Regulation of Olfactory Stem Cells and Neurogenesis by Primary Cilia and the Hedgehog Pathway

by

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ABSTRACT

The olfactory epithelium (OE) is one of the few tissues to undergo constitutive neurogenesis throughout the mammalian lifespan. It contains multiple cell types including olfactory sensory neurons (OSNs) that are replaced by two populations of basal stem cells – frequently dividing globose basal cells (GBCs), and quiescent horizontal basal cells (HBCs). Strikingly, the mechanisms that control stem cell activation and proliferation, during normal OE homeostasis and in response to injury remain largely unexplored. My data indicate that HBCs possess primary cilia, cellular organelles that regulate the activity of multiple signaling pathways, including Hedgehog (HH) signaling. The HH pathway is required for the formation, survival, and activity of stem/progenitor cell populations in several adult tissues, making it an attractive candidate for HBC regulation. The goals of this dissertation are: 1) to investigate the role of primary cilia in HBC-driven olfactory neurogenesis and 2) to explore the contribution of HH signaling to HBC function.

My data indicate that HBC-specific deletion of the ciliary gene *Ift88* effectively abrogates primary cilia. Interestingly, the loss of HBC cilia did not affect HBC cell numbers or maintenance of the adult OE, but dramatically impaired the regeneration of OSNs following injury. Furthermore, the loss of cilia during development resulted in region-specific decreases in neurogenesis, implicating HBCs in in OE establishment. These results demonstrate a novel role for primary cilia in olfactory neurogenesis and suggest a role for primary cilia in HBC activation, proliferation, and differentiation.

My data also demonstrate that HH pathway components are expressed in the adult OE. Specifically, the HH transcription factors, *Gli2* and *Gli3* are selectively expressed in HBCs, implicating HH signaling in the control of HBC function. Constitutive *Gli2* activation in HBCs resulted in hyperproliferation and loss of neuronal cells, and also impaired OE regeneration following injury. Surprisingly, constitutive *Gli2* repression in HBCs did not alter OE maintenance, but did impair injury-induced OE regeneration. These results suggest a novel role for HH signaling, and specifically *Gli2*, in HBC regulation during OE homeostasis and regeneration. Overall, this work defines novel roles for primary cilia and HH signaling in HBC-mediated regulation of the mammalian OE.

CHAPTER I:

Introduction

1.1 Abstract

An appreciation of olfaction, or the sense of smell, and its importance across species dates back to ancient civilizations. This appreciation has grown with recent advances in technology and olfactory research. The sense of smell relies on olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) that project compartmentalized organelles, called cilia into the nasal cavity. Cilia contain various odorant receptors to which odorants bind and propagate a signal to the central nervous system (CNS), where the sense of smell is perceived. OSNs are unique neurons in that they both directly contact the external environment and the brain. While this direct contact allows OSNs to detect odors, it also makes them susceptible to environmental toxins leading to cell death. Upon their death, OSNs are replenished by basal stem cells in the OE leading to constitutive regeneration and neurogenesis. However, several forms of olfactory dysfunction can arise from various perturbations of OSNs, including disruption of olfactory cilia, cell death, or odorant signal transduction pathways. Some olfactory disturbances are treatable but more severe dysfunctions lack therapeutic remedies. In order to develop novel treatments for olfactory dysfunction, understanding the mechanisms of OE maintenance and uncovering additional etiologies of olfactory dysfunction are imperative. Many questions concerning the maintenance of the OE remain unanswered, including the

precise mechanisms and signaling pathways necessary for the cell division, cell fate, and cell differentiation of the basal stem cells. Various signaling pathways, including the cilia-dependent Hedgehog (HH) pathway, play important roles in adult stem cell function. In this chapter, I review: 1) the olfactory system itself, 2) the role of cilia in olfactory function, and 3) the role of cilia-dependent HH signaling as a potential novel regulator of olfactory maintenance and regeneration.

1.2 Olfaction: An Evolutionarily Conserved Sense

1.2.1 Importance and Mechanism of Smell

Olfaction, or the sense of smell, is an evolutionarily and historically important function. The importance of smell ranges across species and extends to prehistoric times. In early medicine, odor and taste were used often as a diagnosis of disease and for animals and prehistoric humans, olfaction was and remains important for survival in obtaining food, finding a mate, and avoiding predators (Doty, 2015). From ancient civilization to present-day, smell has been a large part of the quality of life of humans through the use of fragrances in religious rites and celebrations, as well as for skin care and food (Doty, 2015). In mammals, olfaction has been linked to behaviors related to alarm, feeding, mating, predator-prey relationships, social status appraisal, and species recognition through the social and emotional associations of certain smells (Doty, 2015). A major advance has been the discovery that the loss of smell is the first, or among the first, sign of common neurodegenerative diseases like Alzheimer's disease and Parkinson's disease (Doty, 2003a; Smutzer et al., 2003; Doty, 2015), making olfactory

testing an important early indicator of some neurodegenerative disorders. Olfaction has varying uses and levels of importance across species and this is due in part to the evolution of smell.

Evolution of Smell

Olfaction began in an aquatic environment in which fish detected water-soluble odorants and evolved to incorporate both water-soluble and volatile odorants in four-limbed animals due to their dual environments on land and in water (Hoover, 2010). Eventually mammals evolved, and unlike other non-mammal vertebrates, used an organ, the nose, to detect volatile odors. Within the nose are nasal turbinates that help push odorants within the inhaled air into the olfactory epithelium (OE) via the orthonasal route (Shepherd, 2004). In humans, odorants also reach the OE via the retronasal route from the back of the oral cavity through the nasopharynx, allowing for smell perception of foods and liquids (Shepherd, 2004). Further evolution of humans was characterized by the increasing importance of other senses, like vision, due to the adoption of an erect posture which moved the nose away from the ground and closer to the face, allowing for the eyes to have more depth perception (Sarafoleanu et al., 2009). Although other senses have become important for humans, the sense of smell has not become irrelevant, but its importance has become associated with aspects that diverge from other mammals.

The basic organization of the olfactory system of humans and mice is similar with only some differences in its structure, making experimental information about the function of the mouse olfactory system applicable to humans (Harkema et al., 2012). A schematic of the mouse olfactory turbinates and resulting coronal sections can be seen in Fig. 1-1. From rodents to humans, there is a reduction in the proportion of functional odorant

receptor (OR) genes to total OR genes. In mammals, volatile odors are detected via ORs found in specialized primary cilia (small organelles important for cell function) that project from olfactory sensory neurons (OSNs) in the OE. While mice have approximately 1,300 – 1,500 OR genes with 1,100 that are functional (Young et al., 2002; Zhang and Firestein, 2002), humans only have 350 functional OR genes of the approximate 1,000 in the genome (Glusman et al., 2001). However, this reduction in humans is compensated by the larger capacity of human brain processing via the integration of various areas of olfactory perception in the brain (Sarafoleanu et al., 2009). This combination of odor detection in the main olfactory system and odor perception in the cerebrum is the basis for olfactory transduction.

Olfactory Transduction

The main olfactory system detects odorants in the nasal cavity via OSNs and the resulting information is sent to various regions of the CNS. OSNs are bipolar neurons with a single dendrite that projects to the surface of the OE and ends in a structure called the dendritic knob. Basal bodies within the dendritic knob project cilia towards the nasal cavity into mucous of the OE. The human OE contains approximately 50 million OSNs with 8-20 cilia (Sarafoleanu et al., 2009) compared to 6-10 million OSNs in the mouse OE (Firestein, 2001). In most mammals and humans, lengths of OSN cilia are approximately 50µm, while in non-mammalian vertebrates such as frogs, they can be as long as 200µm (Menco and Morrison, 2003). These cilia are enriched in ORs and other signaling components that mediate the initial transduction events in the cell (Firestein, 2001). The family of ORs was initially identified as a large multigene family of olfactory-specific G protein-coupled receptors (GPCRs) in the rat (Buck and Axel, 1991). They exhibit

extensive sequence diversity within their transmembrane domains (the presumed ligand binding sites), allowing for the detection of a wide range of chemicals in the environment (DeMaria and Ngai, 2010). In addition to the ORs, a second family of receptors called trace amine-associated receptor (TAAR) family are also expressed in a unique subset of OSNs and are thought to recognize volatile amines (Liberles and Buck, 2006). The receptors within the multitude of OSN cilia increase the sensory surface area of the OE, priming the olfactory system for signal detection.

The signaling cascade for the sense of smell starts with the binding of an odorant to its receptor (Fig. 1-2A). Only volatile materials that are soluble in the mucous and that interact with specific OSNs produce odors (Turin and Yoshii, 2003). Odorant molecules pass through the nasal cavity, dissolve in the mucous and bind to their corresponding olfactory receptors. Upon odorant binding, the activated OR couples through the G-protein, $G\alpha_{olf}$, which is a $G\alpha_s$ isoform enriched in OSNs (Belluscio et al., 1998). Activated $G\alpha_{olf}$ then activates type III adenylyl cyclase (ACIII) (Wong et al., 2000), which catalyzes the production of cyclic AMP (cAMP) (Fig. 1-2A). The increase in intracellular cAMP opens a cyclic nucleotide-gated (CNG) channel (Brunet et al., 1996), leading to an influx of sodium and calcium ions, and depolarization of the neuron (Fig. 1-2A). The initial depolarization is further amplified by the subsequent activation of calcium-activated chloride channels and, due to the low concentration of extracellular Cl^- in the mucus, the efflux of Cl^- from the cell (Stephan et al., 2009) (Fig. 1-2A). The odor-induced depolarization in the olfactory cilia spreads throughout the neuron, resulting in the opening of voltage-sensitive ion channels in the sensory neuron's axon hillock, the firing

of action potentials, and the release of neurotransmitter at the synaptic terminal (DeMaria and Ngai, 2010).

At the other end of OSNs, a single, unbranched axon projects to the olfactory bulb (OB), a structure in the forebrain that serves as the first relay of olfactory information. The axons of OSNs in the periphery together comprise the olfactory nerve (the first cranial nerve). Once the axons reach the OB, they form synapses with the dendrites of projection neurons within discrete structures known as glomeruli (DeMaria and Ngai, 2010) (Fig. 1-2B). Each OSN in the OE expresses just one allele of a single OR (Chess et al., 1994; Serizawa et al., 2003) – known as the "one receptor, one neuron" rule (DeMaria and Ngai, 2010). Although OSNs expressing a given OR are distributed broadly throughout the OE, their axons converge to specific glomeruli in the OB in a spatially invariant pattern (Ressler et al., 1993; Vassar et al., 1993; Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). The projection neurons of the OB, the mitral and tufted cells, extend their axons to the olfactory cortex which is comprised of several anatomically distinct areas including the piriform cortex, olfactory tubercle, anterior olfactory nucleus, and specific parts of the amygdala and entorhinal cortex (Zou et al., 2001) (Fig. 1-2C). Communication with the limbic system allows for interplay between smell and various processes like taste, memory and behavior.

1.2.2 Development and Anatomy of the Olfactory Periphery

The sensory organs of the vertebrate head derive from the sensory placodes and the cranial neural crest (NC) during embryogenesis. Placodes are discrete areas of thickened non-neural epithelium that give rise to the paired sensory organs including the olfactory system (Katoh et al., 2011). The olfactory organ has been shown to arise from

a combination of the olfactory placode and cranial NC cells (Katoh et al., 2011). The role of the olfactory placode in olfactory development was first experimentally demonstrated in amphibians when obstruction of the olfactory placode disrupted the development of the OB (Burr, 1916). The importance of NC cells in the development of the olfactory system was demonstrated in rats with a mutation in the *Pax6* gene in which impaired migration of midbrain crest cells into the frontonasal mass led to the loss of the nasal placode (Osumi-Yamashita et al., 1997). Olfactory progenitors are believed to arise from the placode (Chuah et al., 2003) but some studies have revealed they might also arise from the NC (Katoh et al., 2011).

During development, neural stem cells are important for early neurogenesis and morphogenesis of the OE (Gokoffski et al., 2010). Apical and basal progenitors divide and differentiate into various cells resulting in a pseudostratified epithelium of five basic cell types (Chuah et al., 2003; Murdoch and Roskams, 2007). These cell types are morphologically and biochemically distinguishable. From the apical surface down, sustentacular (SUS) cells, mature and immature olfactory sensory neurons (OSNs), Bowman's gland (BG) duct cells, globose basal cells (GBCs), and horizontal basal cells (HBCs) make up the OE (Fig. 1-3). In addition to the five basic cell types, microvillar cells (MVCs) have also been observed in the OE (Moran et al., 1982; Morrison and Costanzo, 1990; Miller et al., 1995). In human and other mammalian embryos, there is a dramatic increase in mitosis in the OE immediately before the first emergence of olfactory axons at stage 13 (28 days) (Chuah et al., 2003). At this stage in development, mitosis in the OE is similar to the cell division observed in the developing neural tube (Chuah et al., 2003). The epithelial cells undergo interkinetic nuclear migration (Sauer, 1937) as G1-

and S-phases take place in deeper parts of the epithelium, and the cells migrate superficially to complete mitosis at the apical epithelial surface (homologous to the ventricular surface of the neural tube) (Smart, 1971; Chuah et al., 2003). Later in development, once the basal cell layer is established, a shift of mitotic activity occurs to the base of the OE (Smart, 1971; Chuah et al., 2003). The precise relationship between the apical and basal progenitor populations has not been clarified but neurons are being generated even at times when mitoses are concentrated apically (Chuah et al., 2003).

The most detailed studies on the development of the various cell types in the OE have been done in rodents. In mice, the OE begins to form at embryonic day 10 (E10), which is shortly after the placode begins to invaginate (Cuschieri and Bannister, 1975; Cau et al., 2000; Murdoch and Roskams, 2007). Within the olfactory placode, the order and progression of pro-neural genes that regulate olfactory neurogenesis have been revealed by genetic analysis of basic helix-loop-helix (bHLH) family proteins which are expressed in GBCs (Guillemot et al., 1993; Cau et al., 1997; Cau et al., 2002). This genetic analysis has led to the current model in which a pool of rapidly proliferating transit amplifying cells expressing *Ascl1* (formerly Mash1) give rise to *Ngn1*- and *NeuroD*-positive immediate neuronal precursors (Duggan et al., 2008). While maturing GBCs that migrate from the apical surface clearly contribute to organogenesis (Cau et al., 2002), the precise role of HBCs is unclear. Small numbers of p63-expressing cells, which are thought to be HBC precursors, first appear at E14, after the emergence of all other cell types (Packard et al., 2011b). HBCs settle to the basal lamina, begin to express Keratin5 (K5), and become dormant between ~E16 and P10 (Packard et al., 2011b). Due to their late emergence, HBCs are not believed to participate in the establishment of the OE.

However, evidence from a lineage-trace mouse model suggests that upon their establishment in the OE at postnatal day 10 (P10), HBCs do participate in neurogenesis to a lesser extent (Iwai et al., 2008).

The first cells to differentiate are neurons and slightly later, there is a major transition in the appearance of the epithelium, which coincides with the shift of mitotic activity from the apical epithelium to the basal region (Chuah et al., 2003). At this time, the various cell types become morphologically distinct (Chuah et al., 2003). The OE is separated from the lamina propria (LP), the underlying region, by a basement membrane called the basal lamina (Fig. 1-3A). The developing LP contains olfactory ensheathing cell (OEC) precursors and immature OECs which ensheath OSN axons (Murdoch and Roskams, 2007). The postnatal LP contains axon bundles, OECs, connective tissue, blood vessels, and Bowman's glands (Murdoch and Roskams, 2007). The highly vascularized LP is supplied by the ethmoidal artery which is distinct from sphenopalatine supply of the respiratory mucosa (Ding and Xie, 2015a). The resulting high perfusion rates in nasal tissue allow for rapid absorption and systemic distribution of substances that penetrate the OE, however, this high perfusion can also allow for toxins in the bloodstream to come in contact with xenobiotic-metabolizing enzymes in the olfactory mucosa (Ding and Xie, 2015b).

OSNs

OSNs make up 80-85% of the epithelial cells and are densest in the posterior dorsal part of the OE (Menco and Morrison, 2003). They are comprised of mature and immature sensory neurons. Mature OSNs are identified by their expression of olfactory marker protein (OMP) (Danciger et al., 1989), and just below and blending in with the

basal cells are the immature OSNs identified by the expression of GAP43 (Verhaagen et al., 1989) and β-tubulin type III (Roskams et al., 1998).

SUS cells

The supporting cells of the OE, SUS cells, make up 12-15% of the epithelial cells and span the apical olfactory epithelium tapering basally where they attach by foot-like processes known as endfeet to the basal lamina (Menco and Morrison, 2003) and make contacts with basal stem cells (Joiner et al., 2015). They are identified by a number of antigens including SUS1 (Hempstead and Morgan, 1983) and SUS4 (Goldstein and Schwob, 1996), as well as more defined markers like keratin 18, E-cadherin (Packard et al., 2011a), and transcription factors like Sox2 and Pax6 (Guo et al., 2010; Packard et al., 2011b). SUS cells are covered with microvilli that extend into the mucus and intermingle with olfactory cilia (Menco and Morrison, 2003). They are phagocytic and like CNS glia, provide structural support (Dennis et al., 2015). The SUS cells make multiple contacts with the OSNs and may release materials in and/or absorb material from the mucus (Menco and Morrison, 2003). They express high levels of enzymes known to participate in metabolic modification of toxicants, suggesting a glia-like protective role of the neuronal environment (Dennis et al., 2015). Some of these enzymes that SUS cells express include several cytochrome P450 isoforms (Ling et al., 2004) and glutathione S-transferase (GST) (Weech et al., 2003; Whitby-Logan et al., 2004).

The Notch receptor, a component of the Notch pathway that is involved in a variety of cell fate decisions during development, is expressed in adult glia (Rodriguez et al., 2008) and in the OE, while *Notch1* is known to be expressed in basal cells, *Notch2* (Carson et al., 2006; Rodriguez et al., 2008) and *Notch3* (Rodriguez et al., 2008), are

expressed in SUS cells. Studies have suggested that maintained expression of *Notch* receptors may be required to permanently establish fate in a differentiated cell type and that transient activation of the *Notch* pathway is sufficient to promote gliogenesis (Morrison et al., 2000; Tanigaki et al., 2001). One study investigated the role of *Notch* in SUS cells and found that the loss of *Notch2* leads to a decrease in expression of *P450s* and *GST*, as well as GST activity, that is accompanied by neurodegeneration of OSNs (Rodriguez et al., 2008). These findings support the idea of SUS cells having a neuroprotective role in the OE and show that *Notch* is important for this role.

BG cells

Like SUS cells, BG cells, are involved in metabolism of xenobiotic compounds (Ding and Dahl, 2003). The acinar and duct cells of BGs contain many xenobiotic-metabolizing enzymes but their localization is species dependent (Ding and Xie, 2015a). BGs found in the LP are responsible for the mucous secretion at the epithelial surface and the glands along with their duct complex, which enters the OE, allows for the olfactory cilia to be embedded in mucous (Menco and Morrison, 2003).

MVCs

There is approximately one MVC per SUS cell in the OE, making up about 1% of the olfactory epithelial cells (Miller et al., 1995). They have neuron-like morphology and are observed in the apical part of the OE (Dennis et al., 2015). Although their function and cell-lineage is unknown, there have been several studies that attempt to address the identity and role of MVCs in the OE. Multiple types of MVCs have been observed in the OE, including 1) microvillous OSNs with an axon projecting to the OB (Moran et al., 1982)

displaying a morphology similar to that of the microvillous sensory neurons found in the vomeronasal organ that express transient receptor channel C2 (TRP C2) (Liman et al., 1999) and 2) microvillous non-OSNs that lack a basal process or axon (Carr et al., 1991). Within the non-neuronal microvillous cell populations, a subset express the TRP M5 channel (Lin et al., 2007; Hansen and Finger, 2008) or phospholipase C beta-2 (PLCβ2), TRP C6 and the type 3 inositol triphosphate receptor (IP3R3) (Elsaesser et al., 2005). A study that looked at the TRP5-expressing MVCs found that these cells also express choline acetyltransferase and are able to synthesize and potentially release Ach which modulates activities of OSNs and SUS cells (Ogura et al., 2011). Additionally, a study that focused on IP3R3-expressing MVCs found that these cells make contacts with nerve fibers and a subset are responsive to ATP, substance P and some odorants (Hegg et al., 2010). However, they do not possess neuronal or glial markers and do not project processes to the OB (Hegg et al., 2010). These findings suggest that IP3R3 MVCs might be a type of solitary chemosensory cell which respond to odorants at concentrations classified as irritants and may transfer sensory information via the nerve fibers (Hegg et al., 2010). These cells were also found to be a possible regulatory link between degenerating OSNs and olfactory stem cells (Jia and Hegg, 2012). Additional studies need to be performed to reveal the true classification and roles of MVCs.

GBCs and HBCs

The GBCs and HBCs are the stem/progenitor cell populations of the OE and make up approximately 5% of the epithelial cells. GBCs are a heterogeneous population of actively cycling cells (Jang et al., 2014), while HBCs are a relatively homogeneous population of quiescent cells (Holbrook et al., 1995; Carter et al., 2004). HBCs resemble

the basal cells of the adjacent respiratory epithelium and envelop the olfactory axons as they exit the OE (Mackay-Sim, 2003). They are identified by their expression of a variety of antigens including keratins 5 and 14 and ICAM-1 (Holbrook et al., 1995). GBCs are round cells located more superficially than HBCs that are not seen in the respiratory epithelium (Goldstein and Schwob, 1996). A type of GBC that is located above the HBC layer but extends a slender process to contact the basal lamina has been interpreted as an intermediate form between HBCs and GBCs (Graziadei and Graziadei, 1979; Holbrook et al., 1995). The functions of HBCs and GBCs will be addressed in greater detail in the next section of this chapter. Together, these cells are important for the olfactory system to properly function.

1.2.3 Olfactory Dysfunction

Olfactory disorders arise from various disturbances, having a large impact on one's quality of life. They can be classified as (i) *anosmia* (absence of smell), (ii) *partial anosmia* (ability to perceive some odorants), (iii) *hyposmia* (decreased sensitivity to odorants), (iv) *hyperosmia* (abnormally acute olfactory function), (v) *dysosmia* (distorted smell perception), (vi) *phantosmia* (sensation in absence of odor stimulus) and (vii) *presbyosmia* (absence of smell due to aging) (Murphy et al., 2003). These impairments can occur from (i) nasal passage obstruction, (ii) damage to the OE and/or (iii) damage to the central nervous system (Murphy et al., 2003).

There are numerous reported etiologies for olfactory disturbance including various drugs, endocrine or metabolic disorders, industrial debris, viral and bacterial infections, lesions of the nose or airway blockage, neoplasms, neurological disorders, nutritional or metabolic imbalances, psychiatric disorders, and pulmonary diseases (Murphy et al.,

2003). Additionally, a normal decline in olfactory function due to aging afflicts approximately 25% of the population over 65 years old and this estimation is largely underrepresented (Murphy et al., 2002). If sensory information cannot be properly detected in the OE, transduced in the OB, or processed in the cerebrum, olfactory disturbances will occur.

Disturbance to OSNs

Impairments to the OE are often associated with a loss of OSNs. Due to the contact of OSNs with damaging agents in the inhaled air, the olfactory system is vulnerable to insult. There are some defense mechanisms that the system employs in order to counteract this vulnerability. These mechanisms include 1) the detection of irritants by the nerve endings of the trigeminal nerve to trigger a flight response and change in breathing rate and/or pattern to minimize the entry of the irritant into the airways (Hastings and Miller, 2003) and 2) the metabolism of these xenobiotics through the presence of phase I and phase II enzymes – cytochrome P450s, glutathione, and related enzymes (Reed, 1993). However, these protective systems can fail and result in olfactory deficits after some toxic exposures (Hastings and Miller, 2003). Exposure to various compounds at certain concentrations can affect the histology and function of specific cells in the OE (for review, see ref. Hastings and Miller, 2003). Additionally, there is a net loss of OSNs over time due to the fact that the capacity to match OSN death with regeneration is compromised as a function of age (Conley et al., 2003). This compromise can be attributed to the dramatic decrease in basal stem cell proliferation with age, with olfactory cell proliferation decreasing ~10 to 15 times after birth during the first 3 months of life (Legrier et al., 2001; Ducray et al., 2002). Upper respiratory infections can also lead to

loss of OSNs, a decrease in OSN cilia, and respiratory infiltration (Murphy et al., 2003), which ultimately lead to olfactory dysfunction.

Disturbance to Signaling Machinery

Disturbances to smell can also be associated with dysfunction in the signaling machinery of OSNs. Channelopathies, a class of disorders that are associated with mutations in genes that encode ion channel subunits or regulatory proteins (Kass, 2005) are associated with olfactory disturbances. Mice that lack the potassium channel, $K_v1.3$, were shown to be "super-smellers" with increased discrimination of odor molecules and increased odor threshold (Fadool et al., 2004; Biju et al., 2008). This phenotype is most likely associated with the altered axonal targeting of OSNs exhibited in $K_v1.3$-null mice (Biju et al., 2008). Additionally, the loss of the sodium channel, $Na_v1.7$, in mice led to a decrease in odor-guided behaviors, odor discrimination, and innate avoidance towards predator odor (Weiss et al., 2011). The OSNs in these $Na_v1.7$-nulll mice still evoke action potentials but fail to initiate synaptic signaling to the projection neurons in the OB (Weiss et al., 2011). A more recent manifestation of diseases related to olfactory dysfunction is the group of disorders associated with a dysfunction in cilia, termed ciliopathies, which will be discussed in greater detail later in this chapter.

Blockage and Disturbance to the CNS

Nasal and sinus disease as well as intranasal and intracranial tumors can obstruct the nasal cavity leading to blockage of airflow or disturbance to the CNS resulting in a loss of olfaction (Murphy et al., 2003). Also, some viruses can infiltrate the CNS and influence central structures independent of peripheral damage (Baker and Genter, 2003).

Psychiatric disorders such as schizophrenia and chronic hallucinatory psychoses are associated with altered olfactory function affecting the CNS (Smutzer et al., 2003). Head trauma can cause olfactory dysfunction and the likelihood of anosmia is dependent on the severity of the injury (Costanzo et al., 2003). Traumatic olfactory dysfunction may be caused by: 1) sinonasal contusions or fractures without direct damage to the olfactory apparatus, 2) tearing or shearing of olfactory nerve filaments, or 3) contusion or hemorrhage within the olfactory-related brain regions (Costanzo et al., 2003).

Current Therapies

Meaningful treatments are available for some, but not all, patients with an olfactory disturbance. The prognosis for patients suffering from long-standing total loss of smell due to upper respiratory illness or head trauma is poor. There are, however, several treatments and surgical procedures to help the improvement of smell, but the prognosis for recovery seems to be better for patients with less severe olfactory dysfunction (Murphy et al., 2003). For loss of smell due to obstruction of the airway, surgery and steroid therapy can reduce the obstruction and improve the sense of smell (Murphy et al., 2003). However, there is no current treatment for congenital olfactory dysfunction or loss of smell due to malformations of the olfactory bulbs and stalks (Murphy et al., 2003). In general, olfactory dysfunction due to sensorineural causes such as upper respiratory illness or head trauma is difficult to treat. Prognosis for recovery appears to be better for patients with less severe hyposmia than for those with anosmia, which may reflect the less extensive damage into the basal layer of the epithelia and possibly less fibrosis around the cribriform plate through which the olfactory nerves pass (Murphy et al., 2003). The use of basal stem and progenitor cells as a viable therapeutic strategy has been proposed

but is still in very early stages of investigation (Krolewski et al., 2011). Furthermore, a more complete understanding of olfactory stem cell function could aid in the development of future therapies.

1.3 Adult Neurogenesis

1.3.1 Distinct Areas of Adult Neurogenesis

Stem cells have varying potencies and capabilities ranging from totipotent stem cells that can give rise to a full organism, to pluripotent stem cells that can give rise to every cell of the organism except the trophoblasts of the placenta, to multipotent stem cells that are believed to give rise to only cells of the organ from which they are derived (Gage, 2000). Neurogenesis occurs from the self-renewal and differentiation of neural stem cells (NSCs), which can give rise to cells other than themselves through asymmetric cell division (Gage, 2000). NSCs exist in the developing and adult nervous system of all mammalian organisms, however, areas of continuous adult neurogenesis have been identified in the olfactory epithelium (OE), the subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the hippocampus (Altman and Das, 1965; Gage, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002; Lie et al., 2004).

In the adult OB, neural stem cells in the SVZ, a layer of dividing cells located along the lateral wall of the lateral ventricles, proliferate and give rise to neuroblasts which then migrate in the rostral migratory pathway and differentiate into granule and periglomerular neurons (Doetsch, 2003; Lie et al., 2004). There are four main cell types in the SVZ; neuroblasts, astrocytes, immature precursors, and ependymal cells (Doetsch et al.,

1997). The dominant cell in the rodent SVZ is the dual-functioning astrocyte which can serve as both a neural stem cell (NSC) and a supporting cell that interacts with other astrocytes to promote neurogenesis (Lim and Alvarez-Buylla, 1999).

In the adult hippocampus, neural progenitors located in the SGZ, a germinal layer in the hippocampus located between the granule cell layer and hilus of the dentate gyrus, proliferate and give rise to dentate gyrus granular neurons (Doetsch, 2003; Lie et al., 2004). Foci within the SGZ contain astrocytes, which extend basal processes under the blades of the dentate gyrus and an apical process into the granule cell layer and dividing immature Type D cells, which express markers for neuronal differentiation, newly generated neurons, and endothelial cells (Palmer et al., 2000; Seri et al., 2001). As in the SVZ, SGZ astrocytes are the primary precursors of neurons and divide to generate granule neurons via Type D cells (Seri et al., 2001).

Various studies suggest that factors and mechanisms that regulate proliferation, migration, differentiation, and cell survival during development are reactivated in the injured adult environment, allowing for tissue repair and neuroplasticity (Lie et al., 2004). Transplantation studies suggest that adult neural stem/progenitor cells from different regions are not fate-restricted by intrinsic programs but by extrinsic cues derived from the local environment (Lie et al., 2004). The adult olfactory system is an example of extraordinary plasticity in its ability to respond to injury through the replacement of neurons by olfactory stem cells (Joel, 2003).

1.3.2 Constitutive Neurogenesis in the Olfactory Periphery

Early evidence that OSNs could regenerate after damage was seen in primates in 1960 (Schultz, 1960). Studies have shown that the type of repair seems to correlate to the degree or extent of initial damage (Mackay-Sim, 2003). The discovery of the co-existence of mitotic cells, immature sensory cells, mature sensory cells, and dying cells suggested that sensory cells are continually replaced (reviewed in Mackay-Sim, 2003). This hypothesis was further supported by other investigators proving that the OE is a system of renewal and regeneration (Graziadei and Graziadei, 1979; Graziadei and Monti Graziadei, 1983). This survival process allows olfactory function to continue throughout the lifetime of an organism. In the mouse, this neurogenesis occurs during three phases: embryonic establishment (E10-P0), postnatal expansion (P1-P30), and postnatal maintenance (P30-death) (Murdoch and Roskams, 2007).

GBCs and HBCs

The precise mechanisms controlling regeneration and homeostasis in the OE are unclear; however, there are several proposed models. Regeneration and neurogenesis is thought to occur through the differentiation of GBCs, HBCs, or a combination of both (Graziadei and Graziadei, 1979; Calof and Chikaraishi, 1989; Leung et al., 2007; Iwai et al., 2008; Jang et al., 2014), but there still remains controversy over which cell type is the true stem cell population. GBCs are neurogenic and bi-potential since they can produce both OSNs and SUS cells (Murdoch and Roskams, 2007). Several studies show that GBCs are neuronal precursors that exist as a heterogeneous population, compartmentalized by their expression of distinct transcription factors. In the GBC lineage cascade, *Ascl1* (*Mash1*) expression lies upstream of *Neurogenin1* (*Ngn1*) which lies upstream of, but also overlaps with, *NeuroD1* expression (Cau et al., 1997; Manglapus et

al., 2004; Packard et al., 2011a; Krolewski et al., 2013). *NeuroD1* is eventually switched off prior to the onset of postmitotic differentiation, and low levels of *NeuroD1* are sometimes found in immature OSNs (Packard et al., 2011a). HBCs are multipotent progenitors with a more quiescent “stem-cell” like behavior (Murdoch and Roskams, 2007). The transcription factor, p63, was found to be a regulator of HBC dormancy and activation and is highly expressed in the basal cell layer of skin and other stratified epithelia (Packard et al., 2011b). Additionally, the basal cell cytokeratins K5/K14 which mediate adhesion to the basal lamina and in the OE are distinctly expressed in HBCs (Holbrook et al., 1995) and are among the genes directly regulated by p63 (Packard et al., 2011a). When p63 is downregulated, HBCs are thought to be activated (Packard et al., 2011a) and can differentiate into GBCs (Leung et al., 2007), suggesting that they are GBC precursors and replenish the GBC population upon damage.

Other Possible Stem Cells

In addition to the basal cells of the OE, the human olfactory mucosa contains a stem cell known as an “ectomesenchymal” stem cell with properties of mesenchymal and neural stem cells (Delorme et al., 2010). It is grown *in vitro* from whole mucosa which includes cells of the OE and LP and has the capability of differentiating into neurons, astrocytes, oligodendrocytes, and non-neural cell types such as cardiac and skeletal muscle, chondrocytes, osteocytes, liver cells, and fat cells (Murrell et al., 2005; Murrell et al., 2008; Delorme et al., 2010; Wetzig et al., 2011). It is possible that the ectomesenchymal stem cell in the adult olfactory mucosa is derived from the ectomesenchymal stem cell that migrates out from the cranial neural crest during

embryogenesis which give rise to multiple neural and non-neural tissues of the head (Mackay-Sim et al., 2015).

Lesion Models

In order to study the restoration of the OE, transgenic mouse models are used to study the lineage of cells as they divide and differentiate into various cell types in the OE. Particularly, the use of Cre-Lox fate mapping to track genetic lineage of gene expression with lesion models of the OE has uncovered some insight to this restoration (Murdoch and Roskams, 2007). The main lesion models used include a surgical bulbectomy and a chemical/pharmacological lesion.

A bulbectomy is the removal of the OB, which in rodents induces a retrograde wave of apoptosis in OSNs within 24-36 hours of lesion (Cowan and Roskams, 2004). This apoptosis stimulates mitosis in local progenitors and OSNs are generated from adult OE-residing progenitors, with a rapid increase in the number of immature OSNs by 10 days and a return of mature OSNs by 3 weeks (Verhaagen et al., 1990). This lesion model limits axon-retargeting and allows the analysis of OSN regeneration only (Schwob et al., 1992). A chemical/pharmacological lesion leaves the OB available for retargeting, but destroys multiple cell types. Tritonx100, ZnSO4, MeBr gas and the thyroid drug methimazole are effective in inducing widespread OE cell loss and stimulating regeneration of multiple lineages (Harding et al., 1978; Schwob et al., 1995; Bergman and Brittebo, 1999). If damage is too severe, regeneration can be incomplete or respiratory epithelium can replace the OE (Jang et al., 2003) (Xie et al., 2010). After MeBr treatment, proliferation initially occurs at 1-2 days, peaks at 1 week and continues for up to 4 weeks with the OE almost fully restored by 6 weeks (Murdoch and Roskams, 2007).

Methimazole (MMI) is currently used over the more toxic MeBr and works by binding to SUS and BG cells, causing OE destruction via the formation of toxic metabolites mediated by P450s (Brittebo, 1995; Bergman et al., 2002), depleting the GBCs and sparing the HBCs in the mouse OE (Brittebo, 1995; Packard et al., 2011b).

Evidence from these lesion models so far has shown that GBCs frequently divide and, during neuronal-specific lesions, are the lead contributor to replacing OSNs through differentiation (Caggiano et al., 1994; Huard et al., 1998) (Fig. 1-4A-B). However, evidence has also shown that when there is a severe lesion that results in the death of not only OSNs, but SUS cells and often GBCs, HBCs, which are the more quiescent cell population, become proliferative and differentiate into multiple cell types to regenerate the OE (Carter et al., 2004; Leung et al., 2007; Iwai et al., 2008) (Fig. 1-4A, C). Within 18 hours of unilateral MeBr exposure, *p63* levels in HBCs are reduced in the lesioned side of the OE compared to unlesioned side and during the next day, some HBCs proliferate and lose all detectable *p63* expression while retaining keratins 5 and 14 and ICAM-1 expression (Schnittke et al., 2015). These data suggest that p63 prevents the activation and multipotency of HBCs. During olfactory neurogenesis, GBCs go through the following stages, 1) multipotent stem cells (Chen et al., 2004), 2) multipotent progenitors that can generate multiple epithelial cells in response to injury (Huard et al., 1998) and express, 3) transit amplifying cells which are committed to neurons (Guillemot et al., 1993), and 4) immediate neuronal precursors which undergo a final division and differentiate into neurons (Calof and Chikaraishi, 1989; Cau et al., 1997). Following injury, residual GBCs express *Sox2* and *Pax6* (Guo et al., 2010), when the cells are considered to be multipotent stem cells, and then go on to express *Ascl1* (formerly known as *Mash1*), as

they become transit amplifying cells, then *Neurog1* and *NeuroD1* as followed by differentiation of post-mitotic daughter cells into immature OSNs (Cau et al., 1997; Manglapus et al., 2004). While some of the transcription factors that are turned off or on during the division and differentiation of HBCs and GBCs are known, the molecular regulation of these cells, including proliferation in response to injury and cell fate determination during differentiation, remains largely unexplored (Doty, 2003b; Manglapus et al., 2004).

1.3.3 Regulation of Olfactory Neurogenesis

Various regulatory factors are thought to regulate neurogenesis of the OE including positive regulator factors that sense a need for more OSNs and stimulate mitosis and differentiation and a reduction in negative feedback from mature OSNs to inhibit additional OSN production (reviewed in Murdoch and Roskams, 2007; Mackay-Sim et al., 2015). Positive regulators include the leukemia inhibitory factor (LIF), a mitogen that contributes to proliferation in embryonic and postnatal OE *in vitro* and *in vivo*, the fibroblast growth factor (FGF), transforming growth receptor α (TGFα), and epidermal growth factor (EGF), which binds to and signals through the EGF receptor, EGFR), and are most prominently expressed in HBCs and SUS cells (reviewed in Murdoch and Roskams, 2007). Factors that negatively regulate OSN genes include TGF-β superfamily of growth factors made up of TGF-β, activins, and BMPs (Murdoch and Roskams, 2007). These proteins are highly conserved and play active roles during differentiation and development of variety of tissues and signal via a serine-threonine kinase receptor complex (reviewed in Murdoch and Roskams, 2007). Additionally, increased BMP signaling can inhibit neurogenesis and was found to induce respiratory epithelial character at the expense of

sensory epithelial character during development (Maier et al., 2010). ATP and the peptide NPY were also shown to play a role in basal cell proliferation via SUS cells (Jia and Hegg, 2010).

The Notch and Wnt signaling pathways have also been proposed to play important roles in the neurogenesis of the OE. The Notch signaling pathway regulates cell proliferation, cell fate, differentiation, and cell death (Kopan, 2012). Notch is a cell-surface receptor that transduces short-range signals by interacting with transmembrane ligands (Kopan, 2012). In the olfactory system, Notch signaling is thought to play a key regulatory role in GBC differentiation (Manglapus et al., 2004). It is known to stimulate expression of Hes1 and Hes5, proteins seen in the GBCs of recovering olfactory epithelium, and *Notch* is expressed by some GBCs (Manglapus et al., 2004). The Wnt signaling pathway plays critical roles during embryonic development and in the regeneration of adult tissues and regulates cell fate determination, motility, polarity, and stem cell renewal (reviewed in Komiya and Habas, 2008; Rodriguez-Gil and Greer, 2008; Clevers and Nusse, 2012). Wnt proteins are secreted glycoproteins that bind to the extracellular domain of the Frizzled receptor to initiate signaling (Komiya and Habas, 2008). The pathway exists as a canonical pathway which is β-catenin-dependent and a non-canonical pathway which is β-catenin-independent (Komiya and Habas, 2008). Wnt signaling is important in adult stem cells of the intestine, skin and hematopoietic system (Grigoryan et al., 2008). In the OE, Wnt signaling was found to augment GBC proliferation and prevent transit amplifying neuronal progenitor cells from further differentiation into mature OSNs (Chen et al., 2014). Additionally, Wnt proteins were seen in the OECs in the LP and in adult glomeruli indicating a role in axonal guidance (Rodriguez-Gil and Greer, 2008). Although there have

been advances in olfactory research, there are still many unanswered questions, including the precise signaling involved in neurogenesis.

1.4 Cilia: Important Signaling Antennas

1.4.1 Motile and Primary Cilia

Cilia are specialized cellular compartments or organelles found in most mammalian cell types with varying important roles. Many unicellular organisms and some specialized vertebrate cells contain multiple, motile cilia which beat in a coordinated and polarized manner for locomotion, feeding, sensation, or to drive directional fluid flow across tissues (Brooks and Wallingford, 2014). In vertebrates, including humans, most cells possess or are capable of generating single, non-motile primary cilia, which serve as critical regulators of signal transduction during development and homeostasis (Brooks and Wallingford, 2014).

The primary cilium, which was first named by Sergei Sorokin (Sorokin, 1968) is a single, non-motile organelle that projects from the cell surface of most mammalian cell types during growth arrest (Satir et al., 2010). Primary cilia act as communication scaffolds to coordinate signaling pathways that are critical in embryonic and postnatal development as well as in adult tissue homeostasis (Pedersen et al., 2008). Signaling in cilia mediate key cellular processes including cell migration, differentiation, and/or re-entry into the cell cycle, specification of the plane of cell division, and apoptosis. Cilia respond to mechanical stimulation (bending of the cilium) and chemosensation (detection of a specific ligand, growth factor, hormone, or morphogen) (Satir et al., 2010).

Development and Maintenance of Cilia

The assembly of primary cilia is tightly coupled to the cell cycle. It occurs from the distal end of the mother centriole as cells enter growth arrest and then sheds shortly before cells enter mitosis (Quarmby and Parker, 2005; Plotnikova et al., 2009). During interphase of the cell cycle, a modified centriole migrates to the plasma membrane and attaches to a Golgi-derived vesicle forming the basal body which templates the nucleation of the axoneme, the structural core of the cilium (Sorokin, 1968). The basal body is composed of 9 sets of microtubules and serves as the microtubule organizing center (MTOC) from which axonemal tubules project (Burton, 1992). These specialized structures result in various functions in the different cell types. Most motile cilia have an additional central microtubule pair resulting in a 9+2 configuration while primary cilia are usually immotile and lack a central pair resulting in a 9+0 configuration with the exception of primary motile cilia present on the embryonic node (Singla and Reiter, 2006). In contrast to those of motile 9+2 cilia, the axonemes of non-motile primary cilia lack key elements involved in ciliary motility, including the central pair of microtubules and the outer and inner dynein arms that power microtubule sliding to produce motility (Satir and Christensen, 2007).

Assembly of the axoneme is mediated by intraflagellar transport (IFT) (Pedersen et al., 2008). IFT was first described in the alga *Chlamydomonas reinhardtii* (Kozminski et al., 1993) and is essential for the construction and maintenance of cilia in all species (Singla and Reiter, 2006; Pedersen et al., 2008). Its basic mechanisms are widely conserved not only between cilia types but also often between species (Sung and Leroux, 2013). Since cilia lack the necessary components for protein synthesis, structural

components of the cilium and cargo must be synthesized in the cell and carried into cilia through the IFT system (McEwen et al., 2008). IFT is a bidirectional motility system localized between the outer doublet microtubules and the ciliary membrane by which particles are moved into and out of the cilium by molecular motors (Rosenbaum and Witman, 2002): 1) a kinesin motor responsible for anterograde transport toward the distal plus end of the cilium, and 2) a dynein motor that mediates retrograde transport of cargo out of the cilium and back into the cell (Hirokawa et al., 1998) (Fig. 1-5). Motors associate with 2 distinct complexes of IFT proteins: complexes A and B, which comprise a set of highly conserved proteins (Cole et al., 1998; Cole, 2003) (Fig. 1-5). Besides IFT complex proteins, there are a number of other proteins involved in cilia establishment, maintenance, and particle entry and movement. The BBSome (Loktev et al., 2008; Scheidecker et al., 2014) is a group of eight conserved proteins affected in human Bardet-Beidl syndrome (BBS) (Zaghloul and Katsanis, 2009), a pleiotropic disorder characterized by obesity, polydactyly, retinal dystrophy, and cystic kidneys. It is proposed to interact with IFT complexes and participate in particle entry and movement (Liew et al., 2014; Williams et al., 2014). When some of these ciliary proteins are defective, they are a major cause of human diseases and developmental disorders known as ciliopathies (Veland et al., 2009).

Growing Roles of Cilia and Disease

Defects in cilia function lead to various human diseases, termed ciliopathies, which are a diverse class of congenital diseases with 23 recognized syndromes caused by mutations in at least 89 different genes (van Reeuwijk et al., 2011). These diseases are highly pleiotropic and the degree of penetrance can vary between patients (McIntyre et

al., 2013). Various impairments can occur including blindness, hearing loss, altered nociception, and anosmia as well as renal and liver cysts, airway distress, and hydrocephaly (reviewed in van Reeuwijk et al., 2011)

Evidence that dysfunction of non-motile cilia play a role in human disease was first considered after the orthologous protein of human polycystin-1 (PC1), which is mutated in autosomal dominant polycystic kidney disease (ADPKD) type 1, was shown to be expressed in ciliated neurons of *C. elegans* (Barr and Sternberg, 1999). Proteins of mutated genes in cystic kidney diseases were later found to localize to primary cilia, including the IFT protein IFT88/Polaris (Pazour et al., 2002b). The significance of primary cilia in signaling became clear upon examination of a hypomorphic mutant mouse, *Tg737*^{orpkRpW}, which is deficient in the homolog of IFT88 (Lehman et al., 2008). The mouse was developed as a model for human autosomal recessive polycystic kidney disease (ARPKD) because they eventually developed polycystic kidneys. It was found that upon development of ARPKD in these mice, like other models of the disease, PC1, PC2 and the transient receptor potential (TRP) Ca^{2+} channel protein complex were mislocalized or absent from primary cilia (Pazour et al., 2002a; Yoder et al., 2002). These mice were also later found to be anosmic (McIntyre et al., 2012). Another complex of recessive cystic kidney diseases is nephronophthisis (NPHP) which results from defects in the genes that encode for Nephrocystin (Nphp) 1-9 (reviewed in Veland et al., 2009). Other ciliopathies include Joubert syndrome in which mutations in *ARL13B* were found in patients (Cantagrel et al., 2008) and Meckel syndrome, in which mutations in *NPHP* were found (Craige et al., 2010).

Cilia and Olfactory Dysfunction

Olfactory dysfunction due to ciliary defects can occur by 2 separate mechanisms: 1) a complete loss of olfactory cilia and 2) a defect in protein trafficking leading to a loss in olfactory signaling (McEwen et al., 2008). The first documented case of a human patient with anosmia presumably due to ciliary defects was in 1975 in which the patient had OSNs devoid of cilia (Douek et al., 1975). Since then, olfactory deficits were shown to occur in 2 different pleiotropic diseases, BBS and Leber Congenital Amaurosis (LCA). There have been observations that patients with BBS are frequently unable to smell (reviewed in Fliegauf et al., 2007). Mice that lack BBS1 or BBS4 have a severely reduced olfactory ciliated border and defects in the highly specialized ciliated dendritic knobs (Kulaga et al., 2004). Additionally mice that lack BBS8 also show decreased olfactory function (Tadenev et al., 2011). LCA is a congenital retinal dystrophy and can occur due to mutations in several proteins of varying function, including mutations in the centrosomal/basal body protein CEP290 (reviewed in McEwen et al., 2008). Mutations in CEP290 resulted in severely impaired olfactory function despite a self-described normal sense of smell which was due to mislocalization of G-proteins rendering the signaling pathway nonfunctional (McEwen et al., 2007).

1.4.2 Cilia in Adult Neurogenesis and Tissue Homeostasis: Implications in Signaling Pathways

Recent research has shown a growing role for cilia in stem cell proliferation and differentiation. Primary cilia are required for the formation of adult neural stem cells in the hippocampal denate gyrus during development, and also play an essential role in the initiation of mesenchymal stem cell differentiation (Tummala et al., 2010; Amador-Arjona et al., 2011). There is also evidence that human embryonic stem cells (hESCs) possess

primary cilia, which contain important components of the HH pathway (Kiprilov et al., 2008). Quiescent stem cells in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the denate gyrus were shown to arise in late embryonic stages and respond to SHH during their differentiation into various cell types (Ahn and Joyner, 2005). Additionally, primary cilia were found to be required for proper proliferation and neurogenesis in a specific subregion of the postnatal ventricular-SVZ as well as for proper HH signaling (Tong et al., 2014).

In regards to differentiation, cilia play important roles in various signaling pathways essential for proper cell differentiation during development and adult homeostasis. These pathways include Platelet-Derived Growth Factor (PDGF), Sonic Hedgehog (SHH) and Wnt (reviewed in Gerdes et al., 2009), which is important in adult stem cells of the intestine, skin and hematopoietic system (Grigoryan et al., 2008). In the adult skin, a loss of IFT88 from basal cells resulted in proliferation of cells in the interfollicular epidermis but not the follicle, and an overall abnormal differentiation in skin homeostasis (Croyle et al., 2011). Conversely, in the hippocampus, when cilia were removed postnatally from neural stem cells (NSCs) via genetic deletion of the IFT gene, *Ift20*, the number of neural stem cells (NSCs) remained the same in mutants compared to controls with an overall reduction in proliferation in the SVZ and a reduction in neurogenesis (Amador-Arjona et al., 2011). In the adult kidney of mice, loss of the kinesin motor, Kif3a, resulted in cyst formation and increased proliferation following an injury (Sharma et al., 2013).

Mice with defective IFT proteins and mutations in BBS genes exhibit phenotypes resembling those observed in mutants of the non-canonical Wnt pathway (reviewed in Goetz and Anderson, 2010), which is also known as the polar cell polarity (PCP)

pathway (Klein and Mlodzik, 2005). The primary cilium was proposed to act as a switch between canonical and non-canonical Wnt signaling pathways but the connection between cilia and Wnt signaling is controversial (Corbit et al., 2008; Huang and Schier, 2009). The PCP pathway is a conserved signaling pathway for coordinated polarization of cells within the plane of an epithelial cell layer (reviewed in Hildebrandt et al., 2011). Core planar cell polarity proteins are sorted asymmetrically along the polarization axis and the protein IFT88/Polaris is required for establishing epithelial planar cell polarity (Jones et al., 2008). Proper positioning of ciliary basal bodies and the formation of polarized cellular structures are disrupted in mice with mutant ciliary proteins (reviewed in Hildebrandt et al., 2011). The HH pathway, which will be discussed in greater detail in the next section of this chapter, has been implicated in the maintenance of stem/progenitor cells in many adult tissues (reviewed in Dessaud et al., 2008; Balbuena et al., 2011; Petrova and Joyner, 2014). Further investigation of these signaling pathways in the OE could answer many open questions pertaining to the regulatory signaling that occurs during neurogenesis and restoration of the OE.

1.5 The Hedgehog (HH) Signaling Pathway

1.5.1 Canonical HH Signaling Mechanisms

The Hedgehog (HH) signaling pathway is critical for normal embryonic development and plays important roles in adult tissue maintenance. HH was first discovered in a genetic screen aimed to understand the body segmentation of *Drosophila* (Nusslein-Volhard and Wieschaus, 1980), in which mutant embryos developed were

phenotypically reminiscient of a Hedgehog animal, due to the lack of segment boundaries. The core components of the HH pathway were also initially identified in *Drosophila* (Nakano et al., 1989; Alcedo et al., 1996) and are conserved in vertebrates (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Goodrich et al., 1996). The HH ligand is first synthesized as a 45kDa precursor that undergoes autocatalytic cleavage to generate a 19kDa processed form that is modified by the covalent attachment of a cholesterol molecule to the C-terminus (Porter et al., 1996) and addition of palmitate, a saturated fatty acid, (Chamoun et al., 2001) to the N-terminus in order to attain its final secreted form. In *Drosophila* and in mammals, the protein, Dispatched, is specifically required for the controlled release of the modified HH ligand (Burke et al., 1999). When HH is secreted, it acts in a concentration- and time-dependent manner to initiate various cell responses such as cell survival, proliferation, and cell fate specification and differentiation.

HH signal transduction is initiated at the plasma membrane of HH-responsive cells where it interacts with its 12 transmembrane protein receptor, Patched (Ptc in Drosophila; Ptch1 in vertebrates) (Chen and Struhl, 1996; Marigo et al., 1996; Stone et al., 1996) which is facilitated by the Ihog/Cdo family of co-receptors (McLellan et al., 2006; Tenzen et al., 2006; Yao et al., 2006). The binding of HH to Patched sequesters HH by internalizing the Patched/HH complex and targeting it to lysosomes for degradation (Gallet and Therond, 2005). This event also increases the expression and activation of Smoothened (Smo) (Chen and Struhl, 1996; Denef et al., 2000; Ingham et al., 2000) via a conformational change (Fan et al., 2012) which results in the downstream signaling cascade that functions to regulate the HH transcription factors.

