Molecular regulation of neural stem cell quiescence by FoxO transcription factors

Draijer, S.

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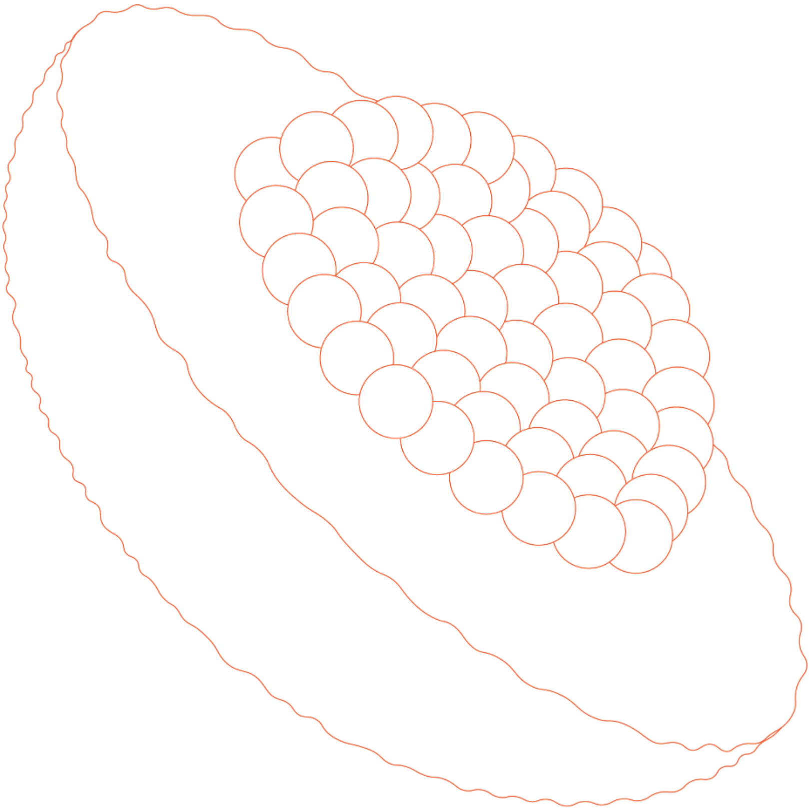
Chapter 1
Introduction



Introduction

When neurons were first recognized as independent functional units in the late nineteenth century, it was thought their number was fixed (Colucci-D'Amato et al., 2006). In 1913, Santiago Ramón y Cajal wrote *"Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."* In the 1960s, however, Altman and Das found evidence of the formation of new neurons (i.e. neurogenesis) in the brains of rats and other mammals (Altman and Das, 1965). This sparked the search for the source of these neurons.

This source remained obscure until 1989 when Sally Temple identified a population of stem cells in the adult mouse brain (Temple, 1989). This was followed by Brent A. Reynolds and Samuel Weiss, who, in 1992, were the first to isolate these stem cells and grow them as spheres in culture, proving their ability to generate neurons (Reynolds and Weiss, 1992). By ingeniously making use of thymidine analog BrdU, used to assess proliferation of tumour cells in cancer patients, Eriksson and colleagues demonstrated the existence of adult neurogenesis in human brains in 1998 (Eriksson et al., 1998). Finally, in 2000, Roy and colleagues managed to isolate stem cells from the human brain, which matured to neurons *in vitro* (Roy, 2000). These studies have consolidated the concept of brain-specific stem cells - neural stem cells - as the source of new astrocytes, oligodendrocytes and neurons in the adult brain. However, tapping this source comes at a cost: it consumes the neural stem cell and leads to a dwindling population shortly after birth. As a survival strategy, the majority of neural stem cells resides in a non-proliferative state known as quiescence where they can persist for a lifetime awaiting signals of activation.

In this thesis, I focus on the molecular regulation of neural stem cell quiescence by transcription factors FoxO3 and FoxO6. Only finely tuned regulation of their cellular behaviour can ensure lifelong neurogenesis. Consumption of neural stem cells leads to an age-related exhaustion of their population but an inability to activate these stem cells renders them useless. Alternatively, neural stem cells are a critical cell-of-origin for brain tumours. Intrinsic regulation of cell cycle and differentiation is, therefore, key to healthy aging. Many of these mechanisms are shared with various tissue-specific stem cells and help to understand the molecular regulation of neural stem cells.

Stem cells

Adult stem cells are multipotent undifferentiated cells with the ability to self-renew (Weissman, 2000) and are present in most mammalian tissues where they help to maintain tissue homeostasis by replacing cells or as a source of highly specialized cell types that repair and regenerate tissues following injury (Li and Clevers, 2010). As the number and/or activity of adult stem cells declines during aging, which results in impaired tissue homeostasis (Goodell and Rando, 2015; Tümpel and Rudolph, 2019), many adult stem cells reside in a non-proliferative state known as quiescence. This allows stem cells to persist over the lifetime of an organism and is thought to protect their DNA from mutations acquired over multiple rounds of cell division (Walter al., 2015). Quiescence is essential for the long-term maintenance of stem cell populations and its loss can lead to stem cell depletion or tumour formation if paired with increased rates of self-renewal (Tümpel & Rudolph, 2019; Orford & Scadden, 2008; Ito & Suda, 2014). In contrast, excessive quiescence can result in the generation of too few progenitor cells necessary to ensure tissue homeostasis (Cheung & Rando, 2013). As such, quiescence is no longer thought as an inactive state of stem cells, but an actively regulated condition dependent on both intrinsic and extrinsic signals (Cheung & Rando, 2013; Tümpel & Rudolph, 2019; Urbán & Cheung, 2021).

