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The Regulation of Smoothened Activity in the Hedgehog Response Pathway

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The Regulation of Smoothened Activity in the Hedgehog Response Pathway

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Abstract

The Regulation of Smoothened Activity in the Hh Response Pathway

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Doctor of Philosophy in Molecular and Cell Biology

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Sonic Hedgehog (Shh) is a vertebrate signaling molecule that is indispensable for patterning during embryogenesis and for the maintenance of adult stem cell populations. Impaired regulation of the Hedgehog (Hh) pathway can lead to birth defects and disease, thus insight into the regulation of signal transduction is critical for understanding developmental mechanisms and for generating new therapeutic strategies.

The function of the ligand, the receptor, and the signal transducer are separated into distinct molecules and the state of pathway activity depends on their interaction. In the absence of the Shh ligand, the canonical receptors, Patched1/2 (Ptch1/2), are potent inhibitors of Smoothened (Smo), the GPCR-like signal transducer of the Hh pathway. The mechanism by which Shh acts through Ptch1/2 to activate Smo and thereby the Hh response remains unclear.

Here we find that *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells have a low level of Hh pathway activity and are unresponsive to exogenously supplied ShhN, unless transfected with *Ptch1,* including forms of Ptch1 lacking antiporter activity required for the inhibition of Smo. However, this Ptch1 requirement can be circumvented by *ShhN* transfection; *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells expressing ShhN have an activated Hh response that requires Smo. Mutant forms of Shh that cannot bind Ptch1/2 or the Shh co-receptors, and thus unable to induce the Hh response when applied to Ptch1/2 proficient cells, are nevertheless potent inducers of the Hh response after transfection into *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells and *in vivo*. Forms of Smo lacking the N-terminal extracellular Cysteine Rich Domain (CRD) retain their sensitivity to Ptch1-mediated inhibition, but can no longer be activated cell-autonomously by Shh. Our findings support a model in which the role of Ptch1/2 as an allosteric inhibitor of Smo is complemented by a role as a facilitator of a Shh-mediated Smo activation event that is independent of Shh (co-) receptors.

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Chapter 1. Introduction

1.1 Cell-cell communication

1.1.1 Signaling molecules in multicellular life

Multicellular life relies on the ability of cells to communicate and coordinate their activities effectively. To accomplish this, multicellular organisms rely on signals sent between cells to elicit specific responses for the purposes of cellular organization and tissue patterning during embryonic development, and for physiological function in adults. Vertebrates have a handful of signaling pathways, some of which are derived from pathways as old as multicellular life itself, which play numerous roles throughout development and beyond. These signaling pathways include: Wnt, TGFβ, BMP, FGF, Notch, and Hedgehog.

During development, ligands often act as morphogens, signals that relay positional information and elicit responses in a dose and time-dependent manner. Morphogen gradients are formed by signaling centers, called "organizers", which secrete signals into the extracellular space and are subsequently shaped by extracellular matrix and proteins that can bind to the signal. Signaling organizers are required for essential morphogenetic changes required to pattern the developing embryo, including gastrulation; which is, as Lewis Wolpert has been famously quoted as saying, "truly the most important time in your life".

The secretion of a signal from a cell or a group of cells is only among the first steps of signal transduction, and a signal must often travel several cell diameters away to be received. Signaling pathways have different mechanisms for the presentation of a ligand to its receptor(s). Although signaling molecules, or ligands, were first thought to be soluble in the extracellular space, it has since been found that they are often affixed to extracellular matrix proteins that can either impede or enhance their interaction with receptors. Ligands in the extracellular space can also interact with secreted extracellular proteins that can modulate their ability to activate signal transduction pathways. Remarkably, tethered ligands that travel through the extracellular space to reach other cells can be carried by "cytonemes", specialized cell extensions that have been implicated in a variety of cell signaling mechanisms both in arthropods and vertebrates. Ligands can also remain fixed upon the membrane of signaling cells through complete association of the plasma membrane, allowing for juxtacrine signaling mediated by cell-cell contacts. Cell-contact mediated signaling is exemplified by cadherin based signaling and the Notch/Delta and Eph/Ephrin pathways. Thus, ligands are under high regulation in the extracellular space as well as on the cell membrane during receptor interactions.

Cellular responses to signaling can include changes in transcriptional output or cytoskeletal arrangement, processes that regulate proliferation, differentiation, cell migration, axon guidance, apoptosis, and other morphogenetic changes that characterize embryonic development. The ability to respond to molecular signals is also an integral part of fully developed animals, and regulate critical processes such as immune system responses, synaptic signaling, and adult stem cell niche maintenance.

Signal transduction pathways are a symphony of complex molecular interactions, with each step in the pathway providing a layer of regulation against inappropriate activation or inhibition. Mutations that undermine the regulation of these signaling pathways can lead to the uncontrolled cell proliferation that characterizes cancer. Accordingly, the molecular processes that govern signal secretion, transport, recognition, and transduction have been an important focus for cell biologists for many decades. Despite the gain of tremendous knowledge in the field, the distinct signal transduction mechanism of each pathway is not fully resolved.

1.1.2 Hedgehog signaling in development

The Hedgehog (Hh) signaling pathway is an evolutionarily conserved pathway found in most metazoan life forms. The evolutionary origins of the Hh pathway are old, and primitive forms of various components of the pathway can be found in most forms of life including unicellular choanoflagellates (Adamska *et. al.*, 2007). The Hh pathway is fundamental to many embryonic and physiological processes in arthropods and vertebrates, and model organisms such as *Drosophila melanogaster*, *Mus musculus*, *Danio rerio*, and *Gallus gallus*, have been central in understanding the molecular details of the pathway.

The Hedgehog ligand was first identified in the now famous developmental fruit fly screen performed by Christiane Nüsslein-Volhard and Eric Wieschaus in the late 1970s. The screen used a technique known as “saturation mutagenesis" to isolate genes involved in the formation of the Drosophila body plan (Nüsslein-Volhard & Wieschaus, 1980). Three classes of genes were identified for their role in regulating the process of segmentation during the embryonic development of Drosophila: gap genes, pair rule genes, and segment polarity genes. Hh functions as a segment polarity gene, a type of gene that defines the anterior and posterior polarity of each embryonic segment. Hh mutant larvae have a solid lawn of denticles on the cuticle rather than stripes of denticles on the anterior half of each segment, hence the name “hedgehog”. During segmentation, Hh signals locally to cells within the same segment to induce the expression of another signal, wingless (wg), which signals reciprocally to Hh expressing cells to stabilize the boundary of each segment (Ingham & McMahon, 2001).

Besides segmentation, Hh signaling plays a role in many other aspects of Drosophila development. One process that has been studied extensively, and is often used as an experimental system, is the development of the wing imaginal disc. In the wing imaginal disc of Drosophila larvae, cells in the posterior compartment secrete Hh, which forms a concentration gradient along the anterior-posterior compartment boundary (**Figure 1**). Cells in the anterior compartment of the wing disc respond to the Hh signal. High levels of Hh protein induces the expression of various target genes such as engrailed (en), and patched (ptc), while lower levels of Hh protein induces the expression of decapentaplegic (dpp), a member of the TGFβ family of signaling molecules which goes on to signal to cells of the anterior and posterior compartments (Lecuit *et. al.*, 1996; Nellen *et. al.*, 1996). Expression of these Hh target genes is required to properly pattern the wing disc which goes on to form the Drosophila adult wing (Ingham & McMahon, 2001).

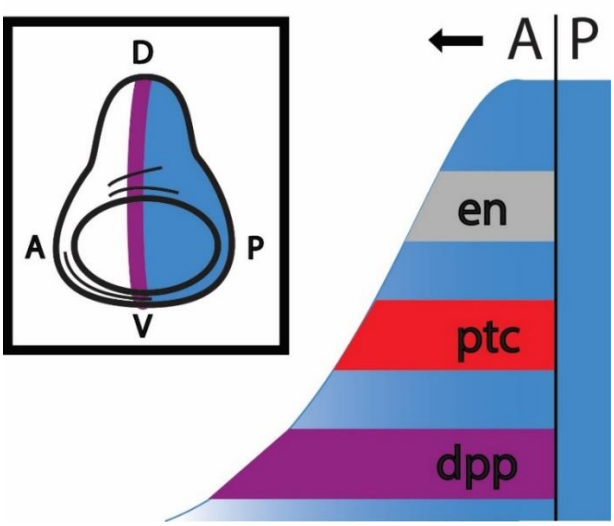

Figure 1 Hh signaling in Drosophila wing imaginal disc

Figure 1. Hh signaling in Drosophila wing imaginal disc

A Hh gradient is required to pattern the anterior/posterior (A/P) axis of the Drosophila wing imaginal disc, the embryonic precursor of the adult wing. Cells in the posterior compartment of the wing imaginal disc express Hh (blue), which signals to cells in the anterior compartment in a concentration-dependent manner along the A/P boundary (purple). High levels of Hh signaling induce the expression of engrailed (en) and progressively lower levels of Hh signaling induce the expression of patched (ptc) and decapentaplegic (dpp).

Vertebrates have three Hh paralogs: Indian Hedgehog, Desert Hedgehog, and Sonic Hedgehog. All three Hh ligands play mostly non-overlapping roles in vertebrate development, and as such are differentially expressed in the embryo. Indian Hedgehog (Ihh) functions in vasculogenesis, hematopoiesis, and the proliferation and differentiation of the endochondral skeleton (Dyer *et. al*., 2001; Lanske *et. al*., 1996; Chung *et. al*., 2001). Desert Hedgehog (Dhh), the most closely related to Drosophila Hh, is required for the maturation of the testes, including the proliferation and differentiation of Leydig cells (Zou *et. al*., 2012). Sonic Hedgehog (Shh), the most extensively studied of the Hh ligands, is broadly expressed and plays multiple roles during embryogenesis and adulthood. The fundamental cellular mechanisms that are regulated by Shh makes this ligand indispensable for vertebrate embryonic development and adult stem cell niche maintenance. Shh is involved in the development of the central nervous system, cerebellum, bone and cartilage, eye, hair, lung, muscle, and limbs, and functions redundantly with Ihh to generate left/right asymmetry (Ingham & McMahon, 2001).

One extensively studied developmental mechanism regulated by Shh is the anterior/posterior (A/P) patterning of the limb. Limb buds form from the proliferation of lateral plate mesoderm cells induced by FGF8 signaling. The formation of the apical ectodermal ridge (AER), a specialized epithelium found on the distal tip of the limb bud, is then sequentially induced by FGF10 and maintains the proliferation of the underlying mesenchyme (Johnson & Tabin, 1997). A population of distal, posterior mesenchyme cells in the limb bud, the Zone of Polarizing Activity (ZPA), being to express Shh which goes on to form a concentration gradient along the (A/) axis of the distal limb. The gradient of Hh signaling induces the formation of posterior limb structures and digits. Experimental manipulation of the limb bud has shown that Shh is required and sufficient for this polarizing activity, as ectopic expression of Shh or application of Shh soaked beads to the anterior limb bud induces the ectopic formation of posterior digits in a dose dependent manner (Yang *et. al*., 1997). Perturbations of Hh signaling during development of the limb leads malformations of the adult limb, including loss or gain of digits.

Shh has also been comprehensively studied for its role in the development of the central nervous system. The developing central nervous system arises from an embryonic structure known as the

neural tube, quite literally a tube of neuroepithelium derived from the neural plate. Early in development, ectodermal cells are exposed to a BMP4 signal that causes a differentiation into epithelial cells. However, during neurulation, an underlying structure made of axial mesoderm, the notochord, secretes proteins in the extracellular space that antagonize the BMP4 ligand: Chordin, Noggin, and Follistatin (Wilson *et. al.,* 1997). Inhibition of the BMP4 signal causing the dorsal ectoderm to thicken into the neural plate. The neural plate then folds inwardly, and the two neural plate borders create "hinges" by undergoing apical constriction. Eventually the borders of the neural fold join together to create the overlying ectoderm and the underlying neural tube closes. The cells within the neural tube must then be patterned into a stereotypical arrangement of neuronal subtypes that function in the central nervous system (Alaynick *et al.*, 2011).

The notochord, located ventral to the neural tube, generates an early ventral to dorsal Shh gradient. This initial signaling event induces the formation of the floor plate, the most ventral aspect of the neural tube midline. Experimental manipulation have revealed the inductive power of the notochord, as removal of the notochord results in a failure of proper neural tube patterning, and grafting a second notochord to the neural tube results in the formation of a second ectopic floor plate in the neural tube (Placzek *et al.*, 1990; Van Straaten *et. al*., 1985). The floor plate then expresses Shh, and contributes to the formation of the ventral to dorsal Shh concentration gradient within the neural tube. The Shh gradient has been shown to reach a significant distance from the floor plate, and has been indirectly visualized with a Shh:GFP fusion protein (Chamberlain *et. al*., 2008). Interestingly, Shh has been shown to localize to the basement membrane that surrounds the neural tube, possibly indicative of the mechanism required to transport Shh to responding cells. Within the ventral half of the neural tube, Shh induces the expression of various transcription factors, such as Nkx2.2, Olig2, and Pax6, in a concentration and time-dependent manner (Briscoe *et. al*., 2009) (**Figure 2**). These transcription factors work in combination to specify five ventral neuronal subtypes, including motor neurons and interneurons, found along the dorsal-ventral axis (Roelink *et. al*., 1994; Roelink *et. al*., 1995; Jessell *et. al*., 2000). Besides its role as a morphogen in neuronal patterning, Shh also acts as an attractive cue for commissural neurons that lie in the dorsal neural tube. Dorsal commissural neurons send their axons to the ventral neural tube using netrins and Shh as a guidance cue, and perturbance of its reception results in misplaced axons (Charron *et. al*., 2003).

Because of the well-characterized patterning generated by Shh in the neural tube, this has become a powerful system for analyzing the molecular mechanism of the Hh pathway. The neural tube is a structure which is routinely analyzed in animal embryos with mutations in presumptive Hh pathway components, as disruption of normal patterning in the ventral neural tube is indicative of a disruption in the Hh pathway. Additionally, the introduction of exogenous DNA coding for Hh pathway mutants in the neural tube is feasible through electroporation. This is a strong experimental approach for piecing together the molecular interactions that define the Hh pathway in an *in vivo* system.

