TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Entwicklungsgenetik

Examining the role of Notch signalling in adult hippocampal stem cell maintenance

Dissertation von

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1 Zusammenfassung

Im Erwachsenenalter werden im Gyrus Dentatus des Hippocampus fortlaufend neue Nervenzellen aus neuralen Stammzellen gebildet. Die Erhaltung und Differenzierung neuraler Stammzellen muss in einem ausgewogenen Verhältnis stehen, um die hippocampale Neurogenese während der gesamten Lebenszeit aufrechtzuerhalten. Frühere Studien haben gezeigt, dass der Transkriptionsfaktor *Sox2* bei der Erhaltung hippocampaler Stammzellen im erwachsenen Gehirn eine wichtige Rolle spielt. Auch der Notch Signalweg wurde bereits mit der Stammzellerhaltung in verschiedenen Stammzell-Systemen in Verbindung gebracht. In der vorliegenden Arbeit wurden mit Hilfe der Expressionsregulation von *Sox2* Signalwege identifiziert, welche für die Erhaltung adulter hippocampaler Stammzellen von Bedeutung sind und die Frage behandelt, ob *Sox2* an der hippocampalen, durch den Notch Signalweg bestimmten Stammzellerhaltung im erwachsenen Gehirn beteiligt ist.

Immunohistochemische Analysen, bei denen Reportertiere für den Notch Signalweg verwendet wurden, zeigten dass dieser in vielen der SOX2 positiven Stammzellen in der SGZ des adulten Gyrus Dentatus aktiv ist und *in vitro* Analysen isolierter hippocampaler Stammzellen bewiesen, dass der Notch Signalweg die *Sox2*-Expression begünstigt. EMSA und ChIP Analysen belegten, dass RBPJK und NICD an den *Sox2*-Promotor in adulten hippocampalen Stammzellen binden. Dies zeigt, dass *Sox2* ein direktes Zielgen des Notch Signalweges in adulten hippocampalen Stammzellen ist.

Der konditionale Knockout von RBPJK, eines Transkriptionsfaktors des *Notch* Signalweges, in hippocampalen Stammzellen in vivo führte zu einer signifikanten Abnahme der Anzahl SOX2 positiver radial-glia ähnlicher Stammzellen des Typs 1 und Zellen des Typs 2. Drei Wochen nach Induktion der Rekombination wurde zudem eine erhöhte Proliferation und Differenzierung von rekombinierten Stammzellen zu unreifen Nervenzellen festgestellt. Nach zwei Monaten waren Proliferation und Neurogenese stark vermindert. Dies zeigt, dass im Hippocampus von Mäusen, bei denen *Rbpjκ* ausgeknockt wurde, keine oder kaum mehr neurale Stammzellen vorhanden sind, die neue Körnerzellen generieren können.

Darüber hinaus war die Aktivität des kanonischen Wnt Signalweges in vielen SOX2 positiven Zellen in der SGZ des adulten Gyrus Dentatus zu beobachten. Weitere

Analysen zeigten, dass der Wnt/ β-catenin Signalweg die *Sox2* Expression positiv reguliert.

Aus den in dieser Arbeit präsentierten Daten kann der Schluss gezogen werden, dass der Notch Signalweg eine wichtige Rolle bei der Erhaltung adulter neuraler Stammzellen spielt, sehr wahrscheinlich durch die Regulation von *Sox2*.

2 Abstract

During adulthood, neural stem cells continuously give rise to new neurons in the dentate gyrus of the hippocampus. The maintenance and differentiation of neural stem cells have to be tightly balanced in order to sustain hippocampal neurogenesis throughout lifetime. Previous studies have demonstrated that the transcription factor *Sox2* is necessary for the maintenance of hippocampal stem cells during adulthood. In this study, the regulation of the expression of the stem cell associated gene *Sox2* was used to identify signalling pathways that are important for adult hippocampal stem cell maintenance. The Notch signalling pathway has previously been implicated in stem cell maintenance in several stem cell systems. The present study addressed the question whether *Sox2* is involved in Notch signalling mediated adult hippocampal neural stem cell maintenance.

Immunohistochemical analysis, using reporter animals for the Notch signalling pathway, revealed that Notch signalling is active in many of the SOX2 positive stem cells in the adult SGZ of the dentate gyrus. Subsequent *in vitro* analysis of isolated adult hippocampal stem cells showed that Notch signalling promotes *Sox2* expression. EMSA and ChIP analysis revealed that RBPJκ and the intracellular domain of the Notch receptor (NICD) are bound to the *Sox2* promoter in stem cells, indicating that *Sox2* is a direct target of the Notch pathway in hippocampal stem cells. Hippocampal stem cell specific conditional knockout of the transcription factor *Rbpjκ*, a down-stream mediator of Notch signalling, in hippocampal stem cells *in vivo*, lead to a significant reduction in the number of SOX2 positive type 1 radial glia like stem cells and type2 cells. The loss of stem cells was accompanied by increased proliferation and differentiation of recombined stem cells into immature neurons three weeks after induction of recombination. At a later point in time, at two months, proliferation and neurogenesis was largely diminished, indicating that the hippocampus of *Rbpjκ* deficient mice is depleted of neural stem cells capable of generating new granule neurons.

Additionally, canonical Wnt pathway activity was found in many SOX2 positive cells in the SGZ of the adult dentate gyrus. Further analysis suggested that Wnt/β-catenin signalling positively regulates *Sox2* expression.

Taken together these data suggest that Notch signalling plays an essential role for adult neural stem cell maintenance most likely through the regulation of *Sox2*.

3 Introduction

3.1 Adult neurogenesis and the hippocampal neurogenic niche

The term “adult neurogenesis” describes the generation of new functional neurons in the adult mammalian brain. The phenomenon of adult neurogenesis was first reported by Joseph Altman in the 1960s (Altman and Das, 1965). The newly born neurons arise from certain cell populations, called neural stem cells, which reside in distinct regions in the adult brain. Neural stem cells are undifferentiated cells which have the unique capability to self-renew and to differentiate into all major cell types of the central nervous system (CNS) which comprise neurons, astrocytes and oligodendrocytes (“multipotency”).

So far, the birth of new neurons in the adult mammalian brain has consistently been observed in only two neurogenic niches: the subventricular zone of the lateral ventricles in the forebrain and the dentate gyrus of the hippocampus (Zhao et al., 2008). Evolutionary, the hippocampus is one of the oldest structures in the brain. It is located in temporal lobe of the cerebral cortex and consists of two main anatomical structures: the cornu amonis (CA) and the dentate gyrus. The CA is divided into the CA1 to CA3 region based on anatomic boundaries and the expression of certain proteins which exhibit specific expression in the different hippocampal subregions (Zhao et al., 2001). The dentate gyrus possesses a v-shaped morphology, the space between the two blades being called hilus. The dentate gyrus consists mainly of granule neurons constituting the granule layer. The subgranular zone (SGZ) lying immediately below the granule layer harbours the stem cells of the hippocampus. The molecular layer, which is located above to the granule layer, consists mainly of the dendrites of the granule neurons of the granule layer.

Neural stem cells of the hippocampus are located in the SGZ and exhibit a morphology resembling that of radial glia during development. Therefore, they are referred to as radial glia like stem cells. They show several features common to astrocytes comprising a light cytoplasm containing few ribosomes, the presence of characteristic intermediate filaments and the expression of glial fibrilary acidic protein (GFAP) (Seri et al., 2001). They project their radial process through the granule layer. Additionally, they possess endfeet which make contact with the vasculature (Filippov et al., 2003).

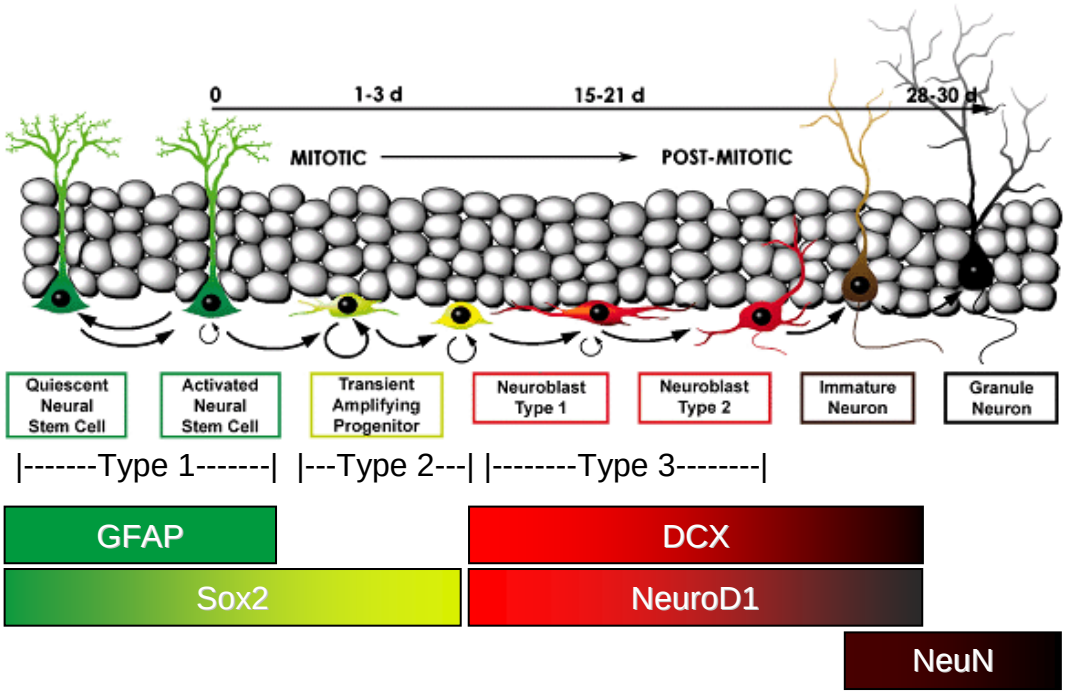

Figure 1: Schematic representation of the different cell stages during adult hippocampal neurogenesis. Radial glia like stem cells or type 1 cells give rise to highly proliferative transient amplifying or type 2 cells. These cells become neuronally fate determined and differentiate into neuroblasts or type 3 cells. Neuroblasts become post-mitotic and migrate into the granule layer where they terminally differentiate into mature granule neurons and get integrated into the hippocampal neuronal circuitry. At the bottom, characteristic proteins which are expressed by distinct cell types in the dentate gyrus are indicated (modified from Encinas et al., 2006).

Seri and co-workers described that GFAP positive radial glia like cells act as neural stem cells in the adult dentate gyrus (Seri et al., 2001) and that these cells are the primary precursors for the generation of new dentate granule neurons. These neurons are generated through a stereotypical sequence: Radial glia like stem cells or type 1 cells are thought to be quiescent or divide at least very rarely (Morshead et al., 1994). Upon division, they generate neural progenitors. Those fast dividing cells are called transient amplifying cells or type 2 cells (Steiner et al., 2006). They do not express GFAP any more. During the course of neurogenesis, these type 2 cells become neuronally fate committed and start to differentiate and express characteristic proteins like DCX or NeuroD. These immature neurons or neuroblasts cease to proliferate and become postmitotic. They continuously mature and migrate into the granule layer of the dentate gyrus. After around 30 days, they express markers of mature dentate granule neurons like NeuN and Calbindin and become integrated into the existing neuronal network.

Adult hippocampal neurogenesis is correlated with hippocampus-dependent memory and learning tasks, and consequently impaired hippocampal neurogenesis is associated with decreased learning and memory performance (Dupret et al., 2008; Imayoshi et al., 2008; Zhang et al., 2008; Garthe et al., 2009; Jessberger et al., 2009). Impaired hippocampal neurogenesis is also correlated with increased anxiety behaviour, indicating that adult neurogenesis contributes to hippocampal function (Bergami et al., 2008; 2009; Clelland et al., 2009; Revest et al., 2009).

As described above, adult neurogenesis is an extended developmental process consisting of multiple steps. Each step has to be carefully controlled in order to generate new neurons at a rate which ensures proper hippocampal function. For example, maintenance and the differentiation of stem cells have to be tightly balanced. Impaired maintenance and precocious differentiation of neural stem cells on the one hand would lead to a depletion of the stem cell pool and decreased neurogenesis in the long run. On the other hand, increased neural stem cell maintenance at the expense of neurogenesis would also fail to supply appropriate rates of newly generated neurons that are crucial for proper hippocampal function.

Until today, it is not fully understood how this balance between maintenance of neural stem cells and differentiation is kept. Recently, several molecules have been identified which are involved in the control of adult hippocampal neural stem cell maintenance. These molecules comprise factors that positively regulate neural stem cell maintenance, e.g. the polycomb factor *Bmi-1* (Molofsky et al., 2003; Fasano et al., 2009), the transcription factor Tailless (*Tlx*) (Shi et al., 2004; Liu et al., 2008; Zhang et al., 2008), the basic helix loop helix (bHLH) proteins *Id1* (inhibitor of differentiation) and *Id2* (Jung et al., 2009), but also factors which negatively influence neural stem cell maintenance, e.g. the tumour suppressor gene *p53* (Gil-Perotin et al., 2006; Meletis et al., 2006).

3.2 *Sox* gene family

SRY (sex determining region Y)-box 2 (*Sox2*) is one of the genes that is most closely associated with neural stem cell activity across many species during different developmental stages (Zappone et al., 2000; Avilion et al., 2003; D'Amour and Gage, 2003; Komitova and Eriksson, 2004; Suh et al., 2007; Fong et al., 2008). SOX proteins are transcription factors of the high mobility group (HMG) box family. So far,

30 different SOX proteins have been identified with 20 of them present in mammals. They are classified according to sequence homologies within and outside the HMG domain into eight groups: Sox A – Sox H. The most reliable signature for all non-SRY *Sox* members is the conservation of the key amino acid motif RPMNAFMVW within the HMG domain. SOX proteins are spatiotemporal ‚omnipresent'. They are, for example, expressed during blastocyste formation, gastrulation, germ layer formation and formation of the hematopoietic and nervous system, skeleton, gonads, spleen, heart, blood vessels, and melanocytes (Kamachi et al., 2000). Functionally, SOX proteins are very diverse as they maintain stem cell characteristics and a pluripotent state, influence cell death, survival and proliferation, influence cell fate decisions and consecutive lineage progression and participate in terminal differentiation processes (for review see (Wegner and Stolt, 2005)). SOX proteins can be post-translationally modified. These modifications comprise phosphorylation, which increases DNA binding affinity and transcriptional activity, acetylation, which also increases DNA binding affinity and promotes nuclear localisation and SUMOylation (SUMO, small ubiquitin-related modifier). The effects of SUMOylation are substrate specific and can change the sub-cellular localization of proteins, alter protein-protein interactions or protein activity (Taylor and Labonne, 2005).

As explained above, the main characteristic of all SOX proteins is the presence of the HMG domain, which is used for DNA binding and is highly conserved throughout eukaryotic species. SOX proteins bind to the minor groove in DNA. The binding of SOX proteins to DNA induces a strong bend in the DNA, enabling the contact between transcription factors bound to distant regions thereby supporting the formation of an enhanceosome. SOX proteins bind to DNA as monomers (Sox B group), facultative dimers (Sox E group) or constitutive dimers (Sox D group) (for review see (Wegner, 2005)). The binding of SOX protein dimers alters DNA conformation differently from monomers (Wegner, 2005).

Besides their HMG box, SOX proteins either possess a carboxy-terminal trans-activation (TAD) or repression domain. A third important structural feature of SOX proteins is a region that can include a part of the HMG domain and is used for interaction with a partner factor. SOX proteins are believed to act in concert with a specific partner protein (for review see (Kamachi et al., 2000)). The current model assumes that if a SOX protein alone binds to SOX binding sites on the DNA through its HMG box, this interaction is unstable. Only the association of the SOX protein with

a partner protein, which binds to the DNA near the SOX binding site, helps to stabilize the SOX protein. Only then can the SOX protein accomplish its activity of transcriptional activation or repression.

The B1 subgroup comprises three members of mouse *Sox* transcription factors, *Sox1*, *Sox2* and *Sox3*. They share more than 90% amino acid residue similarity in their HMG box domain. They all possess a C-terminal transactivation domain; yet current evidence suggests that at least SOX2 can also act as a transcriptional repressor (Kuwabara et al., 2009). SOX2 exhibits a serine-rich region at its C-terminus which is important for transactivation (Nowling et al., 2000).

3.2.1 Expression of *Sox2* in the CNS

In the adult brain, SOX2 is expressed in the two neurogenic niches, the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampal dentate gyrus (Komitova and Eriksson, 2004). As explained before, neural stem cells in both neurogenic niches are believed to be a subpopulation of glial fibrillary acidic protein (GFAP) positive astrocytes (Doetsch et al., 1999; Seri et al., 2001; Doetsch, 2003; Fukuda et al., 2003). Within the hippocampal dentate gyrus, SOX2 is expressed at the base of the granule layer, the subgranular zone (SGZ). SOX2 expression can be detected in GFAP positive, slowly dividing radial glia like stem cells (type1 cells). Most of the proliferating cells, indicated by BrdU incorporation, express SOX2 suggesting that transit amplifying cells (type 2 cells), the immediate progeny of neural stem cells, express SOX2 (Ferri et al., 2004; Steiner et al., 2006). *Sox2* expression is down-regulated when type 2 cells become neuronally fate committed and start to express inducers of neuronal differentiation such as the bHLH transcription factor NeuroD1 (Gao et al., 2009). Within the subventricular zone of the lateral ventricle, GFAP positive astrocytes residing in the subventricular zone express SOX2, suggesting that SOX2 is expressed by stem cells. Like in the SGZ, most of the proliferating cells express SOX2. SOX2 positive cells can also be found in the rostral migratory stream (RMS) leading from the lateral ventricle toward the olfactory bulb. Migrating neuroblasts within the RMS express the membrane antigen PSA-NCAM. Most, if not all of the PSA-NCAM positive neuroblasts are negative for SOX2. SOX2 expression can also be found in non-radial astrocytes, sparse cells within differentiated regions like in pyramidal cells in the cerebral cortex, in

differentiated neurons in the striatum and the periventricular nuclei of the thalamus (Ferri et al., 2004).

Recent studies have begun to shed light on the regulatory sequences controlling *Sox2* expression in different stem cell populations and in the developing and adult brain. There are two known enhancers of the *Sox2* gene named Sox2 regulatory region 1 (SRR1) and Sox2 regulatory region 2 (SRR2), which exert their function in pluripotent embryonic stem cells and multipotent neural stem cells during development (Tomioka et al., 2002; Miyagi et al., 2004). SRR1 is located approximately between 3.5 -4.0 kb upstream, SRR2 approximately between +3.5 and +4.0 kb downstream of the transcriptional start site of the *Sox2* gene. Studies by Sikorska and colleagues (Sikorska et al., 2008) showed that the SRR1 and SRR2 enhancers are unmethylated and associated with acetylated histone H3 in undifferentiated human NT2/D1 teratocarcinoma cells which strongly express *Sox2* and have the potential to differentiate into astrocytes and neurons. By contrast, in neurons, SRR1 and SRR2 were methylated and histones were deacetylated consistent with the lack of expression of *Sox2* in most neurons. It was shown that SRR1 is conserved between mammals and chicken (N2 enhancer) (Uchikawa et al., 2003).

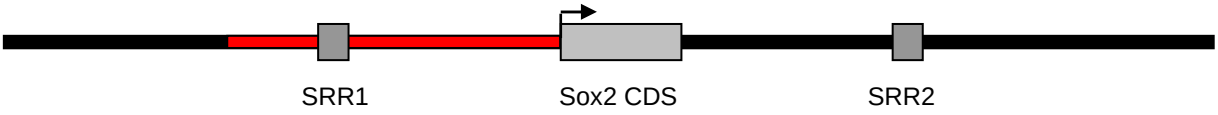

Figure 2: Location of SRR1 and SRR2 enhancers within the *Sox2* promoter. The red promoter fragment indicates the 5.7 kb region that was shown to be essential for telencephalic expression of a *Sox2*-transgene. SRR1 and SRR2 are known enhancer regions of *Sox2* (SRR = Sox regulatory region).

Zappone and co-workers have shown that the 5.7 kb region upstream of the *Sox2* transcription start site is essential for telencephalic expression of a β-geo reporter (Zappone et al., 2000). At 12.5 days post coitum (d.p.c.), reporter expression was confined to the dorsal telencephalon but to some extend also to the lateral ganglionic eminence. At 14.5 d.p.c., intense reporter staining was observed in the ventricular zone, formed by proliferating, undifferentiated cells that will give rise to neurons and glia. Expression of the β-geo reporter persists into adulthood in cells of the subventricular cells of the lateral ventricles and in cells in the subgranular zone of the hippocampal dentate gyrus, i.e., regions where neural stem cells persist and give rise

to new neurons during adulthood. This expression pattern suggested that the 5.7 kb region of the *Sox2* promoter is sufficient to control expression in telencephalic neural stem cells (Zappone et al., 2000). Indeed, cells which were isolated from the periventricular zone of the telencephalon and which showed expression of the reporter could be cultured as neurospheres. Those cells were able to generate secondary and tertiary neurospheres and could give rise to neurons, astrocytes and oligodendrocytes under differentiating conditions, suggesting that the 5.7 kb *Sox2* promoter region confers expression in multipotent neural stem cells. Zappone and co-workers further narrowed down the promoter region essential for telencephalic expression to a region between 5.7 and 3.3 kb upstream of the *Sox2* transcription start site, which comprises the SRR1. They reported that an 800 nt region between the 5.7 and 3.3 kb upstream of the *Sox2* transcription start site confers telencephalic expression specificity. This 800 nt region encompasses a DNaseI hypersensitive site, which is usually indicative of active transcriptional regulation. SRR1 activity in embryonic NSCs is controlled by the neural POU domain transcription factors BRN1 and BRN2 (Catena et al., 2004). Analysis revealed that SRR1 contains a predicted binding site for SOX2 (unpublished data). Similar to the cooperative regulation of the SRR1 activity, SRR2 activity in ES cells is mainly controlled by OCT3/4-SOX2 complexes (Tomioka et al., 2002). Furthermore, it was shown that the same SRR2 core sequence, which is important for expression in ES cells, can also drive expression in neural stem cells (Miyagi et al., 2006). However, the OCT3/4 protein is not expressed in the brain. Miyagi and co-workers have shown that the POU domain proteins BRN1, BRN2, BRN4 and OCT6 can bind to SRR2 and may substitute for OCT3/4 (Miyagi et al., 2004).

The signalling pathways upstream of *Sox2* expression are only poorly characterized. FGF2 signalling exerts mitogenic effects on neural progenitors during development and adulthood (Palmer et al., 1995; Kuhn et al., 1997; Palmer et al., 1997; Yoshimura et al., 2001)). It was shown during embryonic development at E13.5 that *Sox2* expression is enhanced by FGF signalling (Bani-Yaghoub et al., 2006). A link between FGF2 signalling and *Sox2* expression has been also observed in other stem cell niches. Mansukhani and co-workers have shown that *Sox2* is positively regulated by FGF signalling in osteoblasts (Mansukhani et al., 2005). The authors found that FGF2-induced activation of *Sox2* expression inhibited osteoblast differentiation, indicating that FGF signalling via *Sox2* expression contributes to the maintenance of

the precursor cell stage. The LIF/STAT3 pathway is essential for self-renewal of mouse ESCs (Niwa et al., 1998; Matsuda et al., 1999; Raz et al., 1999). At the onset of gliogenesis at embryonic day E18, CNTF and LIF signalling increased the number of SOX2 GFAP expressing astrocytes (Bani-Yaghoub et al., 2006).

3.2.2 *Sox2* mutants and function of SOX2 in neural stem cells

SOX2 was shown to be important for neural stem cell maintenance during embryonic development as well as in the adult neurogenic niches. All members of the SoxB1 subgroup are highly expressed by neural stem cells in the mammalian CNS during development (Graham et al., 2003). SOX2 positive cells isolated from the E13.5 ventricular zone and grown as neurospheres were able to generate SOX2 positive secondary neurospheres (Bani-Yaghoub et al., 2006), demonstrating that SOX2 positive cells have the capability to self-renew. As neural stem cells become neuronally fate committed and start to differentiate into neurons, *Sox2* expression becomes down regulated. It was shown that overexpression of SOX2 by in ovo electroporation in chicken inhibits neuronal differentiation (Bylund et al., 2003; Graham et al., 2003). Conversely, inhibition of *Sox2* expression results in the exit of the cell cycle and concomitant neuronal differentiation.

Ablation of *Sox2* expression resulted in embryonic lethality shortly after the implantation stage (Avilion et al., 2003). To investigate the function of *Sox2* at later embryonic and postnatal stages, Ferri and colleagues generated *Sox2* compound heterozygous mice. These mice carry a *Sox2* null allele (Zappone et al., 2000; Avilion et al., 2003) and a regulatory mutation caused by the deletion of a neural cell specific enhancer (Zappone et al., 2000). The analysis of these hypomorphic mutants shows that significant reduction of *Sox2* expression levels result in many neurological defects and signs of neurodegeneration. Phenotypically these defects comprise feet clasping, epileptic spikes in cortical and hippocampal electroencephalographic recordings and a circling behaviour of the animals (Ferri et al., 2004). The latter one has also been observed in rodent transgenic animals of Huntington's disease (Hodgson et al., 1999). On the morphological side, many neurons showed signs of neurodegeneration like intraneuronal aggregates of neurofilaments and, though less abundant, of ubiquitinated proteins. Most importantly, *Sox2* hypomorphic mutants show a posteriorly and medially reduced cortex, a progressive anteroposterior

reduction of the corpus callosum, a decrease of the anterior thalamus, a dorsal striatum and septum, and a lateral and third ventricle enlargement, which points at a defect in fetal neurogenesis. Indeed, *Sox2* hypomorphic mutants exhibit fewer proliferating cells in both neurogenic niches. Consequently, the number of newly generated neurons is significantly reduced in the hippocampus of *Sox2* mutant mice. Interestingly, the cause for decreased neurogenesis seems to be impaired maintenance of neural stem cells. *Sox2* hypomorphic mutants exhibit a severe decrease in the number of GFAP positive radial glia like stem cells in the SGZ of the dentate gyrus, suggesting that *Sox2* is crucial for adult hippocampal stem cell maintenance. Decreased neurogenesis is also apparent in the SVZ of *Sox2* mutant mice. Recently, *Sox2* was conditionally knocked out in NESTIN positive adult hippocampal neural stem cells (Favaro et al., 2009). Loss of *Sox2* expression caused loss of radial glia like stem cells in the dentate gyrus, indicating the importance of *Sox2* for adult hippocampal neural stem cell maintenance. Consequently, *Sox2*-deficient neurosphere cultures could not be maintained over time. The authors were able to partially rescue the NSC exhaustion in *Sox2*-deficient neurosphere cultures by either applying conditioned medium from wildtype *Sox2*-expressing NSC cultures or by lenti-virally overexpressing SOX2. They identified *sonic hedgehog* (*Shh*) as a direct target of SOX2 and could demonstrate that application of SHH on *Sox2*-defcient neurosphere cultures partially restores the neurosphere forming capacity. In accordance with the observation that *Sox2* is important for stem cell maintenance, Suh and colleagues have shown that SOX2 expressing cells in the adult SGZ constitute the neural stem cell population. Isolated SOX2 positive cells have the potential to generate neurons and astrocytes, thus demonstrating their multipotentiality (Suh et al., 2007). Furthermore, the authors found that SOX2 positive cells could give rise to new SOX2 positive cells, indicating that *Sox2* expressing cells have the capacity to self-renew and act as a primary source for adult neural stem cells. Increased proliferation of SOX2 positive cells is coupled with the generation of neuronal precursor cells.

