

KAP1-Paupar lncRNA Chromatin Regulatory Complex Controls Subventricular Zone Neurogenesis

A thesis submitted to the Department of Physiology, Anatomy and Genetics, in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

by

Farah Alammari

Linacre College, University of Oxford, United Kingdom

Michaelmas 2019

ABSTRACT

Neurogenesis continues postnatally in two major neural stem cell (NSC) niches, the subventricular zone (SVZ) and dentate gyrus of the hippocampus. NSCs in the SVZ exhibit some biological features of glia, especially astrocytes. However, it is still not clear how NSCs in the SVZ can maintain stem cell self-renewal properties and the potential to generate transit amplifying progenitor cells (TAPs) that give rise to neuroblasts. Neuroblasts migrate through the rostral migratory stream (RMS) to the olfactory bulbs where they differentiate into neurons. In this project, I investigated the function and mode of action of a new KAP1-lncRNA chromatin regulatory complex in the control of postnatal and adult neural stem cell (NSC) self-renewal and neurogenesis.

KAP1 is an important epigenetic regulatory protein that is required for embryonic brain development and adult brain function. I have demonstrated a broad expression of KAP1 in the normal postnatal/adult SVZ neurogenic lineage. I then uncovered for the first time that KAP1 influences postnatal and adult SVZ-OB neurogenesis. I showed that KAP1 is important for SVZ NSC identity maintenance, and functions to protect NSCs from mature astrocytic fate. In addition, KAP1 loss-of-function reduced the number of newborn neurons in the olfactory bulb and delayed their morphological differentiation**.** On the molecular level, I showed that KAP1 regulates the expression of members of the BMP signalling pathway to control SVZ NSCs maintenance and lineage commitment.

LncRNAs are a new class of regulatory molecules thought to be important in brain development and function. Several thousand lncRNAs are expressed in the SVZ, yet only a very small number have been studied and shown to have function in vivo. Nuclear lncRNAs act as regulators of gene expression and many of these bind chromatin regulatory complexes. They may act either as guides to recruit associated proteins to their target genes or molecular scaffolds to control ribonucleoprotein complex assembly and function. Here, I show that KAP1 physically interacts with the lncRNA *Paupar*, a CNS expressed and chromatin-associated lncRNA that is transcribed upstream of the gene encoding the Pax6 transcription factor, in the SVZ. This was similar to the N2A cell line where *Paupar*, KAP1 and Pax6 together form a ribonucleoprotein complex (Pavlaki et al., 2018).

Interestingly, I found that even though KAP1 and *Paupar* showed a similar phenotypic result of reduction in the number of neurons arriving to the OB, they displayed a different functional role in regulating NSC behaviour in the SVZ. This suggests that even if they exist in the same ribonucleoprotein complex, they function on different molecular mechanism pathways to regulate different set of genes. *Paupar* maintains stem cell quiescence and restrict lineage progression from stem cells to TAPs by regulating the expression of key stem cell genes like Sox2, Hes1, and EGF as well as genes known for controlling neural growth and differentiation. To conclude, I discovered two interacting epigenetic regulators that control SVZ NSC and neurogenesis.

ACKNOWLEDGMENTS

My time in Oxford has been an intense and rewarding journey, full of enjoyment and challenges. The support, guidance and belief I had from my family, friends, mentors and colleagues gave me courage and strength to strive forward and achieve my dreams.

Dr. Francis Szele has been a great mentor and provided a constant support from the time I applied to his lab to the final stages of my DPhil training. He gave me complete guidance and, at the same time, allowed me to be independent in my research and supported my ideas. In addition, he created a great working environment, and encouraged new collaborative opportunities.

The members of my lab have been a wonderful source of scientific inspiration and personal support. Special thanks to Dr. Bin Sun, a former senior graduate student in Szele lab, who acted as my day-by-day mentor when I first joined the lab. He also helped me with many ideas related to my project and provided all the technical support I needed during these years. Dr. Abeer Alshammari, who helped me to settle and cope and was always by my side when I first arrived in Oxford. Dr. Julie Davies for all the technical advice she gave me, Dr. Blanca Torroba, Dr. Martin Ducker, Dr. Penelope Fouka, Dr. Yichen Li, Dr. Anna Hoerder-Suabedissen, Mr. Chenguang Qu for being such amazing lab colleagues.

I am thankful for the support I had from my friends during these years. Each one of them played a significant role in my life. Sarah Aref, for all the conversations, fun, travels, laughs, and dances we had! Hessa Almasoud, for the support and encouragement. Safa Alhujaily, for being a good listener and an amazing friend.

Finally, I want to thank my family. Mom and Dad, I was able to reach this dream because of them, and no words are enough to express my gratitude. Hisham, Danah, Mohammed, Abdulaziz, and Fai for their love and support. Essam, for his consistent care and love. My one and only sister, Muneerah, the most amazing person in my life, I don't know how my life would be without her.

January 2020, Oxford

AUTHORSHIP STATEMENT

I primarily designed the project in this thesis under the supervision of Prof. Francis Szele.

In Chapter 3, Dr. Bin Sun performed the following: BrdU injections of adult mice, electroporation procedure to knockdown KAP1 in the postnatal SVZ, immunohistochemistry, and quantification of Mash1 and Ki67 in GFP+ cells in the postnatal SVZ. Dr. Martin Ducker performed the migration assay experiment. Dr. Francis Szele quantified the morphological differentiation of neurons in the OB.

In Chapter 4, Dr. Bin Sun performed all the postnatal electroporation to knockdown Paupar and performed the immunohistochemistry and quantification of the number of GFP+ cells in the OB 7 dpe, Mash1 and Ki67 in GFP+ cells 24 hpe, GFAP in GFP+ cells 3 dpe, TdTomato+ cells in the postnatal SVZ. Dr. Francis Szele quantified the morphological differentiation of neurons in the OB.

In Chapter 5, Dr. Ioanna Pavlaki at the University of Bath performed the RNA-IP experiment.

All other experimental procedures, quantifications and analyses were done by me with experimental advice from colleagues in the laboratory.

Copyright © 2020 by Farah Alammari

All rights reserved

TABLE OF CONTENTS

Abstract 3

Acknowledgment 4

Authorship Statement 6

Table of Contents 7

List of Figures 13

List of Tables 15

Abbreviations 16

Chapter 1

Introduction 18

1.1 Stem cells & the stem cell niche 19

1.1.1 Definition of stem cells 19

1.1.2 Considerations when studying stem cell behaviour 20

1.2 Postnatal & Adult SVZ 22

1.2.1 Subventricular zone development 22

1.2.2 The SVZ neurogenic lineage and niche 24

1.2.3 Heterogeneity of the SVZ niche 28

1.2.4 Molecular regulation of neurogenesis in the SVZ 29

1.2.5 Neuroblasts migration in the postnatal and adult brain 31

1.2.6 The SVZ in the human brain 33

1.3 Physiological regulation of neurogenesis 36

1.3.1 Soluble factors 36

1.3.2 Cell- bound signals 37

1.3.3 Morphogens 38

1.4 Epigenetic regulation of Neurogenesis 38
1.4.1 DNA modification 39
1.4.2 Histone modification 39
1.4.3 Chromatin remodelling 41

1.5 KRAB domain-associated protein 1 (KAP1) 43
1.5.1 KRAB-ZFPs 43
1.5.2 KRAB domain-associated protein 1 (KAP1) introduction 44
1.5.3 KAP1 protein structure 45
1.5.4 KAP1: Guardian of the Genome 47
1.5.5 KAP1 function in the brain 49

1.6 Long non-coding RNAs (LncRNAs) 51
1.6.1 Introduction to lncRNAs 51
1.6.2 Genomic context of lncRNAs 52
1.6.3 LncRNAs mechanism of action 53
1.6.4 Functionality of lncRNAs 54
1.6.5 LncRNAs regulation in development 55
1.6.6 lncRNAs in Disease 58

1.7 Research Questions 60

Chapter 2
Materials and Methods 63

2.1 Animals 64
2.2 Tamoxifen and BrdU administration 66
2.3 Electroporation 66
2.4 Histology and immunohistochemistry 67
2.5 Imaging and quantification 70
2.6 TUNEL assay 70
2.7 Neurosphere assay 71

2.8 NSC nucleofection 73
2.9 RT-PCR 74
2.10 Plasmids, transformation of chemically competent bacteria, and DNA extraction 76
2.11 FISH 77
2.12 RMS spheroid migration assay 78
2.13 Chromatin-immune precipitation assay 80
2.14 RNA-Immuno precipitation assay 82
2.15 Statistics 83

Chapter 3
KAP1 in postnatal and adult SVZ neurogenesis 84

3.1 Introduction 85
3.1.1 Molecular biomarkers in postnatal/adult SVZ neurogenic lineage 85
3.1.2 KAP1 regulates proliferation & differentiation 89
3.1.3 KAP1 in regulates cell migration 91

3.2 Results 93
3.2.1 KAP1 expression in postnatal and adult SVZ 93
3.2.2 Loss of SVZ KAP1 impairs postnatal OB neurogenesis 97
3.2.3 KAP1 disrupts morphological differentiation of newborn neurons in the OB 100
3.2.4 KAP1 is dispensable for neuroblast migration in vitro 102
3.2.5 KAP1 loss impairs neural stem cell maintenance in pSVZ 104
3.2.6 KAP1 loss does not induce cell death in the SVZ or OB 107
3.2.7 Generation of KAP1 cKO mice model 109
3.2.8 KAP1 regulates NSC identity maintenance in the SVZ 111
3.2.9 Loss of KAP1 impaired neurosphere generation from the SVZ 114

3.3 Discussion 115
3.3.1 Broad expression of KAP1 in SVZ stem and progenitor cells 115

3.3.2 Inducible conditional knockout mice model for KAP1 119
3.3.3 KAP1 is required for SVZ-OB neurogenesis 120
3.3.4 KAP1 in regulating neuroblasts migration 124
3.3.5 KAP1 in regulating SVZ NSC maintenance 125
3.3.6 Neurosphere assay to analyse NSC behaviour in vitro 127

3.4 Conclusion 130

Chapter 4

The long non-coding RNA Paupar regulates postnatal SVZ neurogenesis 131

4.1 Introduction 132
4.1.1 LncRNA expression in the brain 132
4.1.2 LncRNAs in CNS development 133
4.1.3 Transcriptional regulatory functions of lncRNAs 134
4.1.4 The lncRNA Paupar 136
4.1.5 The transcription factor Pax6 139
4.1.6 The interaction between the lncRNA Paupar and KAP1 141

4.2 Results 144
4.2.1 The expression of the lncRNA Paupar in postnatal SVZ NSCs 144
4.2.2 Loss of Paupar impairs postnatal OB neurogenesis 146
4.2.3 Paupar loss disrupts morphological differentiation of newborn neurons in the OB 149
4.2.4 The lncRNA Pauapr regulates SVZ neurogenesis 151
4.2.5 Paupar KD does not cause cell death in the SVZ and the OB 155
4.2.6 Investigating the role of Paupar on neurosphere generation and differentiation in vitro 157

4.3 Discussion 159
4.3.1 LncRNA Paupar expression in the SVZ 159

4.3.2 Postnatal OB neurogenesis is impaired after the loss of Paupar 162
4.3.3 The lncRNA Paupar is required to maintain SVZ NSC quiescence 165
4.3.4 The role of Paupar on neurospheres generation and differentiation 168

4.4 Conclusion 170

Chapter 5
Molecular mechanisms underlying KAP1 and Paupar regulation 171

5.1 Introduction 172
5.1.1 Transcription regulation by KAP1 172
5.1.2 Recruitment of KAP1 to the genome 173
5.1.3 KAP1 and Paupar function on a common set of target genes 174
5.1.4 Diverse effects of KAP1 and Paupar on target genes 175
5.1.5 Bone Morphogenic Protein (BMP) Signalling 176

5.2 Results 178
5.2.1 The lncRNA Paupar directly binds to KAP1 in the SVZ 178
5.2.2 Selective activation of gene expression after KAP1 knockdown in SVZ NSCs 180
5.2.3 Optimization of ChIP-seq experiment in SVZ derived-NSCs 184
5.2.4 Gene expression changes after Paupar KD in the SVZ 186

5.3 Discussion 189
5.3.1 physical association between KAP1 and Paupar in SVZ-derived neurospheres 189
5.3.2 Selective activation of target genes after KAP1 knockdown 191
5.3.3 KAP1 has diverse roles in transcription regulation 193
5.3.4 The lncRNA Paupar in regulating SVZ neurogenesis 195

5.4 Conclusion 198

Chapter 6

General Discussion **199**

6.1 Overview of the current work **200**

6.2 Limitations of the current study **204**

6.3 Future directions **208**

6.3.1 Identify cell subtype specific functions of KAP1 and define functional cooperation between KAP1 and Paupar in SVZ neurogenesis 208

6.3.2 Define phosphorylation dependent control of KAP1 function during neurogenesis 209

6.3.3 KAP1 and Paupar in glioblastoma 211

Appendix **213**

References **216**

Word count: ~ 46,332

LIST OF FIGURES

Chapter 1

Figure 1.1	The adult SVZ niche	27
Figure 1.2	Multiple regions of the developing brain neuroepithelium contribute to the adult SVZ	29
Figure 1.3	Neuroblasts migration through the rostral migratory stream to the olfactory bulb	33
Figure 1.4	Cytoarchitecture of the human adult subventricular zone	35
Figure 1.5	Epigenetic regulations of neural stem cells by DNA methylations and histone modifications	42
Figure 1.6	KAP1 structure, post-translation modifications, and binding proteins	47
Figure 1.7	Genomic location of lncRNAs	52
Figure 1.8	LncRNAs can act in cis or in trans to regulate gene expression	54

Chapter 3

Figure 3.1	Adult neurogenesis in the SVZ and OB	88
Figure 3.2	KAP1 expression in postnatal and adult mice brain	96
Figure 3.3	Neurogenesis decreases in the RMS/OB after KAP1 knockdown	99
Figure 3.4	KAP1 knockdown cause defective differentiation of newborn neurons in the OB	101
Figure 3.5	KAP1 knockdown does not affect neuroblasts migration in the RMS	103
Figure 3.6	Neural stem cells maintenance defect in the postnatal SVZ after knockdown of KAP1	106
Figure 3.7	KAP1 knockdown does not cause apoptosis in the SVZ and OB	108
Figure 3.8	Conditional knockout of KAP1 in adult SVZ	110
Figure 3.9	KAP1 is required for NSC identity maintenance in the adult SVZ	113
Figure 3.10	Fewer secondary neurospheres could be generated after knockout of KAP1 in the SVZ of adult mice	114

Chapter 4

Figure 4.1	Cis and Trans mode of actions of lncRNAs	135
Figure 4.2	Mechanisms of lncRNA-chromatin association	136
Figure 4.3	Conservation of Paupar	138
Figure 4.4	Paupar regulation during neuronal differentiation of mouse ES cells after treatment with RA	139
Figure 4.5	Gene Ontology annotation of Paupar-regulated genes	139
Figure 4.6	Paupar promotes KAP1 chromatin binding and H3K9me3 Deposition	143
Figure 4.7	Expression of the lncRNA Paupar in the SVZ	145
Figure 4.8	Neurogenesis decreases in the OB after *Paupar* knockdown	148
Figure 4.9	Paupar knockdown cause defective differentiation of newborn neurons in the OB	150
Figure 4.10	Paupar regulates SVZ lineage progression	154
Figure 4.11	Paupar knockdown does not cause apoptosis in the SVZ/OB	156
Figure 4.12	Paupar KD does not impair neurospheres self-renewal and multipotency abilities	158

Chapter 5

Figure 5.1	KAP1 multiprotein complex and recruitment to the genome	174
Figure 5.2	Paupar directly binds the chromatin regulatory protein KAP1 in SVZ-derived cells	179
Figure 5.3	Loss of KAP1 activates BMP signalling genes	183
Figure 5.4	KAP1 chromatin occupancy within the regulatory region of ZFP382	185
Figure 5.5	Loss of Paupar downregulates key stem cell genes	188

Chapter 6

Figure 6.1	Proposed model of KAP1-Paupar lncRNA chromatin regulatory complex in regulating subventricular zone development	203
Figure 6.2	KAP1 S473 phosphorylation regulates the association with Paupar	210

LIST OF TABLES

Chapter 1

Table 1.1	LncRNAs that have been studied and the mutation strategy used	57

Chapter 2

Table 2.1	Primers for genotyping	65
Table 2.2	List of primary antibodies used for immunohistochemistry	69
Table 2.3	Components of Neurobasal+	72
Table 2.4	Components of Neurobasal+ growth factor	72
Table 2.5	List of primary antibodies used for fate potential Immunohistochemistry	73
Table 2.6	Primer list of RT-qPCR	74
Table 2.7	Primers for PCR	77

Chapter 3

Table 3.1	Major molecular biomarkers used to identify cells based on proliferative neurogenic and gliogenic capacity	87

Appendix

Table 1	Supplemental recipes for ChIP solutions	213
Table 2	Supplemental recipes for common solutions	215

ABBREVIATIONS

BLBP	Brain lipid binding protein
BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
CC	Corpus callosum
CDKI	Cyclin-dependent kinase inhibitor
ChIP	Chromatin-immunoprecipitation
cKO	Conditional knockout
CSC	Cancer stem cells
CSF	Cerebrospinal fluid
DAPI	4',6-diamidino-2-phenylindole
Dcx	Doublecortin
ESC	Embryonic stem cell
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GCL	Granule cell layer
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GL	Glomerular layer
Glast	Glutamate aspartate transporter
H3K9me3	Histone 3 lysine 9 trimethylation
HDAC	Histone deacetylases
ISH	In *situ* hybridization
KAP1	KRAB domain-associated protein 1
KD	Knockdown
KRAB	Krüppel associated box domain
lncRNA	Long non-coding RNA
LRC	Label retaining cells
LV	Lateral ventricle
NeuN	Neuronal nuclei
NSC	Neural stem cells
OB	Olfactory bulb
Paupar	Pax6 Upstream Antisense RNA
Pax6	Paired box protein Pax-6
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PHi3	Phosphorylated histone H3
PSA-NCAM	Polysialylated neural cell adhesion molecule
qPCR	Quantitative PCR
REST	RE-1 silencing transcription factor
RMS	Rostral migratory stream
RNAi	RNA interference
SEM	Standard error of the mean
SEZ	Subependymal zone

SGZ	Subgranular zone
shRNA	Short hairpin RNA
str	Striatum
SVZ	Subventricular zone
TMX	Tamoxifen
Trim28	Tripartite motif-containing 28
TrxG	Trithorax group
UTR	Untranslated region
ZFP	Zinc finger proteins

Chapter 1

Introduction

1.1	**Stem cells & the stem cell niche**	**19**
1.2	**Postnatal & Adult SVZ**	**22**
1.3	**Physiological regulation of neurogenesis**	**36**
1.4	**Epigenetic regulation of Neurogenesis**	**38**
1.5	**KRAB domain-associated protein 1 (KAP1)**	**43**
1.6	**Long non-coding RNAs (LncRNAs)**	**51**
1.7	**Research Questions**	**60**

1.1 Stem cells and the stem cell niche

1.1.1 Definition of stem cells

Stem cells are generally identified to be clonogenic cells that are capable of self-renewal and multilineage differentiation. Stem cells exist in various mammalian tissues through our development and in some tissues, they persist during adulthood. The earliest stem cells are totipotent, which extend from the zygote to the inner cell mass of the blastocyst; soon after, totipotent stem cells give rise to primitive germ-line stem cells and somatic stem/progenitor cells (Weissman, 2000) . Stem cells are described by their functional characteristics which causes difficulties in identifying whether a cell is a stem cell or not. The cell must by manipulated for experiments, which may alter their behaviour (Potten and Loeffler, 1990). In order to categorize a cell as a stem cell, all the following criteria must be identified: a) proliferation, b) self-maintenance or self-renewal, c) production of differentiated progeny of multiple lineage, d) regeneration of tissue after injury (Potten and Loeffler, 1990, Weissman, 2000).

Proliferation is the process of symmetric or asymmetric cell division. In the symmetric division of stem cells, the cell can generate identical daughter stem cells (symmetric division to expand) or differentiated cells (symmetric terminal division). It is triggered when additional stem cells are needed, for example to regenerate an injured tissue. Asymmetric division gives rise to one stem cell and one progenitor or differentiated cell and it maintains the stem cell population baseline (Potten and Loeffler, 1990, Lajtha, 1979b).

Differentiation can be defined as the qualitative changes of the cell phenotype which occur due to changes in gene expression and what lead to functional competence. It might be recognized by changes in cell morphology, protein composition, enzymatic

activity, firing action potentials in a nerve cell or contraction of muscle cells (Lajtha, 1979a, Potten and Loeffler, 1990). Finally, the potency of stem cells is defined by their ability to produce multiple lineage progeny. Totipotent embryonic stem cells (ESCs) can give rise to any cell in the organism, while tripotent adult neural stem cells (aNSCs) can give rise to neurons, oligodendrocytes, and astrocytes (Potten and Loeffler, 1990, Weissman, 2000).

1.1.2 Considerations when studying stem cell behaviour

Ideally, a cell is identified as a stem cell once it possesses the four defining criteria of stem cells. However, due to experimental limitation, only some of these criteria may be satisfied (Potten and Loeffler, 1990). For example, a stem cell at any point may not exhibit stemness trait, i.e. when a quiescent stem cell does not proliferate but retains the ability to do so. In addition, proving some of the stemness criteria like self-renewal or regeneration of injured tissues may require manipulation of these cells and possibly altering their behaviour (Potten and Loeffler, 1990, Pastrana et al., 2011). In practice, identifying proliferation and differentiation can be achieved by morphological analysis combined with immunohistochemical labelling of cell markers (Pastrana et al., 2011, Codega et al., 2014, Doetsch, 2003b, Doetsch, 2003a).

Transit amplifying progenitors (TAPs) are a population of proliferating cells situated between the stem cells and their mature differentiated progeny. Thus, they share some properties with stem cells and some properties with mature cells (Potten and Loeffler, 1990). In the subventricular zone (SVZ), TAP cells express markers of more differentiated cells like Mash1 and Dlx2, but preserve the ability to proliferate (Ihrie and Alvarez-Buylla, 2011). The hallmarks of TAPs are their high proliferative capacity, limited self-renewal and self-maintenance, imminent differentiation, and more restricted

potency (Lajtha, 1979b, Potten and Loeffler, 1990). Although stem cells are heterogenous in their marker and cell cycle dynamics, they are still different from TAPs. For example, neural stem cells comprise two functionally distinct population, quiescent (qNSCs) and activated (aNSCs) which differ greatly in their cell cycle status and lineage kinetics and molecular signature (Codega et al., 2014). Interestingly, it is possible to aNSC to switch to the qNCS state or vice versa (Codega et al., 2014). Stem cells have longer G1 phase and a shorter S phase compared to TAPs. Thus, when examining proliferative cells, the heterogeneity in the proliferative cell population that exist in the SVZ must be taken into consideration when analysing the data.

Stem cells are usually present in specific niches that influence their behaviour. Niches are microenvironments that consist of stem cells, their progeny, extracellular matrix, support cells, blood vessels, and external and internal soluble factors (Ihrie and Alvarez-Buylla, 2011, Fuchs and Segre, 2000). Examples of stem cell niches include the intestinal crypts for intestinal stem cells, bone marrow for hematopoietic stem cells, and subventricular zone, and subgranular zone of the dentate gyrus for neural stem cells (Fuchs and Segre, 2000). Due to the dynamic expression of stem cell markers and lack of specific markers, and the heterogeneity of stem cells make it difficult to identify them in vivo using only marker expression analysing. Thus, to study stem cells behaviour, it is important to examine them based on their morphology and location in the context of their niche (Ihrie and Alvarez-Buylla, 2011, Pastrana et al., 2011). Moreover, stem cells behaviour like self-maintenance and multipotency can be better examined on a population level. A recent study showed that in the SVZ niche, NSCs stemness is maintained at a population scale. Individual NSCs are activated then depleted which required the SVZ niche to activate additional qNSCs to maintain the neurogenic output (Calzolari et al., 2015).

Studying the relationship between NSCs and their progeny on a single-cell basis is difficult to accomplish on a population scale. Recent techniques can be used to achieve this like live cell imaging (Ortega et al., 2011), unique labelling by tagging of DNA (Goings et al., 2004), or single-cell RNA sequencing (Zywitza et al., 2018) In this thesis, I examined NSCs at their progeny at a population scale in the SVZ niche using a combination of cell markers, morphological and functional studies.

1.2 Postnatal & Adult SVZ

1.2.1 Subventricular zone development

In the mouse embryo, neurogenesis starts around embryonic day E9-10. Neuroepithelial cells lining the neural tube start to convert into radial glial cells (RGCs) in the ventricular zone. RGCs become the stem cells that give rise to all glial cells and neurons in the forebrain. They apically contact the developing ventricles (neural tube cavity) and extend a long process to the pial surface basally, and express astroglial markers like brain lipid-binding protein (BLBP), glutamate transporters, Vimentin, and Nestin (Kriegstein and Alvarez-Buylla, 2009). The neocortex rises from the pallial ventricular zone where the basal ganglia rises from the subpallial ventricular zone. RGCs change from neuroepithelial symmetric division to asymmetric neurogenic division (Kriegstein and Alvarez-Buylla, 2009). Four regions of the SVZ are distinguished during development based on cell composition, date of embryonic emergence, size, transcription factors expression, which are the medial ganglionic eminence (MGE), the lateral ganglionic eminence (LGE), the caudal ganglionic eminence (CGE), and the fetal neocortical SVZ. Each of these regions are composed of progenitor cells that generate

different types of neurons and glial cells that migrate to different regions of the brain (Brazel et al., 2003)

In the ventricular zone and embryonic subventricular zone (eSVZ), cortical neurogenesis continues to produce neurons in an inside-out direction, starting from deep cortical layers to superficial layers (Brazel et al., 2003). After birth, the RGCs in the VZ and underlying SVZ give rise to glial cells and neural stem cells over the first 2-3 postnatal weeks (Kriegstein and Alvarez-Buylla, 2009, Fuentealba et al., 2015).The postnatal SVZ (pSVZ) thickness enlarges in the first postnatal weeks and shrinks during the 3-4 postnatal weeks, at this stage it is difficult to distinguish it from adult SVZ (Peretto et al., 2005). Neural stem cells that reside in the pSVZ continue to produce undifferentiated NSCs that satisfy all the NSCs criteria like: self-maintenance, proliferation and lineage differentiation (Alvarez-Buylla and Lim, 2004, Doetsch, 2003b). Postnatal neurogenesis persist to adulthood and occurs in two major niches, the postnatal SVZ (pSVZ) and the dentate gyrus (DG) of the hippocampus (Kriegstein and Alvarez-Buylla, 2009). In this project, I explore only postnatal and adult neurogenesis in the SVZ and not the DG.

1.2.2 The SVZ neurogenic lineage and niche

The postnatal SVZ niche is transient and changes into a mature adult SVZ niche. Almost all studies focused on studying the niche components of the aSVZ (Lim and Alvarez-Buylla, 2014, Ihrie and Alvarez-Buylla, 2011). Therefore, I will describe the adult SVZ niche and will point out the key differences between aSVZ and pSVZ niches (Ihrie and Alvarez-Buylla, 2011).

The aSVZ niche consists of cellular and non-cellular components (Figure 1.1). The cellular components of the niche include ependymal cells, type B1 astrocytes or neural stem cells, type B2 niche astrocytes, microglia, and endothelial cells. The non-dividing multiciliated ependymal cells lining the ventricle and their motile cilia regulate the flow of cerebrospinal fluid (CSF) (Mirzadeh et al., 2008, Ihrie and Alvarez-Buylla, 2011). The adult NSCs in the SVZ are called type B1 cells, they share similar characteristics of glial astrocytes and express similar glial markers including the glial-fibrillary acidic protein (GFAP), brain lipid-binding protein (BLBP), and glutamate aspartate transporter (GLAST) (Doetsch et al. 1997). They have bipolar morphology with an apical process contains a non-motile primary cilium, which extends between ependymal cells into the CSF, and a basal process contacting the basal lamina and the vascular network that underlie the SVZ (Ihrie and Alvarez-Buylla, 2011). Type B2 cells, or niche astrocytes, are located close to the underlying striatal parenchyma, they express GFAP and thought to be non-neurogenic (Ihrie and Alvarez-Buylla, 2011, Doetsch et al., 1997).Type B1 cells exist in a quiescent or activated state (Lim and Alvarez-Buylla, 2016). Activated NSCs express the epidermal growth factor receptor (EGFR) and are highly proliferative, while the quiescent type-B1 cells lack EGFR and exist in the G_0 cell cycle phase. Activated B1 cells give rise to the transit amplifying progenitors (type C cells) that

express the transcription factors Ascl1 (or called Mash1) and Dlx2. Type C cells give rise to neuroblasts (Type A cells) that express doublecortin (DCX) and polysialylated neural-cell-adhesion molecule (PSA-NCAM). Neuroblasts migrate to the OB where they integrate into the layers of the OB and differentiate into granule or periglomerular interneurons (Lim and Alvarez-Buylla, 2016), and function in odour discrimination and long-term olfactory memory (Figure 1.1) (Butti et al., 2014).

Astrocytes are known for their "house-keeping" function and in providing viable environment for neurons including, providing nutrients and structural support around the synapses, removing excess potassium and neurotransmitters, maintenance of the blood brain barrier, release molecules that are important for neuronal survival (Wang and Bordey, 2008).

Oligodendrocytes are the myelination cells in the niche that produce and maintain the insulating sheath of nerve axons (Bradl and Lassmann, 2010). Other niche component is the microglia cells, they functions to determine the level of the inflammation, and sense signals from the surrounding environment, and have regulatory effect on neurogenesis (Sato, 2015). In addition, NSCs and TAPs physically contact the blood vessels, which function to secret factors that are important for stem cell maintenance (Ottone and Parrinello, 2015, Ottone et al., 2014).

Non-cellular niche components include extensive basement membrane and extracellular matrix (ECM) components, including heparin sulphate proteoglycans (HSPGs) and laminin, which appear to contact all cell types in the aSVZ. In addition to other components, like neurotransmitters, GFs, enzymes, and cytokines. The ECM can regulate the availability of growth factors like fibroblast growth factors (FGF) and

hence effect the proliferative signalling and neurogenesis (Ihrie and Alvarez-Buylla, 2011).

Both the pSVZ and aSVZ produce neurons that integrate in the OB circuit as their final destination (Lois and Alvarez-Buylla, 1994), however, there are few features that are different between them. Following postnatal weeks, the SVZ becomes greatly thinner and reduced from ~300 µm at birth to <100 µm in aSVZ (Fiorelli et al., 2015). Moreover, injections of 3H-thymidine in juvenile and adult rats indicated that pSVZ has more neurogenic potential than the aSVZ, and most neurons originated during the first three weeks of life (Bayer, 1983). In addition, ependymal cells on the wall of the ventricles are born between E14-E16 and play a role in the transport of CSF and in brain homeostasis. In early pSVZ, a subpopulation of RGCs transform into ependymal cells and become mature later on in the first postnatal week. That suggest that the CSF flow and other functions of the ependymal cells differ between the early pSVZ and aSVZ. This might be associated with the formation of chemo-repellent and chemo-attractant gradients observed in the aSVZ (Spassky et al., 2005). Lastly, astrocytic glial tube chains of migrating neuroblasts occur in the aSVZ and is not distinguishable in the pSVZ until about the third postnatal week (Peretto et al., 2005). Therefore, the pSVZ niche gradually transform from eSVZ-like to an aSVZ-like in the first three postnatal weeks. However, despite these differences, the pSVZ and aSVZ lineage progression is the same.

In this project, I worked on both the postnatal and the adult SVZ to address my research question. In chapter 3, expression of KAP1 in the adult SVZ lineage was confirmed and then functional examination of NSCs function with respect to KAP1 expression was done in the pSVZ and aSVZ. In chapter 4 and 5, I used only the pSVZ niche to

investigate the role of *Paupar* and the molecular mechanisms underlying both KAP1 and *Paupar*.

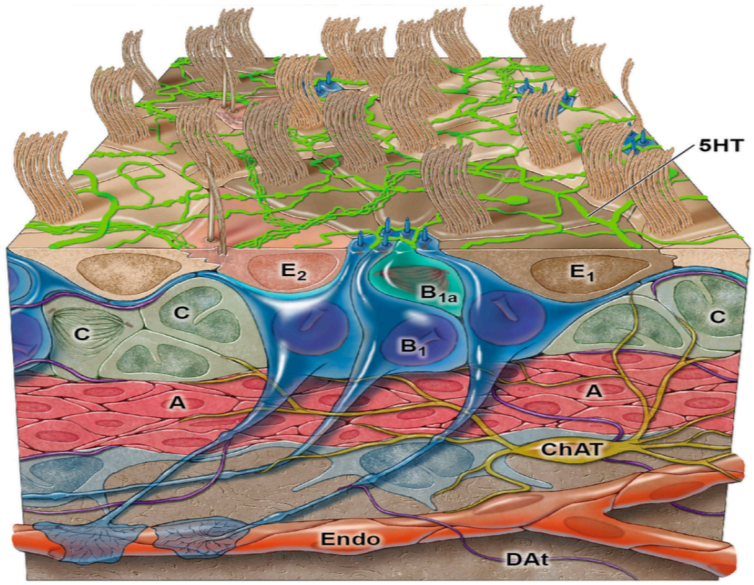


Figure 1.1 The adult SVZ niche

NSCs in the SVZ or B1 cells (dark blue) give rise to activated B1 cells that actively divide and generate transit amplifying progenitors or (type C cells: green). Type C cells undergo 3-4 rounds of mitosis and then generate neuroblasts (type A cells: pink). Neuroblasts are isolated from the striatum by niche astrocytes (B2 cells: light blue). Bl cells contact the ventricle with an apical process that bears a primary cilium. The VZ is also composed of multi-ciliated and bi-ciliated ependymal cells (E1 and E2 cells: brown), that together with the apical ending of the B1cells form a pinwheel structure. The basal process of the B1 cells contacts the endothelial cells lining the blood vessels (red). Image adapted from (Lim and Alvarez-Buylla, 2014)

1.2.3 Heterogeneity of the SVZ niche

During embryogenesis, NSCs correspond to RG, while in the adult brain, a subpopulation of type B1 astrocytes function as NSCs. Neuroepithelial cells and RGs reside in different locations of the developing brain and become regionally specified for producing specific neuronal subtypes (Kriegstein and Alvarez-Buylla, 2009). Studies have also showed that postnatal RGCs and adult B cells in the SVZ become heterogeneous and regionally specified, and neuroblasts migrating in the RMS are heterogeneous before they arrive to the OB (Kohwi et al., 2005, Hack et al., 2005). These studies suggest that the expression of different transcription factors in the SVZ or the RMS results in the generation of different subtypes of neurons. For example, Pax6 is expressed in a subpopulation of neuroblasts and is important to generate dopaminergic periglomerular neurons and a subpopulation of superficial granule cells (Kohwi et al., 2005, Hack et al., 2005).

Different territories of transcription factor expression are present in the developing brain and this regional expression is preserved in the postnatal brain (Kriegstein and Alvarez-Buylla, 2009, Young et al., 2007, Kohwi et al., 2007). Lineage tracing experiments in transgenic mice, using Cre recombinase under the control of different transcription factor promoters expressed in different regions of the embryonic and postnatal SVZ, suggest that different neuronal subtypes in the OB are derived from progenitors in distinct locations in the germinal niche (Young et al., 2007). For example, calbindin + and dopaminergic periglomerular cells in the OB are derived from regions in the SVZ that express Gsh2 transcription factor. Other subpopulations of the calretinin+ cells are derived from progenitors that expressed Emx1, which is a marker of the pallial region of the developing telencephalon (Young et al., 2007, Kohwi et al., 2007) (Figure 1.2).

Taken together, these studies suggest that the NSCs in the SVZ are heterogenous and regionally specified for the generation of distinct subtypes of OB neurons.

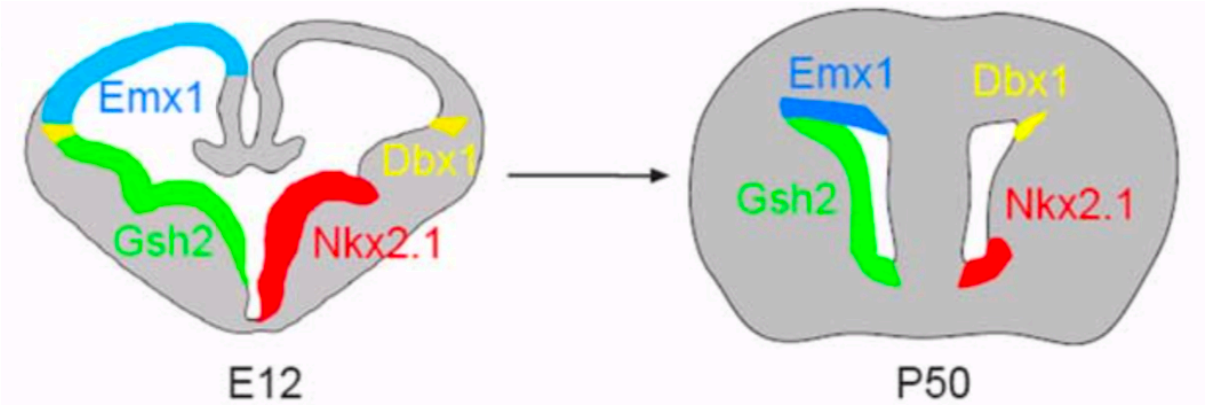


Figure 1.2 Multiple regions of the developing brain neuroepithelium contribute to the adult SVZ

Schematic depicting the different embryonic neuroepithelium domains targeted by Cre mice and their contribution to generate aSVZ. Image adapted from (Young et al., 2007).

1.2.4 Molecular regulation of neurogenesis in the SVZ

SVZ neurogenesis is regulated by many factors to guarantee the proper generation of neurons in the OB. These factors control NSC maintenance, cell cycle, differentiation, migration, cell death, etc

NSC maintenance Neural stem cells self-renewal and multipotency in the SVZ is regulated by many extrinsic and intrinsic components and signalling pathways. For example, Sox2 is a DNA binding transcription factor that is known to play a role in controlling stem cell and progenitor cell fate (Sarkar and Hochedlinger, 2013). In the SVZ, Sox2 is expressed in B1 cells and physically interacts with the homeobox protein PRX2 which acts as a co-activator for Sox2. Depletion of PRX2 in adult mouse NSC culture lead to reduction of their self-renewal ability (Shimozaki et al., 2013).

Moreover, extracellular signalling from the neurogenic niche like ependymal cells, endothelial cells, and choroid plexus also play a role in NSC maintenance (Lim and Alvarez-Buylla, 2016). A study showed that bone morphogenic protein (BMP) signalling is important for NSC maintenance. A mutation of LRP2, a receptor that is expressed in the ependymal cells and plays a role in balancing signals provided by SHH and BMP4, results in impaired proliferation of NSC and decrease in the number of neuroblasts that reach the OB (Gajera et al., 2010). Another study showed Betacellulin (BTC), a member of the EFG family that is secreted by endothelial cells, increased neural stem cell proliferation, neuroblast generation and enhance OB neurogenesis when infused into the lateral ventricle (Gomez-Gaviro et al., 2012). Other molecules like the pigment epithelium–derived factor (PEDF) is secreted by both ependymal and endothelial cells and showed to promote NSCs self-renewal and maintenance (Ramirez-Castillejo et al., 2006).

Lineage commitment In SVZ neurogenesis, glial versus neuronal lineage commitment must be determined. Many transcription factors such as Mash1, Pax6, and Olig2 play major roles in this fate choice. A study revealed that neural progenitor cells with mutation in the transcription factor Mash1 in postnatal mice have defective neurogenesis and oligodendrogenesis indicating its role in lineage commitment (Parras et al., 2004). Moreover, Pax6 physically interact with Brg1-containing BAF complex in adult progenitor cells and together they program neural fate. Deletion of either Pax6 or Brg1 caused the NSC to convert to the ependymal lineage while the migrating neuroblasts convert to glial lineage (Ninkovic et al., 2013). Another transcription factor, Olig2, is expressed in both type B1 and type C cells and it functions is important for fate determination of SVZ progenitor cells toward astrocytic and oligodendrocytic

lineages (Marshall et al., 2005). Extracellular ligands play a role as well in regulating fate choice. For example, in the adult SVZ, platelet-derived growth factor receptor (PDGFR) is expressed in B cells that generate neurons and oligodendrocytes. Conditional KO of PDGFR in B cells showed disruption in oligodendrogenesis but not neurogenesis (Jackson et al., 2006) . Future studies will further identify this interplay between different regulatory mechanisms and lineage commitment.

1.2.5 Neuroblast migration in the postnatal and adult brain

One of the most interesting processes in the SVZ-OB system is the directional "chain" migration of neuroblasts through the rostral migratory stream (RMS) (Figure 1.3). In the postnatal rodent brain, neuroblasts (just 10-30 μm long) migrate over a long distance through the RMS (5-8 mm) at average speeds of ~70-80 μm/hr before they differentiate into neurons in the olfactory bulb (OB) (Nam et al., 2007, Lois and Alvarez-Buylla, 1994, Alvarez-Buylla and Garcia-Verdugo, 2002, Doetsch and Alvarez-Buylla, 1996).These neuroblasts are organized into cords of cells, or chains, in which these cells migrate close to each other without radial glial or axonal guidance (Doetsch and Alvarez-Buylla, 1996). During the first three postnatal weeks, type B2 astrocytes give rise to glial tunnels that covers the forming neuroblast chains (Peretto et al., 2005). The RMS carries the neuroblasts to the OB where they switch from the chains and migrate radially as individual cells to more superficial layers and differentiate into GABAergic neurons in the granule cell layer or dopaminergic neurons in the periglomeruli (Alvarez-Buylla and Garcia-Verdugo, 2002). Functional new GABAergic neurons are first identified as early as 3 days after arrival in the OB (Panzanelli et al., 2009).

The chain migration process of neuroblasts is regulated by the action of both extrinsic and intrinsic signals, cell adhesion/extracellular matrix, growth factors, and neurotransmitters (Ghashghaei et al., 2007). For example, the polysialated neural cell adhesion molecule (PSA-NCAM) is important to guide neuroblasts chain migration in the RMS. Mice deficient in PSA-NCAM showed inability of some neuroblasts to migrate normally (Ono et al., 1994). Other important signal is the directional flow of the cerebrospinal fluid (CSF) in the lateral ventricle that helps in establishing a concentration gradient that supports the migration of neuroblasts (Sawamoto et al., 2006).

Interestingly, neuronal injury in the adult brain cause a transient change in the migration of neuroblasts, which is characterized by reduced migration to the OB and increased migration to the site of injury (Ramaswamy et al., 2005, Goings et al., 2004). For example, after cortical injury, the expression of PSA-NCAM, that is expressed by the majority of cells in the SVZ, was increased dramatically after the lesion, which suggests its involvement in neuroblast migration (Szele and Chesselet, 1996). Another study investigated the role of the tumour necrosis factor-α-converting enzyme, also known as ADAM17/TACE in brain injuries (Geribaldi-Doldan et al., 2018, Dominguez-Garcia et al., 2019). ADAM17 was involved in the glial/neuronal fate decision of neural progenitor cells (NPCs) through its ability to facilitate the release of the epidermal growth factor receptor (EGFR) ligand, transforming growth factor alpha (TGFα), thus regulating EGFR activation, which is known to contribute to glial cells differentiation in injured areas. Inhibition of ADAM17 facilitated neurogenesis within the lesion by promoting the migration and survival of neuroblasts from neurogenic regions and by inducing the differentiation of neural progenitor cells towards the neuronal lineage

(Geribaldi-Doldan et al., 2018). Taken all together, this shows that the SVZ is an accessible and great system for neurodevelopmental mechanisms discovery.

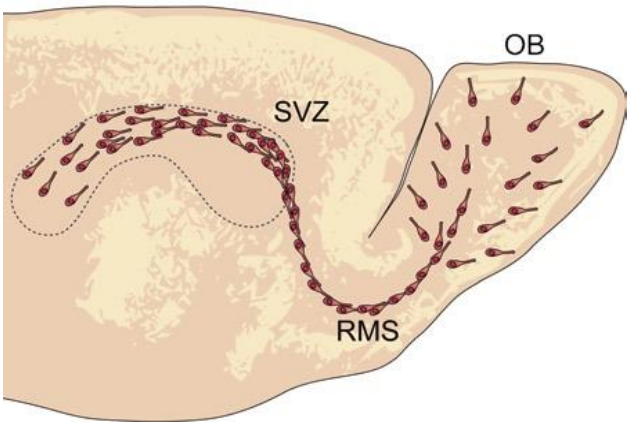


Figure 1.3 Neuroblasts migration through the rostral migratory stream to the olfactory bulb

Neuroblasts are generated in the SVZ. They aggregate and form chains that migrate tangentially along the rostral migratory steam (RMS) to the olfactory bulb (OB). Once they reach the OB, they switch from the chains and migrate radially as individual cells to more superficial layers and differentiate into neurons.

1.2.6 The SVZ in the human brain

A major reason behind the interest in investigating the process of adult neurogenesis is the belief that a similar process exists in humans, and this could have important implications for neurogenerative therapies in the future. This theory was investigated extensively for the past decades using several approaches. The cytoarchitecture of the human SVZ is different from rodents. It is composed of four layers that vary in thickness, cellular composition and densities throughout the lateral ventricles wall. The layer bordering the lateral ventricles wall (layer I) consist of a monolayer of ependymal cells with apical microvilli that are parallel to the ventricular surface and have basal expansions that interact with astrocytic processes in the hypocellular layer (layer II). Adjacent to the hypocellular layer is a ribbon of cells (layer III) that is composed of

astrocytes. The last layer (layer IV) is a transitional zone between the astrocytes and the brain parenchyma. One of the main differences between human and rodents SVZ is the presence of the hypocellular gap while in rodents the astrocytes are not separated from the ependymal layer (Sanai et al., 2004, Quinones-Hinojosa et al., 2006) (Figure 1.4). A group isolated astrocyte from this ribbon and showed that they can proliferate in vivo and form neurospheres with self-renewal and multipotent abilities (Sanai et al., 2004). A subsequent study also showed robust proliferation and migration in the human SVZ and RMS (Curtis et al., 2007). On the other hand, another study showed that the SVZ contain an extensive migrating immature neuron before 18 months of age ,but, contrary to previous reports, it subsides in older children, and is nearly gone by adulthood (Sanai et al., 2011). During this limited time window, no neuroblasts were found in the human OB (Sanai et al., 2011, Wang et al., 2011). Taken together, these studies indicate that neurogenesis maybe age restrained in the human SVZ.

Evaluation of the nuclear bomb test-derived carbon ^{14} (^{14}C) in genomic DNA is another method to study neurogenesis. This method takes advantage of the increased level of atmospheric ^{14}C during the atomic bomb testing between 1940s to 1960s. Cells that were dividing during these years would be expected to have increased level of ^{14}C in their DNA. A study done using ^{14}C birth dating method concluded that there is limited to no postnatal neurogenesis in human OB (Bergmann et al., 2012), supporting previous data (Wang et al., 2011, Sanai et al., 2011).

Moreover, the existence of RMS-OB pathway in the adult human forebrain is highly controversial (Bergmann et al., 2015). A study showed that neuroblasts, that express doublecortin (DCX), neural cell adhesion molecule (PSA-NCAM), and βIII-tubulin exist in the anterior ventral SVZ and RMS but don’t appear to form chains and a proof

of physical structure connecting the SVZ to the OB is still lacking (Wang et al., 2011, Quinones-Hinojosa et al., 2006). A non-RMS migratory pathway has also been described, in which new neurons are added to the prefrontal cortex instead of the OB (Paredes et al., 2016, Sanai et al., 2011). Exciting data show that neurons that express calretinin are added to the adult human striatum, a region adjacent to the SVZ that controls movement and cognition, but is still not clear if these neurons were generated in the striatum or originated from the SVZ (Ernst et al., 2014). Moreover, Neurogenesis in adult mammals is restricted to two active zones while non-mammalian vertebrates have many regions in the brain with active neurogenesis (Kaslin et al., 2008). Neurons are added to the OB in rodents in contrast to songbird, where they are being added to the striatum (Alvarez-Buylla et al., 1994). Therefore, it seems that phylogenetic differences play a major difference in adult neurogenesis. In conclusion, the mechanism of neurogenesis in adult human remain to be investigated.

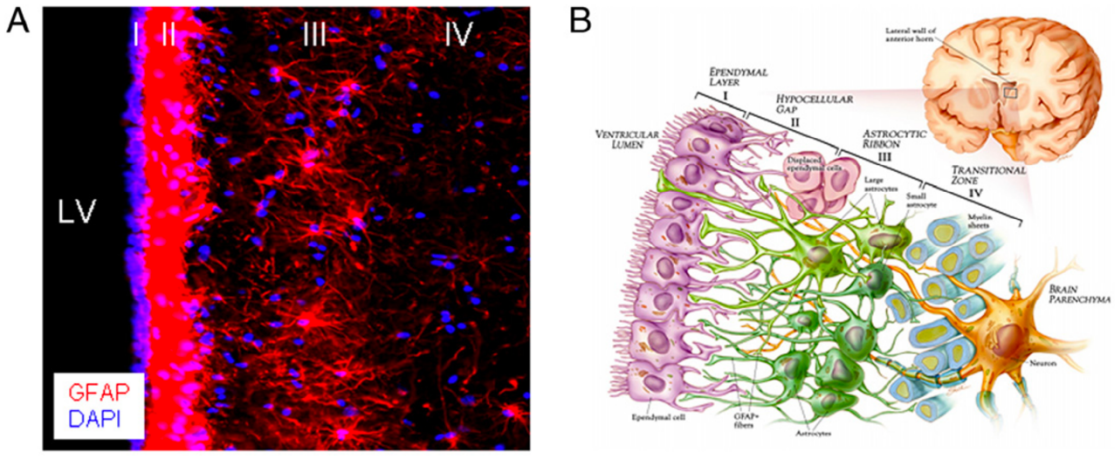


Figure 1.4 Cytoarchitecture of the human adult subventricular zone

A)Immunohistochemistry and (B) schematic drawing of the human SVZ showing the four layers: ependymal layer, hypocellular gap, astrocytic ribbon, and transitional zone. Figure adapted from (Quinones-Hinojosa and Chaichana, 2007)

1.3 Physiological regulation of neurogenesis

The pSVZ niche can be regulated by many signalling molecules and pathways that can influence the behaviour of SVZ cells. These signals can be paracrine signals that are produced locally, endocrine signals that originate from distant regions and uses the circulation system to transport, or autocrine signals that act on the same signalling cells in which they were produced. Example of pSVZ niche signals are soluble factors, cell bound signals, and morphogens. These signals are transduced across SVZ cells membrane and function on the molecular level by changing the transcription of target genes or by epigenetic modification which results in behaviour changes of these cells. These signalling molecules are required for NSC maintenance, proliferation, and fate determination, in addition to their role in effecting SVZ regionalization by influencing the specification of NSCs (Fiorelli et al., 2015). Below, I will briefly explain some of the major SVZ niche signals and their transduction pathways.

1.3.1 Soluble factors: Soluble factors like growth factors, cytokines, nutrients, neurotrophic factors can influence the cellular behaviours of the pSVZ niche. Growth factors (GF) and their receptors are expressed in many cell types in the SVZ including, fibroblast growth factors (FGFs), epidermal growth factor (EGF), insulin-like growth factors, platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and more. GF receptors have intracellular tyrosine kinase activity that facilitates signalling through mitogen-activated protein kinase (MAPK) and phosphoinsitide-3-kinase (PI3K) pathways.

The family of FGF consist of 23 proteins. Members of this family play important roles in neuronal developments by controlling proliferation, migration and chemoattractant

activity (Galvez-Contreras et al., 2012). FGF2 in particular is produced by glial cells and binds to different receptors: FGFR-1, FGFR-2, FGFR-3, FGFR-4. Following ligand binding and FGFR dimerization, it phosphorylates specific tyrosine residues, leading to the docking of adaptor proteins and the activation of RAS/RAF/MAPK and P13K/AKT pathways (Galvez-Contreras et al., 2012). FGF2 signalling in the pSVZ is mitogenic to NSCs and their progenitors (Mudo et al., 2009, Frinchi et al., 2008, Galvez-Contreras et al., 2012). In SVZ cells culture, FGF2 is used to maintain NSCs (Pastrana et al., 2011).

SVZ cells exposed to EGF in cell culture grow to form neurospheres with self-renewal and multipotent abilities (Pastrana et al., 2011). Following EGF binding to its receptor, it facilitate downstream signalling though activation of PI3K, MAPK, and JAK/STAT pathways (Citri and Yarden, 2006). Infusion of EGF caused type C cells to retain activated NSCs phenotype and become highly proliferative (Doetsch et al., 2002). Moreover, enhanced EGFR signalling in vivo results in the increase of NPC pool and reduction of NSC number and self-renewal through EGFR-mediated regulation of Notch signalling (Aguirre et al., 2010).

1.3.2 Cell-bound signals: In contrast to soluble factors mentioned above, when specific signals are bound to the cell surface, they are transduced when ligands on one cell stimulate receptors on the other cells and thus requires cell-cell contact. Some of the major signals transduced upon these cells contact including, cell adhesion molecules (e.g. Integrins and Cadherins), Ephrins and Ephrins receptors, and Notch signalling.

The notch family of proteins are cell-surface receptors that are activated upon the binding of their ligands, Delta and Jagged. Signalling through Notch receptors requires cell-cell contact. When the ligand binds to Notch receptors, the intracellular portion of the receptors is cleaved and enters the nucleus, when it mediates the transcription of

downstream targets (Imayoshi et al., 2010). Notch signalling is required for the maintenance of neural stem cells and regulation of neurogenesis in embryonic and adult brains (Imayoshi et al., 2010, Imayoshi et al., 2013). Since Notch signalling is known to regulate NSC identity and self-renewal whereas EGFR effect NPC proliferation and migration, a study showed a functional cell-cell interaction between these two pathways that is important in maintaining the balance between these cell populations in the SVZ (Aguirre et al., 2010). Moreover, Notch signalling was shown to regulate activation of NSCs, while this function was compensated in quiescence NSCs (Basak et al., 2012, Giachino et al., 2014, Giachino and Taylor, 2014).

1.3.3 Morphogens: Morphogens are signalling molecules that are secreted from a distant source of a tissue and spread away to act on cells directly to induce distinct cellular response in a concentration gradient manner (Tabata and Takei, 2004, Wartlick et al., 2009). These include Sonic hedgehog (Shh), Wnt family member Wingless (Wg), Bone Morphogenic proteins (BMPs), and more. The role of morphogens during development has been extensively studied. Of note, adhesion molecules and GFs (like IGFs and FGFs) can function as morphogens. In the postnatal brain, morphogens can function as mitogens as seen with IGFs, Shh, Wnts, and FGFs, as well as in fate determination as observed with Wnts, Shh, and BMPs (Faigle and Song, 2013). I will elaborate on BMP signalling more in Chapter 5 due to its relevance to this thesis.

1.4 Epigenetic regulation of neurogenesis

Epigenetics is the study of alterations in gene expression that do not involve changes in the underlying DNA sequence (Montalban-Loro et al., 2015, Jaenisch and Bird, 2003). It was first proposed by Conard Waddington in 1942 (Waddlngton, 1942) and since

then different types of epigenetic regulations were discovered, such as DNA methylation (Goll and Bestor, 2005), post-translational modifications of histones (Kouzarides, 2007), and noncoding RNAs (ncRNA) (Mattick and Makunin, 2006, Zaratiegui et al., 2007).These types of modifications are related and sometimes work together to establish many layers of control (Bird, 2007) and any dysregulation of these modification will effect gene expression and result in a new phenotype. In the SVZ, epigenetic modifications play an important role in regulating neurogenesis (Figure 1.5).

1.4.1 DNA methylation includes the addition of a methyl group to the fifth carbon of cytosine in DNA molecules (5mC). In mammalian genes, CpG dinucleotides are arranged in clusters called "CpG islands" and are often methylated and found in regulatory regions such as the promoter and exon regions of the genes (Montalban-Loro et al., 2015). DNA methylation is catalysed by DNA methyltransferase enzymes (DNMT) and repress gene expression either by preventing the binding of transcription factors to the regulatory regions or by attracting DNA methyl binding domain proteins (MBDs), that recruit chromatin remodelling molecules to modulate the chromatin surrounding the genes and establish an inactive chromatin state (Bird, 2002). Mice deficient in MBD1 have decreased neurogenesis and impaired spatial learning. MBD-/- neural stem cells have defective differentiation and increased genomic instability (Zhao et al., 2003). DNA methylation marks can be reversed by DNA hydroxymethylation (5hmC) which is catalysed by enzymes of the TET family (Montalban-Loro et al., 2015).

1.4.2 Histone modifications In eukaryotic cells, a nucleosome is a basic unit of DNA sequence wound around a histone octamer which includes two H2AH2B dimers

and a H3H4 tetramer (Montalban-Loro et al., 2015). The N-terminal tail of histone proteins are subject to post-translation modification that control the chromatin structure around it. These modifications are on different amino acid residues and include methylation, acetylation, phosphorylation, and ubiquitination marks. These modifications are divided into active and repressive marks according to how they affect transcriptional activity (Bannister and Kouzarides, 2011). For example, the histone acetyltransferases add an acetylation mark on lysine residues of histones, allows the recruitment of many transcriptional machinery, and generally associated with active chromatin states. While the histone deacetylases (HDACs) remove acetyl groups and cause condensation of the chromatin (Marmorstein and Trievel, 2009). A study shows that HDAC2 conditional knockout mice have defective adult neurogenesis (Jawerka et al., 2010), and SVZ derived neurospheres that were treated with HDAC inhibitors show an increase in neuronal differentiation (Siebzehnrubl et al., 2007).

Histone methylation is catalysed by histone methyltransferases (HMTs) and is associated with active and repressed chromatin states. Trimethylation of lysine (K) 27 and lysine 9 of histone H3 (H3K27me^3 and H3K9me^3) is associated with gene repression, while H3K4 methylation is associated with active genes (Montalban-Loro et al., 2015). EZH2, a component of the PRC2 complex, functions as a methyltransferase of H3K27, and its role in adult neurogenesis has been investigated. It is expressed in neural progenitor cells and mature neurons and deletion of EZH2 result in the reduction of cell proliferation and the number of neurons. Conditional knockout of EZH2 results in defect in spatial learning and memory (Zhang et al., 2014). Another example is the mixed-lineage leukaemia 1 protein (Mll1), which is a histone methyl transferase that adds a methylation mark on H3K4. Knockout of Mll1 in NSCs caused defective

neurogenesis. Mll1 is associated with the promoter of Dlx2, a downstream regulator of neurogenesis. In Mll1 mutated SVZ cells, the chromatin surrounding the promoter of Dlx2 become bivalently marked with the histone methyltransferase H3K4me3 and the histone demethylase H3K27me3 and so the Dlx2 gene cannot activate properly (Lim et al., 2009).

1.4.3 Chromatin remodelling is the dynamic organization and modification of the chromatin to allow access of the genomic DNA to different regulatory transcriptional machinery proteins. There are five families of chromatin remodellers that have been studied so far: NuRD/Mi-2/CHD, SWI/SNF, ISWI, INO80 and SWR1.These protein complexes have a common ATPase domain that allow them to reposition and modify the nucleosomes from the energy they gain after ATP hydrolysis (Wang et al., 2007).

Studies have shown that the mammalian SWItch/Sucrose Non-Fermentable (mSWI/SNF) , also known as BRG1/BRM associated factor (BAF) chromatin modifier family regulate adult neurogenesis (Sokpor et al., 2017). In a recent study, they show that BAF170 subunit of the BAF complex is expressed in radial glial-like (RGL) progenitor cells and in mature astrocytes. Conditional deletion of BAF170 in the DG depletes the RGL cell pool and promotes the differentiation of astrocytes (Tuoc et al., 2017). Another study showed that the BRG1 subunit of the BAF complex interacts with Pax6 in adult neural progenitor cells. Deletion of either Pax6 or Brg1 in the SVZ causes the neural stem cells convert to the ependymal lineage while migratory neuroblast convert to different glial lineages (Ninkovic et al., 2013). In conclusion, these studies indicate that epigenetic modification play a critical role in neurogenesis.

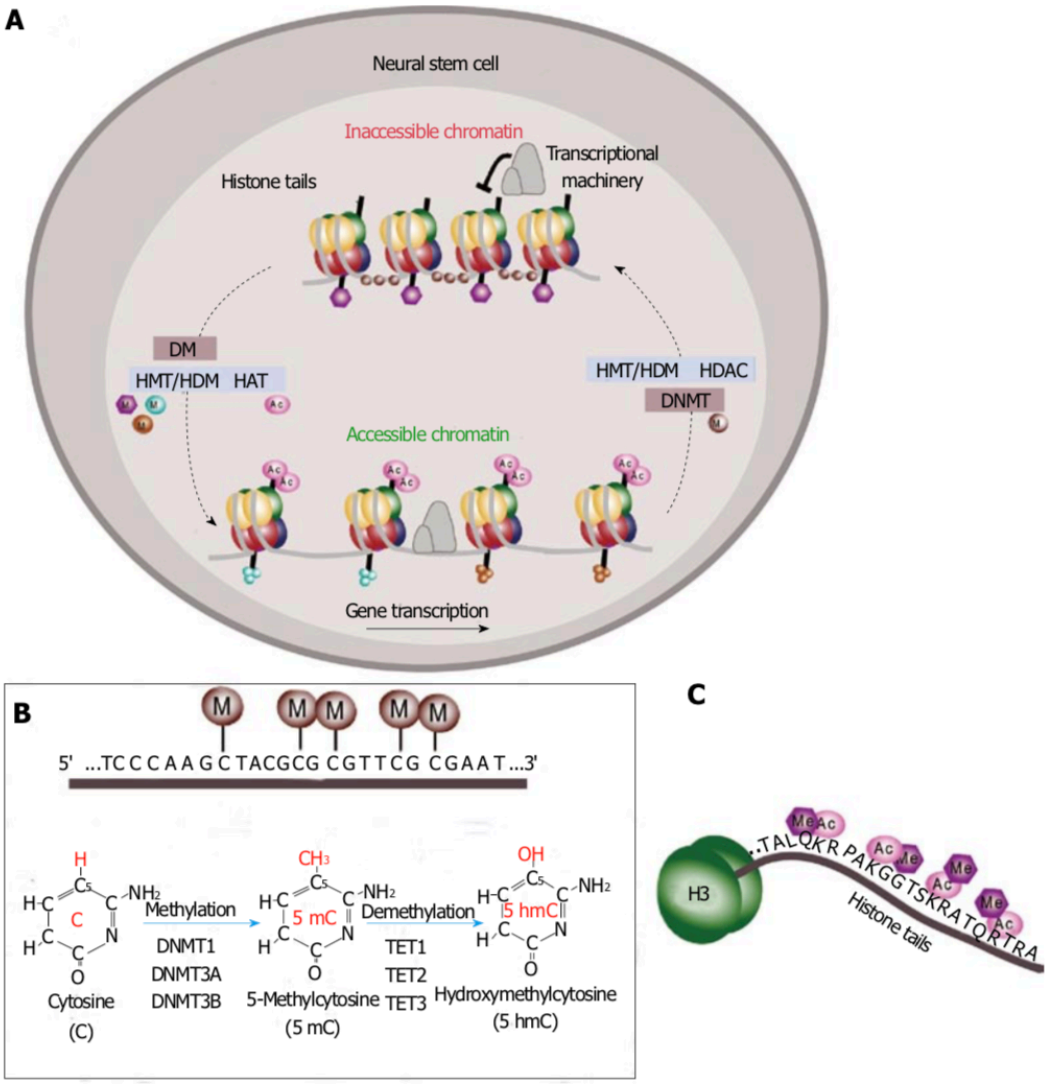


Figure 1.5 Epigenetic regulations of neural stem cells by DNA methylations and histone modifications

A: Chromatin condensation caused by adding of methyl groups (M) to CpGs, and by histone methylation reaction which is catalysed by histone methyltransferases (HMTs). This reaction is reversed by histone demethylases (HDMs).Chromatin decondensation is mediated by histone acetyltransferases (HATs). Histone deacetylases (HDACs) mediate the reverse process and induce chromatin condensation.
B:DNA methylation at the CpG dinucleotides in gene regulatory regions. The reaction is mediated by DNA methyltransferases (DNMTs) and reversed by ten-eleven translocation (TET) family of enzymes that convert 5mC into 5hmC.
C: Histone tail showing multiple site of modifications as methylation (Me) and acetylation (Ac).Figure adapted from (Montalban-Loro et al., 2015).