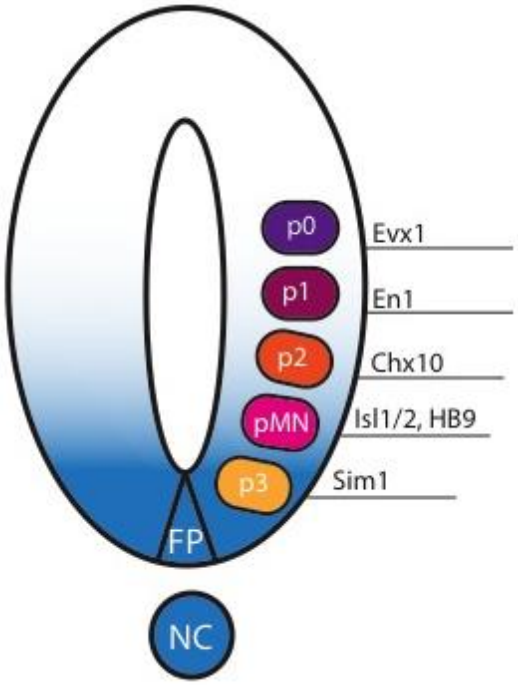

Figure 2 Hh Signaling in the developing vertebrate central nervous system

Figure 2. Hh signaling in the developing vertebrate central nervous system

A Shh gradient is required to pattern the dorsal/ventral (D/V) axis in the neural tube, the embryonic precursor of the central nervous system. A ventral to dorsal Shh gradient (blue) is generated by the notochord (NC) and the floor plate (FP) of the neural tube. Various concentrations of Shh induce expression of transcription factors that are required for the differentiation of distinct neuronal progenitors. Depicted in the diagram are the five neural progenitor domains (p3-p0), and the terminally differentiated neuronal factors they express (Sim-Evx1).

A role for Hh signaling in the maintenance of adult stem cell populations has also become apparent. Throughout the life of a multicellular organism, cells must be replenished as they go undergo senescence and death. Pools of stem cells that can regenerate different cell types must be maintained in a specialized microenvironment rich with signals that maintain their pluripotency. Various signals have been shown to be required in adult stem niches throughout the body, these include Shh, Wnts, and BMPs. Shh has been implicated as one of the factors required for the maintenance of hematopoietic stem cell populations in bone marrow (Adams & Scadden, 2006). Shh is also required for the maintenance of stem cells in the subventricular zone of the forebrain, and in the interfollicular epithelium where it regulates the hair follicle cycle (Gonzalez-Perez *et. al.*, 2014; Millar *et. al.*, 2002).

1.1.3 Hedgehog signaling in disease

Because Shh plays an instructional role in cellular responses required during vertebrate development and adulthood, it is unsurprising that disruption of Hh signaling can lead to a wide array of disorders. Deregulation of the pathway can lead to birth defects and disease later in life; thus, insight into Hh signal transduction is critical for not only understanding developmental mechanisms, but also for generating new therapeutic strategies.

Disruption of Hh signaling during embryonic development can lead to various malformations. One such malformation is Holoprosencephaly (HPE), a disorder that results from the incomplete cleavage of the embryonic forebrain, the prosencephalon, into two cerebral hemispheres. The prosencephalon begins to develop between the fifth and sixth week of human pregnancy, and

perturbation of signaling pathways required for its patterning results in neural and craniofacial defects. Holoprosencephaly (HPE) can present itself in varying degrees of abnormalities, with the most severe resulting in a single-lobed brain and cyclopia. Other, less severe, complications of HPE include craniofacial defects such as clefting of the lip and palate along the midline and neurological impairment such as anosmia (lack of functioning olfaction) (Belloni *et. al*., 1996; Chiang *et. al*., 1996; Roessler *et. al*., 1996). HPE occurs in about 4 out of 100,000 live human births, and a high fraction of cases have been linked to defects in Hh signaling. HPE can be caused by loss of function mutations in genes required to activate the Hh pathway. Mutations in the *Shh* genomic region that either limit Shh expression, or prevent the ability of the Shh ligand to interact with its receptors have been linked to HPE (Nanni *et. al*., 1999; Wallis & Muenke, 2000). Additionally, mutations in the coding-region of the Shh co-receptors, Gas1 and Cdo, which prevent Hh pathway activation have been identified in patients suffering from HPE. (Pineda-Alvarez *et. al*., 2012; Ming *et. al*., 2002).

Because Shh is required for proper limb development, another consequence of Hh pathway perturbation is defects in limb and digit patterning. Ectopic activation of the Hh pathway during limb development results in polydactyly, a gain of digits on the vertebrate limb. Polydactyly can occur in conjunction with other skeletal deformities in the limb, and is the most common congenital hand and foot defect in humans. Preaxial polydactyly is a disorder that is characterized by the formation of an extra digit on the anterior region of the limb (Zguricas *et. al*., 1999). Ectopic formation of digits in patients with preaxial polydactyly has been correlated to ectopic expression of Shh. Animal models for preaxial polydactyly exhibit dual expression of Shh in the anterior and posterior regions of the limb bud (Sharpe *et. al*., 1999). Studies into familial cases of preaxial polydactyly have identified mutations in the enhancers that control the expression of Shh in the limb, which can result in the expansion of Shh expression (Sagai *et. al.*, 2004; Maas & Fallon, 2005).

Pallister Hall Syndrome is characterized by a variety of abnormalities including syndactyly, polydactyly, unusual skull shape, short limbs, congenital heart disease, and abnormalities of the palate, tongue and jaw (Bale, 2002). Inactivation of the Hh pathway is a fundamental cause of this syndrome. Patients with Pallister Hall syndrome have mutations within the *Gli3* gene, which encodes one of the transcription factors that regulate the transcriptional Hh response (Kang *et. al*., 1997). Although Gli3 can function as both an activator and repressor of the Hh pathway, Gli3 has been identified as one of the main transcriptional repressors of the pathway. Pallister Hall Syndrome patients have mutations in Gli3 that yield a constitutive transcriptional repressor of the pathway. These mutations generate a truncated form of Gli3, about 75 kDa, that resembles the proteolytically processed transcriptional repressor form of Gli3, Gli3R (Kang *et. al*., 1997).

Unfortunately, the Hh pathway can play a devastating role in the formation of tumors and cancer. Animal models have shown that inappropriate Hh pathway activation, which can occur at different molecular points, is sufficient to produce tumors (Barakat *et. al.*, 2010). Mutations that cause untimely activation of the Hh pathway have been shown to be a preliminary cause of a variety of cancers, including breast, lung, pancreatic, gastrointestinal and colorectal cancers (Berman *et. al*., 2003; Karhadkar *et. al*., 2004; Mukherjee *et. al*., 2006; Qualtrough *et. al*., 2004; Thayer *et. al*., 2003; Watkins *et. al*., 2003). Mutations that allow inappropriate Hh pathway activation can affect the signal transducer Smooothend (Smo). Oncogenic mutations of Smo, such as SmoM2, generate forms of Smo that can escape the Ptch1/2-inhibitory signal (Xie *et. al.*,

1998). Mutations that inactivate the canonical receptor Patched1 (Ptch1), and eliminate its ability to inhibit the signal transducer Smo, have also been identified in several types of tumors (Hahn *et. al*., 1996; Johnson *et. al.,* 1996).

Basal cell carcinoma (BCC) is the most commonly diagnosed form of skin cancer in adults. Mutations that affect the Hh pathway have been shown to be causatively correlated to BCC, accounting for 90% of cases (Epstein, 2008). BCC patients have been shown to have both gain of function mutations in Smo as well as inactivating mutations in Ptch1 (Xie *et. al.,* 1998; Epstein, 2008). Moreover, it has been shown that a combinatorial effect of genetics and environmental exposure to carcinogens, such as UVA and UVB, increases incidence of BCC (Aszterbaum *et. al*., 1999). Medulloblastoma is the most prevalent and devastating type of childhood brain cancer, and 50% of childhood cases show signs of upregulated Hh signaling (Kool *et. al*., 2012). Like BCC patients, a high percentage of patients afflicted with medulloblastoma have been shown to have either gain of function mutations in Smo or loss of function mutations in Ptch1 (Raffel *et. al*., 1997). Rhabdomyosarcoma, a type of cancer that originates in skeletal muscle tissue, is one of the most common types of soft-tissue sarcomas in children. The role of Shh in myogenesis has been characterized in several animal models, and activation of the Hh pathway has been shown to play a role in the development of rhabdomyosarcoma (Merlino & Helman, 1999). Gorlin syndrome, also known as basal-cell nevus syndrome, is a genetic disease characterized as a predisposition to BCC, medulloblastoma, rhabdomyosarcoma, and other types of sarcomas. Unsurprisingly, one of the genetic causes of Gorlin Syndrome is heterozygous inactivation of Ptch1 (Bale, 2002)

Mutations that increase production of the Shh ligand can also play a role in tumor formation (Barakat *et. al*., 2010). The causative effects of increased Shh production in tumor growth have been linked mostly to paracrine, or non-cell autonomous, signaling. Tumor cells that express high levels of Shh can signal to surrounding stroma tissue, such as blood vessels, which can then form a supportive environment for the tumor cells (Yauch *et. al.,* 2008: Bijlsma & Roelink, 2010). Shh has also been identified as a migrational cue for tumor cells. Glioblastoma tumor stem cells migrate towards sources of Shh in culture, and Shh has been found to promote the cell adhesion, migration and invasion of hepatocellular carcinoma cells (Uchida *et. al.,* 2011; Chen *et. al.,* 2013). Thus paracrine Hh signaling during tumor formation has the unfortunate consequence of creating a permissive microenvironment that nourishes tumor growth and migration.

Due to the undeniable link between active Hh signaling and tumor formation, medical treatments that target components of the Hh pathway are being developed. As the signal transducer, and ultimately the gate-keeper, of the pathway, Smo is a popular target for therapeutic drugs. As of 2014, two small molecule antagonists that inhibit Smo activity have been approved for clinical trials to treat advanced BCC in patients. Currently, vismodegib, also known by its commercial name Everidge, remains the only commercially approved drug that targets the Hh pathway for the treatment of BCC.

1.2 The Hedgehog signal

1.2.1 Synthesis and secretion of Sonic Hedgehog

The first steps of the Hh pathway begin in the ER of Shh-expressing cells. Before Shh is even sent out into the extracellular space, it must undergo a proteolytic cleavage and a series of lipid-modifications that render it a membrane-tethered ligand. After Shh is synthesized and its signal sequence cleaved, it exists as a 45 kDa precursor protein that contains two distinct domains: an amino-terminal signaling domain, and a carboxy-terminal intein-like domain. The 45 kDa precursor Shh protein undergoes an auto-proteolytic cleavage catalyzed by its carboxy-terminal domain in the ER (Hall *et. al.*, 1997; Porter *et. al.*, 1996ab). The cleavage occurs between highly conserved glycine and cysteine residues, and is resolved by the addition of a cholesterol moiety to the carboxy-terminal glycine on the amino-terminal domain (the Shh ligand). No other role for intein-like carboxy-terminal domain of Shh has been identified (Lee *et. al.*, 1994; Porter *et. al.*, 1995; Roelink *et. al.*, 1995). Cholesterol modified Shh becomes further processed by an acyltransferase appropriately called Skinny Hedgehog, which attaches a palmitoleoyl moiety to its amino-terminal domain (Pepinsky *et. al.*, 1998; Chamoun *et. al.*, 2001) (**Figure 3**).

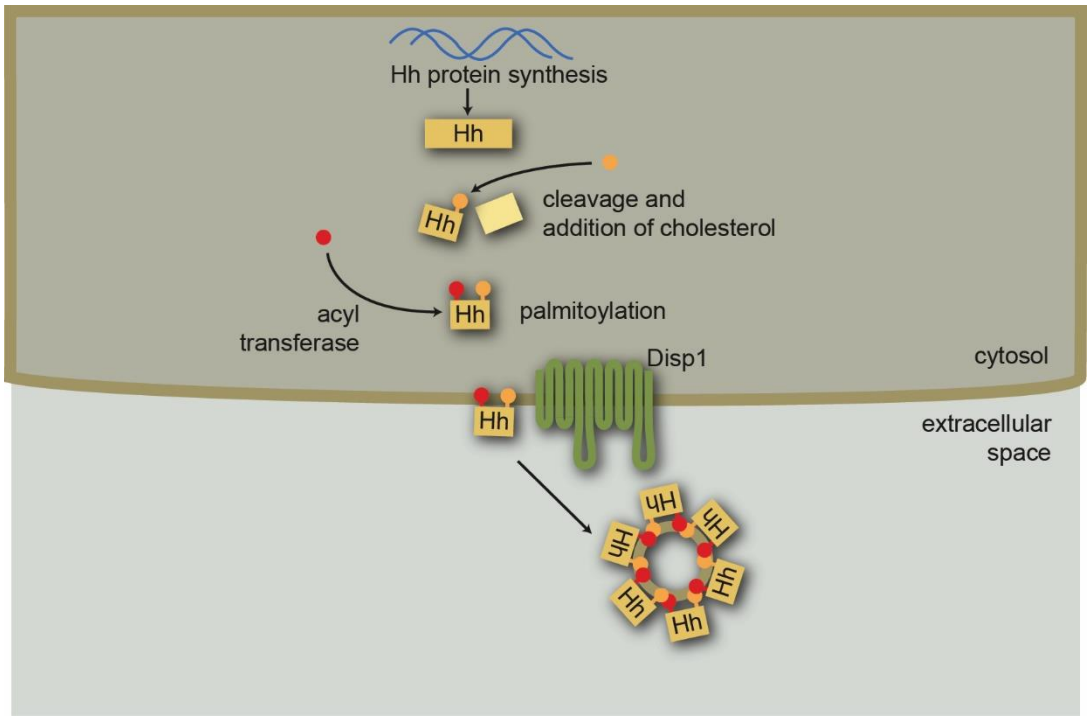

Figure 3 Shh synthesis and secretion

Figure 3. Shh synthesis and secretion

After Shh is synthesized in expressing cells, it undergoes an autoproteolytic cleavage mediated by its carboxy-terminal intein-like domain. This cleavage is resolved with the addition of a cholesterol moiety on the carboxy-terminal end of the signaling domain. The signaling domain of Shh then becomes further modified by an acyltransferase, Skinny Hedgehog, which attaches a palmitoleoyl moiety on its amino-terminal end. The lipid-modified Shh is then secreted via Dispatched1.

At this point in its synthesis, the fully processed Shh ligand is membrane bound (Peters *et. al.,* 2004). While the role that lipid modifications play in regulation of the Shh ligand is not entirely clear, it has been shown that the cholesterol modification greatly enhances its association with the membrane. Lipid modifications may also regulate the cellular localization of Shh in expressing cells, as the cholesterol tail on Shh promotes its association to lipid rafts containing Caveolin and Flotillin-1 (Chen *et. al.,* 2004). Removal of both lipid modifications results in the inability of Shh to localize to specialized lipid rafts (Chen *et. al.,* 2004).

