

Unraveling the Role of *Pten* in Cortical Stem Cell Lineage Progression using MADM

by

Oswaldo Antonio Miranda Romero

August, 2025

*A thesis submitted to the
Graduate School
of the
Institute of Science and Technology Austria
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy*

Committee in charge:
Sandra Siegert, PhD, Chair
Simon Hippenmeyer, PhD
Bennett Novitch, PhD
Dr. med. univ., Dr. rer. nat. Johann Danzl



The thesis of Osvaldo Antonio Miranda Romero, titled *Unraveling the Role of Pten in Cortical Stem Cell Lineage Progression using MADM*, is approved by:

Supervisor: Simon Hippenmeyer, ISTA, Klosterneuburg, Austria

Signature: _____

Committee Member: Johann Danzl, ISTA, Klosterneuburg, Austria

Signature: _____

Committee Member: Bennett G. Novitch, University of California, Los Angeles,
California, United States of America

Signature: _____

Defense Chair: Sandra Siegert, ISTA, Klosterneuburg, Austria

Signature: _____

Signed page is on file

© by Osvaldo Antonio Miranda Romero, August, 2025
All Rights Reserved

ISTA Thesis, ISSN: 2663-337X

ISBN: 978-3-99078-063-3

I hereby declare that this thesis is my own work and that it does not contain other people's work without this being so stated; this thesis does not contain my previous work without this being stated, and the bibliography contains all the literature that I used in writing the dissertation.

I accept full responsibility for the content and factual accuracy of this work, including the data and their analysis and presentation, and the text and citation of other work.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee, and that this thesis has not been submitted for a higher degree to any other university or institution.

I certify that any republication of materials presented in this thesis has been approved by the relevant publishers and co-authors.

Signature: _____

Osvaldo Antonio Miranda Romero

August, 2025

Signed page is on file

Abstract

Cortical development has been studied for many decades and with each passing year, we learn more about the processes that govern the tightly regulated developmental program. Up until recently, the field has struggled to gain reliable access to progenitors at the single-cell level, limiting the resolution at which we can study cortical development *in vivo*. Therefore, much of the data we have on mammalian cortical development is at the cell population level. The subsequent pages of this thesis describe novel elements of cortical development in mice; acquired using a candidate gene approach in conjunction with Mosaic Analysis with Double Markers (MADM) to achieve single cell resolution. In this thesis, I briefly provide an overview of the state of the field, describe the methodology used to quantify morphological characteristics of neurons and glia, and delve into the role of *Phosphatase and tensin homologue on chromosome ten (Pten)* in neurogenesis and gliogenesis in the developing mouse cortex. By using MADM to generate genetic mosaics, in which only a small fraction of cells are mutated, to study population-level changes, we can bypass the early postnatal lethality reported in *Pten* conditional knockouts (cKOs) using *Emx1-Cre*. We used *Emx1-CreERT2* to induce clonal deletion of *Pten*, and quantify RGP neurogenic output at the single progenitor level. *Pten*-mutant neuron populations within the mosaics were significantly expanded. In the latter parts of this thesis, we describe how *Pten* deletion leads to an increase in the abundance of astrocytes. We availed ourselves of the flexibility offered by the MADM system to generate *Pten* mosaics within a series of distinct knockout tissues to test for the interactions between *Pten* and a battery of genes involved in distinct pro-gliogenic signaling pathways. Our survey of *Pten*'s interactions with genes in key signaling pathways reveals that ERK1/2 are the critical effectors downstream of EGFR signaling essential for gliogenesis. Altogether, our results demonstrate that *Pten* plays critical and distinct roles throughout cortical RGP lineage progression.

Acknowledgments

I would like to start this section by thanking Dr. Simon Hippenmeyer. Simon has provided so much support and guidance; this project would not be what it is without him. During my time in the group, he has provided me with mentorship, the scientific freedom to explore, as well as numerous opportunities to present this work at international conferences. I would also like to thank the Austrian Academy of Sciences for awarding me a 2-year DOC fellowship (DOC26253).

Working with the Hippenmeyer group members has been a privilege. I thank all current and former members, as each of you have played a role in my time at ISTA. I would especially like to thank Ana, Fabrizia, and Raquel for the countless laughs and for putting up with my antics. Thank you Florian and Carmen for the spectacular support you have provided over the years. Thank you Giselle, Nici, and Melissa for your guidance and advice. Thank you all for the great chats, outings, and good company. This work was supported by numerous interns and rotation students. Thank you Vanessa Mick, Simon Schnabl, Sophie Gobeil, and Eda Arslantas,

ISTA has top-notch facilities, run by great staff. I would like to thank the Imaging and Optics Facility and the Preclinical Facility for their constant support. I would like to thank Anna Heger, our colony manager, in particular for her truly exceptional efforts given the complex genetics this project required.

I would like to thank Prof. Cristian Ruiz Rueda, Dr. Dana Harmon, Dr. Ranmal Samarasinghe, Prof. Samantha Butler, and Prof. Bennett Novitch. Each of you has played a crucial role in my development as a scientist, be it providing me with my very first research position, or giving me the opportunity to join your group as a bachelor's student with no experience in neuroscience. You all provided invaluable guidance and mentorship during the respective periods in my career. Thank you to the members of the Ruiz Rueda lab, Butler lab, and Novitch lab for your support and encouragement.

I want to thank my committee members, Prof. Bennett Novitch and Prof. Johann Danzl for their continued support, considerable time investment, and encouragement throughout the years. I would also like to thank the committee chair, Prof. Sandra Siegert, for her time.

Lastly, I owe a great debt of gratitude to my family and friends. Making the decision to join ISTA for my doctoral work took a heavy emotional toll on the family members I had to leave behind at home. Thank you Mom and Ron for being model parents, who instilled in me the values and ethics I carry with me today, and for always supporting and encouraging me in the pursuit of my goals. They taught me what it is to be a good person, and have always worked hard to make sure I could take every opportunity to be a better person and scientist. I want to thank my sister Adriana, for always radiating love and positivity. Thank you Dad for your encouragement and for making the tough but necessary decisions to progress in life. Mama Con and Abuelita Rita for always sending me their love and encouraging words. Finally, I would like to thank my Austrian family. To all the friends I made in this PhD journey, thank you for your support, love and friendship. Danai, thank you for your support, patience, and for always being there. Canelo, thank you for your loyalty, companionship, and for accepting us as we are.

About the Author

Osvaldo Antonio Miranda Romero completed his BSc in Cell and Molecular Biology at the California State University, Northridge. He began his research career in the lab of Dr. Cristian Ruiz Rueda, studying the prevalence of antibiotic resistant bacteria in public water sources in the greater Los Angeles area. Osvaldo then joined Dr. Bennett Novitch's group at UCLA through the CIRM-CSUN-UCLA Stem Cell Scientist Training Program. His work focused on characterizing human cerebral organoids derived from Rett Syndrome patient stem cells. In 2019, Osvaldo joined the ISTA PhD program, and affiliated with the Hippenmeyer group in 2020. His main research interest is to gain a better understanding of the molecular mechanisms that govern mammalian brain development. Osvaldo received support from the Austrian Academy of Sciences (ÖAW) in the form of a two-year DOC Fellowship. During his PhD, Osvaldo presented his work at numerous international conferences, including the FENS, EMBO Israel, Gordon Research Conference on Neural Development, and Cold Spring Harbor.

List of Publications

1. **Miranda, O.A.***, Cheung, G.*, and Hippenmeyer, S. (2024). Morphological Analysis of Neurons and Glia using Mosaic Analysis with Double Markers. *Neuronal Morphogenesis. Methods in Molecular Biology*, vol 2831. (Used in full in Chapter 3. Reproduced with permission from Springer Nature)

Contributions

Simon Hippenmeyer, Giselle Cheung, and Osvaldo A. Miranda Romero conceived and designed the manuscript in Chapter 3. Osvaldo A. Miranda Romero and Giselle Cheung equally contributed to the writing and figures. All authors read, edited, and approved the final manuscript.

For Chapters 4 and 5: Simon Hippenmeyer conceived and supervised the study. Osvaldo A. Miranda Romero designed and conducted experiments, analyzed data, and generated the figures. Ximena Contreras bred *Pten* mice and quantified G/R neuron ratios and a portion of the clonal data. Anna Heger generated stock mouse lines and Carmen Streicher generated a portion of the experimental mice. Vanessa Mick, Sophie Gobeil, and Simon Schnabl performed experiments under the supervision of Osvaldo A. Miranda Romero.

Table of Contents

Abstract	i
Acknowledgments	iii
About the Author	v
List of Publications	vi
Contributions	vi
Table of Contents	vii
List of Figures	ix
List of Abbreviations.....	xi
1 Chapter 1 – Introduction	1
1.1 THESIS OUTLINE.....	1
1.2 THE MAMMALIAN CEREBRAL CORTEX	2
1.3 CORTICAL DEVELOPMENT THROUGH THE LENS OF NEURAL STEM CELL LINEAGE PROGRESSION.....	4
1.4 APPLYING MOSAIC ANALYSIS WITH DOUBLE MARKERS TO STUDY GENES AND GENETIC INTERACTIONS AT THE SINGLE PROGENITOR LEVEL.....	8
1.5 PTEN FUNCTIONS IN HEALTH AND DISEASE	10
2 Chapter 2 – Thesis Objectives	14
3 Chapter 3 – Morphological Analysis of Neurons and Glia using Mosaic Analysis with Double Markers.....	16
3.1 AUTHOR CONTRIBUTIONS	16
3.2 ABSTRACT	17
3.3 INTRODUCTION	18
3.4 MATERIALS	19
3.4.1 <i>Reagents and equipment</i>	19
3.4.2 <i>Microscopes and software</i>	19
3.5 METHODS.....	19
3.6 PREPARATION OF EXPERIMENTAL TISSUE CONTAINING MADM-LABELLED CELLS	20
3.6.1 <i>Generation and cryosectioning of MADM brains</i>	20
3.6.2 <i>Immunostaining to enhance endogenous MADM signals</i>	20
3.7 NEURON SOMA AND ASTROCYTE VOLUME MEASUREMENTS	20
3.7.1 <i>Image acquisition</i>	20
3.7.2 <i>Image processing for neuron and astrocyte volume measurements</i>	21
3.8 ANALYSIS OF DENDRITIC SPINES OF MADM-LABELLED NEURONS	22
3.8.1 <i>Image acquisition</i>	22
3.8.2 <i>Image deconvolution</i>	22
3.8.3 <i>Reconstruction of dendrites and dendritic spines</i>	23
3.8.3.1 <i>Dendritic spines detection (Automated mode)</i>	23
3.8.4 <i>Classification of dendritic spines</i>	23
3.8.5 <i>Data extraction and statistical analysis</i>	24
3.9 ASTROCYTE SHOLL ANALYSIS	24
3.9.1 <i>Image acquisition</i>	24
3.9.2 <i>Image deconvolution</i>	25
3.9.3 <i>Render Astrocyte Filaments</i>	25
3.10 NOTES	26
4 Role of <i>Pten</i> in Cortical Neurogenesis at Single Cell Resolution using MADM	35

4.1	SUMMARY	35
4.2	INTRODUCTION	36
4.3	NEUROGENESIS IN MAMMALS	36
4.4	PTEN MOUSE MODELS.....	38
4.5	RESULTS	39
4.5.1	<i>Experimental Design for Sparse Deletion of Pten in the Developing Mouse Cortex</i>	39
4.5.2	<i>Pten Deletion Results in an Expansion of Cortical Neuron Populations with Significant Morphological Alterations</i>	41
4.5.3	<i>Pten is Cell-Autonomously Required in Individual RGP's during Neurogenesis</i>	44
4.5.4	<i>Deep Layer Neuron Populations are Selectively Expanded in Pten-MADM Mice</i>	49
4.6	DISCUSSION	53
5	Uncovering the Role of <i>Pten</i> during Cortical Gliogenesis	55
5.1	SUMMARY	55
5.2	INTRODUCTION TO MAMMALIAN GLIOGENESIS	55
5.2.1	<i>EGFR's Key Role in Cortical Gliogenesis</i>	56
5.2.2	<i>JAK/STAT's Influence on the Onset of Astrocyte Production</i>	57
5.2.3	<i>NOTCH Signaling as a Regulator of Gliogenesis</i>	58
5.2.4	<i>ERK Signaling as a Pro-Gliogenic Pathway in the Mouse Cortex</i>	58
5.3	PTEN IN ASTROCYTE DEVELOPMENT AND DISEASE	60
5.4	RESULTS	60
5.4.1	<i>Experimental MADM Breedings for Analyzing the Role of Pten in Gliogenesis</i>	60
5.4.2	<i>Pten Mutation Expands Glial Production in the Mouse Neocortex</i>	62
5.4.3	<i>Pten Deletion Prolongs the Gliogenic Period and Results in Larger Astrocyte Clones</i>	65
5.4.4	<i>Pten-Mutant Astrocytes are Morphologically Distinct Compared to Control</i>	67
5.4.5	<i>Bulk-RNAseq of Isolated Astrocyte Population Reveals Dereglulation in Key Signaling Pathways</i>	69
5.4.6	<i>Egfr Requirement during Gliogenesis is overridden by Pten Deletion</i>	77
5.4.7	<i>Pten Deletion leads to Over-Activation of Key Signaling Pathways</i>	80
5.4.8	<i>Deletion of Jak/Stat and Notch Signaling Components Does Not Rescue Pten-Mutant Astrocyte Phenotype</i>	86
5.4.9	<i>Pten-Mutant Astrocyte Expansion Depends on an Intact MAPK Signaling Pathway</i>	95
5.5	DISCUSSION	102
6	Conclusion	106
7	Methods	107
7.1	MOUSE HUSBANDRY AND LINES	107
7.2	PREPARATION OF MADM-LABELED TISSUE	107
7.3	IMMUNOLABELING OF MADM-LABELED TISSUE.....	108
7.4	IMAGING AND ANALYSIS OF MADM-LABELED TISSUE	108
7.5	MADM CLONAL ANALYSIS	109
7.6	STATISTICAL ANALYSIS.....	109
7.7	SINGLE-CELL SUSPENSION AND FACS OF MADM-LABELED TISSUE.....	109
7.8	LIBRARY PREPARATION AND SINGLE CELL SEQUENCING.....	110
7.9	SINGLE-CELL SEQUENCING ANALYSIS	110
	References	112

List of Figures

Figure 1. Most prevalent malformations of the neocortex.....	3
Figure 2 Cortical stem cells follow a stereotyped program during corticohistogenesis.	5
Figure 3. Neurons project to subcortical or intracortical targets depending on which cortical laminae they reside in.	7
Figure 4 The genetic principle of MADM.	8
Figure 5 Experimental paradigms for genetic interaction experiments with MADM.	10
Figure 6 Canonical PTEN signaling cascade.	12
Figure 7 Relevant steps to measure the volume of a neuron cell body.	28
Figure 8 Volume measurements of neuron soma and astrocyte.	29
Figure 9 3D Cropping and deconvolution of dendritic segments.	30
Figure 10 Segmentation and reconstruction of dendritic spines.	31
Figure 11 Reconstruction and analysis of dendritic spines.	32
Figure 12 Reconstruction and analysis of astrocyte filaments.	33
Figure 13. Experimental paradigms to study <i>Pten</i> at single cell resolution using MADM. ...	40
Figure 14. <i>Pten</i> deletion results in a cell autonomous increase in the mutant neuron populations in the neocortex.	42
Figure 15. <i>Pten</i> cell-autonomously controls neuron cell size.	43
Figure 16. Deletion of <i>Pten</i> in early RGP results in larger symmetric proliferative clones..	46
Figure 17 <i>Pten</i> is cell-autonomously required for constraining neurogenic clonal unit size..	47
Figure 18. <i>Pten</i> -mutant neurons are more abundant in the deep layers.	51
Figure 19. Neurons with deep layer identities are more abundant in <i>Pten</i> mutants than control at P0.	52
Figure 20. Experimental paradigms to study <i>Pten</i> at single cell resolution using MADM. ...	61
Figure 21. <i>Pten</i> deletion results in a cell-autonomous increase in the astrocyte population at P21.	63
Figure 22. <i>Pten</i> cell-autonomously controls the astrocyte intermediate progenitor pool size.	64
Figure 23. Perturbations to <i>Pten</i> expression lead to a prolonged gliogenic period as well as a skew toward larger astrocyte clones in the mouse cortex.	66
Figure 24. Astrocyte morphology is significantly altered by <i>Pten</i> deletion.	68
Figure 25. Labeling and isolation of highly purified astrocyte population.	71
Figure 26. P0 and P4 astrocyte DEGs differ significantly between the two time points.	72
Figure 27. Prominent deregulation of cell cycle genes in <i>Pten</i> ^{-/-} astrocytes at P0.	73
Figure 28. <i>Pten</i> ^{-/-} astrocytes at P4 show only moderate transcriptional deregulation.	74
Figure 29. STRING network analysis reveals several central signaling pathways as possible mediators of <i>Pten</i> -mutant astrocyte overproliferation.	76
Figure 30. Breeding schemes test for <i>Egfr</i> dependence in <i>Pten</i> astrocyte phenotype.	78
Figure 31. <i>Egfr</i> Requirement during Gliogenesis is overridden by <i>Pten</i> Deletion.	79
Figure 32. Deletion of <i>Pten</i> and <i>Egfr</i> shifts relative abundance of cell types toward non-projection neuron population.	83
Figure 33. Genes from several critical pro-gliogenic signaling pathways are enriched in <i>Pten/Egfr</i> double mutant aIPs.	85
Figure 34. Breeding schemes test for <i>Notch</i> dependence in <i>Pten</i> astrocyte phenotype.	88
Figure 35. Breeding schemes test for <i>Stat3</i> dependence in <i>Pten</i> astrocyte phenotype.	89
Figure 36. Breeding schemes test for <i>Stat5</i> dependence in <i>Pten</i> astrocyte phenotype.	90

Figure 37. Breeding schemes test for <i>Jak2</i> dependence in <i>Pten</i> astrocyte phenotype.	91
Figure 38. Breeding schemes test for <i>Jak1</i> dependence in <i>Pten</i> astrocyte phenotype.	92
Figure 39. <i>Pten</i> -mutant astrocytes expand independently of <i>Jak/Stat</i> and <i>Notch</i> signaling. ..	93
Figure 40. Breeding schemes test for <i>Erk1</i> and <i>Erk2</i> dependence in <i>Pten</i> astrocyte phenotype.	98
Figure 41. <i>Erk1/2</i> are key mediators of astrocyte population expansion in <i>Pten</i> knockout populations.	99
Figure 42. <i>Pten</i> modulates cortical RGP output at distinct stages of lineage progression. ..	104

List of Abbreviations

4EBP1	Eukaryotic Translation-Initiation Factor 4E-binding Protein 1
aIP	Astrocyte Intermediate Progenitor
AKT	Protein Kinase B
ASD	Autism Spectrum Disorder
bHLH	basic Helix Loop Helix
BLBP	Brain Lipid-Binding Protein
bMIPC	basal Multipotent Intermediate Progenitors
Chr.	Chromosome
Cre	Cyclization Recombinase
cKO	Conditional Knockout
CNS	Central Nervous System
CP	Cortical Plate
CTIP2	COUP-TF-interacting protein 2
DEG	Differentially Expressed Gene
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular Signal-Regulated Kinase
FACS	Fluorescence-Activated Cell Sorting
GBM	Glioblastoma Multiforme
GE	Ganglionic Eminence
GFP	Green Fluorescent Protein
GO	Gene Ontology
GOF	Gain of Function
GOI	Gene of Interest
GSK3	Glycogen Synthase Kinase 3
iDEA	Integrative Differential Expression and Gene Set Enrichment Analysis
IP	Intermediate Progenitor
JAK	Janus Kinase
LIS1	Lissencephaly-1
LOF	Loss of Function

MADM	Mosaic Analysis with Double Markers
MAPK	Mitogen-Activated Protein Kinase
mTOR	Mammalian Target of Rapamycin
NESC	Neuroepithelial Stem Cell
NICD	Notch Intracellular Domain
NDEL1	Nuclear distribution gene E-like homolog 1
OBNB	Olfactory Bulb Neuroblast
OPC	Oligodendrocyte Precursor Cells
oRG	Outer Radial Glia
PCA	Principle Component Analysis
PCF	Preclinical Facility
PHTS	PTEN Hamartoma Tumor Syndrome
PI3K	Phosphoinositide 3-kinase
PIP₂	Phosphatidylinositol-4,5-biphosphate
PIP₃	Phosphatidylinositol-3,4,5-triphosphate
PIP-seq	Particle-Templated Instant Partition Sequencing
PDK1	3-phosphoinositide-dependent Kinase 1
PTEN	Phosphatase and Tensin homologue on chromosome ten
RGP	Radial Glia Progenitor
RTK	Receptor Tyrosine Kinase
RTKI	Receptor Tyrosine Kinase Inhibitor
S6K	p70 Ribosomal Protein S6 Kinase
STAT	Signal Transducer and Activator of Transcription
SVZ	Subventricular Zone
tdTomato	Tandem dimer Tomato
UMAP	Uniform Manifold Approximation and Projection
VZ	Ventricular Zone

1 Chapter 1 – Introduction

1.1 Thesis Outline

This thesis consists of one large project divided out into multiple chapters. The unifying aim is to gain a clearer understanding of the molecular processes that govern cortical stem cell lineage progression at single cell resolution using genetic mosaic analysis in the mouse cortex *in vivo*. The first chapter provides a primer on the cerebral cortex and gives a broad overview of the process of cortical development, as the field currently understands it. It also introduces the MADM technology, a powerful genetic manipulation tool and a key method in the project as a whole. The first chapter ends with an introduction of *Pten*, a potent tumor suppressor among other biological functions. *Pten* is the major gene of interest of this study.

The second chapter is an outline of the objectives of this thesis. I touch on the major questions we pose and provide details regarding how we attempt to answer them.

The third chapter consists of a manuscript published in *Methods in Molecular Biology*, vol 2831. In it, we describe the methods we use for assessing neuronal morphology and spine density using MADM. The chapter also covers the steps for quantifying astrocyte volume and branching morphology. Such methods provide valuable insights into the morphological complexity of diverse cell types in the brain.

The fourth chapter consists of unpublished data covering the role of *Pten* during cortical neurogenesis in mice. *Pten* is a known tumor suppressor gene, and mutations in humans often lead to a slew of intellectual deficits and oftentimes to macrocephaly. Given that *Pten* mutations result in a larger brain, I expected that *Pten* plays a significant role in cortical stem cell lineage progression. I used MADM genetic mosaic mice to precisely dissect the contributions of *Pten* at the single progenitor level. I also used mice carrying tamoxifen-inducible Cre to understand whether *Pten* controls clonal unit output using MADM.

The fifth chapter expands on the previous line of study to later stages of development associated with gliogenesis. It is clear that *Pten* deletions often accompany glioma occurrence in humans, but whether and how *Pten* plays a role in cortical gliogenesis during development is still unclear. I extended the previous analysis using mosaic mice to study cortical astrocyte clone size, astrocyte progenitor abundance, and to pin down the pathways contributing to gliogenesis in normal and *Pten* mutant conditions. All of this work was possible with the help of lab technicians, interns, and rotation students under my supervision.

The sixth chapter wraps up the results in the thesis with a discussion. The discussion puts into context all of the work contained within the body of this work.

The seventh and final chapter describes the methods used in the experimental portions of this thesis. If a method originates from a previous publication, the publication is cited and the method is summarized in brief to provide clarity for the reader.

1.2 The Mammalian Cerebral Cortex

The neocortex is evolutionarily the newest part of the brain, and is responsible for higher order cognitive functions, sensory information processing, and consciousness. It is composed of a large assortment of neuronal and non-neuronal cell types. The neuronal population can be divided into two subclasses: excitatory pyramidal neurons and inhibitory interneurons. Pyramidal neurons are born from a relatively limited pool of radial glia progenitors (RGPs) in the ventricular zone (VZ) in the dorsal forebrain (Villalba et al., 2021). Pyramidal neurons project intra-cortically and subcortically, functionally connecting distinct brain regions.

Inhibitory interneurons are born in the ganglionic eminences (GEs) from a distinct pool of progenitors lining the ventricular wall. These multipotent progenitors sequentially produce a diverse array of interneurons (Wamsley & Fishell, 2017) that later migrate tangentially to and infiltrate the neocortex (Bandler et al., 2017). Tangential migration is a fascinating phenomenon during which interneurons born in the GEs migrate from the ventral telencephalon into the dorsal telencephalon during the last third of embryogenesis. Upon reaching the cortex, interneurons switch from tangential to radial migration and distribute themselves throughout the cortical laminae (Wamsley & Fishell, 2017). Interneurons function to modulate the local activity of cortical pyramidal neurons. The mechanisms that orchestrate cell fate decisions and govern the corticodevelopmental program remain poorly understood. Such processes are key to understanding not only how our brains are constructed, but are also clinically relevant. Cortical malformations arise from deviations from the nominal developmental program. Cortical malformations like macrocephaly, lissencephaly, and microcephaly can bring with them a series of complications such as epilepsy, Autism Spectrum Disorder (ASD), intellectual disabilities, and can even be fatal (Barkovich et al., 2015; Kanekar & Gent, 2011) (Figure 1).

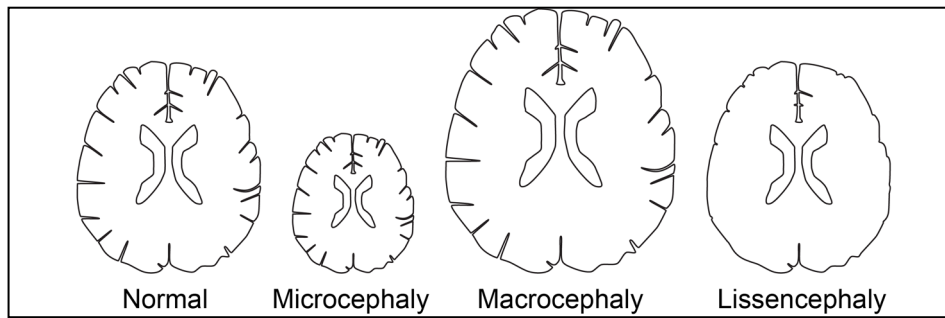


Figure 1. Most prevalent malformations of the neocortex.

Left to right: A normally developed cortex is comprised of gyri and sulci, expanding the surface area of the cortex without increasing the size of the cranium. Aberrations resulting in microcephaly and macrocephaly perturb the size of the cortex, without really altering the overall structures therein. Lissencephaly describes a brain without the gyri and sulci found in normal brains, reducing the cortical surface area.

Microcephaly occurs when the entire brain is too small but is otherwise morphologically normal. It can be caused by a wide array of conditions, both genetic and environmental. A striking example of microcephaly can be seen in newborn babies of Zika patients who became infected during pregnancy, most notably in Brazil (Qian et al., 2017). Mothers bitten by mosquitos carrying the Zika virus were infected, along with their unborn children. Adults experience flu-like symptoms, but the virus crossed the placental barrier, and thus the developing embryo also acquired the virus. Studies using human pluripotent stem cell-derived organoids have shown that the Zika virus can infiltrate the VZ, infect progenitors, attenuate cell divisions, and result in cell death leading to an early depletion of the progenitor pool and reduced organoid growth (Qian et al., 2016; Tang et al., 2016; Watanabe et al., 2017). Early differentiation or progenitor death due to distinct genetic conditions can also lead to a similar result, a depletion of the progenitor pool and an overall decrease in brain size.

Macrocephaly, on the other hand describes an abnormally large brain. Human patients with macrocephaly frequently present with mutations to genes that when mutated are known to cause this cortical malformation. Some such genes include *Pten*, *Cdkn1c*, and *Aspm* (Chen et al., 2015; Groszer et al., 2001; Hippenmeyer, 2023). *Aspm* is a crucial regulator of brain size, and gain of function (GOF) mutations often lead to macrocephaly and ASD (Tsai et al., 2023). A recent study found that *Aspm* GOF led to higher neuron density in the cortex and a greater cortical area (Singh et al., 2025). They report that the increases neuron numbers originate from an expanded secondary progenitor pool; they claim the progenitors resemble outer basal radial glia (oRGs). Finally, they report hyperconnectivity across cortical areas in their GOF mice, hinting at possible network level abnormalities that could help explain the high incidence of

ASD in patients with *ASPM* mutations. Even with such detailed studies, we remain uncertain about the processes that lead to macrocephaly at the single progenitor level, providing an avenue for further research.

Gyrencephalic mammals have cortical folds that increase the cortical surface area to optimize the limited volume provided by the cranium. Humans born without such folds have a smooth, or lissencephalic, brain. Lissencephaly is the outlier from the three conditions described in this introduction, since it is characterized by a change in gross brain topology and not a change in size. One well-studied cause of lissencephaly is due to migratory defects in nascent neurons that cannot properly complete their radial migration to the cortical plate (Hansen & Hippenmeyer, 2020; A. H. Hansen et al., 2022; Hippenmeyer, 2014; Hippenmeyer et al., 2010; Reiner et al., 1995). Lissencephaly-1 (LIS1) is a key component of a complex that regulates nuclear migration (Morris, 2000), a process required by migrating neurons (Hippenmeyer, 2014). Nuclear distribution gene E-like homolog 1 (NDEL1) associates with LIS1 and a dynein motor protein (Niethammer et al., 2000; Sasaki et al., 2000). In 2010, Hippenmeyer and colleagues reported that LIS1 mutation delayed cortical neuron migration, but is not essential for migration. This finding confirms a similar function of *Lis1* in mammals as previous reports from *Drosophila* models (Liu et al., 2000). Curiously, they report that *Ndel1* deletion resulted in an inability of neurons to migrate to target layers without perturbing astrocyte migration patterns (Hippenmeyer et al., 2010).

In summary, these studies show that, while we know much about population level requirements of certain genes during cortical development, we lack single cell resolution to truly dissect the specific actions of a gene's functions in individual cells.

1.3 Cortical Development through the Lens of Neural Stem Cell Lineage Progression

The cerebral cortex is a remarkably complex and diverse structure tasked with numerous higher-level cognitive functions. Cortical development begins with the expansion of the neuroepithelium, a pseudostratified layer of tissue composed of neuroepithelial stem cells (NESCs) (Bishop et al., 2000), which initially divide symmetrically to amplify the progenitor pool. NESCs later lose tight junctions but retain their apical adherens junctions and thereby transform into radial glia progenitors around embryonic day 9-10 (E9-10) (Gotz & Huttner, 2005; Howard et al., 2008). RGPs have both an apical and basal process anchoring the cell to the ventricular and pial surfaces of the cortical tissue, respectively (Villalba et al., 2021). RGPs reside in the VZ of the developing cortex, and are responsible for generating the excitatory

neurons and astrocytes of the *Emx1* lineage (Beattie & Hippenmeyer, 2017; Di Bella et al., 2024; Hippenmeyer, 2023) (Figure 2).

Previous work from our group has shed light on the deterministic program that governs RGP lineage progression in the developing cortex. At the early stages of cortical development, until E11.5, a majority of RGPs continue to divide symmetrically. At E12, RGPs switch from symmetric proliferative divisions to asymmetric neurogenic divisions, resulting in an RGP and a neuron or an intermediate progenitor (IP). RGPs are able to generate neurons in two ways, either through direct neurogenesis or indirect neurogenesis (Borrell & Gotz, 2014). Direct neurogenesis is a more ancient form of producing neurons (Villalba et al., 2021), whereby one RGP divides and produces one neuron and a daughter RGP. Direct neurogenesis is the main mode of neuron generation in reptiles and birds, though birds do have some indirect neurogenesis (Villalba et al., 2021). In contrast, indirect neurogenesis occurs when an RGP divides and produces an IP and a daughter RGP. Mammalian brains rely heavily on indirect neurogenesis to produce the expanded cortex necessary for the higher cognitive functions of this clade (Kriegstein et al., 2006; Villalba et al., 2021). The expanded progenitor pool also brings about a new region in the developing cortex, the subventricular zone (SVZ) (Martinez-Cerdeno et al., 2016).

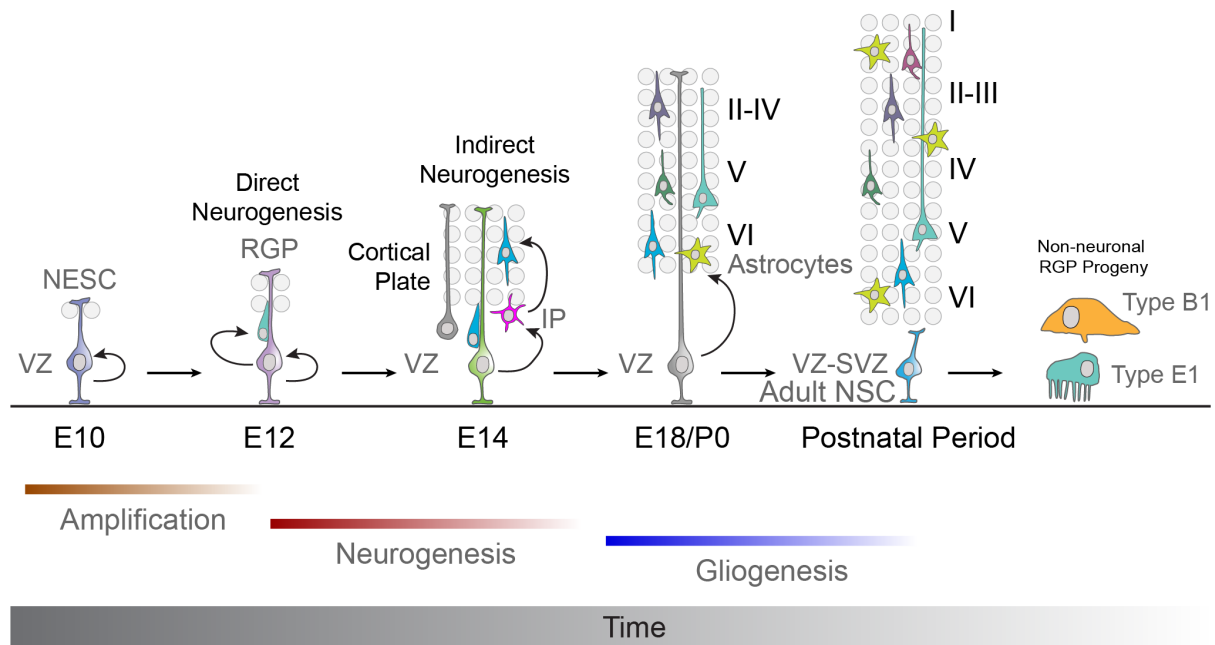


Figure 2 Cortical stem cells follow a stereotyped program during corticohistogenesis.

The cortex begins as a layer of neuroepithelial stem cells, which divide symmetrically to enlarge the progenitor pool. These later give rise to RGPs, which in turn generate the excitatory pyramidal neurons found in the cortex either through direct or indirect neurogenesis. RGPs then transition from neurogenic to gliogenic divisions, during

which they produce astrocytes and oligodendrocytes. Postnatal progenitor populations exist within the mouse cortex and give rise to olfactory bulb interneurons as well as a few non-neuronal cell types.

During the early rounds of indirect neurogenic divisions, RGPs produce the first IPs, which migrate basally to establish the SVZ (Haubensak et al., 2004). IPs are multipolar and lack apicobasal processes anchoring them in place (Haubensak et al., 2004; Noctor et al., 2004). IPs reside in the SVZ and from here divide symmetrically to produce two daughter neurons, thereby amplifying the overall output of that single RGP (Villalba et al., 2021). This basic sequence of lineage progression also holds true in gyrencephalic animals like the ferret and fetal monkey, though gyrencephalic development relies more heavily on other progenitor types like outer radial glia (Schmechel & Rakic, 1979; Voigt, 1989).

Previous work using Mosaic Analysis with Double Markers (MADM) has shown that in mice each RGP produces a unit of 8-9 neurons during their neurogenic phase (Gao et al., 2014). Such precise accounting provides not only a qualitative framework of neurogenesis, but also a quantitative deterministic framework.

The first pyramidal neurons migrate radially using existing RGP basal processes to guide them basally, where they form the subplate, the deep portion of the cortical plate (CP) (Beattie & Hippenmeyer, 2017; Gao et al., 2014; Marin et al., 2010; Taverna et al., 2014). Waves of newly born neurons subsequently migrate to generate the next layer, thereby giving rise to the canonical 6-layer cortex in an inside out manner (Rakic, 2009). Superficial layer neurons (layers II/III and IV) form intracortical networks either locally (in the immediate vicinity or ipsilaterally) or contralaterally. Neurons that project contralaterally end up forming the corpus callosum, a bundle of projections connecting the two hemispheres. Neurons residing in the deep layers (layers V and VI) project subcortically to the thalamus, brainstem and spinal cord (Figure 3).

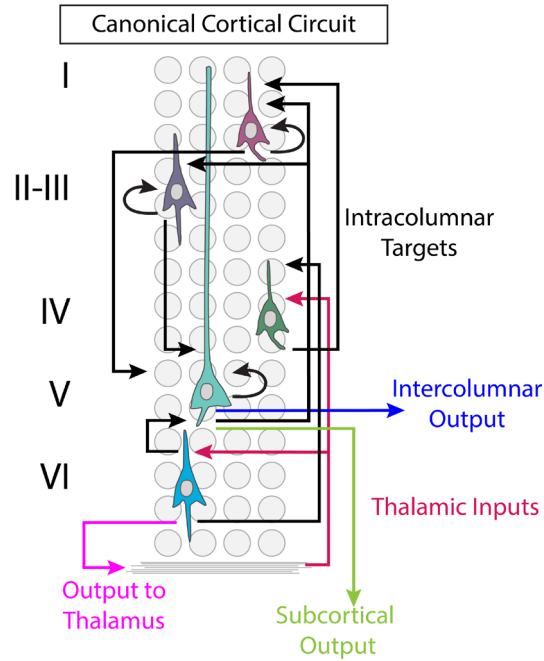


Figure 3. Neurons project to subcortical or intracortical targets depending on which cortical laminae they reside in.

Early born neurons in layers 5 and 6 project subcortically to the thalamus, brainstem, and spinal cord. Later born neurons in layers 2/3 and 4 project locally to intra- and inter-columnar targets as well as contralaterally.

Upon completion of the neurogenic phase, RGP must rapidly switch to produce the macroglia found in the cortex (Costa et al., 2009). During gliogenesis, RGP produce astrocytes and oligodendrocytes, cell types that help maintain neurons and support the existing neuronal circuitry (Chung et al., 2015; Costa et al., 2009; Freeman & Rowitch, 2013). To achieve this, they undergo a neurogenic to gliogenic switch (Bayraktar et al., 2014; Kriegstein & Alvarez-Buylla, 2009), during which approximately 16% of RGP complete the transition to gliogenesis (Gao et al., 2014). The fraction of RGP which do not transition to gliogenesis are predicted to exit the cell cycle by undergoing a terminal neurogenic division (Gao et al., 2014).

Previous studies have touched on this subject, showing that there are many instructive genetic and epigenetic cues that promote or hinder this transition (Amberg et al., 2019; Beattie & Hippenmeyer, 2017). For instance, studies have shown quite conclusively that EGFR signaling is a prerequisite for acquiring gliogenic competence in the cortex (Kornblum et al., 1997; Sibilio et al., 1998). More recently, Amberg and colleagues reported that PRC2 also plays a role in maintaining progenitors throughout gliogenesis (Amberg et al., 2022). Glial-competent RGP then generate multipotent glial progenitors, which according to new data, can produce oligodendrocytes, astrocytes, and olfactory bulb interneurons (Gao et al., 2025).

Previous reports notwithstanding, the mechanisms behind the decision to undergo this switch remain elusive. While we now have a quantitative framework of cortical stem cell lineage progression, questions remain regarding which molecular and genetic mechanisms govern a progenitor's behavior throughout cortical development.

1.4 Applying Mosaic Analysis with Double Markers to Study Genes and Genetic Interactions at the Single Progenitor Level

MADM is a genetic lineage tracing technology in mice that allows for the sparse simultaneous genetic manipulation and stable fluorescent labeling of a cell and all its progeny (Zong et al., 2005). The MADM system is composed of two cassettes, a GT cassette containing a split *Gfp-tdTomato* allele and a TG cassette containing a split *tdTomato-Gfp*. The marker genes within each cassette are separated by a LoxP site (Figure 4A) and were inserted into each pair of the 19 autosomes in mice (Contreras et al., 2021). MADM events occur in actively dividing cells, as it relies on Cre-mediated interchromosomal recombination to reconstitute one copy of each of the split marker genes while the cell prepares to divide (Figure 4B). *Cre* expression can be targeted to specific tissues by placing it under the control of tissue-specific drivers. In this project, we place it under the control of *Emx1* to label the excitatory pyramidal neurons and glia produced by RGP in the neocortex (Gorski et al., 2002). Once the chromosomes have recombined, the dividing cell segregates the chromosomes in one of two modes, either G2-X or G2-Z, and generates stably labeled progeny.

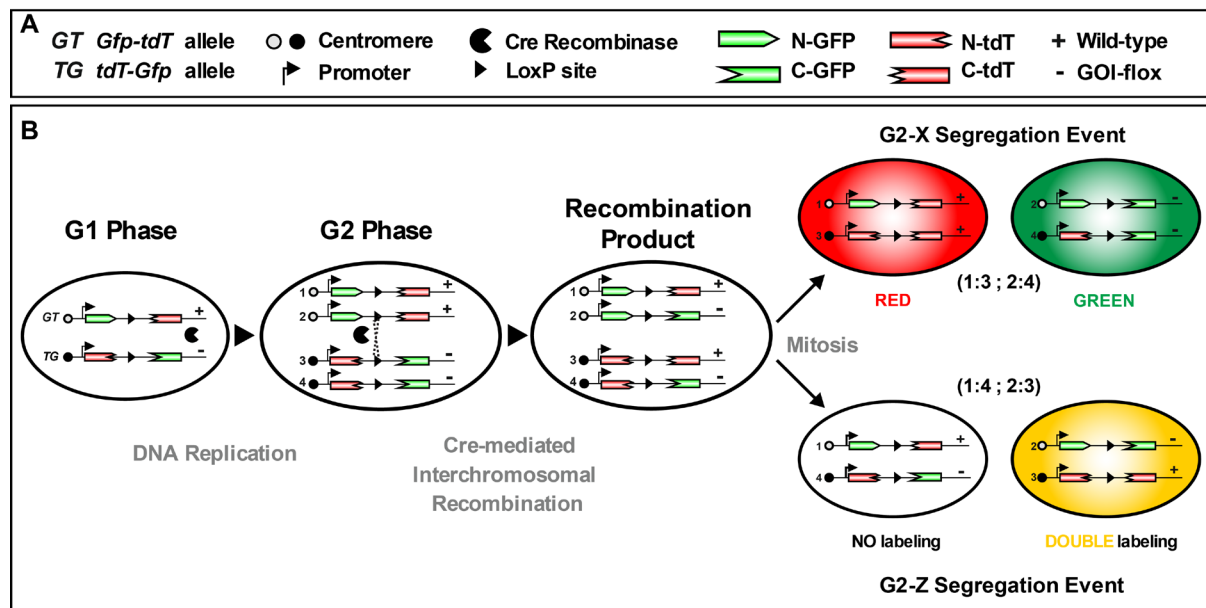


Figure 4 The genetic principle of MADM.

MADM is made up of a series of genetic components. (A) The components of MADM. MADM requires two cassettes (GT and TG) and Cre-recombinase. (B) MADM events occur in the presence of Cre-recombinase, and occur sparsely throughout the dividing population in the target tissue. The results are labeled cells with desired genetic manipulation. (Figured modified from Contreras et al., 2022)

Given that we can link a floxed allele for a gene of interest (GOI) distal to the MADM cassette, it is not only a powerful tool for lineage tracing but also for loss of function (LOF) experiments (Figure 5). Previous works from the lab have elegantly highlighted MADM's ability to parse out cell-autonomous and non-cell-autonomous roles for a variety of genes not only in the developing cortex (Amberg et al., 2022; Beattie et al., 2017; Laukoter, Beattie, et al., 2020), but also in other brain regions as well (Cheung et al., 2024). Additionally, the sparseness of MADM labeling and genetic manipulation permits one to bypass any lethality issues found in conditional knockout models (cKO).

Since MADM labeling and genetic manipulation occurs in a very sparse manner, GFP+ mutant cells reside in a phenotypically normal environment. Though, for dosage sensitive genes, it is important to note that the entire tissue will be heterozygous, and which could contribute to any phenotypes observed in the MADM-labeled cells. Nonetheless, one can quantitatively determine the cell autonomous role of a GOI by quantifying any observable phenotypes in mutant populations within the genetic mosaic (Figure 5B) (Hippenmeyer et al., 2010). Conventional cKO mouse models by definition delete the GOI in the entire target tissue, meaning mutant cells are surrounded by other mutant cells, and so the cKO model blurs the line between cell-autonomous and tissue-wide functions. By performing a subtractive analysis of the data from a sparse mosaic LOF with that of a cKO, one can determine the true cell-autonomous and non-cell-autonomous role, or tissue wide requirement, for that gene.

