Role of Sp transcription factors in adult neurogenesis

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Table of contents

1 Zusammenfassung........................................................................................................ 1

2 Abstract .......................................................................................................................... 3

3 Introduction ..................................................................................................................... 5
  3.1 Adult neurogenesis and neurogenic niches of the adult brain ........................................ 5
    3.1.1 Neurogenesis in the adult dentate gyrus ................................................................. 5
    3.1.2 Neurogenesis in the adult subventricular zone ....................................................... 8
  3.2 Sp family transcription Factors ................................................................................... 10
    3.2.1 Sp1 ......................................................................................................................... 11
    3.2.2 Sp3 ......................................................................................................................... 11
    3.2.3 Sp4 ......................................................................................................................... 13
  3.3 FHOD1 as a possible downstream target of Sp3 ......................................................... 17
  3.4 Objective of this study ............................................................................................... 18

4 Materials .......................................................................................................................... 20
  4.1 Chemicals, media and supplements ............................................................................. 20
  4.2 Commercial kits .......................................................................................................... 21
  4.3 Antibodies .................................................................................................................. 21
  4.4 Primers and oligonucleotides ..................................................................................... 22
  4.5 Organisms .................................................................................................................. 23
  4.6 Plasmids .................................................................................................................... 23
  4.7 Buffers and solutions .................................................................................................. 23
  4.8 Other materials ......................................................................................................... 26
  4.9 Equipment .................................................................................................................. 27

5. Methods .......................................................................................................................... 28
  5.1 Cell culture methods ................................................................................................... 28
    5.1.1 Culturing of HEK 293T cells .................................................................................... 28
    5.1.2 Passaging HEK 293T cells ..................................................................................... 28
    5.1.3 Transient transfection of HEK 293T cells ............................................................... 28
  5.2. RNA methods ........................................................................................................... 28
    5.2.1 RNA isolation from brain tissue ............................................................................. 28
    5.2.2 DNase treatment ..................................................................................................... 28
    5.2.3 cDNA synthesis ..................................................................................................... 29
5.2.4 Quantitative real time PCR (QPCR) ................................................................. 29

5.3 Protein methods .................................................................................................. 29
  5.3.1 Isolation of proteins ....................................................................................... 29
  5.3.2 Western blot analysis .................................................................................... 30

5.4 Cloning methods .................................................................................................. 31
  5.4.1 Cloning of microRNA constructs ................................................................. 31
  5.4.2 Transformation of bacteria .......................................................................... 32
  5.4.3 Plasmid DNA isolation ................................................................................. 32

5.5 Knockdown experiments ...................................................................................... 32
  5.5.1 Virus production .......................................................................................... 32
  5.5.2 Stereotactic injections .................................................................................. 33
  5.5.3 Animals ......................................................................................................... 34
  5.5.4 Perfusions .................................................................................................... 34

5.6 Immunoflorescence experiments ........................................................................ 35
  5.6.1 Preparation of brain slices .......................................................................... 35
  5.6.2 Immunohistochemistry ................................................................................. 35
  5.6.3 Immunocytochemistry ................................................................................ 35
  5.6.4 Confocal microscopy ................................................................................... 36

5.7 Analysis of phenotypes ....................................................................................... 36

6 Results .................................................................................................................. 38

6.1 Sp3 and Sp4 but not Sp1 are expressed in the dentate gyrus of adult brain ........ 38
  6.1.1 Sp3 is continuously expressed during adult hippocampal Neurogenesis ...... 40
  6.1.2 Sp4 expression is initiated in later stages of adult hippocampal neurogenesis .... 43

6.2 Sp3 might be involved in maturation of newborn neurons in DG .................... 45
  6.2.1 Knockdown of Sp3 in adult newborn neurons of dentate gyrus ..................... 45
  6.2.1.1 Efficiency of Sp3 micro RNA in vitro and in vivo ...................................... 45
    6.2.1.2 Knock down of Sp3 in newborn neurons in the dentate gyrus increases dendritic length and branching at 14dpi ................................................................. 47
    6.2.1.3 Increase in the dendritic length and branching persists at 28dpi in Sp3 knockdown cells ............................................................................................................. 51
    6.2.1.4 Expression of stage specific markers reveals accelerated maturation in immature neurons upon knockdown of Sp3 ................................................................. 53
  6.2.2 FHOD1 might be a downstream target of sumoylated Sp3 in adult DG ....... 56
6.2.2.1 Transcriptome profiling reveals differentially regulated genes in the DG of sumoylation deficient Sp3kiki mice .............................................................. 56

6.3 Sp4 might be involved in migration and dendritic development of newborn neurons during adult neurogenesis ................................................................. 58

6.3.1 Knockdown of Sp4 in adult newborn neurons of dentate gyrus ................. 58

6.3.1.1 Efficiency of Sp4 micro RNA in vitro and in vivo .................................. 58

6.3.1.2 Loss of Sp4 in newborn neurons increases dendritic length but not dendritic branching at 14dpi ............................................................. 60

6.3.1.3 Dendritic length and branching does not differ upon Sp4 knockdown at 28dpi .................................................................................................................. 62

6.3.1.4 Knockdown of Sp4 delays the migration of newborn neurons along granule cell layer of dentate gyrus ......................................................... 64

6.3.1.5 Knockdown of Sp4 does not affect survival of newborn neurons between 14 and 28dpi ............................................................................................................. 66

6.3.1.6 Knockdown of Sp4 delays the migration of newborn neurons in the SVZ–RMS- OB neurogenesis in adult brain at 5dpi .............................................. 67

7 Discussion ................................................................................................................................. 74

7.1 Regulation of adult neurogenesis ..................................................................................... 74

7.2 Potential impact of Specificity Proteins on adult hippocampal neurogenesis ........ 75

7.3 Expression pattern of Specificity Proteins in adult hippocampal neurogenesis....... 77

7.4 Identification of specificity proteins as regulators of dentritic development during adult hippocampal neurogenesis ................................................................. 79

7.5 Impact of Specificity proteins on migration during adult hippocampal neurogenesis.. 84

7.6 Role of Specificity Proteins in human disease ................................................................. 88

8 Literature index.......................................................................................................................... 93

9 Acknowledgements.................................................................................................................. 103

10 Erklärung................................................................................................................................ 104

11 Curriculum Vitae ...................................................................................................................... 105
For my parents
1. ZUSAMMENFASSUNG


von Sp4 in der neuronalen Migration während der adulten Neurogenese hin. Die Literatur weiß auf eine Rolle der Sp-Transkriptionsfaktoren in Neuroentwicklungserkrankungen, neurodegenerativen und neuropsychiatrischen Erkrankungen einschließlich der Depression und der Schizophrenie hin.
Die Ergebnisse dieser Studie können dazu beitragen, die molekularen Mechanismen der molekularen und zellulären Pathologie in diesen Erkrankungen zu verstehen.
2. ABSTRACT

Adult neurogenesis is defined as the generation of new functional neurons from neural stem cells in the environment of adult brain. There are two neurogenic regions in the adult rodent brain: subgranular zone (SGZ) of the dentate gyrus and subventricular zone (SVZ) of the lateral ventricles. Stem cells of these pools give rise to newborn neurons that are able to differentiate and integrate into the existing neuronal network of the adult brain. Genetic programs that are involved during this process of neuronal development are important to understand the regulation of adult neurogenesis. Transcription factors are one of the main regulators during the development of newborn neurons in the adult dentate gyrus. In this study, I investigated the role of Sp transcription factor family in the regulation of adult neurogenesis.

Sp transcription factors are known as Specificity Proteins (also Trans Acting Transcription Factors) which include three major members called Sp1, Sp3 and Sp4. Results of this study showed that Sp3 and Sp4 are expressed in the dentate gyrus of the hippocampus in adult mice. Constant Sp3 expression is observed in all stages of neurogenesis process, from early progenitors till differentiated neurons. On the other hand, Sp4 was found to be not expressed in the early stages, but started to get expressed with neuronal differentiation in later stages of neurogenesis.

In addition, using retrovirus mediated knockdown approach; I conducted loss of function studies in the newborn neurons of the dentate gyrus for both Sp3 and Sp4 in vivo. Results of knockdown experiments suggest a similar role for both Sp3 and Sp4 being involved in the regulation of dendritic development during adult hippocampal neurogenesis. Interesting findings however include that knockdown of Sp3 affected both dendritic length and branching whereas knockdown of Sp4 affected only dendritic length but not branching at 14 days after the injection of retrovirus coding the siRNA for either Sp3 or Sp4. According to our knowledge, our study is the first which describes a discrimination in the regulation of dendritic length vs dendritic branching in adult neurogenesis. Another interesting finding is that upon Sp4 knockdown, newborn neuron migration is delayed both in SGZ and in SVZ neurogenesis, which suggests an additional role for Sp4 in neuronal migration during adult neurogenesis.

Considering the literature that is pointing the association of Sp transcription factors with neurodevelopmental / neurodegenerative diseases / disorders including major depression and
schizophrenia, the results of this study also contribute to understand the molecular mechanisms underlying the molecular and cellular pathologies in these disorders.
3. INTRODUCTION

3.1. Adult neurogenesis and neurogenic niches of the adult brain

Within the established structure of the adult brain, there are specific regions that serve as neural stem cell pool. New neurons are constantly being created from this stem cell pool throughout life. The process of generating new functional neurons from neural stem cells in the environment of adult brain is known as “adult neurogenesis”. There are two neurogenic regions in the adult rodent brain; subgranular zone (SGZ) of the dentate gyrus and subventricular zone (SVZ) of the lateral ventricles (Colucci-D.Amato et al. 2006, Ming et al. 2011).

3.1.1. Neurogenesis in the adult dentate gyrus

Dentate gyrus (DG) is a sub-structure within the hippocampal formation. Densely packed granule neurons form a v-shaped structure that is called dentate granule cell layer (GCL) and surrounds the hilus between the blades. The neurogenic zone in the DG lies beneath the granule cell layer, therefore named as subgranular zone (Zhao et al. 2008). Radial glia-like stem cells are located in SGZ with their radial processes spanning the granule cell layer. In addition to the morphology, characteristic markers like Nestin, Sox2 and GFAP are used to distinguish these stem cells (Seri et al., 2001). Similar to development, generation of new neurons in the adult brain progresses through a number of stages. Radial and non-radial precursors produce progenitor population. Having a high proliferative capacity, progenitor cells are able to generate neuroblasts, majority of them expressing immature neuronal marker DCX. Neuroblasts undergo few rounds of cell division and then exit the cell cycle. At the neuroblast stage cells start to migrate into granule cell layer. Maturation of newborn neurons progresses with the establishment of the axon and dendrites, a stage that is no more proliferative (Reviewed in Ming et al, 2011). In early stages, progenitor cells are activated by tonic GABA, which is released by surrounding DG interneurons (Tozuka et al 2005, Ge et al. 2006). Maturation and integration of the newborn neurons to DG continues as they start to develop synaptic GABAergic input, which is followed by glutamatergic input and mossy fiber output in later stages of neuronal development (Esposito et al 2005, Toni et al 2007 and 2008). The formation of a functional neuron from the stem cells in the DG of adult mouse brain under physiological conditions takes approximately 7 weeks (Zhao et al. 2006).

Dorsal hippocampus is involved in learning and memory whereas ventral hippocampus is involved in affective behavior. Whether hippocampal neurogenesis is contributing to
hippocampal function has been tested by manipulating neurogenesis in the adult brain. Several studies have reported effects on spatial memory-related functions like spatial navigation-learning, spatial pattern discrimination and long term spatial memory retention (Stone et al 2011, Marin-Bugin et al 2012, Aimone et al 2011, Jessberger et al 2009, Deng et al 2010). Moreover, trace conditioning, contextual fear conditioning and clearance of hippocampal memory traces has been reported to be affected by the manipulation of adult hippocampal neurogenesis (Koehl et al 2011, Shors et al 2001, Deng et al 2010). Impairment of adult neurogenesis has been associated as well to antidepressant actions in several studies but this effect seems to be restricted to a certain type of antidepressants (Reviewed in Sahay et al 2007).
Figure 1: Adult hippocampal neurogenesis. Subgranular zone of the dentate gyrus is serving a niche for adult neural stem cells that is depicted as radial glia-like cell in (1). Radial glia like cells give rise to progenitor cells that are highly proliferative (2). Progenitor cells go out of the cell cycle and start expressing immature neuronal markers, a stage called neuroblast (3). Immature neurons start to develop neuronal properties like axons and dendrites (4). Maturation and synaptic integration occurs in the last stage of adult neurogenesis (5). ML: molecular layer; GCL: granule cell layer; SGZ: subgranular zone. (Modified from Ming and Song, 2011)
3.1.2. Neurogenesis in the adult subventricular zone

The other region where neurogenesis persists throughout life in the adult brain is subventricular zone of lateral ventricles. Radial glia-like stem cells reside close to the ventricle wall in SVZ. Different from the neuroblasts in DG where the cells are migrating a relatively short distance, newborn neuroblasts in SVZ migrate over a long distance towards olfactory bulb on a route termed rostral migratory stream (RMS) (Lois et al. 1996). The expression of immature neuronal marker DCX starts with the neuroblast stage and lasts until the cells reach the OB as immature neurons. This part of the migration in the RMS is called “chain migration”. When the cells reach the OB, “radial migration” takes place from the center of the OB towards glomeruli and the neurons finally mature and integrate to the network of olfactory bulb as GABAergic granule neurons or GABAergic periglomerular neurons (Lledo et al. 2006).

SVZ-OB neurogenesis is necessary for long-term structural maintenance of the olfactory bulb. In addition, short-term olfactory memory and certain pheromone-induced behaviors have been reported to be associated by SVZ-OB neurogenesis (Lazarini et al. 2011, Feierstein et al. 2010).
Figure 2: Adult olfactory bulb neurogenesis. Subventricular zone of the lateral ventricles is the second neurogenic region in the adult brain. Neural stem cells are lying in the subventricular zone and called as radial glia-like cells, which are quiescent (1). Radial glia like cells give rise to transiently amplifying cells (2). Highly proliferative population of neuroblasts start to express immature neuronal markers (3). Neuroblast migration occurs from subventricular zone towards olfactory bulb, on a route called rostral migratory stream (4). When the cells reach olfactory bulb, they start to integrate to the existing circuitry as granule cells or periglomerular neurons (5). (Modified from Ming and Song, 2011)
3.2. Sp family transcription factors

Adult neurogenesis is strictly regulated by extrinsic and intrinsic factors. Extrinsic regulators include neurotransmitters, growth factors, cytokines, hormones, neurotrophins, and several signaling pathways including Notch, Wnts, BMPs and Shh. Intrinsic factors comprise certain transcription factors and epigenetic regulators. Transcription factors have the power to confer cell identity and to regulate stage specific genetic programs. Therefore, their study may help to understand intrinsic molecular mechanisms of neural stem cell development and stem cell regulation in the adult brain. Transcription factors can be expressed in a cell-, time-, space-, stage- or condition-dependent manner, which makes them interesting candidates for adult neural stem cell development. Several major transcription factor families have been reported to be involved in the regulation of adult neurogenesis so far, including bHLH transcription factors, Sox Family transcription factors, Foxo transcription factor family, CREB/ATF family transcription factors, and a number of them are waiting to be investigated. In this study, I investigated the role of Sp transcription factor family in adult hippocampal neurogenesis.

Sp Transcription factor family and Krüppel-like Factors (KLFs) are closely related transcription factors comprising three Cys$_2$His$_2$ zinc-finger motifs within their structure, a domain known to bind to GC-rich DNA sequences. The genome-wide abundance of GC rich sequences (i.e. CpG islands) on the promoters of the genes makes these transcription factors potential regulators for a wide range of genes. Sp originally stands for “Specificity Protein” and Sp transcription factors, known as “Trans-acting transcription factors”, include five closely related proteins named Sp1, Sp2, Sp3, Sp4, and Sp5 (Harrison et al. 2000). Although other members are reported, namely Sp6-Sp8, they do not possess sequence similarity to other members of Sp transcription factors therefore are not considered within Sp family in general. Sp1-Sp5 share a conserved DNA binding domain at the C terminus and Sp1, Sp2, Sp3, and Sp4 has a glutamine rich N terminal region which acts as transactivation domain in case of Sp1 (Suske 1999, Hagen 1994). Moreover, most closely related members of the Sp transcription factor family are Sp1, Sp3, and Sp4 according to the very high conservation of their nucleotide sequence among mammalian species. Although Hagen et al. (1992, 1994) reported that Sp1, Sp3 and Sp4 recognize and bind to the same consensus sequence, which is the GC box; more detailed analysis on different loci in the
past years showed that the affinity of different Sp transcription factors to GC boxes might differ, mostly due to the posttranslational modifications (Reviewed in Bouwman et al 2002).

3.2.1. Sp1

Sp1 is one of the first transcription factors cloned and characterized in mammalian species in 1980s. Expression of Sp1 is reported to be ubiquitous in murine tissues. Straight knockout studies in mice indicated non-overlapping functions to different Sp transcription factors. Sp1 knockout is lethal in mice and the embryos die at embryonic day 10. Although development of the heart, eye, and limbs appeared to be normal in some embryos, Marin et al reported a high phenotypic heterogeneity between Sp1 mutant embryos. Further analysis in chimeras showed elimination of Sp1-/- cells in chimeras. Therefore, lack of Sp1 is thought to cause a general cellular defect (Marin et al 1997). Numerous studies have reported elevated Sp1 expression levels in human cancers including breast cancer, thyroid carcinoma and pancreatic cancer (Wang 2011). Parallel to this finding, Shao and coworkers reported that Sp1 is regulatory by G1-specific cyclins, possessing a role in cell cycle progression (Shao 1995). Several downstream targets identified so far include cell cycle regulated genes like B-myb, thymidine kinase, and dihydrofolate reductase; oncogenes like vascular endothelial growth factor, urokinase plasminogen activator and its receptor; and tumor suppressor genes like p27kip1, p21WAF1/CIP1, p16INK4 and pp2a-c (Lin 1996, Wang 2011).

3.2.2. Sp3

In contrast to Sp1 deficient mice that die at embryonic stage, Sp3 knockout mice develop until birth but die shortly after birth due to respiratory failure (Marin 1997, Bouwman 2000). Expression of Sp3 is reported to be ubiquitous as well. In Drosophila SL2 cell line, which is an Sp-free system, Sp3 was acting as a clear repressor for an Sp1 induced reporter. Co-transfection of Sp1 together with Sp3 lacking the DNA-binding domain showed that DNA binding mutant of Sp3 cannot repress Sp1 mediated induction of the reporter. Since the DNA binding domain of Sp3 is necessary for repressive function of Sp3, competitive binding model has been postulated as repression mechanism. These results altogether suggest that Sp3 might act as a repressive member of the Sp transcription factor family in general and the repression effect is due to competition of Sp1 and Sp3 for the common binding sites on DNA (Hagen 1994).

