Role of Deubiquitylating Enzyme USP9X in Neural Progenitor Fate Determination

Susitha Premarathne

B.BMed.Sci (Hons)

School of Natural Science
Griffith University

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Abstract

During brain development, neural progenitors (NPs) need to balance their self-renewal with differentiation, in order to maintain the NP population while establishing the complex tissue architecture of the brain. NP fate is under the close scrutiny of plethora of fate determination factors, which can be divided into two groups based on their site of origin namely, intrinsic and extrinsic fate determinants. To date, a number of intrinsic factors, such as cell polarity and adhesion, and extrinsic factors including Notch, WNT and mTOR signaling pathways have been shown to regulate NP fate specification. Despite a modest understanding of how individual fate determinant pathways influence NP fate, number of significant and fundamental questions remains to be answered; many of which centre on the integration and regulation of distinct determinant factors. The current study focused on understanding how the posttranslational modification, deubiquitylation, contributes to NP fate determination. Conditional deletion of the deubiquitylating enzyme, Usp9x from mouse NPs results in perinatal lethality and diffused cortical cellular architecture during late embryonic stages signifying its importance in NP fate specification (Stegeman et al., 2013). In light of this previous study, the overarching aim of this project was to identify roles, if any, of Usp9x in neural progenitor fate specification.

Conditional deletion of Usp9x from mouse NPs, using Nestin-Cre donor mice, transiently perturbed the intrinsic fate determinant factors cell adhesion and apical-basal cell polarity in apical progenitors. Usp9x-depleted apical progenitors detached from the ventricular zone and prematurely differentiated into Tbr2⁺ intermediate NPs. Interestingly, perturbed adherens junction and cell polarity proteins were restored to their normal expression levels and pattern at later stages. However β-catenin, which is an Usp9x substrate in some cell contexts, was a notable exception. In contrast to other adhesion and polarity proteins, β-catenin protein levels were elevated in the absence of Usp9x throughout embryonic neural development. Our analysis revealed that Usp9x physically interacts with the β-catenin destruction complex in developing mouse brains. Loss of Usp9x disrupted the composition of the destruction complex functionally impairing the targeting of Serine 33/37/ Threonine 41 phosphorylated β-catenin for ubiquitin mediated degradation. This identified Usp9x as the first deubiquitylating enzyme capable of regulating the destruction complex composition. Accumulated levels of β-catenin activated the Wnt signaling pathway in NPs. In-vitro depletion of Usp9x alone was sufficient to activate Wnt reporter gene transcription cell
autonomously. Concomitantly, increase in Notch signaling was detected in Usp9x-depleted brains. The protein levels of the E3 ubiquitin ligase Itch, a common substrate of Usp9x, and Numb, a well-established Notch antagonist, were significantly reduced in Usp9x-depleted brains. Usp9x and Numb proteins extensively co-localized in NPs and a co-immunoprecipitation analysis confirmed the physical interaction between Usp9x and Numb in embryonic mouse brains. Reflecting the reduced Itch and Numb protein levels, the Notch intracellular domain protein levels were significantly increased in Usp9x-depleted brains corresponding to the increased Notch signaling. Even though both Notch and Wnt signaling pathways were significantly increased in Usp9x-depleted NPs and Usp9x-depleted apical progenitors prematurely differentiated into intermediate NPs, overall neurogenesis and cortical lamination were relatively intact in the cerebral cortices of Usp9x-depleted brains. However, depletion of USP9X from human NP cell line, ReNcell VM, resulted in a cell-cycle arrest at G0/G1 phase. The G0/G1 cell cycle arrest corresponded with reduced mTORC1 signalling as measured by decreased p-S6 expression. Loss of USP9X significantly reduced levels of the canonical mTORC1 complex protein RAPTOR in ReNcell VM cells. Co-immunoprecipitation confirmed a physical interaction between Usp9x and Raptor proteins in embryonic mouse brains. Reduced Raptor and p-S6 protein levels were also observed in E12.5 mouse cortices. Neurospheres generated from E18.5 Usp9x-depleted mouse NPs were reduced in size, similar to Raptor-null neurospheres.

The data presented herein showed that Usp9x is capable of regulating NP fate through multiple intrinsic and extrinsic fate regulatory mechanisms. Importantly, this study highlights the temporal and context specific regulation of NP fate by Usp9x, functionally confirming Usp9x as a stem cell regulator.
Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

___________________________
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Abbreviations

AJ  - Adherens junctions
AP  - Apical progenitors
APC - Adenomatous polyposis coli
aPKC - atypical protein kinase C
bHLH - basic helix-loop-helix
BMP - Bone morphogenetic proteins
BP  - Basal progenitor
BSA - Bovine Serum Albumin
Cas3 - Caspase3
CBF1 - C-promoter binding factor 1
Cdc42 - Cell division control protein 42
cHLH - basic helix-loop-helix
CK1 - casein kinase 1
CP  - Cortical plate
CT  - Cortical tubers
DC  - Destruction complex
Dcx  - Doublecortin
Deptor - DEP domain containing mTOR-interacting protein
DFFRX - Drosophila fat facets related X
dH2O  - Distilled H2O
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<th>Abbreviation</th>
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<td>Dickkopf-related protein 1</td>
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<td>DLG</td>
<td>Discs Large</td>
</tr>
<tr>
<td>Dll-1</td>
<td>Delta like 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modification of Eagle’s Medium</td>
</tr>
<tr>
<td>DUB</td>
<td>Deubiquitylating enzymes</td>
</tr>
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<td>Dvl</td>
<td>Dishevel</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
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<td>Electrogenerated chemiluminescence</td>
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<td>Exchange factor for Arf6</td>
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<td>EGFR</td>
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<td>eIF4E-BP</td>
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<tr>
<td>Ngn</td>
<td>Neurog</td>
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<tr>
<td>Ngn1/2</td>
<td>Neurog 1/2</td>
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<tr>
<td>NICD</td>
<td>Notch intracellular domain</td>
</tr>
<tr>
<td>NP</td>
<td>Neural progenitor</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimal Cutting Temperature</td>
</tr>
<tr>
<td>pAkt</td>
<td>Phosphorylated active Akt</td>
</tr>
<tr>
<td>Par</td>
<td>Partitioning defective</td>
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<td>Paired box 6</td>
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<td>Paraformaldehyde</td>
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<td>PH3</td>
<td>Phospho-Histone 3</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
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<tr>
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<td>+</td>
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<tr>
<td>PP</td>
<td>Preplate</td>
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<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate 40kDa</td>
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<tr>
<td>Protor1/2</td>
<td>Protein observed with rictor 1 and 2</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>PTMs</td>
<td>Post translation modifications</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
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<td>Ras-Related C3 Botulinum Toxin Substrate 1</td>
</tr>
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<td>Raptor</td>
<td>Regulatory-associated protein of mTOR</td>
</tr>
<tr>
<td>RGC</td>
<td>Radial glial cells</td>
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<tr>
<td>Rheb</td>
<td>Ras homologue enriched in brain</td>
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<tr>
<td>RhoA</td>
<td>Ras Homolog Family Member A</td>
</tr>
<tr>
<td>Rictor</td>
<td>Rapamycin-insensitive companion of mTOR</td>
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<td>Revolutions per minute</td>
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<td>Receptor-like tyrosine kinase</td>
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<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<td>SEN</td>
<td>sub-ependymal nodules</td>
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<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
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<td>Sin1</td>
<td>Stress-activated map kinase-interacting protein 1</td>
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<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
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<td>Subplate</td>
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<td>SVZ</td>
<td>Sub ventricular zone</td>
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<td>Threonine</td>
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<td>T-box brain protein 2</td>
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<tr>
<td>TCF</td>
<td>T cell factor</td>
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<tr>
<td>TJ</td>
<td>Tight junction</td>
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<tr>
<td>TSC</td>
<td>Tuberous sclerosis</td>
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<tr>
<td>TUNNEL</td>
<td>Terminal transferase dUTP nick-end labeling</td>
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<tr>
<td>USP</td>
<td>Ubiquitin-specific protease</td>
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<tr>
<td>Usp9x</td>
<td>Mouse homolog of Ubiquitin Specific Peptidase 9, X-Linked</td>
</tr>
<tr>
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<td>Human homolog of Ubiquitin Specific Peptidase 9, X-Linked</td>
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<td>Usp9x conditionally knockout mice</td>
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<td>Littermate control mice</td>
</tr>
<tr>
<td>VZ</td>
<td>Ventricular zone</td>
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<tr>
<td>WB</td>
<td>Western blot</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>WM</td>
<td>White matter</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula Occludens-1</td>
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CHAPTER 1

INTRODUCTION
1.0 Introduction

1.1 Principal classes of mammalian neural progenitors

The cerebral cortex is the centre for processing and regulating higher cognitive behaviours and somatosensory skills in mammals and the most evolutionary recent part of the mammalian brain (Rodriguez et al., 2012; Kaas, 2013). The cerebral cortex is mainly comprised of neurons, which are the functional and structural subunit of the brain and glial cells. Importantly, all these cellular subtypes are generated from neural progenitors (NPs) during embryonic development, except for hippocampal granular cells and interneurons within the olfactory bulb, which continue to be generated in the adult with an exception in humans (Ming et al., 2005). In the developing mammalian neocortex there are several NP subclasses, which can be classified according to their mitosis location, apical-basal polarity and proliferative capacity (Tavera et al., 2014; Homem et al., 2015).

The mammalian neocortical wall has its own intrinsic polarity. The inner surface lines the ventricles and is therefore exposed to extrinsic factors present in the cerebral-spinal fluid, while the outer surface contacts the basal lamina. Relative to this tissue polarity, NPs are categorized into two principal classes, based on the location of their nuclei during mitosis. Apical progenitors (AP) undergo mitosis at the ventricular zone (VZ), which is the luminal surface of the cortical wall, while basal progenitors (BP) undergo mitosis abventricularly in the subventricular zone (SVZ).

NPs are also classified according to the extent of their cell polarity, which can be determined based on the presence of distinct apical and basal membrane domains and the existence of apically or basally directed processes exhibiting the same membrane constituent similar to their domain of origin (Fietz et al., 2011). In terms of proliferative capacity, NPs fall into two principal classes: NPs that undergo multiple rounds of cell division before terminally differentiating into postmitotic cells and those that divide once, producing terminally differentiated daughter cells (Fietz et al., 2011; Tavera et al., 2014).

Although various subtypes of NPs exist within these principal progenitor subclasses, lack of specific markers has hampered the identification and characterisation of specific progenitor pools. Nevertheless, NP heterogeneity underpins the genesis of diverse neuronal
and glial cell types and intricate cytoarchitecture of the mammalian cerebral cortex (Becher et al., 2010).

1.2 Mouse cortical neural progenitors

According to the NP classification described above three principal subclasses of NPs reside in the developing mouse neocortex: (1) neuroepithelial cells, (2) radial glial cells (RGCs) and, (3) intermediate neural progenitors (INPs) (Figure 1).

Neuroepithelial cells are derivatives of neural ectoderm epithelial cells, which form the neural tube. Neuroepithelial cells are polarised along their apical-basal axis and span the entire width of the neural tube (Figure 1.1b). The apical domain of neuroepithelial cells forms the lumen of the neural tube, while the basal domain rests on the overlying basal lamina. Adherens junctions (AJs) are located at the apical most end of the lateral membrane and separate the apical and basal domains while linking neighbouring neuroepithelial cells, forming an AJs belt along the VZ (Aaku-Saraste et al., 1996). Initially, neuroepithelial cells undergo symmetric proliferative cell divisions to expand their number. As a neuroepithelial cell progresses through the cell cycle (asynchronously) its nucleus migrates along the apical-basal axis, with S-phase always occurring at the pial surface and mitosis at the apical surface of the VZ. This process is known as interkinetic nuclear migration (INM) (Figure 1.1c) and as a result neuroepithelial tissue appears as a pseudostratified epithelium (Kosodo et al., 2011).

At the onset of neurogenesis, around embryonic day 10.5 (E10.5), neuroepithelial cells start to express astrogial markers such as glial fibrillary transporter (GFAP) and astrocyte-specific glutamate receptors (GLAST), and transcription factors such as Pax6, while down regulating some epithelial characteristics including tight junctions and their associated proteins Occludin and Zonula Occludens-1 (ZO-1) initiating the transformation into RGCs (Aaku-Saraste et al., 1996; Aaku-Saraste et al., 1997; Englund et al., 2005). Although RGCs adopt an astrogial lineage they retain some prominent stem cell-like features such as apical-basal polarity, AJs and INM (Figure 1.1c).
Figure 1.1 Temporal development of neural progenitor subtypes in embryonic mouse brain. (a) Schematic representation of a coronal embryonic mouse brain section. Red insert represents the area focused in this schematic. (b) Prior to neurogenesis, neuroepithelial cells (NEs) are the predominant neural progenitor subtype in neocortex. NEs expand their cell number by undergoing series of symmetric self-renewing divisions. (c) At the onset of neurogenesis NEs differentiate into radial glia cells (RGCs). RGCs span through the cortical wall positioning the apical domain at the lumen of the lateral ventral (LV) while its basal process affixes to the basal laminar. During the G1-phase of the cell cycle, the nuclei of RGCs migrate towards the pial surface, being at the pial surface during S-phase and migrate back to the apical surface during the G2-phase to undergo mitosis (M) at the apical surface. This nuclear translocation is known as interkinetic nuclear migration. (d) During mid-neurogenic period RGCs give rise to transient amplifying intermediate neural progenitor cells (INPs). INPs detached from the ventricular zone (VZ) and migrate towards the basal boundary of the VZ forming new proliferative region, sub ventricular zone (SVZ). In addition to generating neurons, RGCs also act as a scaffold for the migrating neuroblasts. (e) Represents the post-mitotic cells at the end of neurogenesis, specifically neurons and terminally differentiated RGCs into astrocytes.
In contrast to neuroepithelial cells, RGCs have an apical (apical endfoot) and basal process, and are capable of undergoing symmetric as well as asymmetric cell division (Ever et al., 2005; Gotz et al., 2005). At the cellular level, symmetric versus asymmetric cell division can be determined by the equal versus unequal distribution of fate determinant factors such as apical domain, AJs, centrioles, or proteins such as Numb, between daughter cells. The distribution pattern of fate determinants depends on the mitotic cleavage plane in NPs such that if it occurs vertically, relative to the ventricle lumen, it results in symmetric cell division, while oblique or horizontal cleavage planes result in asymmetric cell divisions (Kosodo et al., 2004; Gotz et al., 2005).

During the mid-neurogenic period, RGCs transition from symmetric proliferative divisions to asymmetric neurogenic divisions. Asymmetric division of a RGC produces a daughter cell with a similar fate to the mother cell to maintaining RGC number. The other daughter cell inherits a more restricted fate differentiating into either an INP or a post-mitotic neuron or glial cell (Homem et al., 2015). In contrast, RGC could generate neurons directly by symmetric neurogenic division or indirectly by producing two INPs which are eventually differentiate into neurons (Laguesse et al., 2015). Although the underlying mechanism(s) initiating this molecular switch remains largely unknown, two recent studies have suggested that this switch is under the control of a temporally expressed key-set of transcription factors (Gao et al., 2014; Okamoto et al., 2016). From this developmental stage onwards, the neocortex starts expanding horizontally as well as vertically due to nascent neurogenic divisions of RGCs. In addition to neurogenesis, RGCs act as a scaffold that aid migrating immature neuroblasts to reach their designated location (Figure 1.1d). Therefore, in accordance with the radial unit theory, RGCs are the ultimate structural subunit of the neocortex (Rakic, 2007).

Newly synthesised INPs migrate away from the VZ to form a new proliferative zone at the basal surface of the VZ, known as the SVZ (Figure 1.1d). INPs divide in a self-consuming manner at the SVZ, undergoing one or two symmetric self-renewal cell divisions prior to terminally differentiate into two post-mitotic neurons. The consecutive symmetric cell divisions of INPs help to double the neuronal production, hence INPs are also referred to as transient amplifying progenitors (Miyata et al., 2004; Noctor et al., 2004). With the loss of their ventricular attachment, INPs lose apical-basal polarity and become spherical in shape (Pontious et al., 2008). Nevertheless, INPs maintain an intrinsic polarity by mechanisms such
as unequally distributing the epidermal growth factor receptor (EGFR) (Sun et al., 2005). Although the underlying mechanism(s) initiating INP differentiation is yet to be elucidated, several transcriptional regulators such as insulinoma-associated protein 1 (INSM1) and T-box brain protein 2 (Tbr2) seem to be involved (Englund et al., 2005).

### 1.3 Regulation of Neural Progenitor Fate

#### 1.3.1 Temporal and spatial regulation

During brain development, NPs need to balance their self-renewal with differentiation, in order to maintain the stem population while establishing the complex tissue architecture of the brain. (Sun et al., 2014; Taverna et al., 2014; Homem et al., 2015). During embryonic development NP fate is both temporally and spatially regulated. This temporal regulation of NP fate is well demonstrated during murine corticogenesis. The mouse cortex is a complex, highly organised, six-layered multicellular structure. Preplate formation is the initial step of mammalian corticogenesis. The preplate is mostly formed by the neurons generated by the early APs, prior to the onset of neurogenesis (Figure 1.2). At the onset of neurogenesis, the preplate divides into a superficial marginal zone and a more deeply located subplate. The cortical plate, which later gives rise to the multilayered neocortex, develops between the marginal zone and the subplate (Figure 1.2). In the mature neocortex, distinct populations of pyramidal neurons are in different layers. Neurons in each cortical layer have unique morphological features, express different complements of transcription factors, and ultimately carry out different functions. Lineage tracing experiments have shown that these pyramidal cortical neurons generate in a temporally coordinated manner (Figure 1.2) (Tarabykin et al., 2001; Zimmer et al., 2004; Colasante et al., 2015). This coordinated sequential neuronal differentiation indicates a direct correlation between NP fate and embryonic stage, suggesting neural progenitor fate is driven temporally by a genetically inherited intrinsic mechanism. Due to recent developments in single cell sequencing techniques several transcriptomic analyses successfully predicted several genes involved in temporal patterning of NPs (Kawaguchi et al., 2008; Lai et al., 2008; Ohtsuka et al., 2011; Okamoto et al., 2016) further strengthening the notion that NP fate is temporally regulated by a genetically inherited intrinsic mechanism.
**Figure 1.2 Mouse corticogenesis.** These schematics represent the presumptive areas of the murine cortex at different developmental stages. Corticogenesis initiates around E11.5 in embryonic mouse brain and spans till E18.5, end of embryonic development. Apical progenitors in the ventricular zone (VZ) produce the first wave of neurons which forms the preplate (PP). Preplate is later divide into marginal zone (MZ) and subplate (SP), delaminating the cortical plate (CP). Cortical plate is the last plate to form in corticogenesis, which give rise to the multilayered neocortex. Mouse neocortex is comprised of six different layers with each layer comprised of a specific neuronal subtype to carry out a specific function. Neurons in the deep layers were produced by the neural progenitors in the VZ and neurons in the superficial layers were mostly produced by the intermediate neural progenitors in the sub ventricular zone (SVZ). Neocortex develops in an inside-out manner, in which a new born neuroblast has to pass through later born neurons at the deep layers. New born neuroblast migrates through intermediate zone (IZ) to their destined cortical layer, while the marginal zone (MZ) is the predecessor of the layer 1 of the cortex. WM- white matter (Molyneaux et al., 2007)
Spatial regulation of NP fate is the other factor that influences the heterogeneity of cortical neurons. It has been hypothesized that progenitor subtypes exist within the VZ and SVZ NP pools; however, lack of specific markers to segregate these NP subtypes has hampered their identification (Kohwi et al., 2013). Despite the lack of specific markers, results from transplantation and lineage tracing studies provide evidence for the existence of progenitor heterogeneity. The heterogeneity of the telencephalic NPs was best illustrated in the primordial hippocampal/hippocampal neuroepithelium. The hippocampal neuroepithelium is localized at the caudomedial edge of the dorsal telencephalic VZ, adjacent to the cortical hem (Khalaf-Nazzal et al., 2013). According to lineage tracing studies, hippocampal neuroepithelium can further divide into three main regions depending on the neuronal subtypes produced by the NPs. The three regions are ammonic neuroepithelium, dentate neuroepithelium and fimbrial gliopithelium (Khalaf-Nazzal et al., 2013). NPs in the ammonic neuroepithelial give rise to the pyramidal and large neurons of the stratum oriens and the radium, while NPs in the dentate neuroepithelium give rise to the granular neurons of the dentate gyrus. NPs at the fimbrial gliopithelium produce hippocampal glial cells (Tole et al., 1997; Grove et al., 1999). A subpopulation of NPs at the ganglionic eminence produce GABAergic inhibitory interneurons compared to other dorsal NPs, which produce excitatory neurons (Cai et al., 2013; Wang et al., 2016). These studies strongly suggest that fate of a NP is influenced by its spatial location.

1.3.2 Neural progenitor fate determinants

NP fate is constantly under the scrutiny of temporal and spatial regulation throughout brain development. Not surprisingly, a number of fate determinant factors are involved in this regulation. Despite a modest understanding of how individual fate determinant pathways influence NP fate, several significant and fundamental questions remain to be answered; many of which centre around the integration and regulation of individual fate determinant pathways (Fietz et al., 2011; Munji et al., 2011; Sun et al., 2014; Taverna et al., 2014; Homem et al., 2015).

NP fate determinant factors can be divided into two groups based on their site of origin namely, intrinsic and extrinsic fate determinants. Intrinsic fate determinant factors encompass cellular functions such as cell cycle, polarity and INM, cellular structures like
INTRODUCTION

AJs, primary cilium, centrioles and mitotic spindle, transcription factors, and small RNAs (Knoblich, 2008; Toledano et al., 2008; Kohwi et al., 2013). The extrinsic factors are comprised of extracellular signaling pathways such as Notch, Wnt, Bone morphogenetic proteins (BMP) and mechanistic target of rapamycin (mTOR) (Caronia et al., 2010; Dong et al., 2012; Cloetta et al., 2013; Draganova et al., 2015). For the purpose of this dissertation this review will focus on the intrinsic factors AJs and cell polarity, and extrinsic factors Notch, Wnt and mTOR singling pathways.

1.3.3 Intrinsic fate determinants

1.3.3.1 Cell polarity and neural progenitor fate

Apical-basal polarity with a specialised apical membrane domain is one of the hallmark of NPs (Gotz et al., 2005; Fietz et al., 2011; Taverna et al., 2014). The apical-basal polarity of NP is reflected by the asymmetric distribution of cytoplasmic structures such as centrosome and Golgi apparatus, and cellular structures such as apical primary cilium and, also by the distribution pattern of plasma membrane proteins such as Prominin-1 (CD133) (Aaku-Saraste et al., 1997; Chenn et al., 1998). Although basal progenitors do not exhibit any of these features, they do exhibit an intrinsic polarity evident by the unequal distribution of EGFR (Li et al., 2003; Sun et al., 2005). Cell polarity is the underlying basis of symmetric versus asymmetric mitotic divisions of NPs. The progenitor fate of a NP is dependent on the inheritance of the apical domain where many intrinsic fate determinants such as Notch1, tetradecanoyl phorbol acetate-inducible sequence 21 (Tis21), Numb and Par3 are concentrated (Fietz et al., 2011; Shimojo et al., 2011; Sun et al., 2014; Homem et al., 2015). In contrast, several studies have claimed that the basal domain influences NP fate (Chenn et al., 1995; Cayouette et al., 2001; Gotz et al., 2005; Sabherwal et al., 2009; Jabes et al., 2011). While several other studies suggest that NP fate is dependent on the inherited ratio of apical to basal domain (Cabernard et al., 2009). Regardless, it is clear that apical-basal polarity plays a crucial role in determining the NP fate.

NPs achieve apical-basal polarity through the action of three evolutionarily conserved protein complexes. These are, the apical Crumb complex, and Par (Partitioning defective) complex and the basal Scribble complex (Lin et al., 2000; Toledano et al., 2008). The Par
complex comprises four proteins namely Par6, Par3, atypical protein kinase C (aPKC) and RhoA-GTPase protein Cdc42 (Manabe et al., 2002; Cappello et al., 2006a; Imai et al., 2006; Costa et al., 2008). The Crumbs complex comprises Crumbs, PALS1 and PATJ proteins (Bulgakova et al., 2009). The basally located Scribble complex consists of Scribble, Discs Large (DLG) and Lethal Giant Larvae (LGL) proteins. These apically and basally located protein complexes interact with each other and regulate downstream targets involved with establishing and maintaining cell polarity (Assemat et al., 2008; Leong et al., 2009).

Several studies have established the importance of these polarity proteins in NP fate determination. Studies by Ghosh et al. (2008) and Ossipova et al. (2009) showed that deletion of the Par complex protein aPKC, from chicken or frog neuroepithelial cells, disrupted their polarity and AJ, subsequently increasing premature neural differentiation. Paradoxically, Imai et al. (2006) showed that ablation of aPKCλ from mouse NPs, using Nestin-Cre donor mice, did not affect their differentiating potency although the apical-basal polarity and AJs were disrupted in the aPKCλ-null brains. In addition, polarity proteins could influence NP fate through extrinsic fate regulatory signaling pathways. Conditional deletion of Par3 polarity protein from mouse NPs abrogated Notch signaling increasing neural differentiation (Bultje et al., 2009). In Drosophila neuroblast and mammalian cultured epithelial cells, aPKC phosphorylates the Notch antagonist Numb, prompting their basal localization (Smith et al., 2007). In Xenopus embryos Par1 phosphorylates the E3 ubiquitin ligase Mindbomb (Mib) targeting it for degradation, subsequently decreasing Notch signaling (Ossipova et al., 2009). These studies provide evidence of three principal mechanisms by which cell polarity could influence NP fate. First, by controlling the mitotic spindle orientation, which dictates the symmetric versus asymmetric cell divisions of NPs (Siller et al., 2009). Secondly, polarity can influences NP fate through the AJs. Although AJs can independently act as a fate determinant (Section 1.3.3.2), data from polarity protein knockout mouse models demonstrated the structural and functional interconnection between AJs and polarity. Therefore polarity proteins could regulate NP fate through AJs utilizing its downstream fate regulatory mechanism (Costa et al., 2008; Ghosh et al., 2008; Asami et al., 2011). Lastly, polarity could influence NP fate by directly regulating extrinsic signaling pathways such as Notch signaling as mentioned above (Etienne-Manneville et al., 2003; Asami et al., 2011; Boroviak et al., 2011). Although under experimental paradigms these three mechanisms may appear to function independently, these mechanisms converge to regulate NP fate determination.
1.3.3.2 Adherens junction and neural progenitor fate

AJs are the predominant intercellular cell-cell adhesion structures in NPs. AJs are constructed on a foundation of homophilic interaction between cadherin clusters on adjacent NPs. While the extracellular domain of the cadherin proteins establishes homophilic interactions, the intracellular domain interacts with an array of proteins linking AJs to the actin cytoskeleton. The Armadillo repeat protein β-catenin binds to the intracellular domain of cadherin and α-catenin, primarily connecting cadherin to the actin cytoskeleton. Several other actin binding proteins such as AF-6 and p120 catenin are also involved in this process (Figure 1.3b) (Lu et al., 2001; Junghans et al., 2005; Katayama et al., 2011).

AJs form at the most apical end of the basolateral membrane of NPs, demarking the apical and basal domains (Paridaen et al., 2013). After the initial contact, cadherin proteins accumulate into the nascent AJ site forming a plaque of AJs at the apical surface of the VZ (Adams et al., 1998). These concentrated AJs not only connect adjacent NPs but also anchor them to the VZ. The VZ of the neocortex acts as a stem niche, creating a self-sustained pro-proliferative microenvironment and AJs prevent NPs detaching from this stem cell niche (Figure 1.3a) (Sheng et al., 2009). When the focal adhesion proteins αE-cadherin and β1-ingenin were ablated from embryonic mouse NPs, the mutant NPs detached from the VZ and prematurely differentiated into neurons (Leone et al., 2005; Stocker et al., 2009). Corroborating the above studies, over expression of N-cadherin prevents the differentiation of early NPs in embryonic mouse brains (Rousso et al., 2012).
**Figure 1.3 Structures of the Adherens Junction (AJ).** (a) Schematic illustration of how AJs connect neural progenitors at the ventricle zone. The ventricular zone by itself acts as a stem cell niche in developing neocortex. (b) Schematic representation of the basic structure of AJs. Shown here are the cadherin-catenin complex and Nectin-AF-6 complex, and their potential integration with the actin cytoskeleton. (a- modified with permission from Buchman et al. (2007), b- modified with permission from Niessen (2007)).

Conditional deletion of AJ structural proteins N-cadherin and β-catenin from mouse NPs, using Nestin-Cre donor mice, results in cortical dysmorphism (Zhadanov et al., 1999; Adachi et al., 2007; Wrobel et al., 2007; Chilov et al., 2011). This tissue dysmorphism is associated with unregulated NP cell divisions arising from the disrupted NP polarity, as AJs are needed for the establishment and maintenance of NP polarity (Section 1.3.3.1) (Martiens et al., 2009; Paridaen et al., 2015). In addition to maintaining NP tissue architecture, AJs could also influence NP fate by regulating NP cell cycle. Premature cell cycle exit and abventricular mitosis are the most common phenotypes observed in NPs following AJ disruption. These phenotypes have been observed when expression of AJ structural proteins N-cadherin (Chalasani et al., 2011), β-catenin (Chenn et al., 2003) or regulators such as Kinesin-associated protein 3 (KAP3), Cell division control protein 42 (Cdc42), Ras-Related C3 Botulinum Toxin Substrate 1 (Rac1) and Ras Homolog Family Member A (RhoA) were altered in cortical NPs (Lin et al., 2000; Teng et al., 2005; Cappello et al., 2006b; Katayama et al., 2011). Although the exact cause for this cell cycle dysregulation remains unknown, the involvement of AJs in mitotic spindle orientation may be involved as seen in APC
(Adenomatous polyposis coli) knockout Drosophila neuroepithelium cells (Lu et al., 2001). Interestingly, similar phenotypes were observed when the polarity protein Lgl-1 (Klezovitch et al., 2004) and Notch signaling regulatory protein Numb (Rasin et al., 2007) were conditionally deleted from embryonic mouse NPs, suggesting AJs work closely with other intrinsic and extrinsic factors in this process.

Similar to polarity proteins, AJs also interact with several extrinsic signaling pathways. The Notch signaling pathway plays a critical role in maintenance of progenitor number and the switch from proliferative to neurogenic cell division (Section 1.3.4.1). Both Notch1 receptor and its ligand Delta like 1 (Dll1) are distributed around the AJs at the apical domain in NPs. Following an asymmetric division nascent neurons and BPs inherit lower levels of Notch receptors compared to the daughter cell which retains a NP fate (Mizutani et al., 2007; Dong et al., 2012). N-cadherin also stabilizes the expression of Dll1 ligand in NPs promoting lateral inhibition in the surrounding NPs (Mizuhara et al., 2005). Correspondingly, downregulation of Notch signaling is important for the downregulation of N-cadherin, in order to initiate the detachment of differentiated NPs from VZ (Hatakeyama et al., 2014). Conditional deletion of N-cadherin or its regulator Rho A, increase the hedgehog signaling activity in NPs (Ruiz i Altaba et al., 2002; Palma et al., 2005; Feijoo et al., 2011). Although the exact mechanism how AJs and hedgehog pathway functionally connect is poorly understood, two recent studies suggest that tissue tension sensed by the AJs may biomechanically induce hedgehog pathway (Yao et al., 2015). The AJ structural protein β-catenin is also the co-activator of the canonical Wnt pathway, another key extrinsic signaling pathway involved in NP fate determination (Section 1.3.4.2).

1.3.4 Extrinsic fate determinants

1.3.4.1 Canonical Notch signaling pathway

Pathway overview

Notch signaling mediates juxtacrine signaling between adjacent cells. Notch signaling is activated when Notch ligands Jagged (Jag) or Dll1, bind to a Notch receptor expressed in an adjacent cell (Figure 1.4). Both Notch receptors and their ligands are single pass
transmembrane proteins. Ligand binding induces conformational changes in Notch receptor, exposing its extracellular cleavage site 2 to the metalloprotease ADAM10. Cleaved Notch extracellular domain remains bound to the ligand. Subsequently, this receptor-ligand complex undergoes endocytic recycling in the signal sending cell, which is dependent on the ubiquitylation of the Notch ligand by the ubiquitin ligase Mib. At the intramembrane surface of the signal receiving cell, γ-secretase cleaves the Notch receptor at cleavage site 3 releasing the transcriptionally activate Notch intracellular domain (NICD). In the absence of Notch activation, Notch target genes are maintained in an actively repressed state through the formation of transcriptional complexes involving CSL transcription factors and various corepressors. Once the cleaved NICD translocates into the nucleus, through a poorly understood mechanism, it associates with the CSL transcriptional complex causing conformational changes to the CSL transcription complex, which then replaces its corepressors with coactivators initiating the transcription of Notch target genes (Figure 1.4) (Pierfelice et al., 2011; Guruharsha et al., 2012).

**Figure 1.4 Notch signal transduction.** Notch ligand (Dll1) expression in terminally differentiated cells, adjacent to NPs, is promoted by proneural genes such as Mash1 and Ngn2. Notch ligands are activated by ubiquitin-mediated endocytosis facilitated by E3 ubiquitin ligase Mind bomb1. Once activated Notch ligand binds to Notch receptors at the adjacent cell. Upon ligand binding, γ-secretase cleaves the Notch receptor releasing the transcriptionally activated Notch intracellular domain (NICD). Subsequently, NICD transferred to nucleus where it binds to of the CSL transcription complex, replacing its corepressors with coactivators initiating the transcription of Notch target genes Hes1 and Hes5. Hes1 and Hes5 then repress the expression of proneural genes and Dll1, thereby
maintaining the progenitor characteristics. Numb can inhibit this signal transduction by targeting NICD for ubiquitin mediate proteasomal degradation (modified with permission from Shimojo et al., 2011).

Notch signaling in neural progenitor fate

The most characterised and prevailing function of Notch signaling in NPs is the inhibition of neuronal differentiation and maintenance of progenitor characteristics, through lateral inhibition (Figure 1.4) (Kageyama et al., 2008). In developing mammalian brains Notch1 and Notch2 are expressed in NPs, while Notch ligands Dll1 are expressed in differentiated INPs or post-mitotic neurons. Once Notch signaling transduction is initiated, the NICD bound CSL transcription complex initiates transcription of Hes1, Hes5 and Hey genes in NPs. These genes encode inhibitory basic helix-loop-helix (bHLH) proteins, which subsequently repress the function of proneural bHLH proteins such as Asc1, Mash2 and Neurog 1/2 (Ngn 1/2) (Yoon et al., 2005). In addition, several NP specific Notch target genes such as CyclinD1 (Ronchini et al., 2001), ErbB2 (Patten et al., 2006) and Fatty acid binding protein 7 (Fabp7) (Anthony et al., 2005) have been identified in recent years.

Lateral inhibition is the fundamental mechanism by which early mouse NPs maintain their progenitor fate. As reported previously, conditional deletion of Notch receptors - Notch1 and Notch2 or its ligand Dll1 from developing mouse brains results in the premature differentiate of early NPs into neurons (Itoh et al., 2003; Kageyama et al., 2008). The lateral inhibition theory was challenged when several studies reported that pro-neuronal genes Ngn2 and Mash2 and the Notch ligand Dll1 are expressed in early NPs prior to neurogenesis (Guillemot et al., 1993; Bettenhausen et al., 1995). However real time image analysis conducted by Kageyama et al. (2008) clarified this growing doubt on lateral inhibition showing that the expression of proneural genes Ngn2 and Dll1 and anti-neurogenic Hes1 oscillates in NPs and their oscillatory patterns were inverse to each other. Oscillation of Hes1 expression is necessary for NP cell cycle progression since sustained Hes1 expression inhibits cell cycle progression. Ngn2 expression is regulated by Hes1 expression, while Dll1 expression is induced by Ngn2 and repressed by Hes1 expression. Thus these two opposite phase oscillations are required to activate Notch signaling between adjacent NPs (without the
need of differentiated cells) and the cycling levels of Ngn2 prevents terminal differentiation of Hes1 decreased cells (Kageyama et al., 2008; Shimojo et al., 2011).

Growing evidences suggests that Notch signaling has multiple roles in developing mammalian brains. Notch-induced glial differentiation was one of the first non-lateral inhibitory function of the Notch signaling pathway discovered in NPs. Conditional deletion of the DNA binding protein RBP/J, from cortical NPs using Nestin-Cre donor mice, caused mild defects in neurogenesis, mostly due to decreased NP numbers, but significantly increased oligogenesis (Taylor et al., 2007). Similarly, continuous Notch signaling is required for switching from neurogenesis to oligodendrocyte precursor production in embryonic zebrafish spinal code RGCs (Kim et al., 2008). Based on these two studies Pierfelice et al. (2011) in their review suggest that Notch signaling is required for promoting NPs towards oligodendrocyte precursors fate but inhibits subsequent oligodendrocyte differentiation. On the contrary, Notch signaling integrates with the JAK signal transducer and STAT signaling pathway to promote the astrocytic lineage in mouse cortical NPs (Yoshimatsu et al., 2006). In fact, the astroglial protein BLBP encoding gene Fsbp7 is a direct Notch signaling target in mouse RGCs (Anthony et al., 2005). Towards the end of embryonic development most RGCs terminally differentiate into astrocytes. Therefore, this study suggests that by upregulating glia proteins in RGCs Notch signaling may prime the astrocytic differentiation of RGCs during early development stages.

Notch signaling is also involved in establishing NP heterogeneity in developing mammalian brains. As described oscillatory cycles of Hes1, Dll1 and Ngn2 could result in heterogeneity even between adjacent NPs during early cortical development. Complementing this notion, single cell transcriptomic analysis of mouse cortical NPs identified two distinct AP cell types with respect to their Notch signaling activity (Kawaguchi et al., 2008). A similar observation was made by Basak et al. (2007) while trying to identify putative neural stem cells in the mouse neural tube based on Hes5 expression level using a GFP transgene reporter under control of the Hes5 promoter. The multipotency and self-renewability of the NPs correlated with their Hes5 expression level, in which the highest Hes5 expressing cells were multipotent and proliferative, and lower Hes5 expressing cells even failed to proliferate. Furthermore, Notch signaling differs between apical and basal progenitors, with respect to its signal transduction pattern. In APs Notch signaling is transduced through the transcriptional regulator C-promoter binding factor 1 (CBF1), but Notch signaling transduces via a CBF1
independent mechanism in INPs (Mizutani et al., 2007). Though Notch signal transduction appears relatively direct compared to other extrinsic signals, the numerous modulators are involved with fine-tuning the amplitude and timing of Notch activity in NPs. The fate regulatory characteristics of these modulators will be discussed in the Section 1.5.3.

