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TUBERIN REGULATION IN NEURAL TISSUE AND CELL FATE

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By

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ABSTRACT

Mutations in the *TSC2* gene, coding for the tumour suppressor protein Tuberin, lead to formation of benign tumours in systems like the brain. Some data supports Tuberin being an essential regulator of neural development and playing a role in cell fate decisions.

I hypothesized that Tuberin is an essential regulator of neural cell fate decisions in the cerebellum and mutations in Tuberin may fuel the expansion of a stem-like population of cells in the childhood malignant brain cancer Medulloblastoma (MB). I found that Tuberin levels were tightly regulated in select regions of the brain. Culture of primary cerebellum stem/progenitor cells was performed. I demonstrated that Tuberin levels are downregulated throughout cell differentiation. Tuberin levels are high in the stem-like population in MB cells. This supports that Tuberin is an essential regulator of differentiation in stem-like progenitor population. Coaxing this stem-cell population to undergo functional differentiation in MB is one potentially exciting area for new cancer therapy.

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LIST OF ABBREVIATIONS

ED - Embryonic Day

TS - Tuberous Sclerosis

TSC - Tuberous Sclerosis Complex

TSC1 - Tuberous Sclerosis 1 (Gene)

TSC2 - Tuberous Sclerosis 2 (Gene)

mTOR - Mammalian Target of Rapamycin

mTORC1 - Mammalian Target of Rapamycin Complex 1

mTORC2 - Mammalian Target of Rapamycin Complex 2

PBS - Phosphate Buffered Saline

DAB - 3, 3- Diaminobenzidine

PFA- Paraformaldehyde

CNS - Central Nervous System

APC- Allophycocyanin

EGL- External Germinal Layer

FGF- Fibroblast Growth Factor

EGF- Epidermal Growth Factor

NGF- Nerve Growth Factor

MEFS- Mouse Embryonic Fibroblasts

CSC- Cancer Stem Cells

TIC- Tumour Initiating celss

GBM- Glioblastoma Multiforme

MB-Medulloblastoma

CSF- Cerebrospinal Fluid

PNET- Primitive Neuroectodermal Tumour

SHH- Conic Hedgehog

GFAP- Glial Fibrillary Acidic Protein

Dsh- Dishevelled

Ptch- Patched

Smo- Smoothened

HPE- Holoprosencephaly

INTRODUCTION

I. **Tuberin: A Tumour Suppressor**

It is crucial to maintain cellular homeostasis within a growing population of cells in the body. The process of cellular growth involves a series of controlled and regulated divisions. There are many different factors that keep the process of cellular growth and cellular death in balance. Genes that achieve this purpose are called tumour suppressor genes. A tumour suppressor gene is mainly defined as an anti-oncogene; thereby, preventing cells from becoming cancerous[1]. Generally, a tumour suppressor gene hinders cell proliferation and slows down the cell cycle. Mutations that lead to uncontrolled cell growth, which with further cooperating mutations, can lead to cancer formation [1].

For tumour suppressor genes to lose their inhibitory function, the requirements for the "two hit hypothesis" must be met. Established by Alfred Knudson, the "two hit hypothesis" proposes that tumour suppressor genes must lose both functional copies to contribute to the oncogenic process[2,3]. The first mutation that occurs would result in a germ line mutation which would then be passed on to the somatic cells of the embryo. During mitosis, another mutation, a "second hit", occurs in a single cell. If these somatic mutations accumulate, an abrogation in the tumour suppressor gene will occur[3,4]. Knudson came upon this discovery when studying Retinoblastoma cells where he found that in a sporadic Retinoblastoma, if the two alleles are wild type then two mutations will be required to cause disease. On the other hand, if the alleles were heterozygous, then one mutation will be required to result in disease formation[3,4]. The gene byproducts of tumour suppressors can be affected when the suppressor's function is abrogated.

A key pathway that regulates cellular growth and proliferation is the Mammalian Target of Rapamycin (mTOR)[5,6]. One of the upstream regulators of mTOR is a heterodimer complex called the Tuberous Sclerosis Complex (TSC). This heterodimer is made up of Tuberin, encoded by the gene *TSC2* and Hamartin encoded by the *TSC1* gene[6,7]. Mutations that occur in these tumour suppressor genes can lead to tumour formation. An example that has been well studied is the disease Tuberous Sclerosis (TS). This disease is usually passed on by genetic inheritance through parents that possess germ cell mutations. The transmission of TS can happen through spontaneous genetic mutations called *de novo* mutations. Such spontaneous mutations occur more frequently. Statistically, 60-70% of patients do not exhibit any genetic inheritance from sporadically caused mutations[7]. Under circumstances where there is heterozygous inactivation in either genes, *TSC1* or *TSC2*, it will be inadequate to cause severe disease phenotypes in patients[7]. Conversely, it is imperative that second hit somatic mutations are behind serious disease manifestation to arise. These mutations depict Knudson's Tumour Suppressor model[2,8]. While the protein products of these genes work together it is known that mutations in the *TSC2* gene are more frequent and result in more severe phenotypes than mutations occurring in the *TSC1* gene[9].

The *TSC2* gene is situated on chromosome 16p13.3 and constitutes of 42 exons overall and 41 of them code for the protein, Tuberin[8]. *TSC1* is found on chromosome 9q34 and constitutes 23 exons [8,10]. The protein Tuberin has a molecular mass of 200 kDa, Hamartin is smaller with a molecular mass of 130 kDa. Interestingly, the molecular mass of the TSC is roughly 450 kDa which is bigger than Tuberin and Hamartin forming independently, implying the presence of additional members of the complex are yet to be identified[11,12]. The TSC is formed through Hamartin interacting with Tuberin at the N-terminus (**Figure 1**). Hamartin and

Tuberin interactions function to support and stabilize Tuberin by preventing Tuberin's degradation by HERC1 ubiquitin ligase[13,11]. The primary characterized functional domain of Tuberin is the GTPase activating protein (GAP) domain residing in the C-terminal portion of the protein. Tuberin GAP function is directed toward the GTP binding protein Ras homolog enriched in the brain (Rheb), which itself has GTPase activity and is responsible for negatively regulating mTOR.
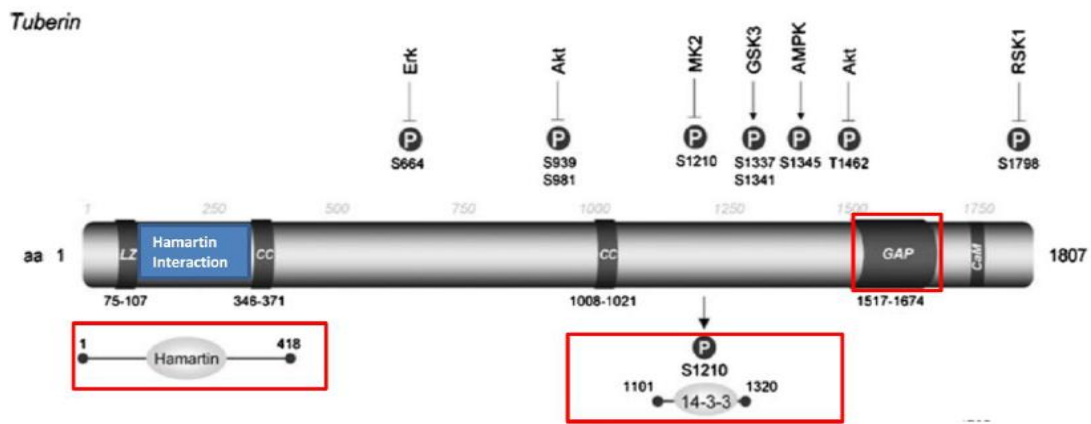
Figure 1. Tuberin Structure Showing the Functional Domains
Highlighted with a red box at amino acid (aa) 1-418 is the Hamartin binding domain, at aa 1101-1320 is the 14-3-3 binding region and the functional GAP domain is indicated at aa 1517-1672. Two coiled-coil (cc) domains, a leucine zipper (LZ), and a calmodulin binding region (CaM) are indicated as boxes on the diagram itself. Phosphorylation sites are indicated by a P with either an inhibitory or activating modification and the primary kinase indicated above. Adapted from Rosner *et al.* 2008[8].[12]

II.   TSC and mTOR Signalling

Tuberin and Hamartin are indispensible tumour suppressor proteins needed in controlling cellular growth via the mTOR pathway[6]. Tuberin function is largely affected by post-

translational modifications occurring via nutrient signaling such as growth factors (ERK) and insulin (PI3K/Akt) and direct sensing of ATP levels (AMPK)[14,15] (**Figure 1**). These signals are then transmitted specifically to the downstream effector mTOR or directly to the cell cycle machinery to regulate cell size, proliferation, and protein synthesis[6] (**Figure 2**).
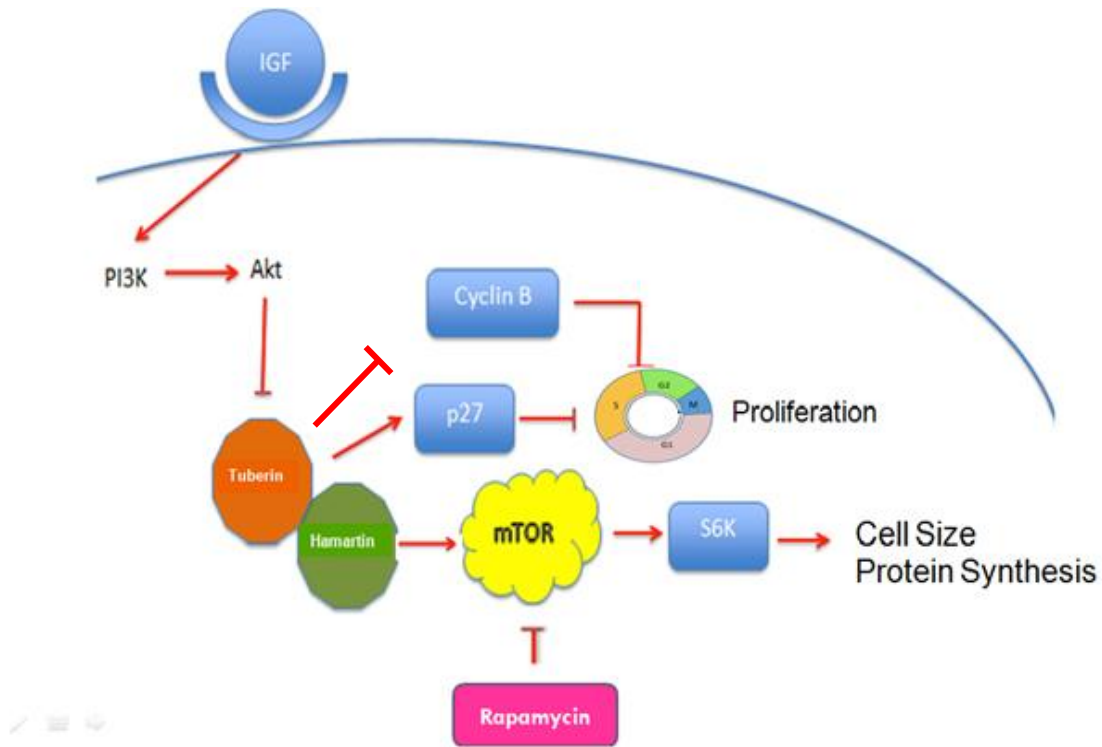
Figure 2: mTOR Pathway

Cartoon depicting the links between the TSC (Tuberin and Hamartin) with the mTOR pathway and cell cycle regulation. Upstream signaling from the insulin growth factor receptor (IGF) is one representative feed from a nutrient pathway.

mTOR is a large protein and has a molecular mass of 289 kDa and belongs to the superfamily phosphatidylinositol kinase-related kinase (PIKK)[16]. Based on research conducted on yeast, *Saccharomyces cerevisiae*, the Target of Rapamycin (TOR) was discovered. *TOR1* and

TOR2 are the two major genes that form the TOR proteins. Researchers concurred that these two genes have been known for their Rapamycin sensitivity; however, recent research has demonstrated that TOR2 possesses Rapamycin insensitivity features. Being insensitive to the drug, Rapamycin, TOR2 acts as a roadblock in decreasing TS symptoms[17]. In mTORC1, mLST8 can bind to mTOR to facilitate its kinase activity. Also, mTOR can bind with rictor (Rapamycin insensitive), and associate with SIN1 and mLST8 to form mTORC2 and preserve its integrity[17].[12]

mTOR is the downstream effector of PI3K/Akt signal transduction pathway. This pathway is initiated when ligands or hormones bind to the IGF receptor on the cell membrane surface. Once binding of the ligand to the receptor takes place, phosphorylation of PI3K instigates a regulation cascade that creates a positive feedback loop amongst all the elements in the pathway[6]. If mTOR is misregulated due to the mutation in *TSC2* or *TSC1*, certain acute phenotypic manifestations will be visible and diseases become dominant. Amongst these diseases are: Tuberous Sclerosis, autism, and Alzheimer's disease, etc.[8]. [12]

Tuberin also directly regulates the cell cycle by enhancing the functional activity of p27, a cyclin-dependent kinase (CDK) inhibitor. Previous studies had shown that Tuberin expression levels in cells have a huge impact on the cytoplasmic or nuclear localization of p27[7]. This suggests that the CDK inhibitor, p27, works as a tumour suppressor by inhibiting CDK activity[7],[12]. Tuberin is also capable of regulating mitotic onset binding directly to the mitotic cyclin, Cyclin B1, and controlling the timing of the nuclear transport of the protein, a fundamental step that triggers mitosis[18]. Collectively, Tuberin functions as a pivotal mechanism of translating information about the nutrient status in a cell. When energy levels are low, Tuberin is regulated

and inhibits growth and proliferation. Conversely, Tuberin is inactivated in situations of high nutrient levels to permit growth and cell division[18].

III.   Brain Development and Neurogenesis

Brain development.

The first stage of embryonic development from a blastula is gastrulation, or the reorganization of cells into the three germ layers; ectoderm, mesoderm, and endoderm[19,20,21]. Gastrulation is followed by organogenesis which is a stage initiated by the development of the neural tube which is the structure from which the CNS originates[21]. Neural tube formation is elicited by signals from a structure that arises from the mesoderm called the notochord[21]. The CNS and the brain arise from the ectoderm[20,21]. Environmental signals inhibit neural specific tissues from becoming other ectoderm structures such as the epidermis. Two critical signaling pathways that dictate patterning of the neural plate, which acts as the foundation of the CNS and arises from the ectoderm, are the Wnt pathway, responsible for CNS patterning, and the Bone Morphogenic Protein (BMP) pathway[22]. During neurulation the cells located around the neural plate undergo invagination and folding to form the neural tube[22]. The neural tube is characterized by polarization alongside the ventral-dorsal axis[23]. The spinal neurons exist in the dorsal area of the neural tube whereas the motor neurons are located in the ventral area where they receive external signals[19,23]. The ventral pattern and dorsal pattern are induced by the notochord and epidermis, respectively[23]. This patterning is critical for dictating the continued development of the CNS as a whole and forming the spinal cord and the brain.

Neurogenesis.

Neurogenesis is the cell biology process by which different neural structures are formed through the production of neurons and glial cells from a neural stem cell and underlying progenitor cells[24,19]. The development of the human brain begins at the third week of gestation[25]. However cell fate, or the concept of what the cells will become, is partly determined by the polarity of the neural stem cell and the position by which it lies in the developing embryo. One of the key processes in neural development is the process of neural induction that occurs at the blastula stage. Throughout neural induction, cells found in the embryonic ectoderm are instigated to differentiate and to specialize into cells that contribute to the overall formation of the CNS[19,26]. Neural induction sets the stage for cells to take specific routes in response to external signals. Throughout the developmental process the stem and progenitor cells will receive cues from the changing environment to guide the differentiation decisions determining the number and type of neural cells formed[19]. The two routes that cells can differentiate into are glial cells or neuronal precursor cells. As the cell fate decision is being made, the cells become committed to a certain type of cell which down the road will be differentiated into a neuron or a mature glial cell type[26]. The events that take place while the embryo is still developing are quite important due to the fact that critical CNS structures are formed at the end of that period[25].

