p53 is a hub for the integration of multiple vascular quiescence signals in the subventricular zone stem cell niche

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Abstract

Neurogenesis is the process by which new neurons are produced from neural stem cells (NSCs) in the brain. In the adult mammal, the subventricular zone (SVZ) and subgranular zone constitute the two neurogenic areas that continue to produce neurons over the life of the animal. SVZ NSCs (type b cells) reside in a niche which provides them with a host of signals that co-ordinate their behaviour. Type b cells are maintained in a quiescent, non-dividing state, in part through contact dependent signals supplied by vascular endothelial cells. I performed a mass spectroscopy screen to identify factors expressed by endothelial cells that enforce NSC quiescence, and showed that ephrin B2 and BMP4 are the main endothelial quiescence signals in vitro. To ascertain how these signals are transduced, I performed RNA sequencing of NSCs grown in co-culture with endothelial cells. This showed p53 as a key transducer of endothelial quiescence signals. Both ephrin B2 and BMP4 were found to be dependent on p53 for their quiescence activity. Interestingly, enquiry into signalling downstream of p53 showed that proliferative arrest is independent of its canonical effector p21. In contrast, p53 was found to regulate levels of EGFR, and BMP4 caused a reduction in EGFR which is abrogated upon loss of p53. Thus, p53 may control NSC quiescence at least in part through controlling levels of EGFR in response to endothelial BMP4. Despite indications that p53 is important for transducing endothelial quiescence signals in vitro, acute loss of p53 in type b cells in vivo had no effect on the number of proliferating type b cells. Finally, I describe work performed establishing super-resolution imaging
techniques to enquire into morphological changes following type b cell activation. Thus, this thesis makes a contribution to our understanding of the regulation of neural stem cell quiescence by the SVZ vascular niche.
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<td>a6b1</td>
<td>integrin alpha 6 beta 1</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helic</td>
</tr>
<tr>
<td>Ara-C</td>
<td>cytosine-b-D-arabinofuranoside</td>
</tr>
<tr>
<td>Ascl1</td>
<td>Achaete-scute homolog 1</td>
</tr>
<tr>
<td>bEND</td>
<td>immortalised mouse cerebral endothelial cells</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BTC</td>
<td>Betacellulin</td>
</tr>
<tr>
<td>CDKN1a/p21</td>
<td>cyclin dependent kinase inhibitor 1A</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>ChAT</td>
<td>acetylcholine releasing choline acetyltransferase</td>
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<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>Cre</td>
<td>Cre recombinase</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>D2L</td>
<td>D2-like</td>
</tr>
<tr>
<td>DE</td>
<td>differentially expressed</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco’s modified Eagle’s media</td>
</tr>
<tr>
<td>DNMT3a</td>
<td>DNA (cytosine-5)-methyltransferase 3A</td>
</tr>
<tr>
<td>EBSS</td>
<td>Earle’s Balanced Salt Solution</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>ephrin</td>
<td>eph receptor-interacting protein</td>
</tr>
<tr>
<td>Ephs</td>
<td>erythropoietin-producing human hepatocellular receptors</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>Foxo</td>
<td>forkhead box</td>
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</table>
GABA  gamma-aminobutyric acid
GAP  GTPase activating protein
GEF  guanine nucleotide exchange factors
GFAP  glial fibrillary acidic protein
GFP  green fluorescent protein
GLAST  glial high-affinity glutamate transporter
GN  granule neurons
GPI  glycosylphosphatidylinositol
HBSS  Hank’s buffered salt solution
Id  inhibitor of DNA binding
IGF2  Insulin-like growth factor 2
ILK  integrin-linked kinase
IPA  ingenuity pathway analysis
ITGA6  integrin alpha-6
K/G  kanamycin/gentamicin
KD  knock down
KO  knock out
LVCP  lateral ventricle choroid plexus
LVCPsec  LVCP secretome
MAPK  mitogen activated kinase
mass spec  mass spectroscopy
mRNA  messenger RNA
MT5-MMP  membrane type-5 matrix metalloprotease
NOS  nitric oxide synthase
NT3  Neurotrophin-3
PBS  phosphate buffered saline
PCR  polymerase chain reaction
PFA  paraformaldehyde
PGNs  periglomerular neurons
PLL  poly-L-lysine
POMC  proopiomelanocortin
Q-PCR  quantitative PCR
RAP  rap GTP-binding protein
RNA  ribonucleic acid
RMS  Rostral migratory stream
RNA seq  RNA sequencing
rSMAD  receptor associated SMAD
RTK  receptor tyrosine kinase
SGZ  subgranular zone
shRNA  short hairpin RNA
siRNA  silencing RNA
Sox2  sex determining region Y (SRY) box 2
STED  Stimulated emission depletion microscopy
STORM  Stochastic optical reconstruction microscopy
SVZ   Subventricular zone
TGF  transforming growth factor
VCAM1  vascular cell adhesion protein 1
WT  wild type

Declaration of originality

I, Timothy Davies, assert that everything contained within this thesis is my own work, and that all other work that has been referred to within this thesis has been appropriately referenced.

Copyright declaration

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Thanks to Dr Owen Sansom and Tam Jamieson at the Beatson Cancer Centre Glasgow for the gifting of brains from p21\textsuperscript{floxfloxfloxflox} and p21\textsuperscript{-/-} mice.
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Chapter 1 - Introduction

Neurogenesis

Neurogenesis is the process by which new neurons are generated from stem and progenitor cell populations in the brain. Following histological analysis of the brain it was concluded that neurogenesis is restricted to embryonic development and that shortly after the postnatal period the production of new neurons stops (Ramon y Cajal S, 1913–14).

The existence of neurogenesis in the adult brain was first shown by Joseph Altman and Gopal D. Das when they injected thymidine-H³ into rats and observed labelled cells in the dentate gyrus, and germinal zones in the third and lateral ventricles (Altman and Das, 1965). There are currently considered to be two neurogenic areas in the adult brain, the subgranular zone of the hippocampus and an area lining the lateral ventricle termed the subventricular zone (Bond et al. 2015), although evidence is growing for a third neurogenic region in the hypothalamus (Takiar et al, 2012; Li et al, 2012; Mcnay et al, 2012).

The existence of adult neurogenesis is of particular interest to scientists and clinicians because of the limited capacity of the central nervous system (CNS) to repair itself following injury. Loss of function is due in large part to the death of neurons, which due to their post-mitotic nature are unable to divide to re-populate the lost population. Stem cells are proliferative and multipotent,
meaning, given the right treatment, they can potentially act as source of any of the many different neuron types that might be lost through injury.

There are two possible approaches for the utilization of stem cells for repair: introduction of exogenous stem cells into the damaged area, or the mobilisation of endogenous stem cell populations. The second approach seems feasible given that following injury, proliferation increases in the SVZ (Ramaswamy et al, 2005), and it has even been reported that following cortical ischaemic injury neuroblasts generated in the SVZ home to the site of injury and differentiate into mature neurons (Kreuzberg et al, 2010). The goal then, is to try to enhance and potentiate the post-injury response, such that it leads to a greater functional recovery for those who suffer CNS damage. To do this we must first understand the means of regulation of subventricular neural stem cells, both the cell intrinsic aspects and the non-cell autonomous signals received from their environment.

An overview of the subventricular zone stem cell niche

The subventricular zone (SVZ) contains a population of neural stem cells termed type b cells that occupy an environment which provides them with a multitude of signals that regulate their behaviour. This environment is termed the “niche” (Lim et al, 2016). The majority of the type b cells residing in the niche are in a non-dividing, i.e. quiescent state (Codega et al. 2014). Once type b cells become ‘activated’ they begin proliferating and progress through their lineage. The cells give rise to transit amplifying progenitor cells termed ‘type c’. These cells in turn give rise to neuroblasts or ‘type a’ cells which migrate away from the SVZ.
through a migratory channel known as the rostral migratory stream (RMS) to the olfactory bulb, where they differentiate into neurons and integrate into the existing neural circuitry (Introduction Figure 1).

Type b cells are radial in morphology. At the apical aspect of the SVZ they intercalate with ependymal cells that line the ventricle, extending a primary cilium into the ventricular space (Codega et al. 2014). The cells also extend a basal process which makes contact with endothelial cells that comprise a vascular network closely associated with the niche (Shen et al. 2008).
Introduction Figure 1. Lineage progression and marker expression of SVZ neural stem cells. The majority of type b cells exist in a quiescent state. In the quiescent state type b cells have high expression of glial markers such as GFAP and GLAST and have high levels of glycolytic and lipid metabolism. Once activated, cells begin to proliferate and progress through their lineage, upregulating markers of activation such as nestin and EGFR. Protein synthesis rates increase and cells express proteins associated with progression through the cell cycle, such as Ki67. Activated type b cells give rise to a population of intermediate progenitors, or type c cells. After several rounds of division these cells give rise to neuroblasts, or type a cells, which are the migratory progenitor that, in rodents, move through the rostral migratory stream to the olfactory bulb, where they fully differentiate and integrate into the existing neuronal circuitry. At the neuroblast stage, cells begin to upregulate genes associated with their neuronal fate, such as DCX. (Adapted from Ottone and Parrinello, 2015; Llorens-Bobadilla, and Martin-Villalba, 2017; Codega et al, 2014)
Type b cells, discovery and characterisation

The neural stem cells of the SVZ were first isolated as self-renewing neurospheres that could be cultured in the presence of epidermal growth factor (EGF) and fibroblast growth factor (FGF) and were multi-potent, able to give rise to neurons and astrocytes (Reynolds and Weiss, 1992). Later, ultrastructural characterisation by electron microscopy defined the SVZ progenitor cell types and established the nomenclature used today (Doetsch et al, 1997). Antimitotic elimination of the type a and type c cells revealed that the astrocyte-like type b population are able to completely regenerate the other progenitor populations, indicating that they are the NSC of the SVZ (Doetsch et al, 1999). What has been unclear until recently is what relationship type b cells have to the radial glia of embryonic development. That is, whether type b cells constitute a population ‘left over’ from embryonic development or if there is a specific adult NSC population defined during the embryonic stage. Recent lineage tracing of radial cells in embryonic development suggests that type b cells arise during a window of two days from E13.5 to E15.5 and remain quiescent until becoming activated postnatally (Fuentealba et al, 2015).

Type b cell quiescence

It has long been known that that the SVZ NSCs are largely quiescent, with only a small fraction proliferating at any given time (Morshead et al, 1994). Unlike quiescent stem cells, activated type b cells express the EGF receptor EGFR and expand in response to in vivo infusion of the EGF ligand (Weickert, et al, 2000; Doetsch et al, 2002). Several more recent studies have attempted to further
characterise the differences between the activated and quiescent type b populations. Codega and colleagues used a Fluorescence-activated cell sorting (FACS) approach to distinguish the different SVZ cells using a combination of specific markers (Codega et al, 2014). They used a mouse model in which green fluorescent protein (GFP) expression is driven by the glial GFAP promoter, and combined this with the marker CD133 to isolate type b cells from the ependymal cells (CD133+ GFAP-), astrocytes (GFAP+ CD133-), type c (GFAP- CD133-) and type a (GFAP- CD133-) populations, employing a fluorescently-conjugated form of the EGF ligand to distinguish between EGFR+ and EGFR- populations. They showed that EGFR+ cells express proliferative markers such as Ki67 and form colonies in vitro, conversely, EGFR- cells for the most part do not express proliferative markers or form colonies in vitro, although over time they are able to ‘activate’ in vitro and begin to form colonies. Microarray analysis of the differences between the two cell types showed that they are indeed molecularly distinct, with the EGFR- cells sharing a common signature with other quiescent cell types.

More recently approaches combining FACS with a single cell RNA sequencing have revealed heterogeneity within the quiescent type b population (Llorens-Bobadilla, 2015). The authors in this study used CD133 in combination with the astrocyte marker glial high-affinity glutamate transporter (GLAST) to sort type b cells prior to RNA seq. By using pseudo-temporal ordering to define a lineage progression within the pool of cells the group revealed the existence of two quiescent states, termed dormant and primed. The dormant state is
characterized by increased glial marker expression and glycolytic and lipid metabolic processes. Conversely, the primed state is associated with higher levels of protein synthesis, greater RNA content and expression of genes associated with progression through the cell cycle (Llorens-Bobadilla, and Martin-Villalba, 2017; see also Introduction Figure 1). This population of cells resembles similar such primed quiescent states described in other stem cells such as muscle satellite cells (Rodgers et al, 2014).

As well as helping to define the transcriptional changes that could be important for the transition of type b cells from quiescence to activation, this study helps elucidate the heterogeneity present in the type b population. In fact, type b cell heterogeneity has been the focus of a number of recent studies and is an important emerging aspect of these cells that should be considered.

**Type b cell heterogeneity**

Transcriptional subdomains are known to exist along the rostro-caudal and dorso-ventral axis of the SVZ. These areas are defined during embryonic development but persist in the adult (Fuentealba et al, 2015). Lineage tracing has shown NSCs generate different types of olfactory bulb interneurons depending on where they reside within these SVZ subdomains. In the lateral wall, GSX2 expressing NSCs from the dorsal portion give rise mostly to superficial granule neurons (GN) (López-Juárez et al, 2013), whilst Gli1 expressing NSCs in the ventral portion give rise mostly to deep GNs (Merkle et al, 2014). Note that both domains of the lateral wall give rise to both GNs as well
as periglomerular neurons (PGNs). In addition, several small, ventral “microdomains’ have been identified. For example, Nkx2.1 expressing cells give rise to deep GNs and some PGNs (Delgado and Lim, 2015). In addition, ventral microdomains have also been identified that give rise to novel interneuron types located in different areas of the olfactory bulb. Nkx6.2 expressing cells give rise to type 1-4 neurons (Merkle et al, 2014) and Zic expressing cells to type 1-3 neurons (Merkle et al, 2014). Type 1 neurons integrate into the granule cell layer and are smaller and more ramified than other GNs, type 2 and 3 integrate into the mitral cell layer and type 4 neurons into the external plexiform layer (Chaker et al, 2016). In the dorsal SVZ, Emx1 expressing cells give rise to PGNs and superficial GNs (Young et al, 2007), whilst cells derived from PAX6 and SP8 domains are largely subtypes of PGNs (López-Juárez et al, 2013). Finally, Neurog2 and Tbr2 expressing domains give rise to glutamaterigic juxtaglomerular interneurons (Brill et al, 2009).

Differences in the behaviour of NSCs from different SVZ subdomains remains relatively unexplored, but differences in the location of NSCs in the SVZ should be considered when comparing studies that may have targeted certain subpopulations. In addition, primary NSCs isolated from SVZ subdomains have been shown to retain differences in their identity in vitro (Delgado et al, 2016), highlighting the need for consistency during SVZ preparations.
Introduction

Figure 2. Niche factors involved in the regulation of SVZ neurogenesis and their origin within the niche. Type b cells span the niche, receiving signals from a number of cell compartments. Apically, cells intercalate with ependymal cells lining the ventricle, which supply them with a number of regulatory factors. In this way, they are also exposed to soluble signals secreted by the choroid plexus into the cerebrospinal fluid. Basally, cells make contact with endothelial cells of the vasculature, receiving soluble and contact dependent signals important for maintaining the cells in an undifferentiated state. A number of neuronal populations innervate the SVZ, releasing a variety of neurotransmitters that have been shown to have an impact on neurogenesis. Finally, type b cells receive important negative feedback signals from their progeny. Green arrows indicate factors that have an overall positive impact on neurogenesis, red arrows indicate factors that act to suppress or constrain neurogenesis. See the accompanying table for further details. (Adapted from Ottone and Parrinello, 2015).
**Compartment** | **Signal** | **Effect** | **Citation**
--- | --- | --- | ---
Choroid plexus | IGF2 | Promotes proliferation of SVZ progenitors | Lehtinen et al, 2011; Ziegler et al, 2012
Amphiregulin | Promotes proliferation of NSCs in vitro | Falk and Frisen, 2002
TNF alpha | Promotes proliferation of progenitors in the dorsoventral SVZ | Tropepe et al, 1997
Choroid plexus secretome | Promotes type b clonal expansion | Silva-Vargas et al, 2016
Neuronal projections | Dopamine | Promotes or suppresses proliferation of SVZ progenitors | Höglinger et al, 2004; Kippin et al, 2005
Acetylcholine | Promotes proliferation of type b cells | Paez-Gonzalez et al, 2014
Nitric oxide | Suppresses proliferation of SVZ progenitors | Romero-Grimaldi et al, 2008
Serotonin | Promotes proliferation of type b cells | Tong et al, 2014
Beta-endorphin | Promotes proliferation of Nkx2.1+ NSCs | Paul et al, 2017
Ependymal cells | Noggin | Promotes or suppresses neurogenesis | Lim et al, 2000; Colak et al, 2008
PEDF | Promotes self-renewal and activation of type b cells | Ramírez-Castillejo, et al, 2006
N-cadherin | Maintains quiescence of type b cells | Porlan et al, 2014
Vascular endothelial cells | Blood borne factors | Promotes self-renewal, suppresses differentiation of NSCs in vitro | Shen et al, 2004
PEDF | Promotes self-renewal and activation of type b cells | Ramírez-Castillejo, et al, 2006
Betacellulin | Promotes proliferation of type b cells | Gómez-Gavirio et al, 2012
NT3 | Promotes quiescence of type b cells | Delgado et al, 2014
Jagged1 | Promotes quiescence through induction of stemness | Ottone et al, 2014
Ephrinb2 | Promotes quiescence through suppression of proliferation | Ottone et al, 2014
BDNF | Promotes type a cell migration | Snapyan et al, 2009
SDF | Promotes type a cell migration | Kokovay et al, 2005
BMP4 | Promotes quiescence of NSCs in vitro | Mathieu et al, 2008
EGL7 | Promotes type b quiescence through potentiation of dll4 signalling | Bicker et al, 2016
Type a cells | GABA | Suppresses proliferation of type b cells | Liu et al, 2005
Type b cells | BMP2/4 | Suppresses neurogenesis | Lim et al, 2000
Dil1 | Maintains quiescence of type b cells through juxtacrine signalling | Kawaguchi, 2012

**Introduction Figure 3. A list of niche factors, their effect on neurogenesis and the relevant citations**
Mechanisms of control of type b cell proliferation

The control of type b cell behaviour has two aspects: cell intrinsic control such as transcription factor expression, and control by the niche through extrinsic signals. Since the focus of this thesis is on type b cell quiescence and proliferation, I will focus on mechanisms of control that have been implicated in the regulation of type b and progenitor cell division. First, I will give an account of the cell intrinsic mechanisms that have been identified, and second, a description of niche signals providing extrinsic control (see also Introduction Figures 2 and 3).

Cell intrinsic mechanism of proliferation control

Transcription factors

Sex determining region Y (SRY) box 2 (Sox2) is a well-known marker of both embryonic and adult progenitor cells. It is a transcription factor that has been shown to be important in the maintenance of neural progenitor identity (Graham et al, 2003). Sox2 is associated with a more proliferative state (Marqués-Torrejón et al, 2013), it is broadly expressed in proliferating progenitors such as activated type b and type c, but is present in smaller proportion of quiescent type b cells (Codega et al, 2014).