Recently, Kuwabara and colleagues identified several overlapping binding sites for SOX2 and LEF, a transcription factor involved in mediating Wnt signalling activity, in the promoter of the neuronal differentiation gene *NeuroD1* (Kuwabara et al., 2009). The authors found that SOX2 was bound to these binding sites in neural stem cells and was inhibiting *NeuroD1* expression. However, SOX2 was replaced by LEF

proteins from the *NeuroD1* promoter in immature neurons, thus enabling the Wnt signalling pathway to activate *NeuroD1* expression in these cells. These results raise the intriguing question, which signal or factor is maintaining *Sox2* expression in neural stem cells?

3.3 Notch signalling

Notch signalling is an evolutionary highly conserved signalling pathway. It is involved in many processes during development but is also important for tissue homeostasis during adulthood. The Notch receptor itself is a single-pass trans-membrane receptor. It transduces signals coming from trans-membrane ligands.

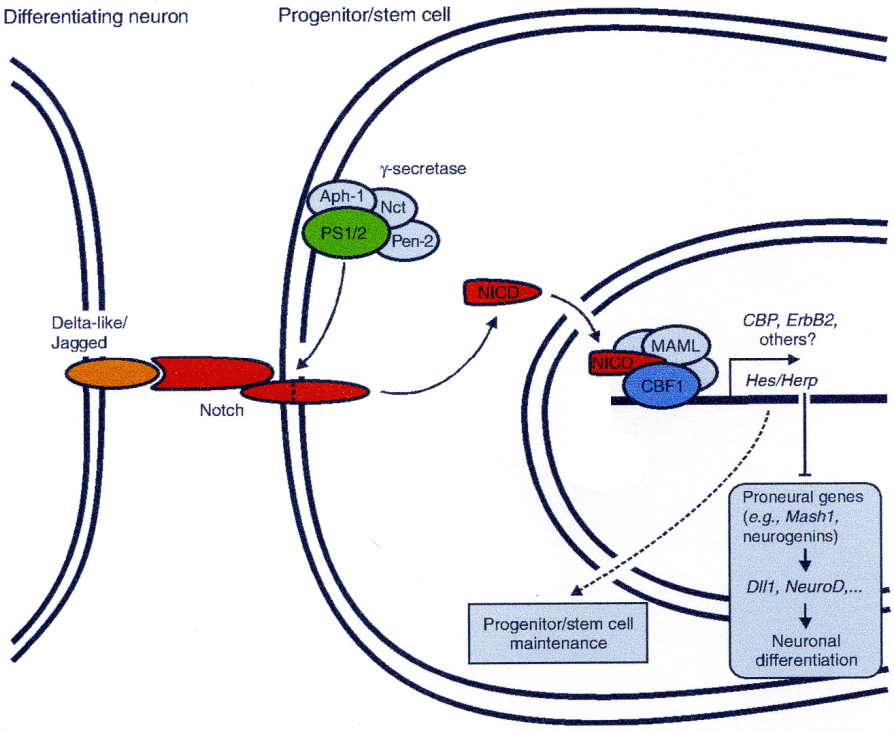

Figure 3: The Notch signalling pathway and its role in neural stem cells maintenance. Upon binding of one of the ligands (Delta-like, Jagged) to the membrane bound Notch receptor, the Notch receptor itself is cleaved by the multi-enzyme complex γ-secretase. This cleavage leads to the release of the Notch intracellular domain (NICD) which in turn translocates to the nucleus of the cell. In the nucleus, NICD binds to the transcription factor RBPJκ, also known as CBF1 or CSL. Binding of NICD to RBPJκ results in the assemblance of a transcriptional activation complex and the expression of Notch target genes like the Hairy and Enhancer of Split (*Hes*) genes. Hes genes act as transcriptional repressors and are believed to inhibit the expression of proneural genes thereby counteracting differentiation and maintaining neural stem cells in an undifferentiated state (modified from Yoon and Gainano, 2005).

Thus, cell-cell contacts are prerequisite to activate Notch signalling. Vertebrates possess four different Notch receptors (*Notch 1* to *Notch 4*) and five different ligands (*Dll1*, *Dll3*, *Dll4*, *Jag1* and *Jag2*).

Signal transduction via the Notch pathway can be divided into several distinct steps. The Notch signal cascade is activated upon contact of one of the ligands with the Notch receptor. This contact is accomplished by the EGF repeats of the Notch receptor and the ligands. The interaction between ligand and receptor renders the "heterodimerization domain" (HD), a part of the negative regulatory region (NRR) of the Notch receptor, accessible to specific enzymes. The precise mechanism is still unknown. These conformational changes of the HD are the "activation switch" of the receptor. The HD harbours the main cleavage site for both furin (S1) and ADAM metalloproteases (S2). The latter one is part of the multi-enzyme complex called gamma-secretase. The gamma-secretase cuts the Notch receptor at a specific aminoacid residue at the S2 cleavage site within the HD domain, which leads to the release of the Notch intra-cellular domain (NICD) and its translocation from the inner side of the plasma membrane into the nucleus. NICD itself consists of five different segments comprising seven tandem-ankyrin repeats, the transactivation domain (TAD), a glutamine-rich OPA domain, a proline-, glutamine-, serine- and threonine rich PEST domain and a RAM23 domain (Wharton et al., 1985; Tamura et al., 1995; Radtke et al., 2005). It was shown that the RAM23 domain contains the nuclear localization signals (NLS) and is, in combination with the ankyrin-repeats, responsible for the direct interaction with the transcription factor RBPJκ, also known as CBF1 or CSL (Tamura et al., 1995; Hsieh et al., 1996). The region between the ankyrin-repeats and the PEST domain constitutes the TAD of NICD (Kurooka et al., 1998).

In the nucleus, NICD binds to the transcription factor RBPJκ, which belongs to the CSL-protein family (CSL, CBF1 (human), Suppressor of Hairless (Drosophila), Lag-1 (C. elegans)). RBPJκ is the only known transcription factor in the Notch signalling pathway. The RBPJκ protein is a ubiquitously expressed and highly conserved nuclear 60 kD DNA-binding protein with 75% amino acid similarity between human and Drosophila (Furukawa et al., 1995). Knockout models for *Rbpjκ* in mice exhibited a lethal phenotype in embryonic development at day 10.5 of gestation. Conditional knock-out of *Rbpjκ* in the hematopoietic cells resulted in a block in T-cell development and ectopic development of B-cells in the thymus (Han et al., 2002). In the absence of NICD, RBPJκ is believed to be bound to Notch target promoters

acting as a transcriptional repressor (for review see (Bray, 2006)). However, constitutive promoter occupancy by RBPJκ, in contrast to human cells, seems not to be the case in Drosophila cells (Krejci and Bray, 2007). The transcriptional repression by RBPJκ seems to be mediated by various mechanisms. Biochemical studies suggest that RBPJκ can interact directly with histone deacetylase-containing protein complexes. Different interactions between RBPJk and co-repressor complexes have been described. These complexes comprise SMRT/mSin3A/HDAC-1 (SMRT: Silencing Mediator for Retinoic acid and Thyroid hormone receptor; mSin3a: mammalian Sex-lethal interactor 3A; HDAC-1: histone deacetylase-1), NCor/mSin3a/HDAC-1 (NCor: Nuclear receptor co-repressor) (Kao et al., 1998), CIR/SAP30/HDAC-2 (CIR: Recepin, CBF interacting co-repressor; SAP30: Sin3A-associated protein) (Hsieh et al., 1999) or SHARP/CtBP/CtIP (SHARP: SMRT and HDAC associated repressor protein; CtBP : C-terminal binding protein; CtIP: CtBP interacting protein) (Oswald et al., 2005).
Once the Notch receptor has been proteolytically cleaved and translocated into the nucleus, it strongly and primarily binds to RBPJκ with its RAM domain and only weakly with its ankyrin repeats, thereby displacing the transcriptional co-repressors and recruiting transcriptional co-activators (Tamura et al., 1995). These co-activators comprise, among others, the glutamine-rich protein Mastermind. The ternary complex of RBPJκ, NICD and Mastermind recruits additional transcriptional co-activators like the histone acetyl-transferase (HAT) p300 to activate Notch target genes by promoting the assembly of initiation and elongation complexes (Wallberg et al., 2002). Additional identified factors which bind to NICD in the activation complex are PCAF and GCN5 (Kurooka and Honjo, 2000). The assembly of the transcriptional activation complex does not only lead to the activation of Notch target genes, but also to the degradation of NICD. Mastermind recruits factors like the cyclin-dependent kinase-8 (CDK8) which phosphorylates NICD and transforms it into a substrate for the nuclear ubiquitin ligase Fbw7/Sel10. This process results in PEST domain-dependent degradation, thus limiting Notch signalling activity.

Notch signalling regulates cell fate decisions in many tissues and controls neuronal function and development. The Notch signalling pathway has been implicated in stem cell maintenance in several stem cell systems (Hitoshi et al., 2002; Chojnacki et al., 2003; Duncan et al., 2005; van Es et al., 2005).

Previous studies analyzed *Notch* and *Rbpjκ* knockout mice during embryonic development (de la Pompa et al., 1997). *Notch1* mutant embryos died around embryonic day 11. The authors found that expression of the Notch target gene *Hes5* is reduced in mutant animals. This is particularly interesting as it was recently shown that neural progenitors in the adult brain can be identified by *Hes5* expression (Basak and Taylor, 2007). Furthermore, de la Pompa and co-workers found that loss of *Notch* or *Rbpjκ* expression resulted in the up-regulation of neuronal differentiation genes like *Neurogenin 2* or *NeuroD* and precocious differentiation (de la Pompa et al., 1997). Embryonic neural stem cells which were deficient of *Rbpjκ* could generate primary neurospheres but exhibited a significant reduction in the number of secondary and tertiary spheres, indicating the importance of RBPJκ-mediated signalling for the maintenance of the self renewing capacity of stem cells (Hitoshi et al., 2002). These results support the notion that Notch signalling is important for neural stem/progenitor cell maintenance during embryonic development. Loss of *Rbpjκ* appears to cause more severe impairments compared to the loss of function phenotype of *Notch*, most likely due to functional redundancy or compensation effects between different *Notch* genes. Similar to the *Notch* phenotype, *Rbpjκ* mutant embryos show decreased expression of genes like *Hes5* and increased expression of genes like *NeuroD*, being suggestive of precocious differentiation (de la Pompa et al., 1997).

However, most of the studies about the role of Notch-/RBPJκ-mediated signalling in neural stem cell maintenance were conducted during embryonic development and much less is known about the involvement of Notch signalling in stem cell maintenance during adulthood. Stump and colleagues found expression of several ligands and receptors of the Notch signalling pathway in both neurogenic niches in the postnatal brain (Stump et al., 2002). In the early postnatal SVZ, loss of Notch signalling resulted in the decreased ability of isolated neural stem cells to generate secondary neurosphere numbers, indicating that loss of Notch signalling affected the self-renewing capacity of neural stem cells (Nyfeler et al., 2005). Similarly, Chojnacki and co-workers demonstrated that knockdown of *Notch1* in adult hippocampal neurospheres resulted in reduced secondary sphere formation, suggesting impaired self-renewal of neural stem cells (Chojnacki et al., 2003). Activation of Notch signalling results in increased numbers of neural stem cells in the brain of adult rats (Androutsellis-Theotokis et al., 2006). However, the authors could show that RBPJκ-

independent mechanisms mediated the Notch signal in neural stem cells *in vitro* and *in vivo*. In this case, Notch activation was followed by phosphorylation of Akt kinase, mTOR and STAT3 on Ser 727, and by subsequent induction of *Hes3* and *Shh* in a temporally controlled order.

Classical targets of the Notch signalling pathway are the Hairy and Enhancer of split genes (*Hes*). These genes code for basic helix-loop-helix (bHLH) transcription factors that act as transcriptional repressors. *Hes* genes exhibit a characteristic amino-terminal bHLH domain, through which they form dimers and bind to DNA. Unlike other bHLH proteins, they have a characteristic proline residue in the middle of the basic region and show higher binding affinity to the N box (CACNAG) than to E box (CANNTG) sequences in the DNA. Furthermore, HES proteins posses a carboxy-terminal tetra-peptide WRPW, which interacts with the co-repressor TLE/Grg, a homolog of the Drosophila *Groucho* gene, and which recruits histone deacetylases, thereby inactivating the chromatin and transcription of Notch target genes (Paroush et al., 1994; Dawson et al., 1995). Mammals possess seven different *Hes* genes, but not all of them are Notch target genes. *Hes1*, *Hes3* and *Hes5* are highly expressed by neural stem cells. However, there is no evidence that Notch signaling is regulating *Hes3* expression (Nishimura et al., 1998). It was shown that *Hes1* and *Hes5* counteract differentiation and neurogenesis in the CNS (Ross et al., 2003; Hatakeyama et al., 2004; Kageyama et al., 2005; Yoon and Gaiano, 2005)). Misexpression of HES1, HES3 or HES5 in the embryonic brain inhibited neuronal differentiation and promoted the maintenance of radial glia (Ishibashi et al., 1994; Hirata et al., 2000; Ohtsuka et al., 2001). Conversely, *Hes1* and *Hes5* mutant embryos exhibited impaired maintenance of radial glial cells and premature differentiation into neurons (Ishibashi et al., 1995; Hatakeyama et al., 2004). *Hes1*; *Hes5* double knock-out mice exhibited an even more severe decrease in the number of radial glia and increased neuronal differentiation compared to the single mutants, suggesting a redundant role of *Hes1* and *Hes5* during CNS development (Ishibashi et al., 1995; Ohtsuka et al., 1999; Cau et al., 2000; Hatakeyama et al., 2004). Furthermore, *Hes1*; *Hes5* double knock-out mice showed impaired neurosphere formation and impaired neural stem cell maintenance in the developing brain (Ohtsuka et al., 1999; Ohtsuka et al., 2001). However, in *Hes1*; *Hes3*; *Hes5* triple knock-out mice neuroepithelial cells are still initially formed but not maintained over time. These data suggest that the initial generation of neuroepithelial cells is

independent of Hes gene activity. Target genes of these HES proteins include other bHLH genes such as *Mash1* or *Ngn2*. *Hes1* represses *Mash1* expression through two different mechanisms. First, HES1 can repress *Mash1* expression by directly binding to the *Mash1* promoter (Chen et al., 1997). Second, activator bHLH proteins like MASH1 form a heterodimer with another activator bHLH factor called E47 and promote neuronal differentiation of neural stem cells. HES1 forms a non-functional heterodimer with E47, thus sequestering E47 from *Mash1* and inhibiting formation of MASH1-E47 complexes (Sasai et al., 1992).

Interestingly, the Notch receptor and the Delta ligand are not expressed at the neuroepithelial stage, when the initial *Hes1* and *Hes3* expression occurs, suggesting that *Hes1* and *Hes3* may be regulated by a Delta/Notch signaling independent mechanism (Hatakeyama et al., 2004). The expression of *Hes5* starts at around E8.5 and is accompanied by down regulation of *Hes3* expression and starting *Delta* and *Notch* expression. It remains to be analyzed which signals regulate *Hes1* and *Hes3* expression at the stages before E8.5.

3.4 The objective of this study

The molecular mechanisms, which control the maintenance of stem cells in the adult brain, are still not fully known. In order to understand better how adult neural stem cells are maintained this study aims to identify molecular pathways that are involved in this important process. To this end, it was analyzed whether the Notch signalling pathway is involved in adult hippocampal stem cell maintenance and the long-term consequences of ablated Notch signalling on the neural stem cell population in the adult hippocampus were examined. Additionally, it should be assessed whether Notch function in neural stem cell maintenance is RBPJκ dependent. Furthermore, it was investigated whether the expression of the stem cell associated gene *Sox2* is linked to Notch/RBPJκ pathway activity.

4 Materials and Methods

4.1 Materials

4.1.1 Organisms

4.1.1.1 Mice

C57BL/6	Wildtype inbred strain (Charles River, Wilmington, US-MA)
Tg(Cp-EGFP)25Gaia	Transgenic Notch reporter (Duncan et al., 2005) (Charles River, Wilmington, US-MA)
B6.Cg-Tg(BAT-lacZ)3 Picc/J	Transgenic canonical Wnt reporter (Maretto et al., 2003) (Jackson Laboratory, Bar Harbor, US-ME)
GLAST::CreERT2; *Rbpjκ* $^{loxP/loxP}$; R26::EYFP	Inducible conditional *Rbpjκ* knockout mice (Jonas Frisén, Karolinska Institute, Stockholm, Sweden)

4.1.1.2 Cell lines

mNSCs	Adult hippocampal mouse stem/progenitor cells were grown as an adherent culture (Ray and Gage, 2006).
mNSCs	Adult hippocampal primary mouse stem cells were grown as neurospheres.
293T	HEK cells (human embryonic kidney cells)
DG75	Burkitt's lymphoma cell line (Ben-Bassat et al., 1977)
SM224.9	*Rbpjκ* knock-out cell line (Maier et al., 2005)

4.1.1.3 Bacteria

DH5α	F-ΔlacU169 (σ80 lacZ) ΔM15 hsdR17 recA1 endA1 supE44 gyrA96 thi-1 relA1 (Hanahan, 1983)

4.1.2 Software

Software	Reference
FluoView 1.7	Olympus, Hamburg, Germany
Genomatix	Genomatix Software GmbH, Munich, Germany
Leica Application Suite AF	Leica Microsystems, Wetzlar, Germany
NetPrimer	PREMIER Biosoft International, CA, USA
StepOne Software	Applied Biosystem Deutschland GmbH, Darmstadt, Germany
Vector NTI	Invitrogen, Karlsruhe, Germany

Table 1: Software used in this study

4.1.3 Chemicals

All chemicals used in this work were, if not stated otherwise, purchased from Sigma-Aldrich (Deisenhofen, Germany), Biomol (Hamburg, Germany), Biorad (Munich, Germany), Fluka (by Sigma-Aldrich, Deisenhofen, Germany) , Invitrogen (Karlsruhe, Germany), Kodak (Stuttgart, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Riedel de Haen (Seelze, Germany), Serva (Heidelberg, Germany). Reagents for molecular biology were purchased from Applied Biosystems (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), New England Biolabs (Frankfurt am Main, Germany), PeproTech (Hamburg, Germany), Promega (Mannheim, Germany) and Roche (Mannheim, Germany).

MilliQ water was used for the generation of solutions (Millipore, Schwalbach, Germany).

All restriction enzymes and their respective buffers were purchased from Fermentas (St. Leon-Rot, Germany), Roche (Mannheim, Germany) and New England Biolabs (NEB) (Frankfurt am Main, Germany).

Master-Mixes for PCR and quantitative real-time PCR were purchased from Eppendorf (Hamburg, Germany) and Agilent (Boeblingen, Germany)

4.1.4 Commercial kits

Kit-name	Manufacturer
DNA Maxi Prep	Promega, Mannheim, Germany
DNA Mini Prep	Macherey-Nagel, Düren, Germany
Dual luciferase assay	Promega, Mannheim, Germany
ECL solution for Western blots	GE Healthcare, Munich, Germany
PCR Master Mix	Eppendorf, Hamburg, Germany
qPCR Master Mix	Agilent, Böblingen, Germany
RNeasy Kit	Qiagen, Hilden, Germany
cDNA Kit SuperScript III	Invitrogen, Karlsruhe, Germany
Gel extraction	Qiagen, Hilden, Germany

Table 2: Commercial kits used in this study. In each case, the manufacturer's protocol was followed.

4.1.5 Buffers and solutions

Annealing buffer for EMSA:	Tris/HCl, pH 7.4	10 mM
	$MgCl_2$	10 mM
	NaCl	50 mM
APS solution:	Ammoniumperoxodisulfat	0.1 g/ml
Binding buffer for EMSA:	HEPES, pH 7.9	40 mM
	EDTA, pH 8.0	4 mM
	KCl	800 mM
	Ficoll	16 %
Borate buffer:	Boric Acid (MW: 61.83)	3.0915 g
	Dissolve in H_2O	500 ml
	Adjust pH 8.5	

Buffer A:	HEPES, pH 8.0	10 mM
	KCl	10 mM
	EDTA	0.1 mM
	EGTA	0.1 mM
	DTT	2 mM
	Protease inhibitor tablet	1 / 50 ml
Buffer B:	HEPES, pH 8.0	10 mM
	KCl	10 mM
	EDTA	0.1 mM
	EGTA	0.1 mM
	DTT	2 mM
	NaCl	400 mM
	IGEPAL	1 %
	Protease inhibitor tablet	1 / 50 ml
2 M $CaCl_2$:	$CaCl_2$ in H_2O	2 M
Cryoprotect solution:	Glycerin	250 ml
	Ethylene Glycol	250 ml
	0.1 M Phosphate buffer	500 ml
Heparin solution:	dissolved in Ampuwa-H_2O	10 mg/ml
HBS (2x):	NaCl	8.00 g
	KCl	0.37 g
	Na_2HPO_4 x 7 H_2O	201 mg
	Glucose	1.00 g
	HEPES / 500 ml	5.00 g
	NaOH, adjust to pH 7.05	
Laemli buffer (5x):	SDS	15 %
	Glycerol	50 % (v/v)
	Mercaptoethanol	15 %
	Bromphenaolblau	1.5 %

LB medium:	Bacto-Trypton	10 g/l
	Bacto-yeast extract	5 g/l
	NaCl	10 g/l
	pH 7.0; autoclave (120 °C, 20 min)	
LB agar:	Bacto-Trypton	10 g/l
	Bacto-yeast extract	5 g/l
	NaCl	10 g/l
	Bacto-agar	15 g/l
	pH 7.0; autoclave (120 °C, 20 min)	
LB^{amp} agar plates:	LB agar + Ampicillin	0.1 mg/ml
Loading dye (6x):	Tris-HCl, pH 7.5	10 mM
	Glycerol	50 %
	EDTA	100 mM
	Xylencyanol	0.25 %
	Bromphenolblau	0.25 %
PAGE buffer (10x):	1M MES, pH 7.2	195.24 g
	Tris Base	121 g
	20 % SDS	100 ml
	0.5 M EDTA, pH 7.3	40 ml
	H_20	add up to 2 l
PAGE gels (10 %):	Separation gel:	
	H_20	1.8 ml
	1M Lower Tris, pH 8.8	1.9 ml
	10 % SDS	50 µl
	TEMED	5 µl
	10 % APS	25 µl
	Collection gel:	
	MQ	2.27 ml

	1M Upper Tris, pH 6.8	0.375 ml
	40 % Acryl amide	0.3 ml
	10 % SDS	20 µl
	TEMED	3 µl
	10 % APS	30 µl
PAGE gel (native, 6 %):	H_2O	27.77 ml
	10x TBE, pH 8.3	4 ml
	30 % PAA	8 ml
	10 % APS	0.2 ml
	TEMED	30 µl
PBS (10x):	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4	8 mM
	KH_2PO_4	1.4 mM
	Adjust to pH 7.4	
4% PFA:	PFA	40 g
	Dissolve in MilliQ H_2O	500 ml
	NaOH	till clear
	0.2 M PO_4-Buffer	500 ml
	Adjust to pH 7.4	
0.2 M Phosphate buffer:	Sodium phosphate monobasic	16.56 g
	Sodium phosphate dibasic	65.70 g
SDS (10x):	SDS	10% (v/v)
	Adjust to pH 7.2 (HCl)	
SDS-PAGE transfer buffer:	Tris base	0.25 M
	Glycin	1.92 M
	SDS	1 % (w/v)

Sodium citrate buffer:	Sodium Citrate	0.01 M
	Adjust to pH 6.0	
Stripping solution:	Tris / HCl pH 6.8	62.5 mM
	SDS	2% (v/v)
	Mercaptoethanol	14.5 M
30% sucrose:	Sucrose	30 %
	Dissolved in 0.1 M	
	Phosphate-buffer	
TAE-buffer (5x):	Tris, pH 8.3	90 mM
	Acetic acid	90 mM
	EDTA	2.5 mM
TBS (10x):	Tris	250 mM
	NaCl	1.37 M
	KCl	26 mM
	Adjust to pH 7.5	
TBS++:	TBS	1x
	3 % BSA (v/v)	3 % (v/v)
	Triton-X100	0.25 % (v/v)
TBST:	TBS	1x
	BSA	3 % (v/v)
	TritonX-100	0.25 % (v/v)
Tris-HCl:	TRIZMA Base	0.1 M
	Adjust pH (HCl)	

4.1.6 Antibodies

4.1.6.1 Primary antibodies

Name	Species	Dilution	Reference
Alpha-Tubulin	mouse	1:1000	Sigma, Munich, Germany
BrdU	rat	1:500	AbD Serotec, Düsseldorf, Germany
β-Actin	mouse	1:10000	Abcam, Cambridge, UK
β-galactosidase	rabbit	1:2000	Cappel/MP Biomedicals, Illkirch, France
βIII-tubulin	mouse	1:3000	Sigma, Munich, Germany
DAPI	-	1:1000	Sigma, Munich, Germany
DCX	goat	1:250	Santa Cruz Biotechnology, Heidelberg, Germany
DCX	guinea pig	1:1000	Millipore, Schwalbach, Germany
GFAP	guinea pig	1:1000	Zytomed Systems, Berlin, Germany
GFAP	rabbit	1:500	Dako, Hamburg, Germany
GFAP	mouse	1:1000	Sigma-Aldrich Chemie GmbH, Munich, Germany

GFP	chicken	1:1000	Aves Labs, Tigard, US-OR
KI67	rabbit	1:1000	Novocastra, Newcastle upon Tyne, UK
NeuN (clone A60)	mouse	1:100	Hybridoma Bank, Iowa, US-IA
NeuroD	goat	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany
Notch1	rabbit	2 µg	Abcam, Cambridge, UK
PARP (C2-10)	mouse	1:2000	Santa Cruz Biotechnology, Heidelberg, Germany
PCNA	mouse	1:500	Santa Cruz Biotechnology, Heidelberg, Germany
RBP1F1	rat	2 µg	Non-commercial, from E. Kremmer, Helmholtz Zentrum München, Germany
RBPJκ (D20)	goat	2 µg	Santa Cruz Biotechnology, Heidelberg, Germany
Sox2	goat	1:1000	Santa Cruz Biotechnology, Heidelberg, Germany

Sox2	rabbit	1:1000	Chemicon via Millipore, Schwalbach, Germany

Table 3: Primary antibodies used in this study

4.1.6.2 Secondary antibodies

Name	Species	Dilution	Reference
Alexa 488	According to primary antibody	1:250	Jackson Laboratory, Bar Harbor, US-ME
Cy3	According to primary antibody	1:250	Jackson Laboratory, Bar Harbor, US-ME
Cy5	According to primary antibody	1:250	Jackson Laboratory, Bar Harbor, US-ME
FITC	According to primary antibody	1:250	Jackson Laboratory, Bar Harbor, US-ME
HRP-conjugated IgG	According to primary antibody	1:10000	Jackson Laboratory, Bar Harbor, US-ME

Table 4: Secondary antibodies used in this study

4.1.7 Plasmids and oligonucleotides

4.1.7.1 Plasmids

Name	Properties	Reference
pGL3-Sox2	Luciferase vector, Amp^r	This work
pTOPFlash	Luciferase vector, Amp^r	(Moon et al., 2004)
phEF-Renilla	Luciferase vector, Amp^r	(Nakashima et al., 2005)
pBOSE-NICD	Expression vector, Amp^r	NICD cloned into MCS of pEF-BOS vector (Mizushima and Nagata, 1990)
pCMX-RBP-J/R218H	Expression vector, Amp^r	(Kato et al., 1997)
pcDNA3-β-catenin-S33Y	Expression vector, Amp^r	β-catenin-S33Y cloned into MCS of pcDNA3 (Invitrogen)
pBOSE-Sox2	Expression vector, Amp^r	Sox2 cloned into MCS of pEF-BOS vector (Mizushima and Nagata, 1990)

Table 5: Plasmids used in this study

4.1.7.2 Oligonucleotides

4.1.7.2.1 Primers for RT-PCR

Name	Sequence (5' – 3' direction)
Dll1	Forward: CCTCGTTCGAGACCTCAAGGGAG Reverse: TAGACGTGTGGGCAGTGCGTGC
Dll3	Forward: CACGCCATTCCCAGACGAGTGC Reverse: GCAGTCGTCCAGGTCGTGCT
Jagged1	Forward: CCTGCCAGTGCCTGAATGGACG

	Reverse: GGCTGTCACCAAGCAACAGACCC
Jagged 2	Forward: ACCGTGACCAAGTGCCTCAGGGCA Reverse: GAGCGGAGCCCACTGGTTGTTGG
Notch1	Forward: GCTGACCTGCGCATGTCTGCCATG Reverse: CATGTTGTCCTGGATGTTGGCATCTG
Notch2	Forward: CACCTTGAAGCTGCAGACAT Reverse: TGGTAGACCAAGTCTGTGATGAT
Notch3	Forward: ATATATATGGAGTTGCTCCCTTCC Reverse: GGCTTTGAGCAGACAAGACCCCTT
Notch4	Forward: GGAAGCGACACGTACGAGTCTGG Reverse: CAACACCCGGCACATCGTAGGT
RBPJκ	Forward: TGGCACTGTTCAATCGCCTT Reverse: AATCTTGGGAGTGCCATGCCA
Hes1	Forward: ACACCGGACAAACCAAAGAC Reverse: GTCACCTCGTTCATGCACTC
Hes5	Forward: AGATGCTCAGTCCCAAGGAG Reverse: TAGCCCTCGCTGTAGTCCTG
Frzd1	Forward: CAGAGCGGGCAGCAGTACAA Reverse: GCGCGGGCAGGAGAACTT
GSK3β	Forward: CAGCAGCCTTCAGCTTTTGG Reverse: CCGGAACATAGTCCAGCACCAG
β-catenin	Forward: GCTGATTTGATGGAGTTGGACATGG Reverse: GCCAAACGCTGGACATTAGTGG
TCF3	Forward: TACCCCTTCCTGATGATTCCAGAC Reverse: GGAGAAGTGGTCGTTGCTGTAGGT
TCF4	Forward: GCATCAGGACTCCAAAAAGGAAGA Reverse: TTCCCATAGTTATCCCGTGCAGAC
LEF1	Forward: AGACACCCTCCAGCTCCTGA Reverse: CCTGAATCCACCCGTGATG
Sox2	Forward: GAGTGGAAACTTTTGTCCGAGA Reverse: GAAGCGTGTACTTATCCTTCTTCAT
GAPDH	Forward: GACCCCTTCATTGACCTCAAC Reverse: CTTCTCCATGGTGGTGAAGA

Table 6: Primers for RT-PCR. All primers were used at a concentration of 10 µM.