1.5 KRAB domain-associated protein 1 (KAP1)

1.5.1 KRAB-ZFP

In eukaryotic cells, transcriptional control is a very complex process which involves the regulation of many transcription factors (TFs) and cofactors to assemble multi-protein complexes and initiate or repress the transcription of target genes. The important feature that TFs have is the presence of DNA-binding motifs that allow their binding to a specific regions of the promoter of target genes, this interaction is important to regulate the functions of these genes (Lupo et al., 2013).

One of the largest class of DNA-binding motifs in TFs is the zinc finger (ZF). They are small protein domains in which zinc contribute to their stability. They are structurally diverse and play roles in various cellular processes (Krishna et al., 2003). Many members of the zinc finger domains have been identified. The most studied C2H2 zinc finger motif (also called Kruppel-like) is composed of 28-30 amino acids and it is stabilized by a zinc ion that is coordinated by two cysteines and two histidine residues. The folded structure of zinc finger consist of an alpha helix that mediates its binding to DNA through non-covalent interaction between three amino acids and three bases of DNA (Krishna et al., 2003, Lupo et al., 2011). They contain from 1 to more than 30 ZF motifs, and variations in the amino acid sequence of the finger domain increase their ability to bind to multiple different proteins, RNAs, and DNA-RNA hybrids. Most C2H2 zinc finger proteins have conserved domains that contribute to their functions like the well-studied KRAB domain (Kruppel-Associated Box) (Lupo et al., 2013).

The Krüppel-associated box (KRAB) domain is located in the N-terminal sequence of C2H2 zinc finger protein, and it binds to different corepressor proteins and/or

transcription factors through protein-protein interactions to cause repression of target genes. KRAB domain is considered as a powerful transcriptional repressor domain and the KRAB-ZFPs are one of the largest family of transcription regulators that play roles in cell proliferation, cell differentiation, embryonic development and apoptosis (Friedman et al., 1996, Urrutia, 2003).

1.5.2 KRAB domain-associated protein 1 (KAP1) introduction

KRAB domain-associated protein 1 (KAP1), also identified as transcriptional intermediary factor 1 beta (TIF1β) or tripartite motif-containing 28 (TRIM28) was recognized as an interacting protein for Krüppel-associated box zinc finger proteins (KRAB-ZFPs) in 1996 (Friedman et al., 1996, Iyengar and Farnham, 2011). KAP1 binds to the KRAB repressor domain of zinc finger TFs and was originally classified as a transcriptional corepressor. KAP1 is a critical regulator of many aspects of cellular physiology including tumorigenesis, immune response, virus replication, and DNA damage response (Cheng et al., 2014b, Kim et al., 1996). KAP1 is located in many cellular compartments including the heterochromatin, euchromatin and the cytoplasm, which implicate its involvement in many cellular processes (Ryan et al., 1999).

Global knockout of KAP1 in mice embryo lead to embryonic lethality due to their inability to undergo gastrulation which suggests its role in normal development (Cammas et al., 2000). Whereas conditional knockout deletion of KAP1 in the adult forebrain show a stress and anxiety like behaviour (Jakobsson et al., 2008). KAP1 is also involved in regulating self-renewal genes in embryonic stem cells (ESCs) (Hu et al., 2009), and required for mouse ESC differentiation (Cammas et al., 2007, Cammas et al., 2002). Moreover, KAP1 play major roles in tumour progression which suggest the development of anti-KAP1 drugs for cancer therapy (Silva et al., 2006, Beer et al.,

2002, Yokoe et al., 2010, Wang et al., 2005). For example, a study showed that endogenous KAP1 interacts with MDM2, a major regulator of p53 tumour suppressor gene activity. When KAP1 is depleted, that stimulates p53 transcriptional activity and increases apoptosis which suggests the use of KAP1 as a drug target for p53 re-activation in cancer cells (Wang et al., 2005) . Taken together, these diverse functions of KAP1 suggest its important impact and warrant further future studies on this vital coregulator.

1.5.3 KAP1 protein structure

KAP1 is a member of TIF1 family of protein that includes TIF1α, TIF1β, TIF1γ and TIF1δ. All members of the family share almost similar structure, they are composed of N-terminal tripartite motif (TRIM), RBCC domain that is composed of RING finger, 2 B-box zinc fingers and a coiled coil region. In addition, they all share a central TIF1 signature sequence (TSS), HP1 binding domain (HP1BD), a C-terminal domain that is composed of PHD and bromodomain (known as PB domain). Unlike other TIF1 family members, KAP1 does not have a nuclear receptor domain (Cheng et al., 2014b, Iyengar and Farnham, 2011) (Figure 1.6).

The RBCC domain of KAP1 was known to be required for its recruitment to KRAB-ZFP binding sites (Peng et al., 2007). However, a study has done a functional analysis of KAP1 recruitment to the genome and found that deletion of RBCC domain disrupts KAP1 binding to the 3' exon of ZF genes but KAP1 can still bind to the promoter of target genes (Iyengar et al., 2011), suggesting additional mechanisms for its recruitment to the genome. The TSS domain is next to the RBCC domain and its function has not been identified yet.

HP1 binding domain of KAP1 facilitates its binding to HP1 and this KAP1-HP1 interaction is believed to play an important role in heterochromatin formation and gene silencing. For example, KAP1-HP1 interaction on mesoderm-specific genes (MEST) is essential to establish a heterochromatin structure characterized by H3K9 trimethylation. When this interaction was disrupted, KAP1 was released from the promoter of MEST genes and caused an activation of MEST expression (Riclet et al., 2009).

The PHD and bromodomain at the C-terminal region recognize histone tails and recruit histone modifiers like CHD3/NuRD complex, and histone methyltransferase SET domain bifurcated 1 (SETDB1) that are required for KAP1 mediated silencing of genes (Schultz et al., 2002, Schultz et al., 2001).

KAP1 is subject to different types of post-translational modification (PTM) on its C-terminal domain. KAP1 is SUMOylated on six lysine residues on its PB domain and is required for its repressive function by directly recruiting SETDB1 histone methyltransferase and the NuRD complex by SUMO interacting motifs (Ivanov et al., 2007, Li et al., 2007, Mascle et al., 2007). KAP1 also undergoes phosphorylation, a study reported that phosphorylation of KAP1 at tyrosine residues 449,458 and 517 by Src family of tyrosine kinases (SFKs) prevents the interaction of KAP1 and HP1 with heterochromatin causing chromatin structural changes around target genes (Kubota et al., 2013). SFKs are known to regulate tumour cell growth and differentiation, and these findings imply that KAP1 is involved in SFK-mediated tumour transformation.

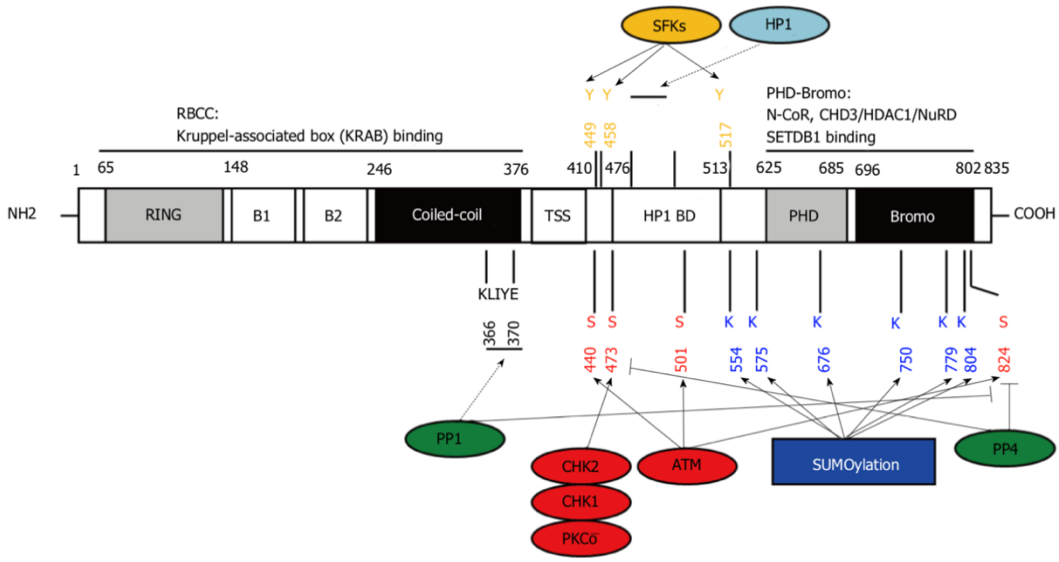


Figure 1.6 KAP1 structure, post-translation modifications, and binding proteins

RBCC: RING, B1, B2, and coiled-coil; TSS: TIF signature sequence; HP1BD: HP1 binding domain; PHD: Plant homeo domain. SUMOylation sites (blue); Serine phosphorylation sites targeted by kinases (red) or antagonized by phosphatases (green); Tyrosine phosphorylation sites (orange) targeted by kinases. Figure adapted from (Cheng et al., 2014b).

1.5.4 KAP1: Guardian of the Genome

KAP1 is implicated in the regulation of many mechanisms including the repression of endogenous retroviruses, DNA damage repair, and apoptosis. Endogenous retroviruses (ERVs) are viral elements in the genome that are derived from a previous retroviral infection. When the viral DNA integrated into the genome, it becomes fixed and inherited through generations. ERVs are distributed within the DNA of all cells, constitute ~1% of the genome and serve a vital role in gene expression and regulation (Nelson et al., 2003). Aberrant ERV expression is implicated in several neurological disorders (Christensen, 2016). KAP1 was found to contribute to the regulation of ERV expression in embryonic and adult tissues. Deletion of KAP1 led to the loss of histone 3 lysin 9 trimethylation (H3K9me3), a hallmark of KAP1 repression mechanism, and caused upregulation of endogenous retroviruses in mouse embryonic stem cells but not

in embryonic fibroblasts (Rowe et al., 2010), suggesting that KAP1 has distinct roles in regulating ERV depending on the cell type. Similar mechanism was also observed in neural progenitor cells cultured from embryonic mice brain (Fasching et al., 2015) and in somatic adult tissues (Ecco et al., 2016). Excitingly, a recent study demonstrated around 10,000 ERVs that act as a docking sites for KAP1 in human neural progenitor cells, which resulted in heterochromatin formation and repression of ERV and subsequently neighbouring protein-coding genes that are important for brain development (Brattas et al., 2017).

KAP1 has also been reported in regulating DNA repair. DNA damage activates ataxia-telangiectasia mutated (ATM) which is known to phosphorylates KAP1 on serine 824 and this results in the removal of SUMOylation mark on KAP1 and upregulation of genes involved in cell cycle arrest or apoptosis in response to genotoxic stress (Li et al., 2007). Further study from the same group indicated the role of protein phosphatase 1 (PP1) in dephosphorylation of KAP1 serine 824 while promoting its SUMOylation to reverse the effect of ATM and return KAP1 to its repressive effect on target genes (Li et al., 2010). This suggests that KAP1 repressive role is being balanced and regulated between phosphorylation and SUMOylation modifications of its domain.

Finally, KAP1 was suggested to regulate apoptosis. A study showed that KAP1 cooperate with MAGE proteins, which are aberrantly expressed only in melanomas and other malignancies, in the suppression of p53-mediated apoptosis and survival of cancer cells (Yang et al., 2007). Taking together, these studies suggest that implication of KAP1 in different cellular processes.

1.5.5 KAP1 function in the brain

KAP1's role in the brain has not been investigated thoroughly. A few studies focused on KAP1 function in regulating endogenous retroviruses in neural progenitor cells (Fasching et al., 2015, Brattas et al., 2017), glioma progression (Qi et al., 2016a, Qi et al., 2016b), and behavioural stress responses in mice models (Jakobsson et al., 2008).

Exciting study by Jakobsson et al. reported that KAP1 is expressed in high levels in the mouse hippocampus. Knockout of KAP1 in adult mice brain exhibit anxiety-like behaviour and stress-induced alteration in spatial learning and memory based on experiment performed on two paradigms, the open field (OF) and the elevated plus maze (EPM). Surprisingly, microarray analysis detected a subtle difference between the KAP1 cKO and control mice, with only 30 gene were dysregulated including some imprinted genes. The heterogeneity of the sample harvested through dissection of the whole hippocampus contains both KAP1-positive and KAP1-deleted cells may attribute to this difference. Other reason could be that KAP1 play only a cofactor role rather than a major transcriptional regulator in this condition. This is surprising since KAP1 was shown to bind to 7000 sites genome wide in ChIP analysis performed on human tumour cell line (O'Geen et al., 2007). Moreover, the genes that were shown to be dysregulated in Jakobsson et al. microarray analysis did not provide a straightforward explanation of the phenotype observed in this study. Additionally, chromatin analysis revealed changes in the histone modification states of these genes after KAP1 deletion. In conclusion, their model suggest that KAP1 epigenetically regulate the expression of neuronal genes in the hippocampus and thereby controlling response to behavioural stress and anxiety (Jakobsson et al., 2008).

Two studies from the same group investigated the expression and the role of KAP1 in glioma cell proliferation and progression. KAP1 expression was significantly higher in glioma patients and was associated with poor overall survival rate. Knocking down of KAP1with shRNA in glioma cells lead to increase p21 expression and cause a cell cycle arrest at G1 phase. In a xenograft model, down regulating of KAP1 suppress tumour growth and proliferation (Qi et al., 2016b). Moreover, they observed that KAP1 is a direct target of microRNAs. Both miR-491 and KAP1 were aberrantly expressed in glioblastoma tissues. Downregulation of miR-491 lead to increase the expression of KAP1 and promote glioma cells proliferation (Qi et al., 2016a). These studies warrant future studies to use KAP1 as a diagnostic marker and a therapeutic target for glioma treatment.

1.6 Long non-coding RNAs (LncRNAs)

1.6.1 Introduction to lncRNAs

Sequencing of the human genome provided a surprise when it revealed that only ~20,000 genes are encoding proteins, making < 2% of the total genomic sequence, while a significant portion of the genome are non-coding RNAs (ncRNAs). Noncoding transcripts are divided into two classes, house-keeping ncRNAs and regulatory ncRNAs. Housekeeping ncRNAs include ribosomes, small nucleolar RNA, and transfer RNAs. Regulatory ncRNAs include microRNAs (miRNAs), endogenous small interfering RNAs, Piwi-associated RNAs, and long non-coding RNAs (lncRNAs) (Wilusz et al., 2009, Kung et al., 2013, Mercer et al., 2009, Ponting et al., 2009, Wei et al., 2018).

Long noncoding RNAs (lncRNAs) are more than 200 nucleotides transcripts without any protein coding potential. Similar to mRNAs, they are transcribed by RNA polymerase II, contain splicing sites, introns and exons, but without an open reading frame (ORF) in their sequence (Ponting et al., 2009, Wei et al., 2018). They are also known for their secondary structure which allows protein binding at many sites or for specific DNA-RNA binding (Wei et al., 2018). They are known to regulate gene expression on a transcriptional and post-transcriptional level and by different modes of action (Wei et al., 2018). lncRNAs are highly expressed in the brain and play major roles in brain development, neural maintenance and differentiation (Ramos et al., 2016, Mercer et al., 2008).

In 2008, Mercer et al. identified hundreds of lncRNAs that are expressed in the brain by in situ hybridization method. The expression of these lncRNAs differ according to their anatomical location, cell type, and subcellular locations (Mercer et al., 2008). The

lncRNA *Evf2* is expressed in the ventral forebrain and was shown in a vivo knockout study to regulate the development of GABAergic neurons. Another example is the lncRNAs *MALAT-1* and *Neat1*, they are both localized in the nucleus and play a role in regulating alternative splicing of pre-mRNA by modulating serine/arginine splicing factor phosphorylation (Tripathi et al., 2010). While the muscle-specific long non-coding RNA,linc-MD1, is expressed in the cytoplasm of myoblasts and plays an important role in muscle differentiation (Cesana et al., 2011).

1.6.2 Genomic context of lncRNAs

One way to classify lncRNAs is according to their genomic location, as in from where in the genome they are being transcribed. These lncRNAs can be classified into five different groups: Stand-alone lncRNAs, which are transcribed from a specific sequence that does not overlap with a protein-coding genes; Antisense transcripts, transcribed opposite to the sense DNA sequence; Pseudogenes, transcribed from genes that lost their coding potential due to a mutation; Intronic, transcribed from an intron sequence; Intergenic, lncRNAs that are promoter-associated and enhancer associated transcripts (Kung et al., 2013, Mercer et al., 2009) (Figure 1.7). This genomic context categorization does not provide any information about their function or conservation.

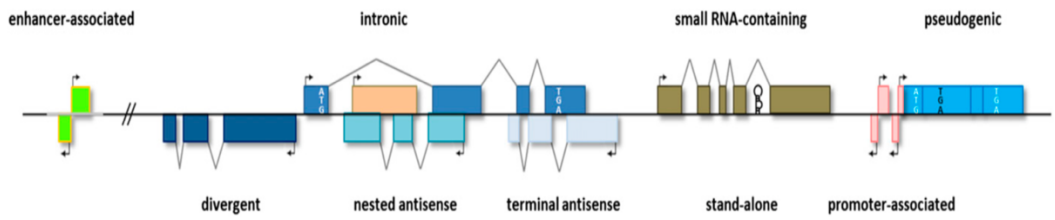


Figure 1.7 Genomic location of lncRNAs

lncRNAs may be transcribed from enhancers, promoters, pseudogenes, or antisense to other genes with different types of overlaps, from complete (nested), to partial (terminal),to none (divergent). They could be stand-alone transcript unit, and may also host small RNAs within their transcript unit (black hairpin). Figure adapted from (Kung et al., 2013)

1.6.3 LncRNAs mechanism of action

LncRNAs have different mechanisms to regulate gene expression including chromatin modification, transcription and post-transcription regulations.

Chromatin modification: lncRNAs can modify the chromatin structure by recruiting chromatin remodelling factors to a specific DNA sequence. For example, Hox transcript antisense RNA (*HOTAIR*), is expressed across the HOXC locus and is known to act in trans to recruit polycomb chromatin remodelling complex (PRC2) to modify the chromatin and repress the transcription of HOXD locus ,which is 40 kb away from its site of synthesis (Rinn et al., 2007). Another example is the lncRNA *Xist*, which acts in cis to inactivate X-chromosome by also recruiting PRC2 complex to modifying the chromatin and silence the gene (Zhao et al., 2008, Brown et al., 1992).

Transcriptional regulation: Some lncRNAs are transcribed from the promoter of specific genes and function to recruit RNA binding proteins to these genes promoter to regulate their expression (Wang et al., 2008). Other lncRNAs can function as transcriptional coregulators and cooperate with transcription factors to regulate target gene expression. The lncRNA *Evf2* is transcribed from Dlx-5/6 enhancer and acts as a coactivator by recruiting the transcription factor Dlx2 to Dlx-5/6 gene and induce its expression (Feng et al., 2006).

Post-transcriptional regulation: LncRNAs can function post transcriptionally by acting as antisense transcripts and regulate mRNAs splicing, translation and degradation. They can form RNA duplexes and cover some elements in mRNA. For example, the Zeb2 antisense lncRNA was observed to effect the alternative splicing of Zeb2 mRNA. It complements the intronic region in the 5` UTR in the mRNA and prevents the splicing and the translation of the Zeb2 protein (Beltran et al., 2008). In

conclusion, lncRNAs seem to have many functions and mechanism that haven't been discovered yet and more research in the lncRNA word will reveal their importance in cell biology (Figure 1.8).

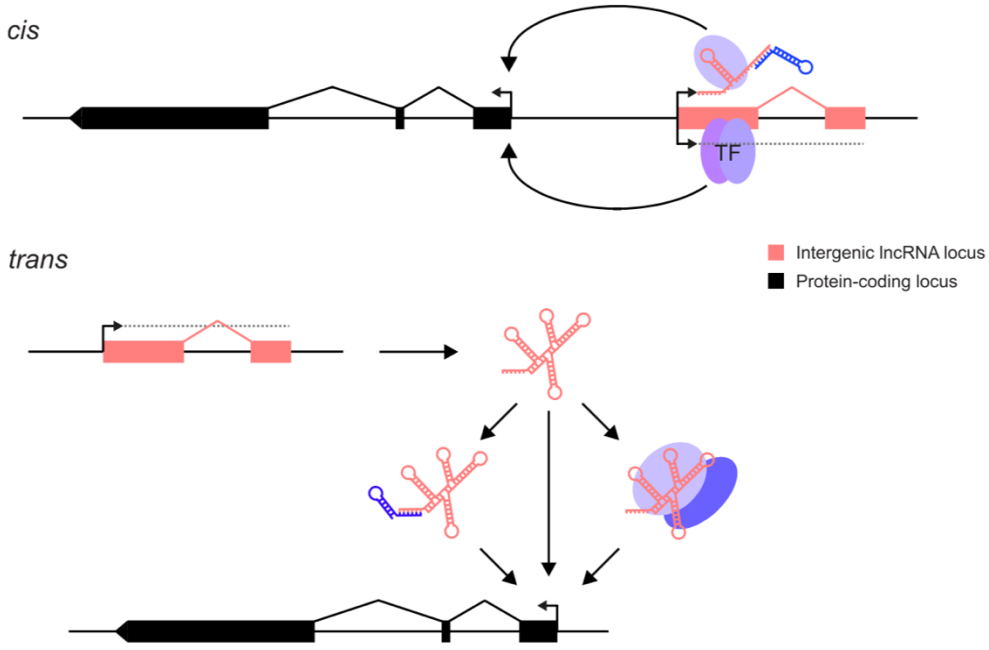


Figure 1.8 LncRNAs can act in cis or in trans to regulate gene expression

LncRNAs (pink) act in cis to regulate the expression of their neighbouring protein-coding genes (black), or in trans to regulate the expression of distant protein-coding genes. The RNA my act independently, or through base pairing with other RNA (blue), or by binding to transcription factors (purple).Figure adapted from (Bassett et al., 2014)

1.6.4 Functionality of lncRNAs

Even though lncRNAs are extremely abundant, they were initially classified as transcriptional noise or "junk" DNA. The low sequence conservation of lncRNAs fuelled the debate about their function and suggests low evolution pressure and biological importance (Mercer et al., 2009, Ponting and Belgard, 2010, Ponting et al.,

2009). However, the observation that large number lncRNAs play major roles during development (Amaral and Mattick, 2008), and many lncRNAs are specifically expressed during embryonic stem cell differentiation (Dinger et al., 2008), and in the brain were shown to exhibit specific subcellular localization (Mercer et al., 2008). In addition to their binding of transcription factors and involvement in different types of transcriptional regulation suggests that they have explicit biological functions (Mercer et al., 2009, Ponjavic et al., 2009). Second, unlike protein-coding genes which are highly conserved and must preserve their ORF, lncRNAs can only have shorter stretches of sequence that are conserved to sustain their functional domain and structure (Pang et al., 2006). For example, the lncRNA *Xist* is known for its function in X chromosome inactivation but only a short sequence of its length is conserved, and despite this, PRC2 targeting region in Xist is defined in different species. So functionally and high conservation of the sequence may not always correspond to each other (Hendrich et al., 1993, Nesterova et al., 2001, Zhao et al., 2008).

1.6.5 LncRNAs regulation in development

Even though lncRNAs functions have been investigated thoroughly in many studies, the extent of their contribution to normal cellular processes in development, differentiation, and disease are still not clear. In many models, no or only subtle phenotypic effects were observed after loss-of-function studies of lncRNAs with high expression in certain tissues, such as *Malat11*, *Visc-2*, and *Neat1* (Table 1.1) (Bassett et al., 2014, Oliver et al., 2015). In contrast, the lncRNA *Fendrr* was shown to be an essential regulator in early embryonic development (Grote et al., 2013, Sauvageau et al., 2013). *Fendrr* is a lateral mesoderm-specific lncRNA and important for proper heart and body wall development in the mouse. Loss of *Fendrr* in mouse embryo caused upregulation of many transcription factors needed for cardiac differentiation, accompanied with

reduction in H3K27 methylation marks and PRC2 occupancy at their promoters (Grote et al., 2013). Importantly, Knockdown of *Fendrr* using shRNA method did not cause any effect, instead, replacement of the first exon with a stop signal sequence caused the observed phenotype (Grote et al., 2013). Another group used a different knockout method, they replaced the *Fendrr* locus with a lacZ cassette, and this also lead to embryonic lethality (Sauvageau et al., 2013). Nevertheless, the later study observed different phenotype: lung and gut developmental defect as well as heart (Sauvageau et al., 2013). One possible explanation of this difference in the phenotype is the knockout method used by Sauvageau et al. which could have disrupted the potential regulatory DNA sequence in the *Fendrr* locus and so the genomic locus function has been affected as well and lead to an overt phenotype. These studies demonstrated how crucial it is to use the appropriate loss-of-function method to understand the function of lncRNAs.

lncRNA name	Organism	Mutation strategy	Reported animal phenotype	RNA-based rescue?	Reference
Xist	*Mus musculus*	~15 kb replaced with a *neo* expression cassette	Females inheriting paternal allele were embryonic lethal; males fully viable	No	(***Marahrens et al., 1997***)
Xist	*Mus musculus*	Inversion of Exon 1 to intron 5	Embryonic lethality of paternally inherited allele	No	(***Senner et al., 2011***)
H19	*Mus musculus*	Replacement by *neo* cassette	Slightly increased growth	No	(***Ripoche et al., 1997***)
roX	*Drosophila melanogaster*	Deletions of *roX1* or *roX2*	None, except when in combination: male-specific reduction in viability	Yes	(***Meller and Rattner, 2002***)
Kcnq1ot1	*Mus musculus*	Promoter deletion	Growth deficiency for paternally inherited mutation	No	(***Fitzpatrick et al., 2002***)
Airn	*Mus musculus*	Premature transcriptional termination	Growth deficiency for paternally inherited mutation	No	(***Sleutels et al., 2002***)
Evf2	*Mus musculus*	Premature transcriptional termination	None	N/A	(***Bond et al., 2009***)
BC1	*Mus musculus*	Replacement of promoter and exon by *PgkNeo* cassette	Vulnerable to epileptic fits after auditory stimulation	No	(***Zhong et al., 2009***)
Neat1	*Mus musculus*	3 kb Promoter and 5′ deletion	None	N/A	(***Nakagawa et al., 2011***)
Tsx	*Mus musculus*	2 kb Promoter and exon 1 deletion	Smaller testes and less fearful (males)	No	(***Anguera et al., 2011***)
Malat1	*Mus musculus*	Deletion	None	N/A	(***Eissmann et al., 2012***)
Malat1	*Mus musculus*	*lacZ* insertion and premature transcriptional termination	None	N/A	(***Nakagawa et al., 2012***)
Malat1	*Mus musculus*	3 kb Promoter and 5′ deletion	None	N/A	(***Zhang et al., 2012***)
Hotair	*Mus musculus*	Deletion	Spine and wrist malformations	No	(***Li et al., 2013***)
Hotdog* and *Twin of Hotdog	*Mus musculus*	Large (28 Mb) translocation by inversion	Loss of *Hoxd* expression in the cecum	N/A	(***Delpretti et al., 2013***)
Fendrr	*Mus musculus*	Replacement of exon 1 with transcriptional stop signal	Embryonic lethal around E13.75	Yes (majority of embryos)	(***Grote et al., 2013***)
Fendrr	*Mus musculus*	Locus replacement with *lacZ* cassette	Perinatal lethality	No	(***Sauvageau et al., 2013***)
Peril	*Mus musculus*	Locus replacement with *lacZ* cassette	Perinatal lethality	No	(***Sauvageau et al., 2013***)
Mdgt	*Mus musculus*	Locus replacement with *lacZ* cassette	Reduced viability and reduced growth	No	(***Sauvageau et al., 2013***)
15 other lncRNA loci	*Mus musculus*	Locus replacement with *lacZ* cassette	None	N/A	(***Sauvageau et al., 2013***)

Table 1.1 LncRNAs that have been studied and the mutation strategy used. Figure adapted from (Bassett et al., 2014)

1.6.6 lncRNAs in Disease

Glioblastoma multiforme (GBM) is the most common and highly malignant type of brain tumour, it occurs in 7.2 per 100,000 adults per year. The lncRNA *HOTAIR*, Hox transcript antisense intergenic RNA, is a well-studied example of lncRNAs function in tumorigenesis (Ramos et al., 2016). *HOTAIR* expression was upregulated in GBM samples, and its expression was associated with glioma grade and considered as a prognostic factor in glioblastoma multiforme patients (Zhang et al., 2013). *HOTAIR* directly interact with EZH2 (component of the PRC2 complex) and together they control cell cycle progression. Knockdown of *HOTAIR* or EZH2 prevent cell cycle progression in glioma cells (Zhang et al., 2015a). In an orthotopic model, *HOTAIR* knockdown inhibited GBM cell invasion and migration (Zhou et al., 2015). Furthermore, bromodomain and extraterminal domain protein (BET), which directly binds to *HOTAIR* and regulates its expression, have emerged as a class of therapeutic targets in glioblastoma and other cancer types. Treatment with BET inhibitors reduces GBM growth as well as *HOTAIR* expression (Pastori et al., 2015).

Dysregulation of lncRNAs play a vital role in neurodegenerative diseases

The pathological mechanism underlying Alzheimer's disease (AD) have not been understood yet. The main identified characteristics are defects in neurons which lead to their loss and the accumulation of neurofibrillary tangles and senile plaques (SPs), which consist of β-amyloid (Aβ). β-amyloid are produced after the cleavage of amyloid precursor protein by β-amyloid precursor protein cleaving enzyme (BACE1) and they cause toxicity when excessively accumulated (Wei et al., 2018).

The lncRNA BACE1-AS is transcribed antisense of the BACE1 gene and highly expressed in AD patients' brain. BACE1-AS functions to cover a specific site on BACE1 mRNA that is known to be targeted by miR-485 which causes its inhibition, and so preventing its silencing and increase the stability of the BACE1 mRNA. This allows to more Aβ production and more SPs accumulation in the brain of AD patients which worsen the development of the disease (Faghihi et al., 2010). Another group performed microarray expression analysis on lncRNAs in the hippocampus of AD rat model, and identified around 300 significantly dysregulated lncRNAs that are involved in AD pathogenesis and development (Yang et al., 2017). In conclusion, the emerging role of lncRNAs in brain diseases suggest the importance of further investigating their mechanism.

1.7 Research Questions: Investigating the role of KAP1 and lncRNA in SVZ neurogenesis

The overall aim of this project is to investigate the function and mode of action of a new KAP1-lncRNA chromatin regulatory complex in the control of postnatal and adult neural stem cell (NSC) self-renewal and neurogenesis. NSCs in the SVZ produce functional neurons throughout life and are considered as an accessible and important system to understand neurodevelopmental mechanisms. Elucidating the properties of NSCs may also provide insights for their use in the treatment of neurodevelopmental disorders and brain injuries. However, the molecular mechanisms regulating how NSCs are maintained and activated to regulate neurogenesis are not fully understood. While many studies have been carried out to investigate the role of epigenetic regulators in NSC function and lineage progression, our understating is still incomplete. I combined functional studies in the mouse subventricular (SVZ) NSCs niche using inducible knockout and in vivo knockdown electroporation, with molecular analysis using neural stem cells dissected from the SVZ and cultured in vitro, to generate important new understandings of NSCs and neurogenesis of the postnatal/adult brain. The specific aims investigated in this thesis are:

Aim 1: Identify cell subtype specific expression of KAP1 and define the specific effect of KAP1 loss of function on SVZ lineage progression at different developmental ages (postnatal and adult)

KAP1 is an essential epigenetic regulatory protein that is required for embryonic brain development and adult brain function. KAP1$^{-/-}$ mice die prior to gastrulation, hypomorphic KAP1 mouse mutant have defects in the development of the central

nervous system, while conditional deletion of KAP1 in the adult forebrain induces behavioral vulnerability to stress (Cammas et al., 2000, Jakobsson et al., 2008). The function of KAP1 in the SVZ has not been investigated before. Thus, this thesis aims to investigate the role of KAP1 in SVZ neurogenesis and answer the following questions. Given that KAP1 is ubiquitously expressed throughout development, Is KAP1 expressed in a specific individual cell subtype or in the whole neurogenic lineage? Second, given the role that KAP1 play in embryonic stem cell proliferation and differentiation (Cheng et al., 2014a), does it also impact upon SVZ NSCs activation, proliferation, or fate choice? The aforementioned research questions will be address in detail in chapter 3.

Aim 2: Confirm the expression of the lncRNA *Paupar* in the SVZ, and Identify the functional role of *Paupar* in SVZ lineage progression

lncRNAs are a new class of regulatory molecule thought to be important in brain development and function. Several thousand lncRNAs are expressed in the SVZ, making the niche a potential hot-spot of lncRNA function (Ramos et al., 2013). Yet, only a very small number of SVZ lncRNAs have been studied and shown to have function in vivo. Thus, I aim to investigate the following: considering that many lncRNAs are differentially expressed during SVZ neurogenesis, suggesting they regulate lineage progression (Ramos et al., 2013). Does the lncRNA *Paupar* expressed in the SVZ as well? Moreover, given that *Paupar* is transcribed upstream of the gene encoding PAX6 transcription factor, and was shown to control its expression (Vance et al., 2014). PAX6 is known to regulate SVZ fate choices via a Brg1-contaning BAF chromatin remodelling complex (Ninkovic et al., 2013). In addition, *Paupar* was shown to physically interact with KAP1 and PAX6 proteins (Pavlaki et al., 2018). Does

Paupar have a functional in vivo role in SVZ neural lineage progression and fate choice? These questions will be investigated in detail in Chapter 4.

Aim 3: Investigate the molecular mechanism underlying KAP1 and the lncRNA *Paupar* function in the SVZ

Numerous nuclear lncRNAs act as regulators of gene expression and many of these bind chromatin regulatory complexes. They may act either as guides to target associated protein to their sites of expression or molecular scaffolds to control ribonucleoprotein complex assembly and function. In neuroblastoma cells, the lncRNA *Paupar* in particular was shown to mediate a mechanism of KAP1 genomic recruitment, through promoting the assembly of *Paupar*-KAP1-PAX6 ternary complex, that is distinct from the well-described KRAB-ZNF association (Pavlaki et al., 2018, Vance et al., 2014). Given this, I aimed to answer the following questions: Does the lncRNA *Paupar* and KAP1 physically interact in vivo in the SVZ similar to N2A cells? Moreover, considering that *Paupar* and KAP1 were shown to play a key role in regulating the expression of key neural genes in cancer cells, do they regulate common or distinct set of neural stem cell genes in normal condition that could explain the observed in vivo phenotype? Finally, how individual signalling pathways are coordinated is a major question in developmental biology. One potential mechanism is through proteins that regulate them in common. As I will describe in Chapter 5, KAP1 knockdown resulted in upregulation of the activity of some members of the BMP and Notch signalling pathways. Overall, my thesis revealed that KAP1 and lncRNA contribute to normal SVZ neurogenesis and therefore have the potential to guide the design of new regenerative medicine treatment for neurodevelopmental diseases and brain injury.

Chapter 2

Materials and Methods

2.1	**Animals**	**64**
2.2	**Tamoxifen and BrdU administration**	**66**
2.3	**Electroporation**	**66**
2.4	**Histology and immunohistochemistry**	**67**
2.5	**Imaging and quantification**	**70**
2.6	**TUNEL**	**70**
2.7	**Neurosphere assay**	**71**
2.8	**NSC nucleofection**	**73**
2.9	**RT-PCR**	**74**
2.10	**Plasmids, transformation of chemically competent bacteria, and DNA extraction**	**76**
2.11	**FISH**	**77**
2.12	**RMS Spheroid Migration Assay**	**78**
2.13	**Chromatin-immune precipitation assay**	**80**
2.14	**RNA-Immuno precipitation assay**	**82**
2.15	**Statistics**	**83**

2.1 Animals

Species and ages of mice used for the neurosphere assay and electroporation are indicated in each chapter.

KAP1$^{fl/fl}$ mice from the Jackson laboratory. A targeting vector designed by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire) containing a *loxP* site flanked PGK-NEO cassette. This cassette was inserted upstream of exon 4 of the targeted gene, and another *loxP* site was inserted downstream of exon 14. This construct was electroporated into embryonic stem (ES) cells. Correctly targeted ES cells were injected into C57BL/6 blastocysts and the resulting chimeric animals were crossed to transgenic mice expressing Cre recombinase under the control of the cytomegalovirus promoter. The mice that retained *loxP* site flanked exons 4 through 14 were then backcrossed to C57BL/6J for 10 generations.

Nestin-CreERT2-YFP mouse was a kind gift from Dr. Ian Tomlison at University of Oxford (Lagace et al., 2007). To obtain YFP/Nestin-CreERT2$^{Cre/+}$; KAP1$^{fl/fl}$ mice, KAP1$^{fl/fl}$ mice were mated with YFP/Nestin-CreERT2$^{Cre/+}$ mice to get YFP/Nestin-CreERT2$^{Cre/+}$; KAP1$^{fl/+}$ mice. YFP/Nestin- CreERT2$^{Cre/+}$; KAP1$^{fl/+}$ mice were then mated with KAP1$^{fl/fl}$ to obtain YFP/Nestin- CreERT2$^{Cre/+}$; KAP1$^{fl/fl}$.

Genotyping procedure was as follows. DNA was extracted from mouse ear clips with E.Z.N.A. Tissue DNA Kit (Omega bio-tek, D3396-02). For polymerase chain reaction (PCR), 5µl of 50µl extracted DNA was mixed with primers, 2µl 10x REDTaq PCR Reaction Buffer, 0.5µl deoxynucleotide Mix (10mM each, Roche 11 581 295 001), 0.5µl 50mM $MgCl_2$ (Invitrogen P/N y02016), 2µl REDTaq DNA Polymerase (Sigma

D4309) and nuclease-free water (Qiagen 1039498). The primers for PCR were as follows:

Table 2.1 Primers for genotyping

Primer name	Primer sequence
Nestin-CreERT	
oIMR1084	GCG GTC TGG CAG TAA AAA CTA TC
oIMR1085	GTG AAA CAG CAT TGC TGT CAC TT
oIMR7338	CTA GGC CAC AGA ATT GAA AGA TCT
oIMR7339	GTA GGT GGA AAT TCT AGC ATC ATC C
KAP1	
Forward	CA GGG CCT TGT TTA TTT GG
Reverse	AAC CTT GGC CCA TTT ATT GA
YFP	
oIMR4982	AAG ACC GCG AAG AGT TTG TC
oIMR8546	GGA GCG GGA GAA ATG GAT ATG

PCR program for genotyping:

94°C 4min, 94°C 30sec, 60°C 1min, 72°C 1min (35 cycles), 72°C 10min, 4°C hold.

PCR products were separated by agarose gel electrophoresis (2.5% agarose for $KAP1^{fl/fl}$ mice or 1.5% agarose for other mice in 1x TAE buffer with 5μg ethidium bromide/100 mL) at 100V for 60min. Results were visualized under ultraviolet light and images were taken with a Bio-Rad UV transilluminator.

2.2 Tamoxifen and BrdU administration

Tamoxifen (Sigma T-5648) was dissolved in corn oil (Sigma C-8267) with 5% ethanol at 37°C for several hours to prepare a 50 mg/ml concentration. The tamoxifen solution was warmed to 37°C before injection. Adult (10-12 weeks old) mice were given one gavage injection per day for 5 days (180 mg/kg body weight) (Lagace et al., 2007). Mice were sacrificed at indicated time points after the last injection.

5-bromo-2'-deoxyuridine (BrdU, Sigma B5002) was dissolved in 0.9% normal saline (10mM NaOH) at 37°C to final stock concentration of 10 mg/ml. For mouse injections, 50 mg/kg (5ul/g) of BrdU solution was injected intraperitoneally (Comte et al., 2011).The injection frequency and times were indicated elsewhere in every experiment. BrdU injections were done by Dr. Bin Sun.

2.3 In vivo electroporation

The electroporation procedure was conducted in P0-P2 C57BL/6 mice as described in (Boutin et al., 2008, Chesler et al., 2008, Fernandez et al., 2011). Plasmids used for postnatal electroporation were prepared at 2-3 ug by Endotoxin Free Plasmid Maxi Kit. Before use, 1% fast green in PBS was mixed with the plasmid solution at 1:10 for tracing. Electroporation pipettes were made using Steolting 5/1, 1brl, GF glass pipettes pulled on a micropuller. The electroporation procedure took less than 5 minutes per pup from anaesthesia to recovery stage. First, pups were anesthetized by hypothermia for 2 mins. ~2µl of plasmid solution was injected into the lateral ventricle via the glass capillary. Pups were then subjected to electrical pulses (-100V, 50ms ON with 850ms intervals for 5 cycles) (BTX ECM 830) by tweezer electrodes coated with conductive

gel. After surgery, pups were allowed to recover in a heated chamber set to 36°C for 15-20 minutes before being taken back to the dam.

2.4 Histology and immunohistochemical staining

At sacrifice, animals were first overdosed with Pentobarbital, perfused with cold normal saline and fixed with cold 4% PFA before brains were carefully dissected out of the skull. Brains were post-fixed overnight in 4% PFA at 4°C then cryoprotected in 30% sucrose in 0.1M phosphate buffer (PB) and kept at 4°C for 48 hrs. Brains were frozen down on dry ice and sectioned into cryoprotectant solution on Leica sliding microtome. 30 μm- thick serial coronal sections were obtained from the olfactory bulbs till the rostral hippocampus. The sections were put in 48-well plates containing cryoprotectant and stored at -20°C.

Free-floating fluorescent immunohistochemistry was performed over two days at room temperature. Up to three free-floating brain sections were placed in porous well inserts in 12-well-plates (2- 3ml of solution/well) to facilitate transfer of sections between wells.

Brain sections were washed 3x10 min in PBS (pH 7.4), 1x15min in 50mM glycine in PBS, 3x10 min in PBS and then blocked in PBS+ (10% donkey serum, 0.1% Triton-X in PBS) for 1hr. Sections were then incubated in primary antibodies at 4°C overnight (see Table 2.2). The next day, sections were washed with PBS 3x10 min, and incubated with species-appropriate fluorophore-conjugated secondary antibodies (1:1000 in PBS+) for 1hr in the dark and then washed again 3x10 min in PBS. Brain sections were treated with 4',6-diamidino-2-phenylindole (DAPI, 1:1000) for 15min and washed with 0.1M PB for 3x10min. Sections were mounted on SuperFrost Plus microscope slides

with FluorSave Reagent. Slides were then dried overnight at room temperature and stored at 4°C in darkness.

Secondary antibodies conjugated to Alexa Fluor 488, 568, and 647 (Invitrogen) raised against the host species of the primary antibodies were used as appropriate. Negative controls lacking primary but including secondary antibodies were carried out for all experiments.

For DLX2 and BrdU immunohistochemistry, an antigen retrieval step was applied before primary antibody incubation. Brain sections were first washed for 10 min with PBS three times and incubated in 2M HCl at 37°C for 1hr. The rest of the steps were the same as the immunohistochemistry described above. Biotinylated and streptavidin-conjugated secondary antibodies were used to amplify the fluorescence signal.

Table 2.2 List of primary antibodies used for immunohistochemistry

Antigen	Dilution	Host	Supplier
GFP	1:500	Chicken	Aves 1020
Mash1	1:100	Mouse	BD Bioscience 556604
Ki67	1:500	Rabbit	Abcam 16667
Dcx	1:100	Goat	Santa Cruz 80661
GFAP	1:500	Rat	Invitrogen 130300
NeuN	1:400	Mouse	Millipore
Pax6	1:700	Rabbit	Biolegend 901301
KAP1	1:1000	Mouse	Millipore
Dlx2	1:50	Rabbit	Millipore
BRDU	1:400	Sheep	Abcam B1893
BRDU	1:400	Rat	Novus NB500-169
GFAP	1:5000	Chicken	Abcam ab4674
Sox2	1:500	Rabbit	Millipore AB5603
Sox2	1:200	Goat	R&D systems AF2018
S100β	1:200	Rabbit	Dako Z031129-2

2.5 Imaging and quantification

For Fluorescent IHC staining slides, images were taken using confocal microscopy (Zeiss LSM 710 laser scanning microscope) for Z- stack imaging using 40X oil immersion (Zeiss Plan-NeoFluar 40X1.3 Oil DIC) and 20X (Zeiss Plan-Neofluar 20X/0.50) objectives. Four excitation lasers; 405, 488, 568, and 647 nm were used to excite DAPI, Alexa Fluor 488, 568, and 647 fluorophores, respectively. 2µm intervals were used for generating Z-stacks.

For in vivo analysis, 3-5 representative sections from anterior to posterior SVZ of adult and postnatal brains (approximately Bregma +1.70 mm to -0.58. mm) were imaged and quantified. In every brain section, dorsal, lateral and ventral areas of SVZ were analysed. For double and triple staining analysis, at least 4 optical layers of Z stack were imaged. The co-localisation was examined in single optical planes through the Z-axis of every individual image. All images shown represent single optical planes, unless stated otherwise, with orthogonal views also displayed in the same figure. All images were acquired using the same acquisition parameters for animals in the same experiment. Images were analysed with ImageJ.

2.6 TUNEL assay

I used In Situ Cell Death Detection Kit, TMR red by Roche (Cat. No. 12 156 792 910). It is a kit for detection and quantification of apoptosis (programmed cell death) at single cell level, based on labelling of DNA strand breaks. I used protocol and reagents provided by the supplier.

Briefly, fixed tissue sections preserved in cryoprotectant were washed with PBS for 30 mins and then permeabilized with 0.1% Triton X–100, then 0.1% sodium citrate for 2 mins. Next, I prepared TUNEL reaction mixture using the reagent supplied by the kit according to the protocol. TUNEL reaction mixture contains 50 ul of enzyme solution (terminal transferase (TdT)) and 450 ul label solution (TMR-dUTP) was added on each tissue section (3 sections/condition) and incubated for 60 mins at +37°C in a humidified incubator in the dark. During incubation period, TdT catalyses the addition of TMR-dUTP at free 3′-OH groups in single- and double-stranded DNA breaks that occur in early stages of apoptosis. Samples were then washed with PBS and analysed under the confocal microscopy.

Negative controls and positive controls were also included in each experimental setup. For negative control: Incubated fixed and permeabilized cells in 50 μl/well Label Solution (without terminal transferase) instead of TUNEL reaction mixture. For positive control: incubated fixed and permeabilized cells with micro- coccal nuclease or DNase I recombinant (3000 U/ml– 3 U/ml in 50 mM Tris-HCl, pH 7.5, 1 mg/ml BSA) for 10 min at +15 to +25°C to induce DNA strand breaks prior to labelling procedures.

2.7 Neurosphere assay

Mice were sacrificed by overdose intraperitoneal injection with Pentobarbital and brains were dissected out and section into coronal plane sections with McIlwain tissue chopper. Under laminar flow hood, the SVZ was dissected in neurobasal + medium (see Table 2.3) and transferred into an Eppendorf tube and spun for 30 seconds. I removed the supernatant and added 500-800 ul of Accutase for 7 mins in room temperature while pipetting to dissociate the SVZ tissue into single cells. Next, I centrifuged at 1200 rpm

for 5 mins. I removed the supernatant and resuspended the cell pellet with 1 ml of neurobasal+ medium with growth factors (see Table 2.4). I counted the cells using haemocytometer and plated the cells at a final concentration of 100,000 cells/ml. Four days later, cells were dissociated and cultured again (100,000 cells/ml) for four days to generate secondary neurospheres that were imaged and analysed. Neurospheres were cultured in Poly (2-hydroxyethyl methacrylate) (Poly-HEMA) coated 6-well plates.

Table 2.3 Components of Neurobasal+

component	Amount	Final concentration	Catalogue #
Neurobasal medium	500 ml	475 ml	GiBCO 21103
B27 supplement	50x	10 ml	GiBCO 17504
L glutamine	100x	5 ml	Invitrogen 25030
Pen/Strept	100x	5 ml	GiBCO 15140-122

Table 2.4 Components of Neurobasal+ growth factor

component	Amount	Final concentration	Catalogue #
Neurobasal+	25 ml		
EGF (100 ug/ml)	5 ul	20 ng/ml	Sigma
FGF (100 ug/ml)	5 ul	20 ng/ml	R&D system

Neurosphere differentiation assay was performed in 96-well plates. Plates were coated with poly-D-lysine and kept at 4°C overnight. Next day, plates were washed 3x for 5 mins with distilled water and coated with 10 ug/ml laminin in PBS for 1 hour in 37°C incubator and then removed before plating the cells. Secondary neurospheres were dissociated by Accutase and plated in the coated plate with neurobasal+ medium and without growth factors for 6-7 days.

For fate potential immunohistochemistry, differentiated neurospheres were fixed with 4% paraformaldehyde (PFA) for 15 mins, washed with PBS for 3x for 5 mins, blocked with PBS+ (5% donkey serum in PBS) for 1 hour and incubated with anti-Tuj1 and anti-GFAP primary antibodies in PBS+ at 4°C overnight. On the next day, neurospheres were washed with PBS for 3x for 5min and incubated with secondary antibody (Alexa 488 anti-Rat and Alexa 568 anti-Rabbit) for 1hr (see Table 2.5). Then, wells were washed 3x for 5 mins in PBS and added 4',6-diamidino-2-phenylindole (DAPI, 1:1000) for 15min and washed with 0.1M PB for 3x for 5min. Wells were mounted with FluorSave Reagent and imaged under the EVOS microscopy.

Table 2. 5 List of primary antibodies used for fate potential immunohistochemistry

Antigen	Dilution	Host	Supplier
GFAP	1:400	Rat	Invitrogen 130300
Tuj1	1:1000	Rabbit	COVANCE MMS-435p

Alexa Fluor dyes from Invitrogen were used for secondary antibodies.

2.8 Neural stem cell nucleofection

Neural stem cell nucleofection was performed following the protocol of LONZA (VPG-1004). 4-5 x 10^6 dissociated cells from secondary neurospheres were mixed with 100µl nucleofection solution (82 µl of Nucleofector Solution + 18 µl of supplement) and mixed with 5 ug DNA. The mixture was then transferred into a cuvette supplied by the kit and transfection was carried out under program A-033. 500 ul of warm medium was added into the cuvette and the samples were then transferred by the supplied kit pipette into an Eppendorf tube. 1 ml of medium was added. Cells were then centrifuged at 1200

rpm for 5 min and suspended with fresh medium before plated in a 6-well poly-HEMA coated plate.

2.9 RT- PCR

Secondary neurospheres were collected for total RNA isolation using RNeasy Mini Kit following the manufacturer instructions. I first removed the DNA using RNase-Free DNase Set (Qiagen 79254) and then performed reverse transcription reaction by SuperScript III RT kit (Invitrogen 18080) to make cDNA. To make first-strand cDNA synthesis reaction, I mixed 1µg RNA with 1µl 250 ng/µl random primers, 1µl 10mM dNTP and RNase free H_2O, and incubated the mixture at 65°C for 5min and then on ice for 1min. I added to the reaction mixture 4 µl 5x First-Strand Buffer, 1µl SuperScript III RT, 1µl RNaseOUT Recombinant RNase Inhibitor, 1µl 0.1M DTT and incubated the final mixture at 25°C for 5min, 55°C for 1hr, and 70°C for 15min. The cDNA was run on real-time quantitative PCR with SYBR Green PCR Master Mix. I normalized the target mRNAs to GAPDH housekeeping gene.

Table 2.6 Primer list of RT-qPCR

Gene	Sequence (5'-3')
KAP1	CGGCGCTATGGTGGATTGT
	GGTTAGCATCCTGGGAATCAGAA
Paupar	ACCATCACCCCTGCAATAGTTC
	AGCAGCCTTTGAGCCATCTC
BMP2	GGGACCCGCTGTCTTCTAGT
	TCAACTCAAATTCGCTGAGGAC
BMP6	AGAAGCGGGAGATGCAAAAGG
	GACAGGGCGTTGTAGAGATCC
SMAD1	GCTTCGTGAAGGGTTGGGG
	CGGATGAAATAGGATTGTGGGG
SMAD4	ACACCAACAAGTAACGATGCC
	GCAAAGGTTTCACTTTCCCCA
SMAD5	TTGTTCAGAGTAGGAACTGCAAC
	GAAGCTGAGCAAACTCCTGAT

CREBB	GGCTTCTCCGCGAATGACAA
	GTTTGGACGCAGCATCTGGA
FAST1	GACCTGGCCTCAACTTACACT
	TGTCATGCCGTAGGTATCTCTT
STAT3	CAATACCATTGACCTGCCGAT
	GAGCGACTCAAACTGCCCT
Notch1	GATGGCCTCAATGGGTACAAG
	TCGTTGTTGTTGATGTCACAGT
Notch2	ATGTGGACGAGTGTCTGTTGC
	GGAAGCATAGGCACAGTCATC
Hes1	CCAGCCAGTGTCAACACGA
	AATGCCGGGAGCTATCTTTCT
Hes5	AGTCCCAAGGAGAAAAACCGA
	GCTGTGTTTCAGGTAGCTGAC
DLL1	CAGGACCTTCTTTCGCGTATG
	AAGGGGAATCGGATGGGGTT
Nrg1	ATGGAGATTTATCCCCCAGACA
	GTTGAGGCACCCTCTGAGAC
Hey1	GCGCGGACGAGAATGGAAA
	TCAGGTGATCCACAGTCATCTG
Hey2	AAGCGCCCTTGTGAGGAAAC
	GGTAGTTGTCGGTGAATTGGAC
Mash1	GCAACCGGGTCAAGTTGGT
	GTCGTTGGAGTAGTTGGGGG
Neurog2	AACTCCACGTCCCCATACAG
	GAGGCGCATAACGATGCTTCT
NeuroD1	ATGACCAAATCATACAGCGAGAG
	TCTGCCTCGTGTTCCTCGT
Pax6	CACCGCCCTCACCAACAC
	TGCATAGGCAGGTTGTTTGC
Igfbp5	AAAGCAGTGTAAGCCCTCCC
	TCCCCATCCACGTACTCCAT
Mst1	TGCTATACCTTGGACCCGGA
	TCAAACACCACCTGGTCTGG
Mab2	ACAGCGTCTGGCTACCTTTC
	ATCATCTTGACCACGTCCCG
P21	CCTGGTGA TGTCCGACCTG
	CCA TGAGCGCA TCGCAA TC
P57	CGAGGAGCAGGACGAGAA TC
	GAAGAAGTCGTTCGCA TTGGC
P27	TCAAACGTGAGAGTGTCTAACG
	CCGGGCCGAAGAGA TTTCTG
Cdkn2a	CAGACCGACGGGCATAGCTTCA
	GGATTTAGCTCTGCTCTTGGGATTGG
SOX2	GCGGAGTGGAAACTTTTGTCC
	CGGGAAGCGTGTACTTATCCTT
E2F2	TTGGGAATAGCTCAGAGGCAG
	AGGTCTGCTCGGCATTCATC

EGF	AGCATCTCTCGGATTGACCCA
	CCTGTCCCGTTAAGGAAAACTCT

2.10 Plasmids, transformation of chemically competent bacteria and DNA extraction

shKAP1 and shPaupar constructs were a kind gift from Dr. Keith Vance at University of Bath. To obtain KAP1 or Paupar sequence, a plasmid containing cDNA for KAP1 or Paupar was purchased from Clonetech and the sequence was amplified by PCR. After purification, both KAP1 or Paupar PCR amplicon and pBS-U6-CMV-EGFP plasmids were digested by restriction enzymes followed by ligation steps. This was done by Dr. Keith Vance laboratory.

After receiving the plasmids, small amount of the plasmid was mixed with DH5α chemically competent E.Coli (Invitrogen) and 2µl ligation enzyme. The cells were then incubated on ice for 30 minutes and heat-shocked (42°C) in a water bath for 20 seconds. Next, they were incubated again on ice for 2 minutes and then cultured in Lysogeny broth (LB) at 37°C for 1 hour. 200µl of the culture was seeded onto LB agar plates containing 100µg/ml Ampicillin at 37°C for 8-12 hours. After successful transformation, single colonies were picked, seeded into 3 ml of LB broth + 100µg/ml Ampicillin, and cultured for 6-8 hours before DNA was extracted (Qiagen MiniPrep) and sent for sequencing with appropriate primers (see Table 2.7). In addition, the picked colonies were seeded onto another LB agar + Ampicillin plate, to allow for reseeding the colonies with the correct sequence into starter cultures for 6 hours then expansion cultures (250ml's LB broth + Ampicillin) for 18 hours. DNA from those cultures was extracted using Qiagen Endo-Free MaxiPrep Kit.

Table 2.7 Primers for PCR were as follows

shKAP1-A FOR	TCGAGTTCTGGTCAATGATTCAAGAGACCATTGACCAGAACTCGACTTTTTC
shKAP1-A REV	TCGAGAAAAAGTCGAGTTCTGGTCAATGGTCTCTTGAATCATTGACCAGAACTCGA
shKAP1-B FOR	AACAGTCTACTGTAATGTTTCAAGAGACCATTACAGTAGACTGTTCTTTTTC
shKAP1-B REV	TCGAGAAAAAGAACAGTCTACTGTAATGGTCTCTTGAAACATTACAGTAGACTGTT
shPaupar-165 FOR	GGCAGTAATTACAAATTATTCAAGAGACAATTTGTAATTACTGCCCTTTTTC
shPaupar-165 REV	TCGAGAAAAAGGGCAGTAATTACAAATTGTCTCTTGAATAATTTGTAATTACTGCC
shPaupar-408 FOR	AAGAGCAGTCACAATATATTCAAGAGACATATTGTGACTGCTCTTCTTTTTC
shPaupar-408 REV	TCGAGAAAAAGAAGAGCAGTCACAATATGTCTCTTGAATATATTGTGACTGCTCTT

2.11 Fluorescence in situ hybridization (FISH)

I used Stellaris RNA FISH custom design kit to detect Paupar transcript in neurospheres.

A set of RNA FISH Probes comprised of up to 48 singly labelled oligonucleotides designed to selectively bind to either the lncRNA Paupar or GAPDH. Stellaris RNA FISH Probes bound to target RNA produce fluorescent signals that permit detection under fluorescence microscopy.

First, I centrifuged a suspension of 2 -5 x 10^6 cells in a 15 mL conical tube at 1200 rpm for 5 mins. I aspirate the supernatant and washed the cells with 1 ml 1x PBS and

centrifuged again to pellet the cells. Cells were then fixed in 1 ml fixation buffer (provided by the supplier) while inverting the tube and incubated at room temperature for 10 mins. I centrifuged to pellet cell suspension. Aspirated fixation buffer, and washed cells three times with 1 ml of 1x PBS. I then resuspend cells in 1 mL of 70% ethanol for at least 1 hour at 4 °C to permeabilize the cells. Next, I centrifuged to pellet cells and aspirated 70% ethanol and gently resuspended cells in 500 µL of Wash Buffer A (provided by the supplier). Centrifuged to pellet cells and aspirated Wash Buffer A and resuspend cells in 100 µL of Hybridization Buffer containing either the lncRNA Paupar or GAPDH probes (labelled with Quasar 570 fluorophore). I then incubated the cells in the dark at 37 °C overnight (~16 hours).

Next day, I centrifuged to pellet cells and aspirated about 50% of the Hybridization Buffer containing probe. Added 500 µL of Wash Buffer A. Centrifuged to pellet cells and aspirated the solution. Resuspend cells in 500 µL of Wash Buffer A and Incubated in the dark at 37 °C for 30 minutes. I then Centrifuged to pellet cells and aspirated Wash Buffer A and resuspend cells in 500 µl of DAPI nuclear stain (1X Wash Buffer A consisting of 5 ng/mL DAPI) to counterstain the nuclei and incubated in the dark at 37 °C for 30 minutes. Centrifuged to pellet cells and aspirated DAPI nuclear stain. Resuspended cells in 500 µL of Wash Buffer B (provided by the supplier). Centrifuged to pellet cells and aspirated Wash Buffer B. Resuspend cells in a small drop (approximately 30 µL) of Vectashield Mounting Medium. Placed 5-10 µL of cell suspension on a clean glass microscope slide and then placed an 18 x 18 mm square #1 cover glass over the cells to spread the solution. Allowed to dry and then imaged under confocal microscopy. All centrifuge steps were done at 200x g for 2 mins at room temperature.

2.12 RMS spheroid migration assay

RMS spheroid migration assay was done by Martin Ducker (DPhil candidate in the lab). P2-P4 CD1 wild type mice pups were culled and their brains removed and sliced into 300µm sections. To dissect out the RMS tissue the olfactory bulb and anterior most 2-3 sections were collected in ice cold HBSS (Gibco 10888-022). Under a dissection microscope and using 27G syringe needles the RMS was carefully dissected and transferred into an 2ml Eppendorf tube on ice. The RMS can be identified as the translucent, triangular region at the centre of the olfactory bulb sections.