The population of cells located in the wall of the developing neural tube, are neuroepithelial cells which ultimately differentiate into different cell types such as neurons and glial cells. As the process of neurogenesis proceeds, the cells within the neural tube undergo a series of events that involves reorganization and rearrangement of the cells within the neural tube[27,23]. This series of rearrangements allows the population of proliferating progenitor cells to go through neural divisions either symmetrically or asymmetrically[28]. It is crucial that the

process of neural tube formation does not go wrong because any defects in this process will cause neurological defects in the embryo such as Spina Bifida[29]. 1 in 2000 newborns experience neural tube defects in the US[29]. Patterning of the neural tube is a result of environmental signals that are secreted by certain molecules[19,23,30]. The ventral axis patterning is governed by Sonic Hedgehog signaling which eventually leads to the expression of transcription factors that give neural progenitor cells their integrity[31]. On the other hand, BMP and Wnt signaling pathways govern the patterning of the dorsal region of the neural tube[27,23,32]. All in all, neurogenesis is a crucial process throughout mammalian brain development. Furthermore, the activity of neurogenesis is higher in regions of the brain like the cerebellum which regulates motor control[33].

Implications of Tuberin in neural development.

While not well-understood, Tuberin has been implicated in development. Floricel *et al*. have conducted experiments that show when *TSC2* is mutated *in vivo*, neurological development especially in the brain is negatively affected[34]. They found that neurite outgrowth was inhibited within the PC12 cell line[34]. Additionally, in a separate study by Choi *et. al.*, they demonstrated that axons that formed were disordered. This was mainly observed when *TSC2* or *TSC1* were knocked down. In the absence of *TSC2* or *TSC1*, axonal outgrowth increased in the cells. Under conditions where these genes were overexpressed, axon formation was repressed[35]. Furthermore, it has been demonstrated that the knockdown of *TSC2* or *TSC1* led to an increase in the size of cells in the postmitotic neurons and dendritic spines[36]. Collectively, these findings imply the intriguing possibility that Tuberin is important in the process of neural cell fate decisions.

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IV.   A Brief Overview of Cerebellum Development

The cerebellum is a brain structure situated in the hindbrain specifically in the anterior region[37]. It is responsible for regulating sensory-motor processes[37]. Of the vast majority of neurons that make up the brain, about half of them are located in the cerebellum[37,38]. The rudimentary structures of the cerebellum start forming at six weeks of embryonic development within the human brain. CNS development begins very early in embryogenesis and it is the last process to be completed after birth. On average, the brain is fully developed at 20 years of age. In the cerebellum there is a heterogenous population of neurons. Pre-cerebellar neurons are neurons found in the rhombic lip of the hind brain[39]. Through the process of nucleokinesis, these cells migrate to designated places of the cerebellum by allowing their nucleus to move[39,40]. Through several different processes of chemotactic agents, the movement of these cells is guided[39].

There are several layers of neuronal cells that make up the cerebellum and migrate to designated locations termed the nuclear layer and the molecular layer[41] **(Figure 3)**. The molecular layer is composed of Purkinje cells that are found between compact granule cells and Purkinje cell dendrites[41]. The Purkinje cells are a group of the largest inhibitory GABAergic neurons that process inhibitory information relayed to them by granule cell fibers. Granule cells are located in the granular layer and they are classified as excitatory glutamatergic neurons. They process inhibitory information received from Golgi neurons. Granule cells form axons that penetrate to the molecular layer and make connections with dendrites of the Purkinje cells[42]. Granule cell precursors usually reside in the External Germinal Layer (EGL) where they later migrate from the rhombic lip to the pial surface during development. The EGL disappears when the cerebellum is fully developed and the granule cells are found within the IGL[43].
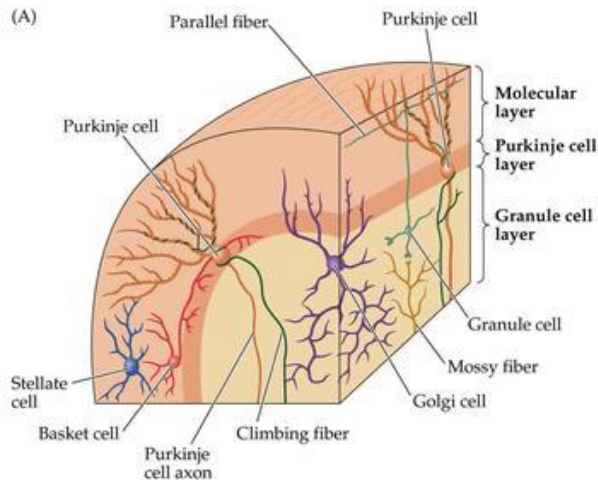
Figure 3. Illustration Depicting the Neuronal Cells that Make up the Cerebellum

Within the cerebellum, the neuronal cells reside in various layers such as the granule cell layer, purkinje cell layer, and the molecular layer. Adapted from Tabata *et al.*[44]

V.   Stem Cells Have Distinctive Properties

A stem cell is present at various stages during development. It is an undifferentiated cell that has the potential to differentiate or develop into different types of cells, tissues, and organs in the body[45]. The stem cell population both creates an initial structure and it works as an internal repair mechanism in the body by continuously dividing and replenishing damaged cells[46]. There are two ways in which cells can divide; asymmetric cell division and symmetric cell division[47,48]. In asymmetric cell division, the parent stem cell divides into two daughter cells one of which is a stem cell while the other is a non-stem cell that has the potential to specialize and differentiate into a specific cell type such as a brain cell, or blood cell, etc. In a symmetric cell division, the parent stem cell produces two new stem cells[48,49,50]. **(Figure 4)**.
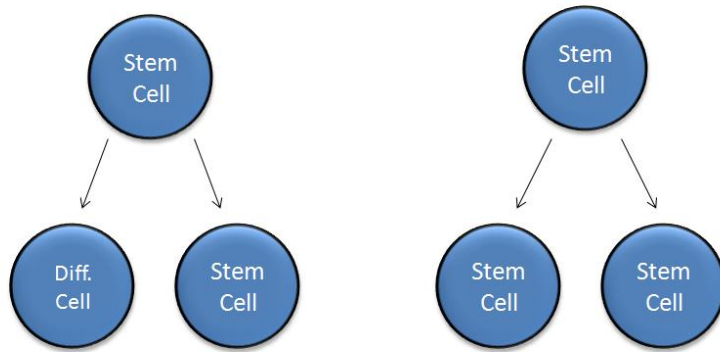
Figure 4. Mechanisms of Stem Cell Division[50]

Illustration depicting the two different models of stem cell division. Stem cells can divide asymmetrically, producing a daughter cell that is differentiated and another that is a stem cell. In symmetric cell division the daughter cells produced are identical to the original parent cell. Adapted from Shahrayari *et. al.* 2013.

In the asymmetric division model, it is believed that homeostasis is controlled at a single cell level by having the parent cell produce one stem cell and one differentiated cell[48,51,46]. One problem that arises from the asymmetric division model is that in case of damage or injury, the stem cell pool may exhaust the ability to self-renew. This problem is solved by the presence of symmetric cell division model which controls and maintains homeostasis at a cell population level as compared to a single cell level[46].

The symmetric division model involves two different types of division which includes a proliferation division and a differentiation division[50,46]. As mentioned above, proliferation leads to the formation of two stem cells while differentiation gives rise to two differentiated specialized cells from unspecialized progenitors[46]. The proliferation or differentiation division is determined by certain signals or quos that are received by the stem cells from the surrounding

environment. Proliferation and differentiation decisions must be controlled in order to maintain homeostasis and balance control[46]. If these decisions are not controlled, stem cell expansion will be tipped off balance. In addition, abnormal control of asymmetric division may lead to formation of cancerous tumours due to build up of undifferentiated cells[46,47]. The main role of stem cells in tissue is to maintain homeostasis through its major properties; differentiation and self-renewal. Disease can be a serious result of fluctuations in the balance of such stem cell populations[50].

To classify a cell as "stem" cell, it must possess the two important stem cell characteristics. The first characteristic is that the cell is unspecialized[45]. This feature means that these cells are able to continuously divide and self-renew[52]. The second feature of stem cells is that they are able to become committed and specialize into specific types of cells under certain situations physiologically and experimentally[52, 45].

Stem cells are divided into two main classes; embryonic stem cells as well as adult stem cells[45]. An embryonic stem cell forms when the fertilized egg divides. These cells are present in humans at the first 3-5 days of development at the blastocyst stage[45]. Cells at this stage are the ones necessary to give rise to the organism as a whole. Adult stem cells are those that are found in already developed tissues[45,48]. The main task of these cells is to repair damaged tissue. Adult stem cells work to replenish specialized cells that are dead[46]. The brain, bone marrow, blood, muscle, and skin, are the tissues with the most work done on adult stem cells[45,46].

The existence of adult neural stem cells was a topic of much debate until a finding by Nottebohm demonstrated that neurogenesis exists in the adult brain of vertebrates[53]. Neural stem cells are stem cells that are found within the CNS which possess the ability of self renewal[54].

Mammalian neural stem cells were first isolated by Reynolds and Weiss in 1992[55]. In their studies, Reynolds and Weis investigated whether cells isolated from the stratium and grown in culture were able to proliferate and divide in the absence of the epidermal grow factor (EGF). These researchers have concluded that in order to promote the growth and proliferation of isolated cells either from an adult or embryonic mouse brain sample, EGF is required. EGF allows for the formation of free floating spheres which were eventually called "neurospheres". Neurospheres are composed mainly of glia and neuron cells[55]. These spheres also express the neural stem cell marker, Nestin[56]. Reynolds *et al.* have also confirmed the stem cell identity of the neurospheres by conducting experiments demonstrating that a neurosphere can form from a single cell even when the neurospheres are dissociated[55]. This neural cell isolation was achieved by building on information and discoveries made previously in 1983 by a group of researchers. Raff and colleagues discovered a type of cell that has the capacity to differentiate into a fibrous astrocyte and an oligodendrocyte[57]. This cell was derived from the optic nerve of a rat that was 7 days old. Their findings suggested that from a single glial progenitor cell *in vitro*, two different types of cells were able to form[57]. Being able to isolate neural stem cells and producing other cell types in culture can aid in contributing to finding a cure in patients suffering from neurological disorders.

Since the discovery by Reynolds and Weiss, many researchers have begun studying mammalian adult neural stem cells. In 1993, Luskin and colleagues were able to report that cells in the adult subventricular zone (SVZ), exhibited continuous proliferation which in turn led to the production of neurons in the olfactory bulb[58]. This suggests that within the SVZ there is a population of neural progenitor cells that is continuously proliferating. Furthermore, the ependymal region of the brain has also been shown to possess characteristics of neural cells.

Researchers have proved that ependymal cells are stem cells because they elicit neuron production when the CNS is exposed to injury[53].

The first neural stem cell extraction conditions characterized neurosphere assay as a method of culturing and retaining stem cell characteristics[55]. This assay also began to teach a lot about the conditions that maintain stemness in the brain as the neurospheres must be provided with specific media and given necessary growth supplements[55]. For neural stem cells fibroblast growth factor (FGF) and EGF are important in maintaining stemness. In addition, the nerve growth factor (NGF) is needed to aid in producing neurons[55]. With today's technology and laboratory techniques cells can be extracted and cultured from nearly any region of the adult vertebrate brain.

Among the different factors like FGF and EGF that have proven important in dictating the stemness of the neural stem cell population, the Notch pathway plays a role in regulating cell fate decisions and cell self-renewal[59]. The Notch pathway is highly conserved and involved in important cellular decisions such as proliferation and differentiation especially during embryogenesis and CNS formation[59,60]. In the brain, the neural progenitor cell population is sustained through the Notch pathway[59]. This pathway is composed of 4 protein homologues; Notch1, Notch2, Notch3, and Notch4 that interact with the Notch receptor along with other ligands that start a cascade of proteolytic cleavage events that result in Notch translocation into the nucleus of the cell where interactions with downstream effectors begins to control the cell proliferation, differentiation, and cell fate decisions[59,60]. Tuberin is involved in the Notch pathway as an upstream effector[61]. In a study conducted by Ma *et. al.* in 2010, it was shown that the mTOR pathway can be linked to an upregulation of the Notch pathway[61,62]. Some of their findings revealed that when mTOR activity is increased, cell differentiation was impeded. To

support the fact that when mTOR is hyperactive it leads to Notch upregulation, they proved that in mouse embryonic fibroblasts (MEFs) with hyperactive mTOR, Jagged1, the Notch ligand, was also found at increasing levels[61]. Furthermore, experiments performed to inhibit the Notch pathway were found to abolish tumour formation in the cells where mTOR was active[61].

Studying stem cells and their ability to differentiate into specific cell types is critical. Stem cells can provide scientists with some insight into how to cure certain diseases by investigating how these cells contribute to development. Overtime, research findings can contribute to curing diseases such as diabetes, neurological diseases, burns, etc. Studying stem cells can provide limitless opportunities for healing disease.

VI.   Brain Tumours and the Cancer Stem Cell Model

The cancer stem cell (CSC) hierarchal model proposes that a subpopulation of cells existing in the tumour cell mass is capable of driving tumour progression. As a result of the cells' ability to self-renew and differentiate, this subpopulation of cells leads to the regeneration of the cancer causing cells leading to tumourigenesis[63,64]. On the other hand, the stochastic tumour model hypothesizes that within a tumour, all the cells possess tumorigenic properties or can be transformed to become tumorigenic[64,65]. There is evidence to support that both models may exist, and indeed some cases where combinations of the two may co-occur, but certainly there is strong evidence to support that some tumours arise only from a distinct population of cells that possess stem cell properties[63,65]. Some researchers refer to these cells as 'tumour-initiating cells (TIC)' while others continue to use the more controversial CSC term. These TIC/CSCs have certain properties which include self-renewal and differentiation into specific tissue cells[66, 64]. In 1994, John Dick, a Canadian researcher, was one of the first scientists to discover the presence of CSCs in certain leukemia models. In his study, he demonstrated using severe combined

immunodeficient mouse model, that cancer cells isolated using cell surface markers that express a $CD34^+ CD38^-$, were able to form tumours when introduced into the mice blood system. These tumours were representative of the tumour heterogeneity seen in patients with acute myeloid leukemia[67].

CSCs can lead to the formation of aggressive malignant tumours in the brain. One form of cancer that follows this model is glioblastoma multiforme (GBM). These tumours generally originate from neural stem cells located within the brain[68,69]. GBM usually occurs as a result of a multitude of mutations and can occur both during childhood and during adulthood. The diversity of mutations in this tumour makes the cancer causing cells a challenge to target with chemotherapy[70,71,72]. It is believed that the neural stem cells that are the origin of this tumour are located in the SVZ of the brain. In previous studies, cancer cells were isolated from the tumours in GBM[71]. These tumour cells were able to self renew and produce more tumours which makes this disease a recurrent disease even when exposed to conventional therapy[72].

VII.   Medulloblastoma: Childhood Cancer

Medulloblastoma (MB) is the most common fast-growing neuroectodermal pediatric malignant brain tumours and is the number one cause of mortality in children diagnosed with brain cancer[73,74]. According to the American Brain Tumor Association, 400 patients are diagnosed with MB each year with the majority of patients being children up to 10 years of age[74]. Some of the symptoms that are associated with Medulloblastoma are; lack of co-ordination and balance, vomiting, headaches, behavioural changes, and double vision[73,74]. Some treatments have been developed to alleviate these symptoms in young children[73,74].

