

## Human Latrophilin-2 is Expressed in the Cytotrophoblast and Syncytiotrophoblast of Placenta and in Endothelial Cells

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**Abstract:** Latrophilin-2 is a member of the family of adhesion-GPCRs that is characterised by a long N-terminus which contains motifs identified in proteins involved in cell adhesion. We were interested in determining the expression pattern of human latrophilin-2 and to perform a biochemical characterisation of this protein. The expression pattern of latrophilin-2 was analysed in human organs, tissues and cell lines. RT-PCR analyses detect a very strong signal for latrophilin-2 in human placenta and *in situ* hybridisation further showed that latrophilin-2 is predominantly expressed in the cytotrophoblast and syncytiotrophoblast. Moreover, latrophilin-2 expression is visible in adherent cells with a remarkably strong signal in microvascular endothelial cells (MVEC) and in human umbilical vein endothelial cells (HUVEC). Deglycosylation experiments using glycosidase F demonstrated that the N-terminal fragment of human latrophilin-2 is highly glycosylated. Using specific antibodies and latrophilin-2 stable cell lines we could show that human latrophilin-2 is cleaved into a 135 kDa N-terminal and a 70 kDa C-terminal fragment. It was also possible to detect the N-terminal fragment of latrophilin-2 in cell culture supernatant of HUVEC indicating that endogenous latrophilin-2 is expressed on the protein level in human vascular endothelial cells and that post-translational modification and generation of a 135 kDa N-terminal fragment takes place. The role of this fragment in the activation of the transmembrane domain of latrophilin-2 or in other cellular processes remains to be elucidated.

**Key words:** Adhesion-GPCR, latrophilin-2, placenta, endothelial cell, glycosylation

### INTRODUCTION

The latrophilin sub-family of adhesion-GPCRs<sup>[1,2]</sup> consists of the three members-latrophilin-1, latrophilin-2 and latrophilin-3-that are characterised by a galactose-binding lectin domain (GBL) and an olfactomedin domain (OLF). Hence, adhesion-GPCRs are thought to be natural chimeras of cell adhesion proteins and signal transducing G-protein coupled receptors.

While most is known about latrophilin-1 (LEC2), the member identified first<sup>[3-5]</sup>, much less is known about latrophilin-2 (LEC1) and latrophilin-3 (LEC3). Human latrophilin-2 is alternatively named LPHN2, lectomedin-family member 1 (LEC1), or latrophilin homologue 1 (LPHH1)<sup>[6,7]</sup>. The rat homologue is called calcium-independent receptor of  $\alpha$ -latrotoxin

(CIRL-2)<sup>[8]</sup>, whereas the bovine latrophilin-2 was described by Matsushita *et al.*<sup>[9]</sup>.

Besides the cell adhesion domains at the N-terminus, a further characteristic feature of the adhesion-GPCRs is a GPCR proteolysis site (GPS) which is located about 60 residues upstream of the first transmembrane domain (Fig. 1). The GPS consists of about 50 amino acids, including a box of four cysteine residues and a cleavage site characterised by a leucine-threonine motif. At this site, the adhesion-GPCRs are cleaved constitutively into two fragments<sup>[4,10,11]</sup>. The GPS site was first described by Krasnoperov *et al.*<sup>[4]</sup>. Cleavage results in an N-terminal fragment that corresponds to the cell-adhesion domain and a C-terminal fragment that represents the seven transmembrane domain. So far, for the latrophilins, cleavage has only been shown for latrophilin-1<sup>[12]</sup> but

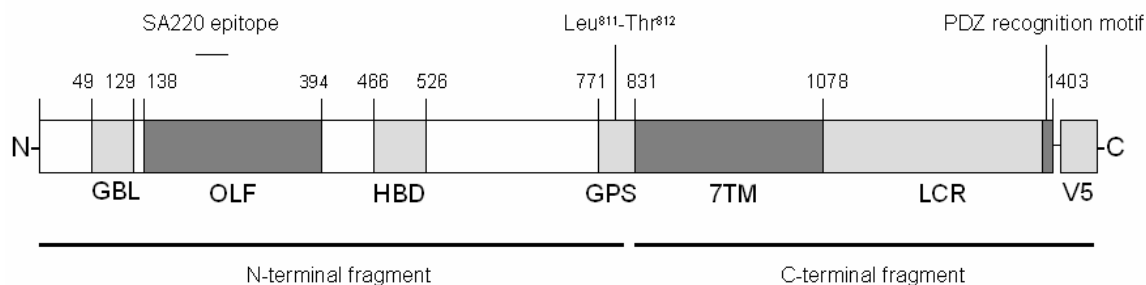


Fig. 1: Protein domains of human latrophilin-2 and peptide epitope. Abbreviations used: GBL, galactose-binding lectin domain; OLF, olfactomedin-like domain; HBD, hormone-binding domain; GPS, GPCR proteolysis site; 7TM, 7 transmembrane domain; LCR, latrophilin family cytoplasmic region. The peptide epitope used for generation of the antiserum SA220 at residues 259-274, the GPS at Leu<sup>811</sup>-Thr<sup>812</sup> and the PDZ recognition motif at the extreme C-terminus are indicated. A V5 tagged is fused at the C-terminus of human latrophilin-2. After proteolytic processing, the affinity purified antiserum SA220 recognises the N-terminal fragment and the anti-V5 antibody detects the C-terminal fragment

the GPS is conserved in latrophilin-2 (Leu<sup>811</sup>-Thr<sup>812</sup>) (Fig. 1).

Although the C-terminus of latrophilin-2 has typical features of a GPCR, coupling with small G-proteins has not been demonstrated. For latrophilin-1, an association with G-protein G $\alpha$ o has been demonstrated in transfected COS cells<sup>[5]</sup> and latrophilin-1 co-purifies with G $\alpha$ q<sup>[13]</sup>. However, the C-terminus of latrophilin-2 might be involved in signal transduction and in the organisation of membrane-associated signalling complexes<sup>[14]</sup> as it contains the PDZ recognition motif S/T-X- $\Phi$ , where  $\Phi$  is a large and hydrophobic amino acid. It has been shown that Shank proteins interact with this PDZ recognition motif of latrophilin. Shank proteins contain several additional protein interaction motifs including ankyrin repeats and Src homology (SH3) domains and might be involved in signal transduction and in mediating the interaction of adhesion-GPCRs with the cytoskeleton<sup>[14]</sup>.

