

RESEARCH ARTICLE

Lgr4 Promotes Glioma Cell Proliferation through Activation of Wnt Signaling

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Abstract

The key signaling networks regulating glioma cell proliferation remain poorly defined. The leucine-rich repeat containing G-protein coupled receptor 4 (Lgr4) has been implicated in intestinal, gastric, and epidermal cell functions. We investigated whether Lgr4 functions in glioma cells and found that Lgr4 expression was significantly increased in glioma tissues. In addition, Lgr4 overexpression promoted while its knockdown using small interfering RNA oligos inhibited glioma cell proliferation. In addition, Wnt/ β -catenin signaling was activated in cells overexpressing Lgr4. Therefore, our results revealed that Lgr4 activates Wnt/ β -catenin signaling to regulate glioma cell proliferation.

Keywords: Lgr4 - Wnt - β -catenin - glioma - cell proliferation

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Introduction

Glioma belongs to the most common primary malignant brain tumor (astrocytic, oligodendroglial, oligoastrocytic and ependymal origin) (Ostrom et al., 2011; Gigineishvili et al., 2013). It accounts for almost 80% of primary malignant brain tumors, usually leading to poor survival compared to other types of brain tumors (Bondy et al., 2008).

GPR48, also known as leucine-rich repeat (LRR)-containing G protein-coupled receptor 4 (LGR4), is a member of the G protein coupled receptor (GPCR) family of proteins (Kudo et al., 2000; Loh et al., 2001). The GPCR family containing seven transmembrane domains function as the receptors for various classes of ligands, such as growth factors, chemokines and peptide hormones, and have become major targets for pharmaceutical development (Lagerström et al., 2008; Lappano et al., 2011; Stevens et al., 2013). Several GPCRs, including chemokine (C-X-C) receptors and thyroid-stimulating hormone receptor, have also been found to contribute to carcinogenesis (Xing et al., 2003; Brewer et al., 2007; Obermajer et al., 2011; Sengupta et al., 2012).

Lgr4 gene deletion have been shown to cause developmental defects in multiple organs, including male reproductive tracts, spermatogenesis, bone formation and hair follicle development, as well as reduced embryonic growth (Mazerbourg et al., 2004; Mohri et al., 2008; Luo et al., 2009; Li et al., 2010; Qian et al., 2013). In addition, recent studies identify members of the R-spondin family as ligands for LGR4, directly linking this GPCR to the Wnt/ β -catenin signaling (de Lau et al., 2011; Glinka et al.,

2011). Moreover, Lgr4 expression is up-regulated in p27-deficient colon cancer cells, and forced over-expression of Lgr4 promotes the invasive and metastasis potential of HCT116 cells (Gao et al., 2006). Furthermore, Lgr4 expression is inversely correlated with the expression of p27 in human colon carcinomas and associated with lymph node metastasis of these tumors, suggesting that dys-regulation of Lgr4 contributes to tumor invasiveness and metastasis (Gao et al., 2006). In this study, for the first time, we will investigate the functions of Lgr4 in glioma progression.

Materials and Methods

Cell culture and tissue samples

Glioma cells (SHG-44 and U251) were obtained from American Type Culture Collection (Rockville, MD). Cells were culture in RPMI 1640 medium supplemented with 10% fetal bovine serum. Glioma tissues and adjacent tissues from glioblastoma patients were collected from routine therapeutic surgery at our department. All samples were obtained with informed consent and approved by the hospital institutional review board.

siRNA, RNA extraction and Real-time Analysis

Small interfering RNA oligos (siRNA) targeting Lgr4, b-catenin or negative controls were obtained from Dharmacon Company (USA). Cells were seeded on to 6-well plates then transfected with 50nM siRNA oligos. Total RNAs were isolated from tissues or cells by TRIzol reagent, and reverse transcriptions were performed by Takara RNA PCR kit (Takara, China) following the

