Notch2 signaling in development and cancer

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Jan Stephan Tchorz

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Table of contents

1. Summary 1

2. General introduction 2

2.1. The Notch signaling pathway 2

2.2. Notch signaling in intrahepatic bile duct development and Alagille syndrome 3

2.3. Notch signaling in brain development and glioblastoma multiforme 5

2.4. Aim of this study and contributions 8

3. Results 10

3. 1. Paper 1 10
3.1.1. Abstract 11
3.1.2. Introduction 12
3.1.3. Materials and methods 14
3.1.4. Results 16
3.1.5. Discussion 19
3.1.6. Acknowledgements 22
3.1.7. Figure legends 22
3.1.8. Figures 27

3.2. Paper 2 35
3.2.1. Abstract 36
3.2.2. Introduction 36
3.2.3. Materials and methods 38
3.2.4. Results 41
3.2.5. Discussion 45
3.2.6. Acknowledgements 48
3.2.7. Figure legends 48
3.2.8. Figures 52

4. Concluding remarks 61

5. Acknowledgements 65

6. References 66

7. Appendix 78

1. Summary

Notch signaling via cell to cell interaction is a key mechanism in regulating proliferation, cell fate decisions and survival of various cell types in both invertebrates and vertebrates. Among the four Notch receptors (Notch1-4) described in mammals, most studies focused on Notch1, while Notch2 functions are poorly understood. In humans, *Notch2* mutations are associated with a variety of developmental disorders and tumor formation in several tissues. In the liver, *Notch2* deletion was shown to cause Alagille syndrome (AGS), which is characterized by impaired intrahepatic bile duct (IHBD) development. In glioblastoma multiforme (GBM), the most aggressive form of CNS tumors, *Notch2* is amplified and high Notch2 levels correlate with poor prognosis. However, the exact role of *Notch2* in these human pathologies is not established. In order to investigate the function of *Notch2* in AGS and GBM at the level of cells, tissues and organs, I generated transgenic mice that allow for tissue-specific expression of activated Notch2 (Notch2ICD), which mimics ligand-induced activation of Notch2 signaling.

To address the function of Notch2 signaling in AGS and IHBD development, Notch2ICD expression was induced in hepatoblasts. Hepatoblasts are the bipotential progenitors in the liver that give rise to either hepatocytes or biliary epithelial cells (BECs) that undergo tubulogenesis to form IHBDs. I observed that ectopic Notch2ICD expression in hepatoblasts induces biliary epithelial cell (BEC) differentiation, tubulogenesis of IHBDs, and BEC survival. These findings shed light on the role for *Notch2* in AGS, since they provide an explanation why AGS patients with *Notch2* mutations suffer from impaired IHBD development.

It is believed that GBM originates from glioma stem cells (GSCs) which can derive from developmentally stalled neural stem cells (NSCs). Thus, I addressed whether Notch2 plays a role in regulating NSC proliferation and differentiation, possibly predisposing NSCs to become GSCs and eventually GBMs. Therefore, I generated mice that ectopically express activated Notch2 in NSCs and compared the induced molecular alterations to those in GSCs from GBM cell lines and primary GBM biopsies. I show that key features of GSCs, such as increased proliferation and

astrocytic lineage commitment, are induced by ectopic Notch2 signaling in NSCs. Aberrant Notch2 expression may therefore predispose NSCs to become GSCs that give rise to GBMs. Moreover, Notch2 signaling enhanced survival of GBM cells, possibly explaining the increased aggressiveness of GBMs with high Notch2 levels. Therefore, blockade of Notch2 signaling may interfere with GBM cell survival, and the formation and proliferation of GSCs and thus be of therapeutic benefit for the treatment of GBMs, for which no cure is available yet.

2. General introduction

2.1. The Notch signaling pathway

Notch was first described by T.H.Morgan in 1917 in a Drosophila strain characterized by notched wing blades (1). More than 50 years later, the first review on *Notch* by T.Wright started with the sentence: "If one was asked to choose the single, most important genetic variation concerned with the expression of the genome during embryogenesis in *Drosophila melanogaster*, the answer would have to be the *Notch* locus" (2). Although this statement might have been regarded as an exaggeration 40 years ago, today the Notch pathway is one of the most extensively studied developmental pathways with more than 10.000 publications listed in Pubmed. *Notch* was shown to encode a surface receptor with an extracellular domain containing epidermal growth factor (EGF)-like repeats (3,4). Because *Notch* mutants display abnormalities in neural fate decisions, it was originally classified as a neurogenic gene (5). However, it was soon discovered that hardly any tissue in *Drosophila* is unaffected by *Notch* mutations and it is now clear that Notch signaling plays a conserved role in a large variety of developmental processes from C.elegans to humans (6). Mammals express four Notch receptors (Notch1-4) and five ligands (Delta-like1, Delta-like3, Delta-like4, Jagged1 and Jagged2). Both Notch receptors and Notch ligands are single-pass transmembrane receptors. After its synthesis in the endoplasmatic reticulum (ER), the Notch receptor is transported through the secretory pathway to the trans-Golgi network, where the Notch receptor precursor

protein undergoes intramolecular cleavage by a furin-like convertase (S1 cleavage) to form heterodimers, composed of an extracellular and a transmembrane subunit (7-9). Ligand-binding to Notch receptors on neighboring cells leads to the proteolytic cleavage of the extracellular region of the Notch transmembrane subunit by ADAM-type metalloproteases (S2 cleavage) (7-9). The S2-cleavage triggers a subsequent cleavage within the Notch transmembrane domain by a multiprotein protease complex known as γ-secretase (S3-cleavage), releasing the Notch intracellular domain (NotchICD) (8,10). Then, NotchICD translocates into the nucleus and converts RBPjκ from a transcriptional repressor into a transcriptional activator (11). In cooperation with the mastermind-like I (MAML1) adaptor protein, the NotchICD/RBPjκ complex activates Notch effector genes, such as *Hairy* and *Enhancer of Split* homologs (e.g., *Hes1 or Hes5*) and other basic helix-loop-helix (bHLH) transcription factors (12-14). Besides the NotchICD/RBPjκ mediated (canonical) signaling, few Notch functions involving non-canonical signaling pathways have been described, such as the activation of the AKT pathway regulating cell survival and the blockade of myogenesis which both occur in a RBPjκ independent manner (15-17). Since Notch signaling is activated upon cell to cell contact, it is a critical pathway in regulating cell fate decisions, proliferation and survival. Thus, dysregulation of Notch signaling is implicated in a large variety of developmental defects, including many tumors (15,18,19).

2.2. Notch signaling in intrahepatic bile duct development and Alagille syndrome

Bile plays an essential role in fat digestion and transportation of metabolized and detoxified liver products. After bile synthesis in hepatocytes, bile passes from the liver to the gall bladder via bile ducts. During digestion, bile is then released from the gall bladder into the intestine where it emulsifies fats. Dysfunction of the biliary system is a significant cause of liver diseases, morbidity and mortality in humans. While the large extrahepatic bile ducts (EHBDs) develop by branching of the primitive hepatic diverticulum, the smaller IHBDs represent the largest component of the biliary tree and form within the developing liver (20-24). During liver development in mammals, the bipotential hepatic precursors termed hepatoblasts give rise to

either hepatocytes or BECs. Hepatoblasts in close contact to the portal mesenchyme differentiate into BECs, whereas lobular hepatoblasts become hepatocytes. Upon differentiation of periportal hepatoblasts into BECs, these cells form a continuous single cell layer around the portal vein, called ductal plate. During ductal plate remodeling, BECs begin to form tubular structures at distinct sites of the ductal plate, while ductal plate BECs that did not contribute to tubulogenesis gradually disappear. Tubular structures mature into proper IHBDs and connect to the biliary tight junction network that transports bile acids from lobular hepatocytes towards the bile ducts (20-24). During early postnatal development, bile ducts mature, are subsequently incorporated into the portal mesenchyme and dilate into the biliary tree (21,22,25,26). Although it has been suggested that local cues from the portal mesenchyme trigger differentiation of hepatoblasts into BECs and are crucial for IHBD development, the exact molecular mechanisms underlying these processes are poorly understood.

Our understanding of mechanisms regulating IHBD development has improved since the discovery of *Jagged1* and *Notch2* mutations in AGS patients. AGS is a rare hereditary multisystem disorder that accounts for a wide range of developmental abnormalities in liver, heart, eye, skeleton and kidney. The abnormalities in liver development are characterized by neonatal jaundice, impaired differentiation of IHBDs, and chronic cholestasis (27-30). Since haploinsufficiency of *Jagged1* as well as *Notch2* mutations cause AGS, Jagged1-Notch2 signaling was suggested to be a key pathway in regulating IHBD development (31). Mice heterozygous for the *Jagged1* and *Notch2* null alleles recapitulate most of the developmental abnormalities seen in human AGS, including IHBD development defects (32). Jagged1 is expressed in the portal mesenchyme, while hepatoblasts express Notch2 receptors (33,34). Thus, cell to cell contacts between Jagged1 expressing portal mesenchyme cells and Notch2 expressing hepatoblasts could possibly explain the locally restricted differentiation of BECs during IHBD development. However, the presence of a ductal plate in mice with liver-specific ablation of *Notch2* leads to the assumption that Notch2 signaling is dispensable for BEC differentiation. Since these mice displayed disorganized IHBDs, it was believed that Notch2 regulates ductal remodeling, IHBD maturation and IHBD maintenance (31,35,36). Deletion of Notch1 in the liver showed that

Notch1 is not required for IHBD development (35,37). In contrast, Notch1ICD expression induced BEC markers in cultured hepatoblasts. However, it was not sufficient to induce mature BECs (38). Thus, the role of Notch2 during BEC fate specification, the induction of tubulogenesis and survival of BECs is still controversial. To better understand IHBD development and the phenotype seen in AGS patients, reliable Notch2 gain-of-function studies are required.

2.3. Notch signaling in brain development and glioblastoma multiforme

In vertebrates, the central nervous system (CNS) emerges from the neural tube which is formed during early embryonic development. The neural tube is an epithelial structure consisting of neuroepithelial cells, which differentiate into numerous types of neurons and supportive (glia) cells present in the mature CNS. Neurons are generated during early embryonic life from multipotent NSCs close to the ventricle. After their final mitotic division they migrate to their destinations where they terminally differentiate and integrate into the brain circuitry. Glial cell generation occurs at late embryonic and early postnatal stages in the proliferating subventricular zone. Brain morphogenesis is finally achieved by a concerted action of proliferation, differentiation and migration of neurons and glial cells (39-43). Neurogenesis is not only observed during brain development, but continues in the adult brain throughout life (44,45). Adult neurogenesis is mainly restricted to subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus. In the SVZ, adult neural stem cells give rise to neuroblasts which then migrate through the rostral migratory stream (RMS) into the olfactory bulb, where they finally mature and integrate into olfactorial circuits. Adult neural stem cells in the SGZ mainly become Doublecortin (Dcx) expressing neuroblasts that migrate into the granule cell layer (GCL) of the dentate gyrus where they mature into NeuN expressing granule neurons. Adult neurogenesis is controlled by a large variety of locally defined regulatory networks, including the Notch1 pathway (46-48).

The exact role of Notch signaling during neural development is best understood in Drosophila, where Notch was shown to inhibit differentiation by lateral signaling and to regulate cell fate decisions by inductive interactions (49,50). Notch signaling in mammals is more complex, since they express four Notch receptors (compared to only one in Drosophila) and genetic manipulations in mammals are more laborious than in fruit flies. Although our understanding of Notch signaling during mammalian neurogenesis has grown in recent years, much more detailed insights are required. Notch target genes such as *Hes* and *Herp* (Hes-related protein) (12,16) antagonize proneural genes like *Mash1* and neurogenins (51). This antagonism largely blocks early neuronal gene expression and therefore accounts for the inhibitory effect of activated Notch1 on neurogenesis (46,48-50,52). Consistent with this finding, null mutations in either *Notch1* or its target gene *Hes1* lead to precocious neuronal differentiation in the mouse embryo, indicating that Notch1 signaling is important in maintaining the embryonic NSC pool (52-54). While neurogenesis is largely blocked by Notch1 activation, gliogenesis was shown to be increased (55). Recently, it was demonstrated that Notch1 signaling accounts for the delayed generation of astrocytes during brain development by regulating the demethylation of astrocyte-specific genes (56). Another study argues that Notch1 first specifies glial progenitors and then promotes astrocyte versus oligodendrocyte fate decisions (57).

Besides the conserved role of Notch1 in regulating cell fate decisions in the developing CNS, it was shown that activated Notch1 increases the proliferation of NSCs (58) and the upregulation of antiapoptotic genes (59). In adult mice, Notch1 regulates proliferation and differentiation of adult NSCs in the dentate gyrus (46). While Notch1 is well studied during both embryonic and adult neurogenesis, the functions of other Notch receptors in these processes are poorly understood. *Notch2* ablation caused early lethality in embryos with widespread cell death in the developing CNS, while Notch3 and Notch4 mutant mice were viable and showed no obvious phenotype in the CNS (60-63). Notch2 receptors are expressed throughout the embryonic (64,65) and adult NSC niche (46,65). Glial-specific *Notch2* ablation revealed that Notch2 is required for the monolayer formation and morphogenesis of Bergmann glia (66). Moreover, activation of Notch2 signaling inhibits the differentiation of cerebellar granule neurons in mice

(67). The requirement of Notch2 signaling for glial development and its inhibitory effect on neuronal differentiation in the cerebellum suggests a similar function for Notch2 as it was shown for Notch1. However, a role for Notch2 signaling in NSCs of the developing or adult brain is not established.

Since the Notch signaling pathway is crucial for the concerted regulation of proliferation, differentiation and survival in the CNS, dysregulation of this pathway is implicated in many CNS tumors, including GBMs (18,19,68). GBMs are glial tumors (gliomas) and represent the most common incurable brain tumor in adults. The mean survival of patients with high grade GBMs lies within months and more than 70% of GBM patients die within 2 years of diagnosis (69). Primary GBMs usually form in the cerebral white matter, grow quickly and can become very large before producing symptoms. Thus, GBMs are often diagnosed in advanced stages that make surgical removal difficult and cure impossible. Rarely, secondary GBMs can form following degeneration of lower grade gliomas and are more common in younger patients (70,71). Since gliomas were first recognized and treated in the mid-19th-century, a tremendous amount of data was published concerning possible origins and therapies of GBM. However, despite all the advances in tumor imaging, surgery, chemotherapy and radiation therapy, survivability of GBM could not be significantly increased (70,71). New treatment options were envisioned since the discovery of a highly tumorigenic subpopulation of stem-like cells, termed glioma stem cells (GSCs), within surgical isolates of GBMs (71-73). GSCs are resistant against cytotoxic drugs and radiation therapy, have a high migration potential, and only few GSCs suffice to induce GBM formation in xenograft models (68,70). Thus, GSCs potentially account for the aggressiveness of GBMs and the high rate of recurrence after surgery. It is believed that GBMs originate from GSCs that can derive from developmentally stalled NSCs (68,71,73). However, the understanding of molecular events causing GSC formation, proliferation, differentiation and survival is very limited, but desperately needed in order to validate new targets that might improve GBM therapy. Frequent mutations in GBMs are observed in genes encoding for EGFR, TP53, PTEN, ARF and INK4A (68). In addition, it was recently reported that most GBMs express high levels of Notch2*, while* loss of *Notch2* is frequently found in oligodendroglioma and

correlates with a much better prognosis. (74). While the role of Notch2 signaling in GBM formation is poorly understood, there is evidence that Notch1 regulates proliferation and survival of GBM cells (75). Moreover, inhibition of Notch signaling using γ-secretase inhibitors sensitizes GSCs to radiation therapy (76) and inhibits xenograft growth (76,77). It is therefore possible that Notch2 and Notch1 function similarly in regulating GSC proliferation and survival, possibly explaining the increased aggressiveness of GBMs with high Notch2 levels (74). Gliomas are classified and named according to the cell type they mainly resemble. While GBMs belong to the class of astrocytomas which have an astrocytic cellular identity, oligodendrogliomas mainly contain oligodendrocytes (78). Notch1 directs astrocyte versus oligodendrocyte fate decisions in NSCs (57). Notch2 is highly expressed in GBMs and frequently deleted in oligodendrogliomas (74,79). A possible function of Notch2 signaling in directing glial fate decisions similar to Notch1 might therefore contribute to the cellular identity of glial tumors.

A general functional redundancy for the activated domains of Notch1 and Notch2 was postulated based on domain swap experiments, although this study omits analysis within the CNS (80). However, this postulation was challenged since activated Notch1 and Notch2 have opposite effects on embryonal brain tumor growth (81) and on mesothelioma cell survival (82). Thus, the specific functions of Notch2 signaling during brain development and GBM remain to be addressed.

