Sall1 regulates neuronal differentiation and progenitor cell maturation in the developing forebrain

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Sall1 is a zinc finger containing putative transcription factor that is robustly expressed during mammalian embryogenesis. In humans, the developmental disorder Townes Brocks Syndrome is associated with mutations in the SALL1 gene. Sall1-deficient animals die at birth due to kidney deficits; however, its function in the nervous system has not been characterized. During embryonic development Sall1 is expressed by cortical progenitor cells. In late embryonic and postnatal stages Sall1 is expressed by oligodendrocytes and glia, as well as regions of adult neurogenesis. In the absence of Sall1 forebrain derived structures, the cerebral cortex and olfactory bulbs, were specifically decreased in size, while no gross alterations in midbrain development was observed. This study investigated the cellular mechanisms of Sall1 function in the developing cortex. Alterations in progenitor cell number and the rate of neuronal differentiation were observed during cortical development in Sall1-mutant animals compared to controls. In the absence of Sall1 more cells are committed to early-born cortical structures, at the expense of the progenitor population. In addition, from mid-neurogenesis fewer cells are committed to later-born structures. Together, these findings suggest that early in development Sall1 promotes a progenitor state, and from mid-neurogenesis Sall1 promotes a neural fate. Furthermore, I propose that Sall1 regulates the

transition from an early cortical progenitor cell to an intermediate progenitor cell. These findings suggest that Sall1 regulates cortical neurogenesis and progenitor cell maturation in the developing cortex.

Sall1 is also expressed by peripheral and central components of the developing olfactory system. Alterations in neurogenesis and mitral cell production were observed in Sall1-deficient olfactory bulbs. In addition, the olfactory nerve failed to extend past the ventral-medial region of the olfactory bulb in Sall1-mutant animals. Intrinsic patterns of neurogenesis were observed during olfactory development in control animals and in Sall1-mutant animals, these patterns of neurogenesis were disrupted. These findings suggest a role for Sall1 in regulating neuronal differentiation and maturation in the developing olfactory system. Together, these findings suggest a conserved role for Sall1 in regulating neurogenesis and cellular maturation in the developing cortex and olfactory bulb.

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1.0 INTRODUCTION

The adult cortex is a six layered structure that controls complex functions, including motor coordination, auditory, visual, and somatosensory processing as well as cognition (reviewed in Rosner, 1970). Appropriate regulation of cell number, cell type specification, laminar position, and circuit formation is essential for the normal functioning of the mature nervous system. Alterations in cortical development can lead to a variety of gross cortical malformations and psychiatric disorders including lissencephaly, periventricular heterotopia, microcephaly, epilepsy, autism, and schizophrenia (Arnold, 1999; Schwartzkroin and Walsh, 2000; Pilz et al., 2002; Polleux and Lauder, 2004; Lian and Sheen, 2006). During development, progenitor cells proliferate and differentiate in a temporally and spatially regulated manner to produce the diverse population of neural cell types that constitute the mature cortex. Progenitor cells undergo multiple decisions during development. They must first decide when to proliferate and what type of division to undergo; a proliferative division will produce two progenitor cells, a differentiative division will produce a differentiated cell and a progenitor cell, while a terminal differentiative division will produce two differentiated cells. If progenitor cells undergo a differentiative division, the fate of the progeny and the laminar position of the new cell within the nervous system must be determined. Intrinsic and extrinsic factors influence these decisions (reviewed in Pearson and Doe, 2004). Identifying the mechanisms that regulate progenitor cell decisions

during development is essential for understanding how the cerebral cortex is normally assembled and how disruption of these events can lead to anatomical and behavioural abnormalities.

This study investigates the role of the Sall1 (Spalt1) gene in cortical development. Sall1 is a zinc-finger containing putative transcription factor that is highly expressed in the developing nervous system and peripheral organs (Kuhnlein et al., 1994; Kohlhase et al., 1996; Ott et al., 1996; Kohlhase et al., 1999a; Buck et al., 2000; Kohlhase et al., 2000; Buck et al., 2001; Ott et al., 2001; Kohlhase et al., 2002a). Mutation of Sall1 in humans results in the developmental disorder Townes-Brocks syndrome, characterized by imperforate anus, as well as ear, limb, renal, and auditory abnormalities, and, to a lesser extent, cognitive deficits (Townes and Brocks, 1972; Cameron et al., 1991; Kohlhase et al., 1998; Doray et al., 1999; Surka et al., 2001; Botzenhart et al., 2005; Borozdin et al., 2006; Botzenhart et al., 2007). This study suggests a role for Sall1 in regulating neuronal differentiation and progenitor cell maturation in the developing cerebral cortex and olfactory bulb. Characterization of the role of Sall1 in progenitor cell regulation will aid in the understanding of cognitive deficits associated with Townes-Brocks syndrome and other psychiatric disorders associated with neural developmental abnormalities.

1.1 CORTICAL DEVELOPMENT

1.1.1 Cortical patterning during development

The nervous system arises from a region of specialized tissue, the neural plate, which is specified during gastrulation. The neural plate undergoes a series of morphological changes, in a process

termed neurulation, resulting in formation of the neural tube (reviewed in (Colas and Schoenwolf, 2001)). It is hypothesized that a series of graded morphogenetic signals result in the segmentation of the neural tube into four domains, the forebrain, midbrain, hindbrain, and spinal cord (reviewed in (Foley and Stern, 2001)). The developing forebrain (or telencephalon) arises from the anterior segment of the neural tube, the anterior neural ridge. The dorsal forebrain gives rise to cells destined for the cortex and olfactory bulb, and the ventral forebrain gives rise to cells destined for the basal ganglia, cortex and olfactory bulb.

The adult cortex is a complex structure that contains spatially separated populations of cells that define discrete cortical areas along the rostral-caudal axis. These areas are histologically, molecularly, and functionally distinct; e.g. rostral areas control motor functions, whereas caudal areas control visual and somatosensory functions. Signaling centers established early in cortical development are hypothesized to impart regional cortical identity by inducing graded expression of transcription factors in progenitor cells, such as Emx2 and Pax6 (Sur and Rubenstein, 2005; Rash and Grove, 2006). Fibroblast growth factor 8 (FGF8) expression at the anterior neural ridge is hypothesized to inhibit expression of Emx2, inducing a low rostral to high caudal gradient of Emx2 expression and establishing the rostral-caudal axis (Fukuchi-Shimogori and Grove, 2003; Garel et al., 2003). Alterations in FGF8 or Emx2 expression induce expansion of rostral areas at the expense of caudal cortical areas (Bishop et al., 2000; Fukuchi-Shimogori and Grove, 2003; Garel et al., 2003). Pax6 is expressed in a high rostral to low caudal gradient (Stoykova and Gruss, 1994). In Pax6 mutant mice caudal areas are expanded at the expense of rostral areas (Bishop et al., 2000). Furthermore, an interaction between Pax6 and Emx2 has been proposed whereby expression of Emx2 is required to establish a Pax6 gradient and vice versa

(Muzio et al., 2002). In the absence of Pax6 or Emx2 alterations in progenitor cell proliferation and neuronal differentiation are observed (Cecchi et al., 1999; Estivill-Torrus et al., 2002; Bishop et al., 2003; Quinn et al., 2007). Taken together these findings suggest that Pax6 and Emx2 regulate rostral-caudal cortical patterning in an opposing manner and that expression of these genes is critical for progenitor cell regulation.

Similar to their role in establishing a rostral-caudal identity, signaling centers and key transcription factors are hypothesized to impart a dorsal-ventral telencephalic identity. Expression of Wnt and Bone morphogenetic proteins (BMP) family members in the dorsal midline is hypothesized to impart a dorsal cortical identity (Tole et al., 2000). Pax6 expression in the dorsal cortex is required to establish a dorsal cortical identity, and in the absence of Pax6 molecular makers normally restricted to the ventral telencephalon extend into the dorsal cortex (Toresson et al., 2000; Yun et al., 2001). Gsh2 expression in the ventral telencephalon has been implicated in imparting a ventral identity. In Gsh2 mutant mice, expression of dorsal cortical markers extends into the ventral telencephalon (Toresson et al., 2000; Yun et al., 2001). Sonic hedgehog expression in the medial ganglionic eminence is required to induce expression of the transcription factor Nkx2.1, which imparts a ventral identity on the progenitors in the medial ganglionic eminence (Xu et al., 2005). Thus, expression of the transcription factors Pax6, Gsh2, and Nkx2.1 is required to establish dorsal and ventral cortical identities. These findings suggest that establishment of signaling centers is critical to induce transcription factor expression in progenitor cells and to impart dorsal-ventral regional identity during early development.

1.1.2 Neural cell type specification in the developing cortex

The mature cortex is composed of three cell types, neurons, oligodendrocytes, and astrocytes, and generation of these cell types is regulated in a temporal and spatial manner. Neurons are primarily born from E10.5 to E17.5, and production of glial cell types, astrocytes and oligodendrocytes, is initiated at the end of the neurogenic period (Bayer and Altman, 1991; Price et al., 1997; Takahashi et al., 1999; Levers et al., 2001). Inhibitory and excitatory neurons are derived from spatially segregated progenitor cell populations. Excitatory neurons arise from dorsal progenitor cells and migrate radially to their laminar position in the cortical plate (Gorski et al., 2002; Gupta et al., 2002). Interneurons arise ventrally and tangentially migrate to their laminar position in the cortex (Anderson et al., 1997; Anderson et al., 2002). A ventral to dorsal, rostral to caudal gradient of neurogenesis also influences cell type generation (Bayer and Altman, 1991). Furthermore, excitatory neurons and astrocytes are born from progenitor cells in the dorsal cortex in a temporally regulated manner. Interestingly, studies of cultured progenitor cells determined that early cortical progenitor cells have the potential to produce neurons, astrocytes and oligodendrocytes, suggesting the presence of a common precursor progenitor cell with the potential to generate all three neuronal cell types of the cortex (Davis and Temple, 1994; Qian et al., 1997; Qian et al., 1998; Qian et al., 2000).

Cortical neurogenesis initiates at E10.5 when a subpopulation of progenitor cells in the dorsal cortex differentiate, producing excitatory neurons primarily destined for the preplate. Cells destined for the preplate are born from E10.5 to E13.5 (Figure 1) (Price et al., 1997; Sheppard and Pearlman, 1997). Previous studies have identified that the transcription factors Foxg1, p73, Coup-TFI, Emx1/2, and Tbr1 are critical regulators of preplate neurogenesis and cell fate

(Yoshida et al., 1997; Hevner et al., 2001; Chen et al., 2002; Meyer et al., 2002; Shinozaki et al., 2002; Bishop et al., 2003; Hanashima et al., 2004; Meyer et al., 2004). At ~E13.5, the fate of newly generated neurons changes to a cortical plate fate (Price et al., 1997; Levers et al., 2001). These neurons destined for the cortical plate radially migrate away from the progenitor population and split the preplate into two structures, the marginal zone, superficial, and the subplate, deep (Figure 1) (Marin-Padilla, 1978). The cortical plate is a laminar structure containing six layers of cells that exhibit distinct molecular characteristics, which is patterned in an inside out manner with deep layers born before more superficial layers (Figure 1) (Angevine and Sidman, 1961; Hevner et al., 2003; Molyneaux et al., 2007). The transcription factors Fezl and Coup-TFI have been implicated in the generation and survival of cortical layer V and IV neurons, respectively (Zhou et al., 1999; Chen et al., 2005; Molyneaux et al., 2005). The secreted protein Reelin and its downstream targets have been shown to influence the migration of cortical plate neurons and subsequently their deep to superficial position within the cortical plate (reviewed in Gupta et al., 2002).

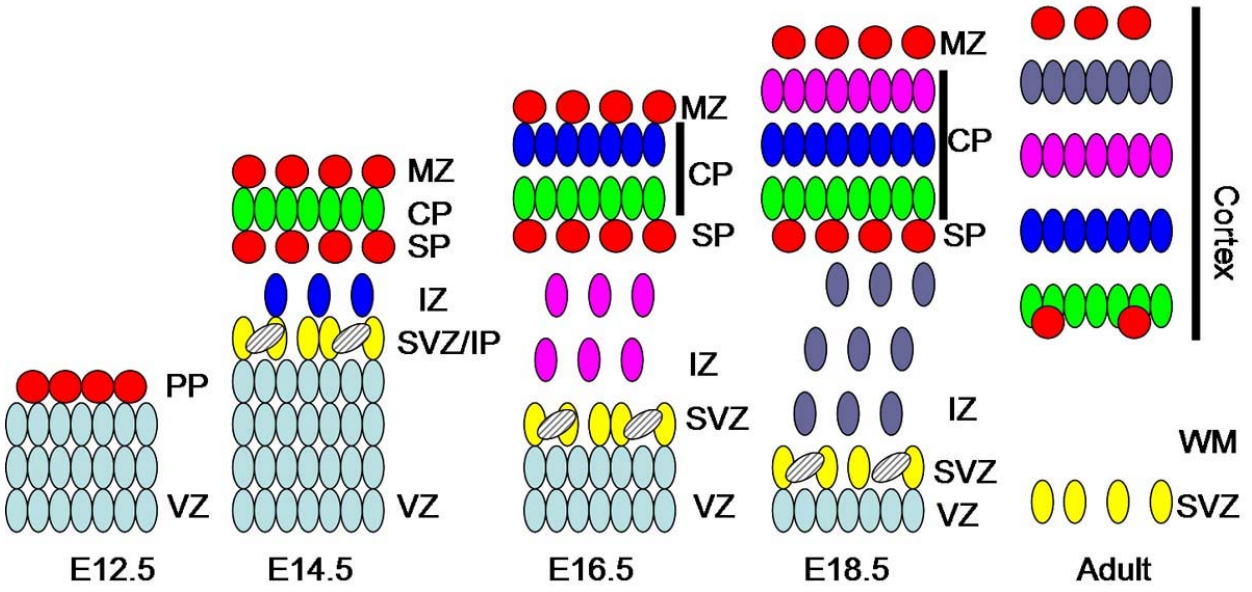

Figure 1. Corticogenesis in the dorsal cortex.

Cortical neurons are generated from E10.5 to E18.5 in the mouse. Progenitor cells, located in the ventricular zone (light blue cells), proliferate and differentiate to populate the cortex. The first structure generated during cortical development is the preplate (red cells). Waves of migrating neurons destined for the cortical plate, split the preplate by E14.5 to form the marginal zone, superficial, and the subplate, deep. The cortical plate forms in an inside out manner, with inner-most layers populated before the outer ones, ultimately, giving rise to the six layered structure of the adult cortex. At E14.5 a secondary proliferative population arises, the subventricular zone. The subventricular zone is a mixed population containing a newly characterized progenitor population, intermediate progenitor cells (yellow cells), as well as migrating neurons from the ventral telencephalon (stripped cells). VZ: ventricular zone; PP: preplate; SVZ: subventricular zone; IP: intermediate progenitor cell; IZ: intermediate zone; SP: subplate; CP: cortical plate; MZ: marginal zone; WM: white matter.

Progenitor populations in the ventral telencephalon give rise to inhibitory interneurons destined for the basal ganglia, olfactory bulb, and cortex. Cells born in the ventral telencephalon tangentially migrate into the dorsal cortex (Anderson et al., 1997; Anderson et al., 2002). Recent lineage analysis has determined that molecularly distinct interneuron populations arise from progenitor populations that can be distinguished early in neurogenesis by their transcription factor expression (Nery et al., 2002; Stenman et al., 2003; Xu et al., 2004; Butt et al., 2005;

Miyoshi et al., 2007). For example, in the lateral ganglionic eminence, Dlx1/Isl1-positive cells give rise to cortical interneurons, whereas Dlx1/ER81-positive cells give rise to interneurons destined for the olfactory bulb (Stenman et al., 2003). Parvalbumin- and Somatostatin-positive interneurons arise from Nkx2.1-positive progenitor cells in the medial ganglionic eminence, whereas Calretinin-positive interneurons arise from the caudal ganglionic eminence (Xu et al., 2004; Butt et al., 2005). Temporal specification of interneuron populations has also been suggested, as cells derived from the caudal ganglionic eminence give rise to Somatostatin- and Parvalbumin-positive cells prior to E13.5, whereas Calretinin-positive cells are born from E14.5 (Nery et al., 2002; Xu et al., 2004). Some studies have suggested that similar to excitatory neuronal populations, interneurons are born in an inside out manner, with interneurons destined for deep cortical layers born before those destined for superficial cortical layers. These studies have indicated that Reelin signaling influences laminar position of interneuron populations (Fairen et al., 1986; Yabut et al., 2005; Miyoshi et al., 2007). However, recent evidence indicates that Calretinin-positive populations may be born in an outside-in manner (Rymar and Sadikot, 2007), which suggests that distinct mechanisms may regulate cortical layer position of independent interneuron populations.

Oligodendrocytes are the myelin-producing cells in the cortex. Oligodendrocytes have distinct spatial and temporal origins. Early born oligodendrocytes arise from the preoptic area and the ganglionic eminences; sonic hedgehog signaling and the transcription factor Mash1 are required for their specification (Spassky et al., 1998; Tekki-Kessaris et al., 2001; Kessaris et al., 2006; Nakahira et al., 2006; Parras et al., 2007). A late-born oligodendrocyte population is derived from dorsal progenitor cells; FGF2 signaling is required for their specification (Gorski et al.,

2002; Kessaris et al., 2006; Naruse et al., 2006; Yue et al., 2006). Expression of the cyclin dependent kinase inhibitors $p27^{kip1}$ and $p57^{kip2}$ in the optic nerve and cortex intrinsically regulate oligodendrocyte differentiation through a counting mechanism whereby protein levels increase during successive divisions and oligodendrocyte differentiation occurs when a critical concentration of inhibitor is reached (Gao et al., 1997; Dugas et al., 2007). Astrocytes play multiple roles in the central nervous system, including regulation of synaptogenesis and maintenance of the blood brain barrier (reviewed in He and Sun, 2007). Astrocytes are derived from dorsal progenitor cells that initially give rise to excitatory neurons (Voigt, 1989; Mission et al., 1991; Gorski et al., 2002). Several signaling pathways have been implicated in regulating the onset of gliogenesis, including BMP, ciliary neurotrophic factor/ leukemia inhibitory factor, and epidermal growth factor (EGF)/FGF signaling (Bonni et al., 1997; Mabie et al., 1997; Qian et al., 1997; Mehler et al., 2000; Qian et al., 2000). Basic helix-loop-helix proteins are also implicated in regulating the transition to glial cell production. Early in development, high levels of proneural genes sequester proastroglia factors and repress the progliogenic JAK-STAT pathway (Sun et al., 2001). Furthermore, other studies have shown that gliogenic promoters are methylated early in development but demethylated late in development, allowing gliogenic promoters to respond to extrinsic cues that induce gliogenesis (Takizawa et al., 2001; Namihira et al., 2004; Fan et al., 2005). These findings suggest that neurons, astrocytes, and oligodendrocytes have distinct temporal and spatial origins and complex signaling mechanisms regulate their generation.

1.1.3 Molecular characteristics of progenitor cells

The developing telencephalon is initially composed of a relatively homogenous population of multipotent progenitor cells termed neural epithelial progenitor cells that are arranged in a pseudostratified manner and derived from the neural tube (reviewed in Rakic, 2003). These neural epithelial cells are hypothesized to give rise to radial glia progenitor cells (Alvarez-Buylla et al., 2001). Radial glia are bipolar cells with a distinctive morphology; their cell nucleus is located adjacent to the ventricular surface with a short radial process extending towards the ventricular wall and a long radial process extending to the pial surface (Chanas-Sacre et al., 2000). Radial glia cells were traditionally thought to be supporting cells in the developing cortex, along which newborn neurons migrate to their laminar position (reviewed in Rakic, 2003). However, recent studies have shown that radial glia are progenitor cells that have the capacity to produce neurons and glia and that these progenitors are a primary source of neurons during development (Malatesta et al., 2000; Hartfuss et al., 2001; Noctor et al., 2001; Noctor et al., 2002; Malatesta et al., 2003; Anthony et al., 2004). At the end of neurogenesis radial glia cells transform into astrocytes (Voigt, 1989; Mission et al., 1991).

Radial glia cells express molecular markers characteristic of mature glia cells (glutamate-aspartate transporter (GLAST)/ brain lipid binding protein (BLBp)), as well as markers of progenitor cells (RC2/Nestin). During neurogenesis the molecular characteristics and cellular properties of radial glia cells change (Hartfuss et al., 2001). Hartfuss et al., distinguished subpopulations of radial glia cells based on expression of molecular markers, and observed that during neurogenesis the contribution of these subpopulations to the progenitor population changes (Hartfuss et al., 2001). Interestingly, in contrast to earlier studies (Cai et al., 1997),

Hartfuss et al. observed differences in the rate of proliferation of these subpopulations, suggesting that subpopulations of progenitor cells differ with respect to their cell cycle properties. These findings suggest that distinct progenitor populations are present in the cortex during development that differ with respect to molecular characteristic and cell cycle parameters, and over time the molecular characteristics of the progenitor population changes. However, the contribution of these molecular changes to cortical development is not understood.

1.1.4 Progenitor cell cycle kinetics during neurogenesis

Progenitor division type, cell cycle length, and cell fate change during development. Temporal and spatial regulation of these changes has been extensively studied for the neurogenic period, during which time progenitor cells undergo eleven cell cycles (Takahashi et al., 1995a). Early in development, an expansion of the progenitor population occurs, whereby the majority of progenitor divisions result in a proliferative division, producing two progenitor cells (Figure 2). At this age the proliferative fraction of the progenitor population (P – measure of the number of proliferating cells that re-enter the cell cycle) is 1, whereas the differentiating fraction (Q – measure of the number of proliferating cells that exit the cell cycle) is 0 (Takahashi et al., 1994). As neurogenesis proceeds a subset of progenitor cells switch to a neurogenic division, producing one progenitor cell and one neuron (Figure 2). Upregulation of Tis21, an inhibitor of G1 progression, is associated with this switch from a proliferative to neurogenic division. Furthermore, selective lengthening of the cell cycle is observed in neurogenic progenitors (Iacopetti et al., 1999; Calegari and Huttner, 2003; Calegari et al., 2005). E14.5 is a critical age in the neurogenic period. Prior to E14.5, $Q<0.5$ (Takahashi et al., 1995a), which indicats that the progenitor population continues to expand. However, from E14.5, $Q>0.5$ (Takahashi et al.,

1995a). This change in Q is associated with a switch to terminal divisions, producing two differentiated cells, and depletion of the progenitor population (Figure 2), as P approaches 0 and Q approaches 1 (Takahashi et al., 1994, 1995b; Noctor et al., 2004).

At E14.5, a second proliferative population arises in the cortex, the subventricular zone, which contains intermediate progenitor cells derived from the dorsal ventricular zone and migrating interneuron populations derived from the ventral telencephalon (Figures 1,2) (BoulderCommittee, 1970; Marin and Rubenstein, 2001; Haubensak et al., 2004; Noctor et al., 2004). Recent data suggest that the majority of neurons in the cortical plate are produced via neurogenic divisions of intermediate progenitor cells (Figure 2) (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Intermediate progenitor cells are characterized by abventricular mitosis (where mitosis occurs away from the ventricular surface), and Tbr2 has recently been identified as a molecular marker of intermediate progenitor cells from E14.5 (Englund et al., 2005). While early studies suggested similar cell cycle kinetics for cells in the ventricular and subventricular zone populations, recent analysis of the rate of proliferation of molecularly defined populations have challenged this (Takahashi et al., 1993; Cai et al., 1997; Hartfuss et al., 2001; Calegari and Huttner, 2003; Calegari et al., 2005). Taken together these studies have identified that intermediate progenitor cells produce a large number of cortical neurons; however, molecular mechanisms regulating specification, maintenance and fate of this population have not been identified.

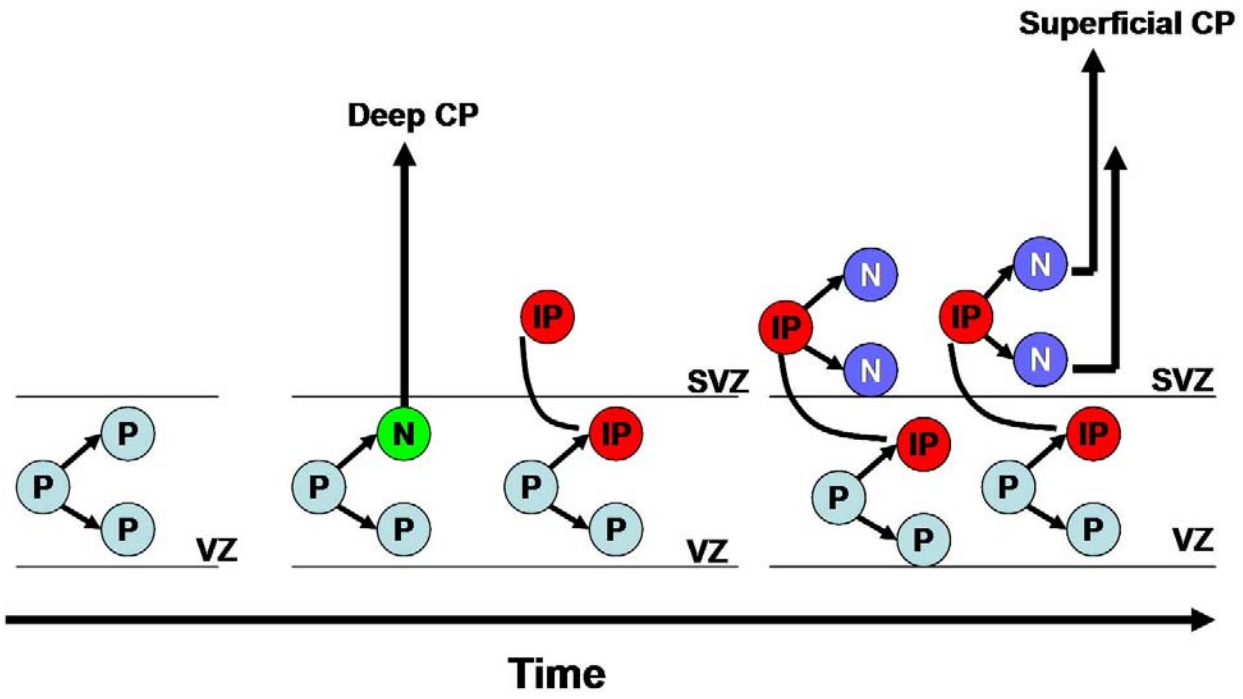

Figure 2. Progenitor cell fate changes during development

During development the progenitor cell division type and progeny fate changes. Early in development an expansion of the progenitor population occurs and the majority of progenitor cells undergo proliferative divisions resulting in progenitor/progenitor divisions. At the onset of neurogenesis neurogenic division are observed resulting in progenitor/neuron progeny. It is hypothesized that these neurons are destined for deep cortical layers. At midneurogenesis, a subset of ventricular zone progenitors undergo a different division type, producing intermediate progenitor cells, which seed the second proliferative population appears, the subventricular zone. Intermediate progenitor cells predominately undergo terminal neurogenic divisions within the subventricular zone, and it is hypothesized that they produce neurons destined for the superficial cortical plate. P: progenitor cell; VZ: ventricular zone; N: neuron; CP: cortical plate; IP: intermediate zone; SVZ: subventricular zone.

1.1.5 Molecular regulation of neuronal differentiation

In both the dorsal and ventral telencephalon basic helix loop helix proteins are implicated in imparting a neuronal identity. The Notch signaling pathway maintains cells in a progenitor like state. Notch is a transmembrane protein that is activated by the ligands Delta, Jagged and Serrate (Artavanis-Tsakonas et al., 1995). Binding of Notch to one of its ligands results in cleavage of

Notch to release the intracellular domain of Notch, which translocates to the nucleus where it forms a complex with RBP-J (Jarriault et al., 1995; Tamura et al., 1995; Kopan et al., 1996; Kidd et al., 1998; Schroeter et al., 1998). This complex activates transcription of the basic helix loop helix proteins Hes1 and Hes5, which are repressors of proneural genes (Ohtsuka et al., 1999). Hes1 and Hes5 inhibit the transcription of proneural genes by interacting with the corepressor Groucho and recruiting histone deacetylase to the promoter of proneural genes (Iso et al., 2001; Yao et al., 2001; Barolo et al., 2002). Hes1 and Hes5 can also form a complex with co-activators of proneural proteins, preventing the co-activator from forming functional complexes with proneural proteins (Akazawa et al., 1992; Sasai et al., 1992).

The basic helix loop helix proteins Neurogenin1/2 (Ngn1/2) and Mash1 have been identified as promoters of neural fate in dorsal and ventral telencephalon respectively (Fode et al., 2000; Parras et al., 2002). It is hypothesized that a transient increase in expression of Ngn1/2 and Mash1 in progenitor cells induces neuronal differentiation. Although the mechanism of action of this transient increase is not well characterized, studies have suggested that it may in part be mediated by expression of another member of the Hes family, Hes6, which binds to Hes1 thereby inhibiting Hes1 function and resulting in neuronal differentiation (Bae et al., 2000; Koyano-Nakagawa et al., 2000). Alternatively, unequal inheritance in daughter cells of apical components of the plasma membrane, including inhibitors of Notch signaling, may regulate the progenitor to neuron fate transition (Zhong et al., 1996; Shen et al., 2002; Gotz and Huttner, 2005). These studies have implicated basic helix loop helix proteins as critical regulators of neuronal differentiation.

1.1.6 Laminar specification in the dorsal cortex

How is cell type identity and laminar fate specified in the cortex? Examination of progenitor cells cultured in isolation demonstrated that during successive divisions a restriction in the potential of progenitors occurs; neurons with a deep fate were produced before neurons with a superficial fate (Shen et al., 2006). However, the molecular mechanisms that induce cell type specification and laminar fate have not been well characterized, and several hypotheses have been proposed. Transplantation studies indicated that environmental signals at the time of entry to final mitosis are critical in determining laminar fate, which suggests that extrinsic cues influence laminar fate (McConnell and Kaznowski, 1991). Expression studies have led to the hypothesis that progenitor cells switch on layer specific markers in the progenitor population prior to migration to the cortex; layer V Otx1-positive neurons arise from an Otx1-positive population in the ventricular zone and Svet/Cux-positive upper cortical layer cells arise predominately from the Svet/Cux expression population in the subventricular zone (Tarabykin et al., 2001; Nieto et al., 2004). During the neurogenic period, progenitor cells undergo 11 cell cycles, and previous studies have suggested that laminar position is determined by cell cycle number. Neurons destined for deep cortical layers (layers VI, V) are born during cell cycles 1-6, and neurons destined for superficial cortical layers (layers IV, III/II) are born during cell cycles 7-11 (Takahashi et al., 1995a; Takahashi et al., 1999; Caviness et al., 2003). During this developmental period, a progressive lengthening of the cell cycle is also observed (Takahashi et al., 1995a; Takahashi et al., 1999; Caviness et al., 2003).

Direct evidence for a role of the cell cycle in regulating cell fate has come from manipulation of p27 expression in cortical progenitor cells. p27 is a cell cycle inhibitor, and in the absence of p27

decreased neuronal output during early cell cycles lead to a decrease in the number of neurons committed to deep cortical layers (Caviness et al., 2003). Consequently, late progenitor populations were increased in number, and more cells were committed to the late-born superficial cortical layers (Caviness et al., 2003). These data also suggest that differentiated cell number does not influence the switch from deep to superficial fate, i.e. that the switch from deep to superficial does not occurs when an appropriate number of cells are committed to a deep fate. Analysis of the Tlx genetic mutant suggests that progressive lengthening of the cell cycle, rather than simply cell cycle number alone, can influence the switch from making deep to superficial cortical neurons (Roy et al., 2004). Other studies have suggested that a combination of extrinsic signals, which change over time, accompanied by a progressive restriction of developmental potential of progenitor cells, induce a change from deep to superficial cortical fate (Bohner et al., 1997). Taken together, these studies suggest that both cell cycle length and extrinsic cues influence neuronal fate in dorsal progenitors. However, molecular mechanisms regulating cell type specification are not well characterized.

1.1.7 Consequences of disruption of cortical development

Human neural developmental disorders have been linked to alterations in progenitor cell proliferation, cell migration, and synapse formation. Through molecular genetic analyses, functional roles for genes associated with disease pathology have been identified, leading to a better understanding of disease etiology. Microcephaly is a developmental disorder in which alterations in neural cell proliferation are observed, resulting in a decreased brain size and mental retardation (Woods, 2004). Mutations in the abnormal spindle-like microcephaly-associated (ASPM) gene have been implicated in this disease (Bond et al., 2002; Bond et al., 2003). ASPM

is expressed by cortical neural progenitor cells, and downregulation of ASPM is associated with the transition from proliferative to neurogenic divisions (Kouprina et al., 2005; Fish et al., 2006), which suggests that disease pathology may result from premature transition from a proliferative to differentiative division. Bilateral frontoparietal polymicrogyria is a disorder associated with altered cortical structure and small gyri (Piao et al., 2002; Piao and Walsh, 2004). Patients exhibit mental retardation, developmental delay, and seizures (Piao et al., 2002; Piao et al., 2004). Mutations in the G protein–coupled receptor 56 (GPR56) are associated with this disorder (Piao et al., 2004). GPR56 is expressed by cortical progenitors suggesting a role in regulating progenitor cell proliferation or patterning, although the mechanism of action of GPR56 has not been identified to date (Piao and Walsh, 2004). Lissencephaly is a neuronal migration disorder characterized by gyri alterations, thickened cortex, with altered cortical layering, and heterotopias, resulting in mental retardation, epilepsy, and cerebral palsy (reviewed in Cardoso et al., 2002). Reelin and the downstream components of this signaling pathway have been implicated in this disorder, and molecular genetic analysis has identified a role for these genes in multiple aspects of cell migration, including cell motility, somal translocation, dynein function, and microtubule polymerization (Kato and Dobyns, 2003). Alterations in cell number and synaptic connections have been observed in patients with autism, schizophrenia, and epilepsy, however, the molecular mechanisms underlying these alterations are not well established (Lewis and Levitt, 2002; Courchesne et al., 2003; Guerrini et al., 2003; Levitt et al., 2004; Courchesne and Pierce, 2005; Geschwind and Levitt, 2007). These findings highlight the importance of progenitor cell regulation and neuronal migration in appropriate formation of the cortex.

1.2 THE SALL GENE FAMILY

The Sall gene was originally identified in Drosophila and described as a homeotic gene that promoted head and tail development (Jurgens, 1988). Four mammalian members of this gene family have been identified, Sall1, Sall2, Sall3, and Sall4 (Kohlhase et al., 1996; Ott et al., 1996; Kohlhase et al., 1999a; Buck et al., 2000; Kohlhase et al., 2000; Ott et al., 2001; Al-Baradie et al., 2002; Kohlhase et al., 2002a; Kohlhase et al., 2002b). These genes are conserved both within and between species (Ott et al., 1996; Ott et al., 2001; Camp et al., 2003; Sweetman and Munsterberg, 2006). In addition, overlapping patterns of expression are observed during embryonic development (Kohlhase et al., 1996; Ott et al., 1996; Buck et al., 2000; Buck et al., 2001; Ott et al., 2001; Kohlhase et al., 2002a; Camp et al., 2003; Sato et al., 2003; Barembaum and Bronner-Fraser, 2004; Elling et al., 2006). Distinct developmental disorders with overlapping phenotypes are associated with mutation or deletion of members of this gene family in humans (Kohlhase et al., 1998; Kohlhase et al., 1999a; Al-Baradie et al., 2002; Kohlhase et al., 2002b). Early studies focused on the expression pattern of these genes and the mutational spectrum associated with disruption of these genes in humans (Kohlhase et al., 1996; Ott et al., 1996; Kohlhase et al., 1998; Kohlhase et al., 1999a; Buck et al., 2000; Kohlhase et al., 2000; Buck et al., 2001; Ott et al., 2001; Al-Baradie et al., 2002; Kohlhase et al., 2002a; Kohlhase et al., 2002b; Camp et al., 2003; Kohlhase et al., 2003). However, more recent studies have begun to elucidate the molecular mechanisms of action of this gene family (Netzer et al., 2001; Cantera et al., 2002; Kiefer et al., 2002; Netzer et al., 2002; Kiefer et al., 2003; Sweetman et al., 2003; Sato et al., 2004; Bohm et al., 2006; Lauberth and Rauchman, 2006; Netzer et al., 2006; Sakaki-Yumoto et al., 2006; Wu et al., 2006; Yamashita et al., 2007). These studies have identified that Sall family members are important developmental regulators.

1.2.1 Expression of members of the Sall gene family

Members of the Sall gene family have been identified in diverse organisms from Drosophila to humans (Jurgens, 1988; Kuhnlein et al., 1994; Kohlhase et al., 1996; Ott et al., 1996; Kohlhase et al., 1999a; Farrell and Munsterberg, 2000; Kohlhase et al., 2000; Buck et al., 2001; Ott et al., 2001; Al-Baradie et al., 2002; Kohlhase et al., 2002a; Kohlhase et al., 2002b; Camp et al., 2003; Toker et al., 2003; Onai et al., 2004). In the mouse, temporal expression of members of the Sall gene family is observed during development. Sall4 is expressed from the 8-cell embryo stage (Elling et al., 2006). Expression of Sall1 and Sall3 is not observed until E7 (Buck et al., 2001; Ott et al., 2001), and Sall2 is expressed from E8.5 (Kohlhase et al., 2000). Expression of Sall1, Sall3, and Sall4 is observed in the developing neural tube; however, in contrast to Sall1 and Sall3, Sall4 is not expressed by the midbrain/hindbrain boundary (Ott et al., 1996; Buck et al., 2000; Ott et al., 2001; Elling et al., 2006; Sakaki-Yumoto et al., 2006; Warren et al., 2007). Sall family members are temporally activated in dorsal cortical progenitor cells; Sall1 and Sall4 expression is observed from E9.5/E10.5, but Sall3 expression does not extend into this population until E13.5 (Ott et al., 1996; Ott et al., 2001; Sakaki-Yumoto et al., 2006; Warren et al., 2007). Sall1, Sall2, and Sall3 are also expressed by progenitor populations in the spinal cord at E11.5/E12.5 (Ott et al., 2001; Sato et al., 2003). Outside the central nervous system Sall gene expression is predominately observed in the urogenitial ridge, tail, and limb buds (Ott et al., 1996; Ott et al., 2001; Sato et al., 2003; Elling et al., 2006; Sakaki-Yumoto et al., 2006; Warren et al., 2007). Expression of the Sall gene family in the central nervous system and peripheral organs is conserved across species from Xenopus to humans (Hollemann et al., 1996; Kohlhase et al., 1996; Onuma et al., 1999; Farrell and Munsterberg, 2000; Farrell et al., 2001; Kohlhase et al., 2002a; Camp et al., 2003; Kohlhase et al., 2003; Barembaum and Bronner-Fraser, 2004).

These findings indicate that the Sall family is widely expressed throughout development. In the adult human and mouse, Sall1, Sall2, and Sall3 are expressed in the brain, ovary, testis, and kidney (Kohlhase et al., 1996; Ott et al., 1996; Kohlhase et al., 1999; Kohlhase et al., 2000; Kohlhase et al., 2002a; Ma et al., 2002). Sall4 expression in the adult is only observed in the testis (Kohlhase et al., 2002a).

Thus, members of the Sall gene family are expressed during development in a temporally and spatially regulated manner. Expression of Sall family members continues in the adult. These findings suggest that members of the Sall gene family may be important regulators of development.

1.2.2 Molecular characterization of the Sall gene family

The Sall gene encodes a zinc finger protein comprised of three double C_2H_2 zinc finger pairs with an additional single C_2H_2 zinc finger associated with the second pair (Figure 3) (Kuhnlein et al., 1994). Several mammalian members of this gene family have an additional pair of zinc fingers (*, Figure 3). In addition, a single C_2HC zinc finger is observed at the N-terminus in mammalian members of this family, which is absent in Drosophila (white oval, Figure 3). Sall has proline and glutamine rich regions at the C-terminus characteristic of known activator sequences, and alanine and proline rich regions also at the C-terminus characteristic of known repressor sequences (Courey and Tjian, 1988; Mermod et al., 1989; Licht et al., 1990; Han and Manley, 1993; Kuhnlein et al., 1994), which suggests that Sall may act as a transcriptional activator or repressor.

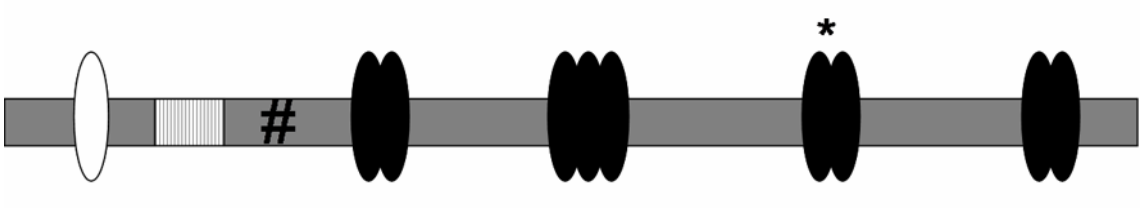

Figure 3. Schematic of Sall protein structure

Sall genes encode a series of 7-9 C_2H_2 zinc fingers arranged in pairs (black ovals). The zinc finger pair * is absent in Drosophila and some mammalian members of the family. Mammalian members have an additional C_2HC zinc finger at the N terminus (white oval). A glutamine rich region is observed at the N terminus (Striped box). # indicates the position of the majority of mutations observed in Townes-Brocks syndrome. N-terminus is left; C-terminus is right.

The majority of evidence suggests that Sall1 functions as a transcriptional repressor. Sall1 can localize to heterochromatin, although independent studies have identified different regions of the Sall1 gene that regulate this localization to heterochromatin (Netzer et al., 2001; Kiefer et al., 2002; Lauberth and Rauchman, 2006; Netzer et al., 2006; Sakaki-Yumoto et al., 2006; Yamashita et al., 2007). The N-terminus of Sall1 is a potent transcriptional repressor in vitro, and a 12 amino acid motif at the N-terminus is required to mediate Sall1 repression (Kiefer et al., 2002; Lauberth and Rauchman, 2006; Netzer et al., 2006). Interestingly, this 12 amino acid motif is absent in Drosophila Sall (Lauberth and Rauchman, 2006), suggesting potential differences in mechanisms for action for members of the Sall gene family across species. Sall1 is believed to mediate transcriptional repression by recruitment of a histone deacetylase complex, which also requires the extreme N-terminus of the Sall1 protein (Kiefer et al., 2002; Lauberth and Rauchman, 2006). A second domain of the Sall1 protein capable of mediating repression has been identified that requires the first and second pairs of C_2H_2 zinc fingers (Netzer et al., 2006). Furthermore, the extreme C-terminus zinc finger pair of Sall1 has been shown to bind A/T rich sequences in vitro, although this interaction was not assessed for repression or activation ability

(Yamashita et al., 2007). While the N-terminus of Sall1 is sufficient to localize to heterochromatin and to mediate repression, this region does not contain a nuclear localization signal and can only enter the nucleus when fused to a GAL4 DNA binding domain containing a nuclear localization sequence (Kiefer et al., 2002). Studies have shown that both Sall1 and Sall4 can activate a Wnt responsive promoter in vitro; which suggests that in some contexts Sall proteins may act as a transcriptional activator (Ma et al., 2002; Sato et al., 2004). Sall4 has been identified as a transcriptional activator of the transcription factor Pou5f1 and the polycomb gene Bmi1 (Zhang et al., 2006; Yang et al., 2007). The N-terminus of the Sall4 protein is required for this activation (Zhang et al., 2006), although the N-terminus of Sall4 can also function as a transcriptional repressor (Bohm et al., 2007). It is not known if the differences in activation/repression function of members of the Sall gene family are context specific.

Studies have suggested that members of the chick Sall gene family can form homo- and hetero-dimers in vitro, and the N-terminal glutamine rich region (striped box, Figure 3) is required to mediate this interaction (Sweetman et al., 2003). Furthermore, expression of either cSall3 or truncated cSall1 resulted in relocation of cSall1 from the nucleus to the cytoplasm, which suggests that Sall3 may regulate Sall1 function by altering the cellular localization of Sall1 (Sweetman et al., 2003). In addition, expression of a truncated Sall1 can interfere with the cellular localization of Sall4 in vitro (Sakaki-Yumoto et al., 2006), and this truncated protein can interact with all members of the Sall gene gamily (Kiefer et al., 2003). Taken together, these studies indicate that members of the Sall gene family can interact; which suggests a mechanism to regulate function in regions where overlapping expression is observed, and that expression of a truncated Sall1 may interfere with endogenous Sall1 and/or Sall4 function.

1.2.3 Functional roles of members of the Sall gene family

Functional roles for members of the Sall gene family have been established in a number of systems and species. The Sall gene family was initially characterized in Drosophila, where it was identified as a homeotic gene that promoted head over trunk development (Jurgens, 1988). A dual role for Sall in Drosophila tracheal development has been proposed, whereby early in development it suppresses tracheal development, and later, it is required to induce dorsal branch identity and for the migration of cells within the dorsal trunk branch in response to Wingless (Wnt) signaling (Kuhnlein and Schuh, 1996; Chihara and Hayashi, 2000). In the developing wing Sall is expressed in response to Decapentaplegic (mammalian homolog of BMP), Hedgehog, and Wingless signaling, and regulates expression of the transcription factors Knirps and Iroquis (de Celis et al., 1996, 1999; de Celis and Barrio, 2000). A role for Sall in neuronal differentiation in the developing thorax has been proposed, whereby Sall expression must be eliminated from proneural cells in order for differentiation to proceed, and overexpression of Sall prevents neuronal differentiation (de Celis et al., 1999). In the developing central nervous system, Sall mutant flies exhibited alterations in neuronal differentiation and cell adhesion (Cantera et al., 2002). In Caenorhabditis elegans Sall (sem-4) is required for the differentiation of subsets of serotonergic and GABAerigic motor neurons (Basson and Horvitz, 1996). Sall also directly regulates the Hox gene egl5 in precursors and differentiating cells and directly represses the Lim homeobox gene mec-3 in differentiating cells, thereby regulating touch neuron generation (Toker et al., 2003). Taken together, these findings suggest roles for Sall in neuron differentiation, migration, and cell adhesion.