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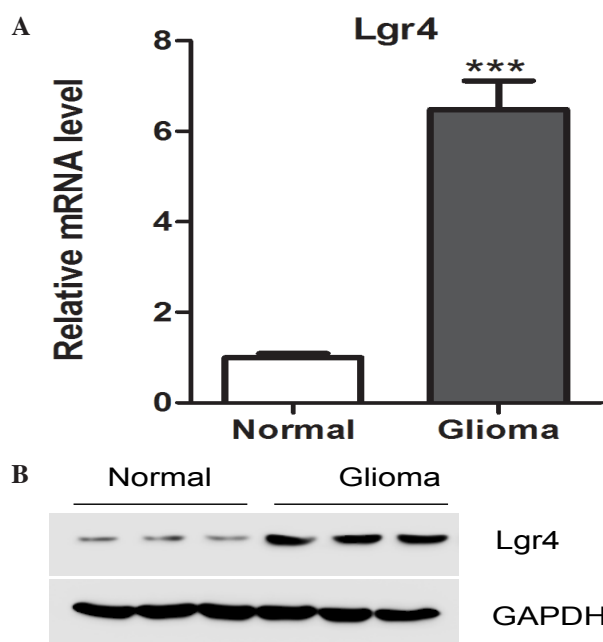


Figure 1. Expression Levels of Lgr4 in Glioma Tissues. (A-B) Representative Lgr4 expression was determined by TaqMan real-time PCR and western blot in human glioma tissues and pair-matched adjacent noncancerous brain tissues (Normal)

manufacturer's instructions. In order to quantify the transcripts of the interest genes, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Japan) on Light Cycler 480 (Roche, Switzerland).

BrdU Assays

A cell proliferation enzyme-linked immunosorbent assay (BrdU kit; Beyotime) was used to analyze the incorporation of BrdU during DNA synthesis following the manufacturer's protocols. All experiments were performed in triplicate. Absorbance was measured at 450 nm in the Spectra Max 190 ELISA reader (Molecular Devices, Sunnyvale, CA)

Western blot

Cells or tissues were harvested and lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM 2-ME, 2% w/v SDS, 10% glycerol). After centrifugation at 20000×g for 10 min at 4 °C, proteins in the supernatants were quantified and separated by 10% SDS PAGE, transferred to NC membrane (Amersham Bioscience, Buckinghamshire, U.K.). After blocking with 10% nonfat milk in PBS, membranes were immunoblotted with antibodies as indicated, followed by HRP-linked secondary antibodies (Cell Signaling). The signals were detected by SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) according to manufacturer's instructions. Anti-Lgr4 and β -catenin antibodies were purchased from Abcam Company (USA). Protein levels were normalized to lamin B (Abcam, USA) or GAPDH (Santa Cruz, USA).

Statistical analysis

Data are expressed as the mean \pm SEM from at least three separate experiments. Differences between groups were analyzed using Student's t-test. A value of $p < 0.05$ was considered statistically significant.

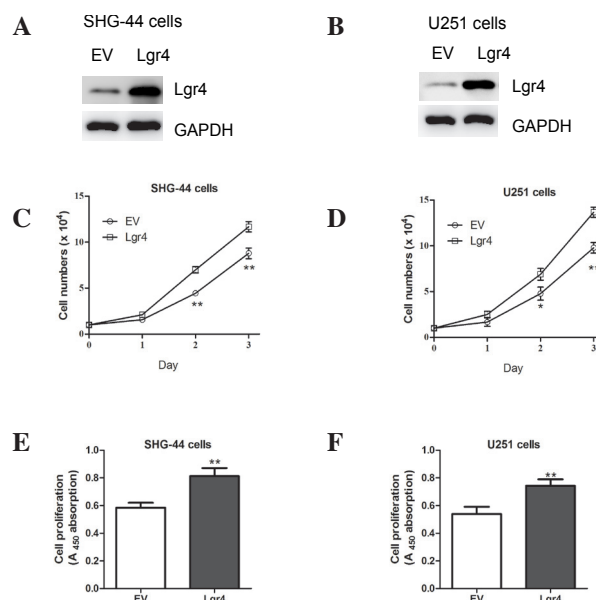


Figure 2. Overexpression of Lgr4 Promotes Glioma Cell Proliferation. (A-B) Lgr4 expression was determined by western blot in SHG-44 and U251 cells transfected with plasmids containing empty vector (EV) or Lgr4. (C-D) The growth curve of SHG-44 and U251 cells after Lgr4 transfection compared to controls (EV). (E-F) The cell proliferative potential (BrdU) was determined in SHG-44 and U251 cells. A450 absorption was assayed after transfection for 36 hr

Results

Lgr4 expression levels were up-regulated in glioblastoma patients

Firstly, to examine whether the Lgr4 is differentially expressed in human glioma, its expression level was determined using TaqMan real-time PCR in 25 pairs of glioma tissues and pair-matched adjacent noncancerous tissues. Our results demonstrated that the mRNA level of Lgr4 was significantly increased in glioma tissues in comparison with the adjacent noncancerous tissues (Figure 1A). We then employed western blot with anti-Lgr4 antibody to detect the protein content of Lgr4 in those clinic samples. The results showed the increased protein expression of Lgr4 in glioma samples (Figure 1B).