1.3.4.2 Canonical Wnt signaling pathway

Pathway overview

Canonical Wnt signaling is an evolutionarily conserved pathway involved in regulating numerous developmental events including cell proliferation, specification, differentiation and migration (Liu et al., 2010). The key regulatory step of Wnt signal transduction is the maintenance of its co-transcriptional activator β-catenin (Li et al., 2012).

Wnt signaling transduction is initiated by binding of Wnt ligands to the frizzled (FZD) and low-density-lipoprotein-related protein 5/6 (LRP5/6) co-receptor complex. In Wnt unstimulated cells, cytoplasmic β-catenin protein level is kept low by an oligomeric protein complex known as the destruction complex (DC) (Figure 1.5). Upon Wnt signaling activation the DC is deactivated through a molecular mechanism(s) not yet fully resolved. To date nine models have been proposed on DC inactivation including Axin turnover, Disheveled (Dvl)-Axin hetero polymerization and membrane sequestration of Axin1 (Figure 1.5) (Zeng et al., 2005; Schwarz-Romond et al., 2007; Fiedler et al., 2011). By far the most pragmatic model was proposed recent by Clevers and colleague (Li et al., 2012). This study showed that the DC remains intact for some time after Wnt activation and continues to phosphorylate β-catenin, but failed to ubiquitylate β-catenin due to the dissociation of the E3 ubiquitin ligase β-TrCP. The accumulating β-catenin within the DC subsequently impaired DC function (Li et al., 2012). Regardless of the mechanism, Wnt stimulation inhibits the DC function, saturating the cytoplasmic β-catenin pool. Accumulated cytoplasmic β-catenin then enters into the nucleus in a concentration dependent manner (Fagotto, 2013). In the nucleus β-catenin activates Wnt target genes in association with T cell factor/Lymphoid enhancer factor-1 (TCF/LEF) family transcription factors (Figure 1.5) (Valenta et al., 2012; Fagotto, 2013).
The DC comprises two scaffolding proteins Axin and APC, two kinases glycogen synthase kinase-3β (GSK-3β) and casein kinase-1 (CKI) and an E3- ubiquitin ligase β-TrCP (Figure 1.5). In addition to these core components, several other proteins such as Protein phosphatase 2 and Dvl interact with the DC in a context and/or temporal specific manner (Stamos et al., 2013). Assembly of the DC is initiated when APC and Axin simultaneously bind to free floating cytoplasmic β-catenin. Despite a decent understanding of the molecular interactions involved in this step, how these interactions mediate DC assembly remain largely unknown. (Burgess et al., 2011; Stamos et al., 2013; Kunttas-Tatli et al., 2014; Pronobis et al., 2015). Following this initial binding to β-catenin, the serine and threonine kinases GSK-3β and CK1 then bind to the scaffolding protein Axin. CK1 initially phosphorylates β-catenin at Serine 45 (S45) priming GSK-3β mediated phosphorylation at Threonine 41(T41), S37 and S33. In addition, phosphorylation of APC by GSK-3β and CK1 within the DC increases its affinity to β-catenin by 150 fold further enhancing its binding to β-catenin. The E3 ligase β-TrCP then binds to Axin, APC and the short peptide region of β-catenin containing the S33 and S37, subsequently polyubiquitylates β-catenin within the DC. APC plays a vital role in this process by removing phosphorylated β-catenin from Axin and presenting it to β-TrCP. Polyubiquitylated β-catenin then presents to the proteasome, where it undergoes proteolytic degradation (Figure 1.5) (Li et al., 2012; Stamos et al., 2013; Kunttas-Tatli et al., 2014). Therefore the DC efficiently targets free floating cytoplasmic β-catenin for proteasomal degradation, thereby helping to maintain vanishingly low levels of β-catenin in Wnt unstimulated cell, despite the gene being continuously transcribed.
**Figure 1.5 Wnt signal transduction.** (A) In the absence of Wnt cytoplasmic β-catenin forms a complex with Axin, the tumour suppressor adenomatous polyposis coli gene product (APC), casein kinase 1 (CK1), and glycogen synthase kinase3β (GSK3β) known as destruction complex (DC). β-catenin is phosphorylated by CK1 and GSK3β within the DC. Phosphorylated β-catenin is then recognized and ubiquitylated by the E3 ubiquitin ligase β-TrCP targeting β-catenin for proteasomal degradation. (B) Binding of Wnt ligands to frizzled (FZD) and low-density-lipoprotein-related protein 5/6 (LRP5/6) co-receptor complex activate the canonical Wnt signaling. Disheveled (Dvl) translocates the DC to LRP receptors and mediates its phosphorylation. This disrupts the phosphorylation and subsequent ubiquitylation of β-catenin allowing it to accumulate in the cytoplasm. Accumulated cytoplasmic β-catenin enters into nucleus in a concentration dependent manner where its binds with T cell factor/lymphoid enhancer factor-1 (TCF/LEF) transcription factors and activates Wnt responsive genes (modified with permission from MacDonald et al., 2009).

**Wnt signaling in neural progenitor fate**

Wnt signaling has diverse, but context and temporal-dependent functions in the developing brain. Prior to neurogenesis, Wnt signaling controls the dorsal-ventral and anterior-posterior tissue patterning of the neural tube, therefore demarcating the future
telencephalon. Concurrent with neural tube closure, which occurs around E9.5, Wnt3a, Wnt4 and Wnt7 are expressed in NPs at the anterior region of the neural tube marking the future cerebral cortex (Parr et al., 1993). Disruption of Wnt signaling, by deleting \( \beta\)-catenin, prior to neurogenesis results in embryonic lethality and defective anterior-posterior axis formation as marked by the absence of expression of anterior specific genes Hex and Hex1 and mislocalized posterior specific genes Cerberus-like and Lim1 (Huelsken et al., 2000). Overexpression of transcriptionally active \( \beta\)-catenin in mouse neural tube prior to neurogenesis increases the expression of dorsal telencephalic NP markers Pax6 and Pax7, and expand their expression towards ventral telencephalon at the expense of ventral NP markers Nkx6.1 and Olig2 (Yu et al., 2008). Although other signaling pathways such as sonic hedgehog and BMP (Le Dreau et al., 2012) are also involved in dorsal-ventral patterning of the neural tube, these studies indicate that Wnt signaling plays a pivotal role in this process.

Wnt signaling is expressed ubiquitously in mouse neocortex throughout brain development. Wnt reporter mice in which the \( \beta\)-galactosidase reporter is driven by TCF/LEF binding elements showed that Wnt signaling is active in neocortical NPs in a high-medial to low-lateral manner (Backman et al., 2005). Electroporation of a destabilized GFP variant under the control of a \( \beta\)-catenin responsive promoter (TOPdGFP) or Axin2 promoter during the neurogenic period revealed that Wnt signaling is highly active in RGCs and INPs. Its activity is downregulated in differentiating NPs and migrating neuroblasts, but restored in cortical neurons 24 hours after they enter into cortical plate (Woodhead et al., 2006; Mutch et al., 2010). Constitutive activation of Wnt signaling in mouse NPs, by over expressing transcriptionally active \( \beta\)-catenin under the Nestin promoter, resulted in enlarged cortices owing to the overproduction of RGCs (Chenn et al., 2002). Similar phenotype was observed when Wnt signaling was activated by overexpression of \( \beta\)-catenin/Lef1 fusion protein in RGCs (Machon et al., 2007). Even though these overexpression studies suggest that Wnt signaling favours RGC self-renewal, several tissue wide \( \beta\)-catenin deletion studies suggest the contrary. Conditional deletion of \( \beta\)-catenin from developing mouse brains using Nestin-Cre and D6-Cre donor mice resulted in the premature differentiation of APs into INPs (Machon et al., 2003; Junghans et al., 2005). In both of these \( \beta\)-catenin mutant mice AJs were significantly disrupted, which prompted Junghans et al. (2005) to conclude that in early neural progenitors \( \beta\)-catenin-mediated cell adhesion is more important than its transcriptional activity. The discrepancy between overexpression and deletion studies may be partly due to the dual functionality, adhesion and transcription, of \( \beta\)-catenin in NPs. To resolve this issue,
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Woodhead et al. (2006) cell-autonomously deleted β-catenin from NPs to prevent the tissue wide deletion of β-catenin by in-utero electroporating Cre recombinase into β-catenin floxed NPs. Cell-autonomous deletions of β-catenin from early NPs resulted in their premature differentiation into neurons without affecting the AJs (Woodhead et al., 2006). Similarly, when Wnt signaling was induced by in-utero electroporation of Wnt3a into NPs during the early neurogenic period (E13.5), a significant increase the Pax6-positive RGCs was observed (Munji et al., 2011). These studies revealed that cell autonomous Wnt signaling plays an important role in maintaining early NP identity. Furthermore, inhibition of Wnt signaling in early NPs by over expressing the Wnt inhibitor T cell factor (ICAT) (Shin et al., 2014) or Dickkopf-related protein 1 (DKK1) (Munji et al., 2011) also prevented the self-renewal of NPs, supporting the importance of Wnt signaling in maintaining early NP fate.

As neurogenesis progresses Wnt signaling promotes the neuronal differentiation of NPs. Overexpression of the oncogenic (transcriptionally active) form of β-catenin under humanised GFAP (hGFAP) promoter during the mid-neurogenic period (E14.5), significantly increased neuronal production (Poschl et al., 2013). Similarly, Wnt7a overexpression in E13.5 mouse cortical tissue and β-catenin overexpression in cortical NP ex-vivo cultures increased the overall neuronal production (Hirabayashi et al., 2004) suggesting Wnt signaling differentiated APs into neurons without progressing through INPs. These results complement findings from the transcriptionally active β-catenin over-expression studies, which proposed that Wnt signaling prevents RGCs differentiation into INPs during neurogenic periods (Chenn et al., 2002; Wrobel et al., 2007). However, contradicting these studies Kuwahara et al. (2010) reported that overexpression of stabilized β-catenin in RGCs by retroviral transfection, during the early neurogenic period increases INP differentiation at the expense of RGCs. To reconcile these contradictory finding, Munji et al. (2011) conducted an elegant experiment by over expressing Wnt3a in embryonic mouse brain during the early neurogenic period and showed that ectopic expression of Wnt3a promoted the expansion of RGCs during early neurogenic periods (E12.5), however during mid and late neurogenic periods Wnt signaling promoted the differentiation of INPs into neurons. In this same study, inhibiting Wnt signaling by electroporating DKK1 into NPs, prevented the self-renewal of RGCs during early neurogenic period and INPs differentiation into neurons during the mid-neurogenic period (Munji et al., 2011). Therefore, this study concluded that in early NPs Wnt signaling promotes self-renewing proliferative cell divisions, but induces neuronal differentiation during mid-late neurogenic period by promoting the terminal differentiation of INPs.
1.3.4.3 mTOR signaling pathway

Pathway overview

The mammalian target of rapamycin (mTOR) is an evolutionarily conserved atypical serine/threonine kinase belonging to the phosphatidylinositol-3 kinase (PI3K) family. mTOR constitutes the catalytic core of two well-characterised signaling complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Figure 1.6). The two complexes are defined by their binding components, exclusively by two scaffolding proteins namely, regulatory-associated protein of mTOR (Raptor) and rapamycin-insensitive companion of mTOR (Rictor). In addition to their catalytic mTOR subunit, several other proteins such as mammalian lethal with sec-13 (GβL), DEP domain containing mTOR-interacting protein (Deptor), and the Tti1/ Tel2 are common to both mTORC1 and mTORC2 complexes. In contrast, proline-rich Akt substrate 40 kDa (PRAS40) only binds to mTORC1, while mammalian stress-activated map kinase-interacting protein 1 (Sin1) and protein observed with Rictor 1 and 2 (Protor1/2) only bind to mTORC2 (Figure 1.6) (Laplante et al., 2012; Cloetta et al., 2013). As the name implies the small compound Rapamycin inhibits the activity of mTORC1, however prolonged Rapamycin exposure also inhibits mTORC2 activity, but not in all cell types (Sarbassov et al., 2006).

The mTORC1 pathway integrates inputs from five major intracellular and extracellular cues - growth factors, stress, energy status, oxygen, and amino acids. mTORC1 controls many major cellular processes including protein and lipid synthesis and autophagy. Heterodimers between Tuberous sclerosis 1 (TSC1) and TSC2 (TSC1/2) proteins serve as signaling hub integrating signals from all the inputs, except amino acids. The TSC1/2 complex possesses a GTPase activating site for the Ras homologue enriched in brain (Rheb) GTPase. GTP bound Rheb directly interacts with mTORC1 and simulates its kinase activity. TSC1/2 negatively regulates mTORC1 activity by converting Rheb into its inactive GDP-bound state. Once activated mTORC1 phosphorylates its downstream target proteins such as eIF4E-binding protein (eIF4E-BP) and S6 kinase1 (S6K1), the best characterised effectors of the mTORC1 signaling pathways (Figure 1.6). Phosphorylated eIF4EB-BP dissociates from eIF4E allowing it to recruit the translation initiation factor eIF4G into the 5’ end of most mRNAs initiating their translation. Similarly, phosphorylated S6K1 promotes mRNA translation by phosphorylating or binding to multiple protein targets including eEF2K, S6K1 Aly/Ref like target, CBP80, eIF4B, and the best characterised target of the S6K1 is 40S
ribosomal protein S6 (S6) (Ma et al., 2009). Substantially less is known about the mTORC2 pathway. Unlike mTORC1, mTORC2 is insensitive to nutrients but does respond to growth factors, such as insulin, through a molecular mechanism not yet fully understood. Although some suggest that the TSC1/2 complex is involved in this upstream signaling modulation of mTORC2 pathway (Huang et al., 2008), others oppose this suggesting that mTORC2 is activate through a PI3K variant instead (Dalle Pezze et al., 2012). Once activated, mTORC2 also phosphorylates its down-stream target proteins. The best characterised target of mTORC2 pathway is the kinase, Akt (Figure 1.6). Phosphorylated active Akt (pAkt) then phosphorylates its multiple target proteins involved in the regulation of multiple cellular processes such as metabolism, survival, apoptosis, growth, and proliferation (Sarbassov et al., 2005).

**Figure 1.6 mTOR signal transduction.** The mTORC1 and mTORC2 have distinct protein compositions and are stimulated by different upstream stimuli. Tuberous sclerosis 1 (TSC1) and TSC2 heterodimer serve as a hub integrating upstream stimuli. GTP bound Rheb directly interacts with mTORC1 and simulates its kinase activity. Once activated mTORC1 phosphorylates its downstream target proteins such as eIF4E-binding protein (eIF4E-BP) and S6 kinase 1 (S6K1) that have critical roles in protein translation. TSC1/2 complex could convert Rheb into its inactive GDP bound state, therefore negatively regulates the mTORC1 activity. In contrast mTORC2 is activated independently from TSC1/2 complex. Once activate, mTORC2 also phosphorylates its down-stream target protein active kinases Akt that
controls multiple cellular processes such as metabolism, survival, apoptosis, growth, and proliferation (modified with permission from Talboom et al., 2015)

mTOR signaling in neural progenitor fate

The mTOR pathway is one of the key extrinsic fate determinants of several different stem cell types including NPs (Russell et al., 2011). The current knowledge of mTOR signaling pathway in NPs has been collected by studying the pathophysiology of the tuberous sclerosis complex (TSC) formation. The fundamental cause of TSC formation is the hyper-activation of mTOR signaling due to the mutations in either TSC1 or TSC2 genes (Au et al., 2004; Cassetty, 2004). TSC is characterised by the formation of unique benign lesions known as hamartomas. Hamartomas are found in multiple organs including the central nervous system. Hamartomas in the brain cause severe tissue dysplasia such as cortical tubers (CT) and sub-ependymal nodules (SEN) in patients (DiMario, 2004; Maria et al., 2004). CT is defined as developmental abnormalities of the cortical cytoarchitecture such as defective cortical lamination and the presence of dysplastic neurons, large astrocytes, and a unique cell type known as giant cells (DiMario, 2004). Both CTs and SENs can be detected in embryonic brains and express NP specific markers such as vimentin and GFAP suggesting these lesion may have originated from NPs (Lee et al., 2003; Ess et al., 2005). Indeed conditional deletion of Tsc1 from mouse embryonic NPs, using Emx1-Cre or hGFAP-Cre donor mice, recapitulates the TSC neuropathological lesions. Deletion of Tsc1 from mouse NPs using Nestin-Cre donor mice results in premature differentiation of APs into INPs at the expense of neurogenesis, which may be responsible for the deregulated cortical lamination in the mutants (Way et al., 2009; Magri et al., 2011). Although the conditional deletion of Tsc1 from mouse NPs was perinatally lethal, a single dose of rapamycin was able to rescue these mutants suggesting mTORC1 plays a vital role in NP fate determination and TSC etiopathogenesis (Anderl et al., 2011). Conditional deletion of Raptor using the same Nestin-Cre donor mice also resutls in perinatal leathlity. In contrast to Tsc1 mutant, Raptor-depleted brains did not show the symptoms of TSC until later developmental stages, but still showed severe microcephaly and cortical tissue dysplasia, while the mutant NPs failed to differentiate into neurons (Cloetta et al., 2013). Inhibition of mTORC1 signalling in neurospheres, by addition of rapamycin or deleting RAPTOR, decreased proliferation of NPs without affecting the self-renewing capacity of the NSCs (Sato et al., 2010). When Rictor was conditionaly
deleted using Enx1-Cre donor mice, mutant mice survived although they displayed symptoms of microcephally similar to Raptor-depleted brains (Carson et al., 2013). Although the cortical lamination was normal in Rictor-null brains, neurons were smaller and oligodendrocyte number significantly reduced (Carson et al., 2013). Collectively, these studies validate the importance of mTOR singaling in brain development and NP fate specification.

1.4 Post-translational modifications in neural progenitor fate

As discussed in the previous section, multiple intrinsic and extrinsic pathways regulate NP fate specification. Proteins are the principle signal transduction elements of these pathways. In NPs these intrinsic and extrinsic signaling pathways are integrated into a one sophisticated molecular network. The most common way signal integration occurs is through the crosstalk of their signal transducing elements. Post translational modifications (PTMs) are one of the mechanisms that can integrate signal transducing elements from different pathways (Nijman et al., 2005; Hayward et al., 2008; Lebrilla et al., 2009; Wang et al., 2014). Compared to other regulatory mechanisms, such as epigenetic changes and microRNA, PTMs produce more rapid responses (Lebrilla et al., 2009). Additionally, proteins are often modified through a combination of PTMs, the majority of which are reversible, enabling proteins to quickly switch between pathways or functions. This diverse and the dynamic capability of PTMs enable the integration of multiple pathways during NP fate specification (Kikuchi et al., 2006; Wang et al., 2014). Although, more than 20 different types of PTMs have been identified to date, this dissertation will focus on the ubiquitin pathway and specifically, on deubiquitylation.

1.4.1 The Ubiquitin Proteasome System

Ubiquitylation is an enzymatic process that involves the bonding of an ubiquitin protein to a substrate protein. Ubiquitin is a 76 amino acid long polypeptide that is highly conserved from yeast to mammals. The best characterised function of ubiquitylation is to target proteins for proteasomal degradation (Komander et al., 2009; Kawabe et al., 2011). In addition, ubiquitylation regulates many other cellular processes including vesicular
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trafficking, cell-cycle control and DNA repair (Joberty et al., 2000; Hurley et al., 2006; Stegmuller et al., 2010).

Ubiquitin is attached to its target protein through a thiol-ester bond formed between the C-terminal glycine residue of ubiquitin and a lysine residue on the substrate. This thiol-ester covalent attachment is processed by a cascade of enzymes; E1-ubiquitin activating enzyme, E2- ubiquitin conjugating enzyme, E3- ubiquitin ligase enzyme (Figure 1.7) (Pickart et al., 2004). Protein substrates may be ubiquitylated on a single or several lysine residues, resulting in monoubiquitylation or multi-monoubiquitylation, respectively. Monoubiquitylation or multi-monoubiquitylation of a protein can alter its trafficking and/ or sub-cellular localization, and can also regulate cellular functions such as DNA repair, endocytosis and signal transduction (Sadowski et al., 2012). Proteins are also subject to polyubiquitylation by attaching additional ubiquitin moieties to lysine (K) residues of the previously attached ubiquitin. Ubiquitin contains seven K residues at positions 6, 11, 27, 29, 33, 48, and 63, and all can be utilized during polyubiquitin chain formation (Peng et al., 2003; Passmore et al., 2004). Depending on the number of K residues utilised, polyubiquitylation can generate either linear or branched polyubiquitylated chains with different topologies. Substrates inherit different fates, depending on the K residue utilised for polyubiquitin chain formation. Polyubiquitylation through K-48 or K-11 targets proteins for proteasomal degradation, while K63-linked polyubiquitin chains regulate signal transduction and endocytosis. Polyubiquitylation involving K-6, K-27 K-29, and K33 is implicated in multiple cellular functions including DNA repair, cell cycle regulation, autophagy and signal transduction (Figure 1.7) (Passmore et al., 2004).
**Figure 1.7 Ubiquitin-protease system.** Ubiquitylation is the post-translational modification process of attaching ubiquitin proteins on to a substrate protein. Ubiquitin is attached to its target protein by an enzyme cascade reaction; E1-ubiquitin activating enzyme, E2- ubiquitin conjugating enzyme, E3- ubiquitin ligase enzyme. Proteins could be to either monoubiquitylated or polyubiquitylated. Proteins polyubiquitylated through Lysine 48 (K-48) or K-11 are getting targeted for proteasomal degradation. Proteins polyubiquitylated through K-11, K-63 or by linear polyubiquitin chains regulate many cellular functions such as DNA repair, cell cycle regulation, autophagy and signal transduction. Deubiquitylating enzymes (DUBs) reverse these modifications by un-conjugating ubiquitin proteins from their substrates (modified with permission from Vucic et al., 2011).

### 1.4.2 Deubiquitylating Enzymes

Similar to other PTMs, ubiquitylation is reversible, a process which is mediated by deubiquitylating enzymes (DUBs). DUBs are a group of, predominantly, cysteine proteases that cleave the thiol-ester bond of the ubiquitin-protein complex, thereby opposing the effects of the ubiquitin pathway (Figure 1.8). Thus DUBs are the final arbitrators of an ubiquitylated protein, and hence determine its fate. DUBs have been implicated in several important cellular processes including cell growth, differentiation and development in many biological systems. Not surprisingly, defects in DUB activity or expression have been implicated in the etiopathogenesis of numerous cancers and neurodegenerative diseases (Wilkinson, 1997; Komander et al., 2009).
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Figure 1.8 Deubiquitylation is a post-post-translational modification. The substrates presenting to deubiquitylating enzymes (DUBs) are post-translationally modified by ubiquitylation. However, most of these substrates were post-translationally modified by kinases or hydroxylases prior presenting for ubiquitylation. Therefore, deubiquitylation is a post-post-translation modification and its enzyme kinetic activity (K4) is governed by the kinetic properties of the upstream events (K1, K2 and K3).

The human genome encodes an estimated 98 DUBs, which can be classified into five classes based on their catalytic domain. The five classes include ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases, Machado-Joseph disease proteases, ovarian tumor proteases and metalloprotease-JAMM motif containing proteases (Todi et al., 2011).

USPs are the most common and diverse subset of DUBs (Nijman et al., 2005). Evolutionarily, USP numbers expanded in parallel to the increase in E3 ligases suggesting an intimate relationship between the two protein classes (Semple et al., 2003). The catalytic domain of USPs is flanked by two well conserved motifs known as Cys and His boxes. However, the size of the catalytic domain varies extensively between USPs due to the large unrelated sequences interspersed between the two motifs. Apart from this catalytic domain USPs share very little structural homology and display very high substrate specificity (Komander et al., 2009). DUBs sit at the end of an enzymatic cascade of PTMs (Figure 1.8). Most protein substrates undergo at least two different PTMs, commonly phosphorylation followed by ubiquitylation, before becoming a substrate for a DUB. Therefore, deubiquitylation is a post-post-PTM and the fate of a substrate is governed by the kinetic properties of the upstream enzymatic events (Figure 1.8). More importantly, PTMs induced by DUBs result in quantitative changes, meaning as the final arbiter of a substrate’s fate DUBs regulates their absolute number, location or function rather than an all or non-response (Murtaza et al., 2015). The experiments described in this dissertation focus on the
DUB, Ubiquitin Specific Peptidase 9, X-Linked (Usp9x) and its functions during NP fate specification.

1.5 Ubiquitin Specific Peptidase 9, X-Linked

A gene trap study conducted to identify murine genes expressed during gastrulation and neurulation first identified Usp9x in mouse embryonic stem cells (ESC). Due to its protein sequence similarity to the Drosophila fat facets (faf) with an exception of the last 80 amino acids, Usp9x was first named as Fam (fat facets in mouse) (Wood et al., 1997). Concomitantly, human homologues of faf were identified using adult human testis cDNA library and mapped to Xp11.4 and Yq11.2 on the X and Y chromosomes, respectively. Again due to high sequential similarity to faf, Usp9x was first labelled as DFFRX (Drosophila fat facets related X) (Jones et al., 1996). Faf and its homologues DFFRX and Fam were later renamed as Usp9x to reflect the DUB class to which they belong. In addition to its remarkable evolutionary conservation, USP9X is one of two X-linked DUBs (USP26 is the other) and one of the few X-linked genes capable of evading X-chromosome inactivation in humans (Dirac et al., 2010; Deng et al., 2014). However, Usp9x does not evade X-chromosome inactivation in mice (Jones et al., 1996), which is not surprising as X inactivation mechanisms are different between rodents and humans (Deng et al., 2014). Phylogenetic analysis revealed that Usp9x is evolutionarily conserved to an extent similar to developmental regulatory proteins such as Pax6 and β-catenin and housekeeping proteins such as β-actin and ARF-1. Despite being a much larger protein, Usp9x is more highly conserved than developmental regulators such as Notch1, Sonic Hedgehog, and EGF and EGFR (Khut et al., 2007).

To date, eight alternatively spliced USP9X mRNA variants have been reported. Only three variants are capable of encoding a protein, while the protein encoded by the shortest transcript variant undergoes nonsense mediated protein decay (Aken et al., 2016). Although expression analysis suggests the other two mRNA variants encode functional isotypes with slightly different molecular weights (2554 and 2559 amino acids) (Wood et al., 1997), to date there is no evidence verifying the existence of both. USP9X encodes an ubiquitin-like domain near the N-terminal and a USP catalytic domain closer to its C-terminal (Murtaza et al., 2015). USP9X is capable of deubiquitylating monoubiquitylated substrates as well as K-48,
K-63 and K-29 linked polyubiquitylated substrates (Mouchantaf et al., 2006; Marx et al., 2010). To date more than 35 USP9X interacting proteins have been identified, most of which are substrates (Murtaza et al., 2015). However, the regulation of these substrates by USP9X is context specific.

USP9X has been identified as a putative stemness gene. That is, USP9X is one of 34 commonly expressed genes with higher expression in undifferentiated embryonic, hemopoietic, neural and hair bulge stem cells (Blanpain et al., 2004). Furthermore, proteomic and microarray analysis conducted on several mouse and human ESCs and several cancer stem cells have also detected significantly increased levels of USP9X in these stem cell pools (Sato et al., 2012).

1.5.1 Usp9x in brain development

Usp9x is essential for early embryonic development. Usp9x depleted preimplantation mouse embryos failed to develop into blastocysts (Pantaleon et al., 2001). Faf mutant Drosophila syncytial embryos fail to initiate cellularisation and polarity (Fischer-Vize et al., 1992), while Usp9x depleted gastrulating Xenopus embryos failed to form mesoderm (Dupont et al., 2009) highlighting the evolutionary conserved function of Usp9x in early embryonic development stages. Until the post-gastrulation period Usp9x is robustly expressed in mouse embryos, however its expression becomes more complex from mid-gastrulation until birth (Wood et al., 1997). The neocortex is one of the prominent sites that maintain a high Usp9x expression from mid-gastrulation onwards. In fact, Usp9x mRNA expression is significantly higher in E13.5 embryonic mouse brains compared to peripheral tissues such as heart, kidney, and liver (Xu et al., 2005). Usp9x is robustly expressed in NPs at the VZ of mouse neocortices with an increased expression gradient towards the apical domain (Jolly et al., 2009; Stegeman et al., 2013), where it co-localised with the fate determinant factor Notch1 and partially co-localized with the AJ proteins N-Cadherin (Figure 1.9) (Jolly et al., 2009). Usp9x is also expressed in NPs of the Zebrafish cephalic central nervous system (Khut et al., 2007). In the adult brain, Usp9x expression is significantly higher in the SVZ of the cortex and sub-granular zone of the hippocampus, the only neurogenic sites of the adult brain (Xu et al., 2005; Oishi et al., 2016). This temporal and spatial expression pattern of Usp9x suggests Usp9x may have a role in NP regulation.
Indeed, over expression of Usp9x in mouse ESC-derived NPs promote the formation of polarised neural rosettes associated with an increase in NP self-renewal (Jolly et al., 2009). Conversely, conditional deletion of \textit{Usp9x} from mouse NPs, using \textit{Nestin-Cre} donor mice, results in perinatal lethality, demonstrating that Usp9x is essential for mouse brain development (Stegeman et al., 2013). Histological analysis revealed disorganisation of the cells at the VZ and SVZ of Usp9x-ablated brains at later developmental stages (Figure 1.9) (Stegeman et al., 2013). This tissue dysplasia suggests deletion of \textit{Usp9x} may have dysregulated NP fate. Interestingly, when Usp9x was conditionally deleted from embryonic NPs using \textit{Emx1-Cre} donor mice, the resultant mutants survived through their adulthood without obvious major complications (Stegeman et al., 2013; Oishi et al., 2016). Intriguingly the hippocampi of the Usp9x mutants were significantly smaller, while the SVZ and the cerebral cortex appeared to be relatively normal (Stegeman et al., 2013). Analysis of cell populations within the Usp9x null hippocampus revealed reduced NPs, neuroblast and neuronal numbers suggesting a critical role for Usp9x in adult hippocampal NP fate specification (Oishi et al., 2016). Although these studies did not explore the molecular mechanism(s) whereby Usp9x regulates NP fate, collectively these studies; (1) identify Usp9x as a NP fate determinant, (2) corroborates the concept of \textit{Usp9x} as a stemness gene and, (3) illustrate the cell specific function of Usp9x.

As described in Section1.4, PTMs could integrate multiple fate determinant pathways by modifying their signal transducing elements. Several Usp9x substrates belong to this exclusive class of signal transducing elements. Therefore, Usp9x regulation of NP fate may, in part, involve modification of these signal transducing elements.
1.5.2 Usp9x regulation of intrinsic neural progenitor fate determinants

AJs and apical-basal polarity are two prominent intrinsic factors regulating NP fate determination (Section 1.3.3). The AJ structural proteins AF-6 and β-catenin are substrates of Usp9x, at least in cultured epithelial cells (Taya et al., 1998; Taya et al., 1999; Kanai-Azuma et al., 2000; Theard et al., 2010). USP9X was colocalized and immunoprecipitated with AF-6 at the AJs of polarised MDCKII cells (Taya et al., 1998) and in developing mouse eyes lens fibres and neural retina (Kanai-Azuma et al., 2000). However, only the over expressed catalytic domain USP9X was able to rescue AF-6 from its ubiquitin mediated proteasomal degradation (Taya et al., 1998). β-catenin also co-localised with USP9X in MDCKII cells (Taya et al., 1998). Overexpressed USP9X catalytic domain interacts with full-length β-

Figure 1.9 Identifying Usp9x as a novel neural progenitor fate regulatory factor. (a) Usp9x is highly expressed at the apical surface of E14.5 mouse neural progenitors as marked by Nestin staining (A and C), where it co-localized with known NP fate determinant factors N-cadherin (B) and Notch (C). (b) Nestin-cre mediate deletion of Usp9x resulted in disrupted ventricular and subventricular zones (VZ/SVZ) at late embryonic stages (E18.5). VZ and SVZ of the conditional knockout mice show a diffused cellular architecture (B) compared to its litter mate controls (A). C and D are higher magnification images of A and B, respectively. Abbreviations – VZ - ventricular zone, SVZ – Subventricular zone. Scale bars = A and B-50µm, C and D – 40 µm. (modified with permission from Jolly et al., 2009; Stegeman et al., 2013).
catenin and increases β-catenin protein half-life in cultured L cells (Taya et al., 1999). However, the USP9X catalytic domain failed to bind truncated forms of β-catenin, which lack Axin and APC binding sites. Since catalytic domains of USP proteins are well conserved and the USP9X catalytic domain failed to bind to the truncated β-catenin lacking ubiquitylation sites suggest that this interaction between USP9X catalytic domain and β-catenin proposed by Taya et al. (1999) may be non-specific. However, when Usp9x was ablated from preimplantation mouse embryos, AF-6 and β-catenin protein levels were significantly reduced within 24 hours disrupting the AJs (Pantaleon et al., 2001). Although AF-6 expression was restored after 72 hours it was mislocalized to a region around the nucleus. β-catenin protein level remained very low except for that at sites of cell-cell contact (Pantaleon et al., 2001) suggesting a functional connection between Usp9x and β-catenin in these cells. Correlating with this study interaction between full length USP9X and β-catenin was recently reported in MCF-7 breast cancer cell lines. Knockdown of USP9X using siRNA reduce the β-catenin protein levels in MCF-7 cells. In the same study full length USP9X protein was immunoprecipitated with β-catenin using β-catenin overexpressed HEK293 cells (Ouyang et al., 2016). These studies suggest that Usp9x is capable of regulating adherens junctions by directly manipulating the expression of its structural components β-catenin and AF-6.

USP9X co-localises with β-catenin/E-cadherin heterodimer in the epithelial cell line T84 but interestingly, this interaction was apparent only when cells were sub-confluent (Murray et al., 2004). Similarly, USP9X was detected in multiple Golgi trafficking vesicles in L-cell fibroblasts transfected with E-cadherin, undergoing AJ remodelling (Murray et al., 2004). These data suggested Usp9x may be important for the trafficking of the cadherin-catenin heterodimer to the apical-basal boundary of cells undergoing polarisation, and that Usp9x may play a role in de novo AJ formation. Consistent with this notion, studies have shown that Usp9x is essential for de novo tight junction (TJ) formation. The Usp9x substrate exchange factor for Arf6 (EFA6) facilitates de novo TJ formation. Usp9x transiently increases EFA6 protein levels at de novo TJ sites by opposing its proteasomal degradation. This transient surge of EFA6 level initiates TJ formation at primordial epithelial junctions (Theard et al., 2010). Furthermore, knockdown of USP9X impairs TJ biogenesis in polarised MDCKII cells and over expression of EFA6 was able to rescue depletion of Usp9x (Theard et al., 2010). Therefore, these studies suggest that Usp9x could influence NP fate by regulating the establishment of AJs.
Cellular apical-basal polarity was upregulated when Usp9x was modestly overexpressed in ESC derived NPs supporting a role for Usp9x in polarity regulation (Jolly et al., 2009). Although this study did not address the underlying molecular mechanism, Usp9x has been implicated in the regulation of Par polarity complex proteins. MARK4 (Par-1 homologue) is a component of the Par protein complex, which is activated by the LKB1 kinases (Par-4 homologue) (Goldstein et al., 2007). AMP-activated protein kinase NUAK1 and MARK4 are both substrates of Usp9x. Deubiquitylation of these two kinases promotes their activation by the LKB1 kinases suggesting Usp9x may able to regulate apical-basal polarisation by regulating the expression of these two kinases (Al-Hakim et al., 2005; Al-Hakim et al., 2008) (Al-Hakim et al., 2005; Al-Hakim et al., 2008).

These studies suggest Usp9x is capable of regulating the intrinsic fate determinants AJ and polarity, and provide a number of molecular mechanisms through which it could regulate these two intrinsic factors.

1.5.3 Usp9x regulation of extrinsic neural progenitor fate determinants

Usp9x has been implicated in regulating multiple extrinsic signaling pathways including Notch, Wnt and mTOR, which control important aspects of NP fate determination.

Usp9x is the most implicated DUB in Notch pathway regulations, due to its ability to regulate Notch modulators in both signal sending and receiving cells (Zhang et al., 2012). Usp9x can promote Notch signaling by enhancing ligand activation in signal sending cells. Ubiquitylation of the ligand’s intracellular domain, by the E3 ligases Neuralized or Mib, is essential to activate the membrane tethered Notch ligands. Once ubiquitylated, the ubiquitin-binding adaptor protein Epsin mediates the endocytosis of the inactive Notch ligands. Within the endocytic vesicle Notch ligands become activated either because endocytosis is directly associated with receptor activation, or it releases the inactive ligand into a specific membrane compartment that renders its activation (Pierfelice et al., 2011). The first evidence of Usp9x regulation of Notch receptor activation came from a genetic study of mutations affecting *Drosophila* photoreceptors (Chen et al., 2002; Overstreet et al., 2004). In *Drosophila* photoreceptors Faf (*Drosophila* Usp9x homolog) prevents the ubiquitin-induced degradation of Liquid facets (*Drosophila* Epsin homolog) enhancing the efficiency of ligand
internalization (Overstreet et al., 2004; Wang et al., 2004). In addition to its ligand activation function in Notch signal sending cells, Mib is also expressed in mature postmitotic neurons and regulates their morphology and synaptic activity (Mertz et al., 2015). Two studies have shown Usp9x physically interacts with Mib at the postsynaptic density (Choe et al., 2007; Mertz et al., 2015). Yeast two-hybrid analysis of Mib, using embryonic Zebrafish cDNA library also predict the interaction between Usp9x and Mib (Tseng et al., 2014). Paradoxically, Usp9x may also attenuate Notch signaling by targeting NICD for ubiquitin-mediated degradation via the E3 ubiquitin ligase Itch (McGill et al. (2003). Usp9x and Itch partially colocalize, in COS-7 cells, at the trans-Golgi network and in peripheral vesicles and, their physical interaction was confirmed by co-immunoprecipitation. Binding of Usp9x to Itch, prevents the latter’s autoubiquitylation and proteasomal degradation (Mouchantaf et al., 2006).