2.4. Aim of this study and contributions

The aim of this thesis was to characterize the role of Notch2 signaling during brain and liver development and related disorders, such as GBM and Alagille Syndrome, respectively. Therefore, the development of an experimental mouse model that enables tissue-specific activation of Notch2 was a prerequisite for the studies performed during my PhD studies.

Using this novel mouse model, I aimed to characterize the role of Notch2 signaling in AGS. Since *Notch2* mutations were found in many AGS patients that suffer from impaired differentiation of intrahepatic bile ducts (IHBDs) (30), I wanted to dissect the effect of Notch2 signaling during IHBD development. Therefore, I specifically expressed Notch2ICD in hepatoblasts to study the potential of Notch2 signaling in BEC differentiation, tubulogenesis into IHBDs and survival. Possible functions of Notch2 in these steps of IHBD development could explain why Alagille patients lack properly developed bile ducts and make major contributions for the understanding of liver development in general.

Based on the finding that *Notch2* was amplified in GBMs and high Notch2 levels correlated with poor prognosis (74), I wanted to dissect the oncogenic potential of Notch2 in GBM formation and progression. Since GSCs that can derive from developmentally stalled NSCs are believed to initiate GBM formation, I aimed to identify a possible role of Notch2 signaling in NSC to GSC transition. Therefore, I studied the molecular consequences of ectopic Notch2 signaling in NSCs and compared these to the molecular alterations observed in GSCs from GBM cell lines and primary GBM biopsies. Besides a further understanding of the mechanisms leading to GBM formation and progression, this study could provide substantial new insights into brain development and regulation of adult neurogenesis.

Jochen Kinter helped with the biochemical analysis for paper1 shown in figure 1. Dimitri Cloëtta and Mercedes Tome performed the *in vitro* analysis on Notch2 signaling in NSCs in paper2 (Figure 4 and 6). Balasubramanian Sivasankaran and Roland Huber generated the genetically modified GBM cell lines and performed western blot analysis for figure 5 and 6, respectively (paper2). Michal Grzmil performed the correlation analysis in primary GBM biopsies for figure 7 in paper2.

3. Results

3.1. Paper1

Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice

Jan S. Tchorz [1] , Jochen Kinter [2] , Matthias Müller [3], Luigi Tornillo [4], Markus H. Heim [5] and Bernhard Bettler [1]

[1]Department of Biomedicine, Institute of Physiology, University of Basel, Switzerland
[2]Department of Biomedicine, Division of Neurology, University of Basel, Switzerland
[3]Novartis Institute for Biomedical Research, Novartis Pharma AG, Basel, Switzerland
[4]Institute for Pathology, University Hospital Basel, Basel, Switzerland
[5]Department of Biomedicine, Division of Gastroenterology and Hepatology, University of Basel, Switzerland

Abbreviations

IHBD, intrahepatic bile duct; BEC, biliary epithelial cell; Notch2ICD, Notch2 intracellular domain; AGS, Alagille syndrome; RMCE, recombinase-mediated cassette exchange; DBA, dolichos biflorus agglutinin; HNF, hepatocyte nuclear factor; ZO1, zona occludens 1

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3.1.1. Abstract

Intrahepatic bile duct (IHBD) development begins with the differentiation of hepatoblasts into a single continuous biliary epithelial cell (BEC) layer, called ductal plate. During ductal plate remodeling, tubular structures arise at distinct sites of the ductal plate, forming bile ducts that dilate into the biliary tree. Alagille syndrome patients, which suffer from bile duct paucity, carry *Jagged1* and *Notch2* mutations, indicating that Notch2 signaling is important for IHBD development. To clarify the role of Notch2 in BEC differentiation, tubulogenesis and BEC survival, we developed a mouse model for conditional expression of activated Notch2 in the liver. We show that expression of the intracellular domain of Notch2 (Notch2ICD) differentiates hepatoblasts into BECs, which form additional bile ducts in periportal regions and ectopic ducts in lobular regions. The additional ducts in periportal regions are maintained into adulthood and connect to the biliary tight junction network, resulting in an increased number of bile ducts per portal tract. Remarkably, Notch2ICD expressing ductal plate remnants were not eliminated during postnatal development, implicating Notch2 signaling into BEC survival. Ectopic ducts in lobular regions did not persist into adulthood, indicating that local signals in the portal environment are important for maintaining bile ducts. *Conclusion*: Notch2 signaling regulates BEC differentiation, the induction of tubulogenesis during IHBD development and BEC survival.

3.1.2. Introduction

During liver development both hepatocytes and biliary epithelial cells (BECs) arise from common bipotential progenitors called hepatoblasts (20,23,24). Hepatoblasts in the liver parenchyma differentiate into hepatocytes, whereas those adjacent to the portal mesenchyme differentiate into BECs. Early intrahepatic bile duct (IHBD) development in both humans and rodents is characterized by the formation of the so-called ductal plate, a single continuous cell layer containing BECs. During ductal plate remodeling tubular structures arise at distinct sites of the ductal plate and are subsequently incorporated into the portal mesenchyme. Postnatal non-tubular ductal plate remnants get eliminated while the tubular structures become mature IHBDs that dilate into the biliary tree (21,22,25,26). It is generally assumed that the periportal environment is required for BEC differentiation and tubulogenesis, but the exact mechanisms underlying these processes are poorly understood. Several factors contributing to bile duct development have recently been identified, including Notch2 (21,31,83). The Notch signaling pathway is highly conserved throughout evolution and plays an important role in cell fate determination via cell-cell contacts. Mammals express four Notch receptors (Notch1-4) with five ligands (Dll1, Dll3, Dll4, Jagged1 and Jagged2). Ligand-binding to Notch receptors on neighboring cells leads to the proteolytic processing and translocation of the Notch intracellular domain (NotchICD) into the nucleus. NotchICD then forms a complex with RBPjκ, leading to the transcriptional activation of Notch effector genes, such as *Hairy* and *Enhancer of Split* homologs (e.g. *Hes1*) (49,84).

Alagille syndrome (AGS) is a rare hereditary multisystem disorder caused by haploinsufficiency of Jagged1 (OMIM #118450) (27-29) as well as Notch2 mutations (OMIM#610205) (30). AGS patients display a wide range of developmental abnormalities in liver, heart, eye, skeleton and kidney. The abnormalities in liver development are characterized by neonatal jaundice, impaired differentiation of IHBDs and chronic cholestasis (27-29). Mice with a haploinsufficiency for *jagged1* (85) or a liver-specific ablation of *jagged1* (86) exhibit no IHBD abnormalities. However, mice heterozygous for the *jagged 1* and *Notch2* null allele

recapitulated most of the developmental abnormalities seen in human *Alagille* syndrome, including IHBD development defects (32). Similarly, liver-specific deletion of *Notch2* using AlbCre mice caused severe defects in IHBD development, characterized by disorganized tubular structures accompanied by portal inflammation, portal fibrosis and foci of hepatocyte feathery degeneration in adulthood (35,36). The formation of a ductal plate and the presence of BECs in these mice suggested that Notch2 is dispensable for BEC differentiation and mostly required for IHBD development. However, a role for Notch2 signaling in BEC differentiation cannot be excluded, since AlbCre-mediated *Notch2* ablation experiments are not entirely conclusive. It is known that albumin expression begins around E13.5 and that AlbCre-mediated gene ablation occurs progressively with age (87). Thus, as pointed out by Geisler and colleagues (35), partial *Notch2* ablation or embryonic Notch2 levels may still allow differentiation of hepatoblasts into BECs, which starts around embryonic day 15. Unfortunately, mice with a complete ablation of Notch2 die before the onset of BEC differentiation (60), which prevented studying possible effects of Notch2 signaling in this process. Supporting that Notch signaling plays a role in BEC differentiation, it was shown that expression of activated Notch1 in cultured hepatoblasts represses hepatocyte differentiation and induces the expression of BEC markers (38). However, expression of activated Notch1 did not produce cells with the morphological characteristics of mature BECs and did not result in the formation of tubular structures. Notably, conditional ablation of *Notch1* in the liver revealed that Notch1 signaling is dispensable for IHBD development (35,37). Thus, the role of Notch2 during BEC fate specification, the induction of tubulogenesis and survival of BECs is still controversial. For this reason we wanted to further address the role of Notch2 during IHBD development by generating mice that express Notch2ICD in hepatoblasts, which mimics ligand-induced activation of Notch2. These mice not only allowed us to study the role of Notch2 signaling in its cognate portal environment, but also at ectopic lobular sites. Lobular Notch2ICD expression shed light on the inherent potential of Notch2 signaling in hepatoblasts, since it dissociated Notch2 signaling from local cues in the portal environment.

3.1.3. Materials and methods

Generation of N2ICD and N2ICD/AlbCre mice

Recombinase-mediated cassette exchange (RMCE) was used to introduce the cDNA encoding the intracellular domain of Notch2 (Notch2ICD; Swiss Prot #O35516, aa1699-aa2470) with a C-terminal human 5xMyc-tag into the modified Rosa26 locus of Balb/c mouse embryonic stem (ES) cells (Figure 1A). The Myc-tag allows immunohistochemical detection of transgenic Notch2ICD protein expression. This anti-Myc antibody (A-14, Santa Cruz) is specific for the human Myc epitope and exhibits no cross-reactivity with the endogenous mouse c-Myc in immunohistochemical experiments (Supplementary Figure 1). The RMCE plasmid contained a pBS-SK(+) backbone, with FRT sites flanking the chicken β-actin (CAGS) promoter (88), a floxed transcriptional STOP cassette (89), the Notch2ICD-5xMyc cDNA and a HSVtkNeo cassette. ES cells with cassette exchange events were identified by PCR. ES cells with preserved karyotypes were used for blastocyst injection as described (90). Chimeric mice were mated with Balb/c mice. Mice with the conditional Notch2ICD-Myc transgene (N2ICD mice) were identified by genotyping. N2ICD mice were born at a Mendelian ratio and exhibited no overt phenotype. N2ICD mice were crossed with AlbCre mice expressing Cre-recombinase under the liver-specific albumin promoter (87). Single-transgenic AlbCre and N2ICD littermates and wildtype mice were used as controls in our experiments. Genotyping of the mice was performed by TaqMan analysis. In brief, small tail biopsies were digested in proteinase-K containing lysis buffer overnight at 55°C. The 1:10 diluted digested samples were then genotyped by TaqMan-PCR using the following 5'-3' primers and probes: Cre-fw (gccgcgcgagatatgg), Cre-rv (gccaccagcttgcatgatc), Cre-probe (Fam- ccgcgctggagtttcaataccgg-Tamra), Rosa26-N2ICD-fw (atatccgcggtggagatcaa), Rosa26-N2ICD-rv (tagaccaggctgggctaaa), Rosa26-N2ICD-probe (MGB-cggtaccagatctc-Tamra). Southern blots with genomic liver DNA were done using a 1.2kb hybridization probe derived from the neomycin resistance gene using a standard protocol (Figure 1A,B). All animal experiments were performed in accordance with governmental guidelines and approved by the veterinary office of Basel.

Immunohistochemistry, Western blots and quantitative real-time PCR (RT-PCR)

Mice were sacrificed by cervical dislocation and livers were removed. Samples for protein, DNA and RNA analysis were immediately frozen at -80°C. Samples for histological examination were fixed overnight with 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4°C. Tissue used for ZO1 staining was freshly frozen and postfixed in methanol at -20°C for 10min. 10µm thick cryostat sections were mounted, washed in PBS and incubated for 1h in blocking solution (2% BSA and 0.2% Triton X-100 in PBS) followed by overnight incubation at 4°C in blocking solution containing primary antibodies or dolichos biflorus agglutinin (DBA, biotinylated or FITC coupled, Vector Laboratories, Burlingame, CA). The primary antibodies used were rabbit anti-HNF4α (H-171, Santa Cruz, Santa Cruz, CA), rabbit anti-HNF1β (H-85, Santa Cruz), goat anti-Myc (A-14, Santa Cruz), rabbit anti-ZO1 (Zymed, San Francisco, CA), rabbit anti-Ki67 (Novocastra, UK) and rat anti-Notch2 (C6551.6DbHN, Developmental Studies Hybridoma Bank, Iowa City, IA). After rinsing in PBS, sections were incubated in blocking solution containing secondary antibodies for 1h at RT. The secondary antibodies used were Cy3-conjugated donkey anti-rabbit and Cy5-conjugated donkey anti-sheep, (Jackson Immunoresearch, UK). 4µm thick paraffin-embedded tissue sections were rehydrated and antigen retrieval was performed in citrate buffer (10mM, 0.01% Tween20, pH6.0) for 20min. Incubation with the primary antibodies was performed as described above. Immunostaining was completed using the Vectastain ABC Kit (Vector Laboratories). Cellular colocalization of immunohistochemical markers was quantified (≥3 mice per genotype and age) using Volocity Software (Improvision, UK). Immunoblotting and light cycler quantitative RT- PCR was performed as described (91).

3.1.4. Results

Liver-specific transgenic expression of Notch2ICD

To study the role of Notch2 signaling during IHBD development, we developed a mouse model for conditional expression of Notch2ICD in hepatoblasts (Figure 1A). We generated N2ICD mice harboring a Notch2ICD-Myc fusion construct downstream of a floxed STOP cassette and crossed them with AlbCre mice (87), which yielded N2ICD/AlbCre mice. The expression of Cre recombinase from the albumin promoter leads to the excision of the STOP cassette and the expression of the Myc-tagged Notch2ICD protein. In the embryonic liver, the onset of albumin expression in hepatoblasts occurs around E13.5, when bipotential hepatoblasts begin to differentiate into either hepatocytes or BECs (22). Successful excision of the STOP cassette in genomic N2ICD/AlbCre liver DNA was confirmed by Southern blot analysis (Figure 1B). Western blot analysis of liver homogenates confirmed the expression of the Myc-tagged Notch2ICD protein in N2ICD/AlbCre mice (Figure 1C). N2ICD/AlbCre livers exhibited a 2-fold increase in the mRNA level of the Notch2 target gene *HES1*, thus confirming that the Myc-tagged Notch2ICD protein is signaling-competent (Figure 1D).

Notch2ICD regulates differentiation of hepatoblasts into BECs

To study the fate of Notch2ICD expressing cells, we performed co-immunostaining studies at different time points during embryonic and postnatal development. At E16.5 transgenic Notch2ICD expression, detected by Myc-staining, was observed in a mosaic pattern in both periportal and lobular regions (Figure 2A). 96% of Notch2ICD expressing cells were positive for the hepatoblast marker HNF4α and 70% expressed HNF1β, a transcription factor mediating BEC fate (Figure 2B) (92). At this developmental stage, BECs in both N2ICD/AlbCre and control mice showed negligible DBA staining, a lectin that specifically binds to mature BECs (93). This is consistent with previous reports showing that at E16.5 BECs are still at an immature stage (33). At E18.5 the number of HNF1β+/Myc+ cells increased to more than 80%, whereas the number of HNF4α+/Myc+ cells decreased to ~30%, indicative of a differentiation of HNF4α+/HNF1β+ cells to HNF4α-/HNF1β+ cells (Figure 2B). HNF1β+ cells close to the periportal environment

were strongly DBA+, while those in lobular regions were weakly DBA+ (Figure 2A). Notably, both Myc+ and Myc- BECs within the portal region were DBA+ (data not shown). At birth (P0), HNF1β+/Myc+ cells in the lobules became strongly DBA+, suggesting maturation of HNF1β+/DBA- to HNF1β+/DBA+ cells in a portal-to-lobular manner (Figure 2A). During early postnatal development the number of HNF1β+/Myc+ cells further increased and peaked at P4, while the number of HNF4α+/Myc+ cells decreased to less than 5% at P4. This suggests a progressive differentiation from HNF4α+/HNF1β+ into HNF4α-/HNF1β+ cells. Interestingly, a small subset of Myc+ cells remained HNF4α+/HNF1β- and their number increased progressively from P4 to P90 (Figure 2A, left panels). Due to the misspecification of lobular Myc+ hepatoblasts into HNF4α-/HNF1β+/DBA+ BECs cells, the number of HNF4α+ cells was extremely low at P4, but increased progressively with age until a normal level of about 60% was reached at P90 (Figure 2C). We therefore assessed proliferation in N2ICD/AlbCre mice by Ki67 staining (Supplementary Figure 2A, B). Increased proliferation was observed at P4 and P90 but not at E18.5. Proliferation was restricted to morphologically identified hepatocytes, whereas proliferation in DBA+ BECs was negligible (Supplementary Figure 2A). In addition, co-staining for Myc and Ki67 showed that Notch2ICD expression did not alter proliferation (data not shown). The postnatal increase in hepatocyte proliferation in N2ICD/AlbCre mice resulted in increased liver weight (Supplementary Figure 2C). We assume that the increased postnatal proliferation of hepatocytes in N2ICD/AlbCre mice compensates for the extremely low number of hepatocytes seen at P4 (Figure 2C). In summary, our data support that Notch2ICD expression in bipotential hepatoblasts promotes their progressing differentiation from HNFα+/HNF1β+/DBA- to HNF4α-/HNF1β+/DBA- cells and eventually to HNF4α-/HNF1β+/DBA+ BECs (Figure 5).