The lipid modified Shh ligand requires a dedicated mechanism for secretion and long-range signaling that involves the function of the pathway component Dispatched1 (Disp1). Disp1 is a twelve transmembrane domain protein homologous to the Resistance-Nodulation-Division (RND) superfamily of proton-driven transporters (Nies *et. al.,* 1995). RNDs form trimers and mediate the secretion of small lipophilic and amphipathic molecules, such as antibiotics and lipids (Nikaido *et. al.,* 2001; Nikaido *et. al.,* 2009). Although Disp1 has two vertebrate paralogs, Disp2 and Disp3, only Disp1 has been shown to play a role in Hh signaling (Nakano *et. al.,* 2004; Tian *et. al.,* 2004).

While the mechanism of Shh secretion via Disp1 is not understood, it is known that the Drosophila homolog of Disp1, dispatched (disp), is required for the basolateral secretion of fully processed Hh in the wing imaginal disc (Burke *et. al.,* 1999: Callejo *et al.,* 2011). Similarly, it has been shown that Disp1 mediates the basolateral release of fully processed Shh *in vitro,* and is required to form a gradient from source cells (Etheridge *et. al.,* 2010). Cells that lack functional Disp1 activity accumulate Shh, and mice that lack Disp1 display evidence of disrupted Hh signaling (Caspary *et. al.,* 2002; Kawakami *et. al.,* 2002; Etheridge *et. al.,* 2010). Consequently, Disp1 is critical for functional Hh signaling *in vivo* (Caspary *et. al.,* 2002; Kawakami *et. al.,* 2002; Ma *et. al.,* 2002).

Disp1 contains a sterol-sensing domain (SSD), a domain frequently found in proteins that regulate lipid homeostasis (Kuwabara and Labouesse, 2002). Interestingly, Disp1 only acts on cholesterol modified Shh. It has been shown that soluble ShhN (the amino-terminal signaling domain lacking the cholesterol moiety) can bypass the Disp1-mediated secretion mechanism, whereas the absence of the palmitoleoyl moiety does not affect Disp1-mediated secretion (Burke *et. al.,* 1999; Ma *et. al.,* 2002; Etheridge *et. al.,* 2010). While it is tempting to speculate that the

SSD of Disp1 is required for the recognition of cholesterol-modified Shh, there is no direct evidence that supports this conclusion.

Along with Disp1, Scube2, a secreted glycoprotein, has been shown to be required for the release of cholesterol modified Shh from the cell surface of expressing cells (Tukachinsky *et. al.,* 2012; Jakobs *et. al.,* 2014). Scube2 contains a CUB domain, which functions as a regulator of proteolytic activity in serine proteases and metalloproteinases, and is required for efficient secretion of fully processed Shh (Tukachinksy *et. al.,* 2012). The cholesterol moiety on Shh is required for its interaction with Scube2, and it has been suggested that fully processed Shh is handed off from Disp1 to Scube2 during secretion (Tukachinsky *et. al.,* 2012).

A Member of the A Disintegrin and Metalloproteases (ADAMs) protein family, ADAM17, has also been implicated in the release of fully processed Shh from the membrane of producing cells (Dierker *et. al.,* 2009). It has been shown that the release of lipid modified Shh is increased by the expression of ADAM17 *in vitro*, and it has been suggested that the release of the biologically active Shh ligand requires this sheddase mechanism (Dierker *et. al.,* 2009; Ohlig *et. al.*, 2011).

Taken together, these findings demonstrate that the cell-autonomous release of fully processed Shh requires an intricate interaction with various components.

1.2.2 Sonic Hedgehog in the extracellular space

Once Shh enters the extracellular space, it can mediate both short and long-range signaling. How the dual lipid-modified Shh ligand travels several cell diameters away from source cells has been one focus of Hh pathway research.

One hypothesis is that Shh travels in a lipophilic complex through the aqueous extracellular environment. In the Drosophila wing disc, as well as in cultured Drosophila cells, Hh has been demonstrated to co-localize to high density lipoprotein particles (Panakova *et. al.,* 2005). Both Hh and Shh can be found associated in complexes about six times the molecular weight of the monomeric ligand in the supernatant of culture cells (Chen *et. al.,* 2004; Zeng *et. al.,* 2001). These finding suggests that Shh ligands travels through the extracellular space as multimers that are packaged into lipoprotein particles.

The lipid modifications of Shh appear to play a role not only in the formation of multimeric complexes, but also in its movement through the extracellular space. The palmitoleoyl moiety of Shh is required for the formation of multimeric complexes, as well as the establishment of concentration gradients in mice (Chen *et. al.,* 2004). Forms of Shh missing the palmitoleoyl moiety, ShhC25S, have been shown to have decreased activity *in vitro* (Taylor *et. al.,* 2001). Conversely, Hh lacking the palmitoleoyl modification appears to spread further away from sources cells in wing imaginal discs (Callejo *et. al.,* 2006). Thus, the function of the palmitoylation in the movement of Shh in the extracellular space has not been resolved. The role of the cholesterol modification in the formation of Shh concentration gradients in the extracellular space is also not clear. Expression of soluble ShhN, which lacks the cholesterol modification, in Drosophila wings discs results in larger domains of Hh pathway activity (Burke *et. al.,* 1999), suggesting that cholesterol restricts the range of the Hh ligand. However in

vertebrates, loss of the cholesterol moiety on Shh results in limited movement and activity (Lewis *et. al.,* 2001).

The transport of Shh through the extracellular space, as well as its reception on the surface of target cells, requires the function of extracellular proteins and extracellular matrix components (**Figure 4**). Heparan sulfate proteoglycans (HSPGs) are elements of extracellular matrix, usually enriched in basement membranes, and are ubiquitous on the extracellular surface of cells. In Drosophila, proteins found at the core of HSPGs, dally and dally-like, are required for Hh signaling (Lum *et. al.,* 2003; Desbordes *et. al.,* 2003; Han *et. al.,* 2004). The enzymes that modify HSPGs are also a required components of the Hh pathway; mutant clones lacking tout-velu (ttv), a glycosyltransferase required for Heparan sulfate polymerization, have impaired Hh distribution in the Drosophila wing disc (Bellaiche *et. al.,* 1998). HSPGs, and the enzymes that modify their glycosaminoglycan chains, are also required for the distribution and activity of Shh (Lin *et. al.,* 2004). In vertebrates, core HSPG proteins, Glypican 3, Glypican 5, and Perlecan, are involved in the binding of Shh in the extracellular space. Interestingly, Glypican 5 has been demonstrated to be a positive regulator of Hh signaling, while Glypican 3 is a negative regulator of Hh signaling; indicative of the complex roles that HSPGs play in the movement and reception of the Shh ligand (Li *et. al.,* 2011; Capurro *et. al.,* 2008; Yan *et. al.,* 2008; Palma *et. al.,* 2011). Like in Drosophila, glycosyltransferases that modify glypicans are involved in regulating vertebrate Hh signaling. In mice, hypomorphic mutations of the glycosyltransferase Ext1 gene result in elevated Hh signaling by Ihh in developing chondrocytes, suggesting that in this context HSPGs negatively regulate Hh signaling (Koziel *et. al.,* 2004). In Zebrafish, the level of expression of Ext homologs Ext1A and Ext1C are themselves modulated by Hh pathway activity in the somites (Siekmann and Brand, 2005).

A major regulator of Shh in the extracellular space is Hedgehog Interacting Protein (Hhip), a vertebrate specific component of the Hedgehog pathway (Chuang & McMahon, 1999). Hhip is a single pass transmembrane glycoprotein that possesses a Hh-binding domain that closely resembles the binding domain of the canonical Shh receptors Ptch1/2. Hhip sequesters Shh in the extracellular space and consequently impedes Hh pathway activation. Recently it was shown that Hhip can leave its site of synthesis and negatively regulate Hh signaling non-cell autonomously (Kwong *et. al.,* 2014). Hhip itself is a target of the Hh pathway, and its expression is upregulated by Hh signaling (Chuang & McMahon, 1999). This provides a negative feedback in the pathway, and is an important component that shapes the Shh gradient in morphogenetic fields such as the neural tube (Holtz *et. al.,* 2013).

The complex movement of morphogens across fields of cells may require specialized filopodia called cytonemes. Cytonemes are actin based cellular extensions that have been shown to carry signals across several cell diameters. Evidence that cytonemes function in long-range Hh signaling has been found in Drosophila and in vertebrates (Ramirez-Weber and Kornberg, 1999; Kornberg & Roy, 2014; Sanders *et. al.,* 2013). In the Drosophila wing disc, cytonemes that carry the Hh ligand as well as cytonemes that carry the Hh receptor patched (ptc) and the Hh co-receptor ihog have been identified (Kornberg, 2014). Amazingly, cytonemes in developing chick limb buds have been observed in real time. These highly dynamic cytonemes carry Shh "particles" to target cells as far away as 150 mm (Sanders *et. al.,* 2013). Cytonemes emanating from Shh responding cells were also identified, these were observed to carry the Shh co-

receptors, Boc and Cdo. Stabilized interaction between cytonemes carrying the Shh ligand and those carrying the Shh co-receptors occurred over a long distance (Sanders *et. al.*, 2013). Some biologists, like Dr. Thomas Kornberg, hypothesize that cytonemes require extracellular matrix components like HSPGs to guide them to target cells.

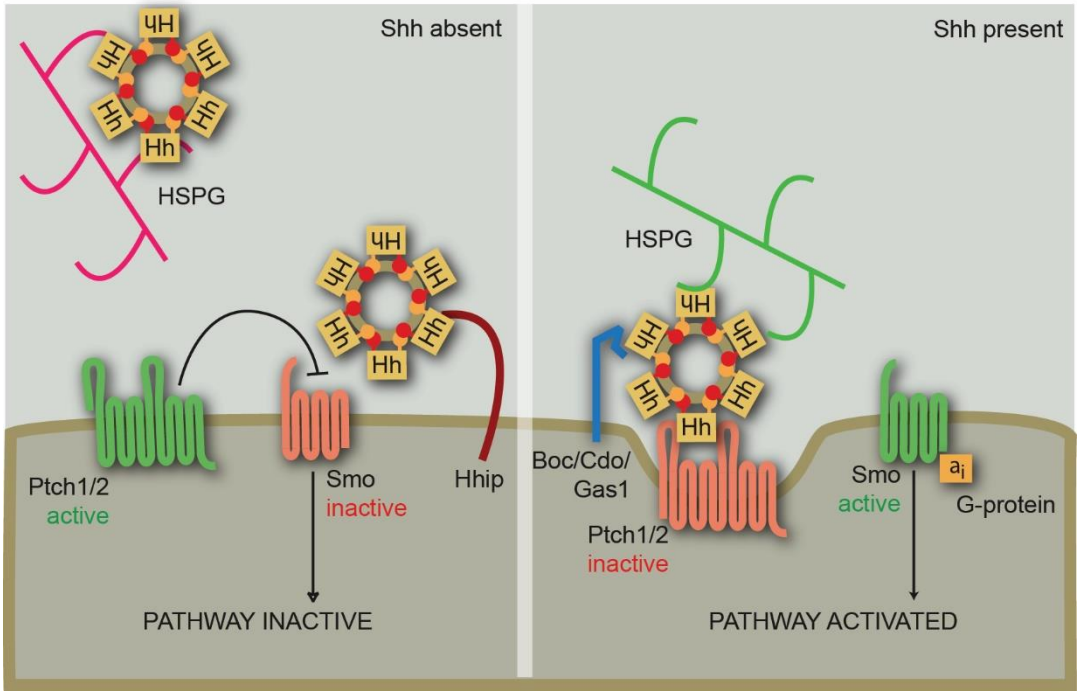

Figure 4 Shh in the extracellular space

Figure 4. Shh in the extracellular space

Shh interacts with many molecules in the extracellular space and at the cell surface. Pictured above, Shh interacts with Heparan Sulfate Proteoglycans (HSPGs) which both positively and negatively regulate Hh signaling. Shh also binds and is sequestered by Hedgehog-interacting protein (Hhip), a cell surface glycoprotein which contains a binding domain similar to the Ptch1 Shh binding domain (L2). Under favorable conditions, Shh binds to the "receptorsome" on the cell surface of target cells. The "receptorsome" contains the canonical receptors, Ptch1/2, as well as the co-receptors Boc, Cdo, and Gas1. The binding of Shh to the "receptorsome" initiates Hh signal transduction.

1.2.3 Receptorsome

As Shh reaches its target cells, it must interact with and bind to a receptor complex to transduce its signal across the membrane. While the Drosophila genome contains one canonical receptor, Patched, vertebrates contain two: Ptch1 and its homolog Ptch2 (Nakano *et. al.*, 1989; Carpenter *et. al.*, 1998; Motoyama *et. al.*, 1998; Lewis *et. al.*, 1999). Ptch1/2 are 12 transmembrane domain proteins which are, like Disp1, homologous to the RND family of proton driven transporters.

Ptch1/2 contain a sterol sensing domain (SSD), a putative proton transporter domain with highly conserved amino acids, and a Hh binding domain (loop 2) (Kuwabara *et. al.,* 2002; Taipale *et. al.,* 2002). All three Hh ligands are able to bind to both receptors with similar affinity (Carpenter *et. al.*, 1998).

When Shh is absent from the extracellular space, Ptch1/2 inhibit Smo, the signal transducer of the Hh pathway. This inhibitory mechanism is often referred to as ligand-independent antagonism because its effects occur independent of the ligand in the extracellular space. The exact mechanism by which Ptch1/2 inhibits Smo in the absence of Shh is still a mystery, although it has been shown that inhibition occurs substoichiometrically and likely not as a direct interaction (Taipale *et. al.,* 2002). Other RND members that contain an SSD, such as NPC1, are able to regulate the cellular movement of cholesterol and can transport lipids, such as oleic acid, across the membrane when expressed in bacteria (Davies *et. al.,* 2000). Mutations of highly conserved residues within the putative proton transporter domain (located in the fourth transmembrane domain) of Ptch1/2, Ptch1D499A or Ptch2D469A, decreases the ability of these proteins to inhibit Smo. One hypothesis is that Ptch1/2 pumps an inhibitory sterol across the membrane (Taipale *et. al.,* 2002; Alfaro *et. al.*, 2014). Deletion of the Hh binding domain in Ptch1, Ptch1Δ L2, disrupts the ability of Ptch1 to recognize Shh in the extracellular space, resulting in the inability to cell-autonomously activate the pathway *in vitro* and *in vivo* (Mullor and Guerrero, 2000; Briscoe *et. al.*, 2001; Taipale *et. al.*, 2002). Consequently, two central domains of Ptch1/2 function appear to be the proton driven transporter domain and the ligand binding domain.