As described in Section 1.5.1, ablation of Usp9x results in decreased β-catenin levels in multiple mammalian cell lines and preimplantation mouse embryos. However these studies failed to report the Wnt activity in these cells except for the MCF-7 cells cancer cell. Ablation of Usp9x decreased the expression of decoy death receptors due to the decreased Wnt activity (Ouyang et al., 2016). Nevertheless, two independent mass spectrometric analyses of HEK293 cells predict that USP9X could be a part of the DC suggesting a novel intersection between Usp9x and Wnt signaling (Major et al., 2007; Li et al., 2012). In addition to its regulation of Notch signaling, the USP9X substrate Epsin also potentiates Wnt signaling in HT-29 human colon cancer cells by preventing the ubiquitin induced proteasomal degradation of Dvl-2 (Chang et al., 2015). Similarly, in addition to its function in Notch signaling, USP9X substrate Mib can also enhance Wnt signaling by promoting the internalisation of a Wnt receptor RYK (receptor-like tyrosine kinase) in both HEK293 and HeLa cells (Chang et al., 2015).

Usp9x also regulates mTOR signaling in mouse C2C12 myoblasts. Ablation of Usp9x from C2C12 myoblasts, using Usp9x specific siRNAs, increases mTORC1 activity without affecting mTORC2 activity. Interestingly, when Usp9x was ablated from differentiating myoblasts, mTORC2 activity was significantly upregulated without affecting mTORC1 activity (Agrawal et al., 2012) highlighting the cell context specificity of Usp9x. This study failed to detect or predict the component(s) of the mTOR signaling pathway regulated by Usp9x in C2C12 cells. However, Raptor and very small amounts of Rictor protein were
immunoprecipitated with Usp9x, when full length USP9X was ectopically expressed in HEK293 cells (Agrawal et al., 2012).

1.6 Hypothesis and Aims

Usp9x is highly expressed in NPs and is capable of regulating multiple intrinsic and extrinsic pathways involved in NP fate regulation. Therefore Usp9x is ideally situated to integrate extrinsic signals with intrinsic responses during fate determination. Not surprisingly, conditional deletion of Usp9x from embryonic mouse NPs using Nestin-Cre donor mice resulted in perinatal lethality highlighting its importance in embryonic brain development. Histological analysis conducted on the Usp9x ablated brains during later embryonic stages (E16.5 onwards), showed a diffused cellular architecture at the VZ and SVZ (Stegeman et al., 2013), however this was conducted at low levels of magnification. Earlier embryonic time points have not been examined leaving open the question of which cell types are affected by loss of Usp9x and when? Therefore the overarching aim of this project was to investigate the role of Usp9x in NP fate specification and to identify the intrinsic and/or extrinsic mechanism Usp9x regulates in this process.

1.6.1 Aims and Hypotheses

It is hypothesized that Usp9x regulates NP fate by regulating intrinsic (adherens junction and apicobasal polarity) and/or extrinsic (Notch, Wnt and mTOR signaling) fate determinant factors.

To address the hypothesis the following aims were addressed;

Aim 1 - Determine the role of Usp9x in AJ and apical-basal cell polarity regulation in NPs. This aim will be address by comparing the expression levels and localisation of AJ and cell polarity proteins between control and Usp9x depleted embryonic mouse brains using immunohistochemical, WB and co-IP analyses.
Aim 2 - Determine the role of Usp9x in Wnt signaling regulation in NPs. The expression of Wnt signaling in Usp9x depleted brains will be assessed by employing qRT-PCR reactions. WB, immunohistochemical and co-IP analyses will be employed to determine the molecular mechanism(s) behind this regulation. In addition, USP9X-depleted *in-vitro* human NP cells (ReNcell-VM) and HEK293 cells will be used to elaborate the underpinning molecular mechanism(s).

Aim 3 - Determine the role of Usp9x in Notch signaling regulation in NPs. The same approach and techniques used in Aim 2 will be employed to address this aim.

Aim 4 - Determine the role of Usp9x in mTOR signaling regulation in NPs. The regulatory function of Usp9x in mTOR signaling will be address using USP9X-depleted *in-vitro* human NP cell line, ReNcell VM and neurospheres generated from E18.5 Usp9x depleted cortical NPs.

Aim 5 - Analyse how the loss function of Usp9x affects NP fate specification process in Usp9x-depleted brains. This aim will be address using quantitative immunohistochemical analyses targeting different NP and neural progenies in the Usp9x-depleted brains. In addition, an EdU birth dating experiment will be employed to assess the cell cycle dynamics of Usp9x-depleted neural progenitors and simple lineage tracing purposes.
CHAPTER 2

Materials and Methods
2.1 Generating *Usp9x* conditional knockout mice

2.1.1 Animal ethics

Use of experimental animals was approved under the ethical clearances of BPS/02/11, ESK/06/14 and BPS/03/14 granted by the Griffith University Animal Ethics Committee. Mice were housed and handled in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the protocols were approved by the Institutional Animal Care and Use Committee of Eskitis Institute for Drug Discovery, Griffith University.

2.1.2 Genotyping mouse embryos using polymerase chain reaction

The *Usp9x* conditional knockout mouse model was developed using the Cre/loxP recombination system as published by Stegeman et al. (2013). To targetedly delete *Usp9x* from embryonic mouse NPs, *Usp9x*\(^{loxP/loxP}\) female mice were crossed with heterozygous males expressing Cre recombinase under control of the Nestin promoter (Dubois et al., 2006). Since *Usp9x* is an X-linked gene, all female offspring inherit one *Usp9x* floxed gene from the mother and a wild type *Usp9x* gene from the father. Therefore, female offspring would be heterozygous for *Usp9x* expression. On the other hand, all male offspring inherit a single *Usp9x* floxed gene. As the *Nestin-Cre* male mice are heterozygous for the Cre-recombinase gene, only half would receive the Cre transgene, potentially resulting in the *Usp9x* knockout genotype. For this reason, in this study males inheriting the Cre transgene were used as the experimental specimens (*Usp9x*\(^{+/}\)), while males within the same litter lacking the Cre transgene were used as controls (*Usp9x*\(^{+/+}\)). To identify the *Usp9x* null genotype offspring, two polymerase chain reactions (PCRs) were performed on tail tip DNA. The first PCR was to detect the male-specific Sry gene (Berta et al., 1990). The second PCR was targeted to the Cre transgene. All males inheriting the Cre gene were considered to be *Usp9x*\(^{+/}\) mice.
2.2.1 Tissue Digestion Step

Tissue digestion was performed using REDExtract-N-Ampa Tissue PCR Kit (Sigma) according to the manufacturer’s instructions. In brief, DNA was extracted from tail tip tissue by adding 100 µl DNA extraction buffer. Tissue preparation solution was left at room temperature for 10 minutes (min) and then heated to 98°C in a heat block for a further 3 min. 100 µl of Neutralisation Buffer was added to inhibit the digestive activity.

2.2.2 Primers

The following primers were purchased from Sigma and used in PCRs during the course of this study.

Table 2.1 Primer Sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequences</th>
<th>Annealing Temperature</th>
<th>Expected amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cre</td>
<td>Forward</td>
<td>CTGACCGTACACCAAAATTTGCCTG</td>
<td>53°C</td>
<td>389 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATAATCACAACATCTTCAAGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SRY</td>
<td>Forward</td>
<td>GAGGCACAAGTTGGCCCAGCA</td>
<td>60°C</td>
<td>266 bp</td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>GGTTCTGTGTCCACTGCAGAAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### 2.2.3 Polymerase chain reaction cycling conditions

Cycling conditions for Cre PCR:

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial template denaturation</td>
<td>98</td>
<td>30</td>
</tr>
<tr>
<td><strong>35X cycles</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Template denaturation</td>
<td>92</td>
<td>5</td>
</tr>
<tr>
<td>Primer annealing</td>
<td>53</td>
<td>30</td>
</tr>
<tr>
<td>Extension</td>
<td>68</td>
<td>30</td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>30</td>
</tr>
<tr>
<td>Hold</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

Cycling conditions for Sry PCR:

<table>
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<tr>
<th>Cycle</th>
<th>Temperature (°C)</th>
<th>Time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial template denaturation</td>
<td>98</td>
<td>30</td>
</tr>
<tr>
<td><strong>35X cycles</strong></td>
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</tr>
<tr>
<td>Template denaturation</td>
<td>92</td>
<td>5</td>
</tr>
<tr>
<td>Primer annealing</td>
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<td>Extension</td>
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<tr>
<td>Final extension</td>
<td>72</td>
<td>30</td>
</tr>
<tr>
<td>Hold</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

### 2.2.4 Polymerase chain reaction mix

20 μl PCR reactions (Table 2.2) were performed on DNA extracted from the tail tip tissues (Section 2.2.1). Both Cre and Sry PCR were performed on a Veriti thermal cycler (Thermo Fisher) using REDExtract-N-Ampa Tissue PCR Kit (Sigma) according to the manufacturer’s instructions. Refer to Section 2.2.2 for primer sequence and Section 2.2.3 for
cycling conditions of each PCR reaction. PCR products were run on a 1% agarose gel containing 0.5 μg/ml ethidium bromide at 100 volts for 90 min in TAE buffer. Gels were then photographed under UV light using Kodak gel logic 200 imaging system.

<table>
<thead>
<tr>
<th>Table 2.2 PCR reaction mix</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent</strong></td>
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<tr>
<td>DNA</td>
</tr>
<tr>
<td>Red PCR mix</td>
</tr>
<tr>
<td>Primer F (10 µM)</td>
</tr>
<tr>
<td>Primer R (10 µM)</td>
</tr>
<tr>
<td>Extraction Buffer</td>
</tr>
<tr>
<td>Neutralizing Buffer</td>
</tr>
<tr>
<td>dH₂O</td>
</tr>
</tbody>
</table>

2.3 Immunohistochemistry

2.3.1 Solutions

Phosphate Buffer Saline (PBS) was made by dissolving one PBS tablet (Sigma) in 200 ml distilled H₂O (dH₂O) (pH7.4).

High Salt PBS was made by dissolving 18 g of Sodium Chloride (Sigma) in 1 L of PBS.

15% Sucrose Solution was made by dissolving 15 g of sucrose (MERCK) in 1xPBS up to 100 ml.

30% Sucrose Solution was made by dissolving 30 g of sucrose (MERCK) in 1xPBS up to 100 ml.

4% Paraformaldehyde (PFA) was made by dissolving 2 g of PFA powder (Scharlau) in 1x PBS up to 50 ml, at 60°C and stored at -20°C.
1% Sodium dodecyl sulphate (SDS) was made by dissolving 2 g of SDS powder (Invitrogen) in 1x PBS up to 200 ml.

2% Bovine Serum Albumin (BSA) was made by dissolving 2 g of BSA powder (Sigma) in 1x PBS up to 100 ml.

2.3.2 Embryo collection

Mice were time mated, and mating was ascertained by detection of a vaginal plug. The day on which plugs were detected was designated E0.5. Mice were euthanized by cervical dislocation on the day of embryo collection. Embryos were removed from extra embryonic membranes and washed with dH2O. Embryos were decapitated and the bodies stored at -20°C for genotyping.

2.3.3 Tissue Fixation, Cryoprotection and Sectioning

Dissected embryonic heads were drop-fixed in 4% PFA at room temperature for 90 min. Tissues were then washed three times with PBS and then either processed for cryoprotection or left over-night at 4°C in the final PBS wash. For cryoprotection, tissues were sequentially immersed in 15% sucrose, 30% sucrose and half 30% sucrose and half Tissue-Tek Optimal Cutting Temperature (OTC) compound (Sakura), respectively at 4°C. The cryoprotected heads were mounted in OTC and flash frozen using liquid nitrogen. Tissues were flash frozen to reduce ice crystal formation and minimize any morphological damage. Frozen tissues were then sectioned at 10 μm thickness using a Leica CM3050-S cryostat and mounted on microscope slides (Super Frost Plus; Menzel-Glasher, Thermo Fisher Scientific), before proceeding with the desired histological analysis.

2.3.4 Tissue preparation for Immunohistochemical analysis

Day 1: Brain sections were washed with PBS for 15 min and then treated with 1% SDS for 4 min at room temperature. Treating sections with 1% SDS increase the permeability of the tissue membranes allowing antibody to reach intracellular antigens and unmask cryptic antigens in the tissue (Brown, D. et al., 1996). The tissue sections were washed three times with PBS for
5 min each, and then blocked with 2% BSA for 15 min at room temperature. Primary antibodies diluted in 2% BSA (Table 2.3), were then applied and allowed to incubate on the section overnight at 4°C.

Day 2: Primary antibodies were removed and sections washed twice with high salt PBS for 5 min followed by a 5 min PBS wash. Sections were then incubated with secondary antibody diluted in 2% BSA (Table 2.3) for 1 hour at room temperature. The secondary antibody was aspirated and sections were washed again with two 5 min high salts PBS washes followed by a 5 min PBS wash. Then tertiary antibody, diluted in 2% BSA, was applied and incubated for 1 hour at room temperature. Sections were washed again with two 5 min high salt PBS washes and one 5 min PBS wash. After the final wash, DAPI dissolved in the Prolong gold mounting media (Invitrogen) was applied, slides were coverslipped and sealed with nail polish. Sections were stored at 4°C in a light tight container until required.

2.3.5 EdU labelling

5-Ethynyl-2´-deoxyuridine (EdU) (Life Technologies) was dissolved in sterile PBS at a concentration of 10 mg/ml. EdU injection was performed as previously described (Ferland et al., 2009). Briefly, a single dose of EdU (50 mg/kg body weight) was injected intraperitoneally into pregnant females at E12.5 or E14.5. After 3 hours, embryos were harvested and processed for immunostaining as described in Section 2.3.4. To improve the immunodetection of nuclear staining, antigen recovery process was performed by incubating the sections with 10 mM sodium citrate (pH 6.0) buffer for 15 min in a steamer (>99°C inside the steamer). After the antigen recovery process, sections were washed with PBS before blocking with 2% BSA for 1 hour at room temperature. EdU detection was performed after immunostaining using Click-it EdU Alexa 594 imaging kit (Life Technologies) according to the manufacturer’s instructions. EdU positive cells were analysed either by confocal fluorescent microscope (Section 2.4) or Flow cytometry analysis using Beckman Coulter CyAn ADP.
2.3.6 Tunnel assay

Terminal transferase dUTP nick-end labelling (TUNEL) assay was performed to assess cell death in brain sections at desired embryonic stages using DeadEnd Fluorometric TUNEL system (Promega) according to the manufacturer’s instructions. Briefly, sections were permeabilised with 20 μg/ml Proteinase K solution for 10 min. Permeabilised sections were post fixed by immerse in 4% PFA and afterwards incubated in the TUNEL reaction mix for 1 hour at 37°C in a humidified atmosphere, in the dark. For positive controls, sections were incubated with DNase I (3000 IE/ml) to induce DNA strand breaks. TUNEL reactions mix lacking Terminal Deoxynucleotidyl Transferase recombinant enzyme was used as a negative control. TUNEL positive cells were analysed by confocal fluorescent microscope (Section 2.4).

2.3.7 Antibody Dilutions

Table 2.3 Antibody Dilutions used for immunohistochemistry analysis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Working Concentration</th>
<th>Source</th>
<th>Catalogue Number</th>
</tr>
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<tbody>
<tr>
<td>Primary Antibody</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USP9X</td>
<td>Rabbit</td>
<td>1/250</td>
<td>Bethyl Laboratories</td>
<td>A301-351A</td>
</tr>
<tr>
<td>USP9X</td>
<td>Mouse</td>
<td>1/100</td>
<td>Millipore</td>
<td>MABE352</td>
</tr>
<tr>
<td>p-PKC ζ</td>
<td>Rabbit</td>
<td>1/300</td>
<td>Santa Cruz</td>
<td>sc-12894-R</td>
</tr>
<tr>
<td>Prominin-1 (CD133)</td>
<td>Rat</td>
<td>1/200</td>
<td>Millipore</td>
<td>MAB4310</td>
</tr>
<tr>
<td>PAR-3</td>
<td>Rabbit</td>
<td>1/300</td>
<td>Millipore</td>
<td>07-330</td>
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<tr>
<td>PAR-6 (PARD6A)</td>
<td>Rabbit</td>
<td>1/300</td>
<td>Santa Cruz</td>
<td>sc-25525</td>
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<td>aPKCλ</td>
<td>Rabbit</td>
<td>1/100</td>
<td>Santa Cruz</td>
<td>sc-11399</td>
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<tr>
<td>Scribble (C-20)</td>
<td>Goat</td>
<td>1/200</td>
<td>Santa Cruz</td>
<td>sc-11049</td>
</tr>
<tr>
<td>β-Catenin</td>
<td>Mouse</td>
<td>1/1000</td>
<td>BD Transduction</td>
<td>610153</td>
</tr>
<tr>
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### MATERIALS AND METHODS

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<td>Abcam</td>
<td>Ab15580</td>
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**Secondary Antibody**

| Biotinylated Goat Anti-rabbit | Goat | 1/200 | Abcam | ab6720 |
| Streptavidin, Alexa Fluor 488 | Mouse | 1/400 | Abcam | ab10020 |
| Alexa 594 (Goat-anti-mouse)   | Goat  | 1/1000| Invitrogen | A11005 |
| Cy3 (Rabbit-anti-Rat)         | Rabbit | 1/400 | Millipore | AP136C |
| Alexa 488 (Chicken-anti-goat) | Chicken | 1/200 | Invitrogen | A-21467 |
MATERIALS AND METHODS

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<th>1/400</th>
<th>Invitrogen</th>
<th>A-11034</th>
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</table>

2.4 Microscopy of sections

Tissue specimens were visualised on an Olympus FV1000 (Tokyo, Japan) fluorescence microscope and images were taken using the FV10-ASW 3.0 Viewer programme.

2.5 Quantitative Analysis

For all the experiments, at least three brain sections from at least three different animals were analysed per genotype. The quantification of NPs and neurons was performed on neocortical sections of Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> brains at corresponding anatomical levels, by staining for Sox2 and Doublecortin (Dcx), respectively. The cortex is defined as the area from the cortical hem to the pallial-subpallial boundary in each confocal image. The thickness of the germinal layer was measured in a confocal z-stack image stained with Sox2 by first drawing a line at right angles to the ventricular surface, from the apical surface to the pial surface. The length of this line was measured by the ImageJ 1.60s software (National Institute of Health) and considered as the total cortical thickness. A second line was drawn from the apical to basal side of the Sox2<sup>+</sup> band with the same angle to the ventricular surface and this length was considered as the thickness of the band of NPs. The width of the NP enriched germinal layer was calculated as a proportion to the overall thickness of the cerebral cortex at five different points along the rostral-caudal axis in each image and the mean value of this proportion was used for all the statistical analysis (Section 2.10). The same analytical method was conduct for the DCX<sup>+</sup> cortical plate (Haubst et al., 2004).

The quantification of mitotic cells at the ventricle was performed by counting the number of Phospho-Histone 3 positive (PH3<sup>+</sup>) cells and divided by the length of the rostral-caudal axis of the lateral ventricle. Cells undergoing mitosis in an abventricular position were counted separately by identifying PH3<sup>+</sup> cells, which were located five nuclear lengths away from the ventricular surface along the dorsal-ventral axis (Smart, 1976).

Tbr2<sup>+</sup> and EdU<sup>+</sup>/Tbr2<sup>+</sup> INPs were counted semi-blindly with Adobe Photoshop Cs5 (Adobe Systems, San Jose, CA). In briefly, green, red and blue channels were used to identify
Tbr2+, EdU+ and DAPI stained cells, respectively. Tbr2+ nuclei were selected using the pixel-selection tool then counted using the measurement tool. The number of Tbr2+ cells were normalised against the total area of the neocortex. The data was presented as the number of cells/µm². EdU+/Tbr2+ cells were selected by merging the green and red channel using the image calculation tool. The number of double label cells was counted using the measurement tool and normalised against the total number of EdU+ cells. The same analytical method was used to count the EdU+/Ki67+ cells.

Lamination markers Stab2+ and Ctip2+ cells were counted semi-blindly with Adobe Photoshop Cs5 (Adobe Systems, San Jose, CA) as described above. The number of Stab2+ and Ctip2+ cells was normalised against the total number of cells in the cortex as marked by DAPI, and the data were presented as a ratio. Tbr1+ mature neuronal cell number was counted semi-blindly with Adobe Photoshop Cs5 (Adobe Systems, San Jose, CA) as described above. The number of Tbr1+ cells were normalised against the total are of the neocortex.

2.5.1 Cell cycle analysis

For all experiments, at least three coronal brain serial sections from at least three different animals were analysed per genotype. Brain sections were chosen at the mid-anterior level of the brain. Newly generated Tbr2+ INPs were assessed by staining EdU pulsed E12.5 and E14.5 brains sections with Tbr2 antibodies. Stained cells were counted semi-blindly using Adobe Photoshop Cs5 (Section 2.5) (Adobe Systems, San Jose, CA). Newly generated INPs are EdU+/Tbr2+ and the data was presented as the ratio of newly generated IPs to the total number of Tbr2+ INPs present in the section.
2.6 SDS PAGE and Western Blotting

2.6.1 Reagents and Solutions

SDS Lysis buffer was made by mixing 10% (4 ml of 100% stock) glycerol, 1% (2 ml of 1% stock) SDS, 10 mM (1 ml of 2 mM stock) Tris pH 7.4 and 10 µl/ml Sigafast Protease Inhibitor (Sigma-Aldrich) in 20 ml of dH2O.

2x SDS-PAGE Loading Buffer was made by mixing 10 mM (4 ml of 0.5 M stock) Tris pH 6.8, 4% (8 ml of 1% stock) SDS, 20% (4 ml of 10% stock) glycerol and 20-30 µL of Bromophenol blue in 20 ml of dH2O. 100 mM (1:10 dilution from a 1 M stock) of DDT was added just prior to use.

SDS Running Buffer was made by mixing 14.4 g glycine, 3.05 g Tris, and 10 ml of 10% SDS in 1 L of dH2O.

2x Transfer Buffer was made by mixing 28.8 g glycine, 6.10 g Tris, 200 ml Methanol in 1 L of dH2O.

10x TBS was made by adding 24.23 g Tris pH 7.4-7.6, 80.06 g NaCl in 1 L of dH2O. The pH of the solution was adjusted by adding concentrated HCl.

TBS Tween was made by mixing 1 M TBS, 1 ml Tween 20 in 90 ml of dH2O.

Ponceau S Stain Solution was made by adding 0.1% Ponceau S and 1% acetic acid in 50 ml of dH2O.

2.6.2 Brain Collection and Preparation

Timing of embryonic development and isolation of embryos was determined as above (Section 2.3.2). The brains were dissected but only the cerebral cortex was used for the analysis. The dissected tissues were collected into a 1.5ml microfuge tube (Eppendorf) and immediately flash frozen using liquid nitrogen, then stored at -80°C until the completion of genotyping.
2.6.3 Protein Extraction

Flash frozen brains were taken from -80°C, washed three times with 5 ml of cold PBS prior to adding the SDS-PAGE lysis buffer. Tissues were homogenized by mechanical pipetting and sonication. The sheared DNA and insoluble cellular debris were pelleted by centrifuging the tissue lysate at 4°C at 14000 revolutions per minute (RPM) for 20 min. The supernatant was carefully extracted and transferred into a fresh 1.5 ml microfuge tube (Liu et al., 2014).

2.6.4 Protein Estimation

Protein concentration was estimated using a Bicinchoninic acid (BCA) kit (Thermo Scientific Pierce) according to the manufacturer’s instructions. 20 µg of protein from each brain was loaded into wells on SDS-PAGE gels.

2.6.5 SDS preparation

SDS-PAGE loading buffer was prepared as described in Section 2.6.1. Estimated protein volume (as described in Section 2.6.4) along with an equal amount of 2x SDS-PAGE loading buffer was added to 1.5 ml microfuge tubes. Tubes were then heated for 5 min at 95°C promoting the further denaturation of the proteins by increase the binding affinity of the SDS and activating the reducing agent. Samples were either stored at -20°C or loaded directly into 6-10% SDS-PAGE gel. 2.5 µl of pre-stained protein ladder (Thermo Scientific) was loaded into the first well of the gel to determine the position of the targeting protein and running distance of the gel. Samples were then run at 100 volts for 120 min, with the gel immersed in SDS running buffer.

2.6.6 Western blot

Gel was removed from the caster and transferred onto a piece of blotting paper soaked with transfer buffer, while the transfer rig was assembled as per manufacturers’ instruction
MATERIALS AND METHODS

(BioRad). Transfer was performed at 100 volts over 3 hours at 4°C. Gel was removed from the caster and transferred onto a piece of blotting paper soaked with transfer buffer, until the transfer rig was assembled. At the end of the transfer, membrane was separated from the cassette and blotted dry on a blotting paper. Then, either stored at 4°C between two blotting papers in a clip-seal bag or processed immediately for immunodetection. The membrane was then blocked with TBS blocking buffer (Licor), if using fluorescence secondary antibody or, 5% skim milk, if using horseradish peroxidase (HRP) conjugated secondary antibody, for 1 hour to avoid non-specific binding of the antibody. Next immunoblotting was commenced (Section 2.6.7).

2.6.7 Immunoblotting

Primary antibody (Table 2.4) diluted in the TBS blocking buffer (Licor) or 5% skim milk, depending on the use of fluorescent or HRP tagged secondary antibodies, was added to the membrane and incubated over night at 4°C on a rocking platform. The following day the membrane was washed three times with TBST buffer and the secondary antibody (Table 2.4), diluted in the TBS blocking buffer (fluorescent tagged) or 5% skim milk (HRP tagged), was added and incubated for 1 hour at room temperature. Then the membrane was washed three times with TBST buffer on an orbital shaker. After the last wash, Electrogenerated chemiluminescence (ECL) substrate (Millipore) was added, and the membrane was visualized on a VersaDoc Imaging station (BioRad). Membranes probed with fluorescence tagged secondary antibodies were imaged on the Odyssey FC Imaging station (Licor). Refer to Section 2.6.8 for reprobing procedures.
Table 2.4 Antibody Dilution used for Western blot analysis.

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2.6.8 Reprobing membrane

After visualization, the membrane was washed with TBS Tween to rinse off the ECL reagent and then re-blocked for 10 min at room temperature. Procedures were re-performed as per Section 2.6.7.

2.7 Immunoprecipitation

2.7.1 Solutions

**IP Lysis buffer** – 20 mM HEPES (pH 7.8), 400 mM KCl, 5 mM EDTA, 0.4% NP40, 10% glycerol, 1 mM DTT, protease and phosphatase inhibitor cocktails (Cell Signalling) (Azzolin et al., 2014).

**Binding buffer** – 20 mM HEPES (pH 7.8), 100 mM NaCl, 2.5 mM MgCl₂, 1% Triton X-100, 5% glycerol, .05% NP40 (Azzolin et al., 2014).

2.7.2 Co-immunoprecipitation

Frontal cortices were extracted from embryonic mouse brains as described in Section 2.5. Extracted cortical tissues were lysed with IP lysis buffer (Section 2.7.1). Immunoprecipitation (IP) was conducted using Pierce co-immunoprecipitation kit (Thermo Scientific, Cat# 26149) according to manufacturer’s instructions. In brief, cortical tissue lysate was pre-cleared to avoid unspecific binding by incubating in a spin column containing control agarose resin for 120 min at 4°C. Pre-cleared lysate was then incubated in a spin column containing AminoLink Plus coupling resin attached with 5-10 µg of primary antibody. Resin bound prey protein complex was eluted using binding buffer (Section 2.7.1). For every IP an IgG control was performed. The eluted protein complex was then run on SDS-PAGE, and later western blot (WB) analysis was conducted to identify the protein in the prey protein-complex.
2.8 Transcriptomic Analysis

2.8.1 Microarray Analysis

For each genotype, four embryos were used for both microarray and Quantitative real time polymerase chain reaction (qRT-PCR) analyses. Frontal cortices of the each animal were dissected, and meninges removed before snap freezing with liquid nitrogen. Flash frozen tissues were stored at -80°C until further use. Total RNA was isolated using the RNeasy Micro kit (Qiagen). Total RNA concentration was determined using the NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies) and RNA integrity was confirmed using the Agilent 2100 Bioanalyzer RNA 600 NanoChip (Agilent). Initially, 250 ng of total RNA from each sample was amplified using the Illumina RNA TotalPrep Amplification kit (Ambion) following the manufacturer's instructions. The in-vitro transcription reaction was conducted for 18 hours including the labelling of the cRNA by biotinylation. Labeled and amplified material (1.5µg/sample) was hybridized to Illumina MouseRef-8 v2.0 Expression BeadChips (Illumina) at 55°C for 18 hours according to the Illumina BeadStation 500x protocol. Arrays were washed and then stained with 1 µg/ml cyanine3-streptavidin (GE Healthcare). An Illumina Beadstation was used to scan the arrays. Samples were initially evaluated using the BeadStudio software from Illumina. Quality control reports were satisfactory for all samples. Data was processed and quantile normalised using R/BioConductor lumi package as described previously (Cook et al., 2011). Differentially expressed transcripts were identified using the limma package -linear models for microarray (Smyth, 2005), at p ≤ 0.05. Functional over-representation analysis was determined using right-tailed Fisher's exact test using Ingenuity Pathway Analysis (IPA) 8.5 (Ingenuity Systems).

2.8.2 Quantitative real time Polymerase chain reaction

RNA was extracted from telencephalic tissue as described in the Section 2.8.1. For each genotype, four embryos were used. Reverse transcription was performed using the High capacity cDNA reverse transcription kit (Applied Biosystem). A 1 µg of RNA was reverse transcribed with random primers. Synthesised cDNA was diluted 1/100 with RNase/ DNase free water and 10 µl of these dilutions was used for each qRT-PCR reaction containing 10 µl of 2x SensiFAST mix and 0.8 µl of 10 µM concentration forward and reverse primers (Table
2.5. qRT-PCR was performed in a Rotor-Gene 3000 (Corbett Life Science). The data was analysed with Linregpcr analysis software (Heart Failure Research Centre, Netherland). The relative expression was calculated by using the delta Ct method, and the housekeeping gene GAPDH was used a relative standard (Schmittgen and Livak, 2008). All the samples were tested in triplicate.

**Table 2.5 Primer Sequences**

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### 2.9 Mammalian tissue culture

#### 2.9.1 Cell Counting

Cell counts were performed on single cell suspension by diluting 10 μL of the suspension with 10 μL of trypan blue (Sigma-Aldrich). 10μL of the mixture was then loaded onto a haemocytometer and counted under the 10X magnification using an Olympus CKX41 microscope (Olympus).
2.9.2 ReNcell-VM cells

Immortalized human NP cell line ReNcell-VM was obtained from Millipore (Darmstadt, Germany) and maintained as described Donato et al. (2007). In brief, ReNcell-VM cells were cultured in Dulbecco’s Modification of Eagle’s Medium (DMEM) (Life Technologies) with 2 mM L-Glutamine (Life Technologies), gentamicin 50 µg/ml (Life Technologies), 1X B27 neural cell supplement mix (Life Technologies), 10 units/ml Heparin, 20 ng/ml EGF (Chemicon Cat # GF144) and 20 ng/ml FGF (Chemicon Cat # GF003). Flasks were treated with mouse 0.5 mg/ml mouse laminin 3 hours prior to cell seeding. Media was changed every two to three days and cells were passaged upon achieving 70-90% confluence using TrypLE enzyme solution (Life Technologies).

2.9.3 Generation of inducible USP9X knock-down ReNcell-VM

To generate ReNcell-VM cells with doxycycline-inducible knock-down (KD) of USP9X, cells were transduced with a lentiviral vector (Brown, C. Y. et al., 2010) containing either a scrambled short hairpin RNA (shRNA) sequence 5’ACTACCGTTGTTATAGGTGTTCAAGAGACACCTAACAA CGGTAGT3’ with no homology to any known gene, or shRNA targeting Usp9x sequences 5’GCCATAGAAGGCACAGGTAGT3’. Successfully transduced pools of ReNcell-VM cells were isolated by FACS based on green fluorescence protein (GFP) expression (Brown, C. Y. et al., 2010). These transduced pools were used in subsequent experiments. Induction of shRNA expression was achieved by supplementing the media with 1 μM doxycycline (Sigma Aldrich).

2.9.4 Generation of inducible USP9X knock-down HEK293 cells

HEK293 cells were cultured in DMEM (Life Technologies) medium supplemented with 10% fetal calf serum and 1% Penicillin Streptomycin. A pool of four USP9X short interfering RNA (siRNAs) obtained from Millenium Science (SMARTpool ON-TARGETplus USP9x siRNA Cat# L-006099-00-0005) was used to knockdown USP9X. Scrambled siRNA (ON-TARGETplus Non-targeting Pool Cat# D-001810-10-05) was used as control. USP9X
siRNA interference was conducted using Thermo Scientific DharmaFECT Transfection Protocol (Thermo Scientific) in accordance with the manufacturer’s instructions. In brief, HEK293 cells were seeded at 100,000 cells/well into a 6 well plate 24 hours prior to transfection. 50 nM final working concentration of siRNA was achieved by diluting the stock siRNA in Serum-Free OptiMEM media (Life Technologies). 10 μl of DharmaFECT (Millenium Science) reagent was used as the transfection reagent. Cells were harvested or used for other biochemical assays 72 hours after the initial transfection.

### 2.9.5 Chemical activators and inhibitors

WP1130 (Cayman) was used to chemically inhibit USP9X deubiquitylating activity in ReNcell-VM. Cells were treated with WP1130 for 4 hours prior to biochemical analysis and DMSO was used as vehicle control. Wnt3a (R&D system) and DKK1 (Sigma Aldrich) was used to activate and inhibit Wnt signaling in-vitro, respectively (Table 2.6). Cells were treated with Wnt3a or DKK1 for 1 hour prior to harvest. Activation or inhibition of the Wnt pathway was confirmed by qRT-PCR analysis (Section 2.8.2).

<table>
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</tr>
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</tbody>
</table>

Table 2.6 Chemical inhibitors and activators

### 2.9.6 Luciferase assay

USP9X expression was depleted (KD) in HEK293 cells using USP9X siRNA as described in Section 2.9.4. USP9X KD HEK293 cells were then transfected with plasmids pGL3-TOP and pGL3-FOP containing TCF luciferase-reporter construct (Molenaar et al., 1996) using Lipofectamine 2000 reagent (Invitrogen). Wnt3A or DKK1 added to the cells 24 hours after the transfection. 1 hour after the Wnt3a and DKK1 treatment, cells were lysed using luciferase lysis buffer (Promega). To measure the luciferase activity, 10 μl of cell lysate was transferred to a black 96-well Optiplate (Perkin Elmer). Luciferase activities were measured...
using the Dual-Luciferase-reporter assay system (Promega) according to the manufacturer’s instructions. Transfection efficiency was normalised using the co-transfected Renilla luciferase activity. The data were presented as the mean ± standard error of the mean (SEM) of the ratio between the TOP/FOP reporters. All the transfection experiments were performed in triplicate.

2.9.7 Neurospheres assay

Isolation and culture of NPs from the E18.5 cortex was performed as previously described (Homan et al., 2014). Briefly, cortices were extracted from E18.5 mice using dissection media made of cold Hanks Buffered Salt solution (Invitrogen), 10 mM HEPES (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 0.5% glucose (Sigma-Aldrich), and 1 x penicillin/streptomycin (Invitrogen). Extracted cortices were then digested using digestion mix contain 1:1 mixture of PAP (L15 Media, 30 U/ml papin, 0.24 mg/ml cysteine, 40 μg/ml DNase) and Ovomucoid (L15 media, 0.86 mg/ml ovomucoid trypsin inhibitor, 0.5 mg/ml BSA, 40 ug/ml DNase) at 37°C for 45 min. Tissue were then homogenised with a flame polished pipette. Tissue digestion was then neutralized by adding cultural media without supplements. Neutralised digestion mix was then centrifuged at 1000 RPM and resultant supernatant was aspirated. Remaining cells were resuspended in 1 ml of full neurosphere culture media (DMEM, 2% B27, 20 ng EGF, 20 ng FGF, 1% Penicillin Streptomycin) before transferring into a flask. Spheres were passaged every four days based on the average neurosphere diameter.

2.10 Statistical Analysis

Statistical analysis was performed using Prism 5 (GraphPad Prism). For all analysis at least three biological repeats were assessed. Statistical analysis was perform using one-way ANOVA and Tukey’s post-hoc tests, for experiments containing more than two samples, or with a Student T-test for data sets with only two samples. The error bars on all the graphs represent SEM. All the values were presented as mean ± SEM.
CHAPTER 3

Usp9x regulation of intrinsic fate determinants – Cell polarity and Adherens Junctions
3.1 Introduction

Cell adhesion and apical-basal polarity are key intrinsic fate determinant factors of NPs (Taverna et al., 2014). AJ s are the predominant cell-cell adhesive structures in NPs. During NP fate specification, AJ s and polarity function very closely to balance symmetric versus asymmetric cell divisions (Stocker and Chenn, 2015). The Usp9x substrates AF-6 (Taya et al., 1998) and β-catenin (Taya et al., 1999) are key structural components of AJ s. Ablation of Usp9x significantly decreases the β-catenin and AF-6 protein levels in polarised MDCK and embryonic stem cells, perturbing their cell adhesion (Taya et al., 1998; Taya et al., 1999; Murray et al., 2004). In addition, Usp9x colocalized with cadherin-catenin heterodimers at multiple protein trafficking points in sub-confluent epithelial cells suggesting Usp9x may also be involved in AJ establishment (Murray et al., 2004). Usp9x also activates the polarity regulatory proteins NUAK1 and MARK4 in polarised cells (Al-Hakim et al., 2005; Al-Hakim et al., 2008), and apical-basal polarity was upregulated in Usp9x overexpressed embryonic stem cell derived NPs (Jolly et al., 2009) suggesting that in addition to AJ s, Usp9x could also regulates NP cell polarity. In light of these studies, the aim of this chapter was to determine the functional consequence, if any, of Usp9x deletion on apicobasal polarity and adhesion in mouse embryonic NPs, and what affect this may have on their fate.

