THE ROLE OF NOTCH2 GENE IN HUMAN MALIGNANT GLIAL BRAIN TUMOURS

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Balasubramanian Sivasankaran
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Prof. Dr. Eberhard Parlow

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Summary

Background: Glioblastoma multiforme (GBM), astrocytoma (A) and oligodendroglioma (OG) are the neoplasms of the glial lineage in the Central Nervous System (CNS). Among them, GBM occurs at the highest frequency and shows the shortest patient median survival time of some 10 months as compared for instance to the survival time of OG of about 10 years. Genetically, OG differs from GBM by the frequent combination of loss of heterozygosity (LOH) on chromosomes 1p and 19q, which is associated with more favourable prognosis in OG patients. However, the clinical significance of LOH on 1p in other glioma subtypes remained unknown.

Methods and Results: We identified a subgroup of GBM with LOH on centromeric chromosome 1p together with longer survival. The minimally lost area(s) in both GBM and OG converged at the *NOTCH2* locus on 1p11 and positively correlated with prognosis in GBM as well as in OG patients. Comparison between gene expression of *NOTCH2* and the genetic status at the *NOTCH2* locus on chromosome 1p11 supported the hypothesis of a loss of function alteration of *NOTCH2* in tumours. However, many GBMs do not display deletions at the *NOTCH2* locus on 1p11 and do express the NOTCH2 gene. Abundant expression of components of canonical NOTCH signaling in these tumors and a positive correlation between *NOTCH2* transcripts with the target gene *HES-1* (P=0.0001) indicated that functional NOTCH signaling in glioma is mainly driven by NOTCH2. In addition, we defined *TNC*, the gene for the cell migration factor tenascin-C as a novel target gene for NOTCH signaling. We further showed that activation of NOTCH signaling was indeed promoting TNC-dependent glioma cell motility. Thus, together with the ability to increase proliferation, canonical Notch signaling turned out to be critical for glioma progression. We also found that non-canonical Notch signaling was associated with the maintenance of tumorigenic potential of the GBM cells in soft agar culture. In addition, Notch2 had a pro-survival effect on GBM cells by upregulating anti-apoptotic proteins Bcl-2 and Mcl-1, independently of the canonical pathway. Finally, defective degradation pathway of Notch receptors in GBM cells led to slow receptor turnover, thereby providing additional contribution to the oncogenic function of Notch2.

Conclusion: This study identified aberrant multi-facetted oncogenic behaviours of Notch proteins, in particular of Notch2, in GBM. This provided a molecular basis for the higher aggressiveness of Notch2-positive GBM compared to Notch2-negative GBM or OG, and suggested Notch2 as a sensible target for new therapeutic approaches against GBM.

Introduction

In the year 2000, malignant tumours were responsible for 6.2 million deaths out of 56 million worldwide from all causes, while 5.3 million men and 4.7 million women developed a malignant tumour according to World Health Organization (WHO) (Stewart, 2003).The report also revealed that cancer has emerged as a major public health problem in developing countries, matching industrialized nations. In Switzerland approximately 25% of deaths are caused by cancer (Quinto, 2004). In spite of phenomenal progress in basic and clinical research, current treatment results only in a modest prolongation of life. Exposure to carcinogens in tobacco smoke (Witschi et al., 1995) or in food (Johnson, 2002) or UV light (Fisher and Kripke, 1977) results in genetic alterations that target genes involved in the regulation of cell-cycle, survival and genome integrity. Genetic alterations such as gene amplification, gain of function mutation or ectopic expression due to gene translocation result in activation of oncogenes that accelerate the cell cycle preferentially during the G1/S phase, but also during the G2/M. Loss of heterozygosity (LOH), loss of function mutations (Pihan and Doxsey, 2003) and transcriptional silencing result in inactivation of tumour-suppressor genes (Herman and Baylin, 2003; Merlo et al., 1995). These types of genetic alterations are believed to cooperate in the promotion of tumour development.

Acquired genetic alterations are clonally selected if they convey a growth advantage leading to progressive conversion of normal into neoplastic cells (Nowell, 1976). Self-sufficient growth signals, insensitivity to anti-growth signals, unlimited replicative potential, evasion of apoptosis and sustained angiogenesis are all considered to be hallmarks of cancer cells (Hanahan and Weinberg, 2000). A cancer cell no longer induces efficient cell cycle arrest and apoptosis in response to mutations in cell cycle, DNA repair and pro-apoptotic genes. Germline mutations present in cancer genes, such as *NF-1 & -2, PTEN, TSC1/2, MLH1/MSH2* (Turcot syndrome), *TP53* (Li-Fraumeni syndrome) and *Rb* (He et al., 1995) result in hereditary predispositions to cancer (familial cancer syndromes) (Fearon, 1997) whereas somatic mutations give rise to the prevalent sporadic tumours. Such genes involved in gliomagenesis are *Rb* (He et al., 1995), *CDKN2A* (Labuhn et al., 2001), *CDKN1B* (Alleyne et al., 1999), and *HDM2* (Vogelstein and Kinzler, 2004).

1.1 Clinical presentation of brain tumours

Gliomas, tumours of glial origin, are the most common neoplasms of the central nervous (CNS) that include oligodendroglioma, astrocytoma and glioblastoma **(Annex-2)** (Holland, 2001). Oligodendrogliomas have an incidence of 3 in $1x10^6$ population/year with a mean survival of 10 years (Kleihues and Sobin, 2000). Pilocytic astrocytomas are benign tumours of WHO grade I that rarely progresses to more advanced stages and the patients are cured if the tumour can be completely resected (Zhu and Parada, 2002). Astrocytoma WHO grade II and anaplastic astrocytomas WHO grade III infiltrate into the normal brain. The incidence rates of grade II and III astrocytomas are 10-15 in $1x10^6$ population/year. The mean survival is 7 years in grade II and less than 5 years in grade III atstrocytomas (Burger et al., 1985; Leighton et al., 1997; Philippon et al., 1993; Prados et al., 1992). Glioblastoma (GBM) is the most malignant form of tumour with 36 in $1x10^6$ population/year as occurrence rate. The patients mean survival is less than 10 months (Ohgaki and Kleihues, 2007). Surgical resection is difficult in these tumours. Despite advances in surgical and medical neuro-oncology, their prognosis remains poor (Ohgaki and Kleihues, 2005). Like tumours of other cellular types, they also show resistance to chemo- and radio-therapy (Shapiro et al., 1989). However, in contrast to other tumour types, they rarely metastasize (Giordana et al., 1995), but show diffuse infiltration and rapid invasion of neighbouring brain structures (Burger et al., 1988).

1.2 Purpose of this work

The core intention of this work is to identify and understand the key molecular genetic mechanisms, which are crucial. That would eventually pave a way for finding effective diagnostic and therapeutic strategies to improve patient prognosis.

a. Understanding the mechanism of gliomagenesis

Frequent genetic alterations targeting the chromosomes 1p, 7p, 10p and 10q, 11p, 17p, 19q and 22q have been observed in gliomas (Merlo, 2003). Genes located within these alterations may be responsible for initiation and progression of glioma. Identification of underlying genes and associated pathways within these chromosomal

aberrations will shed light on their biological and clinical significance. In the past two decades, progress in the field of molecular neuro-oncology revealed that the most relevant genes involved in gliomagenesis are those encoding growth factor receptors (e.g. *EGFR*), components of the cell cycle machinery (*Rb*, *Cdk4*, and the Cyclin-dependent kinase inhibitor *CDKN2A*/$p16^{INK4a}$), and regulators of apoptosis (*p53*, *HDM2*, $p14^{ARF}$ and *PTEN*) (Maher et al., 2001; Merlo, 2003). Most of the animal models created so far are based on the molecular alterations mentioned above (Holland, 2001; Hu and Holland, 2005). These alterations have been shown to confer a growth advantage, leading to uncontrolled cell proliferation, a high invasive potential and drug resistance.

b. Clinical application

In recent years there has been tremendous progress towards a detailed characterization of genetic alterations that underlie many human tumour types (Hanahan and Weinberg, 2000). The new molecular genetic insights of tumour biology have been exploited with success to identify pliable cancer pathways and use them as targets for low molecular weight compounds that have potential anti-tumourigenic effects. Targeted therapies raise new hopes in the treatment of cancer. Compounds like Gleevec and Iressa have established a paradigm for the treatment of tumours such as chronic myeloid leukemia (CML), gastro-intestinal tumour (GIST) (Capdeville et al., 2002) and non-small cell lung cancer (NSCLC) (Sordella et al., 2004). As the tumour growth is acutely dependent on specific kinases however, only Gleevec has fulfilled its therapeutic promise so far in the chronic phase, but not in the blast crisis of CML. No equivalent drug has been identified yet for interfering with glioma progression, and single drug treatments are likely to be inefficient to treat gliomas, possibly because of the genetic instability that allows a swift adaptation to a therapeutic challenge. Hence, a new strategy is to find crucial molecular genetic alterations during initiation and progression of gliomas that can be exploited for development of new drugs that ought to be used in drug combinations targeting several pathways at once instead of single drug approaches.

1.3 Central nervous system development

The three major fundamental cell types of the vertebrate central nervous system (CNS) are neurons, astrocytes and oligodendrocytes. This basic triad comprises many distinct sub-types of neurons, of astroglia and of oligodendroglia. The different neural cell types are generated sequentially during the CNS development from ventricular zone neuro-epithelial stem cells (NSC) derived from the embryonic neural tube, with neurogenesis preceding gliogenesis (Battiste et al., 2007; Sugimori et al., 2007; Zhou and Anderson, 2002). These NSC undergo series of symmetric and asymmetric divisions at specific points in time and space to generate progressively more restricted precursors: neuronal restricted precursors that produce only neurons and glial-restricted precursor cells (GPCs) which further lineage into either astrocytic precursor cells (APCs) or oligodendrocyte precursor cells (OPCs), and terminally either differentiate into astrocytes or oligodendrocytes. This results in the correct proportion of cells needed to form a mature nervous system (Holland, 2001; Maher et al., 2001; Wechsler-Reya and Scott, 2001) **(Annex-1)**. At the adult stage, most neurons become post-mitotic, and only a small fraction of stem cells remains undifferentiated. However, many astrocytes or their precursors retain their dividing capacity throughout life. This makes them particularly susceptible to transformation and this is presumably one reason why astrocytic tumours are the most common brain tumours, besides the fact that astrocytes are about ten times more frequent in the CNS compared to neurons (Zhu and Parada, 2002).

1.4 Classification, grading and genetic alteration of gliomas

a. Precursors of gliomas

Gliomas include tumours with predominance of astrocytic (astrocytomas) or oligodendrocytic origin (oligodendroglioma) or which show a mixture of both glial cell types (oligoastrocytoma), or less differentiated glial cells (glioblastoma) **(Annex-2)** (Holland, 2001). These tumours have cellular morphologies and gene-expression patterns similar to astrocytes, oligodendrocytes and their precursors, respectively. The pathways known to be involved in proliferation and differentiation of glial progenitors are altered in gliomas. The cell type from which gliomas originate has not been

definitely established (Sanai et al., 2005). The common understanding is that astrocytoma and oligodendrogliomas arise from respective precursors and that mixed gliomas originate from progenitors of both astrocytes and oligodendrocytes, but this is not completely proven (Holland, 2001). Next, the widely assumed mechanism of gliomagenesis derived from differentiated glia has not been adequately tested. Finally, the concept of de-differentiation of mature glia fails to explain adequately the origin of oligo-astrocytoma. However, the important point is that the origin of a neoplasm is not necessarily reflected in the appearance of its most common cellular component: brain tumours of apparently comparable histological structure can exhibit vastly different behaviors (Holland, 2001; Merlo, 2003; Ohgaki and Kleihues, 2005; Wechsler-Reya and Scott, 2001).

For decades, adult glia was thought to be the only dividing cells in the postnatal brain, making them the only brain cells susceptible to transformation. Since then, other multi-potent, self-renewing and proliferative populations like Neural stem cells (NSC) and glial cells (GPC) have been identified in multiple regions of the human adult brain that are reservoirs for immature neural cells, i.e. the subventricular zone (Johansson et al., 1999). So, the classic theories regarding gliomagenesis are now being reappraised in the hope of reconstructing a more accurate picture of the origin of gliomas (Johansson et al., 1999; Sanai et al., 2005). Recently, two groups (Galli et al., 2004; Singh et al., 2004) have identified brain tumour initiating, stem-like precursors from human primary glioblastomas. This sub-population seemingly responsible for radio-resistance of glioblastomas (GBM) (Bao et al., 2006), looses its tumourigenic potential upon addition of bone morphogenic protein-4 (BMP4) (Piccirillo et al., 2006). The identification of the cellular origin of gliomas presents an opportunity for improving our understanding of this disease and finding potential therapeutic strategies (Holland, 2001; Maher et al., 2001; Sanai et al., 2005).

b. Classification and grading of gliomas

The seminal system of classification and grading of glial tumours introduced by Bailey and Cushing in the early 20th century was based on the comparison of histological appearances and putative developmental stages of glia (Louis et al., 2001). This was basically due to the consideration that astrocytomas and oligodendrogliomas are looking similar to their normal differentiated counterparts. Later, advancements in molecular histopathology have proven that the seminal concepts were correct and they form the basis for the current World Health Organization (WHO) classification and grading revised in 2000 (Louis et al., 2001). The WHO classification system divides diffusive gliomas into astrocytic tumours, oligodendrogliomas and oligo-astrocytomas. The degree of malignancy, as determined by histopathological criteria, grades them on the scale of I to IV. The clinical outcome of the patients predominantly depends on the tumour grade since gliomas rarely metastasize outside the CNS. Biologically, grade I tumours are benign and can be surgically cured because they do not infiltrate adjacent normal brain tissue. Grade II tumours are low-grade malignancies that may take a long clinical course. Because grade II tumours infiltrate normal surrounding brain tissue, they cannot be cured surgically. Grade III & IV gliomas are highly malignant and diffusely infiltrate normal brain. Around 70% of the grade II gliomas transform into grade III & IV tumours within 5-10 years of diagnosis (Maher et al., 2001) **(Annex-3)**.

Astrocytomas of WHO grades II-IV are the most common CNS neoplasms and represent more than 60% of all primary brain tumours. Glioblastoma multiforme (GBM), also called astrocytoma WHO grade IV is the most malignant form of infiltrating glioma with a very short median survival of 8 to 10 months (Zhu and Parada, 2002). Although identical at the histopathological point of view, GBMs can be divided into two subclasses: primary and secondary GBM based on clinical characteristics. Primary GBM occur in older patients, are very aggressive, highly invasive and arise from a *de novo* process, without a record of a pre-existing low-grade lesion (Ohgaki and Kleihues, 2007). Secondary GBM are usually observed in younger patients and develop progressively from low-grade astrocytoma over a period of 5 to 10

years. Genetic studies of GBM indicate that there are common, but also distinct genetic pathways that lead to these two sub-types (Wechsler-Reya and Scott, 2001). Very recently, the isocitrate dehydrogenase (IDH1) gene has been identified that allows distinction between primary and secondary GBM (Parsons et al., 2008).

c. Genetic alterations associated with gliomas

The median survival is statistically similar between primary and secondary GBM, although secondary GBM have a tendency for longer survival that may be related to younger patient age. Both tumours have equal capacities to proliferate, invade and resist to all therapeutic interventions (Maher et al., 2001; Wechsler-Reya and Scott, 2001). Microvascular proliferation and necrosis are found in primary as well as in secondary GBM. Both are composed of highly infiltrative and less differentiated cells than low-grade astrocytomas. The analysis of mutations indicates that the same genetic pathways (growth and cell cycle regulation) are targeted, but the frequency of specific genetic mutations may differ between the GBM sub-types (Zhu and Parada, 2002).

Primary GBM

Primary GBM are the majority of GBM and develop *de novo* very rapidly, without clinical, radiological, or morphological evidence of a pre-existing low-grade lesion (Labuhn et al., 2001; Maher et al., 2001; Ohgaki and Kleihues, 2005).

In primary GBM, the homozygous deletion of the *CDKN2A* locus, coding for $p16^{INK4A}$ & $p14^{ARF}$ occurs quite frequently, simultaneously affecting pathways mediated by the retinoblastoma protein (Rb) and p53 (Labuhn et al., 2001; Ohgaki and Kleihues, 2007). $p16^{INK4A}$ binds to CDK4 and inhibits CDK4/cyclinD1 complex formation, thereby releasing Rb. The free Rb controls G1-S transition by sequestering E2F transcription factors (Sherr and Roberts, 1999; Zhu and Parada, 2002). In normal cells, an autoregulatory feedback loop regulates both the activity of p53 and the expression of mouse double minute 2 (MDM2, in humans: HDM2). The $p14^{ARF}$ gene product binds to HDM2 and inhibits HDM2-mediated p53 degradation and *trans*-activational silencing

of genes related to cellular processes such as the cell cycle, response to DNA damage and cell death (Kamijo et al., 1998; Picksley and Lane, 1993; Pomerantz et al., 1998).

The gene coding for the growth-promoting epidermal growth factor receptor (*EGFR*) is amplified in 40% of primary GBM and frequently contains the mutated variant 3 (EGFRvIII) consisting of a deletion of exons 2-7 (Ekstrand et al., 1994).

Loss of chromosome 10q is the most common genetic alteration associated with GBM. The tumour suppressor *PTEN,* located on 10q23*,* is genetically inactivated in more than 30% of GBM. The protein and lipid phosphatase PTEN negatively regulates the growth and survival pathway PI3K-Akt by acting on its product phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Sansal and Sellers, 2004; Vivanco and Sawyers, 2002). PTEN also has a protein phosphatase activity, which directly or indirectly dephosphorylates the Thr-383 residue. This allows its C2 domain to associate with a signaling complex in the cell membrane and slows cell migration. This protein phosphatase activity is lacking in GBM cells (Merlo and Bettler, 2004; Raftopoulou et al., 2004). PI3KCA has been mutated in GBM (Samuels et al., 2004). The carboxyl-terminal modulator protein (CTMP) is another negative regulatory component controlling PKB activity (Maira et al., 2001) and the corresponding gene is often epigenetically silenced in GBM (Knobbe et al., 2004). The mutational spectrum has nicely been confirmed by a whole genome sequencing approach in GBM (Parsons et al., 2008).

Recent observations support a plastic model of primary GBM development: either a transformation of adult neural stem cells from the sub-ventricular zones of the brain or de-differentiation of mature astrocytes. Glial progenitor cells (GPC) can develop into neural stem cells in response to exogenous fibroblast growth factor 2 (FGF2), while astrocytes with loss of the *INK4A/ARF* locus can be de-differentiated into neural stem cells in response to epidermal growth factor (EGF) signaling. Thereafter, amplification of the EGFR gene or mutations in *PTEN* can lead to primary GBM (Maher et al., 2001; Wechsler-Reya and Scott, 2001; Zhu and Parada, 2002) **(Annex-4)**.

Astrocytomas & Secondary GBM

The presence of some genetic alterations in both low and high-grade astrocytomas indicates that common mutations are involved in early phases of tumour formation (Maher et al., 2001). For example, patients with the Li-Fraumeni syndrome carry a germ line mutation in the *TP53* gene that encodes the p53 protein, a regulator of cell cycle progression and apoptosis. Li-Fraumeni patients are predisposed to the development of tumours of various lineages, including astrocytomas. More than 60% of all grades of astrocytomas show *TP53* mutations (Ohgaki and Kleihues, 2007). The early occurrence of *TP53* mutations points to the need for astrocytoma cells to evade apoptosis, migrate and survive in a non-adequate environment. However, observations in knock-out mice indicate that the loss of *TP53* alone is not sufficient to initiate astrocytoma formation and additional genetic events are required (Maher et al., 2001; Wechsler-Reya and Scott, 2001; Zhu and Parada, 2002).

The growth factor-receptor tyrosine kinases (GF-RTK) transmit growth and survival signals through the effector GTP-binding protein, RAS. The GF-RTK-RAS signaling cascade is frequently targeted in human cancers rendering cancer cells constitutively independent of exogenous growth factors (Kinzler and Vogelstein, 1996). Platelet-derived growth factor (PDGF) and its receptor are both highly expressed in astrocytomas, indicating that the cells establish an autocrine stimulatory loop. Neurofibromatosis type 1 (NF-1) is a familial cancer syndrome in which patients develop multiple CNS and peripheral tumours. The gene *NF1* codes for a protein that shares homology with the GAP family (RAS GTPase-activating protein), a negative regulator of RAS. Loss of both *NF1* copies, as observed in these tumours, leads to activation of RAS-mediated MAPK. Transgenic mice models over-expressing oncogenic *Ras* in astrocytes lead to the development of astrocytoma (Vogel et al., 1999; Zhu et al., 2005).

In the PI3K/PTEN cancer related pathway, mutations in the *PI3KCA* gene have also been detected in glioblastomas and other forms of brain tumours. These mutations lead to increased lipid kinase activity (Samuels et al., 2004). In addition, the tumour

suppressor gene *PTEN* is mutated or deleted only in 4% of the secondary GBM. These mutations lead to increased PKB/Akt activity, promoting growth and survival (Knobbe et al., 2002). These observations support the hypothesis that p53 and the growth factor signaling pathways are involved in the initiation of low-grade astrocytoma development **(Annex-4)**.

Oligodendrogliomas

Oligodendrogliomas, a major type of gliomas, constitute 5% of all primary brain tumours (Kleihues, 2000). They frequently show sensitivity to chemotherapy, especially to PCV (procarbazine, CCNU and vincristine) (Cairncross and Macdonald, 1988). Molecular genetic studies on oligodendrogliomas revealed that allelic loss at chromosome 1p, which is found in 60-80% of tumours and often accompanied with allelic loss at 19q (Smith et al., 1999), was highly associated with good responsiveness for treatment and better prognosis. The remaining 20-30% of tumours without chromosome 1p loss are resistant to therapy (Cairncross et al., 1998; Ino et al., 2001). Unfortunately, the putative tumour suppressor genes at chromosomes 1p and 19q, obvious keys to investigate the molecular features of the tumour cells, are yet to be identified despite intensive investigations. Several attractive candidate tumour suppressor genes on chromosome 1p include *TP73* (Mai et al., 1998), *RAD54* (Bello et al., 2000; Husemann et al., 1999), *CDKN2C* (Bello et al., 2000), *CAMTA1* (Barbashina et al., 2005) and *CHD5* (Bagchi et al., 2007), but their role in oligodendrogliomagenesis has not been established.

1.5 General biology of tumours highlighting malignant gliomas

a. Evasion from apoptosis

The balance between cell growth and programmed cell death is essential for successful embryonic development and maintenance of normal cellular homeostasis in adult organisms. Perturbation of cellular homeostasis can be a primary pathological event that results in disease, such as cancer. Apoptosis is an evolutionarily conserved cell death program that counteracts tumour growth (Jaattela, 2004; MacFarlane and

Williams, 2004). Loss of normal induction of apoptosis results in increased cell population. Programmed cell death manifests in irreversible damage to cellular constituents through caspase activation and loss of mitochondrial integrity. The major regulators of mitochondrial integrity are the BCL-2 family members, which include both anti-apoptotic and pro-apoptotic proteins (Danial and Korsmeyer, 2004).

An important regulator of apoptosis is the nuclear protein p53 which in response to DNA damage (Vogelstein et al., 2000) initiates the transcription of many genes involved in genetic stability, cell-cycle inhibition, apoptosis (el-Deiry et al., 1993; Harper et al., 1993) and integrates numerous signals that are crucial for the determination of cell survival (Lane and Fischer, 2004; Levine et al., 2004). Mutated cells are normally eliminated by apoptosis. However, inactivation of the cell death pathway results in an enhanced intrinsic apoptotic threshold generating resistance to apoptotic stimulus (Cavenee et al., 1991; Van Meir et al., 1995). This is a critical step in the process of transformation of a cell into a slightly hyper-proliferative cell with growth advantage, following the Darwinian principle for tumourigenesis as formulated in the clonal theory of cancer (Nowell, 1976).

In gliomas, this pathway is de-sensitized from death stimuli at different levels. Examples for this are the loss of p53 function, high expression of anti-apoptotic proteins (Bcl-2, Bcl-xL, Mcl-1) that block the activation of pro-apoptotic proteins such as Bax and the activated PKB/Akt, which inactivates pro-apoptotic protein Bad. This contributes to the suppression of apoptosis and increased cell proliferation in gliomas (Austin and Cook, 2005; Krajewski et al., 1997; Vivanco and Sawyers, 2002). Although there is no correlation between *TP53* gene status and GBM patient survival, p53 mutation may contribute to tumourigenesis as well as progression to malignancy (Shiraishi et al., 2002).

b. Unchecked proliferation

Multicellular organisms have a strong intrinsic proliferative potential. The interplay between mitogenic signals and cell cycle control pathway restrains this proliferative capacity to the appropriate time and place. Loss of restraining mechanisms leads to abnormal proliferation and accumulation of clonal cell populations. Tumour cells differ from their normal counterparts in generating their own growth signals by creating a positive feedback loop, either by autocrine or paracrine stimulation, which reduce their dependence on the surrounding tissues (Hanahan and Weinberg, 2000). Illustrative examples are the production of the platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) in glioblastomas (Fontana et al., 1991; Lokker et al., 2002). Many oncogenes act by mimicking normal growth signaling.

In glioblastomas, the epidermal growth factor receptor (EGFR) is highly expressed (Barker et al., 2001). The EGFRvIII mutation lacking the prominent ligand binding domain, renders it constitutively active. This in turn is activating the PI3K/Akt (Vivanco and Sawyers, 2002) and MAPK pathways (Hunter, 2000). Cell cycle regulatory proteins are often disrupted in order to allow proliferation and avoid the block at the G1 checkpoint (Kastan and Bartek, 2004; Molinari, 2000). Changes in the genes encoding cyclins, CDK, CDK inhibitors (Fischer and Gianella-Borradori, 2003; Hunter and Pines, 1994; MacLachlan et al., 1995), and Rb (Knudson et al., 1975) have been observed at high frequency in glioblastomas (Hanahan and Weinberg, 2000). Historically, the Rb gene gave rise to the concept of the two-step model to inactivate a tumour suppressor gene (Knudson, 1996) by loss of heterozygosity (Cavenee et al., 1991).

c. Sustained angiogenesis

Oxygen and nutrients supplied by the vascular system are crucial for cell function and survival. Angiogenesis, the formation of new blood vessels, is a discrete step in tumour progression that is required for expansion of the tumour mass (Bouck,

1996; Hanahan and Folkman, 1996). Astrocytomas are strongly oxygen-dependent. When grade III astrocytomas progress to grade IV, they show features of hypoxic and necrotic palisades (Blouw et al., 2003). Hypoxia is promoting formation of new blood vessels that supply the tumour with the necessary metabolites. Hypoxic response is triggered to a large extent by the hypoxia inducible factor-1 (HIF-1) (Semenza, 2003), which is over-expressed in human glioblastomas. The most prominent target genes of HIF-1 is the vascular endothelial growth factors (VEGF), which is expressed in the perinecrotic palisading cells (Folkman, 1996). VEGF is also produced by tumour-associated cells like endothelial cells and macrophages, as well as by surrounding stromal cells and regulates endothelial cell proliferation and vascular permeability. The earliest stages of angiogenesis are defined by vasodilatation and an increased vascular permeability of pre-existing capillaries or post-capillary venules in response to VEGF. The vascular basement membrane and the extracellular matrix are locally degraded to allow underlying endothelial cells to migrate into the perivascular space and multiply.

Astrocytic tumour cells do not require neo-vascularization. They acquire their nutrients from existing blood vessels without initiating angiogenesis, but by growing along blood vessels of the vascular-rich brain parenchyma (Brat and Van Meir, 2004). However, when they progress into glioblastomas, they become hypoxic and necrotic, partially due to vessel regression and increased tumour cell proliferation. This initiates hypoxia-induced angiogenesis. As soon as these tumours are re-vascularized, they become extremely aggressive. Expression of tenascin-C, an extracellular matrix glycoprotein, has been found in the wall of the blood vessels and in the extra-cellular matrix (ECM) of GBMs (Chiquet-Ehrismann et al., 1986), suggesting a role of this protein in angiogenesis (Zagzag et al., 1995).

d. Migratory and invasive potential of maliganant gliomas

The ability to widely invade normal brain tissue is a key property of the malignant glial cells. Malignant lesions are usually considered to be metastatic; however, brain tumours differ from these by the fact that they rarely spread to sites outside of the CNS (Maher et al., 2001; Merlo, 2003). The progression is a diffuse,

locally invasive growth into the normal brain coupled with an increase in tumour volume. Moreover, tumours may spread along blood vessels, white matter tracts and grey–white matter interfaces. Invasion does not correlate with the grade, since low-grade astrocytomas already extensively invade normal brain tissue. In the adult CNS, neurons, astrocytes and oligodendrocytes are fixed in position and do not migrate. A number of studies indicate that PTEN can regulate cell shape and movement (Merlo and Bettler, 2004). In addition, the focal adhesion kinase (FAK) mediates signal transduction by integrins and by regulating cell adhesion and migration. Interestingly, FAK is over-expressed in primary GBM (Jones et al., 2001a). Over-expression of PTEN inhibits cell spreading and cell migration induced by integrins, whereas reduction of PTEN levels has the opposite effect (Maier et al., 1999). The inhibitory effects of PTEN can be blocked by the over-expression of FAK, which induces extensive reorganization of the actin cytoskeleton, formation of focal adhesions and directional migration (Jones et al., 2001b). Thus, cells lacking functional PTEN would be expected to manifest increased migration and increased tendency to metastasize.