In mammals, there are three paralogues of HH genes (Echelard et al., 1993): Sonic HH (SHH, the most broadly expressed and best studied HH molecule), Indian HH (IHH, primarily involved in bone differentiation) and Desert HH (DHH, involved in gonad differentiation). Two major differences between HH signaling in *Drosophila* and vertebrates include: 1) while there is a single ancestral gene encoding the transcription factor, *Ci*, in *Drosophila,* three genes comprise this family (*Gli1*, *Gli2*, and *Gli3*) in vertebrates, and 2) the requirement in vertebrates for intraflagellar transport (IFT), which is necessary for the establishment and maintenance of cilia. The first indication of a potential role for cilia in mammalian HH signaling emerged from genetic screens in the mouse which identified mutations in IFT genes that produced patterning defects similar to those of mutations in genes encoding HH pathway components (Huangfu et al., 2003). Both PTCH1 and SMO can localize to primary cilia in a mutually exclusive way, where the binding of HH ligand to PTCH1 allows SMO to move into the cilium where it accumulates and promotes pathway activation through the HH transcription factors (Rohatgi et al., 2007) (Fig. 1-6). The transcription factors then undergo various processing events to either inhibit or activate the pathway (Fig. 1-6).

1.5.2 GLI Processing in HH Signaling

The GLI family of transcription factors mediates most vertebrate HH signaling and it is proposed that cilia are required for the differential regulation of these transcription factors (Caspary et al., 2007). GLI proteins localize to primary cilia and require intact IFT for proper processing and function (Haycraft et al., 2005; Liu et al., 2005). Disruption of the HH pathway results in aberrant cell growth, differentiation and migration with a tissue homeostatic imbalance leading to severe disorders (Mimeault and Batra, 2010). When

the IFT protein, IFT88, and cilia are disrupted, SMO translocation and GLI processing is perturbed, resulting in HH-specific phenotypes (Huangfu et al., 2003; Haycraft et al., 2005; Goetz and Anderson, 2010).

HH signaling results in a change in the balance between the activator and repressor forms of the Ci and GLI proteins by regulating their post-translational proteolytic processing (Fig. 1-7). All GLI and Ci proteins have a Zn-finger DNA-binding domain (Kinzler et al., 1988) and a C-terminal activation domain, while only Ci, GLI2 and GLI3 contain an N-terminal repressor domain (Sasaki et al., 1999; Briscoe and Therond, 2013) (Fig. 1-7). GLI1 has diverged evolutionarily, lacking a transcriptional repressor domain (Park et al., 2000). The post-translational regulation of Ci and GLI proteins is similar, however, each GLI protein has a unique role.

In *Drosophila*, in the absence of HH ligand, the C-terminal domain of Ci undergoes sequential phosphorylation on multiple sites, first by PKA and then by glycogen synthase kinase 3β (GSK3β) and different members of the casein kinase I (CKI) family (reviewed in Hui and Angers, 2011). These events lead to the recruitment of an E3 ubiquitin ligase complex (Jia et al., 2005; Smelkinson and Kalderon, 2006) and ubiquitylation of specific residues targets the modified protein to the proteasome, where the C-terminal transactivation domain is removed by partial degradation (Briscoe and Therond, 2013). The remaining protein, which comprises the N-terminal repressor domain and DNA-binding domains, translocates to the nucleus, binds genomic target sites and represses transcription. In the presence of HH ligand, activation of SMO inhibits the phosphorylation and partial proteolytic processing of Ci, allowing full length Ci to act as a transcriptional activator.

In vertebrates, in the absence of HH ligand, GLI proteins are retained in the cytoplasm by the kinesin-4 protein, KIF7, and suppressor of fused, SUFU, to limit transcriptional activation (Kogerman et al., 1999; Merchant et al., 2004). Like Ci, GLI2 and GLI3 require PKA and an E3 ubiquitin ligase for processing to a transcriptional repressor, but GLI1 functions solely as a transcriptional activator (Hooper and Scott, 2005). Mouse mutant studies suggest that GLI2 is chiefly responsible for the activator function in response to HH signaling, and GLI3 is responsible for the repressor activity (Matise et al., 1998; Litingtung and Chiang, 2000; Wang et al., 2000a; Pan et al., 2006). However, there are instances when GLI2 acts as a repressor (Pan et al., 2006) and GLI3 acts as an activator as seen in the developing somite (Buttitta et al., 2003). Additionally, GLI3 has been shown to act as an activator in spinal cord patterning (Bai et al., 2004). *Gli1,* but not *Gli3* is transcriptionally activated by SHH signaling which creates a positive-regulatory feedback loop (Lee et al., 1997). In mammals, GL1 seems to have only a minor role in amplifying the transcriptional response (Park et al., 2000). It is believed that GLI2 and GLI3 are the primary transducers of HH signaling and GLI1 plays a secondary role since its expression is transcriptionally regulated by GLI2 and GLI3 (Bai et al., 2004) and *Gli1*$^{-/-}$ mice are viable (Park et al., 2000).

1.5.3 HH Signaling in Adult Neurogenesis and Tissue Homeostasis

HH acts as a long-range morphogen to control cell patterning and differentiation in several embryonic tissues (Jiang and Hui, 2008). In addition to its role in normal embryonic development, HH also plays important roles in adult tissue homeostasis. In the adult, HH continues to signal to and regulate several populations of stem and progenitor cells within various organs, including the brain (Lai et al., 2003; Machold et al., 2003; Ahn

and Joyner, 2005; Ihrie et al., 2011), skin, prostate, and bladder and others (reviewed in Petrova and Joyner, 2014). It is also required for the maintenance of stem cells in a range of tissues, from the hair follicle to the haematopoetic system, and is involved in the injury-dependent regeneration of many organs, including the exocrine pancreas, prostate, and bladder (Beachy et al., 2004; Shin et al., 2011; Lim et al., 2014).

Neural Stem Cells

As mentioned in Section 1.3, in the adult mammalian brain, new neurons are generated from transit-amplifying cells (TACs) that are derived from quiescent and self-renewing neural stem cells (NSCs) located in the SVZ of the lateral ventricles and the SGZ of the hippocampal dentate gyrus (DG). *In vivo* evidence that SHH is required for the establishment of stem and progenitor cells in the SVZ and SGZ has come from conditional genetic loss-of-function studies. When HH genes or cilia are abrogated midgestation, there is a severe depletion of progenitors in the olfactory bulb and DG, suggesting that HH signaling is required for the initial establishment of NSCs (Balordi and Fishell, 2007a; Han et al., 2008). HH signaling has also been shown to be required for the continuous maintenance of neurogenesis in the SVZ and SGZ. Studies using small molecules showed that SHH gain or loss of function increases or inhibits proliferation respectively in neurogenic regions and neural stem/progenitor cells (reviewed in Petrova and Joyner, 2014). Inhibition of the pathway by removal of SMO in the majority of adult SVZ NSCs results in reduced neurogenesis (Balordi and Fishell, 2007b; Petrova et al., 2013) while over-activation of the pathway by deletion of *Ptch1* promoted NSC self-renewal at the expense of transit amplifying progenitors and neuron production (Ferent et al., 2014). This reduction in neurogenesis was linked to a shift in NSC division from

asymmetric to symmetric potentially through the induction of NOTCH signaling (Ferent et al., 2014).

Hair and skin

There are multiple populations of lineage-restricted stem cells in the adult mammalian skin (reviewed in Solanas and Benitah, 2013). Like the adult brain, SHH is the main HH ligand present in postnatal skin (Petrova and Joyner, 2014). Mitotically active cells in the epithelial basal layer of the skin constantly produce new interfollicular skin cells (Petrova and Joyner, 2014). During the expansion (anagen) phase of the hair cycle, *Shh* expression is seen in the epithelial cells of the lower end of the hair follicle while the downstream effectors *Gli1* and *Ptch1* are more broadly expressed (Oro and Higgins, 2003). During the resting (telogen) phase when old hairs degenerate and die, *Gli2* and *Gli3* are broadly expressed in both the follicle and in the surrounding dermis (Petrova and Joyner, 2014), while *Gli1* and *Ptch1* expression is restricted to two distinct epithelial stem cell domains in the upper bulge and the lower bulge (Brownell et al., 2011). Inhibition of SHH with the antibody 5E1 in mice blocks the anagen progression and hair re-growth, suggesting that SHH is required for the regenerative function of adult stem cells (Wang et al., 2000b). Conversely, exogenous expression of SHH via adenoviral infection triggers the onset of the anagen phase in resting hair follicles and stimulates hair growth (Sato et al., 1999). Additionally, removal of *Shh* in the hair germ causes defects in proliferation throughout the hair follicle while genetic abrogation of *Smo* or *Gli2* alone in the stem cells reduced their proliferative abilities but did not affect anagen progression, suggesting that SHH is required to maintain the function of hair follicle stem cells but not of the progenitor cells in which it is expressed (Hsu et al., 2014).

Additionally, SHH signaling gain of function mutations are found in human basal cell carcinoma (BCC) (Hahn et al., 1996; Johnson et al., 1996; Reifenberger et al., 1998). Overexpression of *Gli1* and to a lesser extent, *Gli2* are common features of BCC (Dahmane et al., 1997; Grachtchouk et al., 2000; Oro and Higgins, 2003; Hutchin et al., 2005). SMO gain of function or PTCH1 loss of function mutations also give rise to BCC-like lesions when induced either in the mouse interfollicular epidermis or in hair follicle stem cells that move into the interfollicular skin following injury (reviewed in Petrova and Joyner, 2014), suggesting these stem cells can function as tumor-initiating cells for BCC.

Teeth

Rodent incisors continue to grow throughout the life of the animal and require constant repair via stem and progenitor cells that produce amelioblasts to renew the incisor epithelium (reviewed in Petrova and Joyner, 2014). During the growth of the adult rodent incisor, SHH signals to parental *Gli1*-expressing amelioblast stem cells (Seidel et al., 2010). Inhibition of SHH signaling leads to a decrease in amelioblast production and tooth growth but does not deplete the GLI1+ stem cell population (Seidel et al., 2010), suggesting that SHH signaling is not required for stem cell survival but instead for maintaining the ability of stem cells to expand the amelioblast population. Although human teeth do not grow continuously, a multipotent stem cell population within the connective tissue surrounding the tooth was shown to express *SHH, GLI1,* and *PTCH1* (Martinez et al., 2011).

Bone

Indian hedgehog (IHH) is one of the main regulators of chondrocyte proliferation and osteoblast differentiation during development (reviewed in Petrova and Joyner, 2014). Throughout adulthood, HH signaling continues to contribute to maintenance of the bone as administration of the SMO inhibitor, cyclopamine, to adult mice resulted in decreased bone mass and conversely, *Ptch1*$^{+/-}$ mutant mice exhibit a high bone mass density and Ptch1$^{+/-}$ cultured cells showed osteoblast differentiation associated with a loss of GLI3 repressor generation (Ohba et al., 2008).

Respiratory and gastrointestinal

Localized upregulation of HH signaling occurs in response to an injury in the adult lung airways (Petrova and Joyner, 2014). Initially, in the normal adult mouse lung, only a few fibroblasts around the airway express *Gli1*; however, upon lung fibrosis or airway injury, HH signaling is upregulated as seen by an increase in stromal GLI1+ cells (Watkins et al., 2003; Liu et al., 2013b). Additionally, overexpression of *Shh* in the normal adult mouse airway epithelium can induce cell proliferation and lung tissue modifications similar to those seen in injury (Krause et al., 2010).

Shh and *Ihh* are continuously detected throughout the adult gastrointestinal tract of both humans and rodents where it signals to the *Gli*-expressing mesenchyme (reviewed in Petrova and Joyner, 2014). In the stomach of the adult mouse, HH signaling inhibits proliferation (van den Brink et al., 2001) and stimulates the differentiation (van den Brink et al., 2002) of the gastric epithelium. Additionally, the loss of IHH in the intestinal epithelium of the mouse results in a wound healing response in which there is epithelial proliferation and an ultimate loss of smooth muscle and villi (van Dop et al., 2010; Zacharias et al., 2011). When isolated mesenchyme from the intestine was treated

with SHH or IHH ligand, smooth muscle precursors decreased in number and smooth muscle cells increased in number (Zacharias et al., 2011), suggesting that HH signaling is important for smooth muscle differentiation. Overall, these studies propose that HH signaling in the intestine of the adult mouse regulates tissue homeostasis in a concentration-dependent manner.

Taste

The adult fungiform taste papilla is comprised of specialized cell types that reside in a stratified squamous tongue epithelium. This sensory organ contains taste buds, papilla epithelium, and lateral walls that extend into underlying connective tissue to surround a core of lamina propria cells. The taste buds continue to differentiate and turn over postnatally throughout the life span (Beidler and Smallman, 1965; Farbman, 1980). A few studies have suggested that SHH signaling is involved in the maintenance and differentiation of taste cells. SHH ligand is produced by taste bud cells for paracrine signaling to HH-responsive cells in the basal epithelial layer, perigemmal cells, and stromal cells of the papilla and was found that these *Shh*-responding cells contribute to the maintenance of not only filiform and fungiform papillae, but also taste buds (reviewed in Liu et al., 2013a). Additionally, constitutive activation of GLI2 in the tongue epithelium led to a rapid loss of fungiform papillae and taste buds (Liu et al., 2013a), while ectopic expression of SHH induced the formation of taste buds in regions not capable of generating taste buds (Castillo et al., 2014). It was also shown that pharmacological blockade of the HH pathway with the SMO inhibitor, LDE-225, disrupted taste organs including the fungiform papillae and taste buds, and electrophysiological responses to all

taste qualities in mice (Kumari et al., 2015), suggesting that HH signaling is a principal regulator of papilla and taste bud maintenance and taste sensation.

Olfactory Periphery

HH signaling in the olfactory periphery has been limited to a few studies in *Drosophila* and rats and remains largely unexplored. In *Drosophila*, HH signaling was found to play a role in OSN axon targeting that is dependent on Smo and Ihog, a co-receptor for HH ligands (Chou et al., 2010). Differential HH activity in peripheral sensory organ precursors in larva and early pupa creates OSN populations with different levels of Ptc: low-Ptc class and high Ptc class. Genetic knockdown and overexpression experiments showed that removing HH from the periphery results in loss of Ptc expression in high-Ptc OSNs which releases Smo inhibition and causes axon mistargeting similar to loss of Ptc, while brain-derived Hh is required for low-Ptc classes (Chou et al., 2010). Additionally, a recent study suggested a novel cilia-dependent role for Hh signaling in *Drosophila* that occurs in the cilia of OSNs, however, there was no evidence for the role of Hh in the OSNs (Kuzhandaivel et al., 2014). One study investigating HH signaling in the mammalian olfactory periphery found that: 1) *Shh* mRNA is expressed in mitral and tufted cells of the olfactory bulb and 2) cultured OSNs, when exposed to SHH protein, exhibit an increase in axonal branching (Gong et al., 2009), suggesting a role for HH signaling in axon branching of mammalian OSNs. This same study showed transcripts of several HH pathway components not only in the olfactory bulb, but also in the olfactory periphery, suggesting a potential role for HH signaling the adult mammalian olfactory periphery.

1.6 Conclusions

Olfaction is an important part of our everyday life and has evolved to have a large influence on our taste, memory, and behavior. Odorant detection occurs in the olfactory epithelium (OE) which is comprised of various cell types including olfactory sensory neurons (OSNs), which are necessary for the detection of smell, and the stem/progenitor cell populations, globose basal cells (GBCs) and horizontal basal cells (HBCs) (for review, see ref. Murdoch and Roskams, 2007). OSNs are unique in that they both directly contact the external environment and the brain (Doty, 2008), which exposes them and other cells in the OE to insults from toxins, bacteria, and viruses leading to cell death (Duggan and Ngai, 2007). It is known that GBCs and HBCs participate in the restoration of the OE following injury and tissue homeostasis injury (Barber, 1982; Costanzo, 1991; Iwai et al., 2008). However, olfactory dysfunction can still arise from a variety of etiologies. Treatment for more severe cases remains under investigation.

While the cell types involved in the maintenance and regeneration of the OE are known, the signaling pathways that control their activation and proliferation during normal homeostasis, and in response to injury remain largely unexplored. Various signaling factors and pathways have been investigated in the olfactory system including the involvement of Wnt and Notch signaling in the proliferation of GBCs (Cau et al., 2002; Manglapus et al., 2004; Wang et al., 2011; Krolewski et al., 2012; Chen et al., 2014). However, there has been no clear answer to which factors and pathways are directly involved in the regulation (cell division, differentiation, and fate determination) of the stem/progenitor cells, specifically HBCs. The central focus of this dissertation is to

investigate specific factors involved in regulating the activity of HBCs and their contribution to neurogenesis in the OE following an injury.

Cilia have become greatly associated with stem cells in the regulation of their dormancy, proliferation, and differentiation as well as proper adult neurogenesis (reviewed in Irigoin and Badano, 2011). In Chapter II, I provide evidence of primary cilia on HBCs and indicate that OSNs are not the only ciliated cell type in the OE. I use an HBC-specific inducible system to delete cilia specifically from HBCs in order to assess the effects that a loss of HBC cilia have on OE development, homeostasis, and recovery following an injury. Disruption of cilia in systems in which cilia are important for cell proliferation and differentiation alters the activity of signaling pathways that utilize cilia, such as the HH pathway (Kumamoto et al., 2012; Tong et al., 2014). The HH pathway is not only important for the development of various tissues and organs, but also for the formation, survival, and function of stem and progenitor cell populations in several adult tissues (reviewed in Petrova and Joyner, 2014). It is an attractive candidate for regulation of HBCs due to its dependence on cilia in tissue maintenance (Croyle et al., 2011). Although HH signaling has been investigated in the SVZ and olfactory bulb (Balordi and Fishell, 2007a), and in OSN axon branching and targeting (Gong et al., 2009), it is virtually unexplored in the mammalian OE. In Chapter III, I use *lacZ* reporter mice to determine expression profiles of HH components in the OE. Additionally, I utilize an HBC-specific inducible system to target specific components of the HH pathway in order to assess the effects that HH signaling has on OE homeostasis and regeneration following an injury.

1.7 Figures

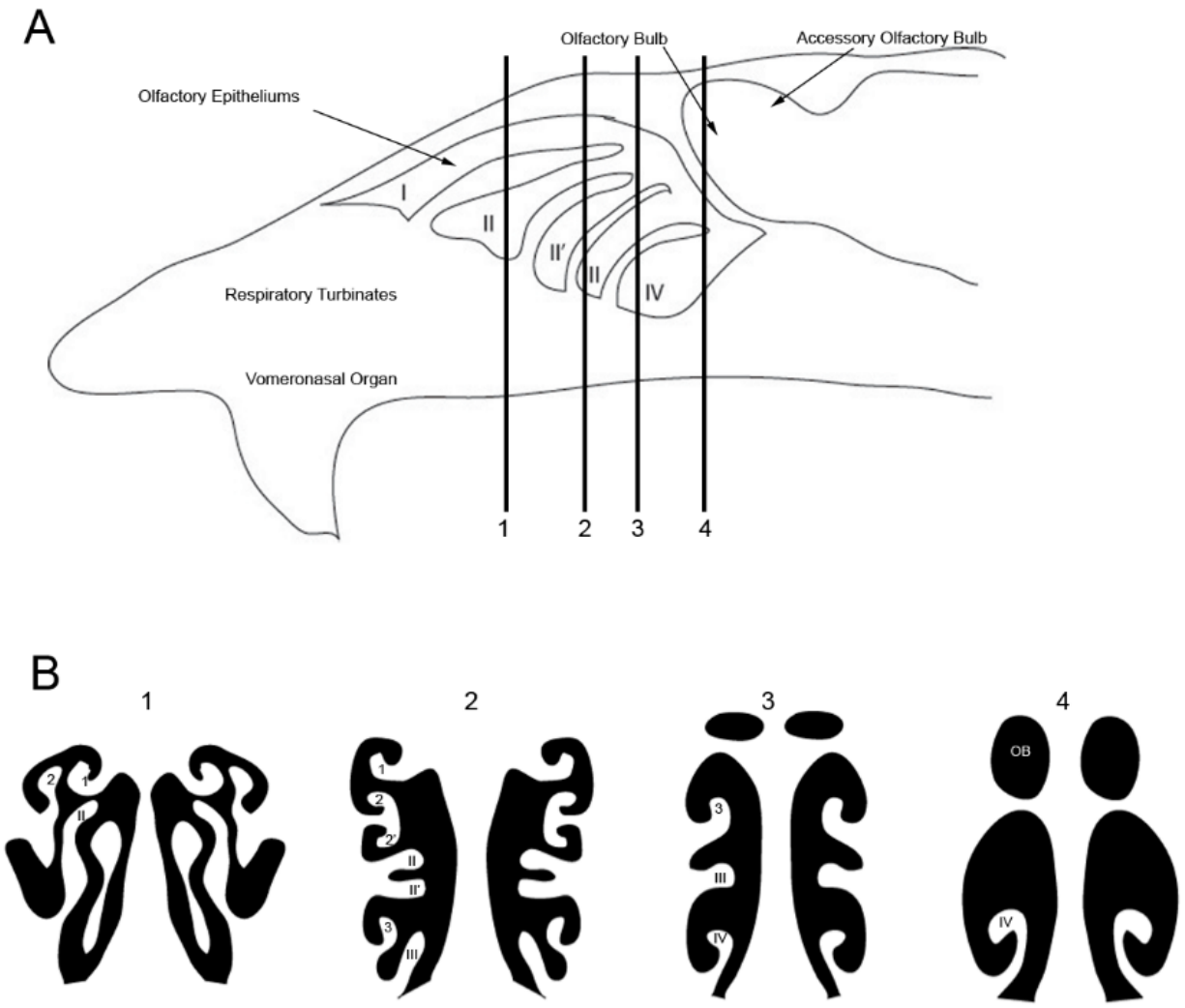

Figure 1-1. Rodent olfactory system. Schematic of the rodent olfactory system adapted from Adam C. Puche. ***A,*** Sagittal view of the mouse head depicts olfactory turbinates I-IV, olfactory bulb, and accessory olfactory bulb. ***B*****,** Resulting coronal sections from hypothetical cuts 1-4. Roman numerals represent endoturbinates and numbers represent ectoturbinates.

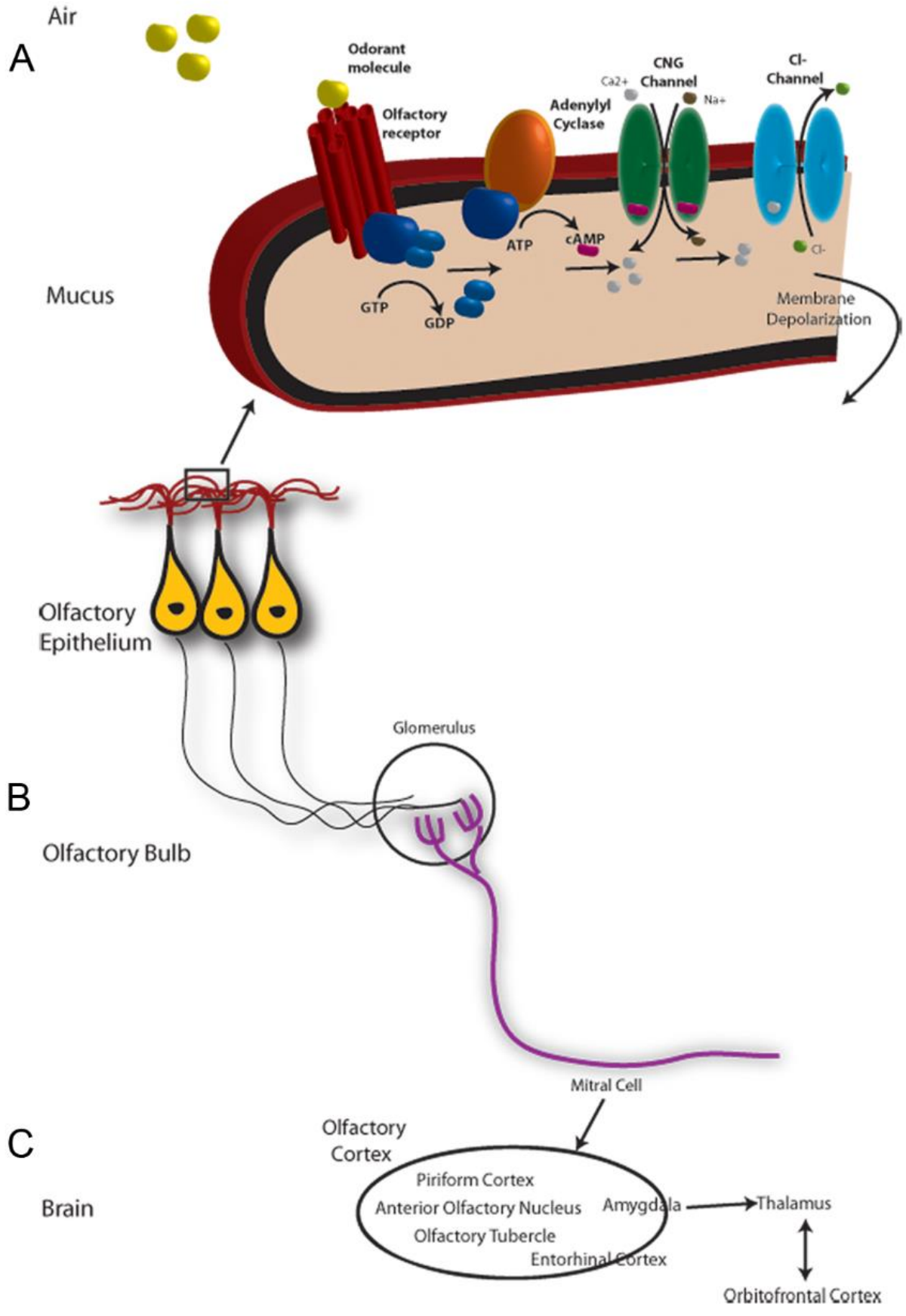
Air
A
Odorant molecule
Olfactory receptor
Adenylyl Cyclase
Ca2+
CNG Channel
Na+
Cl- Channel
Cl-
ATP
cAMP
GTP
GDP
Mucus
Membrane Depolarization
Olfactory Epithelium
Glomerulus
B
Olfactory Bulb
Mitral Cell
C
Olfactory Cortex
Piriform Cortex
Anterior Olfactory Nucleus
Amygdala
Thalamus
Olfactory Tubercle
Entorhinal Cortex
Brain
Orbitofrontal Cortex

Figure 1-2. Olfactory transduction pathway. Schematic of the olfactory transduction pathway. ***A,*** All olfactory signaling machinery is found in the cilia of olfactory sensory neurons (OSNs). Upon an odorant binding to its corresponding olfactory receptor (OR), the G-protein, $G\alpha_{olf}$, is activated resulting in the activation of adenylyl cyclase II (ACIII) and production of cyclic AMP (cAMP). This increase in cAMP opens cyclic nucleotide-gated (CNG) channels leading to an influx of sodium (Na^+) and calcium (Ca^{2+}) ions and depolarization of OSNs. This depolarization is further amplified by activation of calcium-activated chloride (Cl^-) channels. ***B*****,** OSNs project axons to corresponding structures in the olfactory bulb (OB) known as glomeruli, where they form synapses with dendrites of mitral and tufted cells. ***C,*** These projection neurons extend their axons to the olfactory cortex which is comprised of several areas including the piriform cortex, olfactory tubercle, anterior olfactory nucleus, and specific parts of the amygdala and entorhinal cortex.

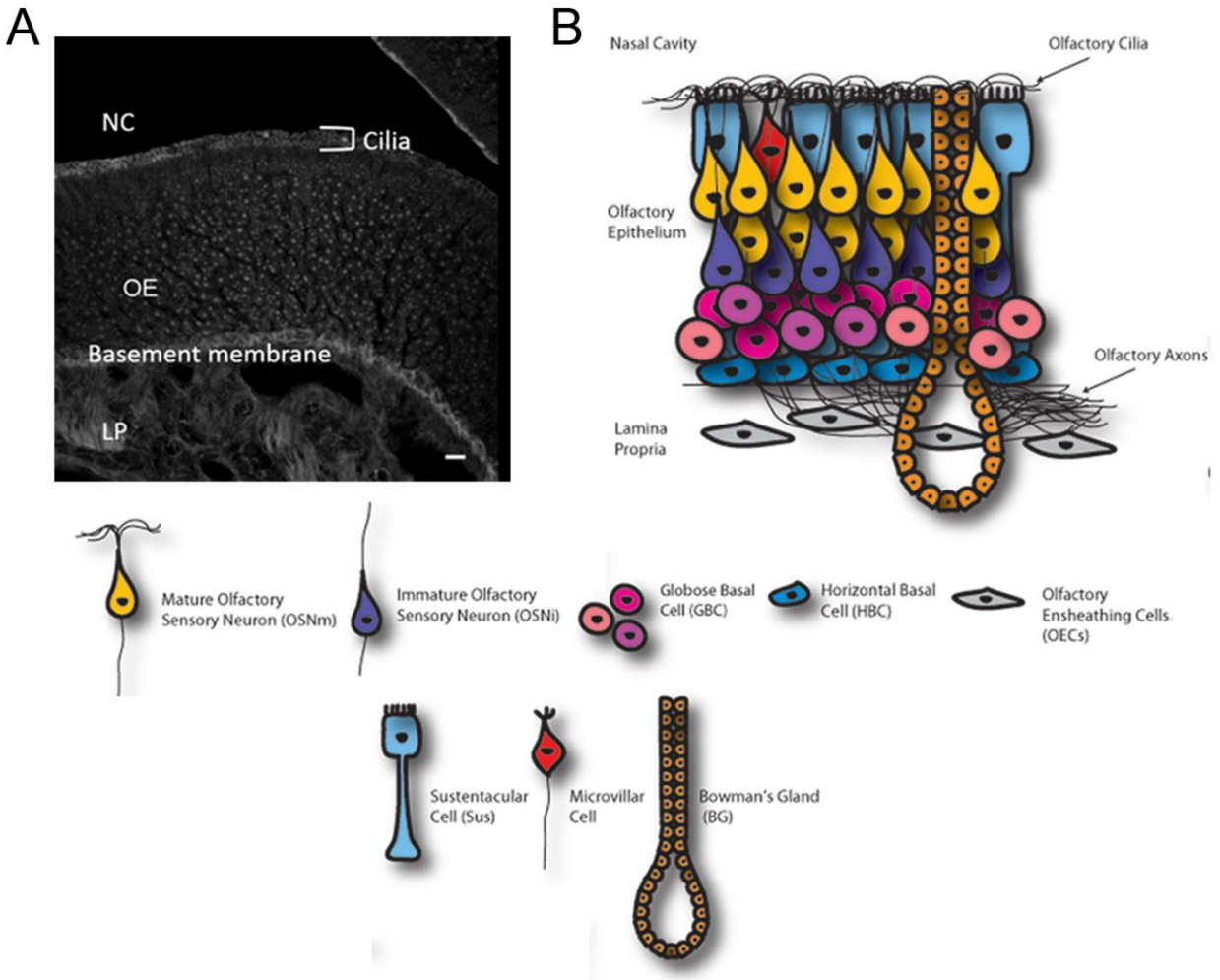

Figure 1-3. Cell composition of the olfactory epithelium. ***A,*** Immunofluorescent image of the mouse OE in grayscale showing the cilia layer of the OE within the nasal cavity and the separation of the OE from the lamina propria (LP) by the basement membrane. ***B*****,** Schematic illustration depicting the various cell types in the OE and LP. Microvillar and SUS cells project microvilli into the nasal cavity, while mature OSNs project cilia. Immature OSNs do not possess cilia but like mature OSN, extend axons into the LP that traverse to the olfactory bulb. Globose basal cells are comprised of a heterogeneous population of cells that can be grouped into two main types of cells due to their expression of distinct transcription factors: immediate neuronal progenitors, transit-amplifying precursors committed to producing neurons, and multi-potent precursors that can produce non-neuronal cells. They lie just below immature OSNs and above horizontal basal cells which lie just against the basement membrane. Olfactory ensheathing cells are found in the lamina propria. NC = nasal cavity, OE = olfactory epithelium, and LP = lamina propria.

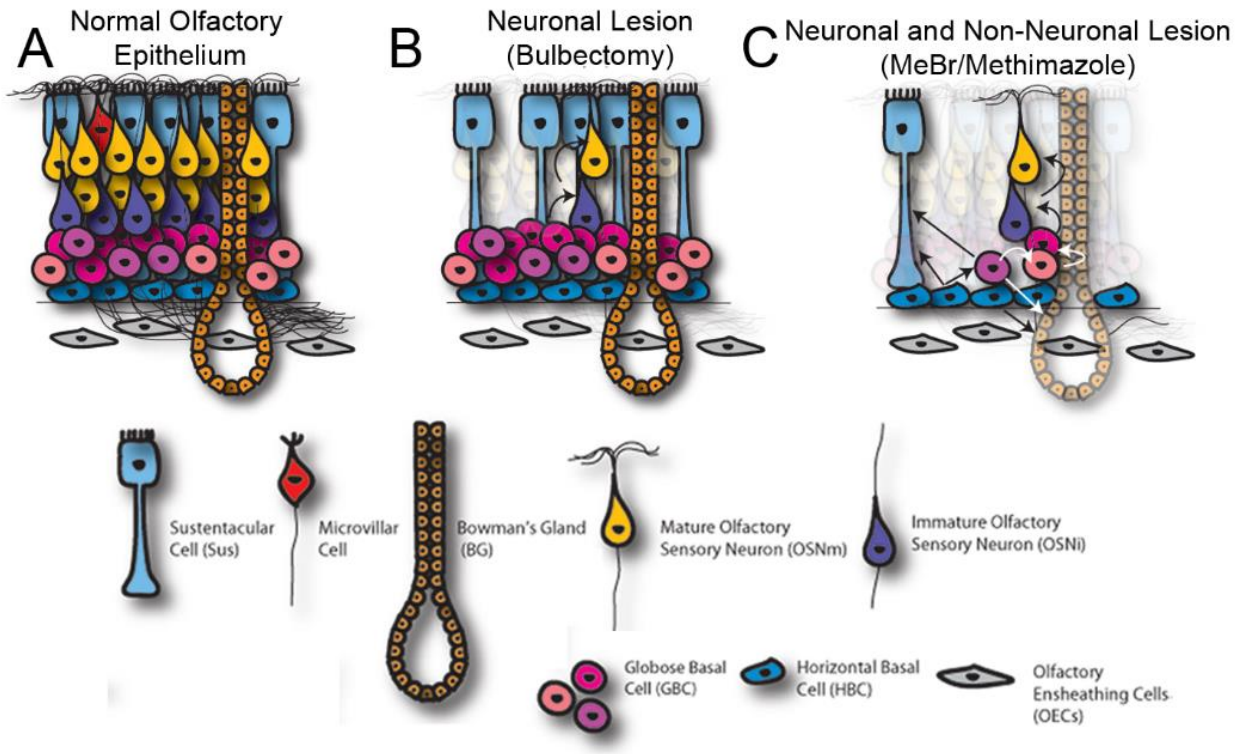

Figure 1-4. Differentiation pathways of olfactory basal stem cells. Schematic illustration depicting various cell types of the OE and the differentiation pathways of basal stem cells at varying levels of injury to the OE. ***A,*** Cell types of the normal, unlesioned OE. ***B*****,** During a neuronal-specific lesion, as seen with olfactory bulbectomy models, GBCs are the lead contributor to replacing OSNs through differentiation. ***C,*** However, in more severe lesion models such as ablation with MeBr or the anti-thyroid drug, methimazole (MMI), in which SUS cells, BG cells, and GBCs are also depleted, HBCs replenish neuronal cells (neuronal precursor GBCs) and non-neuronal cells (multipotent GBCs, SUS cells, and BG cells) of the OE through differentiation. GBCs are believed to differentiate into non-neuronal cells (SUS cells and BG cells) from a sub-population of multipotent cells and neuronal cells (neuronal precursor GBCs and OSNs) from a sub-population of transit-amplifying GBCs. OE = olfactory epithelium.

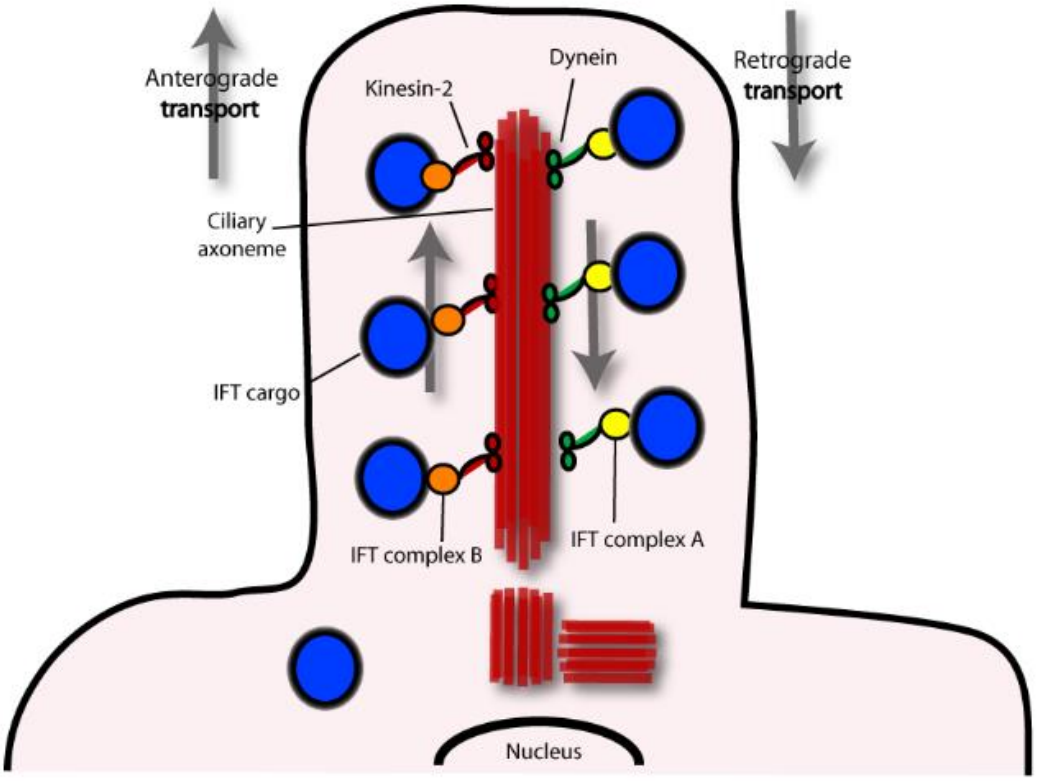

Figure 1-5. Cilia machinery and trafficking. Schematic illustration showing the cilium and its trafficking components adapted from (Hildebrandt et al., 2011). Centrioles form basal bodies, comprised of 9 sets of microtubules, from which the axoneme extends. Intraflagellar transport (IFT) proteins mediate the assembly and maintenance of the cilium by associating with kinesin motors to bring cargo into the cilium for anterograde transport and associating with dynein motors to bring cargo out of the cilium for retrograde transport. Kinesin motors associate with 2 distinct complexes of IFT proteins: complex A for anterograde trafficking and complex B for retrograde trafficking.

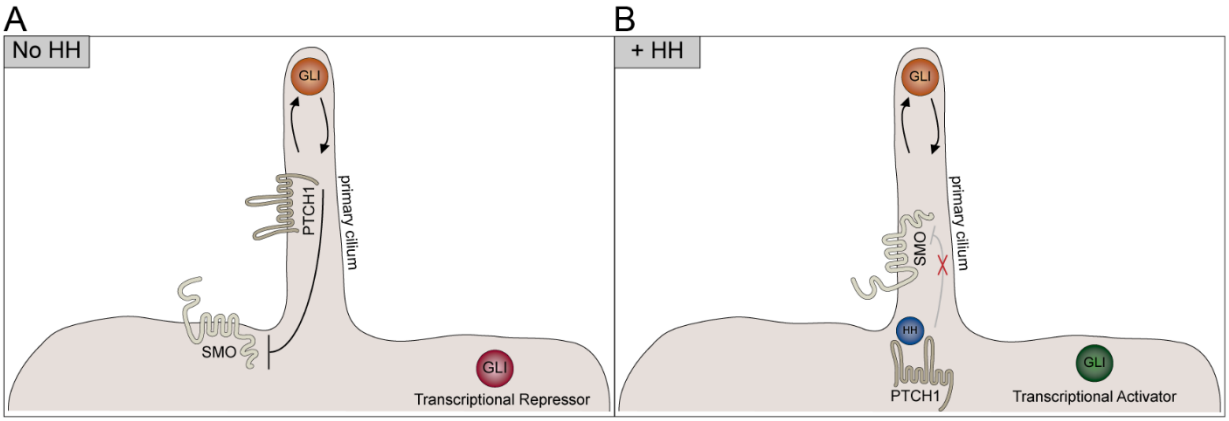

Figure 1-6. Canonical Hedgehog pathway. Schematic illustration depicting the canonical Hedgehog (HH) pathway in mammalians. ***A,*** In the absence of HH ligand, the transmembrane receptor Patched1 (PTCH1), inhibits the transmembrane protein Smoothened (SMO), resulting in the transcriptional repression of HH target genes by GLI transcription factor. ***B,*** In the presence of HH ligands, which bind to PTCH1, de-repressed SMO, which traffics into cilia, allows for the activation of GLI transcription factors and HH target genes.

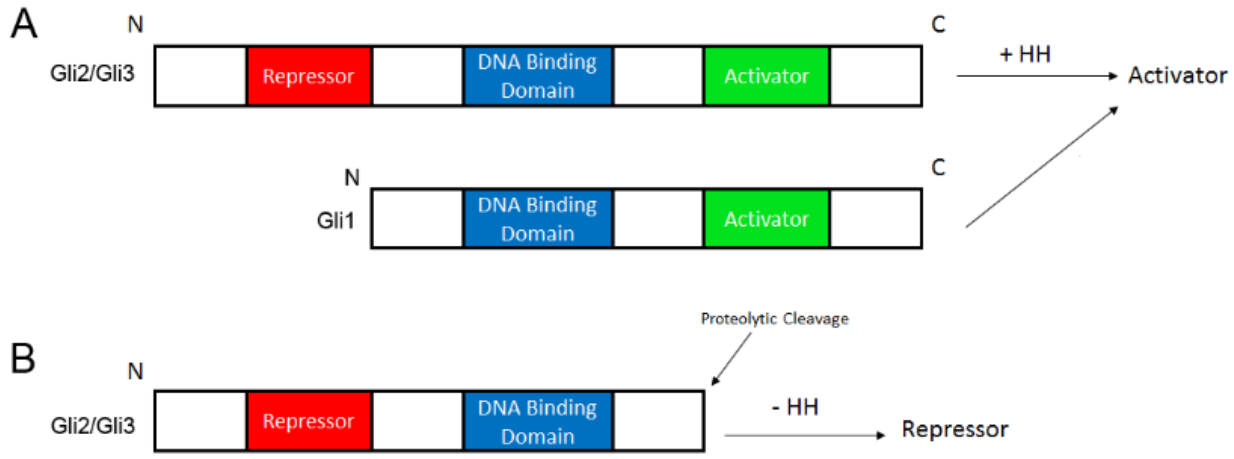

Figure 1-7. GLI processing in the Hedgehog pathway. Schematic illustration showing the processing of GLIs into activators and repressors. ***A,*** All GLI proteins have a DNA-binding domain and C-terminal activation domain while GLI1 has diverged evolutionarily to lack a transcriptional repressor domain. In the presence of HH ligand, activation of SMO inhibits the proteolytic processing of GLI2 or in certain circumstances, GLI3, allowing the full length protein to act as a transcriptional activator. ***B,*** In the absence of HH ligand, GLI3 and to a lesser degree, GLI2, are processed as a transcriptional repressor upon proteolytic cleavage of the C-terminal activation domain. N = N-terminus, C = C-terminus, and HH = hedgehog.

1.8 References

Ahn S, Joyner AL (2005) In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature 437:894-897.

Alcedo J, Ayzenzon M, Von Ohlen T, Noll M, Hooper JE (1996) The Drosophila smoothened gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. Cell 86:221-232.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Alvarez-Buylla A, Garcia-Verdugo JM (2002) Neurogenesis in adult subventricular zone. J Neurosci 22:629-634.

Amador-Arjona A, Elliott J, Miller A, Ginbey A, Pazour GJ, Enikolopov G, Roberts AJ, Terskikh AV (2011) Primary cilia regulate proliferation of amplifying progenitors in adult hippocampus: implications for learning and memory. J Neurosci 31:9933-9944.

Bai CB, Stephen D, Joyner AL (2004) All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Dev Cell 6:103-115.

Baker H, Genter MB (2003) The Olfactory System and the Nasal Mucosa as Portals of Entry of Viruses, Drugs, and Other Exogenous Agents into the Brain. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 549-574. New York, New York: Marcel Dekker, Inc.

Balbuena J, Pachon G, Lopez-Torrents G, Aran JM, Castresana JS, Petriz J (2011) ABCG2 is required to control the sonic hedgehog pathway in side population cells with stem-like properties. Cytometry A 79:672-683.

Balordi F, Fishell G (2007a) Hedgehog signaling in the subventricular zone is required for both the maintenance of stem cells and the migration of newborn neurons. J Neurosci 27:5936-5947.

Balordi F, Fishell G (2007b) Mosaic removal of hedgehog signaling in the adult SVZ reveals that the residual wild-type stem cells have a limited capacity for self-renewal. J Neurosci 27:14248-14259.

Barber PC (1982) Neurogenesis and regeneration in the primary olfactory pathway of mammals. Bibl Anat:12-25.

Barr MM, Sternberg PW (1999) A polycystic kidney-disease gene homologue required for male mating behaviour in C. elegans. Nature 401:386-389.

Beachy PA, Karhadkar SS, Berman DM (2004) Tissue repair and stem cell renewal in carcinogenesis. Nature 432:324-331.

Beidler LM, Smallman RL (1965) Renewal of cells within taste buds. J Cell Biol 27:263-272.

Belluscio L, Gold GH, Nemes A, Axel R (1998) Mice deficient in G(olf) are anosmic. Neuron 20:69-81.

Bergman U, Brittebo EB (1999) Methimazole toxicity in rodents: covalent binding in the olfactory mucosa and detection of glial fibrillary acidic protein in the olfactory bulb. Toxicol Appl Pharmacol 155:190-200.

Bergman U, Ostergren A, Gustafson AL, Brittebo B (2002) Differential effects of olfactory toxicants on olfactory regeneration. Archives of toxicology 76:104-112.

Biju KC, Marks DR, Mast TG, Fadool DA (2008) Deletion of voltage-gated channel affects glomerular refinement and odorant receptor expression in the mouse olfactory system. J Comp Neurol 506:161-179.

Briscoe J, Therond PP (2013) The mechanisms of Hedgehog signalling and its roles in development and disease. Nat Rev Mol Cell Biol 14:416-429.

Brittebo EB (1995) Metabolism-dependent toxicity of methimazole in the olfactory nasal mucosa. Pharmacol Toxicol 76:76-79.

Brooks ER, Wallingford JB (2014) Multiciliated cells. Curr Biol 24:R973-982.

Brownell I, Guevara E, Bai CB, Loomis CA, Joyner AL (2011) Nerve-derived sonic hedgehog defines a niche for hair follicle stem cells capable of becoming epidermal stem cells. Cell stem cell 8:552-565.

Brunet LJ, Gold GH, Ngai J (1996) General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron 17:681-693.

Buck L, Axel R (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65:175-187.

Burke R, Nellen D, Bellotto M, Hafen E, Senti KA, Dickson BJ, Basler K (1999) Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. Cell 99:803-815.

Burr HS (1916) The Effects of the Removal of the Nasal Pits in Amblystoma Embryos. Journal of Experimental Zoology 20:27-57.

Burton PR (1992) Ultrastructural studies of microtubules and microtubule organizing centers of the vertebrate olfactory neuron. Microscopy research and technique 23:142-156.

Buttitta L, Mo R, Hui CC, Fan CM (2003) Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. Development 130:6233-6243.

Caggiano M, Kauer JS, Hunter DD (1994) Globose basal cells are neuronal progenitors in the olfactory epithelium: a lineage analysis using a replication-incompetent retrovirus. Neuron 13:339-352.

Calof AL, Chikaraishi DM (1989) Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. Neuron 3:115-127.

Cantagrel V et al. (2008) Mutations in the cilia gene ARL13B lead to the classical form of Joubert syndrome. Am J Hum Genet 83:170-179.

Carr VM, Farbman AI, Colletti LM, Morgan JI (1991) Identification of a new non-neuronal cell type in rat olfactory epithelium. Neuroscience 45:433-449.

Carson C, Murdoch B, Roskams AJ (2006) Notch 2 and Notch 1/3 segregate to neuronal and glial lineages of the developing olfactory epithelium. Dev Dyn 235:1678-1688.

Carter LA, MacDonald JL, Roskams AJ (2004) Olfactory horizontal basal cells demonstrate a conserved multipotent progenitor phenotype. J Neurosci 24:5670-5683.

Caspary T, Larkins CE, Anderson KV (2007) The graded response to Sonic Hedgehog depends on cilia architecture. Dev Cell 12:767-778.

Castillo D, Seidel K, Salcedo E, Ahn C, de Sauvage FJ, Klein OD, Barlow LA (2014) Induction of ectopic taste buds by SHH reveals the competency and plasticity of adult lingual epithelium. Development 141:2993-3002.

Cau E, Casarosa S, Guillemot F (2002) Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129:1871-1880.

Cau E, Gradwohl G, Fode C, Guillemot F (1997) Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124:1611-1621.

Cau E, Gradwohl G, Casarosa S, Kageyama R, Guillemot F (2000) Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. Development 127:2323-2332.

Chamoun Z, Mann RK, Nellen D, von Kessler DP, Bellotto M, Beachy PA, Basler K (2001) Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. Science 293:2080-2084.

Chen M, Tian S, Yang X, Lane AP, Reed RR, Liu H (2014) Wnt-responsive Lgr5(+) globose basal cells function as multipotent olfactory epithelium progenitor cells. J Neurosci 34:8268-8276.

Chen X, Fang H, Schwob JE (2004) Multipotency of purified, transplanted globose basal cells in olfactory epithelium. J Comp Neurol 469:457-474.

Chen Y, Struhl G (1996) Dual roles for patched in sequestering and transducing Hedgehog. Cell 87:553-563.

Chess A, Simon I, Cedar H, Axel R (1994) Allelic inactivation regulates olfactory receptor gene expression. Cell 78:823-834.

Chou YH, Zheng X, Beachy PA, Luo L (2010) Patterning axon targeting of olfactory receptor neurons by coupled hedgehog signaling at two distinct steps. Cell 142:954-966.

Chuah MI, Schwob JE, Farbman AI (2003) Developmental Anatomy of the Olfactory System. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 115-138. New York, New York: Marcel Dekker, Inc.

Clevers H, Nusse R (2012) Wnt/beta-catenin signaling and disease. Cell 149:1192-1205.

Cole DG (2003) The intraflagellar transport machinery of Chlamydomonas reinhardtii. Traffic 4:435-442.

Cole DG, Diener DR, Himelblau AL, Beech PL, Fuster JC, Rosenbaum JL (1998) Chlamydomonas kinesin-II-dependent intraflagellar transport (IFT): IFT particles contain proteins required for ciliary assembly in Caenorhabditis elegans sensory neurons. J Cell Biol 141:993-1008.

Conley DB, Robinson AM, Shinners MJ, Kern RC (2003) Age-related olfactory dysfunction: cellular and molecular characterization in the rat. Am J Rhinol 17:169-175.

Corbit KC, Shyer AE, Dowdle WE, Gaulden J, Singla V, Chen MH, Chuang PT, Reiter JF (2008) Kif3a constrains beta-catenin-dependent Wnt signalling through dual ciliary and non-ciliary mechanisms. Nature cell biology 10:70-76.

Costanzo RM (1991) Regeneration of olfactory receptor cells. Ciba Found Symp 160:233-242; discussion 243-238.

Costanzo RM, DiNardo LJ, Reiter ER (2003) Head Injury and Olfaction. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 629-638. New York, New York: Marcel Dekker, Inc.

Cowan CM, Roskams AJ (2004) Caspase-3 and caspase-9 mediate developmental apoptosis in the mouse olfactory system. J Comp Neurol 474:136-148.

Craige B, Tsao CC, Diener DR, Hou Y, Lechtreck KF, Rosenbaum JL, Witman GB (2010) CEP290 tethers flagellar transition zone microtubules to the membrane and regulates flagellar protein content. J Cell Biol 190:927-940.

Croyle MJ, Lehman JM, O'Connor AK, Wong SY, Malarkey EB, Iribarne D, Dowdle WE, Schoeb TR, Verney ZM, Athar M, Michaud EJ, Reiter JF, Yoder BK (2011) Role of epidermal primary cilia in the homeostasis of skin and hair follicles. Development 138:1675-1685.

Cuschieri A, Bannister LH (1975) The development of the olfactory mucosa in the mouse: light microscopy. Journal of anatomy 119:277-286.

Dahmane N, Lee J, Robins P, Heller P, Ruiz i Altaba A (1997) Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. Nature 389:876-881.

Danciger E, Mettling C, Vidal M, Morris R, Margolis F (1989) Olfactory marker protein gene: its structure and olfactory neuron-specific expression in transgenic mice. Proc Natl Acad Sci U S A 86:8565-8569.