The dissected RMS tissue was spun down briefly using a bench top centrifuge and the HBSS carefully removed. The tissue was then resuspended in 2ml Accutase and carefully dissociated using a P200 pipette. The Accutase was diluted out by adding the 2ml of cells to 8ml of NB- A+ and the cell suspension was transferred into a 50ml falcon tube and spun down at 300g for 5 minutes, resuspended in 1ml NB-A+ media and counted using a haemocytometer. Typically resulting in approximately 500,000 cells per mouse.

Cells from the RMS (1x 10^6 cells/condition) were nucleofected by LONZA protocol VPG-1004 (see section 2.8) using shRNA scramble control or shRNA to knockdown KAP1. Cells were then suspended with 4ml fresh medium, plated in a single well per condition in a 6 well plate and left to recover at 37°C. After 4 hours the cells were collected and diluted to a concentration of 50,000 cells per ml of NB-A+ and 100ul added to each well of a 96 well ultra low adhesion round bottom plate (Greiner 650970). The culture plate was then sealed with a gas permeable seal (4TI 044850) and incubated overnight at 37°C, 5% CO_2 and spheroids formed overnight.

After RMS spheroids were formed, the migration was studied by embedding the spheroids in Growth Factor Reduced Matrigel (BD 356231). The spheroid plate was removed from the incubator and left to cool to room temperature. The Matrigel was diluted to 50% in ice cold NB- A+ media. The spheroid plate was then briefly chilled in ice and seal removed. 30µl of Matrigel was then carefully trickled down the side of each well using a multichannel pipette and the plate returned to the ice for the Matrigel to settle to the bottom of the well. The plate was then re-sealed and left for ~3 minutes to warm to room temperature before replacing to the incubator for the Matrigel to set. Images of the spheroid we taken after 3 days using EVOS microscopy.

2.13 Chromatin-immunoprecipitation assay

Secondary neurospheres were washed with PBS, centrifuged and then cross-linked by disuccinimidyl glutarate (DSG) crosslinking to a final concentration of 2mM to fix protein-protein interactions followed by 1% formaldehyde to fix protein-DNA interactions. The samples were incubated for 15mins at RT, then the reaction was quenched with 2.5M glycine (0.125M final concentration) for 10mins at RT. Cells were rinsed twice with cold PBS and spun down for 5 min, at 1500rpm, 4°C.

Next, cells were resuspended with 2ml (per IP) cold hypotonic buffer, dounced 10 times and incubated on ice for 10mins. Dounced another 10 times. Spanned at 3500rpm/5min/4ºC. Resuspend nuclear pellet in 200µl (per IP) sonication buffer. Then samples were sonicated to an average size of 200bp (+/- 50bp) (for Bioruptor Pico: 30on/30off – 6 cycles).

Centrifuged sonicated chromatin to pellet the cell debris at 14000rpm/10min/4 ºC and diluted the supernatant 1:10 on ice with IP dilution buffer, kept 1% input.

After that, samples were immunoprecipitated using 5µg of antibody (KAP1, Rabbit, Invitrogen PA5-27648 and unspecific IgG antibody) overnight at 4 ºC on a rotating wheel. Then, beads were prepared as follows: Add 50µl of beads (per IP) in LoBind tubes, add 1ml of blocking solution to the beads, collect beads using magnetic stand and remove the supernatant, repeat wash 2 more times, resuspend beads in 1ml of blocking solution and incubate overnight rotating at 4ºC.

On day 2, I added the IP samples (antibody-bound) to the blocked beads (after removing the supernatant) and incubate for 4 hours on a rotating wheel at 4 ºC. Centrifuged samples at 14000rpm/30sec/4 ºC. Washed the beads with 500µl from each of the wash buffers I, II and III for 5mins rotating. Washed twice with TE pH 8. Transferred the beads in a new LoBind tube. Then proceeded with elution step as follows: Add 250µl of ChIP elution buffer to elute crosslinked chromatin, rotate at RT for 15 minutes, spin down the beads and collect supernatant using the magnetic stand. Reversed crosslinking by adding RNAse (DNAse-free / stock 0.5mg/ml use 2µl in 1ml) and NaCl to a final concentration of 0.25M (50µl of 5M). Incubated samples overnight at 65 ºC.

On day 3, I added 2.5 volumes of 100% ethanol (625 µl for 250 µl) and samples were left overnight at -20 ºC or -80 ºC.

On Day 4, samples were centrifuged at 13000rpm/20mins/4ºC, washed with 400µl of 70% ethanol and resuspended in 100µl of water. Then, samples were incubated with 25µl of Proteinase K + buffer and incubated for 1-2 hrs at 42ºC. Lastly, DNA was

purified using the QIAquick PCR purification kit (Qiagen 28104), and then proceeded with qPCR validation.

To run qPCR, 1ul DNA was mixed with 1ul primers (2uM stock for forward and reverse primers), 10ul Syber Green and 8 ul H_20 to final volume of 20ul. qPCR was run with standard program and the results were calculated by $2^{-\Delta\Delta Ct}$ relative to input.

2.14 RNA- Immunoprecipitation assay

Secondary neurospheres were generated and collected by me then sent to Dr. Iona Pavlaki at University of Bath for RNA-IP. Briefly, cells were cross linked with formaldehyde for 10 mins at RT then the cross-linking was quenched by glycine for 5 mins while shaking. Cells were then washed with cold PBS and spun down for 5 mins at 1000g. Next, nuclei were isolated and lysed by resuspending the cells in nuclear isolation buffer and PBS for 20 mins, centrifuged for 15 mins at 2500 g, resuspended with RIP buffer and split into two fractions (one for mock-IgG and one for antibody-IP). Chromatin was sheared with Turbo DNAse and incubated for 20 mins at RT. EDTA was added to stop the reaction, and then cells were pelleted.

Next, magnetic beads were washed with PBS and suspended in BSA and 5 ug antibody (KAP1, Abcam, 10483 or IgG control) and incubated for 1 hour. Beads+Ab were then recovered and added to the lysate and incubated overnight at 4^{o}C rotating.

On the second day, beads+Ab+complexes were recovered using the magnetic strand and washed with RIP buffer then PBS to wash off unbound material and recovered again with the strand. Proteinase K buffer was then added for 1 hour at 55^{o}C to elute the complexes from the beads. RNA was purified and resuspended in water. Finally, cDNA

was prepared followed by a q-PCR run. Samples: input, IgG, KAP1. Targets: Paupar, u1snRNA (control)

2.15 Statistics

Biological replicate numbers were indicated as n number in every experiment in all chapters. For in vivo experiments, n indicates the animal numbers per each group; for in vitro experiments, n indicates the independent culture numbers. All quantifications were done blinded to experimental conditions. At least 3 images were quantified per animal and considered technical replicates. The mean of technical replicates was used as one biological replicate. Numerical data were presented as mean ± standard error of the mean. Differences between two groups were assessed using Student's t-test. For differences between three groups, analysis of variance (ANOVA) with Tukey post-hoc tests were employed as appropriate. For significant difference, $p<0.05$ was marked as *, $p<0.01$ as **, $p<0.001$ as ***. Statistical analyses and graphical representations were performed in Microsoft Excel and GraphPad Prism.

Chapter 3

KAP1 in postnatal and adult SVZ neurogenesis

3.1	**Introduction**	**85**
3.2	**Results**	**93**
3.3	**Discussion**	**115**
3.4	**Conclusion**	**130**

3.1 Introduction

3.1.1 Molecular biomarkers in postnatal/adult SVZ neurogenic lineage

The postnatal and adult SVZ niche contain four main cell types: Astrocytes/NSCs (Type B cells), transit progenitor cells (type C cells), neuroblasts (type A cells), and ependymal cells. Molecular markers for different neurogenic stages have been identified for cell type specification, lineage tracing and cell sorting (Ihrie and Alvarez-Buylla, 2011, Ming and Song, 2011). Briefly, type B cells express GFAP and Sox2.They give rise to transit amplifying progenitor cells (Type C cells) which express Mash1, and Dlx2. Type C cells give rise to migratory neuroblasts (Type A cells) that express PSA-NCAM and Dcx and migrate through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into neurons. Ki67 and PH3 can be used as markers of the nuclei of proliferating cells. S100β marks astrocytes and ependymal cells, CD24 marks migratory immature neurons and ependymal cells, CD31 (PECAM-1) marks the vascular endothelial cells, β III-tubulin marks neurons and O4/CNPase marks oligodendrocytes (Zhang and Jiao, 2015, Ming and Song, 2011, Doetsch et al., 1997) (Table 3.1) (Figure 3.1). However, a few questions still remain unclear:

Quiescent NSCs vs active NSCs: It is still not clear how we can accurately distinguish quiescent and active NSCs. The major difference between them is the cell cycle state and therefore the best method so far to identify quiescent stem cells is by their incorporation and retention of the thymidine analogue (e.g. EdU, BrdU) (Cheung and Rando, 2013). In the SVZ, EGFR is used to distinguish active from quiescent NSCs. Using this marker, several gene expression differences have been described between these two groups (Codega et al., 2014). In addition, Nestin and GLAST are used to identify NSCs and their promoters are used to label them with GFP, however, studies

showed that Nestin is preferentially expressed in active NSCs while Glast is expressed in quiescent NSCs (Codega et al., 2014, Lagace et al., 2007). Nevertheless, more markers need to be discovered to accurately identify cell types within the SVZ neural cell lineage.

Adult NSC vs. niche astrocytes: The glial nature of adult NSCs is reflected in their molecular signature similarities with niche astrocytes. Type B cells express astroglial markers such as glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter (GLAST), and brain-lipid-binding protein (BLBP). However, neonatal radial-glia (RG) cells transform to ependymal cells or into type B cells which serve as NSCs throughout adulthood. These NSCs (type B cells) preserve their epithelial morphology of a primary cilium, and the primary cilium is surrounded by ependymal cells having a pinwheel-like structure, which is the most reliable way to distinguish them from niche astrocytes. (Mirzadeh et al., 2008, Ihrie and Alvarez-Buylla, 2011).

Based on this comparison, one interesting question is whether KAP1 is specifically expressed in one cell type or all the neural lineage in the SVZ. Thus, I will begin the work in this chapter with KAP1 expression analysis in the SVZ using immunohistochemistry.

Table 3.1 Major molecular biomarkers used to identify cells based on proliferative neurogenic and gliogenic capacity (Zhang and Jiao, 2015)

BLBP	Brain Lipid Binding Protein is a member of the fatty acid-binding proteins (FABPs) family. They are cytoplasmic proteins that are responsible for fatty acids intake and transportation. BLBP is a marker for radial-glial cells.
BrdU	5-bromo-2-deoxyuridine, is a thymidine analogue that is used to label cells in the S phase in the cell cycle. The schedule of administration and tissue collection combined with double-labeling with neural markers determines identification of BrdU+ cells.
Dcx	Doublecortin, is a protein that enables microtubule polymerization. It is expressed in migrating neuroblasts and immature neurons.
Dlx2	Member of the distal-less (Dlx) family of genes. Pro-neurogenic factor in adult SVZ and its expression is specific to cells in early neuronal commitment prior to differentiation.
GFAP	Glial Fibrillary Acidic Protein is a marker for astrocytes. It is an intermediate filament protein that functions to maintain astrocytic mechanical strength.
GLAST	Astrocyte-specific glutamate transporter and a marker for glial cells. It is expressed in most of S100β positive cells. It is expressed by mouse embryonic day 13 and persists throughout adulthood.
Ki67	Nuclear protein marker for actively dividing cells. It is expressed in all cell cycle stages
Mash1	Mammalian achaete scute homolog (also known as Ascl1) is a transcription factor that is highly expressed in transit amplifying progenitor cells (type C cells) in addition to a small population of NSCs that have neurogenic potential. Mash1 is important for neuronal differentiation
Nestin	Neuroepithelial stem cell protein, is an intermediate filament protein that is expressed in adult NSCs and immature transit progenitor cells.
NeuroD	Neurogenic differentiation transcription factor. It is expressed in later stages of the neuronal lineage and serve as a differentiation marker for neurogenesis.
NeuN	Neuronal nuclear antigen that is known as a marker of most neurons.
Pax6	Paired box 6 is a member of the paired box family of transcription factors. It functions to control NSC proliferation and differentiation by regulating downstream gene expression. Pax6 is co-expressed with GFAP/Nestin positive cells, yet there are also a small number of cells that express both Pax6 and Dcx or PSA-NCAM. It collaborates with Dlx2 to maintain and specify neuronal subtypes in the adult brain.
PCNA	Proliferating Cell Nuclear Antigen. It is expressed in all cell cycle stages. Its expression increases in G1 and S phases and decreases in G2 and M phases. Important for cell replication and considered as a proliferation marker that marks actively dividing NCSs.
PH3	Phosphohistone H3, is expressed in late G phase and in entire M phase of the cell cycle. It is used to subgroup proliferating and mitotic states.
PSA-NCAM	Polysialylated Neural Cell Adhesion Molecule is highly expressed in migrating neuroblasts in the SVZ. Most PSA-NCAM positive cells are also positive for DCX, NeuN and NeuroD but negative for GFAP, indicating that PSA-NCAM is expressed in later stages in adult neurogenesis.
S100β	Calcium binding protein β, is a member of the S100 family that is important for cell cycle regulation and cells differentiation. It is used to discriminate cells that are losing their NSCs potential from GFAP/Nestin positive cells.
Sox2	Sex Determining Region Y Box 2 is a member of Sox family of transcription factors that are known to play important roles in mammalian development. Sox2 is highly expressed during development and considered as a marker for NSCs. Sox2 is important for cell proliferation and differentiation. Sox2 positive cells can generate identical cells and also differentiated cells which indicate that they have multipotent and self-renewal abilities.
Class III tubulin	The Tuj1 antibody detects this cytoskeletal protein in young neurons and is co-expressed with Dcx-positive and PSA-NCA-positive cells.
Vimentin	It is an intermediate filament protein. Similar to GFAP, it is expressed in radial glial cells and immature astrocytes.

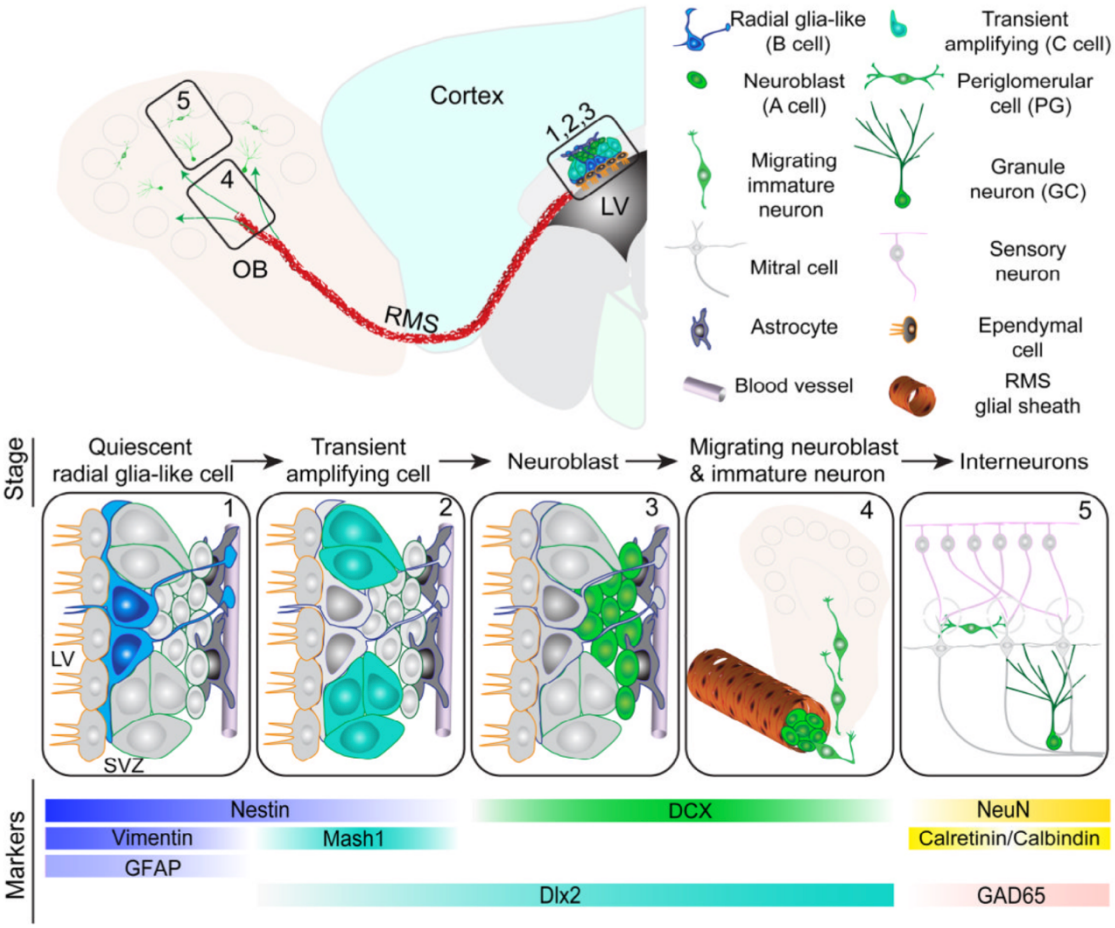


Figure 3.1 Adult neurogenesis in the SVZ and OB

Different cell types can be found in the adult SVZ, RMS, and OB. The neurogenic lineage includes quiescent NSC (radial glia-like), transit amplifying cells and neuroblasts. These individual cell types express stage-specific markers during lineage progression. Ependymal cells, blood vessels and astrocytes also reside in the adult SVZ. (modified from Ming and Song 2011).

3.1.2 KAP1 regulates proliferation & differentiation

The postnatal/adult SVZ is composed of a large number of neural stem cells that have two major features: Self-renewal and multipotency (Ihrie and Alvarez-Buylla, 2011). Dysfunction of these features may contribute to gliomagenesis (Sanai et al., 2005). However, the mechanism that control NSCs proliferation and differentiation are still not fully understood. In previous studies, KAP1 has shown to play a role in regulating stem cell development in other developmental systems which makes it a potential target to investigate in the SVZ NSC biology.

KAP1 in regulating cell self-renewal and proliferation

KAP1's role in regulating cell proliferation has been investigated in embryonic stem cells and in cancer cells. KAP1 appears important in regulating self-renewal and proliferation of mouse embryonic stem cell model (Hu et al., 2009, Seki et al., 2010, Cheng et al., 2014a), and KAP1 knockout mice die before gastrulation suggesting its functional importance in very early stages of development (Cammas et al., 2002). Screening with siRNAs in mouse embryonic stem cells (ESs) identified genes that are highly expressed in ES cells and essential for their self-renewal, including KAP1. In this study, they found that KAP1 forms a complex with pluripotency-associated transcription factors like c-Myc and Zfx and together they regulate a common set of genes that are involved in the cell cycle, apoptosis and cancer (Hu et al., 2009). This suggests a unique regulatory network that controls stem cell self-renewal separate from the core model formed by Sox2, Nanog and Oct4. In addition, this suggests that regulatory complexes controlling self-renewal in stem cells may also be active in cancer and be used as anti-cancer targets. Another group showed that phosphorylation of KAP1 at its C-terminal serine 824 in mouse ES cells causes it to colocalize at active chromatin sites, forming complexes with different transcription factors to induce ES cell-specific

genes and enable maintenance of the undifferentiated state (Seki et al., 2010). This suggest a different function of phosphorylated KAP1 as an activator of gene expression rather than its well-known repressor function.

The Human Protein Atlas database reported KAP1 protein expression in different tumour types compared to the adjacent normal tissues. The finding in the database implied that KAP1 protein is upregulated in many tumours (including breast cancer) and down regulated in other tumours (including lung cancers) (Chen et al., 2012, Addison et al., 2015, Czerwinska et al., 2017), suggesting a complex role of KAP1 in cancer. For example, in early-staged lung adenocarcinoma KAP1 interacts with E2F3 and E2F4 transcription factors that are critical for cell proliferation and blocks their growth-inducing activity and so the tumour growth is restrained (Chen et al., 2012). This observation indicates that KAP1 has an anti-proliferative activity in lung cancer.

KAP1 regulates stem cell pluripotency and differentiation

KAP1 is also required in some contexts for stem cell differentiation. As a global transcriptional regulator, it seems that it controls the expression of differentiation genes through cooperation with different transcription factors. This working model was observed in cell types like embryonic stem cells, neurons, myoblasts, and erythroblasts (Fasching et al., 2015, Cammas et al., 2007, Cammas et al., 2004, Cammas et al., 2002, Cheng et al., 2014a, Lee et al., 2016, Singh et al., 2015, Hosoya et al., 2013). For example, KAP1 maintains pluripotency in ES cells by interacting directly with polycomb repressive complex 1 (PRC1) and together they bind the promoter of differentiation-inducible genes and repressed their transcription. In contrast, KAP1 bound the transcribed sequences of pluripotency-associated genes without recruiting PRC1, and derepressed the transcription of these genes (Cheng et al., 2014a). This

suggest a model of KAP1 function as an activator or repressor of genes depending on its protein binding partner. Another interesting example is KAP1 interaction with RE-1 silencing transcription factor (REST), a transcription repressor that regulates gene expression by binding to repressor element 1. Together, they co-regulate neuronal differentiation-related genes like CTNND2 and their depletion promoted neurite outgrowth of mouse embryonic cortical neurons (Lee et al., 2016). Taken together, these studies confirm that KAP1 interactions with other proteins are important for its function. Therefore, one interesting question is whether KAP1 has any role in determining or inhibiting the neuronal differentiation of NSCs in the SVZ, and if KAP1 functions on an epigenetic level to suppress or activate neuronal differentiation genes through binding with other regulators.

3.1.3 KAP1 regulates cell migration

KAP1 function in regulating migration has not been studied extensively. Published work focused only on KAP1 role in regulating breast and lung cancer metastasis, migration and invasion (Wei et al., 2016, Chen et al., 2014).

KAP1 is highly expressed in breast cancer tissues and cell lines. It directly interacts with TWIST1, a transcription factor that is important in regulating cancer development and metastasis and protects it from degradation. The levels of KAP1 and TWIST1 are positively correlated with the aggressiveness of breast cancer. Overexpression of KAP1 in breast cancer cell line promoted cell migration and invasion. While knockdown of KAP1 reduced TWIST1 protein level and caused upregulation E-cadherin and downregulation of N-cadherin which led to inhibition of cell migration. These results

suggest that KAP1 enhances cancer metastasis by stabilizing TWIST1 (Wei et al., 2016). Another group examined KAP1's role in epithelial-to-mesenchymal transition (EMT) a major developmental fate shift that is also implicated in cancer metastasis. In a lung cancer cell line, KAP1 plays a role in TGF β- induced EMT. Silencing of KAP1 impairs its function and leads to decreased cell migration and invasion (Chen et al., 2014). It would be interesting to investigate if KAP1 plays a role in regulating neuroblast migration from the SVZ to the OB in normal and pathological condition.

In this chapter, I aimed to study the following questions: (1) Whether KAP1 is expressed in the SVZ neural lineage? (2) Whether KAP1 loss affects postnatal and adult SVZ NSC activation, proliferation or fate choice?

3.2 Results

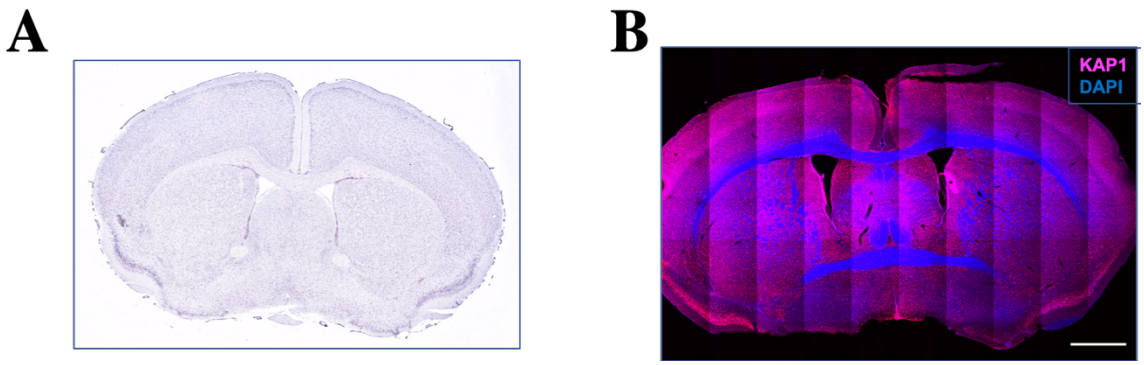
3.2.1 KAP1 expression in postnatal and adult SVZ

In situ hybridization of KAP1 in P56 mouse brain had shown a high and dense expression of KAP1 in the SVZ and the RMS, and also broad expression around the brain (Figure 3.2 A) (data from Allen Brain Atlas). Consistent with this, my immunohistochemistry analysis of P42 mice brains also showed a broad expression of KAP1 in the striatum, cerebral cortex and OB, while in the white matter e.g. corpus callosum, a weak immunostaining of KAP1 was observed (n=3, Figure 3.2 B).

Different cell markers were then co-stained with KAP1 to detect its expression in various cells of the neural lineage. In the SVZ, all examined GFAP+/Sox2+ neural stem cells were KAP1+ (n=3, Figure 3.2 C). To detect if KAP1 is expressed in both quiescent and actively proliferating NSC in vivo, the BrdU-label retaining technique was performed (Ferron et al., 2007). BrdU was injected twice daily for 5 consecutive days in P42 mice, and the mice were sacrificed either 2 hours or 5 weeks after the last injection. Only quiescent NSC in the SVZ can retain BrdU after the 5-week dilution period because of their low cell division frequency. All BrdU-label retaining cells (LRC) were KAP1+, which suggest that KAP1 is expressed in both quiescent and actively proliferating cells (n=3, Figure 3.2 D&E). This was confirmed with co-staining of KAP1 with the Ki67 actively proliferating cell marker (n=3, Figure 3.2 F). In addition, all examined cells that are DLX2+ transit amplifying progenitor cells in the SVZ were KAP1+ (n=3, Figure 3.2 G). In the RMS and OB, KAP1+ nuclei were observed in DCX+ neuroblasts and neurons in the granular layers (n=3, Figure 3.2 H).

Next, in order to confirm KAP1 expression in postnatal brain, different brain regions of age P4 pups were dissected and mRNA levels were measured by RT-qPCR. KAP1 was

broadly expressed in the SVZ, OB, striatum, cerebral cortex and in tertiary neurospheres generated from the SVZ (n=3, Figure 3.2 I). In conclusion, these data suggest that KAP1 is expressed in the entire SVZ lineage.


A
B
KAP1
DAPI
C
KAP1/GFAP/SOX2/DAPI
LV
D
KAP1/BrdU
BrdU
analysis
6 week old
2hrs later
E
KAP1/BrdU
BrdU
analysis
6 week old
5wks later
F
KAP1/Ki67

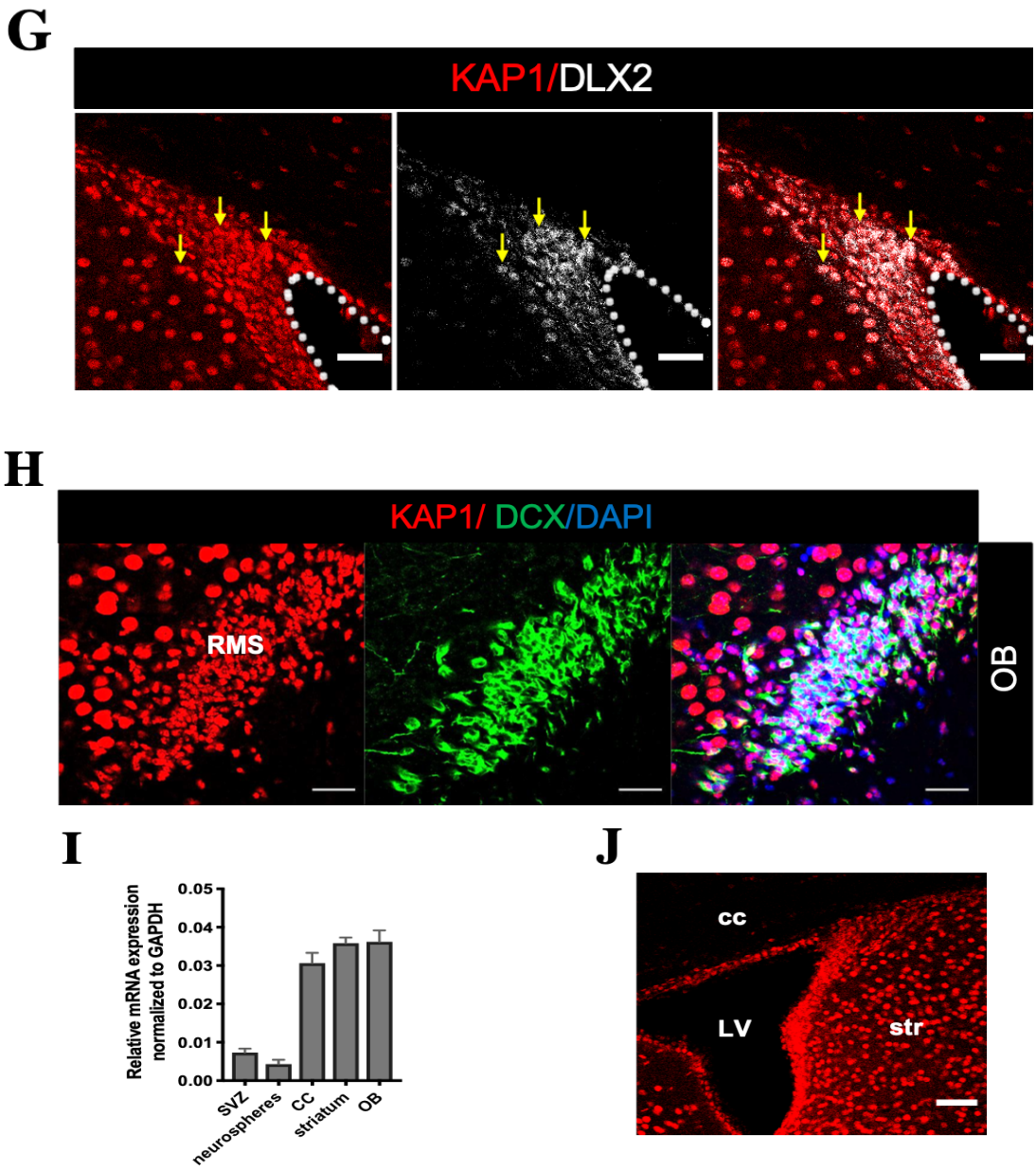


Figure 3.2 KAP1 expression in postnatal and adult mice brain

(A): In situ hybridization of KAP1 in the adult brain (data from Allen Brain Atlas).

(B): Immunostaining shows the broad expression of KAP1 in the P42 mice brain.

(C): Costaining of KAP1 with GFAP and Sox2 in the P42 SVZ (n=3).

(D-E): Costaining of KAP1 and BrdU after 2hrs and 5 weeks BrdU labelling. The arrows indicate the examples of BrdU+ cells (n=3)

(F&G): Costaining of KAP1 with KI67 and DLX2 respectively in the P42 SVZ (n=3).

(H): Costaining of KAP1 with DCX in the P42 OB (n=3)

(I): qPCR analysis of KAP1 expression in different brain areas in postnatal mice brain (n=3).

(J): Immunostaining of KAP1 expression in higher magnification in the SVZ, striatum, and corpus callosum. Scale bar: (B)= 1000 µm , (C-H) =30 µm, (J)= 50 µm.

3.2.2 Loss of SVZ KAP1 impairs postnatal OB neurogenesis

To study the role of KAP1 in postnatal neurogenesis, I electroporated individual constructs that express GFP (scramble, shKAP1-A, and shKAP1-B) into the SVZ of age P1 pups to detect newborn neurons that had migrated from the SVZ to the OB. Pups were sacrificed 7 days later which is the approximate required time for new-born neuroblasts to arrive to the OB from the SVZ. Surprisingly, at 7 dpe the number of GFP+ cells in the OB significantly decreased by 42% with shA and by 63% with shB KAP1 knockdown (scramble: 28,845 ± 2,245/ mm^3, shA: 16,807 ± 1,400/ mm^3, shB:10,955 ± 787/mm^3) (n=3 for all groups, $p< 0.01$ for scramble versus shA, $p< 0.001$ for scramble versus shB)(Figure 3.3 C & D). The comparable GFP signals in the SVZ at 3dpe suggests that this reduced GFP+ cells in the OB was not due to different electroporation efficiencies between control and KD (Data not shown).

Quantification of GFP+ cells in the RMS area in the core of the OB showed a decrease in the knockdown groups compare to the control (scramble: 126,001±4,966 / mm^3, shA: 97,899±11,888/ mm^3, shB: 82,493±6,270/mm^3) (n=3 for all groups, $p=0.09$ for scramble versus shA, $p< 0.05$ for scramble versus shB))(Figure 3.3 E).

Quantification of GFP+ cells in the granular layers of the OB showed a stronger decrease in the number of GFP+ cells compared to the RMS (scramble: 21,405±1,106/ mm^3, shA: 10,438±2,314/ mm^3, shB: 7,044±741/mm^3) (n=3 for all groups, $p<0.01$ for scramble versus shA, $p< 0.01$ for scramble versus shB))(Figure 3.3 F).

Next, in order to further investigate the mechanism of this reduced neurogenesis, brains were collected at earlier time points i.e. 3dpe for analysis in the RMS. There was a reduction in the number of GFP+ neuroblasts in the RMS in the knockdown groups compared to the control group (scramble: 169,871±6,823/ mm^3, shA: 121,621 ±31,917/ mm^3, shB: 129,771±25,403/mm^3) (n=3 for all groups, p=0.05 for scramble versus shA) (Figure 3.3 G & H). Fewer GFP+ cells in the RMS at 3 dpe led to fewer mature neurons in the granular layers at 7 dpe. Furthermore, co-staining with the immature neuroblast marker DCX in both the OB and the RMS showed that all GFP+ cells were DCX+ and therefore that DCX expression was not affected by the knockdown of KAP1. These data suggest that KAP1 is required for the production of newborn OB neurons in postnatal mice.

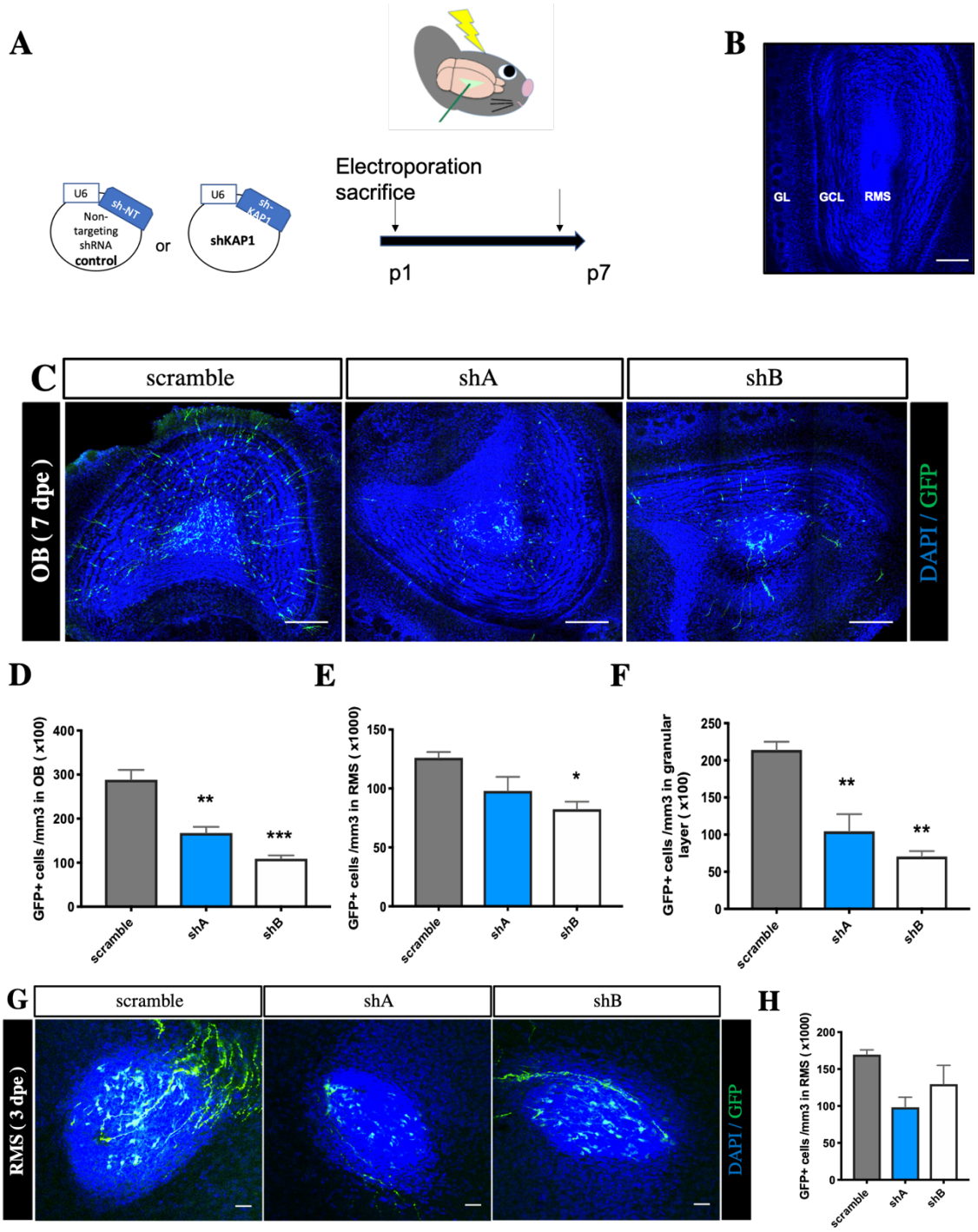


Figure 3.3 Neurogenesis decreases in the RMS/OB after KAP1 knockdown

(A): A schematic diagram of the experimental design.
(B): Representative image of mice postnatal OB.
(C): Immunostaining of GFP+ cells in OB 7 dpe reveals fewer newborn neurons in the OB after KAP1 knockdown using shA and shB constructs compared to the scramble control.
(D-F): Quantification of GFP+ cells in the whole OB section, RMS, and granular layer (n=3 for controls and knockdown groups).
(G-H):Immunostaining and quantification of GFP+ cells in the RMS 3 dpe after KAP1 knockdown (n=3 for controls and knockdown groups). Error bars represent SEM.
* p< 0.05, **p<0.01, ***p<0.001. Scale bar: (B&C) =200 µm, (G) =50 µm.

3.2.3 KAP1 disrupts morphological differentiation of newborn neurons in the OB

Interestingly, electroporating KAP1 shRNA construct in the SVZ altered the morphology of newborn neurons in the OB at 7 dpe. As expected, in the control condition many GFP+ neurons had arrive to the OB granule layer and began morphological differentiation with their processes extending radially towards the pial surface and presenting primary and secondary branching, and dendritic spines which indicate their synaptic integration into the OB circuit (Petreanu and Alvarez-Buylla, 2002). These cells are classified as class I and were similar to a previous morphological classification scheme (Boutin et al., 2010). However, after KAP1 KD, abnormal neuronal morphologies were observed which are classified as class II and class III. Class II cells were rare, they presented a single primary process and short branches. Class III cells had spherical cell bodies with very short processes and no branching, suggesting that they are still migrating but have not differentiated yet (Figure 3.4 A).

Quantification of GFP+ cells showed that the percentage of cells with Class I morphology was 42.0± 1.5% in scramble control and only 16.7±5.6% after KAP1 KD in the shA group and 19.3±2.0% in the shB group ($p<0.01$ for both groups) (Figure 3.4 B). However, after KAP1 KD there were significantly more class III neurons observed in the shA 81.3 ± 5.6% and shB 77.3± 0.3% groups compared to the control 54.7± 2.2% ($p< 0.01$ and $p< 0.05$, respectively) (Figure 3.4 C). These data further suggest that KAP1 affects postnatal neurogenesis by disrupting morphological differentiation of newborn neurons.

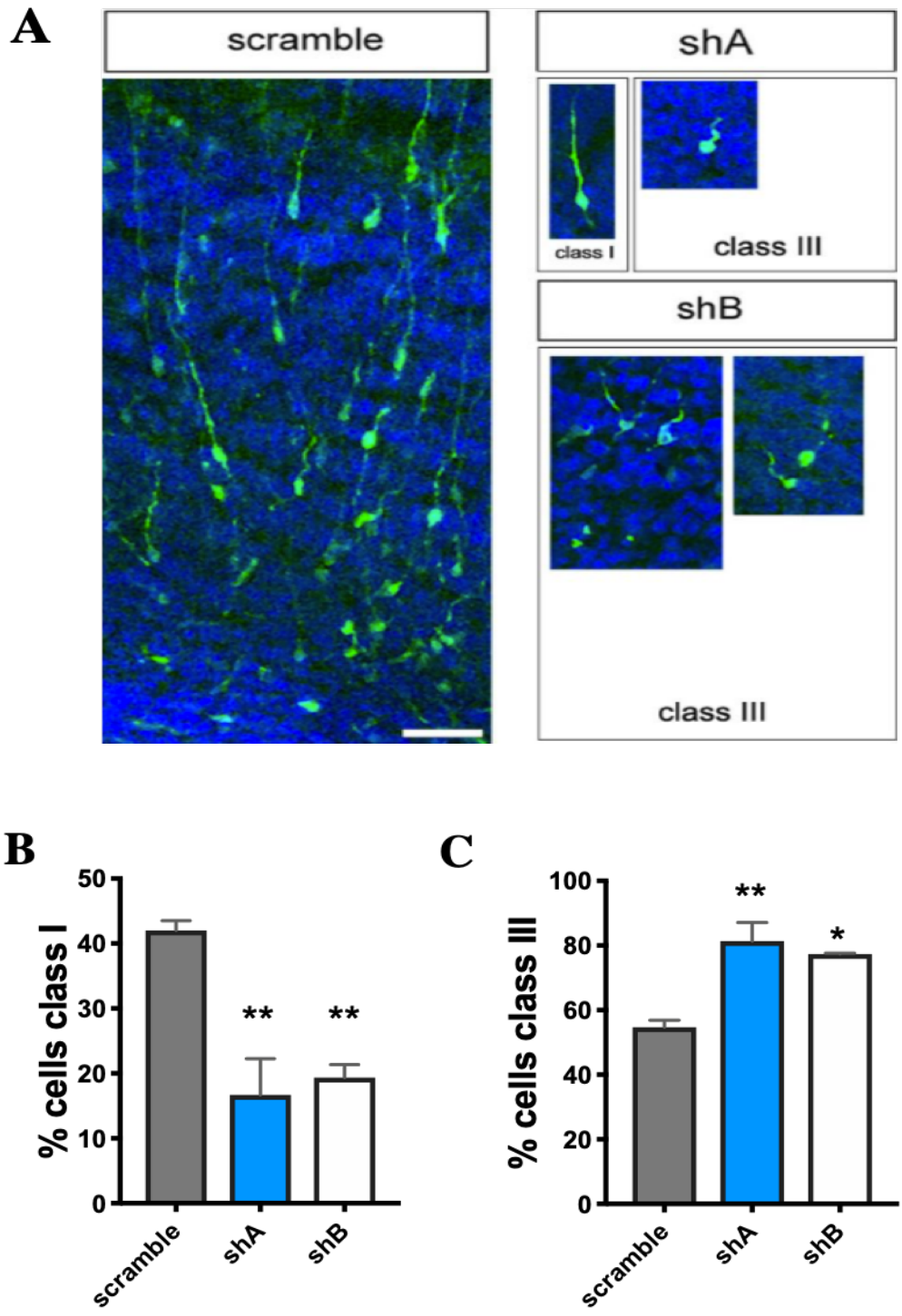


Figure 3.4 KAP1 knockdown cause defective differentiation of newborn neurons in the OB

A) High magnification showing different morphologies of GFP^+ granule layer OB neurons 7 dpe after KAP1 KD. For ease of comparison, neuronal orientations were aligned to vertical. The cells shown in the scramble control group are class I.
B) Quantification of the percentage of cells with Class I morphology 7 days after KAP1 KD. (n=3 for controls and knockdown groups).
C) Quantification of the percentage of cells with Class III morphology 7 days after KAP1 KD. (n=3 for controls and knockdown groups).Error bars represent SEM. **p<0.01. Scale bar =50 µm. The same scale bar is used for all images within each section.

3.2.4 KAP1 is dispensable for neuroblast migration in vitro

Many transcription factors have been found to regulate the migration of neuroblasts. Loss of KAP1 reducing neurogenesis could also be partially explained by a defect in neuroblast migration from the SVZ through the RMS to the OB. In previous studies, KAP1 was shown to regulate migration and invasion of multiple cancer cell lines (Chen et al., 2014, Wei et al., 2016) . However, KAP1 regulation of cell migration during development had not been investigated before. Here, I investigated if KAP1 play such a role which could explain the defective number of new-born neurons arriving to the OB.

In vitro RMS spheroid migration assay was performed by my colleague Martin Ducker (Szele lab DPhil candidate), in which he dissected cells from the RMS of age P2-P4 mice and nucleofected by using shRNA scramble control or shRNA to knockdown KAP1. Cells were plated at 6 well plate and left to recover at 37°C for 4 hours. Then cells were cultured in round bottomed plates to form spheroids, and 12 hours later spheroids were embedded into Matrigel (as described in Chapter 2 materials and methods). We used postnatal day 4 because explant migration is more robust at this age (Ward and Rao, 2005). 3 days after the knockdown, images were taken on EVOS microscope to observe the distance of neuroblasts migration from the Matrigel (Figure 3.5). We did not find an effect of the migration distance of neuroblasts from the explants after KAP1 KD compared to the scramble control (n=3), which suggest that KAP1 does not cause a defect in migration.

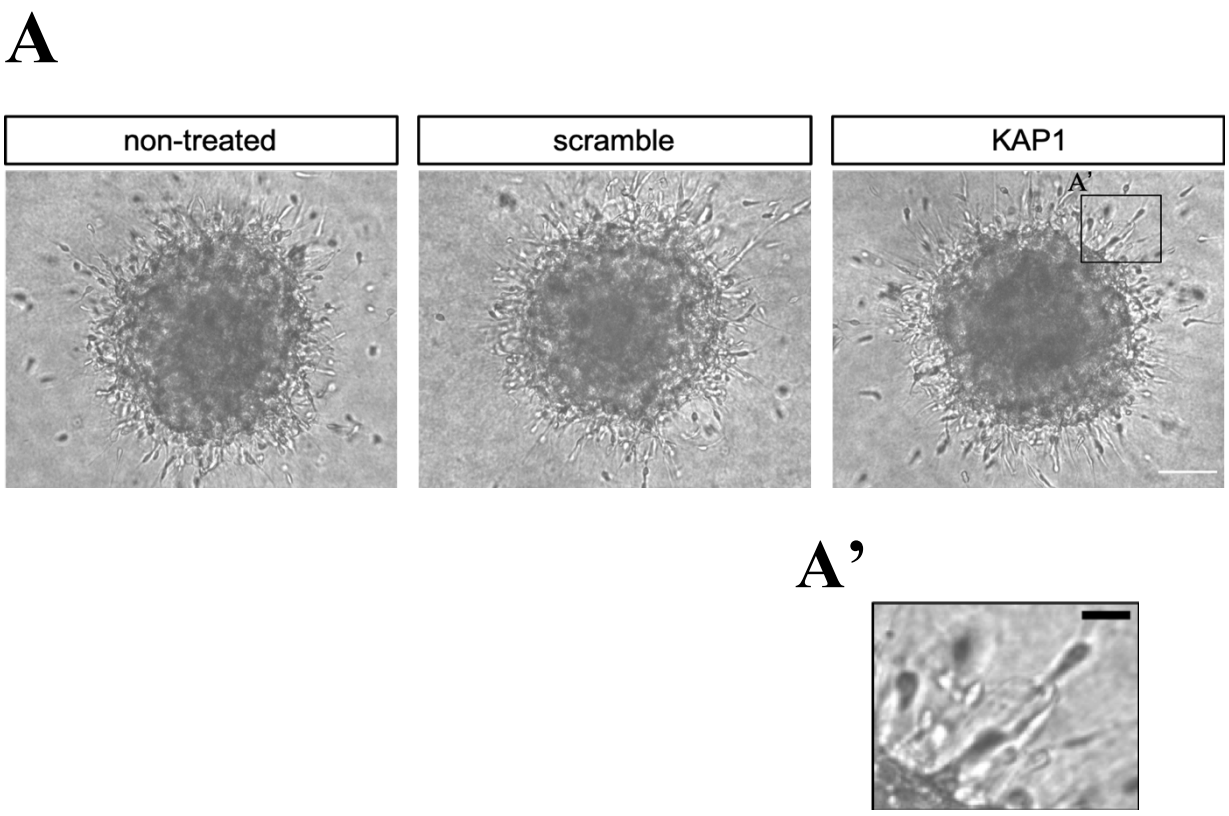


Figure 3.5 KAP1 knockdown does not affect neuroblasts migration in the RMS

A)Spheroids are adhered in Matrigel and shRNAs were used to knockdown KAP1, distance of neuroblast migration was imaged after 3 days. Knockdown of KAP1 did not affect neuroblasts migration from spheroids compared to non-treated control and scramble condition. Images show cells migrating out as chains from an RMS spheroid. Neuroblasts self-organise into chains to radiate out from the spheroid before continuing as single cells. A') higher magnification of neuroblast migration from matrigel. Scale bar = 200 μm

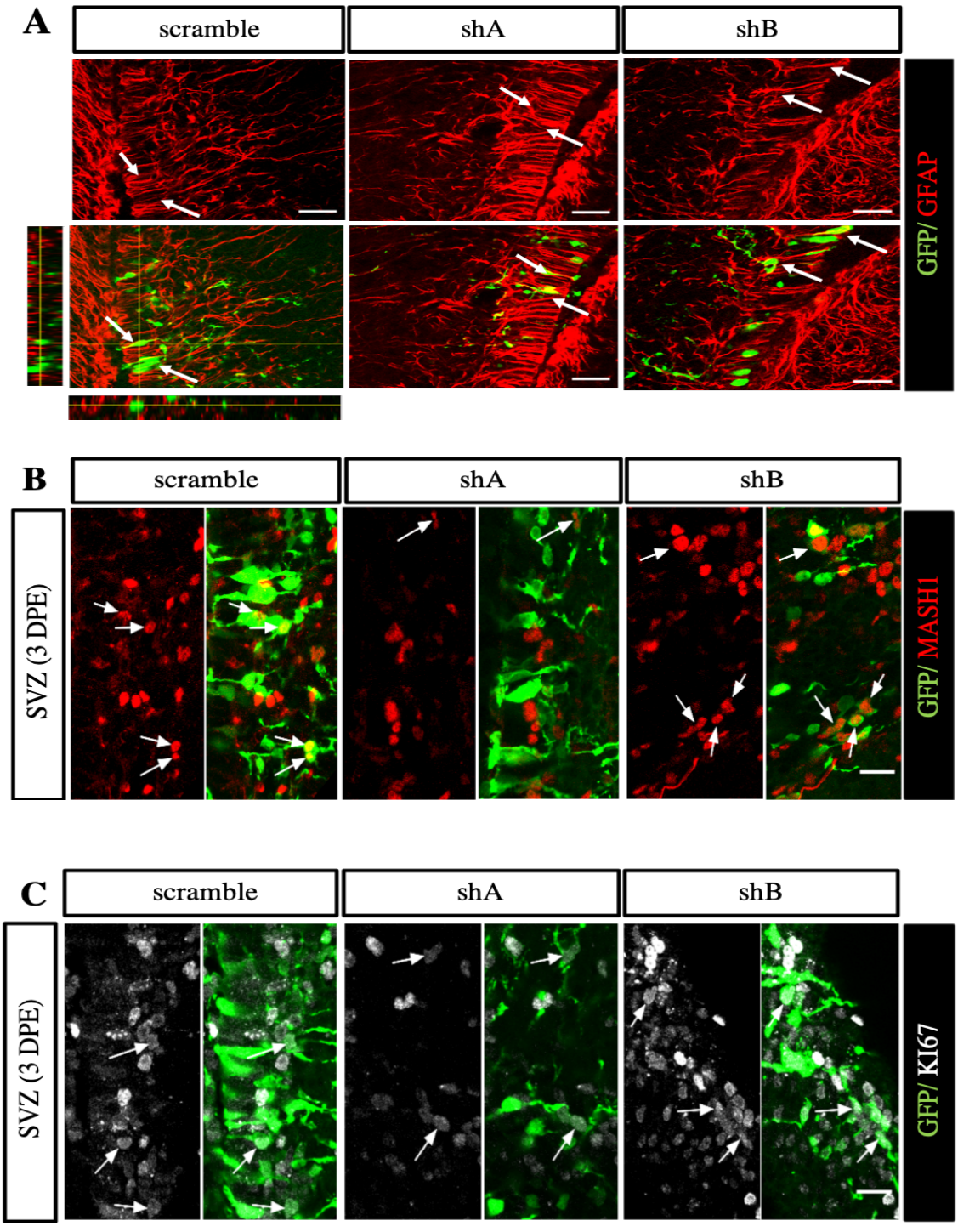
3.2.5 KAP1 loss impairs neural stem cell maintenance in pSVZ

I next decided to study the functional effect of KAP1 loss in SVZ neurogenesis. In vivo electroporation was performed on p1 pups SVZ using a scramble control and two knockdown constructs targeting KAP1, shA and shB, all constructs express GFP. The knockdown efficiency of the shRNA constructs was confirmed in neurosphere cultures dissected from postnatal SVZ (as shown in Chapter 5, Figure 5.3). 3 days post electroporation (dpe), the animals were sacrificed, and brains were collected for analysis. Previous reports suggested that KAP1 is important for the maintenance of embryonic stem cell self-renewal and proliferation (Seki et al., 2010, Hu et al., 2009). In the KAP1 KD SVZ, there was a significant reduction in the expression of the radial glial/neural stem cell marker GFAP (scramble: 78.01±2.23% versus shA: 54.9±1.73%, and shB: 61.66±1.95%) (n=3 for each group, shA $p<0.05$, shB $p<0.01$,Figure 3.6 A). Loss of GFAP could have been due to accelerated activation of NSC into Type C cells. Thus, we hypothesised that more GFP+ cells would express transit amplifying (Type C) cell markers.

Immunostaining of the transit amplifying cell marker Mash1 actually showed a mild but significant decrease among GFP+ cells (scramble: 21.45±1.57% versus shA: 13.22±1.31%, and shB: 19.8±1.78%) (n=3 for each group, shA $p<0.05$, Figure 3.6 B), which was confirmed by slight loss, but not significant, with the transit amplifying progenitor marker DLX2 (scramble: 70.75±2.26% versus shA: 52.74±9.1%, and shB: 66.2±7.3%) (n=3 for each group, Figure 3.6 D).

In addition, immunostaining of the proliferative cell marker KI67 in GFP+ cells in the SVZ did not show a significant difference (scramble: 29.49±2.94% versus shA:

24.8±3.5%, and shB: 37.38±2.42%) (n=3 for each group, Figure 3.6 C).These results suggest that KAP1 KD did not accelerate linage progression but caused a defect in neural stem cell maintenance which may have resulted in a reduction of the neural stem cell pool that lead to a reduction in the number progenitor cells in the SVZ.



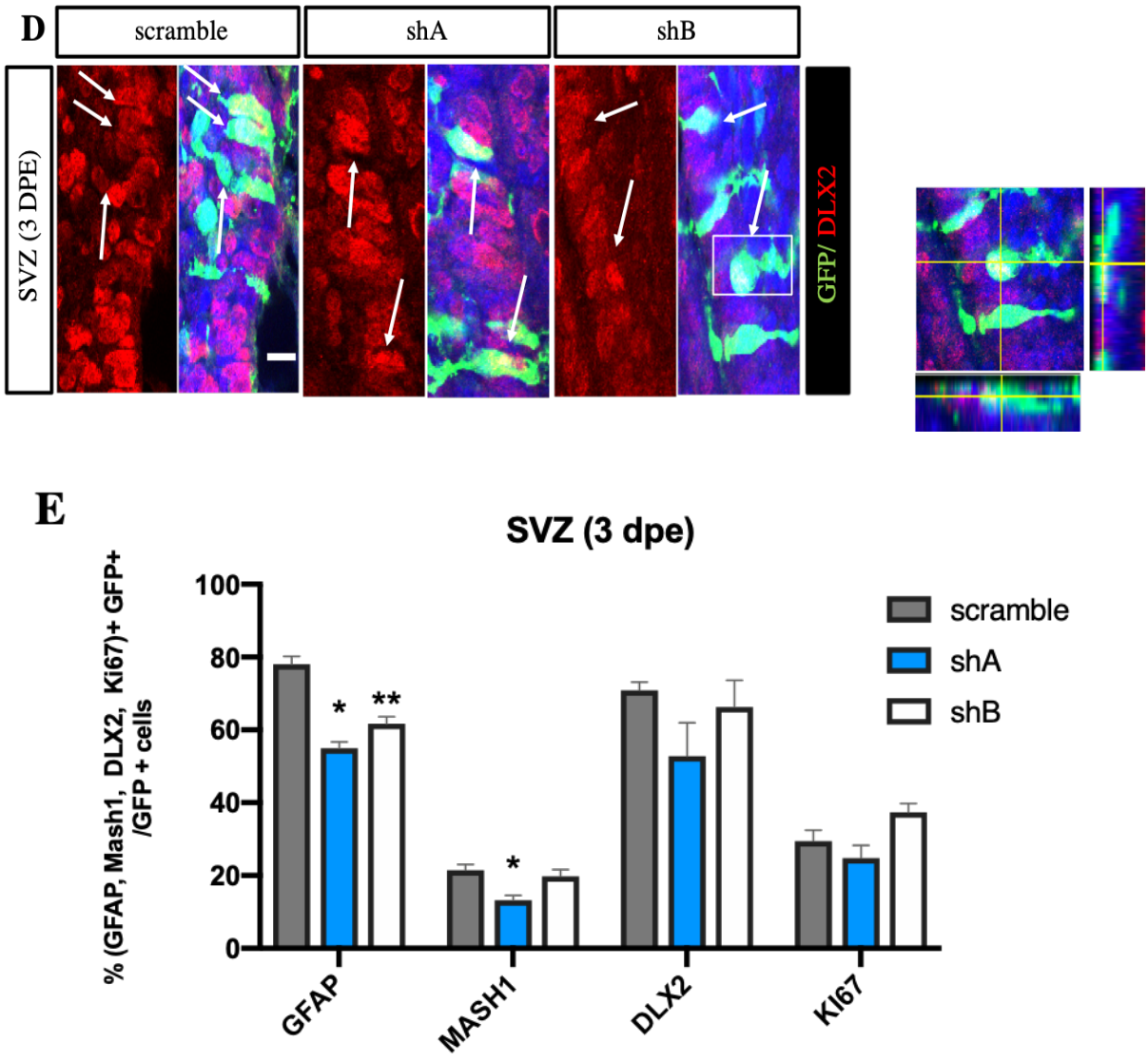


Figure 3.6 Neural stem cells maintenance defect in the postnatal SVZ after knockdown of KAP1

(A): Costaining of GFAP+ and GFP+ cells in the SVZ after KAP1 knockdown using shA and shB constructs compared to the scramble control.

(B): Costaining of MASH1+ and GFP+ cells in the SVZ after KAP1 knockdown using shA and shB constructs compared to the scramble control.

(C): Costaining of KI67+ and GFP+ cells in the SVZ after KAP1 knockdown using shA and shB constructs compared to the scramble control.

(D): Costaining of DLX2+ and GFP+ cells in the SVZ after KAP1 knockdown using shA and shB constructs compared to the scramble control.

(E): Quantification of the percent of GFAP, MASH1, KI67, and DLX2 +GFP+ cells/ total GFP+ cells (n=3 for each group). Error bars represent SEM. * $p< 0.05$, **$p<0.01$, ***$p<0.001$. Scale bar: 30 µm

3.2.6 KAP1 loss does not induce cell death in the SVZ or OB

Cell death is essential for many physiological processes. To investigate whether the reduction of the number of neural stem cells and transit progenitor cells in the SVZ, and the number of neuroblast arriving the OB was due to cell death that caused by KAP1 loss, the TUNEL apoptosis detection method was performed. GFP signals can remain active and stable for a long time after cell death (Lowder et al., 2000) which allow us to quantify the number of GFP+ cells that are also TUNEL+. Quantification was performed in SVZ, RMS, and OB sections at 3 dpe and 7 dpe and showed that there is no difference in cell death (TUNEL + cells) in knockdown conditions compared to control (n=3 for control and KD groups, Figure 3.7). This indicates that the observed reduction in neurogenesis was not due to cell death after KAP1 knockdown.