Finally, MADM can also be used to dissect out interactions between two genes of interest. In addition to the two MADM cassettes, one can breed in two floxed alleles for a second gene of interest (Figure 5C) (Beattie et al., 2017). Previous work from our group has shown that deletion of *Lgll* in the developing mouse cortex results in an overproduction of astrocytes (Beattie et al., 2017). To delve deeper into the mechanism behind the role of *Lgll* in gliogenesis, Beattie and colleagues bred in two floxed *Egfr* alleles, and found that *Lgll*'s functions throughout gliogenesis depend on intact EGFR signaling. This modular approach (Figure 5C) can be used to parse out whether one gene's function depends on that of another. Taken together, MADM presents a powerful tool to conduct in-depth studies of gene function (cell-autonomous and non-cell-autonomous) throughout RGP lineage progression and for the study of interactions between two genes of interest.

MADM Experimental Paradigms									
A control-MADM <i>wild-type</i>				B <i>Gene X</i> -MADM <i>genetic mosaic</i>				C <i>Gene X</i> -MADM- <i>Gene Y</i> -cKO	
			<i>Gene X</i> ^{+/+}				<i>Gene X</i> ^{+/+}		<i>Gene X</i> ^{+/+} <i>Y</i> ^{-/-}
			<i>Gene X</i> ^{+/+}				<i>Gene X</i> ^{+/-}		<i>Gene X</i> ^{+/-} <i>Y</i> ^{-/-}
			<i>Gene X</i> ^{+/+}				<i>Gene X</i> ^{-/-}		<i>Gene X</i> ^{-/-} <i>Y</i> ^{-/-}
			<i>Gene X</i> ^{+/+}				<i>Gene X</i> ^{+/-}		<i>Gene X</i> ^{+/-} <i>Y</i> ^{-/-}
<i>Emx1</i> -Cre Driver									

Figure 5 Experimental paradigms for genetic interaction experiments with MADM.

From one breeding, one can obtain all three genotypes for the experiment. (A) Control-MADM animals serve as internal controls and green-to-red ratio should be 1. (B) *Gene X*-MADM animals can be used to determine the cell-autonomous role of *Gene X*, green-to-red ratio would vary according to phenotype (higher or lower than 1). (C) The *Gene X*-MADM-*Gene Y*-cKO is a sparse mosaic for *Gene X* on within a *Gene Y* cKO tissue. All cells within the target tissue are *Gene Y* cKO and *Gene X* genotype would match the expressed fluorophore as in the MADM paradigm.

1.5 PTEN Functions in Health and Disease

The protein encoded by *Phosphatase and Tensin homologue on chromosome ten* (*PTEN*) is composed of a PIP₂ binding motif, a phosphatase domain, a PDZ binding domain, and a C2 domain that may help recruit PTEN to the inner cell membrane (Rizo & Sudhof, 1998). Canonically, phosphoinositide 3-kinase (PI3K) phosphorylates phosphatidylinositol 4,5-biphosphate (PIP₂) into phosphatidylinositol 3,4,5-triphosphate (PIP₃) (Figure 6). AKT, a prominent protein kinase within the mTOR signaling cascade, translocates to the inner side of the cell membrane, and binds PIP₃ and is subsequently activated by 3-phosphoinositide-dependent kinase 1 (PDK1). Activated AKT then drives key cellular processes like cell proliferation, cell survival, and metabolism by activating downstream effectors like SKP2 and AS160 or by inhibiting other effectors like GSK3 and FOXO (Manning & Cantley, 2007). The mTOR pathway drives protein translation of key mRNAs crucial for cell growth and proliferation through phosphorylation of S6K (p70 ribosomal protein S6 kinase) and 4EBP1 (eukaryotic translation-initiation factor 4E-binding protein 1) (Ma & Blenis, 2009). PTEN's canonical role is to negatively regulate AKT-mTOR signaling by de-phosphorylating PIP₃ into PIP₂, thereby directly antagonizing PI3K activity and reducing the availability of PIP₃ at the inner side of the cell membrane (Worby & Dixon, 2014). Since PIP₃ directly activates AKT isoforms, PTEN is considered a direct antagonist for downstream AKT-mTOR signaling.

Therefore, PTEN is responsible for keeping mTOR signaling functions in check, some of which include translation, cell size, proliferation, and cell survival.

Mouse models with overactive PTEN activity show remarkable resistance to tumor formation (Worby & Dixon, 2014). Loss of PTEN increases PIP₃ abundance, thereby increasing AKT recruitment to the cell membrane and activating mTOR signaling cascade activity (Manning & Cantley, 2007). Activation of AKT by PIP₃ has also been shown to promote cell cycle entry through downstream inhibition of GSK3 β (Massague, 2004). Decreased activity of GSK3 β allows for the activation of cyclin-D, which promotes cell cycle progression (Massague, 2004). PTEN's localization also varies along with the cell cycle. Studies suggest that during S phase, PTEN accumulates at the inner surface of the cell membrane, while during G₀-G₁, PTEN can be found in the nucleus at greater concentrations (Ginn-Pease & Eng, 2003). Deregulation in such central cell signaling pathways present new targets for further investigation.

Disruptions in cortical development can manifest themselves in several ways, including macrocephaly, microcephaly, ASD, and epilepsy (Sacco et al., 2018). In humans, *PTEN* mutations frequently result in macrocephaly, and carry an increased risk of developing tumors, ASD, and glioma (Duerr et al., 1998; Song et al., 2012). PTEN mutations are frequently found in patients with glioma, one of the most commonly occurring cancers among adults with a high rate of drug resistance (Duerr et al., 1998; Wang et al., 1997; Yang et al., 2010). Patients with PTEN mutations also frequently present with PTEN hamartoma tumor syndromes (PHTS). PHTS are series of clinically distinguishable syndromes, Bannanyan-Riley-Ruvacalba, Cowden Syndrome, and Lehermitte-Duclos among others (Hobert & Eng, 2009; Longy et al., 1998; Marsh et al., 1998). Hamartomas are non-cancerous tumor-like growths made from uncontrolled proliferation of the distinct cell types of the areas from which they grow. People living with PHTSs also frequently present with macrocephaly, as well as other neurological deficits like developmental retardation and intellectual disability (Eng, 2003). In summary, PTEN functions make it a key regulator for a variety of cellular processes. Given this key role, it is essential to delineate the precise contributions of PTEN in RGP lineage progression during cortical development at single-cell resolution.

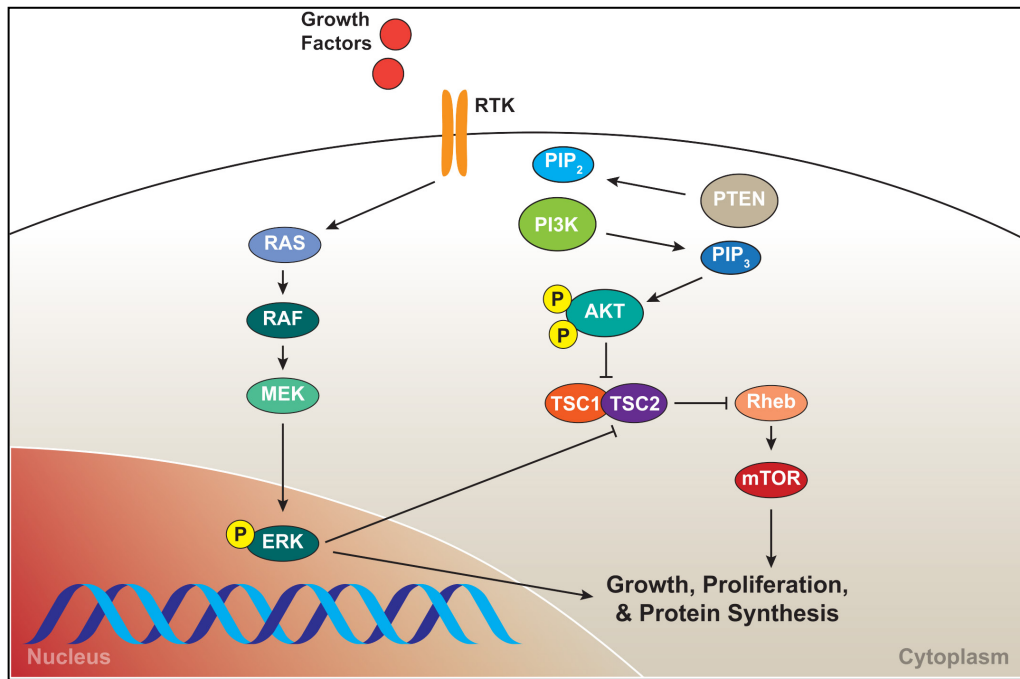


Figure 6 Canonical PTEN signaling cascade.

PTEN localizes at the inner cell membrane and performs key regulatory functions by negatively regulating AKT-mTOR signaling. mTOR signaling activates indispensable cellular programs like cellular proliferation, cell size, and translational activity. Decreased PTEN activity generally results in an overactive mTOR pathway, leading to over-proliferation, increased cell size, and increases cancer transformation risk.

2 Chapter 2 – Thesis Objectives

Cortical development is a tightly regulated developmental program that relies on the precise tuning of gene expression to generate the appropriate cells at the right time. Cortical RGPs are responsible for generating all the pyramidal neurons and macroglia present in the cortex (Hippenmeyer, 2023). Thanks to previous MADM-based clonal analysis using tamoxifen-inducible *Emx1-CreERT2* (Beattie et al., 2020), we now know that RGPs follow a deterministic program during corticohistogenesis. Each individual RGP produces a clonal unit of approximately 8-9 neurons, 16% of those same RGPs then undergo a transition from neurogenesis to gliogenesis (Gao et al., 2014). Our approach focuses on the importance of specific gene function throughout cortical development. MADM-based candidate gene analysis allows us to study gene function at the single-progenitor level.

I aim to understand (1) the contributions of *Pten* during cortical neurogenesis, (2) the role of *Pten* in gliogenesis, and (3) which signaling pathways converge with *Pten* signaling during gliogenesis. In the first aim, we use tamoxifen inducible *Emx1-CreERT2*, which gives us added sparseness and temporal control over labeling, to catalyze clonal MADM events. Using this dataset, I can parse out whether *Pten* helps determine the clonal output of RGPs and use it to understand how *Pten* influences RGP division patterns. I address the second aim by again using clonal analysis to quantify the astrocyte output of individual RGPs, and I leverage advances in single-cell sequencing technologies to gain insights into the molecular correlates involved in gliogenesis in control and mutant tissue. I address the third aim by using the transcriptomic data to guide my experimental approach. I take advantage of the flexibility offered by MADM and perform genetic interaction experiments. The battery of experiments helps clarify through which pathways *Pten* exerts its influence on the gliogenic program.

3 Chapter 3 – Morphological Analysis of Neurons and Glia using Mosaic Analysis with Double Markers

Osvaldo A. Miranda^{1*}, Giselle Cheung^{1*} & Simon Hippenmeyer^{1#}

¹ Institute of Science and Technology Austria (ISTA), Am Campus 1, 3400 Klosterneuburg, Austria

* Denotes equal contribution

Corresponding author: Simon Hippenmeyer (simon.hippenmeyer@ist.ac.at)

The entire contents of this chapter were published as a book chapter and are reproduced in this thesis with permission from Springer Nature.

Miranda, O.A.*, Cheung, G.*, and Hippenmeyer, S. (2024). Morphological Analysis of Neurons and Glia using Mosaic Analysis with Double Markers. *Neuronal Morphogenesis. Methods in Molecular Biology*, vol 2831.

3.1 Author Contributions

Project Management: S.H.; Conceptualization and Design: S.H., O.A.M., and G.C.; Experimental Data Collection and Image Analysis: O.A.M. and G.C.; Data Interpretation: O.A.M. and G.C.; Data Visualization: O.A.M. and G.C.; Manuscript Drafting: O.A.M. and G.C.; Critical Revision of the Manuscript: All authors; Final version was read by all authors and approved to be published.

Morphological Analysis of Neurons and Glia using Mosaic Analysis with Double Markers

3.2 Abstract

Mosaic Analysis with Double Markers (MADM) is a powerful genetic method typically used for lineage tracing and to disentangle cell autonomous and tissue-wide roles of candidate genes with single cell resolution. Given the relatively sparse labeling, depending on which of the 19 MADM chromosomes one chooses, the MADM approach represents the perfect opportunity for cell morphology analysis. Various MADM studies include reports of morphological anomalies and phenotypes in the central nervous system (CNS). MADM for any candidate gene can easily incorporate morphological analysis within the experimental workflow. Here, we describe the methods of morphological cell analysis which we developed in the course of diverse recent MADM studies. This chapter will specifically focus on methods to quantify aspects of the morphology of neurons and astrocytes within the CNS, but these methods can broadly be applied to any MADM-labeled cells throughout the entire organism. We will cover two analyses - soma volume and dendrite characterization - of physical characteristics of pyramidal neurons in the somatosensory cortex, and two analyses - volume and Sholl analysis - of astrocyte morphology.

3.3 Introduction

Neurodevelopmental disorders like autism and fragile X syndrome have been associated with altered neuronal and dendritic spine morphology (Martinez-Cerdeno, 2017; Phillips & Pozzo-Miller, 2015; Rademacher & Eickholt, 2019). Changes in neuronal cell volume affects many intracellular processes like cell signaling and ion diffusion (Hoffmann et al., 2009; Lang et al., 1998) whereas dendritic spines are directly involved in the establishment of synaptic contacts and neuronal communications (Yuste & Denk, 1995). Besides neuronal dysfunctions, glial cell types like astrocytes are often also affected in pathologies (Molofsky et al., 2012; Sloan & Barres, 2014). The extent of the elaborate organization of astroglial fine processes largely reflects their maturation state and in turn their functions within astrocytic networks or with neighboring neurons (Santello et al., 2019; Torres-Ceja & Olsen, 2022). Therefore, in order to understand pathological alterations to neurons and astrocytes, their morphological parameters down to the finest structures are commonly considered and compared.

Mosaic Analysis with Double Markers (MADM) is broadly used to concomitantly label and genetically manipulate target tissues within a mouse (Zong et al., 2005). This is done by Cre-mediated interchromosomal recombination of two reciprocal chimeric marker genes inserted in identical loci on homologous chromosomes. A tissue of interest can be targeted by placing *Cre* under the control of a tissue-specific promoter. Recently, our group published a significant resource (Contreras et al., 2021) detailing the creation of the MADM library, a repository of 19 MADM mouse lines, which allows for MADM analysis of over 95% of the genes in the mouse genome. Such mice have been used to study genes involved in microcephaly (Beattie et al., 2017), epigenetic molecular machinery involved in gene silencing (Amberg et al., 2022), neuronal migration in the neocortex (Andi H Hansen et al., 2022), and in a diverse body of works. MADM presents a significant advantage in studying genes of interest in that only a very small subset of cells are genetically manipulated, bypassing any lethality issues present in more ‘traditional’ conditional knockout (cKO) models. The fluorophores expressed by the reconstituted MADM cassettes, tandem dimer Tomato (tdTomato) and Green Fluorescent Protein (GFP), very efficiently fill even the finest cellular structures, such as dendritic spines (Zong et al., 2005). Additional staining with primary antibodies against GFP and tdTomato further enhances the results of confocal imaging, and is necessary in the case of embryonic tissue. This technology provides the perfect platform for a multifaceted study of the role of a gene of interest, not only in the brain but in any organ in the mouse system.

3.4 Materials

The following is a list of materials and equipment are necessary for the processing, imaging and analysis of samples described in this protocol.

3.4.1 Reagents and equipment

1. Phosphate buffered saline (PBS, Sigma-Aldrich)
2. Triton X-100 (Sigma-Aldrich)
3. Donkey Serum (Thermo Fisher)
4. Chicken anti-GFP (AVES, dilution 1:500)
5. Goat anti-tdTomato (SICGEN, dilution 1:500)
6. Donkey anti-chicken Alexa Fluor 488 secondary antibody (Jackson Immuno., dilution 1:500)
7. Donkey anti-goat Alexa Fluor 568 secondary antibody (Molecular Probes, dilution 1:1000)
8. DAPI (4',6-diamidino-2-phenylindole) (Thermo Fisher Scientific)
9. Glass cover slides (Thermo Fisher Scientific)

3.4.2 Microscopes and software

1. Confocal inverted microscope with camera and 40x or 63x objective: Zeiss LSM-800
2. Confocal acquisition software: ZEN 2.6 (Zeiss)
3. Image analysis and 3D reconstruction software: Imaris v9.9
4. Statistical analysis and data visualization program: GraphPad Prism v8

3.5 Methods

The following protocol lists the steps required to go from tissue preparation, image acquisition to final data visualization with minimal coding experience. Naturally, the coding-inclined can perform the statistical analysis and data visualization using their platform of choice (such as R). Here, we focus on quantitative analysis of neuronal soma volume dendritic spine morphology, and astrocyte morphology.

3.6 Preparation of experimental tissue containing MADM-labelled cells

3.6.1 Generation and cryosectioning of MADM brains

For the generation of MADM-labelled mice and the preparation of cryosections of MADM brains, follow previously published protocols (Amberg & Hippenmeyer, 2021; Beattie et al., 2020). See Notes 1-2.

3.6.2 Immunostaining to enhance endogenous MADM signals

The amplification of endogenous MADM signals is recommended for dendritic spine morphology analysis but not required for neuronal soma volume and astrocyte morphology analysis.

1. Rehydrate and wash mounted cryosections on glass coverslips with PBS.
2. Incubate sections in blocking solution (0.5% Triton-X and 5% donkey serum in PBS) for 2hr at RT. Protect from light.
3. Incubate sections in blocking solution containing primary antibodies overnight at 4oC. Protect from light. The following primary antibodies are recommended: Chicken anti-GFP (AVES, 1:500) and Goat anti-tdTomato (SICGEN, 1:500).
4. Wash in PBS and incubate sections in PBS containing 0.5% Triton-X and secondary antibodies for 2hr at RT. Protect from light. The following secondary antibodies are recommended: Donkey anti-chicken Alexa Fluor 488 (Jackson Immuno. 1:500) and Donkey anti-goat Alexa Fluor 568 (Molecular Probes, 1:500).
5. Wash in PBS and incubate sections in PBS with 2.5% DAPI (4',6-diamidino-2-phenylindole) (Thermo Fisher Scientific) for 10min at RT. Protect from light.
6. Wash in PBS and mount with Mowiol 4-88 with DABCO (Carl Roth) and cover with glass coverslip (24 X 60mm, Thermo Scientific).
7. Allow slides to air dry at 4oC protected from light until used.

3.7 Neuron Soma and Astrocyte Volume Measurements

3.7.1 Image acquisition

The following steps are described for the use of an LSM 800 inverted confocal microscope (Zeiss) operated using ZEN Blue 2.6 software (Zeiss). Adapt accordingly to other confocal microscopes and software.

1. Initiate confocal microscope according to standard operating procedures. Be sure to not over expose the soma, as it can lead to issues with reconstruction later on.

2. Set up the system to acquire Z-stacks so that the entire cell is within the first and last Z-planes. In this analysis, avoid having multiple soma in the field of view (Notes 1-2).

3.7.2 Image processing for neuron and astrocyte volume measurements

The following image processing steps are described for the use of Imaris 9.9.1 (Oxford Instruments). Adapt accordingly to other image analysis software like ImageJ (National Institutes of Health).

1. Use the Imaris File Converter tool to convert source files (.czi in this case) to .ims files.
2. Open Imaris9.9.1 and drag and drop the .ims file into the “Arena”. Click on the file to be analyzed. Then navigate to the “Surpass” tab.
3. On the left side of the screen, select “Add new Surfaces” (Fig 7A orange highlight).
4. Next the boundaries of the cell will be defined under the “Create” tab (Fig 7B blue highlight).
5. If the image is dense with cells, segmentation of only a single cell is recommended. To do this, select the “Segment only a Region of interest” box. Otherwise, skip to 6.
6. To segment this image, click on the blue arrow to continue. In this next step left-click on the arrows to drag the box so that it only contains the soma of the neuron of interest.
7. Select the “Start creation with Slicer View’ box. Then click on the blue arrow on the bottom of this area to continue.
8. On the next page select the source channel (Fig 7B orange highlight), in this example we use the red tdTomato channel. Then click the blue arrow to continue.
9. Next set the threshold for detection of the neuron. Use the same setting for all samples in the experiment. In this example an automatically detected value of 14.1 is used (See Note 3). Should there multiple cells in the image, choose to “Split touching Objects” by selecting the “Enable” box (Fig 7C orange highlight). Click on the blue arrow to continue.
10. On this page filter out any objects smaller than a certain value (number of voxels), such as artifacts. Otherwise just continue.
11. Skip machine-learning object classification, click the green double arrow to finish this process and render the surface of the neuron. (Note 4)
12. To get the target data, go to the tab with the graph icon (Fig 7D orange highlight) and then choose the “Detailed” tab, then in the first drop down menu choose “Specific Values”, and in the second drop down menu select “Volume” (Fig 7D blue highlight).
13. The data is now displayed. In this case the example neuron has a volume of 690 cubic microns. Other metrics from this surface can be viewed by navigating the dropdown menus, such as area, sphericity, etc.

14. This measurement will be recorded and later plotted with Graphpad Prism software (Fig 8).

3.8 Analysis of dendritic spines of MADM-labelled neurons

3.8.1 Image acquisition

The following steps are described for the use of an LSM 800 inverted confocal microscope operated using ZEN Blue 2.6 software. Adapt accordingly to other confocal microscopes and software.

1. Configure confocal software for the following fluorophores: Alexa Fluor 488 (excitation laser 488nm), Alexa Fluor 568 (excitation laser at 561nm) and DAPI (excitation laser at 405nm). See note 5 for an optional step.
2. To image dendritic spines, use objectives and zoom settings to obtain imaging resolution of 0.08 μm /pixel. For example: a 63X oil immersion objective (NA 1.4) with 2.5 x zoom, or a 40X oil immersion objective (NA 1.4) with 4 x zoom.
3. Setup image acquisition parameters for the relevant channel for dendritic spine labeling with pinhole of 1 Airy Unit and optimal laser intensity and master gain (500-600V). To reduce background noise, averaging of acquisition by 2 - 4 times can be used (Note 6).
4. Setup z-stack and tiles to cover area of interest with 0.20 μm z-interval (Note 7).
5. Start image acquisition.
6. After image acquisition, stitch image stacks using “Stitching” function under processing tab.

3.8.2 Image deconvolution

The following image processing steps are described for the use of Imaris 9.9.1. Adapt accordingly to other image analysis software like ImageJ or Huygens (Scientific Volume Imaging).

1. Open .czi image in Imaris and convert to .ims file (Fig. 9A)
2. Choose Crop 3D under Edit and crop into smaller segments of dendritic spines (Fig. 9B). Save as separate .ims files. (Note 8).
3. Open individual .ims files of cropped fragments and choose “Image Processing” under “View”.
4. For each channel, choose “Deconvolution” in the drop down menu.
5. Specify deconvolution parameters according to image acquisition parameters (see Fig. 9C for an example). Image acquisition parameters can be found associated with the each .czi file listed under information tab in ZEN software.

6. Check preview window for quality (Fig. 9D) and press “OK” to perform deconvolution if parameters are correct. Save as new .ims file.

3.8.3 Reconstruction of dendrites and dendritic spines

The following image processing steps are described for the use of Imaris 9.9.1. Adapt accordingly to other image analysis software like Neuronstudio (BioImage Informatics Index).

Segmentation of dendrites (Semi-automated mode)

1. In Imaris, open deconvolved .ims file from “Arena”. In 3D View, press “Add New Filament”
2. Press “Skip automatic creation, edit manually” (Note 9).
3. Select “AutoPath” method, automatic center and diameter correction (Fig. 10B).
4. Set the start of dendrite segment (hold Shift + right mouse click).
5. Move cursor slowly along dendrite segment and mark the end point (hold Shift + left mouse click).
6. Reconstruct 1 dendritic segment per filament (Fig. 10C). As shown in this image, another dendritic branch denoted by white arrows are not segmented and therefore removed in the analysis.

3.8.3.1 Dendritic spines detection (Automated mode)

1. Within “Creation” tab, under “Recompute”, choose “Dendrite Diameters”.
2. For the following automated mode of detection, use automated thresholding or set to specific values (Note 10).
3. Detect spines by setting thinnest diameter and maximum length (Note 11). In this example, default values = 0.156 and 3.9mm are used, respectively.
4. Use automated thresholding for seed points and spine diameter of set to specific values (Note 10 and 11). Proceed and complete the reconstruction wizard following on-screen instructions.
5. An example of a reconstructed dendritic segments with spines are shown (Fig. 10D).

3.8.4 Classification of dendritic spines

1. Under filament tab, select “Classify Spines” tool, a MATLAB window will open.
2. Use default parameters or change accordingly to define different categories of dendritic spines (Note 11). New classes of spines can also be created. In this example, four types of spine morphology are considered: filopodia/dendrite, long thin, mushroom, or stubby.
3. When the correct parameters are set, choose “Classify spines”. An example using default settings are shown (Fig. 10E).

4. All spines are automatically classified based on defined parameters and color coded in viewing panel (Fig. 10F).
5. Save all reconstruction and analysis performed on the dendritic segment by saving the .ims file.
6. An example of a reconstructed dendritic segment is shown in Fig. 11A in stepwise manner.

3.8.5 Data extraction and statistical analysis

1. In the “Arena” of Imaris, create a new folder containing all dendritic fragments files to be analysed.
2. Select folder and choose “New Plot” option.
3. Under plot type, choose desired parameters to be exported. Some basic parameters include: Dendrite length, Dendrite no. spines, Dendrite Spine Density, Spine Length, Spine Neck Max Diameter and Spine Terminal Pt Diameter.
4. For each parameter, choose to display detailed plot and export data as individual .xls files.
5. For each neuron, analyze multiple dendritic segments depending on imaged area and combine dendritic length and spine number per cell as. Example quantifications obtained from 9 neurons each with 1-9 dendritic segments analyzed are shown in Fig. 11B-11H. Individual values and mean $\pm$ SEM are shown in bar plots.
6. For example, from the total dendritic length (Fig. 11B) and spine number (Fig. 11C) sampled, spine density can be determined as the total spine number per 10 μ m of total dendritic length per cell (Fig. 11D).
7. For the analysis of spine parameters, determine the median value of spine length (Fig. 11E), neck diameter (Fig. 11F), and head diameter (Fig. 11G) per cell. Alternatively, distribution of these parameters can also be analysed as histograms and compared.
8. Spine morphology can be compared based on the number of spines of each category per cell (Fig. 11H). The relative proportion of spines belonging to different classes can be determined.
9. Repeat analysis for neurons of different experimental conditions and perform appropriate statistical analysis to compare different parameters.

3.9 Astrocyte Sholl Analysis

3.9.1 Image acquisition

The following steps are described for the use of an LSM 800 inverted confocal microscope (Zeiss) operated using ZEN Blue 2.6 software (Zeiss). Adapt accordingly to other confocal microscopes and software.

1. Initiate confocal microscope according to standard operating procedures. Be sure to not over expose the soma, as it can lead to issues with reconstruction later on.
2. Set up the system to acquire Z-stacks so that the entire cell is within the first and last Z-planes. In this analysis, avoid having multiple astrocytes in the field of view (Notes 1-2).

3.9.2 Image deconvolution

The following image processing steps are described for the use of Imaris 9.9.1. Adapt accordingly to other image analysis software like ImageJ or Huygens (Scientific Volume Imaging).

1. Open .czi image in Imaris and convert to .ims file (Fig. 9A)
2. Open individual .ims files of cropped fragments and choose “Image Processing” under “View”.
3. For each channel, choose “Deconvolution” in the drop down menu.
4. Specify deconvolution parameters according to image acquisition parameters (see Fig. 9C for an example). Image acquisition parameters can be found associated with the each .czi file listed under information tab in ZEN software.
5. Check preview window for quality (Fig. 9D) and press “OK” to perform deconvolution if parameters are correct. Save as new .ims file.

3.9.3 Render Astrocyte Filaments

The following image processing steps are described for the use of Imaris 9.9.1. Adapt accordingly to other image analysis software like ImageJ or Huygens (Scientific Volume Imaging).

1. In Imaris, open deconvolved .ims file from “Arena”. In 3D View, press “Add New Filament”.
2. Under “Creation Preferences” turn off “Start creation with Slicer view” and press next (Fig. 12A blue highlight).
3. Select source channel and continue. Indicate 8.0 μm as the largest diameter for “Starting Points”, and 0.7 μm for “Seed Points”. These values have generated the best results in our analyses.
4. Adjust the “Starting Points Threshold” so that only a single starting point is present in the body of the astrocyte. Additionally, adjust the “Diameter of Sphere Region(s)” to clear seed points within the astrocyte cell body.

5. Adjust “Seed Points Threshold” as necessary, so that, if possible, seed points remain only within the cell of interest.
6. Skip dendrite diameter thresholding, the software’s automatic settings work well. Adjust if necessary.
7. Skip spine detection. Click green double arrow to finish.
8. The filaments should rather faithfully reflect the morphology of the astrocyte. Trimming of excess filaments is common. To do this, click on the “edit” tab (pencil).
9. Select “Branch” under the “Mouse selects”, this instructs the software to highlight the branch distal to its nearest branch point.
10. Delete selected branch with the “delete” key.
11. Once the render is accurate, select the “statistics” tab, depicted by the graph icon.
12. Select the “detailed” tab, under the dropdown menu select “specific values” (Fig. 12C orange highlight). A second dropdown menu will appear, select “filament no. shall intersections” (Fig. 12C blue highlight).
13. The selected data will be displayed, it can then be exported and plotted (Fig. 12D & 12E).

3.10 Notes:

Note 1. It is important to consider sparseness of labelled neurons and astrocytes in order to obtain individual isolated labelled cells for accurate morphological measurements. When neurites or glial processes overlap with another cell, a semi-automated reconstruction to segment out undesired background is required.

Note 2. Thickness of cryosections should be optimized based on the size of neurons or astrocytes to be analyzed and maximal penetration for immunostaining and confocal microscopy. Typically, sections between 30-50µm are used.

Note 3. The same, or very similar, settings should be used to analyze the dataset of a single experiment. This helps ensure consistency in the analysis, BUT manual intervention is sometimes necessary when the software does not get the threshold correct. In this case, increase/decrease the threshold as required to get a proper rendering of the cell.

Note 4. For batch analysis of cells, save the parameters after the first cell is completely processed and go to the magic wand tab and click “Store Parameters for Batch”. This can then be loaded into the program to automatically set the parameters for a batch of .ims files.

Note 5. (Optional step) It is recommended that a low resolution image (with 10-20x objectives) of the entire cell should be taken in 3 different channels in order to capture the neuronal morphology and document MADM-labelling. Subsequent image acquisition of spines should be taken of the relevant channel only to save time.

Note 6. In order to capture fine dendritic spine structures, neuronal cell bodies are often overexposed.

Note 7. During confocal sampling, it is crucial for subsequent deconvolution steps that sampling interval is less than the optimal interval determined automatically by confocal software.

Note 8. Processing in smaller fragments increase the efficiency of deconvolution as well as accuracy of segmentation of dendrites of interest.

Note 9. For dendrite segmentation, automatic creation can also be performed but only recommended for simple images with clearly defined neurites without background.

Note 10. It is important to use the same threshold for the same image and preferably also across different experiments. Therefore, it is important to determine whether automated thresholding or specific values should be used.

Note 11. Dendritic spines of different neuronal types across different brain regions may differ by length and thickness. It is important to adapt to the specific type of neuron of interest. When unsure, it is recommended that the dimension of a number of spines are measured from the same image under “Slice” view.

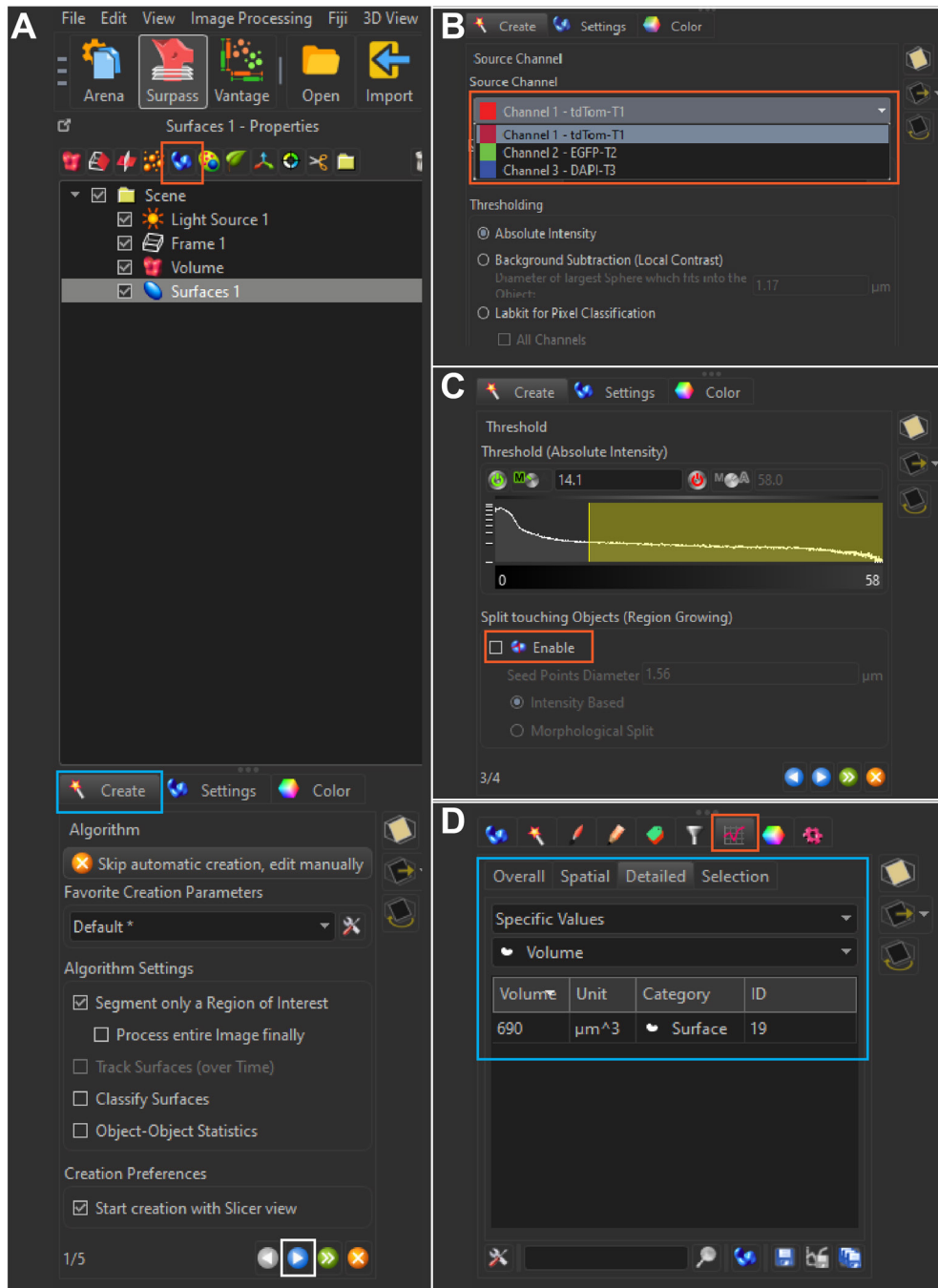


Figure 7 Relevant steps to measure the volume of a neuron cell body.

- Upon importing an .ims file, all processing takes place in the “surpass” tab. The orange box highlights the “create a new surface” function; all subsequent steps will take place under the “create” tab (blue box).
- A source channel must be selected for further analysis; thresholding is always set to “Absolute intensity”.
- Imaris is able to automatically threshold the image, this can be manually adjusted. Additionally, if multiple objects are in close proximity, the “split touching objects” function (orange box) can separate these.
- The resulting data are extensive, even for a straightforward analysis such as this, click on the graph (orange box) and select the “detailed” tab>”specific values”>”volume”. The volume will now be displayed along with the corresponding unit of measurement.

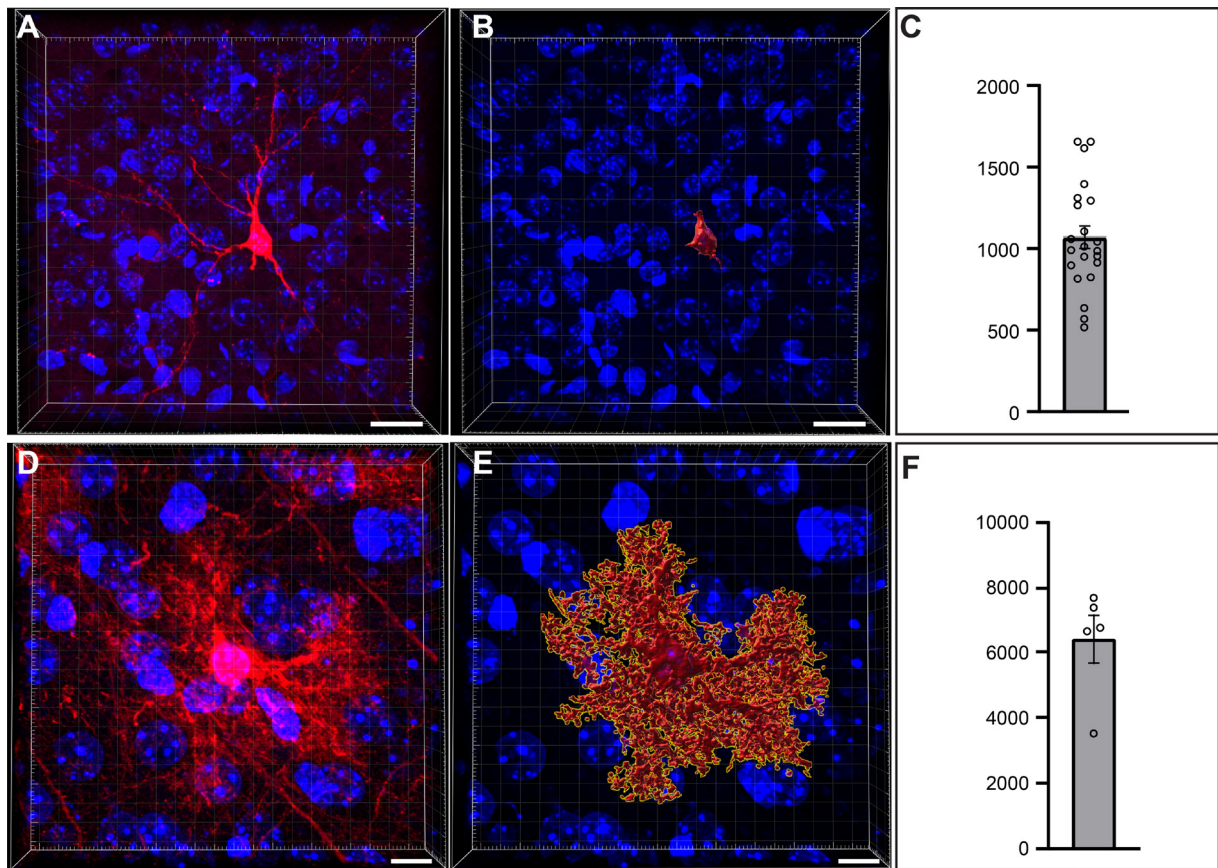


Figure 8 Volume measurements of neuron soma and astrocyte.

A. Source data displayed upon importing of the corresponding .ims file. The red neuron is labeled using MADM, hence the lack of neighboring cells.

B. Once all processing has concluded, Imaris will render the surface; here we have cropped the neurons pre-processing to measure only the volume of the soma.

C. The resulting soma volume data is plotted, in this case using Graphpad Prism.

Plots are shown as mean $\pm$ SEM (n=22 neurons, n=5 astrocytes).

Scale bars = 20μm (A-B); 10μm (D-E).

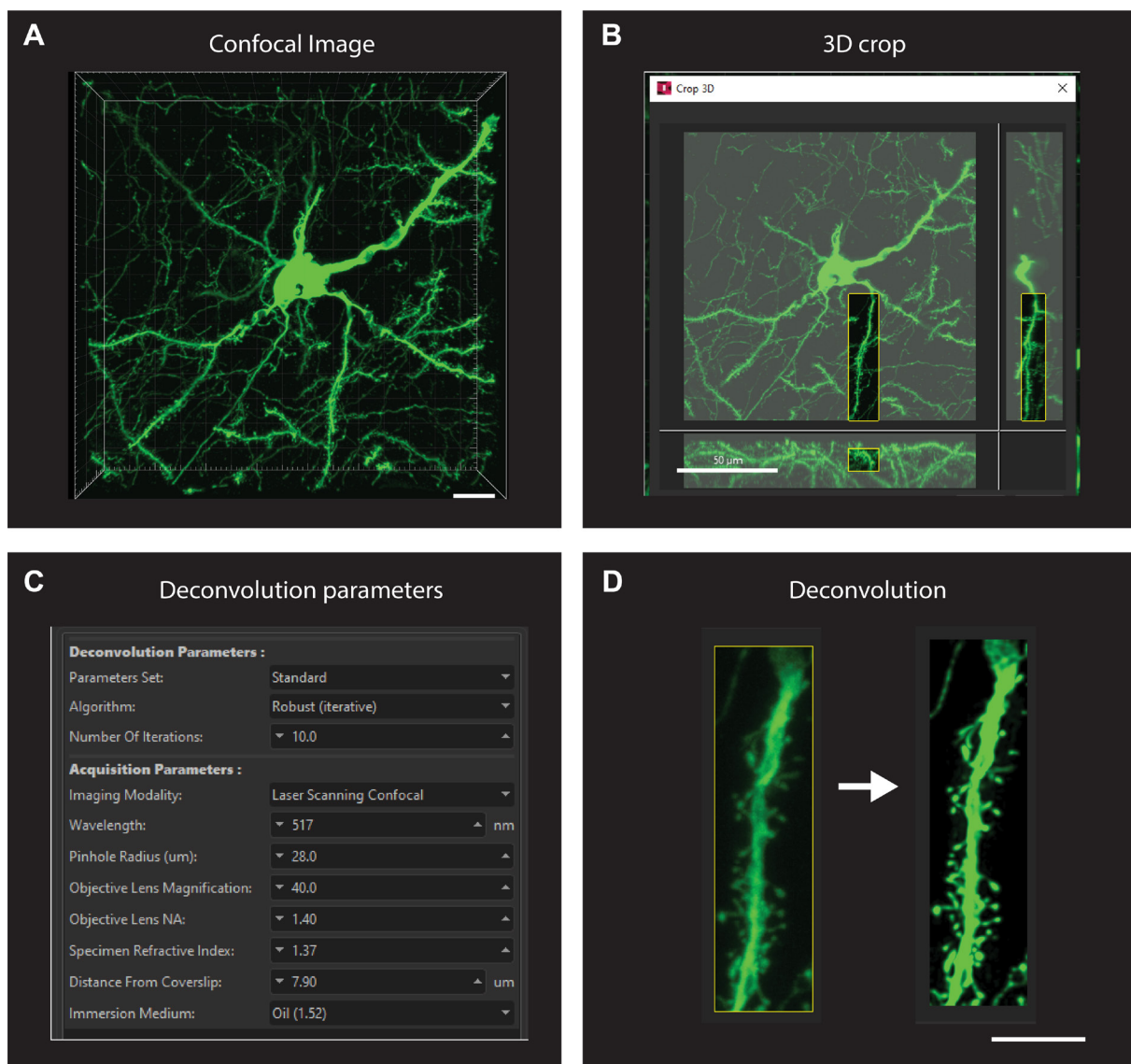


Figure 9 3D Cropping and deconvolution of dendritic segments.

A. Confocal image of a cortical neuron labelled in green GFP fluorescence.

B. 3D Crop window in 3D slice mode with selected area (yellow box) highlighted to be cropped.

C. Deconvolution parameter windows with settings specific to confocal acquisition and processed image.

D. Magnification of region in yellow box shown in (B) before (left) and after (right) deconvolution. Scale bars: 15 μm (A); 50 μm (B); 5 μm (D).