4.1.7.2.2 Primers for quantitative real-time PCR

Name	Sequence (5' – 3' direction)
Sox2-RBPJκ #1	Forward: GGCGAGTGGTTAAACAGAGC Reverse: GCGAGAACTAGCCAAGCATC
Sox2-RBPJκ #3	Forward: GCAGTGAGAGGGGTGGACTA Reverse: GCTCCGCTCATTGTCCTTAC
Sox2-RBPJκ #4	Forward: CAATGGGAGATCGGCTAAAA Reverse: ACAGGCACGGTGGTAGTCAC
Sox2-RBPJκ #5	Forward: CTTGTGTCAGGGTTGGGAGT Reverse: CCTGGCTTCCGTGTCATC
Sox2-UR	Forward: CGCAGGTAAGCAGGGATTTCT Reverse: CGCTTGCTTTTGGAGAGGAAC
Hes1-RBPJκ	Forward: ACACCGGACAAACCAAAGAC Reverse: GTCACCTCGTTCATGCACTC

Table 7: Primers for real-time PCR used in this study. All primers were used at a concentration of 10 µM.

4.1.7.2.3 Primers for genotyping

Primers for genotyping of Tg(Cp-EGFP)25Gaia and BATGAL mice were as follows:

Name	Sequence (5' – 3' direction)
GFP	Forward: CGACCACTACCAGCAGAACA Reverse: TGGTGCAGATGAACTTCAGG
β-galactosidase	Forward: CGGTGATGGTGCTGCGTTGGA Reverse: ACCACCGCACGATAGAGATTC

Table 8: Primers for genotyping used in this study. All primers were used at a concentration of 10 µM.

4.1.7.2.4 Oligonucleotides for EMSA

Oligonucleotides spanning predicted RBPJκ binding sites in the *Sox2* promoter, with respect to the transcription start site, were designed as follows:

Nr.	Position	Strand	Sequence (5' – 3' direction)
1	-4475 to -4461	+	Forward: CTAATTAGCAATGCTGAGAAATT Reverse: CCTTGTTAACTGGAATTTCTCAGC
2	-3746 to -3732	+	Forward: TGAGAAAATAGGTTTTGCTACCG Reverse: TATTTTCTCATTAGAATATTTTT
3	-2538 to -2524	+	Forward: TGGGAGAATGGGGATTGGAGATG Reverse: ATTCTCCCAGGCTTGGCTGTTA
4	-1983 to -1969	-	Forward: CCCGCCCCCAGCCCATTCTCCCA Reverse: CAAGCCCAGGCTGTGGGAGAATGG
5	-1472 to -1458	+	Forward: AGGCTGGGAACAAGGCCTGGTCC Reverse: GTTCCCAGCCTTTTCCTAGGCCGA

Table 9: Oligonucleotides for EMSA used in this study

4.2 Methods

4.2.1 Animals

For all experiments, animals 4-6 between 8 and 12 weeks of age were used. Mice were grouped housed in standard cages under a 12 hour light/dark cycle and had ad libitum access to food and water. B6.Cg-Tg(BAT-lacZ)3Picc/J mice (Maretto et al., 2003) were obtained from Jackson Laboratory (Bar Harbor, US-ME). C57BL/6 mice and Tg(Cp-EGFP)25Gaia mice were obtained from Charles River (Wilmington, US-MA). GLAST::CreERT2 mice (Slezak et al., 2007) exhibit a tamoxifen-inducible Cre-recombinase gene under the control of the promoter of the astrocyte specific glutamate aspartate transporter (GLAST). GLAST::CreERT2 mice were crossed with *Rbpjκ*$^{loxP/loxP}$ mice, in which exon 6 and 7, which code for DNA- and Notch-binding domains, are flanked by loxP sites (Han et al., 2002) and R26::EYFP reporter mice (Srinivas et al., 2001). Tamoxifen was injected daily (2 mg) for 5 consecutive days. For loss of *Rbpjκ* function experiments n= 4-6 animals per group were analyzed.

4.2.2 Tissue processing

Animals were sacrificed using CO_2. Mice were transcardially perfused with phosphate-buffered saline (PBS, pH 7.4) at a speed of 10 ml/minute for 5 minutes followed by 4% paraformaldehyde (PFA) for 5 minutes. The head of the animal was cut and the brain was prepared. Brains were postfixed in 4 % PFA overnight at 4℃ and were subsequently transferred to a 30% sucrose solution. 40 µm thick coronal brain sections were made using a sliding microtome (Leica Microsystems, Wetzlar, Germany).

4.2.3 Mouse embryos

Embryos were dissected from C57BL/J6 wildtype mice. Pregnancy was identified by plug check. The day of the plug was defined as 0.5 dpc.

4.2.4 Cell cultures

Murine adult hippocampal stem/progenitor cells were kept under proliferating conditions in Dulbecco's modified Eagles's medium (DMEM) / F12 medium (Gibco-Invitrogen, Karlsruhe, Germany) supplemented with N2 supplement (Gibco-Invitrogen), glutamine and 1 x penicillin / streptomycin / fungizone (Gibco-Invitrogen) in the presence of 20 ng/ml FGF2, 20 ng/ml EGF (PeproTech GmbH, Hamburg, Germany) and 0.5 µg/ml heparin (Sigma-Aldrich Chemie GmbH, Munich, Germany). Medium and growth factors were renewed every second day (Ray and Gage, 2006).

For neural stem cell cultures, 8-week-old male C57BL/J6 mice were used. Brains were cut coronally through the optic chiasm to dissect the dentate gyrus of the hippocampus. Tissue was prepared and transferred to ice-cold phosphate-buffered saline. Tissue was enzymatically dissociated in 0.7 mg/ml hyaluronic acid and 1.34 mg/ml trypsin in Hank's Buffered Salt Solution (Sigma) supplemented with 2 mM glucose at 37 ℃ for 30 min. The reaction was stopped with bovine serum albumin (0.02 g/ml) and then passed through a 70-mm nylon mesh (Falcon). Cells were centrifuged at 120 g for 5 min, resuspended in 0.9 m sucrose in Hank's Buffered Salt Solution, and centrifuged for 10 min at 300 g. The cell pellet was resuspended in 2 ml

of 4 % bovine serum albumin in Earle's Buffered Salt Solution (AppliChem, Darmstadt, Germany), transferred on top of 12 ml of the same solution, and centrifuged at 150 g for 7 min. Supernatant was removed, and cells were resuspended in DMEM/F-12 culture medium containing B27 supplement (Life Technologies-Invitrogen, Karlsruhe, Germany), 1 m HEPES buffer, 1x penicillin / streptomycin / fungizone (Gibco-Invitrogen, Karlsruhe, Germany), 20 ng/ml epidermal growth factor, and 20 ng/ml basic fibroblast growth factor (PeproTech, Hamburg, Germany). Neural stem cells were cultured as neurospheres. Cultures were supplemented with growth factors every other day. Every fourth day, three-quarters of the medium were changed. Neurospheres were passaged mechanically to single cells by passing the neurospheres through a 5-ml pipette with a 200 µl tip. Cell viability was tested by examining the exclusion of Trypan Blue.

For differentiation experiments, neurospheres were put on poly-d-lysine (20 µg/ml)-coated glass coverslips. The next day, medium was refreshed but without growth factors to allow neurospheres to differentiate. After 5 days, neurospheres were immunofluorescently stained for the expression of specific proteins.

293T human embryonic kidney cells (HEK cells) were grown in DMEM (high glucose + pyruvat) supplemented with 10 % of bovine serum albumin and 1x penicillin / streptomycin / fungizone. Cells were grown on uncoated cell culture plates.

The *Rbpjκ* overexpressing DG75 Burkitt's lymphoma cell line (Ben-Bassat et al., 1977) and the *Rbpjκ* knockout cell line SM224.9 (Maier et al., 2005) were cultured in RPMI 1640 + GlutaMAX -I medium (Gibco, Invitrogen).

4.2.5 Fluorescent immunohistochemistry

Sections were rinsed three times in 0.1 M Tris buffered saline (TBS) for 15 minutes each. Sections were blocked in blocking solution (TBS++) containing TBS supplemented with 3% normal donkey serum, 0.25% Triton-X 100 for 60 minutes. Brain sections were then incubated in TBS++ containing the primary antibody at the appropriate dilutions at 4℃ for 48 hours. Afterwar ds, they were rinsed three times in TBS for 15 minutes each. Sections were blocked in TBS++ for 30 minutes. Then, brain slices were incubated in TBS++ containing the secondary antibody at a dilution of 1:250 for 2 hours at room temperature. Thereafter, brain sections were rinsed three times in 0.1 M Tris buffered saline (TBS) for 15 minutes each. In between,

sections were rinsed in TBS containing a DAPI antibody for 5 minutes. Brain slices were transferred into 0.1 M phosphate buffer and mounted on superfrost slides (Menzel-Gläser, Braunschweig) using Aqua/Polymount (Polysciences Inc., Warrington, USA).

For anti-gen retrieval, sections were incubated in 2N HCL at 37℃ for 30 minutes after the initial three washing steps. Slides were rinsed in 0.1 M borate buffer then washed in TBS six times for a total of 90 minutes. Sections were then blocked as described above.

Alternatively, sections were boiled in 0.01 M citrate buffer at 95℃ for 10 to 20 minutes, allowed to cool down to room temperature and then rinsed in borate buffer and further processed as explained above for the HCl treatment.

Confocal single plane images and Z-stacks were taken on an Olympus, FluoView 1000 (Olympus, Hamburg, Germany) or on a Leica SP5 confocal microscope (Leica Microsystems). The number of SOX2-, SOX2/GFAP-, DCX-, PCNA-, and YFP-expressing cells in the dentate gyrus was determined in every sixth 40 µm section of the dorsal hippocampus. DAPI staining was used to trace the granule cell layer. For normalization, cell numbers were related to the analyzed granule cell layer volume. For phenotyping, all YFP+ cells were analyzed for co-expression with lineage specific markers.

4.2.6 Fluorescent immunocytochemistry

Cells were fixed in 4 % PFA (in PBS) for 10 to 15 minutes at room temperature. Cells were then rinsed three times with TBS. Fixed cells were blocked with 1 % normal donkey serum in TBS supplemented with 0.1 % Triton X-100 (TBS++) for 60 minutes. Blocked cells were incubated with primary antibody in TBS++ at 4℃ over night. The next day, cells were rinsed three times in TBS. Thereafter, cells were blocked in TBS++ for 30 minutes. After blocking, cells were incubated with secondary antibody at a dilution of 1:250 in TBS++ for two hours at room temperature. Then, cells were rinsed in TBS supplemented with DAPI at a dilution of 1:10000 for five minutes at room temperature. Cells were rinsed two times in TBS. Cells grown on cover slips were mounted on microscope slides using Aqua/Polymount (Polysciences Inc., Warrington, USA).

4.2.7 Electroporation

Adherent growing cells were treated with 0.05 x Trypsin for 1 minute and then centrifuged at 300 rcf for 2 minutes at room temperature. Two million cells were resuspended in 100 µl Nucleofector solution and used for one electroporation. The cells were mixed with 10 µg at maximum of the DNA to be electroporated. The DNA/cell mixture was transferred into electroporation cuvettes and the cells were electroporated using a Nucleofector II electroporation device (Lonza Cologne AG, Cologne, Germany). For mouse cells, the program A-33 was selected. Afterwards cells were suspended in fresh medium supplemented with FGF2, EGF and heparin. Medium including all supplements was changed 24 hours after electroporation. Cells were analysed 48 hours after the electroporation.

4.2.8 Calciumphosphate-mediated transfection

This protocol is suitable for cells on cell culture plates with a diameter of 10 cm. Human embryonic kidney (HEK) cells were plated the night before transfection to give 60% to 70% of confluency at the day of transfection. At maximum 10 µg of DNA was added to H_2O to give a receive a total volume of 1095 µl. 155 µl of 2M CaCl2 were added and mixed. Then, 1250 µl of 2x HBS were added dropwise while gently mixing. This mixture was directly added to the cells dropwise through the medium after an incubation time of 2 to 3 minutes.

4.2.9 Transformation of bacteria

Heat-shock competent DH5α or Xl1blue E.coli cells were allowed to thaw on ice. The DNA was added to the bacteria and incubated for 20 minutes on ice. The heat-shock was performed at 42℃ for 30 seconds. Cells were pu t back on ice and 200 µl of LB media was added. Thereafter, cells were put on a shaker at 37℃ for 30 minutes. Cells were plated out on agar plates with the appropriate antibiotics for selection and incubated for at maximum 16 hours over night at 37°C.

4.2.10 Luciferase assay

5x Passive lysis buffer (Promega) and the Promega dual luciferase kit (Promega GmbH) were used for luciferase assays. Cells were lysed in 150 µl of 1x passive lysis buffer. Cells were incubated on a shaker for 10 minutes at room temperature. Afterwards 15 µl of the cell lysate were transferred onto a 96 well plate (Nunc, via Thermo Electron LED GmbH, Langenselbold, Germany) and luciferase activity was measured using a Centro LB 960 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany) with the following settings:

Dispense:	Injector	2
	Volume	50
	Speed	middle
	Meas. Operation	by well
	Repeated operation	yes
Delay:	Duration	2.0
	Meas. Operation	by well
	Repeated operation	yes
Firefly:	Name	Firefly
	Counting Time	5.0
	Meas. Operation	by well

4.2.11 Isolation of DNA

DNA was isolated using the Pure Yield Plasmid Midiprep system (Promega) or the Nucleospin Kit (Macherey-Nagel). The concentration of isolated DNA was measured on a BioPhotometer (Eppendorf AG, Hamburg, Germany).

4.2.12 Isolation of RNA

Total RNA was isolated either using the RNeasy kit (Qiagen) or the Trizol reagent. The manufacturer's protocol was followed when using the RNeasy kit. When using the Trizol reagent, cells were lysed with an appropriate amount of Trizol for five minutes at room temperature. One-fifth volume of chloroform was added, vortexed for

15 seconds and incubated for 2 to 3 minutes at room temperature. Afterwards, the lysate was centrifuged at 12000 g at 4℃ for 15 min utes. The aqueous phase was transferred to a new reaction tube. 0.5 ml of Isopropanol was added per 1 ml of Trizol and the mixture was incubated for 10 minutes at room temperature. Afterwards, the lysate was centrifuged at 12000 g at 4℃ for 30 min utes. The pellet was then washed with 70% of ethanol and centrifuged at 12000 g at 4℃ for 5 minutes. The pellet was allowed to dry for about 5 minutes at room temperature and was then dissolved in RNase-free water and incubated for at least 30 minutes at room temperature. The concentration of isolated RNA was measured on a BioPhotometer (Eppendorf AG, Hamburg, Germany).

4.2.13 cDNA synthesis

cDNA was synthesized using the Superscript III Kit (Invitrogen) following the manufacturer's protocol. DNase (Fermentas) treatment preceding the ligation reaction was performed following the manufacturer's protocol.

4.2.14 Extraction of DNA fragments from agarose gels

DNA fragments were cut out of the agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden) following the manufacturer's protocol.

4.2.15 Cloning procedures

For cloning purposes, restriction endonucleases (10 % (v/v)) and the respective restriction enzyme buffers, alkalic phosphatase (Promega) treatment and T4-ligations (New England Biolabs) were performed according to standard procedures or the manufacturer's protocol. Recognition sites for restriction endonucleases were visualized and the appropriate plasmid maps were generated using the program Vector NTI (Invitrogen).

4.2.16 PCR

4.2.16.1 RT-PCR

PCRs were performed using the MasterMix kit (Eppendorf) following the manufacturer's protocol. PCRs were run on a Mastercycler epgradient S (Eppendorf). Primers were designed using the program Vector NTI (Invitrogen). The initial denaturing step was performed at 94℃ for 3 minutes. The cycles consisted of the denaturing step at 94℃ for 30 seconds, the annealing time for 30 seconds and the extension time for 30 seconds. PCRs were run for 35 cycles.

Gene name	Annealing temp. [℃]	Time [sec]	Extension temp. [℃]
Dll1	59	30	72
Dll3	58	30	72
Jagged1	55	30	72
Jagged 2	58	30	72
Notch1	59	30	72
Notch2	52	30	72
Notch3	58	30	72
Notch4	58	30	72
RBPJκ	52	30	72
Hes1	52	30	72
Hes5	52	30	72
Frzd1	56	45	72
GSK3β	52	30	72
β-catenin	58	30	72
TCF3	52	30	72
TCF4	52	30	72
LEF1	52	30	72
Sox2	56	30	72
GAPDH	52	30	72

Table 10: Parameters for RT-PCR primers used in this study

4.2.16.2 Quantitative real-time PCR

Quantitative RT-PCR was performed on a StepOne instrument (Applied Biosystems) using. For detection, Brilliant II Fast SYBR Green qPCR Master Mix (Agilent, Boeblingen, Germany) was used according to the manufacturer's protocol. The online program NetPrimer was used to design primers for quantitative RT-PCR.

Name/Gene	Denaturing [℃]	Annealing [℃]	Extension [℃]
Sox2-RBPJκ #1	95	58	72
Sox2-RBPJκ #3	95	58	72
Sox2-RBPJκ #4	95	58	72
Sox2-RBPJκ #5	95	58	72
Sox2-UR	95	58	72
Hes1-RBPJκ	95	58	72

Table 11: Parameters for real-time PCR primers used in this study. Time for denaturing and annealing was 30 seconds, time for extension 60 seconds.

4.2.16.3 Genotyping

The initial denaturing step was performed at 94℃ for 4 minutes. The cycles consisted of the denaturing step at 94℃ for 30 seconds, the annealing time for 30 seconds and the extension time for 30 seconds (GFP = 1 min). PCRs were run for 30 cycles. 5 % DMSO were added to the PCR reaction for GFP.

Gene name	Annealing temp. [℃]	Time [sec]	Extension temp. [℃]
GFP	60	30	72, 1 min
β-galactosidase	60	30	72

Table 12: Parameters PCR primers used in this study

4.2.17 Preparation of nuclear protein fractions and Western Blotting

For nuclear cell extracts for western blots, cells were allowed to swell on ice in buffer A (10 mM Hepes pH8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT) for 5

minutes. 30 µl of IGEPAL was added and the cells were vortexed for 10 seconds. Cells were then centrifuged at 10000 rpm at 4℃ for 1 minute. The supernatant constitutes the cytosolic cell fraction and was transferred into a new reaction tube. The pellet was resuspended in 180 µl of IP buffer B (10 mM Hepes pH8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 400 mM NaCl, 1% IGEPAL). The suspension was incubated on a rotor shaker at 4℃ for 15 minutes. Thereafter, the suspension was centrifuged at 10000 rpm at 4℃ for 1 minute. The supernatant constitutes the nuclear cell fraction and was transferred into a new reaction tube. Supernatants were combined for whole cell extract analyses. When analyzed separately, 300 µl of IP buffer A was added to the nuclear fraction to get an isotonic suspension (~ 150 mM NaCl). Extracts were shock-frozen in liquid nitrogen and stored at -80℃. The concentration of isolated protein samples was measured on a BioPhotometer (Eppendorf AG, Hamburg, Germany). To measure the concentration of protein samples, a BCA protein assay (Pierce) or Bradford assay (BioRad) was performed according to the manufacturer's protocol. Western Blotting was performed according to standard procedures. For SDS-PAGE the Mini Protean 3 System (Biorad) was used. Blotting procedures were performed in a tankblot apparatus (Invitrogen). Proteins were blotted on a 0.45 µm BioTrace PVDF-(Polyvinylidenfluorid) membrane (Pall Corporation). PVDF-membranes were blocked in 5 % milk solution (slim milk powder in TBST) for 1 hour at room temperature. Primary antibodies were used in TBST with 3 % BSA. Primary antibody incubation was performed under constant shaking/rolling over night at 4 ℃. Blots were washed three times with TBST. HRP-conjugated secondary antibodies were used at a dilution of 1:1000 in TBST. Secondary antibody incubation was performed under constant shaking/rolling for 1 hour at room temperature. Afterwards, blots were washed three times in TBST and one time in TBS. Protein bands were visualized using ECL solution (Amersham) on ECL hyperfilms (Amersham) and developed in an Agfa-Curix 60 device (Agfa).

4.2.18 Preparation of nuclear protein cell extracts for EMSA

Adherent growing cells were washed with PBS, trypsinized and centrifuged at 300 g for 15 minutes at room temperature. Suspension cultures were spun and the pellet was resuspended in PBS and centrifuged again. Afterwards, the pellet was

resuspended in three to four volumes of buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$). The cells were allowed to swell on ice for 60 minutes. All further steps were performed on ice. The cell suspension was transferred into a 1 ml douncer and was then homogenized. Afterwards cells were centrifuged for 10 seconds at 14000 rps at 4℃. 300 µl of buffer A was added to the pellet and vortexed briefly. The resulting suspension was centrifuged for 10 seconds at 14000 rps at 4℃. The pellet was then resuspended in three volumes if buffer B (20 mM Hepes pH 7.9, 25 % glycerol, 4.2 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA ph 8.5, 0.5 mM DTT, protease inhibitors) and incubated for 30 minutes on ice. Afterwards, the suspension was centrifuged at 14000 rpm at 4℃ for 20 minutes. The supernatant, which contained the nuclear protein fraction, was aliquoted and flash freezed in liquid nitrogen. Aliquots were stored at –80℃. To measure the concentration of protein samples, a BCA Protein Assay Kit (Pierce) or a Bradford assay (BioRad) was used according to the manufacturer's protocol.

4.2.19 Electrophoretic mobility shift assay (EMSA)

Annealing of the oligonucleotides

Equimolar amounts of both single stranded DNA oligonucleotides were mixed in annealing buffer. The mixture was incubated for 10 minutes at 90℃ in a waterbath or a heating block. The mixture was allowed to cool down to room temperature. Dilutions of 625 ng/µl, 250 ng/µl and 25 ng/µl were made, aliquoted and stored at -20℃.

Cold labelling of the annealed oligonucleotides

1 µl of the annealed oligonucleotides with a concentration of 625 ng or 250 ng were used respectively together with 2 µl of NEB Buffer 2, 2 µl of a 500 µM dNTP-mix, containing all four deoxynucleotides, and 2 µl of Klenow enzyme. The total volume was adjusted with Millipore water to 20 µl. The reaction was incubated at 37℃ for 1 hour.

Radioactive labelling of the annealed oligonucleotides

2 µl of the annealed oligonucleotides with a concentration of 25 ng were used respectively together with 2 µl of NEB Buffer 2, 2 µl of a 500 µM dNTP-mix,

containing dATP, dGTP, dTTP, and 2 µl of Klenow enzyme. 5 µl (3000 Ci/mol) of the radioactive isotope ^{32}P in form of the deoxynucleotide α-dCTP was added in the hot lab. The total volume was adjusted with Millipore water to 20 µl. The reaction was incubated at 37℃ for one hour.

Purification of the radioactive oligonucleotide

Purification was performed on Sephadex G50 columns (Phamracia). The columns were washed with two to three ml of TE. The 20 µl labelling reaction was mixed with 30 µl of TE and transferred onto Sephadex G50 columns. 50 µl of TE was added. Then, 100 µl of TE were added and the flow-through was collected separately. In total, 9 fractions were collected. The decays per minute (dpm) of each fraction were measured in a Bioscan QC-2000 radio counter (American Instrument Exchange, US-MA). Fractions five to seven normally contained the highest number dpm and were stored in an appropriate protecting container at -20℃.

Preparation of the reactions

Reactions were performed in siliconized reaction tubes. For supershift experiments, recombinant protein or nuclear extracts and 5 µl of cell supernatant (1F1) containing antibodies directed against RBPJκ were incubated for 40 minutes at room temperature. 9 µl of binding mix together with 2-5 µl of recombinant protein, nuclear extracts or competitor DNA were mixed. The mix was incubated for 5 minutes at room temperature. 2 µl of the radioactive nucleotide was added and Millipore water was added up to 20 µl total volume. The mix was incubated for 30 minutes at room temperature.