Notch2ICD expressing biliary epithelial cells in the lobules form ectopic tubular structures

During ductal plate remodeling, tubular structures forming bile ducts arise at distinct sites of the ductal plate. Findings from AGS patients (27-30) and mouse studies (32,33,35,36,86,94) both implied a role for Notch2 in ductal plate remodeling and tubulogenesis. We therefore examined whether Notch2ICD expression in Alb-Cre/N2ICD mice is able to induce tubulogenesis at ectopic lobular sites. We analyzed tubulogenesis at P4 by costaining for Myc/HNF1β/DBA

(Figure 3A-D) and HNF1β/Hematoxylin (Figure 3E,F). We found that Notch2ICD expressing BECs in P4 N2ICD/AlbCre mice indeed formed ectopic tubular structures in the lobules (Figure 3A-D, Figure 2A). Most of these ectopic tubular structures had a lumen (Figure 3E) and were HNF1β+/DBA+ (Figure 3 C,D). However, some ectopic tubular structures were disorganized and some HNF1β+/DBA+ cells did not participate in tubular structures. Ectopic tubular structures first appeared at P0 and their number increased until P4. At P10 most ectopic tubular structures had disappeared (Figure 2A). These data suggest that Notch2 signaling promotes tubulogenesis, but that it is not sufficient to maintain tubular structures into adulthood.

Transgenic Notch2ICD expression results in an increased number of bile ducts

We next investigated whether the expression of Notch2ICD results in an increased number of bile ducts in the periportal environment. We stained liver sections from P90 N2ICD/AlbCre (Figure 4A) and littermate control (Figure 4B) mice for DBA and Hematoxylin and quantified the number of bile ducts per portal tract. We observed up to 7 bile ducts per portal tract in N2ICD/AlbCre mice, while control mice never showed more than 4 ducts per portal tract. Portal tracts with 5 or more bile ducts were seen in 8.3 % of all N2ICD/AlbCre portal tracts analyzed (Figure 4G). N2ICD/AlbCre mice also showed a significant increase in the mean number of DBA+ ducts per portal tract compared to control littermates (2.20 ± 0.06 versus 1.52 ± 0.04; Mann-Whitney test P=0.0022, n=6 mice per genotype). We next addressed whether Myc+/DBA+ ducts are connected to the biliary canaliculi network, a tight junction network transporting bile from hepatocytes to the bile ducts (95) Myc+/DBA+ ducts stained for the tight junction marker zona occludens 1 (ZO1) at P10 and P90 (Figure 4E,F). This indicates that Myc+/DBA+ ducts are connected to the biliary network, which suggests that these bile ducts are functional. In support of this, Bilirubin, alanine aminotransferase (ALAT), alkaline phosphatase (AP) (Supplementary Figure 3) and gamma glutamyl transferase (GGT) levels (<5U/l for all animals tested) were normal in N2ICD/AlbCre mice.

Myc+/DBA+ ducts in the periportal region of N2ICD/AlbCre mice persisted into adulthood. In contrast, ectopic tubular structures were no longer observed in the lobules of adult mice.

However, some loosely associated Myc+/HNF1β+ cells were still present at P90 in the lobules of N2ICD/AlbCre mice but not of control mice (Figure 4C,D). It appears that the numbers of these cells decreased after P4 and that they lost their DBA marker (Figure 2A). We further observed that ductal plate cells, which normally get eliminated within the first 2 weeks after birth, are still present at P90 in N2ICD/AlbCre mice and that these cells express Notch2ICD (Figure 2A, 4A). This suggests that Notch2ICD promotes BEC survival. However, Notch2ICD expression is clearly not sufficient to maintain bile ducts in the absence of additional portal signals.

3.1.5. Discussion

Our understanding of the mechanisms regulating IHBD development has improved since the discovery of *Jagged1* and *Notch2* mutations in AGS patients (27-30). There is experimental evidence showing that Notch2 signaling is required for tubulogenesis of bile ducts during ductal plate remodeling (32,33,35,36,86,94). Whether Notch2 signaling is required for the differentiation of hepatoblasts into BECs is less clear. Notch2 knock-out studies using AlbCre mice for conditional gene ablation tend to support that Notch2 is dispensable for BEC differentiation (35,36). However, AlbCre-driven gene ablation in embryos is incomplete and occurs progressively with age (87). Therefore, BEC development could still be supported by residual Notch2 signaling. Moreover, Notch1ICD expression in cultured hepatoblasts caused the induction of BEC markers while hepatocyte markers were repressed, pointing to a possible role for Notch signaling during BEC differentiation (38). It is known that periportal hepatoblasts differentiate into BECs whereas lobular hepatoblasts differentiate into hepatocytes (21). One possibility is that cell-cell contacts of Notch2 receptor bearing hepatoblasts and ligand-expressing cells in the portal environment are responsible for the spatially confined formation of BECs. To test this possibility, we developed a novel mouse model that allows for conditional expression of Notch2ICD in bipotential hepatoblasts. We show that Notch2ICD expression not only induces BEC differentiation in periportal but also in lobular regions, clearly demonstrating that Notch2 signaling can induce BEC differentiation remote from periportal cues. Notch2ICD

expression in HNF4α+ hepatoblasts triggers progressing differentiation to HNF4α-/HNF1β+/DBA+ BECs through HNFα+/HNF1β+/DBA- and HNF4α-/HNF1β+/DBA- intermediate stages. While this is clearly the predominant differentiation pathway of Notch2ICD expressing hepatoblasts, we also observed Myc+/HNF4α+ cells with mature hepatocyte morphology in P90 N2ICD/AlbCre mice. These cells could derive from lobular HNF4α-/HNF1β+/DBA+ cells that transdifferentiate into hepatocytes due to the lack of signals required for the maintenance of the BEC phenotype in the lobular environment. Alternatively, these cells might represent a subset of hepatoblasts that were already committed towards the hepatocyte fate at the time when Notch2ICD expression occurred. It is therefore possible that progressing transdifferentiation of HNF4α-/HNF1β+/DBA+ cells into hepatocytes as well as a compensatory proliferation of Notch2ICD expressing hepatocytes contribute to the increase in Myc+/HNF4α+ cells observed from P4 to P90 (Figure 2B).

At P4, N2ICD/AlbCre livers featured multiple ectopic tubular structures, mostly containing a lumen. Remarkably, tubulogenesis of BECs not only occurred in periportal but also in lobular regions, suggesting that Notch2 signaling can promote tubulogenesis in the absence of periportal cues. However, diffusible periportal cues may still be necessary for ectopic BEC differentiation and the formation of ectopic tubular structures. At P10 most ectopic tubular structures in the lobule were lost. In contrast, the additional periportal tubular structures survived into adulthood, which resulted in an increased number of ducts per portal tract. The periportal ducts were connected to the biliary canaliculi network, since Myc+/DBA+ ducts stained for ZO1, thus indicating functional bile ducts. N2ICD/AlbCre mice displayed preserved liver chemistries, supporting that their biliary system functions normally. In summary, our data indicate that signals from the periportal environment are crucial for preserving functional bile ducts in adult livers.

It was proposed that Notch2 signaling is absent in ductal plate cells that do not contribute to bile duct formation and that these cells get eliminated (33). In agreement with this, ductal plate cells were progressively eliminated within the first 2 weeks after birth in control mice.

However, in N2ICD/AlbCre mice Notch2ICD expressing ductal plate cells persisted into adulthood, implicating Notch2 signaling into BEC survival. Notch1ICD was shown to increase survival of neuronal precursor cells by upregulating the anti-apoptotic proteins Mcl1 and Bcl2 (59). It is possible that Notch2 promotes survival via the same mechanism.

The present study shows that transgenic expression of Notch2ICD in hepatoblasts triggers differentiation into BECs, induces tubulogenesis and promotes BEC survival. However, the spatial and temporal profile of Notch2 signaling during normal IHBD development remains to be addressed in more detail. Kodama and colleagues showed that Notch2 and Hes1 expression is restricted to the parts of the ductal plate where tubulogenesis occurs (33). *Hes1* null mice lacked proper ductal plate remodeling despite the presence of a ductal plate (33), similar as mice with a liver-specific *Notch2* ablation (35,36). As reviewed by Frederic Lemaigre (31), these findings suggest that the Jagged1-Notch2-Hes1 cascade induces ductal plate remodeling. However, it is still unclear how Notch2 contributes to the formation of mature bile ducts emerging from the single-layered ductal plate. Our data shows that hepatoblasts differentiate into BECs upon activation of the Notch2 pathway. Jagged1 expression was found within ductal plate BECs (34). Thus, ductal plate BECs expressing Jagged1 could activate Notch2 in adjacent hepatoblasts, differentiating them into BECs. Our data also show that activated Notch2 induces BECs to form tubular structures. It is therefore possible that Notch2 regulates ductal plate remodeling by differentiating neighboring hepatoblasts into BECs, which in turn participate in tubulogenesis.

The main findings of our study are summarized in the model presented in Figure 5. We demonstrate that transgenic Notch2ICD expression in bipotential hepatoblasts leads to their differentiation into BECs and to the formation of additional tubular structures in portal regions and ectopic tubular structures in lobular regions. Additional periportal ducts are connected to the biliary tight junction network and maintained in adult mice. In contrast, ectopic lobular ducts are lost during postnatal development, suggesting a crucial role for the portal environment in mediating bile duct maintenance. Remarkably, ductal plate cells that express

transgenic Notch2ICD persist into adulthood, suggesting that Notch2 also plays a role in BEC survival.

3.1.6. Acknowledgement

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3.1.7. Figure legends

Figure 1. Liver-specific expression of Notch2ICD. (A) Scheme depicting the generation of N2ICD mice, which allow for Cre-mediated expression of Notch2ICD. The RMCE plasmid contains the ubiquitously active chicken β-actin (CAGS) promoter. The CAGS promoter is silenced by a STOP sequence flanked by loxP sites. The Notch2ICD is C-terminally tagged with a human 5xMyc epitope. The CAGS promoter, STOP sequence, the Notch2ICD and a neomycin resistance cassette (neoR) are flanked as a group by FRT sites. Flipase-mediated recombination in modified mouse embryonic stem (ES) cells was used to exchange a hygromycin resistance cassette (hygR) flanked by FRT sites with the Notch2ICD targeting construct in the ROSA26 locus. Correctly recombined ES cells were used to generate N2ICD mice. N2ICD mice were crossed with AlbCre mice, to induce Cre-mediated excision of the STOP cassette flanked by loxP sites, resulting in liver-specific Notch2ICD expression. (B) Site-specific integration of the Notch2ICD targeting construct into the ROSA26 locus and Cre mediated excision of the STOP

cassette was analyzed on Southern blot using a neoR-specific hybridization probe and genomic liver DNA. Recombination of the Notch2ICD targeting construct into the Rosa26 locus yields a 5.6kb EcoRV fragment in N2ICD mice, which is absent in wildtype mice. N2ICD/AlbCre mice display an additional 4.1kb band, indicating excision of the STOP cassette. (C) Western blot analysis of liver homogenates shows that Notch2ICD levels are highly increased in N2ICD/AlbCre mice compared to wildtype and single transgenic N2ICD mice. Myc-tagged Notch2ICD was only present in N2ICD/AlbCre mice, showing that the STOP cassette in N2ICD mice is not leaky. Actin controls for sample loading. (D) Light cycler quantitative real-time PCR for the Notch target gene Hes1 shows a 2-fold increase in Hes1 mRNA levels in N2ICD/AlbCre mice (n=3), confirming that the Myc-tagged Notch2ICD protein is functional. Mean ± SEM, P**≤ 0.01.

Figure 2. Notch2ICD differentiates hepatoblasts into BECs. (A) Liver sections were prepared from mouse embryos (E16.5, E18.5), postnatal (P0, P4, P10) and adult (P90) N2ICD/AlbCre and control mice (n=3-5). Markers for hepatoblasts/hepatocytes (HNF4α) and BECs (HNF1β and DBA) were used to follow differentiation of hepatoblasts into BECs. HNF1β is expressed at early stages of BEC differentiation, while DBA binds to mature BECs. The portal vein is outlined with a dashed line. Immunostaining using an anti-Myc antibody reveals a mosaic expression pattern of the Myc-tagged Notch2ICD in both portal and lobular regions. Co-immunostaining for Myc and HNF4α (left panels) or HNF1β (middle left panels) shows progressing differentiation of Notch2ICD expressing hepatoblasts towards the biliary lineage. HNF1β/DBA co-immunostaining shows that the maturation of BECs occurs in a portal (E18.5) to lobular (P0-P4) manner in N2ICD/AlbCre mice (middle right panels). BEC differentiation through a HNF1β+/DBA- intermediate stage is also seen in control sections (right panels). Ductal plate BECs (arrows) persist to P90 in N2ICD/AlbCre mice, while they are gradually eliminated during postnatal development in control mice. Multiple ectopic tubular structures (arrow heads) are present throughout the liver in P4 N2ICD/AlbCre mice. Ectopic tubular structures and DBA markers gradually disappeared within lobular regions after P4, while periportal extra ducts survive into adulthood (asterisk). (B) The percentage of Myc+ cells expressing HNF4α and HNF1β was

determined by co-immunostaining (E16.5-P90) and quantified. Both HNF4α and HNF1β are co-expressed in Myc+ cells at E16.5-E18.5. From P0 onwards Myc+ cells mostly express the BEC marker HNF1β+. Notably, a small percentage of Myc+ cells remain HNF4α+ and their percentage increases progressively from P4 to P90. (C) The number of HNF4α and HNF1β expressing cells in N2ICD/AlbCre mice normalized to DAPI shows an increase in HNF1β+ cells, accompanied by a dramatic decrease of HNF4α+ cells which peaks at P4. After P4 the number HNF4α+ cells increases progressively until P90. Bar diagrams (B, C) show mean ± SEM. Scale bar 50 μm.

Figure 3. Notch2ICD induces ectopic tubular structures remote from the periportal environment. Liver cryosections from P4 N2ICD/AlbCre mice were stained for Myc, HNF1β and DBA (A-D). Paraffin sections from P4 N2ICD/AlbCre and control mice were stained for the BEC marker HNF1β and Hematoxylin (E, F). (A-D) Ectopic tubular structures containing BECs are present throughout the lobules of N2ICD/AlbCre mice, as demonstrated by HNF1β staining (A). The outlined area was magnified showing HNF1β (B), Myc/HNF1β (C) and HNF1β/DBA (D) stained ectopic tubular structures. (E, F) HNF1ß/Hematoxylin stained N2ICD/AlbCre (E) lobular sections show ectopic tubular structures, mostly containing a lumen. Control mice do not express HNF1ß in the lobules (F). Cell clusters with strong Hematoxylin staining represent hepatic hematopoietic stem cells. Scale bar (A) 200μm, (B-F) 50μm.

Figure 4. Adult (P90) N2ICD/AlbCre mice have an increased number of bile ducts per portal tract. (A-B) Hematoxylin- and DBA-stained paraffin-embedded sections of N2ICD/AlbCre (A) mice and control (B) livers showing 6 and 2 ducts (arrowheads) per tract, respectively. 5 and more bile ducts per tract were only seen in N2ICD/AlbCre mice, but were relatively rare (see Figure 4G for quantification).Some DBA+ ductal plate cells (arrows) were still present in N2ICD/AlbCre mice at P90 (A) but not in control mice (B).(C-D) Ectopic lobular BECs in N2ICD/AlbCre mice remain HNF1β+ (C). No lobular HNF1β+ cells are found in control mice (D). (E-F) Co-immunostaining for Myc, DBA and the tight junction marker ZO1 shows that Myc+ periportal bile ducts are connected to the biliary canaliculi network in both P10 (E) and P90 (F)

N2ICD/AlbCre mice. Areas outlined in D, E and F are enlarged in the corresponding insets. (G) Number of bile ducts per portal tract in N2ICD/AlbCre and control littermate mice (n=6 per genotype). N2ICD/AlbCre mice had up to 7 bile ducts per portal tract, controls had never more than 4. pv, portal vein. Bar diagram (G) shows mean ± SD. Scale bar (A-D) 50µm, (E-F) 100µm.

