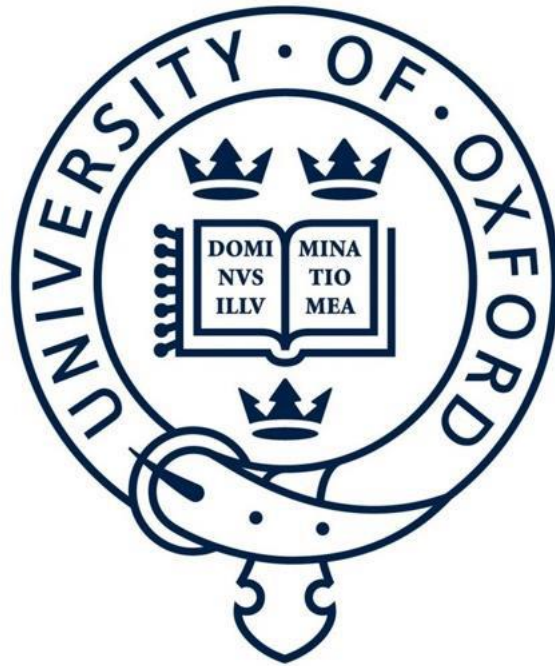


Modelling cerebellar development using human induced pluripotent stem cells



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A thesis submitted for the degree of Doctor of Philosophy
in Interdisciplinary Bioscience

January 2024

To my parents

I. Acknowledgements

As I reach the end of this DPhil I am grateful to so many people. Thank you to all those who have contributed to the science in this thesis, and everything I have learnt and enjoyed along the way.

Firstly, I am very grateful to my supervisor Esther Becker. Thank you for your continuous encouragement and belief in me. Through your guidance and support, I have learnt so much and finish this DPhil a better scientist. To Stuart, Ashley and all of the team at Axol, thank you for your suggestions and advice throughout my DPhil, and for welcoming me into your Cambridge site. Thank you to Emily and Gena for sharing your insights on FOXP2.

I would also like to acknowledge the Oxford interdisciplinary bioscience DTP and the BBSRC who provided my funding and made this work possible.

To the members of the Becker lab, past and present, thank you for all your help and support. You made my time in the lab more fun and I will miss you.

Thank you to my friends and housemates for all the kindness and joy. In particular, I'm very grateful to the residents of Ferry road who made Oxford feel like home, and to Niamh and Phill for adopting me for a wholesome term in Cambridge. To OUCCC and OUTriC, thank you for sharing all the miles and lengths. Your generosity helped me to ride the highs and lows in sport and the lab. I am proud to now be a darker shade of blue.

To my parents and family, thank you for your ever-present love and support. And to Su-Min, thank you for not complaining about the weekends scheduled around cells, and for always believing that I could do this.

II. Abstract

The cerebellum is an important brain region for coordinating a range of motor and non-motor functions, including language. Central to cerebellar function are Purkinje cells, large neurons with complex dendritic trees, that can be identified by high expression of transcription factor *FOXP2* from early in development. However, little is known about the function of *FOXP2* in Purkinje cell development and previous studies have relied on animal models. Human induced pluripotent stem cell (iPSC)-derived cerebellar organoids provide an accessible model for early human cerebellar development. However, current methods show limited Purkinje cell maturity, and it is challenging to track individual cell populations.

This thesis describes the generation of a fluorescent Purkinje cell reporter line based on *FOXP2* expression, to successfully visualise the developing human Purkinje cell population live in cerebellar organoids. In addition, a method for 2D feeder-free dissociated growth of iPSC-derived cerebellar cultures was developed, generating more mature Purkinje cells and widening the application of human cerebellar models to a range of assays.

iPSC-derived cerebellar models were then applied to understanding the role of language-associated gene *FOXP2* in the early human cerebellum. Transcriptomic analysis of isolated *FOXP2*-expressing cells identified putative targets and pathways regulated by *FOXP2* in the developing human cerebellum. These targets were further investigated using *FOXP2* knock-out organoids, identifying a role of *FOXP2* in regulating expression of *ZFHX4*, a gene associated with speech and intellectual disability. In addition, *FOXP2* was found to influence the intracellular calcium response to depolarisation in cerebellar neurons.

Together this work expands the capability of iPSC-derived models for studying early human cerebellar development with a specific focus on Purkinje cells, and highlights potential functions of *FOXP2* in the developing human cerebellum.

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VI. List of abbreviations

2D - 2-dimensions

ACM - *astrocyte conditioned medium*

ASD - autism spectrum disorder

bp - base pair

CAS - childhood apraxia of speech

Cas9 - CRISPR associated system protein 9

ChIP - chromatin immunoprecipitation

CRISPR - Clustered regularly interspaced short palindromic repeat

CS - Carnegie stage

DCN - deep cerebellar nuclei

DM - *Differentiation medium*

ds - double stranded

E - embryonic day

ECM - extracellular matrix

EGL - external granule cell layer

FGF - Fibroblast growth factor

gDNA- genomic DNA

gRNA - guide RNA

GSEA - gene set enrichment analysis

hDN - human differentiated neuron

hESCs - human embryonic stem cells

ID - intellectual disability

IM - *Induction medium*

iPSC - induced pluripotent stem cell

kb - kilobase pair

KD - knock down

KO - knock out

LOH - loss of heterozygosity

MEA - multi-electrode array

mes - mesencephalon
met - metencephalon
MHB - midbrain-hindbrain boundary
NuRD - nucleosome remodelling and deacetylase complex
P - postnatal day
PCA - principle component analysis
PCW - post conception week
RL - rhombic lip
ROI- region of interest
RT-qPCR - reverse transcriptase quantitative PCR
SCA - spinocerebellar ataxia
scRNA-seq - Single cell RNA sequencing
SFEBq - serum-free floating culture of EB-like aggregates
SHH - sonic hedgehog
shRNA - small hairpin RNA
smFISH - single-molecular fluorescent in situ hybridisation
SNA - spontaneous neuronal activity
snRNAseq - single nuclei RNA sequencing
ss - single stranded
SVZ - sub-ventricular zone
USV - ultrasonic vocalisation
VZ - ventricular zone
WT - wild-type
Z - Zebrin II

Chapter 1. Introduction

Although cerebellum literally translates as “little brain”, this name understates the importance of this brain region in a wide range of both motor and non-motor functions (Koziol *et al.*, 2014). The cerebellum is a highly significant structure, containing over 80% of the neurons in the human brain despite forming only 10% of the whole brain mass (Herculano-Houzel, 2010; Herculano-Houzel *et al.*, 2015). To achieve this final form, the process of cerebellar development is complex and prolonged, including massive expansion of neural progenitors, cell migration and refinement of precise circuitry (Leto *et al.*, 2016). As a consequence, the cerebellum is vulnerable to a range of neurodevelopmental disorders such as ataxia, medulloblastoma and autism spectrum disorder (ASD) (Haldipur, Millen and Aldinger, 2022). Generation of induced pluripotent stem cell (iPSC)-derived cerebellar organoids provides an accessible tool to study human cerebellar development. Cerebellar organoids recapitulate features of early development, including generation of neurons from both the glutamatergic and GABAergic cerebellar lineages (Muguruma *et al.*, 2015; Nayler *et al.*, 2021). Therefore, cerebellar organoids have potential to further understanding of specifically-human cerebellar development and changes which occur in neurodevelopmental disorders.

This thesis will focus on the modelling of human Purkinje cell development, and the role played by transcription factor Forkhead box protein P2 (FOXP2) in this process. Therefore, in this chapter, current knowledge of cerebellar development will be summarised, with a focus on Purkinje cells. Next, stem cell-derived models of human brain development will be discussed, including the advantages and limitations of current approaches for generating Purkinje cells. Finally, the expression, function and alternative models for study of *FOXP2* will be reviewed.

1.1 The cerebellum

The cerebellum has a long-established role in motor coordination, with the most prominent cerebellar-associated disorders, such as spinocerebellar ataxias, involving motor dysfunctions. However, the cerebellum also acts in higher cognitive and affective processes, and is connected to a range of other brain regions, including the cerebral cortex (Schmahmann, 2010; Buckner, 2013). Evidence for cerebellar involvement in cognitive functions, such as language, has emerged from neural mapping of connected regions, functional imaging of electrical activity and the study of individuals with damage to their cerebellum (Buckner, 2013; Mariën and Borgatti, 2018).

The cerebellum acts as a modulator: receiving information about a system, comparing it to the expected or intended state, and adjusting the response to achieve that (D'Angelo, 2018). Therefore, the cerebellum provides an automatic response to situations, and where there are deviations in prediction and outcomes the cerebellar model is updated (D'Angelo, 2018). For example, the cerebellum receives sensory information about limb position and pressure, and adjusts the body position accordingly to maintain balance. Although this model of cerebellar processing was first developed for coordination of motor function, it can also be applied to a variety of brain functions (Mariën and Borgatti, 2018). In addition, it provides a model for understanding a range of cerebellar disorders as due to reduced modulation and hence exacerbated changes in outcomes (Schmahmann, 1998).

1.1.1 Cerebellar structure and cell types

The cerebellum is found in the hindbrain and is formed of two hemispheres connected by a central vermis. Inside the cerebellum, an outer cortex surrounds central white matter containing deep cerebellar nuclei (DCN). The cerebellar cortex is highly folded, with the surface area measuring 68-78% of the surface area of the cerebral cortex in humans (Serenio *et al.*, 2020; Zheng *et al.*, 2022). Horizontal folds divide the cerebellum into ten lobules, which are grouped into four transverse zones: the anterior zone (lobules I-V), central zone (lobules VI–VII), posterior zone (lobules VIII-IX), and nodular zone (lobule X) (Larsell, 1970) (Figure 1.1A).

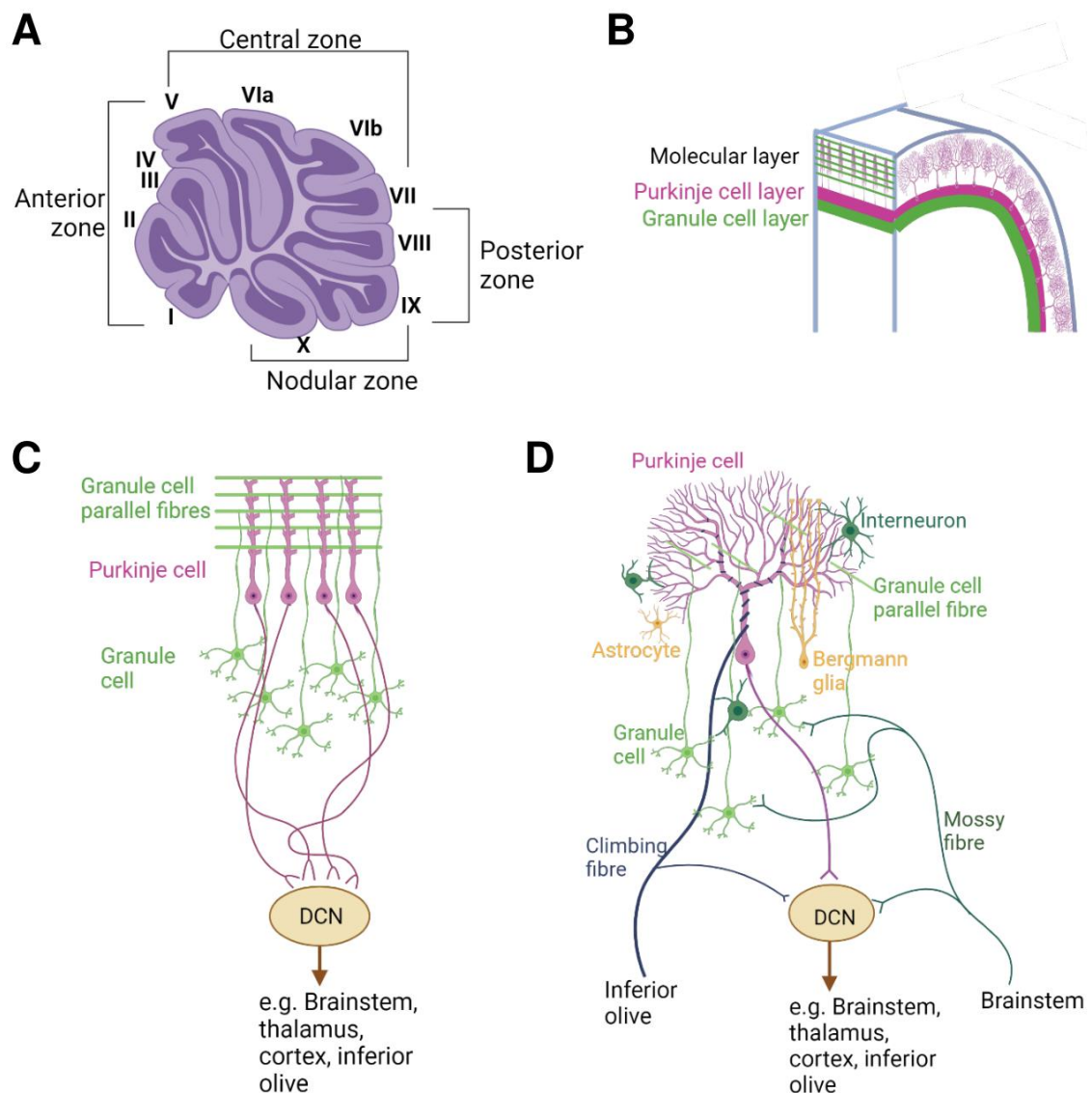


Figure 1.1 Structure of the cerebellum.

- A) The cerebellum is divided into four transverse zones (anterior, central, posterior and nodular) and further into ten lobules (I-X) as shown on the diagram of a sagittal medially-located section from the adult mouse cerebellum.
- B) The cerebellar cortex is formed of three distinct layers. The innermost the granule cell layer, the Purkinje cell layer and outermost the molecular layer.
- C) A coronal view of the major neurons in the cerebellar circuits.
- D) A sagittal view of cerebellar cytoarchitecture. Purkinje cells (pink) receive excitatory afferent inputs from granule cell parallel fibres (light green) and climbing fibres (dark blue), and inhibitory inputs from GABAergic interneurons (dark green). Purkinje cells then output signal from the cerebellar cortex to the deep cerebellar nuclei (DCN).

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The cerebellar cortex has a regular and highly organised cytoarchitecture formed of three layers (Figure 1.1B). The innermost granule cell layer contains granule cell bodies and unipolar brush cells (Figure 1.1C & D). Though unipolar brush cells show uneven distribution across the cerebellum (Mugnaini, Sekerková and Martina, 2011). Next is the Purkinje cell layer, containing the cell bodies of Purkinje cells and Bergmann glia, and the axons of granule cells (Figure 1.1C & D). The outermost molecular layer contains the highly branched dendritic trees of Purkinje cells and the T-shaped axons of granule cells, known as parallel fibres (Figure 1.1C & D). Different populations of interneurons are also distributed across the layers, including Golgi and Lugaro cells in the granule cell layer, candelabrum cells in the Purkinje cell layer, and stellate and basket cells in the molecular layer. In addition, the cerebellar cortex contains glia, including cerebellum-specific Bergmann glia.

Mammals have four cerebellar nuclei, the anterior and posterior interposed nuclei, the medial and the lateral nuclei (Kebschull *et al.*, 2023). Each deep cerebellar nucleus contains both glutamatergic excitatory neurons and GABAergic inhibitory neurons. The GABAergic DCN neurons divide into three classes: projection neurons which extend to the inferior olive, and two groups of small glycinergic neurons which act as local interneurons or project to the cerebellar cortex respectively (Kebschull *et al.*, 2023). GABAergic projection neurons are thought to derive from SOX14+ progenitors whilst the GABAergic glycinergic populations likely arise from PAX2+ progenitors (Kebschull *et al.*, 2023).

The major neuron types acting in cerebellar circuitry are excitatory granule cells and inhibitory Purkinje cells. The cerebellum receives three types of input (D'Angelo, 2018). Mossy fibres project to the cerebellum from the spinal cord, hindbrain and brainstem nuclei to terminate on granule cells (Ito, 1984; D'Angelo, 2018). In turn granule cells stimulate Purkinje cells via parallel fibre synapses (Ito, 1984; D'Angelo, 2018). Climbing fibres originate from the inferior olive, a region of the medulla oblongata within the brainstem, and pair to distinct Purkinje cells (Kano *et al.*, 2018). Additionally, there is a third class of diverse inputs including serotonergic, cholinergic and noradrenergic (Sillitoe and Joyner, 2007). Within the cerebellum, Purkinje cells are central, integrating both excitatory input from mossy and climbing fibres and inhibitory

input from GABAergic interneurons. Purkinje cells are then the sole output neurons of the cerebellar cortex, acting to inhibit the DCN (Sillitoe and Joyner, 2007). Cerebellar DCN contain a mixture of glutamatergic and GABAergic neurons. Glutamatergic excitatory neurons project from the cerebellar DCN to the thalamus, amygdala, brainstem and cerebral cortex (Sydnor and Aldinger, 2021). In contrast, GABAergic DCN inhibitory outputs are mainly directed to the inferior olive creating an inhibitory feedback loop on the cerebellum (Sydnor and Aldinger, 2021). GABAergic DCN neurons also mediate direct feedback within the cerebellum (Sydnor and Aldinger, 2021).

Cerebellar connections are precisely arranged and organised as functional modules (Apps *et al.*, 2018; D'Angelo, 2018). Within a module, Purkinje cells receive climbing fibre input from a specific region of the inferior olive and output to a particular DCN area (Apps *et al.*, 2018). This DCN area also receives inputs from the same region of inferior olive that targets the Purkinje cells (Apps *et al.*, 2018). Mossy fibres also terminate in functionally organised parasagittal zones, although unlike climbing fibres they branch and interact with multiple zones (Apps *et al.*, 2018). Neural mapping has found specific regions of the cerebellum also receive input from and output to the same areas of the cerebral cortex, indicating spatially specialised programmes (Stoodley and Schmahmann, 2018). Broadly the anterior lobules of the cerebellum act in motor functions and connect to the motor cortex, whereas the posterior regions have more high cognitive functions and connect to regions such as the prefrontal cortex and the temporal cortex (Stoodley and Schmahmann, 2010).

1.2 Cerebellar development

1.2.1 Specification of the cerebellum

The cerebellum develops from rhombomere 1 adjacent to the midbrain-hindbrain boundary (MHB). In the early embryo, patterns of transcription factor activity specify the MHB (Figure 1.2). *Orthodenticle Homeobox 2* (*Otx2*) is expressed in the anterior neural tube marking the mesencephalon (mes, midbrain), whilst *Gastrulation Brain Homeobox 2* (*Gbx2*) is expressed in the posterior marking the metencephalon (met, hindbrain) (Acampora *et al.*, 1997; Millet *et al.*, 1999). *Otx2* and *Gbx2* act antagonistically and are required for proper localisation, but not induction, of mes-met genes such as *Fibroblast growth factor 8* (*Fgf8*), *Engrailed 1* (*En1*) and *Wnt family member 1* (*Wnt1*) (Acampora *et al.*, 1997; Li and Joyner, 2001; Su, Kemp and Moens, 2014). From around embryonic day (E) 8.5 in mice the gene pairs *Engrailed* (*En1* and *En2*) and *Paired box* (*Pax2* and *Pax5*) are expressed across the mes-met region, with at least one gene of each pair required for midbrain and cerebellum development (Joyner, Liu and Millet, 2000). By E9.5 *Wnt1* and *Fgf8* are expressed in narrow adjacent rings at the MHB with *Fgf8* to the posterior (Joyner, Liu and Millet, 2000).

Early transplantation experiments demonstrated that tissue at the MHB can induce ectopic midbrain formation in chick forebrain, thus identifying the isthmic organiser (Martinez, Wassef and Alvarado-Mallart, 1991). FGF8 is secreted by the isthmic organiser and is sufficient for induction of mes-met patterning in both chick and mouse studies (Crossley, Martinez and Martin, 1996; Liu, Losos and Joyner, 1999; Martinez *et al.*, 1999). FGF8 activates the Ras-ERK signalling pathway, inducing expression of isthmic factors including *Wnt1*, *En2*, *Pax5* and *Fgf8* and repressing *Otx2* expression (Sato, Joyner and Nakamura, 2004). The duration and intensity of FGF8 signal from the isthmic organiser patterns the different identities of the surrounding midbrain and cerebellum (Sato, Joyner and Nakamura, 2004; Basson *et al.*, 2008; Sato and Joyner, 2009). Levels of FGF signalling are refined by activity of modulators, such as Sprouty RTK Signaling Antagonist 2 (SPRY2), and the isoform of FGF8 present (Basson *et al.*, 2008). In addition, FGF8 is required for cell survival in the midbrain and cerebellum (Chi *et al.*, 2003).

The posterior boundary of the cerebellar anlage is defined by *Hoxa2*, which is expressed caudally from rhombomere 2, the region of the neural tube that will form the hindbrain (Wingate and Hatten, 1999).

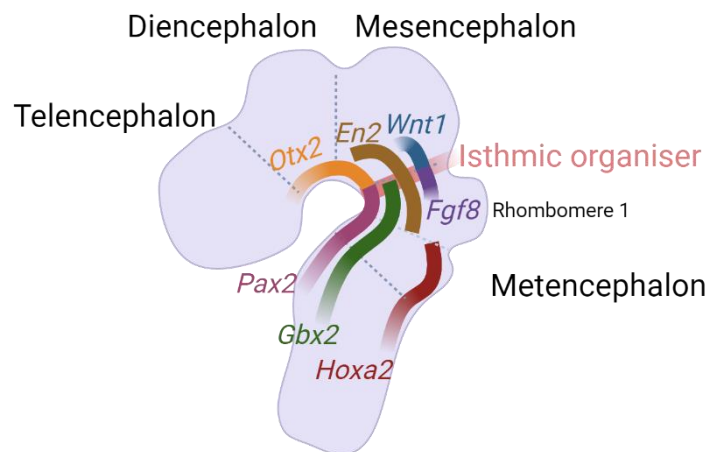


Figure 1.2 Transcription factors pattern the mid-hindbrain boundary.

A combination of transcription factors specify the identity of the midbrain and hindbrain around the isthmic organiser. The diagram shows expression of key factors in the developing mouse neural tube at ~E9.

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1.2.2 Cerebellar germinal zones

Cerebellar neurons develop from two germinal zones (Figure 1.3A). The rhombic lip (RL) gives rise to glutamatergic cells, including unipolar brush cells, granule cells and glutamatergic DCN neurons (Leto *et al.*, 2016). Whilst the ventricular zone (VZ), generates the GABAergic lineage including GABAergic interneurons, Purkinje cells and GABAergic DCN neurons (Leto *et al.*, 2016). Both germinal zones are identified by expression of basic helix-loop-helix transcription factors, with *Atonal bHLH transcription factor 1 (Atoh1 or Math1)* and *Pancreas associated transcription factor 1a (Ptf1A)* marking the RL and VZ respectively (Hoshino *et al.*, 2005; Machold and Fishell, 2005). Specification of the germinal zones is dependent on signalling from the choroid plexus and roof plate (Chizhikov *et al.*, 2006; Machold, Kittell and Fishell, 2007).

In the RL, ATOH1+ cells are generated at different times migrating to form discrete glutamatergic cell types (Machold and Fishell, 2005; Wang, Rose and Zoghbi, 2005). Knock-out and lineage tracing experiments have shown that *Atoh1* is required for migration of progenitors out of the rhombic lip and the generation of glutamatergic cell types (Ben-Arie *et al.*, 1997; Wang, Rose and Zoghbi, 2005). Early rhombic lip derivatives, produced around E9.5-10.5, lose *Atoh1* expression, migrate to the nuclear transitory zone and give rise to glutamatergic DCN neurons (Wang, Rose and Zoghbi, 2005). However, the majority of ATOH1+ cells transit amplify, spreading across the surface of the developing cerebellar to form the external granule cell layer (EGL) (Wang, Rose and Zoghbi, 2005) (Figure 1.3C). The clonal proliferation of granule cell precursors is driven by sonic hedgehog (SHH) secreted by Purkinje cells (Lewis *et al.*, 2004). Expansion of the EGL is responsible for the huge increase in cerebellar volume and leads to foliation of the cerebellar cortex (Corrales *et al.*, 2006) (Figure 1.3D). From around birth, granule cell precursors exit the cell cycle and migrate radially inwards, along Bergmann glia fibres to form an internal granule layer below the Purkinje cell plate (Haldipur, Dang and Millen, 2018)(Figure 1.3E).

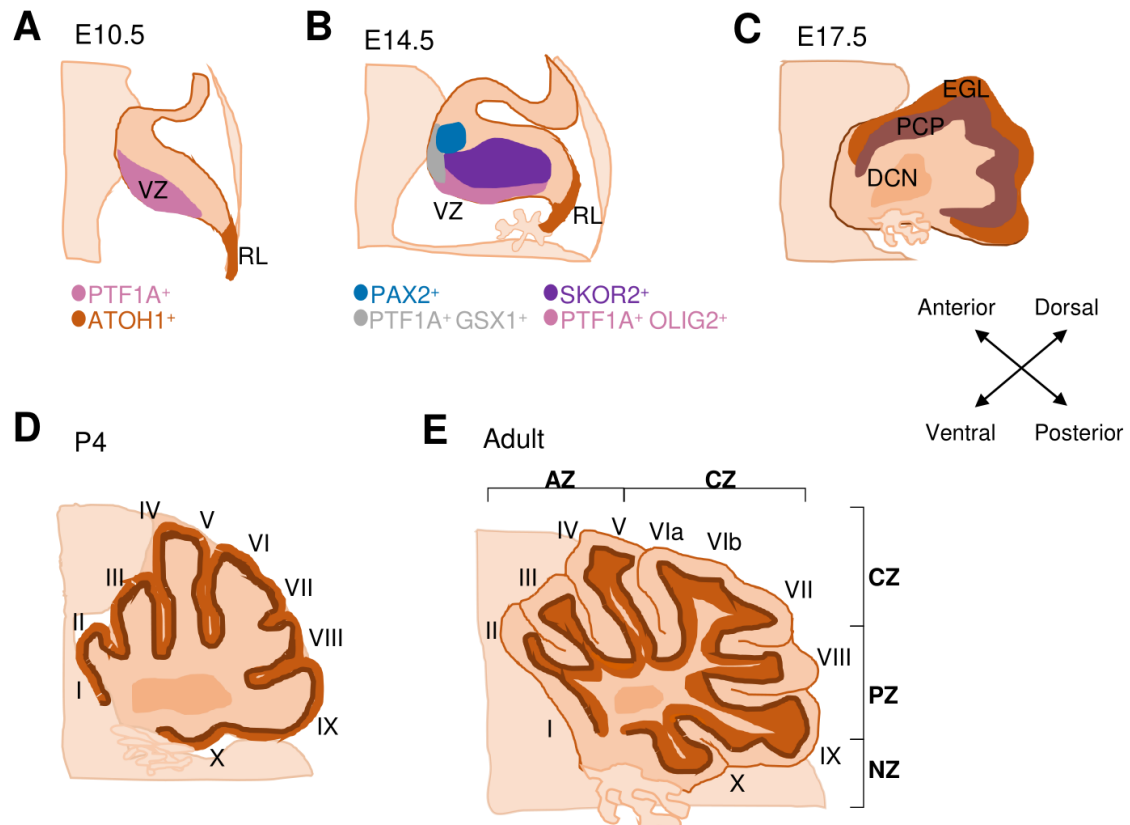


Figure 1.3 Mouse cerebellar development.

Mid-sagittal sections show the stages of mouse cerebellar development over time from embryonic day (E) 10.5 to adult.

- A) At E10.5, two germinal zones are established: the ventricular zone (VZ) and the rhombic lip (RL), which are identified by expression of PTF1A and ATOH1, respectively.
- B) SKOR2⁺ post-mitotic Purkinje cells are generated from PTF1A⁺ OLIG2⁺ progenitors in the VZ. The VZ also gives rise to GABAergic interneurons from PTF1A⁺ GSX1⁺ progenitors.
- C) From E14.5, Purkinje cells migrate outwards from the VZ to form the Purkinje cell plate (PCP). The PCP is initially several layers of cells thick and lies underneath the external granule layer (EGL).
- D) In early postnatal days (P), the PCP spreads to form the characteristic monolayer of Purkinje cells that exists in the adult.
- E) The adult cerebellum consists of ten lobules (I–X) from anterior to posterior that are grouped into four transverse zones (AZ, anterior zone; CZ, central zone; PZ, posterior zone; NZ, nodular zone).

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Similar to the RL, the VZ also generates different populations of neurons over time, and expression of a transcription factor, in this case *Ptf1a*, is important (Hoshino *et al.*, 2005; Pascual *et al.*, 2007). Lineage tracing of *Ptf1a*-expressing cells found labelling in GABAergic DCN neurons, Purkinje cells and some glial cells confirming their origin in the *Ptf1a*⁺ VZ (Hoshino *et*

al., 2005). In the *cerebelless* mutant, loss of *Ptf1a* expression in the cerebellum abolishes production of GABAergic cells from the VZ (Hoshino *et al.*, 2005). Moreover, lineage tracing revealed that in the absence of *Ptf1a*, VZ cells aberrantly migrate to the EGL and adopt features of that region (Pascual *et al.*, 2007). Together, these studies demonstrate that *Ptf1a* is necessary for correct specification of the GABAergic lineage in the cerebellum. In addition to *Ptf1a*, GABAergic progenitors in the VZ express the cell surface marker *Kirre-like nephrin family adhesion molecule 2* (*Kirrel2*), a direct downstream target of PTF1A (Mizuhara *et al.*, 2010).

In the VZ, *Ptf1a*⁺ progenitors give rise to a layer of neurons expressing LIM homeobox proteins, LHX1 and LHX5 (Chizhikov *et al.*, 2006) (Figure 1.3B). At early stages (by E12.5) the PTF1A⁺ layer in the VZ can be divided into two non-overlapping populations: a larger dorsal region expressing *Oligodendrocyte transcription factor 2* (*Olig2*), and a ventral region expressing *GS homeobox 1* (*Gsx1*) (Seto *et al.*, 2014) (Figure 1.3B). Lineage tracing found that *Olig2*⁺ cells give rise to SKI family transcriptional corepressor 2 (SKOR2)-expressing Purkinje cells, whilst *Gsx1*⁺ cells generate GABAergic interneurons (Seto *et al.*, 2014). During development, the *Olig2*⁺ Purkinje cell-producing region decreases in size, disappearing by E14.5 in mice, whilst the *Gsx1* expression expands dorsally, therefore altering the type of neurons generated (Seto *et al.*, 2014). Consequently, Purkinje cells are produced from around E10.5 to E13.5 whilst the majority of GABAergic interneurons are produced later. Gain- and loss-of-function mutants suggest *Gsx1* and *Olig2* acts in opposition, so this transition is driven by *Gsx1* and delayed by *Olig2* (Seto *et al.*, 2014). An early-born population of interneurons form the GABAergic component of DCN (Sepp *et al.*, 2023).

1.2.3 Purkinje cell development

Post-mitotic Purkinje cell precursors migrate from the apical VZ to the basal cerebellar surface to form a layer several cells thick, known as the Purkinje cell plate (Rahimi-Balaei *et al.*, 2018) (Figure 1.3C). The Purkinje cell plate is visible from E14.5 and spreads tangentially as the cerebellar cortex expands, to become a layer of Purkinje cells only one-cell thick by early in postnatal development (Rahimi-Balaei *et al.*, 2018). Plate formation is dependent on Reelin (RELN), an extracellular glycoprotein expressed by glutamatergic cells, first prospective DCN

neurons and later EGL cells (Miyata *et al.*, 2010). In *reeler* mice (*Reln*^{-/-}), initial migration of Purkinje cells occurs apparently normally, however the Purkinje plate fails to form at E14.5 as cells remain tangential to the surface (Goffinet, 1983; Miyata *et al.*, 2010).

The formation of afferent connections to Purkinje cells and development of their dendritic tree, occurs simultaneously in the first three postnatal weeks in mice (Kapfhammer, 2004; Leto *et al.*, 2016) (Figure 1.4). At birth, Purkinje cells are mostly bipolar 'simple-fusiform cells' with primitive apical dendrites that may be bifurcated (Armengol and Sotelo, 1991). In the first phase of dendritogenesis, Purkinje cell dendrites undergo intense remodelling: growth and then retraction of the primitive dendrites, followed by formation of somatic protrusions around the cell body (Armengol and Sotelo, 1991; Sotelo and Dusart, 2009). In the second phase, a single protrusion at the apical pole extends and other branches retract (Kapfhammer, 2004; Sotelo and Dusart, 2009). The apical dendritic tree branches and grows, initially spreading wider and then taller (Sotelo and Dusart, 2009). The formation of Purkinje cell dendritic trees is influenced by both intrinsic factors, such as expression of *Retinoid-related orphan receptor alpha* (*Rora*), and extrinsic factors, including thyroid hormones, tropic factors and stimulation from afferent fibres (Kapfhammer, 2004; Sotelo and Dusart, 2009). The early phase of dendritogenesis is thought to be largely intrinsic, whilst the second phase is also dependent on afferent fibre activity (Sotelo and Dusart, 2009).

Each mature Purkinje cell connects to around 100,000 parallel fibres via synapses on distal dendrites, but to just one climbing fibre via multiple synapses on proximal dendrites (Ito, 1984). However, neonatal Purkinje cells are initially contacted by multiple climbing fibres, which subsequently undergo refinement (Kano *et al.*, 2018) (Figure 1.4). Immature olivocerebellar axons project many fibres to Purkinje cells, such that at postnatal day (P) 2-3 around five climbing fibres connect to each Purkinje cell with equal strength synapses (Kano *et al.*, 2018). During the first postnatal week, one climbing fibre connection becomes strengthened relative to others (Hashimoto and Kano, 2013). The strongest climbing fibre then extends connections to Purkinje cell dendrites whilst other climbing fibre perisomatic connections are lost (Kano *et al.*, 2018). Climbing fibre elimination occurs initially

independently of granule cells, however from around P12 formation of parallel fibre-Purkinje cell connections are influential to this process (Sotelo and Dusart, 2009; Kano *et al.*, 2018).

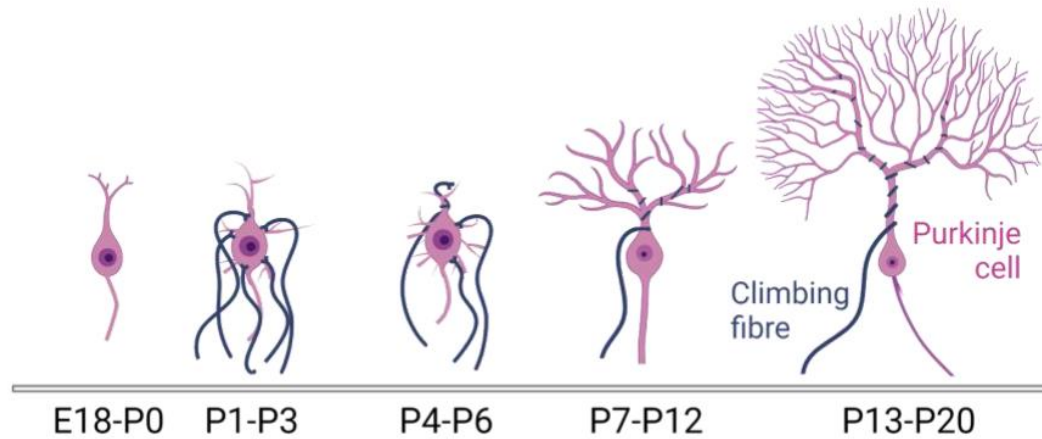


Figure 1.4 Development of Purkinje cell dendritic tree and climbing fibre innervation.

Schematic of Purkinje cell morphology from embryonic day (E) 18 to postnatal day (P) 20 in the developing mouse cerebellum.

Adapted from Kapfhammer, 2004. Created with BioRender.com.

1.2.4 Purkinje cell patterning

The cerebellum has a regular cytoarchitecture of cell layers and circuitry, however, it also displays spatially specialised functions mediated by distinct cell properties and connections (White and Sillitoe, 2013). Purkinje cells are not a homogeneous population instead forming clusters with differences in gene expression and electrical function (reviewed by Apsley and Becker, 2022). During embryonic development, Purkinje cells form a reproducible array of clusters that are first distinguished by timing of birth date and spatial location.

Purkinje cells are generated from the VZ between E10.5 and 13.5 and migrate sequentially to form the Purkinje cell plate (Leto *et al.*, 2016). Early-born Purkinje cells from the posterior VZ migrate only a short distance of around 200 μm and have been observed to move largely tangentially before at change of posture to move Purkinje cell axons perpendicular to the surface (Miyata *et al.*, 2010). In contrast, later born and more anterior Purkinje cells migrate further, a distance of up to 700-800 μm , and move radially towards the basal surface from the VZ (Morales and Hatten, 2006). Additionally, birth dating of Purkinje cells in mouse identified

specific mediolateral clusters formed from cells generated at E10.5, E11.5 and E12.5 respectively (Hashimoto and Mikoshiba, 2003) (Figure 1.5A). In general, more early-born Purkinje cells were found in lateral and dorsal clusters, and more late-born in medial and ventrally located clusters. However, all Purkinje clusters still contained a mixture of birthdates.

At a higher spatial resolution, studies integrating imaging of different markers have identified over 50 embryonic clusters based on location and molecular phenotypes (Fujita *et al.*, 2012b)(Figure 1.5B). Genes used for definition of clusters showed differential expression across Purkinje cells, including *Phospholipase C-beta 4 (Plcb4)*, *EPH receptor 4 (Epha4)*, *Protocadherin-10 (Pcdh10)*, and *Inositol 1,4,5-trisphosphate receptor type 1 (Itpr1)* (Fujita *et al.*, 2012b; Tran-Anh *et al.*, 2020) (Figure 1.5C). However, it is not yet clear how the overall gene expression and functional phenotypes of all of these clusters differ.

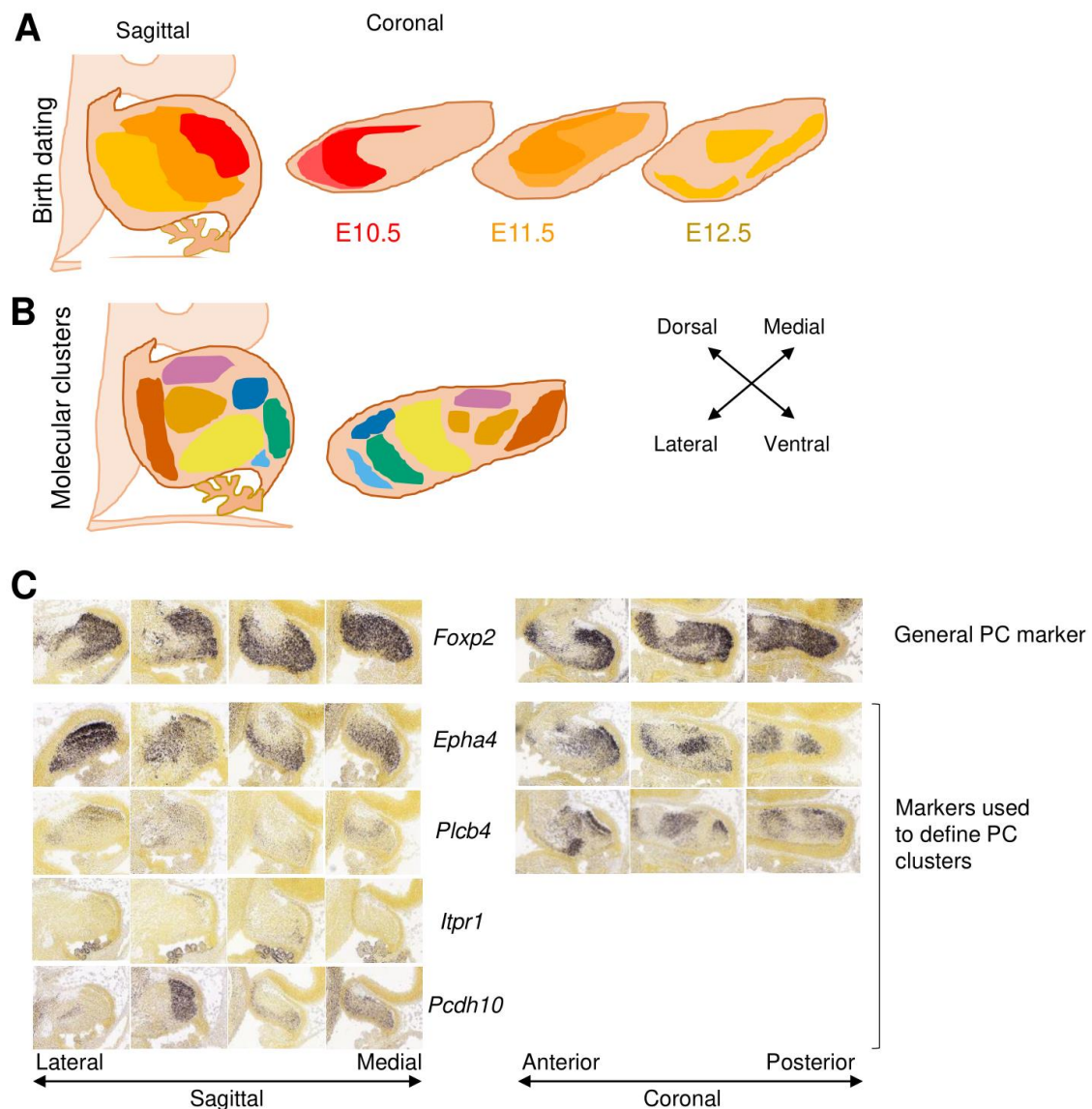


Figure 1.5 Purkinje cell embryonic clusters.

- A) At embryonic day (E) 15.5 Purkinje cell (PC) clusters can be defined by lineage tracing of different Purkinje cell birthdates E10.5 (red), E11.5 (orange), E12.5 (yellow).
- B) Alternatively, E15.5 clusters can be defined by expression of different combinations of molecular markers. Colours reflect different molecular clusters identified by Fujita *et al.*, 2012. Only one hemisphere of the cerebellum is shown in coronal diagrams.
- C) Distribution of Purkinje cell markers at E15.5: *Foxp2* is broadly expressed in most Purkinje cells. *Epha4*, *Plcb4*, *Pcdh10*, and *Itpr1* are differentially expressed and often used as molecular markers to define distinct clusters. Images of sagittal and coronal sections from Allen Developing Mouse Brain Atlas (<https://developingmouse.brain-map.org/> accessed on 7 July 2022) are shown sequentially across lateral-medial and anterior-posterior axis. Coronal sections were not available for *Pcdh10* and *Itpr1*.

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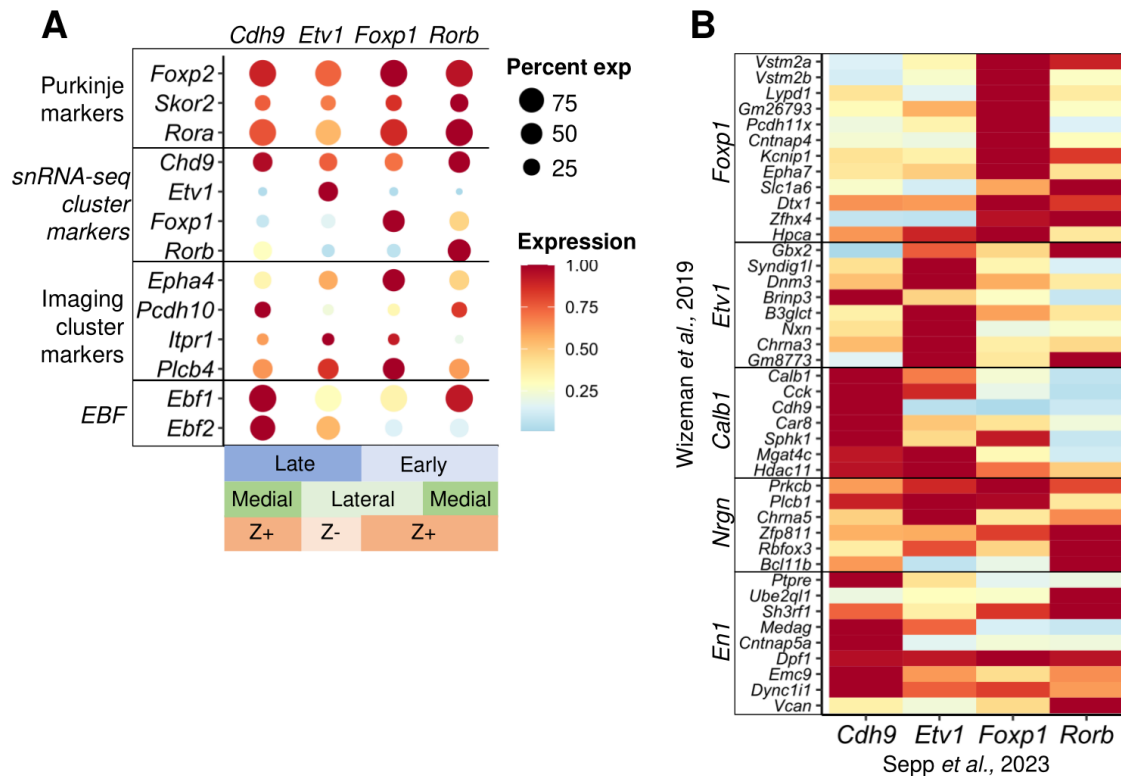


Figure 1.6 Mouse embryonic Purkinje cell clusters defined by single-cell transcriptomics.

- A) Sepp *et al.*, 2023 define four Purkinje cell subtypes in the developing mouse cerebellum, based on transcriptomic differences and labelled by differentially expressed markers *Cdh9*, *Etv1*, *Foxp1*, and *Rorb*. All subtypes show broad expression of Purkinje cell markers (*Foxp2*, *Skor2*, *Rora*) but variable expression of genes previously used in imaging studies to define clusters (*Epha4*, *Pcdh10*, *Itpr1*, *Plcb4*). The different subtypes can also be defined by the combination of *Ebf1* and *Ebf2* expression. In addition, subtypes differ with regards to birth date, location, and predicted adult Zebrin (Z) phenotype. Dot size scales for the percentage of cells in cluster expressing gene. Expression of each gene is normalized across samples.
- B) Correlation between embryonic Purkinje cell subtypes identified by single-cell transcriptomic studies of the developing mouse cerebellum. Wizeman *et al.* 2019 found genes differentially expressed by five Purkinje cell subtypes (y axis). The average expression of these genes is shown across the four Purkinje cell subtypes identified by Sepp *et al.*, 2023 (x axis). Expression indicated by colour scale as (A).

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Single cell RNA sequencing (scRNA-seq) studies of the developing cerebellum have identified four or five embryonic Purkinje subtypes based on gene expression, which were then also related to birth date and spatial location (Wizeman *et al.*, 2019; Sepp *et al.*, 2023). Early-born clusters were identified by high expression of *Foxp1* and *Rorb*, whilst late-born clusters were identified by *Chromodomain helicase DNA binding protein 9* (*Chd9*) and *ETS variant transcription factor 1* (*Etv1*) (Sepp *et al.*, 2023) (Figure 1.6A). Spatially, *Foxp1* and *Etv1* showed

more lateral localisation whilst *Rorb* and *Chd9* expression was more medial (Sepp *et al.*, 2023). Furthermore, clusters were distinguished by expression of *EBF transcription factor (Ebf)* genes, *Ebf1* and *Ebf2* corresponding with spatial and birthdates respectively (Sepp *et al.*, 2023). *Ebf1* was higher in medial clusters (*Rorb* and *Chd9*), whilst *Ebf2* was higher in late-born clusters (*Chd9* and *Etv1*) (Sepp *et al.*, 2023)(Figure 1.6A). A similar combination of Purkinje cell clusters were identified in E13.5 mouse cerebellar scRNA-seq, with clusters also labelled by *Foxp1* and *Etv1*, along with correlation between Wizeman *et al.*, 2019 *Calbindin 1 (Calb1)* and *En1* clusters to the Sepp *et al.*, 2023 *Chd9* cluster (Figure 1.6B).

From ~E18 embryonic clusters of Purkinje cells disperse, and in the adult cerebellum Purkinje cell populations are grouped into parasagittal stripes. The best characterised marker of adult Purkinje cell stripes is Zebrin II (Z), labelling the enzyme Aldolase C (ALDOC) (Brochu, Maler and Hawkes, 1990). Zebrin positive (Z+) and negative (Z-) Purkinje cells form vertical stripes across the cerebellum, however spacing is such that posterior cerebellum contains more Z+ and the anterior more Z- (Apps *et al.*, 2018) (Figure 1.7). In addition to differential zebrin expression, Z+ and Z- Purkinje cells also display other differences in gene expression and electrical phenotypes. Markers including Protein kinase C-delta (PKC δ), Excitatory amino-acid transporter 4 (EAAT4), Phospholipase C-beta 3 (PLC β 3), Neuronal calcium sensor 1 (NCS1), GABA B receptor 2 (GABABR2) are expressed in the Z+ population, whereas others such as PLC β 4, Metabotropic glutamate receptor 1b (mGluR1b), and Neurogranin (NRGN) are expressed in a complementary pattern in the Z- cells (Cerminara *et al.*, 2015). As a consequence of the molecular differences, Z- Purkinje cells have increased intrinsic excitability and a higher rate of both simple and complex spike firing than Z+ cells (Zhou *et al.*, 2014; Nguyen-Minh, Tran-Anh and Sugihara, 2020) (Figure 1.7). Cerebellar functions also show regional differences as a result of the arrangement of connections (Stoodley and Schmahmann, 2010). Therefore, the electrical differences between Purkinje cell subtypes may help mediate different functions. Additionally, the differences in Purkinje cell populations results in selective vulnerability in which functions may be impaired in disease (Cerminara *et al.*, 2015; Hawkes, 2021). For example, greater cell death occurs in the largely Z- anterior cerebellum in patients with autosomal-recessive spastic ataxia of Charlevoix-Saguenay

(ARSACS) and Niemann-Pick type C disease (Sarna *et al.*, 2003; Toscano Márquez *et al.*, 2021).

Whilst the more Z+ posterior cerebellum is heavily affected in the *nervous* mouse mutant (Wassef *et al.*, 1987; Cerminara *et al.*, 2015).

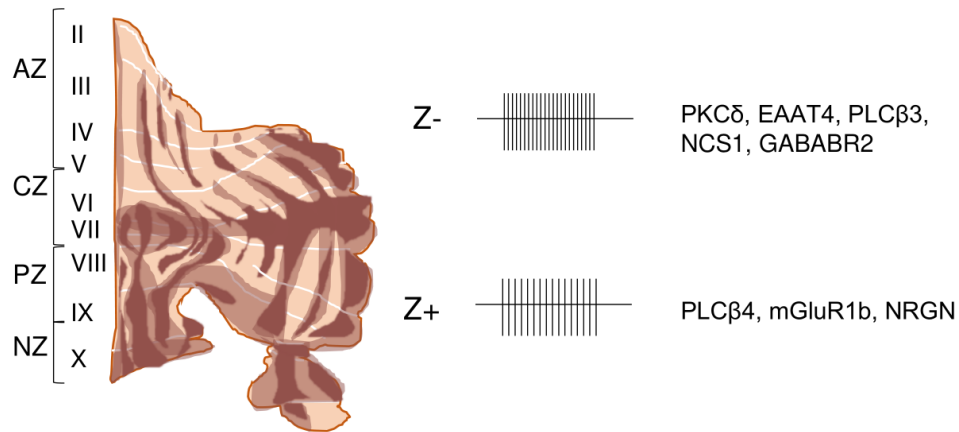


Figure 1.7 Adult Purkinje cell stripes.

Zebrin (Z) stripes define Purkinje cell populations in the adult mouse cerebellum. Stripes are parasagittal with most of the Z+ cells located in the posterior cerebellum. Cerebellar transverse zones (anterior zone, AZ; central zone, CZ; posterior zone, PZ; nodular zone, NZ) and lobules (I-X) are labelled. A complete description of the zebrin map is provided in Apps *et al.*, 2018. Zebrin populations show differences in electrical activity, including simple spike frequency, and gene expression as indicated. Z- markers include Protein kinase C-delta (PKCδ), Excitatory amino-acid transporter 4 (EAAT4), Phospholipase C-beta 3 (PLCβ3), Neuronal calcium sensor 1 (NCS1), GABA B receptor 2 (GABABR2). Z+ markers include Phospholipase C-beta 4 (PLCβ4), Metabotropic glutamate receptor 1b (mGluR1b), and Neurogranin (NRGN).

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scRNA-seq has also expanded knowledge of adult Purkinje cell subtypes. scRNA-seq of samples from 16 regions across the adult mouse cerebellum identified nine Purkinje cell clusters based on gene expression (Kozareva *et al.*, 2021). Of these, seven clusters were Z+ and only two Z-, indicating there may be higher diversity amongst Z+ Purkinje cells (Kozareva *et al.*, 2021).

Greater Z+ heterogeneity may contribute to diverse non-motor functions performed by the posterior cerebellum. In targeted single nuclei RNA sequencing (snRNA-seq) of mouse Purkinje cells, two major populations were identified as Z+ (*Aldoc*+) and Z- (*Plcb4*+) and found to be enriched in the posterior cerebellum and anterior vermis respectively (Chen *et al.*, 2022).

Markers of the two populations also included *Carbonic anhydrase 7* (*Car7*) and *Contactin 5* (*Cntn5*) for Z+ and *Plcb1*, *Ebf2* and *Potassium voltage-gated channel* (*Kcnab1*) for Z- (Chen *et*

al., 2022). Interestingly, transcriptomic changes in response to motor activity and learning were only detected in Z- populations (Chen *et al.*, 2022). There was a shift in the proportion of cells in different subclusters, with an increase in clusters enriched for expression of genes including immediate early gene *Fos*, postsynaptic *Metabotropic glutamate receptor 5* (*Grm5*) and FGF signalling pathway components (Chen *et al.*, 2022). Moreover Z- Purkinje cell function was shown to be essential for associated learning, and dependent on increased FGF signalling (Chen *et al.*, 2022). Thus, understanding how Purkinje cell populations are defined and develop is a necessary part of understanding cerebellar function. Whilst mapping embryonic Purkinje cell clusters to adult stripes is complex, correlation between snRNA-seq studies suggested the early-born *Foxp1* and *Rorb* clusters give rise to subtypes of Z+ Purkinje cells enriched in the cerebellar hemispheres (Sepp *et al.*, 2023). The late-born clusters differed, with the *Cdh9* cluster correlating to a subtype of Z+ Purkinje cells enriched in the posterior vermis, and the *Etv1* cluster with a Z- Purkinje cell subtype (Sepp *et al.*, 2023).

The specialisation of Purkinje cells into different subtypes increases the complexity of modelling cerebellar development *in vitro*. There has not yet been any characterisation of whether iPSC-derived Purkinje cells represent a particular subtype or include multiple clusters, and whether there is any 'birthdate' effect, with differing Purkinje cells generated over time. scRNA-seq studies have increased knowledge of Purkinje cell embryonic clusters, enabling characterisation of how *in vitro* models compare to these. In turn, *in vitro* models may help inform understanding of how Purkinje cell clusters arise, and which features are independent of afferent signalling.

1.2.5 Human specific features of cerebellar development

The previous descriptions have focused on the development of the mouse cerebellum, which is accessible for detailed characterisation and provides a useful model for cerebellar development. Indeed, the human cerebellum shows many similarities, including in the layered cytoarchitecture, overall cerebellar organisation, and many developmental pathways. However, the human cerebellum also shows significant differences to the mouse.

A major difference is in the neuron density and complexity of the adult cerebellum. The number of neurons in the cerebellum is expanded in humans, making up around 80% of the total neurons in the brain in humans compared to only 60% in mouse (Herculano-Houzel, 2010). To fit the additional neurons, the human cerebellum has more complex foliation and a correspondingly increased surface area (Figure 1.8A). Even compared to close evolutionary relatives, the surface area of the human cerebellum is vastly increased, measuring 78% of the neocortex area in human compared to 33% in macaques (Sereno *et al.*, 2020). In addition, the ratio of neuron types in the adult human cerebellum is altered with the granule cell to Purkinje cell ratio of 3000:1 in comparison to 200:1 in mouse (Lange, 1975).

Secondly, human development takes place over years compared to periods of weeks in mouse. Cerebellar development starts by 30 days post conception and continues for two years after birth in humans (Haldipur *et al.*, 2019). In comparison, mouse cerebellar development is completed within three weeks after birth. Importantly, many key processes that occur postnatally in mice occur *in utero* in humans, for example peak granule cell progenitor proliferation and afferent connections innervation, making the human cerebellum particularly sensitive to damage in the final trimester and at birth (Haldipur, Dang and Millen, 2018).

During development, there are also differences in neuron generation in the human cerebellum. Increased precursor populations located in discrete sub-ventricular zones (SVZ) in both germinal zones have been observed in human but not in mice (Figure 1.8B & D). In the mouse VZ, there is a single layer of progenitors expressing SRY-box 2 (SOX2) and marker of proliferation Ki-67 (KI67), and mitosis is also restricted to the ventricular edge (Haldipur *et al.*, 2019)(Figure 1.8B). In comparison, SOX2+KI67+ progenitors expand in the human VZ forming a

SVZ, with mitotic cells observed scattered throughout the VZ and VZ^{SVZ} (Haldipur *et al.*, 2019)(Figure 1.8B). Perhaps as a result of the expanded GABAergic progenitor pool, the peak proportion of Purkinje cells within the cerebellum is around two-fold higher in human than mouse based on the integration of multiple scRNA-seq studies (Sepp *et al.*, 2023) (Figure 1.8C). Another factor in the early peak in Purkinje cell numbers, may be a shift in the proportions of different Purkinje cell subtypes in humans. Transcriptomic analysis of human Purkinje cells identified early- and late-born subclusters identified by differing expression of *EBF1* and *EBF2*, both low in early-born and high in late-born (Sepp *et al.*, 2023). Further analysis of a second human dataset found all four Purkinje cell subclusters as identified in mouse (Aldinger *et al.*, 2021; Sepp *et al.*, 2023). Moreover, spatial localised expression of late-born marker *EBF2* and subcluster markers *ETV1* (lateral) and *CHD9* (medial), was described in 12 post conception week (PCW) human cerebellum as in mouse (Sepp *et al.*, 2023). However, early-born subcluster markers *FOXP1* and *RORB* had widespread expression in 12 PCW human cerebellum (Sepp *et al.*, 2023). Therefore, there is less clear evidence for higher resolution subdivision of human Purkinje cells, however the importance of birth date and location is evident. Combined analysis of multiple snRNA-seq datasets, found an increase in the ratio of early-born to late-born Purkinje cells in human compared to mouse and opossum (Sepp *et al.*, 2023).

Similarly, the human rhombic lip also is divided with SOX2+KI67+ progenitors in the RL^{VZ} and a SOX2-low KI67+ RL^{SVZ} from 10 PCW (Haldipur *et al.*, 2019) (Figure 1.8D). A further striking difference in the human RL is its expanded shape and persistence. The mouse rhombic lip is transient and disappears before birth. In contrast, the human RL elongates at 10 PCW and around 13-14 PCW integrates into the posterior lobule where a proliferative zone persists until 36 PCW (Haldipur *et al.*, 2019). The altered RL-morphology has been implicated in increased risk of cerebellar malformations such as the Dandy-Walker Syndrome, and the long-term presence of RL-progenitors associated with medulloblastoma (Haldipur *et al.*, 2019). Therefore, modelling human cerebellar progenitors and their differentiated neuron progeny, is necessary to study mechanisms and develop treatments for these cerebellar diseases.

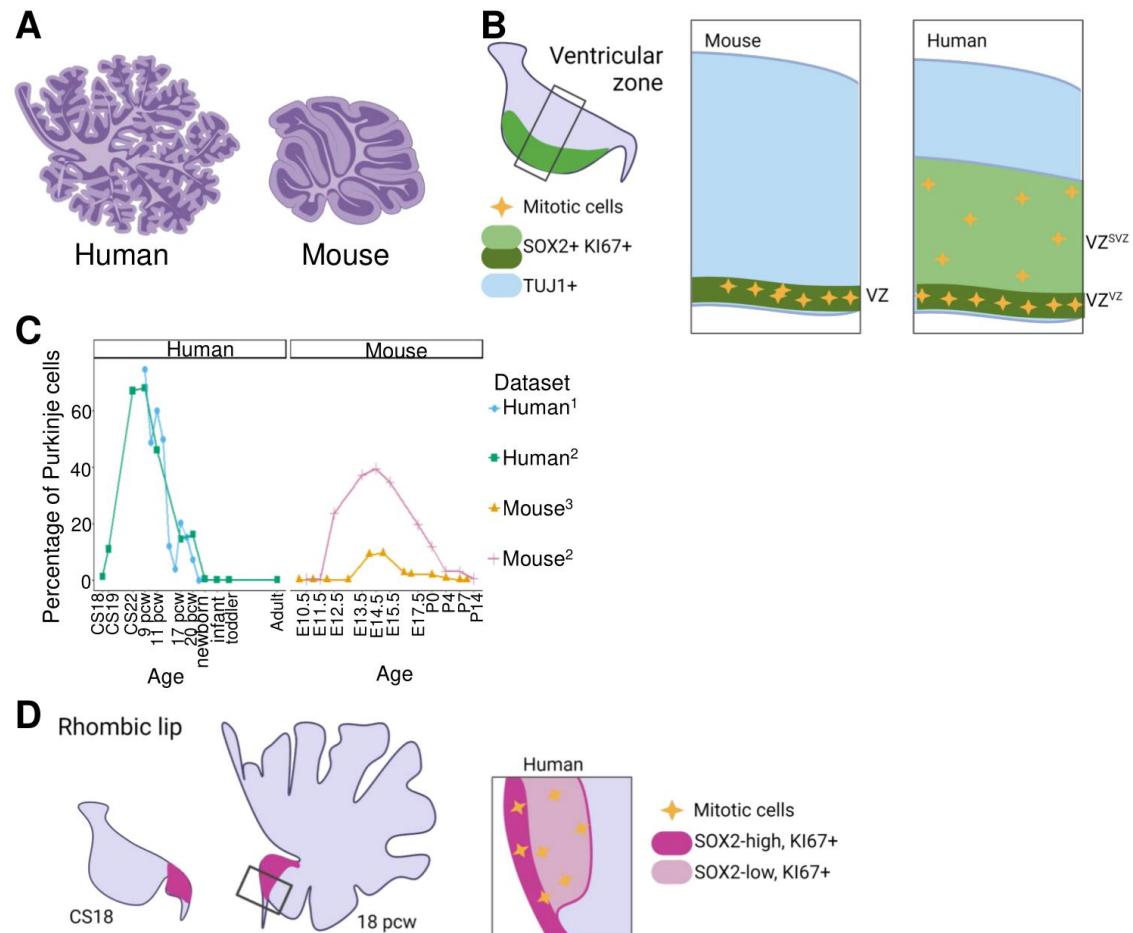


Figure 1.8 Differences between the human and mouse cerebellum.

- A) Schematic of cerebellar foliation in human and mouse.
- B) The human ventricular zone (VZ) contains a subventricular zone (SVZ) formed of SOX2+KI67+ progenitors not found in the mouse cerebellum. Mitosis is localised to a single layer on the ventricular surface in mouse but occurs throughout the VZ in human.
- C) The peak proportion of Purkinje cells during cerebellar development is around two-fold higher in human than mouse. The Purkinje cell population is plotted as percentage of total cells over development. Data from scRNA-seq datasets from human (1 - Aldinger *et al.*, 2021, 2 - Sepp *et al.*, 2023) and mouse (3 - Carter *et al.*, 2018, 2 - Sepp *et al.*, 2023). Timepoints labelled as Carnegie stage (CS), post conception week (pcw), embryonic day (E) or postnatal day (P).

- D) The human rhombic lip (RL) integrates into the posterior lobule and is subdivided into a SOX2-high KI67+ layer and the RL^{SVZ} containing SOX2-low KI67+ progenitor cells.

A, B & D adapted from Haldipur *et al.*, 2019, and created with BioRender.com. C is reproduced with permission from Apsley and Becker, 2022, under a Creative Commons License (CC BY 4.0 DEED).

Finally, RNAseq studies have provided detailed comparison of gene expression between human and other species. Overall, cell types show largely common expression patterns, with some repurposing of gene expression between cell types found across species (Sepp *et al.*, 2023). Only a few genes have been shown to be specifically lost or gained in the human cerebellum. Genes confirmed by single-molecular fluorescent *in situ* hybridisation (smFISH) to be present in human Purkinje cells but absent in mouse included *OSBPL3*, *NCAPG*, *PAQR8*, *MST1*, *DSC2* and *AP1S3* (Sepp *et al.*, 2023). Most of the genes gained in evolution were found to be expressed later in development (Sepp *et al.*, 2023). Interestingly, the subset of genes gained in the human lineage which instead showed high early expression were enriched in germinal zone progenitor populations, including *Piezo type mechanosensitive ion channel component 2* (*PIEZO2*) which showed human-specific expression in VZ progenitors and the posterior VZ. These changes were suggested to contribute to the formation of the human-specific SVZ progenitor pools (Sepp *et al.*, 2023). Therefore, studying the expression of these genes gained in the human astroglia lineages in iPSC-derived cerebellar organoids could provide insight into the development and regulation of germinal zone subventricular zones in the developing human cerebellum.

1.3 Generation of cerebellar organoids from human iPSCs

Primary cultures of cells derived from animal brains can be maintained *in vitro* for periods of days to weeks and recapitulate some *in vivo* processes. In cerebellar research, both dissociated cell cultures and organotypic slices from rodent cerebellum have been used. These systems allow the effect of individual factors to be studied, for example, the role of different exogenous signalling factors in Purkinje cell dendritic tree development (Kapfhammer, 2004). However, primary neuron cultures typically require a supply of embryonic or early postnatal animals and culture survival is limited due to the differentiated state of the starting cells. In addition, animal cells cannot model the full genetic background, cellular timing and differentiation trajectories of the human brain (Sidhaye, 2020).

In addition to the differences between human and mouse cerebellar development highlighted, there is particular demand for human-specific brain models in drug development. Only three

percent of drugs for nervous system disorders entering phase I clinical trials between 2010 and 2017 were successfully launched, indicating the failure of preclinical testing (Dowden and Munro, 2019). Therefore, better preclinical human models are necessary for both studying mechanisms and screening drugs to address neurodevelopmental disorders.

1.3.1 *In vitro* models for human brain development

The isolation of human embryonic stem cells (hESC) provided a breakthrough in the study of human development (Thomson *et al.*, 1998). Capable of differentiating into all three germ layers, hESCs can be used to generate human neurons *in vitro* (Zhang *et al.*, 2001).

Subsequently the ability to reprogram somatic cells to iPSCs provided greater access to human pluripotent stem cells, including the ability to generate patient-derived lines (Takahashi *et al.*, 2007).

Early methods for differentiation of hESCs to neural cells started with formation of hESC aggregates, known as embryoid bodies, before plating in the presence of FGF2 to generate neural rosettes (Zhang *et al.*, 2001). Rosettes showed basic polarity and generation of sequential neural progenitor types, but lacked many neuron types and clear layer organisation (Zhang *et al.*, 2001). Dual SMAD inhibition was also found to promote differentiation of hESCs to neural cells in 2-dimensions (2D) (Chambers *et al.*, 2009). However, these 2D methods had limitations including restricted cell-cell interactions and migration, lack of cell type diversity and short culture periods. Moving from 2D to 3D, designed medium conditions (serum-free floating culture of embryoid body-like aggregates, SFEBq) were used to differentiate embryoid bodies to neural forebrain identity in suspension (Watanabe *et al.*, 2005). Further adaptations of the SFEBq system, including the use of an extracellular matrix Matrigel, led to the first brain organoid models with self-organised polarised epithelium and layered progenitor zones (Eiraku *et al.*, 2011; Kadoshima *et al.*, 2013; Lancaster *et al.*, 2013).

Brain organoid approaches are broadly classified as intrinsic or directed differentiation. The intrinsic or whole-brain organoid approach developed by the Knoblich group, is more reliant on self-induction after initial patterning to neuroectoderm. This leads to a predominantly

forebrain identity, with heterogenous development of other brain regions and patterning centres (Lancaster *et al.*, 2013; Renner *et al.*, 2017). Directed brain organoids use more growth factors to focus differentiation towards specific brain regions, for example forebrain, midbrain or cerebellum (Muguruma *et al.*, 2015; Pasca *et al.*, 2015; Jo *et al.*, 2016; Qian *et al.*, 2016). Regional brain organoids typically have less heterogeneity, both within the organoid and between batches, due consistent signalling from exogenous growth factors making them more suitable for drug screening (Amin and Pasca, 2018). However, the increased use of growth factors often leads to smaller neuroepithelium and may less accurately reflect the self-induction that occurs *in vivo* and this should be considered when using them to study development (Chiaradia and Lancaster, 2020; Sidhayee, 2020).