One of the most effective ways to manage tumours is tissue re-sectioning through surgery and the removal of the tumour mass[73,74,75]. Although surgery aims at removing most of the cancerous mass, there is a high risk of recurrence through the cerebral spinal fluid (CSF)[75,76]. The CSF is one of the pathways through which tumour cells can spread; therefore, it is important to keep track of the nature of the cells that are found in the CSF[76]. Chemotherapy and radiation that target the brain and spinal cord are standard of care and can shrink the tumour mass but are currently not curative.

VIII. MB Neuropathology:

MB is classified as a Primitive Neuroectodermal Tumour (PNET)[77,78]. These embryonal tumours are characterized by immature and undifferentiated neural tissue[78]. MB tumours are found in the cerebellum and the cell of origin is believed to be granule neuron precursors (CGNPs) located in the EGL[78,79,73,77]. The EGL is naturally composed of different types of cells mainly including precursor cells. To make it up to the external layer of the cerebellum, these cells migrate to the surface. Once they reach the surface of the cerebellum, the cells differentiate by going through cell division stages[78]. To form the granular layer, the neurons move inwards. In humans, the EGL, remains present in the cerebellum until 2 years of age[78,42]. The purkinje cells as well as the cerebellar nuclei arise from the subependymal matrix around the fourth ventricle. MB tumours are solid and distinctive[78]. Histologically, cells appear oval and small with little cytoplasmic to nuclear ratio[78]. In a study focusing on 21 MB patients, the differentiation capacity of the cells from 20 samples that were categorized under classic MB, were studied using glial fibrillary acidic protein (GFAP), neurofilaments (NF), S-100 protein (S-100P), and neuron specific enolase (NSE)[80]. The findings from this study showed that the majority of the cells were positive for NSE which is a nonspecific marker for embryonic cells, neuroblasts[80]. This suggests

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that neuroglial cells within the MB tumour are actually undergoing differentiation[80]. The tumour cells are heterogeneous and may contain differentiated cells where the majority is expected to be undifferentiated.

Generally, MB emerges due to genetic mutations that occur in the pathways that are involved in cerebellum formation which lead to developing tumour cells from stem cells. In 2011, a group of researchers studied primary MB samples. Using techniques such as genome profiling in combination with bioinformatics approach, scientists were able to divide MB into four distinct subgroups based on several factors. These factors and categorization was based on the metastatic status of the tumour, DNA copy number aberrations, and histology[81]. Classification of the tumors was also based on age groups. MB is categorized into four different subgroups; WNT, Sonic Hedgehog (SHH), Group 3, and Group 4[81]. Classifying MB into the four different subgroups allows researchers to investigate specific characteristics of cells in each subtype and propose several targeted therapies particular to each subtype. To date, the WNT and SHH pathways are more heavily and well studied compared to Group 3 and Group 4 subtypes[81,82,83].

MB categorized into the WNT subgroup, is a rarely occurring tumor[75,83,84]. The Wnt pathway is an essential pathway that is activated during embryogenesis[75,84]. During development Wnt acts to regulate extremely important processes such as cell fate, embryo patterning, proliferation and cell growth. Protein interactions in the Wnt pathway are essential for tightly regulated cell to cell communication. Wnt misregulation or mutations are implicated in diseases such as cancer[75]. Studying what genetic mutations can lead to Wnt malfunction can be implicated in stem cell fate. The primary receptors found in the Wnt pathway are Frizzled (Fz) and LRP [84]. In a normal cell, Wnt signalling is usually inactive, a destruction complex made up

of Axin and APC secure the transcriptional regulator β-catenin and target it for ubiquitination and proteolytic degradation. Wnt pathway is activated when a Wnt ligand binds to Fz and LRP, a membrane receptor[84,]. The binding processes induces the activation of Dishevelled (Dsh) which leads to the disintegration of the destruction complex causing β-catenin levels to increase in the cytosol and nucleus. When high levels of β-catenin are present, β-catenin accumulates in the cytosol and the nucleus as a result of the lack of degradation by proteosomes[84,85]. In the nucleus, β-catenin binds to the TCF/LEF transcription factor thereby changing the role that TCF/ LEF transcription factor plays as a repressor. Conversely, TCF/ LEF transcription factor activates RNA polymerase that prompts gene transcription. A malfunction in this system that inappropriately activates the Wnt pathway by a Wnt signal can lead to a mutation in one of the few genes that form the destruction complex[84,86].

Another MB subtype is driven by the Shh pathway[75,82]. *SHH* is the gene that encodes for the protein Shh, a morphogen[75]. The Shh pathway is inactivated by the receptor Patched (Ptch) and in turn Smoothened (Smo), the signaling receptor, gets suppressed by Ptch, thereby inhibiting the transcription factor, Gli and the downstream signaling cascade[75,87]. When the Shh ligand is present it binds to Ptch at the cell surface which leads to its degradation of Ptch[75,87]. This results in Smo moving from the intracellular vesicle to the cell surface to initiate Shh signaling and Gli gets activated which can then regulate gene expression[75,88,87]. Historically, the hedgehog gene was identified in 1980 in *Drosophila melanogaster* embryo[89]. Researchers performed genetic screening to identify embryonic lethal loci in Drosophila[89]. They looked for genes that are mutated and influence the segment polarity. One of those genes was the one that encodes for Shh. Shh is responsible for establishing the patterning during embryonic development[89]. It is important in creating the midline of the spinal cord, the brain, and the overall

body pattern etc[75,89]. In addition Shh is essential for the development of the CNS as a whole[90]. It follows that mutations in *SHH*, can lead to severe neurological diseases and congenital defects such as holoprosencephaly (HPE) which results due to an aberration in Shh and incapability of the two brain hemispheres and the eyes to develop normally[90].

Shh affects signaling in the neural precursor cells (CGNPs) which are believed to be the cells of origin of the childhood cancer MB[91]. Interestingly, mutations in *TSC2* can cooperate with the Shh pathway to lead to formation of cancerous tumours[88]. In a study performed by Bhatia *et al.* in 2009, researchers used a transgenic mouse model that was dominant negative for *TSC2* (*TSC2-RGΔ*)[88]. The lack of Tuberin resulted in aberrant proliferation of CGNPs as well as cellular growth was increased indicating abnormalities in cerebellar development. Importantly, the TSC2-null/transgenic mouse model had enhanced susceptibility to the initiation of SHH subtype of MB. Characterization demonstrated that there was an increased number of neural precursor cells along with an increase in mTOR signaling and an inhibition of p27 and GSK3α/β activity [75,88].

IX. Hypothesis and Objectives

I hypothesize that Tuberin protein levels will vary throughout the brain, with levels and activity being higher in regions of peak neurogenesis activity. Tuberin is crucial for brain development and neural cell differentiation. The tumour suppressor protein, Tuberin, halts the cell cycle to allow for asymmetric division and differentiation of cells; therefore, I hypothesize that Tuberin levels are lower in differentiating cells as compared to progenitor cells and it serves to regulate stem cell differentiation. It is important to determine the normal role of Tuberin in neural cell fate; proliferation and differentiation during early development. In addition, I hypothesize that Tuberin plays a critical role in the self-renewal of CSC

populations within MB.

Objectives:

1. **Determine Tuberin Protein Levels During Early Brain Development**

   Immunohistochemistry DAB staining will be performed on specific brain regions critical for neurological development, such as the choroid plexus and the purkinje cell layer, and Tuberin protein levels will be quantified and compared at ED 14.5 and ED 16.5.

2. **Identify the Importance of Tuberin During Cerebellum Formation and Cell Fate Determination using Primary Cerebellum Cells**

   I will optimize the isolation, culturing and differentiation of primary cerebellum cells from Balb/C mouse. I will measure changes in Tuberin mRNA through differentiation and optimize neurosphere experiments to begin to determine the importance of Tuberin in neural stem cell decisions in the cerebellum.

3. **Elucidate the Role of Tuberin in MB CSC Population.**

   Tuberin levels will be manipulated in a MB cell line and properties of the CSC population studied using neurosphere assays and cell surface markers using flow cytometry.

MATERIALS AND METHODS

I.   Animals and Breeding

BALB/c mice (Stock number 000651), were purchased from Charles River Laboratories. The mice were kept in the animal care facility at University of Windsor. The experimental procedures set by the University of Windsor animal care committee were followed as directed in AUPP # 12-18. BALB/c mice were bred in the animal care facility and the F1 generation was obtained. Mice were euthanized at embryonic day (ED) 14.5 and 16.5 using the CO_2 asphyxiation chamber.

All BALB/c mice were housed in the same animal care facility room. Mice were maintained under a 12 hour light 12 hour dark cycle. Female mice were placed in cages separate from the male mice. To increase the chances of the female's pregnancy, mouse shavings from the female's cage were introduced into the male's cage a day before the scheduled breeding date. For embryonic time points females were separated and checked for a copulatory plug the next morning; denoting day 0 of pregnancy.

To acquire postnatal brain samples, the mice were kept together for three nights to ensure the chances of successful breeding. The gestation period for the mice was about 21 days. The mice were checked and monitored throughout the 21 gestation days. Once the pups were born, that day was assigned to be postnatal day 1[92].

II.   Tissue Samples Dissection and Fixation

Mice were sacrificed in the most humane way possible to obtain samples. For postnatal samples, mice were placed in a CO_2 chamber for euthanasia. The CO_2 cylinder is set to 4 psi.

The mice were left in the chamber for 3-6 minutes as per the guidelines from the University of Windsor Animal Care and Use Committee, AUPP#12-18.

Prenatal brain samples were obtained by extraction of the embryos following euthanization of the mother. For both embryonic and postnatal brain samples, the brain was placed in 4% paraformaldehyde PFA solution overnight. Extra embryonic tissues were then removed and embryos were placed in a fresh PFA solution for another night or until the expected tissue rigidity was achieved. Sequentially, the samples are moved through a sucrose gradient starting with a 20% sucrose gradient (200 mL High Purity Water and 40g sucrose) moving up to 30% sucrose gradient (200mL High Purity Water 60g sucrose). The tissue was left in each gradient overnight at 4°C. Once one observes that the brains sunk to the bottom of the tube, the gradient could be changed and the brains are cryoprotected.

III. Tissue Sectioning

The heads were submerged in Shandon M-1 Embedding Matrix (Thermo Electron Corp.; #1310) and then placed in a vacuum for 20 minutes at 10mmHg for fixation purposes. The tissue in matrix was then frozen at -20°C for about 30 minutes and at a temperature of -18°C. Brain tissue was sectioned at 30 μm in the sagittal plane under cold/ freezing conditions using a cryostat (Leica cm3050s). For the postnatal time points, the brain was sectioned in the sagittal plane at 18 μm thickness. Sections were mounted using permount mounting media (Fischer Chemicals; #SP15-100) onto Superfrost Plus slides and stored at -80°C.

IV. Immunohistochemistry (IHC): DAB Staining

Slides containing tissue sections were left at room temperature for 10 minutes to ensure the tissue adhered properly to the slide. The slides were then incubated in 0.3% H_2O_2 for 30 minutes

to block endogenous peroxidase activity. Slides were washed with 1X PBS for 5 minutes and then incubated at room temperature with diluted blocking serum, which prevents non-specific binding, for 20 minutes. The primary antibody, rabbit anti-Tuberin polyclonal antibody (Santa Cruz; #sc-893) was administered to the sections on the slides. The slides were then left at 26°C in a humidity chamber for approximately one hour or left at 4°C overnight. The slides were washed again with 1X PBS for 10 minutes. The secondary antibody was administered to the slides at room temperature for one hour. This was followed by washing in 1X PBS for 10 minutes. The negative control slides were lacking the primary antibody.

At the final stages of staining, DAB, 3,3-Diaminobenzidine substrate solution (Vector Laboratories, PK 61010) with H_2O_2 was used to incubate the slides after the wash in 1X PBS solution for about 30 seconds to 1 minute. Once a brown colour appeared, the slide was placed in 1X PBS in order to stop the reaction and to obtain adequate staining. Sequentially, the sections were dehydrated by submerging them through an ethanol gradient of 70% ethanol, 95% ethanol, and 100% ethanol; followed by submersion in xylene to clear the tissue samples. The slides were placed in each gradient for approximately 10 minutes. Slides were mounted using Permount and then coverslips were applied to the slides.

Leica CTR 6500 microscope was used to obtain pictures of the stained sections using the DFC 425 digital colour camera. Paxinos and Franklin's mouse brain atlas was used to identify anatomical features of the brain[93]. The two main regions that were examined for embryonic time points ED 14.5 and ED 16.5, were the purkinje cell layer, and choroid plexus. The cerebellum was the structure of main focus in the postnatal brain samples. The pictures of the regions were taken at the following objectives; 2.5x, 10x, 20x and 40 x.

V.   Immunohistochemistry (IHC): Quantification and Analysis

Three sections of each sample were quantified using the program ImageJ. A gradient of black and white was used to calibrate the program with white selected as the background colour on the 'step tablet' with a range of 0-250, 250 therefore representing the lightest staining. Representative sections were selected for the background (white region – designated as 250), and the darkest stained region (set to 0). Specific brain regions were compared on this scale by using the "free hand tool" to manually outline the region in the brain. Values that were obtained from control slides were subtracted from the slides containing secondary antibody. This difference reflects the difference in optic density between the control stained sections and the primary antibody stained sections.

VI.   Immunofluorescence and Analysis

Sections were washed with 1X PBS for 5 minutes. Excess PBS was dried with kim wipes and the sections were outlined with a grease pen to prevent the antibodies from mixing and overflowing the sections. A solution consisting of 0.2% Triton diluted in 1X PBS was used to wash the sections for 5 minutes in order to permeabilize the tissue. Following this, the slides were washed in 1X PBS for 5 minutes. Consequently, the slides were incubated in LAP blocker (500 μL of Triton x-100, 0.75g glycine, 1.25 mL FBS, up to 50 mL 1X PBS) for 15 minutes before applying the primary antibodies to the sections in order to prevent nonspecific binding of antibodies (Muller et al, 2009). After taking off the excess LAP blocker, the rabbit anti-Tuberin polyclonal antibody (Santa Cruz Biotechnology; # sc-893) was diluted in the blocking serum with 1:250 μL ratio. The sections were left to incubate in the primary antibody at 4°C overnight. The control slides were treated only with the primary antibody and cover slipped the same day.

The next day, 1X PBS was used to wash the sections from primary antibody residue three times, 5 minutes each. Then secondary antibody was applied to the sections for 30 minutes. The secondary antibody consisted of 500 µL of LAP blocking serum, 1 µL of Alexa Fluor-488 goat anti-Rabbit dye (ThermoFischer Scientific; #1705869) and 1 µL of Hoechst (ThemoFischer Scientific; #62249). Due to Hoechst solution's sensitivity to light, sections and secondary antibody, Texas Red goat anti-mouse (Life Technologies; 1666400) were covered in aluminum foil at all times. The slides were washed again with 1X PBS and cover slipped with anti-fade mounting solution. The light sensitive slides were then stored in an opaque box .

Leica CTR 6500 microscope was used to obtain pictures of the stained sections using the FX360 digital colour camera for better resolution. Leica Application Suite (LAS) AF was the software that was utilized to view fluorescent images. As previously mentioned, the two main regions that were examined were the purkinje cell layer, the choroid plexus, and the cerebellum. The DAPI filter was used to look at the Hoescht stained nuclei of the cells. The GFP filter was used to detect the green dye, Alexa Fluor 488, for the Tuberin expression for immunofluorescence staining. Sections were imaged at 2.5X, 10X, 20X and 40X objectives.

