaOH is added. SDS will denature the proteins and NaOH destroys the basepairing of the DNA strands. Chromosomal and plasmid DNA are now completely denatured (Birnboim & Doly, 1979). In the next step, renaturation is done using potassium acetate (KOAc). The complementary strands of the chromosomal DNA will not find each other efficiently and the chromosomal DNA precipitates. The plasmid DNA, however, will renature to its supercoil conformation. The plasmid DNA is then purified using isopropanol precipitation and washing with EtOH. Finally, the plasmid DNA is dissolved in Bidi.

Next, a control restriction enzyme digest is performed to check the correctness of the construct. Restriction enzymes are used to cut the plasmid DNA in fragments, which are then checked for their size via (analytical) agarose gel electrophoresis.

5.3.8. **DNA concentration measurement via Nanodrop**

The concentration of the isolated plasmids is determined by measuring the absorbance of DNA (260nm) using a spectrophotometer (Nanodrop). The software calculates the concentration of DNA (µg/µL) according to the absorbance. Different parameters to check the purity of the DNA are also given: A260/A280 and A260/A230. The 260/280 ratio, for example, assesses the purity of the DNA and should be around 1.8. Lower ratios can indicate the presence of proteins, phenol or other organic contaminants.

Next, the plasmid was sequenced using primers that span the Etag-KLHL12 sequence (primer I: EtagF, primer II: KLHL12-C terminal F., sequences in attachment) of the KLHL12-YFP construct.

5.3.9. **Bacterial maxipreparation**

Bacterial colonies from the Kan plates, are picked up and transferred to culture flasks containing LB and kan. The bacterial cultures are grown during the day at 37°C with
application of rotation. In the evening, a big culture bottle with LB and kan (150mL total) is prepared and the bacterial culture, grown during the day, is transferred sterile to this culture bottle. Bacteria are grown overnight at 37°C with application of rotation.

5.3.10. Plasmid DNA isolation via Qiagen plasmid purification kit

The bacterial cells of the maxipreparation are first harvested by centrifugation at 4°C. First, the bacterial pellet is resuspended in resuspension buffer to which RNaseA is added, then cells are lysed using a lysis buffer and the DNA is precipitated by adding the precipitation buffer (Website_Qiagen). After centrifugation, the cleared lysate is transferred onto a pre-equilibrated anion-exchange column to which the plasmid DNA is bound. Proteins, RNA and other impurities are washed from the column using a medium salt wash buffer. Plasmid DNA is eluted from the resin using a high-salt elution buffer. Then, the DNA is concentrated and desalted by isopropanol precipitation and washing with EtOH. Finally, the DNA pellet is redissolved in bidi.

5.4. Protein detection

5.4.1. RIPA cell lysis

Cells are first harvested 48 hours post-transfection. Therefore, cells are washed twice with ice-cold PBSA and then scraped from the surface of the 10 cm dish using a spatula. The cells are collected in a 15mL tube and centrifuged (1000rpm, 4°C, 10min). Cell pellet is frozen for at least 1 hour at 70°C, because this helps the lysis process. Then, after thawing of the cell pellet cells are resuspended in 400µL Radio-immunoprecipitation assay (RIPA) buffer to which protease and phosphatase inhibitors are added: aprotinin (2.3mg/mL), leupeptin (10µg/mL), pefabloc (1mM), β-glycerolphosphate (10mM) (some experiment also require the addition of N-ethylmaleimide (NEM) (10mM), an inhibitor of deubiquitinating enzymes). RIPA buffer also contains extra detergents like deoxycholic acid (DOC; 12.7mM). RIPA cell lysis is performed for 1 hour at 4°C using rotation. Finally, lysates are cleared from cell debris by centrifugation (4°C, 10 min, 8000rcf). Proteins are present in the supernatant and should be kept on ice till further handling.

5.4.2. Total lysis

Cells are first washed three times with ice-cold PBSA. Total lysis buffer, supplemented with fresh dithiothreitol (DTT, 50mM), is added to the cells (200µL/well of a 6-well plate). This generates a viscous lysate that is transferred to an eppendorf and is sonicated for 1min. Next, the lysate is denatured at 95°C for 5min and is then centrifuged. The mixture can now be loaded on a polyacrylamide gel.

5.4.3. SDS-PAGE

SDS-PAGE is a technique to separate proteins according to their molecular weight in an electric field. Polyacrylamide gels are formed by a polymerization reaction of monomers and their pore size is determined by the percentage acrylamide/bisacrylamide. Ammonium persulfate (APS) is the catalyzer of the polymerization and tetramethylethlenediamine (TEMED) the initiator. Polyacrylamide gels are composed of two phases: an upper stacking gel (pH=6.8), that concentrates the proteins, and a lower separating gel (pH=8.8), that separates the proteins (Haegeman, 2010-2011). We used 4% stacking gels and 10% separating gels.
Before loading, protein lysates are first mixed with DTT (50mM) and 4xLaemli buffer and denatured for 10min at 37°C in a thermoshaker. Laemli buffer contains SDS, an anionic detergent that denatures proteins and attaches a negative charge to the proteins proportional to their mass. This allows separation of the proteins according to their size. Because of the negative charge, proteins will migrate to the positively charged electrode and are separated by a molecular sieving effect. Then samples were loaded on gel and also a protein ladder (Pageruler prestained protein ladder, Fermentas, Thermo Scientific) was added. Gels are run in electrophoresis buffer at 100V and 250mA for approximately 1.5 hours.

5.4.4. Western blot

After gel electrophoresis, proteins were transferred to a nitrocellulose membrane by electroblotting in transfer buffer at 100V, 250mA for 1 hour (cooling with ice packs). The gel and the nitrocellulose membrane are placed between six Whatman papers (three under the gel and three above the membrane) and two sponges and are placed in a cassette. During the blotting process, proteins are transferred from the gel onto the membrane along an electric field and migrate from the positive to the negative pole. After transfer, membranes are blocked with Licor blocking buffer (50min) to prevent aspecific binding of the antibody. Then, membranes are incubated with primary antibody that recognizes the protein (or the tag attached to the protein) of interest. Next, blots are washed 3x 5min with TBS-T (Tween20 content: 0.05%), to remove unbound antibody. Now the blots are incubated for 45min with secondary antibody, which recognizes the primary antibody and allows visualization of the proteins. Blots are washed 3x 5min with TBS-T (to avoid background fluorescence) before detection on the Odyssey, an infrared imager which allows detection of the fluorescent signal emitted by secondary antibodies.

Alternatively, blots can be also incubated with HRP-conjugated antibodies. After incubation, blots are also washed with TBS-T (see above). For visualization, blots are incubated 1min with Western Lightning Chemiluminescence Reagent plus (1:1 enhanced luminol reagent: oxidizing agent). HRP that is coupled to the antibody will catalyze the oxidation of luminol. The light emission of the oxidized substrate can be detected with the Kodak Imaging station 440 CF (exposure time: 10min).

5.4.5. Stripping

Sometimes we want to remove all the antibodies that are already attached to the membrane, because we want to reprobe it with new antibodies that otherwise could not be used anymore (overlap of signal). Therefore, blots need to be stripped. We incubated the blots with Newblot stripping solution (Licor) in bidi (1:4 or 2:3 ratio) for 10min at RT or first 5min at 37°C and then 5min at RT, depending on the secondary antibodies that were used. Then blots are washed 3x with PBS, blocked for 20min with Licor blocking buffer and reprobed with primary antibody.

