The Effects of MAPK Signaling on the Development of Cerebellar Granule Cells

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The Effects of MAPK Signaling on the Development of Cerebellar Granule Cells

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University Scholar and Honors Thesis

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The granule cells are the most abundant neuronal type in the human brain. Rapid proliferation of granule cell progenitors results in dramatic expansion and folding of the cerebellar cortex during postnatal development. Mis-regulation of this proliferation process causes medulloblastoma, the most prevalent childhood brain tumor. In the developing cerebellum, granule cells are derived from Atoh1-expressing cells, which arise from the upper rhombic lip (the interface between the roof plate and neuroepithelium). In addition to granule cells, the Atoh1 lineage also gives rise to different types of neurons including cerebellar nuclei neurons. In the current study, I have investigated the mechanisms that regulate the proliferation of cerebellar granule cells and the diversification of the Atoh1 lineage. The research presented in Chapter 1 elucidates a previously underappreciated role of FGF/MAPK pathway signaling in granule cell (GC) development and cerebellar morphogenesis. Here, we consider the involvement of MAPK signaling in the development of the Atoh1 lineage of granule cell precursors (GCP) in the cerebellum. Using mice with upregulation of the MAPK intermediate, MEK1, and mice with downregulation of the proliferative readout Etv4 gene, the regulatory effects of MAPK signaling were observed and interpreted through phenotype analysis. The results showed distinct morphological differences between the mutants and the wildtypes, including irregularities in foliation patterning of the central lobe, changes in lobule sizes, and discontinuities in the developing external granular layer (EGL) and internal granular layer (IGL). The measured cerebellar foliation index was indeed increased in MEK gain-of-function (MEK-GOF) mutants, but oddly enough the internal granular layer (IGL) area at maturity was decreased compared to the WT. Further investigation of the MEK-GOF mutants revealed ectopic expression of a neural progenitor gene called Sox2 in the EGL of late stage postnatal mice and
ectopic expression of the MAPK gene *Tlx3*, indicating that GCP continue to proliferate longer than expected due to induction of MAPK activity in non-endogenous tissues. Furthermore, analyses showed that sustained GCP presence in the EGL did not seem to affect total GC number, but may contribute to the foliation and expansion phenotypes in seen in MEK-GOF and may also provide insight for division mechanisms in overexpression of MAPK signaling. In addition to examining the signaling and effects of GCP populations during development, the origin of *Atoh1* neural precursor lineages was investigated in lineage tracing experiments presented in *Chapter II*. While the rhombic lip has historically been identified as the sole progenitor region for *Atoh1* glutamatergic stem cell lineages, we propose an early developmental origin for subpopulations of glutamatergic *Atoh1* cells in the ventricular zone (VZ). Early embryonic analyses of *Atoh1* and Cre expression at E10.5 revealed clonal expansion of a novel *Atoh1* lineage demonstrating oscillating expression from the VZ prior to commitment as deep cerebellar nuclei (DCN).
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CHAPTER I: Overview of Cerebellar Development

INTRODUCTION

Significance of Cerebellar Research

The cerebellum is classically known for controlling coordination and movement, but recent research suggests the cerebellum contributes to a more extensive role in higher cognitive functioning (Ackermann, 2013). The human cerebellum contains a central region called the vermis and two large, bilateral hemispheres connected on both sides of the vermis, for which language and enhanced cognitive functions have been implicated (Fabbro, 2000). In rare genetic cases, malformation of hemispheres primarily leads to lesions and communication defects in an otherwise healthy brain (Fabbro et al., 2003). Other research also indicates that many patients with chronic schizophrenia experience noticeable reductions in cerebellar vermis size, which has related schizophrenic origins to developmental defects from the cerebellar vermis (Ichimiya et al, 2001). Further research in areas of human disease have revealed associations between autism spectrum disorder (ASD) and developmental impairments in lobules VI and VII of the cerebellar hemispheres (Courchesne et al., 1988). Despite extensive research efforts targeting mechanisms behind the developmental consequences from characterized cerebellar morphological impairments, there remains very limited understanding in these areas of human disease.

Another significant area of cerebellar research that requires further investigation involves understanding the specific tumor pathologies behind most common pediatric neurological cancer, medulloblastoma. This type of cancer is caused by a number of identified genetic mutations in granule cells (GC) of the cerebellum, leading to uncontrolled cell division and tumor formation (Salsano et al., 2004). There are many identified mutation subtypes in medulloblastomas, many of which involve the Shh signaling pathway. As more becomes known
from research on medulloblastoma mutations, it has become clear from the few clinical options provided thus far, that tumor-specific molecular therapies are extremely effective, and the ability to identify these treatments will be the future of cancer interventions. Unfortunately, much is still unknown in this realm, and molecular therapies require enhanced understandings for tumor pathology and the genetic underpinnings behind each individual cancer. In order to apply these diverse treatments and improve cancer outcomes, more research must first be done in almost every area leading up to this end-goal. In terms of medulloblastoma, this starts with identifying the mutations for all specific subtypes. Following this, we must build a comprehensive understanding of what leads to disease pathologies, what goes wrong, what is the effect. Finally, these uncovered areas of disease could provide many enhanced opportunities for diagnoses, prevention, screening, and genetic treatment targeting as part of standard practice.

While many studies have enhanced our understanding of refined cerebellar function and cerebellum-related diseases, there is still a need for a more comprehensive understanding of the molecular underpinnings contributing to developmental control and pathologies within the cerebellum. Research in this area of development has the potential to uncover many possible treatment options and an in depth pathological understanding of ailments that affect a significant portion of the population.

**Cerebellar Composition and Structure**

The cerebellum, a significant unit of the hindbrain, is organized into a highly compacted, yet complex structure with several diverse cell types. While there are many different organizational levels of the cerebellum, the two main divisions of internal structure include the cerebellar nuclei (CN) and the cerebellar cortex. The cerebellar cortex covers the surface of the
cerebellum and contains three cellular layers that are crucial for successful development and function of neural circuitry. These three layers in the adult cerebellar cortex include (from most exterior moving inward): the molecular layer (ML), the Purkinje cell layer (PCL), and the internal granular layer (IGL) (Atlman and Bayer, 1997). Each of these layers contain distinct types of neurons, including several GABAergic (inhibitory) interneurons and glutamatergic (excitatory) interneurons, most notably the glutamatergic granule cells (GC). The GC are the most abundant cell type in the mature cerebellum, and GC arise from granule cell precursors (GCP) that proliferate in the external granular layer (EGL) during development. After GCP begin to differentiate into mature GC, they migrate inward to populate the internal granular layer (IGL) of the adult cerebellum indefinitely. The innermost division of the cerebellum, the cerebellar nuclei (also known as the deep cerebellar nuclei (DCN)), contain the neural circuitry fibers that connect afferent and efferent projections to the cerebellar cortex neurons along with integration of signals from within the brain and spinal cord (Armstrong & Hawkes, 2000).

In addition to its diverse abundance of cell types and organizational layers, the cerebellar cortex has a characteristically conserved patterning of lobes defined by discrete layers of cellular composition. In an external frontal view, the cerebellum contains the vermis and two identical, lateral hemispheres on each side (Ben-Arie et al., 1997). However, within this regional distinction, the cerebellum can be further separated into ten lobules formed by gyrifications that radiate inward (Figure 1). While there are some nuanced variations in the advanced organization of cerebellar sub-lobules in rodents and humans, many of the main structural distinctions described are consistent among species. The most common method of organizing and identifying lobules in adult mice is by splitting the sagittal midline of the cerebellum into four lobule-specific transverse zones (Hallonet et al., 1990). As shown in Figure 1, these zones include
(from most anterior to most posterior): the anterior zone, the central zone, the posterior zone, and the nodular zone, which resides underneath the posterior portion of the cerebellum.

As these highly-preserved lobules form throughout development, several characteristic milestones can be temporally observed in these dynamic processes. One cell type in particular, the granule cells, is of particular interest due to the substantial proliferation that occurs in this cell population, along with the regulated differentiation processes that contribute to the cerebellum’s highly-preserved lobule structure. The granule cells (GC) comprise a significant portion of the adult cerebellum, but the GC proliferation mechanisms start fairly late in development, and the composition changes dramatically in this time as well. The granule cell precursors (GCP) are first seen from the rhombic lip (RL) at approximately E14 as these cells tangentially migrate to form the beginning of external granular layer (EGL) (Wang & Zoghbi, 2001). At this point in development, the EGL continues to expand at a significant rate as GCP divide indefinitely. The highest-yield proliferative period for GCP is observed within the first two weeks of postnatal development, but markedly ends when all GCP have exited the cell cycle, differentiated, and migrated laterally into the newly formed internal granular layer (IGL) at postnatal day (P) 15 (Bae et al., 2000).

Perhaps the most notable feature of GCP development is the 1000-fold increase in cerebellar volume as the EGL compensates by expanding and foliating inward, eventually giving rise to the lobules of the adult cerebellum (Broccoli et al., 1999). Each of the ten lobules have a remarkably distinct pattern of foliation induction, position, and general size, but the mechanism driving the signals for these foliation events remain relatively unclear. Since the developmental time period where foliation begins and is completed almost exclusively coincides with major milestones in granule cell development, this evidence suggests a relationship between GCP and

Morgan
cerebellar foliation processes (Herrup, 1983). While it is highly probable that granule cell proliferation and differentiation processes could play a role in inducing and maintaining precise cerebellar foliation cues, the process of identifying a mechanism must be elucidated before this conclusion can be considered. Since the process of foliation and GC expansion in mice occurs predominantly within the first two weeks of postnatal development, this serves as a useful time period for investigation of the mechanisms behind lobule formation cues. By birth (E19.5), the first structural evidence of lobule formation is observed as four main fissures form on the vermis surface (Smeyne et al., 1995). These initial fissures become the basis of five main lobes, including: the anterobasal lobe, the anterodorsal lobe, the central lobe, the posterior lobe, and the inferior lobe (Dahmane et al., 1999). As postnatal development continues, additional fissures form by P8 to become the ten lobules seen in the adult cerebellum (Sillitoe et al., 2007). The distinct lobules continue to grow outward until around P15-P21, but no further gyrifications form after the initial lobulation events described (Cheng et al., 2010).
Figure 1. This schematic shows the ten lobules of the mouse cerebellum. The image labeled A represents an approximately midline sagittal section of the adult mouse cerebellum. The anterior domain (lobules I-V) are colored blue, the central domain (lobules VIa, VIb, and VII) are colored green, the posterior domain (lobule VIII and part of lobule IX) is colored yellow, and the nodular domain (lobule X and part of IX) is colored yellow. Image B in the bottom portion of the figure is a coronal whole mount of the cerebellum. The shaded areas in the middle show different lobules within the vermis (midline) region.

Granule Cell Precursor Development

The gene Atoh1 encodes a basic helix loop helix (BHLH) transcription factor that plays a pivotal role in many aspects of development, including the cerebellar glutamatergic neurons, inner ear sensory cells, dorsal spinal cord, brainstem, and secretory cells of the intestine (Morales
& Hatten, 2006). During development, specifically in the GCP of the cerebellum, expression of Atoh1 promotes cell proliferation (Henver et al., 2006). The GCP universally express Atoh1 as they arise from the rhombic lip (RL) at E14, and expression of Atoh1 is sustained up until differentiation of GCP into mature granule neurons (Karam et al., 2000). In the process of cell cycle exit, the Atoh1 transcription factor has been identified as vitally important in signal pathway regulation and termination of cell cycle activities for differentiation within the EGL, therefore making this a particularly useful gene in investigating GCP development (Puelles et al., 2004).

In order to understand the complex effects of granule cell populations from cerebellar developmental processes, it is important to first consider the many factors contributing to proliferation of granule cell precursors (GCP) in the EGL, as well as granule cell differentiation into the IGL. While there are identified molecular signaling pathways that have been considered for a role in regulating and inducing the proliferative activities of GCP, including Shh, none have been able to account for the developmental complexity and variability seen in GCP proliferative processes. Currently, the Shh signaling pathway has been implicated as the predominantly accepted pathway for regulating GCP developmental processes. Research involving three different Shh LOF knockouts for each of the functional pathway genes Gli1, Gli2, and Gli3 have demonstrated mandatory Shh pathway activities in GCP development (Corrales et al., 2004). Specifically, the Gli1 knockout demonstrated no changes in cerebellar morphology, but Gli1 is necessarily expressed when the Shh pathway is active, this gene is frequently used in determining Shh activity (Bai et al., 2004). Additionally, Gli1 expression is seen in proliferating GCP of the oEGL, but is turned off once differentiated cells exit the EGL. Most important evidence for Shh functional necessity in proper GCP development was found from Gli2
knockouts. These LOF mutants experienced significant morphological changes, including reductions in cerebellar size and GCP number, and a loss of normal foliation patterning in lobules. Furthermore, it was also determined that Gli2 is a mandatory activator for turning on the $Shh$ pathway, and expression of the redundant activator, Gli1, is essential for sustained $Shh$ activity (Corrales et al., 2004).

Some additional pathways that may have some significance in GCP development include the activities of Notch signaling pathway and downstream expression of $Mbh$ genes from the Atoh1 transcriptional cascade, but it is possible that an integration of many pathways contribute to regulated differentiation of specified populations of granule cells (Kawauchi et al., 2008). The size of the EGL and abundance of GC alone may imply a more spatially driven approach to cohort differentiation processes within the foliated lobules and transverse zones of the EGL. When considering $Shh$ signaling in particular, the readout genes Gli1, Gli2, and Ptc1 show consistent expression throughout all transverse zones of the cerebellum but are absent within the distinct central lobe region (Bai et al., 2002). This observation combined with evidence of multipathway contributions in GCP development suggests that GCP development may in fact proceed through an aggregation of population-specific signals with several moving parts (Smeyne et al., 1995). Additionally, previous in-house research efforts from scRNAseq data has revealed evidence of defined PC subtypes, which may ultimately contribute to the observation of diverse developmental programming comprising GCP populations, specifically in the characteristic late differentiation of GC seen in the central lobe and other signaling pathways involved in GCP development (Wizeman et al., 2019). Furthermore, this concept of specified signals in population-dependent GCP differentiation processes may also provide further insight into other vague processes during development that remain relatively unclear. However, we must first
investigate the underlying components of GCP proliferative signaling, specifically in determining whether differential signals for defined regions of GCP can be elucidated in our approach to understanding developmental complexity of GCP.