Several studies have shown that the inhibition mediated by Ptch1/2 can occur non-cell autonomously. Supernatant collected from fibroblasts overexpressing Ptch1 have an inhibitory effect on the Hh pathway when applied to reporter cells (Bijlsma *et. al.,* 2006). In mixed embryoid bodies derived from genome edited mouse embryonic stem cells (mESCs), cells containing endogenous copies of Ptch1 and Ptch2 can inhibit Hh pathway activation non-cell autonomously. This inhibition occurs in the genetic absence of the Shh ligand (Roberts *et. al.,* 2016, manuscript in review). Interestingly, both Drosophila Ptc and vertebrate Ptch1 are transcriptionally upregulated upon activation of the Hh pathway (Nakano *et. al.,* 1989; Goodrich *et. al.,* 1996). One hypothesis about the role of non-cell autonomous inhibition is that Ptch1/2 serve to raise the activational threshold in a field of Hh responsive cells, creating a more graded response to the Shh ligand.

A different type of inhibitory mechanism mediated by Ptch1/2 is ligand-dependent antagonism. It has been observed that the upregulation of Ptc is required to restrict the range of Hh protein in a field of cells (Chen & Struhl, 1996; Briscoe *et. al.,* 2001). Similarly, the upregulation of Ptch1, Ptch2, and Hhip are required to limit the range of Shh in the ventral neural tube (Jeong & McMahon, 2005; Holtz *et. al.,* 2013). The ability of Ptch1/2 to limit the spread of the Shh ligand in the extracellular space can consequently limit Hh pathway activation non-cell autonomously. Although genetics in mice have revealed a role for ligand-dependent antagonism, overexpression of Ptch1 and Ptch1ΔL2 in the chick neural tube fails to reveal non-cell autonomous effects (Alfaro *et. al.,* 2014).

Surprisingly, of the two canonical Hh receptors, only Ptch1 has been shown to be required for vertebrate survival. The expression of Ptch1 is quite broad during development, while the expression of Ptch2 is low and remains limited to the dermis, tests, and cerebellum (Carpenter *et. al.,* 1998; Lee *et. al.,* 2006; Motoyama *et. al.,* 1998ab). Ptch2$^{-/-}$ mice appear to be viable and fertile with no obvious defects, however Ptc$^{-/-}$ Drosophila embryos and Ptch1$^{-/-}$ mice embryos die during embryogenesis and display evidence of widespread Hh pathway activation (Goodrich *et. al.,* 1997; Lee *et. al.,* 2006; Nieuwenhuis *et. al.,* 2006). Nevertheless, Ptch2 has been shown to regulate Smo activity *in vitro* and *in vivo* (Alfaro *et. al.,* 2014; Zhulyn *et. al.,* 2015).

Besides Ptch1/2, Shh also has three identified co-receptors: Boc, Cdo, and Gas1. Boc and Cdo are cell-surface glycoproteins that are part of the Immunoglobulin (Ig)/fibronectin type III-like (FNIII) family, and Gas1, Growth arrest specific-1, is a GPI anchored glycoprotein (Kang *et. al.,* 1998; Kang *et. al.,* 2002; Martinelli & Fan, 2007). All three co-receptors can form complexes with Ptch1 and can bind to Shh with similar affinity, so it is unsurprising that they can function redundantly in some contexts (Izzi *et. al.,* 2011; Tenzen *et. al.,* 2006). While Gas1 has no homologous counterpart in Drosophila, the Drosophila homologs of Boc and Cdo were independently identified using an RNA interference screen to identify new components of the Hh pathway; hence their names Interference hedgehog (Ihog) and brother of Ihog (Boi) (Lum *et. al.,* 2003).

The Boc co-receptor has been shown to be involved in the reception of Shh during development, and is required for myogenesis, cell proliferation, and commissural neuron axon guidance (Kang *et. al.,* 2002; Okada *et. al.,* 2006; Izzi *et. al.,* 2011). Because Boc can bind to Shh in the extracellular space, it has also been shown to sequester the Shh ligand and inhibit Hh pathway activation non-cell autonomously when overexpressed in the chick neural tube (Allen *et. al.,* 2011). Like Boc, Cdo is also involved in the reception of Shh during development and plays a role in Shh-mediated myogenesis (Tenzen *et. al.,* 2006; Zhang *et. al.,* 2006; Kang *et. al.,* 2002). Cdo is particularly important in the patterning of the prosencephalon during development, and mutations that affect the ability of Cdo to interact with Shh have been shown to be a genetic cause of Holoprosencephaly (Kang *et. al.,* 2002; Zhang *et. al.,* 2006). Gas1 is required for cell proliferation in response to Shh in the cerebellum, and has been shown to activate the Hh signaling at low Shh concentrations (Martinelli & Fan 2007; Kang *et. al.,* 2002).

Although the co-receptors have been shown to function redundantly, what the composition of the "receptorsome" is *in vivo*, or whether the components of the "receptorsome" is tissue specific, is not known. Deletion of multiple alleles of the co-receptors *in vivo* leads to a disruption of Hh pathway activity, and mice genetically null for all three co-receptors have no evidence of Hh pathway activation (Allen *et. al.,* 2011). This supports the hypothesis that the co-receptors are required for cell-autonomous activation of the Hh pathway *in vivo*.

An additional molecule that has been identified as a Shh receptor is Megalin, also known as low-density lipoprotein receptor-related protein (LRP)-2. Megalin is co-expressed with Shh in the floor plate of the neural tube, and can mediate the endocytosis of Shh *in vitro* (McCarthy *et. al.,* 2002; Spoelgen *et. al.,* 2005). Evidence suggests that Megalin functions mostly in the central nervous system, and plays a role in patterning the forebrain ventral midline and oligodendrocyte precursor migration and proliferation (Christ *et. al.,* 2012; Ortega *et. al.,* 2012). Additionally,

genetic ablation of Megalin causes forebrain defects reminiscent of those found in $Shh^{-/-}$ embryos (Willnow *et. al.,* 1996). These findings suggest that Megalin is a tissue-specific auxiliary receptor for Shh.

Once Shh is bound to a Ptch1/2-containing receptor complex, it is internalized through a dynamin-dependent mechanism (Incardona *et. al.,* 2000). Endocytosis of Hh ligands has been observed both in Drosophila as well as *in vitro* (Capdevila *et. al.,* 1994; Incardona *et. al.,* 2000). In the Drosophila embryo, Hh target cells contain intracellular vesicles that contain both Ptc and Hh, suggesting that a Hh-receptor complex is internalized by cells that respond to the ligand (Bellaiche *et. al.,* 1998; Burke *et. al.,* 1999; Martin *et. al.,* 2001). In tissue culture cells, ShhN, a soluble form of the Shh ligand that lacks the cholesterol moiety, is efficiently internalized by cells expressing Ptch1 (Incardona *et. al.,* 2000). Megalin has also been shown to be required for the uptake and subsequent intracellular trafficking of ShhN in epithelial cells (Morales *et. al.,* 2006). Internalized Ptch1 and Shh have also been observed to be targeted to the lysosome for destruction (Mastronardi *et. al.,* 2000; Incardona *et. al.,* 2000).

1.3 Smoothened: The Serpentine Transducer

1.3.1 Activation of Smoothened

After Shh interacts with the receptorsome at the cell surface and undergoes internalization, Smo becomes activated. Smo was first identified as a segment polarity gene required for Drosophila embryogenesis. $Smo^{-/-}$ Drosophila embryos lose their ability to respond to exogenous Hh and lose expression of Hh target genes 1-2 hours after gastrulation (Alcedo *et. al.* 1996). Smo is 7 transmembrane domain G-Protein-Coupled Receptor (GPCR)-like protein with a conserved Cysteine Rich Domain (CRD) and high homology to Frizzled (Frz), the receptor and signal transducer of the Wnt pathway (Alcedo *et. al.* 1996). In vertebrates, the role of Smo is epistatic to Ptch1, and is required for Hh pathway activation. $Smo^{-/-}$ mice embryos have a phenotype identical to $Shh^{-/-};Ihh^{-/-}$ embryos: arrested development at somite stages, small, linear heart tube, open gut, and cyclopia (Zhang *et. al.,*. 2001).

Drosophila Smo exists as a constitutive dimer in cells, and this dimerization is mediated by its amino-terminal domain (Zhao *et. al.,* 2007). When Smo is repressed by Ptc, it is localized to the internal membranes of a cell, and its carboxy-terminal domain is locked in a "closed" configuration close to the intracellular loops of the protein (Zhu *et. al.,* 2003; Zhao *et. al.,* 2007). After Ptc internalizes the Hh signal, Drosophila Smo translocates from internal membranes to the cell surface (Zhu *et. al.,* 2003; Denef *et. al.,* 2000). Once Smo becomes enriched on the plasma membrane, 3 arginine clusters on the carboxy-terminal domain of Smo becomes phosphorylated by the kinases PKA and CK1 (Jia *et. al.,* 2004; Zhang *et. al.,* 2004; Apionishev *et. al.,* 2005). This phosphorylation causes the carboxy (C)-terminal tail of Smo to open and cluster with the C-tails of other phosphorylated Smo proteins (Zhao *et. al.,* 2007; Kupinski et al 2013). The modification of the C-tails allow Smo to undergo a dramatic conformational change to an active state (Zhao *et. al.,* 2007; Kupinski et al 2013). Phosphomimetic mutations on the C-tail of Smo have been shown to cause constitutive activity of the Hh pathway (Jia *et. al.,* 2004; Zhang *et. al.,* 2004). In addition to C-tail clustering, Hh pathway activation also promotes the oligomerization

of the amino-terminal and transmembrane domains of Smo, indicating that activated Smo forms higher order clusters (Shi *et. al.,* 2013).

Many of the processes involved in Drosophila Smo activation are conserved in vertebrates. The internalization of Shh by a Ptch1-containing receptor complex allows the segregation of Smo away from Ptch-1 containing vesicles, resulting in the enrichment of Smo at the cell surface (Incardona *et. al.,* 2002). The C-tail of Smo is then phosphorylated by G-protein coupled receptor kinase 2 (GRK2) and CK1 during activation (Chen *et. al.,* 2004; Chen *et. al.,* 2011). The conformational change that Smo undergoes after phosphorylation also requires a rearrangement of its extracellular loops, and the mutation of certain amino acids within the extracellular loops has both activating and inactivating effects (Carroll *et. al.,* 2012)

1.3.2 Canonical Responses

The activation of Smo regulates a wide array of cellular responses to Hh signaling. The activation of Hh target genes, the canonical response, has been the most studied. In Drosophila, the transcriptional activation of Hh target genes is regulated by the transcription factor Cubitus interruptus (Ci). In cells with no active Hh signaling, Ci becomes proteolytically processed into a truncated repressor form through interaction with a multi-protein complex composed of Costal2 (Cos2), Fused, and Suppressor of Fused (SuFu). This inhibitory complex promotes its phosphorylation by PKA, GSK3β, and CK1, through microtubule-dependent and independent processes (Methot *et. al.,* 2000; Wang *et. al.,* 2000). After Ci is phosphorylated, it becomes targeted for proteolytic processing through interaction with a SCF (Skp1, Cdc53, and F-box) ubiquitin ligase complex (Chen *et. al.,* 1999; Wang *et. al.,* 1999; Jia *et. al.,*. 2002; Jia *et. al.,* 2005). The activation of Smo is thought to stop the proteolytic processing of Ci by stopping its interaction with a Cos2-containing complex, allowing full length Ci to accumulate (Zhang *et. al.,* 2005).

In vertebrates, Ci has three homologs, Gli1, Gli2, and Gli3, and the mechanism that regulates their activity is highly conserved. Gli2 and Gli3 are the primary mediators of Hh transcriptional regulation, with Gli2 acting as both an activator and suppressor and Gli3 acting mainly as a repressor. Gli1 is a transcriptional target of Hh signaling and functions as an activator of the response (Ingham & McMahon, 2001). Like Ci, Glis are phosphorylated by PKA when the Hh pathway is off, and this allows interaction with the ubiquitin ligase complex β-TRCP and subsequent proteolytic processing into transcriptional repressor forms (Huntzicker *et. al.,* 2006).

A role for the primary cilium in the regulation of the vertebrate canonical Hh response has become evident. In the absence of Hh signaling, Ptch1 is localized to the primary cilium. Upon Hh pathway activation, Smo, and downstream transcriptional components, translocate to the primary cilium as Ptch1 exits (Corbit *et. al.,* 2005; Wen *et. al.,* 2010; Rohatgi *et. al.,* 2007; Kim *et. al.,* 2009) (**Figure 5**). To enter the cilium, Smo requires a dedicated intraflagellar transport mechanism that moves along the axoneme, a microtubule structure underlying the cilium. Mutations of the intraflagellar transport proteins IFT88, IFT172, and Kif3a, a subunit of the kinesin-II IFT motor, result in mice embryos with defects in Hh-mediated patterning, such as polydactyly and neural tube defects (Huangfu *et. al.,* 2003; Liu *et. al.,* 2005).

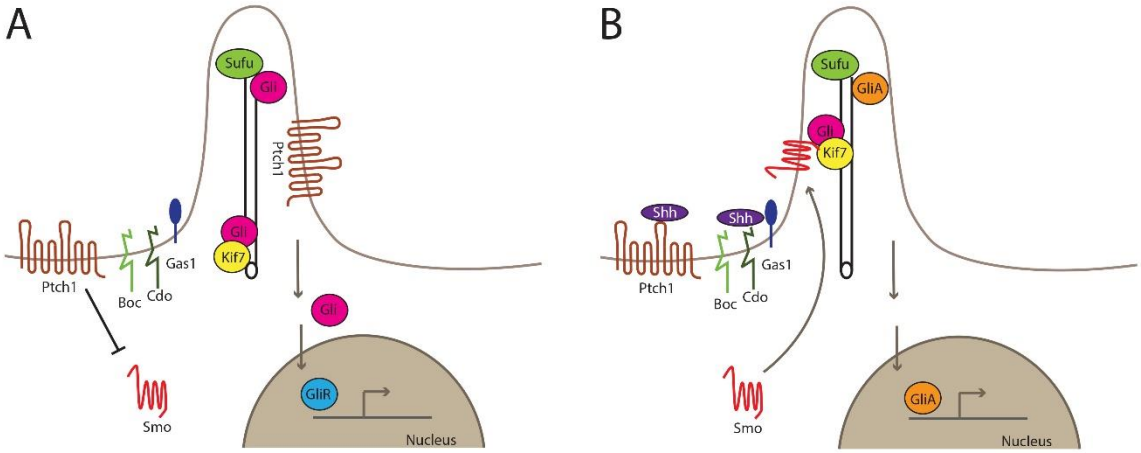

Figure 5 Overview of the Hh transcriptional response

Figure 5. Overview of the Hh transcriptional response

(A) The Hh pathway in its inactive state. In the absence of the Shh ligand, Ptch1/2 inhibits Smo and it is located outside of the primary cilium. SuFu is located in the primary cilium where it, in conjunction with other pathway components, mediates the proteolytic processing of Gli into the transcriptional repressor form (GliR). GliR translocates to the nucleus and prevents the transcription of target genes. (B) The Hh pathway in its activated state. Upon binding to Shh, Ptch1/2 moves out of the primary cilium and release the inhibition of Smo. Active Smo translocates to the primary cilium and prevents the proteolytic processing of Gli, resulting in the accumulation of the transcriptional activator form (GliA). GliA translocates to nucleus and promotes the transcription of target genes.