One of the posttranslational mechanisms that is associated with the repressive function of transcription factors is SUMO (Small Ubiquitin-like Modifier) modification. Structural
investigation of Sp3 transcription factor revealed that lysine 551 residue, which lies within SUMO consensus motif IKEE, can be sumoylated. The sumoylation site lies close to the DNA binding domain of Sp3, therefore was suggested to be directly related to its repressive function. In order to investigate the role of Sp3 sumoylation in vivo, Stielow and coworkers created a knock in mouse model by changing the sumo attachment motif of the Sp3 from IK$^{551}$EE to IK$^{551}$ED. This change in the Sumo consensus site within the Sp3 has been shown to prevent sumoylation of the lysine residue in all three isoforms of Sp3 that are expressed in vivo. The homozygote Sp3 knock in mouse will be presented as Sp3ki/ki in the text (Stielow 2010).

Homozygote and heterozygote Sp3 knock in mice were reported to breed at expected Mendelian frequency and no obvious phenotype was detected. Further molecular analysis in Sp3ki/ki mice and mouse embryonic fibroblasts (MEFs) that are homozygous for the knock in allele showed alterations in gene expression as well as in chromatin state. Gene expression profiling of MEFs derived from E13.5 Sp3wt/wt and Sp3ki/ki littermates revealed upregulation of more than 60 genes and downregulation of 7 genes. It is worth to note that among upregulated genes, there are several regulators like Paqr6, Rims3 and Robo3 that are known to be expressed in the central nervous system. Thus, it makes the Sp3 ki/ki mouse model a suitable tool to further investigate possible sumoylated-Sp3 targets in the mouse brain (Stielow 2010).

Parallel to gene expression alterations, authors reported changes in the chromatin state of some of the corresponding gene promoters. Chromatin immuno-precipitation analysis showed that the promoters of Paqr6 and Rims3 are positive for the histone marks H3K9me3 and H4K20me3 in the wildtype MEFs but this signal is strongly reduced in Sp3ki/ki and Sp3-/- MEFs. The reduction of these repressive histone marks on the promoters of Paqr6 and Rims3 in the Spki/ki MEFs correlate with the increase in their expression level in these cells. Moreover, histone methyltransferase SETDB1, chromatin remodeler Mi-2, and MBT domain proteins L3MBTL1 and L3MBTL2 that are involved in chromatin compaction are found to be enriched on the promoters of Paqr3 and Rims3 but shown to be strongly reduced in Sp3ki/ki and Sp3-/- MEFs. Parallel to these findings, authors reported a strong decrease in the DNA methylation of these promoters in Sp3ki/ki MEFs compared to the wildtype MEFs. All these results strongly suggest that sumoylation of Sp3 is essential to recruit chromatin silencing machinery to the promoters of certain neuronal genes in MEFs (Stielow 2008, 2010).
Sumoylation of Sp3 was barely detectable in the mouse brain using brain extracts in western blot analysis, and authors reported no obvious expression changes of the neuronal genes like Paqr6, Rims or Robo3 in the mouse brain on RNA level. However, it has been postulated that detailed analysis needs to be done in the brain of Sp3ki/ki mice by isolating pure population of neurons to investigate neuronal gene expression changes more precisely (Stielow 2010).

![Figure 3: Model of SUMOylation dependent repression of chromatin by Sp3. Sumoylation of the Sp3 triggers the establishment of the heterochromatin by recruiting repressive chromatin markers Mi-2, MBT-domain proteins and histone methyltransferases including SETDB1 and SUV4-20H). Established heterochromatin structure is maintained by heterochromatic protein 1 (HP1). SUMO, small ubiquitin-like modifier. (Modified from Stielow et al, 2008).](image)

3.2.3. Sp4

Different from Sp1 and Sp3 that are ubiquitously expressed, Sp4 is known to be the brain- and heart- enriched member of the Sp transcription factor family in mammals. Additionally, it has been reported to be homologous to Drosophila buttonhead (btd) gene, which is expressed at the
head region during Drosophila development. In situ hybridization experiments in E10 mouse embryos detected the highest expression of Sp4 in the brain and in the neural tube, and it is kept highly expressed in these tissues in further embryonic stages. Similarly, northern blot analysis showed highest expression in the adult brain and skeletal muscle, and low expression in several other adult tissues (Supp 1996). Taken together, these observations suggest that Sp4 might be one of the transcription factors that is involved in the expression of brain specific genes. Co-transfection experiments to Drosophila SL2 cells indicated that Sp4 is a transcriptional activator like Sp1 and this transcriptional activation can be repressed by Sp3 (Hagen 1995).

Several transgenic mouse lines have been established to study Sp4 function in mice in vivo. Supp and colleagues created an Sp4 mutant mouse line by deleting three zinc fingers within the Sp4 sequence which abolished the DNA binding ability of Sp4. The mutant mice are born normally, but most of them die few days after birth. In addition, surviving mice are reported to be smaller compared to wildtype littermates (Supp 1996). Brain size in the newborn and adult mutant mice did not show any obvious deficiency. Since male Sp4 mutant mice failed to breed, authors pointed to the expression of Sp4 in vumeronasal organs (VNOs) during mouse development, concluding that a possible dysfunction of the VNOs might perturb detection of pheromones and therefore the reproductive behavior (Supp 1996).

Another Sp4 transgenic mouse line was created by Göllner and colleagues by deleting the exons coding the N-terminal activation domain of Sp4, which yielded complete absence of Sp4 mRNA. This mouse line is therefore named as Sp4 null mutant mice (Sp4\textsuperscript{null}). Similar to previous Sp4 mutant mice, Sp4\textsuperscript{null} mice were born normally and most of them died within 4 weeks after birth. The surviving mice were smaller in size, and male Sp4\textsuperscript{null} mice did not breed. One interesting finding in these mice was altered Sp3 levels (approximately two fold upregulation) which might contribute to the phenotype seen in Sp4\textsuperscript{null} mice (Göllner 2001).

Specific deletion of Sp4 in either ventricular or neural crest cell lineages of mice was aimed by Amand and colleagues who created Sp4 conditional knockout mice for this purpose. Crossing of Sp4 conditional knockout mice with appropriate Cre transgenic lines (MLC2v-Cre for ventricular restricted deletion and Pax3-Cre for neural crest restricted deletion of Sp4) showed several deficits in peripheral conduction system, including cardiac arrhythmia. Interestingly, Neurotrophin-3 (NT3) receptor TrkC was significantly downregulated in the heart of the these
knockouts indicating that Sp4 is involved in the regulation of TrkC receptor expression in the heart (St. Amand 2005).

The most recent Sp4 transgenic mouse model was created by Zhou and colleagues where the expression of endogenous Sp4 was blocked by insertion of a cassette into the first intron of Sp4. Inserted cassette in this case contained LacZ sequence flanked by two loxP sites flowed by rat Sp4 cDNA. Due to the insertion of this cassette into mouse, Sp4 expression was reduced to 2-5% of the endogenous levels in the brain. Therefore, this transgenic mouse line is known as Sp4 hypomorphic mice in the literature. The advantage of this mouse line was that the expression of Sp4 can be rescued using Cre recombinase, which allows removal of the LacZ gene and subsequent expression of rat Sp4 cDNA (Zhou 2005).

Detailed analysis of the Sp4 hypomorphic mice presented important findings regarding the Sp4 function in mouse brain in vivo. LacZ expression in the heterozygotes during development indicated Sp4 expression mainly in central nervous system, confirming endogenous Sp4 expression reported previously by using in situ hybridization. In addition, authors reported highest expression levels of the LacZ in the hippocampal CA1 region and dentate gyrus in the adult brain. Expression of LacZ in the adult brain was reported to be restricted to the neuronal layers in the hippocampus and no expression was detected in glial cells. Although there were no major abnormalities found in the hippocampus of Sp4 hypomorphic mice, structural analysis revealed massive vacuolization in the CA3 area and in the hilus of the dentate gyrus. TUNEL staining could not detect altered cell death in these areas. One very interesting observation of the authors was that GFAP staining seemed to be increased in the dentate gyrus but not in CA1 and CA3 regions of the hippocampus. Additionally, microtubules in the dendrites were thicker in the dentate gyrus of the hypomorphic mice, which might indicate alterations of the cytoskeleton in dentate granule neurons (Zhou 2005).

To investigate the molecular mechanisms lying behind the Sp4 dependent alterations, authors carried out gene expression analysis from hippocampus of the hypomorphic mice. Reduction in the mRNA level of over a hundred genes was reported. Interestingly, neurotrophin-3 was shown to be reduced in the dentate gyrus of Sp4 hypomorphic mice over time, with no detectable mRNA levels at 6 months of age. To test the possible effects of hippocampal alterations to hippocampus related behavioral responses in the hypomorphic mice, a series of behavioral tests are performed. Results indicated more anxiety and/or less exploratory behavior to novel environment in open
field test, impaired memory in both contextual and cued fear conditioning tests, and impairment in sensorimotor gating in Sp4 hypomorphic mice. All the structural (vacuolization), molecular (decreased NT3 levels) and behavioral phenotypes could be rescued when Sp4 hypomorphic mice was bred with protamine-cre mice to excise the LacZ allele which allows the expression of a rat/mouse chimeric Sp4 protein in Sp4 hypomorphic mice (Zhou 2005).

Using Sp4<sup>null</sup> mutant mice (mentioned and described in detail within the introduction part of this thesis) Zhou and colleagues analyzed the postnatal development of the dentate gyrus. Expression of mitotic cell markers revealed less cell proliferation in the hippocampus, but not in the cerebellum of postnatal Sp4<sup>null</sup> mice, although both dentate gyrus and cerebellum continue to develop in the early postnatal period. In line with this finding, the size of the dentate gyrus was smaller and the granule cell density was lower in the DG but not in the CA1 and CA3 regions of hippocampus in adult Sp4 mutant mice. The analysis of cell density in hippocampus at the age of 8 months showed similar tendency, the cell density of DG being less in mutants than in control mice whereas the cell density of CA1 and CA3 was not affected. Additionally, the size of the dentate gyrus and the width of the molecular layer, which contains the dendrites of DG neurons are reported to be smaller in the mutants at 8 months of age. These findings strongly suggest a role for Sp4 specifically in the development of the dentate gyrus, but not for other sub regions of the hippocampal formation. The expression of Prox-1, a neuronal marker in the dentate gyrus appeared to be normal in the mutant mice, suggesting that Sp4 is not necessary for the lineage specification of the dentate granule neurons. Moreover, authors reported vacuolization in the hilus and in the CA3 area of hippocampus in the Sp4<sup>null</sup> mice, consistent with the findings reported before in Sp4 hypomorphic mice (Zhou 2007).

In addition to the in vivo findings reported from several mouse models, a couple of in vitro studies are concentrated on the role of Sp4 in neuronal development. Hippocampal cultures that are established from neonatal Sp4<sup>null</sup> mice showed that the dendritic length of the granule neurons are decreased 25% and dendritic branching was decreased around 60% in the cultures established from Sp4<sup>null</sup> mice compared to controls. These findings suggest that Sp4 is needed for the proper development of dendrites in dentate granule cells, in line with the in vivo observations from Sp4<sup>null</sup> mice (Zhou 2007). A separate in vitro study investigated the role of Sp4 in cerebellar maturation. RNAi mediated knockdown of Sp4 resulted in a two-fold increase in the number of dendrites and up to four-fold increase in dendritic branching in cultured rat cerebellar granule
neurons, but total dendritic length was not affected. Authors reported as well that in cerebellar granule cultures, dendritic pruning takes place between day 4 and day 6 in vitro but this was not visible in Sp4 RNAi delivered cells; suggesting that Sp4 is required for dendritic pruning in vitro (Ramos 2007).

Molecular and structural alterations in neurons in the absence of Sp4 underline the importance of this transcription factor for proper development and function of the brain. Not surprisingly, recent association studies showed a link between allelic variants of Sp4 locus and psychiatric disorders including bipolar disorder and schizophrenia (Zhou 2009).

3.3. FHOD1 as a possible downstream target of Sp3

Formin homology-2-domain containing protein 1 (FHOD1) is a member of evolutionary conserved protein family called formins. Formins are scaffold proteins that contribute to actin cytoskeleton remodeling by inducing nucleation and elongation of actin filaments. (Evangelista et al 2003, Wallar et al 2003, Schulte et al 2008). Several studies showed contribution of FHOD1 to the regulation of cytoskeleton, cell elongation, cell migration, and gene transcription. When activated by specific Rho GTPases, formin family proteins contribute to the formation of actin cytoskeleton related structures like stress fibers. FHOD1 particularly is inactive in the cell due to the interaction of its N and C terminal domains. It has been shown that phosphorylation of FHOD1 in its C terminal region by Rho-dependent protein kinase ROCK disrupts this auto-inhibitory interaction of N and C terminals, which indeed activates FHOD1. This activation was shown to contribute to tick stress fiber formation via regulation of actin cytoskeleton in vascular endothelial cells (Takeya 2008). Gasteier and colleagues showed that overexpression of constitutively active form of FHOD1 resulted in cell elongation in HeLa cells, which is induced by enhanced FHOD1-actin fiber formation together with microtubule alignment. Therefore, it has been postulated that FHOD1 might coordinate actin cytoskeleton and microtubule cytoskeleton. Additionally, authors reported that the cell elongation phenotype was also dependent on the activity Rho-ROCK cascade, a signaling cascade involved in the regulation of actin cytoskeleton (Gasteier et al 2005). In addition to cell elongation, FHOD1 is shown to enhance cell migration of WM35 cells to type-I collagen and NIH-3T3 cells to fibronectin in vitro (Koka S. et al 2003). In another study, Hannemann and colleagues reported that FHOD1 is interacting directly with ROCK1 to modulate plasma membrane blebbing, and Src kinase is a key regulator for the interaction of FHOD1 and ROCK1 during this process in HeLa cells (Hannemann et al 2008). I
identified FHOD1 as a downstream target of sumoylated Sp3 in the dentate gyrus of adult mouse brain in this study.

3.4. **Objective of this study**

The overall aim of this study is to investigate the expression profile and the role of the Sp transcription factor family in adult neurogenesis.

Unlike embryonic brain, where many different regions of the brain contain pools of neural stem cells which massively produce neurons and glia; in the adult brain, there are only two brain areas within the established brain circuitry that harbor neural stem cell pools, which are actively engaged in neurogenesis (Zhao et al., 2008). In order to understand the regulation of proper neuronal development within an adult neurogenic niche, it is important to investigate genetic programs that are involved in adult neurogenesis.

Firstly, in the complexity of adult neurogenic niche where neurogenesis occurs; stem cells, progenitor cells, immature neurons, mature neurons and glial cells are present at a certain time in the same region of the brain. Therefore external signals that separately control those stages of neuronal development are found altogether in the neurogenic region (Faigle and Song 2013, Suh et al 2009, Mu et al 2010). This phenomenon makes “internal regulators” of adult neurogenesis (like transcription factors) especially important to be able to understand how adult neurogenesis process is governed internally and specifically for each cell despite a batch of complex external signaling that is going on within the adult neurogenic niche.

Secondly, members of the Sp transcription factor family has been reported previously to have an effect on dendritic development, neuronal development, development of the dentate gyrus, dendritic pruning and finally in several neurodevelopmental disorders and neurodegenerative diseases (Ramos et al 2007 and 2009; Zhou et al 2007, 2009 and 2010; Priya et al 2013) which might indicate a crucial role for Sp transcription factor family in neuronal development and neurological diseases.

Considering the importance of “internal regulatory mechanisms” and the potential role of Sp transcription factor family in neurodevelopment and neurological disease; in this study, I aimed to investigate the role of Specificity Protein (Sp) transcription factors in adult neurogenesis. Loss of function studies for Sp transcription family members are conducted by using *in vivo* miRNA based retroviral labeling strategy in the dentate gyrus. Results of this study propose specific roles
for Sp3 and Sp4 in the establishment of dendritic complexity during stem cell differentiation in the adult brain. Considering molecular pathologies of neurological disorders like major depression, schizophrenia and autism; which are related to proper neuronal function in different brain regions including hippocampus, results of this thesis might contribute to understand the underlying disease mechanisms and therefore might open new ways for their treatment.
4. MATERIALS

4.1. Chemicals, media and supplements

1 kb DNA ladder New England Biolabs
100 bp DNA ladder Fermentas
Acrylamide/Bis Solution (40%, 19:1) Biorad
Albumin from bovine serum (98%) Sigma-Aldrich
Ampicillin Sigma-Aldrich
Bovine serum albumin (2 mg/ml) Pierce
B27 supplement GIBCO/Invitrogen
Chloroform Invitrogen
DAPI dilactate Sigma-Aldrich
DMEM F12 Glutamax GIBCO/Invitrogen
DNA agarose Biozym
DTT Roche
EBSS (Earls Balanced Salt Solution) GIBCO/Invitrogen
ECL Western Blotting Detection Reagents Amersham
EDTA Sigma-Aldrich
EGF (Epidermal Growth Factor) Life Tech
EtBr (1 mg/ml) Roth
EtOH (100 %) Merck
FGF2 (Fibroblast Growth Factor 2) Life Tech
Glucose Sigma
HBSS (Hanks Balanced Salt Solution) GIBCO/Invitrogen
HEPES (1 M) GIBCO/Invitrogen
Hyaluronidase Sigma
Isopropanol (100 %) Merck
Loading dye (6x) BioLabs
NaOH tablets Merck
Normal Donkey Serum Chemicon
Page Ruler Plus Prestained Protein Ladder Fermentas
PFA Roth
ProteinaseK (recombinant, PCR grade) Roche
PSF (100x)  
RNase A (from bovine pancreas)  
Skim milk powder  
Sucrose  
Triton X-100  
TRIzol®  
Trypsin  
Trypsin-EDTA (0.5 %)  

4.2. Commercial Kits

cDNA Kit Superscript III  
DNA Maxi Prep  
DNA Mini Prep/ NucleoSpin Plasmid Kit  
PCR Master Mix  
RNeasy Kit  
SYBR Green ER qPCR Super Mix  
QIAquick Gel extraction  
QIAquick PCR purification Kit  
BCA Protein Assay Kit  
Lipofectamine™ 2000