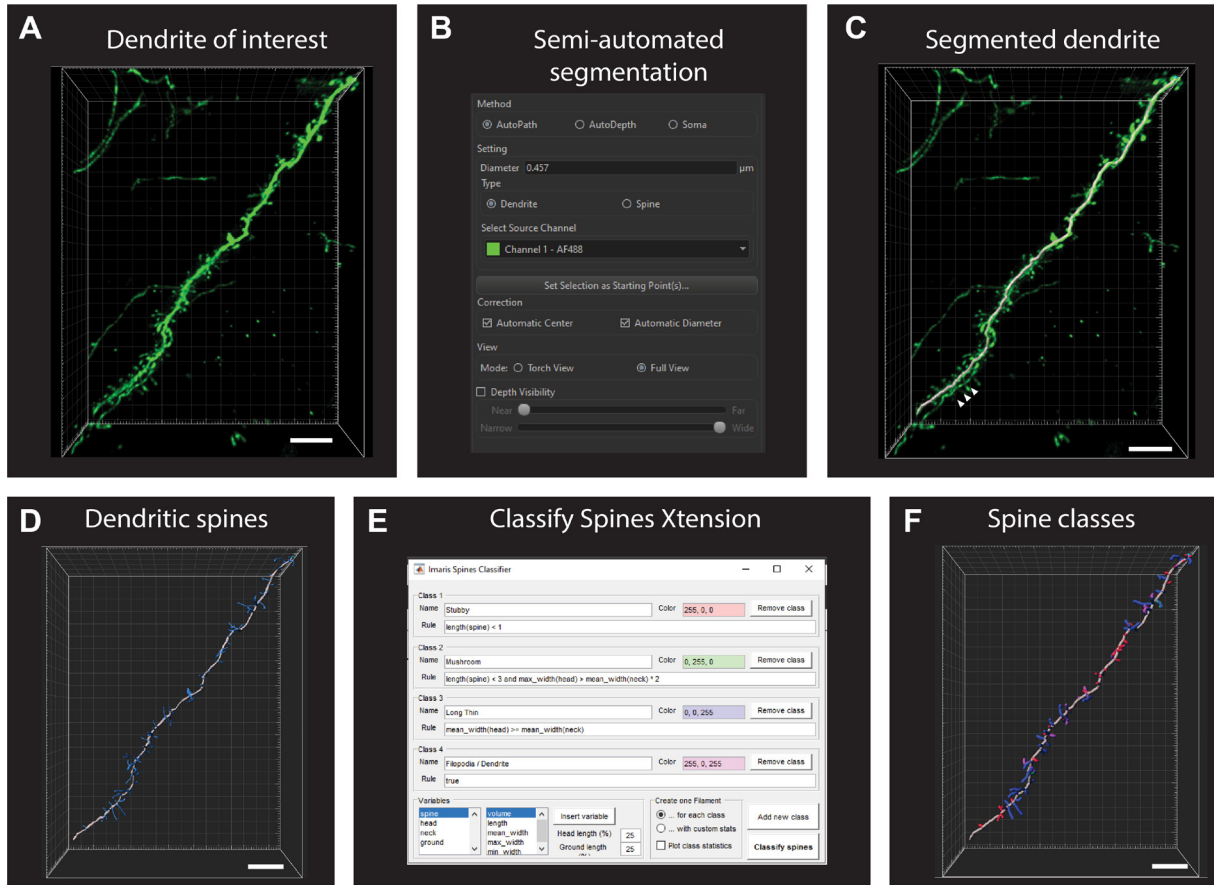


Figure 10 Segmentation and reconstruction of dendritic spines.

A. Deconvolved image of a cropped dendrite of interest.

B. Settings for semi-automated (Auto-Path) method to segment dendrite. Note that “Automatic Center” and “Automatic Diameter” options are selected.

C. Segmented dendrite is shown in thick line. Arrow heads indicate a branched dendritic segment behind the dendrite segment of interest which was not segmented.

D. Filament view of segmented dendrite (grey) with its dendritic spines detected in automatic mode (blue).

E. MATLAB window extension allowing user-defined parameters to classify spines.

F. Classified spines are highlighted in filament view with color codes for different spine class.

Scale bars: 5µm.

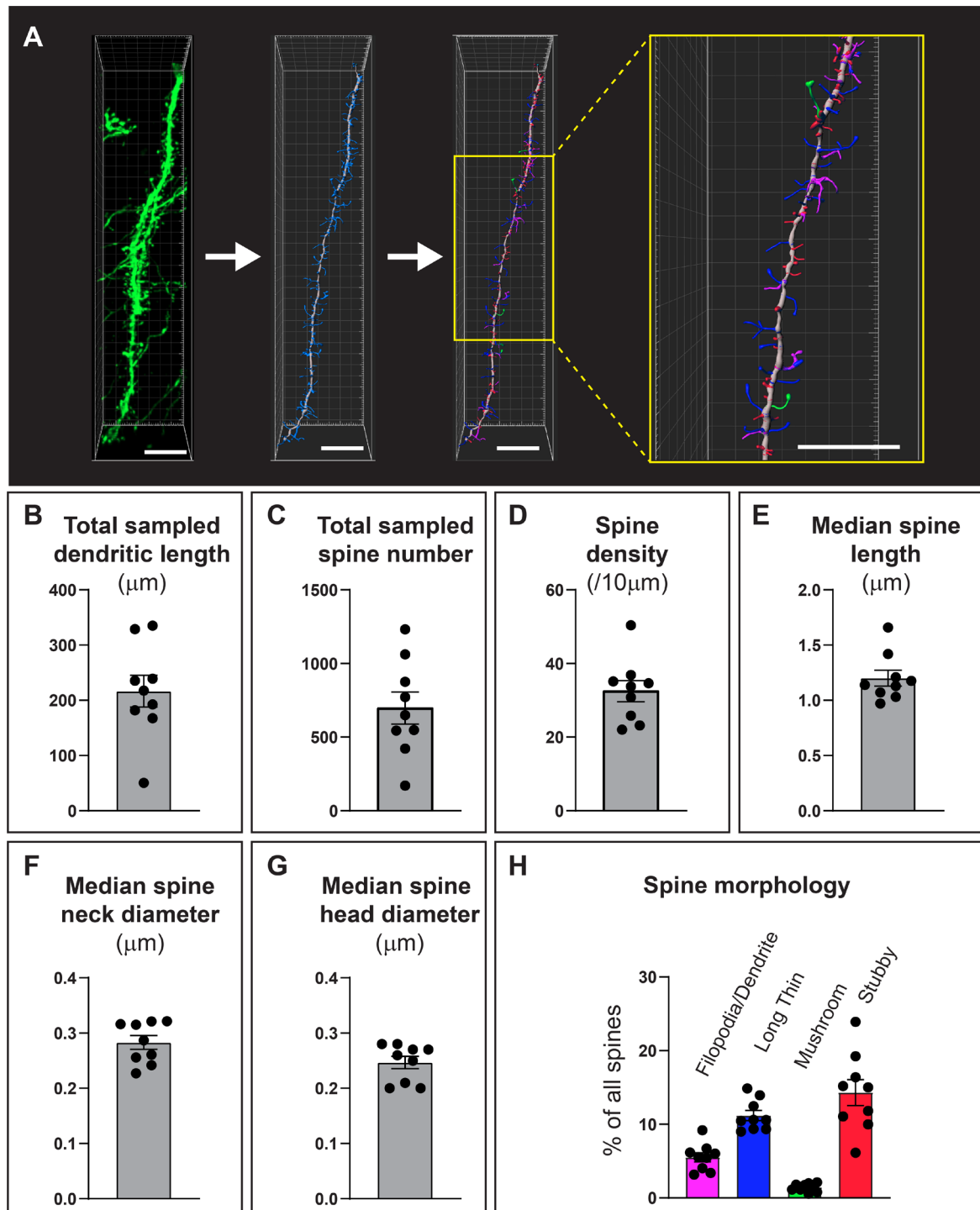


Figure 11 Reconstruction and analysis of dendritic spines.

A. Images showing progressing analysis of a dendritic segment to detect and subsequently classify spines. Magnified view of classified spines in filament view mode is shown in yellow box.

B - H. After extraction from exported parameters total dendritic length per cell (B), total spine number per cell (C), spine density per cell (D), median spine length (E), median spine neck diameter (F), median spine head diameter (G), and % of all spines for each spine class (H) are shown as mean $\pm$ SEM ($n=9$ neurons, 1-9 dendritic segments analyzed per cell).

Scale bars: $7\mu\text{m}$.

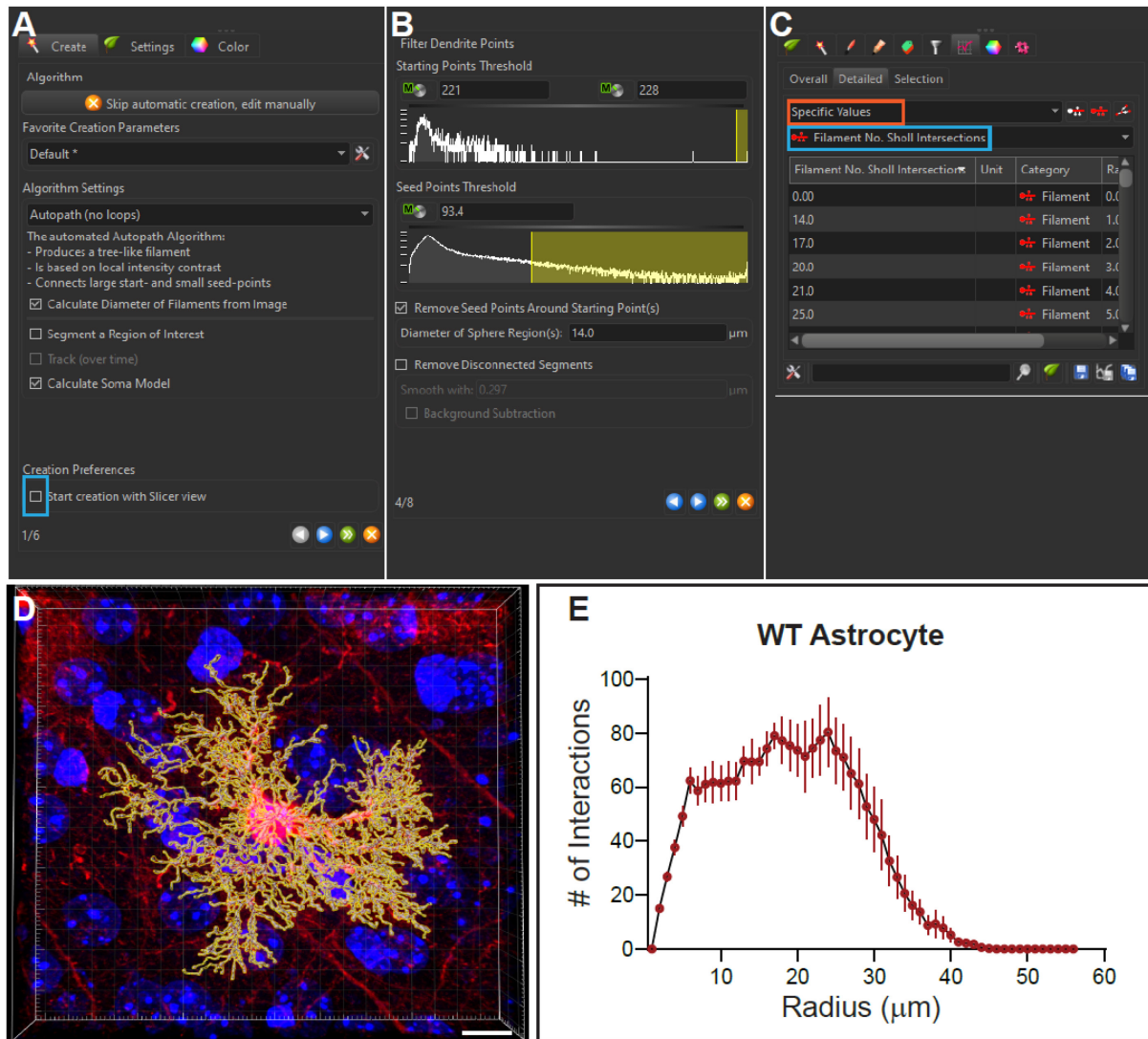


Figure 12 Reconstruction and analysis of astrocyte filaments.

A - B. Images depicting the stages of processing of images for filament detection.

C. The resulting data from this analysis displaying the number of filament intersections and the radius of each sholl boundary.

D. Visual representation of the filaments reconstructed by the Imaris software.

E. Plot of the sholl intersections data. Plots are shown as mean $\pm$ SEM (n=5 astrocytes).

Scale bar = 10 μm

4 Role of *Pten* in Cortical Neurogenesis at Single Cell Resolution using MADM

4.1 Summary

The cerebral cortex is evolutionarily the newest brain region, responsible for the higher order cognitive functions found in mammals like ourselves. The cortex is composed of both excitatory neurons, inhibitory neurons and glial cells. It is rich in cellular diversity and many of the resident cells are generated locally from multipotent progenitors called radial glia progenitors (RGPs). Cortical RGPs produce all the excitatory neurons and macroglia found in the neocortex. Work from our group has revealed that RGPs follow a deterministic developmental program throughout corticogenesis, whereby an RGP progresses in an orderly manner from a proliferative program to a neurogenic phase around E12, and later produces a clonal unit of 8-9 neurons. Nonetheless, the field lacks an understanding of the mechanisms that govern the cortical developmental program.

In this project, I aim to uncover key aspects of RGP lineage progression at the single cell level using MADM. MADM allows us to stably label and genetically manipulate a sparse subset of cells in any tissue *in vivo* with high spatiotemporal resolution. Here, I focus on uncovering the role of *Pten* in RGP lineage progression during cortical neurogenesis. *Pten* is an ideal target, since *PTEN* mutations in humans are known to cause macrocephaly, autism spectrum disorder (ASD), epilepsy, and a high susceptibility to tumors. The key question I sought to address is: where in RGP lineage progression does *Pten* exert its influence? I found that *Pten* plays a significant role in constraining progenitor proliferation and neuronal output during embryonic development. *PTEN* deletion results in shifts to the prescribed symmetric/asymmetric division switch. Finally, *PTEN* constrains clonal unit size, and its deletion results in larger neuron clonal units. Taken together, I show that *PTEN* functions are crucial for generating an appropriately sized cortex.

4.2 Introduction

The cerebral cortex arises from the early neuroepithelium, composed of progenitors with both apicobasal junctions and tight junctions thereby forming a pseudostratified tissue (Villalba et al., 2021). It is these early progenitors that give rise to RGPs and subsequently all excitatory neuronal cell types within the forebrain. RGPs continue to proliferate and expand the progenitor pool until approximately E12, at which point they switch to a neurogenic division mode to generate the neuronal diversity of the cortex (Beattie & Hippenmeyer, 2017). The cortical laminae are generated in an inside out manner, meaning the deep layer neurons are generated first, followed by subsequent waves of neurons, which sequentially populate the next layer. Neurogenesis continues until E16-E17, when the last of the superficial layer neurons are generated. Our group has discovered that RGPs follow a deterministic program during corticogenesis, during which they generate 8-9 neurons (Gao et al., 2014).

Despite this well-described stereotyped framework, the mechanisms which control RGP clonal output remain elusive. For this reason, I employ MADM to determine whether and how *Pten*, a gene that when mutated is known to cause macrocephaly, modulates progenitor output during neurogenesis.

4.3 Neurogenesis in Mammals

By E11, most progenitors in the mouse cortex have acquired the features of RGPs, with neurogenesis beginning between E11.5-12.5. Initially, RGPs generate neurons via the direct neurogenesis route, by dividing asymmetrically to self-renew and generate a neuron. RGPs later give rise to most neurons via IPs, this process is known as indirect neurogenesis (Agirman et al., 2017). Early born neurons migrate radially along the basal processes of RGPs to the deep layers, while later born neurons use this same scaffolding to create the superficial layers, thereby generating the cortical laminae in an “inside-out” fashion. Thanks to recent advances, we are beginning to acquire a better understanding of how RGPs generate the neuronal diversity found in the neocortex.

RGPs initially generate two classes of neurons, corticofugal neurons and commissural neurons (Magrinelli et al., 2022), that populate layer 6 of the cortex. Pearson and colleagues showed that RGPs ramp up *Foxp1* expression during early corticogenesis, and its expression decreases as RGPs transition to generate neurons destined for the upper layers (Pearson et al., 2020). They find that extending the period during which *Foxp1* is expressed results in an extended window of deep-layer neurogenesis (Pearson et al., 2020). Moreover, during deep-

layer neurogenesis, a sparse subset of RGPs are known to express *Fezf2*. *Fezf2* expression in post-mitotic neurons leads to them acquiring the corticofugal identity (Greig et al., 2013). Strikingly, deletion of *Fezf2* in mice results in the absence of large layer 5 neurons (Chen et al., 2005). Instead, the neurons in the presumptive layer 5 express *Tbr1* and project to the thalamus, and not to the brainstem and spinal cord. *Fezf2* activates expression of a series of genes required for deep-layer fate acquisition, such as *COUP-TF-interacting protein 2* (*Ctip2*), which controls differentiation into subcortical projection neurons (Chen et al., 2005). These results together indicate that *Foxp1* provides the competence window for deep-layer neurogenesis, while *Fezf2* expression is necessary for acquiring deep-layer identity while also repressing superficial-layer identity.

RGPs subsequently switch to generate superficial-layer neurons, which mostly project intracortically. Using in-utero electroporation to fate map RGPs and their progeny, Franco et al. showed that RGPs expressing *Cux2* only produce neurons bound for the superficial layers (Franco et al., 2012) and *Cux2* also marks the upper-layer neurons they produced (Huilgol et al., 2023). Interestingly, *Cux2* expression in progenitors was observed as early as E10, significantly before superficial-layer neurogenesis (Franco et al., 2012). These data can be interpreted to mean that from the very earliest stages of corticogenesis, at least two pools of progenitors exist (*Cux2*- and *Cux2*+). The *Cux2*- progenitors generate the lower layer neurons, while the *Cux2*+ progenitors amplify themselves in preparation for the later stages of neurogenesis. These data, however, have been contested and are contradicted by several heterochronic transplantation studies (Frantz & McConnell, 1996; McConnell, 1988).

A more recent study from Oberst and colleagues elegantly tested whether RGPs harvested from an animal of one age, can generate the appropriate neuronal progeny when transplanted into a cortex at either an earlier or a later stage of neurogenesis. Their data suggest that RGPs from E15 cortices, in other words RGPs generating superficial-layer neurons, remain competent to make deep-layer neurons when transplanted into an E12 cortex (Oberst et al., 2019). RGPs were able to respond in a plastic manner due to Wnt signaling provided by their host tissue. Meanwhile, IPs showed that they are indeed committed to generating a specific neuronal identity and remained insensitive to host-tissue signaling cues (Oberst et al., 2019). These data suggest that RGPs retain competency later in their lives, but IPs lack flexibility to produce neurons outside of their respective period of origin.

MADM-based studies also indicate that the vast neuronal diversity found throughout the cortical laminae comes from a single progenitor population, namely from *Emx1*-lineage RGPs (Amberg et al., 2022; Beattie et al., 2017; Gao et al., 2014).

In summary, despite all the advances in developmental neurobiology over the past decades, the mechanisms that govern neurogenesis in the cortex are not fully understood. This gap in our understanding leaves open avenues for new research.

4.4 *Pten* Mouse Models

Given its crucial role in modulating cell proliferation via mTOR signaling, it is essential to understand how PTEN may regulate key aspects of mammalian cortical development. PTEN is ubiquitously expressed throughout the developing embryo. Global homozygous knockout of *Pten* results in early embryonic lethality. Global *Pten* heterozygous knockout results in mild increases in brain size and mild predisposition to tumors, indicating *Pten* is dosage sensitive (Chen et al., 2015; Di Cristofano et al., 1998). Previous studies have delineated the gross effects *Pten* mutations can have on cortical development, but they were limited to studying embryonic or early post-natal timepoints in conditional knockout (cKO) mice due to perinatal lethality. Using a *Nestin-Cre* driver to delete *Pten* within neural progenitor cells *in vivo*, resulted in macrocephaly, hyperactivation of mTOR signaling, and early postnatal death (Groszer et al., 2001). Neurospheres derived from such mice allowed the authors to show that *Pten* deletion results in greater sensitivity to growth factors and an increase in the fraction of cells that transition from G₀-G₁ to the G_{1B} phase of the cell cycle (Groszer et al., 2006).

Pten cKO mice with deletion only in postmitotic excitatory neurons, using the *NEX-Cre* driver, revealed that neurons are hypertrophic and have a significant over-activation of AKT and mTORC1-S6K while heterozygous mutant cells have only a mild effect, again indicating dosage sensitivity (Kazdoba et al., 2012).

It has been shown that *Pten* deletion in the entire *Emx1* lineage results in a drastic macrocephaly as well as death around P10 (Chen et al., 2015). Additionally, the same mouse model showed that homozygous *Pten* deletion results in drastic increases in AKT, mTOR-S6K, and β -catenin activation, while heterozygous *Pten* deletion only increases pathway activity mildly (Chen et al., 2015). While *Pten* has been the subject of extensive study in the past decades, the field still lacks a clear picture of how *Pten* contributes to cortical stem cell lineage progression at the single progenitor level. In this study, we report on the requirement of functional PTEN throughout cortical stem cell lineage progression in mice using MADM. Our MADM-based approach bypasses the mortality of traditional *Pten* cKO mouse models by generating a sparse mosaic knockout of PTEN in the *Emx1* lineage, allowing us to study the trajectories of RGPs, neurons, and astrocytes from development to maturity.

4.5 Results

4.5.1 Experimental Design for Sparse Deletion of *Pten* in the Developing Mouse Cortex

To determine the cell-autonomous function of *Pten* in RGP lineage progression, I used a MADM-based genetic strategy. It consists of phenotypic analysis at single-cell resolution using a control-MADM (MADM-19^{GT/TG}; *Emx1*^{Cre/+}) model with sparse MADM labeling within the *Emx1* lineage in the control tissue (*Pten*^{+/+}) (Figure 13A), and a genetic mosaic *Pten*-MADM (MADM-19^{GT/TG, Pten-flox}; *Emx1*^{Cre/+}) mouse model with sparse *Pten* ablation in a minority of RGPs in a tissue consisting of phenotypically normal cells (Figure 13B). Sparse labeling and genetic manipulation in the cortex is induced by using an established driver to target the *Emx1* lineage, *Emx1-Cre* (Gorski et al., 2002) as well as a tamoxifen-inducible Cre (*Emx1-CreER*) (Kessaris et al., 2006). Experimental animals were generated by crossing parental lines according to the breeding scheme below (Figure 13C). As explained in the introduction, the presence of Cre recombinase catalyzes *trans*-recombination in the target chromosomes of animals carrying the TG and GT cassettes. Except that Cre recombinase in the *Pten*-MADM mouse model also induces *cis*-recombination of the LoxP sites which flank *Pten* exon 5 (Groszer et al., 2001), resulting in a Δ -Exon5 *Pten* allele that, when transcribed and translated, produces an incomplete and inactive PTEN protein. Therefore, in the *Pten*-MADM brains, cells labeled with tdTomato carry *Pten*^{+/+} alleles, cells expressing GFP carry two *Pten*^{-/-} alleles, and yellow cells carry *Pten*^{+/-} alleles. The unlabeled cells within the *Emx1* lineage are also *Pten*^{+/-}, whereas cells derived from other lineages, such as interneurons, are *Pten*^{+/+}.

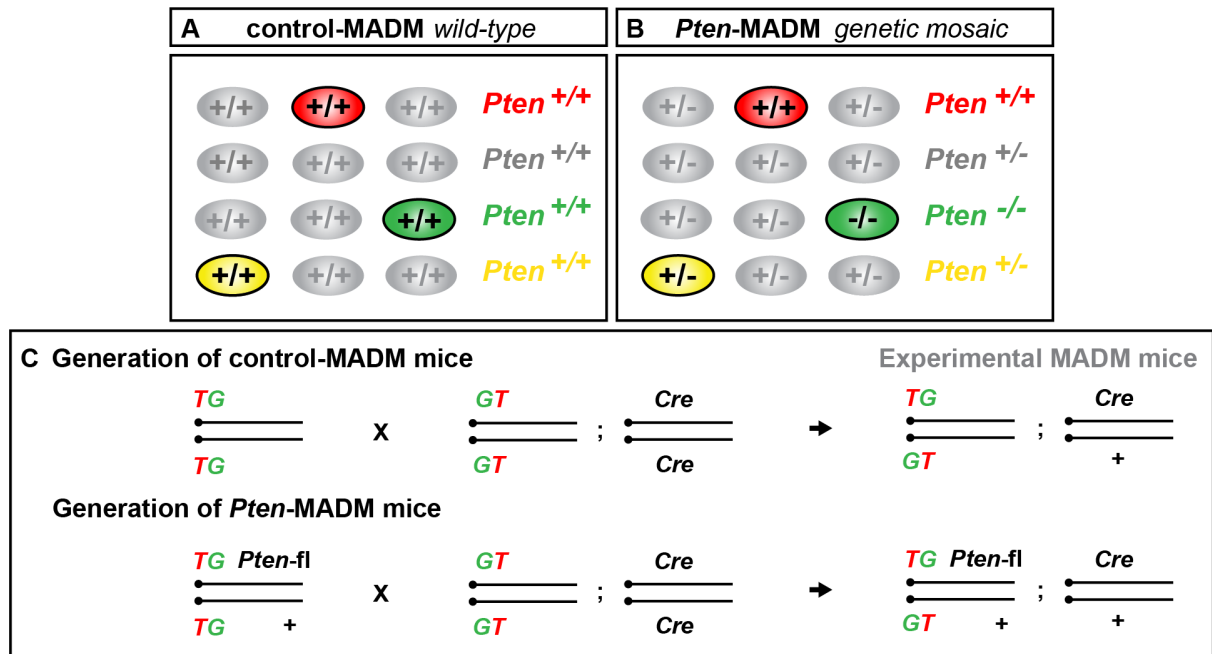


Figure 13. Experimental paradigms to study *Pten* at single cell resolution using MADM.

(A) Control-MADM brains contain sparse labeling without additional genetic manipulation. All cells in the tissue are control (*Pten*^{+/+}).

(B) *Pten*-MADM experimental brains are sparse genetic mosaics. GFP⁺ cells carry *Pten*^{-/-} alleles, while tdTomato⁺ cells carry *Pten*^{+/+} alleles. They therefore serve as additional internal controls. Yellow cells are excluded from our analyses, as they can result from either G2-Z or G1 recombinations.

(C) The breeding schemes for the respective genotypes. Although control-MADM mice can be generated through a dedicated breeding pair, most control-MADM and *Pten*-MADM mice in this study were generated from the same breeding pairs.

4.5.2 *Pten* Deletion Results in an Expansion of Cortical Neuron Populations with Significant Morphological Alterations

In this section of the study, we look at the output of cortical RGPs throughout neurogenesis at single-cell resolution (Figure 14A). We generated control-MADM and *Pten*-MADM mice (Figure 13C) and collected brain tissue at P21. Unlike traditional *Pten* cKO mouse models, our *Pten*-MADM mice survive past the early postnatal stages and have normal lifespans, well past 6 months of age. Since sparse MADM labeling occurs in a random sample of cells within the *Emx1* lineage, we expect GFP⁺ and tdTomato⁺ cell populations to be equal in control-MADM. We therefore quantify many of our analyses using a green-to-red ratio (G/R ratio). We identify neurons simply by morphology, as at P21 their morphology is easily distinguishable from that of astrocytes and oligodendrocytes.

We quantified the neuronal population in both control-MADM and *Pten*-MADM brains at P21. We find that, as expected, the G/R neuron ratio in control-MADM mice is 1.01 (SEM±0.02) (Figure 14D). Meanwhile in *Pten*-MADM mice, we find a G/R ratio of 1.99 (SEM±0.19) (Figure 14G). These data indicate a doubling of the *Pten* mutant neuron population compared to the control neurons in the *Pten*-MADM model.

We find that *Pten*-mutant neurons are morphologically distinct (Figure 15C-D) to their control counterparts (Figure 15A-B). Using the methods described in Chapter 3 of this thesis, we show that *Pten* mutant neurons in our sparse mosaic also show the characteristic hypertrophy reported in other *Pten* mouse models (Kazdoba et al., 2012). We find that the nuclear volume is 712 μm^3 (SEM±26.7) in *Pten*-mutant neurons (Figure 15E), significantly larger than control neurons with 266.2 μm^3 (SEM±26.7) (Figure 15E). Likewise, soma volume was significantly increased in *Pten*-mutant neurons (mean: 3885 μm^3 , SEM±376.3) (Figure 15F). In summary, we find that *Pten* mutations in our sparse genetic mosaic result in phenotypes we expect based on previous *Pten* mouse studies. Using MADM, I will go deeper into the role of *Pten* at the single progenitor level.

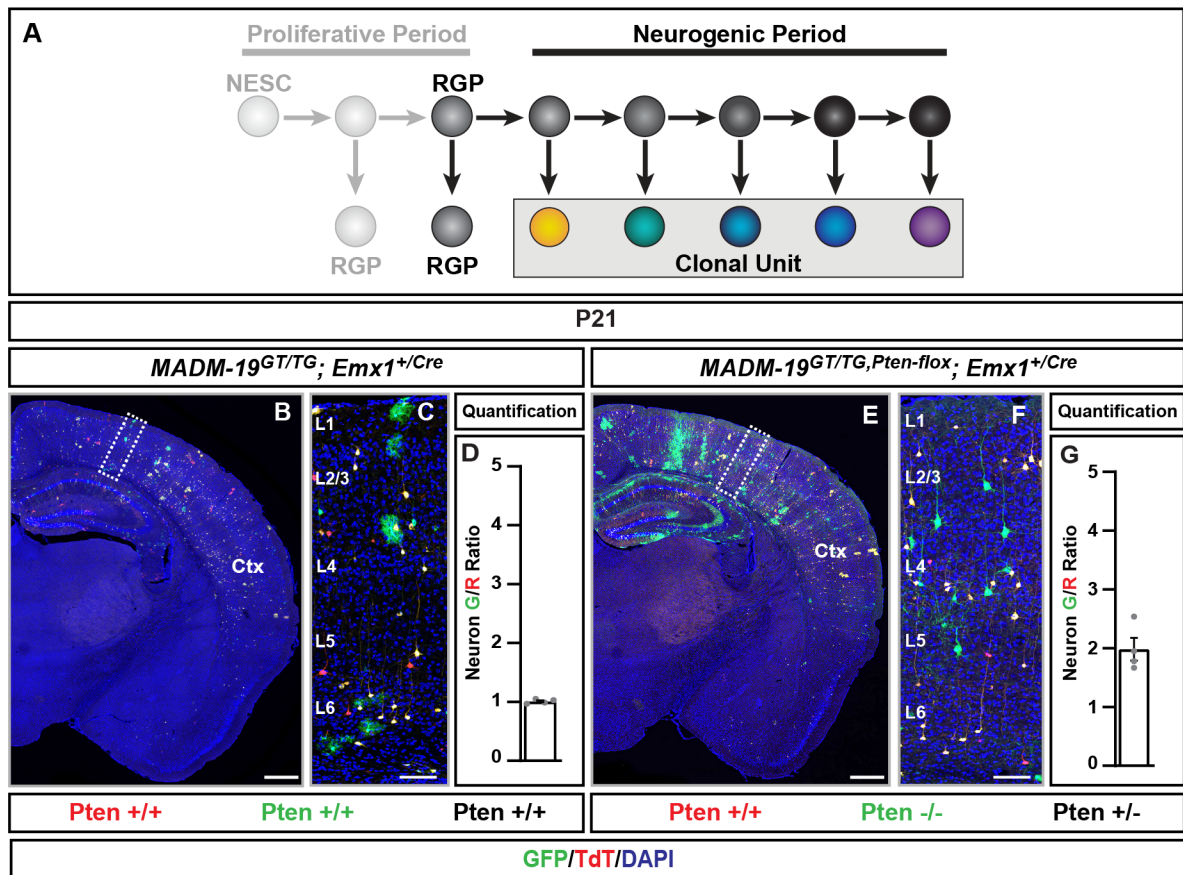


Figure 14. *Pten* deletion results in a cell autonomous increase in the mutant neuron populations in the neocortex.

(A) Schema of radial glia lineage progression in the developing cortex

(B-C) Confocal image of 35 μm thick section of a *MADM-19^{GT/TG}; Emx1^{Cre/+}* hemisphere at 20x magnification. Scale bar indicates 500 μm . Inset image of cortex of 35 μm thick section of *MADM-19^{GT/TG}; Emx1^{Cre/+}*. Scale bar indicates 50 μm .

(D) Quantification of the green to red ratio (G/R) of neurons in *MADM-19^{GT/TG}; Emx1^{Cre/+}* cortices, each dot indicates one animal (minimum of five sections counted per animal). Data shown is mean $\pm$ SEM. (Quantified by Ximena Contreras)

(E-F) Confocal image of 35 μm thick section of a *MADM-19^{GT/TG}; Pten-flox⁺; Emx1^{Cre/+}* hemisphere at 20x magnification. Scale bar indicates 500 μm . Inset image of cortex of 35 μm thick section of *MADM-19^{GT/TG}; Pten-flox⁺; Emx1^{Cre/+}*. Scale bar indicates 50 μm .

(G) Quantification of the green to red ratio (G/R) of neurons in *MADM-19^{GT/TG}; Pten-flox⁺; Emx1^{Cre/+}* cortices, each dot indicates one animal (minimum of five sections counted per animal). G/R ratio is increased, indicating a larger GFP+ mutant neuron population. Data shown is mean $\pm$ SEM. (Quantified by Ximena Contreras)

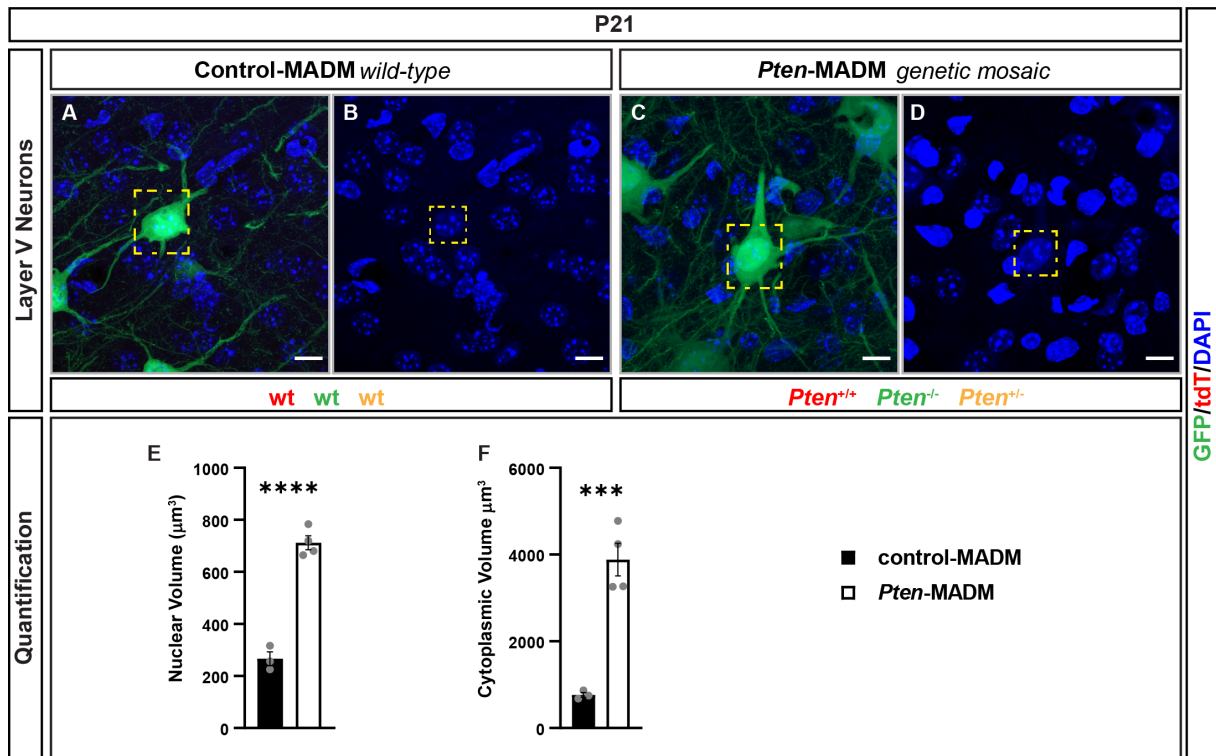


Figure 15. *Pten* cell-autonomously controls neuron cell size.

(A-B) Confocal image of 35 μm thick section of a *MADM-19^{GT/TG}; Emx1^{Cre/+}* neuron at 63x magnification. Scale bar indicates 5 μm .

(C-D) Confocal image of 35 μm thick section of a *MADM-19^{GT/TG,Pten-fl}; Emx1^{Cre/+}* neuron at 63x magnification. Scale bar indicates 5 μm .

(E) Quantification of nuclear volume in GFP+ neurons in *MADM-19^{GT/TG}; Emx1^{Cre/+}* (3 animals, 15 neurons quantified. Mean: 266.2 μm^3 , SEM $\pm$ 26.7) and in *MADM-19^{GT/TG,Pten-fl}; Emx1^{Cre/+}* (4 animals, 20 neurons quantified. Mean: 712 μm^3 , SEM $\pm$ 26.7). Data shown is mean $\pm$ SEM. Two-tailed unpaired t-test, P-value = <0.0001, F = 1.333. ****p<0.0001

(F) Quantification of soma volume in GFP+ neurons in *MADM-19^{GT/TG}; Emx1^{Cre/+}* (3 animals, 15 neurons quantified. Mean: 763.3 μm^3 , SEM $\pm$ 55.31) and in *MADM-19^{GT/TG,Pten-fl}; Emx1^{Cre/+}* (4 animals, 20 neurons quantified. Mean: 3885 μm^3 , SEM $\pm$ 376.3). Data shown is mean $\pm$ SEM. Two-tailed unpaired t-test, P-value = 0.0009, F = 61.7. ***p<0.001

4.5.3 *Pten* has a Cell-Autonomous Function in Individual RGPs during Neurogenesis

Previous data from our lab (unpublished) has shown that *Pten* deletion results in a significant increase in the number of PAX6⁺ RGPs in the developing mouse cortex. I followed up on this finding by generating clonal MADM mice, using a tamoxifen-inducible *Emx1*-CreER recombinase (Kessaris et al., 2006), to understand progenitor division patterns throughout neurogenesis. In MADM clonal analysis, we categorize clones as being either symmetric or asymmetric in nature. Symmetric clones are defined as having 4 or more cells per subclone, while asymmetric clones consist of one majority subclone with 4 or more cells and one minority subclone with 3 or less cells.

Pregnant dams were injected at E11 timepoints to label RGPs during the time in which they divide mostly in a symmetric proliferative manner. I then analyzed tissue at E13 and E16 timepoints. As symmetric clones will contain two subclones of similar size, I quantified clones by counting the number of cells per subclone (green subclone and red subclone) in MADM-19^{GT/TG, Pten^{-fl}; Emx1-CreER^{+/-}} mice and compare the control subclone (tdT⁺) to the *Pten*-mutant subclone (GFP⁺) (Figure 16A-B). I find that during the first 2-3 cell divisions, RGP output remains similar in control (mean = 3.188 cells/subclone, SEM±0.4002) and *Pten*-mutant subclones (mean = 3.625 cells/subclone, SEM±0.4171) (Figure 16C). I then quantified clone size at E16 (Figure 16D), when neurogenesis has nearly finished. *Pten* mutant subclones are significantly larger (mean = 25.56 cells/subclone) than control subclones (mean = 15.11 cells/subclone) (Figure 16E), indicating an increased RGP proliferative potential resulting in larger mutant subclones.

Previous MADM studies established that RGPs produce a defined unit of 8-9 neurons (Gao et al., 2014). By using a tamoxifen inducible CreER recombinase, I was able to assess whether *Pten* plays a role in constraining an RGPs clonal output. We injected pregnant dams at E12.5 to induce MADM events, collected the pups at P21 and analyzed the size and division mode of clones in the somatosensory cortex. Control-MADM clones largely reflected previous reports (Figure 17C), but the unitary output of *Pten* mutant RGPs was significantly larger, approximately 9.5 neurons per clone (Figure 17C). While both control and mutant clones are similarly likely to produce neurons across both upper and lower layers, mosaic clones tend to produce a greater fraction of lower layer neurons (Figure 17F). Finally, we observe that approximately 37% of E12-induced clones in control-MADM are symmetric, about 50% of the *Pten*-MADM clones are symmetric (Figure 17G-H). These results indicate that *Pten* deletion

perturbs the timing of the symmetric-asymmetric switch in RGPs. Taken together, our data provides insights into the individual progenitor dynamics that give rise to the overproduction of neurons in the developing *Pten*-mutant cortex and highlight the importance of *Pten* in controlling the proper timing of lineage progression during development.

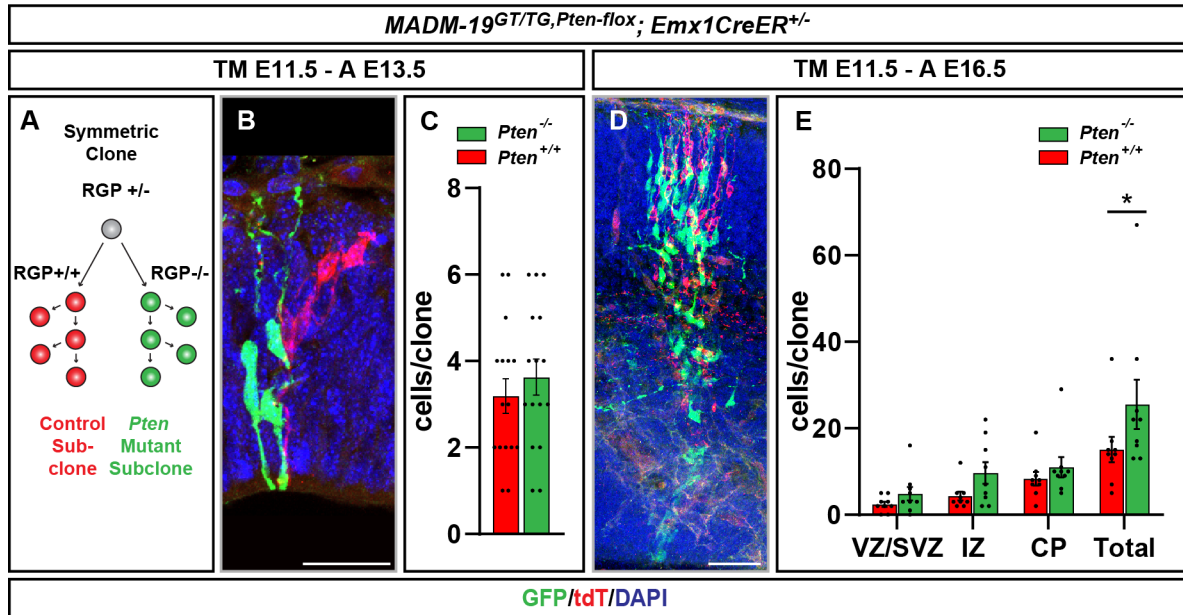


Figure 16. Deletion of *Pten* in early RGPs results in larger symmetric proliferative clones.

(A) Model of symmetric clone resulting from an E11.5 tamoxifen injection. Symmetric clones have two subclones.

(B) Representative image of a symmetric clone from an *MADM-19^{GT/TG}-Pten-^{fl}; Emx1-CreER^{+/-}* mouse cortex at E13.5. Scale bar = 50 μ m. Section cut at 35 μ m.

(C) Quantification of red and green subclones in *MADM-19^{GT/TG}-Pten-^{fl}; Emx1-CreER^{+/-}* mouse cortex at E13.5. Green and red subclones are of similar size at this stage (red subclone mean = 3.188 cells/subclone, SEM $\pm$ 0.4002, green subclone mean = 3.625 cells/subclone, SEM $\pm$ 0.4171). Each dot indicates one clone. Data shown is mean $\pm$ SEM. Unpaired t-test p-value = 0.455, ns.

(D) Representative image of a symmetric clone from an *MADM-19^{GT/TG}-Pten-^{fl}; Emx1-CreER^{+/-}* mouse cortex at E16.5. Scale bar = 50 μ m.

(E) Quantification of red and green subclones in *MADM-19^{GT/TG}-Pten-^{fl}; Emx1-CreER^{+/-}* mouse cortex at E16.5 (red subclone total mean = 15.111 cells/subclone, SEM $\pm$ 2.922, green subclone mean = 25.556 cells/subclone, SEM $\pm$ 5.694). Each dot indicates one clone. Data shown is mean $\pm$ SEM. One-way Anova adjusted p-value = 0.0332. *p<0.05

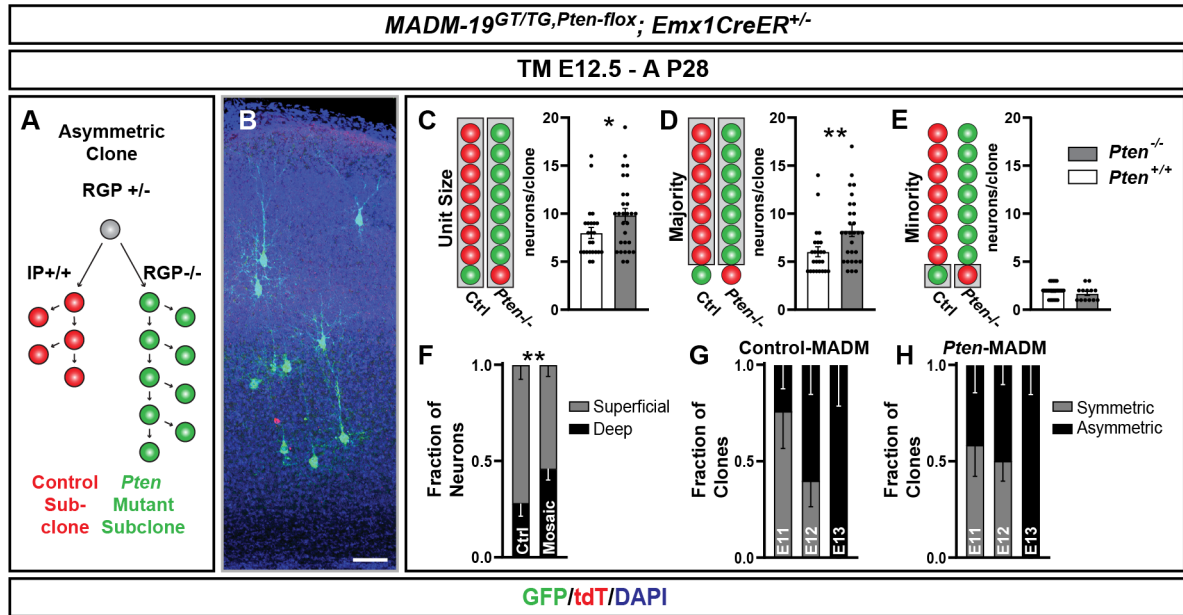


Figure 17 *Pten* plays a cell-autonomous role in regulating neurogenic clonal unit size.