Figure 5. Model of IHBD development in N2ICD/AlbCre mice. Upon Notch2 activation, bile duct development begins with the differentiation of HNF4a+ hepatoblasts into HNF1β+/DBA+ BECs, passing HNF4α+/HNF1β+ and HNF4α-/HNF1β+ BEC differentiation stages. While this is the predominant differentiation pathway of Notch2ICD expressing hepatoblasts, we also observed some Myc+/HNF4α+ cells with mature hepatocyte morphology. These cells could either derive from lobular HNF4α-/HNF1β+/DBA+ BECs that transdifferentiate into hepatocytes or from hepatoblasts that already were committed to become hepatocytes, possibly due to delayed expression of Notch2ICD (dashed arrows). Ectopic Notch2 signaling promotes BEC differentiation and the formation of tubular structures in the lobules, showing that Notch2 signaling can promote tubulogenesis remote from the periportal environment. This is consistent with a conditional lack of Notch2 signaling (Notch2 cKO) disrupting tubulogenesis (*Geisler et al [18], Lozier et al [19], highlighted in grey). Notch2ICD expressing tubular structures are only maintained in the periportal environment. Tubular structures in the lobules are eliminated during postnatal development, presumably leaving behind dedifferentiated HNF1β+/DBA- bile duct cells. This suggests that cues from the periportal environment are required for maintaining bile ducts in adult livers.

Supplementary Figure 1. Anti-Myc immunostaining detects the Myc-tagged transgenic Notch2ICD protein but no endogenous mouse c-Myc. Liver cryosections from embryonic (E16.5, E18.5) N2ICD/AlbCre and control mice were immunostained using an anti-Myc antibody raised against the human c-Myc epitope. Staining for DAPI and Myc shows that the anti-Myc antibody specifically detects Myc-tagged Notch2ICD in N2ICD/AlbCre mice, but no endogenous c-Myc

protein in control mice. Nuclear colocalization of DAPI- and Myc- immunostaining shows that the transgenic Notch2ICD protein efficiently translocates into the nucleus. Scale bar 50µm.

Supplementary Figure 2. N2ICD/AlbCre mice show postnatal compensatory hepatocyte proliferation. (A) Costaining for DAPI, DBA and Ki67 in E18.5, P4 and P90 liver sections from control and N2ICD/AlbCre mice (n=3 per genotype) show proliferation in hepatocytes but not in DBA+ BECs. While hepatocyte proliferation is increased in N2ICD/AlbCre mice at P4 and P90 compared to control mice, no difference was observed at 18.5. (B) Quantification of the immunostained Ki67+ cells in percent of DAPI for control N2ICD/AlbCre mice are shown in a bar diagram. (C) Quantification of the liver weight as a percentage of body weight shows an increased liver weight in P4, P10 and P90 N2ICD/AlbCre mice compared to control mice. Bar diagrams (B, C) show mean ± SEM. P*≤ 0.05, P**≤ 0.01, P***≤ 0.001.

Supplementary Figure 3. Adult N2ICD/AlbCre livers exhibit normal values for bilirubin, alanine aminotransferase (ALAT) and alkaline phosphatase (AP). Blood plasma from adult (P90) control and N2ICD/AlbCre mice (n=5) was analyzed for changes in bilirubin (A), ALAT (B) and AP (C). 1 out of 5 N2ICD/AlbCre mice showed an increased bilirubin value.

3.1.8. Figures

Figure 1

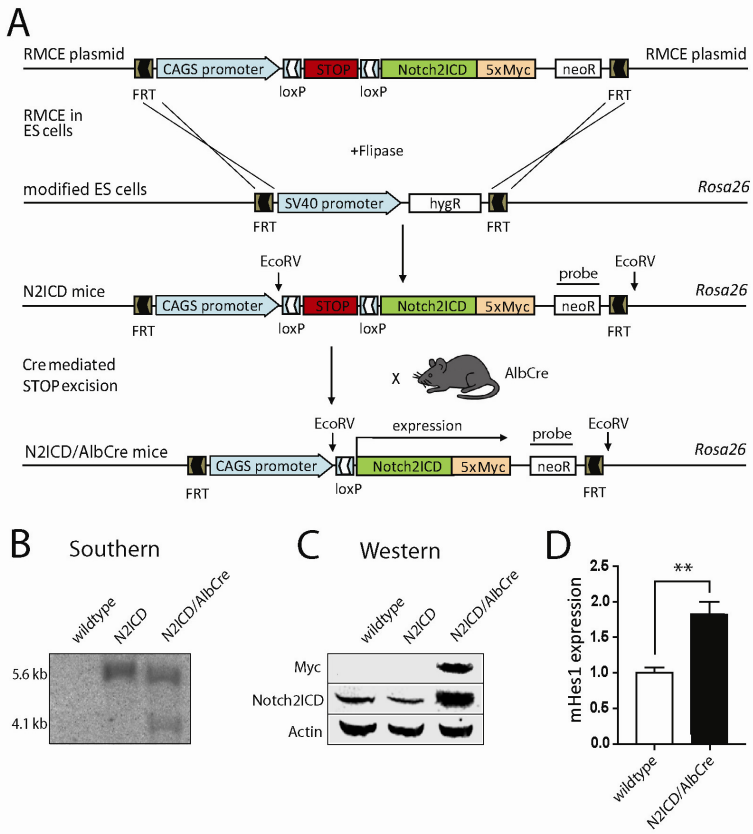
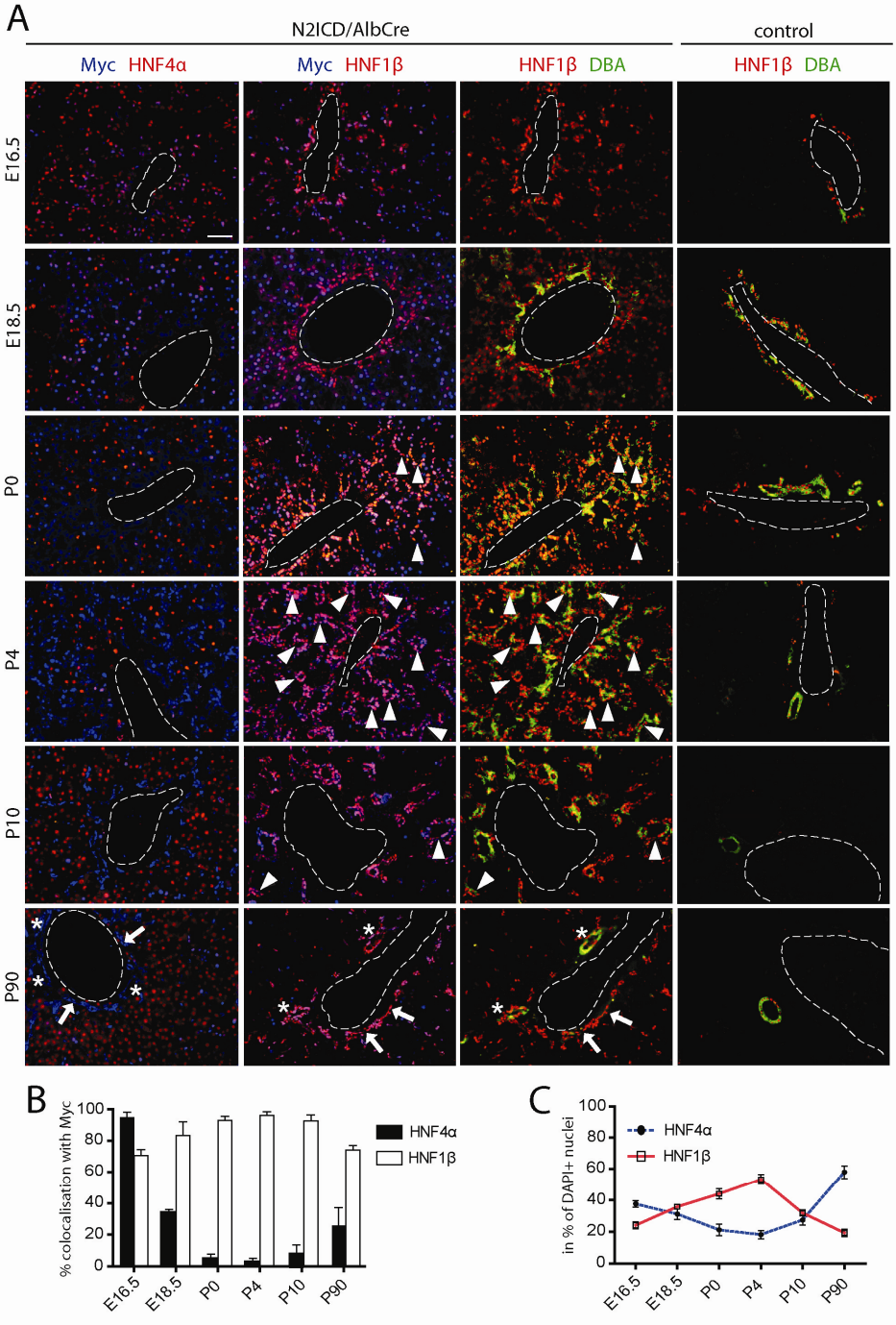


Figure 2


A
N2ICD/AlbCre
control
Myc HNF4α
Myc HNF1β
HNF1β DBA
HNF1β DBA
E16.5
E18.5
P0
P4
P10
P90
B
% colocalisation with Myc
100
80
60
40
20
0
E16.5
E18.5
P0
P4
P10
P90
HNF4α
HNF1β
C
in % of DAPI+ nuclei
100
80
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HNF4α
HNF1β
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E18.5
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P4
P10
P90

Figure 3

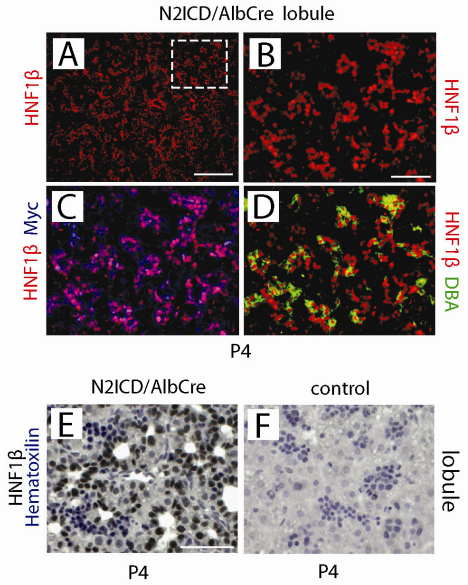

N2lCD/AlbCre lobule
A
B
C
D
HNF1β
HNF1β
HNF1β Myc
HNF1β DBA
P4
N2lCD/AlbCre
control
E
F
HNF1β
Hematoxilin
lobule
P4
P4

Figure 4

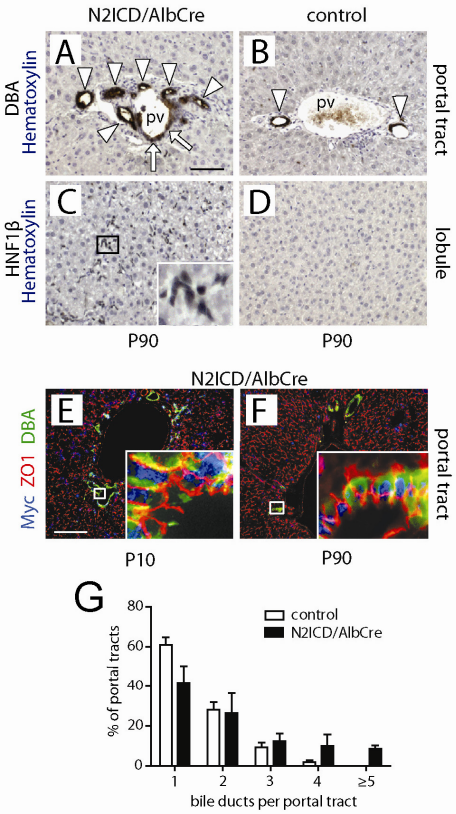
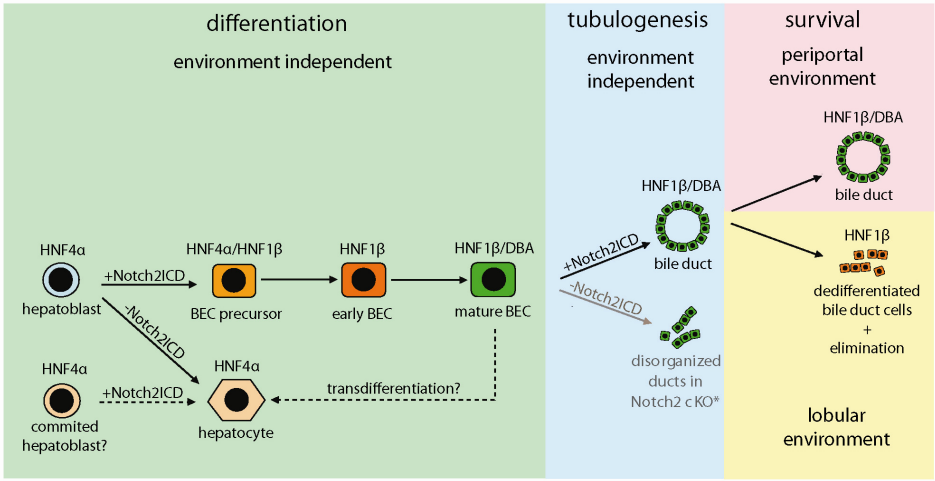
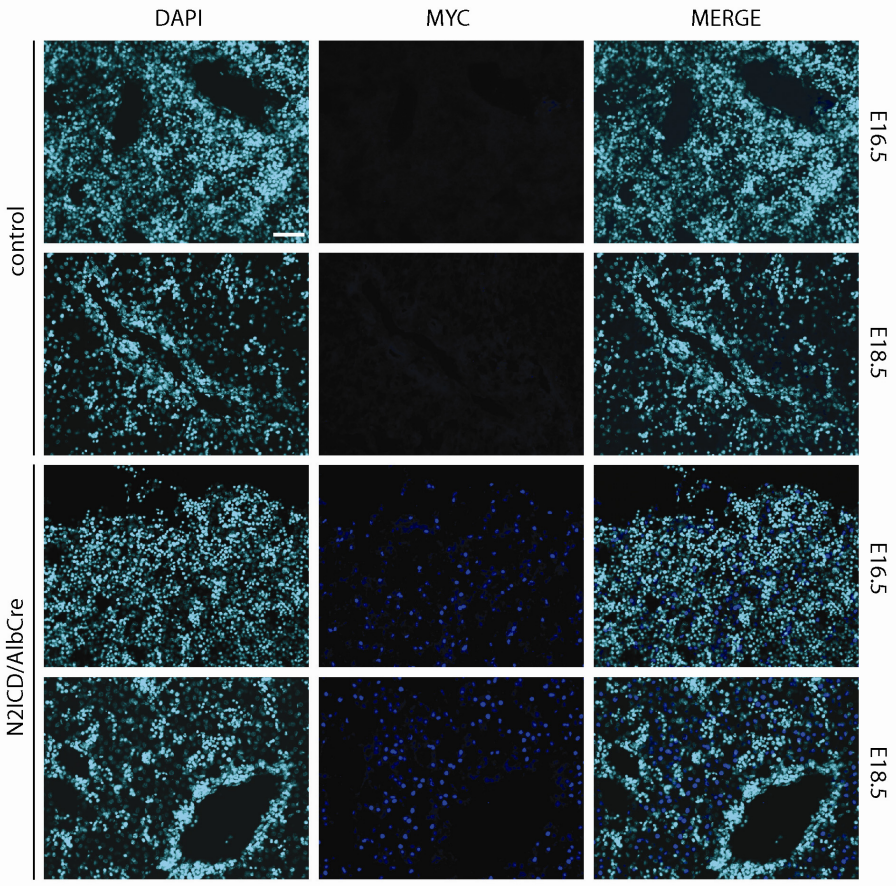

N2ICD/AlbCre
control
A
B
pv
pv
DBA
Hematoxylin
portal tract
C
D
HNF1β
Hematoxylin
lobule
P90
P90
N2ICD/AlbCre
E
F
Myc ZO1 DBA
portal tract
P10
P90
G
control
N2ICD/AlbCre
% of portal tracts
80
60
40
20
0
1
2
3
4
≥5
bile ducts per portal tract