4.3. Antibodies

Primary antibodies

goat anti DCX (1:250)  
mouse anti GFAP (1:1000)  
chicken anti GFP (1:500)  
mouse anti NeuN (1:50)  
goat anti Sox2 (1:500)  
Sp1 (1C6) sc420 mouse  
Sp3 (D-20) sc-644 rabbit  
Sp4 (V-20) sc-645 rabbit  
Sp4 (V-20-G) sc-645-G goat  

Secondary antibodies

GIBCO/Invitrogen  
Serva  
BioChemika  
Sigma  
Roth  
Invitrogen  
Sigma  
GIBCO/Invitrogen  
Invitrogen  
Promega  
Macherey-Nagel  
Eppendorf  
Qiagen  
ABI Prism, Applied Biosystems  
Qiagen  
Qiagen  
Pierce  
Invitrogen  
Santa Cruz Biotechnologies  
Santa Cruz Biotechnologies  
Aveslab  
Chemicon  
Santa Cruz Biotechnologies  
Santa Cruz Biotechnologies  
Santa Cruz Biotechnologies  
Santa Cruz Biotechnologies  
Santa Cruz Biotechnologies
Cy3-rabbit Jackson ImmunoResearch
Cy5-goat Jackson ImmunoResearch
Cy5-rabbit Jackson ImmunoResearch
Fitc-chicken Jackson ImmunoResearch
Fitc-mouse Jackson ImmunoResearch
HRP-mouse Jackson ImmunoResearch
HRP-rabbit Jackson ImmunoResearch
HRP-rat Jackson ImmunoResearch

4.4. Primers and oligonucleotides

**Primers**

FHOD1 QPCR Forward Primer CCTGCTGGTGCACTCAGT
FHOD1 QPCR Forward Primer GCTTTGCTTAGTCCCAGT
Srp14 forward CAGCGTGTTCATCACCCTCAA
Srp14 reverse GGCTCTCAACAGACACTTGT
GAPDH forward GTGTTCCTACCCCCCAATGTGT
GAPDH forward ATTGTCATACCAGAAATGAGCTT

**shRNA oligonucleotides**

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp4miRNA1F</td>
<td>TGCTGTGAAATGGCTGGCAATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp4miRNA1R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp4miRNA2F</td>
<td>TGGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp4miRNA2R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp4miRNA3F</td>
<td>TGCTGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp4miRNA3R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp1miRNA1F</td>
<td>TGCTGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp1miRNA1R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp1miRNA2F</td>
<td>TGGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp1miRNA2R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp1miRNA3F</td>
<td>TGCTGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp1miRNA3R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp3miRNA1F</td>
<td>TGCTGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp3miRNA1R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp3miRNA2F</td>
<td>TGGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp3miRNA2R</td>
<td>CCGTGAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
<tr>
<td>Sp3miRNA3F</td>
<td>TGCTGTGAAATGGTTCACATCTTTGGGTTTGCCACTGAGCAGCCAAAGAGATGCAACCTCAA</td>
</tr>
</tbody>
</table>
Control miRNA sequences are published in Merz and Lie, 2013.

4.5. Organisms

E. Coli (TOP10 a) Provided by the lab
HEK 293T cells (human) Provided by the lab
Mouse strain C57/Bl6 Charles River
Mouse strain Sp3kiki Provided by Suske lab

4.6. Plasmids

pCAG IRES-GFP Jagasia et al., 2009
pCAG RFP Zhao et al., 2006
pKSSP-EmGFP-miR Merz and Lie, 2013

4.7. Buffers and solutions

Agar
Readymade LB agar 32 g/l
Ampicillin 75 μg/ml

0.1 M Borate buffer (pH 8.5)
Boric acid (MW: 61.83 g/mol) 3.0915 g
H₂O 100 ml

0.01 M Citrate buffer (pH 6.0)
Citric acid 0.29 g
H₂O 100 ml

Cryoprotectant solution
Glycin 250 ml
Ethylene glycol 250 ml
0.1 M Phosphate buffer 500 ml

Dissociation media
Solution #1 5 ml
Trypsin 6.7 mg
Hyaluronidase 3.5 mg

2 M HCl
<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>32 % HCl</td>
<td>2.49 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.51 ml</td>
</tr>
<tr>
<td><strong>5x Laemmli</strong></td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Glycerol</td>
<td>25 ml</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>0.75 g</td>
</tr>
<tr>
<td><strong>3 M NaAc (pH 5.0)</strong></td>
<td></td>
</tr>
<tr>
<td>NaAc</td>
<td>12.3 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>50.0 ml</td>
</tr>
<tr>
<td><strong>10x NuPage running buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Tris-base</td>
<td>20 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>379 g</td>
</tr>
<tr>
<td>SDS</td>
<td>20 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>2 l</td>
</tr>
<tr>
<td><strong>10x NuPage transfer buffer</strong></td>
<td></td>
</tr>
<tr>
<td>Tris-base</td>
<td>6.0 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>28.8 g</td>
</tr>
<tr>
<td>MeOH</td>
<td>400 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>1.6 l</td>
</tr>
<tr>
<td><strong>10x PBS (pH 7.2)</strong></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>80.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Na₂PO₄</td>
<td>14.4 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.4 g</td>
</tr>
<tr>
<td>H₂O</td>
<td>800.0 ml</td>
</tr>
<tr>
<td><strong>4 % PFA (pH 7.4 at RT)</strong></td>
<td></td>
</tr>
<tr>
<td>PFA (dissolved by heating)</td>
<td>40 g</td>
</tr>
<tr>
<td>0.2 M phosphate buffer</td>
<td>500 ml</td>
</tr>
<tr>
<td>NaOH</td>
<td>1-2 tablets</td>
</tr>
<tr>
<td>H₂O</td>
<td>500 ml</td>
</tr>
</tbody>
</table>
0.2 M Phosphate buffer
Sodium phosphate monobasic 16.56 g
Sodium phosphate dibasic 65.70 g
H₂O 3 l

Solution #1
HBSS 50 ml
Glucose 2.7 g
HEPES 15 mM
H₂O up to 500 ml, filtered (0.22 µm)

Solution #2
HBSS 25 ml
Sucrose 154 g
H₂O up to 500 ml, filtered (0.22 µm)

Solution #3
HEPES 20 mM
BSA 20 g
EBSS up to 500 ml, filtered (0.22 µm)

Solution #4
NaCl 592 mg
NaCitrate 500 mg
RNase A (from bovine pancreas) 5 mg
NP-40 (Nonidet P-40) 0.15 ml
H₂O up to 500 ml, filtered (0.22 µm)

Solution #5
Citric Acid 7.5 g
Sucrose 0.25 M
H₂O up to 500 ml, filtered (0.22 µm)

30 % Sucrose (4 °C)
Sucrose 150 g
0.1 M phosphate buffer 500 ml

50x TAE
Tris Base 242.0 g
Glacial acetic acid 57.1 ml
0.5 M EDTA (pH 8.0) 100.0 ml
H₂O 842.9 ml

10x TBS
NaCl 80 g NaCl
KCl 2 g
1 M Tris/HCl (pH 7.5) 250 ml
H₂O 750 ml

TBS++
Donkey serum 1.50 ml
10 % Triton X-100 1.25 ml
TBS 50.00 ml

4.8. Other materials

24 well plates Falcon
Aqua-Poly/Mount Polysciences
BioTrace PVDF Pall
Cellstar pipettes Greiner bio-one
Counting chamber (0.0025 mm²) Neubauer
Cover slips Menzel-Glaser
Cryoblock mounting medium Medite Medizintechnik
High Performance chemiluminescence film Amersham
Microliter syringes Hamilton
Miniwells (60 wells) Nunc
PCR tubes Biozym
Reaction tube 15 ml Falcon
Reaction tube 50 ml Falcon
Round bottom tube 14ml Falcon
Safe lock tube 1.5 ml Eppendorf
Safe lock tube 2.0 ml Eppendorf
Superfrost Plus microscope slides Menzel-Glaser
Surgical disposable scalpels Braun
Tissue culture dishes (100x20 mm)  
Tissue culture flasks (25 m$^2$, 75 m$^2$, 175 m$^2$)  
Whatman filter paper

4.9. **Equipment**

*Hardware*

- Centrifuge 5415 D  
- Centrifuge 5417 R  
- Curix60 developing machine  
- Fluorescence microscope DMI 600B  
- GelSystem Mini  
- HeraCell 150 incubator  
- HeraCell Tissue Culture hood  
- Leica SP5 confocal microscope  
- Luminometer Centro LB960  
- Mini Protean® Tetra Cell  
- ND-1000 spectrophotometer (Nanodrop)  
- PowerSupply  
- Rotamax 150  
- Sliding microtome SM 2000R  
- Sorvall Evolution High Speed Centrifuge  
- Thermomixer comfort  
- Trabs-Blot SD Semi-Dry Transfer Cell  
- XCell SureLock Electrophoresis Cell

*Software*

- LAS AF 2.5  
- NTI vector  
- IMARIS software  
- StepOne Real-Time PCR  
- Genomatix

Falcon  
BD BioCoat™  
Whatman  
Eppendorf  
Agfa  
Leica  
Peqlab  
Kendro  
Leica  
Berthold  
Biorad  
Heidolph  
Leica  
Thermo Science  
Eppendorf  
Biorad  
Invitrogen  
Leica  
Invitrogen  
Bitplane  
Applied Biosystems  
Genomatix Software GmbH
5. METHODS

5.1. Cell culture methods

5.1.1. Culturing of HEK 293T cells

Human embryonic kidney 293T cells were cultured in DMEM (1x) +4.5 g/l Glucose/L-Glutamine/Pyrurate supplemented with PSF (5 ml in 500 ml) and 10 % FBS. Cells were incubated on 10 cm petri dishes at 37 °C with 5 % CO2.

5.1.2. Passaging of HEK 293T cells

When confluence was reached, cells were rinsed once with PBS and detached by adding 2 ml trypsine with EDTA. Approx. 5 ml medium were added for neutralization of trypsin. After centrifugation at 1500 rpm for 2 min, supernatant was discarded and cells resuspended in 1 ml medium. 100 μl of cell suspension (1:10) was transferred to a new petri dish containing 8 ml fresh medium.

5.1.3. Transient transfection of HEK 293T cells

One day before transient transfection, cells were detached and neutralized as described above. 10 μl of the cell suspension were diluted with 90 μl medium (1:10) and 15 μl of the dilution were transferred into a Neubauer counting chamber. Four quadrants were counted; one million cells were resuspended in 5 ml medium (DMEM) and were plated on a 10 cm plate and incubated overnight at 37°C.

For transfection the Lipofectamine™ 2000 kit from Invitrogen was used and the transfection was made according to the manufacture’s protocol. After overnight incubation, medium was replaced in the early morning to prevent acidification of the medium. 24 h later, transfection efficiency was assessed by estimating the percentage of cells expressing the co-transfected GFP encoding gene. The medium was removed; cells were washed with 5 ml PBS and scraped of the plate with a sterile spatula. Protein extract preparation is mentioned in protein methods part.

5.2. RNA methods

RNA, the concentration and quality was measured with Nanodrop (nucleic acid program).

5.2.1. RNA isolation from brain tissue

For the microarray experiments and for other in vivo RNA experiments, RNA is isolated using the RNeasy kit (Qiagen) following manufacturer’s instructions.

5.2.2. DNAsse Treatment

For DNAsse treatment, DNAsse kit of Qiagen is used. To produce cDNA, 1 μg of RNA is incubated with 1 μl RQ1-10x reaction buffer and 1 μl RQ1-DNase (total volume of 10 μl was achieved with Ampuwa water) for 30 min at 37°C. After adding 1 μl Stop solution and incubation for 10 min at 65°C, RNA was stored at -80°C.
5.2.3. cDNA synthesis

cDNA was prepared according to the protocol of the SuperScript™ III First-Strand Synthesis SuperMix from Invitrogen and stored for further use at -20°C.

5.2.4. Quantitative real-time PCR (QPCR)

Quantitative RT-PCR was performed using StepOne instrument (Applied Biosystems). Brilliant II Fast SYBR Green qPCR Master Mix (Agilent, Boeblingen, Germany) was used for PCR reactions according to the manufacturer’s protocol. To design QPCR primers, online software called NetPrimer is used.

5.3. Protein methods

5.3.1. Isolation of proteins

Buffers A and C are mixed freshly, while the last three components, DTT, PMSF and protease/phosphatase inhibitors (I), are added just before usage of the buffer. For protease/phosphatase inhibitors a 10x stock was prepared by dissolving one tablet of each inhibitor in 1 ml MilliQ.

Table 1. Buffer A for preparation of cytosolic and nuclear extracts

<table>
<thead>
<tr>
<th>Buffer A Compound</th>
<th>End-c</th>
<th>Stock-c</th>
<th>For 10 ml [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>10.0 mM</td>
<td>0.5 M</td>
<td>200.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.0 mM</td>
<td>0.5 M</td>
<td>20.0</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.1 mM</td>
<td>50.0 mM</td>
<td>20.0</td>
</tr>
<tr>
<td>KCl</td>
<td>10.0 mM</td>
<td>3.0 M</td>
<td>33.3</td>
</tr>
<tr>
<td>MgCl2</td>
<td>1.5 mM</td>
<td>2.0 M</td>
<td>7.5</td>
</tr>
<tr>
<td>DTT</td>
<td>1.0 mM</td>
<td>1.0 M</td>
<td>10.0</td>
</tr>
<tr>
<td>PMSF</td>
<td>1.0 mM</td>
<td>100.0 mM</td>
<td>100.0</td>
</tr>
<tr>
<td>Protease/phosphatase I</td>
<td>1x</td>
<td>10x</td>
<td>1000.0</td>
</tr>
<tr>
<td>MilliQ</td>
<td></td>
<td></td>
<td>8609.2</td>
</tr>
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</table>
Table 2. Buffer C for preparation of cytosolic and nuclear extracts

<table>
<thead>
<tr>
<th>Buffer C Compound</th>
<th>End-c</th>
<th>Stock-c</th>
<th>For 10 ml [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>20.0 mM</td>
<td>0.5 M</td>
<td>400.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.2 mM</td>
<td>0.5 M</td>
<td>4.0</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.1 mM</td>
<td>50.0 mM</td>
<td>20.0</td>
</tr>
<tr>
<td>Glycerol</td>
<td>25.0 %</td>
<td>86.0 %</td>
<td>2900.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>420.0 mM</td>
<td>5.0 M</td>
<td>840.0</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5 mM</td>
<td>2.0 M</td>
<td>7.5</td>
</tr>
<tr>
<td>DTT</td>
<td>1.0 mM</td>
<td>1.0 M</td>
<td>10.0</td>
</tr>
<tr>
<td>PMSF</td>
<td>1.0 mM</td>
<td>100.0 mM</td>
<td>100.0</td>
</tr>
<tr>
<td>Protease/phosphatase I</td>
<td>1x</td>
<td>10x</td>
<td>1000.0</td>
</tr>
<tr>
<td>MilliQ</td>
<td></td>
<td></td>
<td>4718.5</td>
</tr>
</tbody>
</table>

All steps of cytosolic (CE) and nuclear extract (NE) preparation were performed on ice or in 4 °C cold centrifuge to prevent protein degradation.

For protein isolation from cells, volume of cell pellets is estimated and volume of buffers is calculated accordingly. Cells are resuspended in 2 V buffer A and incubated for 15 min. Swollen cells were briefly centrifuged at 1,000 rpm for 1 min. Supernatant was discarded and cells again resuspended in 3 V buffer A* (buffer A with 0.1 % NP-40 or Igepal). After 5 min incubation, CE was obtained in the supernatant by centrifugation at 10,000 rpm for 5 min. CE was frozen at -80°C. Remaining pellet containing cell nuclei was washed once with 500 μl buffer A, while the pellet remained intact, to avoid contaminations with cytosolic components (centrifugation at 500 rpm for 1 min). Pellet was resuspended vigorously in 3 V buffer C (hypertonic) and agitated for 20 min. Afterwards, broken nuclei were centrifuged at 14,000 rpm for 5 min to eliminate DNA and remains of the nuclear membrane and to obtain NE in the supernatant. NE was stored at -80 °C.

5.3.2. Western blot analysis

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. SDS-PAGE is carried out using Mini Protean 3 System (Biorad). Blotting procedures were performed in a tankblot apparatus (Invitrogen). For blotting, 0.45 μm BioTrace PVDF-(Polyvinylidenfluorid) membrane (Pall Corporation) is used. 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 °C. 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).

5.4. Cloning methods

5.4.1. Cloning of microRNA constructs

MicroRNA sequences against Sp3 and Sp4 are designed in silico using online microRNA software of invitrogen. Control microRNA sequence is obtained from invitrogen.

In the first step, miRNA constructs are cloned into pKSSP-EmGFP shuttle vector. Insert (miRNA) preparation is carried out by annealing 60pmol of sense and 60pmol of antisense oligos for each miRNA in 50µl of volume separately, using the following annealing buffer and PCR condition.

**annealing buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-Acetate</td>
<td>100mM</td>
</tr>
<tr>
<td>Hepes pH 7.4 with KOH</td>
<td>30mM</td>
</tr>
<tr>
<td>Mg-Acetate</td>
<td>2mM</td>
</tr>
</tbody>
</table>

**PCR Condition**

- 95°C, 5min
- 70°C, 10min
- 70°C to 4°C: 0.1°C/min decrease
- (storage: -20°C, defreeze at RT)

Phosphorylation of miRNA ends are carried out by using T4 protein kinase (NEB) according to manufacturer’s instructions. Following insert preparation, vector preparation is carried out by BsmBI restriction digestion and dephosphorylation of cut ends by antarctic phosphatase. The vector is then gel purified using Qiagen Kit. Prepared miRNA and vector constructs are ligated with 1:5 vector:insert ratio and transformed into E.coli with heat shock method. Properly cloned constructs are selected by MscI digestion, where MscI is a single cutter if the clone positive.

In the second step, EmGFP-miRNA sequence was cut out from the pKSSP-EmGFP-miR vector with SfiI and PmeI restriction enzymes. CAG-IRES-GFP vector was cut also with Sfi & PmeI. Cut constructs and vector were gel purified by using Qiagen Gel Extraction Kit using manufacturer’s instructions. Gel Purified inserts and the vector are ligated with 1:5 vector/insert ratio at room temperature overnight with the same protocol described above.
5.4.2. Transformation of bacteria

For amplification of plasmid DNA, competent E. coli cells (TOP10) are transformed with 10-100 ng of plasmid DNA. Standard heat-shock transformation protocol is carried out as follows: Competent E.coli is thawed on ice and incubated on ice for 15 min. Heat-shock transformation is done at 42 °C for 45 seconds and immediately placed back on ice for another 15 min. LB medium is added on the bacteria and incubated in a heating block (350 rpm) at 37 °C for 1 h. After short centrifugation at 13,000 rpm, cells are resuspended in 100 µl LB medium and plated on agar plates containing appropriate selection antibiotic. Plates were incubated at 37 °C overnight.