Delorme B, Nivet E, Gaillard J, Haupl T, Ringe J, Deveze A, Magnan J, Sohier J, Khrestchatisky M, Roman FS, Charbord P, Sensebe L, Layrolle P, Feron F (2010) The human nose harbors a niche of

olfactory ectomesenchymal stem cells displaying neurogenic and osteogenic properties. Stem cells and development 19:853-866.

DeMaria S, Ngai J (2010) The cell biology of smell. J Cell Biol 191:443-452.

Denef N, Neubuser D, Perez L, Cohen SM (2000) Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell 102:521-531.

Dennis JC, Aono S, Vodyanoy VJ, Morrison EE (2015) Development, Morphology, and Functional Anatomy of the Olfactory Epithelium. In: Handbook of Olfaction and Gustation, pp 93-108: John Wiley & Sons, Inc.

Dessaud E, McMahon AP, Briscoe J (2008) Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. Development 135:2489-2503.

Ding X, Dahl AR (2003) Olfactory Mucosa: Composition, Enzymatic Localization, and Metabolism. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 51-74. New York, New York: Marcel Dekker, Inc.

Ding X, Xie F (2015a) Olfactory Mucosa: Composition, Enzymatic Localization, and Metabolism. In: Handbook of Olfaction and Gustation, pp 63-92: John Wiley & Sons, Inc.

Ding X, Xie F (2015b) Olfactory Mucosa: Composition, Enzymatic Localization, and Metabolism. In: Handbook of Olfaction and Gustation, 3rd Edition (Doty RL, ed). Hoboken, NJ, USA: John Wiley & Sons, Inc.

Doetsch F (2003) A niche for adult neural stem cells. Current opinion in genetics & development 13:543-550.

Doetsch F, Garcia-Verdugo JM, Alvarez-Buylla A (1997) Cellular composition and three-dimensional organization of the subventricular germinal zone in the adult mammalian brain. J Neurosci 17:5046-5061.

Doty RL (2003a) Odor Perception in Neurodegenerative Diseases. In: Handbook of Olfaction and Gustation, 2nd Edition, pp 479-502. New York, New York: Marcel Dekker, Inc.

Doty RL (2003b) Handbook of olfaction and gustation. New York: M. Dekker.

Doty RL (2008) The olfactory vector hypothesis of neurodegenerative disease: is it viable? Annals of neurology 63:7-15.

Doty RL (2015) Introduction and Historical Perspective. In: Handbook of Olfaction and Gustation, 3rd Edition (Doty RL, ed), pp 1-36. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Douek E, Bannister LH, Dodson HC (1975) Recent advances in the pathology of olfaction. Proceedings of the Royal Society of Medicine 68:467-470.

Ducray A, Bondier JR, Michel G, Bon K, Millot JL, Propper A, Kastner A (2002) Recovery following peripheral destruction of olfactory neurons in young and adult mice. Eur J Neurosci 15:1907-1917.

Duggan CD, Ngai J (2007) Scent of a stem cell. Nat Neurosci 10:673-674.

Duggan CD, DeMaria S, Baudhuin A, Stafford D, Ngai J (2008) Foxg1 is required for development of the vertebrate olfactory system. J Neurosci 28:5229-5239.

Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75:1417-1430.

Elsaesser R, Montani G, Tirindelli R, Paysan J (2005) Phosphatidyl-inositide signalling proteins in a novel class of sensory cells in the mammalian olfactory epithelium. Eur J Neurosci 21:2692-2700.

Fadool DA, Tucker K, Perkins R, Fasciani G, Thompson RN, Parsons AD, Overton JM, Koni PA, Flavell RA, Kaczmarek LK (2004) Kv1.3 channel gene-targeted deletion produces "Super-Smeller Mice" with altered glomeruli, interacting scaffolding proteins, and biophysics. Neuron 41:389-404.

Fan J, Liu Y, Jia J (2012) Hh-induced Smoothened conformational switch is mediated by differential phosphorylation at its C-terminal tail in a dose- and position-dependent manner. Dev Biol 366:172-184.

Farbman AI (1980) Renewal of taste bud cells in rat circumvallate papillae. Cell and tissue kinetics 13:349-357.

Ferent J, Cochard L, Faure H, Taddei M, Hahn H, Ruat M, Traiffort E (2014) Genetic activation of Hedgehog signaling unbalances the rate of neural stem cell renewal by increasing symmetric divisions. Stem cell reports 3:312-323.

Firestein S (2001) How the olfactory system makes sense of scents. Nature 413:211-218.

Fliegauf M, Benzing T, Omran H (2007) When cilia go bad: cilia defects and ciliopathies. Nat Rev Mol Cell Biol 8:880-893.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.

Gallet A, Therond PP (2005) Temporal modulation of the Hedgehog morphogen gradient by a patched-dependent targeting to lysosomal compartment. Dev Biol 277:51-62.

Gerdes JM, Davis EE, Katsanis N (2009) The vertebrate primary cilium in development, homeostasis, and disease. Cell 137:32-45.

Glusman G, Yanai I, Rubin I, Lancet D (2001) The complete human olfactory subgenome. Genome research 11:685-702.

Goetz SC, Anderson KV (2010) The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet 11:331-344.

Gokoffski KK, Kawauchi S, Wu HH, Santos R, Hollenbeck PLW, Lander AD, Calof AL (2010) Feedback Regulation of Neurogenesis in the Mammalian Olfactory Epithelium: New Insights from Genetics and Systems Biology. In: The Neurobiology of Olfaction (Menini A, ed). Boca Raton (FL).

Goldstein BJ, Schwob JE (1996) Analysis of the globose basal cell compartment in rat olfactory epithelium using GBC-1, a new monoclonal antibody against globose basal cells. J Neurosci 16:4005-4016.

Gong Q, Chen H, Farbman AI (2009) Olfactory sensory axon growth and branching is influenced by sonic hedgehog. Dev Dyn 238:1768-1776.

Goodrich LV, Johnson RL, Milenkovic L, McMahon JA, Scott MP (1996) Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes & development 10:301-312.

Grachtchouk M, Mo R, Yu S, Zhang X, Sasaki H, Hui CC, Dlugosz AA (2000) Basal cell carcinomas in mice overexpressing Gli2 in skin. Nat Genet 24:216-217.

Graziadei PP, Graziadei GA (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol 8:1-18.

Graziadei PP, Monti Graziadei AG (1983) Regeneration in the olfactory system of vertebrates. Am J Otolaryngol 4:228-233.

Grigoryan T, Wend P, Klaus A, Birchmeier W (2008) Deciphering the function of canonical Wnt signals in development and disease: conditional loss- and gain-of-function mutations of beta-catenin in mice. Genes & development 22:2308-2341.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463-476.

Guo Z, Packard A, Krolewski RC, Harris MT, Manglapus GL, Schwob JE (2010) Expression of pax6 and sox2 in adult olfactory epithelium. J Comp Neurol 518:4395-4418.

Hahn H et al. (1996) Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. Cell 85:841-851.

Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A (2008) Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. Nat Neurosci 11:277-284.

Hansen A, Finger TE (2008) Is TrpM5 a reliable marker for chemosensory cells? Multiple types of microvillous cells in the main olfactory epithelium of mice. BMC neuroscience 9:115.

Harding JW, Getchell TV, Margolis FL (1978) Denervation of the primary olfactory pathway in mice. V. Long-term effect of intranasal ZnSO4 irrigation on behavior, biochemistry and morphology. Brain Res 140:271-285.

Harkema JR, Carey SA, Wagner JG, Dintzis SM, Liggitt D (2012) Nose, Sinus, Pharynx, and Larynx. In: Comparative Anatomy and Histology, 1st Edition (Dintzis PMTM, ed), pp 71-94. San Diego: Academic Press.

Hastings L, Miller ML (2003) Influence of Environmental Toxicants on Olfactory Function. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 575-592. New York, New York: Marcel Dekker, Inc.

Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK (2005) Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet 1:e53.

Hegg CC, Jia C, Chick WS, Restrepo D, Hansen A (2010) Microvillous cells expressing IP3 receptor type 3 in the olfactory epithelium of mice. Eur J Neurosci 32:1632-1645.

Hempstead JL, Morgan JI (1983) Monoclonal antibodies to the rat olfactory sustentacular cell. Brain Res 288:289-295.

Hildebrandt F, Benzing T, Katsanis N (2011) Ciliopathies. N Engl J Med 364:1533-1543.

Hirokawa N, Noda Y, Okada Y (1998) Kinesin and dynein superfamily proteins in organelle transport and cell division. Current opinion in cell biology 10:60-73.

Holbrook EH, Szumowski KE, Schwob JE (1995) An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. J Comp Neurol 363:129-146.

Hooper JE, Scott MP (2005) Communicating with Hedgehogs. Nat Rev Mol Cell Biol 6:306-317.

Hoover KC (2010) Smell with inspiration: the evolutionary significance of olfaction. American journal of physical anthropology 143 Suppl 51:63-74.

Hsu YC, Li L, Fuchs E (2014) Transit-amplifying cells orchestrate stem cell activity and tissue regeneration. Cell 157:935-949.

Huang P, Schier AF (2009) Dampened Hedgehog signaling but normal Wnt signaling in zebrafish without cilia. Development 136:3089-3098.

Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV (2003) Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature 426:83-87.

Huard JM, Youngentob SL, Goldstein BJ, Luskin MB, Schwob JE (1998) Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. J Comp Neurol 400:469-486.

Hui CC, Angers S (2011) Gli proteins in development and disease. Annual review of cell and developmental biology 27:513-537.

Hutchin ME, Kariapper MS, Grachtchouk M, Wang A, Wei L, Cummings D, Liu J, Michael LE, Glick A, Dlugosz AA (2005) Sustained Hedgehog signaling is required for basal cell carcinoma proliferation and survival: conditional skin tumorigenesis recapitulates the hair growth cycle. Genes & development 19:214-223.

Ihrie RA, Shah JK, Harwell CC, Levine JH, Guinto CD, Lezameta M, Kriegstein AR, Alvarez-Buylla A (2011) Persistent sonic hedgehog signaling in adult brain determines neural stem cell positional identity. Neuron 71:250-262.

Ingham PW, Nystedt S, Nakano Y, Brown W, Stark D, van den Heuvel M, Taylor AM (2000) Patched represses the Hedgehog signalling pathway by promoting modification of the Smoothened protein. Curr Biol 10:1315-1318.

Irigoin F, Badano JL (2011) Keeping the balance between proliferation and differentiation: the primary cilium. Curr Genomics 12:285-297.

Iwai N, Zhou Z, Roop DR, Behringer RR (2008) Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. Stem Cells 26:1298-1306.

Jang W, Youngentob SL, Schwob JE (2003) Globose basal cells are required for reconstitution of olfactory epithelium after methyl bromide lesion. J Comp Neurol 460:123-140.

Jang W, Chen X, Flis D, Harris M, Schwob JE (2014) Label-retaining, quiescent globose basal cells are found in the olfactory epithelium. J Comp Neurol 522:731-749.

Jia C, Hegg CC (2010) NPY mediates ATP-induced neuroproliferation in adult mouse olfactory epithelium. Neurobiology of disease 38:405-413.

Jia C, Hegg CC (2012) Neuropeptide Y and extracellular signal-regulated kinase mediate injury-induced neuroregeneration in mouse olfactory epithelium. Mol Cell Neurosci 49:158-170.

Jia J, Zhang L, Zhang Q, Tong C, Wang B, Hou F, Amanai K, Jiang J (2005) Phosphorylation by double-time/CKIepsilon and CKIalpha targets cubitus interruptus for Slimb/beta-TRCP-mediated proteolytic processing. Dev Cell 9:819-830.

Jiang J, Hui CC (2008) Hedgehog signaling in development and cancer. Dev Cell 15:801-812.

Joel M (2003) Plasticity Within the Olfactory Pathways. In: Handbook of Olfaction and Gustation: CRC Press.

Johnson RL, Rothman AL, Xie J, Goodrich LV, Bare JW, Bonifas JM, Quinn AG, Myers RM, Cox DR, Epstein EH, Jr., Scott MP (1996) Human homolog of patched, a candidate gene for the basal cell nevus syndrome. Science 272:1668-1671.

Joiner AM, Green WW, McIntyre JC, Allen BL, Schwob JE, Martens JR (2015) Primary Cilia on Horizontal Basal Cells Regulate Regeneration of the Olfactory Epithelium. J Neurosci 35:13761-13772.

Jones C, Roper VC, Foucher I, Qian D, Banizs B, Petit C, Yoder BK, Chen P (2008) Ciliary proteins link basal body polarization to planar cell polarity regulation. Nat Genet 40:69-77.

Kass RS (2005) The channelopathies: novel insights into molecular and genetic mechanisms of human disease. J Clin Invest 115:1986-1989.

Katoh H, Shibata S, Fukuda K, Sato M, Satoh E, Nagoshi N, Minematsu T, Matsuzaki Y, Akazawa C, Toyama Y, Nakamura M, Okano H (2011) The dual origin of the peripheral olfactory system: placode and neural crest. Molecular brain 4:34.

Kinzler KW, Ruppert JM, Bigner SH, Vogelstein B (1988) The GLI gene is a member of the Kruppel family of zinc finger proteins. Nature 332:371-374.

Kiprilov EN, Awan A, Desprat R, Velho M, Clement CA, Byskov AG, Andersen CY, Satir P, Bouhassira EE, Christensen ST, Hirsch RE (2008) Human embryonic stem cells in culture possess primary cilia with hedgehog signaling machinery. J Cell Biol 180:897-904.

Klein TJ, Mlodzik M (2005) Planar cell polarization: an emerging model points in the right direction. Annual review of cell and developmental biology 21:155-176.

Kogerman P, Grimm T, Kogerman L, Krause D, Unden AB, Sandstedt B, Toftgard R, Zaphiropoulos PG (1999) Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nature cell biology 1:312-319.

Komiya Y, Habas R (2008) Wnt signal transduction pathways. Organogenesis 4:68-75.

Kopan R (2012) Notch signaling. Cold Spring Harbor perspectives in biology 4.

Kozminski KG, Johnson KA, Forscher P, Rosenbaum JL (1993) A motility in the eukaryotic flagellum unrelated to flagellar beating. Proc Natl Acad Sci U S A 90:5519-5523.

Krause A, Xu Y, Joh J, Hubner R, Gess A, Ilic T, Worgall S (2010) Overexpression of sonic Hedgehog in the lung mimics the effect of lung injury and compensatory lung growth on pulmonary Sca-1 and CD34 positive cells. Molecular therapy : the journal of the American Society of Gene Therapy 18:404-412.

Krauss S, Concordet JP, Ingham PW (1993) A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. Cell 75:1431-1444.

Krolewski RC, Jang W, Schwob JE (2011) The generation of olfactory epithelial neurospheres in vitro predicts engraftment capacity following transplantation in vivo. Exp Neurol 229:308-323.

Krolewski RC, Packard A, Schwob JE (2013) Global expression profiling of globose basal cells and neurogenic progression within the olfactory epithelium. J Comp Neurol 521:833-859.

Krolewski RC, Packard A, Jang W, Wildner H, Schwob JE (2012) Ascl1 (Mash1) knockout perturbs differentiation of nonneuronal cells in olfactory epithelium. PLoS One 7:e51737.

Kulaga HM, Leitch CC, Eichers ER, Badano JL, Lesemann A, Hoskins BE, Lupski JR, Beales PL, Reed RR, Katsanis N (2004) Loss of BBS proteins causes anosmia in humans and defects in olfactory cilia structure and function in the mouse. Nat Genet 36:994-998.

Kumamoto N, Gu Y, Wang J, Janoschka S, Takemaru K, Levine J, Ge S (2012) A role for primary cilia in glutamatergic synaptic integration of adult-born neurons. Nat Neurosci 15:399-405, S391.

Kumari A, Ermilov AN, Allen BL, Bradley RM, Dlugosz AA, Mistretta CM (2015) Hedgehog pathway blockade with the cancer drug LDE225 disrupts taste organs and taste sensation. J Neurophysiol 113:1034-1040.

Kuzhandaivel A, Schultz SW, Alkhori L, Alenius M (2014) Cilia-mediated hedgehog signaling in Drosophila. Cell reports 7:672-680.

Lai K, Kaspar BK, Gage FH, Schaffer DV (2003) Sonic hedgehog regulates adult neural progenitor proliferation in vitro and in vivo. Nat Neurosci 6:21-27.

Lee J, Platt KA, Censullo P, Ruiz i Altaba A (1997) Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development 124:2537-2552.

Legrier ME, Ducray A, Propper A, Chao M, Kastner A (2001) Cell cycle regulation during mouse olfactory neurogenesis. Cell Growth Differ 12:591-601.

Lehman JM, Michaud EJ, Schoeb TR, Aydin-Son Y, Miller M, Yoder BK (2008) The Oak Ridge Polycystic Kidney mouse: modeling ciliopathies of mice and men. Dev Dyn 237:1960-1971.

Leung CT, Coulombe PA, Reed RR (2007) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10:720-726.

Liberles SD, Buck LB (2006) A second class of chemosensory receptors in the olfactory epithelium. Nature 442:645-650.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH (2004) Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annual review of pharmacology and toxicology 44:399-421.

Liew GM, Ye F, Nager AR, Murphy JP, Lee JS, Aguiar M, Breslow DK, Gygi SP, Nachury MV (2014) The intraflagellar transport protein IFT27 promotes BBSome exit from cilia through the GTPase ARL6/BBS3. Dev Cell 31:265-278.

Lim A, Shin K, Zhao C, Kawano S, Beachy PA (2014) Spatially restricted Hedgehog signalling regulates HGF-induced branching of the adult prostate. Nature cell biology 16:1135-1145.

Lim DA, Alvarez-Buylla A (1999) Interaction between astrocytes and adult subventricular zone precursors stimulates neurogenesis. Proc Natl Acad Sci U S A 96:7526-7531.

Liman ER, Corey DP, Dulac C (1999) TRP2: a candidate transduction channel for mammalian pheromone sensory signaling. Proc Natl Acad Sci U S A 96:5791-5796.

Lin W, Margolskee R, Donnert G, Hell SW, Restrepo D (2007) Olfactory neurons expressing transient receptor potential channel M5 (TRPM5) are involved in sensing semiochemicals. Proc Natl Acad Sci U S A 104:2471-2476.

Ling G, Gu J, Genter MB, Zhuo X, Ding X (2004) Regulation of cytochrome P450 gene expression in the olfactory mucosa. Chemico-biological interactions 147:247-258.

Litingtung Y, Chiang C (2000) Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. Nat Neurosci 3:979-985.

Liu A, Wang B, Niswander LA (2005) Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. Development 132:3103-3111.

Liu HX, Ermilov A, Grachtchouk M, Li L, Gumucio DL, Dlugosz AA, Mistretta CM (2013a) Multiple Shh signaling centers participate in fungiform papilla and taste bud formation and maintenance. Dev Biol 382:82-97.

Liu L, Kugler MC, Loomis CA, Samdani R, Zhao Z, Chen GJ, Brandt JP, Brownell I, Joyner AL, Rom WN, Munger JS (2013b) Hedgehog signaling in neonatal and adult lung. Am J Respir Cell Mol Biol 48:703-710.

Loktev AV, Zhang Q, Beck JS, Searby CC, Scheetz TE, Bazan JF, Slusarski DC, Sheffield VC, Jackson PK, Nachury MV (2008) A BBSome subunit links ciliogenesis, microtubule stability, and acetylation. Dev Cell 15:854-865.

Machold R, Hayashi S, Rutlin M, Muzumdar MD, Nery S, Corbin JG, Gritli-Linde A, Dellovade T, Porter JA, Rubin LL, Dudek H, McMahon AP, Fishell G (2003) Sonic hedgehog is required for progenitor cell maintenance in telencephalic stem cell niches. Neuron 39:937-950.

Mackay-Sim A (2003) Neurogenesis in the Adult Olfactory Neuroepithelium. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 93-114. New York, New York: Marcel Dekker, Inc.

Mackay-Sim A, St. John J, Schwob JE (2015) Neurogenesis in the Adult Olfactory Neuroepithelium. In: Handbook of Olfaction and Gustation, 3rd Edition (Doty RL, ed), pp 133-156. Hoboken, NJ, USA: John Wiley & Sons, Inc.

Maier E, von Hofsten J, Nord H, Fernandes M, Paek H, Hebert JM, Gunhaga L (2010) Opposing Fgf and Bmp activities regulate the specification of olfactory sensory and respiratory epithelial cell fates. Development 137:1601-1611.

Manglapus GL, Youngentob SL, Schwob JE (2004) Expression patterns of basic helix-loop-helix transcription factors define subsets of olfactory progenitor cells. J Comp Neurol 479:216-233.

Marigo V, Davey RA, Zuo Y, Cunningham JM, Tabin CJ (1996) Biochemical evidence that patched is the Hedgehog receptor. Nature 384:176-179.

Martinez C, Smith PC, Rodriguez JP, Palma V (2011) Sonic hedgehog stimulates proliferation of human periodontal ligament stem cells. Journal of dental research 90:483-488.

Matise MP, Epstein DJ, Park HL, Platt KA, Joyner AL (1998) Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 125:2759-2770.

McEwen DP, Jenkins PM, Martens JR (2008) Olfactory cilia: our direct neuronal connection to the external world. Curr Top Dev Biol 85:333-370.

McEwen DP, Koenekoop RK, Khanna H, Jenkins PM, Lopez I, Swaroop A, Martens JR (2007) Hypomorphic CEP290/NPHP6 mutations result in anosmia caused by the selective loss of G proteins in cilia of olfactory sensory neurons. Proc Natl Acad Sci U S A 104:15917-15922.

McIntyre JC, Williams CL, Martens JR (2013) Smelling the roses and seeing the light: gene therapy for ciliopathies. Trends in biotechnology 31:355-363.

McIntyre JC et al. (2012) Gene therapy rescues cilia defects and restores olfactory function in a mammalian ciliopathy model. Nat Med 18:1423-1428.

McLellan JS, Yao S, Zheng X, Geisbrecht BV, Ghirlando R, Beachy PA, Leahy DJ (2006) Structure of a heparin-dependent complex of Hedgehog and Ihog. Proc Natl Acad Sci U S A 103:17208-17213.

Menco BPM, Morrison EE (2003) Morphology of the Mammalian Olfactory Epithelium: Form, Fine Structure, Function, and Pathology. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 17-50. New York, New York: Marcel Dekker, Inc.

Merchant M, Vajdos FF, Ultsch M, Maun HR, Wendt U, Cannon J, Desmarais W, Lazarus RA, de Vos AM, de Sauvage FJ (2004) Suppressor of fused regulates Gli activity through a dual binding mechanism. Molecular and cellular biology 24:8627-8641.

Miller ML, Andringa A, Evans JE, Hastings L (1995) Microvillar cells of the olfactory epithelium: morphology and regeneration following exposure to toxic compounds. Brain Res 669:1-9.

Mimeault M, Batra SK (2010) Frequent deregulations in the hedgehog signaling network and cross-talks with the epidermal growth factor receptor pathway involved in cancer progression and targeted therapies. Pharmacol Rev 62:497-524.

Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R (1996) Visualizing an olfactory sensory map. Cell 87:675-686.

Moran DT, Rowley JC, 3rd, Jafek BW (1982) Electron microscopy of human olfactory epithelium reveals a new cell type: the microvillar cell. Brain Res 253:39-46.

Morrison EE, Costanzo RM (1990) Morphology of the human olfactory epithelium. J Comp Neurol 297:1-13.

Morrison SJ, Perez SE, Qiao Z, Verdi JM, Hicks C, Weinmaster G, Anderson DJ (2000) Transient Notch activation initiates an irreversible switch from neurogenesis to gliogenesis by neural crest stem cells. Cell 101:499-510.

Murdoch B, Roskams AJ (2007) Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. J Mol Histol 38:581-599.

Murphy C, Doty RL, Duncan HJ (2003) Clinical Disorders of Olfaction. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 461-478. New York, New York: Marcel Dekker, Inc.

Murphy C, Schubert CR, Cruickshanks KJ, Klein BE, Klein R, Nondahl DM (2002) Prevalence of olfactory impairment in older adults. Jama 288:2307-2312.

Murrell W, Feron F, Wetzig A, Cameron N, Splatt K, Bellette B, Bianco J, Perry C, Lee G, Mackay-Sim A (2005) Multipotent stem cells from adult olfactory mucosa. Dev Dyn 233:496-515.

Murrell W, Wetzig A, Donnellan M, Feron F, Burne T, Meedeniya A, Kesby J, Bianco J, Perry C, Silburn P, Mackay-Sim A (2008) Olfactory mucosa is a potential source for autologous stem cell therapy for Parkinson's disease. Stem Cells 26:2183-2192.

Nakano Y, Guerrero I, Hidalgo A, Taylor A, Whittle JR, Ingham PW (1989) A protein with several possible membrane-spanning domains encoded by the Drosophila segment polarity gene patched. Nature 341:508-513.

Nusslein-Volhard C, Wieschaus E (1980) Mutations affecting segment number and polarity in Drosophila. Nature 287:795-801.

Ogura T, Szebenyi SA, Krosnowski K, Sathyanesan A, Jackson J, Lin W (2011) Cholinergic microvillous cells in the mouse main olfactory epithelium and effect of acetylcholine on olfactory sensory neurons and supporting cells. J Neurophysiol 106:1274-1287.

Ohba S, Kawaguchi H, Kugimiya F, Ogasawara T, Kawamura N, Saito T, Ikeda T, Fujii K, Miyajima T, Kuramochi A, Miyashita T, Oda H, Nakamura K, Takato T, Chung UI (2008) Patched1 haploinsufficiency increases adult bone mass and modulates Gli3 repressor activity. Dev Cell 14:689-699.

Oro AE, Higgins K (2003) Hair cycle regulation of Hedgehog signal reception. Dev Biol 255:238-248.

Osumi-Yamashita N, Kuratani S, Ninomiya Y, Aoki K, Iseki S, Chareonvit S, Doi H, Fujiwara M, Watanabe T, Eto K (1997) Cranial anomaly of homozygous rSey rat is associated with a defect in the migration pathway of midbrain crest cells. Development, growth & differentiation 39:53-67.

Packard A, Giel-Moloney M, Leiter A, Schwob JE (2011a) Progenitor cell capacity of NeuroD1-expressing globose basal cells in the mouse olfactory epithelium. J Comp Neurol 519:3580-3596.

Packard A, Schnittke N, Romano RA, Sinha S, Schwob JE (2011b) DeltaNp63 regulates stem cell dynamics in the mammalian olfactory epithelium. J Neurosci 31:8748-8759.

Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479-494.

Pan Y, Bai CB, Joyner AL, Wang B (2006) Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Molecular and cellular biology 26:3365-3377.

Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui CC, Nakashima M, Joyner AL (2000) Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 127:1593-1605.

Pazour GJ, San Agustin JT, Follit JA, Rosenbaum JL, Witman GB (2002a) Polycystin-2 localizes to kidney cilia and the ciliary level is elevated in orpk mice with polycystic kidney disease. Curr Biol 12:R378-380.

Pazour GJ, Baker SA, Deane JA, Cole DG, Dickert BL, Rosenbaum JL, Witman GB, Besharse JC (2002b) The intraflagellar transport protein, IFT88, is essential for vertebrate photoreceptor assembly and maintenance. J Cell Biol 157:103-113.

Pedersen LB, Veland IR, Schroder JM, Christensen ST (2008) Assembly of primary cilia. Dev Dyn 237:1993-2006.

Petrova R, Joyner AL (2014) Roles for Hedgehog signaling in adult organ homeostasis and repair. Development 141:3445-3457.

Petrova R, Garcia AD, Joyner AL (2013) Titration of GLI3 repressor activity by sonic hedgehog signaling is critical for maintaining multiple adult neural stem cell and astrocyte functions. J Neurosci 33:17490-17505.

Plotnikova OV, Pugacheva EN, Golemis EA (2009) Primary cilia and the cell cycle. Methods in cell biology 94:137-160.

Porter JA, Young KE, Beachy PA (1996) Cholesterol modification of hedgehog signaling proteins in animal development. Science 274:255-259.

Quarmby LM, Parker JD (2005) Cilia and the cell cycle? J Cell Biol 169:707-710.

Reed CJ (1993) Drug metabolism in the nasal cavity: relevance to toxicology. Drug metabolism reviews 25:173-205.

Reifenberger J, Wolter M, Weber RG, Megahed M, Ruzicka T, Lichter P, Reifenberger G (1998) Missense mutations in SMOH in sporadic basal cell carcinomas of the skin and primitive neuroectodermal tumors of the central nervous system. Cancer research 58:1798-1803.

Ressler KJ, Sullivan SL, Buck LB (1993) A zonal organization of odorant receptor gene expression in the olfactory epithelium. Cell 73:597-609.

Ressler KJ, Sullivan SL, Buck LB (1994) Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. Cell 79:1245-1255.

Riddle RD, Johnson RL, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. Cell 75:1401-1416.

Rodriguez-Gil DJ, Greer CA (2008) Wnt/Frizzled family members mediate olfactory sensory neuron axon extension. J Comp Neurol 511:301-317.

Rodriguez S, Sickles HM, Deleonardis C, Alcaraz A, Gridley T, Lin DM (2008) Notch2 is required for maintaining sustentacular cell function in the adult mouse main olfactory epithelium. Dev Biol 314:40-58.

Rohatgi R, Milenkovic L, Scott MP (2007) Patched1 regulates hedgehog signaling at the primary cilium. Science 317:372-376.

Rosenbaum JL, Witman GB (2002) Intraflagellar transport. Nat Rev Mol Cell Biol 3:813-825.

Roskams AJ, Cai X, Ronnett GV (1998) Expression of neuron-specific beta-III tubulin during olfactory neurogenesis in the embryonic and adult rat. Neuroscience 83:191-200.

Sarafoleanu C, Mella C, Georgescu M, Perederco C (2009) The importance of the olfactory sense in the human behavior and evolution. Journal of medicine and life 2:196-198.

Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H (1999) Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 126:3915-3924.

Satir P, Christensen ST (2007) Overview of structure and function of mammalian cilia. Annual review of physiology 69:377-400.

Satir P, Pedersen LB, Christensen ST (2010) The primary cilium at a glance. J Cell Sci 123:499-503.

Sato N, Leopold PL, Crystal RG (1999) Induction of the hair growth phase in postnatal mice by localized transient expression of Sonic hedgehog. J Clin Invest 104:855-864.

Sauer FC (1937) Some factors in the morphogenesis of vertebrate embryonic epithelia. Journal of Morphology 61:563-579.

Scheidecker S, Etard C, Pierce NW, Geoffroy V, Schaefer E, Muller J, Chennen K, Flori E, Pelletier V, Poch O, Marion V, Stoetzel C, Strahle U, Nachury MV, Dollfus H (2014) Exome sequencing of Bardet-Biedl syndrome patient identifies a null mutation in the BBSome subunit BBIP1 (BBS18). J Med Genet 51:132-136.

Schnittke N, Herrick DB, Lin B, Peterson J, Coleman JH, Packard AI, Jang W, Schwob JE (2015) Transcription factor p63 controls the reserve status but not the stemness of horizontal basal cells in the olfactory epithelium. Proc Natl Acad Sci U S A 112:E5068-5077.

Schultz EW (1960) Repair of the olfactory mucosa with special reference to regeneration of olfactory cells (sensory neurons). Am J Pathol 37:1-19.

Schwob JE, Szumowski KE, Stasky AA (1992) Olfactory sensory neurons are trophically dependent on the olfactory bulb for their prolonged survival. J Neurosci 12:3896-3919.

Schwob JE, Youngentob SL, Mezza RC (1995) Reconstitution of the rat olfactory epithelium after methyl bromide-induced lesion. J Comp Neurol 359:15-37.

Seidel K, Ahn CP, Lyons D, Nee A, Ting K, Brownell I, Cao T, Carano RA, Curran T, Schober M, Fuchs E, Joyner A, Martin GR, de Sauvage FJ, Klein OD (2010) Hedgehog signaling regulates the generation of ameloblast progenitors in the continuously growing mouse incisor. Development 137:3753-3761.

Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A (2001) Astrocytes give rise to new neurons in the adult mammalian hippocampus. J Neurosci 21:7153-7160.

Serizawa S, Miyamichi K, Nakatani H, Suzuki M, Saito M, Yoshihara Y, Sakano H (2003) Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. Science 302:2088-2094.

Sharma N, Malarkey EB, Berbari NF, O'Connor AK, Vanden Heuvel GB, Mrug M, Yoder BK (2013) Proximal tubule proliferation is insufficient to induce rapid cyst formation after cilia disruption. Journal of the American Society of Nephrology : JASN 24:456-464.

Shepherd GM (2004) The human sense of smell: are we better than we think? PLoS biology 2:E146.

Shin K, Lee J, Guo N, Kim J, Lim A, Qu L, Mysorekar IU, Beachy PA (2011) Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder. Nature 472:110-114.

Singla V, Reiter JF (2006) The primary cilium as the cell's antenna: signaling at a sensory organelle. Science 313:629-633.

Smart IH (1971) Location and orientation of mitotic figures in the developing mouse olfactory epithelium. Journal of anatomy 109:243-251.

Smelkinson MG, Kalderon D (2006) Processing of the Drosophila hedgehog signaling effector Ci-155 to the repressor Ci-75 is mediated by direct binding to the SCF component Slimb. Curr Biol 16:110-116.

Smutzer GS, Doty RL, Arnold SE, Trojanowski JQ (2003) Olfactory System Neuropathology in Alzheimer's Disease, Parkinson's Disease, and Schizophrenia. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 503-524. New York, New York: Marcel Dekker, Inc.

Solanas G, Benitah SA (2013) Regenerating the skin: a task for the heterogeneous stem cell pool and surrounding niche. Nat Rev Mol Cell Biol 14:737-748.

Sorokin SP (1968) Reconstructions of centriole formation and ciliogenesis in mammalian lungs. J Cell Sci 3:207-230.

Stephan AB, Shum EY, Hirsh S, Cygnar KD, Reisert J, Zhao H (2009) ANO2 is the cilial calcium-activated chloride channel that may mediate olfactory amplification. Proc Natl Acad Sci U S A 106:11776-11781.

Stone DM, Hynes M, Armanini M, Swanson TA, Gu Q, Johnson RL, Scott MP, Pennica D, Goddard A, Phillips H, Noll M, Hooper JE, de Sauvage F, Rosenthal A (1996) The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog. Nature 384:129-134.

Sung CH, Leroux MR (2013) The roles of evolutionarily conserved functional modules in cilia-related trafficking. Nature cell biology 15:1387-1397.

Tadenev AL, Kulaga HM, May-Simera HL, Kelley MW, Katsanis N, Reed RR (2011) Loss of Bardet-Biedl syndrome protein-8 (BBS8) perturbs olfactory function, protein localization, and axon targeting. Proc Natl Acad Sci U S A 108:10320-10325.

Tanigaki K, Nogaki F, Takahashi J, Tashiro K, Kurooka H, Honjo T (2001) Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. Neuron 29:45-55.

Tenzen T, Allen BL, Cole F, Kang JS, Krauss RS, McMahon AP (2006) The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. Dev Cell 10:647-656.

Tong CK, Han YG, Shah JK, Obernier K, Guinto CD, Alvarez-Buylla A (2014) Primary cilia are required in a unique subpopulation of neural progenitors. Proc Natl Acad Sci U S A 111:12438-12443.

Tummala P, Arnsdorf EJ, Jacobs CR (2010) The Role of Primary Cilia in Mesenchymal Stem Cell Differentiation: A Pivotal Switch in Guiding Lineage Commitment. Cell Mol Bioeng 3:207-212.

Turin L, Yoshii F (2003) Structure-Odor Relationships. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 275-294. New York, New York: Marcel Dekker, Inc.

van den Brink GR, Hardwick JC, Tytgat GN, Brink MA, Ten Kate FJ, Van Deventer SJ, Peppelenbosch MP (2001) Sonic hedgehog regulates gastric gland morphogenesis in man and mouse. Gastroenterology 121:317-328.

van den Brink GR, Hardwick JC, Nielsen C, Xu C, ten Kate FJ, Glickman J, van Deventer SJ, Roberts DJ, Peppelenbosch MP (2002) Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. Gut 51:628-633.

van Dop WA, Heijmans J, Buller NV, Snoek SA, Rosekrans SL, Wassenberg EA, van den Bergh Weerman MA, Lanske B, Clarke AR, Winton DJ, Wijgerde M, Offerhaus GJ, Hommes DW, Hardwick JC, de Jonge WJ, Biemond I, van den Brink GR (2010) Loss of Indian Hedgehog activates multiple aspects of a wound healing response in the mouse intestine. Gastroenterology 139:1665-1676, 1676 e1661-1610.

van Reeuwijk J, Arts HH, Roepman R (2011) Scrutinizing ciliopathies by unraveling ciliary interaction networks. Hum Mol Genet 20:R149-157.

Vassar R, Ngai J, Axel R (1993) Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. Cell 74:309-318.

Vassar R, Chao SK, Sitcheran R, Nunez JM, Vosshall LB, Axel R (1994) Topographic organization of sensory projections to the olfactory bulb. Cell 79:981-991.

Veland IR, Awan A, Pedersen LB, Yoder BK, Christensen ST (2009) Primary cilia and signaling pathways in mammalian development, health and disease. Nephron Physiol 111:p39-53.

Verhaagen J, Oestreicher AB, Gispen WH, Margolis FL (1989) The expression of the growth associated protein B50/GAP43 in the olfactory system of neonatal and adult rats. J Neurosci 9:683-691.

Verhaagen J, Oestreicher AB, Grillo M, Khew-Goodall YS, Gispen WH, Margolis FL (1990) Neuroplasticity in the olfactory system: differential effects of central and peripheral lesions of the primary olfactory pathway on the expression of B-50/GAP43 and the olfactory marker protein. J Neurosci Res 26:31-44.

Wang B, Fallon JF, Beachy PA (2000a) Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 100:423-434.

Wang LC, Liu ZY, Gambardella L, Delacour A, Shapiro R, Yang J, Sizing I, Rayhorn P, Garber EA, Benjamin CD, Williams KP, Taylor FR, Barrandon Y, Ling L, Burkly LC (2000b) Regular articles: conditional disruption of hedgehog signaling pathway defines its critical role in hair development and regeneration. The Journal of investigative dermatology 114:901-908.

Wang YZ, Yamagami T, Gan Q, Wang Y, Zhao T, Hamad S, Lott P, Schnittke N, Schwob JE, Zhou CJ (2011) Canonical Wnt signaling promotes the proliferation and neurogenesis of peripheral olfactory stem cells during postnatal development and adult regeneration. J Cell Sci 124:1553-1563.

Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB (2003) Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. Nature 422:313-317.

Weech M, Quash M, Walters E (2003) Characterization of the mouse olfactory glutathione S-transferases during the acute phase response. J Neurosci Res 73:679-685.

Weiss J, Pyrski M, Jacobi E, Bufe B, Willnecker V, Schick B, Zizzari P, Gossage SJ, Greer CA, Leinders-Zufall T, Woods CG, Wood JN, Zufall F (2011) Loss-of-function mutations in sodium channel Nav1.7 cause anosmia. Nature 472:186-190.

Wetzig A, Mackay-Sim A, Murrell W (2011) Characterization of olfactory stem cells. Cell transplantation 20:1673-1691.

Whitby-Logan GK, Weech M, Walters E (2004) Zonal expression and activity of glutathione S-transferase enzymes in the mouse olfactory mucosa. Brain Res 995:151-157.

Williams CL, McIntyre JC, Norris SR, Jenkins PM, Zhang L, Pei Q, Verhey K, Martens JR (2014) Direct evidence for BBSome-associated intraflagellar transport reveals distinct properties of native mammalian cilia. Nature communications 5:5813.

Wong ST, Trinh K, Hacker B, Chan GC, Lowe G, Gaggar A, Xia Z, Gold GH, Storm DR (2000) Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. Neuron 27:487-497.

Xie F, Zhou X, Behr M, Fang C, Horii Y, Gu J, Kannan K, Ding X (2010) Mechanisms of olfactory toxicity of the herbicide 2,6-dichlorobenzonitrile: essential roles of CYP2A5 and target-tissue metabolic activation. Toxicol Appl Pharmacol 249:101-106.

Yao S, Lum L, Beachy P (2006) The ihog cell-surface proteins bind Hedgehog and mediate pathway activation. Cell 125:343-357.

Yoder BK, Hou X, Guay-Woodford LM (2002) The polycystic kidney disease proteins, polycystin-1, polycystin-2, polaris, and cystin, are co-localized in renal cilia. Journal of the American Society of Nephrology : JASN 13:2508-2516.

Young JM, Friedman C, Williams EM, Ross JA, Tonnes-Priddy L, Trask BJ (2002) Different evolutionary processes shaped the mouse and human olfactory receptor gene families. Hum Mol Genet 11:535-546.

Zacharias WJ, Madison BB, Kretovich KE, Walton KD, Richards N, Udager AM, Li X, Gumucio DL (2011) Hedgehog signaling controls homeostasis of adult intestinal smooth muscle. Dev Biol 355:152-162.

Zaghloul NA, Katsanis N (2009) Mechanistic insights into Bardet-Biedl syndrome, a model ciliopathy. J Clin Invest 119:428-437.

Zhang X, Firestein S (2002) The olfactory receptor gene superfamily of the mouse. Nat Neurosci 5:124-133.

Zou Z, Horowitz LF, Montmayeur JP, Snapper S, Buck LB (2001) Genetic tracing reveals a stereotyped sensory map in the olfactory cortex. Nature 414:173-179.

CHAPTER II:

Primary Cilia on Horizontal Basal Cells Regulate Regeneration of the Olfactory Epithelium

2.1 Abstract

The olfactory epithelium (OE) is one of the few tissues to undergo constitutive neurogenesis throughout the mammalian lifespan. It is comprised of multiple cell types including olfactory sensory neurons (OSNs) that are readily replaced by two populations of basal stem cells, frequently dividing globose basal cells (GBCs) and quiescent horizontal basal cells (HBCs). However, the precise mechanisms by which these cells mediate OE regeneration are unclear. Here, we show for the first time that the HBC subpopulation of basal stem cells uniquely possess primary cilia that are aligned in an apical orientation in direct apposition to sustentacular cell endfeet. The positioning of these cilia suggests that they function in the detection of growth signals and/or differentiation cues. To test this idea, we generated an inducible, cell-type specific *Ift88* knockout mouse line (*K5rtTA;tetOCre;Ift88*$^{fl/fl}$) to disrupt cilia formation and maintenance specifically in HBCs. Surprisingly, the loss of HBC cilia did not affect the maintenance of the adult OE but dramatically impaired the regeneration of OSNs following lesion. Furthermore, the loss of cilia during development resulted in a region-specific decrease in neurogenesis, implicating HBCs in the establishment of the OE. Together, these

results suggest a novel role for primary cilia in HBC activation, proliferation, and differentiation.

2.2 Introduction

Neurogenesis occurs from the self-renewal and differentiation of neural stem cells (Gage, 2000). While prevalent during development, neural stem cells are also found in the adult nervous system, in the olfactory epithelium (OE), subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the hippocampus (Gage, 2000; Alvarez-Buylla and Garcia-Verdugo, 2002; Lie et al., 2004). Factors and mechanisms that regulate proliferation, migration, differentiation, and cell survival during development can be active in the adult nervous system, allowing for tissue repair and neuroplasticity (reviewed by Lie et al., 2004). Unlike most sensory systems, the OE is able to reconstitute both neuronal and non-neuronal populations following injury and neuronal death via the action of resident populations of olfactory stem cells (Graziadei and Graziadei, 1979; Calof and Chikaraishi, 1989; Edge and Chen, 2008). Olfactory sensory neurons (OSNs) are unique in that they both directly contact the external environment and the brain. While this direct contact allows OSNs to detect odors, it also exposes the OE to insults from toxins, bacteria, and viruses leading to cell death. Therefore, the capacity for neurogenesis and replacement of OSNs is critical for maintaining this important sensory system.

The OE is composed of OSNs, supporting sustentacular (SUS) cells, Bowman's gland (BG) duct cells, and two groups of basally located stems cells, globose basal cells

(GBCs), and horizontal basal cells (HBCs). GBCs and HBCs are considered progenitor or stem cells of the OE, capable of promoting regeneration and neurogenesis both for tissue homeostasis and in response to injury (Barber, 1982; Costanzo, 1991; Leung et al., 2007). GBCs are a heterogeneous population of actively cycling cells comprised of immediate neuronal progenitors and transit-amplifying precursors committed to producing neurons as well as multi-potent precursors that can produce non-neuronal cells (Jang et al., 2014). HBCs are a homogeneous population of multi-potent, quiescent cells (Holbrook et al., 1995; Carter et al., 2004; Iwai et al., 2008). Following the selective death of olfactory neurons, as occurs in response to an olfactory bulbectomy, GBCs replace OSNs through differentiation, while HBCs remain quiescent (Caggiano et al., 1994; Huard et al., 1998; Leung et al., 2007). In severe lesion models such as exposure to methyl bromide or ablation with methimazole (MMI), in which SUS cells and GBCs are affected, HBCs contribute to the regeneration of the OE (Leung et al., 2007; Iwai et al., 2008). While the cell types involved are known, the precise mechanisms controlling regeneration and homeostasis are unclear. Importantly, the molecular regulation of olfactory basal stem cells, including their activation and proliferation in response to injury and cell fate determination during differentiation, remains largely unknown.

Primary cilia have important roles in cell proliferation, differentiation, and regulation of the cell cycle (reviewed by Irigoin and Badano, 2011). There is growing evidence that primary cilia regulate neurogenesis and/or proper differentiation of adult stem cells into amplifying progenitor cells or glial cells in the SVZ and hippocampus (Amador-Arjona et al., 2011; Kumamoto et al., 2012; Tong et al., 2014). Consequently, when cilia are disrupted in these systems, cilia-dependent signaling pathways such as Wnt and SHH

are also disrupted (Kumamoto et al., 2012; Tong et al., 2014). These findings demonstrate the important role cilia play in signaling pathways essential for proper cell differentiation during development and adult homeostasis.

Here, we show that HBCs possess primary cilia, suggesting a potential mechanism for the molecular regulation of olfactory basal stem cells in the OE. In mice with a HBC-specific cilia deletion, the OE showed limited regeneration following lesion, with a significant reduction of mature OSNs. Additionally, when HBC cilia were removed early in development, specific regions of the OE were found to have a significant reduction in OSNs. These data identify HBC cilia as a previously unrecognized signaling structure in the OE, provide mechanistic insight into the regulation of HBCs during olfactory regeneration, and indicate a potential role for HBCs in OE development.

2.3 Results

Olfactory stem cells possess primary cilia.

ARL13B is a small Ras GTPase that regulates ciliogenesis and cilia function (Cantagrel et al., 2008; Higginbotham et al., 2013; Kasahara et al., 2014; Bangs et al., 2015). While immunostaining the OE of wild-type mice for endogenous ARL13B, we made a fortuitous discovery of what appeared to be cilia projecting from cells located just above the lamina propria (Fig. 2-1). To determine if these primary cilia were on a specific olfactory cell type we immunolabeled the OE for the basal body marker gamma (γ) tubulin and the nuclear transcription factor p63, which labels HBCs (Packard et al., 2011). The staining revealed ARL13B+ cilia projecting from basal bodies located within HBCs (Fig.

2-1A-B). To confirm the presence of cilia using a transgenic marker, we analyzed the OE of mice expressing an ARL13B-GFP fusion protein (*Arl13b-EGFP*^{tg}) (Delling et al., 2013) labeled with a second HBC marker, Keratin5 (K5) (Holbrook et al., 1995). Similar to the endogenous staining, ARL13B:GFP also labels cilia structures projecting from HBCs (Fig. 2-1C). To further characterize HBC cilia, we stained the OE for additional ciliary markers. Adenylyl cyclase III (AC3) is a canonical marker of cilia projecting from OSNs, but also labels cilia present on other cell types including primary neurons and astrocytes (Bishop et al., 2007; Guadiana et al., 2013; Higginbotham et al., 2013). AC3 co-localizes with endogenous ARL13B (Fig. 2-1D) in wildtype mice, as well as ARL13B:GFP (Fig. 2-1E) in transgenic mice. The AC3+ cilia are also associated with p63 labeled HBCs similar to ARL13B (Fig. 2-1F). Finally, we used a second transgenic mouse, *EGFP-Cetn2*, as an additional label for basal bodies (Higginbotham et al., 2004; Bangs et al., 2015). Staining tissue with AC3 antibodies revealed AC3+ projections associated with EGFP-CETN2 labeled basal bodies (Fig. 2-1G). Together, these data suggest that HBCs possess primary cilia and indicate that OSNs are not the only ciliated cell type in the OE.

In the OE, apically located SUS cells possess basal projections, known as endfeet, that juxtapose olfactory basal cells en route to contact with the basal lamina (Doyle et al., 2001). The purpose of these connections has yet to be elucidated; however, it has been proposed that this is a site for communication between basal cells and SUS cells (Jia and Hegg, 2010). In our analysis, we observed that the majority of HBC cilia project from the top of the HBCs towards the SUS cell endfeet. To determine if HBC cilia are associated with SUS cell endfeet, analysis of p63+ HBCs and SUS cells immunolabeled with Keratin18 (K18) (Holbrook et al., 2011) was carried out in *Arl13b-EGFP*^{tg} mice. As seen

in a representative image, cilia projecting from HBCs are in apposition to the endfeet (Fig. 2-1H). To improve the resolution of a single SUS cell endfoot, we analyzed the OE of mice intranasally injected with an adenovirus expressing GFP, in which a small subset of SUS cells are transduced (for methods, see ref. McIntyre et al., 2012). Using this approach, we observed that the ARL13B+ cilium projects into the interstitial space between the HBC and SUS cell (Fig. 2-1I). These data suggest the potential for cilia to act as an antenna for communication between basal cells and SUS cells perhaps in a manner analogous to the immunological synapse (Bromley et al., 2001; Finetti et al., 2011).

Cilia are present on distinct subpopulations of olfactory stem cells.

Given the close proximity of GBCs and HBCs at the base of the OE, we assessed whether GBCs also possess primary cilia. We identified a novel pan-GBC marker to distinguish between HBC and GBC subpopulations. SEC8 is one of eight subunits in the exocyst complex that was initially discovered in the secretory pathway of yeast *S. cerevisae* and proposed to contribute to various mechanisms, including protein synthesis and vesicle trafficking (Wang et al., 2004). Antibody staining for SEC8 in the OE specifically labels a large population of GBCs overlapping with previous identified makers of GBC subpopulations (Fig. 2-2A). Immunostaining with antibodies to either MASH1 (also known as ASCL1) (Manglapus et al., 2004), a basic helix-loop-helix transcription factor that is required in the early stages of olfactory neuron lineage (Cau et al., 1997) or, LSD1 (lysine-specific demethylase 1), a chromatin modifying complex protein (Krolewski et al., 2013) in conjunction with SEC8 confirmed that SEC8 is indeed a pan-GBC marker. All MASH1+ or LSD1+ cells appear to co-express SEC8 (Fig. 2-2A). Approximately 41%

of SEC8+ cells were MASH1+, while approximately 90% of SEC8+ cells were also LSD1+ (Fig. 2-2A, C; N=4). Labeling of the OE with ARL13B and SEC8 antibodies revealed that very few (<5%) SEC8+ GBCs possess cilia, while >70% of K5+ HBCs possess cilia (Fig. 2-2B, D; N=6). These data show that HBCs are the predominant ciliated olfactory basal stem cells and suggest a specific role for cilia in this subpopulation of neural progenitors.

Conditional deletion of IFT machinery results in the specific loss of cilia on HBCs.

To study the cell autonomous function of cilia in HBCs, we used a floxed allele for a gene essential for intraflagellar transport (*Ift88)* and an inducible Cre driver specific to HBCs in the OE (see methods). IFT88 is a critical component of the bi-directional intraflagellar transport (IFT) system (Singla and Reiter, 2006; Pedersen and Rosenbaum, 2008) and conditional knockout of *Ift88* ablates cilia from specific cell types (Croyle et al., 2011; Sharma et al., 2013). Mice in which HBC cilia were ablated (*K5rtTA;TetOCre;Ift88*$^{fl/fl}$ and *K5rtTA;TetOCre;Ift88*$^{fl/\Delta}$) are hereafter referred to as iHBC-IFT88 mice, while phenotypically wildtype mice (*K5rtTA;TetOCre;Ift88*$^{fl/wt}$) are hereafter referred to as control mice.

Control and iHBC-IFT88 mice were administered a doxycycline-containing (dox) diet to activate Cre expression (Gunther et al., 2002; Grachtchouk et al., 2011) at postnatal day 28 (P28), when the OE is mature (Murdoch and Roskams, 2007). One week post dox administration, ~90% of HBCs in mice with both *TetO-Cre* and *K5rtTA* alleles show Cre expression (Fig. 2-3A). Cre expression was not detected in HBCs of mice that lacked the *TetOCre* allele (Fig. 2-3B). More importantly, iHBC-IFT88 mice show a significant loss (88.5%) of ciliated HBCs after 4 weeks of dox (Fig. 2-3C-D, N=5). Interestingly, loss of cilia does not result in a change in the number of HBCs (Fig. 2-3E,

N=5). Immunostaining with the mature OSN marker, olfactory marker protein (OMP), and acetylated α-tubulin (Acet-Tub) revealed no changes in OSN composition (Fig. 2-3F). An increase in cilia loss on HBCs (95.6%) was detected after 8 weeks of dox administration (Fig. 2-4A-B), with no effect on the number of HBCs (Fig. 2-4C), GBCs (Fig. 2-4D-E) and OSNs (Fig. 2-4F-G) (Control mice N=2, iHBCIFT88 mice N=4). These data demonstrate that genetic ablation of *Ift88* efficiently removes cilia from HBCs, without altering HBC quiescence or the normal maintenance of the OE.