Due to the specificity of these processes and the substantial increases in GC populations, many have speculated that the formation of the lobules may occur through temporal cues, spatial cues, or a combination of both. Although a specific developmental mechanism for this process has yet to be identified, one proposed mechanism involves Shh signaling as a spatial and temporal component contributing to cerebellar fissure formation (Corrales et al., 2004). The Shh readout gene, Gli2, was identified in this mechanism as an activator for initiating GCP proliferation in the EGL. Through the ablation of Purkinje cells (PC), which secrete Shh starting at E17.5, a substantial reduction in GCP proliferation and cerebellar foliation was observed. Further investigation also showed that Shh expression is restricted to areas of foliation induction at E17.5. In vitro cultures also showed that Shh sustains GCP proliferation, which further elaborates a connection between GCP development and foliation, but with an emphasis on a Shh-driven mechanism. However, removal of Shh signaling prior to E16.5, before PC start to secrete Shh, shows a complete dysfunction of GCP development, and ultimately a loss of foliation. Similarly, Gli2 knockouts showed delays in foliations, but this may also have been a result of reduced GCP proliferation. While this evidence shows a strong relationship between Shh and cerebellar foliation, it remains unclear as to whether the role of Shh is implicated solely in GCP development, which is ultimately what defines foliation instead. Additionally, presence of Shh is sufficient to induce foliation, but the underlying mechanism is still unclear (Corrales et al., 2004). For this reason, one critical aim of the research presented in this paper is to establish
evidence of a granule cell-driven signaling mechanism for lobule foliation and growth, while also proposing potential molecular contributions for the maintenance of foliation events.

One of the initial signals identified in cerebellar development involves FGF protein signaling (Wanaka et al., 1990). There are many different types of FGF proteins, but the expression of the protein FGF8 in the isthmus in particular appears to be required for the formation and organization of the midbrain/hindbrain boundary and early proliferation (Wang et al., 2005). The FGF8 growth factor acts on cells with a compatible membrane receptor, which transduces a signaling cascade within the cell. This pathway, known as the mitogen activated protein kinase (MAPK) signal cascade, includes many different proteins and effectors that amplify a signal for downstream gene expression. One protein in particular, known as MEK1 and encoded by the gene MAP2K1, acts as an essential component of the MAPK signaling pathway. At the end of this cascade, the downstream signaling molecules enter the nucleus and regulate several important genes for cell proliferation. The Etv4/5 genes in particular encode an important transcription factor that contributes to early cerebellar development and proliferation (Fontanet et al., 2013). By manipulating certain intermediates and processes within MAPK signaling, this pathway serves as a useful method to observe the outcomes and effects of altered FGF proliferative signals in granule cell precursor populations (GCP).

Additionally, some Shh mutations implicated in medulloblastoma subtypes show increased levels of Atoh1 expression, which contributes to aspects of uncontrolled cell growth seen in tumorigenesis (Sidman et al., 1962). Thus, an additional understanding of developmental processes and signaling mechanisms contributing to the Atoh1 lineage of cells in GCP may reveal significant insights in the development of medulloblastoma and other implicated diseases. While Shh has been identified as an influential component of GCP development, there are still
many unknowns in regard to the mechanisms of action, and gaps continue to prevail in the current understanding of alternative signaling contributions. When combined with the knowledge of GCP developmental processes playing a potential role in cerebellar foliation patterning and induction, research in the area of uncovering specific GCP proliferative pathways may lead to a more comprehensive understanding of the cerebellum at large.

**Figure 2.** This figure is a very basic depiction of the MAPK signal cascade in response to fibroblast growth factors binding to the plasma membrane. Each major effector in the pathway is represented in descending order. While *Env4* is not shown, it is one of the many “transcription factors” accounted for in the nucleus portion of the diagram.
Figure 3. This timeline illustrates a summary of significant milestones during cerebellar development. Granule cell neurogenesis initiates at around E14-E15 from the rhombic lip. Between P3 and P12 is when granule cells populate and proliferate in the thickening EGL. By P15, the majority of granule cells differentiate, exit the cell cycle, and migrate to the IGL.

Figure 4. This figure serves as a reference for lobule structure and composition throughout postnatal development in mice. The top row of four images (A) show a full midline sagittal view
of four different stages (P2, P6, P10, P14), labelled from left to right. The P14 image on the far-right labels each lobule I-X. The bottom row of images (B) show a magnified view of the cellular layers for each of the four postnatal stages. The dark purple areas indicate granule cells. This figure shows the gradual accumulation of granule cells in the EGL from P2-P6, and the migration of these granule cells inward to the IGL during P10-P14. (IGL= inner granule layer, EGL= external granule layer, ML= molecular layer).

OBJECTIVES OF THE STUDY

A key component of the research presented here focuses on the potential effects of MAPK signaling on the Atoh1 lineage of GCP in the cerebellum throughout different time points in mouse development, specifically in the central lobe. We aim to understand the phenotypic effects that result from manipulation of MAPK signaling, with the ultimate goal of uncovering the relationship of MAPK in the GCP proliferative processes of the central lobe. In addition to determining a relationship between MAPK signaling and the development of GCP, further analysis in this area may also provide cell cycle insights throughout postnatal development, along with GC differentiation processes. Using the Atoh1 gene as an enhancer, a genetic model was made to upregulate and downregulate the MAPK pathway signaling in the Atoh1 specific GCP populations in the cerebellum. In order to manipulate the Atoh1 lineage of cells, we used the Cre enzyme to constitutively express two genes of interest with the Atoh1 enhancer gene on the Rosa 26 locus. The first mouse model upregulated the MAPK signaling pathway, by constitutively expressing the MEK1 gene, which is responsible for an intermediate protein in the MAPK pathway. In order to model the downregulation of MAPK, the expression of the proliferative Etv4 gene was inhibited by inserting a dominant negative gene (Etv4-DN) that has a DNA-binding domain capable of repressing Etv4 in the central lobe. The repressor gene for Etv4 (Etv4-DN) was inserted on the R26 locus for Cre dependent expression in Atoh1 cells. This dominant negative gene successfully binds to the Etv4 gene, inhibiting its transcription in Atoh1.
cells of the central lobe, and ultimately reducing GCP proliferation from the MAPK pathway. In these mouse models, one component worth noting is the differing domains of expression between the MEK1 and Etv4-DN genes. The Etv4-DN expression is directed towards the central lobe, whereas the MEK1 model is expected to show expression throughout all Atoh1 cells of the cerebellum. For the purposes of this research, we will restrict comparison of MAPK manipulation results to specifically the central lobe regions.

In Chapter I of this study, we investigate the heterogeneity of GCP during development through use of compositional analyses from gene expression data in GCP, that may ultimately provide evidence for identifying specific GCP characteristics. From this, we aim to elucidate a role of MAPK signaling in GCP development. In our efforts to understand the signals driving GCP proliferation, we will investigate the effects of upregulating and downregulating MAPK activity in GOF and LOF models for phenotypic analysis. To further understand whether a specific function of MAPK signaling exists in regulating GCP development, comparisons with already established signaling mechanisms in GCP development is expected explain possible similarities or difference that may be used in ascribing developmental functions for MAPK.

CHAPTER II: Regulation of granule cell progenitors by MAPK signaling

RESULTS

Expression Data from Single-Cell RNA Sequencing Reveals Distinct GCP Subtypes

In initial single-cell RNA sequencing (scRNA seq) analyses of GCP heterogeneity at stages E16.5 and E18.5 of WT mice, four distinct expression profiles became apparent for identifying and separating GCP subtypes into gene-specific clusters (Figure 5, A). The GCP1 profile was mainly enriched for expression of Tlx3, Etv4/5, Dusp4/6, Eya1, Otx2 and Fgf8.
However, GCP1 was the only subtype to be enriched for *Tlx3, Fgf8*, and *Etv4* (Figure 5, B), all of which are involved in MAPK signaling activities. This evidence of *Tlx3* and *Etv4/5* expression initially suggested that input from the signaling MAPK pathway could potentially function in promoting proliferative activities in a portion of granule cells during development, in some capacity. The MAPK pathway is most commonly activated in response to receptor binding of fibroblast growth factors (FGF) from the FGF family of proteins, and the cascade proceeds by initiating transcription of a collection of genes involved in promoting cellular growth and division. The GCP1 subtype also showed enrichment for expression of a specific MAPK growth factor *Fgf8*, which provided additional evidence to suggest that this GCP1 subtype was experiencing MAPK activity.

The remaining subtypes (GCP2, GCP3, and GCP4) had more overlap in expression profiles and showed more overall similarity to one another than with the GCP1 profile of enriched genes. All subtypes showed enrichment for *Atoh1* and *Gli2* as expected, but uniform expression the essential readout gene *Gli1* from *Shh* signaling was not present, and in fact was evidently only expressed in the GCP3 subtype (Figure 5, B). This observation contradicts the widely accepted *Shh* signaling mechanism as a mandatory regulator for GCP development and proliferation. The results found from the scRNAseq data did demonstrate significant enrichment for *Shh* readouts, however these expression profiles were clearly restricted to the GCP3 cluster, and the remaining three GCP subtypes showed essentially no evidence of *Gli1* readout expression from the proposed mechanism of *Shh* signaling. Furthermore, this evidence proposes an alternative concept for GCP development involving different proliferative mechanisms for groups of independent GCPs to proceed in a subtype-specific manner.
In order to confirm the results from the scRNAseq analyses, and also spatially observe the location of the proposed GCP subtypes, immunohistochemistry (IHC) experiments were performed. An IHC experiment performed on Gli1-lacZ transgenic mice at P1 was used to compare the domains of expression for Tlx3 and Gli1, which scRNAseq previously identified as two unrelated genes enriched in GCP1 and GCP3, respectively (Figure 5, D). Results from the experiment showed very limited co-expression of the Tlx3/Gli1 markers, but notably high amounts of Tlx3 expression within the restricted boundaries of the developing central lobe EGL region. At P1 in GCP development, the entirety of Tlx3 expression was observed in GCPs populating the future central lobe region in a highly specific manner. Several days later in GCP development, Tlx3 expression expands beyond the boundaries seen at P1 and localizes in large amounts within the posterior lobe. In contrast, Gli1 expression was seen in fairly moderate amounts throughout the remaining EGL regions, but showed very little traces of expression in the central lobe region where Tlx3 was expressed. These results confirmed what was expected from the scRNAseq expression data while also providing further evidence in favor of identity-dependent growth mechanisms for heterogeneous GCP subtypes. In order to further investigate the validity of our scRNAseq data in Gli1 cluster expression, in situ hybridization (ISH) for X-gal was performed using a Gli1-lacZ reporter transgene, where transcription of the Gli1 enhancer activates cleavage of X-gal by the lacZ beta-galactosidase enzyme and that leads to blue localization. The results supported what had been expected, and localization of X-gal occurred broadly in regions intentionally outside the Tlx3 restricted domain of expression in the central lobe EGL. This indicates that Gli1 expression, and thus Shh signaling activity, exists primarily in the EGL of the posterior and nodular lobes at P1 with some limited expression in the anterior lobe region as well, but demonstrates antagonistic expression in relation to Tlx3 of the central
lobe EGL. From this, it may also be inferred that GCP1 is predominantly contained within the central lobe, and the location of the various subtypes seem to correlate with less specificity in most other cerebellar regions at P1.

Additional experiments performed also confirmed these results. In situ hybridization (ISH) experiments on a lineage of $Ptpn11$ knockout mice at E16.5 demonstrated complete loss of $Etv4$ expression from the central lobe, and IHC experiments with the $Ptpn11$ knockout showed full loss of $Tlx3$ expression in the central lobe as well (Figure 5, F). The $Ptpn11$ gene has essential function in MAPK signal transduction, and this gene encodes the regulatory SHP2 protein for fibroblast growth factor receptors (FGFR) involved in MAPK pathway activation. Loss of $Ptpn11$ would fully inhibit MAPK pathway function, and the transcription of genes that respond to MAPK signal transduction, like $Etv4/5$ and $Tlx3$, would also be prevented as a result. The knockout experiments performed demonstrated this exact finding, indicating that endogenous $Tlx3/Etv4$ expression seen in the GCP of the central lobe is directly related to the input of MAPK signaling activity during early development.

Further analysis of expression data from the GCP subtypes revealed various signaling pathway enrichments with specificity for each of the different subtypes (Figure 5, E). The strongest correlation found from this analysis was for MAPK enrichment in GCP1, which was determined by number and rate of associated pathway genes expressed as well as relative quantity of cells expressing the genes. The overwhelming data supporting the diversity of gene expression seen in GCP subgroups, along with the identification of non-uniform pathway enrichments in different subtypes, suggests that distinct groups of GCP may have characteristic expression patterns that program GCP developmental fates as they proceed through various identity-dependent mechanisms.
Figure 5. Image (A) shows a map of E18.5 scRNA-seq data clustered by gene expression. As shown by the four distinct colors and regions of cells, the data revealed four GCP subgroups. Image (B) shows a display of violin plots that were generated to show the characteristic GCP subgroup-specific markers that were identified through scRNA seq analysis. The red represents a proportionate enrichment of gene markers for GCP 1, green for GCP 2 markers, blue for GCP 3 markers, and purple for GCP 4 markers. In images C and D, IHC results from sagittal sections of Ptpn11 knockouts and WT at E16.5 (C) and P1 (D) are shown. The white arrowheads (D) mark the boundary between Tlx3 (red) and Gli1-lacZ (green) expression, and the brackets (C) demarcate cells with both Tlx3 (red) and Otx2 (green). The corner image within (D) shows X-gal situ hybridization (ISH) results on a comparable section plane of the P1 Ptpn11 knockout. Image (E) contains dot-plots that display pathway enrichments results for each of the GCP subgroups. The comparison diagram in image (F) demonstrates IHC and ISH results for Etv4 expression in WT and Ptpn11 knockout mice from sagittal sections of E16.5 cerebella. Asterisks in the ISH
image of the Ptpn11 knockout indicate the loss of Tlx3, Etv4, and Etv5 expression in the EGL of the central lobe region.

*Alterations of the MEK-ETV signaling cascade affect cerebellar foliation*

The first general analyses performed on the heterozygotic MEK-GOF and Etv4-DN-LOF mutant mice compared initial phenotypic observations of stained tissue sections and whole mount images of specified time points during development for comparison. The selected stages were: P4, P8, P15, and P21, although some E16.5 and E.18.5 samples were also collected to compare the early stages of GCP proliferation. The initial results collected were limited to heterozygote mutants (Genotypes: Atoh1+/Cre;R26+/Etv4-DN, Atoh1+/Cre;R26+/MEK), but it was evident from early on in our observations that these samples had visibly irregular structural and compositional phenotypes at all postnatal stages P1-P21. IHC experiments were performed to characterize the compositional differences in E16.5 and E18.5 mutants, since these stages do not yet have foliations or obvious structural differences that had been used in comparing postnatal mutant phenotypes. Nissl staining of GC in postnatal samples from both types of mutants allowed for general observations to be made in terms of foliation, GC composition in the EGL and IGL, lobule size, and characterization of other general regional patterns when compared to WT samples. These initial observations revealed some unpredictability in the degree and specificity of phenotypic changes in mutants for all the qualities previously mentioned, even among littermates with the same genotype. However, despite these slight variations, the overall pattern of phenotypic changes for each type of mutant remained the same in terms of how the mutants were generally affected. Furthermore, several distinguishable phenotypic patterns were characterized for the upregulation and downregulation of MAPK using
these transgenic models, and the MEK-GOF mutants provided particularly useful insight on identifying a more specific role of MAPK in GCP development.