Achaete-scute homolog 1 (Ascl1) is a transcription factor that is important in the activation of neural stem cells to a proliferative state both in the SGZ and SVZ (Andersen et al, 2014). Ascl1 is required for activation, and blockade of
Ascl1 expression prevents exit from quiescence. Mechanistically, Ascl1 directly regulates the expression of cell cycle genes such as Ccnd2 and Rrm2 in hippocampal NSCs. Interestingly, the return of hippocampal NSCs to a quiescent state requires the degradation of the pro-activating Ascl1, underlining the importance of its role in the transition between quiescent and activated states (Urbán et al, 2016). Whether or not there is such a return to the quiescence state following activation in the SVZ niche is more contentious, with some fate tracing studies suggesting that once activated, type b cells go through several rounds of cell division before becoming depleted (Calzolari et al, 2015). The transcription factor **TLX** is expressed in both adult neurogenic regions and also seems to be important, at least in the SGZ, for regulating the activation of NSCs. This effect seems to be dependent on the activation of p21 by p53 (Niu et al, 2011).

**The inhibitor of DNA binding (Id)** proteins act as negative regulators of basic helix-loop-helix (bHLH) transcription factors. Ids 1, 2 and 3 promote proliferation and self-renewal of NSCs in the embryonic brain (Jung, S, 2010). Id1 was shown to be expressed in type b cells in the adult SVZ and promotes self-renewal of NSC in vitro (Nam, and Benezra, 2009). Interestingly, loss of Ids 1,2 and 3 in the postnatal brain causes detachment of type b cells from the vasculature through bHLH activation of the levels of Rap1GAP, the activating GAP for Rap1. This results in premature differentiation of type b cells. This finding is consistent with other studies demonstrating the importance signals
from the vasculature for maintenance of stemness in type b cells (Shen et al, 2008; Ottone et la, 2014).

Conversely, the forkhead box (Foxo) family of transcription factors have been shown to be important in constraining proliferation of adult NSCs. Loss of Foxos 1,3 and 4 in GFAP+ cells resulted in an increase in the number of proliferating progenitors in the postnatal SVZ, followed by a subsequent depletion of the progenitor pool in adult mice (Paik, et al. 2009).

As previously mentioned, the SVZ contains a number of transcription factor defined subdomains that persist following specification in the developing telencephalon. However, analysis of the impact of differences in the subset of transcription factors has thus far mostly been concerned with differences in the cohorts of neurons produced by NSCs in the various regions (Merkle et al, 2014; Fuentealba et al, 2015). Single cell analyses of SVZ NSCs should, in the future, allow the elucidation of the functional impact of the heterogeneity of transcription factor expression on NSC behaviour, and the responsiveness of cells to niche signals.

Epigenetic regulation

Histone modification seems to play a role in the regulation of neurogenesis. Bmi-1, a member of the polycomb group of chromatin re-modellers is essential for SVZ NSC self-renewal. Loss of Bmi-1 causes an increase in levels of p16Ink4a and suppression of NSC proliferation (Molofsky et al, 2003).
DNA methylation is another epigenetic modification that has been shown to play a role in neurogenesis. The DNA methyltransferase DNA (cytosine-5)-methyltransferase 3A (DNMT3a) methylates the intergenic regions of neurogenic genes in SVZ NSCs, and loss of DNMT3a results in a decrease in neurogenesis in the postnatal olfactory bulb (Wu et al, 2010). Methyl-CpG binding proteins bind to methylated DNA and affect changes to transcription. Loss of Methyl-CpG binding protein 1 results in increased NSC proliferation through increased expression of micro RNA miR-184 (Liu et al, 2010). Methyl-CpG binding protein 2 controls progenitor proliferation by regulating expression of miR-137. Knock down of miR-137 in vivo results in an increase in the number of BrdU incorporating progenitors in the SVZ (Szulwach et al, 2010).

**p21**

Loss of p21 causes an increase in young mice (60 days old), and a decrease in old mice (480 days old), of the number of long term label retaining cells in the SVZ. *In vitro* there is an initial expansion in the number of neurospheres from p21−/− mice, but a decrease in the self-renewal as determined by a decrease in the number of neurospheres upon serial passage (Kippin et al, 2005). More recently p21 has been shown to directly control the expression of BMP2 *in vivo* (Porlan et al, 2013). Increased numbers of label retaining GFAP+ cells were noted in young p21−/− mice, in agreement with the observation by Kippin and colleagues, but it was noted this increase is concomitant with an increase in the number of label retaining cell expressing the mature astrocyte marker s100. p21
therefore seems to play a role in supressing the terminal differentiation and promoting the self-renewal of type b cells. In addition, p21 has been reported to regulate the self-renewal of type b cells by directly regulating expression levels of the transcription factor Sox2 (Marqués-Torrejón et al, 2013).

p53

p53 is one of the best known and most extensively studied proteins in biology. This is due to the important role played by p53 as a tumour suppressor. In fact, loss of p53 function, either through mutation of the p53 gene or through disruption to p53 associated pathways, is a feature in the majority of human cancers (Muller and Vousden, 2013).

Perhaps the best studied role of p53 is as key regulator of the cell’s response to DNA damage. Activation of p53 in response to genetic insults can result in apoptosis (Kenyon, et al 1993), growth arrest (Waldman et al, 1995) or senescence (Xue W et al, 2007). Which outcome prevails, may depend on a number of factors including cellular context, the nature of the insult and the extent of the damage (Vousden, 2002).

In addition to long established roles in the control of apoptosis and the cell cycle, p53 has an emerging role in the regulation of cell metabolism (Bensaad and Vousden, 2007). Cancer cells are known to utilize glycolysis for their energy production rather than oxidative phosphorylation, which is the preferred energy production pathway for cells in aerobic environments. p53 has been shown to
play a role in regulating this metabolic switch, by controlling the transcription of proteins that regulate the glycolytic pathway such as TP53-induced glycolysis and apoptosis regulator (TIGAR); as well as glucose-transporters such as Glut1, which, since glycolysis is a relatively inefficient means of energy production, provide the high levels of glucose that are required by the cell to produce sufficient energy from glycolysis (Puzio-Kuter, 2011).

Another role for p53 is in the control of fatty acid metabolism. Fatty acids are important building blocks for the synthesis of lipids, and cells can generate energy from fatty acids through the process of beta-oxidation. p53 may play role in regulating the oxidation of fatty acids. For example, increased fatty acid oxidation occurs in the livers of wild type p53 mice (Puzio-Kuter, 2011). p53 may exert this control by regulating key enzymes in the beta oxidation process. For example, fatty acid synthase (D’Erchia et al, 2006) and creatine palitoyltransferase, which is important for import of lipids into the mitochondria, where beta-oxidation takes place (Sanchez-Macedo et al, 2013).

Recent studies are also defining a role for p53 in the maintenance of homeostasis in various stem cell compartments (Bonizzi et al, 2012). For example, in the mammary stem cell niche p53 has been shown to control the symmetry of mammary stem cell division (Cicalese, et al, 2009) and in hematopoietic stem cells p53 regulates cell quiescence (Liu et al, 2009). p53 also plays a role in the regulation of neural stem cell behaviour, both in development and in the adult. In development, loss of p53 loss causes an
increase in the number of neurons produced, this occurs at the expense of gliogenesis (Liu et al, 2013). In the adult, p53 loss causes an increase in the number of proliferating cells in the SVZ, and an increase in the proliferation and self-renewal of SVZ derived progenitors in vitro (Meletis et al, 2006; Gil–Perotin et al, 2006). p53 expression has been shown in the GFAP+ type b cells of the SVZ (Meletis et al, 2006; Theus et al, 2010) but it is not clear if loss of p53 results in an increase in the number of proliferating type b cells. That is, it is unclear whether p53 plays a role in the maintenance of quiescence in vivo. Although studies in vitro would suggest p53 does play a role in adult NSC proliferation, papers that have specifically looked at the effect of p53 loss on proliferating type b cells have either shown no effect (Wang et al, 2013) or a trend that does not reach statistical significance (Gil–Perotin et al, 2006). A confounding factor may be that the described studies have all utilized a constitutive p53 knock out system. As previously mentioned, p53 plays a role in developmental neurogenesis, it is therefore possible that the SVZ of an adult mouse lacking p53 already has compensatory changes that would not be seen in an inducible system.

Extrinsic signals, proliferative and anti-proliferative signals from the niche

The cerebrospinal fluid

Type b cells intercalate into the ependymal layer and extend a primary cilium into the ventricular space. They therefore come into contact with a number of factors present in the cerebrospinal fluid (CSF). Insulin-like growth factor 2 (IGF2)
from the CSF has shown to act as a proliferative signal to SVZ NSCs (Lehtinen et al, 2011; Ziegler et al, 2012). Other CSF factors may also have an effect on type b cell proliferation. Amphiregulin expression has been detected in the adult choroid plexus and this factor has been shown to drive proliferation of SVZ NSCs in vitro (Falk and Frisen, 2002). Transforming growth factor (TGFα) has been shown to be expressed in the striatal region (Lazar and Bloom, 1992) and TGFα−/− mice displayed decreased proliferation in the dorsolateral aspect of the SVZ (Tropepe et al, 1997). The TGFβ receptor TGF-BR2 has been shown to be expressed by nestin positive cells in the SVZ, while infusion of TGFβ1 into the ventricle by osmotic minipump caused a decrease in the number of proliferating cells along of the ventricle wall (Wachs et al, 2006). FGF-2 expression has been reported in the choroid plexus and ependymal of rat lateral ventricles (Hayamizu et al, 2001). Recently, Silva-Vargas and colleagues carried out a thorough analysis of the composition of factors secreted by the lateral ventricle choroid plexus (LVCP) into the CSF (Silva-Vargas et al, 2016). They showed that supernatant taken from LVCP explants supports the proliferation of SVZ NSCs in vitro, as well as the clonal expansion of quiescent and activated type b cells and type c cells astutely isolated by FACS from the SVZ. Transcriptional analysis of the LVCP coupled with antibody array proteomic analysis of the LVCP secretome (LVCPsec) revealed expression of a number of factors including lymphokines and cytokines such as CSF-1, CXCL16, CX3Cl1, CCL2/JE and CXCL12. Carriers such as RBP4 and Fetuin-A. Growth factors such as FGF2, TGFβ2, LIF, PLGF and VEGF-A. As well as previously reported LVCP factors such as IGFBP-2, IGFBP-3, Lipocalin-2, IGF2 and IL1B. Interestingly, the
composition of the LVCPsec seems to change with age, with old LVCPsec proving less able to support the clonal expansion of activated NSCs than LVCPsec derived from old mice.

**Neuronal projections**

Dopaminergic projections originating from the substantia nigra and the ventral tegmental area (VTA) neurons are known to innervate the SVZ (Baker et al, 2004). Studies into the effect of the release of dopamine by these projections on the proliferation of SVZ progenitors have produced conflicting results. D2-like (D2L) dopamine receptors have been shown to be expressed on type C cells, and de-nervation resulted in a reduction in proliferation of type C cells, but not of type b cells, which could be rescued by pharmacological activation using a dopamine analogue (Höglinger et al, 2004). Seemingly in opposition to this finding is the observation that use of the D2L receptor antagonist haloperidol results in an increase in proliferating cells in the SVZ, and of neurospheres derived from haloperidol treated animals (Kippin et al, 2005). An explanation put forward for these seemingly contradictory findings is that dopamine may exert different effects on type b and type c cells, perhaps due to differences in receptor expression (Berg et al, 2013; Bjornsson et al, 2015).

Nitric oxide releasing nitergic neurons project into the SVZ, and inhibition of nitric oxide synthase (NOS) results in an increase in EDU incorporating cells
(Romero-Grimaldi et al, 2008). Serotonergic axons innervate the SVZ and make contact with type b cells and ependymal cells.

Type b cells have been shown to express serotonin receptors and infusion with serotonin receptor agonists results in increased SVZ proliferation, conversely, antagonist treatment suppresses proliferation (Tong et al, 2014).

A population of acetylcholine releasing choline acetyltransferase (ChAT)+ neurons have been identified which innervate the SVZ. The activity of these neurons has been manipulated using optogenetics to demonstrate that local release of acetylcholine by these projections is necessary and sufficient to control SVZ proliferation (Paez-Gonzalez, et al, 2014).

Finally, SVZ region-specific innervation has also been reported. A population of hypothalamic neurons has been found that specifically innervates Nkx2.1 expressing type b cells in the anterodorsal portion of the SVZ. The hypothalamus plays a role in the regulation of physiological states, and the group showed that hunger and satiety regulate the proliferation of Nkx2.1 type b cells through alterations in firing of these proopiomelanocortin (POMC) neurons (Paul et al, 2017).

**Ependymal cells**

Type b cells reside in close proximity to ependymal cells, intercalating with them to form characteristic ‘pin-wheel’ structures. The importance of this interaction was demonstrated by deletion of the adhesion protein vascular cell adhesion
protein 1 (VCAM1), which disrupts this pinwheel architecture and leads to the aberrant proliferation and depletion of the quiescent type b population (Kokovay et al, 2012). The adhesion protein N-cadherin has been shown to be important for this interaction and maintains the stem cells in their quiescent state. Cleavage of N-cadherin by the membrane type-5 matrix metalloprotease (MT5-MMP) modulates levels of type b cell proliferation, and is required for the activation following niche injury (Porlan et al, 2014).

Ependymal cells have also been shown to produce several factors that influence neurogenesis. Noggin, an antagonist to BMP signalling is released by the cells (Lim et al, 2000). The role of BMP in SVZ neurogenesis will be discussed later in this introduction. Ependymal cells have also been shown to secrete PEDF which has been shown to promote self-renewal and activation of type b cells (Ramírez-Castillejo, et al 2006).

Fractones

Fractones are small deposits of extracellular matrix (ECM) proteins that can be visualized as punctate structures within the SVZ following labelling with antibodies against proteins such laminin. Fractones have the capacity to bind growth factors and other signalling molecules and present them to cells. A number of papers by Mercier and colleagues have demonstrated this by performing intraventricular injections of FGF2 and BMP4/BMP7 and showing that the molecules bind to fractones and stimulate or suppress proliferation of SVZ cells respectively (Douet et al, 2012; Douet et al, 2013; Mercier and Douet,
2014). Though the ability of fractones to bind to and present exogenously introduced signalling molecules to cells is beyond question, whether or not under normal conditions growth factors bound to fractones play an important role in the regulation of SVZ proliferation has yet to be shown.

Type b cells interact with ECM components of the basal lamina and fractones through integrin receptors (Shen et al, 2008). Signalling through integrins may play a role in controlling proliferation. Indeed, deletion of one of the mediators of integrin signalling integrin linked kinase (ILK) in nestin+ cells of the SVZ resulted in a decrease in the number of proliferating progenitors (Porcheri et al, 2014).

Vascular endothelial cells

At the basal extent of the SVZ there is a dense network of blood vessels that runs parallel to ependymal layer. Type b cells frequently make contact with this plexus through a basal process, and proliferating progenitors are found closely associated blood vessels (Shen et al, 2008; Tavazoie et al, 2008, Mirzadeh, et al; 2008). Transwell assays have shown endothelial cells produce factors that stimulate self-renewal, inhibit differentiation and enhance neuron production of NSCs in vitro (Shen et al, 2004). For example, endothelial cells are a source of PEDF, which as previously noted has the ability to promote self-renewal and activation of type b cells (Ramírez-Castillejo, et al 2006) and Betacellulin (BTC) produced by endothelial cells induces proliferation of NSCs in vitro, while use of a BTC blocking antibody decreased the number of type b cells in vivo (Gómez-Gaviro et al, 2012). Conversely, Neurotrophin-3 (NT3), which is produced by
endothelial cells and the choroid plexus, induces the production of nitric oxide which acts to promote the quiescence and long-term maintenance of type b cells (Delgado et al, 2014).

Contact dependent signalling between type b cells and endothelial cells has also been shown to be important. Loss of jagged 1 and ephrin B2 ligands from endothelial cells in vivo causes aberrant activation of type b cells, leading to over-proliferation and eventual depletion of the quiescent type b population. Mechanistically, ephrin B2 maintains quiescence by suppressing phosphorylation of MAP kinases downstream of mitogenic signals, while jagged 1 promotes stem cell identity by inducing the expression of stemness genes such as Hes5 (Ottone et al, 2014).

**Type a cells**

Type a cells have been shown to spontaneously release gamma-aminobutyric acid (GABA). This acts on GFAP expressing cells in the SVZ to limit their proliferation in a manner which is dependent on epigenetic mechanisms involving H2AX phosphorylation (Liu et al, 2005; Fernando et al, 2011).

I will now discuss in greater depth a selection of the niche signals implicated in the control of type b cell quiescence and proliferation. These signals are focused on in the results section of this thesis and therefore more detail on them will provide useful context for the reader.
Notch signalling and SVZ neurogenesis

Notch activity is important for the maintenance of adult NSCs, and its loss results in a disruption of neurogenesis (Giachino and Taylor, 2014). However, there may be heterogeneity in the response to notch signalling between quiescent and activated type b populations. For example, ablation of notch1 results in loss of activated but not quiescent type b populations (Basak, 2012) and jagged 1 promotes the proliferation and self-renewal of EGFR+ SVZ NSCs \textit{in vivo} (Nyfeler et al, 2005). Conversely, Jagged1 expressed on endothelial cells is important for maintaining a pool of quiescent type b cells (Ottone et al, 2014), and Dll1 segregated during cell division provides important quiescence-enforcing juxtacrine signalling between daughter cells (Kawaguchi et al, 2013). Recently, the expression of EGFL17 was shown to enforce quiescence of type b cells through potentiation of Dll4 signalling (Bicker et al, 2017).

BMP signalling and SVZ neurogenesis

\textbf{Bone morphogenetic proteins (BMPs)} are a family of proteins belonging to the transforming growth factor beta (TGF\textbeta) family. Originally identified for their ability to induce bone formation (Urist, 1965), they have since been found to play important roles regulating a host of developmental processes influencing aspects of cell function such as cell growth, differentiation and apoptosis, as well as playing roles in many tissues in the maintenance of adult tissue homeostasis (Wang et al, 2014). They are synthesised as dimeric pro-proteins which are cleaved by pro-protein convertases into non-covalently bonded N and
C terminal fragments. The C terminal fragment binds to receptors, whilst the N terminal fragment plays an important regulatory role (Harrison et al, 2011).

BMPs can signal through both canonical and non-canonical pathways. In the canonical pathway, the signal is transduced by a heterodimer consisting of two type 1 and 2 type 2 serine/threonine kinase receptors. There are seven type 1 receptors in the TGFβ receptor family, of which three are bound by BMP: BMPR1a also known as Alk3, BMPR1b also known as Alk6 and ActR1a, also known as Alk2. There are four members of the type 2 receptor family, three of which are known to transduce BMP signals: BMPR2, ActR2a and ActR2b. The manner in which the heterodimers are formed varies, with BMPs 2 and 4 preferentially binding to type 1 receptors and recruiting type 2 receptors, and BMPs 6 and 7 binding to type 2 receptors and recruiting type 1 receptors (Wang et al, 2014). The existence of pre-formed heterodimers has also been reported and the manner in which signalling heterodimers are formed has been shown to change signalling pathways downstream (Nohe et al, 2002).

Following assembly of the signalling heterodimer, the constitutively active type 2 receptor subunits phosphorylate the type 1 receptor subunits. This allows phosphorylation of the downstream mediators, receptor associated SMADs (R-SMADs), which in the case of BMPs are SMADs 1,5 and 8. These then bind to the obligate co-SMAD SMAD4, and this complex translocates to the nucleus where it can act as a transcription factor to influence expression of target genes.
Non-canonical signal transduction pathways have also been reported, including P/kc, PI3K/Akt, MAP kinases and Rho GTPases (Derynck et al, 2003).