In the developing kidney, Sall1 expression in the mesenchyme is required for ureteric bud invasion. In Sall1-mutant animals, kidney agensis or severe dysgenesis are observed, resulting in death of Sall1-mutant mice in the perinatal period (Nishinakamura et al., 2001). During kidney development, SALL1 is activated by SIX1, and Six1 mutant mice exhibit a similar kidney phenotype to Sall1-mutant mice (Xu et al., 2003; Chai et al., 2006). Studies of the developing chick limb bud identified that there are contextual developmental requirements for csal1 expression (Farrell and Munsterberg, 2000). Csal1 expression is induced in the distal limb in response to FGF4 and Wnt3a; however, in the proximal limb BMP2 expression was required, in addition to FGF4 and Wnt3a, to induce csal1 expression (Farrell and Munsterberg, 2000). Sall1 has been shown to interact with several proteins, including β-catenin, the telomere repeat binding factor-1, ubiquitin conjugating enzyme 2I, and the small ubiquitin-like modifier-1 (Netzer et al., 2001; Netzer et al., 2002); however, functional consequences of these interactions have not been identified. High levels of SALL1 expression are observed in Wilms tumor, which suggests a role for SALL1 in disease etiology (Ma et al., 2001). These studies suggest a role for Sall1 in kidney and limb development and implicate Sall1 in tumor pathogenesis.

Sall2 mutant mice survive postnatally and appear phenotypically normal, which suggests that Sall2 is dispensable during development (Sato et al., 2003). However, a role for Sall2 in tumor pathogenesis has been proposed. The SALL2 gene overlaps with a chromosomal region associated with ovarian carcinoma (Bandera et al., 1997), and SALL2 expression is observed in Wilms tumor and a subset of synovial sarcomas (Li et al., 2002; Nielsen et al., 2003; Subramaniam et al., 2006). SALL2 inhibits viral DNA replication of the oncogenic polyoma virus; however, binding of Large T Antigen to SALL2 prevents this inhibition (Li et al., 2001).

Furthermore, SALL2 can activate cyclin-Cdk inhibitor p21, which suggests a role in regulation of cell cycle progression. Extensive molecular analysis of Sall3 has not been conducted; however, murine knockout studies suggest a role for Sall3 in palate formation and cranial nerve innervation (Parrish et al., 2004).

Sall4 null mice die shortly after implantation, and in vitro studies suggest that Sall4 is required for embryonic stem cell proliferation (Elling et al., 2006; Sakaki-Yumoto et al., 2006). Sall4 is hypothesized to maintain the pluripotency of embryonic stem cells by activating Pou5f1 transcription and interacting with Nanog (Wu et al., 2006; Zhang et al., 2006). Two further lines of evidence suggest that Sall4 may regulate progenitor cell fate; Sall4 can interact with Cyclin D1, a regulator of the G1 to S phase transition (Bohm et al., 2007), and over-expression of csal4 in neural crest derivatives resulted in stalling of these cells in an undifferentiated state, which suggests that csal4 maintains cells in a progenitor state (Barembaum and Bronner-Fraser, 2004). Sall4 heterozygous animals display partially penetrant abnormalities, including anal dysplasia, ventricular septum deficits, and exencephaly (Sakaki-Yumoto et al., 2006). These malformations were more pronounced and frequent in Sall4+/-Sall1+/- animals, which suggests a genetic interaction between these genes in vivo (Sakaki-Yumoto et al., 2006). Murine and zebrafish Sall4 are regulated by Tbx5 and induce FGF signaling (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006). SALL4 can also be activated in response to Wnt signaling (Bohm et al., 2006). In zebrafish Sall4 is required for pectoral fin outgrowth, and mice expressing a truncated Sall4 protein display limb and heart abnormalities (Harvey and Logan, 2006; Koshiba-Takeuchi et al., 2006). During hematopoiesis, Sall4 is only expressed by hematopoietic progenitor cells; however, Sall4 is constitutively expressed in acute myeloid leukemia, and precursor B-cell

lymphoblastic leukemia and transgenic mice that constitutively express Sall4 develop Myelodysplastic syndrome/acute myeloid leukemia (Cui et al., 2006; Ma et al., 2006). Taken together, these data suggest a role for Sall4 in progenitor cell maintenance, development of the urogenital tract, limb, brain, and heart, and hematopoietic disease pathogenesis.

In summary, these studies suggest a role for the Sall gene family in cell cycle regulation, neural differentiation, and migration in the developing nervous system, limb, kidney, heart and palate. However, the cellular mechanisms of Sall function in vertebrates are not well characterized.

1.2.4 Developmental disorders associated with SALL gene family

Mutation or deletion of three members of the SALL gene family in humans is associated with distinct developmental disorders affecting overlapping structures. Table 1 summarizes the developmental disorders associated with mutation or deletion of members of the human or mouse SALL gene family. Kohlhase et al., isolated two members of the human SALL gene family, SALL1 and SALL2, by PCR amplification of expected homologous regions to the murine Sall gene (Kohlhase et al., 1996). These genes were mapped to 16q12.1 (SALL1) and 14q11.1-q12.1 (SALL2) (Kohlhase et al., 1996). Subsequent studies determined that mutations in SALL1 were associated with Townes-Brocks syndrome, an autosomal-dominant disorder characterized by imperforate anus (86%/77%)(percent of patients with Townes-Brocks syndrome displaying the characteristic/ percent of patients with Townes-Brocks syndrome with confirmed Sall1 mutations displaying the characteristic), as well as ear (67%/91%), limb (60%/91%) and renal (27%/73%) abnormalities, and hearing loss (47%/86%) (Townes and Brocks, 1972; Rossmiller and Pasic, 1994; Kohlhase et al., 1998; Surka et al., 2001). Cognitive deficits,

although not extensively studied, are associated to a lesser extent (8%/14%) with Townes-Brocks syndrome, and include mental delay, learning disabilities, and Attention Deficit Hyperactivity Disorder (Kohlhase et al., 1999b; Marlin et al., 1999; Keegan et al., 2001; Surka et al., 2001; Botzenhart et al., 2005; Borozdin et al., 2006; Botzenhart et al., 2007). The majority of mutations in Townes-Brocks syndrome associated with mutation of SALL1 are located in the N-terminus, resulting in the predicted expression of a truncated protein (#, Figure 3) (Kohlhase et al., 1999b; Marlin et al., 1999; Keegan et al., 2001; Surka et al., 2001; Botzenhart et al., 2005; Botzenhart et al., 2007). Studies have suggested that the glutamine-rich region in the N-terminus, which mediates Sall family member interactions, may be important in disease pathogenesis, with the truncated protein acting in a dominant negative manner interfering with other SALL family members (Kiefer et al., 2003; Sweetman et al., 2003; Sakaki-Yumoto et al., 2006). Supporting evidence of this model has been obtained from examination of transgenic mice; Sall1 null animals do not mimic the classical Townes-Brocks disease phenotype; however, mice expressing a trans-dominant negative Sall1 protein recapitulate characteristics of the human phenotype (Nishinakamura et al., 2001; Kiefer et al., 2003). However, two patients have been identified with typical Townes-Brocks syndrome with mutations before this domain, which suggests that expression of a truncated protein with this glutamine rich region is not essential for the disease phenotype (Botzenhart et al., 2007). A recent study identified that large deletions in the SALL1 gene can result in a milder form of Townes-Brocks syndrome, which suggests that at least some of the phenotypes observed in the patients are due to haploinsufficiency of the SALL1 protein, while a more severe phenotype may be due to expression of a truncated protein that acts in a dominant negative manner (Borozdin et al., 2006).

SALL3 was identified as the third member of the SALL gene family and mapped to 18q23 (Kohlhase et al., 1999a). The SALL3 gene is one of several genes deleted in 18q deletion syndrome (Kohlhase et al., 1999a); other genes deleted include myelin basic protein, galanin receptor and cytochrome b5 (Cody et al., 1997; Strathdee et al., 1997). 18q deletion syndrome is characterized by growth deficiency (80%), midface hypoplasia (50-80%), limb (80%), ear (50-80%), genitourinary abnormalities (30-80%), and cognitive deficits (80%) (Strathdee et al., 1995). A new member of the SALL gene family was recently identified, SALL4, and is localized to 20q13.13-q13.2 (Kohlhase et al., 2002b). The developmental disorder Okihiro syndrome/Duane radial ray syndrome maps to this region, and studies identified mutations in the SALL4 gene in patients with this syndrome (Al-Baradie et al., 2002; Kohlhase et al., 2002b). It is estimated that 90% of cases of this syndrome are sporadic, with the remaining 10% inherited in an autosomal-dominant manner (Gutowski, 2000). Okihiro syndrome is characterized by eye motility abnormalities, as well as ear and limb malformations, hearing loss, and renal malfunction (Gutowski, 2000). Mutations in SALL4 have also been shown to be associated with Holt-Oram syndrome and acro-renal-ocular syndrome, which are syndromes with overlapping phenotypes (Kohlhase et al., 2003). These findings suggest that members of the Sall gene family are critical regulators of development.

Table 1. Sall gene family and associated disease

Mouse gene	Knockout phenotype	Human gene	Associated disease
Sall1	Kidney deficits	Sall1	Townes-brocks syndrome - kidney, limb, anus, ear & cognitive deficits
Sall2	None determined to date	Sall2	No known association
Sall3	Craniofacial abnormalities	Sall3	18q deletion syndrome - growth retardation, dactyl, midface hypoplasia, cognitive deficits & hearing loss
Sall4	Early embryonic lethal, heterozygote exencephaly	Sall4	Okihiro syndrome - eye movement disorders & malformations of the spine, outer ear and forelimbs & kidney and hearing abnormalities

1.3 OUTLINE OF STUDY

This study examines the role of a member of the Sall gene family, Sall1, in cortical development. Studies in Drosophila and the chick suggest a role for members of this family in cell cycle regulation, neuronal differentiation, migration, and cell adhesion (de Celis et al., 1996; Kuhnlein and Schuh, 1996; de Celis et al., 1999; Chihara and Hayashi, 2000; de Celis and Barrio, 2000; Cantera et al., 2002; Barembaum and Bronner-Fraser, 2004). Sall1 is robustly expressed in the developing central nervous system and peripheral organs (Buck et al., 2000; Ott et al., 2001). Sall1-deficient animals die at birth due to kidney deficits (Nishinakamura et al., 2001), which suggests a critical requirement for this gene during development. However, the role of this gene in cortical development has not previously been characterized. Chapter 2 examines expression of Sall1 in cortical development, and characterizes the neural lineages that express Sall1 during development and in the adult. In Chapter 3, the cellular mechanism of Sall1 in cortical development is described. Examination of Sall1-deficient animals suggests that Sall1 regulates the rate of neuronal differentiation and progenitor cell maturation in the developing cortex. Chapter 4 examines the role of Sall1 in the developing olfactory bulb. These studies suggest that, similar to the cortex, Sall1 is required for neuronal differentiation, and that the pattern of neurogenesis in the developing olfactory bulb regulates olfactory nerve extension. Taken together, these studies suggest a conserved role for Sall in regulating neuronal differentiation. In Chapter 5, I discuss the implications of the model of Sall1 function during neural development and the future directions of this study.

2.0 SALL1 EXPRESSION

2.1 INTRODUCTION

Previous studies have examined expression of sall1 mRNA in the nervous system of the developing embryo. Sall1 expression is first observed on E7-E7.5 in the neural ectoderm (Buck et al., 2001; Ott et al., 2001). From E8.5, sall1 is expressed in the telencephalon, diencephalon, hindbrain, and spinal cord (Ott et al., 2001). By E9.5 sall1 is also expressed in the midbrain-hindbrain boundary (Buck et al., 2001; Ott et al., 2001). At E12.5, sall1 is expressed by progenitor cells in the ventricular zone in both the ventral and dorsal telencephalon (Ott et al., 2001). RNAse protection studies indicated that sall1 is strongly expressed in the adult brain (Ott et al., 2001). However, these studies have been limited by the lack of a commercially available Sall1 antibody. The recent development of a Sall1 antibody has facilitated a more detailed study of the temporal and spatial pattern of Sall1 protein expression in the developing and adult cortex. In order to better understand the role of Sall1 in cortical development I examined Sall1 expression in the cerebral cortex from E9.5 to the adult.

2.2 MATERIALS AND METHODS

2.2.1 Animals

Embryos were obtained from timed pregnant CD1 mice from Charles Rivers Laboratories (Wilmington MA). Embryos were collected via cesarean section at embryonic ages from E9.5 to E18.5. The day of vaginal plug was designated as E0.5, the first postnatal day was designated P0.5. Embryos were fixed in either 4% paraformaldehyde (PFA) (pH 7.4) and processed through increasing sucrose gradients for cryosectioning or in Carnoys solution (1:3:6 acetic acid: chloroform: 100% alcohol) and then processed through a butanol series for paraffin sectioning. For postnatal studies, the brain was dissected from the skull, fixed overnight in Carnoys solution and processed as described above. Cryopreserved embryos were embedded in Tissue-Tek O.C.T. compound (EMS, Hatfield, PA) and sectioned at 20µm. Paraffin embedded embryos were sectioned at 10µm. Animal protocols and procedures were approved by Institutional Animal Care and Use Committee at the University of Pittsburgh and adhered to the National Institutes of Health guidelines.

2.2.2 Immunohistochemistry

Paraffin processed sections were deparaffinized in xylene, rehydrated through an ethanol series and washed in 0.1% triton in phosphate buffered saline (PBS) pH 7.4. For diaminobenzidine detection, sections were incubated with 3% hydrogen peroxide in methanol for 10 minutes. Slides were subsequently microwaved in 0.1M sodium citrate solution pH 6.0, rinsed in PBS and blocked in 10% heat inactivated goat serum (Jackson ImmunoResearch, West Grove, PA).

Cryopreserved tissue was rinsed in PBS, blocked in 10% heat inactivated goat serum (HINGS) and incubated with primary antibody overnight at 4°C. Antibodies used were rabbit anti-Aquaporin 4 (1:1000; Chemicon, Temecula, CA); rabbit anti-Calretinin (1:1000; Chemicon); rabbit anti-glial fibrillary acidic protein (GFAP) (1:400; Sigma); guinea pig anti-GLAST (1:4000; Chemicon); rabbit anti-Phospho-histone (1:200; US Biologicals, Swampscott, MA); mouse anti-Sall1 (1:500; PPMX Perseus Proteomics, Tokyo, Japan); rabbit anti-Sox10 (1:500; Cemines, Golden, CO); rabbit anti-Tbr1 (1:1000; (Englund et al., 2005)); rabbit anti-Tbr2 (1:1000; (Englund et al., 2005)). The tissue was subsequently washed with PBS, incubated with the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and then incubated with Vectastain® Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions. Staining was visualized using nickel enhanced diaminobenzidine (Sigma) reaction. Sections were counterstained with nuclear fast red (Vector Laboratories). Slides were then dehydrated through ethanol, washed in xylene and mounted in DPX (Fluka, Sigma). For fluorescent detection of signal the tissue was washed with PBS and incubated with the appropriate Cy3 (Jackson ImmunoResearch) and/or Alex Fluor 488 (Invitrogen, Carlsbad, CA) secondary antibody, and counterstained with 1,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) before mounting in fluormount G (Southern Biotechnology Research, Birmingham, AL). Sections were visualized on a Nikon 400 (Melville, NY) fluorescent microscope, photographed with a Photometrics (North Reading, MA) Cool Snap CCD digital camera and IP Lab software (Biovision Technologies, Exton, PA). Composite images were prepared using Photoshop 7.0 (Adobe Systems, San Jose, CA). Contrast, color, and brightness were adjusted in Photoshop 7.0.

2.2.3 Histological cell type identification

To assign cellular subtypes and boundaries to cortical regions, sections were examined at 40X. The ventricular zone was identified as the cellular region adjacent to the ventricle containing organized parallel arrays of cells with elongated nuclei arranged perpendicular to the ventricular surface. The subventricular zone was composed of a compact layer of cells with round nuclei, adjacent to the ventricular zone. Cells in the subventricular zone were arranged in a disorganized manner compared to the ventricular zone. In this study the progenitor population is defined as the ventricular and subventricular zone. The intermediate zone contained fewer cells than proliferative regions and contained a mixture of cells with round and elongated nuclei. Subsets of cells in the intermediate zone were orientated parallel to the ventricular surface. The subplate was identifiable within the intermediate zone as a layer of cells with large round nuclei, deep to the cortical plate. The cortical plate was evident from E14.5, and identifiable due to an increased packing density of cell nuclei compared to the intermediate zone. Cells in the cortical plate were arranged perpendicular to the ventricular surface in an organized manner. The marginal zone was located superficial to the cortical plate, adjacent to the pial surface. The marginal zone contained a mixture of cells with round nuclei and tangentially orientated nuclei.

2.3 RESULTS AND DISCUSSION

2.3.1 Sall1 expression in developing cortex

Sall1 mRNA is expressed in the central nervous system, including the telencephalon, diencephalon, hindbrain, and spinal cord from E8.5 (Buck et al., 2001; Ott et al., 2001). However, the temporal and spatial pattern of Sall1 protein expression in the developing cerebral cortex has not been described in detail. Expression of Sall1 protein in the early developing telencephalon mimics previously described sall1 mRNA expression. At E9.5 and E10.5, Sall1 protein is expressed by a subset of progenitor cells; expression is highest in lateral and ventral regions; however, over time expression increases in dorsal and medial regions (data not shown). A lateral to medial and ventral to dorsal gradient of development exists within the developing cortex (Bayer and Altman, 1991), and differences in Sall1 expression across this gradient may reflect a natural maturation of progenitor cells. By E11.5, Sall1 is robustly expressed by progenitor cells in the ventricular zone of the dorsal cortex (Figure 4A,D). Weaker Sall1 expression is observed in the medial cortex at this age (*, Figure 4A). By E13.5 robust Sall1 expression in progenitors is observed throughout the cortex extending into the medial cortical wall (*, Figure 4B,E). At mid-neurogenesis, a second proliferative population arises in the cortex, the subventricular zone (BoulderCommittee, 1970). Sall1 was expressed by a subset of cells in the subventricular zone at E17.5 (Figure 4C,F). Throughout development, Sall1 is also expressed by ventral progenitor cells (Figure 4A-C). At E11.5, Sall1 is robustly expressed by cells in the lateral ganglionic eminence and the lateral-medial ganglionic sulcus, with weak expression in the medial ganglionic eminence (Figure 4A). By E13.5, weak Sall1 expression

extends into the medial ganglionic eminence and preoptic area (Figure 4B). Sall1 was not expressed by cells in the subicular neural epithelium during development (S, Figure 4A,B).

Progenitor cells in the ventricular zone proliferate and differentiate to produce cells destined for cortical structures. Sall1 expression was also observed in a subset of cells outside the ventricular zone from E11.5 (Figure 4A,B,E,F). Sall1 expression in this population of cells increased in a ventral to dorsal gradient during development (Figure 4A,B,E,F). In summary, Sall1 is expressed by progenitor cells in the dorsal and ventral telencephalon from E9.5. In addition, Sall1 was expressed by a subset of cells outside the ventricular zone from E11.5. These data suggest that Sall1 may be an important regulator of cortical development.

2.3.2 Sall1 expression in the early postnatal and adult cortex

Sall1 expression in the cerebral cortex continues in postnatal animals and the adult. At P14.5, a subset of cells in the dorsal cortex, hippocampus and dentate gyrus express Sall1 (Figure 5A-C). In the dentate gyrus, Sall1 expression is localized to the sub-granular zone, a region of adult neurogenesis (arrows, Figure 5C). Two populations of Sall1-expressing cells were present in the cortex at this age, small cells robustly expressing Sall1 (arrowheads, Figure 5D) and cells with larger cell bodies weakly expressing Sall1 (arrows, Figure 5D). Interestingly, a number of the small cells strongly expressing Sall1 were closely associated with Sall1 negative cells with large nuclei (yellow arrowheads, Figure 5D). In the adult, Sall1 was expressed by a subset of cells in the dorsal cortex, hippocampus, and dentate gyrus (Figure 6A-D), similar to that described at P14.5. These findings suggest a role for Sall1 in adult neurogenesis.

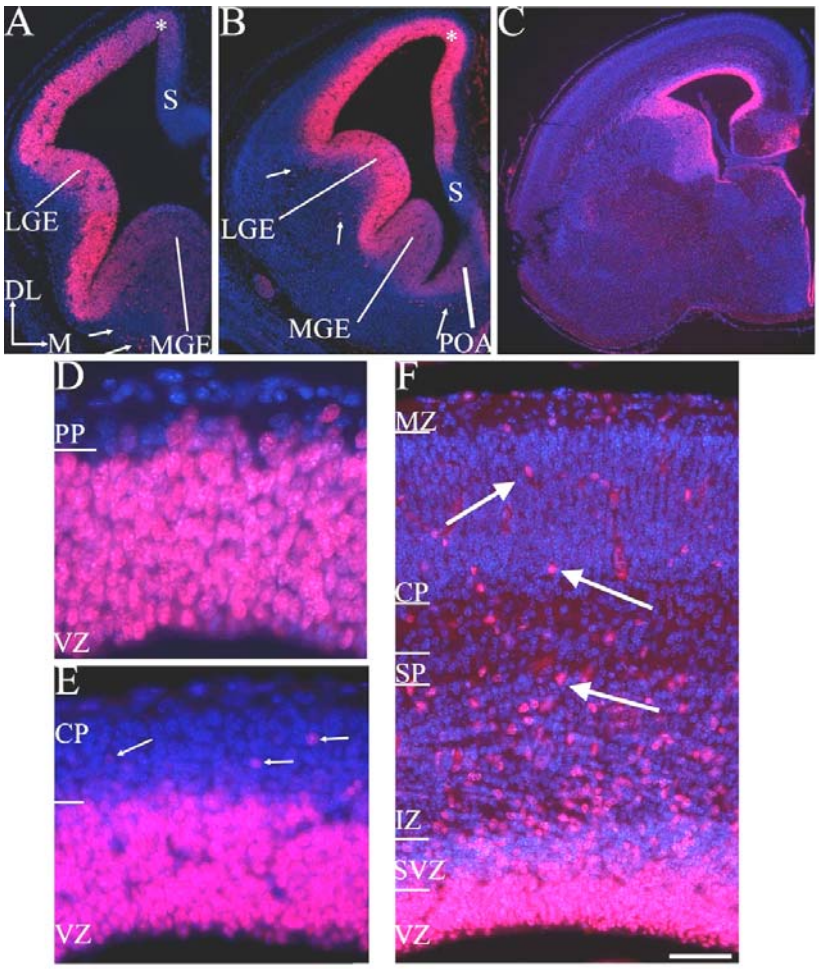

Figure 4. Sall1 expression in the developing telencephalon

Sall1 immunohistochemistry (red) in the developing telencephalon at E11.5 (A,D), E13.5 (B,E), and E17.5 (C,F). Sections are counterstained with DAPI (blue). At E11.5, E13.5, and E17.5, strong Sall1 expression is observed in the progenitor population in the dorsal cortex and lateral ganglionic eminence (A,B,C). Weaker expression is observed in the medial cortex at E13.5 (*,A) and the medial ganglionic eminence at E11.5 and E13.5 (A,B). Sall1 was also expressed by a subpopulation of cells in the differentiating field (arrows, A,B,E,F). Sall1 expression was absent from the subicular neural epithelium (S, A,B). High power examination of the dorsal cortex indicates that Sall1 was expressed by progenitor cells (D-F), and at E17.5, by subsets of cells in the subventricular zone (F). At E17.5, Sall1 was expressed by a number of cells outside the ventricular/subventricular zone in the dorsal cortex (F). DL: dorsal; M: medial; LGE: lateral ganglionic eminence; MGE: medial ganglionic eminence; S: subicular neural epithelium; VZ: ventricular zone; PP: preplate; CP: cortical plate; SVZ: subventricular zone; IZ: intermediate zone; SP: subplate; MZ: marginal zone. Scale bar (in F) represents: 75µm in D; 125µm in E; 200µm in F; 300µm in A; 600µm in B; 1100µm in C.

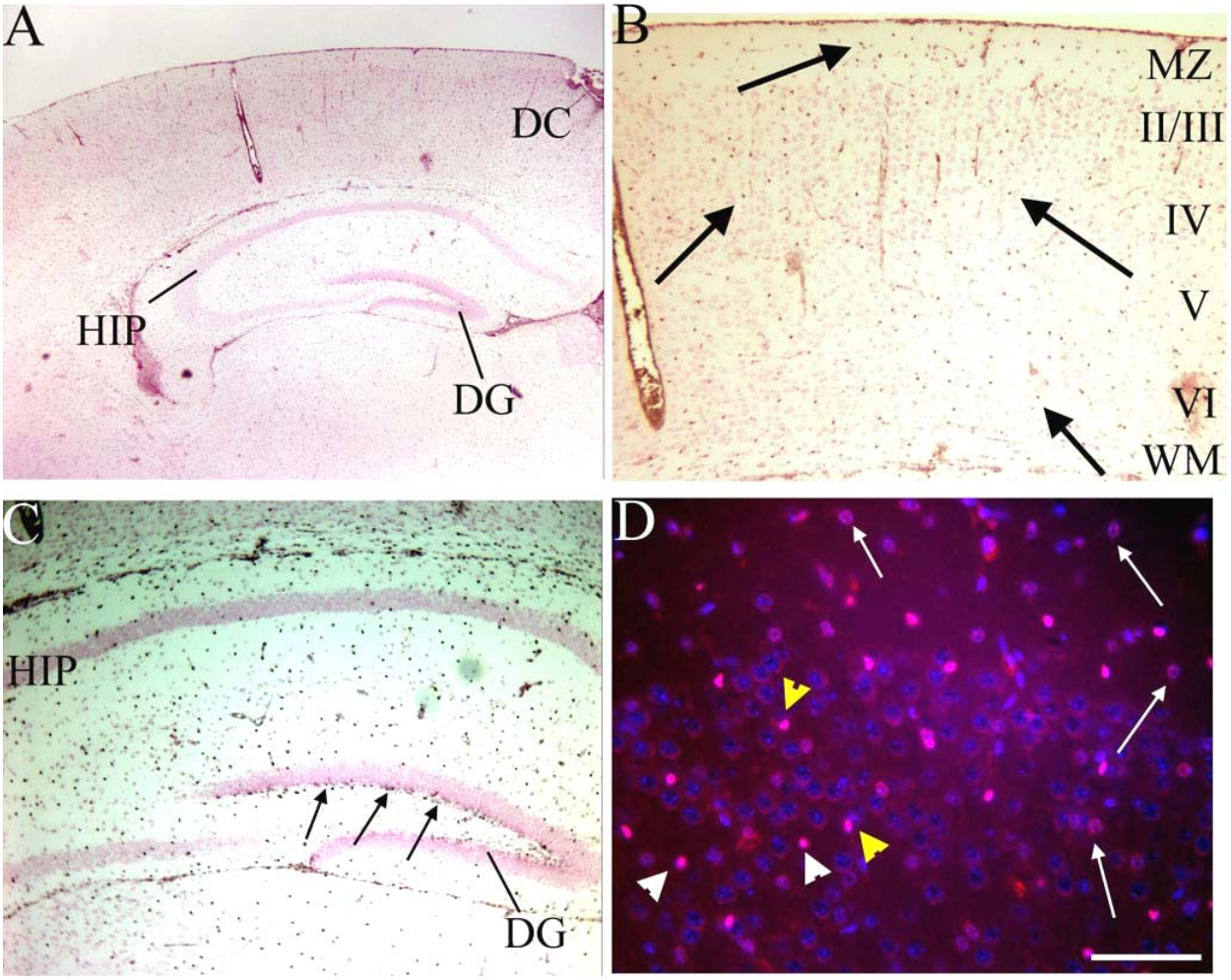

Figure 5. Sall1 expression at P14.5

Sall1 (brown) is expressed by a subpopulation of cells in the dorsal cortex at P14.5 (A,B). Sections are counterstained with nuclear fast red (A-C) or DAPI (D). In the hippocampus and dentate gyrus, a subset of cells express Sall1 (C). Sall1 is expressed by cells in the subgranular zone of the dentate gyrus (arrows, C). In the dorsal cortex, two populations of Sall1-expressing cells were observed, small cells strongly expressing Sall1 (arrowheads, D) and large cells weakly expressing Sall1 (arrows, D). A subset of the small cells strongly expressing Sall1 were closely associated with Sall1-negative cells (yellow arrowheads, D). DC: dorsal cortex; HIP: hippocampus; DG: dentate gyrus; MZ: marginal zone; WM: white matter; II/III, IV, V, VI demarcate cortical layers. Scale bar (in D) represents: 90µm in D; 400µm in B,C; 1350µm in A.

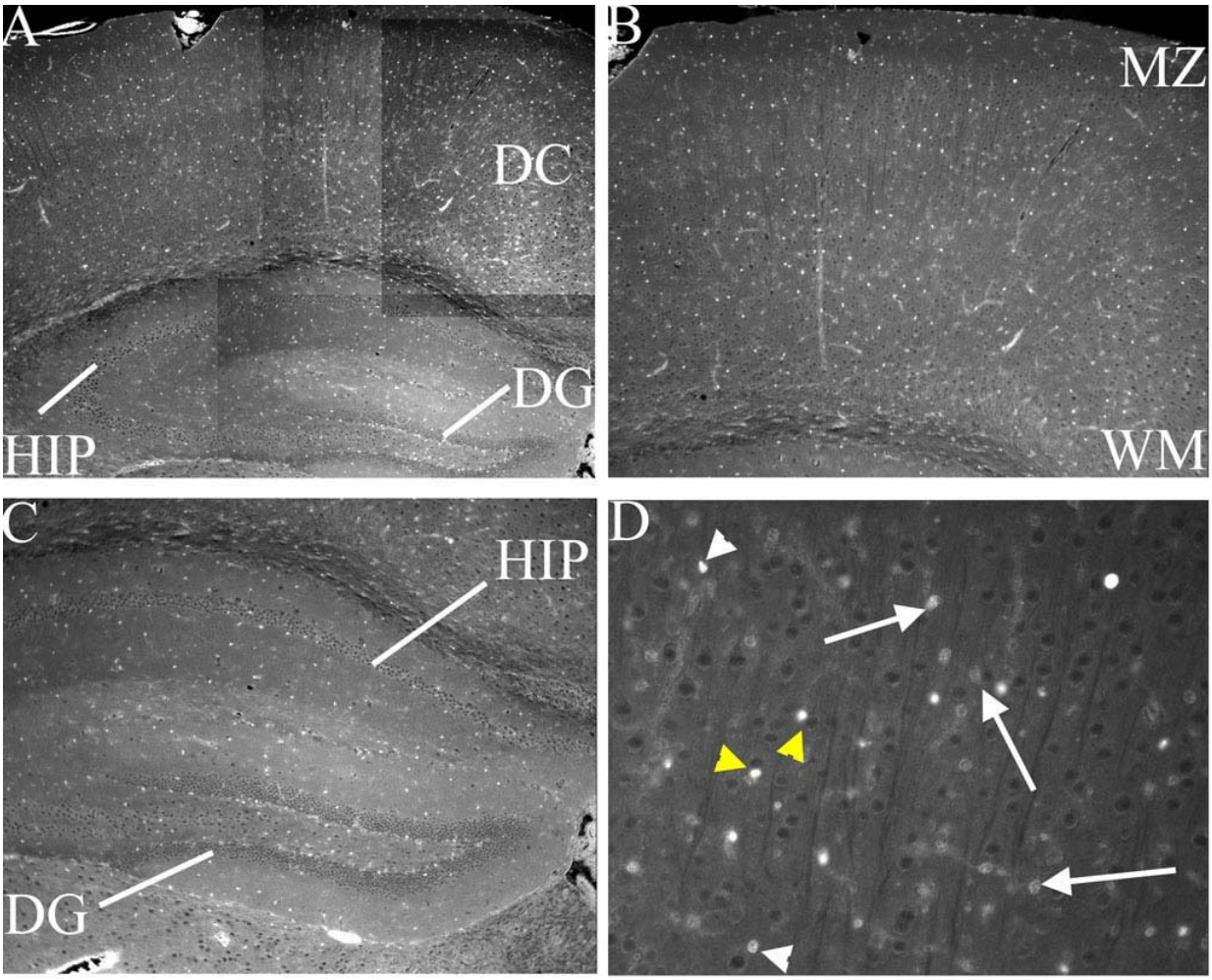

Figure 6. Sall1 expression in the adult

Sall1 (white) is expressed by a subpopulation of cells in the dorsal cortex in the adult (A,B). In the hippocampus and dentate gyrus a subset of cells express Sall1 (C). In the dorsal cortex two populations of Sall1-expressing cells were observed, small cells strongly expressing Sall1 (arrowheads, D) and large cells weakly expressing Sall1 (arrows, D). A subset of the small cells strongly expressing Sall1 were closely associated with Sall1 negative cells (yellow arrowheads, D). DC: dorsal cortex; HIP: hippocampus; DG: dentate gyrus; MZ: marginal zone; WM: white matter. Scale bar (in D) represents: 90µm in D; 400µm in B,C; 1350µm in A.

2.3.3 Sall1 is expressed by neural progenitor cells but not by mature neurons

Sall1 is robustly expressed by cells in the dorsal ventricular zone early in development (Figure 4A,B). At E17.5, Sall1 is expressed by cells in the ventricular zone, as well as a subset of cells in the subventricular zone. The subventricular zone contains a mixed neuronal cellular population; cells arising from the dorsal cortex, destined for a glutamatergic cortical fate, and migrating neurons born in the ventral cortex, destined for a GABAergic cortical fate. To verify that these Sall1-expressing cells were progenitor cells, we examined co-expression of Sall1 and the radial glia marker GLAST at E18.5. GLAST co-localized with Sall1 in the dorsal cortex at this age (Figure 7A), which indicates that Sall1 is expressed by radial glia progenitor cells. Tbr2 is expressed by proliferating cells in the ventricular and subventricular zone, and Tbr2-positive cells have the properties of intermediate progenitor cells (Englund et al., 2005). Furthermore, Tbr2-positive cells are hypothesized to give rise to dorsally derived glutamatergic neurons (Englund et al., 2005). In the ventricular zone, co-expression of Tbr2 and Sall1 was observed (arrows, Figure 7B). In the subventricular zone, Sall1 was expressed by a subpopulation of Tbr2-positive cells (arrowheads, Figure 7B). These data indicate that Sall1 is expressed by radial glia progenitor cells and intermediate progenitor cells in the dorsal cortex, which suggests a role for Sall1 in the regulation of glutamatergic neurogenesis. To determine whether Sall1 was expressed by mature glutamatergic neurons in the dorsal cortex we examined expression of Tbr1, expressed by glutamatergic cells in the subplate and cortical plate (Hevner et al., 2003; Kolk et al., 2005). Sall1 was not co-expressed with Tbr1 (Figure 7C) which suggests that Sall1 is not expressed by mature glutamatergic neurons in the cortical plate.

GABAergic interneurons arise in the ventral telencephalon. Sall1 is expressed by progenitors in the lateral, medial, and caudal ganglionic eminences during development (Figure 4 A,B, data not shown). We observed co-expression of Sall1 and GLAST in the ventral progenitor population (Figure 8A), verifying that Sall1 is expressed by radial glial progenitors that give rise to interneuron populations. To determine whether Sall1 was expressed by mature interneurons, we examined expression of two calcium binding proteins that are expressed by independent interneuron populations, Calretinin and Calbindin (Gonchar and Burkhalter, 1997). We did not observe co-expression of Sall1 and Calretinin (Figure 8B) or Calbindin (Figure 8C). These data suggest that, similar to the dorsal cortex, Sall1 is expressed by ventral progenitors destined for an interneuron fate, but, it is not by subsets of mature interneurons.

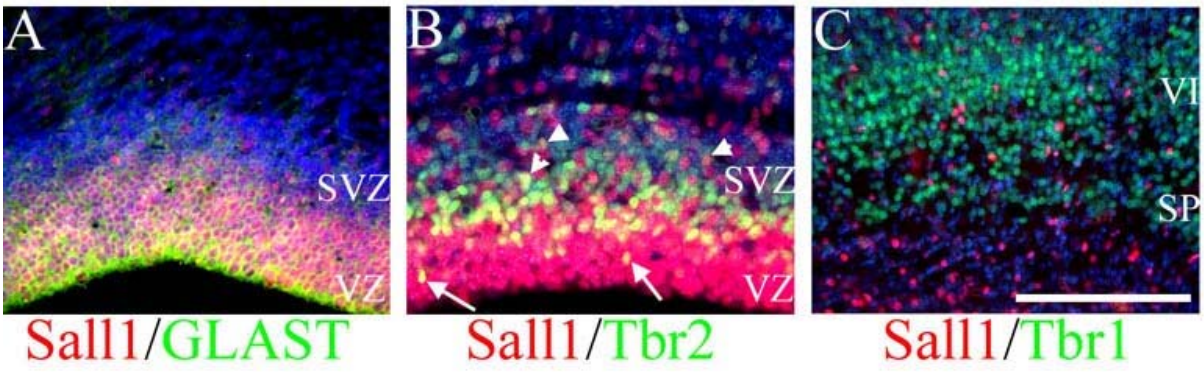

Figure 7. Sall1 is expressed by radial glia and intermediate progenitor cells in the dorsal cortex

Sall1 (red) is co-expressed with the radial glia marker GLAST (green) in the dorsal cortex at E18.5 (A). Co-expression of Sall1 (red) and Tbr2 (green), an intermediate progenitor cell marker, is observed in the ventricular zone (arrows, B) and subventricular zone (arrowheads, B) in the dorsal cortex at E18.5. Sall1 (red) was not co-expressed with the mature glutamatergic marker Tbr1 (green) (C) at E18.5. VZ: ventricular zone; SVZ: subventricular zone; SP: subplate; VI: layer VI. Scale bar (in C) represents: 75µm in B; 100µm in A; 150 in C.

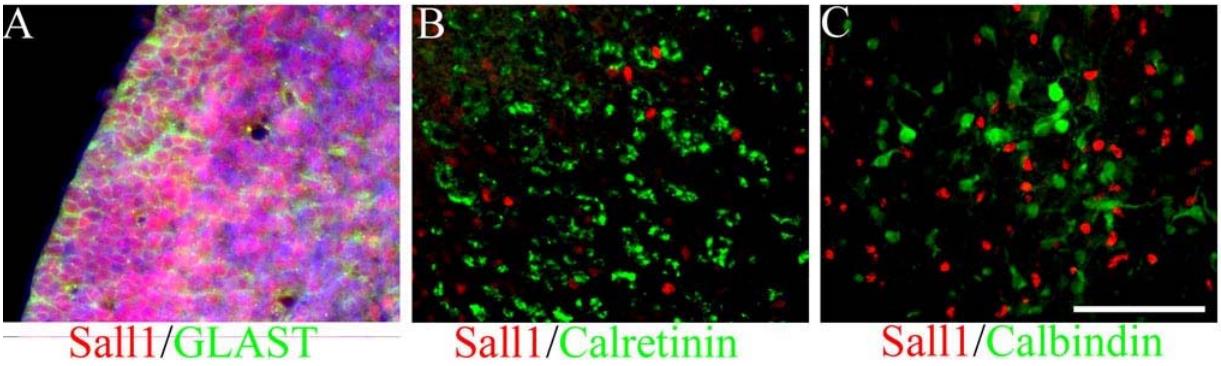

Figure 8. Sall1 is expressed by radial glia cells in the ventral telencephalon

Co-expression of Sall1 (red) and the radial glia marker GLAST (green) is observed in ventral progenitors at E18.5 (A). Sall1 (red) was not co-expressed with the mature GABAergic interneuron markers Calretinin (green) (B) or Calbindin (green) (C) at E18.5. Scale bar (in C) represents: 75µm in A; 100µm in B,C.

2.3.4 Sall1 is expressed by oligodendrocytes and astrocytes

From E11.5, Sall1 was expressed by a subset of cells outside the progenitor cell population. These cells did not co-label with neuronal markers of mature glutamatergic or GABAergic cells. We therefore hypothesized that Sall1 may be expressed by the other major cellular population in the cortex, the glial population. Furthermore, at P14.5 we observed Sall1 expression in a population of cells with small nuclei that were closely associated with cells with larger nuclei, characteristic of glial cells. At E17.5 staining for phospho-histone, a mitotic marker, revealed a number of mitotic cells outside the progenitor population in the differentiating field (arrows, Figure 9A). Mature neuronal cells do not divide, and the only cell type that can divide outside the progenitor population are glial cells. Co-expression of Sall1 and phospho-histone was observed in the dorsal cortex at this age (white arrow, Figure 9A,B), which suggests that Sall1 is expressed by glia cells during development. Two populations of glia cells are present in the cortex, astrocytes and oligodendrocytes. We examined expression of Aquaporin 4 (Figure 9C,D,D') and GFAP (Figure 9E,E'), markers of mature astrocytes, and Sox10 (Figure 9F,F'), a marker of oligodendrocytes (Kuhlbrodt et al., 1998). At E18.5 co-expression of Sall1 and Aquaporin 4 was observed in the ventral cortex (arrows, Figure 9C). At P14.5, Sall1 was co-expressed with Aquaporin 4 in cells adjacent to the lateral ventricles (white arrows, Figure 9D,E) and in cells outside the subventricular zone (yellow arrows, Figure 9D). Co-expression of Sall1 and GFAP was observed at P14.5 in the dorsal cortex (arrows, Figure 9E,E'). In addition, co-expression of Sall1 and the oligodendrocyte marker Sox10 was observed in the dorsal cortex at E18.5 (arrows, Figure 9F,F'). These data suggest a role for Sall1 in the generation and maintenance of glia cell types.

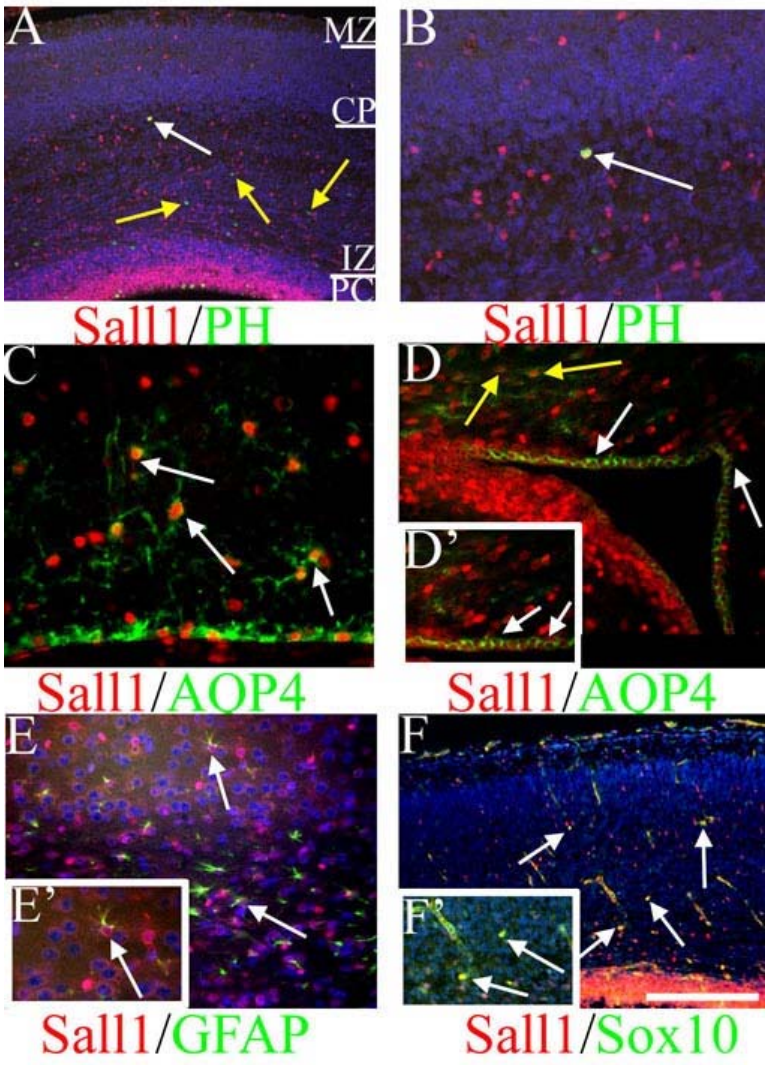

Figure 9. Sall1 is expressed by glia cells during development and in the adult

Sall1 (red) and phospho-histone (green) expression in the dorsal cortex at E17.5 (A,B). Phospho-histone is expressed by a subset of cells outside the progenitor populations (arrows, A). Co-expression of Sall1 and phospho-histone is observed in the dorsal cortex (white arrow, A,B). Sall1 (red) was co-expressed with the mature astrocyte marker, Aquaporin 4 (red), in the ventral cortex at E18.5 (arrows, C) and at P14.5 in cells adjacent to the lateral ventricles (white arrows, D,D') and in the subventricular zone (yellow arrows, D). In the adult Sall1 (red) was expressed by GFAP-positive cells (arrows, E,E'). At E18.5 co-expression of Sall1 (red) and the oligodendrocyte marker, Sox10 (green), was observed in the dorsal cortex (arrows, F,F'). Scale bar (in F) represents: 75µm in C; 90µm in B; 100µm in D,D',E,E',F'; 200µm in A,F.

Figure 10 summarizes the cells types that express Sall1 during development and in the adult. Sall1 is expressed by progenitor cells that give rise to both excitatory neurons and inhibitory interneurons during development, although Sall1 is not expressed by these cells following differentiation. Sall1 is expressed by astrocytes and oligodendrocytes during development and in the adult. In addition, Sall1 is expressed in regions of adult neurogenesis. These data suggest that Sall1 may be an important regulator of neurogenesis and gliogenesis during development and in the adult.