5.4.6. Protein concentration via BCA assay

To measure the protein concentration in samples, we used the BCA protein assay. This assay allows the colorimetric detection and quantitative measurement of total protein content. The assay is based on the reduction of Cu²⁺ to Cu⁺ by protein in an alkaline medium that can be colorimetrically detected by adding bicinchoninic acid (BCA). The formed complex
exhibits a strong absorbance around 562nm and increases almost linear with increasing protein concentrations. A standard serial dilution series, in the buffer in which the assay is performed, should be added to the analysis. This enables us to determine the concentration of the samples starting from the standard curve equation and the absorbance values of the samples. In the BCA kit, two reagents (A and B) are supplied that are mixed in 1:50 ratio. Solution A contains the BCA and solution B contains the copper sulfate (Website_BCA_kit). On a 96-well plate, 12.5µL of samples and standards is pipetted together with 100µL of BCA mix. The plate is then incubated for 30min at 37°C after which absorbance is measured using the Wallac Victor plate reader.

5.4.7. Immunofluorescence

Cells are seeded and transfected on 6-well plates coated with coverslips and 48 hours post-transfection immunofluorescence (IF) experiments are performed. Cells are first fixed with formaldehyde diluted 1:10 in Marks PBS (150mM NaCl, 10mM Na3PO4 pH=7.4) for 20min at RT, after which the cells are washed 3x with PBSA. Then, cells are permeabilized with 3%NCS/1%BSA/0.1% TritonX100 in PBSA for 20 min at RT and washed afterwards with PBSA. Next, cells are blocked with 3%NCS/1%BSA (blocking buffer) in PBSA for 1 hour at RT and then cells are incubated with primary antibody diluted in blocking buffer. Cells are washed again with PBSA and incubated with secondary antibody diluted in blocking buffer. Finally, cells are incubated with DAPI in PBSA for 5min and washed again thoroughly. Coverslips are taken out the 6-well plate and placed on a microscope glass with Mowiol, edges are fixed with nailpolish. Samples can now be visualized using the fluorescence microscope.

5.5. Protein interaction

5.5.1. Co-immunoprecipitation

HEK293T cells are seeded in 10 cm dishes and are transiently transfected, using PEI or calcium phosphate precipitation, with plasmids that code for the proteins of interest. After 48 hours, the cells are harvested and lysed using RIPA buffer (see 5.4.1). Part of the lysate (40µL) is kept separately to check protein expression and the remaining lysate is incubated for 2-4 hours with anti-HA 16B12 or anti-Flag M2 antibody (2µL), which recognize the glycosylated N-terminus of the overexpressed D4R or β-arrestin. Then, protein A beads are added, that recognize the Fc tail of the antibody. The sample together with the beads is rotated ON at 4°C. This allows the formation of bead-antibody-receptor-interacting protein complexes. The beads are then washed 3x with RIPA buffer and proteins are eluted under denaturing conditions (37°C) by adding 4xLaemli buffer (or 5xLaemli buffer to the lysate fraction) supplemented with fresh DTT (together called elution buffer). The denatured samples are loaded on gel and subjected to SDS-PAGE and Western blotting. Appropriate antibodies are then used to visualize the interaction partners of the receptor.

5.5.2. Double immunoprecipitation – ubiquitination assay

In this assay two sequential immunoprecipitations are carried out to remove the contribution of other ubiquitinated proteins to the ubiquitination signal of the receptor. Immunoprecipitation of the receptor was performed as described in section 5.5.1, but NEM was added to the RIPA buffer to inhibit deubiquitinating enzymes. The other difference is that the washed beads are heated three times for 10 min at 37°C in the thermosthaker, after
addition of elution buffer. From the first elute fraction 14µL is kept separately to check IP and ubiquitination of the receptor via WB. The three elute fractions are then mixed and diluted with RIPA buffer (avoiding breakdown of antibody) supplemented with NEM. Next 1.5µL of anti-HA 16B12 antibody is added and incubated again for 2-4 hours. The rest of the procedure is completely equivalent to a regular IP. After elution from the beads, samples are loaded on gel and analyzed via WB. The membrane is then probed with antibodies recognizing ubiquitin, the receptor and the interacting proteins (KLHL12 and β-arrestin2).

5.5.3. BRET

BRET is based on the transfer of energy between a luminescent energy donor (Renilla luciferase, RLuc coupled to protein 1) and a fluorescent energy acceptor (yellow fluorescent protein, YFP coupled to protein 2). This process is mediated via the oxidation of coelenterazine (substrate) by RLuc, which excites RLuc. If RLuc-protein 1 is sufficiently close to the acceptor YFP-protein 2 (<10nm), the energy emitted by RLuc will excite the fluorophore (Poyner & Wheatley, 2010). Consequently the fluorophore emits light at longer wavelengths, which can be monitored by a dual-filter luminometer.

HEK293T cells are seeded on 6-well plates and are transiently transfected with two constructs via the calcium phosphate precipitation method. The first plasmid expresses the D_{4.2}R-RLuc fusion protein and the other KLHL12-YFP. The optimal donor/acceptor ratio has to be optimized by doing saturation BRET experiments. This implies taking constant amounts of D_{4.2}R-RLuc and increasing amounts of KLHL12-YFP. After 48 hours, cells are collected in HBSS buffer, centrifuged and dissolved in fresh HBSS buffer. Then, a BCA assay (see 5.4.6) is performed to dilute the samples to equal protein concentration (a minimum of 0.3mg/mL is required). Then, 80µL of sample is transferred to a black and a white 96-well plate. The black plate is used for fluorescence measurement (excitation 485nm, emission 535nm), the white plate for luminescence (emission 460nm) and BRET 1 measurement (dual emission 460nm and 535nm). Next, 20µL of coelenterazine-h (5µM final concentration) is added to the samples in the white plate and after 1 min BRET1 is measured. After 10 min, the plate is measured for total luminescence. The BRET ratio is calculated (equation) and is plotted against the fluorescence/luminescence signal.

\[
BRET\ \text{ratio} = \frac{\text{long wavelength emission (donor – acceptor pair)}}{\text{short wavelength emission (donor – acceptor pair)}} - \frac{\text{long wavelength emission (donor only)}}{\text{short wavelength emission (donor only)}}
\]

5.6. Functional assays

5.6.1. Ligand binding assay

HEK293S HA D_{4.2}R cells are seeded in giant dishes and are transiently transfected with pcDNA3 or pEtag-KLHL12. 48 hours post-transfection, cells are collected and frozen for at least 1 hour at -70°C. In ligand binding experiments, two assays are performed: a saturation binding assay and a competition binding assay.

First, the saturation binding assay is performed to determine the number of receptors (B_{max}) and the equilibrium dissociation constant (K_D). In this experiment, constant amounts of membrane suspension are incubated with increasing amounts of [³H]spiperone (0.01nM-2.6nM final), together with binding buffer (1xBB) or with a constant high concentration of haloperidol (1µM final). This allows to determine respectively total and non-specific binding at each concentration of radioligand. Samples are incubated 1 hour at 20°C. Then,
receptor-bound [$^3$H]spiperone is harvested on a presoaked (with 0.1% PEI) filter. Filters are washed twice with 50mM TrisHCl pH 7.5. Finally, scintillation fluid is added to the filters. After ON incubation in the dark, the samples are measured with a scintillation counter (Perkin Elmer). The $B_{\text{max}}$ and $K_D$ values are obtained via Scatchard analysis of the results.