The role of BMPs in regulation of neurogenesis in the SVZ is somewhat contentious. Lim and colleagues showed that BMPs 2 and 4 are expressed within the SVZ, and that type b/c cells express BMP receptors BMPR1A and BMPRII. Overexpression of BMP7 in ependymal cells in vivo resulted in a marked decrease in proliferating cells in the SVZ. By using retroviral transduction to express constitutively active BMP receptors in SVZ cultures, the group found that BMP signalling antagonizes neuron production by promoting glial differentiation. Colak et al confirmed that there is indeed active BMP signalling in type b and c cells by staining for the phosphorylated form of the BMP effectors SMADs 1/5/8. However, they found that deletion of SMAD4, the obligate co-effector in the transduction of BMP signalling in GLAST+ type b cells had no effect on numbers of type b or type c cells, nor on type b cell proliferation. The authors did note a decrease in the number of type a cells in the SMAD4−/− animals, concomitant with apparent expression of oligodendrocyte marker Olig2 and migration of oligodendrocytes to the corpus callosum. They therefore concluded that BMP signalling directs neurogenesis by promoting neuronal, at the expense of glial fate. These conflicting reports leave the role of BMP in the SVZ unclear, with one possibility being that BMP signalling has different effects on cells at different stages of the lineage.
Ephrin signalling and SVZ neurogenesis

**Erythropoeitin-producing human hepatocellular receptors (Ephs)** are the largest class of receptor tyrosine kinase receptor. They are involved in the regulation of a multitude of processes in both development and in the adult including axon guidance, cell proliferation and differentiation, cell migration, tissue compartmentalisation among others (Pasquale, 2005). Their ligands, the Eph receptor-interacting proteins (ephrins) are membrane bound and have the notable ability to carry out ‘reverse’ signalling. There are two classes of Eph receptor. There are nine EphA receptors that bind promiscuously to five ephrin-a ligands, and five EphB receptors that bind promiscuously to three ephrin-b ligands (Lisabeth et al, 2013). The receptors Ephb2 and EphA4 are able to bind to ligands of either class. Ephrin-bs have a transmembrane and intracellular region, ephrin-as are attached to the membrane via a glycosylphosphatidylinositol (GPI), which can be severed allowing them to bind receptors at a distance (Bartley et al, 1994).

There have been several studies implicating ephrin signalling in the control of SVZ neurogenesis. The expression of EphB1-3 and EphB4 as well as ephrin B2 and b3 within the SVZ has been demonstrated, and infusion of both recombinant ephrin B2 and recombinant EphB2 causes an increase in SVZ proliferation (Conover et al, 2000). Ephrinb3	extsuperscript{−/−} mice have an increased number of proliferating cells in the SVZ, infusion of recombinant ephrinb3 rescued proliferation to wild type levels (Ricard, et al, 2006). Later work by the group suggested that EphA4 acts as a dependence receptor inducing cell death in the
absence of its ligand ephrinb3 (Furne, et al, 2009). Signalling through EphB3 has been suggested to supress proliferation of progenitors in a manner which is dependent on p53 (Theus et al, 2010). Finally, as previously described in the section on vascular endothelial signals, ephrin B2 expressed on endothelial cells has been shown to supress proliferation of type b cells and maintain cells in a quiescent state (Ottone et al, 2014).

**Semaphorin signalling and SVZ neurogenesis**

**Semaphorins** are a family of ligand originally identified as axon guidance signals in the developing nervous system. There have been 20 semaphorins identified in mammals, which are divided into 5 classes based on their structural properties. Class 3 semaphorins are secreted, class 4-6 semphorins are transmembrane proteins and semaphorin 7A is tethered to the membrane by GPI anchor. Additionally, several of the membrane bound semaphorins can be proteolytically cleaved to yield ligands able to signal at a distance (Worzfeld and Offermanns, 2014).

The main mediators of semaphorin signalling are a class of receptors called the plexins. Plexins are divided into 4 classes, A-D. Class A plexins are activated by class 4 and 5 semaphorins; they are also activated by class 3 semaphorins but this requires the co-receptor neuropillin. Class B plexins are activated by class 4 and 5 semaphorins. Plexin C1 is activated by semaphorin 7A. Finally, plexin D1 is activated by several class 3 semaphorins together with neuropillin, and is
activated by sempahorin 3E and 4A independent of neuropillin binding (Worzfeld and Offermanns, 2014).

The cytoplasmic tail of Plexins contains a GTPase activating protein (GAP) domain. This domain has been shown to activate Rap GTP-binding protein (RAP) (Wang et al, 2012). RAP proteins are small GTPases with similarity to Ras which mediate changes to processes such as proliferation, establishment of cell-cell adhesion, integrin mediated adhesion and the establishment of cell polarity (Gloerich and Bos, 2011). B-class plexins, unlike other plexins have a cytoplasmic PDZ interacting domain through which they can interact with Rho guanine nucleotide exchange factors (GEF) 11 and 12 to induce activation of Rho A and Rho C in a manner which requires Erbb2 and phospholipidase C (Swiercz, et al, 2002; Swiercz et al, 2009).

Semaphorin-plexin signalling has been shown to be important for cortical development, with plexin B2−/− mice demonstrating deficits in cortical neuron layering, cell migration and differentiation (Hirschberg et al, 2010). A role for plexin signalling has also been found in adult neurogenesis. Saha and colleagues demonstrated expression of plexin B2 in several cell types of the adult subventricular zone including GFAP+ type b cells. Following injection of BrdU, plexin B2−/− mice had higher numbers of labelled cells in the lateral ventricle than control mice, suggesting a role for semaphorin-plexin signalling in the control of progenitor cell proliferation (Saha et al, 2012).
Super-resolution imaging

For decades, immunofluorescent labelling coupled with confocal fluorescent microscopy has been the default method for visualizing the spatial localisation of tissue and proteins in vitro and in vivo. However, the resolution of such standard light microscopy techniques is limited. For a long time the only alternative for the visualization of very small, sub-cellular structures, has been electron microscopy (EM). EM has a number of limitations however, which reduce its usefulness. A relatively recent development in microscopy is the emergence of a number of techniques that couple the versatility of immunofluorescent approaches with a level of resolution previously only achievable using non-light-based microscopic techniques. As these techniques become more ubiquitous they will deliver new insight into the biology occurring at the subcellular level. I will now give a brief account of these new approaches.

The amount of detail discernible by a microscope is dependent on its maximum resolution. The resolution is the minimum distance at which two points can still be distinguished from one another. It was thought that the maximum resolution achievable by light microscopy is 200 nm. To understand why this limit exists we should consider the formula devised by Ernst Abbe in 1873 for calculating the maximum resolution of a given microscope:

$$d = \frac{\lambda}{2NA}$$

The formula states that maximum distance resolvable by a microscope is dependent on the wavelength of light $\lambda$ divided by twice the numerical aperture of the microscope. The numerical aperture is a property of the objective which
describes its ability to gather and focus light, and has a theoretical maximum of 1.51 for oil immersion lenses. The resolution limit for light microscopy is therefore determined by the minimum wavelength of light used to illuminate the sample. This effectively set a limit on the resolution attainable by light microscopes of no greater that 200 nm.

Recently, a number of methods have emerged that allow light microscopy to break this 200 nm resolution limit, known as super-resolution. These enable experimenters to use fluorescent microscopy to image subcellular structures in a way that would not have been possible before the advent of these techniques.

There are a number of different approaches to produce super-resolution images, and each has its trade-offs in terms of the maximum resolution that can be achieved, ease of the preparation of specimens, cost, etc. In this project, I have mostly made use of two super-resolution techniques. I will give a brief technical description of these now.

*Stimulated emission depletion (STED) microscopy*

STED largely functions in a similar way to confocal microscopy. The difference is that in addition to the LASER used to illuminate stimulate emission from the sample, an additional STED depletion laser is used to project a donut shape around this point of illumination. The purpose of this depletion donut is to decrease the size of this point of illumination in order to improve the resolution of the resulting image. Using this technique, a resolution of around 50 nm can be
achieved, an improvement of around 4-fold over standard confocal microscopy. The advantage of STED microscopy is that it does not require changes to sample preparation protocols, the only caveat is that it functions much better with the use of certain bright and photostable secondary antibodies such as Abberior STAR635P.

**Stochastic optical reconstruction microscopy (STORM)**

STORM relies on the labelling of specimens using dyes that can be made to stochastically blink on and off. A large number of images are then taken sequentially and an algorithm is used to assemble the images into a super-resolved image. This approach achieves even higher resolution than STED, around 20 nm, but is less amenable to imaging thick samples due to the influence of out of focus light. Formerly, this technique required expensive equipment to establish, but recent work on low cost approaches has the potential to make the technique more widely available (Kwakwa et al, 2016). The sample preparation has also become more straightforward; samples labelled with Alexa Fluor 647 and mounted on coverslips with a mix of vectorshield and glycerol are able to produce good results, eliminating the need for complicated buffers and chambered microscope slides (Kwakwa et al, 2016).
Chapter 2 - Identifying signals from the vasculature that regulate quiescence

Introduction

Type b cells extend a process which contacts the vasculature within the SVZ (Shen et al, 2008). This point of contact is known as a vascular “end-foot”, and it is becoming clear that signals transduced by these end-feet play a crucial role in regulating neurogenesis. Indeed, work performed in our lab has shown that removal of membrane bound signals from endothelial cells leads to premature exhaustion of the stem cell pool (Ottone et al, 2014). One important aspect of this interaction is the control of NSC proliferation. Indeed, genetic deletion of ephrin B2 from the vasculature results in an increase in the number of activated type b cells in the SVZ. However, though significant, this increase in activated cells is somewhat modest. Furthermore, we know that when co-cultured with endothelial cells in vitro NSCs undergo a G1 arrest analogous to the quiescence state observed in vivo (Ottone et al. 2014), but we have noticed that when cultured with efnb2−/− endothelial cells this arrest is only partially rescued. Together these findings suggest that there are other contact dependent signals from the vasculature that play a role enforcing quiescence on type b cells. We therefore wanted to ascertain, in an unbiased way, signals expressed by brain endothelial cells that can induce quiescence in NSCs. Once we have the information about which are the important endothelial signals that enforce quiescence, we can begin to answer the important question of how these
signals are transduced within cells and how they interact to converge on the arrest phenotype.
Figure 1. Mass spectroscopy analysis of membrane proteins expressed by endothelial cells. A) Western blot showing enrichment of membrane proteins and de-enrichment of cytosolic proteins following ultracentrifugation of protein lysates harvested from primary endothelial or bEND cells. B) Venn diagram showing the number of proteins identified by mass spec analysis of the membrane enriched fraction. Following manual curation of the list of proteins...
common to both cell types a table was produced containing proteins identified as membrane bound ligands. C) Effect of recombinant ephrin B2 and semaphorin 4C on proliferation of SVZ NSCs. Plates were coated overnight with ephrin B2 or semaphorin 4c conjugated to human fc, NSCs were grown on coated plates for 24 hrs and pulsed for 3 hours with EdU. Following staining, cells were analysed by flow cytometry. D) Sema4C gene expression levels in primary endothelial cells stably transfected with shRNA for Sema4C. Gene expression levels were analysed by QPCR and levels are normalized to control cells transduced with a scrambled shRNA control plasmid. E) Proliferation of NSCs grown overnight in co-culture with primary endothelial cells expressing shRNA for Sema4C or a scrambled control plasmid. Cells were pulsed for 3 hours with EdU, isolated from endothelial cells by selective trypsinization, stained and then analysed by flow cytometry. Error bars depict standard error of the mean. **P ≤ 0.01 paired student’s t test.
Performing a Mass spec screen of endothelial cells to determine membrane bound signals quiescence

We set out to determine what are the quiescence enforcing signals originating from the vasculature. To answer this, we turned to our in vitro system. We know from previous work that when NSCs are cultured together with endothelial cells the NSCs undergo a proliferative arrest. These cells also upregulate hallmarks of quiescent type b cells such as GFAP and Hes5 and downregulate markers of activated NSCs, such EGFR (Ottone et al, 2014). This in vitro system therefore re-capitulates to some extent the in vivo interaction, as it has also been shown that signals from the vasculature suppress proliferation and enforce the expression of stemness genes (Ottone et al 2014). We therefore decided to analyse the expression of signalling proteins in our cultured endothelial cells, with the ultimate goal of determining signals that are relevant to the regulation of quiescence in vivo.

I decided to analyse the expression in two endothelial cells types: primary mouse brain microvascular endothelial cells and a brain endothelial cell line named immortalised mouse cerebral endothelial cells (bEND). The rationale for this approach is that we observe the co-culture growth arrest phenotype in both endothelial cell types, and therefore whatever signals are responsible are likely to be present in both. By comparing the two cell types and looking only at proteins that the cells have in common, we can exclude proteins that are not involved in the induction of quiescence.
To determine the expression of signalling proteins I performed mass spectroscopy (mass spec) analysis. We know that quiescence signals from endothelial cells are dependent on cell-cell contact (Ottone et al, 2014) so we focused our analysis on cell fractions likely to contain such short-range signalling molecules, namely the membrane and secreted fractions.

I began by performing mass spec analysis of proteins present in the membrane fraction of the endothelial cells. I collected material from the cells and enriched for the membrane fraction by ultracentrifugation of the cell lysate. This produces a pellet containing cell membranes with the cytosolic protein remaining in the supernatant fraction. To confirm that this approach is successful in enriching for membrane proteins I performed western blotting for various markers associated with the different subcellular fractions (Figure 1A). Antibodies to the membrane proteins EGFR and Ephb3 showed an enrichment of these proteins in the membrane fraction when compared to a whole cell lysate. Conversely, the supernatant fraction had virtually undetectable levels of these proteins. Antibodies for the cytoplasmic proteins Erk and tubulin show the converse, that these proteins are present in the supernatant fraction but are absent from the membrane fraction.

I then began to analyse the list of proteins identified. For each endothelial cell type we analysed 3 biological replicates, and looked only at proteins that showed at least some expression in all 3 replicates. Figure 1B contains a Venn diagram showing the number of protein hits identified in each cell type. 1701
proteins were identified in the primary endothelial cells and 1647 in the bEND cells with 1583 proteins found in both cell types. After curating this list of common genes for membrane bound signalling molecules we identified 3 families of membrane bound ligand ephrins, semaphorins and notch ligands.

From the family of ephrin ligands we identified ephrin B2. This was consistent with our previous work wherein we demonstrated the importance of ephrin B2 as a quiescence signal from the vasculature (Ottone et al. 2014).

From the Notch family of ligands, we identified jagged 1 and dll4. As mentioned in the introduction, notch activity is important for the maintenance of adult NSCs. We have previously demonstrated through mechanistic work in vitro, that notch activity acts to promotes stemness through induction of stemness genes. In our hands, notch activity had no effect on NSC proliferation, either in response to recombinant Jagged1 or in the context of endothelial co-culture (Ottone et al, 2014).

The final class of ligands we identified were the semaphorins. We identified semaphorin 4C as the only membrane bound semaphorin present in all samples. To test whether semaphorin 4C could induce proliferative arrest of NSCs we adopted the same assay we use for ephrin B2, which is to coat plates with a chimeric recombinant form of ligand, which consists of the ligand fused to the FC portion the human IgG antibody. Figure 1C is an EDU incorporation assay showing the proliferation of primary NSCs isolated from the SVZ cultured
on coatings of either ephrin B2 and semaphorin 4C ligands. Whereas growth on recombinant ephrin B2 induces a significant growth arrest, growth on recombinant semaphorin 4C showed no effect.

In addition to testing the recombinant ligand I used lentiviral shRNA constructs to knock down semaphorin 4C expression in endothelial cells to see if removal of the ligand could rescue the proliferative arrest observed in co-culture. Figure 1D shows that knock down of around 60% was achieved in Semaphorin 4C shRNA expressing endothelial cells when compared to scrambled shRNA expressing cells. However, as can be seen in Figure 1E, co-culture of NSCs with endothelial cells expressing the semaphorin 4C shRNA showed no difference in growth arrest compared to controls. Together these data show that Semaphorin 4C is not involved in proliferative arrest in response to endothelial signals.

We know through use of transwell assays that the growth inhibitory signals from endothelial cells operate at close range (Ottone et al. 2014). However, it is possible that in addition to membrane bound signalling molecules there is a role played by secreted soluble signals acting at short range. It may, for example, only be possible to achieve meaningful concentrations of these secreted ligands in proximity to the secreting cell. We therefore wanted to know if such signals could be playing a role in our system.
Figure 2. Mass spectroscopy analysis of the secretome of endothelial cells.  

**A** Venn diagram showing the number of proteins identified by mass spec analysis of proteins secreted to primary endothelial cells or bEND cells. Cells were grown for 24 hours in a reduced volume of minimal media which was subsequently concentrated by spin filtration. Following manual curation of the list of proteins common to both cell types a table was produced listing proteins identified as secreted signalling molecules not previously shown to be a proliferative signal.  

**B** Testing of factors for their ability to induce arrest in NSCs. Cells were treated with the recombinant protein for 24 hours. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. Proliferation is presented normalized to untreated control. Error bars depict standard error of the mean. **P ≤ 0.01, ***P ≤ 0.001 paired student’s t test.**
Performing a Mass spec screen of the secretome of endothelial cells to determine short range secreted quiescence signals

To analyse the secretome of endothelial cells I switched cells to minimal media without factors for 24 hours. I then harvested the supernatant from the cells, concentrated it by centrifugation through size exclusion columns and submitted the resultant eluent for mass spec analysis. As with membrane protein analysis we compared 3 biological replicates of bEND and primary brain endothelial cells looking for proteins found to be expressed in all samples.

Figure 2A contains a Venn diagram visualisation of the number of proteins identified in each endothelial cell type. 1887 proteins were identified in bEND cells and 3035 were identified in primary endothelial cells. We identified 1150 proteins common to both cell types. We then curated this list by looking for secreted signalling molecules expressed in both cell types and excluded signals that had previously been reported in the literature to be proliferative. From this process, we generated a list of 6 secreted ligands to test for the ability to induce a proliferative arrest in NSCs.