1.3.2 Cerebellar organoid development and protocol

The first cerebellar organoids cultured ESCs using the SFEBq approach, in medium adapted from a chemically defined composition previously demonstrated to induce rostral hypothalamic identity (Wataya *et al.*, 2008; Muguruma *et al.*, 2010). Cerebellar organoids were initially generated using mouse ESCs, before the protocol was optimised for human ESCs (Muguruma *et al.*, 2010, 2015). The description below will focus on the human protocol timings and factors (Figure 1.9A).

Specification of cerebellar identity required a combination of growth factors added to a chemically defined medium (Muguruma *et al.*, 2010, 2015)(Figure 1.9A). In particular, a brief pulse of FGF2, added at day two of differentiation, was important to patterning a cerebellar fate (Muguruma *et al.*, 2015). FGF2 induces expression of WNT and FGF8 signalling, characteristic of the isthmic organiser *in vivo* (Sato, Joyner and Nakamura, 2004; Muguruma *et al.*, 2015). Moreover, a multiplexed screen of growth factors in brain organoid differentiation also independently identified FGF2 as a driver of cerebellar neuron production (Amin *et al.*, 2023). In addition, insulin acts cooperatively with FGF2 to promote hindbrain identity in cerebellar organoid differentiation (Muguruma *et al.*, 2010). Insulin has been shown to have a caudalising effect on ESC neural differentiation, increasing expression of MHB genes such as *En1* (Wataya *et al.*, 2008; Muguruma *et al.*, 2010). As well as factors targeted at cerebellar

identity, differentiation of EBs to cerebellar organoids uses Transforming growth factor- β inhibitor SB431542, commonly included in brain organoid methods to block meso-endoderm differentiation (Chiaradia and Lancaster, 2020). In addition, anti-apoptotic factor rock inhibitor (Y-27632) is commonly used to support survival in suspension culture for the first week (Watanabe *et al.*, 2007). In mouse cerebellar organoids, SHH inhibitor cyclopamine was required to block ESC-derived ventralising SHH signals and generate SKOR2+ Purkinje cells (Muguruma *et al.*, 2010). However, cyclopamine did not enhance production of KIRREL2+ VZ progenitors in human cerebellar organoids and reduced production of ATOH1+ granule cells precursors, so it is not included for human stem cell derived-cerebellar organoids (Muguruma *et al.*, 2015).

Cerebellar organoids approximately follow the trajectory of *in vivo* cerebellar development: first showing markers of the MHB, then markers of germinal zone progenitors and later markers of specific neuron populations. By day 21 of differentiation, cerebellar organoids express markers of the MHB, such as EN1 and GBX2 (Wurst, Auerbach and Joyner, 1994; Wassarman *et al.*, 1997) (Figure 1.9B). Markers of both cerebellar germinal zones are present by day 35 of differentiation: KIRREL2 marking the GABAergic VZ lineage (Mizuhara *et al.*, 2010) and PAX6 glutamatergic RL derivatives (Engelkamp *et al.*, 1999) (Figure 1.9C). Cerebellar organoids can be maintained in culture for more than 100 days of maturation, using a range of methods discussed in more detail in Chapter 4 (Figure 1.9D). At day 63, amongst other cerebellar cell types, cerebellar organoids contain neurons positive for markers of Purkinje cells: FOXP2 and CALB1 (Jande, Tolnai and Lawson, 1981; Fujita and Sugihara, 2012) (Figure 1.9E). The diversity of cerebellar cell types represented has been confirmed by scRNA-seq of day 90 cerebellar organoids (Nayler *et al.*, 2021). This study annotated clusters for all major cerebellar cell types, including key neuron types Purkinje and granule cells, as well as additional neuron classes and non-neuronal cells, such as endothelial cells and Bergmann glia (Nayler *et al.*, 2021) (Figure 1.9F).

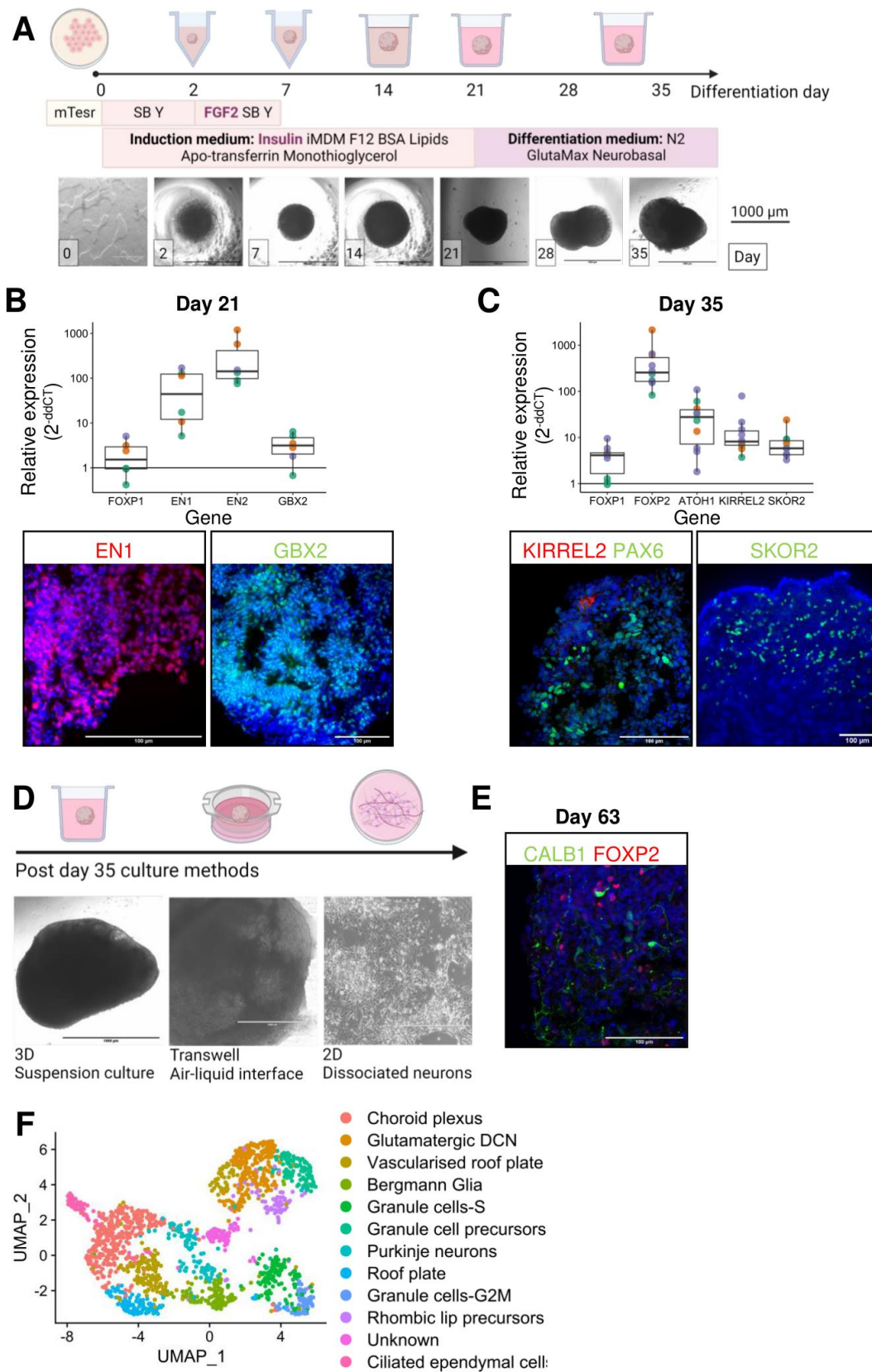


Figure 1.9 Generation of cerebellar organoids from human iPSCs to recapitulate cerebellar development.

Figure 1.9 Generation of cerebellar organoids from human iPSCs to recapitulate cerebellar development.

A) Protocol for generation of cerebellar organoids. Differentiation starts with formation of embryoid bodies from human iPSCs and patterning with growth factors towards cerebellar identity. Key factors include Fibroblast growth factor 2 (FGF2), TGF β inhibitor SB431542 (SB), anti-apoptotic factor rock inhibitor, Y-27632 (Y) and insulin. Full medium compositions are given in section 2.3. Brightfield images show typical organoid development. Scale bar = 1000 μ m.

To monitor cerebellar organoid differentiation, quality control is performed at day 21 (B) and day 35 (C). Gene expression was quantified by RT-qPCR, normalised to housekeeper genes (*ACTIN*, *GAPDH*) and calculated relative to day 0 (iPSCs) as $2^{-\Delta\Delta CT}$. Points represent individual differentiations and colours indicate samples from three different control iPSC lines. Sections of cerebellar organoids were immunostained with antibodies for cerebellar markers and nuclei stained with Hoechst (blue). Scale bars = 100 μ m.

B) At day 21 cerebellar organoids express markers of the mid-hindbrain boundary (*EN1*, *EN2* & *GBX2*) and some early markers of the cerebellar germinal zones (e.g. ventricular zone marker *FOXP1*). Organoid sections were stained using antibodies against EN1 (red) and GBX2 (green).

C) By day 35 markers of cells from both cerebellar germinal zone lineages are upregulated: including GABAergic ventricular zone cells (*KIRREL2*, *SKOR2*, *FOXP1* & *FOXP2*) and glutamatergic rhombic lip derivatives (*ATOH1*). Generation of cerebellar neurons was further shown by staining with antibodies against KIRREL2 (red) and PAX6 (green) or SKOR2 (green).

D) For extended culture, cerebellar organoids can be maintained in 3D either continuing in suspension or at the air-liquid interface, or alternatively dissociated and plated as neurons. These methods are discussed further in Chapter 4.

E) Sections from day 63 cerebellar organoids were stained using antibodies against Purkinje cell markers FOXP2 (red) and CALB1 (green).

F) Nayler *et al.*, 2021 performed scRNA-seq on day 90 cerebellar organoids, identifying 12 clusters representing major cerebellar cell types. DCN, deep cerebellar nuclei; G2M, post mitotic; S, Proliferative.

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Cerebellar organoids therefore provide a model for early human cerebellar development, including mimicking specification of the cerebellar anlage and generation of major cerebellar cell types. The 3D structure of organoids facilitates a high density of cell-cell interactions, including synaptic signalling. Moreover, after initial FGF2 and insulin treatment towards MHB identity, cerebellar organoids are reliant on self-inductive patterning resulting in small luminal rosette structures, offering greater similarity to in vivo architecture than 2D cultures.

Furthermore, several groups have independently grown cerebellar organoids with similar results demonstrating robust reproducibility (Muguruma *et al.*, 2015; Watson *et al.*, 2018; Silva *et al.*, 2020; Chen *et al.*, 2023).

1.3.3 Limitations of current protocols

Cerebellar organoids are useful for study of both developmental processes and disease. However, as with all models, there are limitations and challenges to the application of cerebellar organoids.

Brain organoid models share common challenges, including inherent variability, disorganised tissue architecture, and lengthy culture time. Variability is introduced at multiple levels and should be considered in experimental design. iPSC lines perform differently in differentiation due to different genetic backgrounds, reprogramming methods and previous handling in culture. Therefore, comparison of patient to control lines should ideally involve several clones from multiple donors. Alternatively, isogenic lines provide a common genetic background to isolate mutations of interest. However, generation of multiple lines is expensive and time consuming and therefore not always feasible, for example when generating reporter lines. The majority of work in this thesis was performed using a single well-characterised control iPSC line, AH0-17-3 (Handel *et al.*, 2016). However, the cerebellar organoid protocol was demonstrated using three control iPSC lines (Figure 1.9B & C). Batches of organoids grown in separate differentiations typically also show greater variation, this can partly be addressed by use of defined protocols and quality control, as well as inclusion of organoids from different batches. The presence of multiple cell types is both an advantage and challenge in organoid models, on one hand better reflecting *in vivo* tissues, but also adding variability in analysis (Chiaradia and Lancaster, 2020). The cell type composition varies between individual organoids, even within the same batch adding a high level of heterogeneity to bulk readouts. Therefore, the use of cellular-resolution metrics or isolation of cell populations is required for characterising more subtle phenotypes (Atamian, Córdón-Barris and Quadrato, 2020). This is particularly true when studying a small population, such as the Purkinje cells within cerebellar organoids.

Another challenge to more accurately model *in vivo* development is the formation of tissue architecture. Cerebellar organoids self-pattern to a limited extent forming rosette structures with small lumens that display polarity, for example KIRREL2+ staining is observed within the

centre of rosettes (Muguruma *et al.*, 2015) (Figure 1.9C). However, the tightly organised layered structure of the cerebellum is not recapitulated. The lack of cytoarchitecture makes cell type identification more challenging and means processes such as cell migration cannot be studied. Additionally, the precise circuitry of the cerebellum is not feasible as cell types interact variably. Future approaches, such integration of engineered signalling centres may provide the spatial constraints needed for greater organisation within organoids (Cederquist *et al.*, 2019).

Human brain development requires a period of over two years to complete, similarly brain organoid cultures typically require months for processes such as astrogenesis and neural activity to develop (Pasca *et al.*, 2015; Quadrato *et al.*, 2017). Moreover, current cerebellar organoids reflect only immature stages of cerebellar development even with prolonged culture for over 90 days (Nayler *et al.*, 2021). Therefore, in this thesis different approaches are explored to increase maturity, such as co-culture, 2D plating and chemical treatment.

1.4 *FOXP*s in the cerebellum

FOXP2 was the first gene to be associated with speech and language development, identified over two decades ago (Lai *et al.*, 2001). Following its discovery, research has aimed to uncover how *FOXP2* acts in development of speech to aid wider understanding of speech and language development. However, despite considerable work, there is still relatively little understanding of the molecular targets of *FOXP2* and how downstream pathways contribute to language development (Hoed, Devaraju and Fisher, 2021). In particular, despite evidence for cerebellar changes in response to *FOXP2* disruption, knowledge of the role of *FOXP2* and related *FOXP* family members in the cerebellum is limited. Human iPSC-derived cerebellar organoids provide a platform for investigating *FOXP2* in the early human cerebellum when expression is highest. Therefore, in this section the structure, expression and available models of *FOXP2* function will be reviewed.

1.4.1 *FOXP* family genes and proteins

FOXP2 is a member of the forkhead family of transcription factors, that have important roles in development of a range of organ systems, including the pulmonary, cardiac, neuronal and immune systems (Co, Anderson and Konopka, 2020). As the naming suggests, *FOXP* family members share a common forkhead domain which is highly conserved and binds DNA at a recognised motif sequence (Lai *et al.*, 2001; Stroud *et al.*, 2006). In addition, *FOXP1*, 2 and 4 have a common leucine zipper domain, polyglutamine tract and zinc finger domain (Figure 1.10). The leucine zipper domain facilitates the formation of dimers which are essential for the DNA-binding and transcriptional repression by *FOXP* proteins (Li, Weidenfeld and Morrissey, 2004). *FOXP2* can form homodimers, or heterodimers with *FOXP1* or *FOXP4*, with combinations differentially regulating target genes (Li, Weidenfeld and Morrissey, 2004; Sin, Li and Crawford, 2015). *FOXP2* also interacts with a range of other transcriptional regulators, including the Nucleosome remodelling and deacetylase complex (NuRD) and transcription factors such as Nuclear factor 1 proteins (NFIA/B) and Nuclear receptor subfamily 2 member, NR2F2 (Chokas *et al.*, 2010; Estruch *et al.*, 2018; Hickey, Berto and Konopka, 2019).

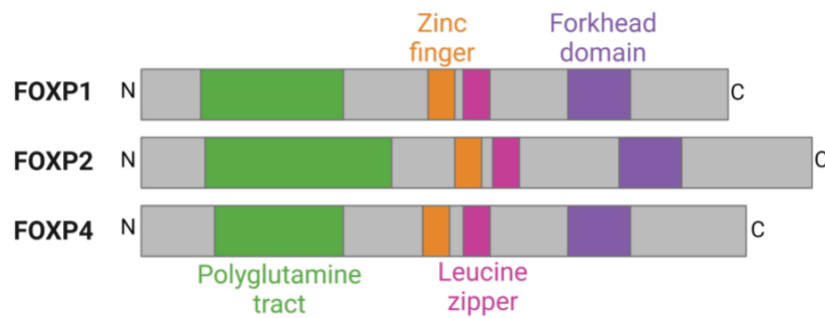


Figure 1.10 FOXP protein structure.

FOXP family proteins, FOXP1, FOXP2 and FOXP4, show conserved structure. All three contain a polyglutamine tract (green), zinc finger (orange), leucine zipper (pink) and forkhead (purple) domains.

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1.4.2 Expression of *FOXP* genes in the developing brain

FOXP1, *2* and *4* all show high expression in the developing brain (Figure 1.11). Earliest expression of *Foxp2* in mouse brain was detected in the area of the future medulla at E11.5, followed by expression in the cerebellum, striatum, medulla and thalamus at E13.5 (Lai *et al.*, 2003). During brain development *Foxp2* is expressed in the cerebellum; basal ganglia, including the substantia nigra and striatum; medulla, specifically the inferior olive; caudate nucleus; amygdala; hypothalamus; thalamus; and deep layers of the cerebral cortex (Ferland *et al.*, 2003; Lai *et al.*, 2003; Fujita and Sugihara, 2012; Co, Anderson and Konopka, 2020). Similar expression patterns have been found in human brain development (Co, Anderson and Konopka, 2020). In the foetal human brain, *FOXP2* is highly enriched in the cerebellum, but is also expressed in several other brain regions including the medulla, hypothalamus, thalamus and caudate nucleus (Lai *et al.*, 2003). Across the mouse brain, *Foxp1* and *Foxp4* show some overlap with *Foxp2*, with expression observed in the cerebral cortex, amygdala, thalamus, striatum, cerebellum and hippocampus (Co, Anderson and Konopka, 2020). However, within brain regions *Foxp* gene expression patterns often differ, for example in the cerebral cortex *Foxp1* is predominantly expressed in layers 3-5 compared to *Foxp2* in layer 5-6, whilst *Foxp4* is transiently more broadly expressed in early postnatal cortex (Co, Anderson and Konopka,

2020). Within the mouse cerebellum, *Foxp1*, 2 and 4 are all expressed in developing Purkinje cells, with *Foxp2* and *Foxp4* maintained in adult Purkinje cells (Ferland *et al.*, 2003; Lai *et al.*, 2003; Tam *et al.*, 2011). *Foxp1* and *Foxp2* have also been detected in the DCN (Ferland *et al.*, 2003; Lai *et al.*, 2003).

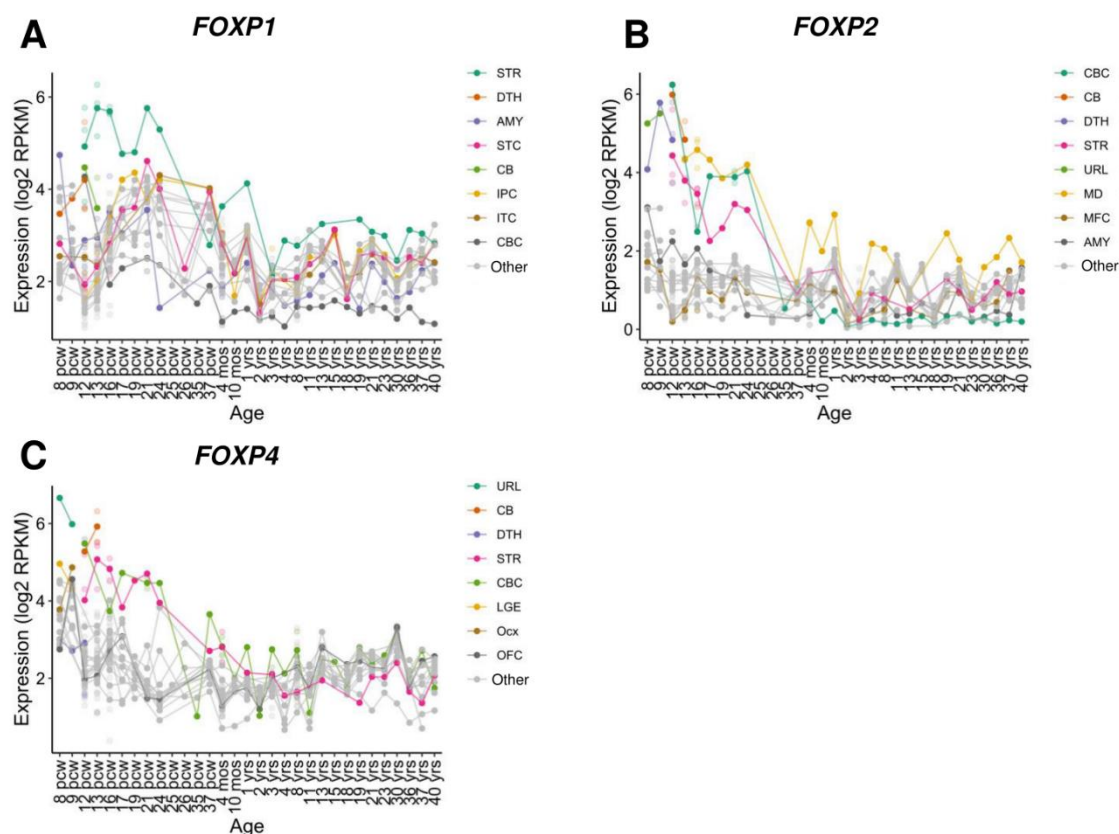


Figure 1.11 Expression of FOXP family genes across the developing human brain.

FOXP genes (A) *FOXP1*, (B) *FOXP2* and (C) *FOXP4*, show high expression in the embryonic human brain. Bulk RNA-seq data from BrainSpan (<https://www.brainspan.org/>). Points indicate expression in single samples, bold points indicate average expression for each brain region over time. Regions with highest expression are highlighted in colour, other brain regions are shown in grey. Timepoints labelled as post conception week (pcw), month (mos) or year (yrs). Brainspan brain regions: AMY, amygdala; CBC, cerebellar cortex; CB, cerebellum; DTH, dorsal thalamus; MD, mediodorsal nucleus of thalamus; MFC, anterior (rostral) cingulate (medial prefrontal) cortex; STR, striatum; URL, upper rhombic lip.

1.4.3 FOXP2 dysfunction in speech disorders

The identification of *FOXP2* resulted from genetic mapping of a severe speech and language disorder (Fisher *et al.*, 1998; Lai *et al.*, 2001). Around half of the members of a large multi-generational family, referenced as the KE family, were affected by an autosomal dominantly inherited speech disorder (Hurst *et al.*, 1990). Affected members showed verbal dyspraxia, difficulty planning and performing oral movements, particularly for more complex sequences (Lai *et al.*, 2001). The disorder was severe, with speech unintelligible for some of those unfamiliar with the affected individuals (Lai *et al.*, 2001). The disorder was mapped to chromosome 7, and then precisely to a point mutation in *FOXP2* (Fisher *et al.*, 1998; Lai *et al.*, 2001). Affected KE family members had a substitution which altered a highly conserved amino acid (R553H) in the forkhead DNA-binding domain of FOXP2 (Lai *et al.*, 2001). Subsequently, additional cases of *FOXP2*-related speech and language disorders have been described, caused by both point mutations or larger genomic changes (Lai *et al.*, 2001; MacDermot *et al.*, 2005). The primary symptom of *FOXP2*-related speech and language disorders is childhood apraxia of speech (CAS) (Morgan *et al.*, 2019). Affected individuals have difficulty with planning and production of speech, including coordinating the required orofacial motor movements (Morgan *et al.*, 2019). However, there are also non-verbal components, such as difficulty with grammar and comprehension (Morgan *et al.*, 2019). For example, affected KE family members struggled with ordering of words and comparisons of objects (Lai *et al.*, 2001). Together these clinical features suggest a role for *FOXP2* in the functioning of neural circuits involved in orofacial motor programming, but also in language generation.

Brain imaging of KE family members has been used to identify structural and functional changes in individuals with the *FOXP2*-R553H speech disorder. In unaffected right-handed individuals, brain activity during spoken language tasks is focused in the left hemisphere. Individuals with the *FOXP2*-R553H mutation generally showed more bilateral activation and pathology (Watkins *et al.*, 2002). Amongst structural changes, affected individuals showed increased grey matter in the posterior lobe of the cerebellum (lobules VIIa Crus I and VIIb–VIIIb), but decreased in the posterior lobe of the right cerebellum (lobule VI) (Watkins *et al.*, 2002; Argyropoulos *et al.*, 2019). Overlapping areas of the cerebellum also showed differential

activation in affected individuals during a spoken non-word repetition task (lobule VIIa Crus I and VIIIb/IX) (Liégeois *et al.*, 2011; Argyropoulos *et al.*, 2019). However, other brain regions, including the caudate nucleus, also showed both structural and functional changes in affected individuals (Co, Anderson and Konopka, 2020). Therefore, cerebellar changes may reflect altered development directly caused by *FOXP2* dysfunction in the cerebellum, or alternatively may have been indirectly caused by altered feedback signals from connected brain regions. To understand why cerebellar changes occur on loss of *FOXP2*, models that can be manipulated and monitored over time are required.

1.4.4 Animal models of *FOXP2* mutations

Following the identification of the KE family mutation, several genetically modified mouse models were generated to examine the effect of global *Foxp2* disruption (Table 1.1). *Foxp2* knock out (*Foxp2*-KO) mice were generated by deletion of the exons encoding the forkhead DNA binding domain, exons 12 to 13 or 14 respectively (Shu *et al.*, 2005; French *et al.*, 2007). Alternatively, a R552H mutation was introduced, as equivalent to the mutation causing the R553H substitution in the KE family, to more closely model patient genetics (Fujita *et al.*, 2008; Groszer *et al.*, 2008). Whilst these models showed some phenotypic differences, homozygous *Foxp2*-KO or *Foxp2*-R552H caused severe motor impairments, reduced weight and premature death (Shu *et al.*, 2005; French *et al.*, 2007). In contrast, heterozygous *Foxp2*-KO or *Foxp2*-R552H mutants were largely healthy, with no changes in baseline motor function and showed few differences to wild-type (WT). However, during more complex motor-tests, heterozygous *Foxp2*-R552H mice did display deficits in motor-skill learning and sensory-motor association learning (Groszer *et al.*, 2008; Kurt, Fisher and Ehret, 2012). To understand the role of *FOXP2* in speech and language development, mouse phenotyping has focused on the ultrasonic vocalisations (USVs) produced by pups in response to separation from their mother. Homozygous *Foxp2*-KO or *Foxp2*-R552H mice displayed a large decrease or complete loss of USV production, despite showing ability to make ultrasonic calls within distress conditions (Shu *et al.*, 2005; Fujita *et al.*, 2008; Groszer *et al.*, 2008; Gaub *et al.*, 2010). Heterozygous mice often showed modest reductions in isolation induced USVs and distress calls in response to

stress (Shu *et al.*, 2005; Fujita *et al.*, 2008; Gaub *et al.*, 2010). Later in development, heterozygous mice also displayed altered development of courtship calls and changes in calls in different social environments (Castellucci, McGinley and McCormick, 2016; Chabout *et al.*, 2016). Therefore, mouse models of *Foxp2* dysfunction somewhat recapitulate a “speech-deficit” like phenotype.

Across most models, homozygous disruption of *Foxp2* led to decreased size and foliation of the cerebellum, whilst heterozygous mice showed no gross cerebellar changes (French *et al.*, 2007; Fujita *et al.*, 2008; Groszer *et al.*, 2008). In one *Foxp2*-KO model, prolonged presence of the external granule layer at P15-17 was observed, accompanied by disrupted radial glia structures which may have led to the impaired granule cell migration (Shu *et al.*, 2005). In homozygous *Foxp2*-KO mice the EGL was 3-5 cells thick at P15-17 and persisted into adult, whilst heterozygous mice had a 1 cell thick EGL at P15-17 which was resolved by adulthood (Shu *et al.*, 2005). However, EGL changes were not described in other *Foxp2* models (French *et al.*, 2007; Fujita *et al.*, 2008; Groszer *et al.*, 2008). Purkinje cell alterations were more commonly observed across *Foxp2* mutant models. Homozygous *Foxp2* disruption by KO or R552H mutation, led to reduced Purkinje cell dendritic tree size (Shu *et al.*, 2005; Fujita *et al.*, 2008), however this was not found in other similar models (Groszer *et al.*, 2008). In the most severe phenotype, homozygous *Foxp2*-KO mice had a disorganised Purkinje cell layer of several cells thick, misalignment of dendritic trees, and some Purkinje cells ectopically located in the granule layer (Shu *et al.*, 2005). Heterozygous *Foxp2* mutants typically showed normal Purkinje cell morphology (Shu *et al.*, 2005). A slight reduction in dendritic tree complexity was found in early development of a *Foxp2*-R552H heterozygous mouse, however normal arborisation was complete by P19 (Fujita *et al.*, 2008). In summary, global *Foxp2* mutants showed deficits in motor function and learning, and alterations in vocal call production, accompanied by some cytoarchitectural changes in the cerebellum.

Table 1.1 Mouse models with global *Foxp2* mutations.

Reference		Shu et al., 2005.	French et al., 2007	Fujita et al., 2008	Groszer et al., 2008. (Gaub et al., 2010. Kurt et al., 2012)		
Model		Global <i>Foxp2</i> knock-out. Ex 12-13 ¹ replaced with a neomycin cassette.	Global <i>Foxp2</i> knock-out in early embryo. Sox-driven, CRE-mediated deletion of ex 12-14 ¹ , leading to a premature stop in ex 15.	Global <i>Foxp2</i> -R552H mutation, corresponding to human R553H ² .	<i>Foxp2</i> mutations generated by ENU mutagenesis.		
					R552H mutation corresponding to human R553H mutation ² .	S321X premature stop codon.	N549K substitution.
Cerebellar phenotypes	Cerebellar size and foliation	No gross changes.	<i>Foxp2</i> ^{-/-} Substantial reduction in size and foliation of cerebellum. <i>Foxp2</i> ^{+/-} no changes.	<i>Foxp2</i> ^{R552H/R552H} reduced foliation. <i>Foxp2</i> ^{+/-R552H} no changes.	<i>Foxp2</i> ^{R552H/R552H} decreased size and foliation. <i>Foxp2</i> ^{+/-R552H} no changes.	<i>Foxp2</i> ^{S321X/S321X} decreased size and foliation. <i>Foxp2</i> ^{+/-S321X} no changes.	<i>Foxp2</i> ^{N549K/N549K} decreased size and foliation. <i>Foxp2</i> ^{+/-N549K} no changes.
	EGL migration, ML thickness, RG alignment	<i>Foxp2</i> ^{-/-} persistent EGL 3-4 cells thick at P15-17; reduced ML thickness; frequent gaps and clumps in RG fibres. <i>Foxp2</i> ^{+/-} delayed EGL of 1 cell thick at P15-17 but resolved in adults; some areas of thinner and less well aligned RG.	<i>Not described.</i>	<i>Not described.</i>	No changes to cytoarchitecture of cerebellum at P21.		
	Purkinje cell alignment, number and dendritic tree	<i>Foxp2</i> ^{-/-} At P17 PCs were disordered, in a layer several cells thick and some ectopically located in granule layer, dendritic trees were less complex and misaligned.	<i>Not described.</i>	<i>Foxp2</i> ^{R552H/R552H} PCs aligned but sparse in folia at P10, less complex PC dendritic trees, reduced synaptic staining on PCs. <i>Foxp2</i> ^{+/-R552H} delayed dendritic tree development, early complexity reduced but recovered by P19. Slightly reduced synaptic staining on PCs.	PCs aligned in monolayer as normal at P21; no changes in dendritic tree observed.		

Reference	Shu et al., 2005.	French et al., 2007	Fujita et al., 2008	Groszer et al., 2008. (Gaub et al., 2010. Kurt et al., 2012)		
Non-cerebellar phenotypes	<i>Foxp2</i> ^{-/-} reduced weight, severe motor impairments, developmental delays, death by P21, large decrease in number of USVs at P6 and P10. <i>Foxp2</i> ^{+/-} reduced weight, developmental delay, reduced number of USVs at P6 and P10.	<i>Foxp2</i> ^{-/-} reduced weight, severe motor impairments, developmental delays, and premature death by P21. <i>Foxp2</i> ^{+/-} no overt differences to WT in weight, righting reflexes or timing of eyes opening.	<i>Foxp2</i> ^{R552H/R552H} reduced weight and growth, reduced brain size, severe motor impairments, large reduction in USVs, death by P21. <i>Foxp2</i> ^{+/-R552H} reduced size, modest impairments to USVs (reduced whistle-type calls and increased short calls). No changes in motor function.	<i>Foxp2</i> ^{R552H/R552H} reduced weight gain, severe motor impairments, complete loss of USVs (isolation), fewer USDs (stress), altered distress calls death 3-4 weeks after birth. <i>Foxp2</i> ^{+/-R552H} no differences in weight, base-line motor function or USVs, reduced motor-skill and delayed auditory-motor association learning, synaptic changes in striatum and cerebellum.	<i>Foxp2</i> ^{S321X/S321X} reduced weight gain, severe motor impairments, death 3-4 weeks after birth. <i>Foxp2</i> ^{+/-S321X} no differences in weight, base-line motor function.	<i>Foxp2</i> ^{N549K/N549K} death by 3-5 months, decreased USVs (isolation) and USDs (stress), altered distress calls <i>Foxp2</i> ^{+/-N549K} fewer USDs (stress), impaired auditory-motor association learning.

Table 1.1 Mouse models with global *Foxp2* mutations.

E, embryonic day; EGL, external granule layer; ex, exon; ML, molecular layer; P, postnatal day; PC, Purkinje cell; RG, radial glia; USDs, ultra-sounds of distress; USVs, ultra-sonic vocalisations; WT, wild-type.

1 - Exons 12-13 encode forkhead DNA-binding domain. 2 - KE family mutation (Lai et al., 2001).

1.4.5 Molecular studies of FOXP2 function

FOXP2 acts as a transcription factor, regulating expression of target genes in combination with other transcription factors and chromatin modifiers. FOXP proteins were initially found to be transcriptional repressors (Shu *et al.*, 2001; Li, Weidenfeld and Morrisey, 2004). FOXP2 has been shown to inhibit expression of target genes via directly binding DNA at forkhead recognition motifs (Hickey, Berto and Konopka, 2019). Furthermore, the activity of FOXP2 in the developing mouse cerebellum was examined using single-nucleus assay for transposase accessible chromatin using sequencing. In Purkinje cells, the chromatin accessibility of the FOXP2-binding motif was found to be negatively correlated with predicted expression of *Foxp2*, indicating a predominantly repressive role (Sarropoulos *et al.*, 2021). However, FOXP2 has also been shown to activate transcription of genes, for example through interaction with NFIA/B transcription factors leading to opening of chromatin (Hickey, Berto and Konopka, 2019).

Targets of FOXP2 have been identified using chromatin immunoprecipitation (ChIP) and differential expression analysis. Though showing limited overlap in identified genes, these studies have suggested some common pathways regulated by FOXP2, including cell migration, cell signalling, regulation of transcription, and nervous system development (Spiteri *et al.*, 2007; Vernes *et al.*, 2007, 2011; Oswald *et al.*, 2017). Terms identified related to neurogenesis included synaptic transmission, neurite outgrowth and axon guidance (Spiteri *et al.*, 2007; Vernes *et al.*, 2007, 2011). Additional experimental evidence supports a role for FOXP2 in neurite outgrowth. Overexpression of FOXP2 in multiple cell lines increased neurite outgrowth in retinoic acid stimulated differentiation (Vernes *et al.*, 2011; Devanna, Middelbeek and Vernes, 2014). Furthermore, *Foxp2*-expressing primary neurons isolated from E16 mouse ganglionic eminences showed increased neurite outgrowth when derived from WT compared to FOXP2-R552H mice (Vernes *et al.*, 2011).

Transcriptional targets of FOXP2 include the upregulation of several retinoic acid receptors and a number of genes involved in neuronal differentiation (Devanna, Middelbeek and Vernes, 2014). In zebrafish, FOXP2 has been shown to bind to the promoter of *Very low density*

lipoprotein receptor (VLDLR), encoding the RELN receptor, and activate transcription (Adam *et al.*, 2016; Mendoza and Scharff, 2017). Moreover knockdown of *Foxp2* in the region of zebrafish striatum involved in song production decreases *VLDLR*, demonstrating a role of FOXP2 in maintaining *VLDLR* expression (Adam *et al.*, 2016). In contrast, genes shown to be repressed by FOXP2 include *CNTNAP2*, *SLIT1*, *CCK*, *NRN1*, *MEF2C* (Vernes *et al.*, 2011; Devanna, Middelbeek and Vernes, 2014; Sin, Li and Crawford, 2015; Chen *et al.*, 2016). In the cerebellum, there is evidence for FOXP2 inhibition of *Contactin associated protein 2 (CNTNAP2)*, with expression increased in *Foxp2*-R552H mice (Fujita *et al.*, 2012a). *CNTNAP2* encodes CASPR, a neurexin transmembrane protein with a range of roles in neuron development and function (Hoed, Devaraju and Fisher, 2021). FOXP1 and FOXP2 have been shown to directly bind regulatory regions of *CNTNAP2*, interestingly with opposite effects, FOXP1 activates transcription whilst FOXP2 inhibits (Vernes *et al.*, 2008; Mendoza and Scharff, 2017).

1.5 Aims of thesis

iPSC-derived cerebellar organoids offer a human-specific model for early cerebellar development, but protocols still pose a number of challenges in studying individual cell types and the relative immaturity of cultures. The aims of this thesis were therefore to develop tools to improve study of iPSC-derived Purkinje cells, by fluorescent labelling of this population and screening of conditions to promote differentiation. Then, to apply iPSC-derived cerebellar models to understanding the role of language-associated gene, *FOXP2*, in cerebellar development.

This thesis describes the following aims:

1. To generate a fluorescent cell-type specific reporter line to label the developing Purkinje cell population in iPSC-derived cerebellar organoids.
2. To further develop current protocols for generation of Purkinje cells from iPSCs, including functional characterisation of cultures.
3. To utilise iPSC-derived cerebellar neuron models to investigate the function of *FOXP2* in the early human cerebellum.

Chapter 2. Materials and Methods

2.1 iPSC culture

Human iPSCs were grown on tissue culture-treated plates coated with Matrigel hESC-qualified matrix (Corning, 354277) and fed daily with mTeSR™1 medium (StemCell, 85850). Wells were passaged when 70-80% confluent and typically split 2-3x per week at a ratio of 1:3-1:8. To passage, cells were incubated in 0.5 mM EDTA (Invitrogen, 15575020) for 3-5 min to cause detachment. The EDTA was then removed and cells gently resuspended into fresh mTeSR medium before transferred to new Matrigel-coated wells. Rock inhibitor (10 μ M Y-27632, Abcam, ab120129) was added to mTeSR™1 medium to promote survival for the first 24 hour after thawing, subsequent culture and passaging was performed without Y-27632. iPSCs were frozen in freezing medium (10% DMSO (Sigma, D2650), 30% embryonic stem cell grade foetal calf serum (ES-FCS, Life Technologies, 16141-079), 60% knock-out DMEM (Gibco, 10829)) at 2×10^6 cells/vial.

The majority of work in this thesis, including the generation of a FOXP2-P2A-mNeon line, used the control iPSC line AH0-17-3 (Handel *et al.*, 2016). This line has been previously characterised and used for differentiation to cerebellar organoids (Handel *et al.*, 2016; Watson *et al.*, 2018; Nayler *et al.*, 2021). Additional control iPSC lines were used to demonstrate the cerebellar organoid differentiation, WTC11 (<https://hpscreg.eu/cell-line/UCSFi001-A>) and CENSO58A (control iPSC line, Axol Bioscience). Collaborators Seon Hye E. Park and Genevieve Konopka from UT Southwestern, USA, kindly provided a *FOXP2*^{-/-} line. The *FOXP2*^{-/-} line was generated from parental WTC11 line using Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated system protein 9 (Cas9) mediated deletion of the whole *FOXP2* gene. The WTC11 line was therefore used for comparison in these investigations.

2.2 Generation of a FOXP2-mNeon reporter line

2.2.1 Sequencing target *FOXP2* locus.

The sequence around the *FOXP2* stop codon was first verified in the AH0-17-3 line before design of CRISPR/Cas9 guide RNA (gRNA) and donor construct. Genomic DNA (gDNA) was extracted from iPSCs using the DNeasy Blood & Tissue Kit (Qiagen, 69504). The desired region to be used for homology dependent repair region was amplified by PCR using primers (Table 2.1) and the Q5® High-Fidelity DNA Polymerase (New England BioLabs, M0491). A 1.5-kilobase pair (kb) PCR product was confirmed by gel electrophoresis. Sanger sequencing of the PCR product was performed using the PCR and supplementary primers (Table 2.1).

2.2.2 Single guide RNA design and testing

gRNAs targeting within 50 base pairs (bp) of the *FOXP2* STOP codon were designed using online tools CCTop (<https://cctop.cos.uniheidelberg.de:8043/>) and CRISPOR (<http://crispor.tefor.net/>). Four candidate gRNAs were selected based on high predicted specificity and efficiency scores, and a low number of off-target sites (Table 2.2). BLAT (<https://genome.ucsc.edu/cgi-bin/hgBlat>) was used to confirm gRNA targeting locations.

To test efficiency in practice, the four designed single guide RNAs (sgRNAs) were individually transfected into iPSCs with Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT, 20925207) as described below. gDNA was extracted from the resulting pools of cells and a 314 bp sequence spanning the *FOXP2* stop codon amplified by PCR (Primers, Table 2.1). The PCR products were Sanger sequenced and resulting traces analysed using TIDE (<http://shinyapps.datacurators.nl/tide/>) to determine editing efficacy (Table 2.2). The gRNA with the highest efficacy was chosen (gRNA sequence AGAGCCTTTATCTGAAGATC).

2.2.3 Preparation of a single-stranded DNA donor construct

The donor construct for homology-dependent repair was designed to contain sequences encoding a flexible linker, a P2A peptide and mNeonGreen, surrounded by 2 homology arms. The homology arms were sequences identical to 637 bp upstream (5') and 660 bp downstream

(3') of the *FOXP2* stop codon. The entire donor construct sequence was purchased as a double stranded (ds) DNA gblock (IDT).

Repair using a single-stranded (ss) DNA construct has lower off-target integration, higher insertion rate at the desired site and lower cytotoxicity (Hornung and Latz, 2010; Renaud *et al.*, 2016; Roth *et al.*, 2018). Therefore, a ssDNA copy of the repair construct was generated using the Guide-it™ Long ssDNA Production System v2 (Takara Bio, 632666) according to manufacturer's instructions. First PCR was used to generate a dsDNA strand incorporating one phosphate-labelled primer (Table 2.1). Then an enzymatic reaction digested the labelled strand to leave ssDNA. Both the dsDNA and ssDNA products were run on a 1% agarose gel to assess size and purity. As prediction of the relative integration efficiency of each strand is unknown, both ssDNA products were made and used in separate transfection reactions.

2.2.4 CRISPR-Cas9 transfection

In preparation for CRISPR/Cas9 transfection, wells of a 24-well plate were coated with Matrigel and one 6-well plate well of iPSCs was treated with 10 μ M Y-27632 for 1 hour. A ribonucleoprotein was prepared by mixing 0.5 μ l Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT, 20925207) and 22 pMole sgRNA per reaction in transfection enhancer buffer (Invitrogen, MPK10096). 1.5 μ g of ssDNA donor or an additional volume of transfection enhancer buffer (mock) was added to make a total volume of 3 μ l and incubated for 20 min at room temperature.

Pre-treated iPSCs were dissociated using 0.5 mM EDTA and resuspended at 2.2×10^7 cell/ml in buffer R (Invitrogen, MPK10096). For each reaction, 10 μ l of cell suspension containing around 2.2×10^5 cells was mixed with the prepared ribonucleoprotein and ssDNA donor solution. Cells were electroporated using the NEON Transfection system (Invitrogen, MPK10096) and 10 μ l Neon pipette tips, according to program HiTrans (1400 V, 20 ms width, 1 pulse). Transfected cells were seeded into one well of the Matrigel coated 24-well plate in mTeSR™1 with 10 μ M Y-27632. On the following day, medium was changed to just mTeSR™1 and cells were fed daily. Once confluent, the pools of transfected cells were harvested. From each well, 50% of

cells were frozen for future isolation of clones, 30% were pelleted for DNA extraction, and 20% were passaged as a back-up.

2.2.5 Checking CRISPR pool for insertion

gDNA was extracted from the pelleted pools of transfected cells using the DNeasy Blood & Tissue Kit (Qiagen, 69504) and eluted in 20 µl of water. To detect whether the desired insertion had occurred, PCR used primers outside the homology arm regions around the *FOXP2* STOP codon (Table 2.1). The PCR products were run on a 1% agarose gel to visualise bands. Samples from untransfected cells showed a clear band ~1.4 kb. In addition, samples from cells transfected with sgRNAs and either ssDNA donor also had a range of larger size bands including at around 2.2 kb indicating insertion of the P2A-mNeon sequence had occurred.

2.2.6 Isolation of clones

To obtain stable reporter lines for use in downstream applications, clonal lines had to be isolated from the pool of transfected cells. A process of colony formation and picking was followed to select cells all derived from a single iPSC.

First, a layer of inactivated mouse embryonic feeder cells was prepared. Irradiated mouse embryonic feeders were plated onto 0.1% gelatin (Sigma, G1393-100ML) at 6.7×10^5 cells per well into 6 wells of a 6-well plate in MEF medium (10% FCS, 1X GlutaMAX (Gibco, 35050061), 0.1% beta-Mercaptoethanol (Gibco, 31350010) in Advanced DMEM (Gibco, 12491)). On the following day, medium was changed to hES medium (20% KO Serum replacement (Gibco, 10828028), 1x Non-essential Amino Acids (Gibco, 11140050), 1x GlutaMAX (Gibco, 35050061), 50 µM beta-Mercaptoethanol (Gibco, 31350010), 1x Pen/Strep (Gibco, 15140122), 5 ng/ml bFGF (BioTechne, 234-FSE/CF) in KO DMEM) with 10 µM Y-27632. The pool of transfected iPSCs were plated onto the feeder layer in a serial dilution. Cells were seeded at 8000, 4000, 2000, 1000 or 500 cells per well. Half-volume medium changes were performed daily until iPSC colonies were ready for picking seven days after plating.

In preparation for picking clones, three 96-well plates were coated with Matrigel and filled with mTeSR™1 with 10 µM Y-27632. The medium on the iPSC colonies was also changed to

mTeSRTM1 with 10 μ M Y-27632 1 hour before picking. Colonies were visualised using a dissection microscope in the tissue culture hood and picked manually using a P200 tip, transferring into individual wells in the prepared 96 well plate. Selected colonies were defined, relatively small and spaced apart from others to reduce the chance of cross contamination between clones. In total 288 colonies were picked.

iPSC clones were fed daily with mTeSRTM1 for seven days until the majority of wells were confluent. The cells were passaged using 0.5 mM EDTA to produce replica plates. From each of the three original 96 well plates, two identical plates were made for freezing clone stocks and the original plate was also maintained for gDNA extraction.

2.2.7 Screening clones

To extract gDNA from the large quantity of clones, an ethanol precipitation protocol was used. Cells were first lysed overnight at 55°C in humidified conditions in lysis buffer (10 mM Tris pH 7.5, 10 mM EDTA, 10 mM NaCl, 0.25% Triton-X-100, 1 mg/ml proteinase K (Sigma, P6556-100mg) in H₂O). The following day, ice-cold precipitation solution (75 mM NaCl in 100% ethanol) was added to precipitate DNA. After around 4 hours, once DNA was visible in most wells, liquid was discarded and the DNA washed twice in 100% ethanol before drying. Finally, gDNA was resuspended in 50 μ l H₂O by incubation overnight and concentration measured using a Nanodrop.

gDNA from 245 clones was screened for successful insertion of mNeon at the *FOXP2* locus by PCR using primers spanning from outside the 5' homology arm to inside the mNeon sequence (Table 2.1). PCR products were run on a 1% agarose gel to visualise bands. 75 clones produced a PCR product and used for the next screening step. Primers spanning outside the homology arms, as described for checking the pool, were used to genotype selected clones. 17 clones appeared homozygous for the insertion with only a higher molecular weight band around 2.2 kb, and 17 clones appeared heterozygous with both a 2.2 kb and WT 1.4 kb band. Sanger sequencing was performed on a selection of clones and verified correct sequences in two homozygous (A6B & B2D) and one WT control (C1G) clones.

2.2.8 Clone expansion and banking

Selected clones were thawed from the previously frozen 96-well plates. Working rapidly, the whole plate was thawed in an incubator and selected wells transferred to a 96-well V-bottomed plate to spin down. After centrifuging at 400x *g* for 3 min, cell pellets were resuspended in mTeSR™1 with 10 µM Y-27632 and transferred to a Matrigel-coated 24-well plate. Clones were then expanded and frozen to generate a master stock.

2.2.9 Quality control on iPSCs

Following the production of master stocks of iPSCs several quality control steps were performed to ensure chromosomal stability and avoidance of contamination. In addition, for the newly generated clones of the FOXP2-P2A-mNeon reporter line, steps were performed to screen for off-target mutations and check for maintenance of pluripotency.

Karyotype analysis: To test for gross chromosomal abnormalities, gDNA from each iPSC stock was analysed and compared to the parental stock. gDNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, 69504) and samples submitted for SNP karyotyping analysis (Infinium Global Screening Array-24 v3.0, Illumina, performed by Life&Brain GmbH).

Mycoplasma: After generating master stocks, iPSCs were tested for mycoplasma using the MycoAlert™ PLUS Mycoplasma Detection Kit (LT07-705). Supernatant was collected from cultured cells around 24 hour after the last medium change and centrifuged at 200 x *g* for 5 min to remove any cell debris. The assay was performed following manufacturer's instructions, with luminescence readings after addition of the reagent (A) and after addition of the substrate (B). A ratio B/A of less than 1, indicating mycoplasma negative, was found for all tested cell stocks.

Off-target site sequencing: To check for the most likely off-target mutations following CRSIPR/Cas9 editing, the top six highest risk off-target sites were identified for the gRNA used (Table 2.3). Primers were designed to amplify regions of approximately 200 bp around these high-risk sites (Table 2.1). PCR amplification and Sanger sequencing was performed on the

FOXP2-P2A-mNeon clones and compared to the parental AH0-17-3 stock. No off-target changes were detected in the sites screened.

Fluorescence-activated cell sorting (FACS) staining for pluripotency markers: iPSCs were harvested using TrypLE (Gibco, 12604013) and fixed in 2% PFA at room temperature for 10 mins. Cells were then centrifuged at 400 x g for 5 min, before resuspending in ice-cold methanol to permeabilise for at least 30 min at -20°C. Next, cells were washed once in FACS buffer (1% BSA in PBS) and incubated with primary antibodies against pluripotency markers TRA-1-60 and Nanog or isotope controls (Table 2.6) in FACS buffer at room temperature in the dark. After 1 hour of staining, cells were washed twice with FACS buffer and analysis performed using a Cytoflex LX analyser (Beckman Coulter).

Trilineage differentiation: To assess pluripotency of the iPSCs after CRISPR/Cas9 editing, a trilineage differentiation was performed according to the STEMdiff™ Trilineage Differentiation Kit (STEMCELL technologies, 05230). In preparation sterile coverslips were placed into 6-well plates and coated with Matrigel. On day 0 iPSCs were detached using TrypLE and resuspended in mTeSR™1 with 10 µM Y-27632. Cells were seeded into a 6-well plate according to differentiation lineage, for mesoderm 500,000 cells/well whilst for ectoderm and endoderm 2,000,000 cells/well were added. The following day, the medium was changed to the appropriate lineage medium. Cells were fed daily until day five for mesoderm and endoderm and day seven for ectoderm. On the final day, coverslips were washed in PBS and fixed in 4% PFA for 20 min at room temperature. Fixed cells were permeabilised for 20 min using 0.4% Triton-X-100 in PBS and incubated in block (5% serum (Normal goat serum, Abcam, ab7481 or Normal donkey serum, Abcam, ab7475), 0.1% Triton-X-100 in PBS) for 1 hour. Primary antibodies (Table 2.6) in diluent (5% serum in PBS) were applied over night at 4°C. Following three washes in PBS, secondary antibodies were incubated for 1 hour at room temperature. Three washes were performed to remove unbound antibody with 1 µg/ml Hoechst (Invitrogen, H1398) in the final wash. Stained coverslips were imaged on a Zeiss-880 laser scanning confocal microscope.

Table 2.1 Primer sequences for FOXP2-P2A-mNeon reporter line generation

Purpose	Name	Fw primer	Rv primer	Location	Product size (bp)	PCR conditions
Sequencing <i>FOXP2</i> homology arms Clone screening: genotyping (Screen 2)	FOXP2_HAs eq	TTGAAAATAGTA CTGGCATCCTGG	CTATGGTGCTTG CACAGATAATG	Chr7: 114689210 (spanning <i>FOXP2</i> STOP codon)	WT <i>FOXP2</i> : 1438 <i>FOXP2</i> -P2A- mNeon: 2245	Q5, Ta = 64°C, E = 45 s
Additional <i>FOXP2</i> sequencing	FOXP2_HAs_midse2	GCCAGTGATTGC AGAGGATG	-	chr7:114,689, 806	NA	NA
Additional <i>FOXP2</i> sequencing	FOXP2_HAs_eq_midseq_rv	-	TTTGCCAGTATA TGAGGCGC	chr7:114,690, 290	NA	NA
FOXP2 gRNA testing	FOXP2_gRNA_test	CAATCCACGTCA AGGAAGAGC	TGCCTGTTGGTA CTGAATCC	chr7:114,689, 787 (spanning <i>FOXP2</i> STOP codon)	314	Q5, Ta = 66°C, E = 30 s
Generation of P2A-mNeon ssDNA repair construct	FOXP2_ssDNA	TTCAACAAACAC CCAATCTAGC	CAGCACAGACTG GAAGCCTA	na	2221	PrimeSTAR Max, Ta = 55°C, E = 11 s
Clone screening insertion (Screen 1)	FOXP2mNeON_screen	TGGGGTTGGAG GGATAATAAACT T	CCATCATTTGGA TTGCCGGT	chr7:114,689, 076 3' <i>FOXP2</i> to mNeon	WT <i>FOXP2</i> : no product <i>FOXP2</i> -P2A- mNeon: 1081	REDTaq pol, Ta = 1 min, E = 3 min
Off-target testing	10_OTUD1	AAGAGCTCTGAA GCCTGCTG	AAGTGTTGGGA TTACAGGCG	Chr10: 23417892	196	Q5, Ta = 68°C, E = 10 s
Off-target testing	8_KBTBD11	GCACCATTTGAC TCCAGCCT	AGCGGAACACA ATAAATGTGGA	Chr8: 2013206	133	Q5, Ta = 59°C, E = 10 s
Off-target testing	10_PDZD8	GCGTTACTGTTG TGGGAGGA	GCCCTTAGAGCT GTCTGCAT	Chr10: 117390874	231	Q5, Ta = 68°C, E = 10 s
Off-target testing	6_COX7A2	GTCCGCTGAGAG TTTCCTCC	TTTGTTTCAGAA AGGCGGGA	Chr6: 75248430	506	Q5, Ta = 68°C, E = 10 s
Off-target testing	X_RP1	GAGTACACCCAA GGCCTGATT	ACACATATACCT TCCCAACACACT	ChrX: 146650781	188	Q5, Ta = 66°C, E = 10 s
Off-target testing	3_ACPP	ACGTTGACCGGA CTTGATG	ACAACTGTTGAG TAGCCTGC	Chr3: 132332323	206	Q5, Ta = 66°C, E = 10 s

E, elongation time; Q5, Q5 high fidelity polymerase (NEB, M0491); REDTaq pol (Sigma, D4309); PrimeSTAR Max (Takara, 632666); Ta, annealing temp (Ta).

Table 2.2 gRNAs designed to target near FOXP2 STOP codon

Sequence of gRNA (PAM)	Direction	CRISPOR specificity score	CTOP efficacy score	Frequency of 0-1-2-3-4 mismatches (mismatches within 12 bp of PAM)	TIDE tested efficiency (%)
CTTGTGAAACCTC AGCGTGA (AGG)	Fw	89	0.79 HIGH	0 - 0 - 0 - 9 - 89 (0 - 0 - 0 - 0 - 0)	13.2
TGATATGTCCCTT CACGCTG (AGG)	Rv	88	0.62 MEDIUM	0 - 0 - 0 - 8 - 63 (0 - 0 - 0 - 0 - 0)	42.8
AGAGCCTTTATCT GAAGATC (TGG)	Fw	63	0.48 LOW	0 - 0 - 3 - 14 - 247 (0 - 0 - 1 - 2 - 3)	63.2
CATTCCAGATCTT CAGATAA (AGG)	Rv	41	0.55 LOW	0 - 0 - 14 - 60 - 400 (0 - 0 - 1 - 5 - 3)	40.7

CRISPOR (<http://crispor.tefor.net/>); CTOP (<https://cctop.cos.uniheidelberg.de:8043/>); PAM, protospacer adjacent motif; TIDE efficiency when tested in iPSCs (<http://shinyapps.datacurators.nl/tide/>).

Table 2.3 Top predicted gRNA off-target sites

Name (closest gene)	Location	Matching bases (), mismatches (-), binding site adjacent to PAM ([])	Mismatches
10_OTUD1	Chr10: 23417892	- -[]PAM	2
8_KBTBD11	Chr8:2013206	- [-]PAM	2
10_PDZD8	Chr10:117390874	---- -[]PAM	4
6_COX7A2	Chr6: 75248430	-- -- -[]PAM	4
X_RP1	ChrX: 146650781	-- -- -[]PAM	4
3_ACPP	Chr3: 132332323	-- [-]PAM	4

PAM, protospacer adjacent motif.

2.3 Differentiation of iPSCs to cerebellar organoids

Cerebellar organoids were generated from iPSCs over a 35-day period following an established protocol (Van Essen *et al.*, 2022). Approaches for extended culture for further maturation were tested in Chapter 4 and depend on the desired downstream application. Table 2.4 lists reagents and their preparation for use in cerebellar organoid differentiation.

2.3.1 Formation of embryoid bodies (day 0)

The first stage in cerebellar differentiation was the formation of embryoid bodies. iPSCs were ready to differentiate after at least one passage after thawing and at 60-70% confluency. Typically, one well of a 6-well plate of iPSCs was enough cells for at least 1.5-2 x 96-well plates of embryoid bodies.

The steps from dissociation of iPSCs to seeding were performed quickly to reduce cell death. In preparation, 96-well ultra-low attachment V-bottom plates (Greiner Bio-One, 651970) were filled with 50 µl/well *Induction medium* (50% Iscoves MDM (Gibco, 31980022), 50% Ham's F12 (Gibco, 31765-027), 7 µg/ml insulin (Sigma, I3536-100MG), 5 mg/ml BSA (Sigma, A4161-5G), 1x Lipid concentrates (Gibco, 11905-031), 450 µM Monothioglycerol (Sigma, M-6145), 15 µg/ml Apo-transferrin (Sigma, T1147), 1x Pen/strep (Gibco, 15140122)), supplemented with 50 µM Y-27632 (Abcam, ab120129) and 10 µM SB431542 (Tocris, 1614/10), and warmed to 37°C in the incubator. An aliquot of 1x TrypLE (Gibco, 12604013) was also prewarmed and tubes prepared for spinning cells, counting cells and freezing a day 0 pellet of cells for RNA extraction.

iPSCs were washed once with PBS and incubated with TrypLE for 3-5 min to detach. Harvested cells were centrifuged at 400 x *g* for 5 min, and the pellet resuspended at in 1 ml *Induction medium* with Y-27632 and SB431542. After cell counting, the volume was adjusted to 2.5 x 10⁵ cells/ml. A multi-channel pipette was used to add 50 µl/well of cell suspension to the prepared 96-well V-bottomed plate, giving a final cell concentration of 1.25 x 10⁴ cells in 100 µl per well. Plates were placed in an incubator undisturbed to allow aggregation to embryoid bodies.

Table 2.4 Medium components for cerebellar organoid differentiation

	Reagent	Supplier & code	Final conc in medium	Preparation and storage of stocks	Stock conc	per 100 ml medium
Induction medium	Iscove's Modified Dulbecco's Medium (MDM)	Gibco 31980022	0.5 x	Stored at 4°C.	1 x	50 ml
	Ham's F-12 Nutrient Mix	Gibco 31765027	0.5 x	Stored at 4°C.	1 x	50 ml
	Apo-transferrin	Sigma T1147-100mg	15 µg/ml	100 mg dissolved in 10 ml distilled H ₂ O. 150 µl and 300 µl aliquots stored at -20°C.	10 mg/ml	150 µl
	Monothioglycerol	Sigma M6145-25ml	450 µM	Stored at 4°C.	1.25 g/ml	3.9 µl
	BSA	Sigma A4161-5G	5 mg/ml	5 g dissolved in 20 ml distilled H ₂ O overnight at 4°C without agitation. 1 ml aliquots stored at -20°C.	250 mg/ml	2 ml
	Chemically Defined Lipid Concentrate	Gibco 11905031	1x	1 ml aliquots stored at -20°C.	100 x	1 ml
	Insulin	Sigma I3536-100MG	7 µg/ml	100 mg dissolved in 10 ml acidic H ₂ O (0.01 M HCl). 75 µl and 150 µl aliquots stored at -20°C.	10 mg/ml	70 µl
	P/S	Gibco 15140122	1x	1-5 ml aliquots stored at -20°C.	100 x	1 ml
Supplements	Y-27632	Abcam ab120129	50 µM	10 mg dissolved in H ₂ O. 10 µl and 50 µl aliquots stored at -20°C.	100 mM	50 µl
	SB431542	Tocris 1614/10	10 µM	10 mg dissolved in appropriate volume of DMSO for lot. 20 µl aliquots stored at -20°C.	20 mM	50 µl
	FGF2	R&D 4114-TC-01M	50 ng/ml	Prepared to 200 µg/ml stock then diluted to 20 µg/ml. 50 µl aliquots stored at -20°C.	20 µg/ml	250 ng/ml in medium
Differentiation medium	Neurobasal	Gibco 21103049	1x	Stored at 4°C.	1 x	97 ml
	GlutaMax	Gibco 21103049	1x	Stored at 4°C.	100 x	1 ml
	N2	Gibco 17502048	1x	1 ml aliquots stored at stored at -20°C.	100 x	1 ml
	Pen/Strep	Gibco 15140122	1x	1-5 ml aliquots stored at stored at -20°C.	100 x	1 ml

2.3.2 Patterning to cerebellar identity (day 2 to 21)

On day 2 of differentiation, FGF2 was added to the cultures to induce a cerebellar identity. First, 20 µl of spent medium was removed from each well. This was replaced with 20 µl of *Induction medium* supplemented with 50 µM Y-27632, 10 µM SB431542 and 250 ng/ml FGF2 (R&D, 4114-TC-01M). Therefore, resulting in a concentration of 50 ng/ml FGF2 in each well.

On day 7, a 1/3-volume medium change was performed using *Induction medium* with no additional supplements. On day 14, organoids were transferred to 48-well ultra-low attachment plates (Greiner Bio-One, 677970) in 200 µl of fresh *Induction medium* per well.

2.3.3 Cerebellar progenitor maturation (day 21 to 35)

After 21 days, medium was switched to *Differentiation medium* (Neurobasal (Gibco 21103-049), 1x GlutaMax (Gibco 35050-061), 1x N2 (Gibco 17502-048), 1x Pen/Strep (Gibco, 15140122)) and a complete medium change performed every 7 days up to day 35.

2.3.4 Long-term growth of cerebellar cultures in 3D (day 35 onwards)

Beyond day 35, organoids can be kept in suspension to over 63 days or transferred to membranes for air-liquid interface culture for over 100 days.

In suspension culture, *Differentiation medium* volume was increased to 300 µl/well and a 1/3-1/2 volume medium change performed 3x per week.

For air-liquid interface culture, transwell membranes (Millipore, PICMORG50) were placed on top of 1 ml of *Differentiation medium* in a 6-well plate well. Using a cut off pipette tip, organoids were transferred onto the membrane and excess medium removed. Five organoids were grown on each transwell. A complete medium change was performed 3x per week.

2.3.5 Long-term growth of cerebellar cultures in 2D (day 35 onwards)

For 2D culture of iPSC-derived cerebellar neurons various medium compositions tested for growth as described in Chapter 4. This section will describe the general procedure for dissociation organoids and plating to 2D.

2D cerebellar cultures were grown in 24 or 96-well tissue coated treated plates, on sterile coverslips or in 96-well CytoView multi-electrode array plates (Axion, M768-tMEA-96B). All plates were coated with 15 µg/ml poly-L-Ornithine (100x stock prepared in distilled H₂O, 100 µl aliquots stored at -20°C Sigma, P2533) in distilled H₂O overnight at 37°C. Plates were then washed thoroughly with distilled H₂O to remove any traces of poly-L-ornithine which can be toxic to cells and dried for at least one hour in a tissue culture hood. When necessary, coated plates were stored at 4°C for up to one week. Next, plates were coated with 20 µg/ml laminin in PBS (Aliquots of 30 µg and 60 µg stored at -20°C, Sigma, L2020) for 3-4 hour at 37°C.

At day 35 organoids were collected into HHGN (1x HBSS (Invitrogen, 14180-046), 2.5 mM HEPES pH7.3-7.5 (Invitrogen 15630-049), 35 mM Glucose, 4 mM NaHCO₃ (Sigma, S6297) in H₂O). Organoids were then incubated in Neuronal isolation enzyme (Thermo Scientific, 88285) for 12-15 min in a water bath at 37 °C, with resuspension half-way through. For dissociation of 10-15 organoids 100 µl of Neuronal isolation enzyme was used, when more cells were required, multiple batches were set up and dissociated cells pooled. Once the organoids appeared slightly fluffy, Neuronal isolation enzyme was carefully removed, and organoids washed in HHGN. Organoids were dissociated by gentle pipetting in 500 µl of *Differentiation medium*. Dissociated cells were pooled into 5 ml Neurobasal medium and centrifuged at 400 x g for 5 min. The cell pellet was resuspended in *Differentiation medium* (or medium being tested e.g. BrainPhys) and plated at 80,000 cells/cm². A 1/3 medium change was performed gently three times per week. For extended culture, 5 µg/ml laminin was added to culture medium after 2-3 weeks.

When Neuronal isolation enzyme was not available, cerebellar organoids were dissociated using Accutase (Sigma, A6964), however viability was decreased to around 50-60% from 70-80%. For this method, organoids were incubated in 300 µl Accutase. After 20 min, 300 µl *Differentiation medium* was added and organoids dissociated manually by pipetting, before transferring cells into 5 ml Neurobasal medium and continuing as above.

To freeze differentiate cells at day 35, organoids were dissociated using Neuronal isolation enzyme as above. Following dissociation and spinning, cells were resuspended in CryoStor

CS10 (StemCell, 07930) for freezing. On thawing, cells were plated as above, but with addition of 10 μ M Y-27632 to *Differentiation medium* for the first 24 hour.

2.4 Analysis of iPSC-derived cerebellar cultures

2.4.1 Immunostaining of fixed frozen organoid sections

Organoids were collected into a PBS wash using a P1000 cut off pipette tip, and then fixed in 4% PFA for 15-20 min at room temperature. After fixing, organoids were washed once in PBS and then transferred into 20% sucrose (Sigma, S9378) to equilibrate overnight at 4°C. The following day, organoids were transferred into moulds, embedded in Optimal Cutting Temperature compound (OCT, Fisher Scientific, KMA-0100-00A) and frozen on dry ice to form cryoblocks for sectioning. Samples were cryosectioned at 10-30 μ m (15 μ m was used for most stainings) onto SuperFrost slides (VWR, 6310108). For quantification of co-expression, organoid sections were distributed across slides so each slide contained non-sequential sections from throughout the organoid.

Before staining, slides were warmed to room temperature and then hydrated in PBS for 10 min. Sections were treated with 0.1 M glycine (Sigma, G8898) for 30 min to quench free aldehyde groups, before permeabilising with 0.3% Triton-X-100 (Sigma, T9284) in PBS (PBST). Next blocking was performed using 2% milk (Millipore, 70166) in PBST for 1 hour, following by incubation with primary antibodies (Table 2.6) in block overnight at 4°C. Unbound antibodies were removed in three washes with 0.05% Tween in PBS and then secondary antibodies applied (1:1000, Alexa Fluor, Invitrogen). After 1 hour incubation, wash steps were repeated and nuclei stained with 1 μ g/ml Hoechst33258 (Invitrogen, H1398) in PBS before mounting in Vectashield (Vector, H-1000). Stained slides were imaged using an Axioplan2 upright fluorescent microscope or a Zeiss-880 laser scanning confocal microscope.