The requirement of the primary cilium in vertebrate Hh signaling is further supported by the identification of two distinct domains on Smo that regulate its entry into the organelle. Smo contains a conserved hydrophobic sequence on its C-tail that controls its localization to the cilium. The mutation of a conserved tryptophan and arginine within this sequence generates a form of Smo (SmoCLD) that does not enter the primary cilium during Hh signaling and fails to activate the transcriptional response *in vitro* and *in vivo* (Corbit *et. al.*, 2005; Aanstad *et. al.*, 2009; Bijlsma *et. al.*, 2012). The extracellular CRD of Smo also plays a role in the movement of Smo during signaling. The mutation of a conserved cysteine within the CRD results in a form of Smo (C151Y) that is unable to translocate to the primary cilium in response to Hh signaling and fails to activate the transcriptional response (Aanstad *et. al.*, 2009.; Bijlsma *et. al.*, 2012). Complete deletion of the CRD (SmoΔCRD) disrupts the regulatory mechanism that prevents Smo from entering the cilium in the absence of Hh signaling. SmoΔCRD is constitutively localized to the cilium and has a high level of baseline transcriptional activity (Aanstad *et. al.*, 2009; Myers *et. al.*, 2013). Despite its constitutive residence in the cilium, SmoΔCRD remains unresponsive to Hh signaling *in vitro* and *in vivo* (Aanstad *et. al.*, 2009; Myers *et. al.*, 2013).

1.3.3 Non-Canonical Responses

While the canonical Hh responses have been well-characterized in a number of systems, the mechanisms that regulate the non-transcriptional, or non-canonical, responses of Smo remain elusive.

A migrational response to the Hh signal has been observed in various *in vivo* and *in vitro* systems. Hh has been shown to mediate the migration of germ cell precursors in Drosophila, and Shh serves as a migrational cue for cells in the developing tooth (Deshpande *et. al.,* 2001; Prochazka *et. al.,* 2015). Shh-responsive neural progenitor cells inside developing zebrafish neural tubes actively sort themselves into appropriate domains along the dorsal-ventral axis (Xiong *et. al.,* 2013). Additionally, cultured fibroblasts display evidence of Smo-mediated migration toward a Shh source (Polizio *et. al.,* 2011). Smo is required for the Shh migrational response, and there is evidence that this response does not require translocation to the primary cilium. Forms of Smo unable to travel to the cilium, SmoC151Y and SmoCLD, are better mediators of the migrational response compared to WT Smo (Bijlsma *et. al.,* 2012). *Kif3*$^{-/-}$ cells, which have impaired intraflagellar transport to the cilium, also mediate stronger non-canonical responses compared to WT cells (Bijlsma *et. al.,* 2012). These findings suggests that distinct pools of Smo in different subcellular locations mediate different Hh responses.

Non-canonical Hh responses appear to link Smo, a putative GPCR, with the activity of heterotrimeric G-proteins. Fibroblast migration towards a source of Shh has been shown to require the small GTPases, Rac1 and RhoA, and elicits the activity of a Gαi/PI3K mechanism (Polizio *et. al.,* 2011). Furthermore, artificial introduction of Smo into frog melanophores mediates Gαi-dependent signaling (DeCamp *et. al.,* 2000). The pertussis toxin (PTX), which inhibit Gαi release and activation, blocks Smo activation in cultured cells, and injection of PTX into developing zebrafish embryos also gives rise to Hh-like phenotypes (Riobo *et. al.,* 2006; Hammerschmidt *et. al.,* 1998). However not all data supports the conclusion that Smo activity requires G-proteins. While the expression of constitutively active Gαi in *Ptch1*$^{-/-}$ fibroblasts decreases transcriptional activity, expression of constitutively active Gαi in the chick neural tube fails to impede Hh signaling (Low *et. al.,* 2008).

There are many other less well-characterized Smo-mediated responses to Hh signaling. Intracellular calcium fluxes in response to Shh have been shown to occur in cultured cells as well as in developing neurons of the neural tube (Belgacem *et. al.,* 2010). Both neurite outgrowth and commissural neuron axon guidance, which require Shh as a cue, are processes that require activated Smo (Bijlsma *et. al.,* 2012; Okada *et. al.,* 2006). Interestingly, it was recently shown that activated Smo can also internalize and promote the degradation of Hhip cell-autonomously in a transcription-independent mechanism (Kwong *et. al.,* 2014).

Based on the literature, it is clear that Smo regulates many different cellular activities. The localization of Smo and its subsequent interactions with downstream effectors are critical factors that regulate its activity.

1.3.4 Modulation of Smo activity

Smo is classified as a proto-oncogene due to its ability to mediate cell proliferation and tissue growth. The cellular mechanisms that regulate Smo activity are complex, and developing strategies to control Smo activity is a goal of many pharmaceutical companies.

Ptch1/2 is a potent inhibitor of Smo activity in the absence of the Shh ligand. The mechanism of Ptch1/2-mediated inhibition is not known, however the hypothesis that Ptch1/2 pumps an inhibitory sterol of Smo is supported by various lines of evidence. The homology of Ptch1/2 with proteins that regulate cholesterol homeostasis has led to the observation that cellular cholesterol levels can alter Smo activity (Myers *et. al.,* 2013). While the effect of cellular cholesterol on Smo may be indirect, by altering the membrane topology and conformation of Smo, sterol synthesis has been shown to play a more direct role in Smo regulation. Smith-Lemli-Opitz syndrome is caused by a deficiency of an enzyme that is part of the cholesterol synthesis pathway, 7-dehydrocholesterol reductase (7DHCR). Smith-Lemli-Opitz patients suffer from many defects characterized by a deficiency in Hh signaling, including cleft palate and polydactyly (Bale, 2010). It has been observed that mutating enzymes that are part of the cholesterol synthesis pathway, including 7DHCR, increases Ptch1/2-mediated non-cell autonomous inhibition (Bijlsma *et. al.,* 2006; Roberts *et. al.,* 2016, manuscript submitted). It has also been shown that Ptch1 overexpression in cultured fibroblasts leads to an accumulation of 3B-hydroxysteroids, (pro-) vitamin D3, in the medium. Addition of this conditioned medium to reporter cells can inhibit their transcriptional response (Bijlsma *et. al.,* 2006). The addition of Vitamin D3 to cultured cells and to developing zebrafish embryos results in the inhibition of Hh pathway activation (Bijlsma et. al. 2006). However, the presumptive Ptch1/2 inhibitor has yet to be identified.

Exogenous small molecules that can modulate Smo activity have been a tool for controlling cancerous cells with elevated Hh pathway activity. Cyclopamine is a steroidal alkaloid that directly inhibits Smo by binding to its heptahelical bundle (Chen *et. al.,* 2002; Incardona *et. al.,* 1998; Incardona *et. al.,*. 2000). Other small molecule inhibitors of Smo include SANT1-4, LY2940680, and vismodegib, and all have been shown to exert their effects through the binding of Smo transmembrane domains (Chen *et. al.,* 2002; Wang *et. al.,* 2013; Rubin *et. al.,* 2006). Smoothened Agonist (SAG), is a chlorobenzothiophene-containing small molecule that can activate Smo by also binding to its heptahelical bundle and presumably altering the conformation of Smo (Chen *et. al.,* 2002).

Recently it has been shown that another type of molecule can affect Smo activity through direct interaction. Oxysterols, specifically 20(S)-hydrocholesterol and 22(S)-hydroxycholesterol, have been shown to activate the transcriptional Hh response, an activity that can be reversed by cyclopamine (Dwyer *et. al.,* 2007). The activation mediated by oxysterols occurs through direct binding with the Smo CRD (Nachtergaele *et. al.,* 2013; Nedelcu *et. al.,* 2013; Myers *et. al.,* 2013). Activation by oxysterols can be impeded by the addition of blocking azasterols, like 22-NHC, or by the removal of the CRD of Smo (Nachtergaele *et. al.,* 2013; Nedelcu *et. al.,* 2013; Myers *et. al.,* 2013). Removal of the Smo CRD not only eliminated binding of oxysterols, it eliminates the ability of Smo to maximally respond to Hh ligands (Aanstad *et. al.,* 2009; Myers *et. al.,* 2013). Remarkably, the oxysterol binding pocket of SmoCRD is structurally analogous to

the binding pocket of FrzCRD, which binds to the lipid modification of Wnt and is required for signal transduction (Janda *et. al.,* 2012; Nachtergaele *et. al.,* 2013; Bhanot *et. al.,* 1996; Dann *et. al.,*. 2001).

1.4 Aims

While many aspects of Hh pathway activation have been discovered through years of research, several questions regarding the activation of Smo remain. The Ptch1/2-mediated inhibition of Smo activity is a central aspect of Smo regulation, however the status of Smo activity in the absence of Ptch1/2 is unknown. Additionally, how the binding and internalization of Shh by a Ptch1/2-containing receptor complex triggers Smo activation is not clear. Although Smo was originally postulated to be the receptor for Hh, direct binding between Smo and Shh has never been shown. While the co-receptors, Boc, Cdo, and Gas1, are required at the membrane to bind the Shh ligand, it is uncertain whether they serve a more direct role in the activation of Smo.

The goal of this dissertation is to uncover the molecular interactions that activate Smo in the presence of Shh. The questions that this dissertation aims to answer are: 1) What is the level of Smo activity in cells devoid of Ptch1/2-mediated inhibition? 2) Can Smo respond to Shh in the absence of Ptch1/2 and the co-receptors? 3) Do the co-receptors play a direct role in the activation of Smo? Using an array of molecular tools, the roles that Ptch1/2 and the co-receptors play in Shh-mediated activation of Smo will be analyzed.

Chapter 2. Materials and Methods

Materials:

Cyclopamine was a gift from Dr. William Gaffield (USDA) (Gaffield *et. al*., 1996). SAG was from EMD Biochemicals (Darmstadt, Germany). Vismodegib was from Genentech. Lipofectamine 2000 reagent was from Invitrogen.

Electroporations:

Hamburger-Hamilton (HH) stage 10 Gallus gallus embryos were electroporated caudally in the developing neural tube using standard procedures (Meyer et. al., 2003). Embryos were incubated for another 48 h following electroporation, fixed in 4% PFA, mounted in Tissue-Tek OCT Compound (Sakura) and sectioned.

Immunofluorescence:

Antibodies for mouse Pax7 (1:10), Mrx2 (1:100), Nkx2.2 (1:10), Shh (5E1, 1:20) were from the Developmental Studies Hybridoma Bank. Rabbit α-GFP (1:1000) was from Invitrogen. In all experiments, Alexa488 and Alexa568 secondary antibodies (Invitrogen) were used at 1:1000. Nuclei were stained with DAPI (Invitrogen).

Expression vectors:

Ptch1 was a gift from Dr. Scott (Stanford University, CA, USA). Ptch1Δloop2 was a gift from Dr. Thomas Jessell (Columbia University, NY, USA). The Gli-luc reporter and the Renilla control were gifts from Dr. H. Sasaki (Sasaki et. al., 1997). The following mutations were created using Quikchange mutagenesis (Stratagene): ShhE90A, ShhH183A, ShhNE90A, ShhNH183A, and SmoΔCRD-CLD. Smoothened constructs were cloned into pMES, a vector that contains Gallus gallus Beta-actin promoter with an IRES GFP.

Cell Culture:

Smo$^{-/-}$ fibroblasts (Dr. Taipale) and *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen). Serum starvation media was composted of DMEM supplemented with 0.5% fetal calf serum (Invitrogen). All mESCs cell lines were maintained under standard conditions without feeder cells, the media used for mESCs was as follows DMEM (Invitrogen) supplemented with 15% fetal calf serum (Invitrogen), 2mM Glutamine, 1X Non-essential amino acids (Gibco), 1X Nucleosides (EDM Millipore), 2 ug/ml Gentamicin, 0.1 mM β-mercaptoethanol, and 1000u/ml LIF. mESCs were neuralized in DFNB media, which was composed of a mixture of 25 % F-12 Hams, 25% DMEM, and 50% Neurobasal medium supplemented with 0.5X B-27 Supplement (Gibco), 2mM Glutamine, 0.1mM B-Mercaptoethanol, and 2 ug/ml Gentamicin.

Fibroblast Derivation:

Ptch1$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ fibroblasts were obtained by transfecting large T antigen from the SV40 virus into *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ mouse ES cells (Alfaro *et. al.*, 2014) grown in ES medium. Cells were then switched to Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS).

Transfection:

Cells were transiently transfected for 24h at 80-90% confluency using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

Gli-Luciferase Assay:

Smo$^{-/-}$, and *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells were plated at a density of $3x10^4$ in 24 well plates and transfected with Gli-luciferase, CMV-Renilla, and specified plasmids 24 hours after plating. Cells were allowed to grow to confluency and then switched to low serum medium (0.5% FBS) alone or with specified concentrations of cyclopamine, vismodegib, or SAG for 16 hours. Cells were subsequently lysed and luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega).

Co-Culture Assay:

Ptch1$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ reporter cells were plated at a density of $1.4x10^5$ in 6-well plates and transfected with Gli-luciferase, CMV-Renilla, with or without a variant of Ptch1. *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$signaling cells were plated similarly and transfected with a variant of Shh 24 hours after plating. Cells were then trypsinized and plated in 24-well plates in specified combinations ($1.5x10^4$ of each type) 24 hours after transfections. Cells were grown to confluency and switched to low serum medium (0.5% FBS) for 16 hours before assaying for luciferase activity.

EB differentiation

mESCs were neuralized using established procedures (Wichterle *et al.*,2002). NEBs were harvested after 5 days in culture, fixed and stained for Isl1/2, Olig2, Nkx2.2 or Pax7 (Developmental Studies Hybridoma Bank). NEBs were mounted in Fluoromount, imaged, and quantified for number of positive nuclei. Number of positive nuclei were normalized for the size of the NEB, and presented as the number in the average NEB.