3.2 Establishing the earliest Usp9x ablated time point in Usp9x<sup>-/-</sup> neural progenitors

In order to investigate the role of Usp9x in AJ s and polarity regulation, the earliest embryonic stage when Usp9x protein completely depleted from Usp9x<sup>-/-</sup> brains was established using immunohistochemical (IHC) and WB analysis. Previous study from our lab showed the splicing of Exon3 and complete depletion of Usp9x proteins in E18.5 Usp9x<sup>-/-</sup> brains (Stegeman et al., 2013). As described in Section 2.1.3, Usp9x<sup>-/-</sup> mice were generated by mating Usp9x<sup>lox/lox</sup> female mice with Nestin-Cre male mice. Under the Nestin promoter Cre recombinase proteins start expressing in NPs around E10.5 (Imai et al., 2006). However, little difference in Usp9x protein level was observed in Usp9x<sup>-/-</sup> brains until E12.5. As described in previous studies, in E12.5 Usp9x<sup>-/-</sup> brains Usp9x showed a robust immune reactivity throughout the neocortex with an enriched expression near the apical surface of the VZ (Xu et al., 2005; Jolly et al., 2009; Stegeman et al., 2013). Compared to its littermate controlled Usp9x<sup>+/+</sup> neocortices, Usp9x immunoreactivity was completely depleted from E12.5 Usp9x<sup>-/-</sup>
neocortices (n=7) (Figure 3.1). This was confirmed using WB analysis of cortical tissue lysates from Usp9x+/Y and Usp9x−/Y brains (n=8) (Figure 3.1). These results established the complete depletion of Usp9x protein from NPs and their subsequent progenies occurs in E12.5 Usp9x−/Y brains.
3.1 Complete depletion of Usp9x protein from E12.5 Usp9x⁻/⁻ neocortices. (A) Coronal sections of three independent E12.5 Usp9x⁺/⁺ and Usp9x⁻/⁻ neocortices stained with Usp9x antibodies. Usp9x immunoreactivity is completely depleted from Usp9x⁻/⁻ neocortices (b, d, f) compared to littermate controls (a, c, d). (B) Westernblot analysis conducted on separate Usp9x⁺/⁺ and Usp9x⁻/⁻ (n=3) cortical tissue confirmed the complete depletion of Usp9x protein from E12.5 neural progenitors and their subsequent progeny. The faint bands observed in the Usp9x⁻/⁻ lanes were likely due to contamination from non-neural tissues such as meninges and blood vessels. Scale bars = 80 µm. LV- lateral ventricle, VZ- ventricular zone.
3.3 Assessing the effect of Usp9x deletion on adherens junctions in E12.5 Usp9x<sup>-/-</sup> neural progenitors

After confirming Usp9x proteins were completely depleted in Usp9x<sup>-/-</sup> neocortices from E12.5 onwards, the effects of Usp9x deletion on NP AJs and polarity were examined using series of IHC and WB analyses. AJs are located at the most apical end of the basolateral membrane of the NPs (Paridaen et al., 2013). The Usp9x substrate AF-6 is a structural component of the AJs. Usp9x rescues AF-6 from proteasomal degradation (Taya et al., 1998; Kanai-Azuma et al., 2000; Pantaleon et al., 2001). AF-6 expression was assessed in Usp9x<sup>-/-</sup> cortices using IHC analysis, and consistent with the previous reports AF-6 staining forms a tight continuous band at the apical surface of the VZ in Usp9x<sup>+/+</sup> brains, where AJs are localized (Figure 3.2A). In contrast, AF-6 immunoreactivity was reduced in the apical surface of Usp9x<sup>-/-</sup> VZ (n=4) (Figure 3.2A) indicating downregulated AF-6 expression in Usp9x<sup>-/-</sup> brains. To confirm this AF-6 downregulation, WB analysis was conducted using E12.5 Usp9x<sup>+/+</sup> and Usp9x<sup>-/-</sup> cortical lysates. Corroborating the previous result, WB analysis showed a significant reduction in AF-6 protein level relative to β-tubulin loading control in Usp9x<sup>-/-</sup> cortical lysates compared to littermate controls (n=4) (Figure 3.2B, C).

The AJ structural protein β-catenin is also a known substrate of Usp9x. Overexpression of the catalytic domain of Usp9x rescued β-catenin from its proteasomal degradation in MDCKII cells, while knockdown of Usp9x using siRNA reduced β-catenin expression in MCF-7 breast cancer cells (Taya et al., 1999; Ouyang et al., 2016). Consistent with previous studies (Kosodo et al., 2004; Cappello, Silvia et al., 2006; Costa et al., 2008; Thompson et al., 2008), strong β-catenin immunoreactivity was observed at the apical surface of Usp9x<sup>+/+</sup> VZ forming a tight band similar to AF-6. Surprisingly, but in contrast to AF-6 expression and previous studies, β-catenin showed a higher immunoreactivity in Usp9x<sup>-/-</sup> brains compared to Usp9x<sup>+/+</sup> brains (n=5) (Figure 3.3A). Closer examination revealed that in Usp9x<sup>-/-</sup> NPs β-catenin expression was significantly high in the cytoplasm and its expression extended into the basolateral membrane compared to constricted apical expression in Usp9x<sup>+/+</sup> NPs. WB analysis confirmed the significantly increased β-catenin protein levels in Usp9x<sup>-/-</sup> neocortices compared to Usp9x<sup>+/+</sup> (n=6) (Figure 3.3B).
Figure 3.2 Reduced AF-6 expression in E12.5 Usp9x<sup>−/−</sup> brains. (A) Coronal sections of two independent E12.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained for AF-6. Compared to Usp9x<sup>+/+</sup> (a, c) AF-6 immunoreactivity was markedly reduced at the apical surface of Usp9x<sup>−/−</sup> VZ (white arrows) (b, d). (B) Western blot analysis showing relatively reduced AF-6 protein levels in E12.5 Usp9x<sup>−/−</sup> cortices (n=4). (C) Quantification of AF-6 protein level relative to β-tubulin. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test, *, p < 0.05.
**Figure 3.3 Increased β-catenin expression in E12.5 Usp9x<sup>−/+</sup> brains.** (A) Coronal sections of two independent E12.5 Usp9x<sup>−/+</sup> and Usp9x<sup>−/−</sup> neocortices stained for β-catenin. Compared to Usp9x<sup>−/+</sup> (a, b) β-catenin immunoreactivity markedly increased in Usp9x<sup>−/−</sup> VZ (white arrows) (c, d). β-catenin expression was clearly increased in the cytoplasm and basolateral membrane of Usp9x<sup>−/−</sup> NPs (yellow arrows). (B) Westernblot analysis showing increased β-catenin protein levels in E12.5 Usp9x<sup>−/−</sup> cortices (n=3). (C) Quantification of β-catenin protein level relative to β-tubulin. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
Perturbed AF-6 and β-catenin expression in $Up{9x}^{-/Y}$ brains suggest perturbed AJs in $Up{9x}^{-/Y}$ NPs. To confirm this notion, further analyses were conducted targeting the core AJ structural component, N-cadherin (Kadowaki et al., 2007; Zhang et al., 2013). Similar to AF-6 expression, N-cadherin immunoreactivity was greatly reduced at the apical surface of the $Up{9x}^{-/Y}$ VZs compared to $Up{9x}^{+/Y}$ (n=4) (Figure 3.4A). Significantly reduced N-cadherin protein levels in $Up{9x}^{-/Y}$ cortices was confirmed by WB analysis (n=3) (Figure 3.4B, C). Taken together, reduced AF-6 and N-cadherin expression and increased but distorted β-catenin expression confirmed the perturbed AJs in E12.5 $Up{9x}^{-/Y}$ NPs.

To further analyse the integrity of the AJs, co-IP was conducted using β-catenin as the bait protein. The same amount of E12.5 $Up{9x}^{+/Y}$ and $Up{9x}^{-/Y}$ cortical lysates were loaded into the each column. At AJs cadherin protein maintains a 1:1 ratio with catenin proteins (Niessen and Gottardi, 2008). Relative to the inputs, similar amounts of N-cadherin protein were immunoprecipitated from E12.5 $Up{9x}^{+/Y}$ and $Up{9x}^{-/Y}$ cortical lysate (Figure 3.5). Surprisingly, despite the decreased N-cadherin expression in E12.5 $Up{9x}^{-/Y}$ neocortices, N-cadherin proteins remained bound to β-catenin (Figure 3.5), suggesting the cadherin-catenin heterodimer is intact in $Up{9x}^{-/Y}$ NPs.
**USP9X REGULATION OF INTRINSIC FATE DETERMINANTS**

**CHAPTER 3**

**Figure 3.4 Reduced N-cadherin expression in E12.5 Usp9x<sup>−/−</sup> brains.** (A) Coronal sections of two independent E12.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained for N-cadherin. Compared to Usp9x<sup>+/+</sup> (a, c) N-cadherins immunoreactivity was clearly reduced at the apical surface of Usp9x<sup>−/−</sup> VZ (white arrows) (b, d). (B) Western blot analysis showing significantly reduced N-cadherin protein levels in E12.5 Usp9x<sup>−/−</sup> cortices (n=3). (C) Quantification of N-cadherin protein level relative to β-tubulin. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
### Figure 3.5 Co-immunoprecipitation of E12.5 neural progenitors’ adherens junction components

Co-immunoprecipitation of N-cadherin from E12.5 Usp9x\textsuperscript{+/Y} and Usp9x\textsuperscript{-/-} cortical lysates, using β-catenin as the bait protein. Relative to inputs, similar amounts of N-cadherin were pulled down from Usp9x\textsuperscript{+/Y} and Usp9x\textsuperscript{-/-} brains suggesting N-cadherin remained binding to β-catenin with great affinity even in E12.5 Usp9x\textsuperscript{-/-} NPs.

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<tr>
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</tr>
<tr>
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<td>β-tubulin</td>
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#### 3.3 Assessing the effect of Usp9x deletion on cell polarity in E12.5 Usp9x\textsuperscript{-/-} neural progenitors

Usp9x could regulate cell polarity through its substrates NUAK1 and MARK4 (Al-Hakim et al., 2005; Al-Hakim et al., 2008). In addition, a modest overexpression of Usp9x in mouse ESC derived NPs, significantly increased their polarity suggesting a direct functional correlation between Usp9x and NP cell polarity. In NPs, AJs are essential for establishing and maintaining apical-basal polarity. Apical polarity proteins Par3 and aPKC physically interact with AJs at the apical domain of NPs (Marthiens and ffrench-Constant, 2009). Thus AJs and polarity function in a mutually inclusive manner during NP fate specification. Due to the functional correlation between Usp9x and NP apicobasal polarity and perturbed AJs observed in E12.5 Usp9x\textsuperscript{-/-}, the next set of experiments were designed to examine the effect of Usp9x ablation on NP polarity.

Similar to its epithelial counterparts, NP progenitor polarity is achieved and maintained by the action of three polarity protein complexes (Assemat et al., 2008). aPKC is part of the apical Par protein complex, which is localized at the AJs of NPs. In mammals there are two
aPKC isotypes, aPKCλ and aPKCζ. Both of these isotypes are expressed in mammalian NPs and have been implicated in NP polarity and AJ formation (Imai et al., 2006; Ghosh et al., 2008). aPKCλ expression was assessed in E12.5 Usp9x⁻/Y brains by IHC analysis. Like AF-6 and N-cadherin expression, aPKCλ showed a reduced immunoreactivity at the apical surface of the Usp9x⁻/Y VZ compared to Usp9x⁺/⁺ (n=3) (Figure 3.6A). WB analysis confirmed the reduced aPKCλ immunoreactivity in Usp9x⁻/Y VZ was due to decreased aPKCλ protein levels (n=4) (Figure 3.6 B, C). Taken together, the IHC and WB analysis of aPKCλ suggest polarity was perturbed in Usp9x⁻/Y NPs. To further confirm this result, IHC analysis was conducted targeting Par3 and Scribble proteins. Par3 is another component of the Par protein complex that structurally attached to aPKC near AJs (Costa et al., 2008). While Scribble is part of the basal polarity protein complex Scribble (Assemat et al., 2008). Similar to aPKCλ expression, Par3 and Scribble immunoreactions were significantly downregulated in Usp9x⁻/Y VZ compared to Usp9x⁺/⁺ (n=3) (Figure 3.7). In addition to these polarity proteins, expression of Prominin1 (CD133), a well-established transmembrane protein localized at the apical domain of the NPs (Florek et al., 2005), was also compared between E12.5 Usp9x⁻/Y and Usp9x⁺/⁺ brains. Similar to other polarity markers Prominin1 expression was also reduced in the Usp9x⁻/Y VZ compared to Usp9x⁺/⁺ (n=3) (Figure 3.8).
Figure 3.6 Reduced levels of aPKCλ in E12.5 Usp9x<sup>−/−</sup> brains. (A) Coronal sections of two independent E12.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained with aPKCλ antibody. Compared to Usp9x<sup>+/+</sup> (a, c) aPKCλ immunoreactivity was reduced from the apical surface of Usp9x<sup>−/−</sup> VZ as indicated by the white arrows (b, d). (B) Western blot analysis showing relatively reduced aPKCλ protein levels in E12.5 Usp9x<sup>−/−</sup> cortices (n=4). (C) Quantification of aPKCλ protein level relative to β-tubulin. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
Figure 3.7 Reduced immunofluorescence staining signals for apical and basal polarity proteins in E12.5 Usp9x<sup>+/Y</sup> brains. (A) Coronal sections of two independent E12.5 Usp9x<sup>+/Y</sup> and Usp9x<sup>−/Y</sup> neocortices stained for apical polarity protein Par3. Compared to Usp9x<sup>+/Y</sup> (a, c) Par3 immunoreactivity was markedly decreased from the apical surface of Usp9x<sup>−/Y</sup> VZ (b, d) as indicated by white arrows. (B) Coronal sections of two independent E12.5 Usp9x<sup>+/Y</sup> and Usp9x<sup>−/Y</sup> neocortices stained for basal polarity protein Scribble. Similar to Par3 expression, Scribble immunoreactivity was greatly reduced in the Usp9x<sup>−/Y</sup> VZ compared to Usp9x<sup>+/Y</sup> VZ (white arrows). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.
Figure 3.8 Reduced immunofluorescence staining signals for Prominin1 expression in E12.5 Usp9x<sup>-/-</sup> brains. (A) Coronal sections of two independent E12.5 Usp9x<sup>+/Y</sup> and Usp9x<sup>-/-</sup> neocortices stained for apical polarity protein Prominin1. Compared to Usp9x<sup>+/Y</sup> (a, c) Prominin1 immunoreactivity was clearly reduced from Usp9x<sup>-/-</sup> VZ (b, d) as indicated by white arrows. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.

Therefore, the combining down regulated polarity and adherens junctional proteins (with an exception of β-catenin upregulation) confirmed that AJs and cell polarity are perturbed in E12.5 Usp9x<sup>-/-</sup> NPs.

3.4 Assessing the effect of Usp9x deletion on adherens junctions and cell polarity in E14.5 Usp9x<sup>-/-</sup> neural progenitors

Expression of these adherens junctional and polarity markers were examined 48 hrs later at E14.5. Similar to E12.5, Usp9x is robustly expressed throughout the E14.5 Usp9x<sup>-/-</sup> cortices and Usp9x protein levels were remained ablated in E14.5 Usp9x<sup>-/-</sup> brains (Figure S3.3). Interestingly, and in contrast to E12.5, no difference was observed for N-cadherin and AF-6 expression staining patterns or immunoreactivities in E14.5 Usp9x<sup>-/-</sup> brains compared to Usp9x<sup>+/Y</sup> (n=3) (Figure 3.9). However, β-catenin immunostaining remained elevated in E14.5
*Usp9x* VZs (Figure 3.10A), similar to its expression in E12.5 *Usp9x* NPs, with increased immunoreactivity at the cytoplasm and basolateral membrane (Figure 3.10A). Increased β-catenin protein level was confirmed by WB analysis (Figure 3.10B, C). Despite the increased β-catenin expression, N-cadherin and AF-6 IHC results suggest that the perturbed AJs observed at E12.5 may have been restored in E14.5 *Usp9x* NPs. Specially the honeycomb like staining pattern of AF-6, observed at the apical surface of *Usp9x* VZs, confirming the restored apical localization of these proteins in *Usp9x* NPs (Figure 3.10).

To further confirm the restored AJs in E14.5 *Usp9x* NPs, co-IP analysis was conducted using E14.5 *Usp9x*+/*Y* and *Usp9x*+/*Y* cortical lysates, and β-catenin as the bait protein. Compared to E12.5 IP result, a relatively similar amount of N-cadherin protein was co-immunoprecipitated with *Usp9x*+/*Y* and *Usp9x*+/*Y* cortical lysates (Figure 3.11). Therefore, consistent with N-cadherin and AF-6 expression, co-IP results also suggest restored AJs in E14.5 *Usp9x* NPs.
Figure 3.9 Immunofluorescence signals for adherens junctional (AJ) markers in E14.5 Usp9x<sup>−/−</sup> brains. Coronal sections of two independent E14.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained with AJ markers N-cadherin and AF-6. No differences were observed for N-cadherin (a-d) and AF-6 (e-h) immunoreactivity or staining pattern between Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains. Honeycomb-like staining pattern was observed in both Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> VZ (white arrows) indicating apically localized AF-6 proteins. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.
Figure 3.10 Increased β-catenin levels in E14.5 Usp9x<sup>−/−</sup> brains. (A) Coronal sections of two independent E14.5 Usp9x<sup>+</sup>/Y and Usp9x<sup>−/−</sup> neocortices stained for β-catenin. Compared to Usp9x<sup>+</sup>/Y (a, b) β-catenin showed increased immunoreactivity in Usp9x<sup>−/−</sup> VZ (white arrows) (c, d). β-catenin immunostaining intensity was increased in the cytoplasm and basolateral membrane of Usp9x<sup>−/−</sup> (yellow arrows). (B) Western blot analysis showing significantly increased β-catenin protein levels in E14.5 Usp9x<sup>−/−</sup> cortices (n=3). (C) Quantification of β-catenin protein level relative to β-tubulin. Scale bars = 80μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.*, p < 0.05.
Figure 3.11 Co-immunoprecipitation of E14.5 neural progenitor’s adherens junction components. Co-immunoprecipitation of N-cadherin using E14.5 $Usp9x^{+/Y}$ and $Usp9x^{-/Y}$ cortical lysates, and β-catenin as the bait protein. Relatively similar amount of N-cadherin and α-catenin protein levels were pulled down from $Usp9x^{+/Y}$ and $Usp9x^{-/Y}$ brains suggesting the catenin-cadherin heterodimer remained intact in E14.5 $Usp9x^{-/Y}$ NPs.

Polarity of the E14.5 $Usp9x^{-/Y}$ NPs was evaluated next using the same polarity markers used for E12.5 analyses. No difference was observed in staining pattern or immunoreactivity for apical polarity proteins aPKC, Par3 and Prominin1, and the basal polarity protein Scribble between E14.5 $Usp9x^{+/Y}$ and $Usp9x^{-/Y}$ brains (n=3) (Figure 3.12). Furthermore, a honeycomb-like staining pattern was observed with aPKC staining at the apical surface of $Usp9x^{-/Y}$ VZs confirming the restored apical localization of these proteins (Figure 3.12). Therefore these results confirm the restoration of apicobasal polarity in E14.5 $Usp9x^{-/Y}$ NPs.
Figure 3.12 Immunofluorescence staining for polarity markers in E14.5 Usp9x<sup>−/−</sup> brains. Coronal sections of E14.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained with polarity proteins. No difference was observed for the apical polarity protein aPKCλ (a, b) expression pattern or intensity between Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains (n=3). Interestingly, honeycomb-like structures were observed in Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains at the apical surface of the VZ confirming the apical localization of the aPKCλ proteins. No difference was observed for the expression pattern or intensity for apical polarity protein Par3 (c, d) or basal protein Scribble (e, f) between 5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains (n=3). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.

To further analyse the effect of Usp9x loss on adhesion and polarity, as suggested by the IHC and WB results, a microarray analysis was conducted using RNA extracted from E12.5 and E14.5 Usp9x<sup>−/−</sup> mouse cortical tissues. At E12.5 over 1000 genes were differentially expressed between the Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains, at a significance level of p<0.05 (n=4 for each genotype). In concordance with the IHC and IB results, the functional annotation of the differentially expressed genes at E12.5 identified the cytoskeleton organisation, nervous
system development and function and, cell to cell signaling interaction as the most significantly affected functions in E12.5 Usp9x+/Y brains (Table 3.1). Despite the apparent restoration of adhesion and polarity in E14.5 NPs, as determined by IHC and IB, nearly 1400 genes were differentially expressed in the Usp9x+/Y brains, at a significance level of p<0.05 (n=4). However, only 147 genes were common to E14.5 and E12.5. At E14.5 the affected functions included tissue morphology and, cell growth and proliferation (Table 3.2). Therefore, the microarray analysis revealed that loss of Usp9x affects the expression of a large number of genes and a range of NP functions, and these differ at E12.5 and E14.5. Additionally, despite the apparent restoration of adhesion and polarity at E14.5, multiple gene networks remained perturbed in the absence of Usp9x.

Table 3.1 Functional annotation of the disrupted genes in E12.5 Usp9x+/Y cortex

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<td>Nervous System Development and Function synaptic transmission</td>
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<td>Cellular Assembly and Organization-reorganization of cytoskeleton</td>
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Microarray was conducted using cortical tissues of E12.5 Usp9x+/Y and Usp9x+/Y brains (n=4). Differentially expressed genes were annotated using the functional annotation tool of Ingenuity pathway analysis software.
Table 3.2 Functional annotation of the disrupted genes in E14.5 Usp9x+/Y cortex

<table>
<thead>
<tr>
<th>Functions Annotation</th>
<th>Category</th>
<th>p-Value</th>
<th>Gene Count</th>
</tr>
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<tbody>
<tr>
<td>Cellular Growth and Proliferation- proliferation of cells</td>
<td>Cellular Growth and Proliferation</td>
<td>0.0000201</td>
<td>202</td>
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<tr>
<td>Cellular Assembly and Organization- organization of cytoplasm</td>
<td>Cellular Assembly and Organization</td>
<td>0.0033</td>
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<tr>
<td>Cellular Development- differentiation of cells</td>
<td>Cellular Development</td>
<td>0.00802</td>
<td>96</td>
</tr>
<tr>
<td>Tissue Morphology- abnormal morphology of embryonic tissue</td>
<td>Tissue Morphology</td>
<td>0.0000319</td>
<td>78</td>
</tr>
<tr>
<td>Post-Translational Modification- phosphorylation of protein</td>
<td>Post-Translational Modification</td>
<td>0.0183</td>
<td>70</td>
</tr>
<tr>
<td>Cell Morphology- formation of cellular protrusions</td>
<td>Cell Morphology</td>
<td>0.00119</td>
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</tr>
<tr>
<td>Cell Signaling- activation of enzyme</td>
<td>Cell Signaling</td>
<td>0.0194</td>
<td>43</td>
</tr>
<tr>
<td>Cellular Organization- formation of plasma membrane projections</td>
<td>Cellular Assembly and Organization</td>
<td>0.0157</td>
<td>32</td>
</tr>
<tr>
<td>Nervous System Development- development of central nervous system</td>
<td>Nervous System Development and Function</td>
<td>0.0198</td>
<td>30</td>
</tr>
<tr>
<td>Embryonic Development- development of brain</td>
<td>Embryonic Development</td>
<td>0.0119</td>
<td>29</td>
</tr>
<tr>
<td>Cell Signaling- activation of Protein kinase</td>
<td>Cell Signaling</td>
<td>0.0165</td>
<td>29</td>
</tr>
<tr>
<td>Tissue Development- formation of actin filaments</td>
<td>Tissue Development</td>
<td>0.00511</td>
<td>28</td>
</tr>
<tr>
<td>Nervous System Development and Function- branching of neurites</td>
<td>Nervous System Development and Function</td>
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<tr>
<td>Embryonic Development- development of neural tube</td>
<td>Embryonic Development</td>
<td>0.0214</td>
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Microarray was conducted using cortical tissues of E14.5 Usp9x+/Y and Usp9x−/Y brains (n=4). Differentially expressed genes were annotated using the functional annotation tool of Ingenuity pathway analysis software.

3.5 Assessing the effect of Usp9x deletion on neural progenitor fate

Taken together, proteomic (IHC and WB analyses) and transcriptomic (microarray analysis) analyses indicated perturbed apical-basal polarity and AJs in E12.5 Usp9x−/Y brains. By connecting adjacent APs, AJs forms a stem cell niche at the apical surface of the VZ in developing mouse brains. Once NPs migrate away from this stem cell niche, their fate is changed either into more fate-restricted INPs or terminally differentiated neurons or glia cells (Gotz and Huttner, 2005; Katayama et al., 2011). Therefore the above results prompted an examination of NP fate in Usp9x−/Y brains.
First, proliferation of \( Usp9x^{+/Y} \) NPs was assessed by measuring the number of NPs undergoing mitosis using phospho-histone H3 (PH3) antibodies. APs undergo mitosis at the apical surface of the VZ, while INPs undergo mitosis at the SVZ (Fietz and Huttner, 2011; Taverna et al., 2014). PH3 antibody recognizes the evolutionary conserved phosphorylated Serine 10 (S-10) residue of the histone H3. During mitosis the S-10 residue of the N-terminal tail of histone H3 is phosphorylated by members of aurora AIR2-Inl1 kinase family. This phosphorylation is crucial for cell-cycle progression in to mitosis (Nowak and Corces, 2004). No difference was observed between E12.5 \( Usp9x^{+/Y} \) and \( Usp9x^{-/Y} \) brains, for the total number of PH3 positive (PH3+) mitotic cells at the VZ (\( Usp9x^{+/Y} = 35 \pm 5.568 \) PH3+ cells; \( Usp9x^{-/Y} = 33.67 \pm 2.404 \) PH3+ cells; \( p = 0.8367, n=3 \)) or for number of PH3+ mitotic cells per unit length of the VZ (\( Usp9x^{+/Y} = 0.04219 \pm 0.004105 \) PH3+ cells/\( \mu m \); \( Usp9x^{-/Y} = 0.04087 \pm 0.002809 \) PH3+ cells/\( \mu m \); \( p = 0.8044, n=3 \)) (Figure 3.13). In contrast, a significant increase was detected for the total number of PH3+ cell undergoing mitosis at an abventricular location in E12.5 \( Usp9x^{-/Y} \) brains compared to \( Usp9x^{+/Y} \) (\( Usp9x^{+/Y} = 3 \pm 0.5774 \) PH3+ cells; \( Usp9x^{-/Y} = 8 \pm 0.9129 \) PH3+ cells; \( p<0.01, n=3 \)). Similarly difference was observed when compared the number of PH3+ mitotic cells per unit length, between E12.5 \( Usp9x^{+/Y} \) and \( Usp9x^{-/Y} \) brains (\( Usp9x^{+/Y} = 0.003637 \pm 0.0006 \) PH3+ cells/\( \mu m \); \( Usp9x^{-/Y} = 0.01284 \pm 0.00124 \) PH3+ cells/\( \mu m \); \( p<0.01, n=3 \)) (Figure 3.13).

Similarly, when E14.5 neocortices were stained with PH3, no difference was observed for total number PH3+ cells (\( Usp9x^{+/Y} = 39.33 \pm 5.783 \) PH3+ cells; \( Usp9x^{-/Y} = 42 \pm 0.577 \) PH3+ cells; \( p = 0.6702, n=3 \)) or for number of PH3+ cells per unit length at VZ (\( Usp9x^{+/Y} = 0.03126 \pm 0.00119 \) PH3+ cells/\( \mu m \); \( Usp9x^{-/Y} = 0.03247 \pm 0.001736 \) PH3+ cells/\( \mu m \); \( p = 0.5984, n=3 \)) between \( Usp9x^{+/Y} \) and \( Usp9x^{-/Y} \) brains. However, significantly higher numbers of PH3+ mitotic cells (\( Usp9x^{+/Y} = 22.67 \pm 4.372 \) PH3+ cells; \( Usp9x^{-/Y} = 39 \pm 0.33 \) PH3+ cells; \( p<0.05, n=3 \)), as well as PH3+ cells per unit length (\( Usp9x^{+/Y} = 0.01021 \pm 0.00337 \) PH3+ cells/\( \mu m \); \( Usp9x^{-/Y} = 0.02815 \pm 0.00043 \) PH3+ cells/\( \mu m \); \( p<0.05, n=3 \)) were detected in \( Usp9x^{-/Y} \) SVZ (Figure 3.14).
Figure 3.13 Increased abventricular PH3-positive cells in the E12.5 Usp9x<sup>+/Y</sup> brains. (A) Coronal sections of E12.5 Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> neocortices stained with phosphohistone3 (PH3) antibody (n=3). (B) No difference was observed for number of PH3<sup>+</sup> cells per unit length at the VZ between Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> brains (yellow arrows). (C) In contrast, the number of abventricularly proliferating PH3<sup>+</sup> mitotic cells per unit length was significantly increased in the Usp9x<sup>-/Y</sup> brains compared to Usp9x<sup>+/Y</sup> brains (red arrows). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
Figure 3.14 Increased phosphohistone3-positive (PH3+) cells at SVZ of the E14.5 Usp9x<sup>−/−</sup> brains. (A) Coronal sections of E14.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained with PH3 antibody (n=3). (B) No difference was observed for number of PH3<sup>+</sup> cells per unit length at the VZ between Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains (yellow arrows). (C) In contrast, the number of PH3<sup>+</sup> mitotic cells per unit length was significantly increased in the Usp9x<sup>−/−</sup> SVZs compared to Usp9x<sup>+/+</sup> SVZs (red arrows). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
To identify the lineage of the abventricularly proliferating PH3+ cells, sections were double stained with the AP marker Pax6 and INP marker Tbr2. In $Usp9x^{-/Y}$ brains, the majority of PH3+ abventricularly proliferating cells also positively stained for Tbr2 (n=3) (Figure 3.15). At E12.5 very few intermediate progenitors were present in the neocortex of $Usp9x^{-/Y}$ mice (Figure 3.15) as expected. The presence of numerous PH3+ and Tbr2+ cells in $Usp9x^{-/Y}$ brains suggests the premature differentiation of APs to INPs.

**Figure 3.15 Increased Tbr2 and Phospho-histone H3 (PH3) double stained cells in E12.5 $Usp9x^{-/Y}$ brains.** (A) Coronal sections of E12.5 $Usp9x^{+/Y}$ (a) and $Usp9x^{-/Y}$ (b) neocortices PH3 and intermediate neural progenitor (INP) marker Tbr2 (n=3). Most of the abventricularly proliferating PH3+ cells in $Usp9x^{-/Y}$ brains were positively stained for INP marker Tbr2 (while arrows). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.

Increased abventricularly proliferating Tbr2+ INPs observed in E12.5 $Usp9x^{-/Y}$ brains suggest an overall increase in Tbr2+ INPs in $Usp9x^{-/Y}$ brains. Total number of Tbr2+ cells was counted in both E12.5 and E14.5 brains and cell number was normalized to the area of the neocortex for each section. Complementing the results in Figure 3.15 the number of Tbr2+ cells within a unit area of the cortex was significantly increased in E12.5 $Usp9x^{-/Y}$ brains ($Usp9x^{+/Y} = 0.3322 \pm 0.02870$ Tbr2+ cells/μm²; $Usp9x^{-/Y} = 0.4174 \pm 0.004897$ Tbr2+ cells/μm²; p<0.05, n=3) (Figure 3.17A, B). However, no difference was found for the Tbr2+ cells within a unit area in E14.5 $Usp9x^{-/Y}$ brains ($Usp9x^{+/Y} = 0.1686 \pm 0.001079$ Tbr2+ cells/μm²; $Usp9x^{-/Y} = 0.1620$...
± 0.003035 Tbr2+ cells/μm²; p = 0.5299, n=3) (Figure 3.16A, C). To further confirm the increased Tbr2+ INPs in Usp9x+/Y brains was due to premature differentiation, Tbr2 immunostaining was conducted on brain sections exposed to a 3 hour EdU pulse. Correlating with the previous results, the number of EdU+ and Tbr2+ double labelled cells was significantly increased in E12.5 Usp9x+/Y brains implying the observed difference was due to the newly synthesised cells (Usp9x+/Y = 35.00 ± 4.589; Usp9x−/Y = 62.25 ± 6.625; p<0.05, n=3) (Figure 3.17A, D). However, as expected no difference was observed in E14.5 Usp9x+/Y brains for EdU+ and Tbr2+ double labelled cells (Usp9x+/Y = 49.63 ± 1.375; Usp9x−/Y = 52.75 ± 0.2500; p = 0.1548, n=3) (Figure 3.17A, E).
Figure 3.16 Increased Tbr2+ intermediate progenitor cells production in E12.5 Usp9x<sup>+/Y</sup> brains. (A) E12.5 and E14.5 Usp9x<sup>+/Y</sup> and Usp9x<sup>−/Y</sup> neocortices expose to 3 hour EdU pulse stained with Tbr2 antibodies (n=3 for each embryonic stage). (B, C) The graph represents the number of Tbr2<sup>+</sup> intermediate neural progenitor cell normalized to the neocortical area. Number of Tbr2<sup>+</sup> cells per unit area was significantly increased in the E12.5 Usp9x<sup>−/Y</sup> brains. (D, E) Proliferative Tbr2<sup>+</sup> intermediate progenitors were presented as the ratio of EdU<sup>+</sup> and Tbr2<sup>+</sup> double labelled cells to all cells that incorporated EdU. Proliferative Tbr2<sup>+</sup> intermediate
cell number was significantly increased in E12.5 Usp9x⁻/⁻ brains. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.*, p < 0.05.

A recent study showed Usp9x is capable of regulating Sox2 expression in glioblastoma cells (Cox et al., 2013). In mouse NPs, N-cadherin expression is also known to be regulated by the Sox2 transcription factor. Sox2 is expressed prior to N-cadherin in neuroepithelial cells and has been shown to activate the N-cadherin expression in this region (Sakane and Miyamoto, 2013). Therefore Sox2 expression was examined in the Usp9x⁻/⁻ brains to ensure morphological difference observed in E12.5 Usp9x⁻/⁻ brains were not due to skewed Sox2 expression. No difference was observed for the Sox2 expression level between Usp9x⁻/⁻ and Usp9x⁺/+ brains at E12.5 or E14.5 (n=3) (Figure 3.17) suggesting dysregulated Sox2 expression was not the cause of the phenotypic differences observed in E12.5 and E14.5 Usp9x⁻/⁻ brains.
Figure 3.17 Sox2-positive (Sox2⁺) neural progenitors expression in E12.5 and E14.5 Usp9x⁺/⁻ brains. (A) Coronal sections of E12.5 (a, b) and E14.5 (c, d) neocortices stained with Sox2 antibodies (n=3 for each embryonic stage). Dotted lines demark the borders of the ventricular zone and cortical plate. (B) Sox2 expression in each section was quantified as the ratio of Sox2⁺ cortical thickness (red double headed arrow) over total cortical thickness (blue double headed arrow). The graph represents the quantified result of Sox2⁺ cells in E12.5 and E14.5 neocortices. No difference was observed for the total Sox2⁺ cells between Usp9x⁺/⁻ and Usp9x⁻/⁻ brains at E12.5 or E12.5. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
3.6 Effect of Usp9x deletion on cortical cytoarchitecture

The EdU birth dating experiments suggested a premature differentiation of Tbr2+ INPs in E12.5 *Usp9x/Y* brains. Therefore, neurogenesis and cortical lamination was examined next to determine whether the dysregulated cell cycle kinetic had perturbed the overall neuronal production in *Usp9x/Y* brains. First overall neurogenesis was explored by examining the expression pattern of Doublecortin (Dcx). Dcx is a microtubule associate protein expressed specifically in migrating neuroblasts (Friocourt et al., 2005). Dcx is known binding partner of Usp9x, and binds to Usp9x outside its catalytic domain. Deletion of Usp9x did not change the Dcx expression level, suggesting Dcx may be involved in Usp9x transportation in immature neuroblasts (Friocourt et al., 2005). Despite the increased Tbr2+ INPs observed in *Usp9x/Y* brains, no difference was observed for the Dcx expression pattern in all tested embryonic stages suggesting an intact overall neurogenesis in *Usp9x/Y* brains (n=3) (Figure 3.18). To further confirm the intact neurogenesis in *Usp9x/Y* brains, E12.5, E14.5 and E16.5 brains sections were stained with the well-established mature neuronal marker Tbr1. Similar to Dcx expression, no difference was noted for the total number of Tbr1+ cells in *Usp9x/Y* brains at all the tested stages (n=3) (Figure 3.20).
USP9X REGULATION OF INTRINSIC FATE DETERMINANTS

A

<table>
<thead>
<tr>
<th>DCX</th>
<th>Usp9x^{+/y}</th>
<th>Usp9x^{-/-}</th>
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<tr>
<td>E18.5</td>
<td><img src="g" alt="Image g" /></td>
<td><img src="h" alt="Image h" /></td>
</tr>
</tbody>
</table>

VZ, LV

B

![Graph showing DCX thickness over time](Graph.png)
Figure 3.18 Doublecortin-positive (Dcx+) neuroblasts expression in Usp9x<sup>+/Y</sup> embryonic brains. (A) E12.5 (a, b), E14.5 (c, d), E16.5 (e, f) and E18.5 (g, h) neocortices stained with Dcx antibodies (n=3 for each embryonic stage). Dotted lines demark the borders of the ventricular zone and cortical plate. (B) The graph represents the Dcx expression in brains as the ratio of Dcx<sup>+</sup> cortical plate thickness (blue double headed arrow) over total cortical thickness (red double headed arrow). No difference was observed for Dcx<sup>+</sup> neuroblasts expression between Usp9x<sup>+/Y</sup> and Usp9x<sup>+/Y</sup> brains in all the tested embryonic stages. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.
Figure 3.19 Tbr1-positive (Tbr1+) neuronal expression in Usp9x−/Y embryonic brains. (A) E12.5 (a, b), E14.5 (c, d) and E16.5 (e, f) neocortices stained with Tbr1 antibodies (n=3 for each embryonic stage). (B) The graph represents the number of Tbr1+ cells expressed in a unit area of the neocortices. No difference was observed for the Tbr1+ neuronal expression between Usp9x+/Y and Usp9x−/Y brains in all the tested embryonic stages. Scale bars = 80 μm. LV-lateral.
ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.