Figure 5

Model of intrahepatic bile duct development in N2ICD/AlbCre mice



Supplementary Figure 1



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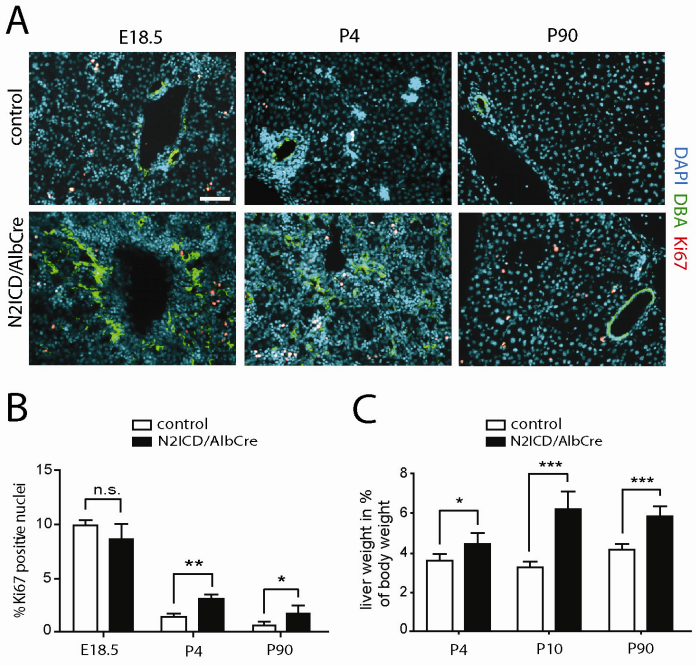
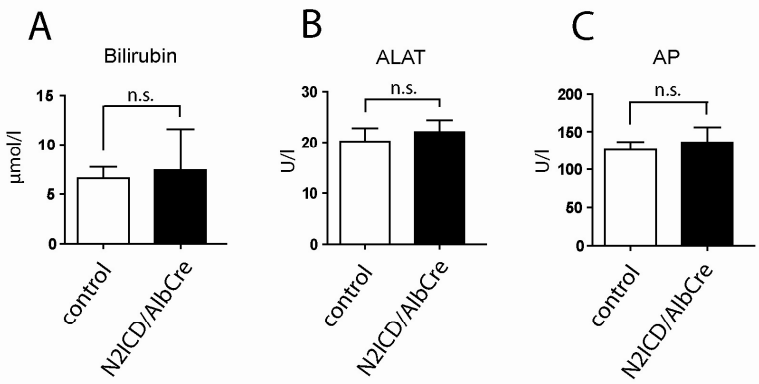


Supplementary Figure 3



3.2. Paper2

Constitutive Notch2 Signaling in Neural Stem Cells Promotes Tumorigenic Features and Astroglial Lineage Entry

Jan S.Tchorz[a], Mercedes Tome[a,+], Dimitri Cloëtta[a,+], Balasubramanian Sivasankaran[b], Michal Grzmil[c], Roland M. Huber[b,c], Franziska Rutz-Schatzmann[a], Frank Kirchhoff[d,e], Nicole Schaeren-Wiemers[f], Martin Gassmann[a], Brian A. Hemmings[c], Adrian Merlo[b] and Bernhard Bettler[a]

[a]Department of Biomedicine, Institute of Physiology, Pharmazentrum, University of Basel, 4056 Basel, Switzerland; [b]Neurosurgery, Department of Biomedicine, University Hospital Basel, University of Basel, 4031 Basel, Switzerland; [c]Friedrich Miescher Institute for Biomedical Research, 4058 Basel, Switzerland; [d]Max-Planck Institute for Experimental Medicine, 37075 Göttingen, Germany; [e]Institute of Physiology, University of Saarland, 66421 Homburg, Germany; [f]Neurobiology, Department of Biomedicine, University Hospital Basel, University of Basel, 4031 Basel, Switzerland

[+] These authors contributed equally to this study.

Correspondence: Bernhard Bettler, PhD, Department of Biomedicine, Institute of Physiology, University of Basel, Basel, Switzerland. Tel: +41-61-2671632; fax: +41-61-2671628; e-mail: bernhard.bettler@unibas.ch

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3.2.1 Abstract

Recent studies identified a highly tumorigenic subpopulation of glioma stem cells (GSCs) within malignant gliomas. GSCs are proposed to originate from transformed neural stem cells (NSCs). Several pathways active in NSCs, including the Notch pathway, were shown to promote proliferation and tumorigenesis in GSCs. Notch2 is highly expressed in glioblastoma multiforme (GBM), a highly malignant astrocytoma. It is therefore conceivable that increased Notch2 signaling in NSCs contributes to the formation of GBM. Here we demonstrate that mice constitutively expressing the activated intracellular domain of Notch2 in NSCs display a hyperplasia of the neurogenic niche and reduced neuronal lineage entry. Neurospheres derived from these mice show increased proliferation, survival and resistance to apoptosis. Moreover, they preferentially differentiate into astrocytes, which are the characteristic cellular population of astrocytoma. Likewise, we show that Notch2 signaling increases proliferation and resistance to apoptosis in human GBM cell lines. Gene expression profiling of GBM patient tumor samples reveals a positive correlation of Notch2 transcripts with gene transcripts controlling anti-apoptotic processes, stemness and astrocyte fate and a negative correlation with gene transcripts controlling proapoptotic processes and oligodendrocyte fate. Our data show that Notch2 signaling in NSCs produces features of GSCs and induces astrocytic lineage entry, consistent with a possible role in astrocytoma formation.

3.2.2 Introduction

Gliomas are the largest group of primary tumors in the brain and include morphologically distinct astrocytomas and oligodendrogliomas. Glioblastoma multiforme (GBM), the most aggressive form of malignant astrocytomas, offers limited therapeutic options with a median survival for GBM patients of less than 14 months (69). Malignant gliomas contain a highly tumorigenic population of glioma stem cells (GSCs) that are defined by their ability to self-renew, to give rise to all cell types within a particular glioma and their resistance to apoptosis

(68,71,73,96-98). Due to their longevity, self-renewal, and sustained proliferative capacity, neural stem cells (NSCs) are a likely source for GSCs (68,71,73,96-98). In addition, the pluripotency of NSCs provides a rationale for the cellular heterogeneity observed in gliomas (69). Dysregulation of genes controlling cell cycle, differentiation and apoptosis in NSCs may therefore promote their transformation into GSCs (68,71,73,96-98). Intriguingly, Notch2 was shown to be highly expressed in GBM and the *Notch2* gene was found to be amplified in many primary GBM cell lines (79). Moreover, elevated Notch2 signaling increased GBM growth and reduced survival in mouse xenograft models (77). In contrast, *Notch2* deletions are frequently observed in oligodendrogliomas and are associated with a more favorable prognosis (74). Together these data support that Notch2 is a prognostic marker and possibly involved in malignant progression. However, whether aberrant Notch2 signaling contributes to NSC transformation and ultimately to gliomagenesis is not known.

Four Notch receptors (Notch1-4) exist in mammals (49). Binding of ligands triggers a proteolytic processing of receptors and translocation of Notch intracellular domains (NICDs) to the nucleus. NICD binding to a transcriptional complex including RBPjκ and MAML1 leads to transcriptional activation of the effector genes *Hairy* and *Enhancer of Split* homologs (e.g. *Hes1 or Hes5*) (49,84). Notch2 regulates morphogenesis of Bergmann glia cells (66) and inhibits granule neuron differentiation in the cerebellum (67). Moreover, Notch2 is expressed throughout the embryonic (64,65) and adult neurogenic regions (46,65). However, while Notch1 signaling was shown to regulate proliferation, cell fate decisions and survival in NSCs (50,52,84,99,100), a role for Notch2 in NSC regulation is not established.

Here, we report that constitutive expression of the intracellular domain of Notch2 (N2ICD) in NSCs in transgenic mice promotes proliferation and prevents neuronal lineage entry. NSCs from these mice exhibit increased proliferation, reduced apoptosis and preferentially differentiate into the astrocytic lineage. Likewise, modulation of Notch2 signaling regulates proliferation and apoptosis in human GBM cell lines. Thus, our data support that aberrant Notch2 signaling contributes to NSC transformation and possibly to GBM formation.

3.2.3 Materials and methods

Mice

N2ICD mice (101) were crossed with heterozygous Nestin-Cre mice (102) to obtain double transgenic Nestin-Cre/N2ICD mice that allow N2ICD expression in the neural lineage (102,103) (Figure 1A,B). Single transgenic N2ICD mice were used as littermate controls. N2ICD mice were crossed with GFAPCre-ER^{T2} (hereafter GCE) mice (104) containing a Cre/LoxP inducible Rosa26-YFP reporter gene (hereafter YFP) (105) resulted in triple transgenic GCE/YFP/N2ICD mice expressing N2ICD in the adult NSC niche and in astrocytes (Figure 3A,C and data not shown). GCE/YFP mice were used as littermate controls. Intraperitoneal administration of tamoxifen (2 mg, Sigma, St. Louis, MO) once daily for 5 consecutive days was used for Cre-mediated excision of floxed STOP cassettes in 8-10 week old GCE/YFP/N2ICD mice and GCE/YFP mice. Mice were sacrificed 7 days after the last tamoxifen injection and perfused with paraformaldehyde. Genotyping was performed with TaqMan-PCR primers (YFP mice: sense 5'-ACAGCTCGTCCATGCCGA-3', antisense 5'-ATCACATGGTCCTGCTGGAGT-3', probe 5'-FAM-TGATCCCGGCGGCGGTCA-TAMRA-3'; N2ICD mice: sense 5'-ATATCCGCGGTGGAGATCAA-3', antisense 5'-TAGACCAGGCTGGGCTAAA-3', probe 5'-VIC-CGGTACCAGATCTC-MGB-3'; Nestin-Cre/GCE mice: sense 5'-GCCGCGCGAGATATGG-3', primer antisense 5'-GCCACCAGCTTGCATGATC-3', probe 5'-FAM-CCGCGCTGGAGTTTCAATACCGG-TAMRA-3'). All animal experimentation was performed in accordance with institutional guidelines approved by the veterinary office of Basel-Stadt.

Immunohistochemistry and Immunoblots

Immunostaining and immunoblots were performed as described (101) with rabbit anti-Caspase-3 (#9062, Cell Signaling), mouse anti-beta-Actin (A5316, Sigma), anti-Bcl-2 (Santa Cruz) and anti-Mcl1 (Santa Cruz), mouse anti-NeuN (Chemicon, Billerica, MA), rabbit anti-GFAP (Dako, Denmark), mouse anti-GFAP (Chemicon), rat anti-Notch2 (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse anti-Myc (SC-40, Santa Cruz), goat anti-Myc (Santa Cruz, Santa Cruz, CA), rat anti-Substance-P (Abcam, UK), rabbit anti-Ki67 (Novocastra, UK), rabbit anti-Gad65/67

(Sigma, Saint-Louis, MO), rabbit anti-Olig2 (Chemicon) goat anti-Sox2 (R&D Systems, Switzerland), goat anti-Dcx (Santa Cruz), chicken anti-GFP (Molecular Probes/Invitrogen Carlsbad, CA), mouse anti-Nestin (Santa Cruz), rabbit anti-CD133 (Santa Cruz), mouse anti-βIII-Tubulin (Tuj1) (Sigma), mouse anti-Myc (Santa Cruz) and rabbit anti-Caspase-3 (Cell Signaling, Beverly, MA) antibodies. The secondary antibodies used were Alexa-conjugated or horseradish peroxidase-conjugated (Jackson Immunoresearch, UK). Image analysis was performed with a Leica DMI6000 fluorescence microscope or a Zeiss LSM510 confocal microscope and ImageJ software (NIH, Bethesda, MD, USA).

Neurospheres

NSC cultures were prepared and maintained as described (106). For determination of neurosphere number and size, 100 µl samples containing comparable numbers of cells from each genotype were transferred into wells of a 96 well plate and grown for 5 days. For *in vitro* differentiation, cells were seeded at a density of $2x10^5$ cells/cm² on coverslips coated with 15 µg/ml poly-L-ornithin and 40 µg/ml laminin. Cells were differentiated in neurosphere medium lacking FGF2, EGF and heparin and fixed after 5 days with 4% PFA.

Stable Expression of N2ICD in GBM Cell Lines

U373 GBM cells (107) were grown and stably transfected as described.(79) N2ICD (nucleotides 5107 to 7425 of the human Notch2 cDNA, AF308601) was expressed from pcDNA3.0-IRES-EGFP, dominant-negative Mastermind-like I (MAML1dn) from pEGFP-N3-MAML1dn (gift of Dr. Aster, HMS, Boston) (108) and GFP from pcDNA3.1-IRES-EGFP as control. For culturing GSC-like spheres from GBM, U373 GBM cells were cultured under the same conditions as neurospheres. Parental U373 cells (wildtype; WT) were used as controls.

RT-PCR Quantification

Total RNA was isolated from whole brain of E16.5 embryos using the SV-total-RNA isolation system (Promega, Switzerland) and used as template to synthesize cDNA with GoScript reverse transcriptase (Promega). Relative quantitative RT-PCR was performed using the Power-SYBR-

Green PCR Master Mix (Applied Biosystems, Switzerland) according to manufacturer's protocol. Data were normalized to GAPDH cDNA. The following primers were used for Notch2 (sense 5'-CCCAAGGACTGCGAGTCAGG-3', antisense 5'-GGCAGCGGCAGGAATAGTGA-3'), Hes1 (sense 5'-CTACCCCAGCCAGTGTCAAC-3', antisense 5'-AAGCGGGTCACCTCGTTCAT-3'), Hey1 (sense 5-'CTTGAGTTCGGCGCTGTGTTCC -3', antisense 5'-GATGCCTCTCCGTCTTTTCCT-3') and GAPDH (sense 5'-TTAGCCCCCCTGGCCAAGG-3', antisense 5'-CTTACTCCTTGGAGGCCATG-3').

Pharmacological Induction of Apoptosis

After growing 6 days *in vitro* (div) neurospheres (2-4 passages, Nestin-Cre/N2ICD and single transgenic N2ICD controls) were dissociated to obtain a single cell suspension. Cells were plated at a density of $5x10^4$ cells/ml in a 6-well plate (BD, Franklin Lakes, NJ) in neurosphere medium for 4 days to allow neurosphere formation. On the fourth day Etoposide (Sigma) was added at a concentration of 20 μM for either 6 or 10 hrs. Untreated neurospheres were used as a control. Neurospheres were harvested by centrifugation at 300g for 5 min and lysed. U373 GBM cells stably transfected either with N2ICD, MAML1dn or GFP were plated at $5x10^4$ cells/ml. After 2 days 20 μM or 40 μM Etoposide was added for 24 hrs and protein extracts were obtained as described above. Apoptosis induction was assessed by immunoblot detection of cleaved Caspase-3 from at least three independent experiments.

Correlation Analysis

For bioinformatics analysis we used absolute expression values from recently published microarray data (109). Briefly, labeled total RNA from two normal brains and 15 GBM samples was hybridized to Affymetrix U133v2.0 Gene. Data-mining was performed using the Genedata Analyst 4.1 package. All samples were quantile normalized and median scaled to correct for minor variation in expression distribution. Expression values for specific genes were used to calculate Spearman correlation coefficients.

3.2.4 Results

N2ICD Expression in Embryos Causes Severe Hyperplasia and Inhibits Neurogenesis

To study the role of Notch2 signaling in the developing brain, we crossed Nestin-Cre mice (102) with N2ICD mice (101) to drive expression of myc-tagged N2ICD in NSCs and their progeny (Nestin-Cre/N2ICD mice) (Figure 1A). Western blot analysis revealed expression of myc-tagged N2ICD in the brain of E18.5 Nestin-Cre/N2ICD mice (Figure 1B). In addition to the expected increase in Notch2 mRNA due to N2ICD transgene expression, Nestin-Cre/N2ICD mice also showed increased mRNA levels for its target genes *Hes1* and *Hey1* when compared to control mice (Figure 1C). This demonstrates that the transgene generates functional N2ICD. E18.5 Nestin-Cre/N2ICD embryos displayed enlarged ventricles and a hyperplasia of the ganglionic eminence, the neurogenic region in the ventral forebrain (Figure 2A). The ganglionic eminence displayed a 3-fold increase in size (Figure 2B) and an enlarged region of NSCs identified by Ki67 (Figure 2C) and Nestin immunostaining (Supplementary Figure 1A). The striatal region of E18.5 Nestin-Cre/N2ICD embryos had an increased cell density and lacked the striasome, a typical porous structure (Figure 2D). Moreover, the striatal region lacked immunostaining for Substance-P and GAD65/67, which are commonly used to outline the striatum in the ventral forebrain (Figure 2D). NeuN immunostaining showed that the ventral forebrain of Nestin-Cre/N2ICD mice is not only devoid of GAD65/67+ interneurons but of mature neurons in general (Supplementary Figure 1B). It therefore appears that the striatal region is mainly populated by an increased number of Nestin+ NSCs failing to develop into neurons. Strikingly, the consequences of constitutive N2ICD expression in NSCs are detrimental, since Nestin-Cre/N2ICD mice die at birth. In summary, the above experiments support a dual role for Notch2 during embryogenesis, both in increasing proliferation and inhibiting neurogenesis.

N2ICD Expression in Adult Mice Increases Proliferation and Inhibits Neuronal Differentiation in Neurogenic Niches

GBM mostly occur in adults and therefore N2ICD expression in adult NSCs more accurately addresses a possible role of Notch2 in gliomagenesis. The perinatal lethality of Nestin-

Cre/N2ICD mice precludes studying a possible role of Notch2 in malignant transformation of adult NSCs. We therefore generated triple transgenic GCE/YFP/N2ICD mice by crossing N2ICD mice with double transgenic GFAPCreER^{T2}/R26R-YFP (GCE/YFP) mice (104,105). These mice allow for tamoxifen-induced N2ICD and YFP expression in adult GFAP+ NSCs and astrocytes (104) (Figure 3A). GCE/YFP mice, which express YFP but not N2ICD upon tamoxifen induction, were used as littermate controls. GCE/YFP/N2ICD mice exhibit a 2-fold increase in Ki67+ proliferating YFP+ cells in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dentate gyrus, two prominent areas of neurogenesis in the adult brain (Figure 3B). This indicates that N2ICD expression increases proliferation in NSCs in the adult, similar as it does during embryogenesis.