After collection of n=5 heterozygote samples (Atoh1+/Cre;R26+/MEK), the predominant phenotype identified for MEK-GOF was an apparent increase in foliation of lobules IV, V, VIa, VIb, and VII that comprise the central lobe. Interestingly enough, this observation was consistently restricted to the central lobe region, but showed no predictable changes in size, density/composition of GC in the EGL or IGL, or placement of the additional lobule foliations for the MEK-GOF mutants. Data collected from different stages of postnatal development also revealed relationships between observable phenotypes and developmental age. The E16.5, E18.5, and P1 samples had not yet undergone final fissure formation, and consequently lacked any visibly repeatable changes in cerebellar structure (data not shown). By P4, we saw clear structural and compositional differences arise in the EGL of MEK-GOF samples, and results from older samples continued to demonstrate similar phenotypic effects. In P4 and P8 samples in particular, additional folia were present in lobules IV-VII, and the EGL in these areas appeared to have thicker, but more uneven layers of GCP distribution. In addition, it appeared as though expansion from additional folia in lobules VIa-VII had reduced the overall size of lobules IV and V while also contributing to gyrification deformities in the structural patterning (Figure 6). By later stages in postnatal development, the expansion of additional folia continued to increase cerebellar size and the observed lobule characteristics remained, but additional changes were observed as well. At P14, proliferation of GCP ceases and the differentiated GC migrate inward to populate the thickened IGL as the EGL disappears. In the P14 MEK-GOF mutants, we observed remains of the EGL, indicating continued proliferation and delayed differentiation of GCP, along with a noticeably thinner IGL (data not shown). This finding was particularly
strange, since the IGL area was expected to increase due to the continued divisions of GCP past the stage of expected differentiation, which would ultimately contribute to the presence of more GC. By P21, the EGL had disappeared in MEK-GOF mutants and no further lobule expansions or foliations were observed, however we did continue to see reductions in the IGL area at full maturity (data not shown). All of the MEK-GOF phenotypic observations mentioned thus far were found from analyzing sagittal sections of the vermis and hemisphere regions, but whole-mount comparisons of the cerebellar cortex confirmed the phenotypes and allowed for 3D spatial observations of cerebellar enlargements (Figure 8, A). Samples from homozygous MEK/MEK mutants were used in later portions of our investigations, and much of the data analyzed has been from double dominant samples with the genotype Atoh1+/Cre;R26 MEK/MEK.

In our comparisons of the heterozygous Etv4-DN-LOF mice, the results appeared to generally match what was expected from downregulation of MAPK pathway transcription of Etv4. Since Etv4 expression is restricted to the central lobe region, our results were limited to observations of phenotypes demonstrated in solely the central lobe. Much like the MEK-GOF observations, there were no significant phenotypes identified in Etv4-DN-LOF mutants prior to approximately P4. However, the Etv4-DN-LOF comparisons in P8 showed more obvious evidence of an almost compacted cerebellar structure, with a seemingly deeper foliation patterning of lobules VIa-VII within the central lobe (Figure 8). Another observed phenotype in the Etv4-DN-LOF mutants that was similar, although opposite, to a MEK-GOF phenotype was in the compensatory deformity of lobules IV and V due to changes in the central lobe. In contrast to what was seen in MEK-GOF, the LOF showed reduction of foliation in lobules IV and V (Figure 6). Additionally, the density and composition of GC in the EGL and IGL of lobules IV and V appeared to be reduced (Figure 6), however specific measurement data was not collected.
for this finding. However, zoomed in images do not show any of compositional irregularities that were observed in the EGL of MEK-GOF mutants. Mutants at P14 and later postnatal stages did not have the deep foliation phenotype that had been observed in the central lobe of P8 mutants, but an obvious thickening of the IGL area in lobules IV and V (supported by measured data in Figure 8, C) had become apparent, despite the overall size reduction that was also found in these lobules. Additionally, by P14 there appeared to be normal differentiation of GCP and appropriate disappearance of the EGL (Figure 8, B). More results were collected from whole-mount images of Etv4-DN-LOF, however these comparisons did not reveal any obvious changes in overall cerebellar size that had been seen in MEK-GOF (Figure 8, A). In examinations of the EGL, statistical analysis of EGL measurements did not reveal any significant compositional changes indicative of cortical reduction or changes in EGL thickness.

In line with our initial hypothesis, we consistently observed a variety of clear, correlational phenotypes in response to genetic upregulation and downregulation of the MAPK pathway within lineages of Atoh1+ GCP. More specifically, the results demonstrated modifications in qualities of GCP proliferation and differentiation in the central lobe region of both the GOF and LOF mutants, providing more evidence in support of our hypothesis. An additional finding of relative significance was the observation of dosage dependency in determining the degree of phenotypic affects. Although visible phenotypic comparisons between heterozygotic and homozygotic mutants are not shown, IHC of heterozygotes and homozygotes confirmed the concept of dosage dependency. One IHC experiment using MEK-GOF mutants with a GFP transgene was performed to observe cells affected by the GOF, and the results a unique patterning of cell clusters that lacked GFP expression, indicating non-unanimous expression of the GOF gene throughout (results not shown). Another IHC experiment comparing
heterozygous and homozygous mutants showed a dosage dependent affect in the degree of Tlx3 ectopic expression (Figure 7). As a result, many of the samples collected and analyzed were from MEK/MEK and Etv4-DN/Etv4-DN homozygotes (double dominant), but the heterozygotes were used at the start of our investigations and were frequently excluded or analyzed separately.

Figure 6. This side-by-side comparison shows the sagittal midline sections of wildtype (WT), Etv4-DN-LOF mutant, and MEK-GOF mutant mice at P8. The mutants used for this comparison are heterozygotes (Genotypes: Atoh1+/Cre;R26+/Etv4-DN, Atoh1+/Cre;R26+/MEK1). The bottom images show a 10x magnification of the Nissl stain to provide an elaborated view of the inconsistent foliation patterns marked in the dashed red box above. The dark purple coloring indicates stained granule cells, and the densely stained areas comprise the EGL. The red arrows mark regions of phenotypic significance in the central lobe. The red arrow in the 10x image of the MEK mutant is used to show the irregular consistency of the GCP in the EGL, with areas of apparent thickness and foliations with thinned cell layers.
Figure 7. Image shown for this diagram are from an IHC experiment performed on MEK/MEK homozygote mutants, +/MEK heterozygote mutants, and a WT. The two full-sized adjacent images are from the WT and MEK/MEK mutant, with the dashed white box showing the reference area used for comparing all three samples in the last set of vertical images. The white arrowheads in the column of magnified images are used to compare the relative abundance of Tlx3 ectopic expression in the heterozygote and homozygote.

Quantitative analysis of cerebellar malformation caused by perturbations of MAPK function

The observable phenotypes consistently found in MEK-GOF and Etv4-DN-LOF mutants across all postnatal stages, particularly the additional foliations in the central lobe of MEK-GOF mutants, required further investigation before any objective patterns could be determined. In order to reliably compare the apparent MEK-GOF and Etv4-LOF phenotypes with the WT, the cerebellar foliation index (CFI) of each sample was initially measured in a number P14 of samples (WT: n=4, MEK-GOF: n=6, Etv4-DN-LOF: n=3). The CFI measures the total lobule perimeter of the sagittal cerebellum, and allows for further interpretations in predicting the degree of cerebellar cortical gyrification (Iwaniuk et al., 2006). The initial comparison of CFI measurements from comparable planes of the hemisphere (lateral) and of the vermis (midline) in late stage (P14) samples confirmed what had been expected from the general observations of
phenotypes. Results showed an overall statistically significant increase in CFI for both the hemisphere and the vermis of MEK-GOF mutants, which was indicative of cerebellar enlargement and lobule expansion in multiple dimensions *(Figure 8, B).* In contrast, statistical analysis of CFI measurements taken from the hemisphere and vermis sections of Etv4-DN-LOF mutants showed no statistical significance, nor did any observable trends arise in measurements collected from either area. However, the results were not completely indicative of whether or not a phenotype for cerebellar size exists in Etv4-DN-LOF mutants, as it has been determined that *Etv4/5* transcripts are restricted to lobules VI-VIII *(Figure 5, F).* Thus, the effects of the Etv4-DN-LOF mutation are expected to be predominantly limited to the central lobe region, which may not be accounted for when incorporated into total measurements of cerebellar size. Alternatively, the MEK-GOF mutation does not appear to be restricted to any particular region of the cerebellum, but does seem to show more pronounced affects within the central lobe region in particular.

In further analyses with P14 mutants, more measurements were taken to investigate density and composition of mature GCs in mutants. While CFI measurements were useful in identifying and characterizing lobule expansions in MEK-GOF mutants, additional types of measurements were performed to compare genotype-specific IGL qualities as well. Initial measurements of the IGL perimeter length were collected from the MEK-GOF and Etv4-GOF mutants, including both homozygous and heterozygous samples, as well as measurement data from WT samples for reference (WT: n=5, MEK/MEK: n=4, +/MEK: n=2, Etv4-DN/Etv4-DN: n=4, +/Etv4-DN: n=6). For a more accurate comparison of relative size and composition across all samples, IGL surface area measurements were also obtained from the samples and then adjusted for proportional comparison of IGL length to IGL area. Additionally, the IGL
measurements and relative IGL proportions were obtained from each lobe and used for further comparisons of GC composition. Multiple sets of statistical analyses across the WT, MEK-GOF and Etv4-DN-LOF samples were performed for: total IGL area in both the hemisphere and vermis, overall IGL proportions from the hemisphere and vermis measurements, and individual IGL proportions in all four lobes (anterior, central, posterior, nodular). Several statistical differences were found for the samples of mutant lobe measurements, specifically in the anterior and central lobes of the MEK-GOF, and also in the central lobe of the Etv4-LOF (Figure 8, C). Despite the visible and numerical enlargement seen in the cerebellar cortex of MEK-GOF mutants, the total IGL area of the mutant was found to be reduced, indicating that GC composition in the IGL of MEK-GOF samples was also decreased. In specific measurement data and IGL proportions collected from the vermis and hemisphere of MEK mutants, t-tests and confidence intervals revealed a statistically significant reduction of the IGL of the MEK-GOF hemisphere in particular (vermis: 24.9 ± 0.7 vs 29.0 ± 0.8, p = 0.12; hemisphere: 18.8 ± 0.3 vs 22.2 ± 0.5, p = 0.02; t.test). Additionally, the relative proportions for the total area and IGL length, which was used in providing comparisons for IGL thickness, was significantly lower in MEK-GOF than controls (data not shown).

After analyzing the GOF/LOF phenotypes and additional results, it was determined that our findings could explain a unique contribution to proliferation and cell cycle regulation in a specific GCP subtype in the central lobe, and it was also concluded that the MAPK signaling pathway has role in GCP development that had been previously under-appreciated. In order to determine whether our findings in MEK-GOF had been described in any similar research performed that might provide further explanations or insights for our observations, previous publications of relevance were reviewed. In the literature search performed, we found that
overexpression of the MAPK pathway in MEK-GOF mutants produced several phenotypes and characteristics that were notably comparable to findings previously reported for experiments on a transgenic Shh-GOF mutant line of mice. Interestingly enough, further insight was gathered from the Shh-GOF mutants through comparisons that were actually directly antagonistic to what we observed in the MEK-GOF mutants. The study performed on Shh-GOF mutants investigated the effects of overexpressing Shh. Results from the Shh-GOF showed measurable increases in IGL area, which was an opposite effect found from our experiments on MAPK overexpression in MEK-GOF mutant models. Additionally, the EGL of Shh-GOF was maintained longer but not changed in any notable way, something that also opposed our findings in EGL characteristics. There were also no observed changes in lobule foliation or expansion, which we had characterized as phenotypes in the MEK-GOF.
**Figure 8.** This figure compares the opposing phenotypes observed in the central lobe region of MEK-GOF mutants and the Etv4-DN-LOF mutants at P14. Starting from the left portion of the comparison diagrams labeled as (A), the column of vertical images (2.0X mag.) show the whole-mount cerebella for a WT sample and the two homozygote mutants. The green double-headed arrows depict the general boundaries of the vermis region in the WT sample displayed. The arrow does not reach the vermis boundaries in the MEK-GOF image, and the arrow extends beyond the vermis boundaries seen in the Etv4-LOF image. The smaller red arrowheads indicate regions of the MEK-GOF mutant where additional folia exist. The two columns in the right portion of (A) are vertical comparisons of Nissl stains (2.5X mag.) in midline sections (vermis) and lateral sections (hemisphere). The red arrowheads in MEK-GOF images indicate additional folia, whereas red asterisks mark regions of absent or decreased foliation in Etv4-LOF. The boxplot graphs shown in (B, C) display data from measurements of cerebellar foliation index (CFI) in the vermis and hemisphere (B) and sample proportions for IGL length relative to total IGL area in individual boxplots made for the four main cerebellar regions (C). The red asterisks show statistically significant results (p<0.05) from a Tukey post-hoc test. The CFI measurement is used to represent EGL length. Colored dots from the boxplots represent measurement data points from an individual sample. The genotype represented by each color is indicated in the plot legend, but the plots in (C) incorporate measurement data from heterozygous mutants +/-MEK and +/-Etv4-DN as well (shown in a different shade of green for +/-MEK and light blue for +/-Etv4-DN).