Second, competition binding experiments are performed to study the affinity of dopamine for the $D_{4.2}$R in the presence of pcDNA3 or KLHL12. Constant amounts of membrane suspension are incubated with a constant amount of [$^3$H]spiperone, but with increasing concentrations of dopamine (0.001nM-10µM). Also here haloperidol (1µM final) is used to determine non-specific binding. The processing and the measurement of the samples is analogous to the saturation binding experiment. The binding of the radioligand is then plotted against the concentration of dopamine, from which the IC$_{50}$ and $K_i$ can be determined.

5.6.2. cAMP assay

HEK293T cells are seeded on 10 cm dishes and transfected with the calcium phosphate method. Cells are reseeded 24 hours post-transfection to 12-well plates (samples in duplicate). The experiment is started 24 hours after reseeding. For this cAMP assay a commercial kit from Cayman is used (cAMP EIA kit, Website_Cayman). The assay is based on the competition between free cAMP and cAMP Tracer (cAMP-acetylcholinesterase conjugate) for a limited number of cAMP-specific rabbit antibody binding sites. Cells are first pretreated in EBSS buffer (20min, 37°C) after which different concentrations of PD (D$_4$R specific agonist) are added to the cells. Next, cells are incubated with forskolin and are lysed after 20 min in 0.1M HCl. Then, protein concentrations are determined via BCA (see 5.4.6) and samples are diluted to equal protein concentrations. Next, 2µL of sample is transferred to a 96-well plate and mixed with 198µL EIA buffer after which the dilutions are transferred to the antibody-coated assay 96-well plate. Also a standard serial dilution series and appropriate controls are added to the assay plate. Finally, cAMP Tracer and antiserum are added to the appropriate wells and the plate is incubated for 18 hours at 4°C. After 18 hours, wells are washed 5x with wash buffer after which Ellman’s reagent is added to the wells. The plate is then placed on the plate shaker for 90 min (in the dark). Finally, the absorbance is measured with the Victor Wallac plate reader. This allows to determine the concentration of cAMP in the different set-ups.

5.6.3. MAPK-phosphorylation assay

HEK293S HA D$_{4.0}$R, HA D$_{4.1}$R or HA D$_{4.24KR}$R cells are seeded on 10 cm dishes and transfected with the calcium phosphate method. Cells are reseeded 24 hours post-transfection to 96-well plates (50000cells/well, 24 wells/setup). In cell Western is started 24 hours after reseeding. Cells are starved in SFM F12 medium, 2 hours before starting. Then, quinpirole is diluted in SFM F12 (10µM final concentration) and added to the cells at different time points: 120’, 90’, 75’, 60’, 45’, 30’, 20’, 10’, 5’, 1’. Then, cells are fixed with formaldehyde in PBSA, washed with TritonX100 washing buffer (4x5min) and blocked with Licor blocking buffer. Next, cells are incubated ON with anti-phospho p44/p42 MAPK and anti-total ERK, diluted respectively 1:1000 and 1:800 in Licor blocking buffer. The next day, cells are washed with Tween20 washing buffer (4x5min), incubated with anti-rabbit green and anti-mouse red RD, diluted 1:800 in Licor blocking buffer with Tween20 (0.2%). Cells are washed 4x 5min with Tween washing solution (0.1% Tween20 in PBSA) and read on the Odyssey.
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**Attachments**

<table>
<thead>
<tr>
<th>Attachment</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Attachment 1: Buffers</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Attachment 2: Tissue culture</td>
<td></td>
<td>75</td>
</tr>
<tr>
<td>Attachment 3: Antibodies</td>
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<td>76</td>
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<tr>
<td>Attachment 4: Markers</td>
<td></td>
<td>76</td>
</tr>
<tr>
<td>Attachment 5: Co-immunoprecipitation protocol</td>
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<td>77</td>
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<tr>
<td>Attachment 6: Double co-immunoprecipitation protocol</td>
<td></td>
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<tr>
<td>Attachment 7: Saturation BRET assay</td>
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<td>Attachment 8: Saturation binding assay</td>
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<td>Attachment 9: Competition binding experiment</td>
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<tr>
<td>Attachment 10: cAMP protocol</td>
<td></td>
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</tr>
<tr>
<td>Attachment 11: MAPK-phosphorylation assay</td>
<td></td>
<td>84</td>
</tr>
<tr>
<td>Attachment 12: Cloning procedure</td>
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<td>85</td>
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</table>
## Attachment 1: Buffers

### Lysis buffers:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RIPA buffer</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>Tris HCl pH=7.5</td>
<td>50 mM</td>
</tr>
<tr>
<td>NP40</td>
<td>1%</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1%</td>
</tr>
<tr>
<td>DOC (add fresh)</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

**Inhibitors:**

- Aprotinin: 2.3 mg/mL; 4.4 U/mg protein (100x stock)
- Pefabloc: 200x stock
- β-glycerolphosphate: 100x stock
- Leupeptin: 1000x stock

**Double colP:**

- NEM: 100x stock

### Total lysis buffer: SDS sample buffer in bidil

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH=6.8 (stock 0.5M)</td>
<td>62.5 mM</td>
</tr>
<tr>
<td>SDS (stock 10%)</td>
<td>2%</td>
</tr>
<tr>
<td>Glycerol (stock 100%)</td>
<td>10%</td>
</tr>
<tr>
<td>Bromophenolblue (stock 1%)</td>
<td>0.1%</td>
</tr>
<tr>
<td>DTT (stock 1M)</td>
<td>50 mM; add fresh</td>
</tr>
</tbody>
</table>

### Denaturation buffers:

#### 4x Laemli: IP (50 mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH=6.8 (stock 1M)</td>
<td>3.1 mL (0.62 mM)</td>
</tr>
<tr>
<td>SDS (stock 10%)</td>
<td>20 mL</td>
</tr>
<tr>
<td>Glycerol (stock 100%)</td>
<td>10 mL</td>
</tr>
<tr>
<td>Bromophenolblue (stock 1%)</td>
<td>0.5 mL</td>
</tr>
<tr>
<td>Bidi</td>
<td>16.4 mL</td>
</tr>
<tr>
<td>DTT (stock 1M)</td>
<td>50 mM; add fresh; 2.5 µL/sample</td>
</tr>
</tbody>
</table>

#### 5x Laemli: Lysate (50 mL)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl pH=6.8 (stock 1M)</td>
<td>7.75 mL (0.155 mM)</td>
</tr>
<tr>
<td>SDS (stock 10%)</td>
<td>25 mL</td>
</tr>
<tr>
<td>Glycerol (stock 100%)</td>
<td>20 mL</td>
</tr>
<tr>
<td>Bromophenolblue (stock 1%)</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>Bidi</td>
<td>Add till total volume is 50 mL</td>
</tr>
<tr>
<td>DTT (stock 1M)</td>
<td>50 mM; add fresh; 2.5 µL/sample</td>
</tr>
</tbody>
</table>
Buffers for Western blot immunodetection:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>10x TBS: 0.5L</strong></td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>12.1g (200mM)</td>
</tr>
<tr>
<td>NaCl</td>
<td>40g (1.37M)</td>
</tr>
<tr>
<td>HCl (stock 37%)</td>
<td>Adjust pH to 7.6</td>
</tr>
<tr>
<td>Bidi</td>
<td>Add till total volume is 500mL</td>
</tr>
<tr>
<td><strong>1x TBST</strong></td>
<td></td>
</tr>
<tr>
<td>TBS (x10)</td>
<td>100mL</td>
</tr>
<tr>
<td>Bidi</td>
<td>900mL</td>
</tr>
<tr>
<td>Tween 20</td>
<td>0.05%</td>
</tr>
<tr>
<td><strong>Blocking buffer: Licor blocking buffer in TBS</strong></td>
<td></td>
</tr>
<tr>
<td>TBS (x1)</td>
<td>50%</td>
</tr>
<tr>
<td>Licor blocking buffer</td>
<td>50%</td>
</tr>
</tbody>
</table>