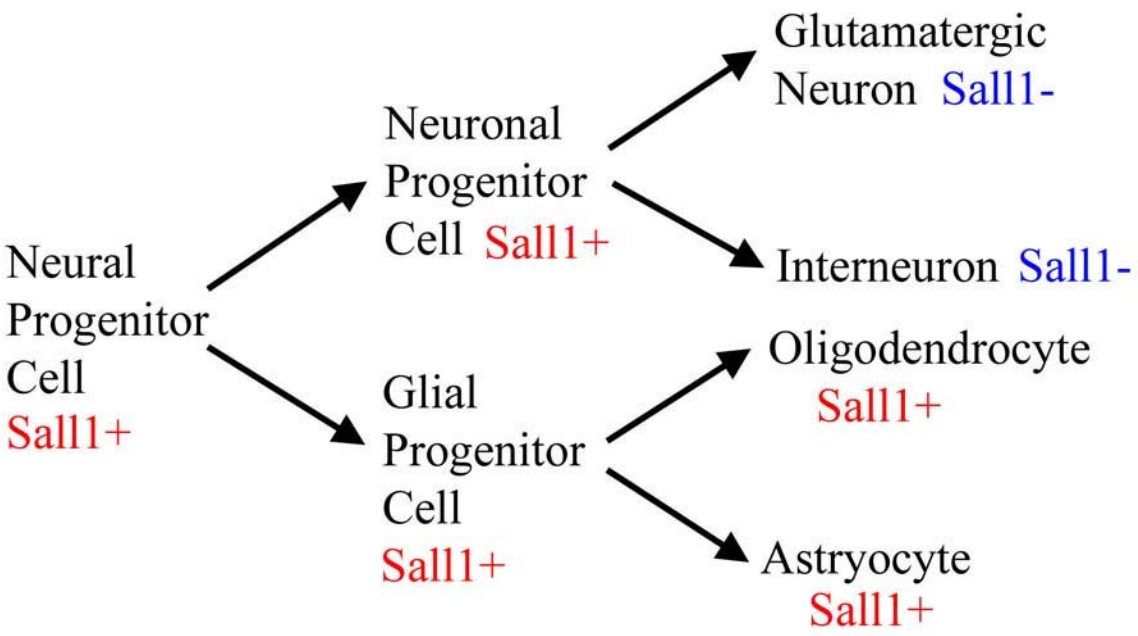

Figure 10. Schematic of Sall1 cell type expression

Sall1 is expressed by neural progenitor cells. These cells become lineage restricted to neuronal populations and glial populations which also express Sall1. Sall1 was not expressed with markers of mature glutamatergic or interneuron populations. Sall1 was expressed by oligodendrocytes and astrocytes.

3.0 SALL1 REGULATES PREPLATE DIFFERENTIATION AND INTERMEDIATE PROGENITOR CELL GENERATION IN THE DEVELOPING CORTEX

3.1 INTRODUCTION

The cerebral cortex is a central component of the limbic system that regulates emotion and behaviour response (Rosner, 1970). Regulation of proliferation, differentiation, migration, and cell survival during development is critical to establish appropriate cell number in the mature cortex. Alterations in cell number, cell type specification, cell migration, and cell connections are associated with gross developmental and neuropsychiatric disorders (Bond et al., 2002; Lewis and Levitt, 2002; Bond et al., 2003; Courchesne et al., 2003; Guerrini et al., 2003; Kato and Dobyns, 2003; Levitt et al., 2004; Courchesne and Pierce, 2005; Kouprina et al., 2005; Piao et al., 2005; Geschwind and Levitt, 2007). Cortical neurogenesis initiates at E10.5, when a subpopulation of progenitor cells in the dorsal cortex differentiate, producing neurons primarily destined for the preplate. Cells destined for the preplate are born from E10.5 to E13.5 (Price et al., 1997; Sheppard and Pearlman, 1997). At ~E13.5, the fate of newly generated neurons changes to a cortical plate fate (Price et al., 1997; Levers et al., 2001). These newborn neurons radially migrate away from the progenitor population and split the preplate into two structures, the marginal zone, superficial, and the subplate, deep (Marin-Padilla, 1978). A correlation between birth-date, the day the progenitor cell leaves the cell cycle, and laminar position in the

cortical plate exists (see Section 1.1.6) (Molyneaux et al., 2007). Neurons destined for deep cortical layers are born first, and those destined for more superficial layers, which are born later, migrate past early born cells to reach their final destination (Angevine and Sidman, 1961).

The preplate is a transient structure in the developing cortex. Cajal-Retzius cells destined for the marginal zone are the first preplate cell type to differentiate on E10.5 and are hypothesized to influence the migration of subsequently derived populations (Sheppard and Pearlman, 1997; Gupta et al., 2002; Kato and Dobyns, 2003). Following preplate split, Cajal-Retzius cells are located superficial to the cortical plate in layer I of the cortex. Cajal-Retzius cells express the glycoprotein Reelin (Ogawa et al., 1995; Schiffmann et al., 1997). Reelin and the downstream components of its signaling pathway influence the migratory behaviour of neurons and are critical regulators of the laminar position of cortical plate neurons. Binding of Reelin to the transmembrane ligands expressed by neurons, very low density lipoprotein receptor (Vldlr), Apolipoprotein E Receptor 2 (ApoER2) and β-integrin, results in phosphorylation of the intracellular protein Disabled-1 (Dab1), activating a downstream signaling cascade which results in changes in the cell cytoskeleton, thereby influencing cell migration of newborn neurons (Rice et al., 1998; D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1999; Dulabon et al., 2000). In the absence of Reelin, Vldlr, ApoER2, or Dab1, migrating neurons destined for the cortical plate fail to split the preplate (Sweet et al., 1996; Sheldon et al., 1997; Yoneshima et al., 1997; Trommsdorff et al., 1999; Gupta et al., 2002). Subsequent waves of differentiating neurons destined for more superficial layers of the cortical plate fail to migrate past earlier born neurons, and thus the cortical plate is populated in an outside-in manner, cortical circuitry is disrupted and mutant mice display ataxia and tremor (Sweet et al., 1996; Sheldon et al., 1997;

Trommsdorff et al., 1999; Gupta et al., 2002). Recent studies have identified distinct spatial origins for Cajal-Retzius cells, the cortical hem, the ventral pallium, and the septum (Meyer et al., 2002; Shinozaki et al., 2002; Bielle et al., 2005). The transcription factor p73 has been identified as a regulator of Cajal-Retzius cells derived from the cortical hem (Meyer et al., 2002; Meyer et al., 2004). The transcription factors Foxg1 (BF1) and CoupTF1 have been identified as regulators of Cajal-Retzius identity. In the absence of Foxg1, neurons adopt a Cajal-Retzius fate instead of a cortical plate fate, which suggests that Foxg1 suppresses Cajal-Retzius fate (Hanashima et al., 2004) and studies suggest CoupTFI must be downregulated for cells to adopt a Cajal-Retzius fate; overexpression of CoupTFI represses a Cajal-Retzius fate in differentiated preplate neurons (Studer et al., 2005). A direct role for the transcription factor Tbr1 in activating Reelin expression has been hypothesized. Tbr1 can activate the Reelin promoter in vitro, and in Tbr1 mutant mice Cajal-Retzius cells appear to be specified but Reelin expression was decreased on a per cell basis compared to controls (Hevner et al., 2001; Chen et al., 2002). These studies have identified that the marginal zone is an important structure influencing the laminar position of differentiating neurons; however, mechanisms regulating the generation of Cajal-Retzius cells and Reelin expression are poorly characterized.

Following the split of the preplate, the subplate is located below the cortical plate. The subplate is a transient structure during development (Luskin and Shatz, 1985; Wood et al., 1992; Robertson et al., 2000). Birthdating studies indicate that the majority of neurons destined for the subplate are born on E11.5-E12.5; however, these cells are not observed in the adult, and it is hypothesized that the majority of subplate cells undergo cell death in the postnatal period, with those remaining becoming part of the deep cortical plate (layer VIb) (Wood et al., 1992;

Robertson et al., 2000). Several transcription factors have been identified that influence subplate formation. Tbr1 and Emx1/2 are hypothesized to influence subplate neurogenesis, although the abnormalities observed in these mutant mice may be complicated by a defective preplate split and altered early patterning deficits (Yoshida et al., 1997; Hevner et al., 2001; Shinozaki et al., 2002; Bishop et al., 2003). CoupTFI is required for the differentiation and survival of subplate neurons (Zhou et al., 1999), and Fezl is required for appropriate specification of subplate neurons (Hirata et al., 2004). A role for the subplate in the establishment of cortical circuitry has been hypothesized. Incoming projections from the thalamus arrive in the cortex at ~E14.5, before the cells with which they will ultimately form connections with have reached their laminar position. These projections make transient synapses on subplate cells while awaiting the maturation of their final targets in the cortex (Ghosh et al., 1990; Ghosh and Shatz, 1993). Analysis of animals with deficient subplate populations also identified alterations in thalmocortical innervation, confirming the requirement for this transient population in the establishment of cortical circuitry (Yoshida et al., 1997; Zhou et al., 1999; Hevner et al., 2001; Shinozaki et al., 2002; Bishop et al., 2003; Hirata et al., 2004).

Few genes have been identified that affect generation or survival of specific populations of cortical plate neurons. The best characterized is the transcription factor Fezl which is required for specification of layer V neurons (Chen et al., 2005; Molyneaux et al., 2005). In the absence of Fezl, layer V neurons are not specified and an increase in layer VI neuronal population is observed, which suggests that Fezl regulates the switch from making layer VI to layer V cells. However, upper cortical layers were not altered, which suggests that appropriate generation of deep cortical layers is not essential for the switch to making superficial layers. The transcription

factor CoupTFI is required for survival of layer IV neurons (Zhou et al., 1999). Previous studies have identified that cell cycle length, extrinsic signals and a progressive restriction of progenitor fate influence cortical plate cell type specification (see Section 1.1.2), although molecular mechanisms regulating the overall differentiation and cell type specification of cortical plate neurons are not well understood.

The type of division a progenitor cell undergoes is an important mechanism in regulating cell number and fate in the cortex. Early in development, an expansion of the progenitor population occurs, and the majority of progenitor cells undergo proliferative divisions, resulting in progenitor/progenitor progeny. At the onset of neurogenesis, neurogenic divisions are observed resulting in progenitor/neuron progeny (Haubensak et al., 2004), and by mid-neurogenesis these represent the predominant division type in the ventricular zone (Noctor et al., 2004). At ~E13.5 an additional proliferative cell division type is observed in the ventricular zone. This division type results in the production of a ventricular zone progenitor cell and an intermediate progenitor cell which migrates to the subventricular zone (Miyata et al., 2004; Noctor et al., 2004). Intermediate progenitor cells predominately undergo terminal neurogenic divisions, resulting in the production of two neurons, although proliferative intermediate progenitor divisions have also been reported, resulting in production of two daughter intermediate progenitor cells (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Expression studies have suggested that the ventricular zone may predominately produce neurons for deep cortical layers, with the subventricular zone predominately producing neurons for superficial cortical layers (Tarabykin et al., 2001; Nieto et al., 2004). However, late neurogenesis has not been followed using live imaging techniques, and the contribution of ventricular zone cells to the intermediate progenitor

population or to cortical neurogenesis has not been characterized during this period. Mechanisms regulating the transition from ventricular zone progenitor cell to intermediate progenitor cell have not been characterized, and questions remain as to how the generation and maintenance of this transient population is regulated.

The Wnt signaling pathway has been implicated in regulating neural progenitor cell differentiation and total cortical cell number (Chenn and Walsh, 2003). Members of the Wnt family bind to Frizzled or to Low density lipoprotein receptors forming a complex which phosphorylates Dishevelled (Yanagawa et al., 1995; He et al., 1997; Bhat, 1998; Kennerdell and Carthew, 1998; Pinson et al., 2000; Tamai et al., 2000). Phosphorylated Dishevelled prevents ubiquitin-mediated degradation of β-catenin, allowing β-catenin to translocate to the nucleus and activate target genes (Aberle et al., 1997; Wodarz and Nusse, 1998; Salic et al., 2000). Analysis of β-catenin signaling in embryonic neural progenitor cells has identified that activation of Wnt signaling at E10.5 results in a decrease in neural differentiation but increased neuronal differentiation when activated at E13.5 (Viti et al., 2003; Hirabayashi et al., 2004; Horn et al., 2007). Furthermore, inhibition of Wnt signaling at E13.5 in vivo results in decreased neurogenesis (Hirabayashi et al., 2004), and it is hypothesized that β-catenin regulation of the proneural gene, Ngn1, mediates this effect (Hirabayashi et al., 2004). However, another study obtained contrasting results at E13.5 using Cre-mediated deletion of β-catenin in vivo in a subpopulation of progenitor cells (Woodhead et al., 2006). Taken together, these studies suggest a temporally regulated role for Wnt signaling in regulating neuronal differentiation, although the complexities of this pathway in modulating neuronal differentiation are not understood.

This study examines the role of Sall1 in cortical progenitor cell regulation. Sall1-deficient animals die at birth due to kidney abnormalities (Nishinakamura et al., 2001). In light of the robust expression of Sall1 in cortical progenitor cells during development, we hypothesized a role for Sall1 in regulating cortical neurogenesis. Our studies show that the cerebral hemispheres are decreased in size in Sall1-mutant animals, and the cortical plate is specifically decreased in size. We observed alterations in progenitor cell number and the rate of neuronal differentiation during development in Sall1-deficient animals compared to controls. Early in development more cells exited the cell cycle and progenitor cell number was decreased in Sall1-mutant animals. From mid-neurogenesis, when cortical plate neurons are born, fewer neurons differentiated and, as a result, the progenitor cell population was increased in number by E18.5 in Sall1-/- animals. We determined that the alterations in progenitor cell number from mid-neurogenesis were due in part to alterations in intermediate progenitor cell number. Our studies suggest that Sall1 regulates the rate of neuronal differentiation. Moreover, we postulate that Sall1 regulates progenitor cell maturation by regulating the transition from an early progenitor cell type to an intermediate progenitor cell type.

3.2 MATERIALS AND METHODS

3.2.1 Animals

Embryos were obtained from matings of Sall1 heterozygote, Sall1 Floxed/Floxed and Emx-Cre Sall1+/- animals (Nishinakamura et al., 2001; Gorski et al., 2002). Genotyping was performed as follows: A tail piece was digested in non-ionic detergent and proteinase K overnight at 56°C. Following heat inactivation at 96°C 50ng of DNA was amplified. For Sall1 PCR the following primers and conditions were used: Msal3-64: AGC TAA AGC TGC CAG AGT GC, Msal3-65: CAA CTT GCG ATT GCC ATA AA, S3NeoRev: GCG TTG GCT ACC CGT GAT AT; 94°C 10 minutes, 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 10 minutes. PCR products were 300 base pairs (bp) for the wild type Sall1 allele and 350bp for the mutant allele. Sall1-flox animals were a gift of Dr. R Nishinakamura and genotyped using the following primers and conditions; Flox2: CCT CTG CCC GAG AGA TCG, Flox3: GGC GCG TCT GAT TTT ATT TTC, Flox4 AGG AAC ACT CAC GAA ATG GGG; 94°C 10 minutes, 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 10 minutes. PCR products were 240bp for the wild type allele and 280bp for the floxed allele. Emx1-Cre animals were a gift of Dr. K. Jones (Gorski et al., 2002) and genotyped using the following primers and conditions: Cre 159: TCG ATG CAA CGA GTG ATG AG; Cre 160: TTC GGC TAT ACG TAA CAG GG; OIMR42: CTA GGC CAC AGA ATT GAA AGA TCT; OIMR43: GTA GGT GGA AAT TCT AGC ATC ATC C; 94°C 10 minutes, 30 cycles of 94°C for 45 seconds, 55°C for 30 seconds and 72°C for 1 minute, followed by 72°C for 10 minutes. PCR products were 300bp for the wild type allele and 400bp for the Cre allele.

Embryos were collected via cesarean section at embryonic ages from E11.5 to E18.5 and processed as described in Section 2.2.1. Pregnant dams were injected with 5-bromo-2-deoxyuridine (BrdU) (50µg/g of body weight), dissolved in sterile 0.9% NaCl, and 0.007M NaOH, at the indicated times before embryo harvest. A single injection at 50µg/g body weight results in labeling of 100% of cells in S phase from 15 minutes to two hours following injection, without cell toxicity (Miller and Nowakowski, 1988; Nowakowski et al., 1989; Takahashi et al., 1992). Short-term BrdU injections were administered 60 minutes prior to embryo harvest.

3.2.2 Measurement of brain size

Brains from E18.5 embryos were dissected from the skull and fixed overnight in 4% PFA. Brains were visualized on a Nikon (Melville, NY) dissecting microscope, photographed with a Photometrics (North Reading, MA) Cool Snap digital camera and IP Lab software (Biovision Technologies, Exton, PA). Images were subsequently imported into Photoshop 7.0 (Adobe Systems, San Jose, CA). Analyses were performed by an observer blind to the genotypes. Total brain length was measured as the distance from the rostral extent of the olfactory bulb to caudal extent of the inferior colliculus. Cortical length was measured as the distance from the rostral extent of the cortex to the caudal extent of the cortex at the medial edge of the olfactory bulb. Midbrain length was measured as the distance from the rostral extent of the superior colliculus to the caudal extent of the inferior colliculus. Surface area was analyzed in similarly defined regions. Statistical analysis was performed using an unpaired t test with InStat 3 software (GraphPad Software, San Diego, CA).

3.2.3 Immunohistochemistry

Immunohistochemistry was performed as described in Section 2.2.2. Antibodies used were mouse anti-BrdU (1:25, Amersham Biosciences, Piscataway, NJ); rabbit anti-Brn2 (1:500, Santa Cruz Biotechnology); rat anti-Ctip2 (1:500, Abcam, Cambridge, MA); rabbit anti-Cux1 (1:1000; Santa Cruz Biotechnology); mouse anti-CSPG (1:5, Developmental Hybridoma Bank); rabbit anti-Pax6 (1:500, Covance); mouse anti-Reelin (1:500, Abcam); mouse anti-Sall1 (1:500, PPMX Perseus Proteomics, Tokyo, Japan); rabbit anti-Tbr1 (1:1000, Englund et al., 2005); rabbit anti-Tbr2 (1:1000, Englund et al., 2005); rabbit anti-Tuj1 (1:1000, Sigma). For Nissl staining, sections were incubated in 0.5% cresyl violet (Sigma), dehydrated through alcohols, washed in xylene and mounted in DPX.

3.2.4 *In situ* hybridization

For preparation of anti-sense digoxigenin-labeled riboprobes Dab1 and Vldlr template DNA (a gift of Dr. T. Curran) were linearized with HindIII and SalI respectively and transcribed with T7 RNA polymerase according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). *In situ* hybridization was conducted as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993) with modifications. Briefly, sections were fixed for 10minutes in 4% PFA, washed in PBS, treated with proteinase K, 1µg/ml in 50mM Tris pH 7.5 and 5mM EDTA, at 37°C for 7 minutes, followed by post-fixation in 4% PFA. Sections were acetylated by incubation in 1.3% triethanolamine, 0.002N HCl, and 0.25% acetic anhydride for 10 minutes, followed by extensive washing in PBS. The probe was dissolved at a concentration of 60ng/ml in hybridization solution (50% formamide, 5XSSC, 5 X Denhardt's solution, 250

μg/ml tRNA and 250 μg/ml of herring sperm DNA) and incubated overnight at 61°C. Sections were washed in 5XSSC at 61°C for 5 minutes, treated with RNAse A, 20 μg/ml, in 0.5M NaCl, 10mM Tris pH7.5, 5mM EDTA buffer, at 37°C for 30 minutes, washed in 2XSSC (30 minutes, 61°C), 1XSSC (30 minutes, 61°C), 0.2XSSC (30 minutes, 61°C), 0.2XSSC (15 minutes, room temperature). Slides were subsequently washed in B1 buffer (0.15M NaCl, 0. 1M Tris pH7.5) and blocked in 10% HINGS in B1 for 1 hour at room temperature. The sections were incubated overnight with anti-digoxigenin antibody (1:2500 in B1 with 1% HINGS). Following washes in B1, sections were incubated in buffer B3 (0.1M Tris pH9.5, 0.1M NaCl, 0.05MMgCl_2). Visualization of antibody was performed by incubating sections with 0.34% Nitro blue tetrazolium chloride and 0.35% 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt dissolved in B3. Following color detection, slides were rapidly dehydrated, washed in xylene, and mounted in DPX.

Radioactive *in situ* hybridization was performed as previously described (Monaghan et al., 1993). For generation of ^{35}S labeled Reelin probe, Reelin template DNA (a gift of Dr. T. Curran) was linearized with HindIII and transcribed with T3 RNA polymerase in the presence of ^{35}S UTP. Paraffin embedded sections were washed in xylene and rehydrated through an ethanol series. Sections were washed in PBS, treated with proteinase K, 1μg/ml in 50mM Tris pH 7.5 and 5mM EDTA, at 37°C for 30 minutes followed by post-fixation in 4% PFA. Sections were acetylated by incubation in 1.3% triethanolamine, 0.002N HCl, and 0.25% acetic anhydride for 10minutes, followed by extensive washing in PBS. Sections were dehydrated through an ethanol series. The probe was dissolved at a concentration of 60ng/ml in hybridization solution (50% formamide, 5XSSC, 5 X Denhardt's solution, 250 μg/ml tRNA and 250 μg/ml of herring sperm

DNA) and incubated overnight at 61°C. Sections were washed in Buffer 1 (50% formamide, 10mM DTT, 0.3M NaCl, 0.01M $NaPO_4$ pH6.8, 0.005M EDTA, 0.02 Tris pH6.8) at 64°C for 60 minutes, followed by Buffer NTE (0.5MNaCl, 0.1M Tris pH8.0, 0.005M EDTA) (37°C 15 minutes). Sections were treated with RNAse A, 20 μg/ml, in Buffer NTE (37°C 30 minutes), washed in Buffer NTE (37°C 15 minutes), Buffer 1 (64°C for 60 minutes), 2XSSC (room temperature 15 minutes), 0.1XSSC (50°C 15 minutes), and 0.1XSSC (room temperature 30 minutes). Sections were counterstained with nuclear fast red and dehydrated through an ethanol series and allowed to air dry. Sections were dipped in Kodak NTB2 emulsion diluted 1:1 with 0.6M NH_4Ac and exposed at 4°C for 7 to 21 days. Sections were developed using Kodak D19 developing solution and Kodakfix at 15°C for 4minutes.

3.2.5 Assignment of cellular morphology

Cellular subtypes and boundaries were assigned as described in Section 2.2.3.

3.2.6 Cell counts

For quantification of cell number in the dorsal cortex stained sections were imaged as described above. Sub-regions within the cortex were histologically distinguished. For all analyses a 150μm region of the dorsal cortex were counted across three sections and the mean of the three sections per animal was compared between genotypes. The analyses were performed by an observer blind to the genotype. For proliferative studies (E11.5, E14.5, and E17.5), BrdU and DAPI stained sections were examined and the number of BrdU-positive and total number of progenitor cells was quantified and analyzed as previously described (Roy et al., 2004). The

labeling index was determined as the number of BrdU-positive cells divided by total progenitor cell number. For birth-dating studies (E18.5 embryos injected with BrdU on E11.5, E12.5, E14.5, or E16.5), BrdU stained sections were examined and heavily labeled BrdU-positive cells were quantified. For quitting fraction studies (E12.5 embryos injected with BrdU on E11.5, E15.5 embryos injected with BrdU on E14.5, and E18.5 embryos injected with BrdU on E17.5) BrdU/Tuj1 stained sections were imaged and BrdU-positive cells were quantified as either single positive for BrdU or double positive for BrdU/Tuj1. The quitting fraction was determined as the proportion of cells that were double positive divided by the total BrdU-positive population. The data subsequently were decoded, and statistical analysis of the results was performed using an unpaired t-test with InStat 3 software.

3.3 RESULTS

3.3.1 The cerebral cortex is decreased in size at E18.5 in Sall1-/- animals

Sall1-deficient animals die at birth due to kidney deficits (Nishinakamura et al., 2001). In light of the robust expression of Sall1 in cortical progenitor cells, we postulated that Sall1 regulates cerebral cortical development. We therefore examined the developing telencephalon in Sall1-deficient animals from E11.5 to E18.5. To determine whether major cortical malformations were present in Sall1-deficient animals, we first examined the gross anatomy of the brain of Sall1-mutant animals at E18.5 (Figure 11A,B). In Sall1-mutant animals, a decrease in size of the olfactory bulbs was observed (Figure 11B). Furthermore, the total length and dorsal surface area of the brain was decreased by 8.4% ($p<0.01$, $n=3$) and 12.2% ($p<0.01$, $n=3$), respectively, in

Sall1-deficient animals compared to controls (Figure 11C,D). Specifically, in Sall1-mutant animals the cerebral cortex (CC, Figure 11A,B) was decreased in length and dorsal surface area by 12.1% ($p<0.01$, n=3) and 17.5% ($p<0.01$, n=3), respectively (Figure 11C,D). However, no alteration in the length ($p=0.93$, n=3) or dorsal surface area ($p=0.49$, n=3) of the midbrain was observed in Sall1-mutant animals compared to controls (MB, Figure 1A-D). Thus, Sall1-mutant animals have a smaller cerebral cortex than controls; however, no gross anatomical abnormalities were evident in the rest of the brain in Sall1-/- animals at E18.5 (Figure 11B). These data suggest that the developing cerebral cortex is particularly sensitive to the loss of Sall1 and support a role for Sall1 in regulating cortical development.

To assess the basis of the decrease in cortical size in Sall1-mutant animals, we histologically examined serial coronal sections of the cortex at E18.5 (n=4) (Figure 11E-J). Alterations in cortical structures were observed in both the dorsal and ventral telencephalon (Figure 11E-H). The cortical plate appeared decreased in size in Sall1-/- animals at all rostral-caudal levels compared to controls (CP, Figure 11F,H,J). In ventral regions the striatum appeared smaller (S, Figure 1F). In addition, an increase in the number of darkly stained cells present in the lateral and caudal ganglionic eminences was observed in Sall1-deficient animals (*,Figure 11F,H), which suggests an increase in the number of progenitor cells in the ventral telencephalon. The hippocampus (H, Figure 11G,H) and dentate gyrus (DG, Figure 11G,H) were identifiable in medial regions in control and Sall1-deficient animals.

We quantified the total number of cells in the dorsal cortex and observed an 8.9% decrease in total cell number in Sall1-deficient animals compared to controls (985.2±29.2 in controls versus

897.8±16.8 in Sall1-/- animals, n=4, $p<0.05$). To determine whether specific populations were disproportionately reduced in Sall1-mutant animals we quantified the number of cells in defined sub-regions of the dorsal cerebral wall. We histologically distinguished the progenitor domain, the intermediate zone, the cortical plate and the marginal zone (Figure 11I,J), as described in the Materials and Methods section. In Sall1-mutant animals, a 14.9% increase in the number of progenitor cells was observed at E18.5 in Sall1-/- animals compared to controls ($p<0.05$, n=4) (Figure 11K). In addition, the cortical plate was decreased in cell number by 25.8% in Sall1-mutant animals compared to controls ($p<0.05$, n=4) (Figure 11K). These data suggest a role for Sall1 in regulating cortical cell number. Alterations in cell survival in Sall1-deficient animals could lead to decreased cell number in the cerebral cortex at E18.5. We examined activated caspase 3 staining at E12.5 (n=2), E14.5 (n=2) and E18.5 (n=4). No gross differences in cell death were observed between Sall1-/- and control animals at these ages, which suggests that Sall1 is not required for cell survival during cortical development. Taken together, these findings suggest that Sall1 regulates cortical neurogenesis or neuronal migration.

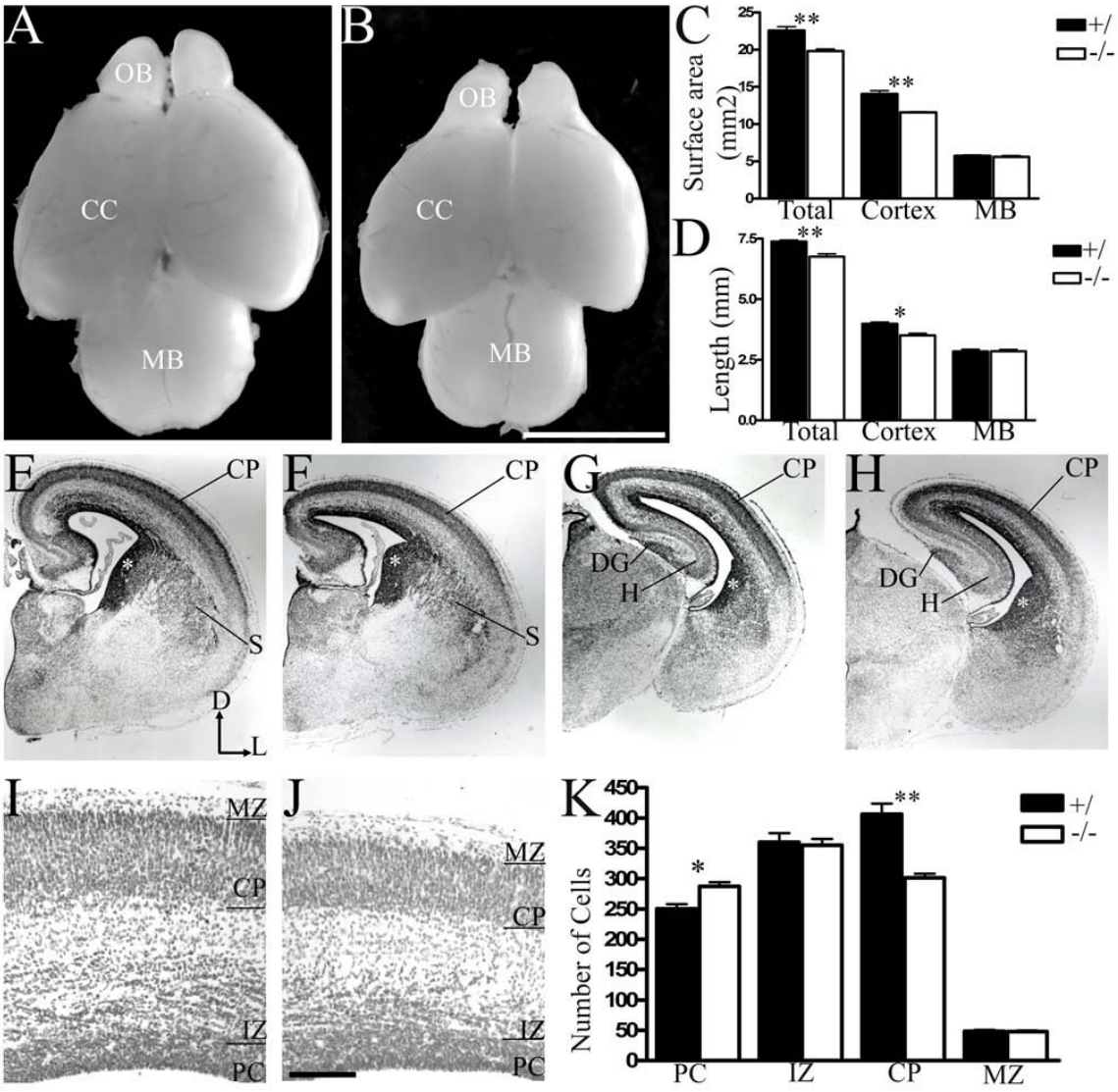

Figure 11. Histological examination of cortical development in control and Sall1-deficient animals.

The cerebral cortex is decreased in size in Sall1-deficient animals (B) compared to controls (A) at E18.5. Measurements of brain surface area (C) and length (D) of control and Sall1-/- animals. Nissl stained coronal sections of the cortex at E18.5 (E-J) in control (E,G,I) and Sall1-/- animals (F,H,J). The cortical plate and striatum are decreased in size in Sall1-deficient animals (E-J). *, Ventral progenitor population (E-H). Quantification of dorsal cell number in control and Sall1-mutant animals (K). *, $p<0.05$; **, $p<0.01$ (K). OB: olfactory bulb; CC: cerebral cortex; MB: midbrain; CP: cortical plate; S: striatum; DG: dentate gyrus; H: hippocampus; PC: progenitor population; IZ: intermediate zone; CP: cortical plate; MZ: marginal zone. Scale bar in B represents: 2500µm in A,B; Scale bar in K represents: 100µm in I,J; 500µm in E-H.

3.3.2 Sall1 regulates preplate formation

Cortical neurogenesis initiates at E9.5, when a subpopulation of progenitor cells in the dorsal cortex differentiate, producing neurons primarily destined for the preplate. Cells destined for the preplate are born from E10.5 to E13.5 (Price et al., 1997; Sheppard and Pearlman, 1997), and at E12.5 a layer of neurons, several cells thick, the preplate, is observed in the dorsal cortex. In order to determine the onset of the deficits observed in Sall1-/- animals, we examined preplate formation at E11.5 and E12.5 using the pan neuronal marker Tuj1 (Figure 12A-C). Two cellular populations were evident in the dorsal cerebral cortex, the preplate (Tuj1-positive) and the progenitor population (Tuj1-negative) (Figure 12A). We quantified the number of neurons and progenitor cells in the dorsal cortex at E11.5 and E12.5 (n=3, E11.5; n=4, E12.5) (Figure 12C). At E11.5, no difference in the number of progenitor cells or neurons was observed in Sall1-/- animals compared to controls. However, by E12.5 a 24.6% increase in the number of neurons in the preplate ($p<0.05$, n=4) was observed in Sall1-mutant animals compared to controls (Figure 12A-C). This increase in the number of neurons is accompanied by a 17.6% decrease in the number of progenitor cells ($p<0.01$, n=4) and a 10.8% decrease in total cell number ($p<0.05$, n=4). These findings suggest that in the absence of Sall1 more neurons differentiate at the expense of re-entering the cell cycle early in cortical development.

From ~E13.5, differentiating cells radially migrate towards the pial surface and split the preplate into the marginal zone, superficial, and the subplate, deep, to form the cortical plate (reviewed in Gupta et al., 2002). To determine whether structures derived from the preplate were also increased in size in Sall1-mutant animals, we examined molecular markers of the marginal zone and subplate at E14.5 and E18.5. Chondroitin sulphate proteoglycan (CSPG) is an extracellular

matrix protein expressed by cells in the subplate and marginal zone (Figure 12D-G) (Sheppard et al., 1991). In Sall1-mutant animals, an increase in CSPG staining was observed in the subplate and marginal zone at E14.5 (n=3) (Figure 12D,E) and E18.5 (n=4) (Figure 12F,G) compared to controls. Reelin is a secreted molecule expressed by Cajal Retzius cells in the marginal zone (Figure 12H-K') (Ogawa et al., 1995; Schiffmann et al., 1997). In Sall1-deficient animals, an increase in the number of Reelin-expressing cells in the marginal zone was also observed at E14.5 (n=4) (Figure 12H,I) and E18.5 (n=5) (Figure 12J,K, arrows, Figure 12J'K') compared to controls. These data suggest that, in the absence of Sall1 more cells are committed to early born structures, the preplate, and its derivatives, the marginal zone and subplate.

The cortical plate can be histologically distinguished from E14.5. In Sall1-mutant animals the cortical plate was decreased in cell number at E18.5 compared to controls. To examine the onset of the decrease in cortical plate size, we quantified the number of cells in the cortical plate at E14.5. We observed a 28.9% decrease in the number of cells in the cortical plate at E14.5 in mutant animals compared to controls (n=3, $p<0.05$; 70.5±5.0 cells in controls versus 50.1±3.5 cells in Sall1-mutants). These data suggest that in the absence of Sall1 fewer cells are committed to the late born structure, the cortical plate, from E14.5.

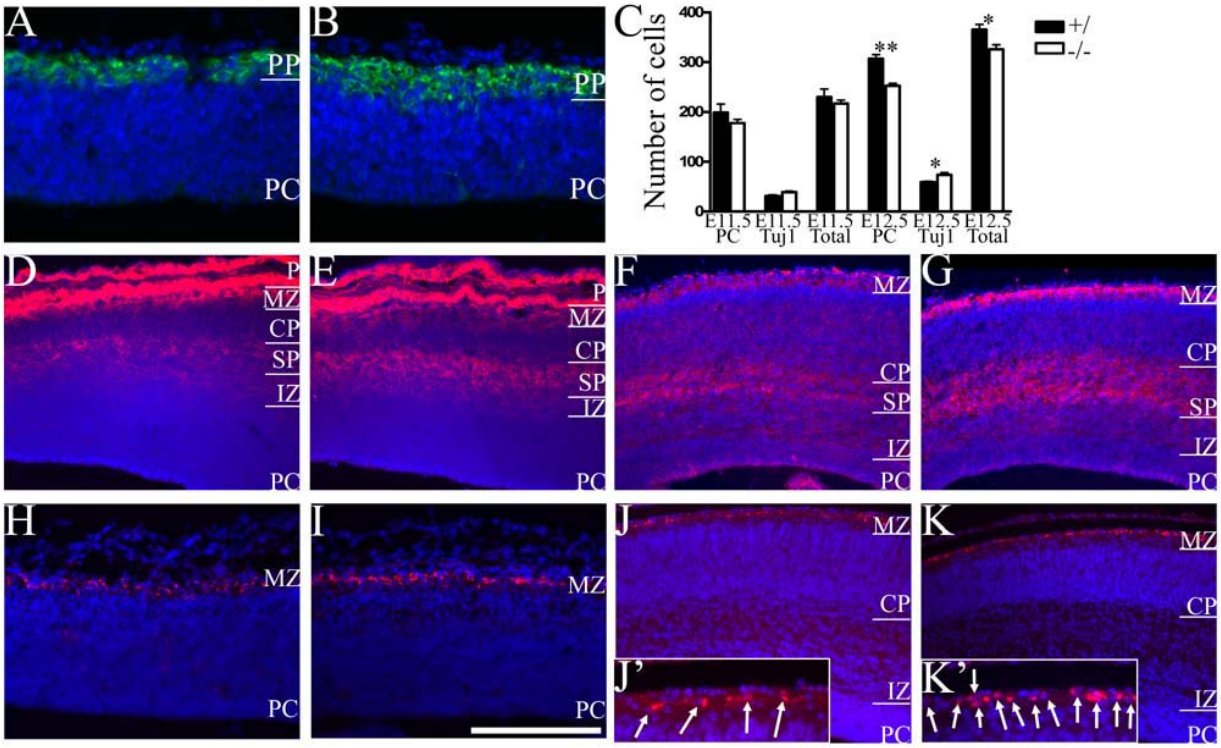

Figure 12. Early neurogenesis in control and Sall1-deficient animals

Tuj1 (green) staining identified an increase in preplate size at E12.5 in Sall1-deficient animals (B) compared to controls (A). Quantification of cortical cell number at E11.5 and E12.5 (C). CSPG (red) immunostaining of the subplate and marginal zone identified an increase in these structures in Sall1-/- animals (E,G) compared to controls (D,F) at E14.5 (D,E) and E18.5 (F,G). Reelin (red) immunostaining identified an increase in Cajal-Retzius cells Sall1-/- (I,K,K') compared to control in controls (H,J,J'), at E14.5 (H,I) and E18.5 (J,J',K,K'). Sections are counterstained with DAPI (blue) (A,B,D-K'). *, $p<0.05$; **, $p<0.01$. PP: preplate; PC: progenitor population; IZ: intermediate zone; SP: subplate; CP: cortical plate; MZ: marginal zone; P: pial surface. Scale bar (in I) represents: 80µm in J'K'; 100µm A,B; 200µm in D,E,H,I,J,K; 400µm in F,G.

3.3.3 Sall1 regulates the cellular level of Reelin expression

Our studies identified an increase in the number of Reelin-expressing Cajal Retzius cells in the marginal zone in Sall1-mutant animals at E14.5 and E18.5. We extended our analysis of this altered Reelin expression by examining the level of Reelin expression on a per cell basis in the marginal zone using radioactive in situ hybridization in control and Sall1-mutant animals. We observed an increase in the level of Reelin expression per cell in Sall1-mutant animals compared

to controls (Figure 13A,B). We quantified this effect and observed a 2.7-fold increase in the level of Reelin expression per cell at E14.5 ($n=2$, $p<0.001$) and a 4.5-fold increase in the level of Reelin expression per cell at E17.5 ($n=2$, $p<0.05$) (Figure 13C). Taken together, these data suggest that Sall1 regulates neurogenesis of Reelin-expressing cells, as well as the level of Reelin expression on a cellular basis.

In order to determine whether downstream components of the Reelin signaling pathway were altered in Sall1-mutant animals, we examined expression of the transmembrane ligand for Reelin, Vldlr, and intracellular mediator of Reelin signaling, Dab-1. Vldlr is expressed by cells in the cortical plate and intermediate zone, and in Sall1-mutant animals an increase in Vldlr expression in the cortical plate was observed compared to controls (Figure 13D,E). Dab-1 is robustly expressed by the majority of cells in the upper cortical plate, and a subset of cells in the lower cortical plate and intermediate zone (Figure 13F). In Sall1-deficient animals, Dab-1 was strongly expressed by the majority of cells in both the upper and lower cortical plate (Figure 13G), which suggests an alteration in the population of neurons expressing Dab-1 in the absence of Sall1. These findings implicate Sall1 in regulating Reelin expression and components of the Reelin signaling pathway.

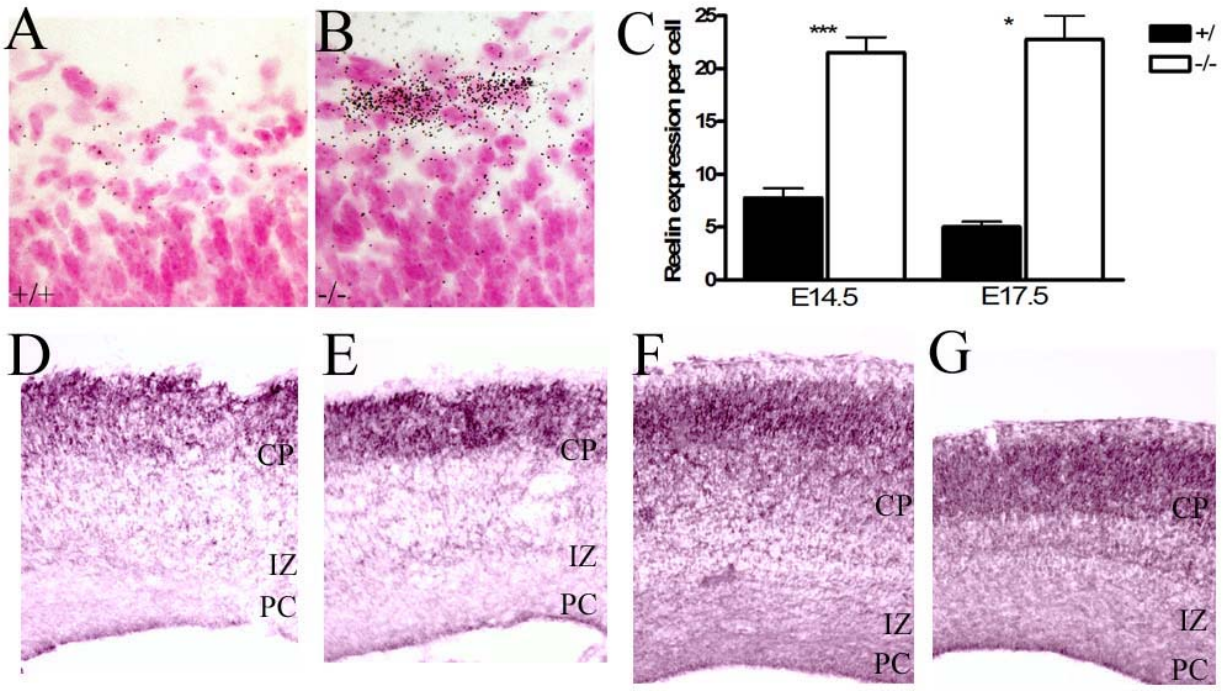

Figure 13. Reelin signaling in control and Sall1-deficient animals

Reelin expression (black) is increased in Sall1 -/- animals (B) compared to controls (A) at E17.5. Sections are counterstained with nuclear fast red. Quantification of Reelin expression on a per cell basis at E14.5 and E17.5. In situ hybridization of Vldlr (D,E) and Dab-1 (F,G) expression (purple) in the cortex at E18.5 in control (D,F) and Sall1-deficient (E,G) animals. PC: progenitor population; IZ: intermediate zone; CP: cortical plate. *, $p<0.05$; ***, $p<0.001$.

3.3.4 Sall1 regulates the number of cells committed to an early cortical fate

Our studies have determined that, in the absence of Sall1, more cells are committed to early born structures, the preplate and its derivatives, the marginal zone, and the subplate, and fewer cells are committed to the later born structure, the cortical plate. The cortex is patterned in an inside-out manner during development, and the Reelin signaling pathway is a critical regulator of layer patterning (Angevine and Sidman, 1961; Gupta et al., 2002). To determine whether the temporal pattern of neurogenesis is preserved in the absence of Sall1, we examined the laminar position of cells born during early neurogenesis (E11.5 and E12.5), mid-neurogenesis (E14.5) and late

neurogenesis (E16.5) at E18.5. In control animals, cells born on E11.5 are predominately located in the upper intermediate zone, within the subplate region, and the marginal zone at E18.5 (Figure 14A, 15A), consistent with previous studies (Price et al., 1997). In Sall1-mutant animals, we observed a 41.0% increase in the total number of cells born on E11.5 compared to controls (13.9±0.9 cells in controls versus 19.6±1.0 cells in Sall1-mutant animals, n=3, $p<0.05$). In Sall1-deficient animals we observed an increase in the proportion of cells born on E11.5 located in the intermediate zone region (n=3, $p<0.05$), accompanied by a decrease in the proportion of cells observed in the cortical plate (n=3, $p<0.05$) compared to controls (Figure 14A,15B). This suggests that, in the absence of Sall1, cells born on E11.5 are selectively committed to a subplate fate. In control animals, the majority of cells born on E12.5 are committed to the subplate (50.6±2.7%) and the cortical plate (29.9±2.2%) (Figure 14B,15C). In Sall1-deficient animals, a 26.3% increase in the total number of cells born on E12.5 was observed compared to controls (33.9±0.8 cells in controls versus 42.8±1.7 cells in Sall1-/- animals, n=4, $p<0.01$). Furthermore, we observed an increase in the proportion of cells born on E12.5 committed to the subplate at E18.5 in Sall1-deficient animals (n=4, $p<0.05$) (Figure 14B,15D). These data indicate that in the absence of Sall1 more cells born on E12.5 are committed to a subplate fate. Taken together, these findings suggest that Sall1 regulates the number of cells committed to an early cortical fate.