Loss of cilia in HBCs results in improper regeneration of the olfactory epithelium following injury.

HBCs are believed to be a quiescent population of cells unless activated by severe injury to participate in OE regeneration (Leung et al., 2007). Methimazole (MMI) is an olfactory toxicant whose metabolites induce cell death of OSNs, SUS cells, and GBCs but spares HBCs in the mouse OE (Brittebo, 1995; Packard et al., 2011). Four weeks after dox administration, iHBC-IFT88 and control mice were given an intraperitoneal injection of MMI (75 mg/kg) to chemically lesion the OE, and analyzed at 8 weeks post-recovery (Genter et al., 1995; Bergman and Brittebo, 1999). The presence of cilia on HBCs remained significantly reduced in iHBC-IFT88 mice after recovery (Fig. 2-5A, C; N=5 for both groups), while no difference in the number of HBCs was observed between iHBC-IFT88 and control mice, as indicated by the number of K5+ cells (Fig. 2-5A, D; N=5 for both groups). Surprisingly, there was a significant reduction in the number of SEC8+ GBCs in iHBC-IFT88 mice compared to controls (Fig. 2-5B, E; N=5 for both groups). In addition, the number of OMP+ mature OSNs was reduced by approximately 75% in iHBC-IFT88 mice (Fig. 2-5F, G; N=5 for both groups). Consequently, the thickness of the OE

was also reduced by approximately 50% (Fig. 2-5H; N=5 for both groups). Interestingly, no change in the number of cleaved caspase-3+ cells was observed between control and iHBC-IFT88 mice 8 weeks post-recovery, suggesting that the reduction of mature OSNs and thickness of the OE was not due to increased cell death (Fig. 2-5I, J; N=5 for both groups).

While the regeneration of neurons was not completely eliminated, the OSNs that returned possess acetylated α-tubulin labeled cilia (Fig. 2-5F). Since IFT88 is required for OSN cilia (McIntyre et al., 2012), the presence of ciliated OSNs in the regenerated OE suggests that these OSNs were derived from progenitors other than HBCs lacking IFT88. In our iHBC-IFT88 mice that were administered dox for 4 weeks, approximately 12% of HBCs still possessed cilia (see results above). Based on our MMI lesion paradigm, it is therefore possible that this remaining 12% of ciliated HBCs, in which IFT88 is present, contributed to regeneration of the OE. However, another possible explanation is that these OSNs were derived from residual GBCs and not from HBCs lacking IFT88. This notion is consistent with MMI lesion, which can leave residual GBCs (Xie et al., 2013). Nonetheless, such significant reduction of OSNs should coincide with a decline in synaptic input into the olfactory bulb (OB), which can be measured by the expression of tyrosine hydroxylase (TH) in the dopaminergic interneurons of the OB (Baker et al., 1993; Baker et al., 1999). Following recovery there was a significant reduction in the intensity of TH in the bulb of iHBC-IFT88 mice compared to control mice (Fig. 2-5K-L; N=4 for both groups) and the glomerular perimeter was significantly reduced. Together, these data indicate that without cilia, HBCs are unable to contribute to the regeneration of the OE,

potentially due to disrupted HBC proliferation, resulting in impaired OSN recovery and olfactory function.

In order to link the observed effects to cilia function, and not off-target effects of IFT88 loss (Robert et al., 2007), we repeated these experiments using a cell-specific knockout of *Arl13b* (Caspary et al., 2007; Larkins et al., 2011). ARL13B is specifically localized to the cilia membrane and is required for proper ciliogenesis (Caspary et al., 2007). Moreover, mutations in *Arl13b*, lead to loss of cilia phenotypes as well as HH-related phenotypes due to the disruption in the localization and ciliary targeting of HH pathway components (Caspary et al., 2007; Duldulao et al., 2009; Larkins et al., 2011). *K5rtTA;TetOCre;Arl13b*$^{fl/fl}$ mice (referred to hereafter as iHBC-ARL13B mice) and *K5rtTA;TetOCre;Arl13b*$^{fl/wt}$ mice (referred to hereafter as control mice) underwent the same MMI lesion paradigm as stated above. After 8 weeks of recovery, ARL13B+ HBC cilia were observed in control mice but not in iHBC-ARL13B mice (Fig. 2-6A). Similar to results observed in iHBC-IFT88 mice, the number of HBCs did not differ between control and iHBC-ARL13B mice (Fig. 2-6B; N=3 for both groups). Additionally, there was a significant reduction (~ 30%) in the number of SEC8+ GBCs (Fig. 2-6C-D; N=3 for both groups) as well an approximate 75% reduction in the number of OMP+ mature OSNs (Fig. 2-6E-F; N=3 for both groups) in iHBC-ARL13B mice compared to controls. Interestingly, the apical surface of the OE of iHBC-ARL13B mice had reduced Acet-Tub labeled OSN cilia. Together, these results not only recapitulate those observed in the iHBC-IFT88 mice but also suggest that *Arl13b* is required for HBC cilia function and regeneration of the OE.

Depletion of HBC cilia results in the impaired development of the OE.

In mice, the OE begins to form at embryonic day 10 (E10) (Murdoch and Roskams, 2007). Small numbers of p63-expressing cells first appear at E14, after the emergence of all other cell types (Packard et al., 2011). Therefore, HBCs are not believed to participate in the establishment of the OE. HBCs settle to the basal lamina, begin to express K5, and become dormant between ~E16 and P10 (Packard et al., 2011). In order to exploit this late development, iHBC-IFT88 mice were administered dox starting at E16 for 4.5-5 weeks to ablate cilia during OE maturation. At P28, iHBC-IFT88 mice had a near complete loss of cilia on HBCs across all regions of the OE compared to control mice (Fig. 2-7A-B; N=5 for both groups), with no difference in the total number of HBCs (Fig. 2-7A, C; N=5 for both groups). Interestingly, the dorsal lateral region associated with ectoturbinates 2 and 2', exhibited a significantly reduced number of OMP+ mature OSNs in iHBC-IFT88 mice (Fig. 2-8A-H). While other regions of the OE also exhibit a reduction in the number of mature OSNs, this reduction was variable along the anterior-posterior axis of the OE and most pronounced near the midpoint of the anterior-posterior axis. Quantitation of OMP+ neurons in the posterior OE revealed a reduction in mature OSNs in the dorsal medial and dorsal lateral regions but not the ventral medial and ventral lateral OE (Fig. 2-8E, I; N=5 for both groups). These data show that cilia on HBCs are important for proper neurogenesis during development of the OE, providing evidence that HBCs participate in the establishment of this tissue that was thought to be limited to GBCs (Treloar et al., 2010).

2.4 Discussion

Within the olfactory system, it is well known that cilia from individual OSNs create a large receptive field in which an OSN can be activated by an odorant (for review, see DeMaria and Ngai, 2010). Until now it was widely believed that neurons were the sole ciliated cell type in the OE. Here, we show for the first time that primary cilia exist on a reserve population of olfactory basal stem cells, the HBCs. Furthermore, utilizing the genetic deletion of two cilia-specific genes in HBCs, we demonstrate that primary cilia are critical for activation of the regenerative capacity of HBCs following chemical lesion. While *Ift88* plays a critical role in ciliogenesis and maintenance, it has also been implicated in G1-S transition in ciliated cells (Robert et al., 2007). However in other studies targeted deletion of *Kif3a*, a kinesin motor important for cilia function, shows similar phenotypic effects observed with loss of *Ift88* suggesting a common mechanism of action through the cilium (Berbari et al., 2009; Wong et al., 2009). In our study we have validated the results observed with deletion of *Ift88* by the targeted removal of *Arl13b*. The loss of *Arl13b* has previously been demonstrated to affect neural development, and mutations in *Arl13b* underlie human ciliopathies (Caspary et al., 2007; Cantagrel et al., 2008; Higginbotham et al., 2012; Zhang et al., 2013). As IFT88 and ARL13B serve different roles in cilia function, the similar phenotypes observed in two different mutant lines supports the conclusion that the loss in regenerative capacity of HBCs is due to dysfunctional primary cilia.

HBCs are long-lived quiescent progenitors that are activated only after extensive lesion of the OE in which SUS cells and GBCs are depleted (Leung et al., 2007). The mechanism by which HBCs become activated remains unknown; however, SUS cell

endfeet have been previously shown to terminate on HBCs (Holbrook et al., 1995). Given that the present findings demonstrate an anatomical localization of the HBC cilia relative to SUS cell endfeet, we propose a model in which HBC cilia act to sense cues from the SUS cells regarding the health of the OE. We propose that this communication occurs at the interface between SUS cell endfeet and HBC cilia (Fig. 2-9A). This paradigm may be analogous to the immunological synapse that forms between a T cell and an antigen-presenting cell functioning to orchestrate signals that drive T cell activation (for review, see Bromley et al., 2001). Following injury to the OE, signaling is conceivably altered between SUS cells and HBCs resulting in activation of the quiescent HBCs (Fig. 2-9B). Without cilia, HBCs are unlikely to detect signals necessary for proliferation and/or differentiation (Fig. 2-9C). Additional support for a SUS/HBC connection in regenerating the OE comes from different lesion models. In OSN-specific lesions, where SUS and GBC cell death is spared, HBCs remain quiescent, and the GBCs are the leading contributor to regeneration (Leung et al., 2007; Makino et al., 2009). However, when lesions result in SUS and GBC cell death, HBCs become active and contribute to regeneration (Leung et al., 2007; Jia et al., 2010). These findings suggest that death of SUS cells stimulates HBCs to proliferate and contribute to regeneration. However, an alternative explanation that cannot be ruled out based on current lesion models is whether signaling occurs between GBCs and HBCs, and how such signaling may contribute to HBC proliferation and OE regeneration. Interestingly, negative feedback signaling is known to occur in the OE, as is observed between neuronal and stem/progenitor cells, in order to control self-replication and differentiation of the stem progenitor cell population (Beites et al., 2005; Gokoffski et al., 2010). It is therefore plausible that such feedback signaling may occur

between GBCs and HBCs via HBC cilia, and that this signaling is lost when lesion results in GBC cell death. In either case, whether the injury results in the release of a stimulatory signal, or the loss of a tonic inhibitory cue between HBCs and other cell types remains to be determined.

Quiescent adult stem cells typically forgo cell cycle decisions in order to respond to transient environment signals sensed through the cilia (Plotnikova et al., 2009). These signals include the Notch, Wnt, and HH pathways, each of which is strongly linked to neurogenesis in the brain (Kumamoto et al., 2012; Tong et al., 2014). The Wnt pathway has been previously implicated in OE proliferation and neurogenesis during development and regeneration (Wang et al., 2011; Chen et al., 2014). In contrast, Notch signaling appears to participate in development of supporting cells but not neurons in the OE (Manglapus et al., 2004; Krolewski et al., 2012). Currently, there is no evidence for the role of the HH pathway in OE development. However, canonical HH signaling is strongly linked to cilia in numerous other systems (for review, see Goetz and Anderson, 2010). Numerous HH pathway components dynamically localize to cilia, while disruption of cilia results in aberrant HH signal transduction and impaired morphogenesis in specific organ systems (for review, see Berbari et al., 2009).

While the signaling pathways may be shared, the phenotypic effects of cilia loss differ between ciliated cell types including various stem cells. In the adult skin, loss of cilia on basal cells resulted in the proliferation of cells in the interfollicular epidermis with subsequent perturbation of epidermal homeostasis (Croyle et al., 2011). In contrast, the conditional ablation of primary cilia postnatally from neural stem cells (NSCs) in the hippocampus led to a reduction in amplifying progenitor cells without altering the number

of quiescent NSCs (Amador-Arjona et al., 2011). In addition, primary cilia in the cerebellum are required for expansion of the neural progenitor pool (Chizhikov et al., 2007). Similarly, a recent report suggested that localized ablation of cilia in NSCs in the ventricular–subventricular zone (V-SVZ) decreases neurogenesis only in the ventral V-SVZ (Tong et al., 2014). These data in NSCs are similar to what we observed with a loss of cilia from HBCs in the OE– no change in the number of HBCs, but a reduction in the neurogenesis of the OE. Together, these data suggest the convergence of a common cilia ablation phenotype between neural stem cells as opposed to epidermis.

During development, NSCs are important for early neurogenesis and morphogenesis of the OE (Gokoffski et al., 2010). While maturing GBCs that migrate from the apical surface clearly contribute to organogenesis (Cau et al., 2002), the precise role of HBCs is unclear. Evidence from a lineage-trace mouse model suggests that upon their establishment in the OE at P10, HBCs do participate in neurogenesis (Iwai et al., 2008). Our data supports the notion of HBC-driven neurogenesis in early postnatal development of the OE and suggests that cilia on HBCs are required for normal OE development. Interestingly, the loss of OSNs observed in the developing OE was regional whereas in the recovering OE it was global. We speculate that these differences can be attributed to the temporal and spatial release of morphogens during development that are synchronized after severe injury to the OE (Vedin et al., 2009). Nevertheless, our data demonstrate a clear role for HBCs and their cilia in the developing OE.

Our finding of cilia on HBCs and their importance in neurogenesis reveals a potential novel mechanism for the etiology of olfactory dysfunction. It is now well recognized that anosmia is a clinical manifestation of ciliopathies, a growing class of

pleiotropic genetic disorders, traditionally associated with the loss of cilia on OSNs. However, the ability to maintain a functional olfactory system throughout life depends on the presence of a stem cell population. While the OE undergoes constant neurogenesis, there is a net loss of neurons over time due to reduced stem cell function (Ducray et al., 2002; Conley et al., 2003). Changes that alter cilia function on HBCs could hasten this decline and contribute to age related olfactory loss. Moreover, ciliopathy patients may show increased susceptibility to chemical insult to the OE. Work here suggests that impaired neurogenesis may be a new unforeseen mechanism for olfactory dysfunction in congenital anosmias.

2.5 Materials and Methods

Mouse strains and Genotyping. All mice were maintained on a mixed genetic background. Transgenic *Arl13b-EGFP*^{tg} mice were provided by David Clapham (Harvard University). *EGFP-Cetn2* mice were obtained from The Jackson laboratory (stock number 008234) (Higginbotham et al., 2004). Conditional deletion of *Ift88* from olfactory HBCs was achieved by the use of a doxycycline-inducible Cre mouse model. This model utilized mice carrying three alleles: (a) a K5-promoter driving expression of the reverse tetracycline transactivator (rtTA) (*K5rtTA*) (Diamond et al., 2000), (b) a tetracycline Operator (TetO) to permit expression of Cre (*TetOCre*) (Mucenski et al., 2003), and (c) a floxed *Ift88*$^{fl/\Delta}$ to ablate cilia (the Δ allele has exons 4-6 deleted from *Ift88*) (Haycraft et al., 2007). K5rtTA and TetOCre mice were provided by Dr. Andrzej Dlugosz (University of Michigan, Ann Arbor, MI). *Ift88*$^{fl/\Delta}$ mice were provided by Dr. Bradley Yoder (University of Alabama at Birmingham, Birmingham, AL). Removal of Arl13b from olfactory horizontal

basal cells was achieved using a similar strategy with a floxed *Arl13b* (exon 2) mouse provided by Dr. Tamara Caspary (Emory University). All mice of either sex were housed and maintained according to the University of Michigan and University of Florida institutional guidelines. All protocols for mouse experimentation were approved by the University of Michigan and the University of Florida Committee(s) on the Use and Care of Animals. Genotyping was performed using primers and PCR parameters from previously published work referenced above.

Doxycycline Transgene Induction and olfactory epithelium lesion. Mice were fed doxycycline chow (200 mg/kg doxycycline, Bio-Serv) and water (200µg/ml doxycycline, 5% sucrose, Fisher) starting at either embryonic day 16 (E16) or postnatal day 28 (P28) and remained on doxycycline diet until the time of euthanasia. Based on an approximate daily food intake of 4 g/mouse and water intake of 6ml/mouse (Bachmanov et al., 2002), mice consumed approximately 2 mg doxycycline / day (0.8 mg in chow and 1.2 mg in water). P28 doxycycline treated *K5rtTA;TetOCre;Ift88*$^{fl/fl}$ mice or *K5rtTA;TetOCre;Arl13b*$^{fl/fl}$ mice and control littermates received an intraperitoneal injection of Methimazole (2-Mercapto-1-methylimidazole, Sigma) (75 mg/kg in sterilized 1xPBS) four weeks after the start of doxycycline diet. Mice were euthanized 8 weeks after methimazole treatment.

Tissue Collection and Preparation. Mice were anesthetized with 30% Fluriso (Isoflurane, VetOne), transcardially perfused with 4% paraformaldehyde (PFA), decapitated, and their heads fixed in 4% PFA for 12-16 hours at 4°C. Tissue was then decalcified in 0.5M EDTA (Fisher)/1xPBS overnight at 4°C, cryoprotected in 10% (1hr), 20% (1hr), and 30% sucrose/1xPBS overnight at 4°C, and frozen in OCT compound

(Tissue Tek). 10-12μm of the olfactory epithelium and olfactory bulb were collected on a Leica CM1860 cryostat.

Immunohistochemistry. For all immunofluorescence, antigen retrieval was used. For antigen retrieval, tissue sections were rinsed in 1xPBS to remove OCT then incubated in Citrate buffer (pH 6.0) for 30min at 90°C, cooled for 20min at RT, then washed with distilled water for 5min. Sections were blocked with 2% donkey or goat serum, 1% BSA in 1xPBS and incubated overnight in primary antibody. Antibodies were used at the following dilutions: mIgG2a anti-p63 (BioCare Medical, 1:200); mIgG2a anti-ARL13B (Neuromab, 1:500); rabbit anti-ARL13B (Proteintech, 1:500); mIgG1 anti-gamma tubulin (Sigma, 1:500); rabbit anti-K5 (Covance, 1:2500); chicken anti-GFP (Abcam, 1:500); rabbit anti-K18 (Abcam, 1:500); mIgG1 anti-MASH1 (BD Pharmingen, 1:100); mIgG2b anti-SEC8 (BD Transduction Laboratory, 1:500); rabbit anti-LSD1 (Abcam, 1:500); goat anti-OMP (Wako Chemicals, 1:1000); mouse anti-Cre (Millipore, 1:500); mouse anti-α acetylated tubulin (Sigma, 1:1000); rabbit anti-AC3 (EnCor Biotechnology, 1:2000), and rabbit anti-TH (Millipore, 1:500). Sections were washed in 1xPBS three times for 5min each at room temperature and then incubated with Alexa Fluor-conjugated secondary antibodies (1:1000) for 1hr at room temperature. Tissue sections were then incubated with DAPI (Invitrogen, 5mg/ml) for 5 min, washed two times with 1xPBS, and then sealed with cover slips mounted with ProLong Gold (Invitrogen).

For detection of Cre, tissue sections were rinsed in 1xPBS to remove OCT, puddled with citrate buffer, and steamed for 10min in a glass jar in a hot water bath. Sections were blocked with 2% donkey or goat serum/5% dry non-fat milk/4% BSA/1% TTX100 in 1xPBS and incubated overnight in primary antibody. For detection of GFP,

tissue sections were rinsed in 1xPBS to remove OCT, puddled with citrate buffer, and steamed for 10min in a glass jar in a hot water bath. Sections were blocked with 2% donkey or goat serum/0.3% TTX100 in 1xPBS and incubated overnight in primary antibody. For triple staining with MASH1, SEC8, and LSD1 or OMP and Acet-Tub, mouse antibodies or OMP were incubated together overnight and LSD1 or Acet-Tub, respectively, were incubated for 1hr the following day.

Image processing and quantification. Images were captured using a Nikon A1R confocal microscope. ImageJ software was used to measure the length of the OE (in µm) in each image, to count specific cell types with the cell counter plugin, and to measure the TH intensity. In order to quantify cell types, 10-15 images were taken from the dorsal medial, dorsal lateral, ventral medial, and ventral lateral regions, across 3-4 sections of the OE. Cell counts were averaged and converted to number of cells/mm of OE. Quantitation was performed for N= 3-6 mice in all control, iHBC-IFT88, and iHBC-Arl13b groups, unless otherwise stated. Reported N-values represent the number of mice examined. Measurements of TH intensity were quantified for all glomeruli in each of 3-4 sections of the OB in 4 iHBC-IFT88 and 4 control mice using Image J software. A blind experimental paradigm was utilized to eliminate bias during image processing and quantification.

Statistics. Statistical significance was determined using an unpaired t test with GraphPad Prism software. Data are presented as mean $\pm$ SEM with two-tailed P values less than 0.05 considered significant.

2.6 Acknowledgments

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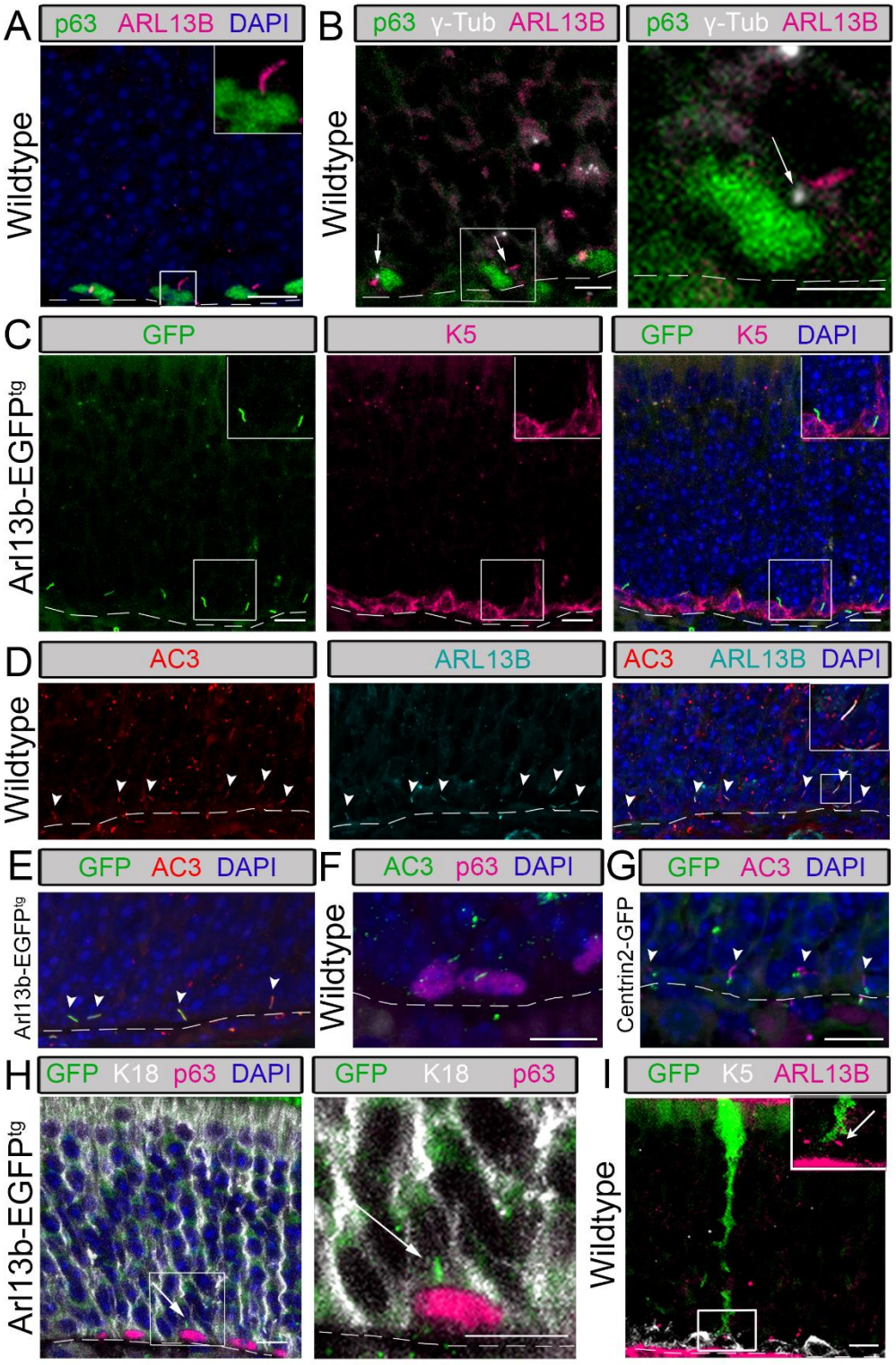
2.7 Figures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Wildtype
B
p63 γ-Tub ARL13B
Wildtype
p63 γ-Tub ARL13B
C
GFP
K5
GFP K5 DAPI
Arl13b-EGFPtg
D
AC3
ARL13B
AC3 ARL13B DAPI
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E
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Arl13b-EGFPtg
F
AC3 p63 DAPI
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G
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Centrin2-GFP
H
GFP K18 p63 DAPI
GFP K18 p63
Arl13b-EGFPtg
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GFP K5 ARL13B
Wildtype

Figure 2-1. HBCs possess primary cilia. Immunofluorescence staining was performed on tissue from the olfactory epithelium of 3-6 week old wildtype, *Arl13b-EGFP*^{tg}, and Centrin2-*E*GFP^{tg} mice. ***A,*** P63-labeled HBCs possess ARL13B-labeled cilia (inset - magnified image). Scale bars = 10µm. ***B,*** Cilia extend from gamma (γ) tubulin-labeled basal bodies (arrows). Low magnification view (left) and high magnification view (right) of ciliated HBC. Scale bars = 5µm. ***C,*** In *Arl13b-EGFP*^{tg} mice, K5-labeled HBCs possess GFP+ cilia (inset – magnified image). Scale bars = 10µm. ***D,*** HBC cilia labeled with canonical cilia markers Adenylate cyclase III (AC3) and ARL13B. ***E,*** In *Arl13b-EGFP*^{tg} mice, AC3 labels GFP+ cilia. ***F,*** P63-labeled HBCs possess AC3-labeled cilia. ***G,*** In Centrin2-*E*GFP^{tg} mice, GFP expressing basal bodies possess AC3+ cilia. ***H,*** In *Arl13b-EGFP*^{tg} mice, GFP+ cilia (arrows) project from p63-labeled HBCs into the interstitial space between HBCs and K18-labeled SUS cell endfeet (inset - magnified image. Scale bars = 5µm. ***I,*** In wildtype mice intranasally infected with adenovirus containing GFP, K5-labeled HBCs possess ARL13B-labeled cilia (see arrow) that project into the interstitial space between an HBC and GFP+ endfoot of a SUS cell (see inset for higher magnified image). Scale bar = 10µm. N=10 total mice for all immunostaining. Dashed lines = basement membrane. HBCs = horizontal basal cells, GFP = green fluorescent protein, K5 = Keratin 5, K18 = Keratin 18, and SUS cells = sustentacular cells.

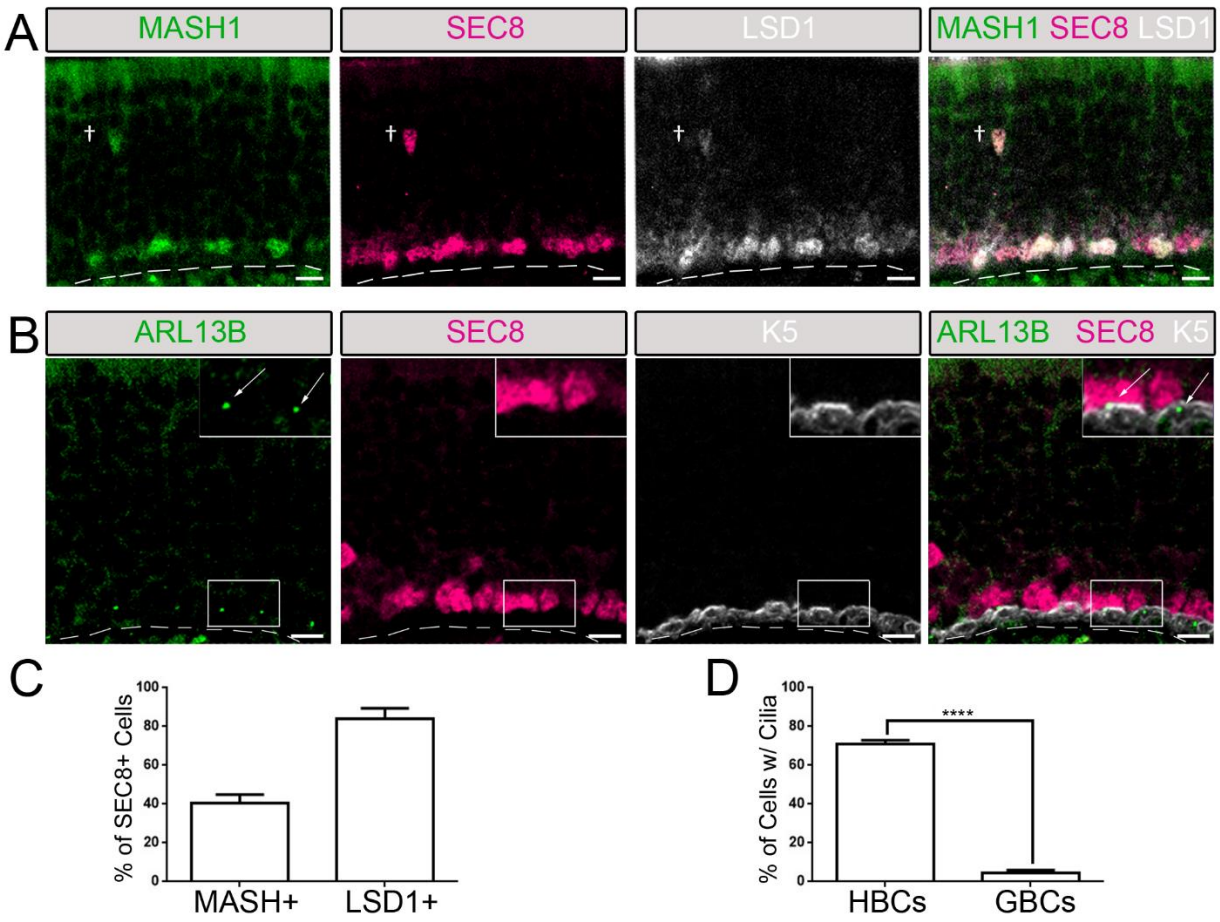

Figure 2-2. HBCs are the predominantly ciliated olfactory basal stem cell. Immunofluorescence staining was performed in the olfactory epithelium of wildtype mice. ***A,*** The canonical GBC marker, MASH1, co-localizes with a subset of SEC8+ GBCs, while, LSD1, co-localizes with a larger subset of SEC8+ GBCs. ***B*****,** Few SEC8-labeled GBCs possess ARL13B-labeled cilia (see arrows) compared to K5-labeled HBCs (inset – magnified image). Scale bars = 10µm. Dashed line = basement membrane. † Occasional migrating GBC. ***C,*** Quantified data of SEC8+ cells that are either MASH1+ (N=4) or LSD1+ (N=4), and ***D,*** the percentage of HBCs (N=6) and GBCs (N=6) that possess cilia. ****P<0.0001 by Student *t* test. Data shown are means ± SEM. HBCs = horizontal basal cells, GBCs = globose basal cells, and K5 = Keratin 5.

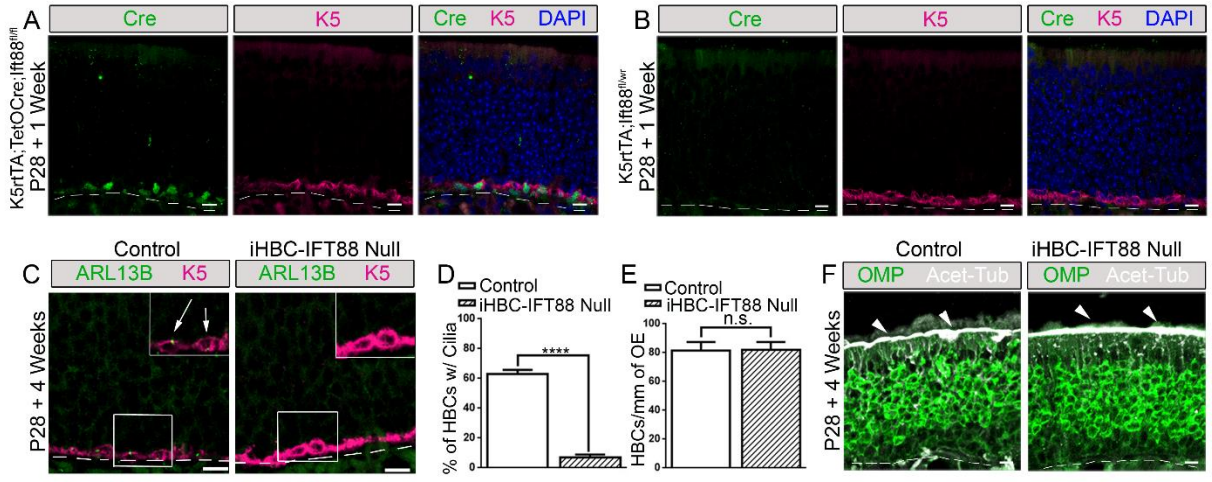

Figure 2-3. Cell-specific deletion of *Ift88* in HBCs results in the loss of HBC cilia with no effects on OSN cilia. Control and iHBC-IFT88 mutant mice (referred to as iHBC-IFT88) were administered a doxycycline (dox) diet at postnatal day 28 (P28) for 1 or 4 weeks and immunofluorescence staining of the olfactory epithelium was performed. After 1 week of dox, ***A,*** Cre is present in K5-labeled HBCs of mice with *K5rtTA* and *TetOCre* alleles but ***B,*** Cre is absent in mice lacking the *TetOCre* allele. After 4 weeks of dox, ***C,*** in control mice but not iHBC-IFT88 mice, K5-labeled HBCs possess Arl13b-labeled cilia (see arrows). See inset for higher magnified image. ***D, E*** Quantified data show the percentage of HBCs that are ciliated in control mice is significantly reduced by ~88.5% in iHBC-IFT88 mice with no significant difference in the number of HBCs per mm of OE in control and iHBC-IFT88 mice after 4 weeks of dox. ***F,*** Acetylated α tubulin (Acet-Tub)-labeled cilia are still present in OMP-labeled mature OSNs (see short arrows). Scale bars = 10μm. Dashed lines = basement membrane. N=5 for both groups. ****P<0.0001, n.s. = not significant by Student *t* test. Data shown are means ± SEM. IFT88 = intraflagellar transport 88, HBCs = horizontal basal cells, cells, OSNs = olfactory sensory neurons, Cre = Cre Recombinase, K5 = Keratin 5, and OMP = olfactory marker protein.

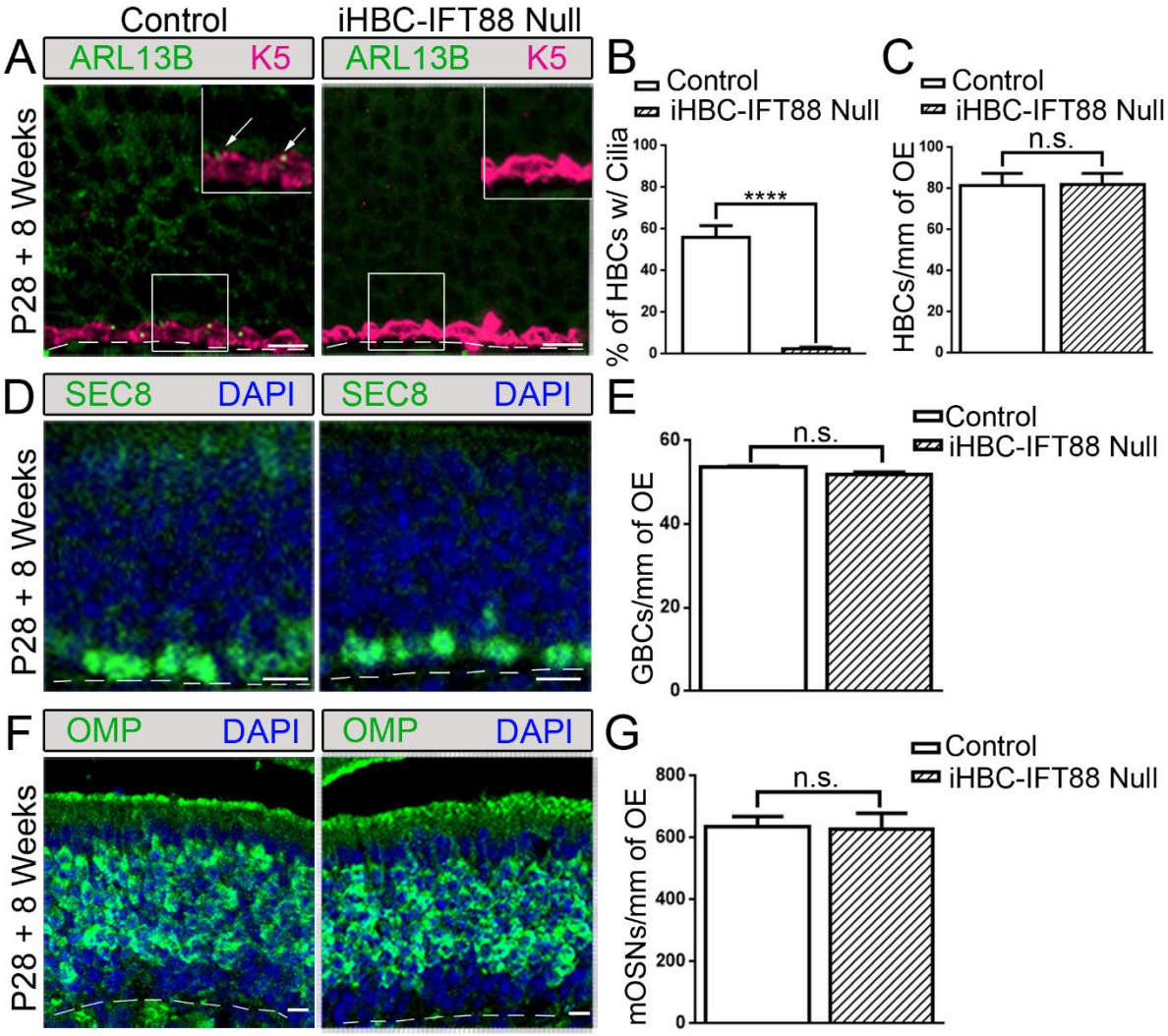

Figure 2-4. Loss of HBC cilia has no homeostatic effects on the cell composition of the olfactory epithelium (OE). Control and iHBC-IFT88 mice were administered a doxycycline diet at postnatal day 28 (P28) for 8 weeks. ***A,*** In control mice, but not iHBC-IFT88 mice, K5-labeled HBCs possess ARL13B-labeled cilia (see arrows) (inset – magnified image). ***B, C,*** Quantified data show a significant loss of ciliated HBCs in iHBC-IFT88 mice with no change in the number of HBCs per mm of OE. ***D,*** SEC8-labeled GBCs in the OE of control and iHBC-IFT88 mice. ***E,*** Quantified data show no significant difference in the number of GBCs per mm of OE in both groups. ***F,*** OMP-labeled mature OSNs in control and iHBC-IFT88 mice. ***G,*** Quantified data show no significant difference in the number of mature OSNs per mm of OE between both groups. Scale bars = 10µm. Dashed lines = basement membrane. N=2 for control mice and N=4 for iHBC-IFT88 mice. ****P<0.0001, n.s. = not significant by Student *t* test. Data shown are means ± SEM. HBC = horizontal basal cells, IFT88 = intraflagellar transport 88, K5 = Keratin 5, GBCs = globose basal cells, OMP = olfactory marker protein, and OSNs = olfactory sensory neurons.

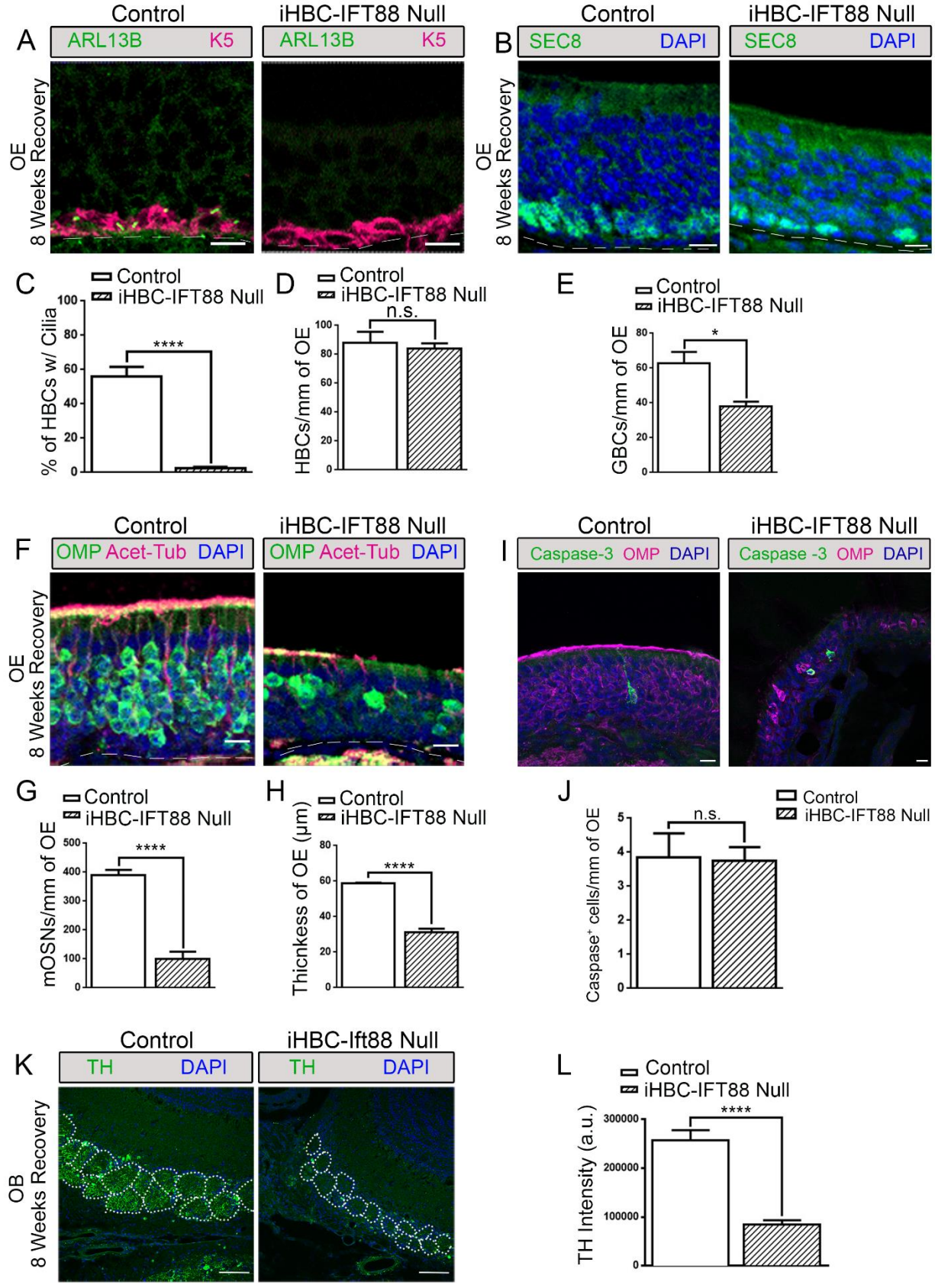
A
Control
iHBC-IFT88 Null
ARL13B K5
ARL13B K5
OE
8 Weeks Recovery
B
Control
iHBC-IFT88 Null
SEC8 DAPI
SEC8 DAPI
OE
8 Weeks Recovery
C
Control
iHBC-IFT88 Null
% of HBCs w/ Cilia
****
D
Control
iHBC-IFT88 Null
n.s.
HBCs/mm of OE
E
Control
iHBC-IFT88 Null
*
GBCs/mm of OE
F
Control
iHBC-IFT88 Null
OMP Acet-Tub DAPI
OMP Acet-Tub DAPI
OE
8 Weeks Recovery
I
Control
iHBC-IFT88 Null
Caspase-3 OMP DAPI
Caspase -3 OMP DAPI
G
Control
iHBC-IFT88 Null
mOSNs/mm of OE
****
H
Control
iHBC-IFT88 Null
Thickness of OE (μm)
****
J
Control
iHBC-IFT88 Null
n.s.
Caspase+ cells/mm of OE
K
Control
iHBC-Ift88 Null
TH DAPI
TH DAPI
OB
8 Weeks Recovery
L
Control
iHBC-IFT88 Null
****
TH Intensity (a.u.)

Figure 2-5. Loss of HBC cilia results in the improper regeneration of the olfactory epithelium (OE) and loss of tyrosine hydroxylase (TH) expression in the olfactory bulb (OB). Control and iHBC-IFT88 mice were administered a doxycycline (dox) diet at postnatal day 28 (P28) for 4 weeks and then given an intraperitoneal injection of 75mg/kg of Methimazole to ablate the OE. Following 8 weeks of recovery, immunofluorescence staining was performed. ***A,*** In the OE of control mice but not iHBC-IFT88 mice, K5-labeled HBCs possess ARL13B-labeled cilia. ***B,*** SEC8-labeled GBCs in the OE of control and iHBC-IFT88 mice. ***C-D,*** Quantified data show the percentage of HBCs that are ciliated in control mice is significantly reduced in iHBC-IFT88 mice with no difference in the number of HBCs per mm of OE between both groups. ***E,*** Quantified data show a significant reduction in the number of GBCs per mm of OE. ***F,*** OMP-labeled mature OSNs and acetylated α tubulin (Acet-Tub)-labeled cilia in the OE of control and iHBC-IFT88 mice. ***G,*** Quantified data show a significant reduction in the number of mature OSNs per mm of OE, and ***H,*** a significantly thinner OE in iHBC-IFT88 mice. ***I - J,*** No difference in the number of cleaved caspase-3 labeled apoptotic cells was observed in the OE of control and iHBC-IFT88 mice. ***K,*** TH expression within glomeruli (dotted circles) in the OBs of control and iHBC-IFT88 mice. ***L,*** Quantified data show that the intensity of TH measured in arbitrary units is significantly reduced in the OBs of iHBC-IFT88 mice. Scale bars = 10μm. Dashed lines = basement membrane. N=4 for both groups. *P<0.05, ****P<0.0001, n.s. = not significant by Student *t* test. Data shown are means ± SEM. HBCs = horizontal basal cells, IFT88 = intraflagellar transport 88, K5 = Keratin 5, GBCs = globose basal cells, OMP = olfactory marker protein, and OSNs = olfactory sensory neurons.

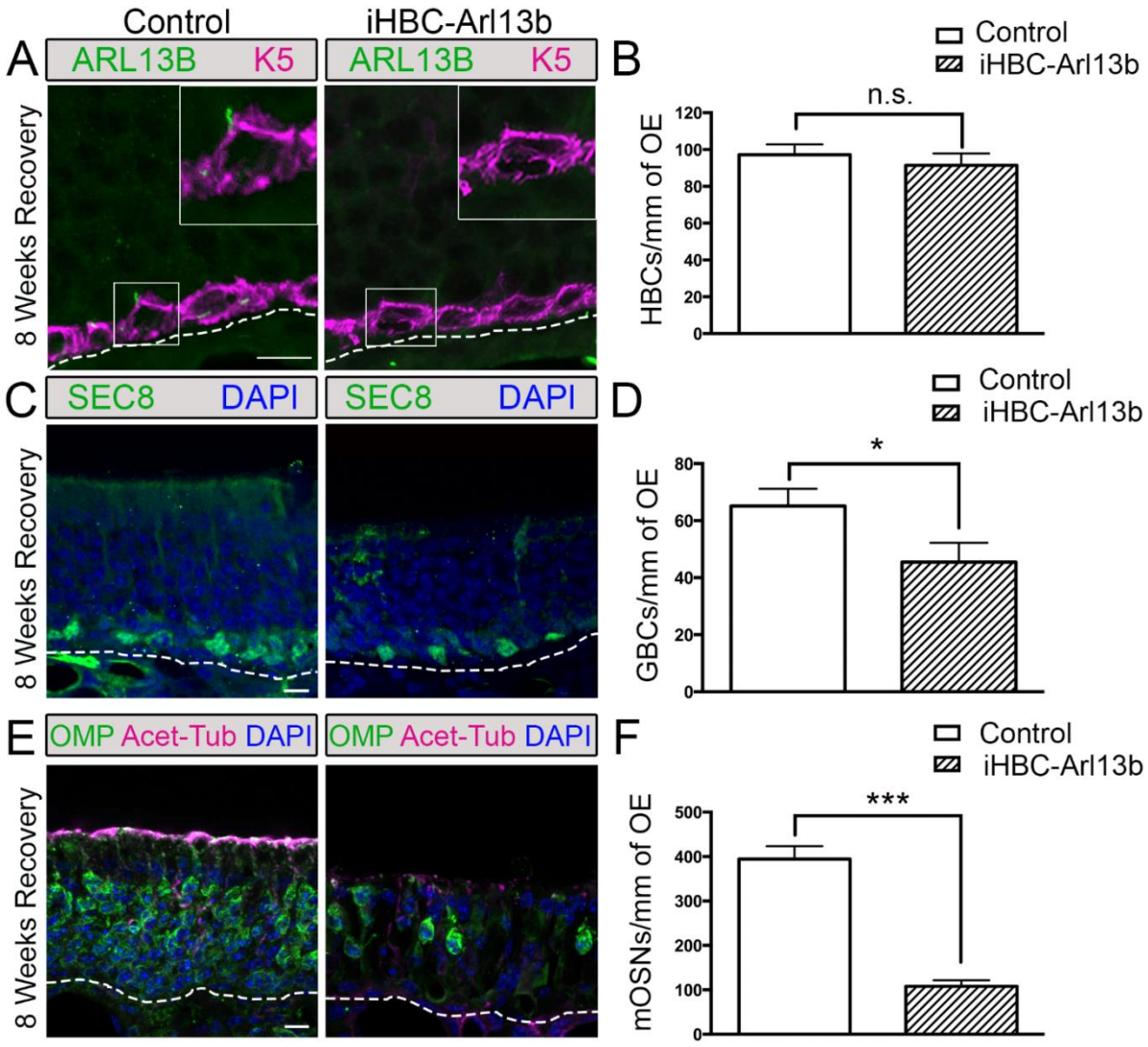

Figure 2-6. **Loss of *Arl13b* in HBCs results in the improper regeneration of the olfactory epithelium (OE).** Control and iHBC-ARL13B mice were administered a doxycycline diet at postnatal day 28 for 4 weeks to induce deletion of *Arl13b*. Mice were then given an intraperitoneal injection of 75mg/kg of methimazole to ablate the OE and allowed 8 weeks of recovery. ***A,*** K5-labeled HBCs in the OE of iHBC-ARL13B mice do not possess ARL13B-labeled cilia. ***B*****,** The percentage of HBCs per mm of OE in both control and iHBC-ARL13B mice. ***C,*** SEC8-labeled GBCs in the OE of control and iHBC-ARL13B mice. ***D,*** Quantified data show a significant reduction in the number of GBCs per mm of OE. ***E,*** OMP-labeled mature OSNs and acetylated α tubulin (Acet-Tub)-labeled cilia in the OE of control and iHBC-ARL13B mice. ***F,*** Quantified data show a significant reduction in the number of mature OSNs per mm of OE. Scale bars = 10μm. Dashed lines = basement membrane. N=3 for both groups. *P<0.05, ***P<0.001, n.s. = not significant by Student *t* test. Data shown are means ± SEM. HBCs = horizontal basal cells, ARL13B = ADP-ribosylation factor-like protein 13b, K5 = Keratin 5, GBCs = globose basal cells, OMP = olfactory marker protein, and mOSNs = mature olfactory sensory neurons.

