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Characterising the interaction between proneural transcription factor ASCL1 and mSWI/SNF chromatin remodelling complexes during human cortical development

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**Characterising the interaction between proneural
transcription factor ASCL1 and mSWI/SNF chromatin
remodelling complexes during human cortical
development**

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and

The Francis Crick Institute

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A thesis submitted for the degree of
Doctor of Philosophy
King's College London

September 2021

Declaration

I, Oana Păun, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

A specialised set of transcription factors called pioneer factors are able to bind their targets in previously inaccessible chromatin and upon binding create accessible regions of DNA. Their activity allows non-pioneer transcription factors to also bind their targets, regulate downstream gene expression and establish gene regulatory networks during development. In the developing mammalian cortex, one of the most illustrative examples of a stable yet versatile system, proneural transcription factors of the bHLH family represent key determinants of neural cell fate and differentiation. Among the proneural proteins, ASCL1 has been proposed to act as a pioneer transcription factor by programming the epigenome and establishing new transcriptional networks during development and cellular reprogramming in both mouse and human models. The mSWI/SNF ATP-dependent chromatin remodelling complexes play critical roles in controlling chromatin dynamics, therefore facilitating rapid transcriptional events. Proper functioning of the mSWI/SNF complexes is essential for the establishment, maintenance and functionality of neural cells during development.

The overlapping activity of ASCL1 and mSWI/SNF remodellers during neurogenesis led us to investigate the hypothesis of a mutual interaction between them. Using an *in vitro* model of human cortical neuronal differentiation from iPSCs, I have established that ASCL1 interacts physically with multiple subunits of the mSWI/SNF complexes. To further characterise this interaction, I investigated whether ASCL1 requires the mSWI/SNF remodellers to regulate its targets. By comparing the DNA binding landscapes of ASCL1 and mSWI/SNF core subunit SMARCB1, I found that

approximately 70% of ASCL1 binding sites are also genomic targets of SMARCB1. This finding suggests that ASCL1 may functionally interact with mSWI/SNF complexes in order to regulate a large subset of its targets.

I then performed reciprocal disruption of ASCL1 and mSWI/SNF assemblies at different time points during corticogenesis to investigate the mutual requirement of ASCL1 for mSWI/SNF recruitment. Correlation of DNA binding and chromatin accessibility in ASCL1 knockout, mSWI/SNF-lacking and wild-type neuronal cells revealed that approximately one third of ASCL1 direct targets are also direct targets of the mSWI/SNF remodellers. In addition, 55% of the ASCL1-dependent genes are also misregulated upon mSWI/SNF removal. Association of ASCL1-mSWI/SNF direct genomic targets with the transcriptional changes observed in the two mutants led to the identification of 61 ASCL1-mSWI/SNF-dependent genes with essential roles during cortical neuronal differentiation whose regulation is linked to the sites where ASCL1 and SMARCB1 bind to regulate chromatin accessibility. However, more than 80% of the ASCL1-mSWI/SNF direct targets represent distal genomic sites with enhancer-specific histone modification signatures. As a consequence, looking at the nearest annotated promoter to associate these genomic regions with their transcriptional output might only explain a subset of the ASCL1-dependent genes. More extensive bioinformatics approaches that take into consideration the 3D organisation of the genome are likely to link these distal regulatory regions with a larger proportion of the ASCL1-mSWI/SNF-dependent genes. Overall, this work advances our understanding of the mechanisms behind ASCL1 pioneer activity. The essential roles of both ASCL1 and mSWI/SNF remodellers during cortical development point towards their interaction having vast implications for human health and disease.

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ADAM10	ADAM metallopeptidase domain 10
aIP	Apical intermediate progenitor
aRG	Apical radial glia
ASCL1	Achaete-scute like 1; Mash 1
ATAC-seq	Assay for transposase accessible chromatin with high-throughput sequencing
ATP	Adenosine triphosphate
BAF	BRG1/BRM-associated factor complex
BCA	Bicinchoninic acid assay
bHLH	Basic helix-loop-helix
bIP	Basal intermediate progenitor
BLBP	Brain lipid-binding protein
BMP	Bone morphogenetic pathway

BP	Basal progenitor
bRG	Basal radial glia
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CGE	Caudal ganglionic eminences
CHD	Chromodomain, helicase, DNA binding
ChIP-seq	Chromatin immunoprecipitation coupled with massively parallel DNA sequencing
CNS	Central nervous system
Co-IP	Co-immunoprecipitation
CP	Cortical plate
CRISPR	Clustered regularly interspaced short palindromic repeats
Ct	Cycle threshold
CTNNB1	Catenin Beta 1
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
dsDNA	Double stranded DNA
EB	Embryoid body
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid

FDR	False discovery rate
FGF	Fibroblast growth factor
GABA	γ -aminobutyric acid
GLAST	Glutamate-aspartate transporter
GO	Gene ontology
gRNA	Guide RNA
GTP	Guanosine triphosphate
HAS	Helicase-SANT
HATs	Histone acetyltransferases
HBSS	Hanks' balanced salt solution
HES	Hairy enhancer of split
hESCs	Human embryonic stem cells
HOPX	Homeodomain-only protein
Id	Inhibitor of differentiation
IF	Immunofluorescence
IGF	Insulin-like growth factor
IGV	Integrative genomics viewer
iN	Induced neuronal
INO80	Inositol requiring 80
Insm1	Insulinoma-associated 1
IP	Immunoprecipitation
IPs	Intermediate progenitors

iPSCs	Induced pluripotent stem cells
iSVZ	Inner subventricular zone
ISWI	Imitation switch
IZ	Intermediate zone
KSR	Knockout serum replacement
LB	Luria broth
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LGE	Lateral ganglionic eminences
LIM-HD	LIM homeodomain
MACS	Model-based analysis of ChIP-seq
MGE	Medial ganglionic eminences
mRNA	Messenger RNA
mSWI/SNF	Mammalian Switch/Sucrose - Nonfermentable
ncBAF	Non-canonical BAF
NDS	Normal donkey serum
NE	Neuroepithelial
NGN1	Neurogenin 1
NGN2	Neurogenin 2
NICD	Notch intracellular domain
NPCs	Neural progenitor cells
NSCs	Neural stem cells

OPCs	Oligodendrocyte precursor cells
oRG	Outer radial glia
oSVZ	Outer subventricular zone
PAX6	Paired Box 6
PBAF	Polybromo-associated BAF complexes
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCW	Post-conceptual weeks
PFA	Paraformaldehyde
PLA	Proximity ligation assay
PR	Progesterone receptor
PTCH1	Patched
PVDF	Polyvinylidene fluoride
pVIM	Phosphorylated Vimentin
qRT-PCR	Quantitative real time polymerase chain reaction
RA	Retinoic acid
REST	Repressor-element-1-silencing
RG	Radial glia
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
ROCK	Rho-associated kinase
RSB	ATAC resuspension buffer

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
Shh	Sonic hedgehog
SMO	Smoothened
SOX	Sry HMG-box
SVZ	Subventricular zone
TALEN	Transcription activator-like effector nucleases
TBR	T-box brain gene
TBS-T	Tris buffered saline Tween
TGF β	Transforming growth factor β
TGS	Tris-glycine-SDS
TLX	T-cell leukemia homeobox
TPM	Transcripts per million
TSS	Transcription start site
TUJ1	Class III β -tubulin
VENs	von Economo neurons
VZ	Ventricular zone
WT	Wild-type
YAP	Yes-associated protein 1

Chapter 1

Introduction

The mammalian cortex is considered the largest site of neural integration in the central nervous system (CNS), where critical integrative and executive functions are performed (Rakic, 2009). All mammals have the outer surface of their brain covered by a six-layered cerebral neocortex. As a consequence, the generation of the mammalian neocortex involves rapid and time-specific transcriptional events, which result from critical interactions between neural transcription factors and their epigenetic modifications during neurogenesis.

This chapter begins with an overview of the mammalian cerebral cortex development and how the pluripotent stem cell (PSC) technology can be used to mimic different neurodevelopmental processes *in vitro*. Next, I will focus on the role of the proneural transcription factor achaete-scute complex homolog-like 1 (ASCL1) and on the epigenetic modifications that occur during neurogenesis, highlighting the roles of the mammalian Switch/Sucrose-Nonfermentable (mSWI/SNF) chromatin re-

modelling complexes. Finally, I will describe specific interactions between chromatin remodellers and pioneer transcription factors, focusing on their roles during development.

1.1 Mammalian cerebral cortex development

The cerebral neocortex is the central region in the mammalian brain, which is responsible for controlling complex cognitive behaviour (Kaas, 2012; Geschwind and Rakic, 2013). It originates from the neuroepithelial (NE) cells located in the most rostral region of the early embryonic mammalian neural tube (Rubenstein et al., 1998). The development and growth of the mammalian cortex relies on the neural stem cells (NSCs) and neural progenitor cells (NPCs), which divide and eventually give rise to post-mitotic neurons and the two main types of microglial cells, astrocytes and oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009). It is therefore essential to understand the mechanisms regulating the stem cell and progenitor populations in order to have a better understanding of how the mammalian cortex develops.

1.1.1 Stem cell and progenitor populations during cortical development

Based on their morphology, gene expression and differentiation potential, there are multiple populations of stem and progenitor cells described in the developing cerebral cortex. Most of these cell populations originate from the sheet of NE cells of the neural tube. NE cells are multipotent stem cells, which initially undergo rapid symmetric divisions (Rakic, 1995), resulting in the lateral expansion of the NE sheet

that, in turn, leads to the closure of the neural tube and initiation of the ventricular system (Martynoga et al., 2012).

At mid-gestation, NE cells generate neurons directly (Haubensak et al., 2004) and, coincident with the start of cortical neurogenesis, they switch to asymmetric differentiative cell division (Götz and Huttner, 2005; Huttner and Kosodo, 2005). This means that one of the two resulting daughter cells becomes either a NE cell or a highly related cell type called apical radial glia (aRG) (Hartfuss et al., 2001), whereas the other daughter cell can become an apical intermediate progenitor (aIP), a basal progenitor (BP) or a post-mitotic neuron. This great variety of cell types leads to the transformation of the neuroepithelium into a heterogenous pseudostratified tissue where different cell types are spatially separated: the apical progenitors (including NE cells, aRGs, and aIPs) are located in the ventricular zone (VZ), the apical-most germinal layer of the cortical wall, whereas the BPs form a germinal layer that is located basally to the VZ and is called the subventricular zone (SVZ). With cells continuously dividing, the cortical wall progressively thickens and elongates, generating a scaffold for neuronal migration called the intermediate zone (IZ) (Rakic, 1972). The newly born neurons therefore use this scaffold to migrate in the basal direction from the VZ and SVZ and accumulate in the future cortical plate (CP) (Figure 1.1) (Florio and Huttner, 2014).

Highly related to the NE cells, aRGs retain a neuroepithelial character. However, the tight junctions between NE cells are replaced by apically located adherent junctions (Aaku-Saraste et al., 1996), while they start expressing astroglial markers such as GLAST (Glutamate-aspartate transporter) and BLBP (Brain lipid-binding protein) (Malatesta et al., 2000), as well as specific transcription factors such as PAX6 (Götz, Stoykova, et al., 1998; Estivill-Torres et al., 2002). Multiple modes of

division have been described for the aRGs. These include symmetric proliferative division, with two aRGs being generated, symmetric differentiative division, with two neurons being generated, or asymmetric division, which results in one aRG and one neuron being generated (Figure 1.1) (Miyata, Kawaguchi, Okano, et al., 2001; Miyata, Kawaguchi, Saito, et al., 2004; Noctor et al., 2004). A more particular type of aRG daughter cell is represented by the aIPs, also known as short neural precursors (Gal et al., 2006; Tyler and Haydar, 2013). These cells downregulate astroglial markers and lose the self-renewal potential, in mouse undergoing only one round of symmetric cell division, which results in a pair of neurons being produced (Figure 1.1) (Gal et al., 2006; Stancik et al., 2010; Tyler and Haydar, 2013).

BPs originate from either NE cells or aRGs, with two distinct types being distinguished: basal intermediate progenitors (bIPs) and basal radial glia (bRGs). bIPs lose the expression of astroglial markers and, during their migration to the SVZ, upregulate the transcription factor TBR2 (Englund et al., 2005; Cappello et al., 2006). Based on their mode of division, bIPs are further divided into neurogenic bIPs and proliferative bIPs. Similar to the aIPs, the neurogenic bIPs undergo one round of symmetric division and generate two neurons, whereas proliferative bIPs (also called 'transit amplifying cells' (Hansen et al., 2010; Lui et al., 2011) or 'transit amplifying progenitors' (Fietz, Lachmann, et al., 2012)) can either generate bRGs or divide symmetrically multiple times before undergoing a symmetric neurogenic division (Noctor et al., 2004; Hansen et al., 2010; Betizeau et al., 2013). On the other hand, most bRGs retain the expression of PAX6 and astroglial markers, while a proportion of them also co-express TBR2 (Betizeau et al., 2013). In a similar way to aRGs, bRGs divide both symmetrically, resulting in two daughter bRGs or two neurons, and asymmetrically, generating one bRG and one neuron or glial cell (Fig-

Figure 1.1) (Hansen et al., 2010; Reillo et al., 2011; Betizeau et al., 2013; LaMonica et al., 2013).

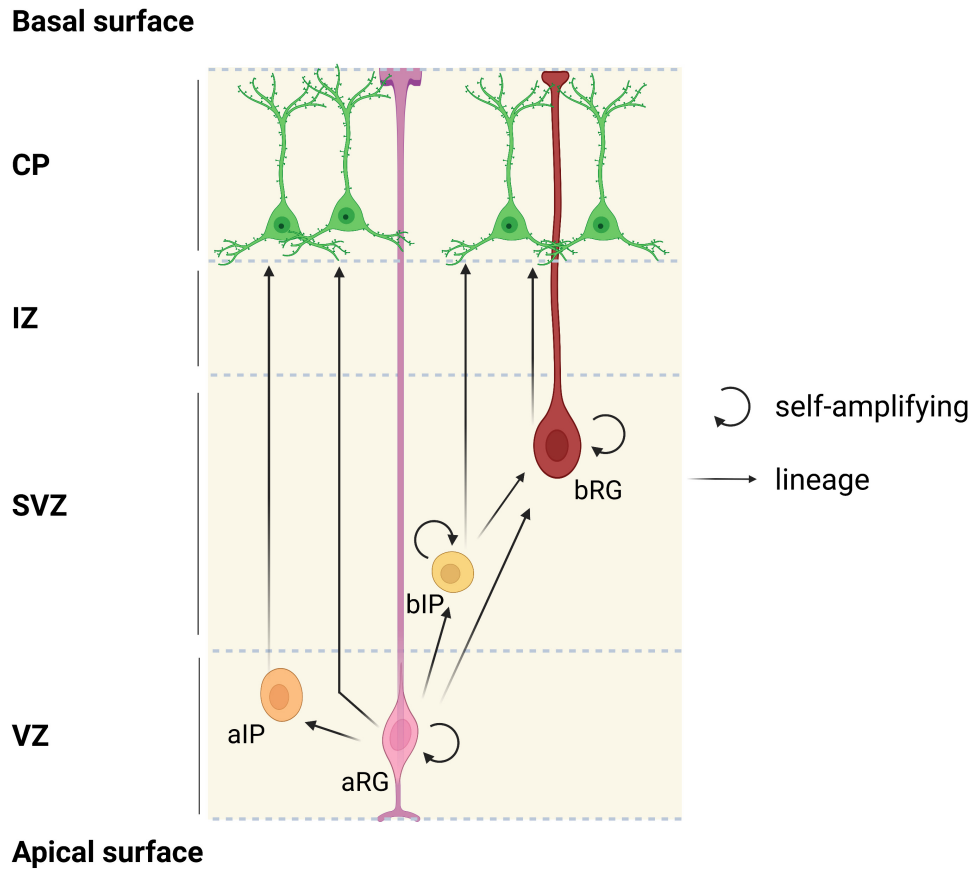


Figure 1.1: Neural progenitor cell types and their lineages.

Diagram showing the neural progenitor cell types and their lineages during the developing mammalian cortex. Both aRG cells and aIPs are located in the VZ. aRG undergo both self-amplification or differentiation into aIPs, BPs or neurons, while aIPs only undergo one round of neurogenic division. bIPs and bRGs are two different types of BPs which reside in the SVZ, both of them being able to self-amplify or generate neuronal cells. In addition, bIPs are also capable of generating bRGs. Figure adapted from (Florio and Huttner, 2014) with permission from the rights holder, Elsevier and created with BioRender.com.

1.1.2 Regulation of progenitor proliferation and neuronal differentiation

1.1.2.1 Extrinsic mechanisms

The balance between progenitor self-renewal and neuronal differentiation, as well as the suppression of the astrocytic lineage during the neurogenic phase, are essential during cortical development. These events are regulated by a complex network of interactions between multiple signalling pathways (Figure 1.3), transcriptional mechanisms and their dynamic expression patterns, different modes of cell division and cell cycle dynamics. A comprehensive discussion of all these complex mechanisms is beyond the scope of this introduction. I will instead focus on the signalling mechanisms and the transcriptional regulation events occurring during early cortical development; these will become relevant when the working model system will be discussed, as well as the experiments that were performed at specific stages during the neurogenesis process.

Notch activity in the dorsal telencephalon is one of the first master regulators during cortical development and it coincides with the onset of neurogenesis, when it promotes the NE to RG transition (Gaiano et al., 2000), as well as inhibits the generation of BPs from RGs (Mizutani et al., 2007). Notch signalling at this stage is supported by the detection of canonical Notch ligands from the Jagged (JAG1 and JAG2) and Delta-like (DLL1, DLL2, DLL4) families (Zhang et al., 2013). Ligand binding to the Notch receptors leads to the cleavage of the receptor by ADAM metallopeptidase domain 10 (ADAM10) and γ -secretase, which releases the Notch intracellular domain (NICD) (Brou et al., 2000; Mumm et al., 2000). NICD then translocates into

the nucleus, where it binds CBF1 or RBPJ co-factor and induces specific transcriptional changes (Figure 1.2), including the activation of Hairy enhancer of split (*Hes*) genes. Among the *Hes* gene family, *Hes1* and *Hes5* represent main downstream effectors of Notch activity (Hatakeyama et al., 2004). The expression of both *Hes1* and *Hes5* oscillates due to an auto-inhibitory feedback loop (Hirata et al., 2002; Haubensak et al., 2004), this way generating oscillatory expression patterns of the basic helix-loop-helix (bHLH) proneural transcription factors (Imayoshi, Sakamoto, et al., 2008; Shimojo et al., 2008). This dynamic expression is critical in controlling proliferation and neurogenic differentiation, with high expression levels of *Hes1* and *Hes5* inhibiting neuronal differentiation and maintaining the long-lasting progenitor potential of the RGs (Kageyama et al., 2008). Conversely, when their expression levels are low, neurogenic differentiation occurs (Hirata et al., 2002; Hatakeyama et al., 2004; Baek et al., 2006; Shimojo et al., 2008).

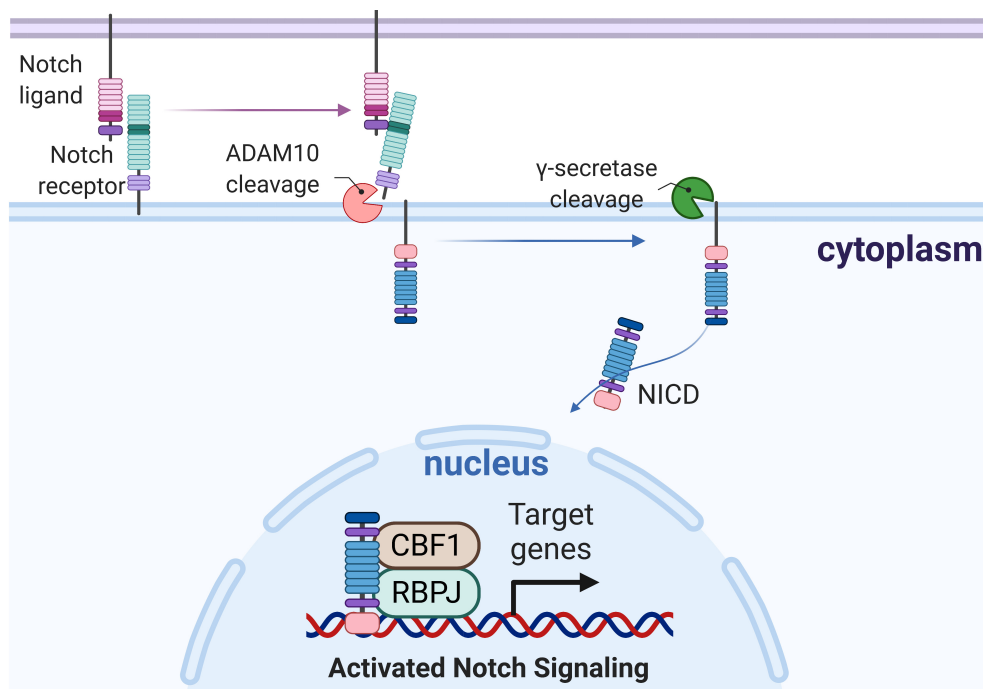


Figure 1.2: Canonical Notch signalling pathway. (Legend next page)

Figure 1.2: Canonical Notch signalling pathway.

Canonical Notch ligands bind to Notch receptors, resulting in sequential cleavages of the receptor by ADAM10 and γ -secretase. The cleavages result in the release of NICD, which translocates into the nucleus, where it binds CBF1 or RBPJ co-factor and regulates gene expression. Figure created with BioRender.com.

Similarly to Notch signalling, fibroblast growth factor (FGF) signalling has been associated with the NE to RG transition (Yoon et al., 2004; Sahara and O'Leary, 2009), while inhibiting the progression of RGs to BPs (Kang et al., 2009). Although many FGF ligands are expressed in the developing brain, FGF10 is considered the most relevant one during the NE to RG transition: *Fgf10* overexpression experiments showed the induction of RG specific markers, whereas the complete lack of *Fgf10* in mouse embryos resulted in NE expansion and delayed neurogenesis (Sahara and O'Leary, 2009). Its role in the regulation of *Hes1* transcription suggests FGF activity is synergistic with and promotes Notch signalling (Yoon et al., 2004; Rash et al., 2011). FGF ligands promote proliferation of cortical progenitors and inhibit neurogenesis (Raballo et al., 2000; Rash et al., 2011; Matsumoto et al., 2017) by regulating the duration of the cell cycle (Lukaszewicz et al., 2005; Lange et al., 2009; Pilaz et al., 2009; Arai, Pulvers, et al., 2011). In addition to its pro-proliferative role, FGF signalling is also involved in the dorsoventral patterning of the cortex, with FGF2 ligand being highly expressed dorsally, but at a low level ventrally. Loss of FGF2 alone changes the dorsal cortex specification (Rash et al., 2011).

Wnt extracellular signalling is tightly regulated in time and space during mammalian development. In the developing dorsal telencephalon a Wnt gradient regulates cell identity along the lateral-medial axis (Machon, Backman, et al., 2007), suggesting that the dose of Wnt activity is involved in cell fate specification during the development of the cortex. While Wnt signalling may not be required for the specification

of RGs (Martynoga et al., 2012), it is a context dependent regulator of neuronal production. Gain and loss of function experiments have shown that, at an earlier stage, Wnt activity promotes the symmetric division of RG progenitors (Chenn and C., 2002; Machon, Bout, et al., 2003; Woodhead et al., 2006; Wrobel et al., 2007). At a later stage, Wnt signalling promotes the maturation of RGs to BPs, as well as the proliferation of newly-generated BPs (Viti et al., 2003; Hirabayashi, Itoh, et al., 2004). In addition, Wnt function has also been linked to the differentiation of BPs into neurons through the direct regulation of bHLH transcription factors *N-myc*, *Neurogenin 1 (Ngn1)* and *NeuroD1* (Hirabayashi, Itoh, et al., 2004; Kuwahara et al., 2014).

Not involved in the specification of RG cells, retinoic acid (RA) signalling has also been shown to play an important role during RG proliferation and further progression downstream the neuronal differentiation lineage. RGs seem to be normally specified in the absence of RA activity, but they are blocked in a state of symmetric division, and fail to generate any neurons (Siegenthaler et al., 2009). RA signalling has also been reported to be a regulator of the *Insulinoma-Associated 1 (Insm1)* (Haushalter et al., 2017), a transcriptional regulator which is both necessary and sufficient for BPs generation (Farkas et al., 2008).

Bone morphogenetic pathway (BMP) signalling represents another important pathway for the regulation of cortical neurogenesis. BMPs are constituents of the transforming growth factor β (TGF- β) superfamily of extracellular morphogens (Derynck and Zhang, 2003; Shi and Massagué, 2003), which have been shown to have multiple roles during cortical development, including patterning, proliferation, survival, differentiation and migration (Rodríguez-Martínez et al., 2012). BMP signalling is initiated upon the binding of a BMP ligand to one of the two types (type I or type II)

of BMP serine-threonine kinase receptor complex (Heldin et al., 1997; Derynck and Zhang, 2003). Although BMPs are able to bind both types of receptors, they have a higher affinity for type I receptors, type of binding known as canonical BMP signalling (Weber et al., 2007; Yadin et al., 2016; Goebel et al., 2019). Upon ligand binding, type I receptors become activated and induce the phosphorylation of different members from the SMAD family of transcription factors (Ebendal et al., 1998), which translocate to the nucleus and modulate target gene expression (Liu and Niswander, 2005; Bond et al., 2012). In the gastrulating mouse embryo, BMP activity is initially suppressed, mainly by FGF and insulin-like growth factor (IGF) signalling pathways (Nishimura et al., 2000; Wilson et al., 2000; Pera et al., 2003), which allows the formation of the neural plate and therefore neural induction (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou, Kelly, et al., 1994). However, following neural induction, BMP signalling is instrumental for the induction of the roof plate and, by interacting with Wnt activity, for the dorsomedial patterning of the telencephalon (Placzek and Briscoe, 2005; Kiecker and Lumsden, 2012; Bond et al., 2012). During the early stages of cortical development, BMP signalling regulates differentiation of cortical progenitors into neurons, as shown by *in vitro* experiments, where the addition of BMP to cortical progenitor cultures results in neuronal differentiation (Lee et al., 1998; Mabie et al., 1999). In addition, deletion of BMP receptors *in vivo* leads to a reduction in neurogenesis in the caudal regions of the CNS (Wine-Lee et al., 2004). There have been suggestions that BMP and Notch pathways may converge in regulating specific targets, such as promoting *Hes3* expression and inhibiting DNA-binding factor genes (*Ids*) (Imayoshi, Sakamoto, et al., 2008). On the other hand, at a later stage, BMPs have been shown to suppress neurogenesis and promote glial differentiation (Gross et al., 1996).

Sonic hedgehog (Shh) signalling has a long-known role in the developing fore-brain. It is a diffusible secreted protein (Echelard et al., 1993; Roelink et al., 1994), which functions through a receptor complex composed of the Patched (PTCH1) and Smoothed (SMO) transmembrane proteins. While Shh activity is critical during the early patterning of the ventral telencephalon (Fuccillo et al., 2004; Hébert and Fishell, 2008; Xu et al., 2010), its function in the developing cortex has not been extensively characterised. However, it has been suggested that Shh activity has an indirect impact on cortical patterning, through regulation of FGF signalling (Hayhurst et al., 2008). Previous studies have also linked Shh activity to growth and folding of the neocortex. Interfering with Shh has a direct effect on the number of progenitor cell populations, with constitutive activation resulting in increased numbers of bRGs and IPs, whereas inhibition of Shh has the opposite effect, leading to significantly less bRGs and IPs (Komada et al., 2008; Wang, Hou, et al., 2016).

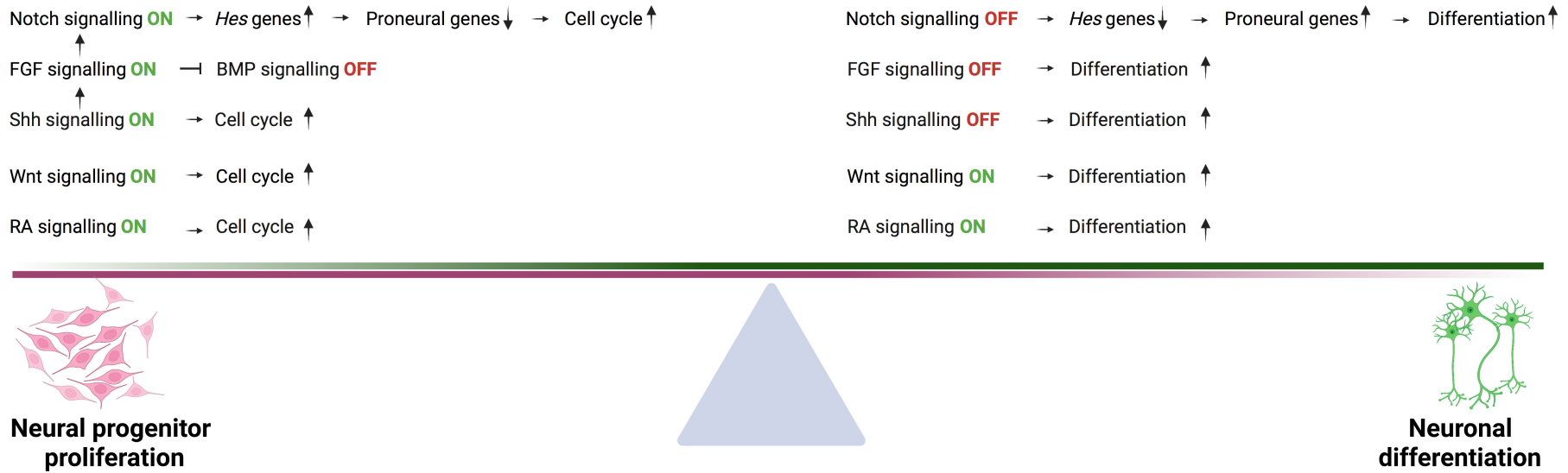


Figure 1.3: Signalling pathways regulating the proliferation-differentiation balance.

Summary of the signalling pathways involved in regulating the balance between progenitor self-renewal and neuronal differentiation in the developing mammalian cortex. Figure created with [BioRender.com](https://www.biorender.com).

1.1.2.2 Transcriptional mechanisms

The complex network of interactions between the different signalling pathways and intrinsic genetic controls ultimately converge towards the precise regulation of gene expression in order to dictate cell fate. Therefore, looking at the transcriptional mechanisms involved in the onset and progression of neurogenesis is important for understanding cortical development. Various transcription factors have been shown to play key roles in the patterning of the cortex, progenitor proliferation and neuronal differentiation.

During the earliest stages of cortical development, the graded expression patterns of a few transcription factors represent one of the key mechanisms required for proper rostral/caudal and ventral/dorsal regionalisation of the mammalian cortex (Ypsilanti and Rubenstein, 2016). Manipulation of these gradients is sufficient to induce changes in the orientation and sizes of specific cortical areas. The paired homeobox factor PAX6 and EMX2 represent dorsal determinants, which are essential for the development of the cortical structures in favour of the choroidal roof and subpallium, as well as to prevent the expansion of the ventral domains (Muzio et al., 2002). Moreover, PAX6 and SP8 promote rostral patterning (Bishop, Goudreau, et al., 2000; Zembrzycki et al., 2015), whereas COUP-TF1 and EMX2 favour caudal identity (Bishop, Goudreau, et al., 2000; Mallamaci et al., 2000; Bishop, Garel, et al., 2003).

The balance between progenitor self-renewal and neuronal differentiation is also modulated by multiple transcription factors, with very specific timings and patterns of expression. Multiple transcription factors have been described as key regulators during progenitor proliferation, with their manipulation resulting in proliferation de-

fects and, in turn, brain growth abnormalities. These include PAX6 (Arai, Funatsu, et al., 2005), the LIM homeodomain (LIM-HD) protein LHX2 (Porter et al., 1997; Bulchand et al., 2001), the winged helix protein FOXP1 (Hanashima et al., 2002), and the T-cell leukemia homeobox (TLX) (Roy et al., 2004). At the early stages of cortical development, PAX6 is a molecular determinant of RG progenitors that regulates their division, molecular phenotype and morphology (Götz, Stoykova, et al., 1998; Warren et al., 1999). PAX6 ablation at this stage results in the expansion of the cell cycle, which in turn leads to a decrease in cortical size (Estivill-Torres et al., 2002; Mi et al., 2013). Asymmetric division is also promoted by PAX6, suggesting that progression of cortical neurogenesis is also PAX6-dependent: mice lacking PAX6 display impaired neurogenesis, explained by an inability of NSCs to exit the cell cycle, correlated with an increase in self-renewal (Heins et al., 2002). Both PAX6 and TLX regulate cell fate in the VZ, as demonstrated by a thickening of the superficial cortex in the absence of the two factors (Molyneaux et al., 2007). PAX6 both directly and indirectly regulates the transcription profiles of approximately 80 different downstream targets, which include other transcription factors, signal transduction proteins or cell cycle regulators (Holm et al., 2007; Visel et al., 2007). A high proportion of these targets are represented by transcription factors with roles in neurogenesis, cortical patterning, or neuronal differentiation, such as *Tbr1*, *Tbr2*, *Satb2*, *AP2-γ*, *NeuroD6*, etc. (Holm et al., 2007; Visel et al., 2007).

Differentiation from RGs to IPs and ultimately to postmitotic neurons seem to be associated with the specific transcriptional network *Pax6* → *Tbr2* → *Tbr1* (Englund et al., 2005; Elsen et al., 2018). This suggests not only that these three transcription factors are all required for the identity of these cell populations, but also that this transcriptional sequence might instruct lineage progression during cortical neuronal

differentiation. Experiments have indeed proven that both TBR1 and TBR2 are responsible for the direct induction of a large number of target genes with key roles in proliferation, differentiation of IPs and differentiation of glutamatergic neurons in the developing cortex (Hevner, 2019). In addition, the PAX6 targets *Neurogenin 2* (*Ngn2*), *Insm1* and *AP2- γ* are involved in the induction of *Tbr2*, an event which is necessary for BP generation and expansion in the mouse neocortex (Arnold et al., 2008; Farkas et al., 2008; Sessa et al., 2008; Pinto et al., 2009).

Ultimately, different sets of transcription factors have been linked to the generation of different types of neurons. PAX6, TBR1, TBR2, EMX1, EMX2, FEZF2, SATB2 are instructive for the acquisition of a glutamatergic neuronal fate, whereas DLX1, DLX2, NKX2.1 regulate the development of GABAergic interneurons (Nord et al., 2015).

The bHLH proneural transcription factors represent a small group of transcription factors, highly conserved across the animal kingdom. During the development of the CNS they are required for both the specification of neural identity of progenitors, as well as for the initiation of the neuronal differentiation programme. Three such bHLH factors, *Ngn1*, *Ngn2*, and *Ascl1* (also known as *Mash1*), are expressed in the developing mouse cortex. Their activity will be explored in detail in 1.3.2.

Another important layer of control during cortical neurogenesis has been described at the post-translational level. A couple of ubiquitin ligases regulate the balance between self-renewal and differentiation via the degradation of their specific targets. For example, during RG division, the TRIM-NHL protein TRIM32 is asymmetrically inherited by only one of the two resulting daughters. The inheriting cell is prone to neuronal differentiation since TRIM32 can act as an ubiquitin ligase and degrade the

pro-proliferative factor C-MYC. In a different manner, upon binding of the Argonaute protein family that incorporates the RISC complex, TRIM32 can activate specific microRNAs that promote neuronal differentiation (Schwamborn et al., 2009). Two other E3 ubiquitin ligases have opposite activities in regulating neuronal differentiation: when HUWE1 is expressed, neuronal differentiation is favoured via the degradation of N-MYC, which would normally activate the Notch ligand DLL3 to promote self-renewal (Zhao et al., 2009); on the other hand, when TRIM11 is expressed, it suppresses neurogenesis via targeted degradation of PAX6 (Tuoc and Stoykova, 2008).

Mammalian cortical development is also epigenetically regulated. This represents a critical level of gene expression control, with chromatin modifications being essential in the intricate and tightly regulated process of neurogenesis. The epigenetic modifications, with a more detailed focus on the ATP-dependent chromatin remodelling complexes, will be discussed in 1.4.

1.1.3 Evolutionary differences in regulation of human cortical development

The six-layered neocortex with a well-developed SVZ makes the human brain a typical mammalian brain (Franchini, 2021). In spite of these overall similarities, there is a huge diversity of brains among the 115 species from 14 mammalian orders. There are specific aspects that differentiate the human brain from other mammals, including other primates, which contribute to its unicity. For instance, the human cerebral cortex is the largest of any primate and contains approximately 16 billion neurons, which is the largest number of cortical neurons of any terrestrial mammal

(Sousa et al., 2017). In addition, the cerebral cortex accounts for more than 75% of the brain mass in humans, which is a larger proportion compared to other mammals (Herculano-Houzel, 2012).

Most of the studies describing the development of the mammalian cerebral cortex have used the mouse as a model organism. While the cortical development in both humans and mice undergoes similar cellular processes and have homologous cell types, sometimes regulated by the same molecular mechanisms (Molnár et al., 2019), it is important to also highlight that there are key differences in complexity between mouse and human neurogenesis. Among these differences, the human neocortex is approximately 1000 times larger than that of a mouse and humans have folded brains, termed gyrencephalic, an evolutionary mechanism that allows an increase in cortical surface without an associated increase in the volume of the skull (Zilles et al., 2013). Mouse cortices are smooth or lissencephalic, which might explain the difference in neuronal density between the two mammals that has been reported to be at least seven times higher in humans compared to a rodent brain (Herculano-Houzel, 2009). This difference in cortical folding highlights one of the most remarkable differences between mice and humans: a striking expansion and remodelling of the SVZ. In gyrencephalic species such as humans, the SVZ is divided into two distinct areas: the inner SVZ (iSVZ), which is considered the equivalent of the rodents SVZ, and an outer SVZ (oSVZ), which is absent in lissencephalic species (Smart et al., 2002; Reillo et al., 2011). This section will highlight some of the developmental particularities of the human cerebral cortex, with a main focus on cellular differences and transcriptional mechanisms that characterise human corticogenesis.

As mentioned above, RGs represent one of the earliest types of cells that emerge

from the neuroepithelium. In the developing human cortex RGs acquire a set of different characteristics compared to rodents. They have different locations, developmental potential and molecular signatures (Molnár et al., 2019). Moreover, additional subtypes of RGs have been described: the outer (or basal) RGs (oRGs or bRGs), and a more recent discovered type of truncated RGs (Fietz, Kelava, et al., 2010; Hansen et al., 2010; Nowakowski et al., 2017). oRGs have a different transcriptional profile that distinguishes them from aRGs (Pollen, Nowakowski, Shuga, et al., 2014; Pollen, Nowakowski, Chen, et al., 2015). They are enriched for genes involved in extracellular matrix formation, migration, extensive proliferation and stemness, including *TNC* (Garcion et al., 2004), *HOPX*, *LIFR* (Yap et al., 2016). Some studies have also reported the presence of oRGs during mouse cortical development, but they are significantly fewer, have a different morphology and are functionally distinct. Mouse oRGs possess only a basal process, whereas the human type has both a basal and an apical process; both mouse and human oRGs express PAX6, SOX2 and phosphorylated Vimentin (pVIM) (Wang, Tsai, et al., 2011), but only in humans they are also positive for TBR2; ultimately, as mentioned above, the human oRGs promote proliferation, while the mouse equivalent cell type undergoes asymmetric neurogenic divisions (Wang, Tsai, et al., 2011; Shitamukai et al., 2011). More extensive transcriptomic analysis revealed a set of genes which are enriched in human but not in mouse oRGs (Lui et al., 2011; Florio, Albert, et al., 2015), which include the secreted growth factors *PDGFD*, *BMP7*, *FAM107A*, as well as the human-specific gene *ARHGAP11B*.

In the human cortex, the oRGs in the oSVZ generate bIPs, which are much more abundant and have more processes compared with mice (Kalebic et al., 2019). Therefore, their number and location within the oSVZ are thought to contribute to

the radial expansion and folding of the human brain (Kriegstein, Noctor, et al., 2006; Baala et al., 2007). While the vast majority of bIPs in lissencephalic rodents proliferate in a self-consuming fashion and generate neurons after one round of division, bIPs in gyrencephalic primates have a considerable proliferative activity (Hansen et al., 2010; Betizeau et al., 2013).

Transcriptome analyses on different cell populations from distinct layers of the developing cortex have revealed that cell-autonomous extracellular matrix (ECM) production might also modulate the proliferative potential of NPCs. For instance, in mouse NPCs the production of ECM constituents in the SVZ is inhibited by the antiproliferative marker TIS21 or BTG2 (Arai, Pulvers, et al., 2011). However, ECM constituents were found to be enriched in the human VZ, iSVZ and oSVZ (Fietz, Lachmann, et al., 2012), which led to the hypothesis that the proliferative potential of human BPs might be influenced by their ability to generate an ECM niche, which stimulates their re-entry into the cell cycle. Supporting this hypothesis, comparative studies between mouse and human have shown that integrin $\alpha\nu\beta3$, a major receptor for ECM constituents, is expressed at a sustained high level in the human oSVZ, while it shows a reduced level of expression in the mouse SVZ (Fietz, Lachmann, et al., 2012). Moreover, manipulating the level of expression in the two species has also suggested that $\alpha\nu\beta3$ may be a major regulator of human BP proliferation (Stenzel et al., 2014).

During cortical neurogenesis, axons from the thalamus migrate through the SVZ to eventually connect with neurons in the CP and form synapses. During their migration, they secrete mitogenic factors in the SVZ (Dehay, Savatier, et al., 2001), which have been suggested to promote cell proliferation. In gyrencephalic mammals such as humans these thalamocortical axons are much more abundant, their

growth cones reside in the IZ for longer periods of time compared to rodents, and therefore secrete more mitogens for longer periods of time. As a result, these mitogenic factors have been shown to promote BP proliferation in the human oSVZ (Dehay and Kennedy, 2007; Reillo et al., 2011), demonstrating that niche-specific regulators such as neuronal inputs from non-cortical regions also contribute to the expansion of the SVZ observed in humans.

As mentioned above, key transcriptional networks involved in neocortical neurogenesis are conserved within all mammals. However, specific transcriptional regulators have different expression patterns in terms of time and space within the two principal germinal zones, the VZ and SVZ, in lissencephalic versus gyrencephalic species. For example, in mouse, BPs show a decrease in *Pax6* expression compared to aRGs (Britz et al., 2006; Kovach et al., 2013), whereas in gyrencephalic species *Pax6* is maintained at a high level in BPs through a mechanism that is not yet known (Bayatti et al., 2008; Betizeau et al., 2013). In addition, the human but not the mouse protein FOXP2 regulates the transition rate of aRGs to bIPs (Tsui et al., 2013). Only human BPs express high levels of the yes-associated protein 1 (YAP). Upon disruption of YAP in human and ferret neocortices, both the abundance and proliferation of BPs was affected (Kostic et al., 2019). Similarly, the homeodomain-only protein (HOPX) was initially identified as a marker for oRGs in the human developing cortex. Further experiments have associated HOPX as a factor with a key role in generating primate-specific oRGs (Vaid et al., 2018). Members of the Sry HMG-box (*Sox*) family of transcription factors have also been described as having different expression patterns in different mammals. For instance, *Sox9*, previously known to be essential for neuronal and glial differentiation (Wegner and Stolt, 2005; Martini et al., 2013; Jo et al., 2014), seem to have an additional role in the proliferation of BPs in gyren-

cephalic species (Güven et al., 2020). While *Sox9* is expressed in the VZ of both lissencephalic and gyrencephalic species, only the latter ones maintain its expression in the SVZ, where it is highly expressed in BPs and regulates their proliferation and ability to re-enter the cell cycle (Güven et al., 2020; Kaplan et al., 2017).