In control animals and Sall1-deficient animals, the majority of cells born on E14.5 are located within the cortical plate at E18.5 (Figure 14C,15E,F). The total number of cells born on E14.5 was similar in Sall1-deficient animals and controls (152.1±4.1 cells in controls versus 163.2±3.4 cells in Sall1-mutant animals, n=3, $p=0.1$). A 21.2% decrease in the proportion of cells committed to a cortical plate fate was observed in Sall1-/- animals compared to controls (n=3,

$p<0.01$). This was accompanied by a 22.0% ($n=3$, $p<0.05$) and 98.9% ($n=3$, $p<0.05$) increase in the proportion of cells in the intermediate zone and progenitor population, respectively (Figure 14C). The majority of cells born on E14.5 located within the intermediate zone at E18.5 were observed below the subplate in Sall1-deficient animals (Figure 15F), which suggests a delay in differentiation or an alteration in migration of this population. No alteration in the total number (167.0±4.7 cells in controls versus 164.4±3.1 cells in Sall1-deficient animals, $n=3$, $p=0.6$) or laminar distribution of cells born on E16.5 was observed at E18.5 (Figure 14D,15G,H). Taken together, these data suggest that the inside-out pattern of neurogenesis is preserved during development, but that the temporal production of neurons may be altered in the absence of Sall1. Furthermore, at E14.5 an alteration in differentiation, migration, or laminar specification of neurons is observed in Sall1-deficient animals.

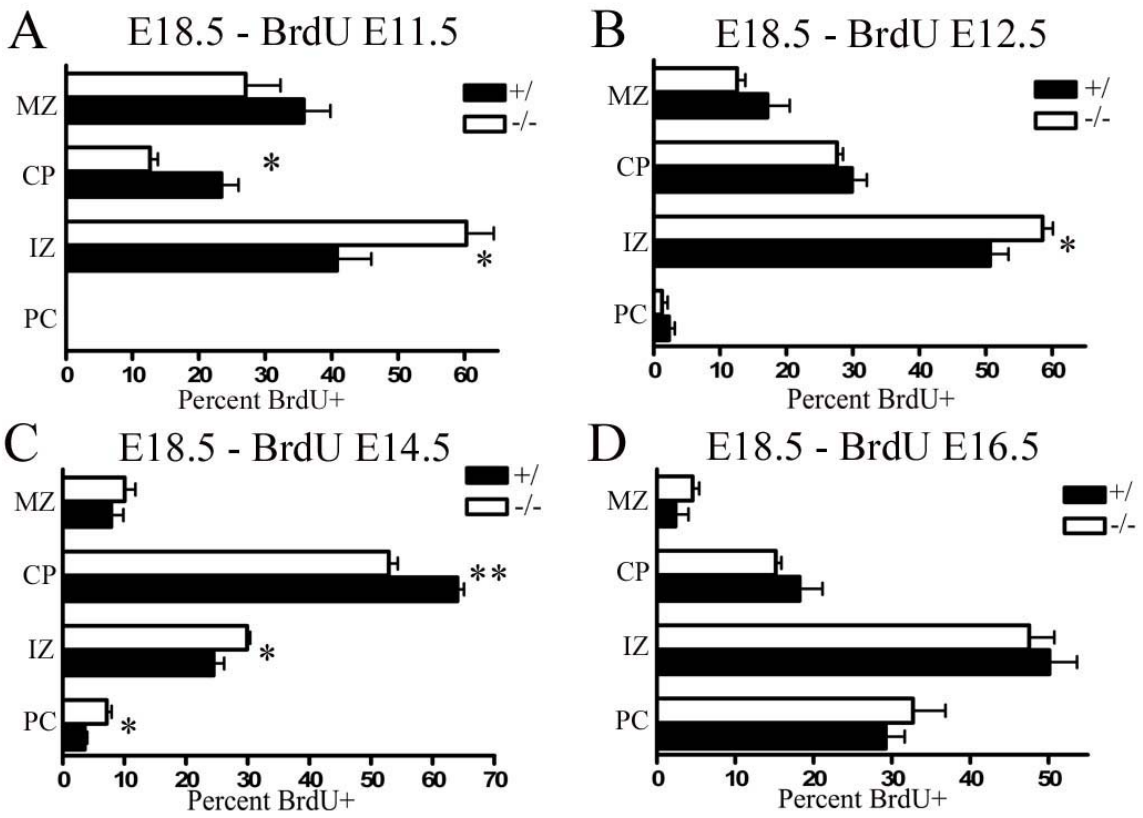

Figure 14. Birth-dating of cells during cortical development

Quantification of the proportion of total cells born on E11.5 (A), E12.5 (B), E14.5 (C) or E16.5 (D) in defined cortical regions at E18.5. *, $p<0.05$; **, $p<0.01$. PC: progenitor population; IZ: intermediate zone; CP: cortical plate; MZ: marginal zone.

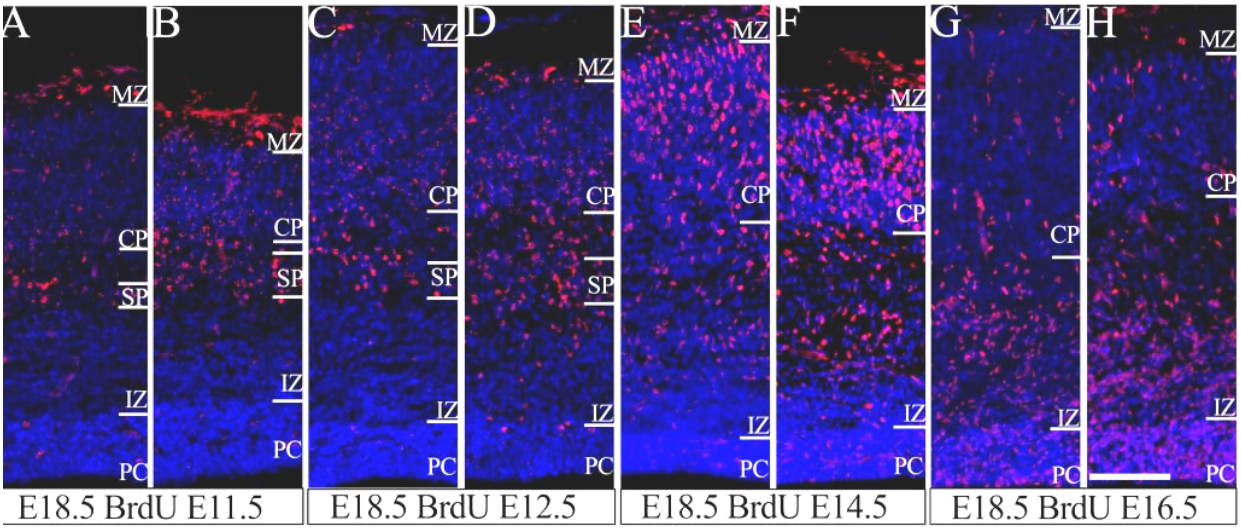

Figure 15. Birth-dating of cells during cortical development

Laminar position of cells born on E11.5 (A,B), E12.5 (C,D), E14.5 (E,F), or E16.5 (G,H) (labeled cells are red) in the cortex at E18.5 in control (A,C,E,G) and Sall1-deficient (B,D,F,H) animals. Sections are counterstained with DAPI (blue). Scale bar (in H) represents: 100μm in A-H

3.3.5 The cortical plate is appropriately specified in Sall1-mutant animals

Our studies identified that, in the absence of Sall1, more cells were committed to an early cortical fate. The later born structure, the cortical plate, was decreased in cell number from E14.5 compared to controls. The cortical plate is a laminar structure containing populations of cells that exhibit distinct molecular characteristics (Hevner et al., 2003; Molyneaux et al., 2007). In order to determine whether cells destined for the cortical plate were appropriately specified in the absence of Sall1 we examined layer specific markers at E18.5. Tbr1 is a transcription factor that is strongly expressed by cells in the subplate, marginal zone, and cortical plate (Bulfone et al., 1995). Within the cortical plate Tbr1 is expressed by cells in layers VI and II/III (Bulfone et al., 1995). At E18.5, cells expressing high levels of Tbr1 were observed in the subplate and layer VI, with weakly expressing Tbr1-positive cells located in the upper cortical plate (Figure 16A).

No difference in the pattern of strong and weak Tbr1-expressing cells was observed between control and Sall1-/- animals, which indicates that deep and upper cortical layers are appropriately specified (n=4) (Figure 16A,B). The C_2H_2 zinc finger protein Ctip2 is highly expressed by cells in layer V and weakly expressed by cells in layer VI at E18.5 (Avram et al., 2000; Arlotta et al., 2005) (Figure 16C). In Sall1-deficient, animals no alteration in the pattern of Ctip2 expression was observed at E18.5 (n=4) (Figure 16D), which suggests appropriate specification of deep cortical plate layers. Brn2 is expressed by layer V and layer II/III cells, as well as cells in the progenitor populations and intermediate zone at E18.5 (He et al., 1989; McEvilly et al., 2002) (Figure 16E). No alteration in the position of Brn2-positive cells was observed in Sall1-deficient animals, which suggests appropriate specification of layer V and II/III cells (n=4) (Figure 16F). The transcription factor Cux1 is expressed by cells in upper cortical layers II/III and IV, as well as cells in the progenitor population and intermediate zone at E18.5 (Figure 16G) (Nieto et al., 2004). In Sall1-mutant animals no difference in the position of Cux1-positive cells was observed at E18.5 (n=4) (Figure 16H), suggesting appropriate specification of upper cortical plate layers. Taken together these data suggest that loss of Sall1 does not alter cell type specification of neurons destined for the cortical plate.

Our data suggest that the cortical plate is appropriately specified in Sall1-mutant animals; however, the number of cells in each layer expressing layer specific markers appeared decreased in Sall1-deficient animals compared to controls (Figure 16A-H). To further assess the decrease in cortical size, we quantified the number of cells in the cortex using Tbr1 immunostaining. A 62.4% increase in the number of Tbr1-positive cells in the subplate was observed in Sall1-mutant animals (n=3, $p<0.01$) (Figure 16E). Within the cortical plate we assigned strong Tbr1-positive

cells to layer VI. In the upper cortical plate, Tbr1-negative cells were assigned as layer V/IV, and Tbr1-positive cells as layers II/III. A decrease in cell number was observed in all cortical layers in the absence of Sall1 compared to controls (Figure 16E). Interestingly, when this decrease was expressed as a percent of total cortical plate cell number no significant difference was observed between wild type and Sall1-mutant animals (Figure 16F). These data suggest that in the absence of Sall1 all cortical layers are proportionally decreased in cell number. In summary, our data suggest that cells in the cortical plate are proportionally decreased but appropriately specified in the absence of Sall1.

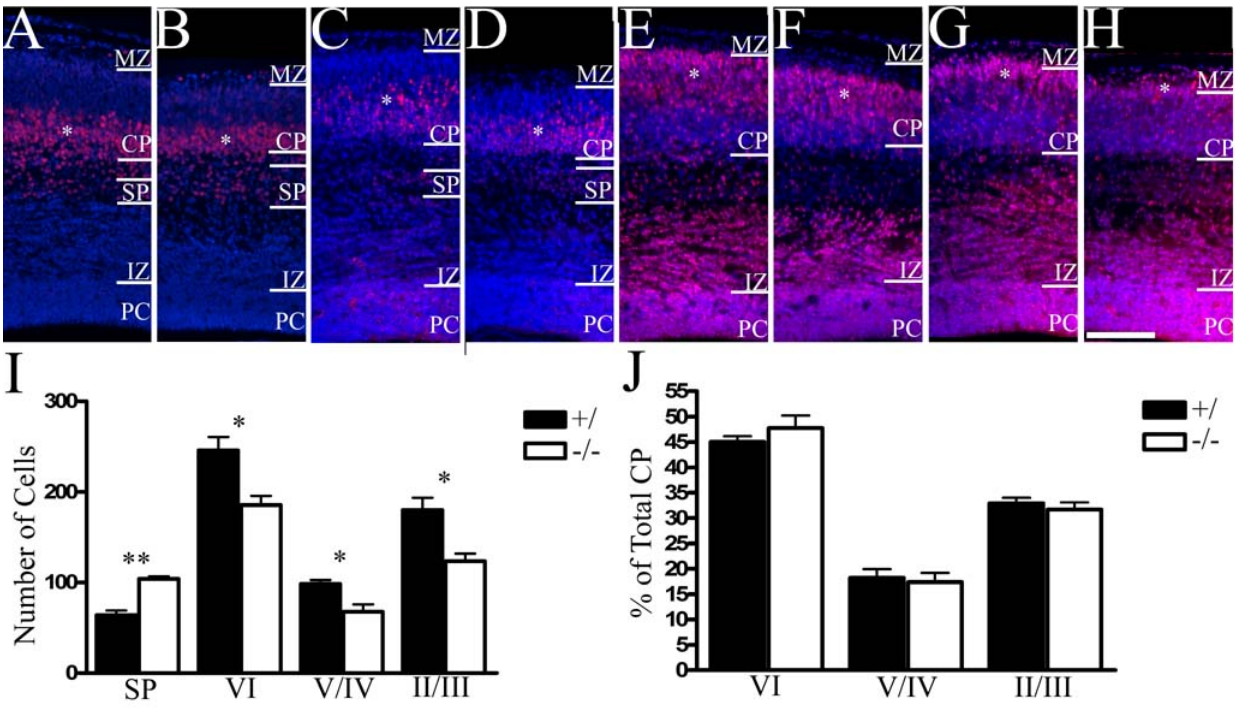

Figure 16. Cortical plate cell type specification and lamination at E18.5

Tbr1 (red) staining of subplate and layer VI (*) cells in control (A) and Sall1-/- animals (B). Ctip2 (red) staining of layer VI and V (*) cells in control (C) and Sall1-/- (D) animals. Brn2 (red) staining of layer V and layer II/III cells (*) in control (E) and Sall1-/- (F) animals. Cux1 (red) staining of layer II/III (*) cells in control (G) and Sall1-/- (H) animals. Sections are counterstained with DAPI (blue). Quantification of cortical plate layer cell number at E18.5 (I). Quantification of the proportion of cells in cortical layers as a percent of total cortical cell number at E18.5 (J). I,J: *, $p<0.05$; **, $p<0.01$. PC: progenitor population; IZ: intermediate zone; SP: subplate; CP: cortical plate; MZ: marginal zone. Scale bar (in H) represents: 100μm in A-H.

3.3.6 Sall1 does not regulate progenitor cell proliferation

We hypothesized that the alterations observed in Sall1-deficient animals were due to a role for Sall1 in regulating progenitor cell proliferation or differentiation. Figure 17 summarizes alterations in progenitor cell number in Sall1-mutant animals during development. Early in development (E12.5) we observed a decrease in the number of progenitor cells in Sall1-mutant

animals (Figure 12C,17). At E14.5 (n=4, p=0.3) and E17.5 (n=4, p=0.3), no significant difference in progenitor cell number was observed in the absence of Sall1 compared to controls (Figure 17). However, by E18.5, an increase in progenitor cell number is observed in Sall1-deficient animals compared to controls (Figure 11K,17). To determine whether Sall1 regulates progenitor cell proliferation, we compared the rate of progenitor cell proliferation using short-term BrdU labeling in control and Sall1-deficient animals from E11.5 to E17.5. The labeling index was determined as the number of BrdU-positive cells over the total number of progenitor cells. No significant difference in the labeling index was observed at E11.5 (n=3, p=0.7), E14.5 (n=3, p=0.2) or E17.5 (n=4, p=0.5) (Figure 18A), which indicates that the number of cells in S phase of the cell cycle is not altered by the absence of Sall1 and suggests that Sall1 does not regulate the rate of progenitor cell proliferation of cortical progenitors.

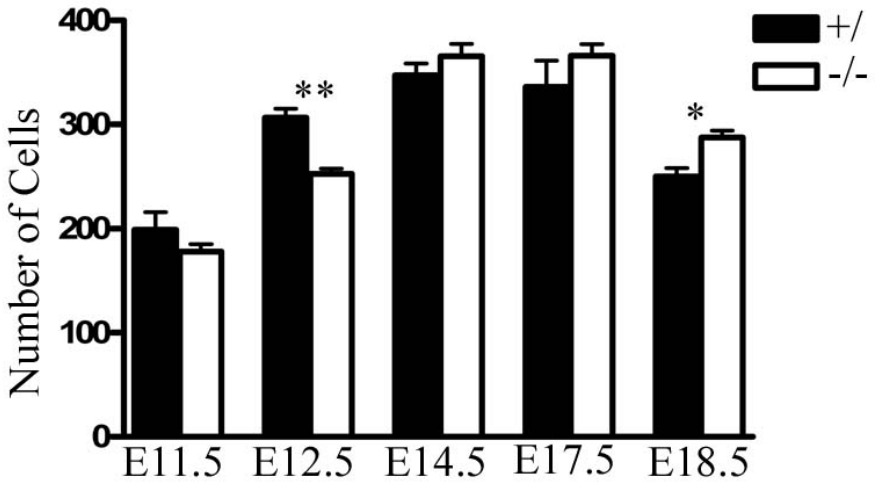

Figure 17. Progenitor cell number during development

Quantification of progenitor cell number at E11.5, E12.5, E14.5, E17.5, and E18.5 in control and Sall1-/- animals. *, $p<0.05$; **, $p<0.01$.

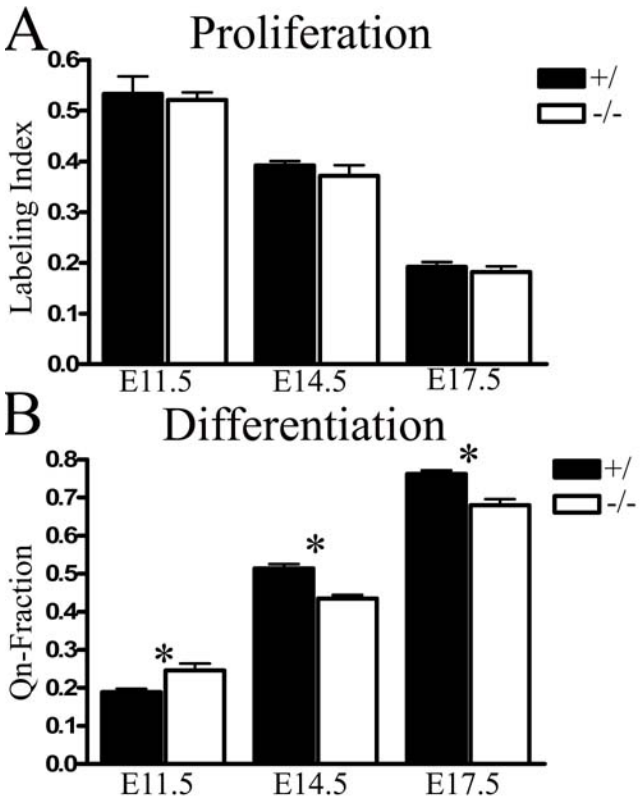

Figure 18. Rate of proliferation and differentiation at E11.5, E14.5 and E17.5.

The labeling index was not altered in Sall1-deficient animals at E11.5, E14.5 and E17.5 compared to controls (A). The Q_n fraction was increased at E11.5, but decreased at E14.5 and E17.5 in Sall1-/- animals compared to controls (B). *, $p<0.05$.

3.3.7 Sall1 regulates progenitor cell differentiation

As Sall1 does not regulate the rate of progenitor cell proliferation we therefore hypothesized that Sall1 may regulate the rate of cell cycle exit. We examined the number of differentiating cells at distinct developmental time points. Pregnant dams were injected with BrdU on either E11.5, E14.5, or E17.5, and embryos were collected 24 hours later. To determine the fraction of cells that had differentiated into neurons we quantified the number of BrdU-positive/Tuj1-positive as a fraction of the total number of BrdU-positive cells. This was termed the neuronal quitting-fraction (Q_n), and Q_n gradually increases from 0 to 1 in control animals from E9.5 to E18.5 (Takahashi et al., 1994). In control animals at E11.5, Q_n was 0.19±0.01 (Figure 18B). At E11.5, in Sall1-deficient animals Q_n was 0.24±0.02, representing a 30.1% increase in differentiation over controls (n=4, $p<0.05$) (Figure 18B). By E14.5, Q_n is 0.51±0.01 in control animals (Figure 18B). However, in Sall1-mutant animals Q_n was 0.44±0.01 at E14.5, representing a 15.9% decrease in the differentiating population (n=3, $p<0.05$) (Figure 18B) and suggesting that more cells are re-entering the cell cycle compared to controls at that age. By E17.5, Q_n increases to 0.76±0.01 in control animals (Figure 18B), as neurogenesis enters its terminal phase, with the majority of cells differentiating and consequent depletion of progenitor cells occurs. In Sall1-deficient animals Q_n was 0.68±0.02 (Figure 16B), representing a 10.5% decrease in Q_n compared to controls (n=4, $p<0.05$). Thus, in the absence of Sall1 early progenitor cells differentiate rather than re-enter the cell cycle, but later in neurogenesis, progenitor cells re-enter the cell cycle rather than differentiating. Consistent with these findings, the total number of progenitor cells was decreased in Sall1-mutant animals at E12.5, but by E14.5 no significant difference in

progenitor cell number was observed between control and Sall1-/- animals. Similarly, at E17.5, Q_n was decreased in Sall1-mutant animals, and by E18.5 an increase in the number of progenitor cells is observed in Sall1-deficient animals compared to controls. These data suggest that Sall1 is a critical regulator of progenitor cell differentiation

3.3.8 Sall1 regulates the number of intermediate progenitor cells during development

Our studies indicate that E14.5 is a critical developmental point when Sall1 switches from promoting a proliferative fate to a differentiative fate. From E14.5, a second proliferative population, the subventricular zone, is observed in the cortex (BoulderCommittee, 1970). This population consists of intermediate progenitor cells, and recent data suggest that the majority of neurons in the cortical plate are produced via neurogenic divisions of intermediate progenitor cells (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). It is difficult to accurately distinguish the ventricular and subventricular zones using histological methods, as extensive mixing of the populations occurs at the boundary of these two populations (Takahashi et al., 1995b). We examined Pax6 immunostaining to molecularly distinguish the ventricular zone population. At E18.5, no significant difference in the number of Pax6-positive cells was observed in the ventricular zone population in Sall1-deficient animals compared to controls (115.8±3.7 cells in controls versus 121.8±1.0 cells in Sall1-mutant animals, n=4, p=0.2). These data indicate that the increase in progenitor cell number at E18.5 in Sall1-deficient animals is not due to an increase in the number of cells in the ventricular zone. To determine whether the alterations in progenitor cell number and neuronal differentiation were associated with changes in subventricular zone progenitor cells, we examined this population throughout development. Tbr2 is a transcription factor that is expressed by intermediate progenitor cells (Englund et al.,

2005). We quantified the number of Tbr2-positive cells in the cortex at E14.5, E17.5, and E18.5 (Figure 19A-E). At E14.5, a 48.1% decrease in the number of Tbr2-positive cells was observed in the cortex in Sall1-mutant animals compared to controls ($n=3$, $p<0.01$, Figure 19A,B,E). By E17.5, no significant difference in the number of Tbr2-positive cells is observed between control and Sall1-deficient animals ($n=3$, $p=0.7$, Figure 19E). However, by E18.5 a 23.0% increase in the number of Tbr2-positive cells was observed in Sall1-mutant animals compared to controls ($n=3$, $p<0.05$, Figure 19C-E). These data suggest that changes in the number of Tbr2-positive intermediate progenitor cells contribute to the alterations in progenitor cell number observed in Sall1-deficient animals. Furthermore, these data suggest that from E14.5 more proliferating cells commit to a Tbr2-positive intermediate progenitor fate as opposed to differentiating into neurons in the absence of Sall1. These findings suggest that Sall1 regulates the transition from an early progenitor state to an intermediate progenitor state, which suggests that Sall1 regulates progenitor cell maturation.

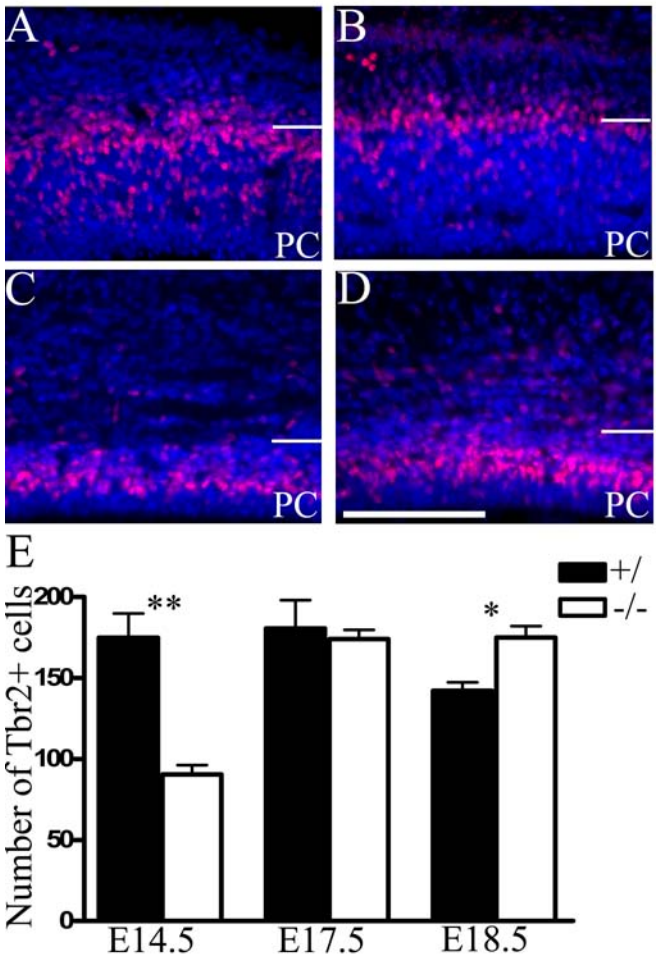

Figure 19. Intermediate progenitor cell number during development

Tbr2 (red) staining at E14.5 (A,B) and E17.5 (C,D) in control (A,C) and Sall1-/- animals (B,D). Sections are counterstained with DAPI (blue). Quantification of Tbr2 cell number during at E14.5, E17.5, and E18.5 (E). *, $p<0.05$; **, $p<0.01$. Scale bar (in D) represents: 100μm in A-D.

3.3.9 Sall1 expression in dorsal progenitor cells regulates progenitor cell maturation

Our results suggest a role for Sall1 in regulating neuronal differentiation in the dorsal cortex. However, alterations in the ventral telencephalon were also observed in Sall1-deficient animals. Interneurons derived from the ventral telencephalon tangentially migrate to their final laminar cortical position through the subventricular zone/intermediate zone and marginal zone (reviewed in Marin and Rubenstein, 2001). Furthermore, dorsal progenitors have been shown to molecularly interact with ventrally derived migrating interneurons (Tiveron et al., 2006), which suggests that differences in ventrally-derived populations may contribute to the alterations in dorsal progenitor cell number in Sall1-deficient animals. To spatially dissect the role of Sall1 in development of the cortex, we examined conditional knockout of Sall1 in the dorsal cortex under the control of Emx1-Cre driven recombination; mutant animals are termed Emx1-Sall1-cKO. Emx1 expression initiates at E9.5, and Emx1 driven Cre recombination is evident from E10.5 (Gorski et al., 2002). Recombination is observed in the majority of excitatory neurons in the cortex but not in interneurons (Gorski et al., 2002). In order to determine whether recombination of the floxed Sall1 allele had occurred, we examined Sall1 expression in the dorsal cortex at E12.5 (Figure 20A,B). In control animals, Sall1 expression extended into the dorsal cortex at E12.5 (Figure 20A); however, in Emx1-Sall1-cKO embryos, strong Sall1 expression was limited to the ventral telencephalon and did not extend past the corticostriatial boundary (n=3) (arrow, Figure 20B). Weak Sall1 expression was observed in the dorsal cortex at E12.5 in Emx1-Sall1-cKO animals, which may represent residual Sall1 protein present in dorsal progenitor cells transcribed prior to the recombination event.

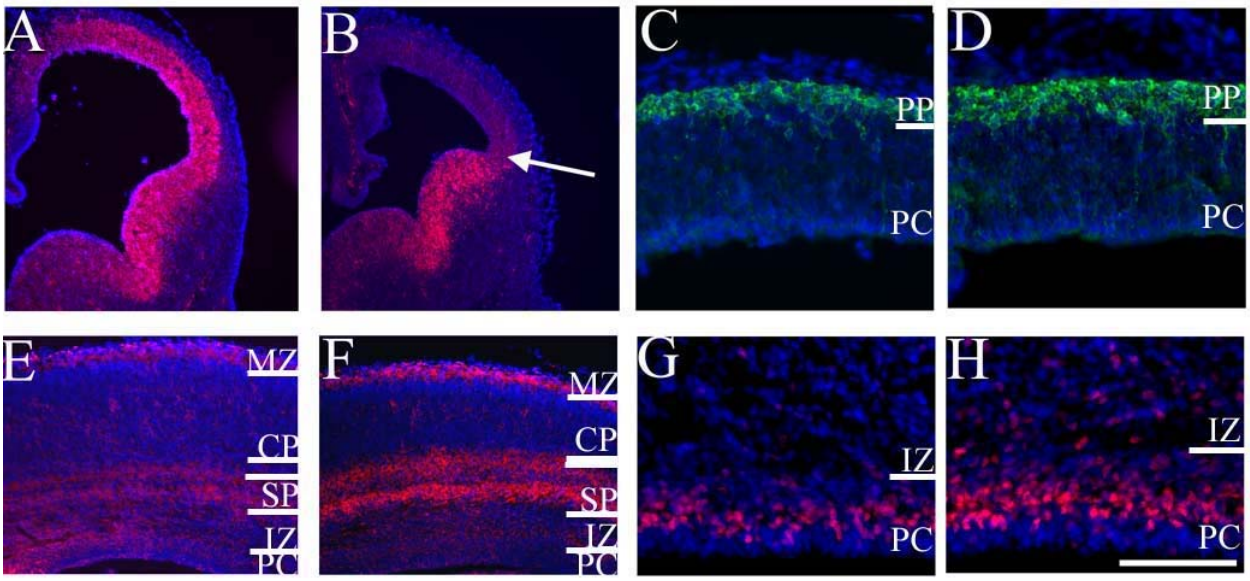

Figure 20. Analysis of dorsally restricted Sall1 knock-out

Sall1 (red) expression in the cortex of control (A) and Emx1-Sall1-cKO (B) animals at E12.5. Strong Sall1 expression does not extend past the corticostriatial boundary in Emx1-Sall1-cKO animals (arrow, B). Tuj1 staining (green) of cortical neurons at E12.5 identified an increase in preplate cell number in Emx1-Sall1-cKO (D) animals compared to controls (C). CSPG (red) immunostaining of the subplate and marginal zone identified an increase in these structures in Emx1-Sall1-cKO (F) animals compared to controls (E) at E18.5. Tbr2 (red) immunostaining at E18.5 identified an increase in intermediate progenitor cells in Emx1-Sall1-cKO (H) animals compared to controls (G). PC: progenitor population; PP: preplate; IZ: intermediate zone; SP: subplate; CP: cortical plate; MZ: marginal zone. Scale bar (in H) represents: 100µm in C,D,G,H; 350µm in E,F; 400µm A,B.

To determine whether gross cortical malformations were present in Emx1-Sall1-cKO animals, we examined the anatomy of the brain at E18.5 in control and Emx1-Sall1-cKO animals. The total length and dorsal surface area of the brain were decreased by 3.6% ($p<0.05$, n=3) and 5.8% ($p<0.05$, n=3), respectively, in Emx1-Sall1-cKO animals (Figure 21A,B). Specifically, the cerebral hemispheres were decreased in length and dorsal surface area by 12.8% ($p<0.05$, n=3) and 12.8% ($p<0.05$, n=3), respectively, in Emx1-Sall1-cKO animals. As expected, no alteration in the length ($p=0.9$, n=3) or dorsal surface area ($p= 0.9$, n=3) of the midbrain were observed

(Figure 21A,B). We quantified the number of cells in the dorsal cortex in Emx1-Sall1-cKO animals and, similar to Sall1-deficient animals, observed an increase in the number of progenitor cells (17.6%, n=3, $p<0.05$) and a decrease in the number of cells in the cortical plate (23.3%, n=3, $p<0.01$) at E18.5 in Emx1-Sall1-cKO animals compared to controls (Figure 21C). Interestingly, in the Emx1-Sall1-cKO, we observed an increase in cell number in the intermediate zone (16.2%, n=3, $p<0.01$) and marginal zone (28.5%, n=3, $p<0.001$) (Figure 21C). While we observed an increase in subplate cell number (in the intermediate zone) and Cajal-Retzius cell number (in the marginal zone) in Sall1-/- animals, we observed no alteration in total cell number in the intermediate zone or marginal zone. Cells born in the ventral cortex migrate through the intermediate zone and marginal zone during development on route to the cortex, suggesting that these ventrally derived migrating populations are decreased in Sall1-/- animals.

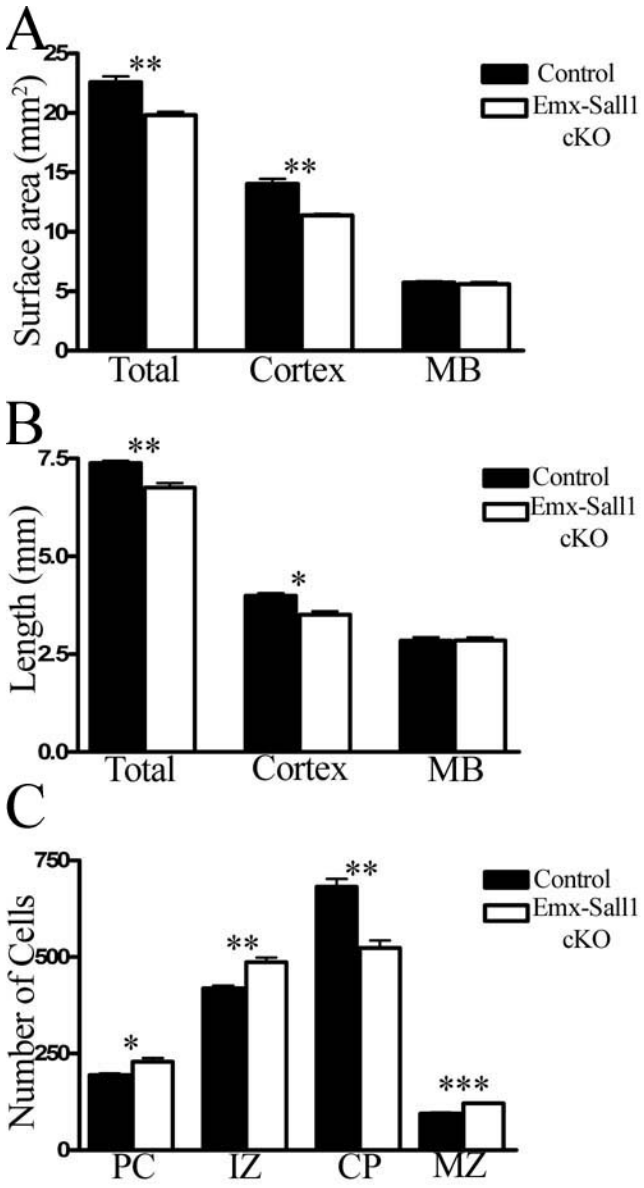

Figure 21. Cortical size in control and Emx1-Sall1-cKO animals

The cerebral cortex is decreased in size in Emx1-Sall1-cKO animals compared to controls at E18.5. Measurements of brain surface area (A) and length (B) in control and Emx1-Sall1-cKO animals. Quantification of dorsal cell number in control and Emx-Sall1-cKO animals (C). MB: midbrain; PC: progenitor population; IZ: intermediate zone; CP: cortical plate; MZ: marginal zone. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

In Sall1-/- animals we observed an increase in the number of neurons in the dorsal cortex at E12.5. To determine whether these early deficits were due to a requirement for Sall1 in dorsal progenitor cells we examined early neurogenesis in Emx1-Sall1-cKO animals. We examined Tuj1 staining in control and Emx1-Sall1-cKO animals to determine whether the increase in preplate size was a consequence of loss of Sall1 in the dorsal cortex. At this age an increase in the number of neurons in the dorsal cortex is observed in Emx1-Sall1-cKO animals compared to controls (Figure 20C,D). These data support a role for Sall1 in regulating differentiation of early cortical progenitor cells in the dorsal cortex. Furthermore, this confirms functional deletion of Sall1 in dorsal progenitor cells driven by Emx1-Cre prior to E12.5. Examination of CSPG staining at E18.5 identified an increase in staining in the subplate and the marginal zone (n=3) (Figure 20E,F), which suggests that similar to Sall1-/- animals, Emx1-Sall1-cKO animals have an increase in subplate and marginal zone populations. These data support our hypothesis for a role for Sall1 in the developmental control of neurogenesis within the dorsal cortex.

Our studies identified a role for Sall1 in the transition from an early progenitor state to an intermediate progenitor state, and by E18.5, we observed an increase in the number of Tbr2-positive intermediate progenitor cells in the dorsal cortex in Sall1-/- animals. Dorsal progenitors molecularly interact with migrating GABAergic interneurons derived from the ventral cortex, and addition of GABA can inhibit differentiation of dorsal progenitor cells in a slice culture model (Haydar et al., 2000; Tiveron et al., 2006), which suggests that the deficits observed in Sall1-deficient animals may be mediated by alterations in migrating ventral populations. In the dorsally restricted Emx1-Sall1-cKO, an increase in the number of Tbr2-positive cells was observed in the dorsal cortex at E18.5 compared to controls (n=3) (Figure 20G,H). These

findings suggest a requirement for Sall1 expression in dorsal progenitor cells to regulate progenitor cell maturation.

3.4 DISCUSSION

Regulation of progenitor cell proliferation and differentiation is a critical determinant of cortical cell number and cell type specification. Alterations in cortical development are associated with a variety of neuropsychiatric disorders, including autism, epilepsy and schizophrenia (Arnold, 1999; Schwartzkroin and Walsh, 2000; Bond et al., 2002; Pilz et al., 2002; Bond et al., 2003; Piao and Walsh, 2004; Polleux and Lauder, 2004; Piao et al., 2005; Lian and Sheen, 2006). Temporal regulation of cortical neurogenesis is observed during development; early born neurons are committed to a preplate fate, and later born neurons are committed to a cortical plate fate. Few molecular determinants of preplate neurogenesis have been identified (Zhou et al., 1999; Hevner et al., 2001; Hanashima et al., 2004; Hirata et al., 2004; Studer et al., 2005). Furthermore, analysis of preplate neurogenesis in mice deficient for identified proteins may be complicated by defective preplate splitting and altered cortical patterning (Hevner et al., 2001; Shinozaki et al., 2002; Bishop et al., 2003; Studer et al., 2005). Recent evidence suggests that the majority of later born neurons in the cortex arise from a newly identified population, intermediate progenitor cells (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). However, the molecular mechanisms regulating the specification and fate of this progenitor population are poorly characterized. Identifying mechanisms that regulate progenitor cell proliferation and differentiation throughout corticogenesis is critical to understand normal cortical development and the consequences of disruption of these processes.

We examined the role of Sall1 in cortical development. Sall1 is robustly expressed by cortical progenitor cells throughout neurogenesis. Sall1-deficient animals die at birth due to kidney deficits (Nishinakamura et al., 2001), and at E18.5 the cerebral hemispheres are decreased in size compared to controls. We identified alterations in neurogenesis and progenitor cell number in Sall1-deficient animals. Our findings suggest a dual role for Sall1 in progenitor cell regulation. Early in development Sall1 regulates the number of cells committed to a preplate fate; in Sall1-/- animals, more cells are committed to the preplate and its derivatives, the subplate and the marginal zone, at the expense of the progenitor cell population. Our studies suggest from midneurogenesis Sall1 promotes a neural fate; in Sall1-deficient animals neuronal differentiation is decreased from E14.5 on, leading to a decrease in cortical plate cell number. This decrease in cortical plate cell number is due, at least in part, to an alteration in the fate of progenitor cells that adopt an intermediate progenitor cell fate, as opposed to differentiating from E14.5 in Sall1-mutant animals. Furthermore, the alterations in Sall1-deficient animals are due to an intrinsic requirement for Sall1 in dorsal progenitor cells. Taken together, our studies suggest that Sall1 is a critical regulator of neuronal differentiation and progenitor cell maturation in the developing cortex.

3.4.1 A dual role for Sall1 in regulating cortical neurogenesis

Cells destined for the preplate and its derivatives the subplate and marginal zone are born from E10.5 to E13.5 (Sheppard et al., 1991; Price et al., 1997). Sall1 is required from as early as E11.5 to regulate cortical neurogenesis. Examination of the onset of deficits in Sall1-deficient animals identified that at E11.5 more neurons differentiate at the expense of re-entering the cell

cycle. Cajal-Retzius cells are predominately born on E10.5 (Sheppard et al., 1991), and in Sall1-deficient animals an increase in the number of Reelin-expressing Cajal-Retzius cells was observed at E14.5 and E18.5. However, quantification of neuronal cell number did not identify an alteration prior to E12.5 in Sall1-deficient animals. An early increase in Cajal-Retzius cell number is likely to exist in Sall1-mutant animals; however, this population represents a small proportion of total cell number, such that it may be beyond the threshold of detection of our quantification. Three spatially distinct origins for Cajal-Retzius cells have been identified; the cortical hem, the ventral pallium and the septum (Meyer et al., 2002; Shinozaki et al., 2002; Bielle et al., 2005). Sall1 is not expressed by cells in the cortical hem at the time of the birth of Cajal-Retzius cells from this structure. Analysis of the dorsally restricted Sall1 knock-out identified an increase in the number of Cajal-Retzius cells. Emx1-Cre expression extends into the ventral pallium but not the septum (Gorski et al., 2002), which suggests that the deficits observed are due to a role for Sall1 in regulating Cajal-Retzius neurogenesis in the ventral pallium. Cells ultimately destined for the subplate are born from E11.5 to E13.5 (Sheppard et al., 1991; Price et al., 1997), and in Sall1-deficient animals an increase in the number of cells born on E11.5 and E12.5 is observed at E18.5. Our studies suggest that these cells are selectively committed to a subplate fate in Sall1-deficient animals. Taken together these findings suggest that Sall1 regulates the number of cells committed to an early dorsal cortical fate.

Cells destined for the cortical plate are born from ~E13.5 (Price et al., 1997; Levers et al., 2001). From mid-neurogenesis, a decrease in the number of differentiating neurons is observed in Sall1-deficient animals, and our data suggest that more cells are re-entering the cell cycle as opposed to differentiating compared to controls. This decreased rate of neurogenesis results in a decrease

in cell number in the cortical plate at E18.5; however, the laminar fate of cortical plate neurons is not altered and cortical layers are proportionally decreased in cell number. These findings suggest that Sall1 regulates neurogenesis but not cell fate specification of cortical plate neurons.

Interestingly, increased early neurogenesis is commonly associated with depletion of the progenitor population late in neurogenesis, and consequentially the cortical plate is decreased in cell number due to the lack of available progenitor cells (Handler et al., 2000; Caviness et al., 2003; Roy et al., 2004; Bedford et al., 2005; Wines-Samuelson et al., 2005). An initial decrease in progenitor cell number is observed in Sall1-deficient animals at E12.5. We postulate that this early decrease in progenitor cell number contributes to the decrease in deep cortical layers. However, from E14.5-E17.5, no alteration in progenitor cell number is observed compared to controls. These findings suggest that the late decrease in cortical plate size is not due to a depletion of the progenitor population and support an independent role for Sall1 in promoting a neural fate late in development. We hypothesize that the late neurogenic role of Sall1 is linked to the requirement for Sall1 in regulating intermediate progenitor cell generation.

It is interesting to speculate a link between Wnt signaling and Sall1 in neuronal progenitor cells. Studies in Drosophila and in chick have shown that Sall1 is activated in response to Wnt signaling (de Celis et al., 1996; Kuhnlein and Schuh, 1996; de Celis et al., 1999; Chihara and Hayashi, 2000; de Celis and Barrio, 2000; Farrell and Munsterberg, 2000). Furthermore, Sall1 has been shown to activate a Wnt responsive promoter and to interact with β-catenin (Sato et al., 2004). Studies of neuronal differentiation have proposed that Wnt signaling regulates neuronal differentiation in a temporal manner (Hirabayashi et al., 2004), although the role of Wnt

signaling at mid-neurogenesis needs to be clarified due to conflicting results from independent studies (Hirabayashi et al., 2004; Woodhead et al., 2006). Similar to our proposed role for Sall1 in neuronal differentiation, early in neurogenesis Wnt signaling promotes a progenitor state, and at mid-neurogenesis Wnt signaling promotes a neuronal state (Hirabayashi et al., 2004), which suggests that Sall1 may regulate or mediate Wnt signaling in neuronal progenitors.

3.4.2 Sall1 regulates progenitor cell maturation

Our observations support a hypothesis of a dual role for Sall1 in regulating neural differentiation. During development progenitor cell division type transitions from a proliferative division (P/P) to a neurogenic division (P/N) to a terminal neurogenic division (N/N) (Figure 22). Our studies suggest that early in development, Sall1 promotes a progenitor fate, by restricting progression from a proliferative division to a neurogenic division, such that in the absence of Sall1, more progenitor cells transition to a neurogenic division type during early neurogenesis. Late in neurogenesis, Sall1 promotes a neural fate, by promoting the transition from a neurogenic division to a terminal neurogenic division, such that in the absence of Sall1 fewer progenitor cells transition to a terminal neurogenic division type (Figure 22).

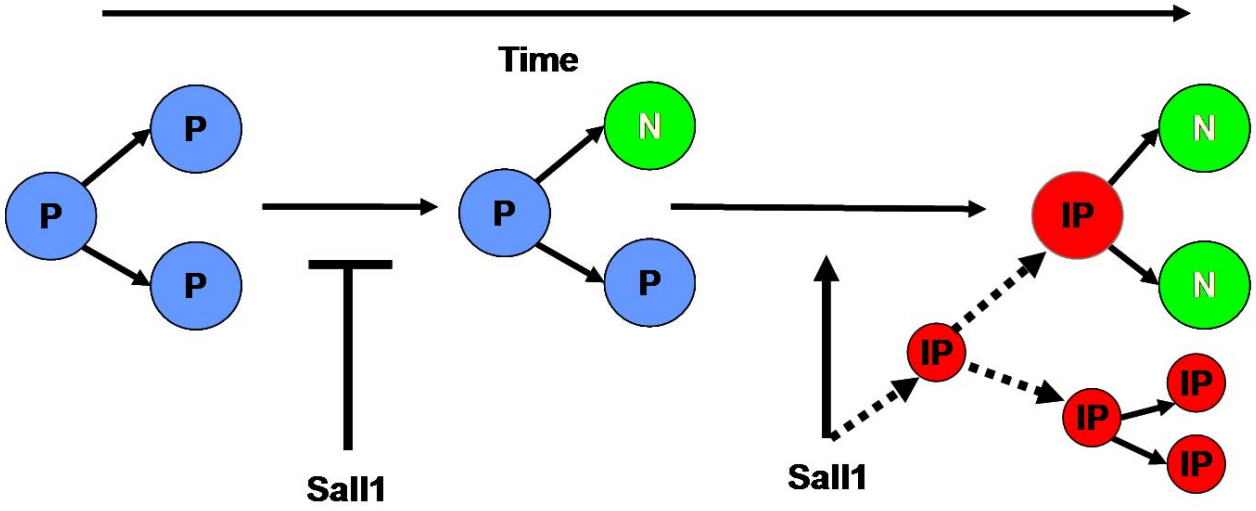

Figure 22. A dual role for Sall1 in regulating neurogenesis

Model of Sall1 regulation of neuronal differentiation. During neural development progenitor cell division type transitions from a proliferative division (P/P) to a neurogenic division (P/N) to a terminal neurogenic division (N/N). Early in development Sall1 restricts the progression from a proliferative division to a neurogenic division. Late in development Sall1 promotes the transition from a neurogenic division to a terminal neurogenic division. We propose that the role of Sall1 in late development is mediated by a role in intermediate progenitor cell regulation. Sall1 may regulate the number of progenitor cells committed to an intermediate progenitor cell fate, or Sall1 may regulate the proliferative capacity of intermediate progenitor cells. P: progenitor cell; N: neuron, IP: intermediate progenitor cell.