5.4.3. Plasmid DNA isolation

Transformed bacteria are cultured at 350 rpm and 37 °C overnight. Cultures were processed with Pure Yield Plasmid Midiprep System following manufacturer’s protocol with the following modifications.

5.5. Knockdown Experiments

5.5.1. Virus Production

Retrovirus production was performed with HEK293 GPG-1F8 cells, which constitutively express the gag and pol genes of MMLV. The VSVG gene is under the control of a tetracycline responsive promoter and can be induced upon withdrawal of tetracycline. The viral RNA is encoded by a transiently transfected minimal retroviral expression plasmid containing the 3’ and 5’ LTR of MMLV, the retroviral packaging signal Ψ, a primer binding site for the retroviral reverse transcriptase and the cDNA encoding the protein of interest. Cells were maintained in basal medium (500 ml DMEM High Glucose/GlutaMax/Hepes with 55 ml FCS, 5ml NEAA (100x) and 5 ml Na-Pyruvate (100x) under double-selection and Tet-repression: Geneticin (100 mg/ml) to select for integrated MMLV genome, puromycin (2 mg/ml) to select for integrated VSVG and doxycycline (10 μg/ml) to repress expression of the VSVG.

The week before the retroviral production, HEK (1F8) cells are expanded to 175cm flasks. We used two confluent 175cm flasks of cells per construct.

On the day of transfection, 2x50ml falcon tubes are filled with 9ml of Opti-MEM media per plasmid without additives. In once, we produced 3 different viruses. To one of the tubes, 360ul of Lipofectamine 2000, and to the second tube, 150ug of plasmid DNA is added. After 10 minutes of incubation at room temperature the contents of the 2x50ml falcon tubes are mixed, and incubated at room temperature for 30 minutes.

During this incubation time, 1F8 cells are dissociated from flasks by using standard trypsin protocol, and resuspended in 30ml OPTI-MEM media containing 10% of FCS. 30ml of this media containing the cell resuspension is mixed with previously prepared 18ml of media containing lipofectamine and DNA mix. The cells are then seeded into 10cm dishes, using 8ml of this prepared mix, and incubated
in a 37C incubator with 5% CO2. After 14 hours of incubation in lipofectamine containing transfection conditions, the media of the cells are refreshed using 11.5ml of equilibrated packaging medium.

Viral harvest is done every second day after this media change, until the third harvest. For harvesting, the supernatant of the transfected cells are collected into 50ml falcon tubes, and fresh packaging media is put on the cells that are incubated further for the next harvest. The collected supernatant was centrifuged at 3500rpm for 15 minutes, and supernatant is filtered through 0.45u low-protein-binding PVDF filter into SW28 ultracentrifuge tubes. The virus particles are precipitated by centrifuging the supernatant at 50000g for 2 hours at 4C. Supernatant is removed and the pellet is gently resuspended in 2ml of TBS-5, and the volume is filled up to 35ml in the ultracentrifuge tube. Viral pellets are collected by centrifuging at 50000g for 2 hours at 4C. After centrifugation, supernatant is removed and the pellet is solved gently in 100ul of TBS-5 buffer. The virus is aliquoted and stored at -80°C freezer until use.

Second and third harvests are proceed the same way, and stored at -80°C freezer.

5.5.2. Stereotactic Injections

8-10 weeks old female mice are put in running wheel containing cages 6 days prior to stereotactic injections. Before injections, sleep and awake solutions are prepared using the following protocol.

Table 3. Sleep Solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Name</th>
<th>Stock concentration (mg/ml)</th>
<th>Dose</th>
<th>Injection Amount ml/kg</th>
<th>Injection Concentration mg/ml</th>
<th>Volume required for Sleep Solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fentanyl</td>
<td>Fentanyl</td>
<td>0.1</td>
<td>0.05</td>
<td>10</td>
<td>0.005</td>
<td>0,25</td>
</tr>
<tr>
<td>Midazolam</td>
<td>Dormicum</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>0.5</td>
<td>0,5</td>
</tr>
<tr>
<td>Medetomidine</td>
<td>Dormitor</td>
<td>1</td>
<td>0.5</td>
<td>10</td>
<td>0.05</td>
<td>0,25</td>
</tr>
<tr>
<td>NaCl Solution (Isotonic)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

Fentanyl (Janssen-Cilag), Dormicum (Roche), Dormitor (Pfizer).
Table 4. Awake Solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Name</th>
<th>Stock concentration (mg/ml)</th>
<th>Dose</th>
<th>Injection Amount ml/kg</th>
<th>Injection Concentration mg/ml</th>
<th>Volume required for Sleep Solution (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buprenorphine</td>
<td>Temgesic</td>
<td>0.3</td>
<td>0.1</td>
<td>10</td>
<td>0.01</td>
<td>0.17</td>
</tr>
<tr>
<td>Adipamezol</td>
<td>Antisedan</td>
<td>5</td>
<td>2.5</td>
<td>10</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Flumazenil</td>
<td>Anexate</td>
<td>0.1</td>
<td>0.5</td>
<td>10</td>
<td>0.05</td>
<td>2.5</td>
</tr>
<tr>
<td>NaCl Solution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.08</td>
</tr>
</tbody>
</table>

Temgesic (Essex Pharma), Antisedan (Pfizer), Anexate (Hexal).

Sleep solution is injected abdominally in depicted dose and animals are left for anesthetizing for 30 minutes. The viruses are stereotactically injected to the dentate gyrus or to the subgranular zone using the coordinates below. Bregma was taken as 0 when referencing all the coordinates.

Table 6. Coordinates of Stereotactic Injections

<table>
<thead>
<tr>
<th></th>
<th>Antero-posterior</th>
<th>Medio-lateral</th>
<th>Dorso-ventral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dentate Gyrus</td>
<td>-1.9mm</td>
<td>+1.6mm/-1.6mm</td>
<td>-1.9mm</td>
</tr>
<tr>
<td>Subgranular Zone</td>
<td>1.25mm</td>
<td>+0.5mm/-0.5mm</td>
<td>-3.2mm</td>
</tr>
</tbody>
</table>

After injections of the retroviruses to the brain, animals are woke up by abdominal injection of the awake solution in doses given in tables 3 and 4.

5.5.3. Animals

The animals that are used for stereotactic injections in this study are 8-12 weeks old female C57BL/6J mice (Charles River, Wilmington, US-MA). Mice are housed in 12 hour light/dark cycle conditions and ad libitum access to water and food.

5.5.4. Perfusions

Animals are sacrificed using CO2. Perfusions are performed transcardially first with phosphate-buffered saline (PBS, pH=7.4) for 5 minutes followed by 4% paraformaldehyde (PFA) for 5 minutes. Brains of the animals are taken out and left overnight in 4%PFA for post-fixation, followed by overnight incubation in 30% sucrose. Brains are kept at 4°C in 30% sucrose solution until further preparations.
5.6. Immunoflorescence experiments

5.6.1. Preparation of brain slices

Brains are frozen using dry ice and cut in 40µm coronal or sagital sections for cell number counting experiments, and in 120µm coronal sections for dendritic morphology experiments, using a sliding microtome (Leica Microsystems, Wetzlar, Germany). Cut brain sections are stored in 96well plates within cryoprotectant solution at -20°C until immunohistochemical analyses are performed.

5.6.2. Immunohistochemistry

Immunohistochemistry is performed with the following order of steps in 6 well plates.

<table>
<thead>
<tr>
<th>Table 7. Immunohistochemical staining procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Step</strong></td>
</tr>
<tr>
<td>wash</td>
</tr>
<tr>
<td>Blocking</td>
</tr>
<tr>
<td>Primary Antibody</td>
</tr>
<tr>
<td>wash</td>
</tr>
<tr>
<td>Blocking</td>
</tr>
<tr>
<td>Secondary Antibody</td>
</tr>
<tr>
<td>wash</td>
</tr>
<tr>
<td>DAPI staining</td>
</tr>
<tr>
<td>wash</td>
</tr>
</tbody>
</table>

Brain sections are mounted using superfrost slides (Menzel-Gläser, Braunschweig) and Aqua/Polymount (Polysciences Inc., Warrington, USA).

5.6.3. Immunocytochemistry

Immunocytochemistry is performed with the following order after removal of the media from the cells that were grown in 24-well plates on ply-D-lysine and laminin coated coverslips and fixation of the cells with 4% PFA for 10 minutes in room temperature.
Table 8. Immunocytochemical staining procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>wash</td>
<td>3 x 15 min</td>
<td>1x TBS</td>
</tr>
<tr>
<td>Blocking</td>
<td>1 hour</td>
<td>2% donkey serum and 0.2% triton-X in TBS</td>
</tr>
<tr>
<td>Primary Antibody</td>
<td>overnight</td>
<td>Primary antibody in blocking solution, at 4°C</td>
</tr>
<tr>
<td>wash</td>
<td>3 x 15 min</td>
<td>1x TBS</td>
</tr>
<tr>
<td>Blocking</td>
<td>30 min</td>
<td>2% donkey serum and 0.2% triton-X in TBS</td>
</tr>
<tr>
<td>Secondary Antibody</td>
<td>2 hours</td>
<td>Secondary antibody in blocking solution, at room temperature</td>
</tr>
<tr>
<td>wash</td>
<td>1 x 15 min</td>
<td>1x TBS</td>
</tr>
<tr>
<td>DAPI staining</td>
<td>1x 8 min</td>
<td>DAPI diluted 1:10000 in 1x TBS</td>
</tr>
<tr>
<td>wash</td>
<td>2 x 15 min</td>
<td>1x TBS</td>
</tr>
</tbody>
</table>

Brain sections are mounted using superfrost slides (Menzel-Gläser, Braunschweig) and Aqua/Polymount (Polysciences Inc., Warrington, USA).

5.6.4. Confocal Microscopy

Confocal microscopy is performed using Leica SP5 confocal microscope (Leica Microsystems).

5.7. Analysis of Phenotypes

Dendritic analysis of the Sp3 KD, Sp4 KD and control cells are carried out by using confocal images of 100µm sections that are taken by using 63x objective of Leica Confocal microscope with step size of 0.5. GFP signal in this was the integrator of cell transduction with microRNA. CAG-RFP virus was injected in order to trace the denritic extension of neurons with high quality since GFP intensity itself was not suitable to trace the dendrites especially in the middle layers of 100µm sections. For dendritic analysis, only GFP (miRNA virus) and RFP (marker virus) double infected cells were considered. The dendritic tree of each double-infected neuron which lies in the middle third of the granule cell layer leaflet was analyzed using IMARIS software, since morphology of the cells at the tips of the dentate gyrus might slightly differ from the general, abundant dentate granule neuron morphology. Dendritic tracing of each neuron is analyzed at the RFP channel. Differences between experimental groups are defined by using standard error of the mean of total dendritic length and total dendrite branch numbers for each of the Sp3 KD, Sp4 and control cells at both 14dpi and 28dpi. All the sections used for this part of the experiment were coronal sections. For comparisons, SEM of the cells is taken.

For migration analysis, confocal images of 40µm sections are used. miRNA infected cell bodies are traced with GFP, and categorized according to their location in the dentate gyrus. The location categories were upper granule cell layer, middle granule cell layer, lower granule cell layer and hilus. According to cell body numbers lying in those zones, statistical analyses were carried out by using
standard error of the mean. For SGZ data coronal brain sections are used. For comparisons, SEM of the animals is taken.

For migration analysis of RMS, also confocal images of 40µm sections are used, but in sagittal sections. Migration of GFP (miRNA) labeled cells and RFP (internal control marker for each animal) cells were analyzed separately. To quantify migration of the cells at 5dpi, SVZ-RMS-OB system was divided into four areas in the analysis. These areas were SVZ, RMS1 (The rising half of the RMS that is facing to SVZ), RMS2 (The descending half of the RMS that is facing to OB), and OB. Percentage of GFP positive cells normalized to percentage of RFP positive cells within each quarter of the SVZ-RMS-OB route for each animal is represented for both Sp4 microRNA GFP and control microRNA GFP injected animals. These two groups were compared to see whether there is a migration deficiency upon Sp4 knockdown. For comparisons, SEM of the animals for each group is taken. Per animal, one 40µm section which covers all the SVZ-RMS-OB route has been used.
6. RESULTS

6.1. Sp3 and Sp4 but not Sp1 are expressed in the dentate gyrus of adult brain

The aim of this study is to investigate the role of specificity proteins (trans-acting transcription factors) in adult hippocampal neurogenesis in mice. To start with, I examined the expression pattern of Sp transcription factor family members in adult hippocampus. As pointed before in the introduction part of this thesis, the closest members of the Sp transcription factor family are Sp1, Sp3, and Sp4 classified according to the similarity in their aminoacid sequence as well as their binding motif on the DNA, which is GC and GT boxes. It is important to note that Sp transcription factors are reported to bind to those consensus sites on the DNA in a competitive manner, making the amount and the activity of these transcription factors critical for the regulation of Sp target promoters in a given cell. The examination of these three transcription factors at the same time is therefore important due to the fact that the presence/absence of these proteins or the ratio between the amount of different Sp members in a certain cell type or at a certain stage of neuronal development might have a regulatory function for common Sp target promoters. Taking this possibility into account I investigated the expression of Sp1, Sp3, and Sp4 in adult hippocampus. I could not detect the expression of Sp1 in the hippocampus, although there were Sp1 positive cells in the forebrain region of the same brain sections. The remaining two members, Sp3 and Sp4 were found to be expressed in all four sub-regions (CA1, CA2, CA3, and dentate gyrus) of the hippocampus. In addition, I observed that certain cells in the molecular layer and in the hilus are not expressing Sp3, contrary to the general assumption that Sp3 is ubiquitously expressed.
Figure 4: Expression of Sp3 and Sp4 transcription factors in the dentate gyrus of adult mice.

(a) Sp3 expression (red) is broadly detected in the molecular layer, dentate granule cell layer and in the hilus of dentate gyrus. Some cells in the molecular layer and in hilus do not express Sp3 (arrowheads). (b) Sp4 expression (green) is detected restrictively in granule cell layer. Very few cells are expressing Sp4 in molecular layer and in hilus. Scale bars 30µm.

I observed that the expression of Sp3 was broader than Sp4 in the subgranular region of the dentate gyrus, suggesting a differential expression pattern in the neurogenic region of the adult hippocampus (Figure 4 and 5). These findings make further investigation Sp3 and Sp4 expression interesting on the regions where they are differentially expressed within the dentate gyrus. For this purpose, I took a closer look at the expression of Sp3 and Sp4 in subgranular zone of the dentate gyrus where progressive generation of new neurons takes place.
Figure 5: Differential Expression of Sp3 and Sp4 in subgranular zone of the dentate gyrus.
Expression of Sp3 (red) (a) and Sp4 (green) (b) transcription factors are shown in the dentate gyrus on the same brain section. Overlap is depicted in (c) (yellow). Sp3 expression is broader than Sp4 expression in the neurogenic region of dentate gyrus. Arrowheads are marking some of the cells that are expressing Sp3 but not Sp4 in the subgranular zone of dentate gyrus. ML molecular layer, HL hilus. Scale bar 40µm.

6.1.1. Sp3 is continuously expressed during adult hippocampal neurogenesis

After detection of a broad expression of Sp3 in dentate gyrus, I further investigated it in more detail in subgranular zone to see Sp3 expression in different stages of neuronal development. In the dentate gyrus of hippocampus, stem cells lie in the subgranular cell layer and continuously give rise to new neurons. The process of adult neurogenesis has several stages which can be discriminated by the morphology of the cells at each stage and by using certain stage specific markers. The primary precursor cell type in this neurogenic lineage is radial glia like stem cells. They are discriminated by a radial process spanning the granule cell layer, and by the expression
of stem cell marker Sox2 and glial marker GFAP. According to the prevailing model, division of the radial glial precursors generates a highly proliferative progenitor population, which can be identified by mitotic cell markers like Ki67. As the cells go out of the mitotic stage and progress through differentiation steps, they start to express immature neuronal markers like DCX and migrate through granule cell layer. Maturation of the cells continues as the cells develop processes and start to express proteins important for synaptic integration. In the mature form, granule cells are positive for the neuronal markers NeuN and Prox-1. I checked the expression of Sp3 in those stages of neuronal development and found that Sp3 expression overlaps with Sox2, DCX and NeuN, suggesting a continuous expression of Sp3 in all stages of neuronal development in the adult hippocampus as depicted in figure 6 below.

Figure 6: Expression of Sp3 during adult hippocampal neurogenesis.

Sp3 (red) is expressed in Sox2 positive progenitor cells, DCX positive immature neurons, and NeuN positive granule neurons of dentate gyrus (depicted in DAPI, gray). Overlay pictures are presented on the right column. Scale bars 10µm.
I took the advantage of retroviral birth-dating as an alternative method to label in vivo newly generated cells in order to study the expression of Sp3 during the development of new dentate granule neurons. The retrovirus that is used in this study is a recombinant form of Moloney murine leukemia retrovirus (MMLV), which needs the breakdown of the nuclear membrane to integrate into the host genome. Virus transduced cells and the progeny of these cells express the transgene coded by the retroviral genome, as well as GFP as a reporter. This allows the tracing of virally transduced newborn neurons in the adult hippocampus (Tashiro et al 2006). Stereotactic injection of GFP reporter retrovirus into the dentate gyrus is capable of transducing proliferating progenitor cells at the time of injection. This approach gives the possibility to time-line the neurogenesis process by sacrificing the animals at different time points after the injection of retrovirus to the brain. Analysis of the newborn cells labeled with GFP reporter retrovirus revealed that Sp3 is expressed from early stages of during adult neurogenesis and remains expressed when the cells mature. 99% of the cells at 2dpi, and every cell that has been analyzed in 7dpi, 14dpi, 21 dpi, 28dpi and 42dpi were positive for Sp3 expression as depicted in figure 7 by immunohistochemistry, suggesting expression of Sp3 in all stages of neuronal development in adult hippocampal neurogenesis.
Figure 7: Sp3 expression in the newborn neurons of adult dentate gyrus.
(a) Representative pictures showing Sp3 expression at different stages of neuronal development in the dentate gyrus. Retroviral birthdating indicates Sp3 expression (red) in 2dpi, 7dpi, 14dpi, 21dpi, 28dpi, and 42dpi in the newborn neurons (labeled with GFP, in green). (b) Quantification of percentage of cells expressing Sp3 at different time points of neuronal development in adult dentate gyrus. Error bars indicate SEM of n=3 animals. (c) Schematic view of Sp3 expression during neurogenesis. Scale bars 10µm.