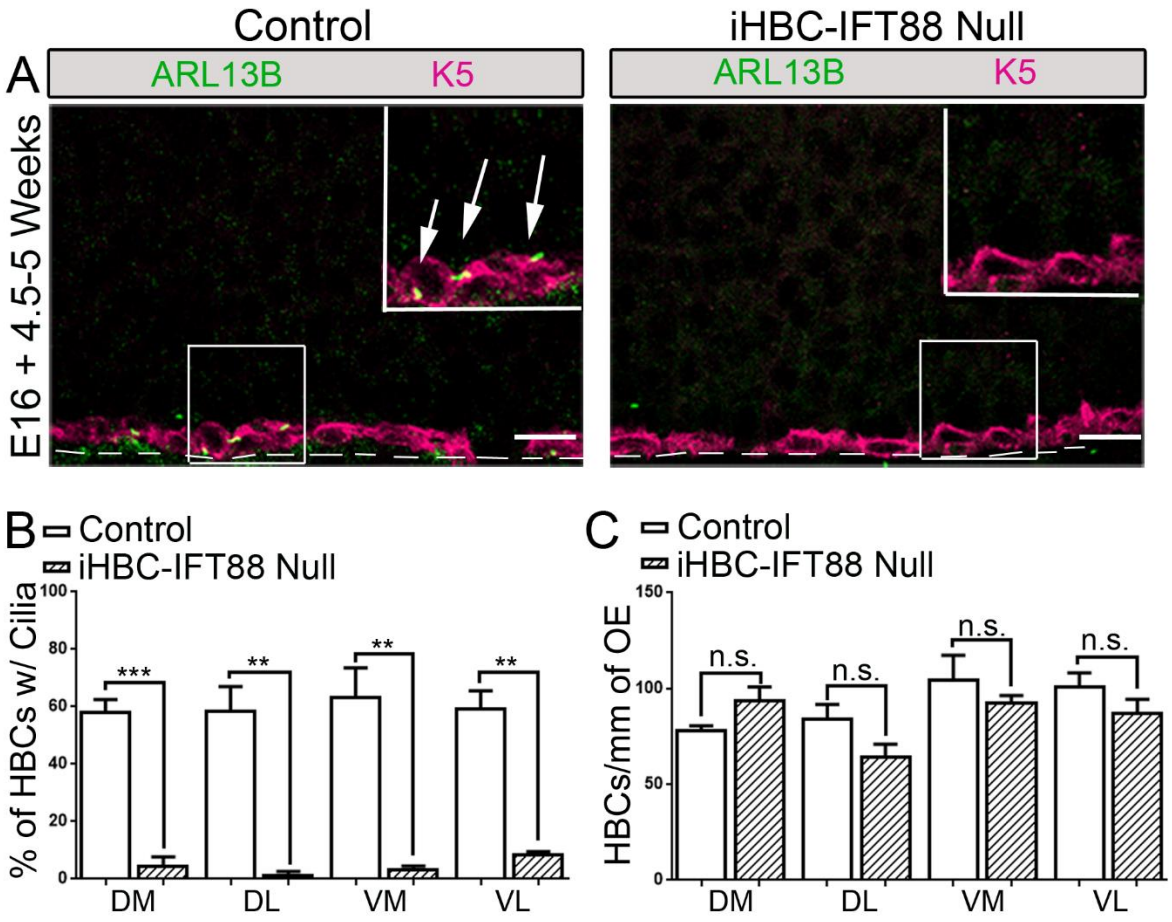

Figure 2-7. Conditional deletion of *Ift88* in HBCs during development of the olfactory epithelium (OE) results in the loss of HBC cilia with no effects on the number of HBCs. Control and iHBC-IFT88 null mice were treated with doxycycline at embryonic day 16 (E16) for 4.5-5 weeks and analyzed at postnatal day 28 (P28) with immunofluorescence staining of the OE. ***A,*** In control mice, but not iHBC-IFT88 null mice, K5-labeled HBCs possess ARL13B-labeled cilia (see arrows) (inset - magnified image). Scale bars = 10µm. Dashed lines = basement membrane. Quantified data show ***B,*** the percentage of HBCs that are ciliated in control mice is significantly reduced in iHBC-IFT88 null mice across all regions and ***C,*** the number of HBCs per mm of OE is similar across all regions in both groups. N=3 for both groups. **P<0.01, ***P<0.001, n.s. = not significant by Student *t* test. Data shown are means ± SEM. HBCs = horizontal basal cells, IFT88 = intraflagellar transport 88, K5 = Keratin 5, DM = dorsal medial, DL = dorsal lateral, VM = ventral medial, and VL = ventral lateral.

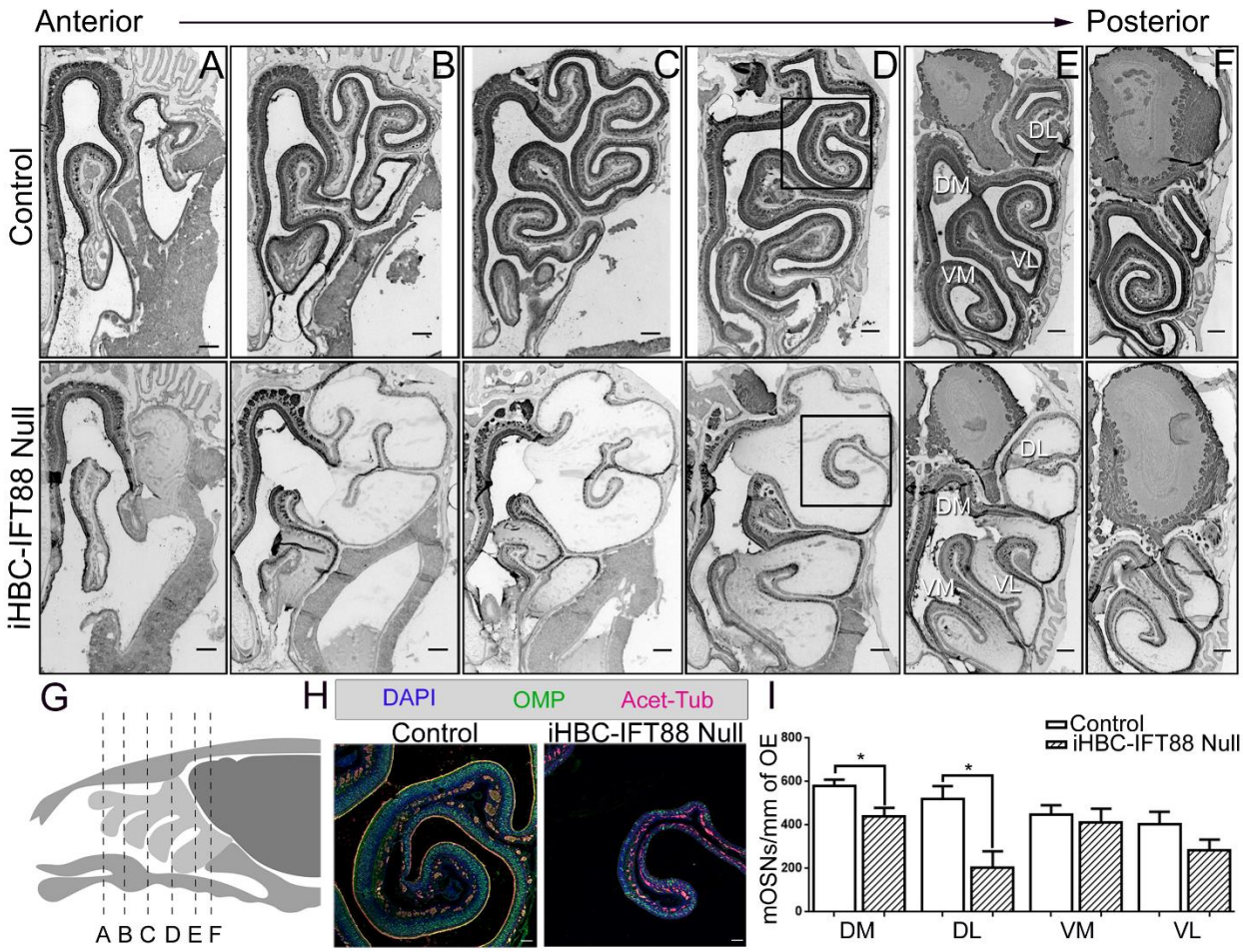

Figure 2-8. Loss of HBC cilia results in the impaired development of regions in the olfactory epithelium (OE). Control and iHBC-IFT88 mice were treated with doxycycline at embryonic day 16 (E16) for 4.5-5 weeks and analyzed at postnatal day 28 (P28) with immunofluorescence staining of the OE. ***A-F,*** OMP-labeled mature OSNs in anterior to posterior sections of control and iHBC-IFT88 mice. Scale bars = 200μm. ***G,*** Illustration depicting the location of sections A-F in a sagittal view of the mouse olfactory organ. ***H,*** Magnified view of boxed region in ***D*** with OMP-labeled mature OSNs and acetylated α tubulin (Acet-Tub)-labeled cilia in the OE of control and iHBC-IFT88 mice. Scale bars = 50μm. ***I,*** Quantified data show the number of mature OSNs per mm of OE in iHBC-IFT88 mice is significantly reduced specifically in dorsal lateral and medial regions of the OE but not in ventral regions. N=5 for both groups. *P<0.05 by Student *t* test. Data shown are means ± SEM. HBCs = horizontal basal cells, IFT88 = intraflagellar transport 88, OMP = olfactory marker protein, OSNs = olfactory sensory neurons, DM = dorsal medial, DL = dorsal lateral, VM = ventral medial, and VL = ventral lateral.

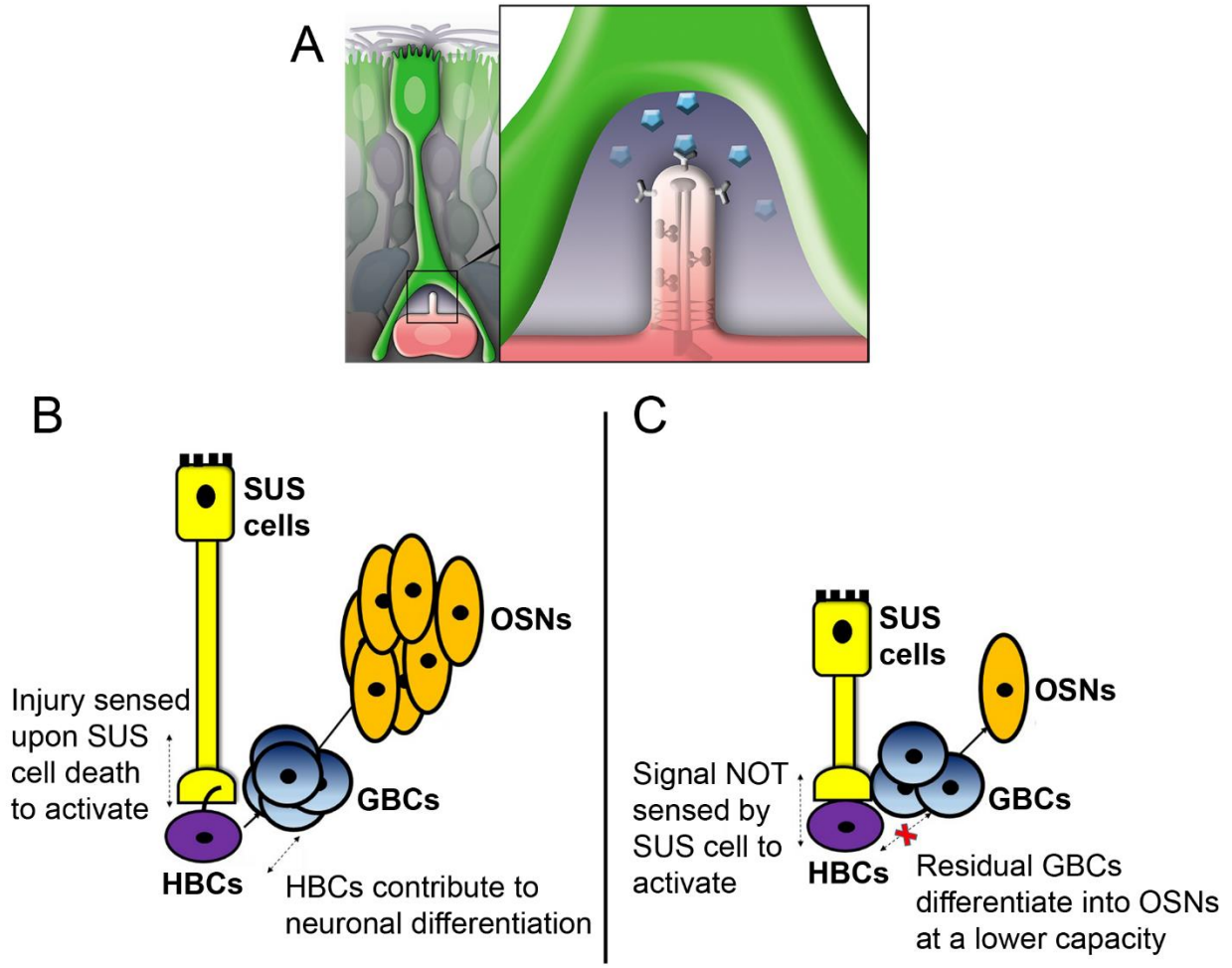

Figure 2-9. Model of HBC cilia-dependent neurogenesis during restoration and development of the olfactory epithelium (OE). ***A,*** Illustration shows that the SUS endfoot may release some signal factor that is received and transduced by HBC cilia. ***B,*** Following an injury, when HBCs possess cilia, SUS cells alert HBCs to the lesion resulting in their activation and production of additional GBCs, which contribute to regeneration of OSNs. ***C,*** Without cilia, HBCs no longer detect a signal from SUS cells, resulting in limited recovery from residual GBCs and a thinner OE. HBCs = horizontal basal cells, SUS cells = sustentacular cells, GBCs = globose basal cells and OSNs = olfactory sensory neurons.

2.8 References

Alvarez-Buylla A, Garcia-Verdugo JM (2002) Neurogenesis in adult subventricular zone. J Neurosci 22:629-634.

Amador-Arjona A, Elliott J, Miller A, Ginbey A, Pazour GJ, Enikolopov G, Roberts AJ, Terskikh AV (2011) Primary cilia regulate proliferation of amplifying progenitors in adult hippocampus: implications for learning and memory. J Neurosci 31:9933-9944.

Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG (2002) Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behavior genetics 32:435-443.

Baker H, Morel K, Stone DM, Maruniak JA (1993) Adult naris closure profoundly reduces tyrosine hydroxylase expression in mouse olfactory bulb. Brain Res 614:109-116.

Baker H, Cummings DM, Munger SD, Margolis JW, Franzen L, Reed RR, Margolis FL (1999) Targeted deletion of a cyclic nucleotide-gated channel subunit (OCNC1): biochemical and morphological consequences in adult mice. J Neurosci 19:9313-9321.

Bangs FK, Schrode N, Hadjantonakis AK, Anderson KV (2015) Lineage specificity of primary cilia in the mouse embryo. Nature cell biology 17:113-122.

Barber PC (1982) Neurogenesis and regeneration in the primary olfactory pathway of mammals. Bibl Anat:12-25.

Beites CL, Kawauchi S, Crocker CE, Calof AL (2005) Identification and molecular regulation of neural stem cells in the olfactory epithelium. Experimental cell research 306:309-316.

Berbari NF, O'Connor AK, Haycraft CJ, Yoder BK (2009) The primary cilium as a complex signaling center. Curr Biol 19:R526-535.

Bergman U, Brittebo EB (1999) Methimazole toxicity in rodents: covalent binding in the olfactory mucosa and detection of glial fibrillary acidic protein in the olfactory bulb. Toxicol Appl Pharmacol 155:190-200.

Bishop GA, Berbari NF, Lewis J, Mykytyn K (2007) Type III adenylyl cyclase localizes to primary cilia throughout the adult mouse brain. J Comp Neurol 505:562-571.

Brittebo EB (1995) Metabolism-dependent toxicity of methimazole in the olfactory nasal mucosa. Pharmacol Toxicol 76:76-79.

Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML (2001) The immunological synapse. Annual review of immunology 19:375-396.

Caggiano M, Kauer JS, Hunter DD (1994) Globose basal cells are neuronal progenitors in the olfactory epithelium: a lineage analysis using a replication-incompetent retrovirus. Neuron 13:339-352.

Calof AL, Chikaraishi DM (1989) Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. Neuron 3:115-127.

Cantagrel V et al. (2008) Mutations in the cilia gene ARL13B lead to the classical form of Joubert syndrome. Am J Hum Genet 83:170-179.

Carter LA, MacDonald JL, Roskams AJ (2004) Olfactory horizontal basal cells demonstrate a conserved multipotent progenitor phenotype. J Neurosci 24:5670-5683.

Caspary T, Larkins CE, Anderson KV (2007) The graded response to Sonic Hedgehog depends on cilia architecture. Dev Cell 12:767-778.

Cau E, Casarosa S, Guillemot F (2002) Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129:1871-1880.

Cau E, Gradwohl G, Fode C, Guillemot F (1997) Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124:1611-1621.

Chen M, Tian S, Yang X, Lane AP, Reed RR, Liu H (2014) Wnt-responsive Lgr5(+) globose basal cells function as multipotent olfactory epithelium progenitor cells. J Neurosci 34:8268-8276.

Chizhikov VV, Davenport J, Zhang Q, Shih EK, Cabello OA, Fuchs JL, Yoder BK, Millen KJ (2007) Cilia proteins control cerebellar morphogenesis by promoting expansion of the granule progenitor pool. J Neurosci 27:9780-9789.

Conley DB, Robinson AM, Shinners MJ, Kern RC (2003) Age-related olfactory dysfunction: cellular and molecular characterization in the rat. Am J Rhinol 17:169-175.

Costanzo RM (1991) Regeneration of olfactory receptor cells. Ciba Found Symp 160:233-242; discussion 243-238.

Croyle MJ, Lehman JM, O'Connor AK, Wong SY, Malarkey EB, Iribarne D, Dowdle WE, Schoeb TR, Verney ZM, Athar M, Michaud EJ, Reiter JF, Yoder BK (2011) Role of epidermal primary cilia in the homeostasis of skin and hair follicles. Development 138:1675-1685.

Delling M, DeCaen PG, Doerner JF, Febvay S, Clapham DE (2013) Primary cilia are specialized calcium signalling organelles. Nature 504:311-314.

DeMaria S, Ngai J (2010) The cell biology of smell. J Cell Biol 191:443-452.

Diamond I, Owolabi T, Marco M, Lam C, Glick A (2000) Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators tTA and rTA linked to the keratin 5 promoter. The Journal of investigative dermatology 115:788-794.

Doyle KL, Khan M, Cunningham AM (2001) Expression of the intermediate filament protein nestin by sustentacular cells in mature olfactory neuroepithelium. J Comp Neurol 437:186-195.

Ducray A, Bondier JR, Michel G, Bon K, Millot JL, Propper A, Kastner A (2002) Recovery following peripheral destruction of olfactory neurons in young and adult mice. Eur J Neurosci 15:1907-1917.

Duldulao NA, Lee S, Sun Z (2009) Cilia localization is essential for in vivo functions of the Joubert syndrome protein Arl13b/Scorpion. Development 136:4033-4042.

Edge AS, Chen ZY (2008) Hair cell regeneration. Curr Opin Neurobiol 18:377-382.

Finetti F, Paccani SR, Rosenbaum J, Baldari CT (2011) Intraflagellar transport: a new player at the immune synapse. Trends in immunology 32:139-145.

Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.

Genter MB, Deamer NJ, Blake BL, Wesley DS, Levi PE (1995) Olfactory toxicity of methimazole: dose-response and structure-activity studies and characterization of flavin-containing monooxygenase activity in the Long-Evans rat olfactory mucosa. Toxicologic pathology 23:477-486.

Goetz SC, Anderson KV (2010) The primary cilium: a signalling centre during vertebrate development. Nat Rev Genet 11:331-344.

Gokoffski KK, Kawauchi S, Wu HH, Santos R, Hollenbeck PLW, Lander AD, Calof AL (2010) Feedback Regulation of Neurogenesis in the Mammalian Olfactory Epithelium: New Insights from Genetics and Systems Biology. In: The Neurobiology of Olfaction (Menini A, ed). Boca Raton (FL).

Grachtchouk M, Pero J, Yang SH, Ermilov AN, Michael LE, Wang A, Wilbert D, Patel RM, Ferris J, Diener J, Allen M, Lim S, Syu LJ, Verhaegen M, Dlugosz AA (2011) Basal cell carcinomas in mice arise from hair follicle stem cells and multiple epithelial progenitor populations. J Clin Invest 121:1768-1781.

Graziadei PP, Graziadei GA (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol 8:1-18.

Guadiana SM, Semple-Rowland S, Daroszewski D, Madorsky I, Breunig JJ, Mykytyn K, Sarkisian MR (2013) Arborization of dendrites by developing neocortical neurons is dependent on primary cilia and type 3 adenylyl cyclase. J Neurosci 33:2626-2638.

Gunther EJ, Belka GK, Wertheim GB, Wang J, Hartman JL, Boxer RB, Chodosh LA (2002) A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. FASEB J 16:283-292.

Haycraft CJ, Zhang Q, Song B, Jackson WS, Detloff PJ, Serra R, Yoder BK (2007) Intraflagellar transport is essential for endochondral bone formation. Development 134:307-316.

Higginbotham H, Bielas S, Tanaka T, Gleeson JG (2004) Transgenic mouse line with green-fluorescent protein-labeled Centrin 2 allows visualization of the centrosome in living cells. Transgenic research 13:155-164.

Higginbotham H, Eom TY, Mariani LE, Bachleda A, Hirt J, Gukassyan V, Cusack CL, Lai C, Caspary T, Anton ES (2012) Arl13b in primary cilia regulates the migration and placement of interneurons in the developing cerebral cortex. Dev Cell 23:925-938.

Higginbotham H, Guo J, Yokota Y, Umberger NL, Su CY, Li J, Verma N, Hirt J, Ghukasyan V, Caspary T, Anton ES (2013) Arl13b-regulated cilia activities are essential for polarized radial glial scaffold formation. Nat Neurosci 16:1000-1007.

Holbrook EH, Szumowski KE, Schwob JE (1995) An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. J Comp Neurol 363:129-146.

Holbrook EH, Wu E, Curry WT, Lin DT, Schwob JE (2011) Immunohistochemical characterization of human olfactory tissue. Laryngoscope 121:1687-1701.

Huard JM, Youngentob SL, Goldstein BJ, Luskin MB, Schwob JE (1998) Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. J Comp Neurol 400:469-486.

Irigoin F, Badano JL (2011) Keeping the balance between proliferation and differentiation: the primary cilium. Curr Genomics 12:285-297.

Iwai N, Zhou Z, Roop DR, Behringer RR (2008) Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. Stem Cells 26:1298-1306.

Jang W, Chen X, Flis D, Harris M, Schwob JE (2014) Label-retaining, quiescent globose basal cells are found in the olfactory epithelium. J Comp Neurol 522:731-749.

Jia C, Hegg CC (2010) NPY mediates ATP-induced neuroproliferation in adult mouse olfactory epithelium. Neurobiology of disease 38:405-413.

Jia C, Roman C, Hegg CC (2010) Nickel sulfate induces location-dependent atrophy of mouse olfactory epithelium: protective and proliferative role of purinergic receptor activation. Toxicol Sci 115:547-556.

Kasahara K, Miyoshi K, Murakami S, Miyazaki I, Asanuma M (2014) Visualization of astrocytic primary cilia in the mouse brain by immunofluorescent analysis using the cilia marker Arl13b. Acta medica Okayama 68:317-322.

Krolewski RC, Packard A, Schwob JE (2013) Global expression profiling of globose basal cells and neurogenic progression within the olfactory epithelium. J Comp Neurol 521:833-859.

Krolewski RC, Packard A, Jang W, Wildner H, Schwob JE (2012) Ascl1 (Mash1) knockout perturbs differentiation of nonneuronal cells in olfactory epithelium. PLoS One 7:e51737.

Kumamoto N, Gu Y, Wang J, Janoschka S, Takemaru K, Levine J, Ge S (2012) A role for primary cilia in glutamatergic synaptic integration of adult-born neurons. Nat Neurosci 15:399-405, S391.

Larkins CE, Aviles GD, East MP, Kahn RA, Caspary T (2011) Arl13b regulates ciliogenesis and the dynamic localization of Shh signaling proteins. Mol Biol Cell 22:4694-4703.

Leung CT, Coulombe PA, Reed RR (2007) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10:720-726.

Lie DC, Song H, Colamarino SA, Ming GL, Gage FH (2004) Neurogenesis in the adult brain: new strategies for central nervous system diseases. Annual review of pharmacology and toxicology 44:399-421.

Makino N, Ookawara S, Katoh K, Ohta Y, Ichikawa M, Ichimura K (2009) The morphological change of supporting cells in the olfactory epithelium after bulbectomy. Chem Senses 34:171-179.

Manglapus GL, Youngentob SL, Schwob JE (2004) Expression patterns of basic helix-loop-helix transcription factors define subsets of olfactory progenitor cells. J Comp Neurol 479:216-233.

McIntyre JC et al. (2012) Gene therapy rescues cilia defects and restores olfactory function in a mammalian ciliopathy model. Nat Med 18:1423-1428.

Mucenski ML, Wert SE, Nation JM, Loudy DE, Huelsken J, Birchmeier W, Morrisey EE, Whitsett JA (2003) beta-Catenin is required for specification of proximal/distal cell fate during lung morphogenesis. J Biol Chem 278:40231-40238.

Murdoch B, Roskams AJ (2007) Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. J Mol Histol 38:581-599.

Packard A, Schnittke N, Romano RA, Sinha S, Schwob JE (2011) DeltaNp63 regulates stem cell dynamics in the mammalian olfactory epithelium. J Neurosci 31:8748-8759.

Pedersen LB, Rosenbaum JL (2008) Intraflagellar transport (IFT) role in ciliary assembly, resorption and signalling. Curr Top Dev Biol 85:23-61.

Plotnikova OV, Pugacheva EN, Golemis EA (2009) Primary cilia and the cell cycle. Methods in cell biology 94:137-160.

Robert A, Margall-Ducos G, Guidotti JE, Bregerie O, Celati C, Brechot C, Desdouets C (2007) The intraflagellar transport component IFT88/polaris is a centrosomal protein regulating G1-S transition in non-ciliated cells. J Cell Sci 120:628-637.

Sharma N, Malarkey EB, Berbari NF, O'Connor AK, Vanden Heuvel GB, Mrug M, Yoder BK (2013) Proximal tubule proliferation is insufficient to induce rapid cyst formation after cilia disruption. Journal of the American Society of Nephrology : JASN 24:456-464.

Singla V, Reiter JF (2006) The primary cilium as the cell's antenna: signaling at a sensory organelle. Science 313:629-633.

Tong CK, Han YG, Shah JK, Obernier K, Guinto CD, Alvarez-Buylla A (2014) Primary cilia are required in a unique subpopulation of neural progenitors. Proc Natl Acad Sci U S A 111:12438-12443.

Treloar HB, Miller AM, Ray A, Greer CA (2010) Development of the Olfactory System. In: The Neurobiology of Olfaction (Menini A, ed). Boca Raton (FL).

Vedin V, Molander M, Bohm S, Berghard A (2009) Regional differences in olfactory epithelial homeostasis in the adult mouse. J Comp Neurol 513:375-384.

Wang S, Liu Y, Adamson CL, Valdez G, Guo W, Hsu SC (2004) The mammalian exocyst, a complex required for exocytosis, inhibits tubulin polymerization. J Biol Chem 279:35958-35966.

Wang YZ, Yamagami T, Gan Q, Wang Y, Zhao T, Hamad S, Lott P, Schnittke N, Schwob JE, Zhou CJ (2011) Canonical Wnt signaling promotes the proliferation and neurogenesis of peripheral olfactory stem cells during postnatal development and adult regeneration. J Cell Sci 124:1553-1563.

Wong SY, Seol AD, So PL, Ermilov AN, Bichakjian CK, Epstein EH, Jr., Dlugosz AA, Reiter JF (2009) Primary cilia can both mediate and suppress Hedgehog pathway-dependent tumorigenesis. Nat Med 15:1055-1061.

Xie F, Fang C, Schnittke N, Schwob JE, Ding X (2013) Mechanisms of permanent loss of olfactory receptor neurons induced by the herbicide 2,6-dichlorobenzonitrile: effects on stem cells and noninvolvement of acute induction of the inflammatory cytokine IL-6. Toxicol Appl Pharmacol 272:598-607.

Zhang Q, Hu J, Ling K (2013) Molecular views of Arf-like small GTPases in cilia and ciliopathies. Experimental cell research 319:2316-2322.

CHAPTER III:

Role of Hedgehog Signaling in the Adult Mammalian Olfactory Epithelium

3.1 Abstract

The olfactory epithelium (OE) is a pseudostratified tissue composed of various cell types including olfactory sensory neurons (OSNs) that are readily replaced by two populations of basal stem cells, frequently dividing globose basal cells (GBCs) and quiescent horizontal basal cells (HBCs). This continuous replacement of OSNs makes the OE one of the few tissues to undergo constitutive neurogenesis throughout the mammalian lifespan. Although the cell types involved in the maintenance and regeneration of the OE are known, the signaling pathways that control their activation and proliferation during normal homeostasis, and in response to injury remain largely unexplored. Previous data indicate that HBCs possess primary cilia (Joiner and Green, et. al., submitted), cellular organelles that regulate the activity of multiple signaling pathways, including the Hedgehog (HH) signaling pathway. The HH pathway is implicated in stem and progenitor cell function in several adult tissues, making it an attractive candidate for the regulation of HBCs. Here, I show that various components of the HH pathway are expressed in cells of the OE. Specifically, I found that the HH transcription factors, GLI2 and GLI3 are expressed in HBCs, suggesting that HH signaling is involved in regulation of HBCs. To test this hypothesis, I utilized two inducible, cell-type specific

mouse models (Fig. 3-1): 1) a constitutively active form of *Gli2* (*K5-rtTA;TetO-Gli2ΔN*) and 2) a dominant negative repressive form of *Gli2* (*K5-rtTA;TetO-Gli2ΔC4)* to disrupt HH signaling specifically in HBCs. The constitutive activation of *Gli2* in HBCs resulted in their hyperproliferation and consequential loss of neuronal cells and also impaired regeneration of the OE following lesion. Interestingly, the repressive form of *Gli2* in HBCs did not affect the maintenance of the adult OE but led to a decrease in regeneration following an injury. Together, these results suggest a novel role for HH and more specifically, *Gli2*, in HBC regulation and HBC-driven neurogenesis.

3.2 Introduction

The olfactory epithelium (OE) is a tissue of regeneration and constitutive neurogenesis (Graziadei and Graziadei, 1979; Graziadei and Monti Graziadei, 1983; Calof and Chikaraishi, 1989). Olfactory sensory neurons (OSNs) within the OE act as a direct link between the CNS and the external environment (Doty, 2008), causing them and other cells to be susceptible to various injuries resulting from inhalation of toxicants or metabolism of drugs (Hastings and Miller, 2003; Murphy et al., 2003). Fortuitously, two groups of presumed stem cells: globose basal cells (GBCs) and horizontal basal cells (HBCs) (Murdoch and Roskams, 2007) are able to replenish the OSNs and other non-neuronal cells in the OE. *In vitro* studies and fate-mapping studies in rodents have shown the varying differentiation pathways of HBCs and GBCs under specific conditions. Although in defined conditions HBCs can generate multiple differentiated neuronal and glial progeny *in vitro* (Carter et al., 2004), GBCs were initially thought to be the main contributor to regeneration of the OE (Treloar et al., 2010). However, in more recent fate-mapping studies, in which GBCs are depleted from a severe injury to the OE, HBCs were shown to be multipotent stem cells that can regenerate neuronal and non-neuronal cells (Leung et al., 2007; Iwai et al., 2008).

HBCs and GBCs have distinct characteristics and expression profiles. HBCs lie in a monolayer against the basement membrane of the OE. They are a quiescent, homogeneous population of cells (Holbrook et al., 1995; Carter et al., 2004; Leung et al., 2007) that were recently found to possess primary cilia that were necessary for proper regeneration of the OE following a severe injury (Joiner et al., 2015). GBCs are a heterogeneous population of cells situated just above HBCs and below immature OSNs

(Packard et al., 2011a; Krolewski et al., 2013). Among the GBCs are transit-amplifying progenitor cells and committed neuronal precursors (Jang et al., 2014). During neurogenesis in the OE, a subset of GBCs that are Ascl1+ (Mash1+) transit amplifying cells primarily give rise to Neurogenin1+ (Ngn1+) and NeuroD1+ immediate neuronal precursors (Duggan et al., 2008; Krolewski et al., 2013), and a recent study suggests that a subpopulation of GBCs are also multipotent progenitors that likely give rise to non-neuronal cells (Jang et al., 2014).

Various growth factors and pathways have been reported to play a role in the proliferation and differentiation of basal stem cells (reviewed in Murdoch and Roskams, 2007). In particular, Notch (Manglapus et al., 2004; Krolewski et al., 2012) and Wnt (Chen et al., 2014) are suggested to play a role in the differentiation of GBCs; however, these studies did not use direct *in vivo* models to perturb specific components of the pathways. The recent finding of cilia on HBCs and its role in regeneration of the OE reveals new potential signaling pathways that could be involved in the regulation of HBCs and neurogenesis in the OE.

Primary cilia are cellular organelles that regulate the activity of multiple signaling pathways, including the HH signaling pathway (for review, see ref. Nozawa et al., 2013). In other tissues, primary cilia have important roles in cell proliferation and cell differentiation (for review, see ref. Irigoin and Badano, 2011). The HH pathway is not only important for the development of various tissues and organs but also for the formation, survival, and function of stem and progenitor cell populations in several adult tissues (reviewed in Petrova and Joyner, 2014). Additionally, there is growing evidence that adult stem cells utilize cilia for adult neurogenesis (Amador-Arjona et al., 2011; Kumamoto et

al., 2012; Tong et al., 2014). In particular, disruption of cilia in these systems alters the activity of signaling pathways that utilize cilia, such as HH (Kumamoto et al., 2012; Tong et al., 2014). Canonical HH signaling occurs through a signal transduction cascade mediated by the GPCR-like protein smoothened (SMO) following ligand binding to the canonical HH receptor patched 1 (PTCH1), resulting in modulation of the downstream GLI transcription factors (Chen and Struhl, 1996; Ingham et al., 2000; Fan et al., 2012). In the absence of ligand, PTCH1 suppresses SMO activity, allowing for GLI3, and to a lesser extent GLI2, to be processed into transcriptional repressors; following HH binding to PTCH1, SMO enters the cilium and promotes the processing of GLI2 into a transcriptional activator and the degradation of GLI3 (for review, see ref. Petrova and Joyner, 2014). Due to its utilization of cilia and the fact that cilia regulate HBC-driven neurogenesis in the OE, the HH pathway is an attractive candidate for the regulation of HBCs. However, the HH pathway remains largely unexplored in the mammalian OE (Chou et al., 2010). Interestingly, a study reported that the HH target gene, *Pax6* (Briscoe et al., 2000), is expressed in HBCs and is essential for the differentiation of HBCs into neuronal cells during regeneration following an MMI-induced injury to the OE (Suzuki et al., 2015), suggesting a potential role for HH signaling in OE maintenance.

Here, I show that components of the HH pathway are expressed in distinct cell populations in the OE, suggesting a potential role for the pathway in olfactory tissue maintenance. Specifically, HBCs express the transcription factors GLI2, and GLI3, suggesting a role for the HH pathway in the molecular regulation of olfactory basal stem cells in the OE. Following expression of a constitutively active form of GLI2 (GLI2ΔN) (Fig. 3-1) in HBCs of mice, HBCs become hyperproliferative with an upregulation of the GBC

marker, SEC8, and a consequential loss of neuronal cells. Additionally, there is incomplete regeneration of the OE following an injury. Conversely, HBC-specific expression of a dominant-negative GLI2 repressor (GLI2ΔC4) (Fig. 3-1) does not change the cell types of the OE during normal tissue homeostasis; however, there is a reduction in regeneration and neurogenesis following an injury. These data identify expression of HH pathway components in specific cells of the OE, implicating a previously unrecognized pathway (HH) in the regulation of OE homeostasis. Additionally, these data provide mechanistic insight into the regulation of HBCs during olfactory regeneration, and indicate a potential role for HH signaling in the differentiation of HBCs during neurogenesis.

3.3 Results

HH components are found in cells of the olfactory periphery.

The HH pathway is an attractive candidate for the regulation of HBCs due to its dependence on primary cilia in tissue maintenance (Croyle et al., 2011). However, in the mammalian olfactory epithelium (OE), a potential role for the HH pathway remains largely unexplored (Gong et al., 2009). To investigate if components of the HH pathway were present in the OE, I analyzed the heads of *lacZ* reporter mice with an X-Gal stain to read out expression of the specific transcript of interest (Fig. 3-2). Tissue from these mice was incubated with an X-Gal substrate that is cleaved by β-Galactosidase, producing a blue precipitate that indicates *lacZ* expression. Compared to control tissue in which no color is present (Fig. 3-2A-C'''), sagittal views of the whole mouse head indicate that most core HH pathway components are found throughout various regions of the brain, including the

cerebellum (Fig. 3-2D, G, J, M). Interestingly, *Gli1*, a general readout of HH activity (Lee et al., 1997) is not expressed in the olfactory bulb (OB) (Fig. 3-2E) and only faintly detected in the olfactory turbinates (bony structures that are comprised of OE) specifically in the stroma (Fig. 3-2F'-F'''). *Gli2*, the common transcriptional activator of the HH pathway (Matise et al., 1998; Pan et al., 2006), is expressed in the dorsal part of the OB (Fig. 3-2H) and throughout all olfactory turbinates in more ventral and anterior areas (Fig. 3-2I'-I'''). The major transcriptional repressor of the HH pathway, *Gli3* (Litingtung and Chiang, 2000; Wang et al., 2000), is not detected in the OB (similar to *Gli1*; Fig. 3-2K) but is detected in the OE more faintly, but in a similar pattern to *Gli2* (Fig. 3-2L'-L'''). Conversely, the HH receptor, *Ptch1,* which, like *Gli1*, is a direct target of the pathway and reads out HH pathway activity (Agren et al., 2004), is expressed throughout the OB (Fig. 3-2N) and in more dorsal and posterior areas of the olfactory turbinates (Fig. 3-2O'-O'''). The expression profiles of these HH pathway components are unusual, especially because *Gli1* and *Ptch1*, which are typically expressed together, do not appear to have the same expression profile in the olfactory turbinates. Interestingly, the expression profile of *Gli2* is typical of the profile of a repressor of the pathway in which the profile is in opposition to the expression profile of *Ptch1* (Goodrich et al., 1996; Marigo and Tabin, 1996; Ruiz i Altaba, 1998). These data demonstrate that HH components are expressed not only in the CNS, but also in the olfactory periphery.

To analyze the expression profile of the HH components more closely, coronal sections of the *lacZ* reporter mice were obtained and incubated with X-Gal substrate (Fig. 3-3). Again, no color developed in control tissue (Fig. 3-3A-D). Similar to the whole mount images (Fig.3-2), *Gli1* is not expressed in the OE, but instead in cells within the underlying

Lamina Propria (LP) (Fig. 3-3E-H). *Gli2* is expressed in basal cells of the OE (Fig. 3-3I-L) and, in some areas, detected in apical cells that appear to be SUS cells (Fig. 3-3L). Similarly, *Gli3* is also expressed in basal cells (Fig. 3-3M-P) and a subset of SUS cells (3-3N, P), but with less intensity and uniformity than *Gli2*. In contrast, *Ptch1* is strongly expressed in a region-specific and "patchy" pattern in apical cells that appear to be OSNs, and possibly at a lower intensity in basal cells (Fig. 3-3Q-T). Together, these results corroborate the whole mount images in Figure 3-2, and also suggest that HH pathway components are expressed in distinct cell types within the mammalian OE.

HH pathway components are present in distinct cell populations of the olfactory epithelium.

In order to confirm the cell types in the OE that express specific HH pathway components, I performed a sequential X-Gal and immunofluorescence stain on tissue from *Gli2*$^{lacZ/+}$, *Gli3*$^{lacZ/+}$, and *Ptch1*$^{lacZ/+}$ mice. Following incubation with the X-Gal substrate, I immunolabeled the OE with either the recently identified marker of GBCs, SEC8 (Joiner and Green, et. al., submitted), and the HBC marker, Keratin 5 (K5) (Holbrook et al., 1995), or the immature OSN marker, growth-associated protein 43 (GAP-43) (McIntyre et al., 2010) and the mature OSN marker, olfactory marker protein (OMP) (McIntyre et al., 2010). Dual staining revealed that *Gli2* is expressed in all K5+ cells but excluded from SEC8+ cells (Fig. 3-4A), while *Gli3* is present in a subset of K5+ cells but not in SEC8+ cells (Fig. 3-4B). Additionally, I found that *Ptch1* is expressed in OMP+ cells but not in GAP-43+ cells or in more basally located cells (Fig. 3-4C). *Ptch1* expression was thought to be present at a lower intensity in immature OSNs and basal cells based on X-Gal staining in sections of the OE (Fig 3-3Q-T), however, it is possible that this low

signal was not resolved in the dual staining. Nonetheless, these data suggest that *Gli2* and *Gli3* are expressed selectively in HBCs of the OE, while *Ptch1* is expressed in a region-specific manner in mature OSNs.

Constitutively active Gli2 in HBCs results in proliferative HBCs.

GLI2 contains both transcriptional activation and repression domains and therefore, full-length GLI2 is a weak transcriptional activator (Sasaki et al., 1999). Deletion of the N-terminal repression domain results in Gli2 protein that functions as a strong transcriptional activator (Gli2ΔN) in cultured cells and mimics overactive SHH signaling in transgenic mice (Sasaki et al., 1999). In order to investigate the role of GLI2 as a transcriptional activator in HBCs, I analyzed *K5-rtTA;TetO-Gli2ΔN* mice (Grachtchouk et al., 2011) which utilize a doxycycline (dox)-regulated mouse model (Gunther et al., 2002). These mice carry two alleles: 1) a promoter specific dox-inducible strain (*K5-rtTA*) (Diamond et al., 2000) and 2) an effector strain (*tetO-Gli2ΔN*) (Grachtchouk et al., 2011). The *K5* promoter drives expression of the reverse tetracycline transactivator (rtTA) and binding of doxy to rtTA allows for binding to the tetracycline operator (tetO) and expression of *Gli2ΔN* specifically in HBCs in the OE.

K5-rtTA;TetO-Gli2ΔN mice (referred to hereafter as iHBC-Gli2ΔN mice) and control littermates (referred to hereafter as control mice) were initially administered a dox diet to activate Gli2ΔN expression in HBCs after P28, when the OE is mature (Murdoch and Roskams, 2007). Mice were treated with dox for 24 hours, 3 days, 5 days, 7 days, or 10 days. Antibody staining for the HBC markers, p63 and K5, revealed that, compared to control mice (Fig. 3-5AI), which exhibit a typical monolayer of HBCs (Holbrook et al., 1995), there is initially no difference in the appearance and number of HBCs in iHBC-

Gli2ΔN mice after 24 hours (Fig. 3-5AII, B) and 3 days (Fig. 3-5AIII, B). However, following 5 days of dox treatment, HBCs undergo a change in morphology as they become more round and GBC-like in appearance (Fig. 3-5AIV). By 7 and 10 days, the population of HBCs has expanded and are no longer a monolayer of cells (Fig. 3-5AV-AVI). This change in appearance following 5 days of dox is reflected in the doubling, and eventual tripling by 10 days, of the number of HBCs (Fig. 3-5B). Consequently, the increase in the number of HBCs, results in an increase in thickness of the HBC layer (Fig. 3-5C). These data suggest that constitutively active GLI2 causes a switch of the HBCs from a dormant state to an active state. When the transcription factor, p63, is downregulated, HBCs are believed to be activated and differentiate into other cell types (Packard et al., 2011a). However, the additional HBCs seen in iHBC-Gli2ΔN mice still express p63 (Fig. 3-5AV-AVI).

In order to determine the proliferative state of the HBCs in these mice, I utilized the antibody Ki67 which should label cells in the G1, S, and G2 phase of the cell cycle but not in quiescent cells in the G0 phase (Scholzen and Gerdes, 2000). Staining the OE of iHBC-Gli2ΔN and control mice after 10 days of dox with Ki67 and p63 revealed that, compared to controls, in which Ki67 is excluded from p63+ HBCs, iHBC-Gli2ΔN mice display an overall increase in Ki67+ cells which are also p63+ (Fig. 3-5D-E). Typically, HBCs do not express Ki67+ due to their quiescence, but in iHBC-Gli2ΔN mice, approximately 40% of p63+ HBCs express Ki67. Together, these data suggest that constitutively active GLI2 induces HBC proliferation leading to a morphological and quantitative change. These findings further suggest that: 1) active HH signaling promotes

HBC proliferation of HBCs, and 2) p63 downregulation is required for subsequent HBC differentiation.

GLI2 localizes to tips of proliferative HBC primary cilia.

The *Gli2ΔN* transgene contains a MYC tag, allowing for its detection and the localization of GLI2 protein (Grachtchouk et al., 2011). As detailed in Chapter II, HBCs possess primary cilia (Joiner and Green, et. al., submitted) and GLI2 protein localizes to the tips of primary cilia in many HH-responsive tissues (Haycraft et al., 2005; Liu et al., 2005). In order to investigate when and where GLI2ΔN protein was expressed, I immunolabled the OE of iHBC-Gli2ΔN and control mice with MYC and ARL13B, a small Ras GTPase that regulates ciliogenesis and cilia function and labels HBC cilia (Joiner et al., 2015). After 24 hours of dox treatment, although I did not see a change in HBCs (Fig. 3-5AII, B), MYC is present in approximately 70% of HBCs in iHBC-Gli2ΔN mice but not in control mice (Fig. 3-6A-B). By 7 days of dox, nearly 90% of HBCs in iHBC-Gli2ΔN mice express MYC but not in control mice (Fig. 3-6A-B). Interestingly, MYC is present in only 5% of HBC primary cilia in iHBC-Gli2ΔN mice after 24 hours of dox, while MYC is present in nearly 20% of HBC primary cilia in iHBC-Gli2ΔN mice following 7 days of dox (Fig. 3-6C). These data suggest a correlation between the presence of GLI2 protein in primary cilia and subsequent HBC proliferation. Additionally, to determine if Gli2ΔN localized specifically to the tips of HBC primary cilia, I stained the OE with the basal body marker gamma (γ) tubulin in addition to ARL13B and MYC. This staining revealed that MYC was present in HBC primary cilia on opposite ends of the γ-tubulin+ basal bodies (Fig. 3-5D). These data demonstrate that Gli2ΔN does localize to the tips of primary cilia in HBCs.

Constitutively active Gli2 in HBCs results in SEC8 upregulation and loss of neuronal cells in the OE.

To determine the status of other cells in the OE following an atypical proliferation of HBCs not triggered by an injury, I analyzed the neuronal GBC and OSN populations of HBC-Gli2ΔN mice. As previously mentioned, SEC8 labels a majority of the heterogeneous population of GBCs (Joiner and Green, et. al., submitted). Surprisingly, analysis of p63+ HBCs and GBCs immunolabeled with SEC8 revealed that although control mice contain a typical layer of SEC8+ GBCs above a monolayer of p63+ HBCs, iHBC-Gli2ΔN mice after 5 and 10 days of dox contain cells co-expressing SEC8 and p63 (Fig. 3-7A). The overall number of SEC8+ cells increases in iHBC-Gli2ΔN mice after 5 and 10 days of dox (Fig. 3-7B). The percentage of p63+ HBCs that express SEC8, which is 0% in control mice, increases to 60% and 90% of HBCs after 5 and 10 days of dox respectively (Fig. 3-7C). These data suggest that SEC8 is upregulated in the HBCs of iHBC-Gli2ΔN mice and that activation of GLI2 mediates this upregulation.

Although SEC8, labels a majority of GBCs, there are other markers for neuronal committed GBCs such, as MASH1 (Cau et al., 1997). The number of MASH1+ GBCs in the OE of iHBC-Gli2ΔN mice was dramatically decreased after 5 and 10 days of dox compared to control mice (Fig. 3-8A-B). Since all MASH1+ cells are also SEC8+ (Joiner and Green, et. al., submitted), any remaining MASH1+ cells in HBC-Gli2ΔN mice could be indicative of SEC8+ cells that do not co-express p63 (Fig. 3-7C). Additionally, GAP-43+ immature OSNs and OMP+ mature OSNs are both reduced in iHBC-Gli2ΔN mice compared to control mice after 5 days of dox (Fig. 3-8C-D). Together, these data indicate that hyperproliferation of HBCs results in a loss of neuronal cells in the OE and suggest

the need for HBC quiescence to be tightly regulated, possibly mediated by *Gli2* expression in HBCs.

Constitutively active Gli2 in HBCs results in improper regeneration of the olfactory epithelium following injury.

HBCs are believed to be a quiescent population of cells unless activated by severe injury to participate in OE regeneration (Leung et al., 2007). Methimazole (MMI) is an olfactory toxicant whose metabolites induce cell death of OSNs, SUS cells, and GBCs but spares HBCs in the mouse OE (Brittebo, 1995; Packard et al., 2011b). MMI injury to the OE is used to study the response of HBCs during restoration of the OE. Due to the lethality of iHBC-Gli2ΔN mice after prolonged dox treatment, I induced the injury after 5 days of dox, when a phenotype in HBCs was seen, and looked at early recovery time points (2 days, 5 days, and 9 days). Importantly, mice continued the dox diet during the recovery period. On the day of the injury, but after 5 days of dox (Day 0), iHBC-Gli2ΔN mice have an increased number of p63+/K5+ HBCs that are no longer restricted to a monolayer of cells as seen in control mice (Fig. 3-9A, E). Control mice contain an increased number of K5+/p63+ inactive HBCs and K5+/p63- active HBCs at 2 days following an injury, while iHBC-Gli2ΔN mice continue to have an even greater number of both active and inactive HBCs (Fig. 3-9B, E-F). By 5 days following injury, the appearance and number of HBCs of control mice have returned to normal while iHBC-Gli2ΔN mice still display an increased number of K5+/p63+ inactive and K5+/p63- active HBCs (Fig. 3-9C, F-G). HBCs are no longer active at 9 days following injury in control mice, but the number of inactive and active HBCs is dramatically increased in iHBC-Gli2ΔN mice (Fig. 3-9D, F-G). Overall, in control mice, the number of active HBCs appears to peak 2 days

after injury and then decreases over time in control mice, while the number of active and inactive HBCs continues to increase in iHBC-Gli2ΔN mice (Fig. 3-9F-G). These data suggest that HBCs in iHBC-Gli2ΔN mice are still able to become active to replenish HBC numbers, but that they remain in a fixed active state that cannot go on to differentiate into other cells of the OE.

To determine whether other cells in the OE are replaced by the activation and differentiation of HBCs, I immunolabeled the OE of the same mice with the neuronal GBC marker, MASH1, immature OSN marker, GAP-43, and mature OSN marker, OMP. After 5 days of dox, and at the time of injury (Day 0), iHBC-Gli2ΔN mice contain decreased numbers of MASH1+ GBCs (Fig. 3-10A, I), as well as GAP-43+ immature and OMP+ mature OSNs (Fig. 3-9B, J-K) compared to control mice. Following 2 days of recovery, both control and iHBC-Gli2ΔN mice exhibit a decrease in the number of MASH1+ GBCs (Fig. 3-10C, I) and immature and mature OSNs (Fig. 3-10D, J-K). By 5 days following injury, in control mice, MASH1+ GBCs return and are greater in number than prior to injury but are still nearly absent in iHBC-Gli2ΔN mice (Fig. 3-10E, I). Additionally, immature OSNs have returned with very few mature OSNs in control mice, while both populations of cells are absent in iHBC-Gli2ΔN mice (Fig. 3-10F, J-K). The number of MASH1+ GBCs returns to normal by 9 days of recovery in control mice, but are absent in iHBC-Gli2ΔN mice (Fig. 3-10G, I). Similarly, the number of immature OSNs increases in control mice by 9 days after injury with an additional return of mature OSNs, while iHBC-Gli2ΔN mice continue to show no return of immature or mature OSNs (Fig. 3-10H, J-K). These data suggest that constitutive GLI2 activation in HBCs impairs their ability to contribute to regeneration of the OE.

Dominant negative repressive Gli2 in HBCs does not alter OE homeostasis.

To investigate the repressive effects of GLI2 on HBCs, I utilized a dominant negative form of GLI2, Gli2ΔC4, in which the activation domain in the C-terminus is truncated (Sasaki et al., 1999). Similar to iHBC-Gli2ΔN mice, I used *K5-rtTA;TetO-Gli2ΔC4* mice which were generated in the laboratory of Dr. Andrzej Dlugosz at the University of Michigan (unpublished), which utilize the same dox-regulated model. However, in addition to the promoter specific dox-inducible strain (*K5-rtTA*) (Diamond et al., 2000), these mice carry the effector strain (*tetO-Gli2ΔC4*) which was generated in the laboratory of Dr. Dlugosz. The *K5* promoter drives expression of the reverse tetracycline transactivator (rtTA) and binding of doxy to rtTA allows for binding to the tetracycline operator (tetO) and expression of *Gli2ΔC4* specifically in HBCs in the OE.

K5-rtTA;TetO-Gli2ΔC4 mice (referred to hereafter as iHBC-Gli2ΔC4 mice) and control littermates (referred to hereafter as control mice) survive longer than iHBC-Gli2ΔN mice; therefore, mice were treated with a dox diet for a longer period of time. Additionally, HBCs are typically quiescent and the constitutively active form of GLI2 promoted proliferation, so I hypothesized that dominant negative GLI2 would not acutely affect OE homeostasis. Therefore, iHBC-Gli2ΔC4 mice and control mice were treated with dox for 36 days and tissue was immunolabeled with p63 and K5 to analyze HBCs, or MASH1 and SEC8 to analyze GBCs. Strikingly, the appearance and number of HBCs (Fig. 3-11A, C) and GBCs (Fig. 3-11B, D) in iHBC-Gli2ΔC4 and control mice were not significantly different. Although, I was unable to detect the transgene in HBCs due to inadequate antibodies, I observed obvious skin and tail phenotypes in iHBC-Gli2ΔC mice, suggesting that the transgene is expressed in *K5*-expressing cells of the skin. These data indicate

that the repressive form of GLI2 does not affect OE homeostasis, which suggests that the HH pathway is either normally inactive or functions mainly as a repressor in the adult OE.