Neural stem cells and their niche

Neural stem cells (NSCs) are the tissue-specific stem cells of the brain and the source of new neurons, astrocytes and oligodendrocytes (Kriegstein and Alvarez-Buylla, 2009). These cells originate from subsets of embryonic radial glia, which acquire a quiescent state at an early developmental stage and largely remain quiescent in the adult brain (Fuentealba et al., 2015; Obernier & Alvarez-Buylla, 2019; Urbán et al., 2019). NSCs reside in mainly two neurogenic niches within the adult brain: the ventricular-subventricular zone (V-SVZ) of the lateral ventricles and the subgranular zone of the dentate gyrus (DG) of the hippocampus (Altman and Das, 1965; Doetsch et al., 1999; Ming & Song, 2011; Zhao et al., 2008).

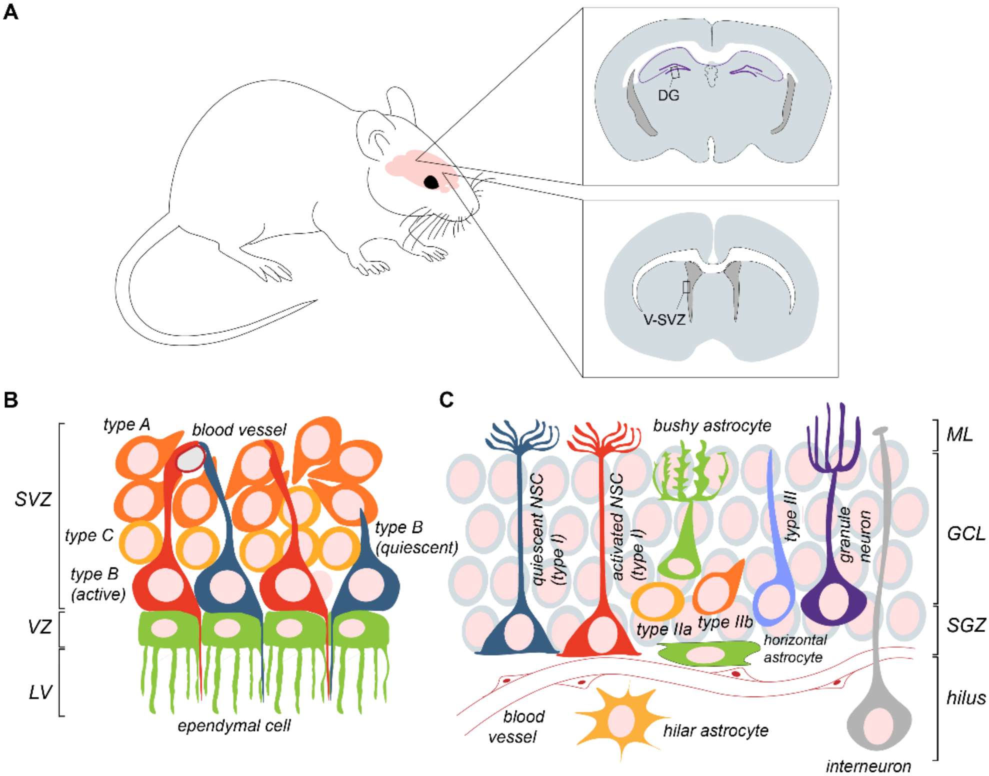


Figure 1. Neural stem cells in the adult neurogenic niches

A) Cartoon showing the location of neurogenic niches in the adult mouse brain: the ventricular-subventricular zone (V-SVZ) and dentate gyrus (DG).

B) Cell types in the V-SVZ: astrocyte-like quiescent and active NSCs (type B cells, blue and red, respectively), transit amplifying cells (type C, yellow), neuroblasts (type A cells, orange) and ependymal cells (green). SVZ, subventricular zone; VZ, ventricular zone; LV, lateral ventricle

C) Cell types in the DG: radial glia-like quiescent and active NSCs (type I cells, blue and red, respectively), intermediate progenitor cells (type IIa/b, yellow/orange), immature granule neurons (type III cells, light purple), mature granule neurons (deep purple), bushy and horizontal astrocytes (green) as well as hilar astrocytes (yellow) and interneurons (grey) in the hilus. ML, molecular layer; GCL, granule cell layer; SGZ, subgranular zone

The V-SVZ is a layer of dividing cells, which extends along the lateral wall of the lateral ventricle (**Figure 1B**). There are four main cell types in the V-SVZ: neuroblasts (type A cells), SVZ NSCs (type B cells), transit amplifying cells (TAP), immature neurons (type C cells) and ependymal cells (Figure 1B; Conover and Notti, 2008; Lim & Alvarez-Buylla, 2016). A single layer of multiciliated ependymal cells separates the SVZ from the lateral ventricle. Neuroblasts born in the SVZ migrate along the rostral migratory stream towards the olfactory bulbs, where they primarily differentiate into interneurons that integrate into the local circuits (Mirzadeh et al., 2008; Lim & Alvarez-Buylla, 2016). The adult dentate gyrus is composed of the molecular layer, granule cell layer and subgranular zone (SGZ), which is the layer of cells directly bordering the hilus (**Figure 1C)**. Within the subgranular zone, radial glia-like NSCs (type 1 cells) give rise to intermediate progenitor cells (type 2a and type 2b cells), which progressively differentiate into immature granule neurons (type 3 cells) and mature granule neurons. The dentate gyrus also contains horizontal and bushy astrocytes (Seri et al., 2004; Bonaguidi et al., 2011), which provide trophic and metabolic support to these new neurons and regulate synaptic transmission (Conover and Notti, 2007).

Whereas these niches are both highly vascularized microenvironments, a key difference is the proximity of SVZ NSCs to cerebrospinal fluid (CSF) in the lateral ventricles (Doetsch, 2003; Obernier and Alvarez-Buylla, 2019). CSF contains ligands and growth factors implicated in the regulation of NSCs, including BMPs, Wnts, Shh, Slits, retinoic acid, IGFs, TGFs and PDGFs (Silva-Vargas et al., 2013). Many of these are secreted by the choroid plexus, a structure mainly consisting of epithelial cells, which floats within the lateral ventricles (Falcão et al., 2012). Additionally, the CSF contains various systemic signals as well as factors produced by ependymal SVZ cells (Silva-Vargas et al., 2013).