All three latrophilin receptors are orphan receptors indicating that their endogenous ligand is unknown. Latrophilin-1 is the best studied of the three receptors. As exogenous ligand, the black spider venom toxin  $\alpha$ -latrotoxin binds to latrophilin-1. It was shown that also latrophilin-2 binds  $\alpha$ -latrotoxin albeit its affinity is about 14 times lower than that of latrophilin-1<sup>[8]</sup>. It has been suggested that latrophilin-2 might be a low affinity receptor for  $\alpha$  latrotoxin<sup>[8]</sup>. Endogenous ligands of adhesion GPCRs are still mainly elusive.

Here, we describe human latrophilin-2 as a gene which was found to be strongly expressed in MVEC, HUVEC and placenta. As it has been shown in the past that latrophilin-1 is cleaved at the GPCR proteolysis site, we were interested in determining if also latrophilin-2 is cleaved into an N- and a C-terminal fragment and we further investigated the post-translational modification of latrophilin-2.

## MATERIALS AND METHODS

**Semi-quantitative RT-PCR:** Reverse transcription (RT) was performed using the Ready-to-Go T-Primed first strand kit (Amersham Biosciences, Piscataway,

New Jersey, USA). 3.3  $\mu$ g total RNA were reverse transcribed in a total volume of 33  $\mu$ L. RT-PCR was performed using 1  $\mu$ L RT product and Ready-to-Go PCR Beads (Amersham Biosciences) in a total volume of 25  $\mu$ L on a Master Cycler (Eppendorf, Hamburg, Germany). For detection of *latrophilin-2* (RefSeq accession number NM\_012301) the forward primer 5'-GGA GAT GTT AGA GAA GGA CA-3' and the reverse primer 5'-AAT ACC TGG CTG GCA TCT CT-3' resulting in a 292 bp PCR product was used. The forward primer lies in the 3' region of the cDNA, the reverse primer in the 3' UTR. As normalisation control, the housekeeping gene *23 kDa highly basic protein* (RefSeq accession number NM\_012423, 23 kDa HBP, alternative name: ribosomal protein L13a) was detected with the forward primer 5'-CCA CCG CCC TAT GAC AAG A-3' and the reverse primer 5'-CAT TCC AGC CAA CCT CGT GA-3' resulting in a 120 bp product. *Latrophilin-2* and *23 kDa HBP* were amplified in parallel. As PCR programme an initial denaturation at 95°C for 2 min and 25 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec were performed. 10  $\mu$ L of each RT-PCR product was analysed on an agarose gel. A 50 bp ladder (Invitrogen, Karlsruhe, Germany) was used as size marker.

**TaqMan analysis:** 5'-Exonuclease fluorogenic quantitative PCR was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). The reaction was carried out in a 25  $\mu$ L reaction volume containing 12.5  $\mu$ L 2 x PCR TaqMan Universal MasterMix (Applied Biosystems) according to the manufacturer's instructions. The cDNA samples from multiple human tissues (Multiple Tissue cDNA Panel) were obtained from Becton Dickinson Biosciences (Heidelberg, Germany). 1 ng of cDNA was used as template. Expression of *latrophilin-2* was detected using the primer/probe combination 5'-CGT GTT TTA TGG GGA GAA ACA-3', 5'-CAG GGG TGA CAC ACA ATC TTT-3' and FAM-GCC AGT TAT GTC ATG CCT TGC ACA-TAMRA

directed against the extreme 3' UTR of the *latrophilin-2* gene. GAPDH was measured with the primer/probe combination 5'-GAA GGT GAA GGT CGG AGT C-3', 5'-GAA GAT GGT GAT GGG ATT TC-3' and FAM-CAA GCT TCC CGT TCT CAG CC-TAMRA. The data were analysed with the comparative  $C_T$  method of relative quantification ( $\Delta\Delta C_T$ -method) according to the manufacturer's instructions<sup>[15]</sup>.  $C_T$  values of *latrophilin-2* (median of triplicate) were adjusted to the corresponding  $C_T$  values of GAPDH. The expression in placenta (calibrator) was set as 1.0 and the other values were calculated accordingly. Experiments were performed three times.

**In situ hybridisation:** Sections from seven weeks old placenta were deparaffinised, rehydrated and fixed in 4% paraformaldehyde. After washing twice with PBS, tissues were digested with proteinase K and washed and hybridised overnight with the sense and antisense probe, respectively, at 65°C. The *latrophilin-2* probe was generated from a PCR product. The oligonucleotide primers 5'-AAT GCC AGG GAT ACA AGT GC-3' and 5'-ATG CAG GCC TAC AAA AAT GG-3' amplify a 1115 bp region spanning the 3' end of the cDNA and the 3' UTR of *latrophilin-2*.

This PCR product was cloned into the pGemTeasy vector (Invitrogen). Orientation of the cloned insert was determined by restriction fragment analysis. Plasmids were linearised with appropriate enzymes to obtain transcription templates for sense (SP6) and antisense (T7) transcripts. *In vitro* run-off transcription was performed using the MAXIscript® SP6/T7 Kit (Ambion, Cambridgeshire, UK) according to the manufacturer's instructions. The reaction mix contained digoxigenin-modified dUTP ribonucleotides for labelling. After 3 hrs, the transcribed RNAs were analysed for purity and size on a 1% denaturing agarose gel. Hybridised probes were detected with the alkaline phosphatase-conjugated anti-DIG antibody and BM Purple as substrate (Roche Applied Science, Mannheim, Germany). After re-fixation, the sections were counterstained for 5 min with Kernecht Rot (Merck, Darmstadt, Germany), mounted and examined by a pathologist. Every seventh section was stained with haematoxylin/eosin.

**Cloning of full length *latrophilin-2* cDNA:** The coding sequence of *latrophilin-2* (LPHN2, RefSeq accession number NM\_012302, 4209 bp) was amplified by a nested RT-PCR approach from BD QUICK-Clone™ II Human Placenta cDNA (BD Biosciences, Clontech) using Pfu Ultra™ High-Fidelity DNA Polymerase (Stratagene, La Jolla, CA, USA). The outer primers were 5'-AGA AAA CCT GGA GAC GGG AT-3' and 5'-AAC TGC TGG AGG TGG TTT TG-3', the inner primers were 5'-GGA TCC GGG ATC AAT AAT GGT G-3' and 5'-GGA TCC AAG ACT TGT AAC

CAG C-3'. The PCR conditions were as follows: 94°C (5 min); 30 cycles: 94°C (30 sec) / 55°C (30 sec) / 72°C (4 min); 72°C (10 min). A-overhangs were created by Taq polymerase and the PCR product was cloned into pcDNA3.1/V5-His-TOPO-TA (Invitrogen) giving rise to pcDNA3.1-LPHN2-V5 which codes for a C-terminally V5- and His-tagged *latrophilin-2*. The integrity of the construct was confirmed by sequencing.