VII.   Primary cerebellum Tissue and Cell Extraction from Balb/c Mice

To begin the tissue extraction procedure, the working station must be sterilized and the dissection equipment must be autoclaved. The mice were first euthanized using a CO_2 chamber. The mouse head was swabbed with 95% ethanol and an incision across the midline of the skin and along the skull was made. In order to be able to expose the skull, the skin was pulled and cut underneath the ears to ease the process of tissue extraction. The skull was peeled off with sharp point forceps carefully without damaging the brain tissue under it. All connections to the brain such as the spinal cord and blood vessels were cut by picking up the brain from the base with

forceps. The brain was placed in a 60mm cell culture tissue dish (Sarstedt AG & Co.; #83.1801) and washed in DMEM media (Sigma Aldrich; # D5796) containing 3% penicillin and streptomycin (Gibco; #1540) for 2 minutes on a shaker.

All steps following this were carried on in a laminar flow hood. In order to extract the cells, the cerebellum tissue must be diced into 1mm^3 pieces using a scalpel blade no. 11. Once, the tissue was minced, it was transferred into a 15mL round bottom tube with DMEM media (Sigma-Aldrich; #D5796). The tissue was triturated using a Pasteur pipette by pipetting up and down gently and cells were spun at 250g for 1 min. The supernatant was removed and 1 mL of 0.05% Trypsin (HyClone; #SH3023601) was added. The tube containing the cells was placed in a 37°C water-bath for 5 minutes. The cells were triturated again and DMEM media was added to inactivate the digestive ability of the Trypsin. The cells were centrifuged at 250g for 5 min and the supernatant was removed, and 2 mL media consisting of 10% FBS was added and the tube was spun for 5 min at 500g. The supernatant was discarded and the cells were dissociated into Neurobasal media (Thermo Fischer Scientific; # 21103-049) with B27 (Thermo Fischer Scientific; # B27), 20ng/ml EGF, and 10ng/ml FGF supplements (Thermo Fischer Scientific; # B27). The cells were then plated onto low adherent 6-well cell culture plates (Corning; #3471) at a density of 5 x 10^4. The cells were monitored and media was replenished every three days. Once the cells start forming neurospheres, they can be collected and plated on adherent 6-well attachment plates and can be used for differentiation assay experiments. The neurosphere formation assay protocol was previously established by Pacey *et al.* (2006).

VIII.   Cell Culture: Primary Cerebellum Cells Assays

Primary cerebellum cells were maintained in serum free Neurobasal Medium supplemented with B27, 20ng/ml EGF (Gibco; #12483) and 10ng/ml EGF (Sigma; #1540). The cells were

seeded at $5\text{x}10^4$ cells per well in an ultra low attachment 6-well plate (Corning; #3471). Cells were incubated at 37°C in 5% CO_2. Every 5-10 days the primary neurospheres that formed were subcultured using 1 mL 0.05% Trypsin to detach the cells (HyClone; #SH3023601). Once the subcultured primary sphere cells were needed for differentiation assays, the cells were provided with medium supplemented with 2% Fetal Bovine Serum (FBS) (Gibco; #12483). To harvest primary, and secondary neurospheres, the cells were seeded at 10000 cells per well in the ultra-low attachment plates. Fresh media was added to the plate every 2-3 days to enrich the most stem-like population of cells. All samples were collected by centrifugation at 1000 rpm for 5 min.

IX.   Cell Culture: DAOY Cell Line Neurosphere Assays

The DAOY cells were seeded at $5\text{x}10^6$ cells per well in a 10 cm cell culture plate. They were incubated at 37°C in 5% CO_2 and maintained in EMEM media supplemented with 10% FBS and 1% penicillin and streptomycin until they reached 80% confluency. To grow cells in neurospheres, cells were subcultured onto low adherent 6-well plates and supplied with neurobasal media and growth factors B27, 20ng/ mL FGF, and 10ng/ mL EGF to enhance neurosphere formation. Once neursopheres were formed, they were subcultured into secondary neurospheres using 0.1 mM EDTA to dissociate the cells. A subset of the DAOY cells were treated with 250 ng/mL of Rapamycin and were then grown into neurospheres. The neurosphere numbers were counted using the Leica M205 FA fluorescence stereo microscope.

X.   Lentivirus Production

Tuberin knock-down vector (pLKO.1. shTSC2) was obtained through (Addgene; # 15478). The production of the VSV-F pseudo typed lentivirus was executed by a HEK293 LentiX cells transient transfection. The transfection was performed with the specific plasmid along with

pMDG, pMDL2, and pRSV packaging plasmids with the aid of Polyethylenimine (PEI) (Sigma Aldrich, # 408719) in a DNA to PEI ratio of 1:3. This was followed by incubation in 5% CO_2 at 37°C for 5 hours. The following day, virus collection was performed and through ultracentrifugation, the collected virus was concentrated at 4ºC for 3 hours. Viral titer determination was done by 293T cells transduction. This process was followed by flow cytometry analysis of eGFP protein expression at 72 hours after transduction. The titred virus was filter sterilized and stored at -80°C.

XI.   Cell Culture: DAOY Cell Line Tuberin knock down

For Tuberin knock down experiments using DAOY cell line, the cells were subcultured in 96- well plates with 100 µl EMEM media without antibiotics or serum overnight and 10µg/mL of polybrene (Santa Cruz sc-134220) was added. 1 µl of shTSC2 virus was then added per well. A subset of cells were treated in the same way with the scrambled sequence vector, PLKO.1, as a control. The following day, the media was removed and cells were washed with 1X PBS and then fresh media containing 2 µg/ mL puromycin (Sigma-Aldrich; #P8833) was added in order to select for cells that have been successfully infected. After giving the cells 1-2 days recovery time; they were then used for experimental assays.

Table 1: Established Cell Lines

Cell line	Source	Disease	Tissue
Primary Cerebellum Cells	Primary Extraction from PN 4 Mouse Cerebellum.	None	Brain/ cerebellum
DAOY- HTB-186	ATCC	Desmoplastic Medulloblastoma	Brain/ cerebellum

XII. Cell Surface Marker Analysis: Flow Cytometry

As previously described, DAOY cells were treated, cultured and collected. Cells were centrifuged and cell pellets were collected to prepare for flow cytometry experiments. For every sample, $5x10^6$ cells were stained with rabbit anti-Tuberin polyclonal primary antibody and Alexa488 rabbit secondary antibody. In addition, cells were labeled for CD133 using CD133/ Allophycocyanin (APC) antibody (eBioscience; #17-1338-42). Cells were left to incubate on ice for 45 min in the dark. Some samples were double labeled with TSC2 and CD133. BD Biosciences Flow Cytometry Transcription Factor Set was used to perform the staining procedure (Pharmingen; # 562574). Once the incubation period was over, the cells were spun at 250g for 5 minutes, washed 2 times with PBS. Cold 1xPBS is used to re-suspend the cell pellets. Samples were analyzed using the BD LSR Fortessa X-20 cell Analyzer.

XIII. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

RNA was extracted from samples using an RNeasy Extraction Plus Mini Kit (Qiagen; #74134) as per manufacturer's protocol. A NanoDrop Spectrophotometer (ND-1000 software

version 3.3.0, Thermo Scientific) was used to measure the concentration and check the purity of the obtained samples. The obtained RNA samples were stored at -80ºC until needed for cDNA synthesis. Superscript II reverse transcriptase (Invitrogen; #100004925) was used to convert RNA to cDNA. ABI Viia7 thermocycler (Applied Biosystems; #278880504) was used to carry out qrt-PCR with Fast SYBR green to detect gene expression levels. The reaction was run for the length of 45 cycles. The primers that were used were specific for each cell type with GAPDH being the internal control at a concentration of 5μM. Specific primers are listed in Table 2 and Table 3. Obtained PCR results were reported as C_T values that are relative to GAPDH. Further data analysis was carried out using Microsoft Excel and the final results are represented as $\log_{10}$ RQ or RQ Values.

Table 2: Mouse Oligonucleotide Primers

Mouse Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	GCACCGTCAAGGCTGAGAAC	GGATCTCGCTCCTGGAAGATG
TSC2	TTGTGAGGAGGTTATGGCCATT	GCAGCTGAACTTCATCTCTGTTGTAG
Nestin	ACCTATGTCTGAGGCTCCCTATCCTA	ACCTATGTCTGAGGCTCCCTATCCTA

Table 3: Human Oligonucleotide Primers

Human Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
GAPDH	GCACCGTCAAGGCTGAGAAC	GGATCTCGCTCCTGGAAGATG
TSC2	GAGAGGAGCCGTGTTTTTTGTG	GACATGCCATGGCCTGGTA
CD133	CAATGACCCTCTGTGCTTGGT	GTGGAAGCTGCCTCAGTTCAG
Nestin	AGAGGGGAATTCCTGGAG	CTGAGGACCAGGACTCTCTA

RESULTS

I.   Tuberin Protein Levels Vary Across Different Regions of the Mammalian Brain

To begin to determine when and where Tuberin protein is expressed across the brain a specific focus on regions related to cerebellum development and general stemness characteristics in the brain were investigated. Brain regions such as the olfactory bulb and cerebral cortex were quantified and compared to the purkinje cell layer and choroid plexus, which are the two regions of interest (**Figure 5 A, B**). These regions actively undergo neurogenesis during embryonic development. Tuberin protein levels in each region were compared at ED 14.5, and ED 16.5, respectively. The brain tissue was sectioned in the sagittal plane and was treated with Tuberin primary antibody and DAB stain using Immunohistochemistry technique (**Figure 5 C, D**). The levels of Tuberin expression seemed to vary throughout the brain regions.

To determine how levels of Tuberin change over different time points in different regions of the brain we first compared the trend of expression in the cerebral cortex (**Figure 6).** Tuberin protein level intensities between the cerebral cortex at ED 14.5 and ED16.5 were not significantly different hence the cerebral cortex represents a good control between these time points (**Figure 6A-C**). As seen with the cerebral cortex, there was no significant change in Tuberin protein levels in the olfactory bulb between ED 14.5 and ED 16.5 (**Figure 7A-C**).

Figure 5. Tuberin Protein Levels at ED 14.5 and ED 16.5 in Different Regions of the Brain.

Immunohistochemistry was performed using Tuberin primary antibody. (**A**) A control image of the full brain lacking the primary antibody. (**B**) Full brain image at 2.5x objective showing all the brain regions; Purkinje Cell layer (PCL), Choroid Plexus (CP), and Cerebral Cortex (CC). Scale bar 1mm (**C**) & (**D**) Tuberin protein levels at ED 14.5 and ED 16.5. The y-axis represents the difference in optic density of the DAB stain which ranges from 0-250 with 0 value representing the lighter DAB stain. The x-axis shows the brain regions; Purkinje Cell Layer, Choroid Plexus, Olfactory bulb, and Cerebral Cortex, that were analyzed.

Figure 6. Depiction of Tuberin Protein Levels at ED 14.5 and ED 16.5 in the Cerebral Cortex.

Immunohistochemistry was performed using Tuberin primary antibody. **(A)** A control image of the cerebral cortex lacking the primary antibody at ED 14.5 and ED 16.5 at 10x objective