(A) Model of asymmetric neurogenic clone resulting from E12.5 tamoxifen injection. Asymmetric clones have a majority (> 3 cells in size) subclone and minority (< 3 cells in size) subclone. Together they constitute the clonal unit.

(B) Representative image of an asymmetric clone from an *MADM-19^{GT/TG}, Pten^{-flox}, Emx1-*CreER*^{+/-}* mouse cortex at P28. Scale bar = 50 μ m. Section cut at 40 μ m.

(C) Quantitative comparison between all asymmetric clones from control mice (*MADM-19^{GT/TG}, Emx1-*CreER*^{+/-}*) and *Pten*^{-/-} clones from *Pten*-MADM mice (*MADM-19^{GT/TG}, Pten^{-flox}, Emx1-*CreER*^{+/-}*). *Pten* mutant clones are significantly larger (mean = 9.862, SEM $\pm$ 0.6763) than control counterparts (mean = 8.00, SEM $\pm$ 0.5679). Each dot indicates one clone. Data shown is mean $\pm$ SEM. Mann-Whitney p-value = 0.0357. *p < 0.05

(D) *Pten*^{-/-} majority subclones (mean = 8.241, SEM $\pm$ 0.6231) are significantly larger than control majority subclones (mean = 6.04, SEM $\pm$ 0.5160). Each dot indicates one clone. Data shown is mean $\pm$ SEM. Unpaired t-test p-value = 0.0061. **p < 0.01

(E) Minority subclones in both *Pten*^{-/-} (mean = 1.621, SEM $\pm$ 0.1351) and control (mean = 1.96, SEM $\pm$ 0.1409) are of roughly equal size. Each dot indicates one clone. Data shown is mean $\pm$ SEM. Unpaired t-test p-value = 0.0793.

(F) Quantification of neuron distribution across deep layers (V-VI) and superficial layers (II/III-IV) reveals that neurons from *Pten*-mutant clones (SL: 56.2%, DL: 43.8%, 95%-CI: 62.3-49.9%) were significantly more concentrated in the deeper layers compared to neurons in control clones (SL: 72.2%, DL: 27.8%, 95%-CI: 78.7-35.4%). Data show is fraction and CI 95%. Fisher's Exact test p-value = 0.0019, **p < 0.01

(G-H) Clones can be categorized as symmetric (two subclones, each greater than 4 cells) or asymmetric (one majority subclone with 4 or more cells, and a minority subclone with 3 or less cells).

(G) At E11 control clones mostly divide symmetrically (76%), at E12 they switch to mostly asymmetric divisions (60%), by E13 all clones are asymmetric (100%). Error bars show lower 95% CI.

(H) *Pten* mutant clones do not follow the same trajectory, instead some enter neurogenic divisions at E11 (41.7%), and half remain in symmetric proliferative mode at E12 (50%). Much like in control, all *Pten* mutant clones were asymmetric by E13 (100%). Error bars show lower 95% CI.

4.5.4 Deep Layer Neuron Populations are Selectively Expanded in *Pten*-MADM Mice

The clonal data provides a lot of clarity about the single-progenitor dynamics that lead to the *Pten* phenotype, but I wanted to follow up on the deep vs superficial layer distribution we found in the clones. Population level MADM (i.e. driven by *Emx1*-Cre) provides the numbers necessary to verify the layer distribution phenotype, I used control-MADM and *Pten*-MADM (Figure 18A,C) to conduct this analysis. Using DAPI staining to differentiate between layers, I subdivided the cortex into the six respective layers which make up the structure (Figure 18B,D). The distribution of neurons in the control-MADM (Figure 18E, black line) condition showed that a relative plurality of neurons reside in layers 2/3 (36.97%) and layer 4 (29.2%), while *Pten*-mutant neurons in *Pten*-MADM brains (Figure 18E, green line) were more evenly distributed among layers 2/3 (23.4%), 4 (21.6%), and 5 (24.0%). Layer 6 in *Pten*-MADM mice contained a significantly larger fraction of green neurons (31.0%).

Interestingly, when comparing the ratio of *Pten*^{-/-} neurons with *Pten*^{+/+} neurons within each layer in the *Pten*-MADM condition, I find that green mutant neurons in layer 5 are preferentially generated (Figure 18F). Layers 2/3 had a G/R ratio very close to one, ratios in layers 4 and 6 were approximately two, while layer 5 had a G/R ratio of four (Figure 18F). This means that in the *Pten* mutant neuron population, more neurons reside in layer 6, but when comparing neuron numbers between control and *Pten* mutants within each layer, I find that layer 5 has the most mutant neurons.

I performed single-cell RNA sequencing on GFP⁺ cells from both control-MADM and *Pten*-MADM cortices at P0 in order to mitigate the negative effects of the dissociation/FACS protocol on neurons at later postnatal stages. I sequenced two samples from control-MADM and four from *Pten*-MADM, the mean cell number is comparable between the two conditions (Figure 19A). All other metrics for genes present (Figure 19B), total RNAs per cell (Figure 19C), and mitochondrial RNA content (Figure 19D) were comparable between the samples, indicating we had acquired data of a good quality. I then used the Seurat package to pool all neurons irrespective of genotype and generated a UMAP with distinct neuronal clusters (Figure 19E).

In order to corroborate the earlier findings, that deep-layer neurons were more abundant in *Pten* mutant populations than in control, I performed a cell-type abundance analysis on the Seurat clusters. I wanted to determine whether the excess cells in the lower layers were of the correct molecular identity. To achieve this I used classical markers for deep and superficial

layer neurons. I find a relative enrichment of clusters “1” and “2” and a relative decrease of clusters “0” and “3” in *Pten* mutant neuron populations compared to control (Figure 19F). Using canonical markers to identify broad cell identity (i.e. superficial vs deep) (Figure 19G-L), I find that the populations in overrepresented clusters “1” and “2” more closely resemble deep layer neurons due to their expression of *Ctip2* and *Fezf2* (Figure 19G,H). The sequencing data provides evidence that the excessive neurons in the deep layers are of the correct identity. These findings bring up new questions for future studies, such as whether *Pten* deletion induces a fate-switch of neurons destined for other layers, or whether the phenotype we observe is simply due to progenitor abundance at specific time points.

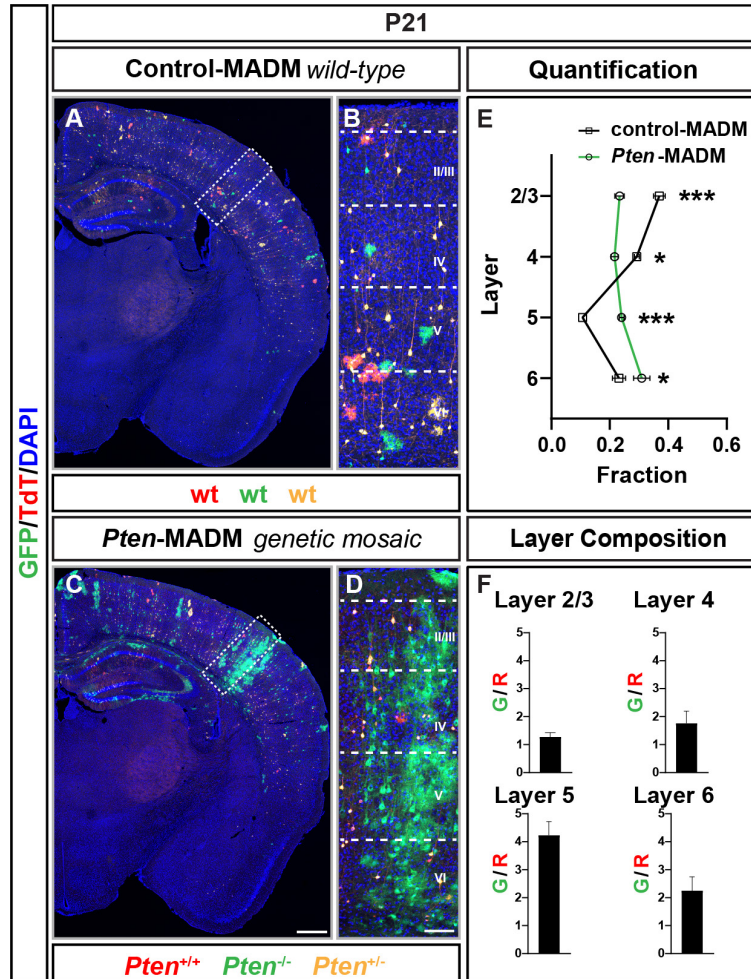


Figure 18. *Pten*-mutant neurons are more abundant in the deep layers.

(A) Confocal image of 35 μ m thick section of a *MADM-19*^{GT/TG}; *Emx1*^{Cre/+} hemisphere at 20x magnification. Scale bar indicates 500 μ m.

(B) Inset image of cortex of 35 μ m thick section of *MADM-19*^{GT/TG}; *Emx1*^{Cre/+}. Scale bar indicates 50 μ m.

(C) Confocal image of 35 μ m thick section of a *MADM-19*^{GT/TG, *Pten*-flox}; *Emx1*^{Cre/+} hemisphere at 20x magnification. Scale bar indicates 500 μ m.

(D) Inset image of cortex of 35 μ m thick section of *MADM-19*^{GT/TG, *Pten*-flox}; *Emx1*^{Cre/+}. Scale bar indicates 50 μ m.

(E) Quantification of the relative distribution of GFP+ neurons in both control-MADM (black line) and *Pten*-MADM (green line). Control-MADM - L6: 23.1%, SEM $\pm$ 2.2, L5: 10.7%, SEM $\pm$ 0.70, L4: 29.2%, SEM $\pm$ 0.9, L2/3: 37.0%, SEM $\pm$ 2.0. *Pten*-MADM - L6: 31.0%, SEM $\pm$ 2.80, L5: 24.0%, SEM $\pm$ 0.8, L4: 21.6%, SEM $\pm$ 1.2, L2/3: 23.4%, SEM $\pm$ 1.60. Two-way Anova: L2/3 adjusted p-value = 0.0001, L4 adjusted p-value = 0.0224, L5 adjusted p-value = 0.0002, L6 adjusted p-value = 0.0185. *p<0.05, **p<0.01, ***p<0.001. Data shown is mean $\pm$ SEM.

(F) Green-to-red ratios of neurons within their cortical laminae. Values shown are mean and SEM. L2/3: mean = 1.274, SEM $\pm$ 0.1583, L4: mean = 1.760, SEM $\pm$ 0.4367, L5: mean = 4.231, SEM $\pm$ 0.4885 L6: mean = 2.249, SEM $\pm$ 0.4930. Data shown is mean $\pm$ SEM.

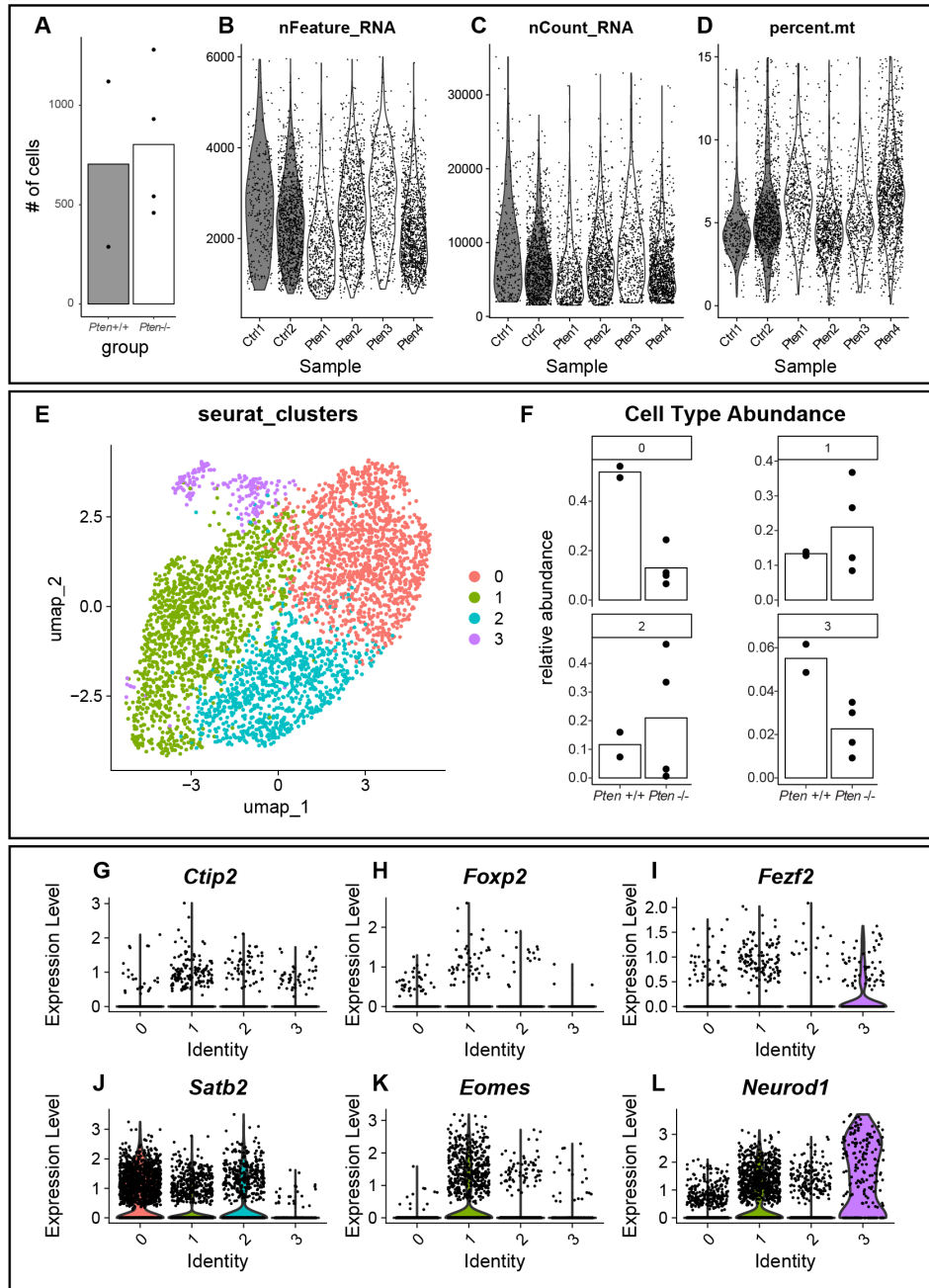


Figure 19. Neurons with deep layer identities are more abundant in *Pten* mutants than control at P0.

(A) Cell counts for the two *Pten*^{+/+} and four *Pten*^{-/-} samples. Each dot represents an individual sample.

(B-D) Violin plots showing (B) unique gene reads, (C) total RNA transcript counts, and (D) percent of reads aligning with mitochondrial RNA, an assay for cell death.

(E) UMAP showing the distinct clusters of neurons present in all samples.

(F) Cell type abundance plots. Each dot represents an individual sample. Bar plots indicate mean.

(G-L) Violin plots showing expression levels for deep (*Ctip2* and *Foxp2*) and superficial (*Satb2* and *Fezf2*) neuronal marker genes. Each dot within a violin plot represents an individual cell.

4.6 Discussion

The mammalian cortex is built from an initial monolayer consisting of a pool of multipotent progenitors. Perturbations to the developmental program often result in critical deficiencies and malformations detrimental to the viability of the organism. One key regulator of development and growth is *Pten*. We have known that *Pten* mutations lead to macrocephaly since the early 2000's, characterized by an enlarged brain with excessive and hypertrophic neurons. Later studies revealed that *Pten* deletion shortened the cell cycle and delayed cell cycle exit (Chen et al., 2015; Groszer et al., 2006; Groszer et al., 2001), but none of these studies had the resolution to determine the cellular contribution of single progenitors. Previous studies into *Pten*'s role during development were limited by the early postnatal lethality of *Pten* cKO in the *Emx1* lineage (Chen et al., 2015). Others used postnatal *Pten* models, like NEX-Cre drivers, to delete *Pten* within postmitotic neuron populations (Kazdoba et al., 2012), but such an approach does not shed light on the developmental role of *Pten*. I sidestepped these limitations by generating sparse mosaics using MADM. This study presents, for the first time, an analysis of *Pten*'s role during cortical stem cell lineage progression at single cell resolution.

My data set expands on previous reports that PTEN-deficient RGP's tended to overproliferate in both mouse cortex and neurospheres (Groszer et al., 2006; Groszer et al., 2001), as well as human organoids (Li et al., 2017). I did so by using MADM clonal analysis to ascertain, at the single-progenitor level, that *Pten*^{-/-} progenitors generate larger proliferative symmetric clones than control counterparts. One long-standing question regarding macrocephaly resulting from *Pten* LOF was whether each RGP increased its individual neuronal output. Once again using the clonal MADM approach, I found that the clonal output of *Pten* mutant RGP's is close to 10 neurons, significantly increased from the canonical clonal unit of 8 neurons found by our group (Gao et al., 2014). The clonal analysis showed that *Pten* deletion resulted in a greater allocation of lower layer neurons than in control. *Pten* therefore appears to play a decisive part in the decision making process during neurogenesis. I report that the macrocephaly found in *Pten* LOF mouse models not only stems from an expansion of the RGP pool, but an increase in the individual RGP output throughout neurogenesis. *Pten* deletion also led to an excessive and preferential production lower layer neurons, skewing the constitution of the cortex toward more subcortically projecting neurons. How *Pten* deletion influences progenitor output remains elusive. Does PTEN truly determine cellular identity? Or is it due to more progenitors being present initially? My data point to the former, given that I

showed that progenitor numbers do not significantly differ until mid-neurogenesis. Further studies might be able to precisely delineate how PTEN influences cell fate specification.

5 Uncovering the Role of *Pten* during Cortical Gliogenesis

5.1 Summary

Cortical gliogenesis begins upon the conclusion of the neurogenic period, whereby RGP undergo a switch from the neurogenic to gliogenic program. Those that do transition from neurogenesis to gliogenesis end up producing the macroglia of the neocortex, namely astrocytes and oligodendrocytes. Astrocytes provide critical support to the neurons in the cortical laminae, and thus are essential for optimal functioning of the neuronal circuitry.

In this section of the thesis, I provide evidence that *Pten* is cell-autonomously required for the nominal production of astrocytes. Using MADM-based clonal analysis, I find that RGP produce larger astrocyte clones than control counterparts. Bulk-seq and scRNA-seq of astrocytes and progenitors at P0 revealed a deregulation in key signaling pathways, which provided valuable new targets to test. Thanks to the flexible MADM system, I was able to test for the requirement of components of various pro-gliogenic signaling pathways during gliogenesis in mosaic *Pten* mutant conditions. I find that, despite literature indicating requirements for Jak/Stat and Notch signaling during gliogenesis, cKOs for *Jak1/2*, *Stat3/5*, and *Notch1* do not rescue the overproduction of astrocytes in *Pten* mutant populations. Finally, I find that *Erk1/2* are required for expansion of the astrocyte population in the absence of *Pten*. The results described in this section provide key insights into the progenitor dynamics and signaling pathways that control astrocyte numbers.

5.2 Introduction to Mammalian Gliogenesis

Cortical glia can be grouped into two broad categories, microglia and macroglia. Astrocytes and oligodendrocytes are the two types of macroglia that help maintain neurons and support the existing neuronal circuitry (Chung et al., 2015; Costa et al., 2009; Freeman & Rowitch, 2013). Macroglia, unlike microglia, are derived from the same cortical progenitors as neurons. To generate macroglia, RGP undergo a neurogenic to gliogenic switch between E16 and E17 (Bayraktar et al., 2014; Kriegstein & Alvarez-Buylla, 2009), during which approximately 16% of RGP transition to gliogenesis (Gao et al., 2014). While there is much to discover about this process, the field has some understanding of the molecular states that progenitors must acquire to generate glia.

Previous studies have shed light on the roles of *Sox9* and *Nfia* during this process. In the developing mouse and chick spinal cord, SOX9 initiates gliogenesis by binding to the *NFIA-e123* enhancer upstream of *Nfia*. This leads to expression of *Nfia*, thereby initiating the

gliogenic period for progenitors (Kang et al., 2012). Then, both SOX9 and NFIA form a complex and subsequently activate a set of progliogenic genes, including *Mmd2* and *Apccdl* (Kang et al., 2012). *Mmd2* was shown to be required for astrocyte precursor proliferation, while *Apccdl* promotes migration of those precursors to their final destinations (Kang et al., 2012).

Olig2 and *epidermal growth factor receptor (Egfr)* are well-known genes crucial for cortical gliogenesis. *Olig2* encodes a basic helix-loop-helix (bHLH) transcription factor expressed by multipotent glial precursors in the SVZ of the dorsal forebrain and not by RGP within the VZ (Gao et al., 2025; Li et al., 2021; Marshall et al., 2005). OLIG2⁺ progenitors in the SVZ exclusively generate glial cells in the rat dorsal pallium (Marshall et al., 2005). Using a combination of MAGIC Marker lineage tracing and *Olig2*^{Cre}, Clavreul and colleagues provide evidence that nearly all cortical astrocytes can be traced back to an Olig2⁺ progenitor (Clavreul et al., 2019). By combining scRNA-seq and lineage tracing analysis, Li and colleagues found that the previously reported *Olig2*⁺ precursors as being central for the generation not only of oligodendrocytes, but also for the majority of cortical astrocytes as well as olfactory bulb neuroblasts (OBNBs) (Li et al., 2021). We now also know that basal multipotent intermediate progenitors (bMIPCs), expressing *Ascl1*, *Egfr*, and *Ki67*, are capable of dividing asymmetrically to self-renew and generate distinct committed IPs for oligodendrocytes (OPCs), astrocytes (aIPs), and OBNBs (OBNB-IPs) (Li et al., 2021).

Li and colleagues also provide further evidence for a subtype of RGP that delaminates from the VZ and translocates into the cortical laminae to generate astrocytes locally (Li et al., 2021), adding to previous evidence for the existence of such progenitors (Clavreul et al., 2019). Local generation of astrocytes in the cortical laminae is not new, though previous data supported the existence of a population of mitotic immature astrocytes (Ge et al., 2012). Unlike the tidy columnar organization of neuron clones, astrocyte clones vary in their spatial characteristics from highly clustered to interspersed and mixed with neighbouring clones (Clavreul et al., 2019). Despite these advances, we still do not have a clear picture of the molecular mechanisms that control cortical astrocyte production. The subsequent parts of this introduction touch on the signaling pathways currently known to be required for gliogenesis.

5.2.1 EGFR's Key Role in Cortical Gliogenesis

EGFR is a receptor tyrosine kinase (RTK) that when bound to a ligand, such as EGF, transduces extracellular signals via intracellular signaling pathways like the MAPK pathway (Holbro & Hynes, 2004). EGFR can itself also be activated by other receptors via Janus kinase 2 (JAK2), a non-receptor tyrosine kinase (Holbro & Hynes, 2004). *Egfr* expression is dynamic, with low

levels of expression until E15, thereafter expression ramps up within the VZ as RGP's prepare to make the gliogenic switch (Sun et al., 2005). Data from multiple early studies suggest that *Egfr* expression is essential for glial cell generation in the mammalian cortex (Burrows et al., 1997; Sibia et al., 1998; Sun et al., 2005). Burrows and colleagues performed elegant retroviral infection experiments to determine whether *Egfr* was instructive in gliogenesis by overexpressing *Egfr* in progenitors in the VZ both *in vivo* and *in vitro* (Burrows et al., 1997). They showed that the extra EGFRs in progenitors led to an increase in cells expressing S100 β and glial fibrillary acidic protein (GFAP) (Burrows et al., 1997), classical astrocyte markers.

Moreover, *Egfr* knockout leads to impairment in the proliferative abilities of primary astrocytes isolated from P1 mice (Sibia et al., 1998). Later studies have shown that the gliogenic program is also sensitive to the dosage of *Egfr* (Zhang et al., 2020; Zhang et al., 2023). Work from our group, as well as collaborators, reaffirms that EGFR is indispensable for gliogenesis, as *Egfr*-cKO cortices have no astrocytes from the *Emx1*-lineage (Beattie et al., 2017; Zhang et al., 2023). Finally, recently published data revealed that aIPs and OBNB-IPs, but not oligoIPs, continue to express *Egfr* (Gao et al., 2025), further cementing *Egfr*'s key role in astrocyte production in the cortex.

5.2.2 JAK/STAT's Influence on the Onset of Astrocyte Production

JAK and signal transducer and activator of transcription (STAT) signaling has also been implicated in astrocyte differentiation. JAKs (JAK1/2) are intermediaries between cytokine receptors, like LIFR and gp130, and STATs. Their function is to detect receptor activation and dimerization and subsequently phosphorylate the host receptor at specific tyrosine residues, which then act as binding sites for Src homology 2-containing signaling proteins like STAT5 and STAT3 to become phosphorylated (Stahl et al., 1995). Once phosphorylated, STATs dimerize and translocate into the nucleus, where they directly activate transcription of target genes (Hu et al., 2021). *Gfap* is a well-known target gene for JAK/STAT signaling, as it has a STAT3 binding site in its promotor region (Bonni et al., 1997; Brenner & Messing, 2021).

Several reports claim that JAK/STAT and their target genes, like *Gfap*, influence the onset of gliogenesis. Indeed, by modulating JAK-STAT activity up or down, one group reported acceleration or inhibition of the onset of gliogenesis, respectively (He et al., 2005). Altering the epigenetic processes that regulate JAK/STAT target gene expression supports the hypothesis that they play a central role in gliogenesis. Precocious expression of *Gfap* in *Dnmt1* cKO mice resulted in early differentiation of progenitors into GFAP⁺ and S100 β ⁺ cells, resembling immature astrocytes (Fan et al., 2005). Therefore, through modulation of

JAK/STAT activity directly and activation of downstream targets, data support that JAK/STATs play an important role in cortical gliogenesis.

5.2.3 NOTCH Signaling as a Regulator of Gliogenesis

NOTCH signaling is also thought to regulate gliogenesis in the murine nervous system (Wang & Barres, 2000). Unlike the previously discussed signaling pathways, NOTCH signaling relies on direct cell-to-cell contact between a cell expressing one of the NOTCH receptors (NOTCH1-4) and a neighbouring cell expressing a ligand (Delta-like ligand 1,3,4 and Jagged 1,2) (Kopan & Ilagan, 2009). When a NOTCH receptor becomes activated by contact with a ligand, the intracellular domain of NOTCH (NICD) is cleaved by proteases and internalized and trafficked to the cell nucleus (Pierfelice et al., 2011). Once inside the nucleus, the NICD associates with RBPJ and MAML proteins to activate transcription of a slew of target genes, like *Hes* genes (Pierfelice et al., 2011). HES1/5 proteins actively repress pro-neural identity by inhibiting pro-neural bHLH genes, like *Neurogenin2* and *Mash1* in the dorsal forebrain (Nieto et al., 2001). Therefore, cells with activated NOTCH receptors are instructed to retain their progenitor identity.

Experiments in the neural crest showed that, in addition to preserving progenitor identity, NOTCH signaling could drive glial cell specification (Morrison et al., 2000). Work in mammalian cells supports the model of NOTCH as a progliogenic signal. Activation of NOTCH receptors in murine neurospheres led to a marked increase in the rate of astrocyte production and differentiation, and consequently an expansion of the astrocyte population (Grandbarbe et al., 2003). NOTCH not only promotes astrocyte production but, in the Zebrafish spinal cord, it is also required for OPC specification. Interestingly, NOTCH signaling later inhibits oligodendrocyte production (Park & Appel, 2003). Such data highlights the importance of understanding how the context in which signaling pathways operate also influences the resulting biological output.

5.2.4 ERK Signaling as a Pro-Gliogenic Pathway in the Mouse Cortex

The extracellular signal-regulated kinase (ERK) pathway is a highly conserved cascade that controls cell proliferation and differentiation in response to extracellular signals (Cargnello & Roux, 2011). The core ERK signaling cascade is activated by RTKs that phosphorylate RAS proteins near the cell membrane, RAS then activates RAF, RAF in turn phosphorylates the active region of MEK1/2, it is the MEKs which finally phosphorylate the ERKs (Cargnello & Roux, 2011). p-ERK1/2, the most well-studied, then directly activate a number of downstream

targets in various subcellular compartments, most notably a slew of nuclear transcription factors called E26 transformation-specific (ETS) transcription factors (Lavoie et al., 2020). Some key functions directly mediated by ERK1/2 include cell proliferation, survival, growth, metabolism, and differentiation (Lavoie et al., 2020).

Given its central role in basic cellular functions, it is no surprise that the ERK signaling pathway has been implicated in several processes during cortical development. ERK1/2 are approximately 83% identical in their amino acid sequence (Boulton et al., 1991; Boulton et al., 1990). They are also functionally redundant in the developing mouse embryo, as ectopic expression of *Erk1* can rescue the severe defects caused by *Erk2* deletion (Fremin et al., 2015). Despite the ability of ERK1 to compensate for the loss of ERK2, *Erk1* deletions themselves seem to be relatively harmless to the developing embryo (Aouadi et al., 2006; Fremin et al., 2015).

Using an *hGFAP-Cre* promotor to delete *Erk2* in the mouse cortex, Samuels and colleagues found that *Erk2* deletion in cortical RGP resulted in a reduced cortical thickness due to a diminished neuronal population throughout the cortical laminae (Samuels et al., 2008). Interestingly, they find an increase in cortical astrocytes (Samuels et al., 2008). A later study focusing on MEK1/2, the kinases directly upstream of ERK1/2, reported contradictory findings as it demonstrated that *Mek1/2* deletion severely disrupted the gliogenic program. Conversely, they find that constitutively active MEK1 led to an expanded astrocyte pool (Li et al., 2012).

Another study deleted both *Erk1/2* in mouse cortex using a *Nestin-Cre* driver to target the neural tissues. They find that deletion of *Erk2* alone results in a decrease in astrocyte numbers, but deletion of *Erk1/2* together exaggerate the phenotype (Imamura et al., 2010). These data indicate that gliogenesis is sensitive to *Erk1/2* dosage. More recent data postulates a context-dependent role for ERK signaling. During neurogenesis, ERK-BMP7-GLI3R signaling actively creates a positive feedback loop by antagonizing sonic hedgehog signaling (Sun et al., 2024). In the late stages of development, p-ERK and GLI3 co-bind to promoters and enhancers to activate transcription of pro-gliogenic genes, like *Egfr* and *Olig2*, and activate *Ccnd1/2* and *Cdk4/6* (Gao et al., 2025). Taken together, these studies have explored the role of ERKs in cortical development and revealed that they are crucial for regulating both the neurogenic and gliogenic developmental programs in mammals. Their precise roles, however, remain unclear due to conflicting data and lack of single-cell resolution in prior studies.

5.3 *Pten* in Astrocyte Development and Disease

As previously introduced in this thesis, PTEN plays significant roles in stem cell renewal and maintenance in cortical progenitors (Ginn-Pease & Eng, 2003; Groszer et al., 2006; Massague, 2004), but less is known about PTEN function in cortical gliogenesis. *Pten* is a difficult gene to study in cortical RGPs using conventional mouse cKO models, as *Pten* cKO using *Nestin-Cre* and *Emx1-Cre* drivers in mice results in perinatal lethality at approximately P0 and P10, respectively (Chen et al., 2015; Groszer et al., 2001). As such, it is hard to follow up on the role of *Pten* in gliogenesis. Many studies which have explored the role of *Pten* in cortical cell types did so using drivers that target postnatal neurons, like *NEX-Cre* (Kazdoba et al., 2012). A more recent study found only moderate increases in S100 β ⁺ and OLIG2⁺ glial cell numbers in the cortices of *Pten* heterozygous mice, possibly indicating *Pten* dosage sensitivity (Chen et al., 2015). Chen and colleagues could not address the molecular mechanism likely responsible for the over production of glial cell types in their mouse model. Does *Pten* influence the decision to transition to gliogenesis? How is the quantitative glial output of an RGP reined in by functional PTEN during gliogenesis?

Pten mutations have not only been implicated in glial development, but also in abnormal expansions of glial populations in adult *Pten* mouse models as well as in humans. Previous reports indicate that *PTEN* mutations can facilitate the formation of astrocytomas (Furnari et al., 1997; Kwon et al., 2008). They report that even *PTEN* haploinsufficiency significantly increased the development of grade 3 astrocytomas (Kwon et al., 2008) and another group found that PTEN's phosphatase activity is required for growth-suppression in *in vitro* glioma cell lines (Furnari et al., 1997). Indeed, *Gfap-Cre* mediated ablation of *Pten* in adult mouse astrocytes results in aberrant proliferation (Fraser et al., 2004). Even though the link between *Pten* mutations and glial tumors is well established, *Pten*'s precise role is not well understood. It is for these reasons that it is essential to understand how *Pten* influences gliogenesis at the single progenitor level during cortical development.

5.4 Results

5.4.1 Experimental MADM Breedings for Analyzing the Role of *Pten* in Gliogenesis

To dissect *Pten*'s cell-autonomous and non-cell-autonomous functions during gliogenesis, I generated one additional genotype to the ones previously discussed in section 4.5.1 of this thesis, the cKO-*Pten*-MADM mouse model. I quantified a variety of phenotypic characteristics

in Control-MADM ($\text{MADM-19}^{GT/TG}; \text{Emx1}^{Cre/+}$), *Pten*-MADM ($\text{MADM-19}^{GT/TG, Pten-flox}; \text{Emx1}^{Cre/+}$), and cKO-*Pten*-MADM ($\text{MADM-19}^{GT, Pten-flox/TG, Pten-flox}; \text{Emx1}^{Cre/+}$) (Figure 20A-C). Mice from all three genotypes could be generated through one single breeding paradigm (Figure 20D, bottom row) and littermates were used when available. Experimental mice used in this chapter range in age from P0 to P28 and ages are explicitly stated in each analysis. The three genotypes used here present us with the opportunity to subtract the phenotype observed in cKO-*Pten*-MADM from that of *Pten*-MADM mutant cells, to parse out the precise cell-autonomous and non-cell-autonomous functions of *Pten* in gliogenesis at the single cell level.

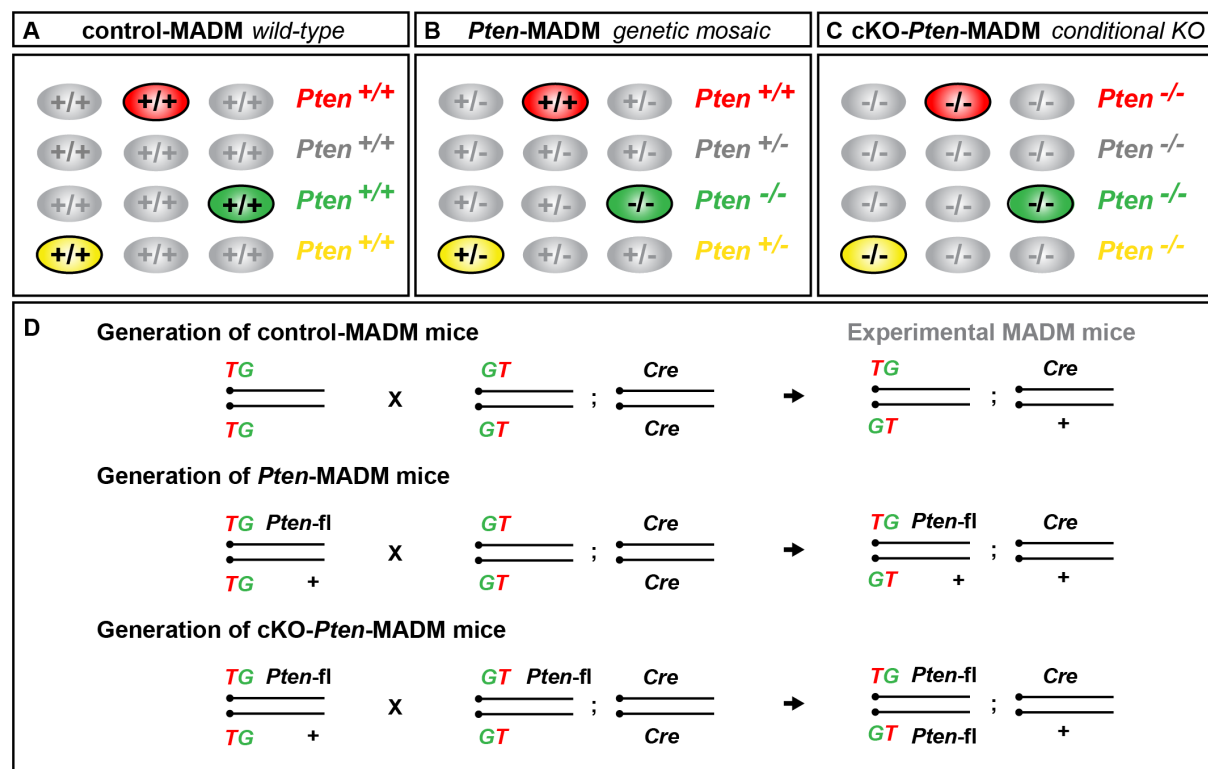


Figure 20. Experimental paradigms to study *Pten* at single cell resolution using MADM.

(A) Control-MADM brains contain sparse labeling without additional genetic manipulation. All cells in the tissue are control for the GOI.

(B) *Pten*-MADM experimental brains are sparse genetic mosaics. GFP⁺ cells carry *Pten*^{-/-} alleles, while tdTomato⁺ cells carry *Pten*^{+/+} alleles. They therefore serve as additional internal controls.

(C) cKO-*Pten*-MADM experimental brains are conditional knockouts for *Pten* in the *Emx1* lineage. All cells, including GFP⁺ and tdT⁺ cells, carry *Pten*^{-/-} alleles.

(D) The breeding schemes for all respective genotypes. Although control-MADM, *Pten*-MADM, and cKO-*Pten*-MADM mice can be generated through dedicated breeding pairs, most control-MADM and *Pten*-MADM mice in this study were generated from the cKO- *Pten*-MADM breeding pairs.

5.4.2 *Pten* Mutation Expands Glial Production in the Mouse Neocortex

A small proportion of the RGP's remaining after neurogenesis must undergo a switch from neurogenesis to gliogenesis. I therefore aimed to study the role of *Pten* during gliogenesis. *Pten*-MADM mice survive well into adulthood, providing an ideal opportunity to quantify the output of *Pten*^{-/-} progenitors during gliogenesis. We utilized Control-MADM (Figure 21A-B) and *Pten*-MADM (Figure 21D-E) to quantify the astrocyte population at P21, cKO-*Pten*-MADM mice do not survive and are therefore not included in the P21 analysis. Astrocytes are easily distinguishable from their morphology alone, and no astrocyte markers were used in these quantifications.

Whereas Control-MADM showed a G/R ratio of approximately 1 (SEM±0.06156) (Figure 22C), we found a 5-fold cell-autonomous increase (SEM±0.3856) in *Pten*-mutant astrocytes in our *Pten*-MADM mice (Figure 22F). I then sought to determine whether the excess astrocytes were a result from an expanded astrocyte progenitor pool. Astrocytes arise from a population of aIPs, which can be labeled with brain lipid-binding protein (BLBP) (Feng et al., 1994). Therefore, I performed a time course from P0 to P7 in all three experimental genotypes (Figure 22A-C), to determine whether aIP abundance was also increased within the *Pten* mutant population. I found significant increases in GFP+BLBP+ mutant cells in the cortex of both *Pten*-MADM (0.03476, SEM±0.00194) and cKO-*Pten*-MADM (0.03476, SEM±0.003501) brains compared to control (0.02267, SEM±0.0012) at P0 (Figure 22D). The BLBP+ aIP pool further increased in both *Pten*-MADM (0.07097, SEM±0.008941) and cKO-*Pten*-MADM (0.08631, SEM±0.01441) compared to control (0.03204, SEM±0.00175) at P7, indicating that the greater astrocyte numbers are, at least partially, the result of an enlarged aIP pool.

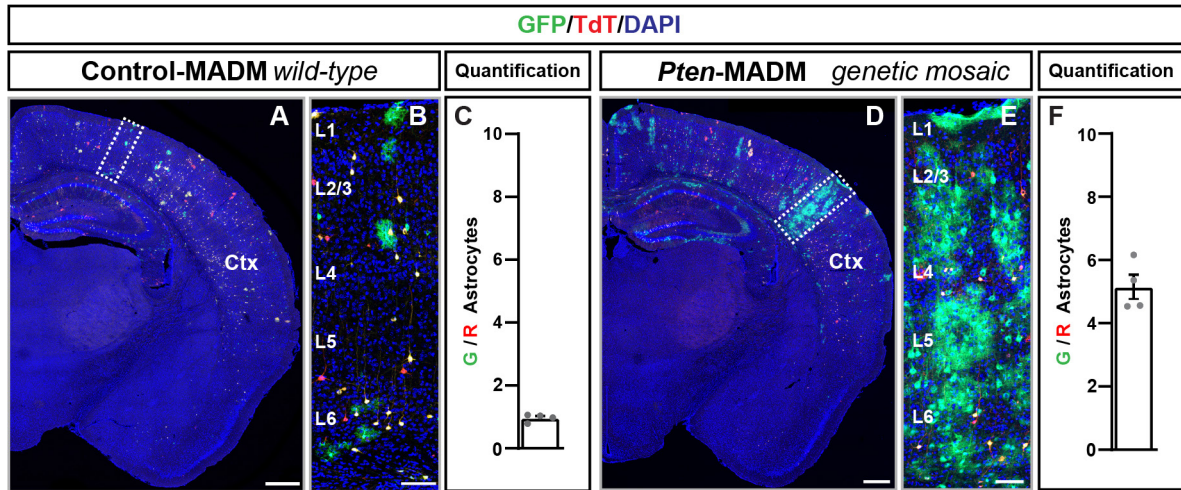


Figure 21. *Pten* deletion results in a cell-autonomous increase in the astrocyte population at P21.

(A-B) Confocal image of 35 μ m thick section of a *MADM-19^{GT/TG}; Emx1^{Cre/+}* hemisphere at 20x magnification. Scale bar indicates 500 μ m. Inset image of cortex of 35 μ m thick section of *MADM-19^{GT/TG}; Emx1^{Cre/+}*. Scale bar indicates 50 μ m.

(C) Quantification of the green to red ratio (G/R) of astrocytes in *MADM-19^{GT/TG}; Emx1^{Cre/+}* cortices, each dot indicates one animal (minimum of five sections counted per animal). Mean = 0.9675, bars show SEM $\pm$ 0.06156. Data shown is mean $\pm$ SEM. (Quantified by Ximena Contreras)

(D-E) Confocal image of 35 μ m thick section of a *MADM-19^{GT/TG,Pten-flox}; Emx1^{Cre/+}* hemisphere at 20x magnification. Scale bar indicates 500 μ m. Inset image of cortex of 35 μ m thick section of *MADM-19^{GT/TG,Pten-flox}; Emx1^{Cre/+}*. Scale bar indicates 50 μ m.