Maturation of progenitor populations is associated with a shift towards neuronal and intermediate progenitor cell divisions, increased neurogenesis, and depletion of the progenitor population (Takahashi et al., 1995b; Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Our findings suggest a role for Sall1 in regulating neuronal differentiation from mid-neurogenesis by regulating intermediate progenitor cell generation. In the absence of Sall1, a decrease in the number of intermediate progenitor cells is observed at E14.5, however, by E17.5 no alteration in intermediate progenitor cell number is observed, and at E18.5 the number of intermediate progenitor cells is increased compared to controls. At E14.5 and E17.5, a decrease in neuronal

differentiation is observed, which suggests that progenitor cells are re-entering the cell cycle and favoring an intermediate progenitor fate over neuronal differentiation. In addition, our studies demonstrate an intrinsic requirement for Sall1 in dorsal progenitors in regulating intermediate progenitor cell generation. Intermediate progenitor cells favor direct terminal differentiation (Noctor et al., 2004). Therefore, the alterations observed in Sall1-/- animals, may reflect a role for Sall1 in regulating the proliferative capacity of the intermediate progenitor population, rather than ventricular zone progenitors selectively committing to an intermediate progenitor state in Sall1-/- animals (Figure 22). This hypothesis predicts that, in the absence of Sall1, intermediate progenitor cells favor expanding the intermediate progenitor population, by re-entering the cell cycle at the expense of differentiating.

Previous studies have suggested that upper cortical layer neurons arise from subventricular zone/intermediate progenitor cells (Tarabykin et al., 2001; Nieto et al., 2004), and at E14.5, the intermediate progenitor cell population is decreased in Sall1-deficient animals. We hypothesize that the decrease in intermediate progenitor cell number at E14.5 leads to the decrease in superficial cortical layers in Sall1-mutant animals at E18.5. In Sall1-deficient animals intermediate progenitor cell number increases from E14.5 to E17.5, and we postulate that ventricular zone progenitor cells switch to an intermediate progenitor division type at the expense of neuronal differentiation of deep cortical layer neurons. Alternatively, intermediate progenitor cells may generate neurons for both deep (born ~E13.5-E14.5) and superficial (born ~E15.5 to E18.5) cortical layers, and the decrease in all cortical plate layers at E18.5 in Sall1-/- animals are due alterations in this progenitor population. The relative contributions of ventricular and intermediate progenitor cells to cortical layers has not been characterized, although live

imaging studies have shown that while ventricular zone progenitor cells are undergoing neuronal differentiation, intermediate progenitor cells are also generating neurons (Noctor et al., 2004). Furthermore, given that at E18.5, intermediate progenitor populations, which are committed to a neuronal fate, are increased in Sall1-deficient animals, upper cortical layers may ultimately be increased in cell number in the adult.

Only one other molecule has been directly identified as a regulator of intermediate progenitor cell generation. Mutation of the FRS2α gene, a mediator of FGF signaling, is associated with perinatal lethality, and decreased cortical plate size (Yamamoto et al., 2005). A decrease in abventricular mitosis and Tbr2-positive intermediate progenitor cells was observed at E14.5 in FRS2α mutant mice (Yamamoto et al., 2005). In Sall1-deficient animals, we observed a decrease in intermediate progenitor cells at E14.5 similar to FRS2α mutant mice. In the developing chick limb bud, Sall1 is induced in response to FGF (Farrell and Munsterberg, 2000). These findings implicate FGF signaling in intermediate progenitor cell generation and suggest that Sall1 may be acting downstream of FGF to regulate intermediate progenitor cell generation.

3.4.3 Sall1 and the Reelin signaling pathway.

Reelin and the Reelin signaling pathway are critical regulators of neuronal migration, and in mice deficient for Reelin or its downstream targets alterations in the laminar position of cortical plate neurons are observed (Sweet et al., 1996; Sheldon et al., 1997; Trommsdorff et al., 1999; Gupta et al., 2002). In Sall1-deficient animals, we observed an increase in Reelin expression in the marginal zone. Cortical plate lamination was not altered in Sall1-deficient animals, and birth-dating studies suggest that the inside-out pattern of neurogenesis is maintained, which suggests

that increased Reelin expression does not influence cortical lamination. In Sall1-deficient animals, the total number of cells born on E14.5 located in the dorsal cortex at E18.5 was not significantly different compared to controls; however, we observed an increase in the proportion of these cells in the progenitor population and intermediate zone, and a decrease in the proportion of cells in the cortical plate, which suggests an alteration in the differentiation or migration of a subset of cells born on E14.5. Examination of the rate of neuronal differentiation at E14.5 identified that fewer cells differentiated into neurons at this age in the dorsal cortex, which suggests that the total number of cells born on E14.5 should be decreased in Sall1-deficient animals compared to controls at E18.5. Taken together, these data suggest an alteration in neurogenesis or migration of ventrally derived populations born on E14.5, which migrate through the progenitor population and intermediate zone at E18.5, or the presence of a very slow proliferating progenitor cell population derived from the dorsal cortex in Sall1-mutant animals. Previous studies have suggested a role for Reelin signaling in the lamination of subpopulations of ventrally derived interneurons (Yabut et al., 2005), and increased Reelin expression in the dorsal cortex may influence the tangential migration of these populations. Future studies will analyze dorsal or ventral restricted conditional knockout of Sall1 and birth-dating at E14.5 to distinguish between these possibilities.

In addition to the changes in Reelin expression, we also observed alterations in downstream components of the Reelin signaling pathway. Binding of Reelin to Vldlr and ApoER2 induces phosphorylation of Dab-1 and activation of a downstream signaling pathway (Rice et al., 1998; D'Arcangelo et al., 1999; Hiesberger et al., 1999; Howell et al., 1999). Interestingly, Dab-1 phosphorylation also leads to ubiquitination and degradation of Dab-1, and in mice deficient for

Reelin, Vldlr or ApoER2 an increase in Dab-1 protein expression is observed (Rice et al., 1998; Trommsdorff et al., 1999; Arnaud et al., 2003). In order to determine whether the increased Reelin expression observed in Sall1-mutant animals has functional implications, it will first be critical to examine protein levels of Dab-1, to determine whether the alterations in Dab1 mRNA expression correspond to an alteration in protein and/or phosphorylated protein level. In Sall1-deficient animals Reelin expression was increased on a per cell basis, which suggests a role for Sall1 in regulating Reelin expression. Interestingly, addition of histone deacetylase inhibitors activates the Reelin promoter in vitro (Chen et al., 2002). In light of the proposed role for Sall1 in recruiting histone deacetylase to mediate transcriptional repression (Kiefer et al., 2002), it is interesting to speculate that Sall1 mediates recruitment of histone deacetylase to repress the Reelin promoter.

Decreased levels of Reelin expression have been observed in patients with autism, bipolar and major depression disorders (Fatemi et al., 2000; Guidotti et al., 2000; Fatemi et al., 2001; Fatemi et al., 2002; Fatemi et al., 2005). Alterations in Reelin expression have also been described in schizophrenic patients, although some studies report increased Reelin expression whereas others report decreased Reelin expression (Fatemi et al., 2000; Guidotti et al., 2000; Fatemi et al., 2001). Interestingly, an association of increased Reelin expression with Alzheimer's disease, frontotemporal dementia, and Parkinson's Disease has been described (Saez-Valero et al., 2003; Botella-Lopez et al., 2006). Although functional consequences of decreased Reelin expression have been well characterized in murine models (Alter et al., 1968; Salinger et al., 2003), the consequences of increased Reelin expression have not. Conditional knockout of Sall1 in the dorsal cortex results in increased Reelin expression and overcomes perinatal lethality of Sall1

null mice. These animals offer a model to examine the functional consequences of increased Reelin expression in the postnatal cortex.

3.4.4 A role for Sall1 in ventral neurogenesis

Although this study focused on the role of Sall1 in dorsal progenitor cell regulation, alterations in both the dorsal and ventral telencephalon were observed in Sall1-deficient animals. In the ventral telencephalon at E18.5, the striatum appeared decreased in size and an apparent increase in progenitor cells in the ganglionic eminences was observed in Sall1-/- animals. These observations suggest that more proliferating cells re-enter the cell cycle at the expense of differentiating into neurons in the ventral telencephalon in the absence of Sall1, similar to late neurogenic phenotype in the dorsal cortex in Sall1-deficient animals. These findings suggest a conserved role for Sall1 in neurogenesis in the developing telencephalon. In Sall1 null animals, an increase in subplate cell number (in the intermediate zone) and Cajal-Retzius cell number (in the marginal zone) was observed; however, total cell number in the intermediate zone and marginal zone was not altered compared to controls, which suggests a decrease in a second population of cells within these regions. Analysis of the dorsally restricted Sall1 knockout (Emx1-Sall1-cKO) identified an increase in total cell number in the intermediate zone and marginal zone in the dorsal cortex compared to controls. Taken together, these data suggest neurons derived from the ventral telencephalon, which migrate through the intermediate zone and marginal zone, are decreased in cell number at E18.5 in Sall1 -/- animals. These findings suggest a role for Sall1 in regulating neurogenesis in the ventral telencephalon.

The consequence of Sall1 disruption in ventral populations was not a focus of this thesis. Future studies will address the role of Sall1 in ventral neurogenesis using conditional mutagenesis to restrict loss of Sall1 to ventral progenitor populations. Preliminary studies have identified that these animals survive postnatally (unpublished observations), and the cellular alterations in these animals are currently being characterized. Alterations in ventral derived populations, including the striatum and cortical interneurons, are associated with a variety of neuropsychiatric disorders, including autism, Tourette's syndrome, obsessive compulsive disorder and bipolar disorder (Sears et al., 1999; Peterson et al., 2003; DelBello et al., 2004; Levitt et al., 2004; Pujol et al., 2004). Thus, conditional ventral knockout of Sall1 may offer an interesting model to dissect the functional consequences of altered ventral neurogenesis in relation to behavioural characteristics of neuropsychiatric disorders.

3.4.5 Conclusions

We identified a critical role for Sall1 in regulating cortical neurogenesis and progenitor cell maturation. In Sall1-deficient animals, more cells are committed to the early born structure, the preplate, and its derivatives, the subplate and the marginal zone, and fewer neurons are committed to the later born structure, the cortical plate. Our data support a dual role for Sall1 in cortical development; early in development Sall1 promotes a progenitor fate, whereas late in development Sall1 promotes a neural fate. Our studies suggest that the late neurogenic role of Sall1 is linked to its role in the generation of the intermediate progenitor cell population. The intermediate progenitor cell population has only recently been identified, and it is hypothesized that this population gives rise to the majority of neurons in the cortex. Thus, deficits in intermediate progenitor cell generation may result in gross alterations in the neuronal

composition of the mature cortex. Molecular determinants of this population are not well characterized; however, this study identified that Sall1 is a critical regulator of the generation and maintenance of intermediate progenitor cells. Characterizing the molecular regulators of this population is critical to understand the normal development of intermediate progenitor cells, as well as the functional consequences of disruption of this population. While cognitive deficits are not well characterized in Townes-Brocks patients, our studies indicate that Sall1 is a critical regulator of cortical development, which suggests that greater consideration should be given to cognitive impairment in these patients.

4.0 SALL1 REGULATES MITRAL CELL DEVELOPMENT AND OLFACTORY NERVE EXTENSION IN THE DEVELOPING OLFACTORY BULB

4.1 INTRODUCTION

The olfactory system is composed of peripheral and central components that are involved in the detection, transmission, and interpretation of scent. Olfactory stimuli are detected within the peripheral olfactory system by olfactory receptor neurons that are topographically organized within the nasal epithelium (Buck and Axel, 1991; Ressler et al., 1993; Vassar et al., 1993; Sullivan et al., 1995). These neurons project to spatially conserved glomeruli in the olfactory bulb (OB) in the central olfactory system, where they synapse with the dendrites of mitral and tufted cells (Ressler et al., 1994; Mombaerts et al., 1996). Information is further transmitted to the olfactory cortex via the lateral olfactory tract, where it elicits a variety of emotional and behavioral responses (reviewed in Stockhorst and Pietrowsky, 2004).

The development, projection pattern, and assembly of the olfactory system has been an area of active research over the last ten years. OB neuronal populations are sequentially produced and have distinct origins. Mitral cells are the first born OB population (Hinds, 1968). These cells arise in the dorsal cortex, and the transcription factor Tbr1 is required for their generation (Bulfone et al., 1998; Puelles et al., 2000; Moreno et al., 2003). OB interneurons arise from an

ER81-positive population in the ventral dorsal lateral ganglionic eminence (LGE) and migrate to the OB via the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1999; Wichterle et al., 2001; Stenman et al., 2003). Previous studies have suggested that the olfactory epithelium and OB appear to be independent developmental domains (reviewed in Lopez-Mascaraque and de Castro, 2002). However, studies in Xenopus suggest that the olfactory placode is required for OB development (Graziadei and Monti-Graziadei, 1992; Byrd and Burd, 1993). In rats, innervation of the OB by the olfactory nerve has been shown to influence the cell cycle kinetics of olfactory progenitors (Gong and Shipley, 1995), leading to the hypothesis that the olfactory nerve extrinsically influences OB morphogenesis.

Insight into the interactions between central and peripheral components of the olfactory system has been gained from murine knockout analyses. FGFR1 is expressed by cells in the olfactory epithelium and OB (Hsu et al., 2001; Hebert et al., 2003). In FGFR1-deficient animals, the olfactory epithelium is normal, and olfactory nerve projections are observed (Hebert et al., 2003). However, in these mutant animals the OB fails to evaginate, yet differentiated OB cells accumulate at the anterior tip of the developing telencephalon. These findings suggest that OB evagination is not required for peripheral innervation. Dlx5 is a transcription factor that is also expressed by the olfactory epithelium and OB (Simeone et al., 1994; Stuhmer et al., 2002). In the absence of this transcription factor, the olfactory nerve fails to reach the OB, yet OB evagination and olfactory neurogenesis occur (Long et al., 2003). However, mitral cells were disorganized and a decrease in interneuron number was observed in these mutant animals (Long et al., 2003). These findings suggest that olfactory nerve innervation is not essential for OB

neurogenesis or evagination but may be required for cellular lamination. Since FGFR1 and Dlx5 are expressed by both peripheral and central components, it is difficult to dissect the precise role of these factors within the developing olfactory system. Recently, two genes have been identified that are only expressed by peripheral (Fez) or central (Arx) components of the olfactory system (Yoshihara et al., 2005; Hirata et al., 2006). Mice deficient for either gene have smaller OBs and similar cellular phenotypes. Most cell types are produced, although interneurons are generated in reduced numbers, and laminar organization is disrupted. In addition, the olfactory nerve fails to appropriately contact the OB, remaining outside the structure in a fibrocellular mass. Interestingly, early deficits in progenitor cell proliferation are observed in Arx-deficient animals, which suggests that olfactory innervation may be influenced by the number of differentiated cells. These findings support a role for cross-talk between peripheral and central components to regulate assembly of the olfactory system during development.

Is appropriate cellular specification required for peripheral innervation? Excitatory and inhibitory neurons in the OB have distinct origins. Excitatory OB neurons have a pallial origin and migrate radially from OB progenitors to form the mitral cell layer (MCL) (Puelles et al., 2000; Moreno et al., 2003). The transcription factor Tbr1 has been implicated in the generation of this population (Bulfone et al., 1998). Tbr1 -/- animals completely lack mitral cells; however, topographic targeting of the olfactory nerve is unaltered. OB interneurons arise from an ER81-positive population in the subpallial dorsal lateral ganglionic eminence and migrate to the OB via the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1999; Wichterle et al., 2001; Stenman et al., 2003). A number of factors that regulate the generation of this diverse population of OB interneurons have been identified,

including Sp8, GSH1/2, Vax1, and Pax6 (Yun et al., 2003; Soria et al., 2004; Hack et al., 2005; Kohwi et al., 2005; Waclaw et al., 2006). However, deficits in the olfactory nerve have not been reported in these mutant animals. In addition, study of Dlx1/2-deficient animals indicates that, even in the absence of GABAergic interneurons, layer organization is maintained and nerve innervation is unaltered (Bulfone et al., 1998). Thus, neither excitatory nor inhibitory neurons alone are required for olfactory nerve targeting.

Sall1 is expressed by cells in both the OB and olfactory epithelium (Ott et al., 2001), although a detailed pattern of its expression has not been described previously. Here, we characterize expression of Sall1 in the developing olfactory system and show that it is expressed in OB progenitor cells and a subset of differentiated neurons. In order to understand the role of this gene during olfactory development, a targeted disruption of Sall1 was examined. Deletion of Sall1 results in perinatal lethality due to kidney deficits (Nishinakamura et al., 2001). In addition, a disorganization of OB laminar structure in Sall1-mutant animals was previously noted (Nishinakamura et al., 2001). Our studies suggest that Sall1 is required to regionally regulate differentiation in the developing OB and, consequently, olfactory axon extension and mitral cell organization.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Embryos were obtained from matings of Sall1 heterozygote animals and genotyped as described in Section 3.2.1 (Nishinakamura et al., 2001). No alterations in olfactory development were observed in Sall1 heterozygous (+/-) animals in our study (data not shown) and therefore these embryos were also used as controls. For Sall1 expression studies, embryos were obtained from timed pregnant CD1 mice from Charles Rivers Laboratories (Wilmington, MA). Embryos were collected via cesarean section at embryonic ages from E13.5 to E18.5 and processed as described in Section 2.2.1. BrdU was administered as described in Section 3.2.1.

4.2.2 Measurement of OB size

The total length of the OB was determined by counting the number of serial sections obtained through the entire OB at E14.5 and E18.5. This was subsequently converted to microns. At E14.5 the length of the OB was 254.4±6.7µm in control animals compared to 246.7±13.3µm in Sall1-mutant animals (n=3, p=0.65). At E18.5 the length of the OB was 626.7±13.3µm in control animals, and 493.3±13.3µm in Sall1-mutant animals (n=3, $p<0.01$). Statistical analysis was performed using an unpaired t-test with InStat 3 software (GraphPad Software, San Diego, CA).

4.2.3 Immunohistochemistry

Immunohistochemistry was performed as described in Section 2.2.2. Antibodies used were mouse anti-BrdU (1:25, Amersham Biosciences, Piscataway, NJ); rat anti-BrdU (1:1000, Abcam, Cambridge, MA); rabbit anti-Calretinin (1:1000, Chemicon, Temecula, CA); rabbit anti-Gad65/67 (1:10,000, Sigma, St. Louis, MO); mouse anti-GAP43 (1:100, Sigma); rabbit anti-GABA (1:1000, Sigma); rabbit ant-Laminin (1:500, Halfter, 1993); rat anti-NCAM (12F11) (undiluted, Chung et al., 1991); rabbit anti-Neuropilin 1 (1:100, Calbiochem, San Diego, CA); rabbit anti-S100β (1:200, Abcam); mouse anti-Reelin (1:500, Abcam); mouse anti-Sall1 (1:500, PPMX Perseus Proteomics, Tokyo, Japan); rabbit anti-Tbr1 (1:1000, Englund et al., 2005); rabbit anti-Tuj1 (1:1000, Sigma); mouse anti-Tyrosine Hydroxylase (1:5000, Sigma). Nissl staining was performed as described in section 3.2.3. For confocal analysis, single optical sections were visualized on a Nikon DF0200 confocal microscope at 60X magnification.

4.2.4 Histological identification of OB regions

To assign cellular subtypes and boundaries to OB regions, sections were examined at 40X magnification. The progenitor population was identified as the cellular region adjacent to the ventricle containing organized parallel arrays of cells with elongated nuclei arranged perpendicular to the ventricular surface. The differentiating population was identified as a region of heavily stained small cells with round nuclei arranged in a disorganized manner, adjacent to the progenitor population. The MCL was evident from E16.5 and contained cells with large oval-shaped nuclei, superficial to the differentiating field and arranged perpendicular to the ventricular surface. At E18.5, the glomerular layer (GL) and granule cell layer (GCL) were

identifiable. The GL was defined as the region containing round cells between the MCL and the olfactory nerve layer. The olfactory nerve layer was identified by the presence of elongated cells, orientated parallel to the ventricular surface and perpendicular to the MCL. The GL, as defined in this study, encompasses the GL and the external plexiform layer. The GCL was located on the ventricular side of the MCL and contained small heavily stained round cells.

4.2.5 Cell Counts and Statistical Analysis

For proliferative studies, BrdU and DAPI stained sections were photographed at 40X magnification. The analysis was performed by an observer blind to the genotype. The outermost boundary of the progenitor population was distinguished at 200X, as described above. To examine regional progenitor cell proliferation, the OB was divided into 6 regions, 3 dorsal and 3 ventral (n=4 per region, Figure 23A). These regions were defined as 50μm wide populations at the medial, central or lateral extent of the olfactory ventricle, and the number of BrdU-positive and total number of cells within each region were quantified, as previously described (Roy et al., 2004). The labeling index was determined, per region, as the number of BrdU-positive cells divided by total cell number. Three sections per animal were counted, and the mean of the three sections per animal was compared (n=4 per genotype). The data were subsequently decoded, and statistical analysis of the results was performed. Within genotype comparisons were performed using paired t-test and between genotype comparisons were performed using an unpaired t-test with InStat 3 software. Based on biological considerations, one-sided testing was used to examine regional differences of within genotype comparisons. For birth-dating studies (E18.5 embryos injected with BrdU on E11.5 or E14.5), BrdU stained sections were imaged at 20X magnification (n=3). A 150μm region in the dorsal-

ventral orientation through the centre of the OB was examined (Figure 23B), and heavily labeled BrdU-positive cells were quantified, as single positive or double positive for Tbr1 (E11.5 injected) or GABA (E14.5 injected). Three sections per animal were counted, and the mean of the three sections per animal was compared (n=3 per genotype). The data were subsequently decoded, and statistical analysis of the results was performed, as described above.

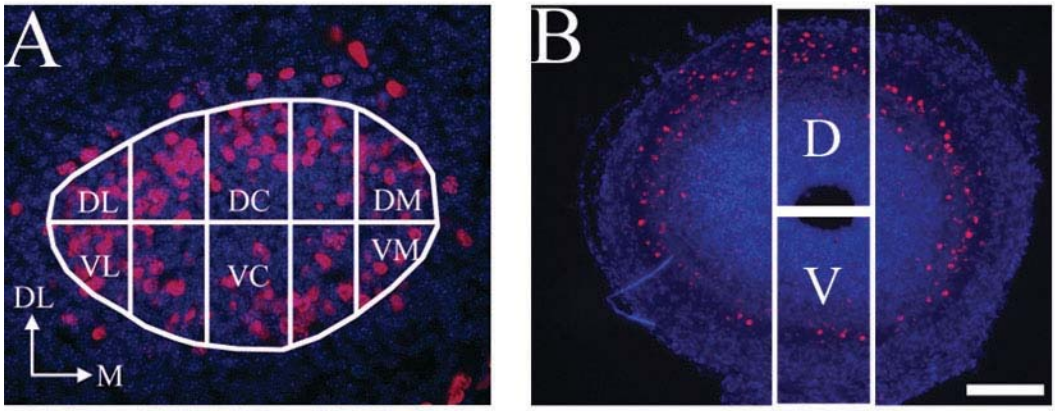

Figure 23. Illustration of regional divisions used in cell counts at E14.5 (A) and E18.5 (B).

At E14.5, the OB was divided into 6 regions, ventral-lateral (VL), ventral-central (VC), ventral-medial (VM), dorsal-lateral (DL), dorsal-central (DC), and dorsal-medial (DM) (A). These regions were defined as 50µm wide populations at the medial, central, or lateral extent of the olfactory ventricle. The progenitor population was identified as the cellular region adjacent to the ventricle containing organized parallel arrays of cells with elongated nuclei arranged perpendicular to the ventricular surface. The number of BrdU-positive cells (red) and total number of cells within each region was quantified. At E18.5 a 150µm region in the dorsal-ventral orientation through the centre of the OB was examined (B). Heavily labeled BrdU-positive cells were quantified in dorsal (D) and ventral (V) regions. Dorsal is up, medial is left, as indicated. Scale bar (in B): 50µm in A; 115µm in B.

4.3 RESULTS

4.3.1 Sall1 is expressed by progenitor cells and subpopulations of neurons in the developing and adult olfactory bulb

Neurogenesis and maturation of olfactory bulb cell types occurs in a precisely timed manner (Altman and Das, 1966; Hinds, 1968; Altman, 1969; Blanchart et al., 2006). In order to more accurately understand the role of Sall1 in the generation of the OB, we first characterized expression of this gene in the developing (n=3 per age) and adult (n=2) OB. Development of the OB is initiated when incoming projections from the olfactory placode interact with progenitor cells in the neural epithelium of the developing telencephalon at ~E11.5 (E13 rat, Gong and Shipley, 1995). These projections are believed to stimulate the differentiation of progenitor cells in the telencephalic neural epithelium to form the OB (Gong and Shipley, 1995). From E9.5, cells in the neural epithelium of the rostral most tip of the developing telencephalon express Sall1 (data not shown). Sall1 is expressed by cells in the olfactory placode from E9.5 (Buck et al., 2001). The olfactory placode gives rise to components of the peripheral nervous system, such as the vomeronasal organ and olfactory epithelium. By E13.5, Sall1 is strongly expressed by supporting cells in the olfactory epithelium and vomeronasal organ and weakly expressed by basal cells in the olfactory epithelium (Figure 24A,B). At E13.5, Sall1 is expressed by OB progenitors (Figure 24C), and expression of this gene in OB progenitors continues throughout development (Figure 24D).

Two major neuronal populations are present in the OB, excitatory projection neurons that comprise the MCL, and inhibitory interneurons that populate the granule cell layer (GCL) and

glomerular layer (GL). These populations are sequentially produced, with excitatory mitral cells born prior to interneuron populations (Altman and Das, 1966; Hinds, 1968; Altman, 1969; Blanchart et al., 2006). Cells destined for the MCL are born from E11.5-E13.5 (Hinds, 1968; Blanchart et al., 2006). At E13.5, Sall1 is expressed by a large number of differentiating cells that are primarily destined for the MCL (D in Figure 24C). By E17.5, Sall1 is robustly expressed by cells in the MCL (Figure 24D,E,F). Interestingly, at E17.5, we observed punctuate staining of Sall1 in mitral cells (arrowheads, Figure 24F), whereas expression in other cell types was diffuse (arrows, Figure 24F). Sall1 has been shown to localize to heterochromatized regions within the nucleus and to interact with histone deacetylases to mediate transcriptional repression (Sato et al., 2004; Netzer et al., 2006; Yamashita et al., 2007). This difference in cellular localization of Sall1 could suggest that it has a distinct functional role in mitral cells at this age.

Interneuron populations arise from the ventral telencephalon and migrate to the OB via the rostral migratory stream (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1999; Wichterle et al., 2001; Stenman et al., 2003). Sall1 is expressed by cells within the rostral migratory stream at E15.5 (Figure 24G). At E17.5, interneurons destined for the GL and GCL are differentiating, and a subpopulation has already reached their final laminar position (Figure 24D). Sall1 is expressed by a subset of cells in the GL and GCL at this age (Figure 24D,E). Expression of Sall1 is maintained in the MCL, GCL, and GL of the adult OB (Figure 24H,I). In summary, Sall1 is expressed by OB progenitors from the onset of olfactory development, and expression continues in a subpopulation of differentiated cells. These data suggest a role for Sall1 in the development and function of olfactory structures.

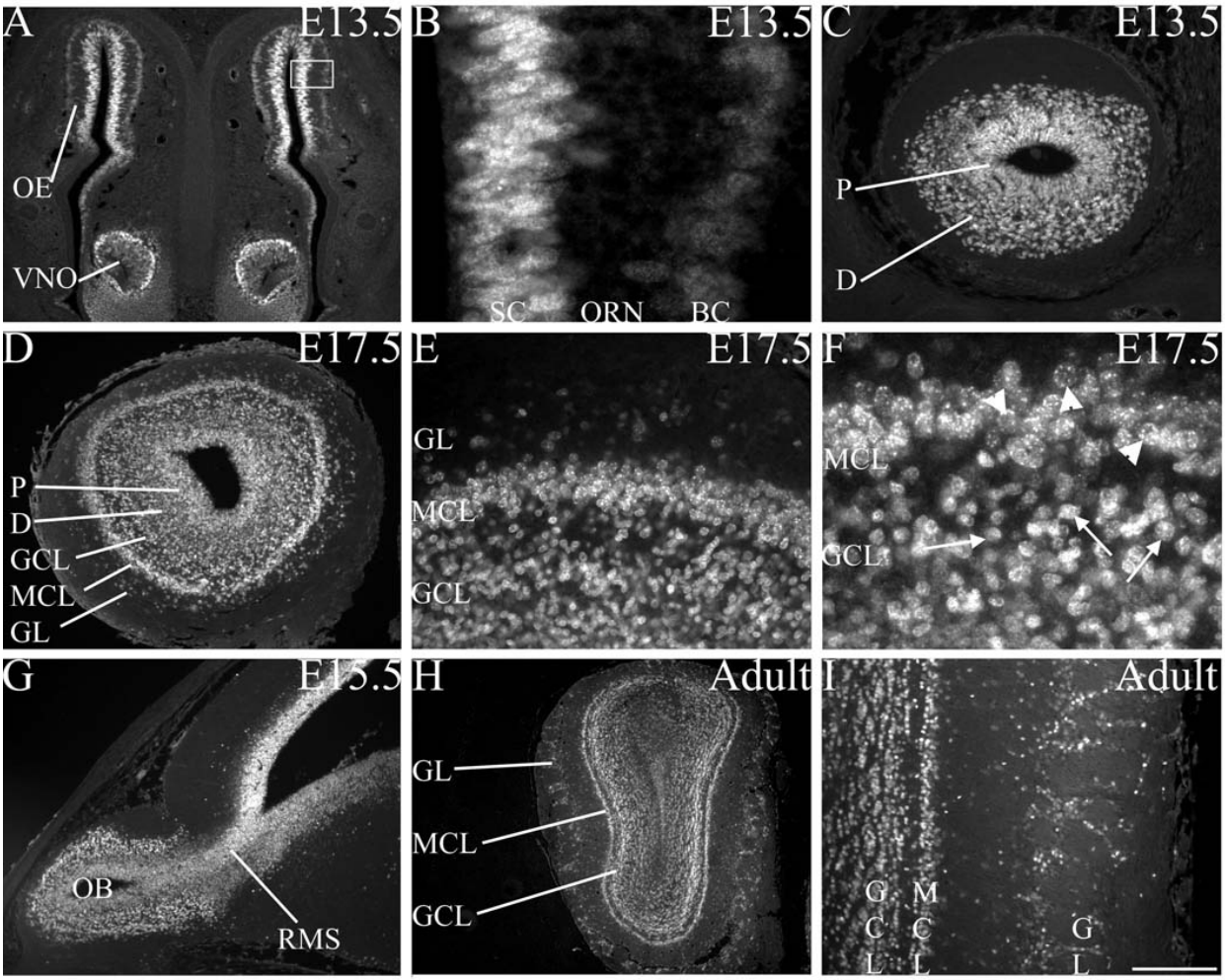

Figure 24. Sall1 expression in the developing and adult olfactory system

Immunohistochemistry of Sall1 expression in coronal (A-F,H,I) and sagittal (G) sections in the developing (A-G) and adult (H,I) olfactory system. Sall1 is expressed by cells in the olfactory epithelium (OE) (A,B) and vomeronasal organ (VNO) (A) at E13.5. Sall1 is strongly expressed by supporting cells (SC) and weakly expressed by basal cells (BC) in the OE (B). At E13.5 (C) and E17.5 (D) Sall1, is expressed by progenitor cells and differentiating cells. In addition, at E17.5 Sall1 is also expressed by cells in the MCL and GCL, and a subpopulation of cells in the GL (D,E). At E17.5, punctuate staining of Sall1 was observed in the MCL (arrowheads, F), while expression in other cellular populations was diffuse (arrows, F). Expression of Sall1 was also observed in the RMS (G). Sall1 expression is maintained in the adult (H,I) in the GL, MCL, and GCL. ORN: olfactory receptor neuron; P: progenitor cell; D: differentiating field; MCL: mitral cell layer; GL: glomerular layer; GCL: granule cell layer; OB: olfactory bulb; RMS: rostral migratory stream. Scale bar (in I) represents: 40µm in B; 70µm in F; 150µm in E; 275µm in C,I; 400µm in A; 425µm in D; 550µm in G; 1000µm in H.

4.3.2 Cellular disorganization in the OB of Sall1-mutant animals

Sall1 is expressed by diverse cellular populations in the developing and adult olfactory system. To determine whether this gene is required for distinct phases of OB development, mice with a targeted disruption of the Sall1 gene were examined. Sall1-deficient (Sall1-/-) animals do not thrive after birth and die shortly thereafter due to kidney deficits (Nishinakamura et al., 2001); thus, Sall1-mutant embryos were examined up to E18.5. An analysis of gross OB morphology at E14.5 and E18.5 was conducted to determine the effect of loss of this gene on OB size. No difference in OB length was observed at E14.5 ($p=0.6$, $n=3$); however, by E18.5 a 21.3% decrease in length of OB ($p<0.01$, $n=3$) was observed in Sall1-mutant animals compared to controls.

To characterize the contribution of Sall1 to distinct cell types, coronal sections of control and Sall1-/- animals were examined from E14.5 to E18.5 (n=3 per age). At E14.5, two distinct histological regions are visible in control OBs, the progenitor population and the differentiating field (Figure 25A,A'). The progenitor population contains organized parallel arrays of cells with elongated nuclei arranged perpendicular to the ventricular surface. The differentiating population contains small cells with round nuclei arranged in a disorganized manner, adjacent to the progenitor population. Both cell types were present in Sall1-mutant animals (Figure 25B,B'). Interestingly, in Sall1 -/- animals the ventricle appeared narrowed, elongated, and extended ventral-medially, which suggests abnormal proliferation or differentiation of cell types in the OB (Figure 25B). This slanting of the ventricle continued to E18.5 (Figure 25D,F). By E16.5, a layer of differentiated neurons is observed outside the differentiating field. Mitral cells represent the major cellular population that has differentiated at this time and can be distinguished by their

large oval-shaped cell body and light staining with Nissl (arrowheads, Figure 25C'), as compared to small intensely stained cell bodies in the differentiating field. We therefore assigned this population as the MCL (Figure 25C,C'). Cells within this population are tightly laminated and arranged perpendicular to the ventricular surface. In Sall1-mutant animals the progenitor population and a population of differentiating cells were evident at this age (Figure 25D). High-power examination of the OB revealed the presence of a cellular population with large oval shaped cell bodies, lightly stained with Nissl, outside the differentiating field (arrowheads, Figure 25D'). However, this population was not tightly laminated, as in controls, and thus was termed MCL-like (MCLl, Figure 25D'). These data suggest an alteration in mitral cell number, specification, or organization in Sall1-mutant animals. By E18.5, a subset of interneurons have migrated past the MCL to reach their laminar position in the GL, superficial to the MCL (Figure 25E,E'). In Sall1-mutant animals at E18.5 it was difficult to histologically distinguish laminar cell types (Figure 25F), although similar to E16.5 a population of disorganized cells with oval shaped cell bodies, lightly stained with Nissl, was present outside the differentiating field at this age (MCLl, Figure 25F'). These observations suggest a role for Sall1 in cell type specification or laminar organization in the OB.

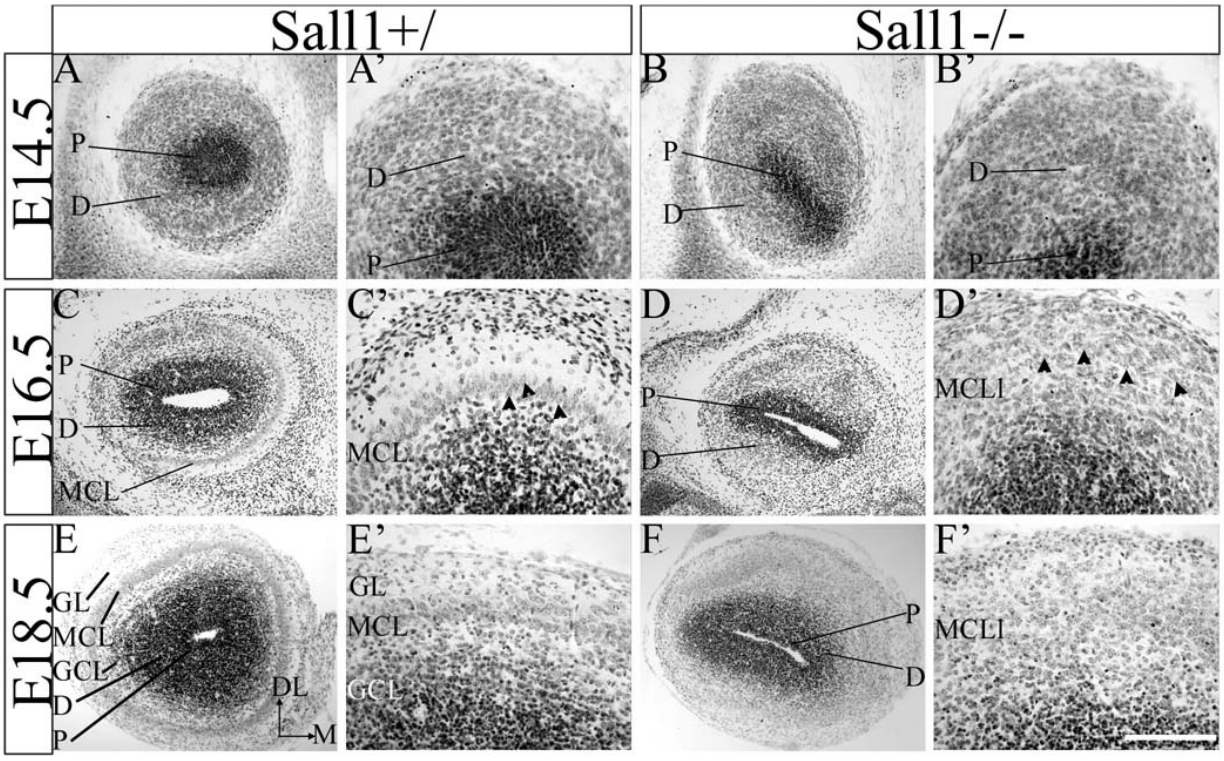

Figure 25. Histological examination of the developing OB in control and Sall1-mutant animals

Nissl staining of Sall1 control (A,A',C,C',E,E') and Sall1-mutant (B,B',D,D'F,F') animals at E14.5 (A-B'), E16.5 (C-D'), E18.5 (E-F'). Differentiated cells are present in wild type (A,A') and Sall1-mutant (B,B') animals from E14.5. From E16.5, a tightly laminated MCL is present consisting of large oval shaped lightly stained cells (arrowheads, C') in control animals (C,C',E,E'). In Sall1-mutant animals large oval cells (arrowheads, D') are present from E16.5, but these cells are not tightly laminated (D,D',F,F'). In the absence of Sall1, the ventricle appears slanted (B,D,F). (A') is higher magnification of (A) etc. Dorsal is top and medial right, as indicated. P: progenitor cell; D: differentiating field; MCL: mitral cell layer; MCLl: mitral cell layer-like; GL: glomerular layer; GCL: granule cell layer; DL: dorsal; M: medial. Scale bar (in F') represents: 100µm in A',B',C',D',E',F'; 200µm in A,B; 300µm in C,D,E,F.

4.3.3 Alterations in cellular lamination are observed in the absence of Sall1

Histological analyses of the developing OB identified alterations in the laminar structure or cell type specification of the OB in the absence of Sall1. To exclude the possibility that these alterations were due to cell death, TUNEL (n=3; E15.5, E18.5) and activated caspase 3 (n=3; E18.5) staining was examined in developing embryos. No gross differences were observed between control and Sall1-mutant animals (data not shown), which suggests that Sall1 is not required for OB cell survival. In Sall1-mutant animals, a disorganized population of cells was present outside the progenitor population from E16.5 (Figure 25D,D',F,F'). To verify these cells were neurons, expression of the pan neuronal marker β-tubulinIII (Tuj1) was examined at E18.5. In control animals, Tuj1-positive cells were present throughout the OB and exhibited a characteristic striated staining pattern in the MCL (Figure 26A). In Sall1-mutant animals, Tuj1-positive cells were present, but the striated staining pattern in the MCL was not observed (n=4) (Figure 26B), which suggests that neurons are differentiating, but that development of the MCL may be compromised.

To identify distinct laminae of the OB and to distinguish between early versus late generated populations, cell type specific markers were examined. Expression of Reelin (Figure 26C-F), a marker of the early born MCL, and Tyrosine Hydroxylase (TH) (Figure 26G,H), a marker of the later born GL, was examined. In Sall1-/- animals, Reelin-positive cells were present in superficial regions of the OB at E18.5 (n=4) (MCLl, Figure 26D), although the staining pattern was not tightly laminated but appeared disorganized in contrast to controls. Reelin expression is first detected in the MCL at E16.5. To determine whether the onset of Reelin expression was normal in Sall1-mutant animals, E16.5 embryos were examined (n=2). Disorganized Reelin-

positive staining was observed in Sall1-/- embryos at E16.5 (MCLl, Figure 26F). These data suggest that in the absence of Sall1, mitral cells are generated but that these cells fail to appropriately organize. In wild type animals, TH is expressed by a subpopulation of cells in the GL, adjacent to the olfactory pial surface (Figure 26G). In Sall1-mutant animals numerous TH-positive cells were detected adjacent to the pial surface at E18.5 (n=3), which suggests appropriate generation and positioning of cells in the GL in the absence of Sall1 (Figure 26F). These findings suggest that Sall1 is required for laminar organization of the MCL.

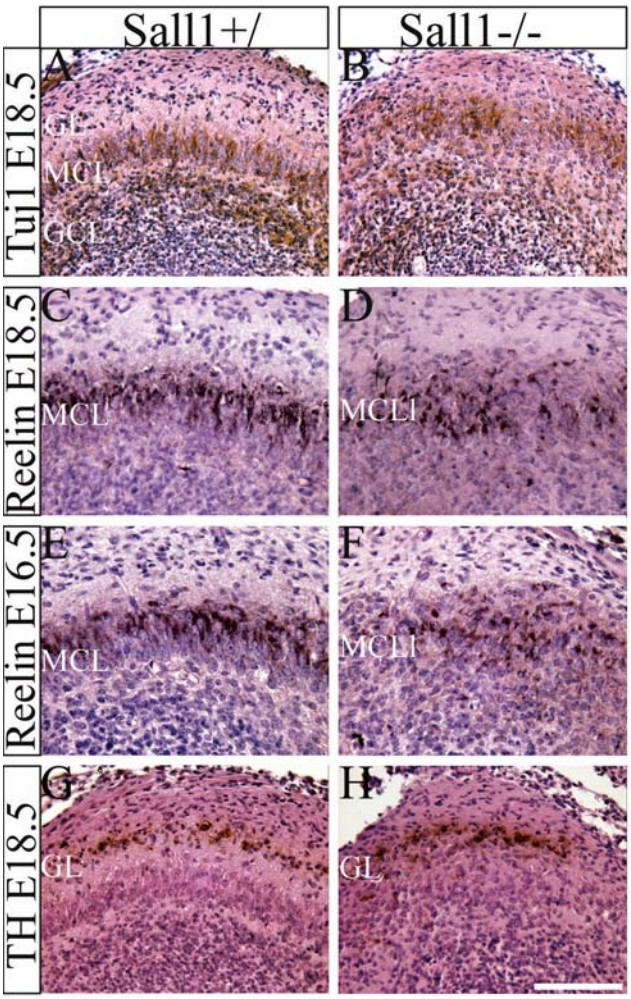

Figure 26. Examination of cell type and layer specification in control and Sall1-mutant animals.

Tuj1 (A,B), Reelin (C-F) and TH (G,H) expression (brown) in Sall1 control (A,C,E,G) and Sall1-mutant (B,D,F,H) animals at E16.5 (E,F) and E18.5 (A-D,G,H). Sections were counterstained with Haematoxylin and Eosin. Cells in the MCL appear scattered and disorganized in the absence of Sall1 (B, MCLl: D,F). The GL is present in superficial regions in Sall1-mutant animals (H). MCL: mitral cell layer; MCLl: mitral cell layer-like; GL, glomerular layer; GCL: granule cell layer. Scale bar (in H) represents: 70µm in D-F; 100µm in A,B,G,H.

In the absence of Sall1, the OB is reduced in size, the olfactory ventricle is elongated and extends ventral-medially, and mitral cells appear disorganized. To further investigate the effect of loss of Sall1 on the MCL, we examined the orientation and organization of this cellular population using expression of Tbr1 to label cell bodies and Reelin to label apical dendrites (n=4) (Figure 27A,B). In control animals, Tbr1-positive cell bodies in the MCL are arranged in parallel arrays and Reelin-positive dendrites extend towards the pial surface (Figure 27A). In the absence of Sall1, some Reelin positive mitral cells dendrites are orientated towards the pial surface, although these dendrites appeared thicker and shorter than in control animals (arrows, Figure 27B). Furthermore, many Reelin-positive mitral cell dendrites were found to be misorientated towards the basal, medial, and lateral surfaces (arrowheads, Figure 27B). These data indicate that, in the absence of Sall1, mitral cells are misorientated and disorganized. Projections from mitral cells to the olfactory cortex via the lateral olfactory tract can be visualized with Calretinin (Figure 27C). Despite the disorganization and misorientation of mitral cells observed in Sall1-/- animals, mitral cell projections via the lateral olfactory tract were present, although somewhat reduced (n=3) (Figure 27D).