showing scale bar 200µm. **(B)** Image of cerebral cortex treated with Tuberin primary antibody at ED 14.5 and ED 16.5. **(C)** Tuberin protein levels quantified at ED 14.5 and ED 16.5. The y-axis represents the difference in optic density of the DAB stain ranging from 0-250 with 0 value representing the lighter DAB stain. The error bars represent the SEM over 9 (N=9) individual experiments. Statistical analysis was performed using single factor ANOVA. $p > 0.05$.
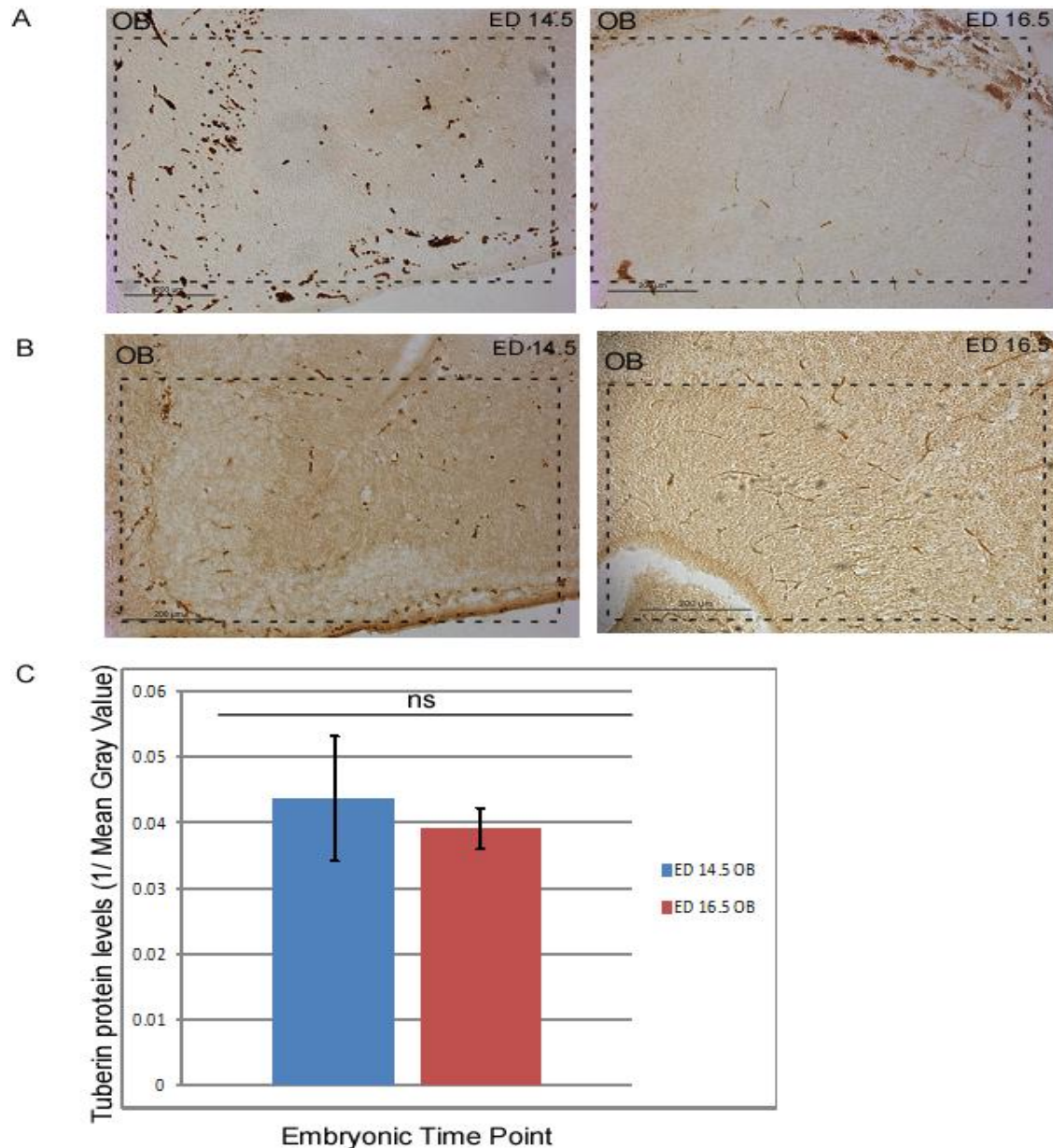
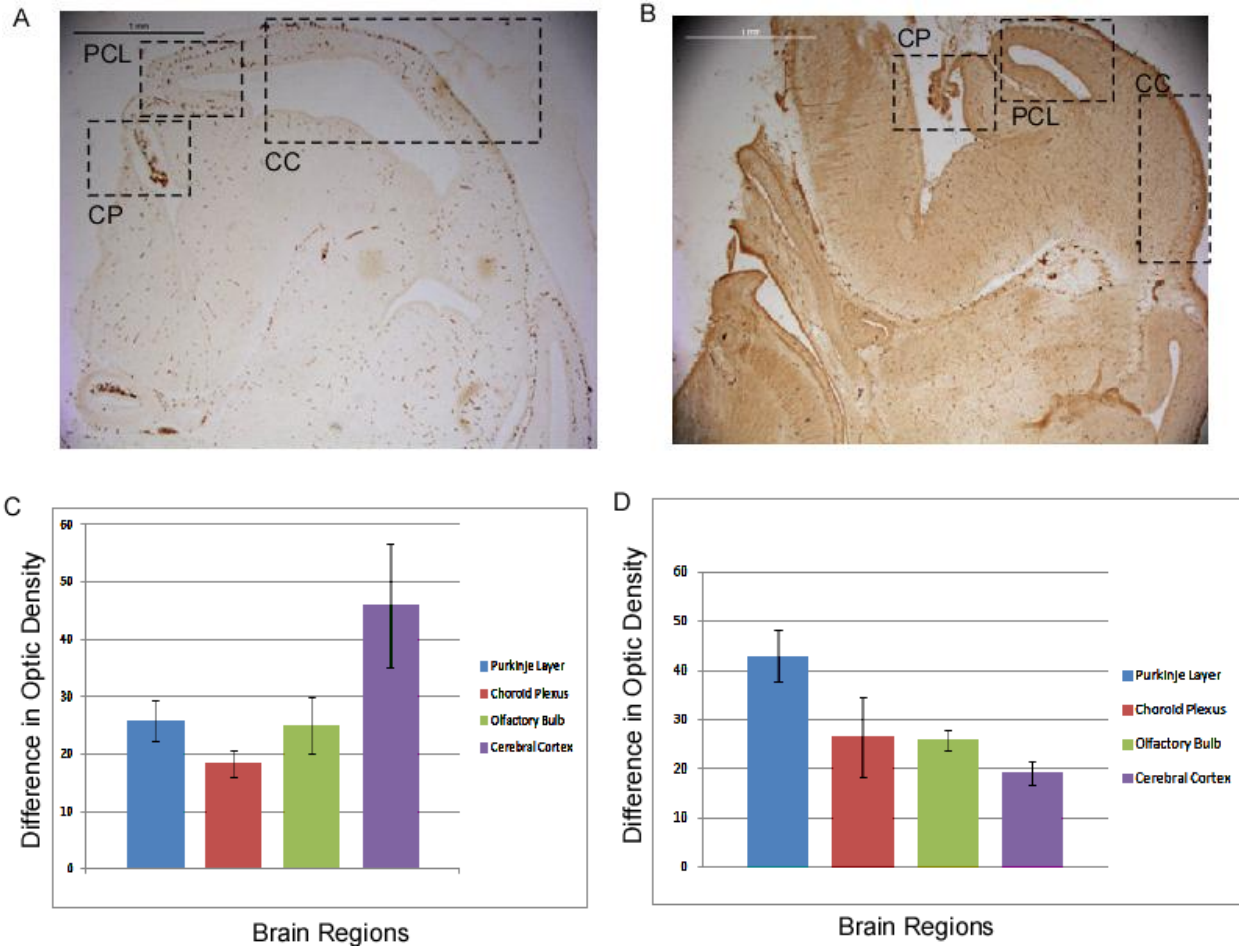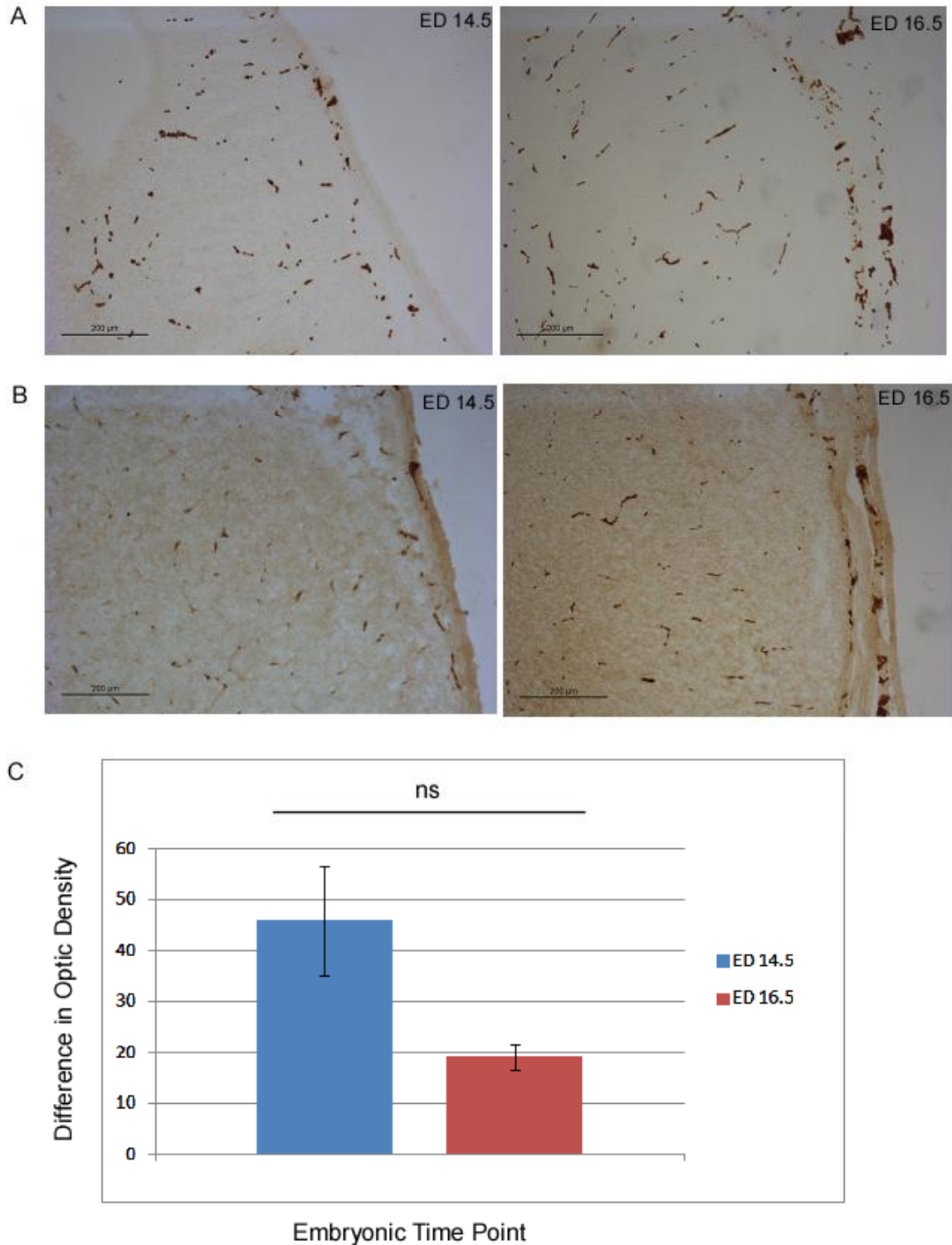**Figure 7. Tuberin Protein Levels at ED 14.5 and ED 16.5 in the Olfactory Bulb.**

Immunohistochemistry was performed using Tuberin primary antibody. **(A)** A control image of the cerebral cortex lacking the primary antibody at ED 14.5 and ED 16.5 at 10x objective

showing scale bar 200µm. **(B)** Image of cerebral cortex treated with Tuberin primary antibody at ED 14.5 and ED 16.5. **(C)** Tuberin protein levels quantified at ED 14.5 and ED 16.5. The y-axis represents the difference in optic density of the DAB stain ranging from 0-250 with 0 value representing the lighter DAB stain. The error bars represent the SEM over 9 (N=9) individual experiments. Statistical analysis was performed using single factor ANOVA. p> 0.05.

**Tuberin Protein Levels are Regulated in Certain Regions of the Brain**

The choroid plexus and purkinje cell layer are brain regions that mark the beginning of the formation of the cerebellum during embryonic development. The choroid plexus was examined to assess Tuberin protein levels. The choroid plexus is made up of specialized cells that are located in the brain's ventricles. These ventricles are characterized by the production of the cerebrospinal fluid. Furthermore, this structure provides the brain with immunological and physical protection by having a blood brain barrier[94]. Immunohistochemistry was performed to determine the levels of Tuberin in the choroid plexus at ED 14.5 and ED 16.5. Control slides were lacking the primary antibody **(Figure 8A)**. Sections at ED 14.5 and ED 16.5 were treated with both primary and secondary antibody **(Figure 8B)**. There was no significant change in Tuberin protein levels in the choroid plexus between embryonic development time points, p>0.05 **(Figure 8C)**.
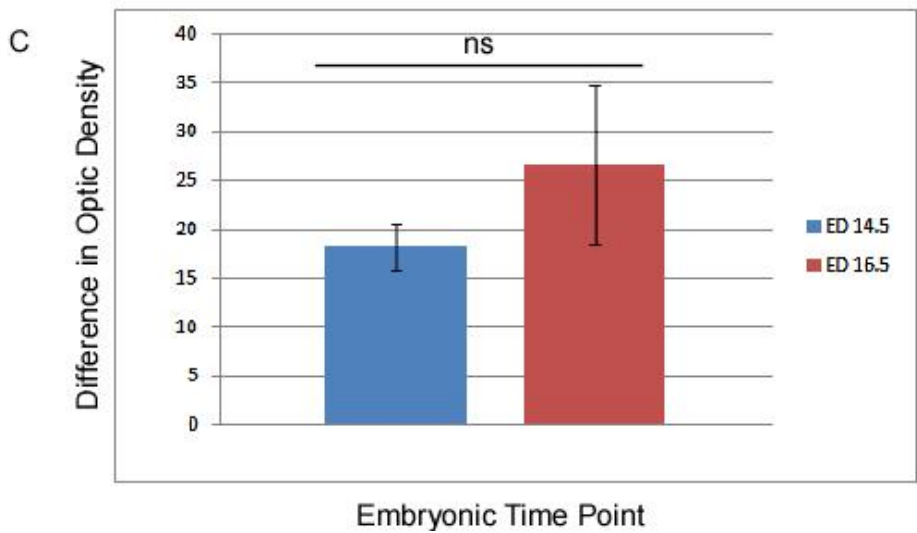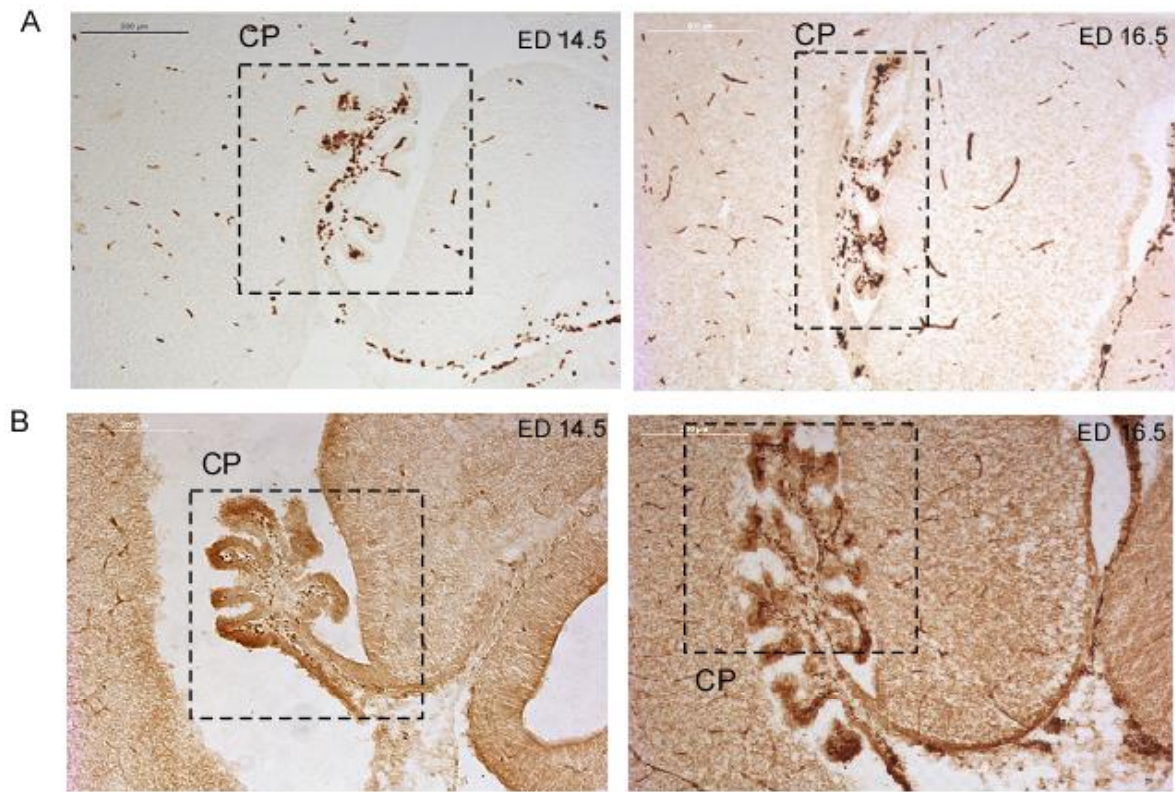
Figure 8. Tuberin Protein Levels at ED 14.5 and ED 16.5 in the Choroid Plexus.

Immunohistochemistry was performed using Tuberin primary antibody. The y-axis represents the difference in optic density of the DAB stain which ranges from 0-250 with 0 value

representing the lighter DAB stain. The x-axis shows the brain region comparing ED 14.5 and ED 16.5. **(A)** A control image of the choroid plexus lacking the primary antibody at ED 14.5 and ED 16.5 at 10x objective showing scale bar 200μm. **(B)** Image of choroid plexus treated with secondary antibody at ED 14.5 and ED 16.5. **(C)** Graph comparing Tuberin protein levels at ED 14.5 and ED 16.5 in the choroid plexus. Error bars represent SEM over 9 (N=9) individual experiments. Statistical analysis was performed using single factor ANOVA. p> 0.05.