**Changes in MAPK affect gene expression in GCPs**

Initial IHC experiments in MEK-GOF were performed to help characterize changes in GCP gene expression within regions that had phenotypes, while also using gene markers to identify the specific qualities and types of cells present in mutants. While trying to determine the regions of mutants experiencing MAPK activation, we hypothesized that there would be evidence of MAPK associated gene expression in the central lobe regions where phenotypes were identified. IHC experiments performed on P4 MEK-GOF were used for identification of Tlx3 expression patterns, which our previous Ptpn11 knockout experiments had already determined would necessarily imply MAPK activity in GCP. Tlx3 is responsible for glutamatergic differentiation during neurogenesis, and was initially thought to be expressed only in post-mitotic, mature granule neurons outside of the EGL (Divya et al., 2016). However, Tlx3
expression is seen in early GCPs first formed at E16.5 during development, and expression is maintained well into postnatal development as well (Divya et al., 2016). In addition, the expression of Tlx3 during postnatal GCP development is restricted to a specific domain comprising most of the central lobe and entirety of the posterior lobe. Therefore, observation of expression patterns for Tlx3 in IHC experiments revealed many valuable insights on aspects of the MAPK pathway in GCP proliferation and also the specific effects and implications of MAPK overexpression.

In the IHC results, Tlx3 was observed in extremely high amounts outside of its expected domain, and instead was seen in almost every portion of the EGL except lobule X of the nodular lobe. In terms of our initial expectations for Tlx3 expression patterns in mutants, almost the exact opposite results were found. The central lobe region of both the vermis and hemisphere showed minimal expression of Tlx3, and the EGL region where the phenotypes were found had apparent patterns of alternating patchiness and then dampened Tlx3 expression (Figure 9). Interestingly enough, the expression of Tlx3 in its endogenous regions of the posterior lobe EGL appeared to be unaffected by MAPK overexpression, however the Tlx3 expression in the developing IGL was reduced in these endogenous regions. Overall, the ectopic expression of Tlx3 was relatively inconsistent throughout the EGL, but phenotypic regions notably had essentially no expression of Tlx3 in the IGL. In observation of other expression patterns, the WT showed that Sox2 expression in the EGL appeared to be very low, and very few Sox2+ cells were co-expressed within the Tlx3 endogenous expression domain of the EGL. Instead, the majority of Sox2 expression would be expected in the developing IGL region. In MEK-GOF, we saw characteristically high amounts of Sox2 ectopic expression throughout the EGL of non-endogenous Tlx3 expression regions, along with clear Sox2/Tlx3 co-expression in the central lobe.
EGL where identifiable phenotypes had been found (Figure 11). In other words, there were no changes in Sox2 expression and insignificant co-expression of Sox2/Tlx3 in the posterior lobe EGL where Tlx3 is normally expressed showed, similar to that of the WT. In the regions with Sox2 ectopic expression, specifically the central lobe EGL, co-expression with Tlx3 was particularly high (Figure 9 and Figure 11). Another experiment performed on E18.5 MEK-GOF mutants had similar expression findings consistent with what we observed in postnatal mutants. At E16.5-18.5, Tlx3 expression is characteristically found within the central lobe EGL region, but this expression domain eventually turns off and shifts to the posterior and central lobe regions that are consistently seen in postnatal mice. Additionally, the expression of Tlx3 at E18.5, much like the postnatal observations described thus far, have very little overlapping expression with Sox2 in the EGL. In IHC of E18.5 MEK-GOF, results once again showed ectopic expression of Tlx3 in non-endogenous EGL regions, and co-localization of Sox2/Tlx3 in these regions as well (Figure 10). We also noticed a markedly high amount of Tlx3/Sox2 co-expression in the Tlx3 endogenous EGL domain, with higher overall ectopic expression of Sox2 throughout. This was not found in prior experiments performed on postnatal mice.

Further analysis of later postnatal stages from P14-P21 also revealed that mutants maintained Sox2 expression in the EGL longer in the mutants than the WT (data not shown). Sox2 is a neural progenitor gene, and as such, is expressed in GCP that are undifferentiated and still actively proliferating. Sox2 expression is also present in Bergmann glia (BG) and Purkinje cells (PC), which is why expression is observed right below the EGL surface of the interior cerebellum. Furthermore, the patterning of ectopic expression of Sox2 in the EGL of MEK-GOF mutants is unexpected, however is a direct result of delayed GCP differentiation and maintenance of GCP neural progenitor activity as indicated by Sox2+ cells in the EGL.
Figure 9. The images in this comparison diagram show IHC results for P4 homozygote MEK-GOF mutants and the P4 WT littermate. The top two images are comparable planes of midline sagittal sections from the vermis, and the bottom two adjacent images show comparable lateral planes of the hemisphere between WT and MEK-GOF. The bold red arrows in the WT identify restricted regions of Tlx3 endogenous expression in the posterior and central lobes. In the MEK-GOF mutant, the bold arrow indicates areas of ectopic expression of Tlx3 beyond the defined boundaries. Although it is not pointed out in the figure, the MEK-GOF image from the vermis
also shows an apparent dampening of Tlx3 expression in endogenous regions with weaker blue fluorescence.

**Figure 10.** The images shown are from an IHC performed on E18.5 a MEK-GOF mutant and a WT littermate. The dashed boxes in the first set of images show the areas that were magnified in the following subsets of images. The red, green, and blue channels are shown in the following sets of images, and the white arrows are used to identify cells that co-express Sox2 and Tlx3 in the MEK-GOF. The white brackets seen in the green channel images compare the relative thickness of Pvalb expression in the WT and MEK-GOF.
**Figure 11.** This magnified comparison shows additional results from the *Sox2/Tlx3/Pvalb* IHC experiment performed on a P4 MEK-GOF mutant and WT littermate. The images are zoomed in on the foliation between lobules VIb at the approximate intersection of the central lobe and posterior lobe regions. The cells in the EGL of the WT show dense labeling of *Tlx3* throughout, whereas the developing IGL forming underneath the EGL is predominantly labeled as red *Sox2* cells with no co-expression with *Tlx3*. The MEK-GOF shows significant co-expression of *Sox2* with *Tlx3* in the EGL, along with more *Sox2* labelling in the IGL and decreased *Tlx3* labeling in both the IGL and EGL.

**MAPK signaling regulates GCP proliferation and differentiation**

After observing EGL expansion and reduction of IGL area in the *MEK/MEK* mutants, combined with the abnormal *Sox2/Tlx3* ectopic expression patterns revealed from IHC experiments, it was then hypothesized that these findings may occur due to changes in cell cycling mechanisms induced by MAPK signaling. In order to test this hypothesis further, several EdU tracing experiments were performed to help identify specific cell cycle characteristics in *MEK/MEK* mutants. Since GCP development initiates fairly late in embryonic development (~E14.5) and normally ends at around P14, we chose to use P5-P8 mutants for observing optimal proliferation. Additionally, we begin to observe many of the most significant phenotypes by these stages. In the tracing experiments, we injected EdU in litters of MEK-GOF and WT mice at P5, samples were collected in sequence at P6, P7, and P8 to determine whether proliferative
activity and GC output change over time (Figure 12). IHC experiments for EdU labeling data were performed using PH3/EdU/NeuroD as markers. EdU is incorporated into newly synthesized DNA from cells in S phase of the cell cycle, which then allows for “tracing” of the cells throughout development. PH3 labels dividing cells, specifically those that are dividing in the mitotic stage of the cell cycle. In contrast, NeuroD is a marker used in labeling differentiated GC that have exited the cell cycle and no longer exhibit progenitor activity.

A general observation across all three stages of WT was that the EGL labeled very high amounts of EdU in the EGL at P6 initially, indicating cell cycle activity, but the labeling for EdU in the EGL progressively diminished and could instead be found accumulating within the IGL by P8 as differentiated cells. The PH3 labeling remained pretty consistently low in the EGL and IGL with no notable changes. NeuroD in the WT showed thinning of the EGL by P8, and the signal density progressively weakened in the EGL as labeling in the IGL increased. The observations in WT samples maintain that P6 progenitor cells of the EGL exist in higher amounts but also contain a high number of recently differentiated cells. Then, these progenitor cells progressively stop dividing and start to differentiate within the EGL by P7/P8. By P8, the overall number of cells in the EGL is reduced, the EGL becomes progressively thinner, and division of progenitor cells has slowed down significantly. The cell cycle qualities demonstrated in these WT samples allowed easy comparisons in the mutants, which ultimately revealed several interesting findings that provided valuable insight as to how the cell cycle may be affected by overexpression of MAPK.

Starting with separate stains for EdU/NeuroD and PH3/NeuroD 24 hr after EdU injections at P6, we immediately observed a very thin, uneven, inconsistent labeling of cells within the phenotypic central lobe EGL (Figure 12). The expression signal for NeuroD seemed
particularly patchy in portions of the EGL and generally reduced throughout this area as well. It was quite difficult to determine whether EdU labeling had changed in the mutant, but overall it was obvious that the density of NeuroD+ differentiated cells, and cell number in general, had been reduced in the EGL of MEK-GOF. However, more PH3+ were found in the mutant than the WT, indicating an increase in number of mitotic progenitor cells throughout the IGL. At P7, labeling of NeuroD in the EGL seemed far more uneven and patchy. However, EdU labeling was noticeably higher in the EGL of the mutant, and also had slightly more PH3 labeling as well, which indicates that comparatively more progenitor cells exist in the mutant. By P8, the EGL thickness and consistency had changed less than was observed in the WT, but there was a reduction in differentiated cells marked by NeuroD. PH3 labeling did not decrease and also remained much higher in the EGL of mutants than the WTs. Surprisingly, EdU labeling was about the same between the WT and mutant, but did not appear to be restricted within the developing IGL as expected. Rather, the labeling of EdU, and also NeuroD, was extremely random inside the interior of the cerebellum, and it was also apparent that there were fewer differentiated cells present in the mutant.

Through analysis of these results, several patterns became obvious. The results found from IGL area measurements were very similar to what was observed in the cell tracing experiments performed on the mutants. The total number of cells appeared to be reduced despite the fact that there were more progenitor cells actively dividing. The unevenness and decreased EGL thickness also matched the observed phenotype from P8 Nissl Stains. However, the reduced thickness of the EGL in mutants conflicted with the results from CFI measurements, since it was found that MEK-GOF mutants experienced higher CFI measurements, indicating EGL expansion leading to an increase in EGL length and surface area from expansion. All of these comparisons
led us to a new hypothesis for explaining the effects of overexpressing MAPK. From the interpretation of the MEK-GOF results, we suggest that MAPK overexpression may potentially induce slower cell cycling activities, while also delaying differentiation of progenitor cells that may contribute to EGL expansion as more GCP divide, and ultimately a lower number of GC in the IGL as a result. We also consider the possibility of MAPK having a role in regulating how and when GCP divide, particularly in the vertical vs. horizontal, or self-renewing vs. neurogenic divisions of stem cell GCP.
Figure 12. The IHC results for the NeuroD/PH3/EdU tracing experiments performed on P6, P7, and P8 MEK/MEK homozygotes along with their littermate WT samples at the same stages are shown above. Each set of adjacent images compares relative planes of sagittal sections from the WT and MEK-GOF mutant. The EdU was injected in live mice at P5, and the amount of time...
between EdU injection and sample dissection is displayed for each stage. The blue color always marks NeuroD+ cells, and shows an uneven and dampened signal in MEK-GOF mutants at all stages (not annotated in images). The two images for each stage display purple labeling for EdU+ cells and then purple labeling for PH3+ cells in the following image, as indicated in each image set.

**DISCUSSION**

*MAPK mutants demonstrate changes in cerebellar size and GCP composition, and expanded lobules with additional folia in the central lobe region*

In the initial scRNA seq data provided, we found four GCP subtypes with variable expression profiles, which led us to further investigate the gene-specific characteristics in each of the GCP subtypes. Collections of enriched genes for each GCP population provided suggestive evidence of differential signaling contributions among the subtypes, with specific genetic evidence relating presence of MAPK activated genes in GCP1. In determining the validity of this finding, we hypothesized that MAPK has a role in GCP proliferation, but this role may be limited to the subtype that we had identified as GCP1. Furthermore, we expected to correlate evidence of MAPK activity to a defined GCP1 spatial region that could provide additional support in favor of combinatory signaling mechanisms contributing to aspects of differential GCP developmental activities.

Our approach for investigating the role of MAPK in GCP involved upregulation and downregulation of MAPK signaling throughout development, for the purpose of determining whether any morphological changes were induced. From observations of MAPK manipulations in our MEK-GOF and Etv4-DN-LOF mutant models, we characterized several restricted phenotypes within lobules of the central lobe region, especially in the MEK-GOF mutants. To summarize these findings, the MEK-GOF mutants with MAPK overexpression experienced
dosage dependent phenotypes, including: increases in cerebellar size, lobule expansions with structural deformities, additional foliations, and inconsistencies in GCP composition. The Etv4-DN-LOF mutants demonstrated less severe phenotypes, but ultimately had reductions in central lobe size, along with deepened gyrifications in lobules VI-VII. From these results, we can clearly correlate the morphological changes to manipulations in MAPK signaling to induced. Thus, we concluded that MAPK has a definitive role in regulating GCP development, but the capacity of this role required further investigations.

Additional analyses that measured GCP composition in the EGL and IGL of mutants also revealed changes in several defining features throughout development. The MEK-GOF mutants demonstrated significant increases in total foliation index of the EGL, which was most likely a result of the observed lobule expansions. However, despite this measurable increase in foliation and total cortical surface area, additional comparisons of EGL area relative to EGL length for determining EGL cross-sectional thickness actually revealed significantly reduced quantifications in GOF mutants. This finding was reflected in the central lobe where the EGL was seemingly thinner and had uneven GC distributions throughout. Furthermore, the lobule expansions and additional foliations seen in the central lobe of mutants certainly explains the total increase in EGL length from CFI measurements, but the opposing reductions from the EGL area measurements (thickness of GCP in EGL) is likely due to the compositional abnormalities in the EGL of the affected central lobe. From this comparison, it can be inferred that upregulating the MAPK pathway causes lobule expansions and foliation defects, however neither of these features are indicative of increased GCP proliferation, but rather reflect no change, or even a reduction, for total number of GCP in the affected central lobe region during development. A specific CFI measurement for the EGL in the central lobe region was not
performed to confirm this observation, but additional measurements and GCP counting efforts may help in defining whether a change in total GCP number actually exists for MAPK GOF mutants. Consequently, EGL measurements from the adjacent IV and V lobules might also provide some insight as to whether our observations of lobule reductions and deformities would reflect lower GCP quantifications in these affected regions. In additional analyses of EGL measurement data from Etv4-LOF mutants, there were no predictable changes observed in measurements, and ultimately there was no indication of significant changes. Since had seen visible evidence of central lobe size reductions in the LOF mutants, we expect that the measurement findings may not accurately reflect the changes seen in mutants. It is possible that the deeper foliations in the central lobe counteract the apparent reductions in size, or it could be that the degree of change in the central lobe was too minor when considering the total measurement data. However, further comparisons in measurements from each lobe could be more useful in determining specific changes.