1.2 *In vitro* models of cortical development

Limited accessibility to human cortical material led to much of the current understanding regarding the molecular mechanisms that regulate the development of the human cortex to be based on studies in different model organisms, particularly rodents. While these foundational studies have revealed general characteristics of vertebrate or mammalian brain development which are shared across taxa, there is still limited knowledge about the specific features of human brain development and disease. Mental health disorders in particular have seen a dismal rate of new therapies in the last century, fact which could be explained by the way findings are translated from animal models to the clinic (Matthews et al., 2005). Several seminal discoveries, including characterisation of human embryonic stem cells (hESCs) (Thomson et al., 1998) and somatic cells reprogramming to a pluripotent state (GURDON, 1962; Takahashi and Yamanaka, 2006; Takahashi, Tanabe, et al., 2007), led to the development of models for human cortical tissue *in vitro* (Zhang et al., 2001; Ying et al., 2003; Gaspard, Bouschet, Hourez, et al., 2008; Chambers, Fasano, et al., 2009; Shi, Kirwan, et al., 2012; Lancaster et al., 2013). These models allow the study of different specificities of human cortical architecture, cell fates and molecular mechanisms. Moreover, advances in gene editing techniques such as zinc finger nucleases (ZNFs), TALEN and CRISPR-Cas9 have provided methods to manipulate

PSCs and investigate mechanisms involved in cortical development and neurological diseases (Hockemeyer, Wang, et al., 2011; Hockemeyer, Soldner, et al., 2009; Jinek et al., 2012; Cong et al., 2013; Xue et al., 2014).

The main parameter for assessing human cortical development *in vitro* is to fully recapitulate the events that occur *in vivo*. As stated in previous sections, cortical neurogenesis is modulated by a combination of intrinsic and extrinsic mechanisms. Therefore, the aim of the *in vitro* studies has been the identification of the right combination of growth factors and small molecules required for the specification, patterning and differentiation of PSCs towards the neurogenic lineage (Ameele et al., 2014). The first step when generating cortical neurons in two-dimensional (2D) adherent cultures consists in promoting ectodermal lineage commitment, while inhibiting both mesoderm and endoderm specification. The ectodermal cells are then pushed towards a neuroectodermal fate, similar to the process of neurulation, to form NE cells that have the ability to self-organise into rosette-like structures. The rosettes resemble the organisation of the early neural tube and even a rough organisation similar to the VZ and SVZ with different cell populations that recapitulate the *in vivo* properties of specific intermediate progenitor cells (Edri et al., 2015; Shi, Kirwan, et al., 2012). Upon expansion of the NE population, the culture conditions are usually switched such that definitive neural cell types and neuronal differentiation are favoured.

The first protocols of PSCs differentiation into dorsal cortical neurons used an intermediate stage of PSCs aggregates called embryoid bodies (EBs). Different studies showed that neural rosettes could be generated in both the presence or absence of serum-supplemented culturing medium and additional growth factors or other inductive signals (Zhang et al., 2001; Chen et al., 2002; Ying et al., 2003; Watanabe

et al., 2005). While these complementary experiments were sufficient as proof-of-concept differentiation methods driving a dorsal cortical neural fate, each of them displayed significant drawbacks and incomplete recapitulation of *in vivo* corticogenesis. For instance, serum-free culturing medium supplemented with antagonists of Shh pathway such as cyclopamine (Chen et al., 2002) were sufficient to inhibit the ventral forebrain identity normally induced by Shh (Ericson et al., 1995), but the method limited the formation of upper layer neurons in favour of the early neuronal subtypes (Gaspard, Bouschet, Herpoel, et al., 2009). In a similar way, although neural induction can occur in the absence of serum and morphogens (Ying et al., 2003), the efficiency of this method is relatively low (Kelava and Lancaster, 2016). Previous work performed in *Xenopus laevis*, which showed the high dependency of germ layer specification on the TGF β superfamily (Piccolo et al., 1999; Whitman and Mercola, 2001), led to the development of a standard method for differentiation of PSCs to cerebral cortex neurons. TGF β members inhibit neural identity in favour of the mesoderm while using the SMADs downstream mediators and regulators (Kretzschmar and Massagué, 1998). Therefore, the synergistic action of two SMAD signalling inhibitors, also called the dual SMAD inhibition method, allowed efficient generation of neural rosettes directly from PSCs, without going through the intermediate EB stage (Chambers, Fasano, et al., 2009). Subsequent addition of retinoids improved dual SMAD inhibition protocol even further, leading to almost 100% efficiency of cortical neuronal induction from PSCs (Shi, Kirwan, et al., 2012). This robust culture system recapitulates cortical development, going through three main checkpoints. First, the PSCs lose their pluripotency and acquire cortical neural tissue identity demonstrated by the upregulation of specific cortical stem and progenitor cell markers such as OTX1 and 2, FOXG1, Nestin, PAX6. These NPCs have the ability to self-organise into rosettes. Secondly, cortical projection neurons and,

later on, astrocytes are generated in a stereotypical temporal order: initially the progenitor cells exit the cell cycle and express neuron specific markers such as Class III β -tubulin (TUJ1); while the neurons mature, they upregulate layer-specific neuronal markers, including TBR1 and CTIP2, specific for deep-layer neurons, CUX1-, SATB2- and BRN2-expressing upper layer-neurons; and ultimately, astrocytes positive for S100 or GFAP specific markers also appear in the culture. Finally, the mature neurons acquire electrophysiological properties, undergo synaptogenesis and form neuronal networks (Figure 1.4) (Shi, Kirwan, et al., 2012).

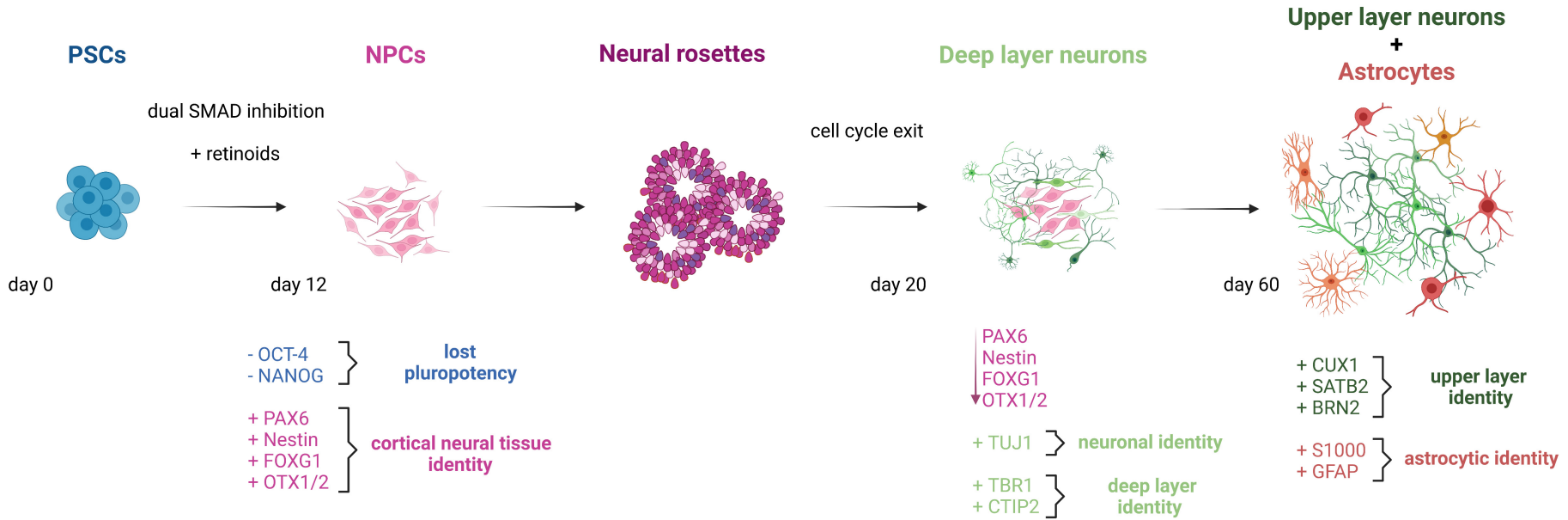


Figure 1.4: Model of cortical differentiation from PSCs.

Summary of the cortical differentiation protocol by (Shi, Kirwan, et al., 2012). PSCs lose their pluripotency and acquire cortical neural tissue identity under dual SMAD inhibition and addition of retinoids to the culture media. The NPCs have the ability to self-organise into rosettes before differentiating into post-mitotic neurons. Around day 20 of cortical differentiation, some progenitors exit the cell cycle and acquire post-mitotic neuronal identity. Neurons found in the culture at this stage express markers characteristic of the deep layers. After day 60, upper layer neurons and astrocytes can also be detected. Figure created with BioRender.com.

The combination of EB method, the absence of serum and tightly controlled culture medium components and cell numbers resulted in a method to generate large rosettes that display elongated lumens and thick apicobasal architecture (Eiraku et al., 2008). This method is considered pioneer work for the generation of three-dimensional (3D) forebrain structures from mouse and human PSCs. The 3D field significantly expanded since then and complex structures such as cerebral organoids, cortical spheroids, or region specific organoids can be derived from PSCs in a dish (Lancaster et al., 2013; Paşca et al., 2015; Qian et al., 2016). 3D structures hold much promise for deciphering the morphological and molecular pathways that underlie human cortical development, as well as for the analysis of specific neurological diseases and potential therapies.

1.3 The proneural factor ASCL1 in neurogenesis

1.3.1 Eukaryotic transcription factors

Cell fate specification in the developing embryo is controlled by precise temporal and spatial patterns of gene expression (Davidson, 2010; Peter and Davidson, 2011). In both prokaryotes and eukaryotes, gene transcription and therefore the protein complement of a cell is controlled by regulatory proteins called transcription factors. They bind to specific DNA sequences called motifs, which reside in gene regulatory elements, in order to promote or inhibit DNA transcription (Voss and Hager, 2014). While prokaryotes and eukaryotes share basic characteristics in gene transcription, eukaryotes have a much larger genome that is tightly packed around histone octamers to form the chromatin, and therefore they developed a more complex and

precise temporal and spatial regulation of gene expression (Kornberg, 1974). The types of motifs recognised by transcription factors are one of the major differences between the two types of organisms: in prokaryotes, transcription factors recognise extended DNA sequences with high affinity and specificity, whereas eukaryotic transcription factors recognise short motifs (6-12 base pairs (bp) long), which indicate it is not only the affinity of transcription factors for DNA, but additional mechanisms that control the way the genome is “read” at one particular time (Spitz and Furlong, 2012).

In order to acquire affinity and specificity, eukaryotic transcription factors display combinatorial occupancy and spatiotemporal activity (Wunderlich and Mirny, 2009). Regulatory enhancer regions typically contain binding sites for different transcription factors. As a result, depending on which and when those factors bind a particular enhancer, this event can lead to different transcriptional outputs (Biggar and Crabtree, 2001). In addition, co-occupancy is sometimes required in order for more transcription factors to be recruited at a particular regulatory element (Wilczynski and Furlong, 2010; Yáñez-Cuna et al., 2013). These discrete and precise patterns of transcriptional activity also imply that a small number of lineage specifying transcription factors have the ability to control the expression of many downstream targets and thus regulate gene expression in a hierarchical manner (Weintraub, 1993; Nutt et al., 1999; Boyer et al., 2005).

1.3.2 Proneural bHLH transcription factors during cortical development

As stated throughout this introduction, despite similarities regarding their morphology, physiology and molecular signature, neurons represent the most diverse cell population of any organism (Bertrand et al., 2002). Considering also the way eukaryotic transcription factors function, it makes development of the mammalian brain one of the most illustrative examples of a versatile network where specific combinations of transcription factors are responsible for creating cell diversity in a spatiotemporal manner (Guillemot, 2007). A small number of proneural genes that encode transcription factors of the bHLH family were described as regulators of neural development in *Drosophila melanogaster* in the late 1970s (García-Bellido, 1979). Their vertebrate orthologs, identified based on sequence conservation with their fly counterparts, are also crucial players in the regulation of neurogenesis. They are both necessary and sufficient to dictate the neural cell fate of progenitors, their subsequent differentiation into neurons, as well as to specify neuronal subtype identities, therefore ensuring the appropriate number of neurons and glia are generated (Bertrand et al., 2002; Ross et al., 2003; Guillemot and Hassan, 2017).

Like other bHLH proteins, proneural factors are made of one HLH domain and a basic domain. The first one consists of two alpha helices connected by a flexible loop and is responsible for dimerization, whereas the latter one is responsible for directing DNA binding (Bertrand et al., 2002). Proneural factors can only bind DNA as homo- or heterodimeric complexes that they form with ubiquitously expressed bHLH proteins, or E proteins. After dimerization, proneural transcription factors only bind specific DNA sequences that contain the core hexanucleotide motif CANNTG, also

known as the E-box (Murre et al., 1989; Johnson et al., 1992). Since dimerization is a prerequisite for DNA binding, factors that interfere with this step are considered passive repressors of proneural factor activity. For instance, in vertebrates, ID proteins only have a HLH domain, but lack the basic domain. As a result, they can act as molecular bait for E proteins and sequester them by forming heterodimers that cannot bind DNA (Massari and Murre, 2000; Yokota et al., 1999). Proneural gene activity is also impeded by the vertebrate HES/HER/ESR proteins that can either inhibit the transcription of proneural genes or prevent the formation of heterodimers with E proteins (Doren et al., 1994; Davis and Turner, 2001). Proneural proteins mainly activate the expression of target genes, and only a few of them, including OLIG2, act as transcriptional repressors (Cabrera and Alonso, 1991; Novitsch et al., 2001). In order to regulate their targets, proneural bHLH factors rely on transcriptional protein complexes, including both other transcription factors and cofactors. In addition, interactions between different proneural proteins also play a critical role for the specificity towards different target genes at different developmental stages or in different cells (Koyano-Nakagawa et al., 2000; Castro, Skowronska-Krawczyk, et al., 2006).

One main characteristic of the proneural transcription factors is represented by their ability to restrict their activity to single progenitor cells and inhibit their own expression in neighbouring cells in order to prevent their differentiation. This process is called 'lateral inhibition' and is achieved through Notch pathway activation: proneural genes directly activate the expression of Notch ligands, specifically *Delta* and *Jagged*, which will drive the Notch signalling cascade in adjacent cells. Notch activity results in the expression of *Hes* genes which will inhibit proneural gene expression, therefore preventing neuronal differentiation (Artavanis-Tsakonas et al., 1999; Ma et

al., 1996; Lewis, 1998; Castro, Skowronska-Krawczyk, et al., 2006). However, HES proteins have autoregulatory activity, which means they are able to bind their own promoters and repress their expression (Shimojo et al., 2008; Takebayashi et al., 1994). As a result, the repressive activity of *Hes* genes over the proneural factors is not stable and leads to a state where proneural genes and *Hes* genes inversely correlate with each other in two to three hour cycles (Imayoshi, Isomura, et al., 2013; Shimojo et al., 2008).

Three of the bHLH proneural factors - *Ngn1*, *Ngn2* and *Ascl1* - are expressed in the RG cells and induce the neurogenesis programme in the developing cortex (Martynoga et al., 2012). *Ngn1*, *Ngn2* and *Ascl1* expression can already be detected around the NE-to-RG transition, although they do not seem to be absolutely required for the acquisition of the RG fate. However, their regulation is one of the key mechanisms that maintains a balance between the number of cells that constitute the progenitor pool and cells that differentiate (Johe et al., 1996; Nieto et al., 2001). Single or double mutant mice for any of these three genes have severe defects in neurogenesis or lack essential sets of neurons and ganglia (Casarosa et al., 1999; Fode et al., 2000; Nieto et al., 2001; Schuurmans et al., 2004; Parras et al., 2007; Mattar et al., 2008; Dixit, Lu, et al., 2011; Dixit, Wilkinson, et al., 2014; Dennis et al., 2017).

1.3.3 ASCL1 during cortical development

Ascl1 expression is observed in both the ventral and the dorsal domains of the embryonic telencephalon. While in the dorsal regions, it shares its proneural activity with *Ngn1* and *Ngn2*, *Ascl1* is the only proneural gene expressed in the ventral te-

telencephalon (Nieto et al., 2001; Britz et al., 2006; Casarosa et al., 1999). In the ventral regions, *Ascl1* is expressed in subcortical progenitors of the VZ and SVZ of the lateral, medial and caudal ganglionic eminences (LGE, MGE, CGE) (Guillemot and Joyner, 1993). Progenitor cells found in these regions generate GABAergic and cholinergic neurons and oligodendrocytes (Bras et al., 2005). In *Ascl1* null mice, the MGE shows a dramatic reduction in size, which is due to a reduction in cell proliferation, as well as lower levels of the Notch ligand DLL1 and downstream Notch effectors such as HES5 (Casarosa et al., 1999). This suggests that ASCL1 plays a critical role in the proliferation of subcortical progenitors, as well as in the specification of both GABAergic interneurons and oligodendrocyte precursor cells (OPCs). Indeed, genome-wide analysis of ASCL1 target sites have shown that many of its target genes are positive regulators of the cell cycle, such as *CyclinD1* (Castro, Martynoga, et al., 2011; Urbán et al., 2016). In addition, *Ascl1* misexpression in specific cellular contexts have also demonstrated its role in progenitor proliferation (Castro, Martynoga, et al., 2011; Li et al., 2014). While promoting the specification of OPC fate, ASCL1 prevents the production of *Dlx*-expressing progenitors, which represses OPC formation and promotes the generation of GABAergic interneurons (Petryniak et al., 2007). Therefore, ASCL1 is able to select between GABAergic neuronal and oligodendroglial target genes and regulate them differently in different cells and at specific times in the embryonic telencephalon.

In the dorsal telencephalon, ASCL1 has different functions compared to its activity in the ventral telencephalon (Britz et al., 2006). Here, *Ascl1* is expressed at low levels in a small number of progenitors in the VZ and SVZ and has different functions compared to its activity in the ventral telencephalon (Britz et al., 2006). Interestingly, the different functions of ASCL1 are associated with different levels of

Ascl1 expression. When expressed in an oscillatory manner it maintains the progenitors in a proliferative state, a stable high expression pattern promotes neuronal differentiation, while a stable low expression pattern allows for other fate determinant factors to dominate (Imayoshi, Isomura, et al., 2013). In cortical progenitors, *Ascl1* shows oscillatory expression with *Ngn1* and *Ngn2*. A switch that leads to stable expression of *Ngn1/Ngn2* results in the cell cycle exit of the progenitors and generation of deep-layer glutamatergic neurons. This suggests ASCL1 is required to maintain the proliferative capacity of cortical progenitors (Wilkinson et al., 2013). During the early stages of neurogenesis, ASCL1 is required for the differentiation of a subset of layer I glutamatergic Cajal-Retzius neurons (Dixit, Lu, et al., 2011). Later on, while differentiated cells start to generate the CP, ASCL1 controls radial migration of the neurons by regulating a small GTP-binding protein called RND3, which inhibits RHOA (Pacary et al., 2011). Similar to its function in the ventral telencephalon, ASCL1 promotes an OPC fate in cortical progenitors at early postnatal stages (Nakatani et al., 2013).

While ASCL1, NGN1, and NGN2 have different functions during mammalian cortical development, they also work together for the regulation of specific events. For instance, ASCL1 functions in a redundant manner with NGN2 to control the timing of neuronal differentiation. They are both required to promote deep-layer neurogenesis during the early stages of cortical development. This is achieved by promoting the expression of *Ctip2*, marker of layer V neurons, while suppressing *Satb2*, which is characteristic for upper layer neurons (Dennis et al., 2017). At later stages during corticogenesis, ASCL1 and NGN2 have the opposite function and repress deep layer neuronal identity, which is achieved through the regulation of other temporal regulators such as *Ikaros* and *Foxg1*. The latter has been shown to switch off the

expression of *Tbr1*, leading to the neurons acquiring an upper layer identity (Kumamoto et al., 2013; Toma et al., 2014). In addition, ASCL1 and NGN2 proneural factors are also involved in controlling the temporal switch from neurogenesis to gliogenesis, their absence resulting in precocious gliogenesis (Nieto et al., 2001; Britz et al., 2006).

Regulation of *Ascl1* during cortical development, which in turn leads to its different expression patterns, is still not fully understood. It is known that Notch targets HES1 and HES5 repress *Ascl1* at the transcriptional level and maintain the NPCs in a proliferative state (Imayoshi and Kageyama, 2014). FGF signalling controls the type of neurons that are generated, i.e. glutamatergic or GABAergic, by dictating which proneural gene is expressed, *Ngn2* or *Ascl1*, respectively (Li et al., 2014). ASCL1 is also regulated at the post-translational level by phosphorylation by proline-directed serine-threonine kinases, such as CDK1 and ERK. When CDK1 phosphorylates ASCL1, it prevents its binding to the DNA and maintains the embryonic NSCs in a proliferative state (Ali et al., 2014). ASCL1 phosphorylation by ERK controls the regulation of downstream targets. Low levels of ERK activation correlated with low levels of ASCL1 phosphorylation lead to the activation of neuronal differentiation genes, whereas high levels of ERK activation correlated with high levels of ASCL1 phosphorylation promote the expression of glial genes (Li et al., 2014). In addition, Akt signalling has been shown to regulate ASCL1 stability in the embryonic brain, suggesting that IGF/Akt signalling might be involved in promoting the proliferative effects of ASCL1 (Oishi et al., 2009).

1.3.3.1 ASCL1 in the human embryonic cerebral cortex

The exact function of ASCL1 during human cortical development has not yet been investigated in detail. Few studies have looked to characterise its expression pattern *in vivo* by using human foetal cerebral cortex (Hansen et al., 2010; Alzu'bi and Clowry, 2019). Early analysis revealed that when oRGs in the oSVZ of the developing neocortex divide asymmetrically they can generate ASCL1-positive neuronal precursors. While some of these cells are also positive for the neural progenitor marker SOX2, they never co-express the Notch effector HES1. Since Notch is known to promote cell division, this suggests that ASCL1-positive cells are committed towards neuronal differentiation. In addition, the inhibition of Notch activity leads to a decrease in the number of SOX2-positive cells and an increase in the number of TBR2- and ASCL1-positive cells. This confirms that Notch restrains neuronal differentiation and suggests that ASCL1 marks the progenitors which will undergo neuronal differentiation (Hansen et al., 2010).

In contrast to the rodent brain, where high levels of *Ascl1* are found in the ventral telencephalon and low levels in the dorsal regions (Guillemot and Joyner, 1993; Britz et al., 2006), in the human embryonic cerebral cortex *ASCL1* is expressed at a constant high level in both the dorsal and ventral telencephalon from 7.5 to 17 post-conceptual weeks (PCW) (Alzu'bi and Clowry, 2019). Although predominantly found in the iSVZ, ASCL1-positive cells are also found in the oSVZ. Most of the cells in the oSVZ are actively dividing cortical progenitors as confirmed by Ki-67, a marker of cell division (Scholzen and Gerdes, 2000). In addition, the vast majority of ASCL1-positive cells co-express PAX6 and/or TBR2, which suggests ASCL1 is involved in the specification of the cortical glutamatergic lineage (Hevner

et al., 2006). Interestingly, ASCL1-positive cells that lack PAX6 and TBR2, express the GABAergic neuronal marker GAD67, leading to the possibility that a small subset of ASCL1-positive progenitors generate GABAergic neurons at a later stage (Alzu'bi and Clowry, 2019). This is consistent with studies that reported a dramatic increase in *ASCL1* and *GSX2* expression between 16 and 19 PCW, the latter being a transcription factor critical for the differentiation of calretinin-positive interneurons of CGE origin (Radonjić et al., 2014).

1.3.4 Pioneer activity of ASCL1

As mentioned in 1.3.1, due to the large size of the eukaryotic genome, DNA is wrapped around histones to form nucleosomes and chromatin (Kornberg, 1974; Kornberg and Lorch, 1999). As a consequence, the association of DNA with nucleosomes to form the heterochromatin limits the ability of transcription factors to interact with the genome (Spitz and Furlong, 2012; Voss and Hager, 2014) and the vast majority of transcription factors require pre-existing accessible chromatin to be able to bind DNA (Guertin and Lis, 2010; Biddie et al., 2011; John et al., 2011). However, to establish new gene regulatory networks when cells differentiate towards different lineages during development, a specialised set of transcription factors called pioneer transcription factors has evolved (Zaret and Carroll, 2011). They are able to bind their target sites in previously inaccessible chromatin and upon binding create accessible regions of DNA (Raposo et al., 2015; Schulz et al., 2015), which allows other non-pioneer transcription factors to bind their targets and regulate downstream gene expression (Magnani et al., 2011; Wapinski et al., 2013; Theodorou et al., 2013). Multiple studies looking at neurogenesis in general, cellular reprogramming and even disease have demonstrated that ASCL1 acts as a pioneer transcription

factor during neuronal differentiation, and is therefore responsible for the generation of multiple regulatory networks within the neurogenic lineage (Raposo et al., 2015; Wapinski et al., 2013; Park et al., 2017).

For instance, an *in vitro* cellular model of neuronal differentiation via *Ascl1* overexpression in NSC cultures has shown that ASCL1 acts as a transcriptional activator at the genome level with most of its binding occurring at distal enhancers, marked by the presence of H3K4me1 and H3K27ac. Interestingly, over half of the ASCL1 binding sites in the enhancer regions of its target genes are found in closed chromatin. Upon binding, ASCL1 induces chromatin opening at those target sites, which allows other transcription factors to bind and regulate downstream target genes (Figure 1.5.A) (Raposo et al., 2015). In addition, the neurons that are generated using this *in vitro* system express *Gad65* and *Gad67*, specific for the GABAergic lineage, and exhibit electrophysiological properties similar to the neurons generated from MGE progenitors of the ventral telencephalon (Martínez-Cerdeño et al., 2012). These similarities suggest that ASCL1 might play its pioneer activity and induce neuronal differentiation in a similar way *in vivo* in the ventral telencephalon (Raposo et al., 2015).

Another characteristic of pioneer transcription factors is their ability to convert different cell types into their specific lineage via cellular reprogramming. Indeed, forced *Ascl1* expression in different cells leads to the acquisition of neuronal properties. For example, ectopic expression of *Ascl1* and *Ngn2* into cultured astrocytes generates fully functional mature neurons (Berninger et al., 2007; Heinrich et al., 2010). ASCL1 alone is also able to reprogram both mouse and human fibroblasts and ESCs into induced neuronal (iN) cells by accessing its cognate target sites in closed chromatin as a pioneer factor (Figure 1.5.B) (Wapinski et al., 2013; Chanda et al., 2014).

The reprogramming efficiency is higher when ASCL1 acts in combination with other transcription factors (e.g. MYT1N, BRN2) (Vierbuchen et al., 2010). However, since neuronal identity can be achieved via *Ascl1* expression only, it suggests that ASCL1 is the key driver of the reprogramming, and MYT1N and BRN2 only enhance the neuronal maturation process but are not required to initiate it (Chanda et al., 2014).

Additional evidence for ASCL1 pioneer activity comes from studies performed in patient derived glioblastoma cell lines. Different levels of *ASCL1* expression in different gliomas (Somasundaram et al., 2005; Phillips et al., 2006; Rousseau et al., 2006; Rheinbay et al., 2013) are a direct regulator of the tumour outcome. Higher levels of *ASCL1* make the tumours more responsive to Notch inhibition, which in turn makes the cells exit the cell cycle, undergo neuronal differentiation and stop the tumour spread (Figure 1.5.C). In contrast, tumours with lower levels *ASCL1* are not responsive to Notch inhibition, which promotes the proliferative capacity of the cells (Park et al., 2017). To induce neurogenesis of the tumour cells, ASCL1 binds nucleosomal DNA and induces the opening of the chromatin around its neuronal differentiation targets (Figure 1.5.C) (Park et al., 2017).

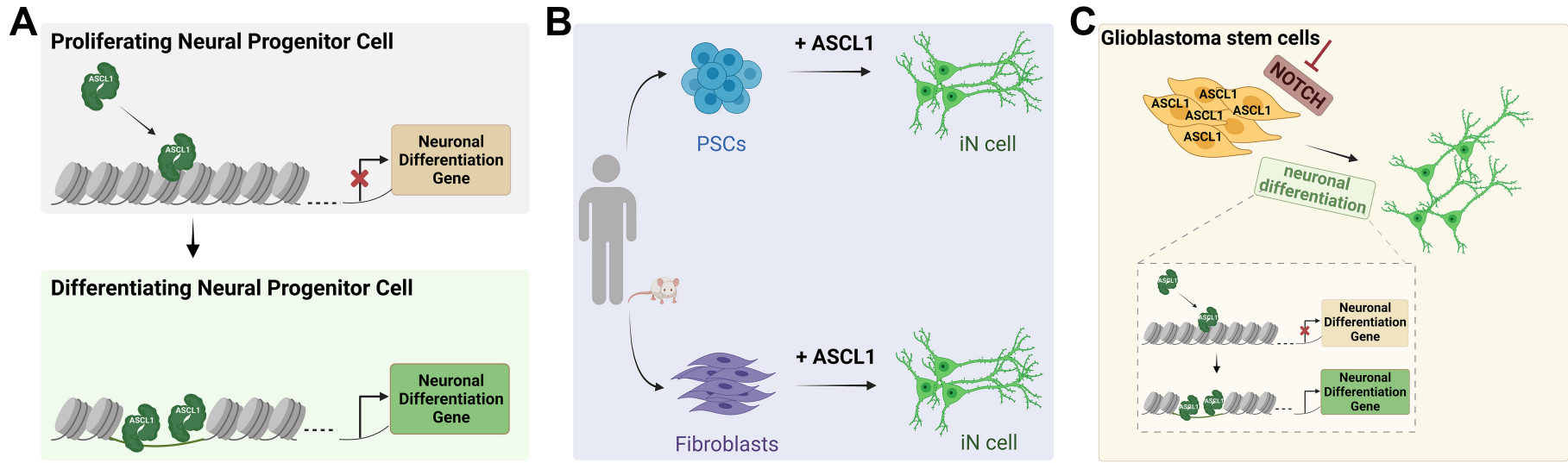


Figure 1.5: Pioneer activity of ASCL1.

(A) When *Ascl1* is overexpressed in proliferating NPCs, it binds its targets found in closed chromatin and, upon binding, it induces a reorganisation of chromatin which leads to the activation of neuronal differentiation genes. Figure reproduced from (Raposo et al., 2015) with permission from the rights holder, Elsevier.

(B) ASCL1 alone is able to reprogram both mouse and human PSCs and fibroblasts into iN cells. Figure modified from (Chanda et al., 2014) with permission from the rights holder, Elsevier.

(C) By acting as a pioneer transcription factor, ASCL1 alone is able to convert glioblastoma stem cells into post-mitotic neurons by making them responsive to Notch inhibition. Figure adapted from (Park et al., 2017) with permission from the rights holder, Elsevier.

Figure created with BioRender.com.

The pioneer activity of ASCL1 has been associated with its DNA binding domain, which is shorter compared to other bHLH transcription factors. As a result, it is likely that ASCL1 can bind its target sites even when the remaining nucleotides are found in nucleosomal structures (Soufi et al., 2015; Guillemot and Hassan, 2017). In cells that are responsive to reprogramming via *Ascl1* overexpression, ASCL1-bound sites have a trivalent histone mark signature comprised of H3K4me1, H3K27ac, and H3K9me3. This suggests this chromatin state might have a role in the recruitment of ASCL1 to these sites (Wapinski et al., 2013). However, how ASCL1 is able to drive chromatin reorganisation upon binding to heterochromatin has not been elucidated yet.

1.4 Epigenetic mechanisms during neurogenesis

As already mentioned, mammalian DNA is tightly packed to form the chromatin. Nucleosomes represent the basic unit of chromatin assembly (Becker and Hörz, 2002; Saha et al., 2006). Each nucleosome consists of ~150 bp of DNA wrapped around a histone octamer composed of two copies of each of the canonical histones H2A, H2B, H3, and H4. They compact the approximately 1.7 metres of mammalian DNA about sevenfold in order to fit into the 5-micrometre cell nucleus (Kornberg, 1974). However, chromatin is not only a way to condense DNA into the nucleus, but also a way to control how DNA is used. Specific mechanisms are therefore required to work in a dynamic fashion and induce developmental programs (Ho and Crabtree, 2010). Epigenetic modifications represent critical regulators of the chromatin structure that control gene expression during embryonic development. Major epigenetic mechanisms that control the assembly and regulation of chromatin include DNA methy-

lation (Suzuki and Bird, 2008), histone modifications (Bannister and Kouzarides, 2011), and ATP-dependent nucleosome remodelling, which I will mainly focus on in this section of the introduction. While there is compelling evidence for the importance of enzymes that modulate DNA methylation and modifications of histones in the embryonic CNS (Hirabayashi and Gotoh, 2010), more recent studies have focused on chromatin remodelling complexes as critical regulators of developmental programs, including neural development (Clapier and Cairns, 2009).

1.4.1 ATP-dependent chromatin remodelling complexes

ATP-dependent chromatin remodelling complexes are multi subunit macromolecular complexes. In order to be defined as part of the complex, the subunits have to show biochemical dedication to the complex, which means they have to form a stable, interlocking association with the other component proteins. In addition, the interactions between the component subunits of a complex can only be disrupted by denaturation (Hargreaves and Crabtree, 2011). Chromatin remodellers have an ATPase derived from the SWI/SNF2 ATPase family as their catalytic module (Eisen et al., 1995). Based on the sequence and structure of the ATPase module, they can be divided into four main families: SWI/SNF (SWItch/Sucrose Non-Fermentable), ISWI (imitation switch) (Corona and Tamkun, 2004; Bartholomew, 2014), CHD (chromodomain, helicase, DNA binding) (Marfella and Imbalzano, 2007), INO80 (inositol requiring 80) (Bao and Shen, 2007; Conaway and Conaway, 2009). The ATPase subunit is divided into two parts, DExx and HELICc, which are separated by a short insertion in remodellers from the SWI/SNF, ISWI and CHD families, and a long insertion in remodellers from the INO80 family. What is different between the different remodellers is the unique domains that flank the ATPase domain. The ATPases within

the SWI/SNF family are flanked by a bromodomain and a helicase-SANT (HAS) domain, ATPases within the ISWI family by a HAND-SANT-SLIDE module, CHD family has tandem chromodomains adjacent to the ATPase, whereas members of the INO80 family have a HAS domain flanking the ATPase (Figure 1.6) (Clapier and Cairns, 2009; Tyagi et al., 2016). Each remodelling complex contains only one ATPase subunit, but each family of chromatin remodellers can build combinatorial assemblies using multiple ATPases via mutually exclusive relationships. Mutual exclusivity also occurs with subunits at other positions within the complex and, together with core subunits present in every complex or accessory subunits that are cell or tissue type specific, each family of chromatin remodellers allows the formation of several hundreds of complexes by combinatorial assembly (Runge et al., 2016). Interestingly, while at some genomic sites remodellers from distinct families co-localise to cooperate or compete (Morris et al., 2014), at different loci they antagonise one another and only complexes from a particular family are able to bind (Parnell et al., 2015).

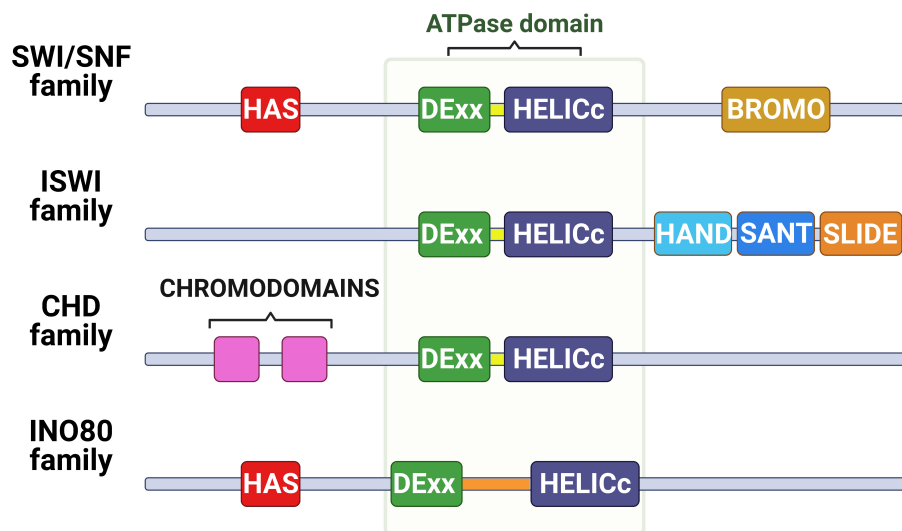


Figure 1.6: Chromatin remodeller families. (Legend next page)

Figure 1.6: Chromatin remodeller families.

Diagrammatic representation of the four families of ATP-dependent chromatin remodelling complexes. The ATPase domain is divided into two parts – DExx (green) and HELICc (purple), which are separated by a short insertion (yellow) in remodellers from the SWI/SNF, ISWI and CHD families, and a long insertion (orange) in remodellers from the INO80 family. The ATPases within the SWI/SNF family are flanked by a HAS domain and a bromodomain; ATPases within the ISWI family are flanked by a HANT-SANT-SLIDE module; tandem chromodomains are found adjacent to the ATPase in remodellers from the CDH family; members of the INO80 family have a HAS domain flanking the ATPase. Figure adapted from (Tyagi et al., 2016) with permission from the rights holder, Elsevier, and created with BioRender.com.

Mammalian cells need chromatin remodellers to regulate all major chromosomal processes: DNA replication, DNA repair, chromosome segregation, regulation of transcription (Clapier and Cairns, 2009). In contrast to DNA methylation and histone modifications, which represent covalent changes, chromatin remodelling represents non-covalent modifications. ATP-dependent chromatin remodelling complexes use the energy derived from ATP hydrolysis to mobilise nucleosomes along the DNA in order to alter the contacts between DNA and histones (Holstege et al., 1998; Becker and Hörz, 2002; Saha et al., 2006). This can be achieved via disruption of the nucleosomes (Ito et al., 2000), moving or sliding of nucleosomes along the DNA (Längst et al., 1999; Hamiche et al., 1999; Whitehouse et al., 1999; Jaskelioff et al., 2000), or via the ejection or exchange of the nucleosomes to a different DNA segment (Lorch et al., 1999; Phelan, Schnitzler, et al., 2000). These remodelling actions can lead to either an increase or a reduction in chromatin accessibility, which in turn will affect the activity of DNA binding proteins such as transcription factors. Depending on the resulting state of the chromatin in terms of accessibility and also on the nature of the binding transcription factors, nucleosome remodelling will have as a final consequence the transcriptional activation or repression of specific genes. For instance, transcriptional repression can be mediated by both a transition of the chromatin from open to a closed state, or through the binding of a repressive transcription fac-

tor to a region of open chromatin. Interestingly, the same chromatin remodelling complex can both promote or inhibit gene expression (Tyler and Kadonaga, 1999; Sudarsanam and Winston, 2000). In addition, the resulting transcriptional outputs are also guided by the type of histone modifying enzymes remodellers work with to regulate chromatin remodelling at gene promoters. When they cooperate with histone acetyltransferases (HATs) gene expression is usually promoted (Grant et al., 1998; Brown et al., 2000), whereas cooperation with histone deacetylases usually leads to gene repression (Struhl, 1998; Ng and Bird, 2000). Despite the set of basic properties shared by all chromatin remodellers and presented above, each family of chromatin remodellers targets distinct sites and engages in a different manner with the genome in order to regulate distinct developmental programs. The next section of the introduction will focus on the SWI/SNF family of complexes and, more specifically, on their role during the development of the mammalian cerebral cortex.

1.4.2 SWI/SNF mammalian complexes

The SWI/SNF family of chromatin remodellers was first discovered in yeast (Neigeborn and Carlson, 1984; Stern et al., 1984; Peterson and Herskowitz, 1992), then identified in *Drosophila*, plants and ultimately in mammals (Tamkun et al., 1992; Khavari et al., 1993; Kwon et al., 1994; Dingwall et al., 1995; Wang et al., 1996; Ho, Ronan, et al., 2009; Kadoch et al., 2013). They are considered conserved from yeast to human since the orthologue complexes contain the key domains in all these species, while some variation exists in the detailed protein composition such as accessory subunits. The mSWI/SNF complexes are 1 to 1.5 MDa macromolecular assemblies of 10-14 different subunits encoded by about 30 different genes. All mSWI/SNF complexes contain the mutually exclusive SMARCA4

(BRG1) or SMARCA2 (BRM) subunits as their ATPase module. They exist in three distinct assemblies: BRG1/BRM-associated factor complexes (BAFs), polybromo-associated BAF complexes (PBAFs), and the newly identified non-canonical BAFs (ncBAFs or GBAFs), which are smaller assemblies that incorporate GLTSCR1 or GLTSCR1L instead of the ARID proteins incorporated in both BAFs and PBAFs (Alpsoy and Dykhuizen, 2018; Ho, Ronan, et al., 2009; Kadoch et al., 2013; Sarnowska et al., 2016; Mashtalir et al., 2018).