(F) Quantification of the green to red ratio (G/R) of astrocytes in *MADM-19^{GT/TG,Pten-flox}; Emx1^{Cre/+}* cortices, each dot indicates one animal (minimum of five sections counted per animal). G/R ratio is increased, indicating a larger GFP+ mutant astrocyte population. Mean = 5.155, bars show SEM $\pm$ 0.3856. Data shown is mean $\pm$ SEM. (Quantified by Ximena Contreras)

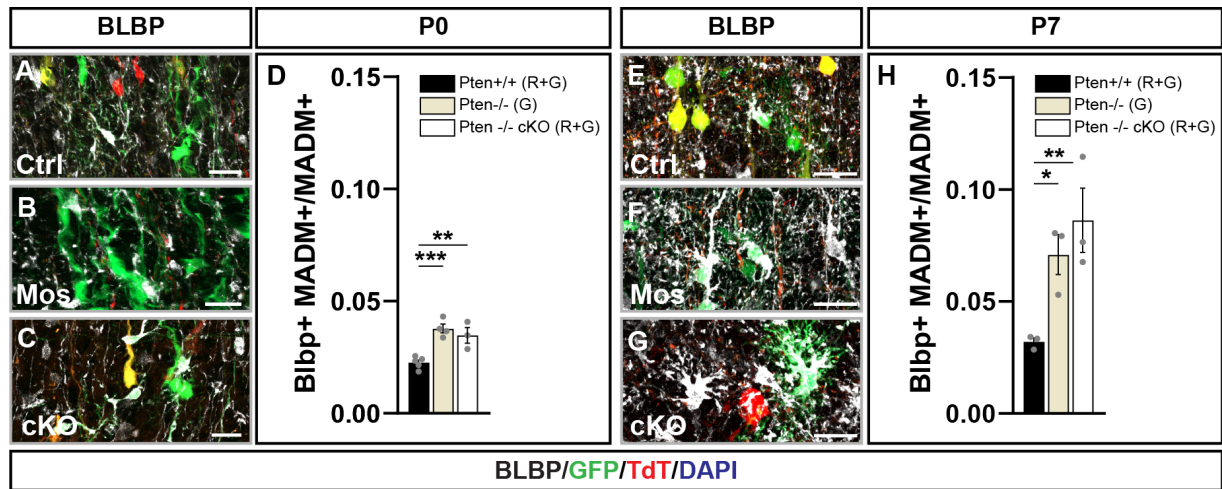


Figure 22. *Pten* cell-autonomously controls the astrocyte intermediate progenitor pool size.

(A-C) Images of BLBP+ aIPs in (A) Control-MADM, (B) *Pten*-MADM, and (C) cKO-*Pten*-MADM cortices at P0. Scale bar indicates 20 μ m.

(D) Quantification of BLBP+ double-positive cells outside of the VZ/SVZ at P0. Cells were quantified manually and divided by the total number of MADM+ cells to attain the fraction of positive cells. Control-MADM and cKO-*Pten*-MADM counts include both GFP+BLBP+ and tdTomato+BLBP+ cells given that all cells are the same genotype. Only GFP+ cells are shown for *Pten*-MADM. BLBP+ mutant cells from *Pten*-MADM (tan bar, mean = 0.03476, SEM $\pm$ 0.00194) and cKO-*Pten*-MADM (white bar, mean = 0.03476, SEM $\pm$ 0.003501) were significantly more abundant than in control (black bar, mean = 0.02267, SEM $\pm$ 0.0012). One-way Anova with multiple comparisons Control vs *Pten*-MADM adj. p-value = 0.0007. Control-MADM vs cKO-*Pten*-MADM adj. p-value = 0.0051. **p<0.01, ***p<0.001. Data shown is mean $\pm$ SEM.

(E-G) Images of BLBP+ aIPs in (A) Control-MADM, (B) *Pten*-MADM, and (C) cKO-*Pten*-MADM cortices at P7. Scale bar indicates 20 μ m.

(H) Quantification of BLBP+ double-positive cells outside of the VZ/SVZ at P7. Cells were quantified manually and divided by the total number of MADM+ cells to attain the fraction of positive cells. Control-MADM and cKO-*Pten*-MADM counts include both GFP+BLBP+ and tdTomato+BLBP+ cells given that all cells are the same genotype. Only GFP+ cells are shown for *Pten*-MADM. BLBP+ mutant cells from *Pten*-MADM (tan bar, mean = 0.07097, SEM $\pm$ 0.008941) and cKO-*Pten*-MADM (white bar, mean = 0.08631, SEM $\pm$ 0.01441) were significantly more abundant than in control (black bar, mean = 0.03204, SEM $\pm$ 0.001750). One-way Anova with multiple comparisons. Control vs *Pten*-MADM adj. p-value = 0.0306. Control-MADM vs cKO-*Pten*-MADM adj. p-value = 0.0091. *p<0.05, **p<0.01. Data shown is mean $\pm$ SEM.

5.4.3 *Pten* Deletion Prolongs the Gliogenic Period and Results in Larger Astrocyte Clones

Gliogenesis is reported to span only the first two postnatal weeks (Clavreul et al., 2019). I performed a time course analysis from P7 to 6 months to determine whether *Pten* deletion perturbed the gliogenic window. I found that the astrocyte population is relatively similar at P7 (Control-MADM: mean = 0.092, SEM $\pm$ 0.013, *Pten*-MADM: mean = 0.127, SEM $\pm$ 0.026), and begins to diverge thereafter (Figure 23A). From P14 (Control-MADM: mean = 0.150, SEM $\pm$ 0.011, *Pten*-MADM: mean = 0.206, SEM $\pm$ 0.011) onwards, the fraction of astrocytes within the overall cell population continued growing from the second to third postnatal weeks (Figure 23A), while the WT astrocyte population plateaued at the second postnatal week. Importantly, a two-way ANOVA analysis shows that there is a significant difference between the two conditions as a whole.

I also observed that *Pten*-mutant astrocytes were often found in close proximity to one another, at times resembling the columnar properties observed in neuron clones. We again took a clonal approach to determine whether RGPs were producing larger clones in the absence of *Pten*. To achieve this, pregnant control-MADM (MADM-19^{GT/TG}; *Emx1CreER*^{+/-}) and *Pten*-MADM (MADM-19^{GT/TG}; *Emx1CreER*^{+/-}) dams were injected with 2 mg tamoxifen at E12.5 to induce labeling of asymmetric clones (Figure 23B-C). I found that *Pten* mutant clones generated larger cohorts of astrocyte progeny at a significantly greater frequency than wild-type counterparts (Fig. 2R). These results suggest that *Pten* plays a crucial role, not only in determining the duration of the gliogenic window, but also in constraining glial output at the clonal level.

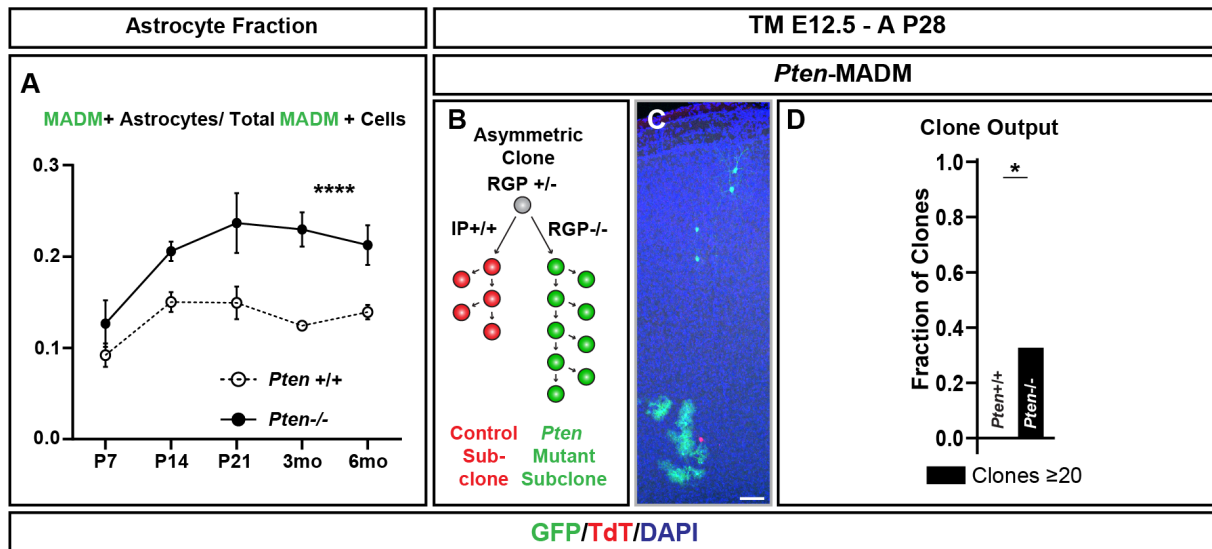


Figure 23. Perturbations to *Pten* expression lead to a prolonged gliogenic period as well as a skew toward larger astrocyte clones in the mouse cortex.

(A) Time course analysis of astrocyte abundance from P7 to 6 months of age. Astrocytes are similarly abundant in both conditions at P7, but begin to diverge at P14. P7: Control-MADM: 0.092, SEM±0.013, *Pten*-MADM: 0.127, SEM±0.026. P14: Control-MADM: 0.150, SEM±0.01, *Pten*-MADM: 0.206, SEM±0.011. P21: Control-MADM: 0.149, SEM±0.018, *Pten*-MADM: 0.237, SEM±0.033. 3mo: Control-MADM: 0.124, SEM±0.003, *Pten*-MADM: 0.230, SEM±0.019. 6mo: Control-MADM: 0.139, SEM±0.008, *Pten*-MADM: 0.212, SEM±0.022. Two-way Anova p-value = <0.0001. ****p<0.0001. Data shown is mean ± SEM.

(B) Astrocyte-containing asymmetric clones were analyzed. An asymmetric clone is defined as having a majority population greater than 4 neurons and a minority population less than or equal or 3 neurons.

(C) Representative image of an astrocyte-containing clone in a MADM-19^{GT/TG, Pten^{fl}}; *Emx1CreER*^{+/-} mouse injected at E12 and collected at P28. Scale bar represents approximately 50 μm.

(D) Quantification of large astrocyte clones (≥20 astrocytes). No large astrocyte clones were observed in the Control-MADM clonal cortices, while approximately 1/3 of all astrocyte clones had 20 or more astrocytes in the *Pten*-MADM clonal cortices. Fisher's exact test p-value = 0.0470, *p<0.05

5.4.4 *Pten*-Mutant Astrocytes are Morphologically Distinct Compared to Control

Given that mTOR is known to control cell size, and that *Pten*-mutant neurons become hypertrophic, I decided to also characterize the morphological changes of *Pten*-mutant astrocytes. Astrocytes from layers II/III were chosen for this portion of the study in order to minimize heterogeneity. I selected isolated astrocytes to facilitate an accurate morphological reconstruction and analysis, and these astrocytes were then imaged with a 63x oil-immersion objective. The z-stacks were then processed using Imaris software for 3D reconstruction. I observed altered astrocyte morphology in our *Pten*-MADM (Figure 24 C-D) model mice compared to Control-MADM mice (Figure 24 A-B). 3D reconstruction and Sholl analysis of *Pten* mutant astrocytes revealed a significantly more complex branching compared to control astrocytes (Figure 24E). In addition to altered branching complexity, I found that cytoplasmic volume was significantly increased in *Pten*-mutant astrocytes (mean = 15810, SEM $\pm$ 1459) compared to control (mean = 9337, SEM $\pm$ 836.2) (Figure 24F), similar to the neuronal hypertrophy associated with *Pten* mutations. *Pten*-mutant astrocytes (mean = 680.4, SEM $\pm$ 24.38) had nuclear volumes twice as large as control astrocytes (mean = 301.4, SEM $\pm$ 17.43) (Figure 24G). These data suggest that *Pten* is critical for astrocyte morphology and for constraining glial output during cortical development, the signaling pathways upon which *Pten* acts to achieve this however, remain unclear.

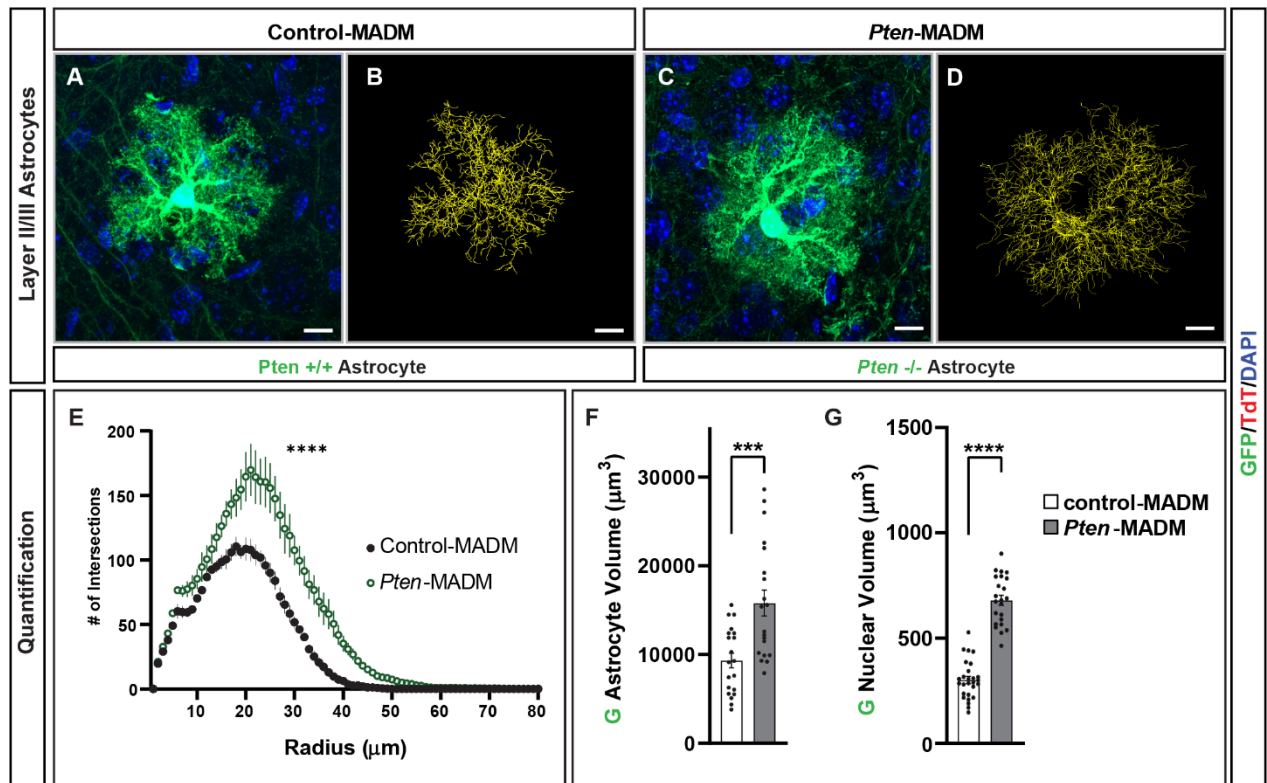


Figure 24. Astrocyte morphology is significantly altered by *Pten* deletion.

(A) Representative 63x image of a *Pten*^{+/+} astrocyte in layers II/III.

(B) Tracing of astrocyte processes using Imaris reconstruction.

(C) Representative 63x image of a *Pten*^{-/-} astrocyte in layers II/III.

(D) Tracing of *Pten*-mutant astrocyte processes using Imaris reconstruction.

(E) Sholl analysis using Imaris software. Data shown is mean ± SEM. Two-way anova p-value for genotype = <0.0001.

(F) Astrocyte cytoplasmic volume was measured using Imaris software. Control-MADM: mean = 9337, SEM±836.2, *Pten*-MADM: mean = 15810, SEM±1459. Unpaired two-tailed t-test: p-value = 0.0004. ***p<0.001. Data shown is mean ± SEM.

(G) Astrocyte nuclear volume was measured using Imaris software. Control-MADM: mean = 301.4, SEM±17.43, *Pten*-MADM: mean = 680.4, SEM±24.38. Unpaired two-tailed t-test: p-value = <0.0001. ****p<0.0001. Data shown is mean ± SEM.

5.4.5 Bulk-RNAseq of Isolated Astrocyte Population Reveals Deregulation in Key Signaling Pathways

As introduced earlier in this chapter, several signaling pathways are reportedly responsible for modulating the onset of gliogenesis (see section 5.2). In order to investigate the molecular changes which may underlie the astrocyte overproduction in *Pten* mutants, I isolated a highly pure astrocyte population from Control-MADM and *Pten*-MADM at P0 and P4 (Figure 25A). This was achieved by crossing our *Pten*-MADM mice with *lacZ* reporter mice (*XGFAP-lacZ*) (Brenner et al., 1994) that express *lacZ* under the control of the human *GFAP* promoter. Such an experimental set up allows us to specifically label cortically derived astrocytes (Amberg et al., 2024; Amberg et al., 2022; Laukotter, Amberg, et al., 2020). I then used FACS to sort and collect MADM-labeled astrocytes (GFP+*lacZ*+) from both Control-MADM and *Pten*-MADM at P0 and P4 (Figure 25A), neurons (GFP+*lacZ*-) were not used for this analysis. Samples from both conditions and time points were sent off for library preparation and Smart-seq2.

The *Pten-flox* allele in the *Pten*-MADM mouse model is not a complete gene deletion, instead only exon 5 is floxed out (Groszer et al., 2001). Therefore, I quantified the *Pten* exon 5 reads in the purified astrocyte samples. The analysis confirmed that *Pten* exon 5 levels were much lower in *Pten*^{-/-} cells compared to *Pten*^{+/+} cells at both P0 and P4 (Figure 25B). Principle component analysis (PCA) using the top 500 variable genes showed that P0 samples clustered by genotype, while P4 samples clustered in a more condensed manner, indicating that transcriptional differences were greater at P0 than at P4 (Figure 25C).

Quantification of differentially expressed genes (DEGs) concurred with the PCA plot, showing greater numbers of DEGs in *Pten* mutant cells at P0 than at P4 (Figure 26A). Despite being the same genotype, P0 and P4 mutant cells did not share many up- (Figure 26B) or down-regulated genes (Figure 26C), suggesting a significant change in the transcriptional landscape between these time points. Two interesting shared DEGs at P0 and P4 included *S100a1*, *Rgcc*, as up-regulated DEGs (Figure 26B). *S100a1* belongs to the same family of genes as the canonical astrocyte marker, *S100β*, while *Rgcc* is involved in cell cycle regulation. Shared down-regulated DEGs include *Ssbp2* and *Spint2*, both tumor suppressors, *Trim67*, which encodes a negative regulator of Ras signaling. P0 astrocytes up-regulated many genes involved in cell cycle regulation (Figure 27A) as well as pro-gliogenic genes (Figure 27B). Both plots contain various genes involved in MAPK signaling (*Pea15a*, *Fgfr1/3*, and *Etv5*), as well as genes downstream of JAK-STAT (*Gfap*) and NOTCH signaling (*Notch2* and *Hes5*). Gene

Ontology (GO)-term analysis of P0 DEGs indicated a significant overrepresentation of genes involved in progenitor proliferation and glial cell fate specification (Figure 27C) and downregulation of genes involved in various processes involved in neurogenesis (Figure 27D). Using the same pipeline as P0, I found deregulation of a handful of genes at P4 annotated as performing functions in progenitors (Figure 28A) as well as glial cell fate specification (Figure 28B). Interestingly, the overrepresented GO terms at P4 did not include progenitor proliferation terms like P0, instead many differentiation terms were present (Figure 28C).

I took the significant DEGs from both P0 and P4, added genes (yellow text) from the several central signaling pathways whose components were present in the DEGs (Figures 27A-B, 28A-B) and performed a STRING network analysis (Figure 29A). STRING network analysis is used to predict functional associations between proteins (von Mering et al., 2003). The P0 (Figure 29B) and P4 (Figure 29C) networks are strikingly different. One key difference is the presence of a cluster of DEGs involved in cell cycle regulation (cluster headed by *Mki67*) in P0 (Figure 29B). This cluster was not found in P4 samples, indicating an acute deregulation of cell cycle genes at P0. According to a betweenness centrality test, *Mapk3* is very central to the P0 network, indicating it interacts with many genes in the network (Figure 29B yellow-green fill). Taken together, the bulk transcriptomic data presented in this section suggests an acute deregulation of key cell cycle genes in astrocyte progenitors present at P0. Finally, the STRING network analysis presented various possible targets, which may be involved in promoting the *Pten*-mutant astrocyte overproduction phenotype.

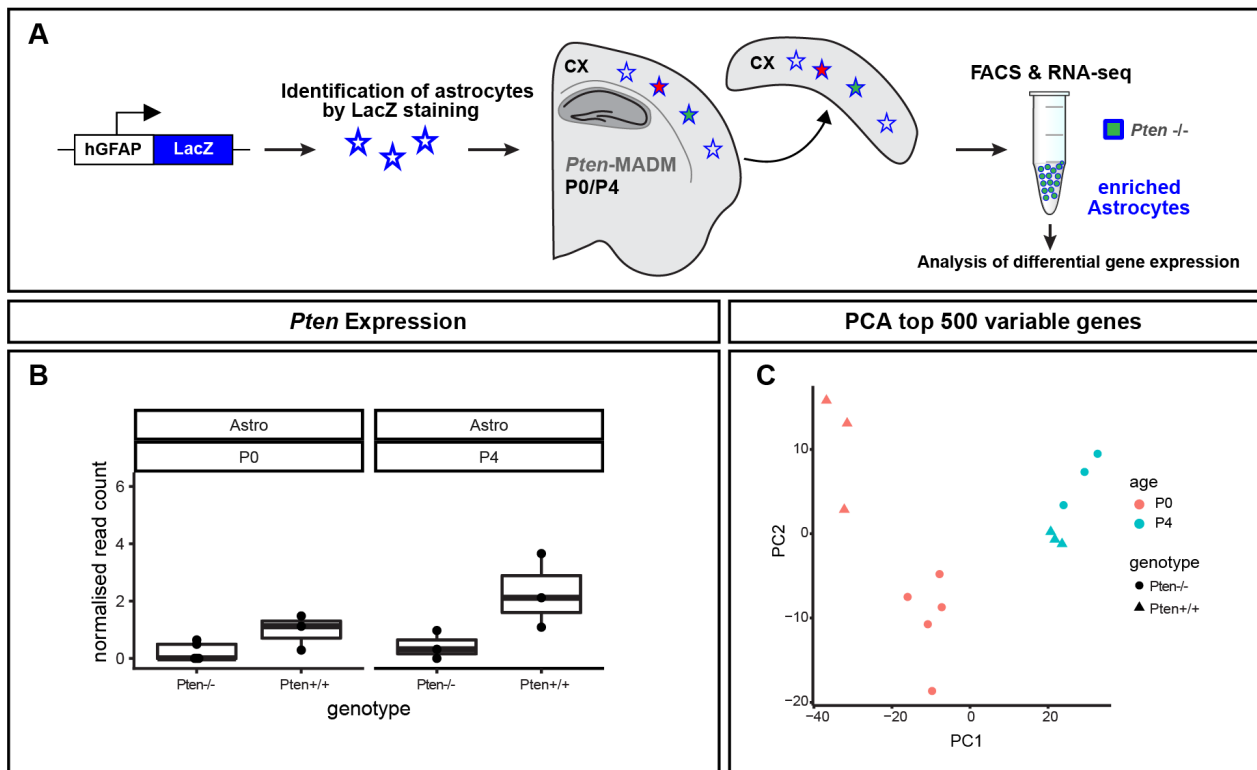


Figure 25. Labeling and isolation of highly purified astrocyte population.

(A) Experimental paradigm to isolate MADM-LacZ double-positive astrocyte population within the mouse cortex. MADM mice were crossed with mice carrying a *LacZ* gene under the control of *hGFAP* promoter. Mice were collected at P0 and P4, the cortex was isolated, and dissociated into a single-cell suspension. Cells were then sorted using FACS and sequenced.

(B) Normalized *Pten* expression at P0 and P4 in purified green astrocytes from control-MADM and *Pten*-MADM. Each dot represents a sample of pooled astrocytes (approximately 500 cells).

(C) PCA plot of control and *Pten*-mutant astrocyte samples at P0 and P4. P0 astrocytes cluster based upon genotype, indicating a significant difference between control and mutant. P4 astrocytes cluster closer together, hinting at a smaller change in the transcriptomic landscape. Each dot/triangle represents a sample of pooled astrocytes (approximately 500 cells).

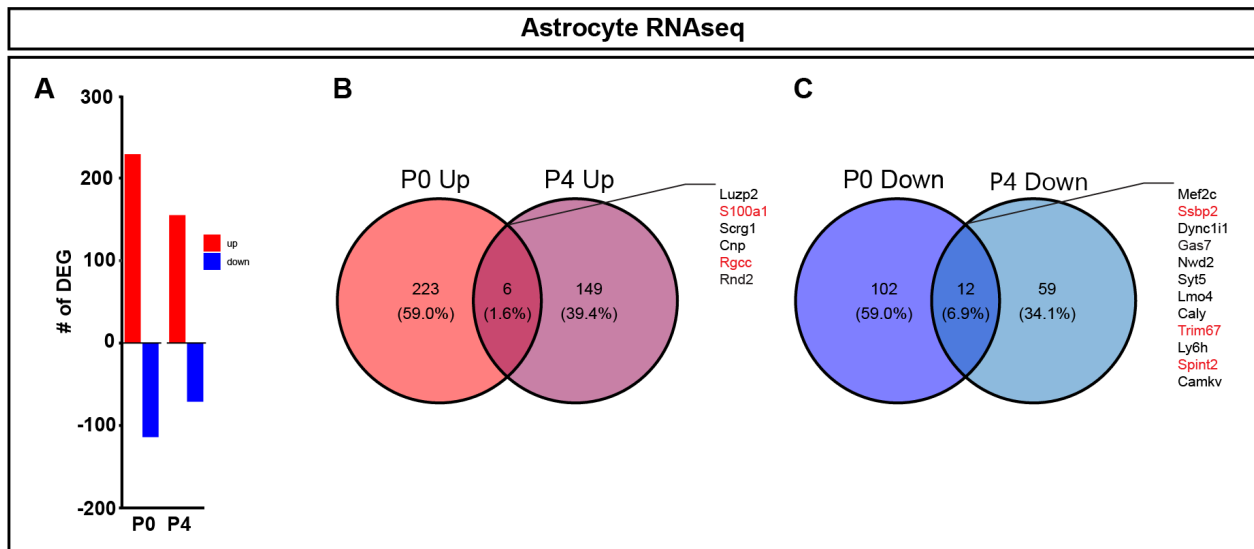


Figure 26. P0 and P4 astrocyte DEGs differ significantly between the two time points.

(A) Number of DEGs ($P_{\text{adj}} < 0.1$, DESeq2) upon comparison of *Pten*^{-/-} mutant (from *Pten*-MADM) cells to *Pten*^{+/+} cells in control-MADM at P0 and P4. “Up” indicates genes up-regulated in *Pten*^{-/-} mutant astrocytes, while “Down” refers to genes down-regulated in *Pten*^{-/-} mutant astrocytes when compared to *Pten*^{+/+} astrocytes.

(B-C) Overlap analysis of DEGs at P0 and P4. *Pten*^{-/-} mutant astrocytes share only a small minority of DEGs between time points. Analysis includes all DEGs from panel A. Numbers indicate number of DEGs, parenthesis indicate percent of genes.

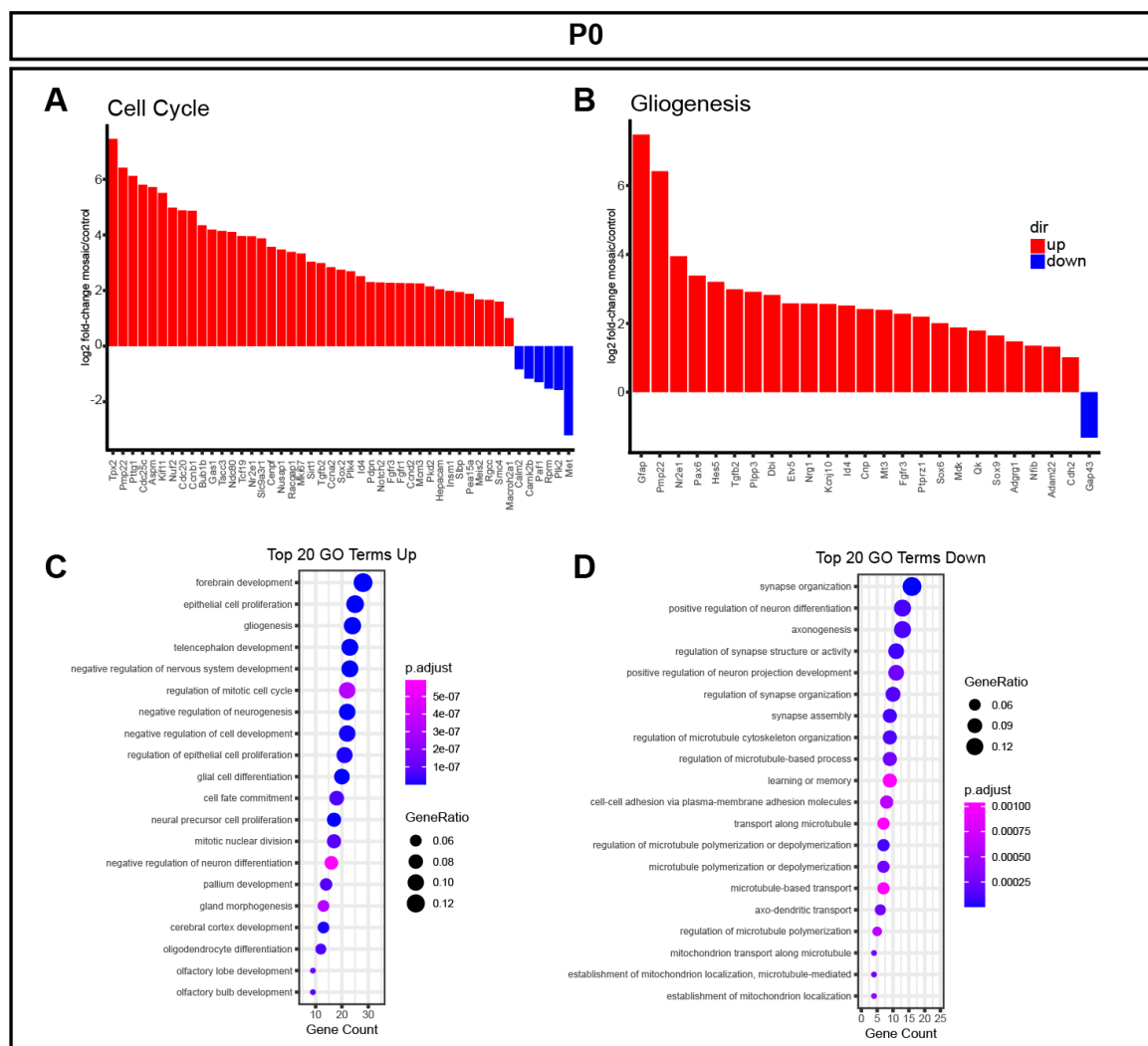


Figure 27. Prominent deregulation of cell cycle genes in *Pten*^{-/-} astrocytes at P0.

(A) Bar plot of cell cycle-related DEGs in P0 astrocytes according to GO annotation.

(B) Bar plot of gliogenesis-related DEGs in P0 astrocytes according to GO annotation.

(C-D) Dot plots of top 20 overrepresented and underrepresented GO terms for P0 astrocytes. Dot size shows the percent of genes within that term that were present. Color fill shows significance using the adjusted p-value.

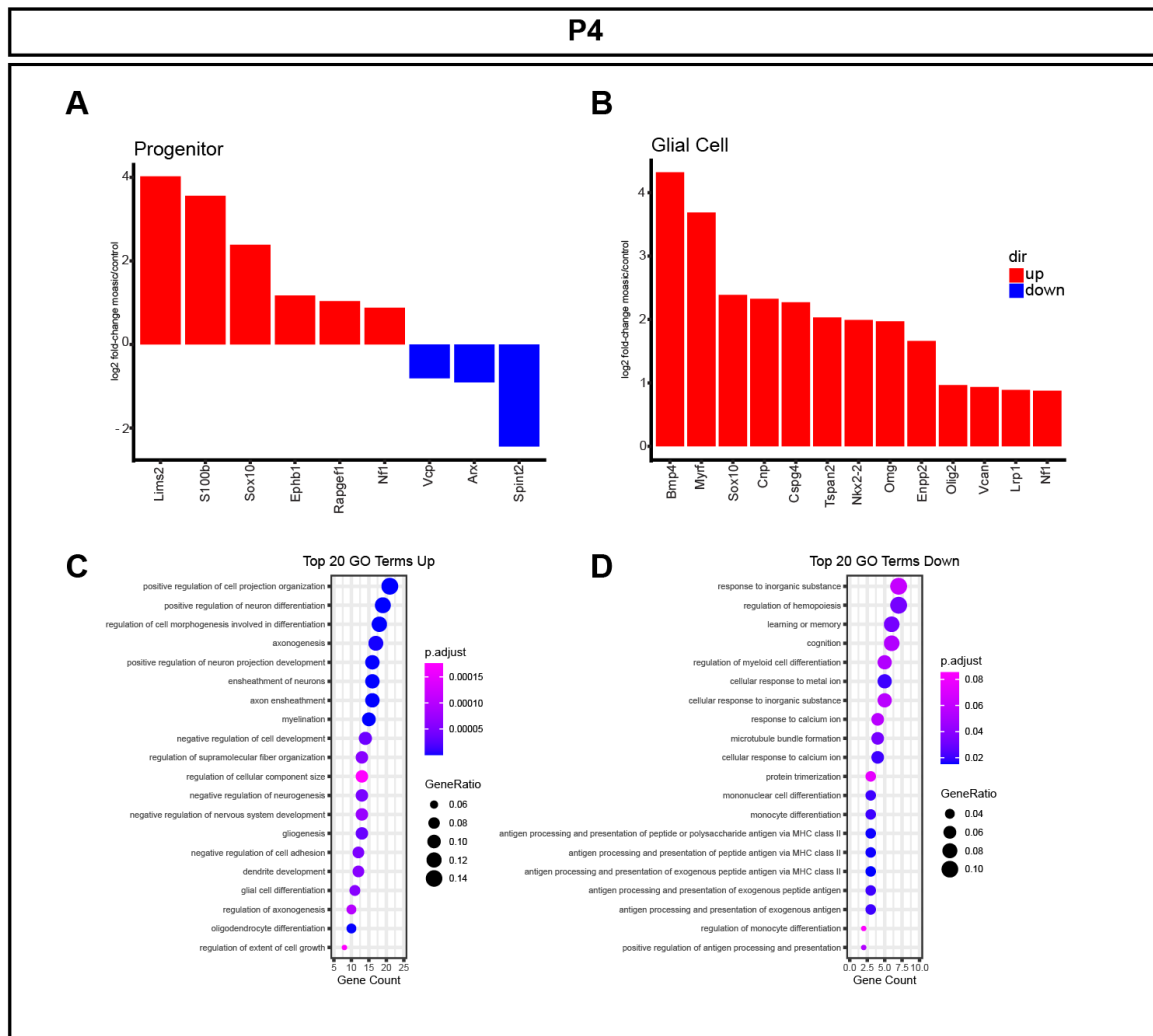
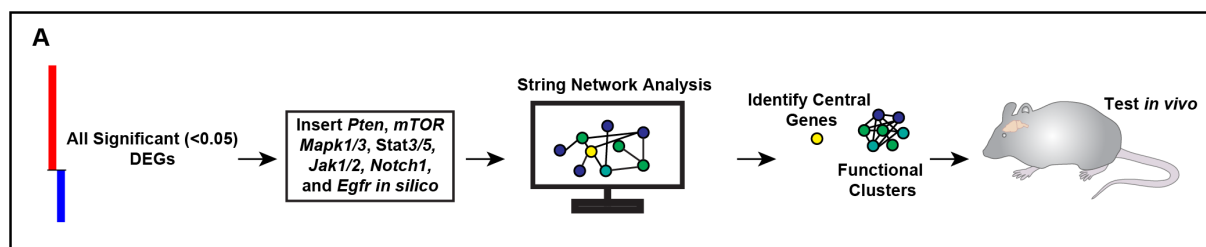


Figure 28. *Pten*^{-/-} astrocytes at P4 show only moderate transcriptional deregulation.

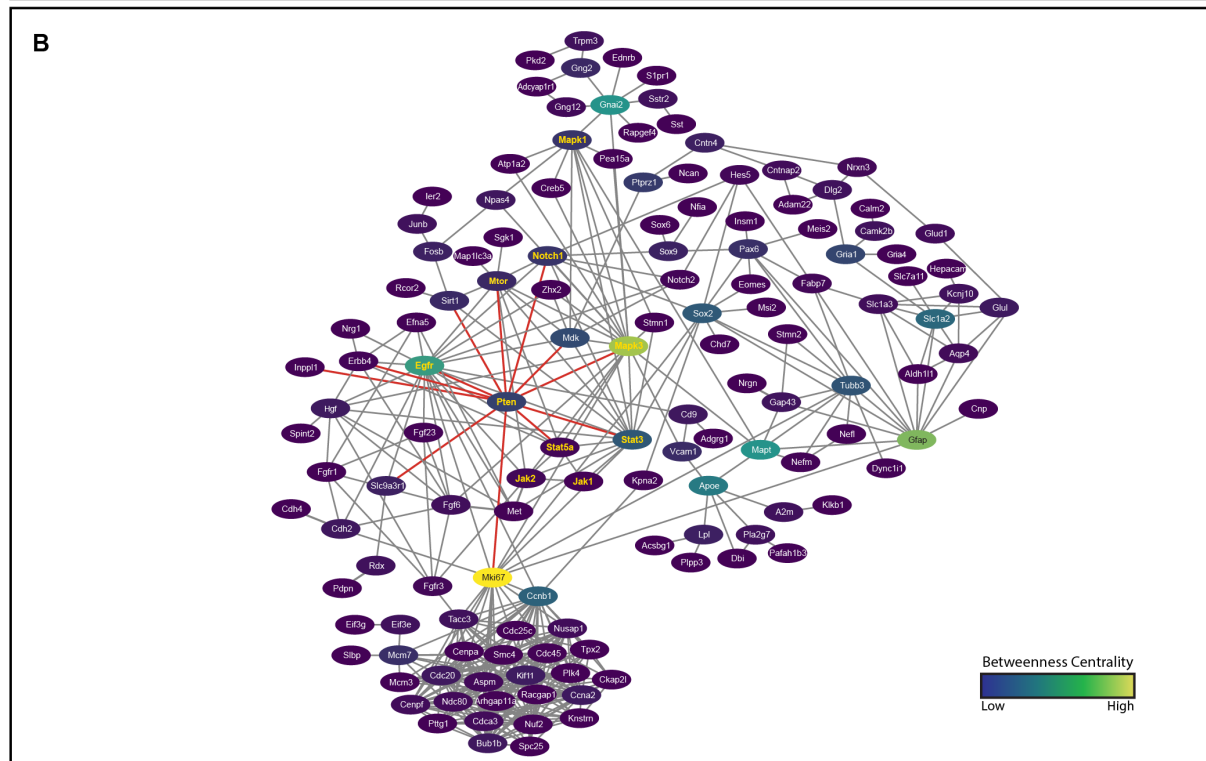
(A) Bar plot of progenitor-related DEGs in P4 astrocytes according to GO annotation.

(B) Bar plot of glial cell-related DEGs in P4 astrocytes according to GO annotation.

(C-D) Dot plots of top 20 overrepresented and underrepresented GO terms for P4 astrocytes. Dot size shows the percent of genes within that term that were present. Color fill shows significance using the adjusted p-value.



P0



P4

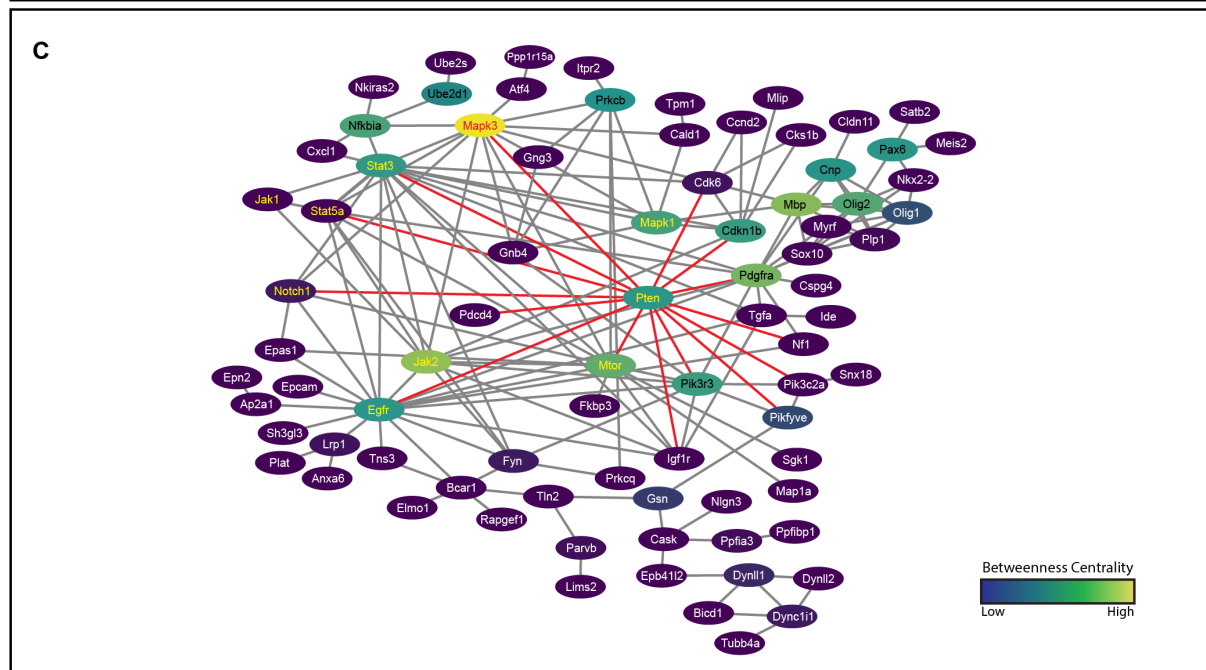


Figure 29. STRING network analysis reveals several central signaling pathways as possible mediators of *Pten*-mutant astrocyte overproliferation.

(A) Experimental setup for STRING network analysis. All genes with $P_{\text{adj}} < 0.05$, as well as a few additional genes of interest, were included in the STRING network input. The network was then used to generate potential targets for study *in vivo*.

(B) P0 STRING network shows a prominent clustering of cell cycle genes with *Mki67* as a highly central node in the network. *Mapk3*, *Gfap*, and *Egfr* are also highly central nodes in the network, though these genes were added *in silico*. Genes added *in silico* are marked with yellow font. Red edges indicate a first-degree neighbor of *Pten*.

(C) P4 STRING network differs greatly from the P0 network. *Mapk3*, *Jak2*, and *Mtor* are highly central nodes in the network, though these genes were added *in silico*. Genes added *in silico* are marked with yellow font (red for *Mapk3* due to fill). Red edges indicate a first-degree neighbor of *Pten*.

5.4.6 *Egfr* Requirement during Gliogenesis is overridden by *Pten* Deletion

Reports from our group, as well as from earlier studies, and the previous network analysis at P0/P4 indicate that *Egfr* is central to glial development (Beattie et al., 2017; Burrows et al., 1997; Sun et al., 2005; Zhang et al., 2020). I assessed whether the astrocyte overproduction observed in *Pten* mutant populations depends on functional *Egfr* by combining a *Pten*-MADM mosaic with an *Egfr* cKO model. To achieve this, parental matings were set up by the Preclinical Facility (PCF) to breed in an *Egfr*-flox allele (Lee & Threadgill, 2009) into mice carrying MADM-19 cassettes and a *Pten*-flox allele (Figure 30A). I then carried out the experimental breedings to generate 4 distinct genotypes (Figure 30B). Control-MADM (MADM-19^{GT/TG}; *Emx1*^{Cre/+}) and *Pten*-MADM (MADM-19^{GT/TG,Pten-flox}; *Emx1*^{Cre/+}) were previously described in the preceding chapter (Figure 13A-B, Figure 31A-B and C-D). Control-MADM-*Egfr*-cKO (MADM-19^{GT/TG,Pten-flox}; *Egfr*^{flox/flox}; *Emx1*^{Cre/+}) is in essence a traditional cKO, as all cells in the *Emx1* lineage lack *Egfr* (Figure 31E-F), with sparse MADM labeling in addition. Green cells in *Pten*-MADM-*Egfr*-cKO (MADM-19^{GT/TG,Pten-flox}; *Egfr*^{flox/flox}; *Emx1*^{Cre/+}) lack *Pten* and all cells in the *Emx1* lineage lack both *Egfr* alleles (Figure 31G-H).

Whereas in control-MADM animals I found the expected density of astrocytes, approximately 5/mm² (Figure 31I, black bar), there were few, if any, astrocytes upon ablation of *Egfr* alone (control-MADM-*Egfr*-cKO) (Figure 31I, orange bar), in line with previously published data from our group (Beattie et al., 2017). These data support claims that EGFR activity is inextricably linked to astrocyte production during normal cortical development. I proceeded to test the hypothesis by quantifying astrocyte density in *Pten*-MADM-*Egfr*-cKO. Strikingly, *Pten*-*Egfr* double knockout astrocytes were not only present, but also significantly increased in our *Pten*-MADM-*Egfr*-cKO model (Figure 31I, blue bar) compared to Control-MADM and Control-MADM-*Egfr*-cKO conditions. These data show the cell-autonomous *Pten* loss of function phenotype (i.e., excessive astrocyte production) is *Egfr*-independent.

