Distinct roles of Tfap2A & Tfap2B in the diversification of GABAergic neuronal subtypes in the cerebellum

Norliyana Zainolabidin

2017

Norliyana Zainolabidin. (2017). Distinct roles of Tfap2A & Tfap2B in the diversification of GABAergic neuronal subtypes in the cerebellum. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/72795>

<https://doi.org/10.32657/10356/72795>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

SINGAPORE

**DISTINCT ROLES OF TFAP2A & TFAP2B
IN THE DIVERSIFICATION OF GABAERGIC
NEURONAL SUBTYPES IN THE CEREBELLUM**

**NORLIYANA ZAINOLABIDIN
SCHOOL OF BIOLOGICAL SCIENCES**

2017

**DISTINCT ROLES OF TFAP2A & TFAP2B
IN THE DIVERSIFICATION OF GABAERGIC
NEURONAL SUBTYPES IN THE CEREBELLUM**

NORLIYANA ZAINOLABIDIN

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2017

ACKNOWLEDGEMENTS

“Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world.” – Albert Einstein.

First and foremost, I thank Nanyang Technological University (NTU) School of Biological Sciences and NTU Research Scholarship for providing me with the opportunity and scholarship to pursue my Ph.D. studies. We also thank the Warwick-NTU Neuroscience Programme for funding this project.

I would like to acknowledge my supervisor, Albert I. Chen, for his invaluable patience and guidance the past four years. He has made it his priority to groom independent individuals to have an inquisitive mind. This thesis would also not be possible without the current and former Chen lab members for their sheer presence, critical inputs and tremendous help. Last but not least, to my husband, Shahril, who is most supportive and understanding.

“I have not failed. I’ve just found 10,000 ways that won’t work.” – Thomas Edison.

TABLE OF CONTENTS

LIST OF FIGURES	i
LIST OF TABLES	ii
ABBREVIATIONS	ii
ABSTRACT	v
1. INTRODUCTION.....	1
1.1. Molecular programs involved in the generation of the cerebellar primodium ..	1
<i>1.1.1. Demarcation of the cerebellar territory</i>	<i>2</i>
<i>1.1.2. Formation of the cerebellar structure</i>	<i>4</i>
1.2. Cerebellar neurogenesis, migration & organisation	8
<i>1.2.1. Generation of cerebellar neural progenitors</i>	<i>8</i>
<i>1.2.2. Schedule of glutamatergic and GABAergic cerebellar neuron production</i>	<i>13</i>
<i>1.2.3. Organisation of local circuits within the cerebellar cortex</i>	<i>16</i>
1.3. Transcription factor activating protein 2.....	20
<i>1.3.1. Molecular profiles of inhibitory versus excitatory neurons</i>	<i>20</i>
<i>1.3.2. Transcription factor activating protein 2, Tfap2A & Tfap2B</i>	<i>21</i>
2. MATERIALS AND METHODS	24
2.1. Animals.....	24
2.2. Histological procedures.....	24
2.3. Mixed cerebellar neuronal culture	24
2.4. Immunohistochemistry & Immunocytochemistry	25
<i>2.4.1. Immunohistochemistry</i>	<i>25</i>
<i>2.4.2 Immunocytochemistry</i>	<i>25</i>
2.5. Chromogenic <i>in situ</i> hybridisation.....	26
2.6. Image and cell count analysis.....	28
2.7. Statistical analysis	28
2.8. Genetic manipulation of <i>Tfap2A</i> and <i>Tfap2B</i> <i>in vivo</i>	29
<i>2.8.1. Generation of knockdown and overexpression plasmid</i>	<i>29</i>
<i>2.8.2. Western blot</i>	<i>31</i>

2.8.3. <i>Production of lentivirus</i>	31
2.8.4. <i>In utero electroporation</i>	32
2.8.5. <i>In vivo transduction</i>	33
2.8.6. <i>In vitro lentiviral transduction</i>	33
3. RESULTS	35
3.1. Gene expression profiles of cerebellar neuronal subpopulations	35
3.1.1. <i>Molecular distinctions between cerebellar cortical inhibitory neurons and excitatory granule cells</i>	35
3.1.2. <i>Validation of microarray data using in situ hybridisation</i>	39
3.2. Characterisation of the expression of Tfap2A and Tfap2B in the developing and mature cerebellum	43
3.2.1. <i>Tfap2A- and Tfap2B-expressing cells follow GABAergic neuronal migration pathway</i>	43
3.2.2. <i>Tfap2A and Tfap2B are expressed in proliferating progenitors and post-mitotic GABAergic neurons</i>	46
3.2.3. <i>Expression of Tfap2A and Tfap2B specifies interneuron cell fate</i>	52
3.2.4. <i>Tfap2A forms embryonic clusters consisting of Purkinje cells</i>	53
3.2.5. <i>The expression of Tfap2 family members define distinct GABAergic neuronal subtypes</i>	55
3.3 Investigating the role of Tfap2A and Tfap2B by genetic manipulation in vivo	60
3.3.1. <i>Testing the efficiency of lentivectors and lentivirus</i>	60
3.3.2. <i>Misexpression of Tfap2A and Tfap2B at E12.5 changes Pax2 and Pax6 population respectively</i>	62
3.3.3. <i>Knockdown of Tfap2B in the ventricular zone at E12.5 affects Tfap2A expression</i>	66
3.3.4. <i>Tfap2B is required for the specification of Pax2-positive interneurons</i>	68
3.3.5. <i>Ptfla does not influence the expression of Tfap2A and Tfap2B in the cerebellum</i>	69
3.3.6. <i>Genetic manipulation of Tfap2A and Tfap2B during postnatal does not affect GABAergic phenotype of cerebellar cortical neurons</i>	71

4. DISCUSSION	76
4.1. Ptf1a, Tfap2A/B and Pax2: sequential expression for GABAergic interneurons in the developing cerebellum	76
4.2. Tfap2A⁺ Purkinje cell clusters suggest an early establishment of topographical organization in the cerebellum	77
4.3. Distinct roles of Tfap2A and Tfap2B in the production of cerebellar GABAergic interneurons	79
5. SUMMARY	83
6. REFERENCES	85

LIST OF FIGURES

Figure 1. Summary of midbrain-hindbrain boundary (MHB) genes crucial in generation of cerebellar primodium.	3
Figure 2. Differentially expressed genes required for the formation of cerebellar structure.	5
Figure 3. Cerebellar neuronal production and the genes involved during development.	11
Figure 4. Molecular mechanisms underlying cerebellar neuronal production.	15
Figure 5. Synaptic circuitry in the cerebellar cortex.	18
Figure 6. Schematic of the RiboTag methodology.	36
Figure 7. <i>In situ</i> hybridisation analysis of known and novel genes identified by microarray analysis.	41
Figure 8. Tfap2A and Tfap2B are expressed by cells originating from the ventricular zone and are detected in the cerebellum at E12.5.	44
Figure 9. Both Tfap2A and Tfap2B are expressed in GABAergic progenitors and post-mitotic GABAergic neurons in the developing cerebellum.	48
Figure 10. Tfap2A labels a subset of Purkinje cells while Tfap2A and Tfap2B label early Pax2 ⁺ interneurons.	51
Figure 11. Tfap2A is expressed in the postnatal cerebellum in some Purkinje cells while Tfap2A and Tfap2B are expressed by migrating interneurons throughout the developing cerebellum.	54
Figure 12. Tfap2A is selectively expressed by small GABAergic interneurons in the medial, interposed and lateral deep cerebellar nuclei.	56
Figure 13. Summary of Tfap2A and Tfap2B expression pattern in the developing cerebellum.	58
Figure 14. Lentivectors are efficient and specific in knocking down respective Tfap2 family members.	60
Figure 15. Overexpression of Tfap2A in the ventricular zone at E12.5 results in an increase of Pax2 ⁺ neurons.	64

Figure 16. The number of Pax2 ⁺ neurons decreases after Tfap2B knockdown in the cerebellar ventricular zone at E12.5.	67
Figure 17. Tfap2A and Tfap2B expression is not influenced by Ptf1a expression.	70
Figure 18. Manipulation of Tfap2A and Tfap2B expression in primary cerebellar neuronal culture and postnatal cerebellum <i>in vivo</i> did not influence neurotransmitter phenotype.	73
Figure 19. GABAergic neuronal identity is not disrupted in the cerebellum of heterozygous Tfap2B mutant mice.	75
Figure 20. Proposed role of Tfap2A and Tfap2B in the specification of cerebellar GABAergic interneurons.	81

LIST OF TABLES

Table 1. Oligonucleotide sequences for RNA probe synthesis.	27
Table 2. Oligonucleotide sequences designed to target Tfap2A and Tfap2B expression.	30
Table 3. Descriptions of the five major cerebellar neuronal subpopulations.	36
Table 4. List of known and novel genes that are enriched in one or more neuronal subpopulations in the cerebellum.	38

ABBREVIATIONS

ABL	Anterobasal Lobe
ADL	Anterodorsal Lobe
ASD	Autism Spectrum Disorder
Atoh1	Atonal homolog 1
bHLH	Basic Helix-Loop-Helix
cDNA	Complementary DNA
CBP	Calbindin protein
CEL	Central Lobe
CMF-PBS	Calcium, magnesium-free PBS

CMV	Cytomegalovirus
CRE	Cre recombinase
CT	Computerised Tomography
DCN	Deep Cerebellar Nuclei
DIG	Digoxigenin
DIV	Days <i>in vitro</i>
dKO	Double knockout
E	Embryonic day
Ef1	Elongation factor 1
EGL	External Germinal Layer
En	Engrailed
EV	Empty Vector
GABA	Gamma-AminoButyric Acid
Gad	Glutamic acid decarboxylase
GFP	Green Fluorescent Protein
HA	Hemagglutinin
HEK	Human embryonic kidney
IGL	Inner Granular Layer
INL	Interior Lobe
IRES	Internal Ribosomal Entry Site
k/d	Knockdown
LSL	Lox-Stop-Lox
MCS	Multiple Cloning Site
mGluR2	Metabotropic glutamate receptor 2
MHB	Midbrain-Hindbrain Boundary
ML	Molecular Layer
MRI	Magnetic Resonance Imaging
Neurod1	Neurogenic differentiation 1
NFH	Neurofilament heavy protein
NTZ	Nuclear Transitory Zone
OE	Overexpression

P	Postnatal day
PC	Purkinje Cell
PCP2	Purkinje Cell Protein 2
PL	Purkinje Layer
PLC β 3	Phospholipase C β 3
PLC β 4	Phospholipase C β 4
POL	Posterior Lobe
pp	Prepyramidal
prc	Preculminate
Ptf1a	Pancreatic transcription factor 1a
PVA	Parvalbumin
rh	rhombomere
RL	Rhombic Lip
RFP	Red Florescent Protein
scr	Scrambled sequence
shh	Sonic hedgehog
shRNA	Short hairpin RNA
SPS	Subpial stream
Tfap2	Transcription Factor AP-2
VZ	Ventricular Zone
WM	White Matter
WT	Wild-type

ABSTRACT

Distinct roles of Tfap2A and Tfap2B in the diversification of GABAergic neuronal subtypes in the cerebellum

The cerebellum plays a critical role in sensory-motor integration and is important for precise coordination of body, limb and eye movements as well as adaptation and learning of motor skills. The ability of the cerebellum to carry out these tasks relies on the faithful production and assembly of various classes of cerebellar neurons into functional circuits during development. The distinct subtypes of inhibitory neurons are important in providing feedforward and feedback inhibition to the cerebellar circuit. To explore the molecular mechanisms underlying the development of cerebellar inhibitory neurons, we examined the involvement of *Tfap2A* and *Tfap2B* in the specification of cerebellar progenitors into distinct inhibitory neuronal subtypes in mice. We show, for the first time, that *Tfap2A* and *Tfap2B* are expressed in distinct subsets of cerebellar inhibitory neurons during embryonic development, and that their expression persists into adulthood. The developmental expression pattern of *Tfap2A* and *Tfap2B* suggests that these transcription factors might play a role in the specification of GABAergic neuronal subtypes in the developing cerebellum and may continue to regulate the maintenance and refinement of inhibitory circuits in the mature cerebellar cortex. We carried out *in utero* electroporation and *in vivo* lentiviral transduction to investigate consequences of manipulating the expression of these transcription factors on specification of GABAergic neuronal subtypes.

The identification and characterisation of genes unique to each neuronal subtype is crucial for a better understanding of the function of individual subtypes. To identify genes that are expressed by specific neuronal subtypes, we examined a database generated by using the Ribo-Tag methodology and microarray technology to profile major cerebellar neuronal types. Analysis of this unpublished microarray database revealed that *Tfap2A* and *Tfap2B* are exclusively expressed by GABAergic neurons, but not glutamatergic neurons in the cerebellum. We confirmed this specific expression pattern using *in situ* hybridisation and immunohistochemistry and showed that *Tfap2A* is expressed in Purkinje projection neurons and Golgi, stellate and basket interneurons while *Tfap2B* is only expressed in Golgi, stellate and basket interneurons. Unlike transcription factors that are transiently expressed

during development, Tfap2A and Tfap2B are expressed early during embryogenesis and remain expressed in adulthood. In order to define the expression of Tfap2A and Tfap2B in different neuronal subtypes during distinct developmental stages, we carried out co-localisation studies using known molecular markers for each neuronal subtype. We observed that both transcription factors are expressed as early as embryonic day (E) 12.5, the onset of cerebellar cortical neuronal production. In addition, Tfap2A⁺ and Tfap2B⁺ cells express molecular markers for early GABAergic precursors such as Ptf1a and Olig2 as well as post-mitotic markers such as ROR α and Pax2. These results suggest that Tfap2A and Tfap2B may serve as determinants for the specification of GABAergic neuronal subtypes and prompted us to assess the function of these transcription factors in the developing and mature cerebellum.

Since Tfap2A and Tfap2B are expressed exclusively in GABAergic neuronal population in the cerebellum, we tested whether manipulation of the expression of these transcription factors individually could alter the molecular phenotypes of these neurons. *In utero* electroporation and lentiviral transduction were performed to study the consequences of over/misexpression or knockdown of *Tfap2A* and *Tfap2B* in the developing and mature cerebellum. Genetic manipulation during early embryogenesis showed that Tfap2B is essential for the specification of Pax2⁺ interneurons and in preventing the specification of Pax6⁺ excitatory neurons. Tfap2A, on the other hand, is capable of inducing Pax2⁺ interneurons when misexpressed in precursor/progenitor neurons. Additionally, we investigated whether Ptf1a, a key determinant of cerebellar GABAergic neuron identity, controls the expression of Tfap2A and Tfap2B. Our results indicate that the expression of Tfap2A and Tfap2B is not dependent on Ptf1a expression and, thus, may represent an independent program in the specification of cerebellar GABAergic neuron diversification. In postnatal experiments, we assessed GABAergic molecular markers such as glutamic acid decarboxylase 67 (Gad67), the enzyme responsible for inhibitory neurotransmitter production. Misexpression studies *in vitro* using primary cerebellar neuronal culture revealed that neither Tfap2A nor Tfap2B was capable of transforming excitatory neurons into GABAergic inhibitory neurons.

In summary, we show that the expression of Tfap2A and Tfap2B molecularly defines GABAergic neuronal subtypes in the cerebellum and, perhaps, other regions of the nervous system and represent an important set of markers for the labelling and monitoring of the development of GABAergic neuronal subpopulations. In addition, we established the role of these transcription factors in the diversification of GABAergic neuronal subtype during embryogenesis.

1. INTRODUCTION

This thesis focuses on the role of Tfap2A and Tfap2B in the specification and diversification of GABAergic cerebellar neuronal subtypes. The generation of the cerebellar primordium during embryogenesis and the current understanding of cerebellar neuronal production will first be introduced. Despite some knowledge on the morphological, electrophysiological and, to some extent, functional properties of cerebellar neurons, little is known about the specification of cerebellar neuronal subtypes and the manifestation of their neuronal phenotypes. Thus, this thesis aims to investigate the roles of Tfap2A and Tfap2B in order to better understand the molecular mechanisms underlying GABAergic neuronal development.

1.1. Molecular programs involved in the generation of the cerebellar primordium

The cerebellum is essential for the control of motor functions and also cognitive functions such as attention and language (Eccles, 1967; Ito, 2006; Schmahmann and Sherman, 1998; Thach and Bastian, 2004). Various developmental and acquired disorders have been linked to the cerebellum such as cerebellar agenesis, cerebellar degeneration, Autism Spectrum Disorders (ASD) and schizophrenia (Andreasen and Pierson, 2008; DiCicco-Bloom et al., 2006; Horak and Diener, 1994; Millen and Gleeson, 2008; Mosconi et al., 2015; Schmahmann and Sherman, 1998; Yu et al., 2015). In a human study that assesses adaptation of stance, control subjects and subjects with cerebellar cortical atrophy were instructed to learn to respond to perturbances during stance (Horak and Diener, 1994). In comparison to a control subject, subjects with cerebellar cortical atrophy cannot anticipate displacement body upon postural disturbances despite multiple trials (Horak and Diener, 1994). Apart from adjustment of posture, the cerebellum has also been reported to be important for fluency in verbal and written communication as seen from a patient with midline cerebellar tumour excision (Schmahmann and Sherman, 1998). These studies, along with many others, link the cerebellum to a wide array of functions, ranging from motor to cognitive functions, in physiological and pathological conditions. Although extensive research has been carried out to characterize the function of individual components of cerebellar circuits, studies of the cerebellum from a developmental perspective are of equal importance for a better understanding of the cerebellar functions.

Therefore, the first sub-chapter serves to introduce the key molecular players that are involved in the generation of the cerebellar primodium.

1.1.1. Demarcation of the cerebellar territory

The development of the three main brain regions, the forebrain, the midbrain (mesencephalon) and the hindbrain (rhombencephalon), are regionalised progressively during embryogenesis (Butts et al., 2014; Harada et al., 2016; Martinez et al., 2013). Due to the topography of the cerebellum on the neural tube, initial studies have considered the cerebellum as the hindbrain (Butts et al., 2014; Harada et al., 2016; Martinez et al., 2013). However, as neural development progresses, the rhombencephalon is segmented into rhombomeres (rh), rh0-rh11, in an anterior to posterior manner and thus, the cerebellum is grouped into rh1 (Wingate and Hatten, 1999; Zervas et al., 2004). Besides topography, the midbrain-hindbrain region can be delineated using segmentation genes such as *En1*, *En2*, *Pax2*, *Pax5*, *Pax8*, *Otx2*, *Gbx2* and *Hoxa2* (Butts et al., 2014; Harada et al., 2016; Martinez et al., 2013; Sillitoe and Joyner, 2007). Investigation of these transcription factors focuses on the importance of these molecular players in the development and patterning of various brain regions including the cerebellum.

Differentially expressed genes in the midbrain-hindbrain boundary (MHB) play a crucial role in establishing tissue boundaries for neighbouring tissues. Transcription factors *Otx2* (Acampora et al., 1997; Broccoli et al., 1999; Di Giovannantonio et al., 2014; Foucher et al., 2006; Katahira et al., 2000; Millet et al., 1999), *Gbx2* (Li et al., 2002; Millet et al., 1999; Su et al., 2014; Sunmonu et al., 2009; Wassarman et al., 1997) and *Hoxa2* (Eddison et al., 2004; Gavalas et al., 1997) are exclusively expressed in the midbrain, cerebellum and hindbrain respectively (Figure 1A) and play crucial roles in delineating these territories. Loss of *Otx2* in mice and zebrafish results in the enlargement of the cerebellum which grows into the midbrain region (Acampora et al., 1997; Foucher et al., 2006). Targeted deletion of *Otx2* in the dorsal midbrain, which is *Gbx2*-negative, also induces the production of cerebellar tissues (Di Giovannantonio et al., 2014). On the other hand, misexpression of *Otx2* beyond the midbrain in mice and chick causes an expansion of the midbrain into the cerebellar territory and thus, impeding cerebellar growth (Broccoli et al., 1999; Katahira et al., 2000). Deletion of *Gbx2*, which is normally expressed in the cerebellum, results in an

expanded midbrain (Li et al., 2002; Millet et al., 1999; Wassarman et al., 1997). This phenotype is, however, rescued in the zebrafish by knocking down *Otx2* expression in the midbrain thus showing the relationship between these two transcription factors (Su et al., 2014). Similar to *Otx2* conditional knockout, ectopic expression of *Gbx2* in the midbrain induces abnormal production of cerebellar tissue in the midbrain (Katahira et al., 2000; Millet et al., 1999; Sunmonu et al., 2009). Interestingly, *Hoxa2* deletion also results in the enlargement of the cerebellum that grows into the hindbrain territory (Gavalas et al., 1997) while ectopic expression of *Hoxa2* in rh1 disrupts specification of cerebellar neurons (Eddison et al., 2004). Thus, these results show an opposing regulation of *Otx2* and *Gbx2* on each other and an inhibitory role of *Hoxa2* on *Gbx2* expression (summarised in Figure 1B). Thus, collectively these genes not only mark the midbrain-hindbrain boundary, but *Otx2* in the midbrain and *Hoxa2* in the hindbrain function to inhibit *Gbx2* for proper positioning and growth of the cerebellar tissue.

Figure 1. Summary of midbrain-hindbrain boundary (MHB) genes crucial in generation of cerebellar primodium.

(A) The midbrain-hindbrain region is demarcated by *Otx2*, *Gbx2* and *Hoxa2* in the midbrain, cerebellum (rh1) and hindbrain respectively (Butts et al., 2014; Harada et al., 2016; Martinez et al., 2013). (B) In the wild type animal, *Otx2* and *Gbx2* inhibit each other to regulate the generation of midbrain and cerebellar tissues (Broccoli et al., 1999; Katahira et al., 2000; Su et al., 2014). *Hoxa2* expressed by the hindbrain also inhibit *Gbx2* to prevent

the inappropriate expansion of cerebellar tissues into the hindbrain region (Gavalas et al., 1997). Abbreviation: rh, rhombomere.

Identification and characterization of genes that promote cerebellar growth, provide insight to the molecular mechanisms that underlies normal cerebellar development. These studies can also help us understand the mechanisms which might be disrupted during cerebellar agenesis or degeneration (Acampora et al., 1997; Di Giovannantonio et al., 2014; Foucher et al., 2006; Gavalas et al., 1997; Katahira et al., 2000; Millet et al., 1996; Sunmonu et al., 2009). The most recent human study which shows complete primary cerebellar agenesis, reports only on clinical and imaging findings (Yu et al., 2015). Patient shows no presence of cerebellar tissues following Computerised Tomography (CT) and Magnetic Resonance Imaging (MRI) scans (Yu et al., 2015). She is also diagnosed with mild to moderate motor impairments such as dysmetria and dysarthria (Yu et al., 2015). Thus, along with genetics studies, we are able to explore strategies to overcome cerebellar disorders. However, the ability to generate cerebellar tissues may not be sufficient to create functional circuits within the cerebellum and the central nervous system. Therefore, information about genes and factors required for the establishment of cerebellar neural circuits is essential.

1.1.2. Formation of the cerebellar structure

Within the cerebellum, spatially expressed genes have been reported to play a role in the formation of the cerebellar structure and are shown to mark specific cerebellar regions which receive distinct neural inputs (Altman and Bayer, 1996; Purves et al., 2004; Sillitoe and Joyner, 2007). *Engrailed (En) 1/2* homeobox genes (Sillitoe et al., 2008; Wilson et al., 2011), *Zebrin II* (Ahn et al., 1994; Brochu et al., 1990), *heat shock protein 25* (Armstrong et al., 2000), *phospholipase C β 3/4* (Marzban et al., 2007; Sarna et al., 2006) and *neurofilament heavy protein (NFH)* (Demilly et al., 2011; White and Sillitoe, 2013) are examples of zonal markers that divide the cerebellum into parasagittal stripes (Figure 2A). Additionally, genes like *En1/2* play important roles in the organization of these parasagittal stripes and formation of the cerebellar structure (Cheng et al., 2010; Orvis et al., 2012; Sillitoe et al., 2008).