2.4.2 Immunostaining of whole fixed organoids

Whole organoid staining used a protocol adapted from Dekkers et al., 2019. Apart from washes, all steps were performed at 4°C. Organoids were fixed in 4% PFA for 45 min with

resuspension midway. Fixed samples were permeabilised with 0.1% Tween in PBS for 15 min, followed by blocking in organoid wash buffer (0.1% Triton-X-100, 0.2% BSA in PBS) for 1 hour. Organoids were incubated with primary antibodies (Table 2.6) in organoid wash buffer overnight. Samples were washed three times for 2 hour in organoid wash buffer with rocking. Secondary antibodies and washes were performed as for primary antibodies, but Hoechst33258 was added for 30 min of the final wash. Organoid tissue was cleared by incubating and mounting samples in glycerol-fructose solution (60% glycerol, 2.5 M fructose in H₂O). Whole organoids were imaged using a Zeiss-880 laser scanning confocal microscope.

2.4.3 Immunostaining of fixed 2D cultures

To stain 2D cerebellar cultures, cells were fixed in 4% PFA for 20 min at room temperature. Samples were permeabilised for 20 min in 0.4% Triton-X-100 in PBS, followed by blocking in 5% serum and 0.1% Triton-X-100 in PBS. After blocking, primary antibodies (Table 2.6) were incubated in 5% serum in PBS, overnight at 4°C. Three PBS washes were performed before incubating secondary antibodies (1:1000, Alexa Fluor, Invitrogen) for 1-2 hour at room temperature. After three more washes in PBS, nuclei were stained using 1 µg/ml Hoechst (Invitrogen, H1398) in the final wash and samples mounted onto slides with Vectashield (Vector, H-1000). Alternatively, mounting medium containing DAPI was used (ProLong Gold, Invitrogen, P36931). Immunostained coverslips were imaged on an Axioplan2 upright fluorescent microscope or Zeiss-880 laser scanning confocal microscope, cultures in 96-well imaging plates (PerkinElmer, Viewplate) were imaged using a Leica DMI8 inverted widefield microscope.

2.4.4 Live imaging of mNeon fluorescence

Z-stack live cell imaging of whole FOXP2-P2A-mNeon organoids used a Leica Mica confocal imaging system. An Olympus IX 81 inverted widefield microscope was used for live cell imaging of 2D cerebellar cultures.

2.4.5 RNA extraction and RT-qPCR

RNeasy Mini or Micro kits (Qiagen, 74104 or 74004) were used for isolation of RNA from iPSC pellets and cerebellar organoids. Around 20-30 organoids were enough for RNA extraction at day 21, 15-20 at day 35 and 8-10 at later timepoints. For 2D cerebellar cultures, 2-3 wells of a 24-well plate were lysed for RNA. RNA was eluted in 16-20 µl RNase-free water and measured using a Nanodrop.

For each reaction, 0.5 -1 µg of RNA was used for generating cDNA with the SuperScript™ III First-Strand Synthesis System kit (Invitrogen, 18080051). cDNA was then diluted 1:10 for use in RT-qPCR. Newly designed primers were tested using a standard curve to determine efficiency and melt-curves checked for a single qPCR product peak (Table 2.5). RT-qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems, 4385612) on StepOne plus Reverse transcription PCR system (Applied Biosciences). Each gene-sample pair was performed in triplicate, with the average value taken from these technical replicates.

Expression of genes of interest (target) was calculated using the dCT method relative to housekeeper genes *ACTIN* and *GAPDH*, $dCT_{\text{target}} = CT_{\text{target}} - \text{mean}(CT_{\text{housekeeper}})$. Statistical tests were performed on dCT values. For comparison of test samples with iPSCs or control lines (reference), ddCT was calculated ($ddCT_{\text{test}} = dCT_{\text{test}} - dCT_{\text{reference}}$).

2.4.6 Western blotting

To extract protein, cerebellar organoids were lysed in ice cold Pierce RIPA buffer (Thermo Scientific, 89900) supplemented with protease (cOmplete™, Roche, 04693159001) and phosphatase (PhosSTOP™, Roche, 04906837001) inhibitors. Organoids were manually disrupted using a needle to improve lysis. At days 50-70, around 6-8 organoids were lysed in 75-150 µl. For 2D cerebellar cultures, cells from 3-4 wells of a 4-well plate were lysed directly in the plate using 100 µl RIPA buffer with inhibitors. After lysis, protein samples were quantified using a Bradford assay (Thermo, 23238) and stored at -80°C.

For Western blotting, protein samples were diluted with NuPAGE™ LDS Sample Buffer (Invitrogen, NP007) containing 25 mM DTT and denatured by heating at 70°C for 10 min. Per

sample, 30-50 µg of protein was loaded on a 4-12% gradient Bis-Tris gel (Invitrogen, NP0335) and run at 200 V for 50 min. Protein was then transferred to a nitrocellulose membrane over 1.5-2 hour at 250 mA. The membrane was blocked with 10% milk in TBST (150 mM NaCl, 10 mM Tris pH8, 0.05% Tween 20) and primary antibodies (Table 2.6) incubated overnight at 4°C, diluted in 3% BSA in TBST. After washing 3 times in TBST, secondary antibodies (HRP-conjugated, GE healthcare) were applied for 1-2 hour at room temperature in 3% BSA in TBST. Unbound antibodies were removed by three washes in TBST. Pierce™ ECL Western Blotting Substrate (Thermo Scientific, 32209) was added to the membrane and signal captured using a Chemidoc MP imaging system.

2.4.7 Multi-electrode array

Day 35 cerebellar organoids were dissociated and plated at 80,000 cells/cm² onto 96-well CytoView multi-electrode array (MEA) plates (Axion, M768-tMEA-96B) as described above (section 2.3.5). Recordings were performed using an Axion Maestro pro machine, between days 41 and 69 of differentiation. MEA recordings were for 5 min intervals, with cells incubated at 37°C and 5% CO₂. Extracellular field potentials were recorded by the 8 electrodes within each well. A threshold of 6-standard deviations above noise was applied for peak classification. In the analysis, wells were classified as active if one or more peaks was recorded in that well during the recording. For active wells, the firing rate described the frequency of peaks within the recording time.

2.4.8 Spontaneous neuronal activity assay

The spontaneous neuronal activity (SNA) assay (Sartorius) provided an overview of activity dynamics. Cerebellar organoids were dissociated and plated at 80,000 cells/cm² onto 96-well ViewPlates (PerkinElmer) as described above (section 2.3.5). To visualise intracellular calcium concentration in neurons, cerebellar cultures were transduced with an Incucyte® Neuroburst Orange Lentivirus (Sartorius, 4736). The Neuroburst Lentivirus encodes a mRuby-based fluorescent indicator calcium, controlled by a *Synapsin* promoter thus providing neuronal specificity. At 24 hour after transduction, 90% of medium was changed and transduced plates were placed in an Incucyte® live cell analysis system within an incubator. The Incucyte enabled

automated non-disruptive imaging over time. Cerebellar cultures were recorded for 60-90 s per well every 24 hours. A few gaps of more than 24 hour between recordings occurred when the Incucyte® system was not accessible.

Incucyte videos were analysed using the Incucyte® Neuronal Activity Analysis Software Module (Sartorius, 9600-0032). The software identified active objects and plotted traces over time showing calcium peaks. The plate layout, including conditions such as different cell lines or medium conditions were integrated into the analysis. Several parameters, such as the mean number of active objects or mean correlation between active objects in a well, could be calculated for each condition. For this work, the number of active objects per well was focused on and this data was extracted from the Incucyte® software and replotted in R.

2.4.9 Calcium imaging using calcium fluorescent dye

The second method for imaging calcium dynamics used a fluorescent dye Calbryte™ 590 AM (AAT Bioquest, 20701) which was non-specific to cell type. Cerebellar cultures grown in 2D were loaded with 2 μ M Calbryte™ 590 AM and 0.04% Pluronic F-127 (AAT Bioquest, 20050) for 1 hour, followed by a complete medium change. To trigger depolarisation, 50 mM KCl (Sigma, A11662-0B) was added manually. Calbryte-loaded cultures were imaged for 2 min at 2 Hz, with KCl or PBS (mock) added at approximately 10 s into recording using a Zeiss-880 laser scanning confocal microscope.

Recordings were analysed in ImageJ and then extracted data processed in R. First, a Weka trainable segmentation classifier (ImageJ plugin) was applied to selected regions of interest (ROI). The classifier selected cell bodies from a maximum intensity projection of the timecourse stack. Next, the mean intensity for each ROI was extracted across the recording. This dataset was loaded into R along the timing of KCl or mock addition. Normalised fluorescence intensity (Df/f_0) was calculated as $Df/f_0 = (f - f_0)/f_0$, where f_0 is the baseline fluorescence calculated as an average of timepoints prior to stimulation and f , is fluorescence at a given time. Frames were also aligned so the frame after stimulation was frame 0. Recordings using mock stimulation (PBS only) were used to determine a minimum df/f_0

response threshold for an active peak. The chosen threshold value was a maximum peak response greater than $\frac{3}{4}$ of mock stimulation ROIs. Active ROIs were also filtered to those which had a peak within 30 s of KCl addition and outliers for parameters maxpeak (maximum df/f_0 value) and area under the curve were excluded using repeated Grubbs' test. Peak response parameters (max peak, area under the curve and timing of max peak) were calculated for each individual active ROI, and then averaged for all ROIs within a recording. To calculate responsive cells, the number of active ROIs was compared to the number of ROIs in total identified for that recording.

2.4.10 Flow cytometry

Flow cytometry analysis was performed on live cells from 2D cerebellar cultures to compare the mNeon+ population across conditions. For each condition, cells from 3 wells of a 24-well plate were washed gently in PBS and harvested using AccuMax (Sigma, A7089). Detached cells were washed off into 5 ml neurobasal and centrifuged at $400 \times g$ for 5 min. Cell pellets were resuspended in FACS buffer (1% BSA in PBS) at 1×10^6 cells/ml and 1 μ g/ml DAPI (Biolegend, 422801) added to stain dead cells. Cerebellar cultures grown from AH0-17-3 cells with no mNeon expression and a sample of cells unstained with DAPI were used as a negative control to position gates.

For staining of organoid samples, cerebellar organoids were harvested at day 63-65 and dissociated as described for live cell sorting (2.6.1). Dissociated cells were fixed and stained following the same procedure as for iPSCs (2.2.9). Prior to incubation with cells, 0.5 μ g of each primary antibody (Table 2.6) was conjugated with a CoraLite® Plus 647 fluorophore using the FlexAble CoraLite® Plus 647 kit (Proteintech, KFA003) and 2.5 μ l of conjugated antibody was added to each cell suspension in 30 μ l of FACS buffer.

Both live and fixed cell samples were analysed using a Cytoflex LX analyser (Beckman Coulter).

Table 2.5 RT-qPCR primer sequences

Target	Fw primer	Rv primer	Source	Primer testing
<i>ACTIN</i>	GCCGCCAGCTCACCATGGATG	CCATCACGCCCTGGTGCCTGG	(Watson <i>et al.</i> , 2018)	ef = 1, R ² = 0.997, Tm = 81°C
<i>ATOH1</i>	TGTTATCCCGTCGTTCAACAAG	TGGGCGTTTGTAGCAGCTC	(Muguruma <i>et al.</i> , 2015)	
<i>CA8</i>	CCTTCACTGCAAGGTGGGAT	TGTTGGCTTGAAGGCTGTGA	Primer blast	ef = 0.861, R ² = 0.998, Tm = 77°C
<i>CALB1</i>	GATTGGAGTTATCACCTGAA	TTCCTCACAGGACTTCAGCT	(Watson <i>et al.</i> , 2018)	
<i>CNTNAP2</i>	GACACAGCTCGTTTACAGCG	AACCCACCAAGTGTAAGGGC	Primer blast	ef = 0.775, R ² = 0.994, Tm = 80°C
<i>EN1</i>	GCTTGTCTCTCTTCTCGTTC	TGGTCAAACTGACTCGCAG	(Watson <i>et al.</i> , 2018)	
<i>EN2</i>	CCGGCGTGGGTCTACTGTA	GGCCGCTTGTCTCTTTGTT	(Muguruma <i>et al.</i> , 2015)	
<i>FOXP1</i>	CTTCAGCTTCTCTGGATCG	GGCAGATCTCTATGCAAGC	Genevieve Konopka	ef = 1.219, R ² = 0.975, Tm = 78°C
<i>FOXP2</i>	TGGCAGCAGAGATGGAAGAT	AGTTGTCTTGTCTGGAG	Genevieve Konopka	ef = 0.988, R ² = 0.981, Tm = 79°C
<i>FOXP4</i>	AATGGTGAGATGAGTCCCGC	TTTGCTGTATTGTTCCCTGG	Primer blast (VC)	ef = 0.977, R ² = 0.999 (VC)
<i>GAPDH</i>	GGAAGGTGAAGGTCGGAGTC	GTTGAGGTCAATGAAGGGGTC	(Nayler <i>et al.</i> , 2021)	ef = 1.135, R ² = 0.978, Tm = 78°C
<i>GBX2</i>	AAAGAGGGCTCGCTGCTC	GGTCGTCTTCCACCTTTGAC	(Nayler <i>et al.</i> , 2021)	
<i>GLRA2</i>	CAGGCCAAATTTAAAGTCTCCA	CGTTTCTGTGACTGATCAAAACTG	Primer blast	ef = 0.918, R ² = 0.984, Tm = 72°C
<i>GRIK3</i>	CAGCAGAGAGAGAGCAGCGTT	GGCTGAGGCTTGTGCTTGAC	Primer blast	ef = 0.932, R ² = 0.981, Tm = 81°C
<i>GRIN3A</i>	TTTGCTGTACGGAGACTTTGC	GATTCGTGGTAGCAGCAGCC	Primer blast	ef = 0.981, R ² = 0.955, Tm = 79°C
<i>ITPR1</i>	CTTGGGCTGGTTGATGATCG	TAGCGGCTTTCCAGAACTGC	(Tolonen <i>et al.</i> , 2023)	
<i>KIRREL2</i>	GGGGCTAGTTCAGTGGACTAA	CACGGGCTAATGTGGAGG	(Watson <i>et al.</i> , 2018)	ef = 1.179, R ² = 0.98, Tm = 81°C
<i>LHX1</i>	AACAGCCTTCACTCGGCCAC	TCGTTGCTACCCGCTTCCTT	Primer blast	ef = 0.996, R ² = 0.987, Tm = 83°C
<i>MAB21L2</i>	TCAGGGAGCGCTATGTGGTG	AACCTTCGGCCTTGACCTC	Primer blast	ef = 0.957, R ² = 0.994, Tm = 85°C
<i>MEIS2</i>	CCAGTCAAATCGAGCAGGTTTTTC	ATCCCCATGTGTTGCTGACC	Primer blast (DR)	ef = 0.9 (DR)
<i>NR4A2</i>	GTAACCTGGCTGAAGCCATGC	GGGGAGAGAAGTGGTGGCAG	Primer blast	ef = 1.001, R ² = 0.984, Tm = 81°C
<i>PAX6</i>	AGCTAGCTCACAGCGGGG	TCTGATGGAGCCAGTCTCGT	(Van Essen <i>et al.</i> , 2023)	
<i>PTGER3</i>	AACCTTGATGTGGAGCGACCA	ATGCAAATTCAGGGAAGCAGGAA	Primer blast	ef = 0.998, R ² = 0.931, Tm = 74°C
<i>RORA</i>	AGATAGAGGGAGTCTCGGAGC	CCGGAGCTGACTCCATGTTT	Primer blast	ef = 1.03, Tm = 77.4
<i>SCN3B</i>	GAGGCGAGAGCCTTGGAAGC	TCCACACACAGGGAAGCAG	Primer blast	ef = 1.029, R ² = 0.996, Tm = 80°C
<i>SKOR2</i>	AGCCCAGTTCACCATCCAT	GCTGTTGTCATCCTTTGTAGATAC	(Watson <i>et al.</i> , 2018)	ef = 1.252, R ² = 0.915, Tm = 74°C
<i>SLC17A6</i>	TCGGCCAGATCTACAGGGTG	GCAGCGGATACCGAAGGAGA	Primer blast	ef = 1.005, R ² = 0.988, Tm = 84°C
<i>SLC6A17</i>	GTTTGCTGTGCTGGGCTTCA	TTGACGTGGGGTGGGATGAG	Primer blast	ef = 1.033, R ² = 0.981, Tm = 79°C

<i>SNCB</i>	TCTACGTCGGAAGCAAGACCC	CCCAGAGAACACAGCTCCTCC	Primer blast	ef = 1.075, R ² = 0.971, T _m = 79°C
<i>SYN1</i>	AGAAACCCAGCCAGGACGTG	GGGCTGGCTCTGGAAGTTG	Primer blast	ef = 1.094, R ² = 0.998, T _m = 83°C
<i>TFAP2A</i>	TTGGGTGCGAGACCGAGAGG	GGGAGATTGACCTACAGTGCCC	Primer blast	ef = 1.048, R ² = 0.995, T _m = 82°C
<i>ZFHx4</i>	CCCTCTCGTTTACTTGGATGTG	CAGCCTGTCAGGGACTTCC	Primer blast (DR)	ef = 0.96 (DR)
<i>mFoxp1</i>	CTACCGCTTCCATGGGAAAT	ACTGTGGTTGGCTGTTGTCA	Genevieve Konopka	ef = 0.94, R ² = 0.983 (VC)
<i>mFoxp2</i>	AAAGCAGCTTGCCTTTGCTA	CACGGGTCTTCCTTGACAT	Genevieve Konopka	ef = 0.906, R ² = 0.980 (VC)
<i>mFoxp4</i>	ATCGGCAGCTGACGCTAAATGAGA	AAACACTTGTGCAGGCTGAGGTTG	Primer blast (VC)	ef = 1.035, R ² = 0.930 (VC)
<i>mGapdh</i>	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGAGGAG	Primer blast (VC)	ef = 0.927, R ² = 0.997 (VC)
<i>mMeis2</i>	TCTCTCCAGCATCTCACACACC	CGATTTGACTGGTCAATCATGGG	Primer blast (DR)	ef = 1.01 (DR)
<i>mZfhx4</i>	GGAGCGATTACAGGGTGCTG	TCTCGTTCGCGCACTCTTTC	Primer blast (DR)	ef = 1.06 (DR)

Vincent Cheng (VC) and Dianali Rodriguez Fernandez (DR) contributed to primer design and testing as labelled.

ef, efficiency; R², R-squared; T_m, Melting temperature of qPCR product, indicated by melt curve peak.

Table 2.6 Primary antibodies

Target	Supplier	Code	Source	Use	Concentration
Actin	Abcam	Ab179467	Rabbit	WB	1:1000
Brachyury	R&D systems	AF2085	Goat	IF	1:100
CALB1	Swant	CB38	Rabbit	IF, WB	1:10,000 (IF), 1:1000 (WB)
EN1	Abcam	ab5732	Rabbit	IF	1:500
FOXA2	R&D systems	AF2400	Goat	IF	1:200
FOXP1	CST	2005	Rabbit	IF, WB, FACS	1:500 (IF), 1:1000 (WB), 1:24 (conjugated, FACS)
FOXP2	Abcam	Ab1307	Goat	IF	1:1000
FOXP2	Sigma	MABE415	Mouse	WB	1:1000
FOXP4	Atlas	HPA007176	Rabbit	IF, WB	1:1000 (IF), 1:1000 (WB)
GBX2	ThermoFisher	H00002637-M01	Mouse	IF	1:100
GFAP	Proteintech	16825-1-AP	Rabbit	IF	1:1000
GFAP	Abcam	Ab7260	Rabbit	IF	1:1000
IgG ⁶⁴⁷	Cell Signalling	2985S	Rabbit	FACS	5 µg/ml
IgM ⁴⁸⁸	Biolegend	401617	Mouse	FACS	15 µg/ml
KIRREL2	R&D	Af2930	Goat	IF	1:250
MAP2	Abcam	Ab183830	Rabbit	IF	1:500
mNeonGreen	Chromotek	32F6	Mouse	IF	1:1000
mNeonGreen	Chromotek	4M2 (test sample)	Rabbit	WB	1:1000
NANOG ⁶⁴⁷	Cell Signalling	5448S	Rabbit	FACS	5 µg/ml
NCAM	Abcam	ab75813	Rabbit	IF	1:200
NESTIN	Sigma	MAB5326	Mouse	IF	1:500
NESTIN	Abcam	Ab22035	Mouse	IF	1:100

PAX6	GeneTex	GTX113241	Rabbit	IF	1:500
PAX6	Abcam	Ab195045	Rabbit	IF	1:500
PCP4	Proteintech	14705-1-AP	Rabbit	IF	1:1000
S100 β	Sigma	SAB4200671	Mouse	IF	1:500
SKOR2	Atlas antibodies	HPA046206	Rabbit	IF	1:500
SOX17	R&D systems	AF1924	Goat	IF	1:100
Synapsin	Abcam	Ab254249	Rabbit	IF	1:500
Synaptogamin	Abcam	Ab13251	Mouse	IF	1:200
Synaptophysin	Abcam	Ab32127	Rabbit	IF	1:1000
TRA-1-60 ⁴⁸⁸	Biolegend	330614	Mouse	FACS	15 μ g/ml
TUJ1	GeneTex	GT1338	Mouse	IF	1:1000
TUJ1	Abcam	Ab78078	Mouse	IF	1:500
ZFHx4	Atlas antibodies	HPA023837	Rabbit	IF, FACS	1:500 (IF), 1:32 (conjugated, FACS)

IF, immunostaining; FACS, flow cytometry; WB, Western blotting.

2.5 Analysis of mouse cerebellar samples

Mouse cerebellar tissue was isolated at three embryonic days (E13.5, E15.5, E18.5) and four postnatal time points (P4, P7, P14 and adult) from WT mice (C57BL/6J OlaHsd). Animals were sacrificed, and cerebella extracted by Esther B. E. Becker and Mohamed F. Ibrahim, under Home Office Project License PP4924146. Vincent Cheng and Dianali Rodriguez Fernandez assisted with the mouse cerebellar RT-qPCR and cryosections.

RNA was extracted using the RNeasy mini kit (Qiagen, 74104) and used to make cDNA as described for organoid samples (section 2.4.5). For samples from E13.5 and E15.5, whole brains from multiple pups in the same litter were used to isolate RNA due to the small size of the cerebellum at this stage. At E18.5, cerebella from multiple pups in the same litter were used to extract RNA. For postnatal timepoints RNA was extracted from the isolated cerebellum of one mouse.

Mouse cerebellar samples for immunofluorescent imaging were fixed in 4% PFA overnight at 4°C and then cryoprotected in 30% sucrose. Mouse sections and immunostaining was performed as for organoid samples (section 2.4.1).

2.6 RNA-sequencing

2.6.1 Dissociation of organoids and isolation of mNeon populations

Organoids at day 63 of differentiation were harvested, washed in HHGN (1x HBSS (Invitrogen, Invitrogen 14180-046), 2.5 mM HEPES pH7.3-7.5 (Invitrogen 15630-049), 35 mM Glucose, 4 mM NaHCO₃ (Sigma, S6297) in H₂O) and incubated in neuronal isolation enzyme (Thermo Scientific, 88285) for 30-40 min at 37°C with resuspension every 10 min. After removal of enzyme, organoids were washed again in HHGN and gently dissociated to single cells in *Differentiation medium* by pipetting. Dissociated cells were centrifuged at 400 x *g* for 5 min and resuspended at 10⁷ cells/ml in FACS buffer (1% BSA (Sigma, A4161-5G) in PBS). To stain dead cells, 1 µg/ml DAPI (Biolegend, 422801) was added. Cells were sorted directly into RLT lysis buffer (Qiagen, 74004) using a BD FACSAria III (BD biosciences).

2.6.2 RNA extraction and quality control

RNA from sorted cell populations was isolated using the RNeasy Micro kit (Qiagen, 74004) according to manufacturer's instructions. Before sequencing, RNA concentration was measured using the Quant-iT RiboGreen RNA kit (Invitrogen, R11490) and the RNA integrity number (RIN) evaluated using High Sensitivity RNA ScreenTape (Agilent, 5067-5579) on a TapeStation. All samples submitted for sequencing had a RIN of 8.9 or above.

2.6.3 Library preparation and sequencing

Library preparation and sequencing was performed by the Oxford Genomics Centre. Library preparation used 22-58 ng of RNA per sample (NEBNext Ultra II Directional mRNA kit, New England BioLabs, E7760). Sequencing was performed on an Illumina NovaSeq6000 platform using 150 bp directional paired end read sequencing. David Sims performed the read alignment and quantification using Kallisto (Bray *et al.*, 2016) with Ensembl release 108. Over 45 million reads were aligned for all samples (45.7 – 52.5 million reads, 83-87% of total reads pseudoaligned).

2.6.4 Transcript quantification and differential expression analysis

Transcript quantifications were imported into R and mapped to genes by tximport (tximport_1.26.1) using a txtgene object (created with makeTxDbFromEnsembl from GenomicFeatures_1.50.3). Genes with more than 10 counts in 4 samples were included in analysis. DESeq2 (DESeq2_1.38.2, Love, Huber and Anders, 2014) was used for differential gene expression analysis using formula ~Differentiation + mNeonpopulation. The *lfcShrink* function type 'apeglm' was applied to remove noise from low expressed genes (Zhu, Ibrahim and Love, 2019). Genes with adjusted p-value (Benjamini-Hochberg method to correct for multiple testing) below 0.05 were determined significantly differentially expressed. Principle component analysis was performed using plotPCA (DESeq2) and results displayed using ggplot2 (ggplot2_3.4.0). For visualising, gene expression was normalised using variance stabilizing transformation (VST), additional plots were made using ggplot2 and Pheatmap (pheatmap_1.0.12).

Developing human cerebellar laser-captured micro-dissected bulk RNAseq data was published by Aldinger *et al.*, 2021. Human developing brain bulk RNA-seq data was downloaded from Brainspan (<https://www.brainspan.org/static/download.html>).

2.6.5 Gene set enrichment analysis

Gene set enrichment analysis (GSEA) was performed on VST normalised expression output from DESeq2 through the online tool (GSEA 20.4.0 on the GenePattern server <https://www.genepattern.org/>) (Mootha *et al.*, 2003; Subramanian *et al.*, 2005). Analysis used 1000 repetitions, permuted on geneset and a false discovery threshold of 0.05 for significant terms. REVIGO was used to summarise GSEA output by semantic similarity (<http://revigo.irb.hr/>) (Supek *et al.*, 2011)). The following optional parameters were used for REVIGO: resulting list size 'small', values associated with GO term = 'Lower value is better' (False discovery rate), species *Homo sapiens*.

2.6.6 Comparison to genesets

Genes differentially expressed between mNeon-sorted samples were compared with genesets to examine enrichment for cerebellar cell types, FOXP2 target genes and neurological disorder associated genes. In this analysis, to focus on the genes showing higher specificity to the *FOXP2* positive or negative population, differentially expressed genes had an adjusted p-value less than 0.05 and an absolute log2FoldChange of more than 0.58, corresponding to an absolute fold change of greater than 1.5x. GeneOverlap (GeneOverlap_1.34.0) was used to perform a Fisher's exact test to determine significance of overlap between genesets.

Conserved markers of cerebellar cell types were published by Sepp *et al.*, 2023, based on snRNA-seq of developing opossum, mouse and human cerebella. FOXP2 target gene lists were from previous FOXP2 studies in human cells: human fetal brain (ChIP)(Spiteri *et al.*, 2007) and SH-SY5Y cells (ChIP) (Vernes *et al.*, 2007) and *in vitro* differentiated human neural progenitors (differential gene expression) (Hickey, Berto and Konopka, 2019). Disorder gene lists were obtained from published datasets previously used for analysis of human cerebellar data (Aldinger *et al.*, 2021). In addition, the Autism spectrum disorder gene list contains genes categorised as class 1 (High confidence) by Simons Foundation Autism Research Initiative (SFARI, <https://gene.sfari.org/>, 01-23-2023 release). CAS genes were compiled from publications that performed whole-genome sequencing of probands with CAS and close family members (Kaspi *et al.* 2022; Eising *et al.* 2019; Hildebrand *et al.* 2020). Genes differentially expressed in autopsy brain samples of individuals with different neurological disorders were published by Gandal *et al.*, 2018.

2.7 Analysis of published scRNA-seq cerebellar datasets

Processed single cell or single nuclei RNAseq data from mouse (Carter *et al.*, 2018; Sepp *et al.*, 2023) and human (Aldinger *et al.*, 2021; Sepp *et al.*, 2023) developing cerebella, and day 90 cerebellar organoids (Nayler *et al.*, 2021), was used for examining cerebellar gene expression. The data from Sepp *et al.*, 2023 was downloaded on 28/01/2022 following bioRxiv release and prior to the final publication. Datasets were loaded and manipulated in R using Seurat (Seurat_4.3.0), SingleCellExperiment (SingleCellExperiment_1.20.0), tidyverse (tidyverse_2.0.0)

and reshape2 (reshape2_1.4.4). Packages Seurat (Seurat_4.3.0), ggplot2 (ggplot2_3.4.3) and RColorBrewer (RColorBrewer_1.1-3) were used for generating plots. CellChat analysis of signalling pathways in the cerebellum used package CellChat (CellChat_1.6.1), following tutorial (<https://github.com/jinworks/CellChat>).

2.8 Statistics

Statistical tests were performed in R. Significant differences between 2 groups were determined using student t-test. For more than 2 groups, ANOVA followed by post-hoc Tukey's HSD test was used to identify significant differences. Unless otherwise stated, a p-value threshold of 0.05 was used to determine significance and p-values are indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Chapter 3. Generation of a Purkinje cell reporter line

3.1 Introduction

Purkinje cells are large inhibitory neurons key to cerebellar function. Playing a central role in cerebellar circuitry, Purkinje cells process inputs from granule cells, climbing fibres and interneurons, and provide the sole output of the cerebellar cortex (D'Angelo, 2018). During development, Purkinje cells are also important to the differentiation of other cell types, as Purkinje cell secretion of SHH drives the expansion of granule cell progenitors (Corrales *et al.*, 2006). Therefore, loss or dysfunction of Purkinje cells underlies many cerebellar disorders (Sarna and Hawkes, 2003; Kapfhammer and Shimobayashi, 2023). During development, Purkinje cells must undergo migration, complex dendritic tree growth and arrangement into stripes of different subtypes (Leto *et al.*, 2016). This complex development is not fully understood and provides many opportunities for problems to arise.

Cerebellar development has been studied in mouse and other animal models, resulting in considerable knowledge of the timing and lineages of cells within the cerebellum (Leto *et al.*, 2016). However, studies of human cerebellar development are understandably more limited. Therefore, many findings defined in the mouse cerebellum, such as the emergence and mapping of Purkinje cell subtypes, have not been characterised in human cerebellar development. Although broadly similar to mouse, the human cerebellum has altered cell type ratios, a significantly longer developmental time and a more complex structure, due to increased foliation and expansion of the cerebellar hemispheres (Haldipur *et al.*, 2019). Human-specific features are important to understand, as they may have a significant role in cerebellar functions, particularly non-motor functions, and susceptibility to disease (Haldipur *et al.*, 2019). A recent study identified a human-specific change to the progenitor population in the ventricular zone, the cerebellar germinal zone which gives rise to GABAergic cell types, including Purkinje cells (Haldipur *et al.*, 2019). In the developing human cerebellum, SOX2+KI67+ progenitors are spread into a subventricular zone (VZ^{SVZ}) and mitosis occurs throughout the VZ and VZ^{SVZ}, rather than localised to the ventricular surface as in mice (Haldipur *et al.*, 2019) (Figure 1.8B). The VZ^{SVZ} is not present in mice and has not yet been

examined in non-human primates, meaning there is a lack models to study the role of this structure in cerebellar development (Haldipur, Millen and Aldinger, 2022). The larger pool of GABAergic progenitors in the VZ^{SVZ}, especially between Carnegie stage (CS)18 - CS23, may contribute to an increased early population of Purkinje cells in humans (Haldipur *et al.*, 2019; Sepp *et al.*, 2023). The peak proportion of cerebellar cells which are Purkinje cells was calculated to be 2-fold higher in human development than in mice (Sepp *et al.*, 2023) (Figure 1.8C). Additionally, a larger proportion of human developing Purkinje cells showed an early-born phenotype (Sepp *et al.*, 2023). Together, the increasing awareness of human-specific features of Purkinje cell development, and the potential insights they may give in disease, highlights the need for more human-based cerebellar models.

Multiple independent groups have shown that human iPSCs can be differentiated into cerebellar organoids which recapitulate features of human cerebellar development (Muguruma *et al.*, 2015; Silva *et al.*, 2020; Nayler *et al.*, 2021; Chen *et al.*, 2023). Cerebellar organoids provide an experimentally tractable tool for studying early human cerebellar development in both healthy and disease states (Ishida *et al.*, 2016; Van Essen *et al.*, 2023). The protocol starts with formation of iPSC aggregates, known as embryoid bodies (Figure 1.9A). These are cultured in suspension with growth factors to induce cerebellar identity (Figure 1.9A). Within 21 days, cerebellar organoids show expression of markers of the mid-hindbrain boundary such as EN1 and GBX2 (Figure 1.9B). Markers of cerebellar neural lineages are present by day 35 of differentiation (Figure 1.9C). VZ marker KIRREL2 is present in cerebellar organoids around day 35, localised to luminal structures. Purkinje cell markers SKOR2, FOXP2 and CALB1 are also induced during cerebellar organoid differentiation (Figure 1.9D & E). Thus cerebellar organoids provide a model of early human Purkinje cell development and these cells are grown within a heterogenous structure including the other major cerebellar cell types (Nayler *et al.*, 2021).

The diversity of cell types within organoids allows more accurate modelling of complex systems, such as the developing brain, than previous 2D approaches. However, the heterogeneity proves difficult in analysis, particularly when changes in individual cell types

may be lost within bulk organoid samples (Atamian, Cerdón-Barris and Quadrato, 2020). In addition, cerebellar organoids lack the architectural cues present in the *in vivo* developing cerebellum, so cell identity cannot be inferred from location. Analysis of specific cell populations in cerebellar organoids typically requires fixing samples, for sectioning and immunostaining or single-cell transcriptomic analysis. However, engineered fluorescent reporter lines can provide a live readout of cellular identity in brain organoids (Lam *et al.*, 2020; Bai *et al.*, 2023; Romero *et al.*, 2023; Sidhaye *et al.*, 2023). Fluorescent labelling of a marker gene's expression enables specific cell populations to be monitored in culture, including over time or across culture conditions (Bai *et al.*, 2023; Romero *et al.*, 2023). Moreover, the labelled population can be isolated by fluorescent sorting for further analysis (Lam *et al.*, 2020; Sidhaye *et al.*, 2023).

The aim when generating a fluorescent reporter line, is to label a specific population without interfering with normal cellular function. Tagging of endogenous proteins directly couples the presence of fluorescence to gene expression. However, the presence of a fluorescent tag can disrupt normal protein function. To avoid this interference, 2A peptides have been applied in bioengineering to facilitate multi-gene co-expression as individual proteins. This has been used for tagging genes of interest or production of protein complexes with controlled stoichiometry (Liu *et al.*, 2017). 2A peptides are 18-20 amino acid sequences, originating in picornaviruses, which enable production of multiple distinct proteins from a single, long open reading frame (Szymczak and Vignali, 2005) (Figure 3.1A). Although described as self-cleaving, 2A peptides have subsequently been found to act via a ribosome skipping mechanism: peptide bond formation is disrupted between a glycine and final proline, causing the upstream protein including the 2A peptide sequence up to the glycine to be released, then translation continues to produce the proline-attached downstream protein (Donnelly *et al.*, 2001a) (Figure 3.1B-D). The use of 2A peptides has been applied in iPSCs to generate fluorescent reporter lines for monitoring a range of brain cell differentiations: neural progenitors labelled by *SOX2* (Balboa *et al.*, 2017; Sidhaye *et al.*, 2023); retinal organoids with *VSX2*, *BRN3b* and *RCVRN* (Lam *et al.*, 2020) or *GNAT2* (Bai *et al.*, 2023); and dopaminergic neurons by *TH* (Überbacher *et al.*, 2019; Yokota *et al.*, 2021).

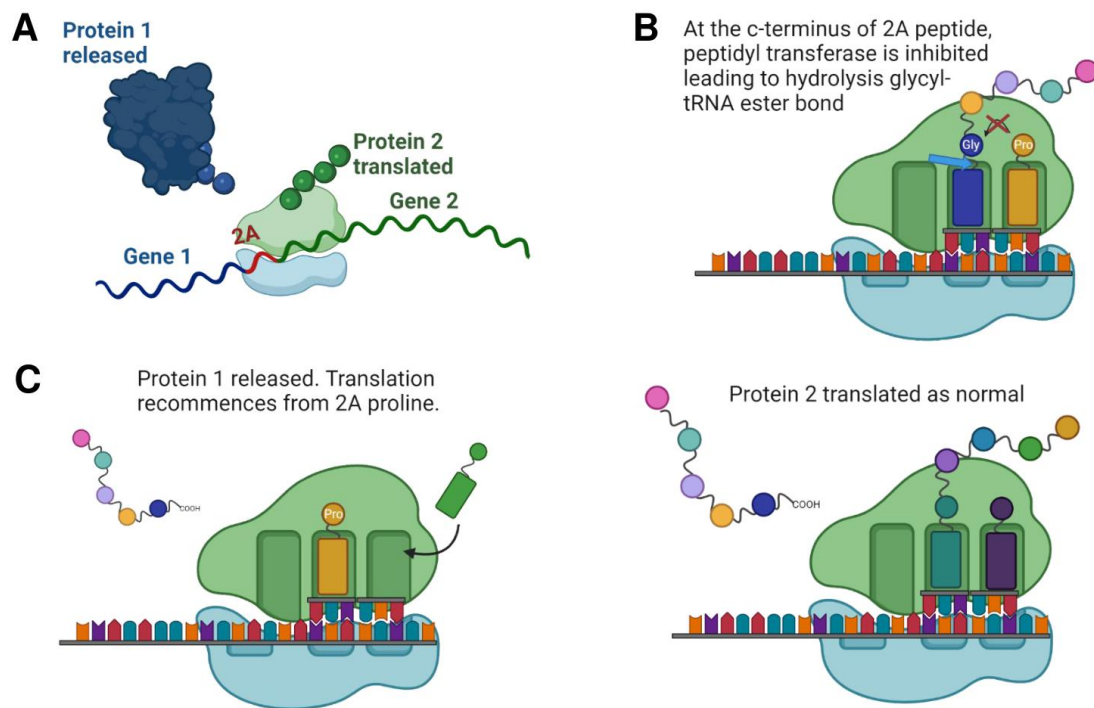


Figure 3.1 2A self-cleaving peptide “ribosome skipping” mechanism.

- A) 2A peptides alter translation, causing release of the nascent protein (protein 1) and allowing translation to continue to produce a second separate protein (protein 2). mRNA containing the 2A peptide sequence (red) between two genes (blue & green), is translated by a ribosome.
- B) The 2A peptide structure inhibits the normal mechanism of translation onto the final proline-tRNA. Instead, the glycine-tRNA ester bond is hydrolysed releasing the glycine and upstream translated protein. A ribosome is shown translating mRNA, within the ribosome the glycine-tRNA (blue) is attached to the nascent protein 1 and proline-tRNA (yellow) cannot form amine bond (red cross). Hydrolysis indicated with blue arrow.
- C) Translation resumes from the 2A peptide proline (central ribosome site), with a tRNA recognising the next codon.
- D) Normal translation of second protein occurs.

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Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated system protein 9 (Cas9) technology has enabled significant advances in our ability to introduce precise genetic changes in cells. A short guide RNA (gRNA) sequence is used to target nuclease Cas9 to a specific DNA sequence (Figure 3.2A). The targeted sequence is complementary to the gRNA, and adjacent to a protospacer adjacent motif (PAM) site with sequence NGG (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012) (Figure 3.2A). Once bound, Cas9 introduces a double-stranded (ds) DNA break (Gasiunas *et al.*, 2012; Jinek *et al.*, 2012). This method can be used to introduce

indels when the DNA is repaired by error-prone non-homology end joining (Cho *et al.*, 2013; Cong *et al.*, 2013; Mali *et al.*, 2013). However, when present, a donor sequence can be incorporated during a cellular DNA repair process known as homology-dependent repair (Mali *et al.*, 2013) (Figure 3.2B). This process requires the donor sequence to contain homology to the regions around the cut site. In this way, CRISPR technology facilitates precise insertion of desired donor sequences into a genome (Figure 3.2B).

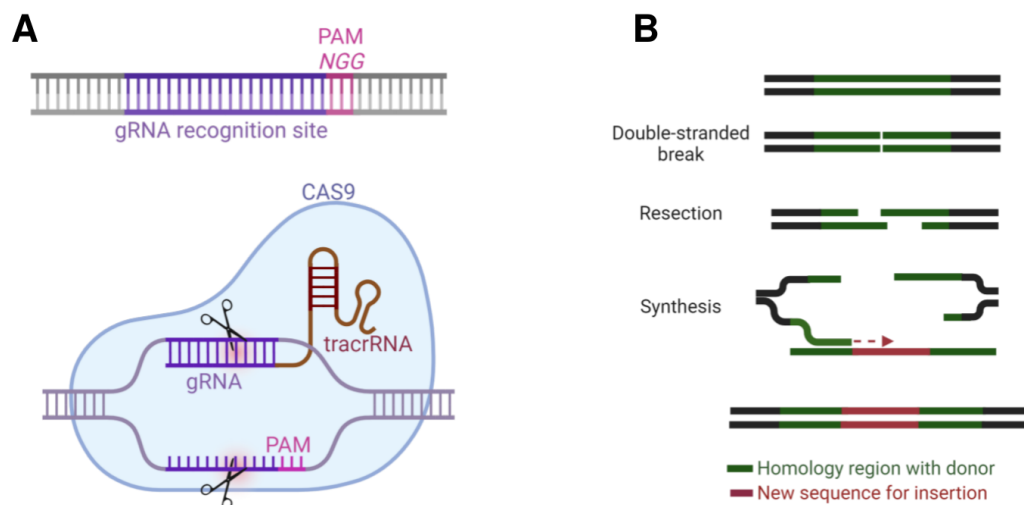


Figure 3.2 CRISPR/Cas9 gene editing.

- A) Cas9 is directed by a guide RNA (gRNA) to a specific DNA sequence where it produces a double-stranded break. The gRNA contains a CRISPR (cr)RNA and a transactivating (tracr)RNA sequence required to form an active complex. The crRNA is complementary to a 20 base pair sequence (purple) which must be adjacent to a PAM motif (pink) with a sequence in the form NGG.
- B) Following the production of a double stranded break by a CRISPR/Cas9 complex, the DNA can be repaired by homology dependent repair (HDR). This process requires a matching DNA template, such as the homologous chromosome or an artificial donor sequence. A new sequence can be introduced during the HDR process if surrounded by sequences homologous to the repair site.

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In designing a strategy for genetic editing, maximising insertion efficiency must be balanced with avoidance of off-target damage and minimising cellular stress. Transient introduction of CRISPR components, by electroporating ribonucleoprotein complexes of Cas9 and gRNA, limits the period of DNA editing due to the degradation of Cas9 (Kim *et al.*, 2014). In contrast, the use of plasmids encoding CRISPR components increases likelihood of off-target effects as the Cas9 nuclease persists for longer and can cause cellular toxicity (Kim *et al.*, 2014; Roth *et al.*, 2018). Similarly, the use of a single-stranded (ss) DNA repair template, as opposed to a dsDNA donor, avoids random insertion at unintended sites (Renaud *et al.*, 2016; Roth *et al.*, 2018). In addition, ssDNA templates have higher insertion efficiency at cut sites and lower toxicity to cells than dsDNA (Hornung and Latz, 2010; Renaud *et al.*, 2016; Roth *et al.*, 2018).

This chapter details the generation of a Purkinje cell fluorescent reporter iPSC line to label developing human Purkinje cells live in cerebellar organoids and provide a tool for studying this population. Selection of an appropriate marker, production of the reporter line using CRISPR/Cas9 technology and subsequent quality control of both the reporter line iPSCs and iPSC-derived organoids are described.

3.1.1 Chapter 3 aims

1. To generate a Purkinje cell reporter line using CRISPR/Cas9 mediated gene editing.
2. To perform quality control on Purkinje cell reporter line iPSCs to check for pluripotency and genetic integrity.
3. To differentiate the Purkinje cell reporter line to cerebellar organoids, performing quality control to assess differentiation and verify live visualisation of a mNeon+ population.

3.2 Results

3.2.1 Selection of *FOXP2* as a marker of Purkinje cells in cerebellar organoids

The first step in generating a fluorescent reporter line was selection of a gene to represent the developing Purkinje cell population. Transcriptomic data from both the developing human cerebellum (Aldinger *et al.*, 2021) and cerebellar organoids (Nayler *et al.*, 2021) was used to select a suitable marker of Purkinje cells. Differentially expressed genes identified as markers of Purkinje cells in scRNA-seq of the developing human cerebellum (Aldinger *et al.*, 2021) were ordered by Purkinje cell enrichment (average $\log_2(\text{fold-change})$) (Figure 3.3A). Next, expression of the top 20 Purkinje cell markers was examined in scRNA-seq data from day 90 cerebellar organoids to compare expression levels (Nayler *et al.*, 2021) (Figure 3.3B). *FOXP2* was identified as a good candidate marker due to its robust expression in cerebellar organoids, good Purkinje cell specificity in scRNA-seq data and previous characterisation as broadly expressed in developing mouse Purkinje cells (Fujita and Sugihara, 2012) (Figure 3.3A & B).

To support the selection of *FOXP2* as the chosen Purkinje cell marker, expression of *FOXP2* was also plotted across the developing brain (Figure 3.3C) and cerebellar cell types (Figure 3.3D). Bulk-RNAseq data from human brain development (Brainspan, <https://www.brainspan.org/>) showed high expression of *FOXP2* in the cerebellar samples (upper rhombic lip, cerebellum, cerebellar cortex), particularly during the earliest weeks of brain development (Figure 3.3C). In addition to the cerebellum, *FOXP2* was also expressed in other brain regions, with high expression in the striatum, thalamus, amygdala and cortex. However, the cerebellum showed especially strong expression of *FOXP2* during embryonic development, the time period which cerebellar organoids most accurately model (Nayler *et al.*, 2021). Within the cerebellum, *FOXP2* showed early and widespread expression in developing Purkinje cells *in vivo* (Figure 3.3D). Previously, multiple studies in mouse cerebellar development have also identified *Foxp2* as a selective marker of Purkinje cells (Fujita and Sugihara, 2012; Wizeman *et al.*, 2019). *FOXP2* expression was reported to be lower but maintained in adult mouse Purkinje cells (Fujita and Sugihara, 2012), suggesting that more mature iPSC-derived Purkinje cells will retain *FOXP2* labelling. Beyond its use as a marker of Purkinje cells, *FOXP2* is also of interest due to its

A

Cell type

01-PC
02-RL
03-GCP
04-GN
05-eCN/UBC
06-ICN
07-PIP
08-BG
09-Ast
10-Glia
11-OPC
12-Committed OPC
13-Endothelial
14-Microglia
15-Meninges
16-Pericytes
17-Brainstem
18-MLI
19-Ast/Ependymal
20-Choroid
21-BS Choroid/Ependymal

Purkinje cell gene

Percent exp

● 75
● 50
● 25

Expression

2
1
0
-1

B

Expression

2.0
1.5
1.0
0.5
0.0

Gene

CA8
ITPR1
CNTNAP4
DAB1
RORA
HS6ST3
FOXP2
TMEM132D
CHST11
EBF1
PTGER3
COL24A1
GRM7
ESRRB
CDH11
PRKG1
OPCM1
BCL11A
FOXP4

C

Expression (log2 RPKM)

6
4
2
0

Age

8 pcw
9 pcw
10 pcw
11 pcw
12 pcw
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A) Expression of Purkinje cell marker genes was plotted in human developing cerebellar scRNA-seq data. Marker genes were identified, and scRNA-seq data provided by Aldinger *et al.* 2021. Point size scales for the percentage of cells in cluster expressing the gene. Colour scale indicates average expression level in cluster

Aldinger *et al.*, 2021 cell clusters: PC, Purkinje cells; RL, Rhombic lip; GCP, Granule cell progenitors; GN, Granule neurons; eCN/UBC, Excitatory cerebellar nuclei neurons/Unipolar brush cells; iCN, Inhibitory cerebellar nuclei neurons; PIP, PAX2+ interneuron progenitors; BG, Bergmann glia; Ast, Astrocytes; OPC, Oligodendrocyte precursor cells; MLI, Molecular layer interneurons; BS, Brainstem.

Continued.

Figure 3.3 *FOXP2* is a Purkinje cell marker expressed in cerebellar organoids.

- B) Expression of the same Purkinje cell marker genes was plotted in day 90 cerebellar organoids. Points represent expression in a single cell, expression shown across all cell types. scRNA-seq data from Nayler *et al.*, 2021. *FOXP2* is highlighted (red box).
- C) *FOXP2* is highly expressed in the cerebellum as well as the thalamus and striatum in the developing human brain. Panel replicated from Figure 1.11B.
- D) *FOXP2* is highly expressed in Purkinje cells from early in human cerebellar development. The DotPlot shows expression of *FOXP2* across cell types and time in the human cerebellum. scRNAseq data from Aldinger *et al.*, 2021. Samples with <10 cells were removed. PCW, post conception weeks.
- E) *FOXP2* was induced during cerebellar organoids differentiation. Expression of *FOXP2* was quantified by RT-qPCR and calculated relative to housekeepers (*ACTIN*, *GAPDH*). Green points represent individual differentiations (n = 3-8), black points show mean expression and error bars measure one standard deviation.
- F) Sections of day 63 cerebellar organoids were stained with an antibody against *FOXP2* (green) and nuclei stained with Hoechst (blue). Scale bar 100 μ m.

Before final selection, expression of *FOXP2* in cerebellar organoids was verified by reverse transcriptase quantitative PCR (RT-qPCR) (Figure 3.3E) and immunostaining (Figure 3.3F). *FOXP2* was strongly induced during differentiation of iPSCs to cerebellar organoids, with RT-qPCR measuring a 160-fold increase in *FOXP2* expression over the first 35 days of differentiation (Figure 3.3E). Characterisation of *FOXP2* protein expression by immunostaining found sparse positive cells from day 35, with more consistent presence of *FOXP2*⁺ cells in organoids from day 49 of differentiation onwards (Figure 3.3F). *FOXP2* expression persisted over to time to beyond 80 days of organoid differentiation.

In summary, *FOXP2* was found to be highly expressed in developing Purkinje cells, distinguishing this cell type within the cerebellum. Moreover, compared to other brain regions, *FOXP2* expression strongly labelled the cerebellum in early human brain development. Cerebellar organoids reflect *in vivo* development with strong upregulation of *FOXP2* expression by day 35 and around the same time as appearance of other markers for cerebellar neuron progenitors.

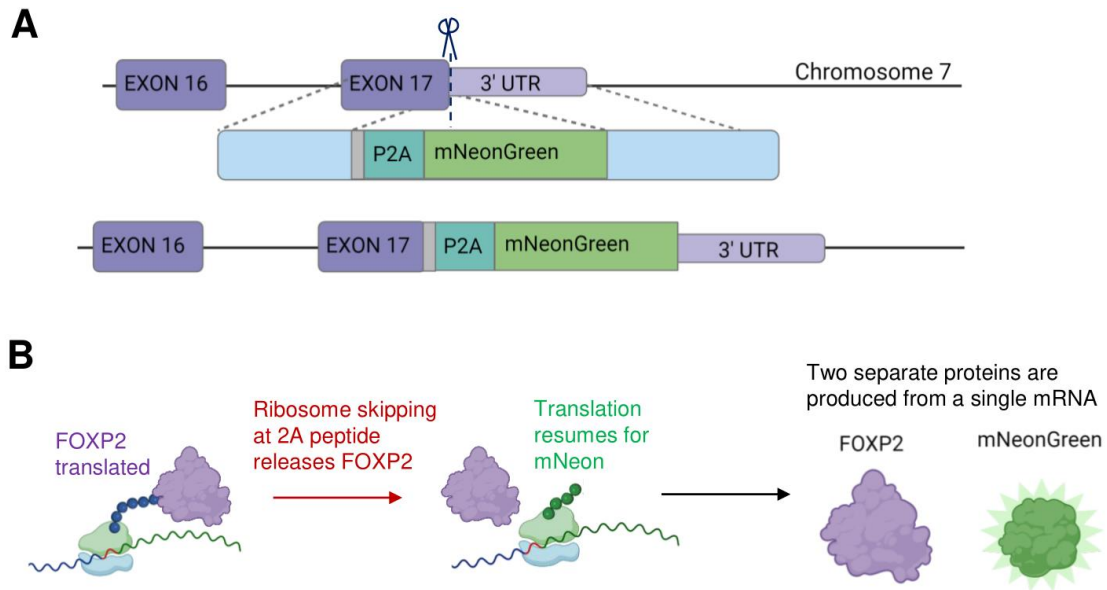


Figure 3.4 Strategy for generation of a *FOXP2* reporter line.

- A) Design of CRISPR strategy for insertion of the sequence encoding P2A-mNeonGreen into the endogenous *FOXP2* locus. A gRNA targets Cas9 (dark blue scissors and dashed line) close to the *FOXP2* stop codon (between exon 17 and 3' untranslated region (UTR)). The donor repair construct contains a P2A-mNeon sequence surrounded by homology arms (light blue) matching the regions adjacent to the *FOXP2* stop codon as indicated by the dashed lines. The result of homology-dependent repair shown below is a FOXP2-P2A-mNeon gene.
- B) The translation of FOXP2-P2A-mNeon mRNA results in separate FOXP2 (blue mRNA, purple protein) and mNeonGreen (green mRNA and protein) proteins due to a ribosome skipping mechanism caused by the P2A 'self-cleaving' peptide sequence (red sequence of mRNA).

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3.2.2 CRISPR strategy and preparation

A CRISPR/Cas9 homology-dependent repair approach was chosen to precisely engineer the Purkinje cell reporter line. The aim was to accurately label *FOXP2*-expressing cells with visible fluorescence, without disrupting normal FOXP2 function. First, to achieve accurate labelling, the design targeted insertion of the fluorescent protein sequence to the 3' end of the *FOXP2* locus, placing the reporter under the control of the endogenous *FOXP2* promoter (Figure 3.4A). Second, as most endogenous promoters drive only weak expression, a brightly fluorescent protein, mNeonGreen (mNeon), was selected for its excellent intensity to increase the likelihood of achieving easily detectable fluorescence (Shaner *et al.*, 2013). Third, a sequence encoding porcine teschovirus-1 (P)2A self-cleaving peptide was included prior to the mNeon sequence to produce separate FOXP2 and mNeon proteins (Figure 3.4B). 2A peptides cause a

ribosome-skipping mechanism during translation, resulting in the production of multiple proteins from a single continuous mRNA (Donnelly *et al.*, 2001a). Compared to other 2A-peptides, P2A shows high cleavage efficiency (Wang *et al.*, 2015). Finally, in order to specifically integrate the desired P2A-mNeon sequence, the repair construct contained two homology arms of approximately 650 base pairs (bp) matching regions adjacent to the *FOXP2* stop codon (Figure 3.4A).

Single guide RNA (sgRNA) sequences were designed to target the region around the *FOXP2* stop codon using online tools (CCTop and CRISPOR). Four sgRNAs were selected based on predicted high efficiency and low off-target impact (Table 2.2). To test efficiency in practise, the candidate sgRNAs were transfected individually with Cas9 into iPSCs. The resulting pools of cells were sequenced across the target cut site and the efficiency of indels quantified (Table 2.2)(Figure 3.5). The highest efficiency sgRNA was then selected for use in reporter line generation.

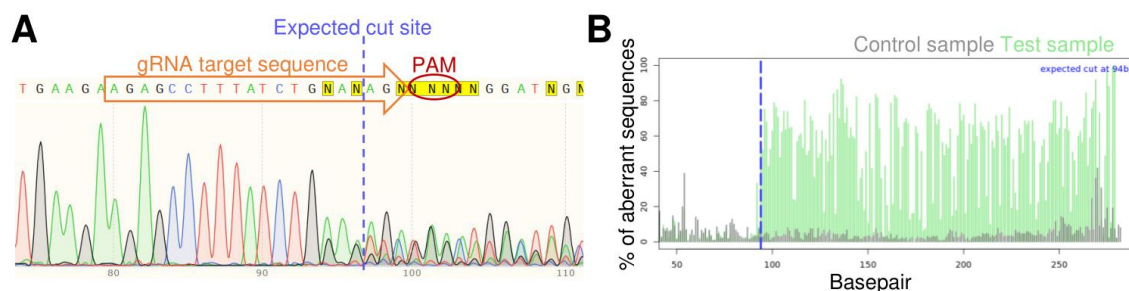


Figure 3.5 Testing efficiency of gRNAs.

The efficiency of gRNA directed CRISPR/Cas9 editing for each of the four designed gRNAs was tested in iPSCs. Genomic DNA was extracted from the pool of transfected cells and sequenced across the target site. Example results are shown for the most efficient gRNA chosen for CRISPR gene editing.

- A sequencing trace from the pool of cells transfected with gRNA and Cas9. A loss of common sequence is seen after the projected cut site, as a result of mutations produced by inaccurate DNA repair following Cas9-mediated DNA cleavage. The gRNA target sequence (orange arrow), expected cut site (dashed line) and PAM motif (red circle) are highlighted.
- Output from TIDE analysis comparing aberrant sequence between control samples (no gRNA transfected, grey) and test samples (with gRNA, green) to quantify efficacy of the gRNA. The blue dashed line shows position of expected cut site.

For high repair efficiency and low cytotoxicity, use of a ssDNA donor construct was chosen. ssDNA was generated by enzymatic digestion, targeting the phosphate-labelled strand of a dsPCR product (Figure 3.6A). There is currently no method for predicting the relative repair efficiency of each DNA strand, so ssDNA constructs for both the forward and reverse strand were prepared. ssDNA product size and purity were checked by gel electrophoresis before use (Figure 3.6B).

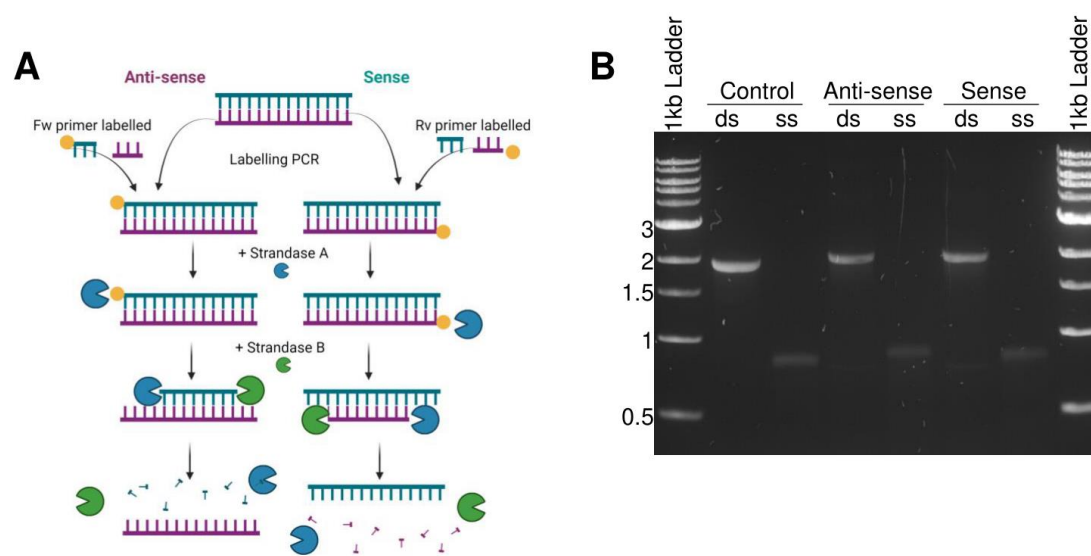


Figure 3.6 Generation of single-stranded DNA donor.

- A) A ssDNA donor was generated for CRISPR/Cas9 gene editing using the Guide-it™ Long ssDNA Production System v2 (Takara Bio, 632666). First, a PCR using one 5'-phosphate labelled primer and one unlabelled primer is used to amplify a double-stranded (ds)DNA template and label one strand. Next, the labelled strand is digested using a two-stage enzymatic digestion, with strandase A and strandase B enzymes, to leave ssDNA. To generate ssDNA copies of each strand, the opposite primer is labelled in two separate reactions. To make the anti-sense strand (purple, left reaction diagram) the forward (Fw) primer is labelled and to make the sense strand (turquoise, right reaction diagram) the reverse (Rv) primer is labelled.
- B) To check the size and purity of the ssDNA donors before transfection, samples of both the dsDNA and ssDNA were run on a 1 % agarose gel. ssDNA copies were produced for both sense and anti-sense strands of the CRISPR donor sequence, along with a positive control sequence provided with the kit. ssDNA products run at approximately half the size of the parental dsDNA (2.1 kilobase pairs (kb)) and show clean bands indicating full digestion.

A created with BioRender.com.

3.2.3 Clone production and screening

The control iPSC line, AH0-17-3, was chosen as the parental line for generating a reporter line as it has been previously characterised and is known to differentiate well into cerebellar organoids (Handel *et al.*, 2016; Watson *et al.*, 2018). To generate the reporter line, AH0-17-3 iPSCs were transfected with a ribonucleoprotein, consisting of the selected sgRNA and Cas9 protein, and the ssDNA donor construct. Genomic DNA (gDNA) was extracted from the resulting pools of electroporated cells. A PCR spanning from sites outside the homology arms (Table 2.1, Genotyping PCR), was then performed to check for successful insertion (Figure 3.7A & B). In WT cells a PCR product of around 1.4 kilobase pairs (kb) was expected, as observed in the sample from untransfected cells (Figure 3.7A & B). Following insertion of the sequence encoding mNeon, a larger PCR product of around 2.2 kb was predicted (Figure 3.7A). Successful insertion was evident from the presence of larger PCR products, including at the predicted size of 2.2 kb, in the cells transfected with both the sgRNA and a ssDNA donor (Figure 3.7B). As both ssDNA donor constructs yielded successful insertion with a similar efficiency, the pool of cells from the anti-sense donor was used for proceeding clone isolation.

To isolate clonal iPSCs derived from single-cells, serial dilution of the CRISPR pool was performed onto CF1 feeder cells. Colonies were then picked and screened for insertion of the P2A-mNeon sequence. Of 288 clones picked, gDNA was successfully isolated from 245 clones, and 75 clones showed the presence of some mNeon insertion, an efficiency of around 30% (Figure 3.7A & C). Selected clones with integration were then further characterised to determine genotype and insertion size using a second PCR (Figure 3.7A & D). From 75 clones with evidence of mNeon insertion, 17 heterozygous and 17 homozygous clones were identified as having insertions of approximately the correct size. Finally, Sanger sequencing was used to confirm the correct sequence and location of the insertion in two clones with homozygous insertion of P2A-mNeon (A6B & B2D) and no editing in a wild-type clone (C1G).

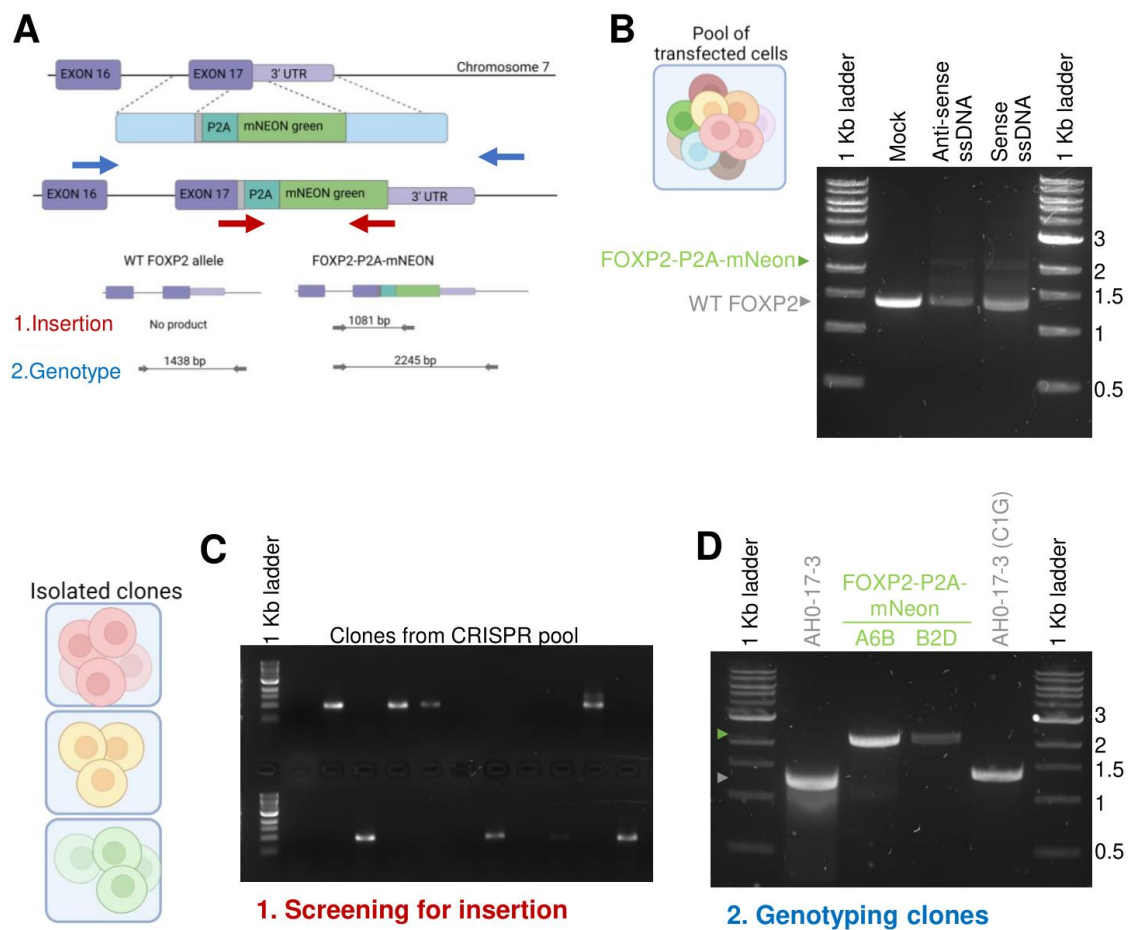


Figure 3.7 Screening of clones for P2A-mNeon insertion.

- A) Diagram showing the CRISPR strategy for generation of the FOXP2-P2A-mNeon reporter line and the primers used for screening clones.
- B) Pools of transfected cells were tested for insertion of P2A-mNeon using PCR. Primers spanning outside the homology regions (2.Genotype in A) were used for amplification and the PCR products visualised on a 1% agarose gel. From untransfected cells, the PCR generated a wild-type (WT) band ~1.4 kilobase pairs (kb). Transfections with either ssDNA donor resulted in some cells with successful insertion detected by an additional band at ~2.2 kb, with similar efficiency. The 1 kb ladder indicates DNA size in kb.
- C) To screen many clones, a PCR was first performed to detect an insertion of the mNeon sequence, using primers from outside the 5' homology region to within the mNeon sequence (1. Insertion in A). PCR products were visualised on a 1% agarose gel, with the presence of a band showing mNeon insertion.
- D) Selected clones with evidence of insertion were further characterised using a PCR spanning outside the homology arms (2.Genotype in A) to determine the size of insertion and genotype. The 1% agarose gel shows products from the parental control AH0-17-3 line, two clones with P2A-mNeon insertion (A6B & B2D) and one unedited clone (C1G) that had been through the CRISPR process as a control.

Panel A and cell diagrams created with BioRender.com.

3.2.4 Quality control of FOXP2-P2A-mNeon iPSC line

Cas9 introduces dsDNA breaks and therefore carries the potential of causing damage to DNA integrity in the form of small indels or larger chromosomal rearrangements (Jinek *et al.*, 2012; Hsu *et al.*, 2013; Pattanayak *et al.*, 2013; Kosicki, Tomberg and Bradley, 2018). These genetic changes may alter cell behaviour, including causing abnormal cell differentiation. Therefore, it was important to screen reporter line clones for genomic damage before their use.

The most likely sites of off-target mutation for the sgRNA used were predicted by gRNA design tools CCTop and CRISPOR. Primers were designed to amplify regions of approximately 200 bp around the six highest risk off-target sites using PCR (Table 2.3). Sanger sequencing was then performed on the amplified off-target regions for both the FOXP2-P2A-mNeon reporter line (clone B2D, used in downstream experiments) and the parental control iPSC line (AH0-17-3). Comparison of sequences found no changes for any of the six sites (Figure 3.8).