Western Blots:

Ptch1$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells were transfected with Shh mutants as indicated. 48 hours after transfection, *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells were rinsed with PBS and lysed with RIPA buffer (150 mM NaCl, 50 mM Tris-Hcl, 1% Igepal, 0.5% Sodium Deoxycholate, and protease inhibitors) for 30 min on ice. Protein lysate was cleared by centrifugation at 13,000g for 30 min at 4 °C. 20 ug of each sample was run on a 15% SDS-PAGE gel and transferred to a 0.45 micron nitrocellulose membrane. Membranes were blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and incubated with a rabbit polyclonal anti-Shh antibody (H160; Santa Cruz Biotechnology) at 1:250. A goat anti-rabbit HRP-conjugated secondary antibody (Biorad) was used at 1:10000.

Genome Editing

TALEN constructs targeting the first exon of mouse Cdo and Gas1 were designed and cloned into the pCTIG expression vectors containing IRES puromycin and IRES hygromycin selectable markers (Cermak *et. al.,* 2011). The following repeat variable domain sequences were generated: Cdo, 5' TALEN: NN HD NI NG HD HD NI NN NI HD HD NG HD NN NN ; 3' TALEN: HD NI HD NI NI NN NI NI HD NI NG NI HD NI NN; Gas1, 5' TALEN: NN NI NN NN NI HD NN HD HD HD NI NG NN HD HD; 3' TALEN: NN NN NI NI NI NI NN NG NG NG NN NG HD HD NN NI. Two CRISPR constructs targeting a double strand break flanking the first exon of Boc were cloned into pSpCas9 vector with an IRES puromycin selectable marker (Ran *et. al.,* 2013). The Boc CRISPRs targeted the following forward genomic sequences (PAM sequences underlined): Upstream of first exon 5' CCTGTCCTCGCTGTTGGTCCCTA 3'; Downstream of first exon 5' CCCACAGACTCGCTGAAGAGCTC 3'.

Mouse embryonic stem cells (mESCs) were plated at a density of $1.0x10^6$ on 6-well plates and transfected with 6 genome editing plasmids the following day. One day after transfection, selective medium (100 ug/mL hygromycin and 0.5 ug/mL puromycin) was added for 4 days. Selective medium was removed and surviving mESC colonies were isolated, expanded and genotyped by sequence PCR products spanning TALEN and CRISPR-binding sites.

Genotyping

PCR screen was performed on extracted gDNA using primers flanking the TALEN and CRISPR-binding sites.
Boc Forward primer: 5' CATCTAACAGCGTTGTCCAACAATG 3'
Boc Reverse primer: 5' GGAGTACTTGGGTGTGGTACA 3'
Cdo Forward primer: 5' GCTGGGATTCTTAGCATTTAAGTT 3'
Cdo Reverse primer: 5'GCCTTGAACTCACAGAGATTCG 3'
Gas1 Forward primer: 5' ACCTGCGTCGCCGCGCTTCTCA 3'
Gas1 Reverse primer: 5'CAGCAGCGCGGCTAGCAT 3'

TOPO cloned PRC products were sequenced to confirm allelic mutations.

A *Ptch1*$^{LacZ/LacZ}$*; Ptch2*$^{-/-}$*; Shh*$^{-/-}$*;Boc*$^{-/-}$*; Cdo*$^{-/-}$*; Gas1*$^{-/-}$ clone was identified. This clone contains a 490 bp deletion of the first exon of the Boc alleles, a 50 bp deletion in the first exon of the Cdo alleles, and a 480 bp insertion and a 200 bp deletion in the first exon of the Gas1 alleles.

Chapter 3. *Ptch1$^{LacZ/LacZ}$; Ptch2$^{-/-}$* cells have low Hh pathway activity and require the Shh binding domain of Ptch1 to activate the pathway in response to extracellular ShhN

3.1 Introduction

Within the Hedgehog (Hh) pathway, the ligand, receptor, and signal transducer are distinct molecules, and the state of the pathway depends on their interaction. In the absence of the Shh ligand, the receptors Patched1 (Ptch1), and its paralog Patched2 (Ptch2), inhibit the signal transducer Smoothened (Smo) sub-stoichiometrically. Ptch1/2 belong to the Resistance, Nodulation, and Division (RND) family of transmembrane transporters, and Ptch1/2-mediated inhibition requires the putative proton anti-porter activity that characterizes these molecules, as shown by mutants lacking conserved amino acids in the putative proton channel (Taipale *et. al.*, 2002; Alfaro *et. al.*, 2014). These Ptch1/2 mutants are greatly reduced in their ability to inhibit Smo, resulting in an increase of Hh pathway activity. In general, RNDs mediate the secretion of small lipophilic and amphipathic molecules (Nikaido *et. al.*, 2001; Nikaido *et. al.*,2009), and it has been proposed that Ptch1/2 re-localizes a Smo-inhibiting sterol (Taipale *et. al.*, 2002).

How Shh binding to a receptor complex, and its subsequent internalization, regulates Smo activation remains unclear. Based on the phenotype of *Ptch1$^{LacZ/LacZ}$* mice embryos, which display evidence of widespread Hh pathway upregulation, the current model of the Hh response postulates that Smo activity is regulated by Ptch1/2-mediated inhibition (Goodrich *et. al.*, 1997). A tenet of this model is that Smo is constitutively active in the absence of Ptch1/2. However, the model does not provide a reasonable explanation as to why at sites where Ptch1/2 expression is very low, the Hh pathway is not necessarily active. This is exemplified in the dorsal neural tube where Ptch1/2 expression is undetectable yet Hh pathway activity is absent. Additionally, embryonic structures in *Ptch1$^{LacZ/LacZ}$* mice that show evidence of Hh pathway upregulation also display an upregulation of the Shh ligand (Goodrich *et. al.*, 1997). Whether the Shh ligand plays a role in the upregulation of the Hh response in this context has not been addressed, and a *Ptch1$^{-/-}$; Shh$^{-/-}$* mouse has never been evaluated. Furthermore, the status of the Hh pathway in the genetic absence of both the canonical receptors *in vivo* has not been assessed.

If the only mechanism regulating Smo activity is the inhibitory activity of Ptch1/2, then the hypothesis is that cells lacking all Ptch1/2 activity (*Ptch1$^{-/-}$;Ptch2$^{-/-}$*) should have a highly upregulated Hh pathway response. However, if there are multiple steps required for the activation of Smo, then simply removing the inhibitory activity of Ptch1/2 will not be sufficient for Hh pathway activation. It is possible that the inhibition of Smo is distinct from its activation, however the activity of Smo in a *Ptch1-/-; Ptch2-/-* cellular environment has never been addressed. Using genome editing and other molecular tools, we will address whether the activation of Smo directly depends on the inhibitory action of Ptch1/2.

3.2 Results

3.2.1 Differentiated *Ptch1$^{LacZ/LacZ}$; Ptch2$^{-/-}$; Shh$^{-/-}$* and *Ptch1$^{LacZ/LacZ}$; Ptch2$^{-/-}$; Shh$^{-/-}$; Boc-/-;Cdo-/-;Gas1-/-* embryoid bodies express ventral neural tube markers

To address potential inconsistencies in the current model of Smo activation, we used Transcription activator-like effector nucleases (TALENs) to generate a mouse embryonic stem cell (mESC) line devoid of Ptch1/2-mediated inhibition. We designed a pair of TALENs that bind within the first exon of Ptch2 and expressed them in an established *Ptch1$^{LacZ/LacZ}$* ES cell line. After transfection and transient selection, surviving mESC clones were selected and genotyped, and a *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* mESC line was established. To further simplify the effects of the ligand on the Hh response, TALENs targeting the first exon of Shh and Disp1 were cloned. Additional genetically engineered mESC lines were generated with null mutations in various Hh pathway components. These mESC lines include: *Shh$^{-/-}$; Disp1$^{-/-}$*, *Shh$^{-/-}$; Disp1$^{-/-}$; Ptch1$^{-/-}$*, and *Shh$^{-/-}$; Disp1$^{-/-}$; Ptch1$^{-/-}$; Ptch2$^{-/-}$* (Alfaro *et. al.*, 2014; Roberts *et. al.*, 2016; manuscript submitted).

The status of the Hh pathway in these mESC lines was initially tested by differentiating these cells into neuralized embryoid bodies, or NEBs. NEBs are spherical, free-floating colonies that have been differentiated such that they can recapitulate the development of the neural tube to some degree. These NEBs can express many of the different neural markers seen during neural tube development and, depending on the state of the Hh pathway in these cells, can differentially express dorsal and ventral markers. To study the effects of the removal of Ptch1/2-mediated inhibition on the expression of neural markers, we compared three genetically engineered mESC lines: *Disp1$^{-/-}$*; *Shh$^{-/-}$*, *Disp1$^{-/-}$,Shh$^{-/-}$*, *Ptch1$^{-/-}$*, *and Disp1$^{-/-}$; Shh$^{-/-}$,Ptch1$^{-/-}$,Ptch2$^{-/-}$* (**Figure 6**). *Disp1$^{-/-}$*; *Shh$^{-/-}$* neuralized embryoid bodies (NEBs) do not express ventral neural markers, but do express Pax7, indicative of an absence of Hh pathway activation (**Figure 6, A, B, G**). *Disp1$^{-/-}$,Shh$^{-/-}$*, *Ptch1$^{-/-}$* NEBs exhibit an increase of Isl1/2 positive nuclei, indicative of a low to intermediate level of Hh pathway activation (**Figure 6, C, D, H**).We find that *Disp1$^{-/-}$; Shh$^{-/-}$,Ptch1$^{-/-}$,Ptch2$^{-/-}$* NEBs express many ventral neural tube markers such as Nkx2.2, Isl1/2, and Olig2, indicative of a high Hh pathway response independent of the Shh ligand (**Figure 6, E, F, I**). This observation suggests that the level of Hh pathway activity in neuralized cells devoid of Ptch1/2-mediated inhibition is high (Roberts *et. al.*, 2016; manuscript submitted).

To assess whether the co-receptors are required for Hh pathway activity in NEBs, we took the *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$*; *Shh$^{-/-}$* mESC line and further modified the genome. Using a combination of TALENs and CRISPRs, we generated a mESC line with null mutations in the first exons of *Boc*, *Cdo*, and *Gas1* (See Chapter 2). *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$*; *Shh$^{-/-}$;Boc$^{-/-}$;Cdo$^{-/-}$;Gas1$^{-/-}$* mESCs were differentiated into NEBs, and we find that these cells express a combination of the ventral neural tube markers Nkx2.2 and Olig2- indicative of high levels of Hh pathway activity (data not shown). In this system we find that Hh pathway activity in the absence of Ptch1/2-mediated inhibition is unaffected by the absence of the co-receptors. This demonstrates that the co-receptors do not play a more direct role in the activation of Smo in the NEB system.

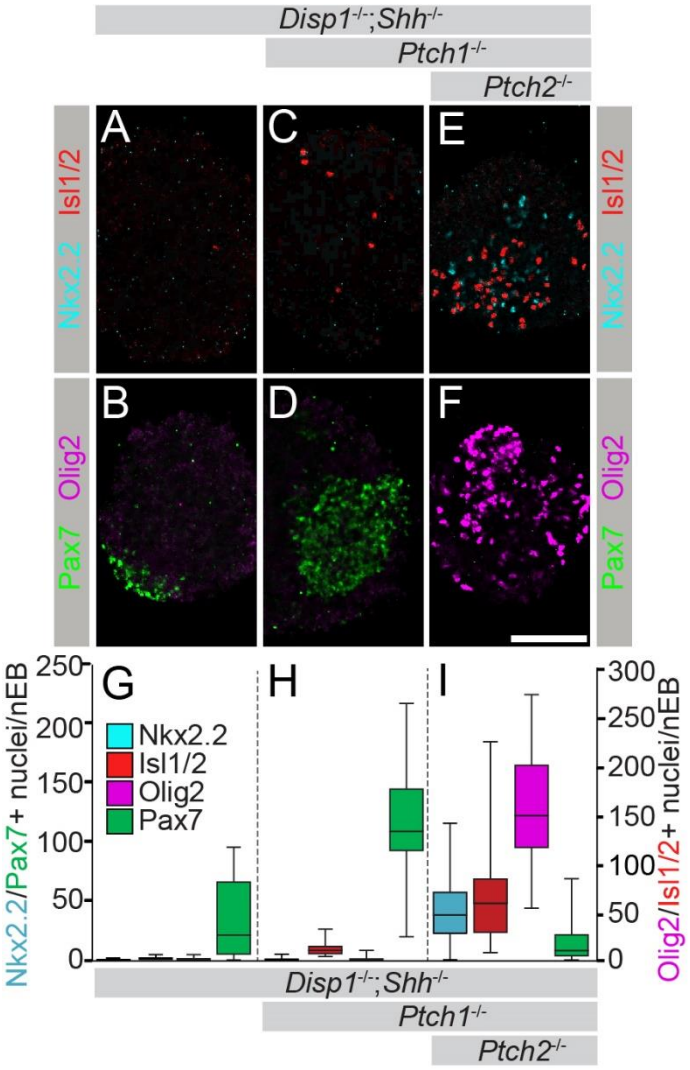

Figure 6 Ptch1LacZ/LacZ;Ptch2-/-; Shh-/- NEBs express ventral neural markers

Figure 6. *Disp1*$^{-/-}$*; Shh*$^{-/-}$*; Ptch1*$^{-/-}$*; Ptch2*$^{-/-}$ NEBs express ventral neural markers

(A, B, G) *Disp1*$^{-/-}$*; Shh*$^{-/-}$
(C, D, H) *Disp1*$^{-/-}$*; Shh*$^{-/-}$*,Ptch1*$^{-/-}$
(E, F, I) *Disp1*$^{-/-}$*; Shh*$^{-/-}$*,Ptch1*$^{-/-}$*,Ptch2*$^{-/-}$
NEBs were cultured for 5 days before fixing and staining for neural markers. Positive nuclei were quantified and normalized for the size of the NEB, and presented as the number in an average NEB. All error bars are s.e.m. (Roberts *et. al.*, 2016; manuscript submitted).