The effects of Lgr4 overexpression on cell growth

In order to assess the effects of Lgr4 on glioma cell growth, the Lgr4 expression plasmid was transfected into SHG-44 and U251 cells and cell growth was examined post-transfection (Figure 2A and 2B). As a result, Lgr4 overexpression significantly increased cell number and promoted proliferation in both cells (Figure 2C and 2F).

Inhibition of Lgr4 blocks the proliferation of glioma cells

As described above, Lgr4 plays a critical role in the proliferation of glioma cells. However, it remained unknown whether inhibiting Lgr4 would reduce cell proliferation. Therefore, cells were transfected with small interfering RNA oligos (siRNA) targeting Lgr4 or negative controls (Figure 3A-3B). The results showed that knockdown of Lgr4 expression reduced the growth of SHG-44 and U251 cells, compared to NC-transfected cells (Figure 3C-3F).

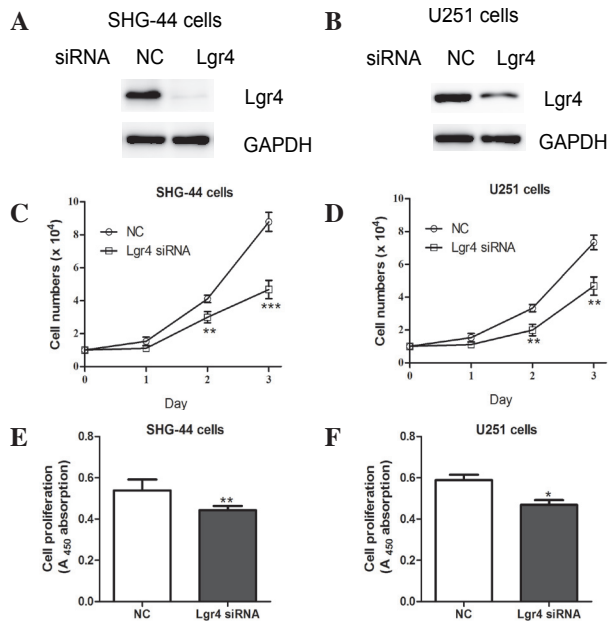


Figure 3. Lgr4 Knockdown Reduces the Proliferation of Glioma Cells. (A-B) Lgr4 expression was determined by western blot in SHG-44 and U251 cells transfected with siRNA oligos targeting Lgr4 or negative controls (NC). (C-D) The growth curve of SHG-44 and U251 cells after transfection with siRNA oligos targeting Lgr4 or negative controls (NC). (E-F) The cell proliferative potential (BrdU) was determined in SHG-44 and U251 cells. A450 absorption was assayed after transfection for 48 hr