The structure of a mammalian cerebellum is highly foliated, but more in the medial (vermis) and less in the lateral (hemisphere) region (Altman and Bayer, 1996; Inouye and Oda, 1980). In the mature cerebellum, the vermis can be divided into ten lobules (I-X) while the hemisphere is divided into four lobules (Altman and Bayer, 1996; Inouye and Oda, 1980). The *En1/2* homeobox genes are important for the production of the distinct medial and lateral foliation patterns in cerebellum (Cheng et al., 2010; Orvis et al., 2012). The cerebellar primodium, which begins with an ovoid structure, is divided during embryogenesis into five primary lobes, namely anterobasal (ABL), anterodorsal (ADL), central (CEL), posterior (POL) and inferior (INL) lobe (Figure 2B) (Altman and Bayer, 1996). Subsequently at embryonic day (E) 16.5, the lobes are foliated by four fissures, preculminate, primary, secondary and posterodorsal fissure (Figure 2B, astericks) (Altman and Bayer, 1996). And at birth, the cerebellum forms two more fissures, preculminate (prc) and prepyramidal (pp) fissures which eventually further divide the lobes (Figure 2B, arrows). Studies on *En2*^{-/-} knockout mouse demonstrates defects in the secondary and prepyramidal fissures formation (Cheng et al., 2010; Orvis et al., 2012). Ablation of both *En1* and *En2* shows a more severe foliation defect, lacking multiple fissures thus indicating the importance of *Engrailed* genes on the formation of cerebellar structure (Orvis et al., 2012). This disruption also severely reduces the growth of the cerebellum which might have been caused by a decrease in cerebellar neuron production and the organisation of the cerebellar circuit (Orvis et al., 2012).

Figure 2. Differentially expressed genes required for the formation of cerebellar structure.

(A) Examples of parasagittal zone markers such as heat shock protein 25, Zebrin II, phospholipase C β 3 (PLC β 3) and PLC β 4 which are influenced by *Engrailed* (*En*) 1/2 homeobox gene, another parasagittal marker (Sillitoe et al., 2008). (B) Formation of the highly foliated cerebellar structure is influenced by *En1/2* (Cheng et al., 2010; Orvis et al., 2012). The red foliated cerebellum illustrates the shape of the cerebellum at E16.5 which consist of five lobes (Altman and Bayer, 1996). The black line delineates the structure of the murine adult cerebellum that consist of ten lobules (I-X) (Altman and Bayer, 1996; Inouye and Oda, 1980). Astericks show primary fissures, arrows show further fissures formation during the course of development creating more lobules. Abbreviation: ABL, anterobasal lobe; ADL, anterodorsal lobe; CEL, central lobe; POL, posterior lobe; INL, inferior lobe; prc, precentral fissures; pp, prepyramidal fissures.

In summary, a set of key molecular players participates in the early development of the cerebellum, starting with the marking of boundaries of the cerebellar region followed by formation of the cerebellar structure (Butts et al., 2014; Cheng et al., 2010; Harada et al., 2016; Martinez et al., 2013; Orvis et al., 2012; Sillitoe and Joyner, 2007; Sillitoe et al., 2008). Generation of the cerebellar primodium is not a discrete mechanism but requires a synergy between its neighbouring brain regions, the midbrain and hindbrain. *Gbx2* transcription factor, that marks the cerebellum, requires the inhibitory properties of *Otx2* and *Hoxa2* to maintain its boundary within the midbrain-hindbrain boundary (Broccoli et al., 1999; Gavalas et al., 1997; Katahira et al., 2000; Su et al., 2014). *En1* and *En2* genes are important in regulating fissure and cerebellar growth (Sillitoe et al., 2008). Although strong links between identifiable cerebellar regions to specific behaviours is still lacking, several identified zonal markers can allow us to investigate the function of cerebellar zones and the circuits within these zones (Altman and Bayer, 1996; Purves et al., 2004; Sillitoe and Joyner, 2007). However, the factors that regulate the expression of these zonal markers and the formation of these parasagittal stripes are not clear. Therefore, we next look at the

molecular mechanisms that regulate the production cerebellar neuronal subtypes which could address some of these questions.

1.2. Cerebellar neurogenesis, migration & organisation

The establishment of the cerebellar territory is followed by the formation of two germinative compartments, rhombic lip (RL) and ventricular zone (VZ), at around E10.5 (Liu et al., 2010; Morales and Hatten, 2006). These compartments are regions where neural precursors are differentiated into excitatory and inhibitory progenitors respectively (Hoshino et al., 2005; Machold and Fishell, 2005; Wang et al., 2005). Progenitors from these germinative zones give rise to various neuronal subtypes respectively (Hoshino et al., 2005; Machold and Fishell, 2005; Wang et al., 2005). Various characteristics regarding the cerebellar neurons, such as molecular phenotype, neurotransmitter type, anatomical position, axonal projection location and electrical physiological properties, have been documented (Altman and Bayer, 1996; Palay and Chan-Palay, 1974). However, it remains elusive how a common pool of progenitors diversifies into different subtypes. Thus, current findings regarding the development of cerebellar neurons and remaining questions are presented in this sub-chapter.

1.2.1. *Generation of cerebellar neural progenitors*

In the embryonic cerebellum, members of basic helix-loop-helix (bHLH) transcription factors, *Math1* and *Ptfla*, distinctly mark the rhombic lip and ventricular zone respectively (Figure 3A) (Hoshino et al., 2005; Liu et al., 2010; Machold and Fishell, 2005; Morales and Hatten, 2006; Wang et al., 2005). These transcription factors are also responsible for the specification of neural precursors into excitatory and inhibitory progenitors respectively (Ben-Arie et al., 1997; Gazit et al., 2004; Hoshino et al., 2005; Millen et al., 2014; Pascual et al., 2007; Wang et al., 2005; Yamada et al., 2014). There are five main cerebellar cortical neurons, Purkinje, Golgi, stellate, basket and granule cells, of which granule cells are the only excitatory neurons while the others are inhibitory neurons (Altman and Bayer, 1996; Wang and Zoghbi, 2001). Beneath the cerebellar cortex, the deep cerebellar nuclei, located in the white matter layer, also consist of both excitatory and inhibitory neurons. Various lineage and genetic studies report that in the absence of *Math1* and *Ptfla*, the respective excitatory and inhibitory neurons fail to be generated (Ben-Arie et al., 1997; Hoshino et al., 2005; Machold and Fishell, 2005; Pascual et al., 2007; Wang et al., 2005). Additionally, neural precursors residing in the rhombic lip will transform into

inhibitory neurons while precursors in the ventricular zone will transform into excitatory neurons (Millen et al., 2014; Pascual et al., 2007; Wang et al., 2005; Yamada et al., 2014).

Math1, also known as *Atoh1* (*atonal homolog 1*), is responsible for the production of excitatory glutamatergic neurons in the rhombic lip as early as E10.5 (Ben-Arie et al., 1997; Gazit et al., 2004; Machold and Fishell, 2005; Wang et al., 2005). Lineage tracing, using genetically engineered mouse which expresses β -galactosidase from *Math1* locus, *Math1*^{LacZ/+}, reveals that the rhombic lip gives rise to subtypes of deep cerebellar nuclei neurons and granule cells in the cerebellum along with other neurons in the brainstem (Wang et al., 2005). On the other hand, in the absence of *Math1*, deep cerebellar nuclei neurons and granule cells are not found in the deep cerebellar nuclei and the external germinal layer (Ben-Arie et al., 1997; Machold and Fishell, 2005; Wang et al., 2005). Thus, these studies demonstrate the importance of *Math1* in glutamatergic neuron production.

Pancreatic transcription factor 1a (*Ptf1a*), on the other hand, is required for the production of inhibitory GABAergic neurons, which include Purkinje, Golgi, stellate and basket cells as well as deep cerebellar nuclei interneurons, in the ventricular zone (Hoshino et al., 2005; Pascual et al., 2007; Yamada et al., 2014). *Ptf1a* mRNA levels are detected at high levels at E12 which decreases during early postnatal stages and are undetected in the adult cerebellum (Hoshino et al., 2005; Pascual et al., 2007). Histological analysis of *cerebelless* mouse, which lacks the expression of *Ptf1a* in the cerebellum, shows an absence of cerebellar cortex in adults (Hoshino et al., 2005). Furthermore, GABAergic neurons are absent in the developing and mature cerebellar cortex and DCN of mutant mice (Hoshino et al., 2005; Pascual et al., 2007). As a result of the loss of GABAergic neurons, production of glutamatergic granule cells is also affected, most likely due to the absence of Sonic hedgehog (Shh) proteins secreted by GABAergic Purkinje cells (Hoshino et al., 2005). Shh has been reported to be a mitogenic factor that is important for the proliferation of granule cells where loss of *Shh* results in the absence of the external germinal layer (Dahmane and Altaba, 1999; Lewis et al., 2004; Wechsler-Reya and Scott, 1999). Therefore, defects in one member of bHLH transcription factors have been shown to not only affect the production of neuronal subtypes but also secondarily affect the presence of other subtypes.

The importance of *Math1* and *Ptf1a* is highlighted recently using two knock-in mouse lines, *Ptf1a^{Math1}* and *Math1^{Ptf1a}*, which ectopically express *Math1* in the ventricular zone and *Ptf1a* in the rhombic lip (Yamada et al., 2014). In this study, the ventricular zone generates glutamatergic deep cerebellar nuclei neurons and granule cells while the rhombic lip produces inhibitory neurons including Purkinje cells and GABAergic interneurons. Thus, these findings suggest that it is not the presence of germinative site, but rather the presence of *Math1* or *Ptf1a* transcription factor in these germinative centres that is critical in determining the glutamatergic or GABAergic cell fate from neural precursors.

Figure 3. Cerebellar neuronal production and the molecular markers that define different stages of neuronal development.

(A) Schedule of cerebellar neuronal production from Math1- (marked in red) and Ptf1a-expressing (marked in blue) germinative zones (Hoshino et al., 2005; Liu et al., 2010; Machold and Fishell, 2005; Morales and Hatten, 2006; Wang et al., 2005). Math1⁺ rhombic lip generates glutamatergic deep cerebellar nuclei (DCN) neurons and granule cells while Ptf1a⁺ ventricular zone produces GABAergic DCN neurons, Purkinje cells and cortical interneurons, all at different time points (Altman and Bayer, 1996; Fink et al., 2006; Leto

et al., 2009; Leto et al., 2006; Muguruma et al., 2015; Seto et al., 2014b; Weisheit et al., 2006; Yamada et al., 2014). (B) Migration pathways are different for distinct classes of cerebellar neurons in the cerebellar primodium. Glutamatergic neurons derived from the rhombic lip (RL) migrate tangentially along the surface of the cerebellum, termed as the subpial stream (SPS) or external germinal layer (EGL), before descending into the nuclear transitory zone (NTZ) or inner granular layer (IGL) (Altman and Bayer, 1996; Hatten and Heintz, 1995). Whereas GABAergic neurons produced from the ventricular zone (VZ) migrate upwards into the white matter (WM) before settling into the cortex (Altman and Bayer, 1996; Leto et al., 2009; Leto et al., 2006; Maricich and Herrup, 1999; Sotelo and Rossi, 2013; Weisheit et al., 2006). (C) Molecular genes responsible for cerebellar neuronal production. Different genes can be used to mark neuronal sub-populations during development and in the mature cerebellum (Bastianelli, 2003; Engelkamp et al., 1999; Fink et al., 2006; Hoshino et al., 2005; Leto et al., 2006; Lumpkin et al., 2003; Maricich and Herrup, 1999; Meguro et al., 1999; Minaki et al., 2008; Morales and Hatten, 2006; Nakagawa et al., 1997; Seto et al., 2014a; Seto et al., 2014b; Weyer and Schilling, 2003; Zhao et al., 2007). Other abbreviations: DCN, deep cerebellar nuclei; IGL, inner granular layer; ML, molecular layer; PL, Purkinje layer.

In conclusion, the presence of Math1 and Ptf1a bHLH transcription factors play crucial roles in the production of excitatory and inhibitory neurons respectively (Ben-Arie et al., 1997; Gazit et al., 2004; Hoshino et al., 2005; Millen et al., 2014; Pascual et al., 2007; Wang et al., 2005; Yamada et al., 2014). Generation of cerebellar neuronal subtypes begins from E10.5 but not all at once (Figure 3A) (Altman and Bayer, 1996; Leto et al., 2006; Sudarov et al., 2011; Yamada et al., 2014). Additionally, the migration route and maturation process of these neurons also differ from one another (Figure 3B). Next, we will discuss developmental events that occur after glutamatergic and GABAergic neurons are produced from the rhombic lip and ventricular zone, respectively.

1.2.2. *Schedule of glutamatergic and GABAergic cerebellar neuron production*

Cerebellar neurons are produced in a specific order, in which the projection neurons precede the local interneurons (Figure 3B) (Altman and Bayer, 1996; Fink et al., 2006; Leto et al., 2009; Leto et al., 2006; Muguruma et al., 2015; Seto et al., 2014b; Weisheit et al., 2006; Yamada et al., 2014). Of the five main cerebellar cortical neuronal subtypes, Purkinje cells are classified as projection neurons because their axons extend out of the cortex while the other neurons are categorised as local interneurons (Altman and Bayer, 1996; Palay and Chan-Palay, 1974). Purkinje cells, the earliest subtype of cerebellar cortical neurons, are produced from the ventricular zone from E10.5 till E13.5 (Yamada et al., 2014). The ventricular zone also gives rise to inhibitory interneurons including Golgi, stellate and basket cells that are derived from the same pool of Ptf1a⁺-neural precursors from E13.5 to E17.5 (Leto et al., 2006; Yamada et al., 2014). The production of these interneurons continues until early postnatal stages where they proliferate in the white matter (Leto et al., 2009; Leto et al., 2006; Yamanaka et al., 2004). It is intriguing how various neuronal subtypes are produced from the same pool of progenitors, yet are able to acquire unique neuronal identities as they differentiate.

Another bHLH transcription factor, *Olig2*, is also expressed in the ventricular zone and is required for the differentiation of GABAergic progenitors into Purkinje projection neurons (Ju et al., 2016; Seto et al., 2014a; Seto et al., 2014b). *Olig2* is transiently expressed in the ventricular zone from E11.5 till E13.5, which coincides with the period of Purkinje cell production (Ju et al., 2016; Seto et al., 2014a). Some *Olig2*-expressing cells in the ventricular zone are Ptf1a⁺ and BrdU⁺, indicating that they are proliferative progenitors while the remaining cells are Lhx1/5⁺ (Ju et al., 2016; Seto et al., 2014a), a marker for post-mitotic GABAergic neurons (Morales and Hatten, 2006; Zhao et al., 2007). The expression of *Olig2* ceases before the onset of early Purkinje cell marker such as *Corl2* and *RORα* (Hamilton et al., 1996; Ino, 2004; Minaki et al., 2008; Nakagawa et al., 1997; Nakatani et al., 2014; Seto et al., 2014a). However, *Olig2* knockout mouse only shows partial reduction of Purkinje cell population (Ju et al., 2016). In this particular study, immunostaining with calbindin protein (CBP), another Purkinje cell marker (Bastianelli, 2003), on E15.5 *Olig2*-null cerebellum shows a significant reduction in Purkinje cells (Ju et al., 2016). With

inference to another study, lineage tracing of *Olig2*^{GFP/+} mouse, which expresses green fluorescent protein (GFP) under *Olig2* promoter, shows only partial colocalisation of GFP⁺ cells with *Corl2* at E13.5 (Seto et al., 2014b) suggesting that it could identify for other neuronal cell types. Although Seto et al. reported no cerebellar abnormalities in the *Olig2* single knockout mouse, a reduction in Purkinje cells and an increase in Pax2⁺ interneurons are observed in *Olig1* and *Olig2* double knockout (dKO) mouse (Seto et al., 2014b). Conjointly, these studies show that *Olig2* is important for the production of Purkinje cell progenitors and also other cerebellar cell types which have yet to be identified (Ju et al., 2016; Seto et al., 2014b). In addition to the role of *Olig2* in Purkinje cell development, *Corl2* has also been reported to promote differentiation and maturation of Purkinje cells (Nakatani et al., 2014). *Corl2* knockout embryo shows a reduction in mRNA expression of several Purkinje cell markers such as Purkinje Cell Protein 2 (PCP2) and calbindin and defects in morphology (Nakatani et al., 2014). Despite ongoing research, it still remains unclear how the same pool of GABAergic progenitors is able to be differentiated into Purkinje projection neurons and interneurons.

As mentioned earlier, GABAergic interneurons, which include Golgi, stellate, basket and deep cerebellar nuclei interneurons, are also generated from the ventricular zone from Ptf1a⁺ progenitors (Hoshino et al., 2005; Yamada et al., 2014). They express Pax2 during its development process while in transit in the white matter (Leto et al., 2009; Leto et al., 2006; Maricich and Herrup, 1999; Weisheit et al., 2006). Pax2-expressing neurons observed in the developing cerebellum coexpress Tuj1, a neuronal marker, and BrdU, indicating that they are also proliferative (Leto et al., 2009; Leto et al., 2006; Maricich and Herrup, 1999). However, when stellate and basket interneurons establish synaptic contacts with their target, Pax2 expression is down-regulated and parvalbumin (PVA), a marker for stellate, basket and Purkinje cells, is up-regulated (Bastianelli, 2003; Maricich and Herrup, 1999; Solbach and Celio, 1991). Pax2 expression can be found in small cluster at the ventral end of the ventricular zone of E12.5 cerebellum which suggests GABAergic neurons are produced early during embryogenesis (Maricich and Herrup, 1999; Seto et al., 2014b). But prior to Pax2 spatial expression, Gsx1, a homeodomain-containing transcription factor, is also expressed at the same end along the Ptf1a⁺ domain at E12.5 (Seto et al., 2014b). As development proceeds, Gsx1 expression spreads dorsally to cover the entire the surface of

ventricular zone by E14.5 (Seto et al., 2014b). And at the same time, Olig2⁺ region recedes which confirms that Purkinje cell production ceases by E13.5 (Seto et al., 2014a; Seto et al., 2014b). However, lineage tracing of *Gsx1*^{GFP/+} mouse shows only partial colocalisation of GFP and Pax2 while *Gsx1*^{GFP/GFP} knockout mouse does not completely remove Pax2⁺ interneurons (Seto et al., 2014b). This suggests that there are other factors which regulate the specification of Pax2⁺ interneurons and to date, complete mechanisms that specify the GABAergic neuronal subtypes have yet to be identified (summarised in Figure 4B).

Various molecular markers have been identified to mark key developmental stages of cerebellar neuronal subtypes (summarised in Figure 3C). Math1 and Ptf1a bHLH transcription factors play crucial roles in the production of excitatory and inhibitory neurons respectively (Ben-Arie et al., 1997; Gazit et al., 2004; Hoshino et al., 2005; Millen et al., 2014; Pascual et al., 2007; Wang et al., 2005; Yamada et al., 2014). However, the subsequent molecular events have still not been determined.

Figure 4. Molecular mechanisms underlying cerebellar neuronal production.

(A) Glutamatergic neurons are derived from Math1-expressing cells from the rhombic lip (RL) and migrate to the external germinal layer (EGL) where they proliferate in transit. Upon entering the state of mitosis, granule cells descend into the inner granular layer (IGL)

where they reside permanently (Ben-Arie et al., 1997; Borghesani et al., 2002; Engelkamp et al., 1999; Gazit et al., 2004; Miyata et al., 1999). (B) GABAergic neurons, on the other hand, are produced from Ptf1a-expressing cells in the ventricular zone (VZ) (Hoshino et al., 2005). Ptf1a-expressing cells can give rise to multiple GABAergic neuronal subtypes however the molecular players involved in the specification of distinct subtypes are still not well understood (Hoshino et al., 2005; Yamada et al., 2014).

1.2.3. Organisation of local circuits within the cerebellar cortex

The specification, of distinct classes of cerebellar neurons during development, is necessary for the assembly of functional cerebellar circuits. Cerebellar excitatory neurons in the cerebellar cortex consist of glutamatergic granule cells and make up more than 90% of the cell numbers in the cerebellum (Andersen et al., 1992; Korbo et al., 1993). The remaining neurons are cerebellar inhibitory neurons consisting of Purkinje, Golgi, stellate and basket cells that use gamma-aminobutyric acid (GABA) neurotransmitter. Despite consisting less than 10% of total neuron in the cerebellum, inhibitory neurons play vital roles in cerebellar function by providing feedforward and feedback inhibition (Eccles, 1967; Ito, 1984). Deficits in cerebellar GABAergic transmission lead to an imbalance between excitation and inhibition which is often associated with neurological disorders, such as schizophrenia and autism spectrum disorder (ASD) (Andreasen and Pierson, 2008; Gao and Penzes, 2015; Nelson and Valakh, 2015; Rubenstein and Merzenich, 2003). ASD mouse models such as *TSC1* and *CNTNAP2* mutants have been reported with abnormal neuronal excitability resulting in autistic-like behaviours such as excessive grooming and abnormal social interactions (Penagarikano et al., 2011; Tsai et al., 2012). Therefore, a functional cerebellar network requires the proper generation and assembly of cerebellar neurons and thus, the generation of appropriate behaviours.

Purkinje cells are specified within the ventricular zone upon expression of *Corl2* which is observed at E12.5 (Minaki et al., 2008; Nakatani et al., 2014). These neurons migrate dorsally into the white matter before they cover the surface of the cerebellar anlage to form Purkinje layer. Consequently, *Pax2*⁺ interneurons, which are also generated from the ventricular zone, transit in the white matter where it continue to proliferate post-natally

(Leto et al., 2009; Weisheit et al., 2006; Zhang and Goldman, 1996). During early postnatal period, Purkinje cells that initially assemble in multiple layers, will assemble in monolayer by postnatal day (P) 5 (Altman and Bayer, 1996; Sotelo and Rossi, 2013). At the same time, Pax2⁺ Golgi interneurons, residing in the inner granular layer, will mature morphologically and form axons and dendrites by P15 (Meguro et al., 1999). On the other hand, stellate and basket interneurons will continue to proliferate in the white matter till P15 before migrating into the molecular layer (Leto et al., 2009; Maricich and Herrup, 1999; Weisheit et al., 2006; Zhang and Goldman, 1996). Thus, these studies demonstrate that inhibitory neurons migrate with an inside-out strategy (Figure 3B).

Granule cells which are generated from the Math1⁺ rhombic lip after E12.5, migrate dorsally to cover the surface of the cerebellar anlage to form external germinal layer (EGL) (Lumpkin et al., 2003; Machold and Fishell, 2005). Upon leaving the rhombic lip, cells express Pax6 and continue to proliferate in the EGL (Lumpkin et al., 2003; Machold and Fishell, 2005). Proliferation of Pax6⁺ granule cells are influenced by Shh protein that is secreted by Purkinje cells in the Purkinje layer (Lewis et al., 2004; Wechsler-Reya and Scott, 1999). Conditional deletion of *Shh* using the *L7-Cre* mouse leads to absence of Shh secretion by Purkinje cell, causing reduced granule cell proliferation in the external germinal layer (Lewis et al., 2004). After proliferation, granule cell progenitors exit the cell cycle, differentiate into granule cells before migrating inwards into the inner granular layer, where they permanently reside (summarised in Figure 4A) (Altman and Bayer, 1996; Hatten and Heintz, 1995). Impairment in granule cell migration has also been reported in *Bdnf*^{ε/-} knockout mice, brain-derived neurotrophic factor which is expressed by granule cells (Borghesani et al., 2002). In the same study, this phenotype is also rescued by exogenous Bdnf *in vitro* (Borghesani et al., 2002). In conclusion, granule cell migration employs an outside-in approach (figure 3B).

To summarise the local cerebellar circuit in brief (Figure 5), the cerebellar cortical neurons, specifically the granule cells and Purkinje cells, receive inputs via the extracerebellar nuclei and deep cerebellar nuclei (Altman and Bayer, 1996; Palay and Chan-Palay, 1974). In turn, granule cells which have axons extended into the molecular layer, will excite Purkinje, Golgi, stellate and basket cell dendrites (Altman and Bayer, 1996;

Palay and Chan-Palay, 1974). Golgi interneurons provide feedback inhibition by inhibiting granule cells to regulate motor coordination as well as fine motor movement (Lu et al., 1998; Watanabe et al., 1998). On the other hand, stellate and basket interneurons provide feedforward inhibition to Purkinje cells to regulate motor learning, motor adaptation and fine motor movement (Dizon and Khodakhah, 2011; Vincent and Marty, 1996). These feedback and feedforward mechanisms regulate the inputs which are eventually, relayed out of the cortex or cerebellum by Purkinje cells. Up until now, various aspects of cerebellar genesis have been presented. From the generation of cerebellar territory, to patterning of the cerebellar structure, production of various neuronal types and the assembly of cerebellar circuit. Although extensive research has been carried out, molecular mechanisms that link these processes are still lacking. What initiates the expression of *Math1* and *Ptf1a*? How do the same pool of progenitors give rise to diverse neuronal subtypes? And which factors coordinate the dynamic organisation of the cerebellar circuits?