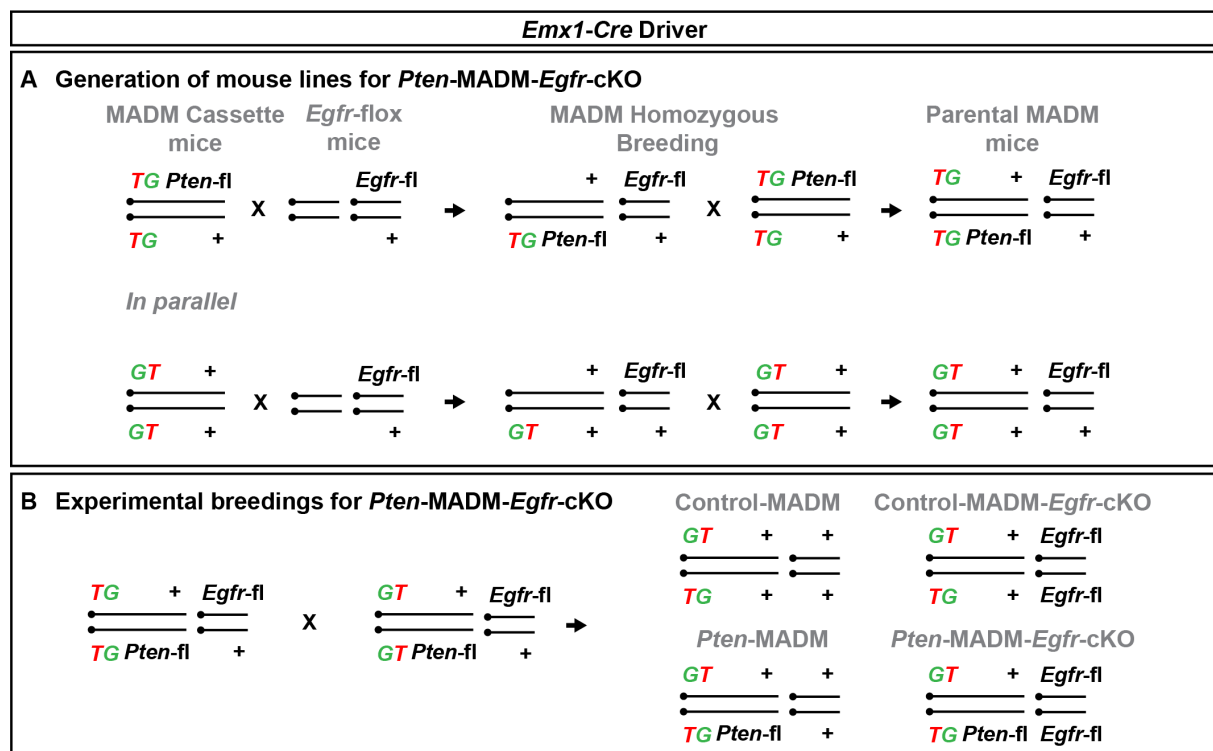


Figure 30. Breeding schemes test for *Egfr* dependence in *Pten* astrocyte phenotype.

(A) Parental breeding scheme. *Egfr*-flox mice were paired with a mate carrying MADM19-*TG*-*Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG*-*Pten* or *GT*) and *Egfr*-flox. F₁ were crossed with MADM19-*TG*-*Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(B) F₂ offspring carrying MADM cassettes and *Egfr*-flox alleles were crossed to generate all four experimental genotypes.

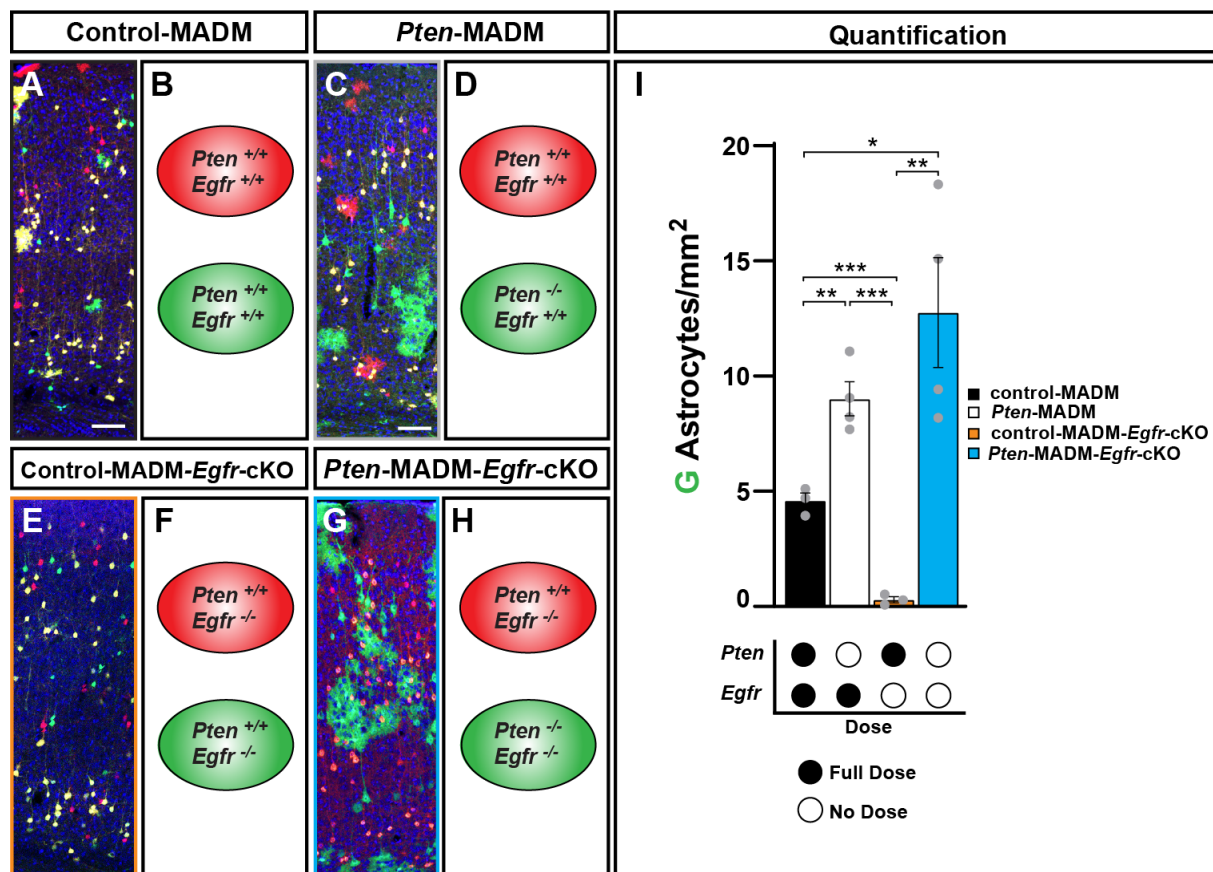


Figure 31. *Egfr* Requirement during Gliogenesis is overridden by *Pten* Deletion

(A, C, E, G) Confocal image 35 μm thick of Control-MADM, *Pten*-MADM, Control-MADM-*Egfr*-cKO, and *Pten*-MADM-*Egfr*-cKO, respectively. Scale bar cortex inset=50 μm .

(B, D, F, H) Cell genotype schemes representing the genotypes of cells within the 4 MADM paradigms, respectively.

(I) Quantification of astrocyte density in the somatosensory cortex of P21 mice of the indicated genotypes. Each dot represents one animal, with a minimum of 5 sections counted per animal. Control-MADM: mean = 4.587 astrocytes/mm², SEM $\pm$ 0.3433; *Pten*-MADM: mean = 9.010 astrocytes/mm², SEM $\pm$ 0.7414; Control-MADM-*Egfr*-cKO: mean = 0.2933 astrocytes/mm², SEM $\pm$ 0.1304; *Pten*-MADM-*Egfr*-cKO: mean = 12.76 astrocytes/mm², SEM $\pm$ 2.386. Multiple unpaired two-tailed t-tests. * p <0.05, ** p <0.01, *** p <0.001. Data shown is mean $\pm$ SEM.

5.4.7 *Pten* Deletion leads to Over-Activation of Key Signaling Pathways

I decided to take advantage of the cellular labeling MADM provides to conduct a set of single-cell RNA sequencing experiments to uncover the transcriptomic changes underlying the *Pten*-MADM astrocyte phenotype and its independence of EGFR requirement. I isolated cortical tissue from Control-MADM, *Pten*-MADM, and *Pten*-MADM-*Egfr*-cKO P0 mice, used FACS to collect GFP⁺ cells from each of the three genetic paradigms with biological replicates and subjected these samples to single cell RNA-Seq using particle-templated instant partition sequencing (PIP-seq) (Figure 32A).

After initial quality control and filtering, I retained comparable numbers of cells per sample for each MADM paradigm (Control-MADM: 704 cells/sample, *Pten*-MADM: 803 cells/sample, *Pten*-MADM-*Egfr*-cKO: 732 cells/sample) (Figure 32B). Standard quality parameters were comparable across all samples (Figure 32C-E). Uniform manifold approximation and projection (UMAP) did not identify noticeable batch effects (Figure 32F). As a first pass analysis I identified projection neuron and non-projection neuron cell-types (progenitors, macroglia and olfactory bulb neuroblasts) using unsupervised clustering in combination with expression analysis of canonical marker genes for projection neurons and non-projection neuron cell types (Figure 32G). Cells were initially categorized as either projection neuron or non-projection neuron (Figure 32H). Analysis of the relative abundance of these broad cell types identified an increase of non-projection neuron cell-types at the expense of projection neurons in *Pten*-MADM and *Pten*-MADM-*Egfr*-cKO compared to Control-MADM (Figure 32I).

To focus the analysis on glial cell types, I removed projection neurons and re-analysed the resulting data set. Partial regression of cell cycle state followed by UMAP, unsupervised clustering, marker expression (Figure 33A-B) and trajectory analyses (Figure 33C) confirmed that oligodendrocytes (Oligo), astrocyte intermediate progenitors (aIP), immature astrocytes (Astro) as well as the common progenitor of all of these cell types (aNSC) were present in all MADM paradigms. Visualizing relative cell type abundance identified significant differences between cell types and genetic paradigm (Figure 33D, ANOVA p-values for cell-type: 0.002 and genotype: 0.0002). To reduce noise, I combined cells per cell type and genotype and found significant increases for almost all cell types in *Pten*-MADM and *Pten*-MADM-*Egfr*-cKO compared to Control-MADM (chi-square test, Figure 33E).

Leveraging the power of the scRNA-Seq data, I sought to identify molecular correlates for the *Egfr* independence of the *Pten*-MADM phenotype. I hypothesized that transcriptomic

changes induced by loss of *Pten* in *Pten*-MADM, are further perturbed by the additional loss of *Egfr*, to allow for the sustained abnormal production of astrocytes in *Pten*-MADM-*Egfr*-cKO. To specifically identify these perturbations I directly compared *Pten*-MADM-*Egfr*-cKO/*Pten*-MADM and identified DEGs in the different cell types. Overall I identified 282 unique DEGs with the vast majority showing high cell type specificity (>92% show significant differential expression in 1-2 cell types only, Figure 33F-G). Finally, we performed Integrative Differential Expression and Gene Set Enrichment Analysis (iDEA) on the scRNA-Seq data to identify possible biological processes connected to these changes. Specifically I focused on a set of GO terms that included signaling cascades previously shown to be involved in *Pten* and/or *Egfr* activity (Figure 29). Besides the expected differences in the *Egfr* signaling, the analysis predicts specific perturbations of MAPK as well as JAK/STAT signaling upon combined loss of *Pten* and *Egfr* (Figure 33H). Taken together, the histological experiments provide evidence for an EGFR-independent gliogenic program in the cortex and based on the scRNA-seq data, I hypothesize that MAPK and/or JAK/STAT signaling are critical for gliogenesis in the absence of EGFR.

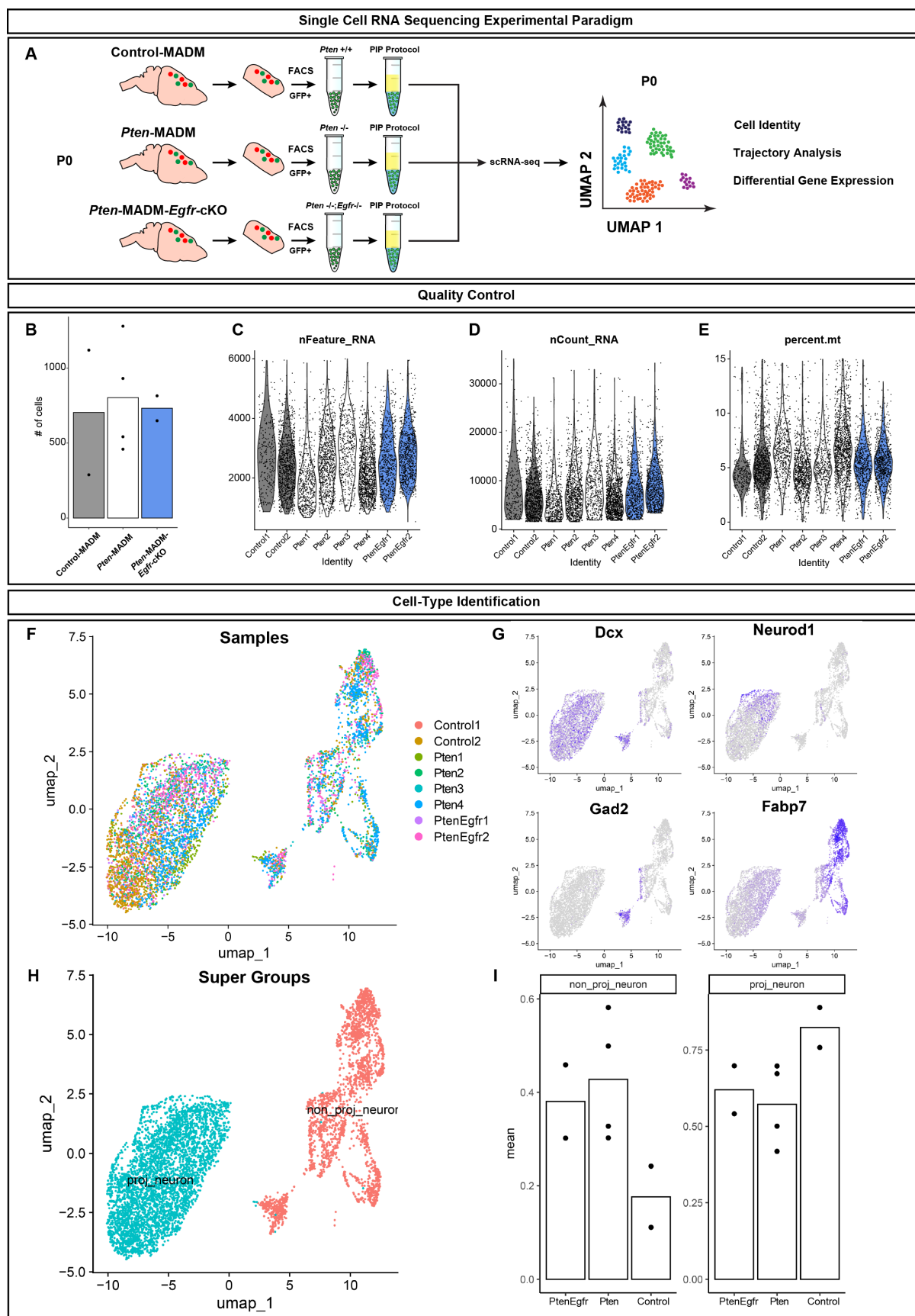


Figure 32. Deletion of *Pten* and *Egfr* shifts relative abundance of cell types toward non-projection neuron population.

(A) Schematic representation of experimental flow. Experimental mice were collected at P0, cortices were extracted and dissociated, and GFP⁺ cells from Control-MADM (3 cortices), *Pten*-MADM (5 cortices), *Pten*-MADM-*Egfr*-cKO (3 cortices) were collected using FACS. GFP⁺ cells were processed according to the PIP-seq protocol, libraries were prepared in-house, and subsequently sent off for sequencing. Single cell sequencing data was processed using the Seurat pipeline.

(B) All three genotypes had a similar average number of cells. Control-MADM: 704 cells/sample, *Pten*-MADM: 803 cells/sample, and *Pten*-MADM-*Egfr*-cKO: 732 cells/sample. Each dot represents one sample, each genotype had a single sample with two pooled cortices.

(C-E) Standard measures of data quality. (C) Total number of unique genes found per cell in each of the individual samples. (D) Number of RNA molecule reads per cell in each of the individual samples. (E) Percent of mitochondrial RNA in each of the individual samples. Mitochondrial RNA is a measure of cell health at the time of collection with lower values indicating higher quality. Each dot represents one individual cell.

(F) Collective UMAP of all cells regardless of genotype. Each sample is represented in one color according to the figure legend. Control-MADM = Control 1-2, *Pten*-MADM = Pten 1-4, *Pten*-MADM-*Egfr*-cKO = PtenEgfr 1-2.

(G) Markers for neurons (*Neurod1* and *Dcx*) were used to identify projection and non-projection neuron cell types.

(H) UMAP with Super Group overlay. “Super Groups” were defined as projection neurons and non-projection neuron cells.

(I) Relative abundance of super groups. Each dot represents a sample from each genotype. Data shown is mean.

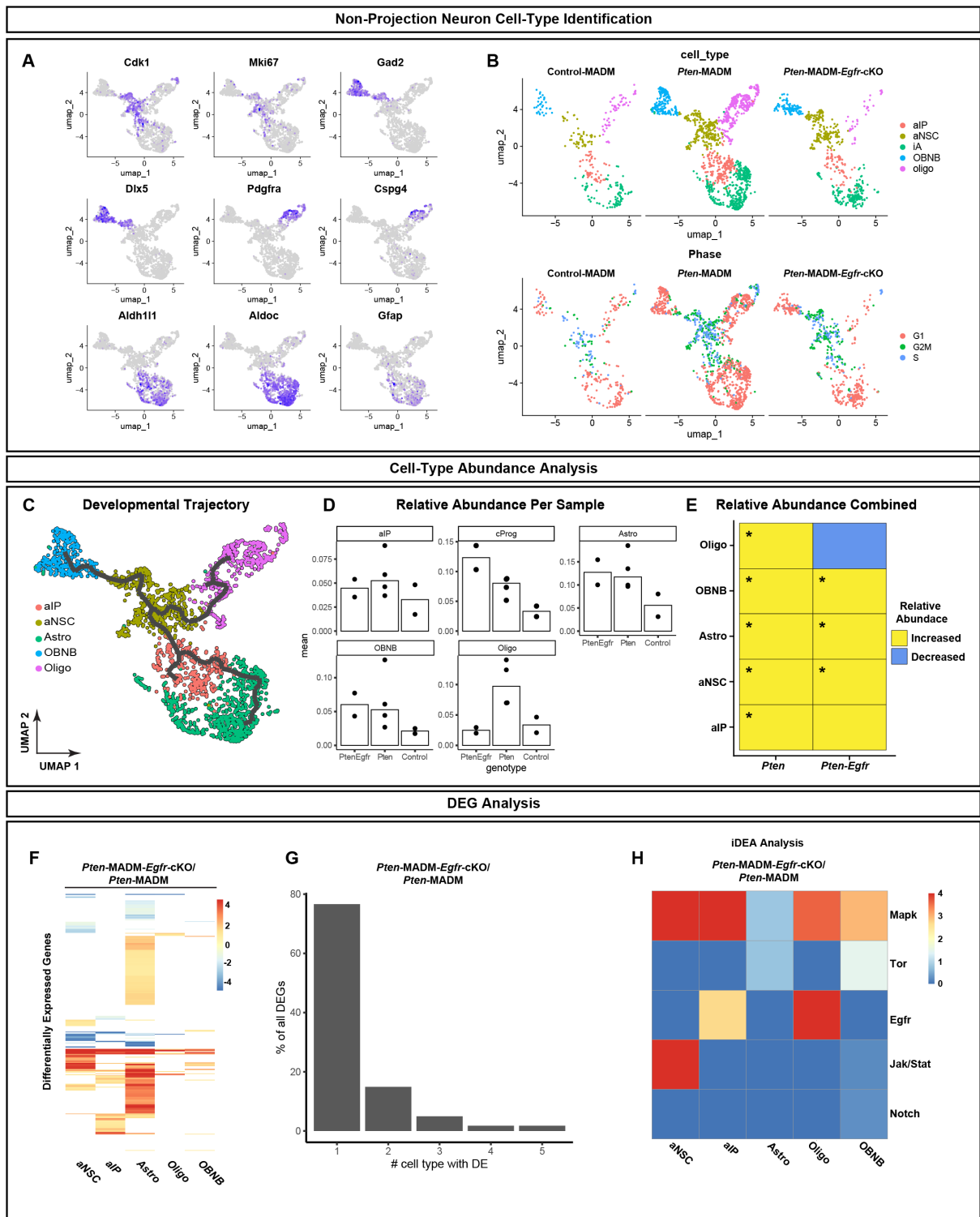


Figure 33. Genes from several critical pro-gliogenic signaling pathways are enriched in *Pten/Egfr* double mutant aIPs.

(A) Non-projection neuron cells were separated from projection neurons. Cell types were identified by using canonical marker genes for the respective cell type. Astrocytes were identified with *Aldoc*, *Aldh1l1*, and *Gfap*. aIPs were *Aldoc*, *Aldh1l1*, and *Mki67* positive. OBNBs express *Gad2* and *Dlx5*, and Oligos express *Cspg4* and *Pdgfra*. aNSCs were identified by their expression of *Mki67* and *Cdk1*.

(B) (Top) UMAP of combined samples per genotype. Each cell type was well represented within each genotype, despite lower numbers in Control-MADM and *Pten*-MADM-*Egfr*-cKO. (Bottom) UMAP showing cell cycle phase. Progenitors, both aNSCs and aIPs, are classified as cycling cells (G2M and S phases) while Astros, OBNBs, and Oligos were found to be postmitotic (G1 phase).

(C) Monocle3 trajectory analysis predicted that all cell types in the dataset originated from the aNSC population. UMAP includes all cells from all genotypes.

(D) Relative cell type abundance shows increases across cell types in both mutant conditions. Each dot represents one sample.

(E) Combined relative abundance data provides enough statistical power to determine significance. Observed significant increases in many cell types in both *Pten*-MADM and *Pten*-MADM-*Egfr*-cKO (yellow with asterisk). Chi-square test for significance. *Pten*-MADM/Control-MADM: OBNB $P_{adj} = 6.87018 \times 10^{-6}$, aIP $P_{adj} = 0.044$, aNSC $P_{adj} = 2.29426 \times 10^{-5}$, Astro $P_{adj} = 4.57553 \times 10^{-7}$, Oligo $P_{adj} = 8.47824 \times 10^{-12}$. *Pten*-MADM-*Egfr*-cKO/Control-MADM: OBNB $P_{adj} = 5.38823 \times 10^{-6}$, aIP $P_{adj} = 1.0$, aNSC $P_{adj} = 3.79944 \times 10^{-16}$, Astro $P_{adj} = 1.24599 \times 10^{-6}$, Oligo $P_{adj} = 0.225800815$. * indicates significance.

(F) Heatmap of up and down DEGs in *Pten*-MADM-*Egfr*-cKO compared to *Pten*-MADM. Score shows significance of up DEG (red) and down DEG (blue).

(G) Quantification of the frequency at which any single DEG appears across cell types. The vast majority (~77%) of DEGs in this analysis were unique to a single cell type.

(H) iDEA analysis of *Pten*-MADM-*Egfr*-cKO/*Pten*-MADM. The analysis indicates that terms related to MAPK signaling are significantly overrepresented across cell types in the *Pten*-MADM-*Egfr*-cKO samples. Score indicates significance (score of 2 = significance).

5.4.8 Deletion of *Jak/Stat* and *Notch* Signaling Components Does Not Rescue *Pten*-Mutant Astrocyte Phenotype

The PIP-seq experiment hinted at deregulation in several signaling pathways, including JAK/STAT and NOTCH. Previous studies also suggest crucial roles for JAK/STAT (Bonni et al., 1997; He et al., 2005) and NOTCH signaling in gliogenesis (Gao et al., 2025; Grandbarbe et al., 2003). To this end, I conducted a series of epistasis experiments, similar to the *Pten-Egfr* experiment found in section 5.4.6 of this thesis, with *Jak1*, *Jak2*, *Stat3*, *Stat5*, *Notch1* to test my hypothesis that the signaling cascades represented in the sequencing data are involved in mediating astrocyte over-proliferation in *Pten*-mutant conditions. Given the sheer number of experiments and genotypes, only the two most relevant conditions from each breeding are shown in the figure for clarity.

Similar to the previous experiment, I introduced a *Notch1*-flox allele into our MADM-19 mouse lines (Figure 34A). The F₂ mice were bred to generate a *Pten* mosaic within a *Notch1*-cKO background (Figure 34B). Due to preliminary results of ongoing experiments in the lab (Casado et al., unpublished), I expected a drop in the astrocyte density in the absence of *Notch1*. GFP⁺ astrocytes (*Pten*^{-/-}) in the *Pten*-MADM condition numbered approximately 8/mm² (Figure 39D, black bar). I observed that GFP⁺ astrocytes (*Pten*^{-/-}, *Notch1*^{-/-}) (Figure 39B-C) in *Pten*-MADM-*Notch1*-cKO (MADM-19^{GT/TG, Pten-flox}; *Notch1*^{flox/flox}; *Emx1*^{Cre/+}) cortices expanded significantly more than in *Pten*^{-/-} populations (Figure 39D, grey bar). These data not only show that *Notch1* deletion does not rescue the *Pten*-mutant over-proliferation phenotype, but also that synthetic effects seem to enhance proliferative capacity and in turn further increase astrocyte output.

Given that literature and our own sequencing analysis implicated JAK/STAT in gliogenesis, I next tested various components of the JAK/STAT signaling pathway. As previously described, I bred in *Stat3*-flox, *Stat5*-flox, *Jak1*-flox, and *Jak2*-flox alleles into our *Pten*-MADM lines (Figures 35-38, respectively). *Pten*-MADM-*Stat3*-cKO (MADM-19^{GT/TG, Pten-flox}; *Stat3*^{flox/flox}; *Emx1*^{Cre/+}) GFP⁺ astrocytes (*Pten*^{-/-}, *Stat3*^{-/-}) (Figure 39E-F) numbered approximately 7.7/mm², similar to *Pten*-mutant astrocytes in *Pten*-MADM (Figure 39G). Likewise, GFP⁺ astrocytes (*Pten*^{-/-}, *Stat5*^{-/-}) (Figure 39H-I) in *Pten*-MADM-*Stat5*-cKO cortices were equally abundant as those in *Pten*-MADM (Figure 39J). The two experiments clearly refute the hypothesis that individual STATs could be involved in promoting the astrocyte over-proliferation I observed in *Pten*-MADM mice, as I observed no rescue in either *Pten*-MADM-*Stat3*-cKO or *Pten*-MADM-*Stat5*-cKO.

I also tested both *Jak1/2* using a similar experimental setup, with some modifications. *Jak2* is on chromosome 19, the same chromosome as *Pten*, this presented a particularly interesting opportunity to test for a *Jak2* requirement at both the whole tissue (*Pten*-MADM-*Jak2*-cKO) and single-cell levels (*Pten*-*Jak2*-MADM). A drawback to *Jak2* being on chromosome 19 is that the only comparison we can make is to GFP⁺ astrocytes from Control-MADM, as it is highly unlikely to get a *Pten*-MADM mouse from these breedings. GFP⁺ and tdTomato⁺ cells in *Pten*-MADM-*Jak2*-cKO are both devoid of *Jak2*, along with the entire tissue, and differ only in *Pten* status (Figure 39K-L). In this paradigm, I found that GFP⁺ astrocytes (*Pten*^{-/-}, *Jak2*^{-/-}) still significantly outnumbered those in Control-MADM (Figure 39M). On the other hand, GFP⁺ and tdTomato⁺ cells in *Pten*-*Jak2*-MADM differ in both *Pten* and *Jak2* status. GFP⁺ cells lack both *Pten* **and** *Jak2* (*Pten*^{-/-}, *Jak2*^{-/-}), while tdTomato⁺ cells have intact alleles for both genes (*Pten*^{+/+}, *Jak2*^{+/+}) (Figure 39N-O). Here, I again find that the *Pten* phenotype dominates, indicating that *Jak2* is not required during gliogenesis (Figure 39P). *Jak1* deletion in combination with *Pten* also yielded negative results (*Pten*-MADM-*Jak1*-cKO) (Figure 39Q-S). For completeness, and to ensure there were no compensatory effects between *Jak1* and *Jak2*, I conducted a double-cKO experiment in conjunction with *Pten*-MADM (*Pten*-MADM-*Jak1/Jak2*-cKO). In this experiment GFP⁺ cells lack *Pten*, *Jak1*, **and** *Jak2* (*Pten*^{-/-}, *Jak1*^{-/-}, *Jak2*^{-/-}), while tdTomato⁺ cells have intact alleles for *Pten* but lack *Jak1*, **and** *Jak2* (*Pten*^{+/+}, *Jak1*^{-/-}, *Jak2*^{-/-}) (Figure 39T-U). Once again, I found a significantly higher density of GFP⁺ (*Pten*^{-/-}, *Jak1*^{-/-}, *Jak2*^{-/-}) astrocytes in the *Pten*-MADM-*Jak1/Jak2*-cKO than in Control-MADM (Figure 39V). Taken together, these analyses suggest that *Pten* mutant astrocytes escape requirements for key components of well-known pro-gliogenic signaling cascades.

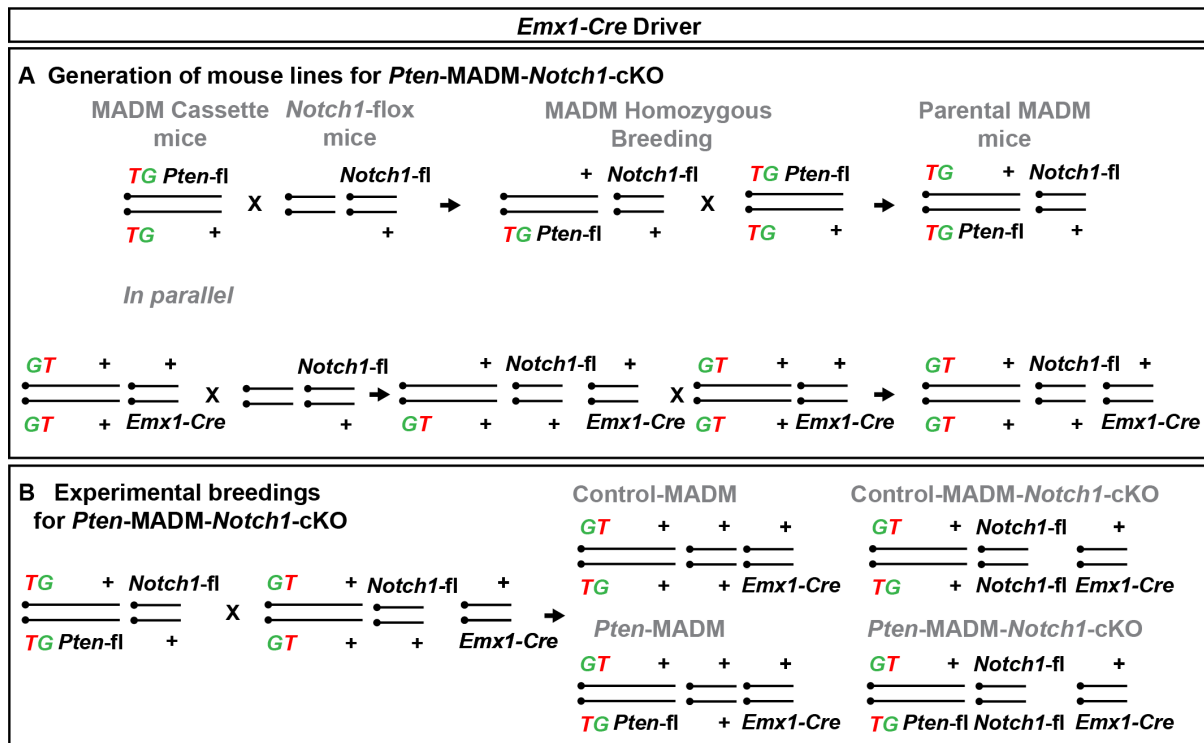


Figure 34. Breeding schemes test for *Notch* dependence in *Pten* astrocyte phenotype.

(A) Parental breeding scheme. *Notch*-floxed mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Notch*-floxed. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(B) F₂ offspring carrying MADM cassettes and *Notch*-floxed alleles were crossed to generate all four experimental genotypes.

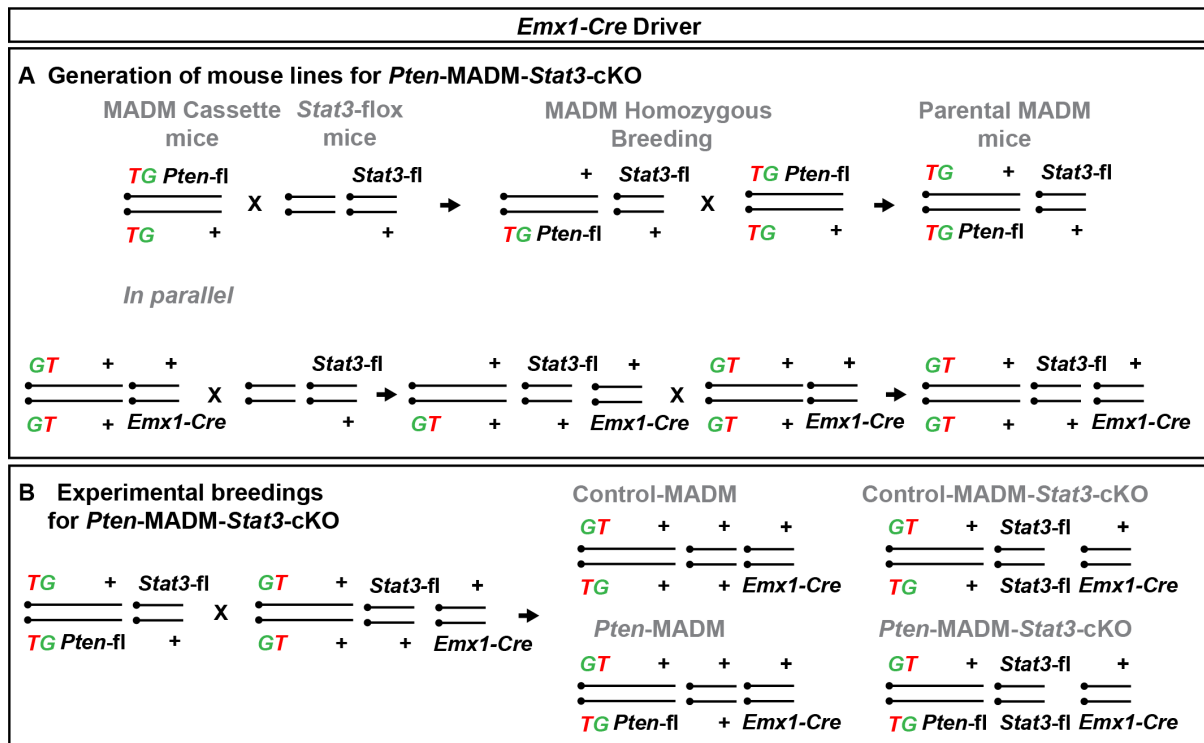


Figure 35. Breeding schemes test for *Stat3* dependence in *Pten* astrocyte phenotype.

(A) Parental breeding scheme. *Stat3*-flox mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Stat3*-flox. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(B) F₂ offspring carrying MADM cassettes and *Stat3*-flox alleles were crossed to generate all four experimental genotypes.

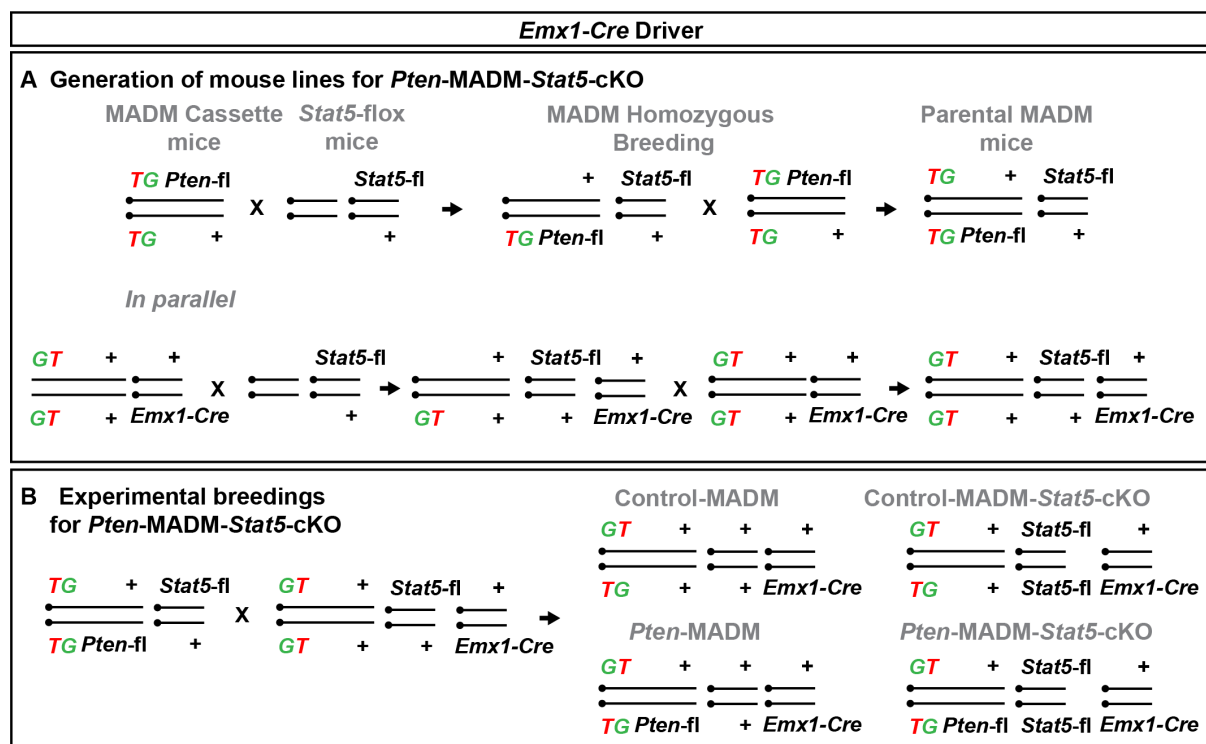


Figure 36. Breeding schemes test for *Stat5* dependence in *Pten* astrocyte phenotype.

(A) Parental breeding scheme. *Stat5*-flox mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Stat5*-flox. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(B) F₂ offspring carrying MADM cassettes and *Stat5*-flox alleles were crossed to generate all four experimental genotypes.

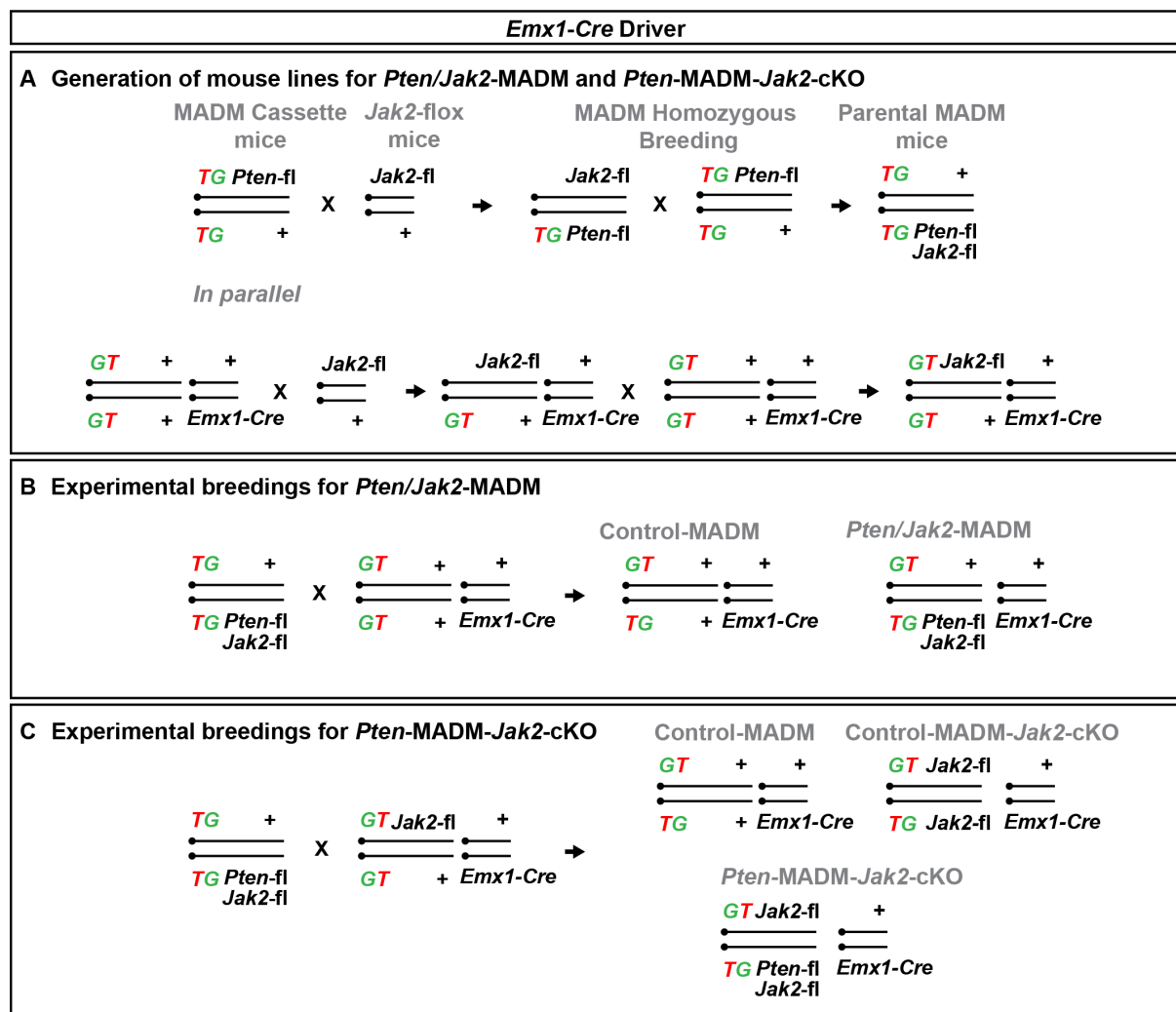


Figure 37. Breeding schemes test for *Jak2* dependence in *Pten* astrocyte phenotype.

(A) Parental breeding scheme. *Jak2*-flox mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Jak2*-flox. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(B) F₂ offspring carrying MADM cassettes and a *TG-Pten*-flox and *Jak2*-flox allele were crossed to generate the two experimental genotypes.

(C) F₂ offspring carrying MADM cassettes and *Jak2*-flox alleles were crossed to generate all four experimental genotypes.

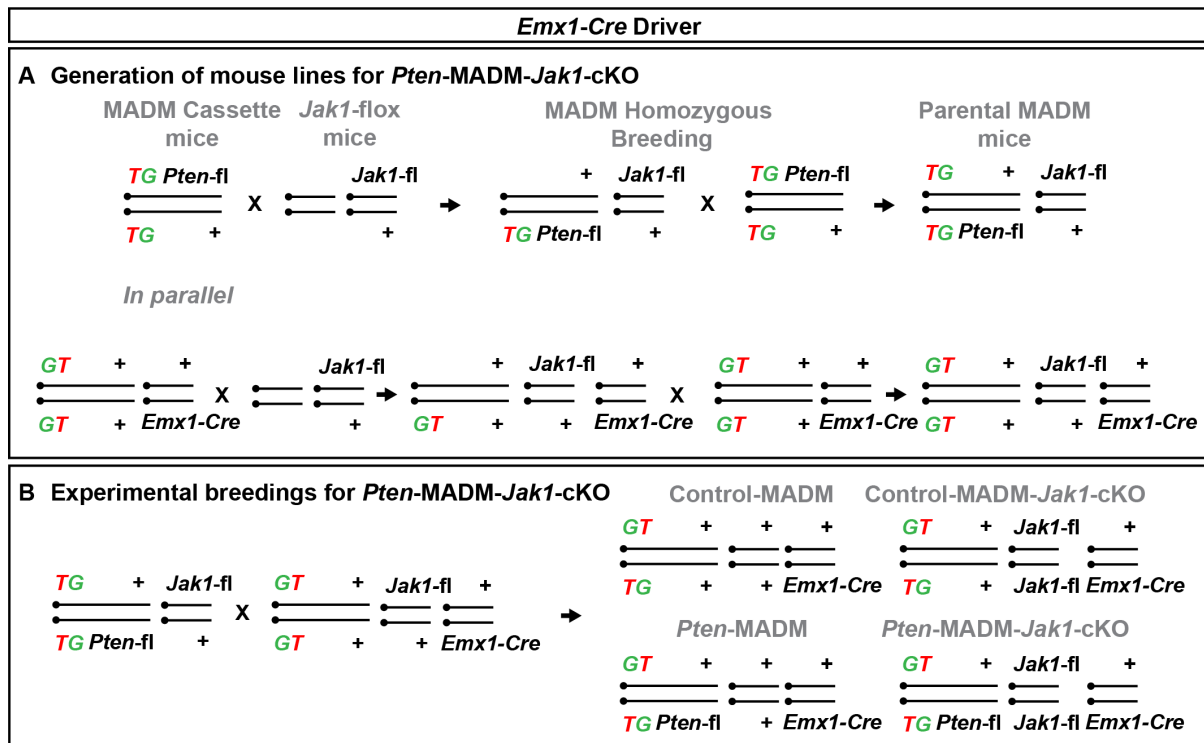


Figure 38. Breeding schemes test for *Jak1* dependence in *Pten* astrocyte phenotype.