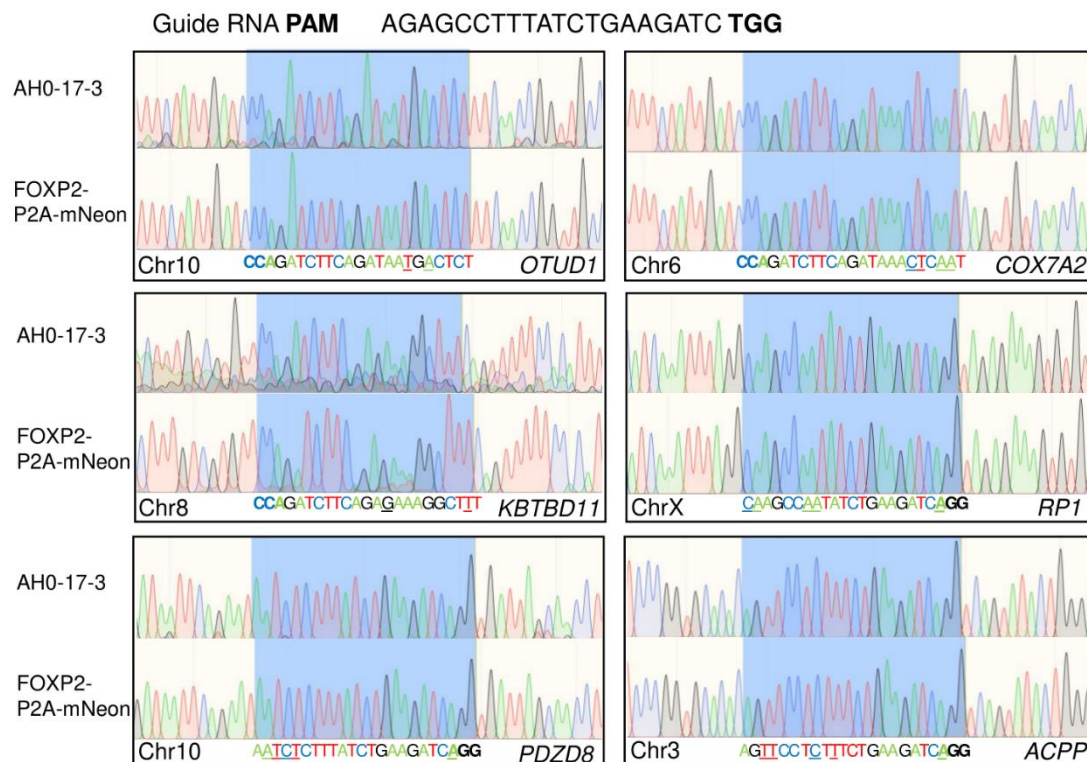


Figure 3.8 Sequencing of top predicted off-target sites for gRNA.

The predicted top six off-target sites for the gRNA used for *FOXP2* editing were sequenced to check for mutations in the FOXP2-P2A-mNeon line (clone B2D) and compared to the parental control AH0-17-3 line. Sites with a close match to the gRNA target sequence and adjacent PAM motif are highlighted in blue. Sequences are shown below with mismatches to gRNA target site underlined and the PAM in bold. Loci are labelled by chromosome and nearest gene.

To look for larger genomic changes, karyotypic analysis was performed using a SNP assay on gDNA from two reporter line clones (A6B & B2D) and the parental control line (Figure 3.9). Unfortunately, all lines showed chromosomal abnormalities: a previously reported minor duplication (Chr 6q14.1 2mb duplication) present when the AH0-17-3 line was isolated (Handel *et al.*, 2016); and an additional more serious loss of heterozygosity (LOH) (Chr17p13-12 13mb LOH). The LOH indicates loss of a region from one chromosome, so only a single copy of these loci are present in the cell. This genomic abnormality is a potential concern as LOH is a common mechanism in tumorigenesis. However, due to a substantial delay in receiving SNP assay results, the FOXP2-P2A-mNeon reporter line clones were used for subsequent experiments along with corresponding stock of parental AH0-17-3, sharing the same LOH. Genomic integrity of future cell stocks of these lines will be closely monitored for any increased genomic instability.

In addition to genetic integrity, it was important that the FOXP2-P2A-mNeon iPSCs maintained expected characteristics of pluripotent stem cells. The quality of starting iPSCs is integral to successful and replicable cerebellar organoid differentiations. First, iPSCs were assessed for stem cell morphology (Figure 3.10A). Human pluripotent stem cells are expected to form tightly packed colonies with smooth bright edges and round cells with a high nuclei: cytoplasmic ratio (Takahashi *et al.*, 2007). Reporter line iPSCs showed identical morphology to the parental control line (AH0-17-3), including densely packed colonies and large nuclei (Figure 3.10A). In addition, over 95% of cells showed expression of both pluripotency markers NANOG and TRA-1-60 (Figure 3.10B). Finally, capacity for differentiation into all three germ lineages was demonstrated, with expression of markers for mesoderm, endoderm and ectoderm respectively in a trilineage differentiation (Figure 3.10C). Together, these results indicate that the FOXP2-P2A-mNeon reporter iPSC line has maintained pluripotency through the CRISPR/Cas9 editing process, and therefore provide a homogenous undifferentiated starting state for differentiation.

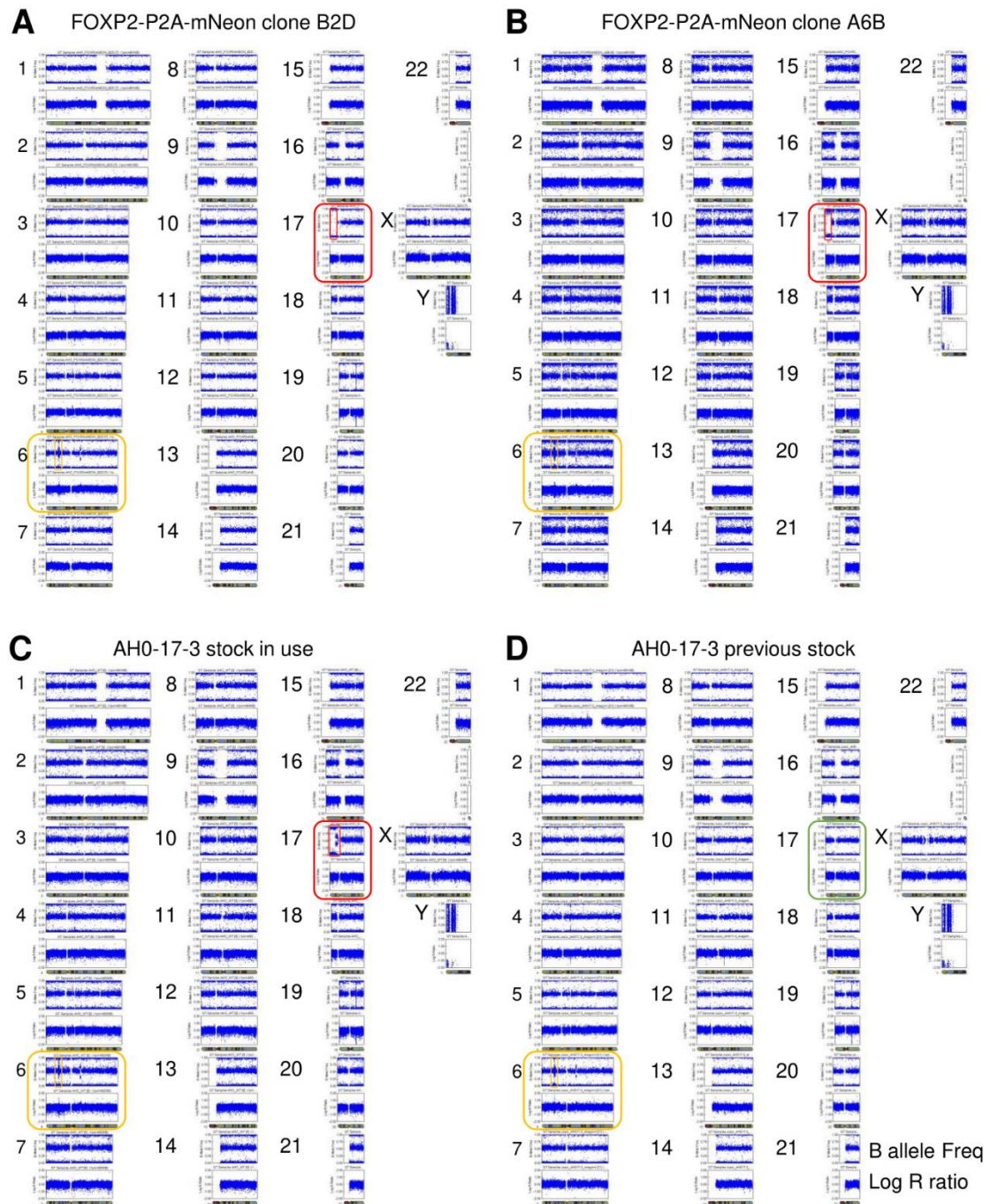


Figure 3.9 Karyograms from SNP analysis.

SNP array analysis on genomic DNA was used to assemble karyograms for the iPSC stocks. Karyotypes are shown for two FOXP2-P2A-mNeon reporter line clones (clone B2D (A) & clone A6B (B)), the parental AH0-17-3 stock used to generate this line (C) and an alternative AH0-17-3 stock (D). The control iPSC line, AH0-17-3, had a small duplication when established (Chr 6q14.1 2mb duplication) indicated by the orange box in all 4 samples (Handel *et al.*, 2016). However, the AH0-17-3 stock used for reporter line generation showed an additional chromosomal alteration on chromosome 17. The red box indicates the Chr17p13-12 13mb loss of heterozygosity (LOH) and Chr17p12-11 6mb duplication. As a result, both FOXP2-P2A-mNeon clones were found to also contain the Chr17p13-12 13mb LOH (red box).

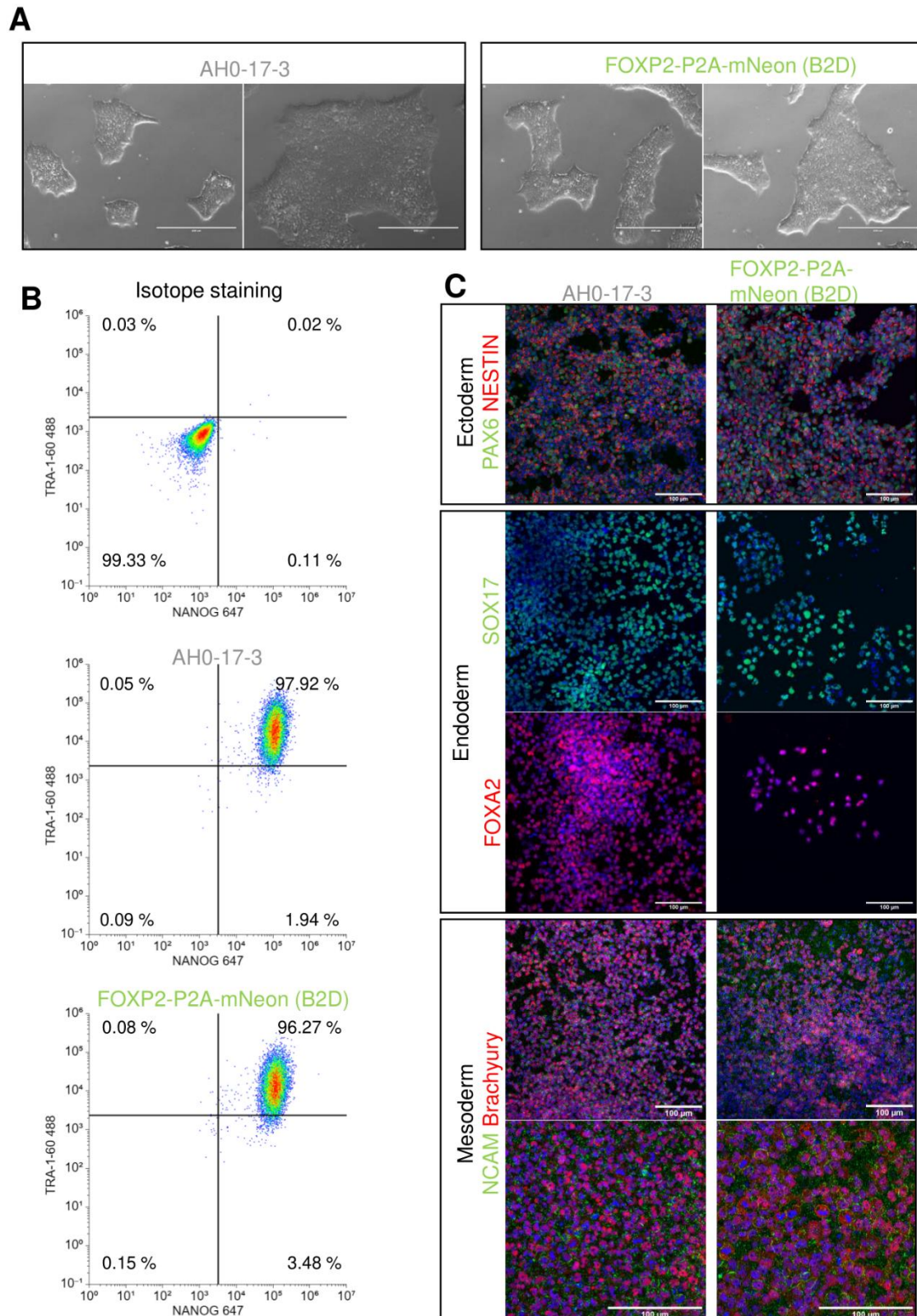


Figure 3.10 FOXP2-P2A-mNeon line maintained pluripotency.

Figure 3.10 FOXP2-P2A-mNeon line maintained pluripotency.

- A) Brightfield images of control parental line (AH0-17-3) and FOXP2-P2A-mNeon reporter line (clone B2D) showing iPSC colonies with bright borders and tightly packed cells with large nuclei. Scale bars are 400 μ m.
- B) Flow cytometry analysis was used to quantify expression of pluripotency markers. iPSCs were stained with fluorescent-conjugated antibodies for TRA-1-60⁴⁸⁸ and NANOG⁶⁴⁷ or with isotope controls IgM⁴⁸⁸ and IgG⁶⁴⁷. Samples stained with isotopes (top) were used to set gating for test antibody staining. The population percentages for each quadrant are indicated. Over 95% of both iPSC lines were double positive for TRA-1-60 and NANOG.
- C) To further confirm pluripotency, a trilineage differentiation was performed with iPSCs of the control parental line (AH0-17-3) and FOXP2-P2A-mNeon reporter line (clone B2D). Differentiated cells were fixed and stained using antibodies against markers for each lineage. Ectoderm cells were stained with antibodies against PAX6 (green) and NESTIN (red), endoderm cells separately with SOX17 (green) and FOXA2 (red), and mesoderm cells with NCAM (green) and Brachyury (red). Nuclei were stained with Hoechst (blue). Scale bars are 100 μ m.

3.2.5 Differentiation of FOXP2-P2A-mNeon iPSCs to cerebellar organoids

Next, the capability of newly generated reporter lines to differentiate into cerebellar organoids was tested. Two FOXP2-P2A-mNeon reporter line clones (A6B & B2D) were differentiated alongside the parental AH0-17-3 line into cerebellar organoids. Both reporter line clones successfully generated cerebellar organoids with typical appearance and growth. Moreover, FOXP2-P2A-mNeon organoids showed successful induction of marker genes used for quality control. For each clone, gene expression at the organoid quality control timepoint was compared to iPSC-state at day 0, showing significant upregulation of marker genes at day 21 (*EN1* and *EN2*), day 35 (*ATOH1*, *FOXP1*, *FOXP2* and *SKOR2*) and day 63 (*ATOH1*, *FOXP1*, *FOXP2* and *SKOR2*) (Figure 3.11A). Gene expression was then compared between organoids from different lines, to see if any changes occurred as a result of the CRISPR/Cas9 editing or clone isolation process. Organoids generated from the parental line (AH0-17-3) and the two FOXP2-P2A-mNeon reporter line clones (A6B & B2D) showed very similar expression of markers (Figure 3.11A). ANOVA found no significant differences in expression between lines for the selected marker genes at day 63, and only a few differences at days 21 and 35 (Figure 3.11A).

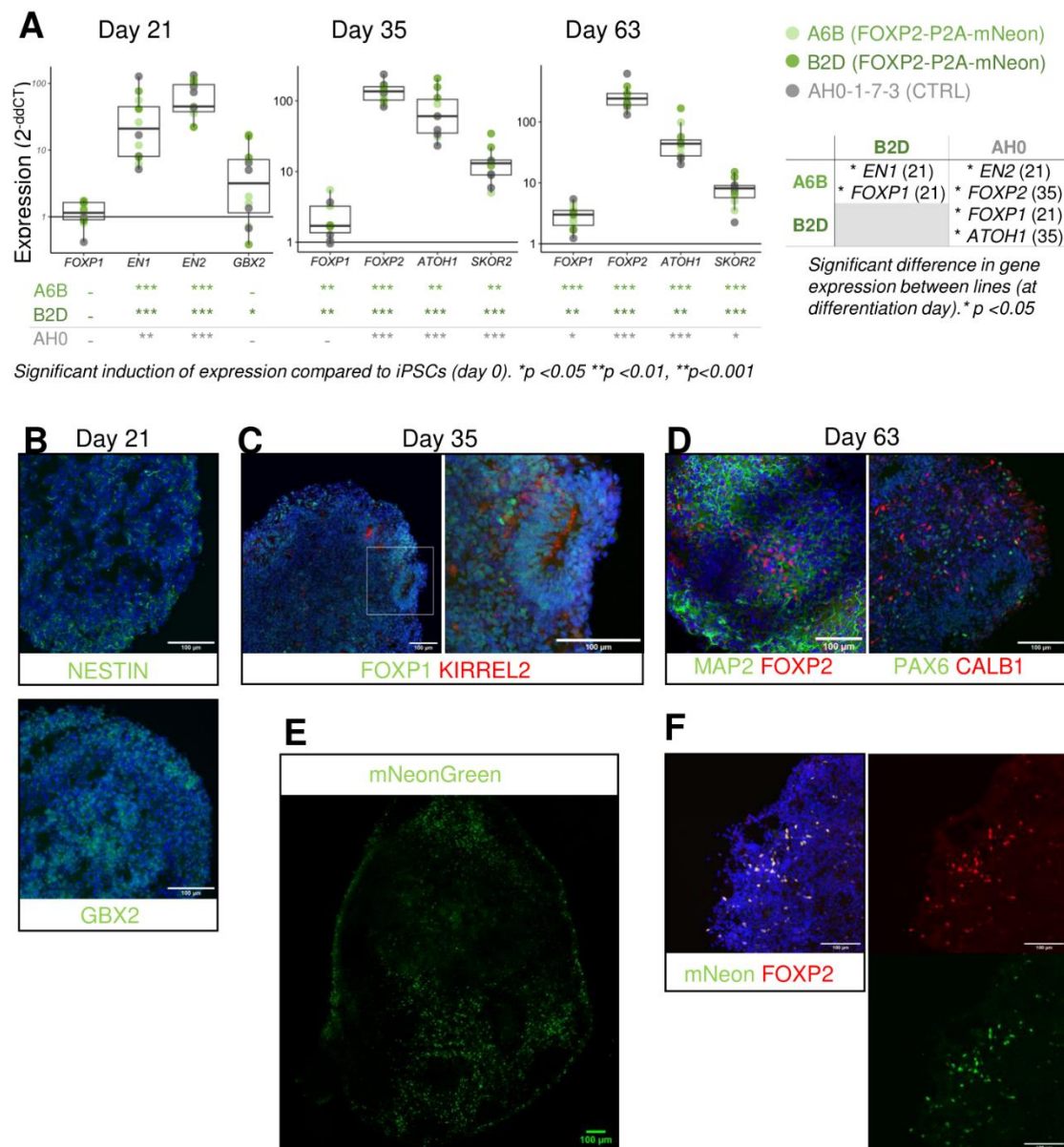


Figure 3.11 FOXP2-P2A-mNeon clones differentiated into cerebellar organoids and mNeon visualised FOXP2 expression.

A) Expression of cerebellar markers was measured at days 21, 35 and 63 of cerebellar organoid differentiation of two FOXP2-P2A-mNeon clones (A6B, light green & B2D, dark green) and the parental control (CTRL) line (AH0-17-3, grey) by RT-qPCR. Expression was normalised to housekeeper genes (*ACTIN*, *GAPDH*) and calculated relative to iPSC state (day 0) as 2^{-ddCT} . Dots indicate individual differentiations, $n = 3-4$. Significant induction compared to iPSCs was assessed using a t-test for each clone at each timepoint and indicated below plots (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, - no significant difference). Significant differences between lines at each timepoint were determined by ANOVA and post-hoc Tukey's HSD, summarised in the table.

Immunostaining for cerebellar markers was performed at the same timepoints. Images are shown for cerebellar organoids generated using FOXP2-P2A-mNeon reporter line clone B2D. Nuclei were stained with Hoechst. Scale bars on all images 100 μm .

Continued.

Figure 3.11 *FOXP2-P2A-mNeon clones differentiated into cerebellar organoids and mNeon visualised FOXP2 expression.*

- B) Markers of neural progenitors (NESTIN, green) and the developing hindbrain (GBX2, green) are widely detected at day 21.
- C) Markers of the cerebellar ventricular zone (FOXP1, green & KIRREL2, red) were detected at day 35.
- D) Markers of Purkinje cells (FOXP2 & CALB1, red), Granule neurons and their progenitors (PAX6, green) and mature neurons (MAP2, green) were imaged at day 63.
- E) Live cell imaging of mNeon fluorescence in a day 58 cerebellar organoid generated from FOXP2-P2A-mNeon clone B2D, showing distribution of *FOXP2*⁺ population.
- F) Immunofluorescent imaging of FOXP2 (red) and mNeon (green) confirms accurate co-expression in sections of a day 63 cerebellar organoid generated using FOXP2-P2A-mNeon clone B2D.

Normal expression of cerebellar markers was further shown by immunofluorescent imaging. This included expression of NESTIN, a general neural progenitor marker, and GBX2, a marker of the MHB, at day 21 (Figure 3.11B). At day 35, FOXP2-P2A-mNeon organoids showed the classic expression of VZ markers: KIRREL2 labelling lumens of rosette structures and nuclear FOXP1 (Figure 3.11C). By day 63 additional markers of Purkinje cells were present such as FOXP2 and CALB1 (Figure 3.11D). The presence of granule cell progenitors or mature granule cells was indicated by PAX6⁺ cells and the organoids showed widespread expression of pan-neuronal marker Microtubule-associated protein 2 (MAP2) (Figure 3.11D). Together these results show that the generated FOXP2-P2A-mNeon line differentiates successfully into cerebellar organoids, with normal induction of markers used to verify cerebellar identity.

3.2.6 mNeon expression provides live visualisation of *FOXP2* expression

Live cell fluorescent microscopy was able to successfully visualise mNeon expression in FOXP2-P2A-mNeon cerebellar organoids (Figure 3.11E). Fluorescent imaging also gave insight into the structure of the organoid, with *FOXP2*⁺ cells showing concentrated localisation around the outer edge of the organoid and in some internal structures rather than even distribution across the organoid. To verify that mNeon was providing an accurate readout of FOXP2 expression, immunostaining was performed for both FOXP2 and mNeon. Complete co-expression of FOXP2 and mNeon was detected in organoid sections (Figure 3.11F). Therefore, mNeon can be reliably used as a proxy for *FOXP2* expression and enables live monitoring of the *FOXP2*⁺ population in cerebellar organoids.

The subcellular localisation of mNeon was nuclear when viewed by both live cell and immunofluorescent imaging (Figure 3.11E & F). FOXP2 is a transcription factor and therefore nuclear localisation signals direct its nuclear localisation (Mizutani *et al.*, 2007). However, the reporter line design linked *mNeon* to *FOXP2* via a sequence encoding a self-cleaving peptide (Figure 3.4A), and isolated mNeon protein would be predicted to show an even cytoplasmic distribution. Therefore, the nuclear location of mNeon raised the concern that the separation of FOXP2 and mNeon was incomplete. To investigate this further, Western blotting was used to determine the molecular weight of FOXP2 and mNeon in two FOXP2-P2A-mNeon reporter line clones (A6B & B2D)(Figure 3.12). In some blots the FOXP2 antibody showed a non-specific band at around 75 kDa, in addition to the FOXP2 band observed around 90 kDa (predicted molecular weight 80 kDa). Therefore several additional samples were included as controls: cerebellar organoids generated from the parental control iPSC line (AH0-17-3); HEK-293 cells previously reported to endogenously express *FOXP2* (Vernes *et al.*, 2006); and cerebellar organoids generated from an iPSC line with both alleles of the *FOXP2* gene knocked-out (*FOXP2*^{-/-}, described in more detail in Chapter 5). As a positive control for mNeon, HEK-293 cells were transfected with a plasmid to express mNeon (pCS2+mNeonGreen-C Cloning Vector #128144). A WT FOXP2 band could be detected in day 63 cerebellar organoids from the parental control line, but not in either clone of the FOXP2-P2A-mNeon line (Figure 3.12A). Instead a higher molecular weight band was observed, with the size suggestive of a FOXP2-mNeon fusion protein (Figure 3.12A). mNeon has a predicted molecular weight of 26 kDa as observed in the HEK293 sample with exogenous mNeon expression (Figure 3.12B). In both of the FOXP2-P2A-mNeon reporter line clones, a much higher molecular weight band for mNeon was again observed, supporting the existence of a FOXP2-mNeon fusion protein (Figure 3.12B). In conclusion, the P2A peptide was found to not be cleaved in either FOXP2-P2A-mNeon reporter line clone, resulting in the presence of a FOXP2-mNeon fusion rather than separated proteins.

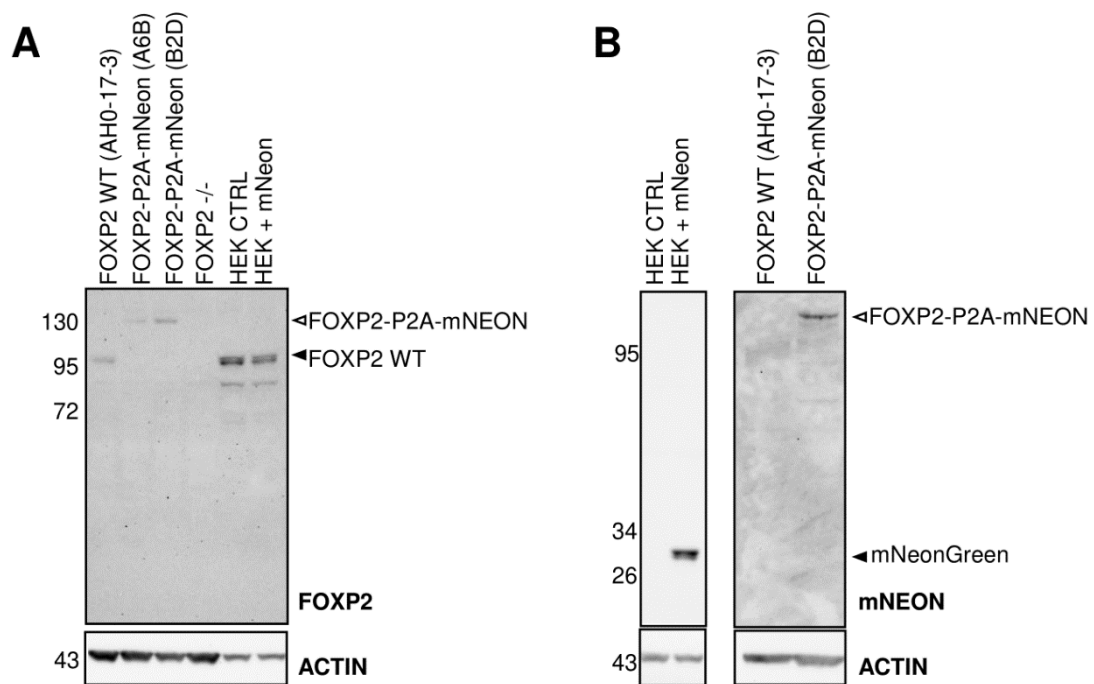


Figure 3.12 FOXP2-P2A-mNeon line shows incomplete cleavage of FOXP2 and mNeon.

- A) Western blot to examine the molecular weight of FOXP2 in samples from control (AH0-17-3) and FOXP2-P2A-mNeon reporter line clones (A6B & B2D). Protein samples were lysed from day 63 cerebellar organoids. A wild-type (WT) band for FOXP2 at around 95 kDa was present in the WT (AH0-17-3) cerebellar organoid and HEK293 cell samples. *FOXP2*^{-/-} samples confirm specificity with no band at this size. The two FOXP2-P2A-mNeon reporter line clones (A6B & B2D) show a larger band around 130 kDa, the size of a predicted FOXP2-mNeon fusion protein. ACTIN is shown as a loading control.
- B) Western blot to examine the molecular weight of mNeonGreen in the FOXP2-P2A-mNeon reporter line. A band around the predicted mNeonGreen molecular weight of 26kDa is detected in HEK293 cells with exogenous expression of mNeon (HEK+mNeon) and absent from untransfected cells (HEK CTRL). A high molecular weight band around 130 kDa was detected in FOXP2-P2A-mNeon organoids suggestive of a FOXP2-mNeon fusion protein.

3.3 Discussion

This chapter presents the production and characterisation of a Purkinje cell-reporter iPSC line, which shows bright mNeon fluorescence when expressing the chosen marker gene *FOXP2*. Live cell fluorescent imaging of cerebellar organoids successfully visualised mNeon expression and immunostaining confirmed this accurately portrayed *FOXP2* expression. Moreover, quality control of cerebellar organoids generated from the *FOXP2*-P2A-mNeon line showed typical upregulation markers indicative of successful differentiation to a cerebellar identity.

In the cerebellum, *FOXP2* is highly expressed in Purkinje cells and therefore in *FOXP2*-P2A-mNeon cerebellar organoids, the mNeon fluorescence labels a putative Purkinje cell population. In a later chapter (Chapter 5), the Purkinje-cell characteristics of this labelled population will be explored in more detail. Labelling the Purkinje cell population is also used in Chapter 4 to screen conditions for increasing the yield of this population in iPSC-derived cerebellar cultures. In future work, the *FOXP2*-P2A-mNeon line could enable physiological experiments on the Purkinje cell population at a single cell level, for example characterising the neuronal activity of Purkinje cells by patch clamping mNeon+ cells.

Quality control measures performed on the *FOXP2*-P2A-mNeon iPSCs showed typical stem cell morphology, pluripotency marker expression and capacity to differentiate into all three germ lineages. However, karyotypic changes which had occurred in the parental line stock were consequently found in the reporter line clones. This highlights the importance of maintaining strict iPSC stock procedures, in particular, the production of a bulk working stock which should undergo all quality control measures before use. Loss of heterozygosity is the complete loss of a stretch of DNA from one chromosome, resulting in a single copy of the loci in this region being present in the cell. The 13 Mb affected region in the *FOXP2*-P2A-mNeon line, at chromosome 17p13-12, encodes hundreds of genes including the tumour suppressor gene *TP53*. *TP53* triggers apoptosis in response to DNA damage, consequently a loss of function mutation in *TP53* has been shown to increase proliferation of human embryonic stem cells (Amir *et al.*, 2017). Reduction of *TP53* was therefore a likely driver of the growth advantage which enabled a clone carrying a spontaneous loss of the 17p13 region to outcompete the

original clone in the parental AH0-17-3 stock. *TP53* knockdown has been found to lead to higher expression of pluripotency marker *OCT4* being maintained following differentiation of human ESCs (Amir *et al.*, 2017). *OCT4* expression was not measured following differentiation of the FOXP2-P2A-mNeon line into cerebellar organoids, however this line differentiated successfully to all three germ lineages in the trilineage differentiation and cerebellar markers were induced normally in this line during cerebellar organoid differentiation. The impact loss of one copy of *TP53* and other genes in the region, could be further assessed by measuring expression of pluripotency markers following differentiation in comparison to multiple control iPSC lines. For subsequent experiments in this thesis, the FOXP2-P2A-mNeon line refers to clone B2D unless otherwise specified.

The CRISPR strategy aimed to avoid altering the FOXP2 protein function by including a P2A peptide, which was predicted to cleave and result in production of individual FOXP2 and mNeon proteins. This strategy has previously been applied to label neural progenitors with a SOX2-P2A-EGF reporter, which produced cytoplasmic EGF fluorescence indicative of successful cleavage (Sidhaye *et al.*, 2023). However, the FOXP2-P2A-mNeon line displayed nuclear localisation of mNeon. Furthermore, Western blotting detected the presence of a FOXP2-mNeon fusion protein in reporter line cerebellar organoids, indicating a failure in P2A cleavage. The efficiency of 2A peptide cleavage has been shown to depend on the upstream protein context (Donnelly *et al.*, 2001b; Szymczak-Workman, Vignali and Vignali, 2012). To mitigate the effect of the FOXP2 protein on P2A cleavage, the sequence encoding a 16 amino acid XTEN linker was included in the design between the C-terminal end of FOXP2 and the P2A peptide. However, other designs recommend a Gly-Ser-Gly linker prior to the P2A peptide to improve flexibility and increase P2A cleavage (Szymczak-Workman, Vignali and Vignali, 2012). A second variable that may influence P2A cleavage is the DNA codons used. In viral replication a clear preference for WT codon sequence was found, with selection for this sequence over others within a few passages (Kjær and Belsham, 2018). However, a range of different DNA sequences encoding P2A have been used successfully in publications, including the sequence used in the FOXP-P2A-mNeon line (Kim *et al.*, 2011; Szymczak-Workman, Vignali and Vignali, 2012; Liu *et al.*, 2017; Li *et al.*, 2019; Sidhaye *et al.*, 2023). To examine the effect of the linker and P2A

codon sequence on cleavage of FOXP2-P2A-mNeon, donor sequence variants could be tested in HEK293 cells which endogenously express *FOXP2*, and the location of mNeon signal compared. Sequences resulting in cytoplasmic mNeon expression, indicative of successful P2A cleavage, could then be used in hiPSCs to generate a new FOXP2-P2A-mNeon line.

The mNeon fusion with FOXP2 had potential to impact on FOXP2 function, for example impairing the binding of FOXP2 to DNA or other transcriptional regulators, or the dimerization, localisation, or post-translational regulation of FOXP2. Although the relatively small size of mNeon, with a molecular weight of ~26 kDa, reduces the likelihood of the fusion physically blocking FOXP2 interactions with DNA or other transcriptional regulators. Additionally, the P2A-mNeon sequence was introduced at the *FOXP2* stop codon and so the fusion protein would contain mNeon at the C-terminus of FOXP2, away from the functional forkhead domain which binds DNA and the leucine zipper domain used for dimerisation. The nuclear localisation of FOXP2 is essential to its' function as a transcription factor and has shown to be dysregulated as a consequence of pathogenic mutations that impair DNA binding (Vernes *et al.*, 2006). The subcellular localisation of FOXP2 was unchanged in the FOXP2-P2A-mNeon reporter line suggesting normal DNA binding capability of the FOXP2-mNeon fusion protein, although this was not directly confirmed. No differences in expression of *FOXP2* were found between the parental WT and FOXP2-P2A-mNeon lines at the time of peak expression in cerebellar organoids at day 63, suggesting transcriptional regulation of *FOXP2* expression was not altered by the mNeon fusion. Together these factors suggest that a FOXP2-P2A-mNeon fusion protein was able to be expressed and localise correctly. Therefore, the FOXP2-P2A-mNeon line was used in the subsequent chapters to label the Purkinje cell population. Following the identification of a reduction in *ZFHX4* expression in *FOXP2*^{-/-} cerebellar organoids (Chapter 5), in the future the expression of this gene could be examined in the FOXP2-P2A-mNeon line to further test for functional impairment of FOXP2 as a result of the mNeon fusion.

3.4 Key findings

- Analysis of single-cell RNAseq datasets found that *FOXP2* expression can be used to identify Purkinje cells from early in their development in both the human and mouse cerebellum.
- CRISPR/Cas9 technology was used to successfully generate a *FOXP2*-P2A-mNeon iPSC line. The reporter line enabled visualisation of *FOXP2* expression in live, unstained cultures.
- *FOXP2*-P2A-mNeon iPSCs displayed pluripotency characteristics including pluripotency factor expression, stem cell morphology and differentiation capacity. No off-target genetic changes were found resulting from CRISPR gene editing, however the reporter line shared a LOH with the parental cell stock.
- *FOXP2*-P2A-mNeon iPSCs differentiated successfully into organoids with cerebellar identity, including expression of markers labelling both glutamatergic and GABAergic cerebellar neurons.

Chapter 4. Optimisation of the cerebellar organoid differentiation protocol for generation of Purkinje cells

4.1 Introduction

In vitro culture models of the brain provide platforms to study mechanisms of development and disease. Additionally, human brain models are needed for preclinical drug development and testing. There is understandably limited access to human brain samples, particularly for developmental time periods, and when available these provide only static snapshots. In comparison, *in vitro* models can be manipulated and assayed in a dynamic manner allowing greater mechanistic insight.

4.1.1 Comparison of current protocols for differentiation of pluripotent stem cells into cerebellar neurons

The first cerebellar organoids adapted the SFEBq approach, directing differentiation of mouse embryonic stem cells in embryoid bodies to cerebellar identity, with a combination of FGF2 and insulin (Muguruma *et al.*, 2010). Subsequently, hESC-derived cerebellar organoids were generated with slight adaptations, including a longer timescale commonly required for human protocols (Muguruma *et al.*, 2015). Within 35 days of differentiation, cerebellar organoids contained rosette structures formed of polarised neuroepithelium (Muguruma *et al.*, 2015). Imaging of markers confirmed cerebellar organoids contain neurons from both germinal zone lineages: glutamatergic rhombic lip derivatives and GABAergic ventricular zone progenitors (Muguruma *et al.*, 2015; Watson *et al.*, 2018)(Figure 1.9C). Following the first publication of human iPSC-derived cerebellar organoids, different groups have made adjustments summarised in Table 4.1. In several studies, 2D co-culture with mouse granule cells has been used to increase maturity of human iPSC-derived cerebellar neurons (Muguruma *et al.*, 2015; Ishida *et al.*, 2016; Sundberg *et al.*, 2018; Watson *et al.*, 2018; Buchholz *et al.*, 2020). However, using mouse feeder cells introduces additional variability, as batch-to-batch differences in mouse cells may influence the behaviour of human iPSC-derived cerebellar cells. In addition, analysis must isolate human cells and exclude mouse cells, which, for example, limits the number of concurrent targets stained for whilst also labelling human cells.

More recent advances have been xeno-free approaches for prolonged culture of iPSC-derived cerebellar neurons (Silva *et al.*, 2020; Nayler *et al.*, 2021). Bypassing the need for mouse cerebellar cells simplifies the experimental procedures, reduces cost and improves reproducibility. In 2D cultures, BrainPhys medium (StemCell) was shown to support survival of iPSC-derived cerebellar neurons in feeder-free conditions (Silva *et al.*, 2020). An alternative method to improve long-term survival is the use of air-liquid interface culture, which has extended culture and maturation of cerebral organoids (Giandomenico *et al.*, 2019). Applying a similar use of transwell culture to cerebellar organoids enabled culture for over 90 days (Nayler *et al.*, 2021). However, despite extended culture, cerebellar organoids grown on transwells show limited Purkinje cell maturation compared to earlier days of differentiation (see section 4.2.1). In contrast, increased Purkinje cell maturity was reported for 2D feeder free cultures (Silva *et al.*, 2020).

Most of the methods for differentiation of human stem cells into cerebellar neurons are based on the early publication from Muguruma *et al.*, 2010 and therefore share common media formulations listed below.

- *Induction medium (IM)*: 50% Iscove's modified Dulbecco's medium, 50% Ham's F12, 7 µg/ml insulin, 5 mg/ml bovine serum albumin, 1% chemically defined lipid concentrate, 450 µM monothioglycerol, 15 µg/ml apo-transferrin and 1x penicillin/streptomycin. (Muguruma *et al.*, 2010)
- *Differentiation medium (DM)*: Neurobasal, 1x GlutaMax, 1x N2*, 1x penicillin/streptomycin. (Watson *et al.*, 2018)
- *Plating medium*: DMEM/F12 with L-glutamine, 1x N2*, 10% FBS, 1x penicillin/streptomycin. (Muguruma *et al.*, 2010)
- *Co-culture medium*: DMEM/F12 with L-glutamine, 1x N2*, 0.5 ng/ml tri-iodothyronine, 100 µg/ml BSA. 4 µM cytosine arabinoside added later. (Muguruma *et al.*, 2010)

*N2 supplement contains Human transferrin, Insulin, Progesterone, Putrescine, and Selenite.

Table 4.1 Summary of methods for generation of cerebellar neurons from pluripotent stem cells.

Publication	Approach	Medium
(Muguruma <i>et al.</i> , 2010)	Differentiation of mouse ESCs to cerebellar organoids. Co-culture of KIRREL2+ cells from day 13 cerebellar organoids, with cells from E15.5 mouse RL.	Day 0-11: <i>IM</i> (modified from (Wataya <i>et al.</i> , 2008)); 20 ng/ml FGF2 added on day 1; 10 μ M cyclopamine added on day 7. Day 11-15: Organoids transferred to collagen-coated transwell in 50% DMEM, 50% F12, 1x N2, 10%, 7 mg/ml glucose. KIRREL2+ cells sorted and re-aggregated at day 13. Day 15+: KIRREL2+ cells mixed with RL cells in <i>Plating medium</i> . Cells grown in <i>Co-culture medium</i> .
(Muguruma <i>et al.</i> , 2015)	Differentiation of hESCs to cerebellar organoids. Co-culture of KIRREL2+ cells from day 35 cerebellar organoids with cells from E15.5 mouse RL.	Day 0-21: <i>IM</i> ; 10 μ M Y-27632 and 10 μ M SB431542 added on day 0 and day 2; 50 ng/ml FGF2 added on day 2; 100 ng/ml FGF19 added days 14-21. Day 21-35: Neurobasal/N2 medium; 300 ng/ml SDF1 added days 28-35 Day 35+: Co-culture as (Muguruma <i>et al.</i> , 2010)
(Ishida <i>et al.</i> , 2016)	Differentiation of human iPSCs to cerebellar organoids. Co-culture of KIRREL2+ cells from day 35 cerebellar organoids with cells from E14.5 mouse RL.	As (Muguruma <i>et al.</i> , 2015) except: Y-27632 increased to 20 μ M on day 0 and 2; no addition of FGF19 or SDF1; addition of 50 ng/ml NT3 and 50 ng/ml BDNF to day 35+ <i>Co-culture medium</i> .
(Watson <i>et al.</i> , 2018)	Differentiation of human iPSCs to cerebellar organoids. Co-culture of mixed cells from cerebellar organoid with cells from E18.5 mouse cerebella.	Day 0-21: As (Muguruma <i>et al.</i> , 2015) except Y-27632 increased to 50 μ M on day 0 and day 2. Day 21- 35: <i>DM</i> . Day 35+: Cells seeded in <i>Plating medium</i> with 1.4 mM L-glutamine and 5 μ g/ml insulin. <i>Co-culture medium</i> with additional 1.4 mM L-glutamine, 5 μ g/ml insulin, 1% penicillin/streptomycin, 50 ng/ml BDNF and 50 ng/ml NT3; 5-10 μ g/ml Laminin added from day 42.
(Sundberg <i>et al.</i> , 2018)	Differentiation of human iPSCs to cerebellar organoids. Co-culture of Thy1+ cells from cerebellar organoids with granule neurons from P5-7 mice.	Day 0-12: Neural induction medium (DMEM/F12, 1x N2, 1x B27, 10 mM nicotinamide, 50 ng/ml noggin, 10 μ M SB-431542, 10 μ M Y-27632, 1.7 μ M CHIR99021); from day 2 noggin increased to 100 ng/ml; 100 ng/ml FGF8b added and nicotinamide removed at day 4; 10 ng/ml bFGF added at days 10-12; cells plated at day 8. Day 12- ~28-32: Neural maturation medium (Neurobasal, 1x B27, 1x N2, 10 ng/ml BDNF, 30 μ M T3). Day ~28-32 – ~32/39: Thy1+ cells plated in neural maturation medium with 1.7 μ M CHIR, 100 ng/ml FGF8b, 10 ng/ml bFGF, 1% BSA. Day ~32+: Co-culture of Thy1+ cells with mouse granule neurons, plated in neural maturation medium with 1% BSA, 2 ng/ml Laminin. 10 μ M Y-27632 added for first 2 days after plating; 4 μ M AraC and 5 ng/ml bFGF added for first 2 weeks.
(Buchholz <i>et al.</i> , 2020)	Differentiation of hESCs to cerebellar neurons. Co-culture of GD3-NCAM1+ hESC-derived	Day 0-12: Neural induction medium (DMEM/F12, 1x NEAA, 1x N2, 1x B27, 2 μ g/mL heparin, 1x Primocin); 10 mM nicotinamide, 50 ng/ml noggin and 1.5 μ M CHIR99021 added at days 0-4; 10 μ M Y- 27632 added at day 0-2 only; Noggin reduced to 10 ng/ml at day 2; 100 ng/ml FGF8b added at days 4-12; aggregates plated at day 6.

	cerebellar cells with mouse P5-7 cerebellar glia and granule cells.	Day 12 - 22: Neurobasal, 1x GlutaMax, 1x N2, 1x B27, 1x Primocin, 10 ng/ml BDNF. Day 22-28+: Co-culture of GD3- NCAM+ human cells with mouse cerebellar glia cells in neural differentiation medium (BME, 1x N2, 1x B27, 0.9% BSA, 0.9% glucose, 0.08% NaCl, 30 nM T3, 1x Primocin, 10 ng/ml BDNF); mouse granule cells added day 65-70.
(Silva <i>et al.</i> , 2020)	Differentiation of human iPSCs to cerebellar organoids. 2D feeder-free culture of dissociated cells from day 35 cerebellar organoids.	Day 0-35: As (Muguruma <i>et al.</i> , 2015). Day 35+: 2D culture in BrainPhys supplemented with 1x SM1, 1x N2-A, 20 ng/ml BDNF, 20 ng/ml GDNF, 1 mM dibutyryl cAMP and 200 nM ascorbic acid.
(Silva <i>et al.</i> , 2021)	Differentiation of human iPSCs to cerebellar organoids using bioreactor.	Media as (Silva <i>et al.</i> , 2020). Culture in 3D for whole period using PBS MINI 0.1 MAG Vertical wheel bioreactors (PBS Biotech).
(Nayler <i>et al.</i> , 2021) (Van Essen <i>et al.</i> , 2022)	Differentiation of human iPSCs to cerebellar organoids. Extended 3D culture at air-liquid interface.	Day 0-35: as (Watson <i>et al.</i> , 2018). Day 35+: culture of organoids at air-liquid interface on PTFE transwell membranes.
(Chen <i>et al.</i> , 2023)	Differentiation of human iPSCs to cerebellar organoids. Extended 3D culture in static suspension or on multi-electrode array plates.	Day 0-35: As (Muguruma <i>et al.</i> , 2015). Day 35+: Continued culture in Neurobasal/N2 medium.

E, embryonic day; ESC, embryonic stem cell; iPSC, induced pluripotent stem cell; P, postnatal day; RL, rhombic lip.

4.1.2 Limitations to current methods for production of iPSC-derived cerebellar neurons

Current approaches for differentiation of human iPSCs to cerebellar neurons successfully generate neurons from both cerebellar lineages (Muguruma *et al.*, 2015). Moreover, iPSC-derived cerebellar organoids have already been applied to studying cerebellar diseases (Ishida *et al.*, 2016; Sundberg *et al.*, 2018; Ballabio *et al.*, 2020; Van Essen *et al.*, 2023) and evolution (Buchholz *et al.*, 2020), highlighting the demand for an *in vitro* model of cerebellar development. However, the cerebellar neurons produced from iPSCs are still relatively immature, comparison of scRNA-seq data suggested at day 90 cerebellar organoids most closely model mid second trimester development (Nayler *et al.*, 2021). In particular, the generation of Purkinje cells has been challenging. *In vivo*, Purkinje cells are large neurons with

highly branched dendritic trees which in humans form millions of synapses (Huang *et al.*, 2014). Purkinje cells are central to cerebellar function and vulnerable in disease, making them a desirable cell type to study which requires an accurate model. Whilst current cerebellar differentiation methods generate cells with branching neurites that stain positive for commonly used Purkinje cell marker CALB1, they fail to recapitulate the stages of Purkinje cell dendritic tree development (Watson *et al.*, 2018; Silva *et al.*, 2020). Moreover, iPSC-derived Purkinje cells lack expression of later stage Purkinje markers. Therefore, protocol optimisation in this chapter will focus on the generation of a greater number of more mature iPSC-derived Purkinje cells.

Several studies have used co-culture, with cells derived from late embryonic or early postnatal mouse cerebella, to increase maturity of iPSC-cerebellar neurons (Muguruma *et al.*, 2015; Sundberg *et al.*, 2018; Watson *et al.*, 2018; Buchholz *et al.*, 2020). However, the introduction of mouse cells adds an additional cause of batch-to-batch variability and increases complexity of analysis as mouse-derived cells must first be excluded. Accordingly, the focus here will be on systems derived only from human iPSCs.

Cerebellar organoids share challenges common to other organoid models including the balance of complexity with variability. An advantage of cerebellar organoids is their complex composition, thus reflecting the multiple cell types present in the *in vivo* cerebellum and allowing cell-cell interactions to be studied. However, this diversity also provides a challenge, as the composition of cell types varies between individual organoids leading to significant variability in bulk analysis methods. As a result, methods to look at gene expression at a single-cell level, or to isolate populations of interest are needed. The FOXP2-P2A-mNeon reporter line generated in Chapter 3, enabled optimisation to focus on the Purkinje cell population of interest.

4.1.3 Approach to developing an optimised protocol for the generation of iPSC-derived Purkinje cells

In protocols based on the Muguruma *et al.*, 2015 approach, after the initial period of directed differentiation using FGF2 and *Induction medium (IM)*, cerebellar organoids are then grown in relatively simple *Differentiation medium (DM)*. Many different neurotrophic factors and hormonal signals are present in the developing cerebellum. However, only a few of these factors are present in *DM*, such as progesterone which is implicated in Purkinje cell dendritic growth and present in the N2 supplement (Kapfhammer, 2004). Consequently, addition of some of the missing signalling factors, such as brain-derived neurotrophic factor (BDNF) and L-3,3',5-triiodothyronine (T3), may improve cerebellar progenitor differentiation (Table 4.2).

Growth of iPSC-derived Purkinje cells is likely to require some form of co-culture, as mouse-derived Purkinje cells show very poor survival in monoculture (Baptista *et al.*, 1994). Cell-cell interactions have been shown to promote *in vitro* survival of mouse Purkinje cells, with granule cell co-culture leading to highest rates of survival and dendritic development (Baptista *et al.*, 1994). The mixture of cell types in cerebellar organoids, including glia and granule cells, are thus likely to help to provide a suitable environment to promote Purkinje cell survival.

Therefore, whilst aiming to increase Purkinje cell differentiation, optimisation is not targeted at generating a pure Purkinje cell culture. In 2D iPSC-cerebellar cultures, granule cells and glial cells from mouse cerebellar have been used to co-culture purified iPSC-derived Purkinje cells (Muguruma *et al.*, 2015; Buchholz *et al.*, 2020). The use of iPSC-derived astrocytes in co-culture with neurons in models of other brain regions has been shown to promote neuronal activity, including increased frequency of spontaneous action potentials and synaptic maturation in iPSC-cortical neurons (Hedegaard *et al.*, 2020). *In vivo*, astrocytes perform numerous functions in support and regulation of neurons in the brain, including secretion of neurotrophic factors, uptake and recycling of ions and neurotransmitters, and promoting synapse maturation (Hedegaard *et al.*, 2020). Therefore, addition of iPSC-derived astrocytes to iPSC-cerebellar cultures may aid survival and promote maturity of neurons.

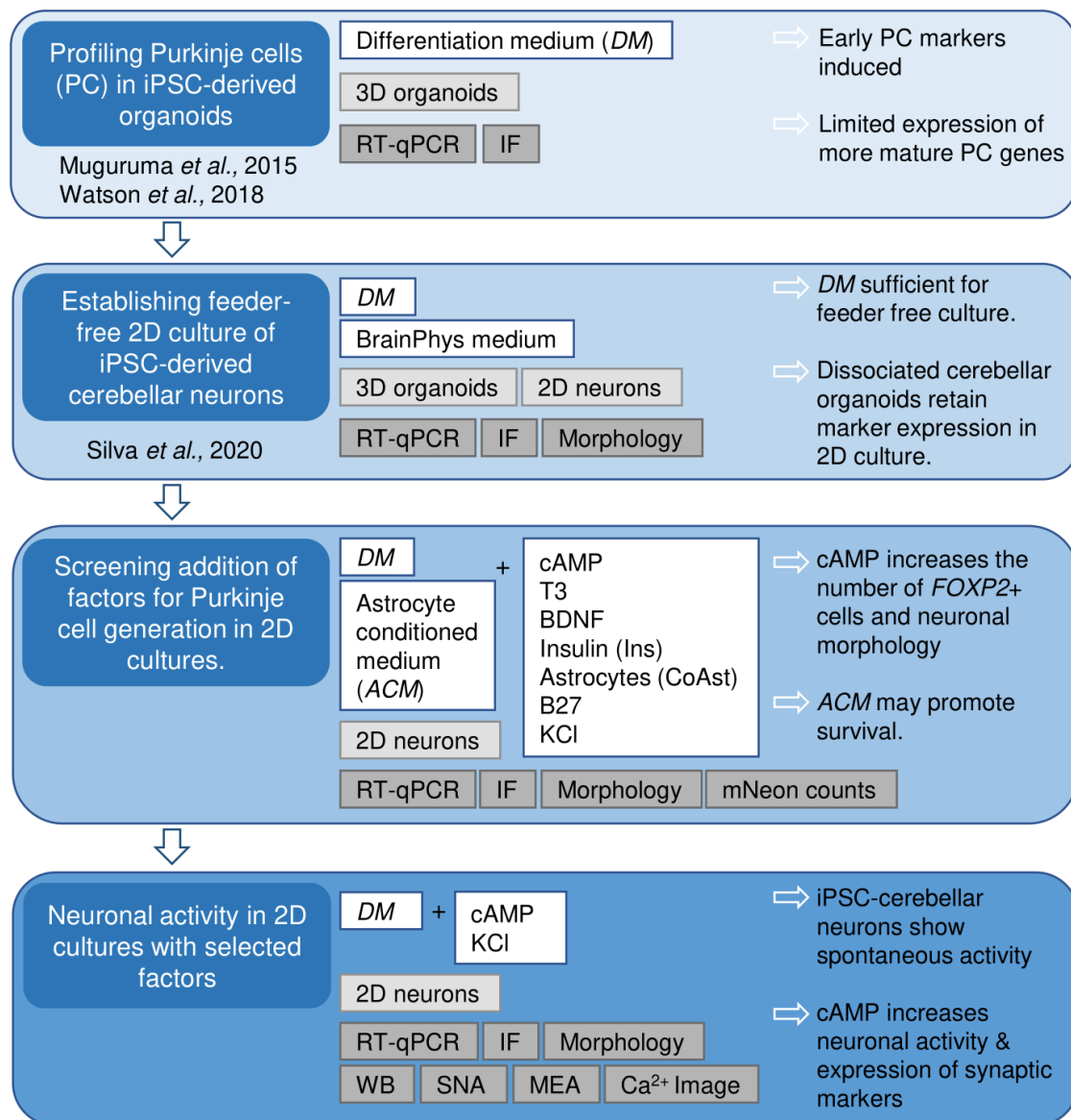


Figure 4.1 Approach to screening factors for improved generation of Purkinje cells from iPSCs.

Schematic showing the stages of protocol characterisation and optimisation described in this chapter. Screening was focused on production of a larger population of more mature Purkinje cells and built on established protocols for cerebellar organoids. For each stage, the growth medium conditions (white boxes), physical culture conditions (light grey boxes) and assays performed (dark grey boxes) are listed and key results are highlighted.

ACM, Astrocyte conditioned medium; CoAst, Astrocyte co-culture; DM, Differentiation medium; IF, immunofluorescent imaging; Ins, Insulin; MEA, Multi-electrode array; SNA, Spontaneous neuronal activity assay; RT-qPCR, Real time quantitative PCR; WB, Western blot.

In summary, generation of cerebellar neurons from human iPSCs shows potential for significant advances in our understanding of disease and development. However, current methods have a number of limitations, such as immaturity and low Purkinje cell number. This chapter aims to improve methods for generation of Purkinje cells from human iPSCs and address some current challenges in cerebellar modelling. First, a new approach for feeder-free 2D culture of iPSC-cerebellar neurons is tested and then used to screen a range of conditions to improve the generation and function of Purkinje cells (Figure 4.1). Finally, different culture approaches are compared and discussed with regards to their usefulness for downstream applications.

4.1.4 Chapter 4 aims

1. To benchmark Purkinje cell generation from human iPSCs using current protocols (Van Essen *et al.*, 2022) in comparison to in vivo gene expression.
2. To screen factors added to post day-35 differentiation medium for improved Purkinje cell production.
3. To characterise neuronal activity in iPSC-derived cerebellar cultures.

4.2 Results

4.2.1 Assessment of Purkinje cells currently generated from human iPSCs

First, to generate a benchmark for protocol optimisation, the current Becker lab protocol for differentiation of human iPSCs into cerebellar organoids was assessed (Nayler *et al.*, 2021; Van Essen *et al.*, 2022). As optimisation sought to better model Purkinje cell development, this characterisation focused on the generation of Purkinje cells. Expression of a panel of Purkinje cell markers was measured throughout differentiation using RT-qPCR and immunostaining (Figure 4.2). Markers of cerebellar VZ progenitors and early Purkinje cells (*KIRREL2*, *LHX1*, *RORA*, *SKOR2*, *FOXP1*, *FOXP2*, *FOXP4*) showed significant induction of expression during cerebellar organoid differentiation (Figure 4.2A). To compare organoid gene expression to normal human cerebellar development, expression of the same panel of markers was plotted in Purkinje cells from developing human cerebellar snRNA-seq data (Sepp *et al.*, 2023). The early Purkinje cell markers showed peak expression by 11 PCW (Figure 4.2B). In contrast, expression of the later markers peaked around birth or postnatally in human Purkinje cells (Figure 4.2B). In cerebellar organoids, expression of the late Purkinje cell markers (*CA8*, *CALB1*, *ITPR1*) was not significantly increased when compared to iPSCs at day 0 (Figure 4.2C). These results confirm that Purkinje cells are generated within cerebellar organoids, however these cells remain relatively immature.

Expression of Purkinje cell markers was further demonstrated by immunostaining of cerebellar organoid sections. The VZ marker *KIRREL2* localised to rosette lumina at day 35 of differentiation as previously reported (Figure 4.2D) (Muguruma *et al.*, 2015). Some *KIRREL2* expression was also observed at day 49 but it was not detected later, matching the trend measured by RT-qPCR and suggesting the early VZ progenitor population may be lost in organoids as cells differentiate further (Figure 4.2A). Purkinje cell markers including *SKOR2*, *CALB1*, *FOXP1* and Purkinje cell protein 4 (*PCP4*) were detected at days 35 and 63 (Figure 4.2D & E). The cytoplasmic marker *CALB1* labelled cells with neuronal morphology including large cell bodies and branching neurites, which are both features of Purkinje cells (Figure 4.2E).

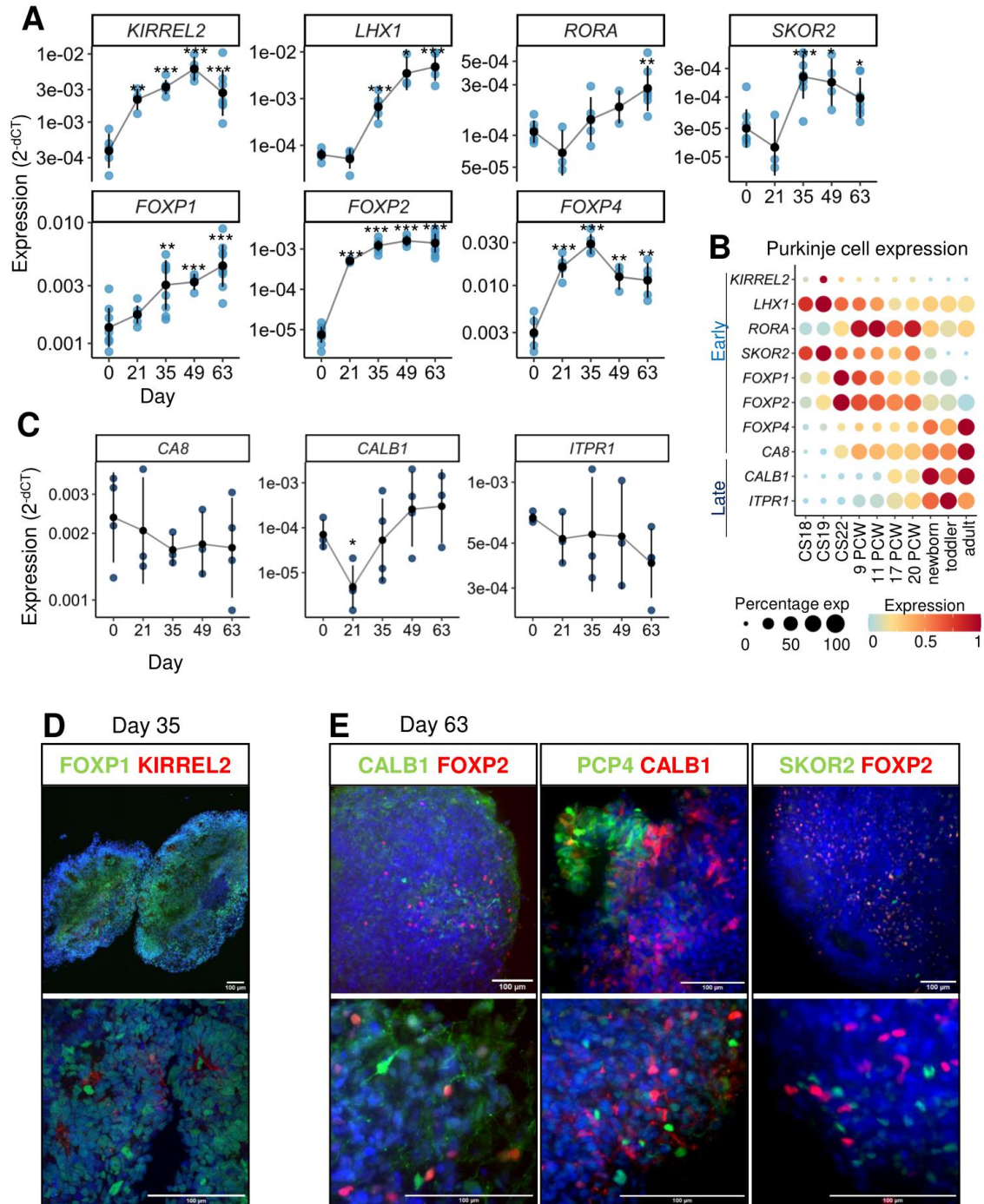


Figure 4.2 Cerebellar organoids show expression of early Purkinje cell markers.

- A) A panel of early Purkinje cell markers were induced during cerebellar differentiation. Gene expression was quantified by RT-qPCR and calculated as $2^{-\Delta CT}$ relative to housekeeper genes (*ACTIN*, *GAPDH*). Coloured dots show individual differentiations ($n \geq 3$) and black dots indicate mean expression. Significant differences in expression at each timepoint compared to day 0 was determined by T-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
- B) Expression of early and late Purkinje cell markers (used in A & C) was plotted in developing human Purkinje cells over time. Human developing cerebellar snRNA-seq data from Sepp *et al.*, 2023. Point size relative to the percentage of cells expressing the gene, colour scale displays average cluster expression normalised for each gene.

Figure 4.2. Cerebellar organoids show expression of early Purkinje cell markers.

- C) Expression of markers for more mature Purkinje cells was not significantly induced during cerebellar organoid differentiation. Quantification by RT-qPCR as above (A).
- D) Sections of day 35 organoids were stained with antibodies against ventricular zone markers FOXP1 (green) and KIRREL2 (red). Scale bars = 100 μ m.
- E) Day 63 cerebellar organoids showed expression of Purkinje cell markers confirming the presence of early Purkinje-like cells. Sections were stained with antibodies against CALB1 (green) and FOXP2 (red), PCP4 (green) and CALB1 (red), and SKOR2 (green) and FOXP2 (red). Scale bars = 100 μ m.

However, staining for combinations of Purkinje cell markers showed low overlap, with relatively few cells expressing multiple markers. This may reflect incomplete differentiation *in vitro*, with cells displaying intermediately identities not necessarily present during *in vivo* development.

In summary, differentiation of iPSCs to cerebellar organoids showed successful upregulation of developing Purkinje cell genes. The mixed population within cerebellar organoids includes dispersed cells with expression of Purkinje cell markers including CALB1, SKOR2 and FOXP2. However, the Purkinje cells produced are immature, with limited expression in cerebellar organoids of marker genes found in later embryonic and postnatal Purkinje cells, such as *ITPR1* and *CA8*.

4.2.2 Recapitulation of 2D feeder-free culture of iPSC-derived cerebellar neurons

Early attempts to generate more mature human iPSC-derived Purkinje cells relied on co-culture with mouse cerebellar granule cells (Muguruma *et al.*, 2015; Watson *et al.*, 2018). Recently, the first feeder-free approach for 2D culture of iPSC-derived cerebellar neurons was published and used BrainPhys medium to support survival (Silva *et al.*, 2020). To test whether this method could be replicated and show any benefits, cerebellar organoids were dissociated at day 35 and plated onto poly-L-ornithine and laminin. For comparison, 2D cell cultures were grown in either BrainPhys with the recommended supplements (SM1, N2-A, BDNF, GDNF, cAMP and ascorbic acid) or in *DM* as used for continued organoid growth in 3D. Although survival decreased over time, cells plated from dissociated cerebellar organoids continued to survive for over four weeks in both medium conditions (Figure 4.3A). Distinct cell morphology was observed between conditions: BrainPhys promoted clumping of cells with long bundles of

connections between, whereas cells grown in *DM* were more evenly spread out but included cells with flatter and less neuronal morphology (Figure 4.3A). Next, immunofluorescent imaging was carried out to determine whether the 2D cultures maintained cerebellar identity. In both BrainPhys and *DM*, cultures contained cells expressing Purkinje cell markers FOXP2 and CALB1 (Figure 4.3B). These Purkinje cell markers were typically not co-expressed, as also noted in 3D organoid sections (Figure 4.2E). However, an increased co-expression of FOXP2 and CALB1 was observed in later stage dissociated cerebellar cultures grown in *DM*, suggestive of a more complete Purkinje cell differentiation (Figure 4.3B). The co-expression of FOXP2 and CALB1 was not seen in cerebellar cultures grown in BrainPhys, and at later time points fewer CALB1+ cells with branched neuronal morphology were seen in this medium (Figure 4.3B). These observations indicated *DM* rather than BrainPhys might be preferable for culture of human iPSC-derived Purkinje cells. In addition, BrainPhys was designed to promote electrical activity in neuronal cultures rather than for long-term maintenance (Bardy *et al.*, 2015), and the use of *DM* allowed greater control over composition.

The range of cell types present in 2D iPSC-cerebellar cultures grown in *DM* was characterised further by immunostaining for additional markers. CALB1+ cells displayed a range of morphologies, including cells with extended branching neurites, which may represent a Purkinje cell-like population (Figure 4.3B & C). However, co-staining for neuronal marker MAP2 found not all CALB1+ cells showed expression of MAP2, suggesting in iPSC-derived cultures CALB1 cannot be relied on individually as a Purkinje cell marker (Figure 4.3C). In addition to Purkinje cells, 2D cultures contained PAX6 signal indicating the presence of granule cell precursors or mature granule cells (Figure 4.3D).