PTEN mutations are found in glioblastomas but rarely in low-grade astrocytic tumours (Knobbe et al., 2002; Sansal and Sellers, 2004). Tenascin-C (TN-C) is an extracellular matrix glycoprotein first identified in gliomas (Bourdon et al., 1983) and high expression is found in grade III and IV astrocytomas (Carnemolla et al., 1999). In some tumour types such as breast cancer, glioma and osteosarcoma, high expression levels of tenascin-C are linked to short patient survival (Tanaka et al., 2000). In many cancers including brain tumours, stromal expression of tenascin-C is increased in higher tumour grades (Leins et al., 2003) and correlates with invasiveness (Orend and Chiquet-Ehrismann, 2006), high vascularisation and a high proliferation index (Godard et al., 2003). In TN-C-deficient mice, oligodendrocyte precursor maturation is accelerated (Garcion et al., 2001). Thus, TN-C promotes cell migration, controls differentiation and is critical for angiogenesis.

1.6 The Notch pathway: role in normal and neo-plastic development in the CNS

a. Discovery of Notch

In *Drosophila*

T.H. Morgan (Morgan, 1917) and O.L. Mohr (Moohr, 1919) discovered the 'Notch' mutation in the fruit fly *Drosophila*, with a phenotype consisting of notches at the wing margin. Further genetic analyses of Notch loss-of-function mutations revealed an embryonic phenotype with an expanded population of neuroblasts at the expense of epidermis cells. These loss-of-function mutations provided the first clue that during neurogenesis, wild-type Notch regulates cell fate decision by preventing ectoderm cells to differentiate into neuroblasts rather than into epidermis. Therefore they qualified as *neurogenic* mutations (Poulson, 1937). Identification of *anti-neurogenic* gain-of-function mutations completed the description of the allelic series of Notch mutations (Brennan et al., 1997; Rebay et al., 1993). In fact, both groups of mutations are dominant and loss or gain of a single genomic copy of the Notch gene is sufficient to perform the hypomorphic and the hypermorphic mutations (Heitzler and Simpson, 1991). Thus, it became clear that the Notch expression level is critical to ensure the subtle balance between neuroblast and epidermal cell fate decision during *Drosophila* development. In addition, the identification of loci that genetically interact by enhancing or suppressing the Notch phenotype such as *DLL* or *Su(H)* further allowed the characterization of genes encoding mediators of Notch signaling (Artavanis-Tsakonas et al., 1999).

Cloning of the gene

Cloning of the *Drosophila Notch* gene revealed an open reading frame encoding a 300-kD type I single-pass transmembrane receptor consisting of 36 epidermal growth factor (EGF)-like tandem repeats and three cysteine-rich Notch/LIN-12 repeats in the extra-cellular domain. The cytpolasmic part contains RAM domain, six tandem ankyrin repeats, a glutamine-rich domain, a proline-, glutamate-, serine-,

threonine-rich (PEST) sequence and a strong transcription activation domain (TAD). A post-translational cleavage of the extra-cellular part at the S1 site occurs within the secretory pathway and a subsequent formation of disulphide bonds between both parts of the Notch receptor chain results in a heterodimeric receptor transported to the cell membrane (Artavanis-Tsakonas et al., 1999; Jarriault et al., 1998).

In vertebrates

The genomes of vertebrate encode four Notch receptor paralogues, (Notch 1-4) with various degrees of similarity with *Drosophila* Notch. Although the overall structures of the four Notch receptors are very similar, they show differences in the extracellular and cytoplasmic parts. The extracellular domains of Notch1 and Notch2 contain 36 EGF-like repeats, while Notch3 and Notch4 have 34 and 29, respectively. All four chains contain three cysteine-rich Notch/LIN-12 repeats. The intracellular parts of all four proteins contain a RAM domain, six-tandem ankyrin repeats, a glutamine-rich domain and a PEST sequence. Notch1 and Notch2 contain a *trans*-activating domain (TAD) domain that is absent in Notch3 and Notch4 (Artavanis-Tsakonas et al., 1999; Radtke and Raj, 2003; Weinmaster, 1997).

b. Notch signaling

Notch signaling defines an evolutionarily highly conserved and ubiquitous intercellular communication mechanism initiated between two neighbouring cells to amplify and consolidate the molecular differences that result in cell fate determination. It acts in two types of local regulation, namely lateral inhibition and inductive signaling essential for organism development (Artavanis-Tsakonas et al., 1995; Artavanis-Tsakonas et al., 1999; Greenwald, 1994). In mammals, Notch receptors are activated by type I transmembrane ligands, known collectively as DSL (Delta, Serrate, and Lag 2) (Artavanis-Tsakonas et al., 1999; Blaumueller and Artavanis-Tsakonas, 1997; Blaumueller et al., 1997). Vertebrate genomes encode five ligands: DLL1, DLL3, DLL4 and JAG1and JAG2. Glycosylation of the receptor as well as the ligands of the EGF repeats by Fringe with glycosyltransferase acivity modulates the receptor-ligand binding interactions and determines which ligand should activate the pathway

(Bruckner et al., 2000; Panin et al., 2002). Following Delta or Jagged binding to the receptor, cleavage at the site S2 releases a membrane-tethered form of the Notch intracellular domain. The latter is a constitutive substrate for S3 cleavage, which releases the soluble Notch intracellular (N-IC) (Artavanis-Tsakonas et al., 1995; Baron, 2003). Then, N-IC translocates to the nucleus where it binds to the transcription factor Su(H)/CSL/RBP-Jκ through the RAM domain and ankyrin repeats. The binding of N-IC recruits histone acetylases and the nuclear protein Mastermind. These events turn the repressor into an activator complex and *trans*-activate target genes such as *hairy / enhancer of Split (HES)-1* or *HES-5* and the *HEY* family of basic Helix-Loop-Helix (HLH) transcription factors (Artavanis-Tsakonas et al., 1999; Baron, 2003; Mumm and Kopan, 2000). HES-1 & HES-5 transcription factors are the negative regulators of neurogenic genes such as those of the *Achaete-Scute* family.

In addition to the canonical pathway, (Ramain et al., 2001) reported an alternative RBP-Jκ-independent pathway signaling through DTX, which represses neural fate in *Drosophila*. The intermediate molecular players of the pathway are not known (Brennan and Gardner, 2002; Martinez Arias et al., 2002). More recently, three new Notch ligands, namley F3/contactin (Hu et al., 2003), NB3 (Cui et al., 2004) and DNER (Eiraku et al., 2005), have been identified. They are signaling through DTX1 during oligodendrocyte maturation, differentiation and Bergmann glia development, respectively.

Moreover, Notch signaling crosstalks with other signaling pathways such as Wnt, TGF-β and JAK-STAT. Notch modulates the Wnt pathway through regulating β-catenin transcriptional activity (Hayward et al., 2005). Wnt regulates Notch signaling by physical interaction with EGF repeats in the extra-cellular part of the receptor (Brennan et al., 1999) and binding of Disheveled in the c-terminal domain of Notch exerts a negative effect (Axelrod et al., 1996). The interaction of the Notch intracellular domain with Smad3 initiates crosstalk between the TGF-β signaling with Notch (Blokzijl et al., 2003). Binding of the Notch target gene HES-1 with Stat3 mediates the crosstalk between Notch and the JAK-STAT signaling pathway (Kamakura et al., 2004). Notch

signaling controls how the cells respond to intrinsic and extrinsic developmental cues that are necessary to unfold the specific developmental program and that affect the implementation of differentiation, proliferation, and apoptotic programs, providing a general developmental tool to influence organ formation (Artavanis-Tsakonas et al., 1999) **(Annex-5)**.

c. Role in CNS development

Among the genes and pathways that regulate development and differentiation of the neural lineage, Notch signaling has been shown to be critical for the maintenance of the neural stem cell (NSC) pool as well as the neuronal and glial differentiation (Gaiano and Fishell, 2002; Lasky and Wu, 2005; Louvi and Artavanis-Tsakonas, 2006). In recent years there has been extensive interest in extending the understanding of the Notch pathway from flies to mammals. This resulted in generation and examination of mouse mutants for Notch receptors, ligands, modulators and effectors (Yoon and Gaiano, 2005). Several studies during the early embryonic stages as well as in the late embryonic and postnatal brain documented the expression of Notch1, Notch2 and Notch3 and their ligands (Del Amo et al., 1992; Higuchi et al., 1995; Lardelli et al., 1994; Lindsell et al., 1996; Weinmaster et al., 1991; Weinmaster et al., 1992). There is some overlapping expression, but also different spatial and temporal patterns, suggesting a different role played by each receptor-ligand combination during neural development (Irvin et al., 2001).

The embryos mutant for *NOTCH1* died at E11, deletion showed precocious neuronal differentiation marked by up-regulation of neuronal markers; *MASH1* and *NeuroD*, defects in somitogenesis, down-regulation of target gene *HES-5* and progenitor pool depletion (low neurosphere frequency) (Conlon et al., 1995; de la Pompa et al., 1997; McCright et al., 2001; Swiatek et al., 1994). This analysis supports the canonical view that Notch signaling is critical for inhibiting aneuronal differentiation and for maintaining a neural progenitor pool. Numerous studies found that rather than simply not inhibiting gliogenesis, Notch signaling actively promotes glial fate such as differentiation into astrocytes (Tanigaki et al., 2001), radial glia in the forebrain and the cerebellum (Gaiano et al., 2000; Yoon et al., 2004), Müller glia in the retina (Furukawa

et al., 2000), and Schwann cells in the neural crest (Morrison et al., 2000).

In contrast, Notch has been found to inhibit the genesis of oligodendroglial cells in the optic nerve (Wang et al., 1998) while promoting the differentiation of neural progenitor cells into oligodendrocytes during interaction with a new family of ligands: F3/contactin and NB-3, through the alternative DTX1 mediated pathway (Cui et al., 2004; Hu et al., 2003). Conditional deletion of *NOTCH1* in the cerebellum leads to premature neuronal differentiation and a subsequent reduction in gliogenesis (Lutolf et al., 2002). This is independent of the role of Notch in the maintenance of undifferentiated neural progenitors.

Notch2 is expressed during brain development in the cerebellum external granule layer and the subventricular zones, where it maintains proliferation and prevents neuronal precursor differentiation (Irvin et al., 2001; Solecki et al., 2001). Notch2 expression in the postnatal brain is restricted to the ventricular germinal zones and dividing immature glial cells (Irvin et al., 2001; Tanaka and Marunouchi, 2003). Unlike *NOTCH1, NOTCH2* mutants undergo widespread cell death in the CNS starting around E9 and there is no change in *HES-5* levels (Hamada et al., 1999). There is no phenotype observed in *NOTCH3* and *NOTCH4* deletion mutant analysis (Krebs et al., 2000; Krebs et al., 2003). Consistent with the *NOTCH1* null phenotype, *DLL1* mutant embryos also showed decreased *HES-5* expression, increased neuronal differentiation and defects in gliogenesis (Grandbarbe et al., 2003; Yun et al., 2002).

Since the Notch signaling cascade is primarily transduced through the transcriptional regulator Cbf1/CSL/RBP-Jκ, Cbf1-/- mutants show altered expression of *HES-5*, *DLL1* and *NeuroD*, suggestive of widespread precocious neuronal differentiation and severe defects in gliogenesis (de la Pompa et al., 1997; Taylor et al., 2007). Mutants for the Notch signaling target gene either *HES-1* (Ishibashi et al., 1995) or *HES-5* resulted in the same phenotype like the receptor and the transcription factor mutants. Double mutants show a far more severe phenotype than *HES-1-/-* and *HES-5-/-* alone, suggesting redundancy such that loss of HES-1 is compensated by HES-5 and vice-versa (Ohtsuka et al., 1999). Presenelin-1 and -2 are part of the gamma-secretase

complex along with nicastrin, Aph1 and Pen-2 that performs S3 cleavage of the Notch receptor. The *PS1-/-* and *PS2-/-* mutant phenotypes during neural development both *in vivo* (Donoviel et al., 1999; Handler et al., 2000) and *in vitro* (Hitoshi et al., 2002) are similar to that found during disruption of positive regulators of Notch. This supports a role of the pathway in neural progenitor maintenance. These works suggest that the role of Notch during vertebrate gliogenesis is more complex than initially taught and also plays a role in neuronal and glial cell differentiation **(Annex-6)**.

d. Role in cancer

Notch signaling impinges on a wide variety of cellular processes in the CNS such as maintenance of stem cells, specification of cell fate, differentiation, proliferation and apoptosis. In line with this, animal models for mutations in the Notch receptor invariably result in developmental abnormalities and thus, human pathologies (Artavanis-Tsakonas et al., 1999). Hence, three functions of Notch are thought to be important in the context of the role in cancer. Notch signaling in the vertebrate nervous system is usually thought to influence the balance between the progenitor cell pool and its differentiating progeny and also to participates in binary cell fate decisions such as glia versus neurons. Finally, it can also induce or enhance terminal differentiation between developmentally related cell types like astrocytes/oligodendrocytes. Therefore, the Notch mediated pathology is a result of abnormality of signaling either during stem cell maintenance, binary cell fate or induction of terminal differentiation (Leong and Karsan, 2006).

The tumourigenic role of Notch receptors in humans was first identified in T-cell acute lymphoblastic leukemia (T-ALL). This is due to the fusion of *NOTCH1* and the *T cell receptor-β chain* (*TCR-β)* loci by the translocation t(7;9) (q34;q34.3). As a result, Notch1-IC is constitutively produced under the control of the *TCR-β* promoter/enhancer (Reynolds et al., 1987). The other T-ALL tumours were shown to have signaling activation either through mutation in the *NOTCH1* gene or inactivation of Sel-10/Fbw-7, which ubiquitinates the Notch1-IC for lysosomal degradation (Malyukova et al., 2007; Weng et al., 2004). Subsequently, the oncogenic form of Notch4 was identified

by a proviral integration experiment using the mouse mammary tumour virus (MMTV) in Czech II mice. Integrated Notch4 forms epithelial tumours (Gallahan and Callahan, 1997). The feline leukemia virus encodes a constitutively active form of Notch2 and forms cat thymic lymphomas in cats (Rohn et al., 1996). An oncogenic function of Notch2 has been reported in human B cell leukemia in which it induces the over expression of the transmembrane glycoprotein CD23, which results in a defect in the initiation of apoptosis (Duechler et al., 2005; Hubmann et al., 2002; Jewell, 2002). The activation of Notch receptors by genomic rearrangement is rare in human tumours, but apparent overexpression of Notch signaling components is common in variety of solid tumours including pancreas (Miyamoto et al., 2003), cervix (Gray et al., 1999), breast (Weijzen et al., 2002) and prostate cancer (Zayzafoon et al., 2004). In breast cancer, the deregulated Notch activity is oncogenic. Notch1 levels are high and 50% of the tumours lost the expression of Numb, the negative regulator of Notch (Pece et al., 2004; Weijzen et al., 2002).

In skin, Notch signaling acts as a tumour suppressor. During skin development, Notch signaling induces the terminal differentiation of keratinocytes by inhibiting Wnt- and Shh-mediated proliferation and inducing the expression of cell cycle inhibitor p21 (Rangarajan et al., 2001). In non-small cell lung cancer (NSCLC), Notch1and Notch2 are frequently expressed, and the *HES-1* level inversely correlates with *ASCL1* (Chen et al., 1997). In small cell lung cancer (SCLC), Notch1 is rarely detectable. Growth is inhibited by high-level overexpression of activated Notch1 & Notch2 through upregulation of p21waf1/cip1 (Sriuranpong et al., 2001; Sriuranpong et al., 2002). Most interestingly, in medulloblastomas Notch1 and Notch2 have opposite effects in a single tumour type. Notch1 is expressed in postmitotic differentiating cells, undetectable in tumour tissue, and it inhibits proliferation upon reexpression. Notch2 was found to be expressed in proliferating cells and is apparently present in tumour lines and associated with shorter patient survival (Fan et al., 2004).

The role of Notch receptors in human gliomas is relatively unknown even though their function during CNS development has started to unravel. Purow et al., (2005) have

found expression of Notch1 and the ligands DLL1 and JAG1 in human glioma primary tissues as well as in established cell lines. Moreover, they also showed that their expression is critical for glioma cell survival and proliferation (Purow et al., 2005). Notch1 promotes neural stem-cell like colonies in human glioma cells *in vitro* (Zhang et al., 2008). In another report, the upregulated expression of *ASCL1* characterizes a progressive type of astrocytoma. There the negative regulator of *ASCL1*, the Notch signaling, seems to be inhibited (Somasundaram et al., 2005). In high-grade gliomas, *PTEN* and *DLL3* expressions suggest that PKB/Akt and Notch signaling are hallmarks of poor prognosis versus better prognosis (Phillips et al., 2006). So, the role of Notch signaling in human tumourigenesis either as an oncogene or a tumour suppressor pathway depends on the cellular context (Radtke and Raj, 2003) **(Annex-7)**.

e. Notch degradation

Small variations in Notch signaling are sufficient to modulate differences in cellular behavior. The pathway is tightly regulated by a variety of molecular mechanisms within and also from outside the signaling pathway at different levels (Schweisguth, 2004). Glycosylation of the receptor on the EGF repeats as well as of the ligands by the glycosyltransferase modulates the receptor-ligand binding interactions and determines which ligand should activate the pathway (Bruckner et al., 2000; Panin et al., 2002). Internalization of membrane receptors and ligands is thought to be associated with signal attenuation. Indeed, endocytosis regulates the steady-state level of receptors, ligands and associated factors at the cell surface. In addition, it can also target receptors for lysosomal degradation (Le Borgne, 2006; Le Borgne et al., 2005). Prior to lysosomal degradation, proteins to be degraded should be tagged by ubiquitination. This process involves the ubiquitin-activating enzyme E1, an ubiquitin-conjugating enzyme of the E2 family and E3-ubiquitin ligases which confer target specificity and associate with both E2 and the substrate to catalyze the transfer of ubiquitin to the substrate (Hershko and Ciechanover, 1998; Hicke, 2001).

Normally, Notch undergoes degradation through two different E3 ubiquitin ligase mediated mechanisms. One is Itch/Su(dx) along with the co-factor Numb which

ubiquitinates the full-length receptor (Cornell et al., 1999; Qiu et al., 2000). The second one is mediated by Sel-10/Fbw-7 which specifically ubiquitinates the N-IC (Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). Recently, DTX, which is known to interact with the intracellular part of the Notch receptor and contains an E3 ubiquitin ligase domain, has been found to degrade Notch together with β-arrestin as an adaptor protein (Mukherjee et al., 2005). Itch/Su(dx) ubiquitinates and regulates plasma membrane associated Notch. Sel-10/Fbw-7 recruits an SCF complex that ubiquitinates nuclear, phosphorylated N-IC, thereby targeting it for degradation by the proteasome. Neur, a RING finger containing E-3 ubiquitin ligase targets DLL for endocytosis and subsequent degradation (Lai et al., 2001; Yeh et al., 2001). LNX ubiquitinates Numb, thereby targeting it for degradation by the proteasome pathway (Nie et al., 2002). In addition to this, receptor-ligand interaction, proteolytic mediated cleavages to activate the receptor and molecular players that transduces the signaling are also regulates the strength of the Notch signaling **(Annex-8)**.

1.7 Key topics

Acquired genetic alterations that cause either activation of oncogenes (Pihan and Doxsey, 2003) or inactivation of tumour-suppressor genes (Herman and Baylin, 2003; Merlo et al., 1995) result in the selection of clones with enhanced growth and progressively change normal cells into neoplastic cells (Nowell, 1976). Such genetic alterations found to be involved in gliomagenesis are targeting genes encoding growth factor receptors (e.g. *EGFR*), components of the cell cycle machinery (*Rb*, *Cdk4*, and the Cdk inhibitor $p16^{INK4a}$), and regulators of apoptosis (*p53*, *HDM2*, $p14^{ARF}$ and *PTEN*) (Maher et al., 2001).

In addition, frequent genetic alterations targeting the chromosomes 1p, 7p, 10p, 10q, 11p, 19q and 22q have been observed in gliomas. Gene(s) located in these chromosomal bands may be responsible for glioma initiation and progression. The patient median survival time differs dramatically between GBM (less than 12 months) and OG (10 years in grade II, 3-4 years in grade III) (Ohgaki et al., 2004; Ohgaki and Kleihues, 2007). Moreover, the combined loss of heterozygosity (LOH) in chromosomes 1p and 19q is highly

prevalent in OG and absent in GBM. This genetic alteration is associated with favorable prognosis and, presumably with a better response to therapy in OG grade III (Cairncross et al., 1998; Ino et al., 2001; Smith et al., 1999). However, the so-called better response could simply be an artifact, since the response criteria have been modified for malignant gliomas, defining stable disease (=no growth) as a response.

Along this line, the recent somatic deletion mapping done in our lab on 26 primary OG and 118 GBM samples, using 43 polymorphic microsatellite markers on chromosome 1 has found that the LOH at *NOTCH2* locus positively predicted patient survival in sub-groups of human glial tumours and the minimally lost areas in OG and GBM converge to *NOTCH2* gene. The rest of the tumour sub-type showed retention at the *NOTCH2* locus and a worse prognosis (Boulay et al., 2007). A major aim of this thesis was to characterize the role of *NOTCH2* in gliomas. To this end, the following aspects were addressed:

a) Is there any gain or loss of function mutation of the *NOTCH2* gene? If so, does it play a role in gliomagenesis?

Approach: Sequencing mutational hotspots in the *NOTCH2* gene as described in *Drosophila* and in human studies.

b) Are the *NOTCH2* gene and its pathway functionaries expressed in glioma primary tumour and cell line samples. If so, what is their role in glioma tumour cell survival, proliferation and migration?

Approach: Studying the expression of *NOTCH2* and pathway functionaries in glioma primary tumour samples and established cell lines using micro-array and western blotting, and by employing pharmacological and molecular level inhibitors to block Notch signaling in glioma tumour cells and study their effect on proliferation and survival.

c). Analyzing the role of Notch2 degradation pathways in gliomas.

Results

2.1 Loss of Notch2 function

The main determinants of prognosis and therapy for highly aggressive and invasive human glial brain tumours are histological classification and WHO grading. The median survival significantly varies between different glioma sub-types. The patient′s median survival is less than 12 months in GBM, while it is 10 years in OG grade II and 3-4 years in anaplastic OG grade III. Loss of heterozygosity (LOH) of the chromosome 1p is highly prevalent in OG and is associated with a favourable prognosis (Ino et al., 2001; Smith et al., 1999). The clinical relevance of loss of heterozygosity of chromosome 1p is not investigated yet in astrocytomas.

We performed a somatic deletion mapping on 26 primary OG and 118 GBM samples, using 43 polymorphic microsatellite markers on chromosome 1 (figure 1). In OG, LOH on chromosome 1p was found in 81% (21/26) of the tumour samples. In contrast, 69% (80/118) of the GBM had retention on 1p, and 31% displayed various deletion patterns with hotspots at markers D1S2845, D1S507, D1S216 and D1S2696. These deletion hotspots were grouped into 10 different haplotypes (figure 1).

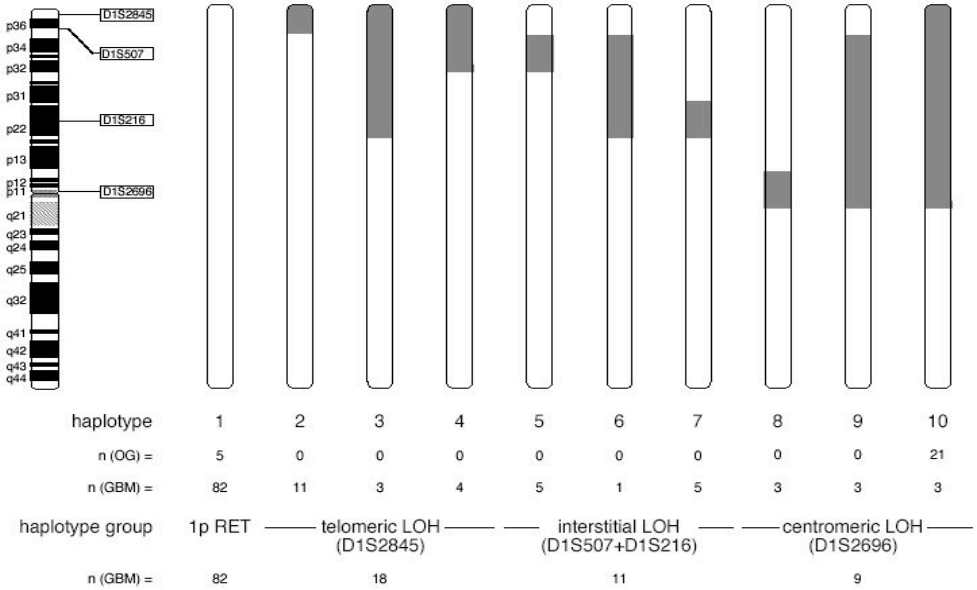


Figure 1. Deletion pattern on chromosome 1p in GBM and OG (Boulay et al., 2007).

Haplotype H1, H2 to H9 and H10 designate no deletion, partial deletion patterns and complete loss of 1p respectively. GBM displayed the entire spectrum of haplotypes H1 to H10 while OG harbored the two haplotypes H1 and H10 (figure 1) (Boulay et al., 2007).

Haplotype H10 significantly differed in survival time compared to H1 (P<0.0007) in OG. Within haplotype H1, OG still had a more favorable prognosis than GBM (OG H1 vs. GBM H1, P<0.02). Even though GBM with and without 1p loss (GBM H1 vs. GBM H2-H10, P<0.3) did not show a difference in survival time, the haplotypes H8-H10, defined by LOH at centromeric marker D1S2696, had a better survival than GBM haplotypes H2-H7, defined by D1S2696 retention (GBM H8-H10 vs. GBM H2-H7, P<0.02) (figure 2).

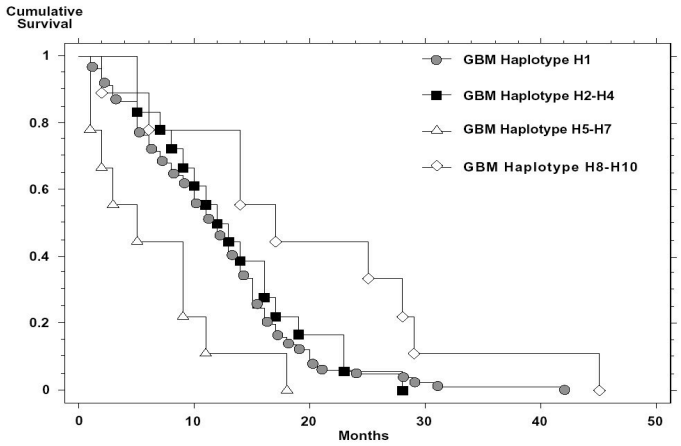


Figure 2. Kaplan-Meier cumulative survival curve for different haplotypes (Boulay et al., 2007).

Based on the telomeric marker D1S2845 status, the haplotypes H2-H7 were further divided: those with retention (GBM H5-H7) showed significantly poorer survival than those with LOH (GBM H2-H4) (GBM H5-H7 vs. GBM H2-H4, P< 0.02). Altogether, GBM with 1p loss were subdivided into 3 categories defined by telomeric (H2-H4, 47%), interstitial (H5-H7, 29%) and centromeric deletions (H8-H10, 24%). GBM with centromeric deletions had the most favorable prognosis (GBM H8-H10 vs. H1, P<0.02), while GBM with interstitial deletions had the worst (GBM H5-H7 vs. H1, P<0.02) and a lower age at diagnosis (50.7). However, survival did not differ between GBM with the prevalent telomeric deletions versus GBM with 1p retention (GBM H2-H4 vs. H1, P<0.5) (figure 2). From this initial mapping, we found that LOH at *NOTCH2* locus

(centromeric) positively predicted patient survival in subgroups of human glial tumours, and that distal LOH was linked with poorer survival (Boulay et al., 2007).

The haplotypes H8-H10 in GBM define a minimally lost area that spans the markers D1S514 and 210WF10 and overlaps the centromeric breakpoint clusters between markers D1S2696 and 210WF10 in OG with haplotype H10 (figure 3). Refinement of deletion mapping in this area has so far been limited by a pericentric duplication on chromosome 1. This duplicates the 5′ part of *NOTCH2* until intron 5 from 1p11 to 1q21.1, which results in the truncated *NOTCH2 N-terminal like (N2N)* gene. Sequence comparison between these genes revealed several single nucleotide polymorphisms (SNP) and microdeletions. Two 5-bp microdeletions from exons 1 and 4 of *N2N* were selected to develop a PCR based assay, the "N2/N2N test", that recognizes either genomic region by size and determines the relative dosage in tumour DNA (Boulay et al., 2007).