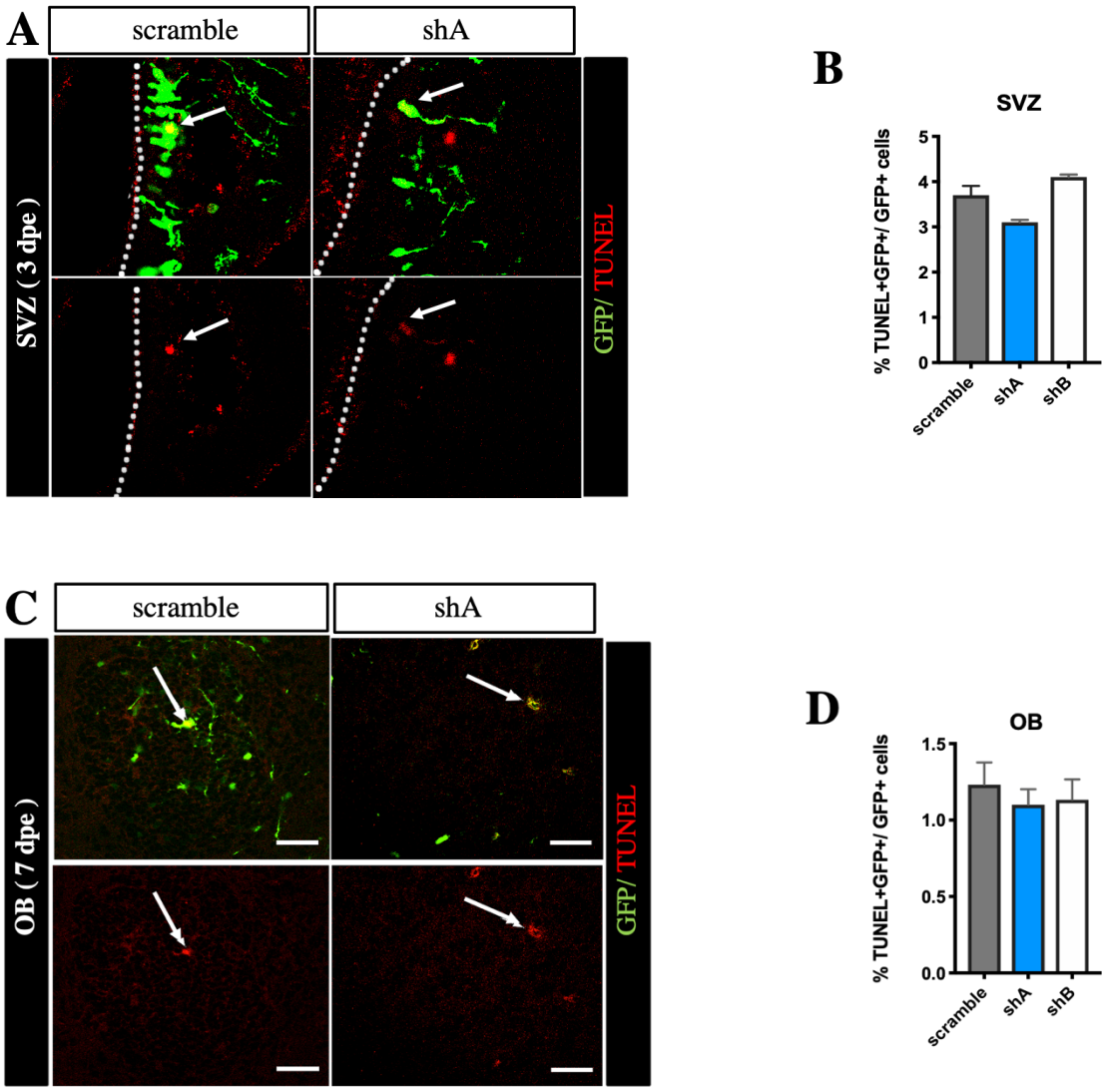


Figure 3.7 KAP1 knockdown does not cause apoptosis in the SVZ and OB

(A-B): TUNEL staining and quantification of TUNEL+GFP+ cells/total GFP+ cells in the SVZ 3 dpe after KAP1 knockdown (n=3 for controls and Knockdown groups).

(C-D): TUNEL staining and quantification of TUNEL+GFP+ cells/total GFP+ cells in the OB 7 dpe after KAP1 knockdown (n=3 for controls and Knockdown groups). Error bars represent SEM. Scale bar = 30 µm

3.2.7 Generation of KAP1 cKO mice

Loss of function results are typically confirmed with multiple tools. Due to the early embryonic lethality of KAP1 knockout mice (Cammas et al., 2000), I decided to use a tamoxifen-induced conditional knockout (cKO) approach (Nagy, 2000) to confirm the postnatal KAP1 effect in adult mice. $KAP1^{fl/fl}$ mice have loxP sites that were inserted in state specific location as reported (Cammas et al., 2000). As expected, genotyping of $KAP1^{fl/fl}$ mice showed a floxed band at ~225 bp, and wild type band at ~ 173 bp (Figure 3.8 B). To conditionally knockout KAP1 specifically in the adult neural stem/progenitor cell lineage. Nestin-CreERT2- yellow fluorescent protein (YFP) mice were used which carry an NSC-specific nestin promoter/enhancer driven Cre recombinase coupled to the estrogen receptor ligand-binding domain (ERT2) (Chen et al., 2009b). Genotyping of the Nestin-CreERT2-YFP mice showed the Cre band at ~ 500 bp, the YFP band at ~320 and the wild type band at ~600 bp. $KAP1^{fl/fl}$ mice were then crossed with Nestin-CreERT2-YFP mice to generate Nestin-CreERT2-YFP; $KAP1^{fl/fl}$ mice (Breeding method is described in Chapter 2 Materials and methods). To induce Cre recombinase, 180 mg/kg body weight of tamoxifen was administered in 8-9 weeks old mice via gavage. 28 days after the last dose, mice were sacrificed, and brain coronal sections were stained with KAP1 antibody to confirm the cKO of KAP1 at the protein level (Figure 3.8 C). A significant decrease of KAP1+ nuclei was observed in the cKO SVZ compared to control. This observation confirmed the Nestin-CreERT2-YFP; $KAP1^{fl/fl}$ mice can be used as a model to study KAP1 functional role in the SVZ.

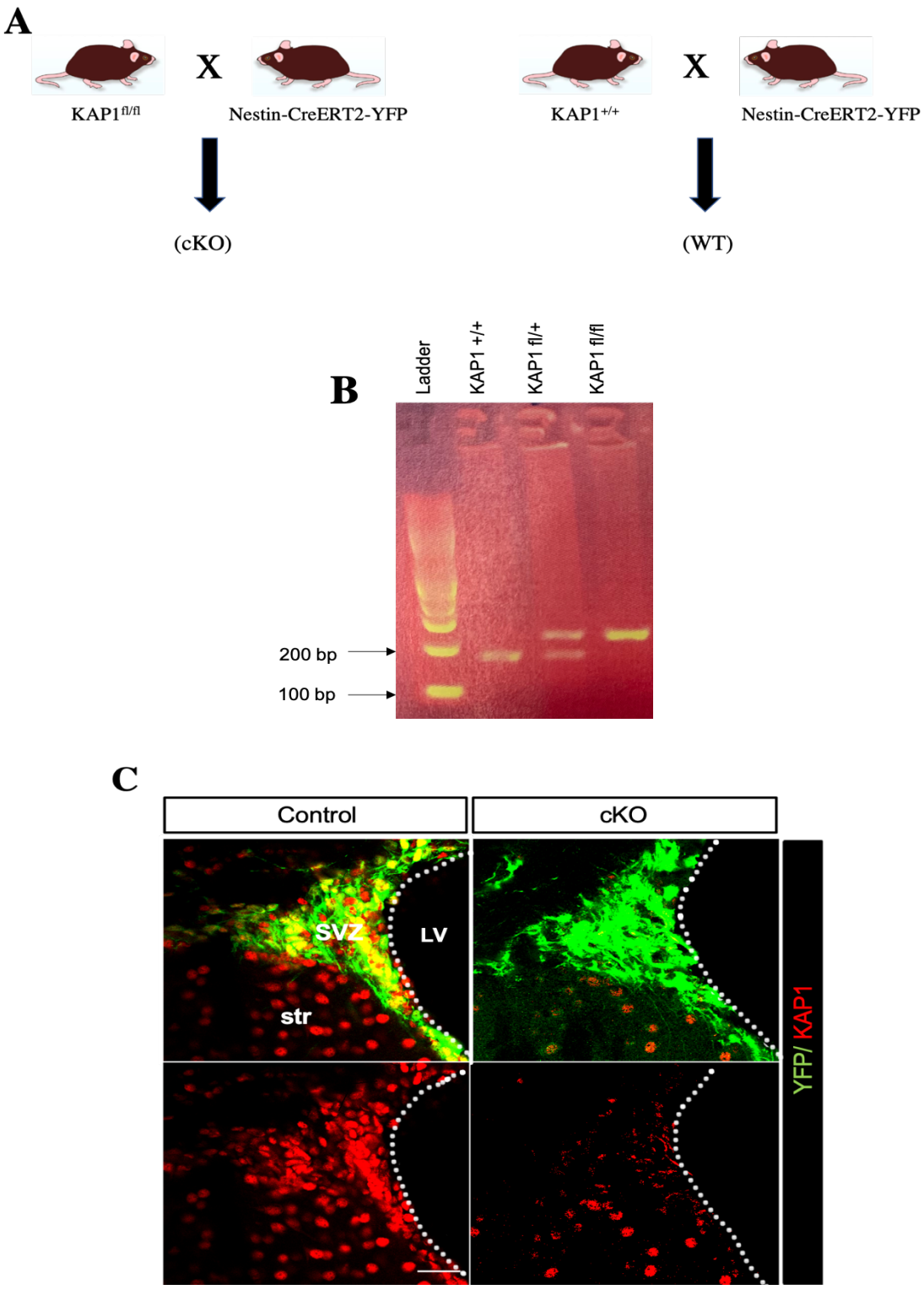


Figure 3.8 Conditional knockout of KAP1 in adult SVZ

A) A schematic diagram of the experimental design.

B) Examples of PCR genotyping of different mouse strains. The floxed band is around 225 bp and the wildtype band is around 173 bp. C) Immunostaining of SVZ shows KAP1 protein level decreases in cKO model compared to wildtype controls. Note that striatal KAP1+ cells under the SVZ could still be detected as expected. Scale bar: 30 μm.

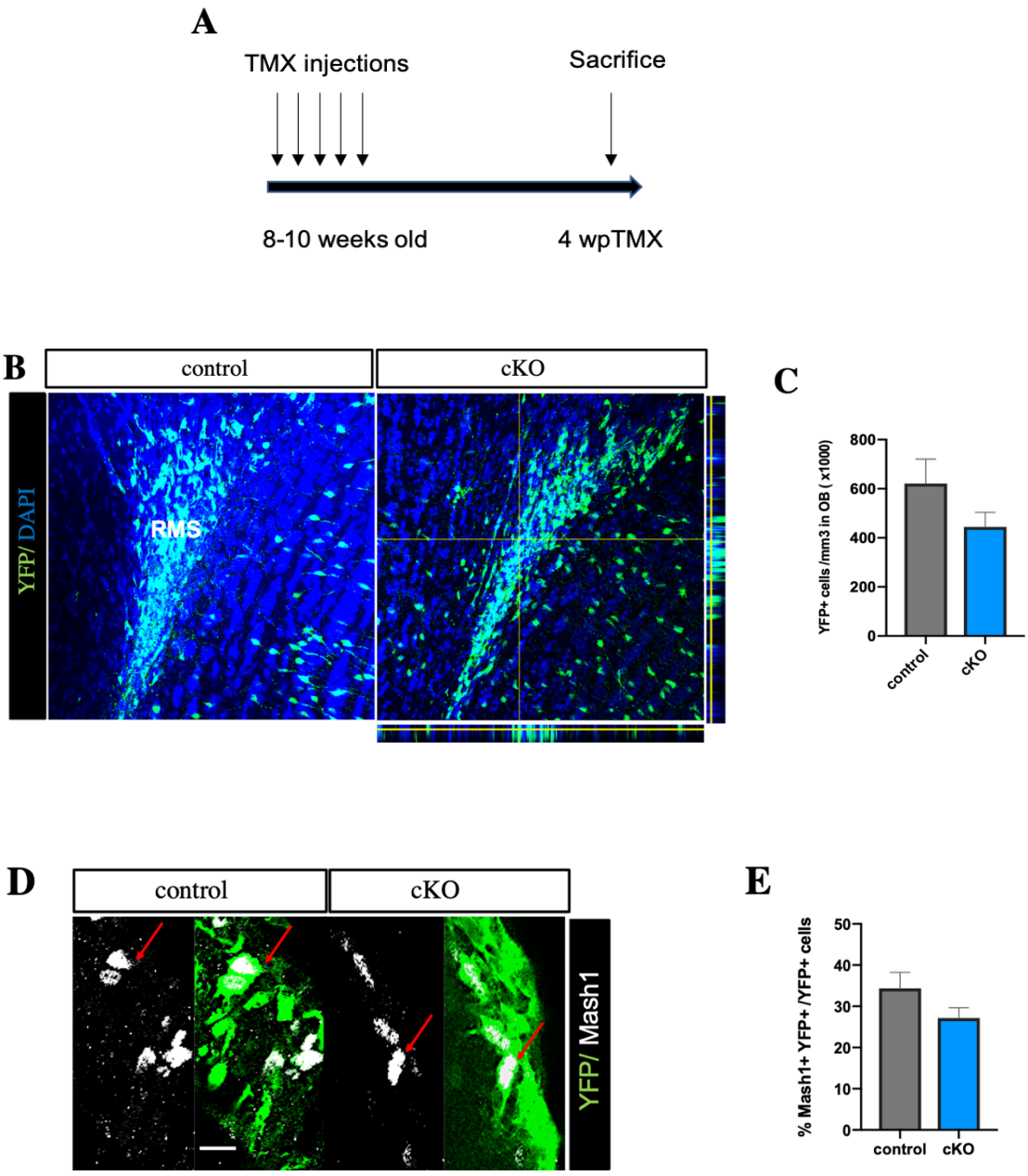
3.2.8 KAP1 regulates NSC identity maintenance in the SVZ

To confirm the knockdown effect, and to study the functional role of KAP1 in the adult brain, I used Nestin-CreERT2-YFP; KAP1$^{fl/fl}$ mice to conditionally knockout KAP1 in SVZ NSCs. Tamoxifen was administered at age 8-10 weeks (one dose per day for five consecutive days) to trace newborn neurons from the SVZ to the OB. 28 days later, neurogenesis was analysed. In partial agreement with the results from postnatal electroporation, after the loss of KAP1, there was a slight decrease in YFP+ cells in cKO OB (443.5±83.9 (x1000)/ mm^3), compared to the control mice (621.2±98.8 (x1000)/ mm^3)(n=3 for control, n=6 for cKO)(Figure 3.9 B&C). These results confirm that KAP1 is important for the production of newborn OB neurons in postnatal and adult mice.

Next, I examined neural stem cells and progenitors in the SVZ. Similar to postnatal mice, immunostaining of the transit amplifying cell marker Mash1 show a mild decrease among YFP+ cells after KAP1 loss (control: 34.3±3.9% versus cKO: 27.1±2.5%) (n=3 for each group, Figure 3.9 D&E). In addition, immunostaining of the proliferative cell marker KI67 in YFP+ cells in the SVZ did not show a significant difference (control: 33.1±9.6% versus cKO: 45.5±2%) (n=3 for each group, Figure 3.9 F&G).

Finally, GFAP+ cells could be either stem cells or niche astrocytes. Thus, in order to characterize neural stem cell in the SVZ, I co-stained S100β and GFAP in YFP cells. S100β marks astroglia maturation stage in NSCs (Raponi et al., 2007). Interestingly, in KAP1 cKO SVZ, the percent of S100β+ cells in GFAP+ YFP+ cells significantly increased (67.3±2.2%) and more cells exhibited the mature astroglia marker S100β compared to control (45.6±3.4%) (p=0.0058, n=3 for each group, Figure 3.9 H&I). These data suggest that after KAP1 loss, there was a defect in NSCs maintenance in the

SVZ which caused the cell identity to shift from stem cells to mature astroglial cells (niche astrocytes) and consequently a reduction of SVZ-OB neurogenesis.



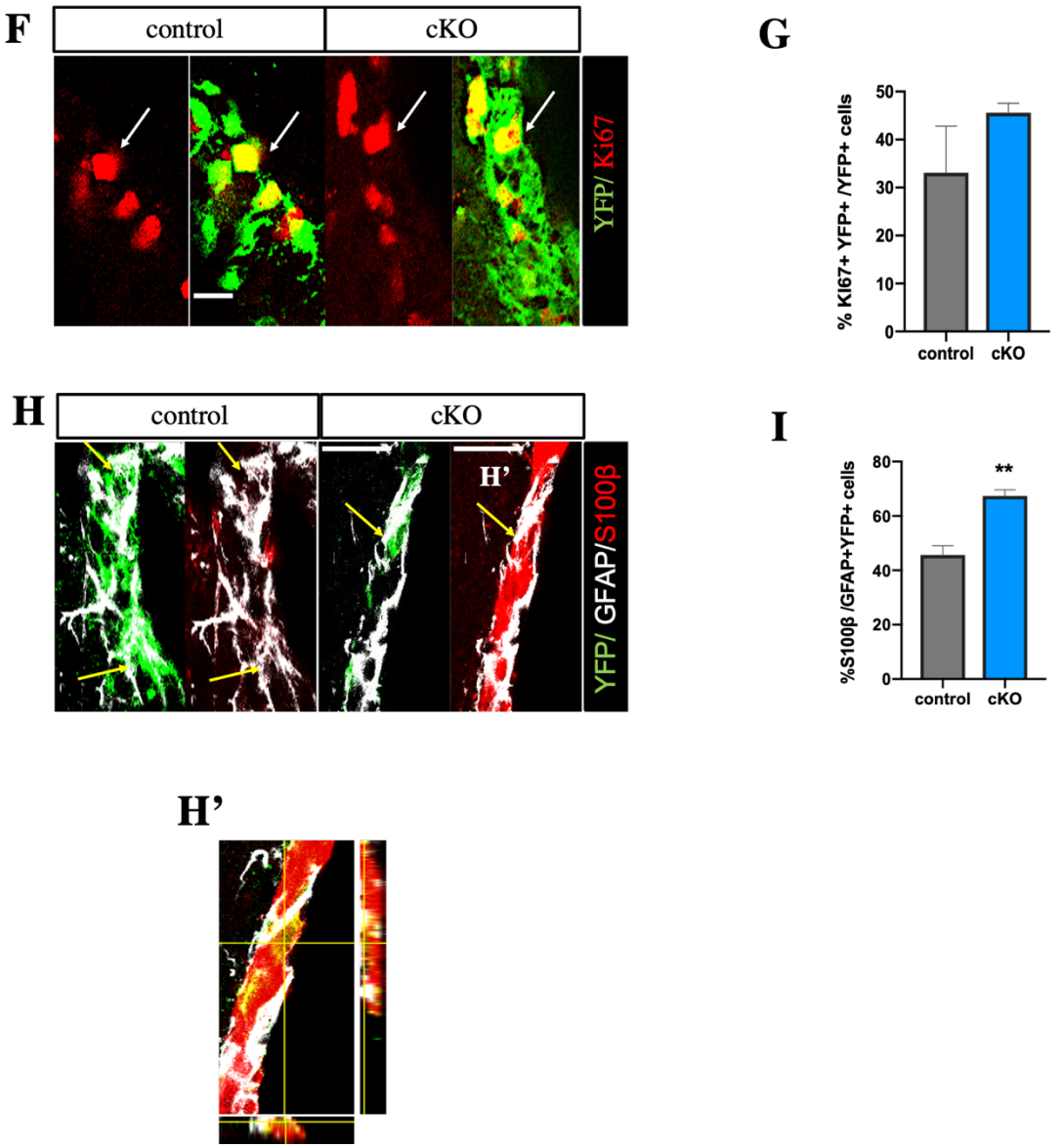


Figure 3.9 KAP1 is required for NSC identity maintenance in the adult SVZ

(A): A schematic diagram of the experimental design.

(B-C): Immunostaining and quantification of YFP+ cells in OB after KAP1 cKO compared to the control (n=3 for control, n=6 for cKO)

(D-E): Costaining and quantification of the % of MASH1+ YFP+ among YFP+ cells in the SVZ after KAP1 cKO compared to the control (n=3)

(F-G): Costaining and quantification of the % of KI67+ YFP+ among YFP+ cells in the SVZ after KAP1 cKO compared to the control (n=3)

(H-I): Costaining and quantification of the % of S100β+ in GFAP+YFP+ cells after KAP1 cKO compared to the control. The yellow arrows indicate GFAP+S100β+ cells in cKO condition compared to GFAP+ S100β- in control condition. The staining indicates NSCs acquire mature astrocytic phenotype after KAP1 knockout (n=3).H': Orthogonal view of the cKO. Error bars represent SEM. **p<0.01. Scale bar: (B): 50 µm, (D,F,H,H')=30 µm

3.2.9 Loss of KAP1 impaired neurosphere generation from the SVZ

To confirm that KAP1 is involved in SVZ NSC identity maintenance, I performed an in vitro neurosphere assay to examine the neurosphere-forming ability of NSCs isolated from the SVZ (Pastrana et al., 2011). I used control group (Nestin-CreERT2-YFP; $KAP1^{+/+}$ mice) and KAP1 cKO group (Nestin-CreERT2-YFP; $KAP1^{fl/fl}$ mice). Tamoxifen was administered at age 8-10 weeks (one dose per day for five consecutive days) and animals were sacrificed 28 days later for in vitro neurosphere assay. Cells were seeded at 100,000/ml in 6-well plates. The ability to generate secondary neurospheres was analysed and showed ~54% fewer neurospheres were generated in the KAP1 cKO group compared to the WT group (n=1, Figure 3.10)

Neurospheres are usually heterogenous and composed of NSCs and neural progenitors which results in diversity of neurosphere size (Pastrana et al., 2011). I did not observe any changes in the average neurosphere diameter between the cKO and the control groups (average size for control: 111.76 μm vs cKO: 112.88 μm) (n=1).

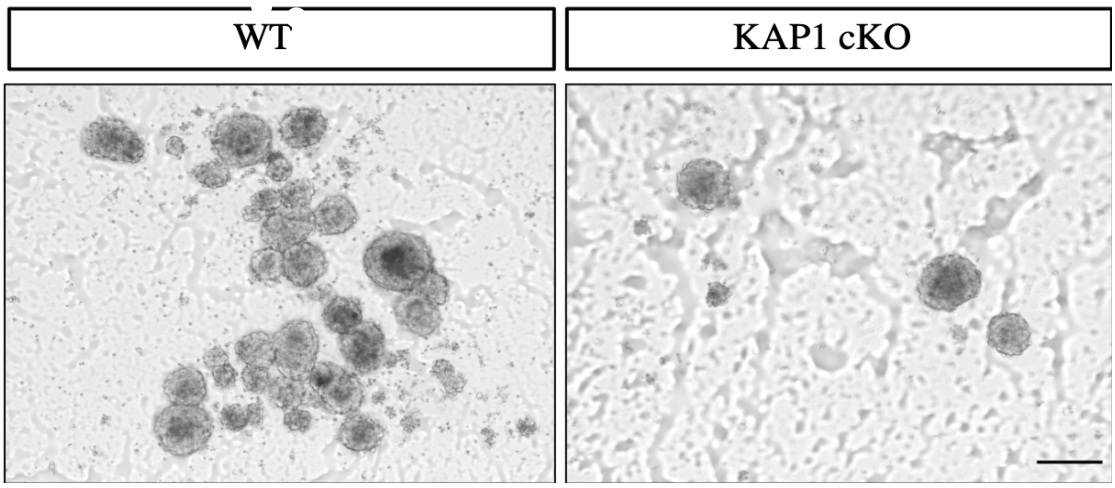


Figure 3.10 Fewer secondary neurospheres could be generated after knockout of KAP1 in the SVZ of adult mice . Tamoxifen was administered at age 8-10 WT and cKO mice. Animals were sacrificed 28 days later for in vitro neurosphere assay. SVZ was dissected and cells were dissociated, cultured with growth factors and seeded at 100,000/ml in 6-well plates. The generated secondary neurospheres were counted and imaged under EVOS microscopy. Scale bar = 200 μm.

3.3 Discussion

In this chapter, I investigated the expression and function of KAP1 in the brain, and specifically in the SVZ. I first showed that KAP1 is broadly expressed in all brain regions, and throughout the neural lineage of the SVZ. Then, I characterized SVZ phenotypes after KAP1 loss in mice. In summary, I found that KAP1 regulates SVZ-OB neurogenesis.

3.3.1 Broad expression of KAP1 in SVZ stem and progenitor cells

KAP1 binds to the KRAB domain of zinc finger proteins and helps in the formation of multiprotein complexes and the recruitment of histone methyltransferases to specific chromatin regions characterized by the repressive histone mark H3K9me3 (Cheng et al., 2014b, Iyengar et al., 2011, Iyengar and Farnham, 2011). In this study, I investigated the temporal and spatial expression of KAP1 in the postnatal and adult mouse brain. I found a broad distribution of the nuclear localized KAP1 in different brain regions and different SVZ neural lineage cell subtypes that were examined. This is not surprising giving the fundamental roles that KAP1 plays in regulating transcription and development.

In order to analyse stage-specific expression patterns of KAP1, I analysed KAP1 expression in the adult and postnatal brain. In the adult brain, I show similar expression patterns of KAP1 in almost all brain regions and neural lineage cell-types using immunohistochemistry (IHC). My results were mostly consistent with other studies. The “Human Protein Atlas” revealed the expression profile of KAP1 in 44 different adult human tissues at both protein and mRNA levels. The protein expression data using IHC showed a very high expression of KAP1 in all tissues examined including different

regions of the brain (Uhlen et al., 2015). Also it revealed that KAP1 protein localizes in the nucleus and associates with specific chromatin regions in 64 different cell lines observed by immunofluorescence confocal microscopy (Thul et al., 2017).

In the postnatal brain (age p4-p5), I analysed KAP1 mRNA levels by qPCR which confirmed its expression in the SVZ, neurospheres, cerebral cortex, and OB. In the embryonic brain, immunohistochemistry of human embryonic brain tissue and cultured human neural progenitor cells showed high expression of KAP1 (Brattas et al., 2017). This study only looked at 8-week-old human embryos, however, RNA-seq reads from different stages of human embryonic tissues revealed that most KRAB-ZFPs, which are bound by KAP1, were expressed during brain development (Brattas et al., 2017) which could also confirm the dynamic expression of KAP1 during brain development. Moreover, Cammas et al. generated a TRIM28 (KAP1) null mouse which developed normally only to the blastocyst stage then failed to gastrulate which indicates its expression and importance at early post-implantation development stage (Cammas et al., 2000).Thus, I can conclude from the results I generated and the published data available the broad expression of KAP1 in different brain regions during brain development. However, some observations need to be further investigated:

1) Despite the broad distribution of KAP1, I also observed weak expression of KAP1 in the corpus callosum. Many studies done on KAP1 indicate its function in stem cell proliferation and differentiation in addition to the many roles it plays in transcription regulation. The corpus callosum is the largest white matter fibre bundle structure in the brain and consists mainly of millions of myelinated axonal projections, astrocytes, and oligodendrocytes. It is considered as the major pathway connecting the brain

hemispheres (Fitsiori et al., 2011). My observation suggests a low expression of KAP1 in glial cells compared to neurogenic cells.

2) By using a BrdU-LRC method, I have observed that KAP1 is also expressed in quiescent stem cells which has not been observed before. Published studies on KAP1 have only shown its enrichment in proliferative embryonic stem cells and its role in their regulation (Cheng et al., 2014a). Also, its expression was confirmed in human and mouse neural progenitor cell culture which are active (Brattas et al., 2017, Fasching et al., 2015). In contrast, in the postnatal and adult SVZ, neural stem cells are relatively quiescent with low proliferation rate to ensure maintenance of the stem cell pool (Kippin et al., 2005, Ponti et al., 2013, Kriegstein and Alvarez-Buylla, 2009, Ihrie and Alvarez-Buylla, 2011). This suggest that KAP1 may play a role in maintaining neural stem cells in a poised state by regulating the transcription of genes. It is important to note that even though many studies have used a BrdU label retaining technique and cell markers to differentiate between quiescent and active neural stem cells, their accuracy is arguable. BrdU is a synthetic nucleoside that is an analogue for thymidine, it incorporates into the DNA of proliferating cells in the S phase and substitutes some of the thymidine molecules. In subsequent cell divisions only slow cycling cells retain BrdU while rapidly dividing cells or transit amplifying cells dilute it out after multiple cell divisions (Zhang et al., 2015b). However, since DNA synthesis can also occur independently from mitosis i.e. during DNA repair, making BrdU a marker of DNA synthesis and not of all cell divisions as assumed (Breunig et al., 2007). In addition, recent studies had isolated quiescent and active NSCs using different combinations of cell markers, GFAP+CD133+Nestin – for quiescent neural stem cells and GFAP+CD133+EGFR+ for activated neural stem cells, and they both differ dramatically in their in vivo cell cycle, lineage kinetics, molecular signature and in vitro

colony-forming abilities (Codega et al., 2014). And so more work should be done to confirm its expression in a specific neural stem cell state.

3) More techniques could be applied to confirm the specific expression of KAP1 in SVZ lineage cell sub-types. One group isolated NSCs and immature neurons by lineage tracing methods using transgenic mice expressing a GFP reporter under the control of the Sox2 and Dcx promoters, respectively. They FACS sorted these cells to identify their expression profile which allowed identification of novel and selective regulators (Bracko et al., 2012). Other groups used a combination of cell surface markers to isolate NSCs by flowcytometry and analysed the transcription of these cells by the single-cell RNA-seq method (Llorens-Bobadilla et al., 2015, Zywitza et al., 2018). If we combine lineage tracing techniques with single-cell transcriptomics that would make a very powerful tool to identify cell states (Kester and van Oudenaarden, 2018). Moreover, immunohistochemistry does not allow us to identify the genomic binding sites of KAP1 on the chromatin, and so in order to examine the binding and localization of KAP1 at a genetic resolution we could use the Chromatin-immunoprecipitation (ChIP) technique (Brattas et al., 2017, Pavlaki et al., 2018). In addition, considering the heterogeneity of the SVZ, the development of single-cell ChIP-seq allow us to identify the binding on an individual cell level (Rotem et al., 2015). Another technique is proximity ligation assay (PLA) which allows examination of the subcellular localization of proteins at single molecule resolution. It depends on the dual binding of detection probes to generate amplified signals which serve as a marker to detect the protein of interest (Soderberg et al., 2006). These techniques would allow us to achieve the specificity and sensitivity required for the detection of endogenous proteins in a subcellular compartment.

4) Mash1, a bHLH transcription factor, is dynamically expressed in intermediate progenitor cells in the SVZ and considered as a well-established marker for type C cells. It is known to promote cell cycle exit and neuronal differentiation (Castro et al., 2011, Kim et al., 2011). In my expression experiment, I did not identify if KAP1 is expressed in Mash1 transit progenitor cells, due to the same antibody host species. Instead, I used Dlx2 as a marker for transit progenitor cells. A recent study has confirmed DLX2 as a pro-neurogenic factor in the adult SVZ and its expression was specific to early neuronal commitment prior to differentiation which makes it a good marker for neuronal progenitors (Gothie et al., 2017).

3.3.2 Inducible conditional knockout mice model for KAP1

Whole-body knockout (KO) mouse, also referred to as constitutive knockouts, are a mouse model in which the gene of interest in permanently inactivated in every cell of the animal at all stages of development. KAP1 null mutants mice develop normally to the blastocyst stage but fail to gastrulate, indicating that KAP1 is required for early post-implantation mouse development (Cammas et al., 2000). The development of the conditional knockout method, in which the gene of interest can be inactivated in a specific cell type in a certain tissue in a temporally controlled manner, can bypass the problem observed with the constitutive knockout. Several groups have used the Cre-loxP system to conditionally knockout KAP1 in the brain (Jakobsson et al., 2008, Brattas et al., 2017). In addition, previous studies used the Nestin-Cre line to induce recombination in adult SVZ NSC based on the fact that Nestin is an intermediate filament protein that is specifically expressed in neural stem cells and their progeny (Lagace et al., 2007, Sun et al., 2014, Chen et al., 2009b, Faiz et al., 2015). By using this line, one question could be raised: whether the knockout affects the behaviour

of adult SVZ NSCs? In order to answer this question, I crossed Nestin-CreERT2-YFP line with KAP1$^{fl/fl}$ mice to knockout KAP1 in SVZ NSCs in a spatiotemporal controlled manner.

3.3.3 KAP1 is required for SVZ-OB neurogenesis

In the postnatal/adult SVZ, cells are born and migrate along the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into granule and periglomerular cells (Alvarez-Buylla and Garcia-Verdugo, 2002, Lledo et al., 2006, Whitman and Greer, 2009). To study the functional role of KAP1 in SVZ-OB neurogenesis in mice, I used two in vivo methods: 1) knockdown of KAP1 by electroporation in postnatal mice and 2) KAP1 conditional knockout in adult mice.

To knockdown KAP1 in NSCs in the SVZ, I used an RNAi approach of electroporating shRNA constructs expressing GFP into the SVZ of P1 pups. At this age, most of the stem cells in the SVZ have radial glial morphology and are the cells that are electroporated. They will express GFP upon electroporating the constructs to allow us to trace the differentiation and migration of cells to the OB. I observed that in both knockdown constructs, the number of GFP+ cells in the RMS at 3dpe and in the OB at 7dpe decreased significantly which suggest reduced neurogenesis upon KAP1 depletion. This method is efficient in selectively labeling progenitors in the SVZ. It is time and cost effective, and widely used for functional studies in postnatal mice with good success rates (Chesler et al., 2008, Boutin et al., 2008) and to a lesser extent in adult mice (Barnabe-Heider et al., 2008). However, several arguments must be considered when explaining the data in this experiment:

1- In vivo knockdown of KAP1 must be confirmed to make an accurate assessment of the results. This can be done by Fluorescence Activated Cell Sorting method (FACS) of GFP+ cells and analysis of mRNA levels of KAP1 by qPCR. I did, however, confirm the efficiency of the knockdown constructs in vitro in neurospheres dissected from postnatal SVZ. Moreover, the difference in the number of GFP+ cells arriving to the OB between the KD conditions compared the control confirms that the effect was dependent upon KAP1 depletion.

2-The OB data were obtained 7 days post electroporation, and the neurons examined might be the ones that generated and migrated to the OB before KAP1 knockdown took an effect. However, this is unlikely to be the case. I have checked the KD effect at different time points in neurosphere culture and found that 24 hrs and 3 days showed the strongest KD effect. I then calculated the number of days that require the neuroblast to generate and migrate to the OB. Ponti et al. studied the cell cycle dynamics of B1 stem cells, intermediate progenitors (C cells) and neuroblasts (A cells) in the SVZ by using thymidine analogue methods. Their results indicate that cell cycle length of B1 cells is: ~17 hrs, C cells: ~18-25 hrs, and A cells: ~18 hrs and they actively divide while migrating (Ponti et al., 2013). Neuroblasts migrate a distance of ~5 mm from the SVZ to the OB at a speed of 70-80 μm /hour (Whitman and Greer, 2009, Sun et al., 2010, Nam et al., 2007). That means that neuroblasts in postnatal brain require ~71 hours to reach the OB. Since the electroporation was done on P1, I assumed the KD effect started from age p2 so it would take neuroblast ~53 hours to generate and ~71 hours to migrate and reach the OB which makes it in total ~ 123 hours (~5 days) or postnatal day 7. So, I can conclude that the neurons analysed on p7 are likely the ones generated from stem cells where KAP1 was depleted.

3-The quantification method I used by counting the absolute number of GFP+ cells in the OB might not be accurate, and instead quantification of the percentage of GFP+ in the OB per total GFP cells should have been done. However, I have chosen this method that has also been used in previous studies (Hartman et al., 2013, Sun et al., 2018), because the electroporation efficiency between the control and KD conditions was similar and GFP signals in the SVZ were comparable. Therefore, it is sufficient to conclude that KAP1 is required for neurogenesis by only quantifying the absolute number of GFP cells in the OB.

4-Instead of knocking down KAP1, I could have conditionally knock it out in postnatal SVZ by electroporating Cre-GFP in $KAP1^{fl/fl}$ postnatal mice to induce recombination which might cause a stronger functional effect of KAP1 (Matsuda and Cepko, 2007, Franco et al., 2011, Hartman et al., 2013). However, I had chosen not to consider this method. Cre induced recombination and degradation of KAP1 takes few weeks while the GFP signal will be invisible at 3-4 weeks post electroporation in postnatal brain (Lacar et al., 2010) which makes this method not practical due to this temporal matter.

5- While studying KAP1 function in OB neurogenesis, I observed a morphological difference in the neuroblasts/neurons arriving the OB in the knockdown groups compared to the control. It might be that the KAP1 effect on OB terminal differentiation and maturation of neurons works in a way similar to loss of NeuroD1 (Boutin et al., 2008, Boutin et al., 2010, Pataskar et al., 2016). Neuroblasts travel along distance via the rostral migratory stream to the OB where they migrate to the granule cell layer (GCL) and glomerular layer (GL) and terminally differentiate into GABA and dopaminergic neurons (Alvarez-Buylla and Garcia-Verdugo, 2002). Little is known

about the factors that induce terminal differentiation. Boutin et al. isolated neuronal precursors and differentiated interneurons from the periglomerular layer of the OB and compared their gene expression profile by microarray. They found that NeuroD1 transcription factor expression coincides with the maturation of neuronal precursor to neurons. Its expression induces neuronal differentiation and its knockdown specifically inhibits terminal maturation of periglomerular neurons (Boutin et al., 2008, Boutin et al., 2010). In chapter 5, I investigated if KAP1 regulates the expression of NeuroD1 which could explain the observed phenotype.

Next, I investigated OB neurogenesis in adult Nestin-CreERT2-YFP; KAP1$^{fl/fl}$ mice given TMX injections between P56 -p63 and scarified 28 days after the first injection. My results show that KAP1 cKO had reduced number of neurons in the OB compared to controls, although non-significant, which is consistent with the postnatal KAP1 KD results. However, one could argue the reason for using Nestin-CreERT2 line instead of GLAST-CreERT2 which also known to label NSCs in the SVZ. As discussed above, nestin is an intermediate filament protein and its expression is a hallmark of neural stem cells. Quiescent NSCs express very low level of nestin, in contrast to activated NSCs in which nestin expression is high (Codega et al., 2014). Nestin expression result in neuronal but not astrocytic progeny and so the advantage of using this line is that we can exclusively label, track and phenotype neural stem cells and their progeny in postnatal/adult SVZ and their long-term contribution to OB neurogenesis (Lagace et al., 2007, Chen et al., 2009b), while in GLAST-CreERT2, the recombination could occur in astrocytes within and outside the SVZ.

In conclusion, the two complementary techniques, shRNA knockdown and conditional knockout, revealed that KAP1 play a role in postnatal/adult OB neurogenesis. Many

questions were raised in order to understand this phenotype. Is it a defect in the migration of neuroblast from the SVZ through the RMS the OB? Or increase in apoptosis caused by KAP1 loss? Or is it a defect in NSC proliferation and differentiation in the SVZ that lead to less neuroblasts arriving to the OB? I will next discuss these possibilities.

3.3.4 KAP1 in regulating neuroblast migration

Cell migration is required for brain development, and defects in migration are known to contribute to neurological diseases like epilepsy and mental retardation (Ayala et al., 2007). Loss of KAP1 caused reduction in the number of neurons arriving to the OB which could partially be a consequence of a defect in neuroblast migration from the SVZ through the RMS to the OB where they differentiate into mature neurons. Studies have shown that KAP1 plays a role in regulating lung and breast cancer metastasis, migration and invasion (Wei et al., 2016, Chen et al., 2014). However, the function of KAP1 in regulating neuroblast migration during brain development has not been explored before. Notch signalling mediates hypoxia-induced tumour cell migration (Sahlgren et al., 2008). And silencing Reelin, which is essential for neocortex lamination, caused a loss of Notch signalling in migratory neurons resulting in migration and morphological defects (Hashimoto-Torii et al., 2008). In this project, I show that KAP1 regulates some members of the Notch signalling in the SVZ (Chapter 5) which suggests that migration could be affected by KAP1 loss. To study this hypothesis, 3D spheroids migration assay was developed in the Szele lab and performed on neuroblasts dissected from the RMS. Transfection to KD KAP1 was performed and no difference in migration was observed between control and KAP1 KD conditions.

This assay is an evolution of the hanging drop assay and it has been designed to be easy to follow and cost effective. A critical feature of it is that it recapitulates the neuroblast chain migration that occurs in vivo, in which the neuroblasts radiating out of the spheroid are able to be self-arranged into chains. Nevertheless, it is important to keep in mind that in vivo cell migration is regulated by a complex cellular architecture and signalling molecule that are impossible to perfectly replicate in vitro which could explain the negative results I got after KD KAP1. An alternative approach would be the use of two-photon time-lapse microscopy which enables imaging of in vivo RMS slices at a fine spatiotemporal scale (Comte et al., 2011, James et al., 2011).

3.3.5 KAP1 in regulating SVZ NSC maintenance

When I knocked down KAP1 in postnatal SVZ NSCs, I found fewer GFAP+ and Mash1+ cells which suggests that the SVZ NSC pool is being affected. Two biological mechanisms are known to be important in maintaining the stem cell pool:1) cell identity (NSCs vs niche astrocytes) and 2) cell cycle (Ihrie and Alvarez-Buylla, 2011). These two mechanisms are regulated by many signalling pathways like A) cyclin/cyclin dependent kinase complexes and their inhibitors, B) intracellular signalling e.g. Notch and BMP signalling, C) post-transcription regulation by TFs and microRNAs. They control NSCs proliferation, differentiation, senescence or entry into quiescence (Salomoni and Calegari, 2010). Interestingly, it seems that KAP1 is involved in some of these processes.

A) For example, the cell cycle inhibitor p21 has been shown to maintain hematopoietic stem cell quiescence and self-renewal. Deletion of p21 in NSCs promotes expansion of stem cells and reduce their quiescence which ultimately leads to their exhaustion, which results in the long term in fewer NSCs that can continue to generate new neurons

(Kippin et al., 2005). P21 can directly inhibit the expression of Sox2, a regulator of NSC maintenance, and so regulate NSC self-renewal and multipotency (Marques-Torrejon et al., 2013). In experiments done on cultured glioma cells, down-regulation of KAP1 increased p21 expression and induced cell cycle arrest at the G1 phase (Qi et al., 2016b).

B) Regulation of gene expression by transcription factors is one of the essential mechanisms that regulate NSCs. For example, I have investigated the expression of Mash1, a transcription factor expressed in transit amplifying progenitor cells and that activates NSCs by targeting cell cycle regulators (Hsieh, 2012). Mash1 was reduced after KAP1 depletion, so it would be interesting to know if KAP1 directly regulates Mash1 on a molecular level in NSCs and/or progenitors. Moreover, the transcription factor Pax6 is important for the differentiation and specification of progenitor cells (Salomoni and Calegari, 2010). Interestingly, in Pavlaki et al., we showed that Pax6 and KAP1 exist in the same multicomponent regulatory complex in N2A cell lines and together they regulate common sets of target genes important for neural proliferation and differentiation (Pavlaki et al., 2018).

C) Cell identity between NSCs and niche astrocytes is a question that remains mysterious (Ihrie and Alvarez-Buylla, 2011). Notch signalling is required for the maintenance of postnatal NSCs (Gaiano et al., 2000). Notch signalling components are expressed in adult NSCs, and mutations in genes encoding Dll1 (Notch ligand), Notch1 (Notch receptor), RBPJk (Notch transcriptional effector), Hes1, Hes3 or Hes5 (RBPJk-induced transcription factors) lead to the depletion of in vivo NSCs (Doe, 2008, Chambers et al., 2001), and reduced neurosphere forming ability in vitro (Chambers et al., 2001). Other studies showed that neural stem cells that were infected with activated

Notch1 (NIC) became periventricular (niche) astrocytes which suggested that Notch signalling promotes a glial identity (Gaiano et al., 2000). In addition, BMP signalling in the SVZ inhibits the differentiation of neurons and oligodendrocytes and promotes astroglial lineage commitment (Bond et al., 2012). In the adult SVZ of the KAP1 cKO model, loss of NSCs stemness and shifting to niche astrocyte was observed, i.e. increased S100β in GFAP+ cells in the SVZ. Thus, it would be interesting to investigate if KAP1 is regulating NSC identity through any of these signalling pathways.

D) Finally, one can argue that reduction in NSCs pool could be attributed to increased cell death caused by KAP1 knockdown. In previous studies, knocking down KAP1 has been associated with induced apoptosis in several cancer cell line (Zhang et al., 2018, Lionnard et al., 2018, Liu et al., 2018). To examine this hypothesis, TUNEL assay was conducted in control and KAP1 KD conditions and I found no evidence of altered cell death in the SVZ, RMS or OB. Therefore, increased cell death cannot account for the reduction of NSCs number observed after knocking down KAP1.

Taken together, the association of these cellular pathways with NSC maintenance and lineage progression raises an interesting question - does KAP1 function in the SVZ by regulating them? I address this in Chapter 5.

3.3.6 Neurosphere assay to analyse NSC behaviour in vitro

The neurosphere-forming assay has been widely used to identify NSCs based on their ability to self-renew and differentiate in vitro (Codega et al., 2014, Pastrana et al., 2011). In this chapter, I used this method to examine the neurosphere-forming ability of NSCs isolated from the SVZ of control and KAP1 cKO mice. The final readout of the assay is the size and number of spheres, whether primary or passaged. My results show

that NSCs isolated from cKO mice generated a smaller number of neurospheres compared to the controls which supports my in vivo observation of reduced NSCs pool after KAP1 knockdown/knockout. Nonetheless, several points must be considered when explaining the data generated from this experiment:

1-**KAP1 knockout model in vitro:** Tamoxifen inducible knockout approach does not guarantee that all my NSCs are completely knocked-out which results in heterogeneity of my culture. Due to this reason, I did an assessment of YFP fluorescence signal in my in vitro culture to estimate the knockout efficiency. I found that in the WT group around 80% of the primary neurospheres generated are YFP+, while in the cKO group around 20-30% were YFP+. Therefore, I can estimate that the neurospheres generated in the knockout condition might be the ones originated from a non-knockout cell dissected from KAP1 cKO SVZ while the cells with homozygous KAP1 knockout have lost (or reduced) neurospheres generation ability which still supports my conclusion.

2-**Clonality and cell density:** Cell density is an important parameter of the neurosphere assay as it has an impact on clonality. Each sphere is derived from a single cell and therefore considered clonal (Pastrana et al., 2011). I used a high cell density ($1x10^5$/ml) which provided a general assessment of the neurospheres generation abilities of the cultured NSCs in both conditions. However, high density culture causes fusion of the neurospheres (Pastrana et al., 2011, Singec et al., 2006). Also, studies suggest that neurospheres can be of a clonal origin only when plated at as low as 0.2 to 20 cells/ul (Ferron et al., 2007, Pastrana et al., 2011). Therefore, I plan in the future to culture my cells at 10 cells/ul to confirm my observation. However, using imaging approaches, Singec at al. argued that neurospheres would still aggregate and fuse even at low densities (Singec et al., 2006). To circumvent this issue, spheres could be cultured in

semi-adherent conditions (i.e. engineered hydrogel matrices) to prevent spheres aggregation and mobility (Cordey et al., 2008).

3-**Neurosphere size:** In normal conditions, heterogeneity exists between neurosphere size which is difficult to interpret. It has been proposed that the size (which ranges from 40-150 μm in diameter) depends on the nature of the clone. Stem cells give rise to large spheres which can be serially passaged while progenitor cells give rise to smaller ones with limited proliferation potential (Pastrana et al., 2011). However, this hypothesis has not been proven yet. In my experiment, I did not observe a change in the neurosphere size between control and the cKO condition. This could be due to the fact that the neurospheres in the cKO condition are the ones generated from the wild type cells rather that the knockout ones as I discussed above.

4-**Neurosphere assay is not an accurate read of the in vivo system:** To evaluate the stem cell self-renewal and proliferation potential of my cells in vitro, I serially passaged the primary neurospheres from control or KAP1 cKO SVZ. FACS purification of neural stem cells and their progeny showed that both neural stem cells and transit amplifying progenitors can generate neurospheres (Pastrana et al., 2011, Louis et al., 2008). However, the neurosphere assay does not reflect the in vivo activity of NSCs but rather it assesses the potential of cells to exhibit self-renewal trait. NSCs and transit amplifying progenitors, which are short-lived cells in vivo, can be serially passaged for a long-term in vitro. This capacity might not be the same when cells are in their in vivo niche. More experiments such as in vivo lineage tracing is important to complement the in vitro assay to identify the differences in stem cell potential.

5-**Differentiation analysis in neurosphere culture**: One question I did not answer is whether KAP1 loss will influence neurospheres differentiation. Future experiments

should involve an overall analysis of neuronal/astrocytic/oligodendrocytic differentiation of NSC on a single cell level after KAP1 knockout.

3.4 Conclusion

In this chapter, I have demonstrated a broad expression of KAP1, a major transcription regulator, in normal postnatal/adult SVZ neurogenic lineage. I then uncovered for the first time that KAP1 has an effect on postnatal and adult SVZ-OB neurogenesis. KAP1 is important for SVZ NSC identity maintenance, and functions to protect the NSC from mature astrocytic fate in the SVZ.

Chapter 4

The long non-coding RNA *Paupar* regulates postnatal SVZ neurogenesis

4.1	**Introduction**	**132**
4.2	**Results**	**144**
4.3	**Discussion**	**159**
4.4	**Conclusion**	**170**

4.1 Introduction

Many long non-coding RNAs (lncRNAs) are expressed in the brain, and emerging data revealed that lncRNAs can have important biological function during central nervous system (CNS) development. However, lncRNA in vivo roles and mechanisms of action remain elusive. In this chapter, I use the SVZ-OB system to characterize the role of the lncRNA *Paupar* in the postnatal mouse SVZ and provide insights into lncRNA function, NSC biology and brain development.

4.1.1 LncRNA expression in the brain

Genome-wide expression profiling of lncRNAs in the brain has been carried out using different methods. The Allen Brain Atlas (ABA) is a large study that maps the expression of genes of the developing and adult mouse brain at a genome-wide scale. The ABA used RNA in situ hybridization (ISH) to visualize the expression of 849 ncRNAs that are expressed in the adult brain, such as *Gomafu* and *Sox2ot*. Careful evaluation of the ABA reveals many dozen lncRNAs which are expressed in the SVZ and RMS. Interestingly, most of the lncRNAs that were identified are involved in the regulation of nearby protein-coding genes (Mercer et al., 2008, Lein et al., 2007). For example, the ABA shows expression of the lncRNA *Evf2* which interacts in trans with the transcription factor Dlx2 to regulate the expression of Dlx6 gene (Feng et al., 2006). Consistent with this function, *Evf2* shows a coincident expression profile with Dlx2, which supports its role in neuronal differentiation (Feng et al., 2006). However, this method is limited to known lncRNAs with available probes and excludes thousands of lncRNAs expressed in the adult brain. Furthermore, it was applied over large brain regions and ignored the complexity of the brain yielding data that does not resolve the cellular diversity within these regions and does not provide a quantitative expression of target genes.

This problem was circumvented with the development of high-throughput sequencing methods. To analyse lncRNA expression patterns in the SVZ neurogenic lineage in vivo while taking into consideration the heterogeneity of the SVZ, Ramos et al. isolated the major cell types of the SVZ using fluorescence activated cell sorting (FACS) followed by in-depth RNA-sequencing analysis. This allowed the identification of thousands of novel lncRNAs that are associated with different neural cell types and cellular processes, as well as the resolution of complex lncRNA loci that were not identified by the ISH method. For example, the lncRNA *Six3os* was shown to play a role in both neuronal and oligodendrocyte differentiation, and knocking down Six3os promoted astrocytic lineage specification in the SVZ (Ramos et al., 2013). While this study is comprehensive, false positive signals should be taken into consideration due to the lack of confirmation by in *situ* hybridization.

4.1.2 LncRNAs in CNS development

One of the first identified lncRNAs in the brain is *Evf-2*, which is transcribed across the enhancer element between the Dlx5 and Dlx6 genes that are known to be important for neuronal differentiation and migration (Feng et al., 2006, Bond et al., 2009). *Evf-2* is expressed in immature neurons and can be induced by sonic hedgehog signalling (Feng et al., 2006). Initial work in cell culture using RNAi suggested that *Evf-2* forms a complex with Dlx-2 to increase the transcriptional activity of the Dlx-5/6 enhancer (Feng et al., 2006). However, later work from the same group using the transcriptional terminator insertion method in mice showed the opposite effect on the expression of Dlx-5/6 (Bond et al., 2009) through changes in DNA methylation at the enhancer region. This may be due to the different techniques used for analysis in these studies. Importantly, these changes can be rescued by *Evf-2* expression from a separated

transgene, which confirms that they are dependent on the lncRNA itself and not DNA-based (Berghoff et al., 2013, Bassett et al., 2014). Another CNS lncRNA example is metastasis-associated lung adenocarcinoma transcript 1 (*Malat1*) which is highly expressed in neurons. Knockdown studies in neuroblastoma cells indicates that *Malat1* controls the expression of genes involved in regulating synapse function. In cultured hippocampal neurons, depletion of *Malat1* leads to reduction in synaptic density (Bernard et al., 2010).

Ramos et al. investigated the lncRNA *Pnky*, a neural-specific lncRNA that is expressed in the nucleus of NSCs. *Pnky* knockdown in the SVZ increases neurogenesis. It interacts with the splicing regulator PTBP1, and together they regulate the expression and splicing of a common set of neuronal differentiation transcripts (Ramos et al., 2015). Nonetheless, the loss of function experiment of *Pnky* was based on RNAi which cannot directly regulate the transcription of a nuclear lncRNA, and so a combination of knockout and rescue experiment by lncRNA can provide a better understanding of the regulatory mechanism of *Pnky* in SVZ neurogenesis. In conclusion, despite the high expression of lncRNAs in the brain, our understanding of their biological function is still in the beginning.

4.1.3 Transcriptional regulatory functions of lncRNAs

A number of lncRNAs control transcription of many genes using different mechanisms of actions. They can function in cis to regulate the transcription of neighbouring genes on the same chromosome, close to their site of synthesis, or function in trans to regulate the transcription of target genes across multiple chromosomes (Vance and Ponting, 2014, Vance et al., 2014) (Figure 4.1). Moreover, lncRNAs may function independently or physically bind to and interact with

transcription factors and chromatin modifiers. Some lncRNAs facilitate the formation of a ribonucleoprotein complex while others function to recruit chromatin-modifying complexes to distal genes through RNA-RNA associations at transcribed loci, directly through RNA-DNA interactions or indirectly through RNA-protein-DNA interactions (Vance and Ponting, 2014, Rutenberg-Schoenberg et al., 2016, Pavlaki et al., 2018) (Figure 4.2). *Haunt*, is an example of a cis-acting lncRNA, which has shown that lncRNA transcripts can accumulate at its site of synthesis and recruits regulatory complexes (Yin et al., 2015). Another trans example is *HOTAIR*, a lncRNA transcribed from the HoxC locus that regulates the activity of HoxD genes in trans while interacting with the chromatin at more than 800 regions (Rinn et al., 2007).

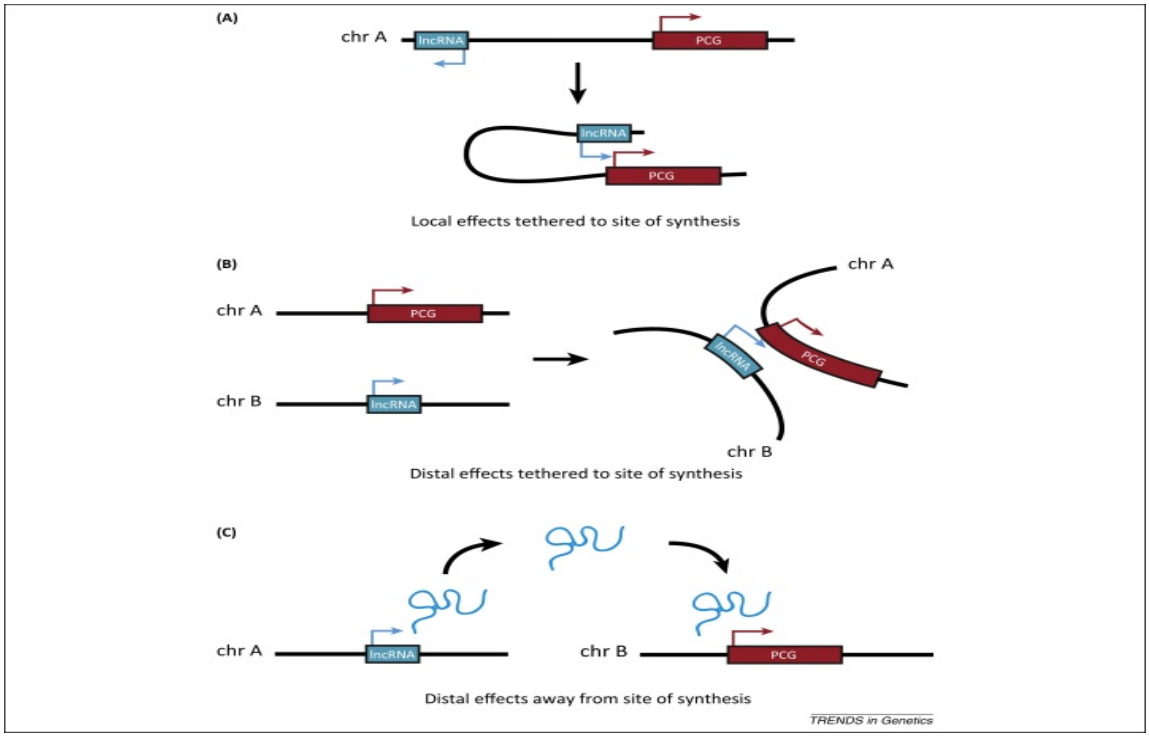


Figure 4.1 Cis and Trans mode of actions of lncRNAs

A) DNA looping brings a lncRNA locus close to an adjacent protein coding gene (PCG) on the same chromosome to regulate its activity. Such lncRNAs act close to their site of synthesis to regulate target genes.
B) Chromatin conformation changes to bring two distant locations into close proximity. LncRNAs function close to their site of synthesis but their target is located on a different chromosome.
C) LncRNAs translocate to different locations and regulate the transcription of target genes on the same or different chromosomes. Figure adapted from (Vance and Ponting, 2014)

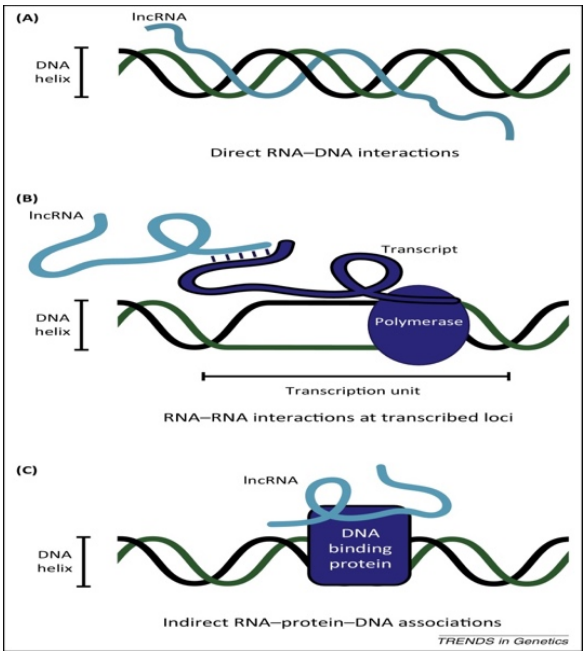


Figure 4.2 Mechanisms of lncRNA-chromatin association

A) Single stranded lncRNAs interact with a double stranded DNA target sequence to form RNA-DNA-DNA structure.
B) lncRNAs base pair with RNA sequence at transcribed loci.
C) Indirect recruitment of lncRNAs to target genes through RNA-protein-DNA associations. LncRNAs may associate with DNA binding transcription factors, non-DNA binding transcriptional cofactors and histone proteins. Figure adapted from (Vance and Ponting, 2014)

4.1.4 The lncRNA Paupar

LncRNAs that are transcribed in the developing mouse central nervous system (CNS) are usually located in close proximity to a transcription factor gene and regulates its transcription (Ponjavic et al., 2009). One of these lncRNAs, which is termed *Paupar* (Pax6 Upstream Antisense RNA), is a single exon, intergenic, vertebrate-conserved, CNS expressed, unspliced and chromatin-associated lncRNA that is located entirely within the *Pax6os1* locus. *Paupar* is transcribed 8.5 kb upstream of the gene encoding the Pax6 transcription factor (Vance et al., 2014), and due to this close proximity, Vance et al. investigated if *Paupar* either controls the expression of Pax6 or regulates

similar biological processes as Pax6. They used shRNA mediated knockdown, microarray profiling, genome-wide mapping and reporter assays to study *Paupar's* mode of action in the regulation of gene transcription in N2A neuroblastoma cell line.

The *Paupar* locus is highly conserved during evolution and expressed in the adult brain (Figure 4.3). While its expression is undetectable in self-renewing ES cells, it is rapidly upregulated after retinoic acid (RA)-induced differentiation of mouse E14 stem cells (Vance et al., 2014) (Figure 4.4). This expression profile is similar to Pax6, which is known to be important for SVZ neural differentiation (Ninkovic et al., 2013, Hack et al., 2004). *Paupar* knock-down in the N2A cell line resulted in significant changes in the expression of genes that are involved in cell cycle control, regulation of synaptic function, and genes involved in chromatin modification and chromosomal organization (Vance et al., 2014) (Figure 4.5).

The relationship between *Paupar* and Pax6 has been further studied and revealed that *Paupar* transcript can act both in cis, to regulate Pax6 expression, and in trans in a transcript-and Pax6-dependent mode. The data shows that the *Paupar* mechanism of action is complex and is driven by three components: down-stream gene expression changes resulting from in cis regulation of Pax6 by *Paupar*; a Pax6- and *Paupar*-dependent trans-acting mechanism to regulate gene expression through physical association of *Paupar* and PAX6 protein; and a genome wide trans-acting Pax6-indepedent function of *Paupar* (Vance et al., 2014).

Paupar's function in vivo and in the SVZ had not been investigated. The expression profile of *Pax6os1,* however, has been identified in SVZ lineage cells (Ramos et al., 2013). *Pax6os1* expression gradually increases during lineage progression from SVZ NSC to neuroblasts, and in vitro differentiation of SVZ NSC is also associated with

upregulated *Pax6os1* expression (Ramos et al., 2013). Interestingly, in the N2A cell line, only *Paupar* and Pax6 but not Pax6os1 are expressed (Vance et al., 2014).