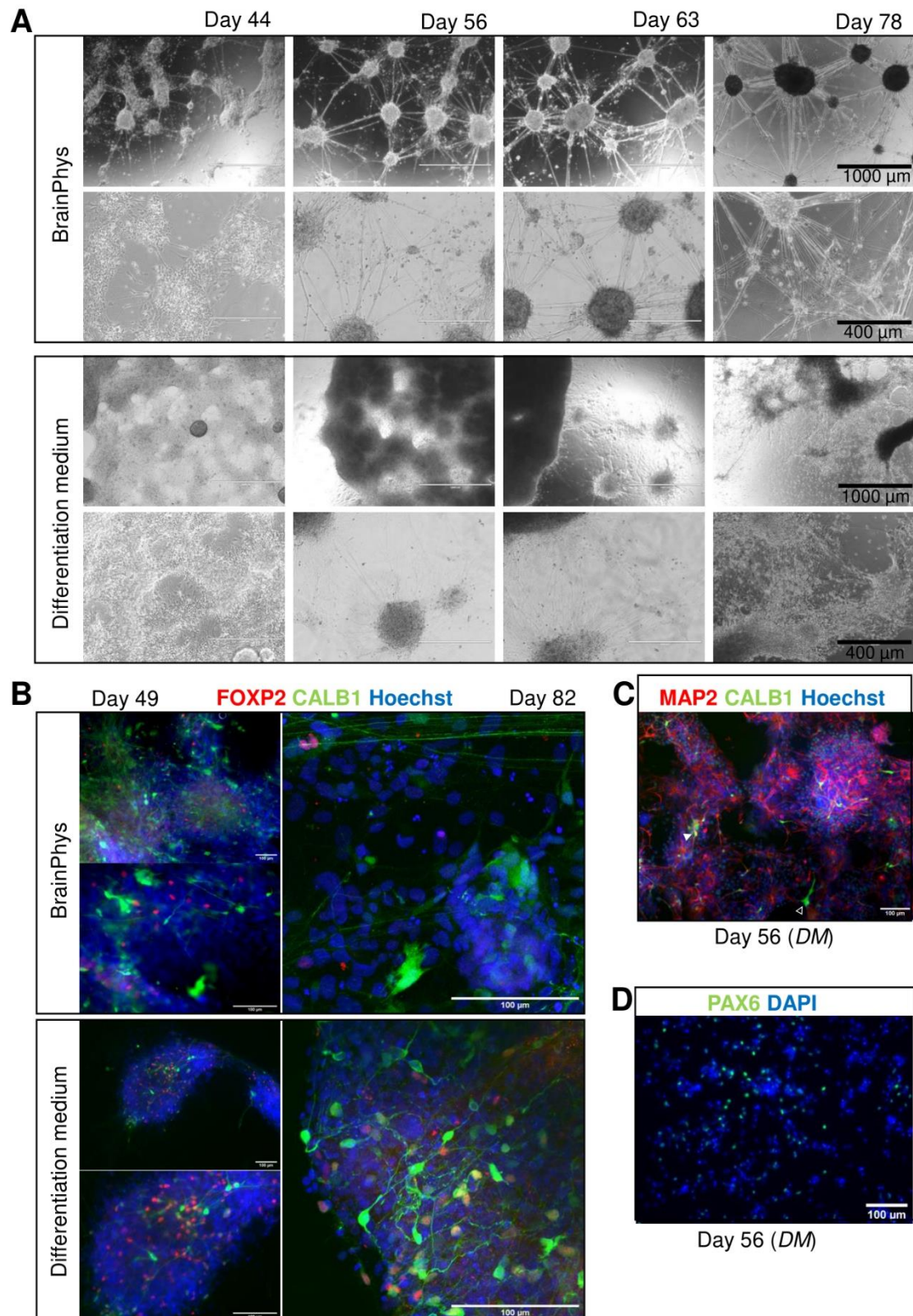


Figure 4.3 Establishment of 2D feeder-free culture from iPSC-derived cerebellar organoids.

- A) Time course of brightfield images from 2D iPSC-cerebellar cultures grown in either BrainPhys or *Differentiation medium* (DM) after organoid dissociation and plating at day 35. Numbers indicate total days of differentiation. Scale bars 1000 μ m and 400 μ m. *Continued.*

Figure 4.3. Establishment of 2D feeder-free culture from iPSC-derived cerebellar organoids.

- B) Comparison of Purkinje cell generation in 2D cultures grown in BrainPhys and *DM*. Coverslips stained with antibodies against FOXP2 (red) and CALB1 (green) at 49 and 82 total days of differentiation. Nuclei stained with Hoechst (blue). Scale bar 100 μ m.
- C) 2D iPSC-cerebellar cultures grown in *DM* were stained for additional Purkinje cell markers. Antibodies were used against CALB1 (green) and neuronal marker MAP2 (red). Nuclei stained with Hoechst (blue). Cells showing Purkinje cell-like CALB1+MAP2+ expression (solid triangle) and non-neuronal CALB1+MAP2- staining (open triangle) were observed. Scale bar 100 μ m.
- D) 2D iPSC-cerebellar cultures also contained cells which stained positive for PAX6 (green) a marker of granule cell precursors and mature granule neurons. Nuclei stained with dapi (blue). Scale bar 100 μ m.

To summarise, the new method for 2D feeder-free culture of iPSC-derived cerebellar neurons was replicable and sustained long term cerebellar cultures (Silva *et al.*, 2020). In addition, growth in *DM*, rather than BrainPhys as used in the published method, was also found to be sufficient for survival of 2D cerebellar cultures. Dissociated iPSC-derived cerebellar cultures grown in *DM* contained both granule cells and Purkinje cells, based on staining with antibodies against markers for these cell types. Furthermore, the 2D culture approach showed promise for the generation of more mature iPSC-derived Purkinje cells based on co-expression of markers.

4.2.3 Screening additional factors for improved 2D cultures

Having demonstrated the feasibility of feeder-free 2D iPSC-derived cerebellar cultures, the next aim was to improve the conditions of this growth. Starting from the known formulation of *DM*, the effect of adding different supplements was tested. In addition to optimising the generation of cerebellar neurons, particularly Purkinje cells, screening aimed to improve the stability and survival of the cultures. Therefore, factors were selected for screening based on literature showing potential for improving neuron survival and Purkinje cell differentiation. This included supplements which are commonly used for iPSC-derived neural differentiations, factors used in dissociated cultures of mouse cerebellar cells, and molecules involved in signalling pathways known to be important in cerebellar development. The factors chosen for testing and motivations for selection are summarised in Table 4.2.

Table 4.2 Factors screened for iPSC-cerebellar neuron culture.

Factor	Concentration	Reason for selection
Ascorbic acid	200 nM	Supplement added to BrainPhys (Silva <i>et al.</i> , 2020). Antioxidant used to promote neuronal differentiation of human iPSCs.
Astrocyte conditioned medium (ACM)	100%	Astrocytes secrete a range of soluble signalling factors which support neuron survival and homeostasis. Non-contact co-culture of astrocytes with dissociated rat cerebellar cells increased Purkinje cell number (Yuzaki, Mikoshiba and Kagawa, 1993), whilst direct co-culture increased cerebellar neuron proliferation (Martinez and Alcantara Gomes, 2005). In cortical iPSC-derived models, astrocytes promoted neural excitability and synaptic maturation (Hedegaard <i>et al.</i> , 2020). Comparison of co-culture with conditioned medium distinguishes the effects of soluble secreted factors.
Astrocyte co-culture	1:8 astrocytes: neurons	
B27	1x	Commonly used supplement for neural differentiation of iPSCs and primary neuronal cultures. Concentrations not published: Vitamin E (DL Alpha Tocopherol Acetate & DL Alpha-Tocopherol), Vitamin A (acetate), BSA, Catalase, Human Recombinant Insulin ¹ , Superoxide Dismutase, Corticosterone, D-Galactose, Ethanolamine HCl, Glutathione (reduced), L-Carnitine HCl, Linoleic Acid, Linolenic Acid, Progesterone ¹ , Putrescine 2HCl, Sodium Selenite, T3 (triiodo-L-thyronine) ¹ .
BDNF	20 ng/ml	Supplement added to BrainPhys (Silva <i>et al.</i> , 2020) and <i>Co-culture</i> medium (Ishida <i>et al.</i> , 2016; Watson <i>et al.</i> , 2018). Neurotrophic factor which acts widely across the nervous system (He <i>et al.</i> , 2013). In the cerebellum, BDNF is expressed in neurons as a survival factor. It also influences Purkinje cell dendritic growth and migration of granule cells (Borghesani <i>et al.</i> , 2002; Kapfhammer, 2004; He <i>et al.</i> , 2013). <i>Bdnf</i> ^{-/-} mice show cerebellar abnormalities, including reduced foliation and disordered Purkinje cells with stunted dendritic trees particularly in early postnatal development (Schwartz <i>et al.</i> , 1997; Carter <i>et al.</i> , 2002). <i>In vitro</i> cerebellar models have provided mixed reports on the effect of BDNF. In cerebellar slice cultures, neither decreased BDNF signalling or addition of BDNF, altered Purkinje cell number or morphology (Kapfhammer, 2004). Instead, in this relatively intact cerebellar environment, BDNF was more important for interneuron development (Kapfhammer, 2004). However in 2D dissociated cerebellar cultures addition of BDNF increased neuron survival (Lärkfors, Lindsay and Alderson, 1996), suggesting it may aid iPSC-derived Purkinje cell culture <i>in vitro</i> .
Dibutyl cAMP	1 mM	Supplement added to BrainPhys (Silva <i>et al.</i> , 2020) and commonly used in neuronal differentiations of stem cells. Acts as a neuronal survival factor via activation of MAPK signalling (Dugan <i>et al.</i> , 1999). Shown to promote granule

		cell survival without high K ⁺ in rat cerebellar <i>in vitro</i> culture (D'Mello <i>et al.</i> , 1993).
GDNF	20 ng/ml	Supplement added to BrainPhys (Silva <i>et al.</i> , 2020). Neurotrophin strongly expressed in Purkinje cells from 25 PCW in human cerebellum (Koo and Choi, 2001). In dissociated rat cerebellar cultures, GDNF treatment increased number and morphology of Purkinje cells (Mount <i>et al.</i> , 1995).
Insulin	5 µg/ml (~10 µg/ml total)	Supplement added to <i>Co-culture medium</i> (Watson <i>et al.</i> , 2018). Insulin is also contained in N2 supplement so is already present at 0.86 µM (~5 µg/ml) in <i>DM</i> . Insulin is used early in cerebellar organoid differentiation as a weak caudalising factor to induce a hindbrain fate (Wataya <i>et al.</i> , 2008). Insulin and IGF1 have been used to promote Purkinje cell dendritic development and survival in mouse dissociated cerebellar cultures (Kapfhammer, 2004). IGF1 increased granule cell survival without high K ⁺ <i>in vitro</i> cultures by activation of phosphatidylinositol 3-kinase (D'Mello <i>et al.</i> , 1993; Miller <i>et al.</i> , 1997).
NT-3	10 ng/ml	Supplement added to <i>Co-culture medium</i> (Ishida <i>et al.</i> , 2016; Watson <i>et al.</i> , 2018). Addition of NT-3 to dissociated rat cerebellar cultures increased Purkinje cell number (Lärkfors, Lindsay and Alderson, 1996).
T3	3 nM	Supplement included in <i>Co-culture medium</i> (Muguruma <i>et al.</i> , 2010, 2015; Watson <i>et al.</i> , 2018) and commonly used in cultures of dissociated mouse cerebella. Thyroid hormone signalling promotes Purkinje cell dendritic development. Addition increased dendritic development in dissociated mouse cerebellar cultures (Heuer and Mason, 2003). Low T3 in rodents delays neuron development, including stunting the growth and branching of Purkinje cells dendritic trees (Heuer and Mason, 2003; Kapfhammer, 2004).
KCl	15 mM (~20mM total)	Neurobasal medium contains 5.3 mM KCl. Higher K ⁺ conditions have been used to maintain primary neurons, including granule cells, <i>in vitro</i> culture (Miller <i>et al.</i> , 1997). Increased K ⁺ concentration causes depolarisation of neurons providing a crude mimic of electrical activity.

1 - used to promote Purkinje cell survival or growth in mouse cerebellar cultures (Kapfhammer, 2004)

To assess the effect of additional factors on iPSC-cerebellar cultures, several metrics were considered: cell morphology and survival was monitored with brightfield microscopy; expression of cerebellar neuron markers was quantified by RT-qPCR and immunostaining and the number of the *FOXP2*⁺ cells was visualised using the *FOXP2*-P2A-mNeon reporter line (Chapter 3).

A) Cell morphology

The impact of factor addition was first determined by observation of the morphology and survival of iPSC-derived cerebellar cultures over time (Figure 4.4A-C). Distinctive morphology was consistently observed upon addition of 1 mM cyclic adenosine monophosphate (cAMP), with cells appearing more neuronal and forming a highly connected mesh (Figure 4.4B). The appearance of cell clumps connected by neurite bundles was reminiscent of the BrainPhys cultures (Figure 4.3A & Figure 4.4B). cAMP was included as a recommended supplement in BrainPhys medium, so may also have been responsible for generating this neuronal morphology in 2D cerebellar cultures grown in BrainPhys. Addition of laminin to the medium from two weeks after plating reduced detachment of cells. However, cultures treated with insulin or B27 had greater cell detachment, often forming aggregates which became separated from the plate surface, so that few cells remaining attached after four weeks of 2D culture (Figure 4.4B). The B27 supplement contains insulin so this may be driving the detachment of cells in both cases.

In brain development, astrocytes develop later typically following neurogenesis. Similar sequential differentiation of neurons and then astrocytes has been reported in brain organoids (Pasca *et al.*, 2015). To examine gliogenesis in cerebellar organoids, sections from timepoints throughout differentiation were stained for Glial fibrillary acidic protein (GFAP). GFAP is expressed across glial cells, including astrocytes, radial glia and ependymal cells (Casper and McCarthy, 2006; Pasca *et al.*, 2015). Expression of GFAP was present in cerebellar organoids from as early as day 21, this early glial population likely represents radial glial precursors of glial and neurons (Figure 4.5). However, GFAP signal is sparse and doesn't show clear star-shaped astrocyte morphology, even in older organoids (Figure 4.5). Any effect of astrocytes generated within cerebellar organoids is therefore limited due to the small population, so addition of extra astrocytes was hypothesised to have the potential to promote neuronal survival and maturation.

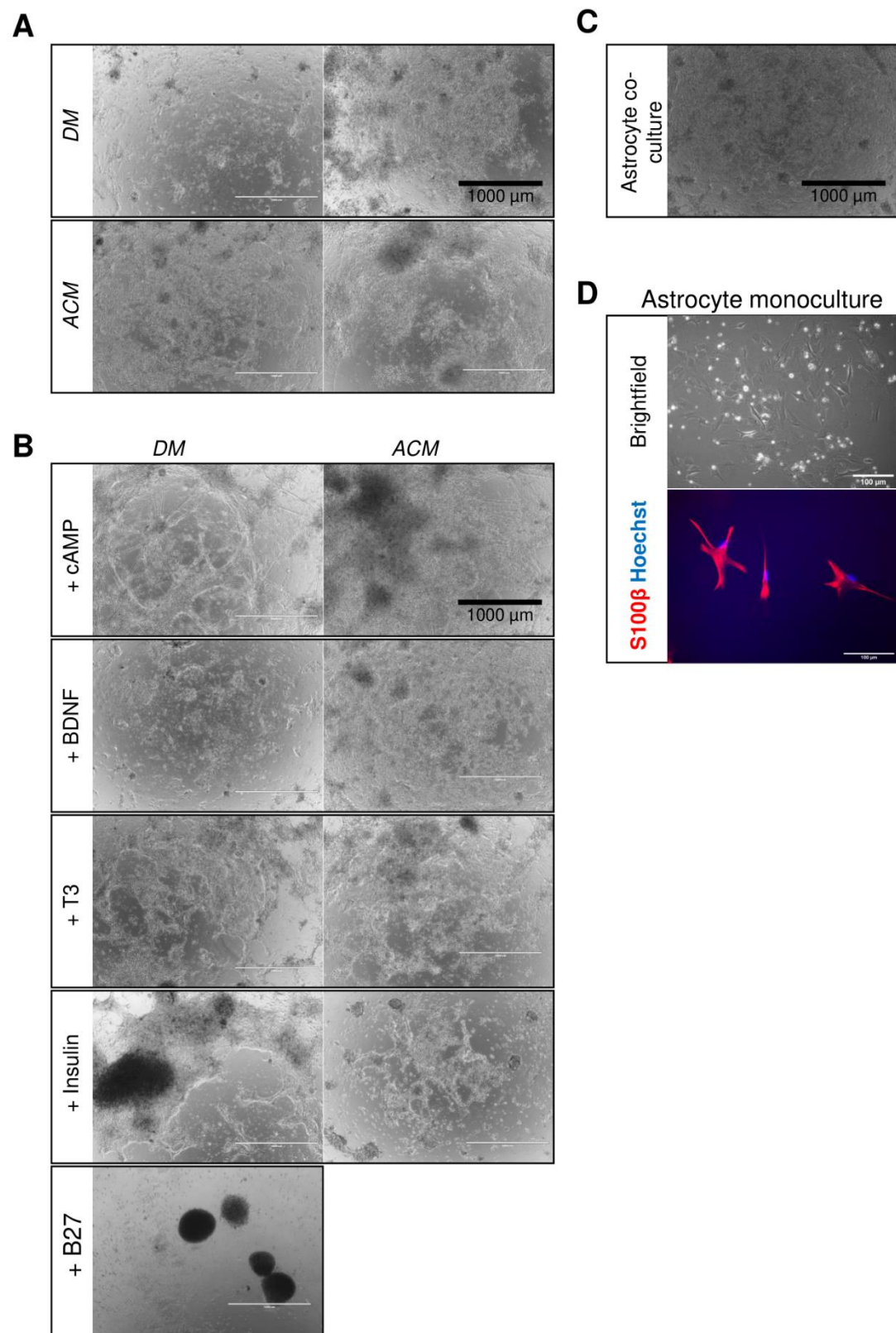


Figure 4.4 Testing addition of individual factors on the growth 2D iPSC-cerebellar cultures.

A) Brightfield images from day 63 of two cerebellar differentiations. Organoids were dissociated, and cells plated to 2D at day 35. 2D iPSC-cerebellar cultures were grown in *Differentiation medium (DM)* or *Astrocyte conditioned medium (ACM)*. Scale bars 1000 μm . *Continued.*

Figure 4.4. Testing addition of individual factors on the growth 2D iPSC-cerebellar cultures.

- B) Different growth factors were added the 2D iPSC-cerebellar cultures to screen their effect. Brightfield images were taken at day 63 of differentiation. Images on the left show cultures where growth factors were added to *DM* and in the right panel to *ACM*. Scale bars 1000 μm .
- C) Representative brightfield image of iPSC-cerebellar cultures co-cultured with iPSC-derived astrocytes. Images taken at day 63. Scale bar 1000 μm .
- D) iPSC-derived astrocytes (Axol Bioscience) used to harvest *ACM*. Brightfield image of astrocytes 6 days after thaw. Immunostaining of astrocytes using glia marker S100 β (red). Scale bars 100 μm .

iPSC-derived astrocytes (Axol Bioscience) were first grown as a monoculture to confirm expected features of successful astrocyte growth. Astrocyte cultures showed flat, spread-out cells with astrocyte-like morphology and expression of S100 β (Figure 4.4D). To assess the effect of factors released by astrocytes, *astrocyte conditioned medium (ACM)* was collected from iPSC-derived astrocyte monocultures over two weeks. iPSC-cerebellar cultures grown in *ACM* appeared to have improved cell survival or proliferation as cultures remained denser than those grown in base *DM* (Figure 4.4A). Direct co-culture with astrocytes also led to more dense cell cultures (Figure 4.4C). These observations suggest astrocyte-secreted factors promote survival or proliferation of the iPSC-derived cerebellar neurons generating more stable cultures. Therefore, *ACM* was used as base medium for some of the subsequent factor testing.

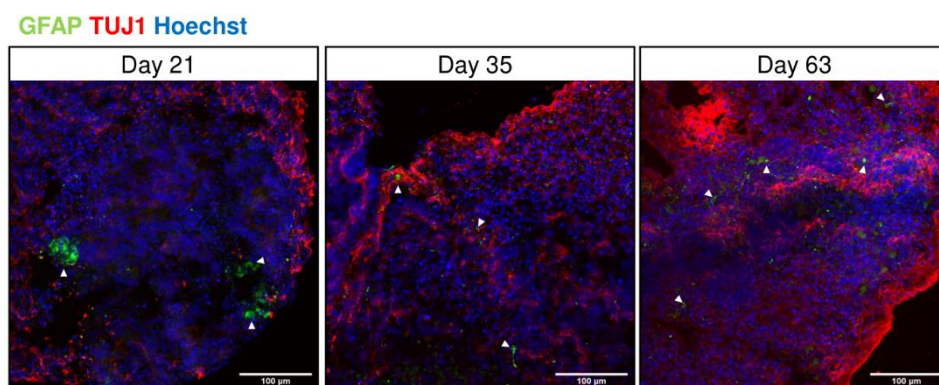


Figure 4.5 Expression of glial marker GFAP in cerebellar organoids.

Sections from cerebellar organoids at day 21, 35 and 63 were immunostained with antibodies for glial marker GFAP (green) and neuronal marker TUJ1 (red), nuclei were stained with Hoechst (blue). Scale bars = 100 μm .

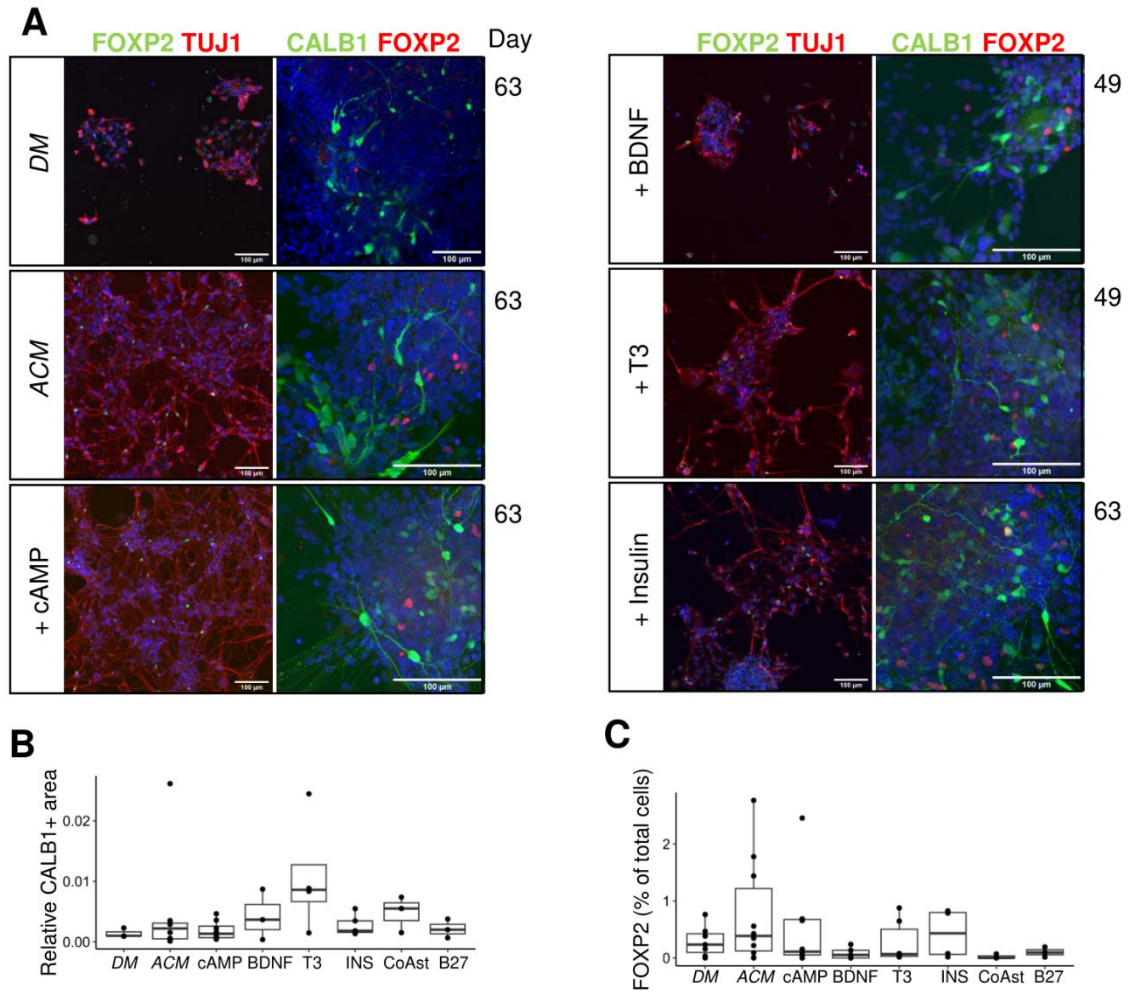


Figure 4.6 Testing addition of factors on Purkinje cell generation in 2D iPSC-cerebellar cultures.

- A) 2D iPSC-cerebellar cultures grown in *Differentiation medium* (DM) with different test factors were stained with antibodies against Purkinje cell marker FOXP2 (green) and neuronal marker TUJ1 (red) or Purkinje cell markers CALB1 (green) and FOXP2 (red). Differentiation day indicated, cells plated at day 35. Nuclei were stained with Hoechst (blue). Scale bars 100 μ m.
- B) Quantification of CALB1+ staining across 2D iPSC-cerebellar cultures grown with different test factors. CALB1+ percentage area normalised by nuclei count. Points represent average values per well, data from ≥ 2 differentiations. No significant differences were identified by ANOVA.
- C) The percentage of cells that were FOXP2+ across different medium conditions. Points represent average values per well, data from ≥ 2 differentiations. No significant differences were identified by ANOVA.
- ACM, astrocyte conditioned medium; CoAst, Co-culture with astrocytes; INS, Insulin.

To determine the identity of cells across conditions, cultures were stained for the neuronal marker class III beta-tubulin (TUJ1), along with Purkinje cell-specific markers FOXP2 and CALB1 (Figure 4.6A). Cells positive for these markers were found across all conditions, confirming that

Purkinje cells were maintained across the dissociated cultures. Quantification found no significant differences in the number of FOXP2+ or CALB1+ cells across different culture conditions (Figure 4.6B & C).

B) FOXP2 population

Immunostaining for markers only gave a static snapshot and quantification had high variation within wells and across wells grown in the same condition. To detect any significant differences between conditions, a higher throughput method was needed to provide sufficient replicates. The FOXP2-P2A-mNeon reporter line provided a tool to easily monitor the *FOXP2*+ Purkinje cell population across conditions and time. Cerebellar organoids generated with the FOXP2-P2A-mNeon reporter line were dissociated and plated at day 35 in different medium conditions. Then mNeon fluorescence was imaged from two to four weeks after plating (Figure 4.7). For these tests *ACM* was used as the basal medium to promote culture stability, so that the effect of additional factors could be better understood. *FOXP2* is a marker of developing Purkinje cells so the live readout of mNeon fluorescence provided a proxy for the Purkinje cell population (Figure 4.7A). As some background autofluorescence was observed, cultures derived from the parental AH0-17-3 iPSC line were used as a negative control (Figure 4.7A & B). Background fluorescence also made automated quantification unreliable, so quantification of images was performed by manual point counting on blinded images.

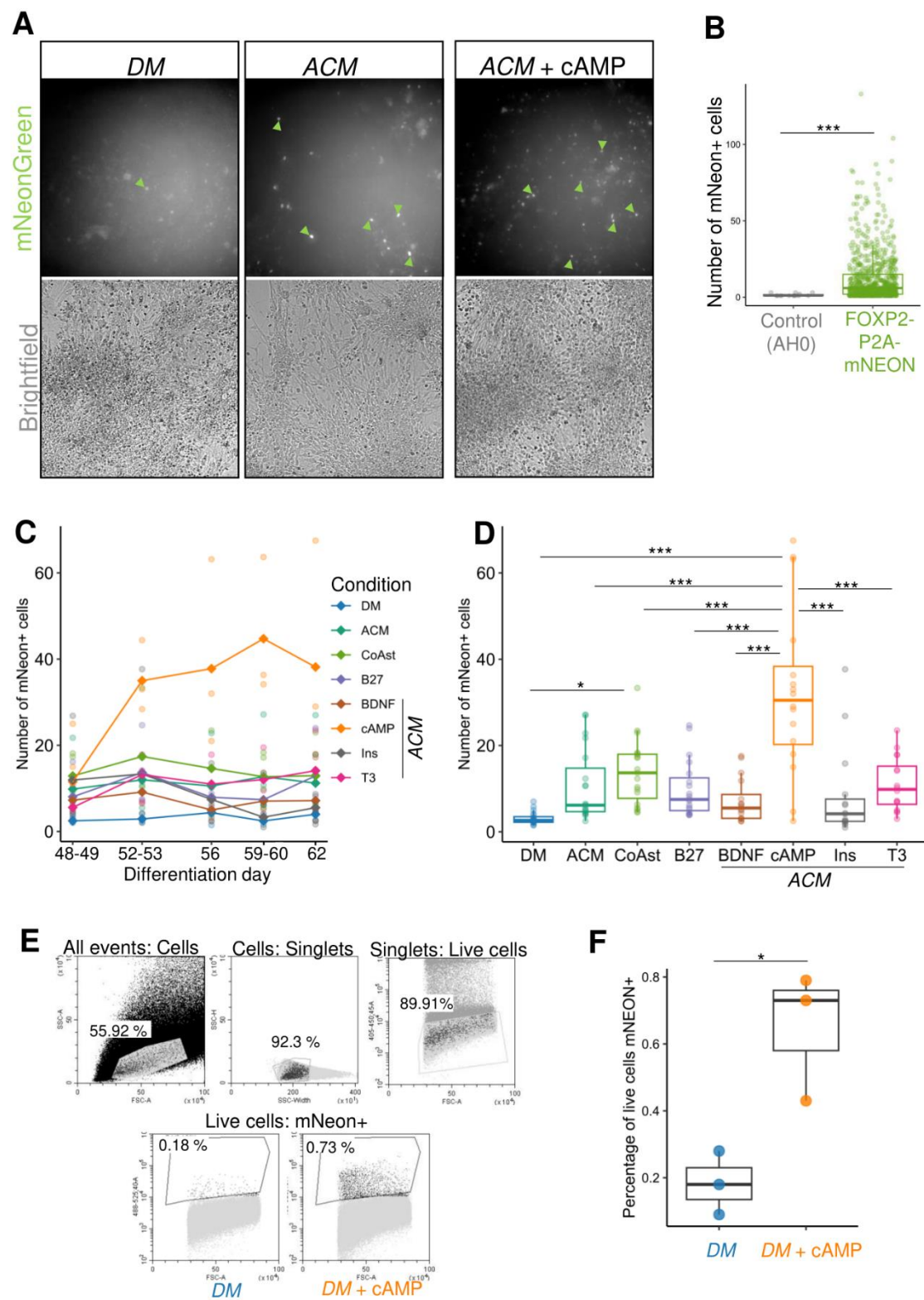


Figure 4.7 Addition of cyclic-AMP increased the FOXP2+ population in 2D iPSC-cerebellar cultures.

Figure 4.7. Addition of cyclic-AMP increased the FOXP2+ population in 2D iPSC-cerebellar cultures.

- A) Live cell images of 2D iPSC-cerebellar cultures grown from the FOXP2-P2A-mNeon line. More mNeon+ cells (examples indicated with green triangles) were seen in cultures treated with 1 mM cAMP compared to *Differentiation medium (DM)* or *astrocyte conditioned medium (ACM)* only. YFP (top) and brightfield (bottom) channel images.
- B) mNeon+ cells could be distinguished from background noise. Counts were compared between cultures from the control parental line (AH0-17-3) and the FOXP2-mNeon reporter line and a significant difference calculated by T-test (** $p < 0.001$). Images were quantified blind and points represent separate images.
- C) The number of mNeon+ cells per image (y-axis) was quantified across time in different culture conditions. Cells were plated at day 35 and cultures were grown in *DM*, with astrocyte co-culture (CoAst), or in astrocyte conditioned medium (ACM). Additional factors were tested by adding to ACM. The x-axis (Day) refers to total differentiation time. Pale points represent individual differentiations ($n=4$) with average counts calculated from 3-6 images across 3-4 wells per condition. Bold diamonds show the mean count for each condition at each timepoint. Ins, insulin.
- D) All timepoints from (C) are combined to show the number of mNeon+ cells per image across different conditions. Points represent average counts per image for an individual differentiation at a timepoint. Significant difference in the number of mNeon+ cells per image across conditions was calculated by ANOVA followed by Tukey's HSD test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
- E) Example gating for quantification of mNeon+ live cells by flow cytometry.
- F) The percentage of live cells expressing mNeon was measured by flow cytometry. Cells grown in *DM* with 1mM cAMP were compared to growth in *DM* alone. Points indicate individual differentiations. A significant difference in mNeon+ population size between conditions was determined by T-test ($n = 3$, * $p < 0.05$).

The majority of tested culture conditions had no effect on the mNeon+ population, matching the quantification of FOXP2+ cells by immunostaining (Figure 4.7C & Figure 4.6C). However, addition of 1 mM cAMP robustly increased the number of mNeon+ cells from 2.5 weeks after plating and this effect persisted for the four weeks of 2D culture monitored (Figure 4.7C). Combining all timepoints, the addition of cAMP significantly increased the number of mNeon+ cells compared to all other conditions (Figure 4.7D). There was also a smaller increase in the number of mNeon+ cells in co-cultures of iPSC-derived cerebellar cells with astrocytes (CoAst, Figure 4.7D). This may be due to the higher cell density observed in these wells, and thus a higher total cell population, however this was not quantified.

The measured increase in the number of mNeon+ cells per image, may reflect a shift in the proportion of cell types present or simply an increase in overall cell density. To determine

whether the addition of cAMP promoted the expression of *FOXP2* and therefore led to a larger proportion of mNeon+ cells, flow cytometry was performed on *FOXP2*-P2A-mNeon iPSC-cerebellar cultures (Figure 4.7E). Control AH0-17-3 cultures were again used as a negative control to set the gating for mNeon fluorescence. The percentage of live cells fluorescent for mNeon was very low with a mean of 0.18% in *DM* (Figure 4.7F). However, addition of cAMP to the culture medium significantly increased this proportion to 0.65% (Figure 4.7F). This confirmed that the increased number of *FOXP2*-expressing cells observed in the presence of cAMP is reflective of an increased proportion of *FOXP2*+ Purkinje cells within the iPSC-cerebellar cultures.

C) Marker gene expression

To determine whether the differences in morphology and *FOXP2*+ population were relevant to broader cerebellar identity, gene expression of further specific markers was compared across conditions using RT-qPCR. To understand the results, expression was plotted in several ways. Firstly, to examine the effects of all the different treatments, expression of selected cerebellar genes was summarised using principle component analysis (PCA) (Figure 4.8A). Differentiated iPSC-cerebellar cultures showed clear separation from undifferentiated iPSCs across PC1, with increased expression of cerebellar marker genes. The 3D cerebellar organoid samples were distinguished from the majority of 2D samples by higher expression of *SKOR2*, *ATOH1*, *FOXP1* and *FOXP2*. In contrast, the 2D dissociated cultures showed increased expression of *RORA*, *CA8* and *ITPR1* compared to 3D organoids.

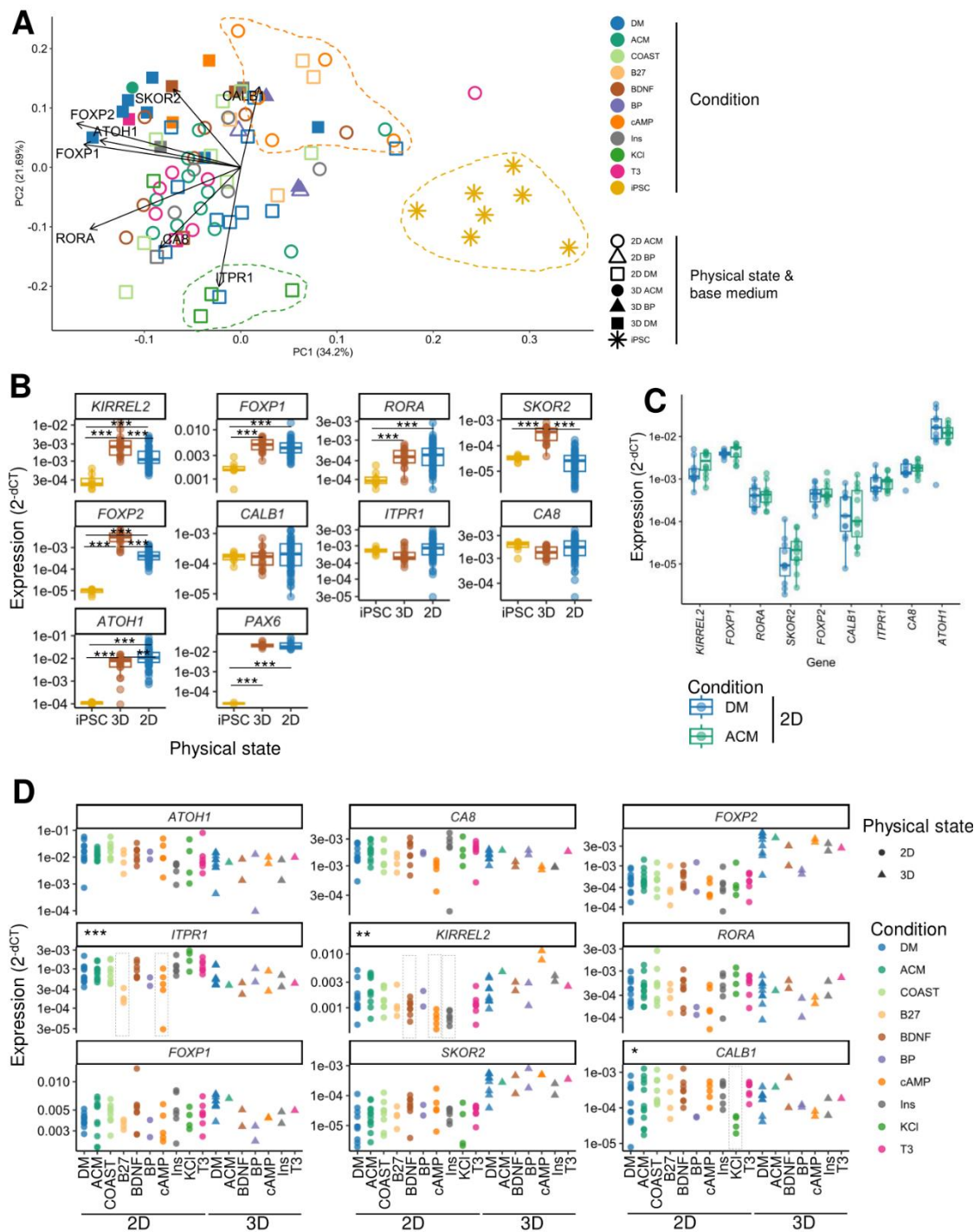


Figure 4.8 Expression of cerebellar marker genes in iPSC-cerebellar cultures across different culture conditions.

Figure 4.8. Expression of cerebellar marker genes in iPSC-cerebellar cultures across different culture conditions.

- A) Expression of cerebellar markers was profiled across iPSC and iPSC-derived cerebellar samples grown in different conditions. Gene expression was quantified by RT-qPCR and dCT values calculated using housekeeper genes (*ACTIN*, *GAPDH*). The selected gene panel contains Purkinje cell markers (*SKOR2*, *CALB1*, *RORA*, *CA8* & *ITPR1*), *FOXP* family genes (*FOXP1* & *FOXP2*) and granule cell progenitor marker (*ATOH1*). Samples were plotted using -dCT values for principle component analysis (PCA). Arrows indicate direction of increasing gene expression. iPSCs (yellow stars, cluster shown by yellow dashed line) were separated from differentiated cells across PC1. 2D cultures (open shapes) and 3D organoids (solid shapes) were separated across PC2. Clustering of samples from 2D iPSC-cerebellar cultures treated with cAMP (orange) and KCl treated (green) are highlighted with dashed outlines. Cells were grown in *Astrocyte conditioned medium* (ACM, circle), *BrainPhys* (BP, triangle), or *Differentiation medium* (DM, square) as the base medium. COAST, Astrocyte co-culture; Ins, Insulin.
- B) Comparison of gene expression between the major clusters from the PCA plot: undifferentiated iPSCs and differentiated cerebellar cultures as organoids (3D) or dissociated and plated to 2D. Different medium conditions within these physical states were pooled together. RT-qPCR data from (A) was replotted as 2^{-dCT} relative expression. Significant differences in expression determined by ANOVA followed by Tukey's HSD test (* $p < 0.05$, ** $p < 0.01$).
- C) Comparison of gene expression between 2D iPSC-derived cerebellar cultures grown in DM and ACM. RT-qPCR from (A) for selected samples was replotted as 2^{-dCT} relative expression. No significant differences in gene expression between the two conditions was found by two-sample T-test ($n = 11$, $p < 0.05$).
- D) Expression of cerebellar marker genes across different medium conditions in iPSC-cerebellar cultures grown in 2D (circles) and 3D (triangles). RT-qPCR data (A) was replotted as 2^{-dCT} relative expression. Samples grown using DM and ACM as basal medium combined as no differences in gene expression were found between these (C). Gene expression across different 2D medium conditions was assessed for any significant differences using ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) followed by Tukey's HSD (conditions boxed). Gene expression significantly decreased in the following listed conditions (compared to conditions in brackets). *ITPR1* - B27 (DM, ACM, COAST, BDNF, Ins, KCl, T3) and cAMP (ACM, COAST, BDNF, Ins, T3, KCl). *KIRREL2* - BDNF (ACM), cAMP (ACM) and Ins (ACM). *CALB1* - KCl (BDNF, COAST, T3).

Cultures grown with different medium factors were largely intermixed and couldn't be distinguished using this panel of gene expression (Figure 4.8A). Overall no large changes in gene expression, along with previously described similarities in morphology and *FOXP2*+ population, indicated there was little benefit to addition of most of these factors for Purkinje cell generation. The exception to this trend was addition of cAMP. The 2D iPSC-cerebellar cultures treated with cAMP were more transcriptionally similar to 3D organoids, with increased *FOXP2* and *SKOR2* expression (Figure 4.8A). The gene expression differences in

cAMP-treated samples to other 2D conditions coincides with previous changes observed, in morphology and *FOXP2*⁺ population. Cultures grown in B27 also show similar expression patterns to cAMP, however, the high cell detachment meant this condition was not followed further (Figure 4.8A). Another, medium condition of note was the increased KCl treatment. Three of the four samples from cerebellar cultures grown in higher KCl were separated from other 2D samples due to increased *ITPR1* expression. This result is consistent with previous studies that have found increased *ITPR1* expression in granule cells grown in higher KCl conditions *in vitro* (Choi, Beaman-Hall and Vallano, 2004).

As the PCA clustering showed the largest differences between the physical culture conditions rather than individual factor treatments, medium treatments were combined and the physical culture conditions (3D organoid or 2D cultures) were compared to undifferentiated iPSCs (Figure 4.8B). Most of the cerebellar markers which showed significant induction of expression in organoids, also showed increased expression in the 2D cultures when compared to undifferentiated iPSCs. This included both ventricular zone markers (*KIRREL2*, *FOXP1*, *FOXP2*, *RORA*) and rhombic lip markers (*ATOH1*, *PAX6*) (Figure 4.8B). Therefore, cerebellar identity was maintained on organoid dissociation and growth in 2D. After plating, dissociated cerebellar cultures exhibited decreased expression of *KIRREL2*, *SKOR2* and *FOXP2* compared to organoids of the same age (Figure 4.8B). Conversely, 2D conditions promoted slight increases in *ATOH1* and *ITPR1* expression as well as a wider range of expression of *CA8*, *RORA* and *CALB1* (Figure 4.8B). The increase in expression of these later markers of Purkinje cells suggests that dissociation and 2D plating may promote neuronal maturation.

For this RT-qPCR analysis, samples were collected from experiments using *DM* and *ACM* as base medium as indicated by shape in the PCA plot (Figure 4.8A). To determine if the conditioned medium altered gene expression, 2D iPSC-derived cerebellar cultures grown in *DM* and *ACM* without any additional factors were compared (Figure 4.8C). None of the cerebellar markers tested showed significant differences in expression when using conditioned medium, indicating any astrocyte secreted factors present were not altering cerebellar

differentiation trajectories, despite the suggested of increased cell survival or proliferation observed from cell density (Figure 4.8C).

Looking more closely at individual treatments, cerebellar markers showed heterogeneous expression with considerable variability between replicates, meaning that few significant differences were found (Figure 4.8D). Amongst 2D samples, *KIRREL2* had significantly increased expression in *ACM* compared to when Insulin, cAMP or BDNF were added as factors, and *ITPR1* expression was decreased in B27- or cAMP-treated cultures (Figure 4.8D). Despite the increased proportion of *FOXP2*+mNeon+ cells shown by flow cytometry, no significant increase in *FOXP2* mRNA was detected in cAMP-treated cultures (Figure 4.8D). This highlights the low resolution of bulk methods such as RT-qPCR when looking for a small subpopulation of cells. In addition, as *RORA* and *ITPR1* are known targets of T3 in Purkinje cells, their expression was examined across conditions (Yu *et al.*, 2015). *ITPR1* expression in 2D cultures showed an upwards trend upon T3 addition, however no significant difference was found (Figure 4.8D).

D) Immunostaining

Finally, the cell types present in 2D iPSC-cerebellar cultures were further characterised by staining with antibodies against selected markers. Based on previous results, comparison was performed between cultures grown in *DM* only or with addition of 1 mM cAMP, or in *ACM*. First, the presence of neurons and glial cells was examined with antibodies for broad neuronal marker TUJ1 and glial marker GFAP (Figure 4.9A). 2D iPSC-cerebellar cultures were predominantly neuronal with widespread TUJ1 staining. GFAP+ cells were rare but were observed in all three growth conditions and showed thick extended processes (Figure 4.9A). No significant difference was found in the size of the GFAP+ population across conditions (Figure 4.9B). Second, 2D iPSC-cerebellar cultures were immunostained for PAX6, a marker of granule cell precursors and mature neurons (Figure 4.9A). Again, PAX6+ cells were present across all three culture conditions and showed no significant difference in number (Figure 4.9C). Finally, the maturity of neurons in the 2D iPSC-cerebellar cultures was assessed using NESTIN, a marker of neural stem cells, and MAP2, a marker of more mature neurons (Figure 4.9A). Both NESTIN and MAP2 were present across culture conditions and labelled distinct

populations of cells as neural progenitors or more mature neuronal cells (Figure 4.9A). MAP2 expression was very similar across conditions, whereas NESTIN was observed less frequently on addition of cAMP, however no significant difference was found (Figure 4.9D & E).

Taken together, the parameters measured identified a benefit from addition of cAMP for 2D iPSC-cerebellar cultures. cAMP increased neuronal morphology, promoted survival and increased the *FOXP2*⁺ Purkinje cell population. Bulk gene expression and immunostaining showed high variability but confirmed the presence of a mixture of cerebellar cell types, including cells showing features of Purkinje cells (*FOXP2*, *CALB1*), granule cells or their precursors (*PAX6*), neural progenitors (*NESTIN*) and glial cells (*GFAP*). In addition, cultures were predominantly neuronal based on imaging of markers MAP2 and TUJ1. Trends in gene expression suggested that 2D dissociated culture increased expression of some more mature Purkinje cell markers (*ITPR1*, *CA8*, *CALB1*, *RORA*), though decreased levels of earlier markers (*KIRREL2*, *SKOR2*, *FOXP2*) in iPSC-derived cerebellar cultures. Addition of cAMP to 2D cultures appeared to somewhat restore expression of earlier Purkinje cell markers and therefore may offer a good compromise system for Purkinje cell generation. Growth with cAMP also generated more stable cultures, with good survival for over four weeks in 2D. The use of astrocyte co-culture or ACM appeared to improve cell survival or proliferation in 2D dissociated iPSC-cerebellar cultures. However, no significant differences were found in expression of Purkinje cell genes by RT-qPCR or immunostaining, and only a small effect on the *FOXP2*⁺ population by mNeon-imaging. These results suggest there is no Purkinje cell-specific benefit to the use of astrocyte co-culture or ACM with iPSC-derived cerebellar cultures. Consequently, the following stages of optimisation focused on the DM + cAMP medium condition which showed the most benefit on Purkinje cell generation.

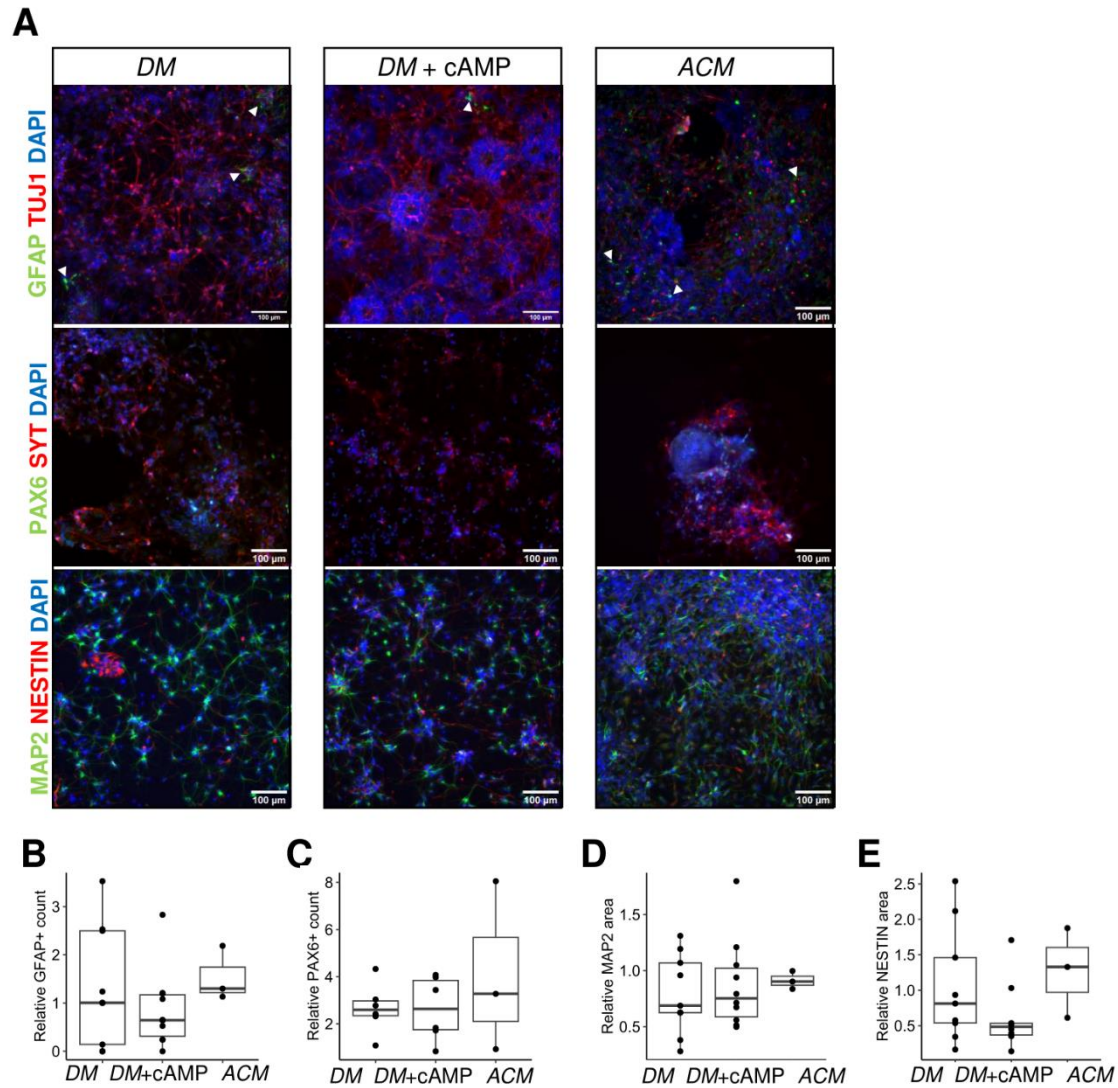


Figure 4.9 Immunostaining of cell type markers present in 2D iPSC-cerebellar cultures.

- A) 2D iPSC-cerebellar cultures grown in different medium conditions were stained with antibodies against glial marker GFAP (green) and neuronal marker TUJ1 (red), granule cell precursor and granule neuron marker PAX6 (green) and synaptic marker synaptotagmin (SYT, red), and mature neuronal marker MAP2 (green) and neural stem cell marker NESTIN (red). Nuclei were stained with DAPI (blue). Cultures were grown in *Differentiation medium* (DM), DM + 1 mM cAMP or *astrocyte conditioned medium* (ACM). Cells were fixed between day 64 and 68 of differentiation. Scale bars 100 μ m.
- B) Quantification of GFAP+ cells across conditions. Relative GFAP count calculated as a percentage of total cells based on DAPI nuclear staining. Data from $n \geq 3$ differentiations.
- C) Quantification of PAX6+ cells across conditions. Relative PAX6 count calculated as a percentage of total cells based on DAPI nuclear staining. Data from $n \geq 3$ differentiations.
- D) Quantification of MAP2+ cells across conditions. Relative MAP2+ area normalised by total cell number based on DAPI nuclear staining. Data from $n \geq 3$ differentiations.
- E) Quantification of NESTIN+ cells across conditions. Relative NESTIN+ area normalised by total cell number based on DAPI nuclear staining. Data from $n \geq 3$ differentiations.

4.2.4 cAMP promotes neuronal activity in iPSC-cerebellar neurons

Establishing a 2D culture system offered the opportunity to study electrical activity in iPSC-derived cerebellar neurons. Human iPSC-derived cerebellar cultures aim to recapitulate cerebellar neuron function and therefore provide a model to study development of neuronal activity and alterations in disease. However, it is challenging to measure neuronal activity within 3D organoids, and so one advantage of establishing a 2D culture system was that it facilitated different assays to study electrical activity in iPSC-cerebellar neurons. Previous studies have reported a calcium response to KCl-stimulated depolarisation and action potential firing in response to current injection, in iPSC-cerebellar neurons grown in BrainPhys (Silva *et al.*, 2020) or in co-culture with cells from mouse rhombic lip (Muguruma *et al.*, 2015). Spontaneous activity generated by GABAergic and glutamatergic synapses was also recorded (Silva *et al.*, 2020). Building on these reports, iPSC-cerebellar cultures were monitored for generation of spontaneous neuronal activity and compared across different growth medium.

Before measuring neuronal activity, the expression of synaptic markers was first examined in the 2D iPSC-derived cerebellar cultures as an indicator of whether neuronal activity was likely to be observed. Based on the increased neuronal morphology observed in the cAMP-treated cultures, the effect of cAMP addition on expression of neuronal activity genes was also compared. Genes were selected from those enriched in the *FOXP2*⁺ population of cerebellar organoids (see Chapter 5) and encode glutamate receptors (*GRIK3* and *GRIN3A*), a glycine receptor (*GLRA2*), a voltage-gated sodium channel (*SCN3B*), and transmembrane transporters active at synapses (*SLC17A6* and *SLC6A17*). All of the neuronal activity genes were found to be expressed in 2D cerebellar cultures by RT-qPCR (Figure 4.10A). Moreover, addition of cAMP significantly increased expression of three of the neuronal activity genes (Figure 4.10A).

Western blotting was used to compare the effect of cAMP treatment on levels of synaptic proteins Synapsin and Synaptophysin (Figure 4.10B). Both synaptic proteins were increased in cultures grown in DM with 1 mM cAMP added (Figure 4.10B). Finally, the localisation of synaptic markers was confirmed by imaging (Figure 4.10C). 2D iPSC-cerebellar cultures had widespread expression of general markers of presynaptic terminals (Synaptophysin and Synaptotagmin) and post-synaptic terminals (Postsynaptic density protein 95, PSD-95)(Figure

4.10C & D). However, higher resolution imaging would be required to show precise localisation of these markers on a synapse to demonstrate the formation of functional synapses in the iPSC-derived cerebellar cultures.

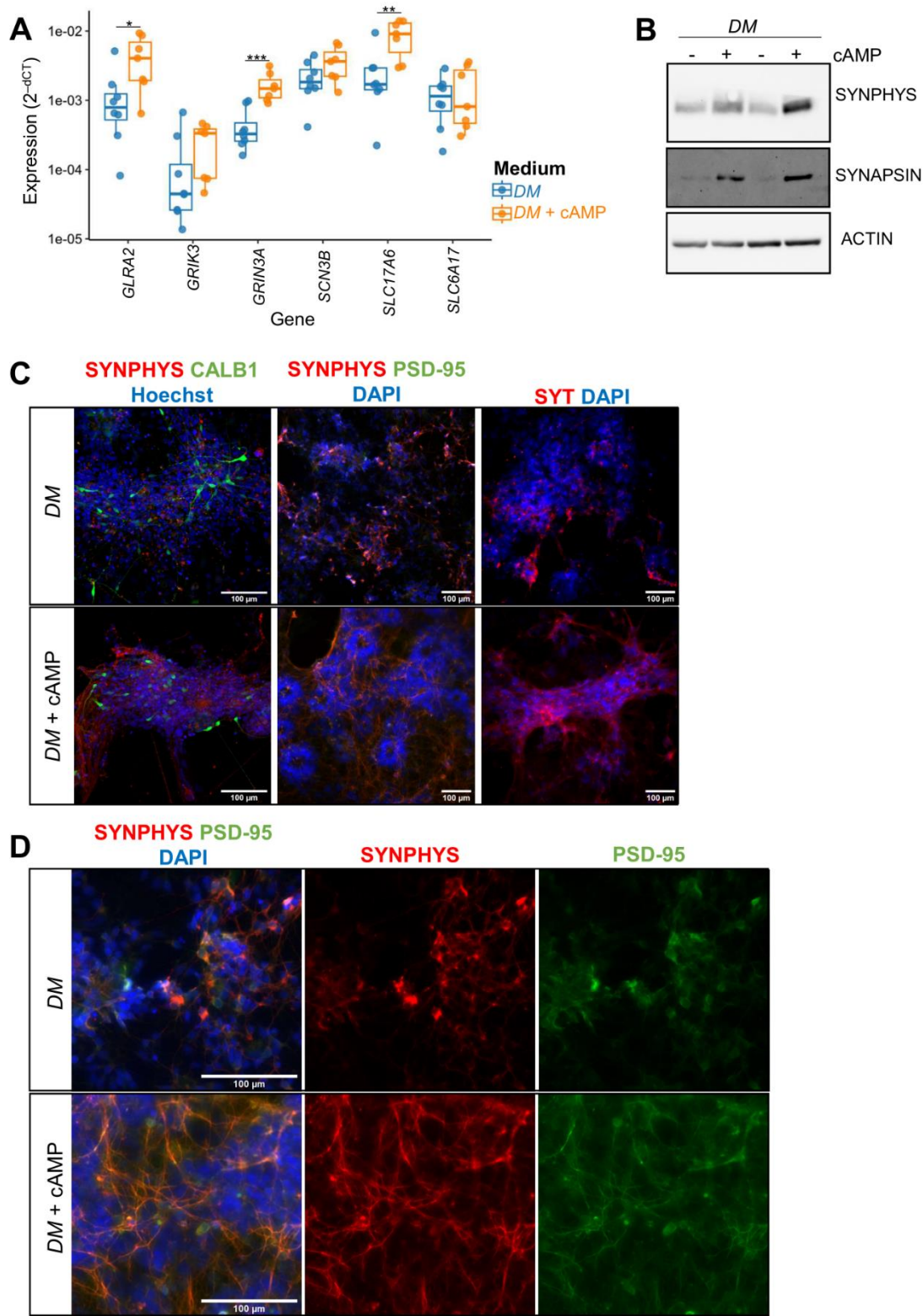


Figure 4.10 2D iPSC-cerebellar cultures expressed synaptic markers.

- A) Genes related to neuronal activity and synaptic transmission were expressed in 2D iPSC-cerebellar cultures. Expression was increased in cultures grown with cAMP added to the base *Differentiation medium (DM)*. Gene expression quantified by RT-qPCR and plotted as $2^{-\Delta CT}$ relative to housekeeper genes (*ACTIN*, *GAPDH*). Points represent individual differentiations. Significant differences in expression determined by T-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
- B) Protein levels of synaptic markers Synaptophysin (SYNPHYS) and Synapsin in 2D iPSC-cerebellar cultures was increased on addition of cAMP to growth medium. ACTIN was used as a loading control.
- C) Synaptic markers were detected in 2D iPSC-cerebellar cultures across different culture conditions using antibodies against Synaptophysin (SYNPHYS), PSD-95 and Synaptotagmin (SYT). Nuclei were stained with Hoechst or DAPI (blue). Scale bars 100 μm .
- D) Individuals channels are shown for presynaptic marker Synaptophysin (SYNPHYS) and post synaptic marker PSD-95. Scale bars 100 μm .

Together these results confirm widespread expression of synaptic markers, as well as expression of genes involved in neurotransmitter signalling and action potential generation, predicting that 2D iPSC-derived cerebellar cultures are capable of spontaneous neuronal activity. Expression of synaptic markers was present in cultures grown in just *DM*, but was increased with addition of cAMP to the growth medium, suggesting a similar increase is likely in the frequency of neuronal activity across conditions.

To test these hypotheses, the presence of neuronal activity in the 2D iPSC-cerebellar cultures was examined. To characterise spontaneous neuronal activity, day 35 cerebellar organoids were dissociated and plated onto a 96-well multi-electrode array (MEA) plate (Figure 4.11A). Each well contained eight electrodes that recorded extracellular field potentials and so could detect the generation of action potentials. Recordings were made for 5 min with cells incubated at 37°C and 5% CO₂. Given the increased expression of neuronal activity markers induced by addition of cAMP, we hypothesised that cAMP may promote the development of neuronal activity. To test whether cAMP had a dose-dependent effect on neuronal activity, cAMP was added to the growth medium at two doses: 1 mM as used in previous testing and at the lower dose of 200 μM . MEA recordings require cells to contact the electrodes which are spaced around the wells. The higher dose of cAMP (1 mM) led to formation of clumps of cells which decreases the likelihood of cells growing on the electrodes (Figure 4.11A). The lower

cAMP dose (200 μ M), altered morphology less and thus produced less clumped cultures so had reduced risk of no cells contacting electrodes (Figure 4.11A).

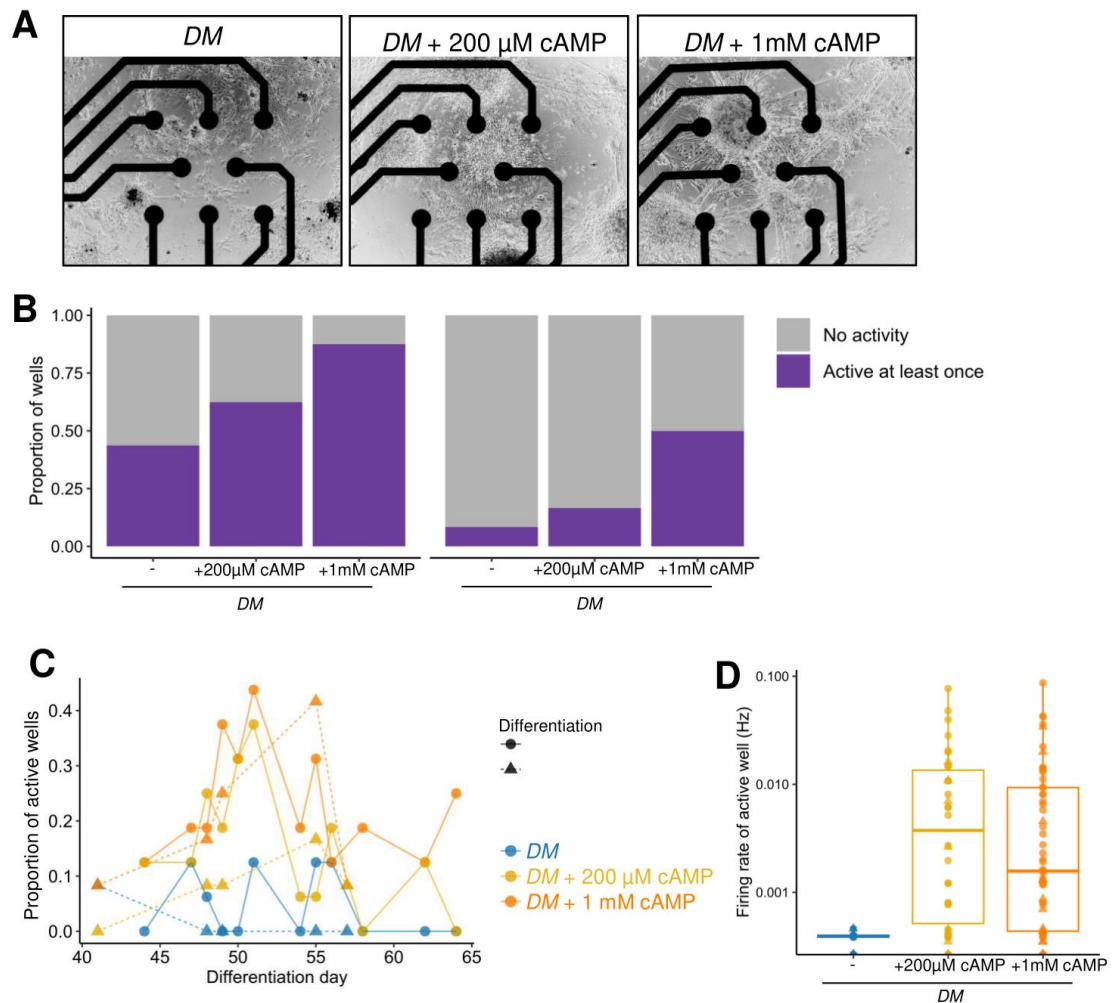


Figure 4.11 2D iPSC-derived cerebellar cultures develop spontaneous neural activity promoted by addition of cAMP.

- Brightfield images of 2D iPSC-cerebellar cultures on 96 well MEA plate grown in different conditions, in *Differentiation medium (DM)* or with two doses of cAMP added.
- Addition of cAMP to medium increased the proportion of wells with detected activity with a dose-dependent effect. Wells were classified as showing activity if one or more peak was recorded. Results from two differentiations shown.
- The proportion of wells active over time, plotted for each medium condition. Data from two differentiations is shown. Cells were plated at day 35. X-axis shows total differentiation day.
- Addition of cAMP to medium also increased the firing rate of active wells. Points represent firing rate for 1 well during 1 recording, data from 2 differentiations combined. Only active wells shown.

The development of spontaneous neuronal activity was detected in all three growth conditions in two separate differentiations (Figure 4.11B). A threshold of six standard deviations above noise was set for detection of activity peaks. Neuronal activity over this threshold was first detected on day 44 and 37 respectively of the two differentiations. Following this, recordings were made regularly (at least four times per week) and signal above the threshold classified as peaks. The proportion of wells that developed neuronal activity showed a dose-dependent response to addition of cAMP. Spikes were detected in 71% of wells grown in 1 mM cAMP across both differentiations (Figure 4.11B). Activity was highest around day 49-56 and the final recordings with detectable activity were at day 64 and 57 respectively for each differentiation (Figure 4.11C). The loss of activity likely reflects deteriorating longer term survival and attachment of the cultures. The frequency of firing in active wells was also increased by addition of cAMP (Figure 4.11D). In 2D iPSC-cerebellar cultures grown in *DM*, at most one peak per well was detected during a recording. In contrast, with addition of cAMP in the medium, repeated firing was detected from multiple electrodes per well reflected in a higher firing frequency (Figure 4.11D). To conclude, MEA recordings measured spontaneous neuronal activity in 2D iPSC-cerebellar cultures with more frequent activity developing when cAMP was added to the growth medium.

The information obtained from MEA was limited by electrode number and cell coverage. Extracellular potential signal rapidly decreases with distance from electrodes so lack of signal at an electrode could be the result of there being no attached cells or silent cells. To capture an overview of activity across the whole culture, a calcium-based reporter assay was used. The spontaneous neuronal activity (SNA) assay uses a fluorescent calcium reporter to provide a readout of calcium concentration in neurons (Figure 4.12A). The Incucyte® Neuroburst Orange Lentivirus (Sartorius) encodes a mRuby-based fluorescent calcium reporter with neuronal specific transcription based on a *Synapsin* promoter. An Incucyte® Live-Cell Analysis system (Sartorius) was used for non-disruptive imaging within the cell culture incubator, capturing brightfield and orange-fluorescent channels (Figure 4.12B). Automated identification of active objects was performed by Incucyte® Neuronal Activity Analysis software module (Sartorius).