Despite overall neurogenesis was intact in Usp9x/Y brains, the increased proliferative Tbr2+ observed in E12.5 Usp9x/Y brain suggest an increase in deep layered neuronal production. Therefore cortical lamination was stereological assessed, using layer specific antibodies. Deep layered neurons were assessed using Ctip2 and Tbr1 antibodies and superficial layered neurons were assessed using Satb2 antibodies. Cortical lamination was assessed using E18.5 brains as at this stage the majority of neurons have reached their specific cortical location (Molyneaux et al., 2007). Interestingly, no difference was detected for number of Ctip2+ (Usp9x+/Y = 8.052 ± 1.946; Usp9x/Y = 9.499 ± 0.9803; p = 0.7285, n=3) (Figure 3.20A, B) and Tbr1+ deep layered neurons in E18.5 Usp9x/Y brains (Figure 3.20 D, E). Similarly, no difference was detected for number of Satb2+ superficial layered neurons (Usp9x+/Y = 16.73 ± 2.744; Usp9x/Y = 19.32 ± 2.468; p = 0.5214, n=3) (Figure 3.20A, C) in E18.5 Usp9x/Y brains as well. Therefore, these results suggest cortical lamination remained intact in Usp9x/Y brains.
**Figure 3.20 Intact cortical lamination in E18.5 Usp9x<sup>-/+</sup> brains.** (A) Coronal sections of E18.5 Usp9x<sup>+/+</sup> and Usp9x<sup>-/-</sup> brains immunostained with deep layered neuronal marker Ctip2 (layer V) and superficial neuronal marker Satb2. (B, C) Number of Ctip2 and Satb2 positive cells were normalised to the total number of cells expressed in the brains marked by DAPI staining. The graph represents the Ctip2<sup>+</sup> and Stab2<sup>+</sup> cells percentages in E18.5 Usp9x<sup>+/+</sup> and Usp9x<sup>-/-</sup> brains. No difference was observed for Ctip2<sup>+</sup> and Stab2<sup>+</sup> cell percentage between E18.5 Usp9x<sup>+/+</sup> and Usp9x<sup>-/-</sup> brains. (D) Coronal sections of E18.5 Usp9x<sup>+/+</sup> and Usp9x<sup>-/-</sup> brains immunostained with deep layered neuronal marker Tbr1 (layer VI). Dotted line demarks the cortical plate (CP). (E) Number of Tbr1<sup>+</sup> cells were normalised to the area of the cortical plate. No difference was observed for the number of Tbr1<sup>+</sup> cells expressed per unit area between E18.5 Usp9x<sup>+/+</sup> and Usp9x<sup>-/-</sup> brains. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.

To ensure early born deep-layered neurons had not died in Usp9x<sup>-/-</sup> brains, apoptosis was explored in Usp9x<sup>-/-</sup> brains using cleaved Caspase3 (Cas3) antibodies and TUNEL assay. Activated Cas3 is only expressed when the death cascade is activated (Arbour et al., 2008), while TUNEL assay marks fragmented DNA in apoptotic cells (Ormerod, 2001). Apoptosis was assessed in Usp9x<sup>-/-</sup> brains at embryonic stages E12.5, E14.5, E16.5 and E18.5. No difference was observed for apoptosis in Usp9x<sup>-/-</sup> brains at the tested embryonic stages with both Cas3 (Figure 3.21) and TUNEL assays (Figure 3.22) (n=3 for each embryonic stages). This result further confirms the unaffected deep and superficial neuronal generation in Usp9x<sup>-/-</sup> brains.
Figure 3.21 Cleaved Caspase 3 stained Usp9x<sup>+/Y</sup> brains. E12.5 (a, b), E14.5 (c, d), E16.5 (e, f) and E18.5 (g, h) neocortices stained with cleaved caspase 3 antibodies (n=3 for each embryonic stage). Caspase staining failed to detect apoptotic cells in Usp9x<sup>+/Y</sup> or Usp9x<sup>−/Y</sup> brains at all the tested embryonic stages. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.
**Figure 3.22 TUNEL assays of Usp9x<sup>−/−</sup> brains.** TUNEL assay on sections from E12.5 (a, b), E14.5 (c, d), E16.5 (f, g) and E18.5 (i, j) neocortices stained (n=3 for each embryonic stage). (e, h) Brain sections exposed to DNaseI were used as the positive controls. No TUNEL positive cells were observed at any tested embryonic stages. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.
3.7 Discussion

3.7.1 Usp9x regulates adherens junction establishment in early neural progenitors

In this chapter the effect of Usp9x deletion on AJs and apical-basal polarity in embryonic mouse NPs was assessed. The Nestin-cre expression resulted in the complete deletion of Usp9x protein and a transient down regulation of both AJ and polarity associated proteins from NPs at E12.5. One interpretation of the transient AJs and cell polarity loss is that Usp9x regulates the establishment rather than maintenance of NP adhesion/polarity similar to its role in other polarised epithelial cells. In cultured polarised MDCK II cells USP9X facilitates de novo tight junction assembly by regulating the temporal and spatial expression of the tight junction protein EFA6B (Theard et al., 2010). The EFA6 protein family is composed of four members EFA6A, B, C and D. EFA6A and EFA6B are the best-characterised isoforms and have similar functionalities in catalysing the Arf6 nucleotide exchanged activity during actin cytoskeleton organisation (Derrien et al., 2002). EFA6A accelerated the formation of the tight junctions by contributing to the reorganization of the apical actin cytoskeleton in MDCK cells, while EFA6B is required for efficient TJ biogenesis. Protein levels of EFA6B increase both temporally and spatially at the initial cell–cell contact site (Luton et al., 2004). USP9X mediated deubiquitylation is responsible for this transient EFA6B increase at the de novo tight junction site (Theard et al., 2010). On the contrary, loss of USP9X did not affect the morphology or function of mature tight junctions in MDCK cells, but it significantly delayed the de novo tight junction formation after a calcium switch suggesting USP9X is important for tight junction establishment rather than its maintenance (Theard et al., 2010). Although RGCs lacks tight junctions they constantly form AJs, therefore it could hypothesised that Usp9x might have a similar role during AJ establishment in NPs. This proposition was further strengthen by a previous study conducted in our lab. In T84 colon epithelial cells USP9X interacted and colocalized with the cadherin-catenin heterodimer at points of protein trafficking in semi confluent cells, in which AJs are still establishing, but no association was detected in confluent cells containing matured AJ complexes (Murray et al., 2004). Similar to previous in-vitro study in MDCK cells, Usp9x co-IP with N-cadherin/β-catenin heterodimer from E12.5 Usp9x⁺/⁻ brains (Figure 3.5), despite N-cadherin and β-catenin protein expression being significantly altered in Usp9x⁺/⁻ NPs at this stage, raising the possibility that Usp9x may regulate the trafficking of N-cadherin/β-catenin heterodimer in NPs. Taken together data from this project and previous in-vitro studies, it could presumed that Usp9x may be involved in AJ
establishment in early NPs. AJs and cell polarity are functionally and structurally interconnected in NPs. Consequently, disrupting AJ will disrupt NP apical-basal polarity or vice-versa (Lin et al., 2000; Imai et al., 2006). Therefore, delayed AJs establishment in Usp9x\(^{+/\gamma}\) NPs must have caused the transient apical-basal polarity disruption in E12.5 Usp9x\(^{+/\gamma}\) NPs.

In addition to serving as the primary cell-cell contacting site in NPs, AJs are also responsible for maintaining cortical tissue integrity making it harder to study AJ regulations in NPs \textit{in-vivo}. To overcome this problem, previous studies used cortical tissue architecture to predict whether the AJ regulatory protein involved with AJ establishment or maintenance similar to our rationalisation above. For example a similar conclusion was reached that MAL-3 is important for AJs and polarity establishment in NPs rather than in maintenance, based upon the transient polarity and AJs disturbance observed in early in MAL-3 knockout NPs (Srinivasan et al., 2008). Likewise, the importance of Cdc42 in AJ establishment and importance of RhoA in AJ maintenance was concluded based on the cortical tissue architectural differences in the Cdc42 and RhoA mutant mice. Conditional deletion Cdc42 and RhoA from mouse NPs using the same \textit{Foxg1-Cre} donor mice had the same effect on AJs and polarity but at different time points. In \textit{Foxg1-Cre X Cdc42} null embryos, the AJ proteins disruption started as early as E9.5–E10.5 (Cappello, S. et al., 2006), in contrast \textit{Foxg1-Cre X RhoA} null embryos AJ proteins disruption did not occur until later developmental stages (Katayama et al., 2011). Based on this temporal difference, these studies concluded that Cdc42 is important for AJ establishment rather than its maintenance, while RhoA is important for AJ maintenance rather than its establishment in NPs (Cappello, S. et al., 2006; Katayama et al., 2011).

Decreased polarity protein expression observed in E12.5 Usp9x\(^{+/\gamma}\) NPs may occur either as a consequence of disrupted AJs or as a direct consequence of Usp9x loss. Usp9x regulates the expression of NUAK1 and MARK4 polarity proteins (Al-Hakim et al., 2008). Deubiquitylation of these two kinases promotes their activation by the LKB1 kinases (Par4 homologues) suggesting Usp9x may able to regulate NP polarity and adhesion through the Par polarity protein complex (Al-Hakim et al., 2005; Al-Hakim et al., 2008). Furthermore ectopic expression of Usp9x in mouse ESC derived NPs, significantly increased their polarity providing evidence of a direct correlation between Usp9x and NP polarity regulation (Jolly et al., 2009). However, in contrast to this current study, AJ proteins such N-cadherin, AF-6 and \(\beta\)-catenin were also increased in these mutant NPs making it harder to determine the exact intrinsic fate regulatory factor regulated by Usp9x.
Another possibility that could have caused the observed transient polarity and adhesion disruption in E12.5 \( Usp9x^{-/} \) NPs is the compensation by another DUB for the loss Usp9x. Such a phenomenon was observed in siRNA mediated USP9X-depleted multiple myeloma cells. Decreased USP9X levels induced apoptosis due to increased degradation of the anti-apoptotic protein Mcl-1, a well-established substrate of USP9X (Trivigno et al., 2012). During later stages upregulated USP24 levels were able to compensate for the USP9X loss and restore the Mcl-1 levels thereby preventing the apoptosis of USP9X-depleted multiple myeloma cells (Peterson et al., 2015). However, the microarray analysis conducted on E12.5 and E14.5 \( Usp9x^{-/} \) brains failed to detect an upregulation of any other DUBs in these embryonic cortices, retracting this hypothesis.

### 3.7.2 Premature differentiation of \( Usp9x^{-/} \) early neural progenitors

The lineage tracing and EdU birthday experiments showed significant increase in Tbr2\(^+\) INP cell number in early \( Usp9x^{-/} \) brains (Figure 3.15, 3.16) without affecting the overall neurogenesis (Figure 3.18, 3.19) or cortical lamination (Figure 3.20). The VZ in embryonic mouse brains acts as a stem cell niche by itself and facilitates the maintenance and expansion of the NP population (Gotz and Huttner, 2005; Taverna et al., 2014). In contrast to other stem cell niches, VZ lacks supporting cells, so adjacent NPs connected through their AJs act as supporting cells. Hence, in addition to their intrinsic fate regulatory function, AJs are vital to maintain the stem cell niche in embryonic mouse brains (Gotz and Huttner, 2005; Stocker and Chenn, 2015). Nascent neurons and INPs need to detach and migrate away from the VZ in order to facilitate their differentiation (Itoh et al., 2013; Homem et al., 2015). Consequently, disrupted AJs will cause the premature differentiation of APs into more fate restricted cell types. Therefore, transiently disrupted AJs and polarity, and increased Tbr2\(^+\) INPs observed in E12.5 \( Usp9x^{-/} \) brains suggest that \( Usp9x^{-/} \) APs prematurely differentiate into INPs. Although we did not conduct lineage tracing experiment directly showing that APs differentiate into INPs, the significant increase in EdU\(^+\)/Tbr2\(^+\) double positive cell number in E12.5 \( Usp9x^{-/} \) brains strongly suggest that \( Usp9x^{-/} \) APs prematurely differentiate into INPs. Another possibility that could have result in this premature differentiation is misorientation of the mitotic spindle in \( Usp9x^{-/} \) NPs. The mitotic spindle is another key element that regulates the symmetric versus asymmetric cell divisions of NPs (Gotz and Huttner, 2005; Fietz and Huttner, 2011; Paridaen et al., 2013). During symmetric cell divisions the spindle is orientated parallel
to the apical-basal axis of the NPs, while during asymmetric division spindle orients more diversely relative to the apical-basal axis resulting in oblique or horizontal cleavages (Siller and Doe, 2009; Paridaen et al., 2013). AJs and apical-basal cell polarity provide planar cue to orient mitotic spindle during NP divisions. Loss of expression or mislocalization of these factors will lead to misorientation resulting alternate NP fate. Disrupted AJs results in misorientation of the mitotic spindle in Drosophila neuroepithelial cells (McCartney et al., 2001) and conditional deletion of the apical polarity protein LGN from mouse NPs mis-orientate the mitotic spindle leading to a significant increase in horizontal or oblique asymmetrical divisions over the vertical symmetric divisions resulting in premature neuronal production (Konno et al., 2008). Since both AJs and polarity were perturbed in E12.5 Usp9x°/NPs disoriented mitotic spindle could have also cause the premature differentiation of Usp9x°/NPs. In the future, this could be addressed by staining for mitotic spindle markers or evaluating the centriole orientation during mitosis.

Interestingly Usp9x deletion only induced the premature differentiation of APs into INP, but did not promote INP differentiation into neurons, since no difference was observed in the total neurogenesis or lamination pattern in Usp9x°/ brains. This could have arisen due to two different reasons (1) increased cell death (2) another molecular mechanism preventing the differentiation of INPs into neurons in Usp9x°/ NPs. No difference was observed between Usp9x°/ and Usp9x°/ brains for both cleaved Cas3 (Figure 3.21) and TUNNEL assays (Figure 3.22) suggesting cell death is unlikely to be the reason for unaffected neuronal numbers observed in Usp9x°/ brains. Interestingly the same phenotype was observed in the aPKCλ conditionally knockout mice. Conditional deletion of aPKCλ from mouse NPs using the same Nestin-Cre donor mice resulted in perturbed AJs and increased abventricular proliferation, but did not affect overall neurogenesis similar to the current study (Imai et al., 2006). In contrast, conditional deletion of aPKCζ from neuroepithelium in the chicken embryonic spinal cord results in significantly affected medial-lateral cell stratification (Ghosh et al., 2008). In addition conditional deletion of MAL-3 from mouse NPs significantly increased neurogenesis despite the transient nature of the polarity perturbation (Srinivasan et al., 2008). Loss of LGN function in the neuroepithelium of the developing mouse causes movement of progenitors out of the VZ forming neural rosettes without affecting the overall neurogenesis (Konno et al., 2008). Therefore, collectively these studies suggest that regardless of the developmental stages, prematurely detached APs inherit different fates based on their genetic manipulation. Two different reasons could have caused this different fate inheritance pattern. First, the initial
genetic manipulation could affect other fate regulatory pathways, which have not been identified or known to be regulated by the particular gene. Or, it could be that this gene may be involved in a certain stage of NP fate specification, and blocking the expression of this gene may favour a particular fate over the other (as explained by Waddington’s epigenetic landscape). Interestingly, several studies have already shown that disruption or blocking the expression of certain AJ or polarity proteins disrupted several extrinsic signaling pathways through a mechanism(s) not yet fully understood. For example, in addition to premature neural differentiation, ablation of N-cadherin from the mouse NPs resulted in decreased Notch signaling in embryonic mouse brains (Hatakeyama et al., 2014) and deletion of RhoA from mouse neural progenitors significantly increases activity of Wnt and sonic hedgehog pathways (Katayama et al., 2011). Although the upregulation of these signaling pathways upon the deletion of AJ or polarity proteins could have been the result of compensatory mechanism or feedback pathways, Usp9x is known to regulate most of these extrinsic signaling pathways. Therefore unaffected neurogenesis and subtle phenotype observed in $Usp9x^{-/-}$ brains could be due to perturbed extrinsic fate determination pathway(s) masking the effect of initial premature differentiation of the early NPs. The status of these extrinsic fate regulatory pathway(s) in $Usp9x^{-/-}$ brains will be discussed in detail in Chapters 4, 5 and 6.
CHAPTER 4

Usp9x regulation of extrinsic fate determinants - Wnt signaling
4.1 Introduction

β-catenin is an evolutionarily conserved multifunctional protein, which was first discovered as a part of the AJ complex in mammalian cells, where it interacts with cadherin and α-catenin linking AJs with the actin cytoskeleton (Ozawa et al., 1989). In addition to this AJ associated β-catenin pool, there are two other β-catenin pools in NPs- free floating cytoplasmic β-catenin pool and transcriptionally active nuclear β-catenin pool (Harrison-Uy and Pleasure, 2012). Regardless of Wnt status, β-catenin is continuously transcribed and translated in NPs (Draganova et al., 2015). The DC maintains cytoplasmic β-catenin levels at vanishingly low levels in Wnt inactive cells (Li et al., 2012; Pronobis et al., 2015a). DC is a dynamic multiprotein entity whose assembly and structure are key to its functions (Pronobis et al., 2015a). The DC is comprised of two scaffolding proteins Axin and APC, two kinases GSK-3β and CKI, and an E3- ubiquitin ligase β-TrCP (Li et al., 2012). Cytoplasmic free floating β-catenin gets phosphorylated and ubiquitylated within the DC before release to the proteasome for degradation (Li et al., 2012; Pronobis et al., 2015a). In Wnt active cells the cytoplasmic β-catenin pool is increased by deactivating the DC Once β-catenin levels saturate the cytoplasm it enters into the nucleus, in a concentration dependent manner, binds to the TCF/LEF transcription factors and transcribes Wnt target genes such as Axin2 and Ccnd1 (Li et al., 2012; Stamos and Weis, 2013).

Until recently it was believed β-catenin’s signal transduction and adherens junctional functions were independent of other. However, several studies have shown that under pathophysiological conditions adherens junctional β-catenin can feed into the Wnt signaling pathway (Kam and Quaranta, 2009; Vlad-Fiegen et al., 2012). However, in Usp9x⁻/⁻ NPs, β-catenin protein level was significantly increased in the presence of both decreased (E12.5) and normal (E14.5) levels of N-cadherin, suggesting increased β-catenin levels may be independent from AJs. Therefore, the main aim of this chapter was to determine the underlying cause for increased β-catenin in Usp9x⁻/⁻ brains and establish the role of Usp9x in β-catenin protein regulation.
4.2 Results

4.2.1 Deletion of Usp9x increases the levels of β-catenin protein in Neural Progenitors

The analysis of β-catenin expression was extended till late embryonic stages to determine whether the increased β-catenin expression observed in E12.5 and E14.5 Usp9x<sup>+/Y</sup> neocortices was temporally specific. Similar to E14.5 Usp9x<sup>+/Y</sup> neocortices, increased β-catenin immunoreactivity was observed in E16.5 and E18.5 Usp9x<sup>+/Y</sup> neocortices (Figure 4.1A, S4.1), with normal N-cadherin expression pattern and immunoreactivity in Usp9x<sup>+/Y</sup> neocortices compared to Usp9x<sup>+/Y</sup> (n=3) (Figure 4.1A, S4.2). Increased total β-catenin protein level in E16.5 Usp9x<sup>+/Y</sup> neocortices was confirmed by WB analysis (n=3) (Figure 4.1B, C). These results indicated that β-catenin expression remained elevated in Usp9x<sup>+/Y</sup> neocortices throughout embryonic development.

Next, to determine the source of the increase in β-catenin protein levels in Usp9x<sup>+/Y</sup> neocortices, β-catenin mRNA expression was assessed by qRT-PCR analysis using RNA extracted from E12.5 and E14.5 neocortices. No difference was observed for Ctnbb1 mRNA expression between Usp9x<sup>+/Y</sup> brains and Usp9x<sup>+/Y</sup> brains at E12.5 or E14.5 (n=3) (Figure 4.2), suggesting an impaired translational or post-translational mechanism. Since β-catenin protein is regulated by the ubiquitin proteasome system (Li et al., 2012) and interacts with Usp9x (Taya et al., 1999; Murray et al., 2004; Ouyang et al., 2016), there was a higher probability of defective post-translational regulation may causing the β-catenin up-regulation in Usp9x<sup>+/Y</sup> brains.
Figure 4.1 Increased β-catenin protein levels in E16.5 and E18.5 Usp9x<sup>-/-</sup> brains. (A) No difference was observed for the N-cadherin staining intensity between Usp9x<sup>+/Y</sup> (a, e) Usp9x<sup>-/-</sup> (b, f) at E16.5 or E18.5. However β-catenin immunoreactivity was markedly increased in both E16.5 (c, d) and E18.5 (g, h) Usp9x<sup>-/-</sup> neocortices compared to Usp9x<sup>+/Y</sup> (representative images of 3 Usp9x<sup>+/Y</sup> versus 3 Usp9x<sup>-/-</sup>). (B) Western blot analysis showing significantly increased β-catenin protein levels in E16.5 Usp9x<sup>+</sup> cortices (n=3). (C) Quantification of
E16.5 β-catenin protein level relative to β-tubulin. Scale bars = 80 μm. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05. LV- lateral ventricle, VZ- ventricular zone.

Figure 4.2 β-catenin mRNA level was unchanged in Usp9x<sup>−/−</sup> brains. Relative Ctnnb1 gene expression in E12.5 and E14.5 brains was measured by qRT-PCR analysis and normalising against GAPDH. No difference was observed for Ctnnb1 expression in Usp9x<sup>−/−</sup> brains at E12.5 or E14.5 compared to Usp9x<sup>+/+</sup> brains (n=3). All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.

As mentioned previously there are three sub-populations of β-catenin in NPs. The next set of experiments was conducted to determine which β-catenin sub-population(s) changed in the absence of Usp9x. In Chapter 3, co-IP results showed that β-catenin remained bound to N-cadherin in E12.5 (Figure 3.5) and E14.5 (Figure 3.11) Usp9x<sup>−/−</sup> brains, suggesting the adherens junctional β-catenin pool was unaffected in Usp9x<sup>−/−</sup> NPs. Therefore, focus was then turned towards examination of the cytoplasmic and nuclear β-catenin pools. Under normal physiological conditions, that is, in the absence of Wnt, the majority of the cytoplasmic β-catenin remained bound to the DC before targeted for to degradation (Li et al., 2012; Azzolin et al., 2014). The DC bound sub-population of β-catenin can be identified by its distinctive phosphorylation at the amino acid residues S33/37 and Threonine 41 (pβ-
catenin33/37/41) (Li et al., 2012; Stamos and Weis, 2013). Interestingly a significant increase in pβ-catenin33/37/41 levels was detected in Usp9x<sup>−/−</sup> brains at E14.5 (n=3) (Figure 4.3). Once β-catenin accumulates in the cytoplasm it enters the nucleus in a concentration gradient manner, and is thereafter regarded as transcriptionally activated β-catenin (Fagotto, 2013). A slight, but significant, increase in the transcriptionally activated form of β-catenin (Tyr654 pβ-catenin) was also detected in Usp9x<sup>−/−</sup> lysates at E14.5 (n=3) (Figure 4.3). These results indicate that cytoplasmic and nuclear β-catenin pools were increased in the Usp9x<sup>−/−</sup> brains. E14.5 cortical tissues lysates were used for these biochemical assays as this was the earliest time when all AJ-associated proteins appeared at normal levels and locations in the Usp9x<sup>−/−</sup> brains. Collectively these results indicated that both the cytoplasmic Ser33/37/Thr41 pβ-catenin and nuclear Tyr654 pβ-catenin pools were significantly increased Usp9x<sup>−/−</sup> brains.
Figure 4.3 Increased phospho-β-catenin protein levels in Usp9x<sup>−/−</sup> brains. (A) Western blot analysis of Ser33/37/Thr41 pβ-catenin and Tyr654 pβ-catenin pools of E14.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortical lysates detected with phospho-specific antibodies. Ser33/37/Thr41 pβ-catenin level was significantly increased in the Usp9x<sup>−/−</sup> brains compared to Usp9x<sup>+/+</sup> (n=3). Similarly, slight but significant increase also observed for Tyr654 pβ-catenin pools in the Usp9x<sup>−/−</sup> brains (n=3). (B) Quantification of Ser33/37/Thr41 and Tyr654 pβ-catenin protein levels relative to β-tubulin. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. **p < 0.01; *p < 0.05.
4.2.2 Absence of Usp9x alters composition of the β-catenin destruction complex

Several studies have reported the physical interaction between USP9X and β-catenin proteins in multiple mammalian cell lines *in-vitro* (Taya et al., 1999; Murray et al., 2004; Ouyang et al., 2016). To test if this interaction occurs in embryonic mouse NPs, co-IP of Usp9x with β-catenin was performed using *Usp9x*+/Y E14.5 neocortical tissue. In accordance with previous reports Usp9x was immunoprecipitated with anti-β-catenin antibodies (Figure 4.4). In contrast to previous studies, which indicated Usp9x positively regulates β-catenin protein levels, significantly increased pβ-catenin33/37/41 and Tyr654 pβ-catenin levels observed in *Usp9x*−/Y brains imply a novel regulatory mechanism in NPs.

![Figure 4.4 Usp9x interacts with β-catenin in embryonic cortical tissue. Usp9x was co-immunoprecipitated by β-catenin antibodies from E14.5 cortical lysates. Detectable amounts of Usp9x proteins were immunoprecipitated from Usp9x+/Y cortical lysates, but not from Usp9x−/Y cortical lysates.](image)

To date, two studies have identified USP9X as a novel component of the β-catenin DC, based on mass spectrometry analysis of Axin binding partners in HEK293 cells (Major et al., 2007; Li et al., 2012). This raised the intriguing prospect that the absence of Usp9x might perturb the composition and/or functionality of the DC which, in turn, might responsible for the increased β-catenin protein levels in *Usp9x*−/Y brains. However, neither published study extended the analysis of Usp9x’s role in the DC beyond its initial identification as an Axin interacting protein. Therefore the interaction between Usp9x and
DC components was tested by co-IP, not only with endogenous Axin, but also with APC from HEK293 cell lysates, both of which were confirmed (Figure 4.5A, B). Next, using the same conditions and endogenous Axin, Usp9x was co-immunoprecipitated along with other components of the DC, including APC, β-TrCP and β-catenin from E14.5 Usp9x+/Y cortical lysates, which are comprised of a large proportion of NPs. These co-IP results, from HEK293 cells and E14.5 cortical tissue, suggest a proportion of Usp9x is located in the DC.

Figure 4.5 Usp9x interacts with destruction complex proteins. USP9X along with other components of the β-catenin destruction complex was co-immunoprecipitated using both Axin (A) and APC (B) antibody from non-transfected HEK293 lysate. (C) Similarly, Usp9x was co-immunoprecipitated with Axin antibody from E14.5 Usp9x+/Y cortical lysate along with other components of the destruction complex.
The increased pβ-catenin33/37/41 observed in $Usp9x^{-/}$ brains suggests a failed/decreased ubiquitylation of β-catenin. Under normal physiological conditions pβ-catenin33/37/41 is ubiquitylated by β-TrCP E3-ubiquitin ligase and rapidly degraded by the proteasome (Li et al., 2012). Therefore, β-TrCP expression was examined in the $Usp9x^{-/}$ brains to determine whether deregulated β-TrCP expression underpinned this phenotype. However, no difference in total β-TrCP protein levels between $Usp9x^{-/}$ and $Usp9x^{+/}$ was observed at either E12.5 (n=3) or E14.5 (n=4) (Figure 4.6). Phosphorylation and ubiquitylation of the β-catenin both occur within the DC. Therefore these results suggested that loss of Usp9x may have affected the assembly, composition and/or activity of the DC.
Figure 4.6 Unaffected β-TrCP protein levels in Usp9x-/- brains. (A) Western blot analysis of β-TrCP expression in E12.5 brains (n=3) (B) Western blot analysis of β-TrCP expression in four E14.5 Usp9x+/+ brains and five E14.5 Usp9x-/- brains. (C) Quantification of β-TrCP protein level relative to β-tubulin. No difference was observed between the Usp9x+/+ and Usp9x-/- brains for total β-TrCP protein level at E12.5 or E14.5 when normalized against β-tubulin. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.
Prior to examining the composition of the DC, expression of each component was examined in $Usp^{9x^{-/Y}}$ brains to determine whether deletion of Usp9x affected the expression of other DC components. No difference was detected for total APC protein levels in both E12.5 and E14.5 $Usp^{9x^{-/Y}}$ neocortices compared to $Usp^{9x^{+/Y}}$ (n=3) (Figure 4.7). Similarly, no difference was observed for total Gsk-3β protein level in both E12.5 and E14.5 $Usp^{9x^{-/Y}}$ brains (n=3) (Figure 4.8). Therefore loss of Usp9x did not affect total levels of DC components.

Figure 4.7 Total APC protein levels are unaffected in $Usp^{9x^{-/Y}}$ brains. (A) Westernblot analysis of E12.5 $Usp^{9x^{+/Y}}$ and $Usp^{9x^{-/Y}}$ cortical lysates for total APC protein expression. (B) Westernblot analysis of E14.5 $Usp^{9x^{+/Y}}$ and $Usp^{9x^{-/Y}}$ cortical lysates for total APC protein expression. (C, D) Quantification of APC protein levels relative to β-tubulin. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.
Figure 4.8 Total Gsk-3β protein levels were unaltered in Usp9x<sup>+/Y</sup> brains (A) Western blot analysis of E12.5 Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> cortical lysates for total Gsk-3β protein expression. (B) Western blot analysis results for E14.5 Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> cortical lysates for total Gsk-3β protein expression. (C, D) Quantification of Gsk-3β protein levels relative to β-tubulin. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.

Next assembly of the Usp9x<sup>-/Y</sup> DC was assessed to determine whether Usp9x deletion affected the assembly of the DC, despite the presence of all the necessary structural proteins in Usp9x<sup>-/Y</sup> brains. DC is static multiprotein complex, therefore its protein composition remains constant during specific cellular conditions (Wnt active versus inactive). By using endogenous proteins under their native conditions, co-IP should able to detect, if there is any, structural difference between Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> DCs (Li et al., 2012; Gerlach et al., 2014). Based on this notion, assembly of the Usp9x<sup>-/Y</sup> DC was assessed by comparing co-
immunoprecipitated endogenous DC components. Co-IP was conducted using an APC antibody, one of the two scaffolding proteins of the DC, with E14.5 \( Usp9x^{-/y} \) and \( Usp9x^{+/y} \) cortical lysates proteins. All the tested DC components were immunoprecipitated with \( Usp9x^{-/y} \) brains \( (n=2) \) (Figure 4.9A), suggesting the absence of Usp9x did not affect the assembly of the DC.

Next the composition of \( Usp9x^{-/y} \) DC was examined next by comparing the amount proteins immunoprecipitated between \( Usp9x^{+/y} \) and \( Usp9x^{-/y} \) brains. Despite increased p\( \beta \)-catenin33/37/4 levels in \( Usp9x^{-/y} \) brains, a similar amount of \( \beta \)-catenin was immunoprecipitated from \( Usp9x^{-/y} \) lysates compared to \( Usp9x^{+/y} \) lysates \( (n=2) \) (Figure 4.9A). Interestingly, but unexpectedly relatively high amounts of Gsk-3\( \beta \) and \( \beta \)-TrCP were co-immunoprecipitated with \( Usp9x^{-/y} \) cortical lysates \( (n=2) \) (Figure 4.9A), implying an increased interaction between Gsk-3\( \beta \) and \( \beta \)-TrCP within the \( Usp9x^{-/y} \) DC’s. To confirm this increased interaction, another co-IP analysis was conducted using \( \beta \)-TrCP antibody on E14.5 cortical lysates. Relatively similar amounts of APC protein levels were immunoprecipitated between \( Usp9x^{-/y} \) and \( Usp9x^{+/y} \) cortical lysates. However, in contrast to APC co-IP relatively higher amount of \( \beta \)-catenin was immunoprecipitated with \( Usp9x^{-/y} \) cortical lysates compared \( Usp9x^{+/y} \) (Figure 4.9B). Increased level of Gsk-3\( \beta \) was detected with \( Usp9x^{-/y} \) cortical lysates, confirming the increased interaction between Gsk-3\( \beta \) and \( \beta \)-TrCP within the \( Usp9x^{-/y} \) DC’s (Figure 4.9B). E14.5 cortical tissues lysates were used for these co-IP assays, as E14.5 was the earliest developmental stage with increased cytoplasmic \( \beta \)-catenin species but normal AJ-associated \( \beta \)-catenin in \( Usp9x^{-/y} \) brains. Taken together these results imply that the composition of the DC is altered in \( Usp9x^{-/y} \) brain, potentially disrupting its function.
Figure 4.9 Relative ratios of β-catenin destruction complex components are altered in Usp9x<sup>−/−</sup> neural progenitors. (A) Components of destruction complex were co-immunoprecipitated by anti-APC antibody from E14.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> cortical lysate. Relatively more β-TrCP and Gsk-3β were immunoprecipitated from Usp9x<sup>−/−</sup> compared to Usp9x<sup>+/+</sup>. β-TrCP image was cropped and reorganised due to the incorrect loading. (B) Similarly, when destruction complex components were immunoprecipitated with anti-β-TrCP antibody, relatively high amounts of Gsk-3β were immunoprecipitated from Usp9x<sup>−/−</sup> compared to Usp9x<sup>+/+</sup>.
4.2.3 Usp9x antagonises Wnt signaling in neural progenitors

Increased β-catenin protein levels in Usp9x<sup>-/+</sup> brains, especially increased Tyr654 pβ-catenin protein levels at E14.5, indicate increased Wnt signaling activity in Usp9x<sup>-/+</sup> brains. Therefore Wnt signaling activity was examined in Usp9x<sup>-/+</sup> brains by qRT-PCR analysis on RNA extracted from E12.5 and E14.5 cortices. Consistent with preceding results a significant increase in Ccnd1 gene (~ 2 fold increase) expression was observed in Usp9x<sup>-/+</sup> brains at E12.5, E14.5 and E16.5 (n=3) (Figure 4.10A). Similar to Ccnd1 expression, the direct Wnt target Axin2 also showed a statistically significant increase, but of a lesser magnitude, in the Usp9x<sup>-/+</sup> brains at all tested embryonic stages (n=3) (Figure 4.10B). Consistent with the qRT-PCR results, Wnt signaling was identified as one of the most significantly affected signaling pathways by Ingenuity Pathway Analysis conducted on the significantly affected genes at E14.5 (Table 4.1). Together these results indicate increased Wnt signaling in Usp9x<sup>-/+</sup> brains.

Figure 4.10 Increased canonical Wnt target gene expression in Usp9x<sup>-/+</sup> brains. qRT-PCR analysis of RNA isolated from E12.5, E14.5 and E16.5 neocortices of Usp9x<sup>+/+</sup> and Usp9x<sup>-/+</sup> brains (n=3 for each embryonic stage). Canonical Wnt signaling target genes Ccnd1 (A) and Axin2 (B) were significantly upregulated in the Usp9x<sup>-/+</sup> neocortices compared to Usp9x<sup>+/+</sup> in all tested embryonic stages. Target gene expression was normalised against housekeeping gene GAPDH. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
Table 4.1 Top most affected pathways in E14.5 \(Usp9x^{-/}\) brains.

<table>
<thead>
<tr>
<th>Ingenuity Canonical Pathways</th>
<th>log(p-value)</th>
<th>Number of Affected Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Epithelial Adherens Junction Signaling</td>
<td>2.22E+00</td>
<td>18</td>
</tr>
<tr>
<td>2 Role of JAK1, JAK2 and TYK2 in Interferon Signaling</td>
<td>1.98E+00</td>
<td>5</td>
</tr>
<tr>
<td>3 Wnt/(\beta)-catenin Signaling</td>
<td>1.95E+00</td>
<td>20</td>
</tr>
<tr>
<td>4 CDK5 Signaling</td>
<td>1.92E+00</td>
<td>14</td>
</tr>
<tr>
<td>5 Dopamine Receptor Signaling</td>
<td>1.88E+00</td>
<td>12</td>
</tr>
<tr>
<td>6 Cdc42 Signaling</td>
<td>1.75E+00</td>
<td>13</td>
</tr>
<tr>
<td>7 p38 MAPK Signaling</td>
<td>1.57E+00</td>
<td>13</td>
</tr>
<tr>
<td>8 Regulation of Actin-based Motility by Rho</td>
<td>1.51E+00</td>
<td>11</td>
</tr>
<tr>
<td>9 Axonal Guidance Signaling</td>
<td>1.43E+00</td>
<td>39</td>
</tr>
<tr>
<td>10 Regulation of the Epithelial-Mesenchymal Transition Pathway</td>
<td>1.37E+00</td>
<td>19</td>
</tr>
<tr>
<td>11 BER pathway</td>
<td>1.33E+00</td>
<td>3</td>
</tr>
<tr>
<td>12 Synaptic Long Term Potentiation</td>
<td>1.27E+00</td>
<td>14</td>
</tr>
<tr>
<td>13 Remodelling of Epithelial Adherens Junctions</td>
<td>1.25E+00</td>
<td>9</td>
</tr>
<tr>
<td>14 HIPPO signaling</td>
<td>1.21E+00</td>
<td>10</td>
</tr>
<tr>
<td>15 Calcium Signaling</td>
<td>1.16E+00</td>
<td>18</td>
</tr>
</tbody>
</table>

*Result from the Ingenuity Pathway Analysis, conducted on the significantly affected genes on E14.5 \(Usp9x^{-/}\) brains.*

Compared to E12.5, E16.5 neocortices are comprised of mixed populations of NPs and fate-restricted neuronal subtypes. Therefore to determine if the observed increased Wnt signaling activity in \(Usp9x^{-/}\) brains included a contribution from NPs, Wnt signaling activity was assessed on a homogeneous NP population, namely, ReNcell VM cells. USP9X proteins were depleted from ReNcell VM cells using doxycycline inducible shRNA against \(USP9X\) (Chapter 6). 72 hours after shRNA induction, USP9X protein was almost completely depleted in these cells (Figure 4.11A) (Chapter 6). Consistent with the *in-vivo* results, USP9X-depleted ReNcell VM cells showed increased \(\beta\)-catenin protein levels (Figure 4.11A) and
increased CCND1 expression (n=3) (Figure 4.1B). Additionally, to test whether USP9X’s DUB activity is required for β-catenin regulation, ReNcell VM cells were treated with the small chemical WP1130, which inhibits USP9X’s DUB activity but does not deplete protein levels (Kushwaha et al., 2015; Liu et al., 2015). Similar to in-vivo and shRNA-induced USP9X depleted ReNcell VM results, increased β-catenin protein levels were observed in WP1130 treated ReNcell VM to a similar level as the proteasome inhibitor epoxomicin, implying USP9X DUB activity is important for its regulation of β-catenin (Figure 4.1C).