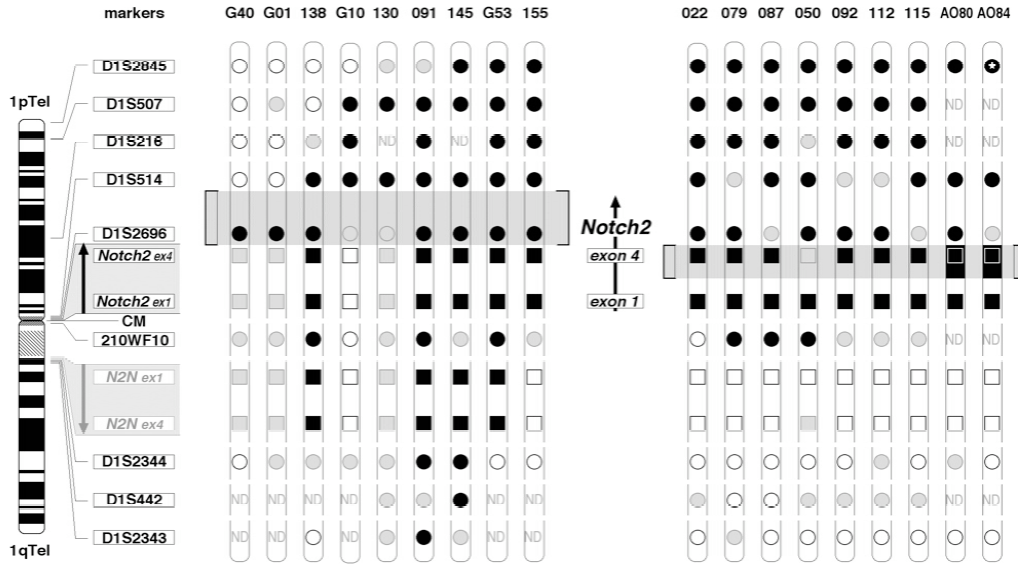


Figure 3. Chromosome 1 pericentric duplication (Boulay et al., 2007).

Calculation of the ratio between *NOTCH2* and *N2N* PCR product levels in DNA from tumour and lymphocytes from the same patient, evaluates the gene copy status at *NOTCH2* relative to *N2N*.

For the OG displaying 1p loss (haplotype H10) (21/21), this test showed imbalance

between the duplicated regions: exon 1 and 4 of *NOTCH2* harbored half the copy number relative to *N2N*, indicating loss of one *NOTCH2* copy. The tumour AO80 and AO84 had almost no PCR product derived from exon 4 of *NOTCH2*. This indicated loss of both *NOTCH2* genomic copies. Real time PCR gave further evidence for the presence of homozygous deletions. This genomic imbalance showed that the breakpoints detected in OG with 1p loss cluster between the duplicated areas (figure 3).

GBM with 1p loss (haplotypes H2-H10) revealed equal copy numbers with the N2/N2N test, and therefore the breakpoints on 1p are telomeric to the pericentric duplication, either towards distal 1p or distal 1q. All analyzed GBM without 1p loss (5/5) also had equal copy numbers of *NOTCH2* and *N2N*. Therefore, OG and GBM display distinct 1p deletion patterns that can be analyzed by the N2/N2N test. The results of the N2/N2N test and fine mapping of centromeric deletions in GBM disclosed a minimal area of loss located between the marker D1S514 and exon 4 of *NOTCH2* (figure 3). These findings support *NOTCH2* as a candidate tumour suppressor gene in OG and in the subgroup of GBM with 1p loss (Boulay et al., 2007).

Next, we decided to study in deep the role of the *NOTCH2* gene in gliomagenesis and to search for loss of function mutation.

Sequencing of *NOTCH2* in glioma primary tissues and in GBM cell lines

In order to find out whether OG (haplotype H10) and the subgroup of GBM (haplotypes H8-H10) (figure 1) have acquired mutations in the remaining copy of *NOTCH2*, the mutational hotspots, as described in *Drosophila* studies (Brennan et al., 1997; Rebay et al., 1993) were sequenced. The hotspots include: the EGF repeats 11 to 14 (blue arrows in figure 4) involved in the interaction with the ligand (ligand-dependent canonical Notch signaling). The green arrows indicate mutations located in the EGF repeats 24 to 29 that impair the interaction with wingless. The intracellular part of Notch2 contains the RAM23 domain, the ankyrin repeats and the trans-activation domain (TAD) (purple arrows in figure 4) that are involved in the interaction with factors and co-factors required for transcription.

Altogether, eight OG and three GBM primary tumour samples (those having LOH at the *NOTCH2* locus) and the cell line Hs683, which expresses OG markers, were sequenced. The Hs683 cell line showed a C-to-A mutation in codon 1711 resulting in the substitution of leucine by methionine.

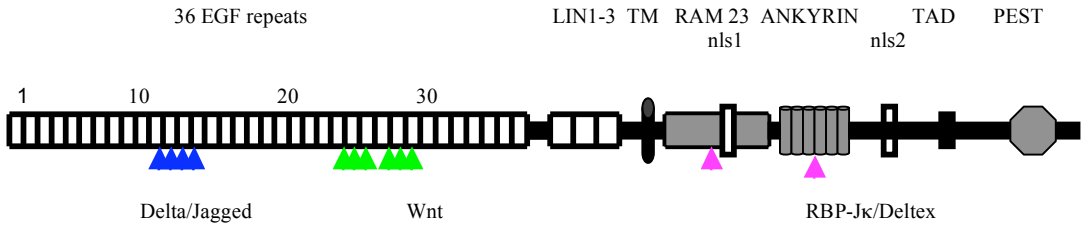


Figure 4. Molecular interactions of Notch2.

Leucine 1711, located within the RBP-Jκ interacting RAM domain, is conserved throughout vertebrate Notch2 proteins as shown in figure 5. A N2-IC *L1711M* mutant expression vector (through site directed mutagenesis in pcDNA3.0 N2-IC) was produced and functionality was checked in the *in vitro* reporter gene assay (figure 31).

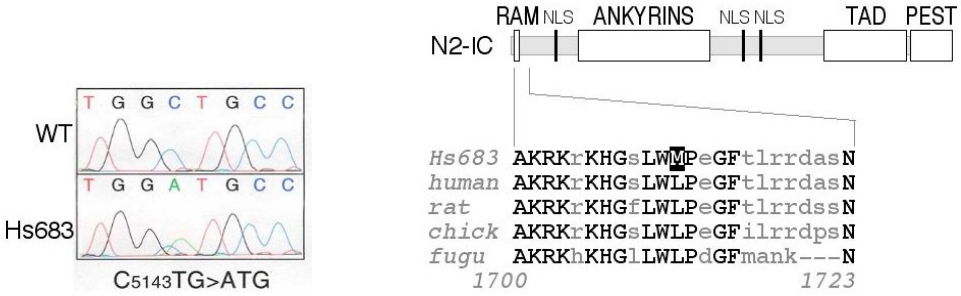


Figure 5. In the Hs683 glioma cell line, sequencing of the NOTCH2 gene shows a mutation targeting the RAM domain through which Notch interacts with transcription factor RBP-Jκ.

We did not find any other mutation in the rest of the samples analyzed, suggesting that mutation is not a main mechanism leading to inactivation of the Notch2 receptor in gliomas. This is in accordance with the previous observation that *NOTCH* genes are rarely mutated in common cancers (Lee et al., 2007). Most probably, *NOTCH2* inactivation is mainly based on homozygous deletions (figure 3) (Boulay et al., 2007).

Conclusion

This initial chromosome 1 deletion mapping identified the minimal area of loss in a subgroup of GBM and in OG, which converges to *NOTCH2*. LOH at the *NOTCH2* locus positively predicted patient survival in subgroups of human glial tumours. Sequencing of primary tumour samples from OG (haplotype H10) and GBM (haplotypes H8-H10) (figure 1) for mutations in the remaining copy of the *NOTCH2* gene has suggested that point mutations may not be the main mechanism of inactivation of Notch2 receptors in gliomas. Since not the entire reading frame of Notch2 was sequenced, we cannot rule out mutations at other regions of the gene. The duplication of the N-terminal of Notch2 (between Notch2 and the pericentromere) does not interfere with the interpretation of sequencing results. Even though Notch2 is a reasonable and attractive candidate suppressor gene, we cannot rule out that another gene in the vicinity of Notch2 is the relevant for gliomagenesis.

2.2 Gain of Notch2 function

Notch expression levels are critical for cell fate determination and have been detected in different transformed cell lines (Miele and Osborne, 1999; Radtke and Raj, 2003). In GBM, it has been shown that the expression of Notch1, the ligands DLL1, JAG1 in primary human glioma tissues as well as in established cell lines is critical for glioma cell survival and proliferation (Purow et al., 2005). Moreover, Notch1 promotes neural stem-cell like colony formation in human glioma cells *in vitro* (Zhang et al., 2008).

We found out that LOH at the *NOTCH2* locus positively predicted patient survival in subgroups of human glial tumours. The other tumour sub-types showed retention (GBM, haplotypes H1-H7) at the *NOTCH2* locus and worse prognosis (figure 1 & 2) (Boulay et al., 2007). Given its critical function in CNS development, Notch may play a role in GBM. Therefore, we decided to study Notch2 expression in different human glial tumours.

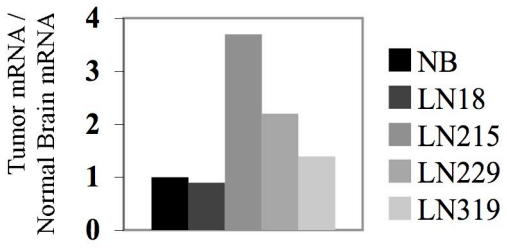
a. Notch2 over-expression in gliomas

NOTCH2 mRNA study on GBM cell lines and brain tumour biopsies

In order to study *NOTCH2* expression in human brain tumours, we analyzed RNA samples extracted from a panel of GBM cell lines and primary brain tumour biopsies using micro-array (in collaboration with Dr. Brian Hemmings at the Friedrich Miescher Iinstitute, Basel, Switzerland). The studied samples include: GBM cell lines (LN18, LN215, LN229 & LN319), 13 primary glioblastomas (GBM-I, grade IV), 3 secondary glioblastomas (GBM-II, grade IV), 8 astrocytomas (A-grade II & III) and 2 control normal brain samples (NB). The probe sets that showed the highest values in the y-axis were selected for further analysis (Morin et al., manuscript in preparation). Changes in the gene expression levels (fold change) for each tumour sample were calculated in comparison to the expression found in normal brain.

GBM cell lines

All four GBM cell lines showed expression of *NOTCH2* mRNA. In the cell lines LN215, LN229 and LN319 the expression were 1.5 to 3 fold higher than in normal brain. Among them, LN215 was the highest expresser, while *NOTCH2* expression was close to that in normal brain in LN18 (figure 6).



Figur 6. Expression of NOTCH2 transcripts in GBM cell lines.

Brain tumour biopsies

All tumours showed higher expression of *NOTCH2* transcripts in comparison to normal brain (figure 7). Astrocytomas were the glioma sub-types expressing the highest level of *NOTCH2* messenger when compared to normal brain (astrocytomas vs. NB, P=0.007). The primary GBMs also showed a statistically significant difference in *NOTCH2* expression (GBM-I vs NB, P=0.022) relative to the control (figure 7).

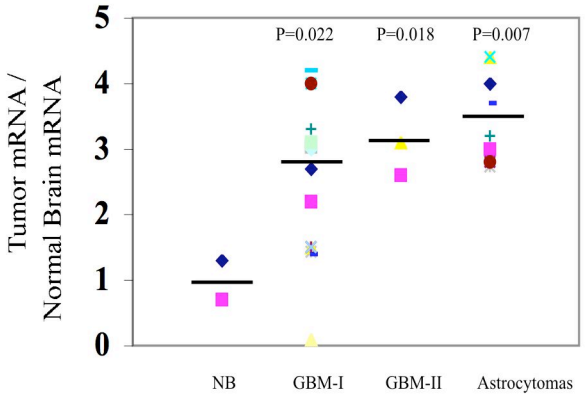


Figure 7. Glial tumours express higher levels of NOTCH2 transcripts than normal brain. The vertical bar is the median value for each group (t-Test: Two-sample assuming unequal variances).

Due to the low occurrence rate, only three secondary GBMs were analyzed. Nevertheless,

the *NOTCH2* transcript levels were significantly higher than in the control (GBM-II vs. NB, P=0.018) (figure 7). The levels of *NOTCH2* expression were significantly higher in astrocytomas than in primary GBM (P=0.02) (figure 7), while no significant difference in *NOTCH2* expression within the tumour types was found (GBM-I vs. GBM-II and GBM-II vs. astrocytomas) (figure 7). This experiment showed that *NOTCH2* is highly expressed in glial brain tumour samples.

Notch2 protein levels in GBM cell lines and brain tumour biopsies

Given the high *NOTCH2* mRNA levels found in glial tumour samples (figure 6 & 7), we analyzed Notch2 protein expression both in cell lines and brain tumour biopsies.

GBM cell lines

Protein extracts from established GBM cell lines were screened for the presence of Notch2 by western blotting. In total, nine tumour cell lines were used, eight of them are derived from glioblastoma and one (Hs683) expresses oligodendroglioma markers (Branle et al., 2002). The Hs683 cell line showed local *NOTCH2* copy loss relative to diploidy (figure 14b) and expressed little Notch2 protein (figure 8). The cell lines U373, U343, LN319 and LN215 expressed higher levels of Notch2, most probably due to genomic amplification (figure 8 & 14b). Lines LN405, LN401 and LN229 expressed high levels of Notch2 without any genomic alteration.

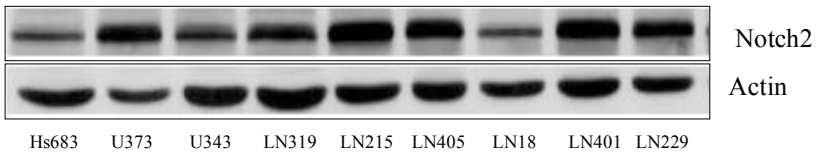


Figure 8. GBM cell lines express high levels of the Notch2 proteins.

Brain tumour biopsies

The human primary brain tumour biopsies were further tested for the presence of the Notch2 protein. The protein extracts from 19 GBM, 7 astrocytomas and a normal brain were analyzed. In this experiment, 63% (12/19) of the GBM and 71% (5/7) of the astrocytoma showed higher Notch2 levels compared to normal brain, consistent with the

micro-array data, where astrocytomas were the highest expressers followed by primary GBM (figure 7 & 9a).

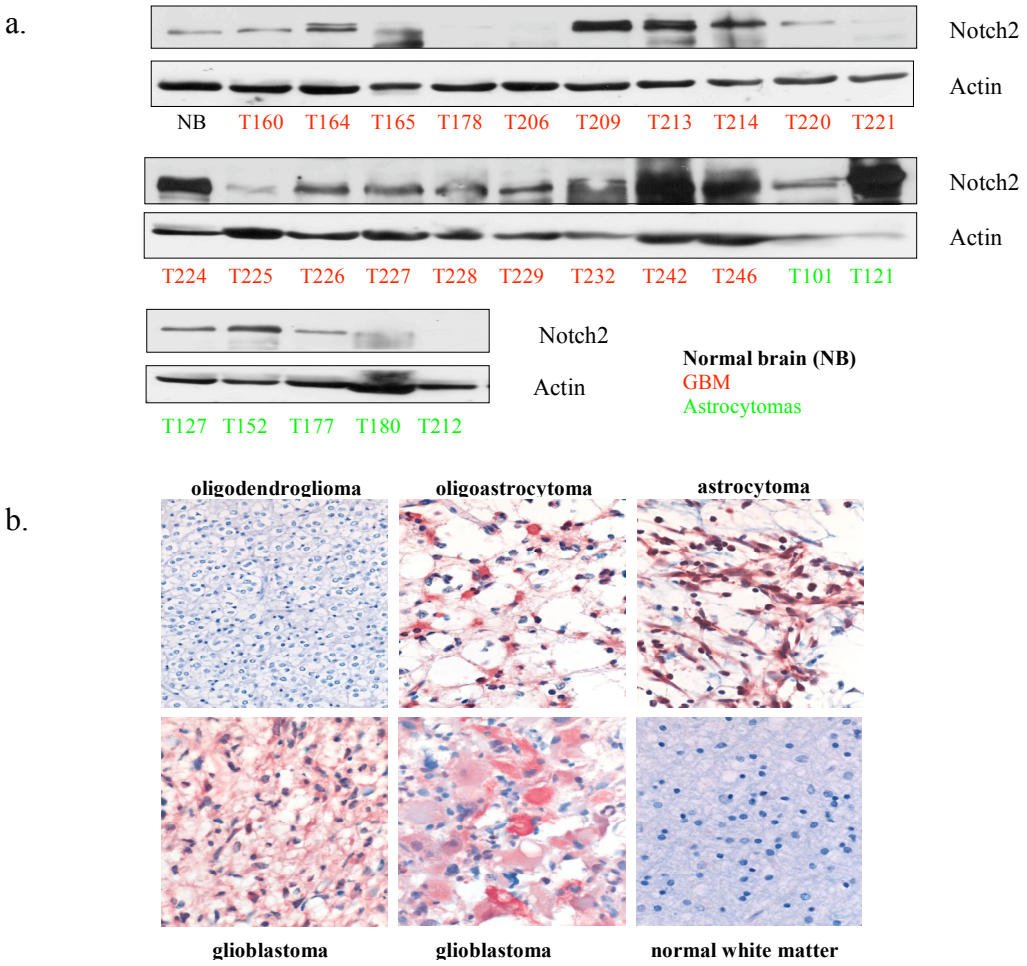


Figure 9a & b. Astrocytic tumours express the Notch2 protein in contrast to normal brain and oligodendrogliomas.

To show the cell specific expression of Notch2, in collaboration with Dr.Markus Tolnay (Institute of Pathology, Basel, Switzerland), immunohistochemical analysis has been performed on human brain tumour biopsies. Glioblastoma, astrocytoma and oligoastrocytoma but not oligodendroglioma biopsies showed Notch2 expression in the tumour cells, while it was absent in the normal brain white matter control (figure 9b). Oligodendrogliomas did not express the Notch2 protein. This was expected since they have LOH at the *NOTCH2* locus (Boulay et al., 2007). Altogether, RNA and protein data from primary tumour biopsies showed that Notch2 is highly expressed in astrocytoma

and GBM, suggesting an active Notch signaling (figure 6, 7, 8, 9a & b).

Expression of Notch2 paralogues

Next, to have a complete overview on the Notch receptors family expression we analyzed the mRNA levels of *NOTCH1* and *NOTCH3* both in cell lines and brain tumour biopsies.

Tumour cell lines

Analysis of the micro-array data showed the expression of the *NOTCH2* paralogues, *NOTCH1 and 3* in the GBM cell lines. In comparison to the normal brain sample, the cell lines LN229 and LN319 showed 1.5 times higher expression of *NOTCH1* and *NOTCH3* respectively (figure 10).

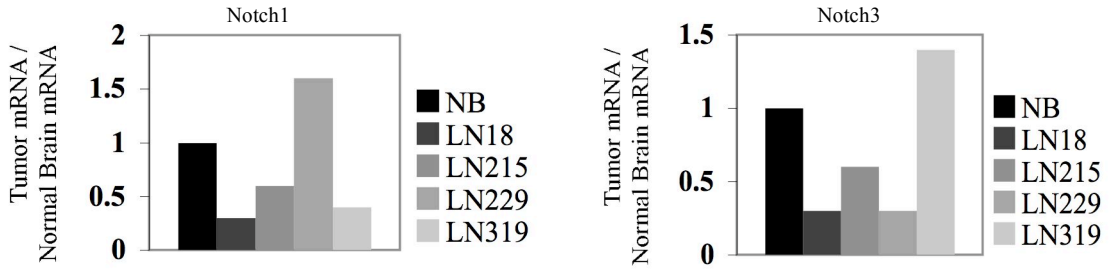


Figure 10. *NOTCH1&3 transcript levels in GBM cell lines.*

Brain tumour biopsies

The same micro-array data set as described above has been analyzed to check the expression of *NOTCH1 and 3* in human glial brain tumour samples. All tumour samples expressed two- to three fold higher *NOTCH1* and two- to four-fold higher *NOTCH3* transcript levels compared to normal brain. Primary GBM and astrocytomas expressed significantly high levels of *NOTCH1* compared to normal brain (P=0.027 & P=0.002, respectively). *NOTCH1* expression in secondary GBM was not different from the normal control (P=1). Within the tumour sub-types, *NOTCH1* expression was significantly different in primary GBM (P=0.03) compared to astrocytomas. No difference has been observed between primary vs secondary GBM and between secondary GBM vs astrocytomas (figure 11).

NOTCH3 expression was significantly higher in primary GBM and in astrocytomas compared to normal brain (P=0.014 & P=0.013, respectively). *NOTCH3* expression in secondary GBM was not different from the normal control (P=0.5).

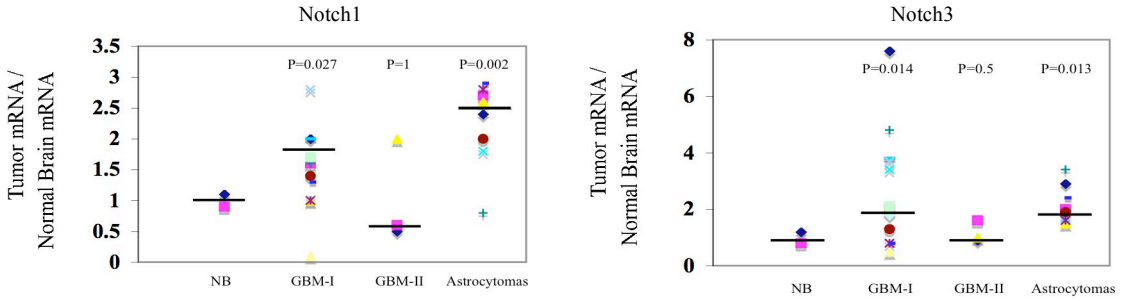


Figure 11. NOTCH1 and NOTCH3 transcript levels in human primary gliomas in comparison to normal brain. The vertical bar is the median value for each group (t-Test: Two-sample assuming unequal variances).

Within the tumour sub-types, *NOTCH3* expression in secondary GBM was significantly different from primary GBM (P=0.02) and astrocytomas (P=0.02). There was no difference between primary GBM vs astrocytomas (P=0.4) (figure 11). These data show an increased *NOTCH1 and NOTCH3* expression, in addition to *NOTCH2*, in the brain tumours.

Given the high Notch1 and Notch3 mRNA levels found in cell lines and glial tumour biopsies, we analyzed their protein expression both in cell lines and brain tumour biopsies.

Tumour cell lines

Protein extracts from established GBM cell lines were screened for the expression of Notch1 and 3 by western blotting. In total, 11 tumour cell lines were used. U373, U343, LN229 and LN319 were the highest expressers of Notch1 while it was low in LN215, LN18 and LN401. We found a high Notch3 expression in all cell lines except in Hs683, the malignant oligodendroglioma line (figure 12). Although the role of Notch1 in proliferation and survival of GBM cells has been studied (Purow et al., 2005), the exact function of Notch2 and Notch3 remains unknown.

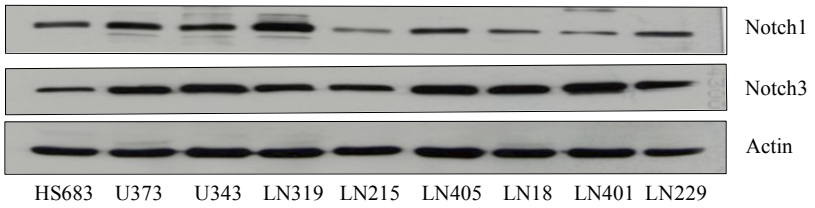


Figure 12. Glial tumour cell lines express higher levels of Notch1 and 3 protein.

Brain tumour biopsies

The human primary brain tumour biopsies were further tested for the presence of Notch1 protein. The protein extracts from 19 GBM, 7 astrocytomas and a normal brain were subjected to western blotting. In this experiment, 73% (12/19) of GBM and 57% (4/7) of astrocytoma showed higher Notch1 levels compared to normal brain and confirmed the micro-array data where primary GBM and astrocytomas showed high Notch 1 expression on the mRNA level (figure 13).

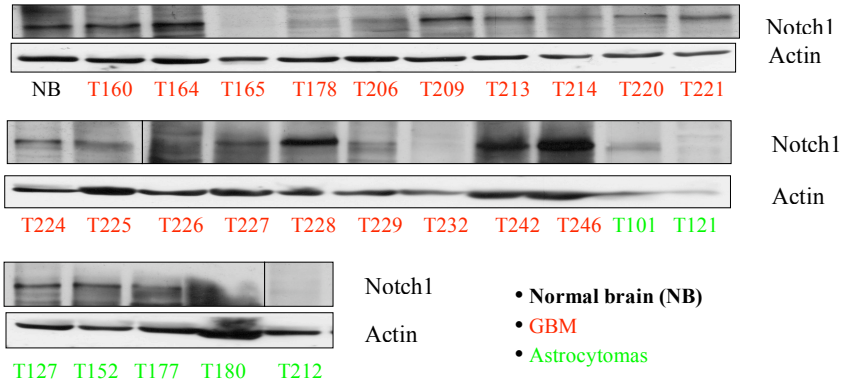


Figure 13. Glial tumours express higher levels of the Notch1 protein.

Basis of Notch2 over-expression: genomic status

The data obtained so far suggest that the human brain tumour and GBM-derived cell lines over-express the Notch2 receptor. In order to elucidate the genetic basis of Notch2 over-expression in glioblastomas, real-time PCR of genomic DNA near the microsatellite marker D1S2696 (figure 14a) located within intron11 of *NOTCH2* was performed in glioma cell lines. Fifty percent (4/8) of the GBM cell lines analyzed harbored a two- to three-fold amplification relative to the diploid status, while only one line (LN18) displayed local haploidy (figure 14b). This prevalence of genomic amplifications at the *NOTCH2* gene is reminiscent of the EGF receptor gene tandem

amplifications observed in 50% of GBM and suggests a gene dosage effect as a possible mechanism of gain of Notch2 activity.

In addition, Hs683, the glioma line expressing OG markers, contains only one *NOTCH2* gene copy per diploid genome, reminiscent of the allelic loss of the *NOTCH2* locus in OGs. Further analysis of these cell lines on Western blots has revealed concordant lower Notch2 protein levels in the cells with genomic deletions (figure 14b), implying that the genomic status at this locus may influence the Notch2 expression level. Similar results were found in *Drosophila* (Heitzler and Simpson, 1991) and in a mouse model where *NOTCH2* haploinsufficiency impairs normal B cell development (Saito et al., 2003).

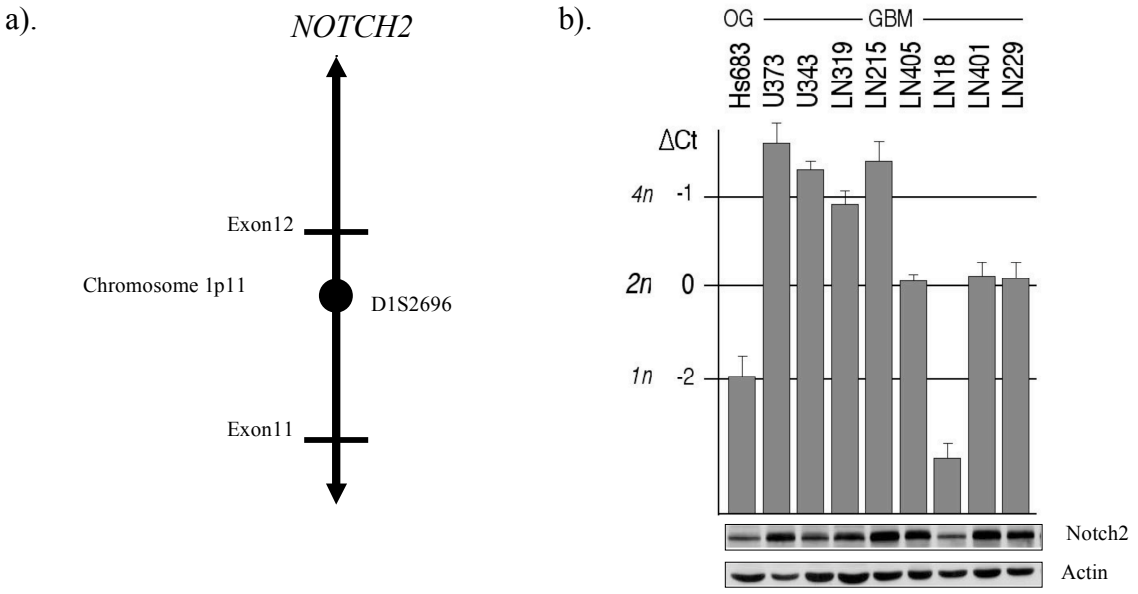


Figure 14. a) Partial map of the NOTCH2 gene b) Correlation of NOTCH2 genomic status and protein expression in GBM cell lines.

Sequencing of *NOTCH2*

In order to find out whether the GBM cell lines acquired gain of function mutations in the *NOTCH2* gene in addition to genomic amplification (figure 14b), sequencing was performed. The sequencing has been carried on the *NOTCH2* mRNA at the mutational hotspots described as gain of function mutations in *Drosophila* (Brennan et al., 1997; Rebay et al., 1993) and in human T-ALL (T-cell lymphoblastic leukemia)

(Weng et al., 2004). These hotspots include the lin-12/Notch repeats (red arrows in figure 15) and the heterodimerization domain (HD) (red arrows in figure 15). Both activate the Notch receptor independent of ligand. The PEST domain in the intracellular part of Notch is involved in the degradation of Notch receptor (blue arrows in figure 15). In figure 15, the green arrow indicates the mutations located in the EGF repeats 24-29 which inactivate the interaction with wingless, resulting in a loss of Notch regulation through Wnt signaling.