Preparation of gels and buffers for SDS-PAGE and WB:

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>4% stacking gel</strong></td>
<td></td>
</tr>
<tr>
<td>Bidi</td>
<td>1.5mL</td>
</tr>
<tr>
<td>Acrylamide (stock 37.5%)</td>
<td>312.5μL</td>
</tr>
<tr>
<td>Tris in HCl pH=6.8 (stock 0.5M)</td>
<td>0.63mL</td>
</tr>
<tr>
<td>SDS (stock 10.0%)</td>
<td>25μL</td>
</tr>
<tr>
<td>APS (stock 10.0%, catalyzing agent)</td>
<td>25μL</td>
</tr>
<tr>
<td>TEMED (initiator)</td>
<td>2.5μL</td>
</tr>
<tr>
<td><strong>10% separating gel</strong></td>
<td></td>
</tr>
<tr>
<td>Bidi</td>
<td>2.35mL</td>
</tr>
<tr>
<td>Acrylamide (stock 37.5%)</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>Tris in HCl pH=8.8 (stock 1.5M)</td>
<td>1.25mL</td>
</tr>
<tr>
<td>SDS (stock 10.0%)</td>
<td>50μL</td>
</tr>
<tr>
<td>APS (stock 10.0%, catalyzing agent)</td>
<td>50μL</td>
</tr>
<tr>
<td>TEMED (initiator)</td>
<td>5μL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1x Laemli: running buffer for SDS-PAGE</strong></td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>60g</td>
</tr>
<tr>
<td>Glycine</td>
<td>288g</td>
</tr>
<tr>
<td>SDS</td>
<td>10g</td>
</tr>
<tr>
<td>Bidi</td>
<td>Add till total volume is 10L</td>
</tr>
<tr>
<td><strong>Transfer buffer for Western blot</strong></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>29g</td>
</tr>
<tr>
<td>Tris</td>
<td>58g</td>
</tr>
<tr>
<td>TDS</td>
<td>3.4g</td>
</tr>
<tr>
<td>MeOH</td>
<td>2L</td>
</tr>
<tr>
<td>Bidi</td>
<td>Add till total volume is 10L</td>
</tr>
</tbody>
</table>
### Ligand binding experiment buffer:

<table>
<thead>
<tr>
<th>10 x Binding buffer (BB)</th>
<th>Concentration (mM)</th>
<th>Concentration (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl</td>
<td>500</td>
<td>60,6</td>
</tr>
<tr>
<td>KCl</td>
<td>50</td>
<td>3,73</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>3,72</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>15</td>
<td>2,21</td>
</tr>
<tr>
<td>MgCl$_2$.6H$_2$O</td>
<td>40</td>
<td>8,13</td>
</tr>
<tr>
<td>Bidi till 1L, adjust pH to 7.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### cAMP assay buffers:

<table>
<thead>
<tr>
<th>10x EBSS buffer: 500mL recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1x EBSS buffer: 100mL recipe</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBMX</td>
</tr>
<tr>
<td>Ascorbic acid</td>
</tr>
<tr>
<td>HEPES (stock 1M)</td>
</tr>
<tr>
<td>CaCl$_2$ (1000x, 1.3M)</td>
</tr>
<tr>
<td>MgSO$_4$ (1000x, 1.2M)</td>
</tr>
<tr>
<td>BCS (2%)</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>NaOH</td>
</tr>
</tbody>
</table>

### In cell Western buffers:

<table>
<thead>
<tr>
<th>Tween washing solution: 500mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tween 20</td>
</tr>
<tr>
<td>PBSA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Triton washing solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X100</td>
</tr>
<tr>
<td>PBSA</td>
</tr>
</tbody>
</table>
Attachment 2: Tissue culture

Tissue culture media:

<table>
<thead>
<tr>
<th><strong>EDTA/trypsin</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>400mL</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>50mL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>10% DMEM</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>1L</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>10% (100mL)</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>10mL</td>
<td></td>
</tr>
<tr>
<td>Penicilline/Streptomyacin</td>
<td>2mL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>10% F12 medium</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>F12</td>
<td>1L</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>10% (100mL)</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>10mL</td>
<td></td>
</tr>
<tr>
<td>Penicilline/Streptomyacin</td>
<td>2mL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>2% FCS DMEM</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>1L</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>2% (20mL)</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>10mL</td>
<td></td>
</tr>
<tr>
<td>Penicilline/Streptomyacin</td>
<td>2mL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>SFM DMEM</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>1L</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>10mL</td>
<td></td>
</tr>
<tr>
<td>Penicilline/Streptomyacin</td>
<td>2mL</td>
<td></td>
</tr>
</tbody>
</table>

Tissue culture recipients, amounts for splitting cells, amounts of seeded cells:

<table>
<thead>
<tr>
<th><strong>Recipient</strong></th>
<th><strong>Surface</strong></th>
<th><strong>Amount medium</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>0.32 cm²</td>
<td>200 µL</td>
</tr>
<tr>
<td>12-well plate</td>
<td>4 cm²</td>
<td>2 mL</td>
</tr>
<tr>
<td>6-well plate</td>
<td>9 cm²</td>
<td>3 mL</td>
</tr>
<tr>
<td>Small scale</td>
<td>29 cm²</td>
<td>3.5 mL</td>
</tr>
<tr>
<td>Medium scale</td>
<td>57 cm²</td>
<td>10 mL</td>
</tr>
<tr>
<td>Small flask</td>
<td>25 cm²</td>
<td>10 mL</td>
</tr>
<tr>
<td>Medium flask</td>
<td>75 cm²</td>
<td>25 mL</td>
</tr>
<tr>
<td>Big flask</td>
<td>175 cm²</td>
<td>50 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Recipient</strong></th>
<th><strong>Amount medium</strong></th>
<th><strong>Splitting:</strong></th>
<th><strong>Splitting:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Small flask</td>
<td>10 mL</td>
<td>2 mL</td>
<td>2 mL</td>
</tr>
<tr>
<td>Medium flask</td>
<td>25 mL</td>
<td>5 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>Big flask</td>
<td>50 mL</td>
<td>10 mL</td>
<td>10 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Recipient</strong></th>
<th><strong>Number of cells (HEK293T, subconfluent)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well plate</td>
<td>50 000</td>
</tr>
<tr>
<td>6-well plate</td>
<td>250 000</td>
</tr>
<tr>
<td>6-well plate with coverslip</td>
<td>200 000</td>
</tr>
<tr>
<td>Medium scale</td>
<td>2 500 000</td>
</tr>
</tbody>
</table>
## Attachment 3: Antibodies