Adult neurogenesis

Following cell cycle entry, activated NSCs either self-renew or commit to more differentiated cell types (Obernier and Alvarez-Buylla, 2019). The transition from proliferative and multipotent NSCs to fully differentiated neurons and glia is called neurogenesis and gliogenesis, respectively. Single cell RNA-sequencing studies in recent years have demonstrated that NSCs transit between multiple states of quiescence and activation before progressively differentiating into intermediate precursor cells, neuroblasts and neurons (Shin et al., 2015; Llorens-Bobadilla et al., 2015; Dulken et al., 2017; Artegiani et al., 2017; Basak et al., 2018). These transitions are characterized by rapid changes in gene expression that are regulated by sequential waves of transcriptional regulators and chromatin remodelling (Beckervordersandforth et al., 2015; Hsieh and Zhao, 2016).

This neurogenic lineage is largely similar between the SVZ and the SGZ as both are characterized by a continuum from quiescence to activation and differentiation (Obernier and Alvarez-Buylla, 2019; Gonçalves et al., 2016). Whereas genes related to BMP, Notch and MAPK pathways are downregulated in the transition from quiescent to activated NSCs, genes related to cell cycle and protein synthesis are upregulated. Importantly, both SVZ and SGZ NSCs switch from glycolysis and lipid metabolism to oxidative phosphorylation during activation (Shin et al., 2015; Llorens-Bobadilla et al., 2015). However, there are some interesting differences between the SVZ and SGZ neural lineage. Neurons born in the SGZ, for example, are excitatory whereas SVZ NSCs give rise to inhibitory neurons (Obernier and Alvarez-Buylla, 2019). Additionally, SGZ NSCs generate differentiated astrocytes but do not seem to produce oligodendrocytes *in vivo* (Rolando et al., 2016).

Studies in rodents demonstrate that adult neurogenesis contributes to olfactory perception, learning and memory, and mood regulation (Bond et al., 2015; Brann & Firestein 2014; Gonçalves et al., 2016; Snyder, 2019). Whereas adult neurogenesis has long been considered to be of similar importance in the human brain (Eriksson et al., 1998; Roy et al., 2000; Wang et al., 2011), a recent study challenged this view as Sorrells et al. (2018) found human hippocampal neurogenesis to drop sharply in children to undetectable levels in adults. However, as subsequent studies revealed human neurogenesis to exist throughout aging (Boldrini et al., 2018; Tobin et al., 2019), conclusions on adult neurogenesis appear to be highly dependent on the used methodological approaches such as brain tissue processing and the use of immunohistochemical markers to detect immature neurons (Paredes et al., 2018; Tartt et al., 2018; Lucassen et al., 2019; Flor-García et al., 2020). Although the levels of neurogenesis in adult humans are very low, this is not unlike other mammals as neurogenesis drops sharply after birth across species (Snyder et al., 2019).

Nevertheless, this does not render adult neural stem cells insignificant in the human brain. Interestingly, patients with Alzheimer's disease show reduced levels of hippocampal neurogenesis, which correlated with memory deficits (Tobin et al., 2019; Moreno-Jimenez et al., 2019). Conversely, whereas neurogenesis in the human SVZ rapidly declines after birth, quiescent NSCs persist (Van den Berge et al., 2010). Quiescent NSCs in the SVZ are considered the cellular origin of glioblastoma, the most aggressive type of brain tumour (Altmann et al., 2019; Matarredona and Pastor, 2019). For instance, SVZ NSCs have been found to acquire somatic mutations and to migrate to the brain parenchyma where they formed brain tumours (Lee et al., 2018).

Neural stem cells in the aging brain

Neurogenesis in the SGZ and SVZ peaks during early life but rapidly declines in adulthood (Kuhn et al., 1996; Maslov et al., 2004; Daynac et al., 2016; Ziebell et al., 2018). Following activation, NSCs undergo limited rounds of cell division before terminally differentiating which leads to a gradual depletion of the NSC population (Encinas et al., 2011; Calzolari et al., 2015; Pilz et al., 2018; Ibrayeva et al. 2021). However, during aging, NSCs become increasingly quiescent, which slows down the rate of depletion (Bouab et al., 2011; Capilla-Gonzalez et al., 2014; Kalamakis et al., 2019; Harris et al., 2021). NSCs grow increasingly quiescent during aging due to changes in, amongst others, proteostasis, asymmetric segregation of proteins, cellular metabolism and mitochondrial function but also because of changes in the neurogenic niche (Audesse & Webb, 2020).

For instance, an age-related accumulation of protein aggregates in old NSCs impairs their ability to be activated and proliferate compared to young NSCs (Leeman et al., 2018). Whereas protein aggregates can be asymmetrically distributed over daughter cells during NSC division to preserve the neurogenic lineage, this relies on an ER membrane that acts as diffusion barrier (Audesse & Webb, 2020). During aging, however, this ER diffusion barrier weakens, which results in a more symmetric distribution of damaged and aggregating proteins following NSC division (Moore et al., 2015). In addition, age-dependent changes in NSC metabolism promote quiescence (Audesse and Webb, 2020). Aged NSCs increasingly utilize glycolysis rather than mitochondrial oxidative phosphorylation, which favours a quiescent state (Stoll et al., 2011; Beckervordersandforth et al., 2017). Conversely, oxidative phosphorylation generates reactive oxygen species (ROS), which can promote proliferation and self-renewal of NSCs (Le Belle et al., 2011).