Dominant negative Gli2 repressor expression in HBCs results in improper OE regeneration following injury.

To explore whether GLI2 must be active in HBCs for basal cells to contribute to OE regeneration, I again utilized MMI injury to the OE of iHBC-Gli2ΔC4 mice. GLI2ΔC4 should be the dominant form of GLI2 expressed in the HBCs of these mice. Because these mice survive longer on dox (compared to GLI2ΔN animals), I analyzed the OE 8 weeks following injury when the OE should be nearly fully recovered (Genter et al., 1995; Bergman and Brittebo, 1999). I initially induced the *Gli2ΔC4* transgene with a dox diet for 7 days which continued throughout the recovery period.

At Day 0 of injury and 8 weeks following injury, iHBC-Gli2ΔC4 and control mice have a similar number of p63+/K5+ HBCs (Fig. 3-12A, C). The number of SEC8+ GBCs at Day 0 is unchanged in iHBC-Gli2ΔC4 compared to control mice however at 8 weeks post injury, there is a decrease in the number of SEC8+ GBCs (Fig. 3-12B, D), suggesting a decrease in the differentiation of HBCs into GBCs. Furthermore, while there are no differences in the number of OSNs at Day 0 of injury, after 8 weeks of recovery, there is a decrease in the number of GAP-43+ immature OSNs and OMP+ mature OSNs (Fig. 3-12E-G). Interestingly, there is not a complete loss of neurons and the OSNs that do return appear in a regionally variable manner. Together, these data suggest that dominant negative GLI2 repressor impairs HBC-driven OE regeneration and that HBCs require GLI2 activation to ensure normal OE regeneration.

Gli2 regulation of SEC8 during the HBC to GBC transition in OE regeneration.

Many of the transcription factors expressed in GBCs, including *Mash1* and *Ngn,* are necessary for OSN development (Cau et al., 1997; Cau et al., 2002). SEC8 is a secretory protein that is thought to be involved in exocytosis acting as a targeting protein for exocytosis or secretion of various proteins (Friedrich et al., 1997). Its loss results in incomplete gastrulation and embryonic lethality (Friedrich et al., 1997). Interestingly, *Sec8* null embryos like *Mash1* null mice, display a loss of *Notch1* (Friedrich et al., 1997; Cau et al., 2002), which is thought to play an important role in GBC differentiation (Manglapus et al., 2004).

I showed that iHBC-Gli2ΔN mice exhibit an upregulation of SEC8 in HBCs while iHBC-Gli2ΔC4 mice have a normal expression profile of SEC8 seen only in GBCs. These observations suggest that the active form of GLI2 is necessary for the HBC to GBC transition of HBCs. To test this hypothesis, I analyzed the expression of p63, K5, and SEC8 in the OE of iHBC-Gli2ΔN mice that had been induced with an MMI injury to the OE. On Day 0 of the injury (5 days of dox), iHBC-Gli2ΔN mice contain increased numbers of p63+/K5+ HBCs and increased numbers of SEC8+ cells that have been upregulated in these HBCs (Fig. 3-13A, E). There is a further increase in SEC8+ cell numbers in iHBC-Gli2ΔN mice 2 days following injury, while control mice show a slight decrease (Fig. 3-13B, E). By 5 days following injury, control mice display increased numbers of SEC8+ cells, while there is a slight decrease in iHBC-Gli2ΔN mice (Fig. 3-13C, E). The number of SEC8+ cells remains normal at 9 days following injury in control mice, but are dramatically increased in iHBC-Gli2ΔN mice (Fig. 3-13D, E). Further analysis of SEC8 expression in either inactive or active cells reveals that: 1) SEC8 is upregulated in a majority of inactive HBCs in the iHBC-Gli2ΔN mice regardless of injury, but only in a small

percentage in control mice soon after injury (Fig. 3-13F), 2) SEC8 is expressed in active HBCs of both control and iHBC-Gli2ΔN mice following injury (Fig. 3-13G), and 3) SEC8 remains upregulated in the active HBCs in iHBC-Gli2ΔN mice, while in control mice, SEC8 is eventually downregulated (Fig. 3-13G). Together these data suggest that GLI2 upregulates SEC8 in HBCs for them to become active but GLI2 must be downregulated to allow for HBCs to differentiate into SEC8+/p63- GBCs (Fig. 3-14).

3.4 Discussion

The HH pathway plays important roles in embryonic development and in adult tissue homeostasis and neurogenesis (reviewed in Petrova and Joyner, 2014). Here, I provide evidence that HH pathway components are expressed in distinct cell populations in the OE. Interestingly, the transcription factor, *Gli1*, which is a transcriptional readout of the HH pathway and often co-expressed with *Ptch1* (Lee et al., 1997; Agren et al., 2004) is absent from the OE. Because *Gli1* expression is dependent on *Gli2* and *Gli3* (Bai et al., 2004) and *Gli1*$^{-/-}$ mice are viable (Park et al., 2000), GLI2 and GLI3 are believed to be the primary transcriptional effectors of HH signaling, while *Gli1* is presumably secondarily activated at the transcriptional level (Sasaki et al., 1999). Therefore, it is possible that GLI1 does not function in the OE, especially if GLI2 and GLI3 act as repressors of the pathway in HBCs, which would preclude GLI1 expression in HBCs. However, different HH pathway components could be upregulated or downregulated in response to OE injury. Particularly, it would be interesting to visualize the expression of *Gli2* transcript following an MMI injury to determine whether *Gli2* downregulation correlates with the HBC to GBC transition. Analysis of *lacZ* reporter mice that are induced with an MMI injury could

verify this concept. Although *Gli* transcripts are present in various cell types of the OE, I do not have information about their corresponding protein levels due to inefficient antibodies. Furthermore, because GLI2 and GLI3 act as both transcriptional activators and repressors (Buttitta et al., 2003), it is important to understand how they are processed in both the normal OE and the injured OE. Future work to analyze GLI protein modifications and levels would help elucidate how these transcription factors function in HBCs.

My data indicate that HBC-specific expression of constitutively active GLI2 promotes hyperproliferation and a subsequent loss of neuronal cells and impaired regeneration. Constitutively active GLI2 as well as full-length GLI2 can drive tumorigenesis, and when expressed in epidermal basal cells of mice, they form tumors characteristic of human basal cell carcinomas (Grachtchouk et al., 2000; Grachtchouk et al., 2011). Interestingly a subset of follicle stem cells are resistant to Gli2ΔN-mediated tumorigenesis, suggesting that there may be cell-specific protective mechanisms in place. In the OE, HBC proliferation is not uniform which could suggest a protective mechanism or could be due to timing of transgene induction. Interestingly, newly formed HBCs in iHBC-Gli2ΔN mice still possess primary cilia, which is not indicative of a proliferative cell (Plotnikova et al., 2009). However, my previous work indicates that the loss of HBC cilia does not stimulate proliferation (Joiner and Green, et. al., submitted), suggesting that these cells may not be as tightly regulated to the cell cycle as other cells. GLI2 trafficking to cilia is necessary for its processing (Haycraft et al., 2005; Humke et al., 2010; Tukachinsky et al., 2010). Additionally, GLI2 transcriptionally activates *cyclin D1* and *cyclin D1*, which function in G1 phase of the cell cycle to promote cell cycle progression

(Mill et al., 2003). It is possible that there is a correlation between the presence of GLI2 in the cilia of HBCs and their proliferative status. Understanding the correlation between proliferative HBCs and ciliated HBCs as well as the presence of GLI2 in primary cilia could provide more insight into the role of primary cilia in the cell cycle regulation of HBCs.

What remains unknown is the fate of neuronal cells that are lost as a consequence of HBC proliferation. If the OE has an intrinsic maximum capacity for the number of cells it can withstand, it is possible that the neuronal cells die at the expense of newly formed HBCs. Analyzing apoptosis in the OE at various time points of dox treatment would confirm what happens to these cells. Additionally, after MMI-induced injury, I observed incomplete regeneration of the OE in iHBC-Gli2ΔN mice, apparently due to a lack of HBC differentiation, which instead appear fixed in an intermediate state. HBCs normally differentiate and replenish the GBC population following injury (Leung et al., 2007; Iwai et al., 2008). In iHBC-Gli2ΔN mice, SEC8 expression, which is regulated by GLI2 appears to be associated with the transition from HBCs to GBCs. However, *Gli2* transcript is not present in SEC8+ GBCs (Fig. 3-4A), suggesting that the expression of SEC8 might only need transient *Gli2* expression for it to be transcriptionally activated. In control mice, SEC8 is upregulated to a lesser extent earlier in the recovery period (Fig. 3-13B-C, F-G), in support of the idea that GLI2 is processed as an activator transiently during regeneration. Analyzing GLI2 transcript and protein following an MMI injury would determine what is seen in its upregulation or downregulation following injury. Although there are no studies that investigate the relationship between SEC8 and GLI2, there are reports on the relationship between GLI2 and the HBC marker and transcription factor, p63. In osteosarcoma cells, p63 induces *Gli2* expression by binding to the *Gli2* promoter,

suggesting that *Gli2* is a direct transcriptional target of p63 (Ram Kumar et al., 2014). Normally, downregulation of p63 is associated with activation and differentiation of HBCs into other cells of the OE following an injury. Furthermore, p63 is thought to act as a promoter of HBC cell renewal and inhibit its differentiation (Fletcher et al., 2011). However, in iHBC-Gli2ΔN mice, despite downregulation of p63 leading to HBC activation, HBCs do not differentiate. Since *Gli2* is a target of p63, when p63 is downregulated, *Gli2* should also be downregulated, but in iHBC-Gli2ΔN mice potentially this downregulation cannot occur leading HBCs to stay in a fixed intermediate HBC/GBC state. Future work investigating the role of SEC8 in this process and whether SEC8 is a direct transcriptional target of *Gli2* would help elucidate the mechanisms controlling HBC differentiation.

As mentioned previously, GLI2 normally functions as an activator and GLI3 as a repressor of the HH pathway, but there are some instances where they can have reverse roles (Buttitta et al., 2003; Bai et al., 2004). Interestingly, expression of dominant negative GLI2 repressor (GLI2ΔC4) did not alter HBC function in the OE of iHBC-Gli2ΔC4 mice during normal tissue homeostasis (Fig. 3-12). However, following an MMI-induced injury these mice exhibited a loss of neurogenesis and regeneration of the OE (Fig. 3-12). These data suggest that GLI2 is processed into a repressor in HBCs in order to maintain their quiescence and upon injury, must be differentially processed into an activator to initiate HBC-driven neurogenesis. Without GLI2 activator function, HBCs are stuck in a quiescent state and are therefore unable to contribute to regeneration of the OE. Because *Gli3* is expressed in a subset of HBCs (Fig. 2-4B), it is also possible that *Gli3* can act as a transcriptional activator. Following injury, GLI3 processing to an activator would initiate HBC activation. Analyzing *Gli3* transcript and GLI3 protein levels following injury as well

as the proliferation of HBCs at early time points during recovery in iHBC-Gli2ΔC4 mice would be useful in further understanding the dynamics of GLI protein function in the OE. Additionally, analysis of a loss of *Gli2* (Corrales et al., 2006) and/or *Gli3* (Blaess et al., 2008) in HBCs using conditional mouse models would elucidate the repressive or activator roles these transcription factors play in regulation of HBCs and neurogenesis and if any redundancies are present.

Finally, an important HH pathway component that is missing in the OE is HH ligand. There are three different HH ligands in the mammalian HH signaling pathway, DHH, IHH, and SHH (Echelard et al., 1993). I was unable to definitively determine which HH ligands are present in the OE and where they are expressed. Due to the proximity of SUS cells and GBCs to HBCs and their cilia, the ligand source could be present in these cells. SUS cells extend projections that terminate in endfeet that contact HBCs via HBC primary cilia (Joiner and Green, et. al., submitted). Importantly, there are examples of ligand-independent HH signaling, specifically in cancer (Onishi et al., 2011; Lei et al., 2013). If HH ligand is not present in the OE, the regulation of HBCs could be occurring in a ligand-independent manner. Future work to look at inhibition of the HH pathway via a SMO inhibitor (Low and de Sauvage, 2010) or complete loss of SMO using conditional mouse models (Long et al., 2001) could provide more insight on whether the regulation of HBCs is ligand-dependent or independent.

In conclusion, the expression of HH pathway components in the OE, specifically in HBCs, and their importance in tissue homeostasis and neurogenesis reveals a potentially novel role for HH signaling in the OE, provides insight into a possible GLI-dependent and

repressive role for HH signaling during homeostasis, and defines a novel mechanism involved in the regulation of olfactory basal stem cells.

3.5 Materials and Methods

Mouse strains and Genotyping. All mice were maintained on a mixed genetic background. Transgenic *K5-rtTA;TetO-Gli2ΔN* (Grachtchouk et al., 2011) and *K5-rtTA;TetO-Gli2ΔC4* mice (unpublished) were provided by Dr. Andrzej Dlugosz (University of Michigan). All mice of either sex were housed and maintained according to the University of Michigan and University of Florida institutional guidelines. All protocols for mouse experimentation were approved by the University of Michigan Committee on the Use and Care of Animals. Genotyping was performed using primers and PCR parameters from previously published work referenced above.

Doxycycline Transgene Induction and olfactory epithelium lesion. Mice were administered doxycycline water (200µg/ml doxycycline, 5% sucrose, Fisher) for the first 3 days of treatment and fed doxycycline chow (1g/kg doxycycline, Bio-Serv) from the beginning of treatment after postnatal day 28 (P28) until the time of euthanasia. Based on an approximate daily food intake of 4 g/mouse and water intake of 6ml/mouse (Bachmanov et al., 2002), mice consumed approximately 2 mg doxycycline / day (0.8 mg in chow and 1.2 mg in water). Dox treated *K5-rtTA;TetO-Gli2ΔN* mice or *K5-rtTA;TetO-Gli2ΔC4* mice and their control littermates received an intraperitoneal injection of Methimazole (2-Mercapto-1-methylimidazole, Sigma) (75 mg/kg in sterilized 1xPBS) five days or 7 days after the start of dox diet respectively. *K5-rtTA;TetO-Gli2ΔN* mice were

euthanized 2, 5, or 9 days after MMI treatment while *K5-rtTA;TetO-Gli2ΔC4* mice were euthanized 8 weeks after MMI treatment.

Tissue Collection and Preparation. Mice were anesthetized with 30% Fluriso (Isoflurane, VetOne), transcardially perfused with 4% paraformaldehyde (PFA), decapitated, and their heads fixed in 4% PFA for 12-16 hours at 4°C. Tissue was then decalcified in 0.5M EDTA (Fisher)/1xPBS overnight at 4°C, cryoprotected in 10% (1hr), 20% (1hr), and 30% sucrose/1xPBS overnight at 4°C, and frozen in OCT compound (Tissue Tek). 10-12μm of the olfactory epithelium and olfactory bulb were collected on a Leica CM1860 cryostat.

X-Gal staining. Tissue sections were rinsed in 1xPBS to remove OCT and then incubated in X-gal solution for 6 hours at 37°C. Sections were rinsed in 1xPBS to remove X-gal solution, incubated in Nuclear Fast Red (Sigma) for 5min at RT, rinsed in water, and rinsed in 75% EtOH, 95% EtOH, 100% EtOH, and Xyelens for 5min each. After drying, sections were sealed with cover slips mounted with Permount (Fisher).

Immunohistochemistry. For all immunofluorescence, antigen retrieval was used. For antigen retrieval, tissue sections were rinsed in 1xPBS to remove OCT then incubated in Citrate buffer (pH 6.0) for 30min at 90°C, cooled for 20min at RT, then washed with distilled water for 5min. Sections were blocked with 2% donkey or goat serum, 1% BSA in 1xPBS and incubated overnight in primary antibody. Antibodies were used at the following dilutions: mIgG2a anti-p63 (BioCare Medical, 1:200); mIgG2a anti-ARL13B (Neuromab, 1:500); rabbit anti-ARL13B (Proteintech, 1:500); mIgG1 anti-gamma tubulin (Sigma, 1:500); rabbit anti-K5 (Covance, 1:2500); mIgG1 anti-MASH1 (BD Pharmingen, 1:100); mIgG2b anti-SEC8 (BD Transduction Laboratory, 1:500); goat anti-OMP (Wako

Chemicals, 1:1000); rabbit anti-GAP-43 (Millipore, 1:500); rabbit anti-Ki67 (Abcam, 1:500); and rabbit anti-MYC (Abcam, 1:500). Sections were washed in 1xPBS three times for 5min each at room temperature and then incubated with Alexa Fluor-conjugated secondary antibodies (1:1000) for 1hr at room temperature. Tissue sections were then incubated with DAPI (Invitrogen, 5mg/ml) for 5 min, washed two times with 1xPBS, and then sealed with cover slips mounted with ProLong Gold (Invitrogen).

For dual X-Gal and Immunofluorescence staining, tissue sections first underwent an X-Gal stain as described above, but instead of incubation in Nuclear Fast Re, sections were puddled with citrate buffer, and steamed for 10min in a glass jar in a hot water bath. Sections were then blocked and stained as described in the immunohistochemistry section above.

Image processing and quantification. Images were captured using a Nikon A1R confocal microscope. ImageJ software was used to measure the length of the OE (in µm) in each image and to count specific cell types with the cell counter plugin. In order to quantify cell types, 10-15 images were taken from the dorsal medial, dorsal lateral, ventral medial, and ventral lateral regions, across 3-4 sections of the OE. Cell counts were averaged and converted to number of cells/mm of OE.

3.6 Acknowledgments

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University of Michigan Center for Organogenesis. A.M.J. was also supported by the Rackham Merit Fellowship from the Rackham Graduate School, University of Michigan.

3-7 Figures

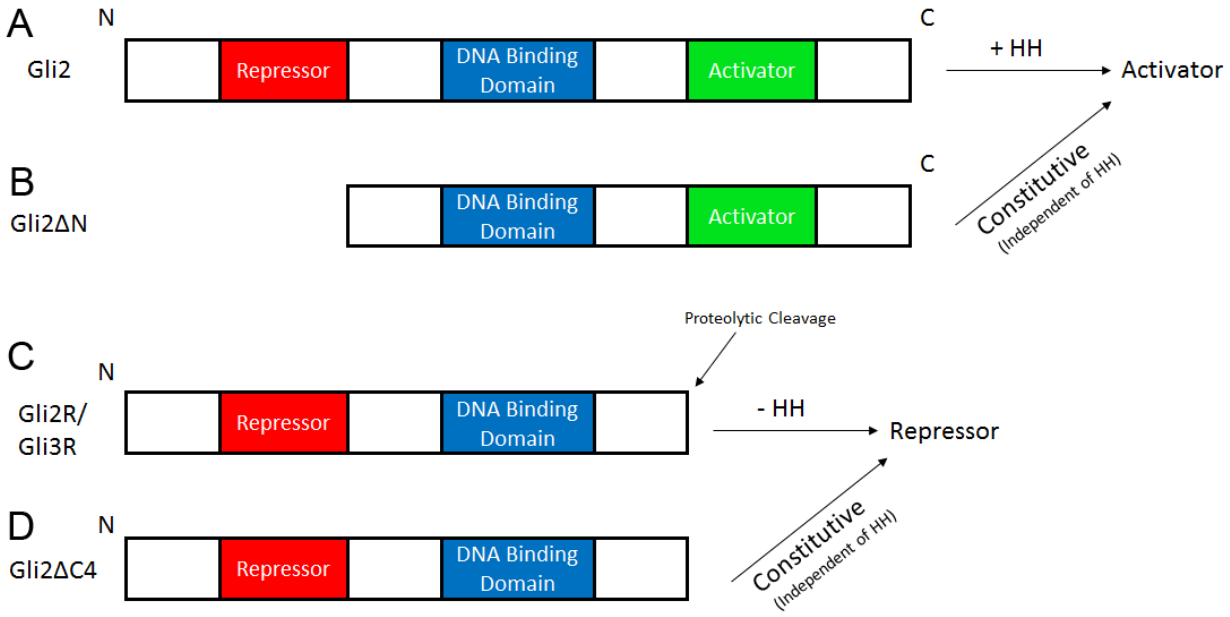

Figure 3-1. GLI2 processing and corresponding genotypes. Schematic illustration of GLI2 constructs used in transgenic mouse models. ***A,*** GLI2 has a DNA-binding domain and C-terminal activation domain, causing it to be a weak activator of the HH pathway in the presence of HH ligand. ***B,*** In the Gli2ΔN construct, when the N-terminal repressor domain is removed, GLI2 can act as a constitutive activator of the HH pathway independent of HH ligand. ***C,*** When the C-terminal activator domain of GLI2 undergoes proteolytic cleavage, it like GLI3, acts as a repressor in the absence of HH ligand. ***D,*** In the Gli2ΔC4 construct, when the C-terminal activator domain is removed, GLI2 acts as a constitutive repressor of the HH pathway independent of HH ligand.

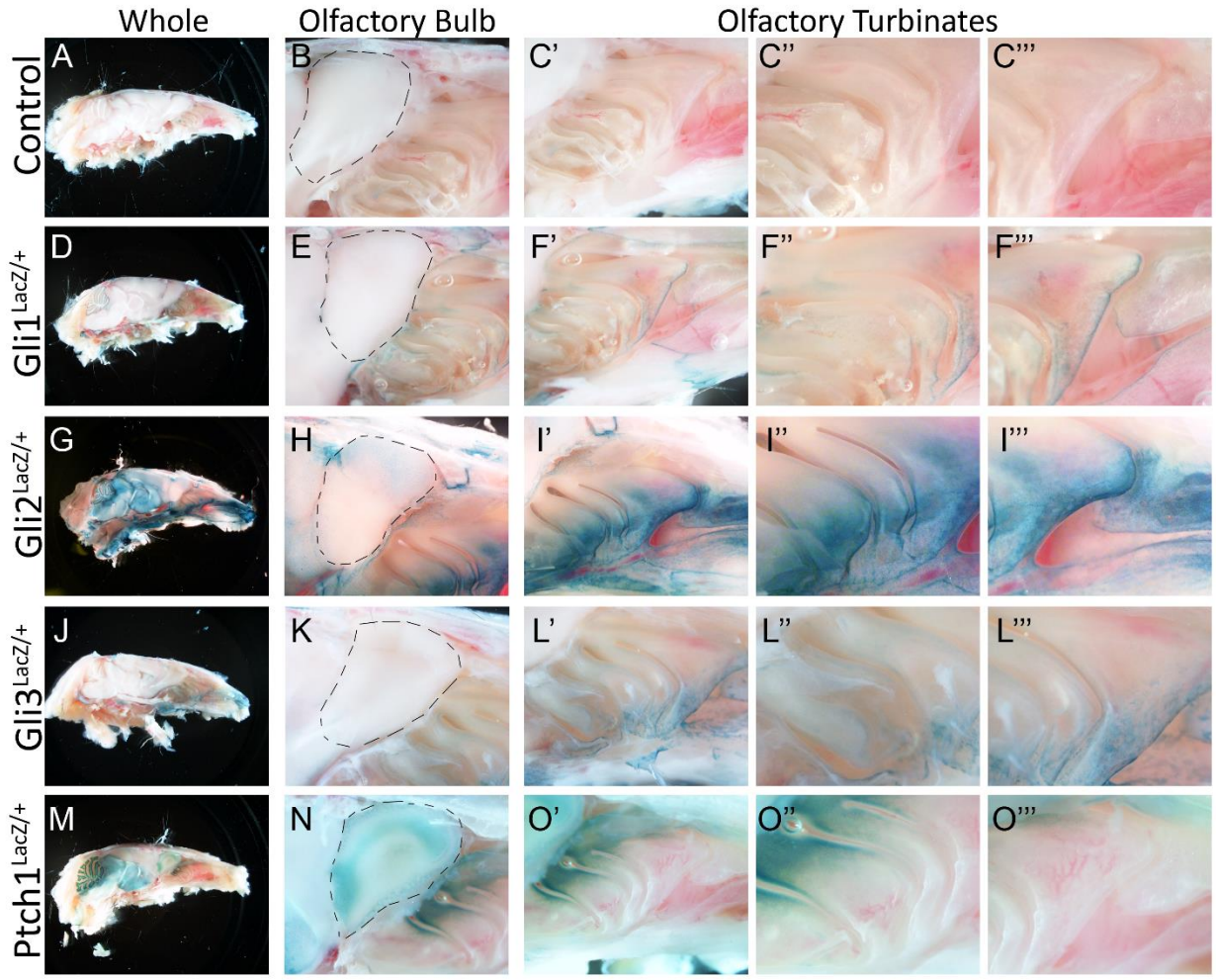

Figure 3-2. Components of the HH pathway are present in the CNS and olfactory turbinates. Whole heads of Control, *Gli1*$^{lacZ/+}$, *Gli2*$^{lacZ/+}$, *Gli3*$^{lacZ/+}$, and *Ptch1*$^{lacZ/+}$ mice were incubated with an X-Gal substrate that is cleaved by β-Galactosidase, producing a blue precipitate that indicates *lacZ* expression. (A-C''') Control mice show no color. (E) *Gli1* is not expressed in the olfactory bulb (OB) but (F'-F''') in the stoma of olfactory turbinates. (H) *Gli2* is expressed in the dorsal OB and (I'-I''') anterior/ventral parts of the olfactory turbinates. (J) *Gli3* is not expressed in the OB but (L'-L''') similar to *Gli2*, is expressed in anterior/ventral parts of the olfactory turbinates at a lesser intensity. (N) *Ptch1* is expressed in the entire OB and (O'-O''') in an opposing gradient to *Gli2* and *Gli3* in posterior/dorsal parts of the olfactory turbinates.

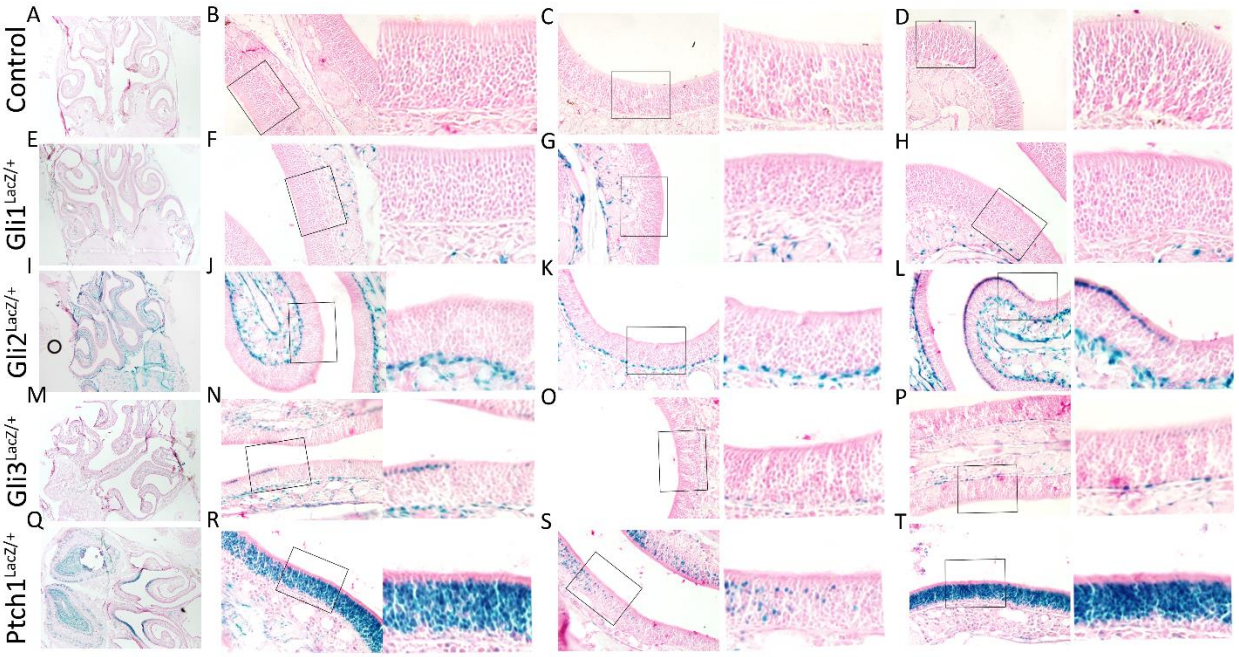

Figure 3-3. Components of the HH pathway are present in specific cells of the OE. Tissue sections from Control, *Gli1*$^{lacZ/+}$, *Gli2*$^{lacZ/+}$, *Gli3*$^{lacZ/+}$, and *Ptch1*$^{lacZ/+}$ mice were incubated with an X-Gal substrate that is cleaved by β-Galactosidase, producing a blue precipitate that indicates *lacZ* expression. (A-D) Control mice show no color in the OE or lamina propria (LP). (E-H) *Gli1* is not expressed in the OE, but in cells of the LP. (I-L) *Gli2* is uniformly expressed in basally located cells in the OE, a majority of cells in the LP, and a subset of SUS cells in the apical OE. (M-P) *Gli3* is sparsely expressed in basally located cells of the OE and cells in the LP, and also a subset of SUS cells. (Q-T) *Ptch1* is expressed with high intensity in a majority of more apically located cells, but not SUS cells, and with lower intensity in basally located cells. The expression pattern is “patchy.”

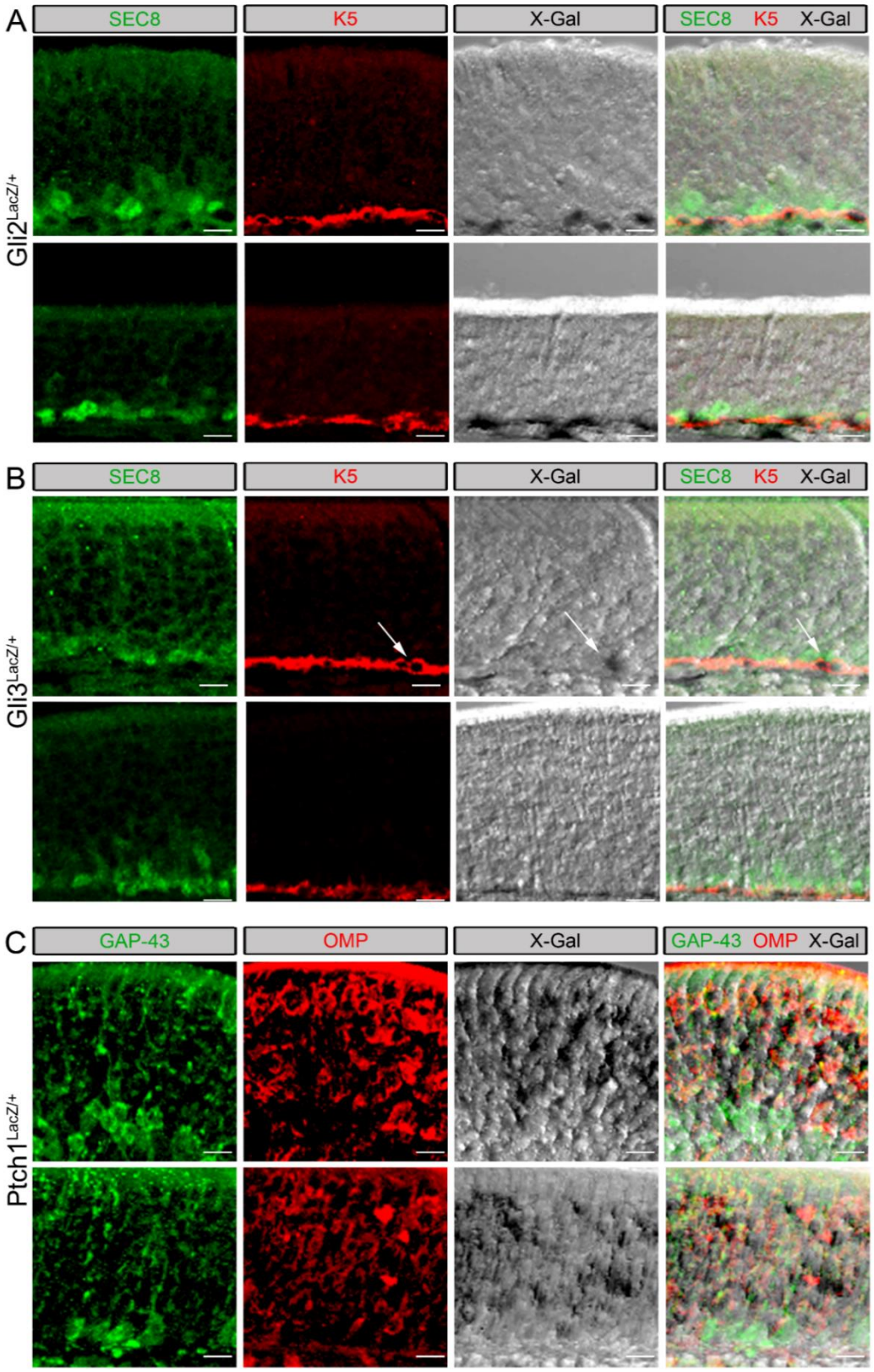
A
SEC8
K5
X-Gal
SEC8 K5 X-Gal
Gli2LacZ/+
B
SEC8
K5
X-Gal
SEC8 K5 X-Gal
Gli3LacZ/+
C
GAP-43
OMP
X-Gal
GAP-43 OMP X-Gal
Ptch1LacZ/+

Figure 3-4. The HH transcription factors *Gli2* and *Gli3* are expressed specifically in HBCs of the OE, while *Ptch1* is expressed in mature OSNs. Dual X-gal and immunofluorescence staining of coronal sections of the OE in *lacZ* reporter mice. (A) *Gli2* is expressed in all K5+ HBCs but not in SEC8+ GBCs. (B) *Gli3* is expressed in fewer K5+ cells (arrow) but also excluded from SEC8+ GBCs. (C) *Ptch1* is specifically expressed in a subset of mature OSNs.

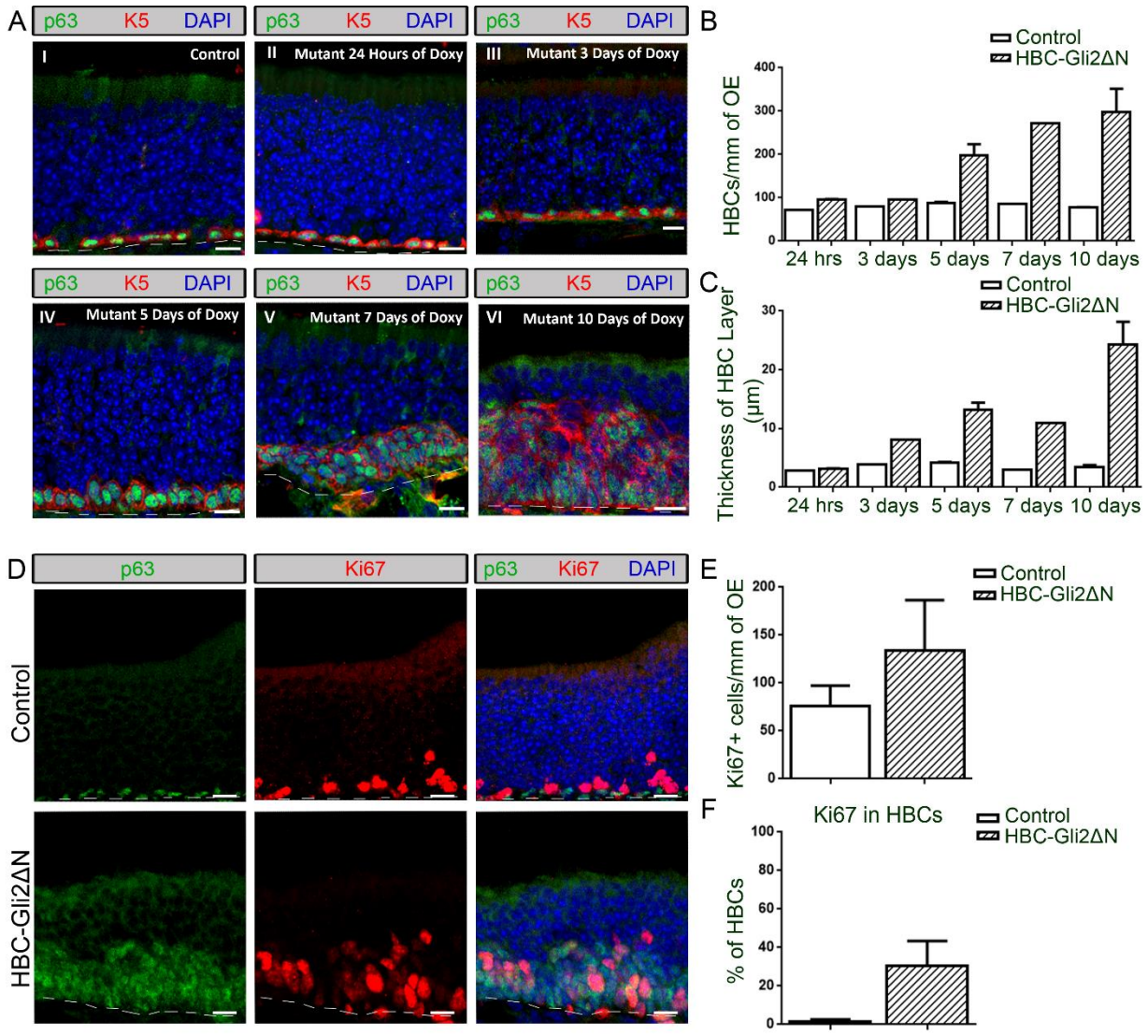

Figure 3-5. Constitutively active *Gli2* in HBCs results in hyperproliferation. (A) Immunofluorescence analysis of p63+/K5+ HBCs in the OE of (I) control mice and iHBC-Gli2ΔN mice treated with dox for (II) 24 hours, (III) 3 days, (IV) 5 days, (V) 7 days, or (VI) 10 days. (B) Quantified data show an increase in the number of HBCs in iHBC-Gli2ΔN mice treated with dox for at least 5 or more days compared to control mice. (C) Quantified data reveal that the thickness of the HBC layer subsequently increases in iHBC-Gli2ΔN mice compared to control mice. (D) While control mice possess p63+ HBCs devoid of Ki67, iHBC-Gli2ΔN mice treated with dox for 10 days, show an increase in the number of Ki67+ cells and Ki67 expression in p63+ HBCs. (E) Quantified data show an overall increase in the number of Ki67+ cells in the OE of iHBC-Gli2ΔN mice compared to control mice. (F) Quantified data show that in iHBC-Gli2ΔN mice, nearly 40% of p63+ HBCs are also Ki67+, while no HBCs express Ki67 in control mice.

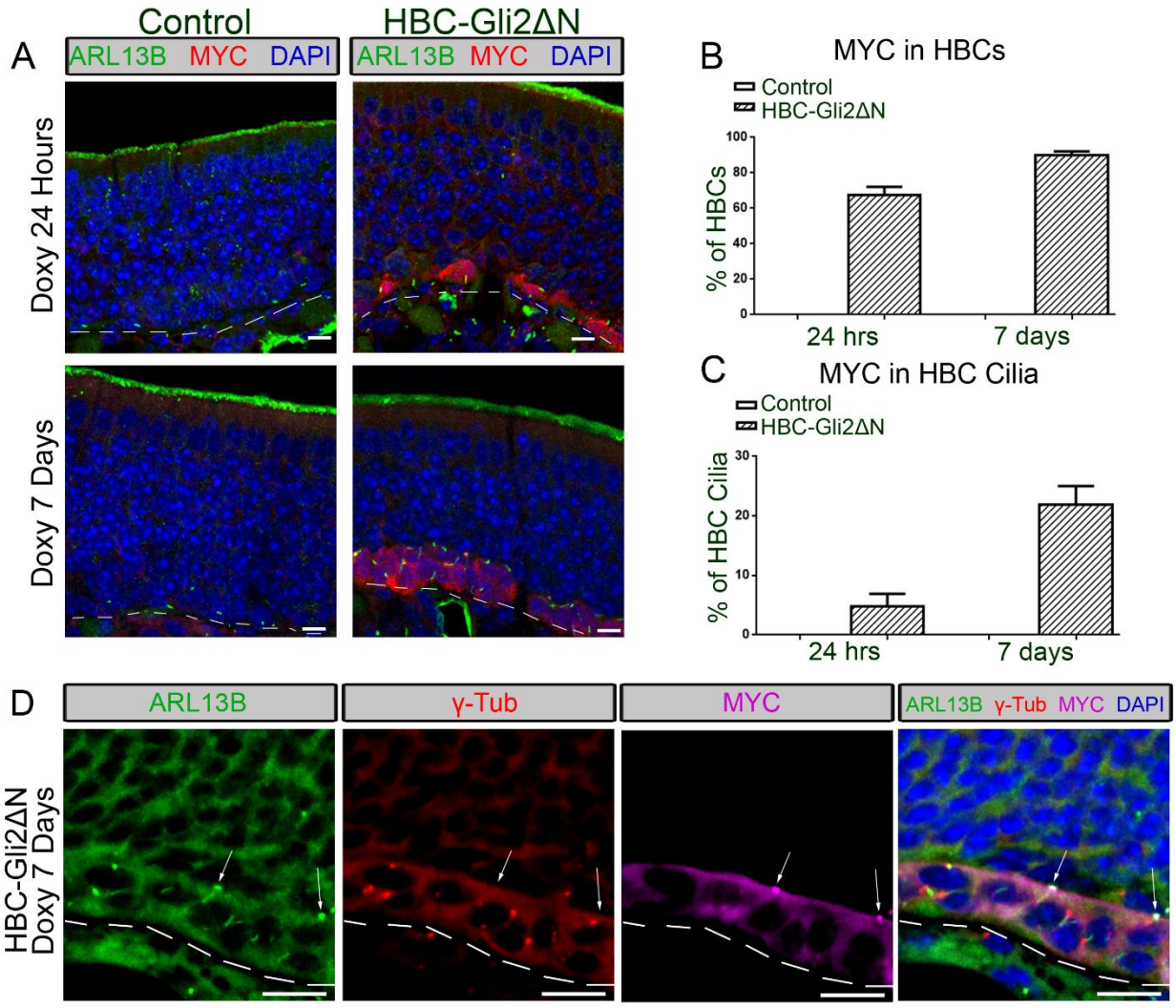

Figure 3-6. GLI2 localizes to tips of HBC cilia. IF analysis of control and iHBC-Gli2ΔN mice reveals (A) expression of MYC in HBCs (indicative of ARL13B+ cells) of iHBC-Gli2ΔN mice after 24 hours or 7 days of dox, which is absent in control mice. (B) Quantified data show that compared to controls, 60% of HBCs express MYC in iHBC-Gli2ΔN mice treated with dox for 24 hours which increases to 90% of HBCs after 7 days. (C) Quantified data show that control mice do not have MYC expression in ARL13B+ HBC cilia, while iHBC-Gli2ΔN mice treated with dox for 24 hours exhibit MYC in ~10% of HBC cilia and those treated for 7 days exhibit MYC in ~25% of HBC cilia. (D) IF analysis shows MYC-labeled GLI2 localizes to tips of ARL13B+ cilia in opposition of γ-tubulin+ basal bodies in MYC-labeled HBCs of iHBC-Gli2ΔN mice treated with dox for 7 days.

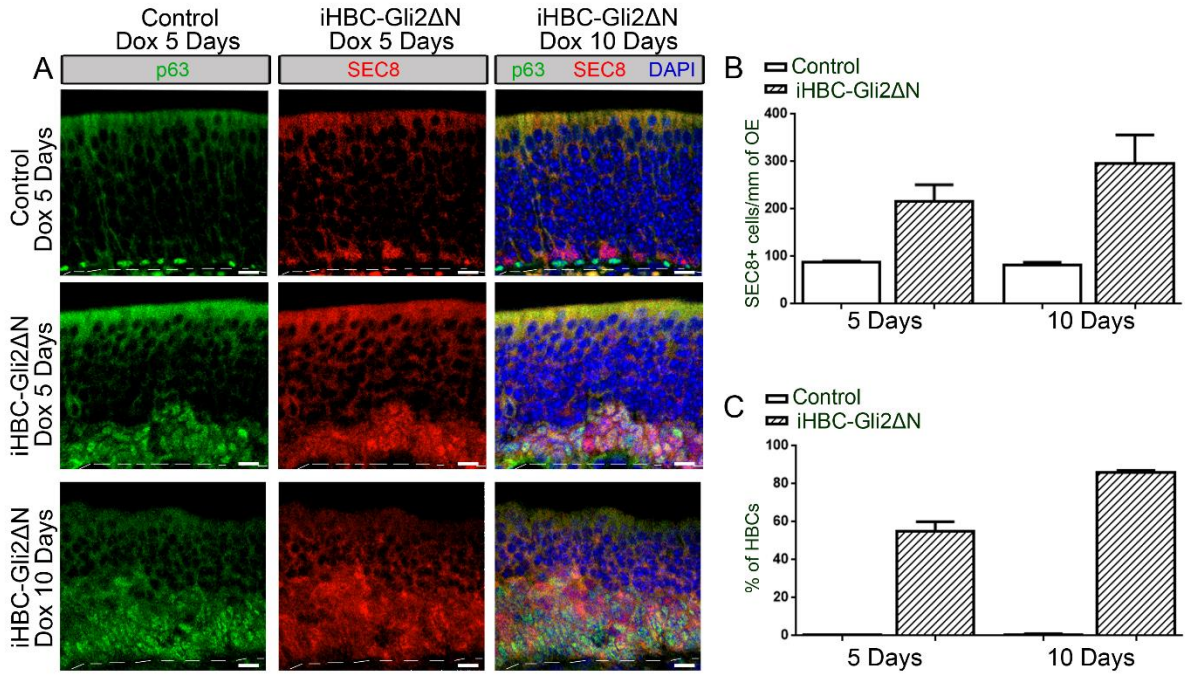

Figure 3-7. Constitutively active *Gli2* in HBCs results in the upregulation of SEC8. IF analysis of control and iHBC-Gli2ΔN mice reveals (A) an upregulation of SEC8 in HBCs of iHBC-Gli2ΔN mice represented by the co-expression of SEC8 in p63+ HBCs treated with dox for 5 or 10 days. (B) Quantified data show the total number of SEC8+ cells in the OE of iHBC-Gli2ΔN mice treated with dox for 5 or 10 days is increased compared to control mice. (C) Quantified data show that while the percentage of HBCs in control mice have no SEC8 expression, 60% of HBCs in iHBC-Gli2ΔN mice treated with dox for 5 days and 90% of HBCs in iHBC-Gli2ΔN mice treated with dox for 10 days have SEC8 expression.

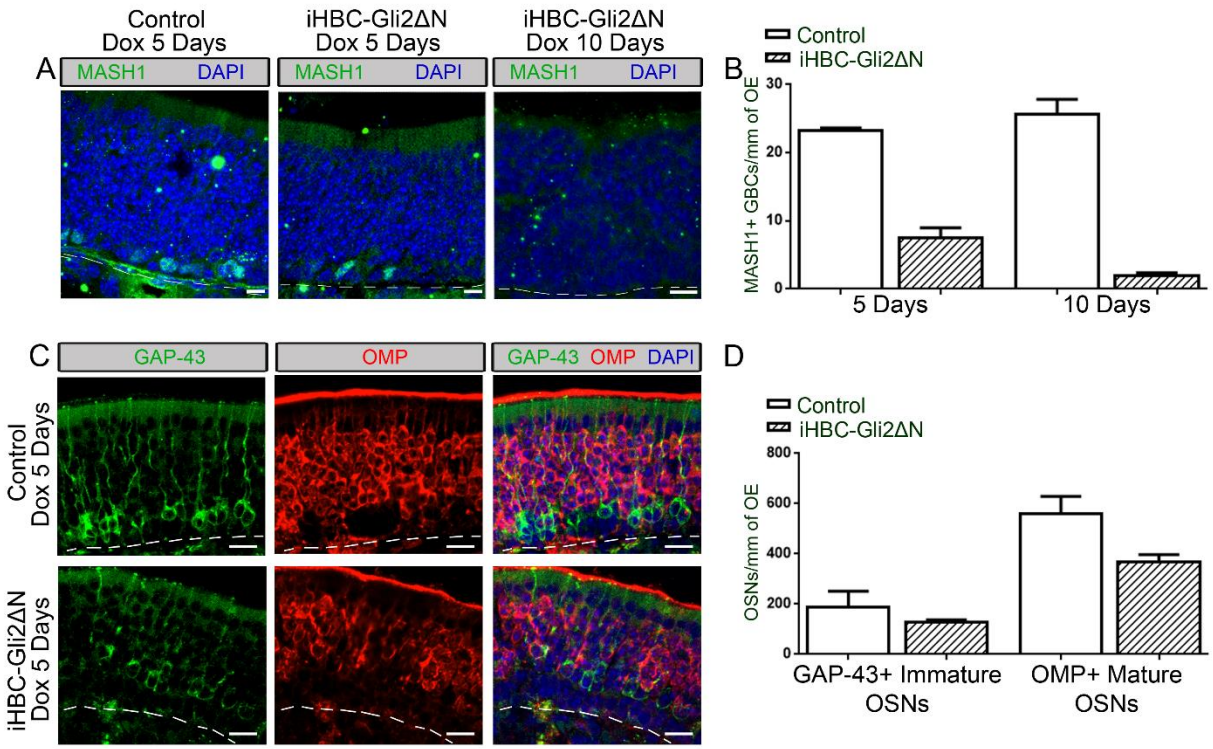

Figure 3-8. Constitutively active *Gli2* in HBCs results in the loss of neuronal cells. (A) IF analysis of control and iHBC-Gli2ΔN mice and (B) quantified data reveal a dramatic reduction in the number of MASH1+ GBCs in iHBC-Gli2ΔN mice treated with dox for 5 or 10 days compared to control mice. (C) IF analysis and (D) quantified data show a loss of GAP-43+ immature and OMP+ mature OSNs in iHBC-Gli2ΔN mice treated with dox for 5 days compared to controls.

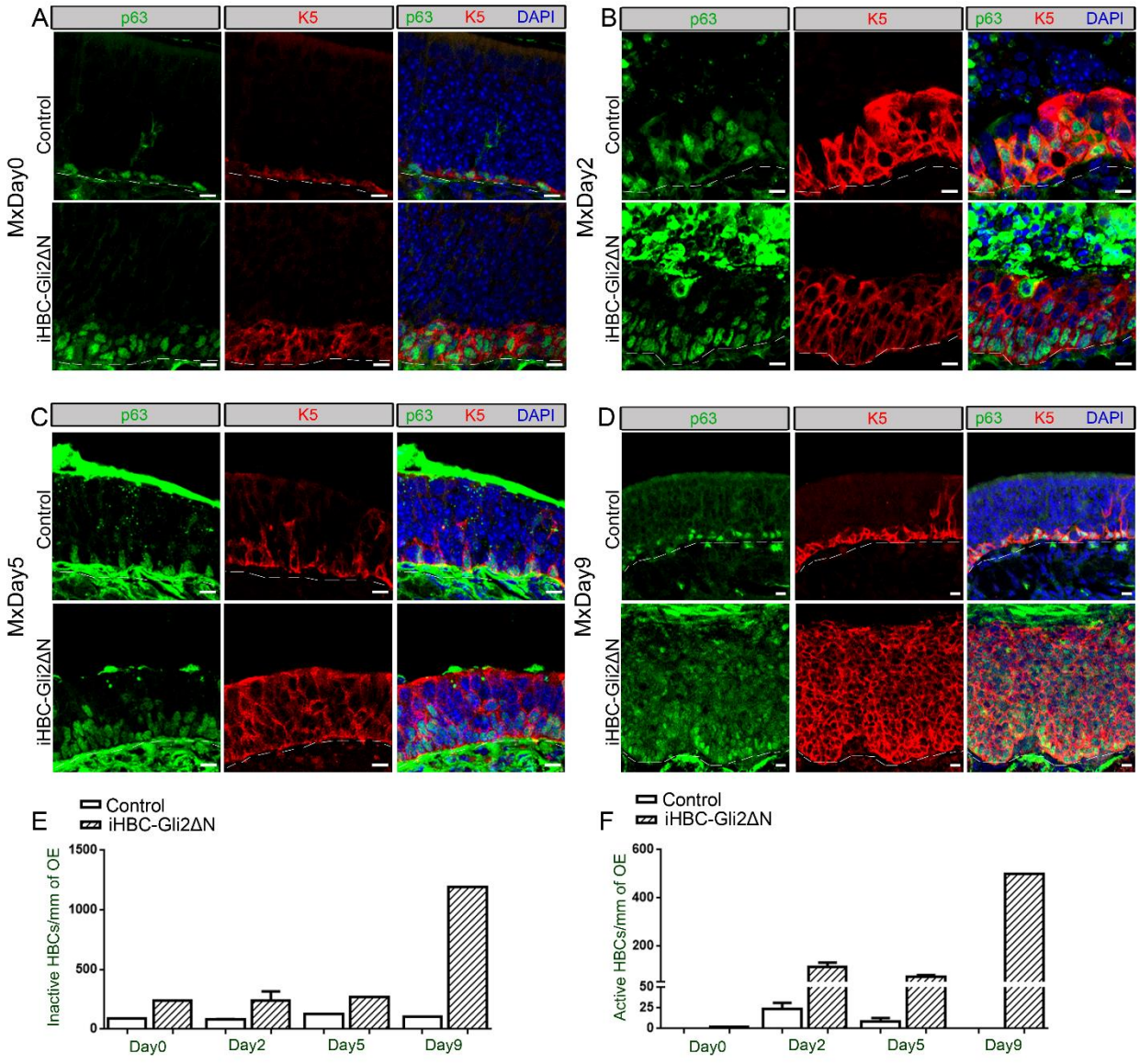

Figure 3-9. Constitutively active *Gli2* in HBCs results in an increase in the number of active and inactive HBCs during the regeneration of the OE. Control and iHBC-Gli2ΔN mice were treated with dox for 5 days and administered an I.P. injection of MMI to induce injury to the OE. IF analysis of p63+/K5+ inactive HBCs and p63-/K5+ active HBCs reveals that (A) at Day 0 of injury, there is an increase in the number of p63+/K5+ inactive HBCs of iHBC-Gli2ΔN mice, which (B) by 2 days following injury, increases in both control and iHBC-Gli2ΔN mice with additional p63-/K5+ active HBCs present. (C) After 5 days of recovery, HBCs of control mice appear to return to an inactive state while iHBC-Gli2ΔN mice continue to possess an increased number of p63+/K5+ inactive and p63-/K5+ active HBCs. (D) At 9 days following injury, HBCs of control mice are similar to Day 0, but the number of p63+/K5+ inactive and p63-/K5+ active HBCs continue to dramatically increase in number. Quantified data of (E) inactive HBCs and (F) active

HBCs reveal that HBCs of both control and iHBC-Gli2ΔN mice become active but while HBCs of control mice eventually become inactive, HBCs of iHBC-Gli2ΔN mice continue to be active.