Figure 5. Synaptic circuitry in the cerebellar cortex.

Excitatory neurons are depicted in red and inhibitory neurons in blue. The cerebellum receives and processes external inputs, and generates output signals which are sent out to various regions of the central nervous system. Cerebellar cortical interneurons provide feedback and feedforward inhibition in order to control inputs and outputs from the cerebellar cortex (adapted from Altman and Bayer, 1996).

1.3. Transcription factor activating protein 2

Gene expression analysis is a valuable tool in defining neuronal cell types and assessing functional relationships between neuronal subtypes. Various experimental datasets, comparing transcriptome analysis of excitatory versus inhibitory neurons, have been carried out to uncover the molecular profile of neuronal subtypes (Borromeo et al., 2014; Jin et al., 2015; Meredith et al., 2013; Russ et al., 2015; Sugino et al., 2006). However, to date, there are limited gene expression studies that characterise the distinct neuronal types in the cerebellum.

1.3.1. *Molecular profiles of inhibitory versus excitatory neurons*

To identify possible gene signatures in distinct cerebellar neuronal type, we can take advantage of datasets from other regions such as the neural tube (Borromeo et al., 2014; Meredith et al., 2013), spinal cord (Russ et al., 2015) and the forebrain (Sugino et al., 2006). Adopting gene expression datasets from Allen Brain Atlas can also identify genes specifically expressed in each of the cerebellar cell types. One of the most exhaustive microarray datasets analysed twelve neuronal populations from different regions of the forebrain (Sugino et al., 2006). Various transgenic mouse lines, which express fluorescent proteins under a specific promoter, are used to isolate specific neurons. For example, *Gad1-GFP* transgenic mouse, which expresses green fluorescent protein under a gene promoter that encodes for an enzyme responsible for catalysing GABA neurotransmitter, is used to isolate inhibitory neurons from the hippocampus. This dataset which describes properties of the neurons isolated, such as its neurotransmitter and firing properties, is useful to make comparison to existing properties of the cerebellar neurons.

As mentioned earlier, bHLH transcription factor *Math1* and *Ptf1a* generate excitatory and inhibitory neurons in the cerebellum respectively (Ben-Arie et al., 1997; Gazit et al., 2004; Hoshino et al., 2005; Millen et al., 2014; Pascual et al., 2007; Wang et al., 2005; Yamada et al., 2014). *Ptf1a* is also required for inhibitory neuronal identity in the cortex (Russ et al., 2015), spinal cord (Glasgow et al., 2005; Huang et al., 2008) and retina (Fujitani et al., 2006; Jin et al., 2015). RNA-seq analysis of *Ptf1a* misexpression in the cortical pyramidal cells results in up-regulation of 2217 genes and down-regulation of 2263

genes (Russ et al., 2015). Comparison of this analysis to other transcriptome analysis such as Sugino et al. (2006), identified 356 of the up-regulated genes to be unique to GABAergic neurons from the cingulate cortex. In addition, Russ et al. (2015) reports that *Ptf1a* has the ability to promote inhibitory identity when overexpressed in excitatory neurons. Moreover, when compared to RNA-seq data from wild-type (WT) versus *Ptf1a* null neural tube, 43% of *Ptf1a*-activated genes are also upregulated in the *Ptf1a*-misexpressed pyramidal cells (Borromeo et al., 2014; Russ et al., 2015). According to the ChIP-seq analysis of wild-type and *Ptf1a* null neural tubes, approximately half of the commonly up-regulated genes are shown to be direct targets of *Ptf1a* (Borromeo et al., 2014). Not surprisingly, some of these genes are well known inhibitory neuron markers in the cerebellum such as *Lhx1*, *Lhx5*, *Pax2*, *Corl2* and *Gad1* (Morales and Hatten, 2006). This list also includes two members of the Transcription Factor AP-2 (*Tfap2*) family of proteins *Tfap2A* and *Tfap2B* which is reported by various studies (Borromeo et al., 2014; Jin et al., 2015; Russ et al., 2015).

Analysis using the Allen Brain Atlas reveals that transcription factors *Tfap2A* and *Tfap2B* are expressed in subsets of GABAergic neurons in the developing and mature cerebellum (Lein et al., 2007). Unlike most transcription factors that are transiently expressed, *Tfap2A* and *Tfap2B* are expressed from embryogenesis until adulthood (Lein et al., 2007; Moser et al., 1997) which raises the possibility that these factors may possess distinct roles during development and in the mature cerebellum.

1.3.2. Transcription factor activating protein 2, Tfap2A & Tfap2B

Tfap2 family is made up of five members, *A-E* (Eckert et al., 2005), in which *Tfap2A* and *Tfap2B* are highly expressed in both the developing and adult rodent cerebellum (Lein et al., 2007; Moser et al., 1997). All five members share similar structures, consisting of a transactivation domain at the N-terminal and a helix-span-helix motif at the C-terminal that is responsible for dimerization and DNA binding (Eckert et al., 2005; Moser et al., 1995). *Tfap2* transcription factors can dimerise in a homologous or heterologous manner with other *Tfap2* family members and subsequently bind to GC-rich regions (G/CCCN₃GGC) thus causing transcriptional activation or repression (Mohibullah et al., 1999; Moser et al., 1995). Due to their high similarity, we were keen to explore the reason for multiple members of this family of transcription factors being expressed in the cerebellar cortex.

Little is known regarding the expression and function of *Tfap2A* or *Tfap2B* in the cerebellum. *Tfap2A* and *Tfap2B* have been demonstrated to play important roles for the development of a variety of tissues including the nervous system, kidney, skeleton, limbs and the eye (Brewer et al., 2004; Jin et al., 2015; Nottoli et al., 1998; Schmidt et al., 2011; Schorle et al., 1996; West-Mays et al., 1999; Zhang et al., 1996; Zhao et al., 2011). *Tfap2A*- and *Tfap2B*-null mice are reported to die perinatally or early post-natally due to various defects during embryogenesis (Brewer et al., 2004; Moser et al., 1997; Schmidt et al., 2011). In the developing nervous system, *Tfap2A* and *Tfap2B* are required for the survival of sympathetic and sensory ganglia progenitors (Schmidt et al., 2011). To the contrary, in the developing mouse retina, *Tfap2A* and *Tfap2B* have been shown to promote the differentiation of GABAergic and glycinergic amacrine cells as well as being downstream effectors of *Ptf1a* (Jin et al., 2015). Thus, these transcription factors appear to regulate proliferation and survival of various cell types and are involved in the specification of distinct cell types during development. In addition to the retina, both *Tfap2A* and *Tfap2B* are expressed in the mouse cerebellum and spinal cord (Kaltschmidt and Jessell, unpublished; Kim et al., 2001; Levine et al., 2014; Moser et al., 1997). In the spinal cord, *Tfap2B* is expressed by motor synergy encoder neurons which comprises of both inhibitory and excitatory neurons (Levine et al., 2014). In the cerebellum, *Tfap2A* and *Tfap2B* have been reported to have distinct temporal expression pattern, however the neuronal subtypes in the cerebellum that express these transcription factors have not yet been determined (Kim et al., 2001; Moser et al., 1997).

In summary, *Tfap2A* and *Tfap2B* are expressed during embryogenesis and throughout adulthood in various tissues including the cerebellum (Brewer et al., 2004; Jin et al., 2015; Moser et al., 1997; Nottoli et al., 1998; Schmidt et al., 2011; Schorle et al., 1996; West-Mays et al., 1999; Zhang et al., 1996; Zhao et al., 2011). Moreover, these transcription factors have the ability to promote generation of GABAergic neurons in the retina (Jin et al., 2015), raising the possibility that these transcription factors control the developmental aspects of cerebellar GABAergic neuronal subpopulations. Could these transcription factors be involved in the specification of GABAergic progenitors into specific GABAergic neuronal subtypes in the cerebellum? Do they orchestrate the assembly

of cerebellar circuits during development? Finally, are they involved in the maintenance of GABAergic neuronal phenotype?

2. MATERIALS & METHODS

2.1. Animals

All experiments involving rodents were approved by the local Institute of Animal Care and Use Committee in accordance with the National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines. All mice used in this study were housed and bred in Agency for Science, Technology and Research (A*STAR) Biological Resource Centre (BRC). C57BL/6J mice were kept in groups of five in a standard plastic cage with free access to food and water.

2.2. Histological procedures

Mice were anaesthetised with 2.5% Avertin (0.025 g/mL 2, 2,2 Tribromoethanol (Sigma #T48402) in 2-Methyl-2-butanol (Sigma #152463)) prior to perfusion with 0.9% saline and 4% paraformaldehyde (Sigma #P6148). Early postnatal tissues were then post-fixed for an hour while adult tissues were post-fixed overnight in 4% paraformaldehyde. Pregnant mice were euthanized using carbon dioxide gas chamber and ensured through cervical dislocation. Embryos were collected in 4% paraformaldehyde and post-fixed for thirty minutes. All tissues were preserved overnight in 30% sucrose (1st base #BIO-1090) and froze in O.C.T compound (Sakura Finetek #4583). All tissues were cryosectioned at 16-20 μ m thick using ThermoScientific Hyrax C20.

2.3. Mixed cerebellar neuronal culture

Early postnatal C57BL/6J mice, between postnatal day (P) 1 to 5, were used for mixed cerebellar neuronal culture. Pups were anaesthetised on ice for at least 5 min before head was decapitated. The cerebellum was removed and placed on a plate of cold calcium, magnesium-free PBS (CMF-PBS) which consists of 2% glucose (Sigma #G8270) and 0.004% NaHCO_3 (Sigma #S5761) in PBS (Science Werke #L0615). The meninges were freed under dissecting microscopes using fine forceps and transferred into a 15 mL tube containing fresh cold CMF-PBS. After dissecting the cerebelli, CMF-PBS was replaced with 1X Trypsin-DNase and the tissues were incubated in the 37°C water bath for 20 min with agitation every 3-4 min. Following that, the digested tissues were centrifuged at 800 xg for 5 min.

The supernatant was carefully removed and pellet washed thrice with CMF-PBS. The tissues were then triturated ten times with 2 mL CMF-PBS and 200 μ L of 10X DNase solution diluted in Basal Media Eagle and 0.339% glucose using 1 mL pipette. The cell suspension was topped with CMF-PBS to 5 mL and then filtered through 70 μ m cell strainer before cells were centrifuged again for 5 min at 1200 xg. Cell pellet was re-suspended with primary culture media containing Basal Media Eagle containing 10% horse serum, 0.48% glucose, 1% penicillin/streptomycin antibiotics and 1X L-glutamine. Subsequently, cells were seeded into 12-well plate containing sterilised round cover glass coated with poly-L-lysine at a density of 200,000 per well. Cultured neurons were incubated in a 37°C humidified incubator with 5% CO₂. Half of the media was replaced with serum-free media comprising of 0.48% glucose (Sigma, #G8270), 1X penicillin-streptomycin antibiotics (Gibco # 15140122), 1X L-glutamine (Sigma, #G7513), 0.5X B27 serum-free supplement (Invitrogen #17504044) and 1X N2 supplement (Invitrogen #17502-048) in Basal Medium Eagle (Science Werke #L0042-500), the following day, and every 2-3 days.

For viral transduction *in vitro*, half of the media was replaced with media containing virus on days *in vitro* (DIV) 1. Similarly, media was replaced every 2-3 days.

2.4. Immunohistochemistry & Immunocytochemistry

2.4.1. Immunohistochemistry

Tissue sections were permeabilised with 0.3% Triton-X (OmniPur #9400) in PBS for 10 min and blocked for an hour with 2% horse serum (Invitrogen #16050122) and 0.1% Triton-X in PBS. Sections were then incubated with primary antibodies overnight in the cold room. On the following day, the tissues were washed with 0.1% Triton-X in PBS for 3 min thrice and incubated with secondary antibodies for an hour. Tissue sections were washed thrice and stained with DAPI before being washed again and mounted with coverslip.

2.4.2. Immunocytochemistry

Cultured neurons were blocked with 2% horse serum and 0.1% Triton-X in PBS then incubated with primary antibodies overnight. On the following day,

immunocytochemistry staining was performed similarly like immunohistochemistry staining explained above.

Primary antibodies used were as follows: rabbit anti-Tfap2B (1:10,000; generous gift from T. Jessell from USCF), rabbit anti-Tfap2A (1:500; Santa Cruz Bio Technology), goat anti-Pax6 (1:500; Santa Cruz Bio Technology) and mouse anti-Pax2 (1:500; Santa Cruz Bio Technology), mouse anti-3B5 (Tfap2A) (2µg/mL; Developmental Studies Hybridoma Bank) and mouse anti-Lhx1/5 (2µg/mL; Developmental Studies Hybridoma Bank), goat anti-Olig2 (1:250; R&D), mouse anti-Calbindin (1:5000; Swant) and goat anti-Parvalbumin (1:5000; Swant), rat anti-GFP (1:1000; Nacalai Tesque) and rat anti-RFP (1:500; Chromotek).

Secondary antibodies used were as follows: donkey anti-rabbit 488, donkey anti-rabbit 647, donkey anti-goat 555, donkey anti-mouse 647, donkey anti-rat 488 and donkey anti-rat 555 (1:1000; Molecular probes).

2.5. Chromogenic *in situ* hybridisation

RNA probe synthesis. Total cerebellar RNA (Clontech #36660) was used to reverse transcribed into complementary DNA and the DNA was used as a template for polymerase chain reaction. Primer sequence was designed against anti-sense strand of target gene and T7 RNA polymerase sequence (5' GCGCTAATACGACTCACTATAGGG 3') was added on the 5' end of the reverse primers to aid in initiation of transcription (see Table 1 for probe sequences). Transcribed RNA was labelled with 11-digoxigenin-UTP (Roche #11175025910).

*Chromogenic *in situ* hybridisation.* Tissue sections were fixed using cold 4% paraformaldehyde for 10 min, washed thrice for 3 min with fresh PBS and permeabilised with Proteinase K (Promega #V3021) for 10 min. Sections were fixed again with room temperature 4% paraformaldehyde for 5 min, then washed and treated with acetylation solution containing triethanolamine (Sigma #108-24-7) and acetic anhydride (Sigma #411000). Sections were washed once more before incubated with hybridisation solution which is made up of 50% formamide (Promega #H5052), 5X SSC (1st base #BUF-3050), 5X Denhardt's solution (Ficoll type 400 (Sigma #F9378), polyvinylpyrrolidone (Sigma

#PVP40) and bovine serum albumin (Sigma #A7906)), 250 µg/mL yeast RNA (Sigma #R6625-5G) and 500 µg/mL salmon sperm DNA (Promega #D1816) at 63.5°C for at least an hour. RNA-labelled probes were diluted to 400-1000ng/mL with hybridisation solution and denatured at 80°C for 5 min. Pre-hybridised sections were incubated with probes and coverslips on in a humidified box for 12-15 hours at 63.5°C.

After overnight incubation with RNA-labelled probes, tissue sections were washed with 5X SSC pre-warmed at 63.5°C and coverslips removed. Two additional washes were carried out using 0.2X SSC for 30 min each at 63.5°C. Tissue sections were then equilibrated to room temperature by incubating with B1 buffer containing 0.1M Tris pH7.5 and 0.15M NaCl, and blocked with 10% sheep serum diluted with B1 buffer for an hour at room temperature. After blocking, sections were incubated with anti-DIG-AP antibody (1:2000; Roche) overnight at 4°C. On the following day, sections were washed with B1 buffer thrice for 3 min each and B3 buffer containing 100mM Tris-HCL pH9.0, 150mM NaCl and 1mM MgCl for 5 min. BCIP/NBT substrate was added onto the slides and incubated for 6-24 hours till the signal appears. Color reaction was stopped with ice-cold methanol and sections were mounted with DakoMount (Dako Cytomation).

Gene	Primer direction	Sequence (5' → 3')
<i>Neurod1</i>	Forward	CTCGGACTTTCTTGCCTGAG
	Reverse	GCGCTAATACGACTCACTATAGGGTGCAGGGTAGTGCATGGTAA
<i>PCP2</i>	Forward	CCCTGCTCCAGAGATGGAC
	Reverse	GCGCTAATACGACTCACTATAGGGGTTTCCTGCGGAAGCTGAGT
<i>Gad1</i>	Forward	GCACAGAGACCGACTTCTCC
	Reverse	GCGCTAATACGACTCACTATAGGGCCAGGATCTGCTCCAGAGAC
<i>Tfap2A</i>	Forward	ACCCCTACAGCCTGAATCCT
	Reverse	GCGCTAATACGACTCACTATAGGGAGGGCCTCGGTGAGATAGTT
<i>Tfap2B</i>	Forward	TCATGCTCTGGAACTCGTG
	Reverse	GCGCTAATACGACTCACTATAGGGACGTGACATTTGCTGCTTTG
<i>Pou3f3</i>	Forward	GGAGCAGTTCGCTAAGCAGT
	Reverse	GCGCTAATACGACTCACTATAGGGCCTCCAGCCACTTGTTTCAGT
<i>Cdh13</i>	Forward	CCCAGAGAACCAGAGACAGC
	Reverse	GCGCTAATACGACTCACTATAGGGACTGGCCCTTCCAGAGTTTT
<i>Mef2C</i>	Forward	TGATCAGCAGGCAAAGATTG
	Reverse	GCGCTAATACGACTCACTATAGGGATGCGCTTGACTGAAGGACT
<i>Lgi2</i>	Forward	TTGCTGGCCTTTTTTCATCTT
	Reverse	GCGCTAATACGACTCACTATAGGGTGGACCGATACACAGCACAT

Table 1. Oligonucleotide sequences for RNA probe synthesis.

List of primers designed to amplify complementary DNA (cDNA) of interest. Amplified cDNA was subsequently used for transcription to generate digoxigenin (DIG)-labelled probe for *in situ* hybridisation. Nucleotide sequences in red represent *T7 RNA polymerase* sequence that is added on the 5' end of the reverse strand of the primer to aid in transcription.

2.6. Image and cell count analysis

Brightfield images from chromogenic *in situ* hybridisation experiment were taken using Zeiss Axio.A1 microscope. Confocal images from immunohistochemistry and immunocytochemistry experiments were captured using Zeiss LSM-710 Confocal Microscope System (Axio Imager Z2 Stand) on ZEN2011. All images were processed with ImageJ (National Institute of Health).

For Tfap2-Pax2 cell count analysis, two sagittal sections were obtained from the vermis region of each of the three brains and cells were counted. Using Image J, specific regions of interest were manually drawn around all nuclei positive for Tfap2A, Tfap2B and Pax2 and were subsequently scored. Immuno-positive neurons were then grouped and percentages were calculated.

Analysis for *in utero* experiments, all transfected cells in the white matter layer from multiple sagittal sections (approximately 10-25 sections, 100-500 cells) were counted for three brains. Cells were scored for the respective molecular markers and percentages were calculated. Experimental groups were compared against control groups.

2.7. Statistical analysis

A non-parametric Mann-Whitney *U* test was carried out in all the experiments that require statistical findings. Data represents mean $\pm$ SEM and a *p* value of 0.05 was used for statistical significance. All data was analysed with GraphPad Prism 6. The statistical data are described in each figure legend.

2.8. Genetic manipulation of Tfap2A and Tfap2B *in vitro* and *in vivo*

2.8.1. Generation of knockdown and overexpression plasmid

For overexpression studies, *pCDH-Ef1a-MCS-IRES-RFP* lentivector was purchased from System Bioscience (#CD531A-2). Plasmid consists of constitutive *Ef1* promoter followed by multiple cloning site (MCS), *Internal Ribosomal Entry Site (IRES)* and *red fluorescent protein (RFP)*. *Tfap2A* cDNA and *Tfap2B* cDNA were purchased from GeneArt Invitrogen and sub-cloned into MCS of the *pCDH* vector. Plasmids were digested with *XbaI* and *NotI* restriction enzymes (New England Biolabs #R0145S and #R3189S), isolated on 1% agarose gel (1st Base #BIO-1000) and extracted using gel extraction kit (Qiagen #28706). Digested *pCDH* and cDNA were ligated using T7 DNA ligase (New England Biolabs #M0202T) at 16°C for 20-22 hours then deactivated by heating at 65°C for 10 min. The ligated plasmids were transformed into ECOSTM X competent cells (Yeastern #FYE610-10VL) as per manufacturer's instruction. The following day, single colonies were picked and grown in LB broth containing 100 µg/mL (Sigma #A9518). Next, plasmid DNA was extracted (Invitrogen #K210011) and send for sequencing to Institute of Molecular and Cellular Biology, DNA Sequencing Facility (IMCB DSF) to identify correctly cloned plasmid.

For knockdown studies, *pLL3.7* lentivector (Addgege #11795) was used to express target short hairpin RNA (shRNA) under *U6* promoter and green fluorescent protein (GFP) reporter under *CMV* promoter. DNA oligonucleotides which will be transcribed into shRNA were designed using Invitrogen BLOCK-iTTM RNAi Designer. It comprised of *XhoI* overhang (5' TCGA 3') on the 5' end of the antisense strand and a short hairpin loop (5' TTCAAGAGA 3') (see table 2). 40 µM of sense and antisense oligonucleotides diluted in 1X T4 DNA ligase buffer (New England Biolabs #M0202) were annealed by boiling at 100°C for 10 min and cooled gradually to room temperature. Subsequently, both the *pLL3.7* and annealed oligonucleotides were digested, separately, with *XhoI* and *HpaI* (New England Biolabs #R0146S and #R0105S). Ligation was carried similar as the overexpression construct. Cloned vectors were sequenced by Institute of Molecular and Cellular Biology, DNA Sequencing Facility (IMCB DSF) to confirm cloned sequence.

As for *Ptf1a* overexpression studies, control (LSL-GFP) and experimental (LSL-*Ptf1a*) plasmids were a gift from Julia Kaltschmidt (Memorial Sloan Kettering Cancer Center/Stanford University) (Russ et al., 2015). The plasmid vector, *pCAG-LSL-Ptf1a-IRES-EGFP*, employs the *Cre-lox* system which requires Cre-recombinase enzyme to mediate recombination in Cre-expressing cells. Construct is driven by *CAG* promoter and consists of *loxP* sites flanking the stop codon (LSL), followed by *Ptf1a* cDNA, *IRES* sequence and *EGFP* cDNA.

shRNA #	DNA strand	Sequence (5' → 3')
scramble	sense	TGCCCTACCACCGAGGTCAATTCAAGAGATTGACCTCGGTGGTAGGGC TTTTTTC
	antisense	TCGAGAAAAAAGCCCTACCACCGAGGTCAATCTCTTGAATTGACCTCG GTGGTAGGGCA
Tfap2Ash1	sense	TGGCAAAGAGGATAATGACATTCAAGAGATGTCATTATCCTCTTTGCC TTTTTTC
	antisense	TCGAGAAAAAAGGCAAAGAGGATAATGACATCTCTTGAATGTCATTA TCCTCTTTGCCA
Tfap2Ash2	sense	TGACAACATTCCGATCCCAATGTTCAAGAGACATTGGGATCGGAATGT TGTCTTTTTTC
	antisense	TCGAGAAAAAAGACAACATTCCGATCCCAATGTCTCTTGAACATTGGG ATCGGAATGTTGTCA
Tfap2Ash5	sense	TGCAAGATCCTTACTCCACGTTTCAAGAGAACGTGGGAGTAAGGATC TTGCTTTTTTC
	antisense	TCGAGAAAAAAGCAAGATCCTTACTCCACGTTCTCTTGAACGTGGG AGTAAGGATCTTGCA
Tfap2Bsh2	sense	TGCATGGACAAGATGTTCTTGATTCAAGAGATCAAGAACATCTTGTC ATGCTTTTTTC
	antisense	TCGAGAAAAAAGCATGGACAAGATGTTCTTGATCTCTTGAATCAAGA ACATCTTGTCATGCA
Tfap2Bsh5	sense	TGGAGAATGTCAAGTACGAAGATTCAAGAGATCTTCGTAATTGACATT CTCCTTTTTTC
	antisense	TCGAGAAAAAAGGAGAATGTCAAGTACGAAGATCTCTTGAATCTTCG TACTTGACATTCTCCA
Tfap2Bsh6	sense	TGCATGAACCTATTGGACCAGTTTCAAGAGAACTGGTCCAATAGGTTC ATGCTTTTTTC
	antisense	TCGAGAAAAAAGCATGAACCTATTGGACCAGTTCTCTTGAACCTGGTC CAATAGGTTTCATGCA

Table 2. Oligonucleotide sequences designed to target Tfap2A and Tfap2B expression.