Totally, 13 GBM cell lines were sequenced at the three lin-12 domain, the heterodimerization domain and the complete intracellular part of the Notch2 receptor covering the RAM23 domain, the ankyrin repeat, TAD and PEST. We did not find any gain of function mutation in any of the cell lines analyzed. These findings suggest that mutations most probably are not a mechanism of activation of the Notch2 receptor in gliomas.

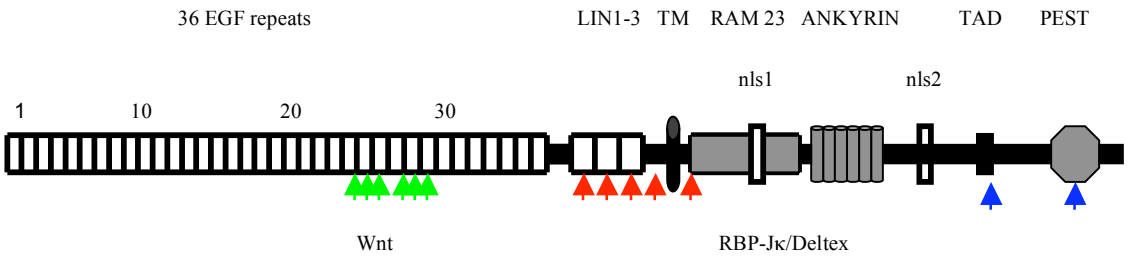


Figure 15. Molecular interactions of Notch2.

Expression of Notch signaling mediators

Since GBM tumour samples highly expressed Notch2 receptors, we next analyzed the expression of Notch ligands that are required to initiate Notch signaling.

Ligands expression

<u>Tumour cell lines</u>

From micro-array data, all four GBM cell lines expressed the ligands *DLL1 and -3, JAG1 and -2*. In comparison to the normal brain sample, the cell lines LN18 and

LN319 expressed nearly 1.5 fold higher levels of *DLL1* (figure 16a). *DLL3 and JAG2* expression was close to normal in all the cell lines. *JAG1* expression was 2 to 3 fold higher in all GBM cell lines analyzed, and LN319 was the highest expresser of them (figure 16b).

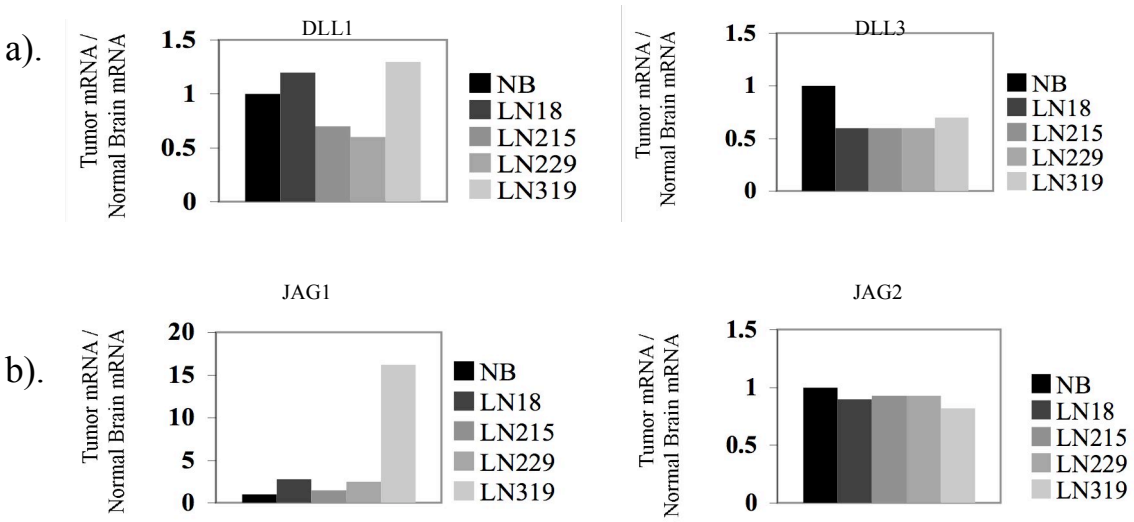


Figure 16. Notch ligand transcript levels in GBM cell lines in comparison to normal brain: a). DLL1 and -3, b). JAG1 and -2.

Brain tumour biopsies

The micro-array data-set described above has also been analyzed for the expression of Notch ligands. In all brain tumours, expression of all four Notch ligands was increased compared to normal brain (figure 17 a & b). *DLL1* was highly expressed in astrocytomas (2-8 fold, P=0.02), while in primary GBM and secondary GBM did not show a significant difference compared to normal brain. Within the tumour subgroups, astrocytomas had significantly higher *DLL1* transcript levels in comparison to primary and secondary GBM (P=0.03). *DLL3*, was increased in primary GBM (2 to 10 fold), secondary GBM (2 to 4 fold), but not significantly compared to normal brain. The astrocytomas had markedly highly high *DLL3* expression (2 to 100 fold, P=0.03) relative to NB. The *DLL3* expression in astrocytomas was significantly different from secondary GBM (P=0.03). Primary GBM had a comparatively higher expression of *DLL1* and *-3* than the secondary GBM. Astrocytomas were the highest expressers of both *DLL1* and *-3* (figure 17a).

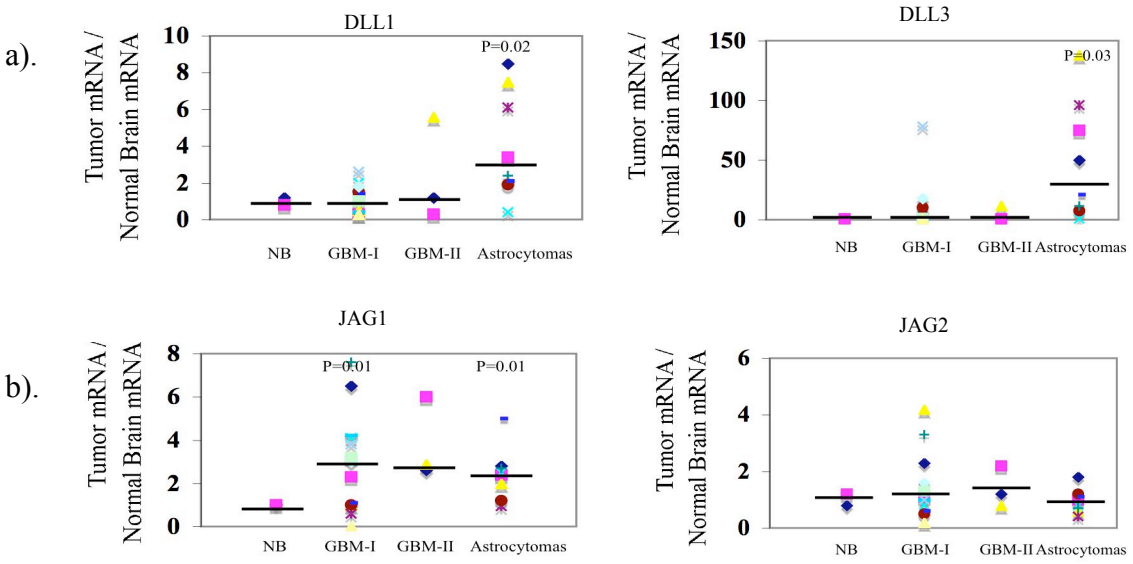


Figure 17. Notch ligand transcript levels in human primary gliomas in comparison to normal brain: a). DLL1 and -3, b). JAG1 and -2. The vertical bar is the median value for each group (t-Test: Two-sample assuming unequal variances).

The serrate family ligands, *JAG1* and *-2* were highly expressed in primary GBM (2 to 4 fold) compared to normal brain. The increase in *JAG1* expression was statistically significant in primary GBM and in astrocytomas (P=0.01). Secondary GBM express relatively low amount of *JAG1* and *-2* compare to primary GBM although still higher than the NB (figure 17b). The *JAG1* and *-2* expression was highly consistent in astrotcytomas.

This experiment identified a differential expression of the Notch ligands within the tumour sub-types. The co-expression of *NOTCH2* either with the *DLL* or *JAG* family ligands suggests an active and ligand specific Notch signaling operative in these tumours.

Given the different ligand mRNA levels found in the GBM cell lines and tumour biopsies, we analyzed their protein expression both in cell lines and brain tumour biopsy.

Tumour cell lines

Next, the established GBM cell lines were analyzed for expression of the Notch ligand proteins: DLL1 and JAG1. We found that DLL1 was present in all the cell

lines with a lower expression in LN405 and Hs683 (figure 18). The expression pattern for JAG1 varied among the cell lines with high expression in U343, U373 and LN71, (figure 18) which were also high Notch2 expressers (figure 8).

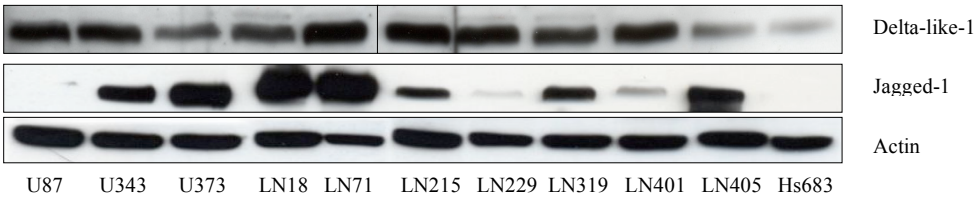


Figure 18. Notch ligand expression in GBM cell lines.

LN18 showed high JAG1 expression (but low Notch2, figure 8) while LN215, LN319 and LN405 showed a moderate expression. Jagged-1 expression was low in LN229 and LN401, and completely absent in U87 and Hs683 (figure 18).

Nuclear signaling

Canonical signaling

The necessary downstream components of Notch signaling are the transcription factor RBP-Jκ and the co-factors MAML1 & 2, which are required to *trans*-activate the Notch canonical target genes. RBP-Jκ regulates transcription by recognizing and binding to the DNA sequence motif "GTGGGAA" located in the promoters of target genes. In the absence of activated Notch, RBP-Jκ represses the transcription of those genes. Activated Notch binds to RBP-Jκ to form a ternary complex with the proteins MAML1 or 2. That turns RBP-Jκ from a repressor into an activator of target gene transcription (Artavanis-Tsakonas et al., 1999; Baron, 2003; Mumm and Kopan, 2000).

To further study the active components of Notch signaling, we analyzed the protein expression of the transcription factor RBP-Jκ in GBM cell lines and brain tumour biopsies.

Tumour cell lines

We found that all the GBM cell lines analyzed express RBP-Jκ. In order to check the *MAML1* and *MAML2* expression, RT-PCR was carried out on the cDNA from cell lines. All the cell lines expressed the co-factor (figure 19).

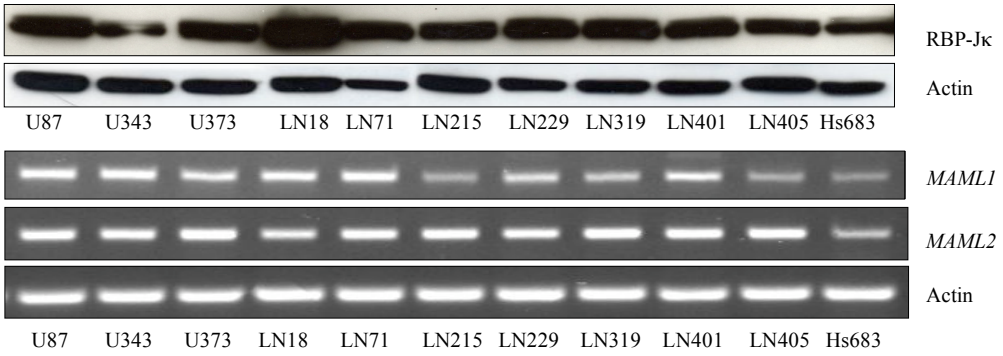


Figure 19. Expression of transcription factor and co-factors required for Notch signaling in GBM cell lines. RBP-Jκ data are from WB; MAML1/2 data are from RT-PCR.

Brain tumour biopsies

To study whether the primary tumour samples express the transcription factor RBP-Jκ, the protein extracts from tumour biopsies were subjected to western blotting. Normal brain did not express RBP-Jκ, while its expression was high in most of the tumour samples analyzed: 74% glioblastomas (14/19) and 71% astrocytomas (5/7) (figure 20).

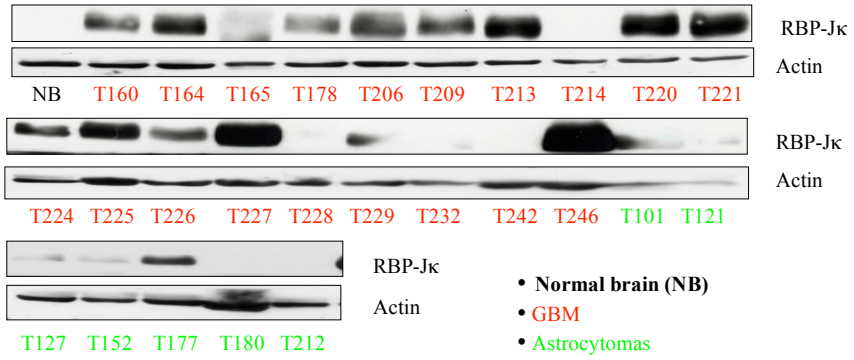


Figure 20. Glial tumours express RBP-Jκ protein.

The presence of transcription factor and co-factors in GBM tumour tissues and in cell lines confirm the possibility of active Notch signaling and provide molecular targets to

interfere with Notch signaling, either by expressing a dominant negative form or by RNA interference.

Non-canonical signaling

In 2001, Ramain *et al*. reported an alternative, RBP-Jκ-independent pathway signaling through DTX. This alternative pathway is able to repress the neural cell fate in *Drosophila*. Nevertheless, the intermediate molecular players of this pathway are not yet known (Brennan and Gardner, 2002; Martinez Arias et al., 2002). Recently, three new ligands of Nocth receptor have been identified in mice: F3/Contactin (Hu et al., 2003), NB3 (Cui et al., 2004) and DNER (Eiraku et al., 2005). They signal through the DTX1 pathway. Moreover it has been reported that in human cervical cancer, DTX1-mediated Notch1 signaling up-regulates the PI3Kinase pathway (Veeraraghavalu et al., 2005), and transgenic expression of *DTX1* inhibits Notch1-mediated mouse mammary tumour (Kiaris et al., 2004). Further, to see whether this alternative pathway is also active in GBM cell lines and brain tumours, we decided to study the DTX mRNA expression.

Tumour cell lines

The cDNA from the GBM cell lines were screened for the expression of *DTX1* using the primer set that covers exon 8-9 at the 3′end of the gene. We found that *DTX1* was expressed, with different levels, in all the cell lines. *DTX1* levels were high in U87, LN215, LN405, moderately expressed in LN18, LN401, Hs683 and comparatively low in LN71 (figure 21).

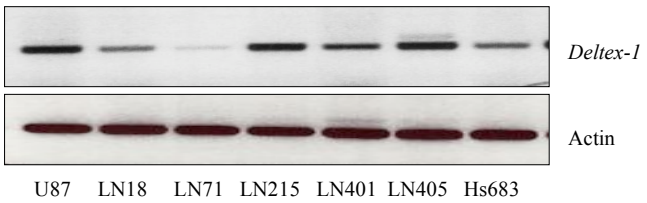


Figure 21. DTX1 expression in human GBM cell lines, using a primer set that covers exon 8-9 at the 3′end of the gene.

Brain tumour biopsies

The micro-array data set had been analyzed to the check the expression of *DTX1* in the primary tumour tissues. All the tumour samples showed high *DTX1* expression when compared to normal brain. *DTX1* mRNA levels were significantly higher in astrocytomas compared to normal brain (P=0.02). In primary and secondary GBM, the higher expression was not significantly different from normal brain.

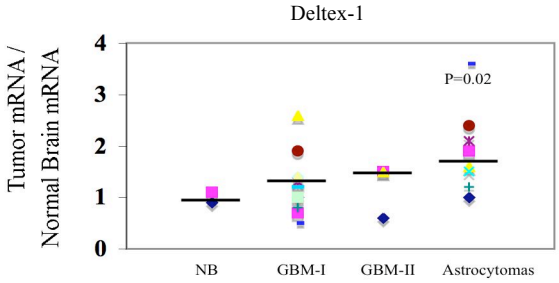


Figure 22. DTX1 expression in human gliomas in comparison to normal brain. The vertical bar is the median value for each group (t-Test: Two-sample assuming unequal variances).

Within the tumour samples, astrocytomas were statistically different from primary GBM (P=0.03) (figure 22). These data suggest that in addition to canonical Notch signaling, Deltex-1 mediated signaling may also be operative in astrocytomas.

Down-stream target gene expression: *HES-1 and HES-5*

HES-1 and *HES-5* are direct targets of Notch signaling (Jarriault et al., 1998) and represent a reliable read-out to monitor Notch signaling. In order to assess the activation status of Notch signaling in brain tumour samples, the levels of *HES-1* and *ESs-5* transcripts were determined.

Tumour cell lines

HES-1 and *-5* expression in the GBM cell lines wa quantified from micro-array data. The results were normalized against the normal brain. In 2/4 GBM cell lines, *HES-1* showed a 2 to 5-fold increased level compared to normal brain. The LN319 cell line was the highest expresser of *HES-1* followed by LN229. *HES-5* was also expressed

in the cell lines analyzed, but of lower levels than normal brain (figure 23).

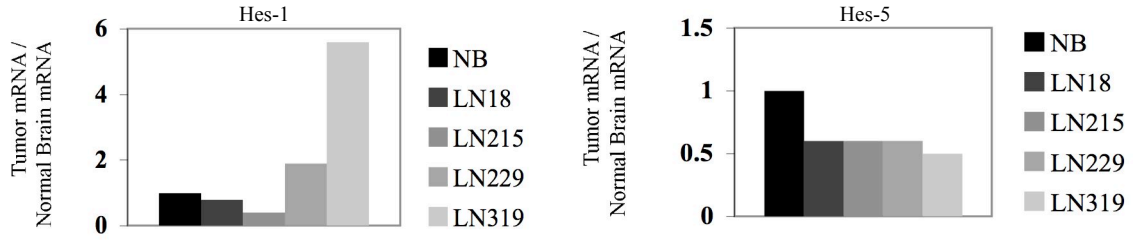


Figure 23. HES-1 and -5 expression in GBM cell line in comparison to normal brain.

Brain tumour biopsies

We analyzed the expression of *HES-1* and *HES-5* transcripts contained in the micro-array data set. When compared to normal brain, the tumours showed different levels of *HES-1* and *HES-5* expression, with a three-fold increase on average (figure 24). All the tumours express the *HES-1* transcript. *HES-1* expression was high in primary GBM while it was very dispersed in secondary GBM and astrocytomas. *HES-5* expression was detectable in most of the tumours screened. The expression levels were less dispersed, and astrocytomas showed the highest (2 to 3 fold) amount compared to primary and secondary GBM.

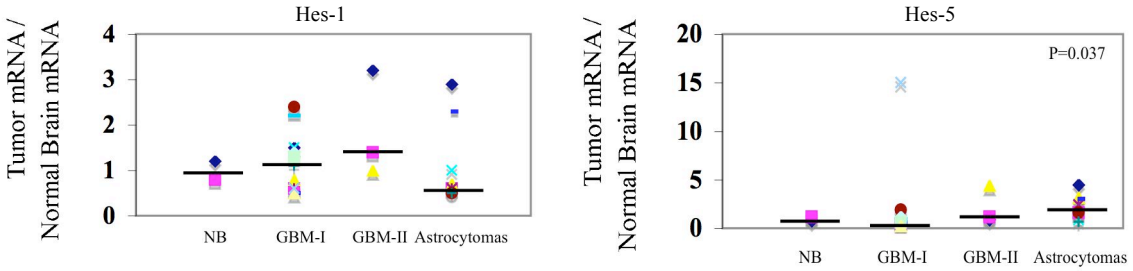


Figure 24. Notch canonical signaling target genes HES-1 and HES-5 expression in gliomas in comparison to normal brain. The vertical bar is the median value for each group (t-Test: Two-sample assuming unequal variances).

These results suggest active Notch signaling in brain tumours, where each tumour subgroup may have a different transcriptional regulation of target genes such as *HES-1* or *HES-5* (figure 24).

Correlation of Notch receptors and target gene expression

The correlation study between Notch receptors and their target gene expression was performed using the micro-array data, to find out which Notch receptor mediates the signaling. We found that in primary glioblastomas, the expression of *NOTCH2 and NOTCH1* significantly correlated with the expression of the target genes *HES-1 and HES-5* respectively (P=0.0001 and P=0.022) (figure 25). In secondary glioblastomas, because of small sample size, correlation analysis not been carried out. In astrocytoma, *HES-1* expression was weakly correlated with *NOTCH1, NOTCH3* and *NOTCH2* expression (not very significant), while *HES-5* correlated moderately with *NOTCH1* and weakly with *NOTCH2* (figure 25). In addition, *NOTCH2/HES-1* correlation was the most significant among the other Notch family members, suggesting that triggering of Notch signaling in GBM is most likely driven by Notch2.

Samples	Genes	*HES-1*		*HES-5*	
		Correlation coefficient*	P-Value	Correlation coefficient*	P-Value
GBM I	*NOTCH1*	0.2965931	0.3251	0.6245946	0.0225
	NOTCH2	0.8707052	0.0001	-0.1794646	0.5574
	NOTCH3	0.3121279	0.2992	0.1670098	0.5855
Astrocytomas	*NOTCH1*	0.3230883	0.435	0.6271685	0.096
	NOTCH2	0.4254356	0.2933	0.3366512	0.4149
	NOTCH3	0.3858183	0.3452	0.0176438	0.9669

Figure 25. Correlation of the relative expression of NOTCH1,2,3 and their target genes HES-1 and -5 in glial brain tumour samples in comparison to normal brain. * Pearson's correlation.

Conclusion

The results obtained from primary tumour samples and established cell lines suggested that *NOTCH2*, its paralogues and their signaling pathway components are highly expressed in these tumour samples compared to normal brain and supported an active and operative Notch signaling in these tumour samples. Each tumour subgroup may have a preferential pattern of expression of *NOTCH2*, ligands and of the transcriptional regulation of target genes such as *HES-1* or *HES-5.* Moreover, the high correlation between *NOTCH2* and *HES-1* expression suggests an oncogenic function of Notch2 in gliomas.

b. Notch and GBM cell invasiveness

We hypothesize that Notch2 is an oncogenic protein, which is over-expressed in GBM primary tumour samples as well as in established cell lines. Some GBM cell lines have shown genomic amplification at the *NOTCH2* locus and higher expression of the Notch2 protein (figure 14b). Activated Notch signaling is known to transform various cell types (Radtke and Raj, 2003); nevertheless the pathway that drives tumourigenesis downstream of Notch signaling is relatively unknown. TN-C is an extracellular matrix glycoprotein (Bourdon et al., 1983) and is a marker for gliomas (Carnemolla et al., 1999). High TN-C expression is associated with a poor patient survival (Tanaka et al., 2000) promotes cell migration, controls differentiation and is critical for angiogenesis (Orend and Chiquet-Ehrismann, 2006). Since Notch2 and TN-C are over-expressed in gliomas, an initial assessment of the parallel expression of these two onco-proteins has shown a striking association. This has impelled us to analyze whether TN-C is the Notch signaling target gene in gliomas.

Co-expression of Notch2 and TN-C in GBM cell lines and in primary tumour samples

Tumour cell lines

The protein extracts from established GBM cell lines were checked for the expression of Notch2 and TN-C. All cell lines except Hs683 and LN18 showed high expression of Notch2 and TN-C (figure 26).

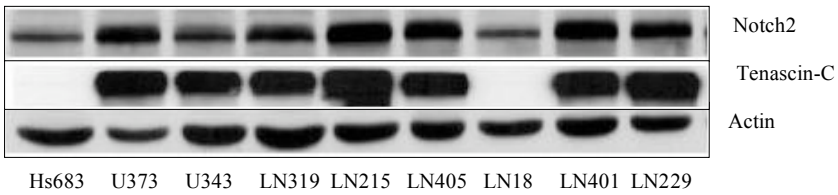


Figure 26. Expression of Notch2 and TN-C in GBM cell lines (western blot).

The cell lines U373, U343, LN319 and LN215 that showed amplification at the *NOTCH2* locus (figure 14b) also expressed high levels of Notch2 and TN-C proteins (figure 26). LN405, LN401 and LN229 were diploid for the *NOTCH2* locus and expressed high

levels of the Notch2 and TN-C proteins as well. However, the cell lines Hs683 and LN18 that displayed haploidy at the *NOTCH2* locus (figure 14b), were low expressers of Notch2 (figure 26) and showed no detectable TN-C expression (figure 26). These cell lines were also expressing the ligands, transcription factor RBP-Jκ and co-factors required to initiate and transmit the Notch signaling (figure 18 & 19). This experiment has shown that expression of Notch2 and TN-C correlates in the GBM cell lines. Both Notch2 and TN-C are oncoproteins implicated in cancer, and the co-expression of these two proteins in GBM indicates a potential molecular link between these two genes.

Brain tumour biopsies

In order to extend and confirm the observation made in GBM cell lines, Notch2 and TN-C protein expression was screened in samples prepared from 19 primary GBM tumours, four oligodendrogliomas (those had LOH at the *NOTCH2* locus) along with normal brain as a control. Most of the GBM tumours analyzed were positive for the two proteins (figure 27).

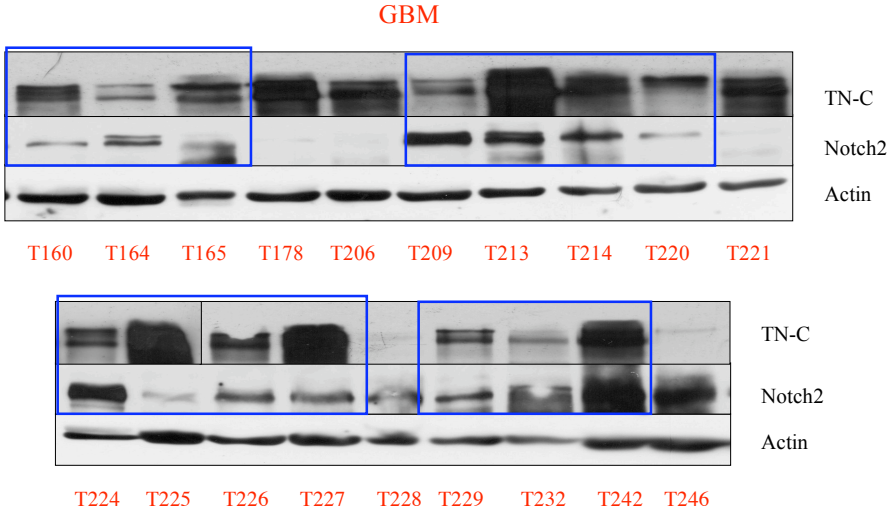


Figure 27. Expression of Notch2 *and* TN-C *in GBM tumour samples. The Boxes in blue show the tumours that co-express* Notch2 *and* TN-C.

TN-C protein expression was found in 90 % (17/19) of the GBM while 80 % (15/19) of the tumours expressed the Notch2 protein. The co-expression of Notch2 and TN-C was found in 60 % (12/19) of the tumours. Notch2 and TN-C expression in oligodendrogliomas was less than or close to normal brain (figure 28).

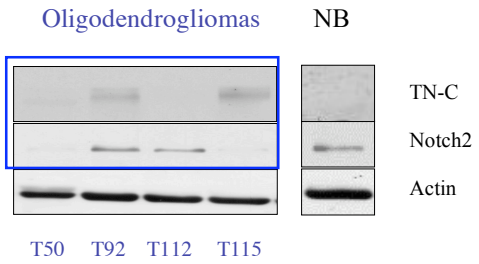


Figure 28. Expression of Notch2 *and* TN-C *in oligodendrogliomas (those with* LOH *at the NOTCH2 locus) and normal brain.*

Further, an immunohistochemical experiment was carried out on the human brain tumour biopsies in collaboration with Dr.Markus Tolnay (Institute of Pathology, Basel, Switzerland). Analysis on glioblastoma, oligodendroglioma and normal brain samples showed that Notch2 and TN-C were highly expressed in the GBM samples while it was undetecable in oligodendrogliomas and normal white matter (figure 29).

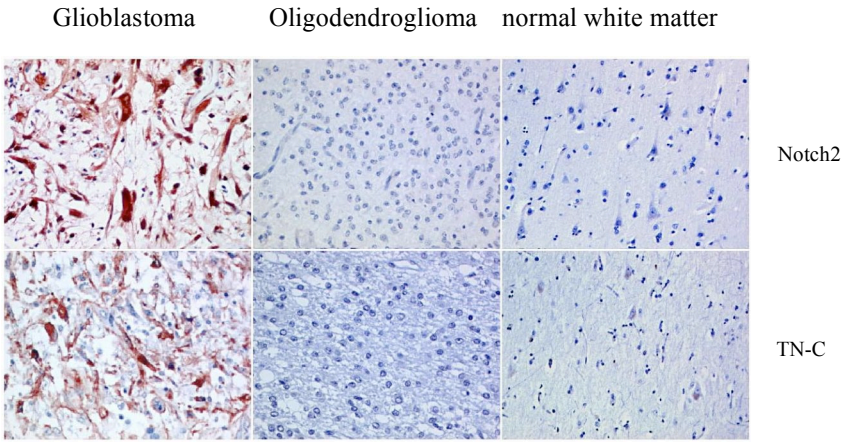


Figure 29. Immnuohistochemical analsis of GBM, OG and normal white matter for Notch2 and TN-C expression.