Gel run

The reaction was run at 130 Volts (6.5 V/cm) on a native 6% polyacryleamide gel for 3 to 4 hours.

Gel drying and exposure

The gel was removed from the glass plates and put on a Whatman paper; then it was covered with a saran wrap and dried on a GD 2000 Vacuum Gel Dryer System (GE Healthcare) at 80℃ for 1 hour. Afterwards, the dry gel was transferred to a

developing cassette and an autoradiographic film (Amersham / GE Healthcare) was put on. The film was exposed at -80℃ over night.

4.2.20 Co-immunoprecipitations

For co-immunoprecipitations, cells were allowed to swell on ice in buffer A (10 mM Hepes pH8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT) for 5 minutes. 30 µl of IGEPAL was added and the cells were vortexed for 10 seconds. Cells were then centrifuged at 10000 rpm at 4℃ for 1 minute. The supernatant constitutes the cytosolic cell fraction and was transferred into a new reaction tube. The pellet was resuspended in 180 µl of IP buffer B (10 mM Hepes pH8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 400 mM NaCl, 1 % IGEPAL). The suspension was incubated on a rotor shaker at 4℃ for 15 minutes. Thereafter, the suspension was centrifuged at 10000 rpm at 4℃ for 1 minute. The supernatant, constituting the nuclear cell fraction, was transferred into a new reaction tube. Supernatants were combined for whole cell extract analyses. When analyzed separately, 300 µl of IP buffer A was added to the nuclear fraction to get an isotonic suspension (~ 150 mM NaCl). 20 µl of supernatants were kept and used as an input control.
30 µl Protein G agarose (slurry, 50 %) (Upstate/Millipore) was centrifuged at 10000 rpm for 1 minute at 4 ℃. The pellet was washed with 200 µl buffer BP (3:1.8; buffer A to buffer B). This washing step was repeated. After this, the supernatant was completely removed from the pelleted agarose beads. Cell extracts were put onto the Protein G agarose beads and were incubated in a rotating shaker for 30 minutes at 4 ℃. After this pre-clearing step, the beads and pre-cleared cell extracts were centrifuged at 10000 rpm for 1 minute at 4 ℃. The supernatant was transferred into a new 1.5 ml reaction tube. Extracts were incubated with 2 µg of an antibody raised against the protein which should be pulled down in a rotating shaker for 2 hours at 4 ℃. Meanwhile, 20 to 50 µl of Protein G agarose beads were washed again with buffer BP as described above. After washing, extracts with coupled antibodies were transferred onto the washed agarose beads and incubated in a rotating shaker over night at 4 ℃.
The next day, the beads were centrifuged at 10000 for 1 minute at 4 ℃. The supernatant was collected as a control for proteins that did not got pelleted by the antibody under the current reaction conditions. The pellet was washed four times with

1 ml of buffer BP, then with 1 ml of PBS. 20 µl from the last washing step were collected and used as a washing control, to check whether some proteins were washed from the beads. The supernatant was removed and 5x Laemmli buffer was added. Samples were incubated for 5 minutes at 95 °C, cooled on ice and then subjected to Western blot analysis.

4.2.21 Chromatin immunoprecipitations

Cross-linking was performed on the first day by adding one tenth (1 ml) of cross-linking solution (for 5x stock: 250 mM Hepes pH 8, 500 mM NaCl, 5 mM EDTA, 2.5 mM EGTA; 1x cross-linking solution: 2 ml 5x cross-linking solution, 6.5 ml H_2O, 1.48 ml formaldehyde (37 %) formaldehyde in water) to cells in growth media. After 10 minutes of incubation at room temperature with gently shaking, one tenth of stop solution (1.25 M glycine, 10mM Tris) was added and incubation was continued for 5 minutes at room temperature. Medium was aspirated. Cell lysis was performed on ice. Cross-linked cells were rinsed twice with 5ml of cold 1x PBS supplemented with 0.5 mM EDTA. Afterwards cells were spun at 2500 rpm for 5 minutes. After addition of 1 ml of lysis buffer (50 ml Lysis buffer 10x: 5 ml 1 M Tris pH8, 12.5 ml Triton x-100 (10 %), 10 ml 0.5 M EDTA, 1.25 ml 0.2 M EGTA, 21.2 ml H_2O; 1x lysis solution: add 1.25 ml Na-butirate,10ml b-glycerophosphate, 500 µl Na-orthovanadate) and incubation on ice for 20 minutes, lysates were spun at 3000 rpm for 4 minutes (nuclei are in the pellet). 1 ml of washing buffer (20 µl 5M NaCl in 1 ml sonication buffer; 500 ml sonication buffer: 471 ml H_20, 5 ml 1 M Tris pH8, 10 ml 5 M NaCl, 1 ml 500 mM EDTA, 1.25 ml 200 mM EGTA, 1.25 ml 4 M NaBut, 10 ml b-glycerophosphate, 500 µl Na-Orthovanadate, 2 tablets of protease inhibitor per 100 ml) was added and lysates were spun at 3000 rpm for 4 minutes. The pellet was resuspended in 600 µl of sonication buffer and 60 µl of 10 % SDS were added. Sonication was performed 3-4 times for 2 minutes at maximum amplitude with an intermediate resting time of10 minutes between the sonication cycles. After sonication, the chromatin was spun at maximum speed for 30 min at 10 ℃. SDS was diluted with sonication buffer to 0.1 %. Then, samples were concentrated in VIVASPIN columns at 3400 rpm at 15 ℃ until the final volume was between 0.5 to 0.8 ml. For preclearing of chromatin, the samples were adjusted to RIPA buffer (for 300 ml RIPA buffer: 158.8 ml H_2O, 75 ml 1 M LiCl, 30 ml 10 % NP-40, 30 ml 10 % DOC, 3 ml 1 M Tris pH8, 600 µl EDTA, 1.5 ml

EGTA, 750 ml NaBut, 300 µl orthovanadate) (final concentrations of 1 % TritonX-100, 140 mM NaCl, 0.1 % DOC), BSA (20 mg/ml; 1 % final concentration) and 1µl salmon sperm (10 mg/ml) were added. 20 µl of sepharose A/G (50:50; previously washed) and 2 µg of appropriate IgG antibody were used per sample. Samples were rotated for 2 hours at 4 ℃. Samples were spun at 3000 rpm for 2 minutes and the supernatant was collected. 50µl (+ 50 µl H_2O; over night at 65 ℃, add 5 µl of proteinase K and incubate for 2 hours at 55 ℃) were used as an input control. 1 to 5 µg of antibody against the protein of interest were used for the immunoprecipitations. Reactions were rotated over night at 4 ℃.

On the second day, BSA (20 mg/ml) was added to a final concentration of 1% together with 1 µl of salmon DNA and a 50:50 mixture of sepharose A/G (previously washed). Samples were rotated for 2 hours at 4℃. Then, samples were spun at 3000 rpm for 2 minutes and the supernatant was discarded. The complex of chromatin-antibody-protein A/G was washed with 3 times with RIPA buffer, 3 times with RIPA buffer supplemented with 1 M NaCl, 2 times with LiCl buffer (181 ml H_20, 2 ml 10 % DOC 10 %, 2 ml 1 M Tris pH8, 5.6 ml 5 M NaCl, 2 ml 100% TritonX-100, 2 ml 10 % SDS, 400 µl 0.5 M EDTA, 250 µl 0.2 M EGTA, 500 µl 4 M NaBut, 4 ml 1 M b-glycerophosphate, 200 µl Na-orthovanadate) and 2 times with 1x TE buffer. Elution buffers 1 (3.3 ml TE, 0.6 ml 10 % SDS, 10 µl Na-But, 80 µl glycerophosphate, 24 µl 5 M NaCl) and II (3.7 ml TE, 0.2 ml 10 % SDS, 10 µl Na-But, 80 µl glycerophosphate, 24 µl 5 M NaCl) were prepared. Elution was performed with 200 µl elution buffer 1 (1.5 % SDS) and samples were rotated for 20 minutes at room temperature. Then, samples were spun at 3000 rpm 2 minutes and supernatants were recovered. Elution, rotation and spinning were repeated with elution buffer II (0.5 % SDS). Afterward, cross-linking was reverted over night at 65 ℃.

On the third day, 2.5 µl of proteinase K (20 mg/ml) were added and the samples were incubated for 2 hours at 55 ℃. After a phenol/chloroform extraction, samples were precipitated with 2.5 volumes of 100 % EtOH, 150 mM NaCl (final concentration) and 2.5 µl of glycogen (20 mg/ml) over night at – 20℃.

On the forth day, samples were spun down at 13000 rpm at 4℃ and the pellets were washed with 80 % of EtOH, dried and resuspended in 30 µl of water or 1x TE buffer. Quantitative real-time PCR was used to analyze the precipitated DNA.

4.2.22 Prediction of transcription factor binding sites (Genomatix)

The genomatix software was used for the prediction of potential binding sites for RBPJκ in the promoter of the *Sox2* gene. The MatInspector and Eldorado algorithms were used for this purpose.

4.2.23 Statistical analysis

Unpaired Student's t-test was used for analysis of most experiments. Before the t-test, an f-test was performed. In those cases in which the f-test resulted in a difference in the variances, a Mann-Whitney-Wilcoxon rank sum test was applied. Differences were considered statistically significant at $p<0.05$. If not stated otherwise, all data are presented as mean ± s.e.m..

5 Results

5.1 Notch signalling is differentially active in SOX2 positive stem cells and neuronally committed cells

The aim of this study is to identify signalling pathways which are involved in the regulation of adult hippocampal stem cell maintenance. *Sox2* is one of the genes which are most closely associated with the stem cell state across many species.

As previously reported, *Sox2* was found to be expressed in neural stem cells in the adult hippocampal neurogenic lineage. Radial glia like cells are considered the primary precursors for new neurons in the adult dentate gyrus. These cells can be identified by their expression of GFAP and a radial process, which spans the granule cell layer. Radial glia like stem cells are referred to as Type 1 cells. Type 1 cells have the unique capability to give rise to highly proliferative transient amplifying type 2 cells. Type 3 cells express characteristic proteins of immature neurons like DCX and constitute the migrating neuroblast population. Virtually all radial glia like stem cells in the SGZ were positive for SOX2, whereas about 60 % of all SOX2 expressing cells co-expressed GFAP and displayed a radial glia like stem cell morphology (Figure 4a). In addition no overlap was found between the neuronal fate determinant NeuroD1 (Figure 4c) and SOX2 expression and only some rare cells that were double positive for the early neuronal marker DCX and SOX2 (Figure 4b), which is consistent with the notion that *Sox2* is expressed in multipotent, undifferentiated cells of the neurogenic lineage.

To further investigate the lineage relationship of SOX2 cells with newly generated neurons in the dentate gyrus 10 to 12 weeks old C57/Bl6 mice were injected intraperitonial (i.p.) with a single pulse of the thymidine-analogon Bromo-deoxyuridine (BrdU) (50 mg/kg bodyweight), which gets incorporated into DNA during the S-phase of DNA replication and thus can be used to label dividing cells and their progeny. Type 1 cells are quiescent or at least slowly dividing. They give rise to undifferentiated Type 2 cells, which are highly proliferative. To analyze whether *Sox2* is expressed in Type 2 cells, the fraction of SOX2 positive cells among the proliferating cells were determined. It was found that over 92 % of all BrdU immuno-reactive cells where also positive for SOX2, two hours after the BrdU injection, indicating that *Sox2* is expressed also by Type 2 cells (Figure 4d). Animals were also

perfused at later points in time after the BrdU injection (1d, 3d and 7d). One day post injection (dpi), the percentage of proliferating SOX2 expressing cells dropped to 80 %.

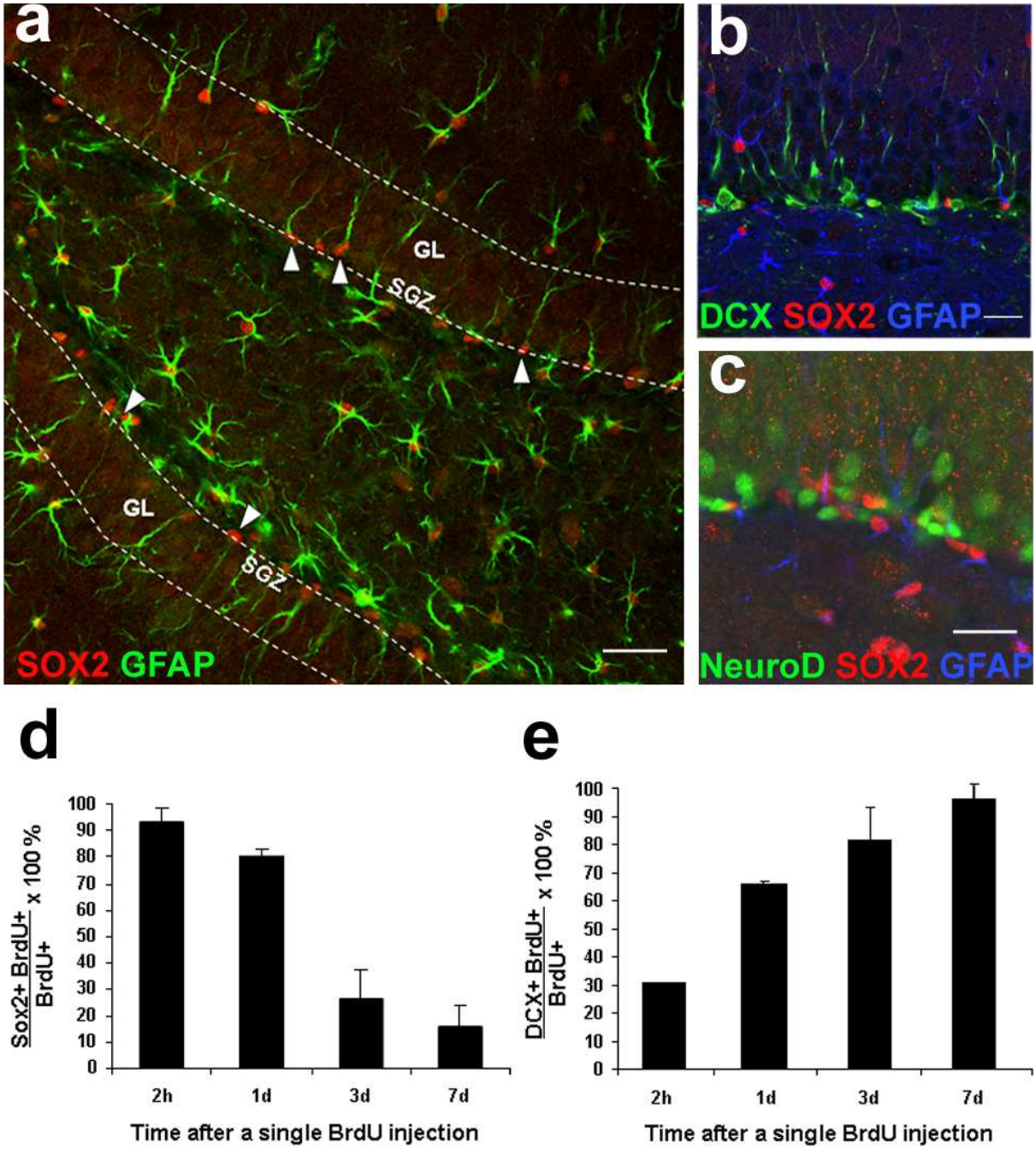

Figure 4: Analysis of SOX2 expression pattern in the adult hippocampal neurogenic niche. (a) SOX2 is expressed by virtually all type 1 stem cells. **(c)** Additionally, highly proliferative type 2 cells, which incorporate BrdU supplied as a short BrdU pulse, express SOX2. Scale bar 30 µm. **(b-e)** During neuronal differentiation, SOX2 becomes down regulated. Despite some rare cells, SOX2 is not expressed by DCX positive cells. SOX2 expression is absent from NeuroD1 positive cells. **(b)** Scale bar 30 µm. **(c)** Scale bar 20 µm. (n = 3 for each point in time).

Three and seven dpi, the percentage was further reduced to 25 % and 15 %, respectively. In contrast the percentage of BrdU cells expressing the early neuronal lineage marker DCX was increasing over time, indicating that proliferating SOX2 cells were giving rise to immature neurons (Figure 4e). Taken together, these results suggest, that SOX2 is expressed in adult hippocampal neural stem and transient amplifying cells early in the neurogenic lineage, whereas its expression is shut off in immature neurons (Figure 5).

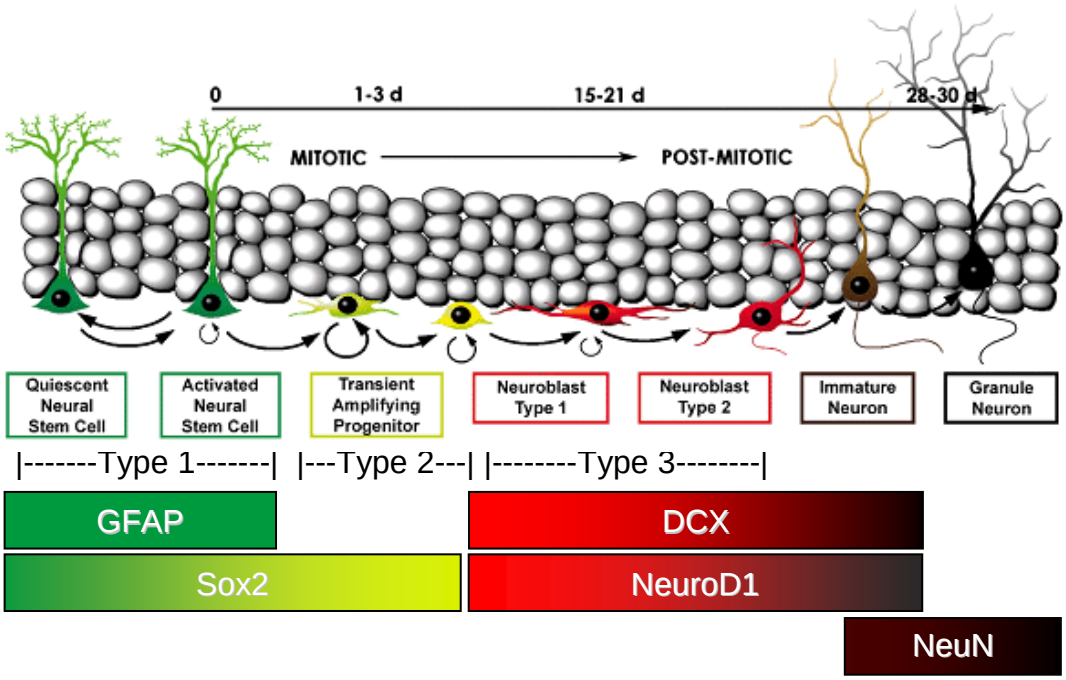

Figure 5: Summary of the expression pattern of SOX2 in the adult hippocampal neurogenic niche. SOX2 is expressed in type 1 stem cells and type 2 cells. As type 2 cells start to differentiate, SOX2 expression is shut down (modified from Encinas et al., 2006).

I sought to identify signalling pathways which are active in SOX2 positive stem cells in the SGZ, but not in neuronally committed cells. To this end, the activity of the Notch signalling pathway in adult hippocampal stem cells was analyzed. In canonical Notch signalling, binding of the ligands to the Notch receptor results in the cleavage of the receptor and liberation of the Notch intracellular domain (NICD). NICD translocates to the nucleus where it binds to the transcription factor RBPJκ and recruits other co-activators like Mastermind. This activation complex then drives target gene expression (Baron, 2003). In this approach, advantage was taken of the Tg(Cp-EGFP)25Gaia Notch reporter mouse line in which an EGFP is controlled by multimerized RBPJκ binding sites. This line has been previously shown to monitor canonical Notch signalling *in vivo* (Duncan et al., 2005; Mizutani et al., 2007).

Analysis of this reporter mouse line found Notch signalling, indicated by EGFP expression, to be active in a subset of SOX2 positive cells in the SGZ (Figure 6a). Among these SOX2 positive cells, many cells co-expressed GFAP and exhibited a radial glia morphology, suggesting that Notch signalling is active in SOX2 GFAP expressing radial glia like Type 1 stem cells.

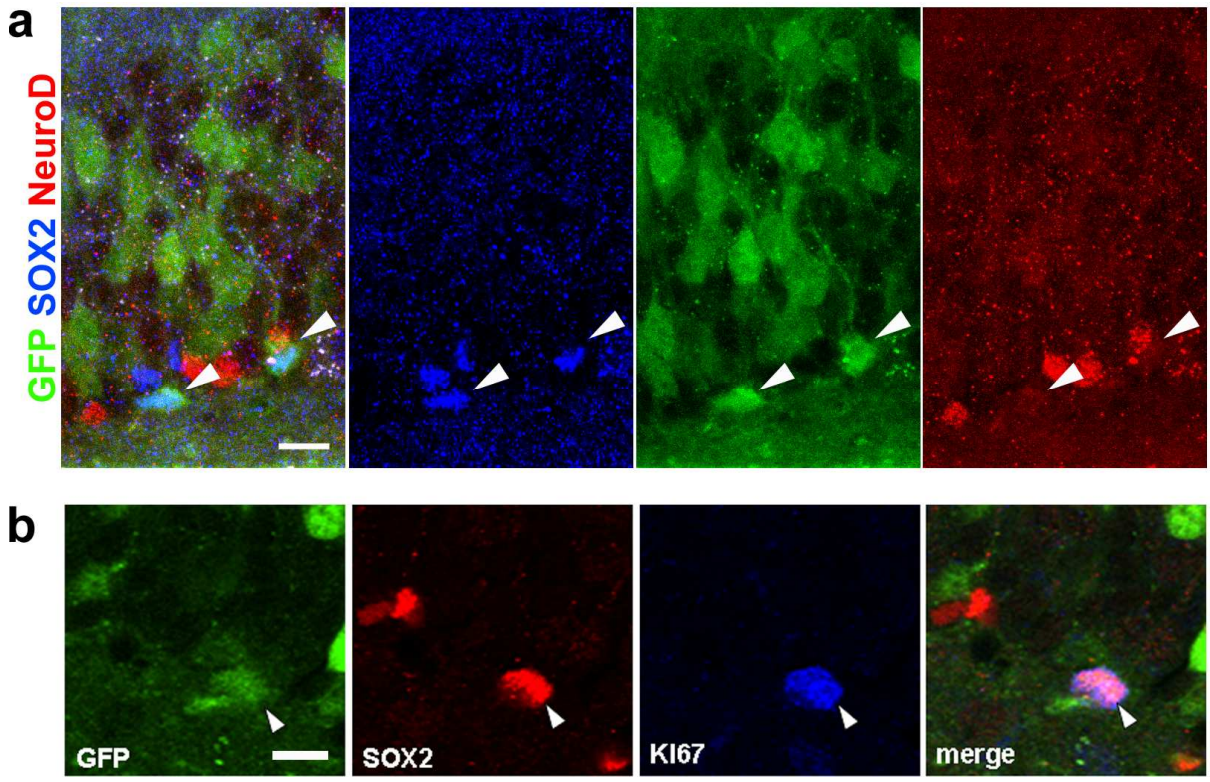

Figure 6: Analysis of adult Tg(Cp-EGFP)25Gaia mice, reporter animals for Notch / RBPJκ signalling. (a) SOX2 expressing cells (blue) in the subgranular zone of the dentate gyrus are positive for the GFP-reporter (green, arrowheads), indicating that Notch / RBPJκ signalling is active in neural stem cells. In contrast, adjacent NeuroD expressing immature neurons (red) are GFP-reporter negative indicating that Notch / RBPJκ signalling is inactive during neuronal fate commitment of neural stem cells. GFP expression in scattered cells throughout the dentate granule cell layer shows that Notch / RBPJκ signalling is also active in a subset of dentate granule neurons. Scale bar 20 µm. **(b)** The GFP-reporter is active in a subset of KI67 expressing proliferating SOX2 positive cells, indicating that Notch / RBPJκ signalling is also active in a subset of type 2 cells. Scale bar 10 µm.

Moreover, GFP expression was found in a subset of proliferating SOX2 positive precursor cells, indicated by immunoreactivity for KI67, suggesting that Notch signalling is also active in highly proliferative SOX2 positive Type 2 transient amplifying precursor cells (Figure 6b). However, no EGFP expression was detected in neighbouring NeuroD positive neuronally committed cells. Kuwabara and co-

workers (Kuwabara et al., 2009) have shown that NeuroD is switched on after loss of *Sox2* expression and concomitant neuronal fate commitment. The activity pattern of the EGFP reporter strikingly resembles the expression of SOX2 in the undifferentiated cell population and suggests that canonical Notch signalling mediated via RBPJκ is active in SOX2 positive stem cells in the SGZ and that the down-regulation of Notch signalling in neuronally committed cells is paralleled by an inactivation of *Sox2* expression in these cells.

Additional EGFP expression was observed in scattered cells throughout the whole granule cell layer of the dentate gyrus (Figure 6a). The activity of the EGFP reporter in scattered cells in the granule layer indicates that canonical Notch signalling is active in mature granule neurons.

5.2 Notch signalling positively regulates *Sox2* expression in adult hippocampal neural stem cells

The observed overlap between active canonical Notch signalling, indicated by EGFP reporter activity and SOX2 expression and the loss of SOX2 expression in EGFP reporter negative neuronally committed cells, raises the possibility that Notch signalling regulates *Sox2* expression in adult hippocampal neural stem cells. To test this hypothesis, Notch signalling was activated in stem cells *in vitro*. For this purpose an established FGF2, EGF responsive adult hippocampal stem/progenitor cell line was used (Ray and Gage, 2006). In line with previous reports, *Sox2* was found to be expressed on RNA as well as on protein level in these cells (Figure 7a, b). Further analysis on the RNA level by RT-PCR revealed, that several components of the Notch signalling pathway, including the ligands (*Dll1*, *Jag1*), the receptors (*Notch 1-4*), down-stream mediators (*Rbpjκ*) and target genes (*Hes1*, *Hes5*), are also expressed in these cells (Figure 7a). Expression of these pathway components alone does not necessarily indicate activity of the pathway itself. Therefore, nuclear levels of NICD were determined. Western blot analysis revealed the presence of the 120 kD NICD in nuclear protein fractions (Figure 7c). The expression of PARP was used to indicate that the nuclear fraction was analyzed, as PARP can only be found in the nucleus. The presence of NICD in nuclear protein fractions suggests that Notch signalling is active in this adult hippocampal neural stem/progenitor cell line.