Oligonucleotides were designed using Invitrogen BLOCKiT™ RNAi Designer, annealed and subsequently cloned into pLL3.7 vector. Nucleotides labelled in red are added onto the 5' end of the antisense strand to create an overhang while the underlined nucleotides create a hairpin structure.

2.8.2. Western blot

Human embryonic kidney 293T (HEK-293T) cells were seeded at a density of 500,000 cells per well in a 6-well tissue culture plate a day before transfection. OPTI-MEM reduced serum media (Gibco) was used to dilute 1 µg of DNA as well as Lipofectamine 2000 reagent (Invitrogen). Lipid-DNA mixture was introduced into the cells and was incubated for 48 hours before protein isolation.

Cells were washed once with PBS before proteins were isolated using M-PER mammalian protein extraction reagent (Pierce) which is supplemented with phosphatase inhibitor tablet (Pierce) and protease inhibitor cocktail (Sigma). Proteins were subsequently quantified via colorimetric assay (Bio-Rad) according to manufacturer's protocol. 20 µg of proteins were used for fractionation by SDS-PAGE and transferred to a PVDF membrane using iBlot® Dry Blotting System (Invitrogen). After incubation with 2% Bovine Serum Albumin (BSA) diluted in TBS-T (10 mM Tris [pH 8.0] , 150 mM NaCl, 0.5% Tween 20) for an hour, this blocking buffer was decanted and primary antibodies were incubated overnight at 4°C.

The primary antibodies used were as follows: rabbit anti-Tfap2A (1:500; Santa Cruz Bio Technology), rabbit anti-Tfap2B (1:500; Santa Cruz Bio Technology), and mouse anti-GAPDH (0.05µg/mL; Sigma). On the next day, membranes were washed with TBS-T thrice, for 10 minutes each before being incubated with appropriate secondary antibodies. The secondary antibodies used were diluted in blocking buffer as follows: anti-mouse IgG fragment-peroxidase antibody produced in goat (1:3000; Sigma) and anti-rabbit IgG fragment-peroxidase antibody produced in goat (1:3000; Sigma). Membrane was washed again, similarly, before being developed with the ECL system (Cell Signaling) according to manufacturer's protocol.

2.8.3. Production of lentiviruses

Third generation lentiviral system which consists of *pRSV-Rev*, *pMDLg/pRRE* and *pMD2.G* plasmids (Addgene) were used to generate the lentivirus. Human embryonic kidney-293T (HEK-293T) cells were co-transfected with the transfer, packaging and envelope plasmids via calcium phosphate co-precipitation method. 10×10^6 cells were

seeded on a 10-cm tissue culture dish in DMEM (Science Werke #L0104-500) supplemented with 10% fetal bovine serum (Gibco #10082147) and 1% penicillin/streptomycin antibiotics (Gibco #15140122) on the morning of transfection day. Transfection was carried out at least six hours upon seeding and media was replaced 12-15 hours upon transfection. Subsequently, media was collected and fresh media was replaced 24 and 48 hours later. Collected media were pooled and filtered through 0.45 µm filter syringe before being concentrated via ultracentrifugation method. Beckman ultracentrifuge SW28 rotor was used to concentrate virus at 19.2k rpm for 2 hours at room temperature. Media was then decanted and allowed to drain by gravity before virus was suspended with cold DPBS. For long term storage, virus were aliquoted and stored at -80°C.

To titre virus, 1 mL of media containing 10×10^3 HEK-293T cells were seeded into 24-well plate a day before transduction. Serial dilutions of virus were made and added onto the cells. Three days post-infection, fluorescent cells were counted and the following equation was used to determine virus titre: $\frac{N_{R1} + N_{R2}}{2} \times \frac{\text{dilution factor}}{10 \times 10^{-3}} \left(\frac{CFU}{mL} \right)$, where N_R is the number of fluorescent cells in each duplicate.

2.8.4. *In utero electroporation*

C57BL/6J pregnant mice were anaesthetized with 2% isoflurane throughout the procedure and were kept warm using a homeothermic blanket. Hair on the abdomen area was shaved and skin cleaned with cotton swabs soaked in 70% ethanol. A small incision (5-8 cm) on the skin through the midline was done and a gauze moistened with 42°C 0.9% saline was placed around the incision. Another incision on the muscle was made and both of the uterine horns carefully placed on the gauze. From time to time, the exposed embryos were moistened with warm saline. 5-7 µL of purified plasmids (1 µg/µL) with Fastgreen Dye (0.1 mg/mL) were delivered into the fourth ventricle using a glass capillary. Electrodes were positioned in between the ventricles and five pulses of 250mV for 50 msec each, were delivered. Uterine horns were returned into the peritoneal cavity and warm saline was added before the lining of cavity and the skin was sutured. Mice were provided with Meloxicam as a post-operative management.

2.8.5. *In vivo transduction*

Early postnatal viral injection. P1 or P2 pups were anaesthetised by wrapping the pups with Kimwipe and covering it with ice for 5 min. Response of the mice were confirmed by testing for surface righting reflex and any kind of reflex. 2.0 μ L of virus was then injected into the cerebellar region using a 35 GA needle. Pups were placed on a warm blanket till it recovers before being returned to the cage with its mother.

Viral injection in adult mice. Mice were placed under general anaesthesia with 2.0% Isoflurane delivered at 0.8-1.5 L/min throughout the procedure. Reflex of the mice were confirmed by pinching its paw. General analgesia, Buprenorphine (0.1 mL/10g of 0.06 mg/mL), and local analgesia, Lidocaine (0.053 mL/10g of 1.33 mg/mL), were injected subcutaneously dorsally on its back and around the incision, respectively. Using the stereotaxic frame, coordinates of injected site was marked with the tip of a syringe needle at anterior-posterior 1.8, medial-lateral 1.0 and dorsal-ventral 2.0 from interaural position and the skull was drilled. 1.0 μ L of virus was infused at a constant rate (200 nL/min) before mouse was sutured and returned to the housing cage. Following this, mice were provided with antibiotics, Meloxicam (1.5 mg/250 mL) and Baytril (15 mg/250 mL) in the water bottle to prevent any infection. All drugs used were prepared by the veterinarian from A*STAR BRC.

2.8.6. *In vitro lentiviral transduction*

Mixed cerebellar neuronal culture. Early postnatal C57BL/6J mice, between postnatal (P) day 1 to 5, were used for mixed cerebellar neuronal culture. Pups were anaesthetised on ice for at least 5 min before head was decapitated. The cerebellum was removed and placed on a plate of cold calcium, magnesium-free PBS (CMF-PBS) which consists of 2% glucose (Sigma #G8270) and 0.004% NaHCO₃ (Sigma #S5761) in PBS (Science Werke #L0615). The meninges were freed under dissecting microscopes using fine forceps and transferred into a 15 mL tube containing fresh cold CMF-PBS. After dissecting the cerebelli, CMF-PBS was replaced with 1X Trypsin-DNase and the tissues were incubated in the 37°C water bath for 20 min with agitation every 3-4 min. Following that, the digested tissues were centrifuged at 800 xg for 5 min. The supernatant was carefully

removed and pellet washed thrice with CMF-PBS. The tissues were then triturated ten times with 2 mL CMF-PBS and 200 μ L of 10X DNase solution diluted in Basal Media Eagle and 0.339% glucose using 1 mL pipette. The cell suspension was topped with CMF-PBS to 5 mL and then filtered through 70 μ m cell strainer before cells were centrifuged again for 5 min at 1200 xg. Cell pellet was re-suspended with primary culture media containing Basal Media Eagle containing 10% horse serum, 0.48% glucose, 1% penicillin/streptomycin antibiotics and 1X L-glutamine. Subsequently, cells were seeded into 12-well plate containing sterilised round cover glass coated with poly-L-lysine at a density of 200,000 per well. Cultured neurons were incubated in a 37°C humidified incubator with 5% CO₂. Half of the media was replaced with serum-free media comprising of 0.48% glucose (Sigma, #G8270), 1X penicillin-streptomycin antibiotics (Gibco # 15140122), 1X L-glutamine (Sigma, #G7513), 0.5X B27 serum-free supplement (Invitrogen #17504044) and 1X N2 supplement (Invitrogen #17502-048) in Basal Medium Eagle (Science Werke #L0042-500), the following day, and every 2-3 days.

For viral transduction *in vitro*, half of the media was replaced with media containing virus on days *in vitro* (DIV) 1. Media was replaced every 2-3 days and culture was fixed with warm 4% PFA/sucrose for 10 min approximately two weeks post-infection. Upon transfection, fixed neurons were washed with PBS for 3 min.

3. RESULTS

3.1. Gene expression profiles of cerebellar neuronal subpopulations

3.1.2 *Molecular distinctions between cerebellar cortical inhibitory neurons and excitatory granule cells*

We analysed an unpublished microarray data which contained the gene expression profiles of the five major neuronal subtypes in the cerebellum (Nathaniel Heintz and Ben Barres, unpublished). Each neuronal subtype was isolated using a mouse line that expressed Cre recombinase enzyme under a neuronal subtype-specific promoter. The *Cre* mouse lines used were *neurogenic differentiation 1* (*Neurod1*), *Purkinje Cell Protein 2* (*PCP2*), *metabotropic glutamate receptor 2* (*mGluR2*) and *LY6/PLAUR domain containing 6* (*LYPD6*) which, respectively, mediated recombination in granule, Purkinje, Golgi and stellate/basket cells (Table 2). Subsequently, the Cre-recombinase expressing mouse was crossed with a RiboTag mouse which allowed the isolation of ribosome-associated mRNA transcripts from a distinct neuronal type. The RiboTag mouse consists of two identical repeats of *Rpl22* exon 4, a gene encoding for a ribosome subunit, of which the first repeat is flanked by *loxP* sites followed by another repeat and hemagglutinin (HA) epitope sequence before the stop codon (Figure 6) (Sanz et al., 2009). Thus, upon crossing the two mouse lines, the wild type *Rpl22* exon 4 was excised and RPL22^{HA}-tagged ribosome was generated. *Rpl22* gene encodes for 60s ribosomal protein L22 that is a component of the 60S subunit of the ribosomes. Consequently, the actively translating polyribosomes present in the Cre-expressing cells were immunoprecipitated using monoclonal antibody against HA and transcripts profiled via Affymetrix microarray.

Cre-driver mouse	Type	Subtype	Cerebellar region	Neuro-transmitter
<i>Neurod1-Cre</i>	Interneuron	Granule	Inner granular layer	Glu
<i>PCP2-Cre</i>	Projection	Purkinje	Purkinje layer	GABA
<i>mGluR2-Cre</i>	Interneuron	Golgi	Inner granular layer	GABA
<i>Lypd6-Cre</i>	Interneuron	Stellate, basket	Molecular layer	GABA

Table 3. Descriptions of the five major cerebellar neuronal subpopulations.

Transgenic mouse lines used to express Cre recombinase (Cre) enzyme under a specific promoter that is enriched in specific neuronal subtypes. Purkinje cell protein 2 (PCP2) is a protein that is expressed solely by Purkinje cells in the cerebellum. Crossing the RiboTag mouse with PCP2-Cre mouse will result in the expression of hemagglutinin-tagged *Rpl22* protein that is exclusively expressed by Purkinje cells.

The GeneChip® Mouse Genome 430 2.0 Array (MOE430) consisted of 45,038 probe sets representing over 39,000 transcripts. During analysis, we considered any expression value less than 100 as negative expression while a fold change of greater than 1 suggested a difference in expression levels between the neuronal cell types. We identified a number of genes to be differentially expressed in glutamatergic and GABAergic neurons. There were 9267 probes exclusively expressed by one or more of the GABAergic neurons and 3171 probes by glutamatergic granule cells. Of the 9267 probe sets expressed by inhibitory neurons, 4392 were highly expressed by Purkinje cells and 632 probes were expressed by all the interneurons, which includes stellate, basket and Golgi cells. For example, genes encoding for glutamic acid decarboxylase (Gad) enzymes which are responsible for catalysing glutamate into Gamma Amino Butyric Acid (GABA) neurotransmitter, *Gad1* and *Gad2*, were enriched in all the inhibitory neuronal subtypes. In another example, *Slc17a7* or *vGluT1*, a gene encoding for vestibular glutamate transporter 2 protein, which is expressed exclusively by glutamatergic neurons, was enriched in granule cells (Hioki et al., 2003). We have also compared our results with published microarray data which identifies gene signatures for inhibitory and excitatory neurons in the cingulate cortex in the forebrain (Sugino et al. 2006). Although they may have used a slightly different microarray chip, all probe sets present in MOE430 chip that are used by Sugino et al. (2006) were present in the MOE430 chip that have been used. Analysis on their microarray data identified 298 probes that were enriched in all three subtypes of GABAergic neurons and 4913 probes that were enriched in layer 5 pyramidal cells of the cingulate cortex. Interestingly, of all the 298 probes identified, all were also enriched in all cerebellar GABAergic neuronal subtypes (out of 1062 probes). However, only 733 probes were enriched in both glutamatergic neurons in the cingulate cortex and the cerebellum (out of 3171 probes). Therefore, to the best of our knowledge, we managed to identify genes from our microarray analysis that have been reported to be expressed by the distinct cerebellar neuronal subtypes (Table 4). However, our results listed many other genes that are unique which have not been reported to play any role in the cerebellum. Among these genes are transcription factors such as *Tfap2A* that is expressed by all the GABAergic neuronal subtypes and *Tfap2B* expressed by GABAergic interneurons (Table 4).

Gene title	Golgi	Granule	Purkinje	Stellate, basket
Granule				
Neurogenic differentiation 1 (Neurod1)	203	23,466	211	3,923
GABA vesicular transporter 1 (vGluT1)	8	434	3	10
GABA receptor, subunit alpha 6 (Gabra6)	5	1,402	4	103
Purkinje				
Purkinje cell protein 2 (PCP2)	370	482	57,566	12,766
Calbindin-28K (CBP)	47	12	14,803	173
RAR-related orphan receptor alpha (RORa)	40	197	1,910	769
Golgi				
Metabotropic glutamate receptor 2 (mGluR2)	10,545	5	8	5
Glycine transporter 2 (GlyT2)	1,598	4	4	9
Neurogranin (Nrgn)	154	4	4	4
Stellate & basket				
LY6/PLAUR domain containing 6 (Lypd6)	49	5	16	12,912
All GABAergic neurons				
Glutamic acid decarboxylase 1 (Gad1)	20,234	1,877	31,643	24,704
Glutamic acid decarboxylase 2 (Gad2)	735	14	431	1,400
Vesicular inhibitory amino acid transporter (VIAAT)	5,846	23	2,365	7,300
Unique genes				
Transcription factor activating protein 2A (Tfap2A)	2,390	32	1,895	7,168
Transcription factor activating protein 2B (Tfap2B)	4,447	39	30	3,913
POU domain, class 3, transcription factor 3 (Pou3f3)	9	5	11	1,169
Cadherin 13 (Cdh13)	359	3	5	5
Myocyte enhancer factor 2C (Mef2C)	32	774	5,911	613
Leucine-rich repeat LGI family, member 2 (Lgi2)	1,379	6	5	167

Table 4. Expression values of known and novel genes that are enriched in one or more neuronal subpopulations in the cerebellum.

Molecular markers such as Neurod1, vGlutT1 and Gabra6 have been established by previous reports to be expressed exclusively by granule cells in the cerebellum (Hioki et al., 2003; Miyata et al., 1999; Zheng et al., 1993). Unique genes such as *Transcription factor activating protein 2* family members, *Tfap2A* and *Tfap2B*, are some of the genes identified from the microarray analysis that are enriched in subtypes of GABAergic neuronal subpopulations.

3.1.2 Validation of microarray data using *in situ* hybridisation

To determine the accuracy of our microarray data, we first analysed published reports and publicly available expression database as well as carry out *in situ* hybridisation. Little is known about the involvement of transcription factors *Tfap2A* and *Tfap2B* in the cerebellum. Earlier studies about *Tfap2* family members report that expression of both *Tfap2A* and *Tfap2B* are found in various regions of the brain, which includes the cerebellum at E13.5 (Moser et al., 1995; Moser et al., 1997). Analysis at E15.5 shows expression of *Tfap2A* and *Tfap2B* in the ventricular zone and white matter of the cerebellum, site of production and migration of GABAergic cerebellar neurons (Moser et al., 1995; Moser et al., 1997). In the adult cerebellum, *Tfap2A* expression is found in the cerebellar cortex which includes the molecular layer, Purkinje layer and inner granular layer (Kim et al., 2001). However, these studies did not assess the cell types that express these transcription factors. Next, we used expression databases such as Allen Brain Atlas and Gensat to identify expression pattern of both transcription factors. Similarly, we found that *Tfap2A* is expressed in neurons in the molecular layer, Purkinje layer and inner granular layer, which could corresponds to stellate and basket cells, Purkinje cells and Golgi cells respectively (Lein et al., 2007). On the other hand, *Tfap2B* is expressed only in neurons in the molecular layer and inner granular layer, which suggests that they are expressed in stellate and basket cells and Golgi cells respectively. Therefore, expression profile provided by Allen Brain Atlas supports our microarray data.

To confirm the results of our microarray data and observations from other expression data, we also performed *in situ* hybridisation on the adult cerebellum (Figure 7). We observed similar spatial expression profiles with Allen Brain Atlas. Expression of *Tfap2A* is detected in the molecular layer, Purkinje layer and inner granular layer, which suggests for stellate and basket cells, Purkinje cells and Golgi cells respectively (Figure 7D). On the other hand, expression of *Tfap2B* is detected in the molecular layer and inner granular layer, presumably expressed by stellate and basket cells and Golgi cells (Figure 7E). Other shortlisted genes were also verified using *in situ* hybridisation of which *Cdh13* (Figure 7G), *Mef2C* (Figure 7H) and *Lgi2* (Figure 7I) are some of the genes that are being studied in the lab. Therefore, our *in situ* hybridisation results support our microarray data where both show enrichment of *Tfap2A* expression in all GABAergic neurons and enrichment of *Tfap2B* expression in GABAergic interneurons.

Figure 7. *In situ* hybridisation analysis of known and novel genes identified by microarray analysis.

(A-C) RNA expression of the known markers such as *Neurod1* (A), *PCP2* (B) and *Gad1* (C) in the adult cerebellum. (D-I) Validation on some of the genes found to be enriched by some of the cerebellar neuronal subtypes, which are detected by the microarray analysis. Expression of *Tfap2A* and *Pou3f3* are found in the molecular layer (ML), Purkinje layer (PL) and inner granular layer (IGL) while expression of *Tfap2B* is found in the molecular layer and inner granular layer only. Genes such as *Cdh13* (G), *Mef2C* (H) and *Lgi2* (I) are only found in either the inner granular layer or Purkinje layer by only one subtype of cerebellar neurons.

From the microarray analysis, we identified differentially expressed genes in the cerebellar cortex such as *Tfap2A* and *Tfap2B* which are highly expressed in subtypes of GABAergic neurons (Table 4). Interestingly, *Tfap2A* and *Tfap2B* are not only expressed in the mature cerebellum, but also in the developing mouse as early as E13.5 (Lein et al., 2007; Moser et al., 1995). Thus, this information led us to speculate that these transcription factors may exert their influence during developmental and postnatal stages. For example, *Tfap2A* and *Tfap2B* could play a role in the specification of GABAergic neuronal subtypes during embryogenesis, but also function to maintain synaptic plasticity during adulthood.

3.2. Characterisation of the expression of Tfap2A and Tfap2B in the developing and mature cerebellum

3.2.1. Tfap2A- and Tfap2B-expressing cells follow GABAergic neuronal migration pathway

Purkinje cells are one of the earliest GABAergic neuronal subtypes born in the ventricular zone between E10.5 and E12.5 (Altman and Bayer, 1996; Leto et al., 2006; Sudarov et al., 2011; Yamada et al., 2014). From the ventricular zone, GABAergic progenitors will transit in the white matter before migrating into the cerebellar cortex (Leto et al., 2009; Maricich and Herrup, 1999; Weisheit et al., 2006; Zhang and Goldman, 1996). Tfap2A⁺ (Figure 8B) and Tfap2B⁺ (Figure 8I) cells are detected in the white matter of cerebellum at E12.5. They are not found in the intermediate domain of rhombomere 1 at E10.5 (Figure 8A, H), the region that forms the cerebellar primodium. Expression of Tfap2A and Tfap2B persists throughout embryogenesis till adulthood (Figure 8G, N). As development progresses and neurons migrate, differences in the spatial organisation of Tfap2A⁺ and Tfap2B⁺ cells become apparent. At E15.5, Tfap2A⁺ cells are detected dorsally beneath the external germinal layer, the ventricular zone and the white matter (Figure 8C). However, Tfap2B⁺ cells are only observed in the ventricular zone and white matter (Figure 8J). This difference becomes distinct as the cerebellum lobulate. Clusters of Tfap2A⁺ cells are found in apical cortex of certain lobules, such as the central lobe, and throughout the white matter of E18.5 cerebellum (Figure 8D). But on the other hand, Tfap2B⁺ cells are only found in the white matter (Figure 8K) suggesting that Tfap2A and Tfap2B are expressed in distinct classes of neurons.

Figure 8. Tfap2A and Tfap2B are expressed by cells originating from the ventricular zone and are detected in the cerebellum at E12.5.

(A-N) Examination of the expression of Tfap2A (green; A-G) and Tfap2B (magenta; H-N) in the developing cerebellum, from E10.5, and the mature cerebellum via immunohistochemistry. (A, H) Tfap2A and Tfap2B are not expressed in the cerebellar

primodium at E10.5 in the intermediate domain of rhombomere 1 (rh1). (B, I) Expression of both Tfap2A and Tfap2B is observed in the underlying white matter (WM) of E12.5 cerebellum where inhibitory neurons transit after exiting the ventricular zone (VZ). Both Tfap2A and Tfap2B are not found, at any point, in the rhombic lip (RL), nuclear transitory zone (NTZ) or external germinal layer (EGL) where excitatory neurons are born and transit during development. (C) Tfap2A expression is detected underneath the EGL at the dorsal end and in the WM which spreads to the ventral end of the E15.5 cerebellum. However, expression of Tfap2B (J) is restricted to the ventral end of the WM unlike Tfap2A (C). (D, K) Spatial expression of Tfap2A in the cerebellum at E18.5 is found in the cortex in a cluster and in the WM but expression of Tfap2B is restricted to the WM only. (E, L) Spatial expression of both transcription factors at P0 is similar to E18.5 (D, K) but cells in the WM are found to be more dispersed. (F, M) In the P10 cerebellum, both Tfap2A and Tfap2B are observed to penetrate the lower half of the molecular layer (ML), in the inner granular layer (IGL) and in the WM but additional Tfap2A expression can also be observed in the Purkinje layer (PL). (G) In the mature cerebellum, Tfap2A expression is detected in the ML, PL and IGL which may represent stellate and basket cells, Purkinje cells and Golgi cells, respectively. On the other hand, expression of Tfap2B (N) is restricted to the ML and IGL. Schematic diagram of the developing and mature cerebellum are drawn for reference. All sections are sagittal view of the cerebellar vermis region. Scalebar = 100 μ m.

To gain more insight to the neuronal population that expresses Tfap2A and Tfap2B, we analysed the spatial expression pattern of these proteins in postnatal cerebellar sections. In P0 cerebellum, the distribution of Tfap2A⁺ and Tfap2B⁺ cells remain similar to E18.5 cerebellum. Expression of Tfap2A is observed in clusters in the cerebellar cortex and sporadically in the white matter (Figure 8E) while expression of Tfap2B is only observed in the white matter (Figure 8L). At a later postnatal stage, Tfap2A⁺ cells are detected in monolayer in the Purkinje layer and in scatters in the molecular layer, inner granular layer and white matter (Figure 8F). But on the other hand, Tfap2B is expressed only in the molecular layer, inner granular layer and white matter (Figure 8M), which suggests that Tfap2A⁺ and Tfap2B⁺ cells are migrating from the ventricular zone into white matter and

the molecular layer. In the mature cerebellum, the expression of Tfap2A (Figure 8G) and Tfap2B (Figure 8N) is no longer observed in the white matter, which indicates the completion of GABAergic neuronal migration (Leto et al., 2009; Maricich and Herrup, 1999; Weisheit et al., 2006). Tfap2A⁺ cells are observed in the molecular layer, Purkinje layer and inner granular layer (Figure 8G), which suggests that Tfap2A is expressed by stellate and basket cells, Purkinje cells and inner granular layer interneurons respectively. Tfap2B⁺ neurons, on the other hand, are found only in the molecular layer and inner granular layer (Figure 8N). Both of the transcription factors are not observed in the rhombic lip, nuclear transitory zone or the external germinal layer, regions where excitatory neurons are born and transit, at any stages examined (Figure 8A-F, H-M). Most importantly, our results support the outward migration of Tfap2A⁺ and Tfap2B⁺ neurons from the ventricular zone into the white matter, inner granular layer, Purkinje layer and molecular layer which resembles the inside-out migration pattern of cerebellar GABAergic neuron.