The purkinje cell layer forms the middle layer of the cerebellum which is crucial for motor control and coordination. Purkinje cells house vital neurons that are important in the cerebellar cortex and contain some of the largest neurons in the brain[95]. Brain tissue was sectioned and controls (secondary antibody only) were obtained for both ED 14.5 and ED 16.5 **(Figure 9A)**. Images for sections treated with both primary and secondary antibodies were obtained at 10x objective for both time points **(Figure 9B)**. Tuberin protein levels were found to significantly increase in the purkinje cell layer at the later time point during embryonic development, p< 0.05 **(Figure 9C)**.
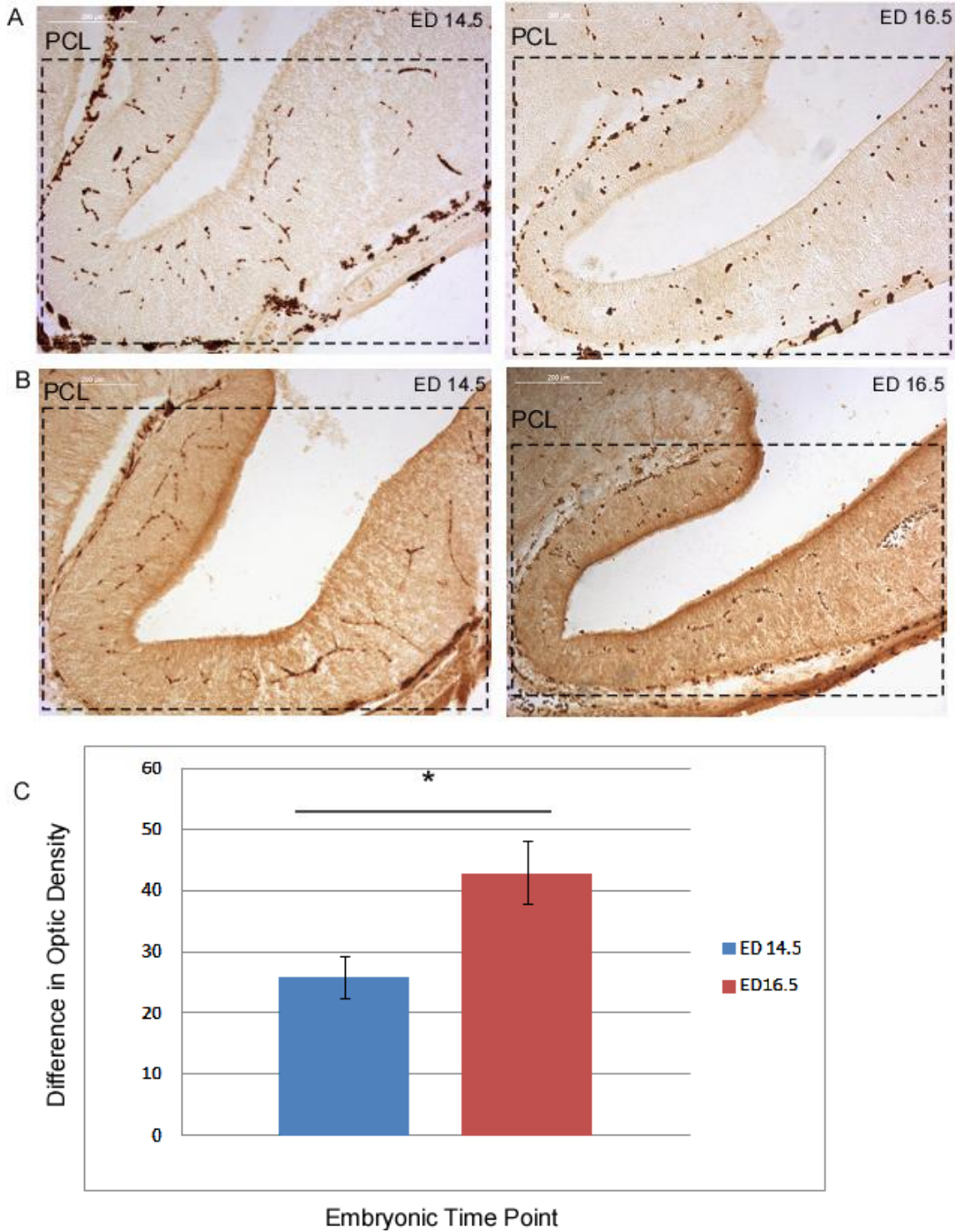
Figure 9. Tuberin Protein Levels at ED 14.5 and ED 16.5 in the Purkinje Cell Layer.

Immunohistochemistry staining was performed using Tuberin primary antibody. **(A)** A control image of the choroid plexus lacking the primary antibody at ED 14.5 and ED 16.5 at 10x

magnification showing scale bar 200µm. **(B)** Image of purkinje cell layer treated with secondary antibody at ED 14.5 and ED 16.5. **(C)** Graph comparing Tuberin protein levels at ED 14.5 and ED 16.5 in the purkinje cell layer. Tuberin levels decreased at ED 16.5 as compared to ED 14.5. The y-axis represents the difference in optic density of the DAB stain which ranges from 0-250 with 0 value representing the lighter DAB stain. The x-axis shows the brain region comparing ED 14.5 and ED 16.5. Error bars represent the SEM for 9 (N=9) individual experiments. Statistical analysis was performed using single factor ANOVA. *p< 0.05.

II. Tuberin mRNA Levels are Regulated during Differentiation and Cell Fate Decisions

To determine endogenous Tuberin mRNA levels in the mouse cerebellum and in stem-like or progenitor cell populations, primary mouse cerebellar cells were extracted and differentiated *in vitro*, and collected for qrt- PCR analysis. The samples were run in a qrt-PCR reaction. Nestin, a stemness marker, was used as a control to verify that the subpopulation of cells is representative of the stem and/or progenitor population[56]. First, an early differentiation time course, 0-24 hours, was investigated. In this time course, no significant pattern was seen for samples run with Nestin **(Figure 10A)** or Tuberin **(Figure 10B)**. The constant levels of Nestin indicate that the cells were not differentiating during this time course. The cells were observed under the microscope and no cellular dendrite extensions were formed at the end of the 24 hours differentiation time point; therefore, a longer differentiation time course was carried out.

After a 72 hours differentiation time course, it was verified that the cells had been differentiated which was indicated by the decrease in Nestin expression from 0-72 hours **(Figure 11A)**. Interestingly, Tuberin mRNA levels also seemed to follow an overall pattern of decreasing as the cells were differentiating **(Figure 11B)**. This result, although not statistically significant, may suggest the possibility of Tuberin's role in regulating stem cell fate decisions in the normal brain especially at early time points of development.

A
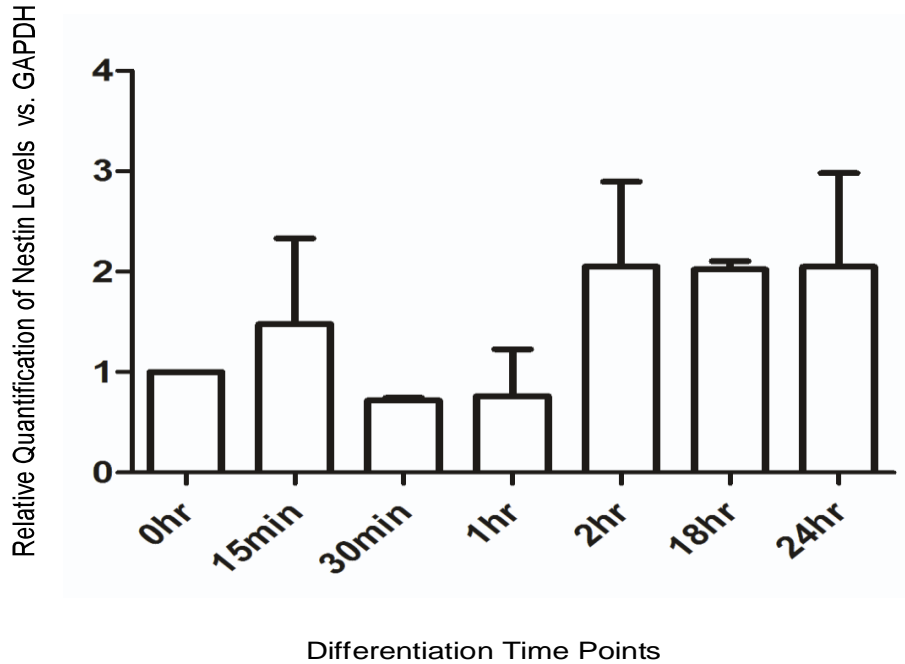
B
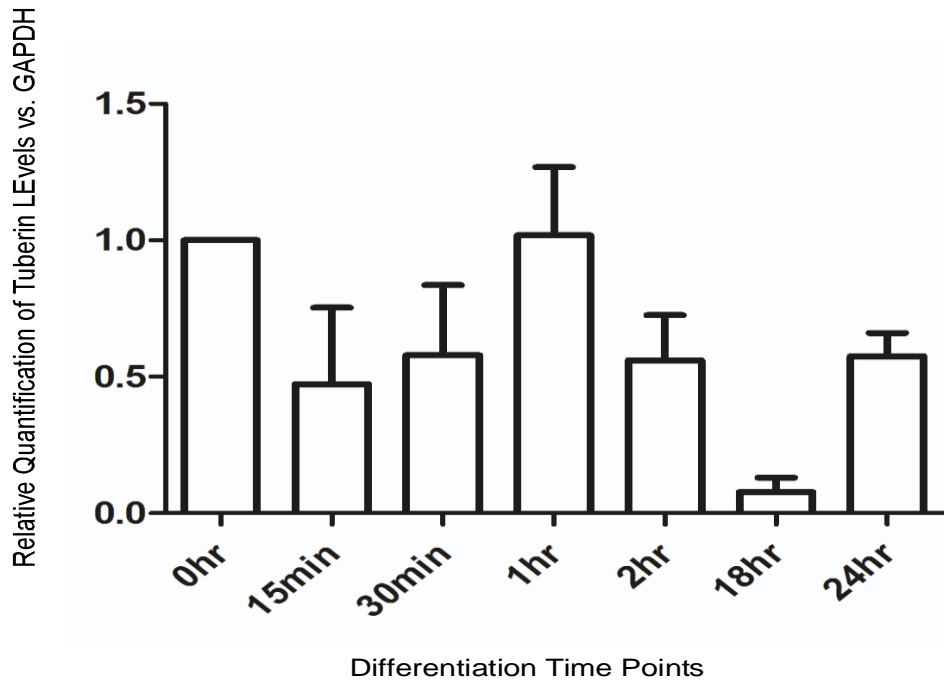
Figure 10. Tuberin mRNA Levels Follow a Decreasing Pattern at Early Stages of Differentiation

mRNA analysis of primary cerebellum cells, post-natal day 4, that were differentiated over 24 hours. **(A)** Nestin mRNA levels were measured using qrt-PCR analysis where data is normalized to GAPDH and data is reported as relative quantification values (RQ). The y-axis represents

Nestin expression levels. **(B)** Tuberin mRNA levels reported as RQ over 24 hours time course for three independent experiments. The y-axis represents Tuberin expression levels. Error bars indicate SEM of three independent qrt- PCR experiments ran in triplicate. mRNA levels are normalized against 0 time point. $p > 0.05$. Statistical analysis was done one-way ANOVA.

A

B

Figure 11. Tuberin mRNA Levels are Regulated throughout Differentiation

mRNA analysis of primary cerebellum cells, post-natal day 4, differentiated over 72 hours **(A)**. Nestin mRNA levels normalized to GAPDH and reported as relative quantification value (RQ).

(B) Tuberin mRNA levels reported as RQ over 0-72 hours time course for three independent experiments. Error bars indicate SEM of three independent qrt-PCR experiments ran in triplicate. mRNA levels are normalized against 0 time point. $p> 0.05$. Statistical analysis was done one-way ANOVA.
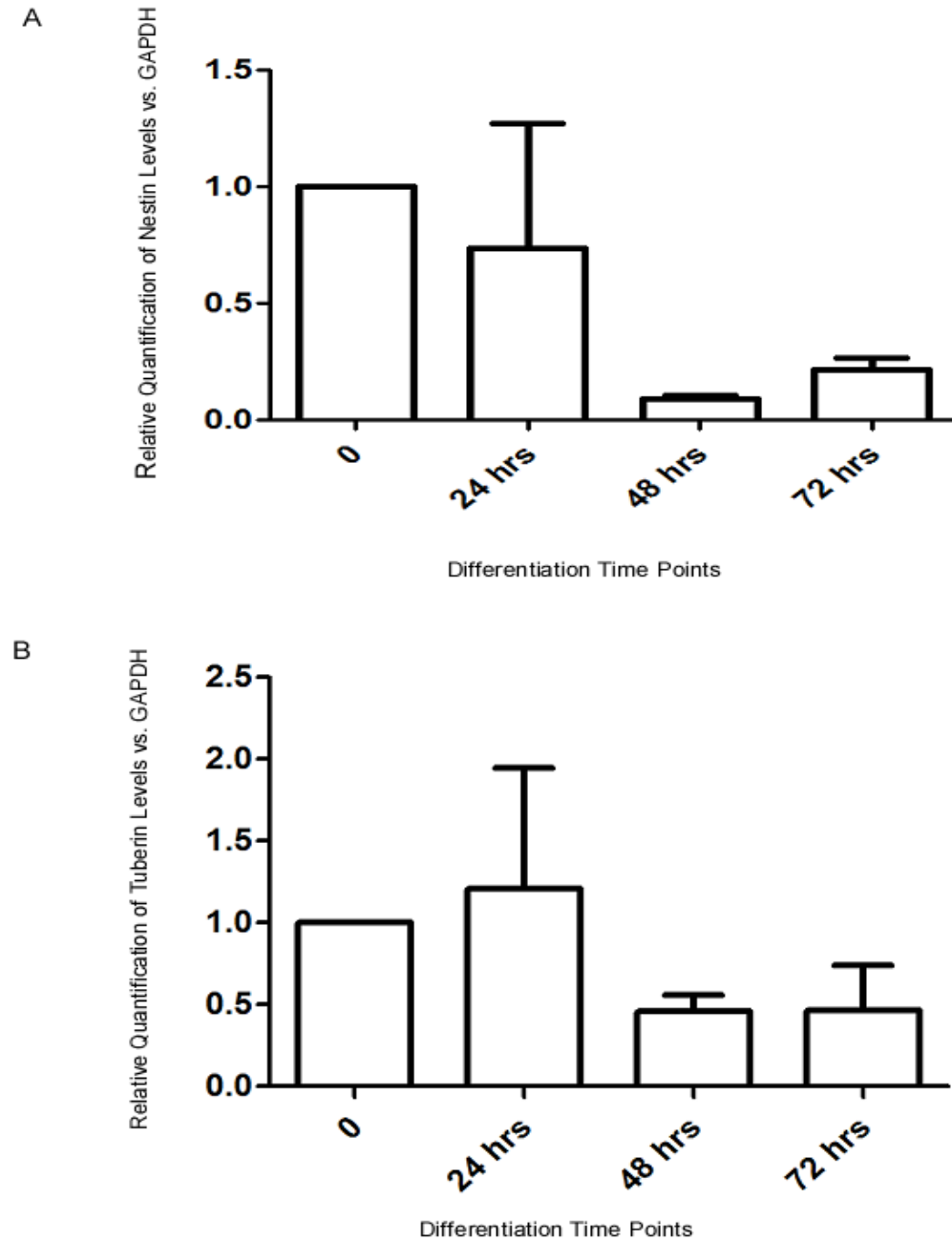
III. Tuberin Expression Increases in the Stem Cell Population in the DAOY Cell Line

Using the Medulloblastoma cell line, DAOY, primary and secondary neurospheres were allowed to form under stem cell conditions **(Figure 12A)**. To look at whether Tuberin expression correlates with the tumour stem cell marker CD133, levels of Tuberin were analyzed in the CD133 positive population (CD133$^+$) using flow cytometry. Neurospheres were dissociated and cells were stained with Tuberin-Alexa 488 and CD133-Allophycocyanin (APC) antibodies, DAOY cells grown in monolayer were used as a negative control for the stem-cell population. In the monolayer, the population of cells positive for CD133 was close to 0% indicating that the stem cell population at that point was very minimal. In the primary neurosphere population, CD133 cell percentage was approximately 18% and the percentage of cells that were labeled for both TSC2$^+$/ CD133$^+$ was about 7%. This percentage increased in the secondary neurosphere population to 18% **(Figure 12B)**. 16% of the cells in the secondary neurospheres remained positive for CD133 alone. In addition, looking at the percentage of cells that were TSC2$^-$/ CD133$^-$, one can see that this population declines as the cells are selected for the more stem-like populations in the secondary neurosphere conditions. These findings suggest that Tuberin is selectively present in stem cell populations, whether Tuberin plays a functional role in cell fate decisions is an intriguing possibility.