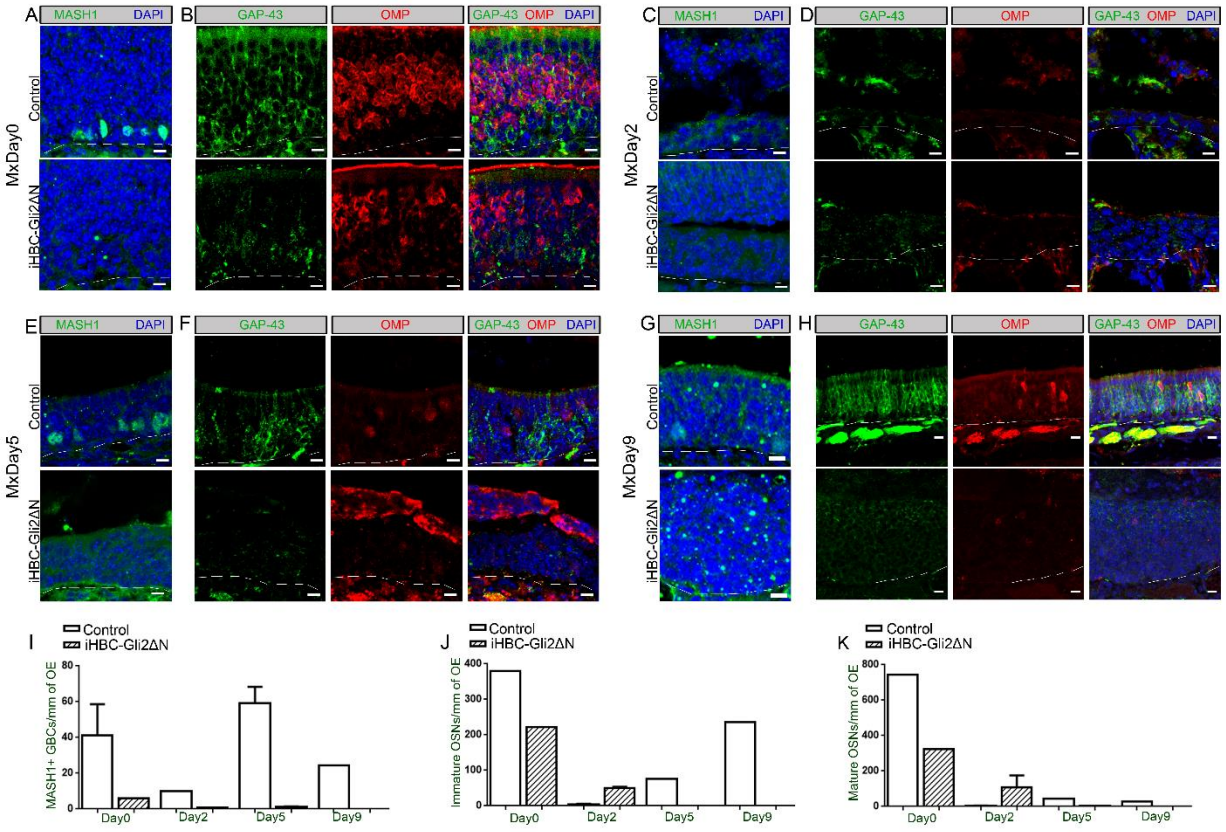

Figure 3-10. Constitutively active *Gli2* in HBCs results in the loss of neurogenesis during the regeneration of the OE. Control and iHBC-Gli2ΔN mice were treated with dox for 5 days and administered an I.P. injection of MMI to induce injury to the OE. IF analysis reveals that at Day 0 of injury, there is a reduction in the number of (A) MASH1+ GBCs and (B) GAP-43 immature and OMP+ OSNs in iHBC-Gli2ΔN mice compared to control mice. By 2 days following injury, there is a decrease in (C) MASH1+ GBCs and (D) GAP-43 immature and OMP+ OSNs in both control and iHBC-Gli2ΔN mice. Following 5 days of recovery, (E) MASH1+ GBCs and (F) GAP-43 immature and OMP+ OSNs return in control mice, but are still absent in iHBC-Gli2ΔN mice. At 9 days following injury, (G) MASH1+ GBCs are still present in control mice but absent in iHBC-Gli2ΔN mice, while (H) there is a further return of GAP-43 immature and OMP+ OSNs in control mice but no OSNs present in iHBC-Gli2ΔN mice. Quantified data of (I) MASH1+ GBCs, (J) immature OSN, and (J) mature OSNs show a marked decrease in neuronal cells of control and iHBC-Gli2ΔN mice following MMI-induced injury that return in control mice but not in iHBC-Gli2ΔN mice.

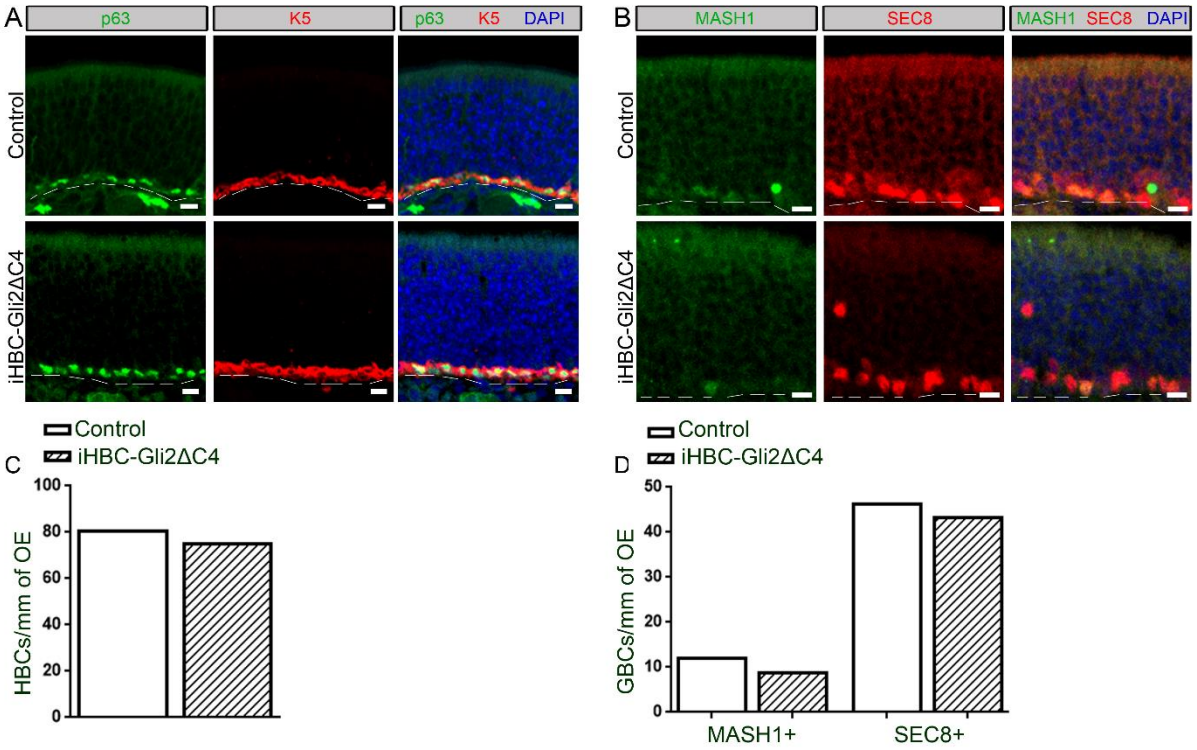

Figure 3-11. Dominant negative repressive *Gli2* in HBCs has no effect on homeostasis of the OE. Following 36 days of dox treatment, (A-B) IF analysis of control and iHBC-Gli2ΔC4 mice and (C-D) quantified data reveal no difference in the number of (A, C) p63+/K5+ HBCs or (B, D) MASH1+ and SEC8+ GBCs.

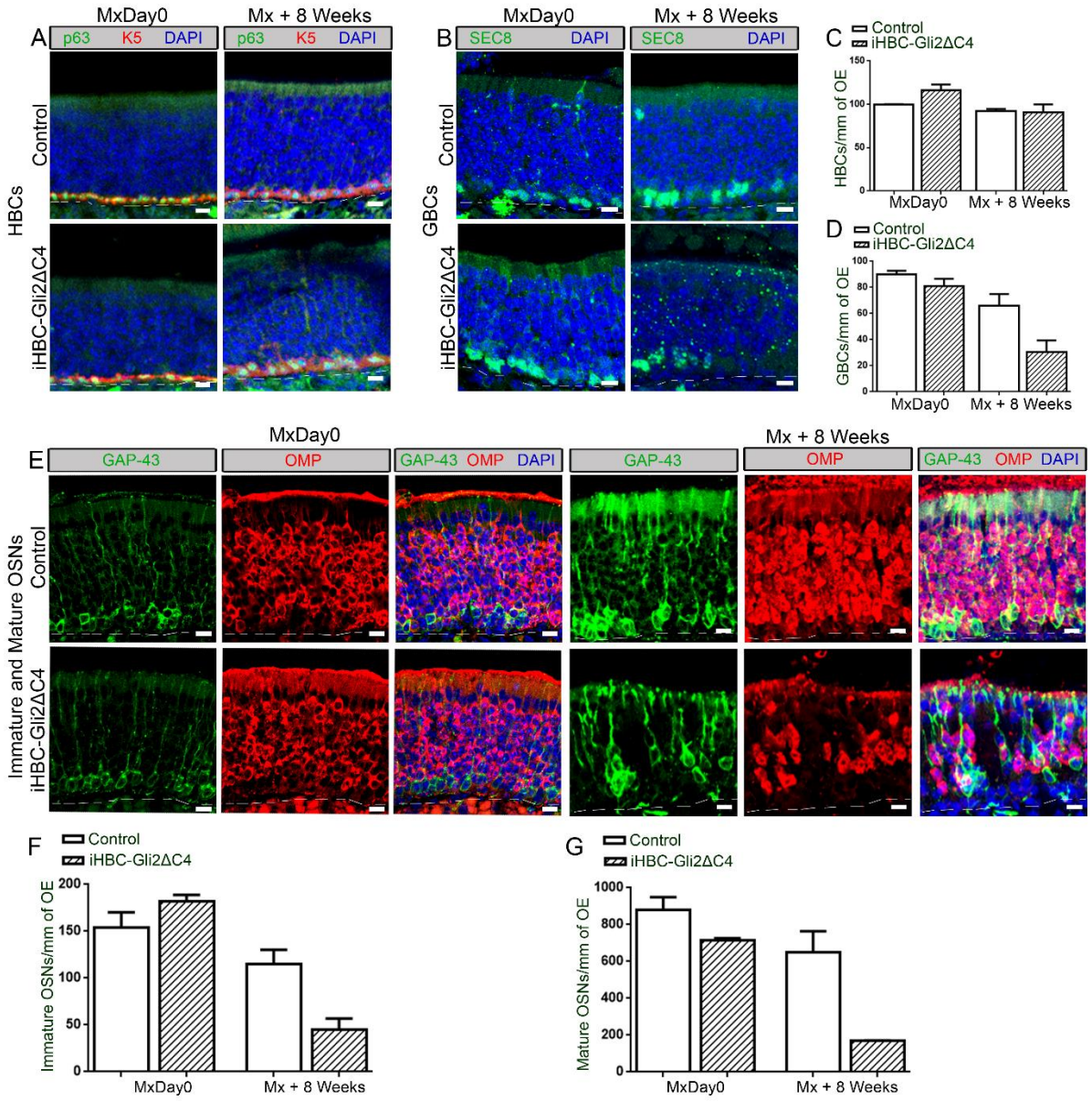

Figure 3-12. Dominant negative repressive *Gli2* in HBCs results in the improper regeneration of the OE. Control and iHBC-Gli2ΔC4 mice were treated with dox for 7 days and administered an I.P. injection of MMI to induce injury to the OE. (A) IF analysis and (C) quantified data reveal that the number of p63+/K5+ HBCs at Day 0 of injury and following 8 weeks is similar between control and iHBC-Gli2ΔC4 mice. (B) IF analysis and (D) quantified data show no difference in the number of SEC8+ GBCs at Day 0 between control and iHBC-Gli2ΔC4 mice but a decrease in GBCs of iHBC-Gli2ΔC4 mice 8 weeks following injury. The number of (E, F) GAP-43 immature and (E, G) OMP+ mature OSNs is unchanged in iHBC-Gli2ΔC4 mice compared to control mice at Day 0 of injury but following 8 weeks of recovery, there is a dramatic decrease in the number of OSNs in iHBC-Gli2ΔC4 mice compared to control mice.

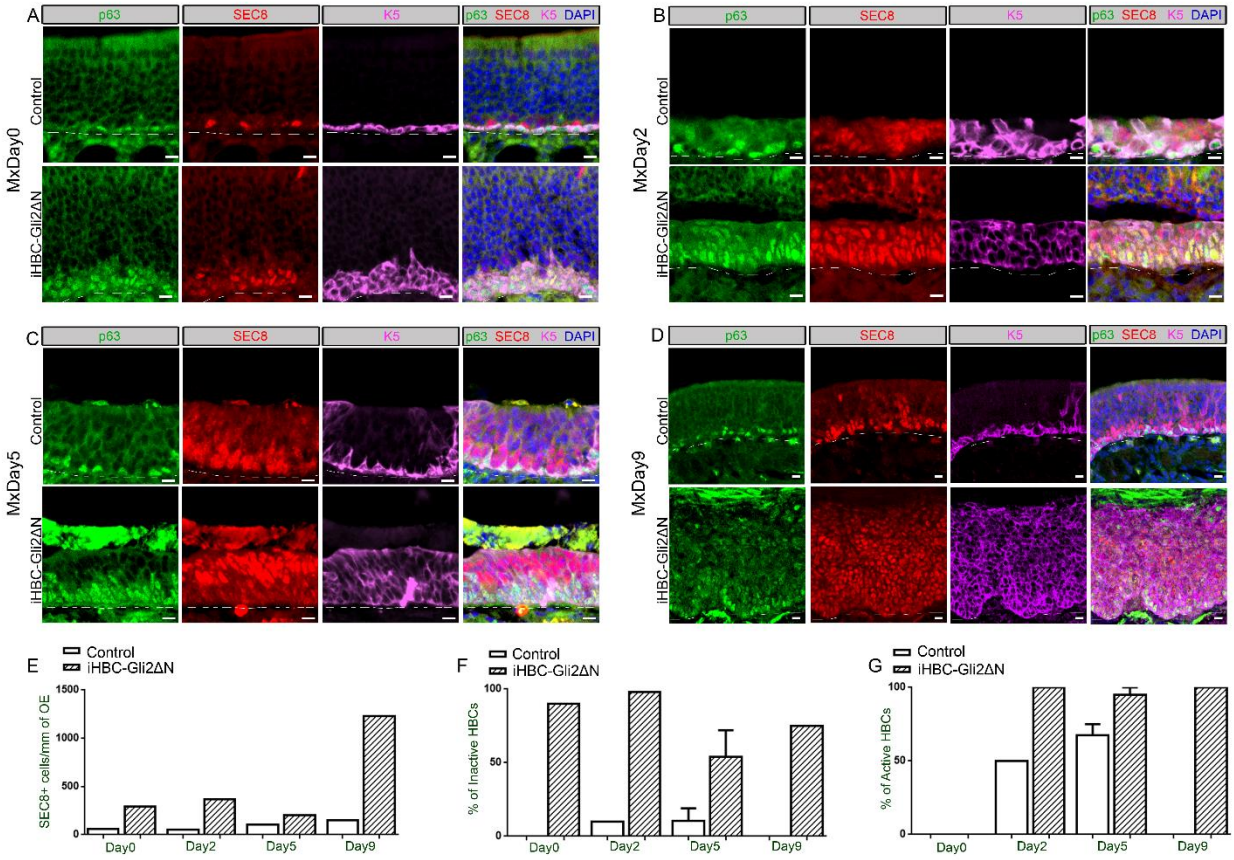

Figure 3-13. Constitutively active *Gli2* in HBCs impedes the differentiation of HBCs during regeneration of the OE. Control and iHBC-Gli2ΔN mice were treated with dox for 5 days and administered an I.P. injection of MMI to induce injury to the OE. (A) At Day 0 of the injury, there is an upregulation of SEC8 in p63+/K5+ inactive HBCs of iHBC-Gli2ΔN mice while control mice possess SEC8+ GBCs that are not co-expressed with K5 or p63. (B) After 2 days of recovery, control mice exhibit some upregulation of SEC8 in p63+/K5+ inactive HBCs and p63-/K5+ active HBCs which is consistently seen in both groups of HBCs in iHBC-Gli2ΔN mice. (C) At 5 days following injury, control mice show a normal appearance of p63+/K5+ inactive HBCs and SEC8+ GBCs while iHBC-Gli2ΔN mice continue to show an upregulation of SEC8 in p63+/K5+ inactive and p63-/K5+ active HBCs. (D) By 9 days of recovery, control mice exhibit normal HBC and GBC expression profiles, while iHBC-Gli2ΔN mice continue to show an increased number of p63+/K5+ inactive and p63-/K5+ active HBCs that co-express SEC8. Quantified data reveal that (E) the total number of SEC8+ cells is dramatically increased in iHBC-Gli2ΔN mice before and following injury compared to control mice, (F) the percentage of inactive HBCs that co-express SEC8 is largely in iHBC-Gli2ΔN mice regardless of injury and (G) a large percentage of active HBCs co-express SEC8 following injury in both control and iHBC-Gli2ΔN mice but only in iHBC-Gli2ΔN mice do active HBCs still express SEC8 9 days following injury.

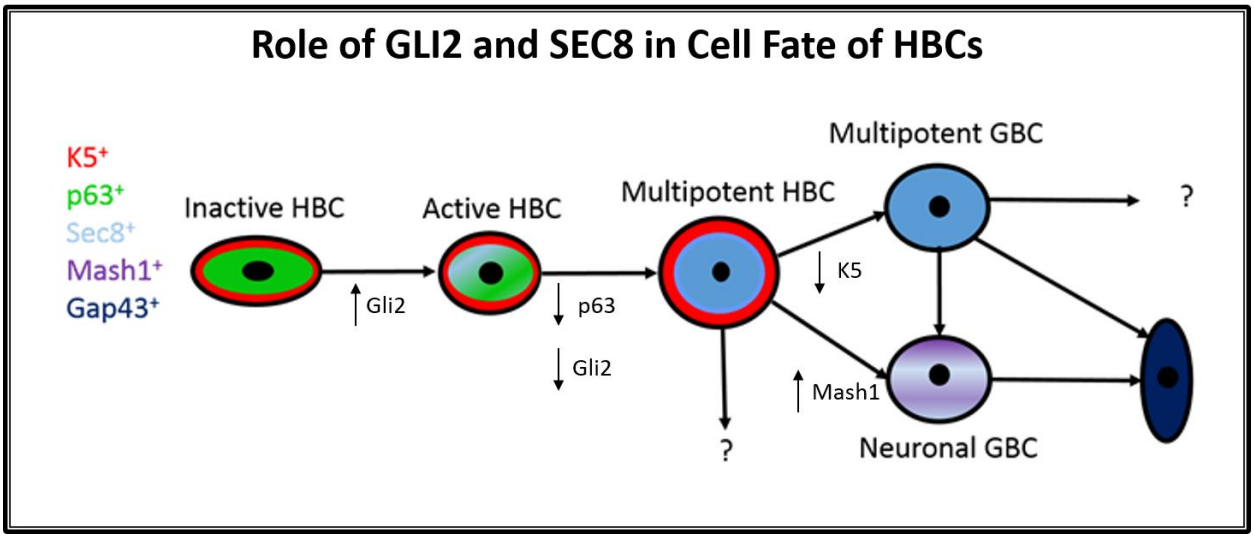

Figure 3-14. Model of GLI2- and SEC8-dependent transition of HBCs to GBCs during neurogenesis in the OE. Normally GLI2 acts as a repressor to maintain HBC quiescence (p63+/K5+). Upon injury, HBCs are activated and GLI2 acts as an activator which upregulates *Sec8* (p63+/K5+/SEC8+). In order for HBCs to become multipotent (K5+/Sec8+) and contribute to differentiation and regeneration in the OE, *p63* must be downregulated which should lead to downregulation of *Gli2*, and subsequent differentiation. However, because the constitutive form of *Gli2* is driven by the *K5* promoter which I propose to be expressed in multipotent cells, HBCs are fixed in an active and multipotent state and unable to differentiate into neuronal cells to replenish the OE.

3.8 References

Agren M, Kogerman P, Kleman MI, Wessling M, Toftgard R (2004) Expression of the PTCH1 tumor suppressor gene is regulated by alternative promoters and a single functional Gli-binding site. Gene 330:101-114.

Amador-Arjona A, Elliott J, Miller A, Ginbey A, Pazour GJ, Enikolopov G, Roberts AJ, Terskikh AV (2011) Primary cilia regulate proliferation of amplifying progenitors in adult hippocampus: implications for learning and memory. J Neurosci 31:9933-9944.

Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG (2002) Food intake, water intake, and drinking spout side preference of 28 mouse strains. Behavior genetics 32:435-443.

Bai CB, Stephen D, Joyner AL (2004) All mouse ventral spinal cord patterning by hedgehog is Gli dependent and involves an activator function of Gli3. Dev Cell 6:103-115.

Bergman U, Brittebo EB (1999) Methimazole toxicity in rodents: covalent binding in the olfactory mucosa and detection of glial fibrillary acidic protein in the olfactory bulb. Toxicol Appl Pharmacol 155:190-200.

Blaess S, Stephen D, Joyner AL (2008) Gli3 coordinates three-dimensional patterning and growth of the tectum and cerebellum by integrating Shh and Fgf8 signaling. Development 135:2093-2103.

Briscoe J, Pierani A, Jessell TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101:435-445.

Brittebo EB (1995) Metabolism-dependent toxicity of methimazole in the olfactory nasal mucosa. Pharmacol Toxicol 76:76-79.

Buttitta L, Mo R, Hui CC, Fan CM (2003) Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. Development 130:6233-6243.

Calof AL, Chikaraishi DM (1989) Analysis of neurogenesis in a mammalian neuroepithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. Neuron 3:115-127.

Carter LA, MacDonald JL, Roskams AJ (2004) Olfactory horizontal basal cells demonstrate a conserved multipotent progenitor phenotype. J Neurosci 24:5670-5683.

Cau E, Casarosa S, Guillemot F (2002) Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129:1871-1880.

Cau E, Gradwohl G, Fode C, Guillemot F (1997) Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124:1611-1621.

Chen M, Tian S, Yang X, Lane AP, Reed RR, Liu H (2014) Wnt-responsive Lgr5(+) globose basal cells function as multipotent olfactory epithelium progenitor cells. J Neurosci 34:8268-8276.

Chen Y, Struhl G (1996) Dual roles for patched in sequestering and transducing Hedgehog. Cell 87:553-563.

Chou YH, Zheng X, Beachy PA, Luo L (2010) Patterning axon targeting of olfactory receptor neurons by coupled hedgehog signaling at two distinct steps. Cell 142:954-966.

Corrales JD, Blaess S, Mahoney EM, Joyner AL (2006) The level of sonic hedgehog signaling regulates the complexity of cerebellar foliation. Development 133:1811-1821.

Croyle MJ, Lehman JM, O'Connor AK, Wong SY, Malarkey EB, Iribarne D, Dowdle WE, Schoeb TR, Verney ZM, Athar M, Michaud EJ, Reiter JF, Yoder BK (2011) Role of epidermal primary cilia in the homeostasis of skin and hair follicles. Development 138:1675-1685.

Diamond I, Owolabi T, Marco M, Lam C, Glick A (2000) Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators tTA and rTA linked to the keratin 5 promoter. The Journal of investigative dermatology 115:788-794.

Doty RL (2008) The olfactory vector hypothesis of neurodegenerative disease: is it viable? Annals of neurology 63:7-15.

Duggan CD, DeMaria S, Baudhuin A, Stafford D, Ngai J (2008) Foxg1 is required for development of the vertebrate olfactory system. J Neurosci 28:5229-5239.

Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, McMahon AP (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75:1417-1430.

Fan J, Liu Y, Jia J (2012) Hh-induced Smoothened conformational switch is mediated by differential phosphorylation at its C-terminal tail in a dose- and position-dependent manner. Dev Biol 366:172-184.

Fletcher RB, Prasol MS, Estrada J, Baudhuin A, Vranizan K, Choi YG, Ngai J (2011) p63 regulates olfactory stem cell self-renewal and differentiation. Neuron 72:748-759.

Friedrich GA, Hildebrand JD, Soriano P (1997) The secretory protein Sec8 is required for paraxial mesoderm formation in the mouse. Dev Biol 192:364-374.

Genter MB, Deamer NJ, Blake BL, Wesley DS, Levi PE (1995) Olfactory toxicity of methimazole: dose-response and structure-activity studies and characterization of flavin-containing monooxygenase activity in the Long-Evans rat olfactory mucosa. Toxicologic pathology 23:477-486.

Gong Q, Chen H, Farbman AI (2009) Olfactory sensory axon growth and branching is influenced by Sonic hedgehog. Developmental dynamics : an official publication of the American Association of Anatomists 238:1768-1776.

Goodrich LV, Johnson RL, Milenkovic L, McMahon JA, Scott MP (1996) Conservation of the hedgehog/patched signaling pathway from flies to mice: induction of a mouse patched gene by Hedgehog. Genes & development 10:301-312.

Grachtchouk M, Mo R, Yu S, Zhang X, Sasaki H, Hui CC, Dlugosz AA (2000) Basal cell carcinomas in mice overexpressing Gli2 in skin. Nat Genet 24:216-217.

Grachtchouk M, Pero J, Yang SH, Ermilov AN, Michael LE, Wang A, Wilbert D, Patel RM, Ferris J, Diener J, Allen M, Lim S, Syu LJ, Verhaegen M, Dlugosz AA (2011) Basal cell carcinomas in mice arise from hair follicle stem cells and multiple epithelial progenitor populations. J Clin Invest 121:1768-1781.

Graziadei PP, Graziadei GA (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol 8:1-18.

Graziadei PP, Monti Graziadei AG (1983) Regeneration in the olfactory system of vertebrates. Am J Otolaryngol 4:228-233.

Gunther EJ, Belka GK, Wertheim GB, Wang J, Hartman JL, Boxer RB, Chodosh LA (2002) A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. FASEB J 16:283-292.

Hastings L, Miller ML (2003) Influence of Environmental Toxicants on Olfactory Function. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 575-592. New York, New York: Marcel Dekker, Inc.

Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK (2005) Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet 1:e53.

Holbrook EH, Szumowski KE, Schwob JE (1995) An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. J Comp Neurol 363:129-146.

Humke EW, Dorn KV, Milenkovic L, Scott MP, Rohatgi R (2010) The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. Genes & development 24:670-682.

Ingham PW, Nystedt S, Nakano Y, Brown W, Stark D, van den Heuvel M, Taylor AM (2000) Patched represses the Hedgehog signalling pathway by promoting modification of the Smoothened protein. Curr Biol 10:1315-1318.

Irigoin F, Badano JL (2011) Keeping the balance between proliferation and differentiation: the primary cilium. Curr Genomics 12:285-297.

Iwai N, Zhou Z, Roop DR, Behringer RR (2008) Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. Stem Cells 26:1298-1306.

Jang W, Chen X, Flis D, Harris M, Schwob JE (2014) Label-retaining, quiescent globose basal cells are found in the olfactory epithelium. J Comp Neurol 522:731-749.

Joiner AM, Green WW, McIntyre JC, Allen BL, Schwob JE, Martens JR (2015) Primary Cilia on Horizontal Basal Cells Regulate Regeneration of the Olfactory Epithelium. J Neurosci 35:13761-13772.

Krolewski RC, Packard A, Schwob JE (2013) Global expression profiling of globose basal cells and neurogenic progression within the olfactory epithelium. J Comp Neurol 521:833-859.

Krolewski RC, Packard A, Jang W, Wildner H, Schwob JE (2012) Ascl1 (Mash1) knockout perturbs differentiation of nonneuronal cells in olfactory epithelium. PLoS One 7:e51737.

Kumamoto N, Gu Y, Wang J, Janoschka S, Takemaru K, Levine J, Ge S (2012) A role for primary cilia in glutamatergic synaptic integration of adult-born neurons. Nat Neurosci 15:399-405, S391.

Lee J, Platt KA, Censullo P, Ruiz i Altaba A (1997) Gli1 is a target of Sonic hedgehog that induces ventral neural tube development. Development 124:2537-2552.

Lei J, Ma J, Ma Q, Li X, Liu H, Xu Q, Duan W, Sun Q, Xu J, Wu Z, Wu E (2013) Hedgehog signaling regulates hypoxia induced epithelial to mesenchymal transition and invasion in pancreatic cancer cells via a ligand-independent manner. Molecular cancer 12:66.

Leung CT, Coulombe PA, Reed RR (2007) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10:720-726.

Litingtung Y, Chiang C (2000) Specification of ventral neuron types is mediated by an antagonistic interaction between Shh and Gli3. Nat Neurosci 3:979-985.

Liu A, Wang B, Niswander LA (2005) Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. Development 132:3103-3111.

Long F, Zhang XM, Karp S, Yang Y, McMahon AP (2001) Genetic manipulation of hedgehog signaling in the endochondral skeleton reveals a direct role in the regulation of chondrocyte proliferation. Development 128:5099-5108.

Low JA, de Sauvage FJ (2010) Clinical experience with Hedgehog pathway inhibitors. J Clin Oncol 28:5321-5326.

Manglapus GL, Youngentob SL, Schwob JE (2004) Expression patterns of basic helix-loop-helix transcription factors define subsets of olfactory progenitor cells. J Comp Neurol 479:216-233.

Marigo V, Tabin CJ (1996) Regulation of patched by sonic hedgehog in the developing neural tube. Proc Natl Acad Sci U S A 93:9346-9351.

Matise MP, Epstein DJ, Park HL, Platt KA, Joyner AL (1998) Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 125:2759-2770.

McIntyre JC, Titlow WB, McClintock TS (2010) Axon growth and guidance genes identify nascent, immature, and mature olfactory sensory neurons. J Neurosci Res 88:3243-3256.

Mill P, Mo R, Fu H, Grachtchouk M, Kim PC, Dlugosz AA, Hui CC (2003) Sonic hedgehog-dependent activation of Gli2 is essential for embryonic hair follicle development. Genes & development 17:282-294.

Murdoch B, Roskams AJ (2007) Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. J Mol Histol 38:581-599.

Murphy C, Doty RL, Duncan HJ (2003) Clinical Disorders of Olfaction. In: Handbook of Olfaction and Gustation, 2nd Edition (Doty RL, ed), pp 461-478. New York, New York: Marcel Dekker, Inc.

Nozawa YI, Lin C, Chuang PT (2013) Hedgehog signaling from the primary cilium to the nucleus: an emerging picture of ciliary localization, trafficking and transduction. Current opinion in genetics & development 23:429-437.

Onishi H, Kai M, Odate S, Iwasaki H, Morifuji Y, Ogino T, Morisaki T, Nakashima Y, Katano M (2011) Hypoxia activates the hedgehog signaling pathway in a ligand-independent manner by upregulation of Smo transcription in pancreatic cancer. Cancer science 102:1144-1150.

Packard A, Giel-Moloney M, Leiter A, Schwob JE (2011a) Progenitor cell capacity of NeuroD1-expressing globose basal cells in the mouse olfactory epithelium. J Comp Neurol 519:3580-3596.

Packard A, Schnittke N, Romano RA, Sinha S, Schwob JE (2011b) DeltaNp63 regulates stem cell dynamics in the mammalian olfactory epithelium. J Neurosci 31:8748-8759.

Pan Y, Bai CB, Joyner AL, Wang B (2006) Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Molecular and cellular biology 26:3365-3377.

Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui CC, Nakashima M, Joyner AL (2000) Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 127:1593-1605.

Petrova R, Joyner AL (2014) Roles for Hedgehog signaling in adult organ homeostasis and repair. Development 141:3445-3457.

Plotnikova OV, Pugacheva EN, Golemis EA (2009) Primary cilia and the cell cycle. Methods in cell biology 94:137-160.

Ram Kumar RM, Betz MM, Robl B, Born W, Fuchs B (2014) DeltaNp63alpha enhances the oncogenic phenotype of osteosarcoma cells by inducing the expression of GLI2. BMC cancer 14:559.

Ruiz i Altaba A (1998) Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. Development 125:2203-2212.

Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H (1999) Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 126:3915-3924.

Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. J Cell Physiol 182:311-322.

Suzuki J, Sakurai K, Yamazaki M, Abe M, Inada H, Sakimura K, Katori Y, Osumi N (2015) Horizontal Basal Cell-Specific Deletion of Pax6 Impedes Recovery of the Olfactory Neuroepithelium Following Severe Injury. Stem cells and development 24:1923-1933.

Tong CK, Han YG, Shah JK, Obernier K, Guinto CD, Alvarez-Buylla A (2014) Primary cilia are required in a unique subpopulation of neural progenitors. Proc Natl Acad Sci U S A 111:12438-12443.

Treloar HB, Miller AM, Ray A, Greer CA (2010) Development of the Olfactory System. In: The Neurobiology of Olfaction (Menini A, ed). Boca Raton (FL).

Tukachinsky H, Lopez LV, Salic A (2010) A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. J Cell Biol 191:415-428.

Wang B, Fallon JF, Beachy PA (2000) Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 100:423-434.

Chapter IV:

Discussion and Future Directions

4.1 A Novel Role for Primary Cilia in the Regulation of Olfactory Horizontal Basal Cells and Neurogenesis

4.1.1 Summary of Key Findings

Data presented in Chapter II demonstrate a novel role for primary cilia in olfactory neurogenesis. I provide evidence that horizontal basal cells (HBCs) in the olfactory epithelium (OE) selectively possess primary cilia. My data support the paradigm that primary cilia are essential regulators of adult tissue homeostasis and neurogenesis (Han et al., 2008; Tummala et al., 2010; Amador-Arjona et al., 2011; Croyle et al., 2011; Kumamoto et al., 2012). Adult stem cells, like HBCs, often remain quiescent in adult tissues unless activated by an injury from which they generate multiple cell types (Veland et al., 2009). Primary cilia are often associated with the G0 stage or quiescent phase of the cell cycle (Plotnikova et al., 2009; Irigoin and Badano, 2011; Kim and Tsiokas, 2011). However, the adult stem cells that exist in a quiescent state make cell cycle considerations secondary to the need to respond to transient environment signals sensed through the cilia (Plotnikova et al., 2009). HBCs appear to work in this fashion – that is, primary cilia are not essential for control of the cell cycle (e.g. proliferation), but are important for sensing external signals that lead to their activation and differentiation. This observation is contrary to what is seen in other epithelia, such as the skin

(Croyle et al., 2011) but is similar to observations in neural stem cells (NSCs) of the SVZ where the loss of cilia also does not change the number of stem cells (Amador-Arjona et al., 2011). These data suggest a tissue-specific role for primary cilia in adult neurogenesis.

I also show that a loss of HBC cilia in iHBC-IFT88 null mice results in reduced neurogenesis during development. It is believed that GBCs and not HBCs contribute to developmental neurogenesis (Guillemot et al., 1993; Cau et al., 1997; Cau et al., 2002). This notion is strongly supported by the fact that HBCs appear late embryonically (Packard et al., 2011b). However, a recent study suggested that HBCs do contribute to neurogenesis during postnatal development (Iwai et al., 2008). My data support this recent finding of HBC-driven neurogenesis during early postnatal development. Interestingly, there are regional differences in this neurogenesis that could be attributed to the temporal and spatial release of morphogens during development (Vedin et al., 2009) or the regional differences of certain transcription factors expressed during development (Duggan et al., 2008).

Furthermore, I introduce SEC8 as a novel marker of GBCs. Several studies indicate that GBCs are neuronal precursors that exist as a heterogeneous population, compartmentalized by their expression of distinct transcription factors. In the GBC lineage cascade, *Ascl1* (*Mash1*) expression lies upstream of *Neurogenin1* (*Ngn1*) which lies upstream of, but also overlaps with, *NeuroD1* expression (Cau et al., 1997; Manglapus et al., 2004; Packard et al., 2011a; Krolewski et al., 2013). *NeuroD1* is eventually switched off prior to onset of post-mitotic differentiation, and low levels of *NeuroD1* are sometimes found in immature OSNs (Packard et al., 2011a). Recently, the chromatin modifying

protein LSD1, was shown to localize with ASCL1 (MASH1) expressing cells, a subgroup of NGN1 and SOX2 expressing cells, as well as a subpopulation of immature OSNs, (Krolewski et al., 2013). I show that SEC8 overlaps with all LSD1-expressing cells and a surplus of cells that are not HBCs or immature OSNs, suggesting that SEC8 labels a larger population of GBCs that span the entire lineage of cells. Interestingly, *Sec8* null embryos, similar to *Mash1* mutant mice, show a loss of *Notch1* (Friedrich et al., 1997; Cau et al., 2002), which is believed to be involved in GBC differentiation. These observations suggest that *Sec8* plays important roles in signaling in GBCs, perhaps through Notch signaling, that is necessary for their neuronal differentiation.

Due to their close association with other cell types in the OE and their expression of epidermal growth factor (EGF) receptor-like immunoreactivity, HBCs are primed to serve as a channel of communication to the GBCs about the status of the OE and other cell types (Carr and Farbman, 1992; Holbrook et al., 1995). My work supports the notion that HBCs and SUS cells make physical contact through HBC primary cilia that project into the interstitial space between SUS cell endfeet and HBCs (Joiner et al., 2015). These endfeet are proposed sites of communication (Jia and Hegg, 2010) and HBC primary cilia may act as a scaffold for this communication.

Overall, my studies support the idea that HBCs contribute to olfactory regeneration and introduce a new role for primary cilia in that regeneration. I also provide evidence of a role for HBCs in OE development that was thought to be limited to GBCs (Doty, 2003; Treloar et al., 2010), and introduce a new marker for GBCs that may be important for their differentiation. What remains unexplored are: 1) the specific mechanisms that primary cilia utilize to regulate HBC proliferation and cell fate determination, 2) a more complete

profile of HBC primary cilia during development, homeostasis, and regeneration, and 3) the identification of potential therapeutic approaches that target HBC primary cilia.

4.1.2 Future Directions

Are Primary Cilia Sensors of Injury that Also Dictate Cell Fate?

It is possible that HBC primary cilia are both detectors of injury and signaling scaffolds for differentiation and cell fate determination. If HBC cilia are detectors of the insult, observing what happens to primary cilia immediately following injury upon activation of HBCs would be informative. Primary cilia could be reabsorbed prior to proliferation and re-established prior to differentiation or maintained throughout HBC activation and differentiation. Ciliary resorption is triggered by the addition of a specific combination of growth factors including PDGFα and either insulin-like growth factor1 (IGF-1), epidermal growth factor (EGF), or fibroblast growth factor (FGF) (reviewed in Kim and Tsiokas, 2011). These factors are positive regulators of neurogenesis in the OE and are most prominently expressed in HBCs and SUS cells (reviewed in Murdoch and Roskams, 2007). Analysis of these factors in the OE of iHBC-IFT88 null mice with and without an injury could provide insight to what possible signals HBCs detect following injury.

Upon cell death, SUS cells may release a signal to HBCs that is transduced through primary cilia, triggering HBC activation. Using an additional lesion model in which SUS cells are ablated, one could determine if there is a direct connection between SUS cell death and HBC activation. Ablation of the OE with NiSO4 selectively causes SUS cell death with a subsequent increase in basal cell proliferation (Jia et al., 2010); however,

the population of basal cells was not defined. Analyzing the OE with specific HBC and GBC markers along with proliferative markers following a lesion with NiSO4 in control and iHBC-IFT88 null mice could test the theory that SUS cell death activates HBCs and whether cilia are important for the detection of injury. Additionally, a specific ablation of SUS cells would provide more direct information about the relationship and communication between SUS cells and HBCs. There exists a transgene, *TetO-DTA* which leads to expression of diphtheria toxin A under control of a tetracycline operator (Lee et al., 1998). SUS cells express K18 specifically in the OE (Holbrook et al., 2011) and if expression of the tetracycline transactivator, rtTA, can be driven under the *K18* promoter (Ye et al., 2001), SUS cells could be specifically ablated to further analyze how HBCs respond. Ideally, this specific ablation of SUS cells should occur in iHBC-IFT88 null mice to determine if primary cilia are in fact necessary for detection of the insult but this breeding scheme would rely heavily on recombination and may not be possible.

Furthermore, cell-fate mapping with the use of either *K5-Cre-tdTomato* mice in which *K5* expressing cells and their progeny express tdTomato or inducible *K5-CreERT2-R26R* mice in which *K5* expressing cells and their progeny express *lacZ*. An inducible model would be ideal to investigate the lineage of HBCs following an injury in order to bypass any possible HBC-driven neurogenesis in development. Therefore $Ift88^{fl/fl}$ mice could be crossed with *K5-CreERT2-R26R* mice or the *K5rtTA;TetOCre;*$Ift88^{fl/fl}$ mice could be crossed with *R26R-tdTomato* mice to lineage trace HBCs in which cilia are ablated. The first cross would utilize a tamoxifen inducible system, while the second cross would utilize the doxycycline inducible system which I successfully utilized to remove cilia (see Chapter II). Both crosses would rely heavily on recombination and therefore, use of an

IFT88 null allele could alleviate some of this burden. Cell-fate mapping would ultimately elucidate what cells are derived from HBCs during regeneration of the OE and developmental neurogenesis. This technique would especially be important for circumventing the residual GBCs that remain after an MMI injury and incomplete loss of HBC cilia.

Interestingly, I noticed an increase in respiratory epithelia in iHBC-IFT88 null mice 8 weeks following MMI injury to the OE (Fig. 4-1). It is unknown whether this epithelia came from infiltration due to a loss of OSNs or from regeneration. Xie, et. al., showed through cell-fate mapping that respiratory regeneration comes from HBCs eight weeks following severe injury with the herbicide, dichlorobenzonitrile (DCBN) when there is a significant loss of GBCs (Xie et al., 2010). Most likely, multiple pathways and signaling factors work together to create the contribution of neurogenesis seen from HBCs. I speculate that during recovery, the first factor likely comes from the SUS cells alerting the HBCs to a severe lesion that requires the activation of both HBCs and GBCs. The second factor could come from GBCs, alerting the HBCs to take on a neuronal fate. Surprisingly, I saw no difference in cell death at 8 weeks post injury in iHBC-IFT88 null mice, but if there is more cell death in iHBC-IFT88 null mice earlier during recovery, this cell death might be necessary to trigger HBCs to regenerate tissue that, without a signal from GBCs, is fated to be respiratory. Analyzing the OE earlier during regeneration for apoptosis, respiratory markers, and using cell-fate mapping would elucidate whether the respiratory epithelia comes from HBCs or is a product of infiltration due to loss of OSNs. Additionally, SEC8 could be involved in communication with GBCs due to its role in exocytosis. Generating a conditional *Sec8* allele, $Sec8^{fl/fl}$, and using the mouse line that expresses

CreER under the GBC promoter, *Ascl1* (*Mash1*), (Ascl1CreER) (Kim et al., 2011) could provide insight into this theory, although MASH1 is only present in a subset of GBCs.

How do HBC Primary Cilia Change with Age and in Ciliopathy Models?

As mentioned before, defects in primary cilia lead to various human diseases, termed ciliopathies, in which mutations of various genes that are involved in the formation and maintenance of primary cilia affect a variety of tissues and organ systems (Singla and Reiter, 2006; Hildebrandt et al., 2011). Ciliary defects also affect the OE, resulting in anosmia, a loss of smell (Kulaga et al., 2004; Tadenev et al., 2011; McIntyre et al., 2012). My identification of primary cilia on HBCs and their importance in neurogenesis reveal new mechanisms for the etiology of olfactory dysfunction. There are numerous reported etiologies for olfactory disturbance including environmental toxins and head injuries. In addition, the normal decline in olfactory function due to aging afflicts approximately 25% of the population over 65 years old and this estimation is thought to be largely underrepresented (Murphy et al., 2002). Although the olfactory basal cells are responsible for the regeneration and restoration of cells in the OE, there is a net loss of neurons over time due to the fact that the capacity to match OSN death with regeneration is compromised as a function of age (Conley et al., 2003). This compromise can be attributed to the dramatic decrease in basal stem cell proliferation with age, with olfactory cell proliferation decreasing ~10 to 15 times after birth during the first 3 months of life (Legrier et al., 2001; Ducray et al., 2002). Similarly, there is an age-related decline in the neurogenesis of the SVZ and the hippocampus (reviewed in Riddle and Lichtenwalner, 2007). Cilia may be associated with this loss of stem cell activation that seems to be a result of specific signals from the OE. If HBC cilia are depleted or become defective with

age, HBCs may become permanently inactive resulting in the inability to recover from an insult to the OE which would lead to a permanent loss of smell. Future work investigating age-related cilia loss or defects could define a mechanism for age-related anosmia. Additionally, analysis in the OE of ciliopathy mouse models in which the OE is ablated to determine if regeneration occurs normally could also translate to human ciliopathies in which HBC cilia may be affected (Kulaga et al., 2004; McIntyre et al., 2012).

Potential Therapeutic Strategies for Olfactory Dysfunction.

With their ability to generate multiple cell types, stem cells are potential therapeutic tools for tissue repair in various diseases including genetic and degenerative diseases as well as aggressive and recurrent cancers (Mimeault et al., 2007). However, stimulating their expansion and differentiation into functional progenitors remains a challenge due to the unknown drivers of differentiation. Olfactory basal stem cells can be cultured into neurospheres and transplanted back into mice in order to repopulate the OE (Jang et al., 2008; Krolewski et al., 2011). Additionally, adenoviral delivery of *Ift88* to mature OSNs in an *Ift88* deficient mouse model that exhibited loss of OSN cilia and resulting anosmia, restored cilia and olfactory function to mice (McIntyre et al., 2012). My preliminary data indicate that combining ablation of the OE via MMI treatment with adenoviral infection results in ectopic gene expression in HBCs (Fig. 4-2). Together, these studies provide a possible therapeutic strategy for olfactory loss that focuses on cilia function and its regulation of olfactory stem cell function. Future work could include analysis of cultured HBCs to study what happens with a loss of ciliary genes via siRNA, subsequent viral infection to rescue defects, and transplantation back into the OE. Additionally, iHBC-IFT88 null mice could be infected with adenovirus containing *Ift88* (McIntyre et al., 2012)

to determine if there is a genetic rescue of cilia and phenotypic rescue of regeneration and olfaction.

4.2 GLI-Dependent Role for Hedgehog Signaling in the Olfactory Epithelium

4.2.1 Summary of Key Findings

Data presented in Chapter III demonstrate a potential role for Hedgehog (HH) signaling in the olfactory epithelium (OE). Various HH components were found in specific cell types of the OE. Interestingly, their expression profiles differ from what is typical, specifically that *Ptch1* and *Gli1*, both of which are direct HH transcriptional targets, display distinct expression patterns. The HH transcription factors *Gli2* and *Gli3* were expressed selectively in HBCs but also in a subset of SUS cells. *Ptch1* appears to be present in a specific set of mature OSNs and at a lower levels in immature OSNS and some basal cells. I show that constitutive activation of *Gli2* in HBCs of iHBC-Gli2ΔN mice results in their hyperproliferation with a resulting loss of neuronal cells. Although during normal homeostasis constitutive *Gli2* has dramatic effects on the composition of the OE, expression of a dominant negative *Gli2* repressor in iHBC-Gli2ΔC4 mice has no effect. These data suggest that GLI2 acts as a transcriptional repressor in the OE. Although GLI2 is typically processed as an activator (Pan et al., 2006), studies have shown GLI2 can act as a repressor (Buttitta et al., 2003). Additionally, I demonstrate that both the activator and repressor forms of GLI2 lead to impaired regeneration following injury in the OE. These data suggest that GLI2 must be properly regulated in order for HBCs to contribute

to regeneration. Overall, these data implicate HH signaling, specifically *Gli2*, in the regulation of HBCs and their contribution to neurogenesis in the OE.

Furthermore, I show an upregulation of the GBC marker, SEC8, in HBCs of iHBC-Gli2ΔN mice, but this observation is not seen when HBCs express the repressive form of *Gli2* in Gli2ΔC4 mice. The SEC8+ HBCs remain in the regenerating OE following injury and appear to be unable to transition to GBCs and eventually differentiate into OSNs. These data suggest that GLI2 and SEC8 are important for regulating HBC transition from dormancy to activation and ultimately differentiation into GBCs. Reports show that *Gli2* is a direct transcriptional target of p63 (Ram Kumar et al., 2014) and that downregulation of p63 in response to injury and subsequent activation could downregulate *Gli2*, driving HBC differentiation. What remains unexplored is: 1) the role *Gli3* plays in regulation of HBCs, 2) whether there are any redundancies between Gli2 and Gli3, 3) a possible non-canonical role for HH signaling in different aspects of olfactory tissue homeostasis, and 4) the link between HBC primary cilia and HH signaling.

4.2.2 Future Directions

GLI Processing in the OE and Possible Redundant Roles

I show that *Gli2* and *Gli3* transcripts are present in HBCs but what remains unknown is how the resulting proteins are processed. It is possible that GLI2 acts as a repressor in HBCs. Normally, HBCs are quiescent but when the constitutively active form of GLI2 is expressed, HBCs become proliferative. If GLI2 was normally processed as a weak activator (Sasaki et al., 1999), it would seem plausible for HBCs to be more proliferative and not quiescent. Because *Gli2* transcript is present in HBCs, it is likely that

GLI2 protein is either targeted for degradation or processed into a repressor. Furthermore, preliminary data show that staining the OE of control mice, iHBC-Gli2ΔN mice, or HBC-Gli2ΔC4 mice with an antibody against GLI2 that detects the C terminus, but not the N terminus (Cho et al., 2008), is seen in HBCs of HBC-Gli2ΔN mice but not control or HBC-Gli2ΔC4 mice (Fig. 4-3). These data further suggest that GLI2 is processed into an N-terminal repressor since the antibody can only detect residues near the C terminus. Additional analysis to detect protein levels of full length GLI2 and GLI3, as well as repressor and active forms would help elucidate the roles of these transcription factors. In order to detect the protein levels specifically in HBCs, cell sorting for Beta-Galactosidase in *Gli2*$^{lacZ/+}$ and *Gli3*$^{lacZ/+}$ mice could be utilized with Western blot analysis combined with qRT-PCR analysis to detect other HH components in HBCs.

My preliminary data indicate that in mice containing an HBC-specific deletion of *Gli2* (*K5-rtTA;TetO-Cre;Gli2*$^{fl/fl}$), there is no change in OE cell composition during normal homeostasis and following an MMI-induced injury to the OE (Fig. 4-4). Unfortunately, because there are no commercially available antibodies to detect the active or repressor forms of GLI2 for immunohistochemistry, it is difficult to determine a loss of *Gli2* in the HBCs of these mice. In order to ensure that proper recombination occurs, *K5-rtTA;TetO-Cre;Gli2*$^{fl/fl}$ mice can be crossed with *Gli2*$^{lacZ/+}$ mice so that cell sorting for Beta-Galactosidase can be used to isolate HBCs and assess recombination efficiency via qRT-PCR. Western blot analysis of the entire OE of *K5-rtTA;TetO-Cre;Gli2*$^{fl/fl}$ lysates can also be used to verify a loss of GLI2 protein. If recombination and subsequent loss of *Gli2* successfully occurs and there is still no phenotype seen with a loss of *Gli2*, *Gli3* could play a redundant role in HH signaling since it is also expressed in HBCs.