6.1.2. Sp4 expression is initiated in later stages of adult hippocampal neurogenesis

As pointed before, I found that the expression of Sp3 is broader than Sp4 in the subgranular zone of the dentate gyrus. I used stage specific markers to identify the cells that express Sp4 in the neurogenic lineage. Interestingly, I did not observe Sp4 expression in the Sox2 positive population, indicating that the stem cells do not express Sp4. Another marker, Tbr2, which is expressed in rapidly dividing precursors, did not show co-expression with Sp4 either. I could detect Sp4 in the DCX positive immature neurons, suggesting that Sp4 expression comes with neuronal maturation and it is kept expressed in mature neurons as indicated by the expression of Sp4 in NeuN positive nuclei (Figure 8).
Figure 8: Expression of Sp4 during adult hippocampal neurogenesis.

Sp4 (red) expression is absent in Sox2 positive and Tbr2 positive progenitor population, which represents the early stages of adult hippocampal neurogenesis. Sp4 expression overlaps with immature neuronal marker DCX and neuronal marker NeuN. Overlay pictures are presented on the right column. Scale bars 10µm.

To determine the fraction of the cells that express Sp4 at different stages of neuronal development, I again took the advantage of retroviral birthdating. In line with my co-expression analysis of Sp4 with different stage specific markers, I could not find almost any cells at 2dpi that expresses Sp4, suggesting that Sp4 is not present in progenitor population in subgranular zone. Although in most of the cells the expression was weak, at 7dpi around 47% of the cells were positive for Sp4. With time, the percentage of the cells that expresses Sp4 was increased. At 14dpi 79% of the cells, at 21dpi 91% of the cells and from 28dpi on virtually all the cells were positive for Sp4 expression (Figure 9). These findings strongly suggest that Sp4 is expressed in later stages of neuronal development during adult hippocampal neurogenesis.
Figure 9: Sp4 expression in the newborn neurons of adult dentate gyrus.
(a) Representative pictures showing Sp4 expression at different stages of neuronal development in the dentate gyrus. Retroviral birthdating indicates lack of Sp4 expression (blue) in early stages of neuronal development (GFP expressing newborn neurons, in green). (b) Quantification of Sp4 expressing cells at different time points of neuronal development in adult dentate gyrus indicating increase in the percentage of Sp4 expressing cells with neuronal maturation, reaching 100% at 42dpi. Error bars indicate SEM of n=3 animals. (c) Schematic view of Sp3 expression during neurogenesis. Scale bars 8µm.

6.2. Sp3 might be involved in maturation of neural stem cells in DG

6.2.1. Knockdown of Sp3 in adult newborn neurons of dentate gyrus in vivo

6.2.1.1. Efficiency of Sp3 microRNA in vitro and in vivo

After determining the expression pattern of Sp family members in the neurogenic lineage of adult hippocampus, I questioned the role of these transcription factors in adult neurogenesis. To investigate the role of Sp3 in adult neurogenesis, I studied the effects of Sp3 knockdown on the development of adult born hippocampal neurons. For this purpose I used retroviral vectors that contain microRNA against Sp3 coding sequence. To start with, three microRNAs were designed against Sp3 in silico and cloned into retroviral plasmid. As a control I used a scrambled microRNA cloned to the same retroviral backbone, which I call as control microRNA from here.
against Sp3 in silico and cloned into retroviral plasmid. As a control I used a scrambled microRNA cloned to the same retroviral backbone, which I call as control microRNA from here on. To determine the knockdown efficiency of the microRNAs I transfected microRNA constructs into the HEK cells together with a mouse Sp3 overexpression vector. According to the western blot analysis 70 hours post-transfection, microRNA-3 was able to reduce the amount of Sp3 to almost zero level whereas control microRNA did not effect Sp3 levels (Figure 10).

![Western Blot Image](image)

**Overexpression in HEK Cells, 70 hours**

Figure 10: Sp3 microRNA efficiencies in vitro.
Three different microRNA constructs (traced by GFP expression) against Sp3 are transfected to HEK cells together with an HA-tagged Sp3 overexpression plasmid. Knockdown efficiency of the microRNAs can be seen by the disappearance of overexpressed Sp3 traced by HA tag. Sp3 microRNA 3 had the most efficient knockdown ability in vitro.

I next examined the efficiency of this microRNA in vivo since there might be some discrepancy between in vitro and in vivo knockdown efficiency. After producing retroviruses from the control microRNA and Sp3 microRNA constructs, I injected the retroviruses into the dentate gyrus of two different groups of animals. At 14dpi animals are sacrificed and the microRNA efficiency was analyzed according to the reduction of the endogenous Sp3 level in Sp3 microRNA positive cells (traced by GFP expression) compared to the control microRNA positive cells (traced by GFP expression). As depicted in figure 11, I could detect less Sp3 expression in 75% of the Sp3 microRNA infected cells; whereas this percentage was 5% in control microRNA infected cells indicating a high knockdown efficiency of the Sp3 microRNA in vivo.
Figure 11: Knockdown efficiency of Sp3 microRNA in vivo.
(a) Injection of the retrovirus encoding the microRNA against Sp3 (traced by GFP expression, in green) was able to reduce the amount of Sp3 protein (in red) at 14dpi in vivo. Scale bars 10µm.
(b) Quantification of Sp3 microRNA efficiency. Error bars indicate SEM of n=3 animals.

6.2.1.2. Knock down of Sp3 in newborn neurons in the dentate gyrus increases dendritic length and branching at 14dpi

In order to understand the importance of Sp3 in neuronal development in adult hippocampal neurogenesis I used a gene knockdown approach using the retroviruses coding microRNA against Sp3, as mentioned before. This viral backbone codes GFP within its sequence, which was used to trace the cells that are infected with the retrovirus. In addition, I always injected microRNA GFP viruses together with an RFP reporter virus, which served as an internal control in all experiments.
Knockdown of Sp3 in vivo results a more complex neuronal phenotype at 14dpi. Control microRNA GFP labelled cells (green) are depicted in (a) whereas Sp3 microRNA GFP labelled cells (green) are in (b). Three representative sections are shown from three different animals for each group. Scale bar 50µm.

To quantify the morphology differences between control microRNA and Sp3 microRNA infected newborn neurons, I used IMARIS software to trace the whole morphology of the dendrites. For morphology analysis I used the cells that are double infected with microRNA GFP and internal control RFP viruses. Morphology of those cells is traced in RFP channel due to weak expression of GFP in microRNA viruses. Since the GFP expression was weak, the distribution of the GFP to the distal part of the dendrites were poor. Therefore I based the morphology analysis on RFP which seemed strongly expressed and equally distributed along the dendrites. On a given coronal section, I excluded the tips of DG and analyzed the morphology of every double infected cell in the upper granule cell layer to exclude cell-selection bias in morphology experiments.

The comparison of control microRNA and Sp3 microRNA infected cells revealed that the average dendritic length of the Sp3 microRNA infected cells (695µm) were almost twice bigger than control microRNA infected newborn neurons (369 µm). Additionally, I found a significant increase in the average number of branch points in Sp3 microRNA infected cells (13.11)
compared to controls (8.5). Dendritic length and branching did not differ between control microRNA infected cells and only RFP infected cells on the same sections, indicating that the control microRNA virus itself doesn’t have an effect on dendritic length and branching. Additionally, Sholl analysis of the infected cells revealed that the overall complexity of the microRNA infected neurons is significantly increased. These results altogether suggest that in vivo knockdown of Sp3 in newborn neurons increases in general the dendritic complexity, both dendritic length and dendritic branching during adult hippocampal neurogenesis at 14dpi (Figure 14).
Figure 14: Effect of Sp3 knockdown on neuronal morphology at 14 dpi in vivo.
(a) MicroRNA mediated knockdown of Sp3 resulted a significant increase in the dendritic length and dendritic branching in newborn neurons at 14dpi. No obvious difference was found between control microRNA infected and internal control RFP infected cells, indicating no effect of the microRNA virus itself on dendritic morphology. (b) Sholl analysis of newborn neurons upon Sp3 knockdown at 14 dpi in vivo. MicroRNA mediated knockdown of Sp3 increased dendritic complexity of the newborn neurons at 14dpi in vivo. Maximum number of average sholl intersections is 5.39 in control micro RNA infected cells whereas this value is 7.76 in the Sp3 microRNA infected cells at 14dpi. Error bars indicate SEM of n=3 animals per group.

6.2.1.3. Increase in the dendritic length and branching persists at 28dpi in Sp3 knockdown cells

Since the knockdown of Sp3 increases dendritic complexity of the newborn neurons at 14dpi, I next questioned whether increased dendritic growth is transient or persistent. For this purpose, I decided to follow the morphology of the microRNA infected cells at a later stage of neuronal development. I injected two sets of animals with control microRNA GFP virus and Sp3
microRNA GFP virus, each time together with the internal control virus RFP. The animals are sacrificed and analyzed at 28dpi like described before for 14dpi.
Morphology analysis revealed that the average dendritic length was increased and average dendritic branching was bigger in Sp3 microRNA infected cells compared to control microRNA infected cells. Although the difference was not as high as I observed at 14 dpi, Sp3 microRNA infected cells were significantly longer than control microRNA infected cells at 28dpi. I next examined the overall complexity of these cells, which is depicted in the Sholl diagram. At 28dpi, dendritic complexity of the Sp3 microRNA infected cells were higher than control microRNA infected cells suggesting that knockdown of Sp3 in newborn neurons results an increase in overall complexity of the dendrites throughout the development of neurons until 28dpi during adult hippocampal neurogenesis (Figure 15).
Figure 15: Effect of Sp3 knockdown on neuronal morphology at 28 dpi in vivo.
(a) Increase in dendritic length and dendritic branching upon Sp3 knockdown in newborn neurons lasted until 28dpi. (b) Sholl analysis of newborn neurons upon Sp3 knockdown at 28 dpi in vivo. Increased dendritic complexity of the newborn neurons lasted until 28dpi in vivo as depicted in the Sholl diagram of control microRNA and Sp3 microRNA infected cells.

6.2.1.4. Expression of stage specific markers reveals accelerated maturation in immature neurons upon knockdown of Sp3

My morphological observations upon Sp3 knockdown in neural progenitor cells showed a structurally more mature phenotype of newborn neurons in 14dpi and 28dpi during adult hippocampal neurogenesis in vivo. I next examined the expression of stage specific markers upon Sp3 knockdown to investigate a possible accelerated maturation phenotype that goes parallel to accelerated structural maturation that I observed on the dendritic tree of these neurons. A summary of the stage specific immunohistochemical markers is given below in figure 16.
Figure 16: Expression of stage specific markers in newborn neurons.

Figure showing different stages of adult neurogenesis with stage specific immunohistochemical markers. Adult neurogenesis stages are illustrated from radial glia like stem cells to mature neuron stage. Radial glia like stem cells express GFAP (Glial Fibrillary Acidic Protein), Sox2 and Nestin. Sox2 and Nestin expression persist in progenitor cells. In highly proliferative neuroblast stage, cells are no longer positive for these stem cell markers. DCX expression starts early in the neuroblast stage and lasts until late immature neuron stage; which makes DCX a suitable marker to trace accelerated maturation phenotypes in later stages of adult neurogenesis. Similarly, calretinin, a calcium binding protein has a restricted expression in the immature neuron stage, which is no more expressed in the mature neurons. Mature neurons can be characterized with another calcium related marker called calbindin. (Modified from von Bohlen and Halbach, 2007)

According to the previous reports on adult neural stem cell maturation in vivo, there are distinct stage specific markers which might discriminate a possible accelerated maturation phenotype at the time points I worked, which are 14 and 28 days after injection of retrovirus. Therefore I examined the expression of certain relevant markers in Sp3 microRNA infected cells, including DCX and calretinin. DCX, also known as doublecortin, is a microtubule-associated protein expressed in migrating neuroblasts and immature neurons (von Bohlen und Halbach, 2011). According to Jagasia et al, nearly 60% of the newborn cells already start to express DCX at 3dpi. At 14 dpi, almost 90% of the newborn cells are positive for DCX and it decreases to 40% at 28dpi as the cells mature (Jagasia et al 2009). Calretinin and calbindin are calcium binding proteins being expressed sequentially during postmitotic phases of adult neurogenesis in DG. Therefore calretinin has a transient expression pattern in immature neuron stage, which is also
DCX positive, and then the expression of calretinin is shut off in mature neurons (von Bohlen und Halbach, 2011). The transient expression of DCX and Calretinin makes these two markers suitable for exploring any acceleration / delay phenotype during neuronal maturation of newborn cells in the DG at the postmitotic stages. In addition, I examined the expression of Egr1, an immediate early gene that starts to get expressed upon neuronal excitation only at later stages of neuronal development. Any accelerated maturation phenotype might increase the percentage of the Egr1 positive proportion of immature neurons for a given time point.

I categorized the calretinin positive cells as low intensity and high intensity calretinin expressing cells. When calretinin starts to get expressed, the intensity is low and when the cells mature further, the intensity gets higher. Interestingly, I found that high calretinin positive cells were significantly higher in the Sp3 microRNA infected cells at 14dpi (Figure 17). This might indicate that upon Sp3 KD, cells are at a more mature stage at 14dpi compared to control cells. The percentage of DCX positive cells and Egr1 positive cells did not differ between Sp3 microRNA infected cells and control microRNA infected cells at 14dpi. I couldn’t detect any difference in DCX and Egr1 expression at 28dpi either. In short, calretinin but not DCX and Egr1 were suitable to discriminate the accelerated maturation phenotype in Sp3 KD neurons.

![14dpi Sp3 Knockdown Calretinin Levels](image)

**Figure 17: Quantification of calretinin expression upon Sp3 knockdown at 14dpi in vivo.** Investigation of calretinin protein levels in control miRNA infected and Sp3 microRNA infected newborn neurons at 14dpi in vivo showed an increase in the high calretinin expressing cells in Sp3 microRNA infected cells.
6.2.2. FHOD1 might be a downstream target of sumoylated Sp3 in adult DG

6.2.2.1. Transcriptome profiling reveals differentially regulated genes in the DG of sumoylation deficient Sp3kiki mice

Morphological analysis of newborn neurons upon Sp3 knockdown showed more dendritic complexity, which might indicate that Sp3 is acting as a repressor for neuronal maturation during adult neurogenesis. In order to identify possible downstream targets of Sp3 in dentate gyrus which might underlie the phenotype that was observed upon Sp3 knockdown, I used the sumoylation deficient Sp3 knock-in (Sp3kiki) mouse model which is described in detail at the introduction part of this thesis. As pointed before, sumoylation of the Sp3 was reported to be related to its repressive function on certain promoters. Therefore I used the Sp3kiki mice first to identify the sumoylated Sp3 targets in the dentate gyrus of adult mice. I dissected the dentate gyrus of 4 Sp3kiki and 4 wildtype mice which is used further for RNA preparation and transcriptome profiling which is performed in collaboration with Dr. Martin Irmler (Experimental Genetics department, Helmholtz Research Center Munich). I identified a set of genes whose expression was altered in the dentate gyrus of Sp3kiki mice. In line with the repressive function of Sp3, most of the differentially expressed genes were upregulated in Sp3kiki mice. The most significantly altered gene was FHOD1 with 5.2-fold upregulation (Figure 18). As described in detail in the introduction part of this thesis, FHOD1 is an actin cytoskeleton related protein, which is reported to be involved in cell migration and cell elongation previously in the literature. Therefore I focused on FHOD1 as a possible downstream target of Sp3 that might underlie the phenotype that I observed upon knockdown of Sp3.
Figure 18: List of altered genes in the dentate gyrus of Sp3ki/ki mice.
Microarray analysis of transcriptome in the dentate gyrus of 4 control and 4 Sp3 ki/ki mice revealed altered expression of Tmem159, Reep6, Dnahc1, Ercc2, Cd6, Got111, Prtn3, Amn, Rbm45, Slc5a5, Fhod1, and Apoc1 genes. Fold change of mutants over controls are depicted on the left column for each gene. Expression level of each gene in every animal is given in a color code which is explained in the legend at the top. Red, up-regulation.

In order to confirm the microarray data on FHOD1 expression with an independent technique, I used quantitative PCR. The same RNA samples that are used for the microarray analysis are used for quantitative PCR analysis. I determined the FHOD1 levels in every control and Sp3kiki RNA samples separately by quantitative PCR. Consistent with the microarray data, I could detect around 5-fold upregulation of FHOD1 in Sp3kiki samples compared to controls (Figure 19), which confirmed the upregulation of FHOD1 in the dentate gyrus of Sp3kiki mice.

<table>
<thead>
<tr>
<th>Mean ratio (mut/wt)</th>
<th>Gene name</th>
<th>Gene symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.54</td>
<td>Tmem159</td>
<td>transmembrane protein 159</td>
</tr>
<tr>
<td>1.37</td>
<td>Reep6</td>
<td>receptor accessory protein 6</td>
</tr>
<tr>
<td>1.32</td>
<td>Dnahc1</td>
<td>dynein, axonemal, heavy chain 1</td>
</tr>
<tr>
<td>1.46</td>
<td>Ercc2</td>
<td>exocision repair cross-complementing rodent repair deficiency, complementation group 2</td>
</tr>
<tr>
<td>1.46</td>
<td>Cd6</td>
<td>CD6 antigen</td>
</tr>
<tr>
<td>1.63</td>
<td>Got111</td>
<td>glutamic-excitoacid transaminase 1-like 1</td>
</tr>
<tr>
<td>1.70</td>
<td>Prtn3</td>
<td>proteinase 3</td>
</tr>
<tr>
<td>1.73</td>
<td>Amn</td>
<td>amionless</td>
</tr>
<tr>
<td>2.17</td>
<td>Rbm45</td>
<td>RNA binding motif protein 45</td>
</tr>
<tr>
<td>2.28</td>
<td>Slc5a5</td>
<td>solute carrier family 5 (sodium iodide symporter), member 5</td>
</tr>
<tr>
<td>5.21</td>
<td>Fhod1</td>
<td>formin homology 2 domain containing 1</td>
</tr>
<tr>
<td>2.37</td>
<td>Apoc1</td>
<td>apolipoprotein C-I</td>
</tr>
<tr>
<td>1.94</td>
<td>Apoc1</td>
<td>apolipoprotein C-I</td>
</tr>
</tbody>
</table>
Figure 19: QPCR confirmation of changes in the Fhod1 level.
Fhod1 upregulation in the DG of Sp3ki/ki mice is confirmed by using QPCR. I could detect a 4.5-fold induction in the dentate gyrus of Sp3kiki mice compared to control mice. Error bars indicate SEM of n=3 animals.