A similar flow cytometry analysis was conducted to investigate the percentage of stem-like cells that were positive for Tuberin along with CD133 in DAOY cells that were manipulated

in vitro. DAOY secondary neurospheres were treated with the mTOR inhibitor Rapamycin or were infected with a PLKO-shTSC2 lentiviral vector targeted against Tuberin mRNA. Neurospheres lacking Rapamycin treatment and TSC2 knock-down, were used as controls. It was observed that the percentage of Tuberin$^+$ and Tuberin$^+$/CD133$^+$ labeled cells, significantly decreased when Rapamycin was added to the neurospheres, this effect was even more pronounced in the secondary neurospheres **(Figure 12C)**.
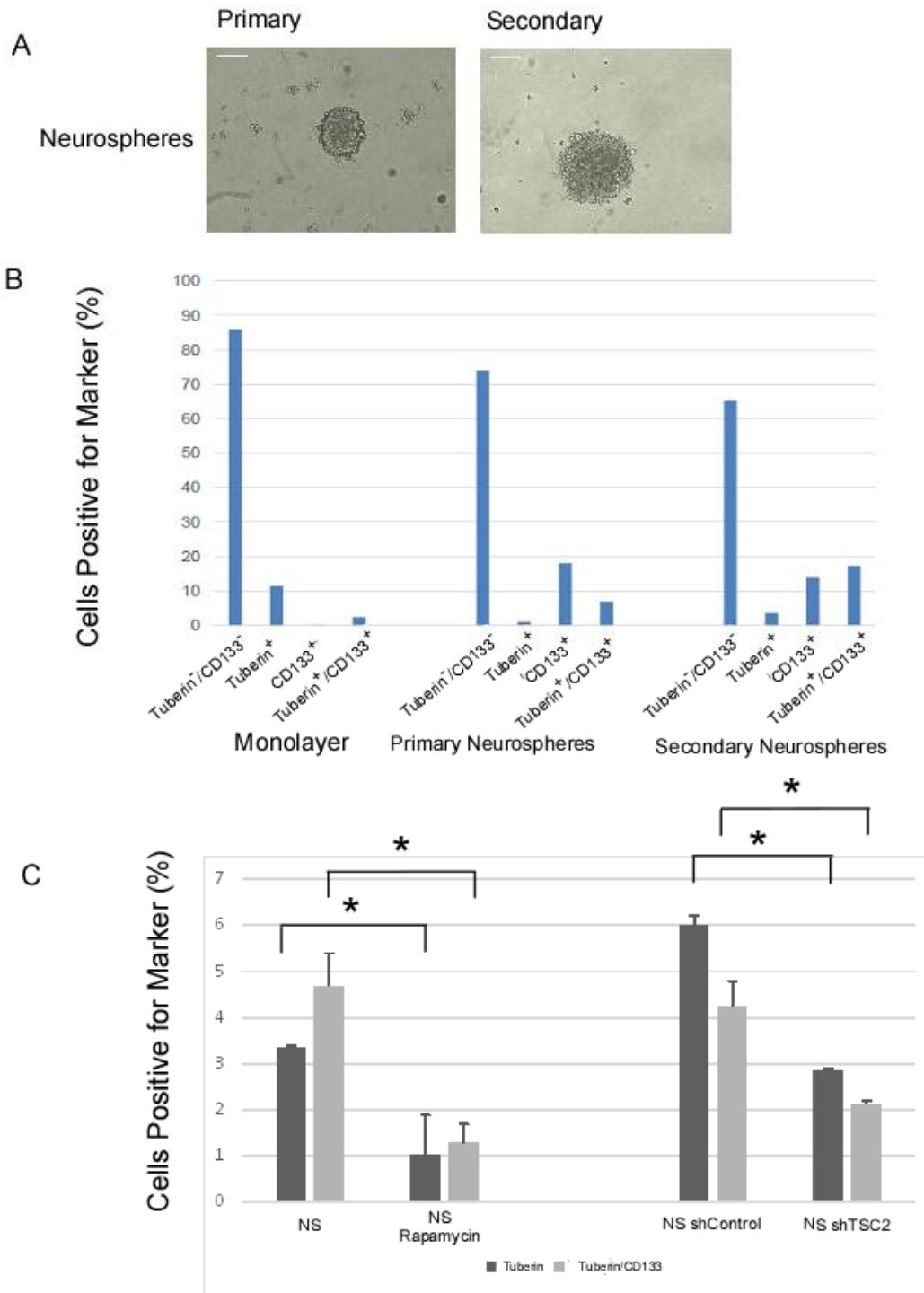
Figure 12. Flow Cytometry Analysis of CD133 and Tuberin Expression in DAOY Neurospheres

DAOY cells were allowed to form primary and secondary neurospheres. The neurospheres were stained with Tuberin and CD133 antibodies. **(A)** Representative images of primary and

secondary DAOY neurosphere growth under necessary growth supplements. Scale bars = 20 pixels **(B)** Flow cytometry results reported as percentage of cells stained for Tuberin$^+$, CD133$^+$ and Tuberin$^+$/CD133$^+$. **(C)** Percentage of secondary neurospheres staining for Tuberin$^+$, and Tuberin$^+$/ CD133$^+$ after treating with Rapamycin and shTSC2. Error bars represent SEM. Unpaired student's t-test. *p< 0.05.

TSC2 Knock-Down Affects Primary Neurosphere Formation

To test the functional importance of Tuberin in neurosphere formation, DAOY cells were seeded into stem-like conditions needed for neurosphere formation and the effects of Rapamycin treatment and Tuberin knock-down, were monitored. Rapamycin treatment did not statistically reduce overall neurosphere numbers **(Figure 13A)**. However, when Tuberin was knocked-down, the number of neurospheres formed significantly decreased **(Figure 13B)**.
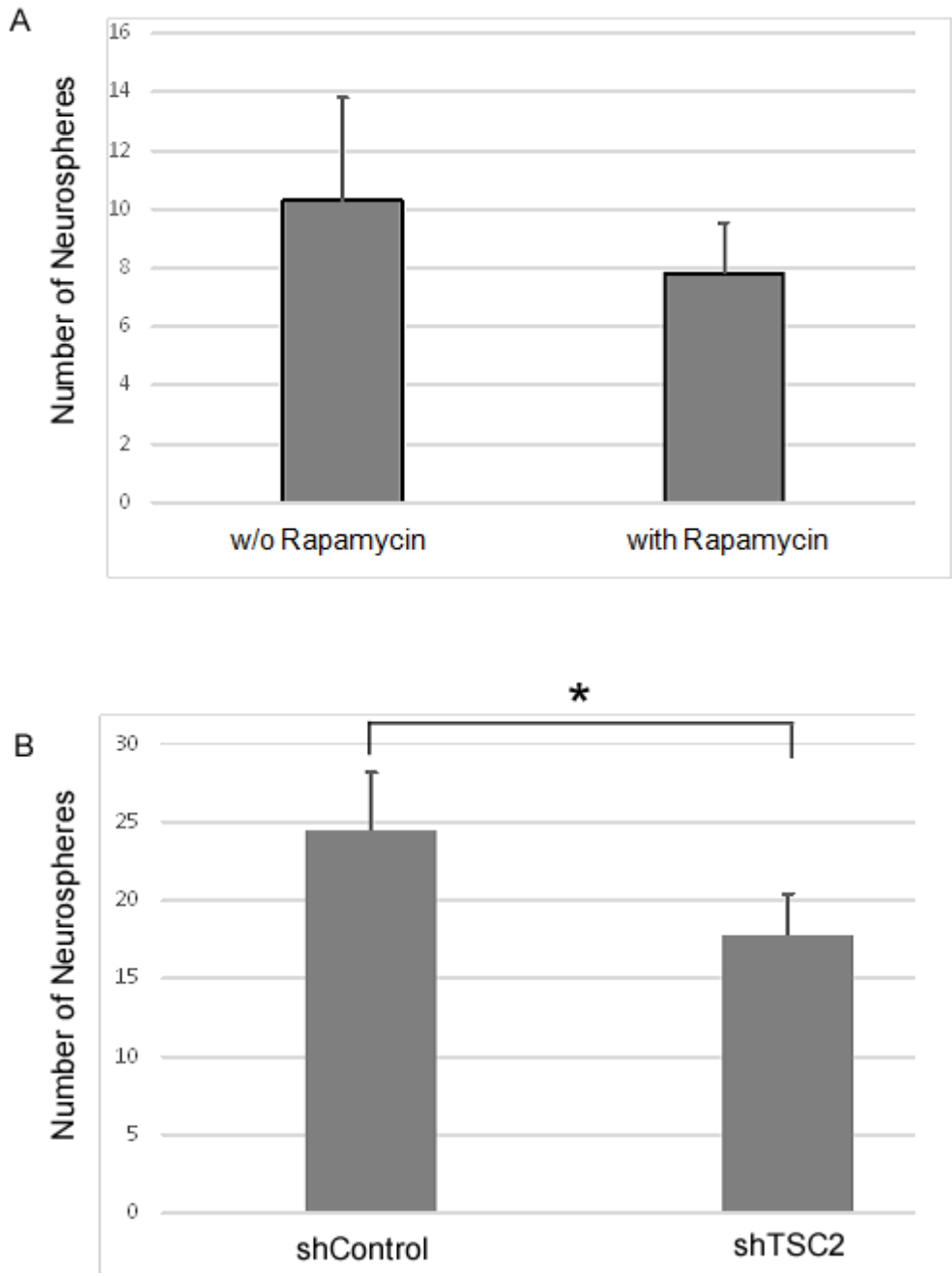
Figure 13. Tuberin Knock-down Decreases the Number of Primary Neurospheres.

DAOY cell**(A)** neurospheres were treated with vehicle control (w/o Rapamycin) and**(B)** TSC2 knock- down. Error bars represent SEM of three independent experiments. Statistical analysis done using student's t- test. * $p < 0.05$.

TSC2 Knock-Down Affects Secondary Neurosphere Formation

To elucidate a functional role for Tuberin on Neurosphere self-renewal, primary neurospheres treated with Rapamycin or shRNA to knock-down Tuberin and then subcultured and seeded into secondary neurosphere formation assays where the number of neurospheres was counted over time. Secondary neurospheres that formed were counted at 4 days, 6 days, and 8 days, respectively. Rapamycin treatment had no statistically significant effect on self-renewal across the six replicates **(Figure 14A)**. Tuberin knock-down resulted in a significant decrease in the number of secondary neurospheres that formed by day 8 **(Figure 14B)**.
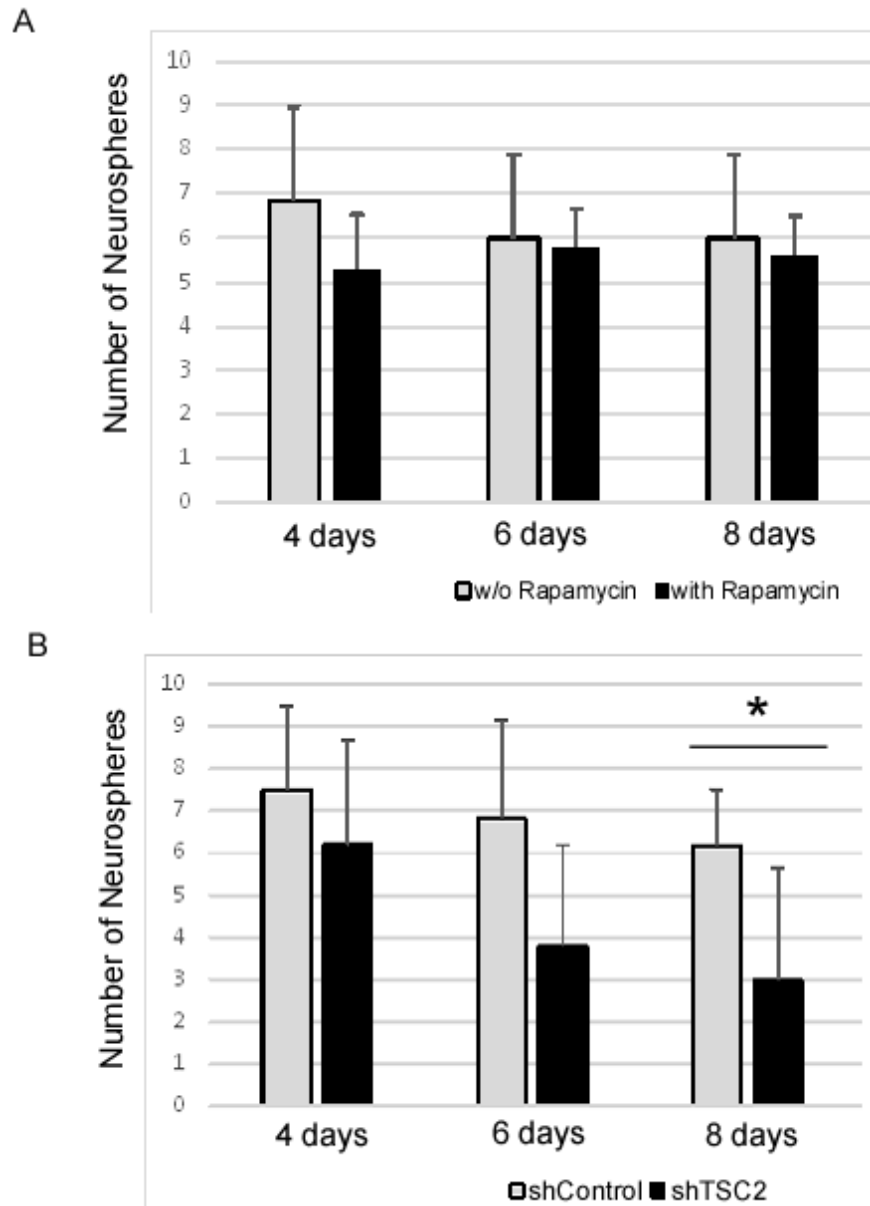
Figure 14. Secondary Neurosphere formation is impaired in the absence to Tuberin.

DAOY cells were subcultured and plated for secondary sphere formation after treatment with **(A)** 250 ng/mL of Rapamycin or vehicle control (w/o Rapamycin) or **(B)** scrambled shControl or shTSC2. Total number of neurospheres are recorded on Y axis over the indicated timepoints. Error bars indicate SEM over six independent experiments. Student's t- test was conducted. * p< 0.05.

DISCUSSION

Cell growth and proliferation are essential for normal development but must be tightly regulated to prevent tumours from forming [97]. This represents a particular challenge during early development when cell growth and proliferation are occurring very rapidly. Childhood cancers, such as those that affect the brain and the blood occur due to an inability to restrict the growth of select cell populations. Today, we know a great deal about specific DNA mutations and deletions that are associated with a plethora of overgrowth disorders; however, proposing suitable treatments for these disorders requires a complete understanding of how these changes impact growth and development. This thesis focuses on resolving some of the biology around the protein Tuberin that is mutated in a number of human tumours, including those found in select childhood brain tumours.

Mutations in the gene encoding Tuberin, *TSC2*, are the most prominent alteration in benign tumours, known as hamartomas, which can form in many organs in the body including the brain[98]. *TSC2* mutations are also found in select brain cancers such as giant cell astrocytoma[99]. In combination with other mutations such as those in the Sonic HedgeHog (Shh) pathway, Tuberin mutations enhance the prevalence of the childhood brain cancer Medulloblastoma (MB)[88]. Understanding how Tuberin regulates the growth of neural cell populations may point to therapeutic directions that can benefit patients with these diseases. Mechanistically, Tuberin is most noted for its roles as a heterodimer with the protein Hamartin, referred to as the Tuberous Sclerosis Complex (TSC) [8].

The most well established role of the TSC is as a negative regulator of the Mammalian Target of Rapamycin (mTOR)[100]. mTOR is highly conserved and it is involved in a plethora of

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cellular processes but is most noted for controlling protein synthesis [101]. Hence the most obvious role of a Tuberin mutation would be to encourage cell overgrowth via a lack of control over protein synthesis and depleting the tumour suppressor roles of Tuberin on the cell cycle. These functions are likely the major drivers of several of the benign hamartomas that form in TSC2 mutated patients. However, astrocytoma and MB are aggressive malignant brain cancers driven at least in part by an aggressive population of cells that harbor stem-like properties. These cells are termed cancer stem cells, which are capable of giving rise to different cell types within the tumour[64]. While cancer stem cell populations may not arise from mutations occurring in normal stem cells, they evolve to possess many of the qualities of a normal stem cell including the ability to self renew. Previous studies done by Kobayashi *et. al.* in 1996, have shown that *TSC2* knock-out mice died at ED 10 due to failure of neural tube closure, suggesting the intriguing possibility that Tuberin may have an active and essential role in the early stages of brain development[102]. One of the key objectives of this work was to determine where and when endogenous Tuberin was found in the developing brain. Should Tuberin play an essential role in neuro- or gliogenesis, we hypothesized that protein levels may be elevated at earlier stages of embryonic development. We focused primarily on the structures that give rise to the cerebellum given that this is the region of the brain where MB is derived.