The mSWI/SNF remodelling complexes exhibit specific steps in their organisation and assembly with essential branching points for the generation of the three distinct types of assemblies (Figure 1.7). The initial core for all members of the complex consists of a homo- or heterodimer made from two SMARCC subunits (SMARCC1 (BAF155) and/or SMARCC2 (BAF170)) and one SMARCD subunit - SMARCD1 (BAF60A) or SMARCD2 (BAF60B) or SMARCD3 (BAF60C) (Figure 1.7). This three subunit core module is required for complex stability and association of the majority of other subunits (Narayanan, Pirouz, et al., 2015; Mashtalir et al., 2018; Schick, Rendeiro, et al., 2019). Moreover, SMARCC1 and SMARCC2 are indispensable for the individual stability of all the component proteins, with their absence resulting in the proteasome-mediated degradation of all known component subunits of the mSWI/SNF remodellers (Chen and Archer, 2005; Sohn et al., 2007). Importantly, all ncBAFs have the same core module that consists of SMARCC1 and SMARCD1, therefore not showing different combinatorial assembly of their core modules. The next point during the complex assembly consists in the addition of the two core subunits SMARCB1 (BAF47) and SMARCE1 (BAF57) to the core module in order to generate the common BAF and PBAF core or the core subunits GLTSCR1 or GLTSCR1L in order to generate the ncBAF core. Therefore, this step represents the

first branching point in the organisation of mSWI/SNF complexes, essential for differentiating between the canonical and non-canonical assemblies. A second branching checkpoint differentiates the canonical complexes into BAFs and PBAFs. This step consists in the assembly of the ARID subunits: ARID1A (BAF250A) and ARID1B (BAF250B) are mutually exclusive subunits that incorporate in BAF assemblies, whereas ARID2 (BAF200) will incorporate in PBAF complexes. The assembly of the ARID proteins is regulated by the SMARCD subunits and it represents the bridge between the core module and the ATPase assembly. Before the incorporation of the ATPase module, an intermediate step contributes even more to the diversification of the three types of mSWI/SNF assemblies: DPF (BAF45) proteins assembly in BAF complexes (DPF1 (BAF45A) or DPF2 (BAF45B) or DPF3 (BAF45C)), BRD7 and PHF10 assembly in PBAF complexes, while BRD9 incorporates in ncBAFs. The last assembly point consists in the association of the ATPase module, which is made of one of the two mutually exclusive ATPases SMARCA2 or SMARCA4 and a few accessory subunits. These are ACTL6 (BAF45) proteins (ACTL6A (BAF45A) or ACTL6B (BAF45B)), SS18 or SS18L1 (CREST) and BCL7 (BCL7A/B/C) subunits in BAF and ncBAF assemblies, and ACTL6, BCL7 and PBRM1 proteins in PBAFs (Figure 1.6) (Mashtalir et al., 2018). In contrast to the yeast SWI/SNF which lacks actin, all mammalian assemblies have at least one actin molecule per complex, which may support the ATPase activity of SMARCA2 and SMARCA4 (Hargreaves and Crabtree, 2011; Mashtalir et al., 2018).

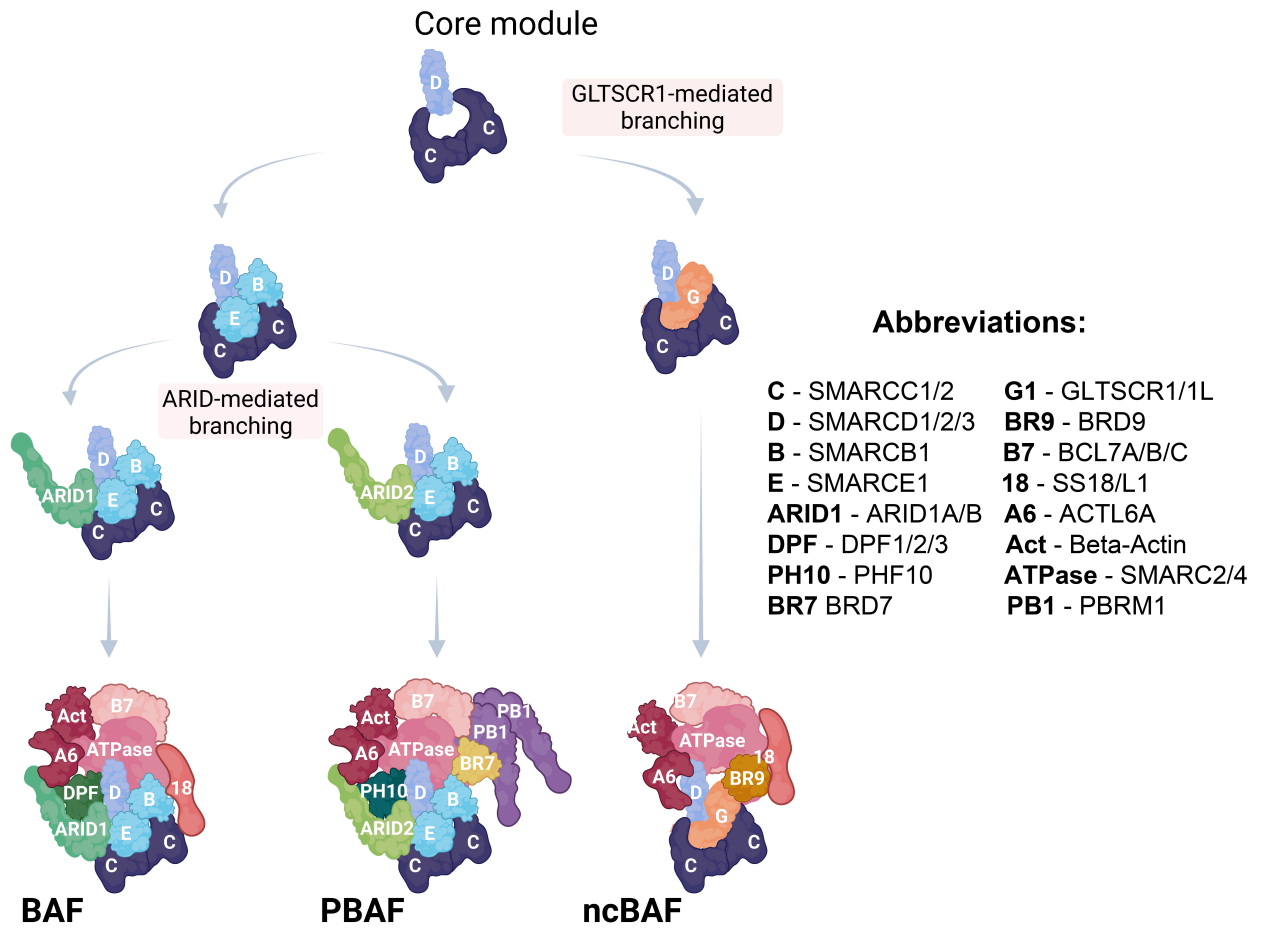


Figure 1.7: Assembly of mSWI/SNF complexes.

All mSWI/SNF complexes have an initial core module composed of two SMARCC subunits and one SMARCD subunit. Incorporation of GLTSCR1 subunits or SMARCB1 and SMARCE1 subunits represents the branching point between ncBAFs and BAF/PBAF assemblies, respectively. Subsequently, incorporation of either ARID1 or ARID2 subunits represents the branching point between BAFs and PBAFs, respectively. Finally, the ATPase module cap (one ATPase and accessory subunits) leads to three different types of mSWI/SNF complexes. Figure adapted from (Mashtalir et al., 2018) with permission from the rights holder, Elsevier, and created with BioRender.com.

The mSWI/SNF chromatin remodellers are involved in essential processes in mammalian cells, including transcriptional regulation, initiation of DNA replication or homologous recombination (Euskirchen et al., 2012). Characteristic to all chromatin remodelling complexes, the core ATPases (SMARCA2 and SMARCA4 in SWI/SNF family) regulate chromatin accessibility at the complex binding sites (Ho, Miller, et

al., 2011; Bao et al., 2015; Miller et al., 2017). While SWI/SNF complexes are not involved in the assembly of the chromatin, they facilitate chromatin accessibility via nucleosome sliding and ejection (Owen-Hughes et al., 1999; Bruno et al., 2003; Fan et al., 2003; Yang et al., 2007). The final outcome of chromatin regulation by the mSWI/SNF members is transcriptional regulation, which can be both gene activation and gene repression, and even a switch between these two modes of action at the same gene (Chi et al., 2003; Clapier and Cairns, 2009; Clapier, Iwasa, et al., 2017). An average of about 300,000 ATPase molecules are present in a mammalian cell, which indicate that binding at a particular genomic site is the cumulated activity of multiple complexes (Hargreaves and Crabtree, 2011). In contrast to yeast where most of the SWI/SNF binding occurs at gene promoters, only a small subset of the mSWI/SNF targets are found within 500bp of the transcription start sites (TSS) with the vast majority of the binding sites being found in intergenic regions where they co-localise with H3K4me1, which marks enhancers and regulatory elements, and less with H3K4me3 that is usually found at promoters (Ho, Ronan, et al., 2009; Ho, Miller, et al., 2011; Euskirchen et al., 2012; Bossen et al., 2015; Barutcu et al., 2016; Wang, Lee, et al., 2017; Kim et al., 2021).

In contrast to previous beliefs that chromatin accessibility is a stable state that requires regulation only at specific stages of the cell cycle, degradation or inhibition of the two ATPases SMARCA2 and SMARCA4 results in rapid changes in chromatin accessibility, mainly losses, demonstrating that mSWI/SNF complexes function in a dynamic fashion by continuously regulating chromatin accessibility (Iurlaro et al., 2021; Schick, Grosche, et al., 2021). Different genomic sites where accessibility is regulated by the mSWI/SNF complexes respond differently to the loss of the ATPase activity. The dependence on mSWI/SNF assemblies is correlated with the levels of

H3K27ac at enhancers: fast responding sites lack H3K27ac but have mono- and demethylated H3K4 instead, enhancers with moderate levels of H3K27ac are less sensitive, whereas super-enhancers with high levels of acetylated histone H3K27 are the most resistant (Iurlaro et al., 2021; Schick, Grosche, et al., 2021; Kubik et al., 2019). As expected, the modifications in chromatin accessibility post degradation or inhibition of the two ATPases are correlated with transcriptional changes. Interestingly, the fast responding sites are associated with binding sites of transcriptional activators such as OCT-4 (POU5F1), SOX2 or NANOG, while the slow loss of chromatin accessibility is correlated with binding sites of repressive transcription factors such as repressor-element-1-silencing (REST or NRSF). In addition, the reversibility of the ATPase inhibition treatments leads to the re-establishment of the wild-type state of chromatin accessibility and transcriptional changes, which suggests that the chromatin landscape is regulated by cell-intrinsic mechanisms rather than epigenetic inheritance (Iurlaro et al., 2021; Hargreaves, 2021).

Chromatin remodellers from the mSWI/SNF family have essential roles during both embryonic development and in the adult organism. In the developing embryo, they regulate stem cell pluripotency, and are critical for cardiac and neural development. For the scope of this introduction, the next section will give an overview on the mSWI/SNF remodellers during mammalian cortical development.

1.4.3 mSWI/SNF complexes during cortical neurogenesis

Chromatin regulators and their involvement in the recruitment of transcription factors are essential for the establishment of gene regulatory networks in response to cellular cues and physiological states in health and disease (Ronan et al., 2013;

Narayanan and Tuoc, 2014; Sokpor et al., 2017). mSWI/SNF chromatin remodellers represent key determinants in the regulation of extrinsic and intrinsic mechanisms that determine the specification and formation of the neocortex. For instance, the mSWI/SNF scaffolding subunits SMARCC1 and SMARCC2 are indispensable for the specification of the neural tissue: their complete ablation at an earlier time point in the rodent brain (E8.5 – E9) abolishes cortical specification, whereas at a later time point (E11.5) leads to severe brain abnormalities that are incompatible with normal cortical functions (Narayanan, Pirouz, et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016). Following cortical specification, signalling pathways known to regulate NPC proliferation, such as Shh, Notch or Wnt, have also been shown to interact with mSWI/SNF remodellers. BRG1-containing assemblies modulate Wnt activity in order to promote the proliferation of progenitors in the telencephalon, whereas Shh signalling, which would normally inhibit proliferation, is suppressed by mSWI/SNF activity (Matsumoto et al., 2006; Zhan et al., 2011; Vasileiou, Ekici, et al., 2015). At a later stage, SMARCC1 and SMARCC2 are also involved in the regulation of BP generation and differentiation. They compete with each other for assembly in mSWI/SNF complexes and SMARCC2-containing assemblies interact with PAX6 to direct its binding to targets that control the generation of BPs. In addition, SMARCC2 controls the number of BPs that are generated in the developing cortex by suppressing the expression of *Tbr2*, which would normally promote neuronal differentiation (Tuoc, Boretius, et al., 2013; Tuoc, Narayanan, et al., 2013). During neuronal differentiation, BRM-containing complexes recruit PAX6 in order to regulate genes such as *Cux1* and *Tle1*, which mark upper layer neuronal identity (Tuoc, Radyushkin, et al., 2009; Georgala et al., 2011; Tuoc, Boretius, et al., 2013; Tuoc, Narayanan, et al., 2013). Moreover, BRM is also involved in controlling the radial migration of neurons during cortical development (Nott et al., 2013).

Unlike *Drosophila* and yeast, BAFs are much more abundant than PBAFs in mammals (Collins et al., 2002). Mammalian BAF complexes have high degree of tissue-specific variability in the component subunits of the complex. As a result, specific BAF assemblies are linked to specific biological processes especially during brain development (Matsumoto et al., 2006; Lessard et al., 2007; Narayanan, Pirouz, et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016; Braun et al., 2021). One such example is the transition from pluripotent stem cells to NPCs and ultimately to post-mitotic neurons, each of this cell types containing specific BAF assemblies with regards to the ATPase domain and three different accessory subunits: ESCs contain the esBAF, NPCs the npBAF and neurons the nBAF (Lessard et al., 2007; Staahl, Tang, et al., 2013). The transition from npBAF to nBAF is essential for vertebrate nervous system development, since interfering with either of the two states is lethal (Hargreaves and Crabtree, 2011). In mice, proliferating neural stem and progenitor cells contain BAF assemblies with either PHF10 or DPF2 zinc finger proteins, the actin related protein ACTL6A and SS18. When progenitors exit the cell cycle and differentiate into neurons, these subunits are replaced by the homologous DPF1 or DPF3, ACTL6B and SS18L1, respectively (Figure 1.8). npBAFs activate Notch activity, which is essential for NPC proliferation and self-renewal (Zhan et al., 2011). Knockdown of the npBAF specific subunits, independent or in combination, leads to premature cell cycle exit and failure to self-renew. Mutant mice with reduced dosage in the npBAF-specific genes show defects in neural tube closure similar to those in human spina bifida. In addition, ablation of ACTL6A leads to a decrease in chromatin accessibility at key neural transcription factors such as SOX2 and ASCL1. Subsequently, this event results in the suppression of cell cycle progression and premature differentiation (Braun et al., 2021). Overexpression of *Dpf1* in rodents results in excessive NPC proliferation in the midbrain and cerebellum.

Moreover, experiments performed in the chick neural tube showed that prolonging PHF10/DPF2 and ACTLA6A past the time they are normally expressed impairs the differentiation of specific neuronal subtypes and leads to a reduction in the size of the spinal cord, whereas continued expression of SS18 in neurons impairs dendritic outgrowth (Lessard et al., 2007; Staahl, Tang, et al., 2013).

On the other hand, preventing the switch impairs neuronal differentiation. The npBAF-nBAF transition is mediated by two microRNAs, miR-9* and miR-124, which bind to the 3'-UTR of *Actl6a* and inhibit its expression by transcript degradation or translational inhibition (Yoo et al., 2009; Carthew and Sontheimer, 2009). These two microRNAs are inhibited in non-neuronal and progenitor cells by REST transcription factor (Conaco et al., 2006). Once cells exit the cell cycle, REST leaves these microRNAs gene loci and non-neuronal transcripts are degraded selectively. In turn, this leads to miR-9* and miR-124 accumulation and *Actl6a* repression in post-mitotic neurons, which allows for the nBAF specific subunits to assemble into complexes (Figure 1.8). Absence of the two microRNAs causes persistent *Actl6a* expression and defective activity-dependent dendritic outgrowth in neurons, whereas their overexpression in NPCs leads to reduced proliferation rates (Yoo et al., 2009).

In addition to their essential roles in the embryonic brain, mutations in mSWI/SNF complex subunits are a major cause leading to both syndromic and non-syndromic intellectual disability, sporadic autism, schizophrenia and amyotrophic lateral sclerosis, highlighting their essential roles during neural specification and development (Morin et al., 2003; Koga et al., 2009; O'Roak et al., 2012; Kleefstra et al., 2012; Neale et al., 2012; Tsurusaki et al., 2012; Wieczorek et al., 2013; Staahl and Crabtree, 2013; Dias et al., 2016; Sokpor et al., 2017; Marom et al., 2017; Vasileiou, Vergarajauregui, et al., 2018).

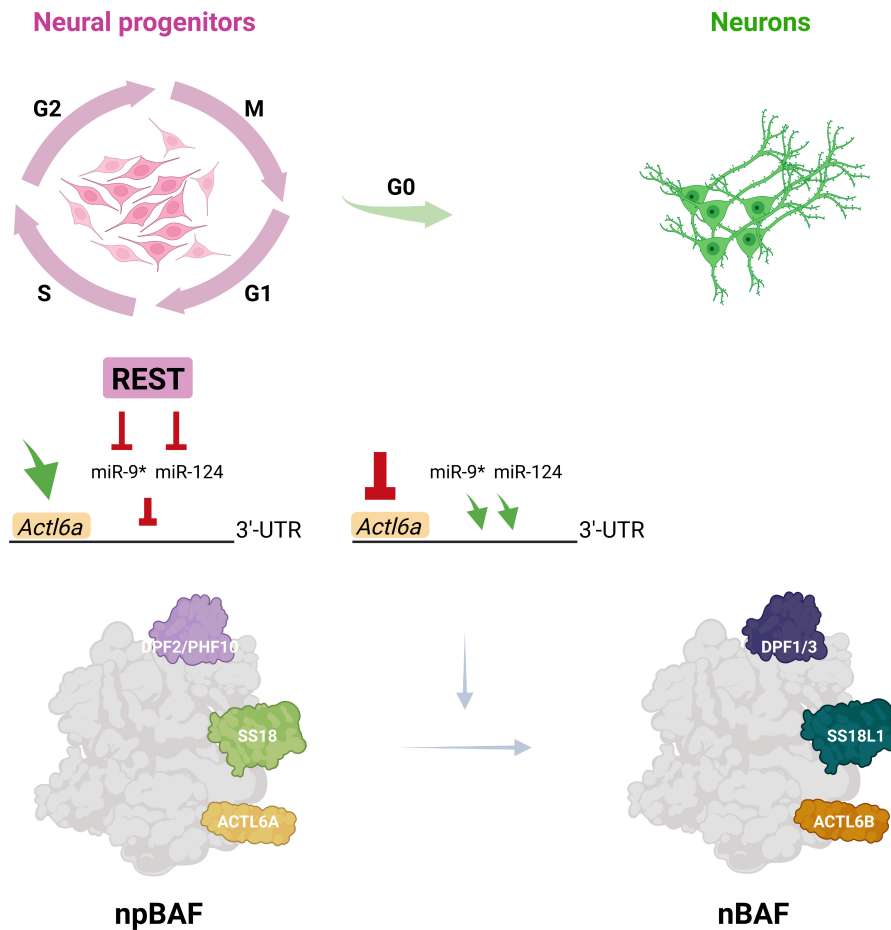


Figure 1.8: npBAF – nBAF switch.

miR-9* and miR-124 bind to the 3'-UTR of *Actl6a* and inhibit its expression. In NPCs, REST inhibits these two microRNAs, which allows the assembly of npBAF complexes that incorporate ACTL6A, SS18 and DPF2/PHF10 subunits. When NPCs exit the cell cycle, REST leaves miR-9* and miR-124 gene loci. miRs de-repression leads to their accumulation, which results in *Actl6a* repression and subsequent substitution by *Actl6b*. ACTL6B, SS18S1 and DPF1/3 proteins incorporate into nBAF specific assemblies in post-mitotic neurons. Figure adapted from (Tang et al., 2013) with permission from the rights holder, Elsevier, and created with BioRender.com.

1.4.4 Interactions between chromatin remodellers and pioneer transcription factors

As already described, pioneer transcription factors are capable of binding nucleosomal DNA and upon binding they create accessible regions of DNA (Raposo et al.,

2015; Schulz et al., 2015). Since regulation of chromatin accessibility is essential for the recruitment of other transcription factors and generation of functional gene regulatory networks, it is important to investigate the ways pioneer transcription factors achieve this essential feature of their activity. It has been proposed that pioneer transcription factors are able to change the chromatin structure by direct interactions with the nucleosomes. For example, FOXA1 and FOXO1 have similar structures to histone H1. This way, it has been suggested they compete with H1 and modulate its displacement at some genomic sites, which in turn leads to the destabilisation of neighbouring nucleosomes (Cirillo, Lin, et al., 2002; Hatta and Cirillo, 2007).

Alternatively, ATP-dependent chromatin remodellers have been shown to assist some pioneer transcription factors at their target sites. For example, members of the GATA family are considered pioneers due to their ability to induce chromatin accessibility (Cirillo, Lin, et al., 2002; Theodorou et al., 2013; Sanalkumar et al., 2014). Binding of different GATA proteins co-localises with SMARCA4-incorporated mSWI/SNF complexes in different mammalian systems. During the differentiation of hematopoietic stem cells into erythrocytes, GATA1 and SMARCA4 bind to the same distal enhancers and induce a global reorganisation of the chromatin structure. In addition, this interaction facilitates the binding of secondary transcription factors such as TAL1 (Hu et al., 2011). In breast cancer cells, during the cell transition from a mesenchymal to an epithelial identity, GATA3 binds inaccessible chromatin and then recruits SMARCA4-assembled mSWI/SNF complexes to shape chromatin architecture (Takaku et al., 2016). The transcription factor SOX10 is essential during the differentiation of neural crest precursors to Schwann cells, oligodendrocytes and melanocytes. mSWI/SNF complexes not only activate, but also assist SOX10 to regulate the expression of *OCT6* and *KROX20*, which are transcriptional regulators

during Schwann cell differentiation (Weider et al., 2012).

Interestingly, there are also instances when chromatin remodellers from different families collaborate in assisting the same transcription factors. For example, INO80 chromatin remodelling complex binds at the same genomic sites as multiple reprogramming transcription factors, including OCT-4, SOX2, KLF4, or Nanog. Following INO80 removal, chromatin accessibility shows a significant decrease at some of these co-bound loci, suggesting INO80 is involved in shaping accessibility at these sites (Wang, Du, et al., 2014). However, subsequent studies performed in mouse ESCs showed that OCT-4 is recruited at target heterochromatic loci, where it recruits SMARCA4-incorporated mSWI/SNF complexes that will open the chromatin. This interaction leads to the stabilisation and further recruitment of OCT-4 and other non-pioneer transcription factors. When SMARCA4 is absent, a severe reduction in OCT-4, SOX2 and Nanog binding is observed, with increased nucleosome occupancy at OCT-4 binding sites. Conversely, when OCT-4 is not present, mSWI/SNF recruitment is also affected, indicating a co-dependency (King and Klose, 2017). Another illustrative example is the interaction between CHD8, mSWI/SNF and progesterone receptor (PR) which is crucial for the activation of PR distal enhancers during cellular differentiation (Ceballos-Chávez et al., 2015).

The studies presented above are illustrative examples of interactions between pioneer factors and ATP-dependent chromatin remodellers that are essential for normal development. Nevertheless, there remains limited understanding about how most pioneer transcription factors are able to bind nucleosomal DNA and regulate chromatin architecture.

1.5 Project Aims

The unique pioneer activity of ASCL1 among the proneural transcription factors, as well as its low sequence binding specificity, point towards an additional mechanism that is required to assist ASCL1 in regulating chromatin accessibility at its targets. Since the function of pioneer factors is essential for the recruitment of non-pioneer transcription factors, which in turn leads to the establishment of functional gene regulatory networks during development, it is important to investigate the mechanisms behind ASCL1 pioneer activity. One of the proposed mechanisms regarding the activity of pioneer transcription factors is their association with ATP-dependent chromatin remodelling complexes. Among the distinct families of chromatin remodellers, the roles of mSWI/SNF complexes during neurogenesis suggest there is an overlapping effect between these remodellers and ASCL1 in the developing neocortex. Therefore, the overall aim of my thesis is to investigate the hypothesis of a mutual interaction between them, which shapes the epigenetic landscape at ASCL1 targets.

- In Results Chapter 1, I will describe the expression pattern of ASCL1 during *in vitro* human cortical neuronal differentiation, highlighting its essential activity at the stage when NPCs exit the cell cycle and differentiate into neurons.
- In Results Chapter 2, I will describe experiments carried out to investigate the physical proximity between ASCL1 and mSWI/SNF remodellers within neuronal cells, as well as the degree to which they share DNA binding landscapes.
- In Results Chapter 3, I will describe the dynamics between the two interacting partners, as well as the transcriptional readout at ASCL1-mSWI/SNF direct targets.

Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Routine maintenance of human iPSCs

The hiPSC line used for the experiments described in this thesis is KOLF2 (HPSI0114i-kolf 2, clone C1), generated and kindly gifted by the Wellcome Sanger Institute's Human Induced Pluripotent Stem Cell Initiative (HipSci) project (www.hipsci.org). The stem cells were generally maintained under feeder-free conditions on Geltrex (ThermoFischer Scientific, A1413201) coated plasticware (Corning) in E8 media (ThermoFischer Scientific, A1517001) + 100U/ml Penicillin/Streptomycin (ThermoFischer Scientific, 15140). Media was changed daily and cell kept at 37°C, 5% CO₂ until approximately 80% confluent, after which point they were passaged or frozen down.

When passaging, cells were washed in dulbecco's phosphate buffered saline (DPBS, ThermoFischer Scientific, 14190-094) and then incubated for 6-7 minutes in 0.5mM ethylenediaminetetraacetic acid (EDTA) pH 8.0 (Invitrogen, 15575-038) in DPBS. EDTA was then removed, cells detached by pipetting 4-5 times into E8 media and replated at a dilution of 1:10.

For freezing, pelleted hiPSCs (centrifuged at 200 x g) were gently resuspended in cryopreservation solution (90% KnockOut Serum Replacement (KSR, ThermoFischer Scientific, 10828010) and 10% dimethyl sulfoxide (DMSO, Sigma, D2650)). 1ml of the cell suspension was dispersed into a 1.5ml cryogenic vial (Thermo Scientific Nunc, 377224) and chilled slowly to -80°C using a cell freezing container following standard procedures (approximately 1°C decreased per minute) before being transferred to liquid nitrogen for long-term storage. In general, from one 10cm dish, 8-10 vials containing 1×10^6 viable cells/ml can be generated.

2.1.2 Neuronal differentiation

The KOLF2 iPSC line was used to perform adherent cortical neuronal differentiations based on the dual SMAD inhibition protocol, which was adapted and optimised (from Shi, Kirwan, et al., 2012 and Deans et al., 2017) by Clementina Cobolli Gigli, Cristina Dias and myself in the Guillemot group. The iPSCs were dissociated in EDTA as described above for usual passaging and plated on Geltrex-coated plates in E8 in a 2:1 ratio on day -1. Usually, after 24 hours (day 0), the culture reached 80-100% confluency, at which point the medium was replaced with neural induction medium (1:1 mixture of N2 medium and B27 medium (composition listed in 2.8.1) supplemented with 10 μ M SB31542 (Abcam, ab120163) and 10nM

LDN193189 (StemCell Technologies, 72147). The neural induction medium was replaced daily for 7 days; on day 7, the NE sheet was washed once with Hanks' Balanced Salt Solution (HBSS, ThermoFischer Scientific, 14170088), detached from the plate using Accutase (Sigma-Aldrich, A6964), incubated at 37°C for 5-7 minutes and replated on Geltrex-coated plates in neural induction medium with 10 μ M Y-27632 ROCK inhibitor (Tocris, 1254/10). The ROCK inhibitor was removed after 24 hours (day 8) and the neural induction medium replaced daily until day 12. On day 12, the NE cells were washed with HBSS and detached again but this time replated on Geltrex-coated plates in neural maintenance medium (1:1 mixture of N2 medium and B27 medium, 2.8.1) with 10 μ M Y-27632 ROCK inhibitor. Similar to day 8, ROCK inhibitor was removed on day 13 and cells were maintained in daily-changed neural maintenance medium until day 23 (with 1:2 passaging on days 15-16 and 19-20 as already described for days 7 and 12). On day 23, the neuroepithelial rosettes were washed with HBSS, detached and plated in a 1:2 ratio in B27 (2.8.1) supplemented with 10 μ M DAPT (Cambridge Bioscience, SM15-10) and ROCK inhibitor on poly-L-ornithine (Sigma-Aldrich, P3655) and laminin (Sigma-Aldrich, L2020) – coated plates. On days 25 and 27, half of the media was replaced with fresh B27 supplemented with 10 μ M DAPT. From day 30 onwards, the neurons were maintained in B27 medium only, replacing only half of the B27 media volume every week.

2.1.3 Generation of CRIPSR-Cas9 targeted neuronal cells

The SMARCC1/2 double knockout (SMARCC1/2^{-/-}) neuronal cells were generated via electroporation with the single cuvette Nucleofector 2b device (Lonza, AAB-1001), using the Mouse Neural Stem Cell Nucleofector Kit (Lonza, VPG-1004) with the A-033 programme. NPCs at day 21 were fed with neural induction medium

(N2B27) with 10 μ M ROCK inhibitor two hours before nucleofection. Cells were then electroporated with two DNA plasmids. The *SMARCC1*-targeting plasmid contains a guide RNA (gRNA) against the first exon of *SMARCC1* under the U6 promoter, the Cas9 sequence driven by the CMV promoter, as well as a neomycin resistance cassette separated from the Cas9 by an T2A peptide (GeneCopoeia, HCP310007-CG040-1). Similarly, the *SMARCC2*-targeting plasmid contains a gRNA against the first exon of *SMARCC2* driven by the U6 promoter and a hygromycin resistance sequence under the Sv40 promoter (GeneCopoeia, HCP310019-SG01-1). Successfully targeted NPCs were selected using neomycin (400 μ g/ml) (Sigma-Aldrich, N1142) and hygromycin (150 μ g/ml) (ThermoFischer Scientific, 10687010).

ASCL1^{-/-} hiPSC KOLF2-C1 cells were kindly supplied by Siew-Lan Ang from the Guillemot lab. They were generated via electroporation of the iPSCs with two plasmids, each containing a gRNA targeting the first exon of *ASCL1*. Single clones were then selected and the knockout confirmed by genotyping using the Illumina MiSeq platform. ASCL1^{-/-} neuronal cells were then generated by performing the neuronal differentiation protocol described in 2.1.2 using the ASCL1^{-/-} iPSCs.

2.2 Molecular Biology

2.2.1 RNA extraction, cDNA synthesis, and qRT-PCR

All RNA samples were collected in RLT lysis buffer (Qiagen, 1053393) added directly to the DPBS-rinsed cell culture plates (ThermoFischer Scientific, 14190-094). RNA was then extracted using the RNeasy Micro Kit (Qiagen 74004) according to

the manufacturer's protocol, with 15 minute on-column digestion with RNase-free DNase I (Qiagen, 79254).

In order to quantify relative gene expression, RNA was converted to cDNA using the Maxima First Strand cDNA Synthesis kit for quantitative real time polymerase chain reaction (qRT-PCR), with dsDNase (ThermoFischer Scientific, K1671). Briefly, between 500ng and 2 μ g of total RNA was treated with dsDNase. After DNase treatment RNA was converted to cDNA using reverse transcriptase with a mix of random hexamer and oligo(dT) 18 primers.

384-well plates were prepared with Taqman Universal qRT-PCR Master Mix (ThermoFischer Scientific, 4304437) and commercially designed primer probes (Table 2.5) following the manufacturer's protocol. Reactions were prepared in triplicate and run on the Lightcycler 480 II thermal cycler (Roche, Switzerland). Data were exported from proprietary Roche software and subjected to statistical testing in Excel (Microsoft, USA) based on the $-2^{-\Delta\Delta C_t}$ or Livak method (Livak and Schmittgen, 2001). Briefly, gene products were normalised to the control (*HPRT* or *UBC*) to generate ΔC_t values, before comparison to the day 0 values to generate the $\Delta\Delta C_t$ values.

2.2.2 Vector cloning

The plasmids which target *SMARCC1* and *SMARCC2* were TOPO TA cloned according to manufacturer's protocol (ThermoFischer Scientific, K457501). Single bacteria colonies were selected and grown in Luria Broth (LB), before DNA was extracted using the EndoFree Plasmid Maxi Kit (Qiagen, 12362) as per manufacturer's protocol. DNA sequences were then confirmed by Sanger sequencing (Genomics Equipment Park, The Francis Crick Institute) using manufacturer's suggested se-

quencing primers (GeneCopoeia, (Table 2.6)).

2.3 Protein Methods

2.3.1 Protein extraction

Cells at different stages were washed with ice-cold DPBS (ThermoFischer Scientific, 14190-094) and lysed in Pierce IP lysis buffer (ThermoFischer Scientific, 87787) supplemented with 1X Protease inhibitor cocktail (ThermoFischer Scientific, 87786), 1X EDTA (ThermoFischer Scientific, 87788) and 1X Phosphatase inhibitor cocktail (ThermoFischer Scientific, 78420). Cells were scraped off the plates and lysed at 4° for 20 minutes under rotation, followed by centrifugation at 17,000 x g for 20 minutes. Supernatant was collected in a new tube and stored on ice for quantification.

2.3.2 Protein quantification

A bicinchoninic acid assay (Pierce BCA Protein Assay Kit, ThermoFischer Scientific, 23225) was used to quantify the protein extract supernatant according to manufacturer's protocol. Bovine serum albumin (BSA, ThermoFischer Scientific, 23209) was used to generate a standard curve and colour change was then quantified using the EnSight multimode plate reader (Perkin Elmer, USA) and analysed using the proprietary software. Following quantification, protein was stored at -80°C until analysed by western blot or subjected to immunoprecipitation.

2.3.3 Western blotting

Variable amounts of protein were prepared for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by dilution with 2X (Sigma S3401-10VL) or 5X (in house made) Laemmli sample buffer and incubation at 95 °C for 5 minutes. Denatured samples were run on 4-15% polyacrylamide gels (Bio-Rad, 4561085) in 1X Tris-glycine-SDS (TGS) running buffer (Bio-Rad, 1610732) at 120-130V. A polyvinylidene fluoride (PVDF) membrane (Bio-Rad, 1704156) was used for sample transfer using the Trans-Blot Turbo Transfer System (Bio-Rad, 17001917) before blocking in 5% milk (Marvel) in Tris Buffered saline Tween (TBS-T, Bio-Rad, 1706435) for 60 minutes. Membranes were incubated with primary antibodies (Table 2.3) diluted in 5% milk (Marvel) in TBS-T overnight at 4 °C with rocking. The next day, membranes were washed in TBS-T, followed by incubation in secondary antibodies (Table 2.4) diluted in 5% milk in TBS-T at room temperature for 60 minutes. TBS-T washes were performed again and signal was generated using enhanced chemiluminescence (ECL) substrate (Amersham, RPN2236) as per manufacturer's instructions. A Hyperfilm ECL (Amersham, 28-9068-36) was developed for signal detection in a dark room.

2.3.4 Co-immunoprecipitation

For co-immunoprecipitation (co-IP) experiments, primary antibodies (Table 2.3) were added to the protein supernatants and incubated with rotation for 2 hours at 4 °C. At the same time, Sepharose coupled with protein G (Sigma, P3296) was blocked with 5% BSA (Sigma, A9647) in precooled DPBS (ThermoFischer Scientific, 14190-094)

for 2 hours with rotation at 4 °C. After three washes with cold DPBS, Sepharose coupled with protein G was added to the protein lysate-antibody mixture and incubated for 90 minutes at 4 °C under rotation. The protein-antibody-sepharose mixture was then washed 5 times in Pierce IP lysis buffer (ThermoFischer Scientific, 87787) and resuspended in 2X Laemmli sample buffer (Sigma S3401-10VL). Samples were then incubated at 95 °C for 5 minutes and stored at -80 °C until western blot analysis.

2.4 Microscopy

2.4.1 Cell and tissue preparation and immunofluorescence

Cells at different time points were plated on glass coverslips coated with geltrex (as described in 2.1). Cultured cells were then fixed with 4% paraformaldehyde (PFA) in PBS (Fisher Scientific, J61899) for 10 minutes at room temperature and washed with DPBS (ThermoFischer Scientific, 14190-094).

Human foetal tissue ranging in age from 12 to 23 PCW from terminated pregnancies was obtained from the joint MRC/Wellcome Trust-funded Human Developmental Biology Resource (HDBR, <http://hdbr.org>, Gerrelli et al., 2015). All tissue was collected with appropriate maternal consent and approval from the Research ethics Committee North East - Newcastle and North Tyneside 1, REC reference 18/NE/0290. For immunostaining experiments, the foetal brains were fixed with 4% PFA in PBS (Fisher Scientific, J61899) for at least 24 hours at 4 °C. After fixation, brains were dehydrated in graded ethanol washes and embedded in paraffin, before being cut and mounted on slides (the Experimental Histopathology Laboratory, the Francis Crick

Institute).

Both cells and tissue were subjected to antigen retrieval in 10mM Na citrate: 10 minutes at 95 °C for cells, and 30 seconds in the microwave for brain tissue. Samples were permeabilized in 0.1% Triton-PBS for 10 minutes at room temperature with rocking, blocked with 10% normal donkey serum (NDS, Jackson ImmunoResearch, 017-000-121) in 0.1% Triton-PBS for 1 hour at room temperature with rocking, and subsequently incubated in primary antibodies (Table 2.3) diluted in 10% NDS in 0.1% Triton-PBS overnight at 4 °C with rocking. The next day, samples were washed 3 times in 0.1% Triton-PBS, and incubated in secondary antibodies (Table 2.4) and DAPI (10 μ g/ml, Sigma, D9564) diluted in 10% NDS in 0.1% Triton-PBS for 90 minutes at room temperature with rocking. Following 3 washes in 0.1% Triton-PBS, samples were mounted in Vectashield mounting medium (Vector Laboratories, H-1000-10).

2.4.2 Proximity Ligation Assay

Proximity Ligation Assay (PLA) was performed using the Duolink In Situ Red Started Kit Mouse/Rabbit (Sigma Aldrich, DUO92101-1KT) according to manufacturer's instructions. Briefly, cells or human foetal brain tissue were subjected to fixation, antigen retrieval, and permeabilization as described above (2.4.1). Samples were then blocked in Duolink Blocking Solution for 60 minutes at 37 °C, followed by incubation in primary antibodies (Table 2.3) diluted in Duolink Antibody Diluent overnight at 4 °C. The next day, samples were washed two times in Duolink Wash Buffer A, followed by incubation with PLA PLUS and MINUS probes for 1 hour at 37 °C, ligation using the Duolink ligase diluted in the 5X Duolink Ligation Buffer for 30 minutes at 37 °C

and amplification using Duolink polymerase diluted in the 5X Duolink Amplification Buffer for 100 minutes at 37°C. All samples were washed twice in Duolink Wash Buffer A and once in Duolink Wash Buffer B, followed by mounting in Duolink In Situ Mounting Medium with DAPI.

2.4.3 Imaging

Immunofluorescence (IF) samples were imaged using a SP5 or SP5 inverted confocal microscope (Leica Microsystems) at a z-section thickness of 1 μ m.

2.5 Next Generation Sequencing

2.5.1 Chromatin immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-seq)

Neuronal cells at different stages were fixed with 2nM di(N-succimidyl) glutarate (Sigma-Aldrich, 80424) in DPBS (ThermoFischer Scientific, 14190-094) for 45 minutes at room temperature on a rocking platform. Three DPBS washes were then performed, before a second 10-minute fixation in 1% methanol-free formaldehyde solution (ThermoFischer Scientific, 28908) in DPBS at room temperature with rocking. The formaldehyde fixation was stopped by adding 1ml of 1.25M glycine (Sigma-Aldrich, 50046), followed by a 5 minute incubation on the rocking platform at room temperature. Cells were scraped off and pelleted by centrifugation at 800 x g for 5 minutes at 4°C. After three more washes with ice-cold DPBS, the cell pellet was snap frozen in liquid nitrogen and stored at -80°C until processing.

To isolate nuclei, pellets were resuspended in 300 μ l of SDS Lysis Buffer (Table 2.8) containing 1X Protease inhibitor cocktail (ThermoFischer Scientific, 87786) and incubated for 30 minutes on ice. The cell suspension was transferred to a 1.5ml Diagenode TGX tube (Diagenode, C30010016) and sonicated for 60 cycles (30 seconds on, 30 seconds off, on High) in a precooled Diagenode Bioruptor Plus Sonication System. 1 ml of Chromatin Dilution buffer (Table 2.9) containing 1X Protease inhibitor cocktail was added to the crosslinked sheared chromatin and centrifuged at 14,000 x g for 30 minutes at 4°C. 60 μ l of soluble chromatin were stored at -20° as input chromatin, while the remaining supernatant was transferred to a protein LoBind tube (Fisher Scientific, 022431081) containing protein G dynabeads (ThermoFischer Scientific, 1004D) – primary antibody (Table 2.3) mix (previously incubated with rotation for 3 hours at room temperature). The chromatin-antibody-dynabeads solution was incubated with rotation at 4°C overnight. Using the magnetic holder to separate the dynabeads, the supernatant was removed and sequentially washed for 5 minutes at 4°C under rotation with 1 ml of Wash buffer A (Table 2.10), Wash buffer B (Table 2.11), Wash buffer C (Table 2.12), twice with TE buffer (Table 2.13). 100 μ l of Elution buffer (Table 2.14) was added after the final wash, followed by a 5 minute incubation at 65°C. Dynabeads were separated using the magnetic holder and the eluted DNA was transferred to a clean 1.5ml tube. The elution step was repeated, resulting in 200 μ l of final DNA. The input chromatin from day 1 was removed from the freezer and the NaCl (Sigma-Aldrich, S5150) concentration was increased to 160mM for all samples. RNase A (ThermoFischer Scientific, EN0531) was added to a final concentration of 20 μ g/ml and all samples were incubated at 65°C overnight to reverse crosslinks and digest contaminating RNA. On day 3, EDTA concentration was increased for all samples to 5mM (Sigma-Aldrich E7889) followed by a 2 hour incubation with 200 μ g/ml Proteinase K (Sigma-Aldrich, AM2546) to digest proteins.

ChIP and input samples were purified using the Monarch PCR & DNA Cleanup Kit (New England BioLabs, T1030L) according to manufacturer's instructions.

DNA fragment size and distribution was determined by Agilent 2100 Bioanalyzer (Agilent, USA) before DNA libraries were prepared by the Advanced Sequencing Facility (the Francis Crick Institute) using the KAPA Hyper Prep Kit (KapaBiosystems, KR0961) as per manufacturer's instructions. ChIP-seq samples were sequenced on the Illumina HiSeq4000 platform (Advanced Sequencing Facility, the Francis Crick Institute) and 100bp single-end reads were generated.

2.5.2 Assay for Transposase Accessible Chromatin and high-throughput sequencing (ATAC-seq)

ATAC-seq experiments were performed using previously-established protocols (Buenrostro, Giresi, et al., 2013; Buenrostro, Wu, et al., 2015; Corces et al., 2017). Briefly, 50,000 neuronal cells at different stages were isolated and pelleted at 500 x g for 5 minutes at 4°C, lysed in 50 μ l of ice-cold ATAC Resuspension Buffer (RSB, Table 2.15) containing 0.1% NP40 (Sigma-Aldrich, 11332473001), 0.1% Tween-20 (Sigma-Aldrich, 11332465001) and 0.01% Digitonin (Promega, G9441) and incubated on ice for 3 minutes. The lysis reaction was stopped with 1 ml of ice-cold ATAC-RSB containing 0.1% Tween-20 (Sigma-Aldrich, 11332465001) and the nuclei extracts isolated by centrifugation at 500 x g for 10 minutes at 4°C. The cell pellet was then re-suspended in 50 μ l of transposition reaction mix (25 μ l 2x TD buffer (Illumina, 20034197), 2.5 μ l transposase (Illumina, 20034197), 16.5 μ l DPBS (ThermoFischer Scientific, 14190-094), 0.5 μ l 1% digitonin (Promega, G9441), 0.5 μ l 10% Tween-20 (Sigma-Aldrich, 11332465001), 5 μ l water (ThermoFischer Scien-

tific, AM9937)) and subsequently incubated at 37 °C for 30 minutes in a thermomixer with 1000 RPM mixing. Transposed DNA was purified using the Zymo Clean and Concentrator-5 kit (Zymo, D4014) according to the manufacturer's protocol and eluted in 21 μ l of elution buffer.