To test the factors, I treated NSCs with the recombinant protein in vitro and determined the proliferation by EdU incorporation assays. We tested two concentrations for each ligand, a low concentration, selected based on study of commonly used concentrations in the literature, and a substantially higher concentration to ensure our screen is robust. It can be seen from Figure 2B
that from our panel of tested ligands only BMP4 was capable of inducing growth arrest in NSCs.
Figure 3. Extracellular matrix contains BMPs that enforce quiescence in NSCs. A, B) Analysis of ECM proteins expressed by endothelial proteins. A) The vasculature of the SVZ was stained using antibodies to laminin. B) Table containing a manually curated list of ECM proteins identified by mass spec analysis as being secreted by primary endothelial cells and bEND cells. C) Proliferation of NSCs grown in the presence of ECM components. NSCs were
grown overnight in a 1:4 mix of Matrigel to media. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. Proliferation is presented relative to alone control. D) Effect of 5 days of culture in Matrigel on the expression of NSC marker nestin. Cells were grown 5 days with Matrigel or without, with or without EGF and FGF and stained with an antibody to nestin. E) Expression of integrin alpha 6 in NSCs transfected with siRNA to integrin alpha 6 or scrambled control siRNA. Protein levels were assayed by western blot. F) Proliferation of NSCs transfected with siRNA to integrin alpha 6 or scrambled control siRNA and co-cultured with endothelial cells for 24 hours. Cells were pulsed 3 hrs with EdU, stained and analysed by flow cytometry. G) Effect of 10 ug/ml GOH3 anti integrin alpha 6 antibody or 50 ug/ml RGD peptide on the proliferation of NSCs grown in co-culture with endothelial cells for 24 hours. Cells were pulsed 3 hrs with EdU, stained and analysed by flow cytometry. H) effect of noggin on the proliferation of NSCs grown in the presence of ECM components. Cells were plated alone or in 1:4 mix of Matrigel to media for 24 hrs in the presence or absence of 1ug/ml noggin. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. Error bars depict standard error of the mean. *P ≤ 0.05 paired student’s t test.
BMPs associated with the extracellular matrix cause growth arrest in NSCs

It has been reported that the SVZ vasculature is associated with ECM which is important for the maintenance of type b-vascular contact (Shen et al, 2008) which can be seen when staining the SVZ with an antibody to the ECM protein laminin (Figure 3A). Within our mass spec data for proteins secreted by endothelial cells we also found the expression of a number of ECM proteins (Figure 3B). Interaction with the vascular basement membrane has been shown to be important for control of NSC proliferation. Indeed, use of a blocking antibody for the laminin binding integrin subunit integrin alpha-6 (ITGA6) results in NSCs moving away from the vasculature and an increase in proliferation (Shen et al. 2008). One interpretation of this finding is that by losing contact with the vasculature contact dependent quiescence signals from the endothelial cells are lost, resulting in an increase in proliferation. However, we wondered if signalling through integrins might also be acting directly to constrain proliferation. Indeed, the deletion of the integrin effector integrin-linked kinase (ILK) in NSCs was found to result in an increase in proliferation (Porcheri et al. 2014).

To test this, I cultured NSCs on a semi-solid layer of extracellular matrix protein (Matrigel). Interestingly, cells grown overnight on the Matrigel layer proliferated significantly less than those grown on poly-L-lysine (PLL) as determined by incorporation of EdU (Figure 3C).
To rule out the possibility that growth arrest in the presence of Matrigel is due to cell differentiation I grew cells on Matrigel or control conditions for 5 days and stained for the presence of the stemness marker nestin. Removal of the growth factors EGF and FGF result in cell differentiation and it can be seen that this results in a decrease in the presence of nestin. In comparison, nestin expression in cells grown on Matrigel is indistinguishable from controls (Figure 3D).

It has been reported that endothelial monolayers grown *in vitro* express laminin and that integrin alpha 6 beta 1 (a6b1) is responsible for the interaction between NSCs and the endothelial basal lamina *in vivo* (Shen et al. 2008). Therefore, to test whether signalling through integrins is having an anti-proliferative effect I took several approaches to disrupt integrin ECM interactions in the context of NSC-endothelial co-culture.

First, I used siRNA to knock down (KD) expression of ITGA6 in NSCs. I tested two sets of siRNAs, the use of both together resulted in substantially reduced expression of ITGA6 (Figure 3E). Despite this reduction we found cells KD for ITGA6 arrested to the same degree on co-culture as cells transfected with a scrambled control siRNA (Figure 3F).

To further test for the involvement of integrin signalling in growth arrest I took alternative approach to disrupting integrin function by blocking to ECM integrin interaction directly. Since Matrigel is heterogeneous in terms of its ECM composition, I wanted our approach to be broad, by targeting a number of
integrin receptors. I therefore used a combination of two blocking approaches. The first is the GOH3 anti-integrin-alpha 6 (ITGA6) blocking antibody used by Shen and colleagues (Shen et al, 2008) and the second is RGD peptides, which are tripeptides that are recognized by a number of integrin species, and produce a competitive inhibition that blocks binding to the ECM by alpha V integrins (Weis and Cheresh, 2011). Blocking of alpha V integrins should prevent interaction with certain ECM components not bound by ITGA6 such as fibronectin. Despite this broader approach the combination of both blocking compounds seemed to have no effect on the growth arrest in co-culture (Figure 3G). Having seen no effect on the growth arrest phenotype in Matrigel despite two loss of function approaches, we concluded that integrin signalling is not responsible for this phenotype.

Matrigel contains small amounts of growth factors. Since we had shown that BMP4 is a potent inhibitor of NSC proliferation we wondered if the growth arrest effect we were observing could be due to small amounts of BMPs present in the culture. To test this, I grew cells in Matrigel in the presence of absence of the BMP4 inhibitor noggin. Figure 3G shows that upon addition of noggin the growth arrest observed in cells in Matrigel is completely abolished, showing that even small amounts of BMPs present in Matrigel are sufficient to produce a growth arrest effect.

In conclusion, we set about to test whether signalling through integrins in the context of co-culture was resulting in a proliferative arrest. I used several
approaches to disrupt integrin signalling but found no effect on the observed arrest, suggesting that integrin alpha 6 signalling does not mediate the endothelial induced arrest, and suggesting the increase in proliferation observed by Shen et al when blocking integrin alpha 6 \textit{in vivo} is due to the loss of quiescence signals rather than loss of integrin signalling.
Figure 4. Ephrin B2 and BMP4 are the main endothelial signals that induce quiescence of NSCs in co-culture. A) The effect of noggin on BMP4 signalling. Cells were grown with or without 5ng/ml BMP4 for 24 hrs in the presence or absence of 1ug/ml noggin. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. B) The effect of noggin on NSC co-cultures. NSCs were grown alone or in co-culture with primary endothelial cells with or without 1ug/ml noggin for 24 hrs. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. C) Effect of the combinatorial removal of ephrin B2 and BMP4 signals from endothelial co-culture on the proliferation of NSCs. NSCs were grown overnight alone or in co-culture with efnb2−/− or efnb2+/− primary lung endothelial cells, and alone or in co-culture with efnb2−/− primary lung endothelial cells in with 1ug/ml noggin. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. D) The effect of combinatorial treatment of NSCs with BMP4 and ephrin B2 on proliferation. NSCs were grown for 24 hrs on plates coated overnight with 8ug/ml efnb2-FC or control plates, in the presence or absence of 5ng/ml BMP4. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. Error bars
depict standard error of the mean. *P < 0.05, **P ≤ 0.01, ***P ≤ 0.001 paired student’s t test.
**Ephrin B2 and BMP4 are the main signals from the endothelial cells that induce quiescence**

We identified from our mass spec screen ephrin B2 and BMP4 as proteins expressed by endothelial cells able to induce proliferative arrest of NSCs *in vitro*. We wanted next to confirm that expression of these two proteins from endothelial cells are responsible for the arrest observed in co-culture.

To confirm that BMP4 produced by endothelial cells induces arrest of NSCs, I performed co-culture experiments with primary endothelial cells in the presence or absence of recombinant noggin, a protein that binds and inactivates BMP4. I first confirmed that noggin is able to fully suppress BMP signalling. To do this I treated cells with BMP4, noggin and a combination of BMP4 and noggin (Figure 4A). I used a concentration of BMP of 5 ng/ml, as this generates a robust arrest which is similar in magnitude to that seen when cells are co-cultured with endothelial cells (Figure 4a, Figure 4b), and is therefore likely to be physiologically relevant concentration. As previously shown cells treated with BMP4 undergo a growth arrest when compared with untreated cells. It should be noted that noggin treated cells proliferate significantly more, albeit with only a modest, ~1.2-fold increase in proliferation. This suggests NSCs secrete a small amount of BMP4 which suppresses their own proliferation, which is consistent with previous studies suggesting type b cells secrete BMP4 *in vivo* (Lim et al, 2000). For this reason, in other experiments involving treatment with noggin I have normalized to a noggin treated control. When comparing cells treated with noggin to cells treated with both BMP4 and noggin it can be seen that there is
no significant difference, indicating that noggin completely blocks the effect of BMP4 on proliferation of NSCs *in vitro*.

**Figure 4B** shows the effect of addition noggin to co-cultures. NSCs grown in co-cultures in the presence of noggin proliferate significantly more than to control co-cultures. Therefore, BMP4 secreted by endothelial cells does indeed cause arrest of NSCs in co-culture.

To confirm the activity of ephrin B2 in co-cultures I utilized lung endothelial cells KO for ephrin B2. We have previously shown that the phenotype is conserved between brain and lung endothelial cells (Ottone et al, 2014). **Figure 4C** shows the result of culturing NSCs together with efnb2\(^{-/-}\) or efnb2\(^{floxflo}\) lung endothelial cells. It can be seen that the cells cultured on efnb2\(^{-/-}\) endothelial cells proliferate significantly more than the ones cultured on efnb2\(^{floxflo}\) endothelial cells. This confirms that in our system ephrin B2 expressed on endothelial cells causes arrest in NSCs in co-culture.

Ephrin B2 and BMP4 were the only signals tested in our screen that were capable of inducing proliferative arrest in NSCs. If these are indeed the most important quiescence signals produced by endothelial cells, then by removing both of these signals in the context of endothelial co-culture should return proliferation to that of NSCs grown alone. To test this, I added noggin to NSC efnb2\(^{-/-}\) endothelial co-cultures (**Figure 4C**). It can be seen from **Figure 4C** that when noggin is added to co-cultures of efnb2\(^{-/-}\) endothelial cells that levels of
proliferation are not significantly different to that of NSCs alone treated with noggin. This therefore shows that when both ephrin B2 and BMP signals are removed from co-cultures that cells proliferate as they would alone and this suggests that these are the only significant endothelial derived quiescence signals, at least in vitro.

We wanted to investigate whether ephrin B2 and BMP4 signals act additively. By determining whether or not they have a cumulative effect we can infer if they are acting through the same or different pathways. If the pathways overlap, we would expect the cells exposed to both signals together to arrest to a similar extent as those treated with each signal separately. On the other hand, if the pathways are separate we would expect an additive effect, with cells treated with both undergoing a greater arrest than those exposed to the individual ligand. Also, since our hypothesis is that ephrin B2 and BMP4 signals together account for the arrest observed when NSCs are co-cultured with endothelial cells, we need to understand if a combination of the two recombinant signals is sufficient to drive a greater level of growth arrest. Figure 4D shows NSCs treated individually with each ligand, as well as the two ligands together. As expected, cells treated with either BMP4 or ephrin B2 proliferate significantly less than control cells. Cells treated with combination of the two ligands shows a trend of lower proliferation when compared to either of the signals alone. Whilst this effect is not significant, and more repeats of this experiment are needed, the data suggests that these ligands signal, at least in part, non-redundantly.
Discussion

Our aim was to perform an unbiased analysis of the proteins expressed by endothelial cells, and in so doing to identify all signals from the vasculature that might play a role in the control of NSC quiescence.

I used mass spec analysis of endothelial cells in vitro to identify membrane bound ligands and secreted ligands, to test for their ability to induce arrest in NSCs. From the candidates tested I identified two proteins, ephrin B2 and BMP4, the recombinant forms of which are capable of inducing arrest. I then showed through loss of function experiments that these proteins act in the context of co-culture to constrain NSC proliferation. These findings are in agreement with the literature. Indeed, we have previously shown that loss of ephrin B2 from the endothelial cells in vivo causes an increase in the proportion of activated NSCs (Ottone, 2014). BMPs have been shown to regulate quiescence in sub-granular zone NSCs, (Mira et al, 2010) and in the SVZ noggin has been shown to antagonize BMPs secreted by NSCs to regulate quiescence (Lim et al, 2000). In vitro BMPs produced by endothelial cells have been shown to result in arrest of SVZ derived NSCs (Mathieu et al, 2008). Building on these observations, our work has shown that these are likely to be the only important quiescence signals from endothelial cells. We know this because firstly, we could identify no other factors expressed by endothelial cells that were capable of inducing NSC arrest and secondly, combinatorial removal of both signals from co-cultures returns proliferation to control levels. With this knowledge of what
the key quiescence signals are we can proceed to ask questions about how these signals are transduced within the cell.

Our finding that NSCs cultured in the presence of ECM components underwent proliferative arrest raised the possibility that signalling through integrins could be sufficient to induce arrest. This would have been interesting given that integrins are traditionally thought to provide a pro-proliferative signal (Kim et al, 2011). Ultimately, we found that noggin treatment of these cultures ablated the growth arrest. This highlights the potency of BMP compounds as inducers of arrest in NSCs and is consistent with what has been shown regarding the ability of ECM to bind to growth factors and form signalling “fractones” (Mercier, 2016). Indeed, it has been shown that BMP4 introduced into the SVZ by intracranial injection binds to fractones and affects NSC proliferation (Mercier and Douet, 2014). The vasculature in the SVZ has been found to be rich in ECM components, however whether endothelial cells are a source of BMP4 or if these fractones play a role in the presentation of endogenously produced BMP4 has not yet been addressed. If it is the case that fractones bind BMP4 produced by endothelial cells this could prove to be an important means by which vasculature contact enforces quiescence.
Chapter 3 - Ephrin2 and BMP4 act through p53 to induce arrest

Introduction

Up to this point our focus in trying to understand the quiescence signalling between endothelial cells and NSCs has been on determining what are the important signals expressed by the endothelial cells. We next wanted to turn our attention to achieving a better understanding of how endothelial quiescence signals are transduced within NSCs, and how downstream changes to gene expression result in the arrest phenotype. I therefore took the approach of exposing NSCs to endothelial signals, either through co-culture or by use of the recombinant protein, and assaying gene expression changes in the cells at the RNA or protein level.
Figure 5. Co-culture induces p53 activity to constrain proliferation of NSCs. A) List of upstream regulators of gene expression changes in NSCs in response to endothelial co-culture. NSCs were grown for 48hrs alone or in co-culture with endothelial cells. The cells were isolated by selective trypsinization, RNA isolated and RNA seq performed. In total 17,414 genes were studied. A list of differentially expressed genes was generated comparing cells alone and in co-culture.

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<th>Top upstream regulators</th>
<th>p-value of overlap</th>
<th>Predicted activation</th>
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<tr>
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B) p53 activity

C) Proliferation

D) p53 gene expression

E) Western Blot
co-culture using a value of $P < 0.05$ using a Wald test adjusted for multiple comparisons (6998 genes), this list was submitted for IPA analysis. The table displays the top 4 hits for which there was a predicted activation direction. **B)** p53 activity in NSCs in response to endothelial co-culture. NSCs were transfected with a p53 reporter luciferase construct and grown alone or in co-culture with endothelial cells for 24 hrs. Cells were harvested and counted, luciferase activity was determined by luciferase assay and the value normalized to viable cell number. **C)** Effect of the removal of p53 on the proliferation of NSCs in co-culture with endothelial cells. p53$^{flox/flox}$ NSCs were infected with an adenovirus expressing Cre recombinase or a control adenovirus. Following complete recombination of p53, cells were co-cultured for 48 hrs with primary endothelial cells. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. p53 flox and null co-cultures are normalized to their respective controls. **D)** Levels of p53 gene expression in NSCs in co-culture with endothelial cells. NSCs were grown for 48 hrs alone or in co-culture with primary endothelial cells. RNA was isolated from NSCs and assayed by QPCR. **E)** p53 protein levels in cells grown alone or in co-culture with endothelial cells. p53$^{-/-}$ or p53$^{flox/flox}$ were grown for 48 hrs alone, and p53$^{flox/flox}$ cells were grown for 48 hrs alone or in co-culture with primary endothelial cells. Protein was isolated and levels were assayed by western blot. All Error bars depict standard error of the mean. *$P \leq 0.05$, **$P \leq 0.01$ paired student’s t test.
**p53 induces arrest in response to endothelial signals**

I began by performing RNA sequencing (RNA seq) to identify changes in transcription between NSCs alone or in co-culture with endothelial cells. To do this I set up triplicate co-cultures using primary brain endothelial cells and NSCs isolated from the SVZ. After 48 hours of co-culture I selectively harvested the NSCs and isolated RNA for RNA seq analysis.

RNA libraries were created with the help of the LMS genomics facility and Sanjay Khadayate, of the LMS bioinformatics facility helped in the analysis of the RNA sequencing data. For the comparison of NSCs grown alone versus NSCs grown in co-culture with endothelial cells we studied a total of 17,414 genes. From this we generated a list of differentially expressed (DE) genes between the two conditions and selecting genes \( P < 0.05 \) using a Wald test adjusting for multiple comparisons (6998 genes). To interrogated which pathways might be involved co-ordinating these gene expression changes I used ingenuity pathway analysis (IPA) software to analyse the DE gene list. **Figure 5A** shows a list of the top 4 ‘top upstream regulators’ as identified by the software, which is generated by comparing the direction of transcriptional changes in the DE gene set with the IPA database. The table displays the most significant ‘upstream regulators’ ranked by \( P \)-value, excluding those that do not have a predicted direction, i.e. are not predicted to be either overall activated or inhibited. Interestingly, I found p53 to be the top hit, with a \( P \)-value of \( 1.23 \times 10^{-44} \) for the overlap between the
DE genes in our data set, and those predicted by the program. The program also predicts that p53 is activated in NSCs in co-culture compared to alone.

To confirm that p53 is activated in NSCs in co-culture I performed a reporter assay using a construct containing a p53 binding element driving expression of firefly luciferase (El-Deiry et al. 1993). Figure 5B shows that cells in co-culture have around two-fold higher p53 activity than those cultured alone.

To determine whether this increase in p53 activity in co-cultures acts to restrict proliferation of the cells I isolated NSCs from the SVZ of p53\(^{\text{flox/flox}}\) mice. I then infected the cells with adenovirus expressing Cre recombinase (Cre) or a control virus. Expression of Cre in p53\(^{\text{fl/fl}}\) cells results in loss of p53, whereas p53\(^{\text{fl/fl}}\) infected with the control virus retain expression of p53. I took cells null for p53 and control cells and performed EdU incorporation assays of the cells alone and in co-culture. Figure 5C shows that cells null for p53 arrest significantly less in co-culture relative to flox control cells. Therefore, p53 null NSCs do not respond as much to quiescence signals from endothelial cells, suggesting under normal conditions p53 acts to restrict the proliferation of cells in response to endothelial derived quiescence signals.

We wondered if expression of p53 could be increasing in response to endothelial co-culture and this could result in the increase in activity. We noted that our RNA seq data showed that in fact p53 levels decrease. To confirm that this is the case I performed gene expression (Figure 5D) and protein analysis
(Figure 5E) in of p53 levels in NSCs grown alone or in co-culture with endothelial cells. Indeed, surprisingly, p53 slightly decrease in co-culture.
Figure 6. Ephrin B2 and BMP4 signal through p53 to induce arrest. A) Effect of the removal of p53 on the proliferation of NSCs in response to ephrin B2 and BMP4. p53<sup>flox/flox</sup> NSCs were infected with an adenovirus expressing Cre recombinase or a control adenovirus. Following complete recombination of p53, cells were grown for 24hrs on plates coated with 8ug/ml efnb2-FC or control plates, with BMP4 or untreated. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. Flox and KO cells are normalized to their respective alone control.

B) Effect of the removal of p53 and BMP4 signalling the proliferation of NSCs in response to endothelial co-culture. p53<sup>flox/flox</sup> NSCs were infected with an adenovirus expressing Cre recombinase or a control adenovirus. Following complete recombination of p53, cells were grown for 48 hrs on alone or in co-culture with primary endothelial cells in the presence or absence of 1ug/ml noggin. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. Flox and KO cells are normalized to their respective alone control. All error bars depict standard error of the mean. *P ≤ 0.05, **P ≤ 0.01 paired student’s t test.
Ephrin B2 and BMP4 signal through p53 to induce arrest

We next wanted to know if ephrin B2 or BMP4 could be signalling through p53 to induce quiescence. To do this, I used cells null for p53 (as previously described) and treated with recombinant ephrin B2 or BMP4. Interestingly, whereas control cells arrest when grown on plates coated with ephrin B2, cells null for p53 show no arrest (Figure 6A), suggesting p53 is essential for arrest in response to ephrin B2.