(A) Parental breeding scheme. *Jak1*-floxed mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Jak1*-floxed. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(B) F₂ offspring carrying MADM cassettes and *Jak1*-floxed alleles were crossed to generate all four experimental genotypes.

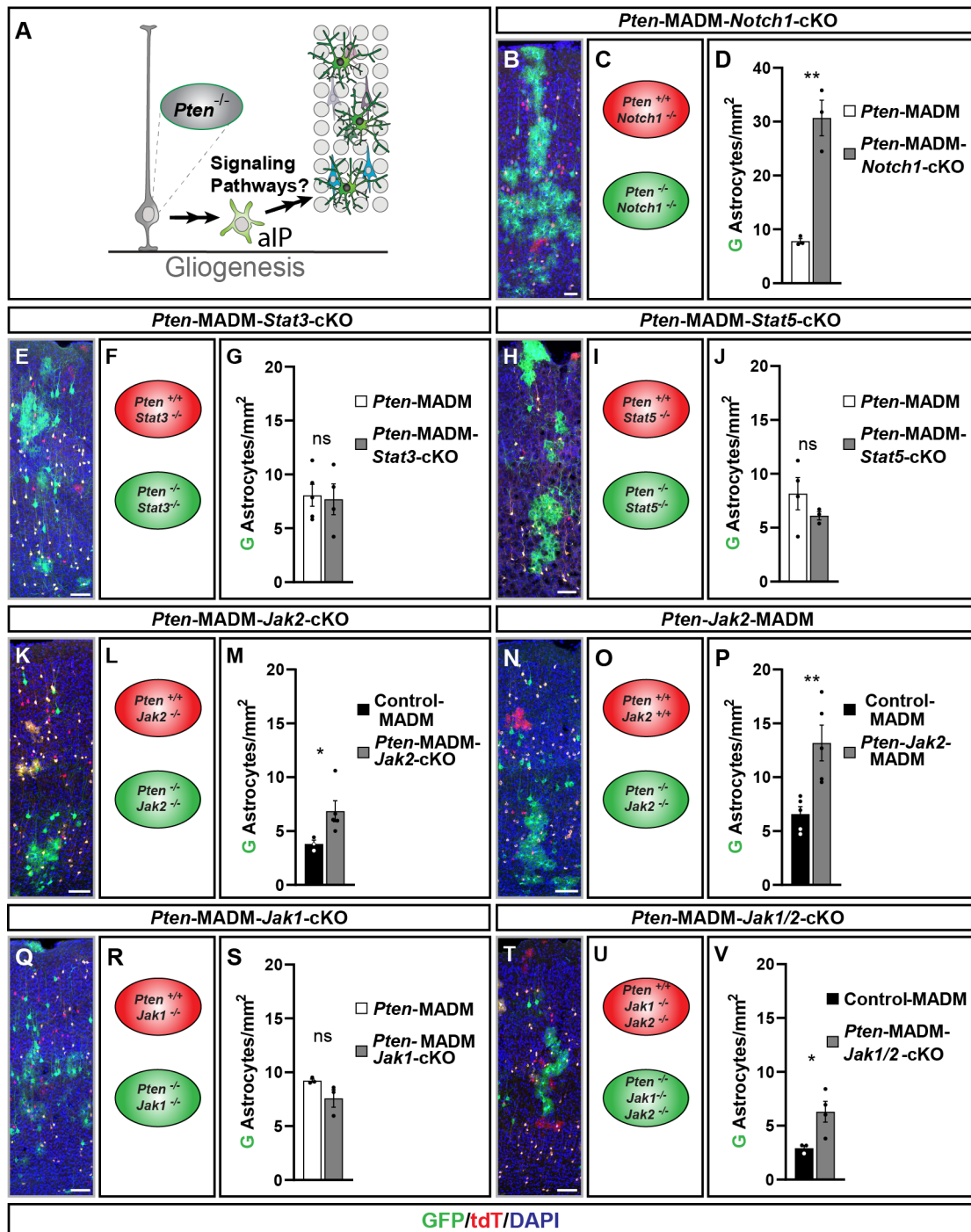


Figure 39. *Pten*-mutant astrocytes expand independently of Jak/Stat and Notch signaling.

(A) Diagram of logic behind the experimental design.

(B, E, H, K, N, Q, T) Confocal images 35 μ m thick of *Pten-MADM-Notch1-cKO*, *Pten-MADM-Stat3-cKO*, *Pten-MADM-Stat5-cKO*, *Pten-MADM-Jak2-cKO*, *Pten/Jak2-MADM*, *Pten-MADM-Jak1-cKO*, and *Pten-MADM-Jak1/2-cKO*, respectively. Scale bar cortex inset=50 μ m.

(C, F, I, L, O, R, U) Schematic representations of genotypes of tdTomato⁺ and GFP⁺ cells within *Pten*-MADM-*Notch1*-cKO, *Pten*-MADM-*Stat3*-cKO, *Pten*-MADM-*Stat5*-cKO, *Pten*-MADM-*Jak2*-cKO, *Pten/Jak2*-MADM, *Pten*-MADM-*Jak1*-cKO, and *Pten*-MADM-*Jak1/2*-cKO, respectively.

(D) Quantification of astrocyte density in the somatosensory cortex of P21 mice of *Pten*-MADM and *Pten*-MADM-*Notch1*-cKO. Each dot represents one animal, with a minimum of 5 sections counted per animal. *Pten*-MADM: mean = 7.825 astrocytes/mm², SEM±0.4883; *Pten*-MADM-*Notch1*-cKO: mean = 30.680 astrocytes/mm², SEM±3.315. Unpaired t-test p-value = 0.0024. **p<0.01.

(G) Quantification of astrocyte density in the somatosensory cortex of P21 mice of *Pten*-MADM and *Pten*-MADM-*Stat3*-cKO. Each dot represents one animal, with a minimum of 5 sections counted per animal. *Pten*-MADM: mean = 8.064 astrocytes/mm², SEM±1.021; *Pten*-MADM-*Stat3*-cKO: mean = 7.698 astrocytes/mm², SEM±1.433. Mann-Whitney test = 0.9048. ns = not significant.

(J) Quantification of astrocyte density in the somatosensory cortex of P21 mice of *Pten*-MADM and *Pten*-MADM-*Stat5*-cKO. Each dot represents one animal, with a minimum of 5 sections counted per animal. *Pten*-MADM: mean = 8.158 astrocytes/mm², SEM±1.494; *Pten*-MADM-*Stat5*-cKO: mean = 6.114 astrocytes/mm², SEM±0.3826. Welch's t-test = 0.2674.

(M) Quantification of astrocyte density in the somatosensory cortex of P21 mice of Control-MADM and *Pten*-MADM-*Jak2*-cKO. Each dot represents one animal, with a minimum of 5 sections counted per animal. Control-MADM: mean = 3.801 astrocytes/mm², SEM±0.3582; *Pten*-MADM-*Jak2*-cKO: mean = 6.852 astrocytes/mm², SEM±0.9736. Mann-Whitney test = 0.0357. *p<0.05.

(P) Quantification of astrocyte density in the somatosensory cortex of P21 mice of Control-MADM and *Pten/Jak2*-MADM. Each dot represents one animal, with a minimum of 5 sections counted per animal. Control-MADM: mean = 6.575 astrocytes/mm², SEM±0.6969; *Pten/Jak2*-MADM: mean = 13.18 astrocytes/mm², SEM±1.652. Mann-Whitney test = 0.0079. **p<0.001.

(S) Quantification of astrocyte density in the somatosensory cortex of P21 mice of *Pten*-MADM and *Pten*-MADM-*Jak1*-cKO. Each dot represents one animal, with a minimum of 5 sections counted per animal. *Pten*-MADM: mean = 9.228 astrocytes/mm², SEM±0.1522; *Pten/Jak2*-MADM: mean = 7.570 astrocytes/mm², SEM±0.8135. Mann-Whitney test = 0.1000. ns = not significant.

(V) Quantification of astrocyte density in the somatosensory cortex of P21 mice of Control-MADM and *Pten*-MADM-*Jak1/2*-cKO. Each dot represents one animal, with a minimum of 5 sections counted per animal. Control-MADM: mean = 2.916 astrocytes/mm², SEM±0.2483; *Pten*-MADM-*Jak1/2*-cKO: mean = 6.296 astrocytes/mm², SEM±0.9638. Unpaired t-test p-value = 0.0332. *p<0.05.

5.4.9 *Pten*-Mutant Astrocyte Expansion Depends on an Intact MAPK Signaling Pathway

The scRNA-seq experiment also suggested that the MAPK signaling pathway could be involved in promoting the expansion of the *Pten*-mutant astrocyte population. To this end, I generated *Pten* mosaics using MADM, within an *Erk1* or *Erk2* cKO tissue (Figure 40A-D). GFP⁺ and tdT⁺ cells in *Pten*-MADM-*Erk1*-cKO are devoid of *Erk1*, along with the entire tissue, and differ only in *Pten* status (Figure 41B-C). In this paradigm, I found that GFP⁺ astrocytes (*Pten*^{-/-}, *Erk1*^{-/-}) are present at similar densities as in *Pten*-MADM (Figure 41D). Similarly, when *Erk2* is ablated in combination with *Pten* (*Pten*-MADM-*Erk2*-cKO) (Figure 41E-F), I found no significant difference in the abundance of GFP⁺ astrocytes (*Pten*^{-/-}, *Erk2*^{-/-}) compared to *Pten*-MADM (Figure 41G). These results indicate that knockout of either *Erk1* or *Erk2* is not sufficient to rescue the over-proliferation found in *Pten*-mutant astrocyte populations.

I tested whether either *Erk* could compensate in the absence of the other. To achieve this, I had to breed in both *Erk1*-flox and *Erk2*-flox alleles into the established *Pten*-MADM parental lines (Figure 40E). Parental lines were then crossed to generate offspring, of which five genotypes were informative (Figure 40F). Control-MADM and *Pten*-MADM samples have been previously described in this thesis (Figure 41 H-K). *Pten*-MADM-*Erk1*-cKO-*Erk2*-het (Figure 41L-M) and *Pten*-MADM-*Erk1*-het-*Erk2*-cKO (Figure 41N-O) test whether a single dose of one *Erk* can compensate for full loss of the other (i.e. whether a single *Erk1* allele compensates for *Erk2* LOF and vice-versa). Interestingly, I find that *Pten*-MADM-*Erk1*-cKO-*Erk2*-het GFP⁺ astrocytes (*Pten*^{-/-}, *Erk1*^{-/-}, *Erk2*^{+/-}) number approximately 10/mm², and thus are not reduced compared to *Pten*-MADM (Figure 41S, orange bar vs white bar). On the other hand, GFP⁺ astrocytes (*Pten*^{-/-}, *Erk1*^{+/-}, *Erk2*^{-/-}) in *Pten*-MADM-*Erk1*-het-*Erk2*-cKO number approximately 3.6/mm², and are significantly reduced compared to both *Pten*-MADM and *Pten*-MADM-*Erk1*-cKO-*Erk2*-het (Figure 41S, blue bar). These data indicate that a single copy of *Erk2* can compensate for *Erk1*-LOF (Figure 41S, orange vs blue bar) and (2) the *Pten* astrocyte over-proliferation phenotype is sensitive to perturbations to *Erk1* and *Erk2*.

Finally, I quantified the triple-KO GFP⁺ astrocytes (*Pten*^{-/-}, *Erk1*^{-/-}, *Erk2*^{-/-}) in *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO. GFP⁺ astrocytes (*Pten*^{-/-}, *Erk1*^{-/-}, *Erk2*^{-/-}) in this paradigm number approximately 1.8/mm², a significant reduction compared to *Pten*-MADM (Figure 41R beige vs white bar). tdTomato⁺ (*Pten*^{+/+}, *Erk1*^{-/-}, *Erk2*^{-/-}) astrocytes in *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO were nearly absent, indicating that *Erk1/2* are essential for gliogenesis (Figure 41S beige bar). This conclusion is further supported when the neuron data are taken into account.

GFP⁺ neurons (*Pten*^{-/-}, *Erk1*^{-/-}, *Erk2*^{-/-}) in *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO did not differ significantly in abundance to neurons in *Pten*-MADM (Figure 41T), indicating a specific pro-gliogenic function for *Erk1/2*. In summary, the *Mapk/Pten* epistasis experiments reveal that proliferation of *Pten*-mutant astrocyte populations depends on intact MAPK signaling, which is essential for glionesis in “normal” *Pten*^{+/+} contexts.

Figure 40. Breeding schemes test for *Erk1* and *Erk2* dependence in *Pten* astrocyte phenotype.

(A) Parental breeding scheme. *Erk1*-flox mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Erk1*-flox. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(B) F₂ offspring carrying MADM cassettes and *Erk1*-flox alleles were crossed to generate all four experimental genotypes.

(C) Parental breeding scheme. *Erk2*-flox mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Erk2*-flox. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(D) F₂ offspring carrying MADM cassettes and *Erk2*-flox alleles were crossed to generate all four experimental genotypes.

(E) Parental breeding scheme. *Erk1/2*-flox mice were paired with a mate carrying MADM19-*TG-Pten* or MADM19-*GT* cassettes. The F₁ offspring were heterozygous for the respective MADM cassette (*TG-Pten* or *GT*) and *Erk1/2*-flox. F₁ were crossed with MADM19-*TG-Pten* and MADM19-*GT* to achieve MADM cassette homozygosity. The F₂ offspring were used for strain maintenance as well as experimental breedings.

(F) F₂ offspring carrying MADM cassettes and *Erk1/2*-flox alleles were crossed to generate all five experimental genotypes.

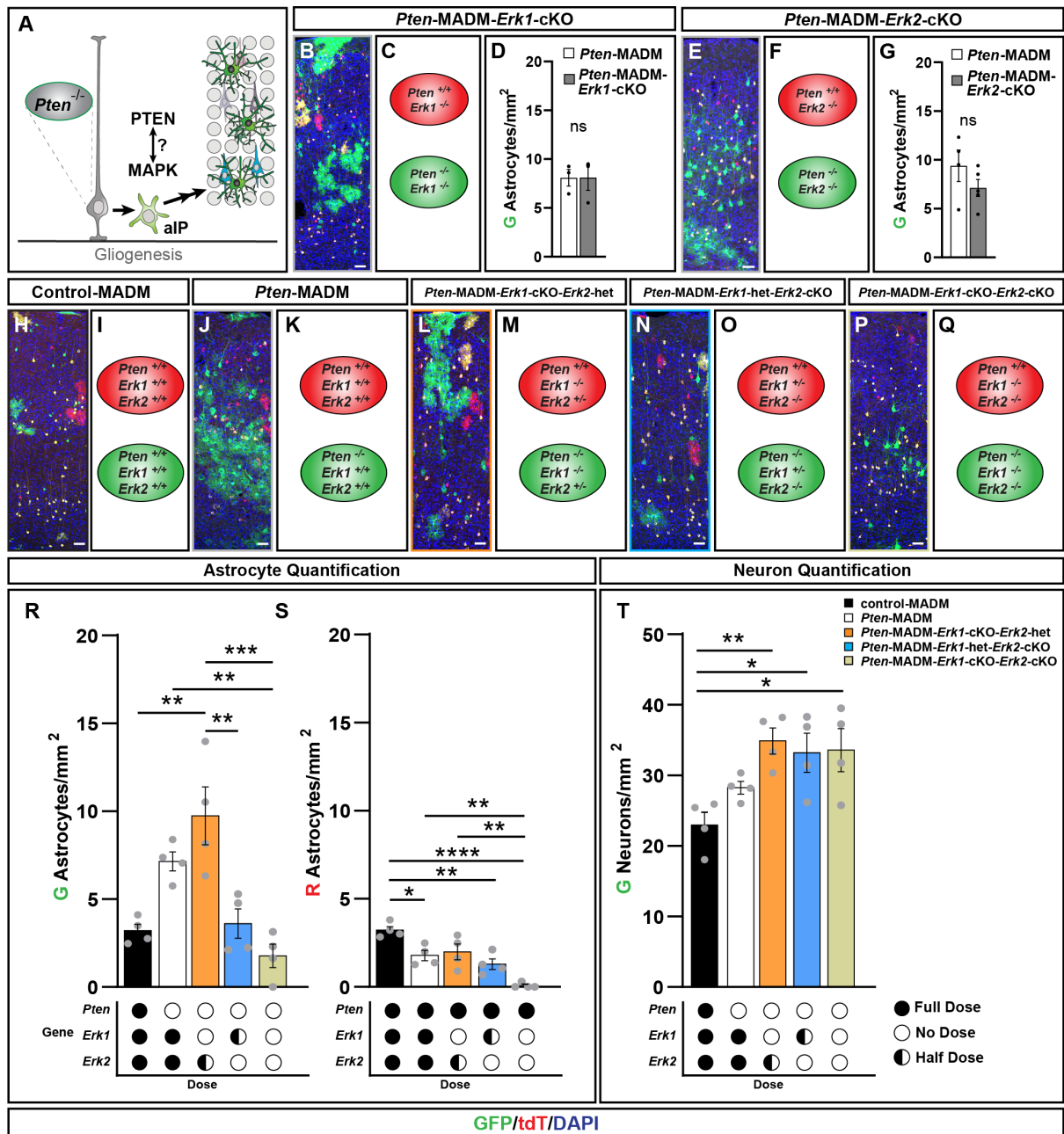


Figure 41. *Erk1/2* are key mediators of astrocyte population expansion in *Pten* knockout populations.

(A) Diagram of logic behind the experimental design.

(B, E, H, J, L, N, P) Confocal images 35 μ m thick of *Pten*-MADM-*Erk1*-cKO, *Pten*-MADM-*Erk2*-cKO, Control-MADM, *Pten*-MADM, *Pten*-MADM-*Erk1*-cKO-*Erk2*-het, *Pten*-MADM-*Erk1*-het-*Erk2*-cKO, and *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO, respectively. Scale bar cortex inset=50 μ m.

(C, F, I, K, M, O, P) Schematic representations of genotypes of tdTomato⁺ and GFP⁺ cells within *Pten*-MADM-*Erk1*-cKO, *Pten*-MADM-*Erk2*-cKO, Control-MADM, *Pten*-MADM, *Pten*-MADM-*Erk1*-cKO-*Erk2*-het, *Pten*-MADM-*Erk1*-het-*Erk2*-cKO, and *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO.

(D) Quantification of astrocyte density in the somatosensory cortex of P21 mice of *Pten*-MADM and *Pten*-MADM-*Erk1*-cKO. Each dot represents one animal, with a minimum of 4 sections counted per animal. *Pten*-

MADM: mean = 8.096 astrocytes/mm², SEM±0.8505; *Pten*-MADM-*Erk1*-cKO: mean = 8.102 astrocytes/mm², SEM±1.287. Unpaired t-test p-value = ns = not significant.

(G) Quantification of astrocyte density in the somatosensory cortex of P21 mice of *Pten*-MADM and *Pten*-MADM-*Erk2*-cKO. Each dot represents one animal, with a minimum of 5 sections counted per animal. *Pten*-MADM: mean = 9.398 astrocytes/mm², SEM±1.608; *Pten*-MADM-*Erk2*-cKO: mean = 7.122 astrocytes/mm², SEM±0.866. Mann-Whitney test = 0.1905. ns = not significant.

(R) Quantification of GFP⁺ astrocyte density in the somatosensory cortex of P21 mice of Control-MADM (mean = 3.209 astrocytes/mm², SEM±0.3687), *Pten*-MADM (mean = 7.148 astrocytes/mm², SEM±0.5423), *Pten*-MADM-*Erk1*-cKO-*Erk2*-het (mean = 9.744 astrocytes/mm², SEM±1.651), *Pten*-MADM-*Erk1*-het-*Erk2*-cKO (mean = 3.612 astrocytes/mm², SEM±0.8310), and *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO (mean = 1.770 astrocytes/mm², SEM±0.6650). Each dot represents one animal, with a minimum of 5 sections counted per animal. GFP⁺ astrocytes in *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO were significantly reduced compared to *Pten*-MADM (one-way Anova, Tukey's correction, p-value = 0.0071).

(S) Quantification of tdT⁺ (*Pten*^{+/+} in all conditions) astrocyte density in the somatosensory cortex of P21 mice of Control-MADM (mean = 3.222 astrocytes/mm², SEM±0.2094), *Pten*-MADM (mean = 1.786 astrocytes/mm², SEM±0.2865), *Pten*-MADM-*Erk1*-cKO-*Erk2*-het (mean = 1.993 astrocytes/mm², SEM±0.4533), *Pten*-MADM-*Erk1*-het-*Erk2*-cKO (mean = 1.291 astrocytes/mm², SEM±0.3045), and *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO (mean = 0.0770 astrocytes/mm², SEM±0.0770). Each dot represents one animal, with a minimum of 5 sections counted per animal.

(T) Quantification of GFP⁺ neuron density in the somatosensory cortex of P21 mice of Control-MADM (mean = 22.97 neurons/mm², SEM±1.810), *Pten*-MADM (mean = 28.26 neurons/mm², SEM±0.8873), *Pten*-MADM-*Erk1*-cKO-*Erk2*-het (mean = 34.88 neurons/mm², SEM±1.858), *Pten*-MADM-*Erk1*-het-*Erk2*-cKO (mean = 33.22 neurons/mm², SEM±2.769), and *Pten*-MADM-*Erk1*-cKO-*Erk2*-cKO (mean = 33.59 neurons/mm², SEM±3.072). Each dot represents one animal, with a minimum of 4 sections counted per animal.

5.5 Discussion

Previous literature reports that MAPK can modulate gliogenic processes during cortical development. Conditional deletion of MEK1/2, key components of MAPK signaling, in the developing cortex has shown that MAPK signaling is necessary for the neurogenic to gliogenic switch (Li et al., 2012). Conflicting data suggests that *Erk2* cKO actually increases astrocyte production in their mouse models (Samuels et al., 2008). Nonetheless, it is evident that MAPK components play a significant role in corticogenesis. Previous studies report both cytoplasmic and nuclear interactions between PTEN and ERK1/2. PTEN's cytoplasmic functions are largely attributed to its role in negatively regulating downstream MTOR signaling (Chung & Eng, 2005; Lee et al., 2018); on the other hand, reports suggest that nuclear PTEN arrests cell cycle progression by downregulating cyclinD1 expression and limiting its exit from the nucleus (Chung & Eng, 2005; Chung et al., 2006; Lee et al., 2018). Follow-up experiments showed that PTEN achieves cell cycle arrest via cyclinD1 by reducing phosphorylation of ERK1/2 and MEK1/2 (Chung et al., 2006). PTEN has been shown to indirectly regulate MAPK signaling via inhibition of protein kinase C (PKC), and that certain isoforms of PKC can be activated by PIP₃ or AKT (Le Good et al., 1998; Toker et al., 1994). These data, along with my experimental results, provide good evidence that deletion of *Pten* leads to direct hyper-activation of MAPK signaling, thereby resulting in an over production of astrocytes in the cortex independently of EGFR status (Figure 42).

Though, we do not yet know exactly in which way, if any, PTEN-MAPK signaling components interact to achieve the astrocyte overproduction we observed. One can speculate that it may occur via some of the reported interactions above, or even further upstream in the pathway. It is also possible that PTEN LOF may result in cells which are hyper-sensitive to external conditions, such as nutrient availability and hormone signaling, due to an overactivation of mTOR signaling (Chung & Eng, 2005; Lee et al., 2018). Additionally, FGFRs and their ligands were repeatedly found in DEG lists in our RNA-seq experiments, possibly hinting at a deregulation of receptor gene expression. This data raises further questions, such as whether the higher FGFR transcript abundance also translates to more receptors at the cell surface, which could in turn lead to runaway MAPK signaling. Experiments to parse out these fine details could provide further clarity, and could have implications in the clinical treatment of certain cancers.

PTEN mutations are implicated in several varieties of cancers, some of which arise from glial cell populations (Fenton et al., 2012). Glioblastoma multiforme (GBM) is one of the

most common intracranial tumors that arises from astrocytes in adults and is associated with, and oftentimes preceded by, PTEN LOF (Kwon et al., 2008; Ozawa et al., 2014). GBM is frequently aggravated by either EGFR amplification or a hyperactivating mutation (An et al., 2018) and oftentimes GBM patients are resistant to receptor-tyrosine kinase inhibitor drugs (RTKI) meant to inhibit EGFR activity (An et al., 2018). The data presented in this section provides evidence that gliogenesis can march on even without EGFR when combined with PTEN LOF, somewhat mirroring the conditions described in PTEN-LOF-induced GBM resistant to EGFR inhibitors.

It is interesting to speculate that future experiments could flesh out the intricacies of the PTEN-MAPK signaling axis in EGFR-independent gliogenesis, which may provide new pharmacological targets for patients with treatment-resistant GBM. Such experiments and their results have the potential to significantly impact the lives of patients receiving treatment for one of the several types of cancers caused by mutations to *PTEN*. Collectively, these data present a novel study on the role of *Pten* throughout RGP lineage progression. For the first time, these data show that the EGFR requirement can indeed be overridden during development. I found that MAPK signaling is the linchpin of the *Pten*-mutant astrocyte phenotype. More generally, I provide a template for studies to determine how LOF of a combination of genes plays together to build the phenotype observed at the macro level using MADM combinatorial analysis.

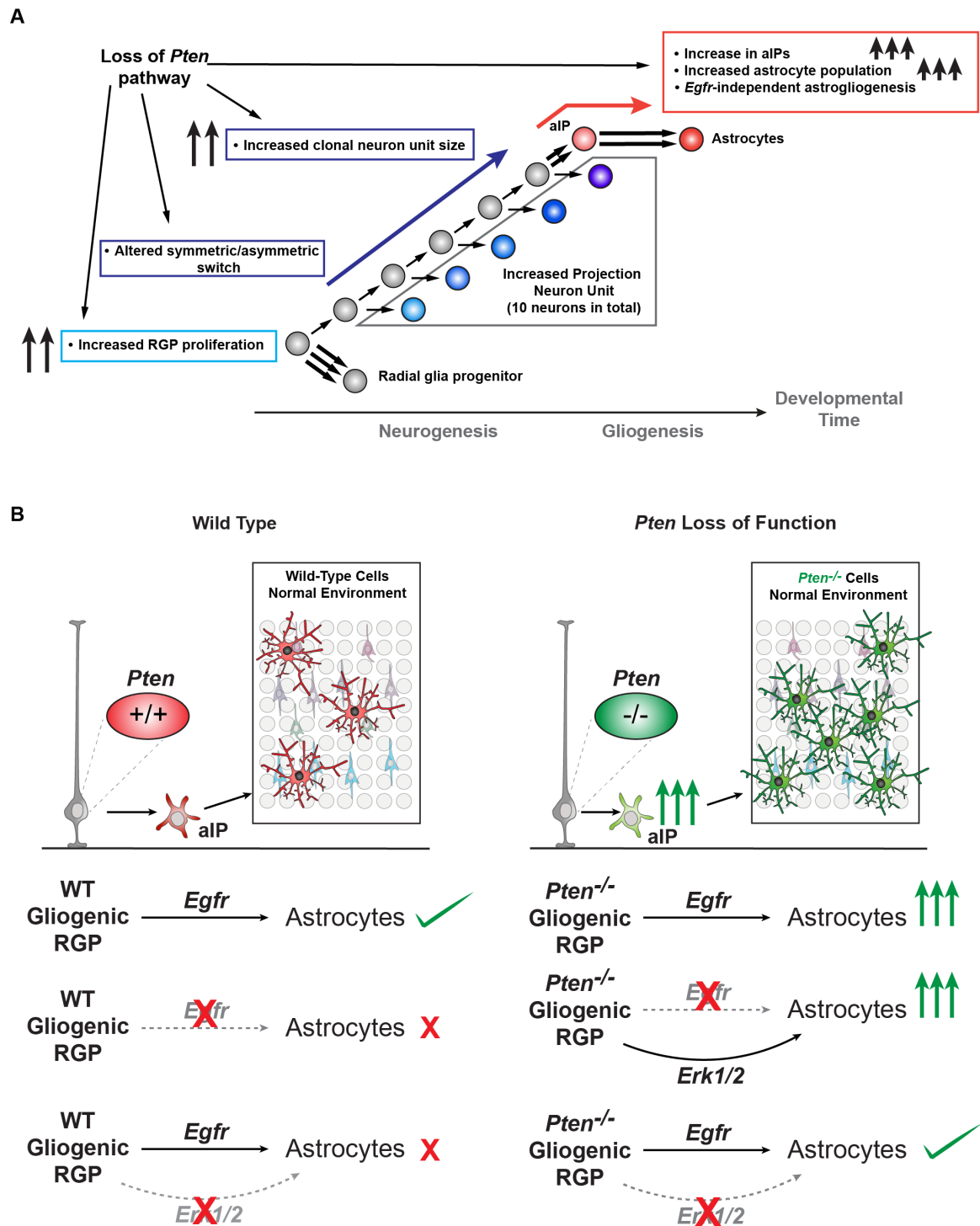


Figure 42. *Pten* modulates cortical RGP output at distinct stages of lineage progression.
 (A) Schematic model of *Pten*'s roles in RGP lineage progression during neurogenesis and gliogenesis.
 (B) Proposed model depicting the genetic interactions driving the *Pten* LOF phenotype.

6 Conclusion

Despite the overwhelming quantity of research into mammalian cortical development, much remains unknown about basic aspects of radial glia lineage progression. This thesis has described some of the main processes in which *Pten* plays a role. Early studies already reported that *Pten* was essential for controlling neural progenitor population size, and humans with mutations in *PTEN* often have enlarged brains. Previous studies on *Pten* found that it is a difficult gene to study due to the early postnatal death of *Pten* cKO animals. By using MADM to generate genetic mosaic, we bypass the perinatal lethality attributed to *Pten* cKOs and are able to assess the progeny of single cortical progenitors. Using MADM, we discover that *Pten* deletion alters the division patterns of progenitors, leading to early differentiation but also a prolongation of symmetric proliferative divisions and increases the overall neuronal output of individual RGPs. We find that not only neuron soma size is increased, but also nuclear volume triples. *Pten* has long been attributed to a susceptibility to glioblastomas. Indeed, we find that astrocyte numbers within the *Pten* mutant population are drastically increased through an amplification of aIPs as well as increased output from RGPs. Taken together, *Pten* controls brain size through negative regulation of progenitor proliferation dynamics, by constraining cell soma and nucleus size, and asserting control of RGP output throughout both neurogenesis and gliogenesis. We performed Bulk RNA-seq and were able to describe the pathways involved in promoting *Pten*-mutant astrocyte population expansion, and used this data as a hypothesis generator to test further candidate genes. By generating *Pten* mosaics within cKO tissues for genes involved in EGFR, MAPK, Jak/Stat, and Notch signaling, we precisely dissect the requirement for each of these pathways. We find that gliogenesis can take place in *Pten*-mutant populations devoid of *Egfr*, despite the common assumption that EGFR is required for gliogenesis. Finally, we report that the expansion of astrocytes in *Pten* mutants requires intact *Erk1/2*. Astrocyte numbers are only impacted by deletion of at least both copies of *Erk2* and one copy of *Erk1*, not vice versa. These results indicate that *Erk2* functions cannot be assumed by the single *Erk1* copy. Deletion of both *Erk1/2* further reduces astrocyte numbers, though we remain uncertain as to how ERK1/2 achieve the astrocyte phenotype in the absence of PTEN. Indeed, further studies delving into the signaling cues which control ERK1/2-mediated astrogliogenesis would be interesting. Altogether, we find that *Pten* plays distinct and significant roles throughout the cortical developmental program, from progenitor pool expansion to gliogenesis.

7 Methods

7.1 Mouse husbandry and lines

All breeding and experiments were performed under a license approved by the Austrian Federal Ministry of Science and Research (BMWFW-66.018/0006-WF/V/3b/2017) and an amendment to that license (2020-0.579.989). The mouse colony was maintained following guidelines approved by the institutional ethics committee, the institutional animal care and use committee, and the preclinical facility (PCF) and the Institute of Science and Technology, Austria (ISTA). Mice with specific pathogen free status according to FELASA recommendations (rodents et al., 2014) were bred and maintained in experimental rodent facilities (room temperature $21 \pm 1^\circ\text{C}$ [mean $\pm$ SEM]; relative humidity 40%–55%; photoperiod 12L:12D). Food (V1126, Ssniff Spezialitäten GmbH, Soest, Germany) and tap water were available *ad libitum*.

Mouse lines with MADM cassettes inserted in Chr. 4, 7, 16, 19 (Contreras et al., 2021), Chr. 11 (Hippenmeyer et al., 2010), *Pten*-flox (Groszer et al., 2001), *Egfr*-flox (Lee & Threadgill, 2009), *Jak1*-flox (Sakamoto et al., 2016), *Jak2*-flox (Krempler et al., 2004), *Stat3*-flox (Moh et al., 2007), *Stat5*-flox (Cui et al., 2004), *Erk1*-flox (Selcher et al., 2001), *Erk2*-flox (Samuels et al., 2008), *Notch1*-flox (Yang et al., 2004), *hGFAP-LacZ* (Brenner et al., 1994), *Emx1*-Cre (Gorski et al., 2002), and *Emx1*-CreERT2^{+/−} (Kessaris et al., 2006) have been described previously.

All histological analyses were carried out in mice with a mixed C57/Bl6, CD1 genetic background, in both male and female mice. Mice were only selected for desired genotype. No sex specific differences were observed in any experiment. The age and genotype of respective animals is available in the respective figures, legends, and supplementary files.

7.2 Preparation of MADM-labeled tissue

Mouse tissue extraction and preparation were done following a previously published protocol (Beattie et al., 2020). In short, mice were anesthetized through an intraperitoneal injection of ketamine/xylazine/acepromazine solution (65 mg, 13 mg, and 2 mg/kg body weight, respectively), unresponsiveness was confirmed by pinching the paw. Anesthetized mice were then perfused with ice-cold phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS. Tissue underwent post-fixation in 4% PFA overnight at 4°C. For embryo tissue collection, pregnant dams were sacrificed by cervical dislocation, embryos were collected, and the heads were dissected and fixed in 4% PFA at 4°C for 48 hrs. Brains were then washed with PBS and transferred to 30% sucrose for 48-72 hours. Excess sucrose was removed and brains

were embedded in Tissue-Tek O.C.T. (Sakura). Embryonic tissue was sectioned at 20µm and directly collected on Superfrost glass slides (Thermo Fisher Scientific). Post-natal tissue with dense MADM labeling was sectioned at 35µm, post-natal tissue with sparse or clonal MADM labeling was sectioned at 45µm, sections were then collected in 24-well dishes (Greiner Bio-one) and stored at -20°C in a cryoprotectant solution (30% v/v ethylene glycol, 30% v/v glycerol, 10% v/v 0.244M PO₄ buffer) until use.

7.3 Immunolabeling of MADM-labeled tissue

Sections on glass slides were first rehydrated 3x with PBS for 15 minutes. Sections were then incubated in blocking solution (10% horse serum, 1% Triton X-100 in PBS) for 1 hr. If necessary, sections underwent antigen retrieval treatment by incubating slides with pre-warmed Citrate Buffer (pH 6) at 85°C for 30 minutes. Primary antibodies were diluted in staining solution (5% horse serum, 0.5% Triton X-100 in PBS) as indicated in the Key Resources Table and 350µl were applied to each slide and incubated overnight at 4°C. The following day, slides were washed 3x with PBS for 5 minutes and incubated with diluted secondary antibodies in staining solution at ambient temperature for 1 hr. Slides were then washed 3x with PBS and incubated with DAPI (1:20,000 in PBS) for 15 minutes. Finally, cover slips were mounted onto the slides with mounting media containing 1,4-diazabicyclooctane (DABCO; Roth) and Mowiol 4-88 (Roth) and stored at 4°C until imaging. Embryonic MADM tissue, up to E18, underwent primary labeling against GFP (Aves Labs) and tdTomato (Sicgen Antibodies). This step is not required for tissue from postnatal timepoints.

BLBP (Chemicon) staining required antigen retrieval using Citrate Buffer (pH 6) at 85°C for 30 minutes. Sections were then incubated for 2 nights with anti-BLBP (rabbit, 1:700 in 5% horse serum, 0.5% Triton X-100 in PBS). Stained sections were then washed 2x with PBS for 15 minutes, the final wash with PBS should be 1 hour long. Sections were then stained with anti-rabbit Alexa Fluor 647 (Molecular Probes) and incubated at room temperature for 1 hour. Sections were then washed 2x with PBS, then incubated with DAPI (1:20,000 in PBS) for 15 minutes. Slides were then mounted as described above.

7.4 Imaging and analysis of MADM-labeled tissue

Samples were imaged using an LSM800 or LSM900 confocal microscopes (Zeiss). Tiled images of regions of interest were acquired for a minimum of four sections per animal per genotype using either 10x or 20x air objectives. Images were initially processed using Zen Blue

software from Zeiss, then imported into Photoshop (Adobe) where the ROI was marked and cell numbers were manually quantified based on marker gene expression.

7.5 MADM clonal analysis

Clonal analysis using MADM was performed as previously described (Beattie & Hippenmeyer, 2017; Beattie et al., 2020; Gao et al., 2014). Briefly, mating cages were set up and females were checked for signs of mating, and subsequently separated from their mates. Pregnant females were each injected with 2mg of tamoxifen (Sigma-Aldrich) dissolved in corn oil (Sigma-Aldrich) at E11, E12, and E13. Embryos were collected by cesarean section and analyzed at E13 and E16. Cesarean sections were performed for pregnant dams who did not give birth by E19. The resulting pups were fostered and maintained until analysis at P28. Clone-containing brains were collected, processed and imaged as described in the manuscripts above.

7.6 Statistical Analysis

Statistical analyses were performed in the Graphpad Prism v10 software. Raw data expressed as proportions was transformed using the $Y = \arcsin(\sqrt{Y})$ formula in order to stabilize variance and prepare for statistical testing. Statistical tests used include: variants of two-tailed unpaired t-test for comparisons between two groups, one-way ANOVA for comparisons between multiple groups or two-way ANOVA for comparisons between groups split by two variables (i.e. time and genotype). For g/r quantifications: A biological sample was defined as the g/r ratio from one animal resulting from the quantification of at least 5 sections/animal in adults and 5-10 sections/animal in embryonic stages. For cell density (cells/mm²) quantifications: A biological sample was defined as the cell density from an animal resulting from the quantification of at least 5 sections/animal in adults [i.e. the number of all GFP⁺ cells in animal “A”/ the sum of the area from all quantified regions in animal “A”]. All figures include information about the test used, as well as the corresponding p-value and other relevant data.

7.7 Single-cell suspension and FACS of MADM-labeled tissue

The protocol followed for generating a single-cell suspension and subsequent FACS of MADM-labeled tissue is already published (Amberg et al., 2024). In short, animals aged P0 were collected and terminated by decapitation. The cortex was then dissected from each brain and placed in cold PBS in 12-well plates while all samples were processed. Single-cell suspensions were generated for individual animals for use as biological replicates. Tissue was dissociated using a kit containing papain with L-cysteine and EDTA (vial 2, Worthington), deoxyribonuclease (DNase) I (vial 3, Worthington), ovomucoid protease inhibitor (vial 4,

Worthington), Dulbecco's modified Eagle's medium (DMEM)/F12 (Thermo Fisher Scientific), Earle's Balanced Salts (EBSS) (Thermo Fisher Scientific), horse serum (HS), and fetal bovine serum (FBS). Worthington reagents were reconstituted according to manufacturer's instructions using the indicated solvents. Isolated cortices were treated with papain-DNase (papain 20 U/ml with 1000 U DNase) and digested for 30 minutes at 37°C in a water bath. Samples then received a solution of EBSS containing 0.67 mg ovomucoid protease inhibitor and DNase (166.7 U/ml) (Solution 2) and the entire solution was triturated to generate a cell suspension and were then centrifuged at 1000 rpm for 5 minutes at ambient temperature. The supernatant was extracted and the remaining cell pellet was resuspended in solution 2. Suspension was then triturated with a p1000 pipette to mechanically dissociate tissue remnants. The suspension was washed with DMEM/F12 and centrifuged at 1500 rpm for 5 minutes at ambient temperature. Cells were resuspended in a final solution of DMEM/F12 with 10 % FBS and 10% HS. Suspensions were kept on ice until sorting.

7.8 Library Preparation and Single Cell Sequencing

Library preparation was performed using Fluent Bioscience's PIPseq T2 Single Cell RNA kit v4.0PLUS (FB0001026) according to the manufacturer's protocol. Libraries were pooled and sequenced on an Illumina NovaSeqX platform at the VBCF NGS Unit (<https://www.viennabiocenter.org/facilities/>). Initial alignment and preparation of count matrices was performed using pipseeker-v3.3.0 and pipseeker-gex-reference-GRCm39-2022.04. Downstream analyses were performed using R v4.3.2 and Seurat v5.0.1.

7.9 Single-Cell Sequencing Analysis

Single cell RNA-Seq:

Figure 19A-D: Data was filtered (parameters: $nFeature_RNA > 500$ & $nFeature_RNA < 6000$ & $nCount_RNA < 40000$ & $percent.mt < 15$), and processed using standard pipelines. UMAP was performed using RunUMAP with $dims = 1:25$, clusters were determined using FindNeighbors with $dims = 1:25$ and FindClusters with $resolution = 0.3$. This analysis identified 7 clusters. Cells from one cluster were not well localized on the UMAP, did not show specific marker genes and were removed (39 cells, 0.6% of total cells).

Cell cycle scoring was performed using Seurat's CellCycleScoring function with genes from https://github.com/hbc/tinyatlas/tree/master/cell_cycle. The difference between G2M and S phase scores was determined and regressed out during scaling. Trajectory analysis was

performed using monocle3 (v1.3.7). Significance of changes in relative abundance were either determined by two-way ANOVA on arcsine transformed values or using chi squared test. Differential expression between cell types was calculated using MAST (v1.28.0). [<https://genomebiology.biomedcentral.com/articles/10.1186/s13059-015-0844-5>]. To draw DEG heatmaps adjusted p-values of significant DEGs (adjusted $p < 0.1$) were transformed to a score ($\log_{10}(\text{adjusted } p) * -1$) and visualized using pheatmap (v1.0.12). Gene Ontology (GO) term analysis was performed using iDEA (v1.0.1) [<https://www.nature.com/articles/s41467-020-15298-6>] on selected GO terms and differential expression statistics (as above). The p-values, as determined by iDEA, were corrected for multiple testing using p.adjust with standard parameters. GO terms were filtered for significant enrichment and grouped using simplifyEnrichment (v1.12.0) [<https://academic.oup.com/gpb/article/21/1/190/7274174>] in a 2-step process. We used the uncorrected p-values to allow for a comprehensive documentation of changes. In the first step, broad GO groups were determined, including a group containing major signaling pathways. GO terms in the signaling pathway group were grouped in a second step using simplifyEnrichment. The smallest adjusted p-value was used for plotting the heatmap.