Our results suggest a role for Sall1 in the lamination and orientation of mitral cells. In the absence of Sall1, TH-positive interneurons destined for the GL can migrate past the MCLl to their appropriate laminar position (Figure 26F). The olfactory bulb contains independent interneuron populations, which can be distinguished by expression of molecular markers (Kosaka et al., 1995; Toida et al., 2000; Parrish-Aungst et al., 2007). To determine whether other olfactory interneuron populations were altered in the absence of Sall1, we examined expression of two independent olfactory interneuron populations, GAD65/67 (n=3) and

Calretinin (n=3), at E18.5. GAD65/67 is expressed by GABAergic cells in the GL and GCL, as well as the projections of these cells through the MCL (Figure 27E). In Sall1-/- animals, GAD65/67 expression was present, although disorganized, throughout this region (Figure 27F). Calretinin is expressed by a non-dopaminergic olfactory interneuron population (Kosaka et al., 1995), and at E18.5 is expressed by olfactory nerve axons and cells within the GL, MCL, and GCL (Figure 27G). In Sall1-mutant animals, similar to the GAD65/67 staining, Calretinin-positive cells were scattered throughout the entire region (Figure 27H). Taken together, these results indicate that interneuron populations are appropriately generated in the absence of Sall1. These data suggest that Sall1 is not required for interneuron specification or migration to the OB via the rostral migratory stream; however, Sall1 expression may be required for laminar organization of subsets of interneurons.

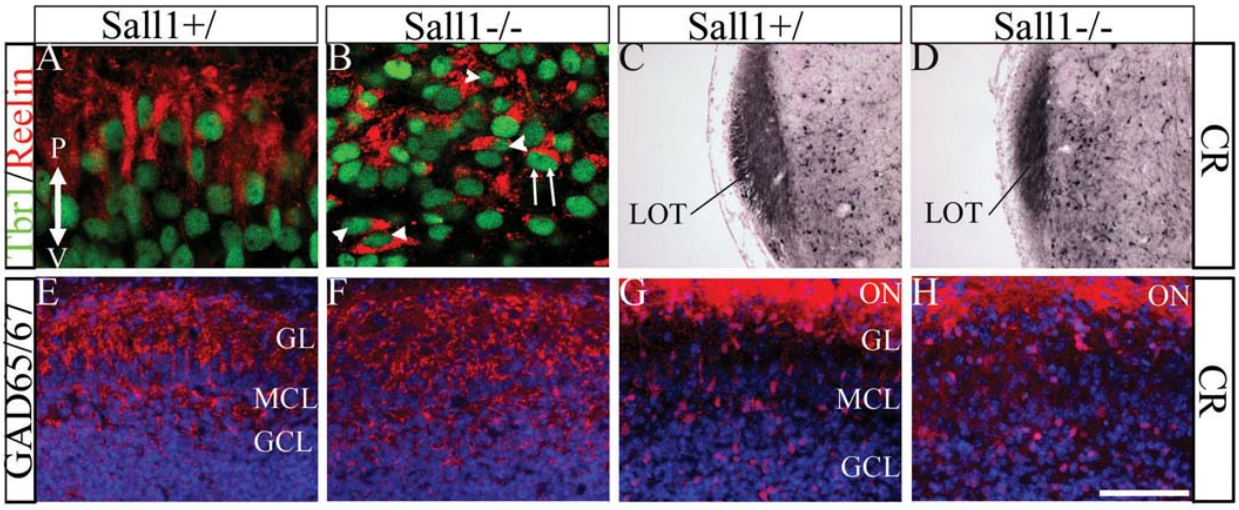

Figure 27. Cellular organization in control and Sall1-mutant animals.

Confocal image of single optical slice of Tbr1 (green) and Reelin (red) expression at E18.5 (A,B). In control animals Reelin-positive dendrites extend towards the pial surface (A). In Sall1-mutant animals, Reelin-positive dendrites orientated towards the pial surface were thicker and shorter than in controls (arrows, B). In addition, many dendrites were misorientated towards the medial, lateral, and basal surfaces in Sall1-/- animals (arrowheads, B). Calretinin immunohistochemistry (black, C,D) indicated the LOT projected to the olfactory cortex in Sall1-deficient animals at E18.5 (D). GAD65/67 (red, E,F) and Calretinin (red, G,H) immunohistochemistry at E18.5 revealed a disorganization of interneuron populations in Sall1 -/- animals (F,H) compared to controls (E,G). Pial (P) surface is up, ventricular (V) surface is down in A,B,E-H, as indicated in (A). Sections were counterstained with nuclear fast red (C,D) or DAPI (E-H). GL: glomerular layer; MCL: mitral cell layer; GCL: granule cell layer; LOT: lateral olfactory tract; ON: olfactory nerve, CR: Calretinin. Scale bar (in H) represents: 30µm in A,B; 75µm in E-H; 200µm in C,D.

4.3.4 The olfactory nerve fails to extend to the dorsal-lateral OB surface in Sall1-/- animals

Defects in olfactory nerve innervation have previously been shown to be associated with MCL deficits and OB disorganization (Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006; Laub et al., 2006). We therefore examined innervation of the OB by the olfactory nerve using GAP43 expression (Figure 28A-D). At ~E11.5 (E13 rat), the olfactory nerve innervates the neural epithelium of the developing telencephalon, and this is believed to stimulate the evagination of the bulb from this region (Gong and Shipley, 1995). By E15.5, the nerve has contacted the ventral surface of the OB and has begun to extend laterally and medially in control animals (arrows, Figure 28A), with a few fibers extending dorsally (*, Figure 28A). In the absence of Sall1, the nerve innervates the ventral surface but does not extend as far medially and laterally as in controls (n=4) (arrows, Figure 28B), although similar to controls a few fibers extend towards the dorsal surface (*, Figure 28B). In wild type animals at E17.5, the nerve has extended laterally, medially, and dorsally (Figure 28C). In Sall1-mutant animals, the nerve remains in the ventral-medial position, failing to extend laterally (n=3), with very few fibers extending dorsally (*, Figure 28D). Furthermore, GAP43 staining demonstrated the presence of glomeruli-like structures in Sall1-mutant animals in the ventral OB with GAP43 at E17.5 (n=3) (arrows, Figure 28E,F), which suggests that where the olfactory nerve is present appropriate innervation is established.

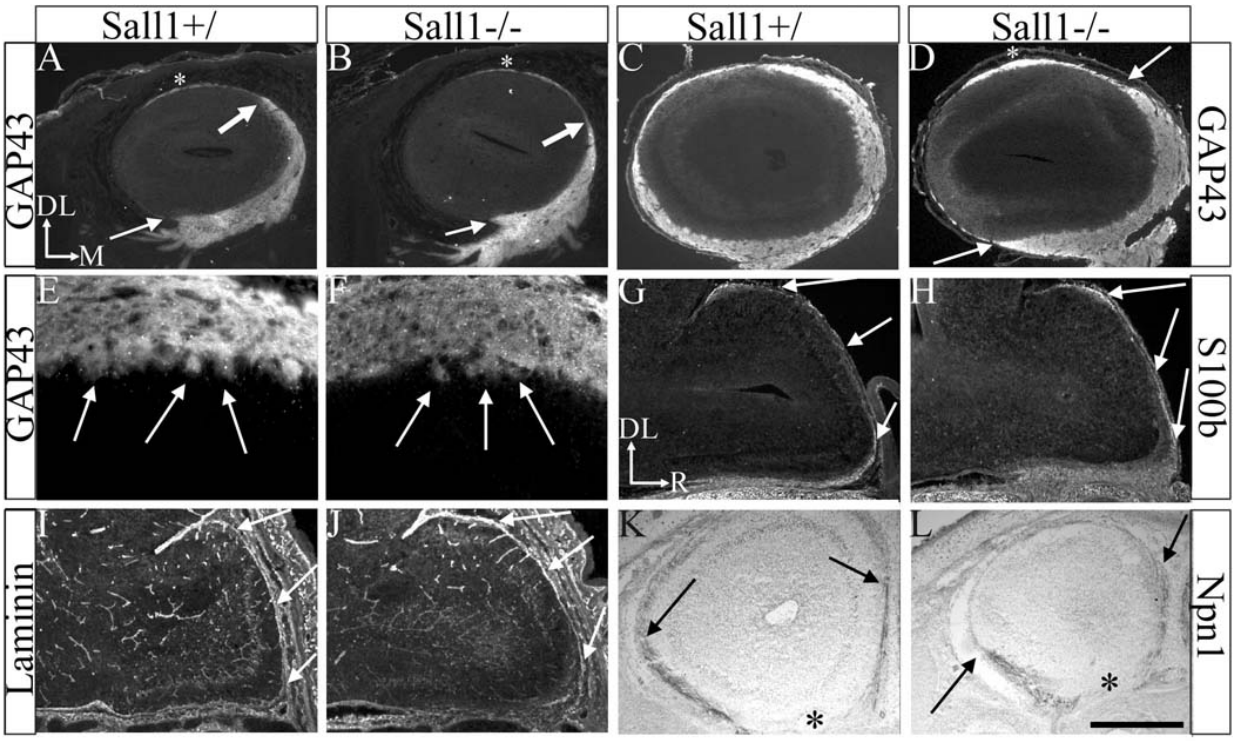

Figure 28. Examination of olfactory nerve extension in control and Sall1-mutant animals.

GAP43 expression at E15.5 (A,B) and E17.5 (C,D) identified a failure of olfactory nerve extension in Sall1-mutant animals (B,D). The arrows demarcate the medial and lateral most extension of the olfactory nerve (A,B,D). At E15.5 a few GAP43-positive fibers extended dorsally in both control and Sall1-/- animals (*, A,B). At E17.5, the olfactory nerve was confined to the ventral-medial OB (arrows, D), with only a few GAP43-positive fibers observed in dorsal regions (*, D). Glomeruli-like structures were observed with GAP43 staining in control and Sall1-deficient animals (arrows, E,F). S100β (G,H) and laminin (I,J) expression identified that olfactory ensheathing cells extended to the dorsal surface in both controls (G,I) and Sall1 -/- (H,J) animals in sagittal sections at E18.5 (arrows). Neuropilin 1 (Npn1)-positive axons were appropriately segregated in control (K) and Sall1-mutant animals (L), avoiding the region corresponding to Semaphorin 3a expression (*, K,L). Dorsal is up and medial is right (A-D), as indicated. Dorsal is up and rostral is right (G-J), as indicated. DL: Dorsal; M: Medial; R: Rostral. Scale bar (in L): 75μm in E,F; 300μm in A-D; 550μm in K,L; 600μm in G-J.

The olfactory nerve is segregated into inner and outer regions (Au et al., 2002), which can be distinguished by expression of NCAM and S100β. NCAM is expressed by the entire olfactory nerve (Chung et al., 1991), while S100β is strongly expressed by olfactory ensheathing cells in

the outer portion of the olfactory nerve (Astic et al., 1998) (Figure 29A). To determine whether Sall1 was required for segregation or layering of the olfactory nerve, we examined expression of these markers in sagittal sections at E18.5 (n=3). In control animals co-expression of NCAM and S100β was observed in the outer region of the olfactory nerve (**, Figure 29A), whereas NCAM alone was expressed within the inner olfactory nerve (*, Figure 29A). In the absence of Sall1, the olfactory nerve was thicker in the ventral regions; however, segregation of NCAM and S100β expression was observed within this region (Figure 29B). These findings indicate that Sall1 is not required for segregation of the olfactory nerve into outer and inner nerve layers.

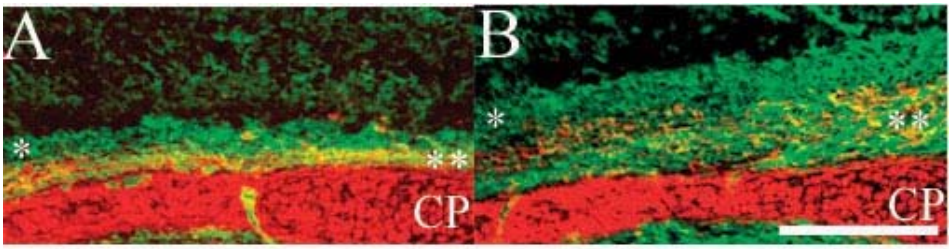

Figure 29. Segregation of the olfactory nerve into outer and inner regions in control and Sall1-mutant animals

NCAM (green) and s100β (red) expression identified appropriate segregation of the olfactory nerve in ventral regions in control (A) and Sall1-/- (B) animals. In control and Sall1-deficient animals co-expression of NCAM and S100β was observed in the outer region of the olfactory nerve (**,A,B), while NCAM alone was expressed within the inner olfactory nerve (*, A,B). CP: cribriform plate. Scale bar (in B): 200μm in A,B.

Olfactory ensheathing cells are hypothesized to produce guidance factors for olfactory nerve innervation (Treloar et al., 1996; Kafitz and Greer, 1999; Tisay and Key, 1999). To determine whether an absence of olfactory ensheathing cells in the dorsal-lateral OB contributed to the observed phenotype, we examined expression of S100β and laminin at E18.5 in sagittal sections (Figure 28E-H). S100β is strongly expressed by olfactory ensheathing cells (Astic et al., 1998),

and at E18.5, S100β-positive cells were observed surrounding the OB, extending from the ventral surface up to the dorsal surface (arrows, Figure 28G). In the absence of Sall1, a layer of S100β-positive olfactory ensheathing cells was observed extending to the dorsal surface (n=3) (arrows, Figure 28H). We confirmed that olfactory ensheathing cells extended the entire surface of the OB in Sall1-mutant animals at E18.5 using the olfactory ensheathing cell marker laminin (Doucette, 1990) (n=2) (arrows, Figure 28I,J). These findings indicate that olfactory ensheathing cells are appropriately specified in the absence of Sall1. Taken together, these data suggest that extension of the olfactory nerve to lateral and dorsal surfaces is dependent on Sall1.

Axon guidance molecules expressed by cells within the olfactory bulb have been shown to guide and sort incoming olfactory nerve fibers (Lin and Ngai, 1999; St John et al., 2002a; Nedelec et al., 2005). Crandall and others hypothesized that the ventral-medial nerve layer is a "critical axon sorting region" during olfactory development (Crandall et al., 2000). In the absence of Sall1, the olfactory nerve contacts the OB but remains in the ventral-medial region, failing to extend laterally or dorsally (Figure 28B,D). We hypothesized that alterations in signaling molecules in this region could account for this phenotype. We identified two key molecules that are expressed in complimentary patterns in the nerve layer of the developing OB, Semaphorin 3a and Neuropilin 1. Semaphorin 3a is expressed by cells in the olfactory nerve layer and is hypothesized to act as a repulsive signal to direct and segregate Neuropilin 1-expressing axons to lateral and medial regions of the central OB (Schwarting et al., 2000; Schwarting et al., 2004). In Semaphorin 3a mutant mice, Neuropilin 1 axons do not segregate and fail to extend to lateral regions in the central and caudal OB (Schwarting et al., 2000), similar to the Sall1-/- phenotype. At E18.5, Neuropilin 1 axons segregated into lateral and medial populations in both control and

Sall1-deficient animals, avoiding the region corresponding to Semaphorin 3a expression (*, Figure 28K,L), although these axons did not extend as far laterally in Sall1-mutant animals as in controls (n=2) (Figure 28K,L). These data indicate that Semaphorin 3a/Neuropilin 1-mediated axonal sorting is relatively normal in Sall1-mutant animals.

The olfactory nerve arises from four distinct zones in the olfactory epithelium, and these zones project to topographically distinct glomeruli in the OB (Ressler et al., 1993; Vassar et al., 1993; Ressler et al., 1994; Mombaerts et al., 1996). To determine whether the failure of the olfactory nerve to extend dorsally and laterally in Sall1-/- animals was a consequence of olfactory epithelium deficiencies, we examined development of this structure and zonal organization of the olfactory epithelium. The olfactory epithelium appears normal in Sall1-mutant animals at E15.5 and E17.5 as visualized by Nissl (Figure 30A-D) and Tuj1 staining (Figure 30E-L). NADPH diaphorase activity is located in zone 1 of the olfactory epithelium (Alenius and Bohm, 2003), which projects to the rostral-dorsal region of the OB (Ressler et al., 1994; Alenius and Bohm, 2003), the region that is deficient in Sall1-mutant mice. No difference in NADPH diaphorase histochemistry was observed at E18.5 (data not shown, n=4), which indicates that altered zonal organization is not responsible for deficiencies observed in Sall1 -/- animals. These data suggest that alterations in olfactory nerve innervation are not due to an intrinsic lack of Sall1 in the olfactory epithelium. Sall1 is not expressed by olfactory receptor neurons, which suggests that the deficits in olfactory nerve extension are due to a non-cell autonomous effect.

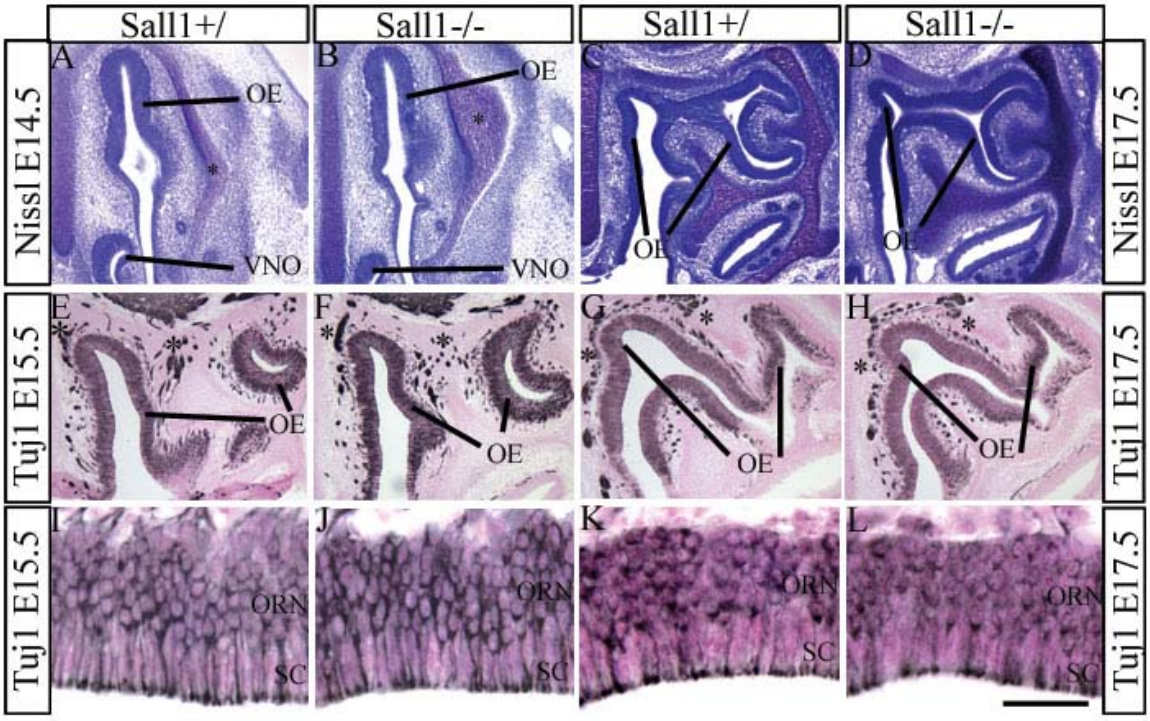

Figure 30. Olfactory epithelium development in control and Sall1-mutant animals

Nissl staining of E14.5 (A,B) and E17.5 (C,D) olfactory epithelium in control (A,C) and Sall1 -/- animals (B,D). * in A,B, demarcates the cartilage primordium of the turbinate bone. Lamination of olfactory receptor neurons appeared normal as visualized with Tuj1 expression in the olfactory epithelium at E15.5 (E,F,I,J) and E17.5 (G,H,K,L) in control (E,G,I,K) and Sall1-mutant (F,H,J,L) animals. The olfactory nerve (* , E-H) projected from the olfactory epithelium to the olfactory bulb in control (E,G) and Sall1 (F,H) deficient animals. OE: olfactory epithelium; VNO: vomeronasal organ; ORN: olfactory receptor neuron; SC: supporting cells. Scale bar (in L): 30μm in I-L; 300μm in A,B,E,F; 350μm in C,D,G,H.

4.3.5 Sall1 regulates the rate of proliferation in the developing OB

One striking phenotype observed in Sall1-mutant animals was the altered shape of the olfactory ventricle, which was elongated and extended ventral-medially from E14.5 (Figure 25B,D,F). In Sall1-deficient animals, very few differentiated cells were observed in the ventral-medial region at this age (Figure 25B). At this time the nerve is localized to ventral-medial regions in control and mutant animals, which suggests that the initial targeting of the nerve is not affected by loss

of Sall1. By E15.5, the olfactory nerve extends dorsally and laterally; however, in Sall1-mutant animals, the nerve remains predominately in the ventral-medial region, with only few fibers extending dorsally. In light of the absence of differentiated cells and failure of nerve extension past ventral-medial regions, we postulated that regional alterations in progenitor cell proliferation or differentiation could account for these deficiencies in Sall1-mutant animals. To investigate this possibility, we first quantified the number of progenitor cells in the OB at E14.5 and observed no difference in total progenitor cell number between wild type and mutant animals (713.8±43.5 progenitor cells in controls versus 764.7±25.4 progenitor cells in Sall1-mutant animals, n=4, p=0.4). We next examined the rate of proliferation of OB progenitors using short term 5-bromo-2-deoxyuridine (BrdU) labeling. To determine if there were regional differences in OB progenitor proliferation, we divided the OB into 6 regions, medial, central, and lateral, in the both the dorsal and ventral OB (Figure 23A). In each region, we calculated the labeling index and compared wild type to mutant animals. In wild type animals, an intrinsic regional difference in the labeling index was observed. The labeling index was higher in dorsal compared to ventral progenitor populations (31.96% (dorsal-central) versus 19.01% (ventral-central), $p<0.05$, n=4, Table 2) and lateral compared to medial progenitor cells (38.98% (dorsal-lateral) versus 30.84% (dorsal-medial), $p<0.05$, n=4; 31.12% (ventral-lateral) versus 23.82% (ventral-medial), $p<0.05$, n=4; Table 2). These regional differences in the labeling index were preserved in Sall1-mutant animals (33.61% (dorsal-central) versus 29.61% (ventral-central), $p<0.01$, n=4; 45.41% (dorsal-lateral) versus 32.48% (dorsal-medial), $p<0.05$, n=4, Table 2; 40.49% (ventral-lateral) versus 23.39% (ventral-medial), $p<0.001$; n=4; Table 2), although the dorsal-ventral gradient was not as robust in Sall1-mutant animals as in controls (12.95% difference dorsal-central versus ventral-central in controls versus 4% difference dorsal-central versus ventral-central in Sall1-mutant

animals, $p<0.05$, n=4). Interestingly, in ventral-central and ventral-lateral regions, where the olfactory nerve failed to extend at E15.5, we saw an increase in the labeling index at E14.5 in Sall1-/- animals compared to wild type littermates (Table 2). These findings suggest that, in the absence of cell death, Sall1 regionally regulates either the rate of proliferation or the rate of cell cycle exit in olfactory progenitors.

Table 2. Labeling index of Sall1+/ and Sall1-/- animals at E14.5

Region of study	Labeling Index ±SEM		% of WT	P value
	Sall1+/	Sall1-/-		
Ventral-medial	23.82±2.41%	23.39±1.68%	98%	0.8870
Ventral-central	19.01±1.13%	29.61±0.84%	155.76%	0.0003
Ventral-lateral	31.12±0.96%	40.49±2.22%	130.10%	0.0088
Dorsal-medial	30.84±2.65%	32.48±1.75%	105.31%	0.6269
Dorsal-central	31.96±2.69%	33.61±1.19%	105.16%	0.5923
Dorsal-lateral	38.98±1.5%	45.41±3.06%	116.49%	0.1090

4.3.6 Sall1 regionally regulates differentiation

To determine the consequences of these changes in the labeling index in control versus Sall1-mutant animals, we labeled a population of proliferating cells on E14.5 using BrdU incorporation and examined the position of these cells at E18.5. Heavily labeled BrdU-positive cells represent the population of cells that differentiated on E14.5; cells that contain more lightly labeled BrdU represent cells that underwent further rounds of cell division, thus diluting the BrdU label. In control animals, heavily labeled BrdU-positive cells are predominately detected in the superficial

GCL, as expected (Figure 31A). Consistent with the observed regional differences in the rate of proliferation, more heavily labeled BrdU-positive cells were located in the ventral OB (65.4% (37.3 cells)) than in dorsal regions (34.6% (19.7 cells)) (n=3, $p<0.05$, Table 3, Figure 31A) in control animals. These findings suggest that the regional differences in the labeling index correlate to regional changes in neurogenesis. In Sall1-mutant animals, the number of cells born on E14.5 and their laminar position in the GCL at E18.5 was similar to controls (n=3, $p=0.4$, Table 3, Figure 31B), which suggests that loss of Sall1 does not alter initial interneuron production or their migration in the rostral migratory stream. However, the ventral-dorsal pattern of neurogenesis was not maintained in Sall1-mutant animals (48.3% (29.2 cells) ventral versus 51.7% (31.2 cells) dorsal, n=3, $p=0.1$, Figure 31B, Table 3). Furthermore, a distinct change in the medial to lateral distribution of labeled cells was observed in Sall1-mutant animals, with very few heavily labeled BrdU-positive cells present in lateral regions (*, Figure 31B). This area contained only lightly labeled BrdU cells, which suggests that they did not differentiate on E14.5 and underwent further rounds of cell division. These findings suggest that the increased labeling index at E14.5 in ventral and lateral regions is associated with decreased neuronal differentiation in Sall1-mutant animals. Furthermore, these data indicate that at E14.5 intrinsic gradients of proliferation and neurogenesis are observed in the OB in controls animals and that in the absence of Sall1 these gradients are disrupted.

Table 3. Cellular birth-dating in Sall1+/ and Sall-/- animals at E18.5

	Sall1+/*	Sall1-/-*	% of WT	P Value
Total: BrdU E14.5	57.0±1.3	60.4±3.4	105.9%	0.3759
Ventral: BrdU E14.5	37.3±1.6	29.2±1.6	78.3%	0.0231
Dorsal: BrdU E14.5	19.7±1.7	31.2±2.0	158.4%	0.0126
Total: BrdU E11.5	5.8±0.8	14.2±1.6	244.8%	0.0102
Ventral: BrdU E11.5	1.7±0.3	4.3±0.3	268.7%	0.0064
Dorsal: BrdU E11.5	4.1±0.6	9.9±1.5	239.0%	0.0266

* Values indicate the number of BrdU-positive cells ± Standard Error of the Mean

Olfactory interneuron populations are born from E14.5 (Hinds, 1968), and the olfactory bulb contains distinct interneuron populations (Kosaka et al., 1995; Parrish-Aungst et al., 2007). GABAergic cellular populations represent the largest interneuron population in the olfactory bulb (Parrish-Aungst et al., 2007). To verify that cells born on E14.5 differentiated into interneurons, we examined the proportion of cells born on E14.5 that were GABAergic at E18.5 (BrdU+/GABA+). In control animals, 72.4±1.8% of cells born on E14.5 expressed GABA at E18.5. In Sall1-mutant animals, 84.4±0.7% of cells born on E14.5 expressed GABA at E18.5. These findings indicate that GABAergic cells are the major population born on E14.5 in control and Sall1-mutant animals. Interestingly, a 16.6% increase in the proportion of GABAergic cells born on E14.5 was observed in the absence of Sall1 ($p<0.01$, $n=3$). These data suggest that Sall1 may influence the relative proportion of differentiating interneuron subtypes.

In the absence of Sall1 alterations in OB development are already apparent from E14.5, which suggests a role for Sall1 in MCL differentiation. To examine this possibility, we performed birth-dating studies on E11.5 animals, when MCL cells are born, and examined the position of heavily labeled cells at E18.5. In control animals, heavily labeled cells are located in the MCL, as expected, and encompass the entire OB. In Sall1-deficient animals, labeled cells were located outside the differentiating field, in the MCLl (Figure 31D). More heavily labeled BrdU-positive cells were observed in dorsal regions (70.7% (4.1 cells)) than ventral (29.3% (1.7 cells)) in control animals (n=3, $p<0.01$, Table 3, Figure 31C). Similar to control animals, more labeled cells were found dorsally in Sall1-mutant animals (69.7% (4.3 cells) dorsal versus 30.3% (9.9 cells) ventral, n=3, $p<0.05$). Interestingly, labeled cells were noticeably absent from the ventral-medial region (*, Figure 31D). In addition, a 144.8% increase in the number of cells born on E11.5 was observed in Sall1-deficient animals compared to control littermates (n=3, $p<0.05$, Table 3, Figure 31D). This increase in the number of cells born on E11.5 in Sall1-deficient animals was observed in both dorsal and ventral populations (Table 3).

To verify that the cells born on E11.5 differentiated into mitral cells in the absence of Sall1, we examined co-expression of Tbr1, a MCL marker (Figure 31E), and BrdU. The percent of cells that differentiate on E11.5 that express Tbr1 at E18.5 was similar in Sall1-/- (95.4±1.6%) and control animals (94.5±1.0%) (n=3, $p=0.6$). In addition, we quantified the total number of Tbr1-positive cells in dorsal-ventral regions at E18.5 and observed a 27.5% increase in the total number of Tbr1-positive cells in Sall1-mutant animals compared to controls (494.2±6.5 in controls versus 630.2±12.3 in Sall1-mutant animals, n=3, $p<0.001$, Figure 31E,F). This is accompanied by a distinct reduction in the number of Tbr1-positive cells in the ventral-medial

region (n=4) (*, Figure 31F). These findings indicate that Sall1 does not regulate mitral cell fate specification, but regionally regulates mitral cell neurogenesis in the developing OB. We therefore hypothesize that Sall1 is temporally and spatially required to regulate cellular differentiation in the developing OB.

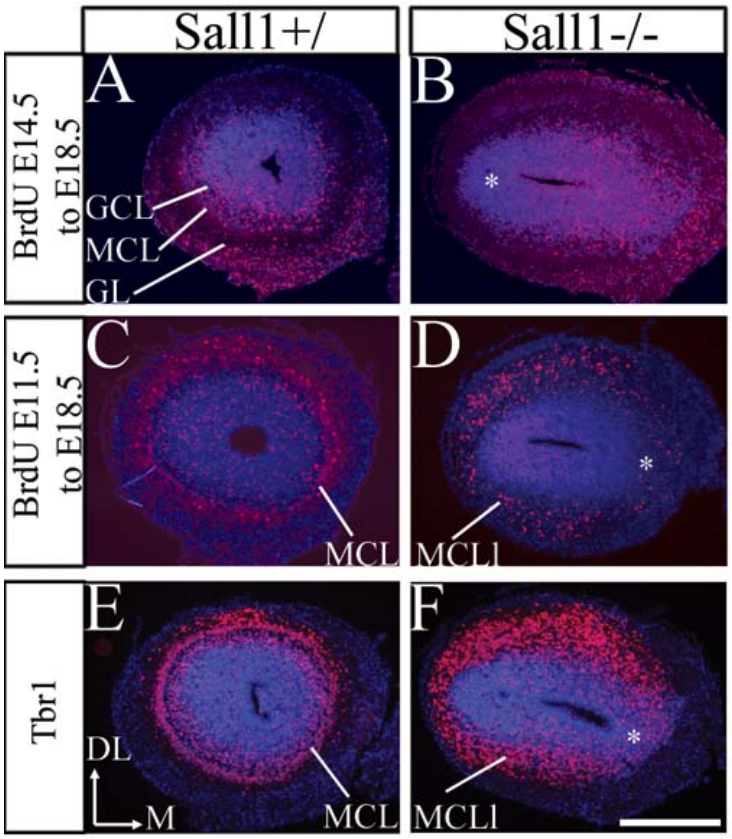

Figure 31. Examination of position and number of early (E11.5) and later (E14.5) born cells in the developing OB at E18.5.

BrdU staining (red) of E18.5 embryos labeled with BrdU on E14.5 (A,B). In control animals, heavily labeled BrdU-positive cells are found throughout the circumference of the OB, with highest concentration in the ventral OB (A). In Sall1-/- animals, heavily labeled cells are located in ventral and dorsal populations but are absent from lateral regions (*,B). BrdU staining (red) of E18.5 embryos labeled with BrdU on E11.5 (C,D). In control animals heavily labeled cells are located in the MCL, although more cells are present dorsally than ventrally (C). In Sall1-/- animals, many more heavily labeled cells were observed in the MCLl in ventral, dorsal, and lateral regions than in control animals; however, very few heavily labeled cells were present in the medial OB (*,D). Staining with the mitral cell marker Tbr1 (red) confirmed that in Sall1-mutant animals (F), more mitral cells were present in the OB at E18.5 than in controls (E), and that the medial OB contained very few mitral cells. Sections were counterstained with DAPI. Dorsal is up and medial is right, as indicated. MCL: mitral cell layer; MCLl: mitral cell layer-like; GL, glomerular layer; GCL: granule cell layer; DL: dorsal; M: medial. Scale bar = 300um.

4.4 DISCUSSION

Olfactory stimuli are detected by neurons within the olfactory epithelium. These neurons project in a topographic pattern to spatially conserved glomeruli within the OB to relay sensory information (Buck and Axel, 1991; Ressler et al., 1993; Vassar et al., 1993; Sullivan et al., 1995). Within the olfactory bulb inhibitory and excitatory neuronal populations modulate the information received from the periphery and further transmit it to the olfactory cortex. Excitatory and inhibitory OB neuronal populations are sequentially produced and arise from distinct progenitor cells populations in the dorsal and ventral telencephalon respectively (Hinds, 1968; Lois and Alvarez-Buylla, 1994; Bulfone et al., 1995; Doetsch and Alvarez-Buylla, 1996; Bulfone et al., 1998; Wichterle et al., 1999; Puelles et al., 2000; Wichterle et al., 2001; Moreno et al., 2003; Stenman et al., 2003). Molecular regulators of interneuron neurogenesis have been identified (Yun et al., 2003; Soria et al., 2004; Hack et al., 2005; Kohwi et al., 2005; Waclaw et al., 2006); however, only one factor, Tbr1, has been implicated in neurogenesis of the excitatory OB neuronal population (Bulfone et al., 1998). Furthermore, cellular and molecular mechanisms regulating innervation of the OB from the periphery are not well understood.

This study examined the role of the Sall1 gene in the development of the olfactory system. We determined that Sall1 is expressed by progenitor cells and sub-populations of differentiated neurons in both the peripheral and central olfactory system. Interestingly, we observed a difference in cellular localization of Sall1 in mitral cells at E17.5 compared to other OB cell types, which suggests a distinct functional role for Sall1 in mitral cells. Sall1-mutant animals die at birth and have smaller OBs. We identified regional alterations in the pattern of proliferation and neurogenesis in the developing olfactory bulb in the absence of the Sall1. Our findings are

consistent with a role for Sall1 in the temporal and spatial regulation of cellular differentiation during olfactory development. An increase in the number of early born neurons, mitral cells, was observed in the absence of Sall1. In addition, this cellular population was disorganized and misorientated. Despite the alterations in the mitral cell population, projections of these neurons to the olfactory cortex, via the lateral olfactory tract were present, although reduced in size. We postulate that the decreased size of the OB in Sall1-mutant animals contributes to the reduction in size of the lateral olfactory tract, although, it is possible that not all of the misorientated mitral cells project to the olfactory cortex.

Interneuron populations were specified in Sall1-deficient animals, and TH-positive cells migrate past the MCLl to their appropriate laminar position. However, alterations in the laminar position of GABAergic and Calretinin populations were observed in Sall1-/- animals. These alterations in laminar position may be a consequence of the disorganized MCL or may reflect a role for Sall1 in the lamination of distinct subsets of interneuron populations. We did not observe an absence of any distinct interneuron population in the absence of Sall1. However, more cells born at E14.5 differentiate into GABAergic subtypes, which suggests that a shift in interneuron populations may exist in Sall1-deficient animals.

In Sall1-mutant animals the olfactory nerve contacted the OB but failed to extend to dorsal and lateral olfactory bulb regions. We hypothesize that the altered olfactory nerve extension is a consequence of Sall1's role in olfactory bulb neurogenesis, and that the disorganization of mitral cells is due to altered peripheral innervation.

4.4.1 Sall1 regulates olfactory bulb neurogenesis

Sall1 is required from as early as E11.5 to regulate olfactory neurogenesis. One of the first deficits observed in the absence of Sall1 is an increase in number of neurons produced at E11.5 relative to control animals. Mitral cells are born from E11.5 to E13.5 in mice (Hinds, 1968; Blanchart et al., 2006), and birth-dating studies indicate that these cells are over-produced in mutant animals. At E18.5, we observed spatial differences in the position of mitral layer cells born on E11.5 in mutant animals. In control animals, BrdU birth-dating studies indicated mitral cells born on E11.5 were localized in dorsal, ventral, medial and lateral positions at E18.5. However, significantly more cells were localized in dorsal regions than ventral. In Sall1-mutant animals, more mitral cells were born on E11.5; nonetheless, the dorsal-ventral pattern of neurogenesis was maintained. However, very few mitral cells were observed in the ventral-medial OB at E18.5. This decrease in cell number in ventral-medial OB is obvious from E14.5, when very few neurons were observed in ventral-medial regions and the ventricle was elongated and extended ventral-medially. These findings suggest that Sall1 regulates mitral cell neurogenesis during olfactory development.

From E14.5, cells destined for the granule cell layer and glomerular cell layer are born (Hinds, 1968). Our birth-dating studies indicate that cells born on E14.5 in control animals are predominantly localized in ventral, medial, and lateral regions on E18.5, with fewer cells born dorsally. Consistent with our findings, a recent study identified a number of factors, including Dlx1, GAD67, ER81 and Sp8, that are expressed in a “ventrolateral crescent” of the OB ventricular/subventricular zone at E15.5 (Long et al., 2007), which verifies that these interneuron populations are predominately localized to this region at E15.5. In Sall1-mutant animals, cells

born on E14.5 were localized in dorsal, medial and ventral regions but were noticeably absent from lateral regions at E18.5. Furthermore, the ventral-dorsal/medial-lateral pattern of neurogenesis was altered in Sall1-deficient animals. We hypothesized that regional alterations in cellular proliferation or differentiation could account for the observed differences in Sall1-deficient animals. We found that there is an intrinsic difference in the labeling index in dorsal/ventral and medial/lateral progenitor cells in control animals at E14.5. The labeling index is higher in dorsal than ventral and lateral than medial regions. Since there is no significant difference in the number of progenitor cells in dorsal versus ventral, or lateral versus medial regions (data not shown), these findings suggest that there are fewer cells in S-phase of the cell cycle in ventral and medial regions, and that therefore the cell cycle is longer. Previous studies in the cerebral cortex have shown that the cell cycle lengthens as progenitor cells mature and increased neuronal output is associated with lengthening of the cell cycle (Takahashi et al., 1999; Caviness et al., 2003). Consistent with these findings, increased neuronal differentiation (E14.5 to E18.5) is associated with a decrease in the labeling index at E14.5 in ventral regions in control animals. In Sall1-mutant animals, decreased neuronal differentiation (E14.5 to E18.5) in the ventral OB is associated with an increased labeling index at E14.5, compared to controls, in the ventral-central and ventral-lateral OB. Taken together, these observations suggest that progenitor cells in ventral-central and ventral-lateral regions continue to proliferate as opposed to differentiate in the absence of Sall1. We therefore hypothesize that Sall1 is required to temporally and spatially regulate olfactory neurogenesis.

4.4.2 Interactions between peripheral and central components of olfactory system

Complex interactions between peripheral and central components of the olfactory system have been described (Mombaerts et al., 1996; Hebert et al., 2003; Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006). Furthermore, it has been shown that the type of neuron produced in the OB is not critical for olfactory nerve innervation, since neither projection neurons nor GABAergic neurons are required for topographic targeting (Bulfone et al., 1998). The olfactory nerve appropriately exits the olfactory epithelium and contacts the OB in Sall1-deficient animals, which suggests that initial olfactory nerve development in not dependent on Sall1; however, from E15.5, the olfactory nerve fails to target to lateral and dorsal regions. Sall1 is not expressed by olfactory receptor neurons in the olfactory epithelium. In addition, no alterations in olfactory epithelium development were observed in Sall1-mutant animals, which suggests that the deficits observed are due to a requirement for Sall1 expression in the OB. Yoshihara and others hypothesize that radial glial cells may be instructive in the guidance of the olfactory nerve (Yoshihara et al., 2005). In Sall1-mutant animals no alterations in radial glial fibers were observed early in development at the time when the first differences in olfactory ventricular shape and olfactory nerve innervation were observed (E14.5-E15.5) (data not shown, n=2, E12.5; n=4, E14.5; n=3 E15.5). Furthermore, at all ages examined radial glial fibers extended to the pial surface. Although we cannot exclude a role for radial glial in the initial process of olfactory nerve innervation, the absence of altered radial glial fibers at the onset of the Sall1-mutant phenotype would suggest that they are not required for targeting or extension of the olfactory nerve to at least the lateral and dorsal olfactory surface. Previous studies have suggested that olfactory ensheathing cells produce guidance factors that influence peripheral innervation (Treloar et al., 1996; Kafitz and Greer, 1999; Tisay and Key, 1999). Interestingly, in Sall1-

mutant mice, olfactory ensheathing cells were present and extended to the dorsal surface of the OB. Our data suggests that olfactory ensheathing cells are not sufficient to induce olfactory nerve extension.

Studies in Xenopus have shown that the timing of production of ventral and dorsal olfactory neurons differs (Fritz et al., 1996). Cells destined for the ventral OB are born first and this differentiation appeared to be independent of olfactory innervation. However, cells destined for the dorsal OB are born later, and this wave of cellular differentiation appeared to be dependent on olfactory innervation. In rats, rostral to caudal differences in the timing of glomeruli formation have also been identified (Bailey et al., 1999). We observed differences in the pattern of neurogenesis of cells born on E11.5 in control animals. More cells born at this age are destined for the dorsal OB than the ventral OB, which suggests that dorsal populations are born first in early olfactory neurogenesis. This would suggest that early olfactory neurogenesis in mice is opposite to that in Xenopus. However, more cells born at E14.5 are destined for ventral regions. The regional differences in proliferation and differentiation are apparent prior to extension of the olfactory nerve to the dorsal surface at E14.5. It is interesting therefore to speculate that these regional differences in the rate of proliferation/neurogenesis in the ventral/dorsal regions in control animals influence olfactory nerve extension. In support of this hypothesis, in Sall1-mutant animals, a significant increase in the labeling index in progenitor populations (on E14.5) and a decrease in the number of neurons produced (by E18.5), was observed compared to controls, in ventral-central and lateral regions. By E15.5, the olfactory nerve had failed to extend past the ventral-central domain towards the ventral-lateral OB in Sall1-mutant animals, which suggests that regional neurogenesis may influence olfactory nerve

extension. Moreover, we propose that, like Xenopus, early olfactory neurogenesis is independent of peripheral innervation, whereas an interaction between the olfactory nerve and the OB is required during late neurogenesis (from E14.5). Consistent with this hypothesis, early born neurons (mitral cells) are generated in a variety of mutant mice that lack peripheral innervation, but varying degrees of disturbance in the later born interneuron populations are observed (Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006). We postulate that OB neurons produce trophic factors that influence innervation, as has been observed in other systems (Tucker et al., 2001; Markus et al., 2002; Zhou et al., 2004), and Sall1 dependent regulation of cellular differentiation is required to influence olfactory nerve extension. A number of axon guidance molecules, including NCAM, OCAM, Ephrin, and Ephrin receptors, are expressed in the developing OB (Treloar et al., 1997; Kafitz and Greer, 1998; St John et al., 2002b; Cutforth et al., 2003; Treloar et al., 2003). However, knock-out analysis of mice deficient for these molecules does not result in olfactory nerve axon extension abnormalities, similar to those observed in Sall1-mutant animals (Treloar et al., 1997; Cutforth et al., 2003; Walz et al., 2006). Based upon expression and knockout analyses, the most likely candidates were the Semaphorin 3a/Neuropilin 1 proteins (Schwarting et al., 2000; Schwarting et al., 2004). However, our studies indicate that these molecules were unchanged in Sall1-deficient animals. Although previous studies have shown that mitral cells are not required for topographic targeting, we cannot exclude the possibility that an over production of mitral cells in lateral and dorsal regions is inhibitory to olfactory nerve extension in Sall1-mutant animal. Previously, a model has been proposed in which two overlapping gradients, located in dorsal and ventral regions of the OB, specify topographic olfactory nerve targeting (Gierer, 1998; St John et al., 2002a). Our findings indicate that intrinsic patterns of neurogenesis exist in the dorsal/ventral OB, which could

establish a molecular gradient of guidance cues. These patterns of neurogenesis are altered in Sall1-/- animals. We therefore hypothesize that regulation of olfactory neurogenesis is required for olfactory nerve extension and that the abnormalities observed in Sall1-deficient animals are a consequence of the altered pattern of neurogenesis.

4.4.3 Olfactory nerve innervation is required for cellular lamination of the OB

A correlation between MCL lamination and altered olfactory nerve innervation has been previously observed (Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006; Laub et al., 2006). In Dlx5-, Arx- and Fez-deficient animals, olfactory nerve innervation was altered and mitral cells were disorganized, with apical dendrites misorientated (Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006). Long et al. proposed that olfactory innervation is not required for olfactory cell type generation, although, peripheral innervation may be required for lamination of olfactory cell types (Long et al., 2003). However, it has also been proposed that disorganization of mitral cells may be a consequence of altered interneuron generation (Yoshihara et al., 2005; Hirata et al., 2006). Interneuron subtypes were specified in Sall1-mutant animals, which suggests that interneuron specification may not be required for mitral cell organization. In mice deficient for the transcription factor Klf7, the OB was decreased in size and altered cellular lamination was observed (Laub et al., 2006). Moreover, the olfactory nerve only partially innervated the OB, in the ventral-medial region, and the shape of the olfactory ventricle was altered, similar to Sall1-/- animals. In a subset of Klf7-/- mice that survived postnatally, focal restoration of cellular lamination was observed in regions where the olfactory nerve innervated the OB (Laub et al., 2006). In Sall1-mutant animals, we observed similar alterations in lamination of olfactory cellular population. Although we cannot exclude an

intrinsic requirement for Sall1 in mitral cell organization, taken together, these data suggest that olfactory nerve innervation is required for olfactory cellular lamination.