This experiment shows that Notch2 and TN-C were co-expressed in glioblastomas while both were absent in oligodendrogliomas and normal brain. Notch2 and TN-C are onco-proteins implicated in cancer, and co-expression of these two proteins in GBM primary tumour biopsies indicates a potential molecular link between these two genes.

RBP-Jκ binding motif in TN-C promoter

Notch2 signaling requires that upon the ligand-dependent activation of proteolytic cleavages, the intracellular domain of Notch is released from the membrane and translocates to the nucleus to trans-activate target genes such as *HES-1* or *HES-5*. Notch2 acts as a trans-activator of gene transcription in association with the DNA-binding transcription factor CSL/RBP-Jκ. This has suggested the hypothesis that Notch2 together with RBP-Jκ may activate *TN-C* gene transcription. Based on this hypothesis we speculate that the *TN-C* gene promoter contains a *cis*-acting element responsive to RBP-Jκ. Such a sequence motif has been described to be recognized by and to respond to RBP-Jκ activation: GTGGGAA (Artavanis-Tsakonas et al., 1999). Scanning for this motif in the vicinity of the *TN-C* gene transcription initiation site (in collaboration with Dr. JL Boulay) revealed the existence of a potential RBP-Jκ responsive element located at -80 with respect to the *TN-C* gene transcription initiation site.

```
                                  RBPJk                                                                TATA
hHES5 cCggccAtTggCcg---cggAgtGTGGGAAcGGccGcggcGcccGgactcCagGcgCCa----gGccGccGc-cCgCgccTATATAggGcgtcggcGcgcggGgccggtGt
mHES5 gCcgccAtTggCcg---ccgAgtGTGGGAAcGGccGcggcGcccGgacccCagGcgCCg----gGccGctGc-cCgCgccTATATAggGctggggtGc-tggGgccag-Gt
hHES1 cCtcccAtTggCtgaaagttActGTGGGAAaGaaaGtttgGgaaGtttcaCacGagCCgttcgcGt-GcaGt-gCcCagaTATATAtaGa--ggccGcca-gGgcctagGg
mHES1 cCtcccAtTggCtgaaagttActGTGGGAAaGaaaGtttgGgaaGtttcaCacGagCCgttcgcGt-GcaGt--CcCagaTATATAtaGa--ggccGcca-gGgcctgcGg
hTnC  cCtttaAtTcgCcaactgaaAaaGTGGGAAaGgatGtctgG-agGcgaggC--GtcCCattacaGagGaaGgagCtC-gcTATATA-aGc----caGccaaaGttggctGc
mTnC  cCtttaAcTcaCcaactgåaAaaGTGGGAAaGgatGtctgG-agGcgaggC--GtcCCattacaGagGaaGgagCtC-ccTATATA-aGc----ctGccaaaGctggctGc
```

Figure 30. Alignment of the canonical Notch target genes and the tenascin-C, promoter sequences.

Interestingly, the alignment of 100 bp of the promoter sequences of the established human and murine Notch target genes *HES-1* and *HES-5* with those of human and murine *TN-C* revealed two highly conserved regions: the TATA box, located at -30 bp, and the RBP-Jκ binding motif GTGGGAA located at -80 (figure 30). The conservation in sequence and position of these two elements supports the possibility that RBP-Jκ may indeed bind to the TN-C promoter and promote its transcription under the control of Notch signaling. Together with our observation that Notch2 is the most likely Notch protein, which activates Notch signaling in GBM, we hypothesized that Notch2 signaling *trans*-activates *TN-C* in GBM.

The RBP-Jκ binding motif a functional element of the *TN-C* promoter

To establish the functionality of the RBP-Jκ binding motif identified in the *TN-C* promoter (figure 30), a series of reporter gene assays in the Hs683 cell line was carried out by Martin Degen in Prof. Ruth Chiquet's lab at the FMI in collaboration with our lab. This series of reporter gene assays consisted of co-transfection of plasmids expressing activated Notch constructs together with plasmids expressing the luciferase reporter gene (driven by the proximal 100 base pairs of the *TN-C* promoter that contain the RBP-Jκ binding motif) into a glioma cell line. The initial reporter gene assay experiments were conducted in cell line LN319 with a high TN-C expression, and the results were reproducible.

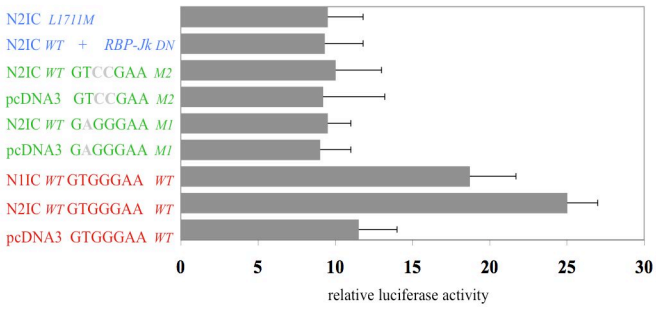


Figure 31. TN-C- promoter reporter gene assay.

Co-expression of either *Notch1-IC* or *Notch2-IC* and a luciferase reporter gene driven by 100 base pairs of wild-type *TN-C* promoter resulted in a 2-fold induction of luciferase reporter activity compared to the pcDNA vector control (figure 31). Introduction of *cis* mutations described to prevent RBP-Jκ binding in the TnC promoter (Tamura et al., 1995) abrogated Notch-dependent induction of luciferase activity (figure 31). Introduction of the *trans* mutation L1711M found in Hs683 *Notch2-IC*, or of the dominant negative RBP-Jκ (Chung et al., 1994) no longer allowed Notch-dependent induction of luciferase activity (figure 31). Altogether, the expression of activated Notch receptor, mutation in the *cis*-element and expression of the dominant negative mutants of *trans*-acting factors have proven that the *TN-C* promoter contains a functional RBP-Jκ responsive element that is regulated by canonical Notch signaling.

Sequencing of the *Tenascin-C* promoter in LN18 and Hs683

Although the reporter gene assay on the *TN-C* promoter was performed in Hs683 cells, these cells express basically no tenascin-C (figure 26). To verify that the absence of tenacin-C protein in Hs683 and also in LN18 is not due to a mutation in the *TN-C* promoter (~300bp) and more precisely in the RBP-Jκ binding motif, the *TN-C* promoter was sequenced. We did not find mutations in the RBP-Jκ binding motif (figure 32). This has suggested that the absence of TN-C protein in Hs683 and LN18 is not related to RBP-Jκ binding motif in the Tenascin-C gene.

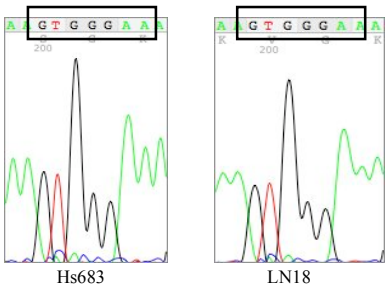


Figure 32. Sequencing of Tn-C promoter in Hs683 and LN18 GBM cell lines.

Abrogation of Notch signaling results in down regulation of TN-C

<u>The Notch2-TN-C link in the GBM cell line LN319</u>

In order to show that abrogating Notch signaling down-regulates the TN-C expression, the GBM cell line LN319, one of the highest expressers of Notch2 and TN-C, was stably transfected with a plasmid expressing a dominant negative (DN) mutant of the Notch signaling mediator MAML1. This mutation consists of the deletion of the C-terminal domain of the MAML1 protein such that it competes with wild-type MAML1 in a dominant-negative way (Weng et al., 2003).

The protein extracts as well as RNAs from the stably transfected cell lines mentioned above and from the parental control cell line were analyzed for the expression of TN-C. MAML1 (DN) over-expression resulted in a decreased expression of TN-C protein and mRNA (figure 33 a & b). Down-regulation of TN-C was dramatic in the MAML-1 (DN) mutant expressing line. This experiment has supported that TN-C expression is induced

by Notch signaling in GBM through the canonical pathway, and that *TN-C* carries a functional RBP-Jκ binding motif.

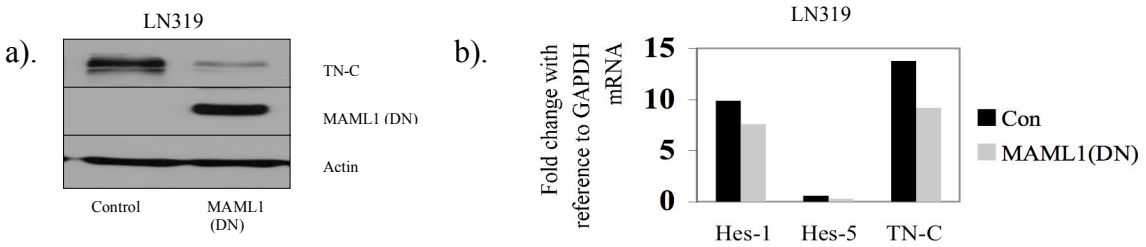


Figure 33. Abrogation of Notch signaling in the LN319 cell line by dominant-negative MAML1: a) down-regulation of Tn-C expression (western blot); b) down-regulation of the canonical Notch target genes: transcripts of Hes-1, Hes-5 and TN-C (Real time PCR).

<u>The Notch2-TN-C link in HEK293 cells</u>

To independently verify that TN-C expression regulated by Notch2 at the transcriptional level is not specific to certain cell types, HEK293 cells stably expressing the Notch2-IC were stably co-expressed with the dominant negative co-factor MAML1 or shRNA against RBP-Jκ. The RNAs from the three resulting stable cell lines were analyzed for the expression of *HES-1* and *TN-C*.

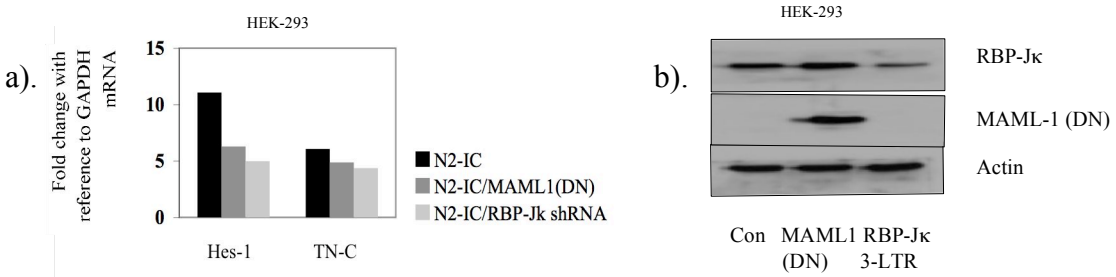


Figure 34. Abrogation of Notch signaling and down-regulation of TN-C expression in HEK293-Notch2-IC cells. a).Real time PCR, b).Western blot.

Expression of the dominant-negative MAML1 as well as down-regulation of RBP-Jκ resulted in down-regulation of the *TN-C* transcript (figure 34a). This experiment has confirmed the results from the GBM cell lines that Notch canonical signaling regulates *TN-C* at the transcriptional level, and that is independent of specific cell type.

Down-regulation of TN-C inhibits the migration of GBM cell line

Next, to check whether down-regulation of TN-C in GBM cell lines interferes with migration capacity, the parental LN319 and the LN319 line stably expressing the MAML1 (DN) mutant were subjected to a trans-well migration assay. Compared to the parental line, the MAML-1 (DN) mutant expressing cell line showed a reduced migration rate (figure 35a). This experiment has shown that canonical Notch signaling acts as a regulator of GBM cell migration. Inhibition of canonical Notch signaling using γ-secretase is also inhibited the migration of LN319 cell line (figure 35b).

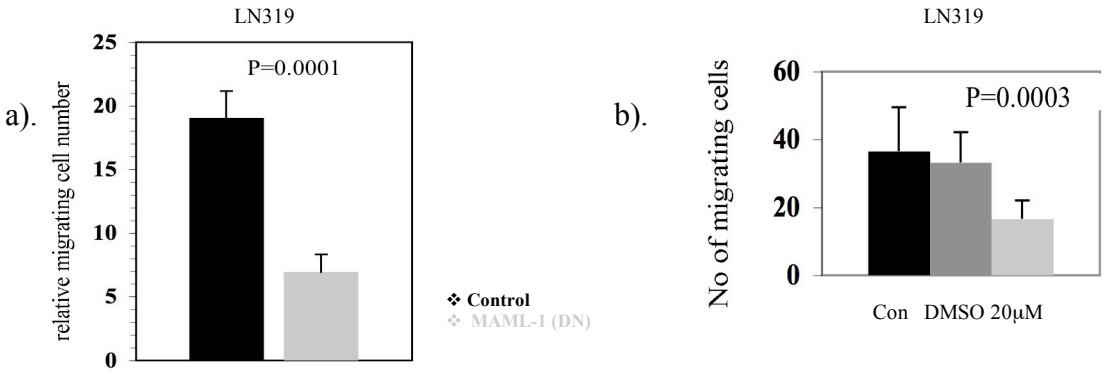


Figure 35. Blocking of Notch signaling inhibits the migration of GBM cell line. a). dominant-negative MAML1, b). γ-secretase inhibitor DAPT at 20μM concentration for three days (t-Test: Two-sample assuming unequal variances).

Conclusion

We found the co-expression of the two oncogenic proteins Notch2 and Tn-C in glioma primary tumour samples and in a GBM cell line. Furthermore, we could prove that Notch signaling can *trans*-activate the *TN-C* gene and that the molecular inactivation of the Notch signaling abrogates the TN-C expression and inhibits GBM cell migration. From these data, we can conclude that Notch signaling in GBM induces TN-C-dependent cell invasiveness, thereby providing a mechanism through which oncogenic Notch2 promotes the neoplastic phenotype.

c. Anti-apoptotic function of Notch2

In the previous sections, the over-expression of Notch2 and its pathway components in the GBM primary tumour samples as well as in established cell lines have been described. Furthermore, the role of Notch2 in inducing tenascin-C (TN-C) expression and migration of the GBM cells has been elucidated. Developmental studies have shown that Notch signaling is important for the maintenance of the stemness character, proliferation, survival and differentiation of the neural stem cells (Hitoshi et al., 2002). Notch1 signaling in neural stem cells is known to promote cell survival independent from the canonical Notch signaling pathway, by inducing anti-apoptotic proteins Bcl-2 and Mcl-1 (Oishi et al., 2004). Several lines of evidence suggest that alterations of Notch signaling contribute to tumourigenesis, and firmly establish that Notch expression and signaling are altered in spontaneous tumours and in tumour models (see introduction). The inability to undergo apoptosis through physiological mechanisms and resistance to therapeutically induced apoptosis are well-recognized features of the transformed phenotype in many human malignancies. Therefore, we decided to study the role of Notch2 in regulating apoptosis in brain tumour cells.

Notch2 induces anti-apoptotic proteins: Bcl-2 & Mcl-1 expression

Notch2 expression protects human B-cell leukemic cells from cell death (Duechler et al., 2005; Hubmann et al., 2002), and Notch2 inactivation in a mouse model leads to widespread cell death in the CNS (Hamada et al., 1999). Moreover, Notch1 induces the anti-apoptotic proteins Bcl-2 and Mcl-1 to promote neural stem cell survival independent of the canonical signaling (Oishi et al., 2004). The anti-apoptotic proteins Bcl-2 and Mcl-1 prevent apoptosis at several levels, either by sequestering death-driving cysteine proteases called caspases (apoptosome) or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF (apoptosis-inducing factor) into the cytoplasm (Tsujimoto, 1998). Based on these data, we decided to stably transfect the HEK-293 cell line over-expressing Notch2-IC to study the expression of the anti-apoptotic proteins Bcl-2 and Mcl-1 as possible targets of Notch2. Bcl-2 and Mcl-1 expression was induced by the stable expression of N2-IC compared to the vector control (figure 36a). Next we stably expressed the shRNA against *NOTCH2* in the HEK-293 cell

line and analyzed the expression of the two anti-apoptotic proteins Bcl-2 and Mcl-1. Down-regulation of Notch2 resulted in a decreased expression of Bcl-2 and Mcl-1 (figure 36b). These two experiments have shown that Notch2 regulates expression of the two anti-apoptotic proteins Bcl-2 and Mcl-1, and that the Notch2 signaling pathway appears to exert an anti-apoptotic effect in different cell types.

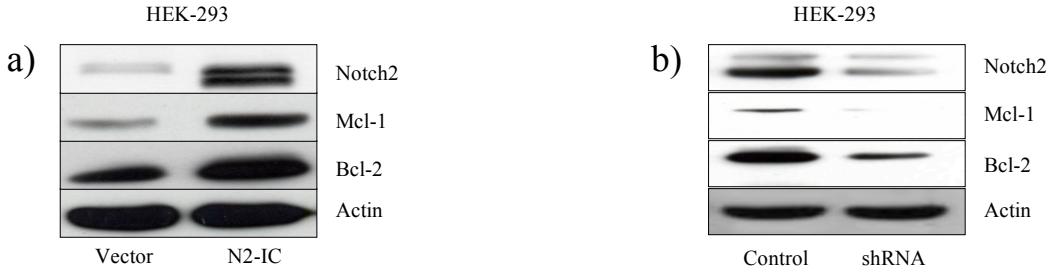


Figure 36. Regulation of Bcl-2 and Mcl-1 expression by Notch2 in HEK-293 cells, a) over-expression of N2-IC induces Bcl-2 and Mcl-1 expression and b) down-regulation of Notch2 reduces expression of Bcl-2 and Mcl-1.

Mode of Bcl-2 and Mcl-1 induction by Notch2

In order to elucidate how and at which level Notch2 signaling regulates Bcl-2 and Mcl-1 expression, we used the HEK-293 cell line stably expressing the Notch2-IC for further experiments. To understand whether the Notch2 signaling pathway regulates *Bcl-2* and *Mcl-1* at the transcription level, the HEK-293 cell line stably expressing Notch2-IC was treated with actinomycin-D at 0.5μg/ml or 1μg/ml for 36 hours to block transcription. Total RNA was isolated, and semi-quantitative PCR for the expression of *Bcl-2, Mcl-1* and *HES-5*, was carried out on the cDNA prepared from the samples. *HES-5*, being a canonical target gene highly induced upon Notch2-IC expression (figure 37), was used as a positive control for transcriptional regulation. HEK293-N2-IC

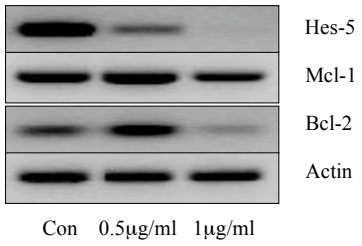


Figure 37. Actinomycin-D blocks the transcription of Hes-5 and Bcl-2, but not of Mcl-1.

Blocking transcription in the HEK293 cell line stably expressing Notch2-IC resulted in down-regulation of *HES-5* at both 0.5μg/ml and 1μg/ml of actinomycin-D (figure 37). *Bcl-2* down-regulation was more pronounced in 1μg/ml of actinomycin-D. There was a slight change in the level of *Mcl-1* expression. From this experiment, we can conclude that *Bcl-2* and *HES-5* are regulated at the transcriptional level.

Next, to understand whether a post-translational mechanism is involved in the regulation of *Bcl-2 and Mcl-1* expression, the HEK-293 cell line stably expressing Notch2-IC was treated with the proteasome inhibitor MG132 at 1.0 μM concentration for 12 hours.

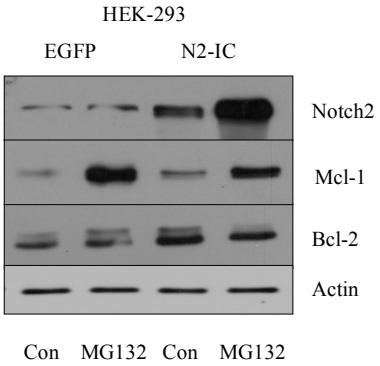


Figure 38. Notch2 regulates the stability of Mcl-1 protein.

At the end of the treatment, the protein extracts were subjected to western blotting. As shown in figure 38, there was a low accumulation of Mcl-1 in the Notch2-IC expressing cell line compared to the vector control showing that Notch2 slowed down the degradation of Mcl-1 and stabilized it. *Bcl-2* expression did not change after the proteasome inhibitor treatment. From these experiments we can conclude that Notch2 is regulating the expression of Bcl-2 and Mcl-1 at different levels.

Expression of Bcl-2 and Mcl-1 in GBM cell lines

Since Notch2 is highly expressed in brain tumours and seems to regulate the expression of the anti-apoptotic proteins Bcl-2 and Mcl-1, the level of these anti-apoptotic proteins expressed in the GBM cell lines were studied. The protein extracts were subjected to western blotting. Both Bcl-2 and Mcl-1proteins were highly expressed in the cell lines. Bcl-2 was highly expressed in LN229, LN18, U373, U343 and LN71

while it was low in U87. Mcl-1 showed a moderate to high expression in U87, U343, U373, LN71 and LN229, while it was comparatively low in LN18 (figure 39).

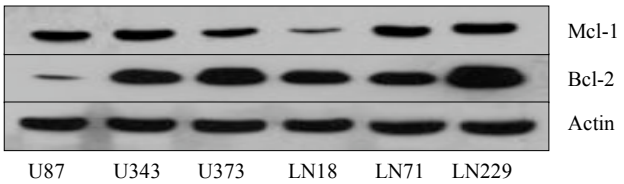


Figure 39. Anti-apoptotic proteins expression in GBM cell lines.

This analysis has shown that the GBM cell lines co-express Notch2, Bcl-2 and Mcl-1, and that Notch2 may regulate them.

Down-regulation of Notch2 decreases the expression of Bcl-2 and Mcl-1 in GBM and in other cancer cell lines

In order to show that Notch2 regulates Bcl-2 and Mcl-1 not only in GBM cells, but also in other types of cancer, GBM cell lines (LN18, U343 and U373), lung (Calu-6) and cervical cancer (Hela) lines stably expressing the shRNA against *NOTCH2* were created. Their protein extracts were screened for the down-regulation of Notch2 and the anti-apoptotic proteins Bcl-2 and Mcl-1. Notch2 expression was highly down-regulated in LN18, U343, U373 and Hela, and only slightly down-regulated in Calu-6.

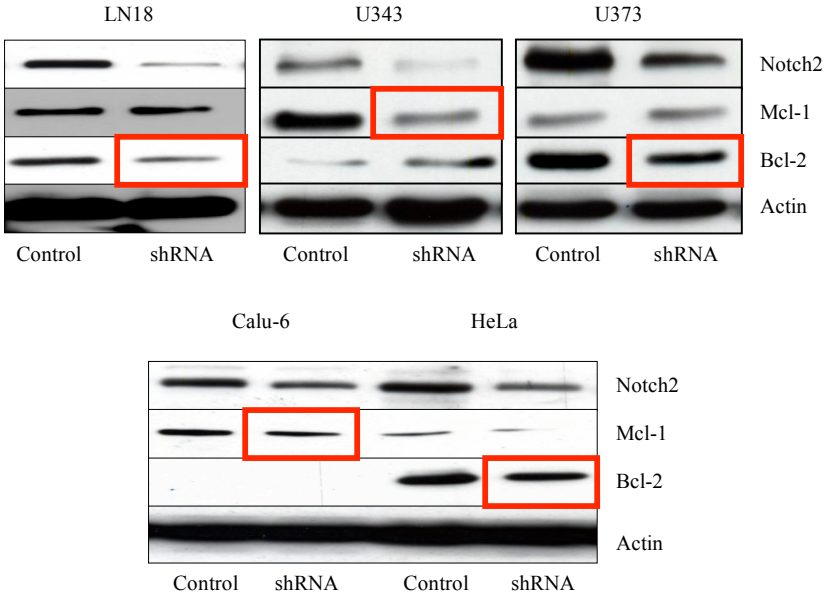


Figure 40. Down-regulation of Notch2 in GBM cell lines and other cancer lines using shRNA against NOTCH2. Red box shows the down-regulation of Bcl-2 and Mcl-1.

Depending on the cell line, expression of either one of the anti-apoptotic proteins was reduced during the down-regulation of Notch2 as seen in the figure 40. Bcl-2 was reduced in LN18, U373 and Hela. U343 and Calu-6 showed a reduction in Mcl-1. This experiment has shown that Notch2 regulates Bcl-2 and Mcl-1 expression in cancer and in normal cells differently.

Induction of Bcl-2 and Mcl-1 by Notch2 is not through the canonical and the DTX1 mediated signaling

Next we studied whether the induction of Bcl-2 and Mcl-1 by Notch2 signaling goes through the RBP-Jκ mediated canonical signaling or the DTX1 mediated non-canonical pathway. To study the canonical signaling, the cell lines HEK293-N2-IC, LN18 and U373 were stably transfected with either the *MAML1 (DN)* mutant or shRNA against *RBP-Jκ*. To understand whether the non-canonical pathway was responsible for the induction of Bcl-2 and Mcl-1, the cell lines U373 and HEK293 were stably transfected with the human *DTX1* tagged with myc. *RBP-Jκ* down-regulation by shRNA was highly effective in the three cell lines. The *MAML1 (DN)* mutant construct was efficiently expressed in the three cell lines. In the cell lines HEK293-Notch2-IC, LN18 and U373, Bcl-2 and Mcl-1 expression did not change both in the *MAML1 (DN)* mutant and in the RBP-Jκ down-regulated lines compared to the control (figure 41).

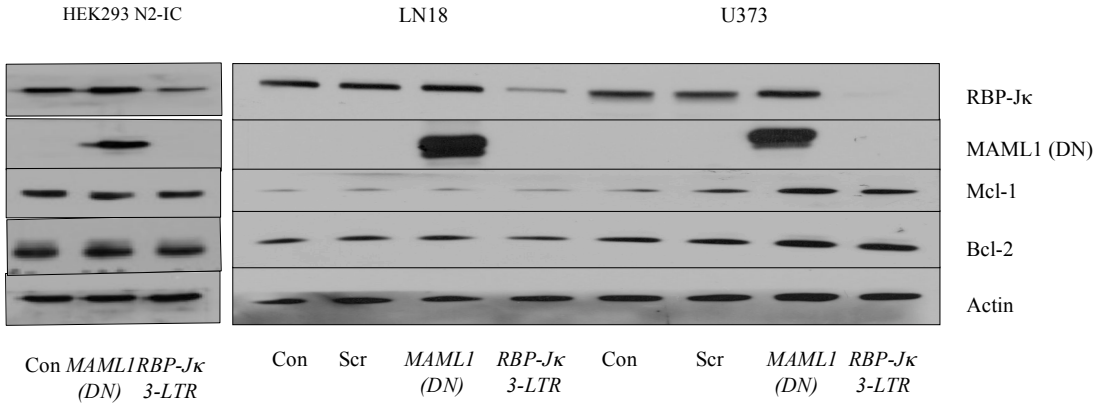


Figure 41. Bcl-2 and Mcl-1 expression is not regulated by Notch canonical signaling (western blot).

Over-expression of human *DTX1-myc* in U373, HEK293 did not influence Bcl-2 and Mcl-1 expression (figure 42). These experiments suggest that both the canonical as well

as the non-canonical pathways do not regulate the expression of Bcl-2 and Mcl-1, and that Notch2 may regulate Bcl-2 and Mcl-1 expression by interacting with other factors.

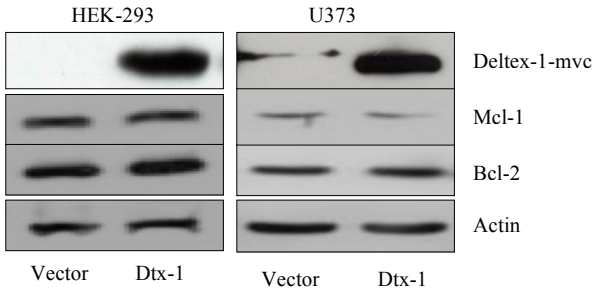


Figure.42. Bcl-2 and Mcl-1 expression are not regulated by Deltex-1 pathway (western blot).

Down-regulation of either Bcl-2 or Mcl-1 increases the background apoptosis in GBM cell lines

Since the down-regulation Notch2 resulted in lower levels of either Bcl-2 or Mcl-1 in GBM cell lines, and since these two proteins are involved in the regulation of programmed cell death, we analyzed whether Notch2 down-regulation increases the background apoptosis. We found an increase in the background apoptosis of the cell lines compared to the control. The increase in the background apoptosis was directly related to the decrease in the expression of the anti-apoptotic proteins Bcl-2 and Mcl-1.

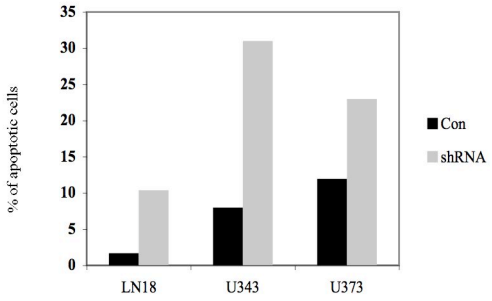


Figure 43. shRNA mediated Notch2 down-regulation increases background apoptosis.

This experiment showed that the down-regulation of either one of the anti-apoptotic proteins shifts the balance towards the pro-apoptotic pathway, which resulted in increased background apoptosis (figure 43).