**Figure 4.11 Increased β-catenin protein level and CCND1 expression in USP9X depleted ReNcell VM cells.** (A) Doxycycline treatment depleted USP9X protein levels 72 hours after treatment in ReNcell VM cells harbouring Usp9x-targeted shRNA compared to the cells harbouring nonsense shRNA (Scrambled). β-catenin expression was significantly increased in the USP9X depleted ReNcell-VM. (B) Increased CCND1 expression in USP9X depleted ReNcell-VM. (C) Chemical inhibition of USP9X deubiquitylating activity increased β-catenin protein levels. Inhibition of USP9X deubiquitylating activity in ReNcell VM cells by WP1130
increased total β-catenin levels to a similar level as the proteasome inhibitor epoxomicin. DMSO was used as the vehicle control. Treating with epoxomicin significantly increased the β-catenin protein level compared to its empty vehicle control in lane 1. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.

To determine if Usp9x depletion directly regulated Wnt signaling, TCF-TopFlash reporter gene assay was performed using HEK293 cells, a well-established in-vitro system to study canonical Wnt signaling. USP9X expression was depleted from HEK293 cells using siRNA specific for USP9X mRNA. Similar to shRNA transfected ReNcell VM cells, complete depletion of USP9X protein was observed 72 hours after the siRNA transfection in HEK293 cells (Figure 4.12A). Nonsense siRNA transfected HEK293 cells (Scr) were used as experimental controls. Consistent with the results from Usp9x−/Y brains, pβ-catenin33/37/41 protein level was clearly increased in the USP9X-depleted HEK293 cells (n=3) (Figure 4.12A). Next, HEK293 cells were transfected with the TCF-TopFlash construct 48 hours after initial siRNA transfections and results were then taken 24 hours after the TopFlash construct transfection (72 hours after the initial siRNA transfection). Increased luciferase activity was observed in the USP9X-depleted HEK293 compared to Scr (n=3) (Figure 4.12B), consistent with the increased Wnt target gene expression in-vivo and USP9X-depleted ReNcell VM cells. Luciferase activity was significantly increased in both Scr and USP9X-depleted HEK293 cells, when treated with exogenous Wnt3a. However, no difference was observed between Wnt3a treated USP9X-depleted and Scr HEK293 cells (Figure 4.12B). Furthermore, when cells were treated with the Wnt signaling inhibitor DKK1, luciferase activity was significantly reduced in the Scr cells, but did not affect the luciferase signal of the USP9X-depleted HEK293 cells. Indeed, luciferase activity in the DKK1 treated USP9X-depleted HEK293 cells remained at the same level as untreated cells (Figure 4.12B). DKK1 antagonizes canonical Wnt signaling by inhibiting LRP5/6 interaction with Wnt and by forming a ternary complex with the transmembrane protein KREMEN that promotes internalization of LRP5/6 (Niida et al., 2004), suggesting USP9X capable of directly activating Wnt target gene expression. In parallel, increased luciferase activity in USP9X-depleted HEK293 cells when treated with Wnt3a suggest Wnt induction is not at its full extent in USP9X-depleted HEK293 cells. A similar phenotype was observed in USP9X-depleted ReNcell VM cells when treated with exogenous Wnt3a for 1 hour (n=3) (Figure
4.12C). Similar to previous results, *CCND1* expression was significantly increased in both Scr and USP9X-depleted ReNcell-VM, however no difference was observed between Wnt3a treated USP9X-depleted ReNcell VM and Scr ReNcell VM (Figure 4.12C).

**Figure 4.12 USP9X opposes Wnt signaling.** (A) USP9X protein is depleted and Ser33/37/Thr41 β-catenin protein is increased in HEK293 cells 72 hours after the siRNA transfection. (B) TCF-TopFlash reporter activity was significantly increased in the USP9X siRNA transfected HEK293 (KD) compared to scrambled nonsense siRNA (Scr) transfected cells. TopFlash reporter activity was increased in both KD and Scr cells when treated with Wnt3a, but no difference was observed between the Wnt3a treated KO and Scr cells. Treating with the Wnt antagonist DKK1, significantly reduced reporter activity only in the Scr HEK293 cells. (C) CCND1 expression was increased in USP9X-depleted ReNcell VM cells compared to the nonsense shRNA transfected cells (Scr). CCND1 expression was increased in both USP9X-depleted and Scr ReNcell VM cells when treated with Wnt3a, but no difference was observed between the Wnt3a treated USP9X-depleted and Scr ReNcell-VMs. All data are
shown as the means ± SEM. Statistical significance was assessed by one-way ANOVA, followed by Tukey’s post-test. ***, p <0.001; **, p < 0.01; *, p < 0.05.

4.3 Discussion

Wnt signaling is one of the prominent extrinsic factors influence in NPs fate regulation (Mutch et al., 2010; Poschl et al., 2013; Draganova et al., 2015). Central to Wnt signaling is the ubiquitin-proteasome mediated proteolytic degradation of its co-transcriptional activator β-catenin, which occurs through the DC (Li et al., 2012; Pronobis et al., 2015a). In addition to ubiquitylation, cells employ deubiquitylation to regulate the amplitude and frequency of Wnt signaling (Table 4.2).

Table 4.2 Deubiquitylating enzymes implicated in Wnt signaling pathway.

<table>
<thead>
<tr>
<th>Wnt Pathway component</th>
<th>Deubiquitylating enzyme</th>
<th>Effect on the Wnt signaling</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DVL</td>
<td>Cylindromatosis tumour suppressor gene (CYLD)</td>
<td>Negative regulation</td>
<td>(Tauroiello et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>USP14</td>
<td>Negative regulator</td>
<td>(Jung et al., 2013)</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>USP4</td>
<td>Positive regulator</td>
<td>(Zhao et al., 2009)</td>
</tr>
<tr>
<td>Axin1/2</td>
<td>USP34</td>
<td>Positive regulator</td>
<td>(Lui et al., 2011)</td>
</tr>
<tr>
<td>APC</td>
<td>TRABID</td>
<td>Positive regulator</td>
<td>(Tran et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>USP15</td>
<td>Negative regulator</td>
<td>(Huang et al., 2009)</td>
</tr>
<tr>
<td>Frizzled Receptors</td>
<td>USP8 (UBPY)</td>
<td>Positive regulator</td>
<td>(Mukai et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>USP6</td>
<td>Positive regulator</td>
<td>(Madan et al., 2016)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>USP7 (incorporate with RNF220)</td>
<td>Positive regulator</td>
<td>(Ma et al., 2014)</td>
</tr>
<tr>
<td></td>
<td>USP47</td>
<td>Positive regulator</td>
<td>(Shi et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>USP4</td>
<td>Positive regulator</td>
<td>(Yun et al., 2015)</td>
</tr>
<tr>
<td></td>
<td>USP9X</td>
<td>Positive regulator</td>
<td>(Taya et al., 1999; Murray et al., 2004; Ouyang et al., 2016)</td>
</tr>
</tbody>
</table>

Most DUBs positively regulate Wnt signaling pathway by rescuing/stabilizing its signalosome function downstream or upstream to the destruction complex. The exceptions are CYLD and Usp14, which stabilize DVL, a negative regulator of Wnt signaling pathway and Usp15, which stabilizes APC expression within the destruction complex.
4.3.1 Usp9x regulates Wnt signaling through β-catenin destruction complex

Elevated levels of β-catenin proteins were detected in Usp9x/Y brains at all embryonic stages from E12.5 onward (earlier stages were not examined, as complete depletion of Usp9x protein was observed only from E12.5). In E12.5 Usp9x/Y NPs, when several critical AJ components were downregulated (Figure 3.2 and 3.4) and in E14.5 Usp9x/Y NPs, when these AJ markers were restored to their normal expression level and pattern (Figure 3.9), β-catenin protein levels remained increased suggesting Usp9x regulates β-catenin independently from its AJ function. This was confirmed by co-IP analyses showing the AJ β-catenin pool remained bound to the N-cadherin in Usp9x/Y brains at both stages (Figure 3.5 and 3.11). In fact the results presented in this chapter identify a novel Wnt signaling regulatory mechanism in NPs, in which a DUB controls β-catenin level by regulating the composition and presumably the function of the DC.

Previously the DC has been portrayed as a relatively static entity targeting cytoplasmic β-catenin for ubiquitin-mediated proteasomal degradation (Xing et al., 2003). However, recent evidence has redefined the DC as a dynamic multiprotein entity with varying component stoichiometry, which alters its composition and structure, thereby defining its functional activity (Gerlach et al., 2014; Pronobis et al., 2015b). Two independent mass spectrometry analyses following an Axin protein pull down of HEK293 cell lysates identified USP9X as a potential component of the DC (Major et al., 2007; Li et al., 2012). In this study we were able to co-IP Usp9x not only with Axin, but also with APC, using endogenous proteins in all instances, from both HEK293 and mouse embryonic cortical tissue lysates confirming the previous reports and extending them an in-vivo relevance.

During the Wnt-off state, phosphorylation of β-catenin at S33/37/T41 primes it for ubiquitylation by β-TrCP within the DC. The significant increase in pβ-catenin33/37/41 levels in Usp9x/Y brains suggests two things, (1) that the components required to progressively phosphorylate β-catenin are present and functioning in the DC and, (2) that either the progression of phosphorylated β-catenin to, or the activity of the ubiquitylation machinery within the DC is hindered in the absence of Usp9x. Interestingly, no difference was observed in total β-TrCP levels in the Usp9x/Y brains (Figure 4.6). Further, APC co-immunoprecipitation suggested all DC components were present in the Usp9x/Y brains rejecting defective assembly of Usp9x/Y DC (Figure 4.9). Increased association between β-TrCP and GSK-3β (Figure 4.9A, B), and β-TrCP and β-catenin (Figure 4.9B) was observed.
in the Usp9x<sup>−/−</sup> brains. These results imply a failure of pβ-catenin33/37/T41 ubiquitylation machinery within the DC, which raises the possibility that Usp9x regulates this transition. Increased β-catenin protein levels in WP1130 treated ReNcell VM to the same level when proteasome was inhibited strongly suggest the importance of deubiquitinase activity of Usp9x for this regulatory step. However, the exact molecular mechanism how Usp9x regulate the ubiquitylatory mechanism within the DC is yet to be elucidated as little is known about the sequential biochemical reactions involved. However, a recent study demonstrated that during the Wnt-on state, β-TrCP monoubiquitylates Gsk-3β (Gao et al., 2014). This monoubiquitylation did not result in Gsk-3β degradation nor affect its enzymatic activity, but increased the association between Gsk-3β and β-TrCP which suppressed β-catenin recruitment to β-TrCP, leading to long-term inhibition of β-catenin ubiquitylation (Gao et al., 2014). The increased Gsk-3β and β-TrCP co-immunoprecipitations with APC in Usp9x<sup>−/−</sup> brains raise the possibility that the dissociation of the β-TrCP from Gsk-3β might have failed in the absence of Usp9x. This was supported by the observation that more Gsk-3β was co-immunoprecipitated with β-TrCP from the Usp9x<sup>−/−</sup> brains (Figure 4.9). Similarly, significantly higher levels of S33/37/T41 pβ-catenin were observed in the cancer cell line SW480, which carries a truncated form of APC, and β-TrCP and Gsk-3β remained bound to the truncated APC (Yang et al., 2006). Our study raises a potential role for Usp9x in the regulating the ubiquitylation activity within the DC, and consequently β-catenin protein stability and Wnt signaling.

Several other DUBs have been implicated in Wnt signal regulation (Table 4.2). However, most of these DUBs rescue/stabilize DC components upstream or downstream to the DC, and thereby positively regulate Wnt signaling pathway. USP34 rescues Axin1 from its ubiquitin induced proteasomal degradation in HEK293 cells (Lui et al., 2011). Interestingly, when USP34 was depleted from HEK293, instead of depleting Wnt signaling due to excessive Axin degradation it increased Wnt activity drawing the conclusion that USP34 acts downstream of the DC (Lui et al., 2011). The DUB Trabid binds and stabilizes APC in HEK293, SW480 and HCT-116 cells clonal cancer lines. However, similar to USP34, depletion of Trabid also resulted in increased Wnt activity in HEK293 cells. Epistasis experiments showed that Trabid acts downstream of the DC and may affect the association or activity of the TCF-β-catenin transcription complex (Tran et al., 2008). In contrast, USP15 rescues APC within the DC from its ubiquitin mediated proteasomal degradation cooperating with the COP9 signalosome (Huang et al., 2009). Similar to the current study, conditional
deletion of COP9 significantly increases β-catenin protein levels in HeLa cells specifying its negative regulatory effect in Wnt signaling (Huang et al., 2009). In addition, three other DUBs have been reported to directly interact with β-catenin, namely Usp7, Usp4 and Usp47 (Ma et al., 2014; Shi et al., 2015; Yun et al., 2015). All these DUBs interact with β-catenin downstream of the DC, thereby rescuing β-catenin from its proteasomal degradation. Unlike current study, deletion of any of the above DUBs, resulted in decreased β-catenin levels. Collectively these previous studies strongly indicate that Usp9x does not act downstream of the DC and most likely regulates Wnt signaling within the DC or upstream. The luciferase assays showed that antagonism of LRP receptors by DKK1 did not change Wnt activity in USP9X-depleted HEK293 cells, implying Usp9x regulates Wnt signaling downstream of receptor activation. Increased β-catenin protein level observed in the ReNcell VM treated with DUB inhibitor WP1130 strongly suggests that USP9X regulation occurs at the post-translational level and highlights the importance of Usp9x’s deubiquitylating activity in this process. Therefore, taken together results from this chapter strongly suggest that Usp9x regulates Wnt signaling through DC, and observation of a consistent phenotype in three different mammalian systems suggests this mechanism is well conserved.

4.3.2 Usp9x is a negative regulator of Wnt signaling in neural progenitors

Deletion of Usp9x affects the β-catenin DC composition preventing the degradation S33/37/T41 pβ-catenin. Accumulating S33/37/T41 pβ-catenin saturates the DC disrupting its function leading to increased cytoplasmic β-catenin level, subsequently activating the Wnt signaling evident by the increased Wnt target genes Ccnd1 and Axin2 in Usp9x-/- brains, and CCND1 expression in USP9X-depleted ReNcell VM and increased luciferase activity in USP9X-depleted HEK293 cells. A similar phenomenon was reported in a recent study conducted by Li et al. (2012), proposing a novel mechanism for increased β-catenin upon Wnt activation. This study showed that upon Wnt activation in HEK293 cells, β-TrCP dissociates from the DC leading to S33/37/T41pβ-catenin accumulation. Saturation of S33/37/T41pβ-catenin within the DC eventually disrupts its function, preventing the targeting of free floating cytoplasmic β-catenin for degradation (Li et al., 2012). In line with this study, results presented in this chapter suggest Usp9x negatively regulates Wnt signaling through the DC. However, the ability of exogenous Wnt3a to increase Wnt activity in USP9X-depleted ReNcell VM and HEK293 cells suggests Wnt signaling is not fully activated in
USP9X-depleted cells. This may explain the relatively low Axin2 mRNA expression observed in \( Usp9x^{-/} \) brains, whereas \( Ccnd1 \) expression could be influenced by other signaling pathways, and this will be discussing further in Section 5.3.1.

Prior to the current project, three studies have reported interaction between Usp9x and \( \beta \)-catenin. In contrast to the current project, these studies reported Usp9x as a positive regulator of \( \beta \)-catenin (Table 4.2). The discrepancy could be due to several reasons. The initial report claiming USP9X as positive regulator of \( \beta \)-catenin was conducted in MDCK cells, in which \( \beta \)-catenin was rescued from its proteasomal degradation by overexpression of the catalytic domain of USP9X (Taya et al., 1999). However, the USP9X catalytic domain failed to bind truncated forms of \( \beta \)-catenin, which lack Axin and APC binding sites (Taya et al., 1999). Since catalytic domains of USP proteins are well conserved and the USP9X catalytic domain failed to bind to the truncated \( \beta \)-catenin lacking Axin and APC binding sites raises questions about the substrate specificity of USP9X in these experiments. The study conducted by Murray et al. (2004) also reported interaction between USP9X and \( \beta \)-catenin in sub confluent T84 cells. In this study, full length USP9X interacted with the cadherin-catenin homodimer within multiple transportation vesicles implying a sub population of Usp9x interacts with the AJ-bound \( \beta \)-catenin, which was discussed in detail in Chapter 3 Section 3.7, suggesting different molecular mechanism for Usp9x and \( \beta \)-catenin interaction. Interestingly, a recent study identified an interaction between full length Usp9x and \( \beta \)-catenin in MCF-7 and MDA-231 breast cancer cell lines (Ouyang et al., 2016). Contrary to the current project, knockdown of USP9X in the MCF-7 breast cancer cell line resulted in decreased \( \beta \)-catenin protein levels. Although the authors did not comment on specific Wnt target genes, they claimed Wnt signaling was reduced in the USP9X knockdown MCF-7 cells by demonstrating decreased decoy death receptors expression. In addition, the authors used \( \beta \)-catenin overexpressed HEK293 cells instead of MCF-7 cells to demonstrate the interaction between USP9X and \( \beta \)-catenin. The different results from these two studies could be due to cell type specific function of Usp9x. Usp9x induces tissue specific responses including opposing effects on same biological process in different cell types (context specific regulation of Usp9x will be discussed in detail in Chapter 7) (Murtaza et al., 2015).
CHAPTER 5

Usp9x regulation of extrinsic fate determinants – Notch signaling
5.1 Introduction

Wnt signaling promotes proliferative self-renewing cell divisions during early neurogenic periods, but induces neuronal differentiation during mid-late neurogenic period by promoting terminal differentiation of INPs (Mutch et al., 2010; Draganova et al., 2015). Despite the increased Wnt signaling activity observed in Usp9x/Y brains from E12.5 onwards, no overt difference in neuronal numbers, cortical lamination or NP populations (with an exception of E12.5 Usp9x/Y brains) were observed in Usp9x/Y brains. The activation of Wnt signaling is relatively small and may be accountable for the less dramatic phenotype observed in Usp9x/Y brains (section 4.3.2) compared with other reports of Wnt activation (Munji et al., 2011; Li et al., 2012; Draganova et al., 2015). However, given that Usp9x is capable of regulating multiple extrinsic fate regulatory pathways in addition to Wnt (Murtaza et al., 2015), other signalling pathways were examined in Usp9x/Y brains.

Notch signaling has been shown to oppose Wnt signaling in several biological systems including ESCs and NPs. Membrane tethered NICD negatively regulates levels of transcriptionally active cytoplasmic β-catenin in mouse ESCs, NPs and cardiac progenitor cells, titrating Wnt response in these stem cells (Kwon et al., 2011). In addition Notch modulator E3 ubiquitin ligase Itch can oppose Wnt signaling either by promoting endocytosis of Frizzled4 receptor (Mossinger et al., 2012) or targeting phosphorylated DVL for ubiquitin-mediated degradation (Wei et al., 2012). Moreover Notch is one of the key extrinsic NP fate determinant (Yoon and Gaiano, 2005; Hatakeyama and Kageyama, 2006; Lowell et al., 2006), and Usp9x is the most implicated DUB in Notch pathway regulations, due to its ability to regulate Notch modulators in both signal sending and receiving cells (Zhang et al., 2012; Murtaza et al., 2015). Therefore the aim of this chapter was to determine the underlying molecular mechanism(s), if any, of Usp9x in Notch signal regulation in NPs.
5.2 Results

5.2.1 Notch signaling is upregulated in Usp9x<sup>−/−</sup> neural progenitors

To evaluate Notch signaling activity in Usp9x<sup>−/−</sup> brains, qRT-PCR analysis was conducted using the same neocortical RNA used for Wnt signaling analyses. Similar to Wnt signaling target gene Axin2, Notch signaling target genes Hes1 and Hes5 were significantly, but slightly increased in Usp9x<sup>−/−</sup> brains at all tested embryonic stages (n=3 for each embryonic stage) (Figure 5.1A, B). Compared to E12.5, E16.5 neocortices are comprised of mixed populations of NPs and fate-restricted neuronal subtypes. Therefore, to determine if the observed increased Notch signaling activity in Usp9x<sup>−/−</sup> brains included a contribution from NPs, Notch signaling activity was measured in a homogeneous NP population, namely the USP9X-depleted ReNcell VM (Chapter 4.2.3). Similar to the in-vivo results, Hes1 expression was significantly increased in the USP9X-depleted ReNcell VM (n=3) (Figure 5.1C).

Figure 5.1 Notch signaling activity is increased in Usp9x depleted neural progenitor cells. qRT-PCR analysis was conducted on RNA extracted from neocortical tissues of Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains at the embryonic stages indicated. Hes1 and Hes5 gene expression was normalised against the housekeeping gene GAPDH. Both Hes1 (A) and Hes5 (B) expression was significantly increased in the Usp9x<sup>−/−</sup> brains compared to Usp9x<sup>+/+</sup> brains at embryonic stages E12.5, E14.5 and E16.5 (n=3). (C) Hes1 expression was significantly increased in the USP9X-depleted ReNcell VM. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *p < 0.05.
Next to determine the underlying molecular mechanism(s) for the increased Notch signaling, expression pattern and level of key Notch signaling components were assessed. First, NICD expression level in neocortical tissue lysates was assessed by WB analysis. Consistent with the qRT-PCR results, a significant increase in NICD protein level was observed in \( \text{Usp9x}^{+/\text{Y}} \) brains at E12.5 and E14.5 (\( n=3 \)) (Figure 5.2). Taken together, qRT-PCR and WB results confirmed increased canonical Notch signaling in \( \text{Usp9x}^{+/\text{Y}} \) brains.

**Figure 5.2 Increased levels of Notch intracellular domain in \( \text{Usp9x}^{+/\text{Y}} \) brains.** Western blot analysis showing increased Notch intracellular domain (NICD) protein levels in E12.5 (A) and E14.5 (B) \( \text{Usp9x}^{+/\text{Y}} \) brains compared to \( \text{Usp9x}^{+/+} \) brains (\( n=3 \)). (B, D) Quantification of relative NICD protein levels after normalisation to \( \beta \)-tubulin. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.* \( p < 0.05 \).
5.2.2 BLBP expression is significantly increased in $Usp9x^{+/Y}$ neural progenitors.

BLBP is a well established Notch signaling target specific for NPs (Anthony et al., 2005). Ectopic expression of NICD in mouse NPs specifically upregulates BLBP expression compared to other NP markers such as Nestin and RC2 (Gaiano et al., 2000). BLBP protein expression was assessed in $Usp9x^{+/Y}$ brains using IHC and WB analyses to further confirm the increased Notch signaling in $Usp9x^{+/Y}$ NPs. Consistent with the preceding results, BLBP immunoreactivity was markedly increased in at the VZs of E12.5 $Usp9x^{+/Y}$ brains (n=3). However no difference was observed for the Nestin (n=3) immunoreactivity between $Usp9x^{+/Y}$ and $Usp9x^{-/Y}$ cortical sections indicating increased BLBP expression was not due to increased number or size of NPs (Figure 5.3 A, S). Increased BLBP expression in E12.5 $Usp9x^{+/Y}$ brains was confirmed by WB analysis (n=3) (Figure 5.3 B).
Figure 5.3 BLBP expression is increased in E12.5 Usp9x<sup>−/−</sup> brains. (A) Coronal sections of E12.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained with BLBP and Nestin. (a, b) Compared to Usp9x<sup>+/+</sup> brains, BLBP showed higher immunoreactivity in Usp9x<sup>−/−</sup> brains (white arrows). (c, d) No difference was observed for Nestin expression between Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains. (B) Westernblot analysis detected increased BLBP expression in Usp9x<sup>−/−</sup> neocortices compared to Usp9x<sup>+/+</sup> (n=3). (C) Quantification of BLBP protein level relative to β-tubulin. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *<sup>+</sup>, p < 0.05.

Increased BLBP expression in E14.5 (n=3) (Figure 5.4A) and E16.5 (n=4) (Figure 5.4B) Usp9x<sup>−/−</sup> brains was confirmed by WB analyses. Increased BLBP expression in Usp9x<sup>−/−</sup> brain is consistent with increased Notch target gene expression observed in Figure 5.3.
Towards the end of neurogenesis, Notch signaling coordinates the astroglial differentiation of RGCs (Mizutani et al., 2007). Although BLBP is commonly used as an early NP marker (RGC), it is a specific astroglial marker. Therefore, increased BLBP expression could also indicate a premature differentiation of Usp9x<sup>−/−</sup> RGCs into astrocytes. To assess the astrocytic component of Usp9x<sup>−/−</sup> brains, sections were co-stained with BLBP and the astrocytic marker GFAP. Increased BLBP immunoreactivity was observed even in E18.5 Usp9x<sup>−/−</sup> brains (n=3) (Figure 5.5) consistent with increased Notch signaling observed in early Usp9x<sup>−/−</sup> brains. However, no difference was observed for GFAP expression pattern or immunoreactivity between Usp9x<sup>−/−</sup> and Usp9x<sup>−/−</sup> brains at E12.5 (n=3) or E18.5 (n=3) (Figure 5.5). This result indicates that increased the BLBP expression in Usp9x<sup>−/−</sup> brains is not due to premature differentiation into astrocytes.
Figure 5.5 Astroglial differentiation is normal in the Usp9x-/-Y brain. (A) Coronal sections E12.5 Usp9x+/Y and Usp9x-/-Y neocortices co-stained with BLBP and the astrocytic marker GFAP. No difference was observed for the GFAP expression (a, b) between Usp9x+/Y and Usp9x-/-Y brains, despite increased BLBP expression (c, d) in Usp9x-/-Y brains. (B) Similar pattern of GFAP (g, h) and BLBP (i, j) staining was observed between E18.5 Usp9x+/Y and Usp9x-/-Y brains. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.
5.2.3 Usp9x regulation of Notch signalling

The next set of experiments sought to determine how Usp9x regulates Notch signaling in the embryonic mouse NPs. The increased NICD expression in Usp9x<sup>−/−</sup> brains could be a result of (1) increased receptor activation and/or (2) impaired NICD degradation. Expression of several Notch modulators, including some known Usp9x substrates, was investigated in Usp9x<sup>−/−</sup> brains. Mib is a positive regulator of Notch signaling. Mib increases Notch signaling by increasing ligand activation in signal sending cells (Mertz et al., 2015). Under some conditions, Usp9x binds and stabilises Mib (Choe et al., 2007; Mertz et al., 2015). Therefore in this scenario depletion of Usp9x would decrease Mib protein levels resulting in decreased Notch receptor activation. Nonetheless, due to the counterintuitive result with Wnt signaling Mib levels were assessed in Usp9x<sup>−/−</sup> brains. No difference was observed in Mib expression in Usp9x<sup>−/−</sup> brains at E12.5 or E14.5 (n=3) (Figure 5.6), suggesting changes in NICD expression was not due to the altered activation of Notch receptors.

![Figure 5.6 Mindbomb expression is unchanged in Usp9x<sup>−/−</sup> brains. (A, C) Westernblot analyses showing equivalent levels of Mindbomb protein expression between Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> brains in E12.5 and E14.5, respectively. (B, D) Quantification of Mindbomb](image_url)
proteins in E12.5 and E14.5 neocortices normalised against β-tubulin. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.

The E3 ligase Itch ubiquitlyates and targets NICD for proteasomal degradation (Qiu et al., 2000). Itch is also a substrate of Usp9x. Usp9x opposes Itch autoubiquitylation and proteasomal degradation (Mouchantaf et al., 2006). Itch expression was assessed in Usp9x<sup>−/−</sup> brains, and was found to be significantly reduced in both E12.5 (n=3) and E14.5 (n=3) Usp9x<sup>−/−</sup> brains (Figure 5.7). This result suggests that increased Notch signaling in Usp9x<sup>−/−</sup> NPs may be, in part, due to the failure of Itch to target excess NICD for degradation.

**Figure 5.7 Decreased Itch protein levels in Usp9x<sup>−/−</sup> brains.** (A, C) Westernblot analyses showing relatively decreased Itch protein levels in Usp9x<sup>−/−</sup> neocortical tissues at E12.5 (A) and E14.5 (C) compared Usp9x<sup>+/+</sup>. (B, D) Quantification of Itch protein in E12.5 and E14.5 neocortices normalised against β-tubulin. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
5.2.4 **Numb is a novel Usp9x binding partner**

Numb is another well-established, prominent modulator of Notch signaling. During asymmetric cell divisions Numb is inherited by the daughter cell, which remains as a progenitor by inhibiting Notch signaling and subsequent neuronal differentiation (Rasin *et al.*, 2007). In the inherited daughter cell, Numb inhibits Notch signaling by targeting NICD for ubiquitin mediated degradation (McGill and McGlade, 2003). The premature differentiation of early *Usp9x*/*Y* NPs (Figure 3.15 and 3.16) and increased NICD levels prompted an investigation into Numb distribution in *Usp9x*/*Y* brains. Spatial distribution of Numb was assessed by IHC analysis; higher Numb immunoreactivity was observed at the VZ of the *Usp9x*/*Y* brains, however Numb immunoreactivity was clearly reduced, if not completely depleted, from *Usp9x*/*Y* brains at all tested embryonic stages (n=3 for each genotype) (Figure 5.8A). The reduced Numb protein levels in E12.5 and E14.5 *Usp9x*/*Y* brains were confirmed by WB analysis (Figure 5.8B, C). Collectively these results imply a direct correlation between Usp9x and Numb protein levels in neural cells.
Figure 5.8 Decreased Numb protein levels in Usp9x<sup>−/−</sup> brains. (A) Coronal sections of E12.5 (a, b), E14.5 (c, d) and E18.5 (e, f) Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained with Numb antibodies. Numb immunoreactivity was greatly reduced in Usp9x<sup>−/−</sup> brains at each tested embryonic stage (a, c, e) compared to Usp9x<sup>+/+</sup> brains (b, d, f). (B, C) Westernblot analyses showing reduced Numb protein levels in E12.5 and E14.5 Usp9x<sup>−/−</sup> neocortices. Numb protein level was quantified by normalising against β-tubulin. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. * < 0.05.
To further investigate the direct correlation between Usp9x and Numb protein expression in the developing brain, E14.5 wild-type brain sections were co-stained with Usp9x and Numb antibodies. Usp9x and Numb proteins were extensively co-localised throughout the VZ of E14.5 neocortices (n=3) (Figure 5.9A) suggesting these proteins may physically interact in embryonic NPs. Co-IP was conducted using E14.5 wild-type neocortical tissue with Usp9x antibody to examine whether Usp9x physically interacts with Numb proteins. Reflecting the co-localization results, a strong interaction between Usp9x and Numb proteins was observed (Figure 5.9B). These results strongly suggest Usp9x binds and regulates Numb protein levels in embryonic mouse brains.

Figure 5.9 Usp9x interacts with Numb in embryonic mouse brains. (A) Coronal sections of E14.5 neocortices immunostained with Usp9x (a) and Numb (b) antibodies. (c) Shows the extensive co-localization of Usp9x and Numb proteins, most notably at the apical domain of the VZ. (B) Co-immunoprecipitation analysis conducted on E14.5 cortical lysates using Usp9x antibody, immunoprecipitated both Numb and Itch proteins. (C) Co-immunoprecipitation conducted on E14.5 cortical lysates using Numb antibodies immunoprecipitated Usp9x and Itch proteins confirming these proteins forms a complex. Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.
5.3 Discussion

5.3.1 Increased Notch signaling in Usp9x⁻/⁻ brains

In this chapter the effect of Usp9x deletion on Notch signaling in embryonic mouse brains and ReNcell VM was assessed. Similar to Wnt singling, an increase in Notch signaling was observed in Usp9x⁻/⁻ brains from E12.5 onward, demonstrated by increased Hes1 and Hes5 Notch target genes. In addition, increased NICD and BLBP protein levels observed in Usp9x⁻/⁻ brains, further confirms the increased Notch signaling. Interestingly, several studies reported that in addition to Wnt signaling, Notch signaling could also upregulate Ccnd1 expression in mammalian cells. Ectopic Notch1 expression in RKE cells and Jagged1 expression in TN breast cancer cells increased CCND1 expression through CSL transcription factor-mediated pathways (Cohen et al., 2010; Ling and Jolicoeur, 2013). Pulse activation of Notch signaling in mouse ESCs during neural induction period also significantly increased Ccnd1 transcription (Das et al., 2010). In light of these reports and the increase in Notch target gene expression observed in Usp9x⁻/⁻ brains, it may suggest that the relatively higher Ccnd1 expression, compared to Axin2, in Usp9x⁻/⁻ brains (Figure 4.10) result as a synergetic effect of increased Notch and Wnt signaling.

On the other hand, Usp9x⁻/⁻ brains showed a slight, but significant, increase in Hes1 and Hes5 expression similar Wnt target gene Axin2. As discussed in Chapter 3 the slight increase in Axin2 may imply a partial activation of Wnt singling in Usp9x⁻/⁻ brains. Based on the expression similarity, it could assume that the slight increase in Hes1 and Hes5 expression may also be due to a partial activation of the Notch signaling in Usp9x⁻/⁻ brains. In contrast to Wnt, Notch signaling cannot be activated by adding exogenous ligands, presenting difficulties testing this hypothesis even in the in-vitro USP9X-depleted ReNcell-VM. However novel approaches such as micro-bead (Beckstead et al., 2009) and mechanogenetic (Seo et al., 2016) could be applied to activate Notch signaling, but due to time constraints and technical limitations we were unable to utilize these techniques in the current study.

Although the slight induction of Hes genes suggest a partial activation of Notch signaling in Usp9x-depleted NPs, NP specific Notch target gene BLBP (Anthony et al., 2005) expression was significantly increased in Usp9x⁻/⁻ brains starting from E12.5 (Figure 5.3-5.5). Notch signaling activates Fabp7 expression through its canonical Suppressor of Hairless pathway (Patten et al., 2006). Even though increased BLBP expression could have resulted
by the increased astroglial differentiation and/or cellular hypertrophy (Chambers et al., 2001; Takahashi et al., 2004), normal Nestin and GFAP expression does not support those conclusions. Therefore significantly increased BLBP expression not only confirms the Notch signaling activation in Usp9x<sup>y</sup> brains but also suggests that slight induction in Hes genes is enough to initiate a physiological response in Usp9x<sup>y</sup> brains.

5.3.2 Usp9x interacts with Notch signaling modulators Numb and Itch in neural progenitors

To date, three E3 ubiquitin ligases Sel10, Itch, and Cbl target different forms of NICD for ubiquitylation. Sel10 exclusively polyubiquitylates the nuclear NICD pool targeting it for proteasome mediated degradation (Gupta-Rossi et al., 2001; Wu et al., 2001), while Cbl polyubiquitylates membrane tethered NICD targeting it for lysosomal degradation (Jehn et al., 2002). Itch is an evolutionarily conserved negative regulator of Notch signalling, which polyubiquitylates membrane tethered NICD through K-48 (Qiu et al., 2000) or K-29 (Chastagner et al., 2008) linked polyubiquitin chains targeting NICD for proteasomal or lysosomal degradation, respectively. Usp9x rescue Itch from its auto-ubiquitylation mediated proteasomal degradation (Mouchantaf et al., 2006). Therefore, reduced Itch expression in Usp9x<sup>y</sup> brains is consistent with current literature and, may be accountable for increased NICD and Notch signaling.

However, other Itch interacting molecules play an important role during Notch regulation, particularly the Notch inhibitor, Numb. Numb attenuates Notch signaling by promoting the polyubiquitylation mediated degradation of membrane tethered NICD. Numb interacts with the cytosolic domain of Itch and facilitates the binding of Itch to membrane tethered NICD (McGill and McGlade, 2003). In addition several other Itch independent mechanisms have also been proposed for Numb’s inhibitory effect on Notch signaling. In Drosophila sensory precursor cells, Numb antagonises Notch signaling by inhibiting the plasma membrane localization of Sanpodo. Sanpodo is required for Notch signaling in these cells and Numb binding mediates the endocytosis of the Sanpodo along with bound Notch, inhibiting Notch signaling (Cotton et al., 2013). Numb could also inhibit Notch signaling by controlling the post-endocytic trafficking of Notch receptors through its interactions with the AP-2 adaptor complex and α-adaptin (Smith et al., 2004; McGill et al., 2009). Furthermore,
Numb could inhibit Notch signal transduction by directly binding to the NICD, and promoting its polyubiquitylation mediated degradation independently from Itch (Berdnik et al., 2002; McGill and McGlade, 2003). These studies suggest that reduced Numb protein alone, could have increased Notch signaling in Usp9x<sup>−/−</sup> brains.

Unlike Itch, Numb is polyubiquitylated by the E3 ligases Murine double minute 2 (Mdm2) (Sczaniecka et al., 2012) and LNX (Nie et al., 2002) targeting it for proteasomal degradation. To our knowledge no other DUB has been reported to interact with Numb. During this project, a strong physical-interaction between Usp9x and Numb protein was detected by co-localization and co-IP assays. Concurrently, Numb protein level was significantly reduced in Usp9x<sup>−/−</sup> brains implying a functional correlation between Usp9x and Numb protein. Taken together these results identify Usp9x as the first DUB capable of regulating Numb protein expression, at least in developing mouse brains.

In addition to inhibiting Notch signaling, Numb plays a pivotal role in maintaining cortical tissue architecture by regulating AJs and polarity of NPs (Li et al., 2003; Rasin et al., 2007). Conditional deletion of Numb from mouse cortical NPs, disrupts their AJs and polarity similar to Usp9x (Chapter 3). In contrast to the Usp9x<sup>−/−</sup> phenotype, Numb knockout mice displayed a more prominent phenotype including severe cortical dysplasia and hydrocephalus, which persists through later developmental stages (Li et al., 2003; Rasin et al., 2007). The phenotypic difference and restored AJs and polarity in Usp9x<sup>−/−</sup> brains, suggests that depleted Numb protein level is not responsible for the perturbed AJ and polarity in Usp9x<sup>−/−</sup> brains.

**5.3.3 Does Usp9x “buffer” the Notch and WNT signaling output in neural progenitors?**

Wnt and Notch signaling are key extrinsic NP fate determinants. Recent studies showed that rather than operating as two separate pathways, Wnt and Notch signaling configured into an integrated molecular network during development (Hayward et al., 2008; Munoz-Descalzo et al., 2012; Collu et al., 2014). Co-activation by Notch and Wnt signaling is required to activate the *vestigial* gene transcription, which is required for wing formation (Klein and Arias, 1999). This Notch and Wnt signaling co-operative regulation of gene expression has also been reported during endothelial cell differentiation in vertebrates (Yamamizu et al., 2010). Another common mechanism of Notch and Wnt signaling
integration arises from their transcriptional dependent interactions, in which expression of one signaling pathway activates or represses the other. For example, during vertebrate somitogenesis activation of Wnt signaling primes Notch activation through LEF1 transcriptional regulation of Dll1 (Galceran et al., 2004).