5 μ l of the cleaned transposed DNA was used for library amplification (12 cycles) using the NEBNext HiFi 2X PCR Master Mix (New England BioLabs, M0541S) and previously designed ATAC-seq barcoded primers (Table 2.7) (Buenrostro, Giresi, et al., 2013). PCR reactions were cleaned-up with KAPA pure beads (Roche, 07893271001) at 1.8X beads versus sample ratio. Prior to sequencing, DNA fragment size and distribution and library concentration were determined by Agilent 2100 Bioanalyzer (Agilent, USA) and QubitTM dsDNA HS assay (ThermoFischer Scientific, Q32851), respectively. ATAC-seq samples were subsequently sequenced on the Illumina HiSeq4000 platform (Advanced Sequencing Facility, the Francis Crick Institute) and 50bp paired-end reads were generated.

2.5.3 RNA-seq

Total RNA was extracted as described above (2.2.1) and 500ng was submitted to the Advanced Sequencing Facility (The Francis Crick Institute) to be assessed for RNA integrity (RIN) quality control using Caliper LabChip GX (Perkin Elmer, USA). Samples that passed quality control (have a RIN above 9) were used for library preparation using the KAPA mRNA polyA HyperPrep Kit (Illumina, KR1352) and subsequently sequenced on the Illumina HiSeq4000 platform (Advanced Sequencing Facility, the Francis Crick Institute) to generate 100bp paired-end reads.

2.6 Bioinformatic analysis

Alignment, generation of visual files, peak calling and differential analysis were performed by Harshil Patel, BABS (Bioinformatics and Biostatistics Facility, the Francis Crick Institute).

2.6.1 Acquisition of publicly available datasets

Several published ChIP-seq datasets were integrated into the analyses. They were downloaded as raw fastq files and analysed by Harshil Patel, BABS, in line with the in-house generated ChIP-seq datasets (described in 2.6.2).

2.6.2 ChIP-seq analysis

Raw reads obtained for each sample were adapter-trimmed using Trim Galore (version 0.5.0) (Martin, 2011), followed by genome-wide mapping to the human hg19 genome using BWA (0.7.17 – r1188) with default parameters (Li and Durbin, 2010). Duplicate reads that were identified with the MarkDuplicates picard tool (version 2.1.1) were then excluded together with reads that were discordant, mapped to different chromosomes, did not map properly or had >1 mismatches. Genome-wide peak calling was performed with MACS2 peak-calling algorithm (version 2.1.2) (Zhang et al., 2008) with default “–broad” parameter used for SMARCB1, SMARCA4, H3K4me1, H3K4me3 and H3K27ac ChIP-seq datasets and “–narrow_peak” for ASCL1 ChIP-seq datasets. The annotatePeaks.pl program from HOMER (version 4.8) (Heinz et al., 2010) was used to annotate peaks relative to hg19 genomic features down-

loaded from UCSC. Peak overlap between replicates was performed (described in 2.6.5.1) and peaks detected in at least two replicates were considered ‘true’ peaks used for downstream analyses. For data visualisation purposes, the BedGraph tracks representing the signal per million mapped reads generated with BEDTools (version 2.27.1) (Quinlan and Hall, 2010) were converted to BigWig files, which are supported by various bioinformatics softwares for downstream processing (Kent et al., 2010).

2.6.3 ATAC-seq analysis

Raw reads obtained for each sample were adapter-trimmed using Trim Galore (version 0.5.0) (Martin, 2011), followed by genome-wide mapping to the human hg19 genome using BWA (0.7.17 – r1188) with default parameters (Li and Durbin, 2010). Sequences of mitochondrial origin represent a known issue that is specific to ATAC-seq libraries (Montefiori et al., 2017). Therefore, the reads that mapped to mitochondrial DNA were excluded, in addition to discordant reads, reads that did not map properly or had mismatches. Genome-wide peak calling was performed with MACS2 peak-calling algorithm (version 2.1.2 (Zhang et al., 2008), with default “–broad” parameter. The annotatePeaks.pl program from HOMER (version 4.8) (Heinz et al., 2010) was used to annotate peaks relative to hg19 genomic features downloaded from UCSC. Similar to the ChIP-seq datasets analysis, the BedGraph tracks were converted to BigWig files for data visualisation purposes.

2.6.3.1 Differential accessibility analysis

In order to perform differential accessibility analysis, the regions identified across all replicates of each condition were merged, resulting in a union set of intervals that represent a consensus set of peaks. A standardised DESeq2 analysis script within the R programming environment (version 3.4.1) was used to assess the overall differential accessibility between two sets of consensus peaks (Love et al., 2014). To determine differential accessible regions, a combination between standard false discovery rate (FDR) threshold ≤ 0.05 (Benjamini-Hochberg multiple testing correction) and a significant fold change ≥ 1.5 was used as the significance threshold.

2.6.4 RNA-seq analysis

Adapter trimming was performed using cutadapt (version 1.9.1) (Martin, 2011). Subsequent mapping and gene-level counting of the sequence reads with respect to the human hg19 genome RefSeq genes from the UCSC Table Browser (Karolchik et al., 2004) was performed with the RSEM package (version 1.3.0) (Li and Dewey, 2011) in conjunction with the STAR algorithm (version 2.5.2) (Dobin et al., 2013).

2.6.4.1 Differential gene expression analysis

DESeq2 package (version 1.12.3) (Love et al., 2014) within the R programming environment (version 3.4.1) was used to perform differential gene expression analysis. To determine differentially expressed genes, a combination between FDR threshold ≤ 0.05 (Benjamini-Hochberg multiple testing correction) and a significant fold change ≥ 2 was used as the significance threshold. Identified differentially ex-

pressed genes were subjected to gene set enrichment analysis using GSEA (gseamsigdb.org) with the Biological processes option.

2.6.5 Functional analyses and data visualisation

2.6.5.1 Peak intersection

To directly calculate overlaps and differences between CHIP-seq and ATAC-seq datasets, I designed a custom script using the R programming language (version 4.0.0). The inputs of the script functions are two text (.txt) files, each of them defining a set of peaks by the chromosome, as well as the start and end coordinates of the peaks. An algorithm for computing the peak intersection as an intersection of intervals (the start and end coordinates of each peak represent the ends of the intervals) is then applied. Different functions can be used for calculating the overlap and the difference between the datasets. The requested result is then written in a new text (.txt) file with the same format as the input files in order to facilitate downstream analyses. For this project, specific bioinformatic analyses required minor modifications of the script (i.e. support for configurable overlap thresholds was added in order to obtain detailed insights into the significance of the overlaps).

2.6.5.2 IGV visualisation

CHIP-seq and ATAC-seq datasets were visually explored using the Interactive Genomics Viewer (IGV) (version 2.6.3) (Robinson et al., 2011; Thorvaldsdóttir et al., 2013). Images of genomic regions used as proof of principle to prove binding and/or accessibility dynamics within different genotypic conditions were exported as .svg

files and cropped with Adobe Illustrator (version 24.1.3) (Adobe, USA).

2.6.5.3 Global data visualisation

All global visualisation of specific ChIP-seq and ATAC-seq datasets (density heat maps) was performed using deepTools (version 3.5.0) (Ramírez et al., 2016). The combination of the following two commands was used to generate coverage heatmaps that display an average of normalised read density for specific subsets of peaks:

```
computeMatrix reference-point -S (.bigWig) <> -R <.bed> <> \  
-o <files.gz> -b 1000/3000 -a 1000/3000 --referencePoint center
```

```
plotHeatmap -m <files.gz> -o <Heatmap.svg> \  
--colorMap # --heatmapHeight # --heatmapWidth #
```

2.7 Statistics

Data are presented as the mean \pm standard error of the mean (SEM) with unpaired Student's t-test used to determine statistical significance (GraphPad Prism and R). Significance is stated as follows: $p > 0.05$ (ns), $p < 0.5$ (*), $p < 0.1$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). Details of statistical analyses are found in the figure legends.

2.8 Reagents

2.8.1 Media composition

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
DMEM/F-12	ThermoFischer Scientific	11320033	-	97%	485ml
N-2 Supplement	ThermoFischer Scientific	17502001	100X	1X	5ml
GlutaMAX-I Supplement	ThermoFischer Scientific	35050-038	100X	1X	5ml
Penicillin/Streptomycin	ThermoFischer Scientific	15140122	10,000U/ml (100X)	100U/ml (1X)	5ml

Table 2.1: N2 media composition (500 ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
Neurobasal Medium	ThermoFischer Scientific	21103049	-	96%	480ml
B-27 Supplement	ThermoFischer Scientific	17504044	50X	1X	5ml
GlutaMAX-I Supplement	ThermoFischer Scientific	35050-038	100X	1X	5ml
Penicillin/Streptomycin	ThermoFischer Scientific	15140122	10,000U/ml (100X)	100U/ml (1X)	5ml

Table 2.2: B27 media composition (500 ml)

2.8.2 Antibodies

Target	Supplier	Catalogue Number	Application	Species	Dilution
ACTL6A	Abcam	ab131272	IF/WB	Rabbit	1:100/1:1000
ACTL6B	Abcam	ab180927	IF/WB	Rabbit	1:50/1:1000
ARID1A	Bethyl	A301-041A	IP/WB	Rabbit	3 μ g/1:1000
ASCL1	BD Biosciences	556604	IF/IP/PLA/WB	Rabbit	1:100/3 μ g/1:100/1:500
ASCL1	Abcam	ab211327	WB	Rabbit	1:500
ASCL1	Abcam	ab74065	ChIP	Rabbit	10 μ g
CTIP2	Abcam	ab18465	IF	Rat	1:300
FOXG1	Abcam	ab18259	IF	Rabbit	1:50
GAPDH	Santa Cruz	sc-47724	WB	Mouse	1:500
GAPDH	Cell Signaling Technology	D16H11	WB	Rabbit	1:2000
Ki-67	Invitrogen	550609	IF	Mouse	1:100
Nestin	Abcam	ab22035	IF	Mouse	1:250
PAX6	Covance	PRB-278P-100	IF	Rabbit	1:3000
SATB2	Abcam	ab51502	IF	Mouse	1:50
SMARCA4	Santa Cruz	sc-10768	WB	Rabbit	1:500
SMARCB1	BD Biosciences	612110	IF/WB	Mouse	1:100/1:50
SMARCB1	Abcam	ab12167	ChIP	Rabbit	10 μ g
SMARCC1	Abcam	ab72503	WB	Rabbit	1:1000
SMARCC2	Bethyl	A301-038A	IF/WB	Rabbit	1:200/1:1000
SOX2	Santa Cruz	sc-17320	IF	Goat	1:500
SS18	Cell Signaling Technology	21792S	IF/WB	Rabbit	1:100/1:1000
SS18L1	Proteintech	12439-1-AP	IF/WB	Rabbit	1:100/1:1000
TBR1	Abcam	ab31940	IF	Rabbit	1:200
TBR2	Abcam	ab23345	IF	Rabbit	1:500
TUJ1	Covance	MMS-435P	IF	Mouse	1:2000
ZO-1	Zymed Laboratories	61-7300	IF	Rabbit	1:200
Normal Rabbit IgG	Cell Signaling Technology	2729S	ChIP/IP	Rabbit	10 μ g/3 μ g
Normal Rabbit IgG	Cell Signaling Technology	3900S	IP	Rabbit	3 μ g
Normal Mouse IgG	Cell Signaling Technology	5415S	IP	Mouse	3 μ g

Table 2.3: Primary antibodies

Target	Supplier	Catalogue Number	Application	Species	Dilution
Alexa Fluor anti-rabbit IgG	Invitrogen	A21206 (488)	IF	Donkey	1:500
Alexa Fluor anti-mouse IgG	Invitrogen	A21202 (488)	IF	Donkey	1:500
Alexa Fluor anti-rat IgG	Invitrogen	A21208 (488)	IF	Donkey	1:500
Alexa Fluor anti-rabbit IgG	Invitrogen	A21207 (594)	IF	Donkey	1:500
Alexa Fluor anti-mouse IgG	Invitrogen	A21203 (594)	IF	Donkey	1:500
Alexa Fluor anti-rat IgG	Invitrogen	A21209 (594)	IF	Donkey	1:500
Alexa Fluor anti-rabbit IgG	Jackson ImmunoResearch	711-606-152 (647)	IF	Donkey	1:500
Alexa Fluor anti-mouse IgG	Jackson ImmunoResearch	715-606-151 (647)	IF	Donkey	1:500
Alexa Fluor anti-goat IgG	Invitrogen	A21447 (647)	IF	Donkey	1:500
Anti-Rabbit Immunoglobulins HRP	Dako	P0448	WB	Goat	1:2000
Anti-Mouse Immunoglobulins HRP	Dako	P0161	WB	Rabbit	1:1000
Anti-Rabbit IgG HRP	Rockland	18-8816-31	WB	Rabbit	1:1000
Anti-Mouse IgG HRP	Rockland	18-8817-31	WB	Mouse	1:1000

Table 2.4: Secondary antibodies

2.8.3 Primers and probes

Gene	Supplier	Assay_ID	Catalogue Number
<i>ACTL6A</i>	ThermoFischer Scientific	Hs00188792_m1	4221182
<i>ACTL6B</i>	ThermoFischer Scientific	Hs00211827_m1	4331182
<i>ASCL1</i>	ThermoFischer Scientific	Hs00269932_m1	4331182
<i>DPF1</i>	ThermoFischer Scientific	Hs01050569_m1	4351372
<i>DPF2</i>	ThermoFischer Scientific	Hs01091979_g1	4351372
<i>DPF3</i>	ThermoFischer Scientific	Hs00247174_m1	4351372
<i>HPRT1</i>	ThermoFischer Scientific	Hs02800695_m1	4331182
<i>HuC/D</i>	ThermoFischer Scientific	Hs00956610_mH	4331182
<i>Ki-67</i>	ThermoFischer Scientific	Hs00606991_m1	4331182
<i>MAP2</i>	ThermoFischer Scientific	Hs00258900_m1	4331182
<i>PAX6</i>	ThermoFischer Scientific	Hs00240871_m1	4331182
<i>PHF10</i>	ThermoFischer Scientific	Hs01097834_m1	4331182
<i>SMARCA2</i>	ThermoFischer Scientific	Hs01030858_m1	4331182
<i>SMARCA4</i>	ThermoFischer Scientific	Hs00231324_m1	4331182
<i>SMARCC1</i>	ThermoFischer Scientific	Hs01024797_m1	4331182
<i>SMARCC2</i>	ThermoFischer Scientific	Hs00900813_g1	4351372
<i>SOX2</i>	ThermoFischer Scientific	Hs01053049_s1	4331182
<i>SS18</i>	ThermoFischer Scientific	Hs00194558_m1	4331182
<i>SS18L1</i>	ThermoFischer Scientific	Hs00988004_m1	4351372
<i>TUJ1</i>	ThermoFischer Scientific	Hs00964962_g1	4331182
<i>UBC</i>	ThermoFischer Scientific	Hs00824723_m1	4331182

Table 2.5: qRT-PCR Taqman probes

Name	Sequence
<i>SMARCC1.F</i>	TTCTTGGGTAGTTTGCAG
<i>SMARCC1.R</i>	GCGTACTTGGCATATGAT
<i>SMARCC2.F</i>	TTCTTGGGTAGTTTGCAG
<i>SMARCC2.R</i>	CGCCATTCGCCATTCAGG

Table 2.6: Sanger sequencing primers for *SMARCC1/2* electroporation plasmids

Name	Sequence
Ad1_noMX	AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTCAGATGTG
Ad2.1_TAAGGCGA	CAAGCAGAAGACGGCATAACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGT
Ad2.2_CGTACTAG	CAAGCAGAAGACGGCATAACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGT
Ad2.3_AGGCAGAA	CAAGCAGAAGACGGCATAACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGT
Ad2.4_TCCTGAGC	CAAGCAGAAGACGGCATAACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGT
Ad2.5_GGACTCCT	CAAGCAGAAGACGGCATAACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGT
Ad2.6_TAGGCATG	CAAGCAGAAGACGGCATAACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGT
Ad2.7_CTCTCTAC	CAAGCAGAAGACGGCATAACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGT
Ad2.8_CAGAGAGG	CAAGCAGAAGACGGCATAACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGT
Ad2.9_GCTACGCT	CAAGCAGAAGACGGCATAACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGT
Ad2.10_CGAGGCTG	CAAGCAGAAGACGGCATAACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGT
Ad2.11_AAGAGGCA	CAAGCAGAAGACGGCATAACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGT
Ad2.12_GTAGAGGA	CAAGCAGAAGACGGCATAACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGT
Ad2.13_GTCGTGAT	CAAGCAGAAGACGGCATAACGAGATATCACGACGTCTCGTGGGCTCGGAGATGT
Ad2.14_ACCACTGT	CAAGCAGAAGACGGCATAACGAGATACAGTGGTGTCTCGTGGGCTCGGAGATGT
Ad2.15_TGGATCTG	CAAGCAGAAGACGGCATAACGAGATCAGATCCAGTCTCGTGGGCTCGGAGATGT
Ad2.16_CCGTTTGT	CAAGCAGAAGACGGCATAACGAGATACAAACGGGTCTCGTGGGCTCGGAGATGT
Ad2.17_TGCTGGGT	CAAGCAGAAGACGGCATAACGAGATACCCAGCAGTCTCGTGGGCTCGGAGATGT
Ad2.18_GAGGGGTT	CAAGCAGAAGACGGCATAACGAGATAACCCCTCGTCTCGTGGGCTCGGAGATGT

Table 2.7: ATAC-seq primers with barcodes for pooling (Buenrostro, Giresi, et al., 2013)

2.8.4 Buffers and Solutions

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
Tris-HCl, pH 7.5	In house ¹	-	1M	50mM	2.5ml
EDTA	Sigma	E7889	0.5M	10mM	1ml
SDS	ThermoFischer Scientific	AM9820	20%	1%	2.5ml
Water	ThermoFischer Scientific	AM9937	-	-	44ml

Table 2.8: ChIP SDS Lysis Buffer (50ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
Tris, pH 7.5	In house ¹	-	1M	25mM	1.25ml
EDTA	Sigma	E7889	0.5M	5mM	0.5ml
Triton X-100	Sigma	T8787	100%	1%	0.5ml
SDS	ThermoFischer Scientific	AM9820	20%	0.1%	0.25ml
Water	ThermoFischer Scientific	AM9937	-	-	47.5ml

Table 2.9: ChIP Chromatin Dilution Buffer (50ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
HEPES, pH 7.9	Sigma	H3375	0.5M	50mM	5ml
NaCl	Sigma	S5150	5M	140mM	1.4ml
EDTA	Sigma	E7889	0.5M	1mM	0.1ml
Triton X-100	Sigma	T8787	100%	1%	0.5ml
Sodium deoxycholate	Sigma	30970	10%	0.1%	0.5ml
SDS	ThermoFischer Scientific	AM9820	20%	0.1%	0.25ml
Water	ThermoFischer Scientific	AM9937	-	-	42.25ml

Table 2.10: ChIP Wash Buffer A (50ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
HEPES, pH 7.9	Sigma	H3375	0.5M	50mM	5ml
NaCl	Sigma	S5150	5M	500mM	5ml
EDTA	Sigma	E7889	0.5M	1mM	0.1ml
Triton X-100	Sigma	T8787	100%	1%	0.5ml
Sodium deoxycholate	Sigma	30970	10%	0.1%	0.5ml
SDS	ThermoFischer Scientific	AM9820	20%	0.1%	0.25ml
Water	ThermoFischer Scientific	AM9937	-	-	38.65ml

Table 2.11: ChIP Wash Buffer B (50ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
Tris, pH 8.0	In house ¹	-	1M	20mM	1ml
EDTA	Sigma	E7889	0.5M	1mM	0.1ml
LiCl	Sigma	L7026	8M	250mM	1.56ml
NP-40 Alternative	Sigma	18896	100%	0.5%	0.25ml
Sodium deoxycholate	Sigma	30970	10%	0.5%	2.5ml
Water	ThermoFischer Scientific	AM9937	-	-	44.59ml

Table 2.12: ChIP Wash Buffer C (50ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
Tris, pH 8.0	In house ¹	-	1M	10mM	0.5ml
EDTA	Sigma	E7889	0.5M	1mM	0.1ml
Water	ThermoFischer Scientific	AM9937	-	-	49.4ml

Table 2.13: ChIP TE Buffer (50ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
Tris, pH 7.5	In house ¹	-	1M	10mM	0.5ml
EDTA	Sigma	E7889	0.5M	1mM	0.1ml
SDS	ThermoFischer Scientific	AM9820	20%	1%	2.5ml
Water	ThermoFischer Scientific	AM9937	-	-	46.9ml

Table 2.14: ChIP Elution Buffer (50ml)

Component	Supplier	Catalogue Number	Stock Concentration	Final Concentration	Volume
Tris-HCl, pH 7.4	In house ¹	-	1M	10mM	0.5ml
NaCl	Sigma	S5150	5M	10mM	0.1ml
MgCl ₂	In house ¹	-	1M	3mM	0.15ml
Water	ThermoFischer Scientific	AM9937	-	-	49.25ml

Table 2.15: ATAC RSB Buffer (50ml)¹Media preparation STP, The Francis Crick Institute

Chapter 3

Results Chapter 1: Identifying the Expression Patterns of ASCL1 and mSWI/SNF Complexes during the Early Stages of Human Cortical Development

Due to the high complexity and heterogeneity of the human embryonic cortical niche, as well as the limited experimental accessibility, investigating expression patterns is challenging *in vivo*. The development of methods to generate hESCs (Thomson et al., 1998), as well as technologies to reprogram adult somatic cells to a pluripotent state (GURDON, 1962; Takahashi and Yamanaka, 2006; Takahashi, Tanabe, et al., 2007) represent the pioneer work which led to modelling human cortical develop-

ment *in vitro* (Zhang et al., 2001; Ying et al., 2003; Gaspard, Bouschet, Hourez, et al., 2008; Chambers, Fasano, et al., 2009; Shi, Kirwan, et al., 2012; Lancaster et al., 2013). Stem cell models of the human cerebral cortex allow the generation of specific cell populations in a temporal fashion, provide a way to model human cortical diseases such as spinal muscular atrophy, Parkinson's or Alzheimer's diseases, motor neuron disease, etc. *in vitro*, as well as patient specific iPSCs which can be further differentiated into cortical implants to explore cell-based therapies (Ebert et al., 2009; Yagi et al., 2011; Shi, Kirwan, et al., 2012). Due to the high potential of these models, several methods to generate cerebral cortex in adherent 2D cell cultures or organoid 3D cultures have been explored. These methods differ in the signalling pathways that are manipulated, pattern different regions of the brain and therefore some of them have more advantages than others to be used for specific applications and research questions (reviewed in Kelava and Lancaster, 2016).

To investigate the expression pattern of the proneural transcription factor ASCL1, as well as specific mSWI/SNF complex compositions in the developing human cortex, I focussed on a differentiation method that generates human cortical neurons from hiPSCs. This protocol has been adapted from Shi, Kirwan, et al., 2012 and Deans et al., 2017 and optimised in the Guillemot lab. In this chapter I will first describe the identity of the cells generated using this protocol. Following the validation of the *in vitro* model system, I will present the expression patterns of both ASCL1 and mSWI/SNF component proteins which are specific to different combinatorial assemblies that are of particular relevance for this project. Since most of the studies investigating the role of ASCL1 as well as mSWI/SNF remodellers in neurodevelopment have been performed in rodents, characterising their expression in our human model system was absolutely required before performing experiments regarding their func-

tion or regulation. In addition, when possible, some of the *in vitro* results have been confirmed *in vivo* using human foetal cortex at different developmental stages.

3.1 *In vitro* working model characterisation

In order to derive human cortical stem and progenitor cells that will differentiate into neurons from all six layers of the human neocortex, I used a monolayer culture system (Figure 3.1.A) that is based on previously described protocols (Chambers, Fasano, et al., 2009; Chambers, Qi, et al., 2012; Shi, Kirwan, et al., 2012; Deans et al., 2017). To begin with, hiPSCs were plated at high density (Figure 3.1.B) in order to prevent their differentiation into neural crest cells, as it has been reported for neural differentiations that use low densities of PSC to start with (Chambers, Fasano, et al., 2009). Once the hiPSC cultures achieved a confluency between 80% and 100%, I induced their neural differentiation by combining retinoic signalling and dual inhibition of SMAD signalling. Retinoids are absolutely necessary for an efficient differentiation of PSCs into cortical stem cells (Shi, Kirwan, et al., 2012), whereas SMADs are the mediators and regulators of the TGF β pathway, which would normally inhibit neural identity in favour of the mesoderm (Kretzschmar and Massagué, 1998), as well as BMP signalling, which would normally favour the trophectoderm and ectoderm fates in the developing embryo (Chambers, Fasano, et al., 2009). Therefore, I used two small molecules to block SMAD signaling: SB431542, which inhibits the Lefty/Activin/ TGF β pathways by preventing the phosphorylation of the ALK4, ALK5, and ALK7 receptors, and LDN-193189, which is a BMP-inhibitory compound (Chambers, Qi, et al., 2012) (Figure 3.1.A). As a result, a rapid neuralization is already observed by day 7 following dual SMAD inhibition: cells form a NE layer

(Figure 3.1.B), lose their pluripotency network as shown by the downregulation of the pluripotency associated marker OCT-4 (Figure 3.1.C), while they retain SOX2 expression (Figure 3.1.D) and upregulate the human neuroectoderm marker PAX6 (Zhang et al., 2010) (Figure 3.1.D). The PAX6-positive neuroectodermal cells also express general neural progenitor markers such as Nestin, FOXG1 (Figure 3.1.E-F) and, subsequently, TRB2, a marker of IPs (Figure 3.1.G). PAX6 expression in the progenitor cells, followed by TBR2 upregulation demonstrates that our *in vitro* system recapitulates the cortical glutamatergic lineage (Hevner et al., 2006). In addition, the specificity of dorsal patterning of the cultures was confirmed by the absence of ventrally or caudally expressed transcription factors: Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) performed by Clementina Cobolli Gigli in the Guillemot lab in collaboration with the Francis Crick Proteomics STP did not reveal the presence of ventral or caudal markers such as DLX1, HOXB4 or ISL1 at any of the timepoints checked throughout the neural differentiation process (data not shown).

Between days 15 and day 20 of neuronal differentiation, the hiPSCs-derived cortical progenitor cells formed polarized neuroepithelial rosettes that resemble the *in vivo* cortical neuroepithelium (Figure 3.2.B). Cells expressing both SOX2 and Nestin are found near a central lumen which is positive for the ZO-1 protein (Figure 3.2.C), normally found at adherent junctions in the cortex (Götz and Huttner, 2005). As *in vivo*, there is also a second population of progenitors positive for Ki-67 and TBR2 (Figure 3.2.C) that represents BPs. While most of the cells are still proliferating at this stage (Figure 3.2.F), some of the progenitors exit the cell cycle and differentiate into neurons, as shown by the expression of the neuronal markers *HuC/D*, *TUJ1* and *MAP2* (Figure 3.2.G). Since the investigation of expression patterns as

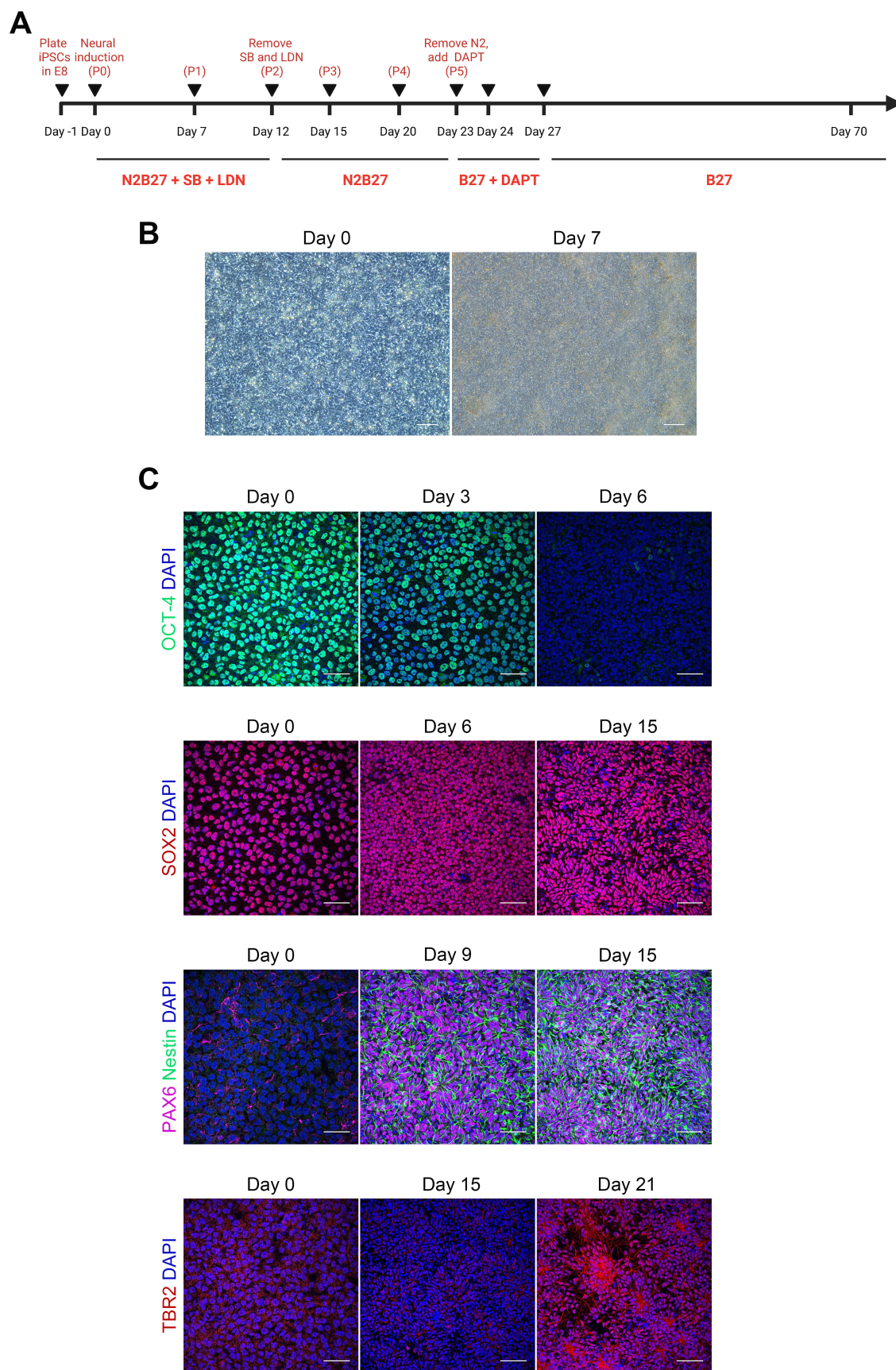


Figure 3.1: NPC stage characterisation during *in vitro* neuronal differentiation. (Legend next page)

Figure 3.1: NPC stage characterisation during *in vitro* neuronal differentiation.

(A) Diagram of the *in vitro* model of iPSC-derived human cortical neurons. hiPSCs were initially plated at high density in E8 medium (day -1). When cells reached 80-100% confluency at day 0, medium was switched to N2B27 supplemented with SB and LDN inhibitors (neural induction). Daily media change with N2B27 supplemented with SB and LDN was performed until day 12, when the two inhibitors were removed from the N2B27 media. At day 23, NPCs were plated in B27 only supplemented with the Notch inhibitor DAPT; B27-DAPT was added (half volume media change) on days 25 and 27. Following day 27, neurons were kept in B27 alone, which was added to the culture once per week (50% volume media change). The arrows between P1 and P5 represent the days when cells were passaged. Diagram created with BioRender.com.

(B) Representative images of hiPSCs at day 0 which had reached the optimal confluency for neural induction (left) and cells forming a NE-like layer at day 7 (right). Scale bars, 50 μ m.

(C-G) Immunofluorescence images during the early stages of the neuronal differentiation process demonstrating the specificity of the *in vitro* working model. **(C)** The pluripotency marker OCT-4 is downregulated, with no protein being detected in the cell culture by day 6. **(D)** SOX2, a common marker of hiPSCs and NPCs is persistent in the culture, expressed by all cells between day 0 and day 15. **(E-F)** NPC-specific markers such as PAX6, Nestin and FOXG1 are upregulated in the culture once the cells lose their pluripotent identity. **(G)** The subsequent upregulation of the intermediate progenitor marker TBR2 (after day 15) shows this *in vitro* protocol recapitulates the cortical glutamatergic lineage (as it follows the much earlier PAX6 upregulation). Scale bars, 50 μ m.

well as more complex analyses are difficult to interpret in a heterogeneous population, we decided to synchronize the cell population by adding DAPT while passaging the progenitors at day 23. DAPT is a γ -secretase inhibitor that blocks Notch activity (Figure 1.2) (Dovey et al., 2001) and accelerates neuronal differentiation. As a result, most of the cortical progenitors exit the cell cycle, as demonstrated by the rapid downregulation of *Ki-67*. NPC markers *SOX2* and *PAX6* are also downregulated following DAPT addition, but they show a slower downregulation compared to *Ki-67* (Figure 3.2.F), perhaps because their mRNA shows increased stability. Combined with the increased mRNA levels of *HuC/D*, *TUJ1*, and *MAP2*, Notch inhibition promotes cell cycle exit of the NPCs and generation of post-mitotic neurons (Figure 3.2.G). Similar to the development of the human cortex *in utero*, following the DAPT-mediated Notch inhibition the post-mitotic neurons acquire identities specific for the six layers of the neocortex, in a temporal manner: deep layer, early born projection neurons expressing CTIP2 and TBR1 can be observed earlier in the cul-

ture (Figure 3.2.D), whereas later-born, upper layer neurons expressing SATB2 are usually observed after day 60 of neuronal differentiation (Shi, Kirwan, et al., 2012) (Figure 3.2.D-E).

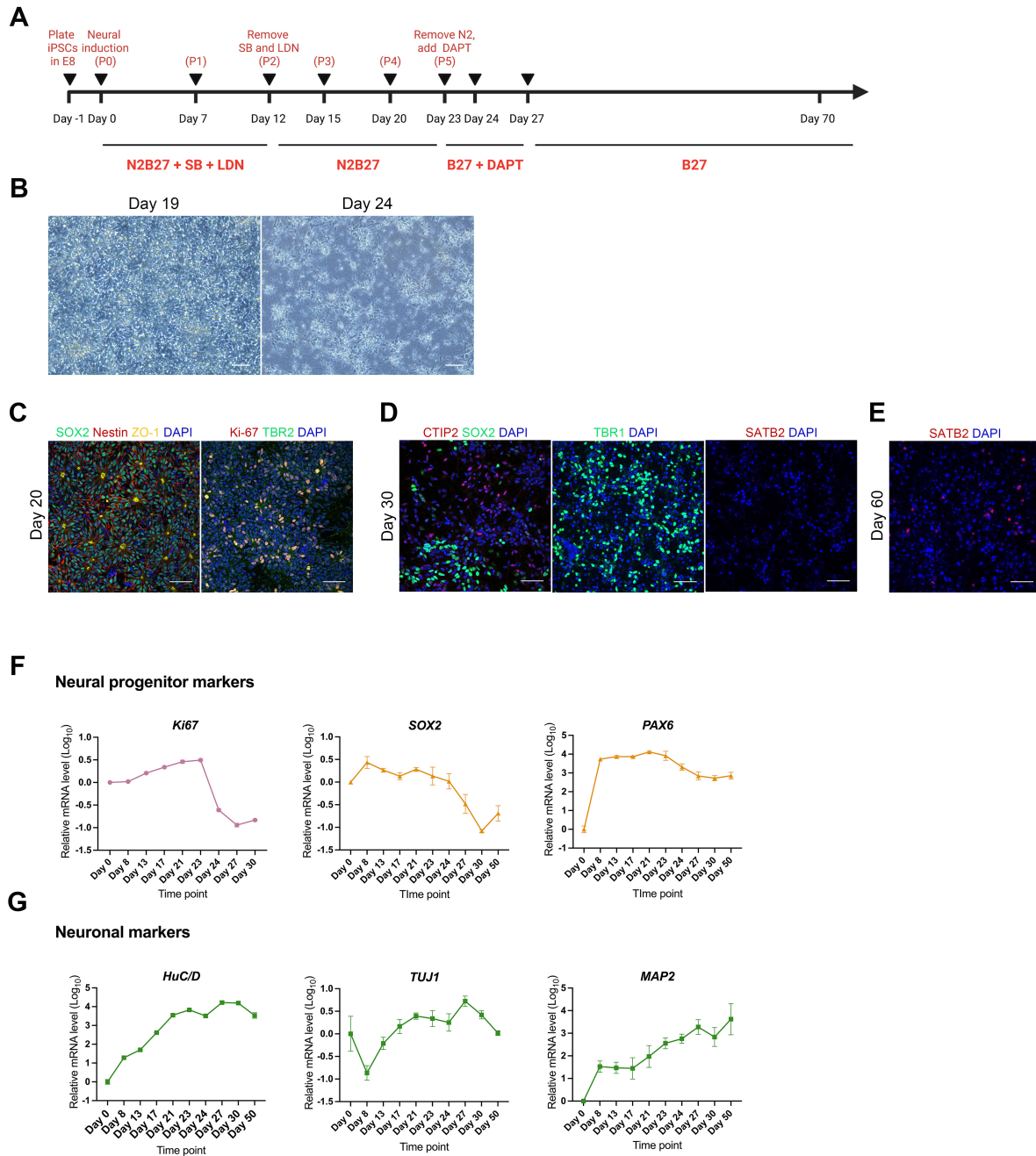


Figure 3.2: NPC-neuronal transition characterisation during *in vitro* neuronal differentiation. (Legend next page)

(A) Diagram of the *in vitro* model of iPSC-derived human cortical neurons. Diagram created with BioRender.com.

(B) Representative images of NPCs at day 19 forming neuroepithelial rosettes (left) and neuronal cells at day 24, after a 24 hour DAPT treatment (right). Scale bars, 50 μ m.

(C-E) Immunofluorescence images during the later stages of the neuronal differentiation process demonstrating the specificity of the *in vitro* working model. (C) Day 20 SOX2-Nestin double positive cells are found around central cells expressing the ZO-1 marker (left). A cell population expressing Ki-67 and TBR2 at day 20 resembles the BPs from the developing cortex (right). (D) At later stages following DAPT addition (day 30), only small subpopulations of cells are still positive for the NPC marker SOX2, while the vast majority of remaining cells in the culture upregulate markers of the deep layer projection neurons such as CTIP2 and TBR1. Upper layer marker SATB2 is detectable in the culture at day 60 (E). Scale bars, 50 μ m.

(F-G) qRT-PCR analysis of the expression of NPC markers (*Ki67*, *SOX2*, *PAX6*) (F) and neuronal markers (*HuC/D*, *TUJ1*, *MAP2*) (G) at different time points during the neuronal differentiation process. Data represents mean \pm SEM of 3 biological replicates.

3.2 ASCL1 expression pattern

Once I confirmed that *in vitro* dual SMAD inhibition, followed by DAPT-mediated Notch inhibition neural differentiation protocol recapitulates the early events of human cortical development from *in vivo*, I investigated the expression pattern of *ASCL1*. Cells were collected for RNA extraction every three days. Although most of the cells have a progenitor identity before DAPT addition and also most of them exit cell cycle and differentiate into neurons immediately after Notch inhibition, the cell cultures are not 100% homogenous at these time points: some neurons can already be detected before DAPT addition, while Ki-67- or PAX6- positive cortical progenitors still persist in the culture post Notch inhibition (Figure 3.3.A). In order to address this issue, I decided to collect cells at more time points around this stage (day23): day 21, day 23, day 24 (24 hours post DAPT), day 25 (48 hours post DAPT), day 27 (4 days post DAPT), and perform qRT-PCR. At the mRNA level, *ASCL1* starts to be expressed from day 15 of neural induction. However, the main expression peak is observed between 24 and 48 hours following DAPT addition, after which time point

ASCL1 is downregulated. This expression pattern was also confirmed at the protein level by western blot analysis (Figure 3.3.B). However, while the protein is already absent from the cells by day 30 of the neuronal differentiation process, intermediate levels of mRNA were still observed in neurons at later time points. This discrepancy between the mRNA and protein expression could suggest either the inhibition of translation or the rapid degradation of *ASCL1* protein once this is produced (Shou et al., 1999; Viñals et al., 2004; Urbán et al., 2016).

Taking advantage of the heterogenous population of cells at 24 hours post Notch inhibition, I was able to investigate the identity of cells expressing *ASCL1*. By performing immunofluorescence experiments using both *in vitro* differentiated neuronal cells and human foetal cortices, I confirmed previously reported analyses showing that *ASCL1* co-localises with SOX2- or PAX6-positive actively dividing cortical progenitors, but not with deep layer neuronal markers such as CTIP2 (Alzu'bi and Clowry, 2019) (Figure 3.3.C). Overall, *ASCL1* expression coincides with the appearance of post-mitotic neurons in the culture (after day 15), shows a main expression peak around the time most of the neural cells exit cell cycle, and then is downregulated by the time the vast majority of cells already acquired a neuronal identity. This expression pattern suggests *ASCL1* might be required to promote neuronal differentiation within the human cortical glutamatergic lineage.