Whereas NSCs are able to return to quiescence following their activation, these cells reside in a shallow quiescence compared to cells that have remained dormant since birth (Urbán et al., 2016). Recently, research carried out by Guillemot's group (Harris et al. (2021) revealed that a growing number of hippocampal NSCs return to shallow quiescence during aging and that this population increasingly contributes to neurogenesis while the dormant cells enter into deeper levels of quiescence with age. Harris et al. show that the balance between pro-activation transcription factor Ascl1 and E3 ubiquitin ligase Huwe1, which degrades Ascl1, shifts towards quiescence when expression of Huwe1 increases during aging. Alternatively, the Bonaguidi lab (Ibrayeva et al., 2021) identified two subpopulations of NSCs: one that is Ascl1-positive and one that is Nestin-positive. Whereas Ascl1-positive NSCs rapidly divide and deplete in early adulthood, the Nestin-positive NSCs are more quiescent, have a higher capability for symmetrical self-renewal and persist throughout life. As such, the Ascl1+ NSC population rapidly declines in number during early adulthood, Nestin+ NSC population persists throughout life at approximately one fifth of the population in the young adult hippocampus. Ibrayeva et al. then show that tyrosine-protein kinase Abl1 is a pro-aging factor that increases with age. This is in line with an *in vivo* imaging study by Jessberger's group (Bottes et al., 2021), which showed the existence of two Ascl1+NSC and Gli1+ NSC subpopulations. Similarly, Ascl1+ NSCs went through several rounds of cell division, depleted shortly after entering the cell cycle, and accounted for the majority of postnatal and young adult neurogenesis. Gli+ NSCs, however, remained quiescent, increased their self-renewal with age, and contributed most to neurogenesis in the long term.

Apart from these intrinsic changes, aging of the neurogenic niche also affects NSC quiescence. Astrocytes, microglia, neurons, endothelial cells and surrounding NSCs secrete signalling molecules that regulate NSC quiescence. Whereas the levels of pro-neurogenic signalling molecules (e.g. IGFs, FGFs, EGFs and WNTs) in the niche decline during aging (Enwere et al., 2004; Shetty et al., 2005; Okamoto et al., 2011), the levels of quiescence-promoting BMP4 and BMP6 increase in the NSC niche (Yousef et al., 2015). Additionally, the composition of the CSF changes during aging and promotes quiescence of SVZ NSCs (Silva-Vargas et al., 2016). During aging, a pro-inflammatory NSC niche also promotes quiescence (Kalamakis et al., 2019; Dulken et al., 2019). For instance, neurogenic niches in old brains are infiltrated by T cells that secrete interferon-γ, which inhibits NSC proliferation (Dulken et al., 2019).

Regulation of neural stem cell quiescence and self-renewal

In line with the above, regulation of NSC quiescence and self-renewal is essential to prevent the early exhaustion of the NSC population and to sustain adult neurogenesis (Urbán et al., 2019; Obernier and Alvarez-Buylla, 2019). Numerous extracellular signalling molecules, including Wnt, BMP, Notch, Shh, GABA and various growth factors (e.g. insulin-like growth factors (IGFs), fibroblasts growth factors (FGFs), epidermal growth factors (EGFs) regulate NSC fates (Faigle and Song, 2013; Obernier and Alvarez-Buylla, 2019). These molecules activate signalling pathways, which differentially control NSC fates. Whereas Wnt signalling promotes NSC proliferation and neuronal differentiation (Gao et al., 2021; Muroyama et al., 2004) BMP signalling promotes quiescence and astrocytic fates (Mira et al. 2010; Bond et al., 2012).

Several transcription factors regulate NSC fates, including ASCL1, p53, p57, REST, TLX, NFIX, OLIG2, and those belonging to the FoxO family (Andersen et al., 2014; Meletis et al., 2005; Furutachi et al., 2013; Mukherjee et al., 2016; Niu et al., 2011; Martynoga et al., 2013; Mateo et al., 2015; Renault et al., 2009; Paik et al., 2009). For instance, Ascl1 is a pro-neural basic helix-loop-helix transcription factor that promotes the activation of quiescent NSCs (Andersen et al., 2014). Transcriptional repressor Hes1, a downstream effector of Notch signalling, drives cyclical expression of *Ascl1* to promote proliferation of NSCs whereas sustained expression of *Ascl1* leads to differentiation (Imayoshi and Kageyama, 2014; Sueda et al., 2019). ASCL1 proteins, in turn, are negatively regulated by E3-ubiquiting ligase HUWE1 and ID4 (Urbán et al., 2016; Blomfield et al., 2019). Transcription factor FoxO3 promotes quiescence by activating transcriptional programs of cell cycle arrest (e.g. p21, p27), glucose metabolism (e.g. *G6pase, Glud1*), DNA repair (e.g. *Gadd45*) and apoptosis (e.g. *Bim*) (Renault et al., 2009; Paik et al., 2009; Yeo et al., 2013; Webb et al., 2013).

More recently, FoxO3 has been found to promote a network of autophagy-related genes to control neuronal morphology and to regulate proteostasis in adult NSCs (Schäffner et al., 2018; Audesse et al., 2019). Interestingly, by competing for DNA-binding sites within shared target genes, FoxO3 and Ascl1 regulate the balance between NSC quiescence and activation (Webb et al., 2013).

Additionally, epigenetic mechanisms control NSC fates by regulating chromatin remodelling, histone modifications, DNA methylation and non-coding RNAs (Ma et al., 2010; Hsieh and Zhao, 2016). For instance, Bmi1, a core component of the PRC1 chromatin-remodeling complex, is required for self-renewal of NSCs by repressing *p16Ink4a* expression (Molofsky et al., 2005). Similarly, EZH2, the catalytic subunit of PRC2, represses expression of genes involved in the PTEN-Akt-mTOR pathway through H3K27 methylation and promotes NSC proliferation (Zhang et al., 2014). In contrast, nucleosomal binding proteins Hmgn1 and Hmgn2 enhance active chromatin states to promote stem cell identity of NSCs (Garza-Manero et al., 2019). Hmgn2-deficient NSCs lose H3K9 acetylation marks and spontaneously differentiate into neurons. However, differentiation into the oligodendrocyte lineage is impaired in the absence of Hmgn1 and Hmgn2 as this leads to increased EZH2 occupancy and elevated levels of repressive H3K27 tri-methylation at the *Olig1* and *Olig2* genes.