In certain cases, including the development of the anterior neural tube (Motoyama et al., 2003), skeletal development (Mo et al., 1997), and development of the lung, trachea, and esophagus (Motoyama et al., 1998) *Gli2* and *Gli3* play redundant roles. However, this redundancy is unexplored in the adult. In order to elucidate the possible redundant role of *Gli2* and *Gli3* specifically in HBCs, a conditional knockout of *Gli3* can be utilized. The laboratory of Dr. Alex Joyner has already generated a *Gli3*$^{fl/fl}$ mouse (Blaess et al., 2008). Successful breeding of *K5-rtTA;tetO-Cre* mice with *Gli3*$^{fl/fl}$ mice will produce an inducible HBC-specific *Gli3* conditional null mouse (*K5-rtTA;tetO-Cre;Gli3*$^{fl/fl}$). If no phenotype is seen in normal homeostasis or following an MMI-induced injury, mice with a conditional loss of both *Gli2* and *Gli3* (*K5-rtTA;tet-OCre;Gli2*$^{fl/fl}$*;Gli3*$^{fl/fl}$) could also be generated. Again, for analysis of loss of GLI2 and GLI3 proteins, *lacZ* can be introduced as one allele (for either *Gli2* or *Gli3*) and subsequent cell sorting can be performed. Additionally, cell-fate mapping with a tdTomato reporter can be used to determine what cells, if any, stem from HBCs that lack *Gli2*, *Gli3* or a combination of both following an injury and during normal tissue homeostasis.

Non-Canonical Role of Hedgehog Signaling in the Olfactory Epithelium

There are a few examples where a subset of the components of the canonical HH signaling pathway regulate various basic cellular processes seemingly independent of the full pathway. PTCH1 has been implicated in cell cycle regulation through interaction with cyclin B1 which acts at the G2/M checkpoint and is required for cell cycle progression (Barnes et al., 2001). Additionally, although not commonly described as non-canonical HH signaling, a role for the GLI transcription factors independent of the traditional HH ligand/SMO-signaling cascade has also been reported, particularly in cancer, where other

signaling pathways appear to directly regulate GLI proteins (reviewed in Stecca and Ruiz, 2010). RAS, MAPK, and PI3/AKT signaling have all been implicated in the modulation of GLI proteins as well as receptor tyrosine kinase (RTK) signaling (Aberger et al., 2012). The growth factors, EGFR, TGFβ, and IGF2 were also shown to be implicated in SMO-independent but GLI-dependent signaling (Aberger et al., 2012).

Although I have not examined the expression of *Smo*, it is believed to be expressed in the OE (Gong et al., 2009). Preliminary data from mice treated with the SMO antagonist, LDE-225 (Pan et al., 2010), a HH pathway inhibitor, showed no change in HBCs or the cell composition of the OE during natural turnover and in response to an MMI-induced lesion (Fig. 4-5), suggesting that the regulation of HBCs and OE regeneration is not HH ligand- or SMO-dependent. These findings were further supported by my data from mice containing an HBC-specific deletion of *Smo* (*K5-rtTA;TetO-Cre;Smo*$^{fl/fl}$), in which there was no change in the cell composition of the OE during natural turnover (Fig. 4-6). *Smo* could still play a role upon injury to the OE, therefore, an MMI-induced injury in *K5-rtTA;TetO-Cre;Smo*$^{fl/fl}$ mice would be important to verify this idea. Furthermore, analyzing mice that express the constitutively active form of *Smo*, *Smo-A1*, (Hallahan et al., 2004) to determine if there is a similar phenotype to iHBC-Gli2ΔN mice could also confirm the role of *Smo* in HBC regulation and olfactory regeneration. Together, these data suggest a SMO-independent and possible GLI-dependent role for HH in regulation of HBCs and olfactory neurogenesis. Additional mice need to be analyzed to confirm these preliminary findings.

An important component of the pathway that needs further investigation is the HH ligand and its source. SHH is the only member of the HH family that continues to be

expressed in the adult brain (Traiffort et al., 2010). Under normal conditions, neurons appear to be the main SHH-secreting cell type (as opposed to SHH-responding cells, which are primarily of glial nature), however, following injury, SHH expression has been reported in reactive astrocytes (reviewed in Petrova and Joyner, 2014). Along with other neuronal populations, it is possible that anterograde movement of SHH protein along axons allows the delivery of the protein to both the SVZ and striatum (reviewed in Petrova and Joyner, 2014). My preliminary data show that through *in situ hybridization*, SHH ligand might be present in SUS cells (Fig. 4-7A), however, in *Shh-Cre;Rosa26R-TdTomato* mice, in which *Shh* expressing cells and their progeny express TdTomato, clusters of cells including SUS cells, OSNs, and GBCs are positive for TdTomato (Fig. 4-7B-C). If SUS cells express *Shh*, it would coincide with the possible communication between SUS cells and HBC cilia. However, due to studies demonstrating secretion of SHH in neurons and that SHH protein can move along axons along with the detection of *Shh* in the OB (Gong et al., 2009), it is possible that the source can come from the bulb or OSNs. Analyzing the OE of *Shh-Cre;Rosa26R-TdTomato* mice or using *in situ hybridization* for the detection of *Shh* transcript following an injury could also elucidate the source of ligand in the OE.

What remains unknown is if the regulation of HBCs occurs in a true HH ligand-independent manner. The use of conditional *Ptch1*$^{fl/fl}$ (Ellis et al., 2003; Uhmann et al., 2007)mice could be used to specifically delete *Ptch1* from HBCs to support the theory that regulation of HBCs occurs in a HH-independent manner. Inhibition of SHH ligand with the antibody 5E1, can be administered to mice via an I.P. injection (O'Toole et al., 2011) to determine if normal OE homeostasis and regeneration following an injury is

disrupted. Additionally, HBCs can be cultured and treated with either 5E1 or SHH ligand to see if differentiation is inhibited or initiated respectively via immunofluorescence or qRT-PCR. In these cultured cells, other growth factors that have been implicated in GLI-dependent signaling can also be utilized to inhibit or drive differentiation. Furthermore, the GLI small-molecule inhibitor, GANT61 (Mazumdar et al., 2011), can be used *in vivo* or in cultured cells to specifically inhibit GLI activity to further elucidate the possible GLI-dependence of HBC regulation and regeneration in the OE.

Canonical Role for Hedgehog Signaling in the Olfactory Epithelium

Interestingly, in mice treated with LDE-225, there was a reduction in attractive odor behaviors with no effect on aversive behaviors, specifically the fear-inducing compound from fox feces, TMT (Fendt and Endres, 2008) (Fig. 4-8). Various studies suggest that HH signaling is important for axon branching and targeting of OSNs to the OB and as before-mentioned, mature OSNs express *Ptch1*. It is possible that inhibiting the HH pathway affects the targeting of OE to affect specifically attractive odor behaviors. OSNs that express specific odorants target specific areas of the OB. The olfactory epithelium can be divided into two non-overlapping areas: a dorsal zone and a ventral one which can map to corresponding dorsal and ventral areas of the OB (Kobayakawa et al., 2007). Different odors were shown to map to different areas of the OB, for example, TMT activated glomeruli in the ventral domain of the OB (Kobayakawa et al., 2007). When OSNs from a specific domain were genetically deleted, mice became attracted to smells that were usually aversive, including TMT and certain attractive odors such as urine and peanut butter became aversive (Kobayakawa et al., 2007).

Because *Ptch1* is expressed in OSNs with regional variability, it is most likely that LDE-225 treatment affected only those neurons that express *Ptch1* which could coincide with OSNs that express specific odorant receptors responsible for detection of attractive odorants as opposed to aversive ones. Further analysis of additional odorants and specific domain markers of the OB to determine if there is still activity (TH expression) in mice treated with LDE-225 would help further elucidate the role of HH signaling in olfactory behavior. Additionally, analyzing a loss of *Ptch1* expression following LDE-225 via treatment in *Ptch1*$^{lacZ/+}$ mice would be important to ensure that treatment is effectively inhibiting the HH pathway in OSNs since *Ptch1* is a known readout of the pathway. Furthermore, cell-specific knockouts of other HH components in mature OSNs via the *OMP-Cre* allele and SUS cells via *K18-Cre* allele could provide additional information about the role of the HH signaling pathway in the OE.

Together, these experiments could elucidate a GLI-dependent role for the HH pathway plays in regulation of HBCs and olfactory neurogenesis and the HH ligand-dependent role in olfactory transduction and behavior.

What Happens to Hedgehog Pathway Components in Mice that Lack HBC Cilia?

The HH pathway is tightly linked to primary cilia (Haycraft et al., 2005). Because HBCs possess cilia and the transcription factor GLI2, which localizes to tips of cilia, it would be important to investigate what happens to GLI2 and other HH components in iHBC-IFT88 null and iHBC-ARL13B null mice. Loss of *Arl13b* and *Ift88* have been shown to affect the HH pathway (Haycraft et al., 2007; Larkins et al., 2011). These null mice can be crossed with *lacZ* reporter mice that express specific HH components to determine what happens to the components after a loss of Arl13b or Ift88. It would be interesting to

see what happens in iHBC-IFT88 null and iHBC-ARL13B null mice that are crossed with iHBC-Gli2ΔN mice and administered an injury to the OE. Without cilia, the constitutively active form of GLI2 could drive activation of HBCs regardless of the inability to detect the injury. Additionally, because *Sec8* is upregulated following injury to the OE, conditional deletion of *Sec8* or overexpression of *Sec8* in HBCs of control mice or cilia deficient mice could also elucidate its role and sufficiency in mediating the HBC to GBC transition following an injury to the OE. Without *Sec8*, HBCs may not be able to differentiate and regenerate the OE but its overexpression in iHBC-IFT88 null and iHBC-ARL13B null mice could bypass the loss of HBC cilia, allowing for proper differentiation. Furthermore, Notch and Wnt signaling pathways have been implicated in olfactory neurogenesis so analyzing these pathways along with HH *in vivo* or in cultured cells that either possess cilia or lack cilia can further elucidate what drives certain cell fates in the OE.

Overall, data presented in this dissertation demonstrate that HBCs, the presumed stem cell population of the OE, possess primary cilia and HH components that are important for HBC regulation and contribution to neurogenesis in the OE. My results provide novel insight on the mechanisms involved in regeneration of the OE by the introduction of a new role for cilia and the HH pathway in the olfactory periphery and the proposal of a GLI-dependent regulation of HBC activation and differentiation. There is most likely interplay between cilia and the HH pathway in regulating HBCs. Therefore, if olfactory dysfunction arises due to the loss of basal stem cell activity from loss of cilia due to a diseased state like a ciliopathy or aging or from a disruption in GLIs, restoration of cilia function or HH signaling components could be an important therapeutic tool.

Ultimately, these results establish a platform for future investigation of olfactory stem cell regulation.

4.3 Figures

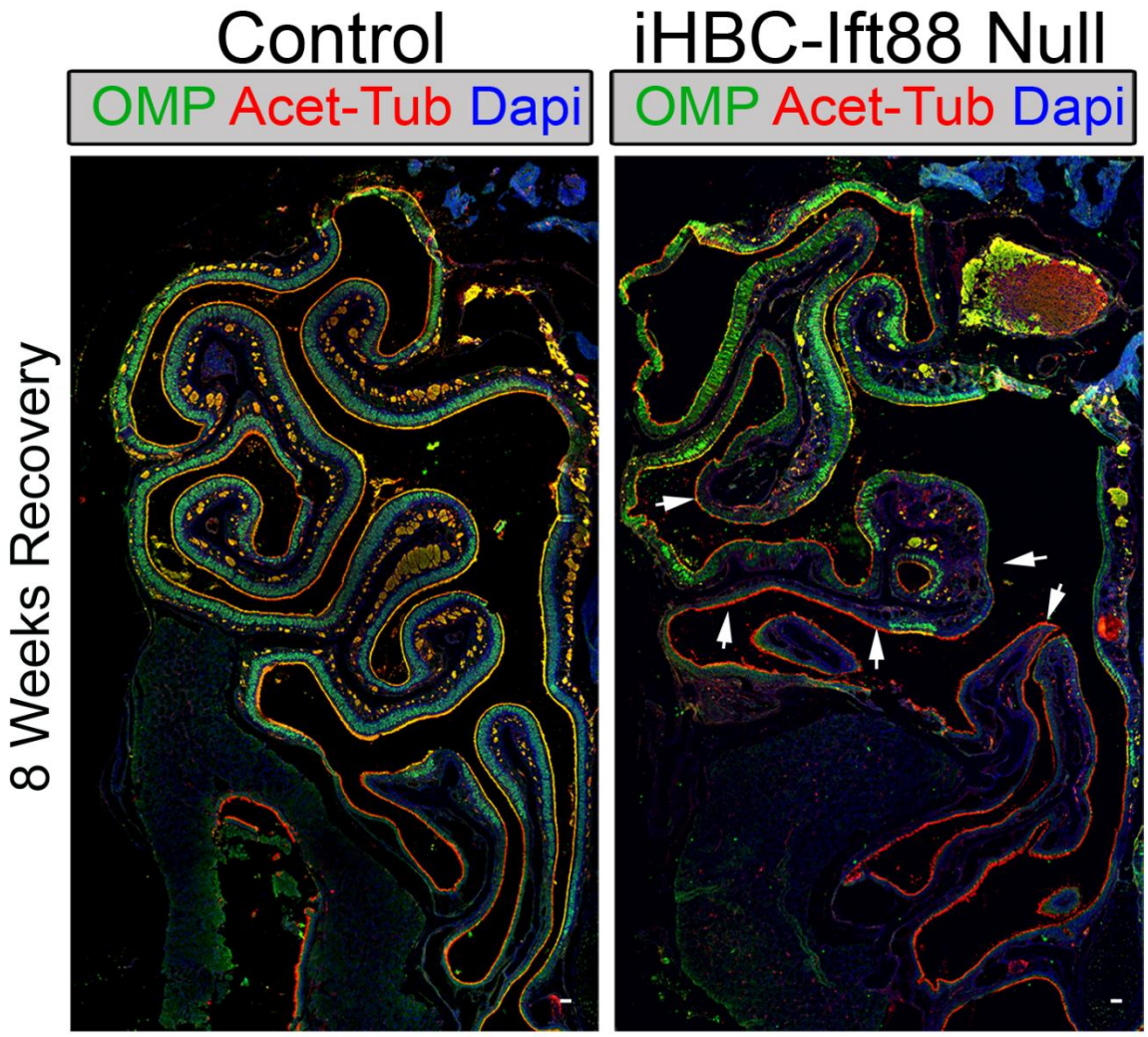

Figure 4-1. Loss of HBC cilia results in respiratory infiltration. Whole sections of the OE of control and iHBC-IFT88 null mice show respiratory infiltration (arrows) in iHBC-IFT88 null mice where Acet-tub labeled cilia are present in areas that lack OMP-labeled mature OSNs.

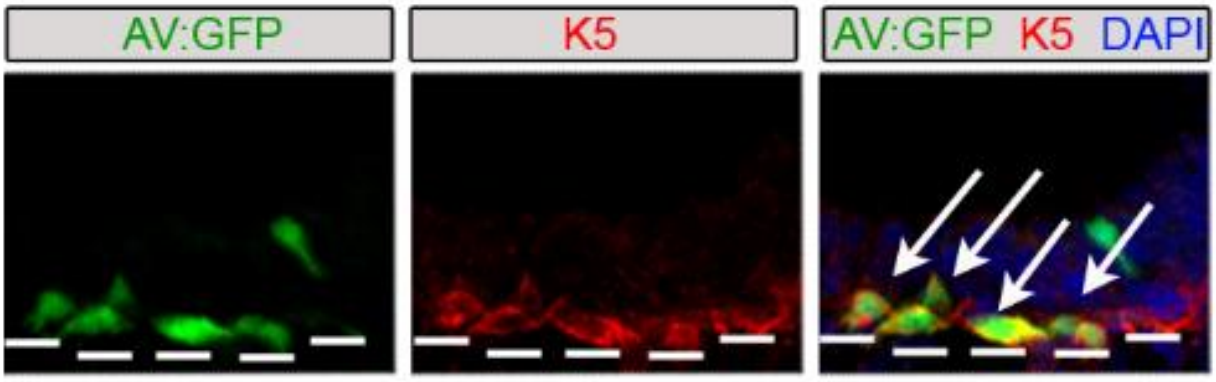

Figure 4-2. HBCs are amenable to adenoviral infection. Two days post MMI-induced injury to the OE, K5+ HBCs are infected with adenovirus (AV) containing GFP, AV:GFP (see arrows).

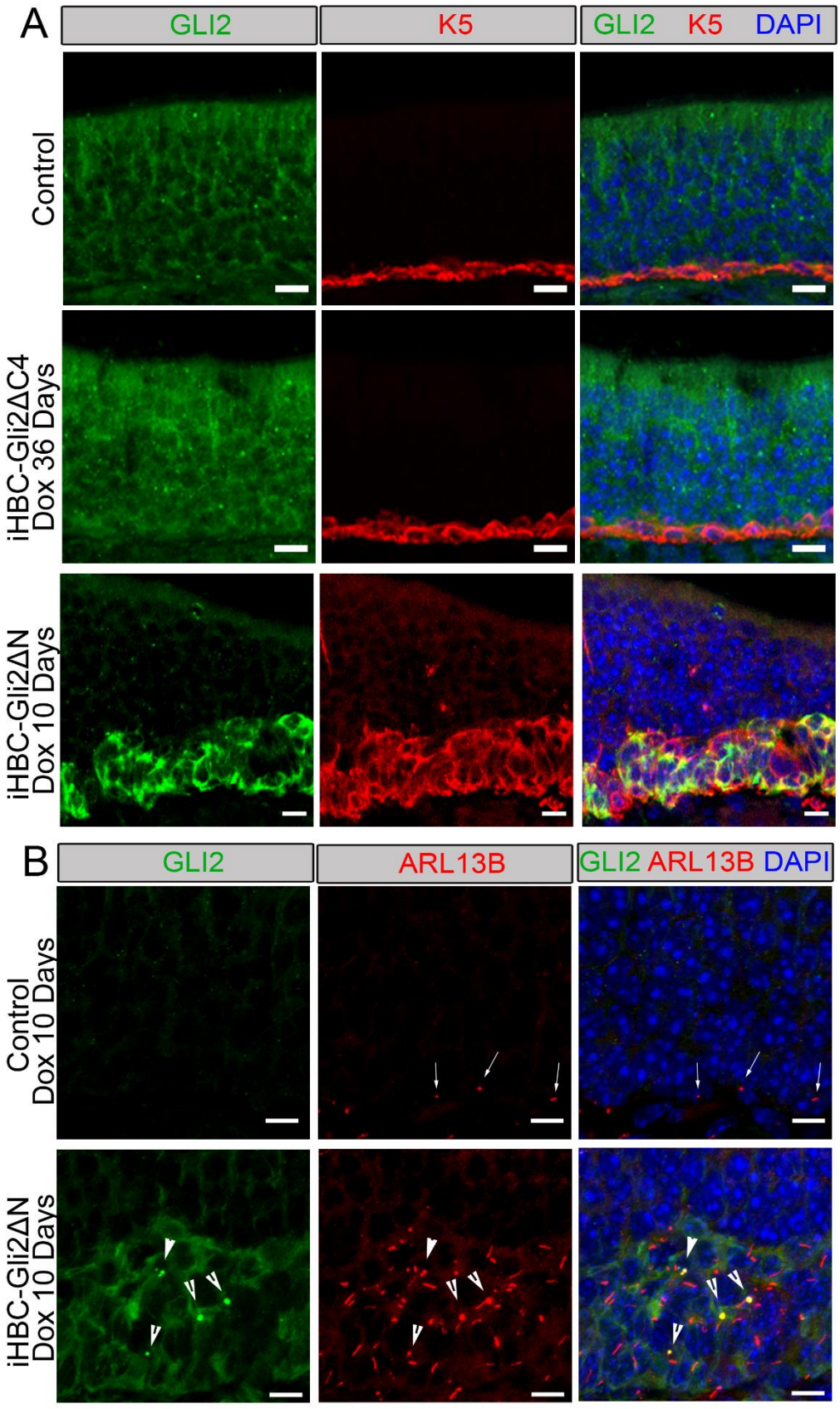
A
GLI2
K5
GLI2 K5 DAPI
Control
iHBC-Gli2ΔC4
Dox 36 Days
iHBC-Gli2ΔN
Dox 10 Days
B
GLI2
ARL13B
GLI2 ARL13B DAPI
Control
Dox 10 Days
iHBC-Gli2ΔN
Dox 10 Days

Figure 4-3. GLI2 antibody detects Gli2ΔN in cilia of HBCs but not endogenous GLI2 or Gli2ΔC4. Immunofluorescence analysis reveals (A) GLI2 antibody is co-expressed with K5+ HBCs of iHBC-Gli2ΔN mice treated with dox for 10 days but not control or iHBC-Gli2ΔC4 mice treated with dox for 36 days and (B) GLI2 antibody localizes to cilia of K5+ HBCs (see arrows) in iHBC-Gli2ΔN mice treated with dox for 10 days but not control mice.

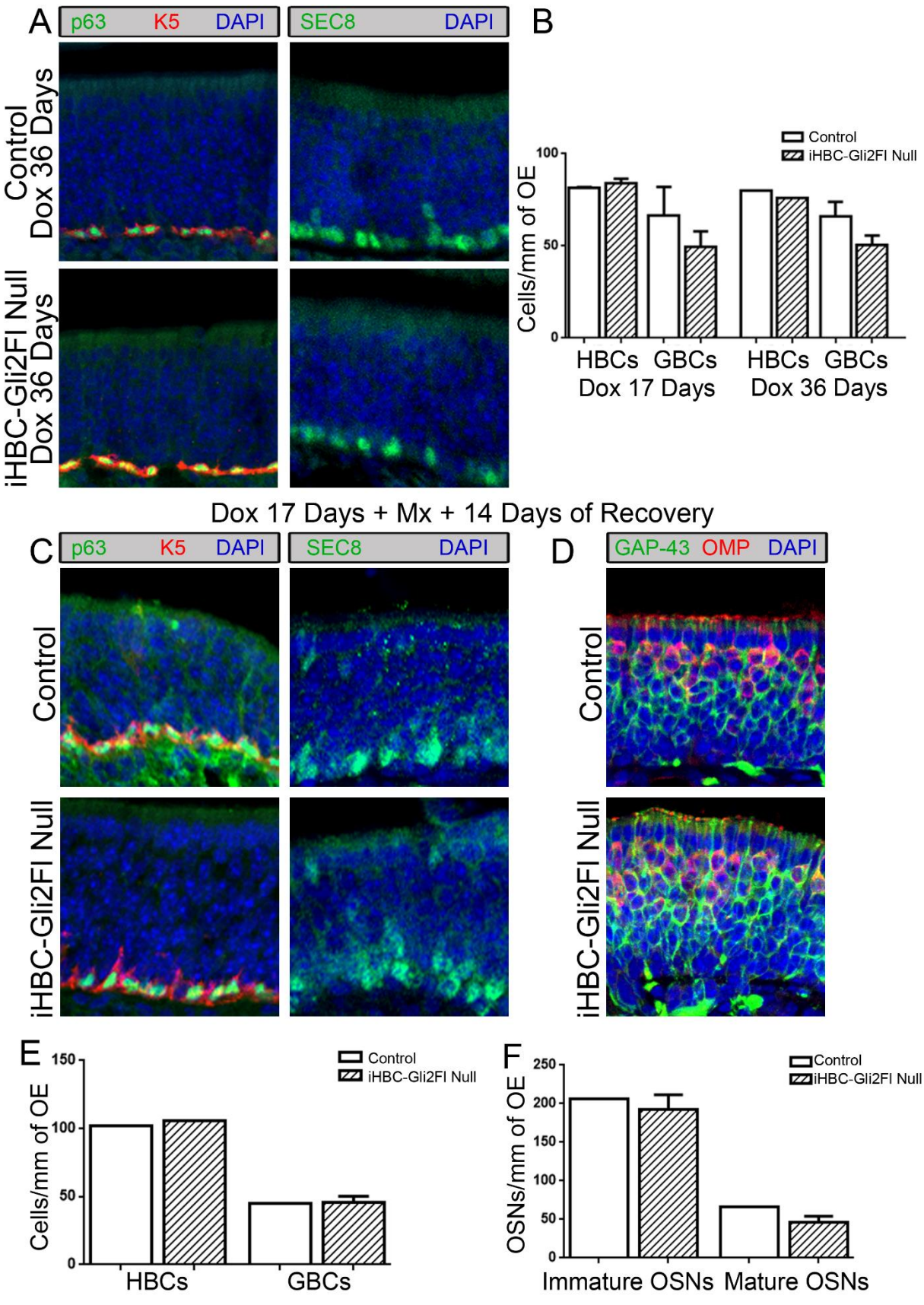
A
p63 K5 DAPI
SEC8 DAPI
Control Dox 36 Days
iHBC-Gli2FI Null Dox 36 Days
B
Control
iHBC-Gli2FI Null
Cells/mm of OE
100
50
0
HBCs GBCs HBCs GBCs
Dox 17 Days Dox 36 Days
Dox 17 Days + Mx + 14 Days of Recovery
C
p63 K5 DAPI
SEC8 DAPI
Control
iHBC-Gli2FI Null
D
GAP-43 OMP DAPI
Control
iHBC-Gli2FI Null
E
Control
iHBC-Gli2FI Null
Cells/mm of OE
150
100
50
0
HBCs GBCs
F
Control
iHBC-Gli2FI Null
OSNs/mm of OE
250
200
150
100
50
0
Immature OSNs Mature OSNs

Figure 4-4. Conditional loss of *Gli2* in HBCs has no effect on homeostasis or regeneration of the OE. (A) Immunofluorescence analysis and (B) quantified data reveal that iHBC-Gli2 null mice treated with dox for 17 or 36 days show now difference in the number of p63+/K5+ HBCs and SEC8+ GBCs compared to control mice. In iHBC-Gli2 null and control mice treated with dox for 17 days and then administered an MMI-induced injury to the OE, (C, E) the number of p63+/K5+ HBCs and SEC8+ GBCs is similar as well as (D, F) the number of GAP-43+ immature and OMP+ mature OSNs.

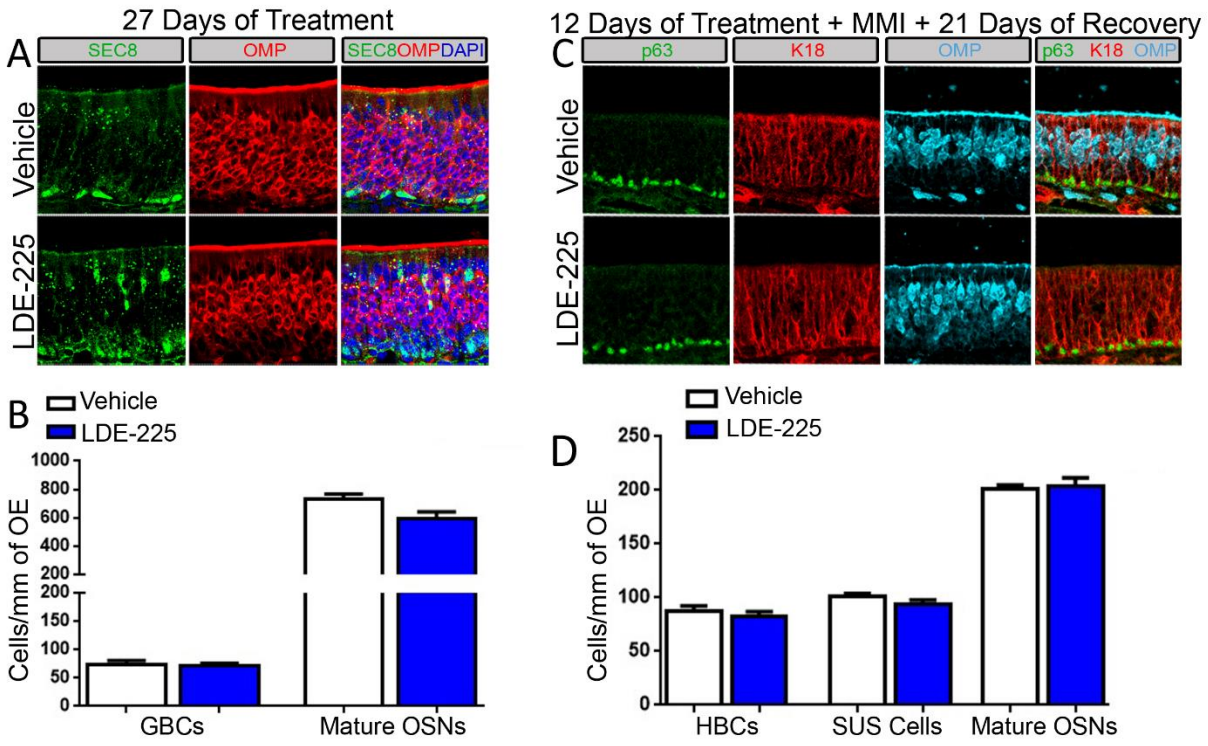

Figure 4-5. The selective SMO inhibitor, LDE-225, has no effect on homeostasis or regeneration of the OE. Mice treated with LDE-225 for 27 days exhibit (A-B) no difference in the number of Sec8+ GBCs or OMP+ mature OSNs compared to vehicle treated mice. Mice treated with LDE-225 for 12 days, administered an MMI-induced injury to the OE, and then allowed to recover for 21 days, exhibit (C-D) no difference in the number of p63+ HBCs, K18+ SUS cells, and OMP+ mature OSNs compared to vehicle treated mice.

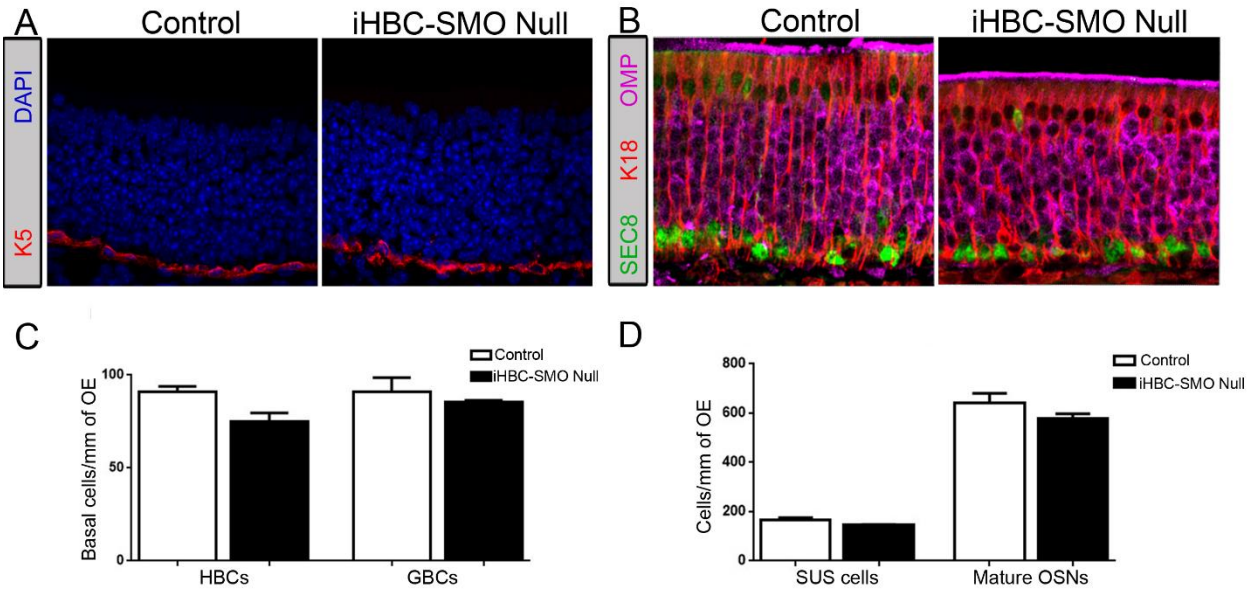

Figure 4-6. Conditional loss of *Smo* in HBCs has no effect on homeostasis of the OE. In iHBC-SMO null mice treated with dox for 19 days there is (A,C) no difference in the number of K5+ HBCs and (B, C-D) no difference in the number of SEC8+ GBCs, K18+ SUS cells, and OMP+ mature OSNs compared to control mice.

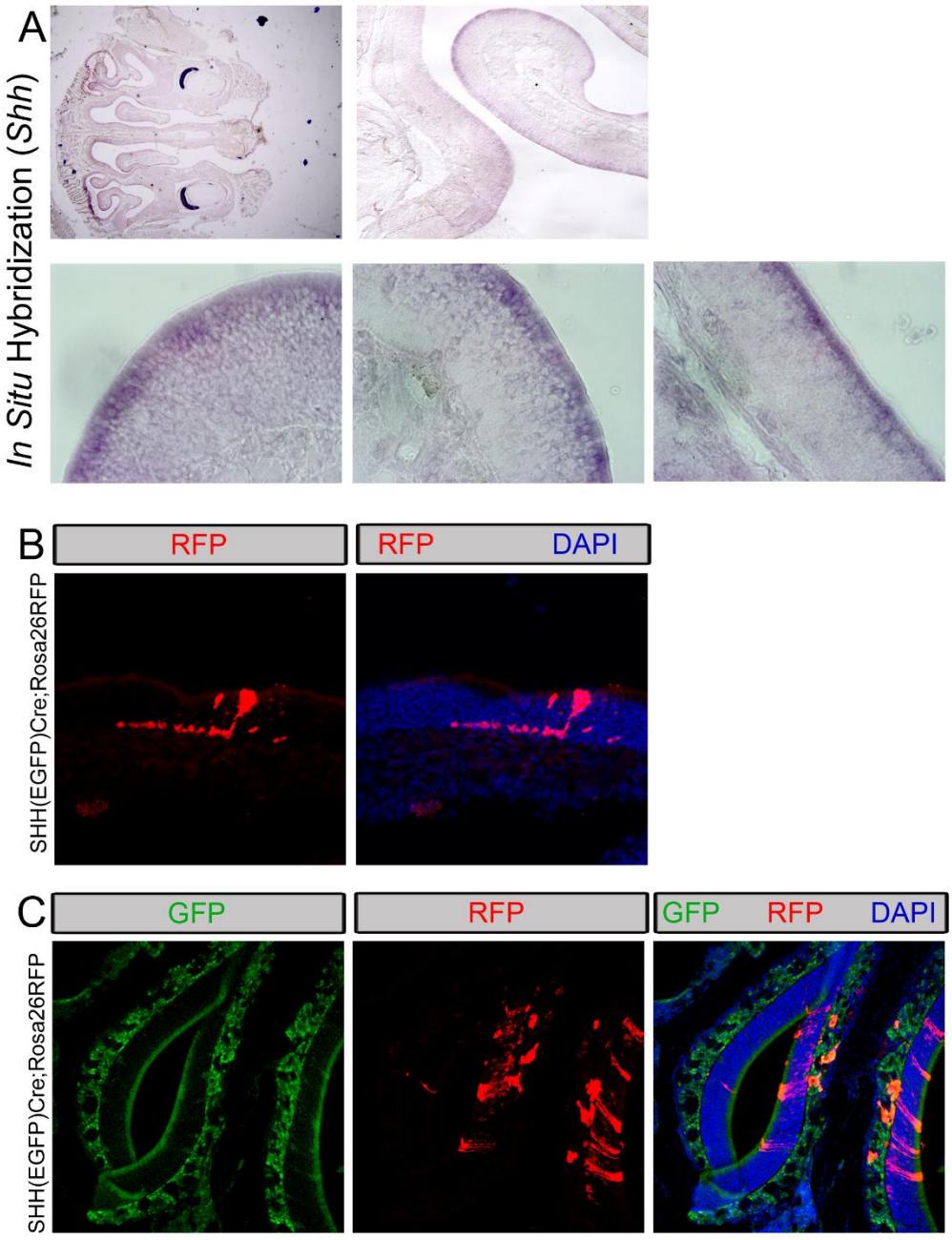

Figure 4-7. *Shh* might be present in SUS cells of the OE. (A) *In situ* hybridization of control tissue with RNA probe against *Shh* shows expression of the transcript in the tooth bud (internal control) and SUS cells of the OE. In *ShhCreER;Roas26RFP* mice, (B) RFP signal is seen in basal cells and SUS cells while (C) GFP is expressed specifically in SUS cells, suggesting they are the source of SHH, and RFP is expressed in SUS cells, OSNs, BG cells, and basal cells, suggesting they are the progeny.

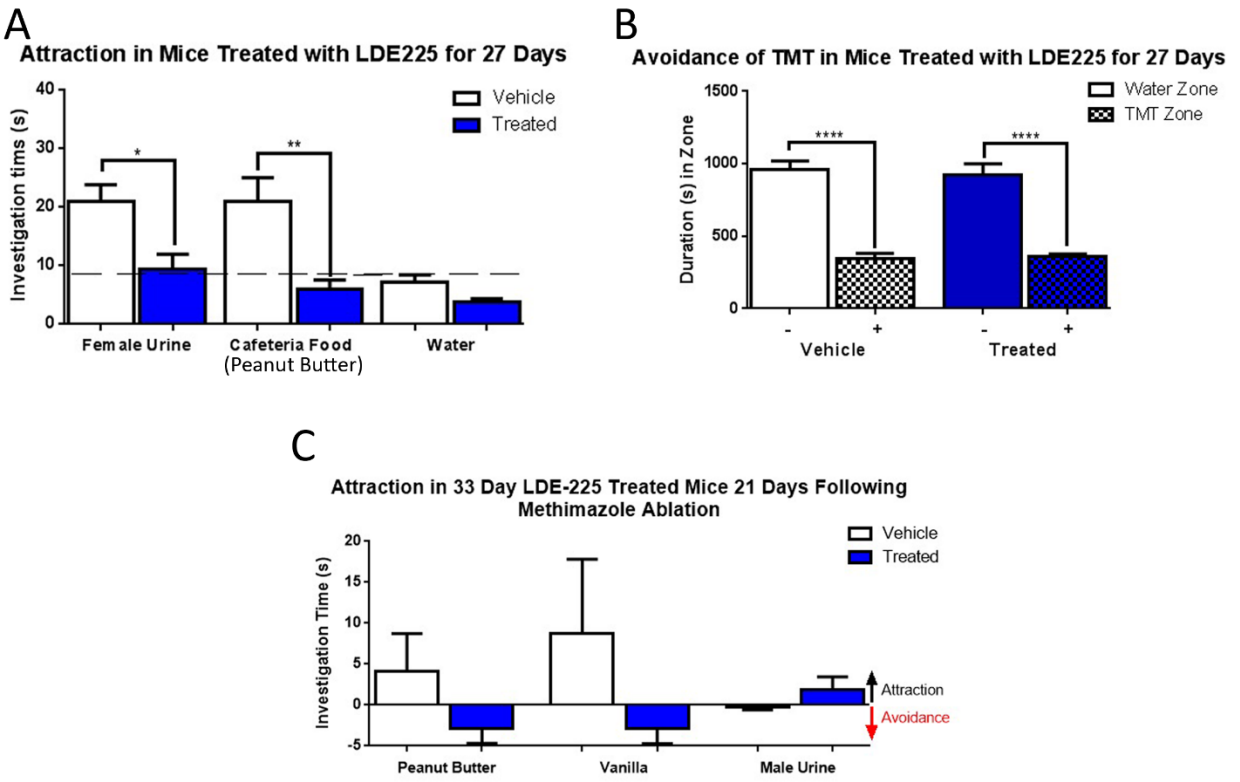

Figure 4-8. The selective SMO inhibitor, LDE-225, reduces attractive behaviors in mice during homeostasis and following injury. (A) Mice treated with LDE-225 for 27 days show a reduction in the investigation time for the attractive odors, urine and peanut butter, but (B) show no difference in avoidance of the component of fox feces, TMT, compared to vehicle treated mice. (C) Mice treated with LDE-225 for 12 days, administered an MMI-induced injury to the OE, and then allowed to recover for 21 days, exhibit a reduction in the investigation time for the attractive odors, peanut butter and vanilla compared to vehicle treated mice.

4.4 References

Aberger F, Kern D, Greil R, Hartmann TN (2012) Canonical and noncanonical Hedgehog/GLI signaling in hematological malignancies. Vitamins and hormones 88:25-54.

Amador-Arjona A, Elliott J, Miller A, Ginbey A, Pazour GJ, Enikolopov G, Roberts AJ, Terskikh AV (2011) Primary cilia regulate proliferation of amplifying progenitors in adult hippocampus: implications for learning and memory. J Neurosci 31:9933-9944.

Barnes EA, Kong M, Ollendorff V, Donoghue DJ (2001) Patched1 interacts with cyclin B1 to regulate cell cycle progression. EMBO J 20:2214-2223.

Blaess S, Stephen D, Joyner AL (2008) Gli3 coordinates three-dimensional patterning and growth of the tectum and cerebellum by integrating Shh and Fgf8 signaling. Development 135:2093-2103.

Buttitta L, Mo R, Hui CC, Fan CM (2003) Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. Development 130:6233-6243.

Carr VM, Farbman AI (1992) Ablation of the olfactory bulb up-regulates the rate of neurogenesis and induces precocious cell death in olfactory epithelium. Exp Neurol 115:55-59.

Cau E, Casarosa S, Guillemot F (2002) Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129:1871-1880.

Cau E, Gradwohl G, Fode C, Guillemot F (1997) Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124:1611-1621.

Cho A, Ko HW, Eggenschwiler JT (2008) FKBP8 cell-autonomously controls neural tube patterning through a Gli2- and Kif3a-dependent mechanism. Dev Biol 321:27-39.

Conley DB, Robinson AM, Shinners MJ, Kern RC (2003) Age-related olfactory dysfunction: cellular and molecular characterization in the rat. Am J Rhinol 17:169-175.

Croyle MJ, Lehman JM, O'Connor AK, Wong SY, Malarkey EB, Iribarne D, Dowdle WE, Schoeb TR, Verney ZM, Athar M, Michaud EJ, Reiter JF, Yoder BK (2011) Role of epidermal primary cilia in the homeostasis of skin and hair follicles. Development 138:1675-1685.

Doty RL (2003) Handbook of olfaction and gustation. New York: M. Dekker.

Ducray A, Bondier JR, Michel G, Bon K, Millot JL, Propper A, Kastner A (2002) Recovery following peripheral destruction of olfactory neurons in young and adult mice. Eur J Neurosci 15:1907-1917.

Duggan CD, DeMaria S, Baudhuin A, Stafford D, Ngai J (2008) Foxg1 is required for development of the vertebrate olfactory system. J Neurosci 28:5229-5239.

Ellis T, Smyth I, Riley E, Graham S, Elliot K, Narang M, Kay GF, Wicking C, Wainwright B (2003) Patched 1 conditional null allele in mice. Genesis 36:158-161.

Fendt M, Endres T (2008) 2,3,5-Trimethyl-3-thiazoline (TMT), a component of fox odor - just repugnant or really fear-inducing? Neuroscience and biobehavioral reviews 32:1259-1266.

Friedrich GA, Hildebrand JD, Soriano P (1997) The secretory protein Sec8 is required for paraxial mesoderm formation in the mouse. Dev Biol 192:364-374.

Gong Q, Chen H, Farbman AI (2009) Olfactory sensory axon growth and branching is influenced by sonic hedgehog. Dev Dyn 238:1768-1776.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463-476.

Hallahan AR, Pritchard JI, Hansen S, Benson M, Stoeck J, Hatton BA, Russell TL, Ellenbogen RG, Bernstein ID, Beachy PA, Olson JM (2004) The SmoA1 mouse model reveals that notch signaling is critical for the growth and survival of sonic hedgehog-induced medulloblastomas. Cancer research 64:7794-7800.

Han YG, Spassky N, Romaguera-Ros M, Garcia-Verdugo JM, Aguilar A, Schneider-Maunoury S, Alvarez-Buylla A (2008) Hedgehog signaling and primary cilia are required for the formation of adult neural stem cells. Nat Neurosci 11:277-284.

Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK (2005) Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet 1:e53.

Haycraft CJ, Zhang Q, Song B, Jackson WS, Detloff PJ, Serra R, Yoder BK (2007) Intraflagellar transport is essential for endochondral bone formation. Development 134:307-316.

Hildebrandt F, Benzing T, Katsanis N (2011) Ciliopathies. N Engl J Med 364:1533-1543.

Holbrook EH, Szumowski KE, Schwob JE (1995) An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. J Comp Neurol 363:129-146.

Holbrook EH, Wu E, Curry WT, Lin DT, Schwob JE (2011) Immunohistochemical characterization of human olfactory tissue. Laryngoscope 121:1687-1701.

Irigoin F, Badano JL (2011) Keeping the balance between proliferation and differentiation: the primary cilium. Curr Genomics 12:285-297.

Iwai N, Zhou Z, Roop DR, Behringer RR (2008) Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. Stem Cells 26:1298-1306.

Jang W, Lambropoulos J, Woo JK, Peluso CE, Schwob JE (2008) Maintaining epitheliopoietic potency when culturing olfactory progenitors. Exp Neurol 214:25-36.

Jia C, Hegg CC (2010) NPY mediates ATP-induced neuroproliferation in adult mouse olfactory epithelium. Neurobiology of disease 38:405-413.

Jia C, Roman C, Hegg CC (2010) Nickel sulfate induces location-dependent atrophy of mouse olfactory epithelium: protective and proliferative role of purinergic receptor activation. Toxicol Sci 115:547-556.

Joiner AM, Green WW, McIntyre JC, Allen BL, Schwob JE, Martens JR (2015) Primary Cilia on Horizontal Basal Cells Regulate Regeneration of the Olfactory Epithelium. J Neurosci 35:13761-13772.

Kim EJ, Ables JL, Dickel LK, Eisch AJ, Johnson JE (2011) Ascl1 (Mash1) defines cells with long-term neurogenic potential in subgranular and subventricular zones in adult mouse brain. PLoS One 6:e18472.

Kim S, Tsiokas L (2011) Cilia and cell cycle re-entry: more than a coincidence. Cell Cycle 10:2683-2690.

Kobayakawa K, Kobayakawa R, Matsumoto H, Oka Y, Imai T, Ikawa M, Okabe M, Ikeda T, Itohara S, Kikusui T, Mori K, Sakano H (2007) Innate versus learned odour processing in the mouse olfactory bulb. Nature 450:503-508.

Krolewski RC, Jang W, Schwob JE (2011) The generation of olfactory epithelial neurospheres in vitro predicts engraftment capacity following transplantation in vivo. Exp Neurol 229:308-323.

Krolewski RC, Packard A, Schwob JE (2013) Global expression profiling of globose basal cells and neurogenic progression within the olfactory epithelium. J Comp Neurol 521:833-859.

Kulaga HM, Leitch CC, Eichers ER, Badano JL, Lesemann A, Hoskins BE, Lupski JR, Beales PL, Reed RR, Katsanis N (2004) Loss of BBS proteins causes anosmia in humans and defects in olfactory cilia structure and function in the mouse. Nat Genet 36:994-998.

Kumamoto N, Gu Y, Wang J, Janoschka S, Takemaru K, Levine J, Ge S (2012) A role for primary cilia in glutamatergic synaptic integration of adult-born neurons. Nat Neurosci 15:399-405, S391.

Larkins CE, Aviles GD, East MP, Kahn RA, Caspary T (2011) Arl13b regulates ciliogenesis and the dynamic localization of Shh signaling proteins. Mol Biol Cell 22:4694-4703.

Lee P, Morley G, Huang Q, Fischer A, Seiler S, Horner JW, Factor S, Vaidya D, Jalife J, Fishman GI (1998) Conditional lineage ablation to model human diseases. Proc Natl Acad Sci U S A 95:11371-11376.

Legrier ME, Ducray A, Propper A, Chao M, Kastner A (2001) Cell cycle regulation during mouse olfactory neurogenesis. Cell Growth Differ 12:591-601.

Manglapus GL, Youngentob SL, Schwob JE (2004) Expression patterns of basic helix-loop-helix transcription factors define subsets of olfactory progenitor cells. J Comp Neurol 479:216-233.

Mazumdar T, Devecchio J, Agyeman A, Shi T, Houghton JA (2011) Blocking Hedgehog survival signaling at the level of the GLI genes induces DNA damage and extensive cell death in human colon carcinoma cells. Cancer research 71:5904-5914.

McIntyre JC et al. (2012) Gene therapy rescues cilia defects and restores olfactory function in a mammalian ciliopathy model. Nat Med 18:1423-1428.

Mimeault M, Hauke R, Batra SK (2007) Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clin Pharmacol Ther 82:252-264.

Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, Heng HH, Chik KW, Shi XM, Tsui LC, Cheng SH, Joyner AL, Hui C (1997) Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Development 124:113-123.

Motoyama J, Liu J, Mo R, Ding Q, Post M, Hui CC (1998) Essential function of Gli2 and Gli3 in the formation of lung, trachea and oesophagus. Nat Genet 20:54-57.

Motoyama J, Milenkovic L, Iwama M, Shikata Y, Scott MP, Hui CC (2003) Differential requirement for Gli2 and Gli3 in ventral neural cell fate specification. Dev Biol 259:150-161.

Murdoch B, Roskams AJ (2007) Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. J Mol Histol 38:581-599.

Murphy C, Schubert CR, Cruickshanks KJ, Klein BE, Klein R, Nondahl DM (2002) Prevalence of olfactory impairment in older adults. Jama 288:2307-2312.

O'Toole SA et al. (2011) Hedgehog overexpression is associated with stromal interactions and predicts for poor outcome in breast cancer. Cancer research 71:4002-4014.

Packard A, Giel-Moloney M, Leiter A, Schwob JE (2011a) Progenitor cell capacity of NeuroD1-expressing globose basal cells in the mouse olfactory epithelium. J Comp Neurol 519:3580-3596.

Packard A, Schnittke N, Romano RA, Sinha S, Schwob JE (2011b) DeltaNp63 regulates stem cell dynamics in the mammalian olfactory epithelium. J Neurosci 31:8748-8759.

Pan S et al. (2010) Discovery of NVP-LDE225, a Potent and Selective Smoothened Antagonist. ACS medicinal chemistry letters 1:130-134.

Pan Y, Bai CB, Joyner AL, Wang B (2006) Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Molecular and cellular biology 26:3365-3377.

Petrova R, Joyner AL (2014) Roles for Hedgehog signaling in adult organ homeostasis and repair. Development 141:3445-3457.

Plotnikova OV, Pugacheva EN, Golemis EA (2009) Primary cilia and the cell cycle. Methods in cell biology 94:137-160.

Ram Kumar RM, Betz MM, Robl B, Born W, Fuchs B (2014) DeltaNp63alpha enhances the oncogenic phenotype of osteosarcoma cells by inducing the expression of GLI2. BMC cancer 14:559.

Riddle DR, Lichtenwalner RJ (2007) Neurogenesis in the Adult and Aging Brain. In: Brain Aging: Models, Methods, and Mechanisms (Riddle DR, ed). Boca Raton (FL).

Sasaki H, Nishizaki Y, Hui C, Nakafuku M, Kondoh H (1999) Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Development 126:3915-3924.

Singla V, Reiter JF (2006) The primary cilium as the cell's antenna: signaling at a sensory organelle. Science 313:629-633.

Stecca B, Ruiz IAA (2010) Context-dependent regulation of the GLI code in cancer by HEDGEHOG and non-HEDGEHOG signals. Journal of molecular cell biology 2:84-95.

Tadenev AL, Kulaga HM, May-Simera HL, Kelley MW, Katsanis N, Reed RR (2011) Loss of Bardet-Biedl syndrome protein-8 (BBS8) perturbs olfactory function, protein localization, and axon targeting. Proc Natl Acad Sci U S A 108:10320-10325.

Traiffort E, Angot E, Ruat M (2010) Sonic Hedgehog signaling in the mammalian brain. Journal of neurochemistry 113:576-590.

Treloar HB, Miller AM, Ray A, Greer CA (2010) Development of the Olfactory System. In: The Neurobiology of Olfaction (Menini A, ed). Boca Raton (FL).

Tummala P, Arnsdorf EJ, Jacobs CR (2010) The Role of Primary Cilia in Mesenchymal Stem Cell Differentiation: A Pivotal Switch in Guiding Lineage Commitment. Cell Mol Bioeng 3:207-212.

Uhmann A, Dittmann K, Nitzki F, Dressel R, Koleva M, Frommhold A, Zibat A, Binder C, Adham I, Nitsche M, Heller T, Armstrong V, Schulz-Schaeffer W, Wienands J, Hahn H (2007) The Hedgehog receptor Patched controls lymphoid lineage commitment. Blood 110:1814-1823.

Vedin V, Molander M, Bohm S, Berghard A (2009) Regional differences in olfactory epithelial homeostasis in the adult mouse. J Comp Neurol 513:375-384.

Veland IR, Awan A, Pedersen LB, Yoder BK, Christensen ST (2009) Primary cilia and signaling pathways in mammalian development, health and disease. Nephron Physiol 111:p39-53.

Xie F, Zhou X, Behr M, Fang C, Horii Y, Gu J, Kannan K, Ding X (2010) Mechanisms of olfactory toxicity of the herbicide 2,6-dichlorobenzonitrile: essential roles of CYP2A5 and target-tissue metabolic activation. Toxicol Appl Pharmacol 249:101-106.

Ye L, Chan S, Chow YH, Tsui LC, Hu J (2001) Regulated expression of the human CFTR gene in epithelial cells. Molecular therapy : the journal of the American Society of Gene Therapy 3:723-733.