4.4.4 A conserved role for Sall during development

We identified a role for Sall1 in regulating neurogenesis during murine olfactory development. A similar role for Sall has been identified in Drosophila. In the developing thorax expression of Sall must be eliminated in sensory organ precursor cells in order for these cells to differentiate (de Celis et al., 1999), which suggests that Sall inhibits differentiation. Consistent with this role for Sall in regulating differentiation, early in olfactory neurogenesis, we observed an increased production of mitral cells in the absence of Sall. Taken together, these data suggest a conserved role for Sall1 in regulating neural differentiation.

Our data indicate the olfactory nerve penetrates the OB in Sall1-mutants, and we observed glomeruli-like structures in regions where the nerve contacts the OB. Since Sall1 -/- animals die at birth, it is not possible to determine if these are functional connections or if the reduced innervation present in these animals leads to abnormalities in olfactory perception. However, previous studies have indicated that loss of olfactory input in neonatal rats is associated with weight loss, and altered social interaction and behavior (Hofer, 1976; Stewart et al., 1983). Deficits in the central nervous system have not been described in detail in patients with SALL1 mutations, Townes Brocks syndrome. Our studies suggest that perturbed SALL1 function in these patients could alter olfactory perception.

5.0 DISCUSSION

Identifying mechanisms that regulate progenitor cell proliferation and differentiation is critical to understanding the normal development of the cerebral cortex as well as the consequences of disruption of these processes in relation to neuropsychiatric disorders. Temporal and spatial regulation of cortical cell type specification is observed in the developing cortex, and the contribution of independent progenitor populations to distinct cell types is an area of active research. Recent studies have challenged a number of long-standing hypotheses in the field of developmental neurobiology. Traditionally, radial glia cells were hypothesized to be supporting cells within the developing cortex (reviewed in (Rakic, 2003)); however, recent studies have identified that radial glia are progenitor cells that give rise to cortical neurons (Malatesta et al., 2000; Hartfuss et al., 2001; Noctor et al., 2001; Noctor et al., 2002; Malatesta et al., 2003; Anthony et al., 2004). In addition, the subventricular zone was traditionally classified as a source of cortical glia cells; however, recent analyses have identified that the embryonic subventricular zone contains a newly characterized population of progenitor cells, intermediate progenitor cells, which give rise to a large proportion of neurons in the cortex (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Thus, the field of developmental neurobiology is undergoing a period of change where traditional views of cortical development are being challenged and exciting new theories of cortical neurogenesis are emerging.

Previous studies have suggested roles for members of the Sall gene family in cell cycle regulation, proliferation, neuronal differentiation, migration, and cell adhesion (Basson and Horvitz, 1996; de Celis et al., 1999; Li et al., 2001; Cantera et al., 2002; Gupta et al., 2002; Toker et al., 2003; Barembaum and Bronner-Fraser, 2004; Elling et al., 2006; Sakaki-Yumoto et al., 2006). Furthermore, sall1 mRNA is robustly expressed in developing nervous system (Buck et al., 2000; Ott et al., 2001). However, the function of Sall1 in the development of the mammalian nervous system has not been examined. Our studies suggest that the cerebral cortex and olfactory bulbs are particularly sensitive to loss of Sall1, whereas no gross anatomical abnormalities were observed in the midbrain of Sall1-deficient animals. We therefore restricted our analyses of Sall1 function to the developing forebrain. Our studies suggest that Sall1 regulates neuronal differentiation and progenitor cell maturation in the developing cerebral cortex and olfactory bulb.

We initially characterized Sall1 protein expression in the developing cortex. Sall1 protein expression in the early developing cortex mimics previous reports of sall1 mRNA expression. Interestingly, coincident with a lateral to medial and ventral to dorsal gradient of neurogenesis, Sall1 expression was highest in lateral and ventral regions at E9.5/E10.5, but expression increased in medial and dorsal regions from E11.5. We postulate that Sall1 expression across this gradient may reflect a natural maturation of the progenitor population. Robust expression of Sall1 was observed in the lateral ganglionic eminence, but weaker expression was observed in the medial ganglionic eminence and preoptic area. Olfactory interneurons and striatial interneurons are derived from the lateral ganglionic eminence, and, at E18.5, alterations in both of these populations were observed in Sall1-deficient animals. Weaker Sall1 expression in the

medial ganglionic eminence and preoptic area may indicate that Sall1 does not play a critical role in the development of cells derived from these populations, subsets of interneurons and early born oligodendrocytes destined for the cortex.

In the developing cortex Sall1 was expressed by progenitor cells that generate glutamatergic neurons and inhibitory interneurons but not by mature neurons, which suggests a role for Sall1 in the generation but not maintenance of these populations. Sall1 was also expressed by radial glia progenitor cells at E18.5, which give rise to astrocytes. Moreover, Sall1 was expressed by mature astrocytes, which suggests a role for Sall1 in their generation and function. At E18.5 Sall1 was also coexpressed with a marker of oligodendrocytes in the dorsal cortex. Oligodendrocytes have two distinct temporal and spatial origins (Spassky et al., 1998; Tekki-Kessaris et al., 2001; Gorski et al., 2002; Kessaris et al., 2006; Nakahira et al., 2006; Naruse et al., 2006; Yue et al., 2006; Parras et al., 2007). Early born oligodendrocytes are derived from the preoptic area and late born oligodendrocytes arise from the dorsal cortex. Further analysis will be necessary to determine whether Sall1 is expressed by both populations of oligodendrocytes. Sall1 expression continues in the early postnatal and adult cortex. In particular, robust Sall1 expression was observed in the subgranular zone of the dentate gyrus, a source of glutamatergic neurons in the adult, which suggests a role for Sall1 in regulating adult neurogenesis.

Our studies have identified changes in the progenitor cell population in Sall1-mutant animals. Early in development progenitor, cells favor neurogenic divisions; from midneurogenesis, progenitor cells favor re-entering the cell cycle; and late in development an expansion of the intermediate progenitor population is observed in Sall deficient animals compared to controls.

The molecular characteristics of progenitor cells change over time (Hartfuss et al., 2001), although these molecular changes have not yet been associated with alterations in progenitor division type or cell fate specification. We have defined the cellular changes in the progenitor cell population in a temporal manner in Sall1-mutant animals. It would be interesting to examine the molecular characteristics of the progenitor cell population during neurogenesis to determine if a correlation exists between the defined molecular characteristics of progenitor cells and the cellular deficits observed in Sall1-deficient animals.

Progenitor cell maturation is associated with a transition from a proliferative division, to a neurogenic division to a terminal neurogenic division. Recent studies have identified a role for intermediate progenitor cells, which predominately undergo terminal neurogenic divisions, in generating the majority of cortical neurons (Haubensak et al., 2004; Miyata et al., 2004; Noctor et al., 2004). Thus, deficits in intermediate progenitor cell generation may result in gross alterations in the neuronal composition of the mature cortex. Our studies suggest that Sall1 is a critical regulator of intermediate progenitor cell generation. At mid-neurogenesis, while total progenitor population number is unchanged, intermediate progenitor cells are decreased by 50%. However, our data suggests that from E14.5, Sall1 promotes a neural fate via regulation of the intermediate progenitor population, such that in the absence of Sall1 more proliferating cells re-enter the cell cycle as intermediate progenitor cells at the expense of differentiating into neurons. It is hypothesized that intermediate progenitor cells predominately give rise to superficial cortical neurons, whereas deep cortical neurons arise from the ventricular zone (Tarabykin et al., 2001; Nieto et al., 2004). However, live imaging studies have identified that at midneurogenesis, when intermediate progenitor cells are differentiating into neurons, progenitor cells in the ventricular

zone are also generating neurons (Noctor et al., 2004). The relative contribution of these two progenitor populations to the cortical plate has not yet been characterized. It would be difficult to lineage trace the ventricular zone population; as the intermediate progenitor population arises from this population traditional lineage tracing techniques would be limited. Retroviral labeling of progenitor cells in control animals at E13.5, E14.5, and E16.5, followed by live imaging analysis could be used to follow ventricular zone and intermediate progenitor cell divisions, as well as the contribution of their progeny to deep and superficial cortical layers. Similar analyses of Sall1-mutants at E13.5 and E14.5 would distinguish between the hypotheses that exist for the alterations observed from E14.5 in Sall1-deficient animals. Deep cortical layers are most likely decreased in size in Sall1-mutant animals due to the decrease in progenitor cell number observed at E12.5. In addition we hypothesize that in Sall1-deficient animals, more cells adopt an intermediate progenitor fate from E14.5 on, which suggests that ventricular zone progenitor cells may selectively commit cells to an intermediate progenitor fate at the expense of neuronal differentiation. This change in progenitor fate may contribute to the decrease in deep cortical layers. Alternatively, intermediate progenitor cells may also generate neurons for deep cortical layers, such that in Sall1-/- animals, the decrease in deep layers is due to the early decrease in intermediate progenitor cell generation.

Live imaging analysis of retroviral-labeled cells suggests that the majority of intermediate progenitor cells favor a direct terminal differentiation (Noctor et al., 2004). Alterations in intermediate progenitor cell number from E14.5 on may reflect a role for Sall1 in regulating the proliferative capacity of the intermediate progenitor population, rather than ventricular zone progenitors selectively committing to an intermediate progenitor state in Sall1-/- animals. This

hypothesis predicts that, in the absence of Sall1, intermediate progenitor cells favor expanding the intermediate progenitor population, by re-entering the cell cycle at the expense of differentiating. Comparison of live imaging of labeled cells in control and Sall1-/- animals would clarify the mechanisms of decrease in deep cortical plate neurons, initial decrease and subsequent increase in intermediate progenitor population in Sall1-deficient animals, as well as the contribution of intermediate progenitor cells to deep versus superficial cortical fate.

Wnt signaling has been characterized as promoting a neuronal fate at mid-neurogenesis (Hirabayashi et al., 2004), as is observed in Sall1-mutant animals, although it has not been assessed whether this occurs via intermediate progenitor cell generation. It would be interesting to analyze members of the Wnt signaling pathway for their role in regulating intermediate progenitor cell generation. FGF signaling has been implicated in intermediate progenitor cell generation (Yamamoto et al., 2005). In light of the role of Wnt and FGF signaling in activating Sall gene expression in other systems, it is interesting to speculate that Sall1 may be a critical mediator of these signaling cascades (de Celis et al., 1996; Kuhnlein and Schuh, 1996; de Celis et al., 1999; Chihara and Hayashi, 2000; de Celis and Barrio, 2000; Farrell and Munsterberg, 2000). Identifying mechanisms that regulate progenitor cell fate is critical to understand the molecular basis of normal neuronal differentiation and the consequences of dysregulation of neuronal differentiation in developmental disease pathology, and to further the use of stem cell therapies in treatment of neurological disorders. It will initially be important to identify molecules that regulate the transition from a ventricular zone progenitor cell to an intermediate progenitor cell. Fluorescent-activated cell sorting of the Pax6-positive ventricular zone population from the Tbr2-positive intermediate progenitor population followed by array analysis

of these two populations would identify differences in gene expression, which could then be assessed in culture for their relative contributions to generation and function of these populations.

Our data support the hypothesis that early in development Sall1 promotes a progenitor state, such that in the absence of Sall1 neurons differentiate at the expense of the progenitor population. Our studies suggest that Sall1 regulates neurogenesis of early born neuronal populations. In the absence of Sall1 more cells are committed to an early neuronal fate, the preplate and its derivatives, the subplate and marginal zone, compared to controls. Interestingly, we identified an increase in the early born mitral cell population of the olfactory bulb. Mitral cells also arise from dorsal progenitor populations, which suggests a conserved role for Sall1 in regulating early cortical neurogenesis of excitatory neurons. A similar role for Sall has been identified in Drosophila in the developing thorax, where Sall expression must be eliminated for sensory organ precursor cells to differentiate (de Celis et al., 1999). Furthermore, overexpression of csall4 maintains cells in an undifferentiated state, which suggests that the cellular function of csal4 is to maintain a progenitor fate (Barembaum and Bronner-Fraser, 2004). Taken together, these findings suggest a conserved role for members of the Sall gene family in regulating early neuronal differentiation of dorsal progenitor cells.

Our studies indicate that Sall1 influences interneuron development. Interneurons destined for the striatum, cortex, and OB are derived from ventral progenitor populations. At E18.5 an increase in ventral progenitor populations was observed, accompanied by a decrease in the number of neurons in the striatum, similar to the late phenotype observed in the dorsal cortex. These

findings suggest a conserved role for Sall1 in regulating neurogenesis of dorsal and ventral progenitor populations. Interneurons destined for the olfactory bulb are born from E14.5 on, and arise in the dorsal lateral ganglionic eminence, and migrate to the OB via the rostral migratory stream. Although, the total number of cells born on E14.5 was not altered in Sall1-mutant animals compared to controls, we observed a shift in the percent of these cells that expressed GABA. These findings suggest a role for Sall1 in regulating interneuron subtype specification. It will therefore be interesting to examine interneuron subtype specification in the dorsal cortex and striatum of Sall1-mutant animals. Future studies will examine characterize the role of Sall1 in regulating neurogenesis and cell type specification of neuronal populations destined for the striatum and dorsal cortex.

How does Sall1 mediate gene regulation in cortical progenitors? Studies have identified that in vitro Sall1 can activate or repress gene transcription (Netzer et al., 2001; Kiefer et al., 2002; Sato et al., 2004; Lauberth and Rauchman, 2006). The role of Sall1 as a transcriptional repressor has been best characterized; a twelve-amino acid motif at the N-terminus mediates transcriptional repression by recruitment of a histone deacetylase complex (Lauberth and Rauchman, 2006). In vitro and in vivo studies suggest that histone deacetylases regulate neurogenesis and lineage progression (Cunliffe, 2004; Hsieh et al., 2004; Shen et al., 2005; Yamaguchi et al., 2005; Siebzehnrubl et al., 2007). Although direct comparison of these studies and Sall1 cortical studies are difficult due to differences in timing of experiments, some correlations can be made. In vitro analysis of E14.5 neural progenitor cells suggest that inhibition of histone deacetylase activity induces neuronal differentiation; however, these cultures were examined after 8 days (Balasubramaniyan et al., 2006). Our studies indicate that from E14.5 to E17.5, Sall1 regulates

intermediate progenitor cell generation, and by E18.5 more intermediate progenitor cells are observed in the dorsal cortex of Sall1-deficient animals. In the absence of cell death it is likely that these progenitor cells would differentiate into neurons, and thus, if Sall1-deficient animals survived postnatally, an increase in late-born neurons would be observed, consistent with the in vitro studies of inhibition of histone deacetylase activity. In vivo studies also suggest that histone acetylase activity is an important regulator of oligodendrocyte differentiation and myelination during early postnatal development (Shen et al., 2005). In vitro studies support a role for histone acetylase activity in promoting glial lineage progression over neuronal differentiation in postnatal and adult progenitor cells (Marin-Husstege et al., 2002; Hsieh et al., 2004). Characterization of glial populations was difficult in Sall1 null animals as these cells predominately arise postnatally; however, it will be interesting to compare the glial phenotype in conditional Sall1 knockouts to those observed with inhibition of histone acetylase activity.

We postulate that Sall1 may regulate neural gene expression in vivo via recruitment of a histone deacetylase complex as has been observed in vitro. Temporal analysis of Sall1 function in cultured neuronal progenitor cells will allow us to examine this hypothesis. Our model predicts that overexpression of Sall1 in early cortical progenitors would maintain these cells in a progenitor state, whereas overexpression of Sall1 in midneurogenic progenitors would induce neuronal differentiation. Addition of histone deacetylase inhibitors, as well as comparison of wild type Sall1 with mutant Sall1-deficient for recruitment of histone deacetylase, will allow us to determine whether the molecular mechanism of Sall1 function in cortical progenitors is mediated by recruitment of histone deacetylase complex.

Related members of the Sall gene family are expressed in overlapping regions during cortical development. Sall1, Sall2, and Sall4 are expressed by early dorsal progenitor populations (unpublished observations) (www.stjudebgem.org/) (Ott et al., 1996; Ott et al., 2001). However, Sall3 expression does not extend into the dorsal cortex until E13.5, and late in development, Sall4 expression is eliminated from dorsal progenitors (www.stjudebgem.org/) (Ott et al., 1996; Ott et al., 2001). In vitro studies have determined that members of the Sall gene family can interact, and that expression of Sall3 can alter the cellular localization of Sall1 (Sweetman et al., 2003). In light of the extension of Sall3 expression into dorsal progenitors at E13.5, it is interesting to speculate that an interaction between Sall1 and Sall3 may mediate the change in Sall1 function observed at E14.5 and regulate intermediate progenitor cell generation. Furthermore, it is possible that interactions between Sall1 and Sall2 or Sall4 may regulate Sall1 function. In vivo studies have identified a genetic interaction between Sall1 and Sall4, whereby developmental malformations are more pronounced in Sall1+/-Sall4+/- animals than in Sall4+/- animals (Sakaki-Yumoto et al., 2006). These studies also have implications for human disease manifestation of SALL disorders and may explain the overlapping phenotypes observed between associated diseases. Taken together, these studies suggest that coordinate regulation of Sall family members may have important functional consequences. Future studies will dissect the contribution of members of this family to neural progenitor cell regulation in vitro and in vivo. Initial studies will determine whether members of the Sall gene family are expressed in overlapping progenitor populations and the fate of these populations in vitro. Analysis of the coordinate effect of members of this family will also be examined by expressing combinations of the Sall gene family in primary neuronal cell culture isolated at distinct time points during development. Finally, in utero electroporation will be used to examine the effect in vivo of

coordinate expression of multiple family members. These studies will dissect on a cellular basis the role of members of the Sall gene family in progenitor cells.

To investigate whether Sall family members have conserved functional roles, I compared the roles of Sall1 and Sall3 during olfactory bulb development (Chapter 4, Appendix A). Within the olfactory bulb Sall1 and Sall3 are expressed by largely independent populations. Sall1 is robustly expressed by progenitor cells, differentiating neurons, mitral cells, and a subset of interneurons. Sall3 is robustly expressed by interneurons in the glomerular layer and a subset of cells in the progenitor, differentiating, mitral cell layer and granule cell layer populations. Sall3 is not expressed by progenitor cells that give rise to the mitral cell population and, as expected, mitral cell development was not disrupted in Sall3-deficient animals. Although disorganization of olfactory interneuron populations was observed in both Sall1 and Sall3-mutant animals, our studies suggest that the cellular mechanism of these alterations differs. We identified that Sall1 regulates neurogenesis of olfactory interneuron populations, whereas Sall3 regulates terminal maturation of interneurons destined for the glomerular layer, specifically tyrosine hydroxylase-positive interneurons (Appendix A), which suggests that these genes function at distinct differentiation steps during olfactory interneurogenesis. These data suggest independent mechanisms of action of members of the Sall gene family during olfactory development. These data suggest that Sall family members may have distinct functional roles in regions where they are expressed by independent populations. In contrast to the developing olfactory bulb, Sall1 and Sall3 appear to be expressed in overlapping progenitor populations in the dorsal cortex, which supports our hypothesis that they may functionally interact to regulate cortical neurogenesis.

Our studies have identified that Sall1 is a critical regulator of neuronal differentiation and progenitor cell maturation during development. Furthermore, we have identified the cellular consequences of deletion of Sall1 in the cortex at E18.5. Future studies will examine the consequences of altering progenitor cell maturation by analyzing the cellular and behavioural abnormalities in Sall1 conditional knockout animals in the adult. At E18.5, an increase in intermediate progenitor cells is observed in Sall1-deficient animals. It will be interesting to examine the consequences of this increased progenitor population using conditional mutagenesis. These progenitors are committed to a neuronal fate; however, they will ultimately differentiate in a more mature cortical environment than control intermediate progenitor cells, which may alter their fate. If these cells differentiate into superficial neurons, then the superficial cortical plate will be increased relative to a decreased deep cortical plate in Sall1-deficient animals. The functional consequences of such an alteration would be interesting to analyze. Preliminary studies suggest that the behavioural consequences of disrupting Sall1 in the dorsal and ventral telencephalon differ (unpublished observations). Preliminary observations suggest that loss of Sall1 in the dorsal cortex is associated with overall growth retardation and decreased cortical size. In addition, excessive grooming behaviour and hyperactivity were observed, which are characteristic of developmental neuropsychiatric disorders, including Attention Deficit Hyperactivity Disorder and Obsessive Compulsive Disorder (Goldman et al., 1998; Mataix-Cols et al., 2005; Sagvolden et al., 2005; Heyman et al., 2006). Mice deficient for ventral Sall1 do not display any gross behavioural characteristics. Future studies will comprehensively examine cellular alterations and behaviour in these animals. These preliminary studies suggest that dorsal restricted loss of Sall1 will offer an interesting insight into the behavioural consequences of altered cortical development. These studies will aid in the characterization of cognitive deficits

associated with Townes-Brocks syndrome, as well as neuropsychiatric disorders associated with disruption of cortical neurogenesis.

Expression studies suggest a role for Sall1 in adult neurogenesis. Recent studies have identified similar molecular and cellular mechanisms of progenitor cell regulation at midneurogenesis and in the adult dentate gyrus (Hevner et al., 2006), which suggests a conserved pathway of differentiation of these populations of progenitor cells. We therefore hypothesize that, similar to midneurogenesis, Sall1 promotes neurogenesis in the adult via generation of intermediate progenitor cells. It is difficult to predict the adult phenotype in Sall1 animals as it is currently not known whether Sall1-mutant intermediate progenitor cells can ultimately differentiate into neurons. However, analysis of Emx1-Sall1-cKO animals will determine whether the expanded progenitor population observed at E18.5 differentiates into neurons postnatally. In the Sall1 conditional adult we would initially predict an increase in the intermediate progenitor population. This population may continuously expand or this population may initially expand and then differentiate, resulting in an increase in newborn adult neurons. Interestingly, previous studies have associated enriched environment housing, which enhances sensory, cognitive, and motor stimulation in rodents, with increased adult neurogenesis and integration of newborn neurons into cortical circuitry (Nilsson et al., 1999; van Praag et al., 1999; Brown et al., 2003; Komitova et al., 2005; Nithianantharajah and Hannan, 2006). In light of our hypothesized role for Sall1 in regulating adult neurogenesis, it will be interesting to determine if enriched environment housing can stimulate neurogenesis of the predicted expanded intermediate progenitor population, or enhance the integration of newborn neurons into cortical circuitry in Sall1 conditional animals.

These studies will offer an insight into the role of Sall1 in adult neurogenesis, as well as the effect of genetic and environmental manipulation on adult neurogenesis.

Our findings suggest a role for Sall1 in regulating neuronal differentiation and progenitor cell maturation. We propose a dual role for Sall1 in neural progenitor cell regulation; early in development, Sall1 promotes a progenitor fate, and late in development Sall1 promotes a neural fate. These findings come at an exciting time in developmental neurobiology where new theories of neurogenesis are being explored. The recently identified intermediate progenitor population is hypothesized to give rise to the majority of neurons in the cortex (Haubensak et al., 2004). Our studies suggest that Sall1 is a critical regulator of the generation of this population. Adult conditional Sall1 animals offer a unique model to examine the cellular and behavioural abnormalities associated with altered intermediate progenitor cell populations. These studies will provide novel insight into the normal role of intermediate progenitor cells, as well as the functional consequences of dysregulation of cortical progenitor cells. Together, these studies will aid in the understanding of cognitive deficits associated with Townes-Brocks syndrome, as well as neuropsychiatric disorders associated with disruption of cortical neurogenesis.

APPENDIX A: SALL3 IS REQUIRED FOR THE TERMINAL DIFFERENTIATION OF DOPAMINERGIC OLFACTORY INTERNEURONS

A.1 ABSTRACT

Sall3 is a zinc finger containing transcription factor and a member of the Sall gene family. Members of the Sall gene family are highly expressed during development. Sall3-deficient mice die in the perinatal period due to dehydration and display alterations in palate formation and cranial nerve formation (Parrish et al., 2004, Mol Cell Biol 24(16):7102-7112). We examined the role of Sall3 in the development of the olfactory system. We determined that Sall3 is expressed by both peripheral and central components of the olfactory system. Sall3 deficiency specifically alters formation of the glomerular layer. The glomerular layer is hypocellular, due to a decrease in the number of interneurons. The lateral ganglionic eminence and rostral migratory stream develop normally in Sall3-deficient animals, suggesting that Sall3 is not required for the initial specification of OB interneurons. Fewer GAD65/67-, Pax6-, Calretinin- and Calbindin-positive cells are detected in the glomerular layer, accompanied by an increase in cells positive for these markers in the granule cell layer. In addition, a complete absence of tyrosine hydroxylase expression is observed in the OB in the absence of Sall3. However, expression of Nurr1, a marker of dopaminergic precursors, is maintained, indicating that dopaminergic precursors are present. Our data suggests that Sall3 is required for the maturation of olfactory interneurons and specifically for the terminal differentiation of olfactory bulb dopaminergic neurons.

A.2 INTRODUCTION

The olfactory bulb (OB) is a laminar structure containing two distinct neuronal populations. Excitatory projection neurons populate the mitral cell layer (MCL) and interneuron populations are located in the glomerular layer (GL) and granule cell layer (GCL), superficial and deep to the MCL respectively. OB neuronal populations are sequentially produced and have distinct origins. Mitral cells are the first born OB population (Hinds, 1968). These cells arise in the pallium and the transcription factor Tbr1 is required for their generation (Bulfone et al., 1998; Puelles et al., 2000; Moreno et al., 2003). OB interneurons arise from an ER81-positive population in the subpallial dorsal lateral ganglionic eminence (LGE) and migrate to the OB via the rostral migratory stream (RMS) (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1999; Wichterle et al., 2001; Stenman et al., 2003).

Previous studies have determined that OB interneurons are continuously generated throughout life (Altman and Das, 1966; Hinds, 1968; Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996); however, mechanisms that regulate the generation of this diverse cellular population are not well characterized. OB interneurons begin to express mature interneuron markers as they terminally differentiate and radially migrate in the OB. The OB is comprised of chemically distinct interneuron populations; at least three subtypes have been identified characterized by GABA, Calretinin and Calbindin expression (Kosaka et al., 1995; Toida et al., 2000). Cells in the GCL homogenously express GABA (Parrish-Aungst et al., 2007). Within the GL GABA, Calretinin and Calbindin are expressed by ~20-53%, ~20-27% and ~10% of neurons respectively (Kosaka et al., 1995; Parrish-Aungst et al., 2007). The

remaining interneuron populations in this layer have not yet been chemically distinguished. The GABAergic population can be further divided into dopaminergic and non-dopaminergic populations, characterized by the expression of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine biosynthesis. ~80% of dopaminergic cells are also GABAergic (Kosaka et al., 1998; Hack et al., 2005; Parrish-Aungst et al., 2007) and 95% of TH-positive cells in the GL are Pax6-positive (Hack et al., 2005). Little overlap is observed between the TH/Pax6 populations and the Calretinin- (~2% overlap with Pax6)/Calbindin- (~10% overlap with Pax6) positive populations (Kosaka et al., 1995; Kosaka et al., 1998; Hack et al., 2005; Waclaw et al., 2006). In addition, examination of the temporal specification of OB interneurons has identified overlap in the timing of birth of these chemically diverse populations (Tucker et al., 2006). These findings suggest that intrinsic factors play an important role in OB interneuron specification, as diverse interneuron populations born at the same time would be exposed to similar extrinsic cues.

Insight into the mechanisms that regulate production of interneuron populations destined for the OB has been obtained from analyses of murine knockout models (Stenman et al., 2003; Yun et al., 2003; Soria et al., 2004; Yoshihara et al., 2005; Waclaw et al., 2006). These studies have demonstrated that interneuron generation can be divided into the following steps; specification of interneuron progenitors within the LGE, entry from the LGE to the RMS, migration in the RMS, exit from the RMS to the OB, radial migration within the OB, and terminal differentiation. The transcription factors Sp8 (Waclaw et al., 2006) and GSH1/2 (Stenman et al., 2003; Yun et al., 2003) are required for the initial specification of OB progenitors in the LGE. In addition, Vax1 is necessary for the transition of cells from the LGE to the RMS (Soria et al., 2004). The secreted

protein Slit has been implicated in the migration of interneurons via the RMS (Hu, 1999; Wu et al., 1999; Chen et al., 2001) and the homeobox protein ARX is required for the entry of interneurons from the RMS to the OB (Yoshihara et al., 2005). Furthermore, Pax6 has been implicated in the generation of dopaminergic OB interneurons (Hack et al., 2005; Kohwi et al., 2005). Thus, we are beginning to understand components of OB cell type generation and migration within the RMS, however, mechanisms regulating terminal differentiation within the OB are not well characterized.

We examined the role of Sall3 in the development of the olfactory system. Sall3 is one of four mammalian members of the Sall gene family, which are widely expressed throughout development, in the peripheral and central nervous system, as well as in peripheral organs (Kohlhase et al., 1996; Ott et al., 1996; Kohlhase et al., 1999a; Buck et al., 2000; Kohlhase et al., 2000; Buck et al., 2001; Ott et al., 2001; Al-Baradie et al., 2002; Kohlhase et al., 2002a; Kohlhase et al., 2002b). Distinct developmental disorders with some overlapping characteristics are associated with mutation or deletion of members of this gene family in humans (Kohlhase et al., 1998; Kohlhase et al., 1999a; Al-Baradie et al., 2002; Kohlhase et al., 2002b). SALL3 is one of several genes deleted in 18q deletion syndrome, characterized by hearing loss, mental retardation, mid-facial hypoplasia, delayed growth and limb abnormalities (Wilson et al., 1979; Mahr et al., 1996; Strathdee et al., 1997; Kohlhase et al., 1999a; Jayarajan et al., 2000; Verhoeven et al., 2006). The Sall genes are zinc finger containing transcription factors (Kuhnlein et al., 1994). They have been shown to localize to heterochromatin (Netzer et al., 2001; Sato et al., 2004; Sakaki-Yumoto et al., 2006) and it is hypothesized that they can mediate repression via recruitment of histone deacetylase (Kiefer et al., 2002; Lauberth and Rauchman, 2006).

Members of the Sall gene family can interact at the protein level (Kiefer et al., 2002; Sweetman et al., 2003; Sakaki-Yumoto et al., 2006) and have been implicated in diverse cellular processes including, cell cycle regulation, cell fate specification, neuronal differentiation, migration and cell adhesion (Jurgens, 1988; Kuhnlein and Schuh, 1996; de Celis et al., 1999; Cantera et al., 2002; Franch-Marro and Casanova, 2002; Toker et al., 2003; Barembaum and Bronner-Fraser, 2004; Bohm et al., 2007). These data suggest the members of the Sall gene family are important developmental regulators.

We have previously shown that Sall3 (previously named msal1) is expressed during olfactory development (Ott et al., 1996; Ott et al., 2001), although a detailed description has not previously been described. Here we demonstrate that Sall3 is expressed by cells within the OB and olfactory epithelium. Within the OB Sall is expressed by progenitor cells and subpopulations of differentiated neurons. Sall3-deficient animals die perinatally (Parrish et al., 2004), and we have examined development of the olfactory system to P0.5. We have identified alterations in interneuron development in Sall3-mutant animals. Our data suggests that Sall3 is required for the terminal differentiation of dopaminergic interneurons in the developing OB.

A.3 MATERIALS AND METHODS

A.3.1 Animals

Sall3 heterozygous animals were obtained from matings of Sall3 heterozygous mice (129SVJx C57BL/6J) from the 12th generation backcross to C57BL/6J. Embryos were obtained from

matings of Sall3 heterozygote animals and genotyped as previously described (Parrish et al., 2004). For Sall3 expression studies embryos were obtained from timed pregnant CD1 mice from Charles Rivers Laboratories. Embryos were collected via cesarean section at embryonic ages from E13.5 to E18.5. The day of vaginal plug was designated as E0.5, the first postnatal day was designated P0.5. Embryos were fixed in either 4% paraformaldehyde (PFA) (pH 7.4; Sigma, St. Louis, MO) and processed though increasing sucrose gradients for cryosectioning or in Carnoys solution (1:3:6 acetic acid: chloroform: 100% alcohol) and then processed though a butanol series for paraffin sectioning. P0.5 pups were first perfused with 4% PFA and then processed as described above. Cryopreserved embryos were embedded in Tissue-Tek O.C.T. compound (EMS, Hatfield, PA) and sectioned at 20μm. Paraffin embedded embryos were sectioned at 10μm. No alterations in the olfactory system were observed in Sall3+/- animals in our study (data not shown) and thus these animals were also used as controls. Animal protocols and procedures were approved by Institutional Animal Care and Use Committee at the University of Pittsburgh.

A.3.2 Measurement of OB size

The cortex and OB of P0.5 pups were dissected from the head and visualized on a Nikon (Melville, NY) dissecting microscope, photographed with a Photometrics (North Reading, MA) Cool Snap digital camera and IP Lab software (Biovision Technologies, Exton, PA). Images were imported into Photoshop 7.0 (Adobe Systems, San Jose, CA) to measure the length of the OB. Statistical analysis of the results was performed using an unpaired t test with InStat 3 software (GraphPad Software, San Diego, CA).

A.3.3 Immunohistochemistry

Paraffin processed sections were deparaffinized in xylene, rehydrated through an ethanol series and washed in 0.1% triton in phosphate buffered saline (PBS) pH 7.4. For diaminobenzidine detection, sections were incubated with 3% hydrogen peroxide in methanol for 10 minutes. Slides were subsequently microwaved in 0.1M sodium citrate solution pH 6.0, rinsed in PBS and blocked in 10% heat inactivated goat serum (Jackson ImmunoResearch, West Grove, PA). A list of the antibodies used and their details is provided in table 1. Primary antibodies were incubated overnight at 4°C. Cryopreserved tissue was rinsed in PBS, blocked in 10% heat inactivated goat serum and incubated with primary antibody overnight at 4°C. Antibodies used were β-galactosidase (1:500; Abcam, Cambridge, MA); c-Fos (1:20,000; Oncogene, San Diego, CA); Calretinin (1:1000; Chemicon, Temecula, CA); Calbindin (1:1000; Abcam); ER81 (1:5,000; Convance, Berkeley, CA); Gad65/67 (1:10,000; Sigma); Neurofilament 160 (1:200, Sigma); Pax6 (1:500, Convance); Reelin (1:500; Abcam); Tuj1 (1:1000; Sigma); Tyrosine Hydroxylase (1:5000; Sigma). For each antibody used a negative control was included where the sections were incubated without the primary antibody but with the secondary antibody. The tissue was subsequently washed with PBS, incubated with the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) and then incubated with Vectastain® Elite ABC kit (Vector Laboratories) according to the manufacturer's instructions. Staining was visualized using nickel enhanced diaminobenzidine (Sigma) reaction. Sections were counterstained with Haematoxylin and Eosin (Fisher Scientific, Pittsburgh, PA) or nuclear fast red (Vector Laboratories). Slides were then dehydrated through ethanol, washed in xylene and mounted in DPX (Fluka, Sigma). For fluorescent detection of signal the tissue was washed with PBS and incubated with the appropriate Cy3 and/or Cy2 secondary antibody (Jackson ImmunoResearch),

and counterstained with 1,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) before mounting in fluormount G (Southern Biotechnology Research, Birmingham, AL). All of the antibodies tested reproducibly produced similar results and mimicked previously published expression patterns from our laboratory and others in the developing and adult brain. For Nissl staining, sections were incubated in 0.5% cresyl violet (Sigma), dehydrated through alcohols, washed in xylene and mounted in DPX (Fluka, Sigma).

A.3.4 *In situ* hybridization

The Sall3 probe has previously been described (Ott et al., 2001). To generate a Nurr1 probe, RNA was obtained from the cerebral cortex of an E18.5 wild type embryo using the RNeasy Protect Mini Kit (Qiagen, Valencia, CA) and reversed transcribed using Advantage RT-for-PCR Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Nurr1 was amplified from this cDNA using the Advantage cDNA PCR Kit (Clontech) with primers (Forward: 5'-CCCGCTTCTCAGAGCTACAG -3' and Reverse: 5'-AGACCCTCATTGGAGGGAGA -3') designed against nucleotides 5587 to 6234 of the *Mus musculus* Nurr1 gene (MMU86783). This PCR product was cloned into pCR-II-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The Nurr1 clone was verified by sequencing. For preparation of anti-sense digoxigenin-labeled riboprobes, Nurr1 template DNA was linearized with BamH1 and transcribed with T7 RNA polymerase according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). *In situ* hybridization was conducted as previously described (Schaeren-Wiemers and Gerfin-Moser, 1993) with the following modifications. The sections were treated with proteinase K, 1µg/ml in 50mM Tris pH 7.5 and 5mM EDTA, at 37°C for 3 min (E13.5-E16.5), 7 min (E17.5-P0.5) or 15 min (adult), followed by post-fixation in 4% PFA prior

to pre-hybridization. The hybridization buffer was modified to contain 50% formamide, 5XSSC, 5 X Denhardt's solution, 250 μg/ml tRNA and 250 μg/ml of herring sperm DNA. The probe was dissolved at a concentration of 60ng/ml in hybridization solution. Hybridization and post-hybridization washes were performed at 61°C. A post-hybridization treatment with RNAse A, 20 μg/ml, in 0.5M NaCl, 10mM Tris pH7.5, 5mM EDTA buffer, at 37°C for 30mins was also added. Following color detection slides were rapidly dehydrated, washed in xylene and mounted in DPX.

A.3.5 Illustrations

Stained sections were visualized on a Nikon (Melville, NY) fluorescent microscope, photographed with a Photometrics Cool Snap digital camera and IP Lab software. Composite images were prepared using Photoshop 7.0. Contrast, color and brightness were adjusted in Photoshop 7.0.

A.3.6 Histological identification of OB regions

To assign cellular subtypes and boundaries to OB regions, sections were examined at 40X magnification. The progenitor population was identified as the cellular region adjacent to the ventricle containing organized parallel arrays of cells with elongated nuclei arranged perpendicular to the ventricular surface. The differentiating population was identified as a region of heavily stained small cells with round nuclei arranged in a disorganized manner, adjacent to the progenitor population. The MCL was evident from E16.5 and contained cells with large oval nuclei, perpendicular to the ventricular surface and superficial to the differentiating field. From,

P0.5 the GL and GCL were identifiable. The GL was defined as the region containing round cells between the MCL and the olfactory nerve layer. The olfactory nerve layer was identified by the presence of elongated cells, orientated parallel to the ventricular surface and perpendicular to the MCL. The GL as defined in this study encompasses the GL and the external plexiform layer. The GCL was located on the ventricular side of the MCL and contained small heavily stained round cells.

A.3.7 Quantification of glomerular cell number

For quantification of cell number within the GL, P0.5 Nissl stained OB sections were photographed at 40X magnification as described above. The analysis was performed by an observer blind to the genotype. The GL was defined as described above. The boundary of the MCL and outer nerve layer were distinguished at 200X. The total number of cells in a 150μm wide window within this region was quantified and analyzed as previously described (Roy et al., 2004). Three sections per animal were counted. The data was subsequently decoded and statistical analysis of the results was performed using an unpaired t test with InStat 3 software. Sall3 control animals had 125.83±1.848 cells and Sall3-mutant animals had 84.750±5.351 at P0.5 ($n=4$, $p<0.001$).

A.4 RESULTS

A.4.1 Sall3 is expressed by olfactory progenitors and interneuron populations

Previous studies have shown that Sall3 is expressed in the developing OB (Ott et al., 1996; Ott et al., 2001); however, a detailed temporal expression pattern has not previously been described. In order to understand more precisely the role of Sall3 in the olfactory system we first characterized its expression in the developing and adult OB using *in situ* hybridization. OB neurogenesis initiates on E11.5 in the mouse. At this time, peripheral projections from the olfactory placode contact progenitor cells in the rostral neural epithelium (Gong and Shipley, 1995). These projections are believed to induce differentiation of this population of progenitor cells and induce OB morphogenesis (Gong and Shipley, 1995). Sall3 was not expressed by cells in the olfactory placode (Ott et al., 2001). The olfactory placode gives rise to components of the peripheral nervous system, such as the vomeronasal organ and olfactory epithelium. By E13.5 Sall3 was expressed by a subpopulation of cells in these structures (arrowheads, Figure 32A). Expression of Sall3 in the neural epithelium of the rostral most tip of the developing telencephalon was not detected until E11.5 (Ott et al., 1996; Ott et al., 2001). From E13.5, Sall3 is expressed by OB progenitors (P, Figure 32B) and expression is maintained in this population throughout development (P, Figure 32C).

Two major olfactory neuronal subtypes are sequentially produced during development, excitatory projection neurons and inhibitory interneurons. Excitatory neurons are born from E11.5 to E13.5 and populate the MCL (Hinds, 1968). At E13.5 Sall3 is expressed by a small subset of differentiating cells destined for the MCL (arrowheads, Figure 32B). By E17.5, Sall3 is

expressed by a few cells in the MCL (arrowheads, Figure 32D). Inhibitory interneurons arise from an ER81-positive population in the LGE and are born from E14.5 on (Hinds, 1968; Stenman et al., 2003). In the developing cerebral cortex expression of Sall3 is initially restricted to the ventral telencephalon and from E12.5 strong expression is observed in the LGE (Ott et al., 1996; Ott et al., 2001). Following initial specification within the LGE, neuroblasts destined for the OB migrate to the OB via the RMS. Sall3 expression is observed in the RMS during development (Figure 32E). Within the OB, interneuron populations are located in the GCL and GL. At E17.5 interneurons destined for these layers are differentiating and a subset has already reached their final laminar position. Similar to E13.5 relatively few differentiating cells express Sall3 at E17.5 (arrowheads, Figure 32C). However, Sall3 is robustly expressed by a large number of cells in the GL (Figure 32C,D) and a subpopulation of cells in the GCL (arrows, Figure 32D). Expression of Sall3 is maintained in the MCL, GCL and GL of the adult OB (Figure 32F,G). In summary, Sall3 is expressed in developing olfactory structures from E11.5. Expression is observed in olfactory bulb progenitors during development and in subpopulations of differentiated cells, predominately by cells in the GL. These data suggest a role for Sall3 in the development and maintenance of olfactory structures.

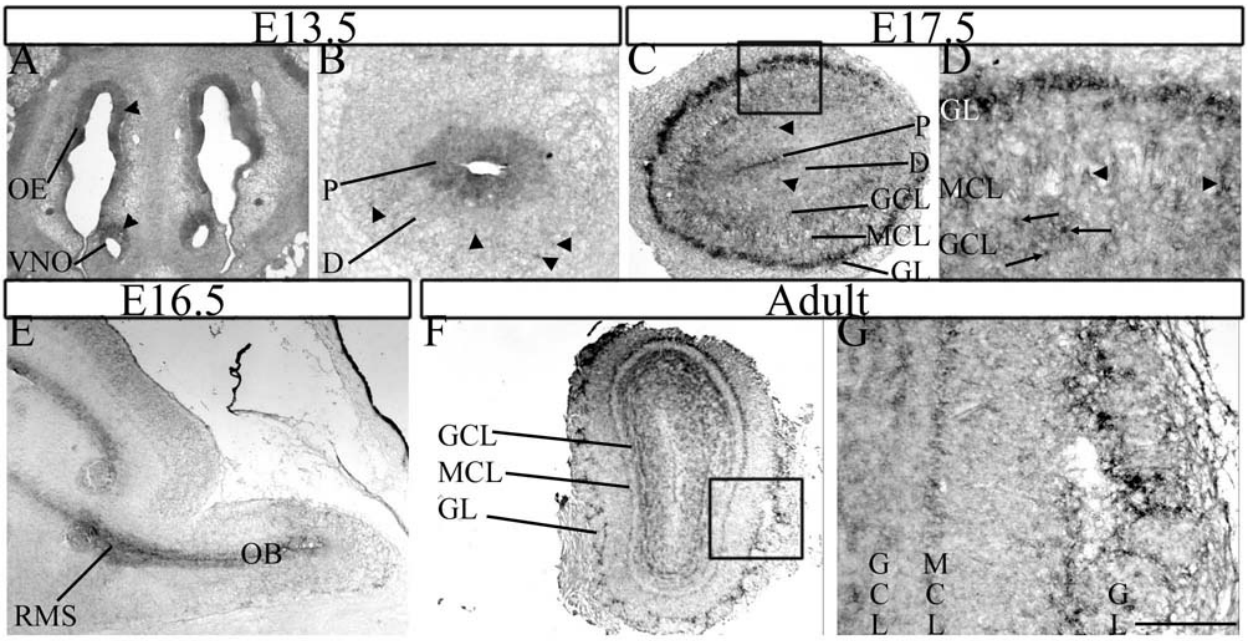

Figure 32. Sall3 expression in the developing and adult olfactory system.

In situ hybridization of Sall3 expression in the developing (A-E) and adult (F,G) olfactory system in coronal (A-D, F,G) and sagittal (E) sections. Sall3 is expressed in the OE and VNO at E13.5 (arrowheads, A). In the olfactory bulb Sall3 is expressed by progenitor cells and a subpopulation of differentiating (arrowheads) cells at E13.5 (B) and E17.5(C). At E17.5 Sall3 is additionally expressed by cells in the GL, MCL (arrowheads) and GCL (arrows) (D). Sall3 expression is observed in the RMS at E16.5 (E). Expression of Sall3 is maintained in the GL, MCL and GCL in the adult (F,G). (D) is boxed area in (C). (G) is boxed area in (F). OE: olfactory epithelium; VNO: vomeronasal organ; P: progenitor populations; D: differentiating field; GCL: granule cell layer; MCL: mitral cell layer; GL: glomerular layer; RMS: rostral migratory stream; OB: olfactory bulb. Scale bar (in G): 100µm in D; 200µm in B,G; 350µm in C; 400µmin A,E; 800µm in F.