Conclusion

From these experiments, it is clear that Notch2 signaling activates the anti-apoptotic protein Bcl-2 at a transcriptional level and Mcl-1 at a post-translational level. Furthermore, Notch2 is not able to induce Bcl-2 and Mcl-1 through the RBP-Jκ or DTX1 mediated pathway. In comparison to normal HEK-293 cells, Notch2 activates either one of the anti-apoptotic proteins in cancer cells, but the reason behind this selectivity is not yet clear. Nevertheless, down-regulation of either one of the anti-apoptotic proteins increases the background apoptosis.

d. Interfering with Notch signaling in GBM cell lines

The activation of the Notch receptor upon ligand binding is determined by two proteolytic cleavages. The first cleavage at the site S2 releases the membrane-tethered form of the Notch intracellular domain and is mediated by the ADAM metalloprotease (Brou et al., 2000). The second cleavage at the cytoplasmic site S3 is mediated by presenilin-dependent γ-secretase activity, which releases the soluble Notch intracellular (N-IC) (Schroeter et al., 1998) **(Annex-5)**. Then, N-IC translocates to the nucleus where it binds to partners of the canonical signaling such as the transcription factor CSL/RBP-Jκ, the nuclear protein Mastermind and trans-activates target genes such as the *hairy/ enhancer of Split (HES)-1* or *HES-5* transcription factors (details in the introduction) (Artavanis-Tsakonas et al., 1999). In addition, an alternative RBP-Jκ-independent pathway signaling through DTX has been identified in *Drosophila* (Ramain et al., 2001) and is a positive modulator of Notch signaling. Recently in mammals, three new Notch ligands F3/contactin (Hu et al., 2003), NB3 (Cui et al., 2004) and DNER (Eiraku et al., 2005) have been identified and found to signal through DTX1 during oligodendrocyte maturation, differentiation and Bergmann glia development.

The expression analysis on GBM primary tumour biopsies and cell lines for the Notch signaling pathway described in section 7.2.1 suggested an active Notch signaling in cancer cells. Hence, we investigated the effect of blocking canonical as well as alternative Notch signaling through a molecular and pharmacological approach in GBM cell lines.

Canonical Notch signaling

<u>Molecular inactivation of Notch signaling</u>

To inhibit the canonical Notch signaling in GBM cell lines, stable lines were created expressing either a dominant-negative MAML1 construct or shRNA targeted against the human RBP-Jκ. A BrdU incorporation assay was used to measure the proliferation rate of GBM lines. The U373 GBM cell line, expressing either the MAML1 dominant negative mutant or shRNA against RBP-Jκ resulted in 20% inhibition of

proliferation. The MAML1 dominant negative mutant in the LN319 cell line and shRNA targeted against RBP-Jκ in the U251 cell line lead to a 20-25% reduction in proliferation.

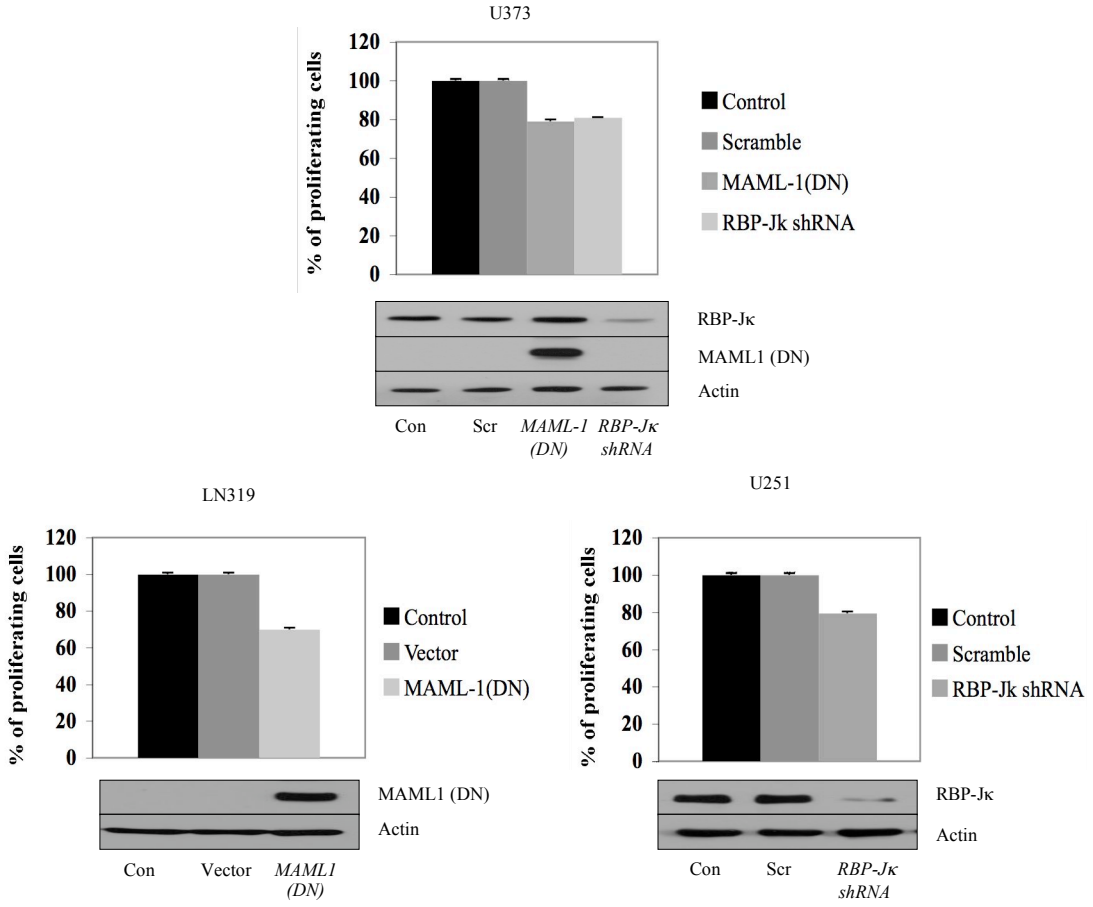


Figure 44. Effect on proliferation in GBM cell lines expressing the dominant-negative MAML1 or targeted down- regulation of RBP-Jκ.

This experiment showed that molecular inhibition of the Notch canonical signaling pathway in GBM cell lines blocked 20-25% of cell proliferation (figure 44). No cell death (data not shown) was observed in GBM cells due to inhibition of Notch signaling using either MAML1 dominant negative or RBP-Jκ shRNA.

Pharmacological inactivation of Notch signaling

Next, the GBM cell lines were treated with DAPT, an inhibitor of the γ-secretase, at two different concentrations for 72 Hrs. At the end of the treatment, the BrdU incorporation rate was measured as readout of Notch signaling inhibition. Each cell line reacted differently to the γ-secretase inhibitor treatment.

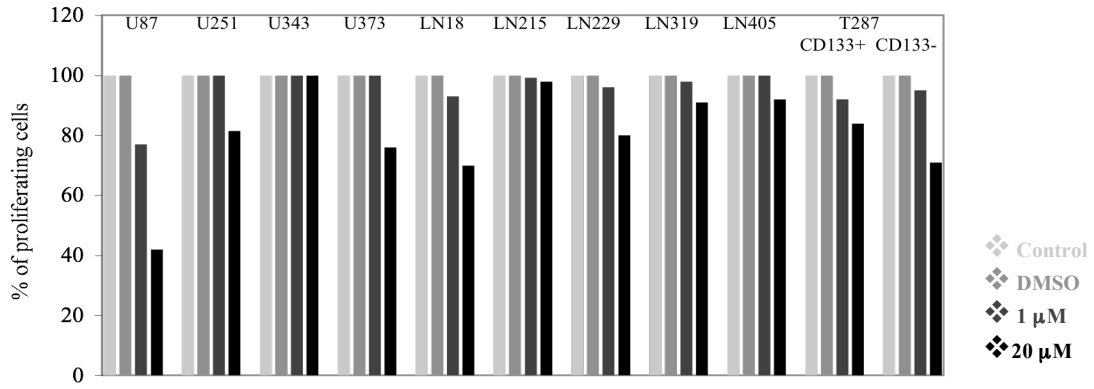


Figure 45. Effect of the γ-secretase inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-Butyl Ester) on GBM cell lines. The results are the average of two experiments.

Cell lines U87, U251, U373, LN18 and LN229 showed varied levels of inhibition of proliferation in response to inactivation of Notch signaling. U87 was the most responsive among them. LN319 and LN405 were moderately responsive to the inhibitor, while U343 and LN215 were non-responsive to γ−secretase inhibition. We also included an *ex-vivo* tumour culture: T287 enriched in CD133+ cancer stem cell like cells population and the CD133- population. Both populations were partially responsive towards γ-secretase inhibition to a similar extent (figure 45). This experiment showed that pharmacological inhibition of Notch signaling in GBM cells results in slight to moderate inhibition of proliferation.

Down-regulation of canonical target genes

In order to confirm that the expression of the dominant negative (DN) MAML1 mutant interferes with Notch signaling in the GBM cell lines, the LN319 and U373 cell lines stably expressing MAML1 (DN) were analyzed for target gene expression (HES-1).

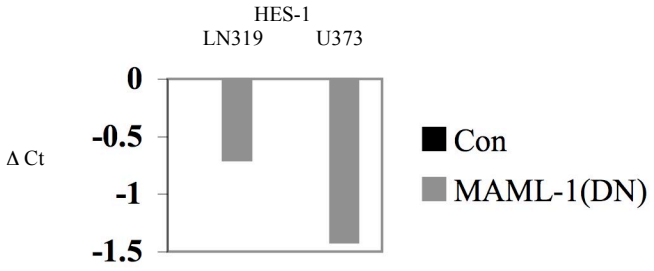


Figure 46. LN319 and U373 cell lines expressing MAML1 (DN) form in that Notch target gene HES-1 transcript was down-regulated (Real time PCR).

The figure 44 shows the expression of MAML1 (DN) in LN319 and U373 cells at the protein level. In these two cell lines, the expression of Notch canonical target gene *HES-1* was quantified by real-time PCR. The ΔCt value for HES-1 was calculated relative to GAPDH expression. The non-transfected parental LN319 and U373 cell lines were considered Δ *HES-1*=0. *HES-1* was found to be down-regulated in *MAML1 (DN)* mutant expressing lines (figure 46). With this experiment, we confirmed that GBM cell lines possesse an active Notch signaling that drives the canonical target gene *HES-1* and can be molecularly interfered.

Non-canonical Notch signaling

To block the non-canonical Notch signaling, a stable cell line expressing shRNA targeted against human *DTX1* was created. To study whether DTX1 down-regulation influences pathway activation, as demonstrated for a cervical cancer (Veeraraghavalu et al., 2005), the U373 cell line was stably transduced with two shRNA targeting the human DTX1 gene and a scramble shRNA as a control. Analysis of these stable lines for a GBM-related cancer pathway like MAP kinase and PI3kinase was performed.

We found a reduction in phosphorylated ERK (P-ERK) level compared to the total amount of ERK (T-ERK), while there was no effect on the P-Akt level (figure 47). This experiment has shown that down-regulation of DTX1 interferes with the cancer related pathway which is inhibiting ERK phosphorylation.

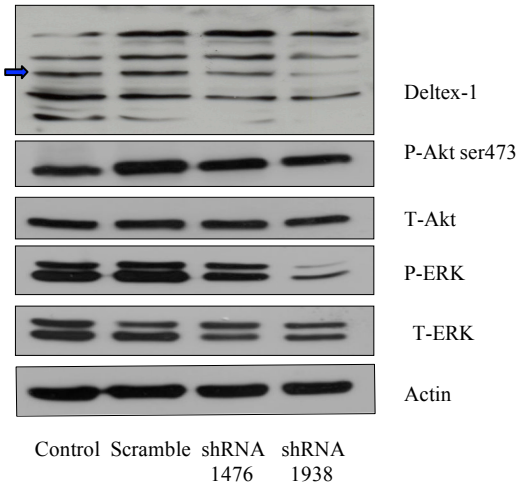


Figure 47. Down-regulation of the human DTX1 in the U373 GBM cell line. Abbreviations: prefix P: phosphorylated protein; prefix T: total protein. The DTX1 antibody shows multiple bands. The ~60 KDa band represent the DTX1 specific protein.

No good antibodies against the human DTX1 protein are available; therefore we generated a rabbit anti-serum for human DTX1. Although multiple protein bands were present, a down-regulated band (blue arrow, ~60 KDa) of the proper size could be identified.

Down-regulation of *DTX1* induces apoptosis and impairs the colony-forming ability of a GBM cell line

Next, we studied the effect on apoptosis due to *DTX1* down-regulation in the U373 GBM cell line. Cell death was measured using annexin-V and PI. We found that the U373 cell line carrying stably *DTX1* down-regulation (shRNA 1938) showed increased apoptosis when compared both to the parental U373 line and to the U373 stably transfected with a scramble shRNA (figure 48).

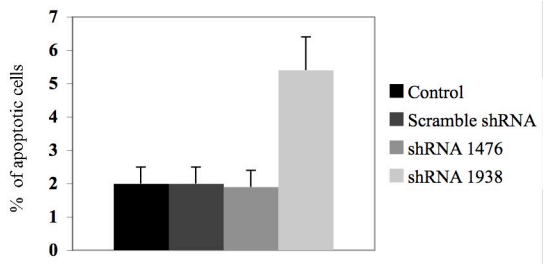


Figure 48. Induction of apoptosis in the DTX1 down-regulated U373 GBM cell line.

This experiment has shown that inhibition of Notch signaling through down-regulation of *DTX1* lead to increased background apoptosis.

Next, we studied the cell growth using a colony formation assay on the U373 cell line carrying *DTX1* shRNA and compare it with the parental line and with the line stably transfected with the scramble shRNA.

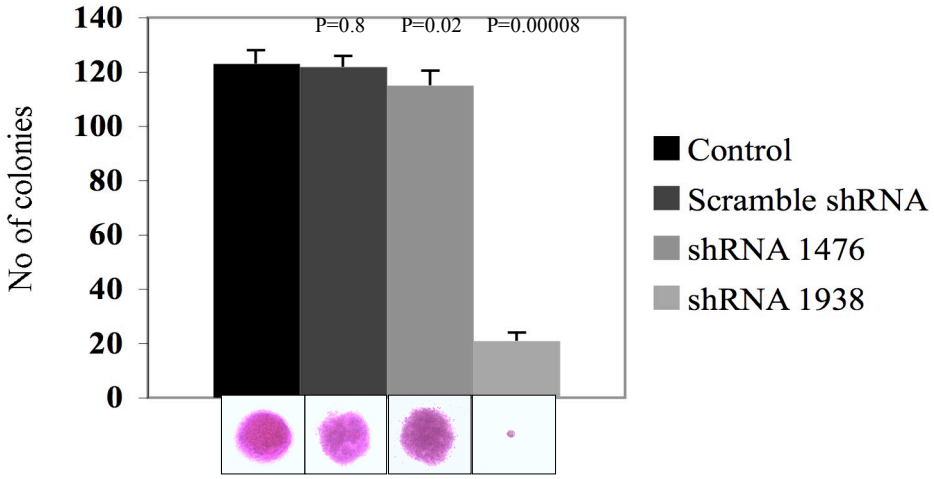


Figure 49. Colony formation in soft-agar shows a marked reduction upon downregulation of DTX1 mRNA. (t-Test: Two-sample assuming unequal variances).

The colonies with a size bigger than 100 μm were counted and the average number was determined between the replicates. The average colony number was significantly reduced in the *DTX1* shRNA1938-expressing line compared to the parental and control shRNA lines (P=0.00008) (figure 49). Furthermore, most of the colonies were smaller in size as shown in figure 49.

The U373 line stably transfected with the shRNA1476 against *DTX1* did show significant differences both in the colony forming efficiency (P=0.02) and in the level of P-ERK compared to the parental and scramble shRNA transfected lines. The colony forming efficiency of shRNA1476 was high compared to shRNA1938 transfected U373 cells; this was most probably due a low efficiency in the *DTX1* down-regulation (figure 47). This experiment has shown that down-regulation of *DTX1* interferes with the colony forming ability of GBM cell lines.

Conclusion

Molecular or pharmacological mediated inactivation of Notch signaling in GBM cell lines has resulted in a partial block of proliferation. Moreover, down-regulation of *DTX1* interferes with the non-canonical Notch signaling pathway and significantly reduced the colony forming efficiency of GBM cells. These experiments suggest that GBM cells possess active Notch signaling that drives canonical as well as non-canonical pathways. Inhibition of γ-secretase may be considered as a possible therapeutic approach to control the development of Notch-positive GBM.

2.3 Notch degradation

Notch signaling is a highly conserved, developmentally regulated pathway that determines cell fate and controls cell growth and proliferation in different cell types. Inactivation of Notch signaling results in developmental defects, whereas activation of this receptor supports anchorage-independent growth *in-vitro* (Artavanis-Tsakonas et al., 1999). Abundant expression of Notch receptors, ligands and pathway components are documented in different cancer types including T-cell acute lymphoblastic leukemia (T-ALL), B-cell leukemia and epithelial tumours such as cervical cancer (Gray et al., 1999), breast carcinoma (Weijzen et al., 2002) medulloblastomas (Fan et al., 2004) and others. In T-ALL, apart from genomic translocation (detail in introduction), mutations targeting the PEST domain and inactivation of *Fbw-7* are the most frequent alterations that stabilize the Notch1 protein and confer constitutive activity (Malyukova et al., 2007; Weng et al., 2004).

Regarding GBM, it has been shown that Notch1 has an effect on proliferation and survival of cancer cells (Purow et al., 2005). Moreover, we found that GBM cell lines have a *NOTCH2* genomic amplification (figure 14b) paralled with a high expression of the Notch2 protein (figure 14b). Nevertheless, we did not find any gain of function mutations in the *NOTCH2* gene in GBM cell lines, and therefore assume tandem duplication/multiplications reminiscent of EGFR amplifications (figure 15). The higher expression of Notch2 is linked to a migratory and apoptotic resistant-phenotype of GBM cells (results sections: 2.2 a & b). The high expression of Notch1 and Notch2 in GBM might be the result of altered degradation of Notch proteins. Therefore, we investigated whether there is an alteration in the degradation of Notch proteins in GBM cell lines.

Expression analysis of Notch degradation pathway components

Fbw-7/Sel-10 is a RING finger type E3-ubiquitin ligase that ubiquitinates the activated form of the receptor, Notch-IC. Ubiquitination by Fbw-7 requires the presence of the PEST domain at the C-terminal end of Notch-IC (Gupta-Rossi et al., 2001; Oberg et al., 2001; Wu et al., 2001). Numb is a negative-regulator of Notch signaling. It is a co-factor of Itch/Su(dx) which ubiquitinates the full-length receptor. Itch is a HECT-domain

containing E3 ubiquitin ligase (Qiu et al., 2000) **(Annex-8)**.

First, we analyzed the expression pattern of genes involved in the degradation of Notch receptors in GBM cell lines. Semi-quantitative PCR (polymerase chain reaction) was done on complementary DNA (cDNA) prepared from GBM cell lines, normal brain (NB) and fetal brain (FB), using cDNA specific primers of the respective genes. FB and NB were the sample controls while actin was taken as a gene expression control. We found that all components required for the degradation of Notch receptors were expressed in the GBM cell lines, fetal brain and in normal brain.

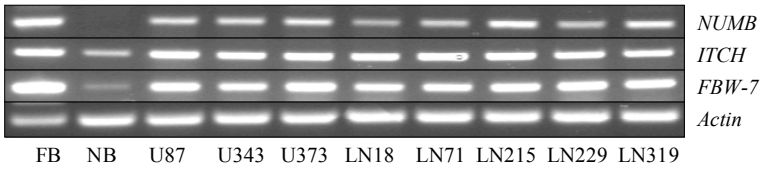


Figure 50. Expression pattern of Notch degradation pathway components in GBM cell lines.

Both *NUMB* and *ITCH* were expressed in all cell lines, but *NUMB* expression was not detected in normal adult brain (figure 50). The absence of *NUMB* in adult brain may be complemented by its paralogue *NUMBL*.

Defective Notch receptor degradation pathway

Since the genes required for the degradation of the full length as well as the activated form of Notch receptors were expressed, we next studied the state of protein degradation in general and Notch receptor degradation in particular in glioblastoma cells lines. For this purpose, two GBM cell lines were treated with an inhibitor of the proteasome-mediated protein degradation pathway, MG132. The protein extracts were collected at two time points, 12 and 24 hours after treatment. Then, western blotting was performed on the collected samples to check for any change in the level of Notch receptors.

In this experiment, c-myc was used as a positive control given its rapid turnover in GBM cell lines. We found an accumulation of c-myc protein in the inhibitor treated samples

confirming the effect of the drug to a block of protein degradation. We did not find any accumulation of Notch1 and Notch2 proteins in the inhibitor treated samples. Actin was used as a loading control (figure 51).

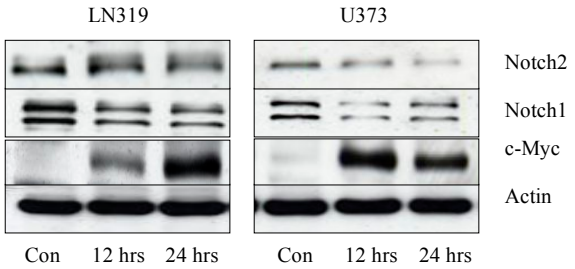


Figure 51. Degradation of proteins effectively blocked by proteosome inhibitor MG132 (5μM) at time points 12 and 24 hrs.

This experiment showed that even though the components required for the degradation of Notch receptors are expressed in GBM cell lines, Notch protein is not subjected to proteasome mediated degradation. These results have suggested that Notch receptors in GBM cell lines do not seem to be subjected to the known degradation pathway.

Loss of *DTX1* expression in GBM cell lines

Since there was no significant change in the level of Notch1 and Notch2 proteins when the GBM cell lines were treated with proteasome inhibitor, we checked whether any other E3-ubiquitin ligase might play a role in the degradation of Notch receptors. In *Drosophila,* DTX is a positive modulator of the Notch pathway driving RBP-Jκ independent Notch signaling. DTX possesses a RING finger domain known to carry ubiquitination function. It has been suspected to be an E3-ubiquitin ligase for the Notch receptor (Mukherjee et al., 2005).

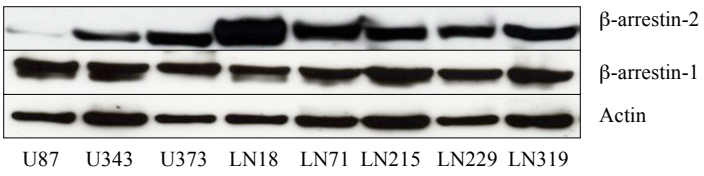


Figure 52. Expression of β-arrestins in human GBM cell lines (western blot).

The DTX action requires arrestins as adaptor molecules to ubiquitinate Notch receptors. There are two human arrestin homologs: β-arrestin-1 & β-arrestin-2. We found that both β-arrestin-1 and β-arrestin-2 were expressed in our GBM cell lines (figure 52).

To proceed further, we studied whether *DTX1* is present in human GBM cell lines. The human DTX1 is highly similar to *Drosophila* DTX. Surprisingly, we did not find mRNA for *DTX1* in any of the GBM cell lines analyzed, while it was expressed in the fetal brain using PCR primers covering exon 1-2 at the 5′ end of the gene (figure 53).

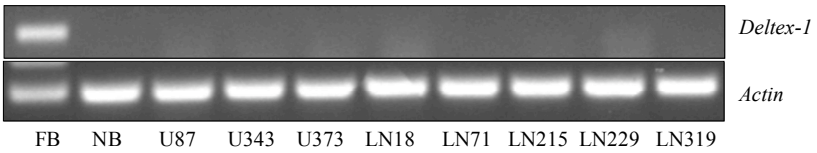


Figure 53. Absence of DTX1 expression in GBM cell lines.

As described in section 2.3, no good antibodies against DTX1 are available. Nevertheless, in GBM cell lines we could detect a possible DTX1 band (60 KDa) with our antibody. This band was down-regulated in GBM cell lines stably transfected with shRNA against DTX1.

The primer used to amplify the *DTX1*, located at the 5′end of the gene covers the protein coding sequence by which it interacts with Notch receptors. With the primers covering the 3′ end of the gene, we found DTX1 transcript in GBM cell lines (figure 21). Since we did not find any mRNA using the 5′ end primers except in the normal brain, we could speculate that alternative splicing forms of DTX1 are present in GBM cell lines. This experiment identified that an E3 ubiquitin ligase involved in the degradation of Notch receptors is not expressed in the GBM cell lines. Hence, the further step was to restore the expression of *DTX1* and study its function in GBM cell lines.

Restoration of *DTX1* expression

<u>Over-expression</u>

We stably expressed human DTX1 in HEK293 (control line) and in the U373 cell line. To see whether introduction of DTX1 initiated the degradation of Notch receptors, these lines were treated with proteasome inhibitor MG132 at 10μM concentration, and cell extracts were collected at two time points.

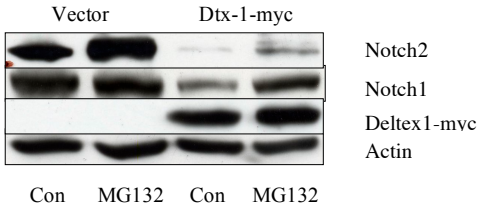


Figure 54. Effect of DTX1-myc expression in HEK293 cells.

Western blot analysis identified the stable expression of DTX1-myc in HEK293 (figure 54) and U373 cell lines (figure 55). We found that DTX1 expression enhances the degradation of Notch receptors compared to the control lines. In the HEK293 cells, the degradation of Notch1 and Notch2 was highly efficient compared to the U373 GBM cell line. Blocking the degradation pathway by using proteasome inhibitor MG132, resulted in the accumulation of the Notch1 and Notch2 receptors (figure 54 & 55) in both cell lines. Here, p21 was used as a control to determine the activity of the proteasome inhibitor MG132.

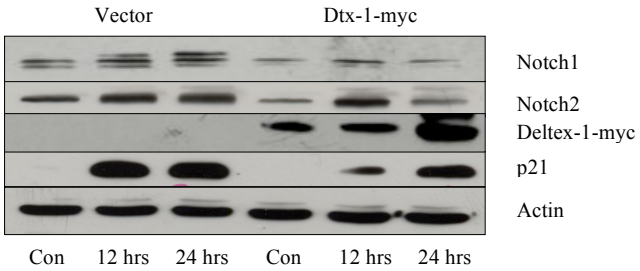


Figure 55. Effect of DTX1-myc expression in U373 cells.

DTX1-myc itself accumulated when the protein degradation was inhibited. This showed that DTX1 levels might be self-regulated. The accumulation of DTX1-myc was more enhanced in the U373 GBM cell line than in the normal line HEK293 (figure 54 & 55)

when protein degradation was blocked. We can conclude that in the GBM cell line, DTX1-myc undergoes a faster turnover than in the HEK293 cell line, leading to a low degradation of targets. From these experiments, we can conclude that most probably the high amount of Notch protein present in GBM cell lines is due to the absence and altered stability of DTX1.

De-repression

To re-induce a proper *DTX1* expression, the HEK293 cell line was treated with the inhibitor of histone deacetylases, trichostatin A (TSA) at 100nM. *DTX1* expression was then analyzed from the cDNA prepared from these samples. We found an up-regulation of *DTX1* transcript in the TSA treated samples compared to the control (figure 56a). This experiment suggests that *DTX1* expression is suppressed through epigenetic modification of DNA, and that gene expression can be re-induced upon the use of epigenetic modulators.

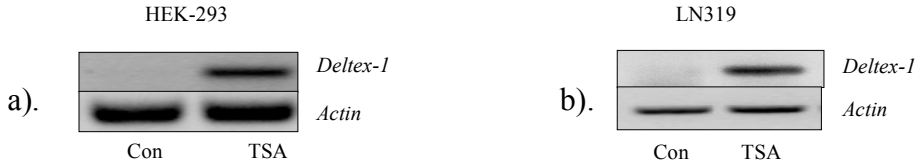


Figure 56. Restoration of DTX1 expression using histone deacetylases inhibitor.

Most probably, the same mechanism may be operative in inhibiting *DTX1* expression in GBM tumour cell lines. To confirm this hypothesis, the LN319 cell line was treated with trichostatin A (TSA) at 1μM for 24hrs, and cDNA was analyzed for the expression of *DTX1*. In TSA treated cells, *DTX1* expression was reinduced confirming that *DTX1* undergoes epigenetic suppression (figure 56b). LN319 required a higher concentration of TSA compared to HEK293 cells.

Relevance for cancer

Next, we studied the effect of a proper DTX1 expression in cancer cell lines. Two important cancer related pathways in GBM downstream of receptor tyrosine kinases (RTKs), namely MAP kinase and PI3kinase, were analyzed. We found that DTX1 expression in HEK293 cells induced the phosphorylation of ERK, while it did not change

the status of Akt phosphorylation. In the U373 GBM cell line, the stable expression of DTX1 resulted in an increase of P-ERK and a decrease of P-Akt (figure 57).

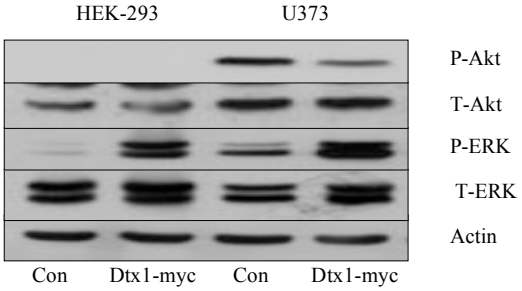


Figure 57. DTX1-myc expression induces P-ERK while reducing P-Akt in GBM cell line.