Next, I tested the proliferation of p53 null cells in response to BMP4. It can be seen from Figure 6A that p53 null cells treated with BMP4 also demonstrate significantly increased proliferation compared to flox control cells, indicating that BMP4 is also acting through p53 to induce arrest. However, in contrast to their response to ephrin B2, p53 null cells treated with BMP4 still undergo proliferative arrest, suggesting BMP4 may have p53 dependent and independent mechanisms of action. This means BMP4 acts only partially through p53.

As the arrest in response to ephrin B2 seems to be completely dependent on p53, whereas BMP4 seems to show p53 dependent and independent mechanisms, I wanted to see if removal of p53 and blockade of BMP4 signalling together would be sufficient to return proliferation to control levels. Indeed, p53 null cells treated with noggin show levels of proliferation indistinguishable from cells alone (Figure 6B).
Figure 7 Investigating potential downstream mediators of the p53 induced proliferative arrest, p21 and cyclin D1. A) List of upstream regulators of gene expression changes in NSCs p53\(^{-/}\) or p53\(^{flox/flox}\) in response to endothelial co-culture. p53\(^{flox/flox}\) NSCs were infected with an adenovirus expressing Cre recombinase or a control adenovirus. Following complete recombination of p53, cells were grown for 48 hrs on alone or in co-culture with

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B) p21 gene expression

C) Proliferation

D) Cyclin D1 gene expression

E) Cyclin D1 gene expression

F) Cyclin D1 gene expression (RNA seq)

G) Cyclin D1 gene expression (Q-PCR)
primary endothelial cells. The cells were isolated RNA seq performed. A list of differentially expressed genes was generated comparing p53\textsuperscript{flox/flox} cells in co-culture and p53\textsuperscript{−/−} cells in co-culture, but excluding those differentially expressed when comparing p53\textsuperscript{flox/flox} cells alone and p53\textsuperscript{−/−} cells alone, using a P < 0.05 cut off. This was then submitted for IPA analysis. The list is the top upstream regulators as ranked by P-value, excluding those without a predicted direction.

**B** Levels of p21 gene expression in response to endothelial co-culture. Cells were grown alone or in co-culture with primary endothelial cells for 48 hrs. Gene expression analysed by QPCR. **C** Effect of loss of p21 on the proliferative arrest of NSCs in response to endothelial cells. NSCs p21\textsuperscript{flox/flox} or p21\textsuperscript{−/−} were grown alone or in co-culture with primary endothelial cells for 48 hrs. Cells were pulsed for 3 hours with EdU, stained and analysed by flow cytometry. **D-E** Expression of cyclin D1 in response to ephrin B2, BMP4 and endothelial co-culture in the presence of noggin. NSCs were grown for 24hrs on plates coated D) with 8ug/ml ephrin B2 or control plates, E) or in the presence or absence of BMP4 and in co-culture in the presence or absence of noggin. Gene expression analysed by QPCR. **F-G** Expression of cyclin D1 in p53\textsuperscript{flox/flox} or p53\textsuperscript{−/−} cells in response to endothelial co-culture. NSCs were infected with an adenovirus expressing Cre recombinase or a control adenovirus. Following complete recombination of p53, cells were grown for 48 hrs alone or in co-culture with primary endothelial cells. Gene expression was analysed by **F** RNA seq and **G** Q-PCR Error bars depict standard error of the mean. *P < 0.05, **P ≤ 0.01, paired student’s t test. RNA seq P value obtained using the Wald test which tests for significance of coefficients in a Negative Binomial GLM, using previously calculated size factors and dispersion estimates.
Investigating potential downstream mediators of the p53 induced proliferative arrest: p21 and cyclin D1

To try and determine how p53 is inducing quiescence in NSCs in response to endothelial signals I performed an RNA seq on p53^{flox/flox} and p53^{−/−} NSCs alone and in co-culture. By doing this we hoped to identify p53 dependent gene changes that might be responsible for the growth arrest.

For the analysis, we created a list of DE transcripts between p53^{flox/flox} and p53^{−/−} co-cultures, but excluded from this list genes that were significantly DE between p53^{flox/flox} and p53^{−/−} cells grown alone (see Figure 7A for Venn diagram). The rationale behind this is that we wanted to identify p53 dependent changes that occur in response to endothelial signals but not changes that occur simply due to the loss of p53 itself. I then ran this list through IPA analysis. Figure 7A shows a table of the top upstream regulators as identified by this analysis, these are the top 3 hits as ranked by P-value for the predicted overlap, excluding those that do not have a predicted direction, i.e. are not predicted to be either overall activated or inhibited. We looked further into these proteins, I will discuss each in turn.

Cyclin dependent kinase inhibitor 1A (CDKN1A or p21) is a very well-known effector of p53 dependent cell cycle arrest, and is classically induced by p53 in response to insults such as DNA damage (El-Deiry et al. 1993). Although not traditionally thought of as a transcription factor, p21 has been shown to regulate the transcription of a number of genes such as BMP2 and SOX2 (Porlan et al,
We therefore wanted to check whether the arrest mediated by p53 is being affected by p21.

To do this I first checked the expression of p21 in NSCs on co-culture. If p21 indeed mediating endothelial quiescence signals its levels should increase. **Figure 7B** shows levels of p21 mRNA and protein in co-culture vs alone. Levels of p21 do not seem to increase, in fact they slightly decrease.

To confirm that p21 is playing no role in the arrest seen in co-culture I isolated SVZ NSCs from the brains of p21\(^{-/-}\) or p21\(^{\text{flox/flox}}\) control mice and performed co-culture experiments. I found that indeed, after 48 hours of co-culture there is no difference in the arrest of cells KO for p21 versus flox control cells (**Figure 7C**). Therefore, although the analysis of upstream regulators of p53 dependent gene changes in co-culture suggest that p21 is driving transcriptional changes, analysis of its effect on proliferation suggests that it does not mediate growth arrest in the context of endothelial co-culture.

CCND1 or cyclin D1 is an important regulator of the G1/S transition. It binds to CDK4/6 creating a complex that is able to phosphorylate Retinoblastoma protein, facilitating a cascade of events that pushes the cell to progress to S-phase. Cyclin expression is therefore necessary for the progression of a cell through the cell cycle. We have previously shown that co-culture with endothelial cells causes a decrease in cyclin D1 in NSCs resulting in arrest, and that this results at least in part from ephrin B2 signals (Ottone et al, 2014).
Indeed, I confirmed by Q-PCR that in my hands cells plated on recombinant ephrin B2 have decreased expression of cyclin D1 mRNA (Figure 7D). I wanted to ask if BMP4 also mediates it’s arrest through decrease in cyclin D1 levels. To do this I treated cells with 5ng/ml BMP4 and performed co-culture experiments in the presence or absence of noggin and looked at cyclin D1 gene expression levels by QPCR. Figure 7E shows that cells treated with BMP4 have significantly decreased levels of cyclin D1 compared with cells alone. This decrease in cyclin D1 can also be seen with cells in co-culture, but when co-cultures are treated with noggin there is a significant increase in cyclin D1 expression. These data suggest that BMP4 produced from endothelial cells in co-culture causes a decrease in cyclin D1 expression.

I next looked at whether cells KO for p53 showed differences in cyclin D1 expression. Figure 7F shows expression data from the RNA seq, it shows that although there is a slight trend towards increased cyclin D1 expression in KO cells in co-culture when compared to floxed cells, this is not significant. To confirm this finding, I set up co-cultures with NSCs flox or KO for p53 and looked at cyclin D1 gene expression, this time by QPCR. Figure 7G shows that, in agreement with the RNA seq data cyclin D1 levels drop both in flox and KO cells in co-culture and there is no significant difference between them. Therefore, cyclin D1 expression decreases in co-culture and in response to both ephrin B2 and BMP4, but this decrease does not seem to be p53 dependent.
Figure 8. Changes to the expression of EGFR in response to p53<sup>flox/flox</sup> or p53<sup>−/−</sup> cells in response to endothelial co-culture. A) NSCs were infected with an adenovirus expressing Cre recombinase or a control adenovirus. Following complete recombination of p53, cells were grown for 48 hrs alone or in co-culture with primary endothelial cells. Gene expression was analysed by RNA seq. Error bars depict standard error of the mean. P value was obtained.
using the Wald test which tests for significance of coefficients in a Negative Binomial GLM, using previously calculated size factors and dispersion estimates. B) EGFR protein levels in $p53^{flox/flox}$ or $p53^{-/-}$ cells in response to endothelial co-culture. $p53^{flox/flox}$ or $p53^{-/-}$ cells were grown for 48 hrs alone or in co-culture with primary endothelial cells. Protein levels were assayed by western blot. C) EGFR protein levels in response to treatment with BMP4. NSCs were grown for 48 hrs in the presence or absence of 5ng/ml BMP4 and protein levels assayed by western blot. D) Levels of EGFR protein in NSCs in co-culture with endothelial cells in the presence or absence of noggin. NSCs were grown for 48 hours alone or in co-culture with primary endothelial cells in the presence or absence of 1ug/ml noggin. Protein levels were assayed by western blot. E) Effect of levels of EGFR protein of removal of p53 in the presence of BMP4. $p53^{flox/flox}$ or $p53^{-/-}$ cells were grown for 48hrs in the presence or absence of 5ng/ml BMP4. Protein levels were assayed by western blot.
Downstream of p53: EGFR

We were interested to see ERBB2 in the list of top upstream regulators in our IPA analysis (Figure 7A). ERBB2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. ERBB2 itself is thought to be an orphan receptor, but it is known to play a role in signal transduction by dimerising with other EGFR family members, such as EGFR (Olayioye, 2001). EGFR is known to be an important marker for SVZ NSC activation (Codega et al, 2014). We therefore wondered if there could be changes to the signalling by the EGFR family in response to endothelial signalling that are dependent on p53.

We noticed that our RNA seq showed that gene expression of EGFR is significantly reduced in co-culture compared to cells alone (Figure 8A). To confirm this at the protein level I performed co-cultures with flox or p53 KO NSCs and looked at EGFR expression levels by western blot. As can be seen in Figure 8B floxed cells in co-culture have decreased levels of EGFR protein compared to cells alone, showing that endothelial signals downregulate EGFR expression in NSCs. Interestingly, p53 KO cells alone show increased levels of EGFR when compared to flox cells, suggesting p53 may play a role in the regulation of EGFR expression. Furthermore, p53 KO cells seem not to downregulate their levels of EGFR to the same extent as flox cells on co-culture. Increased levels of EGFR in cells lacking p53 could be an explanation for the increased proliferation observed upon p53 removal from these cells, and the fact that they do not downregulate their EGFR in co-culture could also explain the fact that cells lacking p53 show less arrest in co-culture when compared to flox
cells. However, we require more repeats of this experiment to perform an accurate quantification of the degree of downregulation of EGFR that occurs in p53 cells, with respect to its own control.

Previous work has shown that NSCs treated with BMP4 enter a quiescent state with decreased expression of transcripts associated with an activated state, such as EGFR (Martynoga et al, 2013). We therefore wanted to know if in our system BMP4 could be causing a decrease in expression of EGFR. We found that, indeed, cells treated with BMP4 show decreased levels of EGFR protein compared to cells alone (Figure 8C).

To check whether BMP4 secreted by endothelial cells is responsible for the downregulation in EGFR levels as a result of co-culture I performed co-culture experiments in the presence or absence of noggin. As can be seen in Figure 8D, co-cultures treated with noggin show increased levels of EGFR when compared to co-cultures without, supporting the idea that BMP4 from endothelial cells causes arrest through a decrease in EGFR expression.

Furthermore, I wanted to ask how the expression of EGFR changes in p53 KO cells in response to treatment with BMP4. From Figure 8E it can be seen that cells lacking p53 do not seem to downregulate EGFR to the same extent as flox cells, and levels of EGFR in KO cells treated with BMP4 are comparable to flox cells alone. This suggests that BMP4 could be acting in a p53 dependent
manner to supress levels of EGFR. Again, this requires further work to provide a quantification of this observation.
Figure 9 Prospective model for the p53 dependent control of NSC quiescence in response to endothelial signals. Ephrin B2 and BMP4 signals from endothelial cells cause arrest in NSCs. EphrinB2 causes arrest through p53, while BMP4 has p53 dependent and independent mechanisms of action. p53 may act to suppress proliferation through inhibiting expression of EGFR (adapted from Ottone and Parrinello, 2015).
Discussion

By performing RNA seq of NSCs in co-culture and analysing the gene expression changes our aim was to identify key upstream regulators of the growth arrest phenotype. We were interested to see that the top predicted upstream regulator as identified by IPA analysis is p53. I then confirmed using p53 reporter assays and p53 loss of function that indeed p53 does play a role in this growth arrest.

It is also interesting to note, that although activity levels of p53 increase in co-culture, that levels of p53 protein do not. However, this is consistent with the literature. Although not much is known about how the choice between the growth arrest and apoptotic function of p53 is made in cells, it has been noted that high levels of p53 protein tend to correlate with apoptotic effects, and lower levels with growth arrest (Ko et al., 1996). We would hypothesise that p53 is undergoing some post-translational modification that results in increased activity, for example phosphorylation. Such modifications are known to be important for regulating p53 function, however as yet we have no evidence to support this.

p53 has been shown to be expressed in GFAP expressing NSCs in the SVZ, and loss of p53 increases proliferation in the SVZ and proliferation of SVZ derived NSCs in vitro (Meletis et al. 2006). When Mathieu and colleagues co-cultured p53 null NSCs with endothelial cells they found that the proportion of Ki67 positive cells increased, which suggests a role for p53 in the transduction
of endothelial quiescence signals (Mathieu et al, 2008). In this study, they also used BMP inhibitor noggin to show that endothelial BMPs produce a proliferative arrest of co-cultured NSCs. However, what is not clear is whether p53 is playing a role in the transduction of the BMP signal, or whether other quiescence signals from endothelial cells are transduced by p53.

Through p53 loss of function experiments we have shown that both ephrin B2 and BMP4 signal through p53 to induce quiescence. By treating p53 null cells with the recombinant signals we showed that the proliferative arrest of ephrin B2 is completely dependent on p53, and that BMP4 seems to induce arrest in a p53 dependent and independent manner. This suggests that ephrin B2 and BMP4 share a common effector in p53, with BMP4 acting through an additional pathway that does not include p53. This is consistent with our finding that the combinatorial treatment of cells with ephrin B2 and BMP4 results in an additive effect.

Next, we turned our attention to pathways downstream of p53. We chose to look at gene expression changes that were specific to co-cultured cells, that were lost upon removal of p53. The rationale being that these changes are likely to be induced by endothelial signals and also p53 dependent. The list of genes was analysed using IPA software to identify upstream regulators, focusing on the most significant hits.

We noted the presence of the well-known p53 effector CDKN1A (p21). The role
typically associated with p21 is that of a cyclin dependent kinase inhibitor, induced by p53 in response to DNA damage, which binds to CDK/cyclin complexes inhibiting progression through the cell cycle (Waldman et al, 1995). p21 has been shown and to regulate the expression of BMP2 to control neural stem cell maintenance and self-renewal in vivo (Porlan et al, 2013). We tested whether p21 could be responsible for the arrest in response to endothelial signals through loss of function and found that it was not. Therefore, the changes in gene transcription noted by the IPA analysis are either falsely attributed to p21, or are due to p21 but do not have an impact on the proliferative arrest we observe in co-culture. Since p21 is traditionally associated with mediating growth arrest in response to DNA damage, it was not unexpected to find that a different pathway is involved in the response to endothelial signals, which in vivo act in the homeostatic regulation of type b cell proliferation. Indeed, p21 independent mechanisms of p53 mediated growth arrest are suggested by the fact that p53 mutants have been generated incapable of inducing growth arrest in spite of strong induction of p21 (Beckerman et al, 2016). However, these p21 independent mechanisms remain relatively unexplored.

Cyclin D1 plays a role in binding to CDK proteins to drive cells through the G1/S transition. As with p21, cyclin D1 is not classically thought of as a mediator of gene expression changes but such a role has been described (Liu et al, 2006; Casimiro et al, 2012). We knew from previous work that co-culture with endothelial cells and treatment with recombinant ephrin B2 causes a fall in cyclin
D1 levels, resulting in growth arrest (Ottone et al, 2014). I confirmed these findings, and showed that BMP4 treatment also decreases cyclin D1 levels, whilst in noggin treated co-cultures cyclin D1 levels are partially rescued. Taken together this suggests that ephrin B2 and BMP4 signals from endothelial cells are both able to suppress levels of cyclin D1. Interestingly, cells null for p53 showed the same decrease in cyclin D1 levels as flox control cells in co-culture. Since we know the growth arrest in response to ephrin B2 is both completely dependent on p53 and requires suppression of cyclin D1 this seems contradictory. It should be noted that the prediction made by the IPA software is based on a prediction of gene changes associated with a particular protein. As mentioned, it has been reported that cyclin D1 can act as a transcriptional co-repressor, it is therefore possible that p53 alters the activity or expression of other co-transcriptional regulators that then impinge on the expression of cyclin D1 associated genes. In this way, expression of genes associated with cyclin D1 could change, without necessitating changes to cyclin D1 levels. Therefore, ephrin B2 signalling suppresses cyclin D1 levels and p53 loss does not, but p53 may alter cyclin D1 activity in a manner independent of levels of cyclin D1 mRNA. It is also worth bearing in mind that the co-culture context is more complex than treatment with the recombinant ligand alone. In co-culture, quiescence signals have their effect in the context of other signals that have the potential to alter NSC behaviour. Therefore, although it is useful to compare treatment with the individual signals to cells in co-culture, it is worth considering that there may be differences in how downstream pathways operate within the two paradigms.
It could be that the observation that p53 null cells arrest less in response to endothelial signals is not due to inherent differences in the intracellular effectors that mediate arrest, but in the expression of transducers of the endothelial signals. We were therefore interested to see in the list of IPA hits the RTK Erbb2. Erbb2 is a member of the EGFR family, and since EGFR is known to be important marker of activated type b cells, and a transducer of mitogenic EGF signals, we looked at EGFR expression in the context of p53 loss. The finding that p53 loss increases the expression of EGFR is interesting. There is indication from work in other systems that p53 is able to regulate EGFR transcription (Ludes-Meyers et al, 1996) and chromatin immunoprecipitation (ChIP) data has shown that p53 binds to p53 response elements on the EGFR promoter (Vaughan et al, 2016). Since signalling through EGFR is so important for driving proliferation of progenitors both in vitro and in vivo (Doetsch et al, 2002), it is tempting to speculate that the increased proliferation of p53 null cells due to increased signalling through EGFR as a result of higher levels of EGFR expression, but pending future gain and loss of function experiments we cannot be certain.

We can therefore put forward a prospective model (Figure 9), wherein ephrin B2 causes arrest in a manner dependent on p53 and BMP4 in a manner which has p53 dependent and independent aspects. We can also suggest that p53 loss may cause an increase in proliferation due to increases in EGFR expression, and that BMP4 may suppress EGFR in a manner dependent on p53.
Chapter 4 - Investigating the role of p53 in vivo

Introduction

Having shown the importance of p53 in controlling the quiescence of NSCs in response to signals from endothelial cells in vitro, we wanted to determine whether p53 also plays this role in vivo. There are two studies of which I am aware that have directly addressed the question of how p53 loss effects neurogenesis in the adult subventricular zone. There are several things to bear in mind regarding these studies, the first being that they resulted in contradictory conclusions. The second is that they all involve constitutive p53 knock out models whereby p53 is lost throughout development. Since there is evidence that p53 plays a role in embryonic neurogenesis (Liu et al., 2013), we cannot be sure what the effect of a lack of p53 during development has on the make-up of the adult SVZ niche.