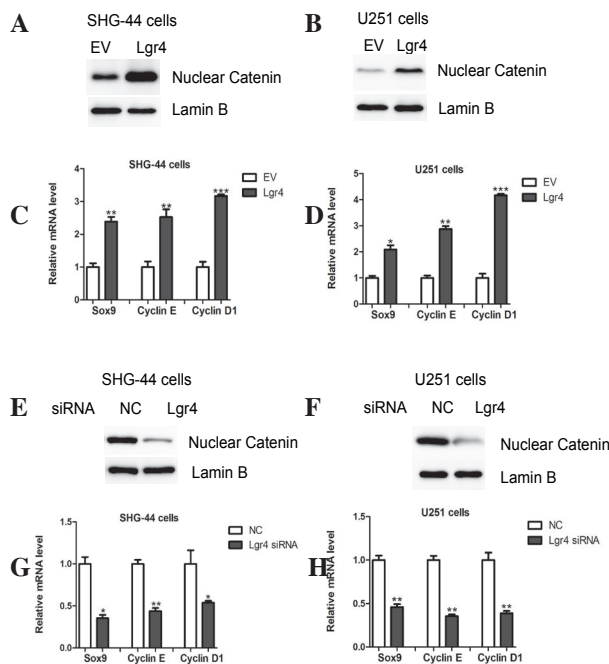


Figure 4. Lgr4 Positively Regulates Wnt Signaling Activation. (A-B) Nuclear β -catenin expression was determined by western blot in SHG-44 and U251 cells transfected with plasmids containing empty vector (EV) or Lgr4. Lamin B levels were measured as a loading control. (C-D) mRNA levels of Sox9, Cyclin E and Cyclin D1 were examined by real-time PCR in SHG-44 and U251 cells overexpressing empty vector (EV) or Lgr4. (E-F) Nuclear β -catenin expression was determined by western blot in SHG-44 and U251 cells transfected with siRNA oligos targeting Lgr4 or negative controls (NC). (G-H) mRNA levels of Sox9, Cyclin E and Cyclin D1 were examined by real-time PCR in SHG-44 and U251 cells transfected with siRNA oligos targeting Lgr4 or negative controls (NC)

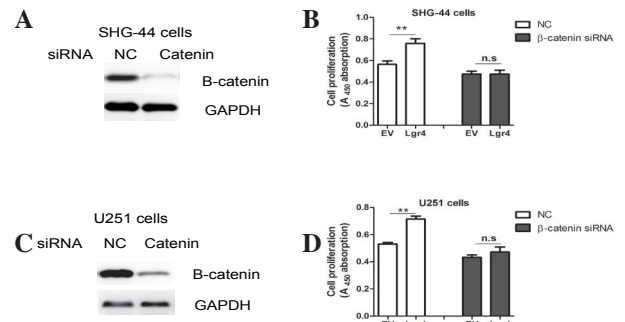


Figure 5. β -catenin Depletion Attenuates the Roles of Lgr4 Overexpression. (A) β -catenin expression was determined by western blot in SHG-44 cells transfected with siRNA oligos targeting β -catenin or negative controls (NC). (B) The cell proliferative potential (BrdU) was determined in SHG-44 cells. Cells were pre-treated with siRNA oligos for 24 hours and then transfected with Lgr4 or empty vector (EV) for another 24 hours. (C) β -catenin expression was determined by western blot in U251 cells transfected with siRNA oligos targeting β -catenin or negative controls (NC). (D) The cell proliferative potential (BrdU) was determined in U251 cells. Cells were pre-treated with siRNA oligos for 24 hours and then transfected with Lgr4 or empty vector (EV) for another 24 hours

Lgr4 activates Wnt signaling in glioma cells

Lgr4 has recently been reported to potentiate Wnt signaling after binding to its ligand R-spondin (de Lau et al., 2011; Glinka et al., 2011). To test whether the Wnt pathway mediates Lgr4 in glioma cells, the nuclear β -catenin abundance was examined. As shown in Figure 4A and 4B, the protein content of nuclear β -catenin was increased in cells overexpressing Lgr4. Besides, several downstream target genes of β -catenin including Sox9, Cyclin E and Cyclin D1 (Chartier et al., 2007; Panza et al., 2013), were also up-regulated by Lgr4 overexpression (Figure 4C-4D). Consistently, we observed a reduced nuclear β -catenin abundance and decreased expression of its target genes in cells depletion of Lgr4 (Figure 4E-4H), indicating that Lgr4 activates Wnt/ β -catenin signaling pathway in glioma cells

The proliferative roles of Lgr4 is dependent on Wnt/ β -catenin signaling

Next, SHG-44 and U251 cells were transfected with siRNA targeting β -catenin or negative controls (Figure 5A and 5C). As a result, knockdown of β -catenin dramatically reduced cell proliferation activity induced by Lgr4 overexpression (Figure 5B and 5D), suggesting that β -catenin is required for the proliferative roles of Lgr4 in glioma cells.