Next, we studied the effect of DTX1 expression on proliferation and apoptosis. We did not find an increase of background apoptosis in the U373 cell line while a slight decrease in proliferation was noticed. We can conclude that a proper DTX1 expression is able to modulate the signaling downstream of RTKs. This is of interest since the U373 cell line, upon DTX1 expression, shows a modulation in the PI3K pathway, which overcomes the lack of PTEN.

Role of DTX1 in different cell types

To assess the effect of DTX1 on the Notch receptor in different cell types, we stably expressed DTX1-myc in the following: HEK293 (normal embryonic kidney line) (figure 54), U373 (a GBM line) (figure 55) and MCF-7 (a breast cancer line) (figure 58).

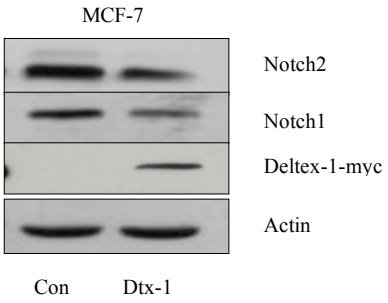


Figure 58. Role of DTX1 is the same in variable cell types.

The protein extracts were subjected to western blotting. The Deltex-myc expression level varied among the cell lines. It was very high in HEK293 (figure 54), moderate in U373 (figure 55) and low in MCF-7 (figure 58). The level of Notch1 and Notch2 in the DTX1-

myc expressing line was decreased when compared to the parental line. From these experiments, we can conclude that lack of a full length DTX1 leads to an accumulation of Notch receptors. We can conclude that DTX1 play a role in the regulation of Notch receptor degradation.

Conclusion

In this section, we studied the role of DTX1 in the regulation of Notch receptor expression in different cell lineages. All components required for Notch degradation, apart from regular *DTX1,* were expressed in GBM cell lines. Over-expression of *DTX1* in control as well as GBM cell lines resulted in an enhanced degradation of Notch receptors. Moreover, we could re-induce the regular *DTX1* expression using epigenetic modulators such as TSA. Since the Dtx1 turnover rate seems to be faster in GBM cells (U373) which express the transgene, we may not see the degradation of Notch receptors in TSA treated samples.

Discussion

The glial cell-derived tumors of the central nervous system (CNS) include glioblastomas (GBM), astrocytomas, oligodendrogliomas (OG) and oligoastrocytomas. The most malignant is GBM that can either develop *de novo* (> 90%) as primary GBM or through progression from low-grade astrocytoma, as secondary GBM (Zhu and Parada, 2002). The incidence rate of GBM is approximately 2-3 per 100,000 people in a year. GBM represents 2% of all cancers. Mean patient survival is less than 10 months (Ohgaki and Kleihues, 2007). On the other hand, OG represents 5% of glial brain tumours. They were found to have combined allelic loss at chromosome arms 1p and 19q in 70-80% of the tumours (Boulay et al., 2007; Kleihues, 2000; Smith et al., 1999). Combined 1p and 19q loss in OG is associated with better prognosis, and possibly better responsiveness to chemotherapeutic treatment with a mean patient survival of about 10 years (Cairncross et al., 1998; Ino et al., 2001). In fact, combined 1p/19q loss results from a translocation t(1;19)(q10;p10) (Griffin et al., 2006; Jenkins et al., 2006). This shows that loss of chromosome 1p is a marker for a subgroup of OG with a more favourable clinical behaviour (Ohgaki and Kleihues, 2007). Despite intense investigations, the putative tumour suppressor gene(s) on chromosome 1p and 19q remains to be identified.

Although surgical resection, radiotherapy and chemotherapy can significantly improve the prognosis of patients with breast, lung or prostate cancer, current treatment results only in a modest prolongation of survival in GBM cancer patients. The purpose of this work is to identify and unwind the molecular intricacy of such clinical differences, which is crucial in order to find new treatment strategies for GBM.

NOTCH2 is a common deletion target in a subgroup of gliomas

Based on somatic deletion mapping of chromosome 1, we found that in a subgroup of OG and GBM, the common deletion at the *NOTCH2* locus was associated with better survival (Boulay et al., 2007). This has suggested a possible causal relationship between *NOTCH2* status and tumour behaviour. *NOTCH2* is located at chromosome band 1p11, near the breakpoint cluster area of OG with 1p/19q loss, suggesting that *NOTCH2* inactivation is associated with the recently described OG

translocation t(1;19)(q10;p10) (Griffin et al., 2006; Jenkins et al., 2006). Allelic loss at 1p microsatellite D1S2696, located in the intron 11 of *NOTCH2,* represents a favourable prognostic marker in GBM as well as in OG. Furthermore, among the glioma with 1p centromeric loss, the N2/N2N test determines the relative genomic dosage of *NOTCH2* at 1p11 compared to *N2N* at 1q21 that allows the distinction between OG and GBM with 1p loss. The N2N gene at centromere represents a highly conserved N-terminal duplication of *NOTCH2*.

Presently, 1p telomeric and subtelomeric molecular markers in combination with 19q markers are used in the identification of OG with 1p/19q loss. Our results show that diagnostic assessment of 1p telomeric markers cannot distinguish between subgroups of prognostically better OG and poor GBM with 1p deletions. In contrast, the N2/N2N test excludes GBM with poor survival in all (21/21) cases. We also found that GBM with interstitial deletions located in the 1p22-32 intervals had the poorest prognosis. This subgroup of GBM may target a suppressor gene that is linked with rapid progression. Interestingly, this interstitial region between telomere and centromere does not contain several proposed candidate genes, in contrast to the region 1p36 with, *TP73* (Mai et al., 1998), *RAD54* (Husemann et al., 1999), *CDKN2C* (Bello et al., 2000), *CAMTA1* (Barbashina et al., 2005) and *CHD5* (Bagchi et al., 2007). In contrast, GBM with deletions at the 1p11-13 intervals have a significantly better prognosis than GBM with interstitial or telomeric deletions patterns and GBM without 1p loss, displaying genetic similarities to OG with 1p loss. Both OG and this subgroup of GBM may target a centromeric gene located on 1p that is linked with a distinct prognostically better glioma pathway.

A classification of OG into two distinct prognostic groups as a function of the 1p/19q status relative to other OG is further supported by the observation that among OG, 1p/19q loss and *TP53* mutations are mutually exclusive events. This suggests that OG with either genetic alteration follow distinct tumour developmental pathways (Bigner et al., 1999). Consistently, genetic profiling of primary OG revealed that both genetic alterations are part of two distinct molecular subgroups of OG (Mukasa et al., 2002). In conclusion, we

have found the breakpoints in OG and in a subgroup of GBM that all converge to the NOTCH2 gene locus. These finding are derived from of somatic deletion mapping and further substantiated by the detection of homozygous deletions in primary OGs. These findings form the basis for the hypothesis of a role for *NOTCH2* in brain tumour development. We further propose the combination of two NOTCH2 genetic markers (DIS2696 and N2/N2N) to robustly diagnose low-grade and malignant OG and to thoroughly estimate patient prognosis.

NOTCH2 sequencing

The sequencing analysis of *NOTCH2* in primary tumour samples and cell lines was based on the NOTCH mutation hotspots described in *Drosophila* (Brennan et al., 1997; Rebay et al., 1993). In Hs683, we found a transversion mutation in *NOTCH2* codon 1711 (CTG to ATG), where leucine is replaced by methionine. Interestingly, this codon lies within the RAM domain, which represents the protein-protein interaction domain for the transcription factor RBP-Jκ. Recreation of this mutation *in vitro* has resulted in functional inactivation of Notch canonical signaling and is similar to a functional inactivation mutation introduced in the RBP-Jκ binding motif "GTGGGAA" (Tun et al., 1994). Apart from this rare mutation, absence of frequent mutations in GBM cell lines and primary tumours confirmed the previous observation that NOTCH genes are rarely mutated in common solid tumours (Lee et al., 2007). We sequenced the complete intracellular domain and the mutational hotspots in the extracellular domain, but not the entire reading frame of *NOTCH2* and can therefore not rule out mutations in other coding and non-coding regions including the promoter of the gene. The duplication of the N-terminal part of *NOTCH2* (between *NOTCH2* and the pericentromere) does not interfere with the interpretation of the sequencing results. Another possibility for impairing gene function may be haploinsufficiency since Notch and Notch2 levels were found to be tightly regulated by gene dosage in *Drosophila* (Heitzler and Simpson, 1991) and murine B-cell development, respectively (Saito et al., 2003). LOH at the NOTCH2 locus may therefore impair protein function. Although NOTCH2 is a reasonable and attractive candidate suppressor gene, we can, however, not rule out that another gene in the vicinity of or even within *NOTCH2* is the targeted gene.

Notch signaling and cancer

Notch signaling represents an evolutionarily conserved pathway operative between two neighbouring cells that controls key stages in development, cell growth and differentiation. Notch signaling amplifies and consolidates the molecular differences through two distinct local regulations: lateral inhibition and inductive signaling (Artavanis-Tsakonas et al., 1999). In the CNS, Notch signaling has shown to be a critical regulator of neural stem cell pool maintenance and differentiation of glial lineage (Gaiano and Fishell, 2002; Louvi and Artavanis-Tsakonas, 2006). Notch signaling actively promotes glial fate such as differentiation into astrocytes (Tanigaki et al., 2001), radial glial cells in the forebrain and the cerebellum (Gaiano et al., 2000; Yoon et al., 2004). In contrast, Notch signaling inhibits oligodendrogliogenesis in the optic nerve (Wang et al., 1998) while promoting the differentiation of neural progenitor cells into oligodendrocytes through an alternative pathway driven by interaction with a new class of ligands such as F3/contactin, NB-3 (Cui et al., 2004; Hu et al., 2003). Animal models for mutations in the Notch receptor invariably result in developmental abnormalities and thus human pathologies (Artavanis-Tsakonas et al., 1999). Notch1 and Notch2 are involved in neoplastic disease (Radtke and Raj, 2003), e.g. leukemia (Ellisen et al., 1991; Hubmann et al., 2002) and skin cancer (Nicolas et al., 2003). In human medulloblastoma, Notch1 and Notch2 have opposite effects (Fan et al., 2004). Notch1 was regarded to be either an oncogene or a tumour suppressor, depending on the cellular context (Radtke and Raj, 2003). This dependability on the cellular context may also be applied to Notch2.

Notch over-expression and effect of its inactivation in GBM cells

We found that GBM retaining the NOTCH2 gene (i.e. haplotypes H1-H7, figure 1 & 2) had a worse prognosis (Boulay et al., 2007). Interestingly, we found amplifications of *NOTCH2* in 4/8 (50%) of GBM cell lines. This may be a possible basis of Notch2 over-expression and is reminiscent of EGFR tandem-like amplification in GBM (Libermann et al., 1985; Wong et al., 1987). This finding raises the hypothesis of a role of *NOTCH2* in brain tumour development. Micro-array based expression analysis of *NOTCH2* and its pathway components in primary tumour samples and in GBM cell lines showed a higher RNA content not only for NOTCH2, but also for NOTCH1 and

NOTCH3 in comparison to normal brain control. We found *DLL1* and *DLL3* to be highly expressed in low-grade astrocytomas, whereas *JAG1* was highly expressed in primary GBM. These findings are in line with several preliminary studies (Phillips et al., 2006; Purow et al., 2005; Somasundaram et al., 2005). However, expression of Notch downstream targets had not been correlated with NOTCH genes in these studies. Our micro-array data show a correlation between expression of *NOTCH1* and *NOTCH2,* and the downstream target genes *HES-1* and *HES-5*. In GBM samples, *NOTCH2* expression was highly correlated with *HES-1, and NOTCH1* more with *HES-5*.

The involvement of HES-1 in astrocytic cell fate determination lends support to a role of NOTCH2 in astrocytic differentiation (Wu et al., 2003). Our micro-array data showed significant correlation between *NOTCH2* and *HES-1* expression in GBM. This is in line with increased expression of *HES-1* observed in GBM (Somasundaram et al., 2005). GBM display a more undifferentiated morphology (Holland, 2001) and express high levels of Notch2 according to our immunohistochemical staining. In this context, it is intriguing that Notch2 was found to be expressed in immature glial cells in the germinal zones of the normal brain (Irvin et al., 2001; Tanaka and Marunouchi, 2003; Wang and Barres, 2000). Moreover, over-expression of dominant-negative MAML1, one of the down-stream effectors of Notch signaling, led to down-regulation of *HES-1* in GBM cells, a finding that has also been observed in T-cell acute lymphoblastic leukemia (T-ALL) (Weng et al., 2003). All these data support the concept that Notch2 directly leads to *HES-1* expression in GBM. In parallel, nestin, a marker of neural precursors and GBM, also possesses a RBP-Jκ responsive element (Shih and Holland, 2006) which implies the possible role of Notch2 in the maintenance of glioma cells in the undifferentiated state. Conversely, the Notch2 loss-of-function mutation in Hs683 cells; a malignant oligodendroglioma cell line represents an inactivation mechanism to inactivate Notch2 in this OG line (together with LOH of the remaining allele). In addition, homozygous deletions detected in primary OG with 1p loss must also be considered to be a mechanism of downregulating Notch2 signaling (Boulay et al., 2007). Thus, OG development appears to be dependent on Notch2 loss in the majority of OGs that also display a 1p loss.

Our results show that inhibition of canonical Notch signaling by *i)* over-expression of dominant-negative MAML1, *ii)* shRNA against RBP-Jκ or *iii)* by pharmacological inhibitors such as γ-secretase inhibitors resulted in the inhibition of GBM cell growth. This is in line with growth inhibition of T-cell acute lymphoblatic leukemia (T-ALL) cells by expression of dominant-negative MAML1 (Weng et al., 2003). In parallel, we found that inactivation of non-canonical DTX1-mediated Notch signaling, using shRNA against *DTX1,* resulted in induction of background apoptosis and decreased colony-forming ability of GBM cells. In cervical cancer, dominant-negative DTX1 also blocked epithelial-mesenchymal transition and E6- and E7- mediated transformation in HaCaT-JAG1 cells, but did not induce apoptosis (Veeraraghavalu et al., 2005).

Role of Notch in GBM cell migration

The key feature of highly malignant glial cells is their invasive nature (Maher et al., 2001; Merlo, 2003), which is among other factors, driven by EGFR, PTEN and FAK (Jones et al., 2001b; Maier et al., 1999). PTEN is inactivated in 50% of GBM (Knobbe et al., 2002; Merlo and Bettler, 2004; Sansal and Sellers, 2004). Tenascin-C is also considered to play an important role in tumour cell migration (Lange et al., 2008). Notch1 and Notch2 proteins, which are expressed in the glial lineage, are inducers of *TN-C*. This association is based on the frequent NOTCH2 gene amplification and high protein expression we found in GBM. The identification of a RBP-Jκ responsive element in a minimal *TN-C* promoter together with the association between RBP-Jκ and TN-C expression in GBM primary samples and cell lines propose a novel mechanism of *TN-C trans*-activation. The molecular cooperation between Notch2 and RBP-Jκ has been observed previously in B cell development, where Notch2- and RBP-Jκ-targeted mice present a common phenotype both lacking a B cell subset (Saito et al., 2003; Tanigaki et al., 2002), and in B cell leukemia, where Notch2 together with RBP-Jκ upregulate *CD23a* transcription (Hubmann et al., 2002). Nevertheless, 5 out of 19 TN-C-positive GBM primary tumours were RBP-Jκ-negative, suggesting that RBP-Jκ-independent regulatory pathways for TN-C might also be operative in GBM. This is supported by the observation in human fibroblasts where the *TN-C* promoter is activated by platelet-derived growth factor (PDGF) and transforming growth factor (TGF)-β (Jinnin et al.,

2004; Jinnin et al., 2006). Since TGF-β signaling promotes PDGF-β-dependent cell proliferation in glioma (Bruna et al., 2007), activation of the *TN-C* promoter by TGF-β signaling may also apply in glioma.

TN-C-deficient mice show compromised proliferation/migration of neural precursors, and accelerated oligodendrocyte differentiation (Garcion et al., 2001). Consistently, GBM lines and biopsies showed strong TN-C expression, while OG did not, although an OG subset has been described as moderately positive (McLendon et al., 2000). Strong TN-C expression is associated with the invasive front in many tumour types and is a diagnostic marker for glioma progression, implying a role for TN-C in tumour promotion (Orend and Chiquet-Ehrismann, 2006). This supports the hypothesis of Notch2/RBPJk/TN-C signaling to be operative in GBM, but not in OG development. From a clinical point of view, conventional therapeutic interventions based on tumour resection and radio- and chemotherapy have only moderately improved glioma patient survival over the past decades (Sanai et al., 2005). In addition to directly targeting TN-C (Merlo et al., 1997; Reardon et al., 2002), our data also show that the use of γ-secretase inhibitors may be of therapeutic value in blocking GBM cell migration. We propose that the Notch/RBPJk/TN-C pathway regulates tumour cell migration, a hallmark of invasive GBM. This molecular cascade provides a novel mechanism through which Notch acts in tumour progression and possibly in normal development of the neuronal and glial cell lineages.

Role of Notch2 in GBM cell survival

One of the hallmarks of tumour cells is their ability to evade cell death (Hanahan and Weinberg, 2000). In GBM, several key signaling components have been implicated in increasing the resistance of tumour cells to pro-apoptotic signals (Merlo, 2003). In this thesis, the focus has been directed on the two anti-apoptotic proteins Bcl-2 and Mcl-1, members of the Bcl-2 family of modular proteins involved in cell death regulation (Danial and Korsmeyer, 2004). In GBM cell lines, we found high expression levels of Bcl-2 and Mcl-1. This is in conformity with previous observations of altered expression of Bcl-2 family proteins in GBM associated with tumour grade and clinical

outcome (Deininger et al., 1999; Fels et al., 2000). Furthermore, we have observed over-expression of Notch2, its ligands and its pathway components in GBM cell lines as well as in primary tissues. High expression of Notch pathway components were also observed in other cancer phenotypes and have been shown to be tumourigenic in several tumour models (Radtke and Raj, 2003). Consistently, we observed that down-regulation of endogenous Notch2 in GBM cells by shRNA resulted in increased apoptosis. By screening for anti-apoptotic proteins down-regulated by Notch2 in GBM cells, we identified Bcl-2 and Mcl-1 to be involved in increased apoptosis in GBM cells. Furthermore, we found that over-expression and down-regulation of Notch2 in HEK293 cells resulted in up- and down-regulation of Bcl-2 and Mcl-1. However, inhibition of downstream canonical Notch signaling using dominant-negative MAML1 or shRNA against *RBP-Jκ* in GBM cell lines as well as in HEK293 cells expressing N2-IC did not alter the expression of Bcl-2 and Mcl-1. This is consistent with the role of Notch proteins in enhancing survival of neural stem cells by up-regulating Bcl-2 and Mcl-1 through a RBP-Jκ independent signaling pathway, which has been the case for Notch1 (Oishi et al., 2004).

It is noteworthy that GBM and progenitor cells share common phenotypical features, including cell morphology and expression patterns (Holland, 2001). Given that Notch2 expression in GBM is reminiscent of Notch2 expression in immature glial cells in the germinal zones of the normal brain (Irvin et al., 2001; Tanaka and Marunouchi, 2003; Wang and Barres, 2000), Notch2 may regulate survival in GBM cells as well. Moreover, Notch2 inactivation in a mouse model led to widespread cell death in the CNS (Hamada et al., 1999). Furthermore, Notch2 protects B-cell leukemic cells from cell death (Duechler et al., 2005; Hubmann et al., 2002). All these findings lend support to the concept of Notch2 in cell survival. Overall, these observations are consistent with the possible role of Notch receptors in tumour cell survival, apart from their role in cell proliferation and progenitor maintenance (Miele and Osborne, 1999).

Notch degradation

We observed strong expression of Notch2 protein in primary GBM samples

and cell lines by immunohistochemistry and immunoblotting. Notch2 appears to have a dual function. On one side, loss of chromosome 1p targets *NOTCH2* at 1p11, which is associated with significantly longer patient survival (mostly in the majority of OG and in a subgroup of GBM) and express low or absent levels of Notch2 protein. On the other side, high expression of Notch2 protein is observed in the majority of GBM which do not display allelic loss at *NOTCH2*, (i.e. haplotypes H1-H7, figure 1 & 2) and have a significantly worse prognosis (Boulay et al., 2007). Notch2 appears to function as an oncogene in the majority of GBM that do not display deletions on centromeric 1p. In half of the GBM cell lines analyzed (4/8), we detected amplifications of *NOTCH2*, but not activating point mutations of *NOTCH2*. This is consistent with the observation that activating mutations of NOTCH are uncommon in solid tumors (Lee et al., 2007). The mutations in the PEST domain found in T-ALL samples enhance the stability of activated N1-IC by preventing the Fbw-7 mediated ubiquitination and degradation (Weng et al., 2004). Several studies have observed higher expression of Notch receptors and their components in different tumour types *in vivo* and *in vitro* (Miele and Osborne, 1999; Radtke and Raj, 2003), including GBM (Purow et al., 2005; Somasundaram et al., 2005). But, the cause for the presence of abundant Notch receptors is not clearly understood. Hence, we put an effort in investigating the role of Notch degradation pathways in GBM cells. Among the genes involved in Notch degradation, *FBW-7, ITCH, NUMB* and β-*ARRB* -1& -2, but not *DTX1*, were found to be expressed. In *Drosophila*, DTX contains a RING finger protein with a E3-ubiquitin ligase for the Notch receptor (Mukherjee et al., 2005). We found that a proteasome inhibitor could not block the degradation of Notch receptors even in the longer time period (24 hours). This finding allows the interpretation that high Notch protein levels are probably due to inefficient or defective degradation of Notch1 and Notch2 receptors in GBM cells. Furthermore, we found that restoration of *DTX1* transgene expression in GBM cell line U373 enhanced the degradation of Notch1 and Notch2, which could be blocked by the proteasome inhibitor. Expressing DTX1 in HEK293 and the breast cancer line MCF-7 cells suggests a ubiquitous role for DTX1 in the degradation of Notch receptors. But what is the mechanism that leads to lack of expression of DTX1 in these distinct tumours? The finding that the histone deacetylase inhibitor TSA restored the endogenous *DTX1* expression in GBM cells suggests that

DTX1 expression is suppressed through epigenetic silencing. What are the functional consequences of a lack of degradation of Notch proteins? The accumulation of Notch receptors might help to enhance the cellular pro-survival or anti-apoptotic program within neoplastic cells, and also enhance tumour cell migration.

Conclusion

We found that a subgroup of GBM and OG showed LOH at the NOTCH2 locus on chromosome 1p11 that is positively correlated with patient prognosis. The tumours that showed retention at the NOTCH2 locus expressed high Notch2 protein levels. Using molecular or pharmacological inhibition, we showed the partial inhibition of proliferation of GBM cells. Inhibition of an alternative Notch signaling pathway using shRNA against *DTX1* increased background apoptosis and reduced colony-forming ability, supporting the oncogene concept of Notch2 in the majority of GBM that do not target the NOTCH2 locus. In GBM cells, we showed that TN-C is a novel canonical target gene of Notch signaling. Inhibition of Notch signaling results in the down-regulation of TN-C protein and GBM cell migration. Moreover, we found that Notch2 expression promotes GBM cell survival through up-regulation of the anti-apoptotic proteins Bcl-2 and Mcl-1, independent of canonical Notch signaling. In addition, we found that DTX1, an E3-ubiquitin ligase for Notch receptors degradation, is not expressed in GBM cells, resulting in abundance of Notch proteins. Restoration of DTX1 expression enhanced the degradation of Notch receptors in GBM cells. Moreover, epigenetic silencing of DTX1 expression in GBM cells can be restored using histone deacetylases inhibitors. In contrast to the 3′ end of the DTX1 gene, which is present in GBM cells, we did not find expression of the 5′ end of the DTX1 gene. We therefore propose that an alternative form of DTX1 is expressed in tumour cells that still has to be molecularly defined. Most likely, an alternative promoter is used in tumour cells that epigenetically silence regular DTX1. This study suggests a role of Notch proteins, of Notch2 in particular, in aberrant cancer signaling, impinging on tumour cell proliferation, survival and migration. These results provide a molecular basis for new therapeutic approaches against GBM.

Materials and methods

Tissue culture

The mutational status of the human glioma cell lines U87, U343, U373, LN18, LN71, LN215, LN229, LN319, LN401, LN405 and Hs683 for the *TP53*, *p16/p14* and *PTEN* genes has been previously established (Ishii et al., 1999). HeLa, MCF-7 and Calu-6 are human cervical carcinoma, breast cancer and colon cancer cell lines respectively. HEK-293, the human embryonic kidney derived non-cancer cell line is used as a control. All these cell lines were cultured at 37°C in 5% CO_2 in a humidified atmosphere. The culture medium was Dulbecco's Modified Eagle Medium (DMEM, No: 10938-025, Invitrogen Corporation, Carlsbad CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, No: S1810, Labforce, Basel, Switzerland), 1% Glutamax solution (v/v) (No: 35050-038, Invitrogen Corporation, Carlsbad CA, USA) and 1% (v/v) antibiotics solution (No: 15240-062, Invitrogen Corporation, Carlsbad CA, USA). Cell detachment was performed with 1x trypsin-EDTA (No: 25300-054, Invitrogen Corporation, Carlsbad CA, USA) for 5 minutes at 37°C.

Western analysis and antibodies

Cells were washed with 1xPBS, scraped in cold 1xSDS sample lysis buffer (2% (w/v) sodium dodecyl sulfate (SDS), 50mM Tris-Hcl pH 6.8, 10% (v/v) glycerol, 0.1M DTT), boiled at 95°C for 5 minutes and stored at –20°C. For the analysis, protein lysates were separated according to their size by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the concentration of the polyacrylamide ranging from 8 to 12% (v/v), depending on the molecular weight to be resolved. The separated proteins were transferred to nitrocellulose membranes (Hybond, ECL, Amersham Biosciences, USA). Subsequently, the membrane were blocked with 5% (w/v) non-fat dry milk in TBS-Tween (10 mM Tris-Hcl, 150 mM NaCl, 0.05% (v/v) Tween 20) and 1% (w/v) BSA. The membranes were probed with the following primary antibodies: Notch-1, Notch-2, Jagged-1 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa city CA, USA), Notch-3 (Abcam, Cambridge, UK), Delta-like-1, Bcl-2, Mcl-1, p21, phospho-ERK (Tyr 204 of p42 and p44), c-myc, β-arrestins-1 and -2, myc-tagged (Santa

Cruz Biotechnology, Santa Cruz CA, USA), Akt, phospho-Akt (Ser-473) (Millipore, Billerica MA, USA), ERK (p42 and p44) (New England Biolabs, Ipswich MA USA), actin (Sigma-Aldrich, SaintLouis MO, USA), anti-TN-C B28-13 (a kind gift from Prof. Ruth Chiquet-Ehrismann, FMI, Basel), anti-RBP-Jκ (Institute of Immunology, Tokyo, Japan), anti-GFP (Roche Diagnostics, Rotkreuz, Switzerland). Primary antibodies were diluted either in TBS-Tween or in 5% (v/v) non-fat dry milk, 1% (w/v) BSA in TBS-Tween overnight at 4°C. Three additional washes with TBS-Tween were performed before incubation with the appropriate secondary antibody (Horseradish peroxidase-conjugated anti-mouse, anti-rabbit, anti-goat and anti-rat immunoglobulins obtained from New England Biolabs, Ipswich MA, USA). Then, after three washes in TBS-Tween, signal detection by super signal (Pierce, Rockford IL, USA) was performed on X-ray films (Fujifilm, Tokyo, Japan). Protein bands were quantified using the software ImageJ (NIH, Bethesda MD, USA).

Anti-DTX-1 serum production

No good antibodies against the human Deltex-1 protein are available; therefore we generated a rabbit anti-serum for human Deltex-1 using two synthetic peptides located in the middle and in the c-terminus of the protein:

1. VRRYMQKVKNPPDEDC
2. PNPGKKFTTARGFPRHC.

Although multiple protein bands were present, a down-regulated band (blue arrow ~60 KDa) of the proper size could be identified using this ani-serum as shown in figure 47.

Proliferation determination by BrdU incorporation

Aliquots of number of required cells were plated to grow for 24 hours. Then the cells were incubated with the respective experimental inhibitors and drugs for the required time. BrdU (bromo-deoxyuridine) was added to the medium at 10μM final concentration and the cells were incubated at 37°C. After one hour, the cells were detached and prepared according to the manufacturer's instructions (BD Biosciences Pharmingen, San Diego CA, USA). Then, the cells were labelled with an anti-BrdU and a DNA staining dye. Combined detection of BrdU incorporation and DNA content was

performed using FACS (fluorescent automated cell sorting) analysis.

Apoptosis assessment by Annexin-V staining

Dead and viable cells were pooled and stained with 1μg/ml PI (propidium iodide) and Annexin-V-FITC, (BD Bioscinces Pharmingen, San Diego CA, USA), according to the instructions of the manufacturer. The cells were incubated at room temperature for 15 min and analyzed by flow cytometry. Annexin-V binds to those cells that present phosphatidylserine on the outer layer of the cell membrane, and PI stains the cellular DNA of those cells with a compromised cell membrane. This allows the discrimination between live cells (unstained with either fluorochrome), apoptotic cells (stained only with Annexin-V) and necrotic cells (stained with both Annexin V and PI). Dead cells were propidium iodide and/or Annexin-V positive.