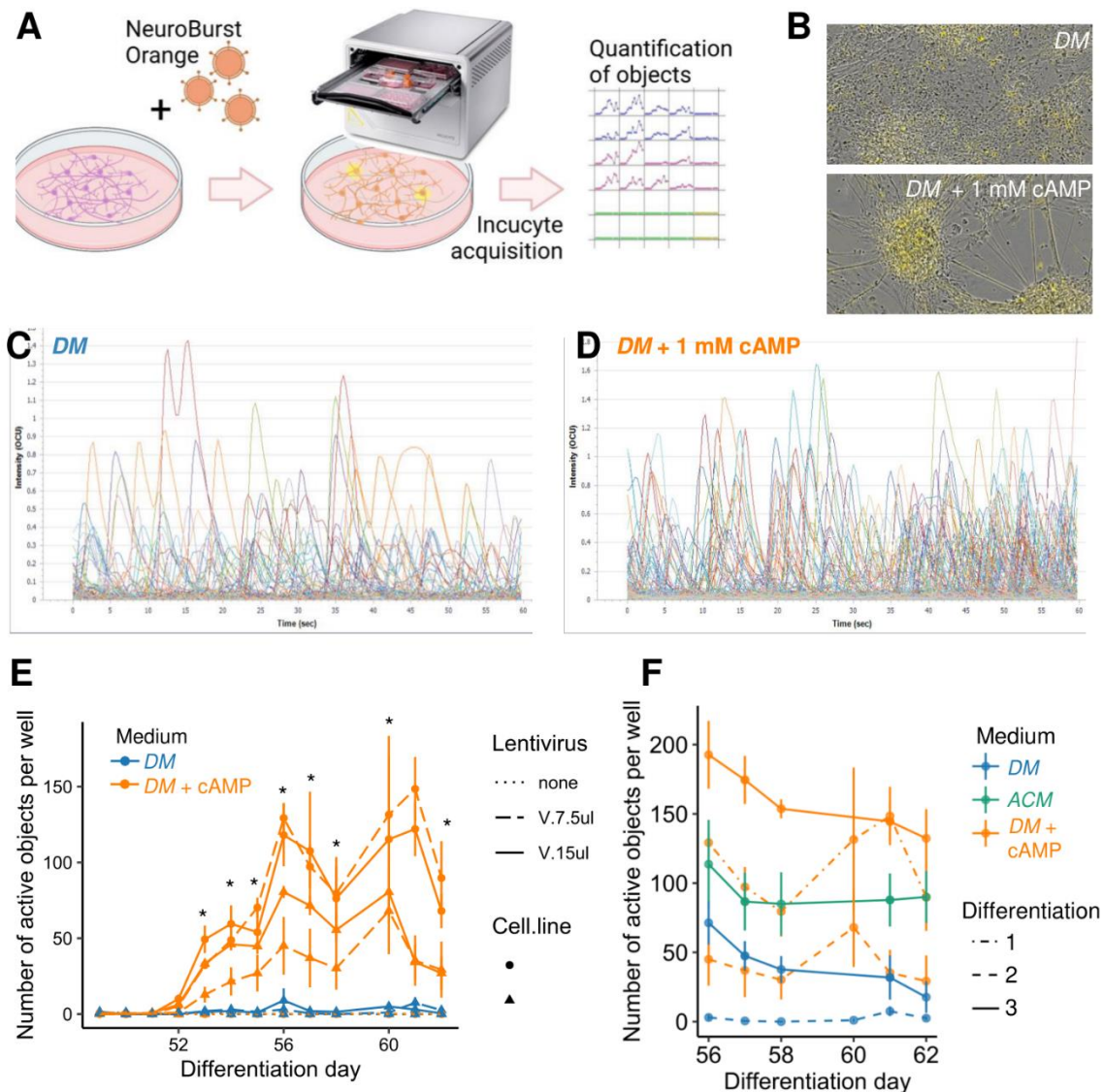


Figure 4.12 iPSC-derived cerebellar cultures develop spontaneous neural activity measured by spikes in intracellular calcium.

- A) Schematic for the spontaneous neural activity assay (SNA). Neuroburst lentivirus was added to cells to introduce a genetically encoded fluorescent calcium reporter. Cultures imaged by an Incucyte imaging system every 24 hours. Quantification of active objects was automated.
- B) Example output Incucyte images with regions of activity (yellow) overlaid onto brightfield images. Differences in morphology of 2D iPSC-cerebellar cultures grown in different medium conditions can be seen. Cultures grown in *Differentiation medium (DM)* or *DM + 1 mM cAMP*.
- C) Example of active objects trace for 2D iPSC-cerebellar cultures grown in *DM*. Colours represent different active objects. Recordings were made for 1 min with time in seconds shown across the x axis. The fluorescent intensity provided a readout of calcium concentration (y axis).
- D) Example of active objects trace for 2D iPSC-cerebellar cultures grown in *DM + 1 mM cAMP*.
Continued.

Figure 4.12. iPSC-derived cerebellar cultures develop spontaneous neural activity measured by spikes in Calcium levels.

- E) 2D iPSC-cerebellar cultures developed spontaneous activity with the earliest activity detected from day 52. The average number of active objects per well is plotted for each condition over time, error bars show standard error across technical replicates. Cells were plated at day 35 and Neuroburst lentivirus added at day 49, total differentiation day shown (x-axis). Line style indicates the volume of lentivirus added, 7.5 μ l lentivirus per well was sufficient to capture activity trends. Activity was compared between cultures grown in *DM* (blue) or *DM* + cAMP (orange). Significant differences in the mean number of active objects between conditions were identified by T-test (*p < 0.05).
- F) The effect of cAMP (orange) in increasing levels of neural activity was replicated across multiple differentiations. Separate differentiations distinguished by linestyle including replotting those shown in E. *Astrocyte conditioned medium* (ACM, green) may also increase activity compared to *DM* (blue). The average number of active objects per well is plotted for each condition over time, error bars show standard error across technical replicates. Cells were plated at day 35 (batch 1 & 2) or 41 (batch 3) and Neuroburst lentivirus added at day 48-49.

To test the SNA assay, cerebellar organoids were dissociated and plated at day 35, followed by lentivirus transduction at day 49. After lentiviral transfection, flashes of fluorescence marking increases in calcium concentration could be visualised and classified as active objects. The fluorescence intensity of these active objects were displayed as traces (Figure 4.12C & D). The SNA assay showed development of spontaneous activity from day 52 which persisted for several weeks consistent with MEA observations (Figure 4.12E & Figure 4.11C). As before, the addition of cAMP significantly increased the amount of neuronal activity, with a greater number of active objects identified per well (Figure 4.12E). For the first test, two doses of Neuroburst lentivirus were trialled. Both lentivirus doses reported a similar level of activity so the lower dose of 7.5 μ l/well was used for subsequent tests (Figure 4.12E). As expected, no activity was detected in the absence of Neuroburst lentivirus. The benefit of cAMP on the level of neuronal activity when compared to *DM* only was replicated across trials, although the number of cells showing activity varied considerably between differentiations (Figure 4.12F). Together, the SNA assay results further demonstrate the development of spontaneous neuronal activity in iPSC-cerebellar neurons and the effect of cAMP in increasing this activity.

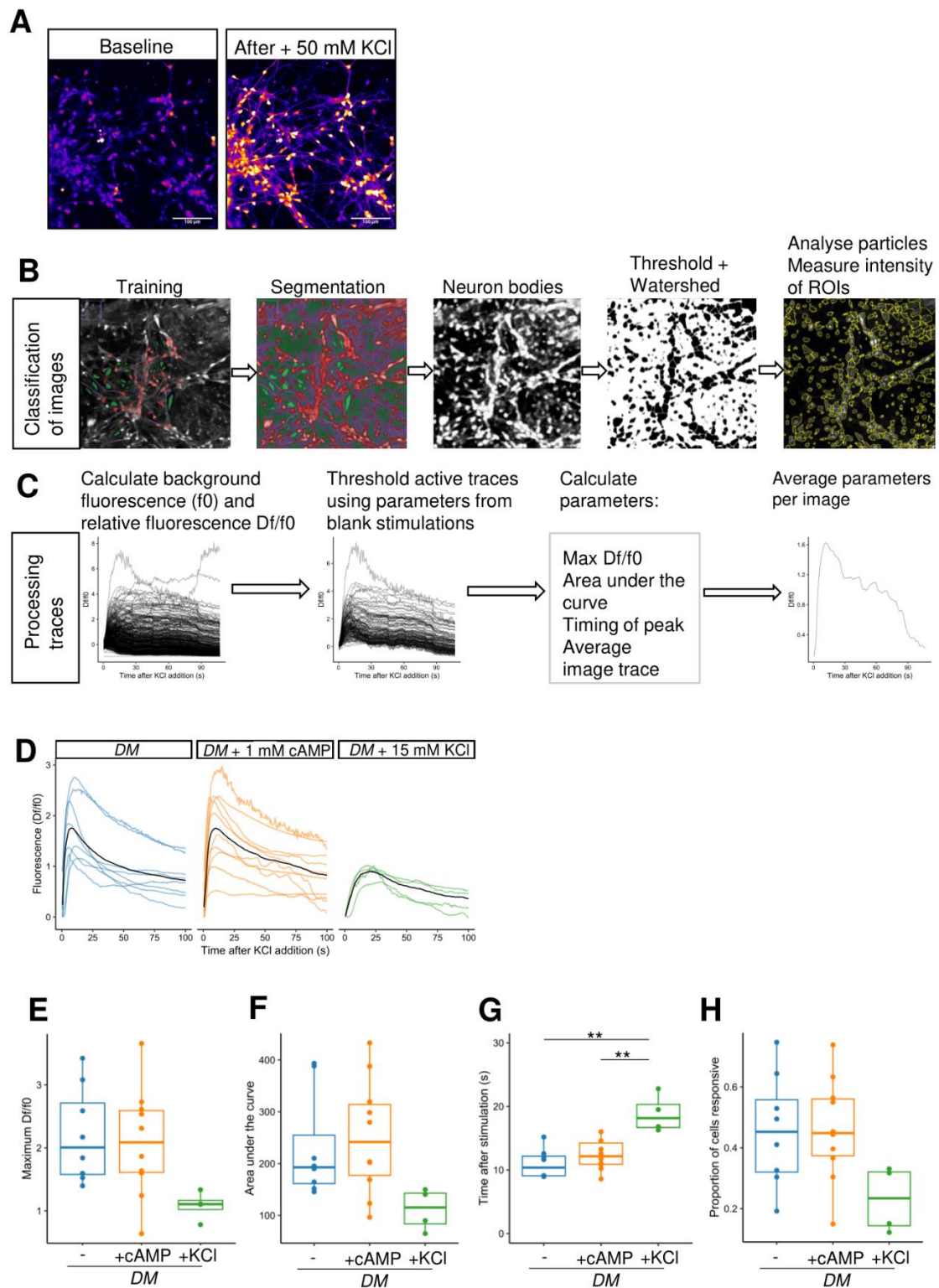


Figure 4.13 2D iPSC-cerebellar cultures show robust response to KCl stimulation.

A) Example images of 2D iPSC-cerebellar cultures stained with calcium sensitive dye Calbryte at frames prior to and at peak response to KCl stimulation. Pseudo coloured to show fluorescent intensity. Scale bar 100 μm .

Continued.

Figure 4.13. 2D iPSC-cerebellar cultures show robust response to KCl stimulation.

- B) Flow chart for extracting regions of interest (ROI) from the recordings of 2D iPSC-cerebellar cultures stimulated with 50 mM KCl. Maximum intensity projections of recordings were segmented using a trained Weka segmentation plugin. The neuron body probability image was then thresholded and watershed applied to select ROI.
- C) Flow chart describing the processing of fluorescent intensity (f) traces for regions of interest. For each trace, background fluorescent (f₀) was calculated as average of frames before stimulation and df/f₀ calculated. Active traces were filtered using thresholds established from recordings of blank stimulations.
- D) Average intracellular calcium concentration following KCl stimulation across different growth conditions. Coloured lines show average fluorescent intensity traces for all active ROIs per recording. Black lines show average trace per condition. Recordings were made at day 56 across 5 differentiations. Cells were grown in *Differentiation medium (DM)*, *DM + 1 mM cAMP* or *DM + 15 mM KCl*.
- In E-G: Parameters describing the fluorescent response. Points represent an average of all active peaks within a well so show response of individual wells. Recordings made across 5 differentiations. Significant differences across growth conditions were calculated by ANOVA then Tukey's HSD (*p<0.05, **p<0.01).
- E) The maximum fluorescence intensity in active cells following KCl stimulation.
- F) Area under the curve of fluorescence response to KCl stimulation in active cells.
- G) The time after KCl addition when peak fluorescence intensity is measured in active cells.
- H) The proportion of cells showing a calcium peak in response to KCl addition across different growth medium conditions. Points represent individual wells, recordings made across 5 differentiations.

Finally, calcium imaging was applied to characterise the response of 2D iPSC-cerebellar cultures to stimulation. Mature neurons express voltage-gated ion channels and show a rapid calcium influx via in response to depolarisation (Simms and Zamponi, 2014). iPSC-cerebellar cultures were loaded with Calbryte 590, a fluorescent calcium indicator which provides a readout of intracellular calcium concentration. Calbryte 590 has a high affinity for calcium and on binding calcium undergoes a 300-fold increase in fluorescent intensity (AATbio). After loading iPSC-cerebellar cultures with Calbryte dye, stimulation with 50 mM KCl triggered a rapid increase in fluorescence intensity confirming the presence of active cells (Figure 4.13A). Moreover, the calcium response was most prominent in cells with neuronal morphology (Figure 4.13A). To quantify the response to KCl stimulation, recordings were analysed using segmentation to identify neuron bodies as regions of interest (ROI) (Figure 4.13B). The mean fluorescence intensity of each ROI was then extracted over the 2-min recording period. The average intensity of frames prior to stimulation were used as background fluorescence and

relative fluorescence (df/f_0) was calculated. Next, blank stimulations using PBS were used to determine thresholds for filtering active peaks (Figure 4.13C). Parameters such as the timing and size of the peak in relative fluorescence, could then be calculated for each active ROI. Results for all ROIs in a recording were averaged, so plots show the average response of all active cells in each recording (Figure 4.13D – H).

The intracellular calcium response to KCl stimulation was compared across iPSC-derived cerebellar cultures grown in different conditions: base *DM* and with addition of 1 mM cAMP or chronic 20 mM KCl. These conditions were selected as cAMP increased the rate of spontaneous activity, suggesting it had potential to alter the response to depolarisation. Hypothesising that neuronal activity might promote the maturation of iPSC-cerebellar neurons, cells were also grown in higher KCl causing a chronically depolarising effect. Average traces of active cells per image show iPSC-derived cerebellar cultures responded to KCl in all conditions indicating that neurons expressing voltage-gated calcium channels are present (Figure 4.13D). Unlike for spontaneous activity, no differences were found on addition of cAMP (Figure 4.13D-H). In contrast, cells grown in 20 mM KCl chronically showed a reduced response to acute 50 mM KCl stimulation (Figure 4.13). These observations were reflected in changes in a number of trace parameters: the peak change of fluorescence and area under the curve trended to decrease in cells cultured in KCl (Figure 4.13E & F), whilst the time to peak was significantly delayed (Figure 4.13G). The proportion of cells responding to KCl with a rapid calcium influx varied considerably between wells in all conditions. No difference in percentage of responsive cells was found on addition of cAMP to medium (Figure 4.13H). However, a lower proportion of cells showed activation when cultured in chronic KCl, though this was not significant (Figure 4.13H).

To summarise the neuronal activity experiments, 2D iPSC-cerebellar cultures were shown to develop spontaneous neuronal activity using both MEA and calcium imaging-based assays. iPSC-cerebellar cultures also showed a strong and rapid increase in intracellular calcium when stimulated by KCl, indicating expression of voltage-gated calcium channels. Growth in medium

containing cAMP promoted development of more frequent spontaneous neuronal activity but did not alter the intracellular calcium response to depolarisation.

4.2.5 Evaluation of 2D vs 3D culture of iPSC-derived cerebellar neurons

In this chapter, a simple method for growing iPSC-cerebellar neurons in 2D has been developed, demonstrating expression of cerebellar markers and development of spontaneous neuronal activity. Whilst screening of conditions included a limited number of Purkinje cell markers, in order to more fully evaluate the generation of Purkinje cells, a larger selection of Purkinje cell markers were profiled across finalised conditions. For comparison, day 56 samples were collected from iPSC-cerebellar organoids and 2D cultures, grown in *DM* and with addition of 1 mM cAMP. Gene expression was then quantified by RT-qPCR. The selected gene panel focused on Purkinje cell markers, but also profiled a couple of genes expressed in granule cell precursors and granule neurons (*ATOH1*, *PAX6*) and synaptic markers as examined across 2D conditions previously (Figure 4.10A) to see if there were major cell type population changes or synaptic changes in 3D organoids grown in different medium.

Average gene expression across conditions was summarised as a heatmap, clustering growth conditions and genes based on similar patterns of expression (Figure 4.14A). As described in earlier analysis, the largest differences in gene expression was dependent on physical state, with the dendrogram dividing 3D organoids from 2D dissociated cultures (Figure 4.14A & Figure 4.8A). Purkinje cell genes increased in 2D dissociated cultures were typically expressed later in human Purkinje cell development (*ITPR1*, *CA8*), but also included earlier expressed Purkinje cell marker *RORA* (Figure 4.2B & Figure 4.14A & B). Early Purkinje cell markers (*SKOR2*, *FOXP2*, *FOXP1*, *LHX1*) tended to show higher expression in 3D organoids (Figure 4.14A). These genes are expressed in Purkinje cell progenitors by 9 PCW and maintained at lower levels in older Purkinje cells during normal human cerebellar development (Figure 4.2B). Together these results suggest an increase in Purkinje cell maturity, perhaps stimulated by altered physical environment, in 2D dissociated cultures. Alternatively, the results may be explained by

a loss of neural progenitors and reduction in newly specified Purkinje cells in 2D dissociated cultures compared to 3D organoids.

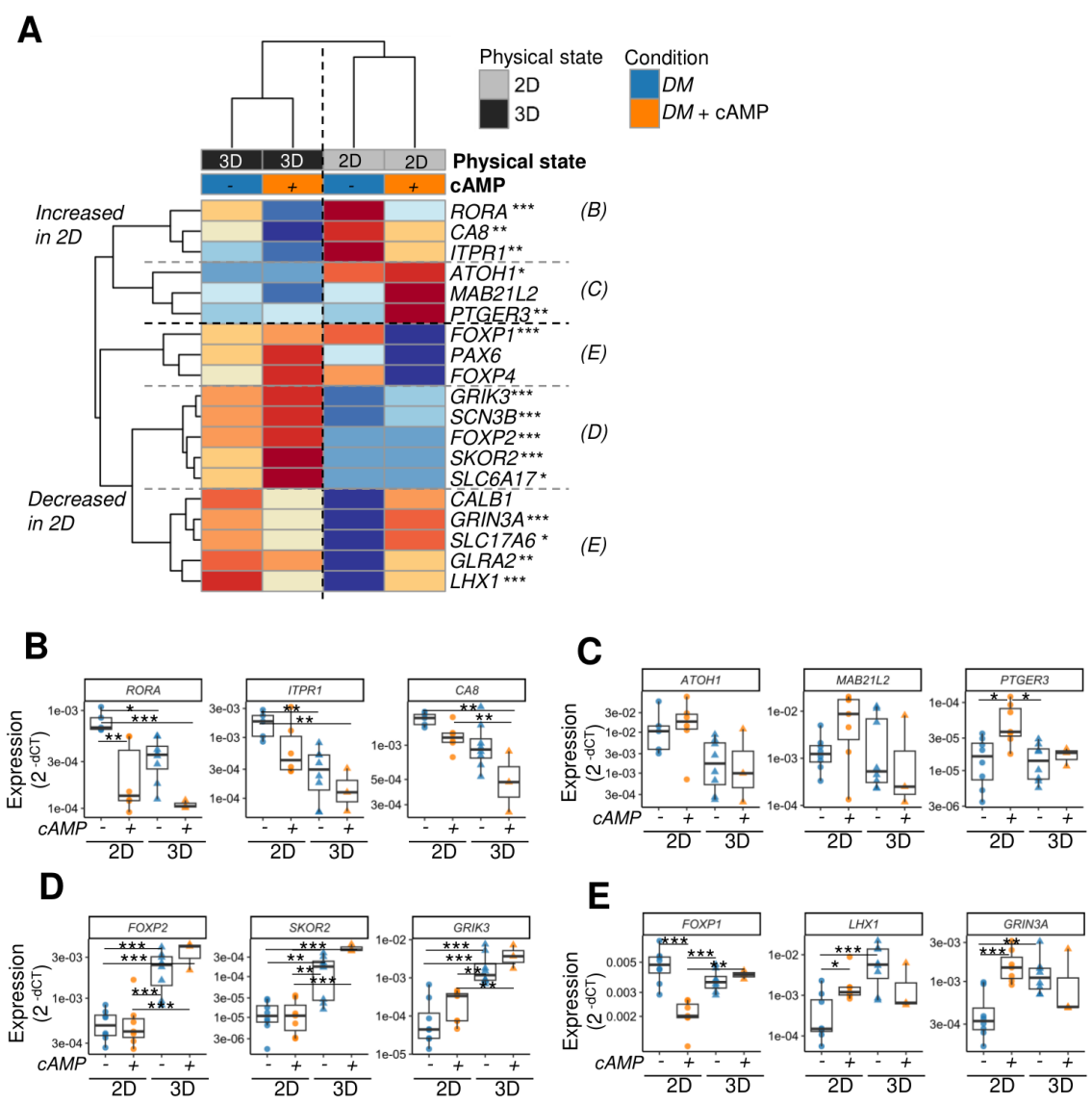


Figure 4.14 Evaluation of culture systems for iPSC-derived Purkinje cells.

The effect of different culture conditions on Purkinje cell generation was evaluated using a large panel of Purkinje cell markers (*CA8*, *ITPR1*, *RORA*, *CALB1*, *FOXP1*, *FOXP2*, *FOXP4*, *LHX1*, *MAB21L2*, *PTGER3*, *SKOR2*). Expression of select granule cell precursor and mature neuron markers (*PAX6*, *ATOH1*) and synaptic markers (*GLRA2*, *GRIK3*, *GRIN3A*, *SCN3B*, *SLC17A6*, *SLC6A17*) was also considered. At day 35 iPSC-derived cerebellar organoids were divided into four conditions: as 3D organoids or dissociated and plated to 2D and either grown in *Differentiation medium* (DM) or DM + cAMP. At day 56 gene expression was measured by RT-qPCR, dCT values were calculated relative to housekeeper genes (*ACTIN*, *GAPDH*). RT-qPCR data for synaptic markers from 2D culture conditions was used previously in Figure 4.10A. *Continued.*

Figure 4.14 Evaluation of culture systems for iPSC-derived Purkinje cells.

- A) Average dCT values for each gene were scaled across condition and displayed as a heatmap. The colour scale shows expression from high expression (red, low dCT) to low expression (blue, high dCT). Significant differences in gene expression across conditions were assessed by ANOVA ($n = 3-8$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$). Genes and conditions were clustered by similarity.

Patterns of gene expression were observed in across different conditions. RT-qPCR data from (A) replotted as 2^{-dCT} , points represent separate differentiations. Significant differences were identified by ANOVA followed by Tukey's HSD ($n = 3-8$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

- B) Purkinje cell markers with increased expression in 2D culture compared to 3D but decreased by addition of cAMP.
C) Genes with increased expression in response to cAMP addition in 2D cultures.
D) Early Purkinje cell markers (*SKOR2*, *FOXP2*) and synaptic markers (e.g. *GRIK3*) had higher expression in 3D organoids than 2D dissociated cultures. No significant effect of cAMP, though trending to increase in both 2D and 3D.
E) Genes with altered expression in response to cAMP dependent on physical state. *FOXP1* was significantly decreased by cAMP addition in 2D, whilst *LHX1* and *GRIK3* are significantly increased by cAMP in 2D but not in 3D.

Patterns of gene expression were then examined in more detail to compare the medium conditions. The cluster of Purkinje cell markers *ITPR1*, *CA8* and *RORA* showed significantly increased expression in 2D dissociated cultures, but decreased expression on addition of cAMP to growth medium in both 2D and 3D (Figure 4.14B). In contrast a second group of genes were expressed most highly in 2D cultures when grown in DM + cAMP (*ATOH1*, *MAB21L2*, *PTGER3*) (Figure 4.14C). Significant changes were only found for *Prostaglandin E receptor (PTGER3)* which is enriched in the cerebellum during human development specifically in Purkinje cells (Sepp *et al.*, 2023), and is later identified as a potential target of *FOXP2* in cerebellar organoids (Chapter 5).

Several markers of early Purkinje cells (*FOXP2*, *SKOR2*, *LHX1*) and synapses (*GRIK3*, *SCN3B*, *GRIN3A*) showed significantly higher expression in 3D cerebellar organoids than 2D dissociated cultures (Figure 4.14D & E). Flow analysis of the *FOXP2*-mNeon+ population reflected these changes in bulk expression. A lower percentage of *FOXP2*+ cells was found in 2D cultures than 3D (0.65% of live cells in 2D with cAMP and ~3% in 3D (Figure 4.7F & Figure 5.5D)), suggesting the changes in bulk gene expression of *FOXP2* result from a decrease in the proportion this cell type. 2D cultures typically had lower expression of neuronal activity and synaptic related genes

(*GRIK3*, *SCN3B*, *GRIN3A*, *GLRA2*, *SLC17A6*) which may reflect the reduced number of cell-cell contacts than in organoids (Figure 4.14D & E).

For Purkinje cell markers, *SKOR2* and *FOXP2*, addition of cAMP caused an increased trend in expression across both 2D and 3D states, but no significant differences were found (Figure 4.14D). Similarly, previous quantification of the mNeon+ population found an increase in the proportion of *FOXP2*+ cells on addition of cAMP in 2D dissociated cultures (Figure 4.7F).

Interestingly, the effect of cAMP on 3D organoids was not always consistent with the effect on 2D dissociated cultures, such as decreasing trends in expression of *LHX1* and *GRIN3A* in 3D despite increasing in 2D on cAMP addition (Figure 4.14E). Therefore, further characterisation should be performed before applying medium conditions optimised for one physical culture state to another.

In conclusion, different methods for growing iPSC-cerebellar cultures influence the gene expression and functional activity. Therefore, compromises may be necessary to study genes of interest, for example 2D culture in *DM* with increased KCl appeared optimal for expression of *ITPR1*, whereas measuring neuronal activity would be aided by addition of cAMP to the culture medium.

4.2.6 Future optimisation approaches informed by single-cell transcriptomic data

Factors selected for screening in this analysis were based on the literature about known signalling pathways involved in Purkinje cell development, identified in rodent cerebellar *in vivo* and *in vitro* studies. However, recent publication of multiple snRNA-seq datasets from the developing human cerebellum provide new resources for identifying important signalling (Aldinger *et al.*, 2021; Sepp *et al.*, 2023). In addition, the development of methods such as CellChat allow prediction and analysis of signalling networks from snRNA-seq data.

To apply a scRNA-seq informed approach to cerebellar culture optimisation, CellChat analysis was performed to identify cell-cell communication networks in developing human cerebellum (Figure 4.15A). For preliminary analysis, a single timepoint of 17 PCW was chosen. At this time point endothelial cells were found to be the dominant source of signalling in the developing

human cerebellum (Figure 4.15A). Incoming signalling to each cerebellar cell type was summarised by pathway, allowing the relative strength of different pathways to be compared (Figure 4.15B). To identify potential soluble factors that could be added to the medium to aid Purkinje cell development, incoming signalling pathways to Purkinje cells were filtered for secreted signalling. Interestingly, the strongest Purkinje cell secreted signalling input pathways were neurotrophin (NT) and insulin growth factor (IGF), which have been previously discussed in playing a role in Purkinje cell development (Figure 4.15C & D). Next, expression of receptors predicted to bind important secreted signalling molecules on Purkinje cells, was examined in day 90 cerebellar organoids (Nayler *et al.*, 2021)(Figure 4.15E). Most receptors showed expression in cerebellar organoids suggesting addition of their corresponding ligand may aid Purkinje cell differentiation, in particular for the most highly expressed receptors addition of WNT5A/B (FZD3), IGF2 (IGFR1 & 2) and NT-3 (NTRK2) (Figure 4.15E).

In addition, genes encoding the T3 receptors (*THRA*, *THRB*) were included in this analysis as hypothyroidism, a lack of T3 signalling, causes stunted Purkinje cell dendritic growth (Heuer and Mason, 2003; Kapfhammer, 2004). Several mouse models have demonstrated that it is the presence of unbound thyroid hormone receptors in the absence of T3 that disrupts Purkinje cell dendritic development (Hashimoto *et al.*, 2001; Morte *et al.*, 2002). Both T3 receptors were expressed in cerebellar organoids, with *THRA* present at a higher level (Figure 4.15E). Given the absence of T3 in current medium conditions (*DM*), the presence of unbound *THRA* may negatively impact Purkinje cell dendritic development in cerebellar organoids.

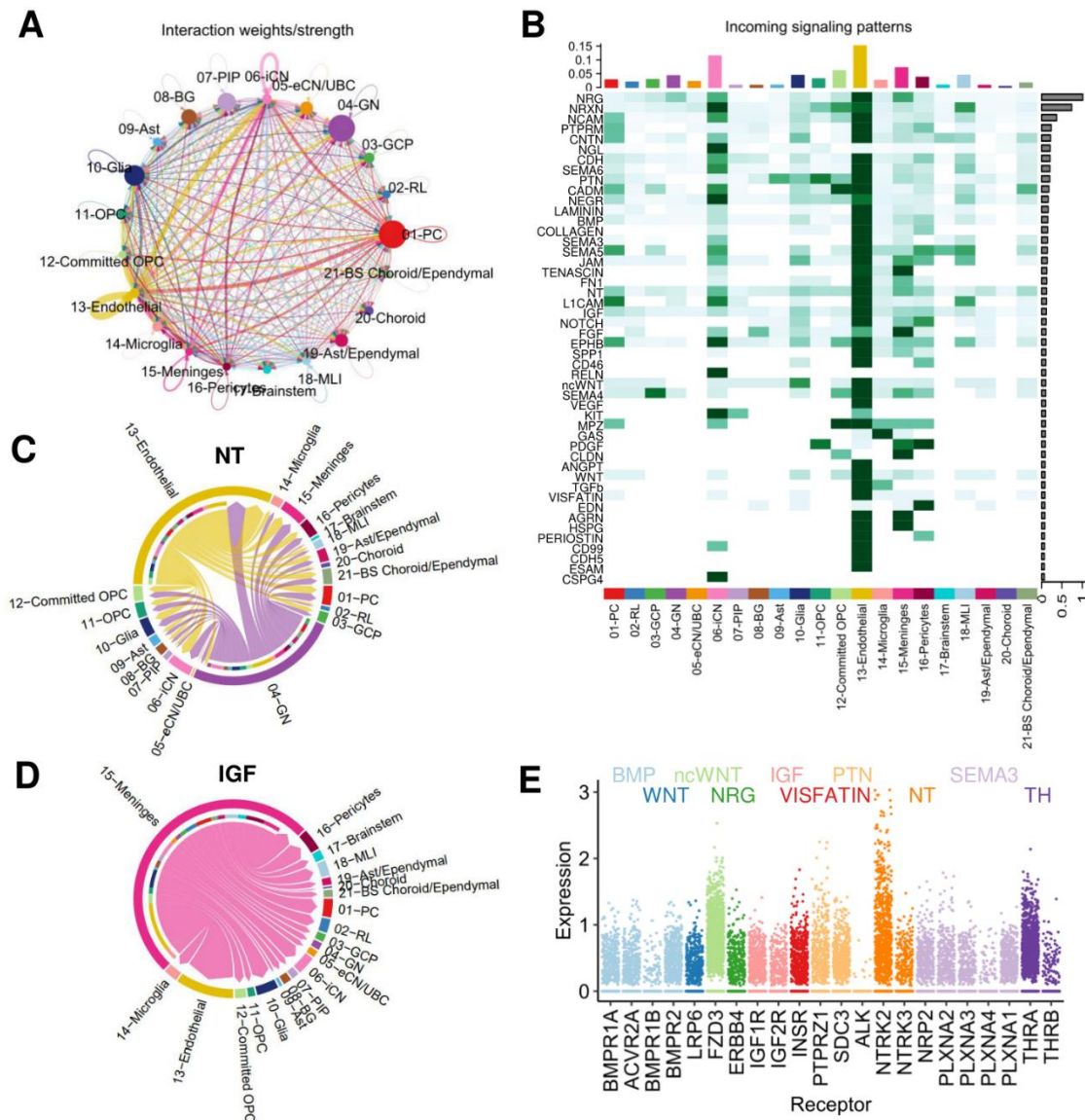


Figure 4.15 CellChat analysis of Purkinje cell input signalling in the developing human cerebellum highlights future avenues for further protocol optimisation.

A) CellChat analysis infers intercellular signalling, using expression of ligands, receptors and cofactors to build a probability-based model of cell-cell communication. An aggregate cell-cell communication network was generated for 17 pcw human cerebellum, using scRNA-seq data from Aldinger *et al.*, 2021. Nodes show different cell types and relative size indicates number of cells. Edges show summed signalling interactions from each cell type, coloured by the sender cell type and thickness is proportional to interaction strength.

Aldinger *et al.*, 2021 cell clusters: PC, Purkinje cells; RL, Rhombic lip; GCP, Granule cell progenitors; GN, Granule neurons; eCN/UBC, Excitatory cerebellar nuclei neurons/Unipolar brush cells; iCN, Inhibitory cerebellar nuclei neurons; PIP, PAX2+ interneuron progenitors; BG, Bergmann glia; Ast, Astrocytes; OPC, Oligodendrocyte precursor cells; MLI, Molecular layer interneurons; BS, Brainstem.

Continued.

Figure 4.15. CellChat analysis of Purkinje cell input signalling in the developing human cerebellum highlights future avenues for further protocol analysis.

- B) Heatmap showing incoming signalling patterns across cell types. Pathways listed in order of decreasing contribution to overall signalling network. Colour intensity indicates relative strength of signalling pathway. Communication by different methods: secreted signalling, ECM-receptor, cell-cell contact and non-protein signalling. The strongest secreted signalling pathways inputting to Purkinje cells are shown in C and D.
- C) Chord diagram for the neurotrophin (NT) pathway shows signalling predominantly from granule neurons (04-GN) and endothelial cells (13-Endothelial). Outer circle sector sizes reflect contribution of that cell type to NT pathway signalling. The inner bar represents the receiving cell types, size proportional to the strength of signal received.
- D) Chord diagram for the IGF pathway with signalling predominantly from the meninges (15-Meninges).
- E) Cerebellar organoid expression of receptors identified as important in receiving secreted signalling pathways in human Purkinje cells. scRNA-seq data from day 90 cerebellar organoids from Naylor *et al.*, 2021. Points show expression in single cells, all cell types combined. Receptors were identified by CellChat as receiving secreted signalling factors in Purkinje cells. In addition, T3 receptors THRA and THRB were included based on literature interest (Kapfhammer, 2004). Colours indicate grouping of signalling pathways.

A preliminary assessment of the effect of T3 on 3D cerebellar organoids was examined using the FOXP2-P2A-mNeon reporter line. From day 35, cerebellar organoids were grown in *DM* alone or supplemented with either 3 nM T3 or 1 mM cAMP. After two weeks (day 49), organoids were dissociated and the mNeon+ Purkinje cell population quantified using flow cytometry (Figure 4.16A). Organoids grown from a control iPSC line were used as a negative control for mNeon fluorescence and unstained cells used to set the DAPI- gate for live cells (Figure 4.16B). For each differentiation, the mNeon+ population in treated samples was normalised to the mNeon population size in organoids grown in *DM* (Figure 4.16C). Addition of T3 was found to significantly increase the proportion of the mNeon+ population in cerebellar organoids by around 1.5-fold (Figure 4.16C). Surprisingly, in contrast to in 2D cerebellar cultures, addition of cAMP significantly decreased the relative mNeon+ population to around half that in *DM* (Figure 4.16C & Figure 4.7F). This contradicts RT-qPCR analysis which found no significant difference in *FOXP2* expression in cerebellar organoids following three weeks growth with cAMP (Figure 4.14D). Further characterisation of T3 treated cerebellar organoids is needed to verify the effect on increasing the Purkinje cell population, including testing other

marker expression. However, this approach is promising for further development of the protocol for growth in 3D.

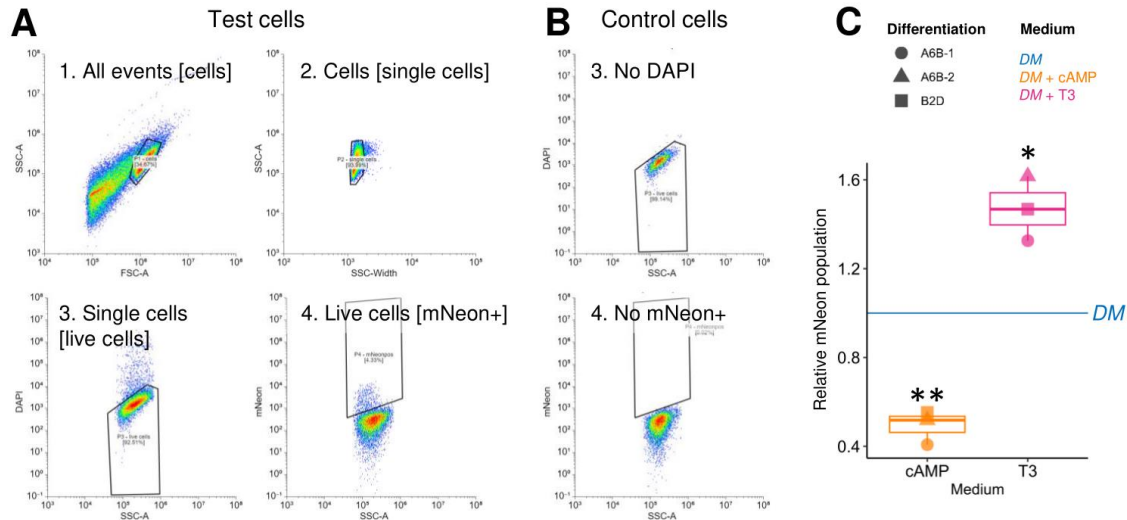


Figure 4.16 Addition of T3 increased FOXP2+ population in cerebellar organoids.

Cerebellar organoids were generated from the FOXP2-P2A-mNeon line (two differentiations with clone A6B, one with clone B2D). From day 35 to 49, organoids were grown in *Differentiation medium* (DM) or DM supplemented with 1 mM cAMP or 3 nM T3. At day 49, organoids were dissociated and analysed by flow cytometry to quantify the FOXP2+ population.

- Flow cytometry gating workflow first selected cells from debris based on visible light scattering (1). Next single cells were gated based even side and forward light scatter (2). DAPI staining was used to exclude dead (DAPI+) cells (3). Finally, the population of mNeon+ cells was quantified (4). Each plot shows only events selected in the previous.
- To set the gates used in (A) negative control samples were measured. A sample of cells were not stained with DAPI to give position of the DAPI- live cell population (3). Organoids generated with control iPSCs (WTC11) were used to set the threshold for mNeon+ (4).
- Per differentiation, the percentage of mNeon+ cells in each treatment was normalised to the mNeon+ population in the organoids grown in DM only. Growth of cerebellar organoids in cAMP decreased the mNeon+ population, whilst growth in T3 increased the mNeon+ population. To determine if each treatment significantly altered the relative mNeon population a one sample student's T-test was performed (* $p < 0.05$, ** $p < 0.001$).

4.3 Discussion

Published protocols for the differentiation of iPSCs into cerebellar organoids generate cerebellar neuron progenitors from both germinal zone lineages within 35 days (Muguruma *et al.*, 2015; Van Essen *et al.*, 2022). However, it has been challenging to progress from this point to model mature cerebellar neurons. This chapter tested different approaches for the growth of iPSC-derived cerebellar cultures, aiming to generate more mature neurons, in particular Purkinje cells. An adapted approach for 2D feeder-free culture of iPSC-derived cerebellar cells generated mixed cultures, including Purkinje cells, granule cells and glia, and showed development of spontaneous neuronal activity. In comparison to long-term culture in 3D, these dissociated iPSC-cerebellar cultures showed higher expression of later Purkinje cell markers (*ITPR1*, *CA8*, *RORA*), though at the cost of reduced expression of early Purkinje cell markers (*SKOR2*, *FOXP2*), suggesting the method promoted Purkinje cell maturity.

4.3.1 Improved conditions for 2D culture of iPSC-cerebellar neurons

Following a recently published protocol (Silva *et al.*, 2020), 2D feeder-free cultures of iPSC-cerebellar neurons could be maintained in culture for several weeks and expressed cerebellar markers. Whilst Silva *et al.*, 2020 relied on supplemented BrainPhys medium, *DM* was also found to support long term 2D iPSC-cerebellar cultures and offered a cheaper alternative with known composition. To further develop the 2D iPSC-cerebellar culture approach, different factors were screened for enhanced Purkinje cell production and maturation.

The most significant improvements across multiple assays were produced by the addition of cAMP. Growth in the presence of cAMP improved formation of neuronal morphology, expression of synaptic markers and development of spontaneous neuronal activity in iPSC-cerebellar neurons. cAMP is beneficial across many neuronal culture types, it is commonly included in iPSC-derived neuronal differentiation protocols and similar improvements have been reported primary neuron cultures (D'Mello *et al.*, 1993; Dugan *et al.*, 1999; Lepski *et al.*, 2013). However, the mechanism of action is difficult to determine, as cAMP interacts with a complex network of signalling pathways (reviewed by Sassone-corsi, 2012). First, cAMP activates protein kinase A (PKA) which then phosphorylates and activates a large number of

downstream targets, including transcription factor CREB (cAMP response element binding protein). CREB in turn regulates the expression of a large number of target genes that act in neuronal differentiation, including neurotrophins, neuronal transcription factors and immediate early genes (Sakamoto, Karelina and Obrietan, 2011). Second, cAMP can act on guanine nucleotide exchange factor EPAC, which activates small GTPases such as Rap1. Activation of MAPK signalling by cAMP via a B-raf/Rap1 pathway has been shown to increase neuronal survival (Dugan *et al.*, 1999). Finally, cAMP regulates cyclic-nucleotide-gated ion channels. These channels conduct calcium, activating a network of calcium-dependent kinases, and other cations thereby regulating voltage-sensitive channels. This cAMP-mediated increase in calcium currents was also found to be required for improved differentiation of rat neural progenitors *in vitro* (Lepski *et al.*, 2013). In summary, in iPSC-derived cerebellar cultures, cAMP may act by directing gene expression towards neuronal differentiation or increasing the survival of more mature neurons when formed.

Interesting, in 2D iPSC-cerebellar cultures there was some evidence for neuron type-dependent effects of cAMP. No significant changes in markers of granule cell precursors and granule neurons *ATOH1* or *PAX6* were detected in cAMP treated 2D iPSC-cerebellar cultures. Instead cAMP treatment increased the *FOXP2*+ population and expression of early Purkinje cell markers, such as *LHX1* and *PTGER3*. In contrast later Purkinje markers, such as *CA8*, *RORA* and *ITPR1* were decreased. Therefore, it appears that cAMP aids growth of early Purkinje cells in 2D iPSC-cerebellar cultures but may limit maturation of Purkinje cells. Following this hypothesis, in the future it would be interesting to test whether an initial period of culture with cAMP to boost the more immature Purkinje cell population (expressing *FOXP2* & *LHX1*), followed by growth without cAMP could result in a larger population of mature Purkinje cells (expressing *CA8* & *ITPR1*).

Another factor of interest for improving iPSC-cerebellar cultures, was addition of ACM or co-culture with iPSC-derived astrocytes. Observation of cultures grown in ACM suggested that astrocyte-secreted factors induced proliferation or improved cell survival. However, expression of cerebellar marker genes found no significant benefit to Purkinje cell generation or maturity.

Previously, astrocytes have shown significant benefits in promoting survival of purified primary Purkinje cells (Yuzaki, Mikoshiba and Kagawa, 1993; Baptista *et al.*, 1994). However, the iPSC-derived cerebellar cultures consisted of a mixture of cell types with only a small (~0.5-3%) Purkinje cell population based on FOXP2+ cell quantification and already contained some GFAP+ glial cells. Astrocyte co-culture or conditioned medium has been shown to increase neuronal maturity and activity in some iPSC-derived neuronal systems (Hedegaard *et al.*, 2020). However, no increase in markers of later Purkinje cells, neural stem cells (NESTIN) or mature neurons (MAP2) markers was detected. The effect of astrocyte co-culture or ACM on neuronal activity in iPSC-derived cerebellar cultures was not fully completed and preliminary results were mixed, so no conclusions were possible. One consideration for future studies of astrocyte co-culture is the phenotype of the astrocytes used. In primary cultures, conditioning medium with cerebellar-derived astrocytes increased Purkinje cell survival more than astrocytes derived from other brain regions (Yuzaki, Mikoshiba and Kagawa, 1993). Currently iPSC-derived astrocytes, including those used in this chapter, recapitulate astrocytes from the cerebral cortex.

4.3.2 iPSC-cerebellar neurons develop spontaneous neuronal activity

One challenge in the use of 3D organoid-models is the application of assays that have been developed for 2D-systems. For example, plate-based fluorescent assays sensitive to cell number, and multi-electrode array recordings where physical contact is important. Consequently, another advantage of developing a 2D culture approach for iPSC-cerebellar neurons is the ability to perform different assays. This chapter focused on assays for measuring neuronal activity, detecting the development of spontaneous neuronal activity and the presence of active cells responsive to depolarisation in iPSC-cerebellar cultures.

Generation of action potentials and associated intracellular calcium spikes is associated with neuronal maturity and is an important demonstration that functional neurons are generated. MEA and calcium imaging-based assays recorded the development of spontaneous neuronal activity, around two weeks after the dissociation and plating of cells, across multiple separate cerebellar differentiations. Thus, demonstrating that functional activity is consistently

generated using this protocol. In line with previous observations of morphology and synaptic marker expression, frequency of neuronal activity was increased by the addition of 1 mM cAMP in culture medium. In this chapter, development of spontaneous activity was observed slightly earlier than reported in previous studies. Using patch-clamp recording, spontaneous activity was reported in co-cultures of iPSC-derived Purkinje cells with mouse rhombic lip progenitors between days 101-108 (Muguruma *et al.*, 2015), and in iPSC-cerebellar neurons grown in BrainPhys from day 56 (Silva *et al.*, 2020). Both GABAergic and glutamatergic synapses were shown to contribute to this activity in iPSC-cerebellar neurons grown in BrainPhys (Silva *et al.*, 2020) so it would be interesting to see if this finding also applies in *DM* + cAMP medium and the relative synaptic contributions.

In this chapter, neuronal activity was compared across only a few growth conditions. Nevertheless, the SNA assay could be applied to comparison of different cell lines or media compositions. The plate-based format facilitates screening multiple conditions at once, and the automated imaging and analysis requires limited experimenter time. As a result, this assay could provide a useful tool for studying the effect of patient mutations on neuronal activity and testing whether electrical dysfunction may underlie cerebellar disease mechanisms. However, to apply the SNA assay to screening cerebellar cultures in this way further optimisation would be required, as high batch-to-batch variation was observed in the number of active objects despite a consistent number of cells being seeded. The variability in number of active objects may reflect differences in cell survival after plating in which case normalisation to a cell density readout could be included. Another explanation is variation in neuron differentiation efficiency, this could be assessed by immunostaining for neuronal markers after recordings had been completed.

One limitation of the assays performed in this chapter is that the neuronal activity observed in the 2D iPSC-cerebellar cultures was not linked to specific cell types within the mixed population. Based on staining with antibodies for markers of specific neuron types, such as FOXP2 for Purkinje cells and PAX6 for granule cell precursors and granule neurons, these neurons make up a very small percentage of the total culture. In the future, single cell

methods such as calcium imaging or patch clamp electrophysiology could be combined with fluorescent imaging of the FOXP2-P2A-mNeon reporter line to characterise neuronal activity specifically within the *FOXP2*⁺ Purkinje cell population. *In vivo*, Purkinje cells are tonically active with a characteristic simple spike firing pattern that increases in frequency over the first few postnatal weeks to a frequency of 40-50 Hz (Arancillo *et al.*, 2014). This characteristic activity has not been replicated in iPSC-derived models and is likely to be challenging to reproduce, however, some comparative repeated intrinsic firing would indicate the generation of a good Purkinje cell model.

4.3.3 Challenges in screening culture conditions and future approaches

When screening different culture factors, the main challenge was selecting useful readouts given variability between differentiations. Methods such as RT-qPCR and Western blotting quantify bulk gene and protein expression, so expression changes in a small population of cells can be masked by the noise present across the whole population. This problem was illustrated by *FOXP2*, which was expressed in less than 1% of cells in 2D iPSC-cerebellar cultures. Addition of cAMP increased the number of *FOXP2*⁺ cells, as quantified by imaging and flow cytometry using the FOXP2-P2A-mNeon reporter line. However, no significant changes in expression of *FOXP2* mRNA were detected. In addition, bulk measures cannot distinguish between changes in cell type composition and levels of expression within certain cells. Therefore, few significant differences in expression were found using these techniques, and larger changes could only be detected when multiple genes were combined in PCA analysis. Given the heterogeneous mix of cell types present in cerebellar organoids, bulk expression measures do not give sufficient resolution to detect more subtle phenotypes. Therefore, single-cell based approaches, such as flow cytometry, are required for further investigation.

A related challenge was the selection of genes to consider. As the focus of optimisation was the generation of Purkinje cells, screening predominantly relied on Purkinje cell markers, with a couple of granule cell markers and several synaptic markers. Whilst this panel of markers was chosen to be as selective to cell types as possible, many genes are also expressed in different cell types and brain regions. For example, CALB1 is a commonly used Purkinje cell marker,

however it also showed expression in non-neuronal cells. RNA-seq may be required in the future to fully characterise the 2D iPSC-cerebellar cultures, giving a more comprehensive picture of the genes expressed and cell types represented.

The FOXP2-mNeon reporter line was a useful tool for screening conditions that increased the FOXP2⁺ population. The FOXP2⁺ population could be quantified by counting mNeon⁺ cells in images or by flow cytometry. An advantage of flow cytometry was the accurate quantification of the total live cell number in addition to fluorescent cells. However, flow cytometry requires dissociation of cells so screening multiple conditions is time-consuming. In contrast, a plate-based imaging system has potential to be higher throughput, enabling screening more conditions. This would benefit from addition of a nuclear dye to quantify total cell number and automated counting of fluorescent cells. One disadvantage of using a reporter line for screening is the reliance on a single marker which may also be expressed in other cell types, leading to accidental selection of a non-desired cell type. Therefore, it was important to also characterise expression of other Purkinje markers. In the future, a combination of reporter lines may aid further protocol development.

In this chapter, optimisation focused on the addition of single factors. However, with more replicable readouts, combinations of factors could be tested in combination and their individual effects separated by use of linear equations as demonstrated for iPSC-derived microglia (Washer *et al.*, 2022). This could help identify the best combination of factors, since when tested individually survival of more mature neurons may be the limiting factor. Instead a mixture of factors that improve neuronal survival more generally and others that direct Purkinje cell maturation may be required.

The use of transcriptomic data to identify signalling pathways active in *in vivo* human cerebellar development can aid future optimisation of the cerebellar organoid differentiation protocol. This approach has been used successfully to inform improvements to human intestinal organoids (Childs *et al.*, 2023). Analysis of fetal intestinal scRNA-seq suggested altering EGF signalling to more accurately recapitulate the *in vivo* intestinal stem cell niche, and this resulted in more complex budded intestinal organoids with crypt and luminal domains

(Childs *et al.*, 2023). Based on analysis of 17 PCW human cerebellar data, activation of Wnt IGF2 and NT-3 signalling in iPSC-cerebellar cultures warrants investigation. Preliminary testing of T3 addition to 3D cerebellar organoid cultures increased the proportion of FOXP2+ cells. This finding requires further testing but is promising for identification of factors to use in 3D organoid culture to improve Purkinje cell generation in this setting. Interestingly T3 was included in the screen of factors to improve Purkinje cell generation in 2D cerebellar cultures in this chapter and no significant changes seen. However, readouts largely focused on the number of Purkinje cells rather than their morphology, which has been impaired in models of hypothyroidism, so future optimisation could also consider re-examining the potential effect of T3 on Purkinje cell dendritic development in 2D.

4.3.4 Selection of conditions for culture of iPSC-cerebellar neurons dependent on purposes

This chapter has developed a new approach to growth of iPSC-cerebellar cultures in 2D and demonstrated a number of advantages such as increased expression of later Purkinje cell genes and the application of assays to measure neuronal activity. However, comparison of 2D and 3D approaches found limitations to both methods, meaning the experimental purpose must be considered when selecting a method and compromises may have to be made. A few considerations in this decision are laid out below.

Gene of interest - Different physical conditions altered the gene expression in iPSC-cerebellar cultures so when studying the effect of a specific gene, one system with higher expression might be preferable. Focusing on Purkinje cells, later markers such as *ITPR1* and *CA8* were increased in 2D whilst early markers were generally higher in 3D (*FOXP2*, *SKOR2*, *LHX1*), although this was not a complete correlation as *RORA* is expressed early human cerebellum and showed increased expression in 2D iPSC-cerebellar cultures. Addition of cAMP to 2D cultures typically increased the expression of early Purkinje cell markers and general neuronal synaptic markers, but decreased expression of the later markers. As a result, in Chapter 5 when studying the role of *FOXP2*, the 3D organoid culture was predominantly used as the

condition with highest FOXP2 expression. However, where 2D culture was necessary for calcium imaging, cAMP was added to the medium to increase the *FOXP2+* population.

Cell type – This work has focused on Purkinje cells and therefore the relative proportions and maturity of other cell types such as granule cells, astrocytes and neural progenitors has not been characterised in detail in the 2D iPSC-cerebellar cultures.

Assays - 2D cultures can be more easily applied to plate-based assays due to control of cell number and cells are more stably attached to a surface such as an MEA plate. This chapter has demonstrated how 2D cultures can enable characterisation of neuronal activity. In addition, the single layer of cells makes imaging easier, both for brightfield observation of single cell morphology and immunostaining. This allowed screening of growth conditions by imaging mNeon+ cells using a widefield microscope. However, the 3D organoids can still be imaged, either by cryo-sectioning into 2D sections or using extended immunostaining protocols to allow antibody penetration and cleared tissue followed by confocal microscopy.

Cell-cell interactions and tissue structure – cerebellar organoids maintain a partially organised cytoarchitecture, with rosette structures and contrast between the outside and inside of the organoids. This provides the opportunity for modelling of tissue organisation and cell migration, which could be potentially extended by use of localised signalling molecule release. Organoids can also be fused with other organoids or cancer spheroids to examine cell invasion. Furthermore, the far greater number of cell-cell interactions in organoids than in 2D more accurately reflects *in vivo* conditions.

Timing - 3D cerebellar organoid cultures are typically more stable than when dissociated and plated to 2D. Cerebellar organoids can be reliably grown for over 90 days, whereas 2D cultures typically show cell detachment and loss over time, so could only be maintained consistently for four weeks after plating. On the other hand, the option to freeze cells from dissociated organoids at day 35 and thaw and plate them later, increases the flexibility of experimental time and reduces the start-up time required.

Co-culture - When plating dissociated iPSC-derived cerebellar cells it is straightforward to combine other cell types. Co-culture was demonstrated for astrocytes, but it could potentially also use other non-neural cells absent from current cultures such as microglia, creating a more complex model of the cerebellar environment. In contrast, seeding additional cells into organoids has not been tested and is likely to be more difficult to incorporate in controlled numbers.

In summary, establishment of a method for 2D growth of iPSC-cerebellar cultures has expanded the repertoire of assays available. The approach chosen for growing post day 35 iPSC-cerebellar cultures should be considered based on the research priorities including those discussed above.

4.4 Key findings

- Following published protocols, iPSC-derived cerebellar organoids were shown to contain cells which express markers of early Purkinje cells such as *FOXP2* and *SKOR2*. However, there was limited induction of markers of later Purkinje cells including *CA8* and *ITPR1*.
- iPSC-derived cerebellar cultures could be grown in 2D feeder-free conditions, whilst maintaining cerebellar identity. In comparison to 3D organoid culture, 2D iPSC-cerebellar cultures showed a decrease in expression of some earlier Purkinje cell markers (e.g. *FOXP2*, *SKOR2*, *LHX1*), but increased expression of more mature genes including *ITPR1* and *CA8*.
- Addition of cAMP to *Differentiation medium* altered the phenotype of 2D iPSC-derived cerebellar cultures. Growth in 1 mM cAMP changed morphology to appear more neuronal, increased the *FOXP2*⁺ population and increased expression of early Purkinje cell markers (e.g. *PTGER3*, *LHX1*).
- 2D iPSC-derived cerebellar cultures were demonstrated to express synaptic markers, develop spontaneous neuronal activity and show a rapid calcium response to KCl-stimulated depolarisation. Expression of synaptic markers and spontaneous activity were increased by addition of cAMP.

Chapter 5. FOXP2 in human cerebellar development

5.1 Introduction

The discovery of a *Forkhead box protein P2 (FOXP2)* gene variant as the monogenic cause of a dominantly inherited speech and language disorder promised greater understanding of human speech (Lai *et al.*, 2001). However, 20 years later much remains to be understood about the function of FOXP2 and how it acts within gene networks to enable functional speech (reviewed by Hoed, Devaraju and Fisher, 2021). Transcription factor, FOXP2, is highly expressed in the developing human cerebellum, particularly in Purkinje cells, neurons central to cerebellar function. Despite evidence for *FOXP2*-related alterations in the cerebellum from individuals affected by *FOXP2*-disorders and mouse models, *FOXP2* research has been more focused on other brain regions more traditionally linked to speech, such as the striatum. In addition, access to samples from the developing human cerebellum is limited and they cannot be experimentally manipulated. As a consequence, little is known about the function of FOXP2 in the developing human cerebellum.

The mouse cerebellum has provided a model to study the timing and location of *Foxp2* expression during development. Within the cerebellum, expression of *Foxp2* was detected earliest at E13.5, with expression during embryonic mouse development in Purkinje cells and a small subset of DCN neurons (Ferland *et al.*, 2003; Lai *et al.*, 2003). FOXP2 staining was found in almost all Purkinje cells up to P6, with the exception of small parts of the flocculus and nodulus (lobule X)(Fujita and Sugihara, 2012). Several recent scRNA-seq studies have confirmed similar enrichment of *FOXP2* expression in developing Purkinje cells in human cerebella (Aldinger *et al.*, 2021; Sepp *et al.*, 2023). Although broadly present, the level of *Foxp2* expression in Purkinje cells has been observed to differ across the cerebellum (Fujita and Sugihara, 2012; Wizeman *et al.*, 2019). In E13.5 scRNA-seq data, expression of *Foxp2* was found in all Purkinje cell clusters, however levels of *Foxp2* expression showed variation which could be related to Purkinje cell subtypes (Wizeman *et al.*, 2019). Similarly in the adult cerebellum, FOXP2 immunostaining showed transverse stripes of fluctuating expression levels (Fujita and Sugihara, 2012). Cerebellar functions show spatial specialisation related to clusters

of cellular phenotypes and connections. Thus, variation in *FOXP2* expression may contribute to the functional patterning of the cerebellum. Furthermore, the impact of *FOXP2* mutations are likely to vary across cerebellar modules and so affect cerebellar functions to differing extents.

Studies investigating *FOXP2* function can be broadly grouped into observation humans with *FOXP2*-disorders, genetic models of *Foxp2* disruption in animals, and molecular studies using cell lines or tissue samples. In the Introduction, the impact of global *FOXP2* disruption in affected human individuals and animal models was described (1.4.3 & 1.4.4, Table 1.1). Cerebellar alterations will be briefly summarised here. MRI and PET imaging studies have identified structural, activity and functional connectivity differences in certain regions of the cerebellum of individuals with *FOXP2* mutations. In particular, lobule VIIa Crus I (hemisphere) and posterior lobules VI, VIIb and VIIIb were altered in individuals with a *FOXP2*-R553H mutation compared to controls (Watkins *et al.*, 2002; Co, Anderson and Konopka, 2020). In mouse models, homozygous *Foxp2*-KO or *Foxp2*-R552H mutations cause more severe cerebellar phenotypes, whilst heterozygous mutations, as present in human patients, show more nuanced differences. Homozygous global *Foxp2*-KO or *Foxp2*-R552H mutations were typically found to cause reduced size and foliation of the cerebellum (French *et al.*, 2007; Fujita *et al.*, 2008; Groszer *et al.*, 2008). Disruption and delay to Purkinje cell dendritic development was also commonly observed, though often resolved to normal morphology by P21 (Shu *et al.*, 2005; Fujita *et al.*, 2008; Groszer *et al.*, 2008). In mice heterozygous for *Foxp2* mutations no differences were found in size or foliation of the cerebellum (Shu *et al.*, 2005; French *et al.*, 2007; Fujita *et al.*, 2008; Groszer *et al.*, 2008). Only one study observed Purkinje cell changes in heterozygous mice, describing more sparse Purkinje cells with delayed dendritic development (Fujita *et al.*, 2008).

Progressing from global *Foxp2* disruption to region-specific modifications, has enabled the role of different brain regions to be examined. Two studies have disrupted *Foxp2* expression specifically in the cerebellum. In the first, *Foxp2* expression was reduced by ~40% from early in cerebellar development using *in utero* electroporation of a small hairpin RNA (shRNA) targeting *Foxp2* (Usui *et al.*, 2018). *Foxp2*-knock down (KD) resulted in impaired Purkinje cell

dendritic development, and led to a reduced righting reflex and USV production (Usui *et al.*, 2018). Moreover, all of these phenotypes could be rescued by addition of WT-*Foxp2* confirming the causative loss of *Foxp2* (Usui *et al.*, 2018). In a second study, L7-driven cre-mediated deletion of *Foxp2* (*Foxp2*-PC-KO), led to complete loss of *Foxp2* expression in Purkinje cells from early postnatal time (French *et al.*, 2019). *Foxp2*-PC-KO mice displayed no changes to cerebellar architecture or Purkinje cell morphology (French *et al.*, 2019). There was also no gross motor phenotypes, for example, no differences in rotarod performance (French *et al.*, 2019). However, *Foxp2*-PC-KO mice showed impairments in more skilled motor tasks, including more missteps during Erasmus ladder tests and a reduced rate of lever pressing (French *et al.*, 2019). These phenotypes were accompanied by an increased simple spike rate and intrinsic excitability in Purkinje cells (French *et al.*, 2019). Purkinje cell activity also showed reduced correlation with wheel velocity in running mice (French *et al.*, 2019). Together, these studies indicate a role for *Foxp2* in dendritic development and normal electrical regulation in Purkinje cells, thus mediating control of skilled motor function and vocal development. The reduced severity of phenotype in *Foxp2*-PC-KO mice compared to the *Foxp2*-KD, may be reflective of the later timing of disruption (French *et al.*, 2019). *Pcp2* (*L7*) is a specific Purkinje cell marker, expressed exclusively in Purkinje cells and retinal bipolar cells, and has a widely used and well characterised promoter (Oberdick *et al.*, 1990). However, *Pcp2* expression begins from E18.5 and is not widespread in Purkinje cells until postnatal (Oberdick *et al.*, 1993). Therefore, the *L7*-cre deletion of *Foxp2* in *Foxp2*-PC-KO mice was not complete until after Purkinje cells have been generated and started early stages of dendritogenesis. In contrast, the shRNA knock-down of *Foxp2* was performed at E12.5 (Usui *et al.*, 2018). From this comparison, it can be inferred that *Foxp2* has an important role in newly differentiated Purkinje cells, and this could be studied using the immature human Purkinje cells present in cerebellar organoids.

As a transcription factor, studies investigating the molecular pathways of FOXP2 have focused on identifying DNA binding sites and target genes. Several studies have performed FOXP2-ChIP to identify FOXP2 regulated genes across different tissue samples and cell types: SH-SY5Y cells (Vernes *et al.*, 2007), foetal human brain and lung (Spiteri *et al.*, 2007) and foetal mouse brain

(Vernes *et al.*, 2011). However, little overlap was found in FOXP2 targets identified across studies, with no genes found to be in common across all three datasets. Despite the lack of consensus targets, FOXP2-ChIP studies have identified some common pathways including neurogenesis and neurite outgrowth (Hoed, Devaraju and Fisher, 2021). Additional studies have identified FOXP2-regulated genes by measuring differential gene expression in the presence or absence of exogenous *FOXP2* expression. However, to date no studies have performed FOXP2-ChIP in the cerebellum so there is a lack of knowledge of FOXP2 targets in developing Purkinje cells. One validated target of FOXP2 in the cerebellum is the gene encoding Contactin-associated protein-like 2 (CASPR2), *Cntnap2*, which has been shown to be important in Purkinje cell dendritic development (Argent *et al.*, 2020). Expression of *Cntnap2* was increased 1.6-fold in the cerebellum of mice homozygous for the *Foxp2*-R552H mutation and therefore lacking FOXP2 activity, suggesting FOXP2 acts to repress transcription of *Cntnap2* in the WT cerebellum (Fujita *et al.*, 2012a). Previously, the ability of FOXP2 to bind to and repress transcription of the human *CNTNAP2* gene had been demonstrated in SHSY-5Y cells, verifying direct FOXP2 regulation (Vernes *et al.*, 2008).

Following the identification of *FOXP2*, a number of other monogenic causes of CAS have been identified in sequencing studies of affected probands and their families (Kaspi *et al.* 2022; Eising *et al.* 2019; Hildebrand *et al.* 2020). Studying the interaction of these speech-associated genes may give insight into more general mechanisms of speech development. Therefore, expression of a CAS geneset will be examined in *FOXP2*+ cerebellar cells to identify co-expressed genes which could act in cooperation with *FOXP2* in the early cerebellum. Cerebellar organoids provide a system for studying mechanisms of a wider range of neurodevelopmental disorders. For comparison to CAS, genesets associated with other cerebellar-related disorders not directly associated with *FOXP2* will also be studied. Cerebellar malformations are typically diagnosed by neuroimaging and the disease mechanisms are largely not understood (Haldipur, Millen and Aldinger, 2022). For example, Joubert syndrome causative genes relate to primary cilia, however it is not clear how this results in vermis hypoplasia (Valente, Dallapiccola and Bertini, 2013). Some individuals with FOXP2-syndrome also display ASD, however this is relatively rare and non-verbal IQ is typically maintained (Morgan *et al.*, 2019). However, there

is increasing discussion of the potential role of the cerebellum in ASD, hypothesising a loss of precise coordination in a range of functions may result in the speech disruption, impaired social behaviour and motor dysfunction observed in ASD. Direct evidence for the role of the cerebellum in ASD includes structural and functional connectivity changes in some affected individuals (Fatemi *et al.*, 2012; Sydnor and Aldinger, 2021). Finally, the cerebellum is impacted by diseases of neurodegeneration, such as Alzheimer's disease and ataxia. These disorders typically develop in adulthood, however, in the case of spinocerebellar ataxias, there is evidence for changes in Purkinje cell development which underly later alterations in Purkinje cell dysfunction and loss (Leto *et al.*, 2016; Kapfhammer and Shimobayashi, 2023).

In summary, *FOXP2* function is interesting to the broader understanding of speech and language, and the development of brain regions involved (Co, Anderson and Konopka, 2020). Molecular investigations have identified some *FOXP2* targets and point to a role in neurogenesis, synaptic formation and cytoskeletal changes (Hoed, Devaraju and Fisher, 2021). However, there is a lack of consensus between these studies and none have been focused on the cerebellum. Only two studies have examined the effect of *Foxp2* disruption specifically in the cerebellum, indicating a particular role for *Foxp2* in early development including Purkinje cell dendritic tree formation (Usui *et al.*, 2018; French *et al.*, 2019). However, the role of *FOXP2* in the early human cerebellum has not been explored. Differentiation of human iPSCs to cerebellar organoids recapitulates features of early cerebellar development, and therefore provides an ideal model for studying the role of *FOXP2* in early human cerebellar development. In this chapter, expression of *FOXP2* in cerebellar organoids is described and the *FOXP2*-expressing population is characterised by RNA-sequencing. Finally, *FOXP2* knock-out iPSCs are used to investigate the effect of *FOXP2* loss on early cerebellar development.

5.1.1 Chapter 5 aims

1. To describe the expression of *FOXP1*, *FOXP2* and *FOXP4* in mouse and human cerebellar development and compare this to expression in human iPSC-derived cerebellar organoids.
2. To characterise the mNeon+ population from FOXP2-P2A-mNeon cerebellar organoids by RNAseq and identify potential targets of FOXP2 in the cerebellum.
3. To examine putative FOXP2-regulated genes and pathways in FOXP2^{-/-} organoids to investigate the function of FOXP2 in early human cerebellar development.

5.2 Results

5.2.1 Expression of *FOXP* genes in cerebellar development

Cerebellar expression of FOXP2 has previously been described through mouse development with FOXP2 immunostaining seen broadly in Purkinje cells between E14.5 and P6 (Fujita and Sugihara, 2012). To confirm these observations, expression of *Foxp* family genes, *Foxp1*, *Foxp2* and *Foxp4*, was first quantified in developing mouse cerebellum by RT-qPCR (Figure 5.1A). All three *Foxp* genes had highest expression levels in embryonic samples, with expression decreasing in the postnatal cerebellum matching previous reports (Figure 5.1A) (Co, Anderson and Konopka, 2020). Next, immunostaining was performed to examine the location of FOXP2 expression in the developing mouse cerebellum. At E15.5 FOXP2 was detected in the VZ where Purkinje cell progenitors are generated, and in migrating Purkinje cells which move from the VZ (apical surface) towards the pial cerebellar surface (basal) to form the Purkinje cell plate (Figure 5.1B). By E18.5, FOXP2 was observed broadly in Purkinje cells that have migrated to form a multi-layered Purkinje cell plate (Figure 5.1C). There were also sparse FOXP2+ cells in the centre of the cerebellum which likely labelled a subset of DCN neurons (Figure 5.1C). At later timepoints FOXP2 expression was found across the Purkinje cell layer, although expression levels decreased in older samples (Figure 5.1D-F). Interestingly, in lateral sections of P7 cerebellum FOXP2 was absent from the most posterior lobule, despite the presence of FOXP4+ Purkinje cells (Figure 5.1D). These observations were consistent with previous descriptions of little or no FOXP2 in a small population of Purkinje cells in the most lateral, rostral and ventral region (Fujita and Sugihara, 2012). CALB1, a commonly used Purkinje cell marker, was co-expressed with FOXP2 in the outer layers of the Purkinje cell plate at E15.5 and E18.5 (Figure 5.1B & C). However, CALB1 was not expressed with FOXP2 in VZ Purkinje cell progenitors (E15.5, Figure 5.1B) or more apically located FOXP2+ cells of the Purkinje cell plate (E18.5, Figure 5.1C) which may represent more immature Purkinje cells.