3.2.2 *Ptch1*$^{LacZ/LacZ}$*; Ptch2*$^{-/-}$ cells have a low level of Hh pathway activity

Because the differentiation protocol for the formation of NEBs is fairly long, 5 days of aggregation and differentiation, we decided to quantify the Hh pathway activity using a built-in genetic reporter. The *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$; *Shh*$^{-/-}$ line contains a LacZ reporter under the control of the Ptch1 promoter, which itself is a target of the Hh pathway. We took advantage of this self-contained reporter gene and measured LacZ levels in *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$; *Shh*$^{-/-}$ NEBs throughout the course of cell aggregation and differentiation. Surprisingly, we find that initial levels of LacZ expression in these NEBs is low (**Figure 7, A**). LacZ levels in *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$; *Shh*$^{-/-}$ NEBs begin to increase on the third day of differentiation, and this increase in LacZ expression is ligand independent. To ensure that the activity measured was Smo-dependent, we treated fully differentiated NEBs with increasing concentrations of

cyclopamine. We find that LacZ levels are significantly lowered with cyclopamine (with an IC50 of about 30 nM), demonstrating that LacZ expression in this context is indeed Smo-dependent (**Figure 7, B**) (Roberts *et. al.,* 2016; manuscript submitted).

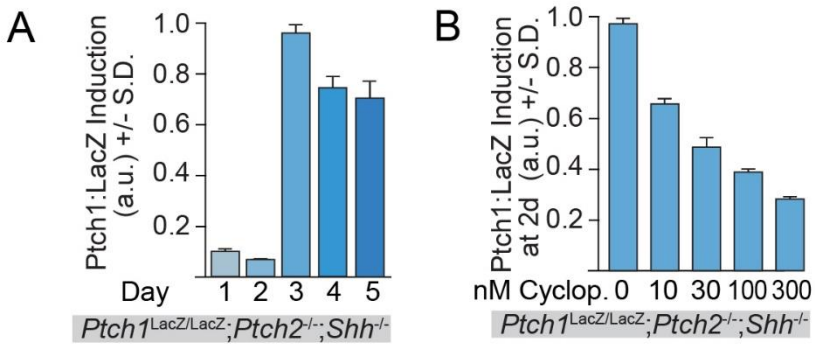

Figure 7 LacZ levels Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-;}$ Shh$^{-/-}$ NEBs are initially low and are sensitive to cyclopamine

Figure 7. LacZ levels Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$; Shh$^{-/-}$ NEBs are initially low and are sensitive to cyclopamine

(A) Ptch1:LacZ levels in *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$*; *Shh$^{-/-}$* NEBs were measured up to 5 days after NEB formation. All error bars are s.e.m. (B) *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$*; *Shh$^{-/-}$* nEBs were cultured in 0-300 nM cyclopamine and Ptch1:LacZ was measured at 72h. Ptch1:LacZ levels in 30 nM cyclopamine were approximately half those of untreated nEBs. All error bars are s.e.m. (Roberts *et. al.*, 2016; manuscript submitted)

Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$; *Shh$^{-/-}$* NEBs show evidence of Hh pathway activation, which concurs with the currently accepted model of the Hh pathway; however these cells require time to differentiate before activating the Hh pathway. Furthermore, at early time points these cells have a low level of pathway activity, despite the absence of Ptch1/2-mediated inhibition. We wanted to further investigate a Ptch1/2-independent activation event in these cells, however mESCs are notoriously difficult to transfect and manipulate using other tools. Therefore we decided to derive a fibroblast cell line from the *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* mESC line to further probe Smo activation events that occur independent of Ptch1/2 activity.

We transfected the *SV40 large T* antigen into *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* mouse embryonic stem cells and derived a fibroblast-like line after LIF withdrawal. We find that this cell line can be efficiently transfected, allowing us to study the transcriptional Hh response using a luciferase reporter under the control of the Hh pathway: Gli-Luciferase (Gli-Luc) (Taipale *et. al.,* 2000). We first assessed the state of the Hh pathway in these cells by testing the effect of two Smo inhibitors, cyclopamine (Chen *et. al.,* 2002; Incardona *et. al.,* 1998) and vismodegib (Rubin *et. al.,* 2006). A dose response curve extending beyond the known IC50 of these drugs resulted in no significant decrease of Gli-dependent Luciferase activity (**Figure 8**). This observation is in

agreement with the observation that early *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$; *Shh*$^{-/-}$ NEBs express low levels of LacZ and are unresponsive to cyclopamine at early time points. This data suggests that the *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ fibroblast-like cells are in a state similar to the early, undifferentiated NEBs. In this particular state, the absence of Ptch1/2-mediated inhibition in these cells is insufficient for Smo activation, and Smo activity is low. One interesting observation made in these fibroblasts is, although cyclopamine has no effect in bringing down Gli-Luc levels, at high concentrations (300 nM), cyclopamine causes a spike of transcriptional activity (**Figure 8, A**). This increase of transcriptional activity is significant and does not occur with the veratramine control (a steroidal alkaloid which has no effect on the Hh pathway). Although we can only speculate about the cause of high Smo activity, it has been observed that cyclopamine can stimulate the entry of Smo to the primary cilium, and perhaps in this context can cause a conformational change of Smo (Wang *et. al.*, 2009)

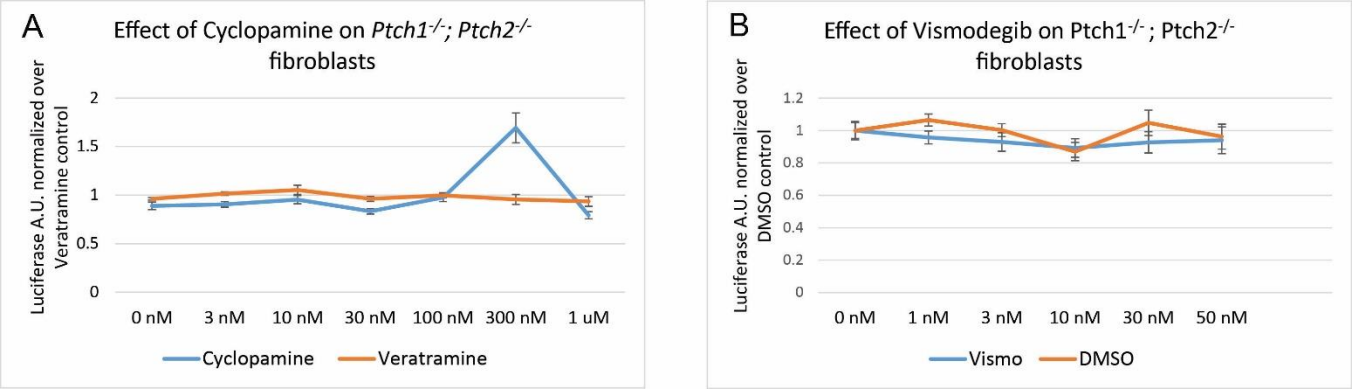

Figure 8 Ptch1$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ *cells are unresponsive to the inhibitors cyclopamine and vismodegib*

Figure 8. *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells are unresponsive to the inhibitors cyclopamine and vismodegib

(A) *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells were transfected with *Gli-luc* and cultured with cyclopamine (0nM-1uM) (blue line) or veratramine control (orange line). Luciferase activity, a measure of the transcriptional Hh response, was measured. Luciferase levels in *Gli-luc* transfected *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells cultured with vehicle control was set at 1. All error bars are s.e.m, n>10.
(B) *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells were transfected with *Gli-luc* and cultured with vismodegib (0-100 nM) (blue line) or vehicle control (orange line). Luciferase activity, a measure of the transcriptional Hh response, was measured. Luciferase levels in *Gli-luc* transfected *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells cultured with vehicle control was set at 1. All error bars are s.e.m., n>10.

The inability of Smo to respond to inhibitors in *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells led us to test whether uninhibited Smo could be activated by SAG (Frank-Kamenetsky *et. al.*, 2002), a small molecule

agonist of Smo. Addition of SAG (1 and 10 nM) to *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells transfected with *Gli-Luc* does not affect Hh pathway activity (**Figure 9**). We decided to test whether overexpressing Smo in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells increases the ability of SAG to modulate Smo, however co-transfection of *Smo* and *Gli-Luc* does not result in Hh pathway activation in the presence of SAG. To test whether SAG modulates Smo in the presence of a Ptch1 inhibitor, we added SAG to *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells co-transfected with *Ptch1*, *Smo*, and *Gli-luc*. Interestingly, we find that in this cellular environment SAG is able to significantly increase the level of Hh pathway activity (**Figure 9**). The finding that SAG activates Smo most effectively when Ptch1 is present in a cell supports the hypothesis that SAG activates Smo by competing with an inhibitor transported by Ptch1. This hypothesis is reinforced by the fact that most known small-molecule modulators of Smo activity bind to the heptahelical (transmembrane) domain of Smo.

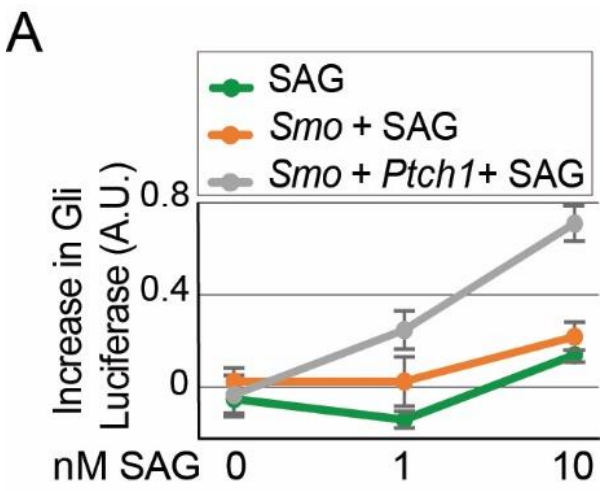

Figure 9. *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells are unresponsive to SAG

(A) *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells were transfected with *Gli-luc* alone (green), together with *Smo* (orange), or with *Smo* and *Ptch1* (grey), and cultured with SAG (0 – 10 nM) and Luciferase levels were measured. Luciferase levels in *Gli-luc* transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells was set at 1. All error bars are s.e.m., n>4.

Figure 9 Ptch1$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ *cells are unresponsive to SAG*

The inability of cyclopamine and SAG to significantly alter the level of Hh pathway activity in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells supports evidence to the hypothesis that the heptahelical domain of Smo is an allosteric site that modulates Smo activity. The true orthosteric site of Smo should be able to regulate the activity of Smo even in the absence of Ptch1/2. This suggests that Smo activation is controlled by an orthosteric site on another distinct domain.

3.2.3 Expression of *Ptch1* with an intact Shh-binding domain in *Ptch1*$^{LacZ/LacZ}$; *Ptch2*$^{-/-}$ fibroblasts restores sensitivity to ShhN in the environment

We find that the level of Smo activity in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells is low, despite the absence of Ptch1/2-mediated inhibition. The addition of SAG to these cells also does not result in activation of the Hh response. In an effort to understand whether Smo could be activated in a Ptch1/2 free

environment, we next sought to assess whether these *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells can respond to extracellular Shh. To accomplish this we used a co-culture assay in which we mixed a distinct pool of signaling cells with a distinct pool of responding cells. We measured the transcriptional response only in *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ "reporter cells" transfected with *Gli-Luc* and grown in a co-culture with mock (*GFP*) or *ShhN* transfected *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells (**Figure 10, A**). ShhN (truncated after G198) is a freely soluble form of Shh that lacks the cholesterol modification but is still able to signal effectively (Porter *et. al.*, 1996). Although the three co-receptors, Boc, Cdo, and Gas1, are present in these cells, we found that *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ reporter cells do not activate the Hh pathway in response to ShhN in the extracellular environment (**Figure 10, B**). Despite the GPCR-like characteristics of Smo, in this context it is unable to function as an extracellular receptor for ShhN.

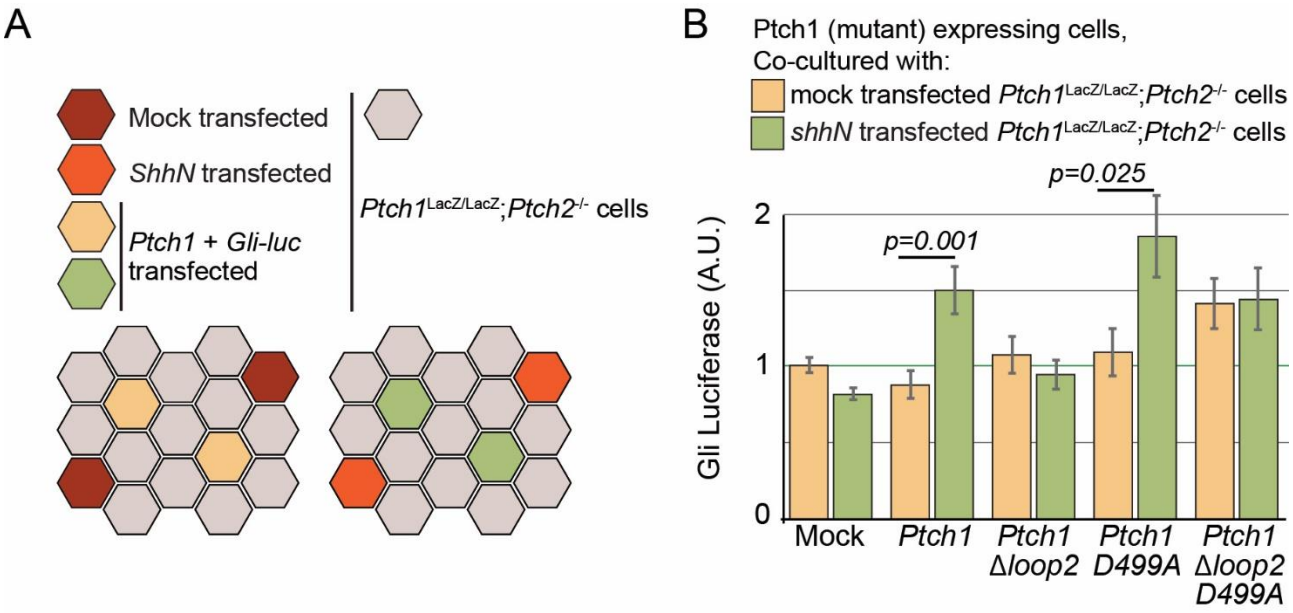

Figure 10 Non-cell autonomous activation by extracellular Shh requires the Shh binding (L2) domain, but not the antiporter activity of Ptch1

Figure 10. Non-cell autonomous activation requires the Shh binding (L2) domain, but not the antiporter activity of Ptch1

(A) Diagram of experiment quantified in B: Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$ reporter cells transfected with *Gli-luc* or *Gli-luc* with a variant of *Ptch1* (beige/green hexagons) were grown in a 1:1 mixed co-culture of *GFP* (Mock) transfected (scarlet hexagons) or *ShhN* transfected Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$ cells (orange hexagons). (B) *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ reporter cells transfected *Gli-luc* alone or with *Ptch1*, *Ptch1ΔL2*, *Ptch1D499A*, or *Ptch1ΔL2-D499A*, co-cultured with *GFP* (Mock) transfected *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ (beige bars) or *ShhN* transfected *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells (green bars). Luciferase levels in *Gli-luc* transfected *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ reporter cells cultured with mock transfected cells was set at 1. All error bars are s.e.m., *p* values (Student T-test, 2 tailed) are indicated were relevant, n>7 (B).