Importantly, signalling pathway components, transcription factors and epigenetic regulators all oscillate in their expression and activity during the day (Zhang et al., 2014; Masri and Sassone-Corsi, 2010). This is the result of rhythmic transcription driven by a molecular circadian clock, present in virtually all cells. The circadian clock is an evolutionary conserved mechanism that orchestrates cellular metabolism and the cell cycle during night and day (Asher and Schibler, 2011; Gaucher et al., 2018). In recent years, the circadian clock has been identified as a regulator of stem cells in various tissues, including the brain (Janich et al., 2013; Weger et al., 2017; Draijer et al., 2019). Whereas NSCs exhibit circadian rhythms in their proliferation, loss of circadian clock components results in the activation and depletion of the NSC population. Interestingly, FoxO3 is a regulator of circadian rhythms in the liver (Chaves et al., 2014) but its circadian function in the brain remains unknown.

FoxO transcription factors

FoxO transcription factors are critical regulators of various adult stem cells populations, including those found in testes (Goertz et al., 2011), bone marrow (Tothova et al., 2007; Miyamoto et al., 2007), muscles (Gopinath et al., 2014; García-Prat et al., 2020) and the brain (Renault et al., 2009; Paik et al., 2009; Webb et al., 2013; Kim et al., 2015, Schäffer et al., 2018; Audesse et al., 2019). Although FoxOs have cell type-specific targets (Webb et al., 2016), they generally promote quiescence and self-renewal in this wide range of tissue-specific stem cells. As such, FoxOs are essential for tissue homeostasis throughout life and are strongly associated with organismal life span (Greer and Brunet, 2005; Martins et al., 2016). For instance, two single-nucleotide variants (SNVs) in the *FoxO3* gene were significantly associated with human longevity and increased *FoxO3* mRNA expression (Flachsbart et al., 2017). By promoting a variety of cellular outputs such as cell cycle arrest, apoptosis and glucose metabolism, FoxOs are at the interface of aging and cancer biology (Salih and Brunet, 2008; Ro et al., 2013; Hornsveld et al., 2018).

FoxO1, FoxO3, FoxO4 and FoxO6 constitute the family of FoxO transcription factors in mammals. FoxO2 is identical to FoxO3 whereas FoxO5 is the fish ortholog of FoxO3 (Carter and Brunet, 2007). FoxOs, or Forkhead transcription factors belonging to the ‘O’ class, share the Forkhead box DNA-binding domain with the larger family of Forkhead transcription factors but are uniquely characterized as being regulated by the PI3K-Akt signalling pathway. Deriving their name from the gene mutation that caused ectopic head structures that resembled a fork in *Drosophila* flies, FoxOs are conserved in both invertebrates and vertebrates, albeit as a single ortholog in invertebrates: dFoxO in *Drosophila* and daf-16 in *C. elegans* (Kaestner et al., 2000). The latter gives it name to the DNA motif that is recognizes by the FoxO forkhead domain: the Daf-16-binding element or DBE with consensus sequence 5′-GTAAACA-3′ (Furuyama et al., 2000). All four isoforms recognize this motif, bind common target genes and appear to be functionally redundant (Biggs et al., 2001; Brunet et al., 1999; Furuyama et al., 2000; Jacobs et al., 2003; Paik et al., 2007).

Nevertheless, knockout studies in mice have revealed their functional diversification (Hosaka et al., 2004; Castrillon et al., 2003; Arden, 2008; Salih et al., 2012). Whereas *FoxO1*-knockout embryos die on embryonic day 10.5 as a consequence of incomplete vascular development, FoxO3, FoxO4 and FoxO6 mice are viable and do not show striking phenotype, though *FoxO3*-kockout females exhibit age-dependent infertility.

In addition, FoxO expression patterns differ. Whereas FoxO1 and FoxO3 are abundantly expressed in many different tissues, FoxO4 is minimally expressed in the brain but abundantly expressed in muscle tissue (Hoekman et al., 2006; Biggs et al., 2001). FoxO6 is highly expressed in the brain particularly in pyramidal neurons within the CA1 and CA3 regions of the hippocampus (Jacobs et al., 2003; Hoekman et al., 2006; Salih et al., 2012). FoxO6 expression in the cortex is clearly decreased between birth and adulthood but remains relatively constant through old age. Salih et al. (2012) also examined non-neuronal tissues but only detected FoxO6 protein in the adult testis. Nevertheless, recent studies have identified FoxO6 expression in the liver, gastric tissue, skeletal muscle, the kidney, lung tissue, breast tissue, skin, craniofacial tissue, the retina and adipose tissue (Kim et al., 2011; Qinyu et al., 2013; Chung et al., 2013; Kim et al., 2014; Hu et al., 2015; Lallemand et al., 2018; Sun et al., 2018; Zhou et al., 2018; Abdalla et al., 2021).

Post-translational regulation of FOXO transcription factors

FoxO proteins consists of four domains (Obsil & Obsilova, 2008): a highly conserved forkhead DNA-binding domain, a nuclear localization signal (NLS), a nuclear export sequence (NES) and a C-terminal transactivation domain (**Figure 2**). Whereas FoxO1 and FoxO3 contain approximately 650 amino acids, FoxO4 and FoxO6 are smaller and have a protein size of about 500 amino acids. Importantly, FoxO6, lacks the C-terminal Akt-dependent site present in the other FoxO isoforms (**Figure 2**).