Since there appeared to be no changes in total GCP output in the MEK-GOF mutants, we then considered whether there was evidence of subsequent changes in total differentiated GC number at maturity. Measurements of IGL area, along with proportions of IGL area/IGL length at P14 were used in comparing the total GC output in mutants. The results revealed significant reductions for total GC number of the central lobe IGL proportions, but also antagonistic increases in GC number from the anterior lobe IGL proportions. While these results may seem contradictory, both of these findings can be reasonably explained by the specific phenotypic observations from the mutants. In GOF mutants, the anterior lobules IV-V showed several deformities that were initially indicative of reduced expansion at the expense of central lobe increases. This was likely the case in terms of overall cortical surface area and length, but the
number of GCP in the anterior lobules remained the same, meaning that the actual composition within the IGL was unchanged despite the reflected increase in IGL proportions. The reduced measurements for IGL length in lobules IV-V made it seem as though GC number had increased, when actually the increase in IGL proportion was likely just reflecting the decreased IGL length relative to an unchanged GC output for this smaller region in mutants. Additional statistical analyses on IGL measurements revealed that the central lobe in Etv4-DN-LOF mutants also had increased proportions of IGL area over IGL length, which once again was considered a potential consequence of reduced IGL length relative to an unchanged number of GC comprising the same IGL area. However, statistical analyses of other central lobe measurements in Etv4-LOF mutants did not reveal evidence supporting significant size reductions in any aspect of the central lobe. While taking all measurement findings from LOF mutants into consideration, it appears as though a true increase in GC output may actually be related to downregulation of the MAPK pathway, despite what was originally expected.

In these collective observations, both mutants repeatedly demonstrated phenotypes that were restricted to the central lobe region, and it became clear that the central lobe has some significance in the general role of MAPK throughout GCP development. The scRNAseq data from a WT had initially indicated an association in GCP1 subtype enrichment for genes endogenously expressed in the central and genes involved in MAPK signaling. The scRNA seq expression data had also revealed that enrichment for Gli1 was only found within the GCP3 subtype, which ultimately suggested that Shh activity was absent in the majority of developing GCPs. The identified genes from the GCP3 cluster seem to correlate with expression patterns found in the anterior or posterior regions. Similarly, Gli1 expression is highest in GCP of the anterior and posterior lobes, but is notably reduced and sometimes completely absent in the
central lobe GCP during development. These findings were confirmed by our own in situ hybridization results and were corroborated by similar reports from many others. As such, the demonstrated lack of Shh signaling activities within the central lobe indicates a substantial gap in how GCP development is currently understood.

In our investigations, we determined clear evidence of MAPK activity in the central lobe where Shh signaling is apparently absent, which led us to further associations between the pathways. The phenotypes that have been reported in Shh-GOF mutants (Corrales et al., 2004) are inherently antagonistic to the phenotypic effects observed in our MEK-GOF mutants, which provides additional context in determining the developmental role of MAPK, but also in better understanding the potential interactions between pathways. In comparing the IGL characteristics between mutants, the Shh-GOF showed increases in IGL thickness, which consequently indicated increases in total GC number at maturity. Furthermore, the IGL in mutants was described as “irregular,” and the most pronounced increases in IGL thickness were identified in the anterior lobules III-V and posterior lobule X, specifically following the normal pattern of Shh expression (Corrales et al., 2004). In comparing IGL characteristics of the MEK-GOF mutants, the exact opposite effect was found. Instead, our mutants demonstrated reductions in the IGL area with unknown changes in total GC output at maturity. The affected central lobe region (lobule VI-VII) in MEK-GOF showed no overlap with the affected anterior/posterior regions found in Shh-GOF. Additionally, some unique observations in MEK-GOF mutants showed no comparable effects in the Shh-GOF. Whereas we found additional folia, irregular EGL composition, and lobule expansions in MEK-GOF, Shh-GOF had no changes in cerebellar morphology and a regular EGL that remained unaffected throughout development. One last comparison revealed similar increases in cerebellar size between both GOF mutants, but
specifications were not collected from either mutants to confirm this observation. With all of the insight from comparisons mentioned thus far, the GOF mutants seem to have opposing mechanisms for regulating proliferation, along with antagonistic patterns of functional control over development in distinct regions.

The seemingly intentional patterning of phenotypes in GOF mutants, along with evidence of counterintuitive proliferation in the respective GOF and LOF models, had also provided suggestive insight for identifying possible developmental functions in MAPK signaling. In particular, the additional folia found in GOF mutants, along with the expansion of lobules VI-VII, both implied changes to aspects of normal GCP proliferation, but not necessarily in terms of increased divisions or increased GC output. Rather, we considered changes in the type of divisions taking place and prolonged cell cycle processes as potential considerations for identifying the developmental functions controlled by MAPK signaling. Comparisons between Shh-GOF mutants and the MAPK GOF mutants revealed several antagonistic relationships in regions of distinct control and also in mechanisms of control. Furthermore, it appears as though investigations into specific characteristics of GCP development from Shh signaling may provide an opportunity to better understand the opposing functional attributes observed in MAPK signaling.

**Overexpressing MAPK can induce ectopic expression and late differentiation of GC**

Another quality that had been investigated in Shh-GOF mutants was whether overexpression of the pathway could induce ectopic expression in the mutants (Corrales et al., 2004). In observations from Shh-GOF mutants, there had been no evidence of ectopic expression, and the only noticeable change at all was for overexpression of Shh in its endogenous
regions, which was the normal expectation. Interestingly enough, our IHC experiments in MEK-GOF mutants once again revealed an opposing finding. We used markers for Sox2/Tlx3/Tfap2b, for which we used Tlx3 expression as evidence of MAPK pathway activities. The expression results from the initial IHC experiment performed in P4 GOF mutants had revealed significant ectopic expression of Tlx3 and the neural progenitor gene Sox2, as well as several other critical findings. The expression of Tlx3 was observed throughout essentially all areas of the EGL (except lobule X), indicating that overexpression of the MAPK pathway can effectively induce MAPK activity in almost all non-endogenous GCPs that normally lack pathway functions.

Additionally, the Tlx3 expression within its normal endogenous domain was seemingly unchanged, meaning that overexpression of MAPK did not increase pathway activation in the GCPs with known pathway functions, going against what would logically be expected. Another pattern found in the central lobe showed alternating levels of Tlx3 expression, specifically in lobules that experienced obvious morphological phenotypes. Although the patterning of expression was seemingly random at first glance, the patchy EGL regions with bright blue Tlx3 expression remained constant from the hemisphere into the vermis, and the areas of EGL with dampened expression followed this same observation. Further investigations of Tlx3 ectopic expression patterns revealed that the patchy areas with highest levels of Tlx3 were always located at fissure formations within the central lobe. This observation was especially interesting, given that these bright Tlx3 pockets were clustered at specific demarcations of extra folia, and also in the corners of shortened lobules in the anterior lobe. Additionally, the pockets of Tlx3 were found at fissure origins for the lobules that had been reduced, especially at the lobule V/VI boundary where the most uneven structural changes are located in mutants. This expression pattern was very much intentional, and the locations of Tlx3 clusters showed localized MAPK
activity specifically in regions with foliation phenotypes and lobule size changes. Furthermore, it seems likely that overexpressing MAPK in non-endogenous areas can induce irregular lobule foliations, which suggests that MAPK may have a specific role in foliation signaling and patterning. Although this has not been proven, it seems quite possible that the patterned regions with highest MAPK activity had driven formations of fissures and invaginations throughout the EGL of the anterior and central lobe. It is still unknown whether this observation can be related to the normal role of MAPK in GC development, but further investigations may reveal insight into how MAPK signaling may function in regulating lobule formations.

In addition to the Tlx3 ectopic expression in mutants, we also found clear ectopic expression of Sox2 as well. More specifically, Sox2 is a neural progenitor gene that marks proliferative cells identified as undifferentiated progenitors. In the context of our observations in mutants, increased Sox2 expression in the EGL indicated high quantities of undifferentiated GCP that maintained their progenitor identity longer than what was normally expected in the WT. However, the ectopic expression of Sox2 was evidently excluded in regions with endogenous Tlx3 expression, and it was collectively observed that overall expression patterns from this domain had not changed at all. In general, we also found that all areas with Tlx3 ectopic expression had also shown ectopic expression of Sox2 as well. In other words, the induced expression patterns for Tlx3 and Sox2 were the same in all instances of ectopic expression, and overlapping expression of both markers was always seen in the affected EGL of mutants.

When all these unique results are combined, we once again observe intentional patterning of MAPK-induced effects in restricted and unrestricted regions of implied regulatory control. This was most significantly observed in the area of endogenous MAPK activity, which was essentially the only region that did not demonstrate any evidence of changes. We alternatively
expected to find evidence of enhanced MAPK activation in this functionally endogenous region, similar to the observations from Shh-GOF. However, the effects of MAPK upregulation actually induced pathway activation into broad regions where additional effects were observed as well. The comparisons made between these specific findings are inherently indicative a regulatory mechanism for controlling MAPK overexpression in regions that normally exhibit MAPK activities. A negative feedback mechanism could explain why essentially no changes were found when MAPK was overexpressed in its own functional domain, while also accounting for the uncontrolled developmental changes and unrestricted expression patterns observed in response to MAPK upregulation within areas that do not have repressive functions.

In comparing several unexpected findings, our successive interpretations imply that MAPK signaling may be involved in regulating developmental control. In particular, the patterning of ectopic expression in MEK-GOF mutants has helped us establish significant relationships that help explain the phenotypic qualities observed in regions with induced MAPK activity. In the central lobe of mutants where we find foliation defects, lobule expansions, and irregularities in GC composition, we also see ectopic expression indicating sustained progenitor activities within MAPK-induced areas of the EGL. This evidence provides possible cause and effect functions for MAPK signaling during GCP development, specifically in understanding how overexpression of MAPK causes the GOF phenotypes found in mutants. Additionally, we observed evidence suggesting that GCPs with endogenous MAPK involvement may have feedback mechanisms for regulating MAPK signaling. Although we have not yet identified how this feedback regulation mechanism functions in the posterior areas of Tlx3 endogenous expression, scRNAseq data from MEK-GOF mutants may reveal key contributors in the regulation events.
Role of MAPK in regulating the cell cycle and GCP divisions of central lobe

From our multi-step investigations of MAPK signaling, we gathered convincing evidence from MEK-GOF mutants that repeatedly implied changes in varied aspects of the cell cycle. The unexpected findings defined in previous analyses ultimately led to proliferative tracing experiments for targeted observation of cell cycle characteristics in mutants. More specifically, we aimed to identify aspects of proliferation in MEK-GOF mutants for further interpretations of how MAPK upregulation might induce changes in cell cycling mechanisms. In the EdU tracing experiments performed, three stages (P6, P7, P8) were collected in 24 hour increments following EdU injections. Observations from each consecutive day following initial EdU labeling of dividing cells allowed for comparisons between relative proportions and spatial distributions of initially dividing cells, as well as comparisons between the relative rates of differentiation throughout all three stages. For visualization of comparative cell characteristics, IHC experiments were performed using additional markers for NeuroD and PH3, labeling for differentiated GC and actively mitotic GCP, respectively.

In the IHC results, general comparisons between all stages of the WTs and GOF mutants had revealed significant changes in morphology and several aspects of compositional irregularities in the EGL of lobules IV-VII. These observations were evident without having to compare the specific expression data, however, there were several notable characteristics found from more refined observations of expression patterns within the EGL of mutants. Starting at P6, the GOF mutants a had thinner, more uneven EGL in the central lobe, with significantly reduced labeling for NeuroD in differentiated GC of the central lobe EGL. Additionally, the labeling for differentiated GC of the IGL was characteristically lower for the central lobe region. Similar features were observed in the central lobe of P7 and P8 mutants, however these later stages had
more irregularities in the central lobe EGL, along with a very low rate of EGL recession by P8 compared to the WT, and the differentiated GC in the central lobe IGL had remained lower and lacked patterning. Although many morphological and compositional characteristics could be elaborated from these results, the main findings interpreted from this experiment were from the specific cell cycle observations in GOF mutants.

From observing the unique EdU expression patterns in mutants, we found several insightful results that allowed for further characterization of cell cycle mechanisms. Throughout all stages P6-P8, the overall amount of EdU labeled cells was collectively increased from start to finish, meaning that the WT labeled more GCPs present in S phase of the cell cycle relative to the number of labeled cycles in mutants. Within the same time frame, WT GCPs cycled through more divisions than the GOF mutant. This finding likely indicates an overall slower rate of divisions in MEK-GOF mutants due to longer cell cycling mechanisms. However, despite the increased EdU labeling in WTs, the MEK-GOF mutants had comparatively more EdU labeling in the central lobe EGL throughout all stages. Even though MEK-GOF mutants may have slower divisions and less proliferation within a given amount of time, it seems as though the mutants also differentiate at slower rates to compensate. Additionally, the sustainment of GCP in the EGL may also contribute to mechanisms behind the lobule expansions or foliation irregularities observed in the central lobe of mutants. Further analysis of PH3 expression revealed findings similar to the observed patterns in EdU labeling. Although there were not very many cells labeled for PH3 in general, it was still quite apparent that MEK-GOF had far more PH3 labeled cells compared to the WT. This comparison was maintained in all three stages, and additional observations of specific PH3 patterns also revealed that the highest proportion of labeled cells was consistently found in the central lobe EGL of mutants.
From the results revealed in our cell cycle tracing experiments, we conclude that upregulating MAPK can induce slower GCP divisions and longer cell cycling activities. However, it was also determined that overexpressing MAPK can decrease differentiation rates by maintaining GCP proliferation within the EGL for longer. When combining these results with the previously identified features in mutants, several additional relationships may provide more context for identifying specific aspects of MAPK signaling in development. For example, we can relate slower divisions and late differentiation of GCP in the EGL to the characteristic lobule expansions and folia observed in mutants. The GC output had been reduced in both the EGL and IGL of the central lobe, yet lobule enlargements and additional folia are observed in regions with high levels of MAPK activity. In contrast, we observe reductions in anterior lobule size and paradoxical increases in GC output within the IGL of these lobules. These similar but opposite effects indicate an inverse relationship between lobule size and total GC output, suggesting that the number of GC produced has not changed significantly, rather the distribution of GCP has created changes during lobule development in mutants.