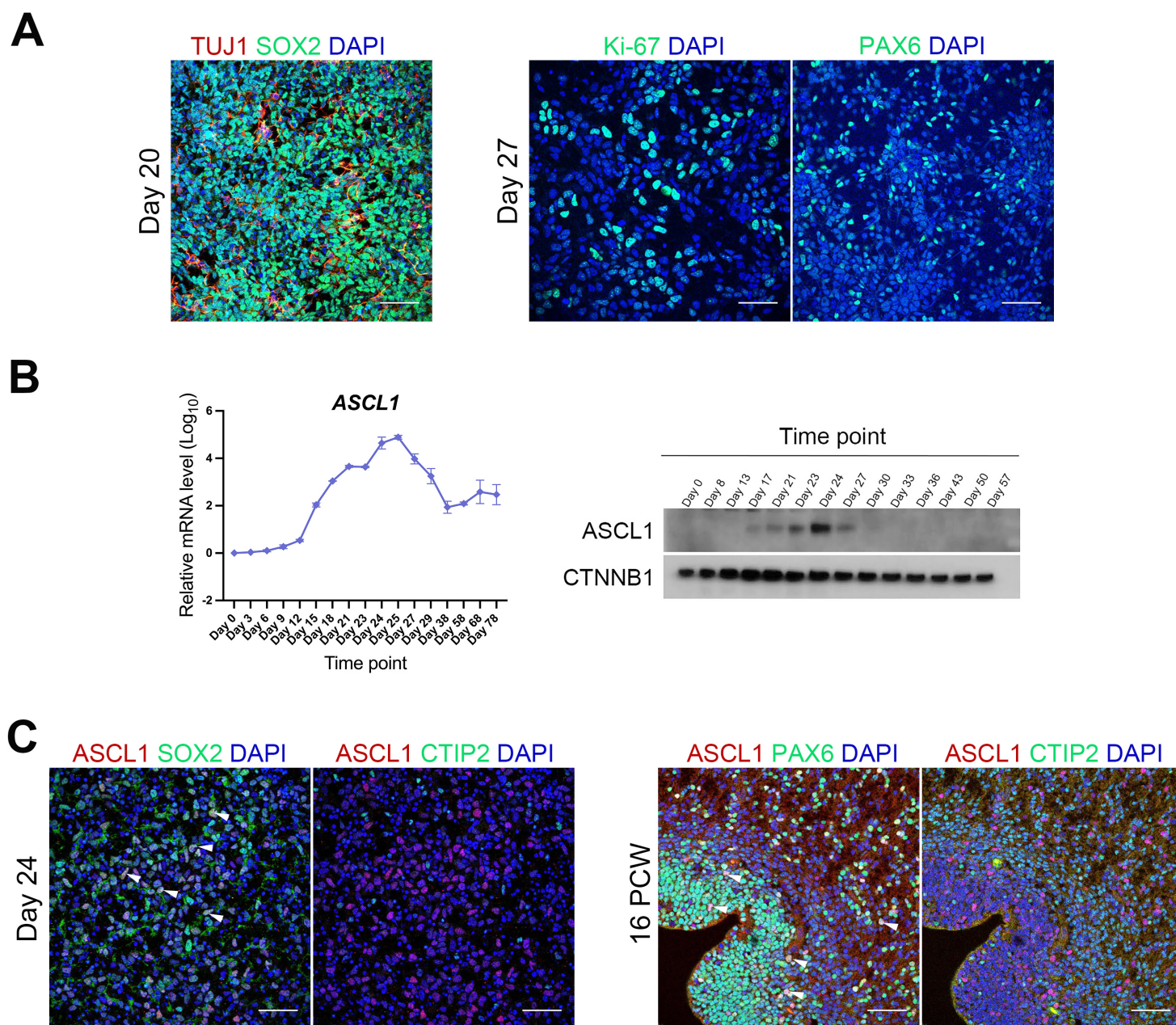


Figure 3.3: Expression pattern of ASCL1.

(A) Immunofluorescence images demonstrating the heterogeneity of the *in vitro* working model around the NPC-neuronal transition stage. TUJ1-positive neurons could already be observed in the culture around day 20, before DAPT addition (left). Small number of actively-dividing NPCs (Ki-67- or PAX6-positive) persist in the culture post Notch inhibition (right). Scale bars, $50\mu\text{m}$.

(B) qRT-PCR (left) and western blot (right) analyses of ASCL1 expression. mRNA data represents mean \pm SEM of 3 biological replicates. Representative CTNNB1 loading control for western blot is included.

(C) Immunofluorescence images showing co-localisation between ASCL1 and NPC markers such as SOX2 or PAX6, or deep layer neuronal markers such as CTIP2 in both *in vitro* (left – neuronal cells 24 hours post Notch inhibition) and *in vivo* (right – human foetal cortex at 16 PCW). Scale bars, $50\mu\text{m}$.

3.2.1 Profiling ASCL1 binding sites

As presented above, during the early stages of human cortical development, ASCL1 is expressed in heterogeneous populations of neural cells: before DAPT addition, most of the cells are actively-dividing NPCs, while from day 24 onwards most of the cells acquire a post-mitotic neuronal identity. Antibody limitations (lack of antibody availability for some immunofluorescence experiments, species limitations for co-localisation analysis) made it difficult to fully interpret ASCL1 expression pattern and draw final conclusions about the association between ASCL1 expression timing and its function. Therefore, in order to further address ASCL1 function, I sought to identify its genomic targets by ChIP-seq. As already described, ASCL1 shows stable upregulation after day 15 of neural induction onwards. The main expression peak during the neuronal differentiation process is observed 24 – 48 hours post Notch inhibition, followed by a rapid decline in expression by day 27. In order to investigate the dynamics of ASCL1 binding I collected neural progenitors at day 20, as well as cells 24 hours following DAPT addition to perform ASCL1 ChIP-seq. The cells at the two selected time points enabled me to describe the DNA binding profile during ASCL1 expression time period and to determine whether ASCL1 has different binding targets at different time points during neuronal differentiation, potentially associated with different functions.

Independent neural inductions from hiPSCs were performed in two separate occasions and cells were collected at day 20 and day 24 during each of the two differentiations. Using input chromatin as control, I first set out to identify the ASCL1 binding events for each of the two time points. A peak was placed in the final *bona fide* set of ASCL1 binding sites used for downstream analyses if that peak was found in

each of the two experimental replicates. As a results, I identified 3592 ASCL1 binding targets in cell cultures at day 20, and 11810 ASCL1 binding targets in neuronal cultures at 24 hours post Notch inhibition.

I next sought to compare the ASCL1 binding sites between day 20 and day 24. A common peak was recorded when a minimum overlap of 1bp (default) was detected. I found approximately 97% of the ASCL1 ChIP-seq peaks from NPCs were also present in day 24 neuronal cell cultures (Figure 3.4.A). Looking at the differences in peak intensity for the peaks identified at both time points, I noticed that all peaks display a higher intensity at the later time point (day 24). I also compared the intensity of peaks identified exclusively at day 24 with the ASCL1 binding profile at the same genomic locations for the cells at day 20. Interestingly, for all ASCL1 binding sites detected only at day 24, I also detected low ASCL1 enrichment at day 20. These low intensity peaks did not have the default threshold intensity to be called 'true' peaks by the utilized software (Figure 3.4.B-C). In order to check for a potential preference in binding between the ASCL1 early binding events and events which could only be detected at the later time point during neuronal differentiation, I looked at the binding sites distribution at the genome level. The mapping of ASCL1 binding targets to various genomic features identified the same pattern for both sets of peaks. Approximately 25% of ASCL1 binding occurs inside genes or at their promoter regions, whereas more than 70% of the binding events are found within intergenic regions or gene introns. The association of ASCL1 binding events to the nearest identified promoters revealed that more than two thirds of the ASCL1 binding occurs at long distances from the promoters for both ASCL1 ChIP-seq datasets – day 20 and day 24 (Figure 3.4.D). This observation, together with the fact that approximately 70% of ASCL1 targets are found within gene introns or intergenic regions, suggests

that ASCL1 binding occurs predominantly at distal regulatory regions, results that recapitulate previous reports from mouse (Raposo et al., 2015).

Taken together, these observations suggests that ASCL1 increase in expression directly correlates with its binding, rather than having a binding activity with different targets at different time points. The lack in binding preference for specific genomic locations at different time points during neuronal differentiation also implies a direct correlation between ASCL1 expression and binding (the highest expression levels detected immediately following DAPT addition correlate with ASCL1 binding its full repertoire of genomic targets), with no dynamic binding activity being detected. Moreover, the increase in ASCL1 expression level and the progressive increase in binding intensity directly correlates with the proportion of cells that have exit the cell cycle and acquired post-mitotic neuronal identity. This correlation reinforces the idea that ASCL1 might be required to promote neuronal differentiation.

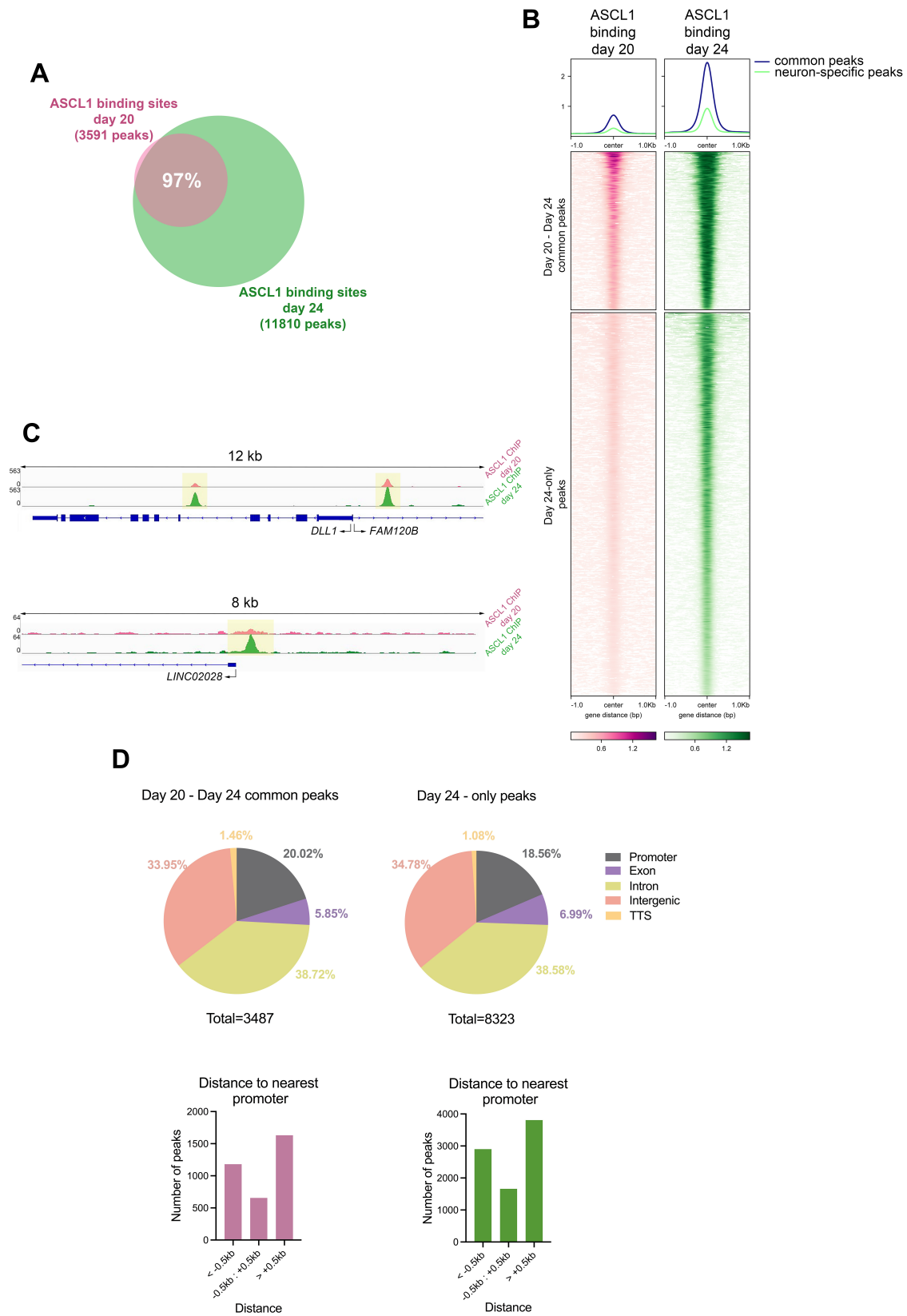


Figure 3.4: ASCL1 binding landscape. (Legend next page)

Figure 3.4: ASCL1 binding landscape.

(A) Venn diagram indicating the overlap of ASCL1-bound sites in NPCs at day 20 compared to neuronal cells at day 24.

(B) Density heat maps showing ASCL1 binding peak intensity at the two selected time points (day 20 – pink and day 24 - green).

(C) ChIP-seq binding profiles showing ASCL1 enrichment at day 20 (pink) and day 24 (green). Top profile shows two examples of peaks which were called at both time points and demonstrates peak intensity is always higher in the cell population where ASCL1 is highest expressed (day 24). Bottom profile exemplifies a binding site that was only detected at day 24, but also shows there is ASCL1 enrichment at day 20, although not enough for the peak calling threshold.

(D) Genomic distribution of the ASCL1 binding sites detected at both time points (left) and the ones detected only at day 24 (right) demonstrates the two peak sets share the same characteristics. Representative ChIP-seq binding profile of two biological replicates. Peak calling performed by Harshil Patel, BABS.

3.2.2 Detecting ASCL1-dependent genes during human cortical development

Another way of correlating ASCL1 function with the identity of cells in the heterogeneous populations was to look at the transcriptional profiles of cells at different time points. Therefore, I extracted RNA at the two time points – day 20 and day 24 – from the same two independent neuronal differentiations used to perform the ChIP-seq experiments presented above. Bulk RNA-seq of these samples was done and, subsequently, Harshil Patel performed differential gene expression analysis between the two time points. I then divided the genes into three different groups: day 20 specific genes, which represent the genes significantly downregulated at day 24 in comparison with day 20; conversely, day 24 specific genes, which represent the genes significantly upregulated at day 24 compared to their expression level at day 20; finally, a third set contains genes expressed at similar levels at both time points (Figure 3.5.A). I then asked how many of the genes in each of the three groups are regulated by ASCL1. More specifically, I was interested in the genes that are misregulated in the absence of ASCL1. In order to be able to perform this analysis, I used

three independent hiPSC clones that lack ASCL1 (ASCL1^{-/-}) generated by Siew-Lan Ang in the Guillemot group using the CRISPR-Cas9 technique (Figure 3.5.B). Similar to the experiments performed for the wild-type cells, I subjected these three hiPSC clones to cortical differentiation and extracted RNA from the neural cells at day 20 and day 24. Following bulk RNA-seq on this material, we performed differential gene expression analysis between wild-type and ASCL1^{-/-} cells at each of the two time points. This analysis allowed me to divide the genes in each of the three groups defined above (NPC-specific, neuronal-specific and common) into ASCL1-dependent and ASCL1-independent genes.

Similarly to the results from the analysis on the ASCL1 expression pattern, as well as from the ChIP-seq analysis, I discovered only a small subset (7.52%) of the day 20 specific genes to be ASCL1-dependent. When I looked at the common group of genes, only a small proportion of them were found to be ASCL1-dependent. Interestingly, even if genes from this group are expressed at relatively similar levels in both day 20 and day 24 neuronal cells, the majority of ASCL1-dependent genes in this group seem to be regulated by ASCL1 only at the later time point (24 hours post Notch inhibition). In contrast, when I investigated the day 24 specific genes, I discovered that almost half of them (44.59%) are misregulated in the absence of ASCL1 (Figure 3.5.C). In agreement with previous reports that stated ASCL1 as a transcriptional activator at the genome level (Raposo et al., 2015), most of the genes found to be ASCL1-dependent seem to be downregulated in its absence. Moreover, our analysis shows that most of these genes are specific to the neuronal time point (24 hours after DAPT addition), which supports the results presented above that suggest ASCL1 has a critical role in regulating cell cycle exit of NPCs and their differentiation into post-mitotic neurons.

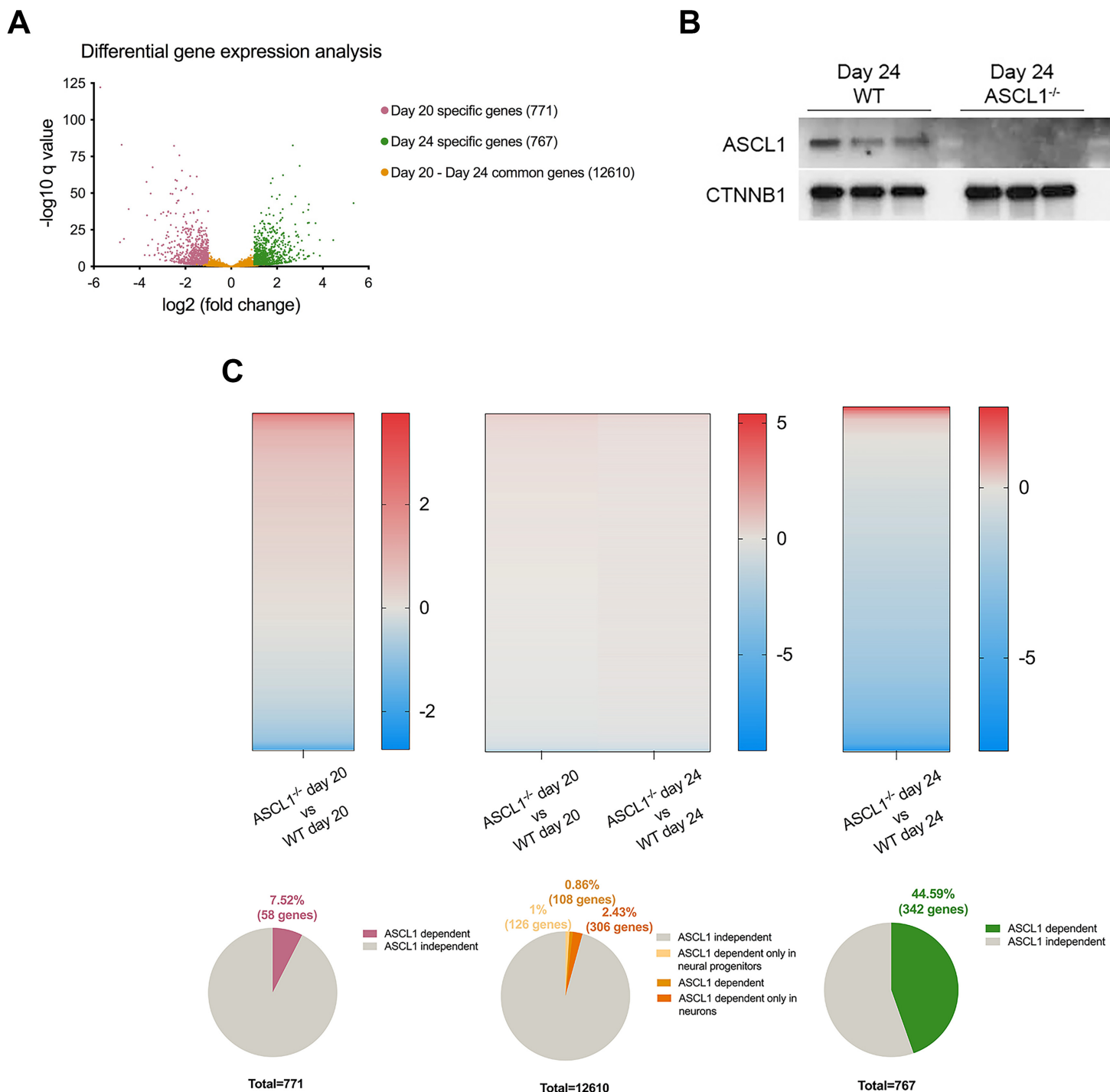


Figure 3.5: Profiling ASCL1-dependent genes.

(A) Volcano plot showing the division of genes expressed at day 20 and day 24 into three subsets: day 20 – specific genes (pink), day 24 – specific genes (green) and genes expressed at relatively similar levels between the two time points (orange).

(B) Western blot for ASCL1 at day 24 during the neuronal differentiation protocol starting from three independent batches of wild-type hiPSCs (left) and from the three independent hiPSC clones that lack ASCL1 (right). Representative CTNNB1 loading control is included.

(C) Division of genes into each of the three subsets into ASCL1-dependent and ASCL1-independent genes based on differential gene expression analysis between wild-type and ASCL1^{-/-} cells at each time point (day 20 or day 24). The heat maps also indicate whether the genes were downregulated (blue) or upregulated (red). Differential gene expression analysis performed by Harshil Patel, BABS.

3.3 mSWI/SNF complexes characterisation during human cortical neuronal differentiation

Most of the studies looking at the composition of mSWI/SNF complexes, as well as the requirement of different subunits at different stages and for different functions in the developing mammalian brain have been performed in rodents (Matsumoto et al., 2006; Lessard et al., 2007; Narayanan, Pirouz, et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016; Braun et al., 2021). Therefore, before performing any functional experiments that involved specific subunits of the mSWI/SNF complexes, I decided to look at their expression patterns in our model system. By combining qRT-PCR, western blot, IF and LC-MS/MS at different time points throughout the neuronal differentiation process, I characterised the patterns of most subunits that can incorporate in mSWI/SNF assemblies. For instance, I found that core subunits of the complex such as SMARCB1, SMARCE1 and SMARCD1 are expressed at similar levels throughout the *in vitro* differentiation process (Figure 3.6.A). Subunits that are present only in one of the three types of mSWI/SNF assemblies (Figure 1.5) (Mashtalir et al., 2018), resemble previously reported patterns. Such subunits include ARID1A and ARID1B, which incorporate in BAF complexes: ARID1A shows a slight increase in NPCs (data not shown) while its expression decreases when cells differentiate into post-mitotic neurons (Pagliaroli et al., 2021), while ARID1B has similar expression levels during the neuronal differentiation process. On the other hand, BRD7 and ARID2, which are core subunits of the PBAF complexes, or GLTSCR1 and BRD9, core subunits specific for ncBAF assemblies, show no variation during neuronal differentiation. These patterns confirm previous lack of evidence from the literature that have not described these subunits as having tissue or cell-specific pat-

terns (Figure 3.6.A). The two ATPases, SMARCA2 and SMARCA4, as well as the core subunits SMARCC1 and SMARCC2 confirm previously described patterns (Ho, Ronan, et al., 2009; Lessard et al., 2007): SMARCA4 and SMARCC1 decrease in expression during cortical neurogenesis. This pattern is correlated with the progressive upregulation of their homologue subunits SMARCA2 and SMARCC2, respectively (Figure 3.6.B-D). These patterns are explained by the fact that both SMARCA4 and SMARCC1 are subunits that incorporate in all mSWI/SNF assemblies at the pluripotent cell stage, whereas during both the progenitor and neuronal stages, SMARCA2 and SMARCC2 are able to assemble within complexes. As a result, both SMARCA2 and SMARCC2 start competing with SMARCA4 and SMARCC1, respectively, for incorporation.

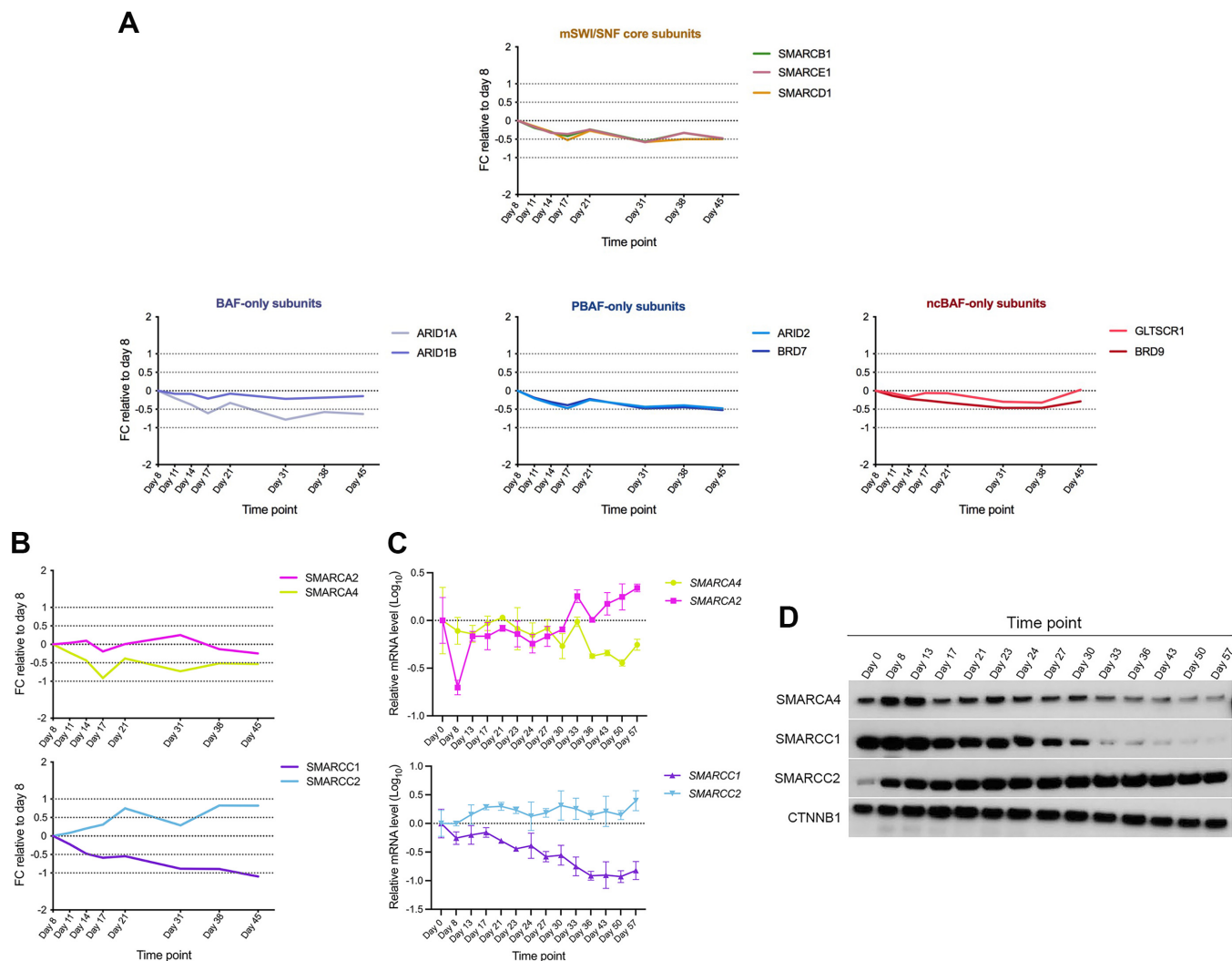


Figure 3.6: mSWI/SNF core subunits during human cortical development.

(A) LC-MS/MS analysis showing the patterns of core subunits present in all mSWI/SNF assemblies (top), as well as core subunits specific to one of the three types of mSWI/SNF remodellers (bottom) - BAF, PBAF and ncBAF - during *in vitro* cortical neuronal differentiation. LC-MS/MS experiment was performed by Clementina Cobolli Gigli in collaboration with the Francis Crick Proteomics STP.

(B-D) LC-MS/MS, qRT-PCR and western blot analyses on the expression patterns of SMARCA2 – SMARCA4 and SMARCC1 – SMARCC2 pairs. mRNA data represents mean \pm SEM of 3 biological replicates. Representative CTNNB1 loading control for western blot is included.

3.3.1 Characterisation of the human npBAF-nBAF subunit switch

The npBAF-nBAF subunit switch was discovered and characterised in mouse (Figure 1.8) (Lessard et al., 2007; Staahl, Tang, et al., 2013; Staahl and Crabtree, 2013; Braun et al., 2021). Whether the same mechanisms are at play during human neurogenesis remains unknown. Therefore, I also characterised the expression patterns of the switching subunits by qRT-PCR and western blot analyses (Figure 3.7). When combined with co-localisation IF analysis, their expression patterns give us a better understanding about the human npBAF-nBAF subunit switch. Quantification of the mRNA level at different time points during neuronal differentiation revealed that *ACTL6A*, *SS18*, *PHF10* and *DPF2* are all expressed in hiPSCs and NPCs. They are progressively downregulated after day 23, when cortical progenitors exit the cell cycle, but their expression is still detectable even at late neuronal time points. Conversely, their paralogs *ACTL6B*, *SS18L1*, *DPF1* and *DPF3* are absent or very lowly expressed in hiPSCs and NPCs, but afterwards upregulated in neurons (Figure 3.7.A).

Based on antibody availability, I further investigated these findings at the protein level for *ACTL6A* – *ACTL6B* and *SS18* – *SS18L1* subunit homologue pairs both *in vitro* and *in vivo*. Western blot analyses were also performed using cell lysates collected at sequential time points during neuronal differentiation. I observed an early (48 hours post DAPT) and sharp downregulation of the NPC specific BAF subunit *SS18*, which is completely abolished in late neuronal cultures. Western blotting for *ACTL6A* and *ACTL6B* showed that these subunits follow the patterns identified in the qRT-PCR assays. *ACTL6B* is completely absent until around day 20 of neural induction,

when neurons start to be generated, and then upregulated in the neuronal cultures. Conversely, ACTL6A protein level decreases progressively following Notch inhibition with DAPT. However, there seems to be an overlap of expression between these two subunits at the neuronal stage (Figure 3.7.B).

To investigate the expression patterns of the BAF complex switching subunits in NPCs versus post-mitotic neurons *in vivo*, I performed co-staining of each subunit with SOX2 and CTIP2, markers of NPCs and deep layer neurons located in layers V and VI (Arlotta et al., 2005; Britanova et al., 2008), respectively, in human foetal cortex at 12 PCW. In the case of SS18 – SS18L1 homologue pair, SS18 mainly co-localises with SOX2 positive cells, though it could also be observed in a subset of CTIP2-positive neurons in the SVZ. Similarly, SS18L1 is mainly expressed in the CTIP2-positive neurons found in the CP, but I could also detect it in some cells in the SVZ. On the other hand, the ACTL6A – ACTL6B homologue pair expression mimics the western blot results obtained from the *in vitro* analysis. While ACTL6B expression specifically overlaps with CTIP2-positive cells that have already migrated from the SVZ into the CP, ACTL6A is mainly expressed in SOX2 NPCs. However, some CTIP2-positive cells from both the SVZ and the CP were also positive for ACTL6A (Figure 3.7.C).

This data shows that the npBAF-specific subunits follow the main patterns described during the rodent developing cortex: they are expressed in pluripotent stem cells and NPCs and mainly co-localise with NPC-specific markers such as SOX2. However, I was not able to see the sharp downregulation of these proteins when the progenitors exit the cell cycle, they still being detectable at later neuronal stages. Co-localisation experiments for both ACTL6A and SS18 show they are also expressed in some CTIP2-positive neurons, which suggests that their detection at later stages during *in*

in vitro neuronal differentiation was not only a consequence of the culture heterogeneity. Looking at the nBAF-specific subunits, both ACTL6B and SS18L1 are rapidly up-regulated following DAPT addition. However, SS18L1 could also be detected at low levels as early as day 8 of the neuronal differentiation protocol, when no post-mitotic neuronal markers were detected with any of the techniques used. Together with the co-staining experiments my analyses do not point in the direction of a sharp subunit switch between SS18 and SS18L1 during human cortical development. However, further investigations would be required to confirm this hypothesis. Interestingly, ACTL6B was the only subunit which could only be detected following DAPT addition and in CTIP2-positive post-mitotic neurons in the CP of the developing human cortex. Co-staining experiments between homologue subunits (ACTL6A-ACTL6B and SS18-SS18L1) would be ideal to investigate whether they co-localise at the cellular level. However, it is not possible to perform this experiment at the moment due to the species limitations of the available antibodies.

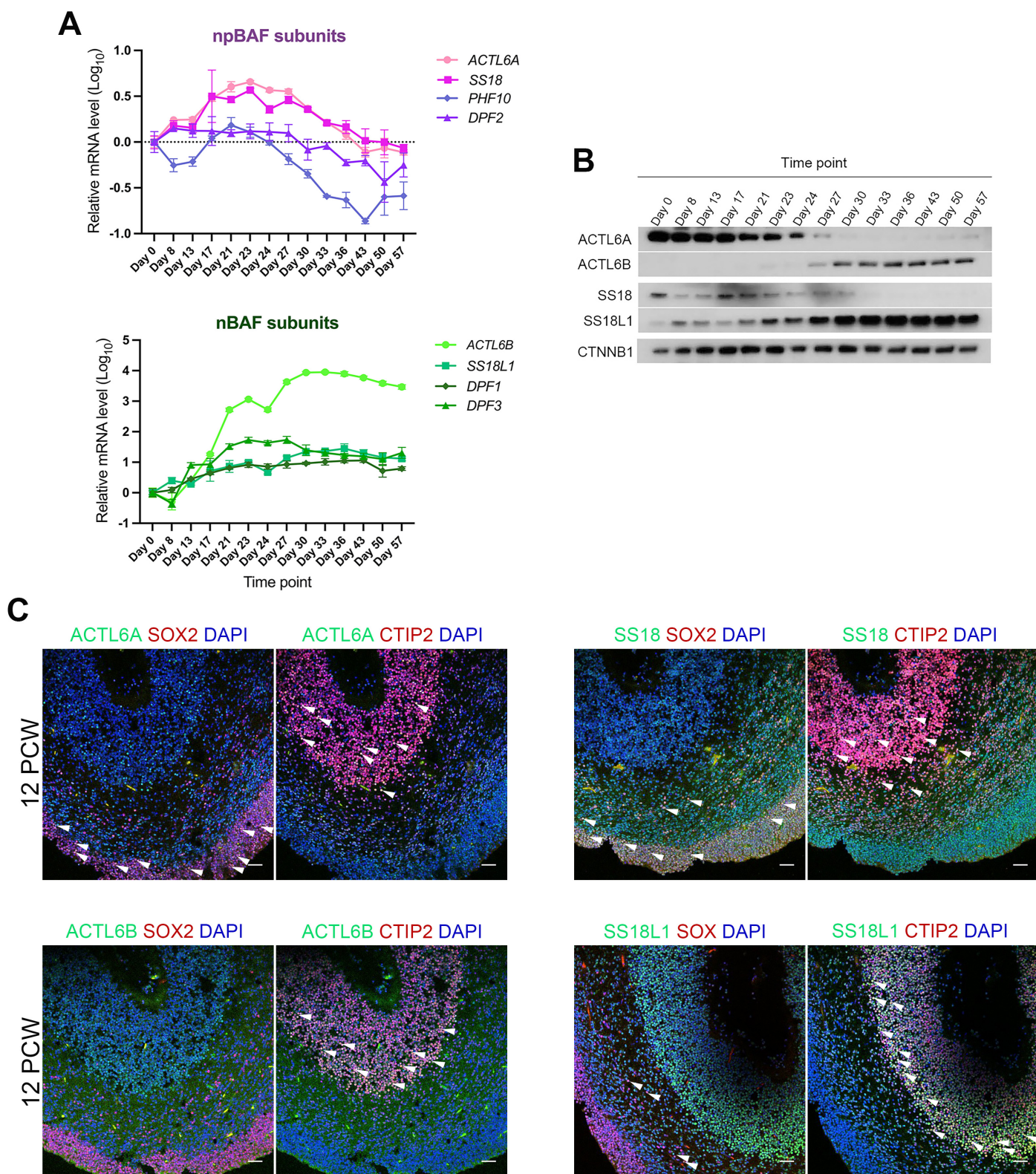


Figure 3.7: Human npBAF-nBAF subunit switch.

(A-B) qRT-PCR and western blot analyses of the npBAF and nBAF subunits. mRNA data represents mean \pm SEM of 3 biological replicates. Representative CTNNB1 loading control for western blot is included.

(C) Immunofluorescence images showing co-localisation between the npBAF subunits ACTL6A and SS18 or their mutually exclusive homologue subunits ACTL6B and SS18L1 and the NPC-specific marker SOX2 or the neuronal marker CTIP2. Scale bars, 50 μ m.

Chapter 4

Results Chapter 2: ASCL1 Interacts with the mSWI/SNF Complexes

Previous studies conducted in rodents revealed the critical roles of ASCL1 in both ventral and dorsal domains of the embryonic telencephalon (Casarosa et al., 1999; Nieto et al., 2001; Britz et al., 2006; Petryniak et al., 2007). Among the three bHLH proneural transcription factors (ASCL1, NGN1 and NGN2), ASCL1 has been shown to have pioneer activity (Wapinski et al., 2013; Raposo et al., 2015; Guillemot and Hassan, 2017). In addition, similar to all eukaryotic transcription factors, ASCL1 shows low sequence specificity and its binding domain is shorter than those of other bHLH proteins that act later during neuronal differentiation (Soufi et al., 2015). These observations point towards an additional mechanism that is required to regulate the chromatin state at ASCL1 targets. Since regulation of chromatin accessibility is essential for the recruitment of other transcription factors and generation of functional gene regulatory networks, it is important to investigate how ASCL1

achieves this essential feature of its activity. ATP-dependent chromatin remodellers have been shown to assist some pioneer transcription factors at their target sites (Hu et al., 2011; Takaku et al., 2016; Wang, Du, et al., 2014; King and Klose, 2017). Most of the mSWI/SNF complex subunits have been shown to regulate distinct processes during neural development. Mutations in these subunits also cause different neurodevelopmental disorders (reviewed in Sokpor et al., 2017; Mossink et al., 2021). Taken together, these pieces of evidence suggest there is an overlapping effect of ASCL1 and mSWI/SNF remodellers in mammalian neurogenesis. Therefore, we hypothesised that there is a mutual interaction between ASCL1 and mSWI/SNF complexes with different possible outcomes: ASCL1 recruits mSWI/SNF complexes to DNA in order to regulate chromatin accessibility and make it accessible to non-pioneer transcription factors and other DNA binding molecules; mSWI/SNF complexes bind the DNA and open the chromatin at ASCL1 target sites; or that both ASCL1 and the mSWI/SNF complexes depend on each other for binding at specific genomic loci where they are responsible for regulating chromatin accessibility.

The strategy I adopted to characterise the putative interaction between ASCL1 and mSWI/SNF complexes, as well as the role of this interaction in human cortical development, was to first look whether there is any physical interaction between them, followed by analyses at the chromatin level in human neuronal cells expressing ASCL1. This approach enabled me to study whether ASCL1 requires the activity of mSWI/SNF remodellers to access its targets.

4.1 Physical interactions between ASCL1 and mSWI/SNF complexes

Having established the expression pattern of ASCL1 in the *in vitro* model of human corticogenesis outlined in the previous chapter (Figure 3.1, Figure 3.2), I used neuronal cells at 24 hours post-DAPT, which show a peak in ASCL1 expression (Figure 3.3), to check ASCL1 interaction with the mSWI/SNF complexes. To test this hypothesis, I conducted co-IP assays. For this experiment, I used SMARCC1 as the mSWI/SNF complex representative subunit. As already mentioned, SMARCC1 is a core subunit of the complex, putatively incorporated in all possible assemblies (Phelan, Sif, et al., 1999; Kadam et al., 2000; Mashtalir et al., 2018). In these assays, SMARCC1 co-immunoprecipitated with ASCL1 (Figure 4.1.B). As a positive control I used the interactions between different subunits that incorporate in mSWI/SNF assemblies (Figure 4.1.A). Based on the definition of protein complexes, as well as the definition of mSWI/SNF chromatin remodellers, when a specific protein of the complex is immunoprecipitated, all the other component subunits are also pulled down. In contrast, I used Catenin Beta 1 (CTNNB1), a protein involved in the regulation of cell adhesion as component of an E-cadherin:catenin complex, as a negative control: as expected, no interaction was recapitulated between ASCL1 and CTNNB1 or between mSWI/SNF subunits and CTNNB1 (Figure 4.1.B). The ASCL1 – mSWI/SNF interaction was also recapitulated when ARID1A was used as the representative subunit of the complex (Figure 4.1.B).

The physical proximity between ASCL1 and mSWI/SNF complexes was further confirmed by proximity ligation assay (PLA) experiments, performed both *in vitro* in 24

hours post DAPT neuronal cells, as well as *in vivo* using 13 PCW foetal cortices. I used a secondary antibody coupled with an anti-mouse PLA probe that recognises ASCL1 primary antibody and another one coupled with an anti-rabbit PLUS PLA probe that recognises the SMARCC2 subunit of the mSWI/SNF assemblies. I took advantage of the physical proximity between SMARCC2 and SMARCB1 subunits of the mSWI/SNF complexes as a positive control (Figure 4.1.C-D). On the other hand, the combination between SMARCC2 and the neuronal marker TUJ1 was used as negative control (Figure 4.1.C). While SMARCC2 is known to be found in the nucleus, TUJ1 is a cytoplasmatic protein. Therefore, as an interaction has not been previously reported, and SMARCC2 and TUJ1 having distinct localisations within the cells, they were considered an appropriate control pair for the experiment. A fluorescent signal was detected when I probed for ASCL1 and SMARCC2 in both *in vitro*-derived neuronal cells as well as in the foetal cortex (Figure 4.1.C-D), which suggests ASCL1 and mSWI/SNF complexes are found less than 40nm apart in ASCL1-expressing neuronal cells.

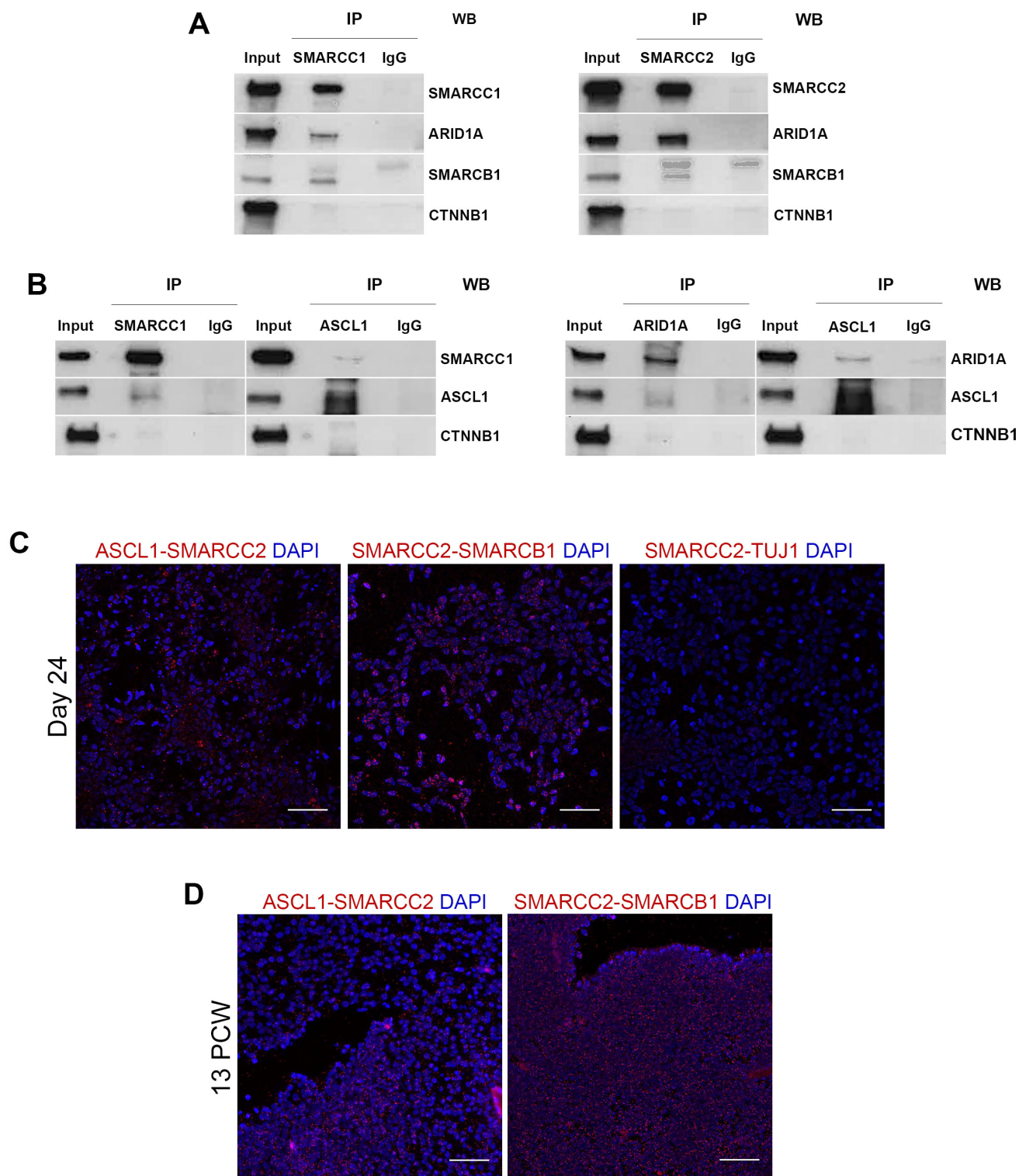


Figure 4.1: Physical interactions between ASCL1 and mSWI/SNF remodellers.