3.2.2. Tfap2A and Tfap2B are expressed in proliferating progenitors and post-mitotic GABAergic neurons

To examine the molecular identity of Tfap2A⁺ and Tfap2B⁺ cells in the developing cerebellum, we compared the expression of Tfap2 with various molecular markers that label distinct cerebellar neuronal subtypes. Olig2, a bHLH transcription factor, is a marker for proliferating and early post-mitotic GABAergic neuron progenitors, specifically Purkinje cells (Ju et al., 2016; Seto et al., 2014a). Another bHLH transcription factor, Pax2, is a well-known marker for GABAergic interneuron progenitors (Maricich and Herrup, 1999). In E12.5 cerebellum, the expression of Olig2 is located in the dorsal region of the ventricular zone (Figure 9C, H) while expression of Pax2 is localised at the ventral end of the ventricular zone (Figure 9B, G), which supports observations made by Seto et al. (2014b). Both Tfap2A (Figure 9D, E) and Tfap2B (Figure 9I, J) are expressed in Olig2⁺ Purkinje cell progenitors and Pax2⁺ interneuron progenitors in the ventricular zone. Previous studies have reported that Olig2⁺ cells in the ventricular zone are derived from Ptf1a⁺ cells and hence represent mitotic GABAergic progenitor neurons (Ptf1a⁺) (Figure 9K) (Ju et al., 2016; Seto et al., 2014a). Co-immunostaining of Tfap2A and Tfap2B in this region showed colocalisation of Tfap2A⁺ and Tfap2B⁺ cells mostly in the white matter with some cells

remaining in the ventricular zone (Figure 9L). Thus, according to the spatial expression, this suggests the sequential expression of *Ptf1a* and *Tfap2* in the developing cerebellum. In addition, overlapping colocalisation of *Tfap2A* and *Tfap2B* is observed, which further implies that *Tfap2A*⁺ and *Tfap2B*⁺ cells mark distinct GABAergic neuronal subtypes.

Figure 9. Both Tfap2A and Tfap2B are expressed in GABAergic progenitors and post-mitotic GABAergic neurons in the developing cerebellum.

(A-E) Tfap2A in the developing cerebellum at E12.5 is expressed in subsets of Olig2⁺ GABAergic progenitors and Pax2⁺ GABAergic interneuron progenitors. Tfap2A⁺ cells (A)

are localised in the white matter (WM) from dorsal to ventral. Dorsal and ventral ends of the ventricular zone (VZ) can be marked by Olig2 (C, H) and Pax2 (B, G) respectively. (D-E) Cropped images of Tfap2A colocalisation with Olig2 (D), which marks Purkinje cell progenitors, show overlapping expression of Tfap2A while colocalisation with Pax2 (E), which marks GABAergic interneuron progenitors, show that all Pax2⁺ cells express Tfap2A. (F-J) Expression of Tfap2B is observed in all Olig2⁺ Purkinje cell progenitors and Pax2⁺ GABAergic interneuron progenitors. (L) Consequently, partial colocalisation of Tfap2A and Tfap2B is observed in the VZ region. Olig2 is expressed by both proliferating Ptf1a⁺ GABAergic progenitors (K) and Lhx1/5⁺ post-mitotic GABAergic neurons in the VZ (O, R). (M-R) Tfap2A (M-O) and Tfap2B (P-R) are expressed by Lhx1/5⁺ cells, which mark post-mitotic GABAergic progenitors in the WM. For figure A-L, filled arrowhead represents colocalisation, empty arrowhead represents no colocalisation. For figure M-R, filled arrowhead represents colocalisation of Tfap2 with Olig2 and Lhx1/5, empty arrowhead represents colocalisation of Tfap2 with Olig2 only. Scalebar = 100 μ m (A-C, F-H), 10 μ m (D, E, I, J, K-R).

We next investigated whether these transcription factors colocalise with Lhx1/5, an early marker for post-mitotic GABAergic neurons (Morales and Hatten, 2006; Zhao et al., 2007). Studies have also reported that Olig2⁺ cells in the ventricular zone can also represent post-mitotic GABAergic neurons (Lhx1/5⁺) (Figure 9O, R) (Ju et al., 2016; Seto et al., 2014a). In the ventricular zone, Tfap2A⁺ cells that are Olig2-positive are also positive for Lhx1/5 (Figure 9N-P) confirming that Tfap2A⁺ cells in the ventricular zone are early post-mitotic GABAergic neurons. In contrast, not all of Tfap2B⁺ cells are positive for Lhx1/5 (Figure 9Q-S), which suggests that Tfap2B⁺ cells in the ventricular zone consist of Olig2⁺/Lhx1/5⁻ mitotic as well as Olig2⁺/Lhx1/5⁺ early post-mitotic GABAergic progenitors. Therefore, this suggests that Tfap2A⁺ and Tfap2B⁺ cells at E12.5 may mark for GABAergic progenitor neurons which are destined for a Purkinje cell fate.

To assess the subtypes of these post-mitotic GABAergic progenitors, we examined the expression of $ROR\alpha$, an early marker for Purkinje cells (Ino, 2004; Nakagawa et al., 1997), and Pax2 at E15.5 (Figure 10). Our results show that Tfap2A marks subsets of $ROR\alpha^+$ Purkinje cells and Pax2⁺ interneurons (Figure 10A-D). But on the other hand, Tfap2B marks only Pax2⁺ interneurons (Figure 10E-H) indicating that Tfap2B is not expressed in Purkinje cells at later stages. Therefore, we wonder if Tfap2B has a suppressive role on Olig2 that prevents Purkinje cell specification or if Olig2 can also induce interneuron specification and not only Purkinje cells. Thus, we prefer to refer to Olig2⁺ cells as mitotic and post-mitotic GABAergic neuron progenitors instead of Purkinje cell progenitors. In summary, our results suggest that Tfap2A and Tfap2B delineate distinct subtypes of cerebellar GABAergic neurons (see summary Figure 13).

Figure 10. Tfap2A labels a subset of Purkinje cells while Tfap2A and Tfap2B label early Pax2⁺ interneurons.

(A-F) At E15.5, Tfap2A is expressed by a subset of ROR α ⁺ Purkinje cells (green) and Pax2⁺ interneurons (blue) (A-C) while Tfap2B is expressed by only Pax2⁺ interneurons (D-F). (G-L) At E18.5, the same Tfap2A⁺ and Tfap2B⁺ subpopulations persist, but with different distributions. (M) Quantification of three Tfap2A⁺ neuronal subpopulations (E15.5: Tfap2A⁺/ROR α ⁺=43 $\pm$ 3%, Tfap2A⁺/Pax2⁺=37 $\pm$ 2%, Tfap2A⁺-alone=20 $\pm$ 5%; E18.5: Tfap2A⁺/ROR α ⁺=35 $\pm$ 1%, Tfap2A⁺/Pax2⁺=42 $\pm$ 3%, Tfap2A⁺-alone=23 $\pm$ 4%). (N) Quantification of two Tfap2B⁺ neuronal subpopulations (E15.5: Tfap2B⁺/Pax2⁺=54 $\pm$ 3%, Tfap2B⁺-alone=42 $\pm$ 3%; E18.5: Tfap2B⁺/Pax2⁺=86 $\pm$ 3%, Tfap2B⁺-alone=14 $\pm$ 3%). (O, P) Analysis of Pax2⁺ neuronal population indicates that most Pax2⁺ interneurons express Tfap2A (E15.5: 97 $\pm$ 1%, E18.5: 89 $\pm$ 4%) and Tfap2B (E15.5: 88 $\pm$ 4%, E18.5: 90 $\pm$ 2%) at both E15.5 and E18.5. Analysis performed on cerebellar vermal region of sagittal sections. Continuous circles indicate colocalization of Tfap2 with ROR α and broken circles indicate colocalization of Tfap2 with Pax2. Values represent mean $\pm$ SEM, n =3 mice, 100-1500 cells, 2 sections per mouse, * p $\leq$ 0.05, ** p $\leq$ 0.01. Scale bar = 100 μ m (A, D, G, J), 10 μ m (cropped images).

3.2.3. Expression of Tfap2A and Tfap2B specifies interneuron cell fate

To explore the role of Tfap2A and Tfap2B during cerebellar neurogenesis, we first examined in detail the relationship between members of the Tfap2 family and Pax2. At E15.5, Tfap2A population is made up of three sub-populations, Tfap2A⁺/Pax2⁺, Tfap2A⁺/ROR α ⁺ and Tfap2A⁺ alone (Figure 10A-C). These sub-populations are also observed at E18.5 (Figure 10G-I), but there is a slight shift in the percentages of these sub-populations, although insignificant. The percentage of Tfap2A⁺/Pax2⁺ neurons increased by 5% while neurons that are Tfap2A⁺-alone decreased by 3% (Figure 10M) suggesting that cells that were previously Tfap2A⁺-alone have become Tfap2A⁺/Pax2⁺ interneurons. There is also no significant change in the percentage of Tfap2A⁺/ROR α ⁺ neurons (Figure 10M), which is expected since the generation of Purkinje cells ends at E12.5 (Chizhikov et

al., 2006; Minaki et al., 2008). Tfap2B population, on the other hand, consists of two sub-populations, Tfap2B⁺/Pax2⁺ and Tfap2B⁺-alone (Figure 10D-F, J-L). A significant increase in the number of Tfap2B⁺/Pax2⁺ neurons from 54% to 86% is observed ($p \leq 0.01$) and concomitantly, cells that are Tfap2B⁺-alone decreased from 42% to 14% (Figure 10N). At every developmental stage we examined (E12.5, E15.5 and E18.5), we observe that almost all Pax2⁺ interneurons are Tfap2A⁺ (Figure 9E, 10A-C, G-I) and Tfap2B⁺ (Figure 9J, 10D-F, J-L). Approximately 92% of Pax2⁺-interneurons are Tfap2A⁺ (Figure 10O) and 89% are Tfap2B⁺ (Figure 10P) at E15.5 and E18.5. Together, our results indicate that Tfap2A and Tfap2B are expressed by GABAergic progenitors committed for interneuron cell fate, and Tfap2A is expressed by progenitors committed for Purkinje cell fate.

3.2.4. *Tfap2A forms embryonic clusters consisting of Purkinje cells*

As previously described, in addition to interneurons, Tfap2A is also expressed by ROR α ⁺ Purkinje cells at E15.5 (Figure 10B). To date, there are approximately ten embryonic clusters of Purkinje cells being reported, which include, Zebrin II and calbindin (Baader et al., 1999; Dastjerdi et al., 2012; Larouche and Hawkes, 2006; Wassef et al., 1985). There are two groups of embryonic cluster markers, early-onset Purkinje cell marker, which disperse its clusters after birth, and late-onset Purkinje cell cluster marker, which form parasagittal stripes in the adult cerebellum (Dastjerdi et al., 2012; Larouche and Hawkes, 2006). To add to this list, Tfap2A⁺ cells also form clusters in the developing cerebellum during embryogenesis (Figure 8D, 10H). This is first observed at E18.5 (Figure 10G-I) which persists till early postnatal (Figure 11A-C). In the medial section of P0 cerebellum, a cluster is observed in the central lobe, which colocalised with ROR α (Figure 11A) and partially with calbindin (Figure 11Bi), an early-onset Purkinje cell cluster marker (Bastianelli, 2003; Wassef et al., 1985). The number of Tfap2A clusters increases laterally and in the most lateral sections, three clusters are detected, in the interior lobe (INL), central lobe (CEL) and anterobasal lobe (ABL) (Figure 11C). Although defined Tfap2A clusters are not observed in other lobes, weak Tfap2A expression is detected (Figure 11Aii, Bii). Despite the weak expression, most, if not all, of the Tfap2A⁺ cells in the Purkinje layer colocalised with ROR α (Figure 11A). However, not all Tfap2A⁺ cells expressed calbindin (Figure 11Bi), and not all calbindin⁺ cells expressed Tfap2A (Figure 11Bii) thus indicating

that Tfap2A labels a distinct cluster of Purkinje cells. But like any other early-onset Purkinje cell cluster marker, Tfap2A clusters dispersed during early postnatal and their expression is found homogenously in all Purkinje cells throughout the Purkinje layer (Figure 11D-F). Therefore, these results suggest that Tfap2A is a novel early-onset Purkinje cell cluster marker.

Figure 11. Tfap2A is expressed in the postnatal cerebellum in some Purkinje cells while Tfap2A and Tfap2B are expressed by migrating interneurons throughout the developing cerebellum.

Tfap2A represents one of the embryonic Purkinje cell cluster markers like calbindin (CBP) and ZebrinII. (A-B) Defined Tfap2A cluster is observed in the central lobe (CEL) in the most medial region of the cerebellum. (Ai, Bi) Cropped images of Tfap2A cluster in the CEL show perfect colocalisation with RORα (Ai) but partial colocalisation with CBP (Bi). (Aii, Bii) Although Tfap2A expression is most defined in CEL, low Tfap2A expression is still observed throughout all lobes and colocalises with RORα and CBP. (C) Sagittal

sections are analysed from medial to lateral and increasing number of clusters are observed laterally. (D, E) At P10, Tfap2A clusters dispersed into single layer of Purkinje cells and colocalise with ROR α (D), CBP (E) and PVA (F). Tfap2A⁺ cells are also observed in the IGL and colocalise with Pax2⁺ interneuron progenitor marker (F). (H) In the mature cerebellum, Tfap2A⁺ cells coexpress parvalbumin in the ML and PL, and Pax2 in the IGL. (G, I) Expression of Tfap2B is not detected in PVA⁺ Purkinje cells in P10 and adult cerebellum, but colocalises with Pax2 in the IGL. Abbreviations: ABL, anterobasal lobe; ADL, anterodorsal lobe; INL, interior lobe; POL, posterior lobe. Scalebar = 100 μ m (A, B, D, E), 10 μ m (F-I).

3.2.5. The expression of Tfap2 family members define distinct GABAergic neuronal subtypes

Analysis of the expression of Tfap2 family members in the postnatal cerebellum provides additional support that they label GABAergic neuronal sub-populations. At P10, some cerebellar neurons have settled in their respective locations while others are still migrating (Leto et al., 2009; Maricich and Herrup, 1999; Yamanaka et al., 2004; Zhang and Goldman, 1996). The expression of Tfap2A is observed in various regions of the cerebellar cortex: in the molecular layer where stellate and basket cells reside, in the Purkinje layer where Purkinje cells settle and in the inner granular layer (Figure 11D, E). In the Purkinje layer, Tfap2A expression colocalised with ROR α (Figure 11D) and calbindin (Figure 11E), indicating that Purkinje cells expressed Tfap2A. Tfap2A expression also colocalised with parvalbumin, a marker for functional stellate, basket and Purkinje cells (Bastianelli, 2003), but only in the Purkinje layer and not in the molecular layer (Figure 11F). The absence in Tfap2A and parvalbumin colocalisation in the molecular layer may suggest that stellate and basket cells are in immature state at P10. Unfortunately, we cannot use Pax2 to confirm the identity of molecular layer interneurons because these interneurons downregulate Pax2 expression upon reaching the molecular layer (Maricich and Herrup, 1999). The scattered distribution of Tfap2A expression in the inner granular layer likely consists of inner granular layer interneurons and migrating stellate and basket interneurons (Figure 11D-F). Analysis in the adult cerebellum shows coexpression of Tfap2A and parvalbumin in the

molecular layer and Purkinje layer and coexpression of Tfap2A and Pax2 in the inner granular layer (Figure 11H) suggesting that Tfap2A are expressed in stellate and basket cells, Purkinje cells and Golgi cells, respectively. On the other hand, expression of Tfap2B is only observed in the molecular layer and inner granular layer postnatally (Figure 11G, I). Colocalisation of Tfap2B with parvalbumin and Pax2 in the adult cerebellum (Figure 11I) indicates that Tfap2B is expressed in the molecular layer by stellate and basket cells and the inner granular layer by interneurons.

Figure 12. Tfap2A is selectively expressed by small GABAergic interneurons in the medial, interposed and lateral deep cerebellar nuclei.

(A-F) Small GABAergic interneurons can be marked by Gad67, an enzyme that catalyses GABA neurotransmitter, and MAP2, a cytoplasmic neuronal marker. Co-staining with Tfap2A (A-C) and Tfap2B (D-F) demonstrate that only Tfap2A are expressed in the deep cerebellar nuclei (DCN) in small GABAergic interneurons. Scalebar = 10 μ m.

To determine if Tfap2A and Tfap2B are expressed outside the cerebellar cortex, we examined their expression in the deep cerebellar nuclei in the adult cerebellum. Only Tfap2A expression (Figure 12A-C), but not Tfap2B (Figure 12D-F), is detected in the medial, interposed and lateral nuclei in the deep cerebellar nuclei. In all three nuclei, Tfap2A is expressed by small Gad67⁺ neurons (Figure 12A-C). Tfap2A also colocalised with Pax2 in the deep cerebellar nuclei (data not shown), which also indicates that Tfap2A exclusively labels small GABAergic interneurons. Expression of Tfap2A is not found in the rhombic lip and nuclear transitory zone during developmental stages, regions where glutamatergic deep cerebellar nuclei neurons are born (Figure 8A-F) thus, supporting the GABAergic origin of Tfap2A⁺ neurons. To date, we are the first to report the expression of Tfap2A in GABAergic interneurons in the deep cerebellar nuclei besides Pax2, Gad67 and GlyT2 (Leto et al., 2006; Maricich and Herrup, 1999; Uusisaari and Knopfel, 2011).

Figure 13. Summary of Tfap2A and Tfap2B expression patterns in the developing cerebellum.

(A) Expression of both Tfap2A and Tfap2B can be detected as early as E12.5 in the developing cerebellum. The ventricular zone where GABAergic neurons are being produced can be marked by Olig2 expression (in black). Intermediary zones show colocalisation of Olig2 with Ptf1a, a marker for mitotic GABAergic progenitors (in shades of grey) and with Lhx1/5⁺, a marker for GABAergic post-mitotic neurons (in shades of blue). (B) Tfap2A is expressed by half of the Olig2-expressing neurons which are post-mitotic. (C) Tfap2B, on the other hand, is expressed by all the Olig2-expressing neurons, which are mitotic and post-mitotic. (D) As development proceeds, Tfap2A⁺ cells express either RORα or Pax2, markers for Purkinje cells and GABAergic interneurons, respectively.

However, Tfap2B⁺ cells only express Pax2. Thus, this suggests that expression of Tfap2A and Tfap2B precede Pax2 expression.

3.3. Investigating the role of Tfp2A and Tfp2B by genetic manipulation *in vivo*

3.3.1. Testing the efficiency of lentivectors and lentivirus

To examine the role of transcription factors *Tfap2A* and *Tfap2B*, we set out to manipulate their expression at various developmental stages. The knockdown construct (k/d) consists of a dual promoter, mouse *RNA polymerase II U6 (U6)* to drive the expression of short hairpin RNA (shRNA) followed by *cytomegalovirus (CMV)* to drive the expression of green fluorescent protein (GFP) (Figure 14A). A scrambled sequence (scr), which does not target any specific region of the mouse genome, is used as a control in the knockdown experiments. The overexpression construct (OE) is made up of *elongation factor 1 (EF1)* promoter that coexpresses *Tfap2A* or *Tfap2B* complementary DNA (cDNA) and red fluorescent protein (RFP) (Figure 14B). The cDNA and RFP sequences are separated by Internal Ribosomal Entry Site (IRES) to allow independent translation of the two proteins (Figure 14B). In a control experiment, an empty vector (EV), which does not contain any cDNA, is used.

Figure 14. Lentivectors are efficient and specific in knocking down respective Tfap2 family members.

(A-B) Knockdown and overexpression constructs used are driven by constitutive promoters, mouse *RNA polymerase II U6* (*U6*) and *elongation factor 1* (*EF1*), respectively. (A) The knockdown vector consists of two promoters, *U6* to drive the expression of short hairpin RNA (shRNA) and *cytomegalovirus* (*CMV*) to drive the expression of green fluorescent protein (GFP) marker. (B) The overexpression vector incorporates Internal Ribosomal Entry Site (IRES) to coexpress red fluorescent protein (RFP) marker with gene of interest. (C) Knockdown and overexpression vectors were co-transfected into human embryonic kidney (HEK)-293T cells and proteins were isolated to investigate the efficiency and specificity of constructs. (D) Lentivirus particles transduced into HEK-293T cells and primary cerebellar neuronal culture show ectopic expression of Tfap2A and Tfap2B respectively. (E-F) In *in utero* electroporation experiments, plasmids were delivered into the fourth ventricle of E12.5 embryos using glass pipettes and embryos were electroporated for five pulses at 250mV for 50 msec each. Electrodes were placed at approximately 45° angle to transfect a larger area of the ventricular zone (F) and plasmids embryos were incubated for three days (E). (G) Electroporation at the fourth ventricle (4v) transfects cells along the ventricular zone (VZ) and the rhombic lip (RL). Upon three days post-transfection, cells transfected at the ventricular zone migrate upwards into the white matter (WM) (yellow arrow) while cells transfected at the rhombic lip migrate along the external germinal layer (EGL) before descending into the white matter (red arrow). Abbreviation: cDNA, complementary DNA; EV, empty vector; ICC, immunocytochemistry; k/d, knockdown; LV, lentivirus; OE, overexpression; scr, scramble. Scalebar = 100µm (G).

Human embryonic kidney (HEK)-293T cells were used to test if both the knockdown and overexpression constructs are efficient and specific in knocking down or expressing Tfap2A or Tfap2B. Three shRNA sequences were designed and cloned, 2Ash1, 2Ash2 and 2Ash3 against Tfap2A and 2Bsh2, 2Bsh5 and 2Bsh6 against Tfap2B. Since HEK-293T cells did not endogenously express Tfap2A or Tfap2B, coexpression was required to test for knockdown efficiency. Each shRNA was co-transfected with either

Tfap2A or Tfap2B into HEK-293T cells. Western blot analysis on the proteins isolated showed that all Tfap2A shRNAs and Tfap2B shRNAs are efficient in knocking down Tfap2A and Tfap2B respectively (Figure 14Ci, 14Ciii). To test if the shRNAs were specific in targeting a specific Tfap2 family member, Tfap2A shRNAs were co-transfected with Tfap2B overexpression construct while Tfap2B shRNAs were co-transfected with Tfap2A overexpression construct. Western blot analysis showed that most of the constructs designed to knockdown Tfap2A expression are specific in suppressing Tfap2A expression but not Tfap2B (Figure 14Cii). Similarly, constructs designed to knockdown Tfap2B are also specific in targeting Tfap2B (Figure 14Civ). Thus, knockdown constructs Tfap2Ash2 and Tfap2Bsh2 and overexpression constructs Tfap2A and Tfap2B were used for genetic manipulation experiments, such as *in utero* electroporation experiments.

To manipulate gene expression in early postnatal or adult mouse, we employed the lentivirus technology. HEK-293T and primary cerebellar neuronal cultures were used to test the efficiency of overexpression lentivirus. Upon misexpressing Tfap2A or Tfap2B in culture, most, if not all, RFP⁺ cells express Tfap2A or Tfap2B respectively (Figure 14D). As mentioned previously, HEK-293T cells do not endogenously express Tfap2A nor Tfap2B while only a very small percentage of cells in primary cerebellar culture express Tfap2A or Tfap2B. Thus, we are unable to test the efficiency of shRNA lentiviral vector. We are also unable to carry out co-transduction in culture because HEK-293T cells are resistant to simultaneous lentivirus infection. Nevertheless, we showed that the lentiviral constructs can suppress Tfap2A and Tfap2B efficiently and specifically, and should not differ much when the same constructs are packaged into lentivirus.