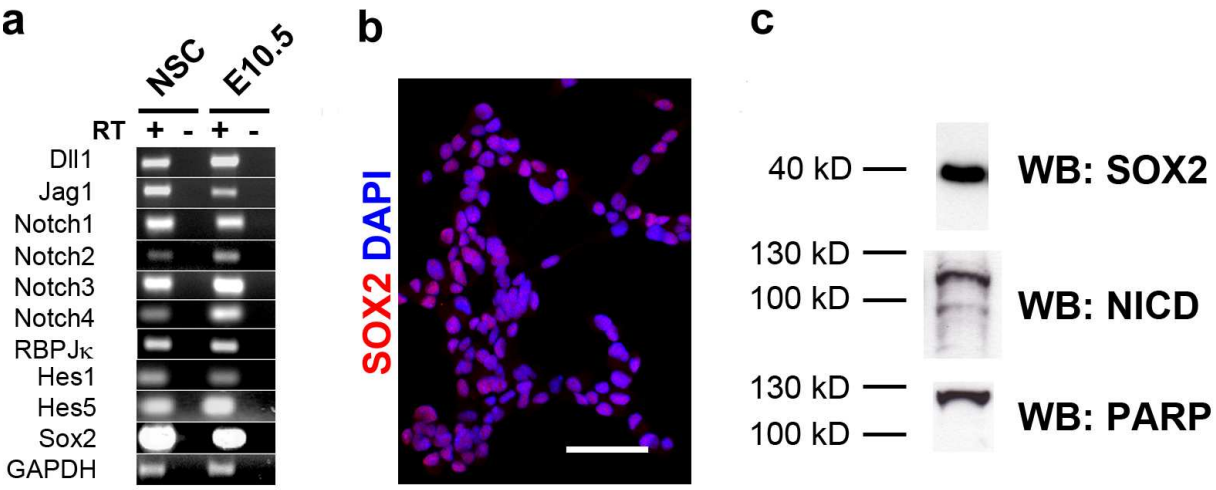

Figure 7: Analysis of adult hippocampal stem cells *in vitro*. (a) RT-PCR analysis demonstrates that adult hippocampal neural stem cells (NSC) express *Sox2* and essential components of the Notch / RBPJκ signalling cascade. RNA isolated from embryonic day 10.5 mouse brain (E10.5) served as a positive control. Delta-like 1 (Dll1), Jagged 1 (Jag1). **(b)** Immunocytochemistry of adult hippocampal neural stem cell cultures shows expression of SOX2 protein (red). DAPI in blue. Scale bar 20 µm. **(c)** Western blot analysis of cultured adult hippocampal NSCs demonstrates expression of SOX2 protein. The presence of a 120 kD Notch intracellular domain (NICD) fragment in the nuclear fraction indicates that the Notch signalling pathway is active. Western blot analysis for the nuclear protein Poly ADP-ribose polymerase (PARP) confirms isolation of the nuclear fraction.

Next, it was analyzed whether Notch signalling promotes *Sox2* expression. To test this, the above-mentioned neural stem/progenitor cells were electroporated with expression vectors for NICD or GFP as a control. Overexpression of NICD mimics the effects of activated Notch signalling. After the electroporation, cells were cultured for 48 hours.

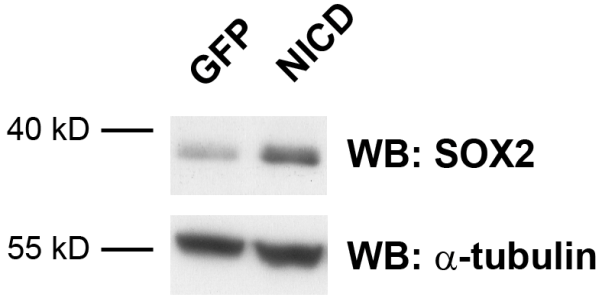

Figure 8: Notch signalling promotes endogenous SOX2 expression. Western Blot analysis of adult hippocampal NSCs following overexpression of NICD or GFP as control. Enhanced activation of Notch signalling by overexpression of NICD increases endogenous expression of SOX2. Loading control: α-tubulin. (n = 3)

Enhancing Notch signalling by the overexpression of NICD resulted in increased endogenous SOX2 protein levels as assayed by Western blot analysis (Figure 8), suggesting that Notch signalling can indeed promote *Sox2* expression in hippocampal stem/progenitor cells. After the promoting effect of Notch signalling on *Sox2* expression was evident, it was investigated whether Notch signalling regulates *Sox2* transcription directly. To this end, the promoter of the *Sox2* gene was analyzed. The MatInspector, Eldorado and DiAlign algorithms from the Genomatix software suite which is available online (www.genomatix.de) were used. The region 5.5 kb upstream of the *Sox2* transcriptional start site has previously been shown to control *Sox2* expression in neural stem cells in the telencephalon during development (Zappone et al., 2000). Suh and colleagues showed that this 5.5 kb promoter region is sufficient to mimic endogenous *Sox2* expression in the adult neurogenic niches (Suh et al., 2007). Therefore, 6 kb upstream of the *Sox2* transcriptional start site were screened for potential RBPJκ binding sites. For this purpose, the V$RBPF matrix family for the search with standard similarity threshold settings was used. The nucleotide profile of this matrix for potential RBPJκ binding sites is gTGGGaa with TGGG being the core sequence used by MatInspector. The Eldorado algorithm predicted 5 putative RBPJκ binding sites within promoter region 6 kb upstream of the transcriptional start site of the *Sox2* gene (Figure 9). The locations of these putative binding sites were #1 (-4475 to -4461), #2 (-3746 to -3732), #3 (-2538 to -2524), #4 (-1983 to -1969) and #5 (-1472 to -1458) with respect to the transcriptional start site.

Predicted Binding site #	Position	Sequence
1	-4475 to -4461	atgcTGAGaaattcc
2	-3746 to -3732	ctaaTGAGaaaatag
3	-2538 to -2524	agccTGGGagaatgg
4	-1983 to -1969	gctgTGGGagaatgg
5	-1472 to -1458	aggcTGGGaacaagg

Table 9: Predicted binding sites for RBPJκ in the 5.5 kb *Sox2* promoter. The MatInspector algorithm from the Genomatix suite was used for predictions. Positions are given relative to the transcriptional start site (+1).

The region 5.5 kb upstream of the *Sox2* transcriptional start site has previously been shown to control *Sox2* expression in neural stem cells in the telencephalon during development (Zappone et al., 2000). Suh and colleagues showed that this 5.5 kb

promoter region is sufficient to mimic endogenous *Sox2* expression in the adult neurogenic niches (Suh et al., 2007).

To determine whether Notch signalling can enhance the activity of the *Sox2* promoter, a reporter construct was generated. In this construct, the firefly luciferase gene is driven by the 5.5 kb promoter region of the *Sox2* gene (5.5 kb *Sox2*-luciferase). Then, the adult hippocampal stem/progenitor cell line was co-electroporated with the 5.5 kb *Sox2*-luciferase together with either expression constructs for NICD or GFP as a control. Overexpression of NICD resulted in an approximately 3 fold increase of the 5.5 kb *Sox2*-luciferase reporter compared to overexpressed GFP (Figure 10). This result shows that Notch signalling enhances *Sox2* promoter activity in adult hippocampal stem cells. Additional co-electroporation of a dominant-negative RBPJκ expression construct, which is unable to bind to DNA (Kato et al., 1997), resulted in decreased NICD induced activation of the 5.5 kb *Sox2*-luciferase. These results suggest that NICD induced stimulation of the *Sox2* promoter is RBPJκ dependent.

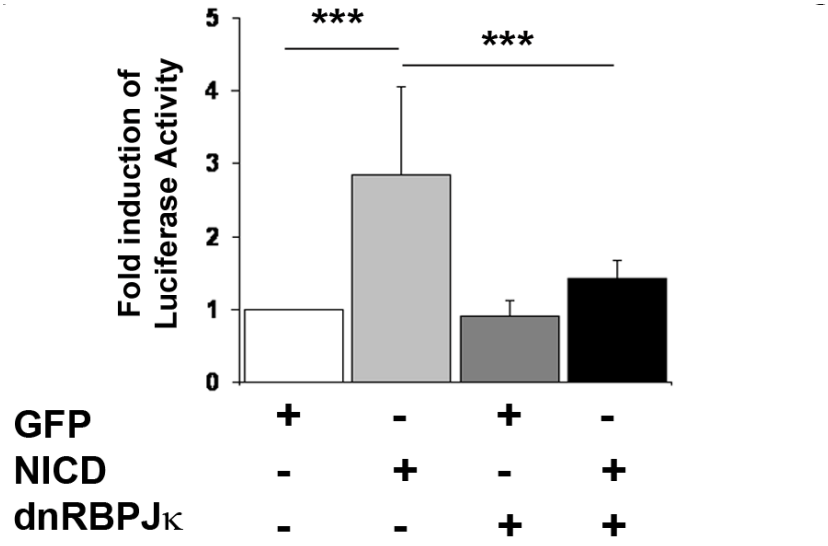

Figure 10: Notch/RBPJκ signalling activates a *Sox2* reporter construct. Luciferase reporter assays in adult hippocampal NSCs. Increased induction of the 5.5 kb *Sox2*-luciferase after overexpression of NICD demonstrates that the *Sox2* promoter is activated by Notch signalling. This activation was inhibited by expression of a dominant negative form of RBPJκ (*** $p<0.001$). (n = 3)

Next, it was analyzed whether *Sox2* is a direct Notch target. Therefore, the predicted RBPJ binding sites in the 5.5 kb *Sox2* promoter were verified. Electrophoretic mobility shift assays (EMSA) with nuclear extracts from adult hippocampal stem/progenitor cells were performed. Oligonucleotides comprising the putative RBPJκ binding sites within the 5.5 kb Sox2 promoter were labelled radioactively with ^{32}P. The radioactive

labelling procedure was repeated several times, but oligonucleotides #2 (-3746 to -3732) and #3 (-2538 to -2524) could not be labeled, although oligonucleotides with a slightly different sequence but also corresponding to the respective RBPJκ binding sites #2 and #3 were used. Oligonucleotides comprising the predicted RBPJκ binding sites #1 (-4475 to -4461), #4 (-1983 to -1969), and #5 (-1472 to -1458) were shifted on the gel after previous incubation with nuclear extracts from adult mouse hippocampal neural stem/progenitor cells (Figure 11). Additional incubation with an antibody directed against RBPJκ (RBP1F1) resulted in a supershifted signal on the gel demonstrating that the observed shift was caused by binding of RBPJκ. Competition experiments, in which the unlabeled wildtype oligonucleotides were used in excess compared to the respective radioactively labelled oligonulceotides, resulted in the loss of the shifted signal. This is caused by the fact that all RBPJκ proteins from the nuclear extracts were bound by the unlabeled nucleotides, which can not be detected on the gel. In competition experiments, mutant oligonucleotides, in which the RBPJκ binding site was altered, were supplied in excess to the reaction. Mutant oligonucleotides failed to compete with radioactively labelled oligonucleotides, demonstrating that the binding of RBPJκ to the oligonucleotides is sequence dependent. Similar results were obtained with recombinant RBPJκ protein and with nuclear extracts from an RBPJκ over-expressing cell line (DG75) (Ben-Bassat et al., 1977).

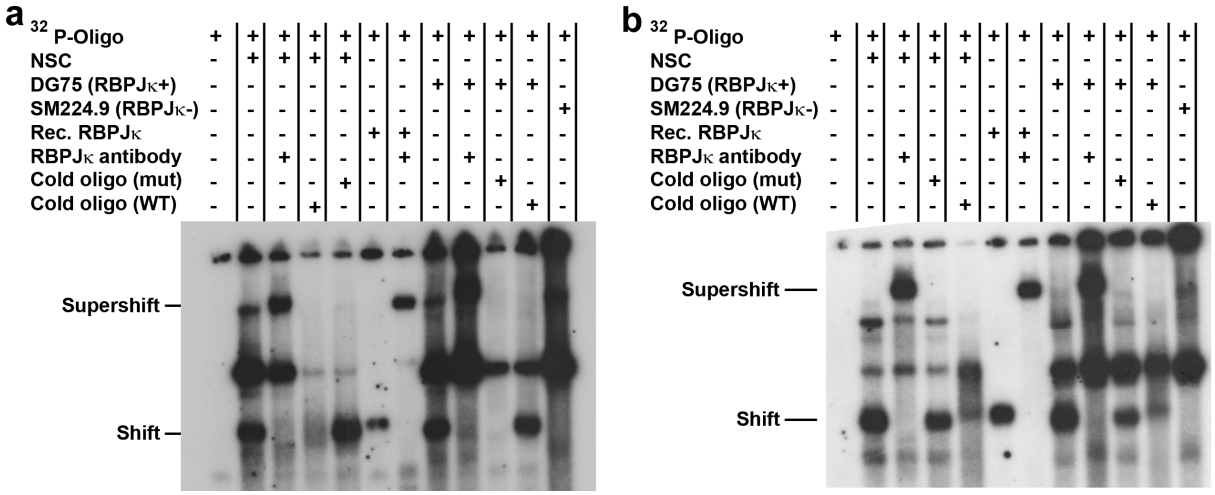

Figure 11: RBPJκ binds to predicted sequences within the *Sox2* promoter. EMSA shows binding of RBPJκ, derived from adult hippocampal neural stem cell (NSC), to predicted RBPJκ binding sites in the 5.5 kb *Sox2* promoter. **(a)** RBPJκ binding site #4 **(b)** RBPJκ binding sites #5

No shift was observed with nuclear extracts from an *Rbpjκ* knockout cell line (SM224.9) (Maier et al., 2005). Taken together, these results show that RBPJκ is present in the nucleus of adult hippocampal stem/progenitor cells and that RBPJκ can bind to at least some of the predicted binding sites in the 5.5 kb *Sox2* promoter.

Next, the presence of Notch signalling pathway components on the endogenous *Sox2* promoter in adult neural stem cells was analyzed. In collaboration with Drs. Anna Bigas and Lluís Espinosa Blay (Stem cells and cancer research program, IMIM hospital del mar, Barcelona, Spain), chromatin immunoprecipitation (ChIP) experiments were conducted to determine whether RBPJκ and NICD are associated with the *Sox2* promoter. Chromatin from adult hippocampal stem cells was precipitated with antibodies specific for RBPJκ and activated NOTCH1 (NICD). ChIP experiments using unspecific IgG antibodies and quantitative real-time PCR with primers flanking an RBPJκ unrelated region served as a control.

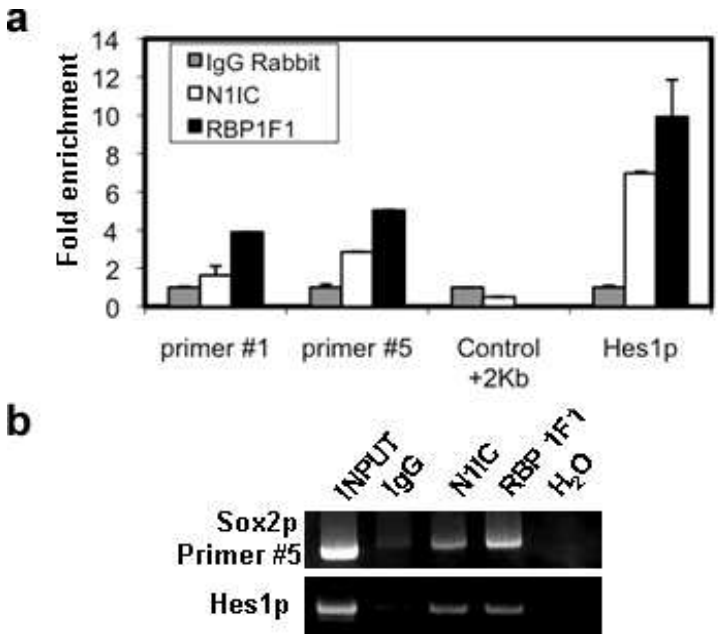

Figure 12: *Sox2* is a direct target gene of Notch/RBPJκ signalling in NSCs. ChIP analysis demonstrates that *Sox2* is a direct target of Notch / RBPJκ signalling in adult hippocampal neural stem cells. PCR primers were designed to surround the predicted RBPJκ binding sites #1 (Primer #1) and #5 (Primer #5) on the *Sox2* promoter. Control primers were designed to flank a Notch/RBPJκ unrelated region 2 kb upstream of the *Sox2* transcriptional start site. The presence of N1IC (NICD) and RBPJκ on the Hes1 promoter served as a positive control. **(a)** Results of qPCR analysis and **(b)** results of endpoint PCR analysis for Primer #5.

Quantitative real-time PCR using primers flanking the predicted RBPJκ binding sites revealed enrichment of RBPJκ and NICD on the predicted binding sites #1 and #5 within the endogenous *Sox2* promoter (Figure 12a, b). The presence of NICD and RBPJκ on the endogenous *Sox2* promoter demonstrates that *Sox2* is a direct transcriptional target of active RBPJκ-mediated Notch signalling in adult hippocampal stem cells.

5.3 Loss of RBPJκ in neural stem cells perturbs hippocampal neurogenesis

The data, which were received from the examination of the canonical Notch signalling pathway activity *in vivo* together with the results from the *in vitro* analysis of the regulation of the *Sox2* promoter by Notch signalling in adult hippocampal neural stem cells, strongly suggest that RBPJκ-mediated Notch signalling is involved in the expression of *Sox2* in adult hippocampal neural stem cells. Given the role of *Sox2* in stem cell maintenance this would also imply RBPJκ-mediated Notch signalling in the maintenance of adult hippocampal neural stem cells.

To test this hypothesis, the Notch signalling pathway was disrupted in adult hippocampal neural stem cells *in vivo*. For this purpose, the BAC transgenic mouse line (GLAST::CREER^{T2}), in which a tamoxifen-inducible Cre recombinase (CreER^{T2}) gene is under the control of the sodium-dependent glutamate/aspartate transporter (GLAST) promoter, was used. As the GLAST promoter is only active in radial glia like stem cells of the hippocampus and in a subset of non-neurogenic astrocytes (Slezak et al., 2007), GLAST::CREER^{T2} mice allow the expression of the CreER^{T2} transgene with relative specificity for adult hippocampal stem cells. In collaboration with Drs. Christian Göritz and Jonas Frisén (Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden), mice were generated which harbour a disrupted Notch signalling pathway in SOX2 positive adult hippocampal neural stem cells by crossing GLAST::CREER^{T2} mice with *Rbpjκ*$^{loxp/loxp}$ mice carrying an R26::EYFP reporter allele (Srinivas et al., 2001) to generate GLAST::CREER^{T2} ; *Rbpjκ*$^{loxp/loxp}$; R26::EYFP mice (*Rbpjκ*-cKO). In these mice, the Notch signalling pathway downstream mediator RBPJκ can be conditionally ablated in adult hippocampal stem cells after administration of Tamoxifen which allows precise timing of the induction of recombination (Figure 13). GLAST::CreERT2; *Rbpjκ*$^{loxp/+}$;

R26::EYFP (control), i.e., mice, in which only one *Rbpjκ* allele can be deleted upon induction of Cre recombinase activity, served as controls.

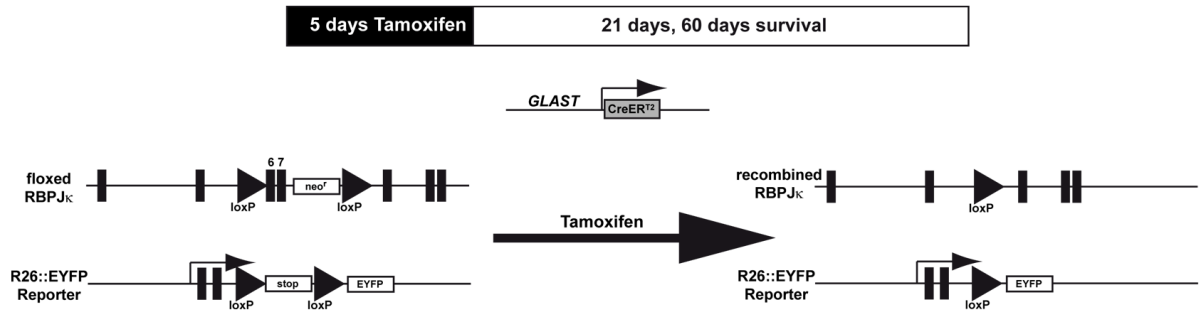

Figure 13: Experimental strategy to study the role of RBPJκ signalling in adult hippocampal stem cells and neurogenesis. GLAST::CreERT2; *Rbpjκ*$^{loxp/loxp}$; R26::EYFP (RBPJκ-cKO) and GLAST::CreERT2; *Rbpjκ*$^{loxp/+}$; R26::EYFP (control) were treated with Tamoxifen on 5 consecutive days to induce recombination in the *Rbpjκ* and the R26::EYFP locus. Animals were analyzed 21 days or 60 days after the final Tamoxifen injection.

12 week old mice were injected with Tamoxifen for five consecutive days to induce recombination in radial glia like neural stem cells. Animals were killed three weeks after the final Tamoxifen injection. The fraction of SOX2 expressing cells among the recombined cells within the SGZ of the dentate gyrus, which could be identified on the basis of YFP expression, was determined. A great reduction of SOX2 positive cells among the recombined cells in *Rbpjκ*-cKO mice compared to control animals was detected (30.32 ± 4.87 % in *Rbpjκ*-cKO vs. 69.60 ± 3.67 % in control, $p < 0.001$) (Figure 14a, b). The number of the quiescent or slowly dividing SOX2 GFAP double positive radial glia like stem cells (type 1 cells) as well as the number of SOX2 positive GFAP negative fast dividing stem/precursor cells (type 2 cells) among the recombined cell population in the SGZ were determined. Both, type 1 stem cells (7.54 ± 0.93 % in *Rbpjκ*-cKO vs. 27.90 ± 3.63 % in control, $p < 0.001$) and type 2 cells (22.78 ± 4.17 % in *Rbpjκ*-cKO vs. 41.70 ± 4.32 % in control, $p < 0.01$) (Figure 14b) were significantly reduced among YFP positive recombined cells in *Rbpjκ*-cKO mice compared to control mice. Most important, the density of SOX2 expressing cells (44390 ± 5153.72 / mm^3 in *Rbpjκ*-cKO vs. 66564 ± 3646.39 / mm^3 in control, $p < 0.01$), of Type 1 neural stem cells (7801 ± 928.78 / mm^3 in *Rbpjκ*-cKO vs. 19466 ± 3831.07 / mm^3 in control, $p < 0.01$), and of Type 2 non-radial stem/precursor cells (36589 ± 4412.97 / mm^3 in *Rbpjκ*-cKO vs. 47097 ± 1230.81 / mm^3 in control, $p < 0.05$) (Figure

14a, c) were significantly decreased in the SGZ of *Rbpjκ*-cKO mice. These findings demonstrate that conditional ablation of *Rbpjκ* leads to a decrease in hippocampal SOX2 positive Type 1 and Type 2 neural stem/precursor cell populations.

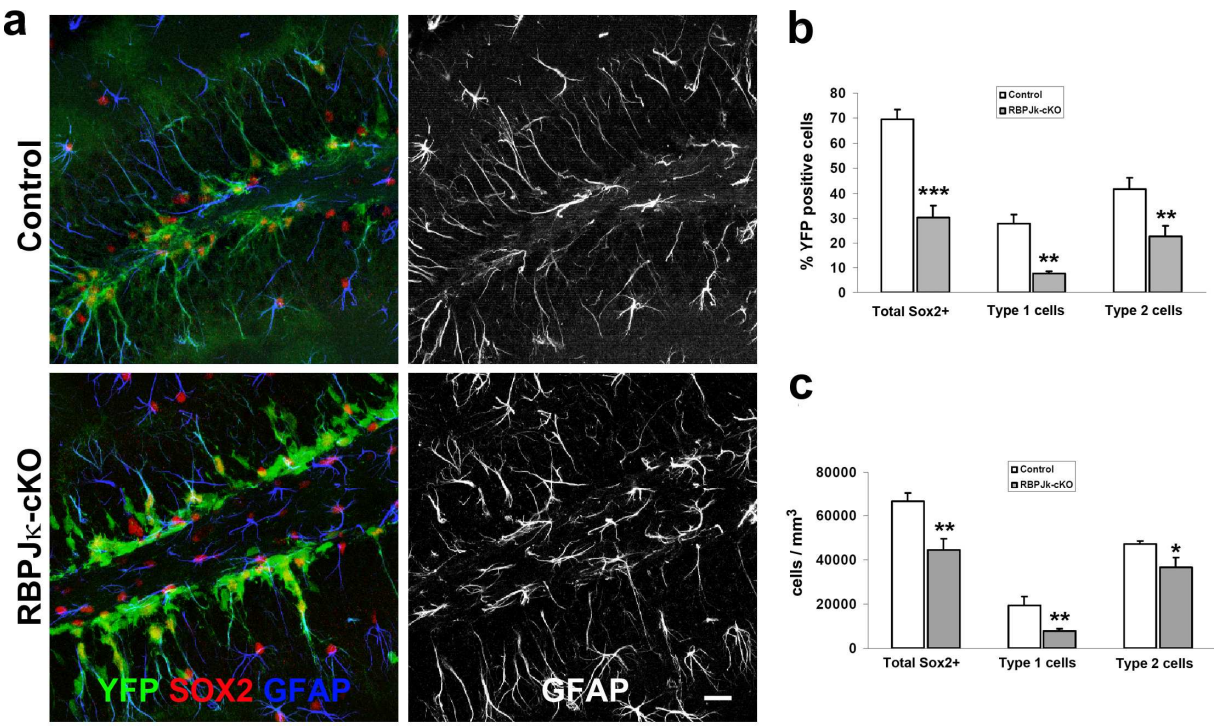

Figure 14: Loss of *Rbpjκ* in radial glia-like stem cells decreases stem cell numbers 3 weeks after Tamoxifen induced recombination. (a) Representative confocal images of *Rbpjκ*-cKO and control mice. SOX2 (red) expression and GFAP positive (blue) radial glia-like processes are strongly reduced within the YFP-positive recombined cell population (green). Furthermore, staining for GFAP demonstrates an overall reduction of radial glia-like stem cells in the dentate gyrus (right panels). In addition, the overall number of YFP-positive cells is increased in *Rbpjκ*-cKO. Scale bar 20 µm. **(b)** The percentage of all SOX2 expressing cells, radial glia like stem cells (Type 1 cells, identified by SOX2/GFAP expression and radial morphology), and non-radial stem cells (Type 2 cells, identified by SOX2 expression), among the recombined cells is significantly decreased in *Rbpjκ*-cKO mice. (**p< 0.01; ***p< 0.001). **(c)** The density of SOX2 expressing cells, radial glia like stem cells (Type 1 cells), and non-radial stem cells (Type 2 cells), in the subgranular zone is significantly decreased in *Rbpjκ*-cKO mice. (*p< 0.05; **p< 0.01). (n = 4 - 6)

Having observed a dramatic reduction of SOX2 positive radial glia like stem cells in *Rbpjκ* conditional knockout animals, the fate of the recombined cells was investigated. Adult hippocampal radial glia like stem cells are quiescent or slowly dividing. It was analyzed whether the disruption of RBPJκ-mediated Notch signalling results in the loss of the quiescent radial stem cell state and the possibility that these quiescent

radial glia like stem cells are recruited into cell cycle and start to differentiate. Indeed, higher numbers of recombined cells (66839 ± 9785 / mm^3 *Rbpjκ*-cKO vs. 46196 ± 5662 / mm^3 in control, p< 0.05), which were identified by YFP-expression, were observed in mutant mice (Figure 15a). The percentage of proliferating cells among the recombined cells was examined by immunoreactivity against PCNA (proliferative cell nuclear antigen), which is expressed during S-phase of the cell cycle, and indeed, it was found to be significantly increased (29.97 ± 1.25 % in *Rbpjκ*-cKO vs. 2.84 ± 1.29 % in control, p< 0.001) in *Rbpjκ*-cKO mice (Figure 15b). Overall proliferation was also markedly increased (31526 ± 5816 / mm^3 in *Rbpjκ*-cKO vs. 2468 ± 797 / mm^3 in control, p< 0.01) in the dentate gyrus of mutant animals (Figure 15b). These findings indicate that ablated Notch signalling results in the loss of quiescent radial glia like stem cells and increased proliferation and cell genesis.