We next analyzed whether N2ICD prevents neuronal differentiation in the adult brain (Figure 3C). Brain sections from GCE/YFP/N2ICD and GCE/YFP mice were co-immunostained for YFP and markers for adult NSCs (Sox2, GFAP; of note GFAP, which is widely used as a marker for astrocytes, also labels NSCs in the dentate gyrus) or neuronal markers (Dcx, NeuN). The dentate gyrus of GCE/YFP/N2ICD mice showed approximately a 2-fold increase in YFP+ cells expressing GFAP/Sox2 (Figure 3C,D). In addition, GCE/YFP/N2ICD mice had a significantly reduced number of YFP+ cells expressing Dcx/NeuN (Figure 3C, D). This shows that Notch2 signaling inhibits neuronal differentiation of adult NSCs in the dentate gyrus.

N2ICD Expression in Neurospheres Increases Proliferation, Promotes Survival and Astrocytic Commitment

To study NSC proliferation and expansion, we prepared neurospheres from the ganglionic eminence of E14.5 Nestin-Cre/N2ICD and littermate control embryos (Figure 4A). Neurospheres from Nestin-Cre/N2ICD NSCs were significantly larger than those from control NSCs (73.1±2.4µm, n=226 neurospheres vs. 42,56±1.08µm, n=224 neurospheres, mean diameter ±SEM, $p<0.0001$), indicating that N2ICD expression increases NSC proliferation in neurospheres (Figure 4A,B), similar as *in vivo*. Next, we used neurospheres to study whether N2ICD regulates NSC fate decisions. We differentiated neurospheres from E14.5 Nestin-Cre/N2ICD embryos *in*

vitro and quantified cells expressing the neuronal marker βIII-Tubulin (Tuj1), the oligodendrocyte marker Olig2 and the astrocyte marker GFAP (Figure 4C). A significantly reduced number of Tuj1+ cells in Nestin-Cre/N2ICD cultures indicates that N2ICD prevents NSCs from efficiently differentiating into neurons (Figure 4C,D). In contrast, differentiated Nestin-Cre/N2ICD NSCs showed a significant increase in GFAP+ astrocytes (Figure 4C,E). Intriguingly, the staining intensity for GFAP in differentiated Nestin-Cre/N2ICD NSCs was visibly increased compared to control (Figure 4C). Increased GFAP protein levels and decreased Tuj1 protein levels were confirmed on immunoblots (Figure 4G). Moreover, the number of Olig2+ oligodendrocytes was significantly decreased in Nestin-Cre/N2ICD NSCs (Figure 4C,F). This supports that Notch2 signaling biases fate decisions in NSCs towards the astrocytic lineage by inhibiting differentiation into neurons or oligodendrocytes.

To test the role of N2ICD in NSC survival and apoptosis, Nestin-Cre/N2ICD and control neurospheres were treated with Etoposide to induce apoptotic cell death. Etoposide is an anti-cancer agent inhibiting topoisomerase II, which induces DNA strand breaks. Etoposide is widely used in chemotherapy, including the treatment of GBM (110). In our experiments, induction of apoptosis was monitored by Caspase-3 cleavage, which is part of the apoptotic signaling cascade (Figure 4H). Our results show that NSCs overexpressing N2ICD have reduced levels of cleaved Caspase-3 compared to control NSCs, supporting that Notch2 signaling prevents apoptosis.

N2ICD Expression in Human GBM Cell Lines Increases Proliferation and Prevents Apoptosis

We studied whether Notch2 signaling regulates proliferation and resistance to apoptosis in GBM cells. To activate or inhibit Notch2 signaling in GBM cells, we stably transfected the human GBM cell line U373 with N2ICD (U373-N2ICD cells) or dominant-negative mastermind-like I (U373-MAML1dn cells), which prevents canonical Notch signaling (79,108). Transfected GBM cells were cultured in neurosphere media under non-adherent condition, which produced GSC-like spheres. Compared to WT spheres, the size of N2ICD-expressing spheres was significantly increased, while the size of MAML1dn-expressing spheres was significantly decreased (WT,

56±1.7µm, n=90 spheres; N2ICD, 111±2.4µm, n=215; MAML1dn, 41±0.5µm, n=291; mean diameter ±SEM, $p<0.0001$) (Figure 5A and B). The presence of GSC markers (Nestin, Sox2, and CD133) in the spheres confirmed that these derived from GSC-like cells rather than from non stem-like GBM cells growing under non-adherent conditions (Figure 5C).

To evaluate whether Notch2 signaling plays a role in GBM cells chemoresistance to cell death, U373-N2ICD and U373-GFP control GBM cells were treated with Etoposide. Both cell lines showed a higher resistance to apoptosis induction compared to NSCs (data not shown). In fact, Etoposide treatment for U373 GBM cells had to be prolonged to 24 hrs to observe apoptosis. As with N2ICD expressing NSCs (Figure 4H), GBM cells expressing N2ICD showed lower levels of cleaved Caspase-3 compared to control GBM cells (Figure 6A). This result supports a role for N2ICD activity in the chemoresistance of glioma cells to apoptosis. In addition, U373-N2ICD GBM cells showed increased levels of anti-apoptotic proteins such as Bcl-2 and Mcl-1 compared to control GBM cells (Figure 6A), in line with the observed resistance to apoptosis. To further investigate whether the prevention of apoptosis in U373 GBM cells is mediated by canonical or non-canonical Notch signaling, we treated U373-MAML1dn and U373-GFP control GBM cells with Etoposide. Caspase-3 cleavage in response to Etoposide was similar in the U373-MAML1dn and control U373-GFP cells, showing that the inhibition of canonical Notch signaling in U373-MAML1dn GBM cells does not increase apoptosis (Figure 6B). Likewise, protein levels for Bcl2 and Mcl1 were similar in U373-MAML1dn and U373-GFP control GBM cells treated with Etoposide (Figure 6B). It is therefore possible that non-canonical Notch signaling prevents apoptosis, consistent with previous reports (111,112). Thus, Notch2 signaling not only increases proliferation of GSC-like cells but also induces chemoresistance of GBM cells to Etoposide treatment, likely by upregulating anti-apoptotic pathways.

Notch2 Levels in Primary Human GBM Correlate with Antiapoptotic, Stem Cell and Glial Markers

Analyzing published microarray data (109), we investigated whether Notch2 expression in primary human GBM samples correlates with the expression of genes regulating cell

differentiation and survival (Figure 7 and Supplementary Table 1). Notch2 expression correlated with expression of the target genes *Hes1* and *Hey1* (rs=0.7 and 0.61). Transcripts for the anti-apoptotic proteins BCL6 and BCL2L12 exhibited correlation with Notch2 transcripts (rs=0.75 and 0.67), while transcripts for the proapoptotic proteins Bax and BCLAF1 showed a negative correlation (rs=-0.56 and -0.52). This further implicates Notch2 into GBM survival. NSC/GSC markers (Sox2 and Nestin) positively correlated with Notch2 expression (rs=0.62 and 0.49), supporting that Notch2 levels correlate with an increase in GSC cells. The expression of the astrocyte markers Vimentin and GFAP highly correlated with Notch2 expression (rs=0.75 and 0.76), in line with Notch2 directing astrocyte versus oligodendrocyte fate decisions. In contrast, transcripts for oligodendrocyte markers (CNP, PLP1 and Olig2) showed a strong negative correlation with Notch2 transcript levels (rs=-0.88, -073 and -0.56). In summary, this correlation analysis further supports that Notch2 signaling contributes to the cellular identity of GBM.

3.2.5 Discussion

Developmentally stalled NSCs are a possible source of GSCs giving raise to malignant glioma (68,71,73,96-98). NSCs inherently have the features of tumor cells (e.g. multipotency, self-renewal capacity, high motility and robust proliferative potential) and therefore are at risk for malignant transformation (68,71,73,96-98). Notch2 is highly expressed in GBM(79) and N2ICD expression in GBM cell xenografts significantly reduced survival (77). In contrast, loss of *Notch2* is frequently found in oligodendroglioma and low Notch2 levels correlate with a much better prognosis (74). Here, we used a transgenic approach to study whether constitutive Notch2 signaling in NSCs plays a role in proliferation, differentiation and apoptosis and possibly contributes to GBM formation. In support of this, we found that Notch2 signaling in NSCs *in vivo* increases cell proliferation and prevents neuronal differentiation, both in embryos and in adult mice. Importantly, N2ICD expression is not sufficient to induce tumors but leads to a prominent expansion of the neurogenic niche. Furthermore, N2ICD expression in cultured NSCs promotes astrocyte differentiation at the expense of neuronal and oligodendrocyte differentiation. The

expression of N2ICD in NSCs also increased the resistance to apoptosis, rendering these NSC less sensitive to the cytotoxic drug Etoposide. Our findings therefore indicate that constitutive Notch2 signaling in NSCs is sufficient to induce key features of GSCs. In addition, they suggest that constitutive Notch2 signaling could promote NSCs to give rise to tumors of the astrocytic rather than the oligodendroglial lineage.

We addressed whether Notch2 signaling is responsible for some of the features of GBM expressing high Notch2 levels. We found that blocking endogenous Notch signaling in cultured GBM cells reduces proliferation while N2ICD expression significantly increases proliferation. Furthermore, we observed that N2ICD expression in cultured GBM cells causes an upregulation of anti-apoptotic proteins and increases cell survival. These results are in line with N2ICD expression increasing proliferation and reducing apoptosis in HSR-GBM1 cells and xenografts thereof (77). Enhanced proliferation and survival of GBM cells with elevated Notch2 levels may therefore provide an explanation for the poor prognosis of GBM with high Notch2 levels and the better prognosis of oligodendroglioma with *Notch2* deletions (74,77). We found that Notch2 expression levels in GBM biopsies positively correlate with the transcript levels of genes controlling stemness (Sox2, Nestin) and astrocyte fate (Vimentin, GFAP) while they negatively correlate with the transcript levels of genes controlling oligodendrocyte fate (Olig2, CNP, PLP1). In addition, Notch2 expression levels in GBM biopsies positively and negatively correlate with the expression levels of antiapoptotic (BCL6, BCL2L12) and proapoptotic (Bax, BCLAF1) markers, respectively.

The enhanced proliferation seen in the presence of Notch2 signaling may stochastically increase the risk of NSCs to acquire somatic mutations and to transit to GSCs. If the increased Notch2 signaling occurs after GSCs are formed this would likely promote GSCs to give rise to astrocytomas, such as GBM, rather than oligodendroglioma. A loss of Notch2 signaling in subsets of expanding GSCs with *Notch2* amplification could explain the formation of mixed oligodendroglioma. In any instance, blockade of Notch2 signaling may interfere with GBM formation and thus be of therapeutic benefit. Unfortunately, pan-Notch inhibitors (e.g. γ-

secretase inhibitors) produce severe side-effects in the intestinal crypt (113). Since genetic deletion of either Notch1 or Notch2 receptors does not result in intestinal phenotypes, a specific therapeutic targeting of Notch2 receptors may still be possible (114). Genentech developed therapeutic antibodies specifically targeting the Adam protease cleavage-site in the Notch1 or Notch2 receptors (115). While the antibody blocking Notch1 signaling efficiently inhibits proliferation of T-cell acute lymphoblastic leukemia cells, no data are available yet for the antibody blocking Notch2 signaling (115). It certainly would be interesting to test such an antibody for the treatment of GBM.

On a different note, the developmental phenotypes seen in mice expressing N2ICD in NSCs shed light on the functions of Notch2 signaling during embryonic and adult neurogenesis. The pronounced hyperplasia of the neurogenic niche during embryonic development and the ensuing lack of a properly developed striatum indicate that Notch2 not only expands the NSC pool but that Notch2 downregulation is important to allow subsequent NSC differentiation. Reduced neuronal differentiation of adult NSCs expressing N2ICD within the SGZ of the dentate gyrus supports that Noch2 downregulation is also important for adult neurogenesis. These findings are in line with Notch2 inhibiting differentiation of cerebellar granule neuron precursors (67) and keeping astrocytes in an immature state (116). Our developmental studies show that Notch2 plays a role in regulating differentiation and proliferation of NSCs in the embryonic and adult brain.

In conclusion, Notch2 signaling in NSCs induces key features of GSCs, such as increased proliferation, reduced apoptosis and induces astrocytic lineage entry. In addition, high/constitutive Notch2 expression may favor the formation of astrocytoma, in particular GBM. Of note, however, high/constitutive Notch2 expression is not sufficient to induce GBM formation, which probably requires the acquisition of additional mutations.

3.2.6 Acknowledgement

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3.2.7 Figure legends

Figure 1. Transgenic expression of N2ICD in the neurogenic region. (A) Breeding scheme for myc-tagged N2ICD expression in NSCs of Nestin-Cre/N2ICD mice. (B) Western blot revealing myc-tagged N2ICD protein in the brains of E18.5 Nestin-Cre/N2ICD mice but not the brains of single transgenic N2ICD littermate control mice. Actin controls for sample loading. (C) RT-PCR shows increased mRNA levels of Notch2 and its target genes Hes1 and Hey1 in the brains of Nestin-Cre/N2ICD mice compared to control mice. Bar diagram shows mean±SD, ***=$p<0.001$, students t-test).

Figure 2. N2ICD expression in NSCs during embryogenesis results in a hyperplasia of the neurogenic region. (A) Cresyl violet stained coronal sections showing hyperplasia of the neurogenic region (ganglionic eminence, GE; outlined by a dashed line) in E18.5 Nestin-Cre/N2ICD mice compared to control mice. E18.5 Nestin-Cre/N2ICD mice also displayed enlarged ventricles (V) and a lack of a properly developed striatum (Str). (B) Quantification of the ganglionic eminence revealed a more than 3-fold increase in size in E18.5 Nestin-Cre/N2ICD mice compared to E18.5 controls. (C) Immunostaining for the proliferation marker Ki67 reveals a highly increased population of proliferating cells in E18.5 Nestin-Cre/N2ICD mice compared to E18.5 control mice. (D) Immunostaining for the marker proteins GAD65/67 (arrowheads) and Substance P (arrows) reveals that Nestin-Cre/N2ICD mice do not express these proteins in the

ventral forebrain of E18.5 mice, in contrast to the striatum of control mice. DAPI staining shows that the typical porous structure of the striasome (arrows in control mice) is lacking in E18.5 Nestin-Cre/N2ICD mice. Bar diagram shows mean±SD, ***=p<0.001, students t-test). Scale bars are (A,C) = 200µm, (D) = 50 µm.

Figure 3. Notch2 regulates proliferation and differentiation of adult NSCs. (A) N2ICD mice were mated with GFAPCreER^{T2}/Rosa26-YFP (GCE/YFP) mice, which yields GCE/YFP/N2ICD mice that express both N2ICD and YFP upon tamoxifen-induced Stop codon excision in adult NSCs. YFP immunostaining in GCE/YFP/N2ICD mice was therefore used to identify cells that constitutively express N2ICD as a result of the GFAP-CreER^{T2}-mediated excision event. YFP+ cells in GCE/YFP mice were used as controls. YFP+ cells expressing a proliferation marker (Ki67) or differentiation markers (Sox2, GFAP, Dcx, NeuN) were used for quantification in (B) or (C), respectively. (B) Quantification of YFP/Ki67+ cells revealed a 2-fold increase in proliferating YFP+ cells in GCE/YFP/N2ICD mice compared to GCE/YFP mice in both the SGZ and SVZ, indicating increased proliferation in the neurogenic niche of adult mice with N2ICD expression. (C) Immunostaining for YFP and NSC markers (Sox2, GFAP; left panels) or neuronal markers (Dcx, NeuN; right panels) in the dentate gyrus in GCE/YFP/N2ICD and GCE/YFP mice. Quantification reveals that GCE/YFP/N2ICD mice have approximately a 2-fold increase in YFP+ cells expressing NSC markers compared to GCE/YFP mice, while the number of YFP+ neurons is strongly decreased (D). This indicates that N2ICD expression increases the number of NSCs while preventing neurogenesis. GCL = granule cell layer. Bar diagrams show mean±SEM, *=p<0.05, **= p<0.01, ***= p<0.01 (Student's t-test). Scale bars are (C) = 25µm.