The study by Meletis and colleagues uses a global constitutive p53 KO mouse model. It shows an increase in proliferating cells in the SVZ using BrdU and PH-3 labelling (Meletis et al, 2006). What is not clear is what the identity of these proliferating cells is, i.e. type b, tybe c or type a. The authors follow up by showing an increase in the proliferation of SVZ neurospheres derived from these mice, but as these cells are thought to be exclusively activated type b or type c like cells, we cannot know what the effect on the quiescent population is.
In the second study, Perotin and colleagues use a similar model to Meletis and colleagues and also observe an increase in the BrdU incorporating cells in the adult SVZ of p53 null mice compared to controls (Perotin et al., 2006). Ultrastructural analysis used to identify the progenitor cell composition of the SVZ in these mice showed an increase in type a and type b cells in the KO animals. Assaying of proliferation of the different cell types by [3H]Thy+ labelling followed by ultrastructural characterisation revealed a significant increase in type c, but not type b or type a cells in the KO animals. Long term labelling experiments used to identify the slow dividing population seemed to suggest a trend toward increased slow dividing cells in the KO SVZ, but this difference was not statistically significant.

From these studies, it can be seen that the question of whether loss of p53 causes an activation of the quiescent SVZ NSC population i.e. whether it is important for transducing endothelial quiescence signals in vivo has not been adequately addressed. We therefore wanted to address this question, using a stem cell specific promoter that is temporally inducible in order to ablate p53 specifically in the stem cell compartment in an animal with an SVZ that has developed normally, without loss of p53.

**p53 is active in radial cells of the SVZ**

Expression of p53 has been previously shown by immunostaining (Meletis et al., 2005). However, staining of the protein in the adult SVZ in normal tissue i.e. non-irradiated has not been widely repeated. Indeed, I was unable to repeat this
staining even using the same antibody as used in this paper. The reason for this is probably that under homeostatic conditions p53 protein levels are maintained at very low levels (Kubbutat et al, 1997). I therefore decided to design an approach whereby I directly visualize p53 activity, avoiding the problem of low levels of protein expression and having the added advantage of telling us whether or not p53 is active in these cells, something which cannot be concluded from visualization of the protein alone. To do this I sub-cloned the p53 responsive element from a p53 reporter luciferase plasmid (El-Deiry et al, 1993), into a lentiviral expression construct such that it drives the expression of GFP in cells. The p53 binding element is PG13-CAT, and was described by Kern et al (Kern et al, 1992) (Figure 10A).
Figure 10. p53 is active in radial cells of the SVZ. A) Schematic for the p53 GFP reporter plasmid. A p53 binding element was sub-cloned from a p53 luciferase reporter (El-Deiry et al., 1993) into a lentiviral plasmid driving expression of GFP. B) p53 activity in the SVZ of mice electroporated with p53 GFP reporter. Mice aged postnatal day 3 were electroporated with a mixture of p53 GFP reporter and tomato CMV plasmids. Mice were collected at 8 weeks and brain sections taken and imaged by confocal microscopy. Representative images of 11 radial cells and 7 cells with astrocytic morphology across 2 brains.
To test the construct, I first infected p53 flox NSCs in vitro. I found that the construct does indeed express in cells in vitro and following recombination of the floxed p53 allele by infection with an adenoviral Cre construct, I was pleased to observe that cells lost their GFP expression. Thus, I created a lentiviral construct whereby GFP expression reports on the activity of p53.

To determine activity of p53 in vivo I introduced the construct by electroporation into the ventricle of adult mice. As a control to determine which cells received the construct I co-transfected a plasmid encoding tomato under the constitutive CMV promoter. Figure 10B shows representative images of transfected cells. Cells lining the ventricle which appeared radial in morphology expressed the p53 reporter construct, conversely cells that had a highly arborized morphology characteristic of an astrocyte expressed the tomato control plasmid, but did not express the p53 reporter construct, consistent with the pattern of p53 protein expression in the SVZ reported previously (Meletis et al, 2006). These observations were based on study of 11 radial cells and 7 cells with astrocytic morphology across 2 brains. This observation is therefore preliminary, and further experiments are required for quantification and statistical analysis.

Establishing an inducible p53 loss system

In setting up an inducible p53 knock out model I encountered several technical hurdles. I will briefly describe the approaches we took, and the difficulties encountered.
Figure 11 Failure of GFAPCreERT2 to recombine p53^{flox/flox}. A) Protocol for recombination of p53^{flox/flox} or p53^{flox/wt} GFAPCreERT2 tomato mice with tamoxifen. B) Images of tissue from GFAPCreERT2 p53^{flox/flox} tomato mice administered with tamoxifen. Tissue was stained with antibodies to GFAP, CD31 and Ki67. C) p53 staining in NSCs isolated from the SVZ of p53^{flox/flox} GFAPCreERT2 tomato mice administered with tamoxifen. Cells were isolated as neurospheres, expanded and plated on laminin coated coverslips for immunostaining. Cells were stained with antibodies for p53.
A GFAP promoter driven CreER system fails to recombine p53\(^{\text{flox/flox}}\)

To generate a line in which p53 can be recombined in type b cells of the SVZ we crossed p53\(^{\text{flox/flox}}\) mice to mice with a transgene encoding a tamoxifen inducible Cre recombinase which is driven by glial fibrillary acid protein (GFAP). To enable us to visualize which cells have induced Cre expression we crossed these this line to mice expressing a tomato fluorescent reporter that is induced following Cre expression. From here these mice will be referred to as p53\(^{\text{flox/flox}}\) GFAPCreERT2 tomato.

To induce recombination, I administered 80mg/kg tamoxifen by intraperitoneal injection for 5 days (Figure 11A). To confirm that GFAP expressing NSCs were indeed undergoing recombination I stained sections containing the lateral ventricles for markers including GFAP (Figure 11B). We noted that radial GFAP expressing cells were positive for tomato expression following tamoxifen administration, indicating that NSCs of the SVZ were inducing Cre expression in response to the administration of tamoxifen. In Figure 11B it be seen that recombination also occurs in astrocytes, identifiable by their highly arborized morphology and GFAP expression. We also observed the labelling of ependymal cells, these can be identified by their location at the ventricular surface, flattened morphology and possession of multiple cilia. The recombination of non-NSC cell types has been reported in other systems where a type b cell promoter has been used to target Cre expression in NSCs, for example GLAST\(^{\text{CreERT2}}\) (Calzolari et al, 2015).
We next wanted to check that in addition to switching on the expression of the tomato gene cells have also recombined their floxed p53 allele. As previously mentioned I was unable to stain for p53 in tissue, therefore I took the approach of performing preparations of the SVZ and staining the cells *in vitro* (Figure 11A). As the staining of p53 works well *in vitro*, we can effectively ascertain the levels of p53 protein *ex vivo*. We were surprised to see that cells p53<sup>flox/flox</sup> that were tomato positive did not lose their p53 expression (Figure 11C).

We hypothesised that the tomato gene lies in an area of the genome more accessible to Cre recombinase. I therefore sought to increase the levels of Cre by optimizing the tamoxifen induction. To do this we sought optimize the administration of tamoxifen. Working together with another PhD student in the lab, Holly Simpson-Ragdale we worked together to test a number of administration protocols, assessing each time the recombination of p53 in NSCs taken from the SVZ. We tried increasing the dose of tamoxifen administered to 100mg/kg, as well as administering tamoxifen by oral gavage, which has been reported to increase the efficiency of tamoxifen dosage. In addition to this we tried the active metabolite of tamoxifen 4-OH tamoxifen, which we hypothesised might result in a greater availability of the active form of tamoxifen to cells in the brain, as it does not require prior metabolic conversion. Finally, we administered tamoxifen and 4-OH tamoxifen to pups aged p10-p14, with the rationale that their blood brain barrier might be more accessible to the tamoxifen. Despite these attempts we were not successful in inducing the loss of p53 with this system.
Viral delivery of Cre recombinase into adult mice

As an alternative to the use of a tamoxifen inducible system, and with the hope that it would induce higher levels of Cre expression in cells, we decided to use a viral system to induce the recombination of p53. To do this I introduced a lentivirus into the lateral ventricle by stereotactic injection. The lentivirus was generated in the lab and contains the Hes5 promoter, another NSC specific promoter, driving the expression of Cre. The intra-ventricular injection of lentivirus posed another problem, that intraventricular injection into the adult brain does not effectively label NSCs. Once I had stained sections of the lentivirus infected mice it became clear that the majority of cells labelled are ependymal cells, with very few to no radial GFAP expressing cells labelled. This was unexpected, given that it has been shown that NSCs extend a primary cilium into the ventricular space and should therefore come into contact with virus in the CSF, however study of the literature showed that this is consistent with what has been previously found (Consiglio et al, 2004).

One alternative to intraventricular injection would have been to inject directly into the SVZ. However, given that we are looking at the role of p53 in control of proliferation, and that injection into the SVZ would inevitably induce an injury response concomitant with increased basal proliferation, we sought an alternative model.
Figure 12. Effect of the acute removal of p53 on the progenitor composition of the SVZ. A) Schematic for the administration of Hes5Cre lentivirus in p53^{flox/flox} or p53^{flox/wt} tomato mice. B) Recombination of neurospheres derived from Hes5Cre injected mice. Two weeks following injection the SVZ of p53^{flox/flox} mice were taken and neurospheres isolated. Following expansion, neurospheres were stained for p53 C) and the number of
p53+ tomato+ cells counted. Error bars depict the standard deviation for 5 fields of view. **D)** Images of p53^{flox/flox} tomato mice injected with Hes5Cre lentivirus and stained with antibodies for GLAST and Ki67. **E)** Quantification of the progenitor make up in p53^{flox/flox} or p53^{flox/wt} tomato mice injected with Hes5Cre lentivirus. Error bars depict standard error of the mean for 3 mice.
Viral delivery of Cre recombinase into postnatal mice

Certain transduction approaches such as electroporation are known to work much more efficiently in the developing than the adult brain (cf. Barnabé-Heider, et al, 2008; Feliciano et al, 2013). Given the difficulty in inducing p53 recombination in type b cells of the adult mice we wanted to know if performing intraventricular injections in postnatal mice would be more successful. I injected mice either p53^{floxed/floxed} or p53^{floxed/wt} tomato at age p3 with lentiviral Hes5Cre. Two weeks post injection I collected the SVZ of p53^{floxed/floxed} mice and isolated NSCs as neurospheres. Following expansion of the cells I stained for p53 (Figure 12A). I was pleased to see that recombination of p53 in the tomato was very high, ~95% (Figure 12 B/C). I then proceeded to collect brains for analysis. I stained sections with antibodies to type b marker GLAST, and marker of proliferation Ki67 (Figure 12D). I then used confocal microscopy to take representative images across the rostrocaudal extent of the SVZ, imaging at two dorsolateral positions along the lateral ventricle. I counted the number of recombined quiescent type b (tomato+, GLAST+, Ki67-), activated type b (tomato+, GLAST+, Ki67+) and type c/type a (tomato+, GLAST-, Ki67+). No difference was found in the number of type b or progenitor cells between p53^{floxed/floxed} or p53^{floxed/wt} mice (Figure 12E).

Discussion

It is clear from literature is that loss of p53 increases proliferation in the SVZ. What is less clear is whether p53 loss causes increased proliferation of type b cells. What is potentially confounding about previous studies is that all of the
knock-out models which have been studied are constitutive (Meletis et al, 2004; Gil-Perotin et al, 2014; Wang et al, 2013), and we know from previous work that changes that lead to exit of type b cells from the quiescent state lead to a depletion of the quiescent type b pool over time (Ottone et al, 2014). For this reason, we chose to establish an inducible knock out system. We firstly confirmed using a p53 reporter that there is activity in the radial cells of the SVZ. This is consistent with the observation by Meletis and colleagues that p53 protein is expressed in GFAP positive cells of the SVZ (Meletis et al, 2004), but since we know from our work in vitro that p53 activity increases independent of increases in p53 protein expression (Figure 5), a reporter for p53 activity is a useful tool. We next went about establishing our knock out system. Our first approach was to use tamoxifen injection into a GFAPCreER tomato line to recombine floxed p53 and a tomato reporter in type b cells. We were disappointed to observe that although the tomato allele recombined efficiently, the floxed p53 allele did not. Indeed, difficulty in recombining p53 with inducible tamoxifen systems, particularly in the brain, has been reported elsewhere (Vooijs, et al 2001). We therefore decided to use a lentiviral approach, which we hoped would drive greater levels of Cre expression and successfully recombine the floxed p53 allele. We were pleased to see that in postnatal mice this approach results in successful recombination of p53 (Figure 12B/C). However, we did not observe an increase in the number of GLAST-, Ki67+ cells that would indicate an increase in type b cell activation, nor an increase in GLAST– Ki67+ type c/type a cells. This is unexpected, given an increase in proliferation in the SVZ has been previously reported in p53 KO mice (Meletis et al, 2006; Gil-
Perotin et al, 2006). However, we have not tried to perform the lentiviral Hes5Cre injection in mice that are p53<sup>wt/wt</sup>. It is possible that there is already an increase in the levels of type b cell activation in our p53<sup>wt/het</sup> control mice. There may therefore not be a great difference in the phenotype between het and homozygous p53 flox mice following recombination. Indeed, p53 haploinsufficiency has been reported (Teoh et al, 2014). We are currently performing experiments to test this, by comparing p53<sup>wt/het</sup> and p53<sup>wt/het</sup> mice.
Chapter 5 - Imaging type b cell-endothelial cell contacts

Introduction

Given the importance of the signals provided by direct vascular contact in maintaining the pool of SVZ NSCs, we feel it is important to understand the nature of these contacts. Whilst it is clear from previous work is that at some point following activation, type b cells lose their radial morphology and differentiate into progeny that lack vascular contacts (Shen et al, 2008), it is not clear at what point in the lineage this loss of contact occurs. Since we know that contact dependent signals from the vasculature provide signals that supress NSC activation (Ottone et al, 2014), it follows that the nature of this contact is altered upon activation. Since a thorough characterisation of the vascular contacts made by activated NSCs in the SVZ is lacking, this question remains unresolved. We can conceive of three possible ways in which the contact is altered upon NSC activation. The first possibility is that the contact is lost by the activated cell. The second is that the contact remains, but that some more subtle change to the morphology of the contact occurs such that the signalling from the endothelial cells is diminished. Lastly, that the contact remains in place, but that changes to the NSC, for example receptor expression, result in alterations to the way in which the signals are transduced.

We wanted to answer two questions. The first is whether or not the contact with the vasculature is maintained in activated NSCs. The second is whether there
are subtle changes to the morphology of this contact. The latter will require high-resolution imaging. I therefore set out to do two things: one, establish an approach to effectively image vascular contacts and two, produce the tools required to perform high resolution images of these contacts.

**Developing an approach to image vascular contacts at standard resolution**

The most widely used marker for SVZ NSCs is GFAP. It is useful because it is expressed in both quiescent and activated NSCs but not in their progeny. GFAP is component of the radial process but it does effectively label cell bodies. To enable us to visualize the entire cell, including the entirety of the cell’s end-foot, we decided to use a mouse strain where the NSCs produce a fluorescent reporter in their cytoplasm. We chose tomato for its brightness. In order to specifically label NSCs I crossed a mouse line in which tomato is activated following the expression of Cre, to a line where a tamoxifen inducible Cre is under the expression of the GFAP promoter (GFAPCreER tomato).

To label the cells I perform a 5-day tamoxifen administration protocol by intraperitoneal injection. **Figure 13A** shows a representative image of NSCs labelled by expression of tomato following tamoxifen administration. **Figure 13B** is a 3-dimensional reconstruction of a series of z-slices obtained by confocal microscopy. In blue, we can visualize the vasculature with the endothelial marker CD31. In green the tomato expressing cell is seen, with an end-foot process that is wrapped around the blood vessel and extends back towards the ventricle.
where the cell body can clearly be seen. This system therefore allows us to label radial NSCs in a way that allows us to visualize both the end-foot process and the cell body.

Following recombination, I stained sections for GFAP to confirm the cells are indeed NSCs, CD31 to label the vasculature and Ki67 which labels proliferating cells and has been shown to be present in activated NSCs (Codega et al, 2014). **Figure 13A** shows a representative image of sections from a tamoxifen injected mouse.

Therefore, I have established a system involving expression of a fluorescent reporter in type b cells, together with immunostaining that allows analysis of the full cell morphology of activated and quiescent type b cells. Activated type b cells make up a small proportion of the total type b population, and due to the need to image a large number of cells in order to build up a representative picture of the differences between the activated and quiescent type b populations, it was not possible to perform such an analysis for inclusion in this thesis. However, this would be interesting to explore in future work.
Figure 13 Development of an approach to image vascular contacts in standard resolution. A) Images of vascular contacts in GFAPCreERT2 tomato mice recombined with tamoxifen. Sections were stained with antibodies to GFAP and CD31. B) 3D reconstruction of NSCs vascular contact in GFAPCreERT2 tomato mice recombined with tamoxifen.
Establishment of super-resolution imaging techniques

We want to be able to discern very subtle changes that may occur to the morphology of the end foot contact, as the cells become activated. To this end I have developed tools to enable the imaging of proteins using super-resolution techniques.

STORM imaging of cytoskeletal proteins in vitro

Since changes in cell shape are mediated in large part through alterations to the cell cytoskeleton any examination of subtle changes to the end-foot should involve imaging of cytoskeletal components. To this end we began a collaboration with the Photonics Group at Imperial College. Together with them we helped develop a low-cost STORM approach which also utilizes a previously-described simplified labelling protocol which makes sample preparation easier (Kwakwa et al, 2016).

Figure 14A shows endothelial cells stained in vitro for the cytoskeletal fibre tubulin. On the left is the summed image, and on the right the super-resolved image. It is clear from the images the increase in resolution that STORM produces, enabling us to discern interactions between individual fibres of the microtubule network.
**STED imaging of the actin cytoskeleton in vitro and in vivo**

We decided that due to its confocal modality, in contrast to STORM, which operates in wide-field, that STED would be a better technique to employ for imaging tissue. I therefore began to develop a STED imaging approach, beginning *in vitro*. Figure 14B shows *in vitro* staining of the filamentous actin using STAR635P conjugated phalloidin in primary NSCs. Again, there is a comparison in the left and right images. On the left is a normal confocal image and the right is the super-resolved image with the STED depletion LASER active.