**Generation of *latrophilin-2* specific antibodies:** The *latrophilin-2* specific antiserum SA220 was generated by immunising rabbits with the peptide GGKTDIDLAVDENGL (amino acids 259-274, Fig. 1) at Eurogentech SA (Seraing, Belgium). After the third bleeding, the polyclonal antiserum was purified by affinity chromatography on the corresponding peptide.

**Cell culture and transient transfection:** Primary human MVEC were prepared from human foreskin as described<sup>[16]</sup>. MVEC were cultivated on BD Matrigel™ Basement Membrane Matrix (Becton Dickinson Biosciences) as described previously<sup>[17]</sup>. Matrigel is a solubilised basement membrane preparation extracted from EHS mouse sarcoma, a tumour rich in ECM proteins. The cell lines HEK-293 and MDA-MB-231 (ATCC, American Type Culture Collection, Manassas, VA, USA) were routinely grown in Dulbecco modified Eagle medium DMEM (Invitrogen) and DMEM/F12 (Invitrogen) respectively, supplemented with 10% foetal bovine serum (Invitrogen) and cultured at 37°C and 5% CO<sub>2</sub>. All cells were cultured free of antibiotics. For transient transfections, Lipofectamine 2000 Reagent (Invitrogen) and Opti-MEM I medium without serum (Invitrogen) were used according to manufacturer's instructions.

**Generation of stable cell lines:** For generation of stable clones, HEK-293 and MDA-MB-231 cells were seeded on 6 well plates at a density of  $2 \times 10^5$  and  $5 \times 10^5$ , respectively. After 24 hrs, the cells were transfected with Lipofectamine 2000 Reagent (Invitrogen) in serum-free Opti-MEM I medium (Invitrogen) according to manufacturer's instructions. For each transfection, 2 µg of pcDNA3.1/V5-LPHN2 or pcDNA3.1/V5 per well were used. After 48 hrs, transfection efficiency was determined with flow cytometry using an anti-V5 antibody. Transfection reactions resulting in more than 20% transfected cells were plated at low densities on 150 mm dishes in the presence of 1 mg mL<sup>-1</sup> G418 (Invitrogen). After 10 to 12 days, single clones were collected and plated in 96 well plates in the presence of G418. The expression of V5-tagged *latrophilin-2* in expanded single clones was analysed via flow cytometry and immunoblot.

**Cell lysates and immunoblotting:** Cells at a confluence of 80-90% were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer (25 mM Tris-HCl, pH 7.6, 1% NP-40, 150 mM NaCl, 1 mM EDTA,

1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM Pefabloc SC (Biomol, Hamburg, Germany) and the protease inhibitor cocktail Complete™ Mini (Roche Applied Sciences). Cell lysates were passed three times through a pipette and centrifuged at 14,000 rpm for 10 min at 4°C. Protein content was measured with the Bradford assay (Bio-Rad Laboratories, Munich, Germany). 16 µg of protein from whole cell lysates were denatured in sample buffer with reducing agent (Invitrogen) for 5 min at 95°C, subjected to 3-8% Tris-acetate gel electrophoresis (Invitrogen) and transferred to nitrocellulose membranes (Millipore, Eschborn, Germany). The membrane was blocked with 5% (w/v) non-fat milk powder in PBS with 0.1% Tween (PBS-T) for 1 hr at RT and probed with an anti-V5 HRP-coupled antibody (Invitrogen) or with the antibody SA220 which is specific for latrophilin-2. HRP-labelled anti-rabbit IgG (Amersham Biosciences) was used as secondary antibody for the detection of latrophilin-2. Signals were visualised using the enhanced chemiluminescence system ECL Plus™ (Amersham Biosciences).

**Glycosidase F treatment:** Supernatants from MDA-MB-231-LPHN2 stable cells (clone 46) and supernatant from HUVEC were concentrated 20-fold by filtration using 50K filters (Millipore). Samples were incubated with 1U µL<sup>-1</sup> of glycosidase F (Roche Applied Science) in denaturation buffer (1% NP-40, 1% 2-mercaptoethanol, 25 mM EDTA) for 18 hrs at 37°C according to manufacturer's recommendations. Control samples were incubated in denaturation buffer without glycosidase F. As recommended by the manufacturer, the glycosidase F treated samples were run with empty lanes between them in order to avoid sample interference. Similar to the cell supernatants, whole cell lysates were treated under the same conditions with denaturation buffer and analysed by Western blot as described. Immunoblots were incubated with the latrophilin-2 specific polyclonal antibody SA220.

**Flow cytometry:** After detachment, cells were washed and fixed for 10 min with 1 x Cellfix solution (Becton Dickinson Biosciences). After washing and centrifugation, samples were permeabilised with 1 x FACS Perm2 solution (Becton Dickinson Biosciences) for 10 min at RT. Afterwards, cells were washed once with cold wash buffer (PBS, 0.5% BSA, 0.1% NaN<sub>3</sub>). The cells were stained in the dark with FITC-conjugated anti-V5 antibody (Invitrogen) for 15 min at RT. Native, non-permeabilised cells were stained directly after detachment with Accutase (PAA Laboratories, Cölbe, Germany). The fluorescently labelled cells were analysed on a FACS Calibur flow cytometer (Becton Dickinson Biosciences) using CellQuest™ software and 50.000 events were acquired.