6.3. Sp4 might be involved in migration and dendritic development of newborn neurons during adult neurogenesis

6.3.1. Knockdown of Sp4 in adult newborn neurons in vivo

6.3.1.1. Efficiency of Sp4 microRNA in vitro and in vivo

As pointed before, I determined the expression pattern of Sp4 in the neurogenic lineage of adult hippocampus, which indicated a neuronal expression of Sp4 in later stages of neurogenesis whereas an absolute lack of expression in the early stages of adult neurogenesis. In order to investigate the role of Sp4 in adult neurogenesis, I followed the same strategy that I took for Sp3, and used the microRNA mediated knockdown approach in vivo. Three different microRNAs were designed against Sp4 in silico, cloned into retroviral plasmid, and transfected into the HEK cells together with a mouse Sp4 overexpression vector to determine the knockdown efficiency of the microRNAs in vitro. Western blot analysis after 70 hours post-transfection revealed that two of the microRNAs were able to reduce the amount of Sp4 efficiently (Figure 20) and I chose the microRNA 2 for further in vivo experiments.
Figure 20: Sp4 microRNA efficiencies in vitro.

Three different microRNA constructs (traced by GFP expression) against Sp4 are transfected to HEK cells together with an HA-tagged Sp4 overexpression plasmid. Knockdown efficiency of the microRNAs can be seen by the disappearance of overexpressed Sp4 traced by HA tag. Sp4 microRNA 2 and 3 had convincing knockdown ability in vitro. MicroRNA 2 is chosen for further in vivo experiments.

For the purpose of determination of microRNA efficiency in vivo I produced retroviruses from the control microRNA and Sp4 microRNA constructs and I injected the retroviruses into the dentate gyrus of two different groups of animals with the same methodology as described for Sp3. At 14dpi, animals are sacrificed and the microRNA efficiency was analyzed according to the reduction of the endogenous Sp4 level in Sp4 microRNA positive cells (traced by GFP expression) compared to the control microRNA positive cells (traced by GFP expression).

According to my retroviral timeline experiments, as described in the results before, Sp4 expression starts when the cells mature and the intensity of the Sp4 is increasing when the cells mature. At 14dpi, 78.6% of the newborn cells are positive for Sp4 and among the positive cells the intensity of the Sp4 differs. Therefore, to determine the reduction of Sp4 level upon knockdown, first I quantified the average intensity of Sp4 in the nucleus for a given Sp4 microRNA positive cell (traced by GFP expression). Secondly, I normalized this intensity to the average Sp4 intensity of the granule cell layer on the corresponding z-step of the confocal scan. This normalization eliminates a possible bias in Sp4 intensity that might result from Sp4 antibody penetration during immunohistochemical staining. For every control microRNA and Sp4 positive cell, this ratio is determined. Then I used these values to compare the intensity of Sp4 in control
microRNA and in Sp4 microRNA positive cells. Analysis of knockdown efficiency using this strategy revealed a 50% reduction of the Sp4 intensity in Sp4 microRNA infected cells compared to control microRNA infected cells at 14dpi (Figure 21).

Figure 21: Knockdown efficiency of Sp4 microRNA in vivo.
(a) Representative pictures of Sp4 microRNA efficiency in vivo. Injection of the retrovirus encoding the microRNA against Sp4 was able to reduce the amount of Sp4 protein (red) at 14dpi in vivo. Scale bar 10µm. (b) Quantification of Sp4 microRNA efficiency. Error bars indicate SEM of n=3 animals.

6.3.1.2. Loss of Sp4 increases dendritic length but not dendritic branching at 14dpi

To investigate the role of Sp4 in neuronal development in adult hippocampal neurogenesis, I followed the same microRNA mediated gene knockdown strategy that I used for Sp3. 6 days prior to stereotactic injections of the retroviruses, adult female mice are put on a cage with running wheel to increase the amount of proliferating cells in the neurogenic niche of adult
hippocampus. Control microRNA and Sp4 microRNA are injected into two separate groups of animals, in both cases an RFP reporter virus is co-injected within a cocktail with microRNA GFP virus. Injected animals are kept on cages containing running wheel until the end of the experiment and for analysis I sacrificed the animals 14 days after injection. The brains are prepared for immunohistochemical analysis as described in the methods part of this thesis. Morphology differences of the dendrites between control microRNA and Sp4 microRNA infected newborn neurons are quantified via IMARIS software. For morphology analysis I used the cells that are double infected with microRNA GFP and internal control RFP viruses. Morphology of those cells is traced in RFP channel due to weak expression of GFP in microRNA viruses. On a given coronal section, I excluded the tips of DG and analyzed the morphology of every double infected cell to exclude selection bias in morphology experiments. Interestingly, I found that the dendritic length of the Sp4 microRNA infected cells were bigger than control microRNA infected newborn neurons at 14dpi, but I could not observe any differences in the number of branch points in Sp4 microRNA infected cells compared to controls. This finding differs from what I observed upon Sp3 knockdown at 14dpi, where both dendritic length and dendritic branching were increased. Sholl analysis showed that the overall dendritic complexity of the Sp4 microRNA infected cells is higher than control microRNA infected cells (Figure 22). These results altogether suggest that in vivo knockdown of Sp4 in newborn neurons increases in general the dendritic complexity, interestingly the dendritic length but not dendritic branching during adult hippocampal neurogenesis at 14dpi.
**Figure 22: Effect of Sp4 knockdown on neuronal morphology at 14 dpi in vivo.**

(a) MicroRNA mediated knockdown of Sp4 resulted a significant increase in the dendritic length but not in dendritic branching in newborn neurons at 14dpi. No obvious difference was found between control microRNA infected cells and internal control RFP infected cells, indicating no effect of the microRNA virus itself on dendritic morphology. (b) Sholl analysis of newborn neurons upon Sp4 knockdown at 14 dpi in vivo. microRNA mediated knockdown of Sp4 increased overall dendritic complexity of the newborn neurons at 14dpi in vivo.

6.3.1.3. Dendritic length and branching does not differ upon Sp4 knockdown at 28dpi in vivo

After the observation that dendritic length but not branching is increased upon Sp4 knockdown at 14dpi I next questioned whether this morphological difference persists. I analyzed the morphology of the Sp4 knockdown cells at 28dpi. Two sets of animals are injected with control microRNA GFP virus and Sp4 microRNA GFP virus, each time together with RFP control virus as an internal control. The animals are sacrificed and analyzed for their morphology at 28dpi as described before for Sp3.
In contrast to Sp3 knockdown data, I could not observe any significant difference in dendritic length and dendritic branching in Sp4 microRNA infected cells compared to control microRNA infected cells at 28dpi. The overall dendritic complexity of Sp4 knockdown cells did not differ according to Sholl analysis at 28dpi. These results suggest that Sp4 knockdown in neural progenitor cells only transiently affect the dendritic development of these neurons, which is not visible at 28dpi anymore (Figure 23).

**Figure 23: Effect of Sp4 knockdown on neuronal morphology at 28 dpi in vivo.**
(a) Analysis of dendritic length and dendritic branching did not show any significant difference between control microRNA infected and Sp4 microRNA infected cells at 28 dpi. (b) Sholl analysis of newborn neurons upon Sp4 knockdown at 28 dpi in vivo. microRNA mediated knockdown of Sp4 did not affect the overall dendritic complexity of the newborn neurons at 14dpi in vivo as depicted in the Sholl diagram.
6.3.1.4. Knockdown of Sp4 delays the migration of newborn neurons along granule cell layer of dentate gyrus

In dentate gyrus, progenitor cells that are identified with the expression of stem cell marker Sox2 are lying in the subgranular zone, close to the hilus of the dentate gyrus. Sox2 positive nuclei of the progenitors can be observed as integrated to or separate from subgranular cell layer. As the maturation and differentiation of the neurons take place, they start to migrate towards the granular cell layer and get integrated to lower, middle or upper third of the granule cell layer. During my analysis of Sp4 microRNA injected coronal brain sections, I observed that in the dentate gyrus, some of the cell bodies of Sp4 infected cells at 14dpi were lying more towards the hilus rather than being integrated in the granule cell layer. Figure 24 below shows representative pictures of these misplaced cells.

![Representative pictures of Control microRNA and Sp4 microRNA infected cell bodies (traced by GFP expression). Control cells are mostly integrated to the granule cell layer (depicted in red), whereas some of the Sp4 knockdown cells appear to lie in the hilus at 14dpi. HL hilus, ML molecular layer. Scale bars 40µm.](image)

Figure 24: Cell body localization of the Sp4 microRNA infected cells in dentate gyrus at 14dpi in vivo. Representative pictures of Control microRNA and Sp4 microRNA infected cell bodies (traced by GFP expression). Control cells are mostly integrated to the granule cell layer (depicted in red), whereas some of the Sp4 knockdown cells appear to lie in the hilus at 14dpi. HL hilus, ML molecular layer. Scale bars 40µm.

After the initial observation of Sp4 microRNA infected cell bodies lying more towards the hilus rather than in subgranular zone at 14dpi, I hypothesized that Sp4 might be involved in the migration of newborn neurons towards granular zone. In order to observe any possible migration deficiency in Sp4 microRNA infected cells on those coronal sections, I divided the granule cell layer into three sub-layers which are lower, middle, and upper granule cell layer. Taking this separation as a reference, I quantified the exact cell body localizations within each third of the granule cell layer. If more than half of a cell nucleus was lying in the hilus compared to two surrounding nuclei at the subgranular zone–hilus border, I considered this cell as lying in the hilus. The nuclei that were separate from subgranular cell layer and lying in the hilus was
considered as lying in the hilus as well. According to this standardization, I quantified the cell body localizations in control microRNA infected, in Sp4 microRNA infected and although I didn’t observe any differences in cell body localization on the brain sections by eye, in Sp3 microRNA infected cells. Quantification of cell body localizations at 14dpi revealed significantly more cells lying in the hilus upon knockdown of Sp4, but not upon knockdown of Sp3 compared to control microRNA infected cells (Figure 25). I also observed tendency to a decrease in the percentage of Sp4 microRNA infected cells in the upper and middle granule cell layer compared to percentage of control microRNA infected cells on those two layers.

Figure 25: Quantification of cell body localizations upon knockdown of Sp3 and Sp4 in the dentate gyrus at 14dpi. Cell body localizations of newborn neurons upon knockdown of Sp3 is not altered significantly in lower, middle and upper granule cell layer and in the hilus. Reduction of Sp4 resulted in significantly more cell bodies lying in the hilus. Although not significant, there was a reduction in the Sp4 microRNA positive cells in the upper granule cell layer compared to control microRNA positive cells which altogether might indicate a possible delay in the migration of newborn neurons upon reduction of Sp4 levels. Error bars indicate SEM of n=3 animals.

After having the first evidence that knockdown of Sp4 in neural progenitor cells might alter cell body localization of the migrating cells at 14dpi, I next examined the cell body localization of Sp4 microRNA infected cells at 28dpi, to see whether this effect lasts till later stages of neuronal development. Interestingly, at this later stage, the percentage of Sp4 microRNA infected that are lying in the lower granule cell layer was significantly more than the percentage of control microRNA infected cells in lower granule cell layer. In addition, in the middle granule cell layer the percentage of Sp4 microRNA infected cells was lower than the percentage of control microRNA infected cells. These results altogether might suggest that Sp4 microRNA infected...
cells are migrating slower through the granule cell layer than control microRNA infected cells. The cells that were infected with Sp3 microRNA did not show any significant alterations in cell body localizations, which served as a negative control strengthening the idea that the migration phenotype is specifically due to downregulation of Sp4 (Figure 26).

![Cell Body Localization at 28dpi](image)

**Figure 26**: Quantification of cell body localization upon knockdown of Sp3 and Sp4 in the dentate gyrus at 28dpi. Cell body localization of newborn neurons upon knockdown of Sp3 is not altered significantly in lower, middle and upper granule cell layer and in the hilus. Reduction of Sp4 resulted in significantly more cell bodies lying in lower granule cell layer and significantly less cell bodies lying in the middle granule cell layer which might suggest a slower migration towards upper granule cell layer. Error bars indicate SEM of n=3 animals.

6.3.1.5. Knockdown of Sp4 does not affect survival of newborn neurons between 14 and 28dpi

To further investigate that the cell body localization alterations upon Sp4 knockdown are not due to altered cell survival, I examined the cell survival between 14dpi and 28dpi upon Sp4 knockdown. A well-defined methodology for survival analysis was followed for this purpose. I injected control microRNA GFP + RFP control virus to 10 animals from the same mixture of the virus, making sure that the initial GFP/RFP ratio that is injected to every animal is the same. Similarly, I injected Sp4 microRNA GFP + RFP control virus to another set of 10 animals, again from the same mixture of the virus. I sacrificed half of the animals from each of the control and Sp4 microRNA injected group at 14dpi and the second half of the animals at 28dpi. If there is no cell death going on between day 14 and day 28 due to the decrease of Sp4, (GFP+RFP)/All RFP ratio in Sp4 microRNA infected animals normalized to control microRNA infected animals should stay the same between day 14 and day 28. As illustrated in figure 27, after the analysis of
cell survival upon Sp4 knockdown, I could not observe any significant changes in the cell survival between 14dpi and 28dpi, supporting the finding that cell body localization alterations are due to the knockdown of Sp4 and not due to alterations in cell survival.

![Survival Analysis in Sp4 Knockdown](image)

**Figure 27:** Cell survival analysis of newborn neurons upon Sp4 knockdown between 14 dpi and 28dpi. Sp4 miRNA GFP and Control RFP viruses are injected together to the dentate gyrus of adult mice and animals are sacrificed at 14dpi and at 28dpi time points. Same strategy is followed for Control microRNA GFP and Control RFP viruses. microRNA GFP and Control RFP double positive cells are indicated as yellow. Only RFP positive cells are indicated as red. The yellow/(yellow+red) ratio did not differ between 14dpi and 28dpi time points for both Control microRNA and Sp4 microRNA groups indicating no significant cell death between 14dpi and 28dpi. Error bars indicate SEM of n=3 animals.

### 6.3.1.6. Knockdown of Sp4 delays the migration of newborn neurons in the SVZ- RMS-OB neurogenesis in adult brain at 5dpi

In the dentate gyrus of hippocampus, newborn neurons are migrating from subgranular layer to the granular zone, a distance that usually is 10-15 cell bodies high. While my results clearly demonstrated a mispositioning of a fraction of Sp4 knockdown cells, the extent to which Sp4 is important for neuronal migration is difficult to judge. In the adult brain there is a second neurogenic niche where progenitor cells are newly generated at the subventricular zone, and migrate a long distance through the RMS to reach the OB. Due to long distance that newborn neurons travel, the SVZ-RMS-OB system is a nice model to use for cell migration analysis. Therefore I aimed to investigate the migration of newborn neurons more clearly upon Sp4 knockdown in the SVZ-RMS-OB neurogenic system.
For this purpose, I injected the control microRNA and the Sp4 microRNA viruses to two separate groups of animals, close to the subventricular zone of lateral ventricles. For each group, I injected the microRNA viruses together with an internal RFP control virus within a cocktail. At 5dpi the animals are sacrificed and the brains prepared and cut sagittal to observe SVZ-RMS-OB route of newborn neurons. For the analysis of the migration, I approximately divided the SVZ-RMS-OB route into four areas of consideration, which are SVZ, RMS1, RMS2, and OB. For quantification of migration, I counted the number of GFP positive cells (infected with Sp4 microRNA-GFP retrovirus) within each quarter of the RMS, and calculated the percentage of the GFP positive cells for each quarter among all GFP positive cells in all quarters. Additionally, on the same brain section, I counted and calculated the percentage of RFP positive cells (infected with Control RFP retrovirus) in the same way, which was internal control. Percentage of GFP positive cells normalized to percentage of RFP positive cells within each quarter of the RMS is represented for both Sp4 microRNA and control microRNA injected animals.

Interestingly, GFP positive cells were more frequently observed in the first half of the RMS in Sp4 knockdown context compared to controls. The number of Sp4 knockdown cells that reached olfactory bulb was relatively less compared to control microRNA injected animals.
Figure 28: Migration analysis of Sp4 KD in SVZ neurogenesis à Control miRNA

Representative confocal image of control miRNA (green) and internal control CAG-RFP (red) infected cells at 5dpi. For migration analysis, the area traveled by newborn neurons are divided into four parts which are SVZ, RMS1 (the half of RMS facing SVZ), RMS2 (the half of RMS facing OB), and OB. For each quarter, green/red cell ratio is considered as normalized cell number per quarter. Normalized cell number for each quarter divided by normalized cell numbers in total (SVZ + RMS1 + RMS2 + OB) represents the migration percentage of miRNA labeled cells to that quarter. Magnified sections of pieces of SVZ, RMS1, RMS2 and OB are shown subsequently. At 5dpi, there are few miRNA infected (green) cells left at SVZ, whereas lots of the cells migrated already through RMS and OB. Scale bars 60µm.
Figure 29: Migration analysis of Sp4 KD in SVZ neurogenesis via Sp4 miRNA

Representative confocal image of Sp4 miRNA (green) and internal control CAG-RFP (red) infected cells at 5dpi. For migration analysis, the area traveled by newborn neurons are divided into four parts which are SVZ, RMS1 (the half of RMS facing SVZ), RMS2 (the half of RMS facing OB), and OB. For each quarter, green/red cell ratio is considered as normalized cell number per quarter. Normalized cell number for each quarter divided by normalized cell numbers in total (SVZ + RMS1 + RMS2 + OB) represents the migration percentage of miRNA labeled cells to that quarter. Magnified sections of pieces of SVZ, RMS1, RMS2 and OB are shown subsequently. At 5dpi, there are still lots of miRNA infected (green) cells left at SVZ and most of them are stacked at RMS1, whereas only few of the cells could migrate to RMS2 and OB. Scale bars 60µm.