3.3.2. Misexpression of Tfap2A and Tfap2B at E12.5 changes Pax2 and Pax6 population respectively

To investigate the role of Tfap2A and Tfap2B during early embryogenesis, plasmid constructs were delivered into the fourth ventricle of E12.5 wild-type embryo and electroporated to transfect cells lining the ventricular zone of the cerebellum (Figure 14E-F). Although attempts were made to transfect cells originating from the ventricular zone, a number of cells from the rhombic lip were also transfected. Embryos were harvested three days later at E15.5 and analysed for several molecular markers. In a wild-type embryo,

neurons that originate from the ventricular zone migrate in an anterior manner into the white matter while neurons that originate from the rhombic lip migrate in a tangential manner over the cerebellar surface before descending into the white matter (Figure 14G) (Chung et al., 2010; Lumpkin et al., 2003; Machold and Fishell, 2005). Therefore, molecular profile of transfected cells in the white matter using immunohistochemistry was carried out to determine the cell fate of the cells transfected at the ventricular zone. Results are presented as the percentage of the number of transfected cells expressing the molecular marker out of total number of transfected cells (marker/GFP or marker/RFP %) for comparison between experimental groups. In a control experiment, RFP-only control or empty vector control (Figure 14Bi) was used.

In RFP-only transfected cells, ~30% of transfected cells are Tfap2A-positive and ~18% are Tfap2B-positive (Figure 16A-H). Experimental expression of Tfap2A increases the percentage of Tfap2A/RFP⁺ population significantly by almost three-fold ($p \leq 0.01$; Figure 15A, B, D), without affecting the expression of Tfap2B (Figure 15E, F, H). Similarly, the experimental expression of Tfap2B increases the percentage of Tfap2B/RFP⁺ population by four-fold ($p \leq 0.01$; Figure 15E, G, H), but not Tfap2A/RFP⁺ population (Figure 15A, C, D). Therefore, misexpression of Tfap2A or Tfap2B *in utero* results in the specific expression of Tfap2A and Tfap2B, respectively.

Figure 15. Overexpression of Tfap2A in the ventricular zone at E12.5 results in an increase of Pax2⁺ neurons.

RFP-only/empty vector control, Tfap2A-overexpression or Tfap2B-overexpression constructs were introduced into neural precursors in the ventricular zone of E12.5 wild-type embryos. Sagittal cerebellar sections were analysed at E15.5 and the number of transfected cells (in green) expressing the respective molecular markers (in red) were counted. Ectopic expression of Tfap2A, but not Tfap2B, results in an increase in Tfap2A⁺ cells (Tfap2A: 84±6% vs 30±4% in controls, $p \leq 0.01$; A, B, D; Tfap2B: 39±5% vs 30±4% in controls,

$p=0.202$; A, C, D; control: $n=3$ mice, 372 cells in 17 sections; 2A OE: $n=3$ mice, 276 cells in 12 sections; 2B OE: $n=3$ mice, 326 cells in 10 sections). (E-H) Similarly, ectopic expression of Tfap2B, but not Tfap2A, results in an increase in Tfap2B⁺ cells (Tfap2B: $82\pm5\%$ vs $18\pm3\%$ in controls, $p\leq0.01$; E, G, H; Tfap2A: $29\pm10\%$ vs $18\pm3\%$ in controls, $p=0.483$; E, F, H; control: $n=3$ mice, 276 cells in 12 sections; 2A OE: $n=3$ mice, 97 cells in 8 sections; 2B OE: $n=3$ mice, 366 cells in 13 sections). (I-T) Consequences of Tfap2A and Tfap2B misexpression on generation of cerebellar neuronal subpopulations. Misexpression of Tfap2A results in an increase in the number of Pax2⁺ cells ($54\pm11\%$ vs $26\pm5\%$ in controls, $p\leq0.01$; I, J, L; control: $n=3$ mice, 347 cells in 13 sections; 2A OE: $n=3$ mice, 130 cells in 14 sections), but misexpression of Tfap2B did not change the number of Pax2⁺ cells compared to controls ($24\pm6\%$ vs $26\pm5\%$ in controls, $p=0.88$; I, K, L; control: $n=3$ mice, 347 cells in 13 sections; 2B OE: $n=3$ mice, 212 cells in 12 sections). Misexpression of Tfap2B, but not Tfap2A, results in a decrease in the number of Pax6⁺ cells (Tfap2B: $1\pm1\%$ vs $16\pm3\%$ in controls, $p\leq0.01$; M, O, P; Tfap2A: $19\pm8\%$ vs $16\pm3\%$ in controls, $p=0.66$; M, N, P; control: $n=3$ mice, 542 cells in 14 sections; 2A OE: $n=3$ mice, 65 cells in 8 sections; 2B OE: $n=3$ mice, 337 cells in 13 sections). Misexpression of Tfap2A and Tfap2B has no impact on the number of CBP⁺ Purkinje cells (Tfap2A: $6\pm2\%$ vs $9\pm3\%$ in controls, $p=0.85$; Q, R, T; Tfap2B: $20\pm6\%$ vs $9\pm3\%$ in controls, $p=0.063$; Q, S, T; control: $n=3$ mice, 365 cells in 15 sections; 2A OE: $n=3$ mice 160 cells in 10 sections; 2B OE: $n=261$ cells in 9 sections). Continuous circles indicate lack of colocalization and broken circles indicate colocalization of transfected cells with respective markers. Abbreviations: VZ, ventricular zone; WM, white matter. Values represent the mean $\pm$ SEM, * $p\leq0.05$, ** $p\leq0.01$. Scalebar = 10 μ m.

To investigate the cell fate of misexpressed neural precursors in the ventricular zone, we analyzed the expression of neuronal markers, Pax2, Pax6 and calbindin, which correspond to interneuron progenitors, granule cells and Purkinje cells, respectively (Bastianelli, 2003; Engelkamp et al., 1999; Maricich and Herrup, 1999). Tfap2A misexpression results in a two-fold increase in the percentage of Pax2⁺ interneurons in Tfap2A-misexpressed cells compared to control ($p\leq0.01$; Figure 15 I, J, L). However, no significant change in the percentage of Pax2⁺ interneurons in Tfap2B-misexpressed cells is

detected (Figure 15 I, K, L). Next, no change is observed in the number of Pax6⁺ granule cells in Tfap2A-misexpressed cells (Figure 15M, N, P) but a significant decrease of over ten-fold is observed in Tfap2B-misexpressed cells when compared to control ($p \leq 0.01$; Figure 15 M, O, P). Additionally, misexpression of Tfap2A or Tfap2B had no impact on calbindin⁺ Purkinje cells (Figure 15 Q-T). Together, we show that the experimental expression of Tfap2A, but not Tfap2B, in the ventricular zone at E12.5 results in an increase in the number of Pax2⁺ interneurons. Conversely, the experimental expression of Tfap2B, but not Tfap2A, results in a decrease in Pax6⁺ population.

3.3.3. Knockdown of Tfap2B in the ventricular zone at E12.5 affects Tfap2A expression

Before assessing the consequences of knockdown Tfap2A or Tfap2B, we analysed the efficiency of knockdown constructs on Tfap2 family members. The knockdown of Tfap2B expression not only results in the suppression of Tfap2B expression ($p \leq 0.01$; Figure 16 E, G, H), but also reduces the expression of Tfap2A ($p \leq 0.05$; Figure 16 A, C, D). On the other hand, knockdown of Tfap2A suppresses only Tfap2A expression ($p \leq 0.01$; Figure 16 A, B, D), but not Tfap2B expression (Figure 16 E, F, H). Together we show that the knockdown of Tfap2A and Tfap2B effectively decreases the number of Tfap2A⁺ and Tfap2B⁺ cells, respectively. We also note that even though Tfap2A and Tfap2B do not induce the expression of each other, knocking down Tfap2B could influence the expression of Tfap2A.

Figure 16. The number of Pax2⁺ neurons decreases after Tfap2B knockdown in the cerebellar ventricular zone at E12.5.

GFP-only/scramble control, Tfap2Ash2, or Tfap2Bsh2 knockdown constructs were introduced in the cerebellar ventricular zone (VZ) at E12.5 and analysed at E15.5. Tfap2A-knockdown cells, and to some extent Tfap2B-knockdown cells, abolishes Tfap2A expression (Tfap2A: 3±2% vs 33±3% in controls, $p \leq 0.01$; A, B, D; Tfap2B: 18±4% vs 33±3% in controls, $p \leq 0.01$; A, C, D; control: $n=3$ mice, 115 cells in 11 sections; 2A k/d: $n=3$ mice, 187 cells in 14 sections; 2B k/d: $n=3$ mice, 391 cells in 22 sections). Tfap2B-

knockdown cells, but not Tfap2A-knockdown cells, reduced Tfap2B expression (Tfap2B: $1\pm1\%$ vs $22\pm6\%$ in controls, $p\leq 0.01$; E, G, H; Tfap2A: $22\pm7\%$ vs $22\pm6\%$ in controls, $p=0.7359$; E, F, H; control: $n=3$ mice, 121 cells in 11 sections; 2A k/d: $n=3$ mice, 176 cells in 12 sections; 2B k/d: $n=3$ mice, 192 cells in 12 sections). (I-P) Knockdown of Tfap2A expression in the VZ does not affect the number of Pax2⁺ cells ($22\pm6\%$ vs $27\pm4\%$ in controls, $p=0.53$; I, J, L; control: $n=3$ mice, 153 cells in 12 sections; 2A k/d: $n=3$ mice, 87 cells in 14 sections) or number of Pax6⁺ cells compared to controls ($27\pm8\%$ vs $34\pm4\%$ in controls, $p=0.29$; M, N, P; control: $n=3$ mice, 241 cells in 13 sections; 2A k/d: $n=3$ mice, 188 cells in 13 sections). However, knockdown of Tfap2B expression results in a significant decrease in number of Pax2⁺ cells ($13\pm3\%$ vs $27\pm4\%$ in controls, $p\leq 0.01$; I, K, L; control: $n=3$ mice, 153 cells in 12 sections; 2B k/d: $n=3$ mice, 391 cells in 26 sections), without affecting the number of Pax6⁺ cells ($22\pm4\%$ vs $34\pm4\%$ in controls, $p=0.054$; M, O, P; control: $n=3$ mice, 241 cells in 13 sections; 2B k/d: $n=3$ mice, 211 cells in 12 sections). (Q-T) A scant percentage of CBP⁺ Purkinje cells are transfected, although knockdown of Tfap2B, but not Tfap2A, slightly decreased this subpopulation (Tfap2B: $0.5\pm0.5\%$ vs $4\pm1\%$ in controls, $p=0.0064$; Q, S, T; Tfap2A: $2\pm1\%$ vs $4\pm1\%$ in controls, $p=0.067$; Q, R, T; control: $n=3$ mice, 307 cells in 13 sections; 2A k/d: $n=3$ mice, 206 cells in 14 sections; 2B k/d: $n=3$ mice, 253 cells in 13 sections). Continuous circles indicate lack of colocalization and broken circles indicate colocalization of transfected cells with respective markers. Abbreviations: VZ, ventricular zone; WM, white matter. Values represent the mean $\pm$ SEM, * $p\leq 0.05$, ** $p\leq 0.01$. Scalebar = 10 μ m.

3.3.4. *Tfap2B is required for the specification of Pax2-positive interneurons*

To determine the effects of Tfap2A or Tfap2B knockdown, we examined the status of Pax2, Pax6 and calbindin expression in the transfected cells. We observed a two-fold decrease in the percentage of Pax2⁺ interneurons in Tfap2B-knockdown cells when compared to control (Figure 16 I, K, L). No change in Pax2 population is detected in Tfap2A-knockdown cells (Figure 16 I, J, L). There is also no change in Pax6 population upon Tfap2A or Tfap2B knockdown (Figure 16 M-P). In addition, Tfap2A knockdown also

does not alter calbindin population. However, knockdown of Tfap2B results in a slight decrease in calbindin⁺ Purkinje cells when compared to control ($p=0.0064$; Figure 16 Q, S, T). Although this is approximately an eight-fold decrease, this observation may not be meaningful since Purkinje cells do not typically express Tfap2B. Moreover, calbindin⁺ Purkinje cells are scarcely transfected in a control experiment and hence our results suggest that Tfap2A and Tfap2B are unable to affect Purkinje cell population. Therefore, our results demonstrate that knockdown of Tfap2B in the VZ at E12.5 results in a decrease in Pax2 expression, indicating that Tfap2B is essential in the specification of cerebellar GABAergic interneurons.

3.3.5. *Ptf1a* does not influence the expression of *Tfap2A* and *Tfap2B* in the cerebellum

To investigate whether *Ptf1a* can induce the expression of *Tfap2A* and *Tfap2B* similar to the retina, neural tube, spinal cord or cerebral cortex (Borromeo et al., 2014; Jin et al., 2015; Meredith et al., 2013; Russ et al., 2015), we misexpressed *Ptf1a* in the cerebellum at E12.5 (Figure 17). To do this, we used a construct gifted by Julia Kaltschmidt (Memorial Sloan Kettering Cancer Center/Stanford University) which employs the *Cre-lox* system consisting of *CAG* promoter that drives the expression *Ptf1a* expression and *green fluorescent protein (GFP)* (Figure 17A). *loxP* sites flanking the stop codon, abbreviated as LSL, were placed upstream of *Ptf1a* cDNA and hence the presence of Cre recombinase enzyme is required to mediate recombination allowing the expression of *Ptf1a* and *GFP* (Figure 17A). In a control experiment, the plasmid construct, LSL-GFP, which does not carry any cDNA, should not express any ectopic proteins other than GFP (Figure 7Ai). In this experiment, we have employed two strategies, (1) electroporation of single plasmid into *Nestin^{Cre/+}* embryos and (2) co-electroporation of either LSL-GFP or LSL-*Ptf1a* plasmid along with Cre-expressing plasmid driven by *EF1* promoter (*pEF1-Cre*) into wild-type embryos. Similar to earlier experiments, plasmids were incubated for three days and the cerebellum was analysed at E15.5 (Figure 14E).

Figure 17. Tfap2A and Tfap2B expression is not influenced by Ptf1a expression.

(A) Constructs used to express of *Ptf1a* employs a *Cre-lox* strategy driven by *CAG* promoter. A stop codon flanked by *loxP* sites was designed upstream of *Ptf1a* cDNA. Downstream of *Ptf1a* cDNA, *internal ribosomal entry site (IRES)* sequence allows independent translation of *green fluorescent protein (GFP)*. LSL-GFP control is used as a control (Ai) and does not express any ectopic protein other than GFP marker. In this experiment, either cerebelli of *Nestin^{Cre/+}* embryos or wild-type embryos were

electroporated at E12.5 and analysed at E15.5. In cases where wild-types were used, embryos were co-electroporated with Cre-expressing plasmid driven by *EF1* promoter. (B-D) Upon *Ptf1a* misexpression, no significant changes in *Tfap2A* expression in *Ptf1a*-misexpressed cells were observed ($68 \pm 6\%$ vs $47 \pm 12\%$ in controls, $p=0.49$; controls: $n=2$ mice, 131 cells in 6 sections; *Ptf1a*: $n=3$ mice, 173 cells in 6 sections). (E-G) No changes in *Tfap2B* expression in *Ptf1a*-misexpressed cells were also observed when compared to LSL-GFP controls ($33 \pm 5\%$ vs $27 \pm 7\%$ in controls, $p=0.47$; controls: $n=2$ mice, 284 cells in 10 sections; *Ptf1a*: $n=3$ mice, 210 cells in 9 sections). Continuous circles indicate lack of colocalization and broken circles indicate colocalization of transfected cells with respective markers. Abbreviations: VZ, ventricular zone; WM, white matter. Values represent the mean $\pm$ SEM, * $p\leq 0.05$, ** $p\leq 0.01$. Scale bar = 10 μ m.

Surprisingly, we did not observe significant changes in the number of *Tfap2A*⁺ and *Tfap2B*⁺ cells when we experimentally expressed *Ptf1a*, as compared to controls (Figure 17 B-G). In addition, costaining with other molecular markers such as Pax2, Pax6 and calbindin also did not show any difference between control and mis/overexpression of *Ptf1a* (data not shown). Together, this result suggests that the expression of *Tfap2A* and *Tfap2B* in the cerebellum is not dependent on the expression of *Ptf1a* like other regions of the nervous system (Borromeo et al., 2014; Jin et al., 2015; Meredith et al., 2013; Russ et al., 2015).

3.3.6. Genetic manipulation of Tfap2A and Tfap2B in postnatal cerebellar neurons does not affect GABAergic phenotype of cerebellar neurons

We explored the function of *Tfap2A* and *Tfap2B* in the postnatal cerebellum by manipulating gene expression in primary cerebellar neuronal cultures *in vitro*. Since primary cerebellar neuronal cultures consisted mostly of excitatory granule cells, we misexpressed *Tfap2A* and *Tfap2B* individually using lentivirus to test their ability to induce GABAergic phenotypes. Cerebelli of five-day old pups were cultured and transduced with virus after one day *in vitro* (DIV). Cells were fixed approximately 14 days post-infection and analysed for several GABAergic markers such as Gad67 and VIAAT, vesicular

inhibitory amino acid transporter. However, we observed no changes in both Gad67 (Figure 18) and VIAAT (data not shown) expression upon misexpression of Tfap2A or Tfap2B in culture. This suggests that transcription factors Tfap2A and Tfap2B are not responsible for GABAergic neuron phenotype postnatally *in vitro*. Alternatively, this may indicate that the lentivirus infection did not sufficiently misexpress Tfap2A and Tfap2B to cause any modifications to the infected neurons. Manipulation of Tfap2A and Tfap2B in the postnatal cerebellum *in vivo* also did not yield any insight to the ability of Tfap2A and Tfap2B to induce GABAergic neuron phenotypes. The primary reason is that even though lentiviral infection in the adult cerebellum transduced various neurons in the cerebellar cortex such as Purkinje, stellate and basket cells (Figure 18I), the expression of Gad67 is highly variable in control experiments. The expression of Gad67 is observed in some cells (Figure 18J, K) but not in others (Figure 18L). Thus, it is an unreliable strategy to assess the consequences of manipulating Tfap2A and Tfap2B on Gad67 expression.

Figure 18. Manipulation of Tfap2A and Tfap2B expression in primary cerebellar neuronal culture and postnatal cerebellum *in vivo* did not influence neurotransmitter phenotype.

(A-F) Primary neuronal cultures were infected with lentivirus expressing empty vector (EV) (A, B), Tfap2A (C, D) and Tfap2B (E, F), and tested for Gad67, enzyme responsible for catalysing GABA neurotransmitter. Comparison between controls and overexpression shows no significant differences upon misexpression of Tfap2A or Tfap2B (Tfap2A: $40 \pm 10\%$ vs $49 \pm 9\%$ in controls, $p=0.52$; A-D, G; Tfap2B: $48 \pm 10\%$ vs $49 \pm 9\%$ in controls, $p=0.95$; A, B, E, F, H; controls: $n=3$ cultures, 287 cells; Tfap2A OE: $n=3$ cultures, 193 cells;

Tfap2B OE: $n=3$ cultures, 266 cells). (I-L) Cerebellum of wild-type adult mouse was infected with lentivirus expressing GFP construct at P90 and sacrificed thirty days later. Various cerebellar cell subtypes located in the molecular layer (ML), Purkinje layer (PL) and inner granular layer (IGL) express GFP. (J-L) Gad67 colocalisation analysis on infected cerebellum shows inconsistency of Gad67 staining in control tissue. A representative of a Purkinje cell (J) and molecular layer interneuron (K) show expression of Gad67 around the soma. However, in another interneuron, Gad67 expression is not observed around the cell body (L). Arrowhead represents colocalisation of Gad67 expression around the cell soma and along the processes of infected neurons. Values represent the mean $\pm$ SEM, * $p\leq 0.05$, ** $p\leq 0.01$. Scalebar = 10 μ m.

During the course of this thesis project, we also attempted to generate a conditional knockout *Tfap2B* mouse, which uses the *Cre-lox* system, in order to understand the role of *Tfap2B* during the development of cerebellar GABAergic neurons. Intended experiments include an assessment on the specification of GABAergic neuronal subtypes in the cerebellum of mouse lacking Tfap2B through Cre-mediated deletion using *PVA::Cre* (all molecular layer interneurons) (Bastianelli, 2003), *L7::Cre* (Purkinje cells) (Nordquist et al., 1988) or *mGluR2::Cre* (Golgi cells) (Meguro et al., 1999) mouse. *Tfap2B* gene locus which is found on chromosome 1 is edited via homologous recombination. The edited gene comprises of *loxP* sites flanking the functional exon 3 (Figure 19A). A homozygote mouse, also referred to as *Tfap2B^{Flox/Flox}* mouse, would require Cre recombinase enzyme to excise exon 3 of the *Tfap2B* gene and hence knocking out *Tfap2B*. For example, crossing a homologous *Tfap2B^{Flox/Flox}* mouse and *Nestin::Cre* mouse would remove *Tfap2B* gene in all Nestin-expressing neurons in the mouse. However, due to technical difficulties, we were unable to generate homozygote mouse. *In situ* hybridisation of heterozygote *Nestin::Cre*; *Tfap2B^{Flox/Flox}* cerebellum shows no difference in *Gad67* expression pattern (Figure 19E) and also *Tfap2B* expression (Figure 19C) when compared to the cerebellum of *Nestin^{Cre/+}* littermates (Figure 19B, D). This result suggests that a single functional copy of *Tfap2B* is sufficient for the specification of GABAergic neurons and their phenotypes.

Figure 19. GABAergic neuronal identity is not disrupted in the cerebellum of heterozygous *Tfap2B* mutant mice.

(A) Construct of conditional *Tfap2B* knockout using knock in-knock out approach. Cre-expressing mouse is required to mediate recombination and hence deletion of exon 3 in a conditional knockout *Tfap2B* mouse. (B-E) Assessment of *Nestin::Cre* control (B, D) and heterozygote *Nestin::Cre; Tfap2B^{Flox/+}* (C, E) cerebellum via *in situ* hybridisation show similar expression pattern of *Tfap2B* and *Gad67*. Scalebar =100μm.

4. DISCUSSION

GABAergic neurons in the cerebellum which consist of projection Purkinje cells and, Golgi, stellate and basket cells are derived from a common pool of multipotent progenitors (Hoshino et al., 2005; Leto et al., 2006; Yamada et al., 2014). Various studies have been carried out to identify the key factor responsible for the production of GABAergic progenitors. However, the molecular players that regulate the diversification of GABAergic neuronal subtypes is not well defined. In this thesis, we identified and explored the role of Tfap2 family transcription factors in defining GABAergic neuronal subtypes in the cerebellum (Figure 20).

4.1. Ptf1a, Tfap2A/B and Pax2: sequential expression for GABAergic interneurons in the developing cerebellum

Various studies have well-established the role of Ptf1a in the cerebellum which is necessary for the generation of GABAergic progenitors (Hoshino et al., 2005; Pascual et al., 2007; Yamada et al., 2014). In addition, the bHLH transcription factor is also responsible for the production of GABAergic neurons in the spinal cord, cochlear nucleus and retina (Fujitani et al., 2006; Hori and Hoshino, 2012). There is also an emerging evidence that GABAergic neuronal subtypes are defined by their unique expression of transcription factors (Achim et al., 2014; Helms and Johnson, 2003; Wonders and Anderson, 2006). In the cortex, for instance, GABAergic interneurons are derived from Nkx2.1 domain of the medial ganglionic eminence and ventral caudal ganglionic eminence (Cobos et al., 2006; Wonders and Anderson, 2006). Subsequently, GABAergic neuronal subtypes can be differentiated by their distinct combinatorial expression of Dlx1/2, Dlx5/6 and Lhx6 (Cobos et al., 2006; Wonders and Anderson, 2006). In more caudal regions of the nervous system, the differential expression of Lbx1, Lhx1/5, Pax2, Gsh1/2, Dbx2 and GATA2/3 defines GABAergic interneuron subtypes in the spinal cord; Lhx1/5 is expressed by all GABAergic neurons and Pax2 is expressed only by interneurons in the cerebellum (Helms and Johnson, 2003; Hori and Hoshino, 2012). However, in comparison to the cortex and spinal cord, there are fewer molecular markers that distinguish the GABAergic neuronal subtypes in the cerebellum.