**Immunofluorescence analysis:** HEK293-LPHN2, MDA-MB-231-LPHN2-HEK-293 and MDA-MB-231

stably transfected with the corresponding empty vector were plated at 1x 10<sup>5</sup> cells/well on cover slips (12 mm, Roth, Karlsruhe, Germany) in a 24 well dish in the presence of selection medium. After 24 hrs, cells were washed with HBSS with Ca<sup>2+</sup>/Mg<sup>2+</sup> and fixed and permeabilised with pre-cooled (-20°C) methanol:acetone (1:1) for 10 min at RT. After fixation and two washing steps, cells were incubated with blocking solution (HBSS with 0.2% fish gelatine from Sigma, Mannheim, Germany) for 30 min at RT. Staining and dilution of antibodies were performed in blocking solution. Slides were incubated with rabbit anti-V5 antibody (Sigma). After three washing steps with blocking solution, the slides were incubated with goat anti-rabbit rhodamine red-coupled secondary antibody (Invitrogen) at RT in the dark for 30 min. Cells were washed once with HBSS and nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole, Invitrogen) for 5 min at RT. Coverslips were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) onto glass slides. The slides were analysed with the inverted laser scanning-microscope Leica TCS SL (Leica, Solms, Germany) using the Leica LCS multi-color software version 2.585 with 63x magnification.

## RESULTS

**Expression pattern of latrophilin-2 in human organs, tissues and cell lines:** The expression pattern of human latrophilin-2 was investigated in organs, tissues and cell lines. Semi-quantitative RT-PCR and quantitative TaqMan PCR demonstrated strong expression of latrophilin-2 in human placenta and lung (Fig. 2 and 3).

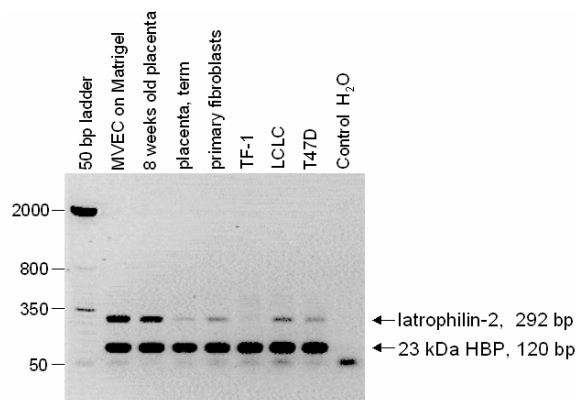


Fig. 2: Semi-quantitative RT-PCR of latrophilin-2 in human tissues and cell lines. RNA was isolated from microvascular endothelial cells (MVEC) cultivated on matrigel for 7 hrs, 8 week old placenta, term placenta, primary human fibroblasts, human erythromyeloblastic TF-1, large cell lung cancer cell line LCLC and from human breast adenocarcinoma cell line T47D. The size of the PCR product using latrophilin-2 specific primer is 292 bp, the size of the PCR product of the housekeeping control gene 23 kDa HBP is 121 bp. A 50 bp ladder was used as size marker. The signal of the 23kDa HBP indicates equal loading in all lanes. Strong signals are detected in MVEC and in 8 week old placenta

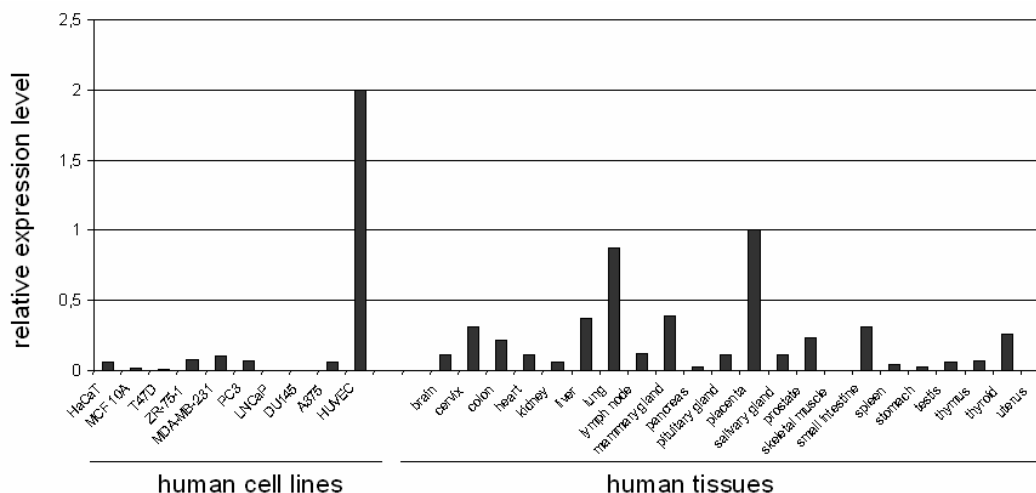


Fig. 3: Quantitative RT-PCR results of *latrophilin-2* expression in human cell lines and tissues. The following cell lines were analysed by TaqMan PCR: human keratinocyte HaCaT, the human breast cancer cell lines MCF 10A, T47D, ZR-75-1 and MDA-MB-231, the human prostate cancer cell lines PC3, LNCaP and DU145, the human melanoma cell line A375 and human umbilical vein endothelial cells (HUVEC). The following human tissues and organs were analysed: brain, cervix, colon, heart, kidney, liver, lung, lymph node, mammary gland, pancreas, pituitary gland, term placenta, prostate, salivary gland, skeletal muscle, small intestine, spleen, stomach, testis, thyroid gland and uterus. The TaqMan probe for *latrophilin-2* lies in the 3' UTR of *latrophilin-2*. The expression values were normalised to GAPDH as internal control and the normalised expression value for placenta was set as 1

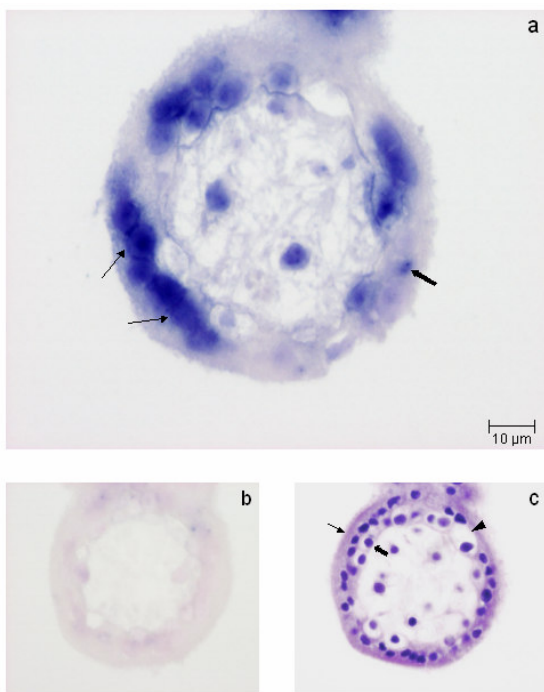


Fig. 4: *In situ* hybridisation with an antisense probe localised in the 3' UTR of *latrophilin-2* on the chorionic villous of a 7 week old human placenta. (a) Staining with the antisense probe demonstrates strong signals for *latrophilin-2* in the cytotrophoblast of the placenta (filled arrow) and syncytiotrophoblast (arrows) and a weak staining of Hofbauer cells in the middle of the stroma of the villous, at 40x magnification. (b) Staining with the sense probe reveals background signals, at 20x magnification. (c) HE staining shows the cytotrophoblast (filled arrow), the syncytiotrophoblast (arrow) and a foetal capillary (arrow head) as well as the Hofbauer cells in the middle of the stroma, at 20x magnification