Soft agar colony formation assay

The 0.6% (w/v) base agar of was prepared in 6 cm culture dishes, using 2.0% (w/v) stock agar solution (Agar Noble, Becton Dickinson, USA) and DMEM (without phenol red) (Invitrogen Corporation, UK) containing 10% (v/v) FCS, 1% (v/v) Glutamine and antibiotics solution. The mixture was poured into the plates, after the proper solidification of the agar, the cells were trypsinized and counted. Then, 0.3% (v/v) top agar was prepared with DMEM containing 5000 cells/plate and poured on the dishes and left at RT until the solidification of the agar. The plates were incubated at 37°C in humidified incubator for 10 to 15 days. At the end of the incubation period, the plates were stained with 0.5 ml of 0.005% (w/v) Crystal violet for at least one hour. Colonies bigger than 50μm were counted using a dissecting microscope. The histograms show the average value and the standard deviation resulting from three independent experiments.

Lentiviral packaging and transduction

HEK-293 cells were seeded at 40% density on 10 cm plates. After 24 hours, the packaging plasmid (pCMV_dr8_91), the plasmid coding for the envelope proteins (pMD2-VSV-G) and vector (pLKO.1-Scr, pLKO.1-puro containing shRNA for *NOTCH2, RBP-Jκ and DTX-1* from Sigma) that carries the gene of interest were transfected on HEK-293 cells using the $CaCl_2$ precipitation method. Within 8 to 10 hours

of the transfection, the medium was changed. At the end of 36 hours the supernatant was collected and centrifuged at 3500 RPM for 10 minutes. The supernatant was passed through a 0.45μm filter. The viral supernatant was mixed with protamine sulphate (at a final concentration of 5μg/ml). Then, the final filtrate was aliquoted and stored in -80°C. The supernatant was titrated on HeLa cells to ascertain the viral transduction efficiency. Then, the GBM cell lines were transduced and selected against the puromycin marker for stable clones.

shRNA sequences:

NOTCH2: "CCGGCCCACTAATAAGTGGTACTATCTCGAGATAGTACCACTTATTAGTGGGTTTTT" (3-UTR)

RBP-Jκ: "CCGGGCTGGAATACAAGTTGAACAACTCGAGTTGTTCAACTTGTATTCCAGCTTTTT" (3-UTR)

DTX-1: "CCGGGACCAAGAAGAAGCACCTTAACTCGAGTTAAGGTGCTTCTTCTTGGTCTTTTT" (1476)

"CCGGCCACTGCTATCTACCCAACAACTCGAGTTGTTGGGTAGATAGCAGTGGTTTTT" (1938)

Gene cloning and sub-cloning

Activated Notch2 (N2-IC, from nucleotides 5107 to 7425 of the *NOTCH2* cDNA sequence AF308601) was cloned into plasmid pcDNA3 (Invitrogen, Carlsbad, CA, USA). The plasmid, pEGFP-N3-*MAML1(DN)* encodes amino acids 12-74 of the human MAML1 gene, is a dominant negative mutant (kind gift from Prof. Jon C. Aster, Brigham Women′s Hospital, USA) (Weng et al., 2003). Human *DTX1*-myc (kind gift from Prof. Kimie Ohta, Keio University, Japan) (Matsuno et al., 1998) was sub-cloned into pcDNA 3.0-IRES-EGFP. The construct dominant-negative *RBP-Jκ* (Kato et al., 1996) was kindly provided by Prof. Tasuku Honjo (Kyoto University, Japan).

Site directed mutagenesis

In the Hs683 cell line, a mutation was found (L1711M) in the *NOTCH2* gene within the RBP-Jκ binding domain (RAM 23) (Tun et al., 1994). Using the site directed mutagenesis kit (Stratagene, La Jolla CA, USA), the same mutation was recreated in the pcDNA3.0-N2-IC construct.

Transfections and stable expression

In order to have stable expression for a given gene, the selected cell lines have been seeded at the required density. After, 24 hours, the cells were transfected with respective expression plasmids using the $CaCl_2$ precipitation method. Eight hours after transfection, the medium was changed and the cells were allowed to recover from the shock. After, the medium was replaced with selection medium containing the appropriate antibiotic at the required concentration. Within 15 to 20 days stable clones started to emerge. They were expanded and analyzed for expression of the transgene. The stable clones were used for further experiments.

Nucleic acid extraction and analysis

Genomic DNA

Genomic DNA was extracted using the genomic DNA purification kit (Qiagen, Hilden, Germany). Real-time quantitative PCR was performed on an ABI Prism sequence 7700 detector (PE Applied Biosystems, Foster, CA, USA) using the following primers:

marker D1S2696

forward : 5′ gaattacatcccaggcaatctga 3′

reverse : 5′ cacacaacaggcccctaatca 3′

probe: 5′ FAM-agcccatgctcattcccactacactgg-TAMRA 3′.

GAPDH

forward: 5′ aatgggactgaggctcccac 3′

reverse: 5′ ttatgggaaagccagtcccc 3′

probe: 5′ FAM-atccaagactggctcctccctgctg-TAMRA 3′.

RNA isolation

Each cell line was grown in 10 cm plates to 90% confluence. The medium was sucked off and the cells were washed with 1x PBS, lyzed in 1.0 mL Trizol (Invitrogen Corporation, Carlsbad CA, USA) and incubated for 3 minutes at room

temperature (RT). The cell lysates were homogenized and after 5 minutes, the cells were transferred into microfuge tubes and left at RT for another 3-5 minutes. 200µl choloroform was added and the tubes were vortexed for 30 seconds and left at RT for 5 min. After centrifugation at 14000 rpm for 15 min at 4°C, the upper (aqueous) phase was transferred into fresh tubes. Genomic DNA and protein contaminations were carefully avoided during this step. RNA was precipitated by addition of 500µl isopropanol. The RNA precipitate was pelleted down at 14000 rpm for 10 min at 4°C. The supernatant was removed and the RNA was washed with 1 mL 75% ethanol and pelleted down at 12000 rpm for 5 min at 4°C, air-dried on ice and re-suspended in 100 µl DEPC-treated H_2O. 5 µl of RNA solution was added to 495 dH_2O. Optical density was measured at 260 and 280 nm. The RNA in the remaining 95µl was precipitated by addition of 9.5 µl 4M LiCl, and 380 µl 100% EtOH. The concentration of RNA was calculated based on the OD_{260nm}, 1 OD unit is equivalent to 40 µg/mL.

cDNA synthesis

The volume corresponding to 5µg of RNA was centrifuged at 14000 rpm at 4°C for 20 min. The supernatant was carefully withdrawn. The pellets were air-dried on ice and re-suspended in 9 µl of DEPC-treated H_2O + lµl of random hexamer oligonucelotides. The RNA samples were denatured at 65°C for 5 min. cDNA synthesis master mix was added as per manufacturer's instructions (Invitrogen Corporation, Carlsbad CA, USA). The cDNA synthesis took place at 50 °C for 1 hour. The Thermoscript-RT was heat-inactivated at 85 °C for 5 min and the RNA template was degraded by 1 µl RNAse H at 37 °C for 20 min. Finally, the sample volume was adjusted to 50 µl by addition of DEPC-treated H_20. PCR actin primers checked the quality of the prepared cDNA.

At the end of the PCR, 12.5µl of each sample was mixed with 2,5 µl of gel loading buffer (GLB) and run on a 1.0% agarose gel to check for amplification of the target template. Once the cDNA quality was checked, it was used with other primer sets to study the expression of different genes.

Program used for amplification

95°C	2 min	
95°C	30 sec	35 cycles
58	1 min	
72	1 min	
72	5 min	

Primers:

MAML1: forward- 5′ ggagaagcaacagtttcagc 3′, reverse- 5′ atctgggttatgccagaagc 3′

MAML2: forward- 5′ tagtatggcaagcatgccac 3′, reverse- 5′ tgggtctcatttgcactgct 3′

DTX1: forward- 5′ ttgagaacgtgctgaaggag 3′, reverse- 5′ atgtccatatcgtaggccgt 3′

forward- 5′ gtactccaatggcaacaaggat 3′, reverse- 5′ ggatccaaactcggtcttgt 3′

HES-1: forward- 5′ cagccagtgtcaacacgaca 3′, reverse- 5′ tcgttcatgcactcgctgaag 3′

HES-5: forward- 5′ gcccaactccaagctgga 3′, reverse- 5′ tggaagtggtacagcagctt 3′

MCL-1: forward- 5′ ttatctctcggtaccttcgg 3′, reverse- 5′ tccagcaacacctgcaaaag 3′

BCL-2: forward- 5′ gatgactgagtacctgaacc 3′, reverse- 5′ tcacatcaccaagtgcacct 3′

NUMB: forward- 5′ ggttaagtaccttggccatg 3′, reverse- 5′ ttgacagccatgaagcagtg 3′

NUMBL: forward- 5′ gtgtgtgaagatgcggtgaa 3′, reverse- 5′ agtgccagaaaacatggca 3′

ITCH: forward- 5′ tgatcctcttggtccattgc 3′, reverse- 5′ cataggctatctgaggtcca 3′

FBW-7: forward- 5′ tgctccctaaagagttggca 3′, reverse- 5′ gtgctgtctgatgtatgcac 3′

ACTIN: forward- 5′ ggtgtaacgcaactaa 3′, reverse- 5′ gcatggagtcctgtggcatccacg 3′

Real-time PCR

Real-time quantitative PCR was performed on cDNA derived from GBM cell lines for *HES-1, HES-5* and *GAPDH* in ABI Prism sequence 7700 detector (PE Applied Biosystems, Foster, CA, USA). Relative expression was determined for each gene in comparison with *GAPDH* as internal control.

GAPDH

forward: 5′ aatgggactgaggctcccac 3′

reverse: 5′ ttatgggaaagccagtcccc 3′

probe: 5′ FAM-atccaagactggctcctccctgctg-TAMRA 3′.

HES-1

forward: 5′ ggacattctggaaatgacagt 3′

reverse: 5′ agcgcagccgtcatctg 3′

probe: 5′ FAM-cctccggaacctgcagcggg-TAMRA 3′.

HES-5

forward: 5′ gcccaactccaagctgga 3′

reverse: 5′ gacgaaggctttgctgtgc 3′

probe: 5′ FAM-gccatctccaggatgtcggcctt-TAMRA 3′.

DNA Sequencing: cDNA and genomic

NOTCH2 cDNAs from glioma cells and primary tumour were sequenced (310 Genetic analyser, applied biosystems, Foster, CA, USA) at mutation hot spots, i.e. EGF repeats 11-14, 24-25, 29 and 32, Lin-12 domains and the entire N2-IC. The *NOTCH2* mutation L1711M found in Hs683 cDNA was confirmed by sequencing genomic DNA.

Transwell migration assays

Transwell migration assays were performed using modified Boyden chamber units with polycarbonate filters of 8µm porosity (Costar, Appleton Woods, Birmingham, UK). The lower side of the filter was coated with 10µg/ml fibronectin for 2 hours at room temperature. The bottom chamber was filled with serum-free DMEM containing 0.1% (w/v) BSA plus/minus increasing amounts of purified human TN-C. The cells (10^4 cells/well in serum-free DMEM) were plated in the upper chamber and incubated for 16 hours at 37°C. After removal of the remaining cells from the upper surface of the filter, migrated cells at the bottom of the filter were fixed with 3.7% (v/v) formaldehyde in PBS

and stained with 0.1% (w/v) crystal violet. For each condition, the cells in five fields of three independent experiments were counted.

Statistical analysis

The significances of RNA and protein expression were established with the "t-Test: Two-sample assuming unequal variances" and correlation was calculated using "Pearson′s correlation" available in Mac Microsoft excel.

Abbreviations

APC: astrocytic precursor cells

BMP4: bone morphogenic protein 4

CDK: cyclin-dependent kinases

cDNA: Complementary DNA

CKI: CDK inhibitors

CML: Chronic myeloid leukemia

CNS: central nervous system

CTMP: carboxyl-terminal modulator protein

ECM: Extra-cellular matrix

EGF: Epidermal growth factor

EGFR: epidermal growth factor receptor

FAK: Focal adhesion kinase

FB: Fetal brain

FGF2: Fibroblast growth factor

GBM: Glioblastoma multiforme

GF-RTK: growth factor receptor tyrosine kinases

GIST: Gastro-intestinal tumour

GPC: glial progenitor cells

HIF-1: Hypoxia inducible factor-1

LOH: Loss of heterozygosity

MDM2: mouse double minute 2

MMTV: Mouse mammary tumour virus

NB: Normal brain

NF-1: Neurofibromatosis type 1

N-IC: Notch intracellular domain

NSC: neural stem cells

NSCLC: Non-small cell lung cancer

OG:Oligodendrogliomas

OPC: Oligodendrocyte precursor cells

PCR: Polymerase chain reaction

PDGF: Platelet derived growth factor

PIP3: phosphatidylinositol(3,4,5)-triphosphate

RB: retinoblastoma protein

RTK: Receptor tyrosine kinase

SCLC: Small cell lung cancer

TAD: Transcription activation domain

T-ALL: T-cell acute lymphoblastic leukemia

TCR-β: T-cell receptor β-chain

TGF-β: Transforming growth factor-β

TSA: Trichostatin-A

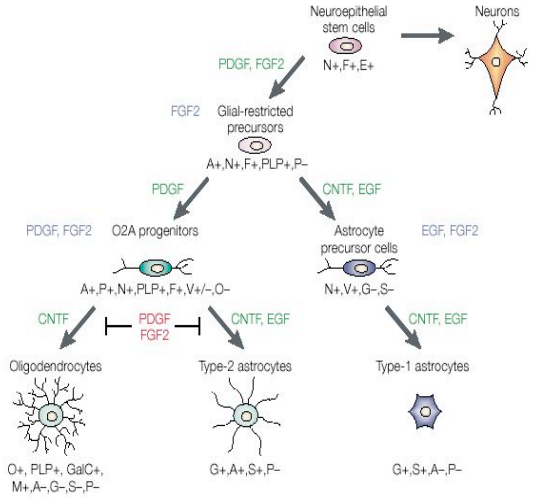
VEGF: Vascular endothelial growth factor

WHO: World Health Organization

Annex-1

CNS cellular differentiation

Multipotent neural stem cells in the ventricular/subventricular zones of the embryonic neural tube give rise to three main cell types: neurons, oligodendrocytes and astrocytes. The expression of specific markers identifies and defines the various stages of neural epithelial stem cell differentiation into astrocytes or oligodendrocytes. The growth factors that are involved in the promotion of specific lineage commitment, inhibition of differentiation, induction of proliferation and maintenance of cells at particular stages highlighted in green, red and blue respectively.



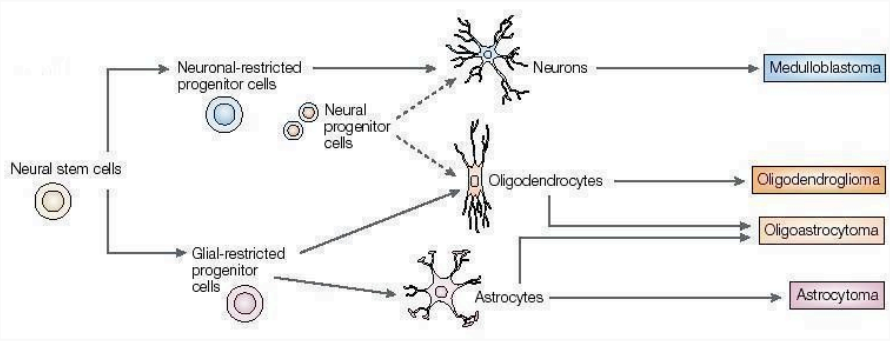
Eric C.Holland, Nature Reviews Genetics, 2001.

The glial-restricted precursors give rise to both astrocyte-restricted precursors and O2A progenitors, which develop into both astrocytes and oligodendrocytes. Platelet-derived growth factor (PDGF) signaling drives cells early in development towards the O2A progenitor cell type and maintain these cells in a proliferating state. Withdrawal of PDGF and fibroblast growth factor 2 (FGF2), and stimulation by ciliary neurotrophic factor (CNTF) and epidermal growth factor (EGF), drives these cells towards astrocyte and oligodendrocyte differentiation. Type 1 and type 2 astrocytes differ in morphology and marker expression. (A=A2B5, E=EGF receptor, F=FGF receptor, G=GFAP, GalC=galactocerebroside, M=myelin basic protein, P=PDGF receptor, PLP=myelin proteolipid protein, O=O4, S=S100, V=vimentin).

Annex-2

CNS glial tumour precursors

It is generally believed that neural stem cells first differentiate into two distinct progenitor cells, which further differentiate into neurons or glia respectively. Glial restricted progenitor cells further differentiate into astrocytes or oligodendrocytes.



Yuan Zhu & Luis F. Parada, Nature Reviews Cancer, 2002.

The classification of neurological tumours is based on their predominant cell type(s). Astrocytomas are composed primarily of astrocytes, oligodendrogliomas are composed primarily of oligodendrocytes and oligo-astrocytomas contain both astrocytic and oligodendroglial components.

Annex-3

Classification of gliomas

In the early 20th century, Bailey and Cushing introduced the seminal system to classify and grade glial tumours that was based on comparison of histological appearances and putative developmental stages of glia. Later, it was the basis for the current World Health Organization (WHO) classification and grading revised in 2000.

WHO grading system

WHO II	Astrocytoma (3-10 yrs)	Oligo-astrocytoma (5-12 yrs)	Oligodendroglioma (8-20 yrs)
WHO III	Anaplastic astrocytoma (2-5 yrs)	Anaplastic oligo-astrocytoma (2-8 yrs)	Anaplastic oligodendrogliomas (2-10 yrs)
WHO IV	Astrocytomas (1-2 yrs)		

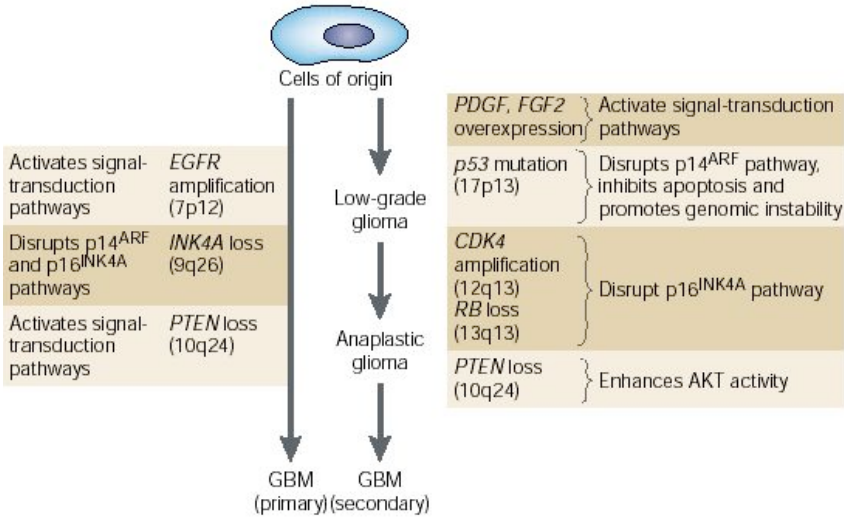
David N. Louis et al., American Journal of Pathology, 2001.

This system divides diffusive gliomas into astrocytic tumours, oligodendrogliomas and mixed oligo-astrocytomas. The degree of malignancy as determined by histopathological criteria, grades them on the scale of I to IV. The clinical outcome of patients entirely depends on the tumour grade since gliomas rarely metastasize outside the CNS. In parentheses survival duration has been given for each tumour type and grade. The survival time dramatically varies between grade-I and IV.

Annex-4

Two pathways to GBM

There are two sub-types of Glioblastoma multiforme (GBM), primary and secondary GBM. Both sub-types differ in their kinetics of gliomagenesis. Primary GBM forms *de novo* without earlier low-grade lesion. In contrary, secondary GBM is a progressive type that progress from low-grade lesion to higher-grade through the acquisition of additional mutations. In practice, GBM diagnosed with no earlier clinical record are considered as primary GBM. The mutations listed in the figure are subsets of those found in these tumours that have some correlation with tumour grade and type. Also, the biological effect of these mutations and changes in gene expression that contribute to gliomagenesis are highlighted.



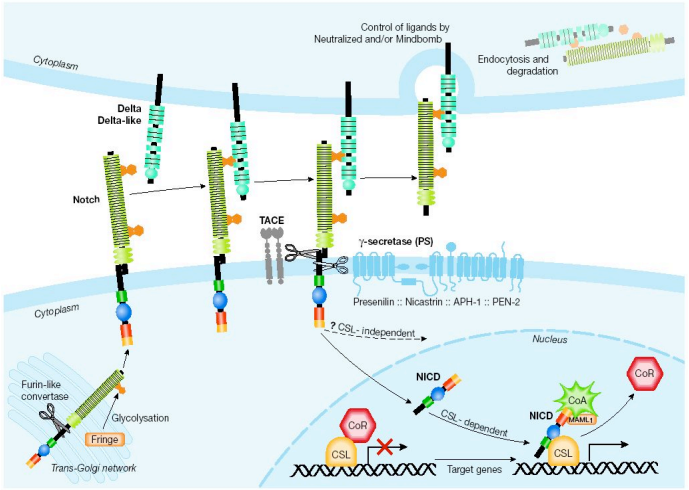
Eric C.Holland, Nature Reviews Genetics, 2001.

The analysis of mutations in both tumour sub-types indicates that the same genetic pathways (growth and cell cycle regulation) are targeted and that the frequency of specific genetic mutations differs between the sub-types. The median survival is statistically similar for both sub-types, which show equal capacity in proliferation, invasion and resistance to therapeutics. Both tumour sub-types are composed of highly infiltrative and more un-differentiated cells than low-grade astrocytomas.

Annex-5

Notch signaling

Notch receptors, single precursor proteins are cleaved by a Furin-like convertase in Golgi and transported to the cell surface as heterodimers. Within the Golgi, Fringe glycosyltransferases modify EGF-like repeats by adding N-acetylglucosamine. The signaling is initiated after ligand-receptor interaction, which induces two sequential proteolytic cleavages. The first one within the extracellular domain is mediated by the metalloprotease TACE (tumour necrosis factor α-converting enzyme).



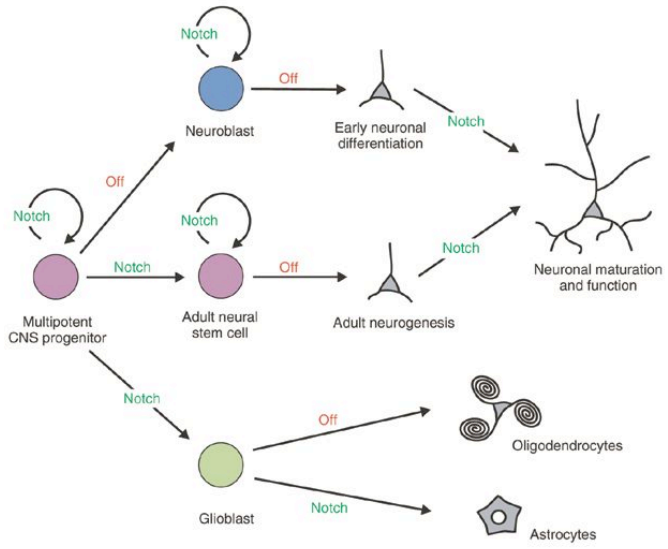
Freddy Radtke et al., EMBO reports, 2005.

The cleaved extracellular subunit of the receptor is 'trans-endocytosed' by the neighbouring ligand-expressing cell. This process seems to be controlled by Neuralized and/or Mindbomb E3 ubiqutin ligases. The second cleavage occurs within the transmembrane domain and is mediated by the γ-secretase activity of the multi-protein complex of presenilins (PS) (Nicastrin, APH-1 and PEN-2). The released Notch intracellular domain (N-IC) translocates into the nucleus and binds to the transcription factor CSL (CBF1 in humans, Supressor of Hairless in *Drosophila* and LAG in *C. elegans*). This interaction displaces the co-repressors (CoR) and simultaneous recruitment of co-activators (CoA), including mastermind-like proteins (MAML1). Receptors modified by Fringe glycosyltransferases cannot mediate signaling via Jagged ligands.

Annex-6

Notch signaling role in CNS development

As in the figure, Notch signaling affects several differentiation stages of neural precursors during development and in *in vitro* culture. The role of Notch signaling in each stage of neural precursor differentiation is revealed by both loss and gain of function studies. Notch signaling is important for the maintenance of self-renewing stem cells. The self-renewing stem cells can give rise to neuronal progenitors, the progression of which to neurons can be inhibited by Notch signal activation. Moreover, during adult neurogenesis Notch signaling involved in the maturation of neurons.



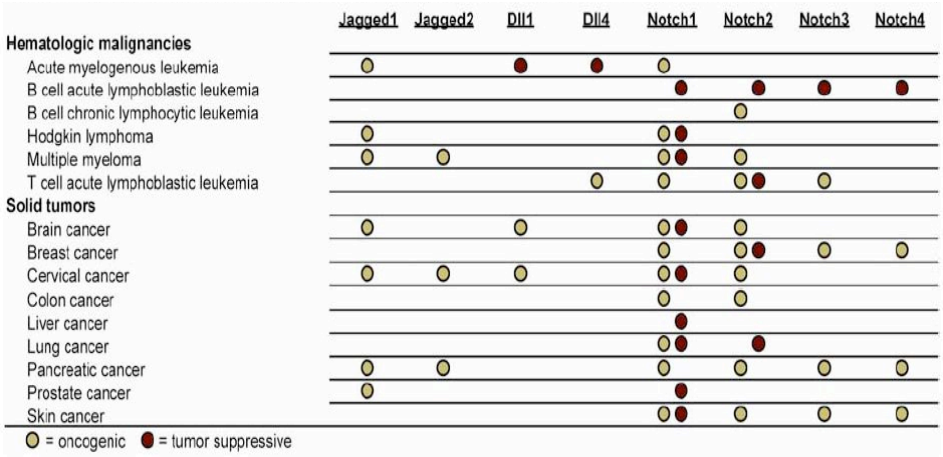
Yoon K & N Gaiano, Nature Neuroscience, 2005.

By contrast, a glial progenitor derived from a stem cell differentiates into an astrocyte with the help of Notch signals. Finally, oligodendrocyte precursors derived from glial progenitors fail to differentiate into mature oligodendrocytes in the presence of active Notch signals.

Annex-7

Notch signaling in cancer

Members of the Notch family of transmembrane receptors play an important role in cell fate determination. A role for Notch in the pathogenesis of hematological and solid malignancies has become apparent in the last decade. Notch signaling modulates numerous cellular functions and microenvironment cues associated with tumourigensis, including proliferation, apoptosis, adhesion, epithelial-to-mesenchymal transition and angiogenesis. From extensive studies across different cell types, it is becoming increasingly evident that Notch signaling can be both oncogenic and tumour suppressive.



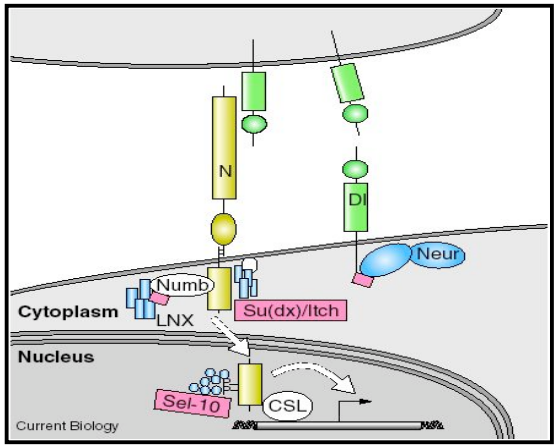
Kevin G. Leong & Aly Karsan, Blood, 2005.

So, it is becoming important to identify the cellular factors that determine the role of Notch signaling in tumourigenesis. Several factors determine the role of Notch signaling whether Notch should promote or suppress tumourigenesis; that includes specific Notch paralog activation (breast tumour, brain tumours), cell type (lung cancer), presence of specific cytokines/growth factor (GM-CSF) and dosage of Notch signaling (T-cell lymphoblastic lymphoma). Work on many different cancer types have advanced our understanding of the dual function of Notch signaling as both oncogene and tumour suppressor. Applying this knowledge to the Notch-related malignancies in the near future will hopefully lead to rational development of clinical therapeutics.

Annex-8

Notch degradation

Notch signaling, a developmental pathway, is tightly regulated by a variety of molecular mechanisms at different levels as marginal oscillation is sufficient to bring out the desired phenotypical changes. Loss and gain of function mutations in the Notch receptor and its pathway components are resulting in developmental defects and cellular transformation. Endocytosis is regulating the steady-state level of receptors, ligands and associated factors at the cell surface level. Lysosome mediated protein degradation is one mechanism that regulates the turnover of proteins in the cells and requires the ubiquitin machinery to ubiquitinate the targeted protein. That includes the ubiquitin activating enzyme (E1), conjugating-enzyme (E2) and E3-ubiquitin ligase.



Eric. C.Lai, Current Biology, 2002.

Notch receptors are undergoing degradation through two different E3-ubiquitin ligase mediated processes. Su(dx)/Itch may ubiquitinate and regulate plasma membrane associated Notch. Sel-10/Fbw-7 recruits an SCF complex that ubiquitinates nuclear and phosphorylated N-IC, thereby targeting it for degradation by proteasome. Neur is targeting Delta for endocytosis and subsequent degradation while LNX is targeting Numb, for degradation by proteasome.

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