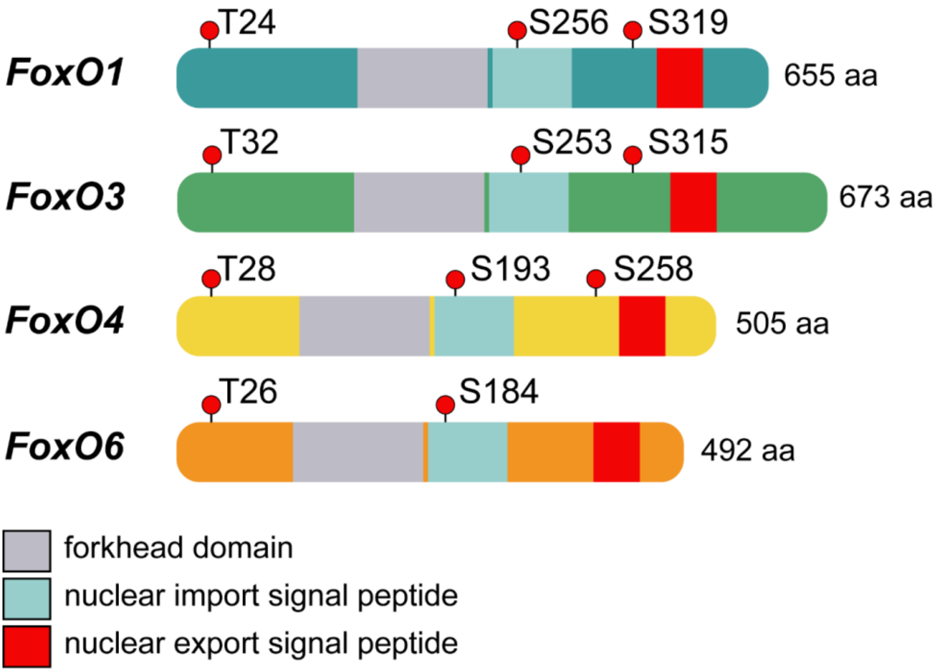


Figure 2. Structure of mouse FoxO1, FoxO3, FoxO4 and FoxO6 proteins
The FoxO transcription factor family consists of four isoforms: FoxO1, FoxO3, FoxO4 and FoxO6. FoxO1, FoxO3 and FoxO4 have three AKT-dependent phosphorylation sites whereas the third AKT-dependent phosphorylation site at the C-terminal end, close to the nuclear export signal peptide, is missing in FoxO6. As a consequence, FoxO6 remains predominantly nuclear following AKT-dependent phosphorylation. T, threonine; S, serine; aa, amino acids

Insulin and growth factors activate receptor tyrosine kinases, including the insulin receptor and insulin-like growth factor receptor (IGFR), which triggers their autophosphorylation and subsequently activates the insulin-receptor substrate (IRS) (Ward and Lawrence, 2009). In turn, the IRS binds and activates phosphoinositide 3-kinase (PI3K), which consists of a regulatory subunit (p85) and a catalytic subunit (p110) (Leevers et al., 1999; Sarbassov et al., 2005). This catalyses the conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3), which, together with PDK1 and mTORC2, activates AKT by phosphorylating its serine-453 residue. Importantly, tumor suppressor protein PTEN antagonizes this conversion and therefore is a crucial inhibitor of AKT activity (Carracedo and Pandolfi, 2008). Akt is a serine/threonine-specific kinase that phosphorylates FoxO1, FoxO3, FoxO4 and FoxO6 (Brunet et al., 1999; Kops et al., 1999; van der Heide et al., 2005). Whereas FoxO1, FoxO3 and FoxO4 have three AKT-dependent phosphorylation site, FOXO6 is missing one (Jacobs et al., 2003). As a consequence, AKT-dependent phosphorylation of FoxO1, FoxO3 and FoxO4 prompts their binding to the 14-3-3 adaptor protein, nuclear exclusion and protein degradation (Van der Heide et al., 2004; Tzivion et al., 2011).

FoxO6, however, remains predominantly nuclear in the presence of serum-containing medium and insulin when FoxO1, FoxO3 and FoxO4 are predominantly cytosolic (Jacobs et al., 2003). Nevertheless, AKT-dependent phosphorylation of Thr26 and Ser184 limits FoxO6 transcriptional activity, independent of nucleo-cytoplasmic shuttling (Van der Heide et al., 2005). The functional consequences of these impaired shuttling abilities, however, still need to be determined. Apart from this Akt phosphorylation motif, FoxO6 also lacks a stretch of three additional serine residues, which are present in the other FoxO isoforms (**Figure 3**). In FoxO1, Ser322 and Ser325 are phosphorylated by CK1 (Rena et al., 2002) whereas Ser329 is phosphorylated by DYRK1a (Woods et al., 2001). As such, FoxO6 likely differs in its regulation from FoxO1, FoxO3 and FoxO4, which could have important implications for aging, stem cell homeostasis and cancer. Additionally, FoxO transcriptional activity, protein stability and nuclear localization are regulated by many other factors, including SGK, CK1, DYRK, p300/CBP, CBP, Set9, SIRT1/2/3, HDAC3, CHIP, MDM2, SKP2, JNK, MST1, AMPK and EGLN2 (reviewed in Van der Heide et al., 2004; Yadav et al., 2018; Brown and Webb, 2018).