(A-B) Post co-IP western blot analyses demonstrating the interactions between different component subunits of the mSWI/SNF complexes (A) and between ASCL1 and SMARCC1 or ARID1A (B). Representative CTNNB1 non-interacting protein was included as a negative control.

(C-D) Immunofluorescence images following PLA experiment that tested the proximity between SMARCC2 and ASCL1 in neuronal cells at day 24 (C) and human foetal cortices at 13PCW (D). SMARCC2-SMARCB1 and SMARCC2-TUJ1 were included as positive and negative controls, respectively. Scale bars, 50 μ m.

4.2 Genome-wide location analysis of ASCL1 and mSWI/SNF remodellers in human iPSC-derived neuronal cells

To further characterise the ASCL1 – mSWI/SNF interaction, I aimed to determine whether ASCL1 requires mSWI/SNF complexes to regulate all its targets, only a subset of them or whether ASCL1 does not require mSWI/SNF activity to bind a significant fraction of its targets. As a first step to address this, I sought to overlap the genomic targets of ASCL1 and of mSWI/SNF remodellers to establish the level at which they share DNA binding landscapes.

4.2.1 Determining mSWI/SNF complexes binding sites

As reported in Results Chapter 1, ASCL1 binding profile seems to correlate with its expression pattern, with a final repertoire of binding sites being detected 24 hours post Notch inhibition. To be able to perform the comparison with the mSWI/SNF binding profile that will be explored in this section, I decided to focus on the set of ASCL1 ChIP-seq peaks identified at day 24. Therefore, on three separate occasions, I collected hiPSC-derived neurons at day 24 and performed ChIP-seq for SMARCB1, which is a core subunit of mSWI/SNF complexes, incorporated in all possible assemblies (Mashtalir et al., 2018). I generated a final set of 26207 SMARCB1 binding sites that contains peaks found in at least two out of the three replicates that were sequenced. This *bona fide* set of SMARCB1 targets was subsequently used for all analyses and comparisons involving mSWI/SNF binding land-

scape. In agreement with previous reports from different mammalian model systems looking at mSWI/SNF binding, we obtained a set of broad peaks with a peak average of 2.3 kb (Figure 4.2.A). In addition, around 70% of the SMARCB1 targets were found within introns or intergenic regions that are located more than 500bp from the nearest promoters (Figure 4.2.B) (Ho, Ronan, et al., 2009; Ho, Miller, et al., 2011; Euskirchen et al., 2012; Bossen et al., 2015; Barutcu et al., 2016; Wang, Lee, et al., 2017).

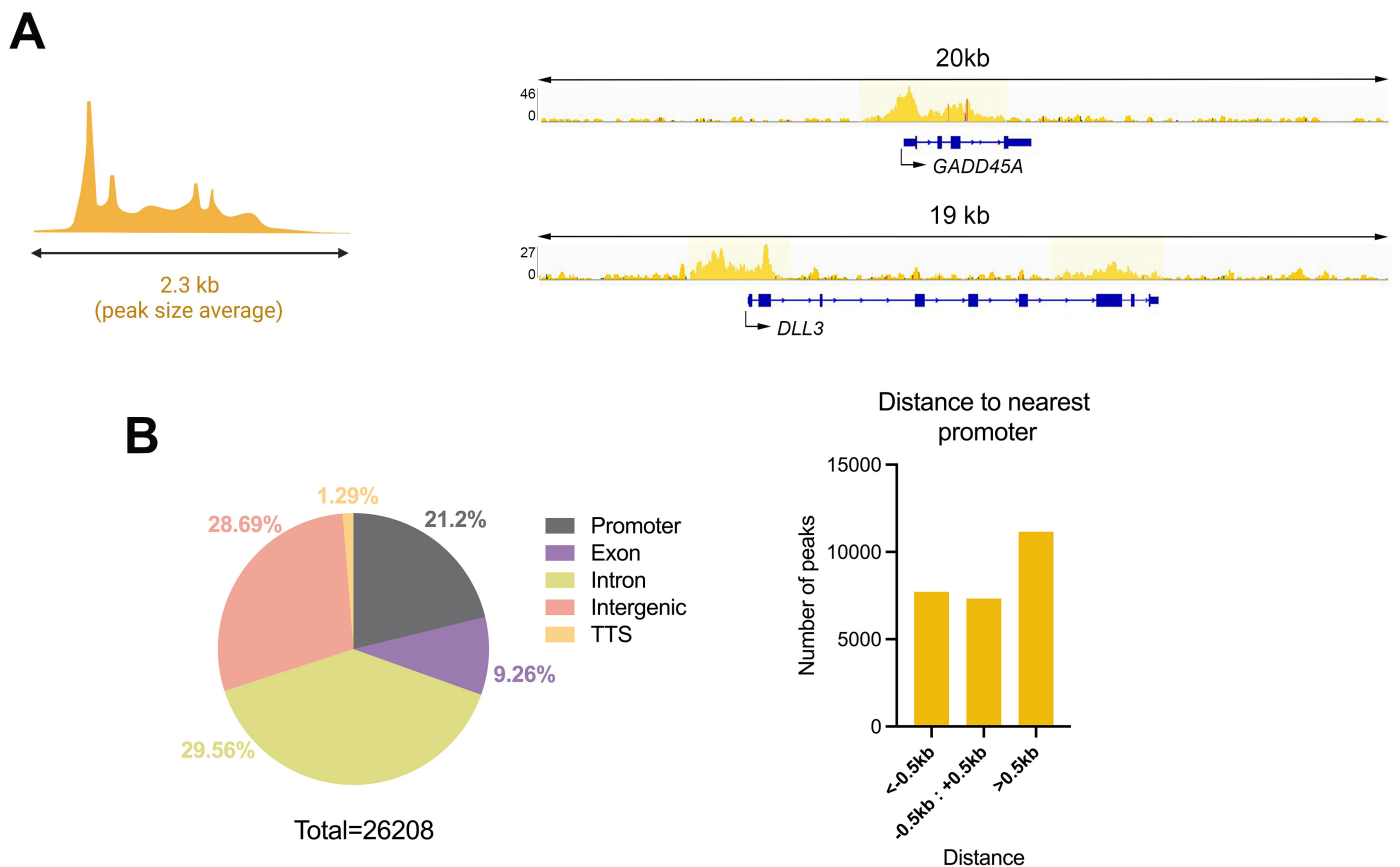


Figure 4.2: SMARCB1 binding landscape.

(A) ChIP-seq binding profiles showing SMARCB1 enrichment.

(B) Genomic distribution of the SMARCB1 binding sites. Representative ChIP-seq binding profile of three biological replicates. Peak calling performed by Harshil Patel, BABS.

4.2.2 ASCL1 and mSWI/SNF remodellers share DNA binding landscapes

Having generated both ASCL1 and SMARCB1 ChIP-seq datasets, I next sought to compare their binding sites by looking at the common peaks in the two sets. To begin with, a common peak was recorded when at least 1bp overlap was detected. Using this criteria, I found that 66.25% of ASCL1 targets are also SMARCB1 binding sites, therefore targets of the mSWI/SNF complexes (Figure 4.3.A-B). However, considering ASCL1 is a transcription factor with classic, narrow peaks that have an average width of 455bp, while mSWI/SNF remodellers have broad binding sites with an average width of more than 2kb, we considered 1bp overlap might also happen by chance. In order to address this question, I performed successive overlaps (Figure 4.3.B, right panel) between the peaks in the two datasets (ASCL1 and SMARCB1) to look at the proportion of the ASCL1 binding sites that are found inside the SMARCB1 peaks. Interestingly, the proportions did not change significantly, with almost 60% out of the 66.25% of the common ASCL1 – SMARCB1 peaks overlapping with at least 90% of the ASCL1 peak proportion (Figure 4.3.B). This result suggests that ASCL1 shares a high percentage of its binding landscape with that of SMARCB1, with the overlap more likely to be specific rather than occurring by chance.

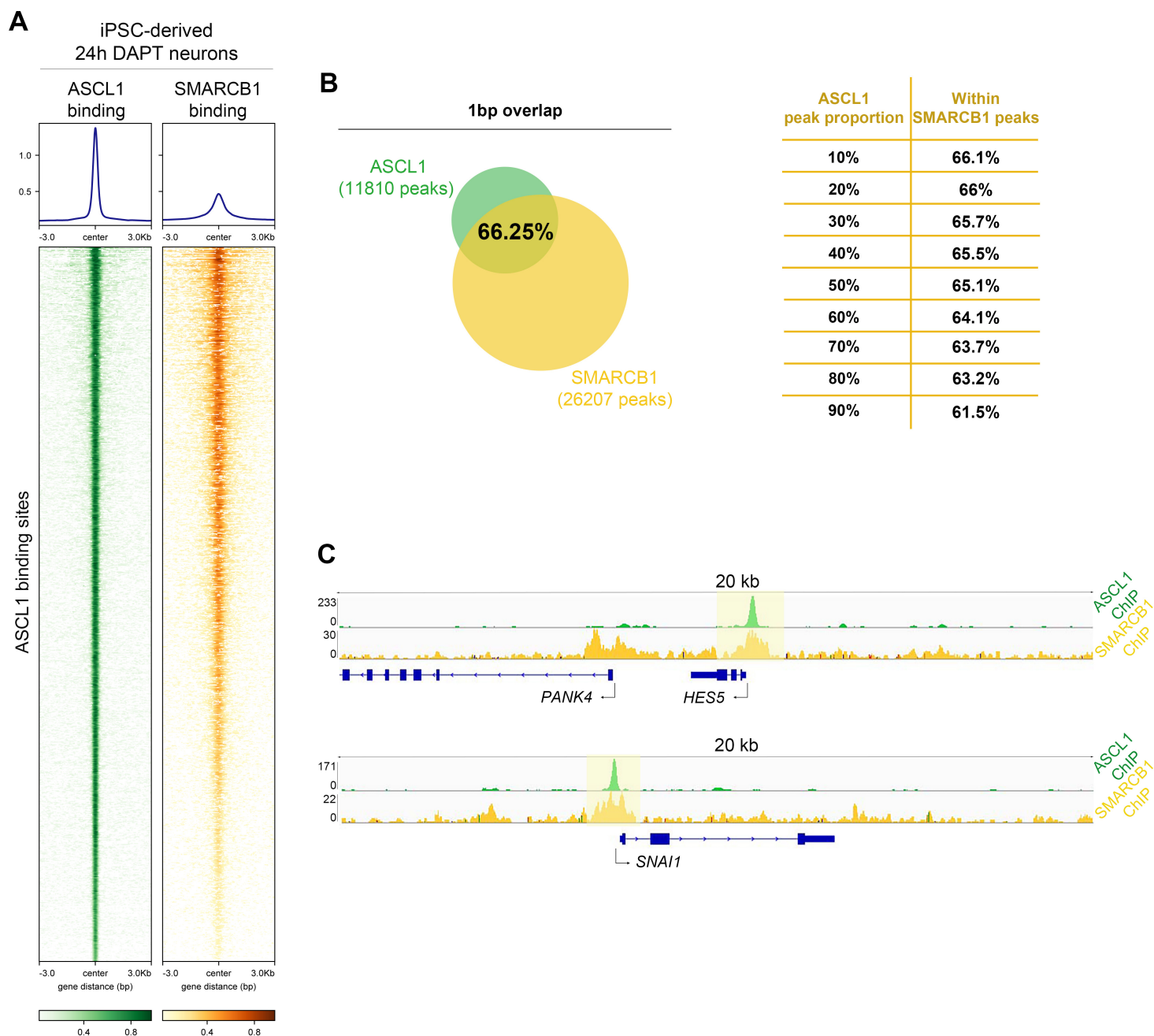


Figure 4.3: ASCL1 and SMARCB1 share DNA binding landscapes.

(A) Density heat maps showing SMARCB1 enrichment at all ASCL1 binding targets at day 24.

(B) Venn diagram indicating the percentage of ASCL1 binding sites which are also targets of the mSWI/SNF complexes. Table on the right shows the overlap percentages between ASCL1 and SMARCB1 binding targets when a specific proportion of the ASCL1 peak surface (10-90%) is considered for the overlap rather than 1bp.

(C) Examples of ChIP-seq binding profiles demonstrating the overlap between ASCL1 and SMARCB1 binding targets. Representative ChIP-seq binding profile of two or three biological replicates. Peak calling performed by Harshil Patel, BABS.

While we were generating the final set of SMARCB1 binding sites based on the criteria used for all ChIP-seq datasets used in this project (i.e. a true peak must be found in at least two replicates), I noticed that most of the ASCL1 peaks were present in all replicates. However, each of the SMARCB1 ChIP replicates had high numbers of unique peaks, which were discarded (based on the criteria set for 'true' peaks). In order to address this issue, I decided to perform a new set of ChIP-seq experiments for the mSWI/SNF complexes. Because the aim is to look at the entire mSWI/SNF binding landscape, I chose SMARCC1 as a second core subunit of the complex that is incorporated in all possible assemblies (Mashtalir et al., 2018). Although different optimization steps were carried out (data not shown), ChIP-seq experiments using the SMARCC1 antibody resulted in the identification of no peaks, suggesting the ChIP was unsuccessful. With no antibodies for mSWI/SNF core proteins being successfully reported in the literature as suitable for ChIP-seq, I decided to generate a second SMARCB1 ChIP-seq dataset as an alternative. However, and also as a way to control for cell-type biased results, this time the ChIP-seq experiment was performed using H9 ESC-derived neuronal cells at day 24. Following sequencing, I obtained a final dataset comprised of 59562 SMARCB1 binding sites. Approximately 90% of the SMARCB1 binding sites found in the previously generated hiPSC-derived neuronal cells dataset were also recovered in this second experiment (Figure 4.4.A). Similar to previous reports found in the literature, as well as to the SMARCB1 ChIP-seq dataset generated from hiPSCs, these binding sites have a broad profile with a width average of approximately 1.7kb and more than 80% of them are found within gene introns or intergenic regions that are located more than 500bp from the nearest annotated gene promoters (Figure 4.4.B). More important, when I compared the ASCL1 binding sites with the SMARCB1 ones from this second set, I found that 78.79% of the ASCL1 peaks are also targets of the mSWI/SNF re-

modellers (Figure 4.4.C-D). This high percentage reinforces the idea of a functional ASCL1 – mSWI/SNF interaction at the DNA-binding level.

Even if I could not find ChIP-seq datasets generated using an antibody for a mSWI/SNF core subunit, there are multiple publicly available ChIP-seq datasets obtained using one of the two ATPase subunits (SMARCA2 or SMARCA4) that can incorporate in mSWI/SNF assemblies. I found two SMARCA4 datasets to be relevant for this project and therefore re-analysed and compared them with my ASCL1 dataset. One of them (Gao et al., 2019) was generated in NPCs derived from human H9 ESCs. Comparison between ASCL1 and SMARCA4 binding landscapes revealed a 41.44% peak overlap (Figure 4.4.C-D). A second SMARCA4 ChIP-seq dataset used was an ENCODE dataset (ENCSR839SFJ) generated from cortical bipolar spindle neurons or von Economo neurons (VENs) derived *in vitro* from the GM23338 hiPSC line. This hiPSC line was derived from human fibroblasts, and treated with 0.5 $\mu\text{g/ml}$ doxycycline hyclate for four days in order to generate bipolar neurons. Comparison between this SMARCA4 dataset and the ASCL1 one that I generated revealed 50% of the ASCL1 peaks to be SMARCA4 targets as well (Figure 4.4.C-D).

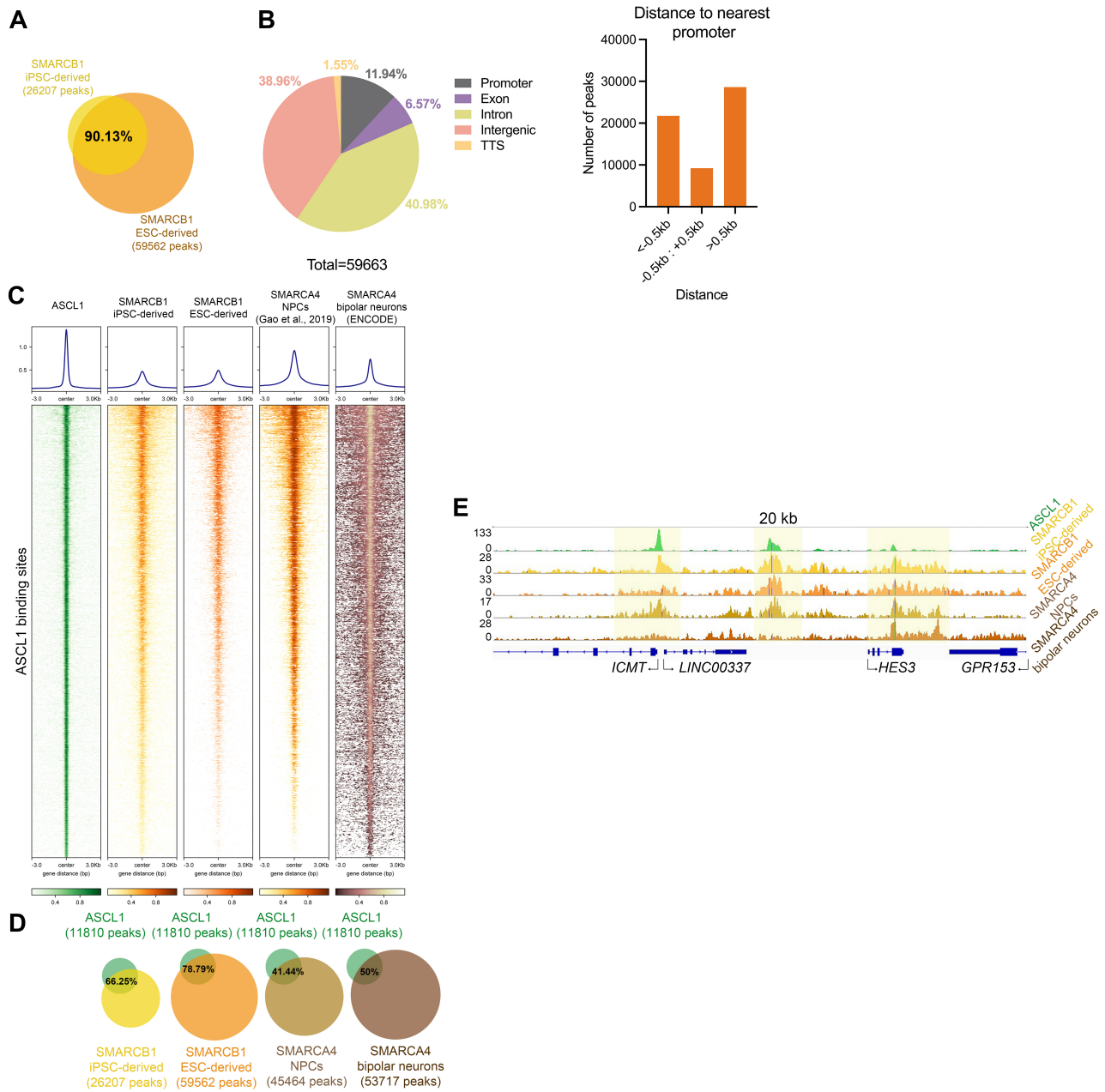


Figure 4.4: ASCL1 shares DNA binding landscapes with multiple mSWI/SNF subunits.

(A) Venn diagram showing the overlap between the two SMARCB1 ChIP-seq datasets that were generated.

(B) Genomic distribution of SMARCB1 binding sites detected in the H9 hESC-derived neurons.

(C) Density heat maps showing SMARCB1 (from the two generated ChIP-seq datasets) and SMARCA4 (in NPCs from Gao et al., 2019 and in hiPSC-derived bipolar neurons from ENCODE) enrichment at all ASCL1 binding targets at day 24.

(D) Each mSWI/SNF density heat map is accompanied by a Venn diagram (bottom) showing the overlap percentage between ASCL1 binding sites and that respective mSWI/SNF binding target dataset.

(E) ChIP-seq binding profile showing three examples of ASCL1 targets (highlighted), which are also binding sites of the mSWI/SNF remodellers in all four datasets used in the analysis. Representative ChIP-seq binding profile of two or three biological replicates. Peak calling performed by Harshil Patel, BABS.

Chapter 5

Results Chapter 3: Chromatin

Regulation at ASCL1 Targets

ASCL1 pioneer activity has been previously described in the context of *in vitro* conducted neuronal differentiation of mouse NSCs via *Ascl1* overexpression (Raposo et al., 2015), cellular reprogramming (Wapinski et al., 2013; Chanda et al., 2014), or pathological conditions such as patient derived glioblastoma cell lines (Park et al., 2017). However, all of the contexts listed above rely on exogenous (Raposo et al., 2015; Wapinski et al., 2013; Chanda et al., 2014) or pathological (Park et al., 2017) levels of ASCL1. The working model used for the experiments presented in this thesis not only recapitulates the early events of human corticogenesis from *in vivo* (Figure 3.1, Figure 3.2), but it also relies on endogenous levels of ASCL1. Therefore, it represents a system closer to the *in vivo* situation to confirm ASCL1 role as a pioneer transcription factor. In this chapter, I first describe ASCL1 pioneer activity via ATAC-seq experiments performed in both wild-type and ASCL1^{-/-} conditions.

Next, I investigate the putative requirement of ASCL1 for mSWI/SNF complexes recruitment or vice-versa to genomic targets. To be able to perform this type of analyses, I performed reciprocal disruption of ASCL1 and mSWI/SNF complexes at different time points throughout the neuronal differentiation process. As the ASCL1^{-/-} cell lines were already available in the Guillemot group (targeted knockout generated by Siew-Lan Ang), I also performed a CRISPR-Cas9-mediated acute mutation of both *SMARCC1* and *SMARCC2*, the core subunits required for assembly of mSWI/SNF remodellers (Narayanan, Pirouz, et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016; Mashtalir et al., 2018; Schick, Rendeiro, et al., 2019). By combining CHIP-seq and ATAC-seq in the mutant cell lines I was able to get a better understanding on the level at which ASCL1 and mSWI/SNF complexes collaborate to regulate chromatin accessibility at ASCL1 target sites, as well as on the level at which ASCL1 requires these chromatin remodellers to bind its targets. In addition, I also performed RNA-seq in both ASCL1^{-/-} and SMARCC1/2^{-/-} cells in order to associate ASCL1-mSWI/SNF dependent genomic targets with transcriptional regulation.

5.1 Investigating the pioneer activity of ASCL1

In order to explore the putative pioneer activity of ASCL1 during the early stages of human cortical development, I investigated its effect on the chromatin landscape. This analysis is based on ATAC-seq data that I generated in both wild-type and ASCL1^{-/-} contexts. As a starting point, chromatin accessibility was explored in neuronal cells 24 hours following Notch inhibition, the time point when ASCL1 is highest expressed. Differential accessibility analysis performed by Harshil Patel us-

ing DESeq2 revealed that 10146 DNA regions showed a decrease in accessibility in ASCL1^{-/-} versus wild-type neurons, while 8509 regions showed an increase in chromatin accessibility in the absence of ASCL1 (Figure 5.1.A). For a better understanding about the time ASCL1 is responsible for regulating chromatin accessibility at specific genomic loci, I performed the same ATAC-seq experiment but this time using NPCs at day 20, one of the first days when ASCL1 protein could be detected in the *in vitro* working model I used (Figure 3.1, Figure 3.2). This time, only 2983 regions showed decreased accessibility, while 1824 sites gained chromatin accessibility in the absence of ASCL1 versus wild-type cells (Figure 5.1.B). Interestingly, when I performed an overlap between the 2983 sites that lost chromatin accessibility in ASCL1^{-/-} NPCs at day 20 and the 10146 sites that lost accessibility in ASCL1^{-/-} neuronal cells at day 24, I identified an 86.7% overlap between them. Similarly, comparison between the 1824 regions with increased chromatin accessibility at day 20 and the 8509 sites that gained accessibility following DAPT addition revealed a 60.9% overlap (Figure 5.1.C). These results agree with the analysis on DNA binding and transcriptional regulation described in Results Chapter 1 and reinforced the idea of an essential role for ASCL1 at the time when NPCs exit the cell cycle to differentiate into post-mitotic neurons, and not earlier.

An additional observation that pointed in the same direction is represented by the identity of cells in terms of their chromatin landscape in the absence of ASCL1. Differential accessibility analysis between wild-type cells at day 24 and wild-type cells at day 20 allowed me to determine the changes in chromatin accessibility that would normally occur between these two time points: more specifically, 8761 genomic loci where chromatin would normally go from an open to a close state, and 11533 regions that would normally experience an opening of the chromatin. Interestingly,

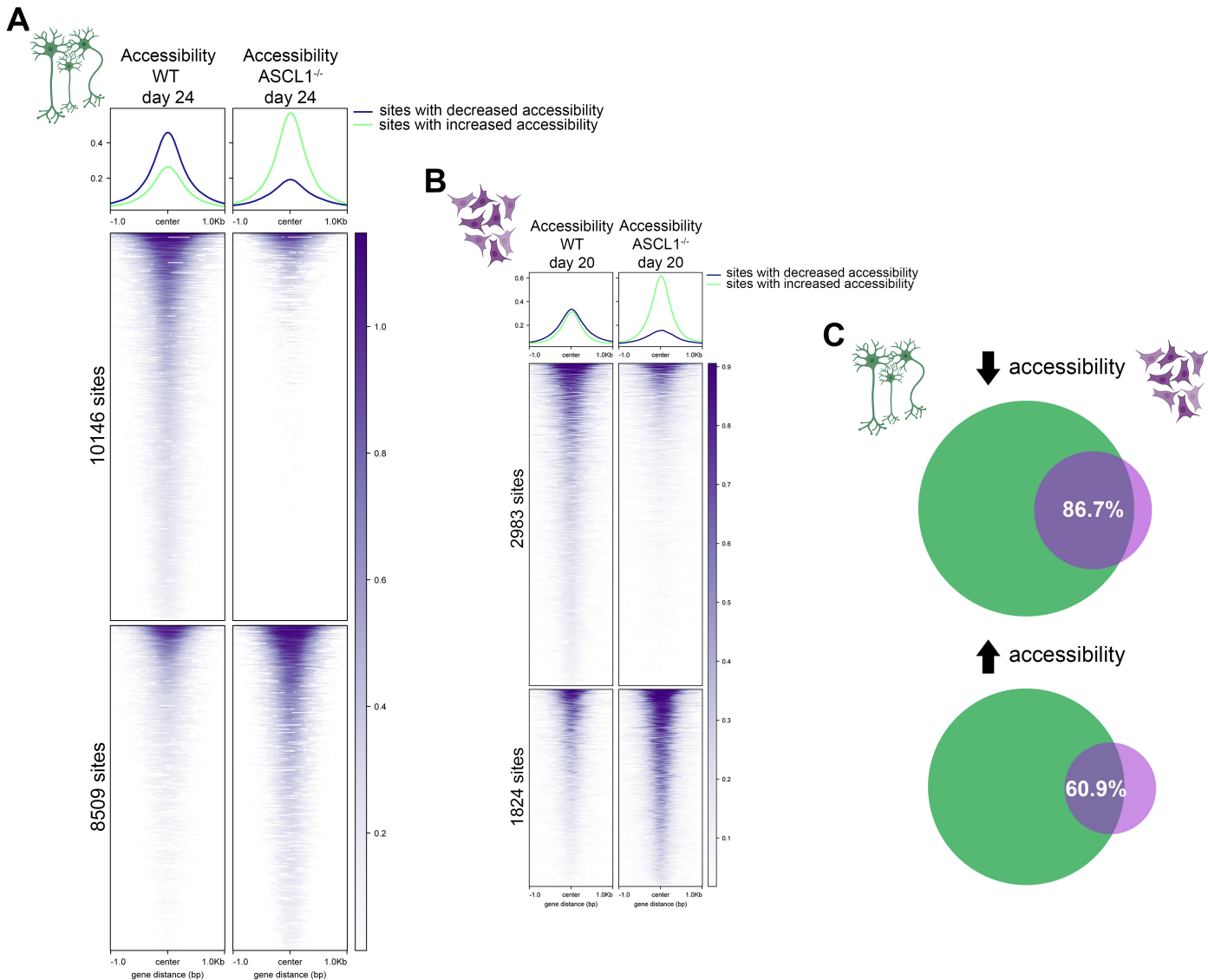


Figure 5.1: ASCL1-dependent accessibility.

(A-B) Density heat maps profiling ASCL1-dependent accessibility in neuronal cells at day 24 (A) and NPCs at day 20 (B). Representative ATAC-seq binding profile of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

(C) Venn diagrams showing the overlap between chromatin regions that lost or gained chromatin accessibility at day 20 (purple) and regions that lost or gained chromatin accessibility, respectively, at day 24 (green).

among the 8509 regions with increased chromatin accessibility upon ASCL1 removal, 3359 (which account for 43% of them) represent accessible sites specific for

NPCs at day 20, which in a wild-type context should have significantly decreased in neuronal cells at day 24. Similarly, 70% (7003 sites) of the 10146 regions which showed a decrease in accessibility in the absence of ASCL1 represent genomic sites that under wild-type conditions should have experienced an increase in chromatin accessibility in neurons at day 24 versus NPCs at day 20 (Figure 5.2). Again, these results provide additional evidence for the critical role of ASCL1 during the differentiation of NPCs into post-mitotic neurons. When ASCL1 is removed, it is unable to bind its target sites where it would normally induce chromatin opening. As a consequence, from the chromatin landscape point of view, the cells seem to be “blocked” in a NPC state, as shown by the high number of DNA regions (8509) that remain open at day 24 while they would be expected to show a decrease in accessibility (as demonstrated by the differential analysis between wild-type cells at day 24 versus day 20).

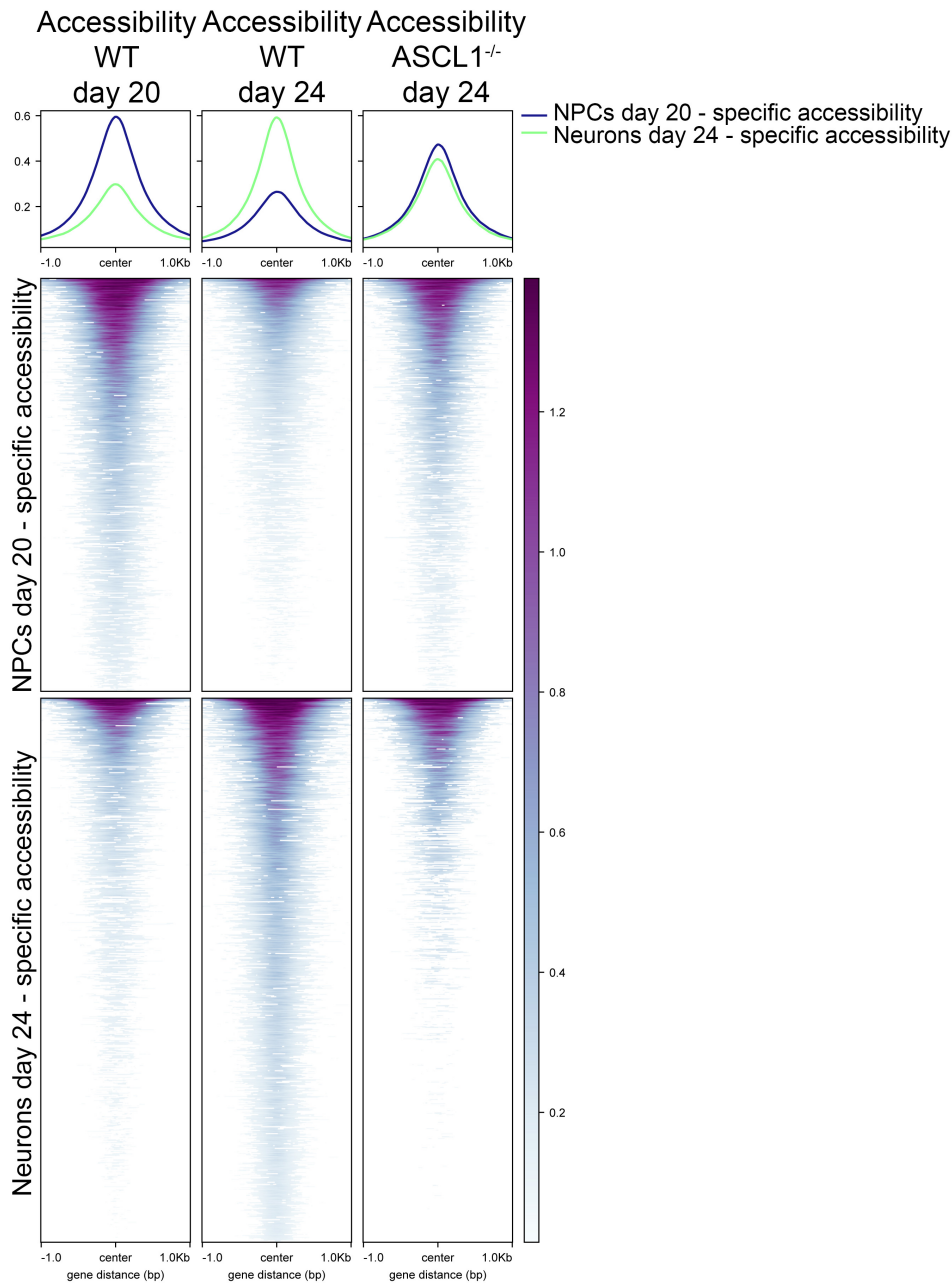


Figure 5.2: ASCL1^{-/-} neuronal cells at day 24 are blocked in a NPC-like stage from a chromatin landscape perspective.

Density heat maps comparing chromatin accessibility in ASCL1^{-/-} neurons (day 24) to wild-type day 20- and day 24-specific accessibility demonstrate that the chromatin accessibility landscape of ASCL1^{-/-} neuronal cells at day 24 resembles the landscape of wild-type NPCs at day 20. Representative ATAC-seq binding profile of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

The analysis described above revealed a high number of genomic loci that show a change in chromatin accessibility in neurons at 24 hours post Notch inhibition in the absence of ASCL1. However, the questions that arise are: how many of these changes in accessibility represent direct effects of ASCL1 loss and how many are indirect consequences? In order to address this, I compared the changes in chromatin accessibility defined by ATAC-seq with ASCL1 binding targets identified by ChIP-seq. I considered a site where ASCL1 is the regulator of accessibility where the region showing a significant change in the state of the chromatin is also an ASCL1 binding site. Conversely, the changes in chromatin accessibility that are not occurring at ASCL1 binding sites were considered indirect effects of ASCL1 loss. This correlation revealed that approximately 25% (2444 peaks) of the sites that show a decrease in chromatin accessibility upon ASCL1 removal are also ASCL1 binding sites. On the other hand, only a small proportion (328 peaks, which account for approximately 4%) of the genomic regions that gain accessibility in the absence of ASCL1 are also ASCL1 binding targets (Figure 5.3.A-B). These results were consistent with previous studies that described ASCL1 as a pioneer transcription factor, reporting it as a positive regulator rather than a repressor of chromatin accessibility, which is responsible for chromatin opening at its target sites (Raposo et al., 2015; Wapinski et al., 2013; Chanda et al., 2014; Park et al., 2017).

Since the main aim of this project was to investigate the mechanisms underlying the role of ASCL1 as a pioneer transcription factor, most of the analyses performed took into consideration only the genomic sites where ASCL1 regulates chromatin accessibility upon its binding. Therefore, in terms of chromatin accessibility and DNA binding, the remaining of this chapter will focus on the 2772 ASCL1 binding sites where ASCL1 regulates chromatin accessibility: the binding targets where ASCL1

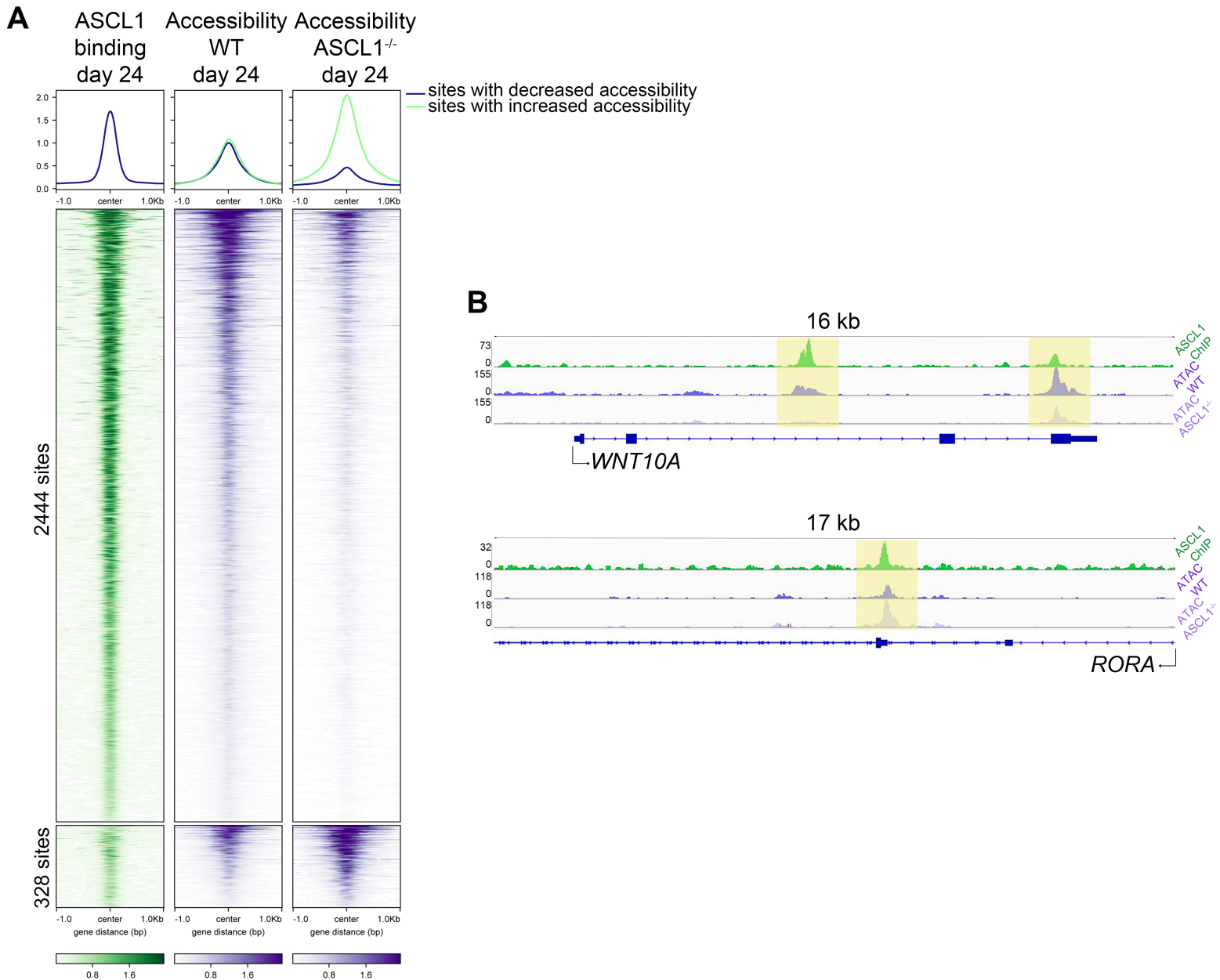


Figure 5.3: ASCL1 direct genomic targets.

(A) Density heat maps profiling the ASCL1 binding targets (green) where it also regulates chromatin accessibility (purple).

(B) ChIP-seq (green) and ATAC-seq (dark and light purple) profiles showing targets where ASCL1 maintains chromatin in an open state (top profile) and targets where ASCL1 represses chromatin accessibility (bottom profile). Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

induces chromatin opening (2444) and the binding targets where ASCL1 represses chromatin accessibility (328). Even if I have already shown the absolute requirement of ASCL1 for shaping the epigenetic landscape at these 2772 genomic regions, I aimed to show that ASCL1 acts as a true pioneer transcription factor during the development of the human cortex. Therefore, I focused on the classic definition of a pioneer factor, which states that these transcription factors are able to bind heterochromatin and upon binding they will induce chromatin opening (Cirillo and Zaret, 1999). In order to explore this definition I took advantage of both ASCL1 DNA binding (ChIP-seq) and chromatin accessibility (ATAC-seq) datasets at the two time points under investigation (day 20 and day 24). Since it has already been shown that ASCL1 binds and maintains an open chromatin state for the 2444 targets described above, I further looked at ASCL1 binding and chromatin accessibility for these regions in NPCs at day 20. Based on this information, I excluded the sites that already had an open chromatin conformation at day 20 but no ASCL1 binding was detected at this time point. This category comprised 36% of the sites under investigation (884 regions) and represents DNA regions where ASCL1 is not responsible for accessing nucleosomal DNA and opening the chromatin, but it is only required to maintain them in an open configuration (referred to as a non-classical pioneer transcription factor (Minderjahn et al., 2020)). Next, I divided the remaining 1560 targets (Figure 5.4.A) into three categories referring to the ASCL1 potential to act as a pioneer transcription factor at each of them. Category A comprises approximately 9% of these sites (139 DNA regions) and represents regions of heterochromatin which are bound by ASCL1 at day 20. Therefore, this ASCL1 targets subset exemplifies the classic definition of a pioneer transcription factor (Figure 5.4.B,E). Category B describes approximately 46% of the 1560 ASCL1 targets (720 sites) and considered the regions of chromatin which are found in a closed conformation at day 20,

but this time with no ASCL1 binding being detected (Figure 5.4.C,F). Since these regions are later found in an open state and also bound by ASCL1, which is also required to maintain accessibility, these regions could also represent sites where ASCL1 acts as a pioneer transcription factor. Because there is a relatively long period of time between the two time points I collected cells at (day 20 and day 24), perhaps ASCL1 bound heterochromatin at these sites sometime between the two time points, but I was not able to catch the exact moment to prove its pioneer function. Conversely, the remaining 45% (701 regions) of the sites where ASCL1 has potential pioneer activity were found to be already bound by ASCL1 at day 20, but these DNA regions were already in open chromatin (category C) (Figure 5.4.D,G). In opposition with targets in subset B, looking earlier at the regions in category C would have been more informative to prove that ASCL1 had bound heterochromatin first. Interestingly, for all targets that were already found in open chromatin at day 20 (category C) ASCL1 is responsible to maintain accessibility even at the earlier time point, which demonstrates one more time that these are indeed targets where ASCL1 regulates chromatin accessibility (Figure 5.4.D,G).