A possible explanation for the differential patterning of GCP observed throughout the affected EGL may involve changes in mitotic division mechanisms induced by MAPK, specifically in terms of the direction of GCP divisions. Specific support for a vertical division mechanism comes from scRNAseq expression data showing Eya1 enrichment in GCP1, which may correlate Eya1 to the MAPK pathway. Recent investigations have suggested a role for Eya1 in promoting vertical divisions, and furthermore could function by facilitating vertical division mechanisms in MAPK proliferation. Other research has suggested that mitotic spindle orientation is controlled by MAPK/ERK signaling functions (Tang, 2011), providing more evidence in support of a MAPK-driven division mechanism for inducing GCP proliferation.
patterns during development. In the context of our findings, it is possible that GCP favor vertical divisions in the central lobe oEGL, creating lobule expansions outward as opposed to transitioning divisions downward into the iEGL. This division mechanism also adequately explains why we observe thinner layers of unevenly distributed GCP within the central lobe EGL, since GCP experience proliferations for cortical expansion at the expense of anticipatory GCP layers differentiating into the IGL. Similarly, anterior lobule size reductions can also be explained by vertical divisions in the fissure formations, for which the aggregates of thick GCP layers at the fissure origin divide internally at the expense of normal lobule expansion. We see a thicker, more irregular EGL at fissure boundaries of anterior lobules, particularly at the fissure bases. We can explain these directional differences in EGL expansions by relating the orientation of each lobule’s vertical division plane perpendicular to the pial surface.

Additional aspects of MAPK division mechanisms may also provide further insight into the enhanced development functions facilitated by MAPK signaling. In the considering types of divisions, it has been determined in previous studies that symmetric divisions are the most common proliferative mechanism for GCPs throughout development (Espinosa, 2008). Two main types of division, proliferative and neurogenic, both exhibit unique cell cycle mechanisms and also demonstrate different proliferative outcomes. Neurogenic divisions (also known as asymmetric divisions) are characterized by rapid proliferations with shortened cell cycle times, and each round of division generates a new neural stem cell and a differentiated neuron. Our scRNAseq data revealed enrichment for neurogenic proliferative genes in the GCP3 subtype, such as Neurod2/6. Furthermore, we have already associated Shh activities and Gli1 enrichment within GCP3, which suggests that Shh signaling may promote a combination of neurogenic divisions and symmetric proliferative divisions. We also observe direct evidence of Shh activity
within the anterior/central lobe regions, which is notably antagonistic to the regions
demonstrating Tlx3 expression found from MAPK signaling. Not only was this restricted
 patterning disrupted from MAPK upregulation, but we also observed phenotypic patterns and
ectopic MAPK pathway expression in an explicitly anterior pattern, exhibiting intentional
overlap within the Shh activity domain. Additionally, we had identified completely opposing
characteristics in Shh-GOF and MAPK GOF models, most notably for the changes in GC output
and proliferation. While Shh GOF mutants only demonstrated Shh activity and Shh enhancement
within its endogenous anterior/central lobe regions, MAPK GOF mutants saw an inverted pattern
of behavior and instead ectopically induced pathway activities in the respective Shh
anterior/central lobe region, without changing activity in its endogenous posterior/central lobe
region. The antagonistic relationship observed between these signaling pathways likely suggests
opposing roles for each pathway in regulating distinct regional domains. However, it is evident
that MAPK-GOF mutants exert MAPK pathway influence in regions controlled by Shh, which
suggests that MAPK upregulation can alter Shh pathway signaling functions and induce
developmental changes. Furthermore, the intrusion of MAPK signaling may disrupt normal
neurogenic proliferative functions promoted by Shh signaling. Instead, the MAPK pathway may
alternatively induce self-renewing (proliferative) division mechanisms, leading to extended cell
cycle activities, reductions in total GC output, and disoriented divisions that further change
compositional integrity of GC.
CHAPTER III: *Temporal Development of the Atoh1 Lineage*

INTRODUCTION

*Atoh1 Lineage of Cells and Origin of GCP*

In cerebellar development, there are two main progenitor regions giving rise to all identified neuronal types in the cerebellum (Legué et al., 2015). The rhombic lip (RL) progenitor domain has been well-studied, and is known to exclusively produce the future glutamatergic cerebellar neurons; including the cerebellar nuclei (CN) and the most abundant cell type in the cerebellum, the granule cells (GC) (Sudarov & Joyner, 2007). During development, the first evidence of RL proliferative activities occurs between E9-E11, which is characterized by *Atoh1* transient expression and production of cerebellar nuclei (CN) (Machold & Fishell, 2005). Following E12, a second lineage of *Atoh1* is initiated as GCP production and tangential migration is observed along the future EGL (Rose et al., 2009). In the rhombic lip, GCP proliferation is specifically regulated and controlled by the basic helix loop helix transcription factor (bHLH), *Atoh1* (Wang et al., 2005). In current research, cells expressing *Atoh1* have been exclusively determined as glutamatergic progenitors derived from the RL, and they are heavily supported as the precursors to the future granule cells (GC) and cerebellar nuclei (CN) of the cerebellum (Gazit et al., 2004). The second progenitor region, the ventricular zone (VZ), is a layer of neural stem cells that arises from the most dorsal portion of the fourth ventricle during development. The VZ is located in the basal portion of the cerebellum and is responsible for the future GABAergic neurons: including the Purkinje cells (PC), interneurons (IN) of the molecular layer (ML), Bergmann glia (BG), and several other remaining neuronal types (Hatten & Heintz, 1995). Both the RL and VZ progenitor regions have been thoroughly studied, and current evidence has defined distinct roles for each domain with no functional overlap in the production
of neuronal cell types. Patterned expression of the bHLH transcription factors *Atoh1* and *Ptf1a* in the RL and VZ, respectively, has been determined to have a defining role in organizing the specific developmental compartments in a non-overlapping manner (Fujiyama et al., 2009). Additionally, *Atoh1* or *Ptf1a* expression has been identified as necessary and sufficient for producing the respective glutamatergic and GABA-ergic neuronal types (Yamada et al., 2014).

As glutamatergic neurons, the CN are classically demonstrated to migrate rostrally from the RL into what is known as the nuclear transitory zone (NTZ). In this process, research has shown that the CN progenitors express of a unique variety of transcription factors in sequence, all of which demonstrate that the RL is both necessary and sufficient for successful expression in vitro (Fink et al., 2006). Such evidence and many more similar findings, spanning many years of research efforts, consistently support an invariable model of separate developmental regions for glutamatergic and GABAergic cerebellar neurons. In opposition to this traditional understanding of cerebellar neurogenesis, one study in particular has provided conflicting evidence of CN having developmental origins from the VZ in addition to the RL (Machold et al., 2011). This research revealed that unique combined expression patterns of transcription factors, including *Lhx2/9* and *Meis2*, are able to distinguish post-mitotic cerebellar nuclei precursors. As such, it was observed that proliferation of cerebellar nuclei precursors from the VZ region initiates immediately after neural tube closure at E9.5. These first neural precursors had differentiated at around E10-E11 when *Meis2* and *Lhx2/9* expression had first been observed within the early anlage of CN. The proposed VZ-derived CN precursors maintained *Lh2/9*, *Meis1/2*, and *Irx3* expression until E14.5 when this population of cells was found to migrate towards the accumulating cohorts of RL pre-cerebellar nuclei (Machold et al., 2011). Although this contradictory finding may have significance worth further considering, the consensus still
upholds the previously established understanding of strictly defined glutamatergic and GABAergic progenitor domains.

Additional findings from studies on gene expression patterns for stages of granule cells (GC) had also provided more evidence demonstrating alternative progenitor origins, but specifically for glutamatergic GC populations. This study was performed on granule cell progenitors (GCPs) from early postnatal mice and had identified spatially restricted, transient expression of cDNA clones against GCP genes (Kuhar et al., 1993). While these results were intriguing, the techniques used for this analysis are somewhat outdated, and postnatal stages of development are overall less insightful for determining developmental origins. Nonetheless, the research performed may have significance for future investigations, and may be worth considering in the context of Atoh1+ GCP populations. In a more relevant study investigating the role of temporal and spatial mechanisms in patterning specific neuronal populations, an additional progenitor domain was identified for a precursor pool of Atoh1+ cells. It was determined that two spatial domains are responsible for patterning Atoh1 populations. One origin was the RL giving rise to DCN and GC, and the second origin was identified as the isthmic organizer domain giving rise to the isthmic nuclei. Additionally, precursors from this domain had been determined to dependently express Lhx9 in response to isthmic FGF signaling (Green et al., 2014). This evidence provides explanations for diverse developmental functions that go against the invariably accepted mechanisms. These novel findings are particularly relevant for investigating neuronal origins, but may also suggest gaps in additional areas of development that have gone unnoticed and remain undiscovered due to already accepted explanations.

The research aims outlined in Chapter I-II described investigations for identifying GCP developmental mechanisms through manipulation and observation of the Atoh1 lineage of GCP.
However, further analysis of *Atoh1* expression prior to GCP proliferation may provide meaningful insights in regard to the well-defined cerebellar progenitor regions and the migratory origins of future neural populations. As more becomes understood about the diverse developmental paths of neural populations through the contributions of advanced analytic techniques, our fundamental understanding of neuronal precursor origins within the cerebellum may necessarily be revisited. Previous in-house analyses of single-cell RNA sequencing data (scRNAseq) revealed a group of glutamatergic cells characterized by expression patterns from genes of the VZ (*Hes1, Vim*) and the RL (*Atoh1, Barhl1*). In the following pseudo-temporal datasets used in patterning the developmental trajectories of cell clusters, the data revealed a genetic transition in cells from the VZ as they migrate and merge into the sub-pial stream of cells derived from the RL (Wizeman et al., 2019). This evidence was suggestive of a VZ domain giving rise to an unidentified lineage of *Atoh1*+ glutamatergic precursors, something that fundamentally opposes the extremely well-defined neuronal origins from distinct progenitor domains. In order to characterize this lineage of *Atoh1* cells and confirm its VZ origin, further investigations throughout various stages of development would be required for locating this precursor population. In particular, early embryonic development prior to E11-E12 has been somewhat understudied thus far, and research in these stages of early neuronal development may be promising for *Atoh1* lineage tracing. Additionally, identifying a novel region of *Atoh1* cells derived outside the undisputed RL origin would provide a more comprehensive understanding of the general developmental outline and overarching functions comprising the cerebellum.
Figure 13. The series of animations in this image show the progenitor regions of the developing cerebellum, following specific cell types through specific stages. As shown in the diagram, orange coloring represents GABAergic neurons from the ventricular zone (VZ), dark green represents granule neurons from the rhombic lip, and light green represents deep cerebellar nuclei (DCN) from the rhombic lip. Granule neurons start to form and migrate tangentially at E14.5, as depicted by the dark green arrow. The external granule layer, in dark green, surrounds the exterior of the cerebellum by P1. After significant proliferation, the granule neurons migrate inward to form the internal granule layer (IGL) by P15-P21.

OBJECTIVES OF THE STUDY

In our investigations of the neuronal origins and processes throughout early development of the cerebellum, we aim to challenge the well-defined hypothesis provided for mechanisms of neuronal specification in compartmentalized progenitor domains. Additionally, we challenge the concept of unanimous progenitor origins, specifically in regard to the current understanding of universal CN progenitor origins derived in the RL region. Instead, we revisit an alternative hypothesis formed from a combination of previous research findings, which states that distinct populations of CN may originate from spatially and temporally independent regions of the VZ not previously accounted for. In our approach, we consider Atoh1 expression from alternative
progenitor regions of E9.5-E11.5 transgenic mice in an effort to determine whether glutamatergic neurons show origins outside of the RL.

The *Atoh1*-lineage arises from the upper rhombic lip (RL), which resides at the interface between the posterior cerebellar VZ and the roof plate of fourth ventricle, and produces diverse cell types, including extra-cerebellar and cerebellar nuclear neurons and granule cells (Machold and Fishell, 2005; Wang et al., 2005). Currently, the mechanisms that generate the remarkable diversity of neurons from the *Atoh1* lineage are still poorly understood. In Chapter III of the research presented, our primary aim is to uncover a novel VZ origin giving rise to a population of *Atoh1* precursors. Ultimately, this type of finding would define a new implied function for progenitor domains, and would also provide an enhanced understanding of early developmental activities in *Atoh1* lineages of cells. Evidence provided from previous scRNAseq data collected on a cluster expressing *Atoh1* was traced to origins within a distinct VZ progenitor domain. Here, we aim to determine whether *Atoh1* cells can be traced to origins outside of the RL by investigating the proposed possibly of an isthmic or VZ domain instead. In *Atoh1* lineage tracing experiments using *Atoh1* as an enhancer for Cre dependent expression of RFP on the R26 locus, we will use observations of RFP labeling to test our hypothesis of *Atoh1* expression. By investigating these *Atoh1*-expressing cells, we aim to analyze the visible distribution of *Atoh1* cells for a comprehensive look at the developmental landscape for glutamatergic lineages.

Furthermore, evidence suggesting a differential domain of origin for *Atoh1* cells outside of the RL would provide a significant contradiction to how progenitor domains are currently understood, which may ultimately initiate efforts to revisit additional aspects of development.
RESULTS

Fate mapping of Atoh1-expressing cells at E10.5

In the Atoh1 lineage tracing experiments performed throughout the early stages of development, we used the RFP reporter gene to observe expression of Atoh1 within the cerebellum. Our investigations were targeted towards E9.5-E11.5 mouse embryos to determine whether Atoh1/RFP expression may exist in the obscurely characterized ventricular zone region. Previous research findings demonstrated CN precursor proliferation from the VZ starting at E9.5 and final migration to the RL populations of CN by E12.5, but our results from the transgenic E9.5 and E11.5 embryos showed no observable RFP expression to suggest evidence of glutamatergic Atoh1+ cells outside of the RL progenitor domain. However, our analysis of the E10.5 embryos showed very clear, consistent expression of RFP from the VZ in what appeared to be clonally produced cell populations (Figure 14).