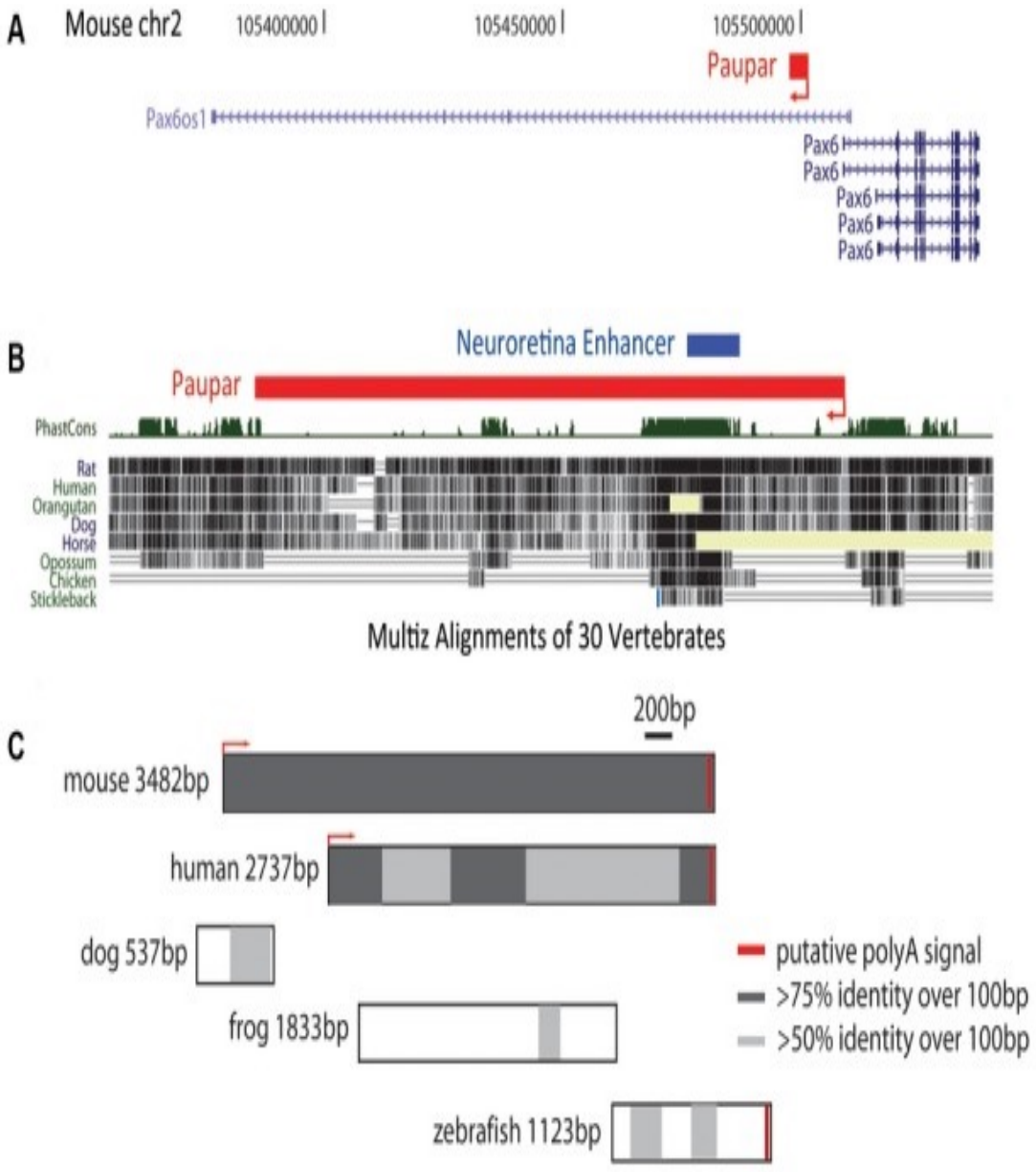


Figure 4.3 Conservation of *Paupar*

A) Illustration of *Paupar* location in *Pax6os1*, upstream of the *Pax6* locus.
B) A view of the mouse *Paupar* locus, indicating its sequence conservation among different species
C) Conservation and relative size of *Paupar* in vertebrates.
(Figure modified from Vance et al. 2014)

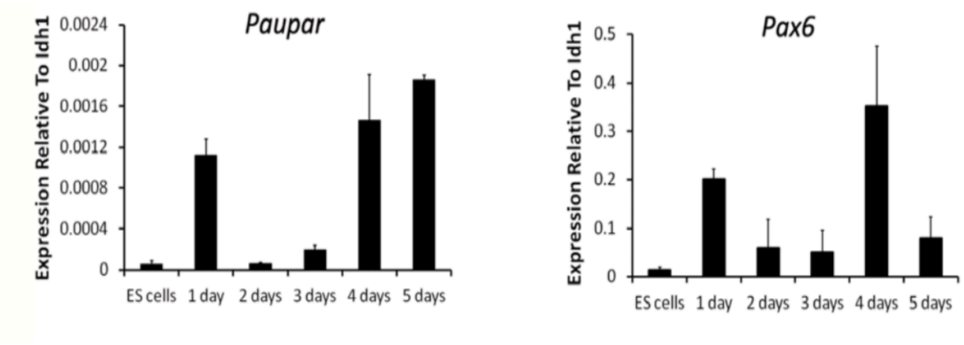


Figure 4.4 Similar to Pax6, *Paupar* is upregulated during neuronal differentiation of mouse ES cells after treatment with RA. (Figure modified from Vance et al. 2014)

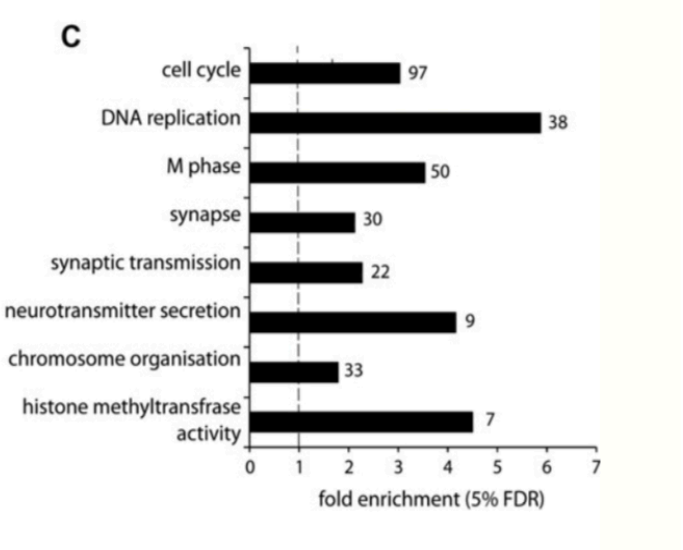


Figure 4.5 Gene Ontology annotation of *Paupar*-regulated genes (Figure modified from Vance et al. 2014)

4.1.5 The transcription factor Pax6

Pax6 is a member of the paired box family of proteins that is responsible for a human eye disease called Aniridia and for the mouse mutant *Small eye*. It is a highly conserved transcription factor that contains two DNA binding domains, a paired domain (PD), and a paired-type homeodomain (HD). Pax6 is important for the development of the CNS, eye, nose, and pancreas. It is expressed spatiotemporally during mammalian brain development and functions upstream of gene networks involved in brain patterning, neuronal circuit formation and neuronal migration. Pax6 homozygous mutant embryos

lack eyes and nose and die after birth. *Small eye* mice carrying Pax6 heterozygous mutations are an extensively used model to study the function of Pax6 (Georgala et al., 2011, Osumi et al., 2008). In the cerebral cortex, Pax6 is highly expressed in radial glia cells and is essential for their differentiation. In embryonic Pax6-deficient mice, cortical radial glia cells had defective morphology as well as altered gene expression pattern and cell cycle characteristic (Gotz et al., 1998).

The SVZ produces different subclasses of OB interneurons throughout life which are thought to become specified depending on which subregions of the lateral ventricle wall they originate from (Weinandy et al., 2011). Proliferating progenitor cells in the adult SVZ express Pax6, however only a small population of the migratory neuroblasts and new interneurons derived from these progenitor cells retain Pax6 expression (Kohwi et al., 2005). To determine the role of Pax6 in OB neurogenesis, Pax6 mutant progenitors from *Small Eye* mice were grafted into wild-type mice. Pax6 mutant progenitors were able to generate migratory neuroblasts but failed to generate OB dopaminergic periglomerular neurons (Kohwi et al., 2005, Hack et al., 2005). These results suggest that Pax6 functions in a cell-autonomous manner and is required for determining dopaminergic neuronal fate in the SVZ-OB. In addition, another study using an inducible knockout model revealed that Pax6 interacts directly with the chromatin remodelling BAF complex in SVZ neural progenitor cells and deletion of either of them causes the NSCs to convert to the ependymal lineage while migratory neuroblast convert to different glial lineages (Ninkovic et al., 2013). These differences in Pax6 function in the SVZ might result from the distinct loss-of-function techniques used, however, it also confirms the important and complicated role of Pax6 in SVZ neurogenesis. Due to the close relationship between Pax6 and *Paupar,* it would be

interesting to investigate whether *Paupar* play a similar function as Pax6 in SVZ neurogenesis.

4.1.6 The interaction between the lncRNA Paupar and KAP1

Despite its major role in epigenetic regulation, the mechanism of KAP1 recruitment to the genome is not fully understood. KAP1 does not have a DNA binding domain and was originally identified to be recruited through its interaction with the KRAB zinc finger (KRAB-ZNF) proteins. However, later studies showed that a mutated KAP1 protein, with disturbed interaction with KRAB-ZFPs, can still bind to target genes (Iyengar and Farnham, 2011), suggesting an alternative mechanism for KAP1 genomic targeting independent from KRAB-ZFPs. Interestingly, our recent published paper in collaboration with Dr. Keith Vance laboratory at University of Bath revealed a novel RNA-protein interaction between KAP1 and the lncRNA *Paupar* (Pavlaki et al., 2018).

As mentioned previously, *Paupar* regulates Pax6 expression in cis and physically interacts with PAX6 protein in trans to control target gene expression (Vance et al., 2014). In subsequent study, it has been shown that *Paupar* also directly interacts with KAP1 in N2A cells and together they regulate the expression of genes important for proliferation and neuronal differentiation. *Paupar* promotes KAP1 chromatin association and the generally repressive H3K9me3 mark deposition through the assembly of a ribonucleoprotein complex containing *Paupar*, KAP1 and Pax6 within the regulatory regions of target genes as illustrated in the model in (Figure 4.6) (Pavlaki et al., 2018). This mechanism is similar to the well-studied lncRNA *HOTAIR*, which was proposed to guide PRC2 to specific locations in the genome and to deposit H3K27me2 mark and silence gene expression (Chu et al., 2011) and the lncRNAs

Prncr1 and *Pcgem1* that interact with the androgen receptor (AR) and associate with non-DNA binding protein to facilitate its gene regulation (Yang et al., 2013).

To further assess the extent to which *Paupar* functions to recruit KAP1 to the genome, *Paupar*-KAP1 genome-wide co-occupancy was mapped and identified many overlaps between *Paupar* and KAP1 binding sites on the chromatin. The majority of these sites are bound by PAX6 and not the 3^{o} UTR of zinc finger proteins (Pavlaki et al., 2018). In addition, *Paupar* genome-wide binding sites are not enriched for sequences that are complementary to *Paupar* itself, rather they are enriched of motifs for neural transcription factors (Vance et al., 2014). This suggest that *Paupar* does not bind to the DNA directly but is targeted to the chromatin through RNA-protein interactions with transcription factors such as PAX6. Taken together, these observations propose that *Paupar* functions as a transcriptional cofactor to promote KAP1-dependent chromatin modulation at target genes in trans through association with a non-KRAB-ZNF transcription factor such as Pax6.

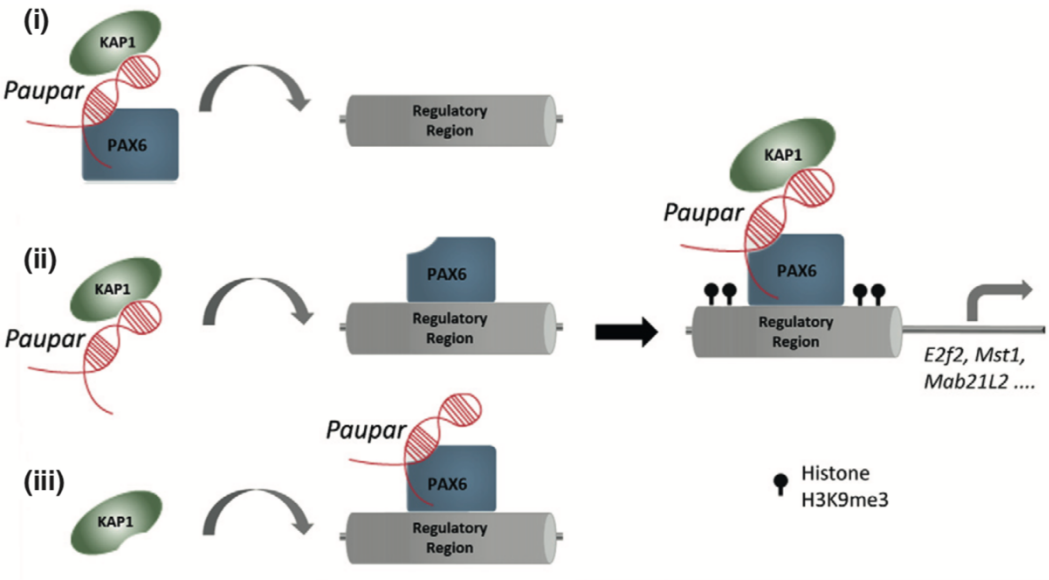


Figure 4.6 *Paupar* promotes KAP1 chromatin binding and H3K9me3 deposition by the assembly of a ribonucleoprotein complex containing *Paupar*, KAP1 and Pax6 within the regulatory regions of target genes

Three potential scenarios to describe the order of assembly of this complex:

(i) The complex forms in the nucleoplasm before binding to the DNA.

(ii) *Paupar* interacts with KAP1 first and then guides it to DNA bound Pax6.

(iii) KAP1 is recruited to a DNA bound Pax6-*Paupar* complex.

This figure is adapted from (Pavlaki et al., 2018).

In this chapter, I am going to identify the expression profile of the lncRNA *Paupar* in the SVZ-OB system. Also, given that it exists in the same ribonucleoprotein complex as Pax6 and KAP1, I will investigate the in vivo functional role of *Paupar* in postnatal SVZ neurogenesis.

4.2 Results

4.2.1 The expression of the lncRNA Paupar in postnatal SVZ NSCs

In the Allen Brain Atlas, there is no probe available for the lncRNA *Paupar*, and therefore I used qPCR on SVZ tissues and SVZ derived neurospheres generated from P4 mouse brain to assess the expression level of *Paupar*. To rule out potential contamination of genomic DNA, minus-reverse transcriptase was used as a control (Minus RT). The Ct values for both SVZ tissues and neurospheres were very small (SVZ tissue: 25.66, Neurospheres: 29.7) (Figure 4.7 A) relative to the internal control GAPDH (Ct:16.8); while in Minus RT control, no signal was detected before cycle 35. This suggests that *Paupar* is expressed in the postnatal SVZ and that the expression level was lower in neurospheres compared to SVZ tissue.

To further examine *Paupar* expression in NSCs, I used FISH, fluorescence in situ hybridization assay, a cytogenic technique used for the detection and localization of RNA sequences within cells. I used FISH to detect *Paupar* expression in neurospheres generated from P4 SVZ mouse brain. I used fluorescent probes, short DNA oligonucleotides (around 20 nucleotides in length), pooled into sets of up to 48 individual probes. The overall binding of multiple oligonucleotides to the same complimentary RNA target results in fluorescent signals that could be visualized by fluorescence microscopy. *Paupar* fluorescence signals were detected to be mostly nuclear localized (colocalize with nuclear DAPI staining) compared to the cytoplasmic localized GAPDH signals, while no signals were detected in the non-probe negative control (Figure 4.7 B & B'). These results confirm that the lncRNA *Paupar* is expressed in SVZ neural stem cells and progenitors.

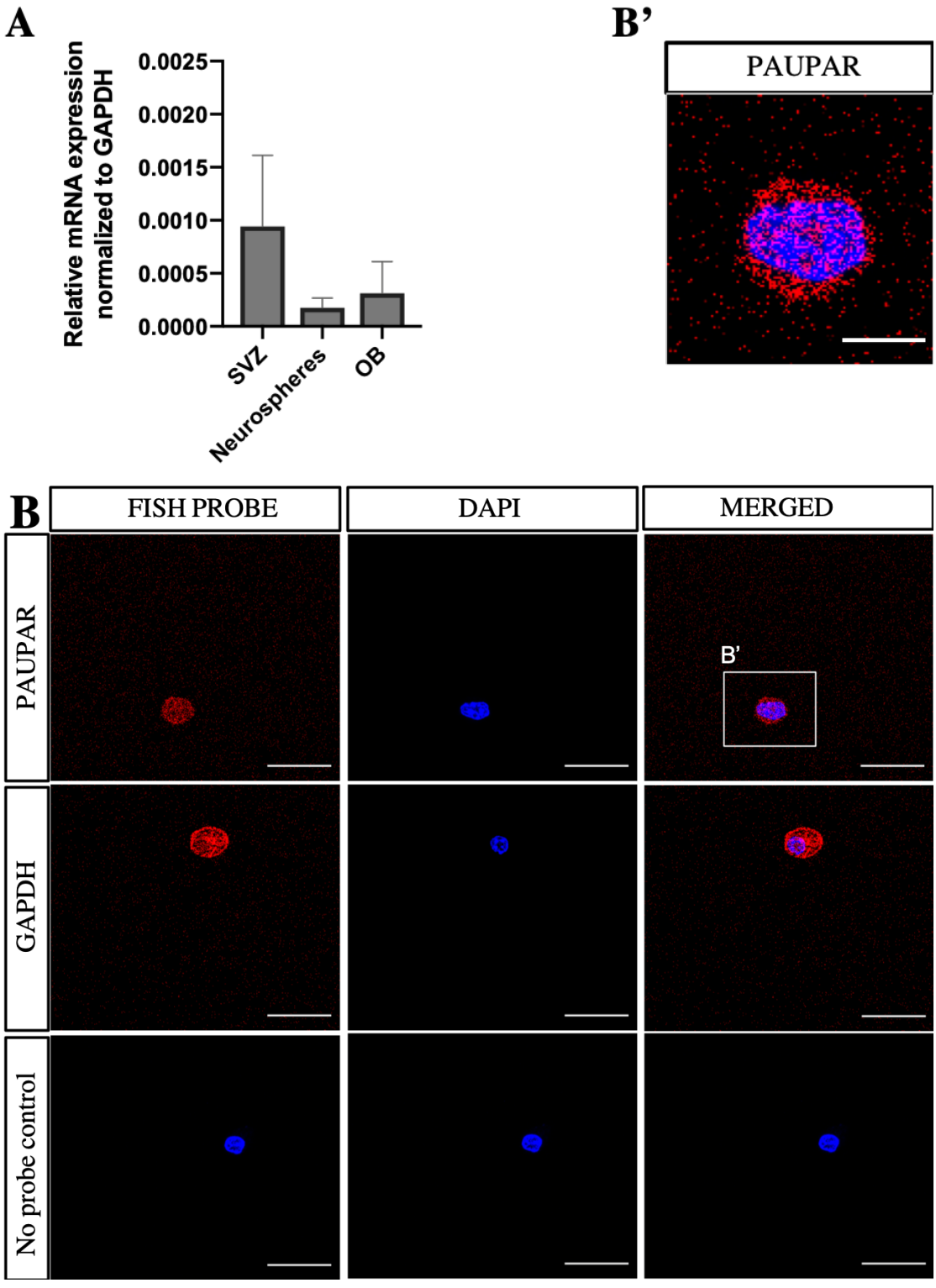


Figure 4.7 Expression of the lncRNA *Paupar* in the SVZ

A)*Paupar* transcript levels were measured in SVZ derived neurospheres, and in SVZ and OB tissues using qRT-PCR. Data was normalized to GAPDH levels. n=3

B) Fluorescence in situ hybridization to detect *Paupar* and GAPDH transcripts level in SVZ derived neurosphere cells. Scale bar=30 µm. n=3. B': Magnified image of *Paupar* colocalized with DAPI for clarity.

4.2.2 Loss of Paupar impairs postnatal OB neurogenesis

Neurogenesis can be assessed by electroporating postnatal neural stem cells in the SVZ and analysing differentiated neurons that have migrated into the OB 7 dpe (Boutin et al., 2008, Chesler et al., 2008).To test whether *Paupar* can regulate OB neurogenesis in vivo, two shRNA knockdown constructs (sh165 and sh408) were used. The knockdown efficiency of shRNA was first tested in P4 SVZ neurosphere culture. Cells were nucleofected with scramble, sh165, or sh408 constructs and collected 3 days post nucleofection for qPCR analysis. Sh165 showed a stronger knockdown effect (~60%) than sh408 (~30%) (as shown in Chapter 5, Figure 5.5). Nucleofection of shRNA expression vectors in vitro targeted ~80% of cells in the culture as assessed by observing GFP signals under the fluorescence microscope. However, I assessed the transcript level in all cells using qPCR. Thus, on a cell-by-cell basis the relative level of *Paupar* knockdown is assumed to be greater than shown.

Similar to the KAP1 experiment, we electroporated individual constructs (scramble, sh165 or sh408) into P1 SVZ and pups were sacrificed 7 dpe to enable newborn neuroblasts to reach the OB. At 7 dpe, the number of GFP+ cells in the OB decreased in the sh165 group by ~ 70% with no significant effect in the sh408 group (scramble: 127,754±6,874/mm^3, sh165: 29,896±4,736/mm^3, sh408: 110,565±5,895/mm^3) (n=3 for scramble, n=5 for sh165 and n=5 for sh408, $p<0.0001$ for scramble versus sh165) (Figure 4.8 B&C). As sh165 depleted *Paupar* expression in vitro more efficiently than sh408, this result is indicative of dose-dependent effects mediated by the *Paupar* transcript.

Co-staining with the immature neuroblast marker DCX revealed that all GFP+ cells in the OB were DCX+ and that the level of DCX expression in cells was not altered by *Paupar* KD (Figure 4.8 D). These results suggest that *Paupar* is required for the production of newborn OB neurons.

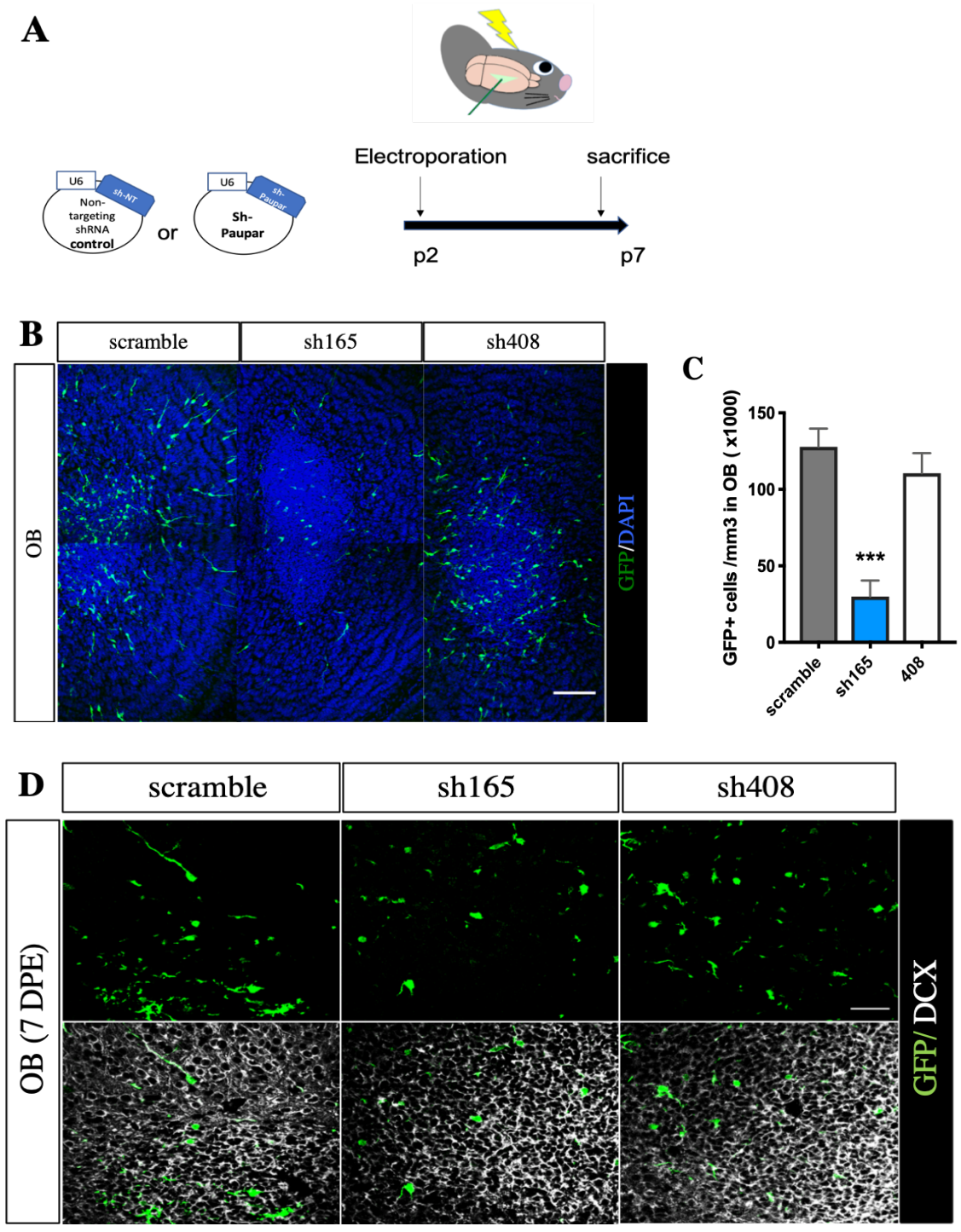


Figure 4.8 Neurogenesis decreases in the OB after *Paupar* knockdown

(A): A schematic diagram of the experimental design.

(B): Immunostaining of GFP+ cells in OB 7 dpe reveals fewer newborn neurons in the OB after *Paupar* knockdown using sh165 and sh408 constructs compared to the scramble control.

(C): Quantification of GFP+ cells in the OB (n=3 for scramble, n=5 for sh165 and n=5 for sh408).

(D): Costaining of DCX+ and GFP+ cells in the OB after *Paupar* knockdown using sh165 and sh408 constructs compared to the scramble control (n=3 for controls and knockdown groups).
Error bars represent SEM.***p<0.001. Scale bar: (B) =100 µm, (D) =30 µm

4.2.3 Paupar loss disrupts morphological differentiation of newborn neurons in the OB

Similar to KAP1, electroporating shRNA construct in the SVZ to knockdown *Paupar* altered the morphology of newborn neurons in the OB 7 dpe. In the scramble controls, many GFP+ neurons arrived in the OB granule layer and begun morphological differentiation with branched processes extending radially towards the pial surface, these are classified as class I (Boutin et al., 2010). By contrast, after *Paupar* KD, abnormal morphologies were observed, which we classified as class II and class III (as explained in Chapter 3). The percentage of electroporated cells with class I morphology was 34±2% in scramble controls but only 8±3% after *Paupar* KD with sh165 and 6±3% in the sh408 group (P= 0.0005 and P=0.0009, respectively; Figure 4.9 A&B). Conversely, after *Paupar* KD there were significantly more class III neurons in the sh165 group (87±4%) as well as in the sh408 group (85±6%) compared to controls (58±5%; P=0.003 and P=0.02, respectively) (Figure 4.9 C). It may be that *Paupar* loss affected the rate of OB interneuron differentiation in a manner similar to the transcription factor NeuroD1 (Boutin et al., 2010, Pataskar et al., 2016). These data suggested that *Paupar* affected postnatal neurogenesis by disrupting morphological differentiation of newborn neurons.

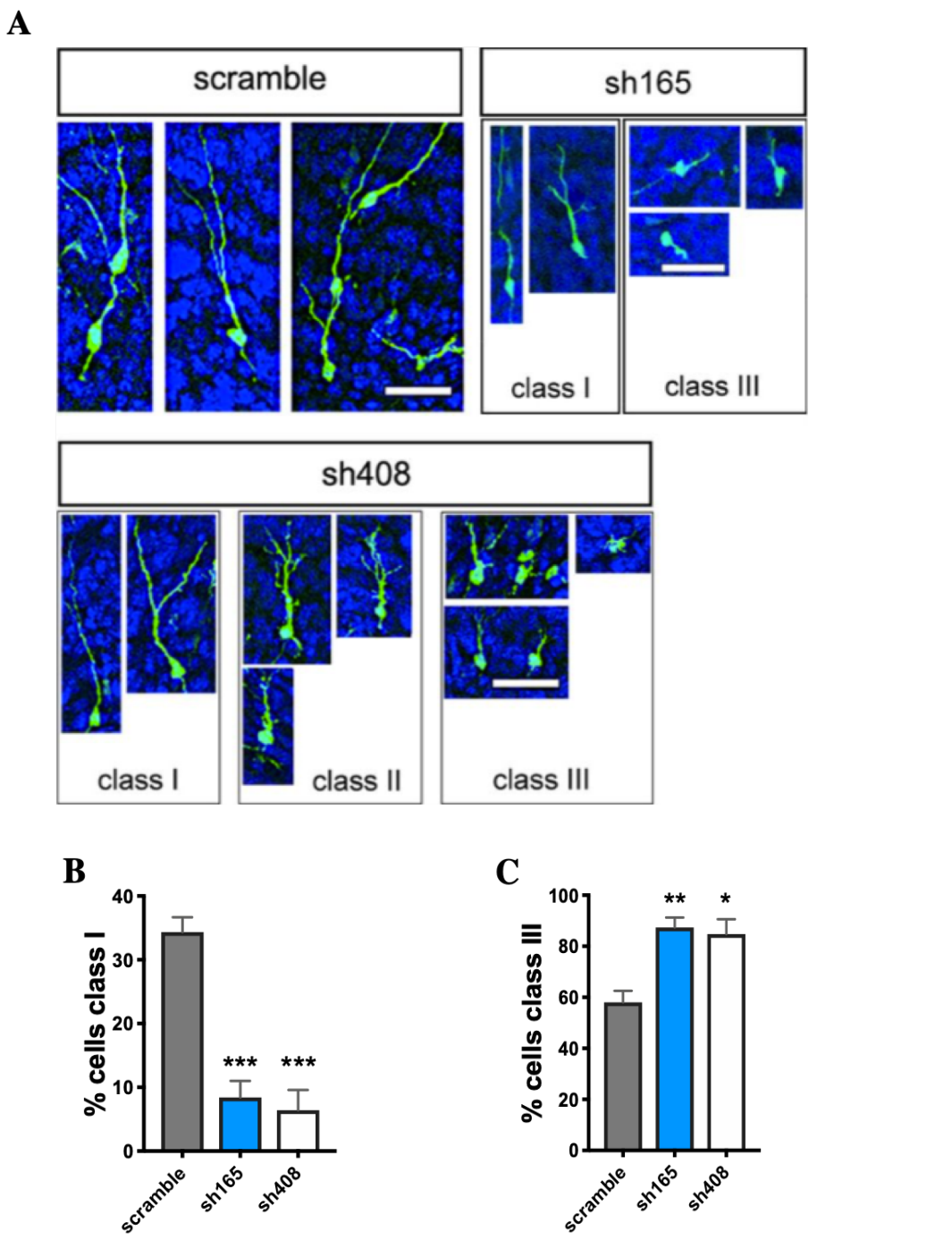


Figure 4.9 *Paupar* KD cause defective differentiation of newborn neurons in the OB

A) High magnification showing different morphologies in GFP^+ granule layer OB neurons 7 dpe after *Paupar* KD. For ease of comparison, neuronal orientations were aligned to vertical. The cells shown in the scramble control group are class I.

B) Quantification of the percentage of cells with Class I morphology 7 days after *Paupar* KD. (n=3 for controls and knockdown groups).

C) Quantification of the percentage of cells with Class III morphology 7 days after pauper KD. (n=3 for controls and knockdown groups). Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001. Scale bar =50 µm. The same scale bar is used for all images within each section.

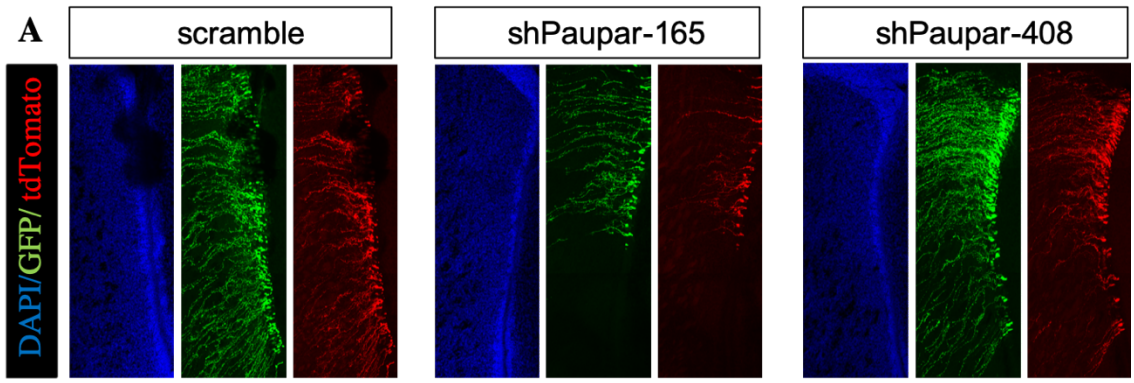
4.2.4 The lncRNA Paupar regulates SVZ neurogenesis

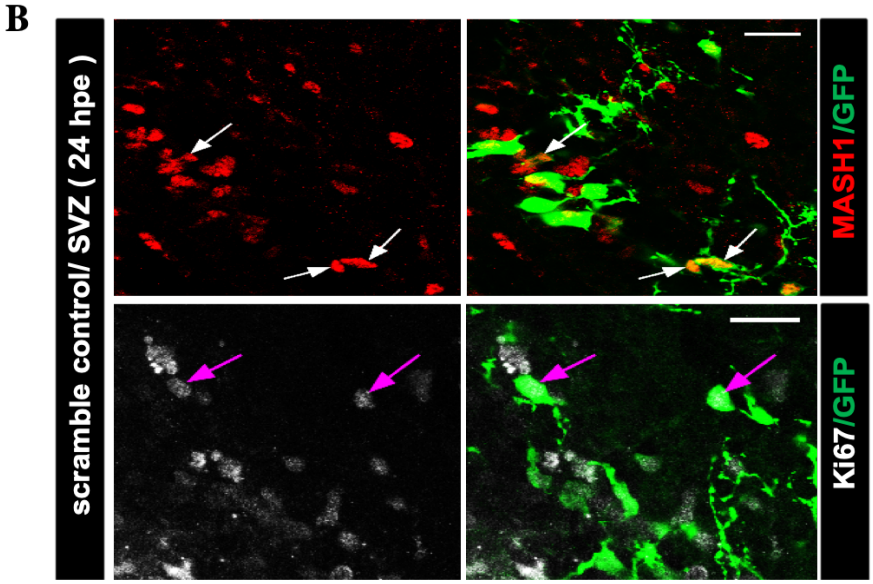
To understand the mechanism of reduced OB neurogenesis and to investigate the functional role of the lncRNA *Paupar* in the SVZ. We electroporated individual constructs (scramble, sh165 or sh408) that express GFP into the SVZ of P1 pups, and brains were collected at early time points i.e. 24 hours post electroporation (hpe) and 3 dpe for analysis in the SVZ.

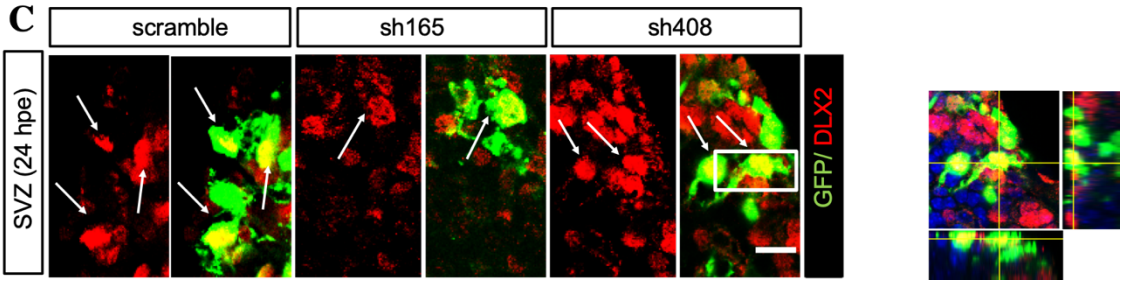
Surprisingly, at 24 hpe, significantly fewer GFP+ cells were found in SVZ in the sh165 condition. Since the constructs' concentrations were matched, we ruled out the possibility of different electroporation efficiencies among scramble, sh165, and sh408. This was confirmed by co-electroporation with a construct expressing tdTomato driven by a different promoter (pCS) which showed a similar effect, suggesting that this was not due to any artificial effect of the sh165 construct itself (Figure 4.10 A). Immunostaining of the transit amplifying progenitor cell marker Mash1 and the proliferative cell marker Ki67 among GFP+ cells revealed ~ 30 - 40% increase in both. For Mash1 (scramble: 26.2±0.7% versus sh165: 41.4±2.3% and sh408: 29.2±4.9%) (n=4 for each group, sh165 $p<0.01$, sh408 $p= 0.629$) and for Ki67 (scramble: 27.1±1.8% versus sh165: 48.3±1.7% and sh408: 34±3%) (n=4 for each group, sh165 $p<0.001$, sh408 $p=0.127$, Figure 4.10 B), suggesting elevated neurogenesis. This was confirmed by immunostaining with the transit amplifying progenitor marker DLX2 which showed ~ 40% increase (scramble: 47.6±0.3% versus sh165: 72.3±2.9% and sh408: 77.9±2.7%) (n=3 for each group, sh165 $p<0.01$, sh408 $p<0.01$, Figure 4.10 C&D).

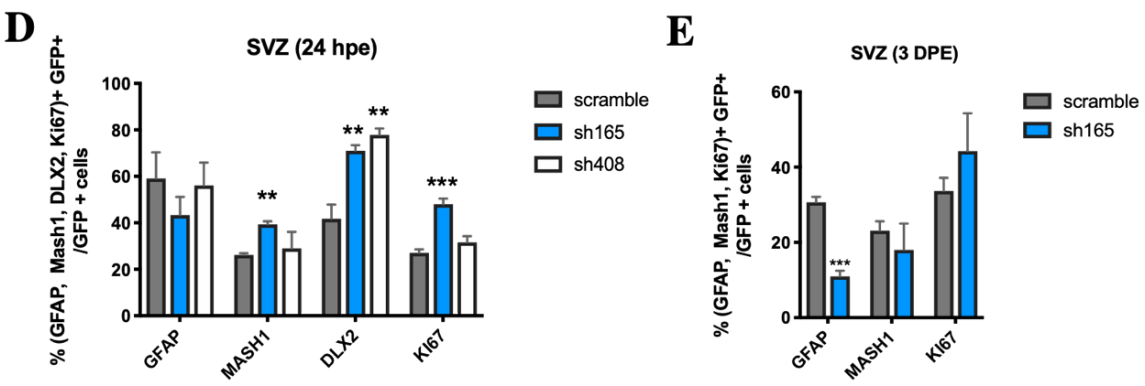
Then we moved to 3 dpe. In line with 24 hpe, fewer GFP+ cells were observed in the SVZ of the sh165 electroporated group. No obvious difference in the number of Caspase3+ cells was detected between the scramble and sh165 groups, indicating that the smaller number of GFP+ cells was not a result of increased cell death. This was confirmed by TUNEL assays in the SVZ at different time points (Result 4.2.5).

Further analysis with the sh165 construct showed 60% reduction in the percent of GFP+ SVZ cells that expressed the radial glial/neural stem cell marker GFAP (scramble: 30.68±1.42% versus sh165: 10.99±1.48%) (n=4 for scramble and n=5 for sh165, $p<0.001$, Figure 4.10 F&E), which suggests there was a depletion of the stem cell pool after the loss of *Paupar*. Taken together, these data suggest that the lncRNA *Paupar* is necessary for postnatal SVZ neurogenesis.


A
scramble
shPaupar-165
shPaupar-408
DAPI/GFP/ tdTomato


B
scramble control/ SVZ (24 hpe)
MASH1/GFP
Ki67/GFP


C
scramble
sh165
sh408
SVZ (24 hpe)
GFP/ DLX2


D
SVZ (24 hpe)
% (GFAP, Mash1, DLX2, Ki67)+ GFP+ /GFP + cells
scramble
sh165
sh408
GFAP
MASH1
DLX2
KI67
E
SVZ (3 DPE)
% (GFAP, Mash1, Ki67)+ GFP+ /GFP + cells
scramble
sh165
GFAP
MASH1
KI67

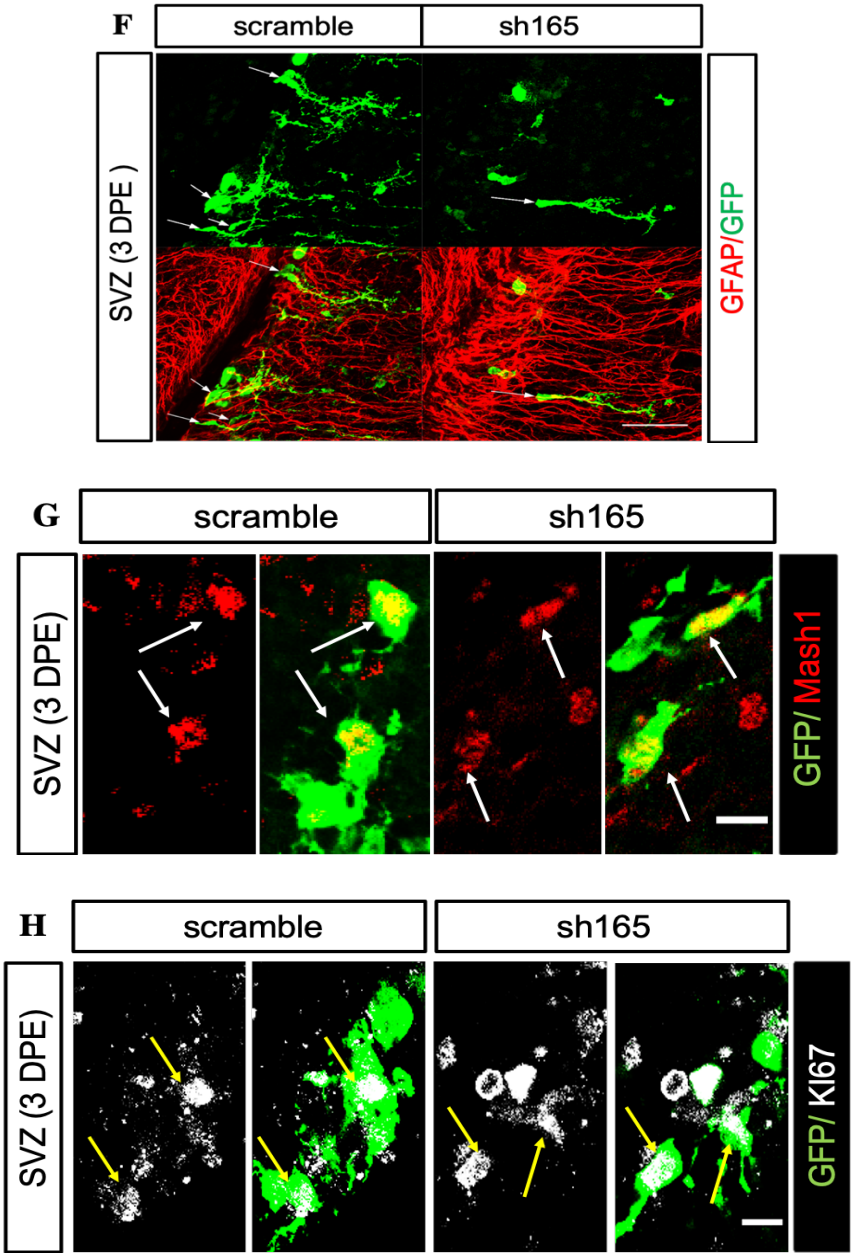


Figure 4.10 *Paupar* regulates SVZ lineage progression

A)Co-electroporation of scramble, sh165 and sh408 constructs expressing GFP with a construct expressing tdTomato driven by a different promoter (pCS) (n=3).

B) Co-staining of GFP+ cells with Mash1 and Ki67 in postnatal SVZ 24 hpe. White arrows point at GFP+MASH1+ cells and purple arrows point at GFP+Ki67+ cells in scramble control condition (n=4 for scramble and n=4 for sh165 and sh408). Results indicate acceleration of proliferation and differentiation at this time point.

C) Co-staining of GFP+ cells with DLX2 in postnatal SVZ 24 hpe (n=3).

D) Quantification of the percent of GFP+ cells that express GFAP, Mash1, Ki67 or DLX2 cells among all GFP+ cells in control, sh165 and sh408 conditions 24 hpe.

E-H) quantification and co-staining of GFP+ cells with GFAP, Mash1 and Ki67 in postnatal SVZ 3 dpe (n=4 for scramble and n=5 for sh165). Error bars represent SEM. **p<0.01, ***p<0.001.
Scale bar: (A) =200 µm, (C&G&H) =30 µm, (B&F) =50 µm.

4.2.5 Paupar KD does not cause cell death in the SVZ and the OB

Emerging evidence has indicated that lncRNAs are key regulators of multiple cellular processes including apoptosis (Lu et al., 2016, Lian et al., 2018). In order to investigate whether the observed phenotype of reduced number of neuroblasts arriving to the OB is due to cell death of NSCs or progenitor cells, TUNEL assay was performed in the SVZ 3 dpe and in the OB 7 dpe. Quantification was performed and showed no significant difference in the number of cells positive for the death marker (TUNEL+ GFP+ cells) in the knockdown condition compared to the scramble control (n=3 for control and KD groups, Figure 4.11), which indicates that the observed reduction in neurogenesis was not due to cell death caused by *Paupar* knockdown.

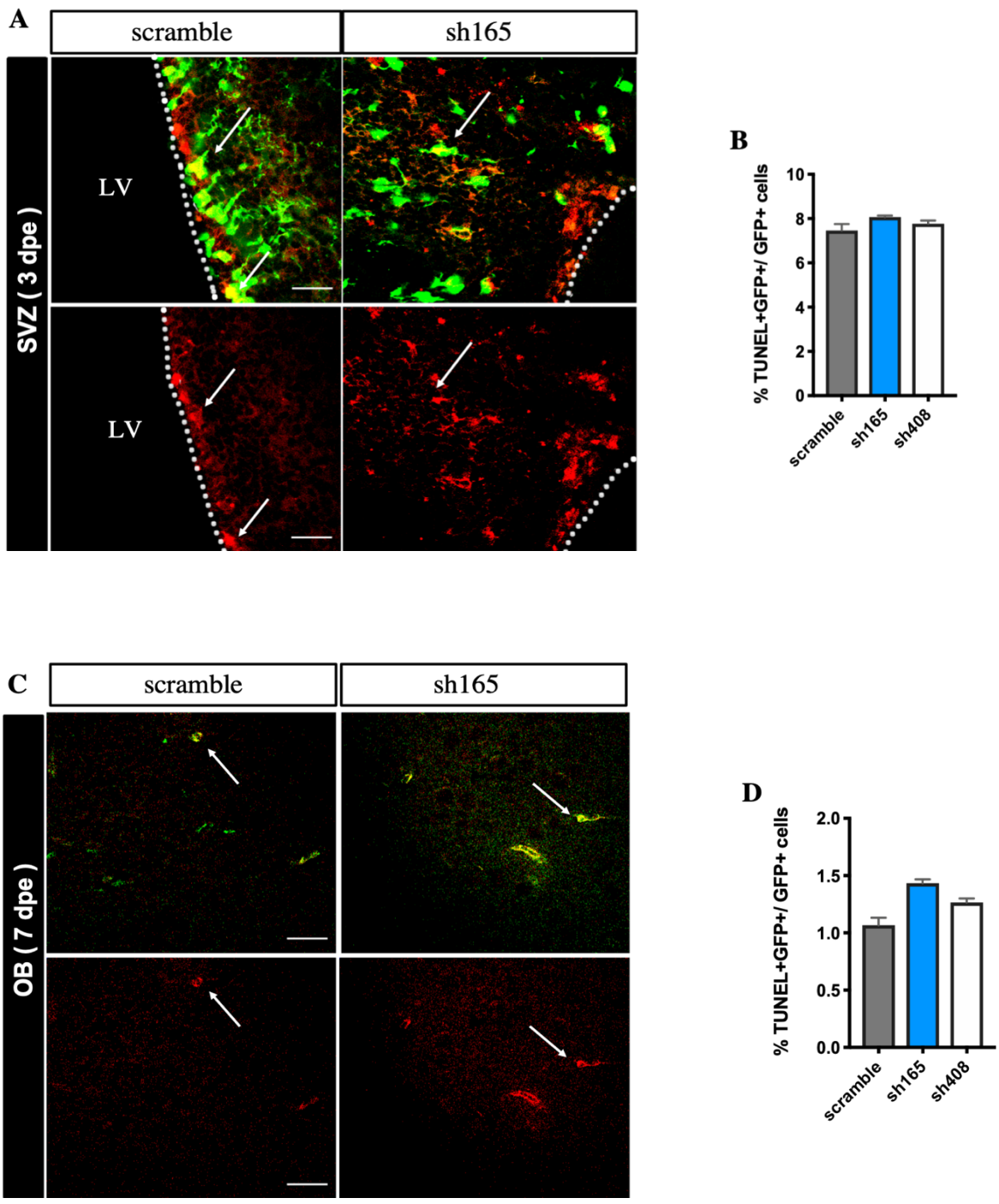


Figure 4.11 *Paupar* knockdown does not cause apoptosis in the SVZ/OB

(A-B): TUNEL staining and quantification of GFP+ cells in the SVZ 3 dpe after *Paupar* knockdown (n=3 for controls and Knockdown groups).

(C-D): TUNEL staining and quantification of GFP+ cells in the OB 7 dpe after *Paupar* knockdown (n=3 for controls and Knockdown groups). Error bars represent SEM. Scale bar = 30 µm

4.2.6 Investigating the role of Paupar on neurospheres generation and differentiation in vitro

To confirm that *Paupar* regulates SVZ NSC self-renewal and differentiation, I performed an in vitro neurosphere assay to analyse NSC properties (Pastrana et al., 2011). *Paupar* shRNA was in vivo electroporated into p1 pups to knockdown *Paupar*. 24 hpe, the SVZ was dissected and cells were dissociated and cultured with growth factors in vitro. 4 days later, neurospheres were generated and there was no obvious change in the number of neurospheres generated per 200,000 starting cells in control compared to the *Paupar* KD condition.

Since results obtained from high density cultures are difficult to interpret due to sphere fusion and to analyse the sphere-forming ability at single cell level, primary neurospheres were dissociated and cultured at clonal density (10 cells/ul) (Pastrana et al., 2011, Singec et al., 2006). Although a significant effect on cell proliferation was observed in vivo, there was no significant effect on the number of neurosphere generated from the KD condition (70.7±2.2 per 2000 cells in the sh165 condition and 64.8±0.6 per 2000 cells in the sh408 condition) compared to the control (68.7±3.3 per 2000 cells) (n=3, Figure 4.12 A&B).

Next, I wanted to investigate the multipotent/differentiating ability of NSCs after *Paupar* KD. I dissociated primary neurospheres and cultured them at low numbers in adherent monolayer cultures which allows us to follow the differentiation process in single cells after the remove of growth factors. 7 days later, cells were differentiating and expressing the characteristic markers of astrocytes (GFAP) and neurons (TUJ1) (n=3) (Figure 4.12 C). I found no obvious difference in the number of differentiated cells in the KD condition compared to the control.

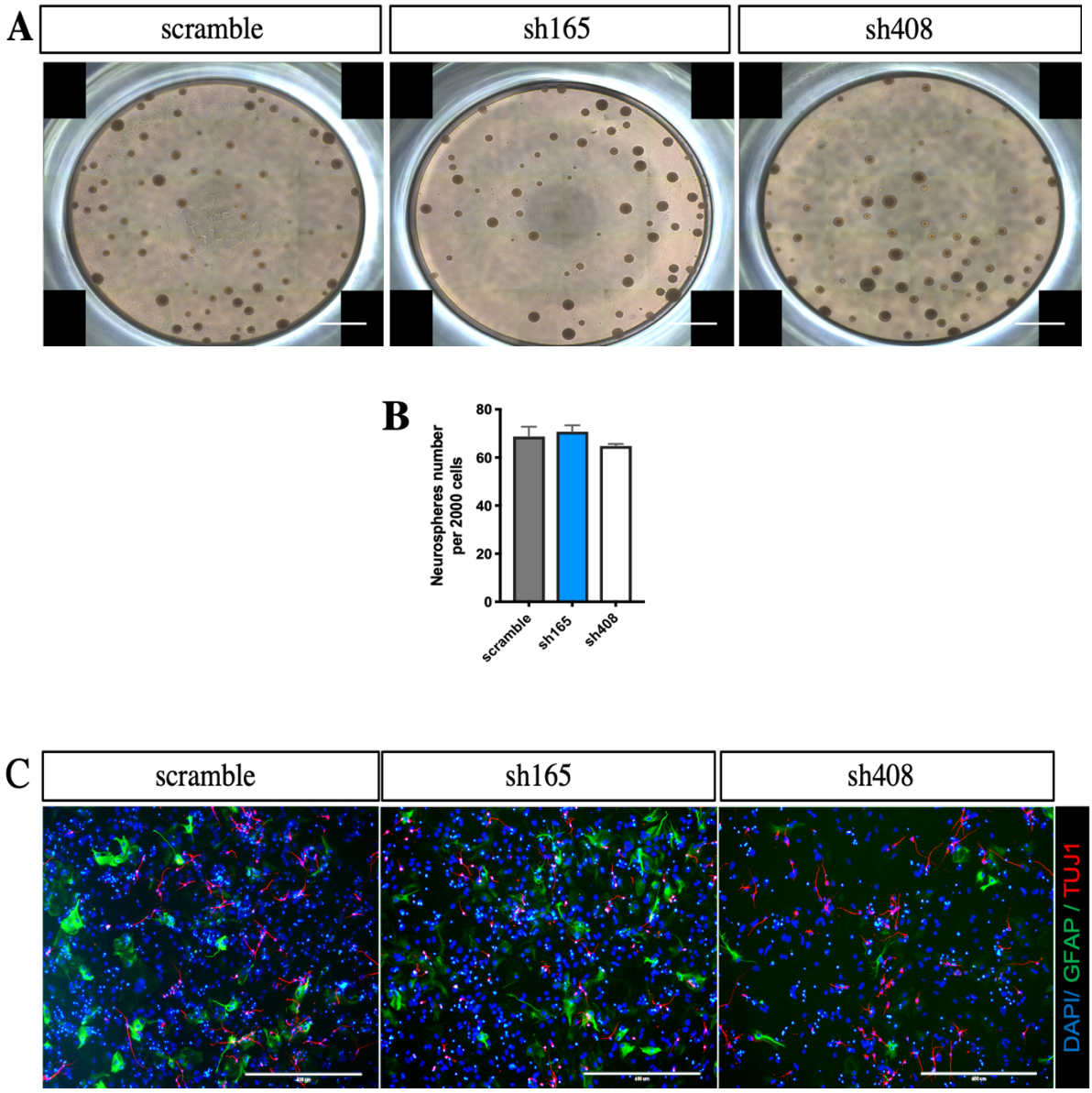


Figure 4.12 The role of Paupar on neurospheres generation and differentiation in vitro

A) Clonal density assay shows no significant change in the number of neurosphere generated from the KD condition compared to the control.

B) Quantification of neurospheres generated per 2000 cells.

C)Differentiation assay show no changes in the number of astrocytes (GFAP+ cells) or neurons (TUJ1+ cells) generated after removing growth factors and differentiating the cells for 7 days in KD condition compared to the control. Neurospheres were imaged and counted from 12 technical replicates per condition and averaged. n=3. Data are shown as mean ± SEM. Scale bar= 400 µm.

4.3 Discussion

LncRNAs are expressed in different cell types. However, unlike protein-coding genes, the in vivo roles of lncRNAs during development and their mechanisms of action remain mysterious. In this chapter, I focused on the lncRNA *Paupar* which is upstream of the transcription factor Pax6. *Paupar* was expressed in both the postnatal SVZ in vivo and in SVZ-derived neurospheres in vitro. Loss of *Paupar* in the postnatal SVZ reduced neurogenesis, probably due to a defect in NSC maintenance.

4.3.1 LncRNA Paupar expression in the SVZ

It has been estimated by RNA-sequencing that around 8,992 lncRNAs are expressed in the SVZ neurogenic lineage (Ramos et al., 2013). However, only a small number of these lncRNAs were observed by in *situ* hybridization (ISH) (as shown in the Allen Brain Atlas). Two reasons could explain this difference: first, RNA-Seq is very sensitive and can identify weakly expressed lncRNAs which leads to uncovering a large number of novel transcripts. Second, there are no probes available in the Allen Brain Atlas for most the 8,992 lncRNAs, which led to underestimation of the total number. On average, lncRNAs are shorter and expressed at lower levels than mRNAs which make them difficult to detect by conventional in *situ* hybridization (Soares et al., 2018). However, one advantage of in *situ* hybridization method is that it avoids false positive signals that can be observed in RNA-Seq. To begin my investigation of *Paupar* function in the SVZ, I first characterized its expression profile and sub-cellular localization in the SVZ.

I confirmed the expression of the lncRNA *Paupar* in postnatal SVZ by qPCR. I found that *Paupar* is more enriched in the SVZ than in SVZ-derived neurospheres and OB.

The reason could be attributed to the heterogeneity of the SVZ which includes neurogenic and non-neurogenic cells. Furthermore, Vance et al. characterized the expression profile of *Paupar* and found that it is highly enriched in adult mouse brain compared to other organs. In addition, it showed a clear correspondence with Pax6 expression profile (Vance et al., 2014), which suggest that it may regulate similar biological function as Pax6. Next, in order to determine the sub-cellular localization of the lncRNA *Paupar*, I applied fluorescent in situ hybridization (FISH) method on neurospheres cultured from postnatal mice SVZ and observed that the *Paupar* transcript is mostly nuclear localized. In support of this, Vance et al. reported that the lncRNA *Paupar*, but not control mRNA, is nuclear-enriched and mainly located in the chromatin of N2A cells (Vance et al., 2014) which is consistent with its function in transcription regulation. Moreover, ENCODE data show that human *Paupar* is located in the nucleus and in chromatin as well (ENCODE Project Consortium, 2012).

Even though the RNA FISH method (with a 48 fluorophore-labelled probe set) used in my experiment was able to identify the subcellular localization of low abundance lncRNAs like *Paupar*, it is still prone to the risk that one or more out of the 48 probes used might show lack of specificity (Cabili et al., 2015, Raj et al., 2008). It is critical in the future to ensure the appropriate controls are included like the use of two differently labelled non-overlapping probes or include knockdown cells to ensure the specificity of the signals detected. Moreover, this result lacks quantitative estimates of the lncRNA *Paupar* signal spots. A recently developed method which uses a modified probe with enzymatic signal amplification is considered to be a more accurate and sensitive alternative for the method used in this experiment (Soares et al., 2018).

Moroever, my qPCR Ct values for *Paupar* in both SVZ tissues and neurospheres were small (SVZ tissue: 25.66, neurospheres: 29.7) relative to the internal control GAPDH (Ct:16.8), which confirms low expression of *Paupar* in postnatal mice brains. It is important to point out that high expression of lncRNAs in specific tissues or cells is not always associated with important biological functions. This is because, unlike protein coding genes, lncRNAs can sometimes be transcriptional "noise" (Ponting et al., 2009). The SVZ niche mostly consist of NSCs and progenitors and not like other brain regions which mainly consist of stable cells like neurons. Therefore, we can assume that overall transcription activity is much higher in the SVZ, and so more errors in transcription of RNA with no biological function are expected. One example is the lncRNA *Visc-2*, which is highly expressed in the SVZ of embryonic brain but its deletion had no detectable functional defect on neurogenesis nor any behavioural phenotype (Oliver et al., 2015). It is also possible that lncRNAs that are expressed at low rates have essential functions that are amplified by interactions with other molecules, like transcriptional regulators of protein-coding genes. In conclusion, *Paupar* low expression rates do not mean it is transcriptional noise, and my functional experiments in the SVZ supports this hypothesis.

Finally, it would be interesting in the future to compare *Paupar* expression through the SVZ neurogenic lineage in vivo. Vance et al. reported that *Paupar* expression was undetectable in mouse self-renewing ES cells, however, its expression was rapidly upregulated after 1 day of treatment with retinoic acid (RA) which induced cell differentiation (Vance et al., 2014). Moreover, Ramos et al. analysed lncRNAs expression through the SVZ neurogenic lineage in vivo. They isolated the major cell types by FACS and performed RNA-sequencing analysing. Surprisingly, *Paupar* expression was not detected in their database, which could be because of its low

expression level. The expression of *Pax6os1*, however, has been identified in the SVZ lineage (Ramos et al., 2013). During lineage progression, *Pax6os1* expression increased from NSC to neuroblasts and in vitro differentiation of SVZ NSCs induced the level of *Pax6os1* (Ramos et al., 2013).

4.3.2 Postnatal OB neurogenesis is impaired after the loss of Paupar

My knockdown experiment indicates that *Paupar* is required for normal postnatal OB neurogenesis in vivo. At 7 dpe, most cells would have migrated from the SVZ to the OB and my quantification supported this in control mice. Interestingly, and in accordance with the *Paupar*-KAP1 physical association (Pavlaki et al., 2018), *Paupar* loss-of-function caused a reduction of the number of newborn neurons in the OB similar to the phenotype observed with KAP1 loss-of-function experiment. Several possibilities could account for the observed phenotype:

1-*Paupar* knockdown could have attenuated neuroblast migration through the RMS. This reduction in migration is sufficient to reduce the rate of OB neurogenesis (Comte et al., 2011), which could explain the observed reduction in the number of neurons arriving to the OB at 7dpe. Previous studies have showed a similar effect; reduction in the lncRNA *HNF1A-AS1* and in the lncRNA *ncRNA-7a* both decreased cell migration (Yang et al., 2014, Derrien and Guigo, 2011), and the later also caused a defect in differentiation (Derrien and Guigo, 2011). Consistent with this, most of the newborn neuroblasts had immature morphological differentiation after *Paupar* KD similar to the phenotype observed with KAP1 (Result 4.2.3).

It maybe that loss of *Paupar* and KAP1 affected the rate of OB neuronal differentiation in a way similar to NeuroD1 as discussed before (Boutin et al., 2010, Pataskar et al., 2016). In addition, knocking out Pax6 in mouse progenitors produced neuroblasts

capable of migrating into the OB but that failed to differentiate into dopaminergic periglomerular and superficial granule cells (Kohwi et al., 2005). Since *Paupar* is transcribed upstream of Pax6 and regulates its expression (Vance et al., 2014), it could be that *Paupar* KD reduced Pax6 expression and caused a defect in neuronal differentiation. In the future, it would be important to examine the direct effect of *Paupar* KD on migration by performing in vitro explant migration assays on neuroblasts dissected from the RMS after knocking down *Paupar*. Moreover, the effect I show here with *Paupar* KD lacks complete cell subtype specification. It would be interesting to examine cell dispersion within the different OB layers and the subtype of these neurons arriving to the OB by examining their neurochemical markers in order to fully understand which cell subtypes are targeted by *Paupar* and understand more its role in neuronal maturation (Figueres-Onate and Lopez-Mascaraque, 2016).