To study normal protein expression in a tissue specific manner in a Balb/C mouse model, IHC staining in cerebellum forming regions, such as the choroid plexus and purkinje cell layer was performed. No significant differences in Tuberin protein levels were observed when comparing various regions of the brain (olfactory bulb, cerebral cortex, choroid plexus, and purkinje cell layer) within a given developmental time point. However, when protein levels within the regions were compared between time points, it was observed that Tuberin protein

levels significantly increased in the purkinje cell layer at ED 16.5. This suggests that between these two critical time points of development and cell fate determination, Tuberin expression is being regulated. How this is related to neurogenesis within this region requires further dissection of the numbers of relative cell populations at ED 14.5 and 16.5 in this particular brain region, and a close up study of the specific stem/progenitor staining for Tuberin. It is possible that the most stem like and/or progenitor population might be increasing or proliferating at this time to give rise to the different distinct layers of the cerebellum. It is documented in a study by Murthy *et. al.* in 2001., that Tuberin expression is relatively high in the brain during brain development especially starting at ED13[103]. Conversely, Tuberin levels were decreasing in other organs in the body including the kidneys, liver, and heart[103]. In these organs, it is possible that the cells were terminally differentiated; therefore, these cells do not need a high level of Tuberin to keep cellular proliferation and growth under control. This may shed light on the significance of Tuberin in regulating growth in mammalian brain development at early embryonic time points especially during neurogenesis.

The increased protein levels of Tuberin observed in the purkinje cell layer may be an indication of its regulatory role in cellular growth especially in the neural stem cells. This tumour suppressor protein may be allowing for cellular proliferation and preventing neural cell differentiation at this time. Tuberin might be needed to instantly increase at a certain stage in development to regulate the proliferation and differentiation process. Analyzing normal Tuberin levels within the purkinje cell layer is important because neurological disorders, such as Autism Spectrum Disorders (ASDs), tend to arise in the purkinje cells of the cerebellum due to *TSC2* mutations[95]. In a recent study, it was found that loss of *TSC2* in the purkinje cells is linked to neurological disorders[95]. This provides evidence for the importance of Tuberin in this region.

The purkinje cell layer arises from the granular precursor neurons in the cerebellum. Those neurons migrate from the granule cell layer, give rise to the purkinje cell layer, and eventually give rise to the molecular layer[104]. We speculate that these granule cells possess stem cell properties and, as they differentiate, they give rise to purkinje cells. Once the purkinje cell layer is formed, Tuberin levels decrease, supporting the hypothesis that Tuberin is required early in development and that Tuberin has a role in regulating both cell growth and differentiation. Further research would be required to specifically mark the purkinje cells and look at normal Tuberin levels at the cell level rather than the tissue as a whole.

In preliminary experiments **(Appendix A Figure 1)**, the purkinje cell layer and choroid plexus were stained with stem cell protein markers, such as Pax6. Pax6 is expressed in stem cells that have not yet differentiated [105]. Those brain sections were co-stained with Tuberin to assess Tuberin co-localization with Pax6 in the stem cell population. Our early data suggests that Tuberin is expressed in the same region as Pax6 expression in the purkinje cell layer. This preliminary result might indicate that in fact Tuberin is expressed in stem cells where it could be playing a role in maintaining that population and regulating differentiation, proliferation, and overall cell fate. Further investigation would require high magnification analysis of each cell to fully observe Tuberin and Pax6 expression together.

Future experiments, utilizing lineage tracing would be extremely useful in assessing Tuberin localization within the neural stem cell population during cerebellum developmental stages. One can label the cell of interest, the granule cell, with a fluorescent protein, which would be Tuberin, and track its differentiation status in the tissue. Tissue harvesting and sectioning at ED 14.5 and ED 16.5 could be undergone and staining with an antibody against Tuberin would reveal Tuberin expression; identifying if Tuberin is present within these neural stem cells. In

addition, it will be easily observed whether Tuberin is important to maintain homeostasis of the neural stem cell population.

Interestingly, regulation by Tuberin in the regions that give rise to the cerebellum may imply its importance in MB, given that MB tumours arise in the cerebellum [88]. MB arises from a subpopulation with cancer stem cell properties [63]. Hence we optimized the isolation of primary mouse cerebellum cells to study the importance of Tuberin during cell fate and differentiation decisions. In a short time course of differentiation (24 hours), no significant change in Tuberin expression was identified however, over a 4 day differentiation time course the mRNA levels of Tuberin significantly decreased over time. This supports the hypothesis that Tuberin is needed early on before differentiation in the stem cell or progenitor population to regulate cellular growth and differentiation. Tuberin expression decrease would facilitate an increase in protein synthesis, allowing for the production of specialty proteins needed for fully functional differentiation. To further validate this hypothesis, experiments involving Tuberin over-expression in cerebellum cells would be required. It has been previously established that Tuberin interacts with a variety of pathways that guide and regulate cell fate and differentiation. In a study of neuroepithelial progenitor cells from *TSC2* homozygous null mice, cells cultured from ED 10.5 had abnormal cell differentiation[106]. In a separate study, Tuberin knock-down prohibited proper axon formation in actively developing neurons. In the cerebellum, an increase in proliferation of the neural precursor cells and a decrease in neuron outgrowth were reported[34]. To further support the regulatory role of Tuberin in these key cellular events, it has been noted by a group of researchers that diseases caused by Tuberin mutations are linked to abnormal differentiation, further supporting the involvement of Tuberin in the regulation of cell fate and differentiation [34].

To investigate the effects of Tuberin on stemness properties, primary extracted cerebellum cells were exposed to lentiviral Tuberin knock-down. Preliminary results from a neurosphere formation assay, showed that neurosphere size increased (**Appendix A Figure 2**). This result supports that the loss of the tumour suppressor protein causes uncontrollable division, whether the increased size is due to proliferation of select populations or truly through an increase in self-renewal needs to be further explored using secondary neurosphere forming assays and cell pair assays.

CD133 has been shown to be expressed in cancer stem cells. Using CD133 as the stem cell marker for the Shh derived Medulloblastoma cell-line, DAOY, cells were cultured under stem cell conditions promoting the formation of neurospheres[96]. Through the use of flow cytometry, the collected neurospheres were sorted from a single cell suspension and stained with specific antibodies. In the monolayer, the CD133$^+$ population was almost non-existent, which is expected since the cells at this stage are less stem-like. The CD133$^+$ cell population is expected to increase as the cells become more stem-like going from monolayer to primary and then secondary neurospheres. In comparison, the percentage of cells that were Tuberin$^+$/CD133$^+$ increased as cells were passaged as neurospheres. This result indicates that Tuberin is present in the stem cell population, consistent with endogenous levels being elevated in select populations at earlier stages of development. Whether Tuberin plays a direct role on the functional stem cell properties cannot be determined from this assay alone.

To elucidate the role of Tuberin in the stem cell population, DAOY cells were manipulated by lentiviral infection to knock-down Tuberin. Cells infected with a lentiviral shRNA scrambled vector served as the control for the experiment. The percentage of cells that were Tuberin$^+$/CD133$^+$ significantly decreased when compared to the control, as expected. There

were fewer neurospheres formed when Tuberin was knocked-down, providing good support that Tuberin is essential for the stem cell population self-renewal. Moreover, a group of cells were treated with Rapamycin, which halts mTOR activity and blocks protein synthesis/cell growth. Through experimental treatment of the cells with Rapamycin, it was observed that there is a decrease in the Tuberin$^+$/ CD133$^+$ population and a decrease in neurosphere numbers, confirming that mTOR regulation is necessary for the stem cell population of self renewal. Our neurosphere experiments confirm that Tuberin plays a critical role in the maintenance of the stem cell population; whether it is through self-renewal, survival or proliferation remains to be further determined. Rapamycin alone did not have a significant effect on neurosphere number which suggests that Tuberin role in stem-cell self-renewal is either not only mediated through mTOR, or that the appropriate stimulus was not provided for testing (ie. a differentiation stimuli). An experiment that could shed light on the mechanism of Tuberin function would be to treat cells that are Tuberin null with Rapamycin to determine whether the cells would be able to self-renew and proliferate or if Rapamycin would completely inhibit their growth with the lack of Tuberin in the cells.

We assess self-renewal through secondary neurosphere formation in Tuberin null cells and Rapamycin treated cells. Over an 8 day period, there was a significant decrease in Tuberin in the secondary neurospheres. A decline in the number of neurospheres was also seen with Rapamycin treatment although findings were not statistically significant. This experiment, supports that Tuberin may be essential in regulating the stem cell population. When Tuberin is knocked-down, the cells start to die or growth is halted due to misregulation of the the mTOR regulatory pathway. To further enhance this result, investigating the activity of mTOR downstream effectors, such as S6K, would provide insight regarding the possible misregulation of mTOR.

Overall, this research emphasizes a potential role for Tuberin in regulating and maintaining the progenitor stem cell population. We have determined that Tuberin is present, and even elevated, in specific regions of the brain at early time points of development when neurogenesis is at its peak. Tuberin seems to be regulating the process of cell fate decisions in instances where it halts cell proliferation and allows for terminal cell differentiation. Assessing Tuberin levels in primary extracted cerebellum cells is one of the few studies that try to link Tuberin regulation with stem cell fate decisions. Being able to correlate Tuberin expression with tumour stem cells, such as those in MB cells, will help imply how these cancer stem cells are driving tumourogenesis. One of the limitations to working with primary extracted cerebellum cells is that they are short lived and extraction is both challenging and time consuming. New advances in technology such as the CRISPR/CAS9 tool, allows researchers to manipulate the genome. Therefore, CRISPR/CAS9 technology would enable us to determine the role of select TSC2 mutations in any cell type selected in the absence of endogenous wild-type protein. This would enable us to find answers faster and more efficiently. The CRISPR/CAS9 system would also allow us to knock-in select *TSC2* mutations *in vivo*. With the advent of this new exciting technology we can take the results obtained herein to a new level to determine whether Tuberin signaling has potential as a therapeutic target for select forms of tumour disorders.

Appendix A
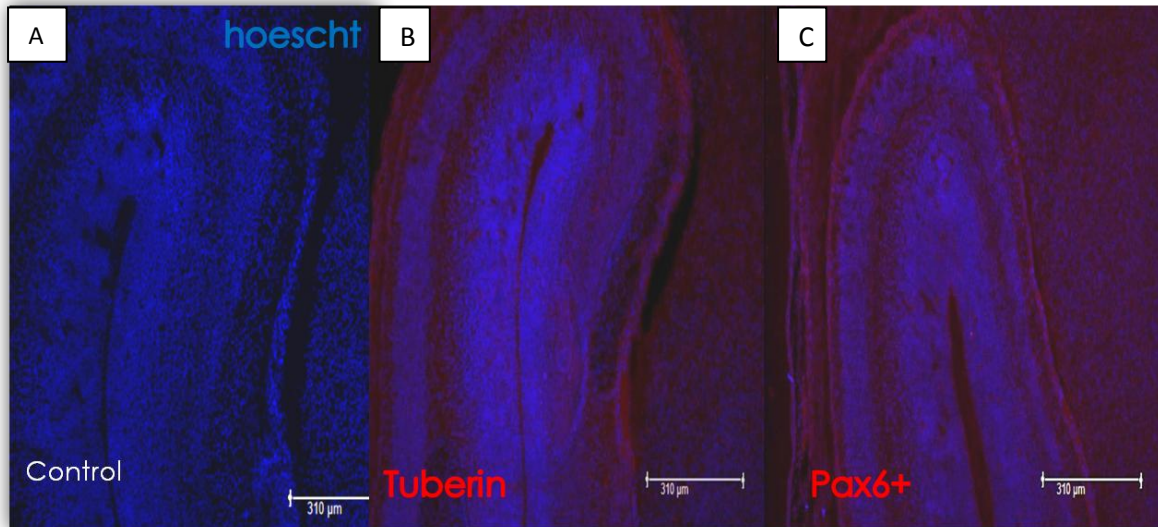
Figure 1. Tuberin and Pax6 are Expressed Around the Purkinje Cell Layer Area.

Purkinje cell layer area at ED 16.5 was stained using Immunofluorescence. **(A)** Control section lacking the primary antibody and expressing DAPI nuclear stain. **(B)** Tuberin expression shown in red around the PCL. **(C)** Pax6 expression shown in red around the PCL. All images were taken with a 10x objective
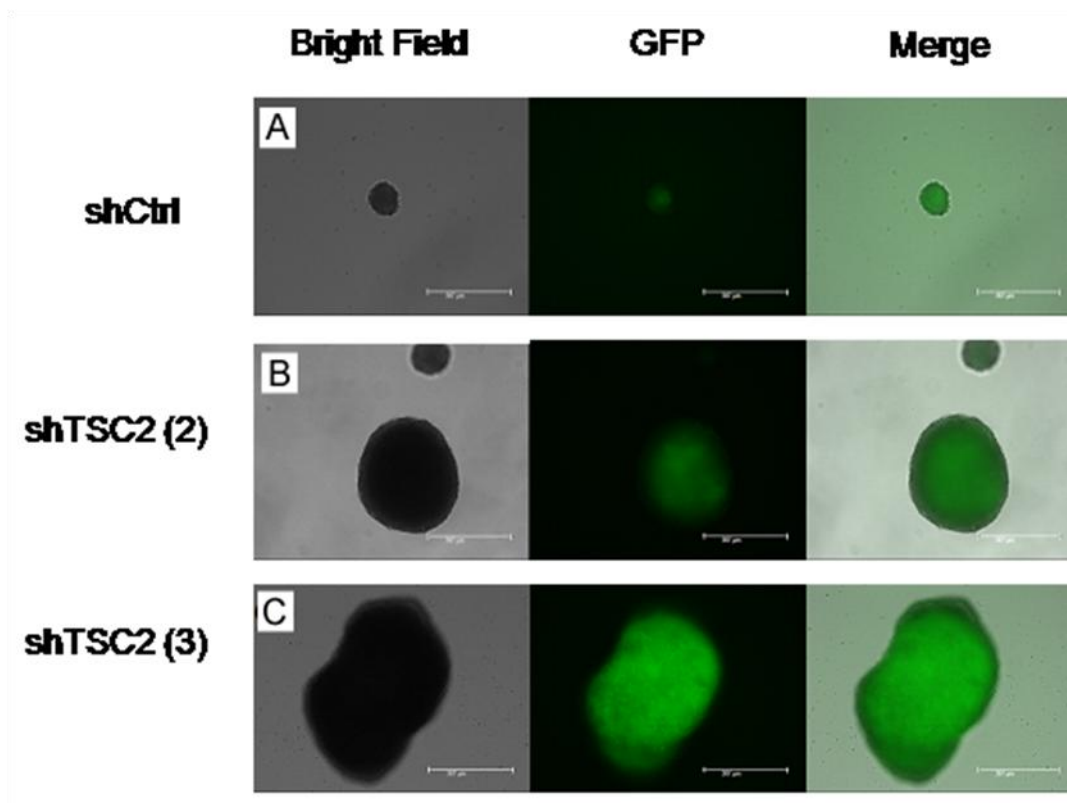
Figure 2. Tuberin Knock-down in Primary Cerebellm Cells shows and Increase in Neurosphere Size

Stem-like cells from the mouse cerebellum at PN=4 were stimulated to form neurospheres. **(A)** Image of neurospheres after shTSC2 control infection. **(B)** Cells treated with shTSC2 infection and **(C)** Cells treated with a different knock-down construct of shTSC2. Images were taken in bright field, GFP, and an overlay of both fields, respectively. Green fluorescence indicates successful knock-down.

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