Discussion

In this study, we demonstrated that Lgr4 expression is up-regulated in glioma tissues. Up-regulation of Lgr4 promoted cell proliferation while inhibition of Lgr4 inhibited glioma cell proliferation. At the molecular level, we revealed that Wnt/ β -catenin signaling plays an indispensable role in mediating Lgr4 activation. Collectively, these findings suggest that up-regulation of Lgr4 may promote the initiation and progression of glioma.

The common hallmark in various human cancer tissues is the nuclear retention of β -catenin, the main effector of the canonical Wnt signaling pathway (Fodde et al., 2001; Casás-Selves et al., 2012). In the absence of Wnt ligands, β -catenin is tightly regulated by a multi-protein complex including: the tumour suppressor APC, the scaffolding protein axin, and the phosphokinases GSK-3 β and CK1 (Fodde et al., 2001; Casás-Selves et al., 2012). Upon ligand activation, the complex is destabilized, allowing for cytoplasmic β -catenin accumulation with subsequent nuclear translocation (Fodde et al., 2001; Moon et al., 2004). In the nucleus, β -catenin acts as an essential cofactor in the context of cell cycle progression, proliferation and survival (Niehrs et al., 2012). For various human cancers, an association between aberrant Wnt signaling pathway activation and the clinical behavior of the respective tumor has been well established (Angers et al., 2009). For instance, in human breast cancer, cytoplasmic and nuclear localization of β -catenin were shown to predict a poor outcome (Deng et al., 2002). In human gliomas, nuclear immunoreactivity of β -catenin is established as a favorable-risk indicator in a subgroup of medulloblastomas (Baryawno et al., 2010). Besides, activating mutations in the β -catenin gene have identified and was shown to be involved in the development of a subset of medulloblastomas (Zurawel et al., 1998). In addition, several extracellular signals were exhibited to promote or repress glioma cell proliferation through regulation of Wnt/ β -catenin (Xie et al., 2004; Baryawno et al., 2010). Therefore, these findings demonstrate the importance of Wnt signaling pathway as a therapeutic target in treatment of glioma.

In summary, the key finding of the current study is that Lgr4 can promote the proliferation of glioma cell lines by activation of Wnt/ β -catenin pathway. Understanding the precise role played by Lgr4 progression will not only advance our knowledge of glioma biology, but also will help determine if Lgr4 has potential as a novel therapeutic target for the treatment of glioma.

References

Angers S, Moon RT (2009). Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol*, **10**, 468-77.

Baryawno N, Sveinbjörnsson B, Eksborg S, et al (2010). Small-molecule inhibitors of phosphatidylinositol 3-kinase/Akt signaling inhibit Wnt/ β -catenin pathway cross-talk and suppress medulloblastoma growth. *Cancer Res*, **70**, 266-76.

Bondy ML, Scheurer ME, Malmer B, et al (2008). Brain tumor epidemiology: consensus from the Brain Tumor Epidemiology Consortium. *Cancer*, **8**, 1953-68.

Brewer C, Yeager N, Di Cristofano A (2007). Thyroid-stimulating hormone initiated proliferative signals converge in vivo on the mTOR kinase without activating AKT. *Cancer Res*, **67**, 8002-6.

Casás-Selves M, Kim J, Zhang Z, et al (2012). Tankyrase and the canonical Wnt pathway protect lung cancer cells from EGFR inhibition. *Cancer Res*, **72**, 4154-64.

Chartier NT, Oddou CI, Lainé MG, et al (2007). Cyclin-dependent kinase 2/cyclin E complex is involved in p120 catenin (p120ctn)-dependent cell growth control: a new role for p120ctn in cancer. *Cancer Res*, **67**, 9781-90.

de Lau W, Barker N, Low TY, et al (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature*, **476**, 293-7.

Deng J, Miller SA, Wang HY, et al (2002). β -catenin interacts with and inhibits NF- κ B in human colon and breast cancer. *Cancer Cell*, **2**, 323-34.

Fodde R, Smits R, Clevers H (2001). APC, signal transduction and genetic instability in colorectal cancer. *Nat Rev Cancer*, **1**, 55-67.

Gao Y, Kitagawa K, Hiramatsu Y, et al (2006). Up-regulation of GPR48 induced by down-regulation of p27Kip1 enhances carcinoma cell invasiveness and metastasis. *Cancer Res*, **66**, 11623-31.