We show in this study that the combinatorial expression of Tfap2A and Tfap2B may distinguish GABAergic projection neurons from interneurons in the developing cerebellum. In the adult cerebellum, Tfap2A is expressed in all GABAergic neurons, whereas Tfap2B is selectively expressed in the interneuronal population (Figure 10). In contrast to Ptf1a and Olig2, whose expression is extinguished by E13.5 (Pascual et al., 2007; Seto et al., 2014a; Ju et al., 2016), the expression of Tfap2 transcription factors persists into adulthood. Our discovery that Tfap2 transcription factors are dynamically and, perhaps, differentially expressed by GABAergic neuronal subtypes in the cerebellum, provides a novel set of molecular markers that can complement a growing list of genetic tools for future characterization and lineage analysis of these neurons. In addition, the sequential expression of Ptf1a and Tfap2A/B precedes the expression of Pax2 in the cerebellum, indicating that Tfap2A and Tfap2B may play a role in the specification of GABAergic neuronal subtypes. There is now a number of mouse lines for the tagging and monitoring of cerebellar GABAergic neurons: *Gad67::GFP* and *Ptf1a::Cre* label all GABAergic neurons (Yamanaka et al., 2004; Pascual et al., 2007; Yamada et al., 2014), *Corl2::GFP* and *Olig2::Cre* mark projection neurons (Nakatani et al., 2014; Seto et al., 2014b; Ju et al., 2016) and *Pax2::GFP* selectively labels GABAergic interneurons (Leto et al., 2006; Weisheit et al., 2006). The emergence of these tools will permit a systematic examination of the development, function as well as molecular profile of cerebellar GABAergic neuronal subtypes.

4.2. Tfap2A⁺ Purkinje cell clusters suggest an early establishment of topographical organization in the cerebellum

Previous studies have identified various early-onset Purkinje cell cluster markers, which are expressed homogeneously shortly after birth, and late-onset Purkinje cell cluster markers, which transform into parasagittal Purkinje cell stripes in the adult cerebellum (Dastjerdi et al., 2012; Larouche and Hawkes, 2006; Sillitoe et al., 2008; Wassef et al., 1985). Calbindin, an early-onset Purkinje cell cluster marker, is detected as early as E15.5 and expression persists into adulthood (Wassef et al., 1985). Like many of the other early-onset markers, calbindin clusters disappear early postnatally and expression is distributed in monolayer (Dastjerdi et al., 2012; Larouche and Hawkes, 2006). On the other hand, late-onset Purkinje cell cluster markers like ZebrinII, which is expressed around E18.5, continue

to form distinction in the postnatal cerebellum as parasagittal stripes (Sillitoe et al., 2009). These stripes, which are topographically mapped, are correlated to specific synaptic connections and perhaps responsible for a particular behavioural function (Apps and Garwicz, 2005; Sillitoe and Joyner, 2007).

In our study, we present *Tfap2A* as an early-onset Purkinje cell cluster marker, where expression is detected as early as E12.5 and persists till adulthood (Figure 8B-G). *Tfap2A* clusters are detected as early as E18.5 (Figure 8D) and colocalise with *ROR α* , an early Purkinje cell marker (Figure 10D, 10P, 11A). Interestingly, colocalisation with calbindin, an early-onset Purkinje cell cluster marker, shows an overlapping expression in the central lobe (Figure 11Bi). This suggests that *Tfap2A* marks subsets of Purkinje cells distinguishable from calbindin⁺ cell clusters. Detailed analysis on P10 and adult cerebellar cortex in lobule VI-VIII, which is derived from the central lobe, shows homogenous expression of *Tfap2A* (Figure 11D-F, H). This finding proposes that *Tfap2A* can bridge the gap between the late-onset Purkinje cell clusters that define adult Purkinje cell stripes. The onset of late-onset clusters such as *Zebrin II* is delayed six to eight days from Purkinje cell generation and the mechanisms regulating its expression is still unclear. Therefore, if we are able to employ genetic inducible fate mapping in mouse, using tamoxifen-dependant Cre recombinase, we may be able to trace Purkinje cells that are born at different timepoints and find the link between expression of early-onset clusters and late-onset clusters. For example, in the inducible *Cre-lox* system, when a Cre-expressing mouse under the promoter of *Tfap2A* crossed to a fluorescent reporter mouse is induced by Tamoxifen, cells that express *Tfap2A* at that point of time will express the fluorescent marker. This will allow tracking of cells and determine effect of *Tfap2A* on expression of late-onset clusters that forms adult Purkinje cell stripes which exhibit unique synaptic projections. Apart from that, it can also determine if the clusters of *Tfap2A* have unique birth dates as compared to other Purkinje cells. Therefore, *Tfap2A* serve as a potential candidate in studying the development of Purkinje cells in the cerebellum.

4.3. Distinct roles of Tfap2A and Tfap2B in the production of cerebellar GABAergic interneurons

Tfap2 family of transcription factors regulates a myriad of developmental processes in the nervous system, kidney, skeleton, limbs, skin and eye (Eckert et al., 2005; Moser et al., 1997; Nottoli et al., 1998; Schorle et al., 1996; Seberg et al., 2017; West-Mays et al., 1999; Zhang et al., 1996). Members of this family exhibit functional redundancy in zebrafish. For instance, Tfap2A and Tfap2E are redundant in their regulation of melanophore development (Van Otterloo et al., 2010). Additionally, Tfap2A and Tfap2C are redundant in their induction of neural crest development (Hoffman et al., 2007; Li and Cornell, 2007). In the mouse, however, members of this family appear to play more discrete roles in developmental processes. For example, Tfap2A is indispensable for craniofacial, eye, and limb development (Nottoli et al., 1998; Schorle et al., 1996); and, Tfap2B is indispensable for kidney development (Moser et al., 1997). Consistently, our results indicate that Tfap2A and Tfap2B play independent roles in the specification of GABAergic interneurons during mouse cerebellar development.

There is growing evidence that, in addition to Ptf1a, Tfap2A and Tfap2B play a crucial role in the specification of GABAergic neuronal subtypes (Figure 15I-L, 16I-L). The expression of Ptf1a in neural precursors directs generation of GABAergic neurons and suppresses generation of glutamatergic neurons in the cerebellum, spinal cord and retina (Glasgow et al., 2005; Hoshino et al., 2005; Nakhai et al., 2007; Pascual et al., 2007). Tfap2A and Tfap2B expression is dependent on Ptf1a in the retina (Jin et al., 2015). Both transcription factors have the ability to promote generation of GABAergic/glycinergic amacrine cells and concomitantly suppress the generation of excitatory photoreceptors and bipolar cells (Jin et al., 2015). However, in the spinal cord, Tfap2B is expressed by motor synergy encoder neurons which consist of both excitatory and inhibitory phenotypes (Levine et al., 2014). In our study, we report that the regulation of Tfap2A and Tfap2B expression in the cerebellum acts independently of Ptf1a pathway (Figure 17). There are a few possible reasons for our observation, (1) manipulation was too quick to detect any consequence on Tfap2A and Tfap2B expression or, (2) cells that endogenously expressed Ptf1a were mostly transfected instead of Ptf1a⁻ cells and thus overexpression of Ptf1a is

futile. Therefore, to confirm that *Ptf1a* does not act upstream of either *Tfap2A* or *Tfap2B*, we suggest targeted expression of *Ptf1a* using mouse genetics. For example, we could electroporate *Ptf1a* expression plasmid in Cre-expressing mouse lines which express Cre recombinase enzyme only in rhombic lip-derived neurons such as *Math1^{Cre/+}* mouse line. Nevertheless, our study shows that misexpression of *Tfap2A* in neural precursors in the ventricular zone results in an increase in *Pax2⁺* interneurons (Figure 15I, J, L) while knocking down *Tfap2B* leads to a decrease in *Pax2⁺* interneurons (Figure 16I, K, L). In addition, misexpression of *Tfap2B* can also results in a decrease in *Pax6⁺* excitatory cells (Figure 15M, O, P). Thus, *Tfap2A* and *Tfap2B* appear to have distinct roles in the specification of GABAergic neuronal subtypes and may serve to delegate *Ptf1a* activities, similar to the retina.

Distinguishing molecular features of GABAergic interneurons versus GABAergic projection neurons in the cerebellum have not be well defined. A set of bHLH transcription factors *Ascl1* and *Ngn1* may be involved in specification of interneurons and projection neurons, respectively. *Ascl1* is required for generation of *Pax2⁺* interneurons, but not Purkinje cells, whereas *Ngn1* is required for generation of Purkinje cells, but not *Pax2⁺* interneurons (Levine et al., 2014; Lundell et al., 2009). Lim homeodomain transcription factors *Lhx1* and *Lhx5* and their cofactor *Ldb1* are required for production of Purkinje cells, but not *Pax2⁺* interneurons (Zordan et al., 2008). Additionally, the transcription factor *Corl2* plays a role in maturation of Purkinje cells (Wang et al., 2011). Besides *Ascl1*, cerebellar GABAergic interneurons are dependent on cyclin D2 and cell-cycle dynamics (Huard et al., 1999; Leto et al., 2011). In this study, we add to this list members of *Tfap2* family of transcription factors whose expression defines cerebellar GABAergic projection versus interneurons, and acts to ensure specification of GABAergic interneurons, but not projection neurons.

Figure 20. Proposed role of Tfap2A and Tfap2B in the specification of cerebellar GABAergic interneurons.

(A) Summary of Tfap2A misexpression in the ventricular zone (VZ), particularly in Ptf1a⁺ (Ptf1a⁺/Olig2⁺) mitotic GABAergic progenitors and neural precursors which do not endogenously expressed Tfap2A, induced Pax2⁺ interneuron production. (B) Knockdown of Tfap2B in the ventricular zone, in Olig2⁺ population that endogenously expressed Tfap2B, reduced population of Pax2⁺ interneurons. (C) On the other hand, misexpressing Tfap2B in the rhombic lip (RL) region where excitatory neurons are derived from, reduced population of Pax6⁺ excitatory neurons. (D) Schematic model of distinct roles of Tfap2A and Tfap2B where we propose that Tfap2B play an essential role in the specification of Pax2⁺ interneurons. Abbreviations: EGL; external germinal layer, WM; white matter.

5. SUMMARY

In this thesis, we present several novel findings regarding diversification of neuronal subtypes in the cerebellum. First, we determined that transcription factors Tfap2A and Tfap2B are expressed in subsets of GABAergic neuronal population. Tfap2A is expressed by all cerebellar cortical inhibitory neurons, which includes Purkinje, Golgi, stellate and basket cells, and deep cerebellar nuclei GABAergic interneurons. On the other hand, Tfap2B is expressed by cerebellar cortical inhibitory interneurons, which comprise of Golgi, stellate and basket cells. We also observed that Tfap2A may be an early-onset Purkinje cell cluster marker. Although function of the early-onset cluster is still unknown, we hypothesize that these clusters may influence late-onset clusters that form Purkinje cell stripes in the adult cerebellum. Based on the spatial and temporal expression analysis, we carried out genetic manipulation studies via *in utero* electroporation and reveal potential roles of Tfap2A and Tfap2B in specifying GABAergic interneurons.

Molecular characterisation of these transcription factors during development demonstrated sequential expression of transcription factors, $Ptf1a \rightarrow Tfap2A/B \rightarrow Pax2$ (summarised in Figure 20). Spatial expression of Tfap2A and Tfap2B along the ventricular zone, which is superior to Ptf1a expression, indicates that Ptf1a expression precedes expression of Tfap2A and Tfap2B. Moreover, molecular profiles of Tfap2A and Tfap2B with Pax2, a marker for GABAergic interneurons, show an increase in Tfap2A/B⁺ cells expressing Pax2 during development. Furthermore, misexpression and knockdown studies further determine roles of transcription factors Tfap2A and Tfap2B in specifying Pax2⁺ interneurons. In short, knocking down Tfap2B in the ventricular zone reduces population of Pax2⁺ interneurons while ectopic expression of Tfap2A increases population of Pax2⁺ interneurons. Although it is more conclusive to state that Tfap2B is essential for the specification of Pax2⁺ interneurons from GABAergic progenitors, Tfap2A is also useful to increase the production of Pax2⁺ interneurons. Ectopic expression of Tfap2B in the rhombic lip region results in a reduction of Pax6⁺ excitatory neurons which suggests possible inhibitory role of Tfap2B on Pax6. Collectively, these findings demonstrate the sequential expression of $Ptf1a \rightarrow Tfap2A/B \rightarrow Pax2$ and the involvement of transcription factors Tfap2A and Tfap2B in the development GABAergic interneurons.

In conclusion, we have identified and characterized a set of genes that is essential for the diversification of GABAergic interneurons, which could be used to further study GABAergic neuronal subtypes. Cerebellar cortical inhibitory interneurons, namely Golgi cells and stellate and basket cells, are important in producing feedback and feedforward inhibition respectively (Eccles, 1967; Ito, 1984). Imbalance between excitation and inhibition have been demonstrated in many neurological disorders such as schizophrenia and autism spectrum disorders. Therefore, the identification of molecular mechanisms involved in organisation of cerebellar circuitry will improve our understanding of the complexities of cerebellar function in health and disease (Andreasen and Pierson, 2008; DiCicco-Bloom et al., 2006; Mosconi et al., 2015).

6. REFERENCES

- Acampora, D., Avantaggiato, V., Tuorto, F., Simeone, A., 1997. Genetic control of brain morphogenesis through Otx gene dosage requirement. *Development* 124, 3639-3650.
- Achim, K., Salminen, M., Partanen, J., 2014. Mechanisms regulating GABAergic neuron development. *Cell Mol Life Sci* 71, 1395-1415.
- Ahn, A.H., Dziennis, S., Hawkes, R., Herrup, K., 1994. The Cloning of Zebrin-II Reveals Its Identity with Aldolase-C. *Development* 120, 2081-2090.
- Altman, J., Bayer, S.A., 1996. *Development of the Cerebellar System: In Relation to Its Evolution, Structure, and Functions.*, 1 ed. CRC Press.
- Andersen, B.B., Korbo, L., Pakkenberg, B., 1992. A quantitative study of the human cerebellum with unbiased stereological techniques. *J Comp Neurol* 326, 549-560.
- Andreasen, N.C., Pierson, R., 2008. The role of the cerebellum in schizophrenia. *Biol Psychiatry* 64, 81-88.
- Apps, R., Garwicz, M., 2005. Anatomical and physiological foundations of cerebellar information processing. *Nat Rev Neurosci* 6, 297-311.
- Armstrong, C.L., Krueger-Naug, A.M., Currie, R.W., Hawkes, R., 2000. Constitutive expression of the 25-kDa heat shock protein Hsp25 reveals novel parasagittal bands of Purkinje cells in the adult mouse cerebellar cortex. *J Comp Neurol* 416, 383-397.
- Baader, S.L., Vogel, M.W., Sanlioglu, S., Zhang, X., Oberdick, J., 1999. Selective disruption of "late onset" sagittal banding patterns by ectopic expression of engrailed-2 in cerebellar Purkinje cells. *J Neurosci* 19, 5370-5379.
- Bastianelli, E., 2003. Distribution of calcium-binding proteins in the cerebellum. *Cerebellum* 2, 242-262.
- Ben-Arie, N., Bellen, H.J., Armstrong, D.L., McCall, A.E., Gordadze, P.R., Guo, Q., Matzuk, M.M., Zoghbi, H.Y., 1997. Math1 is essential for genesis of cerebellar granule neurons. *Nature* 390, 169-172.
- Borghesani, P.R., Peyrin, J.M., Klein, R., Rubin, J., Carter, A.R., Schwartz, P.M., Luster, A., Corfas, G., Segal, R.A., 2002. BDNF stimulates migration of cerebellar granule cells. *Development* 129, 1435-1442.
- Borromeo, M.D., Meredith, D.M., Castro, D.S., Chang, J.C., Tung, K.C., Guillemot, F., Johnson, J.E., 2014. A transcription factor network specifying inhibitory versus excitatory neurons in the dorsal spinal cord. *Development* 141, 2803-2812.

- Brewer, S., Feng, W., Huang, J., Sullivan, S., Williams, T., 2004. Wnt1-Cre-mediated deletion of AP-2alpha causes multiple neural crest-related defects. *Dev Biol* 267, 135-152.
- Broccoli, V., Boncinelli, E., Wurst, W., 1999. The caudal limit of Otx2 expression positions the isthmic organizer. *Nature* 401, 164-168.
- Brochu, G., Maler, L., Hawkes, R., 1990. Zebrin-Ii - a Polypeptide Antigen Expressed Selectively by Purkinje-Cells Reveals Compartments in Rat and Fish Cerebellum. *J Comp Neurol* 291, 538-552.
- Butts, T., Green, M.J., Wingate, R.J., 2014. Development of the cerebellum: simple steps to make a 'little brain'. *Development* 141, 4031-4041.
- Cheng, Y., Sudarov, A., Szulc, K.U., Sgaier, S.K., Stephen, D., Turnbull, D.H., Joyner, A.L., 2010. The Engrailed homeobox genes determine the different foliation patterns in the vermis and hemispheres of the mammalian cerebellum. *Development* 137, 519-529.
- Chizhikov, V.V., Lindgren, A.G., Curre, D.S., Rose, M.F., Monuki, E.S., Millen, K.J., 2006. The roof plate regulates cerebellar cell-type specification and proliferation. *Development* 133, 2793-2804.
- Chung, S.H., Kim, C.T., Jung, Y.H., Lee, N.S., Jeong, Y.G., 2010. Early cerebellar granule cell migration in the mouse embryonic development. *Anat Cell Biol* 43, 86-95.
- Cobos, I., Long, J.E., Thwin, M.T., Rubenstein, J.L., 2006. Cellular patterns of transcription factor expression in developing cortical interneurons. *Cereb Cortex* 16 Suppl 1, i82-88.
- Dahmane, N., Altaba, A.R.I., 1999. Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* 126, 3089-3100.
- Dastjerdi, F.V., Consalez, G.G., Hawkes, R., 2012. Pattern formation during development of the embryonic cerebellum. *Front Neuroanat* 6, 10.
- Demilly, A., Reeber, S.L., Gebre, S.A., Sillitoe, R.V., 2011. Neurofilament Heavy Chain Expression Reveals a Unique Parasagittal Stripe Topography in the Mouse Cerebellum. *Cerebellum* 10, 409-421.
- Di Giovannantonio, L.G., Di Salvio, M., Omodei, D., Prakash, N., Wurst, W., Pierani, A., Acampora, D., Simeone, A., 2014. Otx2 cell-autonomously determines dorsal mesencephalon versus cerebellum fate independently of isthmic organizing activity. *Development* 141, 377-388.
- DiCicco-Bloom, E., Lord, C., Zwaigenbaum, L., Courchesne, E., Dager, S.R., Schmitz, C., Schultz, R.T., Crawley, J., Young, L.J., 2006. The developmental neurobiology of autism spectrum disorder. *J Neurosci* 26, 6897-6906.

- Dizon, M.J., Khodakhah, K., 2011. The role of interneurons in shaping Purkinje cell responses in the cerebellar cortex. *J Neurosci* 31, 10463-10473.
- Eccles, J.C., 1967. Circuits in the cerebellar control of movement. *Proc Natl Acad Sci U S A* 58, 336-343.
- Eckert, D., Buhl, S., Weber, S., Jager, R., Schorle, H., 2005. The AP-2 family of transcription factors. *Genome Biol* 6, 246.
- Eddison, M., Toole, L., Bell, E., Wingate, R.J., 2004. Segmental identity and cerebellar granule cell induction in rhombomere 1. *BMC Biol* 2, 14.
- Engelkamp, D., Rashbass, P., Seawright, A., van Heyningen, V., 1999. Role of Pax6 in development of the cerebellar system. *Development* 126, 3585-3596.
- Fink, A.J., Englund, C., Daza, R.A., Pham, D., Lau, C., Nivison, M., Kowalczyk, T., Hevner, R.F., 2006. Development of the deep cerebellar nuclei: transcription factors and cell migration from the rhombic lip. *J Neurosci* 26, 3066-3076.
- Foucher, I., Mione, M., Simeone, A., Acampora, D., Bally-Cuif, L., Houart, C., 2006. Differentiation of cerebellar cell identities in absence of Fgf signalling in zebrafish Otx morphants. *Development* 133, 1891-1900.
- Fujitani, Y., Fujitani, S., Luo, H., Qiu, F., Burlison, J., Long, Q., Kawaguchi, Y., Edlund, H., MacDonald, R.J., Furukawa, T., Fujikado, T., Magnuson, M.A., Xiang, M., Wright, C.V., 2006. Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. *Development* 133, 4439-4450.
- Gao, R., Penzes, P., 2015. Common Mechanisms of Excitatory and Inhibitory Imbalance in Schizophrenia and Autism Spectrum Disorders. *Curr Mol Med* 15, 146-167.
- Gavalas, A., Davenne, M., Lumsden, A., Chambon, P., Rijli, F.M., 1997. Role of Hoxa-2 in axon pathfinding and rostral hindbrain patterning. *Development* 124, 3693-3702.
- Gazit, R., Krizhanovsky, V., Ben-Arie, N., 2004. Math1 controls cerebellar granule cell differentiation by regulating multiple components of the Notch signaling pathway. *Development* 131, 903-913.
- Glasgow, S.M., Henke, R.M., Macdonald, R.J., Wright, C.V., Johnson, J.E., 2005. Ptf1a determines GABAergic over glutamatergic neuronal cell fate in the spinal cord dorsal horn. *Development* 132, 5461-5469.
- Hamilton, B.A., Frankel, W.N., Kerrebrock, A.W., Hawkins, T.L., FitzHugh, W., Kusumi, K., Russell, L.B., Mueller, K.L., vanBerkel, V., Birren, B.W., Kruglyak, L., Lander, E.S., 1996. Disruption of the nuclear hormone receptor ROR alpha in staggerer mice. *Nature* 379, 736-739.

Harada, H., Sato, T., Nakamura, H., 2016. Fgf8 signaling for development of the midbrain and hindbrain. *Dev Growth Differ* 58, 437-445.

Hatten, M.E., Heintz, N., 1995. Mechanisms of neural patterning and specification in the developing cerebellum. *Annu Rev Neurosci* 18, 385-408.

Helms, A.W., Johnson, J.E., 2003. Specification of dorsal spinal cord interneurons. *Curr Opin Neurobiol* 13, 42-49.

Hioki, H., Fujiyama, F., Taki, K., Tomioka, R., Furuta, T., Tamamaki, N., Kaneko, T., 2003. Differential distribution of vesicular glutamate transporters in the rat cerebellar cortex. *Neuroscience* 117, 1-6.

Hoffman, T.L., Javier, A.L., Campeau, S.A., Knight, R.D., Schilling, T.F., 2007. Tfap2 transcription factors in zebrafish neural crest development and ectodermal evolution. *J Exp Zool Part B* 308b, 679-691.

Horak, F.B., Diener, H.C., 1994. Cerebellar control of postural scaling and central set in stance. *Journal of Neurophysiology* 72, 479-943.

Hori, K., Hoshino, M., 2012. GABAergic neuron specification in the spinal cord, the cerebellum, and the cochlear nucleus. *Neural Plast* 2012, 921732.

Hoshino, M., Nakamura, S., Mori, K., Kawauchi, T., Terao, M., Nishimura, Y.V., Fukuda, A., Fuse, T., Matsuo, N., Sone, M., Watanabe, M., Bito, H., Terashima, T., Wright, C.V., Kawaguchi, Y., Nakao, K., Nabeshima, Y., 2005. Ptf1a, a bHLH transcriptional gene, defines GABAergic neuronal fates in cerebellum. *Neuron* 47, 201-213.

Huang, M., Huang, T., Xiang, Y., Xie, Z., Chen, Y., Yan, R., Xu, J., Cheng, L., 2008. Ptf1a, Lbx1 and Pax2 coordinate glycinergic and peptidergic transmitter phenotypes in dorsal spinal inhibitory neurons. *Dev Biol* 322, 394-405.

Huard, J.M., Forster, C.C., Carter, M.L., Sicinski, P., Ross, M.E., 1999. Cerebellar histogenesis is disturbed in mice lacking cyclin D2. *Development* 126, 1927-1935.

Ino, H., 2004. Immunohistochemical characterization of the orphan nuclear receptor ROR alpha in the mouse nervous system. *J Histochem Cytochem* 52, 311-323.

Inouye, M., Oda, S.-I., 1980. Strain-specific variations in the folial pattern of the mouse cerebellum. *The Journal of Comparative Neurology* 190, 357-362.

Ito, M., 1984. *The Cerebellum and Neural Control*. Raven Press, New York, NY.

Ito, M., 2006. Cerebellar circuitry as a neuronal machine. *Prog Neurobiol* 78, 272-303.

Jin, K., Jiang, H., Xiao, D., Zou, M., Zhu, J., Xiang, M., 2015. Tfp2a and 2b act downstream of Ptf1a to promote amacrine cell differentiation during retinogenesis. *Mol Brain* 8, 28.

Ju, J., Liu, Q., Zhang, Y., Liu, Y., Jiang, M., Zhang, L., He, X., Peng, C., Zheng, T., Lu, Q.R., Li, H., 2016. Olig2 regulates Purkinje cell generation in the early developing mouse cerebellum. *Sci Rep* 6, 30711.