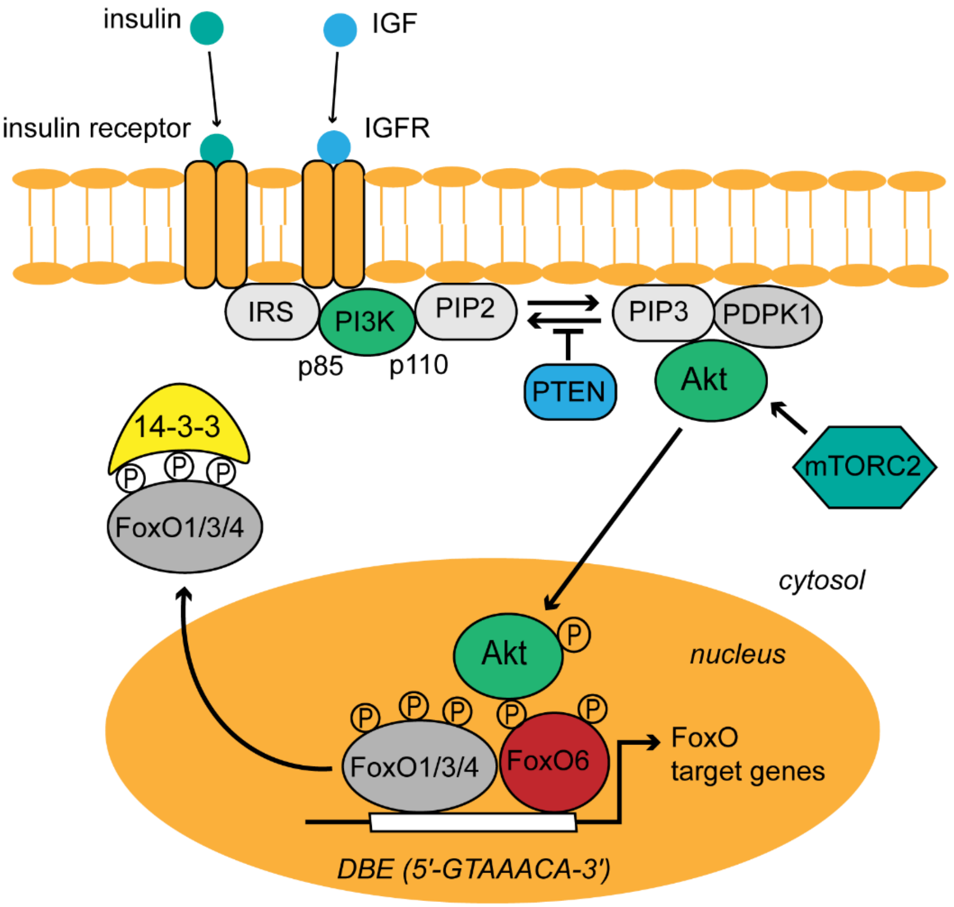


Figure 3. Insulin-PI3K-AKT-FOXO signalling
Binding of insulin and IGF to insulin and IGF receptors results in the phosphorylation of the IRS. In turn, this activates PI3K, a heterodimer of a p85 regulatory and p110 catalytic subunit. Kinase PI3K catalyses the conversion of membrane phospholipids PIP2 into PIP3, which interacts with kinase PDPK1, and activates downstream signalling component AKT. PTEN, in contrast, dephosphorylates PIP3 generating PIP2, and, therefore, inhibits AKT activity. Additionally, AKT, positioned at the membrane by binding to PIP3, can then be activated by mTORC2-dependent phosphorylation. Phosphorylated AKT then translocates to the nucleus where it phosphorylates transcription factors FOXO transcription factors at three sites in the case of FOXO1, FOXO3 and FOXO4 and at two sites in the case of FOXO6. This limits DNA-binding activity of FOXOs to target genes with a recognition motif (DBE) and prompts nuclear exit of FOXO1, FOXO3 and FOXO4 mediated by 14-3-3 scaffolding proteins but only minimally of FOXO6.

IGF, insulin-like growth factor; IRS, insulin response substrate; PI3K, phosphoinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PTEN, Phosphatase and Tensin Homolog deleted on Chromosome 10; PDK1, 3'-phosphoinositide-dependent kinase-1; AKT, Ak strain transforming; mTORC2, mechanistic target of rapamycin complex 2; FOXO, forkhead box O, DBEs; Daf-16 binding element; P, phosphate group.

Aims of this thesis

Quiescence of neural stem cells is key to help neural stem cell populations to persist during life but excessive quiescence impairs their ability to give rise to new neurons. Lifelong neurogenesis, therefore, depends on the balance of quiescence during aging. The necessity for regulation of neural stem cell homeostasis becomes even more apparent when considering brain tumours as neural stem cells are the main cell-of-origin. FoxO transcription factors are key regulators of neural stem cell dynamics but how FoxO6, as the most recently discovered isoform, controls neural stem cells remains unclear. Although FoxO3 is a known neural stem cell regulator, its mode of action is complex and likely involves regulation of circadian rhythms and epigenetic factors such as Hmgn2.

In this thesis, I aim to elucidate the role of FoxO6 in neural stem cell quiescence (**Chapter 2**) as well as in the oncogenic transformation of neural stem cells (**Chapter 3**). Furthermore, I will try to unravel the dynamic regulation of Hmgn2 expression (**Chapter 4**) as well as circadian regulation of neural stem cells (**Chapter 5**) and to determine whether FoxO3 governs this circadian control (**Chapter 6**).

Chapter contents

In **Chapter 2**, we investigated the function of FoxO6 in neural stem cell (NSC) maintenance throughout life. FoxO1, FoxO3 and FoxO4 are required to preserve NSC populations in the aging brain by promoting quiescence and self-renewal. It remains unknown, however, whether FoxO6, an isoform with different nucleo-cytosolic shuttling properties, is a regulator of NSC fates. To this end, we compared the number of stem cells and proliferation in FoxO6 mutant mice with wild-type mice at different ages. Surprisingly, FoxO6 mutant mice do not show the increased cell cycle entry observed in FoxO3 and FoxO1/3/4 combined mutant mice but show a reduced fraction of cycling NSCs. When we crossed FoxO6 and FoxO3 mutant mice with Nestin-GFP mice, we found that lack of FoxO3 and lack of FoxO6 differentially affected NSC proliferation. Subsequently, we isolated NSCs from FoxO6 mutant mice and wild-type mice and found that FoxO6-deficient NSCs were more quiescent *in vitro*. This was accompanied by a transcriptomic signature strongly resembling that of quiescent NSCs. When we cultured NSCs as neurospheres, we found that FoxO6-deficient NSCs formed a higher number of secondary neurospheres, indicative of an increased ability for NSC self-renewal. This also coincided with a quiescent-like transcriptome with increased expression of FoxO3 target genes. This corresponds to a reduced fraction of phosphorylated FoxO3 in NSCs lacking FoxO6, indicating increased FoxO3 activity in these cells. Our study identifies FoxO6 as a novel regulator of NSCs and raises the possibility that FoxO6's ability to restrain FoxO3-dependent quiescence may contribute to a healthy balance between NSC quiescence and activation.