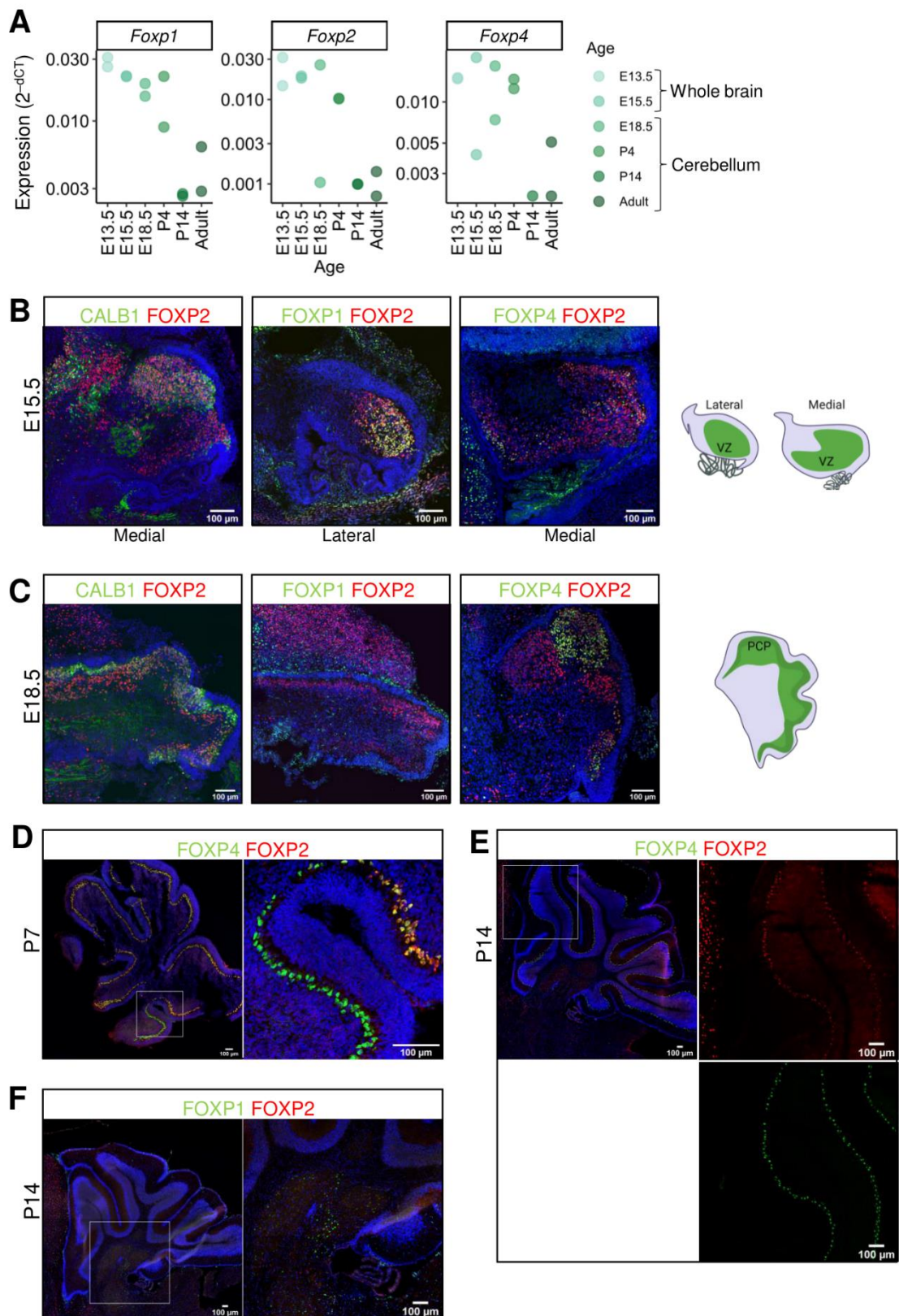


Figure 5.1 *Foxp* family genes are expressed in the developing mouse cerebellum.

Figure 5.1. *Foxp* family genes are expressed in the developing mouse cerebellum.

- A) Expression of *Foxp* family genes was characterised across mouse cerebellar development by RT-qPCR. Expression was calculated as $2^{-\Delta CT}$ relative to housekeeper gene *Gapdh*. At earlier time points (E13.5, E15.5) whole brain samples were used, from E18.5 the cerebellum was isolated for RNA extraction. Points indicate separate biological samples. Time points as embryonic day (E) or postnatal day (P).
- B-F) To examine FOXP protein localisation and co-expression, sections from across cerebellar development were stained with antibodies for FOXP2 (red) with CALB1, FOXP1 or FOXP4 (green). Scale bars = 100 μ m.
- B) At E15.5 FOXP2 is expressed widely in the ventricular zone (VZ) and Purkinje cell population which migrating towards the basal surface. FOXP1, FOXP4 and CALB1 label subgroups of this population. CALB1 and FOXP4 imaged on medial sections, FOXP1 on lateral.
- C) At E18.5 FOXP2 expression in the cerebellum is predominantly localised to the Purkinje cell plate (PCP), with sparse cells in the centre of the cerebellum which may be deep cerebellar nuclei neurons. CALB1 and FOXP4 again label subsets of the Purkinje cells, CALB1 localised to the outer layers and FOXP4 marking distinct clusters. FOXP1 expression is largely absent from the Purkinje cell layer with sparse cells throughout the cerebellum, and expression in the external endothelial cells.
- D) At P7 FOXP2 is present in the majority of Purkinje cells with the exception of the most posterior lobule in lateral sections. Insert location indicated with white box.
- E) At P14 FOXP2 and FOXP4 are expressed widely in Purkinje cells. Expression of FOXP2 is lower than at previous timepoints.
- F) At P14 FOXP1 is only detected in the centre of the cerebellum in a subset of likely DCN neurons.

Differential expression of *Foxp* genes has contributed to defining embryonic Purkinje cell subtypes in transcriptomic analysis of the developing mouse cerebellum (Wizeman *et al.*, 2019; Sepp *et al.*, 2023). Therefore, the co-expression of FOXP1, FOXP2 and FOXP4 was inspected by immunostaining during mouse cerebellar development. In agreement with a published description of *Foxp1* expression at E13.5 (Wizeman *et al.*, 2019), FOXP1 was observed only in more lateral sections at E15.5 and localised to the VZ (Figure 5.1B). At E15.5, in lateral sections FOXP1 and FOXP2 showed high levels of co-expression though some FOXP1-FOXP2+ cells were also present in the anterior cerebellum (Figure 5.1B). FOXP1 was not detected in Purkinje cells at E18.5 or later timepoints, however some expression was observed in the endothelium around the cerebellum and also in central region of the cerebellum, suggesting expression in DCN neurons (Figure 5.1C & F). In contrast, FOXP4 expression was present in both medial and lateral sections at E15.5 and remained expressed over time with high levels in P14 Purkinje cells (Figure 5.1B-E). FOXP4 showed localisation to embryonic

Purkinje cell clusters, particularly at E18.5, where *FOXP4* was present in two posterior and mid anterior clusters, but absent in the most anterior and centrally located *FOXP2*+ Purkinje cells (Figure 5.1C). By P7, *FOXP4* expression appeared more widespread in all Purkinje cells and this was also observed at P14 (Figure 5.1D & E). To summarise, immunostaining confirmed *FOXP2* expression in the majority of developing mouse Purkinje cells across time, though with decreased levels by P14. *FOXP1* and *FOXP4* were also expressed in Purkinje cells. However, *FOXP1* and *FOXP4* showed distinct localisation to different Purkinje cell clusters. Thus supporting previous suggestions that the combination of *FOXP*s present may contribute to defining Purkinje cell subtypes (Wizeman *et al.*, 2019). In addition, sparse *FOXP1* and *FOXP2* cells were present in the central region of the cerebellum indicating expression in subpopulations of DCN neurons.

To determine whether there is a similar expression pattern of *FOXP*s in the human cerebellum, *FOXP* gene expression was examined using published datasets. Expression of *FOXP* family members *FOXP1*, *FOXP2* and *FOXP4* was plotted in snRNA-seq data from the developing human cerebellum (Figure 5.2A) (Sepp *et al.*, 2023). All three genes showed highest expression in the Purkinje cell cluster, with additional expression in the endothelial cell and astroglia clusters (*FOXP1*), and much lower expression in the VZ neuroblast population (*FOXP1* & *FOXP2*) (Figure 5.2A). Purkinje cell expression of *FOXP1* and *FOXP2* peaked at CS22 with *FOXP1* expression considerably decreased by 17 PCW, whilst *FOXP2* expression was sustained throughout the prenatal timepoints (Figure 5.2A). *FOXP4* showed sparser expression in the cerebellum with expression emerging later and increasing over time in Purkinje cells (Figure 5.2A). These expression patterns were confirmed by a separate developing human cerebellar dataset which also identified *FOXP2* as highly and specifically expressed in human Purkinje cells (Aldinger *et al.*, 2021). *FOXP1* expression has been reported to be enriched in an early-born subtype of human embryonic Purkinje cells (Sepp *et al.*, 2023). In light of the localised expression of both *FOXP1* and *FOXP4* in mouse Purkinje cells (Figure 5.1B & C), the expression of *FOXP* genes was plotted across human Purkinje cell subtypes (Figure 5.2B). Sepp *et al.*, 2023 identified two human Purkinje cell subclusters, distinguished as early-born *EBF1/2* low, and late-born *EBF1/2* high (Figure 5.2B). *FOXP2* showed very similar expression across both

Purkinje cell subtypes, indicating broad expression occurs in the human cerebellum as described in mouse (Fujita and Sugihara, 2012) (Figure 5.2B). However, *FOXP1* and *FOXP4* had higher average expression in the early-born subtype (Figure 5.2B).

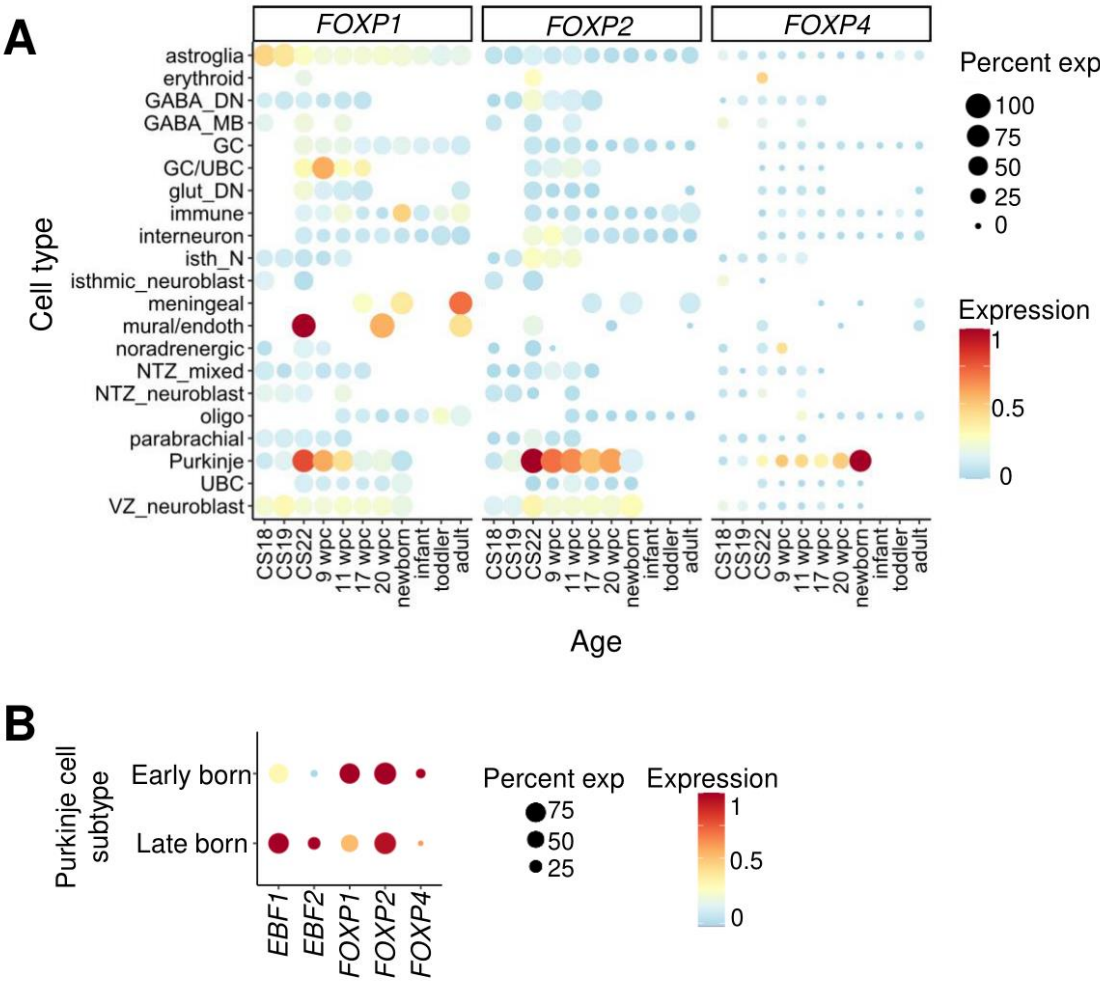


Figure 5.2 *FOXP* family genes are expressed in the developing human cerebellum.

- A) The expression of *FOXP* genes in the developing human cerebellum was plotted across timepoints and cell types. Human cerebellar snRNA-seq data was from Sepp *et al.*, 2023. Point size indicates percentage of cells in cluster with expression and the colour scale shows average expression level for the cluster. Samples with fewer than 10 cells were excluded and left blank.
- B) Sepp *et al.*, 2023 identified two Purkinje cell subtypes distinguished by low and high expression of *EBF1* and *EBF2*, and their timing as early-born or late-born. Expression of the *FOXP* family genes was plotted across Purkinje cell subtypes.

In summary, high levels of *FOXP1*, *FOXP2* and *FOXP4* expression were found in developing Purkinje cells in both mouse and human cerebellum. Immunostaining detected *FOXP2* and *FOXP4* in Purkinje cells broadly across the mouse cerebellum, whilst *FOXP1* showed more lateral localisation. snRNA-seq data from the developing human cerebellum, indicated a similar enrichment of *FOXP* gene expression in developing Purkinje cells. Additionally, in both mouse and human, there was evidence for heterogenous expression of *FOXP1* and *FOXP4* across different Purkinje cell subtypes. Thus, the presence of different combinations of FOXP proteins may contribute to the development of different functional phenotypes in Purkinje cell populations.

5.2.2 Expression of *FOXP* genes in iPSC-derived cerebellar organoids

Differentiation of human iPSCs to cerebellar organoids has previously been shown to recapitulate early stages of cerebellar development (Muguruma *et al.*, 2015; Nayler *et al.*, 2021), and therefore could provide a good model for studying the role of *FOXP2* in the developing human cerebellum. To verify their potential for this purpose, expression of *FOXP* family genes was first characterised in cerebellar organoids. All three *FOXP* genes showed significant upregulation of mRNA expression within the first 21 days of differentiation (Figure 5.3A). Expression of *FOXP2* and *FOXP4* plateaued from around day 49 whilst *FOXP1* continued to increase over the period measured (Figure 5.3A). mRNA levels of *FOXP1* and *FOXP4* were higher than *FOXP2* (Figure 5.3A). Similar trends in FOXP protein levels were confirmed by immunostaining. *FOXP1* and *FOXP4* were observed from day 21 with widespread expression in day 35 cerebellar organoids (Figure 5.3B). *FOXP2* was occasionally present in organoids at day 35, but expression was consistently seen in organoids at day 49 or later (Figure 5.3B). *FOXP2* protein levels as detected by immunostaining therefore appeared later than the timing of the peak of mRNA expression.

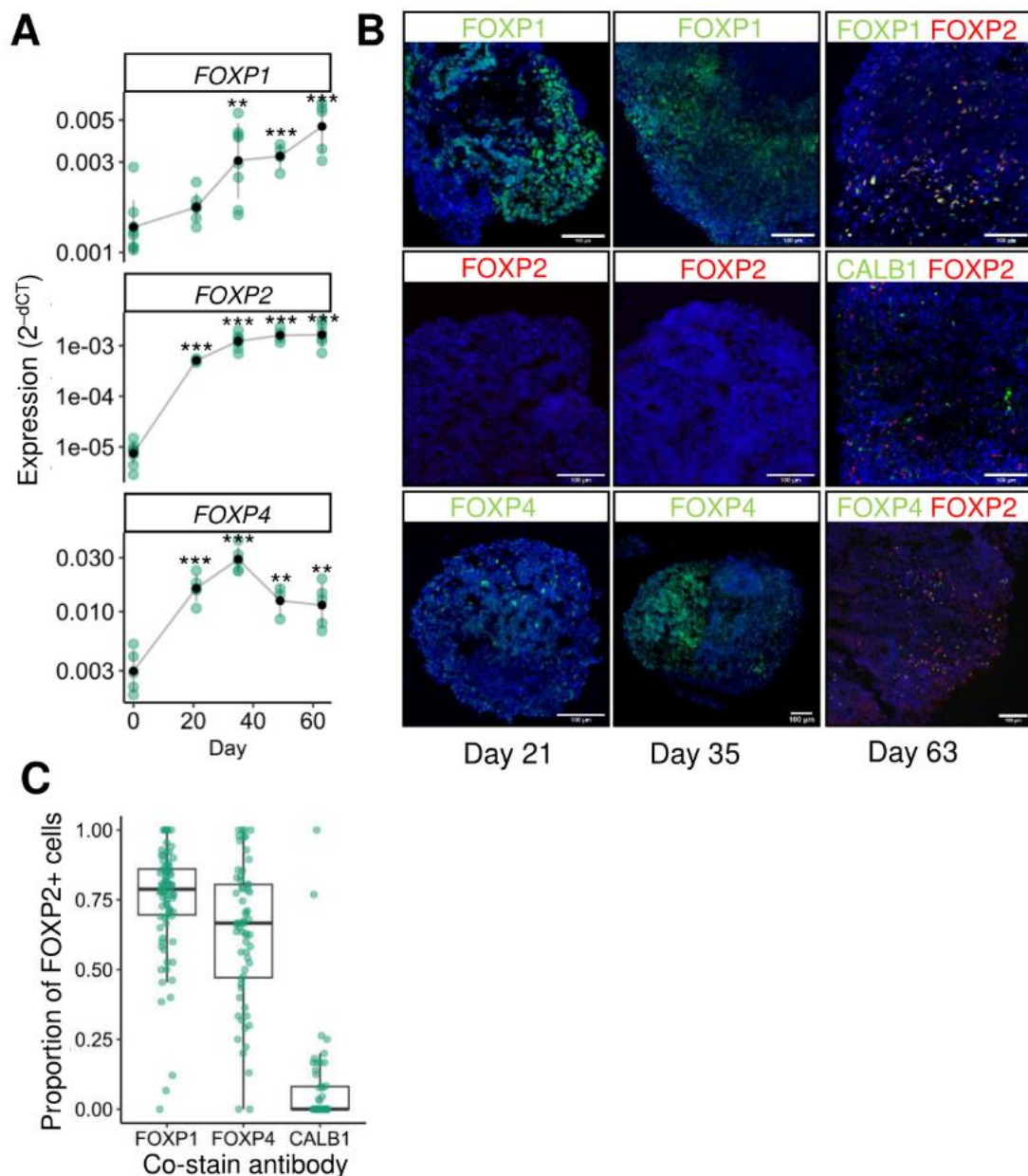


Figure 5.3 Cerebellar organoids show induction of FOXP family genes during differentiation.

- A) FOXP genes significantly increased in expression during cerebellar organoid development. Expression calculated as 2^{-dCT} relative to housekeeper genes (*ACTIN*, *GAPDH*). Significant change in expression compared to day 0 determined using a T-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Points represent individual differentiations, $n = 3-9$.
- B) Imaging of FOXP protein expression across cerebellar organoid differentiation. FOXP1 and FOXP4 could be consistently detected by immunostaining from day 21. FOXP2 was detected from day 49 so signal was absent at day 21 and 35, but present at day 63. Scale bars = 100 μm .
- C) The colocalization of FOXP protein staining was quantified. The majority of FOXP2+ cells in day 63 cerebellar organoids co-expressed FOXP1 and FOXP4, whilst very few expressed CALB1. Points represent separate sections. A minimum of 3 sections were imaged per organoid, for 1-3 organoids per differentiation. Organoids were generated from 5 differentiations.

Formation of dimers is required for FOXP proteins to bind DNA (Li, Weidenfeld and Morrissey, 2004). FOXP protein can form homodimers or heterodimers, and therefore the combination of other FOXP proteins present may influence the function of FOXP2. Additionally, as previously discussed laterally localised expression of *Foxp1* defines a specific subpopulation of Purkinje cells in the developing mouse cerebellum (Wizeman *et al.*, 2019; Sepp *et al.*, 2023). In cerebellar organoids at day 63, FOXP proteins were found to be commonly co-expressed with FOXP2. Of cells staining positive for FOXP2, approximately 75% were FOXP1+ and 63% were FOXP4+ (Figure 5.3C). The presence of both double and single positive cells may be reflective of different Purkinje cell subpopulations in the organoids as occurs *in vivo*. In contrast FOXP2 was rarely co-expressed with CALB in cerebellar organoids (Figure 5.3C). FOXP1 and FOXP4 were also rarely co-expressed with CALB1. This is consistent with immunostaining results in developing mouse cerebellum, where FOXP2+CALB1- cells were observed in early stages the VZ and inner Purkinje cell plate (Figure 5.1B & C). Therefore, suggesting that these cells may reflect more immature Purkinje cells.

To summarise, all three *FOXP* genes were strongly induced during differentiation of human iPSCs to cerebellar organoids as measured by RT-qPCR and immunofluorescent imaging. The function of *FOXP* genes in the early developing human cerebellum can therefore be investigated using iPSC-derived organoids.

5.2.3 Transcriptomic characterisation of *FOXP2*-expressing cells in cerebellar organoids

To investigate the function of FOXP2 in early human cerebellar development, the next aim was to characterise the cellular identity of *FOXP2*+ cells in cerebellar organoids. The *FOXP2* reporter line (FOXP2-P2A-mNeon) generated in Chapter 3 provided a useful tool for investigating FOXP2 function. By fluorescently labelling the *FOXP2*+ cells in cerebellar organoids, the FOXP2-P2A-mNeon line enabled this population to be isolated by flow cytometry (Figure 5.4). The gene expression of *FOXP2*+ and *FOXP2*- populations could then be quantified by bulk RNA sequencing and compared (Figure 5.4).

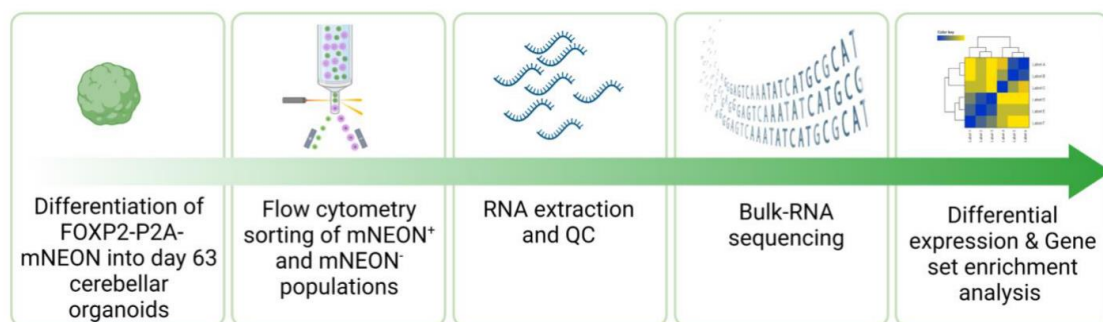


Figure 5.4 Transcriptomic characterisation of *FOXP2*-expressing cells in cerebellar organoids.

Schematic for isolation and transcriptomic analysis of *FOXP2*-expressing cells in cerebellar organoids. The *FOXP2*-P2A-mNeon reporter line was used to grow cerebellar organoids with *FOXP2*-expressing cells fluorescently labelled. At day 63 organoids were dissociated and mNeon⁺ cells isolated by flow cytometry. RNA was extracted from sorted cell populations, checked for quality and sequenced. Differential gene expression and gene set enrichment analysis was performed to characterise the *FOXP2*⁺ cerebellar organoid population.

Cerebellar organoids generated with the *FOXP2*-P2A-mNeon line were harvested at day 63 of differentiation and dissociated to single cells. Day 63 was chosen as a timepoint after *FOXP2* mRNA levels plateau and around the peak of *FOXP2*⁺ cells seen by immunostaining (Figure 5.3B). Next, flow cytometry was performed to isolate mNeon⁺ and mNeon⁻ populations (Figure 5.5). Cells were gated based on light scatter to select cells from debris and exclude clumps (Figure 5.5A & B). DAPI was used to stain dying cells, so DAPI-negative events were selected to sort live cells (Figure 5.5C). From the live cell population, around 3% of cells were isolated as mNeon⁺ (Figure 5.5D). The mNeon gate was set using dissociated control organoids with no mNeon labelling (Figure 5.5D). RNA was then extracted from the sorted mNeon populations and quality control performed before bulk RNA sequencing. All RNA samples used for sequencing had RNA integrity number (RIN) values above 8.9, indicating high quality of RNA and minimal degradation. David Sims performed read pseudoalignment and quantification using Kallisto (Bray *et al.*, 2016). All samples had over 40 million reads successfully pseudoaligned, a good depth of sequencing, providing a global view on gene expression and high statistical power for differential expression analysis. Differential gene expression analysis was conducted using DESeq2 (Love, Huber and Anders, 2014).

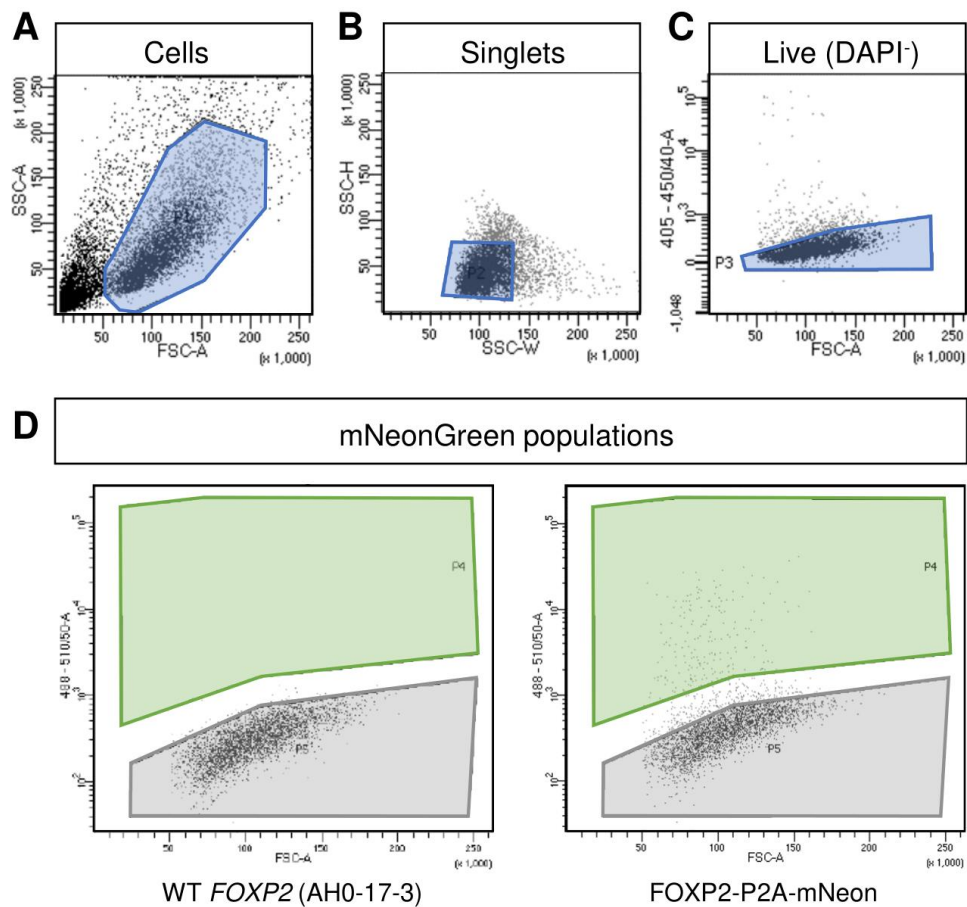


Figure 5.5 mNeon positive and negative populations were isolated from FOXP2-P2A-mNeon cerebellar organoids.

Flow cytometry was used to isolate mNeon+ and mNeon- cells, by a series of gates.

- A) Cells were selected from debris and other particles using a plot of forward scatter (x axis) against side scatter (y axis).
- B) Single cells were then selected based on approximately equal side scatter width (x axis) and side scatter height (y axis).
- C) Live cells were selected based on low signal for DNA-binding dye DAPI (y axis) indicating intact membranes.
- D) Finally mNeon+ and mNeon- populations were selected according to fluorescent intensity from 488 laser excitation (y axis). Gating thresholds were determined using WT cerebellar organoids (left) which had no mNeon expression.

PCA showed clear separation of the mNeon+ and mNeon- samples across PC1 (Figure 5.6A). Indeed, the mNeon population parameter was responsible for 95% variance, with a much smaller effect also visible between individual differentiations in PC2 (Figure 5.6A). The isolation of a *FOXP2*-expressing population was confirmed by examining the differentially expressed genes between mNeon populations. *FOXP2* was significantly increased in the mNeon+ population, with expression over 100-fold higher than in the mNeon- population (Figure 5.6B, *FOXP2* log2FoldChange = 6.82). In total, 2610 genes were significantly increased in the mNeon+ population and 3186 genes were significantly decreased compared to the mNeon- population (adjusted p-value <0.05) (Figure 5.6C). *FOXP* genes *FOXP1* and *FOXP4* also showed significantly higher expression in the mNeon+ samples (Figure 5.6B), consistent with previous co-expression observations from immunofluorescent imaging (Figure 5.3C).

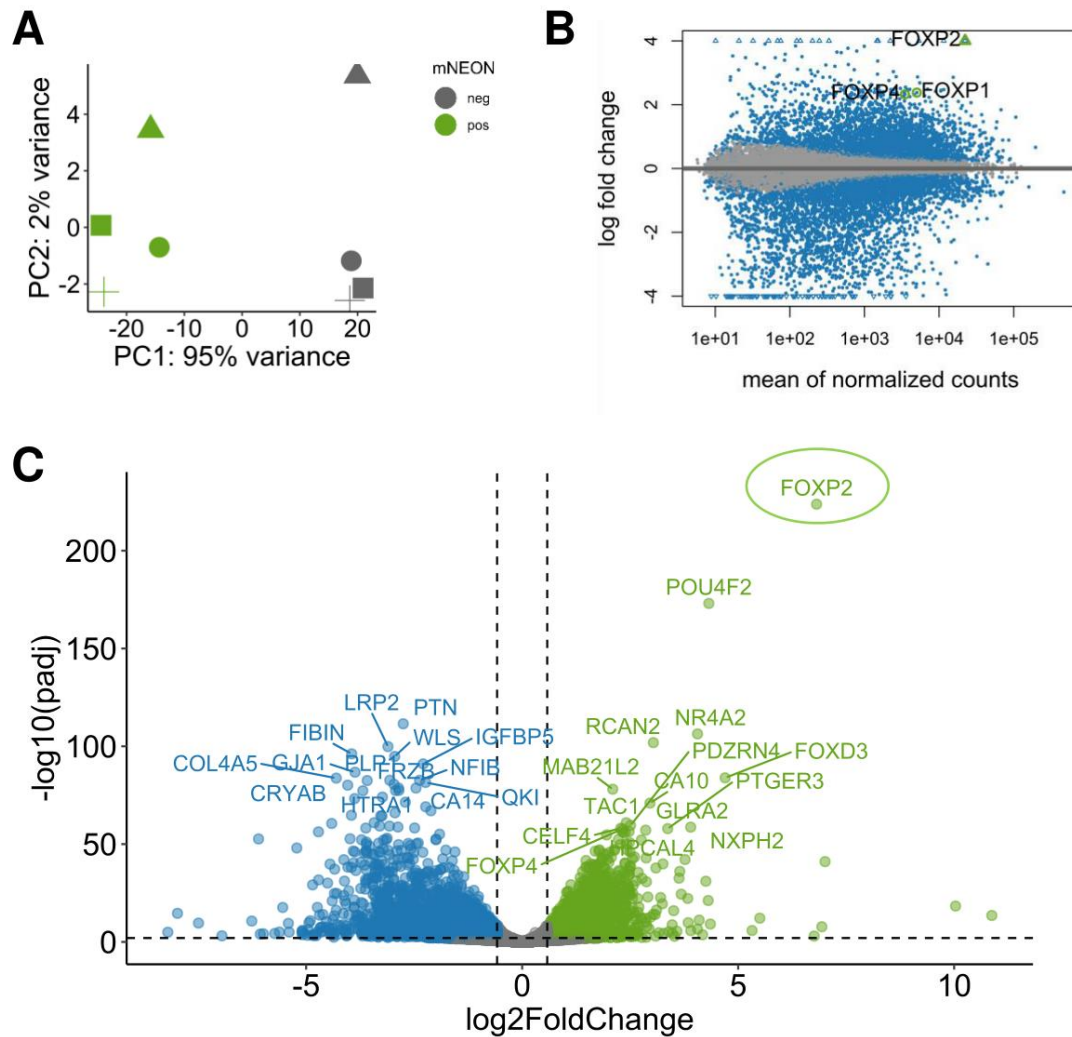


Figure 5.6 Distinct mNeon populations were isolated from cerebellar organoids with strong enrichment for *FOXP2* expression in the mNeon+ samples.

- mNeon+ (green) and mNeon- (grey) populations showed clear separation across the x axis, with PC1 accounting for the majority of variance between samples. Individual differentiations shown by different shapes contribute a small amount of variance across PC2 (y axis).
- DESeq2 was used to identify differentially expressed genes. In the MA plot, log₂FoldChange (M) is shown on the y axis and average expression (A) on the x axis, so genes above the x axis are increased in mNeon+ samples and genes below the x axis decreased in mNeon+ samples. Significantly differentially expressed genes are shown in blue (adjusted p-value < 0.05) and genes not significantly differentially expressed are shown in grey. *FOXP* genes are labelled and highly enriched in the mNeon+ samples. 2610 genes were significantly increased in mNeon+ population and 3186 genes significantly decreased
- Expressed gene were plotted showing differential expression between mNeon populations (log₂FoldChange) across the x axis and increasing significance (-log₁₀(adjusted p-value)) on the y axis. The 15 most significant genes for each direction are labelled. Coloured points indicate significantly differentially expressed genes with adjusted p-value < 0.05 and Foldchange > 1.5 (green) or FoldChange < -1.5 (blue).

To assess the cell-type identity most closely represented by the *FOXP2*⁺ population, the sorted cerebellar organoid samples were compared to published laser capture micro-dissected samples from developing human cerebellum (Aldinger *et al.*, 2021). Using Euclidean distance as a measure of difference, the *FOXP2*⁺ organoid population showed greatest similarity to the Purkinje cell layer (PCL) samples (Figure 5.7A). Meanwhile, the *FOXP2*⁻ organoid population was most similar to the external granule layer (EGL) (Figure 5.7A). These results are consistent with *FOXP2* marking a Purkinje cell-like population and the remaining organoid population containing proliferative progenitors, including granule cell progenitors as found in the EGL. Next, expression of markers of the cerebellar VZ lineages was examined more closely. The *FOXP2*⁺ organoid population showed significantly lower expression of markers of the early VZ (*KIRREL2*, *PTF1A*, *PAX3*) and instead showed increased expression of early Purkinje cell markers (*FOXP1*, *FOXP4*, *LHX1*, *LHX5*, *SKOR2* and *TFAP2A*) (Figure 5.7B). Expression of later Purkinje cell markers was less distinct between sorted populations with a slight significant upregulation of *CA8* and *CALB1* in the *FOXP2*⁺ population (Figure 5.7B). In contrast, markers of GABAergic interneurons (*NFIA* and *NFIB*) were downregulated in the *FOXP2*⁺ population (Figure 5.7B). However, markers of GABAergic DCN neurons (*ZFHX3* and *ZFHX4*) were enriched, suggesting the presence of GABAergic DCN-like neurons in the *FOXP2*⁺ population (Figure 5.7B). Finally, differentially expressed genes across organoid-sorted populations were compared to cell type markers identified in a cross-species developing cerebellar snRNA-seq study (Sepp *et al.*, 2023). The significance of overlap between gene lists was determined using a Fisher's exact test. Genes upregulated in the *FOXP2*⁺ organoid population were enriched for markers of Purkinje cell clusters (Purkinje_defined, Purkinje_differentiating and Purkinje_mature) (Figure 5.7C). Markers of other neuron types were also significantly enriched, including interneuron_defined, GC_defined and isthmic_nuclei, likely as a consequence of many genes showing widespread neuronal expression (Figure 5.7C). In contrast, genes downregulated in the *FOXP2*⁺ organoid population were enriched for markers of non-neuronal cell types (including astrocytes, meningeal cells and glioblasts), glutamatergic lineage neurons (unipolar brush cells (UBC), GC_diff and GCP) and progenitor cells (GCP, progenitor, VZ_neuroblast) (Figure 5.7C). Together, this pattern of gene expression indicates that the *FOXP2*⁺ cerebellar organoid

population represented a developing Purkinje cell-like population. There are also features of DCN neurons so the *FOXP2*+ sorted population may also include this cell type.

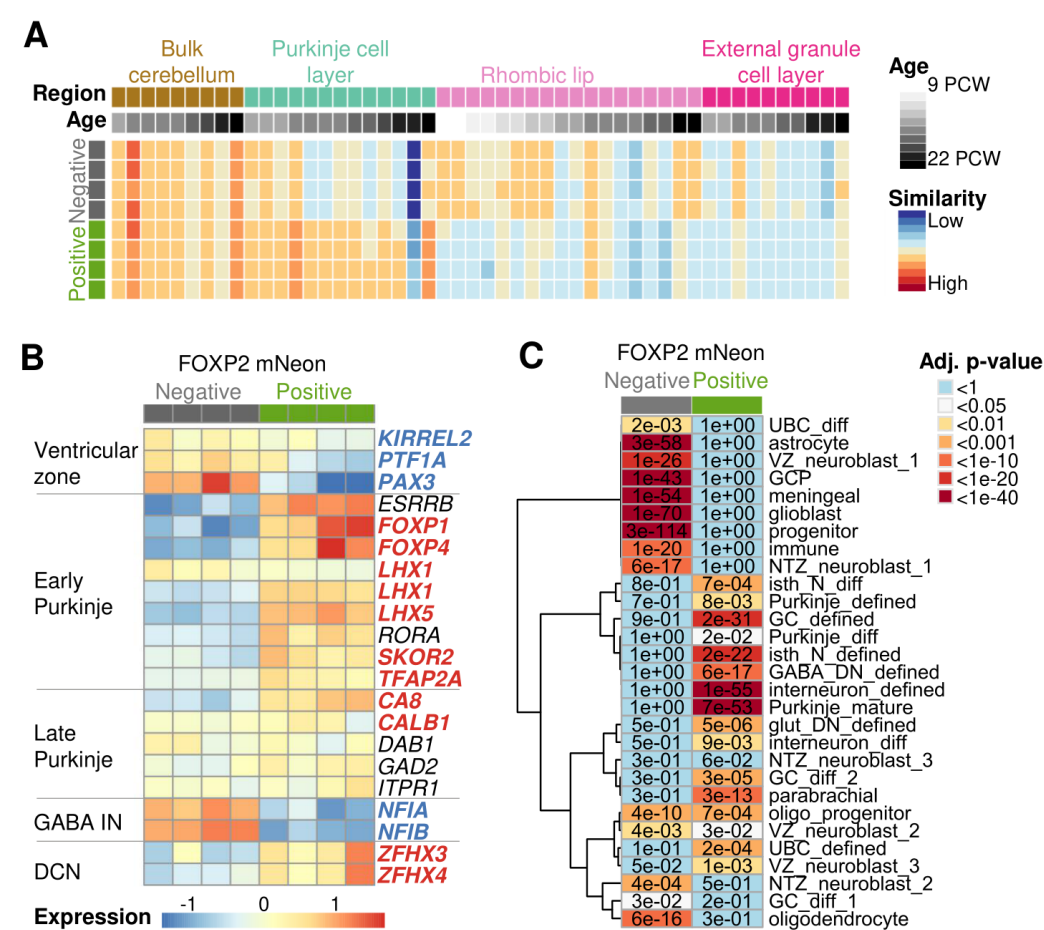


Figure 5.7 The *FOXP2*+ population in cerebellar organoids expressed Purkinje cell markers.

- A) Cerebellar organoid sorted samples were compared to bulk RNA sequencing from developing human laser capture micro-dissected cerebellar regions (Aldinger *et al.*, 2021). mNeon+ samples most closely matched the Purkinje cell layer samples. Colour scale indicate similarity: low Eucidean distance high similarity (red), to high Eucidean distance low similarity (blue).
- B) Expression of selected markers of cerebellar GABAergic cell types across cerebellar organoid populations. Genes showing significant differential expression between mNeon populations are in bold and coloured for increased in mNeon+ (red) or decreased (blue). Expression for each gene scaled across samples, from low (blue) to high (red).
- C) The genes differentially expressed between organoid populations was compared with cerebellar cell type markers. Cross-species consensus cell type marker genes were used from Sepp *et al.*, 2023. The colour scale indicates the significance of overlap between cell-type marker genes and mNeon differentially expressed genes as determined by a Fishers' exact test, Benjamini-Hochberg adjusted p-value shown.

UBC, unipolar brush cells; VZ, ventricular zone; GCP, granule cell progenitors; NTZ, nuclear transitory zone; isth_N, isthmic nuclei; GC, granule cells; diff, differentiating; DN, deep cerebellar nuclei neuron; oligo, oligodendrocyte. PCW, post conception weeks.

Whilst in early brain development *FOXP2* is strongly enriched in the cerebellum, *FOXP2* is also expressed in the striatum, thalamus and layer 5/6 of the cerebral cortex (Figure 1.11B) (Reviewed in Co, Anderson and Konopka, 2020). To study whether the *FOXP2*⁺ cerebellar organoid population showed cerebellar specificity, expression of differentially enriched genes across organoid sorted populations was plotted across different brain regions (Figure 5.8A). Brainspan bulk RNA-seq data from developing human brain samples was used for this analysis, with expression averaged across prenatal timepoints for each region. The majority of the 50 most significantly upregulated genes in the *FOXP2*⁺ cerebellar organoid population displayed broad expression across the developing human brain (Figure 5.8A). However, a subset of genes showed more cerebellar specific expression (*MAB21L2*, *TFAP2A*, *PTGER3* and *LHX1*) (Figure 5.8A). *LHX1* was previously been profiled across cerebellar organoid differentiation and showed significant induction by day 35 (Figure 4.2A). Similar quantification of cerebellar localised genes found significant induction of *MAB21L2* and *TFAP2A* across cerebellar organoid differentiation (Figure 5.8B). Where Allen Brain Atlas *in situ* hybridisation images were available, enriched expression in the cerebellum was confirmed (Figure 5.8C). Both *TFAP2A* and *LHX1* showed strong expression in the cerebellar VZ at E13.5, followed by expression in the Purkinje cell layer (Figure 5.8C). To conclude, whilst the overlap between markers across brain regions makes it difficult to rule out features of non-cerebellar *FOXP2*⁺ cells, transcriptomic analysis supports the cerebellar specificity of the *FOXP2*⁺ cerebellar organoid population.

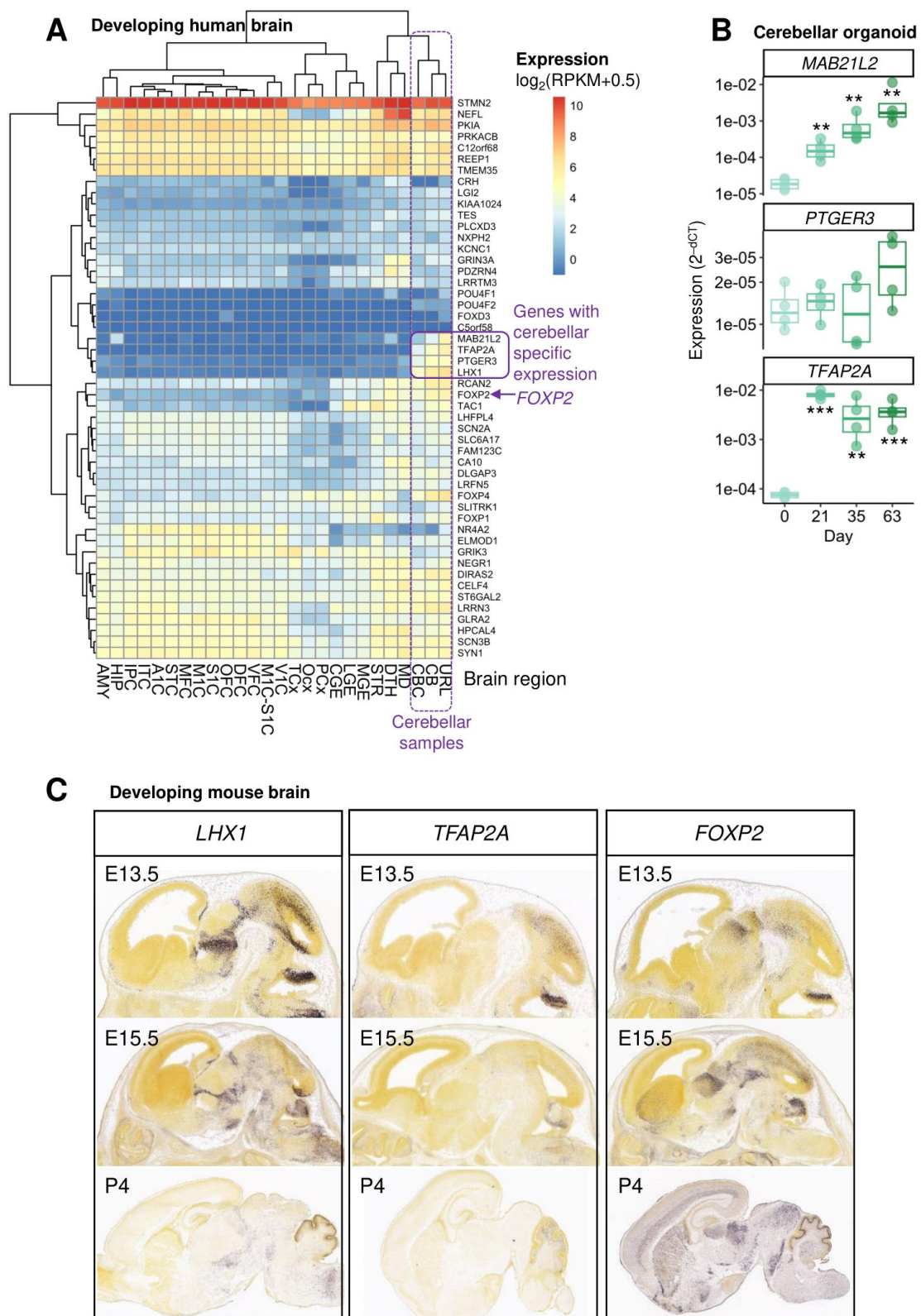


Figure 5.8 Expression of genes enriched in FOXP2+ cerebellar organoid population across the brain.

Figure 8. Expression of genes enriched in *FOXP2*+ cerebellar organoid population across the brain.

- A) Expression of the top 50 genes enriched in the cerebellar organoid *FOXP2*+ population was plotted across the developing human brain. Data from the Brainspan developing human brain resource, expression for prenatal samples (8-37 PCW) averaged for each region. Cerebellar samples highlighted with dotted line. Most genes showed broad brain expression, however some are enriched in the cerebellum (solid line). The colour scale shows expression as $\log_2$ (reads per kilobase per million).
 - B) Selected genes from A with expression localised to the cerebellum were profiled across cerebellar organoid differentiation using RT-qPCR. Expression is plotted as $2^{-\Delta CT}$, calculated relative to housekeeper genes (*ACTIN*, *GAPDH*). *MAB21L2* and *TFAP2A* showed significant increases in expression compared to day 0 by T-test (n =4, *p<0.05, **p<0.01, ***p<0.001).
 - C) Examples of genes significantly enriched in the cerebellar organoid *FOXP2*+ population and which showed cerebellar localised expression in the developing brain. ISH images from Allen brain atlas. E, embryonic day; P, postnatal day
- Brainspan brain regions: AMY, Amygdala; HIP, hippocampus; IPC, posteroventral (inferior) parietal cortex; ITC, inferolateral temporal cortex; A1C, primary auditory cortex; STC, posterior (caudal) superior temporal cortex; MFC, anterior (rostral) cingulate (medial prefrontal) cortex; M1C, primary motor cortex; S1C, primary somatosensory cortex; OFC, orbital frontal cortex; DFC, dorsolateral prefrontal cortex; VFC, ventrolateral prefrontal cortex; M1C-S1C, primary motor-sensory cortex; V1C, primary visual cortex; TCx, temporal neocortex; Ocx, occipital neocortex; PCx, parietal neocortex; CGE, caudal ganglionic eminence; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; STR, striatum; DTH, dorsal thalamus; MD, mediodorsal nucleus of thalamus; CBC, cerebellar cortex; CB, cerebellum; URL, upper (rostral) rhombic lip.

After establishing the cerebellar GABAergic lineage nature of the *FOXP2*+ population, transcriptomic analysis was used to investigate potential downstream pathways of *FOXP2* in the developing human cerebellum. Gene set enrichment analysis (GSEA) was performed on normalised RNAseq data using GO terms and KEGG pathways. GSEA on biological process GO terms identified enrichment for terms related to synaptic processes, neuronal activity and signal transduction in the *FOXP2*+ population (Figure 5.9A & B). Terms identified for the *FOXP2*- population were more varied and related to extracellular matrix (ECM), cytoplasmic translation and connective tissue development (Figure 5.9C). Similar results were obtained using KEGG pathways, although only two pathways were significantly enriched in the *FOXP2*+ population: 'Long term potentiation' and 'Neuroactive ligand receptor interaction'. The top KEGG terms significantly enriched in the *FOXP2*- population were Ribosome; ECM-receptor interaction; Basal cell carcinoma; P53-signalling pathway; Cell cycle.

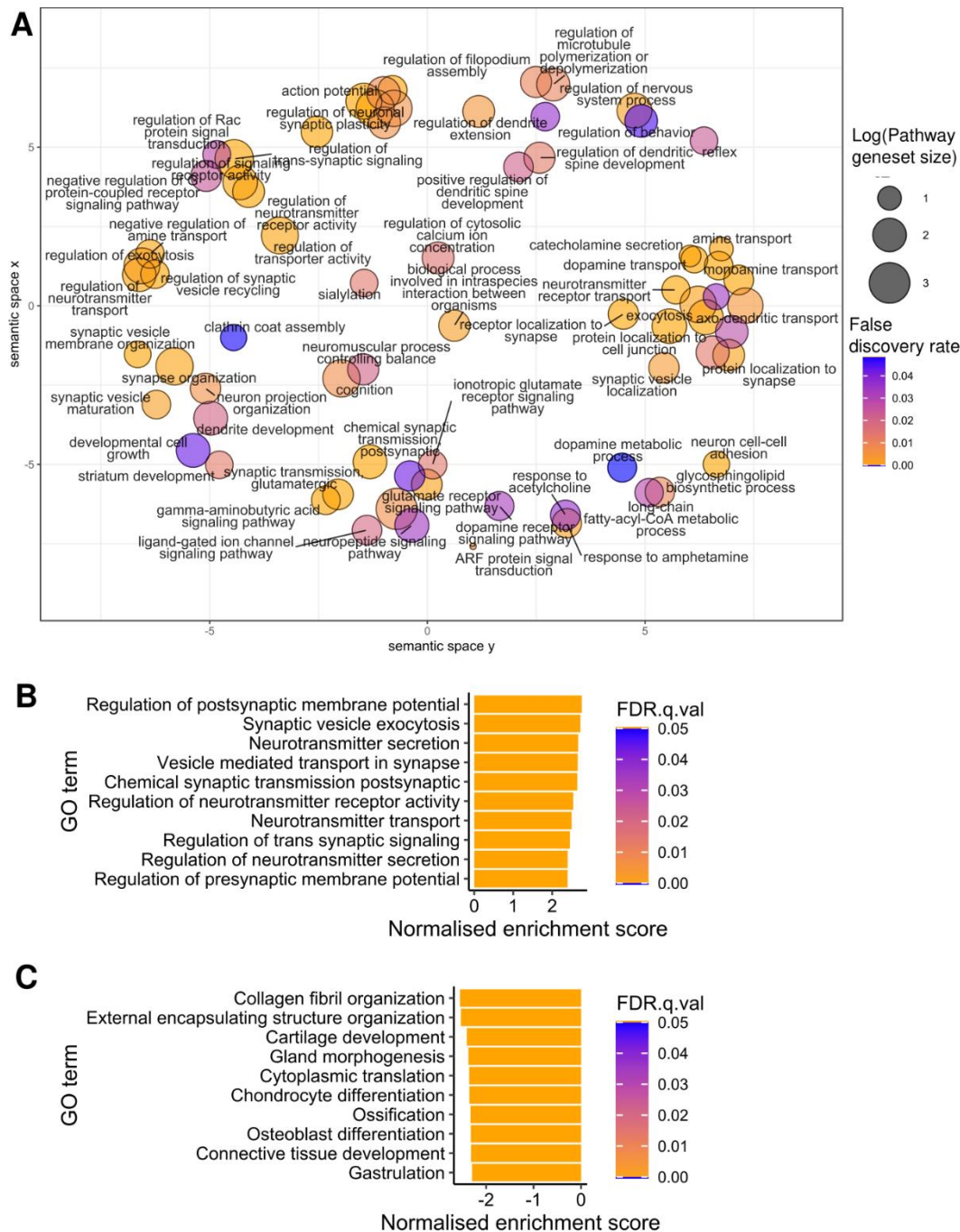


Figure 5.9 Gene expression in the *FOXP2*+ population in cerebellar organoids was enriched for pathways associated with neural activity and synaptic transmission.

- Geneset enrichment analysis was used to identify biological GO terms enriched in the cerebellar organoid *FOXP2*+ population. Significantly upregulated terms were summarised by REVIGO to simplify and cluster by semantic similarity. The colour scale indicates the false discovery rate, a threshold of <0.05 was applied to select for significant pathways. The size of points scales for geneset length.
- The normalised enrichment score for the top 10 biological process terms upregulated in the cerebellar organoid *FOXP2*+ population. Colour scale indicates false discovery rate.
- The normalised enrichment score for the 10 biological process terms most downregulated in the cerebellar organoid *FOXP2*+ population.

In combination these results showed a strong association of the *FOXP2*⁺ population with neuronal activity and synaptic signalling. As confirmed by study of cell identity markers (Figure 5.7) the *FOXP2*⁺ population represents isolation of developing Purkinje cells in cerebellar organoids. Therefore, the enriched pathways reflect processes upregulated in Purkinje cells including those regulated by *FOXP2*. However, previous studies identifying *FOXP2* targets in other brain regions have also identified similar pathways related to neurogenesis and cell communication (Hoed, Devaraju and Fisher, 2021). Thus, suggesting a probable contribution of *FOXP2* to these processes in the Purkinje cells. Together, our results suggest *FOXP2* may form part of a network important for the development of neuronal activity and synapse formation in Purkinje cells, both processes critical to the correct Purkinje dendritic tree development and cerebellar circuit functioning.

5.2.4 Analysis of *FOXP2* targets and neurodevelopmental disorder genes in *FOXP2*-expressing cells in cerebellar organoids

In order to distinguish genes regulated by *FOXP2* from those enriched in Purkinje cells, genes differentially expressed across organoid populations were compared to known *FOXP2* targets. Several published studies have identified *FOXP2* targets using ChIP. However, there is very limited overlap in the results of these studies, so there is currently no consensus list of *FOXP2*-regulated genes (Hoed, Devaraju and Fisher, 2021). Two studies using human neuronal samples were used for comparison to organoid genes. The first study performed *FOXP2* ChIP-chip on human foetal brain and lung (Spiteri *et al.*, 2007). In this analysis, only targets identified in the brain were used. The second study used human neuroblastoma cell line SH-SY5Y stably expressing *FOXP2* (Vernes *et al.*, 2007). Between these published studies only 43 common targets were identified, eight of these were significantly increased in the *FOXP2*⁺ population in cerebellar organoids and nine significantly decreased (Figure 5.10A). There is little literature on these overlapping *FOXP2* targets in cerebellar development. Interestingly, *PAX3* has been identified as a key transcriptional regulator in cerebellar development (Khouri-Farah *et al.*, 2022; Ramirez *et al.*, 2022). *PAX3* expression has been reported in GABAergic cerebellar progenitors in the mouse VZ but was downregulated during differentiation to

Purkinje cells (Ramirez *et al.*, 2022). The absence of PAX3 in differentiated Purkinje cells, resulted in no overlap in expression with Purkinje cell markers, such as SKOR2 and FOXP2, being observed in mouse cerebellum (Minaki *et al.*, 2008; Ramirez *et al.*, 2022). Therefore, a downregulation of PAX3 in the FOXP2+ cerebellar organoid population is reflective of a Purkinje cell differentiation trajectory.

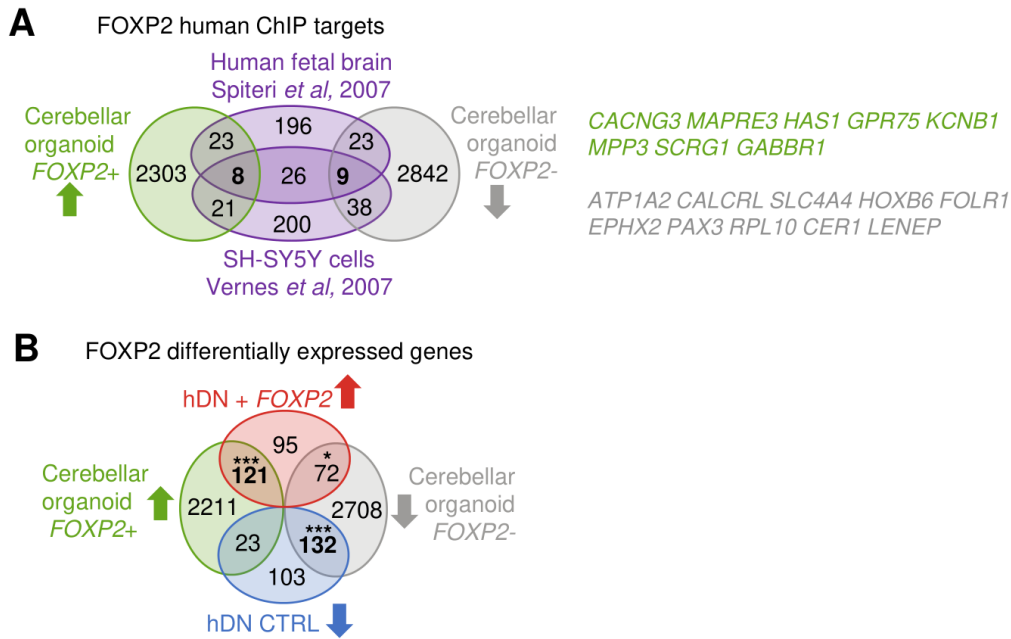


Figure 5.10 Comparison of genes differentially expressed in cerebellar organoid populations with previously identified FOXP2 targets.

- A) FOXP2 targets identified in ChIP studies on neuronal samples (Vernes *et al.*, 2007 & Spiteri *et al.*, 2007) were compared with differentially expressed genes between organoid populations. Significantly differentially expressed genes in the cerebellar organoid populations were defined by an adjusted p-value < 0.05 and absolute(log2FoldChange) > 0.58. Genes listed were present in both ChIP datasets and in cerebellar organoids were significantly increased (green) or decreased (grey) in the FOXP2+ population.
- B) Differentially expressed genes were compared between cerebellar organoid populations and results from Hickey *et al.*, 2019 using human differentiated neurons (hDN). For cerebellar organoids, differentially expressed genes showed significant difference between FOXP2+ and FOXP2- populations (left-right). For the hDNs, differentially expressed genes showed significant difference in expression in hDNs with exogenous expression of FOXP2 compared to hDNs treated with a control lentivirus (top-bottom). Arrows indicate expression of FOXP2 in the different samples. Significance of geneset overlap was determined using a Fisher's exact test (**p < 10⁻³⁰, *p < 10⁻³).

Genes differentially expressed between the *FOXP2*⁺ and *FOXP2*⁻ organoid populations, were also compared with another differential expression analysis (Hickey, Berto and Konopka, 2019) (Figure 5.10B). In this study, human neural progenitors were differentiated to human differentiated neurons (hDNs) in the presence of exogenous *FOXP2* and compared to control cells differentiated to hDNs without *FOXP2* (Hickey, Berto and Konopka, 2019). Neural progenitors were isolated from foetal human cortex and obtained commercially (Lonza, Line #OF4106, PT-2599). In this study the same cell type (hDNs) was compared, and therefore identified genes were regulated directly or indirectly by *FOXP2*. Interestingly a significant number of the genes present in both datasets showed the same response to *FOXP2*: 121 genes were upregulated in both the *FOXP2*⁺ cells of cerebellar organoids and hDNs with exogenous *FOXP2*, and 132 genes were decreased in both (Figure 5.10B). These results suggest there may be common transcriptional responses to *FOXP2* in neuronal differentiation, with a similar network of genes regulated across neuron types. It also highlights a subset of differentially expressed genes from the organoid sorted populations which have been shown independently to respond to *FOXP2*.

FOXP2 was identified as one of the first language associated genes and subsequently the *FOXP2*-related speech and language disorder has been defined, with the core phenotype being childhood apraxia of speech (CAS) (Morgan *et al.*, 2019). As a transcriptional regulator, *FOXP2* is thought to act within a network of gene expression important in speech and language programming and underlying key neurodevelopmental processes. Therefore, the *FOXP2*⁺ cerebellar organoid population was tested for enriched expression of genes associated with neurodevelopmental disorders (Figure 5.11A). Gene sets were chosen from disorders (Alzheimer's disease, cerebellar malformations, intellectual disability (ID), Joubert syndrome, Spinocerebellar ataxia (SCA)) which affect the cerebellum and which were previously examined in human developing cerebellum scRNA-seq (Aldinger *et al.*, 2021). In addition, a list of other genes associated with CAS was compiled from published studies (Kaspi *et al.* 2022; Eising *et al.* 2019; Hildebrand *et al.* 2020). As ASD has been linked to some structural and functional connectivity changes in the cerebellum (Sathyanesan *et al.*, 2019; Sydnor and Aldinger, 2021), a list of ASD-risk genes defined by SFARI (<https://gene.sfari.org/>) were also tested. Disease

gene sets associated with cerebellar malformations, SCA and ASD were significantly enriched among genes upregulated in the *FOXP2*⁺ population (Figure 5.11A). Therefore, the *FOXP2*⁺ population in cerebellar organoids is of interest for studying a number of neurodevelopmental disorders. These results were also reflective of a Purkinje cell-like identity, with significant increased expression of the cerebellar malformation, ID, SCA and ASD genes seen in Purkinje cell clusters of human developing cerebellum (Aldinger *et al.*, 2021). Whilst SCAs are considered neurodegenerative conditions, genes mutated in SCAs are typically involved in normal Purkinje cell function and there is some evidence for a role of altered Purkinje cell development (Kapfhammer and Shimobayashi, 2023). In contrast, genes for the ciliopathy Joubert syndrome showed significant overlap with genes decreased in the *FOXP2*⁺ population (Figure 5.11A). Genes identified as causing Joubert syndrome impair function of the primary cilia and therefore are not specifically associated with Purkinje cells, instead showing enrichment in ciliated cells (a *FOXP2*⁻ population) in scRNA-seq analysis of cerebellar organoids (Nayler *et al.*, 2021). Neither cerebellar organoid population showed enrichment for genes linked to Alzheimer's disease (Figure 5.11A). The early developmental nature of the cerebellar organoid model makes it unsurprising that genes associated with a neurodegenerative disease were not strongly evident and a similar absence of specific cell-type enrichment for Alzheimer-risk genes was found in human foetal cerebellum (Aldinger *et al.*, 2021)

Given the strong enrichment for ASD associated genes in the *FOXP2*⁺ cerebellar organoid population, comparison was also made with an additional dataset profiling differentially expressed genes in post mortem brains of individuals with neurological disorders, including ASD (Gandal *et al.*, 2018) (Figure 5.11B). Cerebellar organoids model early cerebellar specification and neuron generation, and therefore are not an equivalent developmental stage to post mortem brains of adults so comparisons should be made cautiously. However, the dataset from Gandal *et al.*, 2018 provides insight into the directionality of gene expression changes in individuals with neurological disorders, which is lacking from genetic risk factors. In addition, changes in individuals with neurodevelopmental disorders are likely to start during prenatal development, although further differences may accumulate in postnatal brains. Therefore, this comparison may result in identification of correlations for further investigation.

Genes decreased in individuals with ASD were found to be significantly enriched in the *FOXP2*+ organoid population and vice versa (Figure 5.11B). This overlap suggests that the network of genes expressed with *FOXP2*, such as other Purkinje cell genes and genes involved in synaptic signalling, are reduced in the brains of individuals with ASD. This result may reflect a decreased population of GABAergic cells, including Purkinje cells.

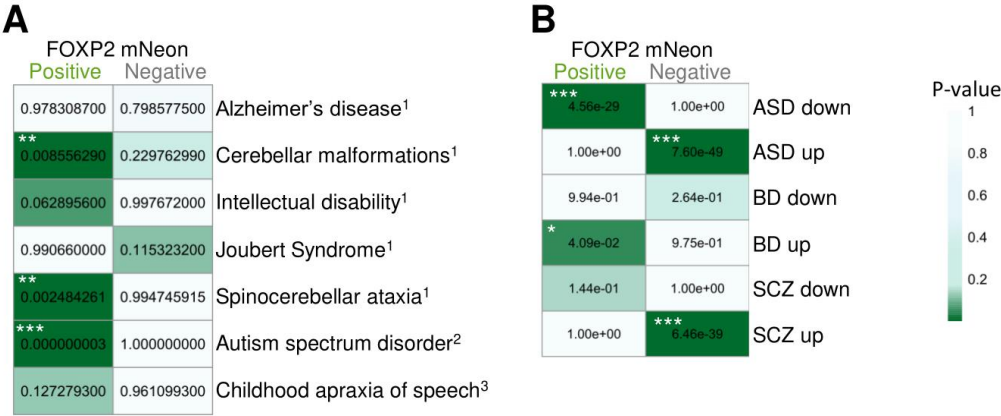


Figure 5.11 An enrichment of genes was associated with neurodevelopmental disorders in *FOXP2*+ population of cerebellar organoids.

- A) Enrichment of disease-associated genes in cerebellar organoid populations was tested by comparing the overlap of differentially expressed genes between sorted populations with disorder genesets. Disorder genesets were collated from multiple sources: 1- previous testing in human cerebellar scRNA-seq, Aldinger *et al.*, 2021. 2-SFARI class 1 risk genes (<https://gene.sfari.org/>). 3 - Kaspi *et al.* 2022; Eising *et al.* 2019; Hildebrand *et al.* 2020.
- B) Enrichment of genes differentially expressed in post-mortem brain samples from individuals with Autism spectrum disorder (ASD), bipolar disorder (BD) and schizophrenia (SCZ). Differentially expressed genes identified by Gandal *et al.*, 2018, genes which were significantly increased in affected individuals compared to healthy controls were classified as up, and those decreased as down.

Significance of overlaps were calculated using a Fisher's exact test, p-values are shown and indicated by colour scale (*p<0.05, **p<0.01, ***p<0.001).