Katahira, T., Sato, T., Sugiyama, S., Okafuji, T., Araki, I., Funahashi, J., Nakamura, H., 2000. Interaction between Otx2 and Gbx2 defines the organizing center for the optic tectum. *Mech Dev* 91, 43-52.

Kim, H.S., Hong, S.J., LeDoux, M.S., Kim, K.S., 2001. Regulation of the tyrosine hydroxylase and dopamine beta-hydroxylase genes by the transcription factor AP-2. *J Neurochem* 76, 280-294.

Korbo, L., Andersen, B.B., Ladefoged, O., Moller, A., 1993. Total Numbers of Various Cell-Types in Rat Cerebellar Cortex Estimated Using an Unbiased Stereological Method. *Brain Res* 609, 262-268.

Larouche, M., Hawkes, R., 2006. From clusters to stripes: the developmental origins of adult cerebellar compartmentation. *Cerebellum* 5, 77-88.

Lein, E.S., Hawrylycz, M.J., Ao, N., Ayres, M., Bensinger, A., Bernard, A., Boe, A.F., Boguski, M.S., Brockway, K.S., Byrnes, E.J., Chen, L., Chen, L., Chen, T.M., Chin, M.C., Chong, J., Crook, B.E., Czaplinska, A., Dang, C.N., Datta, S., Dee, N.R., Desaki, A.L., Desta, T., Diep, E., Dolbeare, T.A., Donelan, M.J., Dong, H.W., Dougherty, J.G., Duncan, B.J., Ebbert, A.J., Eichele, G., Estin, L.K., Faber, C., Facer, B.A., Fields, R., Fischer, S.R., Fliss, T.P., Frensley, C., Gates, S.N., Glattfelder, K.J., Halverson, K.R., Hart, M.R., Hohmann, J.G., Howell, M.P., Jeung, D.P., Johnson, R.A., Karr, P.T., Kawal, R., Kidney, J.M., Knapik, R.H., Kuan, C.L., Lake, J.H., Laramie, A.R., Larsen, K.D., Lau, C., Lemon, T.A., Liang, A.J., Liu, Y., Luong, L.T., Michaels, J., Morgan, J.J., Morgan, R.J., Mortrud, M.T., Mosqueda, N.F., Ng, L.L., Ng, R., Orta, G.J., Overly, C.C., Pak, T.H., Parry, S.E., Pathak, S.D., Pearson, O.C., Puchalski, R.B., Riley, Z.L., Rockett, H.R., Rowland, S.A., Royall, J.J., Ruiz, M.J., Sarno, N.R., Schaffnit, K., Shapovalova, N.V., Sivasay, T., Slaughterbeck, C.R., Smith, S.C., Smith, K.A., Smith, B.I., Sotd, A.J., Stewart, N.N., Stumpf, K.R., Sunkin, S.M., Sutram, M., Tam, A., Teemer, C.D., Thaller, C., Thompson, C.L., Varnam, L.R., Visel, A., Whitlock, R.M., Wornoutka, P.E., Wolkey, C.K., Wong, V.Y., Wood, M., Yaylaoglu, M.B., Young, R.C., Youngstrom, B.L., Yuan, X.F., Zhang, B., Zwingman, T.A., Jones, A.R., 2007. Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445, 168-176.

Leto, K., Bartolini, A., Di Gregorio, A., Imperiale, D., De Luca, A., Parmigiani, E., Filipkowski, R.K., Kaczmarek, L., Rossi, F., 2011. Modulation of cell-cycle dynamics is required to regulate the number of cerebellar GABAergic interneurons and their rhythm of maturation. *Development* 138, 3463-3472.

Leto, K., Bartolini, A., Yanagawa, Y., Obata, K., Magrassi, L., Schilling, K., Rossi, F., 2009. Laminar fate and phenotype specification of cerebellar GABAergic interneurons. *J Neurosci* 29, 7079-7091.

Leto, K., Carletti, B., Williams, I.M., Magrassi, L., Rossi, F., 2006. Different types of cerebellar GABAergic interneurons originate from a common pool of multipotent progenitor cells. *J Neurosci* 26, 11682-11694.

Levine, A.J., Hinckley, C.A., Hilde, K.L., Driscoll, S.P., Poon, T.H., Montgomery, J.M., Pfaff, S.L., 2014. Identification of a cellular node for motor control pathways. *Nat Neurosci* 17, 586-593.

Lewis, P.M., Gritli-Linde, A., Smeyne, R., Kottmann, A., McMahon, A.P., 2004. Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. *Dev Biol* 270, 393-410.

Li, J.Y., Lao, Z., Joyner, A.L., 2002. Changing requirements for Gbx2 in development of the cerebellum and maintenance of the mid/hindbrain organizer. *Neuron* 36, 31-43.

Li, W., Cornell, R.A., 2007. Redundant activities of Tfap2a and Tfap2c are required for neural crest induction and development of other non-neural ectoderm derivatives in zebrafish embryos. *Developmental Biology* 304, 338-354.

Liu, Z.R., Shi, M., Hu, Z.L., Zheng, M.H., Du, F., Zhao, G., Ding, Y.Q., 2010. A refined map of early gene expression in the dorsal rhombomere 1 of mouse embryos. *Brain Res Bull* 82, 74-82.

Lu, H., Prior, F.W., Larson-Prior, L.J., 1998. The role of feedforward and feedback inhibition on frequency-dependent information processing in a cerebellar granule cell, in: Bower, J.M. (Ed.), *Computational Neuroscience*. Springer, US, pp. 453-458.

Lumpkin, E.A., Collisson, T., Parab, P., Omer-Abdalla, A., Haeberle, H., Chen, P., Doetzlhofer, A., White, P., Groves, A., Segil, N., Johnson, J.E., 2003. Math1-driven GFP expression in the developing nervous system of transgenic mice. *Gene Expression Patterns* 3, 389-395.

Lundell, T.G., Zhou, Q., Doughty, M.L., 2009. Neurogenin1 expression in cell lineages of the cerebellar cortex in embryonic and postnatal mice. *Dev Dyn* 238, 3310-3325.

Machold, R., Fishell, G., 2005. Math1 is expressed in temporally discrete pools of cerebellar rhombic-lip neural progenitors. *Neuron* 48, 17-24.

Maricich, S.M., Herrup, K., 1999. Pax-2 expression defines a subset of GABAergic interneurons and their precursors in the developing murine cerebellum. *J Neurobiol* 41, 281-294.

Martinez, S., Andreu, A., Mecklenburg, N., Echevarria, D., 2013. Cellular and molecular basis of cerebellar development. *Front Neuroanat* 7, 18.

- Marzban, H., Chung, S., Watanabe, M., Hawkes, R., 2007. Phospholipase C beta 4 expression reveals the continuity of cerebellar topography through development. *J Comp Neurol* 502, 857-871.
- Meguro, R., Ohishi, H., Hoshino, K., Hicks, T.P., Norita, M., 1999. Metabotropic glutamate receptor 2/3 immunoreactivity in the developing rat cerebellar cortex. *J Comp Neurol* 410, 243-255.
- Meredith, D.M., Borromeo, M.D., Deering, T.G., Casey, B.H., Savage, T.K., Mayer, P.R., Hoang, C., Tung, K.C., Kumar, M., Shen, C., Swift, G.H., Macdonald, R.J., Johnson, J.E., 2013. Program specificity for Ptf1a in pancreas versus neural tube development correlates with distinct collaborating cofactors and chromatin accessibility. *Mol Cell Biol* 33, 3166-3179.
- Millen, K.J., Gleeson, J.G., 2008. Cerebellar development and disease. *Curr Opin Neurobiol* 18, 12-19.
- Millen, K.J., Steshina, E.Y., Iskusnykh, I.Y., Chizhikov, V.V., 2014. Transformation of the cerebellum into more ventral brainstem fates causes cerebellar agenesis in the absence of Ptf1a function. *Proc Natl Acad Sci U S A* 111, E1777-1786.
- Millet, S., Bloch-Gallego, E., Simeone, A., Alvarado-Mallart, R.M., 1996. The caudal limit of Otx2 gene expression as a marker of the midbrain/hindbrain boundary: a study using in situ hybridisation and chick/quail homotopic grafts. *Development* 122, 3785-3797.
- Millet, S., Campbell, K., Epstein, D.J., Losos, K., Harris, E., Joyner, A.L., 1999. A role for Gbx2 in repression of Otx2 and positioning the mid/hindbrain organizer. *Nature* 401, 161-164.
- Minaki, Y., Nakatani, T., Mizuhara, E., Inoue, T., Ono, Y., 2008. Identification of a novel transcriptional corepressor, Corl2, as a cerebellar Purkinje cell-selective marker. *Gene Expr Patterns* 8, 418-423.
- Miyata, T., Maeda, T., Lee, J.E., 1999. NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. *Genes Dev* 13, 1647-1652.
- Mohibullah, N., Donner, A., Ippolito, J.A., Williams, T., 1999. SELEX and missing phosphate contact analyses reveal flexibility within the AP-2[alpha] protein: DNA binding complex. *Nucleic Acids Res* 27, 2760-2769.
- Morales, D., Hatten, M.E., 2006. Molecular markers of neuronal progenitors in the embryonic cerebellar anlage. *J Neurosci* 26, 12226-12236.
- Mosconi, M.W., Wang, Z., Schmitt, L.M., Tsai, P., Sweeney, J.A., 2015. The role of cerebellar circuitry alterations in the pathophysiology of autism spectrum disorders. *Front Neurosci* 9, 296.

Moser, M., Imhof, A., Pscherer, A., Bauer, R., Amselgruber, W., Sinowatz, F., Hofstadter, F., Schule, R., Buettner, R., 1995. Cloning and characterization of a second AP-2 transcription factor: AP-2 beta. *Development* 121, 2779-2788.

Moser, M., Ruschhoff, J., Buettner, R., 1997. Comparative analysis of AP-2 alpha and AP-2 beta gene expression during murine embryogenesis. *Dev Dyn* 208, 115-124.

Muguruma, K., Nishiyama, A., Kawakami, H., Hashimoto, K., Sasai, Y., 2015. Self-organization of polarized cerebellar tissue in 3D culture of human pluripotent stem cells. *Cell Rep* 10, 537-550.

Nakagawa, S., Watanabe, M., Inoue, Y., 1997. Prominent expression of nuclear hormone receptor ROR alpha in Purkinje cells from early development. *Neurosci Res* 28, 177-184.

Nakatani, T., Minaki, Y., Kumai, M., Nitta, C., Ono, Y., 2014. The c-Ski family member and transcriptional regulator Corl2/Skor2 promotes early differentiation of cerebellar Purkinje cells. *Dev Biol* 388, 68-80.

Nakhai, H., Sel, S., Favor, J., Mendoza-Torres, L., Paulsen, F., Duncker, G.I., Schmid, R.M., 2007. Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. *Development* 134, 1151-1160.

Nelson, S.B., Valakh, V., 2015. Excitatory/Inhibitory Balance and Circuit Homeostasis in Autism Spectrum Disorders. *Neuron* 87, 684-698.

Nordquist, D.T., Kozak, C.A., Orr, H.T., 1988. cDNA cloning and characterization of three genes uniquely expressed in cerebellum by Purkinje neurons. *J Neurosci* 8, 4780-4789.

Nottoli, T., Hagopian-Donaldson, S., Zhang, J., Perkins, A., Williams, T., 1998. AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. *Proc Natl Acad Sci U S A* 95, 13714-13719.

Orvis, G.D., Hartzell, A.L., Smith, J.B., Barraza, L.H., Wilson, S.L., Szulc, K.U., Turnbull, D.H., Joyner, A.L., 2012. The engrailed homeobox genes are required in multiple cell lineages to coordinate sequential formation of fissures and growth of the cerebellum. *Dev Biol* 367, 25-39.

Palay, S.L., Chan-Palay, V., 1974. *Cerebellar Cortex*, 1 ed. Springer-Verlag Berlin Heidelberg, Heidelberg, Germany.

Pascual, M., Abasolo, I., Mingorance-Le Meur, A., Martinez, A., Del Rio, J.A., Wright, C.V., Real, F.X., Soriano, E., 2007. Cerebellar GABAergic progenitors adopt an external granule cell-like phenotype in the absence of Ptf1a transcription factor expression. *Proc Natl Acad Sci U S A* 104, 5193-5198.

Penagarikano, O., Abrahams, B.S., Herman, E.I., Winden, K.D., Gdalyahu, A., Dong, H., Sonnenblick, L.I., Gruver, R., Almajano, J., Bragin, A., Golshani, P., Trachtenberg, J.T.,

- Peles, E., Geschwind, D.H., 2011. Absence of CNTNAP2 leads to epilepsy, neuronal migration abnormalities, and core autism-related deficits. *Cell* 147, 235-246.
- Purves, D., Augustine, G.J., Fitzpatrick, D., WHall, W.C., LaMantia, A.-S., McNamara, J.O., Williams, S.M., 2004. Neuroscience, Third ed. Sinauer Associates, Inc., Sunderland, Massachusetts U.S.A.
- Rubenstein, J.L.R., Merzenich, M.M., 2003. Model of autism: increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* 2, 255-267.
- Russ, J.B., Borromeo, M.D., Kollipara, R.K., Bommareddy, P.K., Johnson, J.E., Kaltschmidt, J.A., 2015. Misexpression of ptf1a in cortical pyramidal cells in vivo promotes an inhibitory peptidergic identity. *J Neurosci* 35, 6028-6037.
- Sanz, E., Yang, L., Su, T., Morris, D.R., McKnight, G.S., Amieux, P.S., 2009. Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. *Proc Natl Acad Sci U S A* 106, 13939-13944.
- Sarna, J.R., Marzban, H., Watanabe, M., Hawkes, R., 2006. Complementary stripes of phospholipase C beta 3 AND C beta 4 expression by Purkinje cell subsets in the mouse cerebellum. *J Comp Neurol* 496, 303-313.
- Schmahmann, J.D., Sherman, J.C., 1998. The cerebellar cognitive affective syndrome. *Brain* 121, 561-579.
- Schmidt, M., Huber, L., Majdazari, A., Schutz, G., Williams, T., Rohrer, H., 2011. The transcription factors AP-2beta and AP-2alpha are required for survival of sympathetic progenitors and differentiated sympathetic neurons. *Dev Biol* 355, 89-100.
- Schorle, H., Meier, P., Buchert, M., Jaenisch, R., Mitchell, P.J., 1996. Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* 381, 235-238.
- Seberg, H.E., Van Otterloo, E., Loftus, S.K., Liu, H., Bonde, G., Sompallae, R., Gildea, D.E., Santana, J.F., Manak, J.R., Pavan, W.J., Williams, T., Cornell, R.A., 2017. TFAP2 paralogs regulate melanocyte differentiation in parallel with MITF. *PLoS Genet* 13, e1006636.
- Seto, Y., Ishiwata, S., Hoshino, M., 2014a. Characterization of Olig2 expression during cerebellar development. *Gene Expr Patterns* 15, 1-7.
- Seto, Y., Nakatani, T., Masuyama, N., Taya, S., Kumai, M., Minaki, Y., Hamaguchi, A., Inoue, Y.U., Inoue, T., Miyashita, S., Fujiyama, T., Yamada, M., Chapman, H., Campbell, K., Magnuson, M.A., Wright, C.V., Kawaguchi, Y., Ikenaka, K., Takebayashi, H., Ishiwata, S., Ono, Y., Hoshino, M., 2014b. Temporal identity transition from Purkinje cell progenitors to GABAergic interneuron progenitors in the cerebellum. *Nat Commun* 5, 3337.

Sillitoe, R.V., Gopal, N., Joyner, A.L., 2009. Embryonic origins of ZebrinII parasagittal stripes and establishment of topographic Purkinje cell projections. *Neuroscience* 162, 574-588.

Sillitoe, R.V., Joyner, A.L., 2007. Morphology, molecular codes, and circuitry produce the three-dimensional complexity of the cerebellum. *Annu Rev Cell Dev Biol* 23, 549-577.

Sillitoe, R.V., Stephen, D., Lao, Z., Joyner, A.L., 2008. Engrailed homeobox genes determine the organization of Purkinje cell sagittal stripe gene expression in the adult cerebellum. *J Neurosci* 28, 12150-12162.

Solbach, S., Celio, M.R., 1991. Ontogeny of the calcium binding protein parvalbumin in the rat nervous system. *Anat Embryol (Berl)* 184, 103-124.

Sotelo, C., Rossi, F., 2013. Purkinje Cell Migration and Differentiation, in: Manto, M., Gruol, D.L., Schmahmann, J.D., Koibuchi, N., Rossi, F. (Eds.), *Handbook of the Cerebellum and Cerebellar Disorders*. Springer Science, Dordrecht, Netherlands.

Su, C.Y., Kemp, H.A., Moens, C.B., 2014. Cerebellar development in the absence of Gbx function in zebrafish. *Dev Biol* 386, 181-190.

Sudarov, A., Turnbull, R.K., Kim, E.J., Lebel-Potter, M., Guillemot, F., Joyner, A.L., 2011. *Ascl1* genetics reveals insights into cerebellum local circuit assembly. *J Neurosci* 31, 11055-11069.

Sugino, K., Hempel, C.M., Miller, M.N., Hattox, A.M., Shapiro, P., Wu, C., Huang, Z.J., Nelson, S.B., 2006. Molecular taxonomy of major neuronal classes in the adult mouse forebrain. *Nat Neurosci* 9, 99-107.

Sunmonu, N.A., Chen, L., Li, J.Y., 2009. Misexpression of Gbx2 throughout the mesencephalon by a conditional gain-of-function transgene leads to deletion of the midbrain and cerebellum in mice. *Genesis* 47, 667-673.

Thach, W.T., Bastian, A.J., 2004. Role of the cerebellum in the control and adaptation of gait in health and disease. *Prog Brain Res* 143, 353-366.

Tsai, P.T., Hull, C., Chu, Y., Greene-Colozzi, E., Sadowski, A.R., Leech, J.M., Steinberg, J., Crawley, J.N., Regehr, W.G., Sahin, M., 2012. Autistic-like behaviour and cerebellar dysfunction in Purkinje cell *Tsc1* mutant mice. *Nature* 488, 647-651.

Uusisaari, M., Knopfel, T., 2011. Functional classification of neurons in the mouse lateral cerebellar nuclei. *Cerebellum* 10, 637-646.

Van Otterloo, E., Li, W., Bonde, G., Day, K.M., Hsu, M.Y., Cornell, R.A., 2010. Differentiation of Zebrafish Melanophores Depends on Transcription Factors AP2 Alpha and AP2 Epsilon. *Plos Genetics* 6.

- Vincent, P., Marty, A., 1996. Fluctuations of inhibitory postsynaptic currents in Purkinje cells from rat cerebellar slices. *J Physiol* 494 (Pt 1), 183-199.
- Wang, B., Harrison, W., Overbeek, P.A., Zheng, H., 2011. Transposon mutagenesis with coat color genotyping identifies an essential role for *Skor2* in sonic hedgehog signaling and cerebellum development. *Development* 138, 4487-4497.
- Wang, V.Y., Rose, M.F., Zoghbi, H.Y., 2005. *Math1* expression redefines the rhombic lip derivatives and reveals novel lineages within the brainstem and cerebellum. *Neuron* 48, 31-43.
- Wang, V.Y., Zoghbi, H.Y., 2001. Genetic regulation of cerebellar development. *Nat Rev Neurosci* 2, 484-491.
- Wassarman, K.M., Lewandoski, M., Campbell, K., Joyner, A.L., Rubenstein, J.L., Martinez, S., Martin, G.R., 1997. Specification of the anterior hindbrain and establishment of a normal mid/hindbrain organizer is dependent on *Gbx2* gene function. *Development* 124, 2923-2934.
- Wassef, M., Zanetta, J.P., Brehier, A., Sotelo, C., 1985. Transient biochemical compartmentalization of Purkinje cells during early cerebellar development. *Dev Biol* 111, 129-137.
- Watanabe, D., Inokawa, H., Hashimoto, K., Suzuki, N., Kano, M., Shigemoto, R., Hirano, T., Toyama, K., Kaneko, S., Yokoi, M., Moriyoshi, K., Suzuki, M., Kobayashi, K., Nagatsu, T., Kreitman, R.J., Pastan, I., Nakanishi, S., 1998. Ablation of cerebellar Golgi cells disrupts synaptic integration involving GABA inhibition and NMDA receptor activation in motor coordination. *Cell* 95, 17-27.
- Wechsler-Reya, R.J., Scott, M.P., 1999. Control of neuronal precursor proliferation in the cerebellum by sonic hedgehog. *Neuron* 22, 103-114.
- Weisheit, G., Gliem, M., Endl, E., Pfeffer, P.L., Busslinger, M., Schilling, K., 2006. Postnatal development of the murine cerebellar cortex: formation and early dispersal of basket, stellate and Golgi neurons. *Eur J Neurosci* 24, 466-478.
- West-Mays, J.A., Zhang, J., Nottoli, T., Hagopian-Donaldson, S., Libby, D., Strissel, K.J., Williams, T., 1999. AP-2alpha transcription factor is required for early morphogenesis of the lens vesicle. *Dev Biol* 206, 46-62.
- Weyer, A., Schilling, K., 2003. Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. *J Neurosci Res* 73, 400-409.
- White, J.J., Sillitoe, R.V., 2013. Postnatal development of cerebellar zones revealed by neurofilament heavy chain protein expression. *Frontiers in Neuroanatomy* 7.

- Wilson, S.L., Kalinovsky, A., Orvis, G.D., Joyner, A.L., 2011. Spatially restricted and developmentally dynamic expression of engrailed genes in multiple cerebellar cell types. *Cerebellum* 10, 356-372.
- Wingate, R.J., Hatten, M.E., 1999. The role of the rhombic lip in avian cerebellum development. *Development* 126, 4395-4404.
- Wonders, C.P., Anderson, S.A., 2006. The origin and specification of cortical interneurons. *Nat Rev Neurosci* 7, 687-696.
- Yamada, M., Seto, Y., Taya, S., Owa, T., Inoue, Y.U., Inoue, T., Kawaguchi, Y., Nabeshima, Y., Hoshino, M., 2014. Specification of spatial identities of cerebellar neuron progenitors by *ptf1a* and *atoh1* for proper production of GABAergic and glutamatergic neurons. *J Neurosci* 34, 4786-4800.
- Yamanaka, H., Yanagawa, Y., Obata, K., 2004. Development of stellate and basket cells and their apoptosis in mouse cerebellar cortex. *Neurosci Res* 50, 13-22.
- Yu, F., Jiang, Q.J., Sun, X.Y., Zhang, R.W., 2015. A new case of complete primary cerebellar agenesis: clinical and imaging findings in a living patient. *Brain* 138, e353.
- Zervas, M., Millet, S., Ahn, S., Joyner, A.L., 2004. Cell behaviors and genetic lineages of the mesencephalon and rhombomere 1. *Neuron* 43, 345-357.
- Zhang, J., Hagopian-Donaldson, S., Serbedzija, G., Elsemore, J., Plehn-Dujowich, D., McMahon, A.P., Flavell, R.A., Williams, T., 1996. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature* 381, 238-241.
- Zhang, L., Goldman, J.E., 1996. Generation of cerebellar interneurons from dividing progenitors in white matter. *Neuron* 16, 47-54.
- Zhao, F., Bosserhoff, A.K., Buettner, R., Moser, M., 2011. A heart-hand syndrome gene: *Tfap2b* plays a critical role in the development and remodeling of mouse ductus arteriosus and limb patterning. *PLoS One* 6, e22908.
- Zhao, Y., Kwan, K.M., Mailloux, C.M., Lee, W.K., Grinberg, A., Wurst, W., Behringer, R.R., Westphal, H., 2007. LIM-homeodomain proteins *Lhx1* and *Lhx5*, and their cofactor *Ldb1*, control Purkinje cell differentiation in the developing cerebellum. *Proc Natl Acad Sci U S A* 104, 13182-13186.
- Zheng, T., Santi, M.R., Bovolin, P., Marlier, L.N., Grayson, D.R., 1993. Developmental expression of the alpha 6 GABAA receptor subunit mRNA occurs only after cerebellar granule cell migration. *Brain Res Dev Brain Res* 75, 91-103.
- Zordan, P., Croci, L., Hawkes, R., Consalez, G.G., 2008. Comparative analysis of proneural gene expression in the embryonic cerebellum. *Dev Dyn* 237, 1726-1735.