*Latrophilin-2* has a higher expression in early placenta (8 weeks old) as compared to term placenta (Fig. 2). A strong signal for *latrophilin-2* was detected in differentiated MVEC (Fig. 2) and in HUVEC (Fig. 3). In TF-1, which are derived from peripheral blood leukocytes and which grow in suspension, no signal was detected. By RT-PCR, signals were seen in the adherent lung and breast cancer cell lines LCLC and T47D, respectively (Fig. 2). Weaker signals were observed in various transformed cell lines – e.g. in the breast cancer cell lines MDA-MB-231 and ZR-75-1 (Fig. 3).

**Localisation of *latrophilin-2* in placenta:** In order to determine the cell type in which *latrophilin-2* is expressed in placenta, paraffin-slides of human placenta were hybridised with an antisense probe of *latrophilin-2* (Fig. 4). The corresponding haematoxylin/eosin (HE) stained section of a chorionic villous from a seven week old placenta is shown (Fig. 4c). Interestingly, in the cytotrophoblast, a strong signal for *latrophilin-2* was detected with the antisense probe (Fig. 4a, filled arrow). A prominent staining is also seen in the syncytiotrophoblast (Fig. 4a, arrow). Compared to the cytotrophoblast cells, only a moderate expression of *latrophilin-2* was detected in Hofbauer cells (Fig. 4a: two cells in the middle of the villous stroma). In foetal blood vessels, no *latrophilin-2* expression was observed. The control staining with the sense *latrophilin-2* probe revealed no specific signal in all sections (Fig. 4b).

**Characterisation of post-translational modifications of human *latrophilin-2* protein:** Human *latrophilin-2*



codes for a protein of 1402 amino acids with a calculated molecular weight of 155 kDa. We hypothesised that latrophilin-2 contains a GPCR proteolysis site at amino acid residues 711-831 at which it would be cleaved generating an N-terminal and a C-terminal fragment (Fig. 1) that should be visible under standard denaturing SDS-PAGE conditions. This assumption is based on the overall homology of latrophilin-2 to other adhesion-GPCRs such as rat latrophilin-1 (CIRL-1) which has been shown to be cleaved at the Leu-Thr peptide bond following four distinctly spaced Cys residues<sup>[8,11,12]</sup>. The corresponding consensus amino acid residues Leu-Thr of latrophilin-2 are located at position 811-812 (Fig. 1).

For the functional characterisation of human latrophilin-2, we generated stable cell lines using HEK-293, which are a standard cell line for the expression of GPCRs and MDA-MB-231 giving rise to HEK-293-LPHN2 and MDA-MB-231-LPHN2, respectively. The latter were selected because previously it has been shown that latrophilin-2 is expressed in this estrogen receptor negative human breast cancer cell line<sup>[7]</sup> which we confirmed on the RNA level (Fig. 3). The MDA-MB-231 cell line should therefore contain the machinery for executing the necessary steps for the post-translational modification and the transport of the N-terminal and the C-terminal fragment to the cell membrane. Recombinant human latrophilin-2 was fused with a V5 tag at the C-terminus which should allow us to detect the C-terminal fragment with an anti-V5 antibody. In addition, the affinity purified antiserum SA220 which was generated against a peptide from the N-terminal fragment should allow us to independently detect also the N-terminal fragment (Fig. 1). Several clones of MDA-MB-231-LPHN2 were obtained. In these clones, V5-tagged latrophilin-2 is expressed in different quantities (Fig. 5). In whole cell lysates from MDA-MB-231-LPHN2, the anti-V5 monoclonal antibody detects a specific band at 70 kDa (Fig. 5a). In the stable clones #2 and #10, which have a very high latrophilin-2 expression, additional signals are present at 190 kDa and 200 kDa. No signal is visible in the parental cell line MDA-MB-231 (P) or in MDA-MB-231 transfected with the empty vector control (C). Whereas the 70 kDa signal corresponds to the C-terminal fragment of latrophilin-2, the signals at 190/200 kDa could potentially correspond to uncleaved, partially glycosylated, full length latrophilin-2 extracted from the endoplasmic reticulum or from the cell surface membrane. The affinity purified antiserum SA220 recognises a protein of 135 kDa in whole cell lysates from MDA-MB-231-LPHN2 (Fig. 5b), which is specific for latrophilin-2 since no corresponding staining is seen in the controls. Surprisingly, the full length protein at 190/200 kDa was not stained by the SA220 peptide antiserum. In the parental cell line MDA-MB-231 (P) and in MDA-MB-231 transfected with the vector control (C), non-specific signals are

present at 75 kDa and 190 kDa. Taken together, the data suggest that latrophilin-2 is cleaved into a 135 kDa N-terminal and a 70 kDa C-terminal fragment. The calculated molecular weight is 90 kDa for the N-terminal and 65 kDa for the C-terminal fragment indicating that the N-terminal fragment could be heavily glycosylated which would give rise to additional 45 kDa in the apparent molecular weight.

For human latrophilin-2, nine putative N-glycosylation sites on Asn residues were predicted<sup>[6]</sup>. N-glycosidase F hydrolyses all common classes of Asn-linked glycans from the protein backbone<sup>[18]</sup>. Since complete deglycosylation requires at least partial denaturation of the glycoproteins, while denaturation of N-glycosidase F has to be avoided, the non-ionic detergent NP-40 was used as lysis buffer<sup>[19]</sup>. In order to analyse a potential glycosylation of latrophilin-2, whole cell lysates of MDA-MB-231-LPHN2, clone 46 were treated with glycosidase F or left untreated (Fig. 6). The immunoblot was stained with the latrophilin-2 specific antiserum SA220 that recognises the N-terminal fragment of latrophilin-2. Glycosidase F treatment reduces the size of the extracellular domain from 135 kDa down to 109 kDa indicating that the N-terminal fragment is glycosylated at least at the sites accessible by glycosidase F by 26 kDa.