However the most common avenue for Notch and Wnt integration occurs through crosstalk between their signal transducing elements, which directly affects the signaling output of the pathways. For example, in addition to inhibiting Wnt signaling, Dvl could also inhibit Notch signaling. In Drosophila sensory organ precursor cells Dvl attenuates Notch signaling by promoting Notch endocytosis through a mechanism not yet fully understood (Munoz-Descalzo et al., 2010). Dvl attenuates Notch signaling in Xenopus embryos by inhibiting the CSL transcription factors downstream of Notch receptors (Collu et al., 2012). The Wnt antagonist GSK-3β can either activate or repress Notch signaling depending on the Notch receptor type it phosphorylates. In Neuroblastoma2a and embryonic fibroblasts, phosphorylation of N1ICD by GSK-3β reduced its proteasomal degradation perpetuating Notch signaling (Foltz et al., 2002), while phosphorylation of N2ICD by GSK-3β in NIH-3T3 fibroblast cells inhibits the NICD transcriptional activity (Espinosa et al., 2003). In addition, Itch can negatively regulate Wnt signaling by either promoting endocytosis of the Frizzled4 receptor (Mossinger et al., 2012) or targeting phosphorylated DVL for ubiquitin-mediated degradation (Wei et al., 2012). An elegant study conducted by Kwon et al. (2011) showed that membrane tethered NICD negatively regulates Wnt signaling by targeting transcriptionally activated β-catenin (when DC is inactive) for lysosomal mediate degradation through a mechanism regulated by Numb in NP, ESC and cardiac precursor cells. Several other studies however report that even cleaved NICDs are capable of negatively regulating active β-catenin (Hayward et al., 2005; Acosta et al., 2011).

Results presented in this chapter suggest that Usp9x regulation may also fall into the last category. Contrary to previous studies, increased NICD did not attenuate Wnt signaling in Usp9x−/− NPs. Unlike previous studies, in this current project Notch and Wnt signaling modulators regulated through the common element, Usp9x. Although Usp9x deletion increased NICD and β-catenin levels, that did not fully activate the Notch and Wnt signal pathways; at least for Wnt signaling as evident by higher target gene expression in response to exogenous Wnt3a (Figure 4.12). This may indicate that Usp9x acts like a buffer between Wnt and Notch signaling during NP fate specification. Depletion of Usp9x would have perturbed this balance increasing both signaling pathways (Figure 5.10). Although the
underlying mechanism of this regulation is yet to be elucidated, balancing Notch and Wnt signaling pathway by Usp9x may important in maintaining the resting and/or excitatory state of the progenitors. Under normal physiological condition NPs remain within the stem cell niche in a resting state until fate regulatory pathways intervenes. However, both intrinsic and extrinsic regulatory pathways remain activate at low levels during the resting phase, which is now identified as signaling noise (Ladbury and Arold, 2012; Munoz-Descalzo et al., 2012). Signaling noise is important for maintaining normal biochemical reactions in progenitors/stem cells at the resting state (Ladbury and Arold, 2012). Usp9x might be important to maintain the signaling noise of Notch and Wnt signaling at a physiologically relevant level during the resting phase. During fate specification period, multiple fate determinant pathways act on a single progenitor/stem cell. These fate determinant pathways regulate the cell fate in a permissive manner. Therefore, cell fate is not decided by one single fate determinant pathway, but by the final output of multiple fate determinant pathways based on the time and location of the cell (which is also known as spatial and temporal specific regulation of progenitor fate). In this manner Usp9x may play a vital role during NP fate speciation period, titrating Notch and Wnt outputs based on the final fate decision of the NPs.
Figure 5.10 Usp9x balances Wnt and Notch signaling in neural progenitors. Usp9x is capable of regulating transcriptional co-activators of Notch and Wnt signaling pathways, NICD and β-catenin respectively. Considering the post-posttranslational modification nature, and being the final arbitrary both of NICD and β-catenin, it is possible that Usp9x act as buffer between Notch and Wnt signaling pathways by balancing the absolute numbers of the NICD and β-catenin proteins. In Usp9x<sup>−/−</sup> neural progenitors’ deletion of Usp9x resets the level of this balance and as a consequence both signaling pathways were increased.
CHAPTER 6

Usp9x regulation of extrinsic fate determinants – mTROC1 signaling
6.0 CHAPTER SIX - Usp9x regulation of intrinsic fate determinants - mTOR signaling

6.1 Statement of contribution to co-authorship

This chapter consists of a co-authored manuscript. ‘USP9X deubiquitylating enzyme maintains RAPTOR protein levels, mTORC1 signaling and proliferation in neural progenitors’ which has been re-submitted, with corrections, to ‘Scientific Reports’.

The authors list is as follows: Caitlin R. Bridges*, Susitha Premaratne*, Men Chee Tan*, Devathri Nanayakkara, Bernadette Bellette, Dusan Zencak, Jozef Gecz, Mariyam Murtaza, Lachlan A. Jolly and Stephen A. Wood. * equal contribution.

My contribution to the manuscript / this chapter involved experimental design, conducting experiments, data analysis and manuscript preparation. The data I generated were included in;

- Figure 6.1 C, D
- Figure 6.4 B, C, D and E
- Figure 6.5 K, L
- Figure 6.6 A, C, D, E, F, G, H
- Figure S6.6
- Figure S6.7

Signed: _________________________________ Date:____________

Susitha Premaratne

Countersigned: ___________________________ Date:____________

Supervisor and corresponding author: Associate Professor Stephen A. Wood
6.2 Abstract

The deubiquitylating enzyme USP9X highly expressed in neural progenitors, and essential for neural development in mice. In humans, mutations in USP9X are associated with neurodevelopmental disorders. To better understand the requirement for USP9X in neural progenitors, we studied the effects of altering its expression in, human neural progenitor cell line, ReNcell VM, as well as neural stem and progenitor cells derived from Usp9x conditionally deleted mice. Here we show that even a partial decrease in USP9X protein levels in ReNcell VM cells rapidly resulted in G0/G1 cell cycle arrest. There was a concomitant decrease in mTORC1 signalling, a major regulator of G0/G1 cell cycle progression. Further analyses revealed, (i) the canonical mTORC1 protein, RAPTOR, physically associates with USP9X in embryonic mouse brains, (ii) RAPTOR protein level is directly proportional to that of USP9X, in both loss- and gain-of-function experiments and, (iii) USP9X deubiquitylating activity opposes the proteasomal degradation of RAPTOR. Neurosphere assays confirmed that USP9X is required to maintain the proliferation of neural progenitors but not stem cell self-renewal capacity, similar to both RAPTOR-null and rapamycin treated neurospheres. To our knowledge, USP9X is the first deubiquitylating enzyme shown to stabilize RAPTOR.

6.3 Introduction

The proliferation, self-renewal and differentiation of neural stem cells (NSCs), and their closely related derivatives, neural progenitors (NPs), in both the developing and adult nervous systems, are tightly controlled by extrinsic and intrinsic signals. Extrinsic signals, derived from the stem cell niche or embryonic organising centres, need to be recognised, relayed internally, coordinated and interpreted to ultimately produce a precise cellular response. Cell polarity, adhesion, cycle length and migration all influence the response to extrinsic signals. Concerning the cell cycle, the G0 and G1 phases are particularly involved in dictating the fate of a NP (Blomen and Boonstra, 2007). Specifically, it is the length of the G1 phase that regulates progenitor differentiation. Cells that linger in these phases have increased longevity and retain the capacity to self-renew (Blomen and Boonstra, 2007). Mammalian target of rapamycin (mTOR) pathway responds to growth factors by coordinating an appropriate RNA and protein synthesis response and, as such, is a major
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regulator of G0/G1 cell cycle progression. mTOR is an ubiquitous protein kinase found in two complexes, mTORC1 and mTORC2. The mTORC1 complex, which consists of mTOR, regulatory-associated protein of mTOR (RAPTOR) and mammalian lethal with SEC13 protein 8 (MLST8), responds to growth factor stimulation and links this, along with nutrient availability, to cell growth and division (Fingar et al., 2004; Dibble and Manning, 2013). In particular, mTORC1 response to growth factor stimulation is required for cell cycle progression through the G1 phase of the cell cycle (Fingar et al., 2004).

mTOR is a critical regulator of NSC/NP function in-vivo and in-vitro balancing self-renewal, proliferation, differentiation and maturation (Magri, L. and Galli, 2012), and dysregulation of mTOR signalling gives rise to neurodevelopmental disorders (Dibbens et al., 2013; Scheffer et al., 2014). Hyper activation of the mTOR pathway, due to loss of function mutations in the TSC1 and TSC2 genes, upstream inhibitors of mTOR, gives rise to Tuberous Sclerosis Complex (TSC). TSC is characterized by benign malformations comprised of aberrantly proliferating non-malignant cells of the tissue of origin. In the brain, these lesions are ectopic neurogenic compartments with enhanced proliferation of NPs and their subsequent premature differentiation (Magri, Laura et al.). Conversely loss of mTORC1 function results in decreased NP proliferation. Deletion of RAPTOR, an essential protein of the mTORC1 complex, from NPs of the dorsal telencephalon leads to decreased proliferation but not loss of self-renewal capacity (Cloëtta et al., 2013). Similarly, these cardinal features are seen in ex-vivo cultures of murine NSC/NPs grown as free-floating aggregates, called neurospheres. Inhibition of mTORC1 signalling in neurospheres, by addition of rapamycin or deleting RAPTOR, decreased proliferation of NPs without affecting the self-renewing capacity of the NSCs (Sato et al., 2010). Therefore, mTOR signalling needs to be tightly balanced to maintain homeostasis in NPs.

Another protein well placed to integrate extrinsic signals with the intrinsic responses of NPs, is the ubiquitin-specific protease 9 located on the X-chromosome (USP9X). USP9X is a deubiquitylating enzyme highly expressed in adult and embryonic NPs in-vivo, and in-vitro (Murtaza et al., 2015). Altering USP9X expression levels affects NP function. Moderately increased Usp9x expression in mouse embryonic stem cell-derived NPs in-vitro promotes their self-renewal leading to a large increase in the number of NPs (Jolly, L.A. et al., 2009). Conversely, Nestin-cre mediated deletion of Usp9x from all NPs of the mouse central nervous system disrupted their organisation in the ventricular and sub-ventricular
zones and results in peri-natal lethality (Stegeman et al., 2013). Deletion of *Usp9x* from the dorsal telencephalon only, is compatible with post-natal survival, but results in a dramatic 75% reduction in adult hippocampal size, suggesting NP proliferation is reduced (Stegeman et al., 2013). Mutations in human *USP9X* are associated with several neurodevelopmental disorders including X-linked intellectual disability and autism (Homan et al., 2014). In addition, mutations in Doublecortin that specifically disrupt its ability to interaction with USP9X, result in lissencephaly and severe epilepsy, further highlighting the importance of USP9X function for normal brain development (Friocourt et al., 2005).

Recently, Usp9x has been implicated in mTOR signalling in C2C12 mouse muscle myoblasts (Agrawal et al., 2012). Knockdown of Usp9x in these cells increased mTORC1 activity (Agrawal et al., 2012). Epitope pull-down assays showed that Usp9x associated with mTOR, as well as RAPTOR and RICTOR, signature proteins of the mTORC1 and mTORC2 signalling complexes, respectively (Agrawal et al., 2012). However altered expression of *USP9X* did not affect the level of mTOR protein in HEK293 cells. Here, we show that USP9X is a potent regulator of the mTORC1 signalling in NP/NSCs. Decreasing USP9X levels resulted in a rapid arrest of cultured NPs in G0/G1 of the cell cycle. Further we show that USP9X binds RAPTOR in the developing brain and maintains RAPTOR levels in cultured NPs suggesting RAPTOR is a critical USP9X substrate.

### 6.4 Results

#### 6.4.1 USP9X depletion results in reduced neural progenitor number

To directly test the role, if any, of USP9X in NPs we altered its levels in the immortalized human neural progenitor cell line, ReNcell VM. To deplete USP9X in these cells, lentiviral vectors with doxycycline-inducible expression of shRNAs directed against USP9X were generated (Drabsch et al., 2007). The lentiviral vector also expressed EGFP, which was used to identify and FACS purify, successfully transduced pools of cells, for subsequent experiments. Two independent shRNAs (2193 and 4774, indicating the position of the first base pair of the shRNA in the USP9X open reading frame) efficiently depleted USP9X and these cell lines were chosen for future experiments. Induction of a scrambled shRNA, as well as the addition of doxycycline, had no effect on USP9X protein levels.
Partial loss of USP9X was evident 24 hours after doxycycline addition in 2193 and 4774 cells, and reached maximal levels by 72 hours (Figure 6.1A). To examine the effect of USP9X depletion on ReNcell VM and determine the time of maximum effect, cells were analysed using the xCELLigence system, which measures electrical impedance, and so is proportional to cell number, in real time. Analysis of two biological replicates revealed that the cell index of ReNcell VM cells expressing USP9X shRNAs (2193 and 4774) plateaued approximately 20 hours after doxycycline addition (Figure 6.1B). In 2193 and 4774 cells the decrease in cell index reached statistical significance at 34 hours and 28 hours, respectively. At 24 hours USP9X protein levels were not yet fully depleted (Figure 6.1A). These data indicate that ReNcell VM NPs are particularly sensitive to reduced USP9X levels.

6.4.2 USP9X depletion decreases the proliferation of ReNcell VM cells but does not result in morphological changes, apoptosis or differentiation

The xCELLigence cell index, which measures electrical impedance, does not distinguish between effects on cell proliferation, morphology or cell death. Therefore we examined the effect of USP9X knock-down on these functions. No changes in ReNcell VM morphology were observed, but a clear reduction in the cellular density of USP9X depleted ReNcell VM could be distinguished at 72 hours after doxycycline treatment (Figure S6.1). In addition, lower MTT levels were detected in USP9X depleted ReNcell VM after 48 and 72 hours doxycycline treatment consistent with decreased cell numbers (Figure S6.1B). Analysis of Annexin V (Figure S6.2) and cleaved Caspase 3 (Figure S6.3A) indicated there was no increase in apoptosis, after 12 and 72 hours doxycycline treatment, respectively. Immunoblot analysis failed to detect any change in the level of the astrocytic marker GFAP or neuronal marker βIII-tubulin, indicating that loss of USP9X did not induce the differentiation of ReNcell VM cells to post-mitotic cell fates (Figure S6.3B). Finally, to directly determine the effect on USP9X knockdown on cell proliferation an EdU pulse assay was performed to label cells undergoing DNA synthesis during S-phase. Significantly fewer EdU positive cells were detected in 2193 and 4774 cells treated with doxycycline for 72 hours compared to those expressing the scrambled shRNA (Figure 6.1C, D). These data demonstrate that loss of USP9X decreases the proliferation of ReNcell VM cells but does not alter their morphology, cell death or differentiation.
Figure 6.1 USP9X depletion reduces ReNcell VM proliferation. (A) Doxycycline treatment depleted USP9X protein levels after 24 hours, 36 hours and 72 hours in ReNcell VM cells harbouring USP9X-targeted shRNA (2913 and 4774) but not a nonsense shRNA (Scrambled) or non-transduced cells (Wildtype) (representative of six biological replicates). (B) Reduced Cell index of ReNcell VM cells following addition of 1 μM Doxycycline. xCELLegence real-time measurement of increasing electrical impedance plotted on “Y axis” (“Normalised Cell Index”). Cell Index of doxycycline treated 2193 ReNCell VM cells differed significantly from non-treated after 34 hours (p<0.05), 35 hours (p<0.01) and 36 hours (p<0.001). After 72
hours, the cell index was approximately 50% of untreated 2193 cells. For 4774 cells, the cell index differed significantly at 28 hours p<0.05, 29 hours p<0.01 and 30 hours p<0.001 after treatment. After 72 hours, the reduction in cell index was approximately 61% that of untreated 4774 cells. Error bars represent standard deviation (Wt: wildtype, Scr: Scrambled, +: 1 μM Doxycycline treated, -: No Doxycycline). (C) ReNcell VM cells exposed to doxycycline for 72 hours were labelled with a 6 hour EdU pulse. Scale bar = 50 μm (D) Quantitation of (C) revealed significant fewer EdU-positive cells in doxycycline-treated 2193 and 4774 cells compared to Scrambled. All data are shown as the means ± SEM. Statistical significance was assessed by one-way ANOVA, followed by Tukey’s post-test. ***, p <0.001

6.4.3 USP9X depletion results in G0/G1 cell cycle arrest

To investigate if USP9X depletion affected ReNcell VM cell cycle, flow cytometric profiling of cellular DNA content was conducted. Knockdown of USP9X resulted in an increase in cells at the G0/G1 stage of the cell cycle 72 hours after doxycycline treatment (Figure 6.2A, B). Over three biological replicates the average increase in cells in G0/G1 following USP9X depletion was 12.6% ± 6.3 for “2913” cells (p = 0.0134) and 22.91% ± 8.9 for “4774” cells (p = 0.0065). There was no statistically significant alteration in wildtype or scrambled shRNA cells exposed to doxycycline (Figure S6.4). Removal of doxycycline from the culture medium resulted in restored levels of USP9X and ReNcell VM proliferation (data not shown). These data indicated that USP9X is required for progression of ReNcell VM cells through G0/G1 and/or progression into S-phase. To investigate the molecular mechanism contributing to the accumulation of cells in G0/G1 phase we examined, by immunoblot, the levels of various proteins facilitating the G1/S phase transition. A decrease in the phosphorylated form of retinoblastoma protein (pp-Rb\(^{S780}\)) was detected in USP9X depleted cells, but only after 72 hours of doxycycline treatment. No consistent changes in other proteins including Cyclin D1 or E2F1 were observed at any stage of the time course (Figure S6.4). As the xCELLigence cell index of USP9X depleted ReNcell VM cells plateaued at 24 hours this suggested that decreases in pp-Rb\(^{S780}\) were unlikely to be the mechanism initiating the G0/G1 arrest.
Figure 6.2 Loss of USP9x results in ReNcell VM cell accumulation in the G0/G1 phase of cell cycle. (A) Flow cytometric analysis of ReNcell VM cells after 72 hours of doxycycline treatment (representative of three biological replicates). (B) Quantitation of percentage of cells in each cell cycle phase in (A). All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.* p < 0.05.

6.4.4 USP9X depletion in ReNcell VM cells reduces mTORC1 signalling

It has recently been reported that USP9X interacts with the mTOR signalling pathway (Agrawal et al., 2012), a major regulator of progression through the G1 phase of the cell cycle. mTOR exists in two complexes, mTORC1 and mTORC2, and the activity of each pathway is measured by the phosphorylation of their substrates, S6 and Akt, respectively (Agrawal et al., 2012; Leontieva, O. V. et al., 2012; Leontieva, O. et al., 2014; Rodgers et al., 2014). In the absence of growth factor stimulation, cells arrest in G0. However, re-addition of growth factors promptly activates mTOR signalling. Therefore we examined the activation of mTOR in ReNcell VM cells, by the re-addition of EGF and FGF, in the presence and absence of USP9X. ReNcell VM cells were exposed to doxycycline for 72 hours for maximal knockdown of USP9X, and a further 24 hours in the absence of EGF and FGF to cause G0 arrest. Upon the reintroduction of EGF and FGF there was a rapid increase in p-S6 levels, dependent on the presence of USP9X (Figure 6.3A, D). A failure to induce p-S6, in the absence of USP9X, was observed in each of six biological replicates. There were no obvious or consistent alterations in p-AKT, total S6 or total AKT protein levels across the biological replicates (Figure 6.3A and data not shown). To determine if the decreased mTORC1 signalling occurred with similar kinetics to the observed cell index plateauing in the xCelligence assay (Figure 6.1B), we examined p-S6 protein levels following 24 hours doxycycline treatment. A similar decrease in p-S6 levels in the absence of USP9X was detected at this earlier time point (Figure 6.3B) suggesting this may account for the plateauing cell index detected in the xCelligence assay. Phosphorylation of S6 occurs at the end of the mTORC1 signalling cascade. We therefore examined the protein levels of upstream components of mTORC1, and mTORC2. The levels of total and p-mTOR (Figure 6.3C), pp70S6K and RICTOR (not shown) were not altered in the absence of USP9X following 72 hours of doxycycline treatment, however, a reduction in total RAPTOR levels
was evident (Figure 6.3C, E), and observed in three additional biological replicates (Figure 6.3D, E and Figure S6.5).

**Figure 6.3** mTORC1 activity and RAPTOR levels in ReNcell VM cells are dependent on USP9X. (A) Immunoblot analysis of expression of the mTORC1 and mTORC2 pathways
components induction in USP9X depleted ReNcell VM. Prior to protein harvest, ReNcell VM were expose to doxycycline for 72 hours and deprived for growth factors for additional 24 hours to arrest the cells at G0. Proteins were collected at 0 minutes, 5 minutes and 15 minutes after re-addition of growth factors. Both p-S6 and p-Akt levels increased upon exposure to EGF/FGF (compare 0 to 5 and 15 minutes) indicating both mTORC1 and mTORC2 pathway activation, respectively. However, the extent of induction was markedly reduced in the absence of USP9X (compare plus and minus doxycycline at 5 and 15 minutes in 2193 and 4774). Total S6 and Total AKT were not affected by USP9X depletion (representative of three biological replicates). (B) Diminished mTORC1 activation in ReNcell VM cells, as determined by p-S6 levels, was evident after only 24 hours doxycycline treatment, consistent with the xCELLigence data. (C) RAPTOR protein levels are decreased in USP9X-depleted ReNcell VM cells (2193 and 4774 plus Doxycycline). Phospho and Total mTOR levels were unaffected by the absence of USP9X (representative of two biological replicates). (D) Densitometric quantitation, of immunoblots from two biological replicates, of initial p-S6 induction compared to total S6 levels 5 minutes after addition of EGF/FGF in the absence (dark grey bars) or presence (light grey) of doxycycline. The treatment of cells with doxycycline significantly attenuates the level of p-S6 in 2193 and 4774 cells only. (E) Densitometric quantitation, of immunoblots from three biological replicates, of RAPTOR protein, relative to β-tubulin control, at time 0 hours in (C) in the absence (dark grey bars) or presence (light grey) of doxycycline. The treatment of cells with doxycycline significantly attenuates the level of RAPTOR in 2193 and 4774 cells only. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.*, p < 0.05.

6.4.5 USP9X regulates RAPTOR levels

RAPTOR is the major scaffolding protein of the mTORC1 complex and also binds p70S6 kinase (Hara et al., 2002; Nojima et al., 2003). The level of RAPTOR protein is reportedly regulated by ubiquitylation (Hussain et al., 2013; Choi et al., 2014) raising the possibility that USP9X maintains mTORC1 signalling by opposing RAPTOR degradation by ubiquitin-proteasome system. If so, the levels of substrate, RAPTOR, should correlate with those of the deubiquitylating enzyme, USP9X. We have shown that loss of USP9X correlated with decreased levels of RAPTOR (Figure 6.3C, E, and S6.5). To further investigate the
relationship between these proteins, USP9X was transiently over-expressed in HEK293 cells (due to low transfection efficiency of ReNcell VM cells). Increased levels of USP9X resulted in increased RAPTOR but did not alter the level of other mTORC1 pathway components including mTOR, total S6 or p-S6 (Figure 6.4A). Over-expression of three USP9X mutations, which do not affect their deubiquitylating activity but are associated with human intellectual disability (Homan et al., 2014), also increased RAPTOR levels (Figure 6.4A). The above loss- and gain-of-function approaches, in human ReNcell VM cells and HEK293T cells, respectively, identified a direct correlation between USP9X and RAPTOR protein levels but they do not indicate if USP9X’s deubiquitylating activity is required. Recently a small compound, WP1130, has been identified which inhibits USP9X deubiquitylating activity, but does not affect USP9X protein level (Peterson et al., 2015). In addition, WP1130 rapidly inhibits USP9X within hours, compared with days for RNA knockdown approaches and thereby circumvents possible induction of compensatory deubiquitylating enzyme expression (Peterson et al., 2015). The addition of WP1130 to proliferating ReNcell VM cells under normal culture conditions resulted in a rapid (2 hours) decrease in RAPTOR protein levels but had no effect on p70S6K (Figure 6.4B). As reported by others, WP1130 did not affect the level of USP9X protein, but did deplete another well-established USP9X substrate, MCL-1 (Schwickart et al., 2010) after 4 hours. Therefore, inhibition of USP9X’s deubiquitylating activity leads to a rapid decrease in RAPTOR protein in ReNcell VM cells.

6.4.5 USP9X opposes proteasomal degradation of RAPTOR

The above data are consistent with USP9X opposing the degradation of RAPTOR by ubiquitin-proteasome system. To directly examine this, we measured RAPTOR levels in ReNcell VM cells treated with the proteasome inhibitor, epoxomicin (Meng et al., 1999). Epoxomicin treatment for 4 hours increased RAPTOR levels (Figure 6.4C lane 2) suggesting it is subject to proteasomal degradation in ReNcell VM cells. As shown previously, inhibition of USP9X deubiquitylating activity by WP1130 resulted in depleted RAPTOR levels (Figure 6.4C lane 4). The combination of epoxomicin and WP1130 resulted in intermediate levels of RAPTOR protein compared with epoxomicin or WP1130 alone (Figure 6.4C, compare lane 6 with 2 and 4). These data are consistent with USP9X’s deubiquitylating activity opposing degradation of RAPTOR by the proteasome. However, as WP1130 inhibits deubiquitylating enzymes other than USP9X (Kapuria et al., 2010), proteasomal inhibition experiments were...
Figure 6.4 **USP9X regulates RAPTOR levels and opposes its proteasomal degradation in neural progenitors.** (A) **USP9X over-expression increased RAPTOR protein levels in HEK293 cells.** HEK293T cells were transiently transfected with empty vector (Control), full
length USP9X (WT), and three USP9X mutant variants. Cell lysates were collected 24 hours later. Immunoblot analysis identified increased RAPTOR protein level, but mTOR, total and p-S6 levels were unaffected. (B) Chemical inhibition of USP9X deubiquitylating activity leads to a rapid depletion of RAPTOR protein in neural progenitors. ReNcell VM cells were treated with 5 μM WP1130 and analysed by immunoblot. WP1130 does not deplete USP9X protein levels over 4 hours but results in almost complete loss of RAPTOR after 2 hours. Phospho-p70S6 kinase levels were not affected. Levels of the USP9X substrate MCL-1 were depleted after 4 hours. (C) RAPTOR protein levels are regulated by proteasomal inhibition (epoxomicin) and inactivation of USP9X deubiquitylating activity (WP1130) in ReNcell VM cells. Proteasomal inhibition following 4 hours exposure to 25 nM epoxomicin resulted in increased RAPTOR levels. Inhibition of USP9X deubiquitylating activity with 5 μM WP1130 for 4 hours resulted in decreased RAPTOR. Treatment of ReNcell VM cells for 4 hours with WP1130 followed by 4 hours of epoxomicin resulted in intermediate levels of RAPTOR. DMSO was used as the vehicle control for each experiment. Final concentrations of DMSO were – 0.0025% for epoxomicin; 0.00005% for WP1130; 0.00255% for WP1130 and Epoxomicin. (D) Epoxomicin treatment of 4774 shRNA knockdown ReNcell VM cells resulted in increased RAPTOR protein levels. RAPTOR levels were decreased in doxycycline treated 4774 shRNA cells (+Doxycycline) (representative of two biological replicates). (E) Densitometric quantitation, of immunoblots from two biological replicates, demonstrating a significant increase in RAPTOR protein levels in the presence of the proteasomal inhibitor epoxomicin (Full length blots presented in Supplementary Figure S7). All data are shown as the means ± SEM. Statistical significance was assessed by one-way ANOVA, followed by Tukey’s post-test. *, p < 0.05.

repeated in the 4774 ReNcell VM line to validate a specific role for USP9X in the inhibition of RAPTOR degradation. Similar to WP1130 treated ReNcell VM, USP9X-depleted ReNcell VM had the effect on Raptor levels when expose to epoxomicin (Figure 6.4D, E). Furthermore, qRT-PCR analysis indicated that loss of USP9X did not affect Raptor mRNA levels (Figure S6.6).
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6.4.6 USP9X null neurospheres have reduced neural progenitor proliferation but not stem cell self-renewal

The mTORC1 pathway is well known to regulate murine NP proliferation. Inactivation of mTORC1 signalling in NPs in-vivo, by conditional Nestin-cre mediated deletion of the Raptor gene in mouse brains, resulted in reduced NP proliferation. When mTORC1 signalling is inhibited in NSC/NPs cultured ex-vivo as neurospheres, the reduced NP proliferation manifested as a reduction of neurosphere size (Cloëtta et al., 2013). We therefore performed neurosphere assays on Usp9x<sup>-/-</sup> NSC/NPs derived from late embryonic stage mouse brains following Nestin-cre deletion of Usp9x (Stegeman et al., 2013). Neurospheres contain a heterogeneous population of NSC, NP and differentiated neural cells (Giachino et al., 2009). Upon single cell dissociation and re-culture at very low density, to exclude cell aggregation, only cells with stem cell-like properties reform neurospheres (Reynolds and Weiss, 1996; Rietze et al., 2001; Capela and Temple, 2002; Kim and Morshead, 2003). These NSCs typically represent only ~1-5% of the cells derived from neurospheres (Reynolds and Weiss, 1996; Capela and Temple, 2002; Giachino et al., 2009). In contrast, NPs have very limited capacity to reform spheres over multiple passages. We applied the well-established serial passage sphere-forming assay to identify the effect, if any, that loss of Usp9x had on the long-term self-renewal of the NSC population. In this assay, a set number of single NPs are plated at very low density following each passage (1x10<sup>4</sup> cells/ml) and the number of spheres that form in those cultures then counted. Over several passages, Usp9x<sup>-/-</sup> cultures consistently contained significantly more neurosphere forming cells (2.6% compared to 1.7%; p<0.05; Figure 6.5B). Next we asked if the loss of Usp9x had any effect on the multipotency of the NSCs. As each sphere is derived from a single cell, the cell types a sphere can produce reflects the multipotency of the original sphere-forming cell (Reynolds and Weiss, 1996; Giachino et al., 2009). Spheres were plated at very low density and allowed to differentiate before identifying the three basic neural lineages that are produced from embryonic NSCs, namely, neurons, astrocytes and oligodendrocytes (Figure 6.5C). We scored individual spheres for the presence of all three, two or one lineages as tri-, bi and mono-potent respectively. The majority of spheres (70-73%) were tri-potent, and there were no significant differences between Usp9x<sup>+/+</sup> and Usp9x<sup>-/-</sup> cultures, suggesting loss of Usp9x had no effect on the multipotency of the sphere-forming stem cells (Figure 6.5D). Given the increase in the number of spheres in Usp9x<sup>-/-</sup> cultures, we asked if this resulted into an increase in the absolute number of cells that were produced during culture, however, both
$Usp9x^{+/Y}$ and $Usp9x^{-/Y}$ cultures produced equivalent cell numbers across several passages (Figure 6.5E). This finding had two implication; firstly that the increased percentage of sphere forming cells in $Usp9x^{-/Y}$ cultures translated into increases in the absolute number of sphere forming cells in the cultures (Figure 6.5F); and secondly, that individual neurospheres in $Usp9x^{-/Y}$ cultures must contain fewer cells. Indeed, on average, neurospheres in $Usp9x^{-/Y}$ cultures displayed modest but significant reductions in the number of cells per sphere (30% reduction), which was also reflected in reductions in the average sphere diameter (24% reduction) at the time of passaging (Figure 6.5A, G, H). Therefore, while loss of Usp9x promoted the self-renewal and expansion of the sphere-forming NSC population, the bulk growth of the sphere after formation was compromised. To investigate if this loss of bulk growth could be attributed to altered differentiation behaviour, we used immunofluorescence to reveal the expression of cell-type specific marker proteins to identifying cell types present in the spheres. As it is technically difficult to resolve in the 3D environment of a neurosphere, we dissociated the neurosphere cultures into single cells and plated them onto coverslips. Following attachment overnight and 24 hours growth, cells were fixed and stained for marker proteins of NPs (Sox2), neurons (βIII-tubulin) and astrocytes (GFAP) and nuclei counterstained with DAPI (Figure 6.5I). Using this labelling regime, greater than 99% of all cells were labelled (data not shown). At this time, there was already a 51% reduction in total cell numbers (Figure 6.5J). This reduction was caused solely by a loss of Sox2-positive NPs (67% decrease) with the numbers of astrocytes and neurons being equivalent. To directly test if the absence of Usp9x affected NP proliferation neurospheres were exposed to a 6 hour EdU pulse followed by cell counts (Figure 6.5K, L) or flow cytometric analysis (Figure S6.8). As was observed for ReNcell VM cells, significantly fewer Sox2-positive NPs were proliferating (Edu-positive) in the Usp9x-deleted neurospheres.

In aggregate these data suggest the absence of Usp9x in neurospheres promotes the self-renewal of a rare population of sphere-forming cells that displays the properties consistent with that of NSCs, whilst simultaneously inhibiting the proliferative capacity of the bulk transiently amplifying NP population. This phenotype, of smaller neurospheres and reduced NP proliferation but not their self-renewal, is shared with the central features of both Raptor null or rapamycin treated neurospheres (Cloëtta et al., 2013) and together suggests that Usp9x may also be required to regulate mTORC1 function in NPs in neurospheres.
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**Figure 6.5** Loss of Usp9x promotes stem-cell self-renewal and loss of bulk neural progenitor proliferative capacity. Neurosphere cultures were derived from the cortices of embryonic day 18.5 wild-type (Usp9x<sup>+/Y</sup>; n=4) and knockout (Usp9x<sup>-/Y</sup>; n=5) mice and grown as independent cultures. (A) Representative phase contrast images Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> neurosphere cultures. (B) Usp9x<sup>-/Y</sup> cultures contain a higher percentage of sphere forming cells. The percentage of cells obtained from the dissociation of neurospheres that re-form spheres during subsequent culture at very low density was derived over several passages and an average percentage of sphere forming cells calculated. (C and D) Loss of Usp9x does not affect the multipotency of sphere-forming cells. Neurosphere cultures (passage 2, n=3) were plated as whole spheres at low density and outgrowths stained and scored after 6 days. (C) Representative image of immunofluorescent labelled cultures following immunostaining for cell type marker proteins GFAP: astrocytes; βIII-tubulin: neurons and CNPase: oligodendrocytes. Images show examples of tri-potent outgrowths. (D) The percentage of neurosphere outgrowths displaying tri - bi and mono-potency scored. Percentages were compared by Student t-test and were not statistically different. (E) Usp9x<sup>+/Y</sup> and Usp9x<sup>-/Y</sup> cultures contain equivalent number of total cells. Cell counts conducted at each passage and expressed as an average. (F) Usp9x<sup>-/Y</sup> cultures contain increased absolute numbers of sphere forming cells. Total sphere-forming cells calculated for each culture across several passages and expressed as an average. (G and H) Usp9x<sup>-/Y</sup> neurospheres are smaller in size. The mean number of cells per neurosphere and the mean diameter of neurospheres was calculated at each given passage and expressed as an average. (I and J) Loss of Usp9x decreases NP cell number. Neurosphere cultures (passage 2, n=3 for both Usp9x<sup>-/Y</sup> and Usp9x<sup>-/Y</sup>) were dissociated and plated at very low density onto poly-l-lysine coated plates in media containing EGF and cultures fixed 24 hours later. (I) Representative image of immunofluorescent stained cultures used to quantify cell types; cells labelled for cell marker proteins; Sox2: NP; GFAP: astrocytes; βIII-tubulin: neurons. Cell nuclei counterstained with DAPI. Scale bar = 50 μm. (J) Cell counts used to quantify the numbers of cell types present in cultures. (K and L) Loss of Usp9x inhibits NP cell proliferation. (K) Representative images of neurospheres allowed to attached for 2 hours and then followed by a 6 hours EdU pulse and the immunostained for the NP marker Sox2 (Green) and EdU (Red). (L) A significantly lower percentage of Usp9x<sup>-/Y</sup> NPs (Sox2-positive) were labelled with EdU indicating the absence of Usp9x diminishes NP proliferation. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.* p < 0.05.
6.4.7 USP9X binds RAPTOR and regulates mTORC1 activity *in-vivo*

We next sought to determine if USP9X regulates RAPTOR and the mTORC1 pathway activity *in-vivo*. First, to determine if endogenous Usp9x and Raptor proteins interact during brain development, immunoprecipitation was performed on lysate from the frontal cortex of wild-type embryonic mouse brains, as these are enriched for NPs. A clear interaction between endogenous Usp9x and Raptor proteins was detected, as was a weaker interaction with phospho-p70S6K (Figure 6.6A). Interestingly, no interaction between Usp9x and mTOR was detected under these conditions. In addition immunoprecipitation using anti-Raptor antibodies enriched for Usp9x (Figure 6.6B). Therefore Usp9x and Raptor interacts *in-vivo*. Next the functional consequences of Usp9x depletion on Raptor and mTORC1 signalling *in-vivo* was determined by immunoblot. Significantly lower levels of Raptor and p-S6 protein were detected in *Usp9x*/*Y* brains at E12.5 (Figure 6.6C-E). Similar analysis on neurospheres derived from E18.5 brains confirmed a dependence of p-S6 levels on *Usp9x*/*Y* neurospheres, but interestingly levels of Raptor were not significantly altered (Figure 6.6F-H). These data support a role for USP9X in the maintenance of Raptor and mTORC1 pathway signalling in NPs.
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A

IP- Usp9x

Inputs

IgG

Usp9x

Raptor

Phospho-p70 S6 Kinase

β-tubulin

B

IP- RAPTOR

Inputs

IgG

Raptor

Usp9x

β-tubulin

C

E12.5

Usp9x−/−1

Usp9x−/−2

Usp9x−/−3

Usp9x−/−4

Usp9x−/−1

Usp9x−/−2

Usp9x−/−3

Usp9x−/−4

Usp9x

Raptor

p-S6

β-tubulin

D

E

Relative Raptor protein level

[Graph]

Relative p-S6 protein level

[Graph]

F

E18.5 NSP

Usp9x−/−1

Usp9x−/−2

Usp9x−/−3

Usp9x−/−4

Usp9x−/−1

Usp9x−/−2

Usp9x−/−3

Usp9x−/−4

Usp9x

Raptor

p-S6

β-tubulin

G

H

Relative Raptor protein level

[Graph]

Relative p-S6 protein level

[Graph]
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Figure 6.6 USP9X binds RAPTOR and regulates mTORC1 activity in-vivo. (A and B) Co-immunoprecipitation of endogenous Usp9x and Raptor proteins from E14.5 mouse frontal cortical tissue. (A) Anti-USP9X antibodies precipitated Raptor protein. An interaction with phosho-p70S6 kinase was also detected. The image has been slightly manipulated due to the different exposure time used to capture the inputs and immunoprecipitation bands. Cropped images were put together using Microsoft photo editor. (B) Anti-RAPTOR antibodies precipitated Usp9x protein. (C) Immunoblot analysis of Usp9x, Raptor and p-S6 protein from E12.5 Usp9x+/Y and Usp9x−/Y mouse forebrains (n=4). (D and E) Densitometric analysis of (C) revealed relatively lower levels of Raptor (D) and p-S6 (E) protein levels in Usp9x−/Y brains. (F) Immunoblot analysis of Usp9x, Raptor and p-S6 protein E18.5 derived neurospheres (3 Usp9x+/Y versus 4 Usp9x−/Y). No difference was observed in Raptor protein levels (G) but p-S6 levels were significantly decreased in Usp9x−/Y neurospheres. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.* p < 0.05; ** p < 0.01.