To ensure that the RFP expression seen in the E10.5 transgenic embryos was significant and not an error in the transgene’s Cre enzyme, multiple immunohistochemistry (IHC) experiments were conducted to localize Cre expression in relation to the Atoh1+ RFP. Using antibodies against the Cre protein and Tjap2b, a marker for GABAergic neural progenitors, the results confirmed our initial observations and provided additional information in regard to the transient clonal propagation of Atoh1+ progenitor cells. Data on individual cell counts and co-expression of markers were collected from the IHC experiments, and the findings suggested several implications. Analysis of the blue channel (Cre expression) in the transitory region directly above the VZ indicates showed broad expression of Cre in this traditionally unexpected area. This suggests evidence of an Atoh1 lineage of progenitors arising from the VZ, since the Atoh1 enhancer gene must be expressed in order to activate the Cre enzyme transgene, which is
ultimately expected to constitutively express the RFP reporter gene. However, overlapping expression of RFP and Cre was not frequently seen in the VZ region, which logically contradicts the lineage tracing expectation that Atoh1+ cells will activate Cre dependent RFP expression to enable observation of Atoh1 expression. Similarly, RFP cells present in this same region show some overlapping expression with Cre in comparatively higher proportions, but also demonstrates several instances of expression that is independent of Cre presence. Despite these relatively unclear findings, additional results comparing RFP and Cre expression indicate that as Atoh1+ progenitor cells migrate upwards into the NTZ, almost all of the RFP cells show co-expression with the blue Cre enzyme. This finding was also supported by gradual increases in the proportion of RFP/Cre co-expression as cells appeared to migrate away from the VZ, along with the apparent transitions of co-expression seen from groups of cells demonstrating clonal expansion. We also observed undeniable evidence of RFP/Cre co-expression within the VZ, although not in large amounts, but we consistently saw these same expression patterns throughout each experiment, suggesting that the transgene was in fact effective. While the significance of these results can be supported by the determined reliability of the lineage tracing experiment, the observations of inconsistent variations in Cre/RFP expression and co-expression closer to the VZ still remain unresolved. The basis of these findings, combined with the consistent co-expression of RFP and Cre seen in migrated aggregates of Atoh1+ cells, may indicate a unique proliferative mechanism for VZ-derived Atoh1 progenitor populations.
Figure 14. These two adjacent sets of images show immunohistochemistry (IHC) results for E10.5 Atoh1+/Cre; R26-RFP/+ transgenic mutants. The top images show a full frontal plane of the cerebellum in two different samples of transgenic mice. The white dashed boxes are shown for reference of the zoomed-in set of images below. The red arrows in the bottom images identify the clonal expansion of RFP+ cells from the VZ, as seen by the linear clusters of red cells. The red labeling indicates Cre dependent RFP expression from Atoh1 expressing cells, the blue marker shows cells with activation of the Cre enzyme in Atoh1 cells, and the green marks Tfap2b expression for GABAergic neural progenitor cells. The images demonstrate high levels of RFP/Cre co-expression towards the upper portion of the cerebellum, whereas many cells closer to the VZ exhibit sole expression of either Cre or RFP with few cells expressing both.

Investigating the identity of Atoh1-expressing cells from the VZ

In the same IHC experiments used to analyze the relationship between Cre and RFP expression patterns, we also used Tfap2b as a marker to indicate GABAergic neural progenitors. We chose this marker to ensure that our observation of Atoh1+ cells from the VZ could in fact be identified as glutamatergic Atoh1+ progenitors, and distinguished from the traditional
GABAergic hypothesis. If the *Tfap2b* (green) were to demonstrate co-expression with either the Cre or RFP cells, it would have disproven our evidence and instead confirmed that cells from the VZ are GABAergic. However, the results showed that all Cre+, RFP+, and Cre+/RFP+ cells showed absolutely no expression of *Tfap2b*, which supports the identity of these *Atoh1* cells as glutamatergic precursors derived from the VZ (Figure 15).

While this evidence of *Atoh1* cells lacking GABAergic identity from the VZ may have presented a novel finding in our understanding of developmental origins, the fate and characteristics of this population of cells were still in unknown. Initial evidence showed that none of the proposed *Atoh1* cells showed any overlapping expression with *Tfap2b*, which refuted and discounted the classically supported expectation of these cells would be GABAergic neural progenitors. In further attempts to determine the fate and developmental identity of these uncharacterized *Atoh1* cells, we performed IHC experiments using the *Meis2* marker, which is a transcription factor for CN precursors. In a previous paper describing glutamatergic CN anlages from the VZ, expression of the *Meis2* transcript had been well-defined as a marker for early CN precursor populations. Results from IHC showed that all *Atoh1* cells, as determined by RFP/Cre expression, were exclusively co-expressed with *Meis2*. This finding confirmed the identity of these early *Atoh1* progenitor cells from the VZ as future CN neurons. Despite these convincing findings, we still observed some *Atoh1* cells near the VZ, especially in RFP+ clonal populations, that had not demonstrated expression of *Meis2* as committed CN neural progenitors.
**Figure 15.** The images above show IHC results for the *Atoh1+/RFP* lineage tracing experiments performed at E10.5. The image in the upper left corner labels the VZ progenitor region where cells are arising from in the lower portion of the tissue. The RL progenitor region is also labeled on the opposite side of the labeled VZ, and it tangentially extends upward along the upper surface of the tissue. Red arrows identify the clusters of red *Atoh1+/RFP+* cells that demonstrate apparent clonal expansion from the VZ, but do not co-localize with the green *Meis2* marker. The white arrows show cells starting to co-express *Meis2* and *Atoh1/RFP* closer to the RL region, indicating CN fate commitment.

**DISCUSSION**

*Transient expression and clonal expansion of Atoh1+ cells from the VZ suggests oscillating expression of Atoh1 prior to commitment as CN*

Our *Atoh1* lineage tracing experiments throughout the developmental early stages of embryonic mice revealed a discrete population of *Atoh1* expressing cells arising from the
ventricular zone (VZ), something that had not been demonstrated in previous research. Although the E10.5 was the only stage that demonstrated this result, we are able to provide evidence consistent with what our scRNAseq data had characterized, and we are now able to present additional findings to support the existence of Atoh1+ populations of glutamatergic precursor cells outside of the RL. However, our investigations provided many more insights for determining the identity, characteristics, and developmental properties of this cell population.

Following the initial finding in E10.5 Atoh1 lineage tracing experiments, we looked at E9.5 and E11.5 embryos to investigate the specific developmental timeline for the VZ derived population of Atoh1 cells. Neither of these embryonic stages had demonstrated similar or suggestive evidence of this novel Atoh1 population. This had further indicated that these glutamatergic fated progenitors must necessarily undergo migration and division from the VZ at E10.5 during development, but do not appear any earlier and do not proliferate any later in mouse development. However, further IHC investigations in E10.5 and E11.5 embryos had revealed the migratory route and destination of these cells as they differentiate. Many of the differentiated cells had completed migration and indefinitely populated within the dense region of RL derived cerebellar nuclei (CN) progenitors. Prior to this finding, the cells followed a vertical path with consistent patterns of clonal expansion from the VZ. This pattern was especially strange, since Cre enzyme expression rarely co-localized with Atoh1 expression and was inherently inconsistent from what would be expected. More specifically, the populations undergoing clonal expansion had always demonstrated sole expression of Atoh1 without Cre. Additionally, Cre+ cells in the VZ had shown occasional Atoh1 co-expression, but there were also many instances of independent Cre expression without Atoh1. Once these cells traveled to
the RL pool of progenitors following differentiation, we observed unanimous Cre/Atoh1 co-expression.

From these observations of inconsistent expression patterns and Atoh1 clonal expansion, we concluded that the VZ population of glutamatergic precursors may divide through oscillating Atoh1 expression prior to differentiation and migration into the RL region. This mechanism comprehensively explains our observations of varying patterns of Cre/Atoh1 co-localization, Cre expression without Atoh1, and Atoh1 without Cre in the VZ region, as well as the universal Cre/Atoh1 co-expression in post-mitotic cells after exiting the VZ. Additionally, many bHLH transcription factors have been characterized to have oscillating expression mechanisms, which also suggests that the Atoh1 bHLH transcription factor could also demonstrate similar expression properties. As these cells arise from the VZ, the independent expression of Atoh1 or Cre can be explained by the on and off expression of Atoh1. The precursors divide while Atoh1 is transiently expressed, which we observe through the evidence of clonal expansion. Cells that express only Atoh1 RFP have likely turned off Atoh1 expression, but still demonstrate RFP transgene expression shortly after. Furthermore, the Cre+ cells had likely just turned on Atoh1 expression, and thus activated Cre enzyme expression, but the enzyme had not yet exerted any influence over RFP transgene expression. The instances of cells co-expressing Atoh1/Cre from the VZ showed a point in time where Atoh1 had been turned on long enough to demonstrate RFP transgene expression from Cre activation. Lastly, the Atoh1/Cre expressing cells that are found throughout the expanding pool of RL precursors and within the NTZ are no longer experiencing oscillations of Atoh1 expression, which most likely initiates the precursor’s exit from the cell cycle.
Further results from IHC experiments had helped determine the fate of the Atoh1 precursors from the VZ progenitor domain. None of the Atoh1 lineage showed any co-expression with Tfp2b, which is a marker for GABAergic neural progenitors. Additionally, the CN neural progenitor marker, Meis2, showed exclusive co-localization with Cre and Atoh1 expressing cells within the NTZ and RL. This finding allowed for us to conclude this lineage of Atoh1 precursors had a CN developmental fate. However, we also determined that the precursors do not express Meis2 while clonally expanding, and they ultimately begin to express Meis2 as the cells exit the VZ and enter the NTZ with other fated CN precursors from the RL.

It is very likely that others had not characterized this population of Atoh1 cells due to transient Atoh1 expression patterns, and also the fleeting developmental activities that can only be found at E10.5. Additionally, these glutamatergic cells progressively differentiate within the expected RL region of progenitors as normal, for which this VZ population is otherwise indistinguishable and may easily be classified as RL derived cells.

CHAPTER IV: Future Directions

FUTURE DIRECTIONS

The induction of GCP neurogenesis is largely thought to be from Shh signaling mechanisms, and this proposed pathway has remained the most well-accepted explanation for regulation of GCP proliferative functions during development. In vitro studies have demonstrated mandatory functions of the Shh signals secreted by Purkinje cells (PC) in facilitating proper GCP divisions, and foliation patterning throughout development. However, our evidence from scRNAseq indicates much lower Shh presence than initially expected, with Gli1 expression limited to only the GCP3 subtype. In our investigations modeling upregulation
and downregulation of the MAPK/ERK signaling pathway, we provided overwhelming evidence in support of a previously unidentified role for MAPK/ERK signaling in regulating GCP development. Additional comparisons between the MEK-GOF model for MAPK upregulation and published findings from a Shh-GOF model had revealed a highly suggestive functional correlation in pathway roles that specifically implied reciprocal coordination of pathways during GCP development. Cell cycle experiments in MEK-GOF mutants had revealed far fewer differentiated GC in the EGL and IGL, longer cycling times, delayed rates of differentiation, and significant thinning of the EGL, for which all observations were exclusively found within the anterior and central lobe regions of induced activity. Additional supporting evidence had also implied a mechanism of vertical GCP divisions, which ultimately explained structural irregularities in lobules and cortical expansions in MAPK GOF mutants. Further comparisons between Shh signaling functions and the demonstrated functions in MAPK signaling had suggested distinct differences in the specific GCP proliferation mechanisms facilitated by each pathway during development. Ultimately, we were able to define a role for MAPK signaling within the posterior/central lobe, and we further correlated endogenous Shh pathway functions in opposing regions. Furthermore, we provide evidence to support distinct developmental regions for each pathway. Most significantly, we have also determined that overexpressing MAPK can induce its activity within Shh endogenous regions and exert functional control over normal Shh pathway functions, which causes dysregulation of GCP proliferative mechanisms promoted by Shh signaling.

The implications from findings revealed in this research could provide valuable insight in relating specific pathway functions to characteristics of medulloblastoma subtypes. Mutations in Shh signaling have already been identified in a large majority of medulloblastomas, and still
many features of tumor formation, progression, identification and treatments remain unknown in Shh subtypes. Furthermore, we have just identified a previously unreported role of MAPK/ERK signaling in regulating GC proliferation mechanisms, which may help in identifying new medulloblastoma subtypes with MAPK mutations and other pathway involvements. The identified proliferative functions involved in MAPK signaling, combined with the finding of ectopically induced activation from pathway overexpression, provide valuable insight for a more enhanced understanding of features involved in tumor development and targeted treatment options. We also found evidence of a feedback regulation mechanism in endogenous MAPK regions, which may characterize additional subtype mutations involved in disrupting or downregulating this regulation mechanism, or may even reveal treatments for inducing feedback regulation in targeted tumor regions. The novel findings identified from this research have many unknown implications, creating many additional avenues of investigation with significant potential for understanding cerebellar ailments in human disease.

In our collection of findings, we have also observed clear evidence of MAPK interference in mechanisms of Shh signaling and noted several adverse morphological consequences as well. Our results have also shown a proliferative decrease from MAPK interference in Shh signaling, which provides an interesting comparison to be made between a protective developmental function in MAPK signaling, despite the additional changes induced. One study in particular has investigated a role for WNT3 in suppressing GCP proliferation and inhibiting tumor formation in medulloblastoma Shh subtypes (Sandrine et al., 2013). More specifically, this WNT3 inhibitor was found to indirectly downregulate pro-neural genes in the Shh pathway through non-traditional activation of the MAPK/ERK pathway. The study demonstrated that inhibition of MAPK/ERK signaling had reverted the preventative effects in tumor growth, but ultimately it
was concluded that WNT3 acts a regulator for neurogenesis and has repressive functions in preventing tumor growth (Sandrine et al., 2013).

Another study that investigated a specific CD271 marker used in identifying a Shh subtype of medulloblastoma had found evidence of MAPK/ERK upregulation in a transcriptome analysis of the tumor cell marker. When treating the CD271+ tumor cells with a MEK1/2 inhibitor in vivo and in vitro, CD271+ cells showed less migration, reduced survival rates, fewer stem cells, less proliferation, and the overall levels of CD271+ cells had also been decreased. The study’s findings had implicated a role of MAPK upregulation in Shh medulloblastoma tumors, while also determining a clinically significant MEK1/2 inhibitor treatment for reducing CD271+ tumor cells. The functional data concluded that MAPK signaling had an important role in Shh medulloblastoma tumor cell proliferation, survival, and migration (Liang et al., 2018).

Further support for MAPK pathway involvement in Shh medulloblastoma tumors was determined by a study performed on metastatic (M+) medulloblastomas, where MAPK upregulation had been demonstrated very frequently in M+ tumors (MacDonald et al., 2003). Additionally, the study revealed that inhibition of a receptor known as PDGFA had been involved in reducing MAPK pathway genes in M+ cells, and inhibition of MAPK downstream gene expression was responsible for inhibiting migration in metastatic tumor cells (MacDonald et al., 2003). One final study that investigated the effectiveness of therapeutic Smo receptor inhibitors in treating Shh tumors had found evidence of two drug inhibitors that maintained tumor resistance (Zhao et al., 2015). The first drug inhibitor was a mutation in Sufu that sustained Shh pathway signaling regardless of Smo inhibition, but the second inhibitor was from compensatory MAPK pathway activation that had been responsible for driving tumor growth and metastasis when Shh was inhibited. This study had defined MAPK pathway involvement in
tumor evolution and drug resistance for Shh medulloblastoma tumors and basal cell carcinomas as well (Zhao et al., 2015). From evidence supported in multiple studies, it seems relatively likely that MAPK upregulation has a role in facilitating Shh medulloblastoma tumorigenesis, and may also have a role in directly inducing tumor formation and metastasis. However, evidence is partially conflicting across the entirety of reported findings, which emphasizes the importance of understanding how the mechanisms and contributions of such proliferative pathways during development could influence tumorigenesis, or cell cycle defects.