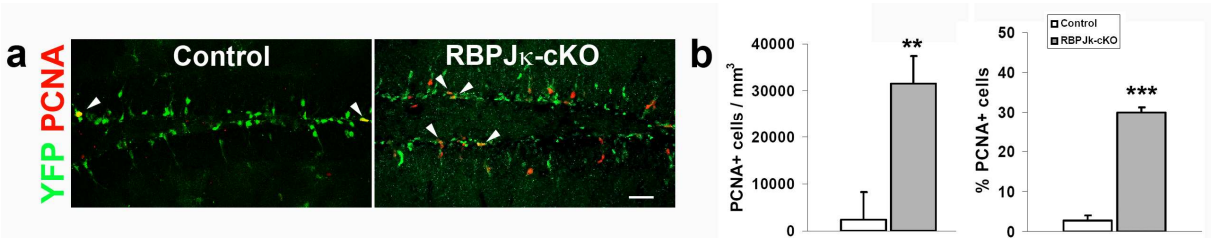

Figure 15: Loss of *Rbpjκ* in type 1 radial glia-like stem cells increases proliferation 3 weeks after induction of recombination. (a) Representative confocal images of *Rbpjκ*-cKO and control mice. The overall number of proliferating cells identified by expression of PCNA (red) as well as the percentage of PCNA positive cells among the YFP-expressing recombined cells (green, arrowheads) is strongly increased in *Rbpjκ*-cKO mice. Scale bar 20 µm. **(b)** Quantification of the density of PCNA positive cells and the percentage of PCNA positive cells among the recombined cells. (**p< 0.01; ***p< 0.001). (n = 3)

Next, it was analyzed whether the reduction of quiescent neural stem cells, increased proliferation and cell genesis would lead to changes in the rate of neurogenesis.

Strikingly, *Rbpjκ*-cKO mice showed a 4-5 fold increase in the fraction of DCX expressing immature neurons among the recombined cells (81.87 ± 5.88 % in *Rbpjκ*-cKO vs. 25.47 ± 5.43 % in control, p< 0.001) (Figure 16a, b). Moreover, the total number of DCX and NeuroD expressing immature neurons was also significantly increased (121345.3 ± 6432.6 / mm^3 in *Rbpjκ*-cKO vs. 16871.2 ± 1908.1 / mm^3 in control, p< 0.001) (Figure 16b, c). Under the assumption, that the number of DCX positive cells provides an estimate of the rate of neurogenesis (Brown et al., 2003), these data demonstrate that loss of *Rbpjκ* in adult neural stem cells results in increased generation of new granule neurons early (3 weeks) after induction of

recombination. These results, together with the findings that the number of SOX2 expressing radial glia like stem cells is reduced in the dentate gyrus of *Rbpjκ*-cKO mice strongly support the view that loss of RBPJκ-mediated Notch signalling shifts the balance from neural stem cell maintenance towards neurogenesis.

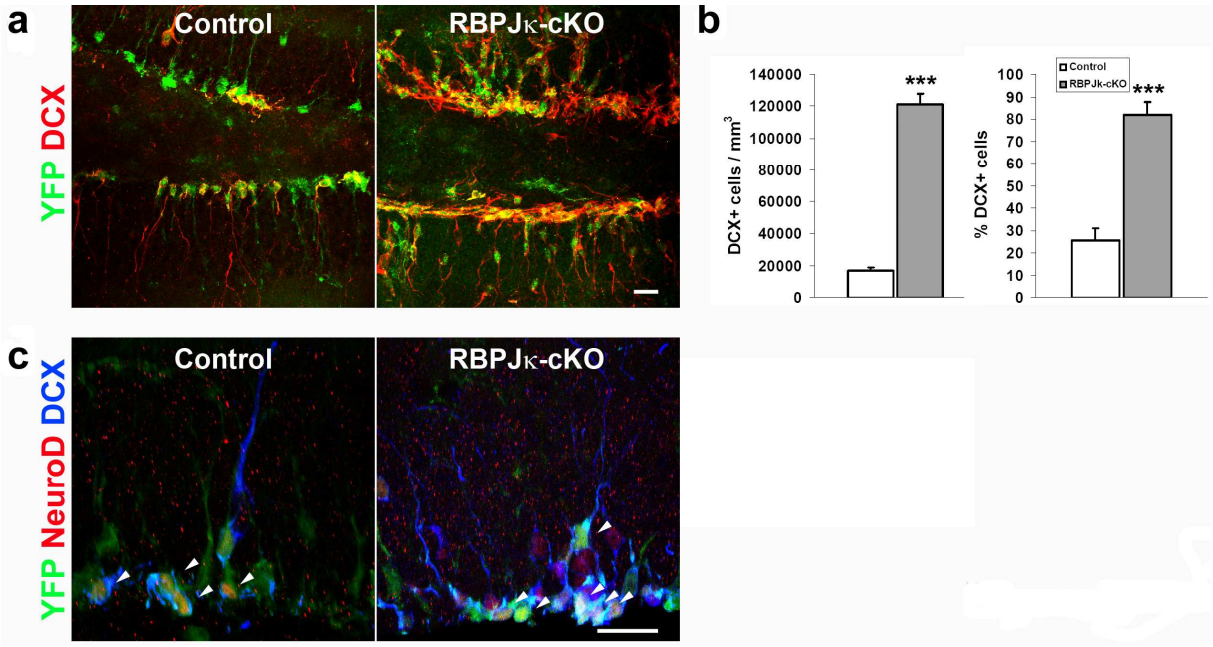

Figure 16: Loss of *Rbpjκ* in type 1 radial glia-like stem cells increases neurogenesis 3 weeks after induction of recombination. (a) Representative confocal images of *Rbpjκ*-cKO and control mice. The overall number of newly generated neurons identified by expression of DCX (red) as well as the percentage of DCX positive immature neurons among the YFP-positive recombined cells (green) is strongly increased in *Rbpjκ*-cKO mice. Scale bar 20 µm. **(b)** Quantification of the density of DCX positive immature neurons and the percentage of DCX expressing cells among the recombined cells. (***p< 0.001). **(c)** The overall number of NeuroD expressing newly generated neurons (red) as well as the fraction of NeuroD positive immature neurons among the YFP-positive recombined cells (green, arrowheads) is strongly increased in *Rbpjκ*-cKO mice. DCX in blue. Scale bar 20 µm. (n = 3)

Apart from the above mentioned findings, a prominent migration phenotype was recognized in *Rbpjκ*-cKO mice. In control animals, YFP-positive recombined cells were predominantly located in the SGZ or the lower third of the granule layer. However, in knockout animals, recombined cells appeared to migrate from the SGZ into the granule layer. It was found that most of the migrating cells were DCX-expressing immature neurons, but interestingly, some of them were SOX2-positive cells and even some of them exhibited a radial glia like GFAP-positive projection. In some cases, recombined cells seemed to travel in “chains” into granule layer.

5.4 RBPJκ is essential for long-term neural stem cell maintenance in the adult hippocampus

Next, it was examined how the shift in the balance from neural stem cell maintenance towards neurogenesis, which was observed in *Rbpjκ*-cKO mice at an earlier point in time, affected adult hippocampal stem cell maintenance and neurogenesis in the long-term. To this end, twelve weeks old *Rbpjκ*-cKO and control animals were injected with TAM on five consecutive days. Animals were analyzed 2 months after the induction of recombination.

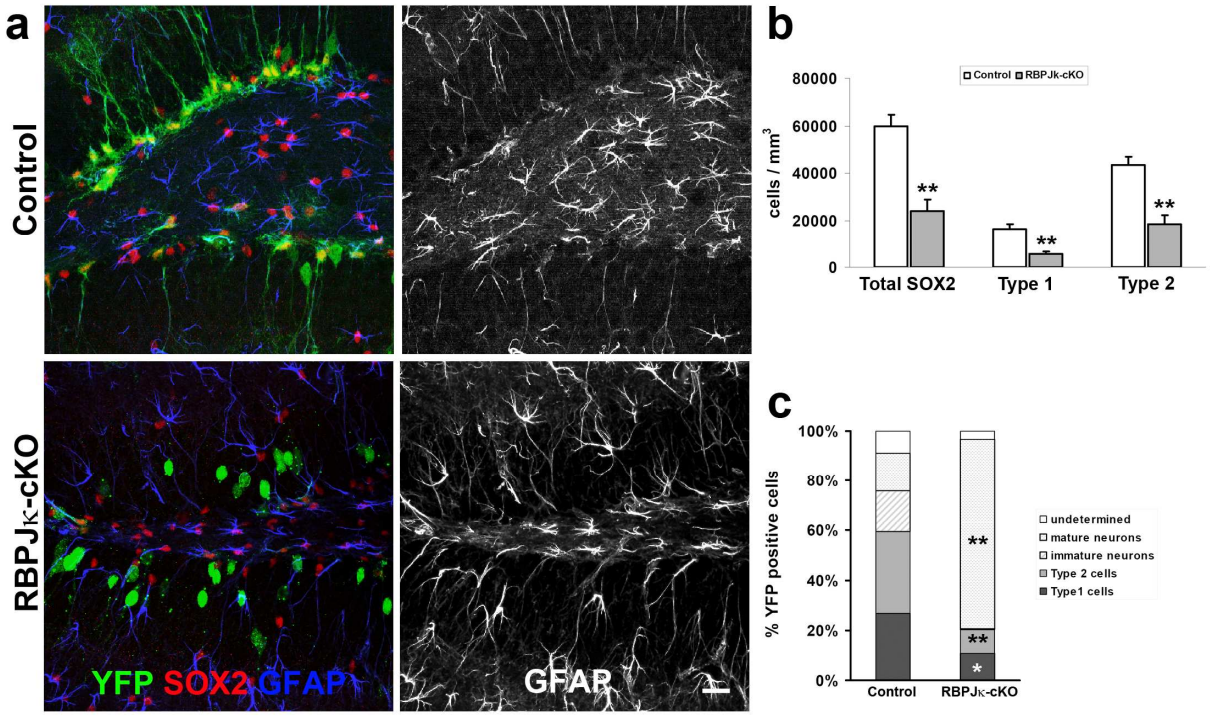

Figure 17: Loss of *Rbpjκ* in radial glia-like stem cells leads to persistent loss of SOX2 expressing stem cells 2 months after induction of recombination. (a) Representative confocal images of *Rbpjκ*-cKO and control mice. *Rbpjκ*-cKO mice exhibit decreased numbers of YFP positive recombined cells (green) that express SOX2 (red) or GFAP (blue). Immunohistochemistry for GFAP reveals a strong reduction in the number of radial glia like stem cells in the dentate gyrus (right panels). The vast majority of YFP-positive recombined cells in *Rbpjκ*-cKO are located in the granule cell layer. Scale bar 20 µm. **(b)** Sox2 expressing cells, radial glia like stem cells (Type 1 cells), and non-radial stem cells (Type 2 cells) are highly significantly reduced in the subgranular zone of the dentate gyrus in *Rbpjκ*-cKO mice. (**p< 0.01). **(c)** Phenotyping of YFP-positive cells demonstrates that the vast majority recombined radial glia like stem cells have left the stem cell compartment. Almost no YFP positive cells express DCX indicating that recombined cells do not contribute to the generation of new neurons 2 months after induction of recombination. (n = 3 - 4)

Rbpjκ-cKO mice exhibited a persistent significant reduction in the numbers of SOX2 positive cells (23927.91 ± 4788.16 / mm^3 in *Rbpjκ*-cKO vs. 59804.71 ± 4851.50 / mm^3 in control, p< 0.01) (Figure 17a, b). SOX2 GFAP positive radial glia like stem cells (5502.98 ± 1004.59 / mm^3 in *Rbpjκ*-cKO vs. 16385.70 ± 2147.41 / mm^3 in control, p< 0.01) (Figure 17a, b) as well as SOX2 non-radial progenitor cells were also severely decreased in *Rbpjκ* deficient mutants.

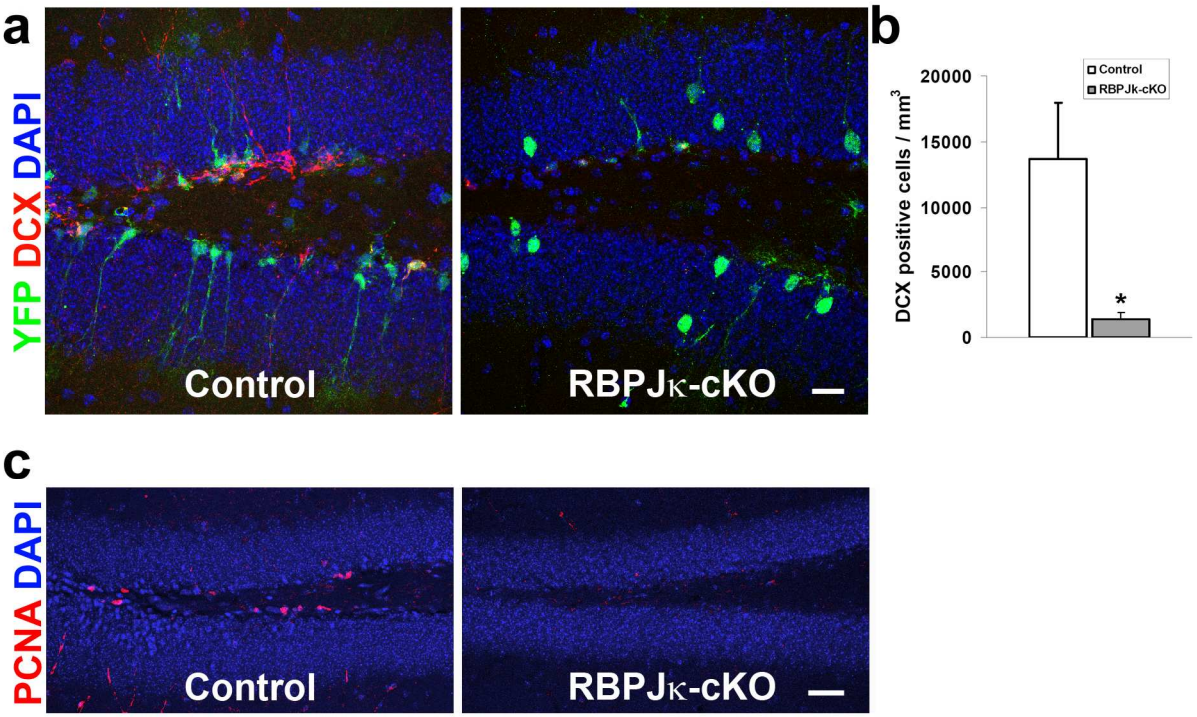

Figure 18: Loss of *Rbpjκ* in radial glia-like stem cells leads to loss of hippocampal neurogenesis 2 months after induction of recombination. (a) Representative confocal images of *Rbpjκ*-cKO and control mice. DCX (red) expressing immature neurons are virtually absent in *Rbpjκ*-cKO mice. YFP in green, DAPI in blue. Scale bar 20 µm. **(b)** The density of DCX expressing immature neurons in the dentate gyrus is significantly reduced. (*p< 0.05). **(c)** PCNA (red) positive, proliferating cells are virtually absent in *Rbpjκ*-cKO mice. DAPI in blue. Scale bar 20 µm. (n = 3 - 4)

However, in striking contrast to the mice, which were analyzed three weeks after the induction of recombination, *Rbpjκ*-cKO mice exhibited two months after the induction of the recombination a pronounced decrease in the rate of neurogenesis. This result was based on the observation that PCNA positive proliferating cells were virtually absent from mutant dentate gyrus of *Rbpjκ*-cKO mice. Furthermore, the number of DCX positive immature neurons was heavily reduced (1408.10 ± 466.10 / mm^3 in

Rbpjκ-cKO vs. 13686.7 ± 4295.8 / mm^3 in control, p< 0.01) in *Rbpjκ*-cKO mice (Figure 18a, b).

Consistent with these results, highly reduced fractions of total SOX2 positive cells, of SOX2 GFAP double positive radial glia like stem cells (10.65 ± 3.32 % in *Rbpjκ*-cKO vs. 26.74 ± 8.20 % in control, p< 0.05) and of SOX2 positive GFAP negative transient amplifying progenitor cells (9.64 ± 2.67 % in *Rbpjκ*-cKO vs. 32.79 ± 3.13 % in control, p< 0.01) (Figure 17c) among the recombined cells were also observed in mutant animals 2 months after induction of recombination. DCX expressing immature neurons (0.19 ± 0.37 % in *Rbpjκ*-cKO vs. 16.31 ± 5.54 % in control, p< 0.01) (Figure 17c) were virtually absent from recombined cells in mutant mice.

Finally, it was analyzed whether the recombined cells in *Rbpjκ*-deficient mice have the capacity to differentiate and mature into new neurons. NeuN is expressed in the hippocampal neurogenic lineage predominantly by mature neurons. The vast majority of YFP-positive recombined cells were NeuN-expressing neurons (76.18 ± 18.43 % in *Rbpjκ*-cKO vs. 15.49 ± 6.22 % in control, p< 0.01) (Figure 17c), indicating that these neurons were likely generated early after the induction of recombination.

Summing up, this data demonstrate that loss of *Rbpjκ* in adult hippocampal neural stem cells leads to a long-term loss of SOX2 expressing radial glia like stem and non-radial transient amplifying progenitor cells, which consequently results in persistent loss of neurogenesis in the adult hippocampal dentate gyrus.

5.5 Wnt/β-catenin signalling is active in adult hippocampal neural stem cells

It is known from the hematopoietic system that Notch signalling cooperates with other signalling pathways during stem cell maintenance (Duncan et al., 2005). In neural precursor cells, NICD and β-catenin were shown to interact (Shimizu et al., 2008). Thus, it was assessed whether Wnt/β-catenin signalling is active in adult hippocampal neural stem cells and potentially interacts with Notch signalling.

In the canonical Wnt signalling, the membrane bound Frizzled-receptors get activated upon binding of a Wnt-ligand leading to the stabilisation of β-catenin, which in turn translocates from the cytoplasm into the nucleus, where it associates with TCF/LEF transcription factors and activates target gene expression.

BATGAL mice, which express a β-galactosidase gene under the control of 7 TCF/LEF-binding sites upstream of a 0.13 kb DNA fragment containing the minimal promoter-TATA box of the siamois gene, were analyzed (Maretto et al., 2003). Reporter activity in the BATGAL mice was found in a subset of SOX2 positive cells in the SGZ in the dentate gyrus (13.93 ± 7.14 % of total SOX2 positive cells were also expressing β-galactosidase) (Figure 19a).

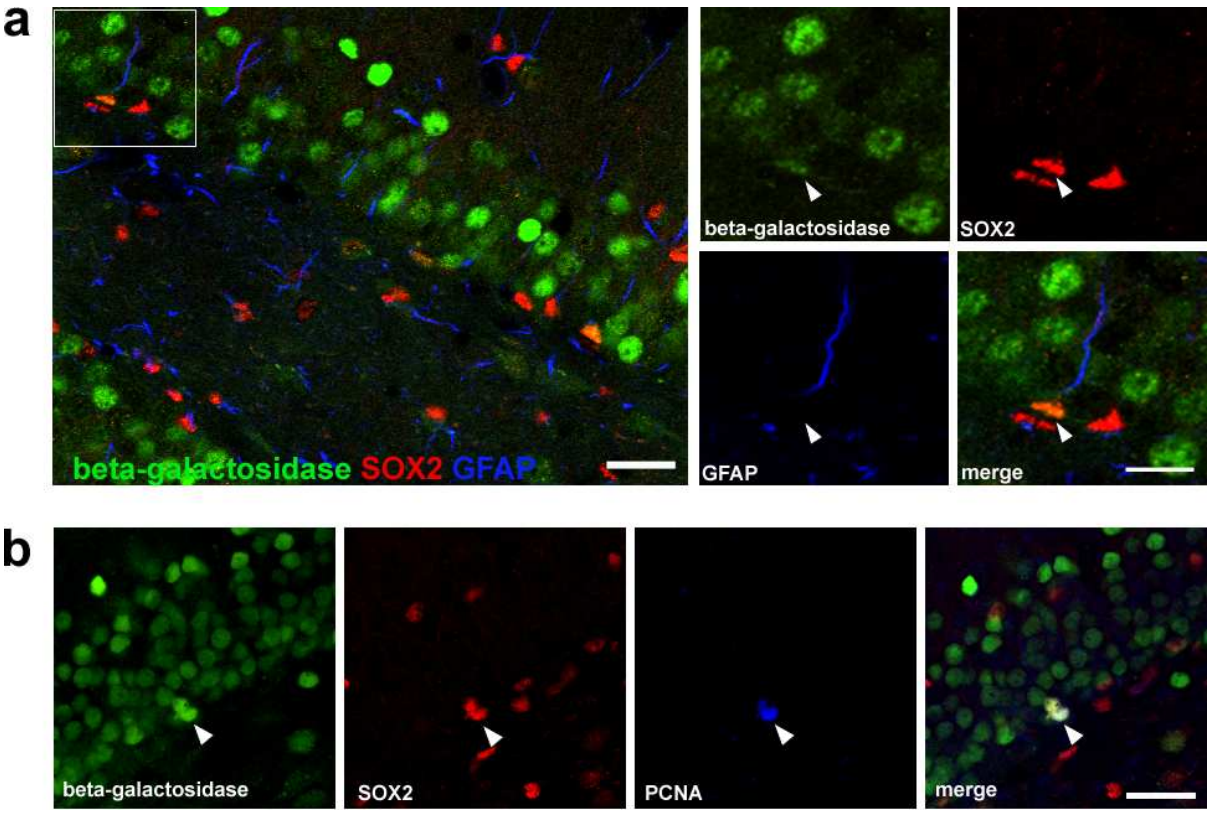

Figure 19: Analysis of adult BATGAL mice, reporter animals for Wnt/β-catenin signalling. (a) SOX2 expressing cells (red) in the subgranular zone of the dentate gyrus are positive for the β-galactosidase-reporter (green, arrowhead), indicating that Wnt/β-catenin signalling is active in neural stem cells. β-galactosidase expression in scattered cells throughout the dentate granule cell layer shows that Wnt/β-catenin signalling is also active in a subset of dentate granule neurons. Scale bar 30 µm, right panels 10 µm. **(b)** A subset of proliferating SOX2 PCNA positive cells also expresses β-galactosidase (green, arrowhead). Scale bar 30 µm.

Furthermore, β-galactosidase expression was detected in a subpopulation of SOX2 GFAP radial glia like stem cells (20.43 ± 1.36 % of total SOX2 GFAP positive cells were also expressing β-galactosidase).

Type 2 cells express SOX2 and show higher proliferative activity than SOX2 positive radial glia like stem cells. BATGAL mice were examined and reporter activity was

found in a subset of SOX2 PCNA positive cells (36.70 ± 15.77 % of total SOX2 PCNA positive cells were also expressing β-galactosidase) (Figure 19b). Taken together, these results indicate that the canonical Wnt-pathway is active in adult hippocampal neural stem cells and transient amplifying neural progenitor cells.

The activity of canonical Wnt signalling in undifferentiated SOX2 positive cells raises the possibility that Wnt signalling regulates *Sox2* expression in adult neural stem cells. To this end, the expression of Wnt pathway components in adult hippocampal stem cells *in vitro* was examined. In line with previous reports, several components of the canonical Wnt signalling pathway were found to be expressed in adult neural stem cells (Figure 20).

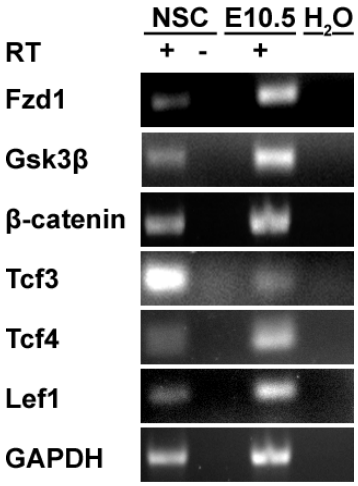

Figure 20: Analysis of adult hippocampal stem cells *in vitro*. RT-PCR analysis demonstrates that adult hippocampal neural stem cells (NSC) express essential components of the Wnt/β-catenin signalling cascade. RNA isolated from embryonic day 10.5 mouse brain (E10.5) served as a positive control. Frizzled 1 (Fzd1), glycerine synthase kinase 3β (Gsk3β), Transcription factor 3/4 (Tcf3/4), lymphoid enhancer binding factor 1 (Lef1).

Next, Wnt signalling was activated in adult hippocampal stem cells to investigate whether active Wnt signalling alters the expression of a *Sox2*-luciferase reporter. Adult hippocampal neural stem/ progenitor cells were electroporated with an expression vector for β-catenin S33Y, a constitutive active form of β-catenin, which can not be phosphorylated by GSK3β, and GFP as a control. Total proteins were isolated 48 hours after the electroporation. Overexpression of the constitutive active form of β-catenin resulted in increased endogenous SOX2 protein levels (Figure 21), suggesting that β-catenin mediated signalling positively regulates *Sox2* expression.

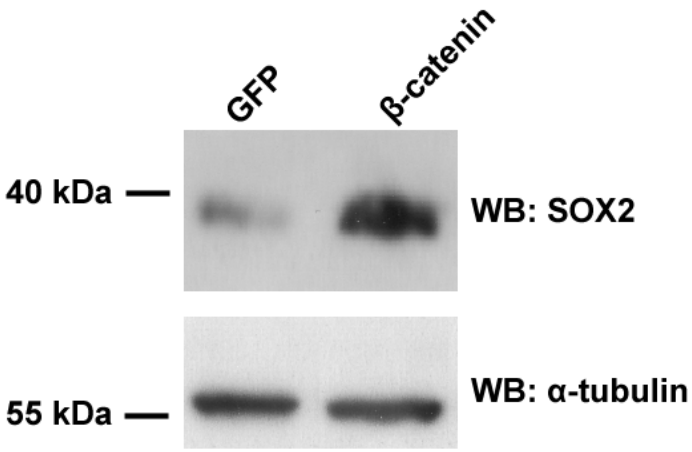

Figure 21: Wnt/β-catenin signalling promotes endogenous *Sox2* expression. Western Blot analysis of adult hippocampal NSCs following overexpression of β-catenin or GFP as control. Enhanced activation of canonical Wnt signalling by over-expression of a constitutive active form of β-catenin increases endogenous expression of SOX2. Loading control: α-tubulin. (n = 3)

To determine whether Wnt/β-catenin signalling can enhance the activity of the *Sox2* promoter, it was investigated whether enhanced activation of Wnt/β-catenin signalling leads to increased *Sox2*-luciferase activity. To this end, adult hippocampal stem cells were co-electroporated with the *Sox2*-luciferase and either a constitutive active form of β-catenin or GFP as a control. Cells were lyzed and the luciferase assay was performed 48 hours after the electroporation. Overexpression of the constitutive active form of β-catenin resulted in a 2.5 fold increase of *Sox2*-luciferase reporter activity (Figure 22a) suggesting that β-catenin mediated signalling positively regulates *Sox2* expression. Furthermore, expression constructs for NICD together with constitutive active β-catenin were co-electroporated to check for potential additive effects of both signalling pathways on the activation of *Sox2* reporter activity. Interestingly, simultaneous enhanced activation of Notch- and Wnt signalling resulted in a 15 fold increase of *Sox2* luciferase activity (Figure 22b) suggesting that Notch- and Wnt signalling act in a synergistic manner when promoting *Sox2* expression.