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Flag HRP Mouse</td>
<td>1/1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-cMyc Rabbit</td>
<td>1/1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-cMyc Mouse</td>
<td>1/1000</td>
<td>Millipore</td>
</tr>
<tr>
<td>Anti-Etag Rabbit</td>
<td>1/2000</td>
<td>Abcam</td>
</tr>
<tr>
<td>Anti-HA Rat</td>
<td>1/2000</td>
<td>Roche</td>
</tr>
<tr>
<td>Anti-HA Rabbit</td>
<td>1/2000</td>
<td>Genetex</td>
</tr>
<tr>
<td>Anti-HA Mouse 16B12</td>
<td>1/2000</td>
<td>Covance</td>
</tr>
<tr>
<td>Anti-Flag Mouse M2</td>
<td>1/5000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-GFP Mouse</td>
<td>1/5000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Anti-phospho MAPK p44/p42</td>
<td>1/1000</td>
<td>Cell signaling</td>
</tr>
<tr>
<td>Anti-total ERK</td>
<td>1/800</td>
<td>Cell signaling</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibodies</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Rabbit Red LT</td>
<td>1/20000</td>
<td>Licor</td>
</tr>
<tr>
<td>Anti-Rabbit Green</td>
<td>1/15000 (WB) or 1/800 (MAPK assay)</td>
<td>Licor</td>
</tr>
<tr>
<td>Anti-Mouse Green</td>
<td>1/15000</td>
<td>Licor</td>
</tr>
<tr>
<td>Anti-Mouse Red LT</td>
<td>1/20000</td>
<td>Licor</td>
</tr>
<tr>
<td>Anti-Mouse Red RD</td>
<td>1/800</td>
<td>Licor</td>
</tr>
<tr>
<td>Anti-Rat Red LT</td>
<td>1/20000</td>
<td>Licor</td>
</tr>
</tbody>
</table>

## Attachment 4: Markers

![PageRuler™ Prestained Protein Ladder, #SM0671](image)

![Lambda DNA/PstI Marker, 24](image)

Range

26 fragments (in bp): 11501*, 5077, 4749, 4507, 2839, 2556*, 2460, 2443, 2410, 1986, 1760, 1159, 1093, 800, 514, 463, 448, 390, 284, 247, 216, 211, 200, 184, 150, 94, 87, 72, 65 (diketo fragment omitted 2.3%).
Attachment 5: Co-immunoprecipitation protocol

RIPA buffer supplemented with inhibitors and DOC (deoxycholic acid) is used, these components are added fresh.

Day 1:

1. Thaw the frozen cell pellet
2. Add 400µL RIPA lysis buffer (+ inhibitors) to cell pellet, pipit up and down until the cells are resuspended
3. Put samples in the rotator at 4°C for 1h
4. Centrifuge at 8000 rcf for 10min, 4°C
5. Transfer the supernatant to new eppendorfs
6. Take 40µL of supernatant and add to new eppendorfs (this is the lysate fraction, to check protein expression)
7. Add 2µL of primary antibody (anti-HA16B12 or anti-Flag M2, depending on the experiment)
8. Put samples in the rotator at 4°C for 2-4h
9. Prepare the beads and add to samples:
   - You need 20µL beads/sample => beads are at 50%
   - Centrifuge the beads at 1000rpm, 1min, 4°C
   - Aspirate supernatant and wash beads 3x 1mL RIPA buffer (+ inhibitors), centrifuge after each washing
   - Aspirate buffer after final wash and add RIPA buffer: number of samples x 30µL
   - Add 35µL bead mixture/sample
10. Put samples in the rotator at 4°C overnight

Day 2:

11. Centrifuge samples at 1000rpm, 1min, 4°C
12. Remove supernatant, wash 3x 1mL RIPA (+ inhibitors), centrifugation after each wash
13. Add elution buffer:
   - Lysate fraction:
     5xLaemli buffer (10µL/sample) + DTT (50mM final concentration, 2.5µL/sample)
   - IP fraction
     4xLaemli buffer (50µL/sample) + DTT (50mM final concentration, 2.5µL/sample)
     Denaturate lysate and IP fraction for 10min at 37°C in thermoshaker
14. Load samples on polyacrylamide gel (4% stacking gel and 10% or 12% separating gel, depending on the type of experiment)
   - 15 lane gel => 20µL sample/lane
   - 10 lane gel => 25µL sample/lane
   - Marker (Pageruler™ prestained protein ladder, Thermo scientific) => 3µL
15. Run polyacrylamide gel in electrophoresis buffer (also called 1x Laemli buffer) at 100V, 250mA (first apply 80V while proteins are moving through the stacking gel, then increase to 100V for the separation)
16. Western Blot: transfer gel on a nitrocellulose membrane. Run in transfer buffer at 100V, 250mA for 1h (use ice to cool!)
17. Add blocking buffer to the membrane (Licor blocking buffer in 1xTBS), incubate 1h
18. Add the desired primary antibody, diluted in Licor blocking buffer in 1xTBS-T. Incubation for 1h at RT (incubation can be also done overnight at 4°C)
19. Wash blots 3x5min with 1xTBS-T
20. Add the desired secondary antibody, diluted in Licor blocking buffer in 1xTBS-T. Incubate for at least 45min – maximum 1h.
21. Wash blots 3x5min with 1xTBS-T
22. Detection on Odyssey
23. Repeat step 18-22 if incubation with other antibodies is necessary.

Attachment 6: Double co-immunoprecipitation protocol

Two types of RIPA buffer are used: RIPA buffer with inhibitors (see attachment 1) and RIPA buffer with inhibitors and NEM (N-ethylmaleimide, an inhibitor of deubiquitinating enzymes).

Day 1:
1. Thaw the frozen cell pellet
2. Add 400μL RIPA lysis buffer (+ inhibitors + NEM) to cell pellet, pipit up and down until the cells are resuspended
3. Put samples in the rotator at 4°C for 1h
4. Centrifuge at 8000 rcf for 10min, 4°C
5. Transfer the supernatant to new eppendorfs
6. Take 40μL of supernatant and add to new eppendorfs (this is the lysate fraction, to check protein expression)
7. Add 2μL of primary antibody (anti-HA16B12 or anti-Flag M2, depending on the experiment) and vortex the samples.
8. Put samples in the rotator at 4°C for 2-4h
9. Prepare the beads and add to samples:
   • You need 20μL beads/sample => beads are at 50%
   • Centrifuge the beads at 1000rpm, 1min, 4°C
   • Aspirate supernatant and wash beads 3x 1mL RIPA buffer (+ inhibitors + NEM), centrifuge after each washing
   • Aspirate buffer after final wash and add RIPA buffer: number of samples x 30μL
   • Add 35μL bead mixture/sample
10. Put samples in the rotator at 4°C overnight

Day 2:
11. Centrifuge samples at 1000rpm, 1min, 4°C
12. Remove supernatant, wash 3x 1mL RIPA (+ inhibitors), centrifugation after each wash
13. Add elution buffer:
   a) Lysate fraction:
      5xLaemli buffer (10μL/sample) + DTT (50mM final concentration, 2.5μL/sample)
      Denaturate lysate and IP fraction for 10min at 37°C in thermoshaker
   b) IP fraction:
      4xLaemli buffer (50μL/sample) + DTT (50mM final concentration, 2.5μL/sample)
Denature IP fraction for 10min at 37°C in thermoshaker. Transfer 12.5µL eluted fraction to a new eppendorf (IP1 fraction), the remaining fraction to another new eppendorf (for IP2). Add again 50µL elution buffer and denature again at 37°C for 10min, add the eluate to the eppendorf for IP2. Denature once more (in total: 3 times).