The collection of studies that have associated Shh medulloblastomas to mechanisms of MAPK upregulation barely seem to scratch the surface of diverse of health consequences, or even in addressing the context of association between proliferative pathway interactions. We have determined from our investigations that the MAPK and Shh pathways have opposing developmental functions, and MAPK clearly exerts some control over Shh functions by inducing changes in characteristics of GC development throughout the anterior/central lobes. However, the exact interactions between the pathways and the context of control exerted by MAPK signaling in relation to the Shh pathway still remain unidentified. While MAPK involvement in GC development and its relationship with the Shh pathway have not been identified in previous reports, there are additional associations that have been described in other contexts of development. A study investigating signaling pathways involved in generating oligodendrocyte progenitors had similarly ambiguous evidence of Shh and MAPK signaling interactions, but further experiments revealed a more refined pathway association. This research reciprocally inhibited Shh signaling to determine effects in FGF2/MAPK activation, and then inhibited the MAPK receptor FGFR1 to observe the effects in Shh pathway activation. It was found from these experiments that MAPK adequately functions independent of Shh signaling, but
alternatively, *Shh* does require MAPK input for pathway functioning producing (Kessaris et al., 2004). *In vivo*, the oligodendrocyte precursors had constant, stable basal levels of MAPK activity that were absolutely required for *Shh* pathway activation. Another important finding from this study had informed that MAPK signaling was cell autonomous in activating the *Shh* pathway (Kessaris et al., 2004). These relationships provide significant context in understanding the potential functions for pathway communications throughout GCP development. However, our research did not demonstrate basal MAPK activation for cell autonomous *Shh* activity, and rather, we observed probable inhibition of *Shh* in MEK-GOF mutants from inducing cell autonomous MAPK activities. If anything, we may suspect an inhibitory effect from MAPK presence in GCP with *Shh* signaling.

The next steps for this study would include further investigations towards uncovering the regulation mechanisms for MAPK signaling. We observed endogenous feedback inhibition when MAPK signaling had been upregulated, but a determined mechanism for relaying negative feedback has not been identified from this. The feedback regulation that had been demonstrated in our mutants may play a potentially significant role in regulating development by relaying communication signals to coordinate GCPs in surrounding areas. In relation to our specific interests, we may be able to apply disruption or absence of this feedback mechanism to failures in developmental processes, like in the instance of medulloblastoma pathogenesis. Furthermore, the knowledge of how feedback regulation works and how it can be turned on/off could influence treatment option that improve medulloblastoma outcomes. In another common pediatric cancer, neuroblastoma, a recently identified inhibitor for MAPK negative feedback regulation has been the basis of several new treatments options. This research was investigating the effects of ALK signaling pathway mutations in neuroblastoma tumor cell lines. From the
results, it was determined that the ALK mutation causes overexpression of an inhibitor for MAPK feedback regulation, which causes uncontrolled cell growth in neuroblastoma tumors. Further suggestions for ALK molecular therapies involved induced inhibition targeting in MAPK pathway regulation. When ALK-inhibition was tested, the MAPK proliferative gene Etv5 and MAPK pathway inhibitors Dusp4/5/6, Spry2/4, MAFF had returned to normal expression levels (Lambertz et al., 2015). These insights are quite valuable in improving treatment options for specific cancer types, which further emphasizes the need for studying the effects and roles of cellular signaling pathways in tumorigenesis. Further analysis could explain specific functions, roles, and mechanisms of control for regulating GCP proliferation.

While we can relate this finding with several other studies that have investigated the functions and contributors for MAPK feedback regulation, many of these studies extend far beyond the bounds of cerebellar development. Thus, more experimental analyses targeting the specific gene expression patterns in mutants may help in relating changes in gene enrichment and the morphological observations. Further investigations using scRNAseq from P4 MEK-GOF and Etv4-DN-LOF mutants will help in comparing the specific changes in expression, which may ultimately help in identifying potential feedback regulators involved in MAPK signaling. The combined data from scRNAseq in MAPK mutants may also reveal insight into signaling mechanism and the mutations involved in subtypes of medulloblastomas. The info on mutant gene expression profiles may also help in correlating features of known mutations for subtypes of medulloblastomas with the dysregulated MAPK genes found in mutants that could lead to formation of medulloblastoma subtypes and further mutations. Ultimately, the goal of scRNAseq in mutants is to uncover specific feedback regulation mechanisms in MAPK signaling through comparisons between endogenous and ectopic GCP populations.
MATERIALS AND METHODS

Animal Studies

All procedures involving animals were approved by the Animal Care Committee at the University of Connecticut Health Center (protocol #101849–0621) and were in compliance with national and state laws and policies. Generation and characterization of the Atoh1creER (Atoh1tm1(cre)Gan/J:#160667) (Yang et al., 2010), R26R FP (B6.Cg-Gt(Rosa)26SorTm9(CAG-tdTomato)Hze/J, #007909) (Madisen et al., 2010), R26RMEK1, R26R Etv4-DN alleles have been described. All mouse strains were maintained on an outbred genetic background. Primer sequences for PCR protocols used in genotyping have been described on the JAX Mice website.

Transgenic mice were made using an Atoh1 enhancer gene with Cre dependent exonuclease enzyme activity, inserted within the coding region. The MEK1 gene sequence was inserted onto the Roas26 locus and loxed by Cre recognition sites. The Cre enzyme is dependently expressed by activation from transcription of Atoh1 in cells. Cre recognizes the “lox” sites that sequester the inserted MEK1 gene on the Rosa26 chromosome locus gene for continuous expression. For Etv4-DN, the same transgenic practices were used in generating this genetically manipulated model. The Etv4-DN gene encodes a repressor protein that binds the transcriptional domain of the Etv4 gene, preventing the transcription and expression of Etv4.

Mouse Breeding: Noon of the day on which a vaginal plug was detected was designated as E0.5 in staging of embryos. In creating a genetic model for the upregulation of MAPK signaling, Atoh1/ Cre heterozygote mice were crossed with R26 +/MEK heterozygote mice to achieve the desired Atoh1+/Cre R26+/MEK genotype. For obtaining homozygote mouse mutant with the genotype Atoh1-Cre;R26MEK1DD/MEK1DD, heterozygote mutants with were crossed
(Atoh1-Cre;R26MEK1DD/+ and Atoh1-Cre;R26MEK1DD/+). In breeding a loss of function model, the Atoh1/+ Cre R26 +/-Etv4-DN genotype was generated by crossing Atoh1/+ Cre heterozygotes with R26 +/-Etv4-DN heterozygotes. Homozygote LOF mice (Atoh1/+CreR26 Etv4-DN/Etv4-DN) were produced from crosses between mutant heterozygotes with the genotype: Atoh1/+CreR26 +/-Etv4-DN. All Cre negative littermates were used as WT controls.

Dissection, Embedding, Preservation and Collection of Tissue

Tissue Perfusion and Dissection: All mice P4 and older required perfusion prior to dissection. The perfusion steps for postnatal samples are as follows: live mice are sedated with an injection of a prepared ketamine/xylazine, dosed at 0.1ul/10g of body weight. Once sedated, a needle is then injected into the lower chamber of the heart, and the body is then flushed with PBS for 5 min, or until the liver is pale. Following removal of blood from the body, the mice are flushed with 4% PFA in PBS for 5 more min, or until the limbs become stiff. Perfused mouse bodies are stored at 4° C until dissection.

For dissection of embryos, the steps are as follows: Embryo tissues are dissected from the uterus of the deceased mother through removal of the placenta, fetal membranes, and yolk sacs. All embryos are placed into PBS prior to removal of all membranes. In the E10.5 samples used, no further sample modifications were made prior to fixation of samples in petri dishes. Fixation solutions with 4% paraformaldehyde in PBS were added to each sample. The embryo dish was stored at 4° C overnight and gently rotated by a Nutator.

Tissue Preservation: The dissected tissue samples (postnatal) were placed in a test tube with approximately 10-15ml of 4% paraformaldehyde in PBS. The tubes were cooled at 4° C overnight and rotated gently on a Nutator. The following day, the dissected tissue(s) were
transferred into a solution of 15% sucrose in PBS and kept at 4° C for approximately 6 hr, or until the sample has sunk to the bottom. The tissue(s) were then transferred into a solution of 30% sucrose in PBS and kept at 4° C for approximately 6 hr again, or until the sample sinks to the bottom. After gradient solution steps have been finished, the samples were embedded in OCT or 7.5% Gelatin (for postnatal tissue) and then stored at -80° C.

_Tissue Collection:_ A cryostat was used to section preserved sample blocks at 10-20 µm thickness, depending on developmental age of the sample. All postnatal samples were sectioned at 20 µm thickness, whereas the E10.5 samples were sectioned at 10 µm thickness instead. A cryostat microtome (Leica) blade was used for sectioning all samples, and the tissue was collected on slides. Sagittal planes were sectioned in many of the samples collected. The cryostat sectioned slides with collected tissue were stored at 4° C.

**Immunohistochemistry**

The slides with tissue collected from cryo-sectioned samples were first rinsed in PBS for 5 min at RT. Gelatin or OCT was removed from the slides with the tissue sections by incubating at 37° C in warmed PBS for 15 min. An antigen retrieval process was used for some types of antibodies and performed as follows: First, 10 mM citric acid with pH 6.0 was heated for approximately 20 min in a steamer. After gelatin has been removed from the slides, the slides were placed in the heated citric acid solution for 1-5 min in the steamer, depending on whether the tissue sample had fluorescent proteins. The slides were removed from the steamer and cooled at RT for about 30-45 min. Following retrieval processes, or if retrieval is not required, slide were rinsed in PBS two or three times at RT. A 250 µl/slide blocking solution containing 10% volume of normal donkey serum and PBS is prepared, and slides are blocked for approximately
45 min at RT in a closed, humid box. Preparation of primary antibodies is performed by making a ~200-250 µl/slide solution with 4% volume of normal donkey serum in PBS, and addition of primary antibodies chilled on ice. After blocking is finished, 200-250µl of the primary antibody solution is added for each slide and then incubated overnight at 4°C in a moist covered box. The following day after incubation, the antibody solution is removed and preserved, and the slides are brought back to RT after approximately 15 min. The slides are rinsed 5 times in PBS, for 5 min each rinse. The 200-250µl/slide secondary antibody solution is performed using 1:500 dilutions of Red 594, Green 488, and Azide 647, with 4% volume of normal donkey serum and PBS. Approximately 250µl of the secondary antibody solution is added for each slide and then incubated for approximately 45 min at RT in a moist box without light. Following incubation, the secondary antibody is discarded from the slides, and the slides are rinsed in PBS 3-4 times. The slides are then counter stained with DAPI (H-3570; 5µl/50ml PBS) for 5 min at RT. The Hoechst/DAPI solution is recycled from the slide for future use and stored at 4°C. Slide are rinsed twice more with PBS, and then individually dried prior to adding a coverslip. A small amount (~50ul) of Fluoromount is added to each slide and then stored at 4°C for drying.

**Nissl histology**

Slides are removed from 4C refrigerator and incubated at 37°C in warmed PBS for approximately 5 min to remove Gelatin or OCT from the slides. The slides are then placed in a Cressyl violet dye overnight for staining of granule cells in the tissue. The following day, the slides are dehydrated in a gradient of ethyl alcohol solutions in order: 90%, diluted 90%, 95%, diluted 95%, and 100%. The slides are then placed in a recycled solution of xylene for approximately 5 min. Then, slides are placed in a second prepared solution of recycled xylene
for another 5 min. Each slide is removed, dried individually, and a small amount of permount is added for placement of a coverslip. The slides dry for 24 hr in a vented hood and stored at RT.

**Single-Cell RNA sequencing**

Brains of mouse embryos were dissected in ice-cold phosphate buffered saline. The dissected tissues were cut into pieces smaller than 1 mm in dimension and transferred to ice-cold MACS Tissue Storage Solution (Miltenyi Biotec, Somerville, MA). For cell dissociation, the storage solution was exchanged with RPMI 1640 Medium (Thermo-Fisher); tissue was pelleted and digested with 500 µl of pre-warmed Accumax (Innovative Cell Technologies) in a 1.5 ml tube at 37 °C for 5–10 min. At the end of digestion, the tissue pieces were dissociated by gentle trituration with a wide-bore pipet tip. The cell suspension was added to a 100 µm cell strainer (Corning, Corning, NY), and it was collected and transferred to 1.5 ml of ice-cold Resuspension Buffer (Lebovitz L15 medium with 2% FBS, 25 mM HEPES, 2 mM EDTA). The cell clumps that were unable to pass through the filter were placed into a new 500 µl pre-warmed Accumax solution, and the digestion and filtering process was repeated twice to maximize the yield of single cells. Following the dissociation, cells were stained with Trypan blue, counted and visualized with Countess II Automatic Cell Counter (ThermoFisher). After single cell suspension, the 10X Genomics Chromium Single Cell Kit v1 (PN-120233) was used to create cDNA libraries. Samples were then sequenced on Illumina NextSeq 500. The raw reads were processed to molecule counts using the Cell Ranger pipeline with default settings (Zheng et al., 2017), which were then processed using the Seurat R package (version 1.4) (Butler et al., 2018; Macosko et al., 2015). Genes that were detected in less than three cells were removed. Cells in which over 5% of the UMIs were mapped to the mitochondrial genes were discarded,
and cells that contained less than 200 or over 4800 genes were considered outliers and discarded. Library-size normalization on the barcodes for UMI-collapsed gene expression were scaled by 10,000 for total number of transcripts per cell and log2transformed. The variable genes were identified using Seurat’s MeanVarPlot function using the following parameters: x.low.cutoff = 0.0; y.cutoff = 0.8. Specific genes for each cluster were identified using the Seurat’s FindAllMarkers function. To refine clustering, the SubsetData function was used to create a new Seurat object containing only a subset of the original cells and cell clustering was reiterated.

**EdU Tracing Click-it Reaction**

For EdU labeling in MEK-GOF, EdU (0.5 mg/ml in PBS) was injected intraperitoneally into live mutants (dosage: 10 μg/g of body weight), and were dissected 24 hr later, 48hr, or 72hr later. EdU labeling was detected with the Click-iT EdU Imaging Kit (Invitrogen, Carlsbad, CA, USA).

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