2-Another possibility was that *Paupar* knockdown cause an increase in apoptosis similar to the effect of the lncRNA-*p21*, which serves as a transcriptional repressor in the p53 pathway and plays a role in triggering apoptosis (Huarte et al., 2010). However, I found no evidence of cell death in the SVZ, RMS or OB using TUNEL staining.

3- KAP1 does not have a DNA binding motif, but Pavlaki et al showed that *Paupar* directly binds KAP1 and operates as a transcriptional cofactor to promote KAP1 chromatin occupancy. One possibility is that the OB phenotype I observed after *Paupar* KD is due to a defect in KAP1 recruitment to the genome and regulation of its target genes rather than an effect caused by *Paupar* loss-of-function. In addition, CHART-seq analysis in N2A cells suggested that *Paupar* occupies a binding site within the Ezh2 gene and regulates its expression (Pavlaki et al., 2018). Ezh2 is a member of the Polycomb repressive complex 2 (PRC2) that mediates gene silencing by

posttranslational histone modification and consist of the enzymes Ezh2, Suz12, RbAp46/48, and Eed (Margueron and Reinberg, 2011). Knockdown of *Paupar* in neurospheres led to reduction of Ezh2 and Eed levels (Data not shown). Interesting data generated from our lab showed that knocking down Ezh2 or Eed lead also to reduction of the number of neurons arriving to the OB similar to KAP1 and *Paupar* (Ezh2 cKO data is in Sun et al. See supp.)(Sun et al., 2018).These observations suggest that *Paupar* may not function independently and that it requires interaction with different protein targets to regulate OB neurogenesis. This provides important insights into the mode of lncRNA-mediated gene regulation.

4-It is important to take into consideration that there are many challenges within the current loss-of-function techniques used to study lncRNAs in the light of different molecular mechanisms they may use and also due to their location (i.e. cytoplasm, nucleus or chromatin-associated). Even though I showed that the lncRNA *Paupar* is nuclear localized, I have used in this experiment an RNAi-based method to knockdown *Paupar*, which is known to function in the cytoplasm and supply double-stranded RNA that acts post-transcriptionally by inhibiting the translation of mature mRNA. Although the presence of active RNAi factors in the nucleus has been reported before (Gagnon et al., 2014), the extent to which nuclear localized lncRNA like *Paupar* can be knocked down in my vivo and in vitro experiments remains elusive, and so another experimental approaches should be used to accurately assess the phenotype observed. This can include genetic manipulation of the lncRNA locus by inserting transcriptional terminator sequences or even deletion of the full-length lncRNA. However, we must take into consideration that *Paupar* is an intergenic lncRNA and so the phenotypes that could arise from genetic manipulation can be attributed either to the loss of *Paupar* or the associated loss of the overlapping DNA regulatory element (Bassett et al., 2014).

Antisense oligonucleotides are an alternative approach. They form DNA/RNA hybrids with nascent RNA transcripts and trigger RNase H-dependent degradation of RNA in the nucleus. This reduces the level of the RNA but the extent of the off-target effects are not fully understood (Sahu et al., 2007). In conclusion, these results identified *Paupar* as a novel lncRNA regulator of OB neurogenesis in vivo and provides important conceptual insights into different modes of lncRNA functions.

4.3.3 The lncRNA Paupar is required to maintain SVZ NSC quiescence

LncRNA *Paupar* regulates cell proliferation and differentiation in N2A cells (Vance et al., 2014). Since N2A is a neuroblastoma cell line, the role of *Paupar* regulation may be different in normal cells in vivo. Here I show that *Paupar* is required for normal postnatal SVZ neurogenesis in vivo. SVZ cells with reduced *Paupar* expression significantly increased proliferation one day following its knockdown. When *Paupar* was depleted, we observed an increase of TAPs at the early time point with a mild reduction of radial glia/stem cells. *Paupar* knockdown could possibly have caused a transition of symmetric to asymmetric division of neural stem cells. At a later time point, our data suggested that stem cells showed a significant depletion due to accelerated lineage progression and decreased symmetric divisions at the earlier time point. These data suggest that *Paupar* may maintain stem cell quiescence and restrict lineage progression from stem cells to TAPs. Moreover, the significant in vivo effect was shown only with the sh165 knockdown construct, while the sh408 construct gave a similar trend but was not significant on TAPs at 24 hpe (except with DLX2). This could be because the sh165 has a stronger knockdown effect on *Paupar* than the sh408 as confirmed in my in vitro neurosphere culture (Chapter 5 results).

In this experiment, I used in vivo electroporation method to knockdown *Paupar* in postnatal SVZ. One consideration of this technique is the risk of having different electroporation efficiency between the control and knockdown groups. I used similar amounts of DNA for each individual pup and interestingly there were dramatically fewer GFP+ cells at different time points only in the sh165 group, the construct with a stronger knockdown effect in vitro. To rule out the possibility of electroporation deficiency in the plasmid, co-electroporation with a td-tomato plasmid driven by a different promoter was done and also resulted in a smaller number of electroporated cells. Also, no obvious increase in apoptosis was observed using the TUNEL assay which suggest that the sh165 construct did not cause cell death. This indicates that the phenotype observed is likely due to a biological effect after *Paupar* KD and is not an artificial effect. One could also argue that GFP expression and stability was affected by sh165. To rule out this issue, I plan to electroporate sh165 at early time points, i.e. less than 24 hours. GFP signals can be observed a few hours after electroporation and this way I should be able to further separate electroporation efficiency from biological effects. Moreover at 3 dpe, the GFP signal in general was weaker in the sh165 group. Since cell proliferation was increased after *Paupar* KD, this could possibility cause an accelerated dilution of GFP. Importantly, the percent of GFP+ cells that expressed GFAP (GFAP+GFP+/GFP+) was reduced in the sh165 electroporated SVZ. This observation controls for different electroporation efficiencies and indicates that *Paupar* regulates NSCs population in the SVZ.

A recent study has suggested a new approach for in vivo electroporation by using mRNA instead of DNA which holds many advantages over the classical DNA electroporation (Bugeon et al., 2017). First, they show a stronger transfection efficiency in vivo using mRNA which led to an over 17-fold increase in the amount of GFP

expressing cells. Since mRNA is much smaller than DNA plasmids, its transfer into the cell was facilitated and permissive. Second, mRNA electroporation leads to faster and more homogenous expression of transgenes because once they are introduced into the cells they can be directly used for translation in the cytoplasm leading to more accumulation of desired protein while DNA needs to enter the nucleus in order to be transcribed into mRNA and then exported to the cytoplasm for translation. The only disadvantage is that the use of mRNA electroporation is limited by the shorter stability of RNA compared to DNA and so it has a transient effect (Bugeon et al., 2017).

Finally, in N2A cells, *Paupar* loss of function disrupts normal cell cycle progression and induces neural differentiation (Vance et al., 2014). *Paupar* binds and modulates the activity of many key stem cell genes like Sox2, Hes1 and E2F2 (Vance et al., 2014). In addition, *Paupar*, KAP1 and Pax6 physically associate on the chromatin within the regulatory region of shared target genes that are known to be important for neuronal proliferation and differentiation like Mab21L2, Mst1, E2f2 and Igfbp5 (Pavlaki et al., 2018). E2F2 is involved in maintaining the differentiated state of post-mitotic neurons in cell culture (Persengiev et al., 2001). E2f2 overexpression promotes cell cycle arrest and inhibits S-phase progression in PC12 neurons (Persengiev et al., 2001). Furthermore, considering the relationship between *Paupar* and Pax6 in N2A cells, I hypothesize that *Paupar* may also be performing its function through regulating of Pax6. Pax6's indispensable role in neuronal differentiation has already been investigated in the SVZ (Jang and Goldman, 2011, Ninkovic et al., 2013). It would be interesting to investigate if *Paupar* also regulates the expression of these genes in vivo in normal condition which could explain the phenotype observed. I am going to talk about *Paupar*'s regulation of target genes in Chapter 5.

4.3.4 Investigating the role of Paupar on neurosphere generation and differentiation in vitro

Paupar knockdown in vitro showed an increase in neurite outgrowth, a well-characterized feature of neuronal differentiation, and also increased the neuronal differentiation marker Tubb3 in N2A cell culture. Cells accumulated in S and G2 phases indicating that *Paupar* is also required for normal passage through S phase and entry into mitosis (Vance et al., 2014). These results are consistent with my in vivo observation of *Paupar*'s role in controlling NSCs proliferation and differentiation.

In order to confirm these observations in my in vitro culture system, I used neurosphere clonal density and adherent monolayer assays which are well established tools to determine the proliferation and differentiation potential of neural stem cells in vitro (Codega et al., 2014, Pastrana et al., 2011). The clonal density assay has a major advantage of giving a quick estimate of the proliferation potential of NSCs in brain. The adherent monolayer system, in contrast, is useful for following differentiation processes in single cells (Walker and Kempermann, 2014). However, in this experiment I didn't observe a change in the number of neurospheres generated in the proliferation assay of the *Paupar* knockdown group compared to the control. In the differentiation assay, neural stem cells were multipotent and were able to differentiate into neurons and astrocytes as shown by immunocytochemistry but did not show any obvious changes in percent of TUJ1+ neuronal marker and GFAP+ astrocyte marker expression in the KD condition. These results contradict my in vivo observation and could be explained by the following:

First, the neurosphere assay is not an accurate readout of the in vivo system. One advantage of the neurosphere assay which can also be a drawback, is that it allows us to evaluate the full potential of the cells when removed from the normal extrinsic cues provided by their in vivo environment. Therefore, a cell that can produce all three major cell types (neurons, astrocytes, and oligodendrocytes) in vitro may not necessarily give rise to them during normal development (Jensen and Parmar, 2006). The differences between the in vivo and in vitro systems may arise because of either the lack of cell-cell interactions, similar to the SVZ niche, or the lack of factors in the culture system. Therefore, it is important to be cautious in interpreting the results and distinguishing between the potential of a cell and its actual behaviour in vivo. Second, due to the heterogeneity of the SVZ and the technical differences in dissecting the tissue, my culture system consisted of GFP+ electroporated cells with reduced *Paupar* expression as well as wild type cells. Together they will merge to form neurospheres and so it will be difficult to analyse accurately the KD effect on neurosphere generation. Moreover, electroporated NSCs are proliferating in the culture system which cause dilution of the plasmid and so reduction in the knockdown effect. In the future I plan to use lentivirus or retrovirus mediated knockdown of *Paupar* which provides a stable integration of the construct into the genome of the host cell.

4.4 Conclusion

In this chapter, the expression and function of the lncRNA *Paupar* has been explored in the postnatal SVZ. I showed that *Paupar* was expressed in the postnatal SVZ and neurospheres and was important for neurogenesis. Loss of *Paupar* increased proliferation one day following its knockdown. When *Paupar* was depleted, we observed an increase of transit amplifying progenitor cells at early time point. *Paupar* knockdown possibly have caused a transition of symmetric to asymmetric division of neural stem cells which explained the depletion of neural stem cell pool observed at later time point. These data suggest that *Paupar* may maintain stem cell quiescence and restrict lineage progression from stem cells to TAPs. Understanding the role of *Paupar* in SVZ neurogenesis can provide new insights about lncRNAs in vivo functional mechanisms.

Chapter 5

Molecular mechanisms underlying KAP1 and *Paupar* regulation

5.1	**Introduction**	**172**
5.2	**Results**	**178**
5.3	**Discussion**	**189**
5.4	**Conclusion**	**198**

5.1 Introduction

5.1.1 Transcription regulation by KAP1

It is well established that KAP1 causes suppression of target gene expression. Biochemical studies on KAP1 suggest that the PHD and bromodomain at the C-terminal region of KAP1 can interact with and coordinate the assembly of a macromolecular complex that contains chromatin remodelling proteins like Mi2α, an isoform of Mi2 protein found in the NuRD (nucleosome remodelling and histone deacetylation) complex, and SETDB1 (SET domain, bifurcated 1), an H3K9me3-specific histone methyltransferase. Together, they create a heterochromatin microenvironment via changes in histone modifications and suppress target genes (Cheng et al., 2014b, Iyengar and Farnham, 2011)(Figure 5.1). However, the addition of an inhibitor of histone deacetylase (HDACs) only partially reduces KAP1 repression of genes, and because only a small fraction of KAP1 binds to Mi2α in vivo, it is thought that Mi2α only plays a minor role of KAP1 suppression (Schultz et al., 2001). In contrast, the H3K9me3 mark by SETDB1 causes a strong genomic binding between KAP1 and HP1 (due to the ability of HP1 to bind to H3K9me3) with the observation that KAP1 and SETDB1 colocalize at thousands of genomic sites, suggests that SETDB1 has the major functional role in the KAP1 repression mechanism (Schultz et al., 2002, Frietze et al., 2010). The bromodomain of KAP1 is important for mediating its interaction with SETDB1, and this interaction depends of SUMOylation at lysines 554, 575, 676, 750, 779, and 804 of KAP1, which also stimulates the H3K9me3 activity of SETDB1 bound to KAP1. While the PHD domain of KAP1 functions as an E3 ligase that SUMOylates the adjacent bromodomain. Thus, the PHD-mediated SUMOylating of the

bromodomain, followed by interactions with SETDB1 provides mechanistic insights for the function of KAP1 PB domain (Ivanov et al., 2007, Zeng et al., 2008).

5.1.2 Recruitment of KAP1 to the genome

Neither KAP1, nor any of the binding proteins in the complex, have DNA-binding domain (DBDs), therefore other proteins are required to recruit KAP1 to the genome. It was originally identified that KAP1 is an interacting partner of two different C2H2 zinc finger proteins, KOX1 and KID-1. Zinc fingers are DNA binding transcription factors and about half of them contain N-terminal KRAB domains that interact with the RBCC domain of KAP1 (as discussed in Chapter 1) (Iyengar and Farnham, 2011). Because KAP1 strongly binds to the 3' end of ZNF genes, one might expect that KAP1 would regulate their expression. Surprisingly, there is no association between the level of KAP1 at the 3' end of ZNFs and the expression of these genes. Binding of the KAP1-SETDB1-H3K9me3 complex to ZNF genes does not reduce the level of the transcript suggesting that the effect of KAP1 on the genome is mostly indirect (Iyengar et al., 2011).

Interesting studies showed that KAP1 can also bind to KRAB domains that are not associated with ZNFs. For example, The KRAB-O (KRAB only) protein can bind to KAP1 and serve as a bridge between KAP1 and the DNA-binding protein SRY (sex-determining region Y) to recruit KAP1 to SRY-binding regions (Peng et al., 2009). Interestingly, KAP1 deleted from the RBCC domain can still bind to the promoter regions of some genes, suggesting that KAP1 can be recruited to certain sites by a novel mechanism independent from the KRAB-ZNF recruitment mechanism. In conclusion, there are two mechanisms for KAP1 recruitment to the genome, one through KRAB-

ZNFs and the other through different binding molecules (Iyengar et al., 2011)(Figure 5.1).

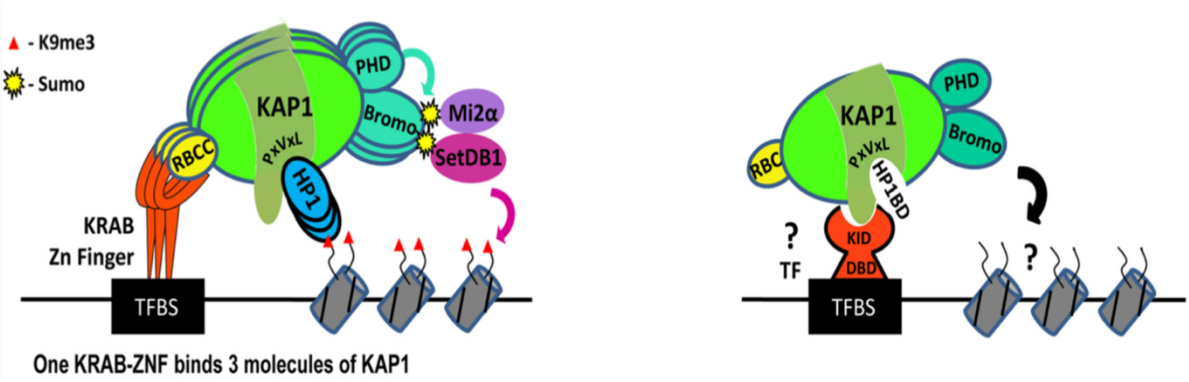


Figure 5.1 KAP1 multiprotein complex and recruitment to the genome Adapted from (Iyengar and Farnham, 2011)

5.1.3 KAP1 and Paupar function on a common set of target genes

As mentioned above, the mechanism of KAP1 recruitment to the genome is not fully understood. Interestingly, Vance et al showed that the lncRNA *Paupar* plays a role in the recruitment of KAP1 to specific sites of the genome that are bound by Pax6 and promote H3K9me3 deposition at these regions. Interestingly, *Paupar* bound sequences are located at gene promoters that are not enriched for KRAB-ZNF transcription factor binding motifs as determined by de novo motif discovery. They identified 46 shared binding sites on the chromatin of N2A cells, and only one of which was colocalized with a ZNF gene. There was also a significant Pax6 enrichment at 11 out of 15 *Paupar*-KAP1 bound regions. Thus, the ribonucleoprotein complex *Paupar*-KAP1-PAX6

regulates the expression of a shared set of target genes that are important for proliferation and neural differentiation like Mab21L2, Mst1, E2f2 and Igfbp5 genes. These results suggest that lncRNAs may function as co-factors to recruit KAP1 to specific target genes and this may involve context-specific association with either KRAB-ZNF or non-KRAB-ZNF containing transcription factors like PAX6 (Vance et al., 2014).

5.1.4 Diverse effects of KAP1 and Paupar on target genes

In contrast to the above, KAP1 and *Paupar* may also acquire independent functions from each other. For instance, *Paupar* knockdown in N2A cells led to disruption of the normal cell cycle profile and induced neuronal differentiation. Its transcript binds and regulates the activity of Hes1, Sox2, and E2F2 genes in trans, in addition to regulating the expression of Pax6 gene in cis, while the expression of these genes was not affected by the loss of KAP1 (Vance et al., 2014, Pavlaki et al., 2018). That supports the above hypothesis that KAP1 uses a KRAB-ZNF or lncRNA-dependent recruitment mechanism to regulate the expression of a different set of genes. Another possibility is that KAP1 could have an H3K9me3-independent function. A recent study had showed that KAP1 interacts with SWI/SNF and Ezh2, in a PRC2-independent manner, and together they activate, rather than repress, genes with roles in breast stem cell maintenance (Li et al., 2017).

In spite of the extensive studies on KAP1 and lncRNAs, KAP1 and *Paupar* molecular mechanisms in the SVZ had not been investigated and it still remains a question how they regulate NSC maintenance in the SVZ. Therefore, in this chapter, I will compare the downstream genes of KAP1 vs *Paupar* and examine whether any of these genes could explain the phenotype I observed in the knockout/knockdown models.

5.1.5 Bone Morphogenic Proteins (BMPs)

The bone morphogenic proteins (BMPs) are a group of morphogens that are important for development of the nervous system (Bond et al., 2012). BMPs are members of the transforming growth factor beta (TGFβ) superfamily of extracellular signalling ligands (Mehler et al., 1997). More than 30 different BMPs were identified across different species and they are divided into subgroups according to structural similarities. One subgroup consists of BMP2 and BMP4 and play a major role in CNS development. Other members like BMP6, BMP7, and BMP9 are important in certain developmental events (Mehler et al., 1997, Bond et al., 2012). In the postnatal SVZ, BMP2, 4, 6, 7, receptor serine threonine kinases BMPRIa, BMPRIb, and BMPRII, and the BMP inhibitor Noggin are all expressed (Bond et al., 2012, Lim et al., 2000, Colak et al., 2008).

BMPs are synthesized as dimers and then secreted from the cell to bind to BMP receptors (BMPRs) on neighbouring cells (paracrine) or on the cell that produced the signals (autocrine) (Bond et al., 2012, Miyazono et al., 2005). BMPRs are composed of type I and type II serine/threonine kinase receptor subunits. BMP ligands bind to both receptors when they are assembled in the membrane but may also bind one receptor and recruit the other to the complex. For example, BMP2 and BMP4 are known to bind to two classes of Type I receptors (BMPRIa and BMPRIb) and one class of Type II receptor (BMPRII). Type II receptors are active kinases which, upon ligand binding, phosphorylates Type I receptor. Type I receptors in turn phosphorylates the intracellular molecules Receptor-regulated Smads (R-Smads; Smad1, 5, 8). R-Smads then bind with co-Smad4 (a common mediator), and the resulting heteromeric complex translocates into the nucleus and interact with transcriptional activators and repressors to regulate

target genes (Miyazono et al., 2005, Bond et al., 2012, Lim et al., 2000, Hegarty et al., 2013).

In the adult SVZ, baseline BMP signalling is important to promote neurogenesis (Colak et al., 2008), however, increased BMP signalling suppress it. Increased BMP signalling reduces proliferation and increases cell cycle exit (Coskun et al., 2001, Lim et al., 2000, Mercier and Douet, 2014). In addition, BMP signalling in the SVZ regulates fate choice by promoting astroglial lineage commitments (Bonaguidi et al., 2005, Gomes et al., 2003) and suppress the differentiation of neurons and oligodendrocytes (Colak et al., 2008, Hegarty et al., 2013, Grinspan et al., 2000, Lim et al., 2000). In contrast, Noggin antagonises the action of BMP signalling on neurogenesis and fate choice (Colak et al., 2008, Lim et al., 2000, Morell et al., 2015).

5.2 Results

5.2.1 The lncRNA Paupar directly binds to KAP1 in the SVZ-derived neurospheres

Mass spectrometry analysis has identified many transcription and chromatin regulatory proteins that bind to *Paupar* in N2A cells. Five of these proteins were validated to be associated with *Paupar* transcript using native RNA-IP experiments in the same cell line. These included KAP1 and RCOR3, a member of the CoREST family that interacts with the REST transcription factor (Pavlaki et al., 2018).

We next performed a native RNA-IP experiment in vivo to confirm the potential association between the endogenous *Paupar* transcript and KAP1 in the SVZ. I dissected NSCs from P4 mice brains and cultured them to generate secondary neurospheres that were sent to Dr. Ioanna Pavlaki at University of Bath to perform the RNA-IP experiment. Whole cell lysates were prepared, and KAP1 was precipitated using an antibody against it. Bound RNAs were then purified and the levels of *Paupar* transcript was detected using qRT-PCR. (Chapter 2; Methods and Materials)

The result revealed that the *Paupar* transcript, but not a U1snRNA non-specific control RNA, was highly enriched using antibodies against KAP1 compared to an IgG isotype control in a native RNA-IP experiment (n=1, Figure 5.2). This result indicates that the endogenous *Paupar* transcript directly associates with KAP1 in SVZ NSCs.

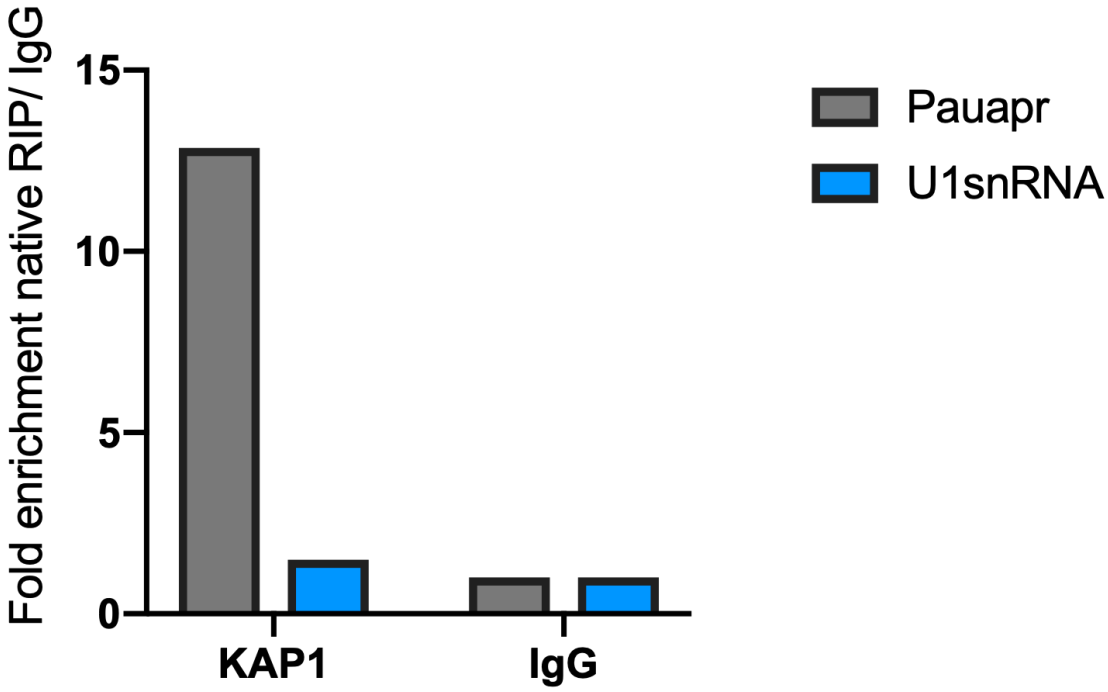


Figure 5.2 *Paupar* directly binds the chromatin regulatory protein KAP1 in SVZ-derived cells.

5.2.2 Selective activation of gene expression after KAP1 knockdown in SVZ NSCs

KAP1 controls the expression of genes important for self-renewal and differentiation of many cell types, including neuronal cells (Iyengar and Farnham, 2011). Microarray analysis from Pavlaki et al identified 1913 differently expressed genes upon KAP1 knockdown in N2A cell line (Pavlaki et al., 2018). 282 of these genes were identified in a different study to be regulated by human KAP1 in Ntera2 undifferentiated human neural progenitor cells (Iyengar et al., 2011). In order to investigate KAP1 role in the SVZ, I cultured neurospheres generated from P4 mouse SVZ, to rule out the potential effects of SVZ cell heterogeneity. Neurospheres do not contain ependymal cells, endothelial cells and microglia but instead comprise of the neural lineage. Cells dissociated from neurospheres were nucleofected with shRNA constructs to knockdown KAP1. 3 days post nucleofection, cells were collected for RNA isolation, cDNA, and qPCR analysis. At this time point, knockdown was confirmed with 70% reduction in the expression of KAP1 (Figure 5.3 A).

Since I have shown that KAP1 is required for NSCs maintenance in the SVZ, I checked the expression of genes that are known to regulate NSCs in the SVZ as well as the expression of KAP1 target genes with known functions in neuronal cells that were identified in the microarray analysis in N2A cells (Pavlaki et al., 2018).

BMP signalling in the SVZ inhibits the differentiation of neurons and oligodendrocytes and promotes astroglial lineage commitment (Bond et al., 2012). I checked the expression of BMP family member genes and their intracellular targets that mediate their downstream effects after KAP1 KD in neurospheres. Interestingly, I found a

significant upregulation in the expression of BMP2, BMP6, SMAD1, and slightly of STAT3 gene expression (Figure 5.3 C). Moreover, another study showed that BMP2 upregulates the expression of Hes5, a downstream Notch signalling target gene, which in turn inhibits the transcriptional activity of the pro-differentiation genes Mash1 and Neurogenin (Nakashima et al., 2001). Interestingly, I also found an induction in the expression of Hes5 after KAP1 KD (Figure 5.3 D), but no changes were observed in Mash1 or Neurogenin2 levels. However, NeuroD1 is a pro-differentiation gene that I show to be significantly downregulated after KAP1 loss (Figure 5.3 E). These results might suggest the presence of crosstalk between BMP and Notch signalling to control downstream pro-differentiation genes. In addition, BMP2 significantly increased the level of the cyclin kinase inhibitor p21 in MCF-7 cell line (Ghosh-Choudhury et al., 2000). In neurospheres KAP1 KD also caused an upregulation of p21 expression (Figure 5.3 F) which could also be mediated by BMP2 signalling.

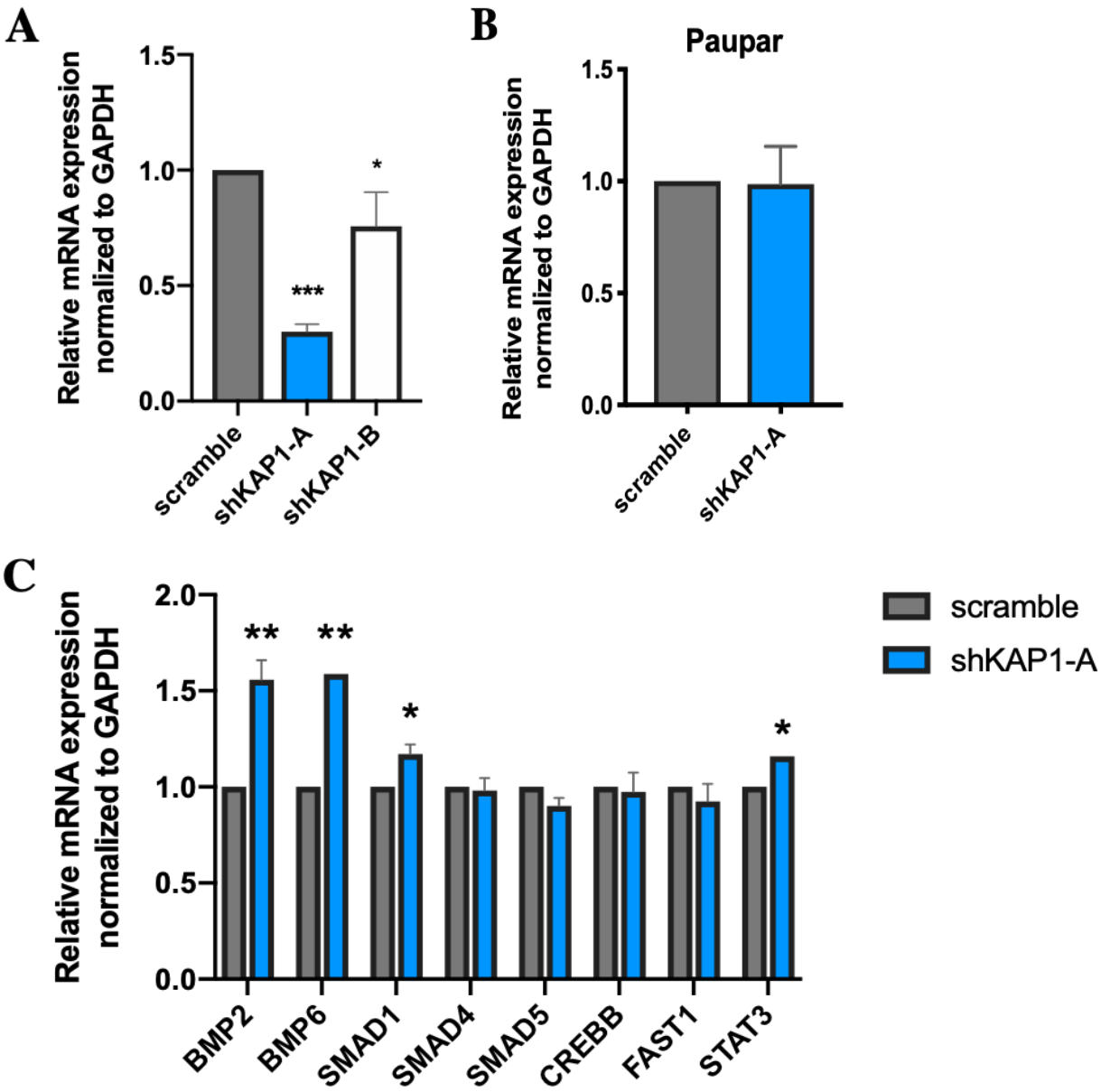
Notch signalling is required for the maintenance of NSCs and for proper control of neurogenesis in the adult brain (Imayoshi et al., 2010). Since Hes-5 was significantly upregulated, I checked the expression of other Notch signalling genes. I found no significant change in the expression of Notch1/Noch2/Hes1/Hes3/ DLL1/Nrg1/Hey1/Hey2 genes (Figure 5.3 D).

As KAP1 shown to control NSC pool and identity maintenance, I checked the expression of cell cycle inhibition genes. No change was detected in the expression of p57/p27/cdkn2a (Figure 5.3 F), and other genes that are known to be regulated by KAP1 in N2A cells (Pax6, Igfbp5, Mst1, Mab) (Figure 5.3 E).

Finally, since KAP1 and *Paupar* exist in the same ribonucleoprotein complex and they physically associate, I checked the expression of *Paupar* after KAP1 KD. There was no

change in *Paupar* expression which suggest that it's not being regulated by KAP1 (Figure 5.3 B).

These data suggest that KAP1 may regulate its downstream genes in an environment and context-specific way and describes an interesting observation in which KAP1 actively repress BMP signalling genes in NSCs.



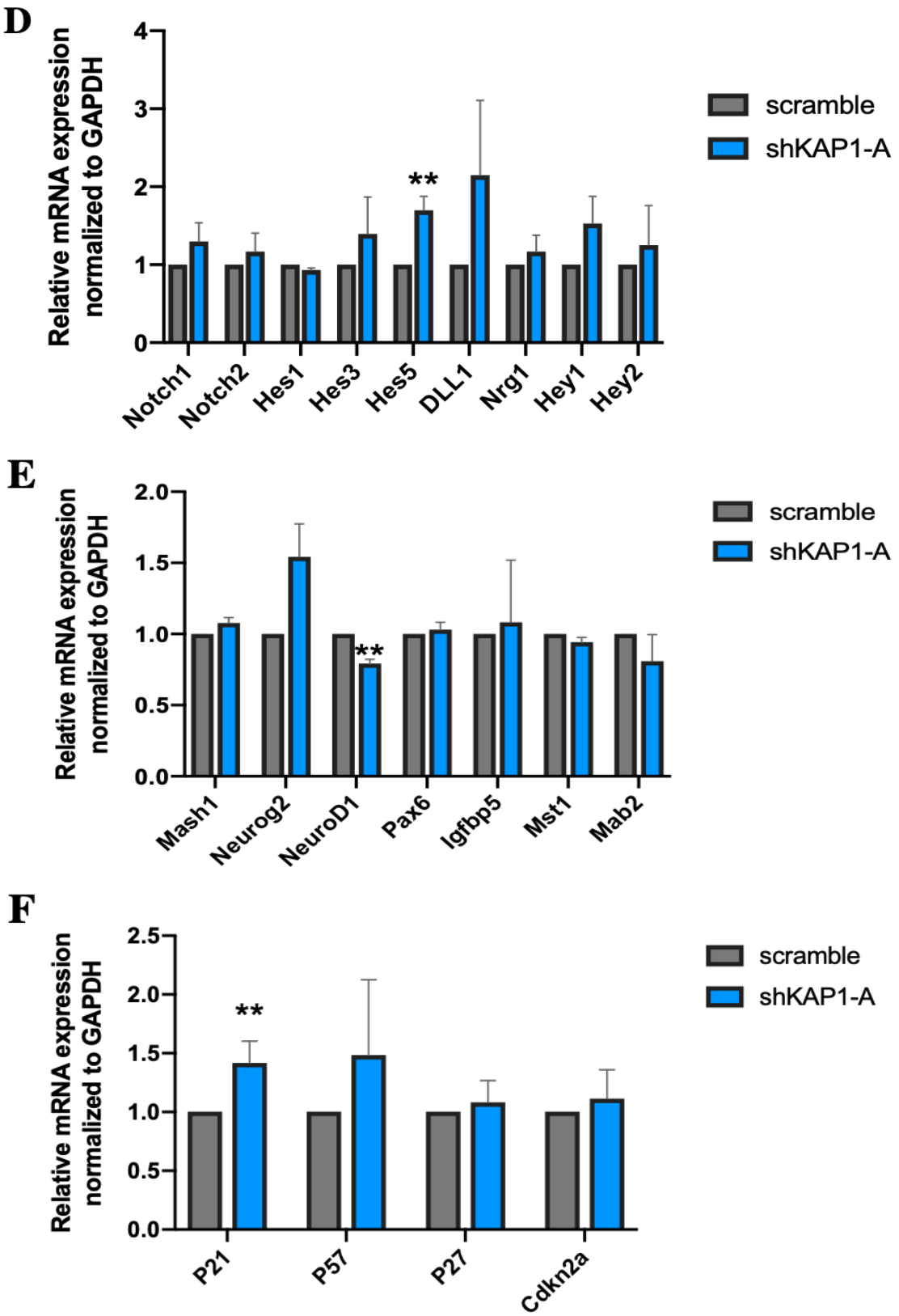


Figure 5.3 Loss of KAP1 activates BMP signalling genes

A)Q-PCR to confirm KAP1 knockdown efficiency in neurospheres 3 days post nucleofection with either shKAP1-A or shKAP1-B (n=3). B-F) Q-PCR of gene expression in neurospheres after KD KAP1 with shKAP1-A compared to scramble control 3 days post nucleofection (n=3 except the ones without error bars n=2). Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001

5.2.3 Optimization of ChIP-seq experiment in SVZ derived-NSCs to investigate KAP1 genome-wide binding

ChIP-seq analysis of KAP1 occupancy in N2A cells showed that KAP1 associated with ~5000 genomic locations compared to input DNA (Pavlaki et al., 2018). KAP1 occupancy is enriched at the promoter regions of target genes and at the 3' UTRs of zinc finger genes, consistent with other studies that investigated human KAP1 genomic occupancy (Iyengar et al., 2011, O'Geen et al., 2007).

To understand how KAP1 controls SVZ neurogenesis on a molecular level, I carried out a ChIP-seq experiment to analyse KAP1 binding genome wide in SVZ cells. Due to the heterogeneity of the SVZ and the existence of different cell subtypes, I did chromatin-immunoprecipitation with either KAP1 antibody or control IgG antibody in secondary neurospheres derived from P4 SVZ mouse brains instead of SVZ tissue. Since KAP1 does not have a DNA-binding motif, I could not use the standard ChIP principle of using only formaldehyde for cross linking. I had to optimize the protocol by adding an extra cross-linking step. The double crosslinking step (adapted from A. Porro and J. Perini) is used to enhance the detection of targets of proteins that do not bind DNA directly, rather binding through other proteins. The first step is to add disuccinimidyl glutarate (DSG) crosslinker which fixes protein-protein interactions and then to add formaldehyde to fix protein-DNA interactions.

Most studies show that KAP1 is recruited to its target genes by binding to the 3' UTR of KRAB-ZNF transcription factors (Iyengar et al., 2011, O'Geen et al., 2007). I therefore measured KAP1 chromatin occupancy at Zfp382 3' UTR as a positive control, which is a good example for high-affinity binding site for KAP1 (Iyengar et al., 2011).

Quantitative PCR analysis of precipitated DNA showed 10 folds occupancy of KAP1 at the Zfp382 3' UTR-positive control site compared to an IgG isotype control (Figure 5.4). Samples were then sent to sequencing and results are currently being analysed by a bioinformatician.

Next, KAP1 interacts with SETDB1 methyltransferase to facilitate H3K9me3 deposition at target sites (Schultz et al., 2002). So, I thought of examining the level of H3K9me3 enrichment at KAP1 binding sites. Unfortunately, the chromatin-immunoprecipitation with H3K9me3 failed to generate positive signals. This could be attributed to low quality of the antibody used.

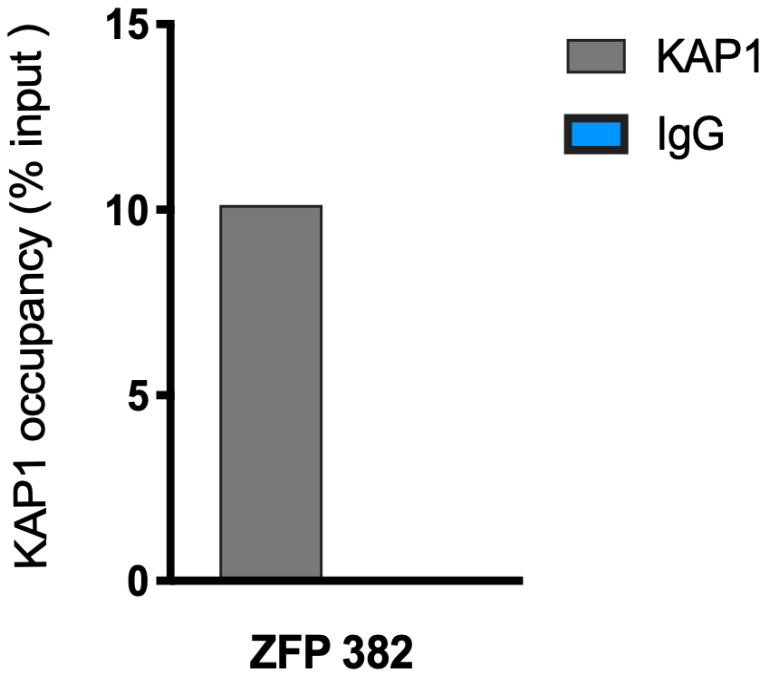


Figure 5.4 KAP1 chromatin occupancy within the regulatory region of ZFP382
ChIP assay was performed in SVZ-derived neurospheres using either KAP1 or an isotype-specific control (n=2)

5.2.4 Gene expression changes after Paupar KD in the SVZ

Paupar is transcribed upstream of the Pax6 gene. Knockdown of *Paupar* disrupts the normal cell cycle profile and induces neural differentiation in neuroblastoma cells. It has been shown that KD of *Paupar* transcript in N2A cells upregulates the expression of Pax6 in cis. In addition, it upregulated the expression of E2F2, which functions in maintaining the differentiated state of post-mitotic neurons in cells (Persengiev et al., 2001), and downregulated the expression of key stem cell genes like Sox2 and Hes1 (Vance et al., 2014).

In the SVZ, I found that *Paupar* maintains stem cell quiescence and restricts lineage progression from stem cells to TAPs. When *Paupar* was depleted I observed an increase of TAPs at early time points with a mild reduction of radial glia/stem cells. At a later time point, the stem cells showed a significant depletion that is explained by the increase in the lineage progression earlier.

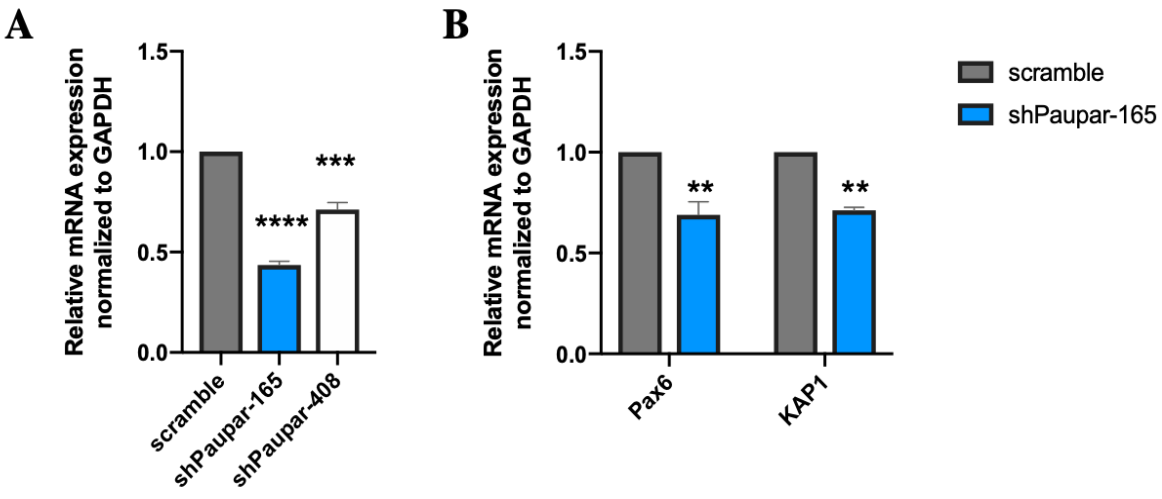
In order to investigate the molecular mechanism underlying *Paupar* KD in the SVZ, I cultured neurospheres generated from P4 mouse SVZ. Cells dissociated from neurospheres were nucleofected with two different shRNA constructs to knockdown *Paupar*. 3 days post nucleofection, cells were collected for RNA isolation, cDNA, and qPCR analysis. At this time point, knockdown was confirmed with 60% reduction in the expression of *Paupar* (Figure 5.5 A).

I first investigated BMP signalling genes. Unlike KAP1, I did not find any changes in the expression of BMP genes like BMP2, BMP4, SMAD4 and SMAD5 (Figure 5.5 C). I then checked the expression of key stem cell genes that were differentially regulated in N2A cells. Similarly, I observed a downregulation of Sox2, EGF, Igfbp5, and Hes1 genes, but not Hes5, and a significant upregulation of E2F2 gene (Figure 5.5 D).

These data confirm the specificity of *Paupar* regulated genes. Moreover, I checked the expression of cell cycle gens that are known to regulate NSCs in the SVZ. I did not observe any changes in the expression of p21, p27, p57, and cdkn2a genes (Figure 5.5 E). Taken together, these data suggest that even though *Paupar* exist in the same ribonucleoprotein complex as KAP1, it regulates different set of target genes.

Since *Paupar* is transcribed very close proximity to Pax6 gene and regulates its expression (Vance et al., 2014), I wanted to determine if *Paupar* regulates Pax6 expression in the SVZ as well. Interestingly, even though in N2A cells there was an upregulation of Pax6 expression after *Paupar* KD, yet I observed a significant reduction in neurospheres (Figure 5.5 B), which suggests that *Paupar* play a different molecular mechanism role in vivo in normal condition compared to neuroblastoma cells.

Finally, since *Paupar* physically interact with KAP1, I thought to examine KAP1 after *Paupar* KD. I found a significant reduction in KAP1 transcript level (Figure 5.5 B) which suggests that KAP1 expression is being regulated by *Paupar*. In conclusion, these data confirm that the lncRNA *Paupar* function as an important regulator of gene expression in neural stem cells.



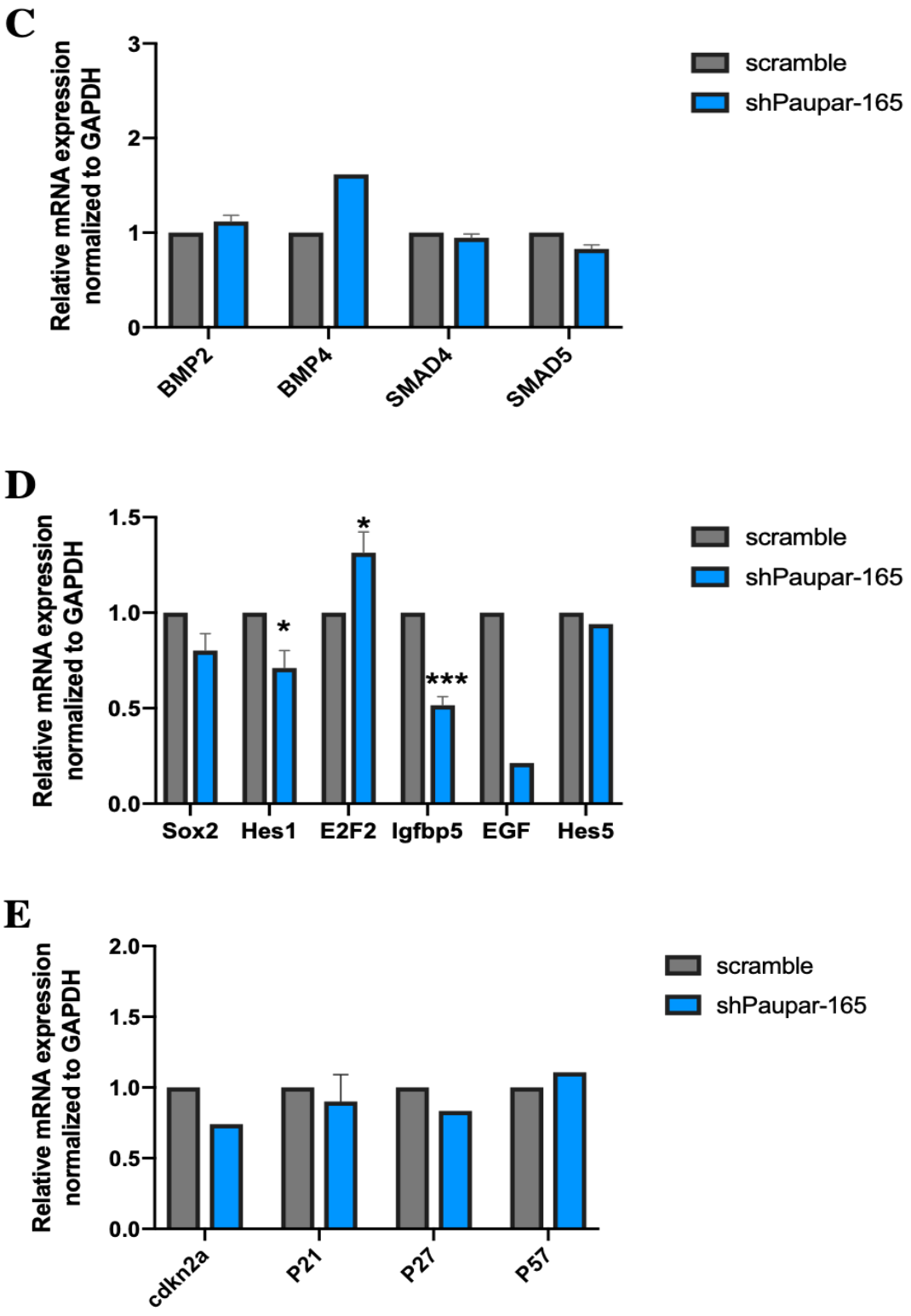


Figure 5.5 Loss of *Paupar* downregulates key stem cell genes

A)Q-PCR to confirm *Paupar* knockdown efficiency in neurospheres 3 days post nucleofection with either sh*Paupar*-165 or sh*Paupar*-408 (n=3)

B-E) Q-PCR of gene expression in neurospheres after KD *Paupar* with sh*Paupar*-165 compared to scramble control 3 days post nucleofection (n=3 except the ones without error bars n=2). Error bars represent SEM. *p<0.05, **p<0.01, ***p<0.001

5.3 Discussion

In this chapter, I investigate the molecular mechanisms underlying the phenotype I observed in the SVZ after KAP1 and *Paupar* knockdown. I found that KAP1 and *Paupar* can regulate the expression level of target genes through different mechanisms involving signalling pathways, cell cycle regulators, and basic helix-loop-helix (bHLH) proteins.

5.3.1 Physical association between KAP1 and Paupar in SVZ-derived neurospheres

Vance et al. showed that the lncRNA *Paupar* associates with the transcription factor Pax6 which helps in recruiting *Paupar* to chromatin sites around the genome (Vance et al. 2014). Since depletion of *Paupar* did not prevent Pax6 chromatin occupancy (Vance et al., 2014), this suggests that *Paupar* may function to recruit transcriptional cofactors to Pax6 and other transcription factors to control gene expression. Moreover, Mass Spectrometry analysis followed by RNA-IP experiments have validated the binding between *Paupar* and KAP1 in the N2A cell line. Together, *Paupar*, Pax6, and KAP1 exist in the same complex in the chromatin (Pavlaki et al., 2018). In order to gain insight about *Paupar* and KAP1 role in brain development, I investigated if this interaction also exists in vivo in the SVZ in normal conditions compared to cancer. Importantly, we show that *Paupar* directly binds KAP1 in SVZ-derived neurospheres. Similar to this, the Polycomb protein complex also shows similar association with a large number of RNAs (Davidovich et al., 2015). This finding suggests the following:

1-*Paupar* recruits KAP1 to the genome in vivo: It is suggested that lncRNAs can recruit chromatin-modifying complexes to specific binding sites in the genome (Vance

and Ponting, 2014). KAP1 is known to be recruited to the genome within the 3' UTRs of ZNF genes by binding to KRAB-ZNF transcription factors (Iyengar et al., 2011, O'Geen et al., 2007). Nonetheless, Vance et al. found that *Paupar* bound sequences are located at gene promoters that are not enriched for KRAB-ZNFs binding motifs as showed using de novo motif discovery (Vance et al., 2014), and proposed that *Paupar* plays a role in recruiting KAP1 to the genome in a KRAB-ZNF-independent mechanism. Another example is the lncRNA *HOTAIR* which was proposed to recruit PRC2 to specific genomic locations and induce H3K27me3 to silence gene expression (Chu et al., 2011). However, recent conflicting results showed that PRC2 does not associate with lncRNAs and that *HOTAIR* can not directly guide PRC2 to the genome (Portoso et al., 2017).

In this experiment, I have shown that KAP1 and *Paupar* physically associate in vivo, similar to the N2A cell line, however mechanistic studies are missing to further define their role. To test this, *Paupar* expression could be depleted by shRNA vectors and then perform ChIP-qPCR on knockdown and control conditions to measure KAP1 occupancy at target genes. If it was depleted, that would confirm *Paupar* recruitment of KAP1.

2-*Paupar* and KAP1 co-regulate a common set of genes: This binding between KAP1 and *Paupar* suggest that they also co-regulate common genes in vivo. In N2A cells, *Paupar* depletion changes the expression of 942 genes (Vance et al., 2014). The intersection of KAP1 and *Paupar* regulated genes identified 244 genes that were effected by both (Pavlaki et al., 2018). Gene ontology analysis of the 244 genes showed that the coregulated genes involved brain development and essential neuronal cell

functions like synaptic transmission. In the next section, I will discuss KAP1 and *Paupar* transcriptional regulation of target genes in the SVZ.

5.3.2 Selective activation of target genes after KAP1 knockdown

In Chapter 3, I observed that after KAP1 loss, there was a defect in NSCs maintenance in the SVZ which caused the cell identity to shift from stem cells to mature astroglial cells and consequently a reduction of SVZ-OB neurogenesis. In this chapter, I studied the molecular mechanism underlying this observation. Interestingly, I found novel target genes to be regulated by KAP1, which had not been described in the literature on KAP1 roles in organ development. I found that KAP1 KD resulted in a differential expression of BMP2, Hes-5, NeuroD1, and P21 which leads me to the following conclusions that could explain the observed phenotype:

1-First, it has been suggested that BMP2 functions as an anti-neurogenic factor. In support of the phenotype I observed, BMP2 expression was shown to alter the differentiation of proliferative neural progenitor cells from neuronal to an astrocytic lineage in the adult SVZ and in embryonic neuroepithelial cells (Gross et al., 1996, Lim et al., 2000, Nakashima et al., 2001). BMP2 also appears to inhibit neurogenesis in vitro in olfactory epithelial cells (Shou et al., 1999). In addition, I show that KAP1 KD upregulated the expression of the Hes-5 gene, a member of the Notch family. It has been reported that Notch activation caused an irreversible switch from neurogenesis to gliogenesis in neural crest stem cells and adult hippocampus progenitors (Tanigaki et al., 2001, Morrison et al., 2000). Interestingly, a study published by Nakashima et al showed that BMP2 unexpectedly enhances the activation of Hes-5 and Hes-1 genes, downstream of Notch, in mouse embryonic neuroepithelial cells and changed their

developmental fate from neuronal cells to astrocytic cells (Nakashima et al., 2001). Since the expression of Hes-5 genes is regulated by Notch as well as BMP2, I hypothesize a cross-talk between BMP and Notch signalling pathways to be regulated by KAP1 in the SVZ. Synergy and antagonism between BMP and Notch signalling have been observed in various cells. For example, Hes1 and Hey1 are induced by BMP4 in myoblast cells and this induction is important for BMP-mediated inhibition of myoblast differentiation (Dahlqvist et al., 2003). Another example is Hey1, which is induced by BMP2 and negatively regulates osteoblast maturation (Zamurovic et al., 2004). However, in contrast to the findings observed in cells from the developing CNS that are described above, BMP2 may play a different role in the peripheral nervous system. BMP2 induced the expression of the pro-neuronal factor Mash1, which enhanced neuronal differentiation in neural crest stem cells (Shah et al., 1996, Lo et al., 1997), which suggests differential cell fate regulation between the CNS and PNS by BMP2. Nevertheless, the relationship between KAP1, BMP2 and Hes-5 still remains to be further investigated in vivo and vitro in postnatal SVZ neurogenesis.

2-Second, transcription factors (TFs) play major roles in cell fate determination. Neurogenesis for example is regulated by pro-neuronal bHLH transcription factors such as Mash1, Neurogenin, and NeuroD (which was downregulated upon KAP1 loss). These TFs regulate multiple aspects of stem cell biology including cell cycle exit, neural versus glial fate determination, subtype specification and survival (Ohsawa and Kageyama, 2008). These bHLH proteins can be negatively regulated by other HLH proteins, such as Hes-1 and Hes-5 genes (Ohtsuka et al., 1999). Hes genes are repressors for gene expression, they bind to their target DNA sequence and recruit histone deacetylases resulting in transcription repression of bHLH factors and so repress the differentiation of cells (Kageyama and Nakanishi, 1997). In the embryonic

telencephalic neuroepithelial study that was done by Nakashima et al, BMP2 induced the expression of Hes-5, which in turn, inhibited the transcription of neurogenic HLH factors such as Mash1 and Neurogenin (Nakashima et al., 2001). This suggest a similar mechanism could exist in the SVZ. In the future, it will be worthwhile to test if KAP1 suppresses NeuroD1 in vivo in the SVZ through BMP and Notch signalling. Nevertheless, taken together, these data suggest a major role of KAP1 in controlling genes important for neuronal differentiation.

3-Finally, SVZ NSC proliferate at a low rate which is regulated by many cell cycle promotors and inhibitors (Cheung and Rando, 2013). For example, p21, a cyclin-dependent kinase inhibitor (CDKI), also called Cdkn1a, antagonizes the actions of Cdk1 and Cdk2. P21 can directly inhibit the expression of Sox2, a regulator of NSCs maintenance, and so regulate NSC self-renewal and multipotency (Marques-Torrejon et al., 2013). In experiments done on cultured glioma cells, down-regulation of KAP1 increased p21 expression and induced cell cycle arrest at the G1 phase (Qi et al., 2016b). Consistent with these observations, I found that KAP1 loss in SVZ NSCs caused an increase in the expression of p21, which could explain the phenotype observed of reduced NSC pool. Taken together, these results suggest that KAP1 functions on different mechanisms to regulate neurogenesis.

5.3.3 KAP1 has diverse roles in transcription regulation

Although it is known that KAP1 regulates the expression of target genes by chromatin remodelling and DNA methylation, the specificity of KAP1 gene regulation in certain conditions is still not clear. My analysis of KAP1 KD in the SVZ derived neurospheres showed upregulation (BMP2, Hes5, P21) and downregulation (NeuroD1) of different

transcripts. This is interesting considering that KAP1 is generally known for its repressive functions. This can be explained by the following:

Context-dependent function of KAP1 in the SVZ: KAP1 is known to interact with histone methyltransferase and HDAC complexes. It was originally proposed that KAP1 binds to ZNFs and recruits HP1 and SETDB1 proteins that marks H3K9me3 (O'Geen et al., 2007, Groner et al., 2010). This creates a heterochromatin environment at target regions and so based on this, it is mostly predicted that KAP1 functions as a repressor of transcription. However, recently KAP1 was shown to interact with and cause transcription activation via other histone methyltransferase (HMTs), HATs, HDACs, and DNA methyltransferases (DNMTs) (Iyengar and Farnham, 2011, Groner et al., 2010, O'Geen et al., 2007). Therefore, KAP1 can function to regulate transcription through several mechanisms, possibly depending on its location, binding partners, and post-translation modifications (PTMs). For example, DNA damage such as double-strand breaks induces ataxia telangiectasia mutated (ATM), which phosphorylates KAP1 and results in loss of SUMOylated KAP1, causing de-repression of KAP1 target genes like *p21, Gadd45a, Bax, Pumam and Noxa* that are involved in apoptosis and cell cycle arrest (Li et al., 2007, Cheng et al., 2014b). This suggests that KAP1 functions according to a balance between a SUMOylated and a phosphorylated state, which influence its repression/activation functions of target genes expression. Moreover, it could be that the HDACs and histone methyltransferase complexes that KAP1 binds to could help in releasing repressor proteins that are bound to target genes. It could be that similar mechanism is applied to SVZ derived NSCs which explains the selective gene activation observed after KAP1 KD.

Formation of various transcriptional complexes/ different KAP1 recruitment mechanisms in the SVZ: Interestingly, it has been reported that different proteins with KRAB domains, but not DNA binding domains, are also capable of functioning as a bridge for KAP1 gene regulation. For example, the KRAB Only (KRAB-O) protein can recruit KAP1 to the sex determination transcription factor SRY to regulate SRY target genes (Peng et al., 2009). It appears that KAP1 is capable of forming different transcriptional complexes with proteins and lncRNAs to recruit it to the genome. Identification of these proteins that KAP1 binds to in SVZ NSCs is required to understand the functional mechanism each complex can play. That could also explain the differential gene regulations that I observed in the SVZ.

Indirect effect of KAP1: Chip-seq experiments followed by RNA-seq have identified thousands of KAP1 binding sites that allowed to observe a genome-wide effect of KAP1. Although some genes were strongly regulated after KAP1 knockdown, the majority of them were not bound by KAP1, suggesting that the effects of KAP1 on the genome is mostly indirect (Iyengar et al., 2011). For example, because KAP1 strongly binds the 3' end of ZNFs, one would expect that KAP1 regulates their expression. Interestingly, there is no change in ZNFs mRNA levels in NTera1 cells upon KAP1 knockdown (Iyengar et al., 2011). In support of this, KAP1 knockdown did not cause a reduction in *Paupar* transcript level in my cells. Thus, KAP1 could also function indirectly in neural stem cells to regulate target genes. To confirm this, a ChIP-qPCR experiment in SVZ derived NSCs on gene loci of BMP2, Hes-5, NeuroD1, and P21 would be necessary to confirm KAP1 binding to their promoter regions.

5.3.4 The lncRNA Paupar in regulating SVZ neurogenesis

The extent to which lncRNAs function to regulate the genome remains not clear. In the N2A cell line, Vance et al. showed that KD of *Paupar*, chromatin-associated CNS expressed lncRNA, resulted in significant changes in the expression of ~900 genes. These genes are involved in cell cycle control, synaptic function, DNA replication and mitosis, chromatin modification and chromosome organization. Furthermore, *Paupar* binds and regulates the expression of Sox2, Hes1, and E2F2 genes in trans, in addition to regulating the expression of Pax6 in cis (Vance et al., 2014). Here, I have used the shRNA knockdown method to understand the action of *Paupar* on gene regulation. Similarly, my finding showed that *Paupar* transcript significantly regulates the expression of key stem cell genes like Sox2, Hes1, and EGF, as well as genes known for controlling neural growth and differentiation like E2f2, Igfbp5, Pax6 genes.