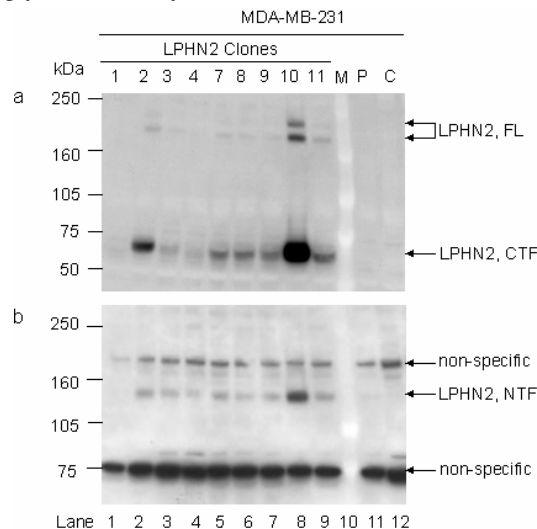


Fig. 5: Detection of latrophilin-2 in stably transfected MDA-MB-231 human breast cancer cells. Whole cell lysates from MDA-MB-231-LPHN2 stable clones 1, 2, 3, 4, 7, 8, 9, 10, 11 (lane 1-9), a protein marker (M, lane 10), parental MDA-MB-231 (P, lane 11), or pcDNA3.1V5 vector control transfected MDA-MB-231 (C, lane 12) were separated on a 3-8% Tris-acetate gradient gel. Each lane contained 16  $\mu$ g of protein. (a) The immunoblot was incubated with an HRP-coupled V5-specific antibody. The anti-V5 antibody detects specific bands at 70 kDa, 190 kDa and 200 kDa. (b) Afterwards, the membrane was stripped and incubated with the latrophilin-2 specific antiserum SA220. The SA220 antiserum detects a specific signal at 135 kDa. Non-specific signals, which are visible at 190 kDa and 75 kDa, are also present in the parental and the vector control transfected MDA-MB-231 cells. Strongest expression of latrophilin-2 was detected in clone 10 using either of the two antibodies

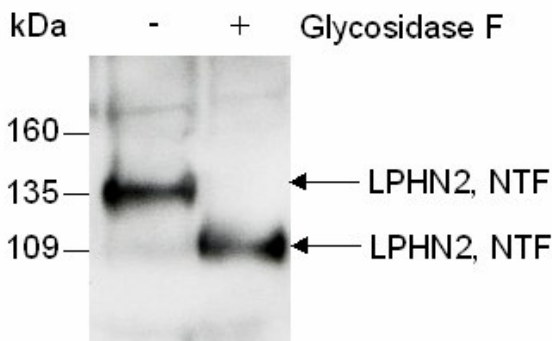


Fig. 6: Deglycosylation of the N-terminal fragment of human latrophilin-2 in whole cell lysates. Cell lysates of clone 46 of MDA-MB-231-LPHN2 were treated with glycosidase F or left untreated. The immunoblot was stained with the latrophilin-2 specific antiserum SA220 that recognises the N-terminal fragment of latrophilin-2. As exemplified for clone 46, treatment with glycosidase F reduces the size of the N-terminal fragment from 135 kDa down to 109 kDa

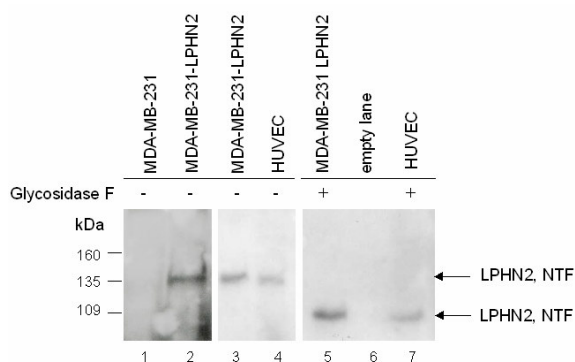


Fig. 7: Deglycosylation of the N-terminal fragment of human latrophilin-2 in cell culture supernatants. Cell culture supernatants from empty vector stably transfected MDA-MB-231 cells (lane 1), from MDA-MB-231-LPHN2, clone 46 (lane 2, 3) and HUVEC cells (lane 4) were left untreated (lane 1-4) or treated with glycosidase F (lane 5, 7). As recommended by the manufacturer, the glycosidase F treated samples were run with empty lanes between them in order to avoid interference of the samples and therefore lane 6 is empty. The immunoblot was stained with the SA220 antiserum that recognises the N-terminal fragment of latrophilin-2. Antiserum SA220 detects the N-terminal fragment of latrophilin-2 in cell culture supernatant of HUVEC (lane 4). Glycosidase F treatment reduces the size of the N-terminal fragment of endogenous (lane 7) and recombinant human latrophilin-2 (lane 6) from 135 kDa down to 109 kDa

In order to study whether the N-terminal fragment might be present in cell culture supernatants we analysed the cell lines which stably express human latrophilin-2 as well as the HUVECs, for which we observed a strong *latrophilin-2* signal on the RNA level (Fig. 3). Cell culture supernatants, from MDA-MB-231-LPHN2, vector control transfected, stable MDA-MB-231 and HUVECs, which were concentrated 20-fold, were analysed by immunoblotting using the SA220 antiserum that recognises the N-terminal fragment of latrophilin-2 (Fig. 7).

Interestingly, shedding of the N-terminal fragment into the culture supernatant is observed. A band at 135 kDa is visible in MDA-MB-231-LPHN2 (lane 2) but not in vector control transfectants (lane 1), similar to the result obtained in Fig. 5b using whole cell lysates. In addition, a 135 kDa band is visible in HUVEC (lane 4) indicating that endogenous latrophilin-2 is expressed on the protein level in endothelial cells and that post-translational modification and generation of a 135 kDa N-terminal fragment takes place on endogenous (lane 4) and recombinant human latrophilin-2 (lane 3). Treatment of cell supernatants with glycosidase F reduces the size of both the recombinant and the endogenous N-terminal fragment of latrophilin-2 in MDA-MB-231-LPHN2 (lane 5) and HUVEC (lane 7), respectively, from 135 kDa down to 109 kDa (Fig. 7).