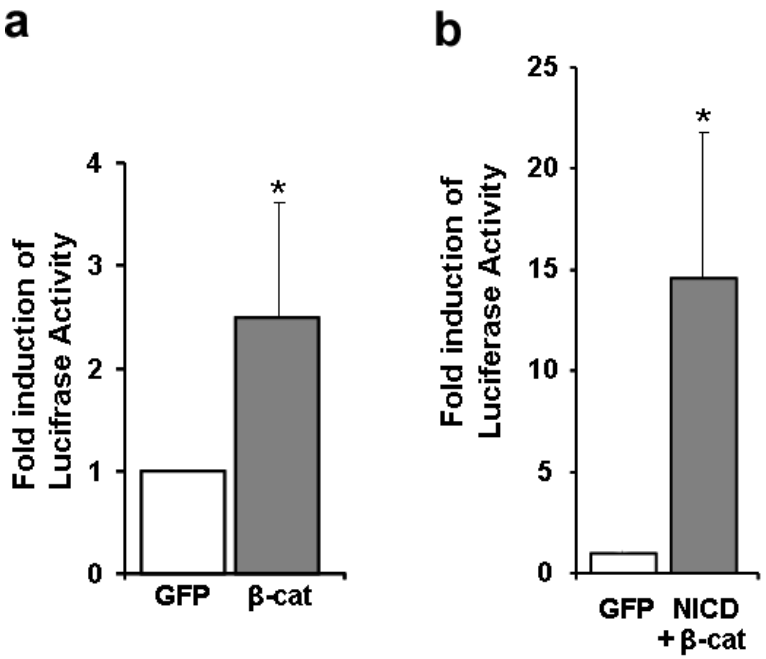

Figure 22: Wnt/β-catenin signalling activates a *Sox2* reporter construct. (a) Luciferase reporter assays in adult hippocampal NSCs. Increased induction of the 5.5 kb *Sox2*-luciferase after overexpression of a constitutive active form of β-catenin (S33Y) demonstrates that the *Sox2* promoter is activated by Wnt/β-catenin signalling. (* $p<0.05$) **(b)** Simultaneous stimulation with NICD and a constitutive active form of β-catenin results in stronger activation of the *Sox2* luciferase reporter compared to the single activation by the Notch or Wnt pathway (* $p<0.05$). (n = 6)

Future experiments will have to analyze the 5.5 kb promoter region upstream of the *Sox2* transcriptional start site for potential binding sites for TCF/LEF. This analysis could help investigating whether *Sox2* expression is directly regulated by Wnt/β-catenin signalling in adult hippocampal neural stem cells.

6 Discussion

The maintenance and differentiation of adult hippocampal neural stem cells has to be tightly balanced and regulated to ensure appropriate supply with new neurons throughout the whole life of the organism. In this study, the role of the Notch signalling pathway in the regulation of the stem cell associated gene *Sox2* and the involvement of Notch signalling in adult hippocampal stem cell maintenance were examined.

Activated Notch signalling was found to induce *Sox2* expression in neural stem cells. Several binding sites for the Notch signalling downstream mediator RBPJκ could be identified in the *Sox2* promoter. Furthermore, RBPJκ and the intracellular domain of the Notch receptor were found to be bound to the *Sox2* promoter in adult hippocampal neural stem cells, indicating that Notch signalling is directly regulating *Sox2* expression. Conditional ablation of *Rbpjκ*, and thus interrupted Notch signalling, in adult hippocampal stem cells resulted in the loss of SOX2 positive radial glia like cells in the SGZ of the dentate gyrus. The reduction in the number of radial glia like stem cells was caused by impaired maintenance of the stem cell population which lead to increased proliferation and increased neurogenesis. At a later point in time after induction of recombination, stem cell numbers were still reduced and proliferation and neurogenesis were more or less undetectable in the hippocampii of Rbpjκ deficient mice, suggesting that the dentate gyrii of RBPJκ-deficient mice were depleted of stem cells which are capable to generate new neurons. The presented results clearly show that RBPJκ-mediated Notch signalling is important for the expression of *Sox2* expression and adult hippocampal stem cell maintenance.

6.1 Notch signalling and *Sox2* expression in neural stem and progenitor cells

Consistent with previous studies, *Sox2* was found to be expressed in adult hippocampal stem and progenitor cells. The fraction of type 1 cells among all SOX2 expressing cells (type 1 and type 2 cells) was determined and it was found that around 37 % of SOX2 positive cells (36.82 ± 5.32 %) in the adult SGZ represent SOX2 GFAP positive type 1 cells. Virtually every GFAP positive radial glia like stem cells exhibited nuclear SOX2 protein. This is in contrast with studies from Bani-

Yaghoub and colleagues, who reported that SOX2 is not expressed in quiescent radial glia (Bani-Yaghoub et al., 2006). However, there are differences between this work and the study by Bani-Yaghoub, as they analyzed embryonic and P0 long-term cell cultures, while this study was conducted in adult brains. Whether these differences can account for the contrary results has to be determined in future experiments. Immuno-fluorescent staining against Nestin was performed and it was found that around 50 percent of all SOX2 GFAP positive radial glia like stem cells were also expressing Nestin. However, it was not further analyzed, whether the SOX2 GFAP Nestin positive subpopulation reflects activated, proliferating stem cells or quiescent/slowly dividing stem cells.

Analysis of RBPJκ-GFP (Tg(Cp-EGFP)25Gaia) mice, i.e. canonical Notch reporter animals, revealed Notch pathway activity in a subset of SOX2 positive cells and SOX2 GFAP double positive radial glia like stem cells. If Notch signalling regulates *Sox2* expression one would expect Notch pathway activity in all SOX2 positive cells. This discrepancy between theoretical expectations and *in vivo* observations could be explained by findings in mouse neural progenitor cells during development. Mizutani and co-workers analyzed RBPJκ-GFP positive cells from Tg(Cp-EGFP)25Gaia canonical Notch signalling reporter mice and found that embryonic NSCs exhibit canonical Notch/RBPJκ signalling whereas more lineage restricted intermediate progenitors (INPs) signal through an RBPJκ independent Notch pathway (Mizutani et al., 2007). The assumption that INPs from developmental stages are the equivalent of *Sox2* expressing type 2 cells in the adult hippocampal niche would suggest that, similar to INPs, *Sox*2 expressing type 2 cells may signal through an RBPJκ independent Notch pathway, which could explain why there is only limited overlap between the RBPJκ-GFP reporter and SOX2 positive cells.

Recently, Shimojo and co-workers demonstrated by real-time imaging that the Notch target gene Hes1 exhibits oscillatory expression in dorsal telencephalic neural progenitors during development. (Shimojo et al., 2008; for review see Kageyama et al., 2008), which suggests that Notch signalling is only periodically active. In case this observation would also apply for adult hippocampal neural progenitor cells, analysis of Notch pathway activity on immuno-fluorescently stained brain sections, i.e. in a static manner, would only deliver “snapshots” of cells which exhibit Notch pathway activity, but could never reveal the complete pathway activity pattern. Thus, oscillatory activity of Notch signalling in adult neural stem cells could also provide a

possible explanation for the small overlap between RBPJκ-GFP reporter activity and SOX2 expression. Future studies should investigate whether Notch signalling and its target genes exhibit oscillatory activity in adult hippocampal stem cells.

Furthermore, the sensitivity of the Tg(Cp-EGFP)25Gaia reporter mice might not be sufficient to monitor the whole activity pattern of canonical Notch signalling in the adult hippocampus. Taken together, the findings discussed above may help explaining the lack of complete overlap between the canonical Notch reporter and SOX2 expressing cells.

The results clearly show that activation of the Notch signalling pathway in hippocampal neural stem cells leads to increased *Sox2* expression. Together with the results from the EMSA and ChIP experiments, *Sox2* could be identified as a direct and novel target of canonical RBPJκ-mediated Notch signalling. These results are in line with findings from Das and colleagues, who showed that inhibition of Notch signalling in retinal cells lead to reduced levels of *Sox2* expression (Das et al., 2006). Interestingly, Taranova and co-workers show that SOX2 directly binds to the *Notch1* promoter and transcriptionally regulates and increases *Notch1* expression in ES cells and embryonic CNS tissue (Taranova et al., 2006). These data together with the results of this work suggest a positive feedback loop between *Notch1* and *Sox2*. Whether this is the case in adult hippocampal stem cells awaits further examination.

6.2 Notch signalling and stem cell maintenance

Multiple studies have linked Notch signalling to stem cell maintenance in the developing nervous system. Roles for Notch signalling in stem cell maintenance in several stem cell compartments of the adult organism have also been proposed (Yamamoto et al., 2003; Duncan et al., 2005; Blanpain et al., 2006; Song et al., 2007)). However, conflicting results have been obtained, e.g. in the hematopoietic system, with regard to the essential role of Notch signalling for stem cell maintenance (Varnum-Finney et al., 2000; Stier et al., 2002; Maillard et al., 2008). Maillard and colleagues demonstrated that hematopoietic progenitors either expressing dominant-negative Mastermind-like1 (DNMAML) or lacking RBPJκ achieved stable long-term reconstitution when grafted into irradiated hosts and showed a normal frequency of progenitor fractions enriched for long-term HSCs. These studies suggested that RBPJκ-independent Notch signalling plays a prominent role in adult haematopoietic

stem cell maintenance. Previous reports regarding the role of Notch signalling in adult neural stem cells have focused on manipulating the notch receptor. In addition, Androutsellis-Theotokis and co-workers have revealed a role of RBPJκ-independent Notch signalling in the control of stem cell proliferation and survival in the adult central nervous system (Androutsellis-Theotokis et al., 2006). Hence the contribution of the canonical Notch signalling pathway, i.e. Notch/RBPJκ signalling, to adult neural stem cell regulation remained unknown.

This study demonstrates that conditional knockout of RBPJκ in Type 1 cells of the hippocampal neurogenic lineage leads to the loss of hippocampal stem cells. This demonstrates that RBPJκ-dependent signalling mechanisms play a major role in the regulation of adult neural stem cells. It also parallels the situation in the developing CNS where loss of *Rbpjκ* in NESTIN expressing cells of the fetal forebrain results in reduced stem cell numbers, reduced neurosphere forming capacity and increased precocious differentiation (Gao et al., 2009).

The *in vitro* data on the regulation of *Sox2* expression by the canonical Notch signalling pathway suggest that loss of RBPJκ-mediated Notch signalling caused reduced *Sox2* expression which resulted in impaired maintenance and increased differentiation of hippocampal stem cells. Recent studies analyzed Nestin-Cre-mediated conditional knockout of *Sox2* in adult hippocampal type 1 neural stem cells (Favaro et al., 2009). Loss of SOX2 expression in type 1 cells lead to the loss of the stem cells and resulted in decreased proliferation and a complete loss of neurogenesis. Similarly, conditional knockout of RBPJκ in type 1 cells lead to the loss of SOX2 GFAP double positive neural stem cells in the adult SGZ and resulted in the loss of neurogenesis in the long run. Hence, the phenotype of loss of SOX2 expression in type 1 cells strikingly resembles the phenotype of loss of RBPJκ expression in hippocampal neural stem cells reported in this study. These results support the notion that *Sox2* is important for adult hippocampal stem cell maintenance and that the loss of *Sox2* expression caused or at least contributed to the loss of type 1 stem cells in the SGZ. Future experiments should try to rescue the phenotype by overexpressing *Sox2* in RBPJκ-deficient hippocampii. However, it is unlikely that *Sox2* is the only downstream mediator of Notch function in adult hippocampal neural stem cells. *Hes1* and *Hes5*, for example, have been shown to play important roles in stem cell maintenance during embryonic development (for review see (Yoon and Gaiano, 2005)). The contribution of *Hes1* and *Hes5* to adult

hippocampal neural stem cell maintenance is still poorly understood and has to be explored. Thus, concomitant and separate overexpression of the Notch pathway downstream mediators *Hes1* and *Hes5* to rescue the *Rbpjκ* loss of function phenotype in NSCs should also be taken in consideration.

RBPJκ recruits transcriptional co-repressors in the absence of active Notch signalling thereby inhibiting the expression of Notch/RBPJκ target genes (for review see (Bray, 2006)). Hence, it is possible that the observed phenotype in *Rbpjκ* knockout mice is a consequence of derepression of Notch/RBPJκ target genes rather than caused by loss of RBPJκ-mediated Notch signalling. Albeit this possibility it can not be fully excluded, the findings that canonical Notch signalling is active in neural stem cells *in vitro* and *in vivo* suggest that RBPJκ targets in neural stem cells are transcriptionally activated rather than repressed. Furthermore, the demonstration that RBPJκ and NICD are bound to the *Sox2* promoter *in vitro* suggest that RBPJκ activity on *Sox2* expression *in vivo* is most likely activated through Notch/RBPJκ signalling. This view is supported by the phenotype of the conditional knockout of the *Notch1* receptor in adult hippocampal stem cells, which comprises the loss of the radial glia like stem cell population (Eisch AJ, personal communication) and increased differentiation of stem cells into immature neurons (Breunig et al., 2007). The fact that loss of *Notch1* in neural stem cells strongly resembles the consequences of ablated *Rbpjκ* strongly supports the idea that rather the activation of Notch/RBPJκ signalling downstream target genes and not the repression of RBPJκ targets due to the inactivity of Notch is involved in the maintenance of neural stem cells.

Deleting the only known down-stream mediator of Notch signalling, e.g. *Rbpjκ*, is the most efficient approach to analyzing the involvement of the canonical Notch pathway in adult neural stem cell maintenance as different Notch receptors are being expressed in the adult hippocampal neurogenic niche (Irvin et al., 2001; Stump et al., 2002) and could compensate for loss of other Notch receptor members. This view of potential redundancy between Notch receptors is supported by studies on *Notch1* and *Rbpjκ* knockout during mammalian development as the *Rbpjκ* knockout exhibits a similar but more severe phenotype compared to the loss of *Notch1* (Oka et al., 1995; de la Pompa et al., 1997).

However, a downside of this approach is that Notch-independent RBPJκ signalling can not be distinguished from classical Notch-activated RBPJκ signalling. Hori and colleagues reported that RBPJκ and Ptf1a, a bHLH transcription factor essential for

the generation of GABAergic inhibitory interneurons in the dorsal spinal chord, cerebellum and retina, associate in non-classical transcriptional activator complexes to induce GABAergic specification in the neural tube of chick and mouse in a Notch-independent way (Hori et al., 2008). Thus, we cannot fully exclude that some of the observed effects are the consequence of Notch-independent RBPJκ signalling. Astrocytes in non-neurogenic regions express high levels of SOX2, but the vast majority of them show no RBPJκ activity indicated by the absence of EGFP expression in *Rbpjκ*-GFP reporter mice. The GLAST::CreERT2 transgene will also be expressed and activated upon TAM stimulation in these astrocytes. Unexpectedly, most of the recombined non-neurogenic astrocytes located outside the dentate gyrus still expressed *Sox2* after ablation of *Rbpjκ*. A possible explanation for this finding may be that *Sox2* is differently controlled in neurogenic radial glia like cells and non-neurogenic astrocytes and that *Sox2* expression in the latter does not depend on Notch/RBPJκ pathway activity. This issue could be resolved by analysis whether RBPJκ and NICD can be found on the *Sox2* promoter in non-neurogenic astrocytes.
I also observed some recombined cells in the SGZ of the dentate gyrus which were still expressing *Sox2*. It needs further examination to investigate whether in these cells only the YFP reporter but not the *Rbpjκ* locus has recombined. Future analysis should also verify whether the expression levels of RBPJκ protein are reduced in these cells. Furthermore, the expression levels of the Notch target genes *Hes1* and *Hes5* should also be analyzed after conditional ablation of *Rbpjκ* in type 1 cells as the roles these proteins play in adult hippocampal stem cell maintenance are still not fully known. The above-proposed rescue experiments of type 1 cells by overexpression of *Hes1* and/or *Hes5* should contribute to understand Hes protein function in adult neural stem cells better. ChIP experiments for RBPJκ in adult hippocampal stem cells followed by microarray analysis should shed more light on the signalling network downstream of RBPJκ and may supply valuable information to explain the phenotype of the conditional RBPJκ knockout.

6.3 Notch signalling and migration

Conditional knockout of *Rbpjκ* was accompanied by a prominent migration phenotype. Homozygous *Rbpjκ* knockout mice exhibited ectopic location of some recombined SOX2 positive cells in the granule layer and many recombined cells which expressed

DCX were located in the upper third of the granule layer. Reelin, an extra-cellular matrix-associated glycoprotein, and its receptor are expressed in radial glial cells and Cajal-Retzius neurons during development (Gaiano et al., 2000; Hartfuss et al., 2003). Reelin has previously been implicated in the radial migration of neurons during development (Bar et al., 2000; Magdaleno and Curran, 2001; Rice and Curran, 2001; Tissir and Goffinet, 2003; Kanatani et al., 2005; Soriano and Del Rio, 2005; D'Arcangelo, 2006). Reelin promotes a radial glial phenotype of neural precursor cells and has been shown to act as an attractant for radial glial fibre orientation to generate a proper scaffold for migrating immature granule neurons in the developing dentate gyrus (Forster et al., 2002; Frotscher et al., 2003; Zhao et al., 2004; Forster et al., 2006). Recently, Notch signalling has been shown to mediate Reelin activity in the developing cerebral cortex (Hashimoto-Torii et al., 2008). Interestingly, Reelin is also expressed in the adult hippocampal neurogenic niche (Figure 23) and controls the positioning of dentate granule neurons (Heinrich et al., 2006), raising the possibility that Notch signalling may cooperate with Reelin signalling during migration of newly born granule neurons to control positioning of newborn neurons in the granule layer of the dentate gyrus.

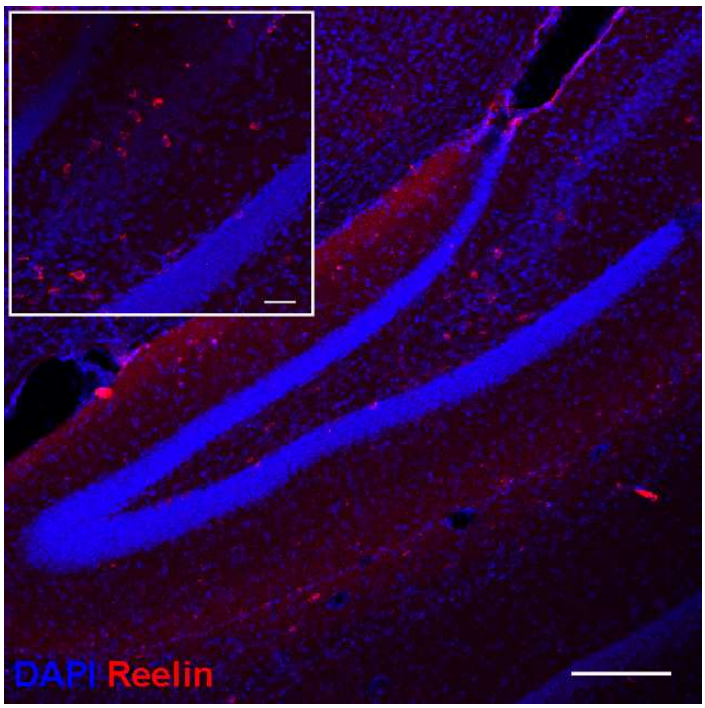

Figure 23: Reelin is expressed in the adult hippocampus. Reelin (red) is expressed by cells in the SGZ of the dentate gyrus as well as by cells in the hilus and CA3 region. DAPI in blue, Scale bar 155 µm; scale bar of inset 40 µm.

Keilani and Sugaya could demonstrate that activation of Reelin signalling activates the Notch pathway, promotes the expression of the Notch target gene BLBP (brain lipid binding protein) and induces a radial glia-like phenotype in human neural progenitor cells (Keilani and Sugaya, 2008). Additionally, Keilani and Sugaya could demonstrate by co-immunoprecipitation that NICD and Disabled1 (*Dab1*), an adapter protein of the Reelin signalling cascade, physically interact, indicating a concrete link between both pathways. Dab1 has been shown to inhibit ubiquitination (Park et al., 2003) raising the possibility that Dab1 might also inhibit the ubiquitination and degradation of NICD thereby stabilizing NICD and promoting Notch signalling activity. Sibbe and colleagues found reduced levels of NICD, HES5 and BLBP in the postnatal hippocampus of Reelin-deficient *Reeler* mice that do not develop the characteristic radial glial scaffold in their dentate gyrus. Furthermore, the authors could show that rescue of the before mentioned hippocampal phenotype in *Reeler* mice by Reelin is Notch-dependent, suggesting that Notch signalling is mediating Reelin function during the development of the dentate gyrus (Sibbe et al., 2009).
Future experiments should take a closer look at the interaction between the Notch pathway and Reelin signalling in the adult dentate gyrus and might provide explanations for the observed migration phenotype of recombined cells in conditional *Rbpjκ* knockout mice.

6.4 Integration of Notch signalling with other signalling pathways

Factors controlling stem cell maintenance have to ensure the proliferative capacity and the undifferentiated, multipotent state. There is increasing evidence that stem cell maintenance in different systems, for example in the neurogenic niches of the brain, the bone marrow and the bulge of the hair follicle, is achieved through the interplay and close interaction of multiple signalling pathways (for review see (Fuchs et al., 2004)). It is therefore likely that Notch signalling interacts with other pathways in the regulation of NSC maintenance in the adult hippocampus. Previously, the glycoprotein 130 (gp130) associated neurotrophic cytokine ciliary neurotrophic factor (CNTF) has been implicated in neural stem cell maintenance during embryonic development and in the adult brain (for review see (Bauer and Patterson, 2006)). Recently, the analysis of CNTF-deficient animals revealed reduced numbers of radial glia like neural stem cells, decreased proliferation and generation of new neurons

(Muller et al., 2009). Treatment of neurosphere cultures prepared from adult forebrain with CNTF increased the number of NSCs as indicated by enhanced secondary neurosphere formation and upregulated expression of stem cell markers including *Sox2*. Conditional ablation signal transducer and activator or transcription 3 (*Stat3*) in GFAP expressing cells resulted in a reduction in neurogenesis similar to the one observed in CNTF-deficient mutants, suggesting that CNTF-induced STAT3 signalling is important for stem cell maintenance. Foshay and colleagues have demonstrated that STAT3 directly binds to the *Sox2* promoter in ES cells (Foshay and Gallicano, 2008) and promotes the expression of SOX2 target genes (Tanaka et al., 2004). Chojnacki and co-workers showed that CNTF-activated gp130 signalling signalling increases *Notch1* expression in the developing brain (Chojnacki et al., 2003). *Hes1* and *Hes5* expression seemed not to be involved in gp130-activated Notch signalling. Taken together, these findings suggest that CNTF-activated gp130 signalling induces the Notch and STAT3 pathway, which in turn promote the expression of *Sox2* to maintain neural stem cells.

Another interacting pathway for Notch signalling in stem cell maintenance may be Shh signalling. Previous studies have shown that Shh signalling is active in the radial glia like population of the dentate gyrus (Ahn and Joyner, 2005). In addition, enhanced Shh signalling can promote stem cell proliferation. Shh signalling has previously been implicated in proliferation (Wechsler-Reya and Scott, 1999; Lai et al., 2003). Androutsellis-Theotokis and co-workers have demonstrated that Notch receptor activation induces the expression of *sonic hedgehog* (*shh*) through activation of several cytoplasmic signals, including the serine/threonine kinase Akt, the transcription factor STAT3 and mammalian target of rapamycin (mTOR), thereby promoting the survival of neural stem cells (Androutsellis-Theotokis et al., 2006).

Previous studies identified the transcription factor *Gli2*, a key downstream mediator of Shh signalling (Sasaki et al., 1999), as a regulator of *Sox2* expression in telencephalic neuroepithelial cells and proposed a mechanism in which a Gli2-Sox2 signalling cascade activates *Hes5* expression, thereby maintaining cells in an undifferentiated state (Takanaga et al., 2009). Whether Shh regulates *Sox2* directly in adult neural stem cells is presently unknown. Interestingly, *Shh* was identified as a direct target of SOX2 in adult hippocampal neural stem cells (Favaro et al., 2009). Loss of *Sox2* in radial glia like stem cells of the hippocampus results in the depletion of neural stem cells. Favaro and co-workers found that pharmacological reactivation

of Shh signalling partially rescued the loss of neural stem cells observed in their study on conditional loss of *Sox2* expression in adult hippocampal neural stem cells. In this work, *Sox2* was identified as a novel target gene of RBPJκ-mediated Notch signalling. Taken together, these results would suggest a signalling cascade in which the Notch pathway promotes *Sox2* expression, which in turn enhances Shh signalling. These findings raise the possibility that *Sox2* may contribute to stem cell proliferation via activation of Shh signalling. This pathway in turn could reinforce stem cell maintenance through a positive feedback loop between Sox2 and Shh signalling, given the observation that Shh signalling can positively regulate *Sox2* expression in fetal neuroepithelial cells.

In this study, the role of β-catenin mediated Wnt signalling in the regulation of *Sox2* expression was also investigated, as this pathway has been recently implicated in adult hippocampal stem cell maintenance *in vitro* (Wexler et al., 2009). Putative binding sites for LEF/TCF transcription factors in the 5.5 kb *Sox2* promoter region were found and it could be shown that β-catenin-mediated signalling is able to increase endogenous SOX2 protein levels. These findings are in line with studies in retinal cells which show that inhibition of Wnt signalling results in reduced expression levels of *Sox2* (Van Raay et al., 2005; Das et al., 2006). Agathocleous and colleagues also found Wnt signalling to be involved in the control of *Sox2* expression in retinal progenitors (Agathocleous et al., 2009). In their model of retinal progenitor cell maintenance, Wnt signalling activates *Sox2* expression in progenitor cells, which in turn counteracts proneural gene expression. Taken together, *Sox2* expression seems to be under the control of a β-catenin mediated signalling pathway in neural stem/progenitor cells.

Synergistic activation of *Sox2* expression was observed after simultaneous stimulation of Notch and Wnt signalling pathways compared to the effects evoked by separate stimulation of the pathways. This result would suggest some form of cross-talk between both pathways. Most of the findings on cross-talk between the Notch and Wnt pathway suggest that these pathways fulfil opposing actions (Axelrod et al., 1996; Uyttendaele et al., 1998). However, studies in D. melanogaster found that both pathways synergistically activate gene transcription (Couso et al., 1995; Wesley, 1999), which is in line with the observed synergistic activation of *Sox2* expression by Notch and Wnt signalling.

Surprisingly, the analysis of B6.Cg-Tg(BAT-lacZ)3Picc/J mice, i.e. reporter animals for the canonical Wnt pathway, revealed that canonical Wnt signalling, indicated by reporter activity, is only active in a subset of SOX2 positive cells and SOX2 GFAP double positive radial glia like stem cells. The fact, that there is only limited overlap between the canonical Wnt/β-catenin reporter, whose activity relies on cooperative binding of TCF/LEF and β-catenin, and SOX2 expressing cells could be explained by TCF/LEF-independent β-catenin signalling. Recently, TCF/LEF-independent Wnt/β-catenin signalling has been implicated in the control of differentiation of epidermal stem cells (Pálmer et al., 2008). Future studies have to determine whether β-catenin-activated *Sox2* expression depends on TCF/LEF transcription factors or not, for example by analyzing *Sox2* expression after simultaneous overexpression of β-catenin and dominant-negative TCF/LEF proteins in adult hippocampal stem cells.
Previous data has shown that canonical TCF/LEF-dependent Wnt signalling will strongly promote neuronal fate determination/differentiation (Lie et al., 2005). These findings would suggest that TCF/LEF-dependent β-catenin signalling would promote differentiation of NSCs whereas TCF/LEF-independent β-catenin signalling would contribute to the expression of *Sox2* and the maintenance of an undifferentiated state.