Whilst there was no significant overlap between the CAS gene set and the genes differentially expressed in organoid populations (Figure 5.11A), given the strong CAS phenotype in individuals with *FOXP2* mutations, other CAS genes were examined in more detail (Figure 5.12A). Three CAS genes were significantly decreased in expression in the *FOXP2*+ cerebellar organoid population and seven were significantly increased (Figure 5.12A). To consider the role of Purkinje cells in CAS, the expression of CAS genes upregulated in the *FOXP2*+ cerebellar organoid population was plotted across cell types in the developing human cerebellum (Figure

5.12B). None of the examined genes showed highest expression in the Purkinje cell cluster, with most showing fairly broad expression across clusters (*CHD3*, *GNAO1*, *SPAST*, *TAOK2*)(Figure 5.12B). *RBFOX3*, the gene encoding mature neuron marker NeuN, displayed granule cell-enriched expression and was absent from other cell types including Purkinje cells as previously reported in immunostaining studies (Wolf *et al.*, 1996; Weyer and Schilling, 2003)(Figure 5.12B). *Meis homeobox 2* (*MEIS2*) and *Zinc Finger Homeobox 4* (*ZFHX4*) showed highest expression in the isthmus nuclei, GABAergic DCN neurons and the nuclear transitory zone (Figure 5.12B). *FOXP2*+ cells in subpopulations of the DCN have been previously described (Fujita and Sugihara, 2012) and were observed in developing mouse cerebellar sections (Figure 5.1F). Therefore, *MEIS2* and *ZFHX4* were chosen for further investigation of their potential interaction with *FOXP2* in the developing cerebellum.

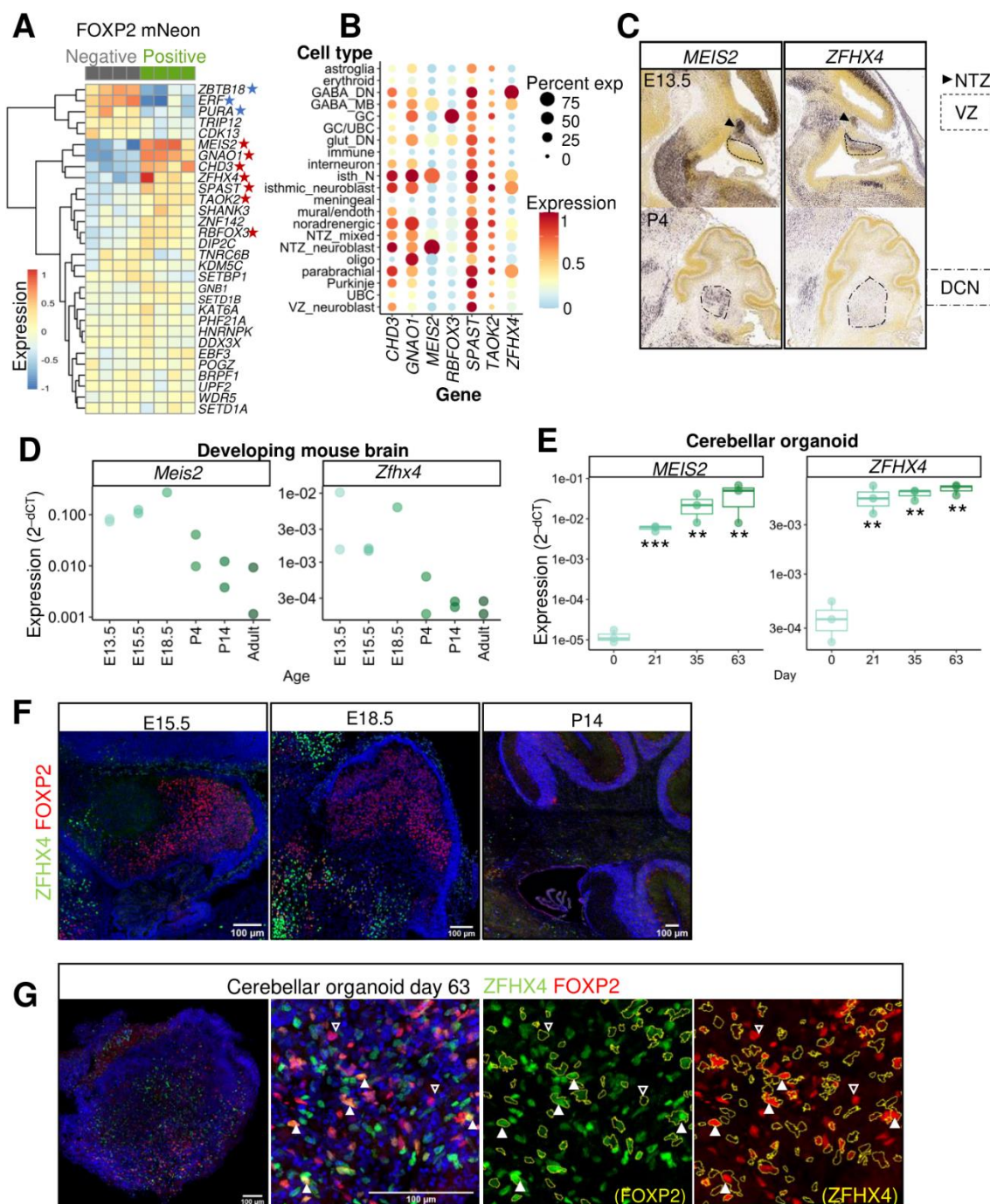


Figure 5.12 Expression of genes associated with childhood apraxia of speech in FOXP2+ population of cerebellar organoids.

- A) Expression of childhood apraxia of speech (CAS) associated genes were compared across cerebellar organoid sorted populations. Genes significantly increased in the FOXP2+ population (red stars) and significantly decreased (blue stars) are indicated. CAS genes were collated from Kaspi *et al.* 2022, Eising *et al.* 2019 and Hildebrand *et al.* 2020.
- B) Expression of CAS genes enriched in the FOXP2+ cerebellar organoid population was plotted across cell types in the developing human cerebellum. snRNA-seq data from Sepp *et al.*, 2023. Point size indicates the percentage of cells expressing gene and colour scale shows average gene expression normalised across cell types. DN, deep cerebellar nuclei neuron; MB, midbrain; GC, granule cells; UBC, unipolar brush cells; isth_N, isthmus nuclei; oligo, oligodendrocyte.

Continued.

Expression of genes associated with childhood apraxia of speech in FOXP2+ population of cerebellar organoids.

- C) The expression pattern of selected CAS genes, *Meis2* and *Zfhx4*, in developing mouse cerebellum. ISH images from the Allen brain atlas. At embryonic day (E)13.5 both are expressed in the nuclear transitory zone (NTZ, arrow), with additional expression of *Zfhx4* only in the ventricular zone (VZ, dashed line). At postnatal day (P)4, expression is localised to the deep cerebellar nuclei (DCN, dashed line).
- D) Quantification of expression of selected CAS genes across mouse cerebellar development by RT-qPCR. Expression was calculated as $2^{-\Delta CT}$ relative to housekeeper gene *Gapdh*. Samples at E13.5 and E15.5 were whole brain, from E18.5 onwards the cerebellum was isolated. Points represent biological samples (n = 2).
- E) Expression of selected CAS genes across cerebellar organoid differentiation. Expression was calculated as $2^{-\Delta CT}$ relative to housekeeper genes (*ACTIN*, *GAPDH*). Both genes show significant induction of expression in organoids compared to iPSCs by T-test (n = 4, *p<0.05, **p<0.01, ***p<0.001). Points represent individual differentiations.
- F) Mouse cerebellar section from different stages of development were stained with antibodies against FOXP2 (red) and ZFH4 (green) to compare expression. Nuclei were stained with Hoechst (blue). Scale bars = 100 μ m.
- G) Immunostaining for FOXP2 (red) and ZFH4 (green) in sections of day 63 cerebellar organoid. Yellow outlines show cells expressing the other marker on single channel images. Examples of double positive cells shown with filled arrows and of FOXP2+ZFH4- cells with open arrow. Scale bars = 100 μ m.

Firstly, ISH images were used to confirm the expression pattern of *Meis2* and *Zfhx4* as localised to the nuclear transitory zone and DCN, with some lower VZ expression of *Zfhx4* in early development (Figure 5.12C). Next, their expression over mouse cerebellar development was quantified using RT-qPCR (Figure 5.12D). Both *Meis2* and *Zfhx4* had highest expression in the embryonic samples, suggesting a possible function in development (Figure 5.12D). Expression was also characterised in cerebellar organoids and both genes showed robust induction during cerebellar differentiation (Figure 5.12E). The cerebellar expression profile of ZFH4 was also characterised using immunostaining in both developing mouse cerebellum and cerebellar organoids. In E15.5 mouse cerebellum, ZFH4 expression was observed in the anterior most region of the VZ (Figure 5.12F). This is the site of GABAergic interneuron generation, including the GABAergic DCN neurons. Subsequently, ZFH4 expression was localised to the DCN at E18.5, including some co-expression with FOXP2 (Figure 5.12F). Sparse ZFH4 expression was also observed in the DCN at P14 (Figure 5.12F). Cerebellar organoid sections were similarly stained for ZFH4 and FOXP2 (Figure 5.12G). The presence of ZFH4+ cells was frequent at day 63, with a high overlap in expression with FOXP2 as expected from the RNA-seq data.

However, both *FOXP2*+*ZFHX4*- and *FOXP2*-*ZFHX4*+ cells were also detected. These observations, in combination with previous characterisation of *FOXP1* and *FOXP4*, indicate that the *FOXP2*+ cerebellar organoid population is not entirely homogenous, and may represent multiple Purkinje cell subtypes and DCN neurons. However, this also highlights the need to consider the function of *FOXP2* and other CAS-associated genes in GABAergic DCN neurons as well as Purkinje cells.

5.2.5 Comparison of *FOXP2* knock-out and wild-type cerebellar organoids

Characterisation of the *FOXP2*+ population in cerebellar organoids identified potential downstream targets of *FOXP2* in the early human cerebellum, including pathways involved in neurogenesis and synaptic signalling. To investigate the mechanisms of *FOXP2* and validate some of these potential targets, cerebellar organoids were generated from iPSCs with the *FOXP2* gene deleted (*FOXP2*^{-/-}) and compared to wild-type (WT) organoids.

FOXP2 knock-out iPSCs were generated from the WTC11 control iPSC line (<https://hpscreg.eu/cell-line/UCSFi001-A>) by collaborators Seon Hye E. Park and Genevieve Konopka. For this, a pair of gRNAs were designed to target Cas9 to sites in the 5' UTR and 3' UTR, leading to deletion of the complete *FOXP2* locus (Figure 5.13A). To confirm the genotypes of the newly obtained lines, primers were designed to amplify sequences containing the gRNA target sites in the 5' and 3' UTRs, in addition to an exon sequence from the middle of the *FOXP2* locus (Figure 5.13A). PCR products were obtained from all three primer pairs when using gDNA from WT cells, whereas for *FOXP2*^{-/-} cells, no PCR products were made (Figure 5.13B). However, a PCR using the 5' UTR forward primer and 3' UTR reverse primer generated an approximately 500 bp product in the *FOXP2*^{-/-} cell samples, whilst in WT cells these sites were separated by the entire *FOXP2* gene so no product was formed (Figure 5.13B). Genotyping PCRs identified one WT clone and one homozygous *FOXP2* knock-out clone (*FOXP2*^{-/-}) used for subsequent experiments.

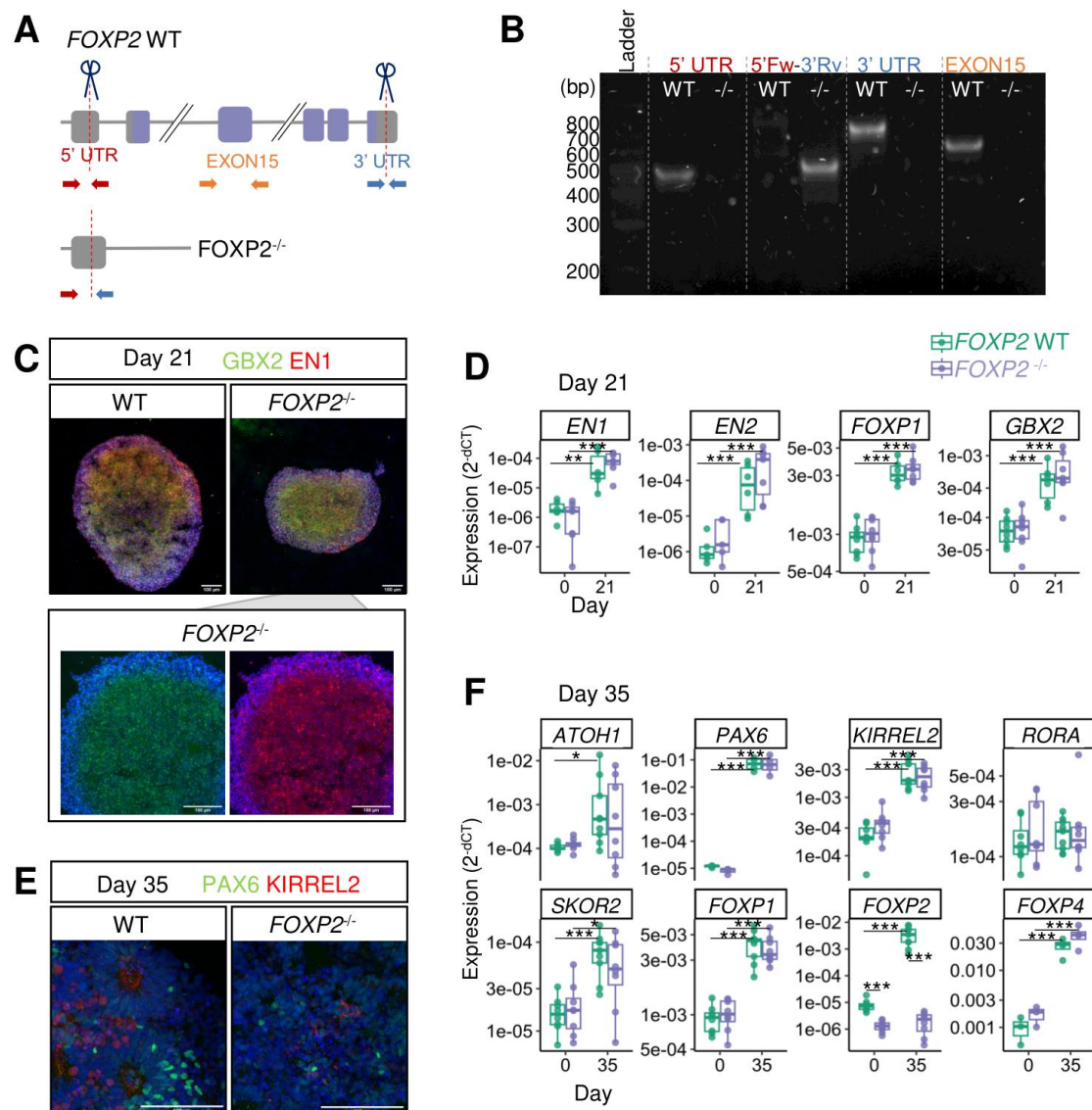


Figure 5.13 *FOXP2*^{-/-} iPSCs differentiated into cerebellar organoids with typical expression of cerebellar markers.

- A) Strategy for knock-out of the whole *FOXP2* coding sequence using gRNAs targeting sites in the 5' and 3' untranslated regions (UTRs, grey blocks). Diagram shows Exons (purple blocks) and gRNA target sites (red dashed lines). The second diagram shows *FOXP2* locus after gene editing. Arrows show primer pairs used for genotyping, spanning the gRNA target site in the 5' UTR (red) and 3' UTR (blue) and a mid-gene exon (Exon 15, orange).
- B) PCRs confirmed the genotype of the *FOXP2*^{-/-} iPSC line, showing loss of the whole *FOXP2* gene. No product was made for the primer pairs at the 5' UTR, exon15 or 3' UTR in the knock-out line due to deletion. Instead an ~500 base pair (bp) product was made for the 5'UTR forward - 3' UTR reverse primer combination in the knock-out. The left-most lane contains a 100 bp ladder, PCR products were run on a 3% agarose gel.
- C) *FOXP2* WT and *FOXP2*^{-/-} organoids successfully differentiated, including expression of mid-hindbrain boundary markers (MHB) EN1 (red) and GBX2 (green) at day 21. Nuclei were stained with Hoechst (blue). Scale bars = 100 μm.

Continued.

Figure 5.13 *FOXP2*^{-/-} iPSCs differentiated into cerebellar organoids with typical expression of cerebellar markers.

- D) Cerebellar organoids generated from both the *FOXP2* WT and *FOXP2*^{-/-} lines showed significant induction of MHB genes (*EN1*, *EN2*, *GBX2*) and VZ marker *FOXP1* at day 21 compared to day 0 by T-test (*p<0.05, **p<0.01, ***p<0.001). Expression calculated as 2^{-dCT} relative to housekeeper genes (*ACTIN*, *GAPDH*). Points represent samples from individual differentiations.
- E) *FOXP2*^{-/-} organoids expressed markers of both key cerebellar neuron lineages. Antibodies were used against GABAergic marker KIRREL2 (red) and glutamatergic marker PAX6 (green). Scale bars = 100 μm.
- F) Both the *FOXP2* WT and *FOXP2*^{-/-} lines showed significant induction of cerebellar marker genes at day 35 by RT-qPCR. *FOXP2* mRNA was significantly reduced in the *FOXP2*^{-/-} line.

After confirming the identity of the *FOXP2*^{-/-} and WT lines, iPSCs were differentiated into cerebellar organoids. Samples were collected at day 21 and 35 of differentiation to test whether these new lines successfully differentiated into cerebellar identity. At day 21, both the WT and *FOXP2*^{-/-} organoids stained for widespread expression of MHB markers *EN1* and *GBX2* (Figure 5.13C). The successful induction of MHB identity was further confirmed by RT-qPCR (Figure 5.13D). Both lines showed significant upregulation of early VZ marker *FOXP1* in addition to MHB markers *EN1*, *EN2* and *GBX2* (Figure 5.13D). No significant differences in expression of these genes were found between the lines at day 0 or day 21. Similarly, at day 35 WT and *FOXP2*^{-/-} organoids stained positively for expression of both cerebellar neuron lineages, VZ marker KIRREL2 and RL marker PAX6 (Figure 5.13E). By RT-qPCR, *FOXP2*^{-/-} organoids showed significant induction of VZ lineage markers (*KIRREL2*, *SKOR2*, *FOXP1*, *FOXP4*) and RL lineage marker (*PAX6*) at day 35 compared to iPSCs (Figure 5.13F). With the exception of *FOXP2*, which was confirmed absent from the *FOXP2*^{-/-} cells, no significant differences in cerebellar marker gene expression was found between lines at day 35 (Figure 5.13F). These results suggest, that despite *FOXP2* expression in newly generated Purkinje cells from the VZ, *FOXP2* is not necessary for early stages of lineage specification and neuron differentiation for either germinal zone in the developing human cerebellum.

As *FOXP2*^{-/-} iPSCs successfully generated cerebellar organoids, further comparison was performed at day 63 to coincide with the RNA sequencing of the *FOXP2*-sorted populations and around the peak of *FOXP2* expression in WT organoids. Expression of a panel of genes was

measured in WT and *FOXP2*^{-/-} organoids by RT-qPCR (Figure 5.14A). Selected genes included other *FOXP* family genes and markers of cerebellar cell types - granule cells, DCN neurons and Purkinje cells. Additionally, *CNTNAP2* was included as a previously characterised target of *FOXP2*, which increased in the cerebellum of *FOXP2*-R552H mice and has been shown to be important in Purkinje cell dendritic and synaptic development (Fujita *et al.*, 2012a; Argent *et al.*, 2020). Finally, a number of genes related to synaptic signalling and membrane potential regulation that were enriched in the *FOXP2*⁺ cerebellar organoid population were profiled. Overall, *FOXP2*^{-/-} cerebellar organoids showed no major changes in gene expression, as only two genes showed significantly altered expression in *FOXP2*^{-/-} organoids compared to WT (Figure 5.14A). CAS-associated gene and DCN marker, *ZFHX4*, was significantly decreased in *FOXP2*^{-/-} organoids (Figure 5.14A). In contrast, *SLC6A17*, encoding a presynaptic amino acid transporter, was significantly increased compared to WT (Figure 5.14A). No significant changes in the *FOXP* genes, cerebellar markers, or *CNTNAP2* were found, although several genes showed considerable variability (Figure 5.14A). The complete knock out of *FOXP2* was confirmed again by Western blotting (Figure 5.14B). However, in agreement with the RT-qPCR results, no differences in expression of several cerebellar markers was found by Western blotting, including no changes in levels of other *FOXP* family members *FOXP1* and *FOXP4* (Figure 5.14B). To confirm the effect of *FOXP2* knock-out on *ZFHX4* expression in cerebellar organoids, flow cytometry was performed on WT and *FOXP2*^{-/-} organoids using fluorescently conjugated antibodies against either *ZFHX4* or *FOXP1* (Figure 5.14C). Across three pairs of samples, *ZFHX4* mean fluorescent intensity was consistently decreased in *FOXP2*^{-/-} organoids (Figure 5.14C, T-test, *p* = 0.06), as predicted from the RT-qPCR results (Figure 5.14A). The difference in *FOXP1* mean fluorescent intensity across genotypes was more variable and no clear difference was observed, again matching RT-qPCR results (Figure 5.14A & C). In summary, deletion of *FOXP2* lead to few molecular changes in cerebellar organoids, however, decreased expression of another CAS-associated gene, *ZFHX4*, was quantified at both mRNA and protein levels.

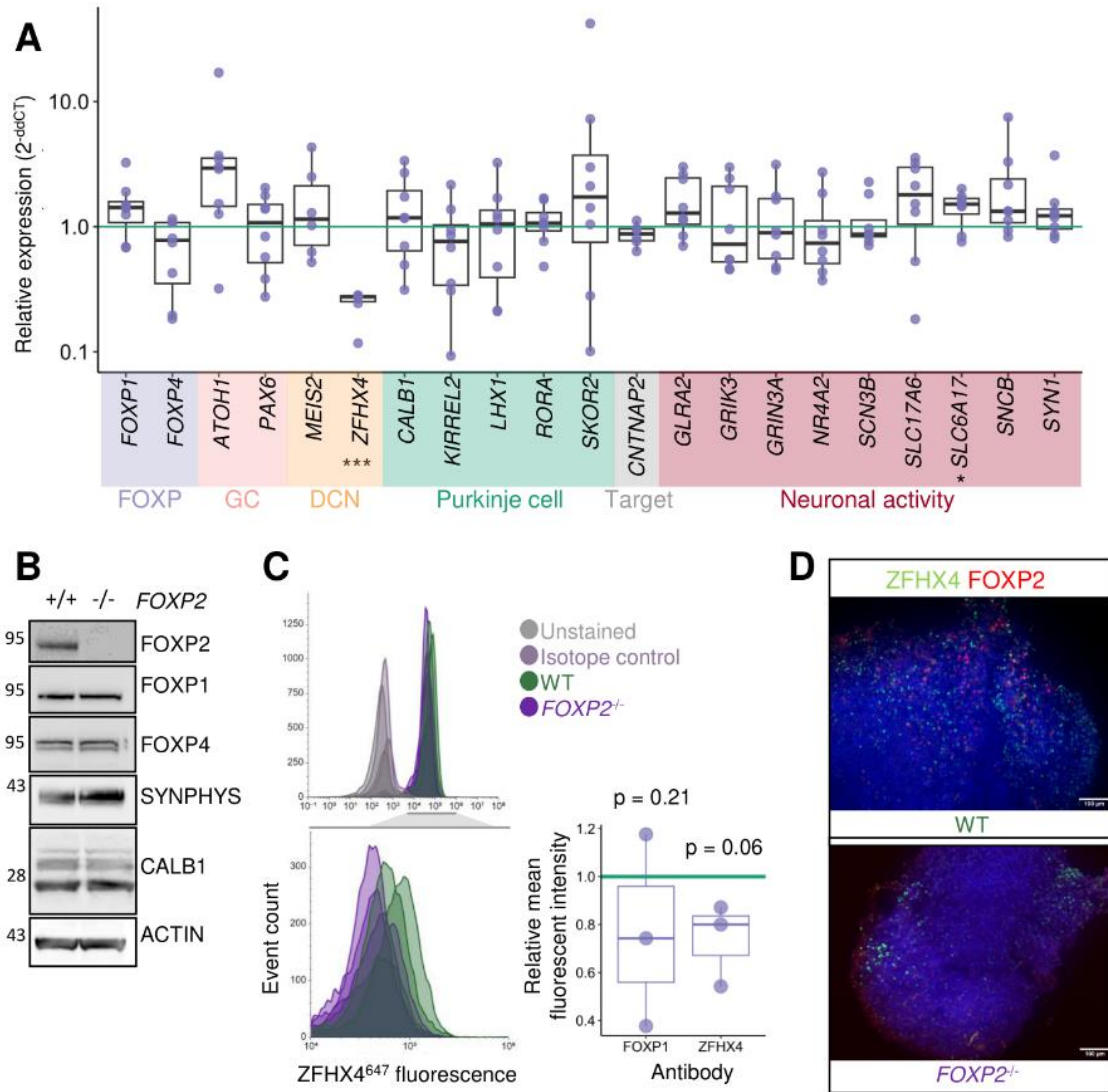


Figure 5.14 *FOXP2*^{-/-} cerebellar organoids showed limited differences in bulk gene expression.

- A) Expression of selected genes was measured by RT-qPCR in *FOXP2* WT and *FOXP2*^{-/-} cerebellar organoids at day 63. The panel of genes included *FOXP* family genes; markers of cerebellar cell types granule cells and their precursors (GC), deep cerebellar nuclei neurons (DCN) and Purkinje cells (PCs); a gene repressed by *FOXP2* in the cerebellum (*CNTNAP2*); and genes related to neuronal activity and synaptic signalling. Expression in *FOXP2*^{-/-} organoids is plotted relative to *FOXP2* WT organoids from the same differentiation batch (2^{-ddCT}). The green line indicates WT expression (2^0). Dots show individual differentiations. Significant differences in expression were determined by T-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).
- B) Protein levels of selected markers were compared between *FOXP2* WT and *FOXP2*^{-/-} organoids at day 63 by western blot. Representative blot, no differences apart from *FOXP2* knock-out were observed in 3 replicates.

Continued.

Figure 5.14 *FOXP2*^{-/-} cerebellar organoids showed limited differences in bulk gene expression.

- C) Flow cytometry was performed on WT and *FOXP2*^{-/-} organoid samples to quantify populations of FOXP1+ or ZFH4+ cells. Organoids were dissociated at day 63 – 65, fixed and stained using fluorescently conjugated antibodies against either FOXP1 or ZFH4. Flow analysis recorded fluorescent intensity of cells (x axis), ZFH4 shown as an example in the left column. Samples of dissociated cells that were unstained or stained with isotype anti-rabbit IgG⁶⁴⁷ antibody (grey) were included as a negative control. The mean fluorescent intensity was measured for each sample and values for *FOXP2*^{-/-} organoids were normalised to WT organoids from the same differentiation and stained concurrently. For each target, significance of decreased mean fluorescence intensity in *FOXP2*^{-/-} sample was calculated by T-test significantly decreased expression p-values show (n = 3).
- D) Sections of day 63 cerebellar organoids were immunostained with antibodies against ZFH4 (green) and FOXP2 (red). Nuclei were stained with Hoechst (blue). Scale bar = 100 µm.

Transcriptomic analysis of the *FOXP2*⁺ cerebellar organoid population highlighted enrichment of pathways relating to neuronal activity and synaptic signalling. To probe the functional effect of FOXP2 on development of neuronal activity, calcium imaging was performed on 2D cerebellar cultures derived from *FOXP2*^{-/-} and WT iPSCs. By immunostaining, no differences were observed between WT and *FOXP2*^{-/-} iPSC-derived cerebellar cultures (Figure 5.15A). Staining of cultures from both genotypes showed widespread neuronal marker expression (MAP2, TUJ1) with lower levels of neural progenitors (NESTIN), sparse glial cells (GFAP) and both glutamatergic and GABAergic cerebellar neurons (PAX6 & CALB1 respectively) (Figure 5.15A).

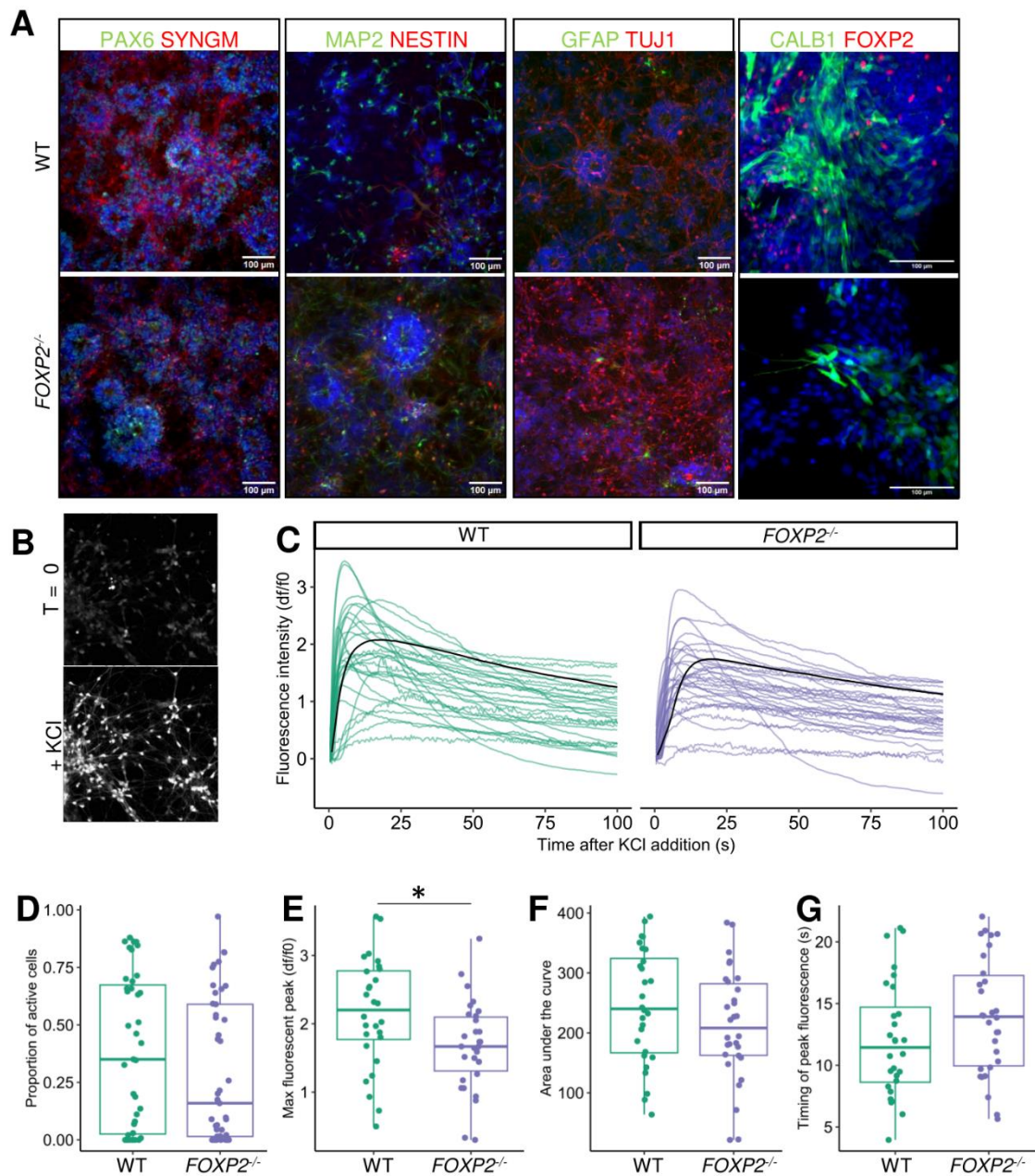


Figure 5.15 Reduced amplitude of depolarisation-triggered calcium peak in FOXP2^{-/-} cerebellar cultures.

- A) iPSC-derived WT and FOXP2^{-/-} cerebellar cultures were stained using antibodies for PAX6 (granule cells and their precursors), SYNGAP1 (synaptic marker), MAP2 (mature neurons), NESTIN (neural stem cells), GFAP (glia), TUJ1 (neurons), CALB1 and FOXP2 (Purkinje cells). Scale bar 100 μ m.
- B) Representative frames before (T=0) and after (+KCl) 50 mM KCl stimulation of iPSC-derived cerebellar cultures.
- C) Relative fluorescent response to KCl stimulation showing intracellular calcium peak. Traces show average response for all active cells in a recording. Black line indicates average trace for all active cells per genotype. Fluorescence calculated relative to background levels prior to KCl addition as df/f0.

Continued.

Figure 4.15 Reduced amplitude of depolarisation-triggered calcium peak in *FOXP2*^{-/-} cerebellar cultures.

- D) The proportion of cells in each recording which displayed a calcium peak in response to KCl stimulation are plotted across genotype. Active cells were determined using minimum peak thresholds based on 'blank' recordings where PBS only was added. Points represent recordings from different wells across six differentiations.
- E-G) The maximum amplitude (E), area under the curve (F) and peak timing (G) of the calcium response was compared across genotypes. Points represent average values for all active cells in a recording. Data from across six individual differentiations. A T-test was applied to determine if values showed significant difference across genotype (*p<0.05).

Cultures were treated with a calcium dye to provide a fluorescent readout of calcium concentration and imaged for 2-minute recordings. Spontaneous calcium activity was observed across both genotypes. However, this spontaneous activity had a small amplitude and was recorded too infrequently in this set up to accurately quantify and compare across lines. Therefore, characterisation focused on the response to stimulation, 50 mM KCl was used to depolarise neurons and trigger a voltage-gated calcium influx (Figure 5.15B & C). Fluorescent traces were extracted for each cell and thresholds based on 'blank' recordings (PBS addition) used to determine actively responding cells. There was a large variation in the proportion of active cells between recordings and no significant difference between genotypes (Figure 5.15D). However, when comparing traces of active cells *FOXP2*^{-/-} cells typically showed a calcium response with slightly smaller and later peak amplitude (Figure 5.15C). The relative peak of fluorescence was significantly decreased in *FOXP2*^{-/-} cells and this difference was observed in five out of six individual differentiation pairs (Figure 5.15E). No significant differences were found in the area under the curve or timing of peak fluorescence between genotypes (Figure 5.15F & G). To summarise, iPSC-derived cerebellar cultures showed a reduced calcium response to KCl depolarisation in the absence of *FOXP2*. Further experiments are necessary to examine whether there is any difference in basal calcium levels or membrane potential between genotypes, which may influence the amplitude of response. However, these results suggest that there may be *FOXP2*-dependent regulation of expression or function of voltage-gated calcium channels in cerebellar neurons.

5.3 Discussion

This chapter has demonstrated the use of cerebellar organoid to model and probe processes of early human cerebellar development. The function of *FOXP2*, a gene associated with development of speech and language, was first profiled in mouse, human and organoid cerebellar development and then studied using fluorescent reporter and knock out iPSC lines.

Expression of *FOXP2* was found to be consistent with published reports, with widespread expression in Purkinje cells and their progenitors by E15.5 onwards (Ferland *et al.*, 2003; Lai *et al.*, 2003; Fujita and Sugihara, 2012). *FOXP2* expression levels in Purkinje cells decreased postnatally and there was a distinct absence in the posterior lobule in the most lateral sections at P7 (Fujita and Sugihara, 2012). *FOXP2* expression was also observed in a subpopulation of DCN neurons (Ferland *et al.*, 2003; Lai *et al.*, 2003; Fujita and Sugihara, 2012). However overall, *FOXP2* was found to be a broad and highly expressed marker of Purkinje cells in embryonic development. Given the functional interactions with other FOXP proteins (Li, Weidenfeld and Morrissey, 2004), the expression of *FOXP1* and *FOXP4* was also characterised in the cerebellum. As literature has previously described, *FOXP1* was found to be restricted to a lateral Purkinje cell population (Wizeman *et al.*, 2019; Sepp *et al.*, 2023). High Purkinje cell expression of *FOXP4* has been described (Tam *et al.*, 2011), but it has not previously been studied in Purkinje cell subtypes. Interestingly, *FOXP4* showed differential expression across embryonic mouse Purkinje cells, suggesting it may also contribute to defining specific Purkinje cell subtypes. By P7, *FOXP4* appeared to be present in all Purkinje cells, including the small population lacking *FOXP2*. As FOXP proteins can form heterodimers, the combination present may result in different FOXP2-regulation of transcription across Purkinje cell subtypes. Moreover, developing Purkinje cells with different levels of *FOXP1* and *FOXP4* may be more vulnerable to *FOXP2* dysfunction which could be responsible for the localised sites of cerebellar structural changes in individuals with *FOXP2* mutations. Structural changes have been identified in the hemisphere of lobule VIIa (crus I) and posterior lobules VII-IIIb (Co, Anderson and Konopka, 2020). In mice these regions may coincide with a Z+ posterior hemisphere Purkinje cell population associated with the early-born *Foxp1+* Purkinje cell embryonic subtype (Sepp *et al.*, 2023). Both *FOXP1* and *FOXP4* showed higher expression in the human early-born embryonic

Purkinje cell subtype, leading to the speculative suggestion that regions with *FOXP1* and *FOXP4* expression could be more affected by *FOXP2* loss. However, more spatially mapping of Purkinje cell subtypes in humans is needed to test for overlap with sites of *FOXP2*-related structural changes.

To validate cerebellar organoids as a suitable model for studying *FOXP* genes, their expression was characterised across differentiation. Cerebellar organoids showed robust induction of *FOXP1*, *FOXP2* and *FOXP4* expression at both an mRNA and protein level. Imaging of *FOXP2* in organoids sections found a high, but not exclusive, level of co-expression with both *FOXP1* and *FOXP4*. This overlap in expression reflected cerebellar *in vivo* expression and suggests that Purkinje cell subtypes may also be present in cerebellar organoids. Whilst in mouse cerebellum *FOXP1*+ Purkinje cells are limited to the more lateral regions, the relatively high proportion of *FOXP1*+*FOXP2*+ cells in cerebellar organoids may reflect an increase in the early-born *FOXP1*+ Purkinje cell subtype in human development. Recent cross-species comparison of embryonic Purkinje cell subtypes, found an increase in the proportion of Purkinje cells with an early-born signature in human compared to mouse (Sepp *et al.*, 2023). The early-born Purkinje cell subtype was characterised by high expression of *FOXP1* and lower expression of *EBF1* and *EBF2* (Sepp *et al.*, 2023). An increase in this early-born Purkinje cell population has been suggested to drive the expansion in peak Purkinje cell numbers during cerebellum development, which is around two-fold higher in human than mouse (Sepp *et al.*, 2023).

In order to further characterise the *FOXP2*+ population in cerebellar organoids, the *FOXP2*-P2A-mNeon reporter line (Chapter 3) was used to isolate the fluorescent *FOXP2*+ population and compare this to non-labelled cells using RNA sequencing. Transcriptomic analysis confirmed successful isolation of a highly *FOXP2*-enriched population, which also had significantly increased expression of other *FOXP* family genes and early Purkinje cell markers. The *FOXP2*+ cerebellar organoid population showed significant upregulation of *SKOR2*, *TFAP2A* and *LHX1* which have been identified as distinguishing Purkinje cells across mammalian species in snRNA-seq (Sepp *et al.*, 2023). Expression of *TFAP2A* has been reported in GABAergic neurons in the VZ, a subset of embryonic Purkinje cells and later broad Purkinje cell expression

in the adult mouse cerebellum (Zainolabidin *et al.*, 2017). In addition, TFAP2A was found to be expressed in a subset of GABAergic DCN neurons and in interneurons in the cerebellar cortex (Zainolabidin *et al.*, 2017). Misexpression of *Tfap2A* increased the population of PAX2+ interneurons in developing mouse cerebellum, suggesting a role in regulating the generation of this cerebellar GABAergic neuron type (Zainolabidin *et al.*, 2017). *SKOR2* is expressed specifically in Purkinje cells and is necessary for Purkinje cell differentiation (Nakatani *et al.*, 2014). Purkinje cells in *Skor2*^{-/-} mice show correct early specification of GABAergic cells in the VZ, but by P0 show signs of disrupted Purkinje cell differentiation, such as ectopic expression of interneuron and glutamatergic markers (Nakatani *et al.*, 2014). *Skor2*^{-/-} mice also showed impaired Purkinje cell migration and dendritic development (Nakatani *et al.*, 2014). *LHX1* has also been shown to be important in early Purkinje cell specification, with almost complete loss of Purkinje cells in *Lhx1/Lhx5* double knock out mice (Zhao *et al.*, 2007). In summary, the *FOXP2*+ cerebellar organoid population shows a significant enrichment for genes that are both highly expressed in and required for successful differentiation of Purkinje cells.

However, there was also upregulation of some genes associated with the DCN, suggesting the *FOXP2*+ population in cerebellar organoids may contain a mixture of Purkinje-like cells and cells with features of DCN neurons. *In vivo*, both Purkinje cells and GABAergic DCN neurons arise the VZ, with DCN neurons produced with other GABAergic interneurons from the more anterior VZ and Purkinje cells from the more posterior (Mizuhara *et al.*, 2010; Seto *et al.*, 2014). PAX2 is expressed in the developing cerebellum prior to P15 in mouse, expression is localised to GABAergic interneurons, including DCN GABAergic neurons, and a stem cell population in the white matter which produces basket and stellate cells (Maricich and Herrup, 1999). PAX2 is not expressed in Purkinje cells and was not significantly altered between organoid sorted populations. However, other markers more specific to the DCN *ZFHX3*, *ZFHX4* and *MEIS2* were significantly increased in the *FOXP2*+ organoid population.

Immunofluorescent imaging of organoid sections stained with antibodies for FOXP2 and ZFHX4 confirmed the presence of co-expressing cells. Recent snRNA-seq analysis of the developing human cerebellum identified a *ZFHX4*+ early population of GABAergic interneurons, which also

showed *MEIS2* expression (Sepp *et al.*, 2023). Moreover, smFISH in 12 PCW human cerebellum confirmed a *ZFHX4*+ population in the forming DCN (Sepp *et al.*, 2023).

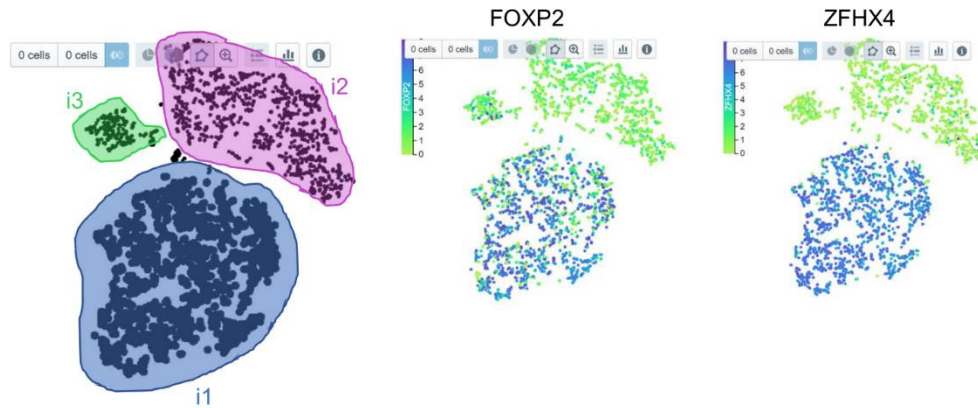


Figure 5.16 Expression of *FOXP2* and *ZFHX4* in human inhibitory DCN neurons.

t-SNE plot showing three clusters of GABAergic DCN neurons identified by (Kebschull *et al.*, 2020). i1 represents GABAergic projection neurons, i2 and i3 are glycinergic cell types. *FOXP2* and *ZFHX4* show high expression (blue) in the i1 cluster compared to lower expression (green) in clusters i2 and i3. Human inhibitory DCN neuron data from (Kebschull *et al.*, 2020) and available at <https://cellxgene.cziscience.com/d/b8032d54-592d-4b46-b308-fea0ac59013b.cxg/>.

In another transcriptomic study focused on the DCN, *ZFHX4* was highly expressed in the inhibitory neuron population projecting to the inferior olive (Kebschull *et al.*, 2020).

Furthermore, *FOXP2* expression was also highest in the *ZFHX4*+ GABAergic DCN cluster in the same study (Figure 5.16). Several markers previously discussed in Purkinje cells have also been reported in the DCN, including *TFAP2A* and *FOXP1* (Zainolabidin *et al.*, 2017; Co, Anderson and Konopka, 2020). The overlap of markers across cell types and bulk sequencing data makes it difficult to ascribe a certain cell type identity to the *FOXP2*+ cerebellar organoid population.

The *FOXP2*+ population may reflect a mixture of Purkinje-like cells and GABAergic DCN projection neuron-like cells, or alternatively consist of a population with features of both cell states due to incomplete specification. However, modelling the *FOXP2*+ GABAergic cell types of the cerebellum remains interesting for studying the cerebellar targets of *FOXP2*.

Furthermore, these results highlight the expression of *FOXP2* in the DCN and thus the possible contribution altered DCN activity in *FOXP2*-disorders, which has not previously been investigated.

To identify potential downstream pathways from *FOXP2*, GSEA was performed to identify processes upregulated in the *FOXP2*⁺ cerebellar organoid population. Terms that significantly increased were related to neuronal activity, including synaptic signalling, neurogenesis and regulation of membrane potential. These results are caveated as the approach used comparison of the isolated *FOXP2*⁺ population, predicted to be neuronal, with the remaining heterogenous organoid population, containing both neural and non-neural cell types. Therefore, a slight enrichment of neural related pathways is expected. However, similar terms have previously been identified when summarising lists of *FOXP2* targets identified by ChIP (Hoed, Devaraju and Fisher, 2021), suggesting that these remain pathways of interest. In future studies, the use of dual reporter lines to label the wider neuronal population in parallel to the cell type of interest could aid comparison between different neuron types. For example, a dual *SOX2*-*SYN1* reporter line has been used to characterise neural progenitor and mature neuron populations in cerebral organoids (Sidhaye *et al.*, 2023).

Following the identification of the mutation in *FOXP2*, and specifically in the forkhead DNA-binding domain, in the affected KE family members, a number of studies sought to identify genes regulated by *FOXP2* using ChIP (Vernes *et al.*, 2006, 2007; Spiteri *et al.*, 2007). However, these studies showed very little overlap in target identification, and also had no significant enrichment in the *FOXP2* sorted cerebellar organoid populations. In contrast, significant overlap was found with a dataset of *FOXP2* differentially expressed genes in a different *in vitro* neural differentiation model (Hickey, Berto and Konopka, 2019). Together, this indicates there may be a common network of *FOXP2* regulated genes involved in neuronal differentiation which is not specific to cell type. This is not uncommon, for example in snRNA-seq of human cerebellum, granule and Purkinje cells shared 56-58% of dynamic genes due to common pathways of neuronal differentiation (Sepp *et al.*, 2023).

The *FOXP2*⁺ cerebellar organoid population also showed enrichment for genesets associated with several neurodevelopmental disorders, highlighting that modelling this cerebellar cell population can be important to understanding of disease. Cerebellar malformations, ASD and SCA genes were significantly increased in the *FOXP2*⁺ population. Additionally, genes

differentially expressed in brains of individuals with ASD showed significant overlap with the sorted organoid populations. Two snRNA-seq studies of the developing human cerebellum also identified a significant enrichment of ASD-associated genes in genes expressed in Purkinje cells compared to other cerebellar cell types (Aldinger *et al.*, 2021; Sepp *et al.*, 2023). The contribution of the cerebellum to language development, social and effective functions, as well as coordination of motor functions, can all relate to symptoms observed in ASD (Sydnor and Aldinger, 2021). Moreover, neuroimaging studies have described some alterations in cerebellar structure, reduced lateralisation of connectivity and reduced GABAergic activity in ASD (Sydnor and Aldinger, 2021). In the cerebral cortex an increased excitation to inhibition ratio, the balance of glutamatergic to GABAergic signalling, has been shown in individuals with ASD (Sydnor and Aldinger, 2021). From studying individuals with ASD and models of the disorder, there is also evidence for a possible reduction in GABAergic signalling in the cerebellum, based on reduction in the number and size of Purkinje cells, decreased expression of enzymes involved in GABA synthesis and a reduction in the number of Purkinje cell synapses (Sathyanesan *et al.*, 2019; Sydnor and Aldinger, 2021). Genes decreased in ASD brains overlapped significantly with genes increased in the *FOXP2*⁺ cerebellar organoid population, whilst genes increased in ASD showed significant overlap with genes decreased in the *FOXP2*⁺ population. This correlation suggests a possible loss of GABAergic *FOXP2*⁺ cerebellar neurons or synaptic connections in the cerebellum of individuals with ASD. Whilst further understanding is still required, there is accumulating evidence for a role of the cerebellum and specifically the *FOXP2*⁺ GABAergic cell types in ASD. Further examination of the functions of genes associated with ASD and enriched in the *FOXP2*⁺ cerebellar organoid population could provide useful mechanistic hypotheses.

To investigate *FOXP2* targets and downstream pathways, cerebellar organoids were generated from *FOXP2*^{-/-} iPSCs and compared to WT organoids. Overall, *FOXP2*^{-/-} cerebellar organoids showed no major changes in growth or bulk gene expression. Of the genes profiled, *ZFHX4* showed the most significant change, with a consistent decrease in *FOXP2*^{-/-} cerebellar organoids compared to WT. *ZFHX4* was selected for characterisation from genes significantly enriched in the *FOXP2*⁺ organoids population, as both a marker of GABAergic DCN neurons

and due to association with CAS (Eising *et al.*, 2019; Sepp *et al.*, 2023). Loss of function mutations in *ZFHX4* have been identified as likely pathogenic in an individual with CAS and in several individuals with intellectual disability and a distinct facial phenotype including ptosis (Palomares *et al.*, 2011; Eising *et al.*, 2019). *ZFHX4* is expressed in the developing brain, liver and muscle (Nogami *et al.*, 2005; Hemmi *et al.*, 2006). It has been suggested to act in neuronal differentiation, including in neural precursors, and has been shown to promote maintenance of a stem cell-like state in glioblastoma (Hemmi *et al.*, 2006; Chudnovsky *et al.*, 2014). In the cerebellum, *ZFHX4* has been identified as a marker for early-born interneurons which contribute to the DCN (Nogami *et al.*, 2005; Sarropoulos *et al.*, 2021; Sepp *et al.*, 2023). Interestingly, *ZFHX4* was identified as a potential FOXP-interacting factor in a mass spectrometry screen in HEK cells (Estruch *et al.*, 2018). Moreover, *ZFHX4* has been shown to interact and regulate CHD4, a core component of NuRD, with which FOXP2 also interacts (Chokas *et al.*, 2010; Chudnovsky *et al.*, 2014). Therefore, *ZFHX4* and FOXP2 could interact directly or via a larger complex of transcriptional regulators around NuRD. The consequences of any interaction between these two transcription factors are not known. In addition, *ZFHX4* has not yet been identified as a direct FOXP2 target in any FOXP2-ChIP studies, so further evidence is also required to understand how *ZFHX4* may be regulated by FOXP2 at a transcriptional level.

Transcriptomic analysis of the *FOXP2*⁺ population in cerebellar organoids found enrichment for pathways related to neuronal activity and synaptic signalling. To test for changes in neuronal functionality on *FOXP2* loss, KCl-stimulated calcium imaging was used to monitor capacity for depolarisation and consequently a voltage-gated calcium influx in iPSC-derived cerebellar cultures. *FOXP2*^{-/-} cerebellar cultures displayed a smaller amplitude of peak response to KCl stimulation. Changes in the relative fluorescent peak may reflect higher resting calcium concentration or a greater resistance to depolarisation due to hyperpolarisation of the membrane. To better understand the reduced responsiveness of *FOXP2*^{-/-} cultures, future experiments using patch-clamp electrophysiology could provide information on the resting membrane potential. In mouse Purkinje cells, *Foxp2* knock out caused increase intrinsic excitability and an increase in simple spike rate (French *et al.*, 2019). However, in D1 spiny

projection neurons in the striatum, *Foxp2* knock out caused hypoexcitability, thought to be mediated via increased expression of *Foxp1* and downstream *Kcnk2*, leading to increased K⁺ leak current (Ahmed *et al.*, 2023). Measurement of the *KCNK2* expression and rate of K⁺ leak currents would therefore also be interesting, to explore whether a similar mechanism occurs in the cerebellar cultures.

Despite the complete loss of *FOXP2* and the decrease in *ZFHX4* expression described, *FOXP2*^{-/-} cerebellar organoids showed only very subtle phenotypes. One explanation for this is the relatively small contribution of the *FOXP2*⁺ population to WT organoids, with *FOXP2* expressed in only 3-5% of cells. Changes in only a small proportion of the organoid population may therefore be too minor to detect, particularly using bulk expression analysis. Alternatively, there may be compensatory action of other genes in response to the lack of *FOXP2*. FOXP proteins show overlapping patterns of expression and have been suggested partially able to replace each other's functions. In mice with regional knock out of *FOXP* genes, *FOXP1* has been shown to compensate for the loss of *FOXP2* in the striatum (Ahmed *et al.*, 2023). In *FOXP2*^{-/-} cerebellar organoids no significant changes were found in *FOXP1* and *FOXP4* at the level of mRNA or protein, however, their presence may still be functional replacement for *FOXP2*.

5.4 Key findings

- In the developing cerebellum FOXP2 was expressed broadly in Purkinje cells and in a subset of GABAergic DCN neurons. FOXP1 and FOXP4 showed localised expressed in specific clusters of Purkinje cells in mouse embryonic development.
- Cerebellar organoids showed a significant induction in *FOXP* gene expression across differentiation and had a high level of co-expression of FOXP2 with FOXP1 and FOXP4.
- A strongly *FOXP2*-enriched population of cells was isolated from cerebellar organoids and characterised by RNA-sequencing. The *FOXP2*⁺ population was significantly enriched for *FOXP1* and *FOXP4*.
- Expression of cell type markers suggested that the *FOXP2*⁺ cerebellar organoid population showed features of Purkinje cells and GABAergic DCN neurons. Significantly enriched genes also included genes with expression localised to the cerebellum.
- Pathway analysis identified a significant upregulation of processes related to neuronal activity and synaptic signalling in the *FOXP2*⁺ cerebellar organoid population.
- The *FOXP2*⁺ cerebellar organoid population also showed enrichment for genes associated with disorders including cerebellar malformations, SCA and ASD. Whilst not significant as a geneset overall, a number of CAS genes were also individually significantly upregulated.
- *FOXP2*^{-/-} cerebellar organoids showed typical expression of cerebellar markers and potential FOXP2 targets identified from profiling the *FOXP2*⁺ population were largely unchanged. However, a significant reduction was found in the expression of transcription factor *ZFHX4* and *FOXP2*^{-/-} cultures showed a reduced amplitude calcium response depolarisation.

Chapter 6. Discussion and future perspectives

This thesis has described the development and application of iPSC-derived models for studying human cerebellar development. The generation of a fluorescent reporter line labelling *FOXP2* expression in Chapter 3, enabled the live tracking and isolation of a Purkinje cell population from heterogeneous iPSC-derived cerebellar cultures. Characterisation of the *FOXP2*+ cerebellar organoid population identified potential downstream genes and pathways regulated by *FOXP2*. A selection of these genes and processes were followed up using cerebellar organoids generated from *FOXP2*^{-/-} iPSCs. In addition, the *FOXP2*-P2A-mNeon reporter line contributed to the screening of conditions focused on improving maturity of human Purkinje cell generation from iPSCs. Through this work, a simplified feeder-free 2D method was developed for human iPSC-derived cerebellar cultures, resulting in more mature Purkinje cells and allowing a range of plate-based assays, including recording neuronal activity.

6.1 Generation of a cell-type specific reporter line enabled study of Purkinje cells in cerebellar organoids

In Chapter 3, the successful generation of a *FOXP2* reporter line was described. CRISPR/Cas9-mediated homology-dependent repair was used to introduce the *mNeon* sequence at the endogenous *FOXP2* locus in human iPSCs. Following differentiation, *FOXP2*-P2A-mNeon cells provided a live readout of *FOXP2* expression in cerebellar cultures. The fluorescent labelling provided a metric for screening of growth conditions, described in Chapter 4, as the Purkinje cell population could be quantified by live imaging or flow cytometry. Furthermore, isolation of the mNeon+ cells by flow cytometry, enriched for *FOXP2*+ cells and enabled detailed transcriptomic analysis of this Purkinje cell population as described in Chapter 5.

Cell type-labelling reporter lines are advantageous for studying complex organoid cultures, immediately distinguishing the cell type of interest from a mixed population. In the future, the construction of reporter lines for additional cerebellar cell types could provide greater insight into the dynamic cell populations in organoids. The use of dual reporter lines could allow cell differentiation trajectories to be monitored, similar to the *SOX2-SYN1* dual reporter line used to characterise neuronal development in cerebral organoids (Sidhaye *et al.*, 2023). In Chapter

5, *FOXP2*⁺ cerebellar cells were compared to all other cells (*FOXP2*⁻) in cerebellar organoids. Based on cell cluster identification in scRNA-seq of cerebellar organoids, the *FOXP2*⁻ population likely contains a mix of neuronal and non-neuronal cell types such as ciliated cells, endothelial cells and glia (Nayler *et al.*, 2021). Therefore, the *FOXP2*⁺ population enriched for neuronal cells and consequently neuronal-related genes and pathways were found to be significantly increased. Combining *FOXP2* with a pan-neuronal marker, such as *MAP2* or *SYN1*, would also allow comparison of *FOXP2*⁺ cells with only other neuron populations to detect more inter-neuronal type specific changes. Alternatively, simultaneous visualisation of the different cerebellar lineages, such as addition of *ATOH1*-driven fluorescence marking granule cell precursors, would be valuable in future development of the cerebellar organoid protocol.

Reporter lines are limited by the specificity of the marker gene used. Whilst *FOXP2* is highly enriched in Purkinje cells, there is also expression in GABAergic DCN neurons and in brain regions close to the cerebellum such as the medulla (Co, Anderson and Konopka, 2020). Therefore, in cerebellar organoids the *FOXP2*⁺ labelled population was found to likely include features of both Purkinje cells and GABAergic DCN neurons. Moreover, when testing new culture conditions, it was important to also include other measurements of cerebellar identity to verify whether factors altered differentiation so considerably that non-cerebellar *FOXP2*⁺ cells were generated. The complexity of biology means few cell types can uniquely distinguished by a single gene. However, cell-type labelling reporter lines still remain very useful, as long as the limitations to labelling specificity are considered.

Whilst the work in this thesis was focused on the function of *FOXP2* in the cerebellum, the newly generated *FOXP2* reporter line could also provide a useful tool for studying *in vitro* models of other brain regions. For example, in the striatum *FOXP2* is highly expressed in dopamine receptor 1 spiny projection neurons, whilst in the cerebral cortex, for which numerous organoid models have been developed, *FOXP2* is specific to layer 5-6 neurons (Co, Anderson and Konopka, 2020).

6.2 Improvements to cerebellar organoid protocol

Chapter 4 described the process of testing different conditions for growth of iPSC-derived cerebellar cultures, with the aim to improve Purkinje cell generation and maturity. scRNA-seq analysis of cerebellar organoids ascribed a Purkinje cell cluster, however this lacked high expression of many commonly used Purkinje cell markers, such as *FOXP2*, *SKOR2*, *RORA* and *LHX1* (Nayler *et al.*, 2021). As a result, Chapter 4 started with a detailed characterisation of the Purkinje cell differentiation achieved using the current xeno-free protocol for long-term organoid culture (Van Essen *et al.*, 2022). Cerebellar organoids were generated and maintained in a 3D format for up to 63 days. From the selected panel of Purkinje cell markers, the majority of early markers showed significant induction during cerebellar organoid differentiation, whilst later markers were less convincingly expressed. This analysis confirmed generation of cells reflective of early human Purkinje cells and provided a benchmark for protocol optimisation.

Next, a recently published method for 2D feeder-free growth of iPSC-derived cerebellar cultures was successfully trialled (Silva *et al.*, 2020). As 2D culture appeared to slightly increase maturity of Purkinje cells, optimisation then focused on further tuning of these conditions. A simple medium of *Differentiation medium (DM)* with 1 cAMP was identified as promoting differentiation of human Purkinje cells. Addition of cAMP in 2D iPSC-derived cerebellar cultures was shown to increase the *FOXP2*⁺ labelled population, increase expression of synaptic markers and result in a more neuronal morphology. This simple medium with defined composition is suggested as an alternative to the commercial BrainPhys medium previously published (Silva *et al.*, 2020).

Development of the 2D culture approach allowed greater flexibility for analysis of iPSC-derived cerebellar neurons, enabling plate-based assays for measuring neuronal activity. Spontaneous neuronal activity was seen to develop in iPSC-derived cerebellar cultures by recording of both extracellular field potential by MEA and intracellular calcium responses by fluorescent imaging assays. In addition, the development of spontaneous neuronal activity was shown to be promoted by addition of cAMP to growth medium. Together, these results defined and demonstrated growth conditions for the consistent generation of functional cerebellar

neurons from human iPSCs. Building on this work using control iPSCs, measurements of neuronal activity in iPSC-derived cerebellar cultures could in the future be used to study the effect of patient mutations on cerebellar neuron excitability. One limitation to the assays used was that they measured whole population or all neurons activity. However, given the heterogeneous cell types present in iPSC-cerebellar culture, future work should examine electrical activity of individual cell populations. This could be done by focusing calcium imaging or patch-clamp recording on mNeon-labelled Purkinje cells derived from the *FOXP2* reporter line.

Throughout the process of cerebellar differentiation protocol optimisation several lessons were learnt about effective screening and assessment of treatments. The additional dissociation and plating step increased batch-to-batch variability between differentiations making it difficult to assess the impact of different factors without sufficient replicates. Another challenge was selection of a representative, but manageable number of parameters to assess conditions. Methods such as comparison of morphology and live cell imaging of mNeon+ cells had higher throughput than sample analysis by immunostaining and RT-qPCR. Therefore, for more efficient development in the future, initial screening may use predominantly longitudinal live cell approaches such as observation of survival, morphology and the mNeon+ population, before more detailed characterisation of selected conditions. In addition, the signalling present *in vivo* is complex and a combination of factors are likely to achieve the best results. Combinatorial testing can be analysed using linear regression to determine individual effects, however this relies on reliable readouts (Washer *et al.*, 2022). Finally, the 3D organoid culture system maintained a number of advantages such as more stable long-term culture and greater tissue organisation. Moreover, a number of markers expressed from early in Purkinje cell development, such as *FOXP2* and *SKOR2*, remained higher in 3D organoids. As a result, the organoid system should not be abandoned. Flow cytometry-based quantification of mNeon+ *FOXP2*-expressing cells could aid screening of different medium conditions for improvements of Purkinje cell generation in 3D.

6.3 The role of *FOXP2* in the early human cerebellum

The promise for the study of human cerebellar development using iPSC-derived cerebellar organoids was demonstrated in Chapter 5. A combination of evidence points to an important role for *FOXP2* in cerebellar development and Purkinje cell function. First, structural and neuronal activity differences have been described in the cerebellum of individuals affected by *FOXP2*-driven disorders (Watkins *et al.*, 2002). Second, global and Purkinje cell-specific *Foxp2* mouse mutants have characterised alterations in Purkinje cell dendritic development and electrical activity (Shu *et al.*, 2005; Fujita *et al.*, 2008; Usui *et al.*, 2018; French *et al.*, 2019). More qualitatively, several recent scRNA-seq studies have identified *FOXP2* as having highly enriched Purkinje cell expression, particularly early in human development (Aldinger *et al.*, 2021; Sepp *et al.*, 2023). However, relatively little is known about *FOXP2* function in early stages of cerebellar development, when *FOXP2* expression peaks and Purkinje cells differentiate and mature. Protocols for cerebellar differentiation of human iPSCs model the specification of the cerebellum and initiation of Purkinje cell differentiation, and thus provide the ideal system to study the role of *FOXP2* in these processes.

To study the function of *FOXP2* in the developing human cerebellum, cerebellar organoids were generated from the *FOXP2*-P2A-mNeon iPSC line, dissociated and sorted for the mNeon+ *FOXP2*-expressing population. Transcriptomic characterisation of this *FOXP2*+ population confirmed a predominantly Purkinje cell identity. However, *FOXP2* was not required for Purkinje cell generation, as *FOXP2*^{-/-} organoids showed no significant changes in panel of Purkinje cell markers. Likewise, human patients with *FOXP2* mutations and the majority of mouse models also show no significant reduction in number of Purkinje cells (Co, Anderson and Konopka, 2020). A lack of observed differences in Purkinje cells in *FOXP2*^{-/-} cerebellar organoids is also reflective of their immaturity, meaning it was not possible to compare processes such as dendritic development which has been impaired in *Foxp2*-KO mouse models (Shu *et al.*, 2005; Fujita *et al.*, 2008; Usui *et al.*, 2018). Upregulation of some GABAergic DCN markers was also present in the *FOXP2*+ population indicating a possible mixture of cell types. Interestingly, *FOXP2*^{-/-} cerebellar organoids did show a reduction in *ZFHX4* but not in *MEIS2*, both markers of the GABAergic DCN neurons. Therefore, differences in DCN development and

function warrant further investigation in relation to the role of *FOXP2* in cerebellar function. GABAergic neurons in the DCN have an important contribution to negative feedback on the cerebellum both via the inferior olive and within the cerebellum, so disruption to this cell population would also impair cerebellar function. Another consideration is the identification of *Zfmx4* in a subpopulation of Purkinje cells in scRNA-seq analysis of the developing mouse cerebellum (Wizeman *et al.*, 2019). *Zfmx4* was found to be a significant marker of a *Foxp1*⁺ Purkinje cell cluster and was confirmed to also have increased expression in an early-born Purkinje cell clusters in a second study (Sepp *et al.*, 2023) (Figure 1.6B). Therefore, decreased *ZFHX4* in *FOXP2*^{-/-} cerebellar organoids may suggest a shift in Purkinje cell subtypes, reducing the population showing an early-born *FOXP1*⁺ cluster phenotype. However, there was no significant change found for *FOXP1* in the *FOXP2*^{-/-} organoids, so further study is required to support this hypothesis. The early-born Purkinje cell phenotype is of particular interest in human development as an expansion of this Purkinje cell subtype was found in snRNA-seq analysis of the developing human cerebellum (Sepp *et al.*, 2023).

Differential gene expression analysis found enrichment for pathways related to synaptic signalling and neuronal activity in the *FOXP2*⁺ cerebellar organoid population, suggesting a role for *FOXP2* in promoting neuronal communication. Further evidence for this function was observation of a reduced voltage-gated calcium response in *FOXP2*^{-/-} cerebellar cultures. In future studies, it would be interesting to apply patch-clamp recording to iPSC-derived cerebellar cultures. This would allow more detailed characterisation of individual cells, comparing the *FOXP2*⁺ population to other, using *FOXP2*-P2A-mNeon iPSC-derived cerebellar cultures. Patch-clamp analysis could also investigate the mechanisms behind the reduced calcium response in *FOXP2*^{-/-} cells, such as measuring the baseline membrane potential and recording action potentials. Another interesting observation from the transcriptional analysis of the *FOXP2*⁺ cerebellar population, was correlating changes in differentially expressed genes with a published *FOXP2* differential expression study. This observation suggested that there may be common neuronal networks regulated by *FOXP2*.

The *FOXP2*-labelled cerebellar organoid population was also enriched for genes associated with ASD. Further study of this population may therefore be interesting for study of possible mechanisms behind ASD such as a shift in excitability, particularly as there is increasing evidence for the involvement of the cerebellum in this disorder (Sydnor and Aldinger, 2021). Whilst there was no significant enrichment for CAS-associated genes overall, several showed significant enrichment in the *FOXP2*⁺ population. Enriched genes included *ZFHX4* described above. Mutations in *ZFHX4* are responsible for cases of CAS and intellectual disability (Palomares *et al.*, 2011; Eising *et al.*, 2019). Expression of multiple genes related to speech development were found to be expressed in cerebellar organoids, including *ZFHX4* and *FOXP2* showing linked expression. This work demonstrates the potential for further investigation of language-associated gene networks in the developing human cerebellum, using iPSC-derived models.

In the future, wider transcriptomic characterisation of *FOXP2*^{-/-} cerebellar organoids by RNA-sequencing could aid broader identification of *FOXP2*-regulated genes in the early human cerebellum. A more detailed examination of possible Purkinje cell subtypes is needed to test the hypothesis that these are present cerebellar organoids and the proportions may be shifted as a result of *FOXP2* loss. This could be tested by simultaneous immunofluorescent imaging of *FOXP1*, *FOXP2* and *FOXP4*, or other subtype markers such as *RORB*, *ETV1* and *CHD9*.

6.4 Future perspectives

In this thesis, the differentiation of human iPSCs was successfully used to model early human cerebellar development and give insight into targets regulated by language-associated gene *FOXP2* in the cerebellum. The generation of a Purkinje cell reporter line and new methods for culturing iPSC-derived cerebellar neurons developed in this work, can be used to discover more about human Purkinje cell development and function in the future. Following the recent publication of histological and snRNA-seq analysis of the human cerebellum (Haldipur *et al.*, 2019; Aldinger *et al.*, 2021; Sepp *et al.*, 2023), iPSC-derived models of human cerebellar development will provide systems to probe mechanisms and test functional differences suggested by these datasets.

Chapter 7. References

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