**Cellular localisation of human latrophilin-2:** In order to analyse the cellular localisation of V5-tagged latrophilin-2, HEK-293 cells were transiently transfected with pcDNA3.1V5-LPHN2, fixed, permeabilised and stained with a FITC-labelled anti-V5 antibody (Fig. 8). By FACS analysis, 56% of the transiently transfected HEK-293 cells express either full-length latrophilin-2 or the C-terminal fragment (Fig. 8b) when compared to vector transfected cells (Fig. 8a). LPHN2 cells (Fig. 8f), but not permeabilised vector control cells (Fig. 8e), revealed a staining with the anti-V5 antibody in 30% of cells indicating that the C-terminal fragment which contains the V5-tag is located towards the cytosol (Fig. 8f). A signal for latrophilin-2 was only visible in 30% of the stable transfected MDA-MB-231 cells. This could be due to the fact that this clone is not homogeneous and that some cells express latrophilin-2 below the level of detection with the V5-antibody. Unfortunately, the specificity of the affinity purified antiserum SA220 directed against the C-terminal fragment of latrophilin-2 (Fig. 5, non-specific bands) was not sufficient to perform flow cytometry or immunofluorescence analysis.

To visualise the membrane localisation of latrophilin-2, an immunofluorescence analysis was performed on fixed and permeabilised HEK-293-LPHN2 (Fig. 9b) and MDA-MB-231-LPHN2 (Fig. 9d) and the corresponding vector control HEK-293 (Fig. 9a) and MDA-MB-231 cells (Fig. 9c) using fluorescently labelled anti-V5 antibodies. A strong membrane staining for latrophilin-2 was detected in HEK-293-LPHN2 cells (Fig. 9b) compared to control cells (Fig. 9a). Similarly, in MDA-MB-231-LPHN2 cells a strong signal was detected in the membrane demonstrating expression of latrophilin-2 in the membrane (Fig. 9d). In addition, staining of intracellular vesicles or of the endoplasmic reticulum was detected in these cells.

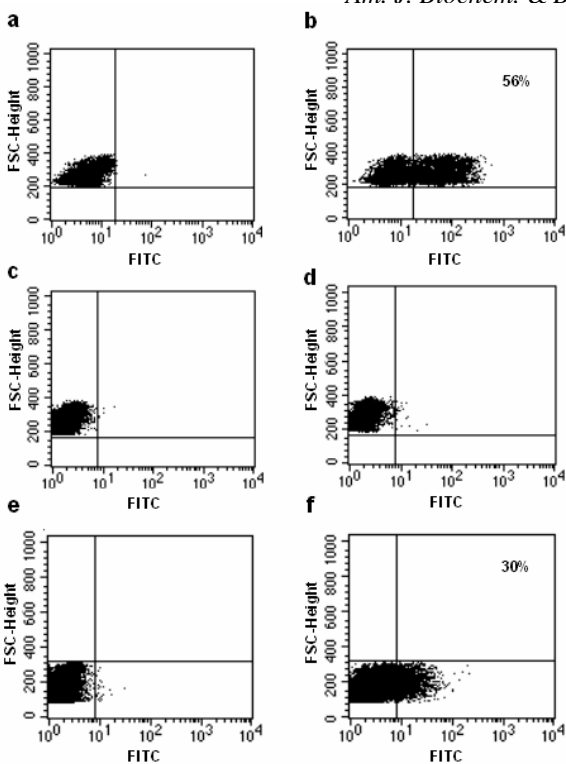


Fig. 8: Expression of V5-tagged human latrophilin-2: HEK-293 cells were either transiently transfected with (a) vector control pcDNA3.1V5 or (b) with pcDNA3.1V5LPHN2. The cells were fixed, permeabilised and stained with an anti-V5 FITC-labelled antibody. Flow cytometric analysis revealed that 56% of HEK-293 cells transiently transfected with pcDNA3.1-LPHN2 cells are positive for latrophilin-2. MDA-MB-231 empty vector pcDNA3.1 stable cell line (c, e) and MDA-MB-231-LPHN2, clone 10 (d, f). The native cells (c, d) or the fixed and permeabilised cells (e, f) were stained with an anti-V5 FITC-labelled antibody. Flow cytometry detects V5-tagged latrophilin-2 in 30% of fixed and permeabilised MDA-MB-231-LPHN2 cells whereas only background staining is visible in native cells

## DISCUSSION

Latrophilin-2 is a member of the adhesion-GPCR family that are characterised by a long N-terminus which contains adhesion domains such as the galactose binding lectin domain or the olfactomedin domain (Fig. 1). Considering the expression pattern of latrophilin-2, we found a strong signal in endothelial HUVEC and MVEC. Strong latrophilin-2 expression was visible in several adherent cells but not in the suspension cells TF-1 and PBL (Fig. 2). With *in situ* hybridisation latrophilin-2 expression was detected in the cytotrophoblast (Langhans' layer) of human placenta. The cytotrophoblast is the inner cell layer of placenta (Fig. 4a) and consists of distinct cells which form a continuous layer at the surface of the villi in the early phase of the pregnancy. A derivative of the cytotrophoblast is the outer layer, the syncytiotrophoblast, which consist of a mass of multinucleated cytoplasm formed by coalescence of cells derived from the cytotrophoblast. Interestingly, these cells show a high expression of latrophilin-2

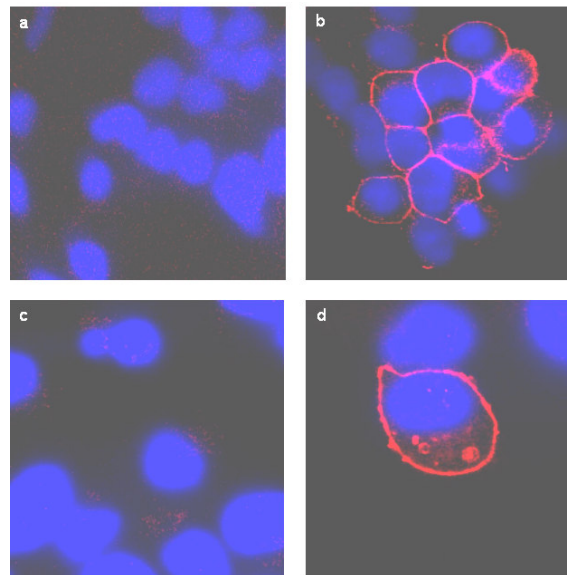


Fig. 9: Cell surface localisation of latrophilin-2. HEK-293 (a, b) and MDA-MB-231 (c, d), stably transfected with empty vector pcDNA3.1 (a, c) or with pcDNA3.1V5-LPHN2-(b, d). The stable clones are HEK293-LPHN2, clone 14 and MDA-MB-231-LPHN2, clone 10. The cells were stained with rhodamine red-labelled anti-V5 antibody, counterstained with DAPI and analysed with confocal microscopy at 40x magnification