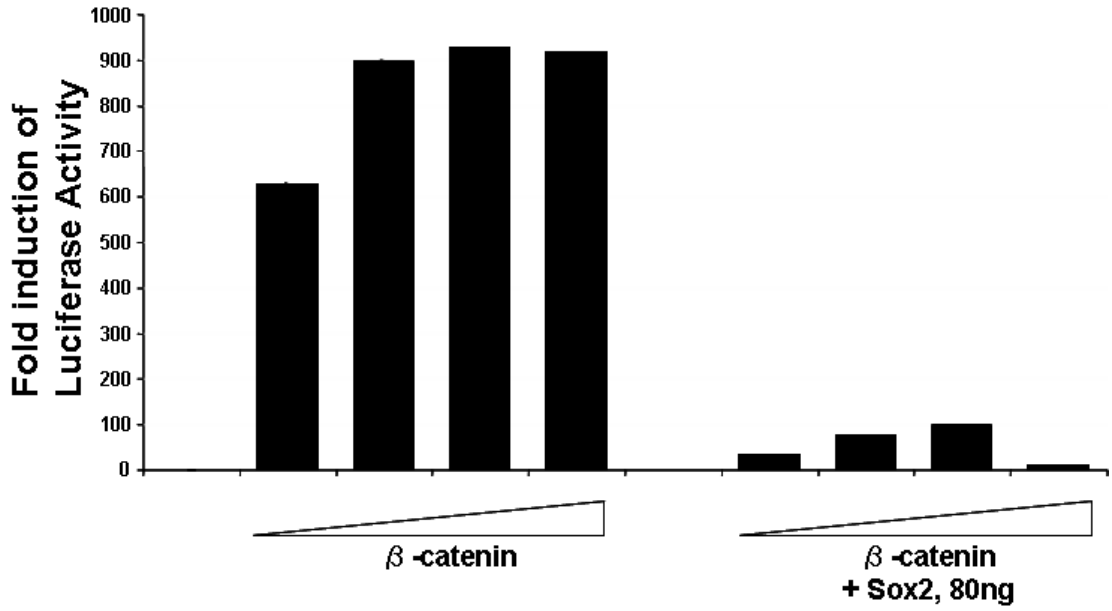

Figure 24: Sox2 interferes with canonical Wnt signalling. Overexpression of SOX2 decreases the β-catenin-induced activation of the TOPFlash luciferase, suggesting that SOX2 interferes with canonical Wnt signalling. Figure kindly provided by Dr. Lucia Berti.

Therefore, it was investigated whether SOX2 may interfere with TCF/LEF-mediated canonical Wnt signalling to counteract differentiation.

Co-transfection of adult hippocampal neural stem/progenitor cells *in vitro* with *Sox2* decreased the β-catenin induced activation of the Super8xTOPFlash luciferase reporter (Veeman et al., 2003) (Berti L, unpublished results, Figure 24), which exhibits a firefly-luciferase gene under the control of eight copies of TCF/LEF binding sites. It can therefore act as a reporter for canonical β-catenin mediated Wnt signalling.

These results suggest that SOX2 has the capacity to interfere with β-catenin mediated Wnt signalling and may inhibit the expression of canonical Wnt signalling targets.

Additionally, it was determined whether SOX2 directly interferes with β-catenin signalling. To this end, co-immunoprecipitations were performed in adult hippocampal neural stem/progenitor cells with antibodies raised against SOX2 or β-catenin, respectively. SOX2 could be pulled down and in subsequent Western blot analyses β-catenin could be detected (Figure 25a).

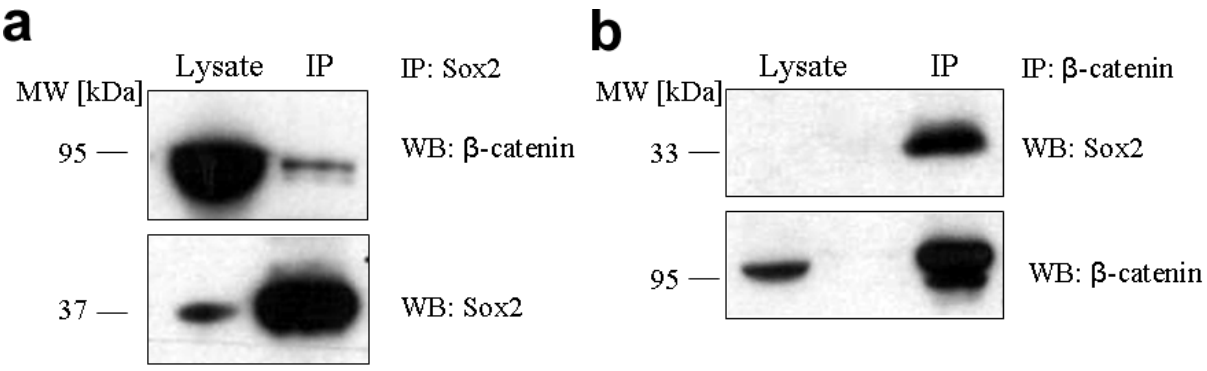

Figure 25: Sox2 and β-catenin interact in adult hippocampal neural stem cells. Co-immunoprecipitations suggest that Sox2 and β-catenin may directly bind to each other in adult NSCs. **(a)** Immunoprecipitation for Sox2 and concomitant Western blot for β-catenin and Sox2. **(b)** Immunoprecipitation for β-catenin and concomitant Western blot for Sox2 and β-catenin.

After β-catenin has been pulled down, SOX2 was detected in Western blot analyses (Figure 25b).

These results suggest that SOX2 directly binds to β-catenin and potentially inhibits β-catenin mediated Wnt signalling. This finding is supported by previous studies (Zorn et al., 1999; Kan et al., 2004; Mansukhani et al., 2005) and suggests that SOX2 is counteracting differentiation by direct binding to β-catenin and hindering it to

associate with TCF/LEF transcription factors on target promoters, thereby inhibiting the expression of Wnt signalling regulated proneural genes.

The notion that SOX2 counteracts canonical TCF/LEF-mediated Wnt signalling is supported by a recent study that analyzed the regulation of the proneural transcription factor NeuroD expression, which controls differentiation/ neuronal fate determination in hippocampal neurogenesis. The expression of NeuroD is controlled by sequences in the *NeuroD* promoter which are recognized by TCF/LEF transcription factors and SOX2 (Kuwabara et al., 2009). Activation of Wnt/β-catenin signalling drives neuronal fate commitment of neural stem cells in the adult hippocampal neurogenic niche by stimulating β-catenin TCF/LEF signalling and activation of *NeuroD* transcription. Kuwabara and co-workers have also shown that SOX2 competes with β-catenin for binding to SOX2/LEF sites in the *NeuroD1* promoter (Kuwabara et al., 2009). In neural stem cells, which do not express *NeuroD*, the authors found SOX2 to be bound to the *NeuroD1* promoter on these overlapping SOX2/LEF sites whereas in immature neurons, SOX2 was displaced by β-catenin on these sites, suggesting that SOX2 inhibits *NeuroD1* expression in neural stem cells. These results raise the possibility that Notch signalling promotes the expression of SOX2 which in turn competes with β-catenin mediated LEF-dependent Wnt signalling in neural stem cells and hinders β-catenin to activate its target genes.

Taken together, these results suggest that Notch/RBPJκ/Sox2 pathways and Wnt/β-catenin/NeuroD1 signalling cascades play important roles in the regulation of the balance between neural stem cell maintenance and neural fate commitment in the adult hippocampal neurogenic niche. Loss of Notch/RBPJκ signalling would lead to decreased *Sox2* expression levels and would result in increased levels of free, SOX2-unbound stabilized β-catenin, which in turn could activate the expression of differentiation genes like *NeuroD1* (Figure 26). These results also indicate that SOX2 seems to inhibit β-catenin mediated differentiation at various levels.

This study provides novel evidence that the Wnt and Notch signalling pathways cooperatively promote the expression of the endogenous *Sox2* gene in adult hippocampal neural stem cells. These results are supported by recent findings in fetal neural stem cells which implicated the cooperative action of Notch/RBPJκ and β-catenin signalling in the regulation of *Hes1* expression and inhibition of differentiation (Shimizu et al., 2008). Furthermore, Notch and Wnt signalling have been linked with *Sox2* expression and the maintenance of Müller glia in the mammalian retina (Das et

al., 2006). Future studies should aim to identify downstream targets of SOX2, as SOX2 seems to play an important role in adult hippocampal stem cell maintenance and inhibition of differentiation.

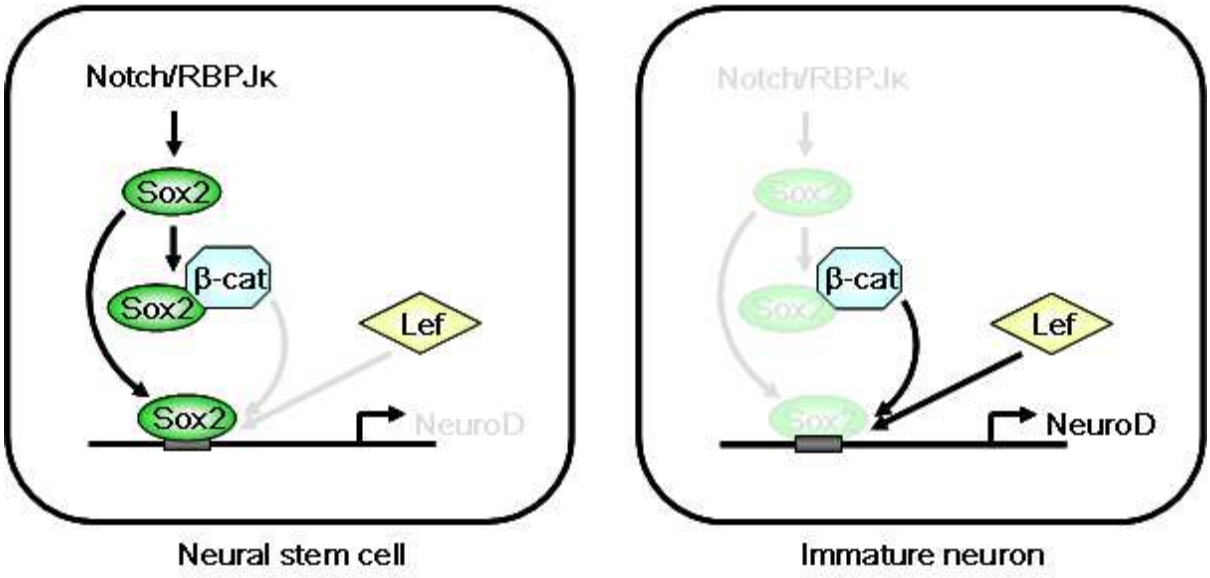

Figure 26: Proposed model for the regulation of *NeuroD* by the Notch/RBPJκ - Sox2 pathway and β-catenin/Lef signalling in neural stem cells and immature neurons. Notch/RBPJκ signalling promotes *Sox2* expression in neural stem cells. SOX2 counteracts transcription of the neuronal differentiation gene *NeuroD* through different mechanisms. SOX2 binds to overlapping SOX2/LEF sites (grey box) in the *NeuroD* promoter thereby preventing the binding of β-catenin and LEF transcription factors and initiation of *NeuroD* transcription. Additionally, SOX2 directly interacts with β-catenin thereby sequestering it and preventing *NeuroD* expression. In immature neurons, Notch/RBPJκ signalling is inactive and consequently *Sox2* is not expressed. The absence of SOX2 allows β-catenin and Lef transcription factors to initiate *NeuroD* expression and progression of differentiation.

Another level of interaction between these two pathways could be the induction of expression of ligands. Wnt signalling has been shown to activate Jagged1-induced Notch signalling through β-catenin in colorectal cancers (Rodilla et al., 2009). Intriguingly, Jagged1 has been shown to play a central role in the NSC niche in the SVZ for maintaining a population of NSCs in the SVZ during the early postnatal period (Nyfeler et al., 2005). Additionally, Jagged1 is also expressed in adult hippocampal neurogenic niche, raising the possibility that at least the effect of Notch signalling on *Sox2* expression and hippocampal stem cell maintenance may be induced by Jagged1. Further analysis should aim to identify the Notch ligand expressing cells and reveal how Wnt ligands are regulated in the adult dentate gyrus.

6.5 Notch signalling, *Hes* genes and the control of quiescence and astrocytic properties in stem cells

Sox2 could be identified as a downstream target of Notch/RBPJκ signalling in adult neural stem cells. Because of the crucial role of *Sox2* for stem cell maintenance (Favaro et al., 2009) we propose that inhibition of the Notch/RBPJκ/Sox2 pathway is a major contributor to the loss of stem cells. It seems however unlikely that *Sox2* is the only essential target of Notch/RBPJκ signalling in neural stem cell maintenance. *Sox2* has been mainly linked to the proliferative capacity of neural stem cells (Ellis et al., 2004; Favaro et al., 2009) and the maintenance of the undifferentiated state (Kuwabara et al., 2009). The role of *Sox2* in maintenance of a quiescent or slowly proliferative state is unknown. In this regard it is interesting to mention that RBPJκ-mediated Notch signalling controls quiescence of ependymal cells in the adult brain and that members of the *Hes* family of transcription factors, namely *Hes1*, have been found to control quiescence in primary cells and tumor cells *in vitro* (Sang et al., 2008). *Hes1* and the closely related Transcription factor *Hes5* are Notch RBPJκ target genes. They are prominently expressed in the adult neurogenic niches (Stump et al., 2002; Crews et al., 2008) and have been implicated in the control of neural stem cell maintenance during embryonic development (Ishibashi et al., 1995; Ohtsuka et al., 1999; Hatakeyama et al., 2004; Basak and Taylor, 2007). These observations raise the intriguing possibility that RBPJκ-mediated Notch signalling may contribute to the control of quiescence in adult hippocampal neural stem cells through the regulation of Hes1 expression. In line with this notion, RBPJκ and NICD have been found on the *Hes1* promoter in adult hippocampal stem cells (Covicova and Lie, unpublished results).

FoxO signalling has been linked with the control of quiescence in haematopoietic stem cells (Tothova and Gilliland, 2007; Tothova et al., 2007). Furthermore, Foxo signalling has been shown to positively regulate cell cycle inhibitors like p21 (Rathbone et al., 2008). Recently, FoxO1 has been shown to interact with Notch signalling in the inhibition of myoblast differentiation (Kitamura et al., 2007). Interestingly, FoxO1 was bound to RBPJκ and recruited to the *Hes1* promoter. This interaction was required for Notch induction of *Hes* genes via RBPJκ. As different FoxO proteins, including FoxO1, are expressed in the adult hippocampal neurogenic niche (Hoekman et al., 2006), it would be interesting to analyze whether this interaction between Notch/RBPJκ and FoxO proteins can be found in neural stem

cells. Indeed, preliminary results suggest that protein complexes containing both Foxo and RBPJκ can be found in neural stem cells of the neurogenic niches in the adult brain (Schwarz and Lie, unpublished results). Intriguingly, signalling cascades involving Forkhead box O (*FoxO*) transcription factors have been implicated in the regulation of hormonal, nutrient, and stress responses (Tothova and Gilliland, 2007; Tothova et al., 2007). Hence, the potential interaction of RBPJκ and FoxO in the regulation of neural stem cell maintenance may provide a signalling module which controls the response of neural stem cells to changes in hormonal, nutrient and stress signals.

Adult hippocampal neural stem cells exhibit besides their quiescence and multipotency several characteristics of astrocytes (Seri et al., 2001). Several studies linked Notch signalling with the induction of gliogenesis (Turner and Cepko, 1987; Hojo et al., 2000; Morrison et al., 2000; Wang and Barres, 2000; Gaiano and Fishell, 2002). Gaiano and co-workers retrovirally overexpressed Notch1 and a marker gene in the embryonic SVZ and found that radial glia in the fetal brain became labelled by the marker gene. Many of the transduced cells became SVZ astrocytes with stem cell characteristics postnatally (Gaiano et al., 2000). Moreover, a Reelin/Notch1/RBPJκ signalling cascade has previously been shown to promote a radial glial phenotype in embryonic neural progenitor cells through the induction of brain lipid binding protein (BLBP) expression (Keilani and Sugaya, 2008). These results indicate that Notch signalling is also involved in conferring the astrocytic character of stem cells in the adult neurogenic niches.

6.6 Role of Notch signalling in mature granule neurons

Previous research has revealed an involvement of the canonical Notch signalling pathway in cortical neurite growth and neuronal maturation during embryonic development (Franklin et al., 1999; Sestan et al., 1999; Redmond et al., 2000). A more recent study linked Notch signalling with dendritic arborisation of maturing neurons in the adult hippocampus (Breunig et al., 2007). EGFP activity was found in scattered cells throughout the granule cell layer in dentate gyrii of *Rbpjκ-EGFP* reporter animals, indicating that RBPJκ signalling is active in mature granule neurons. Furthermore, most of the recombined YFP-expressing cells in homozygous conditional *Rbpjκ* knockout animals were positive for the neuronal marker NeuN,

which is predominantly expressed by mature neurons. Virtually no overlap was detected between the immature neuronal marker DCX and NeuN, suggesting that *Rbpjκ*-deficient neurons can reach a mature stage. However, whether these neurons exhibit a fully developed dendritic tree, receive synaptic input and are integrated into the hippocampal network requires further validation. Future experiments should analyze, whether these essential steps are controlled by a Notch/RBPJκ dependent mechanism.

6.7 Notch signalling, hippocampal function and aging

Notch signalling has been linked to learning and memory abilities (Yu et al., 2001; Costa et al., 2003; Wang et al., 2004). It has been shown that loss of one allele of *Notch1* results in deficits in spatial learning and memory without affecting other forms of learning, motor control or exploratory activity (Costa et al., 2003). Interestingly, it was also found that loss of one allele of the downstream mediator RBPJκ result in similar specific spatial learning and memory deficits. The present study linked Notch/RBPJκ signalling to the maintenance of neurogenesis. Given the role of neurogenesis in cognition, learning and memory, it will be interesting to investigate whether the cognitive deficits in heterozygous *Rbpjκ/Notch1* knockout mice are the consequence of impaired neurogenesis.

Hippocampal neurogenesis has also been correlated with hippocampal function (Drapeau et al., 2003). Conditions increasing hippocampal neurogenesis seem to enhance learning and memory abilities. Consequently, the strong decline in the generation of new dentate granule neurons in the aged hippocampus is thought to contribute to the age-related hippocampal dysfunction. Previous *in vitro* analysis on stem cells frequencies (Walker et al., 2008), histological studies in primates (Aizawa et al., 2009), and analysis by magnetic resonance spectroscopy (Manganas et al., 2007) have revealed that the stem cell pool in the aged dentate gyrus is reduced, suggesting that impaired neural stem cell maintenance may play a role in the age-related decline in hippocampal neurogenesis and function. Given the fact that Notch/RBPJκ signalling is important for adult hippocampal stem cell maintenance, these findings raise the intriguing possibility that constitutive and progressive loss of Notch/RBPJκ signalling pathway activity may contribute to the age-related impaired maintenance of the hippocampal stem cell pool which may cause the decrease in

cognitive function in the aged brain. Future experiments should analyze Notch/RBPJκ pathway activity in the aged hippocampus of, for example, Tg(Cp-EGFP)25Gaia canonical Notch reporter mice. Recent findings by Crews and co-workers support the notion that Notch/RBPJκ activity is altered in the aged hippocampus and might contribute to the age-related cognitive decline (Crews et al., 2008). The authors analyzed A53T α-synuclein transgenic mice, a model for age-related neurodegeneration, and reported a pronounced reduction in hippocampal neurogenesis. Interestingly, the decrease in the number of newly generated hippocampal granule neurons was accompanied by a significant reduction in *Notch1* expression, by reduced levels of nuclear NICD and by decreased *Hes5* expression. This raises the question whether (re)activation of Notch signalling in neural stem cells by overexpression of Notch ligands in the aged hippocampus may be a promising way to prevent the age-related cognitive deficits and maintain proper hippocampal function. Additionally, further experiments are needed to analyze the behavioural phenotype of animals, which exhibit perturbations of Notch/RBPJκ activity in the adult hippocampal neurogenic niche.

Potent promoters of aging are reactive oxygen species (ROS), by-products of oxygen metabolism that cause macromolecular damage to DNA and proteins and have been implicated in neurodegenerative diseases (for review see (Emerit et al., 2004)). Bheeshmachar and colleagues have shown that blocking Notch signalling in T cells leads to the accumulation of ROS and results in decreased T cell survival (Bheeshmachar et al., 2006). The small molecule *Sirtuin 1* (*Sirt1*), a protein deacetylase, has been linked to oxygen metabolism, cellular stress response and aging (Han et al., 2008). Sirt1 has been found to be important for mouse embryonic stem cell maintenance *in vitro* (Han et al., 2008) and has been shown to directly bind to the Notch target gene *Hes1* (Takata and Ishikawa, 2003) and inhibit neuronal differentiation of adult neural stem cells by repression of the expression of the proneural gene *Mash1* (Teng et al., 2009). Taken together, these findings suggest a link between Notch signalling and ROS-mediated aging processes and suggest that loss of Notch signalling does not only lead to the loss of adult neural stem cells and consequent loss of neurogenesis, but may also directly contribute to aging processes and cognitive impairments.

The results of the studies discussed above and the findings presented in this work suggest that the analysis of Notch/RBPJκ pathway activity during aging could help

define new therapeutic avenues for the treatment of age-related cognitive decline. Understanding the molecular mechanisms which are essential for the maintenance of proper hippocampal function is the prerequisite to influence these mechanisms and thereby prevent or slow down age-related cognitive decline.

7 Abbreviations

Akt	Protein kinase B
Amp^R	Ampecillin-resistence
APS	Ammonium persulfate
bHLH	Basic helix-loop-helix
Bmi-1	Polycomb ring finger oncogene
bp	Basepair(s)
BrdU	Bromodesoxy uridin
BSA	Bovine serum albumin
c	Centi (10^{-2})
℃	Degrees Celsius
Cdk	Cyclin dependent kinase
cDNA	copy DNA
ChIP	Chromatin immuno precipitation
Ci	Curie
Cir	Corepressor interacting with RBPJ
CNS	Central nervous system
Cntf	Ciliary neurotrophic factor
CTBP	C-terminal binding protein
CtIP	C-terminal interacting protein
Dab1	Disabled 1
DAPI	4′,6-Diamidino-2-phenylindol
Dll	Delta-like
DNA	Desoyxribonucleic acid
(d)ntP	(Deoxy) nucleotide triphosphate
DOC	Deoxycholic acid
dpc	Days post coitum
dpm	Decays per minute
DCX	Doublecortin
Dsh	Disheveled
DTT	Dithiothreitol
E	Embryonic day
E.coli	Escherichia coli

EDTA	Ethylendiamintetraacetat
EGF	Epthelial growth factor
EGTA	Ethylene glycol tetraacetic acid
EMSA	Electrophoretic mobility shift assay
et al.	et alteri
FGF	Fibrillary growth factor
FITC	Fluorescein
Foxo	Forkhead box O
Fzd-receptor	Frizzeled-receptor
g	Gram(s), gravitation
GABA	γ-Aminobutyric acid
GFAP	Glial fibrillary acidic protein
(E)GFP	(enhanced) green fluorescent protein
Glast	Glutamate aspartate transporter
GSK3β	Glykogen synthetase kinase 3 β
HAT	Histone acetyle transferase
HD	Heterodimerization domain
HDAC	Histone deacetylases
Hek cell	Human embryonic kidney cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hes	Hairy and enhancer of Split
HMG box	High monility group box
IRES	Internal ribosomal entry side
Id	Inhibitor of differentiation
Jag	Jagged
kD	Kilo Dalton
l	Liter
LB	Luria Bertani
LEF	Lymphoid enhancer factor
Lif	Leukemia inhibitory factor
LV	Lateral ventricle
M	Molar, mol
m	Milli (10^{-3})

µ	Micro (10^{-6})
Mash1	Achaete-scute complex-like 1
MCS	Multiple cloning site
MES	2-(N-morpholino)ethanesulfonic acid
min.	Minute(s)
mNSC	Neural stem cells from mouse
mTOR	Mammalian target of rapamycin
Na-But	Sodium butyrate
Ncor	Nuclear receptor corepressor
NICD	Notch intracellular domain
Ngn	Neurogenin
NRR	Negative regulatory region
nt	Nucleotides
Oct	Octamer binding protein
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly [ADP-ribose] polymerase
PBS	Phosphate buffered saline
Pcaf	P300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaktion
Pest domain	Proline (P), glutamic acid (E), serine (S) and threonine (T) rich domain
PFA	Paraformaldehyd
pH	Potentium hydrogenii
PSA-NCAM	Polysialic-acidic-neural cell adhesion molecule
Ptf1α	Pancreas transcription factor 1 α
Ram domain	RBPJκ-associated module
RBPJκ	Recombination signal binding protein
RMS	Rostral migratory stream
Ros	Reactive oxygen species
rpm	Rounds per minute

RNA	Ribonucleic acid
RT	Room temperature
Sap30	Sin3A-associated protein
SDS	Sodium dodecyl sulfate
SGZ	Subgranular zone
SHARP	Smart/hdac1 associated repressor protein
Shh	Sonic hedgehog
Sirt1	Sirtuin 1
SMRT	Silencing Mediator for Retinoic acid and Thyroid hormone receptor
SRR	Sox regulatory region
Stat3	Signal transducer and activator of transcription
SVZ	Subventrikular zone
TAD	Trans-activation domain
TCF	T-cell factor
TE	Tris EDTA
TEMED	N,N,N´,N´-Tetramethylethylendiamin
TGF	Transforming growth factor
tle/grg	Transducin-like enhancer of split / groucho related gene
TLX	Tailless
Tris	Tris-(hydroxymethyl-) aminomethan
TBS	Tris buffered saline
Tween-20	Polyoxyethlensorbitanmonolaurat
V	Volt
(v/v)	Volume to volume
Wnt	Wingless
WT	Wildtype
(w/v)	Weight to volume
Y	Tyrosin
YFP	Yellow fluorescent protein

8 Literature index

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10 Erklärung

Ich erkläre an Eides statt, dass ich die der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Examining the role of Notch signalling in adult hippocampal stem cell maintenance

Am Lehrstuhl für Genetik unter der Anleitung und Betreuung durch

Univ.-Prof. Wolfgang Wurst

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 5 angegebenen Hilfsmittel benutzt habe.

- Ich habe die Dissertation in dieser oder ähnlicher Form in keinem anderen Prüfungsverfahren als Prüfungsleistung vorgelegt.

- Ich habe den angestrebten Doktorgrad **noch nicht** erworben und bin **nicht** in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

- Die Promotionsordnung der Technischen Universität München ist mir bekannt.

München den,…… ……

11 Lebenslauf

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