We then moved on to imaging *in vivo*. As a proof of principle for confirm that we are able to image the type b cell radial process in tissue we decided to label the cell with GFAP. Figure 14C shows the radial process of a SVZ NSC in green, making contact with a blood vessel labelled with laminin, in red. Above is the standard confocal image and below is the super resolved STED depleted image. The resolution improvement is as apparent *in vivo* as *in vitro*. In the super-resolution image the process can be seen to be wrapped around the blood vessel, with more detail of the individual intermediate filament strands visible.
Figure 14 Development of super-resolution imaging techniques. A) STORM imaging of tubulin in endothelial cells. Primary endothelial cells were grown on coverslips and stained with antibodies for β-tubulin. Imaging was performed using a custom STORM set up, see methods section for details. B) STED imaging of filamentous actin in NSCs. Cells were grown on coverslips and stained with phalloidin conjugated to Abberior STAR635P. Imaging was performed using a custom STED set up, see methods section for details. C) STED imaging of vascular contacts ex-vivo. Brain tissue sections were stained with primary antibodies to laminin and GFAP, with an Abberior STAR635P secondary antibody used to label GFAP. STED imaging was performed with a Leica TCS SP8 STED 3X' system. Images are maximum projections of a confocal stack. D) Labelling of filamentous actin in cells stably expressing SNAP and CLIP tagged-LifeAct using cell permeable dyes in 3D collagen cultures. Cells were stably transfectected with lentiviral CLIP conjugated LifeAct. Cells were then plated in 3D collagen cultures and stained using CLIP505 dye.
Counterstaining was performed with conjugated phalloidin. Imaging was performed by confocal microscopy.
Producing versatile constructs to tag cytoskeletal proteins

The caveat with using super-resolution techniques is that they require the use of fluorescent dyes. This is because the brightness and photostability of the fluorescent molecule is important, and therefore fluorescent proteins are usually not suitable for this purpose. We wanted to generate tagged constructs that would allow us to label cytoskeletal components with a variety of fluorescent dyes. I cloned the peptide LifeAct, which binds to filamentous actin (F-actin), fused to SNAP and CLIP tags. These are short peptide sequences that when coupled with a tagged dye results in the rapid and specific covalent labelling of the tagged protein. There are several advantages to the use of SNAP tagging over the use of fluorescent fusions coupled with antibody labelling, but perhaps the greatest, is the availability of membrane permeable tagged dyes. This is demonstrated in Figure 14D where NSCs, lentivirally transduced with CLIP or SNAP tagged LifeAct, were plated in 3-dimensional collagen cultures. Membrane permeable CLIP505 dye was added to label the cells. The cultures were then fixed and stained with conjugated phalloidin. It can be seen that the labelling of the F-actin by the LifeAct matches very well to the labelling produced by the phalloidin. This construct therefore allows the labelling of actin in living cells using fluorescent dyes that are amenable to super-resolution imaging. The use of these next generation tags opens up new imaging possibilities. Indeed, Lukinavicius and colleagues used this approach to perform super-resolution imaging ex vivo, introducing SNAP tags into animals by electroporation and then staining and imaging living tissue (Lukinavicius et al, 2013).
Discussion

Compared to the developing nervous systems the neural stem cells of the adult SVZ remain relatively under-characterised. For example, how the position of the radial glia cell changes within the neurogenic zone over time, the division mode of the cell i.e. symmetric versus asymmetric and what happens to radial glial basal contacts have all been investigated in the developing brain (Götz, and Huttner, 2005). In contrast, these elements of type b cell behaviour remain unclear. It is not known, for example, at what point during lineage progression type b cells lose their basal contact. Part of the difficulty in studying these elements of the adult neural stem cell behaviour has been the lack of good markers for differentiating between quiescent and activated type b cells, as well as the relative scarcity of proliferating stem cells in the adult SVZ as compared to quiescent stem cells and their proliferating progeny. Given the important role played by contact dependent signalling regulating the progression from quiescence to activation in type b cells (Ottone et al, 2014) we decided it was important to understand more about the nature of this contact, and how it might potentially change. I went about developing an approach to visualize type b cell contacts with the goal of better characterising potential differences in the end-foot contact between quiescent and activated cells.

I generated a mouse line allowing the permanent labelling of cells with the bright fluorescent protein tomato, using the best characterised marker for SVZ cells GFAP. This approach has the advantage that the reporter will be equally well expressed in quiescent stem cells and their activated progeny. This is in contrast
to the GFAP-GFP system in which activated cells downregulate levels of GFAP and therefore GFP (Dulken et al, 2017, Figure 4), making visualization of small structures such as cell processes difficult. However, since cells are permanently labelled the tomato positive population is also made up of type c and type a progenitors, thus requiring post-staining for GFAP. The advantage of this genetic approach to staining with GFAP directly is that since the tomato reporter is expressed cytoplasmically, the entire cell is labelled, including the cell body and the entire end-foot (Figure 13B). This is in contrast to labelling using an anti-GFAP antibody, which due to the fact GFAP is a cytoskeletal element, mostly labels the cell process and not the cell body or the entire end-foot. Activated stem cells were then marked by labelling with antibodies to Ki67, a protein known to label actively cycling type b cells (Codega et al, 2014). The proportion of cycling type b cells is known to be low (Morshead et al, 1994). It is also likely that transition states exist on the path from quiescence to activation; this has already been shown on the transcriptional level (Llorens-Bobadilla et al, 2015). Therefore, imaging enough activated type b cells to gain a representative picture of their cell morphology is challenging. Although it was not possible to include such an analysis in this thesis, we hope future work will yield a better understanding of the role of the end-foot contact in the transition from quiescence to activation.

As has been mentioned, contact between type b cells and the vasculature is refractive to the process of activation. Yet, type b cells eventually activate and progress through their lineage. It must therefore be that their interaction with the
endothelial cells changes. This change could take the form of a complete loss of contact, or could be more subtle, such as a change in the morphology or molecular composition of the end-foot, such that signal transduction is altered. In tandem with the above approach of trying to determine whether or not activated type b cells retain their vascular contact, we wanted to develop tools to probe potential changes to the structure of the end foot.

I began by developing a STED based imaging approach, at first developing the labelling strategy in vitro. Although STED imaging can be performed with routinely-used fluorophores such as Alex Fluor 647, a new generation of dyes developed for use in super-resolution imaging are considered a better choice. Indeed, I could produce good results with the STAR635 and STAR635P dyes produced by Abberior. I next performed STED imaging in tissue sections using an anti-GFAP antibody and STAR635P secondary as a proof of principle. I could produce a marked increase in resolution and improved detail in the cell projection imaging on a commercial Leica TCS SP8 STED 3X' system. We hope to use this approach to perform ex vivo imaging of sub-cellular structures in the future.

I also worked to produce an in vitro STORM imaging approach. This approach utilizes a low-cost STORM set up and a previously-described, simplified labelling to reduce cost and make sample preparation much easier (Kwakwa et al, 2016). STORM imaging offers greatly increased resolution over standard confocal microscopy (around 10-fold) and superior resolution to STED approaches.
(around 2-fold). Such an imaging approach could in theory be used ex vivo, but due to the wide field nature of STORM it is not feasible to image deep into tissue, making alternative methods preferable for imaging tissue. We therefore intend to use STORM imaging in vitro when greatly improved resolution is desirable, such as studying receptor-ligand interactions or receptor clustering.

Finally, I developed tools to genetically express tagged versions of cytoskeletal elements that could be used to perform imaging with fluorescent dyes. Labelling of genetically-encoded proteins is usually performed with genetically-encoded, conjugated fluorescent proteins such as GFP. The disadvantage of these fluorescent proteins is that they have poor spectral properties, i.e. they are dim and easily bleached compared to fluorescent dyes. This becomes an even greater problem if sub-cellular resolution is required, as super-resolution approaches involve the use of powerful lasers that make imaging with fluorescent proteins next to impossible. A better approach therefore, is to manufacture genetically encoded proteins that are conjugated to tags that can be combined with appropriately conjugated dyes. SNAP and CLIP tags offer rapid and highly specific labelling, are smaller than conventional fluorescent proteins and can be combined with a variety of conjugated dyes, making them highly versatile. Membrane permeable dye conjugates also exist, making live and ex vivo super-resolution imaging possible (Lukinavicius et al, 2013) As a proof of principle I created SNAP and CLIP conjugated LifeAct, which is a short peptide that binds to filamentous actin (Wombacher and Cornish, 2011). I sub-cloned this fusion construct into a lentiviral vector, which can be used to create stably
expressing cell lines, or introduced in vivo. I used a 3-dimensional collagen-based in vitro system to test the suitability of using cell permeable dyes to label actin using these lentiviral SNAP/CLIP LifeAct constructs in tissue. Co-staining with phalloidin showed that the labelling of F-actin worked very well, and we hope to utilize such an approach in the future. For example, these lentiviral vectors could be used to infect cells of the SVZ by stereotactic injection. ex vivo tissue sections could then be stained using cell permeable SNAP dyes, allowing visualization of cell processes in living tissue. If an infusion of cytosine-β-D-arabinofuranoside (Ara-C) on to the cortex was used to eliminate cycling progenitors prior to collection of the tissue (Doetsch et al, 1999), then it would be possible to image in real time how vascular contacts change as cells become activated.
Chapter 6 - Summary and future directions

Summary

I sought to better understand how quiescence signals from the vasculature cause arrest of SVZ NSCs. I began by looking in an unbiased way at the expression of signalling molecules produced by endothelial cells and asked which of those we identified were capable of causing arrest. I identified BMP4 and ephrin B2 as the molecules able to cause arrest. This finding is consistent with the literature (Ottone et al, 2014; Lim et al, 2000). I found that ablating both signals from endothelial cells completely rescued the proliferative arrest in the context of NSC endothelial co-culture, from this we concluded that the two signals identified are the main signals responsible for arrest, at least *in vitro*.

I next looked at how these signals are transduced in NSCs. I identified p53 as an important mediator of proliferative arrest in response to endothelial signals, and showed that it plays a role in mediating arrest in response to both ephrin B2 and BMP4. My analysis of the gene expression of p53−/− NSCs suggested that p53 may exert control of proliferation by regulating levels of EGFR.

Finally, I wished to better understand the nature of the contacts made between type b cells and endothelial cell *in vivo*, and how they might change as the stem cells activate. To this end I established a number of imaging models and techniques. I set up a tomato reporter model to enable imaging of cell contacts, the analysis of which is still on-going. I also established approaches that allow both *in vitro* and *in vivo* imaging of structures at super-resolution.
Significance and future directions

There are a multitude of niche signals that contribute to the regulation of type b cells in the SVZ. It seems that a balance of pro and anti-proliferative signals is necessary to ensure balanced levels of cell division. This is typified by the vascular niche, which provides both soluble proliferative (Shen et al, 2004), and contact dependent anti-proliferative signals (Ottone et al, 2014). The loss of signals, for example removal of contact dependent quiescence signals from endothelial cells, disrupts this balance, producing aberrant proliferation and eventual depletion of type b cells (Ottone et al, 2014). Since control of proliferation involves the influence of multiple, often conflicting signals, understanding the complete complement of signals that type b cells are exposed to, as well as how these signals integrate within the cells, is crucial for an understanding of how this proliferative balance is achieved.

Probing the mechanisms of this balance is important not only for our understanding of the biology of adult neurogenesis, but also useful if we wish to utilize neural stem cells for their therapeutic potential. SVZ progenitor proliferation is known to increase following cortical injury (Ramaswamy et al, 2005) and some have suggested that neurons derived from the SVZ that have honed to the site of injury can even differentiate and integrate into the existing circuitry (Kreuzberg et al, 2010). Gaining a better understanding of this potential endogenous repair process is clearly the first step to facilitating improvements to recovery following brain injury, but understanding the homeostatic mechanisms
of proliferation control, and how those are altered following injury is also crucial. For example, if it is possible to augment the integration of SVZ derived cells into the injured area to improve recovery as some have been attempting (Okano et al, 2007), it may then be desirable to further remove proliferative brakes from SVZ stem cells in order to transiently increase the supply of cells to the injured site. Clearly much more needs to be understood about how cells derived from the SVZ can help or hinder the response to injury under a number of different injury contexts, but the prospect of utilizing endogenous stem cell populations to potentiate the regenerative response to injury is an exciting one.

To identify the quiescence signalling molecules expressed by endothelial cells, I performed mass spec analysis of the membrane and secreted cellular fractions of brain endothelial cells. This data could be a useful resource for identifying other endothelial factors that play a role in controlling other aspects of type b behaviour. For example, PEDF released by endothelial cells promotes stem cell self-renewal (Ramírez-Castillejo, et al 2006) and SDF1 from the vasculature has been shown to play an important role in migration of progenitors out of the SVZ (Kokovay et al, 2001). Both of these proteins show up in our mass spec analysis. It could therefore be that within the list of signalling molecules we have identified, that there are some with important yet undiscovered functions in neurogenesis. It would be interesting to screen them in vitro, looking at their effects in different aspects of NSC behaviour, such as cell motility or self-renewal capacity.
From my screen, we identified ephrin B2 and BMP4 as the proteins that are capable of inducing arrest. Both ephrin B2 and BMP4 have been previously implicated in the enforcement of quiescence in NSCs (Ottone et al, 2014, Mathieu et al, 2008). But, it was interesting to discover that, at least in the in vitro co-culture context, removal of both signals rescues proliferation to control levels. It would be tempting to speculate that these are therefore likely to be the most important quiescence signals in vivo, but this approach has limitations that must be considered. Firstly, SVZ NSCs cultured in vitro are known to be different from those found in vivo. This is highlighted by a recent study comparing SVZ NSCs freshly isolated using FACS approaches to their in vitro counterparts (Dulken et al, 2017). It is therefore possible that cells in vitro may have differences in the expression of receptors of signalling effectors, which alters the way in which they sense and transduce extrinsic signals compared to their in vivo counterparts. Secondly this approach is, by its nature, intentionally reductionist. We know that in vivo SVZ stem cells exist in an environment in which they are supplied with a raft of signals. Many of these signals likely interact in a way that alters their effect or potency. Therefore, just because we have tested a signal for its effect on SVZ NSCs in vitro, and found no effect, does not mean that we can with absolute certainty conclude that it does not play a role in vivo. It could be that the signal requires the presence of a signal derived from another niche component, for its effect.

We identified p53 as an important transducer of quiescence signals from endothelial cells. p53 has been implicated in the control of proliferation of SVZ
NSCs (Meletis et al, 2006, Gill-Perotin et al, 2006). However, what role it plays in signal transduction is less clear. Studies have suggested a role for p53 in the arrest in the context of endothelial co-culture (Mathieu et al, 2008) and in transduction of signals by Ephb3 (Theus et al, 2010). Here we show p53 activity is activated by endothelial co-culture and that this activity is important for the transduction of the ephrin B2 and BMP4 quiescence signals in vitro. The ability of recombinant ephrin B2 to cause arrest is completely ablated following loss of p53. In contrast, the effect of BMP4 is only partially lost, suggesting the existence of p53 dependent and independent mechanisms. Ephrin B2 is known to play a role in controlling type b cell quiescence in vivo (Ottone, et al, 2014). It is therefore possible that p53 is mediating this effect, and this would be interesting to test in vivo. The role of BMP signalling in the SVZ niche seems less clear, with a study suggesting BMPs can suppress proliferation (Lim et al, 2000) and another that BMP signalling does not (Colak et al, 2008). It should be noted with this latter study that a SMAD4 knock out approach was used, not only will this affect TGF signalling in these cells, which also employs SMAD4 as a co-effector, but also ignores the potential effects of SMAD-independent mechanisms of BMP signalling which have been demonstrated (Derynck and Zhang, 2003). Nevertheless, if BMP is an important quiescent signal in vivo, the production of BMP4 by vascular endothelial cells has not yet been demonstrated. An interesting experiment might therefore be to generate an inducible endothelial BMP4 knock out model, which could demonstrate both the importance of endogenously produced BMP4 in the maintenance of type b cell quiescence, and confirm its production by the vascular endothelium.
From my gene expression analysis of p53 null cells both alone and in co-culture I made the interesting observation that p53 seems to be regulating EGFR expression levels in NSCs, which I then confirmed on the protein expression level. That p53 has been implicated in the control of EGFR expression in other systems (Ludes-Meyers et al, 1996; Vaughan et al, 2016) lends weight to our hypothesis that this could be a fundamental mechanism of NSC proliferative control. Future work will involve confirmation of the link between p53 and EGFR, for example by overexpressing EGFR in the context of co-culture to rescue proliferative arrest, and, conversely by knocking down EGFR in p53 null cells to reduce their increased proliferation that is observed.

p53 loss has been shown to increase proliferation of cells in the SVZ (Meletis et al, 2006), however which population is responsible for the increase in proliferation remains unclear. Since our *in vitro* work suggested an important role of p53 in NSC quiescence *in vitro*, we wanted to show the effect of the acute loss of p53 in type b cells *in vivo*. After finding a technique which allowed the efficient acute recombination of the floxed p53 allele, we were surprised to see no effect in the activated type b population when comparing homozygote and heterozygote p53 flox mice. One possible explanation for this negative result is that p53 heterozygote mice are haploinsufficient, a hypothesis, supported by similar observations in other systems (Teoh et al, 2014). This will be tested in future work, in which we will compare mice null for p53 with mice with wild type levels. Certainly, it is surprising that p53 does not play a role in type b quiescence *in vivo*, given that ephrin B2 expressed by endothelial cells had been
shown to be an important enforcer of type b quiescence (Ottonne et al, 2014), and that loss of p53 renders cells completely refractive to the effect of ephrinb2, at least in vitro.

Another aspect of type b cells behaviour that is important to understand is how their morphology and position within the niche changes. Type b cells span the length of the niche, a large number having both apical and basal contacts. They therefore integrate a variety of sometimes conflicting signals along their extent (Fuentealba et al, 2012). If their position changes, it would be expected that the host of signals to which they are exposed will also change. It has been shown for example, that as the cells activate they are found in closer proximity to the vascular plexus (Shen et al, 2008). At first this might seem counter intuitive. After all, we have described a number of quiescence signals that derive from endothelial contact. However, it is known that proliferative signals are also released from the vascular plexus. For example, endothelial cells release factors that promote stem cell self-renewal (Shen et al, 2004) and the blood brain barrier is more permeable in the SVZ plexus allowing access of blood borne factors that may promote proliferation (Tavazoie et al, 2008). If the nature of the endothelial contact, and therefore the quiescence signals to which the type b cells are exposed remains constant, but the position of the cell body changes, it is conceivable that the balance of pro and anti-proliferative signals to which the cells are exposed changes. Thus, potentially favouring a more proliferative phenotype. One alternative is that as the cells activate, the nature of their contact with the vasculature changes. Since even type c cells seem to make
small contacts with the vasculature (Shen et al, 2008) it is probable that rather than a complete loss of contact as cells transition from the quiescent to activated state, that there is a more-subtle alteration in the morphology of the contact which affects the manner or degree to which quiescence signals from the vasculature are transduced within the cell. These potential differences between quiescent and activated type b cell morphology have not been well studied. This is probably due to a combination of poor markers to differentiate quiescent from activated, as well as activated from type c cells, and the fact that the activated type b population is rare, particularly when compared to the total number of proliferating progenitors within the SVZ. The number of studies that have recently been performing single cell RNA seq of the SVZ will likely contribute to a better understanding of which markers can be used to study the different cell types.

The other challenge for any study attempting to define changes in the morphology of activated cells is be able to image a sufficient number of cells to gain a complete picture of what might be a transitionary process. Indeed, single cell studies have further subdivided the quiescent and activated states into early and late stages (Dulken et al, 2017; Llorens-Bobadilla, 2015). It is not clear if the morphology or position within the niche is different as cells transition from early to late stages of quiescence or activation, but it would be interesting to investigate this. The development of new spatial transcriptomics approaches may allow us to link position to transcriptomic heterogeneity (Stahl et al, 2016).
The final chapter of this thesis describes work undertaken to try and answer some of these questions of what morphological and positional changes are characteristic of the transition from quiescence to activation. From the work I have performed imaging known type b markers such as GFAP, it is clear that to image the full morphology of the cell a constitutively expressed marker is needed. This has the advantage of being present everywhere in the cell, and of not being tied to a specific cell state, such as GFAP which is downregulated upon cell activation. The combination of a GFAP-driven tomato reporter together with staining for type b markers to confirm cell identity is quite effective. As has been previously mentioned, imaging sufficient activated cells is a technical challenge to this approach, and for this reason an analysis of cell contacts could not be performed for inclusion in this thesis. However, this would be an interesting avenue for future exploration.