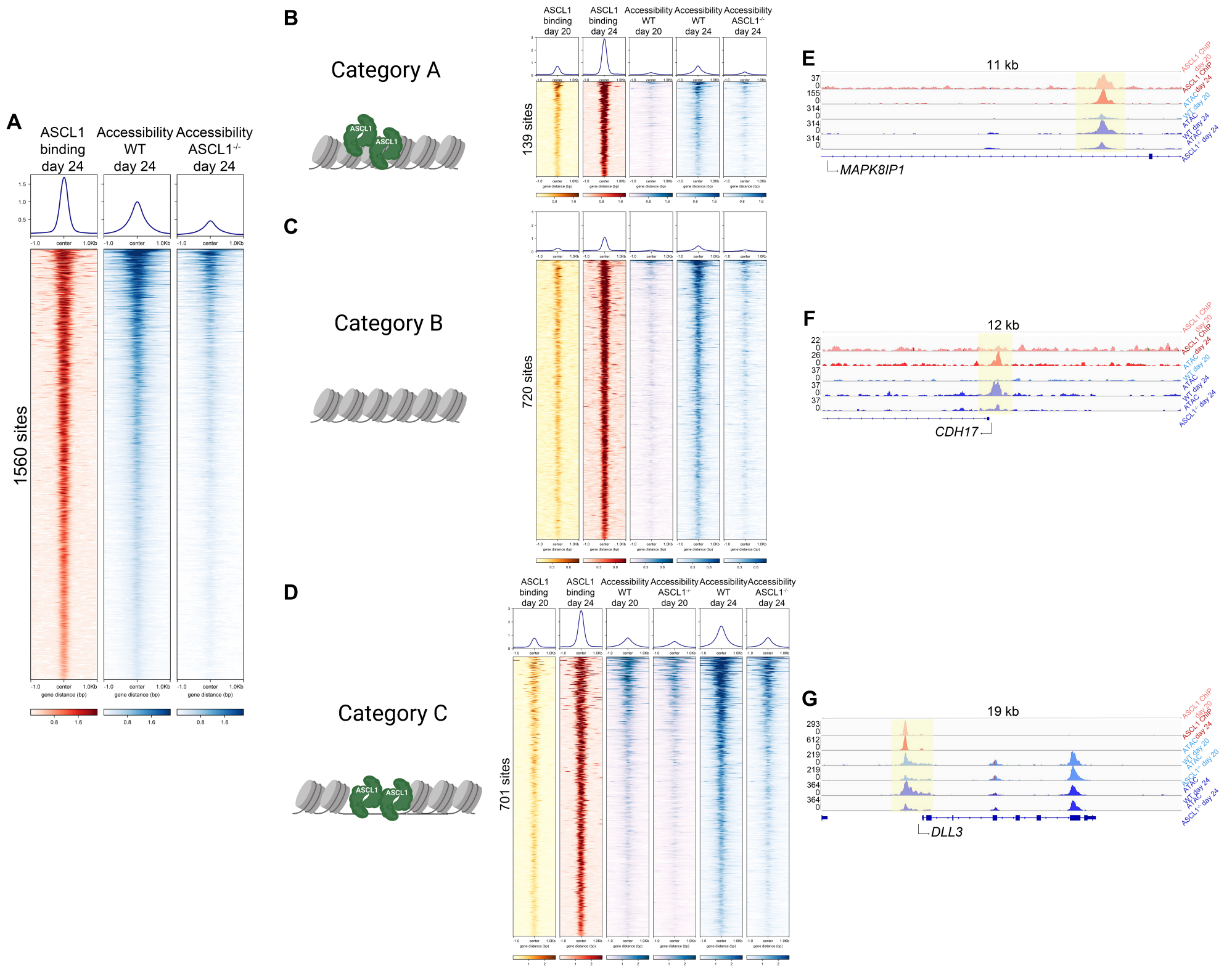


Figure 5.4: ASCL1 pioneer activity. (Legend next page)

Figure 5.4: ASCL1 pioneer activity.

(A-D) Density heat maps profiling chromatin accessibility (blue) at both day 20 and day 24, wild-type and ASCL1^{-/-}, at different categories of ASCL1 binding targets (yellow and red). (A) All ASCL1 targets with putative pioneer activity. (B) Subset of ASCL1 targets where it acts as a classic pioneer transcription factor. (C) ASCL1 binding targets (red heat map) found in open regions of chromatin at day 24, where ASCL1 regulates accessibility. These DNA regions were closed at day 20 and no ASCL1 binding was detected at those targets, although it seems it does start to accumulate (yellow heat map). (D) ASCL1 binding targets detected at both time points (day 20 and day 24), where ASCL1 maintains the chromatin in an open configuration (blue heat maps).

(E-G) ChIP-seq (top two pink IGV tracks) and ATAC-seq (blue IGV tracks) profiles showing an example for each category of ASCL1 targets described in (B-D): B-E, C-F, D-G represent the density heat maps – example profile pairs. Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

5.2 Removal of the mSWI/SNF complexes

5.2.1 Optimisation of the SMARCC1/SMARCC2 double knockout protocol

In order to pursue further the ASCL1 – mSWI/SNF interaction described in Results Chapter 2, I decided to combine transcriptome analysis, DNA binding and chromatin accessibility analysis following the removal of one or the other member of the interaction. For this, I looked into methods to block the activity of the mSWI/SNF chromatin remodellers that could be applied to ASCL1^{-/-} hiPSCs. Previous studies showed that two core subunits – SMARCC1 and SMARCC2 – are responsible for regulating the assembly of the mSWI/SNF complexes. Upon their removal the other subunits are rapidly degraded and the complexes are prevented to assemble, resulting in the complete absence of all mSWI/SNF complexes from a cell (Narayanan, Pirouz, et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016; Mashtalir et al., 2018; Schick, Rendeiro, et al., 2019). Based on this information, I aimed to perform an experiment

where I entirely inactivate mSWI/SNF by removing both SMARCC1 and SMARCC2 from the cells, and then investigate the effects it had on ASCL1 activity. However, removal of both these subunits results in synthetic lethality, which made the analysis of these cells quite challenging. Therefore, I looked into inducing acute depletion of SMARCC1 and SMARCC2 such that there is a significant decrease in their protein levels, but I would still be able to collect enough cells before they undergo apoptosis. Looking into the literature, I decided to use the CRISPR-Cas9 method and purchased two plasmids for this experiment: a Cas9 expression plasmid that also has gRNA that targets the first exon of *SMARCC1*, as well as a neomycin resistance cassette, and one that has a gRNA that targets the first exon of *SMARCC2* and a hygromycin resistance cassette. With the aim of collecting neuronal cells 24 hours post Notch inhibition, I decided to electroporate the cells at day 20 during the neuronal differentiation process, followed by the addition of the two antibiotics 24 hours after electroporation such that I could select only the cells that possibly acquired mutations against both *SMARCC1* and *SMARCC2*. However, while performing this experiment most of the cells were dying at day 23, such that there were not enough cells left to passage and collect at day 24. As a next trial, I decided to electroporate the cells one day later (day 21). This time, there was a considerable number of cells that had a relatively wild-type morphology at day 23. As the protocol implies (2.1.2), these cells were passaged at day 23 onto laminin-coated dishes while DAPT was added. Surprisingly, not only the cells electroporated with the plasmids targeting *SMARCC1* and *SMARCC2*, but also the control cells (electroporated with a GFP expressing plasmid or the ones that only underwent the electroporation process with no plasmids) acquired a different morphology compared to the wild-type cells and died (Figure 5.5.B – top panel). These results suggested that the combination of electroporation, substrate change (from geltrex to laminin), and

media change (from N2B27 alone to B27 supplemented with DAPT) during a short period of time (72 hours) was making the cells very sensitive and decreased their survival chances. The morphology of all cells was similar between the electroporated and non-electroporated cells at day 23, so I decided to set day 21 as the electroporation day, but to change some of the later steps. Since DAPT addition is critical for the homogeneity of the culture, I changed the protocol such that the cells were no longer passaged at day 23 onto laminin-coated dishes, but instead only the media was still switched to B27 supplemented with DAPT (Figure 5.5.A). In order to avoid any possible changes in the cell identity between neurons plated on gel-trex versus neurons plated on laminin, all cells collected for this analysis (wild-type, *ASCL1*^{-/-}, *SMARCC1/2*^{-/-}) were cultured under the same conditions. Under these experimental conditions, there were enough cells with a relatively normal-looking morphology that survived until collection at day 24 (Figure 5.5.B – bottom panel). More important, western blot analyses of wild-type and *SMARCC1/2*^{-/-} electroporated cells revealed a significant decrease in the protein levels of both SMARCC1 and SMARCC2 following electroporation. All of the additional mSWI/SNF subunits that I probed for showed a decrease in their protein levels following SMARCC1 and SMARCC2 downregulation (Figure 5.5.C). This analysis reproduced results from previous studies (Narayanan, Pirouz, et al., 2015; Bachmann et al., 2016; Nguyen et al., 2016; Mashtalir et al., 2018; Schick, Rendeiro, et al., 2019) and confirmed the absence of mSWI/SNF assemblies in the neuronal cells. Although additional analyses regarding the identity of the neuronal cells lacking both SMARCC1 and SMARCC2 would be required (discussed in 6.5), IF analysis of these cells revealed they still had a neuronal identity as shown by the expression of the neuronal marker TUJ1 (Figure 5.5.D).

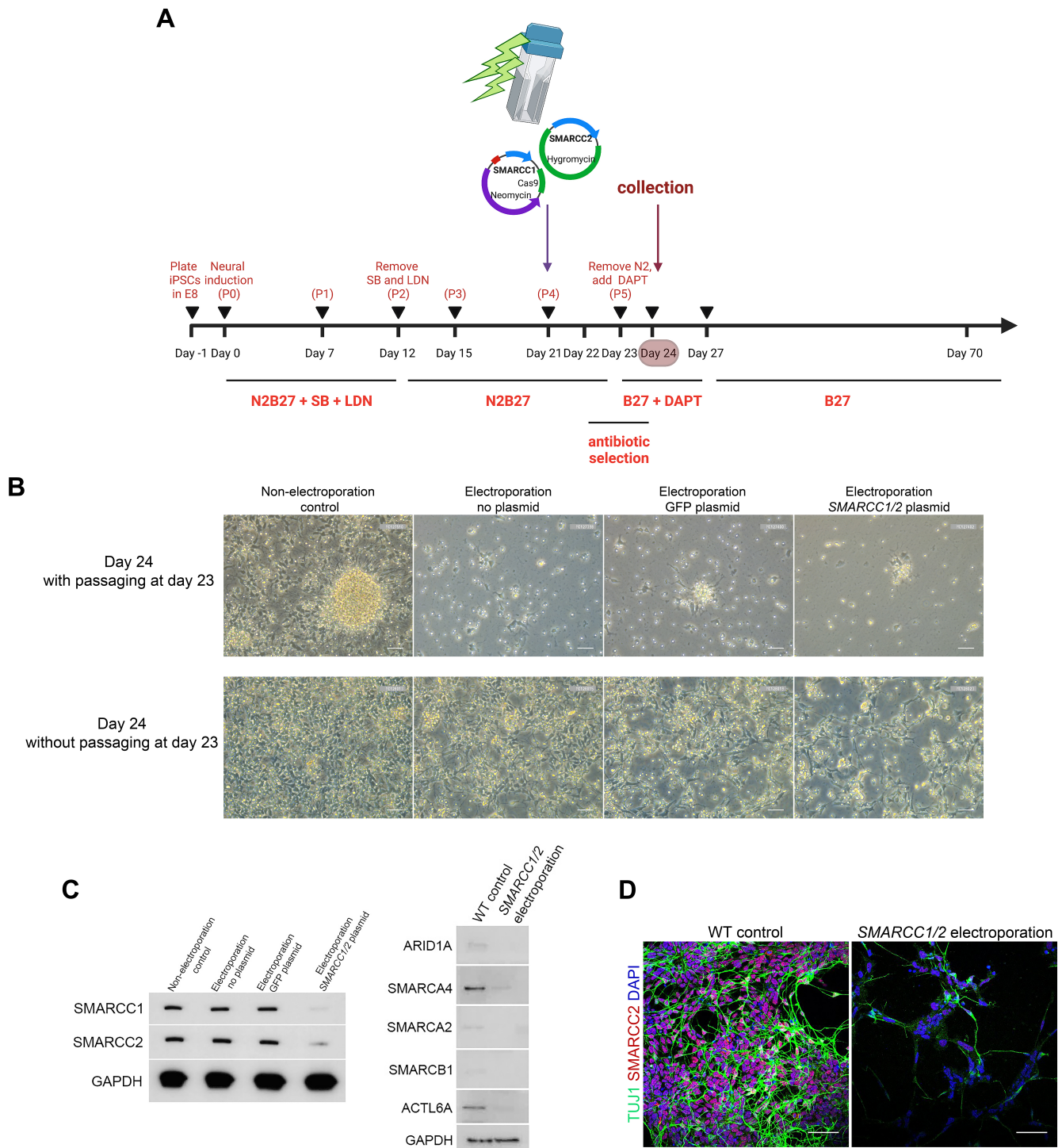


Figure 5.5: Neuronal cells lacking SMARCC1 and SMARCC2. (Legend next page)

Figure 5.5: Neuronal cells lacking SMARCC1 and SMARCC2.

(A) Diagram of the protocol used to knockout *SMARCC1* and *SMARCC2* in the neuronal cultures. NPCs were electroporated at day 21 with two plasmids that contain gRNAs against either *SMARCC1* or *SMARCC2*, Cas9 protein and one antibiotic resistance cassette (either neomycin or hygromycin). 24 hours after electroporation, cells were fed with antibiotic-containing N2B27. At day 23, the culture medium was switched to B27 only supplemented with DAPT and the two antibiotics. At day 24, cells were collected for different experimental procedures. Diagram created with BioRender.com.

(B) Representative images of the electroporated cells at day 24, with (top panel) and without (bottom panel) passaging at day 23. Scale bars, 50 μ m.

(C) Western blot for SMARCC1 and SMARCC2 (left panel) on the collection day (day 24) for cells that underwent electroporation with the plasmids targeting *SMARCC1* and *SMARCC2* and their respective controls. Right hand side panel shows the protein levels of other mSWI/SNF subunits (ARID1A, SMARCA2, SMARCA4, SMARCB1, ACTL6A) in the *SMARCC1/2*^{-/-} neuronal cells compared to wild-type. Representative GAPDH loading control is included.

(D) Immunofluorescence images showing that the *SMARCC1/2*^{-/-} cells still express neuronal markers such as TUJ1. Scale bars, 50 μ m.

5.2.2 Neuronal cells lacking mSWI/SNF complexes show major changes at the transcriptome level and chromatin accessibility landscape

Before analysing the effect of removing the mSWI/SNF complexes on ASCL1 activity, I performed RNA-seq and ATAC-seq on these mutant cells. The absence of chromatin remodellers has a big impact on the transcriptome as well as on the accessibility landscape. Differential gene expression analysis between wild-type and *SMARCC1/2*^{-/-} neurons at the same stage (24 hours following Notch inhibition) revealed a total number of 5679 misregulated genes, with approximately half of them (2860 genes) being significantly downregulated while the other half (2819 genes) significantly upregulated in the mutant neurons (Figure 5.6.A). Prevention of the mSWI/SNF complexes assembly also led to major changes in chromatin accessibility. More specifically, the mSWI/SNF absence affected accessibility at approximately 50% of the 58003 open sites that were initially identified in wild-type neuronal cells. In contrast to studies that reported the ablation of both *SMARCC1* and *SMARCC2*

results mainly in loss of chromatin accessibility (Schick, Grosche, et al., 2021), and in agreement with my transcriptome analysis, differential accessibility analysis revealed similar numbers of regions with loss or gain in accessibility – 14608 sites and 13945 sites, respectively. These regions with significant changes in accessibility following SMARCC1 and SMARCC2 removal were further divided into direct and indirect targets of the mSWI/SNF chromatin remodellers. This division relied on the same strategy used to define the direct genomic targets of ASCL1 – a genomic region where the mSWI/SNF complex binds and where it regulates chromatin accessibility was considered a mSWI/SNF direct genomic target. Using this strategy, I identified 9969 direct targets of the complex (Figure 5.6.B).

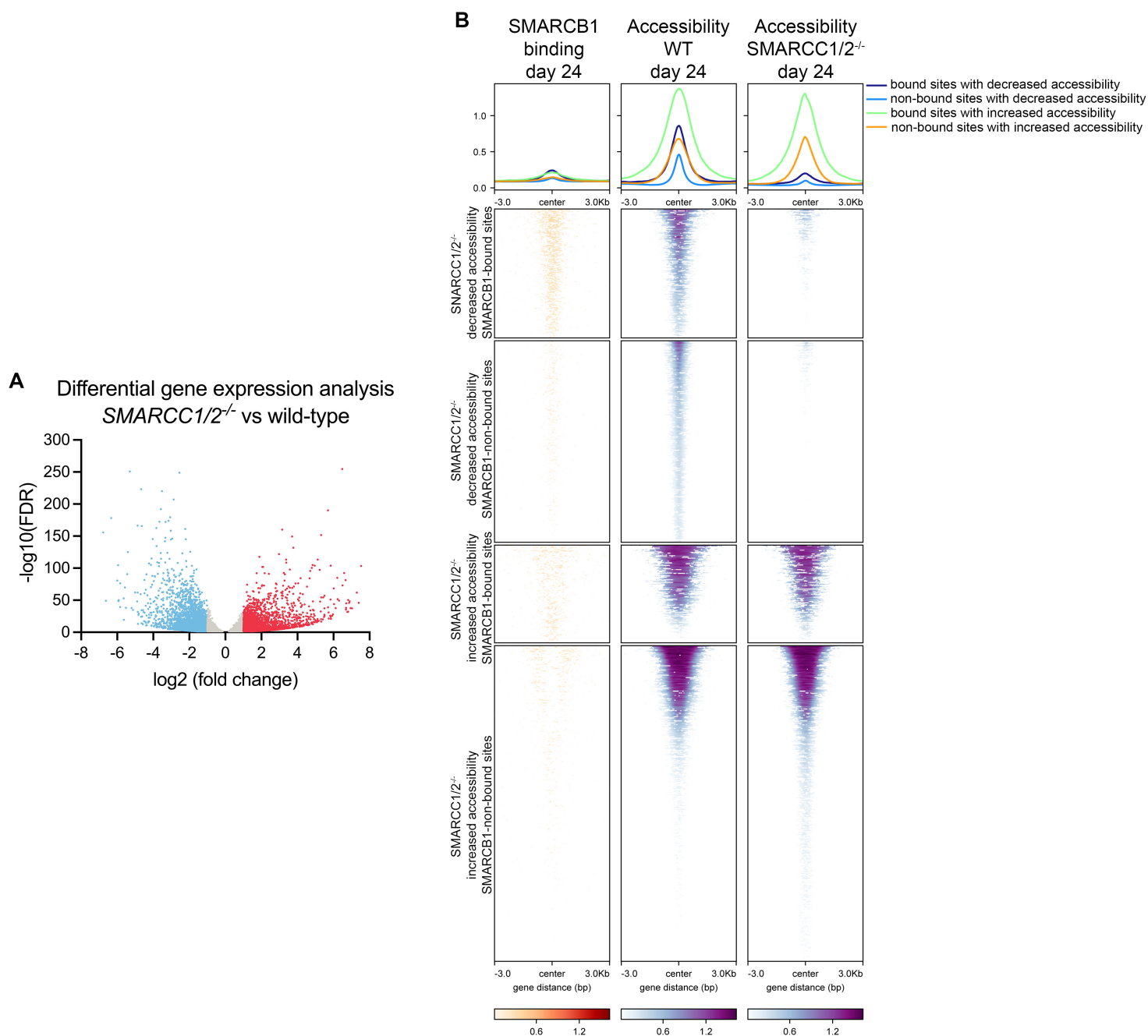


Figure 5.6: *SMARCC1/2*^{-/-} cells show major changes at the transcriptome level and accessibility landscape.

(A) Volcano plot that shows the genes misregulated in mSWI/SNF-lacking cells compared to wild-type neuronal cells at day 24. Differential gene expression analysis performed by Harshil Patel, BABS.

(B) Density heat maps that divide mSWI/SNF-dependent accessibility into direct and indirect targets of mSWI/SNF remodellers. The regions with mSWI/SNF dependent accessibility coupled with SMARCB1 binding at the exact same sites were considered direct mSWI/SNF targets, while the regions with mSWI/SNF dependent accessibility where no SMARCB1 binding was detected were classified as mSWI/SNF indirect targets. Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

5.3 Division of ASCL1 target sites into mSWI/SNF dependent and independent sites

Association of ChIP-seq and ATAC-seq datasets from neuronal cells at 24 hours post DAPT addition led to the identification of 2772 genomic regions where ASCL1 binds and regulates chromatin accessibility. For the purpose of this project, these 2772 sites were defined as direct targets of ASCL1. To further characterise the ASCL1 – mSWI/SNF interactions I previously described (physical interactions by co-IP and PLA – 4.1, and overlap in their binding landscapes – 4.2.2) , I aimed to divide the ASCL1 target sites into mSWI/SNF dependent and mSWI/SNF independent. Therefore, I overlapped the ASCL1 direct targets with the mSWI/SNF direct targets defined and identified above. This analysis revealed that approximately one third of the ASCL1 direct targets are also mSWI/SNF direct targets. More specifically, I identified 760 DNA sites bound by both ASCL1 and SMARCB1, which show a significant decrease in accessibility following removal of any of the two interacting partners. On the other hand, only 16 sites where both ASCL1 and SMARCB1 bind showed an increase in chromatin accessibility upon disruption of the ASCL1 – mSWI/SNF interaction (Figure 5.7.A-B).

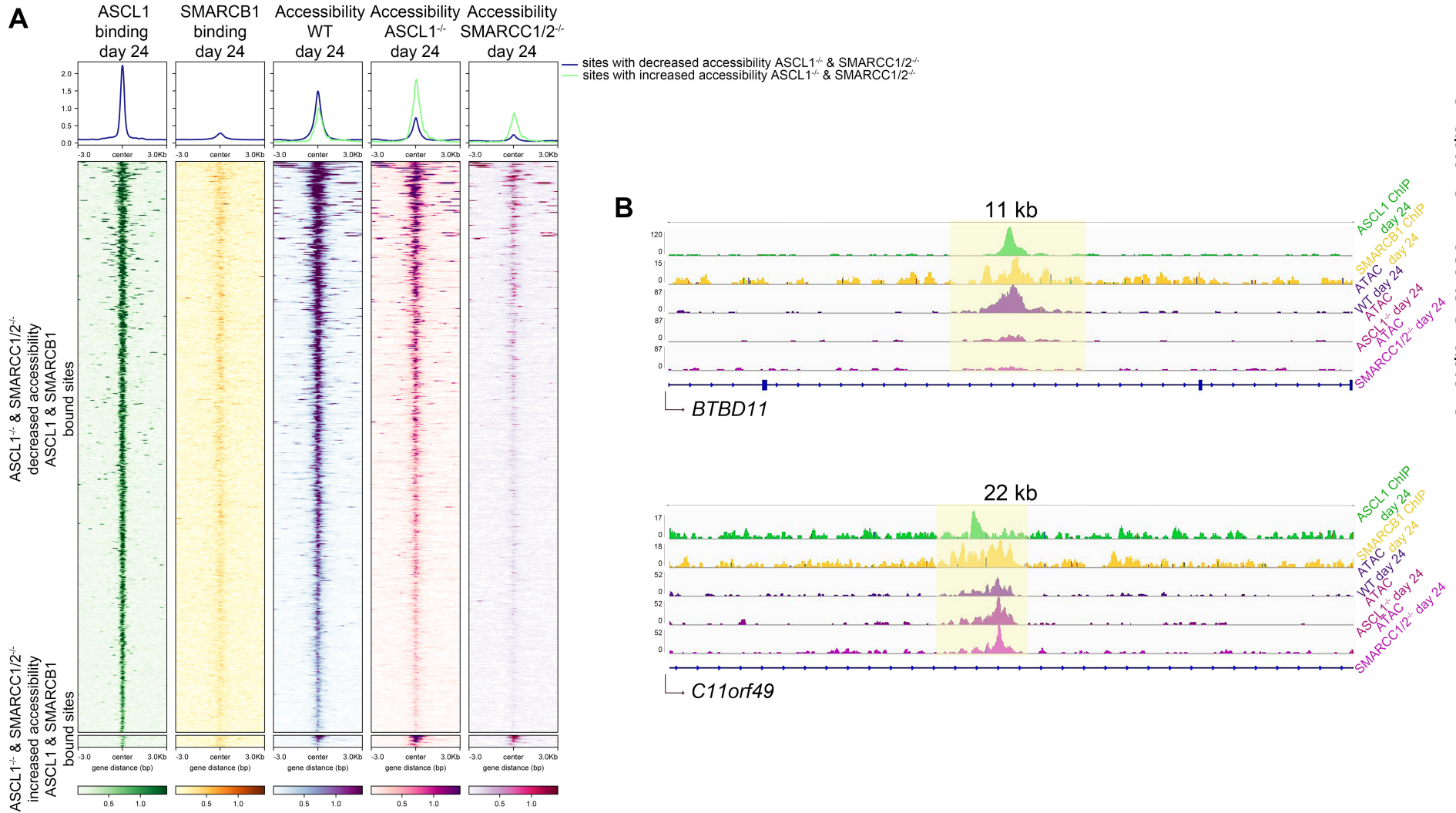


Figure 5.7: ASCL1-mSWI/SNF direct genomic targets.

(A) Density heat maps profiling the ASCL1-mSWI/SNF direct genomic targets: sites bound by both ASCL1 and SMARCB1, where chromatin accessibility is dependent on both ASCL1 and the mSWI/SNF remodelers.

(B) ChIP-seq (green and yellow) and ATAC-seq (purple) profiles showing an example of ASCL1-mSWI/SNF direct target where the two factors maintain chromatin in an open state in wild-type cells (top profile); conversely, an example of ASCL1-mSWI/SNF direct target where the two factors act as repressors of chromatin accessibility (bottom profile).

Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

5.3.1 Investigating ASCL1 recruitment at ASCL1 – mSWI/SNF dependent sites

Once I identified a set of genomic regions where both ASCL1 and mSWI/SNF complexes are required to regulate chromatin accessibility upon their binding, I sought to investigate the recruitment dynamics between them at these target sites. I performed additional ChIP-seq experiments, but this time I looked at binding sites of either ASCL1 or SMARCB1 when their respective interacting partner was missing: ASCL1 ChIP-seq in SMARCC1/2^{-/-} neuronal cells and SMARCB1 ChIP-Seq in ASCL1^{-/-} neuronal cells following Notch inhibition. However, a common challenge when looking at ChIP-seq data between different experimental conditions has been the lack of a quantitative scale. Although efforts into designing methods that would enable this type of analysis have been reported into the literature (such as the addition of exogenous spike-ins or methodological alterations to establish relative scales (Liu et al., 2013; Galen et al., 2016; Dickson et al., 2020)), the overall perception is that there are still unaddressed challenges which only make these alternative methods semi-quantitative. Out of caution, I decided not to perform differential binding analysis for the ChIP-seq data, but to look at the peaks using a “presence-absence” approach. Specifically, for all genotypes included in the analysis (wild-type, ASCL1^{-/-}, SMARCC1/2^{-/-}) two or three biological replicates were used for ChIP-seq. As already mentioned above, during the analysis, a peak was placed in the final set of binding sites only if it was present in at least two biological replicates. Subsequently, by overlapping the *bona fide* sets of peaks, I was able to see which of them have been lost in each experimental condition. By using this approach, I identified 4924 SMARCB1 binding targets that were lost in the absence

of ASCL1, which account for approximately 20% of the SMARCB1 binding sites identified in wild-type neuronals.

While performing the same analysis between wild-type and mSWI/SNF complex lacking cells, I faced an additional challenge. A recent study that was looking to characterise the npBAF – nBAF switch in the mouse developing brain reported that ACTL6A npBAF subunit binds the *Ascl1* locus (Braun et al., 2021). In addition, there are direct ATP-dependent interactions between mSWI/SNF complexes and PRC complexes independent of chromatin which keep the PRC1 and PRC2 repressive complexes away from the mSWI/SNF bound loci. Interference with mSWI/SNF activity leads to PRC accumulation and, in turn, downregulation of the target genes (Stanton et al., 2017; Weber et al., 2021). Not only this observation is also valid in our system (Figure 5.8.A), but it could also have a direct effect on the results of the recruitment mechanisms analysis. To be specific, the method I used to prevent the assembly of the mSWI/SNF complexes required a relatively long period of time (72 hours) to induce downregulation of SMARCC1 and SMARCC2 and, in turn, the degradation of all the other component subunits. The absence of a master regulator of chromatin accessibility, the long period of time, in combination with the electroporation process and antibiotic treatment, not only could cause a change in the cell identity (further discussed in 6.5), but could also lead to a significant downregulation of ASCL1 (Figure 5.8.B) with no mSWI/SNF complexes to bind its locus and prevent PRC accumulation. In consequence, I observed a global loss in ASCL1 binding, which might be a reflection of a long period of time when ASCL1 was absent from the neuronal cells, rather than the precise recruitment dynamics between the two factors - ASCL1 and mSWI/SNF. While I am confident that this side effect does not influence the results of the other analyses that were performed (since I al-

ways associated the changes in chromatin accessibility observed upon the removal of mSWI/SNF remodellers with sites of SMARCB1 binding from the wild-type context), it might not be conclusive when investigating the binding dynamics between ASCL1 and SMARCB1. Additional methods to interfere with the mSWI/SNF activity are currently ongoing (discussed in 6.5). However, I employed an additional strategy to analyse the already generated ASCL1 ChIP-seq datasets from SMARCC1/2^{-/-} neuronal cells. Since I used an acute mutation method to eliminate the mSWI/SNF remodellers during neuronal differentiation, there were always differences in terms of the SMARCC1 and SMARCC2 protein levels remained after electroporation and antibiotic treatment between the replicates. In turn, the variability in the mutation efficiency led to variable levels of ASCL1 protein still expressed at day 24 (Figure 5.8.B). As a result, I decided to consider that an ASCL1 binding site was lost in the absence of mSWI/SNF complexes only if that ASCL1 binding site was missing from all biological replicates. In this way, only the immediate effects of mSWI/SNF removal over the ASCL1 binding dynamics were taken into account, the ones I consider to be the most relevant for the characterisation of the ASCL1-mSWI/SNF interaction. This approach revealed that approximately 55% of the ASCL1 binding sites from wild-type neurons were no longer called in any of the ASCL1 ChIP-seq replicates from SMARCC1/2^{-/-} cells.

Based on these results, I looked to define the recruitment mechanisms between ASCL1 and mSWI/SNF remodellers at their direct targets (Figure 5.9.A), investigating a few specific scenarios: 1) if ASCL1 recruits mSWI/SNF to certain genomic locations, SMARCB1 binding will be lost in the absence of ASCL1, but no change in ASCL1 binding will be observed at those sites in the absence of mSWI/SNF remodellers; 2) conversely, if mSWI/SNF complexes recruit ASCL1 at some DNA loci,

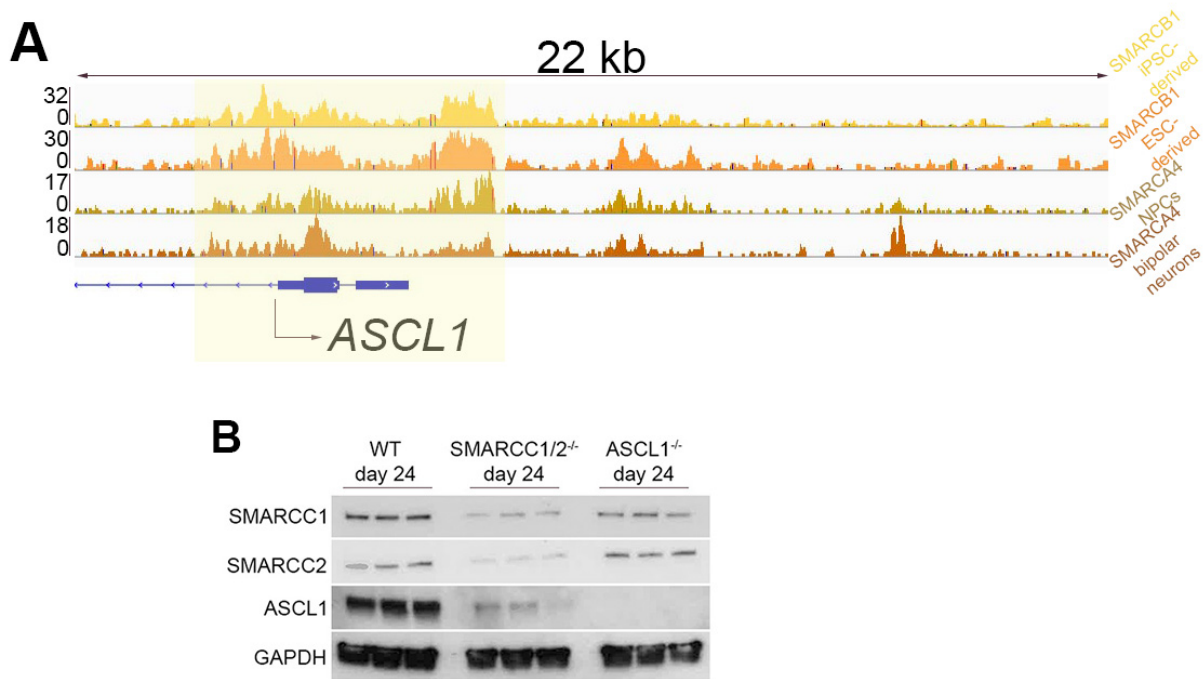


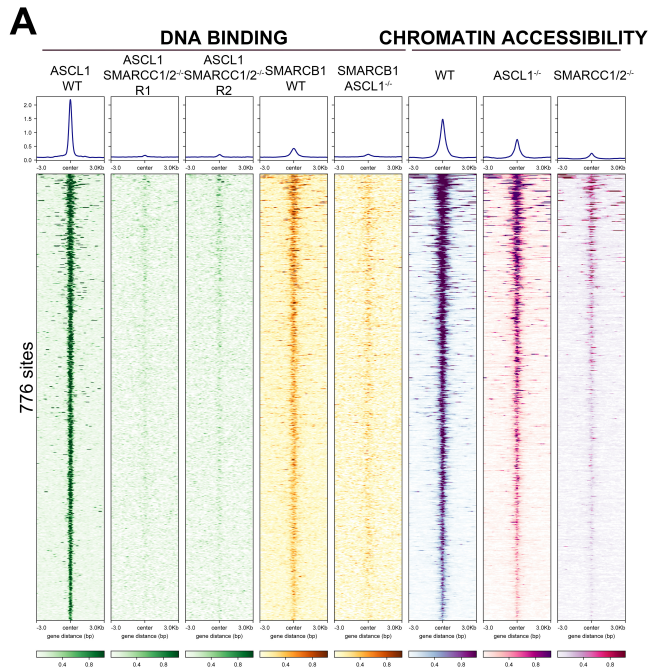
Figure 5.8: mSWI/SNF removal affects ASCL1 expression.

(A) mSWI/SNF ChIP-seq binding profiles at ASCL1 locus. Representative ChIP-seq binding profiles of two or three biological replicates. Peak calling performed by Harshil Patel, BABS.

(B) Western blot for SMARCC1, SMARCC2 and ASCL1 for the three genotypes included in the analysis (wild-type, SMARCC1/2^{-/-}, ASCL1^{-/-} neurons). Representative GAPDH loading control is included.

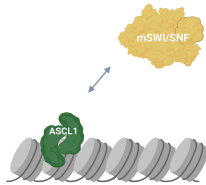
ASCL1 ChIP peaks will be lost upon mSWI/SNF removal, but SMARCB1 binding will be unaffected at those regions in the absence of ASCL1; 3) if the two factors depend on each other for binding, both ASCL1 and SMARCB1 binding will be lost in the absence of their binding partner at those genomic regions; 4) if the ASCL1-mSWI/SNF interaction is only required to regulate chromatin accessibility at specific targets, but the two interacting partners do not depend on each other for binding, neither ASCL1 nor SMARCB1 binding will be lost at those sites (Figure 5.9.B). With these four scenarios in mind, I investigated ASCL1 binding in mSWI/SNF-lacking neurons and SMARCB1 binding in ASCL1^{-/-} cells at the ASCL1-mSWI/SNF direct

target sites. This analysis revealed unique loss of SMARCB1 binding at approximately 10% of the targets, unique loss of ASCL1 ChIP-seq peaks only at approximately 40% of the targets, loss of both ASCL1 and SMARCB1 binding at 25% of the targets and unaffected ASCL1 and SMARCB1 binding at approximately 25% of their direct targets. These numbers suggests that for more than one third of the ASCL1 – mSWI/SNF direct target regions mSWI/SNF remodeller is the first member of the interaction to bind the chromatin and then it recruits ASCL1. While at 25% of ASCL1 – mSWI/SNF dependent DNA regions there seem to be a synergistic binding, with the two members of the interaction being dependent on each other for binding, at a similar number of targets the two interacting partners seem to bind independently of each other, with their interaction being absolutely required only to regulate chromatin accessibility. Finally, I also discovered a small number of DNA regions (approximately 10% of the ASCL1-mSWI/SNF direct targets) where ASCL1 seems to first bind heterochromatin and then recruit mSWI/SNF remodellers to those targets.

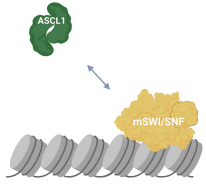


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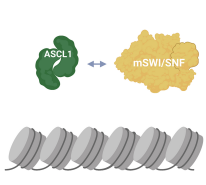
Case scenario 1
(10%)



Case scenario 2
(40%)



Case scenario 3
(25%)



Case scenario 4
(25%)

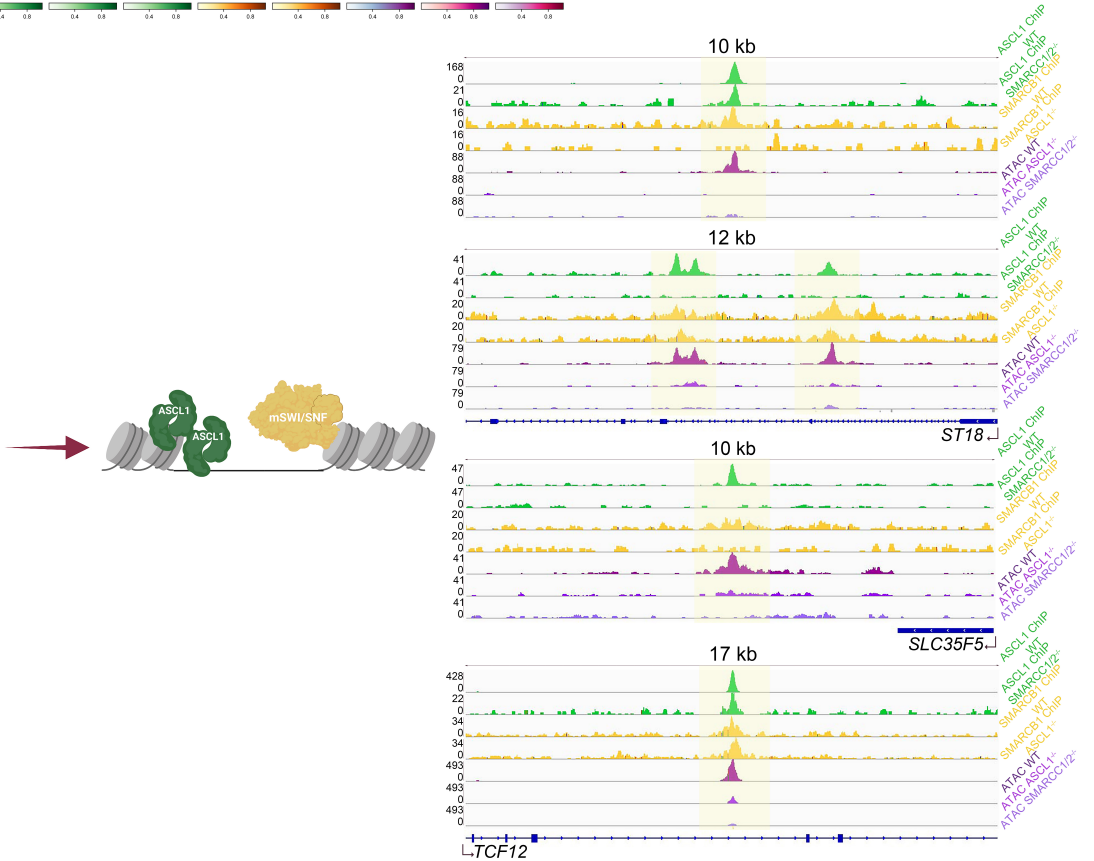
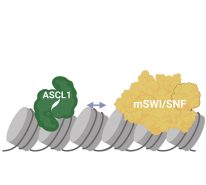


Figure 5.9: ASCL1-mSWI/SNF recruitment dynamics. (Legend next page)

Figure 5.9: ASCL1-mSWI/SNF recruitment dynamics.

(A) Density heat maps profiling ASCL1 binding in the absence of mSWI/SNF remodelers and SMARCB1 binding in ASCL1^{-/-} neuronal cells at ASCL1-mSWI/SNF direct targets.

(B) Recruitment scenarios between ASCL1 and mSWI/SNF complexes at ASCL1-mSWI/SNF direct targets (left). ChIP-seq and ATAC-seq profiles showing examples for each scenario (right). Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

5.4 Transcriptional readout of ASCL1 target sites

The main aim of this project was to investigate the mechanisms behind ASCL1 ability to regulate chromatin accessibility. In addition, I also aimed to correlate the genomic sites where ASCL1 is required to maintain the chromatin in a specific state with their transcriptional readout. More specifically, I wanted to identify which genes are regulated by ASCL1 following its pioneer activity. Profiling ASCL1-dependent genes during human cortical neurogenesis (3.2.2) revealed that most ASCL1-dependent genes are specific to the neuronal time point I explored in this project (day 24 of neuronal differentiation or 24 hours post Notch inhibition). Differential gene expression analysis between ASCL1^{-/-} and wild-type neuronal cells at day 24 led to the identification of 1468 genes that are misregulated in the absence of ASCL1. In agreement with previous studies that described ASCL1 as a transcriptional activator (Raposo et al., 2015), two thirds of the misregulated genes decreased in expression upon ASCL1 removal while one third of these genes were upregulated in ASCL1^{-/-} neurons (Figure 5.10.A).