24. Dilute fraction for IP2 with RIPA lysis buffer 850µL (+ inhibitors + NEM)
25. Incubate with 1.5µL of antibody (anti-HA16B12)
26. Prepare the beads and add to samples:
   - You need 20µL beads/sample => beads are at 50%  
     => so take for at least two samples extra
   - Centrifuge the beads at 1000rpm, 1min, 4°C
   - Aspirate supernatant and wash beads 3x 1mL RIPA buffer (+ inhibitors + NEM)
   - Aspirate buffer after final wash and add RIPA buffer: number of samples x 30µL
   - Add 35µL bead mixture/sample
27. Put samples in the rotator at 4°C overnight

Day 3
28. Centrifuge samples at 1000rpm, 1min, 4°C
29. Remove supernatant
30. Wash 3x with 1mL RIPA (+inhibitors)
31. Add elution buffer:
   IP2 fraction: 4xLaemli buffer (50µL/sample) + DTT (50mM final concentration, 2.5µL/sample) => Denaturate for 10min at 37°C in thermoshaker
32. Load samples on polyacrylamide gel (4% stacking gel and 10% or 12% separating gel, depending on the type of experiment)
   - 15 lane gel => 20µL sample/lane
   - 10 lane gel => 25µL sample/lane
   - Marker (Pageruler™ prestained protein ladder, Thermo scientific) => 3µL
33. Run polyacrylamide gel in electrophoresis buffer (also called 1x Laemli buffer) at 100V, 250mA (first apply 80V while proteins are moving through the stacking gel, then increase to 100V for the separation)
34. Western Blot: transfer gel on a nitrocellulose membrane. Run in transfer buffer at 100V, 250mA for 1h (use ice to cool!)
35. Add blocking buffer to the membrane (Loric blocking buffer in 1xTBS), incubate 1h
36. Add the desired primary antibody, diluted in Loric blocking buffer in 1xTBS-T. Incubation for 1h at RT (incubation can be also done overnight at 4°C)
37. Wash blots 3x5min with 1xTBS-T
38. Add the desired secondary antibody, diluted in Loric blocking buffer in 1xTBS-T. Incubate for at least 45min – maximum 1h.
39. Wash blots 3x5min with 1xTBS-T
40. Detection on Odyssey
41. Repeat step 18-22 if incubation with other antibodies is necessary.
Attachment 7: Saturation BRET assay

The experimental procedure is lined out per day.

Monday:
HEK293T cells are seeded in 6-well plates

Tuesday:
Calcium phosphate transfection of cells: constant amounts of receptor, increasing amounts of KLHL12-YFP. Samples are prepared in duplicate.

<table>
<thead>
<tr>
<th></th>
<th>W0</th>
<th>W1</th>
<th>W2</th>
<th>W3</th>
<th>W4</th>
<th>W5</th>
<th>W6</th>
<th>W7</th>
<th>W8</th>
<th>W9</th>
<th>W10</th>
<th>W11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor (µg)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Acceptor (µg)</td>
<td>/</td>
<td>0.1</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.7</td>
<td>0.8</td>
<td>1.0</td>
<td>1.1</td>
<td>1.2</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>pcDNA3 (µg)</td>
<td>1.8</td>
<td>1.7</td>
<td>1.6</td>
<td>1.4</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>0.8</td>
<td>0.7</td>
<td>0.6</td>
<td>0.4</td>
<td>/</td>
</tr>
<tr>
<td>Ratio (acceptor/donor)</td>
<td>/</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>3.0</td>
<td>3.5</td>
<td>4.0</td>
<td>5.0</td>
<td>5.5</td>
<td>6.0</td>
<td>7.0</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Wednesday:
Let cells grow, experiment 48h post-transfection

Thursday:
- Collect cells with HBSS buffer – 500µL/well (supplemented with MgCl₂ and CaCl₂)
- Centrifuge cells 10min at 1000rpm
- Aspirate HBSS buffer and add new HBSS buffer: from 225µL-525µL (depending on size pellet) => we used 425µL
- BCA: 12.5µL standards/samples + 100µL of mixed BCA buffer (A + B) => in duplicate on 96-well plate
- Incubate 96-well plate 30 min at 37°C => read on Victor => calculate protein concentration
- Dilute samples in HBSS buffer to have equal protein concentration in each sample => minimum 0.3mg/mL => to have minimum 20µg of protein
- Transfer 80µL of cell suspension to black plate (duplicate/triplicate/quadruplet depending on volume of samples) for fluorescence measurement (excitation 485nm, emission 535nm)
- Transfer 80µL of cell suspension to white plate (duplicate/triplicate/quadruplet depending on volume of samples) for luminescence and BRET 1 measurement
- Add 20µL coelenterazine-h (5µM final concentration, stock:1mM => 25µM => 5µM final) and after 1h measure BRET 1 (dual emission 460nm and 535nm). Wait 10min and measure total luminescence (emission 460nm).
Attachment 8: Saturation binding assay

Materials:

- 1x BB (10xBB stock)
- Wash buffer: 50mM TrisHCl pH 7,7
- Haloperidol 2µM:
  Stock = 1mM => 2µM => 1µM in sample
  Dilute 500x: (total 48tubes/setup; 50tubes x 250 x 2 setups = 25000µL=25mL):
  50µl stock + 24950µl 1xBB
- $[^3]$H] Spiperone:
  7.02µL in 10mL ≈ 10nM
  Take 50µl + 3mL scintillation fluid (3x): to determine exact concentration
  Make a dilution series of spiperone

<table>
<thead>
<tr>
<th>Sample</th>
<th>Final conc (nM)</th>
<th>Conc (nM)</th>
<th>Volume Spiperone</th>
<th>V_BB(mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2,6</td>
<td>10,4</td>
<td>7,02 µL</td>
<td>10mL</td>
</tr>
<tr>
<td>B</td>
<td>2,0</td>
<td>8</td>
<td>1,9mL A</td>
<td>0.600</td>
</tr>
<tr>
<td>C</td>
<td>1,4</td>
<td>5,6</td>
<td>1,3mL A</td>
<td>1.200</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>4</td>
<td>0.9mL A</td>
<td>1.600</td>
</tr>
<tr>
<td>E</td>
<td>0,6</td>
<td>2,4</td>
<td>0.577mL A</td>
<td>1.923</td>
</tr>
<tr>
<td>F</td>
<td>0,4</td>
<td>1,6</td>
<td>0.385mL A</td>
<td>2.115</td>
</tr>
<tr>
<td>G</td>
<td>0,2</td>
<td>0,8</td>
<td>0.192mL A</td>
<td>2.308</td>
</tr>
<tr>
<td>H</td>
<td>0,15</td>
<td>0,6</td>
<td>0.144 mL A</td>
<td>2.356</td>
</tr>
<tr>
<td>I</td>
<td>0,1</td>
<td>0,4</td>
<td>0.0961 mL A</td>
<td>2.404</td>
</tr>
<tr>
<td>J</td>
<td>0,06</td>
<td>0,24</td>
<td>0.0577mL A</td>
<td>2.442</td>
</tr>
<tr>
<td>K</td>
<td>0,04</td>
<td>0,16</td>
<td>0.038mL A</td>
<td>2.462</td>
</tr>
<tr>
<td>L</td>
<td>0,01</td>
<td>0,04</td>
<td>0.0096mL A</td>
<td>2.490</td>
</tr>
</tbody>
</table>

Total: 2.5mL

Protocol:

- Membrane preparation in 1x BB (ice-cold)
  - Cell pellet of giant dish: resuspend in 14mL 1xBB
  - Mix with polytron 2x10”, wash polytron with 1xBB
  - Transfer cells to sorvall tube and centrifuge 20’ 4°C at 17000 rpm
  - Remove supernatans and add 1,5 mL 1xBB, resuspendend pellet by pipetting up and down. Mix with polytron for 5”
  - BCA concentration determination: 300µg membrane necessary
- Dilute 300µg membrane protein to 12,5mL with 1xBB. Homogenize and transfer 250µl to 48 ligand binding tubes (300*10$^{-3}$mg/12.5mL= 0.024mg/mL, so in 250µL => 0.006mg/tube)
- In each ligand binding tube:
  500µL 1xBB (to determine total binding) or
  500µL Haloperidol (to determine non-specific binding)
250µL Membrane suspension
Total: 1mL per tube
- Vortex
- 1u RT
- Equilibrate filter in 0.1% PEI solution
- In machine: harvest, wash with twice with 50mM TrisHCl pH 7.7
- Transfer filter to scintillation tubes and add 3mL scintillation fluid
- Keep ON in dark, measure next day with scintillation counter (Perkin Elmer)

**Attachment 9: Competition binding experiment**

**Materials:**

- 1x BB (10xBB stock)
- Wash buffer: 50mM TrisHCl pH 7.7
- Spiperone (48 mL necessary, if samples are done in sixfold) \(\rightarrow\) fresh: 83.7 Ci/mmol
  
  \[1.1 \mu L \text{ in } 55 \text{ ml } 1xBB = 800pM\]
  
  Take 50µl + 3mL scintillation fluid (3x): to determine exact concentration

- Haloperidol 4µM
  
  Stock = 1mM \(\rightarrow\) 4µM \(\rightarrow\) 1µM final concentration
  
  13µl stock + 3237µl 1xBB (for 2X6 samples with 250 µl)

- Dopamine: First prepare 40M stock maken = 7,5856mg/mL (we weighed 4.4mg dopamine \(\rightarrow\) add 580µL bidi)
  
  Dilute 100x: 400µM stock = 10µl in 990µl
  
  Dilution series (calculated for 5 setups in sixfold) \(\rightarrow\)
  
  \(6\times2\times250\mu L = \text{minimum } 3 \text{ mL } \rightarrow 4 \text{ mL}\)

<table>
<thead>
<tr>
<th>Scintillation tubes</th>
<th>Dopamine</th>
<th>1x BB (µL)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.8.9.10.11.12</td>
<td>400µl F</td>
<td>3600</td>
<td>0,013nM (=4x 0.0032nM)</td>
</tr>
<tr>
<td>85.86.87.88.89.90</td>
<td>400µl stock</td>
<td>3600</td>
<td>40µM (=4x 10µM)</td>
</tr>
<tr>
<td>73.74.75.76.77.78</td>
<td>400µl H</td>
<td>3600</td>
<td>4µM (=4x 1µM)</td>
</tr>
<tr>
<td>61.62.63.64.65.66</td>
<td>400µl I</td>
<td>3600</td>
<td>400nM (=4x 100nM)</td>
</tr>
<tr>
<td>49.50.51.52.53.54</td>
<td>400µl J</td>
<td>3600</td>
<td>40nM (=4 x 1nM)</td>
</tr>
<tr>
<td>37.38.39.40.41.42</td>
<td>400µl K</td>
<td>3600</td>
<td>4nM (=4 x 1nM)</td>
</tr>
<tr>
<td>25.26.27.28.29.30</td>
<td>400µl L</td>
<td>3600</td>
<td>0,4nM (=4x 0,1nM)</td>
</tr>
<tr>
<td>13.14.15.16.17.18</td>
<td>400µl M</td>
<td>3600</td>
<td>0,04nM (=4x 0,01nM)</td>
</tr>
<tr>
<td>1.2.3.4.5.6</td>
<td>400µl N</td>
<td>3600</td>
<td>0,004nM (=4x 0,001nM)</td>
</tr>
</tbody>
</table>

Remark: tubes 91.92.93.94.95.96 \(\rightarrow\) add haloperidol, no dopamine (non-specific binding)
Protocol:

- Membrane preparation in 1x BB (ice-cold)
  - Cell pellet of giant dish: resuspend in 14mL 1xBB
  - Mix with polytron 2x10'', wash polytron with 1xBB
  - Transfer cells to sorvall tube and centrifuge 20' 4°C at 17000 rpm
  - Remove supernatans and add 1,5 mL 1xBB, resuspendend pellet by pipetting up and down. Mix with polytron for 5''
  - BCA concentration determination: 600µg membrane necessary

- Dilute 600µg membrane protein to 25mL with 1xBB. Homogenize and transfer 250µl to 48 ligand binding tubes ( 600*10^{-3}mg/25mL= 0.024mg/mL, so in 250µL => 0.006mg/tube)

- In each ligand binding tube:
  250µL dopamine (dilution dependent)
  **250µL 1xBB (to determine total binding)** or
  **250µL Haloperidol (to 91.92.93.94.95.96 to determine non-specific binding)**
  250µL Membrane suspension
  Total: 1mL per tube

- Vortex
- 1u RT
- Equilibrate filter in 0.1% PEI solution
- In machine: harvest, wash with twice with 50mM TrisHCl pH 7.7
- Transfer filter to scintillation tubes and add 3mL scintillation fluid
- Keep ON in dark, measure next day with scintillation counter (Perkin Elmer)

Attachment 10: cAMP protocol

The experimental procedure is lined out per day.

**Monday:**
Seed HEK293T cells on 10 cm dishes

**Tuesday:**
Calcium phosphate transfection of cells:
- HA D₃.₂R (5µg) + pcDNA3 (5µg)
- HA D₄.₂ R(5µg) + KLHL12 (5µg)
- Cells untransfected

**Wednesday:**
Reseed cells on 12-well plate (200.000 cells/well)

**Thursday:**
Experiment:
1. Prepare EIA buffer, wash buffer standards, tracer, antiserum (can be day before)
2. Prepare 1xEBS buffer (Earle’s balanced salt solution)
3. Pretreatment of cells in EBSS buffer – 800ul/well – 20min at 37°C
4. Add 100µl of PD (10x stock) (or EtOH diluted in EBSS diluted to wells without agonist): dilution series (final concentrations): 10µM, 1µM, 100nM, 10nM, 1nM – incubate 20 min at 37°C
5. Add 100µl of forskolin (100µM stock >final 10µM) – incubate 20 min at 37°C
6. Aspirate off medium and drug and lyse cells:
   100µl of 0.1M HCl > incubate for 20 min at room temperature
   - Scrape cells off the surface
   - Dissociate the mixture by pipetting up and down until the suspension is homogeneous, and transfer to eppendorf
   - Centrifuge at 1000g for 10 min
   - Transfer supernatant to new eppendorf
10. Measure protein concentration with BCA; at least 1mg/ml is recommended
11. Make sample dilutions (2µl/198µl EIA) in a normal 96-well plate, then transfer to the frozen antibody assay plate. Columns 1 is reserved for plate controls (blank, non-specific binding, B₀ and total activity), column 2-3 for standards (S1-S8).
12. Add tracer and antiserum according to booklet (Cayman website) > incubate the plate 18h at 4°C

Friday:
13. Rinse wells 5 times with 300µl cold wash buffer
14. Add 200µL Ellman’s reagent to all wells and 5 µL tracer to total activity well
15. Cover with foil and place on rotator for 90-120 min (in the dark)
16. Read on Victor plate reader (405-420nm)

Attachment 11: MAPK-phosphorylation assay

The experimental procedure is lined out per day.