6.5 Discussion

USP9X is highly expressed in, and regulates the function of, NPs both in-vivo and in-vitro (Jolly, L.A. et al., 2009; Stegeman et al., 2013; Oishi et al., 2016). Previously, increased expression of Usp9x was shown to regulate NP self-renewal by promoting apical-basal cell polarity and altering the architecture of NP cultures derived from mouse embryonic stem cells (Jolly, L.A. et al., 2009). Here we show USP9X is also required to maintain proliferation of the human NP line ReNcell VM, in a cell autonomous manner, and this is probably mediated via facilitating mTORC1 pathway signalling.

Inducible knock down of USP9X demonstrated that ReNcell VM cells are exceptionally sensitive to USP9X depletion. Within 24 hours of initiating USP9X knockdown, proliferation of the ReNcell VM cells halted (Figure 6.1) despite the presence of mitogenic quantities of EGF and FGF. Considering that USP9X protein levels were not completely depleted at this time (in some experiments USP9X was only 50% depleted) and, that ReNcell VM have a cell cycle time of approximately 30 hours, this indicates that even partial depletion of USP9X results in a rapid and comprehensive arrest of proliferation. Although apoptosis can quickly alter cell numbers, and the anti-apoptotic protein MCL-1 is a
USP9X substrate in some cellular contexts (Schwickart et al., 2010), no alterations in Annexin V or cleaved caspase 3 levels were detected, indicating no increase in apoptosis (Figure S6.2, S6.3). Nor did USP9X depletion promote the differentiation of ReNCell VM to post-mitotic neuronal or glial lineages (Figure S6.3). Instead the NPs accumulated in the G0/G1 phase of the cell cycle (Figure 6.2). This rapid response was not due to alterations in G1/S check-point proteins, although a delayed decrease in p-Rb levels was observed.

The mTOR pathway regulates progression through the G0/G1 stage of the cell cycle (Fingar et al., 2004) and Usp9x influences mTORC1 and mTORC2 pathway activity in the C2C12 mouse myoblast cell line (Agrawal et al., 2012). Here we show that USP9X is required for mTORC1, but not mTORC2, activity in ReNcell VM human NPs. The depletion of USP9X levels, loss of mTORC1 signalling, as indicated by failure to induce p-S6, and ReNcell VM cell-cycle arrest, all occurred with very similar kinetics strongly suggesting these NPs rely on USP9X-dependent mTORC1 activity for proliferation.

Post-translational modification of mTOR proteins, including by ubiquitylation, allow the mTOR pathway to respond rapidly to changing extra-cellular signals. Both p-mTOR and RAPTOR are targeted for proteasomal degradation by poly-ubiquitylation (Ghosh et al., 2008; Hussain et al., 2013). Here we present several lines of evidence identifying the canonical mTORC1 scaffold protein, RAPTOR, as a probable USP9X substrate in NP/NSCs. Firstly, we show that endogenous Usp9x and Raptor proteins co-immunoprecipitate from mouse embryonic brain tissue enriched for NPs (Figure 6.6A, B). Usp9x did not interact with mTOR under the same conditions. The much weaker interaction detected between Usp9x and phospho-p70S6K (Figure 6.6A) may be direct or indirect via Raptor, which binds phosphor-p70S6K to recruit it to the mTORC1 complex (Nojima et al., 2003). The absence of any alteration in phospho-p70S6K levels following the inhibition of USP9X activity (Figure 6.4B), suggests Usp9x does not directly regulate phospho-p70S6K.

Second, inducible USP9X-specific shRNA-mediated knockdown (Figure 6.3C, E, S6.5), and transient over-expression (Figure 6.4A) of full length USP9X resulted in a concomitant decrease and increase in RAPTOR protein, respectively. Use of the chemical WP1130, which rapidly inhibits USP9X’s deubiquitylating activity but does not deplete USP9X protein levels, confirmed a clear and rapid loss of RAPTOR (Fig. 4C). The near complete depletion of RAPTOR protein within 2 hours strongly suggests that USP9X regulation occurs at the post-translational level. This is supported by the observation that the
loss of MCL-1, a bona fide USP9X substrate, occurred only after 4 hours in the same experiment (Figure 6.4C). However, as WP1130 inhibition is not restricted to USP9X, we cannot rule out the possibility that other DUBs, in addition to USP9X, might also regulate RAPTOR stability. To address this we analysed ReNcell VM cells with inducible USP9X-specific shRNA-mediated knockdown and these produced similar results to WP1130 (Fig 4D,E) indicating USP9X plays a significant, if not exclusive, role in maintaining RAPTOR levels in NPs.

An obvious hypothesis is that USP9X’s deubiquitylating activity opposes RAPTOR’s degradation at the proteasome. Data presented in Figure 6.4C and 6.4D confirmed that inhibition of the proteasome increased RAPTOR protein levels in ReNcell VM cells as observed in HEK293T cells (Hussain et al., 2013), and that inhibiting the proteasome, in the absence of USP9X activity partially rescued RAPTOR levels. USP9X is the first deubiquitylating enzyme shown to stabilize, components of the mTOR pathway namely, RAPTOR. Although another deubiquitylating enzyme, UCH-L1 has been shown to impact upon mTORC1 signalling it does so indirectly by affecting assembly of the mTORC1 complex (Hussain et al., 2013). Unlike USP9X, UCH-L1 has no effect on the protein levels of mTORC1 complex components including RAPTOR.

USP9X’s regulation of mTOR signalling appears to be cell context specific. Recently it was shown in C2C12 muscle myoblasts that epitope-tagged USP9X weakly associated with mTOR and both RAPTOR and RICTOR in HEK293T cells and its depletion in C2C12 myoblasts accelerated their differentiation to myotubes (Agrawal et al., 2012). In contrast to our results in NPs, Usp9x depletion from C2C12 cells led to increased mTORC1 activity, in response to growth factor stimulation. Interestingly, while Usp9x depletion in C2C12 cells altered the downstream mTORC1 and mTORC2 effectors, p-S6 and p-AKT, respectively, no alteration in the levels of the upstream proteins mTOR or RAPTOR, were detected. Therefore in C2C12 myoblasts, the regulation of mTOR signalling by Usp9x may be indirect. Cell context specific roles of USP9X have also been observed in other systems (Schwickart et al., 2010; Perez-Mancera et al., 2012; Cox et al., 2014). Although Usp9x has been identified as a potential regulator of stem cell function (Van Hoof et al., 2006), decreasing Usp9x levels does not overtly diminish the proliferation of other progenitor/stem cells including, embryonic stem cells (Nagai et al., 2009), T-cell progenitors (Park et al., 2013) or pancreatic progenitors (Perez-Mancera et al., 2012). In contrast, the data presented here indicates that
both cultured human NPs and, those in mouse neurospheres, are particularly sensitive to USP9X levels.

mTOR signalling is a major regulator of NP function in both development and disease (Magri, L. and Galli, 2012). Our data establish that USP9X facilitates mTORC1 signalling in NPs in cultured human NPs, neurospheres and developing mouse brain. Our data also reveals the phenotype of Usp9x null neurospheres is very similar to that reported for rapamycin treated (i.e. mTORC1 inhibited) and Raptor depleted neurospheres (Cloëtta et al., 2013) and is therefore consistent with Usp9x stabilisation of mTORC1 signalling in mouse NPs as well. Loss of either Usp9x or Raptor resulted in smaller neurospheres but did not affect their capacity for serial passage indicating they affected proliferation of NPs without inhibiting the self-renewal capacity of the sphere-initiating NSC. Likewise rapamycin treatment of neurosphere cultures resulted in reduced NP proliferation (and reduced neurosphere size) without affecting the stem cell state (Sato et al., 2010), and rapamycin delivery to the adult sub-ventricular zone neurogenic niche specifically depleted the transiently amplifying NPs but not the stem cell population (Paliouras et al., 2012). These data are consistent with retention of transiently amplifying NPs in the G0 stage of the cell cycle, a phenomenon reported for adult neural stem cells exposed to rapamycin (Paliouras et al., 2012). However, we did not detect a significant decrease in Raptor protein in Usp9x-null neurospheres, as assessed by immunoblot (Figure 6F, G) as observed in the other NP systems tested. This may be due to the non-synchronised nature of cycling NPs or the presence of mitogens as initial experiments on asynchronously growing ReNcell VM cells in the presence of mitogens also failed to detect significantly altered p-S6 levels in, at least by immunoblot (data not shown).

Finally, we observed that the loss of Usp9x resulted in more neurosphere-initiating stem cells at each passage, despite fewer proliferating NPs. This suggests that mTORC1 is not the only pathway regulated by USP9X in NPs, as inhibition of Raptor function in neurospheres did not produce a similar increase in sphere-forming cells (Cloëtta et al., 2013). In addition, in ES cell-derived NPs in-vitro it is a moderate increase in Usp9x levels which promotes NP self-renewal (Jolly, L. A. et al., 2009). Indeed, USP9X regulates components of multiple pathways important to NP fate including the Notch, Wnt and TGF-β pathway (Murtaza et al., 2015) and the final outcome is very much dependent on the type of NP.
Consistent with a context-specific role for USP9X in the regulation of NP self-renewal we recently reported that loss of Usp9x has a differential effect on quiescent versus proliferating NSCs in the postnatal mouse hippocampal subgranular zone (Oishi et al., 2016). The differential dependence of NPs and sphere-forming NSCs on Usp9x may reflect a similar molecular mechanism. As Usp9x regulates brain development in mice (Stegeman et al., 2013) and is associated with several human neurodevelopmental disorders (Brett et al., 2014; Homan et al., 2014) the precise role of Usp9x in NPs and stem cells warrants further investigation.

6.6 Materials and Methods

6.6.1 Generation of inducible Usp9x knock-down ReNcell VM cell lines

The human neural stem cell line ReNcell VM was obtained from Millipore (Darmstadt, Germany) and maintained as described (Donato et al., 2007). To generate ReNcell VM with doxycycline-inducible knock-down of USP9X, cells were transduced with a lentiviral vector (Brown et al., 2010) containing either a “scrambled” shRNA sequence: 5’ACTACCGTTGTTAGGTGTTCAAGAGACACCTA TAACAACGGTAGT3’ with no homology to any known gene, or shRNA targeting two independent USP9X sequences “2193” 5’GCTTGATCCTTCCCTGTTAAC3’ (the first base pair is 2193 in the human USP9X sequence) or “4774” 5’GCCATAGAAGGCACAGGTAGT3’. Successfully transduced pools of ReNCell VM were isolated by FACS based on constitutive expression (Brown et al., 2010). These transduced pools were used in subsequent experiments. Induction of shRNA expression was achieved by supplementing the media with 1 μM Doxycycline.

6.6.2 Functional analysis of ReNcell VM

xCELLigence Assays – The xCELLigence system for real-time cell viability monitoring was used as described (Limame et al., 2012). Briefly, ReNcell VM cells were plated at 5 x 10^3 per well and 1 μM doxycycline added the following day. The plate was analysed by xCELLigence (Roche, Basel, Switzerland) in real time over 5 days of doxycycline treatment.

MTS Assay – The MTS assays were performed according to the manufacturer’s instructions (Promega).
Annexin V Apoptosis Assay - Cells were seeded at 2 x 10^5 cells/ml and treated with 1 μM doxycycline for 12 hours. After 12 hours treatment, cells were harvested by centrifugation at room temperature for 5 minutes at 0.4 RCF and subjected to Annexin V assay as per the manufacturer’s instructions (Trevigen, Gaithersberg, USA).

Cell Cycle Analysis - Cells were seeded at 2 x 10^5 cells/ml and treated with 1 μM doxycycline 12 hours later. Cells were in log phase at time of harvest. Cells were washed with HBSS and centrifuged for 5 mins at 0.3 RCF before suspension in 1 ml HBSS and fixation at 4°C for 1 hour in 100% cold ethanol. Cells were stained with Propidium Iodide staining solution (3.8 mM Sodium Citrate, 50 μg/ml PI in DPBS) and 50 μl of RNase A stock solution (10 μg/ml RNase A in DPBS) was added. Tubes were stored at 4°C until Flow cytometry analysis using a Becton Dickinson FACS Aria.

6.6.3 Molecular analyses

Immunoblotting - Cells were lysed with RIPA lysis buffer (150 mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50 mM Tris, and pH 8.0) supplemented with 100 μl/mL protease inhibitor. Cell lysate was then processed for immunoblotting and visualised with ECL substrate as described (Stegeman et al., 2013). The following antibodies have been used: Primary Antibodies: Cell Signalling Technologies (Danvers, MA): Rabbit-Cyclin D136kDa (1/1000), Mouse-IgG1 mono Cyclin E (HE12) 48-56kDa (1/1000), Mouse-IgG2b-mon Cyclin A (BF683) 55kDa (1/2000), Rabbit-poly Caspase-317, 19, 35 kDa (1/1000), Rabbit Cleaved Caspase-3 (Asp175)17,19kDa (1/2000), Rabbit-poly MCL-1 (1/500) L.40kDa S. 32kDa. Purified Rabbit N-terminal (Kanai-Azuma et al., 2000) (1/2000), Bethyl Labs (Montgomery, TX): Rabbit USP9x C terminal–290kDa (1/2000) Sigma Aldrich (St Louis, MO): Mouse Beta-Tubulin (1/2000), Trevigen (Gaithersberg, USA): Rabbit GAPDH (1/2000). Secondary Antibodies: Life Technologies (Mulgrave, AUS): Rabbit HRP (1/5000), Mouse HRP (1/5000). Millipore (Darmstadt, Germany): Rabbit HRP (1/5000), Mouse HRP (1/5000).
**Immunoprecipitation** – Protein lysate was isolated from the frontal cortex of mid-gestation embryonic mouse brain to enrich for neural progenitors. The isolated tissues were lysed by sonication in the presence of the IP lysis buffer – 20 mM HEPES pH 7.8, 400 mM KCl, 5 mM EDTA, 0.4% NP40, 10% glycerol, 1 mM DTT, protease and phosphatase inhibitor cocktails (Cell Signalling, Danvers MA). Immunoprecipitation was conducted using Pierce co-immunoprecipitation kit (Thermo Scientific, Rockford, IL) according to manufacturer’s instructions. Immunoprecipitated proteins and total protein extracts were then subjected to immunoblot analysis.

**Over-expression of USP9X** - Transient expression of wild-type USP9X, and variant forms associated with intellectual disability, in HEK293T cells was as previously described (Homan et al., 2014). Briefly, cells were transfected using Lipofectamine 2000 reagent as per manufactures instructions (Life Technologies). Cell lysates were collected after 24 hours and subjected to immunoblot analysis.

### 6.6.4 Neurosphere Culture

Isolation and culture of NPs from the E18 cortex was as previously described (Giachino et al., 2009; Jolly et al., 2013). All neurosphere-based assays were conducted in at least biological triplicate, with each replicate representing a neurosphere culture derived from individual *Usp9x*+/Y or *Usp9x*−/Y embryos. Averages of the replicates results are reported. Sphere forming assays were initiated by dissociating neurospheres and isolating single cells by passing dissociated cells through a 0.75 µm cell filter (BD Biosciences, San Jose, CA). Single cells were replated at very low density (1x10^4 cells/mL) and cultured for 6 days. The number of spheres was calculated, and subsequently dissociated again to generate single cells for sphere forming assays. Number of cells per sphere was derived using total cell counts and total sphere number counts at each passage. At least 100 spheres were imaged per replicate and analysed using ImageJ to determine average sphere diameters. For the identification of cell types, dissociated single cells from neurospheres were plated onto poly-l-lysine (Sigma, St Louis, MO) coated coverslips (Menzel-glasser, Thermo Fisher Scientific) at 1x10^4/cm² in the presence of EGF and cultured for 24 hours at which point cells were fixed for immunofluorescent staining. The number of neuronal, astrocytes and progenitor cells was counted. Oligodendrocyte differentiation was negligible (<1%) and not included. At least 200 cells were counted per replicate (total n: *Usp9x*+/Y=1299; *Usp9x*−/Y=756). For analysis of multipotency, single cells derived from neurosphere dissociation and then plated at very low density...
density. Following 6 days culture, whole neurospheres were plated onto poly-l-lysine coated coverslips at low density (30 spheres/ 35mm well) and cultured for a further 6 days. Neurosphere outgrowths were fixed and immunofluorescently stained for the presence of terminally differentiated cells namely neurons, astrocytes and oligodendrocytes. Neurosphere outgrowths were scored as uni-, bi and tri-potent based on the presence of one, two or all three cell types in the outgrowths, respectively. At least 20 outgrowths were scored per replicate (total n: Usp9x+/Y = 81; Usp9x−/Y = 68). All data points represent the average of replicate means and error bars represent standard deviation of replicate means. Statistical significance derived using unpaired Student’s t-test assuming equal variance and set to p<0.05.

6.6.5 Immunofluorescence

Cultured cells were fixed with 4% PFA for 15 minutes at room temperature and blocked and permeabilised using 10% PBST and Donkey Serum, respectively, for 1 hour at room temperature. Primary and secondary antibodies were incubated in PBST-3% Donkey Serum overnight at 4°C and 1 hour at room temperature respectively at the following dilutions; mouse anti-CNPAs, (1:2000; Chemicon, Germany), rabbit anti-GFAP, goat anti-GFAP, mouse anti-βIII-tubulin, rabbit anti-βIII-tubulin (all at 1:300; Sigma-Aldrich), rabbit anti-Pax6 (1:200; Chemicon, Germany), donkey anti-sheep Alexa Fluor 555, donkey anti-rabbit Alexa Fluor 488/555/647 and donkey anti-mouse Alexa Fluor 488/555/647 (1:1000; Invitrogen), donkey anti-chicken Cy3 (Jackson Laboratories, Bar Harbour, ME). Cells were counterstained with DAPI and mounted with slow-fade mounting media (Invitrogen). Non-specific staining was controlled by using secondary-only controls. Fluorescence was viewed using the Axioplan2 microscope (Carl Zeiss, Jena, Germany) fitted with an HBO 100 lamp (Carl Zeiss, Jena, Germany). Images were captured using an Axiocam Mrm camera and Axio Vs40 v4.5.0.0 software (Axiovision, Germany).

6.6.6 Animal Use

This study was performed with the approval of both, the Griffith University Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia and the Australian Commonwealth Office of the Gene Technology Regulator as well as under regulations of the South Australian Animal Welfare Act 1986, and in strict accordance with the Australian Code of Practice for the Care of Animals for Scientific
Purposes, 2004. The study was approved by the Women’s and Children’s Health Network (WCHN) Animal Ethics Committee (Approval Number: 750/06/2011 and 888/06/13). All experiments were carried out in accordance with the approved guidelines.

6.7 References


USP9X REGULATION OF mTORC1 SIGNALING


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6.8 Supplementary data

Figure S6.1 Deletion of USP9X reduces ReNcell VM culture density but does not overtly affect cell morphology. (A) USP9X was depleted in ReNcell VM cells by 72 hours culture in presence of 1μM doxycycline. Bright field microscopy of Wildtype (A, A’), Scrambled (B, B’), 2193 (C, C’), and 4774 (D, D’) ReNcell VM clones. Cell density was reduced in 2193 +Dox (C’) and 4774 +Dox (D’) cultures with no pronounced effect on Wildtype (A’) or Scrambled (B’) cultures. (B) MTT Assay analysis of ReNcell VM cells after (a) 48 hours and (b) 72 hours treatment with 1 μM doxycycline showing reduction in proliferation in 2193 +Dox and 4774 +Dox clones. * p <0.05; ** p<0.01. Error bars = standard error of the mean.
**Figure S6.2 Annexin V assay.** Analysis of apoptosis in ReNcell VM cells treated with 1μM doxycycline using Annexin V-Biotin (Streptavidin APC) and Propidium Iodide. Dot Plot of untreated ‘No Doxycycline’ and treated ‘1μM Doxycycline’ ReNcell VM after 12 hours. Shown are Viable cells in lower left quadrant, Early Apoptotic in lower right quadrant (Annexin V Biotin/Streptavidin APC 647-A positive), Late Apoptotic in upper right quadrant (Annexin V Biotin/Streptavidin APC 647-A positive and Propidium Iodide positive). Cells were treated with 1μM Doxycycline for 12 hours before collecting for analysis of Early Apoptotic events. Values represent percentage of cells in each quadrant. Change in percentage of early apoptotic cells after treatment are as follows: Wildtype (0.48%), Scrambled (0.93%), 2193 (1.56%) and 4774 (3.18%).
**Figure S6.3 USP9X depletion after 72 hours does not result in increased apoptosis or differentiation.** (A) Immunoblot analysis of Caspase-3 levels in USP9X-depleted RenCell VM cells after 72 hours 1μM doxycycline treatment. (B) Immunoblot for βIII-tubulin and GFAP in USP9X depleted RenCell VM after 72 hours 1μM doxycycline.
Figure S6.4 Expression of the G0/G1 phase cell cycle proteins in USP9X-depleted ReNcell VM (A) Flow cytometric analysis of ReNcell VM cells after 72 hours of doxycycline treatment. Representative of three biological replicates. (B) Immunoblot analysis of whole cell lysate showing expression levels of G1-S Phase cell cycle proteins in USP9X depleted...
ReNcell VM cells 24 hours, 36 hours and 72 hours after 1 μM doxycycline addition. No change in Cyclin D1, E2F1 or total Retinoblastoma protein (Rb) protein expression in USP9X depleted lines was observed. Reduction of phosphorylated Rb (S780) in USP9X depleted ReNcell VM cells were observed after 72 hours. All loading controls are β- tubulin 55 kDa.

Figure S6.5 Reduction of RAPTOR expression in USP9X depleted ReNCell after EGF/FGF stimulation of 0, 5 and 15mins. (A and B) Two biological replicates of EGF/FGF stimulation experiment demonstrating reduction in RAPTOR protein levels in USP9X depleted ReNcell VM cells. (C) Representative reduction of USP9X depletion after 72 hours doxycycline treatment. Panel (A) and Panel (C) are from the same biological replicate.
**Figure S6.6** RAPTOR mRNA level was unchanged in USP9X-depleted ReNCell VM. Relative RAPTOR gene expression in USP9X-depleted ReNCell VM was measured by qRT-PCR analysis, and normalising against GAPDH. No difference was observed for RAPTOR expression in USP9X-depleted ReNCell VM (n=3). All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test.

**Figure S6.7** FACS analysed of EdU incorporated neurosphere cells. Neurospheres were exposed to a 6 hour EdU pulse. Neurospheres were dissociated into single cell, EdU incorporated cell were measured using FACS analysis. A significantly lower percentage of Usp9x<sup>-/-</sup> NPs were labelled with EdU indicating the absence of Usp9x diminishes NP proliferation. All data are shown as the means ± SEM. Statistical significance was assessed by the unpaired Student’s t test. *, p < 0.05.
CHAPTER 7

General discussion and Future directions
7.1 Usp9x regulation of multiple signalling pathways

Data gathered during this project showed that conditional deletion of Usp9x from NPs significantly affected three signaling pathways. Loss of Usp9x increased Notch and Wnt signaling, but decreased mTORC1 in embryonic Usp9x<sup>-/-</sup> brains and USP9X KD human NP cell line ReNcell VM. In the interim, previous study from our lab showed that TGF-β signaling was also decreased in Usp9x<sup>-/-</sup> brains, in postmitotic neurons during axonogenesis (Stegeman et al., 2013). Collectively, these results suggest that Usp9x simultaneously regulates multiple signaling pathways during mouse embryonic brain development, demonstrating that Usp9x is capable of influencing NP fate specification through various different mechanisms. What remains to be elucidated is a mechanism(s) of how Usp9x is able to integrate signals from multiple pathways. Based on synthesis of the current literature on Usp9x, there are several possible hypotheses worth investigating. Data from several studies suggest the existence of multiple pools of Usp9x maintained within different cellular compartments. For example, during de novo tight junction formation in epithelial cells Usp9x protein levels transiently increase only at the primordial tight junction, without altering Usp9x levels in cytoplasmic and nuclear regions of the cell (Theard et al., 2010). Although Usp9x is expressed throughout the soma and neurites in mature neurons, only a portion of Usp9x localised at the end of neurites interacts with Dcx (Friocourt et al., 2005). Somewhat similar to the current project, Usp9x depletion reduced total β-catenin protein levels in pre-implantation mouse embryos without affecting the AJ-associated β-catenin pool (Pantaleon et al., 2001). Although the locations of Usp9x sub-pools have been poorly defined, these studies provide clear evidence of their existence. Therefore, it could be assumed that different Usp9x sub-pools independently regulate multiple signalling pathways described above in NPs, and depletion of Usp9x will have a different effect on each signaling pathway based on the regulatory function of Usp9x within these pathways. Alternatively, PTMs of Usp9x provides another avenue by which Usp9x could regulate multiple signaling pathways. A large phospho-proteome analysis of mouse ESCs identified three phosphorylation sites in Usp9x (Li et al., 2011), suggesting Usp9x undergoes post-translationally modification. Although the functional consequences or the kinase(s) involved in this process have not yet been identified, it is possible that any of the above signaling pathways could promote the phosphorylation of Usp9x, which may in turn alter other pathway(s) as compensatory or feedback response. This may suggest that there is a hierarchical or temporal order to Usp9x regulation of these pathways.
signaling pathways. However, further experiments are warranted prior to drawing any conclusions. Future research needs to be conducted focusing on (1) which signaling pathway(s) post-translationally modify Usp9x and (2) how PTMs of Usp9x affect other signaling pathways, if at all.

7.2 Context-specific regulatory function of Usp9x

The context- and temporal-specific regulatory function is another striking feature of Usp9x that has been repeatedly encountered during this project. In particular, Usp9x appears to have the capacity to both facilitate and inhibit a signalling pathway depending on which component it regulates. Data present in this project showed that in embryonic mouse brains, Usp9x abrogates Wnt signaling through the DC. In contrast, Usp9x positively regulates Wnt signaling in multiple epithelial cell lines and the MCF-7 breast cancer cell line by directly binding and rescuing β-catenin from its proteasomal degradation (Taya et al., 1999; Pantaleon et al., 2001; Ouyang et al., 2016). In Usp9x/Y brains, deletion of Usp9x decreased Itch and Numb protein levels, suggesting that in NPs the role of Usp9x is to oppose Notch signalling in the signal receiving cells. However, others have shown that Usp9x could promote the Notch signaling at the signal sending cell level by rescuing Mib and Epsin from proteasomal degradation (Overstreet et al., 2004; Wang and Struhl, 2004). Data from the current project showed that depletion of Usp9x decreased mTORC1 signaling in Usp9x/Y NPs, in contrast to increased mTORC1 activity observed in Usp9x-depleted mouse C2C12 myoblast (Agrawal et al., 2012). Therefore, it will be critical to define what circumstances determine whether Usp9x has an overall positive or negative effect on each individual pathway.

In this project, we further demonstrated the context specificity of Usp9x by showing how the consequence of Usp9x deletion differs between cell types. Even though Notch, Wnt and mTORC1 signaling pathways being perturbed in a similar manner, both USP9X-depleted ReNcell VM and neurospheres derived from E18.5 Usp9x/Y NPs showed significantly reduced NP proliferation in contrast to NPs in Usp9x/Y brains where reduced no change in proliferation was observed. However, we do not exclude the possibility that different culturing conditions and/or the complexity of the multicellular architecture of Usp9x/Y brains
could account for this phenotypic difference. Temporal specific regulation of Usp9x could have also contributed to the observed differences in proliferation rate in NPs, in particular the difference in cell cycle kinetics between E18.5 Usp9x+/Y NP derived neurospheres and E12.5 NPs in the Usp9x+/Y brains. Context and temporal specific regulation of NPs by Usp9x, in vivo, has been demonstrated in our recent publications as well. When Usp9x was deleted from dorsal telencephalic NPs by mating Usp9x<sup>flox/flox</sup> females with Emx1-Cre donor male mice, Usp9x knockout mice (Emx1-Cre x Usp9x<sup>+/Y</sup>) survive through the adulthood. However, the size of the hippocampus, one of the two adult neurogenic sites of the murine brains, of the Emx1-Cre x Usp9x<sup>+/Y</sup> brains was significantly reduced (Stegeman et al., 2013; Oishi et al., 2016). NPs in the Emx1-Cre x Usp9x<sup>+/Y</sup> hippocampus were failed to generate neuroblast and there were significantly few quiescence NPs as well (Oishi et al., 2016). In contrast, SVZ, the other adult neurogenic site of the murine brains, of the Emx1-Cre x Usp9x<sup>+/Y</sup> brains were relatively intact and the NPs in Emx1-Cre x Usp9x<sup>+/Y</sup> SVZ were able to generate neuroblast as well. Phenotypic differences between sub granular zone and SVZ in Emx1-Cre x Usp9x<sup>+/Y</sup> brains and to the Usp9x<sup>+/Y</sup> embryonic brains highlights the context and temporal specific role of Usp9x in NPs.

Context specific regulation of Usp9x has been reported in several other biological systems and tissues as well. For example, Usp9x can inhibit TGF-β signaling by protecting the E3 ubiquitin ligase SMURF1 from proteasomal degradation, which in turn induces TGF-β receptor degradation (Xie et al., 2013). In contrast, Usp9x can also facilitate TGF-β signaling by deubiquitylating the mono-ubiquitylated form of Smad4, which inhibits its ability to form signal transducing complexes with receptor SMADs (Dupont et al., 2009). Cancer studies also highlight the context specific regulatory functions of Usp9x. Usp9x is capable of acting as either a tumour suppressor or an oncogene, depending on the cancer type (Murtaza et al., 2015). In a murine Kras activation model of pancreatic cancer, depletion of Usp9x rapidly accelerates progression to highly aggressive pancreatic ductal adenocarcinoma, exhibiting tumour suppressor function of Usp9x (Perez-Mancera et al., 2012). Clinical data from human pancreatic ductal adenocarcinoma patients indicated USP9X also acts as a tumour suppressor in humans. In contrast, USP9X promotes anti-apoptotic functions in human follicular lymphomas, colon adenocarcinoma and small cell lung carcinomas by inhibiting the proteasomal degradation of anti-apoptotic protein MCL-1 (Schwickart et al., 2010; Glaser et al., 2012; Trivigno et al., 2012). Additionally, recent studies conducted in our lab showed that
USP9X promotes the proliferation of several head and neck cancer cell lines by regulating different extrinsic signaling pathways depending on their molecular origin. USP9X increased the cell cycle progression as well as upregulating Notch signaling in SS15 (tongue), CAL27 (tongue) and Detroit 562 (pharynx) head and neck cancer lines. In contrast, in the FaDu (pharynx) cell line, USP9X increased cell cycle progression and mTORC1 signaling (Nanayakkara et al., 2016). Although it is a challenging task to understand the cell context specific regulatory function of Usp9x, a major advancement would be to identify the exact binding sites of substrates to Usp9x and to establish whether Usp9x PTMs affect these interactions.

7.3 USP9X as a human neurodevelopmental disorder gene

Mutations in human USP9X are associated with several neurodevelopmental disorders including X-linked intellectual disability (ID) (Homan, C.C. et al., 2014; Reijnders et al., 2016). Interestingly, adult Emx-1-Cre x Usp9x<sup>x<sup>y</sup></sup> brains phenocopy some structural abnormalities reported in ID patients, such as hypoplastic corpus callosum and enlarged ventricles (Stegeman et al., 2013; Reijnders et al., 2016). Biochemical analysis of fibroblasts from these patients showed increased β-catenin protein levels similar to data presented in this thesis. In addition at least 14 USP9X substrates including ITCH, NUAK1, SMAD4, SMURF1 and Dcx have been implicated in ID, suggesting USP9X may act as a nexus point for an ID regulatory pathway. To date, 24 different missense and protein truncation mutations have been identified in male ID patients ((Homan, C. C. et al., 2014) and unpublished data) and 13 different missense mutation and four different protein truncation mutations have been identified in female ID patients (Reijnders et al., 2016). Although all these patients present with ID, the severity of the disorder varies between each mutation (Reijnders et al., 2016; unpublished data). One reason for this phenotypic variance between patients could be the differences in the USP9X interactome. Future studies should therefore focus on investigating the differences in the interactome for each mutation, which would also provide additional detail on the context and temporal specific regulatory mechanisms of USP9X. This could be achieved by either conducting co-IP using patient-derived fibroblasts or mutation-induced HEK293 or ReNcell VM cells (using CRISPR/Cas9 gene manipulation).
7.4 Conclusion

In summary, data gathered during this project showed that Usp9x can regulate multiple intrinsic (adhesion and polarity) and extrinsic (Wnt, Notch and mTORC1) NP fate determinants. Conditional deletion of Usp9x from embryonic mouse NPs resulted in perturbed adhesion and cell polarity in early NPs, which recovered later in development. Usp9x deletion increased the Notch and Wnt extrinsic signaling pathways and down regulated mTORC1 signaling in both mouse and human ReNcell VM cells. Depletion of Usp9x from mouse NPs did not result in a dramatic phenotypic difference in $Usp9x^{-/}$ neocortex compared to alterations reported in neocortices harbouring perturbed AJs and polarity (Salomon et al., 1997; Rolls et al., 2003; Klezovitch et al., 2004; Kadowaki et al., 2007; Sakane and Miyamoto, 2013), increased Notch (Itoh et al., 2003; Kageyama et al., 2008), increased Wnt (Munji et al., 2011; Draganova et al., 2015), or decreased mTORC1 signaling (Cloetta et al., 2013). This suggests the phenotype of $Usp9x^{-/}$ brains may be the summation of multiple, possibly opposing (as discussed in Section 5.3.3), perturbed fate regulatory pathways. Results from the current project and the previous study by Jolly et al. (2009) demonstrated that it is difficult to elucidate the Usp9x regulatory function in NPs by either simple loss of function or overexpression studies. To overcome these hurdles, future studies should be conducted using fragments of Usp9x to restrict the extent of the Usp9x interactome, followed by an investigation of specific effects of each lost interaction. USP9X point mutations identified in the ID patients could also be used to molecularly dissect Usp9x regulation of NPs. To date no direct evidence has shown that ID associated mutations in Usp9x influence NP regulation, however based on new evidence from this project this would be an interesting hypothesis for further investigation of Usp9x-associated ID. Nevertheless, the data presented in this project showed that Usp9x is capable of regulating NP fate through multiple intrinsic and extrinsic fate regulatory mechanisms. Importantly, this study also highlights the temporal and context specific regulation of NP fate by Usp9x, functionally confirming Usp9x as a stem cell regulator.
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APPENDICES

Appendix A – Supplementary Figures

Figure S3.1 Decreased Usp9x immunoreactivity in E12.5 Usp9x<sup>-/-</sup> brains (n=3). E12.5 10µm coronal neocortical sections stained with Usp9x antibodies (green) and nuclei were counter stained with DAPI (blue). Usp9x immunoreactivity was completely depleted from Usp9x<sup>-/-</sup> cortices. Scale bars = 80 µm. LV- lateral ventricle, VZ- ventricular zone.

Figure S3.2 Decreased AF-6 immunoreactivity in E12.5 Usp9x<sup>-/-</sup> brains with appropriate negative controls (n=3). AF-6 immunoreactivity was markedly reduced in Usp9x<sup>-/-</sup> brains (b, e, h) compared to Usp9x<sup>+/+</sup> brains (a, d, g). (c, f, i) Represent the equalling negative control
panels, which process and captured under the same settings and conditions, but stained without the primary antibody (AF-6).

Figure S3.3 Complete depletion of Usp9x protein from E14.5 Usp9x<sup>−/−</sup> neocortices. (A) Coronal sections of three independent E14.5 Usp9x<sup>+/+</sup> and Usp9x<sup>−/−</sup> neocortices stained with Usp9x antibodies. Usp9x immunoreactivity is completely depleted from Usp9x<sup>−/−</sup> neocortices (b, d, f) compared to littermate controls (a, c, d). (B) Western blot analysis conducted on
separate Usp9x^{+/Y} and Usp9x^{−/Y} (n=3) cortical tissue confirmed the complete depletion of Usp9x protein from E14.5 neural progenitors and their subsequent progeny. The faint bands observed in the Usp9x^{−/Y} lanes were likely due to contamination from non-neural tissues such as meninges and blood vessels. Scale bars = 80 µm. LV- lateral ventricle, VZ- ventricular zone.
Figure S4.1 Increased β-catenin expression in E16.5 and E18.5 Usp9x<sup>+/+</sup> brains (n=3). E16.5 (a-f) and E18.5 (g-l) 10µm coronal neocortical sections stained with β-catenin antibodies. β-catenin immunoreactivity was markedly increased in the Usp9x<sup>+/+</sup> neocortices.
compared to Usp9x+/Y at both stages (n=3). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.

Figure S4.2 Normal N-cadherin expression and immunoreactivity in E16.5 Usp9x+/Y brains (n=3). E16.5 10μm coronal neocortical sections stained with N-cadheirn antibodies. No difference was observed for N-cadherin immunoreactivity or expression pattern between Usp9x+/Y (a, c, e) and Usp9x−/Y (b, d, f). Scale bars = 80 μm. LV- lateral ventricle, VZ- ventricular zone.