Sox2, Hes1, EGF, Igfbp5: In the SVZ, I found that *Paupar* maintains stem cell quiescence and restrict lineage progression from stem cells to TAPs. In support of this, I found that *Paupar* KD downregulated the expression of Sox2, Hes1, EGF, and Igfbp5 genes. They play well characterized and major roles in neural stem cell maintenance and neurogenesis which could explain the observed phenotype (Pevny and Nicolis, 2010, Kageyama et al., 2008, Tropepe et al., 1999, Arsenijevic et al., 2001). Moreover, Vance at al. also identified *Paupar* binding sites and their analysis revealed enrichment of *Paupar* peaks in Sox2, Hes1, Hes5, and Nanog stem cell genes, as well as neuronal gene categories and insulin secretion genes (Vance et al., 2014).

E2F2 and KAP1: The role of the E2F2 gene in neurogenesis has been less studied. I found that E2F2 was upregulated upon *Paupar* KD in neurospheres, similar to N2A cells (Vance et al., 2014). E2F2,a cell cycle regulator, plays a pro-survival role in retinal development (Chen et al., 2009a), and functions to maintain post-mitotic neurons in a

differentiated state in cell culture (Persengiev et al., 2001). E2F2 overexpression in PC12 neurons promotes cell cycle arrest and prevents S-phase progression (Persengiev et al., 2001). Furthermore, *Paupar* KD in N2A cells caused the accumulation of cells in S and G2 phases of the cell cycle (Vance et al., 2014).Therefore, upregulation of E2F2 after *Paupar* KD may have contributed to the observed phenotype. Importantly, I observed a reduction in the level of KAP1 transcript upon *Paupar* depletion which suggests that *Paupar* regulates the expression of KAP1 gene.

Pax6: It has been shown that *Paupar* is targeted to the genome through RNA-protein interactions with different neural transcription factors including Pax6 in N2A cells. The published data indicate that *Paupar* regulates the expression of Pax6. *Paupar* and Pax6 regulate the expression of common and distinct genes, and together the co-occupy specific genomic sites (Vance et al., 2014). Given this, I sought to determine if *Paupar* regulates Pax6 expression in the SVZ as well. While in N2A cells they observed an upregulation of Pax6 expression after transient reduction in *Paupar* RNA levels, I observed an opposite effect after *Paupar* KD in the neurospheres.

Neural progenitor cells reduce Pax6 expression as they become neurogenic (Hsieh and Yang, 2009) and Pax6 can auto-regulate its own expression (Aota et al., 2003, Manuel et al., 2007). Together, these data explain Pax6 downregulation after *Paupar* KD and supports the phenotype observed. Also, it suggests that *Paupar* plays a different role on the SVZ compared to N2A cells.

5.4 Conclusion

In conclusion, I identified novel regulatory mechanisms in SVZ NSCs. Surprisingly, even though KAP1 and *Paupar* exist in the same ribonucleoprotein complex, they seem to function on different regulatory mechanisms to regulate different set of target genes. KAP1 controlled BMP signalling directly to maintain NSCs fate. While *Paupar* function to regulate the expression of key stem cell genes like Sox2, Hes1, and EGF, as well as genes known for controlling neural growth and differentiation like E2f2, Igfbp5, Pax6 genes to maintain stem cell quiescence and restrict lineage progression from stem cells to TAPs.

Chapter 6

General Discussion

6.1	**Overview of the current work**	**200**
6.2	**Limitations of the current study**	**204**
6.3	**Future directions**	**208**

In this project, I investigated the roles of two epigenetic regulators, KAP1 and the lncRNA *Paupar* in postnatal and adult SVZ neurogenesis. Even though KAP1 and *Paupar* exist in the same ribonucleoprotein complex, they were shown to have different functional effects in the SVZ. KAP1 is required for SVZ NSC identity maintenance, and functions to inhibit the NSC from acquiring mature astrocytic fate. In contrast, *Paupar* was shown to maintain stem cell quiescence and restrict lineage progression from stem cells to TAPs. On the molecular level, I hypothesize that KAP1 controls BMP signalling directly to maintain NSCs fate. *Paupar*, in turn, functions to regulate the expression of key stem cell genes like Sox2, Hes1, and EGF, as well as genes known for controlling neural growth and differentiation. Taken together, these results suggest important roles of KAP1 and *Paupar* in SVZ neurogenesis.

6.1 Overview of the current work

Even though there was a common belief that neurons and glial cells were derived from different pools of progenitor cells and that no new neurons can be produced in the adult brain. This assumption was challenged by the discovery of neurogenesis in two niches, the SVZ and the SGZ, which were shown to contain neural stem cells. Interestingly, stem cells in these regions exhibit some biological markers and structural similarities of astrocytes (Ihrie and Alvarez-Buylla, 2011, Kriegstein and Alvarez-Buylla, 2009). However, the molecular mechanisms that regulate the differences between NSCs and astrocytes remain mysterious. SVZ NSCs can proliferate and enter the neurogenic

lineage to produce neurons while astrocytes are known for their supportive functions (Wang and Bordey, 2008).

Members of the BMP signalling pathway are responsible for SVZ NSC maintenance and lineage commitment. They promote astroglial lineage commitment of EGF-amplified cells from the embryonic and postnatal SVZ (Gross et al., 1996, Mehler et al., 2000). BMP signalling inhibits precursor cells in the SVZ from producing neuroblasts while inducing the differentiation of glial cells (Lim et al., 2000). In addition, BMP signalling induces the expression of RE1 silencer of transcription/neuron-restrictive silencer factor (REST/NRSF) which in turn supresses the expression of neuronal genes during the life of the astrocyte (Kohyama et al., 2010). In addition, Notch signalling in neural crest stem cells and adult hippocampal progenitors caused a switch from neurogenesis to gliogenesis (Tanigaki et al., 2001, Morrison et al., 2000). Importantly, a cross-talk between Notch and BMP signalling was observed in different cells, for example, BMP4 was shown to induce Hes1 and Hey1 members of the Notch family which causes inhibition of myoblast differentiation (Dahlqvist et al., 2003). Consist with this, a study done in mouse embryonic neuroepithelial cells showed that BMP2 directly induces the activation of Hes-5, downstream of Notch, which in turn, inhibited the transcription of neurogenic transcription factors such as Neurogenin and changed cell fate from neuronal to astrocytic cells (Nakashima et al., 2001). In my work, I showed that loss of KAP1 lead to increased expression of the S100β marker in GFAP+ cells in the SVZ and also caused misexpression of BMP2, Hes5, and NeuroD1 genes. This suggests that cross-talk between BMP and Notch signalling regulates the impairment of NSC fate which could be mediated by KAP1.

Moreover, KAP1 loss also upregulated the expression of p21, a cyclin-dependent kinase inhibitor. P21 can directly inhibit the expression of Sox2 and control NSC self-renewal and multipotency (Marques-Torrejon et al., 2013). In line with my observation, down-regulation of KAP1 in glioma cells increased p21 expression and induced cell cycle arrest (Qi et al., 2016b). Interestingly, BMP2 significantly upregulated the expression of p21 which in turn inhibited estradiol-induced proliferation of human breast cancer cells (Ghosh-Choudhury et al., 2000). Taken all together, the identification of KAP1 regulatory pathways in this thesis has provided a better understanding of how KAP1 can regulate the stemness of NSCs in the SVZ niche.

The lncRNA *Paupar* is a related epigenetic regulator investigated in this thesis. I have shown that *Paupar* physically associate with KAP1 in SVZ-derived neurospheres, similar to N2A cell line where *Paupar*, KAP1 and Pax6 together form a ribonucleoprotein complex, as shown by our collaborator K. Vance (Univ. Bath) (Pavlaki et al., 2018). My observation suggests that *Paupar* may recruit KAP1 to the genome in NSCs and possibly function in a KRAB-ZNF-independent way. This is in line with recent studies that proposed different scenarios of lncRNAs recruiting chromatin-modifying complexes to specific binding sites (Vance and Ponting, 2014). KAP1 was thought to be recruited to the genome only by binding to KRAB-ZNF transcription factors (Iyengar et al., 2011, O'Geen et al., 2007). Yet, Vance et al. found that *Paupar* recruits KAP1 to gene promoters that are not enriched for KRAB-ZNFs binding motifs (Vance et al., 2014).

Given this physical interaction between *Paupar* and KAP1, I worked toward understanding if they function similarly. Interestingly, I found that even though they showed a similar phenotypic result of reduction in OB neurogenesis, they displayed

different functional roles in regulating NSC behaviour in the SVZ which lead to reduction of neurons arriving to the OB at later time points. This suggests that even if they exist in the same ribonucleoprotein complex, they function on different molecular mechanistic pathways to regulate different sets of genes (Figure 6.1).

My data indicate that *Paupar* maintains stem cell quiescence and restricts lineage progression from stem cells to TAPs by regulating the expression of key stem cell genes as well as genes known for controlling neural growth and differentiation. This result is consistent with previous observations of many CNS- expressed lncRNAs that also resulted in reduction of neurogenesis after their knockdown (Lin et al., 2014, Ng et al., 2013, Chalei et al., 2014, Bond et al., 2009). These results provide strong evidence for the biological function of lncRNAs in CNS development and challenge the previous assumption that lncRNA's are transcriptional "junk". Taken all together, I showed for the first time that KAP1-*Paupar* lncRNA chromatin regulatory complex controls specific aspects of subventricular zone development.

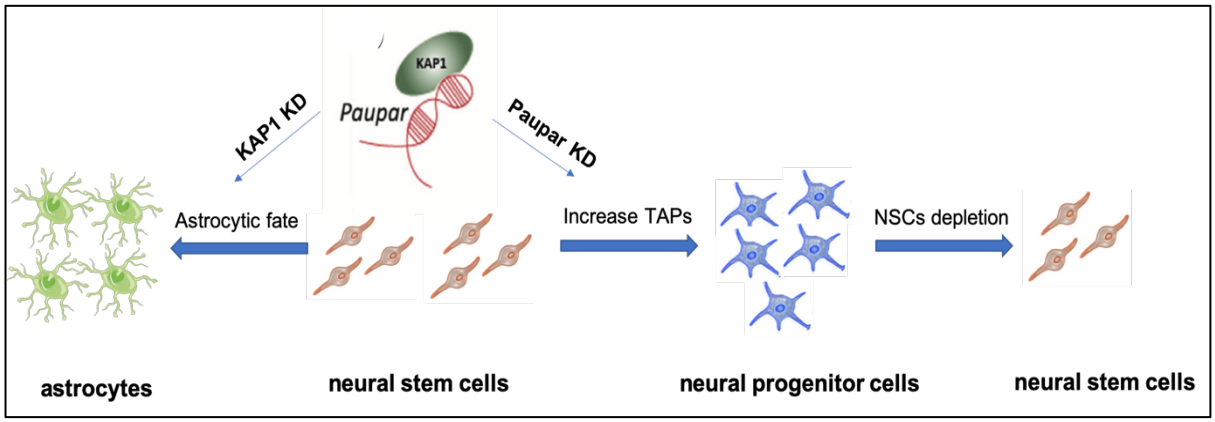


Figure 6.1 Proposed model of KAP1-*Paupar* lncRNA chromatin regulatory complex in regulating subventricular zone development

KAP1 and *Paupar* exist in the same ribonucleoprotein complex. They were shown to have different functional roles in regulating SVZ neurogenesis. KAP1 is required for SVZ NSC identity maintenance, and functions to protect the NSC from acquiring a mature astrocytic fate. While *Paupar* maintains stem cell quiescence and restricts lineage progression from stem cells to TAPs.

6.2 Limitations of the current study

Despite the work I have done to investigate the role of KAP1 and *Paupar* in SVZ neurogenesis, I recognize some possible limitations in the work that I discuss below. I plan to explore these limitations and address them in my future studies.

First. I used an inducible Cre/LoxP transgenic mouse model for the functional analysis of KAP1 in this thesis which allowed quantifiable assessment of the contribution of nestin-expressing stem cells to SVZ-OB neurogenesis. It is important to take into consideration that it is an incomplete knockout system (Chen et al., 2009b) and so the biological effect observed may be underestimated. The surrounding normal non-knockout cells may have repaired the defect in the niche which could be the reason of some of the negative data observed in this thesis rather than a subtle role of KAP1. Moreover, I have used Nestin-CreERT2-YFP; KAP1$^{fl/fl}$ reporter mice that expresses yellow fluorescent protein (YFP) upon Cre-mediated recombination, however, the association between knockout and the YFP marker is not always accurate and we could not always confirm that Cre-recombination occurred successfully in both floxed alleles. The knockout of the floxed-stop cassette in one allele is sufficient to express YFP marker, while the homozygous recombination and knockout of the target genes in the

two alleles may have been needeeded for full functional effects. To resolve this problem, a Nestin-CreERT2-YFP; KAP1$^{fl/-}$mice model should be generated which consists of one floxed allele and one constitutive knockout allele as follows:

1-Cross KAP1$^{fl/+}$ with a universal Cre line (like Rosa26, CAG promoter driving Cre) to knockout one allele of KAP1 in all the animals including germ cells. Cre; KAP1$^{-/+}$ will be crossed with KAP1$^{fl/fl}$ to generate KAP1$^{fl/-}$ without the universal Cre knock-in.

2- The mating between KAP1$^{fl/-}$ and Nestin-CreERT2-YFP; KAP1$^{fl/fl}$ produces Nestin-CreERT2-YFP; KAP1$^{fl/-}$. With this line, we can make sure that the YFP marker will label homozygous knockout cells.

CRISPR/Cas9 technology is another method for genetic manipulation. By delivering a complex composed of Cas9 nuclease and a guide RNA into the cell, it can cause DNA double-strand break at a specific DNA sequence and so genomic editing could be achieved (Sander and Joung, 2014). This method allows for rapid generation of transgenic mice models and could be used to also resolve the issue of heterozygous knockouts.

Second, I used electroporation and nucleofection methods for my in vivo and in vitro knockdown studies to deliver shRNA plasmids into the cells. However, one should keep in mind that the dilution of the shRNA and its transient effect could interfere with lineage analysis. For example, KAP1 knockdown causes defects in neuronal differentiation, but this is be due to a lineage shift towards a glial lineage. To confirm this, in vitro clonal differentiation assay could be used to test stem cell pluripotency; however, it may not be applicable with shRNA plasmids. One solution for this problem

is the use of lentivirus or retrovirus mediated knockdown of KAP1 which provides a stable integration of the construct into the genome of the host cell. Moreover, cellular barcoding is an alternative method available for the purpose of lineage tracing and fate mapping. Individual cells are labelled with a unique nucleic acid sequence (barcodes) that can be tracked through time and space (Szele and Cepko, 1996, Goings et al., 2004). The advantage of this method is that the barcodes are permanent, and a large population of cells can be efficiently and cost-effectively traced at the individual level (Kebschull and Zador, 2018).

Third, different loss-of-function methods could be used to study the role of lncRNAs, however, their distinct genomic loci make it difficult sometimes to apply these methods. For example, I used RNAi to knockdown *Paupar* in vivo and in vitro. Yet, it is important to take into consideration that RNAi is usually used to knockdown protein-coding transcripts and functions post-transcriptionally to inhibit the translation of mRNA in the cytoplasm, whereas *Paupar* is expressed and functions in the nucleus. Although some active RNAi factors were observed in the nucleus, it remains unclear how nuclear lncRNAs are affected by them. Another method is to manipulate the lncRNA locus by inserting exogenous sequences like the transcriptional terminator sequence, loxP, or selection cassettes. However, this manipulation might affect the spacing between DNA regulatory elements such as promoters and enhancers which can cause changes in phenotypes. Another method includes mutation or deletion of the full lncRNA locus or its promoter sequence, but these methods are not always successful. Deletion of the promoter may affect the expression of some protein-coding genes which the lncRNA shares the same promoter with, also some lncRNAs are initiated within enhancers, and disrupting the lncRNA promoter could cause changes in gene expression.

Antisense oligonucleotides could be an alternative method for studying lncRNA function. They form DNA/RNA complexes with the RNA transcript in the nucleus and trigger RNase H-dependent disruption of the RNA. However, off-target effects of this method are still not clearly understood. Recent molecular biology techniques like the transcription activator-like effector (TALE) and the clustered regularly interspersed palindromic repeats (CRISPR) allow the recruitment of transcriptional activators or repressors to the genome and interfere with lncRNA transcription, but the effect of these factors on the transcription of neighbouring genes must be taken into consideration (Bassett et al., 2014).

Fourth, I have concluded that in the adult SVZ of KAP1 cKO mice, loss of NSCs stem-ness and a shift to niche astrocytes was observed, I showed increased expression of the S100β marker in GFAP+ cells. S100β labels niche and parenchymal astrocytes and ependymal cells (Ihrie and Alvarez-Buylla, 2011, Raponi et al., 2007). However, it has also been observed that S100β is expressed in quiescent NSC when compared to active NSC in the SVZ (Codega et al., 2014). Therefore, it could be that the increase in the S100β marker expression that I observed is due to cell defect in exiting the quiescent state instead of shifting of NSC into an astrocytic lineage. So far there are not enough specific markers that can accurately differentiate between NSCs and mature astrocytes. The most reliable way is by observing cell morphology which can be achieved by whole mount preparation method which allow the pinwheel structure of the LV wall to be clearly imaged (Codega et al., 2014) and in the future this should be carried out.

Finally, it is important to take into consideration that even though KAP1 increased BMP signalling, and at the same time induced astrocytic fate, causality between the molecular mechanism and the cellular phenotype observed was not proven and cannot be

supported by this study alone. That is KAP1 was certainly responsible for the phenotype observed, however, my data is lacking causative proof that KAP1 promotes the astrocytic fate shift via BMP signalling. In order to prove the association, KAP1 knockdown could be accompanied by inhibition of BMP signalling before astrocytic fate is assessed.

6.3 Future directions

In this thesis, the roles of KAP1 and *Paupar* in SVZ neurogenesis were studied. Their interaction is described in Chapter 5 and in our study published by (Pavlaki et al., 2018). More work could be done in the SVZ to further investigate the mechanistic and functional cooperation between KAP1 and *Paupar*. For future work, the following will be carried out.

6.3.1 Identify cell subtype specific functions of KAP1 and define functional cooperation between KAP1 and Paupar in SVZ neurogenesis

KAP1 is expressed throughout the whole SVZ neural-lineage as shown in (Chapter 3, Result 3.2.1), which suggests that it may affect neurogenesis at multiple points. I showed that KAP1 is necessary for SVZ NSC identity maintenance, and its loss causes NSC to shift to a mature astrocytic fate and eventually leads to an inhibition of new-born neurons arriving from the SVZ to the OB. This has led to hypothesize that KAP1 depletion may affect several stages during SVZ lineage progression. KAP1 may regulate different genes during lineage progression and therefore I plan to identify the

specific function of KAP1 in different SVZ cell subtypes using functional studies and single cell transcriptomics.

I will electroporate the SVZ of P1 C57Bl/6 pups with KAP1 targeting shRNA-GFP expression vector and a scramble control. Electroporated GFP+ cells expressing the shRNA will then be FACS sorted at 1, 3, and 7 days post electroporation (dpe). At 1 dpe primarily NSCs are electroporated, at 3 dpe NSC and transit amplifying progenitors and by 7 dpe these cells and migrating neuroblasts will be knocked down. The 10X Genomics platform will then be used to prepare 3000 single cells per sample for transcriptomics analysis, in collaboration with Dr Rory Bowden at the Oxford Genomics Centre. Libraries will be sequenced to classify different cell subtypes in the SVZ based on the expression signature. Comparison between control and KAP1 KD will then be made to define cell subtype specific functions of KAP1 in NSC self-renewal and SVZ lineage differentiation.

Next, I will further study *Paupar* function. *Paupar* knockdown in the SVZ reduces the number and alters the morphology of new-born neurons that have migrated to the OB in a pattern similar to KAP1. In addition, *Paupar* lncRNA physically interacts with KAP1 in vivo, but whether this physical interaction is required for their SVZ neurogenesis regulation remains to be tested directly. Since I have shown that KAP1 and *Paupar* regulate different molecular pathways, this predicts that the reduction of both will result in a greater effect on SVZ neurogenesis than loss of either one alone. To test this, I will electroporate KAP1 and *Paupar* targeting shRNA expressing constructs to knockdown both in the SVZ of postnatal mouse. Then I will carry out a detailed characterization of SVZ proliferation and lineage progression analysis at different time points using

phenotype marker immunohistochemistry and label retaining techniques to describe *Paupar*-KAP1 functional cooperation in the regulation of SVZ neurogenesis.

6.3.2 Define phosphorylation dependent control of KAP1 function during neurogenesis

KAP1 phosphorylation at two major sites, S473 and S824, acts as a molecular switch to facilitate cofactor interactions and mediate KAP1 gene regulatory functions. For example, PKC dependent S473 phosphorylation disrupts KAP1-HP1 binding, thus activating cellular proliferation genes (Chang et al., 2008). Preliminary RNA-IP data generated from our collaborator Dr. Keith Vance shows that *Paupar* strongly interacts with the phospho-mimetic KAP1 S473D mutant but does not bind non-phosphorylatable KAP1 S473A in N2A neuroblastoma cells. In contrast, S824 phosphorylation does not effect *Paupar* binding (Figure 6.2). This interesting data shows for the first time that lncRNA-protein interactions can be regulated by phosphorylation and suggests that KAP1 S473 phosphorylation may be required for the formation of KAP1-*Paupar* chromatin regulatory complex in the SVZ.

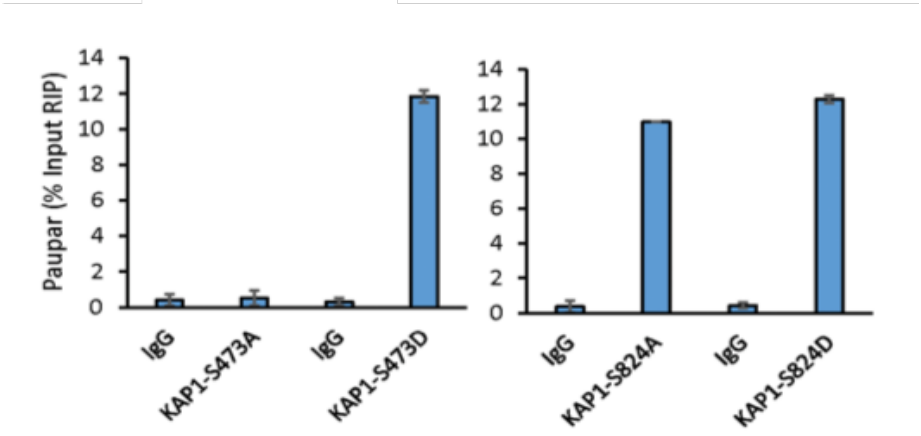


Figure 6.2 KAP1 S473 phosphorylation regulates the association with *Paupar*

Paupar strongly binds KAP1 S473D phosphomimetic but not a S473A non-phosphorylatable mutant in a RNA-IP. Phosphorylation at S824 has no effect on *Paupar*-KAP1 binding.

To identify the function of KAP1 S473 phosphorylation in SVZ lineage progression, I will test whether electroporation of phospho-mimetic and non-phosphorylatable KAP1 mutants affect neurogenesis and if they can rescue cell type specific effects of KAP1 knockdown identified above in my first future plan aim. Constructs expressing wild type, S473D and S473A KAP1 mutants will be co-expressed with control scrambled shRNAs or shRNAs targeting endogenous KAP1 in the SVZ of postnatal mice. A thorough in vivo cellular characterization using immunohistochemistry will then be performed to investigate how phospho-mimetic and non-phosphorylatable KAP1 affect neurogenesis and rescue cell type specific effects of KAP1 knockdown.

I also hypothesize that KAP1 S473 phosphorylation coordinates the differential activation or repression of specific gene sets needed for NSC self-renewal and neurogenesis. To test this, the same constructs will be transfected in vitro in neurospheres generated from the SVZ and then used to investigate phospho-KAP1 regulation with RT-qPCR. These experiments will define the function of KAP1 S473 phosphorylation in SVZ lineage progression and the molecular basis of phospho-KAP1 S473 control of gene regulatory programmes important for neurogenesis.

6.3.3 KAP1 and Paupar in glioblastoma

Glioblastoma is a highly malignant type of brain tumour closely associated with the adult SVZ (Lim et al., 2007). It is believed that progenitor cells that originate from SVZ NSC can transform into cancer stem cells (CSC) (Sanai et al., 2005, Bardella et al., 2016). The molecular differences between SVZ NSC and glioblastoma CSC is still not

clear. A study identified the transcriptome and chromatin modification in glioblastoma CSC and NSC (Rheinbay et al., 2013). However, their work was done on human ES cell-derived NSC, which may not represent the behaviours and features of NSC in vivo.

It has been shown that KAP1 expression was significantly higher in glioma patients and was associated with poor overall survival rate. Knockdown of KAP1 in glioma cells lead to increased p21 expression and caused cell cycle arrest. In a xenograft model, downregulation of KAP1 inhibits tumour growth and proliferation (Qi et al., 2016b). Additionally, it has been suggested that KAP1 is a direct target of microRNAs. The miR-491 and KAP1 were abnormally expressed in glioblastoma tissues. Inhibition of miR-491 lead to increased KAP1 expression and induced glioma cells proliferation (Qi et al., 2016a). I showed that KAP1 negatively regulates members of the BMP signalling in the SVZ. It would be interesting to know if this negative regulatory relationship is preserved in glioblastoma.

On the other hand, many lncRNAs have been identified to be associated with glioma. For example, lncRNA *HOTAIR* expression was upregulated in glioblastoma samples, and its expression was related with glioma grade and considered as a prognostic marker in glioblastoma patients (Zhang et al., 2013). *HOTAIR* interacts with EZH2 in glioma cells, and knockdown of *HOTAIR* or EZH2 prevent cell cycle progression.(Zhang et al., 2015a). In an orthotopic model, knocking down *HOTAIR* inhibited glioblastoma cell invasion and migration (Zhou et al., 2015). Indeed, the heterogeneity of glioblastoma causes differences in lncRNAs expression in stem cell population. More analysis of SVZ NSC and CSC should provide better insights into lncRNA expression and function in tumorigenesis. Importantly, KAP1-*Paupar* interaction was initially observed in N2A neuroblastoma cell lines and identified their regulation of neuronal genes (Pavlaki et al.,

2018, Vance et al., 2014). In the future, it would be interesting to investigate the functional role of KAP1 and *Paupar* in glioblastoma progression in human brain tissue sections from glioblastoma cases and controls as well as primary cultures. Many questions have raised from this thesis and pursuing them will provide more insights toward an understanding of the cell biology of SVZ neurogenesis.

APPENDIX

Table 1 Supplemental recipes for ChIP solutions

Buffer	Stock	Amount
Cold Hypotonic Buffer		**For 50ml:**
10 mM Tris-HCl pH 7.2	1M	500µl
10 mM KCl	1M	500µl
1.5 mM $MgCl_2$	1M	75µl
0.5% NP40	10%	2.5ml
H_2O	-	46.425ml
1x Protease Inhibitor Cocktail	-	20µl / ml
Sonication Buffer		**For 25ml:**
50 mM Hepes KOH pH 7.9	1M	1.25ml
10 mM EDTA pH 8	0.5M	500µl
1% SDS	10%	2.5ml
H_2O	-	20.75ml
1x Protease Inhibitor Cocktail	-	20µl / ml
IP Dilution Buffer		**For 50ml:**
10 mM Hepes KOH pH 7.9	1M	500µl
1% Triton X-100	20%	2.5ml
150 mM NaCl	5M	1.5 ml
	0.5M	10 ul

1 mM EDTA pH 8 0.1% SDS 1x Protease Inhibitor Cocktail H_2O	10% - -	500 ul 20 ul/ml 44.9 ml
Wash Buffer I 0.1% SDS 1% Triton X-100 2 mM EDTA 20 mM Tris-HCl pH 8.1 150 mM NaCl H_2O	 10% 20% 0.5M 1M 5M -	**For 50ml:** 500µl 2.5ml 200µl 1ml 1.5ml 44.3ml
Wash Buffer II 0.1% SDS 1% Triton X-100 2 mM EDTA 20 mM Tris-HCl pH 8.1 500 mM NaCl H_2O	 10% 20% 0.5M 1M 5M -	**For 50ml:** 500µl 2.5ml 200µl 1ml 5ml 40.8ml
Wash Buffer III 0.25 M LiCl 1% NP-40 1% Sodium Deoxycholate 1 mM EDTA 10 mM Tris-HCl pH 8.1	 Mw 42.4 10% (or 100% stock) 10% 0.5M 1M	**For 50ml:** 0.53g 5ml (or 500µl) 5ml 100µl 500µl
TE Buffer 10 mM Tris-HCl pH 8.1 1 mM EDTA H_2O	 1M 0.5M -	 500µl 100µl 49.4ml
ChIP Elution Buffer 1% SDS 0.1M $NaHCO_3$ H_2O	 10% 1M -	**For 10. ml:** 1 ml 1 ml 8 ml
Blocking Solution BSA tRNA	 20mg / ml 10 mg / ml	**For 1ml:** 10µl 20µl

PBS or IP dil. buffer	-	970µl
Proteinase K Treatment: **5x Proteinase K buffer** 50mM Tris-HCl ph 7.5 25mM EDTA 1.25% SDS **For ChIP, use per IP:** 98µl H2O 25µl 5x Prot K Buffer 2µl Prot K	 1M 0.5M 10%	 **For 1ml:** 50µl 50µl 125µl

Table 2 Supplemental recipes for common solutions

Buffer	Abbreviation	Amount
PBS 10X 1M pH 7.4 Sodium chloride Potassium choloride Potassium dihydrogen phosphate Sodium phosphate dibasic anhydrous	 NaCl KCl KH2PO4 Na2HPO4 dH2O	 80 g 2 g 2.4 g 14.4 g to 1000 ml
PB 0.1 M pH 7.4 **Monobasic Solution A (0.2 M)** Sodium phosphate monobasic **Dibasic Solution B (0.2 M)** Sodium phosphate dibasic anhydrous	 NaH2PO4 dH2O Na2HPO4 dH2O	 27.8 g to 1000 ml 28.4 g to 1000 ml
PB (0.1 M) Monobasic Solution A (0.2 M) Dibasic Solution B (0.2 M)	 NaH2PO4 Na2HPO4 dH2O	 140 mL 360 mL to 1000 mL

Cryoprotectant Sucrose Ethylene glycol PB 0.1 M		 300 g 300 mL to 1000 mL
PFA 4% Paraformaldehyde PBS 0.1 M		 20 g to 1000 mL

REFERENCES

ADDISON, J. B., KOONTZ, C., FUGETT, J. H., CREIGHTON, C. J., CHEN, D., FARRUGIA, M. K., PADON, R. R., VORONKOVA, M. A., MCLAUGHLIN, S. L., LIVENGOOD, R. H., LIN, C. C., RUPPERT, J. M., PUGACHEVA, E. N. & IVANOV, A. V. 2015. KAP1 promotes proliferation and metastatic progression of breast cancer cells. *Cancer Res,* 75**,** 344-55.

AGUIRRE, A., RUBIO, M. E. & GALLO, V. 2010. Notch and EGFR pathway interaction regulates neural stem cell number and self-renewal. *Nature,* 467**,** 323-7.

ALVAREZ-BUYLLA, A. & GARCIA-VERDUGO, J. M. 2002. Neurogenesis in adult subventricular zone. *J Neurosci,* 22**,** 629-34.

ALVAREZ-BUYLLA, A. & LIM, D. A. 2004. For the long run: maintaining germinal niches in the adult brain. *Neuron,* 41**,** 683-6.

ALVAREZ-BUYLLA, A., LING, C. Y. & YU, W. S. 1994. Contribution of neurons born during embryonic, juvenile, and adult life to the brain of adult canaries: regional specificity and delayed birth of neurons in the song-control nuclei. *J Comp Neurol,* 347**,** 233-48.

AMARAL, P. P. & MATTICK, J. S. 2008. Noncoding RNA in development. *Mamm Genome,* 19**,** 454-92.

AOTA, S., NAKAJIMA, N., SAKAMOTO, R., WATANABE, S., IBARAKI, N. & OKAZAKI, K. 2003. Pax6 autoregulation mediated by direct interaction of Pax6 protein with the head surface ectoderm-specific enhancer of the mouse Pax6 gene. *Dev Biol,* 257**,** 1-13.

ARSENIJEVIC, Y., WEISS, S., SCHNEIDER, B. & AEBISCHER, P. 2001. Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. *J Neurosci,* 21**,** 7194-202.

AYALA, R., SHU, T. & TSAI, L. H. 2007. Trekking across the brain: the journey of neuronal migration. *Cell,* 128**,** 29-43.

BANNISTER, A. J. & KOUZARIDES, T. 2011. Regulation of chromatin by histone modifications. *Cell Res,* 21**,** 381-95.

BARDELLA, C., AL-DALAHMAH, O., KRELL, D., BRAZAUSKAS, P., AL-QAHTANI, K., TOMKOVA, M., ADAM, J., SERRES, S., LOCKSTONE, H., FREEMAN-MILLS, L., PFEFFER, I., SIBSON, N., GOLDIN, R., SCHUSTER-BOECKLER, B., POLLARD, P. J., SOGA, T., MCCULLAGH, J. S., SCHOFIELD, C. J., MULHOLLAND, P., ANSORGE, O., KRIAUCIONIS, S., RATCLIFFE, P. J., SZELE, F. G. & TOMLINSON, I. 2016. Expression of Idh1R132H in the Murine Subventricular Zone Stem Cell Niche Recapitulates Features of Early Gliomagenesis. *Cancer Cell,* 30**,** 578-594.

BARNABE-HEIDER, F., MELETIS, K., ERIKSSON, M., BERGMANN, O., SABELSTROM, H., HARVEY, M. A., MIKKERS, H. & FRISEN, J. 2008. Genetic manipulation of adult mouse neurogenic niches by in vivo electroporation. *Nat Methods,* 5**,** 189-96.

BASAK, O., GIACHINO, C., FIORINI, E., MACDONALD, H. R. & TAYLOR, V. 2012. Neurogenic subventricular zone stem/progenitor cells are Notch1-dependent in their active but not quiescent state. *J Neurosci,* 32**,** 5654-66.

BASSETT, A. R., AKHTAR, A., BARLOW, D. P., BIRD, A. P., BROCKDORFF, N., DUBOULE, D., EPHRUSSI, A., FERGUSON-SMITH, A. C., GINGERAS, T. R., HAERTY, W., HIGGS, D. R., MISKA, E. A. & PONTING, C. P. 2014. Considerations when investigating lncRNA function in vivo. *Elife,* 3**,** e03058.

BAYER, S. A. 1983. 3H-thymidine-radiographic studies of neurogenesis in the rat olfactory bulb. *Exp Brain Res,* 50**,** 329-40.

BEER, D. G., KARDIA, S. L., HUANG, C. C., GIORDANO, T. J., LEVIN, A. M., MISEK, D. E., LIN, L., CHEN, G., GHARIB, T. G., THOMAS, D. G., LIZYNESS, M. L., KUICK, R., HAYASAKA, S., TAYLOR, J. M., IANNETTONI, M. D., ORRINGER, M. B. & HANASH, S. 2002. Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med,* 8**,** 816-24.

BELTRAN, M., PUIG, I., PENA, C., GARCIA, J. M., ALVAREZ, A. B., PENA, R., BONILLA, F. & DE HERREROS, A. G. 2008. A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. *Genes Dev,* 22**,** 756-69.

BERGHOFF, E. G., CLARK, M. F., CHEN, S., CAJIGAS, I., LEIB, D. E. & KOHTZ, J. D. 2013. Evf2 (Dlx6as) lncRNA regulates ultraconserved enhancer methylation and the differential transcriptional control of adjacent genes. *Development,* 140**,** 4407-16.

BERGMANN, O., LIEBL, J., BERNARD, S., ALKASS, K., YEUNG, M. S., STEIER, P., KUTSCHERA, W., JOHNSON, L., LANDEN, M., DRUID, H., SPALDING, K. L. & FRISEN, J. 2012. The age of olfactory bulb neurons in humans. *Neuron,* 74**,** 634-9.

BERGMANN, O., SPALDING, K. L. & FRISEN, J. 2015. Adult Neurogenesis in Humans. *Cold Spring Harb Perspect Biol,* 7**,** a018994.

BERNARD, D., PRASANTH, K. V., TRIPATHI, V., COLASSE, S., NAKAMURA, T., XUAN, Z., ZHANG, M. Q., SEDEL, F., JOURDREN, L., COULPIER, F., TRILLER, A., SPECTOR, D. L. & BESSIS, A. 2010. A long nuclear-retained non-coding RNA regulates synaptogenesis by modulating gene expression. *EMBO J,* 29**,** 3082-93.

BIRD, A. 2002. DNA methylation patterns and epigenetic memory. *Genes Dev,* 16**,** 6-21.

BIRD, A. 2007. Perceptions of epigenetics. *Nature,* 447**,** 396-8.

BONAGUIDI, M. A., MCGUIRE, T., HU, M., KAN, L., SAMANTA, J. & KESSLER, J. A. 2005. LIF and BMP signaling generate separate and discrete types of GFAP-expressing cells. *Development,* 132**,** 5503-14.

BOND, A. M., BHALALA, O. G. & KESSLER, J. A. 2012. The dynamic role of bone morphogenetic proteins in neural stem cell fate and maturation. *Dev Neurobiol,* 72**,** 1068-84.

BOND, A. M., VANGOMPEL, M. J., SAMETSKY, E. A., CLARK, M. F., SAVAGE, J. C., DISTERHOFT, J. F. & KOHTZ, J. D. 2009. Balanced gene regulation by an embryonic brain ncRNA is critical for adult hippocampal GABA circuitry. *Nat Neurosci,* 12**,** 1020-7.

BOUTIN, C., DIESTEL, S., DESOEUVRE, A., TIVERON, M. C. & CREMER, H. 2008. Efficient in vivo electroporation of the postnatal rodent forebrain. *PLoS One,* 3**,** e1883.

BOUTIN, C., HARDT, O., DE CHEVIGNY, A., CORE, N., GOEBBELS, S., SEIDENFADEN, R., BOSIO, A. & CREMER, H. 2010. NeuroD1 induces terminal neuronal differentiation in olfactory neurogenesis. *Proc Natl Acad Sci U S A,* 107**,** 1201-6.

BRACKO, O., SINGER, T., AIGNER, S., KNOBLOCH, M., WINNER, B., RAY, J., CLEMENSON, G. D., JR., SUH, H., COUILLARD-DESPRES, S., AIGNER, L., GAGE, F. H. & JESSBERGER, S. 2012. Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. *J Neurosci,* 32**,** 3376-87.

BRADL, M. & LASSMANN, H. 2010. Oligodendrocytes: biology and pathology. *Acta Neuropathol,* 119**,** 37-53.

BRATTAS, P. L., JONSSON, M. E., FASCHING, L., NELANDER WAHLESTEDT, J., SHAHSAVANI, M., FALK, R., FALK, A., JERN, P., PARMAR, M. & JAKOBSSON, J. 2017. TRIM28 Controls a Gene Regulatory Network Based on Endogenous Retroviruses in Human Neural Progenitor Cells. *Cell Rep,* 18**,** 1-11.

BRAZEL, C. Y., ROMANKO, M. J., ROTHSTEIN, R. P. & LEVISON, S. W. 2003. Roles of the mammalian subventricular zone in brain development. *Prog Neurobiol,* 69**,** 49-69.

BREUNIG, J. J., ARELLANO, J. I., MACKLIS, J. D. & RAKIC, P. 2007. Everything that glitters isn't gold: a critical review of postnatal neural precursor analyses. *Cell Stem Cell,* 1**,** 612-27.

BROWN, C. J., HENDRICH, B. D., RUPERT, J. L., LAFRENIERE, R. G., XING, Y., LAWRENCE, J. & WILLARD, H. F. 1992. The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus. *Cell,* 71**,** 527-42.

BUGEON, S., DE CHEVIGNY, A., BOUTIN, C., CORE, N., WILD, S., BOSIO, A., CREMER, H. & BECLIN, C. 2017. Direct and efficient transfection of mouse neural stem cells and mature neurons by in vivo mRNA electroporation. *Development,* 144**,** 3968-3977.

BUTTI, E., CUSIMANO, M., BACIGALUPPI, M. & MARTINO, G. 2014. Neurogenic and non-neurogenic functions of endogenous neural stem cells. *Front Neurosci,* 8**,** 92.

CABILI, M. N., DUNAGIN, M. C., MCCLANAHAN, P. D., BIAESCH, A., PADOVAN-MERHAR, O., REGEV, A., RINN, J. L. & RAJ, A. 2015. Localization and abundance analysis of human lncRNAs at single-cell and single-molecule resolution. *Genome Biol,* 16**,** 20.

CALZOLARI, F., MICHEL, J., BAUMGART, E. V., THEIS, F., GOTZ, M. & NINKOVIC, J. 2015. Fast clonal expansion and limited neural stem cell self-renewal in the adult subependymal zone. *Nat Neurosci,* 18**,** 490-2.

CAMMAS, F., HERZOG, M., LEROUGE, T., CHAMBON, P. & LOSSON, R. 2004. Association of the transcriptional corepressor TIF1beta with heterochromatin protein 1 (HP1): an essential role for progression through differentiation. *Genes Dev,* 18**,** 2147-60.

CAMMAS, F., JANOSHAZI, A., LEROUGE, T. & LOSSON, R. 2007. Dynamic and selective interactions of the transcriptional corepressor TIF1 beta with the heterochromatin protein HP1 isotypes during cell differentiation. *Differentiation,* 75**,** 627-37.

CAMMAS, F., MARK, M., DOLLE, P., DIERICH, A., CHAMBON, P. & LOSSON, R. 2000. Mice lacking the transcriptional corepressor TIF1beta are defective in early postimplantation development. *Development,* 127**,** 2955-63.

CAMMAS, F., OULAD-ABDELGHANI, M., VONESCH, J. L., HUSS-GARCIA, Y., CHAMBON, P. & LOSSON, R. 2002. Cell differentiation induces TIF1beta association with centromeric heterochromatin via an HP1 interaction. *J Cell Sci,* 115**,** 3439-48.

CASTRO, D. S., MARTYNOGA, B., PARRAS, C., RAMESH, V., PACARY, E., JOHNSTON, C., DRECHSEL, D., LEBEL-POTTER, M., GARCIA, L. G., HUNT, C., DOLLE, D., BITHELL, A., ETTWILLER, L., BUCKLEY, N. & GUILLEMOT, F. 2011. A novel function of the proneural factor Ascl1 in progenitor proliferation identified by genome-wide characterization of its targets. *Genes Dev,* 25**,** 930-45.

CESANA, M., CACCHIARELLI, D., LEGNINI, I., SANTINI, T., STHANDIER, O., CHINAPPI, M., TRAMONTANO, A. & BOZZONI, I. 2011. A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell,* 147**,** 358-69.

CHALEI, V., SANSOM, S. N., KONG, L., LEE, S., MONTIEL, J. F., VANCE, K. W. & PONTING, C. P. 2014. The long non-coding RNA Dali is an epigenetic regulator of neural differentiation. *Elife,* 3**,** e04530.

CHAMBERS, C. B., PENG, Y., NGUYEN, H., GAIANO, N., FISHELL, G. & NYE, J. S. 2001. Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. *Development,* 128**,** 689-702.

CHANG, C. W., CHOU, H. Y., LIN, Y. S., HUANG, K. H., CHANG, C. J., HSU, T. C. & LEE, S. C. 2008. Phosphorylation at Ser473 regulates heterochromatin protein 1 binding and corepressor function of TIF1beta/KAP1. *BMC Mol Biol,* 9**,** 61.

CHEN, D., PACAL, M., WENZEL, P., KNOEPFLER, P. S., LEONE, G. & BREMNER, R. 2009a. Division and apoptosis of E2f-deficient retinal progenitors. *Nature,* 462**,** 925-9.

CHEN, J., KWON, C. H., LIN, L., LI, Y. & PARADA, L. F. 2009b. Inducible site-specific recombination in neural stem/progenitor cells. *Genesis,* 47**,** 122-31.

CHEN, L., CHEN, D. T., KURTYKA, C., RAWAL, B., FULP, W. J., HAURA, E. B. & CRESS, W. D. 2012. Tripartite motif containing 28 (Trim28) can regulate cell proliferation by bridging HDAC1/E2F interactions. *J Biol Chem,* 287**,** 40106-18.

CHEN, L., MUNOZ-ANTONIA, T. & CRESS, W. D. 2014. Trim28 contributes to EMT via regulation of E-cadherin and N-cadherin in lung cancer cell lines. *PLoS One,* 9**,** e101040.

CHENG, B., REN, X. & KERPPOLA, T. K. 2014a. KAP1 represses differentiation-inducible genes in embryonic stem cells through cooperative binding with PRC1 and derepresses pluripotency-associated genes. *Mol Cell Biol,* 34**,** 2075-91.

CHENG, C. T., KUO, C. Y. & ANN, D. K. 2014b. KAPtain in charge of multiple missions: Emerging roles of KAP1. *World J Biol Chem,* 5**,** 308-20.

CHESLER, A. T., LE PICHON, C. E., BRANN, J. H., ARANEDA, R. C., ZOU, D. J. & FIRESTEIN, S. 2008. Selective gene expression by postnatal electroporation during olfactory interneuron neurogenesis. *PLoS One,* 3**,** e1517.

CHEUNG, T. H. & RANDO, T. A. 2013. Molecular regulation of stem cell quiescence. *Nat Rev Mol Cell Biol,* 14**,** 329-40.

CHRISTENSEN, T. 2016. Human endogenous retroviruses in neurologic disease. *APMIS,* 124**,** 116-26.

CHU, C., QU, K., ZHONG, F. L., ARTANDI, S. E. & CHANG, H. Y. 2011. Genomic maps of long noncoding RNA occupancy reveal principles of RNA-chromatin interactions. *Mol Cell,* 44**,** 667-78.

CITRI, A. & YARDEN, Y. 2006. EGF-ERBB signalling: towards the systems level. *Nat Rev Mol Cell Biol,* 7**,** 505-16.

CODEGA, P., SILVA-VARGAS, V., PAUL, A., MALDONADO-SOTO, A. R., DELEO, A. M., PASTRANA, E. & DOETSCH, F. 2014. Prospective identification and purification of quiescent adult neural stem cells from their in vivo niche. *Neuron,* 82**,** 545-59.

COLAK, D., MORI, T., BRILL, M. S., PFEIFER, A., FALK, S., DENG, C., MONTEIRO, R., MUMMERY, C., SOMMER, L. & GOTZ, M. 2008. Adult neurogenesis requires Smad4-mediated bone morphogenic protein signaling in stem cells. *J Neurosci,* 28**,** 434-46.

COMTE, I., KIM, Y., YOUNG, C. C., VAN DER HARG, J. M., HOCKBERGER, P., BOLAM, P. J., POIRIER, F. & SZELE, F. G. 2011. Galectin-3 maintains cell motility from the subventricular zone to the olfactory bulb. *J Cell Sci,* 124**,** 2438-47.

CORDEY, M., LIMACHER, M., KOBEL, S., TAYLOR, V. & LUTOLF, M. P. 2008. Enhancing the reliability and throughput of neurosphere culture on hydrogel microwell arrays. *Stem Cells,* 26**,** 2586-94.

COSKUN, V., VENKATRAMAN, G., YANG, H., RAO, M. S. & LUSKIN, M. B. 2001. Retroviral manipulation of the expression of bone morphogenetic protein receptor Ia by SVZa

progenitor cells leads to changes in their p19(INK4d) expression but not in their neuronal commitment. *Int J Dev Neurosci,* 19**,** 219-27.
CURTIS, M. A., KAM, M., NANNMARK, U., ANDERSON, M. F., AXELL, M. Z., WIKKELSO, C., HOLTAS, S., VAN ROON-MOM, W. M., BJORK-ERIKSSON, T., NORDBORG, C., FRISEN, J., DRAGUNOW, M., FAULL, R. L. & ERIKSSON, P. S. 2007. Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. *Science,* 315**,** 1243-9.
CZERWINSKA, P., SHAH, P. K., TOMCZAK, K., KLIMCZAK, M., MAZUREK, S., SOZANSKA, B., BIECEK, P., KORSKI, K., FILAS, V., MACKIEWICZ, A., ANDERSEN, J. N. & WIZNEROWICZ, M. 2017. TRIM28 multi-domain protein regulates cancer stem cell population in breast tumor development. *Oncotarget,* 8**,** 863-882.
DAHLQVIST, C., BLOKZIJL, A., CHAPMAN, G., FALK, A., DANNAEUS, K., IBANEZ, C. F. & LENDAHL, U. 2003. Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. *Development,* 130**,** 6089-99.
DAVIDOVICH, C., WANG, X., CIFUENTES-ROJAS, C., GOODRICH, K. J., GOODING, A. R., LEE, J. T. & CECH, T. R. 2015. Toward a consensus on the binding specificity and promiscuity of PRC2 for RNA. *Mol Cell,* 57**,** 552-8.
DERRIEN, T. & GUIGO, R. 2011. [Long non-coding RNAs with enhancer-like function in human cells]. *Med Sci (Paris),* 27**,** 359-61.
DINGER, M. E., AMARAL, P. P., MERCER, T. R., PANG, K. C., BRUCE, S. J., GARDINER, B. B., ASKARIAN-AMIRI, M. E., RU, K., SOLDA, G., SIMONS, C., SUNKIN, S. M., CROWE, M. L., GRIMMOND, S. M., PERKINS, A. C. & MATTICK, J. S. 2008. Long noncoding RNAs in mouse embryonic stem cell pluripotency and differentiation. *Genome Res,* 18**,** 1433-45.
DOE, C. Q. 2008. Neural stem cells: balancing self-renewal with differentiation. *Development,* 135**,** 1575-87.
DOETSCH, F. 2003a. The glial identity of neural stem cells. *Nat Neurosci,* 6**,** 1127-34.
DOETSCH, F. 2003b. A niche for adult neural stem cells. *Curr Opin Genet Dev,* 13**,** 543-50.
DOETSCH, F. & ALVAREZ-BUYLLA, A. 1996. Network of tangential pathways for neuronal migration in adult mammalian brain. *Proc Natl Acad Sci U S A,* 93**,** 14895-900.
DOETSCH, F., GARCIA-VERDUGO, J. M. & ALVAREZ-BUYLLA, A. 1997. Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. *J Neurosci,* 17**,** 5046-61.
DOETSCH, F., PETREANU, L., CAILLE, I., GARCIA-VERDUGO, J. M. & ALVAREZ-BUYLLA, A. 2002. EGF converts transit-amplifying neurogenic precursors in the adult brain into multipotent stem cells. *Neuron,* 36**,** 1021-34.
DOMINGUEZ-GARCIA, S., CASTRO, C. & GERIBALDI-DOLDAN, N. 2019. ADAM17/TACE: a key molecule in brain injury regeneration. *Neural Regen Res,* 14**,** 1378-1379.
ECCO, G., CASSANO, M., KAUZLARIC, A., DUC, J., COLUCCIO, A., OFFNER, S., IMBEAULT, M., ROWE, H. M., TURELLI, P. & TRONO, D. 2016. Transposable Elements and Their KRAB-ZFP Controllers Regulate Gene Expression in Adult Tissues. *Dev Cell,* 36**,** 611-23.
ERNST, A., ALKASS, K., BERNARD, S., SALEHPOUR, M., PERL, S., TISDALE, J., POSSNERT, G., DRUID, H. & FRISEN, J. 2014. Neurogenesis in the striatum of the adult human brain. *Cell,* 156**,** 1072-83.
FAGHIHI, M. A., ZHANG, M., HUANG, J., MODARRESI, F., VAN DER BRUG, M. P., NALLS, M. A., COOKSON, M. R., ST-LAURENT, G., 3RD & WAHLESTEDT, C. 2010. Evidence for

natural antisense transcript-mediated inhibition of microRNA function. *Genome Biol,* 11**,** R56.
FAIGLE, R. & SONG, H. 2013. Signaling mechanisms regulating adult neural stem cells and neurogenesis. *Biochim Biophys Acta,* 1830**,** 2435-48.
FAIZ, M., SACHEWSKY, N., GASCON, S., BANG, K. W., MORSHEAD, C. M. & NAGY, A. 2015. Adult Neural Stem Cells from the Subventricular Zone Give Rise to Reactive Astrocytes in the Cortex after Stroke. *Cell Stem Cell,* 17**,** 624-34.
FASCHING, L., KAPOPOULOU, A., SACHDEVA, R., PETRI, R., JONSSON, M. E., MANNE, C., TURELLI, P., JERN, P., CAMMAS, F., TRONO, D. & JAKOBSSON, J. 2015. TRIM28 represses transcription of endogenous retroviruses in neural progenitor cells. *Cell Rep,* 10**,** 20-8.
FENG, J., BI, C., CLARK, B. S., MADY, R., SHAH, P. & KOHTZ, J. D. 2006. The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. *Genes Dev,* 20**,** 1470-84.
FERNANDEZ, M. E., CROCE, S., BOUTIN, C., CREMER, H. & RAINETEAU, O. 2011. Targeted electroporation of defined lateral ventricular walls: a novel and rapid method to study fate specification during postnatal forebrain neurogenesis. *Neural Dev,* 6**,** 13.
FERRON, S. R., ANDREU-AGULLO, C., MIRA, H., SANCHEZ, P., MARQUES-TORREJON, M. A. & FARINAS, I. 2007. A combined ex/in vivo assay to detect effects of exogenously added factors in neural stem cells. *Nat Protoc,* 2**,** 849-859.
FIGUERES-ONATE, M. & LOPEZ-MASCARAQUE, L. 2016. Adult Olfactory Bulb Interneuron Phenotypes Identified by Targeting Embryonic and Postnatal Neural Progenitors. *Front Neurosci,* 10**,** 194.
FIORELLI, R., AZIM, K., FISCHER, B. & RAINETEAU, O. 2015. Adding a spatial dimension to postnatal ventricular-subventricular zone neurogenesis. *Development,* 142**,** 2109-20.
FITSIORI, A., NGUYEN, D., KARENTZOS, A., DELAVELLE, J. & VARGAS, M. I. 2011. The corpus callosum: white matter or terra incognita. *Br J Radiol,* 84**,** 5-18.
FRANCO, S. J., MARTINEZ-GARAY, I., GIL-SANZ, C., HARKINS-PERRY, S. R. & MULLER, U. 2011. Reelin regulates cadherin function via Dab1/Rap1 to control neuronal migration and lamination in the neocortex. *Neuron,* 69**,** 482-97.
FRIEDMAN, J. R., FREDERICKS, W. J., JENSEN, D. E., SPEICHER, D. W., HUANG, X. P., NEILSON, E. G. & RAUSCHER, F. J., 3RD 1996. KAP-1, a novel corepressor for the highly conserved KRAB repression domain. *Genes Dev,* 10**,** 2067-78.
FRIETZE, S., O'GEEN, H., BLAHNIK, K. R., JIN, V. X. & FARNHAM, P. J. 2010. ZNF274 recruits the histone methyltransferase SETDB1 to the 3' ends of ZNF genes. *PLoS One,* 5**,** e15082.
FRINCHI, M., BONOMO, A., TROVATO-SALINARO, A., CONDORELLI, D. F., FUXE, K., SPAMPINATO, M. G. & MUDO, G. 2008. Fibroblast growth factor-2 and its receptor expression in proliferating precursor cells of the subventricular zone in the adult rat brain. *Neurosci Lett,* 447**,** 20-5.
FUCHS, E. & SEGRE, J. A. 2000. Stem cells: a new lease on life. *Cell,* 100**,** 143-55.
FUENTEALBA, L. C., ROMPANI, S. B., PARRAGUEZ, J. I., OBERNIER, K., ROMERO, R., CEPKO, C. L. & ALVAREZ-BUYLLA, A. 2015. Embryonic Origin of Postnatal Neural Stem Cells. *Cell,* 161**,** 1644-55.
GAGNON, K. T., LI, L., CHU, Y., JANOWSKI, B. A. & COREY, D. R. 2014. RNAi factors are present and active in human cell nuclei. *Cell Rep,* 6**,** 211-21.

GAIANO, N., NYE, J. S. & FISHELL, G. 2000. Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron,* 26**,** 395-404.
GAJERA, C. R., EMICH, H., LIOUBINSKI, O., CHRIST, A., BECKERVORDERSANDFORTH-BONK, R., YOSHIKAWA, K., BACHMANN, S., CHRISTENSEN, E. I., GOTZ, M., KEMPERMANN, G., PETERSON, A. S., WILLNOW, T. E. & HAMMES, A. 2010. LRP2 in ependymal cells regulates BMP signaling in the adult neurogenic niche. *J Cell Sci,* 123**,** 1922-30.
GALVEZ-CONTRERAS, A. Y., GONZALEZ-CASTANEDA, R. E., LUQUIN, S. & GONZALEZ-PEREZ, O. 2012. Role of fibroblast growth factor receptors in astrocytic stem cells. *Curr Signal Transduct Ther,* 7**,** 81-86.
GEORGALA, P. A., CARR, C. B. & PRICE, D. J. 2011. The role of Pax6 in forebrain development. *Dev Neurobiol,* 71**,** 690-709.
GERIBALDI-DOLDAN, N., CARRASCO, M., MURILLO-CARRETERO, M., DOMINGUEZ-GARCIA, S., GARCIA-COZAR, F. J., MUNOZ-MIRANDA, J. P., DEL RIO-GARCIA, V., VERASTEGUI, C. & CASTRO, C. 2018. Specific inhibition of ADAM17/TACE promotes neurogenesis in the injured motor cortex. *Cell Death Dis,* 9**,** 862.
GHASHGHAEI, H. T., LAI, C. & ANTON, E. S. 2007. Neuronal migration in the adult brain: are we there yet? *Nat Rev Neurosci,* 8**,** 141-51.
GHOSH-CHOUDHURY, N., GHOSH-CHOUDHURY, G., CELESTE, A., GHOSH, P. M., MOYER, M., ABBOUD, S. L. & KREISBERG, J. 2000. Bone morphogenetic protein-2 induces cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein in estradiol-treated MCF-7 human breast cancer cells. *Biochim Biophys Acta,* 1497**,** 186-96.
GIACHINO, C., BASAK, O., LUGERT, S., KNUCKLES, P., OBERNIER, K., FIORELLI, R., FRANK, S., RAINETEAU, O., ALVAREZ-BUYLLA, A. & TAYLOR, V. 2014. Molecular diversity subdivides the adult forebrain neural stem cell population. *Stem Cells,* 32**,** 70-84.
GIACHINO, C. & TAYLOR, V. 2014. Notching up neural stem cell homogeneity in homeostasis and disease. *Front Neurosci,* 8**,** 32.
GOINGS, G. E., SAHNI, V. & SZELE, F. G. 2004. Migration patterns of subventricular zone cells in adult mice change after cerebral cortex injury. *Brain Res,* 996**,** 213-26.
GOLL, M. G. & BESTOR, T. H. 2005. Eukaryotic cytosine methyltransferases. *Annu Rev Biochem,* 74**,** 481-514.
GOMES, W. A., MEHLER, M. F. & KESSLER, J. A. 2003. Transgenic overexpression of BMP4 increases astroglial and decreases oligodendroglial lineage commitment. *Dev Biol,* 255**,** 164-77.
GOMEZ-GAVIRO, M. V., SCOTT, C. E., SESAY, A. K., MATHEU, A., BOOTH, S., GALICHET, C. & LOVELL-BADGE, R. 2012. Betacellulin promotes cell proliferation in the neural stem cell niche and stimulates neurogenesis. *Proc Natl Acad Sci U S A,* 109**,** 1317-22.
GOTHIE, J. D., SEBILLOT, A., LUONGO, C., LEGENDRE, M., NGUYEN VAN, C., LE BLAY, K., PERRET-JEANNERET, M., REMAUD, S. & DEMENEIX, B. A. 2017. Adult neural stem cell fate is determined by thyroid hormone activation of mitochondrial metabolism. *Mol Metab,* 6**,** 1551-1561.
GOTZ, M., STOYKOVA, A. & GRUSS, P. 1998. Pax6 controls radial glia differentiation in the cerebral cortex. *Neuron,* 21**,** 1031-44.
GRINSPAN, J. B., EDELL, E., CARPIO, D. F., BEESLEY, J. S., LAVY, L., PLEASURE, D. & GOLDEN, J. A. 2000. Stage-specific effects of bone morphogenetic proteins on the oligodendrocyte lineage. *J Neurobiol,* 43**,** 1-17.
GRONER, A. C., MEYLAN, S., CIUFFI, A., ZANGGER, N., AMBROSINI, G., DENERVAUD, N., BUCHER, P. & TRONO, D. 2010. KRAB-zinc finger proteins and KAP1 can mediate

long-range transcriptional repression through heterochromatin spreading. *PLoS Genet,* 6**,** e1000869.
GROSS, R. E., MEHLER, M. F., MABIE, P. C., ZANG, Z., SANTSCHI, L. & KESSLER, J. A. 1996. Bone morphogenetic proteins promote astroglial lineage commitment by mammalian subventricular zone progenitor cells. *Neuron,* 17**,** 595-606.
GROTE, P., WITTLER, L., HENDRIX, D., KOCH, F., WAHRISCH, S., BEISAW, A., MACURA, K., BLASS, G., KELLIS, M., WERBER, M. & HERRMANN, B. G. 2013. The tissue-specific lncRNA Fendrr is an essential regulator of heart and body wall development in the mouse. *Dev Cell,* 24**,** 206-14.
HACK, M. A., SAGHATELYAN, A., DE CHEVIGNY, A., PFEIFER, A., ASHERY-PADAN, R., LLEDO, P. M. & GOTZ, M. 2005. Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nat Neurosci,* 8**,** 865-72.
HACK, M. A., SUGIMORI, M., LUNDBERG, C., NAKAFUKU, M. & GOTZ, M. 2004. Regionalization and fate specification in neurospheres: the role of Olig2 and Pax6. *Mol Cell Neurosci,* 25**,** 664-78.
HARTMAN, N. W., LIN, T. V., ZHANG, L., PAQUELET, G. E., FELICIANO, D. M. & BORDEY, A. 2013. mTORC1 targets the translational repressor 4E-BP2, but not S6 kinase 1/2, to regulate neural stem cell self-renewal in vivo. *Cell Rep,* 5**,** 433-44.
HASHIMOTO-TORII, K., TORII, M., SARKISIAN, M. R., BARTLEY, C. M., SHEN, J., RADTKE, F., GRIDLEY, T., SESTAN, N. & RAKIC, P. 2008. Interaction between Reelin and Notch signaling regulates neuronal migration in the cerebral cortex. *Neuron,* 60**,** 273-84.
HEGARTY, S. V., O'KEEFFE, G. W. & SULLIVAN, A. M. 2013. BMP-Smad 1/5/8 signalling in the development of the nervous system. *Prog Neurobiol,* 109**,** 28-41.
HENDRICH, B. D., BROWN, C. J. & WILLARD, H. F. 1993. Evolutionary conservation of possible functional domains of the human and murine XIST genes. *Hum Mol Genet,* 2**,** 663-72.
HOSOYA, T., CLIFFORD, M., LOSSON, R., TANABE, O. & ENGEL, J. D. 2013. TRIM28 is essential for erythroblast differentiation in the mouse. *Blood,* 122**,** 3798-807.
HSIEH, J. 2012. Orchestrating transcriptional control of adult neurogenesis. *Genes Dev,* 26**,** 1010-21.
HSIEH, Y. W. & YANG, X. J. 2009. Dynamic Pax6 expression during the neurogenic cell cycle influences proliferation and cell fate choices of retinal progenitors. *Neural Dev,* 4**,** 32.
HU, G., KIM, J., XU, Q., LENG, Y., ORKIN, S. H. & ELLEDGE, S. J. 2009. A genome-wide RNAi screen identifies a new transcriptional module required for self-renewal. *Genes Dev,* 23**,** 837-48.
HUARTE, M., GUTTMAN, M., FELDSER, D., GARBER, M., KOZIOL, M. J., KENZELMANN-BROZ, D., KHALIL, A. M., ZUK, O., AMIT, I., RABANI, M., ATTARDI, L. D., REGEV, A., LANDER, E. S., JACKS, T. & RINN, J. L. 2010. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. *Cell,* 142**,** 409-19.
IHRIE, R. A. & ALVAREZ-BUYLLA, A. 2011. Lake-front property: a unique germinal niche by the lateral ventricles of the adult brain. *Neuron,* 70**,** 674-86.
IMAYOSHI, I., SAKAMOTO, M., YAMAGUCHI, M., MORI, K. & KAGEYAMA, R. 2010. Essential roles of Notch signaling in maintenance of neural stem cells in developing and adult brains. *J Neurosci,* 30**,** 3489-98.
IMAYOSHI, I., SHIMOJO, H., SAKAMOTO, M., OHTSUKA, T. & KAGEYAMA, R. 2013. Genetic visualization of notch signaling in mammalian neurogenesis. *Cell Mol Life Sci,* 70**,** 2045-57.