Finally, I have described establishment of a number of super resolution imaging approaches. Such techniques are in their relative infancy, but hold the promise of allowing greater insight into biological systems through the combination of high levels of resolving power with the versatility and ease of use of fluorescent microscopic techniques. Through our collaboration with members of the Photonics group at Imperial College we have helped develop an easy and low-cost approach to perform STORM imaging in vitro (Kwakwa et al, 2016). I have also optimized STED imaging techniques for use both in vitro and in vivo and which we hope to make use of in future projects. Finally, I generated proof-of-principle constructs which allow the tagging of expressed proteins with next-
generation SNAP and CLIP tags. I have shown these tags to be highly versatile allowing the use of cell permeable dyes to label cells in 3-dimensional culture systems, and therefore also have the potential to be utilized ex-vivo, an exciting potential future application.
Chapter 7 - Materials and methods

Cell culture

*Isolation of adherent neural stem cells from the subventricular zone*

For experiments using cells grown in adherent conditions postnatal animals a litter of mice between ages p6 and p15 were used. The technique for isolation was as follows. The brains were removed and placed in dissection media consisting of Hank's buffered salt solution (HBSS) without calcium or magnesium supplemented with 2.76 μM HEPES and 48 μg/ml kanamycin/1μg/ml gentamicin (K/G). A McIlwain tissue chopper was used to section the brain and a thin slice of the lateral wall was removed from each ventricle. This tissue was pooled and digested by incubation in HBSS + 0.25% trypsin and 20μg/ml DNAse for 2 minutes at 37°C. To achieve a single cell suspension the tissue was triturated by pipetting up and down 10 times with a p1000 pipette. The pellet was then washed twice with media consisting of Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Finally, the pellet was re-suspended in DMEM F12 supplemented with 3% FBS, 20 μg/ml epidermal growth factor (EGF) and K/G and plated at a density of 1x10^6 cells/16ml on Poly-L-Lysine in an incubator set to 37°C, 5% CO₂. After 48 hours the cells were fractionated, i.e. non-adherent cells were removed by rinsing the plate several times with media.
Subculture of adherent neural stem cells

Following fractionation cells were cultured in complete media consisting of DMEM F12 supplemented with 0.25% FBS, 20 μg/ml EGF, 10 μg/ml fibroblast growth factor (FGF), bovine pituitary extract, 1x N2 supplement and K/G. When confluency was reached the cells were split using accutase and re-plated on PLL coated plates.

Isolation of subventricular zone neurospheres

The lateral walls of the lateral ventricles of 3 adult mice aged 8-12 weeks were dissected as above. This tissue was then digested by incubation in 2.5ml Earle’s Balanced Salt Solution (EBSS) + 50 units papain and 300 units DNAse for 30 minutes at 37°C. 2.5 ml of EBSS was added and the mixture was triturated by pipetting 10x with a 5ml serological pipettes. The pellet was then resuspended in 7.5 ml of complete media containing DMEM F12 supplemented with 20 μg/ml EGF, 10 μg/ml fibroblast growth factor (FGF), 4ug/ml heparin, 1x B27 supplement and K/G and grown in an incubator set to 37°C, 5% CO₂.

Sub-culture of subventricular zone neurospheres

When neurospheres were large enough to be split they were spun and re-suspended in 200 μl of accutase. Following a 10-minute incubation at room temperature 900 μl of complete media was added and the solution was triturated 10x with a p1000 pipette. The cells were counted and plated at a density of 10 cells/μl.
Culture of primary endothelial cells

C57BL/6 mouse brain microvascular endothelial cells were purchased from Cell Biologics (Cat: c57-6023). Cells were grown on plates coated with attachment factor protein (Thermo Fisher) for 30 minutes at 37°C in endothelial cell growth media (Lonza, cat: CC-3162). When cells reach confluency, they were split using trypsin and re-plated in a 1:3 ratio.

Culture of primary lung endothelial cells

Cells were cultured at 33°C, 5% CO₂ in DMEM GlutaMAX media (Thermo Fisher) supplemented with 10% FBS, 1x non-essential amino acids (Thermo Fisher), 50 U/ml Interferon γ (IFNγ), human vascular endothelial growth factor (hVEGF), and pen/strep on uncoated plates.

Culture of bEND cells

Cells were cultured at 33°C, 5% CO₂ in DMEM GlutaMAX media (Thermo Fisher) supplemented with 10% FBS and K/G on uncoated plates.

Co-culture experiments

To ensure a uniform monolayer of endothelial cells plates were pre-coated coated with 1:100 solution of laminin in plain media for 30 minutes at 37°C. Primary endothelial cells were plated at a density of 20x10³ cells/cm² and left to form a monolayer overnight. The next day NSCs were plated alone or on the monolayer at a density of 33x10³ cells/cm². After 2 days, the NSCs were
removed by selective trypsinization. To do this the media was removed and replaced with trypsin, the monolayer was monitored under a microscope whilst tapping the plate until the point where the NSCs begin to detach but before the monolayer was disrupted, the cells were then collected for the experiment. Where compounds such as noggin were added to the co-cultures they were added following plating of the NSCs and replenished after 24 hours by further addition into the media.

*EdU incorporation assay*

To assay proliferation the cells were incubated with 10 μM EdU for 3 hours in normal culture conditions. The EdU was then labelled using the Click-iT™ EdU Alexa Fluor™ 647 Flow Cytometry Assay Kit (Thermo Fischer) and DNA was stained propidium iodide (PI). First, RNA was digested by resuspending the cell pellet in a solution of 1 mg/ml RNAse A in phosphate buffered saline (PBS) and incubating for 3x 10 minutes at room temperature, vortexing in between. The cells were then washed and re-suspended in PI in PBS at a concentration of 10 μg/ml. The cells were analysed on a LSR II flow cytometer (BD).

*Retroviral and lentiviral infection of cells in vitro*

To produce retrovirus and lentivirus 5x 10⁶ of phoenix or 293T cells were plated respectively/ 10cm plate the day before transfection. Lipofectamine 2000 reagent (Thermo Fisher) was used to transfect the packaging cells. 1.5 ml of Opti-MEM media (Thermo Fisher) was mixed with 30 ul of lipfectamine 2000
reagent. For retroviral infections 10ug of the retroviral plasmid DNA was diluted in 1.5 ml of Opti-MEM, for Lentiviral infection 10ug of the lentiviral plasmid, 7.5 ug of Δ8.9 gag/pol plasmid and 5ug of VSVG plasmid were added to 1.5ml Opti-MEM. The solutions were incubated for 5 minutes at room temperature and then the 1.5ml of the Opti-MEM + lipofectamine solution was added to 1.5 ml of the Opti-MEM containing diluted DNA and incubated at room temperature for 20 minutes. The transfection mixture was added to 7ml of Opti-MEM supplemented with 10% FBS and the cells were incubated overnight. The transfection mix was removed the following morning and 6ml of normal media was added to the cells. After 24 hours, the supernatant was removed and replaced with 6ml of normal media, the supernatant was filtered through a 0.45 µm pre-washed with PBS and supplemented with 0.2mg/ml polybrene. This was added to the cells for 3 hours and then replaced with normal media. This can be repeated for up to three days as necessary to achieve a desired level of transduction. The cells were then selected for using the relevant antibiotic.

**siRNA transfection**

Cells were transfected in 6 celll format. siRNA was diluted in 100 ul of plain media withoutserum or antibiotics. 12 ul of Hiperfect (Qiagen) was added and the solution incubated at room temperature for 10 mins. The transfection mixture was then added drop-wise to cells and cells incubated overnight. The next morning the transfection media is replaced with fresh normal media.
Mass spectroscopy

Sample preparation for mass spectroscopy of membrane proteins

Primary endothelial or bEND cells were grown to confluency in 15 cm plates. The plate was then washed 2x with ice cold PBS and harvested by scraping with 0.1M Na₂CO₃ with protease inhibitors (Sigma, cat: P8340) and phosphatase inhibitors (Sigma, cat: P5726 and P0044). These were snap frozen and stored at -80°C until fractionation. For fractionation, the lysate was adjusted to 1mM MgCl₂ and 125 U of benzonase was added to digest nucleic acid and reduce the viscosity. Following 15 minutes incubation on ice the lysate was sonicated, 3x 30 second pulses were used on a Biorupter sonicator (Diagenode) set to high power. The membrane fraction was then pelleted by spinning at 100,000g for 30 minutes. The supernatant was saved and the pellet was washed in lysis buffer. The spinning and washing steps were repeated a further 2x in order to remove cytosolic contaminants. After the final spin the pellet was resuspened in 6M urea, 2M thiourea.

Sample preparation for mass spec of the secretome

Primary endothelial or bEND cells were grown to confluency in 15 cm plates. Once confluency was reached the cells the media was removed and replaced with 10 ml of basal media without factors or FBS. The next day the supernatant was harvested and concentrated by spinning in a vivaspin 2 3000 MWCO column (GE Healthcare) quantified using Bradford assay and loaded per well on a 4-15% TGX precast gel.
Further processing of the samples and the raw data processing was performed by Alex Montoya, Peter Faull and Holger Kramer of the mass spectrometry facility at the London Institute of Medical Sciences (LMS):

**Sample digestion**

An in-solution digestion procedure was used. Samples were normalized with 400 ul 8M urea in 0.1M Tris-HCL (10mM CaCl2, pH 7.8) to a final urea concentration of at least 6M. A reduction was performed with 10mM dithiothreitol for 30 mins at 40°C, alkylated with 50mM iodoacetamide and diluted with 0.1M Tris-HCl CaCl2, pH7.8) to a final urea concentration of 2M. Samples were digested with 2.5µg of trypsin for 18 hours at 37°C. Finally, samples were acidified with 1% Trifluoroacetic acid and de-salted with a C18 reversed phase stage tip procedure and dried with a centrifugal vacuum drier.

**Peptide concentration assay**

To re-dissolve, 0.1% TFA was added and samples shaken for 30 mins at 1200rpm, followed by sonication with an ultrasonic water bath for 10 mins and centrifugation for 10 mins at 14,000 rpm 5°C. To determine peptide concentrations the Pierce Quantitative Fluorometric Peptide Assay was used according to the manufacturer’s instructions. LC-MS/MS analysis was performed using 1.0 µg of total total digest which was injected on to a trap column (Acclaim PepMap 100 C18, 100 µm x 2 cm) for desalting and concentration at 8 µL/min in 2% acetonitrile, 0.1% TFA. An analytical column
was used to elute peptides (EASY-Spray PepMap C18, 75 µm x 25cm). Peptide separation was performed using a stepped 90-minute gradient of 4-25% buffer B for 60 minutes and 25-65% buffer B for 30 mins (buffer b: 80% acetonitrile, 0.1% formic acid). The eluted peptides were analysed with the LTQ Velos which was operating in positive polarity with the data-dependent acquisition mode. Fragmented ions were determined using an initial MS1 survey scan at 30,000 resolution (m/z: 200), and ion trap collisional induced dissociation for the top 10 most abundant ions. MS1 and MS2 scan AGC targets were set to 1x10^6 and 1x10^4 for maximum injections times of 500 ms and 100 ms respectively. Survey scan m/z range was set to 350-1500 and a normalized collision energy of 35%. Charge state rejection was enabled for +1 ions, the minimum threshold for triggering fragmentation was 500 counts.

Data processing

For data processing MaxQuant software (version 1.5.8.3) was used with database searches performed by the in-built Andromeda search engine versus the Uniprot Mus musculus database (27/06/2016). For protein identification, a reverse decoy approach was used, with a 1% false discovery rate. For the search missed cleavage was set to two, fixed modification of cysteine carbamidomethylation and variable modifications of methionine oxidation, protein N-terminal acetylation and asparagine de-amidation. The label-free quantification was applied, with a minimum LFQ ratio count of 2. A one-minute match time limit, and a twenty-minute alignment time limit was used with the match-between-runs function. Processing of the protein and peptide
identifications and relative quantifications produced with Maxquant were processed using Excel.

**Immunostaining**

*Staining of cells on coverslips*

Cells were fixed for 15 minutes at room temperature with 4% paraformaldehyde (PFA) in PBS. If the antigen was intracellular then the cells were permeabilized by incubation for 10 minutes at room temperature with 0.5% triton x-100 in PBS. The cells were blocked by incubation with 10% animal serum in PBS for at least 30 minutes, with the species of the serum matched to the host species in which the secondary was raised. The primary antibody was diluted in blocking solution and the cells were incubated overnight at 4°C. Following washing the cells were incubated for 30 minutes at room temperature with secondary antibody diluted in blocking solution. To stain the nucleus cells were incubated in a solution of DAPI in PBS for 15 minutes at room temperature and finally mounted in ProLong gold (Thermo Fisher).

*Staining of tissue*

Mice were perfused and the brains left in 4% PFA at 4°C for 2 hours. A vibratome was used to section the brain in 60 μm increments. Sections containing the SVZ were blocked/permeabilized by incubating for 90 minutes at room temperature in a solution contain 10% animal serum and 1% triton x-100 in PBS. The antibody was diluted in 10% animal serum and 0.1% triton x-100 in PBS and the incubation was performed overnight at 4°C. The following day the
sections were washed 3x 10 minutes in 0.1% triton x-100 in PBS and incubated in secondary antibody in blocking solution for 1 hour at room temperature. Following washing a 15 minute incubation with DAPI was used to strain the nuclei and the sections were mounted in ProLong Gold.

**Staining for STORM**

The initial staining was performed as above, samples were then labelled with Alexa Fluor 647 and the coverslips mounted in a mix of 1 part Vectashield to 6 parts Glycerol in 50 mM TRIS pH 8.

**Staining for STED**

The preparation of samples for STED follows the standard staining protocols above but uses the STAR635P secondary antibody (Abberior).

**Imaging**

Unless otherwise stated imaging was performed using an SP5 or SP8 Leica confocal microscope.

**Superresolution imaging**

Details of the imaging set up for the STORM imaging is contained in the paper Kwakwa et al, 2016) and was custom-built as a product of the collaboration with members of the Biophotonics group at Imperial College London.
in vitro STED imaging was performed using a custom STED set up. The set up follows a standard STED set up with the following alterations: The STED LASER is a Ti-Sapphire 780 nm with a pulse duration of 150 fs stretched with a 100 m fibre to 300 ms. The excitation LASER is supercontinuum via microstructured optical fiber from 500 nm to 1000 nm. The donut depletion pattern was produced with SLM. For full details see: [Lenz et al., 2014]. For ex vivo STED imaging an off the shelf Leica TCS SP8 STED 3X' system was used.

**Western blotting**

Cells are collected using RIPA buffer containing Protease inhibitor (Sigma, P8340) and phosphastase (Sigma, P5726 and P0044) inhibitors. For lysis the samples are incubated on ice for 15 minutes and vortexed every 5 minutes. They are then centrifuged at max speed in a table top centrifuge set at 4°C and the supernatant transferred to a fresh tube.

To determine the protein concentration Bradford style quantification was performed using the Biorad Protein Assay Reagent (Biorad), following the standard protocol. Samples were then prepared such that an equal amount of protein was loaded in each well, the amount of which varied depending on the antibody used, but was in the range of 15-50 µg.

Gels were prepared by addition of the reagents listed in Table 1. Gels were run in 1x Tris/glycine/SDS buffer (National Diagnostics) at 80 mV until samples reached the resolving gel, at which point the voltage was increased to 120 mV. For the transfer a PVDF membrane was used with Tris/glycine buffer (National Diagnostics) with 20% methanol (proteins under 100 kDa) or 10% methanol (proteins over 100 kDa) and performed at 4°C for 90 minutes using a constant
current of 300 mA. Membranes were blocked in 5% milk in TBST for 1 hour at room temperature and incubated overnight at 4°C in blocking solution.

The following day the membrane was washed 3 times for 10 minutes in TBST and then incubated for 1 hour in HRP conjugated secondary antibody (GE Healthcare) in blocking solution. Following washing of the membrane Luminata western assay substrate (Merck) added to the membrane and images taken.

Table 1. Reagents for the preparation of western blot gels.

<table>
<thead>
<tr>
<th>Resolving gel</th>
<th>4%</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>2 ml</td>
<td>3 ml</td>
<td>4 ml</td>
<td>5 ml</td>
<td>6 ml</td>
</tr>
<tr>
<td>4x Tris-HCL-SDS pH 8.8</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>APS</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.13 ml</td>
<td>8.13 ml</td>
<td>7.13 ml</td>
<td>6.13 ml</td>
<td>5.13 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>15 ml</td>
<td>(volume for 2x 1.5 mm gels)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stacking Gel</th>
<th>4 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>666 ul</td>
</tr>
<tr>
<td>4x Tris-HCL-SDS pH 6.8</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>APS</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>3.024 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

RNA isolation

For QPCR RNA was extracted using TRI reagent (Sigma) followed by alcohol precipitation using the following protocol:

400 µl of TRI reagent was added to each 35-mm dish of cells and the TRI reagent left for 2 minutes at room temperature. This is collected and 80 µl of chloroform was added, the mixture was vigorously shaken and left for 15 minutes at room temperature. The sample was spun for 15 minutes at 4°C max
speed and the top phase removed and placed in a new tube. 200 µl of isopropanol and 50µg/ml GlycoBlu Coprecipitant (Thermo Fisher) was added to precipitate the RNA and the sample left at room temperature for 10 minutes at room temperature. The sample was then spun at max speed for 15 minutes at 4°C. The supernatant was removed and the pellet washed with 400 µl of 75% ethanol. The sample was spun a final time, the supernatant removed and the pellet air dried before resuspension in 20 µl of RNAse free H₂O.

Reverse transcription

Reverse transcription was performed using the iScript gDNA Clear cDNA Synthesis Kit (Biorad), following the standard protocol.

Quantitative PCR

QPCR reaction mixtures were set up using the following mixture:

Table 1. Reagents for the preparation of QPCR mix.

<table>
<thead>
<tr>
<th></th>
<th>µl</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>8.5</td>
</tr>
<tr>
<td>MESA Blue qPCR MasterMix (Eurogentec)</td>
<td>10</td>
</tr>
<tr>
<td>QPCR primer (10 µM stock)</td>
<td>0.5</td>
</tr>
<tr>
<td>cDNA</td>
<td>1</td>
</tr>
</tbody>
</table>

The threshold cycle (Ct) for each sample was normalized to a house keeping control gene and compared with an internal control sample (ΔΔCt method).
RNA sequencing

For RNA sequencing RNA was isolated using RNeasy mini columns (Qiagen) following the standard protocol. Library preparation and sequencing was performed by the genomics facility at the London Institute of Medical Sciences. Libraries were prepared using the Truseq mRNA stranded kit and quality control checks were performed by Qubit and Bioanalyser analysis. Libraries were then pooled together and run on a MiSeq Nano Flow Cell (V2 reagents) (Single Read 26 cycles) to check the balance of the libraries within the pool, library balance was adjusted where necessary. Clonal clusters of each library were then amplified onto an Illumina Flow Cell using the Illumina cBot system and sequenced on a HiSeq 2500 (v4 chemistry) as a Paired-End 100bp run.
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