In Chapter 3, we examined whether FoxO6 is involved in the oncogenic transformation of neural stem cells (NSCs) by analysing its transcriptome and the relationship between FoxO6 expression and glioma progression. Gliomas are malignant and aggressive brain tumors that essentially remain incurable. A subpopulation of cancer stem cells drive tumor recurrence. This population is characterized by a relatively quiescent, sustained self-renewal ability and distinct metabolic profiles. As such, FoxO6-deficient NSCs exhibit transcriptomic signatures of glioma stem cells. We found that genes encoding for key enzymes in the glycolysis pathway were upregulated in NSCs lacking FoxO6. The increase in the rate of glucose uptake and preferential production of lactate, known as the "Warburg effect" is common in cancer cells. We also found the upregulation of isocitrate dehydrogenases *Idh1* and *Idh2*. These genes are not only frequently mutated in glioma but also FoxO target genes that support the metabolic requirements of cancer cells. When we examined *FOXO6* expression in glioma patients, we found that low *FOXO6* expression is associated with poor clinical outcome in lower grade glioma but not in glioblastoma. Since *IDH1* and *IDH2* are frequently mutated in lower grade glioma but rarely in primary glioblastoma, the mutational status of the tumor could indicate a context-specific role of FOXO6. We suggest that FOXO6 can act as a tumor suppressor, both as an effector protein and by limiting FOXO-dependent expression of target genes promoting maintenance of cancer stem cells or target genes with oncogenic mutations such as *IDH1*.

In **Chapter 4**, we studied the spatial expression and regulation of *Hmgn2*, a gene that was strongly down-regulated in FoxO6-deficient neural stem cells (NSCs). *Hmgn2* encodes for a nucleosomal binding protein that epigenetically regulates stem cell identity. Its expression is highly enriched in intermediate neural progenitor cells but its role in the gene regulatory network remains unknown. We confirmed the expression dynamics of Hmgn2 in the NSC lineage *in vivo* and found the majority of proliferating neural stem/progenitor cells

(NSC/NPCs) expressing Hmgn2. During aging, the reduction of this population of HMGN2+ NSC/NPCs was mainly responsible for the reduction in total proliferating cells. Interestingly, we found that NSC/NPCs in the postnatal dentate gyrus were HMGN2-positive. As such, Hmgn2 is an epigenetic regulator that bridges the adult and developing neurogenic niches. To determine how *Hmgn2* expression is regulated in the NSC niches, we examined ChIP-seq datasets of FOXO3, ASCL1 and OLIG2 in NSCs. This showed occupancy of all three transcription factors at overlapping genomic locations within the *Hmgn2* intron. Therefore, we suggest that Hmgn2 has a central position within the NSC gene regulatory network in the developing, adult and aging brain.

In **Chapter 5**, we reviewed literature on circadian rhythms in NSC fates. Circadian rhythms are the day/night oscillations present in the physiology and metabolism of most organisms. A cellular circadian clock that drives cyclic gene expression is present in most mammalian cell types, including tissue-specific stem cells. Recent studies have demonstrated that neural stem cells, the source of new astrocytes, oligodendrocytes and neurons in the adult brain, to proliferate according to a circadian rhythm. Here, we reviewed emerging evidence for an important role of the circadian clock in neural stem cell maintenance. Importantly, loss of the circadian clock resulted in a loss of quiescence and an accelerated exhaustion of neural stem cell populations, leading to age-related defects. We propose that the NAD^+-dependent deacetylase SIRT1 limits NSC exhaustion by promotes the activity of the cellular circadian clock. As SIRT1 is a key sensor of energy metabolism, this is important for understanding the effects of metabolic challenges and circadian challenges, such as jet lags and shift work, on neural stem cell maintenance.

In **Chapter 6**, we examined the circadian function of FoxO3 in neural stem cell fates. Many metabolic processes and cell cycle transitions show daily or circadian rhythms generated by a cellular circadian clock. FoxO3 is at the interface of metabolism and the cell cycle and therefore an essential regulator of neural stem cells (NSCs), the source of new cells in the adult brain. Interestingly, FoxO3 controls circadian rhythms in the liver via directly regulating *Clock* expression. Whether FoxO3 regulates circadian rhythms in NSCs, however, remains unknown. To this end, we cultured NSCs and examined circadian oscillations of clock gene *Bmal1* expression following transfection with siRNA against FoxO3. This resulted in a clear loss of circadian rhythmicity, as well as a downregulation of *Clock* expression. Single cells transfected with

FoxO3 siRNA, however, did show circadian rhythmicity, indicating that FoxO3 is required for circadian synchronization of individual cellular clocks. Interestingly, we found rhythmic binding of circadian clock components to the *FoxO3* gene, indicating that FoxO3 itself is a clock-controlled gene and central to the circadian gene regulatory network. When we examined proliferation of hippocampal NSCs in FoxO3 mutant and wild type mice during the day, we observed a circadian oscillation in both genotypes. However, FoxO3 mutant mice showed a delayed peak in proliferation. Such a phase shift can result in misalignment between cell cycle dynamics and metabolism in NSCs. We suggest that FoxO3 preserves the NSC population by modulating the circadian clock and could be important in healthy aging despite metabolic or circadian challenges.

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