(Fig. 4). The syncytiotrophoblast of the villi is essential for erosion and penetration of the endometrium leading to the assumption that latrophilin-2 may play a role in invasive processes. As cytotrophoblast cells are abundant in early placenta, but are reduced in number as term is approached, high expression of latrophilin-2 in early placenta and less expression in term placenta as seen by RT-PCR analysis (Fig. 2) might correlate with a smaller number of cytotrophoblast cells in the near term or term placenta. The core of the villous contains foetal stroma cells, foetal capillaries and Hofbauer cells. Compared to cytotrophoblast cells, only a moderate expression of latrophilin-2 was detected in Hofbauer cells (Fig. 4).

Immunoblot analysis using antiserum SA220 and an anti-V5 antibody directed against the N-terminal and the C-terminal fragment of latrophilin-2, respectively, demonstrated that latrophilin-2 is cleaved into a 135 kDa N-terminal and a 70 kDa C-terminal fragment (Fig. 5). The apparent molecular mass of 190/200 kDa for full-length latrophilin-2 (Fig. 5a) by far exceeds the predicted molecular weight of latrophilin-2. The 135 kDa N-terminal fragment was sensitive to enzymatic protein deglycosylation by glycosidase F. The removal of Asn-linked carbohydrates increased the electrophoretic mobility of latrophilin-2 from 135 kDa to 109 kDa (Fig. 6) demonstrating that human latrophilin-2 is highly glycosylated indicating that the N-terminus is oriented toward the extracellular space.

The latrophilin-2 specific antiserum SA220 detects the N-terminal fragment of latrophilin-2 in cell culture



supernatant of HUVEC indicating that endogenous latrophilin-2 is expressed on the protein level in human vascular endothelial cells and that post-translational modification and generation of a 135 kDa N-terminal fragment takes place.

For latrophilin-1 it has been shown that proteolysis of adhesion-GPCRs occurs constitutively and intracellularly in the endoplasmic reticulum during their post-translational modification. Latrophilin-1 is cleaved into an N- and a C-terminal fragment by an endoprotease<sup>[20]</sup>. The proteolysis is required for the delivery of mature N- and a C-terminal fragments to the cell membrane whereby the N-terminal fragment still associates with the outer cell membrane. Our findings are compatible with the prediction of a GPCR proteolysis site near the extracellular membrane surface of latrophilin-2<sup>[12]</sup>. In cells stably transfected with the gene, the complete protein of approximately 200 kDa can be found. It is always accompanied by two fragments of 135 kDa and of 70 kDa. The 135 kDa fragment resulting from such a cleavage was at least partially shed into cell culture supernatants of MDA-MB-231-LPHN2 and HUVEC cells. Besides a cell-autonomous function of the cell membrane associated N-terminal fragment of latrophilin-2, a paracrine function of the N-terminal fragment of latrophilin-2 which is present in the cell culture supernatant can also be envisioned.

The flow cytometry analysis of native versus fixed and permeabilised cells revealed a staining of fixed and permeabilised but not native MDA-MB-231-LPHN2 cells with the anti-V5 antibody indicating that the C-terminal fragment which contains the V5-tag is located towards the cytosol. In addition, using fluorescently labelled anti-V5 antibodies and confocal immunofluorescence analysis a strong membrane staining for latrophilin-2 was detected in HEK-293-LPHN2 and MDA-MB-231-LPHN2 cells. Taken together, this indicates that the C-terminal fragment of latrophilin-2 is correctly inserted into the cell membrane, where it might be responsible for protein-protein interactions with its PDZ-recognition motif and the induction of signal transduction processes.

In conclusion, latrophilin-2 is characterised by a strong expression in placenta. It is strongly expressed in endothelial cells (MVEC, HUVEC) and as evident from transfected cells it is localised on the surface membrane of cells.

We could show that latrophilin-2 – similarly to rat CIRL-2<sup>[8]</sup> – is cleaved and that the generated N-terminal fragment is highly glycosylated. We present evidence, that latrophilin-2 belongs to the class of adhesion-GPCRs. Similarly to latrophilin-1, the N- and the C-terminal fragment of latrophilin-2 might behave as separate entities where the N-terminal fragment might be involved in cell adhesion or cell-cell interaction and the C-terminal fragment in intracellular signalling.

Currently, it is unknown which types of signals are conferred on the one hand by the membrane-associated N-terminal fragment and on the other hand by the membrane-localised C-terminal fragment.

So far, latrophilin-2 is an orphan receptor and the endogenous ligand is unknown. As the large N-terminal fragment contains different adhesion domains, latrophilin-2 might be activated by cellular ligands such as adhesion proteins present on other cells or extracellular matrix adhesion molecules, rather than by soluble, secreted ligands which activate most classical GPCRs. Potential ligands of the galactose binding lectin domain are  $\beta$ -galactose containing oligosaccharides on extracellular molecules. The protein olfactomedin was originally identified as component of the mucous layer that surrounds the dendrites of olfactory neurons. The olfactomedin domain at the N-terminal fragment of latrophilin is homologous to this protein family. However, potential ligands of the olfactomedin domain are not known<sup>[21]</sup>. The function of latrophilin-2 is presently unknown but based on the strong expression in human placenta we hypothesise that latrophilin-2 might be involved in cell-cell interaction or adhesion processes in the cytotrophoblasts and syncytiotrophoblast.

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**Abbreviations:** GPCR, G-protein coupled receptor; GPS: GPCR proteolysis site; HBP, highly basic protein; HE, haematoxylin/eosin; HRP, horseradish peroxidase; HUVEC: human umbilical vein endothelial cell; LPHN2, latrophilin-2; MVEC microvascular endothelial cell; RT, room temperature; UTR, untranslated region; 7TM, 7 transmembrane domain.

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