As depicted in figures 28 and 29 above, the GFP/RFP intensity seemed to be more in the SVZ and in the first half of the RMS (RMS1) of Sp4 miRNA injected animals compared to control miRNA injected animals. Upon this observation by eye, I quantified the cell percentages in each quarter as described before to see whether there is a significant delay in the migration of Sp4 miRNA infected cells. According to the quantifications, Sp4 miRNA infected cells were significantly more in the SVZ and in the first half of the RMS. Since Sp4 KD cells are stacked in the first half of the RMS due to a possible migration deficiency, I can observe significantly less Sp4 KD cells that could reach to OB compared to control cells. Quantification of migration attenuation is given in figure 30.
Figure 30: Migration analysis of Sp4 down-regulation in SVZ-RMS-OB system at 5dpi

Quantification of the cell migration from SVZ towards OB at 5dpi is shown. For migration analysis, the area traveled by newborn neurons are divided into four parts which are SVZ, RMS1 (the half of RMS facing SVZ), RMS2 (the half of RMS facing OB), and OB. For each quarter, green/red cell ratio is considered as normalized cell number per quarter. Normalized cell number for each quarter divided by normalized cell numbers in total (SVZ + RMS1 + RMS2 + OB) represents the migration percentage of miRNA labeled cells to that quarter. One section from 3 animals per control and Sp4 KD group were quantified. Error bars indicate SEM of 3 animals for each quarter. Significantly more cells were stacked at SVZ and at the first half of the RMS upon Sp4 KD, which resulted in significantly less cells reaching the OB upon Sp4 KD compared to control miRNA infected cells.
7. DISCUSSION

Neurogenesis has been defined as the generation of newborn neurons from neural progenitors (Kempermann et al. 1998). In mammals, neurogenesis was thought to be present only during development whereas adult brain was believed to be “established”, meaning “neurogenesis-free”. After the discovery of neural stem cells in adult rat brain in 1965 by Altman and colleagues, the possibility that newborn neurons can be formed in the adult brain was re-examined (Altman & Das, 1965). Since then, adult neurogenesis is found to be present in several mammalian species including mice and humans, and defined as generation of newborn neurons within the environment of the adult brain (Szulwach et al. 2010).

7.1. Regulation of adult neurogenesis

During embryonic development, many different regions of the brain contain pools of neural stem cells which massively produce neurons and glia. However, in the adult brain, there are only two brain areas within the established brain circuitry that harbor neural stem cell pools, which are actively engaged in neurogenesis (Zhao et al, 2008). In order to understand the regulation of proper neuronal development within an adult neurogenic niche, it is important to highlight some facts about adult neurogenesis.

First of all, stem cells of these pools exhibit glial properties which are quickly down-regulated upon differentiation into neurons (Bracko et al, 1012). Therefore, several genes that switch their expression from glial to neuronal stages are expected to be important during this transition and have been widely studied to understand the regulation of neuronal development in the adult brain. Important examples of those genes include Sox2 and GFAP, which are expressed in the glial like stem cells of the “early” stages of adult neurogenesis and their expression is shut off when neuronal differentiation progresses in later stages. On the other hand, genes like NeuN, Calretinin and Calbindin are not expressed in the glial like stem cells but their expression starts in later stages of neuronal development. It has been reported that such genes that are stage specifically expressed can be important for the regulation of adult neurogenesis (Reviewed in Ming and Song, 2011).

Secondly, in the adult neurogenic niche where neurogenesis occurs; stem cells, progenitor cells, immature neurons, mature neurons and glial cells are present at a certain time in the same region of the brain. Therefore external signals that regulate those stages of neuronal development are
found altogether in the neurogenic region, representing a complex “external signaling” environment (Faigle and Song 2013, Suh et al 2009, Mu et al 2010). Within this complex signaling environment, different stages of neurogenesis occur at the same time. Therefore progression of those different stages of distinct cells within the same neurogenic niche has to be strictly regulated by internal control mechanisms of each individual cell, illustrating the importance of cell intrinsic programs in adult neurogenesis (Johnson et al, 2009). This phenomenon makes “internal regulators” of adult neurogenesis (like transcription factors) especially important to be able to understand how adult neurogenesis process is governed internally and specifically despite a batch of complex external signaling that is going on within the adult neurogenic niche.

In short, genes that have a switch in their expression during glial to neuronal transition and genes that are involved in cell intrinsic and cell specific response to a complex external signaling environment (like transcriptional regulators) are especially crucial to understand how neurogenesis in the adult brain is regulated. Genes that carry both properties in common were my priority to investigate in my thesis. In this study I have focused on one of the transcription factor families named Specificity Proteins (also known as “Trans Acting Transcription Factors”) whose members are reported to be selectively expressed either in glia or in neurons during development in several studies and I aimed to investigate their potential role in adult neurogenesis.

7.2. Potential impact of Specificity Proteins on adult hippocampal neurogenesis

Similar to development, generation of new neurons in the adult brain progresses through a number of stages. In the adult brain, stem cells which are known to have glial properties give rise to transient amplifying progenitor cells in DG. Having a high proliferative capacity, transient amplifying progenitor cells are able to generate neuroblasts, characterized by the expression of the immature neuronal marker DCX. Neuroblasts undergo few rounds of cell division and then exit the cell cycle. At the neuroblast stage, cells start to migrate into the granule cell layer (Reviewed in Ming et al, 2011). The establishment of the axon and dendrites takes place when differentiation goes on and they appear to be concomitant during the maturation of newborn neurons. Using retroviral labeling of newborn neurons, Zhao et al showed that few of the newborn neurons at 10 and 14 dpi already start to show characteristic morphology of mature granule neurons, although they still carry the properties of immature neurons. The apical dendrites of some immature neurons were observed to reach already the inner molecular layer at
10 dpi. In addition, axonal projections of immature neurons reach the target area within the hippocampus (CA3 area) at 10-11 dpi. Spine formation is started 5 days after, at 16 dpi and authors reported that most of the spine formation is observed within 3-4 weeks after retroviral labelling (Zhao et al, 2006). Integration of the newborn neurons to DG continues as newborn neurons mature (Esposito et al 2005, Toni et al 2007 and 2008). The formation of a functional neuron (that is indistinguishable from an old neuron) from glial-like stem cells in the DG of adult mouse brain under physiological conditions takes approximately 7 weeks (Zhao et al. 2006).

The fact that Sp transcription factors can be expressed in a cell-type specific manner in brain cells, like reported previously for Sp1, being glia specific and Sp4, being neuron specific transcription factors (Henson et al 1992, Hagen et al 1995) is an interesting phenomenon to investigate in adult neural stem cell development. Since there is a switch from glial properties of stem cells to neurons during adult neurogenesis, understanding the expression pattern of Sp transcription factors during this process has a particular importance.

The most closely related members of the Sp transcription factor family are Sp1, Sp3, and Sp4 according to the very high conservation of their nucleotide sequence among mammalian species. Hagen et al. (1992, 1994) reported that Sp1, Sp3 and Sp4 recognize and bind to a similar DNA consensus sequence, which is the GC box. For a particular cell, the factors that affect transcriptional regulation of GC box-containing regulatory regions on DNA are the presence/absence of different Sp transcription factor family members and the amount of their expression within that particular cell. Depending on the cell type, some Sp members can act either as a repressor or as an activator of transcription on the GC boxes. In short, Sp transcription factors can bind to the same DNA binding motif and regulate common downstream target promoters in a dosage-dependent manner via competitive binding of different members to those promoters.

Besides, transcriptional regulation in a stage- and dosage-dependent manner is particularly important in adult neurogenesis because of stage-specific requirements and developmental programs which govern contrasting cell biological processes. These processes include proliferation, growth, cell cycle exit, survival, dendritic development, spine development and development of synaptic output. During these processes, genetic programs are switched on and off according to the developmental stage. Given that Sp transcription factors can target/compete for same sequences but have different output due to their activating / repressing nature they might
be ideally suited to control these switches. Therefore, any possible difference in the ratio between Sp transcription factors at different stages of adult neurogenesis would make sense for the regulation of common Sp target promoters, which might contribute to the allover regulation of adult neurogenesis. This aspect is especially important for those promoters which can be activated by certain members of the Sp transcription factors and can be repressed by other Sp members. An important example for such a regulation has been reported previously by Milagre et al for the regulation of CYP46A1 (neuron specific cytochrome P450) which is encoding the key enzyme involved in the cholesterol catabolism pathway in neurons. Authors differentiated NT2 cells into neurons and examined Sp binding to the promoters of different neuronal genes (Milagre et al, 2012). Human teratocarcinoma NTera2/cloneD1 (NT2) cells are able to generate post-mitotic neurons by retinoic acid (RA) treatment; therefore are used as an important tool to study human neurogenesis in vitro (Megiorni et al, 2005). By differentiating NT2 cells into neurons, Milagre et al observed a decrease in the level of Sp1 transcription factor associated with CYP46A1 promoter together with an increase in CYP46A1 expression. Authors reported a change in the ratio of (Sp3+Sp4) / Sp1 that is associated to the proximal promoter of CYP46A1 upon neuronal differentiation, which serves as a model for how different Sp transcription factor ratios can be associated to the expression of neuronal genes upon neuronal differentiation. Similarly, brain associated genes like reelin, glutamate receptor subunit zeta 1 (GRIN1), glutamate receptor subunit epsilon 1 (GRIN2A) and µ-opioid receptor (MOR) showed similar increase in their expression patterns upon differentiation and the binding of Sp1 was shown to decrease commonly in those promoters, suggesting a common regulation of several neuronal genes by Sp transcription factors (Milagre et al, 2012). Taken together, these points make Sp transcription factor family an interesting candidate for the regulation of adult neurogenesis.

7.3. Expression pattern of Specificity Proteins in adult hippocampal neurogenesis

To study the role of Sp transcription factor family in adult neurogenesis, I first investigated the expression pattern of Sp transcription factors in adult hippocampus. Among the closely related members of Sp transcription factor family, I found the expression of two members, Sp3 and Sp4, in the adult dentate gyrus. The third member, Sp1, was not detectable in the hippocampus with the antibody that I used in immunohistochemistry (mouse Sp1-(1C6), sc-420 Santa Cruz). I could detect Sp1 expression in the frontal cortex, which acts as a positive control that the antibody staining itself was working. I concluded that Sp1 is not expressed on protein level in the adult
hippocampus. Neither in glia nor in DG neurons, could I detect Sp1 expression on protein level with the antibody mentioned above. With the commercially available antibodies I was not able to go into further detail with regard to Sp1 expression in adult hippocampal neurogenesis.

The other two members, Sp3 and Sp4 were detectable in the dentate gyrus on protein level. I observed the expression of Sp3 and Sp4 in the granule cell layer of the dentate gyrus and in some of the cells in hilus and molecular layer. Having a closer look at the expression of Sp3 and Sp4 in the neurogenic region of dentate gyrus, which is called subgranular zone, I observed a differential expression pattern. Sp3 is expressed in the subgranular zone, whereas a lot of the cells that lie in the subgranular zone are lacking Sp4 expression. This finding identifies a cell population that expresses Sp3 but not Sp4 in the neurogenic niche of adult dentate gyrus.

Differential regulation of Sp transcription factors in a cell type- or stage-dependent manner might contribute to cell-type specific or stage-specific expression of downstream target genes. Depending on whether they act as an activator or a repressor for a downstream target, transcriptional regulation via Sp transcription factors might be even more complicated. For example, Sp1 is reported to be an activator in general, which interacts with certain members of transcriptional machinery like TATA-box binding protein (TBP) (Emili et al., 1994) and TBP associated proteins (Hoey et al., 1993; Tanese et al., 1996). Sp3 has been reported to be both an activator and a repressor of transcription, depending on its posttranslational modifications (reviewed in Suske, 1999). Sp4, the neuron enriched member of the Sp transcription factor family, is reported to be a transcriptional activator and a repressor as well depending on the downstream target (Ramos et al., 2009).

There are several examples where the ratio between Sp transcription factors is shown to play a role in transcriptional regulation of downstream targets. The ratio between Sp transcription factors are reported to be altered during differentiation of primary keratinocytes, a phenomenon that is important for the regulation of a number of common Sp transcription factor family target promoters in these cells. In primary keratinocytes, the Sp3/Sp1 ratio is reported to be high, and this ratio is inverted when primary keratinocytes are differentiated via excessive levels of calcium (Apt et al., 1996). This shift in the ratio of Sp3/Sp1 was implicated in the Sp-mediated regulation of p21 promoter upon keratinocyte differentiation (Prowse et al., 1996). Another example is that hypoxia can cause the depletion of Sp3 in C2C12 myoblasts which changes the Sp1/Sp3 ratio in
these cells and yields an upregulation of the Specificity protein target promoters like pyruvate kinase-M and β-enolase (Discher et al., 1998).

Taking these models into account, differential expression of specificity proteins in the neurogenic area of the dentate gyrus might be important for transcriptional regulation of distinct downstream targets during the development of newborn neurons in the adult dentate gyrus. My investigation of expression of Sp transcription factors in detail in subgranular zone revealed that at early stages of neuronal differentiation, radial glia like neural stem cells and progenitors are expressing Sp3 but not Sp4. These stages are known to have glial properties as mentioned before. Two days after birth, where the newborn cells are not yet committed, almost none of the newborn cells express Sp4, whereas almost all of them express Sp3. 7 days after birth, where the committed cells start gaining neuronal properties like developing primary dendrites, Sp4 starts to get expressed with low intensity in approximately half of the newborn cells. This reveals stage specific increase in the ratio of Sp4/Sp3 during neuronal differentiation.

As mentioned before, Sp1 was reported by Henson et al (1992) to be expressed mainly in the glial cells (mostly oligodendrocytes) in human brain by an antibody that they developed in their own laboratory and that is not commercial. In this study, Sp1 was not detectable in neural stem cells with the antibody that I used. In the context of adult neurogenesis, it would be interesting to know whether Sp1 is detectable by such other antibodies in adult neural stem cells and in early stages of neuronal development in adult hippocampus in mice, given the fact that these cell populations exhibit glial cell properties. In addition, it has been reported that Sp4 can repress Sp1 expression. This might be one of the reasons why Sp1 is not detectable in the neurons of hippocampus, where Sp4 is expressed. The switch from Sp1 to Sp4 expression would be a key regulation pattern for common Sp transcription factor target genes during neuronal differentiation. This issue remains to be identified by using several other antibodies for the detection of Sp1 expression in neural stem cells. In the later stages of neuronal development and in the mature neurons of granule cell layer, I see Sp3 and Sp4 expression together in the neurons.

7.4. Identification of specificity proteins as regulators of dentritic development during adult hippocampal neurogenesis

After detection of expression of Sp3 and Sp4 in neurogenic region, I next questioned the role of these transcription factors during the development of newborn neurons. I took the microRNA mediated gene knockdown approach to reduce the level of Sp3 or Sp4 expression from the
beginning on during neuronal development in adult neurogenesis. My analysis on dendritic morphology revealed similar phenotypes upon knockdown of Sp3 or Sp4, consistent with the assumption that they can bind to common motifs on the DNA and regulate common targets. The overall complexity of the dendrites in newborn neurons was increased 14 days after Sp3 or Sp4 knockdown in vivo. This suggests that Sp3 and Sp4 might contribute to dendritic development in adult neurogenesis via acting on at least a subset of common downstream targets. While analyzing the dendritic morphology further in detail upon Sp3 or Sp4 knockdown, I observed that dendritic length was almost doubled in both Sp3 and Sp4 knockdown cells. Interestingly, dendritic branching was increased only in Sp3 knockdown cells but not in Sp4 knockdown cells 14 days after the injection of microRNA retroviruses, compared to control retrovirus. According to our knowledge, my data is the first to imply Sp factors in dendrite development and more importantly the first indication that dendritic length and dendritic branching are controlled by distinct genetic programs in newborn neurons in the adult dentate gyrus.

While analyzing dendritic morphology at a later time point after the knockdown of Sp3 or Sp4, I observed that the reduction of Sp3 caused increased dendritic length and branching compared to controls at 28dpi. However, I could not observe any difference in dendritic length and branching in the Sp4 knockdown cells compared to controls at 28dpi. The difference in the Sp3 and Sp4 knockdown neurons in 14 dpi and 28 dpi can be explained by a simple model described in detail below.

Although I see differences in dendritic complexity at 14dpi for both Sp3 and Sp4 knockdown cells compared to controls, this difference is not visible anymore for Sp4 knockdown cells at 28dpi, whereas the dendritic complexity persists in Sp3 knockdown cells at 28dpi. This phenomenon can be explained by differential regulation of dendritic length and dendritic branching by these two Sp transcription factors. To explain this phenomenon further, one has to focus on the limiting factor of dendritic growing in the granule neurons of the dentate gyrus. In hippocampus, the growth of the dendrites of adult newborn neurons is restricted to the molecular layer; once dendrites reach the hippocampal fissure, they stop growing. At14dpi, dendrites of adult-generated neurons are still elongating and do not reach hippocampal fissure. However, in 28dpi, most of the dendrites have already reached the hippocampal fissure, and stopped elongating further. Taken this information into account, I would expect that Sp4 knockdown phenotype on dendritic length at 14 dpi will not be visible at 28 dpi anymore, since average
dendrite branch number per cell does not differ between Sp4 knockdown cells and control cells for each time point. In other words, dendritic length difference is visible during the elongation of dendrites, namely at 14 days, but it is not visible anymore at 28 days since probably all the existing dendrites reaches hippocampal fissure and stop elongating. My findings might indicate a possible acceleration in the development of the dendrites upon knockdown of Sp4 in adult neurogenesis in mice. Different from Sp4 knockdown, Sp3 knockdown cells showed an increase in dendritic length in both 14 dpi and 28 dpi compared to control cells. Since dendritic branching was increased already at 14 dpi in Sp3 knockdown cells, which means average number of dendrites per cell reaching molecular layer was increased, I observed an increase in average length of the dendrites per cell upon Sp3 knockdown at 28dpi. This finding suggests that knockdown of Sp3 might affect dendritic complexity of the newborn neurons in the long term. What I do not know however is, whether these changes persist or even increase after 28 dpi. In the future it will be interesting to determine whether excessive branches will be pruned or whether Sp3 KD cells will continue to add new branches.
Figure 31: Model representing growth dynamics of newborn neurons upon Sp3 and Sp4 knockdown separately at 14dpi.

Representative figures showing the growth changes of newborn granule cells upon Sp3 knockdown (green cell) and Sp4 knockdown (orange cell) compared to control (black cell) at 14dpi. At this stage, dendritic extensions of control and knockdown cells usually do not reach the hippocampal fissure yet. Therefore dendritic growth is not restricted. At 14 dpi, Sp3 knockdown increased dendritic length and dendritic branching together, whereas Sp4 knockdown yielded an increase only in dendritic length but branch numbers stayed the same compared to control. (ML, molecular layer; HL, Hilus).
Figure 32: Model representing growth dynamics of newborn neurons upon Sp3 and Sp4 knockdown separately at 28dpi.