Figure 4. Notch2 signaling regulates NSC proliferation and differentiation *in vitro.* (A) Nestin-Cre/N2ICD neurospheres overexpressing N2ICD have increased sphere size compared to single transgenic N2ICD control spheres. (B) Quantification of neurosphere diameters showed that the median diameter in Nestin-Cre/N2ICD spheres was increased by 75% when compared to control spheres, suggesting that N2ICD expression increases NSC proliferation. (C) Immunostainings of differentiated Nestin-Cre/N2ICD and control NSCs for neuronal (Tuj1,

arrows), astrocytic (GFAP) and oligodendrocytic (Olig2, arrowheads) markers. Quantification of cells expressing marker proteins in percent of DAPI+ nuclei reveals that N2ICD largely blocks neuronal differentiation (D) and increases the number of astrocytes (E). Furthermore, Nestin-Cre/N2ICD NSCs almost completely lost their ability to differentiate into Olig2+ oligodendrocytes (F). (G) GFAP protein levels were highly increased in Western blot analysis of differentiated Nestin-Cre/N2ICD NSCs, while Tuj1 levels diminished. (H) NSCs overexpressing N2ICD were more resistant to apoptosis. Lower levels of cleaved Caspase-3 were detected in the N2ICD expressing NSCs after Etoposide treatment. Box plot diagrams show median (red line), boxed 25% and 75% percentiles while whiskers mark minima and maxima (Mann-Whitney test, $p<0.0001$). Nestin-Cre mediates floxed Stop codon excision in the entire neural lineage (102). All quantifications are therefore based on the total number of cells. Scale bar (B, C) = 100µm.

Figure 5. Notch2 signaling controls proliferation in GSC-like cells. (A) Compared to spheres derived from the parental U373 cell line (WT), spheres overexpressing N2ICD are increased in size (N2ICD), indicating increased proliferation of GSC-like cells. In contrast, spheres derived from U373 cells expressing MAML1dn, which inhibits canonical Notch signaling, were smaller in size (MAML1dn). (B) Quantification shows that U373-N2ICD spheres have twice the diameter compared to U373-WT spheres, while U373-MAML1dn spheres show a decrease in diameter by 25%. (C) Immunostaining of U373-N2ICD spheres for GSC markers (Nestin, Sox2, and CD133) confirms that the spheres retain stem-like properties. Box plot diagrams show median (red line), boxed 25% and 75% percentiles while whiskers mark minima and maxima (Mann-Whitney test, $p<0.0001$). Scale bar (A) = 100µm, (C) = 50µm.

Figure 6. N2ICD expression increases resistance of U373 GBM cells to apoptosis. (A) U373-N2ICD GBM cells expressing N2ICD are more resistant to Etoposide-induced apoptosis. Less Caspase-3 protein is cleaved in U373-N2ICD GBM cells compared to U373-GFP GBM cells (left panel). U373-N2ICD GBM cells with increased N2ICD protein levels have increased Mcl1 and Bcl2 protein levels when compared to U373-GFP cells, indicating that Notch2 signaling increases

the resistance to apoptosis by upregulation of anti-apoptotic factors. Actins controls for sample loading (right panel). (B) Similar levels of Caspase-3 cleavage are observed in U373-MAML1dn and U373-GFP GBM cells, supporting that the inhibition of canonical Notch signaling does not increase apoptosis in response to Etoposide treatment (left panel). Likewise, Bcl2 and Mcl1 protein levels are similar in U373-MAML1dn compared to U373-GFP GBM cells. Actin protein levels control for sample loading (right panel).

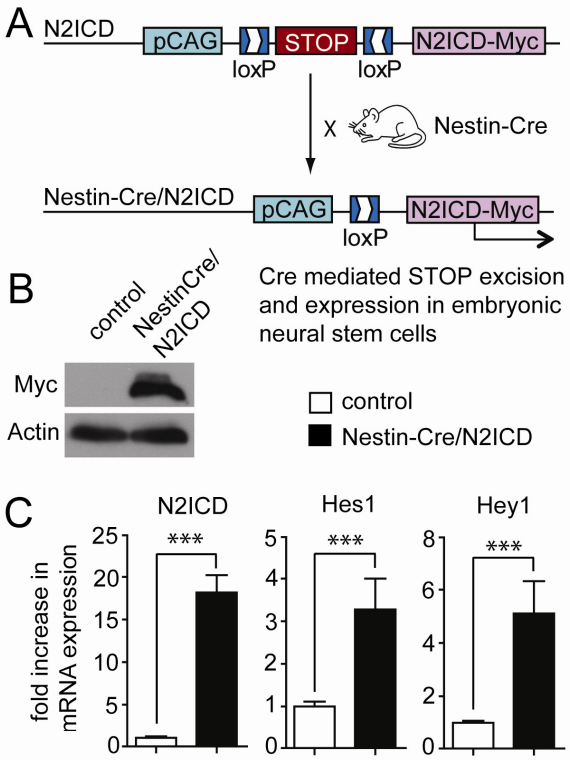
Figure 7. Notch2 mRNA levels in primary human GBM correlate with mRNA levels of genes inhibiting apoptosis, promoting stemness and inducing astrocyte differentiation. Correlation coefficients for Notch2 and genes involved in apoptosis (blue) and differentiation (red/purple) as well as Notch2 targets (green) are shown as Spearman Correlations with a *P* value of less than 0.05. The correlation coefficient (0.98) for another Notch2 probe is included as a positive control.

Supplementary Figure 1. N2ICD expression increases the NSC pool and blocks neurogenesis in the ventral forebrain. (A) Immunostaining for the NSC marker Nestin in the ventral forebrain of E18.5 Nestin-Cre/N2ICD and control mice. Nestin-Cre/N2ICD mice show increased ventricle (V) size and a highly increased density of DAPI+ nuclei (left panels) and increased amounts of Nestin+ NSCs (middle panels) in the ventral forebrain when compared to wt mice. Magnified areas showing the Nestin+ ganglionic eminence (GE) in control mice, which is expanded throughout most of the ventral forebrain in Nestin-Cre/N2ICD mice (right panels). (B) Immunostaining for NeuN, a marker staining mature neurons, shows that E18.5 Nestin-Cre/N2ICD mice have a strong decrease in neurons in the ventral forebrain when compared to E18.5 control mice. This indicates that N2ICD expression in the developing brain increases the Nestin+ NSC pool at the expense of neurogenesis in the ventral forebrain. Scale bar (A,B) = 50 µm.

Supplementary Table 1. Spearman Correlation coefficients of Notch2 (probe 202443_x_at) and selected genes in human GBM.

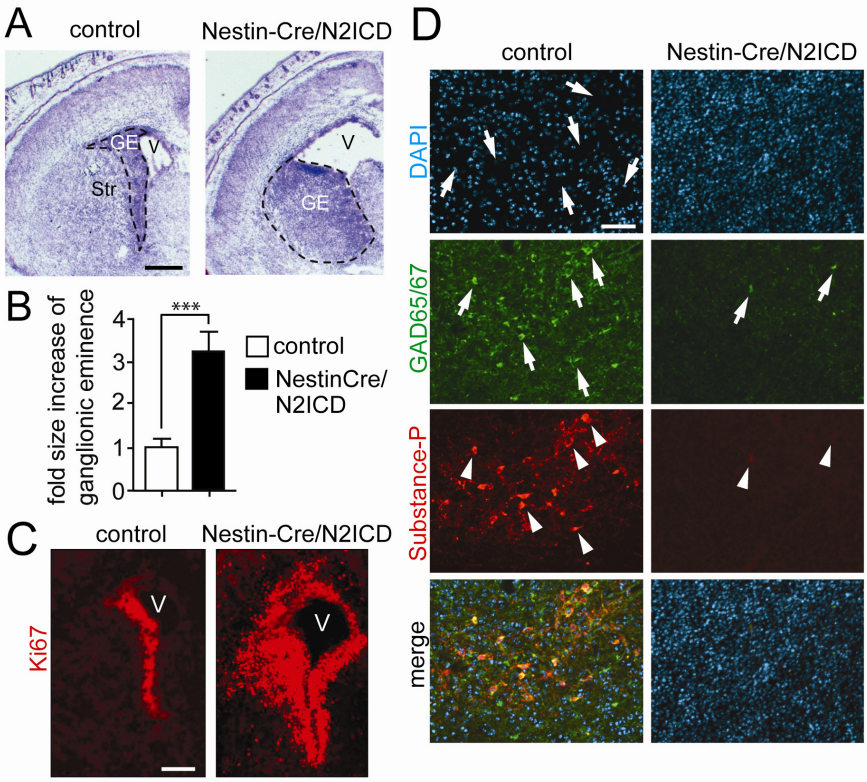
3.2.8. Figures

Figure 1



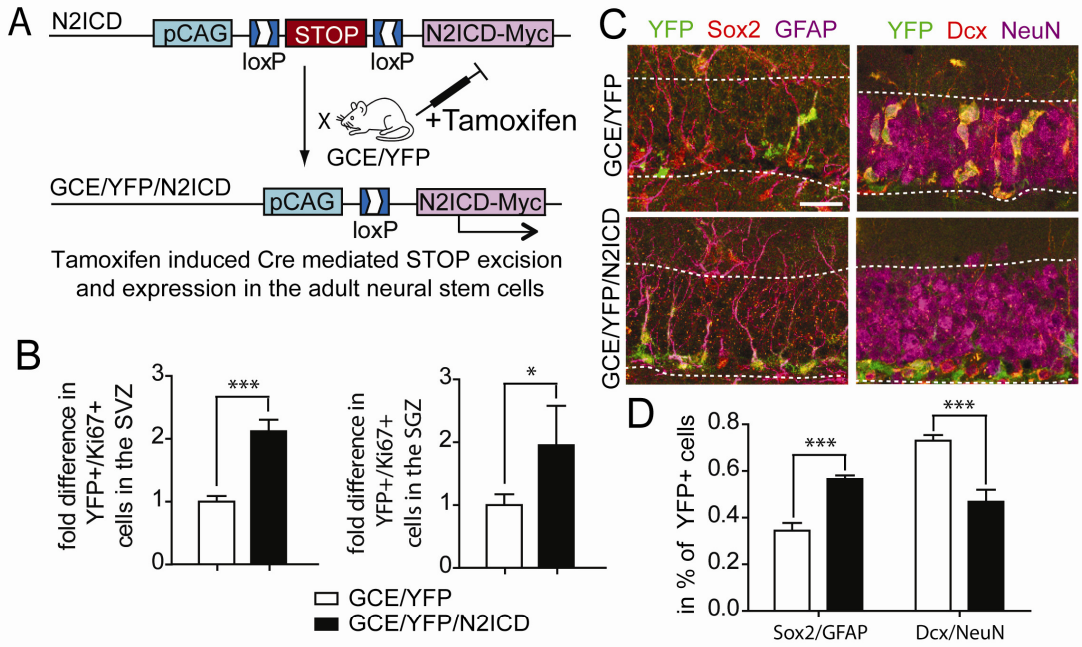
Tchorz et al.

Figure 2



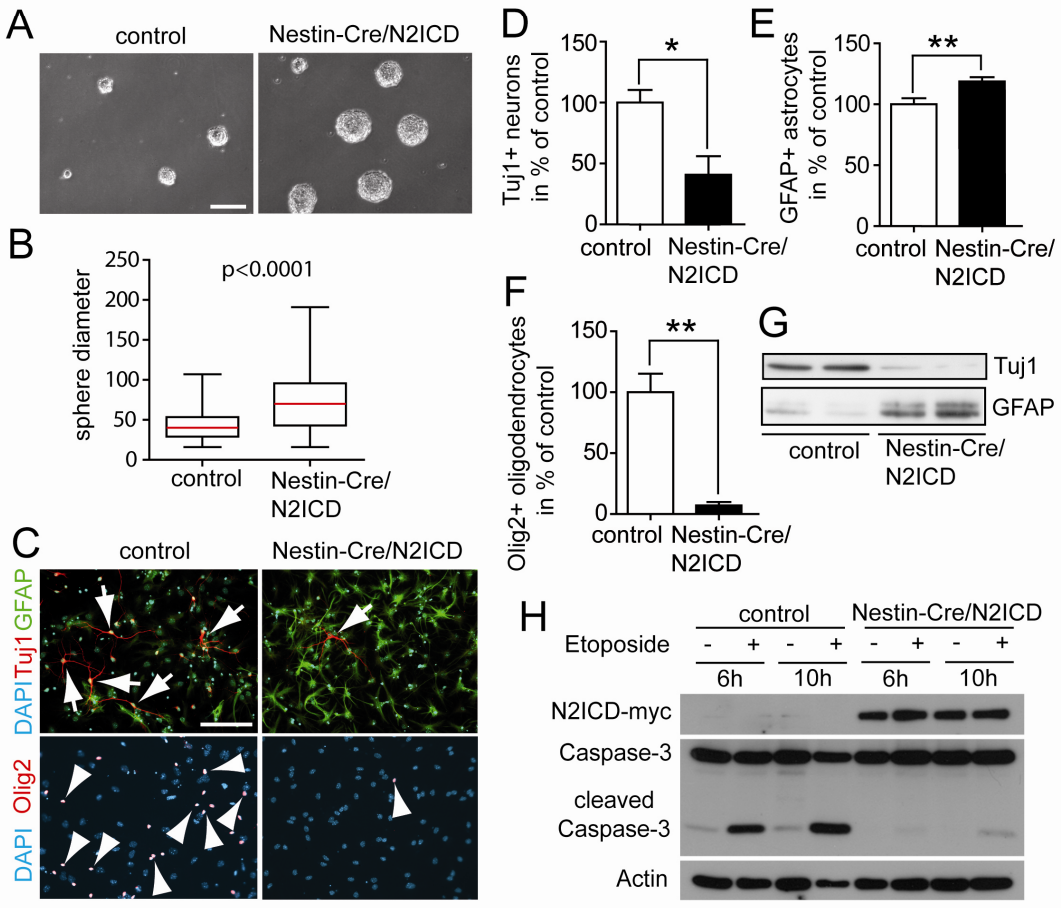
Tchorz et al.

Figure 3



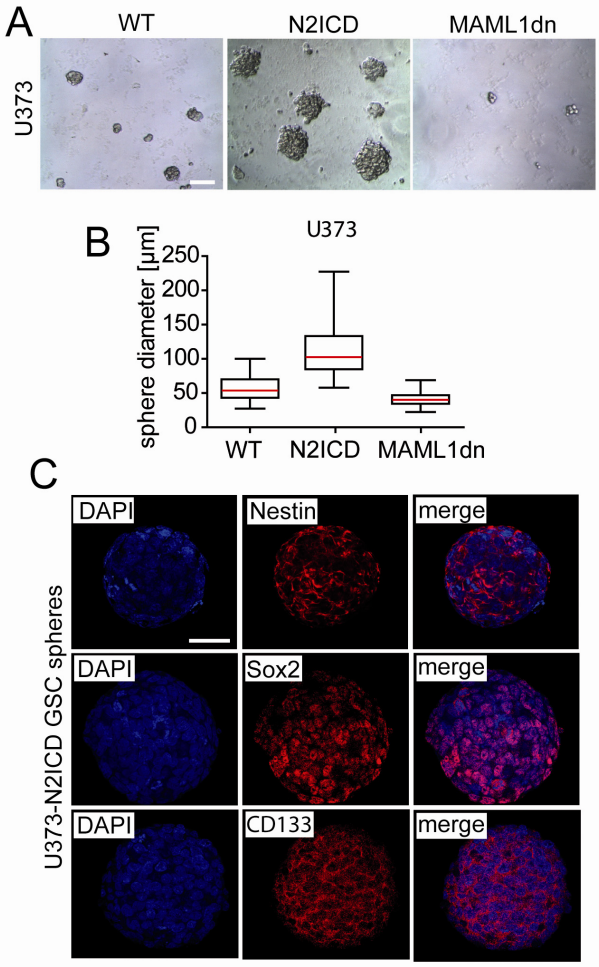
Tchorz et al.

Figure 4



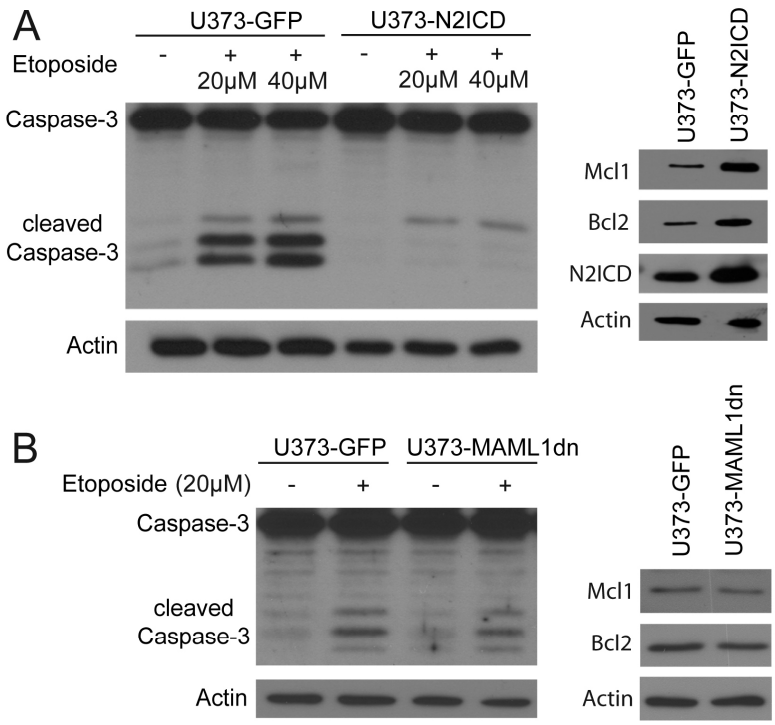
Tchorz et al.