References

- Agirman, G., Broix, L., & Nguyen, L. (2017). Cerebral cortex development: an outside-in perspective. *FEBS Lett*, 591(24), 3978-3992. <https://doi.org/10.1002/1873-3468.12924>
- Amberg, N., Cheung, G., & Hippenmeyer, S. (2024). Protocol for sorting cells from mouse brains labeled with mosaic analysis with double markers by flow cytometry. *STAR Protoc*, 5(1), 102771. <https://doi.org/10.1016/j.xpro.2023.102771>
- Amberg, N., & Hippenmeyer, S. (2021). Genetic mosaic dissection of candidate genes in mice using mosaic analysis with double markers. *STAR Protoc*, 2(4), 100939. <https://doi.org/10.1016/j.xpro.2021.100939>
- Amberg, N., Laukoter, S., & Hippenmeyer, S. (2019). Epigenetic cues modulating the generation of cell-type diversity in the cerebral cortex. *J Neurochem*, 149(1), 12-26. <https://doi.org/10.1111/jnc.14601>
- Amberg, N., Pauler, F. M., Streicher, C., & Hippenmeyer, S. (2022). Tissue-wide genetic and cellular landscape shapes the execution of sequential PRC2 functions in neural stem cell lineage progression. *Sci Adv*, 8(44), eabq1263. <https://doi.org/10.1126/sciadv.abq1263>
- An, Z., Aksoy, O., Zheng, T., Fan, Q. W., & Weiss, W. A. (2018). Epidermal growth factor receptor and EGFRvIII in glioblastoma: signaling pathways and targeted therapies. *Oncogene*, 37(12), 1561-1575. <https://doi.org/10.1038/s41388-017-0045-7>
- Aouadi, M., Binetruy, B., Caron, L., Le Marchand-Brustel, Y., & Bost, F. (2006). Role of MAPKs in development and differentiation: lessons from knockout mice. *Biochimie*, 88(9), 1091-1098. <https://doi.org/10.1016/j.biochi.2006.06.003>
- Bandler, R. C., Mayer, C., & Fishell, G. (2017). Cortical interneuron specification: the juncture of genes, time and geometry. *Curr Opin Neurobiol*, 42, 17-24. <https://doi.org/10.1016/j.conb.2016.10.003>
- Barkovich, A. J., Dobyns, W. B., & Guerrini, R. (2015). Malformations of cortical development and epilepsy. *Cold Spring Harb Perspect Med*, 5(5), a022392. <https://doi.org/10.1101/cshperspect.a022392>
- Bayraktar, O. A., Fuentealba, L. C., Alvarez-Buylla, A., & Rowitch, D. H. (2014). Astrocyte development and heterogeneity. *Cold Spring Harb Perspect Biol*, 7(1), a020362. <https://doi.org/10.1101/cshperspect.a020362>
- Beattie, R., & Hippenmeyer, S. (2017). Mechanisms of radial glia progenitor cell lineage progression. *FEBS Lett*, 591(24), 3993-4008. <https://doi.org/10.1002/1873-3468.12906>
- Beattie, R., Postiglione, M. P., Burnett, L. E., Laukoter, S., Streicher, C., Pauler, F. M., Xiao, G., Klezovitch, O., Vasioukhin, V., Ghashghaei, T. H., & Hippenmeyer, S. (2017). Mosaic Analysis with Double Markers Reveals Distinct Sequential Functions of Lgl1 in Neural Stem Cells. *Neuron*, 94(3), 517-533 e513. <https://doi.org/10.1016/j.neuron.2017.04.012>
- Beattie, R., Streicher, C., Amberg, N., Cheung, G., Contreras, X., Hansen, A. H., & Hippenmeyer, S. (2020). Lineage Tracing and Clonal Analysis in Developing Cerebral Cortex Using Mosaic Analysis with Double Markers (MADM). *J Vis Exp*(159). <https://doi.org/10.3791/61147>
- Bishop, K. M., Goudreau, G., & O'Leary, D. D. (2000). Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. *Science*, 288(5464), 344-349. <https://doi.org/10.1126/science.288.5464.344>
- Bonni, A., Sun, Y., Nadal-Vicens, M., Bhatt, A., Frank, D. A., Rozovsky, I., Stahl, N., Yancopoulos, G. D., & Greenberg, M. E. (1997). Regulation of gliogenesis in the central nervous system by the JAK-STAT signaling pathway. *Science*, 278(5337), 477-483. <https://doi.org/10.1126/science.278.5337.477>
- Borrell, V., & Gotz, M. (2014). Role of radial glial cells in cerebral cortex folding. *Curr Opin Neurobiol*, 27, 39-46. <https://doi.org/10.1016/j.conb.2014.02.007>
- Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radziejewska, E., Morgenbesser, S. D., DePinho, R. A., Panayotatos, N., Cobb, M. H., & Yancopoulos, G. D. (1991). ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, 65(4), 663-675. [https://doi.org/10.1016/0092-8674\(91\)90098-j](https://doi.org/10.1016/0092-8674(91)90098-j)
- Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., Hsu, J., & Cobb, M. H. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science*, 249(4964), 64-67. <https://doi.org/10.1126/science.2164259>
- Brenner, M., Kisseberth, W. C., Su, Y., Besnard, F., & Messing, A. (1994). GFAP promoter directs astrocyte-specific expression in transgenic mice. *J Neurosci*, 14(3 Pt 1), 1030-1037. <https://doi.org/10.1523/JNEUROSCI.14-03-01030.1994>
- Brenner, M., & Messing, A. (2021). Regulation of GFAP Expression. *ASN Neuro*, 13, 1759091420981206. <https://doi.org/10.1177/1759091420981206>

- Burrows, R. C., Wancio, D., Levitt, P., & Lillien, L. (1997). Response diversity and the timing of progenitor cell maturation are regulated by developmental changes in EGFR expression in the cortex. *Neuron*, 19(2), 251-267. [https://doi.org/10.1016/s0896-6273\(00\)80937-x](https://doi.org/10.1016/s0896-6273(00)80937-x)
- Cargnello, M., & Roux, P. P. (2011). Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol Mol Biol Rev*, 75(1), 50-83. <https://doi.org/10.1128/MMBR.00031-10>
- Chen, B., Schaevitz, L. R., & McConnell, S. K. (2005). Fezl regulates the differentiation and axon targeting of layer 5 subcortical projection neurons in cerebral cortex. *Proc Natl Acad Sci U S A*, 102(47), 17184-17189. <https://doi.org/10.1073/pnas.0508732102>
- Chen, Y., Huang, W. C., Sejourne, J., Clipperton-Allen, A. E., & Page, D. T. (2015). Pten Mutations Alter Brain Growth Trajectory and Allocation of Cell Types through Elevated beta-Catenin Signaling. *J Neurosci*, 35(28), 10252-10267. <https://doi.org/10.1523/JNEUROSCI.5272-14.2015>
- Cheung, G., Pauler, F. M., Koppensteiner, P., Krausgruber, T., Streicher, C., Schrammel, M., Gutmann-Ozgen, N., Ivec, A. E., Bock, C., Shigemoto, R., & Hippenmeyer, S. (2024). Multipotent progenitors instruct ontogeny of the superior colliculus. *Neuron*, 112(2), 230-246 e211. <https://doi.org/10.1016/j.neuron.2023.11.009>
- Chung, J. H., & Eng, C. (2005). Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis. *Cancer Res*, 65(18), 8096-8100. <https://doi.org/10.1158/0008-5472.CAN-05-1888>
- Chung, J. H., Ostrowski, M. C., Romigh, T., Minaguchi, T., Waite, K. A., & Eng, C. (2006). The ERK1/2 pathway modulates nuclear PTEN-mediated cell cycle arrest by cyclin D1 transcriptional regulation. *Hum Mol Genet*, 15(17), 2553-2559. <https://doi.org/10.1093/hmg/ddl177>
- Chung, W. S., Allen, N. J., & Eroglu, C. (2015). Astrocytes Control Synapse Formation, Function, and Elimination. *Cold Spring Harb Perspect Biol*, 7(9), a020370. <https://doi.org/10.1101/cshperspect.a020370>
- Clavreul, S., Abdeladim, L., Hernandez-Garzon, E., Niculescu, D., Durand, J., Ieng, S. H., Barry, R., Bonvento, G., Beaupaire, E., Livet, J., & Loulier, K. (2019). Cortical astrocytes develop in a plastic manner at both clonal and cellular levels. *Nat Commun*, 10(1), 4884. <https://doi.org/10.1038/s41467-019-12791-5>
- Contreras, X., Amberg, N., Davaatseren, A., Hansen, A. H., Sonntag, J., Andersen, L., Bernthaler, T., Streicher, C., Heger, A., Johnson, R. L., Schwarz, L. A., Luo, L., Rulicke, T., & Hippenmeyer, S. (2021). A genome-wide library of MADM mice for single-cell genetic mosaic analysis. *Cell Rep*, 35(12), 109274. <https://doi.org/10.1016/j.celrep.2021.109274>
- Costa, M. R., Bucholz, O., Schroeder, T., & Gotz, M. (2009). Late origin of glia-restricted progenitors in the developing mouse cerebral cortex. *Cereb Cortex*, 19 Suppl 1, i135-143. <https://doi.org/10.1093/cercor/bhp046>
- Cui, Y., Riedlinger, G., Miyoshi, K., Tang, W., Li, C., Deng, C. X., Robinson, G. W., & Hennighausen, L. (2004). Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Mol Cell Biol*, 24(18), 8037-8047. <https://doi.org/10.1128/MCB.24.18.8037-8047.2004>
- Di Bella, D. J., Dominguez-Iturza, N., Brown, J. R., & Arlotta, P. (2024). Making Ramon y Cajal proud: Development of cell identity and diversity in the cerebral cortex. *Neuron*, 112(13), 2091-2111. <https://doi.org/10.1016/j.neuron.2024.04.021>
- Di Cristofano, A., Pesce, B., Cordon-Cardo, C., & Pandolfi, P. P. (1998). Pten is essential for embryonic development and tumour suppression. *Nat Genet*, 19(4), 348-355. <https://doi.org/10.1038/1235>
- Duerr, E. M., Rollbrocker, B., Hayashi, Y., Peters, N., Meyer-Puttlitz, B., Louis, D. N., Schramm, J., Wiestler, O. D., Parsons, R., Eng, C., & von Deimling, A. (1998). PTEN mutations in gliomas and glioneuronal tumors. *Oncogene*, 16(17), 2259-2264. <https://doi.org/10.1038/sj.onc.1201756>
- Eng, C. (2003). PTEN: one gene, many syndromes. *Hum Mutat*, 22(3), 183-198. <https://doi.org/10.1002/humu.10257>
- Fan, G., Martinowich, K., Chin, M. H., He, F., Fouse, S. D., Hutnick, L., Hattori, D., Ge, W., Shen, Y., Wu, H., ten Hoeve, J., Shuai, K., & Sun, Y. E. (2005). DNA methylation controls the timing of astrogliogenesis through regulation of JAK-STAT signaling. *Development*, 132(15), 3345-3356. <https://doi.org/10.1242/dev.01912>
- Feng, L., Hatten, M. E., & Heintz, N. (1994). Brain lipid-binding protein (BLBP): a novel signaling system in the developing mammalian CNS. *Neuron*, 12(4), 895-908. [https://doi.org/10.1016/0896-6273\(94\)90341-7](https://doi.org/10.1016/0896-6273(94)90341-7)

- Fenton, T. R., Nathanson, D., Ponte de Albuquerque, C., Kuga, D., Iwanami, A., Dang, J., Yang, H., Tanaka, K., Oba-Shinjo, S. M., Uno, M., Inda, M. M., Wykosky, J., Bachoo, R. M., James, C. D., DePinho, R. A., Vandenberg, S. R., Zhou, H., Marie, S. K., Mischel, P. S., . . . Furnari, F. B. (2012). Resistance to EGF receptor inhibitors in glioblastoma mediated by phosphorylation of the PTEN tumor suppressor at tyrosine 240. *Proc Natl Acad Sci U S A*, 109(35), 14164-14169. <https://doi.org/10.1073/pnas.1211962109>
- Franco, S. J., Gil-Sanz, C., Martinez-Garay, I., Espinosa, A., Harkins-Perry, S. R., Ramos, C., & Muller, U. (2012). Fate-restricted neural progenitors in the mammalian cerebral cortex. *Science*, 337(6095), 746-749. <https://doi.org/10.1126/science.1223616>
- Frantz, G. D., & McConnell, S. K. (1996). Restriction of late cerebral cortical progenitors to an upper-layer fate. *Neuron*, 17(1), 55-61. [https://doi.org/10.1016/s0896-6273\(00\)80280-9](https://doi.org/10.1016/s0896-6273(00)80280-9)
- Fraser, M. M., Zhu, X., Kwon, C. H., Uhlmann, E. J., Gutmann, D. H., & Baker, S. J. (2004). Pten loss causes hypertrophy and increased proliferation of astrocytes in vivo. *Cancer Res*, 64(21), 7773-7779. <https://doi.org/10.1158/0008-5472.CAN-04-2487>
- Freeman, M. R., & Rowitch, D. H. (2013). Evolving concepts of gliogenesis: a look way back and ahead to the next 25 years. *Neuron*, 80(3), 613-623. <https://doi.org/10.1016/j.neuron.2013.10.034>
- Fremin, C., Saba-El-Leil, M. K., Levesque, K., Ang, S. L., & Meloche, S. (2015). Functional Redundancy of ERK1 and ERK2 MAP Kinases during Development. *Cell Rep*, 12(6), 913-921. <https://doi.org/10.1016/j.celrep.2015.07.011>
- Furnari, F. B., Lin, H., Huang, H. S., & Cavenee, W. K. (1997). Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. *Proc Natl Acad Sci U S A*, 94(23), 12479-12484. <https://doi.org/10.1073/pnas.94.23.12479>
- Gao, P., Postiglione, M. P., Krieger, T. G., Hernandez, L., Wang, C., Han, Z., Streicher, C., Papusheva, E., Insolera, R., Chugh, K., Kodish, O., Huang, K., Simons, B. D., Luo, L., Hippenmeyer, S., & Shi, S. H. (2014). Deterministic progenitor behavior and unitary production of neurons in the neocortex. *Cell*, 159(4), 775-788. <https://doi.org/10.1016/j.cell.2014.10.027>
- Gao, Y., Sun, M., Fu, T., Wang, Z., Jiang, X., Yang, L., Liang, X. G., Liu, G., Tian, Y., Yang, F., Li, J., Li, Z., Li, X., You, Y., Ding, C., Wang, Y., Ma, T., Zhang, Z., Xu, Z., . . . Yang, Z. (2025). NOTCH, ERK, and SHH signaling respectively control the fate determination of cortical glia and olfactory bulb interneurons. *Proc Natl Acad Sci U S A*, 122(9), e2416757122. <https://doi.org/10.1073/pnas.2416757122>
- Ge, W. P., Miyawaki, A., Gage, F. H., Jan, Y. N., & Jan, L. Y. (2012). Local generation of glia is a major astrocyte source in postnatal cortex. *Nature*, 484(7394), 376-380. <https://doi.org/10.1038/nature10959>
- Ginn-Pease, M. E., & Eng, C. (2003). Increased nuclear phosphatase and tensin homologue deleted on chromosome 10 is associated with G0-G1 in MCF-7 cells. *Cancer Res*, 63(2), 282-286. <https://www.ncbi.nlm.nih.gov/pubmed/12543774>
- Gorski, J. A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J. L., & Jones, K. R. (2002). Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage. *J Neurosci*, 22(15), 6309-6314. <https://doi.org/10.1523/JNEUROSCI.22-15-06309.2002>
- Gotz, M., & Huttner, W. B. (2005). The cell biology of neurogenesis. *Nat Rev Mol Cell Biol*, 6(10), 777-788. <https://doi.org/10.1038/nrm1739>
- Grandbarbe, L., Bouissac, J., Rand, M., Hrabe de Angelis, M., Artavanis-Tsakonas, S., & Mohier, E. (2003). Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. *Development*, 130(7), 1391-1402. <https://doi.org/10.1242/dev.00374>
- Greig, L. C., Woodworth, M. B., Galazo, M. J., Padmanabhan, H., & Macklis, J. D. (2013). Molecular logic of neocortical projection neuron specification, development and diversity. *Nat Rev Neurosci*, 14(11), 755-769. <https://doi.org/10.1038/nrn3586>
- Groszer, M., Erickson, R., Scripture-Adams, D. D., Dougherty, J. D., Le Belle, J., Zack, J. A., Geschwind, D. H., Liu, X., Kornblum, H. I., & Wu, H. (2006). PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry. *Proc Natl Acad Sci U S A*, 103(1), 111-116. <https://doi.org/10.1073/pnas.0509939103>
- Groszer, M., Erickson, R., Scripture-Adams, D. D., Lesche, R., Trumpp, A., Zack, J. A., Kornblum, H. I., Liu, X., & Wu, H. (2001). Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. *Science*, 294(5549), 2186-2189. <https://doi.org/10.1126/science.1065518>
- Hansen, A. H., & Hippenmeyer, S. (2020). Non-Cell-Autonomous Mechanisms in Radial Projection Neuron Migration in the Developing Cerebral Cortex. *Front Cell Dev Biol*, 8, 574382. <https://doi.org/10.3389/fcell.2020.574382>

- Hansen, A. H., Pauler, F. M., Riedl, M., Streicher, C., Heger, A., Laukoter, S., Sommer, C., Nicolas, A., Hof, B., Tsai, L. H., Rulicke, T., & Hippenmeyer, S. (2022). Tissue-Wide Effects Override Cell-Intrinsic Gene Function in Radial Neuron Migration. *Oxf Open Neurosci*, 1, kvac009. <https://doi.org/10.1093/oons/kvac009>
- Hansen, A. H., Pauler, F. M., Riedl, M., Streicher, C., Heger, A., Laukoter, S., Sommer, C., Nicolas, A., Hof, B., Tsai, L. H., Rulicke, T., & Hippenmeyer, S. (2022). Tissue-Wide Effects Override Cell-Intrinsic Gene Function in Radial Neuron Migration. *Oxford Open Neuroscience*, 1. <https://doi.org/10.1093/oons/kvac009>
- Haubensak, W., Attardo, A., Denk, W., & Huttner, W. B. (2004). Neurons arise in the basal neuroepithelium of the early mammalian telencephalon: a major site of neurogenesis. *Proc Natl Acad Sci U S A*, 101(9), 3196-3201. <https://doi.org/10.1073/pnas.0308600100>
- He, F., Ge, W., Martinowich, K., Becker-Catania, S., Coskun, V., Zhu, W., Wu, H., Castro, D., Guillemot, F., Fan, G., de Vellis, J., & Sun, Y. E. (2005). A positive autoregulatory loop of Jak-STAT signaling controls the onset of astroglialogenesis. *Nat Neurosci*, 8(5), 616-625. <https://doi.org/10.1038/nn1440>
- Hippenmeyer, S. (2014). Molecular pathways controlling the sequential steps of cortical projection neuron migration. *Adv Exp Med Biol*, 800, 1-24. https://doi.org/10.1007/978-94-007-7687-6_1
- Hippenmeyer, S. (2023). Principles of neural stem cell lineage progression: Insights from developing cerebral cortex. *Curr Opin Neurobiol*, 79, 102695. <https://doi.org/10.1016/j.conb.2023.102695>
- Hippenmeyer, S., Youn, Y. H., Moon, H. M., Miyamichi, K., Zong, H., Wynshaw-Boris, A., & Luo, L. (2010). Genetic mosaic dissection of Lis1 and Ndel1 in neuronal migration. *Neuron*, 68(4), 695-709. <https://doi.org/10.1016/j.neuron.2010.09.027>
- Hobert, J. A., & Eng, C. (2009). PTEN hamartoma tumor syndrome: an overview. *Genet Med*, 11(10), 687-694. <https://doi.org/10.1097/GIM.0b013e3181ac9aea>
- Hoffmann, E. K., Lambert, I. H., & Pedersen, S. F. (2009). Physiology of cell volume regulation in vertebrates. *Physiol Rev*, 89(1), 193-277. <https://doi.org/10.1152/physrev.00037.2007>
- Holbro, T., & Hynes, N. E. (2004). ErbB receptors: directing key signaling networks throughout life. *Annu Rev Pharmacol Toxicol*, 44, 195-217. <https://doi.org/10.1146/annurev.pharmtox.44.101802.121440>
- Howard, B. M., Mo, Z., Filipovic, R., Moore, A. R., Antic, S. D., & Zecevic, N. (2008). Radial Glia Cells in the Developing Human Brain. *The Neuroscientist*, 14(5), 459-473. <https://doi.org/10.1177/1073858407313512>
- Hu, X., Li, J., Fu, M., Zhao, X., & Wang, W. (2021). The JAK/STAT signaling pathway: from bench to clinic. *Signal Transduct Target Ther*, 6(1), 402. <https://doi.org/10.1038/s41392-021-00791-1>
- Huilgol, D., Russ, J. B., Srivas, S., & Huang, Z. J. (2023). The progenitor basis of cortical projection neuron diversity. *Curr Opin Neurobiol*, 81, 102726. <https://doi.org/10.1016/j.conb.2023.102726>
- Imamura, O., Pages, G., Pouyssegur, J., Endo, S., & Takishima, K. (2010). ERK1 and ERK2 are required for radial glial maintenance and cortical lamination. *Genes Cells*, 15(10), 1072-1088. <https://doi.org/10.1111/j.1365-2443.2010.01444.x>
- Kanekar, S., & Gent, M. (2011). Malformations of cortical development. *Semin Ultrasound CT MR*, 32(3), 211-227. <https://doi.org/10.1053/j.sult.2011.02.006>
- Kang, P., Lee, H. K., Glasgow, S. M., Finley, M., Danti, T., Gaber, Z. B., Graham, B. H., Foster, A. E., Novitsch, B. G., Gronostajski, R. M., & Deneen, B. (2012). Sox9 and NFIA coordinate a transcriptional regulatory cascade during the initiation of gliogenesis. *Neuron*, 74(1), 79-94. <https://doi.org/10.1016/j.neuron.2012.01.024>
- Kazdoba, T. M., Sunnen, C. N., Crowell, B., Lee, G. H., Anderson, A. E., & D'Arcangelo, G. (2012). Development and characterization of NEX- Pten, a novel forebrain excitatory neuron-specific knockout mouse. *Dev Neurosci*, 34(2-3), 198-209. <https://doi.org/10.1159/000337229>
- Kessaris, N., Fogarty, M., Iannarelli, P., Grist, M., Wegner, M., & Richardson, W. D. (2006). Competing waves of oligodendrocytes in the forebrain and postnatal elimination of an embryonic lineage. *Nat Neurosci*, 9(2), 173-179. <https://doi.org/10.1038/nn1620>
- Kopan, R., & Ilagan, M. X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell*, 137(2), 216-233. <https://doi.org/10.1016/j.cell.2009.03.045>
- Kornblum, H. I., Hussain, R. J., Bronstein, J. M., Gall, C. M., Lee, D. C., & Seroogy, K. B. (1997). Prenatal ontogeny of the epidermal growth factor receptor and its ligand, transforming growth factor alpha, in the rat brain. *J Comp Neurol*, 380(2), 243-261. [https://doi.org/10.1002/\(sici\)1096-9861\(19970407\)380:2<243::aid-cne7>3.0.co;2-3](https://doi.org/10.1002/(sici)1096-9861(19970407)380:2<243::aid-cne7>3.0.co;2-3)
- Krempler, A., Qi, Y., Triplett, A. A., Zhu, J., Rui, H., & Wagner, K. U. (2004). Generation of a conditional knockout allele for the Janus kinase 2 (Jak2) gene in mice. *Genesis*, 40(1), 52-57. <https://doi.org/10.1002/gene.20063>

- Kriegstein, A., & Alvarez-Buylla, A. (2009). The glial nature of embryonic and adult neural stem cells. *Annu Rev Neurosci*, 32, 149-184. <https://doi.org/10.1146/annurev.neuro.051508.135600>
- Kriegstein, A., Noctor, S., & Martinez-Cerdeno, V. (2006). Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat Rev Neurosci*, 7(11), 883-890. <https://doi.org/10.1038/nrn2008>
- Kwon, C. H., Zhao, D., Chen, J., Alcantara, S., Li, Y., Burns, D. K., Mason, R. P., Lee, E. Y., Wu, H., & Parada, L. F. (2008). Pten haploinsufficiency accelerates formation of high-grade astrocytomas. *Cancer Res*, 68(9), 3286-3294. <https://doi.org/10.1158/0008-5472.CAN-07-6867>
- Lang, F., Busch, G. L., Ritter, M., Volkl, H., Waldegger, S., Gulbins, E., & Haussinger, D. (1998). Functional significance of cell volume regulatory mechanisms. *Physiol Rev*, 78(1), 247-306. <https://doi.org/10.1152/physrev.1998.78.1.247>
- Laukoter, S., Amberg, N., Pauler, F. M., & Hippenmeyer, S. (2020). Generation and isolation of single cells from mouse brain with mosaic analysis with double markers-induced uniparental chromosome disomy. *STAR Protoc*, 1(3), 100215. <https://doi.org/10.1016/j.xpro.2020.100215>
- Laukoter, S., Beattie, R., Pauler, F. M., Amberg, N., Nakayama, K. I., & Hippenmeyer, S. (2020). Imprinted Cdkn1c genomic locus cell-autonomously promotes cell survival in cerebral cortex development. *Nat Commun*, 11(1), 195. <https://doi.org/10.1038/s41467-019-14077-2>
- Lavoie, H., Gagnon, J., & Therrien, M. (2020). ERK signalling: a master regulator of cell behaviour, life and fate. *Nat Rev Mol Cell Biol*, 21(10), 607-632. <https://doi.org/10.1038/s41580-020-0255-7>
- Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., & Parker, P. J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science*, 281(5385), 2042-2045. <https://doi.org/10.1126/science.281.5385.2042>
- Lee, T. C., & Threadgill, D. W. (2009). Generation and validation of mice carrying a conditional allele of the epidermal growth factor receptor. *Genesis*, 47(2), 85-92. <https://doi.org/10.1002/dvg.20464>
- Lee, Y. R., Chen, M., & Pandolfi, P. P. (2018). The functions and regulation of the PTEN tumour suppressor: new modes and prospects. *Nat Rev Mol Cell Biol*, 19(9), 547-562. <https://doi.org/10.1038/s41580-018-0015-0>
- Li, X., Liu, G., Yang, L., Li, Z., Zhang, Z., Xu, Z., Cai, Y., Du, H., Su, Z., Wang, Z., Duan, Y., Chen, H., Shang, Z., You, Y., Zhang, Q., He, M., Chen, B., & Yang, Z. (2021). Decoding Cortical Glial Cell Development. *Neurosci Bull*, 37(4), 440-460. <https://doi.org/10.1007/s12264-021-00640-9>
- Li, X., Newbern, J. M., Wu, Y., Morgan-Smith, M., Zhong, J., Charron, J., & Snider, W. D. (2012). MEK Is a Key Regulator of Gliogenesis in the Developing Brain. *Neuron*, 75(6), 1035-1050. <https://doi.org/10.1016/j.neuron.2012.08.031>
- Li, Y., Muffat, J., Omer, A., Bosch, I., Lancaster, M. A., Sur, M., Gehrke, L., Knoblich, J. A., & Jaenisch, R. (2017). Induction of Expansion and Folding in Human Cerebral Organoids. *Cell Stem Cell*, 20(3), 385-396 e383. <https://doi.org/10.1016/j.stem.2016.11.017>
- Liu, Z., Steward, R., & Luo, L. (2000). Drosophila Lis1 is required for neuroblast proliferation, dendritic elaboration and axonal transport. *Nat Cell Biol*, 2(11), 776-783. <https://doi.org/10.1038/35041011>
- Longy, M., Coulon, V., Duboue, B., David, A., Larregue, M., Eng, C., Amati, P., Kraimps, J. L., Bottani, A., Lacombe, D., & Bonneau, D. (1998). Mutations of PTEN in patients with Bannayan-Riley-Ruvalcaba phenotype. *J Med Genet*, 35(11), 886-889. <https://doi.org/10.1136/jmg.35.11.886>
- Ma, X. M., & Blenis, J. (2009). Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol*, 10(5), 307-318. <https://doi.org/10.1038/nrm2672>
- Magrinelli, E., Baumann, N., Wagener, R. J., Glangetas, C., Bellone, C., Jabaudon, D., & Klingler, E. (2022). Heterogeneous fates of simultaneously-born neurons in the cortical ventricular zone. *Sci Rep*, 12(1), 6022. <https://doi.org/10.1038/s41598-022-09740-6>
- Manning, B. D., & Cantley, L. C. (2007). AKT/PKB signaling: navigating downstream. *Cell*, 129(7), 1261-1274. <https://doi.org/10.1016/j.cell.2007.06.009>
- Marin, O., Valiente, M., Ge, X., & Tsai, L. H. (2010). Guiding neuronal cell migrations. *Cold Spring Harb Perspect Biol*, 2(2), a001834. <https://doi.org/10.1101/cshperspect.a001834>
- Marsh, D. J., Dahia, P. L., Caron, S., Kum, J. B., Frayling, I. M., Tomlinson, I. P., Hughes, K. S., Eeles, R. A., Hodgson, S. V., Murday, V. A., Houlston, R., & Eng, C. (1998). Germline PTEN mutations in Cowden syndrome-like families. *J Med Genet*, 35(11), 881-885. <https://doi.org/10.1136/jmg.35.11.881>
- Marshall, C. A., Novitsch, B. G., & Goldman, J. E. (2005). Olig2 directs astrocyte and oligodendrocyte formation in postnatal subventricular zone cells. *J Neurosci*, 25(32), 7289-7298. <https://doi.org/10.1523/JNEUROSCI.1924-05.2005>

- Martinez-Cerdeno, V. (2017). Dendrite and spine modifications in autism and related neurodevelopmental disorders in patients and animal models. *Dev Neurobiol*, 77(4), 393-404. <https://doi.org/10.1002/dneu.22417>
- Martinez-Cerdeno, V., Cunningham, C. L., Camacho, J., Keiter, J. A., Ariza, J., Lovern, M., & Noctor, S. C. (2016). Evolutionary origin of Tbr2-expressing precursor cells and the subventricular zone in the developing cortex. *J Comp Neurol*, 524(3), 433-447. <https://doi.org/10.1002/cne.23879>
- Massague, J. (2004). G1 cell-cycle control and cancer. *Nature*, 432(7015), 298-306. <https://doi.org/10.1038/nature03094>
- McConnell, S. K. (1988). Fates of visual cortical neurons in the ferret after isochronic and heterochronic transplantation. *J Neurosci*, 8(3), 945-974. <https://doi.org/10.1523/JNEUROSCI.08-03-00945.1988>
- Moh, A., Iwamoto, Y., Chai, G. X., Zhang, S. S., Kano, A., Yang, D. D., Zhang, W., Wang, J., Jacoby, J. J., Gao, B., Flavell, R. A., & Fu, X. Y. (2007). Role of STAT3 in liver regeneration: survival, DNA synthesis, inflammatory reaction and liver mass recovery. *Lab Invest*, 87(10), 1018-1028. <https://doi.org/10.1038/labinvest.3700630>
- Molofsky, A. V., Krencik, R., Ullian, E. M., Tsai, H. H., Deneen, B., Richardson, W. D., Barres, B. A., & Rowitch, D. H. (2012). Astrocytes and disease: a neurodevelopmental perspective. *Genes Dev*, 26(9), 891-907. <https://doi.org/10.1101/gad.188326.112>
- Morris, N. R. (2000). Nuclear migration. From fungi to the mammalian brain. *J Cell Biol*, 148(6), 1097-1101. <https://doi.org/10.1083/jcb.148.6.1097>
- 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(5), 499-510. [https://doi.org/10.1016/s0092-8674\(00\)80860-0](https://doi.org/10.1016/s0092-8674(00)80860-0)
- Niethammer, M., Smith, D. S., Ayala, R., Peng, J., Ko, J., Lee, M. S., Morabito, M., & Tsai, L. H. (2000). NUDEL is a novel Cdk5 substrate that associates with LIS1 and cytoplasmic dynein. *Neuron*, 28(3), 697-711. [https://doi.org/10.1016/s0896-6273\(00\)00147-1](https://doi.org/10.1016/s0896-6273(00)00147-1)
- Nieto, M., Schuurmans, C., Britz, O., & Guillemot, F. (2001). Neural bHLH genes control the neuronal versus glial fate decision in cortical progenitors. *Neuron*, 29(2), 401-413. [https://doi.org/10.1016/s0896-6273\(01\)00214-8](https://doi.org/10.1016/s0896-6273(01)00214-8)
- Noctor, S. C., Martinez-Cerdeno, V., Ivic, L., & Kriegstein, A. R. (2004). Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci*, 7(2), 136-144. <https://doi.org/10.1038/nn1172>
- Oberst, P., Fievre, S., Baumann, N., Concetti, C., Bartolini, G., & Jabaudon, D. (2019). Temporal plasticity of apical progenitors in the developing mouse neocortex. *Nature*, 573(7774), 370-374. <https://doi.org/10.1038/s41586-019-1515-6>
- Ozawa, T., Riester, M., Cheng, Y. K., Huse, J. T., Squatrito, M., Helmy, K., Charles, N., Michor, F., & Holland, E. C. (2014). Most human non-GCIMP glioblastoma subtypes evolve from a common proneural-like precursor glioma. *Cancer Cell*, 26(2), 288-300. <https://doi.org/10.1016/j.ccr.2014.06.005>
- Park, H. C., & Appel, B. (2003). Delta-Notch signaling regulates oligodendrocyte specification. *Development*, 130(16), 3747-3755. <https://doi.org/10.1242/dev.00576>
- Pearson, C. A., Moore, D. M., Tucker, H. O., Dekker, J. D., Hu, H., Miquelajauregui, A., & Novitsch, B. G. (2020). Foxp1 Regulates Neural Stem Cell Self-Renewal and Bias Toward Deep Layer Cortical Fates. *Cell Rep*, 30(6), 1964-1981 e1963. <https://doi.org/10.1016/j.celrep.2020.01.034>
- Phillips, M., & Pozzo-Miller, L. (2015). Dendritic spine dysgenesis in autism related disorders. *Neurosci Lett*, 601, 30-40. <https://doi.org/10.1016/j.neulet.2015.01.011>
- Pierfelice, T., Alberi, L., & Gaiano, N. (2011). Notch in the vertebrate nervous system: an old dog with new tricks. *Neuron*, 69(5), 840-855. <https://doi.org/10.1016/j.neuron.2011.02.031>
- Qian, X., Nguyen, H. N., Jacob, F., Song, H., & Ming, G. L. (2017). Using brain organoids to understand Zika virus-induced microcephaly. *Development*, 144(6), 952-957. <https://doi.org/10.1242/dev.140707>
- Qian, X., Nguyen, H. N., Song, M. M., Hadiono, C., Ogden, S. C., Hammack, C., Yao, B., Hamersky, G. R., Jacob, F., Zhong, C., Yoon, K. J., Jeang, W., Lin, L., Li, Y., Thakor, J., Berg, D. A., Zhang, C., Kang, E., Chickering, M., . . . Ming, G. L. (2016). Brain-Region-Specific Organoids Using Mini-bioreactors for Modeling ZIKV Exposure. *Cell*, 165(5), 1238-1254. <https://doi.org/10.1016/j.cell.2016.04.032>
- Rademacher, S., & Eickholt, B. J. (2019). PTEN in Autism and Neurodevelopmental Disorders. *Cold Spring Harb Perspect Med*, 9(11). <https://doi.org/10.1101/cshperspect.a036780>
- Rakic, P. (2009). Evolution of the neocortex: a perspective from developmental biology. *Nat Rev Neurosci*, 10(10), 724-735. <https://doi.org/10.1038/nrn2719>

- Reiner, O., Albrecht, U., Gordon, M., Chianese, K. A., Wong, C., Gal-Gerber, O., Sapir, T., Siracusa, L. D., Buchberg, A. M., Caskey, C. T., & et al. (1995). Lissencephaly gene (LIS1) expression in the CNS suggests a role in neuronal migration. *J Neurosci*, 15(5 Pt 2), 3730-3738. <https://doi.org/10.1523/JNEUROSCI.15-05-03730.1995>
- Rizo, J., & Sudhof, T. C. (1998). C2-domains, structure and function of a universal Ca²⁺-binding domain. *J Biol Chem*, 273(26), 15879-15882. <https://doi.org/10.1074/jbc.273.26.15879>
- rodents, F. w. g. o. r. o. g. f. h. m. o., rabbits, Mahler Convenor, M., Berard, M., Feinstein, R., Gallagher, A., Illgen-Wilcke, B., Pritchett-Corning, K., & Raspa, M. (2014). FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab Anim*, 48(3), 178-192. <https://doi.org/10.1177/0023677213516312>
- Sacco, R., Cacci, E., & Novarino, G. (2018). Neural stem cells in neuropsychiatric disorders. *Curr Opin Neurobiol*, 48, 131-138. <https://doi.org/10.1016/j.conb.2017.12.005>
- Sakamoto, K., Wehde, B. L., Radler, P. D., Triplett, A. A., & Wagner, K. U. (2016). Generation of Janus kinase 1 (JAK1) conditional knockout mice. *Genesis*, 54(11), 582-588. <https://doi.org/10.1002/dvg.22982>
- Samuels, I. S., Karlo, J. C., Faruzzi, A. N., Pickering, K., Herrup, K., Sweatt, J. D., Saitta, S. C., & Landreth, G. E. (2008). Deletion of ERK2 mitogen-activated protein kinase identifies its key roles in cortical neurogenesis and cognitive function. *J Neurosci*, 28(27), 6983-6995. <https://doi.org/10.1523/JNEUROSCI.0679-08.2008>
- Santello, M., Toni, N., & Volterra, A. (2019). Astrocyte function from information processing to cognition and cognitive impairment. *Nat Neurosci*, 22(2), 154-166. <https://doi.org/10.1038/s41593-018-0325-8>
- Sasaki, S., Shionoya, A., Ishida, M., Gambello, M. J., Yingling, J., Wynshaw-Boris, A., & Hirotsune, S. (2000). A LIS1/NUDEL/cytoplasmic dynein heavy chain complex in the developing and adult nervous system. *Neuron*, 28(3), 681-696. [https://doi.org/10.1016/s0896-6273\(00\)00146-x](https://doi.org/10.1016/s0896-6273(00)00146-x)
- Schmechel, D. E., & Rakic, P. (1979). A Golgi study of radial glial cells in developing monkey telencephalon: morphogenesis and transformation into astrocytes. *Anat Embryol (Berl)*, 156(2), 115-152. <https://doi.org/10.1007/BF00300010>
- Selcher, J. C., Nekrasova, T., Paylor, R., Landreth, G. E., & Sweatt, J. D. (2001). Mice lacking the ERK1 isoform of MAP kinase are unimpaired in emotional learning. *Learn Mem*, 8(1), 11-19. <https://doi.org/10.1101/lm.37001>
- Sibilia, M., Steinbach, J. P., Stingl, L., Aguzzi, A., & Wagner, E. F. (1998). A strain-independent postnatal neurodegeneration in mice lacking the EGF receptor. *EMBO J*, 17(3), 719-731. <https://doi.org/10.1093/emboj/17.3.719>
- Singh, S., Kim, H., Ecevitoglu, A., Chasse, R., Ludko, A. M., Sanganahalli, B., Gangasandra, V., Park, S. R., Yee, S. P., Grady, J., Salamone, J., Holly Fitch, R., Spellman, T., Hyder, F., & Bae, B. I. (2025). Autism-associated ASPM variant causes macrocephaly and social-cognitive deficits in mice. *bioRxiv*. <https://doi.org/10.1101/2025.02.17.638753>
- Sloan, S. A., & Barres, B. A. (2014). Mechanisms of astrocyte development and their contributions to neurodevelopmental disorders. *Curr Opin Neurobiol*, 27, 75-81. <https://doi.org/10.1016/j.conb.2014.03.005>
- Song, M. S., Salmena, L., & Pandolfi, P. P. (2012). The functions and regulation of the PTEN tumour suppressor. *Nat Rev Mol Cell Biol*, 13(5), 283-296. <https://doi.org/10.1038/nrm3330>
- Stahl, N., Farruggella, T. J., Boulton, T. G., Zhong, Z., Darnell, J. E., Jr., & Yancopoulos, G. D. (1995). Choice of STATs and other substrates specified by modular tyrosine-based motifs in cytokine receptors. *Science*, 267(5202), 1349-1353. <https://doi.org/10.1126/science.7871433>
- Sun, M., Gao, Y., Li, Z., Yang, L., Liu, G., Xu, Z., Guo, R., You, Y., & Yang, Z. (2024). ERK signaling expands mammalian cortical radial glial cells and extends the neurogenic period. *Proc Natl Acad Sci U S A*, 121(13), e2314802121. <https://doi.org/10.1073/pnas.2314802121>
- Sun, Y., Goderie, S. K., & Temple, S. (2005). Asymmetric distribution of EGFR receptor during mitosis generates diverse CNS progenitor cells. *Neuron*, 45(6), 873-886. <https://doi.org/10.1016/j.neuron.2005.01.045>
- Tang, H., Hammack, C., Ogden, S. C., Wen, Z., Qian, X., Li, Y., Yao, B., Shin, J., Zhang, F., Lee, E. M., Christian, K. M., Didier, R. A., Jin, P., Song, H., & Ming, G. L. (2016). Zika Virus Infects Human Cortical Neural Progenitors and Attenuates Their Growth. *Cell Stem Cell*, 18(5), 587-590. <https://doi.org/10.1016/j.stem.2016.02.016>
- Taverna, E., Gotz, M., & Huttner, W. B. (2014). The cell biology of neurogenesis: toward an understanding of the development and evolution of the neocortex. *Annu Rev Cell Dev Biol*, 30, 465-502. <https://doi.org/10.1146/annurev-cellbio-101011-155801>

- Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., & Cantley, L. C. (1994). Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. *J Biol Chem*, 269(51), 32358-32367. <https://www.ncbi.nlm.nih.gov/pubmed/7798235>
- Torres-Ceja, B., & Olsen, M. L. (2022). A closer look at astrocyte morphology: Development, heterogeneity, and plasticity at astrocyte leaflets. *Curr Opin Neurobiol*, 74, 102550. <https://doi.org/10.1016/j.conb.2022.102550>
- Tsai, K. K., Bae, B. I., Hsu, C. C., Cheng, L. H., & Shaked, Y. (2023). Oncogenic ASPM Is a Regulatory Hub of Developmental and Stemness Signaling in Cancers. *Cancer Res*, 83(18), 2993-3000. <https://doi.org/10.1158/0008-5472.CAN-23-0158>
- Villalba, A., Gotz, M., & Borrell, V. (2021). The regulation of cortical neurogenesis. *Curr Top Dev Biol*, 142, 1-66. <https://doi.org/10.1016/bs.ctdb.2020.10.003>
- Voigt, T. (1989). Development of glial cells in the cerebral wall of ferrets: direct tracing of their transformation from radial glia into astrocytes. *J Comp Neurol*, 289(1), 74-88. <https://doi.org/10.1002/cne.902890106>
- von Mering, C., Huynen, M., Jaeggi, D., Schmidt, S., Bork, P., & Snel, B. (2003). STRING: a database of predicted functional associations between proteins. *Nucleic Acids Res*, 31(1), 258-261. <https://doi.org/10.1093/nar/gkg034>
- Wamsley, B., & Fishell, G. (2017). Genetic and activity-dependent mechanisms underlying interneuron diversity. *Nat Rev Neurosci*, 18(5), 299-309. <https://doi.org/10.1038/nrn.2017.30>
- Wang, S., & Barres, B. A. (2000). Up a notch: instructing gliogenesis. *Neuron*, 27(2), 197-200. [https://doi.org/10.1016/s0896-6273\(00\)00028-3](https://doi.org/10.1016/s0896-6273(00)00028-3)
- Wang, S. I., Puc, J., Li, J., Bruce, J. N., Cairns, P., Sidransky, D., & Parsons, R. (1997). Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res*, 57(19), 4183-4186. <https://www.ncbi.nlm.nih.gov/pubmed/9331071>
- Watanabe, M., Buth, J. E., Vishlaghi, N., de la Torre-Ubieta, L., Taxidis, J., Khakh, B. S., Coppola, G., Pearson, C. A., Yamauchi, K., Gong, D., Dai, X., Damoiseaux, R., Aliyari, R., Liebscher, S., Schenke-Layland, K., Caneda, C., Huang, E. J., Zhang, Y., Cheng, G., . . . Novitsch, B. G. (2017). Self-Organized Cerebral Organoids with Human-Specific Features Predict Effective Drugs to Combat Zika Virus Infection. *Cell Rep*, 21(2), 517-532. <https://doi.org/10.1016/j.celrep.2017.09.047>
- Worby, C. A., & Dixon, J. E. (2014). Pten. *Annu Rev Biochem*, 83, 641-669. <https://doi.org/10.1146/annurev-biochem-082411-113907>
- Yang, X., Klein, R., Tian, X., Cheng, H. T., Kopan, R., & Shen, J. (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev Biol*, 269(1), 81-94. <https://doi.org/10.1016/j.ydbio.2004.01.014>
- Yang, Y., Shao, N., Luo, G., Li, L., Zheng, L., Nilsson-Ehle, P., & Xu, N. (2010). Mutations of PTEN gene in gliomas correlate to tumor differentiation and short-term survival rate. *Anticancer Res*, 30(3), 981-985. <https://www.ncbi.nlm.nih.gov/pubmed/20393024>
- Yuste, R., & Denk, W. (1995). Dendritic spines as basic functional units of neuronal integration. *Nature*, 375(6533), 682-684. <https://doi.org/10.1038/375682a0>
- Zhang, X., Mennicke, C. V., Xiao, G., Beattie, R., Haider, M. A., Hippenmeyer, S., & Ghashghaei, H. T. (2020). Clonal Analysis of Gliogenesis in the Cerebral Cortex Reveals Stochastic Expansion of Glia and Cell Autonomous Responses to Egfr Dosage. *Cells*, 9(12). <https://doi.org/10.3390/cells9122662>
- Zhang, X., Xiao, G., Johnson, C., Cai, Y., Horowitz, Z. K., Mennicke, C., Coffey, R., Haider, M., Threadgill, D., Eliscu, R., Oldham, M. C., Greenbaum, A., & Ghashghaei, H. T. (2023). Bulk and mosaic deletions of Egfr reveal regionally defined gliogenesis in the developing mouse forebrain. *iScience*, 26(3), 106242. <https://doi.org/10.1016/j.isci.2023.106242>
- Zong, H., Espinosa, J. S., Su, H. H., Muzumdar, M. D., & Luo, L. (2005). Mosaic analysis with double markers in mice. *Cell*, 121(3), 479-492. <https://doi.org/10.1016/j.cell.2005.02.012>