Representative figures showing the growth changes of newborn granule cells upon Sp3 knockdown (green cell) and Sp4 knockdown (orange cell) compared to control (black cell) at 28 dpi. The increase in the dendritic length and dendritic branching in Sp3 knockdown cell persists at 28dpi, whereas Sp4 knockdown cells have similar dendritic length and branch numbers at 28 dpi compared to controls. As it can be seen in the figure, growth of dendrites stops when they reach hippocampal fissure. In this case, dendritic branch numbers are the determinant for total dendritic length (sum) at 28dpi. Since Sp3 knockdown cells show more branch numbers already in 14dpi and also in 28 dpi, Sp3 knockdown cells have bigger total dendritic length at 28dpi compared to control cells. However, since Sp4 knockdown didn’t affect the branch numbers compared to control cells neither at 14dpi nor in 28dpi, there are no observable changes between the dendritic length of Sp4 knockdown cells and control cells at 28dpi. (ML, molecular layer; HL, Hilus).

The fact that adult neurogenesis is confirmed in human hippocampus (reviewed by Sierra et al, 2011) makes these discoveries in animal models to be interpreted or related to a broader range of functions or disease phenotypes of the human brain. Hippocampal neurogenesis is found to be related to information processing including spatial discrimination and pattern separation in rodents. Several studies showed that appropriate differentiation of adult neural stem cells into
neurons and integration of those neurons into the existing circuitry is important for information processing in the hippocampus, confirming the connectivity pattern of those newborn cells is crucial for brain functions. During maturation, one of the key steps for the newborn neurons is the development of dendrites to receive appropriate signals from their surrounding environment. Proper dendritic development is especially important for neurotransmitter signaling and for proper integration of the newborn neurons to the existing hippocampal circuitry while the neurons are maturing. Therefore, defining the genes and pathways that are regulators of dendritic development in newborn neurons are of particular importance for information processing in the brain. Moreover, many of these genes and pathways have been found to be linked to neurodevelopmental disorders as well as to neurodegenerative diseases in several studies. DISC1 for example, is one of the genes which, when mutated, causes increased risk for schizophrenia, bipolar disorder and depression. Knockdown studies of DISC-1 in the newborn neurons of dentate gyrus showed that DISC-1 regulates dendritic development. DISC-1 reduction in the newborn neurons causes an increase in dendritic complexity; including both the length and the branch number of the dendrites (Duan X. et al, 2007). These findings support the idea that abnormal increase in the dendritic complexity of neurons in the brain might contribute to the phenotype of mental disorders like schizophrenia. Surprisingly, Sp4 has been defined as a candidate gene for schizophrenia before in several studies, which will be explained in the final part of this thesis.

As described in detail, in this study I define transcriptional regulators Sp3 and Sp4 among the regulators of dendritic development during adult neurogenesis. In addition, one of my interesting findings is that with this study, I define a differential regulation of dendritic length and dendritic branching during the development of newborn neurons in the adult dentate gyrus. According to our knowledge, this discrimination has not been mentioned in the adult neurogenesis literature before and might supply new directions to the understanding of dendritic development during maturation of adult neural stem cells.

7.5. Impact of Specificity proteins on migration during adult hippocampal neurogenesis

In addition to dendritic phenotypes discussed above, I observed a difference in the localization of the cell bodies in Sp4 knockdown cells. In subgranular zone of the dentate gyrus, newborn neurons are located close to hilus. Once they leave the mitotic state, they start to express immature neuronal markers like DCX and they migrate short distance into the granular zone. If
the granular zone is divided into three layers starting from the part that is close to the hilus, the cells are migrating from lower granule cell layer towards upper granule cell layer. One of my observations was that upon Sp4 knockdown at 14dpi, there were a number of cell bodies still lying in the hilus or very close to hilus. Quantification of the localization of cell bodies within the dentate granule cell layer revealed that there were significantly more cell bodies lying in the hilus after the knockdown of Sp4 at 14dpi compared to control. This result might indicate that upon knockdown of Sp4, some of the newborn neurons cannot migrate properly towards granule cell layer or migration of these immature neurons is delayed. To address the question of whether it is a migration delay or not, I analyzed the cell body localizations of the Sp4 knockdown cells at 28dpi. Interestingly, what I observed was that there were significantly more cells in the lower granule cell layer and significantly less cells lying in the middle granule cell layer. I could not see differences in the percentage of the cells lying in the hilus. This result might suggest that upon Sp4 knockdown at 28dpi, the migration of the newborn neurons from lower granule cell layer to middle granule cell layer was delayed and the cells accumulated this time in the lower granule cell layer. Since I did not observe any differences in percentage of the cells lying in the hilus at 28dpi anymore, it might be that Sp4 knockdown cells are probably not losing their migration capability, but rather that their migration is delayed. An alternative explanation for the absence of Sp4 KD cells from the hilus at 28 days could be that proper positioning of the cell body is essential for long term survival of newborn neurons. As a consequence, cells, whose cell body was located in the hilus, might have died between these two time points. To investigate this possibility, I conducted cell survival analysis between these two time points. My results however, suggested no obvious cell death upon Sp4 knockdown between these two time points, which strengthens the idea that knockdown of Sp4 causes a migration delay towards granule cell layer during adult neurogenesis in dentate gyrus.

In adult hippocampal neurogenesis, the route that the newborn neurons are migrating is relatively short compared to the route they migrate throughout the RMS in subventricular zone neurogenesis in adult brain. However, in subventricular zone neurogenesis, newborn neurons start to migrate from subventricular zone towards olfactory bulb, a route named RMS, which allows an easier discrimination of migration differences compared to GCL. Although subventricular zone neurogenesis and hippocampal neurogenesis differs in several molecular and cellular aspects, I used subventricular zone neurogenesis a model to further study the migration
differences upon Sp4 knockdown in newborn neurons. Interestingly, the percentage of the cells that reach olfactory bulb at 5dpi is lower in Sp4 knockdown cells compared to control cells in the case of SVZ neurogenesis. This result might indicate a possible delay in the migration of newborn neurons upon knockdown of Sp4 as well in SVZ neurogenesis. However, it is important to keep in mind that these results might as well indicate that Sp4 might be included in cell survival during maturation of newborn neurons in particular in the OB neurogenesis. Although I could show in the DG that attenuated migration does not affect cell survival, to exclude this possibility particularly in the OB neurogenesis, cell survival analysis upon Sp4 knockdown is also necessary specifically for the OB.

Interestingly, by overexpressing a dominant negative form of CDK5 in newborn neurons of the adult dentate gyrus, Jessberger et al. reported a similar migration attenuation phenotype like Sp4 knockdown in adult hippocampal neurogenesis. CDK5 is a kinase that is involved in several neuronal features like migration, dendritic development, dendritic path finding and learning and memory. Upon retrovirus mediated overexpression of dominant negative CDK5, which is the kinase deficient form of CDK5, it has been reported that newborn neurons of the adult dentate gyrus grew aberrant dendritic processes of decreased length and branching, and that the cell body localizations of these neurons was altered (Jessberger et al., 2008). Sp4 has been suggested to be involved in transcriptional regulation of CDK5 as well as its regulatory subunits, p35 and p39 (Fischer et al., 2003; Ross et al., 2002; Valin et al., 2009). Taking this possibility into account that Sp4 might be involved in the regulation of CDK5, it is not surprising that Sp4 knockdown shares partially redundant phenotype to the previously published function of CDK5 in adult hippocampal neurogenesis by Jessberger et al. Whether migration attenuation phenotype of Sp4 knockdown might be due to altered levels of CDK5 during neuronal development in adult hippocampus has to be investigated further.
Figure 33: Model representing migration dynamics of newborn neurons upon Sp4 knockdown at 14dpi.
Representative figure showing the migration delay of newborn granule cell bodies upon Sp4 knockdown (red cells) compared to controls (black cells) at 14dpi. Newborn neurons that usually are located at the hilus, start to migrate towards granule cell layer when they are maturing. At 14dpi, as can be seen in control cells, newborn neurons already migrated from hilus toward lower GCL, and few cells even migrated further to middle and upper GCL. However, upon Sp4 knockdown significantly more newborn cells are stacked at the hilus compared to controls. There are also almost no cells that could migrate to upper GCL upon Sp4 knockdown. These observations suggest a migration delay of newborn neurons in DG upon Sp4 knockdown at 14dpi. (ML, molecular layer; HL, Hilus; GCL, granule cell layer; DG, dentate gyrus).
Figure 34: Model representing migration dynamics of newborn neurons upon Sp4 knockdown at 28dpi.
Representative figure showing the migration delay of newborn granule cell bodies upon Sp4 knockdown (red cells) compared to controls (black cells) at 28dpi. At this time point, newborn cells are expected to migrate further than in 14dpi. As can be seen in control cells, newborn neurons are migrated further from lower GCL to middle and upper GCL. However, upon Sp4 knockdown there is significantly less number of cells that could migrate from lower GCL to middle GCL. This phenomenon caused an accumulation of newborn cells in lower GCL at 28dpi upon Sp4 knockdown and significantly more newborn cells are stacked at the lower GCL. These observations support the idea that a migration delay of newborn neurons persists in DG upon Sp4 knockdown at 28dpi. (ML, molecular layer; HL, Hilus; GCL, granule cell layer; DG, dentate gyrus).

7.6. Role of Specificity Proteins in human disease

In human brain, several neurodevelopmental and neurodegenerative disorders have been shown to be associated with Sp transcription factors.

One major example of this is the association of Sp4 with bipolar disorder shown by several studies. Bipolar disorder, also known as manic depression, is running with unusual changes in mood, energy, activity levels, and the ability to carry-out day to day tasks. Bipolar disorder
affects about 2.6 percent of the U.S. population at age 18 and older in a year (Kessler, 2005). Zhou et al. (2009) carried out an association study in European Caucasian population to investigate the association of 10 Sp4 mutations with bipolar disorder. They have found that 4 out of these 10 mutations were significantly associated with bipolar disorder. Upon these results, these 10 mutations are screened in Chinese population. Interestingly, 4 mutations (2 of them in common with previous study in European Caucasians) were found to be significantly associated with bipolar disorder. These two independent screenings of two different populations strongly suggest a role of Sp4 in the pathogenesis of bipolar disorder. Another study carried out by Pinacho et al. (2011) has shown that in the postmortem prefrontal cortex and cerebellum tissues of bipolar disorder patients, Sp4 protein levels were significantly reduced compared to controls. This finding suggests a possible contribution of altered Sp4 levels to bipolar disorder pathogenesis.

Another example of mental disorders that is associated with Sp4 is Schizophrenia. Schizophrenia is a mental disease which runs with symptoms like hallucinations, delusions, disorganized thinking and disorganized speech. About 1.1 percent of the population at age 18 or older in a given year has been reported to have schizophrenia (Regier et al., 1993). In 2010, Tam GW and colleagues have identified the genes that are associated with schizophrenia in a Scottish population by comparing DNA copy number variants genome-wide in >90 cases and controls separately. These copy number variants included Sp4, making the Sp4 locus one of the disease loci for schizophrenia. In addition to copy number variation studies, trios families (which include parents and one offspring) are commonly used in association studies when investigating the association of a gene with a disease/disorder. In the study of Zhou et al. (2009), one Sp4 mutation has been found to be significantly associated with schizophrenia in Chinese trios families. The susceptibility allele was found to be present across all three samples, which represents a strong association of this Sp4 allele with Schizophrenia. Additionally, it is known that schizophrenia patients suffer from altered NMDA receptor function. One of the NMDA receptors, the NMDAR1 was found to be significantly reduced in Sp4 hypomorphic mice, in which Sp4 expression is reduced to less than 10%. Therefore, this mouse model has been suggested as a model to study NMDA neurotransmission deficiencies in schizophrenia (Zhou et al., 2010). All these findings strongly suggest an important role of Sp4 in schizophrenia.
Last example of pathologies where Sp transcription factors are found to be involved is Alzheimer’s disease, which is a form of dementia. Cognition, memory, and behavior are altered in Alzheimer patients, which significantly decrease the life quality in elderly population. In US population, it has been reported that every 1 person out of 8 develop Alzheimer’s disease if their age is 65 and older. This ratio goes up to every second person being diagnosed with dementia at the age of 85 and older (Johnson et al., 2009). Alzheimer’s disease is one of the most frequent causes of deaths worldwide. Interestingly, Boutillier et al (2007) reported that Sp3 and Sp4 levels are increased in brains of Alzheimer’s disease patients. This study has been conducted with postmortem brain tissue of Alzheimer’s disease patients, and immunohistochemical results of the CA1 region of the hippocampus and entorhinal cortex revealed an abnormal increase in the levels of Sp3 and Sp4, especially at the neurofibrillary tangles. Moreover, Villa et al (2012) recently reported that Sp4 mRNA levels are significantly increased in the peripheral blood mononuclear cells (PBMC) of living subjects diagnosed with Alzheimer’s disease. Whether the increase in the levels of Sp transcription factors in the brain are due to transcriptional regulation, has to be investigated further. These results altogether support the idea that Sp transcription factors might be involved in the pathology of Alzheimer’s disease.

All these disease connections highlight the importance of Sp transcription factors for proper functioning of neurons and neuronal circuits. Alteration in the levels of Sp transcription factors might underlie several morphological and molecular pathologies seen in these diseases. Altered dendritic complexity and migration phenotypes that I observed in this study might be one of the morphological causes that can contribute to the disease pathology in diseases like schizophrenia and bipolar disorder. In addition, due to their frequent consensus sites throughout the genome, Sp transcription factors can regulate the transcription and the level of a large set of downstream genes, which might significantly contribute to the molecular pathologies that are seen in these neuropsychiatric disorders. I conducted an in silico analysis to determine which promoter sites contain the most binding sites for Sp transcription factors. Taking the consensus GC box sequence (GGCGG), I searched for upstream 1500bp region (from -1000 to +500, taking the transcription start site as 0) of every gene annotated in UCSC Genome browser. GC boxes are most frequently suited on the promoter of genes that are related to calcium signaling, mitochondrial function, and actin cytoskeleton which are the pathways primarily affected or altered in such neurodevelopmental and neurodegenerative disorders. Investigation of these target
promoters might give a clue about the molecular causes that underlie dendritic morphology changes as well as neuronal migration phenotype that I described in this study. Identifying these targets might as well contribute to understand the disease pathologies better in the case of schizophrenia, bipolar disorder and Alzheimer’s disease.

An important example where Sp4 target genes can contribute to the disease pathology can be seen in the case of schizophrenia. Several NMDA receptor subunits are regulated by Sp4. NMDA (N-Methyl-d-aspartate) Receptors are the main glutamatergic receptors responsible for the most excitatory neuronal signaling in the brain. Priya et al (2013) recently reported that 3 of the NMDA receptors, namely GluN1, GluN2A, and GluN2B can be regulated by Sp4, together with some other transcriptional regulators like NRF-1 and NRF-2. Interestingly, authors reported that Sp4 acts in a complementary and concurrent/parallel way to NRF-1 and NRF-2 to regulate the tight balance between energy metabolism and neuronal activity at molecular level. Another study could show the reduced expression of NMDA receptor 1 (NMDAR1) in the Sp4 hypomorphic mice (Zhou et al., 2010). These findings suggest that Sp4 transcription factor might be involved in the regulation of calcium signaling via regulating of one of the main excitatory signaling pathways called glutamatergic signaling.

On the other hand, it has been also shown in cultured neurons in vitro that activation of glutamate receptors causes degradation of Sp transcription factors rapidly via altered calcium influx into the neurons. Authors further conducted a study where they showed that cerebral ischemia/reperfusion injury caused the loss of Sp transcription factor activity in vivo. They also reported that this glutamate-related calcium-dependent effect can be blocked by calpain inhibitors. Calpains are cysteine proteases, responsible for protein degradation upon calcium influx into the neurons. Interestingly, the degradation of Sp transcription factors was blocked when they used calpain inhibitors. They further showed that calpain-1 can cleave Sp3 and Sp4 (Mao et al, 2007). These studies suggest that not only Sp transcription factors can transcriptionally regulate genes that are important for glutamate dependent calcium signaling; but also glutamate dependent calcium signaling itself might regulate the function of Sp transcription factors.

Since calcium dependent signaling is one of the main signaling mechanisms that are altered in several neurodevelopmental and neurodegenerative disorders, understanding mechanisms underlying the molecular pathologies of mental disorders would be of great importance. In this line, the role of Sp3 and Sp4 transcription factors has to be investigated further on molecular
level in neurons in order to be able to explain their contribution to the disease pathologies that they are involved.
8. LITERATURE INDEX


Complex phenotype of mice homozygous for a null mutation in the Sp4 transcription factor gene. Genes to Cells 6, 689-697.


Priya, A., Johar, K., Wong-Riley, M.T.(2013). Specificity protein 4 functionally regulates the transcription of NMDA receptor subunits GluN1, GluN2A, and GluN2B. Biochim Biophys Acta. 2013 Jul 18 (Epub ahead of print)


To begin with, I would like to thank Prof. Wolfgang Wurst, for giving me the opportunity to do my PhD in Institute of Developmental Genetics, in Helmholtz Zentrum München.

Deserving of special mention is my supervisor Prof. Chichung Lie, for supporting me with his knowledge, advice and encouragement. I am grateful to him especially for providing me freedom when I was choosing my thesis research topic in line with my interests and when I was conducting my studies throughout my PhD. Together with the young, dynamic and international “adult neurogenesis” group, this contributed a lot to my personal and scientific assets. I really enjoyed my PhD time in AG_Lie.

I would like to thank to Prof Erwin Grill, for being the chair of my thesis committee.

My special thanks goes to Dr. Katharina Merz for scientific discussions in endless coffee breaks, also for establishing the retroviral knockdown strategy for our lab; and to Dr. Marcela Covic for feeding my deep epigenetics interest during my studies with her knowledge and experience.

Thanks from my heart to my Greek “sister” Marousa, for sharing wonderful Mediterranean flavor of life with me in the middle of Munich both in the lab and during hour stay-together at home. I really enjoyed our time together.

I would also like to thank to my PhD-mates Birgi, Elisabeth, Iris and Tobi for their warm accompany in the office hours and also in several meetings, trips that we attended together.

I would like to take this opportunity to thank all the members of AG Lie. Special thanks for any of you who were making the lab meetings, journal clubs, brainstorming sessions and discussions so much enthusiastic and useful. Thanks to all the anonymous people who were putting chocolates on my desk for me!

In this line I would like to thank Marija, Katrin, Fabi, and Rosi for their technical and administrative support.

And my sincere thanks goes to my parents and my friends for their invaluable support.
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:

**Role of Sp Transcription Factors in Adult Neurogenesis**

am Lehrstuhl für Genetik unter der Anleitung und Betreuung durch

Univ.-Prof. Dr. W. 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.

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