Figure 5



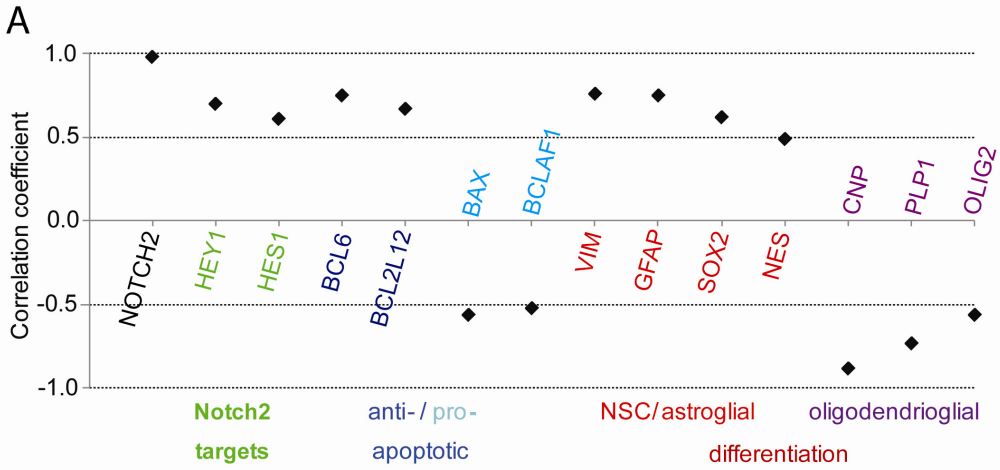
Tchorz et al.

Figure 6



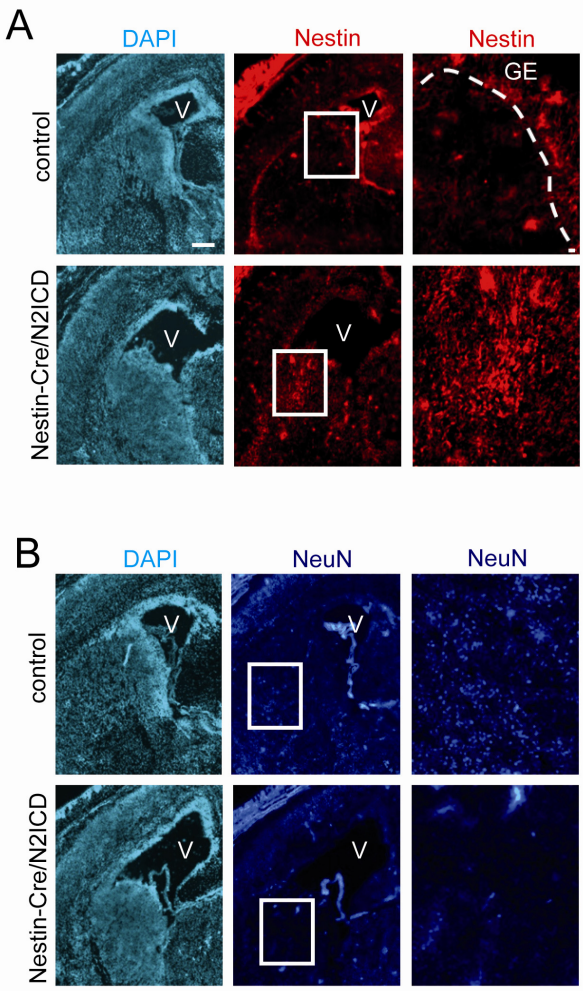
Tchorz et al.

Figure 7



Tchorz et al.

Supplementary Figure 1



Tchorz et al.

Supplementary Table 1

Probe	Gene Symbol	Description	Correlation coefficient	*P*-value
212377_s_at	NOTCH2	Notch homolog 2 (Drosophila)	0.98	<0.001
44783_s_at	HEY1	hairy/enhancer-of-split related with YRPW motif 1	0.70	0.001
203394_s_at	HES1	hairy and enhancer of split 1,	0.61	0.010
203140_at	BCL6	B-cell CLL/lymphoma 6	0.75	<0.001
233110_s_at	BCL2L12	BCL2-like 12 (proline rich)	0.67	0.003
217490_at	BAX	BCL2-associated X protein	-0.56	0.019
239897_at	BCLAF1	BCL2-associated transcription factor 1	-0.52	0.032
201426_s_at	VIM	vimentin	0.75	0.005
229259_at	GFAP	glial fibrillary acidic protein	0.76	0.004
228038_at	SOX2	SRY (sex determining region Y)-box 2	0.62	0.008
229346_at	NES	nestin	0.49	0.047
208912_s_at	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	-0.88	<0.001
210198_s_at	PLP1	proteolipid protein 1	-0.73	<0.001
213825_at	OLIG2	oligodendrocyte lineage transcription factor 2	-0.56	0.020

4. Concluding remarks

In this study, I analyzed the functions of Notch2 signaling during brain and liver development and related disorders, such as GBM and AGS. In both organs, I showed that Notch2 has a conserved role in regulating cell fate decisions and morphogenesis. After establishing a mouse model that allows for tissue-specific Notch2ICD expression, I was able to mimic ligand-induced Notch2 activation in hepatoblasts or in NSCs which enabled functional analysis of Notch2 signaling in these cells and their progeny. Thus, I was able to make major contributions to the understanding of AGS and the possible effects of Notch2 signaling in GBM formation and progression.

Although our understanding of mechanisms regulating IHBD development improved since the discovery of Notch2 mutations and mutations in its ligand Jagged1 in AGS, the exact function of Notch2 during BEC differentiation, IHBD differentiation and BEC survival remained elusive (27-30). Here, I demonstrate that transgenic Notch2ICD expression in bipotential hepatoblasts leads to their differentiation into BECs and to the formation of additional tubular structures in portal regions. The formation of ectopic tubular structures in lobular regions shed light on the inherent potential of Notch2 signaling in hepatoblasts, because it dissociated Notch2 signaling from local cues in the portal environment. Additional periportal ducts are connected to the biliary tight junction network and maintained in adult mice. In contrast, ectopic lobular ducts are lost during postnatal development, suggesting a crucial role for the portal environment in mediating bile duct maintenance. Remarkably, ductal plate cells that express transgenic Notch2ICD persist into adulthood, suggesting that Notch2 also plays a role in BEC survival. The finding that Notch2 regulates BEC differentiation is somehow surprising, since previous studies using Albumin-Cre mediated deletion of Notch2, argued that Notch2 signaling is dispensable for BEC differentiation due to the presence of a ductal plate in these mice (35,36). Since Albumin-Cre driven gene deletion is incomplete in the embryo and starts around E13.5, residual Notch2 levels may account for the presence of ductal plate BECs in liver-specific Notch2 KO mice (35,36,117). Due to the intrinsic potential of Notch2 to drive BEC differentiation, I hypothesized

that Notch2 could be involved in cholangiocarcinoma formation. Cholangiocarcinoma is a rare but very aggressive form a liver cancer that arises from the biliary system (118). Preliminary results from N2ICD/AlbCre mice that were aged for 12 month (data not shown), show that all (7/7) aged N2ICD/AlbCre mice developed lesions and neoplasia that were macroscopically visible, while none of the AlbCre (0/8), N2ICD (0/9) or wt control mice (0/8) showed any abnormalities. Although histological analysis showed signs of neoplasia containing BECs (also termed cholangiocytes), cholangiocarcinomas were not detected. Ongoing experiments using diethyl nitrosamine (DEN) to induce liver cancer in N2ICD/AlbCre and control mice will aim at validating the oncogenic potential of Notch2 signaling in cholangiocarcinoma formation. Upon DEN application, mice normally develop hepatocellular carcinoma (HCC) within 40 weeks (119). It is possible that activated Notch2 signaling in N2ICD/AlbCre mice induces cholangiocarcinoma. This would provide a mouse model for studying cholangiocarcinoma, validation of new mechanisms regulating cholangiocarcinoma formation and investigation of possible treatment options. In addition, future experiments will address the role of Notch2 signaling during liver regeneration which is an essential step following liver failure, tumor resection or small-to-size liver transplantation and impaired in cirrhosis patients. Liver regeneration is characterized by increased proliferation of hepatocytes, endothelial cells and BECs in order to restore the original organ mass (120). Proliferating BECs then transdifferentiate into hepatocytes to restore hepatocyte numbers to about 70-80% (120). It is possible that activated Notch2 signaling in N2ICD/AlbCre mice largely inhibits transdifferentiation of BECs into hepatocytes after partial hepatectomy in these mice. This would help to understand mechanisms regulating liver regeneration and further elucidate the role of Notch2 signaling during liver development.

Before the discovery of adult neurogenesis in the early 1960s (44), the human brain was thought to be a static, fully differentiated organ and it was believed that differentiated glial cells were the cell of origin for GBMs. However, until in the early 1990s, when Reynolds and Weiss reported the isolation of adult NSCs from the adult mouse brain (45), GBM therapy only focused on the bulk mass of tumor cells that were removed by surgery or eliminated by radiation or chemotherapy. The high rate of recurrence and notorious resistance against

radiation and cytotoxic drugs inspired a reevaluation of the gliomagenesis theory and adult NSCs were then hypothesized as being the possible cells of origin for GBM (71,73). This theory was further supported by the discovery of a highly tumorigenic subpopulation of stem-like cells within surgical isolates of GBMs, termed GSCs (71-73). Intriguingly, GSCs resemble most characteristics of NSCs, such as multipotency, self-renewal capacity, high motility, telomerase activity and robust proliferative potential. Likewise, there is increasing evidence that GSCs can derive from NSCs and eventually give rise to GBMs (68,71-73). However, the molecular events leading from NSCs to GSCs are poorly understood.

We recently reported that Notch2 is highly expressed in most GBMs , while loss of *Notch2* is frequently found in oligodendrogliomas and correlates with a much better prognosis (74). However, whether Notch2 plays a role in glioma formation is unknown. For this reason, I addressed whether Notch2 plays a role in regulating NSC proliferation and differentiation, possibly predisposing NSCs to become GSCs and eventually GBMs. Therefore, I generated mice that ectopically express activated Notch2 in NSCs and compared the induced molecular alterations to those observed in GSCs from GBM cell lines and primary GBM biopsies. I showed that key features of GSCs, such as increased proliferation and astrocytic lineage commitment, are induced by ectopic Notch2 signaling in NSCs. Aberrant Notch2 expression may therefore predispose NSCs to become GSCs that give rise to GBMs. Moreover, Notch2 signaling enhanced survival of GBM cells, possibly explaining the increased aggressiveness of GBMs with high Notch2 levels. Moreover, constitutive Notch2 signaling prevents etoposide-induced apoptosis in NSCs and GSCs, likely by upregulating the antiapoptotic proteins Mcl1 and Bcl2 (paper 2). Ongoing studies will further characterize the antiapoptotic function of Notch2 signaling in NSCs and GBM. Blockade of Notch2 signaling may therefore interfere with GBM cell survival, the formation and the proliferation of GSCs and consequently be of therapeutic benefit for the treatment of GBMs, for which no cure is available yet.

Although constitutive Notch2 induces GSC features in NSCs, no GBM formation was observed in 12 month old GCE/YFP/N2ICD mice (paper 2). Therefore, additional somatic mutations may be

necessary to complete the malignant transformation of predisposed NSCs in GSCs. Crossing N2ICD/YFP/GCE mice into a cancer background (e.g. using $PTEN^{-/-}/TP53^{-/-}$ mice (121)) would allow studying the consequence of ectopic Notch2 activation in GBM formation. Since crossing N2ICD/YFP/GCE mice with $PTEN^{-/-}/TP53^{-/-}$ mice (121) is extremely laborious and time-consuming, *in vitro* studies using $PTEN^{-/-}/TP53^{-/-}$ derived NSCs could be performed. Specifically, $PTEN^{-/-}/TP53^{-/-}$ NSCs could be transduced with a virus expressing Notch2ICD, in order to generate artificial GSCs. These cells should then be used for xenograft transplantation to analyze their potential to initiate GBM formation. However, the major drawback of mimicking Notch2 pathway activation in NSCs by the ectopic expression of Notch2ICD is that Notch2 signaling functions independently of γ-secretase cleavage. Therefore, γ-secretase inhibitors cannot be studied in NSCs or GSCs with ectopic Notch2ICD expression.

In addition to addressing the role of Notch2 in GBM formation and progression, my study provides new insights into the ongoing debate concerning a possible functional redundancy of the Notch1 and Notch2 activated domains. Many of our findings for Notch2, such as the regulation of NSC proliferation, differentiation and survival, resemble functions of Notch1 (48-50,122). Kraman and colleagues postulated a general functional redundancy for the activated domains of Notch1 and Notch2 (80). However, their postulation was challenged by other studies that provided evidence that activated Notch1 and Notch2 have opposite effects on embryonal brain tumor growth (81) and on mesothelioma cell survival (82). Interestingly, my findings provide new evidence for a possible redundancy of Notch1ICD and Notch2ICD functions in regulating NSCs behavior. Moreover, recent studies highlighted the importance of Notch1 signaling in regulating cancer stem cells, including GCSs (15,68). In addition, Notch3 activation was shown to induce choroid plexus tumor formation (123). Thus, activation of several Notch receptors seems to have an oncogenic potential in CNS tumors. Therapeutic targeting of Notch signaling might therefore not only be a treatment option for GBM, but also for other CNS tumors.

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CURRICULUM VITAE

Personal data

Surname	Tchorz
Forename	Jan Stephan
Date of birth	16th November 1978
Place of birth	Norden
Nationality	German
Address	Kornhausgasse 2, CH 4051 Basel
Telephone	0041 61 554 9708
Email	jan.tchorz@gmail.com

Education and work experience

03/2010 - current
Project Team Leader
Developmental and Molecular Pathways
Novartis Institute for Biomedical Research
Basel, Switzerland

04/2006 - 03/2010
PhD in Neuroscience (summa cum laude)
Department for Clinical Biosciences, Physiology
Professor Bernhard Bettler
University of Basel, Switzerland

"Notch2 signaling in development and cancer"

06/2005 - 03/2006
Diploma thesis in biology (with honors)
Novartis Institutes for Biomedical Research
Dr. Matthias Müller
Novartis Pharma AG, Basel, Switzerland
In collaboration with Martin Luther University of Halle-Wittenberg, Germany

"Characterisation of exogenous promoters in the Rosa26 locus in mice"

09/2004 - 12/2004
Industrial internship
In-vivo-metabolism, PRBN-S
Dr.Michael Otteneder
Hoffmann La Roche Basel, Switzerland

"Phase-I-metabolism in precision cut organ slices compared to microsomes derived from several organs"

04/2004 - 06/2004
Internship
GPCR group, biochemistry of alimentation
Dr.Frank-Neuschäfer-Rube
University of Potsdam, Germany

"Generation and characterization of a GPCR chimera"

01/ 2001 - 09/2004	Student assistant Internal medicine I, Molecular Hepatology Prof. Bruno Christ and Dr. Hendryk Aurich Martin Luther University of Halle-Wittenberg, Germany
10/2000	Matriculation in biology Martin Luther University of Halle-Wittenberg, Germany
07/2000	Summer school of biosciences Martin Luther University of Halle-Wittenberg, Germany
09/1999 - 07/2000	Alternative civilian service Astrid-Lindgren-School for disabled children, Germany
06/1999	Baccalaureate Meldorfer Gelehrtenschule, Germany

Awards

Kurt-Mothes Prize 2006
Junior Hepatology Prize 2009

Publications

Tchorz JS, Kinter J, Müller M, Tornillo L, Heim MH, and Bettler B**.** Notch2 signaling promotes biliary epithelial cell fate specification and tubulogenesis during bile duct development in mice. *Hepatology* 2009, Sep;50(3):871-9.

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Referees

Dr. Matthias Müller
DMP, NIBR
Novartis Pharma AG, Basel
matthias.mueller@novartis.com
0041798634566

Prof. Bernhard Bettler
DBM, Physiology
Pharmazentrum Basel
bernhard.bettler@unibas.ch
0041612671632

Prof. Markus Heim
DBM, Hepatology
Universitätsspital Basel
markus.heim@unibas.ch
0041612653362