Monday:
Seed HEK293S cells (HA D₄.0R, HA D₄.2R, HA D₄.24KR) on 10 cm dishes.

Tuesday:
Transfect cells with calcium phosphate method (amounts of DNA are given per receptor variant):
- pcDNA3 (10µg)
- KLHL12 (5µg) + pcDNA3 (5µg)
- Flag-β-arrestin2 (5µg) + pcDNA3 (5µg)
- KLHL12 (5µg) + Flag-β-arrestin2 (5µg)

Wednesday:
- Refresh cells with 5mL medium and collect cells to 15mL tube
- Count cells
- Reseed cells to 96-well plate: 50 000cells/well (total volume: 200µL) => 2 rows/sample (24wells)

Thursday: Grow cells for 24h
Stimulate cells with quinpirole (10µM) for different periods of time:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0'</th>
<th>1'</th>
<th>5'</th>
<th>10'</th>
<th>20'</th>
<th>30'</th>
<th>45'</th>
<th>60'</th>
<th>75'</th>
<th>90'</th>
<th>120'</th>
</tr>
</thead>
</table>

Fix cells with 1:10 formaldehyde in PBSA => incubate 20min at RT (fume hood) 100µL/well
Wash cells 4x5min with Triton washing solution (0.1% Triton X100 in PBSA) 200µL/well
Block 90min in Licor blocking buffer 150µL/well
Add 1°Ab: α-phospho MAPK (p44/p42) (1/1000) and α-total ERK (1/8000) in Licor blocking buffer 50µL/well => incubate ON 4°C
Wash cells 4x5min with Tween washing solution (0.1% Tween20 in PBSA)
Add 2°Ab: α-rabbit green (1/800) and α-mouse red RD (1/800) diluted in Licor blocking buffer + 0.2% Tween20
Wash cells 4x5min with Tween washing solution (0.1% Tween20 in PBSA)
Aspirate buffer very well after final wash
Detect on Odyssey

Attachment 12: Cloning procedure

Some additional protocols regarding certain steps of the cloning procedure can be found here.

Nucleo Spin purification protocol:

Add 200µL NT buffer per 100mg agarose
  - KLHL12: 469.6µL (234.8mg)
  - YFP: 518µL (259.1mg)
10min in 50°C thermoshaker
Transfer liquid to column
Centrifuge 1min 11000rcf(g)
Wash with 600µL NT3 buffer
Centrifuge 1min 11000rcf => then once again for 2min 11000rcf
Elute with ~30µL NE buffer => store for 1min at RT (new epp)
Centrifuge 1min 11000rcf

Birnboim plasmid isolation protocol:

1. Add 200µL TEG buffer to bacterial pellet => vortex => shake for 5min at RT (gel room shaker)
2. Add 200µL of NaOH/SDS mix:
   - 0.2M NaOH
   - 1% SDS
   - Bidi
3. Mix softly and store for 5min at RT
4. Add 200µL of 3M KAc => mix softly => store for 5min at RT
5. Add 600µL of Phenol/Chloroform/Isoamyl alcohol (commercial mix)
6. Mix very well
7. Centrifuge 5min at 14000rpm 4°C
9. Add 600µL of isopropanol => mix strongly
10. Centrifuge 15min at 14000rpm 4°C
11. Wash pellet with 500µL 70% EtOH and shake
12. Centrifuge 5min at 14000rpm 4°C
13. Wait until EtOH will evaporate and dissolve in 50µL Bidi

**Qiagen plasmid purification kit:**

Handbook can be downloaded at: http://kirschner.med.harvard.edu/files/protocols/QIAGEN_QIAGENPlasmidPurification_EN.pdf

**Primer sequences (used for sequencing KLHL12-YFP):**

Etag.F - AT GGG TGC GCC GGT GCC GTA
KLHL12-Cterm.F - GTC GTG ACA TCC ATG GGA AC

**Immunofluorescence:** 6-well plate

1. Fix cells with PFA for 20min at RT (1mL/well)
   - 37% formaldehyde diluted 1:10 in Marks PBS
   - 150mM NaCl, 10mM Na₃PO₄ pH=7.4
2. Wash cells 3x with PBSA (1mL/well) => use pipetboy
3. Permeabilize cells with 3%NCS/1%BSA/0.1% Triton X100 in PBSA for 20min at RT (200µL/well)
4. Wash cells 3x with PBSA (1mL/well)
5. Blocking with 3%NCS/1%BSA in PBSA for 1h (200µL/well)
6. Incubate with 1°Ab: anti-GFP mouse 1/1000 in blocking buffer (3%NCS/1%BSA in PBSA) for 1h (150µL/well) (add directly to coverslip)
7. Wash cells 3x with PBSA (1mL/well)
8. Incubate with 2°Ab: anti-mouse alexa fluor 594 1/500 in blocking buffer for 1h (150µL/well) (add directly to coverslip)
9. Wash cells 3x with PBSA (1mL/well)
10. Incubate with DAPI in PBSA for 5min (stock: 1000x) (add directly to coverslip)
11. Wash cells 3x with PBSA (1mL/well)
12. Take a microscope glass and add a drop of Mowiol.
13. Take the coverslip out the well and put it on a microscope glass, with the cells pointing towards the glass
14. Fix the coverslips to the microscope glass with nailpolish, allow to dry
15. Visualize under the fluorescence microscope (objective 60x)
Expression test WB: 6-well plate

1. Wash cells 3x with PBSA (200µL/well)
2. Add lysis buffer (DTT should be added fresh, 50mM DTT in 50mL => 2.5mL) and collect lysed cells with 1mL pipit (viscous) to new eppendorfs
3. Sonicate 1min
4. Denature 5min at 95°C
5. Spin down 5min
6. Load on gel or keep till the day after in the fridge
7. SDS-PAGE
8. Western Blot
9. Blocking with blocking buffer in TBS for 1h
10. Incubation with primary antibody for 1h: anti-GFP 1/1000 diluted in blocking buffer in TBST
11. Wash 3x with TBST for 5min
12. Incubation with secondary antibody for 1h: anti-mouse green 1/1000 diluted in blocking buffer in TBST
13. Wash 3x with TBST for 5min
14. Read on the Odyssee
15. Incubate with anti-GFP HRP 1/1000 diluted in TBST in Licor blocking buffer
16. Incubate with substrate (Western Lightning)
17. Visualize with Kodak

Victor expression test: 6-well plate

1. Aspirate medium of cells
2. Add 0.5mL of HBSS (+ CaCl$_2$, MgCl$_2$) directly to cells, aspirate the buffer with pipit and add again, until the cells are detached. Transfer to an eppendorf.
3. Spin down 7min at 1000rpm 4°C, remove supernatans
4. Resuspend cells in 400µL HBSS
5. Take 80µL of resuspended cells and add to 96-well plate
6. Read on Victor
7. BCA protein quantification:
   - Prepare standards, first standard should be 2mg/mL
   - Weigh an empty eppendorf with analytical balance
   - Add ~2mg of BSA and weigh eppendorf again. Determine the volume of HBSS that should be taken to obtain a concentration of 2mg/mL
   - Make dilution series of standards: 1.5mg/mL, 1mg/mL, 0.75mg/mL, 0.5mg/mL, 0.25mg/mL, 0.125mg/mL, 0.025mg/mL, 0mg/mL
   - Transfer 10µL of standards and samples to 96-well plate, add to each well 190µL of Pierce BCA solution
   - Repeat this for the samples
   - Incubate 30min at 37°C
   - Read on Victor plate reader