A.4.2 The glomerular layer is hypocellular in the absence of Sall3

Sall3 is expressed in the developing and adult olfactory system. To determine whether Sall3 is required for distinct phases of OB development, mice with a targeted disruption of the Sall3 gene were examined. Sall3 homozygous mutant animals (Sall3-/-) die shortly after birth from dehydration and display alterations in palate development and partially penetrant abnormalities in cranial nerves (Parrish et al., 2004). An analysis of gross OB morphology at P0.5 was conducted to determine the effect of loss of Sall3 on OB size. No significant difference in OB length was observed in the absence of Sall3 at P0 (n=4, p=0.9).

To determine if Sall3 was required for the development of distinct cell types coronal sections of control and Sall3-/- animals were examined from E14.5 to P0.5. At E14.5 two distinct histological regions are visible in control OBs, the progenitor population and the differentiating field (Figure 33A,A'). By E16.5 a layer of differentiated neurons is present outside the differentiating field. Mitral cells represent the major cellular population that has differentiated at this time, and can be distinguished by large oval shaped cell body and light staining with Nissl, as compared to small intensely stained cell bodies in the differentiating field. We therefore assigned this population as the MCL (Figure 33C,C'). No gross alterations in lamination or cellular organization were observed in Sall3-mutant animals at E14.5 or E16.5 (Figure 33B,B',D,D') (n=3, E14.5; n=2, E16.5). By P0.5 a well defined MCL is apparent in wild type and Sall3-mutant animals (n=3) (Figure 33E-F'). At this age in control animals a subset of interneurons has differentiated and are located in the GL and GCL (Figure 33E,E'). In Sall3-

deficient animals the GL appeared hypocellular compared to controls (n=3) (Figure 33F,F'). We quantified the number of cells in the GL at P0.5 and observed a 32.6% decrease in glomerular cell number in Sall3-deficient animals ($p<0.001$, n=4). These observations suggest a role for Sall3 in glomerular formation.

Olfactory information is detected by olfactory receptor neurons in the olfactory epithelium, and these neurons project to spatially conserved glomeruli in the OB (Buck and Axel, 1991; Ressler et al., 1993; Vassar et al., 1993; Sullivan et al., 1995). Previous studies have shown that loss of peripheral innervation can lead to a decrease in OB cell number (Cummings and Brunjes, 1997; Fiske and Brunjes, 2001; Mandairon et al., 2003). We therefore investigated if alterations in the pattern of peripheral innervation were present in Sall3 -/- animals. β-tubulinIII (Tuj1) is a pan neuronal marker which can be used to visualize neuronal projections from the olfactory epithelium to the OB (arrows, Figure 33G-J). At E16.5 the olfactory nerve has contacted the OB and extended ventrally, medially and laterally and by P0.5 encompasses the entire OB. No alteration in olfactory nerve innervation was observed at E16.5 (n=2) (Figure 33G,H) or P0.5 (n=4) (Figure 33I,J) in Sall3-mutant animals. Furthermore, protoglomerular structures are observed within the OB in both control and Sall3-/- animals at P0.5 (arrows, Figure 38G,H). These findings suggest an intrinsic role for Sall3 in the development of OB interneurons.

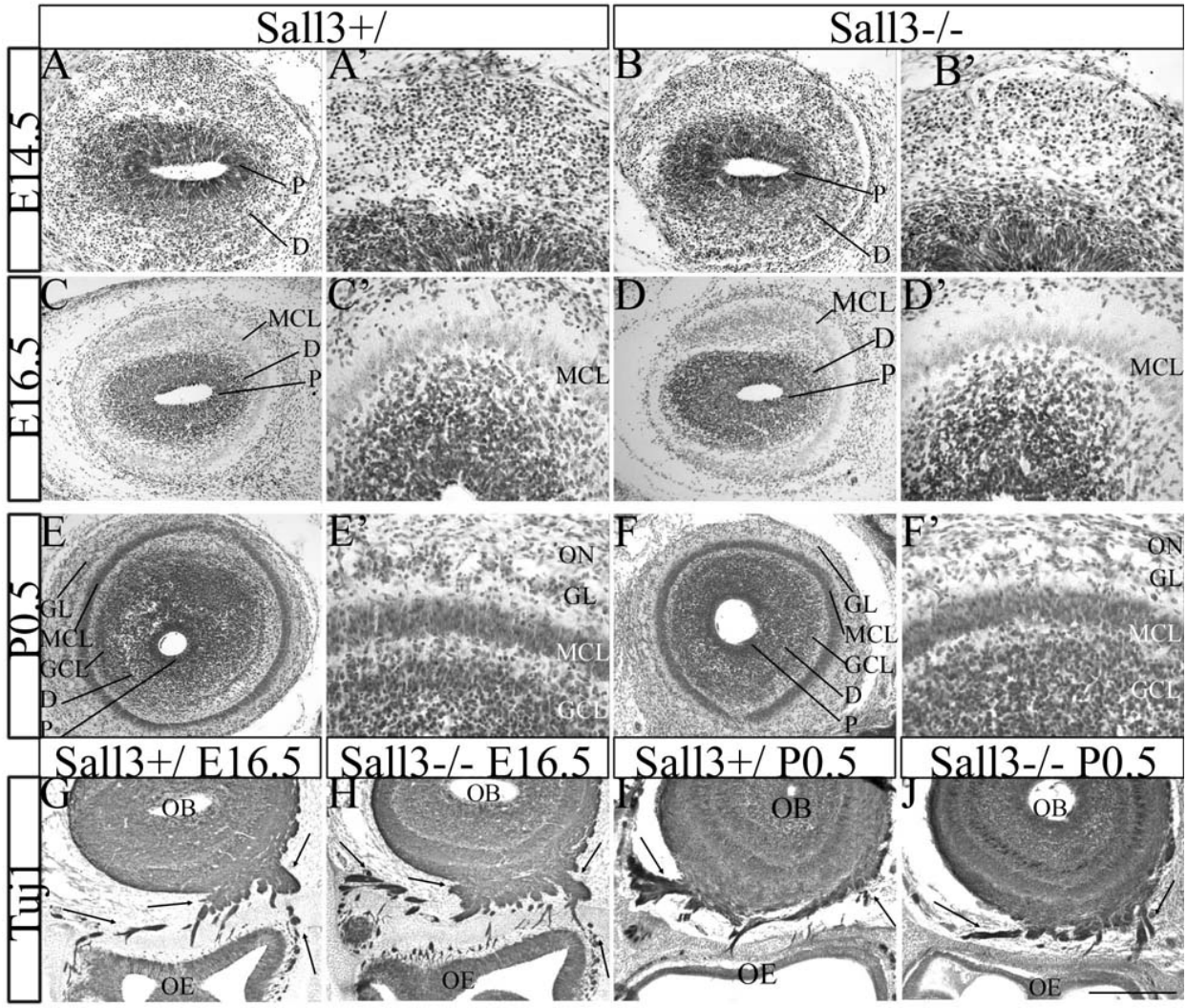

Figure 33. Histological analysis of the developing olfactory system in control and Sall3-mutant animals.

Nissl staining of coronal sections of the olfactory bulb at E14.5 (A-B'), E16.5 (C-D'), P0.5 (E-F') and Tuj1 staining of E16.5 (G,H) and P0.5 (F,J) in control (A,A',C,C',E,E',G,I) and Sall3-/- (B,B',D,D',F,F',H,J) animals. Control animals (Sall3+/) animals represent images of either Sall3+/+ or Sall3+/- animals. No alterations in early olfactory development were observed in Sall3-mutant animals (B,B',F,F'). The GL appeared hypocellular at P0.5 in Sall3-deficient animals (F'). The MCL, GCL and GL were identifiable under high power, by cellular characteristics described in Materials and Methods. Innervation of the olfactory bulb by the olfactory nerve (arrows, G-J) was not altered in Sall3-mutant animals (H,J). P: progenitor populations; D: differentiating field; GCL: granule cell layer; MCL: mitral cell layer; GL: glomerular layer; ON: olfactory nerve; OB: olfactory bulb; OE: olfactory epithelium. Scale bar (in J): 100µm in A',B',C',D',E',F'; 200µm in A,B; 300µm in C,D,G,H; 350µm in E,F,I,J.

A.4.3 Tyrosine hydroxylase expression is absent in Sall3-deficient animals

Histological analysis of the developing OB suggested that interneuron populations in the GL were decreased in the absence of Sall3. To further characterize these alterations a variety of cell type specific markers were examined to distinguish between cellular populations in the OB. Expression of Tuj1 was examined at P0.5. In control animals Tuj1-positive cells are present in the GL, MCL and GCL, and exhibit a characteristic striated staining pattern in the MCL (Figure 34A). Tuj1-positive cells were observed in all three cell layers in the absence of Sall3 at P0.5 (n=3) (Figure 34B). To identify distinct laminae of the OB and to distinguish between early versus late generated populations, expression of Reelin, a marker of the early born MCL (Figure 34C,D), and Tyrosine Hydroxylase (TH), a marker of the later born GL (Figure 34E,F), was examined. Reelin staining indicated that the MCL appeared normal in the absence of Sall3 at P0.5 (n=3) (Figure 34D). However, in Sall3-mutant animals TH-positive cells were absent in the GL (n=6) (Figure 34F), suggesting a requirement for Sall3 in the development of this cellular population. TH expression is first detected in the developing OB at E16.5. To determine if this cellular population was initially produced and subsequently died in the absence of Sall3 we examined expression of TH at this age (Figure 34G,H). No TH-positive cells were observed at E16.5 in Sall3-/- animals (n=3) (Figure 34H). To exclude the possibility that cell death contributed to the phenotype observed in Sall3-deficient animals, we examined expression of activated Caspase 3 in the OB and lateral ganglionic eminences at E16.5 and P0.5. No gross difference in cell death was observed suggesting that Sall3 is not required for cell survival (n=2 per region per age, data not shown). These findings suggest that Sall3 is required for the generation or specification of dopaminergic GL cells.

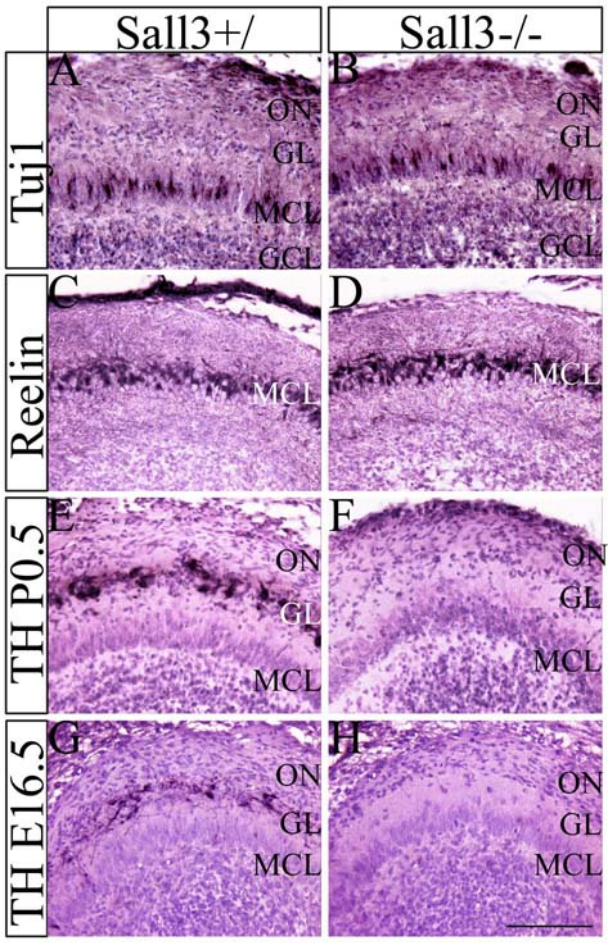

Figure 34. Cell type and layer specification in control and Sall3-mutant animals.

Tuj1 (A,B), Reelin (C,D), TH (E-H) staining (black) in coronal sections of control (A,C,E,G) and Sall3-mutant (B,D,F,H) animals at P0.5 (A-F) and E16.5 (G,H). Sections were counterstained with Haematoxylin and eosin. No alterations in Reelin expression in the MCL were observed in Sall3-deficient animals (D). TH expression in the GL was absent in Sall3-mutant animals at P0.5 (E) and E16.5 (H). TH: tyrosine hydroxylase; GCL: granule cell layer; MCL: mitral cell layer; GL: glomerular layer; ON: olfactory nerve. Scale bar (in H): 100µm in A-H.

A.4.4 TH expression is maintained in the substantia nigra and nigro-striatal pathway

In the absence of Sall3 we observed a decrease in cell number in the GL (Figure 33F'). In addition, Sall3-deficient animals exhibited a complete absence of TH-positive cells in the OB throughout development (Figure 34F,H). TH is also expressed by cells in the substantia nigra and their projections through the nigro-striatal pathway of the midbrain (Figure 35A,C). We have not observed gross histological differences in the developing midbrain in Sall3-deficient mice (Parrish, 2003). Although Sall3 is not expressed by mature cells in the substantia nigra, it is expressed by their progenitors (data not shown). We therefore examined TH expression in the substantia nigra and nigro-striatal pathway to determine if there was a global deficiency in dopaminergic neurons in the brain. Expression of TH was not altered in Sall3-mutant animals (n=3) (Figure 35B,D) (Parrish, 2003), suggesting a distinct function for Sall3 in the generation of OB dopaminergic cells.

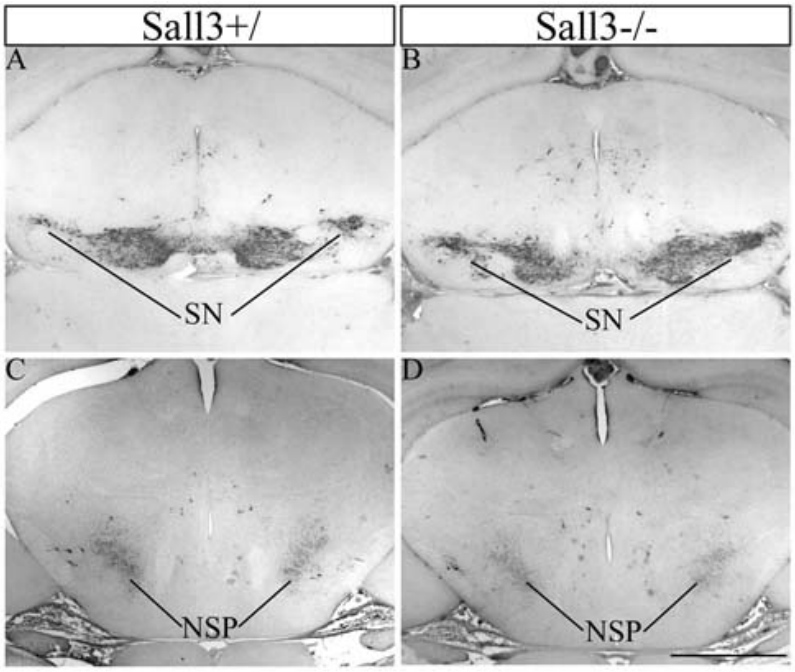

Figure 35. Tyrosine Hydroxylase expression in the midbrain.

TH expression (black) in the midbrain of control (A,C) and Sall3 -/- animals (B,D) at P0.5. TH expression is maintained in the SN and NSP in the absence of Sall3 (B,D). SN: substantia nigra; NSP: nigrostriatal pathway. Scale bar (in D): 800μm in A-D.

A.4.5 Sall3-mutant animals can detect odor

The initiation and maintenance of TH protein expression in the GL is activity dependent (Nadi et al., 1981; Baker et al., 1984; McLean and Shipley, 1988; Biffo et al., 1990; Stone et al., 1991; Baker and Farbman, 1993; Baker et al., 1993). In the absence of Sall3 the OB was innervated from the periphery (Figure 33H,J) and, although hypocellular, glomeruli like structures were observed at P0.5 (arrows, Figure 38H and data not shown). Rodents use olfactory stimuli to locate nipples during feeding (Teicher and Blass, 1976, 1977; Bruno et al., 1980). Previous studies from our laboratory have determined that Sall3-deficient animals are not anosmic as they can locate nipples on anesthetized dams, although Sall3-mutant animals rapidly detached from

the nipples (Parrish et al., 2004). To further verify these findings we examined the expression of the immediate-early transcription factor c-Fos (Figure 36A,B), which is regulated by neuronal activity (reviewed in (Guthrie and Gall, 1995)). Previous studies have shown that TH expression co-localizes with c-Fos expression in the GL and that c-Fos expression is down-regulated, along with TH, when input to the OB is altered (Jin et al., 1996; Liu et al., 1999). c-Fos expression was observed in Sall3-deficient animals at P0.5 (n=2) (Figure 36B) (Parrish et al., 2004). Consistent with the observations of decreased cell number in the GL, c-Fos expression appeared decreased in Sall3-/- animals (Figure 36B). These data suggest that the absence of TH expression in Sall3-/- animals is not due to a lack of input or activity in the OB. Taken together these data suggest that olfactory innervation is not altered in the absence of Sall3 and that despite the absence of TH expression sensory information can be detected by Sall3-mutant mice.

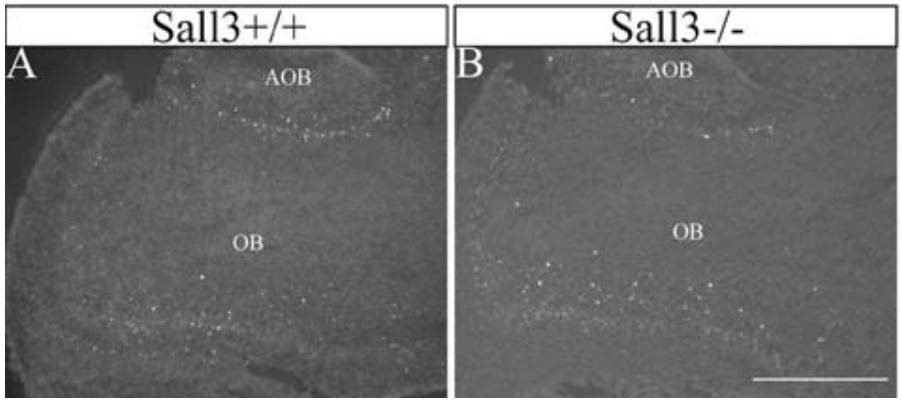

Figure 36. Expression of c-Fos in the OB of Sall3-/- animals

Expression of the immediate early gene c-Fos in the OB at P0.5 in sagittal sections of control (A) and Sall3-mutant (B) animals. c-Fos expression though reduced is present in the absence of Sall3. AOB: accessory olfactory bulb; OB: olfactory bulb. Scale bar (in B): 400μm in A,B.

A.4.6 The LGE and RMS is unaltered in Sall3-deficient animals

Our data suggests that Sall3 is required for the development of a population of OB interneurons. OB interneurons are derived from an ER81-expressing population of cells in the LGE (Figure 37A) (Stenman et al., 2003). No alteration in ER81 expression in the LGE was observed at P0.5 in Sall3-deficient animals (n=3) (Figure 37B), suggesting that Sall3 is not required for the initial specification of these interneuron populations. Cells destined for the OB migrate from the LGE to the OB within the RMS (Luskin, 1993; Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1999; Wichterle et al., 2001). Histological examination of the RMS did not reveal any alterations in this structure in Sall3-/- animals compared to controls at E16.5 (n=2) (Figure 37C,D) or P0.5 (n=4) (Figure 37E,F). Pax6 is expressed by migrating neuroblasts as they travel along the RMS and Pax6 has been shown to be required for TH expression (Hack et al., 2005; Kohwi et al., 2005). Pax6-expressing-neuroblasts were observed in the RMS in Sall3-mutant animals at P0.5, similar to controls (n=4) (Figure 37G,H) suggesting that Sall3 is not required for the differentiation or migration of these neuroblasts within the RMS.

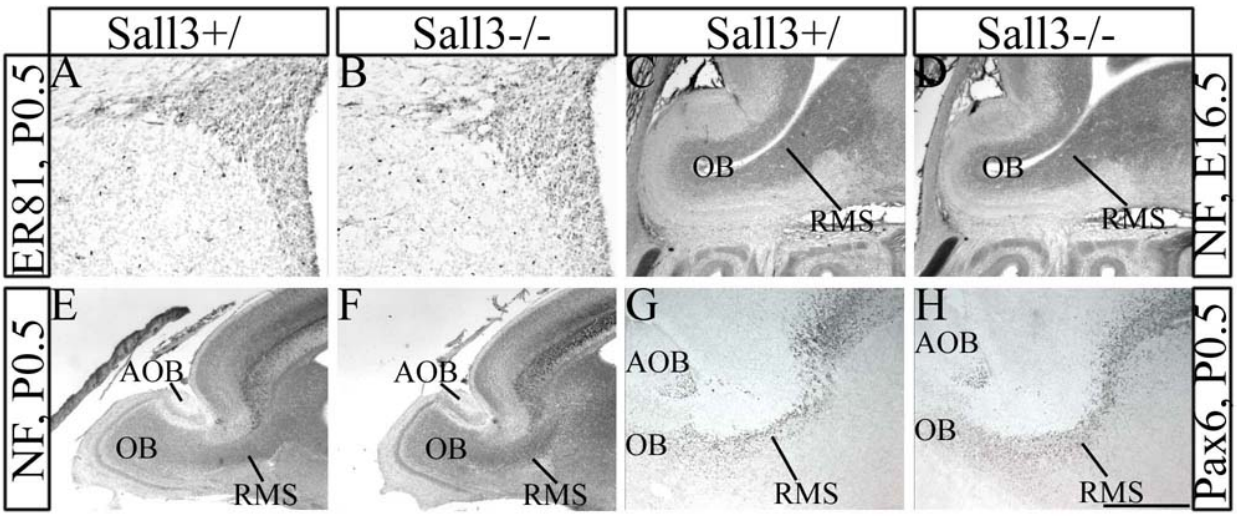

Figure 37. Sall3 is not required for the initial specification of cells within the lateral ganglionic eminence or migration within the RMS.

Expression of ER81 (A,B), neurofilament (C-F) and Pax6 (G,H) in the lateral ganglionic eminence (A-B) and RMS (C-H) of control (A,C,E,G) and Sall3-/- (B,D,F,H) animals at E16.5 (C,D) and P0.5 (A,B,E-H). ER81 expression is unaltered in the lateral ganglionic eminence in Sall3-mutant (B) animals. Histological examination of the RMS did not reveal any abnormalities in Sall3 -/- animals (D,F). Pax6-expressing neuroblasts were observed migrating in the RMS in the absence of Sall3 (H). OB: olfactory bulb; RMS: rostral migratory stream; AOB: accessory olfactory bulb. Scale bar (in H): 150μm in A,B; 400μm in C,D,G,H; 600μm in E,F.

A.4.7 Alterations in other olfactory interneuron subtypes are present in Sall3-mutant animals

Different interneuron subtypes are observed in the OB and can be defined by expression of GABA, Calretinin and Calbindin expression (Kosaka et al., 1995). Expression of these chemical markers is initiated as OB interneurons terminally differentiate and radially migrate in the OB. In Sall3-deficient animals a 32.6% decrease in cell number in the GL is observed, accompanied by an absence of TH expression. TH-expressing cells account for 11% of cells within the GL (Parrish-Aungst et al., 2007), suggesting that alterations exist in other GL cellular populations in

the absence of Sall3. To identify these cellular populations, we examined markers of distinct interneuron subtypes at P0.5. GAD65/67 is expressed by GABAergic cells in the GL (region 1, Figure 38A) and GCL (region 2, Figure 38A), as well as the projections of these cells through the MCL (*, Figure 38A). GAD65/67 is expressed by ~53% of cells within the GL and ~80% of TH-positive cells are also GAD65/67-positive (Kosaka et al., 1998; Hack et al., 2005; Parrish-Aungst et al., 2007). In Sall3-/- animals a mix of GAD65/67-positive and negative cells was observed in the GL, although overall fewer GAD65/67-positive cells were present in this layer (n=3) (region 1, Figure 38B). In addition, more GAD65/67-positive cells were observed in the GCL in Sall3-deficient animals (region 2, Figure 38B). These data suggests that a shift in cell fate specification or an alteration in migration or maturation of interneuron populations exists in the absence of Sall3.

Previous studies have shown that GABA expression overlaps with TH- and Pax6-positive populations in the OB (Hack et al., 2005; Parrish-Aungst et al., 2007). In addition, the majority (95%) of TH-positive cells in the GL are also Pax6-positive, although only 78% of Pax6-positive cells in the GL are TH-positive (Hack et al., 2005). Furthermore, Pax6 has been shown to be required for the generation of dopaminergic TH-positive cells in the OB (Hack et al., 2005; Kohwi et al., 2005). To determine whether the absence of TH expression in Sall3-mutant animals was due to a requirement for Pax6 we examined its expression at P0.5. Pax6 expression is downregulated in the GCL, but is highly expressed in the GL (Hack et al., 2005). In control animals two patterns of Pax6 staining were observed in the GL and GCL, large cells expressing high levels of Pax6, $Pax6^{high}$ (arrows, Figure 38C), and smaller cells expressing low levels of Pax6, $Pax6^{low}$ (arrowheads, Figure 38C). The majority of $Pax6^{high}$-expressing cells were located

in the GL, and Pax6^{low}-expressing cells were observed in the GCL in control animals (Figure 38C). In Sall3-mutant animals very few Pax6^{high}-expressing cells were observed in the GL (n=5) (arrows in GL, Figure 38D). The majority of these cells were located in the superficial GCL (arrows in GCL, Figure 38D). No gross difference in Pax6^{low}-expressing cells in the GCL was observed (arrowheads, Figure 38D). These finding suggest that the absence of TH-positive cells is not due to a requirement for Pax6 expression. In addition, the presence of large Pax6^{high}-expressing cells in the GCL suggests that these cells are ultimately destined for the GL and an alteration in the maturation or migration this cellular population occurs in the absence of Sall3. To determine if radial glia were structurally altered we examined expression of radial glial markers. GLAST expression appeared normal in Sall3 -/- animals at P0.5 suggesting that if a migration deficit is present it is not due to altered radial glia scaffold (n=3, data not shown).

Recent studies have identified that the Ets transcription factor ER81 is expressed by cells in the LGE, RMS and the GL, MCL and superficial GCL, and that OB interneurons are derived from this population (Stenman et al., 2003; Saino-Saito et al., 2007). ER81 expression was unaltered in the LGE (Figure 37B), and the RMS appeared normal in Sall3-deficient animals (Figure 37D,F,H), suggesting that the alterations observed in Sall3-mutant animals are downstream of these events. Mice deficient for ER81 have a decreased number of TH-positive cells in the OB (Saino-Saito et al., 2007), suggesting a role for this transcription factor in the specification of the dopaminergic cellular population. We examined ER81 expression in the OB to determine if alterations in expression of this transcription factor contributed to the deficit in Sall3-mutant animals. At P0.5 the majority of cells within the GL are ER81-positive (Figure 38E). The majority of cells in the GL were also ER81-positive in Sall3-deficient animals, although this

population appeared decreased in number (n=4) (Figure 38F). Calretinin and Calbindin are expressed by independent populations within the OB (Kosaka et al., 1995). In order to determine if Sall3 is required for the generation of non-dopaminergic OB cellular populations we examined expression of these markers in Sall3-deficient animals. Calretinin is expressed by olfactory nerve axons, and cells within the GL, MCL and GCL at P0.5 (Figure 38G). In Sall3-deficient animals Calretinin expression was observed in these layers although fewer positive cells were located in the GL, and more positive cells were located in the GCL (n=4) (Figure 38H). Calbindin is also expressed by interneurons in the GL and GCL (Figure 38I). Similar alterations in the distribution of Calbindin interneuron populations were observed in Sall3-mutant animals, with fewer positive cells in the GL and more positive cells in the GCL (n=3) (Figure 38J). These data suggest that Sall3 is required for the differentiation of dopaminergic expressing cellular populations and non-dopaminergic cellular populations.

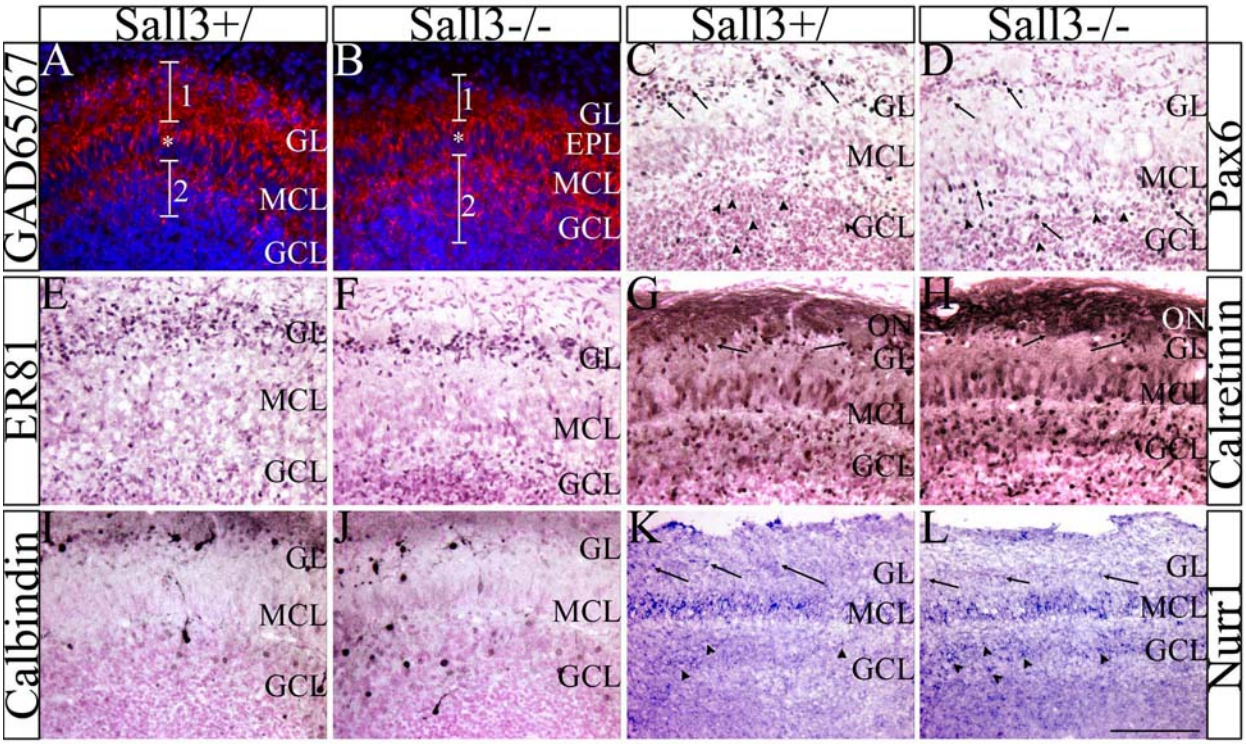

Figure 38. Sall3 is required for the terminal maturation of olfactory interneurons.

GAD65/67 (red) (A,B), Pax6 (black) (C,D), ER81 (black) (E,F), Calretinin (black) (G,H), Calbindin (black) (I,J) and Nurr1 (blue) (K,L) expression in the olfactory bulb in coronal (A-J) and sagittal (K,L) sections at P0.5. Sections were counterstained with DAPI (blue) (A,B) and nuclear fast red (C-I). Fewer GAD65/67-expressing cells were observed in the GL in Sall3-mutant animals accompanied by more positive cells in the GCL (B). 1 and 2, demarcate the GL and GCL respectively; * indicates the GAD65/67-positive projections through the MCL. In control animals, large Pax6high-expressing cells were predominately observed in the GL (arrows, C), and small Pax6low-expressing cells were located in the GCL (arrowheads, C). In Sall3-mutant animals fewer large Pax6high-expressing cells were observed in the GL (arrows, GL, D) and more large Pax6high-expressing cells was observed in the GCL (arrows, GCL, D). No gross difference was observed in the number of small Pax6low-expressing cells (arrowheads, D). A decrease in ER81-expressing cells were observed in the GL in Sall3-mutant animals (F). Similar alterations were observed with Calbindin and Calretinin in Sall3-deficient animals (H,J). Glomeruli like structures were observed in control (arrows, G) and Sall3-mutant animals (H). Nurr1 expression was observed in the GL (arrows), MCL and GCL (arrowheads) of control (K) and Sall3-mutant animals (L), although an increase Nurr1 expression was observed in the GCL in the absence of Sall3 (L). GCL: granule cell layer; MCL: mitral cell layer; GL: glomerular layer; ON: olfactory nerve. Scale bar (in L): 100µm in A-J; 200µm in K,L.

A.4.8 Sall3 is required for the terminal differentiation of dopaminergic olfactory cells

To determine if the absence of TH expression was due to a failure of dopaminergic cellular maturation we examined expression of the transcription factor Nurr1 at P0.5. TH-expressing cells arise from Nurr1-positive progenitor-like cells and in the ventral midbrain Nurr1 is required for TH expression (Backman et al., 1999; Le et al., 1999; Castelo-Branco et al., 2003; Castelo-Branco et al., 2006). Within the OB TH is co-expressed with Nurr1 in the GL (Backman et al., 1999). In control animals at P0.5, Nurr1-positive cells were observed in the GCL (arrowheads, Figure 38K), MCL and GL (arrows, Figure 38K). Nurr1-positive cells were present in all three layers in Sall3-deficient animals (n=3) (Figure 38L). However, fewer Nurr1-positive cells were observed in the GL (arrows, Figure 38L) and more Nurr1-positive cells were observed in the GCL (arrowheads, Figure 38L). Thus, precursors of TH-expressing cells are present in the OB but mature dopaminergic cells are absent suggesting a requirement for Sall3 in the terminal differentiation of Nurr1-positive precursor cells to TH-expressing cells.

A.4.9 Sall3 is not required in a cell autonomous manner for olfactory interneuron development

In Sall3-mutant animals, exon 2 is fused in frame to the β-galactosidase gene (Parrish et al., 2004). This fusion results in a null allele, with deletion of greater than 95% of the coding region of the Sall3 gene. In addition, this construct results in expression of β-galactosidase under the control of the endogenous Sall3 promoter and allows visualization of cells that would normally express Sall3 in Sall3+/- and Sall3-/- animals. To determine if the absence of TH and other

interneuron populations in the GL, observed in Sall3-deficient animals, were due to a cell autonomous effect, we examined expression of β-galactosidase in Sall3+/- and -/- animals. In Sall3+/- animals β-galactosidase-positive cells were observed in progenitor cells, differentiating cells, as well as cells in the GCL, MCL and GL (n=3) (Figure 39A). Within the GL and GCL, β-galactosidase was expressed by a subset of cells (Figure 39A). Similar β-galactosidase expression patterns were observed in Sall3-/- animals (n=3) (Figure 39B). However, in Sall3-deficient animals a decrease in the number of β-galactosidase-positive cells in the GL and an increase in the number of β-galactosidase-positive cells in the GCL was observed (Figure 39B). The presence of β-galactosidase-positive cells in the GL suggests that Sall3-dependent cells can migrate to their laminar position in the absence of Sall3.

In order to further elucidate the interneuron subtypes dependent on Sall3 expression we examined co-expression of Pax6, Calretinin and Calbindin with β-galactosidase in the GL of Sall3+/- and Sall3-/- animals at P0.5. In Sall3+/- animals the majority of Pax6-positive cells were also β-galactosidase-positive (n=3) (arrows, Figure 39C). However, a mix of Calretinin+ β-galactosidase+ (arrows, Figure 39E) and Calretinin+ β-galactosidase- (arrowheads, Figure 39E) cells and Calbindin+ β-galactosidase+ (arrows, Figure 39G) and Calbindin+ β-galactosidase- (arrowheads, Figure 39G) cells were observed within this layer (n=3). In Sall3-deficient animals, while the number of cells in the GL expressing these markers was reduced, the majority of cells expressing Pax6 (arrows, Figure 39D) in the GL were also β-galactosidase-positive (n=3). In addition, similar to Sall3+/- animals a mix of Calretinin+ β-galactosidase+ (arrows, Figure 39F) and Calretinin+ β-galactosidase- (arrowheads, Figure 39F) cells and Calbindin+ β-galactosidase+ (arrows, Figure 39H) and Calbindin+ β-galactosidase- (arrowheads,

Figure 39H) were observed in the GL of Sall3-/- animals (n=3). Thus, Sall3 is not required in a cell autonomous manner for the migration or differentiation of subpopulations of interneurons that display altered laminar position in Sall3-deficient animals. In summary, Sall3 is expressed by diverse interneuron populations at P0.5, and in Sall3-/- animals, subpopulations of cells dependent on Sall3 expression can reach their laminar position in the GL.

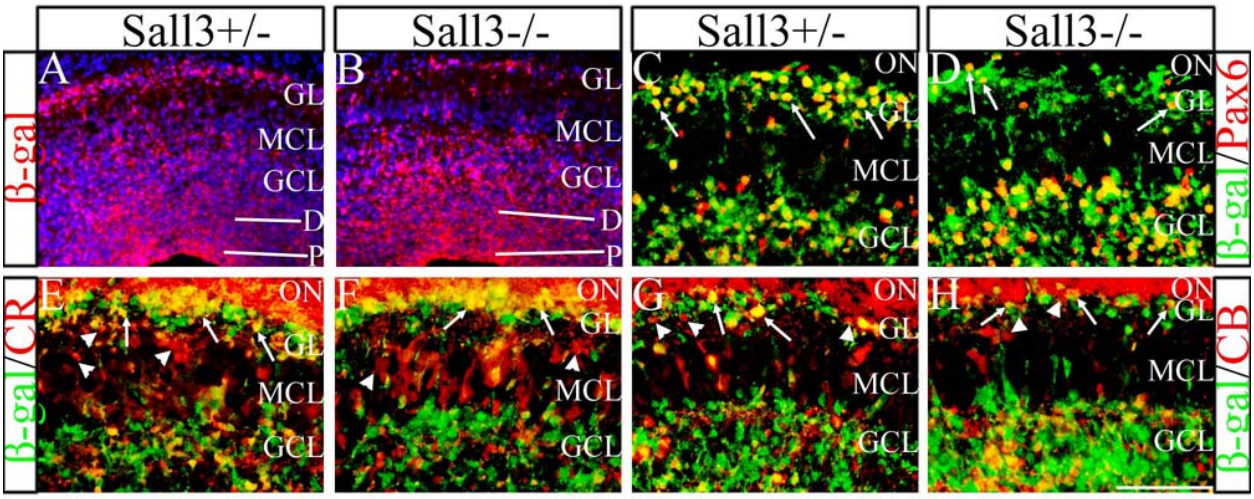

Figure 39. Sall3 is not required in a cell autonomous manner for glomerular olfactory interneuron maturation

β-galactosidase-expressing (red) in Sall3+/- and Sall3-/- animals at P0.5. Expression is observed in progenitors, differentiating cells, GCL, MCL and GL (A,B). Sections were counterstained with DAPI. In Sall3-mutant animals a decrease in the number of β-galactosidase expressing cells in the GL and an increase in β-galactosidase-expressing cells in the GCL is observed. Pax6 (red) (C,D), Calretinin (red) (E,F), and Calbindin (red) (G,H) co-expression with β-galactosidase (green) in the P0.5 olfactory bulb in Sall3+/- (C,E,G) and Sall3-/- (D,F,H) animals. Coexpression of β-galactosidase is observed with the majority of Pax6 (arrows, C) cells in the GL. However, a mix of Calretinin+ β-galactosidase+ (arrows, E) and Calretinin+ β-galactosidase- (arrowheads, E) and Calbindin+ β-galactosidase+ (arrows, G) and Calbindin+ β-galactosidase- (arrowheads, G) is observed in the GL of Sall3+/- animals. In Sall3-mutant animals while the number of Pax6-, Calretinin- and Calbindin-positive cells in the GL is decreased (D,F,H), cells co-expressing β-galactosidase and Pax6 (arrows, D), β-galactosidase and Calretinin (arrows, F) and β-galactosidase and Calbindin (arrows, H) were observed. Calretinin and Calbindin are additionally expressed by olfactory nerve axons, visible as staining superficial to the GL in E-H. P: progenitor populations; D: differentiating field; GCL: granule cell layer; MCL: mitral cell layer; GL: glomerular layer; ON: olfactory nerve; β-gal: β-galactosidase; CR: Calretinin; CB: Calbindin. Scale bar (in H): 150μm in A,B; 100μm in C-H.

A.5 DISCUSSION

Our study examined the role of Sall3 in the differentiation of OB interneuron populations. OB interneurons arise from an ER81-positive population of cells located in the lateral ganglionic eminence and migrate to the OB via the RMS (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1999; Wichterle et al., 2001; Stenman et al., 2003). A number of factors have been identified that influence the initial generation of interneurons from the lateral ganglionic eminence and their migration via the RMS (Hu, 1999; Wu et al., 1999; Chen et al., 2001; Stenman et al., 2003; Yun et al., 2003; Soria et al., 2004; Yoshihara et al., 2005; Waclaw et al., 2006). Previous studies have suggested that cell type specification is intrinsically regulated (Tucker et al., 2006), but few factors have been identified that regulate the terminal differentiation and specification of these cells within the OB.

We have shown that the zinc finger containing transcription factor Sall3 is expressed by olfactory progenitors and a subset of differentiated neurons, predominately by interneurons in the GL. Within OB interneuron populations Sall3 is expressed by chemically diverse populations. In the absence of Sall3 no alteration in OB size was observed at P0.5, however, histological examination of the OB identified a decrease in cell number in the GL. TH expression was absent from the OB throughout development. In addition, we observed a decrease in the number of GAD65/67-, Pax6-, Calretinin- and Calbindin-positive populations in the GL, accompanied by an increase in the number of positive cells in the GCL. In Sall3+/- animals Sall3-expressing cells, assayed by the presence of β-galactosidase expression were observed in the GL and GCL.

In Sall3-deficient animals, β-galactosidase-positive cells were also observed in the GL and GCL, although a decrease in the number of β-galactosidase-positive cells in the GL was observed. This indicates that at least a subpopulation of interneurons dependent on Sall3 can appropriately migrate to their laminar position. Furthermore, this suggests that expression of Sall3 in interneuron populations is not essential for their migration to their laminar position. In addition, the morphology of radial glia appeared normal in Sall3-deficient animals, suggesting that if a migration deficit is present it is not due to alterations in radial glia. While we cannot exclude the possibility that Sall3 is required for the migration of subpopulations of interneurons, we hypothesize that Sall3 is required for the terminal differentiation of OB interneurons.

In Sall3-mutant animals the GL appeared hypocellular and TH expression was absent. Previous studies have implicated Pax6 in the generation of dopaminergic OB cells (Hack et al., 2005; Kohwi et al., 2005). However, Pax6 expression was present in Sall3-mutant animals, suggesting that the absence of TH expression was not due to a requirement for Pax6. Kohwi et al., propose that Pax6 instructs a laminar bias towards the GL, as Pax6 is downregulated by cells in the GCL (Kohwi et al., 2005). $Pax6^{high}$-expressing cells were predominately observed in the GCL in Sall3-deficient animals, suggesting that Pax6 is not sufficient to instruct OB laminar position. Furthermore, the presence of Pax6 expression in Sall3-mutant animals suggests that Sall3 is either downstream of Pax6 signaling pathway or that they represent independent pathways in dopaminergic cellular generation.

In the nigro-striatal pathway TH-positive cells arise from a Nurr1-positive precursor cell (Backman et al., 1999; Castelo-Branco et al., 2003; Castelo-Branco et al., 2006). Interestingly,

in Nurr1 deficient animals, TH expression is absent from the substantia nigra and nigro-striatal pathway but is maintained in the OB (Le et al., 1999). In Sall3-mutant animals, TH expression was maintained in the substantia nigra and nigro-striatal pathway but absent from the OB. This suggests that independent pathways regulate dopaminergic maturation in distinct cortical regions. Expression of the dopaminergic precursor Nurr1 was observed in the GL, MCL and GCL of Sall3-deficient animals. Nurr1-positive cells were present in reduced numbers in the GL, however an increase in Nurr1 expression in the GCL was observed in the absence of Sall3, suggesting that the majority of these cells fail to reach their laminar position and express TH. Previous studies have shown that TH expression is context dependent (Nadi et al., 1981; Baker et al., 1984; McLean and Shipley, 1988; Biffo et al., 1990; Stone et al., 1991; Baker and Farbman, 1993; Baker et al., 1993). Therefore, it is possible that these cells cannot express TH as they are not receiving appropriate signals. In Sall3-deficient animals no alterations in peripheral innervation were observed and expression of the immediate early protein c-Fos was present. In addition, olfactory perception appeared to be present in Sall3-deficient animals (Parrish et al., 2004). We therefore hypothesize that Sall3 is required for terminal differentiation of Nurr1-positive dopaminergic precursors and expression of a mature dopaminergic TH-positive phenotype.

Our studies have demonstrated that Sall3 is required for the terminal differentiation of OB interneurons. We have recently described a role for a related Sall family member, Sall1, in OB development (Monaghan et al., 2006). During development Sall1 and Sall3 are expressed by peripheral and central components of the olfactory system. Interestingly, early in development, Sall1 is expressed prior to Sall3 in overlapping cell types in both the olfactory epithelium and

developing neural epithelium. Sall1-mutant mice die at birth due to kidney deficits (Nishinakamura et al., 2001) and have smaller OBs (Monaghan et al., 2006). The absence of Sall1 specifically alters mitral cell development and olfactory nerve innervation, whereas interneuron subtypes are appropriately specified and mature (Monaghan et al., 2006). These findings indicate that Sall family members regulate distinct steps in olfactory bulb neurogenesis.

Our studies, and those of others, demonstrate that independent mechanisms regulate dopaminergic cell maturation in distinct cortical regions (Le et al., 1999). In Parkinson's disease preferential loss of dopaminergic cells is observed in the substantia nigra pars compacta. Interestingly, however, one of the first symptoms observed in Parkinson's Disease is a loss of olfactory perception (reviewed in (Ponsen et al., 2004)), suggesting that global deficiencies in cellular populations exist. Dopaminergic cells are of potential therapeutic value for the treatment of neurodegenerative disorders, such as Parkinson's Disease (reviewed in (Lindvall and Bjorklund, 2004; Correia et al., 2005; Snyder and Olanow, 2005; Sonntag et al., 2005; Geraerts et al., 2007)). Several factors have been identified that can induce a dopaminergic phenotype from precursor cells, however, these cells do not fully mature (reviewed in (Correia et al., 2005; Sonntag et al., 2005)). Our studies have identified a role for the transcription factor Sall3 in the terminal differentiation and maturation of dopaminergic cells derived from the dorsal ganglionic eminence. It may therefore be of therapeutic value to investigate the potential use of Sall3 in promoting a terminal dopaminergic cellular phenotype.

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