Gigineishvili D, Shengelia N, Shalashvili G, et al (2013). Primary brain tumour epidemiology in Georgia: first-year results of a population-based study. *J Neurooncol*, **8**, 241-6.

Glinka A, Dolde C, Kirsch N, et al (2011). LGR4 and LGR5 are R-spondin receptors mediating Wnt/ β -catenin and Wnt/PCP signalling. *EMBO Rep*, **12**, 1055-61.

Kudo M, Chen T, Nakabayashi K, Hsu SY, Hsueh AJ (2000). The nematode leucine-rich repeat-containing, G protein-coupled receptor (LGR) protein homologous to vertebrate gonadotropin and thyrotropin receptors is constitutively active in mammalian cells. *Mol Endocrinol*, **14**, 272-84.

Lagerström MC, Schiöth HB (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov*, **7**, 339-57.

Lappano R, Maggolini M (2011). G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat Rev Drug Discov*, **10**, 47-60.

Li XY, Lu Y, Sun HY, et al (2010). G protein-coupled receptor 48 upregulates estrogen receptor alpha expression via cAMP/PKA signaling in the male reproductive tract. *Development*, **137**, 151-7.

Loh ED, Broussard SR, Kolakowski LF (2001). Molecular characterization of a novel glycoprotein hormone G-protein-coupled receptor. *Biochem Biophys Res Commun*, **282**, 757-64.

Luo J, Zhou W, Zhou X, et al (2009). Regulation of bone formation and remodeling by G-protein-coupled receptor 48. *Development*, **136**, 2747-56.

Mazerbourg S, Bouley DM, Sudo S, et al (2004). Leucine-rich repeat-containing, G protein-coupled receptor 4 null mice exhibit intrauterine growth retardation associated with embryonic and perinatal lethality. *Mol Endocrinol*, **18**, 2241-54.

Mohri Y, Kato S, Umezawa A, Okuyama R, Nishimori K (2008). Impaired hair placode formation with reduced expression of hair follicle-related genes in mice lacking Lgr4. *Dev Dyn*, **237**, 2235-42.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A (2004). WNT and β -catenin signalling: diseases and therapies. *Nat Rev Genet*, **5**, 691-701.

Niehrs C (2012). The complex world of WNT receptor signalling. *Nat Rev Mol Cell Biol*, **13**, 767-79.

Angers S, Moon RT (2009). Proximal events in Wnt signal transduction. *Nat Rev Mol Cell Biol*, **10**, 468-77.

Obermajer N, Muthuswamy R, Odunsi K, Edwards RP, Kalinski P (2011). PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. *Cancer Res*, **71**, 7463-70.

Ostrom QT, Barnholtz-Sloan JS (2011). Current state of our knowledge on brain tumor epidemiology. *Curr Neurol Neurosci Rep*, **8**, 329-35.

Panza A, Paziienza V, Ripoli M, et al (2013). Interplay between SOX9, β -catenin and PPAR γ activation in colorectal cancer. *Biochim Biophys Acta*, **1833**, 1853-65.

- Qian Y, Liu S, Guan Y, et al (2013). Lgr4-mediated Wnt/b-catenin signaling in peritubular myoid cells is essential for spermatogenesis. *Development*, **140**, 1751-61.
- Sengupta R, Dubuc A, Ward S, et al (2012). CXCR4 activation defines a new subgroup of Sonic hedgehog-driven medulloblastoma. *Cancer Res*, **72**, 122-32.
- Stevens RC, Cherezov V, Katritch V, et al (2013). The GPCR Network: a large-scale collaboration to determine human GPCR structure and function. *Nat Rev Drug Discov*, **12**, 25-34.
- Xie D, Yin D, Tong X, et al (2004). Cyr61 is overexpressed in gliomas and involved in integrin-linked kinase-mediated Akt and beta-catenin-TCF/Lef signaling pathways. *Cancer Res*, **64**, 1987-96.
- Xing M, Usadel H, Cohen Y, et al (2003). Methylation of the thyroid-stimulating hormone receptor gene in epithelial thyroid tumors: a marker of malignancy and a cause of gene silencing. *Cancer Res*, **63**, 2316-21.
- Zurawel RH, Chiappa SA, Allen C, Raffel C (1998). Sporadic medulloblastomas contain oncogenic beta-catenin mutations. *Cancer Res*, **58**, 896-9.