IVANOV, A. V., PENG, H., YURCHENKO, V., YAP, K. L., NEGOREV, D. G., SCHULTZ, D. C., PSULKOWSKI, E., FREDERICKS, W. J., WHITE, D. E., MAUL, G. G., SADOFSKY, M. J., ZHOU, M. M. & RAUSCHER, F. J., 3RD 2007. PHD domain-mediated E3 ligase activity directs intramolecular sumoylation of an adjacent bromodomain required for gene silencing. *Mol Cell,* 28**,** 823-37.

IYENGAR, S. & FARNHAM, P. J. 2011. KAP1 protein: an enigmatic master regulator of the genome. *J Biol Chem,* 286**,** 26267-76.

IYENGAR, S., IVANOV, A. V., JIN, V. X., RAUSCHER, F. J., 3RD & FARNHAM, P. J. 2011. Functional analysis of KAP1 genomic recruitment. *Mol Cell Biol,* 31**,** 1833-47.

JACKSON, E. L., GARCIA-VERDUGO, J. M., GIL-PEROTIN, S., ROY, M., QUINONES-HINOJOSA, A., VANDENBERG, S. & ALVAREZ-BUYLLA, A. 2006. PDGFR alpha-positive B cells are neural stem cells in the adult SVZ that form glioma-like growths in response to increased PDGF signaling. *Neuron,* 51**,** 187-99.

JAENISCH, R. & BIRD, A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet,* 33 Suppl**,** 245-54.

JAKOBSSON, J., CORDERO, M. I., BISAZ, R., GRONER, A. C., BUSSKAMP, V., BENSADOUN, J. C., CAMMAS, F., LOSSON, R., MANSUY, I. M., SANDI, C. & TRONO, D. 2008. KAP1-mediated epigenetic repression in the forebrain modulates behavioral vulnerability to stress. *Neuron,* 60**,** 818-31.

JAMES, R., KIM, Y., HOCKBERGER, P. E. & SZELE, F. G. 2011. Subventricular zone cell migration: lessons from quantitative two-photon microscopy. *Front Neurosci,* 5**,** 30.

JANG, E. S. & GOLDMAN, J. E. 2011. Pax6 expression is sufficient to induce a neurogenic fate in glial progenitors of the neonatal subventricular zone. *PLoS One,* 6**,** e20894.

JAWERKA, M., COLAK, D., DIMOU, L., SPILLER, C., LAGGER, S., MONTGOMERY, R. L., OLSON, E. N., WURST, W., GOTTLICHER, M. & GOTZ, M. 2010. The specific role of histone deacetylase 2 in adult neurogenesis. *Neuron Glia Biol,* 6**,** 93-107.

JENSEN, J. B. & PARMAR, M. 2006. Strengths and limitations of the neurosphere culture system. *Mol Neurobiol,* 34**,** 153-61.

KAGEYAMA, R. & NAKANISHI, S. 1997. Helix-loop-helix factors in growth and differentiation of the vertebrate nervous system. *Curr Opin Genet Dev,* 7**,** 659-65.

KAGEYAMA, R., OHTSUKA, T. & KOBAYASHI, T. 2008. Roles of Hes genes in neural development. *Dev Growth Differ,* 50 Suppl 1**,** S97-103.

KASLIN, J., GANZ, J. & BRAND, M. 2008. Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. *Philos Trans R Soc Lond B Biol Sci,* 363**,** 101-22.

KEBSCHULL, J. M. & ZADOR, A. M. 2018. Cellular barcoding: lineage tracing, screening and beyond. *Nat Methods,* 15**,** 871-879.

KESTER, L. & VAN OUDENAARDEN, A. 2018. Single-Cell Transcriptomics Meets Lineage Tracing. *Cell Stem Cell,* 23**,** 166-179.

KIM, E. J., ABLES, J. L., DICKEL, L. K., EISCH, A. J. & JOHNSON, J. E. 2011. Ascl1 (Mash1) defines cells with long-term neurogenic potential in subgranular and subventricular zones in adult mouse brain. *PLoS One,* 6**,** e18472.

KIM, S. S., CHEN, Y. M., O'LEARY, E., WITZGALL, R., VIDAL, M. & BONVENTRE, J. V. 1996. A novel member of the RING finger family, KRIP-1, associates with the KRAB-A transcriptional repressor domain of zinc finger proteins. *Proc Natl Acad Sci U S A,* 93**,** 15299-304.

KIPPIN, T. E., MARTENS, D. J. & VAN DER KOOY, D. 2005. p21 loss compromises the relative quiescence of forebrain stem cell proliferation leading to exhaustion of their proliferation capacity. *Genes Dev,* 19**,** 756-67.

KOHWI, M., OSUMI, N., RUBENSTEIN, J. L. & ALVAREZ-BUYLLA, A. 2005. Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. *J Neurosci,* 25**,** 6997-7003.

KOHWI, M., PETRYNIAK, M. A., LONG, J. E., EKKER, M., OBATA, K., YANAGAWA, Y., RUBENSTEIN, J. L. & ALVAREZ-BUYLLA, A. 2007. A subpopulation of olfactory bulb GABAergic interneurons is derived from Emx1- and Dlx5/6-expressing progenitors. *J Neurosci,* 27**,** 6878-91.

KOHYAMA, J., SANOSAKA, T., TOKUNAGA, A., TAKATSUKA, E., TSUJIMURA, K., OKANO, H. & NAKASHIMA, K. 2010. BMP-induced REST regulates the establishment and maintenance of astrocytic identity. *J Cell Biol,* 189**,** 159-70.

KOUZARIDES, T. 2007. Chromatin modifications and their function. *Cell,* 128**,** 693-705.

KRIEGSTEIN, A. & ALVAREZ-BUYLLA, A. 2009. The glial nature of embryonic and adult neural stem cells. *Annu Rev Neurosci,* 32**,** 149-84.

KRISHNA, S. S., MAJUMDAR, I. & GRISHIN, N. V. 2003. Structural classification of zinc fingers: survey and summary. *Nucleic Acids Res,* 31**,** 532-50.

KUBOTA, S., FUKUMOTO, Y., AOYAMA, K., ISHIBASHI, K., YUKI, R., MORINAGA, T., HONDA, T., YAMAGUCHI, N., KUGA, T., TOMONAGA, T. & YAMAGUCHI, N. 2013. Phosphorylation of KRAB-associated protein 1 (KAP1) at Tyr-449, Tyr-458, and Tyr-517 by nuclear tyrosine kinases inhibits the association of KAP1 and heterochromatin protein 1alpha (HP1alpha) with heterochromatin. *J Biol Chem,* 288**,** 17871-83.

KUNG, J. T., COLOGNORI, D. & LEE, J. T. 2013. Long noncoding RNAs: past, present, and future. *Genetics,* 193**,** 651-69.

LACAR, B., YOUNG, S. Z., PLATEL, J. C. & BORDEY, A. 2010. Imaging and recording subventricular zone progenitor cells in live tissue of postnatal mice. *Front Neurosci,* 4.

LAGACE, D. C., WHITMAN, M. C., NOONAN, M. A., ABLES, J. L., DECAROLIS, N. A., ARGUELLO, A. A., DONOVAN, M. H., FISCHER, S. J., FARNBAUCH, L. A., BEECH, R. D., DILEONE, R. J., GREER, C. A., MANDYAM, C. D. & EISCH, A. J. 2007. Dynamic contribution of nestin-expressing stem cells to adult neurogenesis. *J Neurosci,* 27**,** 12623-9.

LAJTHA, L. G. 1979a. Stem cell concepts. *Differentiation,* 14**,** 23-34.

LAJTHA, L. G. 1979b. Stem cell concepts. *Nouv Rev Fr Hematol,* 21**,** 59-65.

LEE, N., PARK, S. J., HADDAD, G., KIM, D. K., PARK, S. M., PARK, S. K. & CHOI, K. Y. 2016. Interactomic analysis of REST/NRSF and implications of its functional links with the transcription suppressor TRIM28 during neuronal differentiation. *Sci Rep,* 6**,** 39049.

LEIN, E. S., HAWRYLYCZ, M. J., AO, N., AYRES, M., BENSINGER, A., BERNARD, A., BOE, A. F., BOGUSKI, M. S., BROCKWAY, K. S., BYRNES, E. J., CHEN, L., CHEN, L., CHEN, T. M., CHIN, M. C., CHONG, J., CROOK, B. E., CZAPLINSKA, A., DANG, C. N., DATTA, S., DEE, N. R., DESAKI, A. L., DESTA, T., DIEP, E., DOLBEARE, T. A., DONELAN, M. J., DONG, H. W., DOUGHERTY, J. G., DUNCAN, B. J., EBBERT, A. J., EICHELE, G., ESTIN, L. K., FABER, C., FACER, B. A., FIELDS, R., FISCHER, S. R., FLISS, T. P., FRENSLEY, C., GATES, S. N., GLATTFELDER, K. J., HALVERSON, K. R., HART, M. R., HOHMANN, J. G., HOWELL, M. P., JEUNG, D. P., JOHNSON, R. A., KARR, P. T., KAWAL, R., KIDNEY, J. M., KNAPIK, R. H., KUAN, C. L., LAKE, J. H., LARAMEE, A. R., LARSEN, K. D., LAU, C., LEMON, T. A., LIANG, A. J., LIU, Y., LUONG, L. T., MICHAELS, J., MORGAN, J. J., MORGAN, R. J.,

MORTRUD, M. T., MOSQUEDA, N. F., NG, L. L., NG, R., ORTA, G. J., OVERLY, C. C., PAK, T. H., PARRY, S. E., PATHAK, S. D., PEARSON, O. C., PUCHALSKI, R. B., RILEY, Z. L., ROCKETT, H. R., ROWLAND, S. A., ROYALL, J. J., RUIZ, M. J., SARNO, N. R., SCHAFFNIT, K., SHAPOVALOVA, N. V., SIVISAY, T., SLAUGHTERBECK, C. R., SMITH, S. C., SMITH, K. A., SMITH, B. I., SODT, A. J., STEWART, N. N., STUMPF, K. R., SUNKIN, S. M., SUTRAM, M., TAM, A., TEEMER, C. D., THALLER, C., THOMPSON, C. L., VARNAM, L. R., VISEL, A., WHITLOCK, R. M., WOHNOUTKA, P. E., WOLKEY, C. K., WONG, V. Y., et al. 2007. Genome-wide atlas of gene expression in the adult mouse brain. *Nature,* 445**,** 168-76.

LI, J., XI, Y., LI, W., MCCARTHY, R. L., STRATTON, S. A., ZOU, W., LI, W., DENT, S. Y., JAIN, A. K. & BARTON, M. C. 2017. TRIM28 interacts with EZH2 and SWI/SNF to activate genes that promote mammosphere formation. *Oncogene,* 36**,** 2991-3001.

LI, X., LEE, Y. K., JENG, J. C., YEN, Y., SCHULTZ, D. C., SHIH, H. M. & ANN, D. K. 2007. Role for KAP1 serine 824 phosphorylation and sumoylation/desumoylation switch in regulating KAP1-mediated transcriptional repression. *J Biol Chem,* 282**,** 36177-89.

LI, X., LIN, H. H., CHEN, H., XU, X., SHIH, H. M. & ANN, D. K. 2010. SUMOylation of the transcriptional co-repressor KAP1 is regulated by the serine and threonine phosphatase PP1. *Sci Signal,* 3**,** ra32.

LIAN, Y., YAN, C., XU, H., YANG, J., YU, Y., ZHOU, J., SHI, Y., REN, J., JI, G. & WANG, K. 2018. A Novel lncRNA, LINC00460, Affects Cell Proliferation and Apoptosis by Regulating KLF2 and CUL4A Expression in Colorectal Cancer. *Mol Ther Nucleic Acids,* 12**,** 684-697.

LIM, D. A. & ALVAREZ-BUYLLA, A. 2014. Adult neural stem cells stake their ground. *Trends Neurosci,* 37**,** 563-71.

LIM, D. A. & ALVAREZ-BUYLLA, A. 2016. The Adult Ventricular-Subventricular Zone (V-SVZ) and Olfactory Bulb (OB) Neurogenesis. *Cold Spring Harb Perspect Biol,* 8.

LIM, D. A., CHA, S., MAYO, M. C., CHEN, M. H., KELES, E., VANDENBERG, S. & BERGER, M. S. 2007. Relationship of glioblastoma multiforme to neural stem cell regions predicts invasive and multifocal tumor phenotype. *Neuro Oncol,* 9**,** 424-9.

LIM, D. A., HUANG, Y. C., SWIGUT, T., MIRICK, A. L., GARCIA-VERDUGO, J. M., WYSOCKA, J., ERNST, P. & ALVAREZ-BUYLLA, A. 2009. Chromatin remodelling factor Mll1 is essential for neurogenesis from postnatal neural stem cells. *Nature,* 458**,** 529-33.

LIM, D. A., TRAMONTIN, A. D., TREVEJO, J. M., HERRERA, D. G., GARCIA-VERDUGO, J. M. & ALVAREZ-BUYLLA, A. 2000. Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron,* 28**,** 713-26.

LIN, N., CHANG, K. Y., LI, Z., GATES, K., RANA, Z. A., DANG, J., ZHANG, D., HAN, T., YANG, C. S., CUNNINGHAM, T. J., HEAD, S. R., DUESTER, G., DONG, P. D. & RANA, T. M. 2014. An evolutionarily conserved long noncoding RNA TUNA controls pluripotency and neural lineage commitment. *Mol Cell,* 53**,** 1005-19.

LIONNARD, L., DUC, P., BRENNAN, M. S., KUEH, A. J., PAL, M., GUARDIA, F., MOJSA, B., DAMIANO, M. A., MORA, S., LASSOT, I., RAVICHANDRAN, R., COCHET, C., AOUACHERIA, A., POTTS, P. R., HEROLD, M. J., DESAGHER, S. & KUCHARCZAK, J. 2018. TRIM17 and TRIM28 antagonistically regulate the ubiquitination and anti-apoptotic activity of BCL2A1. *Cell Death Differ*.

LIU, L., ZHANG, L., WANG, J., ZHAO, X., XU, Q., LU, Y., ZUO, Y., CHEN, L., DU, J., LIAN, Y. & ZHANG, Q. 2018. Downregulation of TRIM28 inhibits growth and increases apoptosis of nude mice with nonsmall cell lung cancer xenografts. *Mol Med Rep,* 17**,** 835-842.

LLEDO, P. M., ALONSO, M. & GRUBB, M. S. 2006. Adult neurogenesis and functional plasticity in neuronal circuits. *Nat Rev Neurosci,* 7**,** 179-93.

LLORENS-BOBADILLA, E., ZHAO, S., BASER, A., SAIZ-CASTRO, G., ZWADLO, K. & MARTIN-VILLALBA, A. 2015. Single-Cell Transcriptomics Reveals a Population of Dormant Neural Stem Cells that Become Activated upon Brain Injury. *Cell Stem Cell,* 17**,** 329-40.

LO, L., SOMMER, L. & ANDERSON, D. J. 1997. MASH1 maintains competence for BMP2-induced neuronal differentiation in post-migratory neural crest cells. *Curr Biol,* 7**,** 440-50.

LOIS, C. & ALVAREZ-BUYLLA, A. 1994. Long-distance neuronal migration in the adult mammalian brain. *Science,* 264**,** 1145-8.

LOUIS, S. A., RIETZE, R. L., DELEYROLLE, L., WAGEY, R. E., THOMAS, T. E., EAVES, A. C. & REYNOLDS, B. A. 2008. Enumeration of neural stem and progenitor cells in the neural colony-forming cell assay. *Stem Cells,* 26**,** 988-96.

LOWDER, M., UNGE, A., MARAHA, N., JANSSON, J. K., SWIGGETT, J. & OLIVER, J. D. 2000. Effect of starvation and the viable-but-nonculturable state on green fluorescent protein (GFP) fluorescence in GFP-tagged Pseudomonas fluorescens A506. *Appl Environ Microbiol,* 66**,** 3160-5.

LU, W., HUANG, S. Y., SU, L., ZHAO, B. X. & MIAO, J. Y. 2016. Long Noncoding RNA LOC100129973 Suppresses Apoptosis by Targeting miR-4707-5p and miR-4767 in Vascular Endothelial Cells. *Sci Rep,* 6**,** 21620.

LUPO, A., CESARO, E., MONTANO, G., IZZO, P. & COSTANZO, P. 2011. ZNF224: Structure and role of a multifunctional KRAB-ZFP protein. *Int J Biochem Cell Biol,* 43**,** 470-3.

LUPO, A., CESARO, E., MONTANO, G., ZURLO, D., IZZO, P. & COSTANZO, P. 2013. KRAB-Zinc Finger Proteins: A Repressor Family Displaying Multiple Biological Functions. *Curr Genomics,* 14**,** 268-78.

MANUEL, M., GEORGALA, P. A., CARR, C. B., CHANAS, S., KLEINJAN, D. A., MARTYNOGA, B., MASON, J. O., MOLINEK, M., PINSON, J., PRATT, T., QUINN, J. C., SIMPSON, T. I., TYAS, D. A., VAN HEYNINGEN, V., WEST, J. D. & PRICE, D. J. 2007. Controlled overexpression of Pax6 in vivo negatively autoregulates the Pax6 locus, causing cell-autonomous defects of late cortical progenitor proliferation with little effect on cortical arealization. *Development,* 134**,** 545-55.

MARGUERON, R. & REINBERG, D. 2011. The Polycomb complex PRC2 and its mark in life. *Nature,* 469**,** 343-9.

MARMORSTEIN, R. & TRIEVEL, R. C. 2009. Histone modifying enzymes: structures, mechanisms, and specificities. *Biochim Biophys Acta,* 1789**,** 58-68.

MARQUES-TORREJON, M. A., PORLAN, E., BANITO, A., GOMEZ-IBARLUCEA, E., LOPEZ-CONTRERAS, A. J., FERNANDEZ-CAPETILLO, O., VIDAL, A., GIL, J., TORRES, J. & FARINAS, I. 2013. Cyclin-dependent kinase inhibitor p21 controls adult neural stem cell expansion by regulating Sox2 gene expression. *Cell Stem Cell,* 12**,** 88-100.

MARSHALL, C. A., NOVITCH, B. G. & GOLDMAN, J. E. 2005. Olig2 directs astrocyte and oligodendrocyte formation in postnatal subventricular zone cells. *J Neurosci,* 25**,** 7289-98.

MASCLE, X. H., GERMAIN-DESPREZ, D., HUYNH, P., ESTEPHAN, P. & AUBRY, M. 2007. Sumoylation of the transcriptional intermediary factor 1beta (TIF1beta), the co-repressor of the KRAB Multifinger proteins, is required for its transcriptional activity and is modulated by the KRAB domain. *J Biol Chem,* 282**,** 10190-202.

MATSUDA, T. & CEPKO, C. L. 2007. Controlled expression of transgenes introduced by in vivo electroporation. *Proc Natl Acad Sci U S A,* 104**,** 1027-32.
MATTICK, J. S. & MAKUNIN, I. V. 2006. Non-coding RNA. *Hum Mol Genet,* 15 Spec No 1**,** R17-29.
MEHLER, M. F., MABIE, P. C., ZHANG, D. & KESSLER, J. A. 1997. Bone morphogenetic proteins in the nervous system. *Trends Neurosci,* 20**,** 309-17.
MEHLER, M. F., MABIE, P. C., ZHU, G., GOKHAN, S. & KESSLER, J. A. 2000. Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. *Dev Neurosci,* 22**,** 74-85.
MERCER, T. R., DINGER, M. E. & MATTICK, J. S. 2009. Long non-coding RNAs: insights into functions. *Nat Rev Genet,* 10**,** 155-9.
MERCER, T. R., DINGER, M. E., SUNKIN, S. M., MEHLER, M. F. & MATTICK, J. S. 2008. Specific expression of long noncoding RNAs in the mouse brain. *Proc Natl Acad Sci U S A,* 105**,** 716-21.
MERCIER, F. & DOUET, V. 2014. Bone morphogenetic protein-4 inhibits adult neurogenesis and is regulated by fractone-associated heparan sulfates in the subventricular zone. *J Chem Neuroanat,* 57-58**,** 54-61.
MING, G. L. & SONG, H. 2011. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron,* 70**,** 687-702.
MIRZADEH, Z., MERKLE, F. T., SORIANO-NAVARRO, M., GARCIA-VERDUGO, J. M. & ALVAREZ-BUYLLA, A. 2008. Neural stem cells confer unique pinwheel architecture to the ventricular surface in neurogenic regions of the adult brain. *Cell Stem Cell,* 3**,** 265-78.
MIYAZONO, K., MAEDA, S. & IMAMURA, T. 2005. BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev,* 16**,** 251-63.
MONTALBAN-LORO, R., DOMINGO-MUELAS, A., BIZY, A. & FERRON, S. R. 2015. Epigenetic regulation of stemness maintenance in the neurogenic niches. *World J Stem Cells,* 7**,** 700-10.
MORELL, M., TSAN, Y. C. & O'SHEA, K. S. 2015. Inducible expression of noggin selectively expands neural progenitors in the adult SVZ. *Stem Cell Res,* 14**,** 79-94.
MORRISON, S. J., PEREZ, S. E., QIAO, Z., VERDI, J. M., HICKS, C., WEINMASTER, G. & ANDERSON, D. J. 2000. Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. *Cell,* 101**,** 499-510.
MUDO, G., BONOMO, A., DI LIBERTO, V., FRINCHI, M., FUXE, K. & BELLUARDO, N. 2009. The FGF-2/FGFRs neurotrophic system promotes neurogenesis in the adult brain. *J Neural Transm (Vienna),* 116**,** 995-1005.
NAGY, A. 2000. Cre recombinase: the universal reagent for genome tailoring. *Genesis,* 26**,** 99-109.
NAKASHIMA, K., TAKIZAWA, T., OCHIAI, W., YANAGISAWA, M., HISATSUNE, T., NAKAFUKU, M., MIYAZONO, K., KISHIMOTO, T., KAGEYAMA, R. & TAGA, T. 2001. BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. *Proc Natl Acad Sci U S A,* 98**,** 5868-73.
NAM, S. C., KIM, Y., DRYANOVSKI, D., WALKER, A., GOINGS, G., WOOLFREY, K., KANG, S. S., CHU, C., CHENN, A., ERDELYI, F., SZABO, G., HOCKBERGER, P. & SZELE, F. G. 2007. Dynamic features of postnatal subventricular zone cell motility: a two-photon time-lapse study. *J Comp Neurol,* 505**,** 190-208.

NELSON, P. N., CARNEGIE, P. R., MARTIN, J., DAVARI EJTEHADI, H., HOOLEY, P., RODEN, D., ROWLAND-JONES, S., WARREN, P., ASTLEY, J. & MURRAY, P. G. 2003. Demystified. Human endogenous retroviruses. *Mol Pathol,* 56**,** 11-8.

NESTEROVA, T. B., SLOBODYANYUK, S. Y., ELISAPHENKO, E. A., SHEVCHENKO, A. I., JOHNSTON, C., PAVLOVA, M. E., ROGOZIN, I. B., KOLESNIKOV, N. N., BROCKDORFF, N. & ZAKIAN, S. M. 2001. Characterization of the genomic Xist locus in rodents reveals conservation of overall gene structure and tandem repeats but rapid evolution of unique sequence. *Genome Res,* 11**,** 833-49.

NG, S. Y., BOGU, G. K., SOH, B. S. & STANTON, L. W. 2013. The long noncoding RNA RMST interacts with SOX2 to regulate neurogenesis. *Mol Cell,* 51**,** 349-59.

NINKOVIC, J., STEINER-MEZZADRI, A., JAWERKA, M., AKINCI, U., MASSERDOTTI, G., PETRICCA, S., FISCHER, J., VON HOLST, A., BECKERS, J., LIE, C. D., PETRIK, D., MILLER, E., TANG, J., WU, J., LEFEBVRE, V., DEMMERS, J., EISCH, A., METZGER, D., CRABTREE, G., IRMLER, M., POOT, R. & GOTZ, M. 2013. The BAF complex interacts with Pax6 in adult neural progenitors to establish a neurogenic cross-regulatory transcriptional network. *Cell Stem Cell,* 13**,** 403-18.

O'GEEN, H., SQUAZZO, S. L., IYENGAR, S., BLAHNIK, K., RINN, J. L., CHANG, H. Y., GREEN, R. & FARNHAM, P. J. 2007. Genome-wide analysis of KAP1 binding suggests autoregulation of KRAB-ZNFs. *PLoS Genet,* 3**,** e89.

OHSAWA, R. & KAGEYAMA, R. 2008. Regulation of retinal cell fate specification by multiple transcription factors. *Brain Res,* 1192**,** 90-8.

OHTSUKA, T., ISHIBASHI, M., GRADWOHL, G., NAKANISHI, S., GUILLEMOT, F. & KAGEYAMA, R. 1999. Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. *EMBO J,* 18**,** 2196-207.

OLIVER, P. L., CHODROFF, R. A., GOSAL, A., EDWARDS, B., CHEUNG, A. F., GOMEZ-RODRIGUEZ, J., ELLIOT, G., GARRETT, L. J., LICKISS, T., SZELE, F., GREEN, E. D., MOLNAR, Z. & PONTING, C. P. 2015. Disruption of Visc-2, a Brain-Expressed Conserved Long Noncoding RNA, Does Not Elicit an Overt Anatomical or Behavioral Phenotype. *Cereb Cortex,* 25**,** 3572-85.

ONO, K., TOMASIEWICZ, H., MAGNUSON, T. & RUTISHAUSER, U. 1994. N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. *Neuron,* 13**,** 595-609.

ORTEGA, F., COSTA, M. R., SIMON-EBERT, T., SCHROEDER, T., GOTZ, M. & BERNINGER, B. 2011. Using an adherent cell culture of the mouse subependymal zone to study the behavior of adult neural stem cells on a single-cell level. *Nat Protoc,* 6**,** 1847-59.

OSUMI, N., SHINOHARA, H., NUMAYAMA-TSURUTA, K. & MAEKAWA, M. 2008. Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. *Stem Cells,* 26**,** 1663-72.

OTTONE, C., KRUSCHE, B., WHITBY, A., CLEMENTS, M., QUADRATO, G., PITULESCU, M. E., ADAMS, R. H. & PARRINELLO, S. 2014. Direct cell-cell contact with the vascular niche maintains quiescent neural stem cells. *Nat Cell Biol,* 16**,** 1045-56.

OTTONE, C. & PARRINELLO, S. 2015. Multifaceted control of adult SVZ neurogenesis by the vascular niche. *Cell Cycle,* 14**,** 2222-5.

PANG, K. C., FRITH, M. C. & MATTICK, J. S. 2006. Rapid evolution of noncoding RNAs: lack of conservation does not mean lack of function. *Trends Genet,* 22**,** 1-5.

PANZANELLI, P., BARDY, C., NISSANT, A., PALLOTTO, M., SASSOE-POGNETTO, M., LLEDO, P. M. & FRITSCHY, J. M. 2009. Early synapse formation in developing interneurons of the adult olfactory bulb. *J Neurosci,* 29**,** 15039-52.

PAREDES, M. F., JAMES, D., GIL-PEROTIN, S., KIM, H., COTTER, J. A., NG, C., SANDOVAL, K., ROWITCH, D. H., XU, D., MCQUILLEN, P. S., GARCIA-VERDUGO, J. M., HUANG, E. J. & ALVAREZ-BUYLLA, A. 2016. Extensive migration of young neurons into the infant human frontal lobe. *Science,* 354.

PARRAS, C. M., GALLI, R., BRITZ, O., SOARES, S., GALICHET, C., BATTISTE, J., JOHNSON, J. E., NAKAFUKU, M., VESCOVI, A. & GUILLEMOT, F. 2004. Mash1 specifies neurons and oligodendrocytes in the postnatal brain. *EMBO J,* 23**,** 4495-505.

PASTORI, C., KAPRANOV, P., PENAS, C., PESCHANSKY, V., VOLMAR, C. H., SARKARIA, J. N., BREGY, A., KOMOTAR, R., ST LAURENT, G., AYAD, N. G. & WAHLESTEDT, C. 2015. The Bromodomain protein BRD4 controls HOTAIR, a long noncoding RNA essential for glioblastoma proliferation. *Proc Natl Acad Sci U S A,* 112**,** 8326-31.

PASTRANA, E., SILVA-VARGAS, V. & DOETSCH, F. 2011. Eyes wide open: a critical review of sphere-formation as an assay for stem cells. *Cell Stem Cell,* 8**,** 486-98.

PATASKAR, A., JUNG, J., SMIALOWSKI, P., NOACK, F., CALEGARI, F., STRAUB, T. & TIWARI, V. K. 2016. NeuroD1 reprograms chromatin and transcription factor landscapes to induce the neuronal program. *EMBO J,* 35**,** 24-45.

PAVLAKI, I., ALAMMARI, F., SUN, B., CLARK, N., SIREY, T., LEE, S., WOODCOCK, D. J., PONTING, C. P., SZELE, F. G. & VANCE, K. W. 2018. The long non-coding RNA Paupar promotes KAP1-dependent chromatin changes and regulates olfactory bulb neurogenesis. *EMBO J,* 37.

PENG, H., GIBSON, L. C., CAPILI, A. D., BORDEN, K. L., OSBORNE, M. J., HARPER, S. L., SPEICHER, D. W., ZHAO, K., MARMORSTEIN, R., ROCK, T. A. & RAUSCHER, F. J., 3RD 2007. The structurally disordered KRAB repression domain is incorporated into a protease resistant core upon binding to KAP-1-RBCC domain. *J Mol Biol,* 370**,** 269-89.

PENG, H., IVANOV, A. V., OH, H. J., LAU, Y. F. & RAUSCHER, F. J., 3RD 2009. Epigenetic gene silencing by the SRY protein is mediated by a KRAB-O protein that recruits the KAP1 co-repressor machinery. *J Biol Chem,* 284**,** 35670-80.

PERETTO, P., GIACHINO, C., AIMAR, P., FASOLO, A. & BONFANTI, L. 2005. Chain formation and glial tube assembly in the shift from neonatal to adult subventricular zone of the rodent forebrain. *J Comp Neurol,* 487**,** 407-27.

PERSENGIEV, S. P., LI, J., POULIN, M. L. & KILPATRICK, D. L. 2001. E2F2 converts reversibly differentiated PC12 cells to an irreversible, neurotrophin-dependent state. *Oncogene,* 20**,** 5124-31.

PETREANU, L. & ALVAREZ-BUYLLA, A. 2002. Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. *J Neurosci,* 22**,** 6106-13.

PEVNY, L. H. & NICOLIS, S. K. 2010. Sox2 roles in neural stem cells. *Int J Biochem Cell Biol,* 42**,** 421-4.

PONJAVIC, J., OLIVER, P. L., LUNTER, G. & PONTING, C. P. 2009. Genomic and transcriptional co-localization of protein-coding and long non-coding RNA pairs in the developing brain. *PLoS Genet,* 5**,** e1000617.

PONTI, G., OBERNIER, K., GUINTO, C., JOSE, L., BONFANTI, L. & ALVAREZ-BUYLLA, A. 2013. Cell cycle and lineage progression of neural progenitors in the ventricular-subventricular zones of adult mice. *Proc Natl Acad Sci U S A,* 110**,** E1045-54.

PONTING, C. P. & BELGARD, T. G. 2010. Transcribed dark matter: meaning or myth? *Hum Mol Genet,* 19**,** R162-8.
PONTING, C. P., OLIVER, P. L. & REIK, W. 2009. Evolution and functions of long noncoding RNAs. *Cell,* 136**,** 629-41.
PORTOSO, M., RAGAZZINI, R., BRENCIC, Z., MOIANI, A., MICHAUD, A., VASSILEV, I., WASSEF, M., SERVANT, N., SARGUEIL, B. & MARGUERON, R. 2017. PRC2 is dispensable for HOTAIR-mediated transcriptional repression. *EMBO J,* 36**,** 981-994.
POTTEN, C. S. & LOEFFLER, M. 1990. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development,* 110**,** 1001-20.
QI, Z., CAI, S., CAI, J., CHEN, L., YAO, Y., CHEN, L. & MAO, Y. 2016a. miR-491 regulates glioma cells proliferation by targeting TRIM28 in vitro. *BMC Neurol,* 16**,** 248.
QI, Z. X., CAI, J. J., CHEN, L. C., YUE, Q., GONG, Y., YAO, Y. & MAO, Y. 2016b. TRIM28 as an independent prognostic marker plays critical roles in glioma progression. *J Neurooncol,* 126**,** 19-26.
QUINONES-HINOJOSA, A. & CHAICHANA, K. 2007. The human subventricular zone: a source of new cells and a potential source of brain tumors. *Exp Neurol,* 205**,** 313-24.
QUINONES-HINOJOSA, A., SANAI, N., SORIANO-NAVARRO, M., GONZALEZ-PEREZ, O., MIRZADEH, Z., GIL-PEROTIN, S., ROMERO-RODRIGUEZ, R., BERGER, M. S., GARCIA-VERDUGO, J. M. & ALVAREZ-BUYLLA, A. 2006. Cellular composition and cytoarchitecture of the adult human subventricular zone: a niche of neural stem cells. *J Comp Neurol,* 494**,** 415-34.
RAJ, A., VAN DEN BOGAARD, P., RIFKIN, S. A., VAN OUDENAARDEN, A. & TYAGI, S. 2008. Imaging individual mRNA molecules using multiple singly labeled probes. *Nat Methods,* 5**,** 877-9.
RAMASWAMY, S., GOINGS, G. E., SODERSTROM, K. E., SZELE, F. G. & KOZLOWSKI, D. A. 2005. Cellular proliferation and migration following a controlled cortical impact in the mouse. *Brain Res,* 1053**,** 38-53.
RAMIREZ-CASTILLEJO, C., SANCHEZ-SANCHEZ, F., ANDREU-AGULLO, C., FERRON, S. R., AROCA-AGUILAR, J. D., SANCHEZ, P., MIRA, H., ESCRIBANO, J. & FARINAS, I. 2006. Pigment epithelium-derived factor is a niche signal for neural stem cell renewal. *Nat Neurosci,* 9**,** 331-9.
RAMOS, A. D., ANDERSEN, R. E., LIU, S. J., NOWAKOWSKI, T. J., HONG, S. J., GERTZ, C. C., SALINAS, R. D., ZARABI, H., KRIEGSTEIN, A. R. & LIM, D. A. 2015. The long noncoding RNA Pnky regulates neuronal differentiation of embryonic and postnatal neural stem cells. *Cell Stem Cell,* 16**,** 439-47.
RAMOS, A. D., ATTENELLO, F. J. & LIM, D. A. 2016. Uncovering the roles of long noncoding RNAs in neural development and glioma progression. *Neurosci Lett,* 625**,** 70-9.
RAMOS, A. D., DIAZ, A., NELLORE, A., DELGADO, R. N., PARK, K. Y., GONZALES-ROYBAL, G., OLDHAM, M. C., SONG, J. S. & LIM, D. A. 2013. Integration of genome-wide approaches identifies lncRNAs of adult neural stem cells and their progeny in vivo. *Cell Stem Cell,* 12**,** 616-28.
RAPONI, E., AGENES, F., DELPHIN, C., ASSARD, N., BAUDIER, J., LEGRAVEREND, C. & DELOULME, J. C. 2007. S100B expression defines a state in which GFAP-expressing cells lose their neural stem cell potential and acquire a more mature developmental stage. *Glia,* 55**,** 165-77.
RHEINBAY, E., SUVA, M. L., GILLESPIE, S. M., WAKIMOTO, H., PATEL, A. P., SHAHID, M., OKSUZ, O., RABKIN, S. D., MARTUZA, R. L., RIVERA, M. N., LOUIS, D. N., KASIF, S., CHI,

A. S. & BERNSTEIN, B. E. 2013. An aberrant transcription factor network essential for Wnt signaling and stem cell maintenance in glioblastoma. *Cell Rep,* 3**,** 1567-79.
RICLET, R., CHENDEB, M., VONESCH, J. L., KOCZAN, D., THIESEN, H. J., LOSSON, R. & CAMMAS, F. 2009. Disruption of the interaction between transcriptional intermediary factor 1{beta} and heterochromatin protein 1 leads to a switch from DNA hyper- to hypomethylation and H3K9 to H3K27 trimethylation on the MEST promoter correlating with gene reactivation. *Mol Biol Cell,* 20**,** 296-305.
RINN, J. L., KERTESZ, M., WANG, J. K., SQUAZZO, S. L., XU, X., BRUGMANN, S. A., GOODNOUGH, L. H., HELMS, J. A., FARNHAM, P. J., SEGAL, E. & CHANG, H. Y. 2007. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. *Cell,* 129**,** 1311-23.
ROTEM, A., RAM, O., SHORESH, N., SPERLING, R. A., GOREN, A., WEITZ, D. A. & BERNSTEIN, B. E. 2015. Single-cell ChIP-seq reveals cell subpopulations defined by chromatin state. *Nat Biotechnol,* 33**,** 1165-72.
ROWE, H. M., JAKOBSSON, J., MESNARD, D., ROUGEMONT, J., REYNARD, S., AKTAS, T., MAILLARD, P. V., LAYARD-LIESCHING, H., VERP, S., MARQUIS, J., SPITZ, F., CONSTAM, D. B. & TRONO, D. 2010. KAP1 controls endogenous retroviruses in embryonic stem cells. *Nature,* 463**,** 237-40.
RUTENBERG-SCHOENBERG, M., SEXTON, A. N. & SIMON, M. D. 2016. The Properties of Long Noncoding RNAs That Regulate Chromatin. *Annu Rev Genomics Hum Genet,* 17**,** 69-94.
RYAN, R. F., SCHULTZ, D. C., AYYANATHAN, K., SINGH, P. B., FRIEDMAN, J. R., FREDERICKS, W. J. & RAUSCHER, F. J., 3RD 1999. KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol Cell Biol,* 19**,** 4366-78.
SAHLGREN, C., GUSTAFSSON, M. V., JIN, S., POELLINGER, L. & LENDAHL, U. 2008. Notch signaling mediates hypoxia-induced tumor cell migration and invasion. *Proc Natl Acad Sci U S A,* 105**,** 6392-7.
SAHU, N. K., SHILAKARI, G., NAYAK, A. & KOHLI, D. V. 2007. Antisense technology: a selective tool for gene expression regulation and gene targeting. *Curr Pharm Biotechnol,* 8**,** 291-304.
SALOMONI, P. & CALEGARI, F. 2010. Cell cycle control of mammalian neural stem cells: putting a speed limit on G1. *Trends Cell Biol,* 20**,** 233-43.
SANAI, N., ALVAREZ-BUYLLA, A. & BERGER, M. S. 2005. Neural stem cells and the origin of gliomas. *N Engl J Med,* 353**,** 811-22.
SANAI, N., NGUYEN, T., IHRIE, R. A., MIRZADEH, Z., TSAI, H. H., WONG, M., GUPTA, N., BERGER, M. S., HUANG, E., GARCIA-VERDUGO, J. M., ROWITCH, D. H. & ALVAREZ-BUYLLA, A. 2011. Corridors of migrating neurons in the human brain and their decline during infancy. *Nature,* 478**,** 382-6.
SANAI, N., TRAMONTIN, A. D., QUINONES-HINOJOSA, A., BARBARO, N. M., GUPTA, N., KUNWAR, S., LAWTON, M. T., MCDERMOTT, M. W., PARSA, A. T., MANUEL-GARCIA VERDUGO, J., BERGER, M. S. & ALVAREZ-BUYLLA, A. 2004. Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. *Nature,* 427**,** 740-4.
SANDER, J. D. & JOUNG, J. K. 2014. CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat Biotechnol,* 32**,** 347-55.

SARKAR, A. & HOCHEDLINGER, K. 2013. The sox family of transcription factors: versatile regulators of stem and progenitor cell fate. *Cell Stem Cell,* 12**,** 15-30.
SATO, K. 2015. Effects of Microglia on Neurogenesis. *Glia,* 63**,** 1394-405.
SAUVAGEAU, M., GOFF, L. A., LODATO, S., BONEV, B., GROFF, A. F., GERHARDINGER, C., SANCHEZ-GOMEZ, D. B., HACISULEYMAN, E., LI, E., SPENCE, M., LIAPIS, S. C., MALLARD, W., MORSE, M., SWERDEL, M. R., D'ECCLESSIS, M. F., MOORE, J. C., LAI, V., GONG, G., YANCOPOULOS, G. D., FRENDEWEY, D., KELLIS, M., HART, R. P., VALENZUELA, D. M., ARLOTTA, P. & RINN, J. L. 2013. Multiple knockout mouse models reveal lincRNAs are required for life and brain development. *Elife,* 2**,** e01749.
SAWAMOTO, K., WICHTERLE, H., GONZALEZ-PEREZ, O., CHOLFIN, J. A., YAMADA, M., SPASSKY, N., MURCIA, N. S., GARCIA-VERDUGO, J. M., MARIN, O., RUBENSTEIN, J. L., TESSIER-LAVIGNE, M., OKANO, H. & ALVAREZ-BUYLLA, A. 2006. New neurons follow the flow of cerebrospinal fluid in the adult brain. *Science,* 311**,** 629-32.
SCHULTZ, D. C., AYYANATHAN, K., NEGOREV, D., MAUL, G. G. & RAUSCHER, F. J., 3RD 2002. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev,* 16**,** 919-32.
SCHULTZ, D. C., FRIEDMAN, J. R. & RAUSCHER, F. J., 3RD 2001. Targeting histone deacetylase complexes via KRAB-zinc finger proteins: the PHD and bromodomains of KAP-1 form a cooperative unit that recruits a novel isoform of the Mi-2alpha subunit of NuRD. *Genes Dev,* 15**,** 428-43.
SEKI, Y., KURISAKI, A., WATANABE-SUSAKI, K., NAKAJIMA, Y., NAKANISHI, M., ARAI, Y., SHIOTA, K., SUGINO, H. & ASASHIMA, M. 2010. TIF1beta regulates the pluripotency of embryonic stem cells in a phosphorylation-dependent manner. *Proc Natl Acad Sci U S A,* 107**,** 10926-31.
SHAH, N. M., GROVES, A. K. & ANDERSON, D. J. 1996. Alternative neural crest cell fates are instructively promoted by TGFbeta superfamily members. *Cell,* 85**,** 331-43.
SHIMOZAKI, K., CLEMENSON, G. D., JR. & GAGE, F. H. 2013. Paired related homeobox protein 1 is a regulator of stemness in adult neural stem/progenitor cells. *J Neurosci,* 33**,** 4066-75.
SHOU, J., RIM, P. C. & CALOF, A. L. 1999. BMPs inhibit neurogenesis by a mechanism involving degradation of a transcription factor. *Nat Neurosci,* 2**,** 339-45.
SIEBZEHNRUBL, F. A., BUSLEI, R., EYUPOGLU, I. Y., SEUFERT, S., HAHNEN, E. & BLUMCKE, I. 2007. Histone deacetylase inhibitors increase neuronal differentiation in adult forebrain precursor cells. *Exp Brain Res,* 176**,** 672-8.
SILVA, F. P., HAMAMOTO, R., FURUKAWA, Y. & NAKAMURA, Y. 2006. TIPUH1 encodes a novel KRAB zinc-finger protein highly expressed in human hepatocellular carcinomas. *Oncogene,* 25**,** 5063-70.
SINGEC, I., KNOTH, R., MEYER, R. P., MACIACZYK, J., VOLK, B., NIKKHAH, G., FROTSCHER, M. & SNYDER, E. Y. 2006. Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nat Methods,* 3**,** 801-6.
SINGH, K., CASSANO, M., PLANET, E., SEBASTIAN, S., JANG, S. M., SOHI, G., FARALLI, H., CHOI, J., YOUN, H. D., DILWORTH, F. J. & TRONO, D. 2015. A KAP1 phosphorylation switch controls MyoD function during skeletal muscle differentiation. *Genes Dev,* 29**,** 513-25.

SOARES, R. J., MAGLIERI, G., GUTSCHNER, T., DIEDERICHS, S., LUND, A. H., NIELSEN, B. S. & HOLMSTROM, K. 2018. Evaluation of fluorescence in situ hybridization techniques to study long non-coding RNA expression in cultured cells. *Nucleic Acids Res,* 46**,** e4.

SODERBERG, O., GULLBERG, M., JARVIUS, M., RIDDERSTRALE, K., LEUCHOWIUS, K. J., JARVIUS, J., WESTER, K., HYDBRING, P., BAHRAM, F., LARSSON, L. G. & LANDEGREN, U. 2006. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods,* 3**,** 995-1000.

SOKPOR, G., XIE, Y., ROSENBUSCH, J. & TUOC, T. 2017. Chromatin Remodeling BAF (SWI/SNF) Complexes in Neural Development and Disorders. *Front Mol Neurosci,* 10**,** 243.

SPASSKY, N., MERKLE, F. T., FLAMES, N., TRAMONTIN, A. D., GARCIA-VERDUGO, J. M. & ALVAREZ-BUYLLA, A. 2005. Adult ependymal cells are postmitotic and are derived from radial glial cells during embryogenesis. *J Neurosci,* 25**,** 10-8.

SUN, B., CHANG, E., GERHARTL, A. & SZELE, F. G. 2018. Polycomb Protein Eed is Required for Neurogenesis and Cortical Injury Activation in the Subventricular Zone. *Cereb Cortex,* 28**,** 1369-1382.

SUN, M. Y., YETMAN, M. J., LEE, T. C., CHEN, Y. & JANKOWSKY, J. L. 2014. Specificity and efficiency of reporter expression in adult neural progenitors vary substantially among nestin-CreER(T2) lines. *J Comp Neurol,* 522**,** 1191-208.

SUN, W., KIM, H. & MOON, Y. 2010. Control of neuronal migration through rostral migration stream in mice. *Anat Cell Biol,* 43**,** 269-79.

SZELE, F. G. & CEPKO, C. L. 1996. A subset of clones in the chick telencephalon arranged in rostrocaudal arrays. *Curr Biol,* 6**,** 1685-90.

SZELE, F. G. & CHESSELET, M. F. 1996. Cortical lesions induce an increase in cell number and PSA-NCAM expression in the subventricular zone of adult rats. *J Comp Neurol,* 368**,** 439-54.

TABATA, T. & TAKEI, Y. 2004. Morphogens, their identification and regulation. *Development,* 131**,** 703-12.

TANIGAKI, K., NOGAKI, F., TAKAHASHI, J., TASHIRO, K., KUROOKA, H. & HONJO, T. 2001. Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. *Neuron,* 29**,** 45-55.

THUL, P. J., AKESSON, L., WIKING, M., MAHDESSIAN, D., GELADAKI, A., AIT BLAL, H., ALM, T., ASPLUND, A., BJORK, L., BRECKELS, L. M., BACKSTROM, A., DANIELSSON, F., FAGERBERG, L., FALL, J., GATTO, L., GNANN, C., HOBER, S., HJELMARE, M., JOHANSSON, F., LEE, S., LINDSKOG, C., MULDER, J., MULVEY, C. M., NILSSON, P., OKSVOLD, P., ROCKBERG, J., SCHUTTEN, R., SCHWENK, J. M., SIVERTSSON, A., SJOSTEDT, E., SKOGS, M., STADLER, C., SULLIVAN, D. P., TEGEL, H., WINSNES, C., ZHANG, C., ZWAHLEN, M., MARDINOGLU, A., PONTEN, F., VON FEILITZEN, K., LILLEY, K. S., UHLEN, M. & LUNDBERG, E. 2017. A subcellular map of the human proteome. *Science,* 356.

TRIPATHI, V., ELLIS, J. D., SHEN, Z., SONG, D. Y., PAN, Q., WATT, A. T., FREIER, S. M., BENNETT, C. F., SHARMA, A., BUBULYA, P. A., BLENCOWE, B. J., PRASANTH, S. G. & PRASANTH, K. V. 2010. The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation. *Mol Cell,* 39**,** 925-38.

TROPEPE, V., SIBILIA, M., CIRUNA, B. G., ROSSANT, J., WAGNER, E. F. & VAN DER KOOY, D. 1999. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev Biol,* 208**,** 166-88.

TUOC, T., DERE, E., RADYUSHKIN, K., PHAM, L., NGUYEN, H., TONCHEV, A. B., SUN, G., RONNENBERG, A., SHI, Y., STAIGER, J. F., EHRENREICH, H. & STOYKOVA, A. 2017. Ablation of BAF170 in Developing and Postnatal Dentate Gyrus Affects Neural Stem Cell Proliferation, Differentiation, and Learning. *Mol Neurobiol,* 54**,** 4618-4635.

UHLEN, M., FAGERBERG, L., HALLSTROM, B. M., LINDSKOG, C., OKSVOLD, P., MARDINOGLU, A., SIVERTSSON, A., KAMPF, C., SJOSTEDT, E., ASPLUND, A., OLSSON, I., EDLUND, K., LUNDBERG, E., NAVANI, S., SZIGYARTO, C. A., ODEBERG, J., DJUREINOVIC, D., TAKANEN, J. O., HOBER, S., ALM, T., EDQVIST, P. H., BERLING, H., TEGEL, H., MULDER, J., ROCKBERG, J., NILSSON, P., SCHWENK, J. M., HAMSTEN, M., VON FEILITZEN, K., FORSBERG, M., PERSSON, L., JOHANSSON, F., ZWAHLEN, M., VON HEIJNE, G., NIELSEN, J. & PONTEN, F. 2015. Proteomics. Tissue-based map of the human proteome. *Science,* 347**,** 1260419.

URRUTIA, R. 2003. KRAB-containing zinc-finger repressor proteins. *Genome Biol,* 4**,** 231.

VANCE, K. W. & PONTING, C. P. 2014. Transcriptional regulatory functions of nuclear long noncoding RNAs. *Trends Genet,* 30**,** 348-55.

VANCE, K. W., SANSOM, S. N., LEE, S., CHALEI, V., KONG, L., COOPER, S. E., OLIVER, P. L. & PONTING, C. P. 2014. The long non-coding RNA Paupar regulates the expression of both local and distal genes. *EMBO J,* 33**,** 296-311.

WADDLNGTON, C. H. 1942. The Pupal Contraction as an Epigenetic Crisis in Drosophila. *Proceedings of the Zoological Society of London,* 111**,** 181-188.

WALKER, T. L. & KEMPERMANN, G. 2014. One mouse, two cultures: isolation and culture of adult neural stem cells from the two neurogenic zones of individual mice. *J Vis Exp***,** e51225.

WANG, C., IVANOV, A., CHEN, L., FREDERICKS, W. J., SETO, E., RAUSCHER, F. J., 3RD & CHEN, J. 2005. MDM2 interaction with nuclear corepressor KAP1 contributes to p53 inactivation. *EMBO J,* 24**,** 3279-90.

WANG, C., LIU, F., LIU, Y. Y., ZHAO, C. H., YOU, Y., WANG, L., ZHANG, J., WEI, B., MA, T., ZHANG, Q., ZHANG, Y., CHEN, R., SONG, H. & YANG, Z. 2011. Identification and characterization of neuroblasts in the subventricular zone and rostral migratory stream of the adult human brain. *Cell Res,* 21**,** 1534-50.

WANG, D. D. & BORDEY, A. 2008. The astrocyte odyssey. *Prog Neurobiol,* 86**,** 342-67.

WANG, G. G., ALLIS, C. D. & CHI, P. 2007. Chromatin remodeling and cancer, Part II: ATP-dependent chromatin remodeling. *Trends Mol Med,* 13**,** 373-80.

WANG, X., ARAI, S., SONG, X., REICHART, D., DU, K., PASCUAL, G., TEMPST, P., ROSENFELD, M. G., GLASS, C. K. & KUROKAWA, R. 2008. Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription. *Nature,* 454**,** 126-30.

WARD, M. E. & RAO, Y. 2005. Investigations of neuronal migration in the central nervous system. *Methods Mol Biol,* 294**,** 137-56.

WARTLICK, O., KICHEVA, A. & GONZALEZ-GAITAN, M. 2009. Morphogen gradient formation. *Cold Spring Harb Perspect Biol,* 1**,** a001255.

WEI, C., CHENG, J., ZHOU, B., ZHU, L., KHAN, M. A., HE, T., ZHOU, S., HE, J., LU, X., CHEN, H., ZHANG, D., ZHAO, Y. & FU, J. 2016. Tripartite motif containing 28 (TRIM28) promotes breast cancer metastasis by stabilizing TWIST1 protein. *Sci Rep,* 6**,** 29822.

WEI, C. W., LUO, T., ZOU, S. S. & WU, A. S. 2018. The Role of Long Noncoding RNAs in Central Nervous System and Neurodegenerative Diseases. *Front Behav Neurosci,* 12**,** 175.

WEINANDY, F., NINKOVIC, J. & GOTZ, M. 2011. Restrictions in time and space--new insights into generation of specific neuronal subtypes in the adult mammalian brain. *Eur J Neurosci,* 33**,** 1045-54.

WEISSMAN, I. L. 2000. Stem cells: units of development, units of regeneration, and units in evolution. *Cell,* 100**,** 157-68.

WHITMAN, M. C. & GREER, C. A. 2009. Adult neurogenesis and the olfactory system. *Prog Neurobiol,* 89**,** 162-75.

WILUSZ, J. E., SUNWOO, H. & SPECTOR, D. L. 2009. Long noncoding RNAs: functional surprises from the RNA world. *Genes Dev,* 23**,** 1494-504.

YANG, B., O'HERRIN, S. M., WU, J., REAGAN-SHAW, S., MA, Y., BHAT, K. M., GRAVEKAMP, C., SETALURI, V., PETERS, N., HOFFMANN, F. M., PENG, H., IVANOV, A. V., SIMPSON, A. J. & LONGLEY, B. J. 2007. MAGE-A, mMage-b, and MAGE-C proteins form complexes with KAP1 and suppress p53-dependent apoptosis in MAGE-positive cell lines. *Cancer Res,* 67**,** 9954-62.

YANG, B., XIA, Z. A., ZHONG, B., XIONG, X., SHENG, C., WANG, Y., GONG, W., CAO, Y., WANG, Z. & PENG, W. 2017. Distinct Hippocampal Expression Profiles of Long Non-coding RNAs in an Alzheimer's Disease Model. *Mol Neurobiol,* 54**,** 4833-4846.

YANG, L., LIN, C., JIN, C., YANG, J. C., TANASA, B., LI, W., MERKURJEV, D., OHGI, K. A., MENG, D., ZHANG, J., EVANS, C. P. & ROSENFELD, M. G. 2013. lncRNA-dependent mechanisms of androgen-receptor-regulated gene activation programs. *Nature,* 500**,** 598-602.

YANG, X., SONG, J. H., CHENG, Y., WU, W., BHAGAT, T., YU, Y., ABRAHAM, J. M., IBRAHIM, S., RAVICH, W., ROLAND, B. C., KHASHAB, M., SINGH, V. K., SHIN, E. J., YANG, X., VERMA, A. K., MELTZER, S. J. & MORI, Y. 2014. Long non-coding RNA HNF1A-AS1 regulates proliferation and migration in oesophageal adenocarcinoma cells. *Gut,* 63**,** 881-90.

YIN, Y., YAN, P., LU, J., SONG, G., ZHU, Y., LI, Z., ZHAO, Y., SHEN, B., HUANG, X., ZHU, H., ORKIN, S. H. & SHEN, X. 2015. Opposing Roles for the lncRNA Haunt and Its Genomic Locus in Regulating HOXA Gene Activation during Embryonic Stem Cell Differentiation. *Cell Stem Cell,* 16**,** 504-16.

YOKOE, T., TOIYAMA, Y., OKUGAWA, Y., TANAKA, K., OHI, M., INOUE, Y., MOHRI, Y., MIKI, C. & KUSUNOKI, M. 2010. KAP1 is associated with peritoneal carcinomatosis in gastric cancer. *Ann Surg Oncol,* 17**,** 821-8.

YOUNG, K. M., FOGARTY, M., KESSARIS, N. & RICHARDSON, W. D. 2007. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J Neurosci,* 27**,** 8286-96.

ZAMUROVIC, N., CAPPELLEN, D., ROHNER, D. & SUSA, M. 2004. Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. *J Biol Chem,* 279**,** 37704-15.

ZARATIEGUI, M., IRVINE, D. V. & MARTIENSSEN, R. A. 2007. Noncoding RNAs and gene silencing. *Cell,* 128**,** 763-76.

ZENG, L., YAP, K. L., IVANOV, A. V., WANG, X., MUJTABA, S., PLOTNIKOVA, O., RAUSCHER, F. J., 3RD & ZHOU, M. M. 2008. Structural insights into human KAP1 PHD finger-bromodomain and its role in gene silencing. *Nat Struct Mol Biol,* 15**,** 626-33.

ZHANG, J., JI, F., LIU, Y., LEI, X., LI, H., JI, G., YUAN, Z. & JIAO, J. 2014. Ezh2 regulates adult hippocampal neurogenesis and memory. *J Neurosci,* 34**,** 5184-99.

ZHANG, J. & JIAO, J. 2015. Molecular Biomarkers for Embryonic and Adult Neural Stem Cell and Neurogenesis. *Biomed Res Int,* 2015**,** 727542.

ZHANG, J. X., HAN, L., BAO, Z. S., WANG, Y. Y., CHEN, L. Y., YAN, W., YU, S. Z., PU, P. Y., LIU, N., YOU, Y. P., JIANG, T., KANG, C. S. & CHINESE GLIOMA COOPERATIVE, G. 2013. HOTAIR, a cell cycle-associated long noncoding RNA and a strong predictor of survival, is preferentially expressed in classical and mesenchymal glioma. *Neuro Oncol,* 15**,** 1595-603.

ZHANG, K., SUN, X., ZHOU, X., HAN, L., CHEN, L., SHI, Z., ZHANG, A., YE, M., WANG, Q., LIU, C., WEI, J., REN, Y., YANG, J., ZHANG, J., PU, P., LI, M. & KANG, C. 2015a. Long non-coding RNA HOTAIR promotes glioblastoma cell cycle progression in an EZH2 dependent manner. *Oncotarget,* 6**,** 537-46.

ZHANG, L., LI, H., ZENG, S., CHEN, L., FANG, Z. & HUANG, Q. 2015b. Long-term tracing of the BrdU label-retaining cells in adult rat brain. *Neurosci Lett,* 591**,** 30-4.

ZHANG, P. P., DING, D. Z., SHI, B., ZHANG, S. Q., GU, L. L., WANG, Y. C. & CHENG, C. 2018. Expression of TRIM28 correlates with proliferation and Bortezomib-induced apoptosis in B-cell non-Hodgkin lymphoma. *Leuk Lymphoma,* 59**,** 2639-2649.

ZHAO, J., SUN, B. K., ERWIN, J. A., SONG, J. J. & LEE, J. T. 2008. Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. *Science,* 322**,** 750-6.

ZHAO, X., UEBA, T., CHRISTIE, B. R., BARKHO, B., MCCONNELL, M. J., NAKASHIMA, K., LEIN, E. S., EADIE, B. D., WILLHOITE, A. R., MUOTRI, A. R., SUMMERS, R. G., CHUN, J., LEE, K. F. & GAGE, F. H. 2003. Mice lacking methyl-CpG binding protein 1 have deficits in adult neurogenesis and hippocampal function. *Proc Natl Acad Sci U S A,* 100**,** 6777-82.

ZHOU, X., REN, Y., ZHANG, J., ZHANG, C., ZHANG, K., HAN, L., KONG, L., WEI, J., CHEN, L., YANG, J., WANG, Q., ZHANG, J., YANG, Y., JIANG, T., LI, M. & KANG, C. 2015. HOTAIR is a therapeutic target in glioblastoma. *Oncotarget,* 6**,** 8353-65.

ZYWITZA, V., MISIOS, A., BUNATYAN, L., WILLNOW, T. E. & RAJEWSKY, N. 2018. Single-Cell Transcriptomics Characterizes Cell Types in the Subventricular Zone and Uncovers Molecular Defects Impairing Adult Neurogenesis. *Cell Rep,* 25**,** 2457-2469 e8.