As a first step in correlating these ASCL1-dependent genes with exact genomic loci where specific changes might induce these transcriptional modifications, I looked at which of these genes are bound by ASCL1. Therefore, I looked at the nearest annotated promoter for each of the 11810 ASCL1 binding sites and checked whether that

gene was found among those which are misregulated upon ASCL1 removal. This analysis revealed that the promoters of 701 (47.8%) out of the 1468 misregulated genes from ASCL1^{-/-} cells represent the nearest annotated promoter of a least one ASCL1 binding site (Figure 5.10.B). I next correlated the RNA-seq – ChIP-seq analysis explained above with the ATAC-seq data. I divided the ASCL1 binding sites for which the nearest promoter was the promoter of one of the genes misregulated in ASCL1^{-/-} neurons into targets where ASCL1 also regulates chromatin accessibility and targets where ASCL1 seems to have no effect on chromatin accessibility. This correlation revealed that for 415 out of the 701 genes (59%) that I identified as being bound by ASCL1, the transcription factor is not responsible for regulating chromatin accessibility, suggesting ASCL1 acts as a classic transcription factor at those binding targets (Figure 5.10.D). In contrast, for the remaining 286 ASCL1-dependent genes which have at least one ASCL1 binding site in their proximity (i.e. the promoters of those genes represent the nearest promoters of ASCL1 binding targets) ASCL1 is also required to regulate chromatin accessibility (Figure 5.10.C). Interestingly, enrichment of Gene Ontology (GO) biological process terms for each set of genes did not reveal any striking differences between the two categories, all genes bound by ASCL1, accompanied or not by regulation of chromatin at the binding sites, being involved in biological processes related to neuronal differentiation or cortical development in general (Figure 5.10.C-D).

For the loci associated with 254 of these genes (86.1%) ASCL1 appears to induce chromatin opening (ASCL1 removal led to a significant decrease in chromatin accessibility), while for the DNA regions associated with 41 of these genes (13.9%) ASCL1 seems to act as a repressor (a significant increase in accessibility is observed upon ASCL1 removal). In both cases the changes in chromatin accessibility

correlate with the transcriptional modification for more than 70% of the genes: at sites where ASCL1 maintains the chromatin in an open state I observed a significant decrease in the mRNA level of the nearest annotated genes upon ASCL1 removal; conversely, at sites where ASCL1 represses accessibility, the nearest annotated gene is significantly upregulated in the absence of ASCL1.

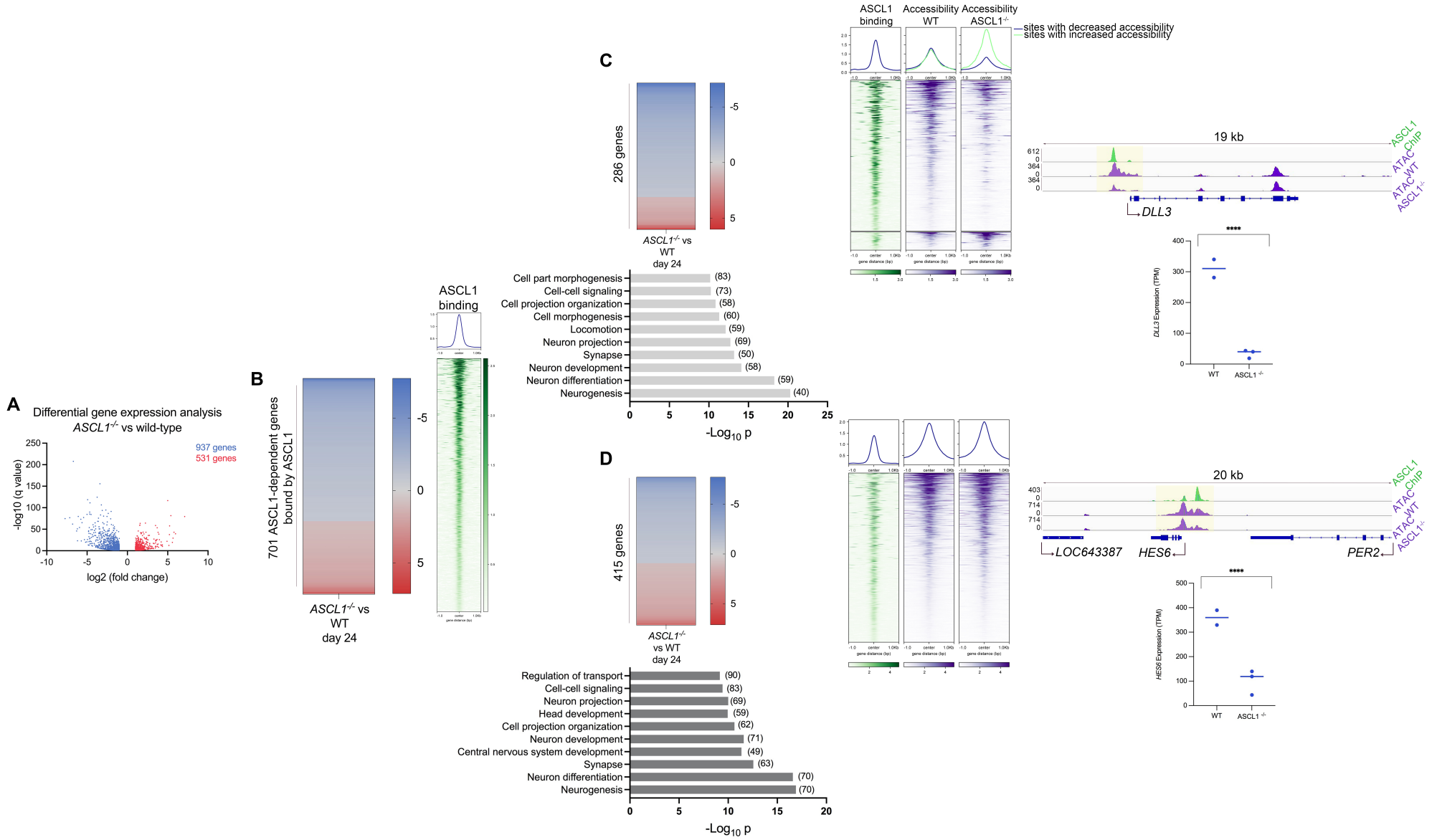
Figure 5.10: Transcriptional readout at *ASCL1* direct targets. (Legend next page)

Figure 5.10: Transcriptional readout at ASCL1 direct targets.

(A) Volcano plot that shows the genes misregulated in ASCL1 knockout (ASCL1-dependent genes) compared to wild-type neuronal cells at day 24. Differential gene expression analysis performed by Harshil Patel, BABS.

(B) ASCL1-dependent genes bound by ASCL1 at day 24.

(C-D) Division of ASCL1 binding targets associated with ASCL1-dependent genes into targets where ASCL1 also regulates chromatin accessibility (C) and targets where ASCL1 acts as a classic transcription factor and does not influence chromatin accessibility (D). Enrichment of GO biological process terms for ASCL1-dependent genes found below each heat map showing the direction of the misregulated genes (downregulated – blue or upregulated – red). ChIP-seq, ATAC-seq example profiles around one gene found on the right hand side of each category. Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

5.5 Division of ASCL1 target genes into mSWI/SNF dependent and independent genes

As already described above, lack of mSWI/SNF complexes from the neuronal cells had major implications not only on the accessibility landscape, but also at the transcriptome level, with around 5000 genes being misregulated upon mSWI/SNF removal. Division of ASCL1 target sites into mSWI/SNF dependent and independent sites revealed that approximately one third of the DNA regions where ASCL1 has pioneer activity are also bound by SMARCB1 and their accessibility is dependent on the mSWI/SNF assemblies. As a last step of this analysis, I aimed to identify the transcriptional readout of the ASCL1 – mSWI/SNF dependent genomic targets. An overlap between the genes misregulated in ASCL1^{-/-} and mSWI/SNF-lacking neuronal cells at 24 hours following Notch inhibition showed that approximately 55% of the genes misregulated upon ASCL1 removal are also misregulated in the absence of mSWI/SNF remodellers. However, because mSWI/SNF absence seems to have a much bigger transcriptional effect in the neuronal cells compared to ASCL1 absence, I only selected the genes that showed the same

direction of misregulation in both $ASCL1^{-/-}$ and $SMARCC1/2^{-/-}$ for downstream correlations. This way, I identified 396 genes which are downregulated upon either ASCL1 or mSWI/SNF complexes removal and 189 genes which are upregulated in both mutants (Figure 5.11.A). To fully integrate these transcriptional events with DNA binding and chromatin accessibility changes, I looked whether the nearest promoters of the ASCL1 – mSWI/SNF dependent genomic sites identified in 5.3 represent the promoters of the genes I identified as being misregulated in both mutants (ASCL1-mSWI/SNF-dependent genes). This analysis revealed that the promoters of 61 genes misregulated in the same direction in both $ASCL1^{-/-}$ and $SMARCC1/2^{-/-}$ neurons represent the nearest promoters of ASCL1 – mSWI/SNF-dependent genomic targets (Figure 5.11.B). In 90% of the cases (55 genes), the genes were downregulated in both $ASCL1^{-/-}$ and $SMARCC1/2^{-/-}$ and at the binding targets associated with these genes ASCL1 and mSWI/SNF remodellers seem to be responsible for chromatin opening (accessibility decreases upon either ASCL1 or mSWI/SNF removal), suggesting the ASCL1 – mSWI/SNF interaction acts as an activator at both accessibility and transcriptional levels. In contrast, in 10% of the cases (6 genes) the genes were upregulated in both $ASCL1^{-/-}$ and $SMARCC1/2^{-/-}$ and at the binding targets associated with these genes ASCL1 and mSWI/SNF remodellers seem to be responsible for chromatin opening (accessibility decreases upon either ASCL1 or mSWI/SNF removal), suggesting the ASCL1 – mSWI/SNF interaction acts as an activator at the chromatin level, but results in transcriptional repression. Interestingly, I identified no genes associated with sites where ASCL1 and SMARCB1 both bind in order to repress chromatin accessibility. Since the ASCL1-mSWI/SNF-dependent genes associated with the ASCL1-mSWI/SNF direct genomic targets represent a subset of the ASCL1-dependent genes described in 5.4, enrichment of GO biological process terms revealed that genes in this subset

are also involved in multiple processes required during neuronal differentiation and cortical development in general.

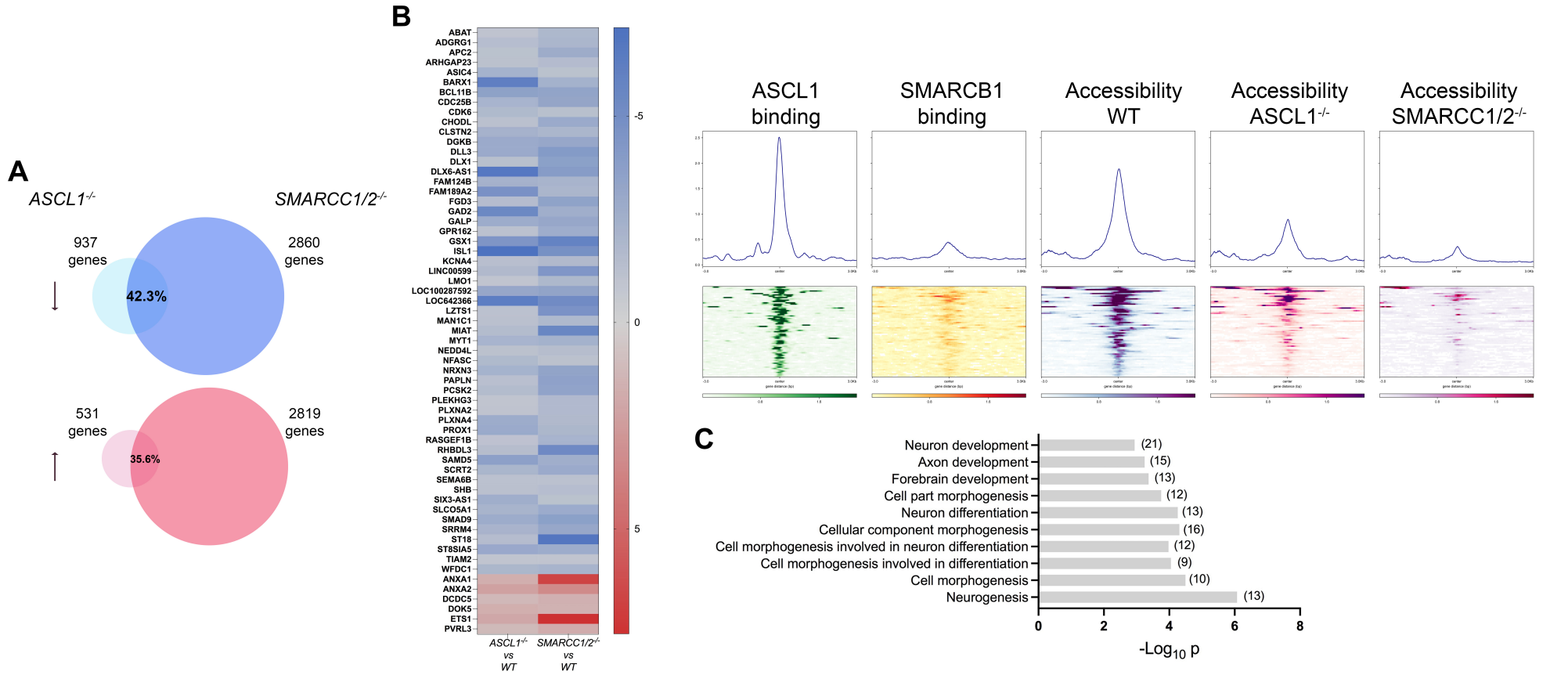


Figure 5.11: Transcriptional readout at ASCL1-mSWI/SNF direct targets.

(A) Venn diagrams showing the percentages of ASCL1-mSWI/SNF-dependent genes misregulated in the same direction (either downregulated or upregulated in both mutants). Differential gene expression analysis performed by Harshil Patel, BABS.

(B) ASCL1-mSWI/SNF-dependent genes whose promoters are the nearest annotated promoters of ASCL1-mSWI/SNF direct genomic targets. Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

(C) Enrichment of GO biological process terms for the ASCL1-mSWI/SNF-dependent genes associated with the ASCL1-mSWI/SNF direct genomic targets.

5.6 ASCL1 - mSWI/SNF direct targets are found at distal regulatory elements

By associating the datasets defining ASCL1 binding (ChIP-seq) and chromatin accessibility (ATAC-seq) in wild-type versus ASCL1^{-/-} neuronal cells I was able to correlate approximately 20% of the ASCL1-dependent genes with the genomic regions where ASCL1 acts as a pioneer transcription factor. Performing the same analysis for the mSWI/SNF complexes (SMARCB1 ChIP-seq experiments, chromatin accessibility differential analysis as well as differential gene expression analysis between wild-type and SMARCC1/2^{-/-} neurons) led to the identification of 61 genes which have at least one ASCL1 – SMARCB1 binding site associated with their promoter and where accessibility is dependent on both ASCL1 and mSWI/SNF chromatin remodellers. However, DNA binding and chromatin accessibility analyses alone showed that ASCL1 regulates chromatin accessibility at approximately 25% of its binding targets. Among the almost 3000 sites, one third seem to also be direct targets of SMARCB1 where mSWI/SNF complexes are responsible for maintaining chromatin accessibility in a specific state (open or closed). In addition, differential gene expression analysis between both wild-type and ASCL1^{-/-} and wild-type and SMARCC1/2^{-/-} cells at day 24 revealed that approximately 40% (585 genes) of the ASCL1-dependent genes are also misregulated in the same direction in mSWI/SNF-lacking cells (either downregulated in both mutants or upregulated in both mutants). Taking into consideration the ChIP-seq – ATAC-seq analysis alone or the transcriptional analysis alone, the final correlation between them seems relatively poor.

An important denominator of the likelihood that a specific genomic region targets

a specific gene is the physical distance between that site and the gene. Even if each of the genomic sites was associated with the nearest promoters of certain genes, I noticed that more than 80% of the *ASCL1* binding targets where it also regulates accessibility are located more than 10kb away from the nearest annotated promoter. This observation suggests these genomic targets either represent distal regulatory elements such as enhancers and super enhancers or are unrelated to the genes misregulated by *ASCL1* knockout or by the absence of both *ASCL1* and the mSWI/SNF remodellers in the neuronal cells.

To investigate the identity of these distal regions, I next looked at the histone marks found at these genomic sites. This analysis relied on three ENCODE datasets looking at DNA binding (H3K4me1 ChIP-seq (ENCSR301AEA), H3K4me3 ChIP-seq (ENCSR849YFO), H3K27ac ChIP-seq (ENCSR905TYC) generated from the same cortical bipolar spindle neurons used for the SMARCA4 ChIP-seq dataset used in 4.2.2. Overlaps between the approximately 3000 binding targets of *ASCL1* where it also regulates accessibility and each of these three histone modification datasets showed that these *ASCL1* target sites are more enriched for H3K4me1 and H3K27ac, which are typically found at distal regulatory elements such as enhancers and super enhancers and less enriched for H3K4me3, which is usually located at gene promoters. This histone modification signature supports the hypothesis that these distal *ASCL1* targets represent distal regulatory elements. Since these targets are found mostly within gene introns or intergenic regions that are located at long distances from the nearest annotated gene promoters, it suggests a different bioinformatics approach could be more accurate in associating the *ASCL1* genomic targets with the genes they regulate (further discussed in 6.6) .

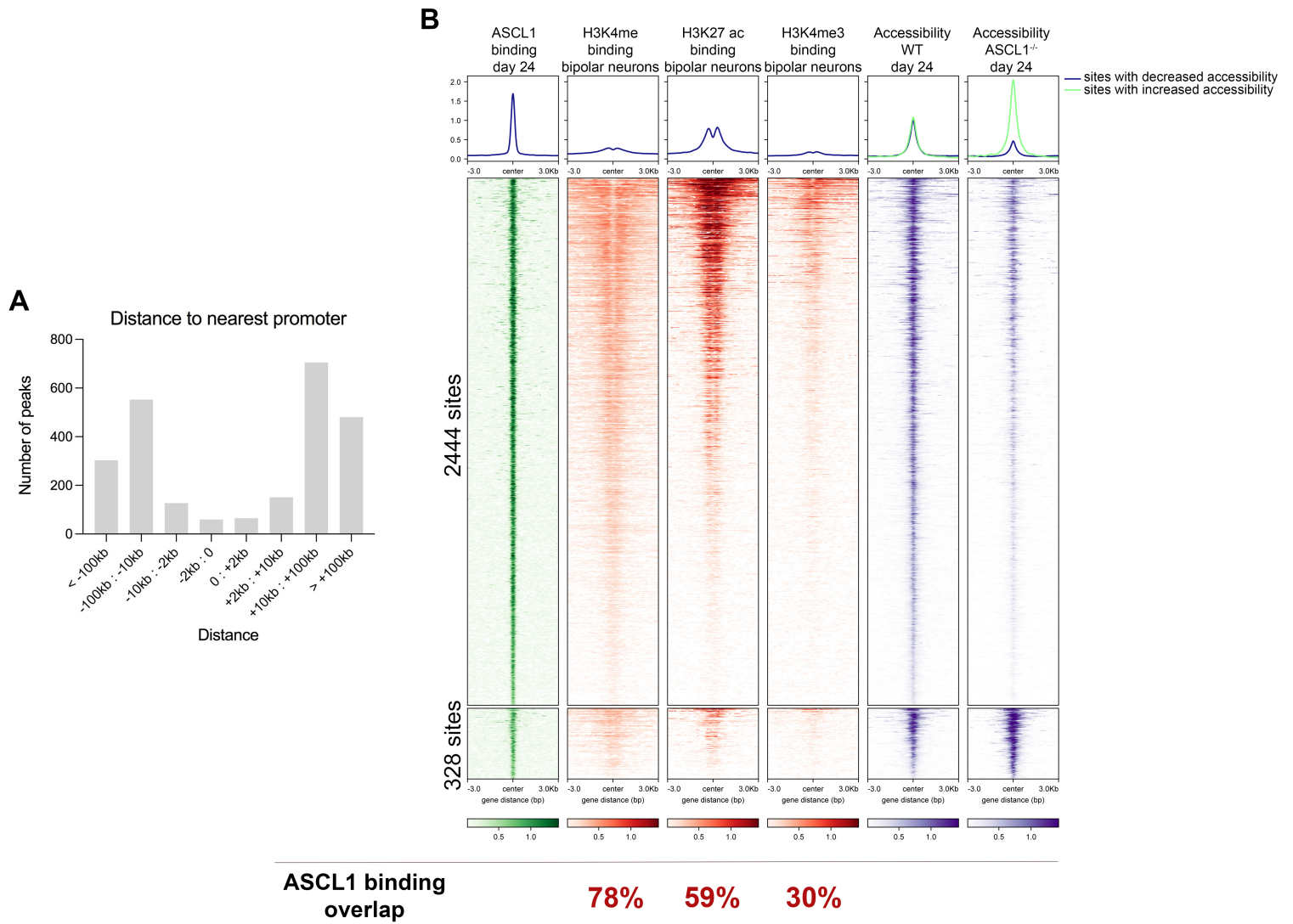


Figure 5.12: ASCL1 direct genomic targets show enrichment of distal regulatory elements-specific histone marks. (A) Analysis showing the distribution of ASCL1 direct genomic targets in relationship with their distance to the nearest annotated gene promoter. (B) Density heat maps showing the enrichment of different histone modification marks (H3K4me, H3K27ac and H3K4me3) at ASCL1 direct genomic targets. The percentage underneath the heat maps shows the exact overlap percentage between ASCL1 direct targets and the respective histone signature. Representative ChIP-seq and ATAC-seq profiles of two or three biological replicates. Peak calling and differential accessibility analysis performed by Harshil Patel, BABS.

Chapter 6

Discussion

6.1 Pioneer activity of ASCL1

Throughout this project, using different methods, I showed the absolute requirement for ASCL1 during cell cycle exit of human NPCs and differentiation into post-mitotic neurons. By combining ChIP-seq experiments for ASCL1 at 24 hours following Notch inhibition in our *in vitro* model of cortical neuronal differentiation (when ASCL1 is highest expressed) and ATAC-seq analysis both in wild-type and ASCL1^{-/-} cells, I was able to identify 2772 DNA regions where ASCL1 regulates chromatin accessibility. These sites account for approximately 25% of ASCL1 binding landscape and represented the first demonstration that pointed towards a pioneer activity of this transcription factor during the early stages of human cortical development. Moreover, correlation between ASCL1 binding and chromatin accessibility landscapes at day 20 and day 24 in wild-type neuronal cells led to the identification of 855 genomic

regions where ASCL1 acts as a pioneer transcription factor: it binds heterochromatin and upon binding induces chromatin opening. These 855 genomic regions account for 35% of all binding targets where ASCL1 acts as a positive regulator of chromatin accessibility. The remaining 1589 DNA regions that are dependent on ASCL1 to maintain them in an open state were already found in open chromatin at day 20. However, even if these 1589 sites were already open in NPCs, they all had a lower degree of accessibility compared to day 24, suggesting ASCL1 binding increases chromatin accessibility at these targets. This analysis represents a strong proof of principle as well as a confirmation that ASCL1 has pioneer activity in an *in vitro* model system that recapitulates the early stages of human cortical development from *in vivo*. However, there are almost 2000 remaining DNA sites that are dependent on ASCL1 in terms of their accessibility state, but for which I could not show ASCL1 binding closed chromatin prior to regulating chromatin accessibility - the expected dynamics for a pioneer transcription factor. The observation that the 1589 ASCL1-dependent regions showed a lower degree of accessibility at the earlier time point compared to day 24 and the lack of evidence that clearly shows the time ASCL1 binds these regions led to two possibilities: 1) the chosen time points at which CHIP-seq and ATAC-seq experiments were performed were not sufficient to explain ASCL1 dynamics at the chromatin level for all sites where it regulates accessibility or 2) at these sites ASCL1 acts as a non-classical pioneer factor, which shares pioneer functions with the classical factors (such as de novo chromatin access, regulation of accessibility or redistribution of partner transcription factors), but lacks the ability to access nucleosomal DNA (Minderjahn et al., 2020). In order to elucidate between these two theories a time course experiment would be required. More specifically, using the ASCL1 expression pattern as a guide, cells would have to be collected before ASCL1 starts to be expressed (around days 14-15 of neu-

ronal differentiation) and then every couple of hours until day 24 (for example every 6 or 12 hours). Subsequent ChIP-seq and ATAC-seq experiments would allow us to investigate ASCL1 dynamics at the chromatin level for all 2772 sites where ASCL1 regulates accessibility. However, at the moment, the ChIP-seq protocol used requires large number of cells, which makes this large scale experiment not feasible.

In order to confirm the pioneer activity of ASCL1 alternative methods could be used. For instance, one way would consist in assessing ASCL1 ability to bind to compacted chromatin *in vitro*. One such method is called electrophoretic mobility shift assay (EMSA) and represents a sensitive method to detect interactions between proteins and nucleic acids (Garner and Revzin, 1986; Fried, 1989; Lane et al., 1992). Initially, we would have to combine ASCL1 protein and nucleosomal coated DNA, followed by electrophoresis through acrylamide or agarose gels. If ASCL1 binds the nucleic acid, this complex will migrate more slowly than a control represented by the corresponding free nucleosomal DNA (Hellman and Fried, 2007).

6.2 Genes expressed at similar levels in NPCs and neurons are ASCL1-dependent only at day 24

As already described above, between day 15 and day 25 of neuronal differentiation, I noticed differences regarding cell identity within the cultures: before Notch inhibition (day 23) some cells differentiate into post-mitotic neurons, but vast majority of cells in the culture have a NPC identity; conversely, following DAPT addition, most NPCs exit cell cycle to become post-mitotic neurons, but some cells retain an NPC identity. An interesting observation between these two time points is represented

by a progressive upregulation in ASCL1 expression. Therefore, I decided to collect RNA and perform bulk RNA-seq at day 20 (one of the first time points when ASCL1 protein could also be detected in addition to upregulation of the mRNA level, which is usually observed a few days before) and day 24, which coincides with the peak in ASCL1 expression at both mRNA and protein level. These experiments allowed me to divide genes into three groups: day 20 specific genes, day 24 specific genes and genes common to both time points. Complementing this analysis with differential gene expression analysis between wild-type and ASCL1^{-/-} cells at the two time points revealed a high proportion (50%) of the day 24 specific genes to be ASCL1-dependent, which is in contrast with the earlier time point (day 20) when only a small number of genes (7.52%) seem to be regulated by ASCL1. However, an interesting observation came from the genes expressed at similar levels between the two time points – most of the genes that are ASCL1-dependent seem to be misregulated only following Notch inhibition. A possible explanation for these results could come from the way differential gene expression analysis is performed. For most of these ASCL1-dependent genes we see a slight upregulation at day 24 compared to day 20 cells. However, because of the heterogeneity of cell cultures at the two time points, as well as the short time window between them (4 days), this upregulation was not significant to place these genes into any of the day 20 or day 24 specific genes, but to keep them into the group of genes expressed at relatively similar levels.

Another possible explanation might be the way ASCL1 regulates these genes. They might not be directly regulated by ASCL1 (such as direct binding of ASCL1 to these genes), but might be targets located downstream of direct ASCL1 target genes. This possibility would imply an indirect regulation by ASCL1.

6.3 Human SWI/SNF complexes

I used the neuronal differentiation system to investigate the expression of the mSWI/SNF complex subunits throughout the neuronal differentiation process. Core subunits of the complex such as SMARCB1, SMARCE1, or SMARCD1 show similar, constant expression patterns. However, most of these subunits show a slight upregulation post DAPT, which might indicate an increase in the total number of mSWI/SNF assemblies that are required for the transition of NPCs to differentiated neurons.

Further investigations carried out *in vitro* suggest that the three subunit switches ACTL6A – ACTL6B, SS18 – SS18L1, PHF10/DPF2 – DPF1/DPF3, previously described in mouse model systems, also occur during human cortical development. At the transcriptional level, all four NPC specific subunits (*ACTL6A*, *SS18*, *PHF10*, and *DPF2*) are expressed in iPSCs and during neurogenesis, up to Notch inhibition. DAPT triggers neuronal differentiation, which is associated with the decrease in the mRNA level of these PSC- and NPC-specific subunits. However, I do not observe a sharp decrease in these esBAF/npBAF subunits, which could indicate a high proportion of NPCs still present in the culture or their persistent expression in post-mitotic neurons. Notch inhibition is also accompanied by significant upregulation of the nBAF specific subunits (*ACTL6B*, *SS18L1*, *DPF1*, and *DPF3*), which are absent or very lowly expressed in PSCs and NPCs.

At the protein level, I could mainly localise SS18 and SS18L1 in isolated populations of progenitors and neurons in the foetal developing cortex, respectively. However, both SS18 and SS18L1 could also be detected in some cells in the SVZ. This observation suggests the presence of an intermediate state when early-born neurons

can express either of the two subunits or both of them. Since each cell contains multiple mSWI/SNF complexes, SS18- and SS18L1-incorporated assemblies could co-exist in the early-born neurons. However, antibody species limitations do not allow co-localisation analyses at the moment. On the other hand, while ACTL6B could be identified only in CTIP2-positive neurons, ACTL6A was mainly expressed in SOX2-positive progenitors, but also in some neurons in both the SVZ and the CP. The same observation was made in western blot experiments, where ACTL6A is significantly downregulated, but still persists at a low level. This analysis suggests that during human cortical development ACTL6B is a neuronal exclusive subunit, while ACTL6A downregulation is not absolutely required for neuronal identity. In order to investigate the exact function of each of these homologous subunit pairs during human cortical development, additional experiments need to be performed. For instance, the alteration of the npBAF – nBAF transition via knockout or knock-down and overexpression of the npBAF specific subunits would be required. These experiments would allow the association of these proteins with the effect they have on NPC proliferation and/or neuronal differentiation.

6.4 Comparison of ChIP-seq datasets for ASCL1 and multiple mSWI/SNF subunits revealed different overlap percentages between their binding landscapes

In order to investigate whether ASCL1 requires the mSWI/SNF complexes to regulate its targets, I overlapped the genomic targets of both ASCL1 and the mSWI/SNF

complexes. When I used the SMARCB1 ChIP-seq datasets I generated using the *in vitro* model system described in 3.1, starting with both hiPSCs and hESCs, I discovered that more than 70% of the ASCL1 ChIP-seq peaks are also binding sites of the mSWI/SNF complexes. However, comparisons with publicly available datasets for SMARCA4 ATPase of the mSWI/SNF complexes, revealed only an approximately 50% overlap between the binding landscapes of the two factors. Looking at the SMARCA4 study in human NPCs (Gao et al., 2019), the method used to derive these cells involved an intermediate embryoid body state, and the final cell population collected for the ChIP-seq experiment was described as being more heterogeneous, with similar numbers of dividing cells and post-mitotic neurons. On the other hand, bipolar spindle neurons represent a very specific class of neurons found only in three regions of the human brain (the anterior cingulate cortex, the fronto-insular cortex, and the dorsolateral prefrontal cortex (Economo, 1926; Watson et al., 2006; Seeley et al., 2012)). Considering the identity of the neurons obtained with our *in vitro* model of cortical neuronal differentiation, significant differences can be observed in the cell identities used to generate the four mSWI/SNF ChIP-seq datasets.

In addition, SMARCA4 is not incorporated in all mSWI/SNF assemblies (with SMARCA2 being a mutually exclusive second ATPase specific for mSWI/SNF chromatin remodellers) (Alpsoy and Dykhuizen, 2018; Ho, Ronan, et al., 2009; Kadoch et al., 2013; Sarnowska et al., 2016; Mashtalir et al., 2018). Overall, with only a subset of mSWI/SNF assemblies incorporated in the analysis when SMARCA4 is used as the representative subunit, as well as with the different identity between the three neuronal cell types, I consider the 50% binding overlap between ASCL1 and SMARCA4 still a positive finding towards our hypothesis. The higher overlap percentage observed in the 24 hours post DAPT neurons, associated with the co-IP and PLA

experiments which suggest a physical interaction and proximity, respectively, are indicative of a co-recruitment mechanism between ASCL1 and mSWI/SNF complex to target sites of regulation.

6.5 New identity of the mSWI/SNF-lacking neurons

In this study, I decided to interfere with the activity of the mSWI/SNF remodellers by preventing their assembly via the ablation of SMARCC1 and SMARCC2 core subunits. The loss of these two proteins represents one of the reported intracomplex synthetic lethalties (Oike et al., 2013; Hoffman et al., 2014; Narayanan, Pirouz, et al., 2015; Mashtalir et al., 2018; Schick, Rendeiro, et al., 2019; Schick, Grosche, et al., 2021) and led to a global reduction in chromatin accessibility as well as to significant changes at the transcriptome level with approximately 5000 genes being misregulated. These results revealed by my analysis agree with previous studies which described that the mSWI/SNF remodellers are absolutely required to maintain the balance between global repressive and active epigenetic programs. Their absence results in a global reduction of active histone marks such as H3K27ac accompanied by a global increase of repressive histone marks such as H3K27me2 and H3K27me3 (Narayanan, Pirouz, et al., 2015; Iurlaro et al., 2021; Schick, Grosche, et al., 2021). The global effects that occur following the loss of mSWI/SNF assemblies can also affect the identity of the cells lacking these remodellers. For instance, differential chromatin accessibility analyses between cells where mSWI/SNF activity was ablated by inducing intracomplex synthetic lethalties and cells where only the ATPase activity of the complexes was inhibited revealed additional chromatin accessibility loss following synthetic lethality. Specifically, complete disruption of

mSWI/SNF remodellers severely affects accessibility at super enhancers in addition to changes that are also observed with the other methods (Schick, Grosche, et al., 2021). Super enhancers represent regulatory elements that control expression of genes that define cell identity (Hnisz et al., 2013). Although additional bioinformatics analyses to investigate the identity of the neuronal cells where I impeded the assembly of mSWI/SNF remodellers will be required, I consider a change in cell identity which may mask or interfere with the results. This idea is also supported by studies that showed mSWI/SNF complexes are indispensable for brain development, with their loss affecting different cell types and processes in the developing mammalian brain, which include proliferation and differentiation of neural progenitors, cell survival or cortical layer formation (Narayanan, Pirouz, et al., 2015).

Taking into consideration the results showed by my analysis, as well as previous reports from the literature, I looked into alternative ways to disrupt the activity of mSWI/SNF remodellers for short periods of time. At the moment, I am carrying on experiments where, instead of using CRISPR-Cas9 to knockout SMARCC1 and SMARCC2 subunits, I use an inhibitor of the catalytic activity of the two ATPases SMARCA2 and SMARCA4 called BRM014 (Papillon et al., 2018). This dual inhibitor acts faster than protein degradation methods and induces similar changes in chromatin accessibility compared to disruption via synthetic lethality. However, it has been shown not to affect accessibility at super enhancers, and therefore not to induce any changes in cell identity. In addition, both chromatin accessibility and transcriptional changes induced by BRM014 are specific to mSWI/SNF complexes, with no effects on the activity of other families of chromatin remodellers (Iurlaro et al., 2021; Schick, Grosche, et al., 2021). In comparison to SMARCC1/2 double knock-out, which has been shown to have increasing numbers of differentially expressed

genes at later time points following the induction of mutation (Schick, Grosche, et al., 2021), BRM014 induces a quick response which provides the ideal conditions to correlate chromatin accessibility and transcriptional regulation. Moreover, the longer period of time required for the degradation of SMARCC1 and SMARCC2 by CRISPR-Cas9 (72 hours) made hard to interpret the binding dynamics between ASCL1 and mSWI/SNF complexes. These remodellers not only that interact with and assist ASCL1 in regulating chromatin accessibility, but also bind its locus such that most of ASCL1 binding was lost after 72 hours from the moment I induced the mutation. However, BRM014 would allow me to investigate the binding dynamics at the genomic sites where ASCL1 – mSWI/SNF interaction is required for chromatin accessibility regulation since only the catalytic activity of the complex is abolished while the assemblies are still physically present at the chromatin level. Moreover, binding activity of other transcription factors that are known to interact with mSWI/SNF complexes such as OCT-4 or REST has been shown to be affected relatively slowly following BRM014 treatment, making this method ideal to investigate the remaining questions of this project (Iurlaro et al., 2021).

6.6 Alternative ways to correlate DNA binding and chromatin accessibility with gene expression

The correlation between DNA binding – chromatin accessibility regulation and gene expression changes between wild-type and ASCL1^{-/-} and wild-type and SMARCC1/2^{-/-} neuronal cells was only able to find a direct relationship for a subset of the ASCL1-dependent genes and genomic regions. The majority of these genomic targets are located more than 10kb away from the nearest annotated promoters and present a

histone modification signature characteristic for distal regulatory elements such as enhancers and super enhancers rather than promoters. As a consequence, the poor correlation between the DNA targets selected by ChIP-seq and ATAC-seq analyses and the transcriptional readout could be explained by the fact that several enhancers could target one single gene or because there are enhancer loops in the genome that can skip the promoters of the proximal genes and target distal ones (Moonen et al., 2020). Alternative ways to link distal non-coding regions to the genes they regulate is to consider the 3D organization of the genome rather than considering it linear. One such method that looks at the 3D contacts between enhancers and promoters and is able to explain how distal regulatory elements located far away from gene promoters in the linear genome are actually found in close physical proximity is represented by Hi-C (Lieberman-Aiden et al., 2009; Rao et al., 2014). More specifically, H3K27ac Hi-C chromatin immunoprecipitation measures the frequencies of 3D looping between enhancers and promoters and therefore correlates more of the distal genomic targets with the genes they regulate (Mumbach, Rubin, et al., 2016; Mumbach, Satpathy, et al., 2017). Alternatively, the Activity-By-Contact (ABC) algorithm is also able to predict enhancer-promoter interactions based on ATAC-seq and H3K27ac ChIP-seq datasets (Fulco et al., 2019). Since I have not generated any Hi-C data and there are no publicly available datasets in the literature that could be used in the context of this project (i.e. looking at human cortical development), the ABC algorithm seems like a more feasible option which I am currently approaching with the Bioinformatics & Biostatistics facility at the Francis Crick Institute.

6.7 Chromatin accessibility regulation at ASCL1 targets that are mSWI/SNF independent

Looking at ASCL1 DNA binding, my analyses showed it acts as a pioneer transcription factor (classical or non-classical) at approximately 25% of its binding targets. However, in trying to decipher the mechanisms behind ASCL1 pioneer activity, my experiments showed evidence for an interaction between ASCL1 and the mSWI/SNF remodellers responsible for chromatin accessibility regulation for about one third of them. In order to investigate the mechanisms that could explain the remaining two thirds of ASCL1 targets that are mSWI/SNF independent, I am planning to look for ChIP-seq datasets of chromatin remodellers from other families and overlap them with the ASCL1 direct genomic targets. This preliminary analyses could set the scene for additional experiments such as ATAC-seq and RNA-seq in wild-type versus ASCL1^{-/-} and wild-type versus the knockout of the candidate remodellers, which could potentially explain the remaining two thirds of the ASCL1 targets. Alternatively, since it is quite difficult to find ChIP-seq datasets derived from human neuronal cells in the developing cortex that also express ASCL1, an ASCL1 ChIP-mass spectrometry experiment could be conducted. By looking at the top ASCL1-interacting proteins, I would be able to select the best candidates and design additional experiments such that I would be able to explain the entire repertoire of ASCL1 targets where it exhibits pioneer activity.

6.8 Conclusions and perspectives

In this thesis I have reported experiments I performed to investigate the mechanisms behind the pioneer activity of ASCL1. This work was based on an *in vitro* model of human cortical neuronal differentiation from hiPSCs (Shi, Kirwan, et al., 2012; Deans et al., 2017) optimised in the Guillemot lab. I first showed the absolute requirement for ASCL1 during cell cycle exit of human cortical NPCs and differentiation into post-mitotic neurons. Next, I confirmed the pioneer activity of ASCL1 during the developing human cortex by identifying ASCL1 binding targets where it regulates chromatin accessibility.

Looking into the literature (Hu et al., 2011; Takaku et al., 2016; Wang, Du, et al., 2014; King and Klose, 2017), I hypothesised that one or more ATP-dependent chromatin remodellers could assist ASCL1 at its target sites. The overlapping effects between ASCL1 and mSWI/SNF complexes in mammalian neurogenesis led me to investigate a putative mutual interaction between ASCL1 and mSWI/SNF complexes. Co-IP and PLA, as well as ChIP-seq experiments showed there is a physical interaction between ASCL1 and mSWI/SNF remodellers, and that approximately 70% of ASCL1 binding sites are also targets of the SMARCB1 core subunit of mSWI/SNF complexes.

Reciprocal disruption of ASCL1 and mSWI/SNF complexes at different time points during neuronal differentiation, coupled with DNA binding by ChIP-seq and chromatin accessibility analyses by ATAC-seq showed that mSWI/SNF remodellers assist ASCL1 at approximately one third of its direct targets. In addition, ChIP-seq experiments in the two mutants (ASCL1^{-/-} and SMARCC1/2^{-/-}) led to the iden-

tification of different recruitment scenarios between ASCL1 and mSWI/SNF complexes. These scenarios are further investigated at the moment using the BRM014 inhibitor (Papillon et al., 2018) as an alternative method to abolish the activity of mSWI/SNF remodellers.

Finally, the association of ASCL1 only and ASCL1-mSWI/SNF direct targets with their transcriptional output was relatively poor, explaining less than half of the genes that are misregulated upon ASCL1 removal. However, the distance between these genomic targets and the nearest associated gene promoters, as well as the histone modification signature at these targets suggest they represent distal regulatory sequences. As a consequence, using the 'nearest annotated promoter' method to associate these genomic regions with their transcriptional output might only explain a subset of the ASCL1-dependent genes. Bioinformatics approaches that take into consideration the 3D organisation of the genome are currently ongoing.

The data presented in this thesis supports the hypothesis of a mutual interaction between ASCL1 and mSWI/SNF remodellers that stays behind the pioneer activity of ASCL1, and certainly merit further work. For instance, the role of ASCL1 in the transition from proliferation to differentiation coincides with the npBAF-nBAF subunit switch, briefly investigated in this thesis. Therefore, altering the npBAF-nBAF switch will allow me to investigate the hypothesis of a functional synergy between ASCL1 and npBAF or nBAF specific assemblies.

The ASCL1-mSWI/SNF targets represent one third of the ASCL1 direct targets. Therefore, in trying to decipher the mechanisms behind ASCL1 pioneer activity for the remaining two thirds of its direct targets would definitely benefit from investigating DNA binding and chromatin regulation activity of ATP-dependent remodellers

from different families using neuronal cells that express ASCL1.

Finally, ASCL1 pioneer activity is considered unique among the different proneural transcription factors. The work presented in this thesis points towards the mSWI/SNF remodellers as being one of the main mechanisms behind ASCL1 pioneer role. Since mutations in genes coding for subunits of mSWI/SNF and chromatin remodellers from the remaining three families represent a major cause of neurodevelopmental disorders (reviewed in Mossink et al., 2021), understanding the roles of chromatin remodellers in mediating ASCL1 pioneer activity has vast implications for human health and disease.

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