We next tested whether the re-introduction of functional Ptch1 into *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ cells repairs their ability to respond to extracellular Shh. We find that transfection of *Ptch1* into *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ reporter cells restores their ability to activate the Hh pathway in response to extracellular ShhN, consistent with the role of Ptch1 in mediating the Hh response (**Figure 10, B**). The putative antiporter activity has been shown to be required for Ptch1-mediated inhibition of Smo (Taipale *et. al.*, 2002; Alfaro *et. al.*, 2014), and we assessed if a form of Ptch1 unable to inhibit Smo, Ptch1D499A, can restore Shh-sensitivity to *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ reporter cells. Remarkably we find that transfection of *Ptch1D499A* restores responsiveness to ShhN in the environment (**Figure 10, B**). This demonstrates that Ptch1 can mediate a Shh-induced activation of the Hh response completely independent of its inhibitory activity. However the Shh-binding domain remains essential for the response, as transfection of forms of Ptch1 lacking the Shh binding (L2) domain (*Ptch1*Δ*L2* and *Ptch1*Δ*L2-D499A*) did not restore the ability of reporter cells to respond to ShhN.

Here we show that the canonical receptor, Ptch1, is required for cells to activate the pathway in response to Shh in the extracellular environment. Many lines of evidence suggest that the primary role of Ptch1 is to inhibit Smo activity in the absence of the Shh ligand. In contrast, these observations demonstrate that Ptch1 has two distinct functions in regulating Smo activity. We show that Ptch1 can activate the Hh pathway in response to extracellular ShhN via a mechanism that is distinct from the release of Smo inhibition.

Chapter 4. Shh mutants activate the Hh pathway independent of extracellular Shh receptors in Ptch1$^{LacZ/LacZ}$; Ptch2$^{-/-}$ cells

4.1 Introduction

Our observation that *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells have a low level of Hh pathway activity seriously question longstanding dogmas about the regulatory mechanism of the pathway. We have shown that, along with having a low baseline level of activity, *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells are unresponsive to extracellular ShhN. Boc, Cdo, and Gas1, work with Ptch1/2 to bind to extracellular Shh, which then undergoes an internalization event *(Marigo et. al., 1996; Incardona et. al., 2002; Allen et. al.,* 2011; Izzi *et. al.,* 2011). It is after this internalization event that the subcellular distribution and activity of Smo changes. However we show that in the absence of Ptch1/2, the co-receptors are unable to mediate this binding and internalization event.

The re-introduction of Ptch1 into *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells restores the cell's ability to activate the pathway in response to extracellular ShhN. Furthermore, we show that this activation event is direct and not simply caused by a release of inhibition, as forms of Ptch1 that cannot inhibit Smo retain their ability to activate the pathway in response to ShhN. This suggests that the activation event itself is mediated by Shh. Because Shh is internalized during signaling, there is a distinct possibility that Shh mediates other important signaling events after internalization. We next want to address how internalized Shh activates the pathway, and whether we can circumvent the binding and internalization of Shh through other means and activate the pathway more directly.

4.2 Results

4.2.1 *Ptch1*$^{LacZ/LacZ}$; *Ptch2*$^{-/-}$ cells can activate the Hh pathway in response to transfected *ShhN*

The observation that Ptch1 is cell-autonomously required to endow cells the ability to respond to extracellular ShhN is consistent with evidence that Ptch1 internalizes Shh during signaling (Incardona *et. al.,* 2000). We wanted to test whether we could circumvent this function of Ptch1 and activate the Hh pathway cell-autonomously. If Shh mediates additional activation events after binding and internalization, then intracellularly localized ShhN may be able to activate the pathway in *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells. We decided to test this hypothesis through transfection of *ShhN*.

We transfected *ShhN* and *Gli-luc* directly into *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells and measured the transcriptional response (**Figure 11**). Amazingly, transfection of *ShhN* into *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells results in a significant increase of Luciferase activity (6-8 fold increase (**Figure 11, A**). To visualize ShhN-dependent activation of the pathway, we transfected either *ShhN* or *GFP* into *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells and stained for LacZ expression (**Figure 11, B**). We found that *ShhN* transfection increased the percentage of LacZ positive cells from 5.8% to 9.5% over control transfected cells. These observations demonstrate that ShhN can cell-autonomously activate the Hh response pathway in the absence of the canonical receptors Ptch1/2. Additionally, these

results show that ShhN can activate the Hh response without affecting Ptch1/2-mediated inhibition of Smo, since these cell lack all Ptch1/2 activity. This also supports our previous observations that the inhibition mediated by Ptch1/2 is distinct from an activation event mediated by ShhN.

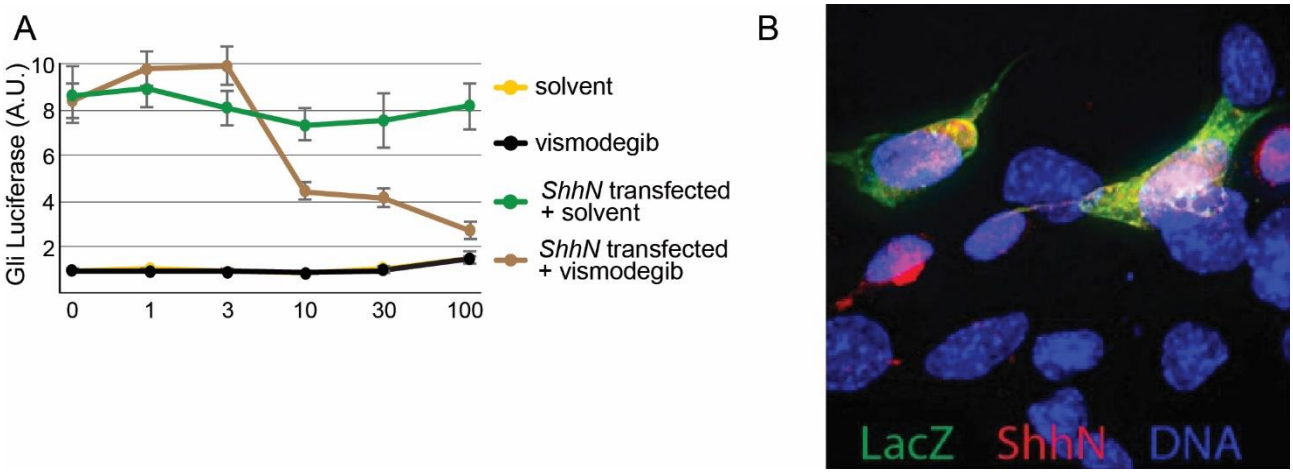

Figure 11 Cell-autonomous activation of the Hh response is independent of Ptch1/2

Figure 11. Cell-autonomous activation of the Hh response is independent of Ptch1/2

(A) *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells were transfected with *Gli-luc* alone (yellow/black) or with *ShhN* (green/brown), and cultured with vismodegib (0-100 nM) (black/brown) or vehicle control (yellow/green). Luciferase activity, a measure of the transcriptional Hh response, was measured. Luciferase levels in *Gli-luc* transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells cultured with vehicle control was set at 1. All error bars are s.e.m., p values (Student T-test, 2 tailed) are indicated were relevant, n>9. (B) *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells were transfected with *GFP* (not shown) or with ShhN. Transfected cells were then stained for GFP and LacZ (beta-galactosidase) or for Shh (red) and LacZ (green).

We next wanted to test whether the activation mediated by ShhN transfection is dependent on Smo activity. We added increasing concentrations of the Smo inhibitor vismodegib (0-100 nM) to *ShhN* transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells and found that vismodegib can lower Luciferase activity with an IC50 of about 10nM (**Figure 11, A**). We also find that addition of vismodegib at a concentration of 100 nM can lower Hh pathway activity almost completely back to the level of mock transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells. This demonstrates that even in the absence of Ptch1/2, activation of the Hh response pathway after ShhN transfection remains Smo dependent.

4.2.2 Shh mutants can activate the Hh pathway cell-autonomously independent of Shh receptor function *in vitro*

Transfection of *ShhN* causes a Smo-dependent activation of the Hh pathway in the absence of the canonical receptors Ptch1/2, however cell-autonomous activation may require other Shh-binding

molecules. The Shh co-receptors, Boc, Cdo, and Gas1 have been shown to function redundantly as obligate receptors in Hh signaling (Allen *et. al.*, 2011; Izzi *et. al.*, 2011; Tenzen *et. al.*, 2006). To assess the involvement of the co-receptors in cell-autonomous activation of the pathway, we utilized a previously characterized ShhN mutant (ShhNE90A) that cannot bind to either Boc, Cdo, or Gas1 (Izzi *et. al.*, 2011). We also cloned a form of ShhN modeled after ShhH183A, which has been shown to have defects in auto-proteolytic processing and non-cell autonomous signaling (Goetz *et. al.*, 2006). This finding is consistent with its predicted inability to bind to Ptch1, due to the disruption of the zinc coordination site (ShhNH183A) (Fuse *et. al.*, 1999; Goetz *et. al.*, 2006). (**Table 1**).

Mutant	Binding	
	Ptch1	Boc, Cdo, Gas1
WT	+	+
E90A	+	-
H183A	-	+

Table 1 Receptor binding partners of WT, E90A, and H183A Shh mutants

Table 1. Receptor binding partners of WT, E90A, and H183A Shh mutants

WT Shh and ShhN binds to all canonical receptors and co-receptors (Ptch1/2, Boc, Cdo, and Gas1). E90A Shh mutants bind the canonical receptors (Ptch1/2) but cannot interact with the co-receptors (Boc, Cdo, Gas1). H183A Shh mutants are predicted to bind to the co-receptors (Boc, Cdo, Gas1) but cannot interact with the canonical receptors (Ptch1/2).

We first tested the ability of ShhNE90A and ShhNH183A to signal non-cell autonomously, or *in trans*, by transfecting *Ptch1* and *Gli-luc* into *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ reporter cells and co-culturing them with *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells transfected with *ShhN*, *ShhNE90A*, or *ShhNH183A* (**Figure 12, A Green**). Reporter cells activated the Hh response when co-cultured with ShhN expressing cells (**Figure 12, B**). Reporter cells did not activate the Hh response pathway when co-cultured with ShhNE90A expressing cells, confirming previously published data that ShhNE90A is unable to mediate paracrine Hh signaling (**Figure 12, B**) (Izzi *et. al.*, 2011). Consistent with its predicted inability to bind to Ptch1, we find that ShhNH183A does not activate the Hh pathway in reporter cells (**Figure 12, B**). This finding supports the notion that activation of the pathway

by ShhN during non-cell autonomous signaling requires the binding of Shh to Ptch1/2 in conjunction with the co-receptors.

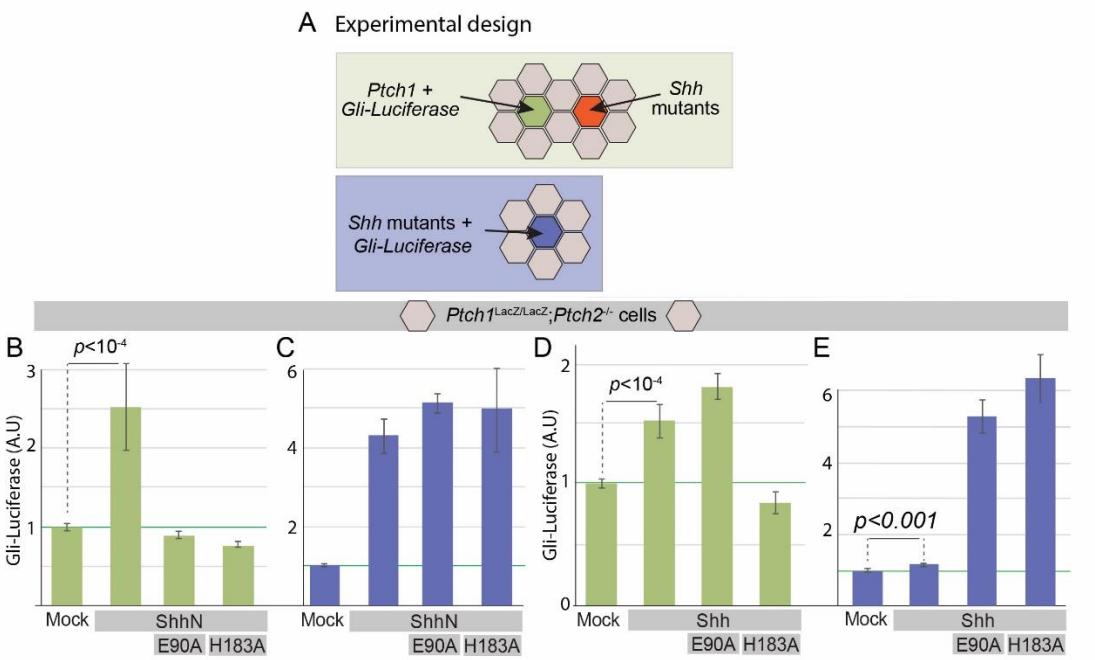

Figure 12 Shh mutants activate the Hh pathway cell-autonomously in Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$ cells

Figure 12. Shh mutants activate the Hh pathway cell-autonomously in *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells

(A) Diagram of non-cell autonomous signaling (green background): Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$ reporter cells transfected with *Gli-luc* and *Ptch1* (green hexagons) were grown in a 1:1 mixed co-culture of *GFP* (Mock) transfected or *Shh* mutant transfected *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells (red hexagons). Diagram of cell-autonomous signaling (blue background): *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells transfected with *Gli-luc* were *GFP* (Mock) transfected or transfected with *Shh* mutants. (B) *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells co-cultured with mock, *ShhN*, *ShhNE90A*, or *ShhNH183A* transfected *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells. Luciferase levels in *Gli-luc* transfected *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells cultured with mock transfected cells was set at 1. (C) *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells were mock transfected or transfected with *ShhN*, *ShhNE90A*, or *ShhNH183A*. Luciferase levels in mock transfected *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells was set at 1. (D) *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells co-cultured with mock, *Shh*, *ShhE90A*, or *ShhH183A* transfected Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$ cells. Luciferase levels in *Gli-luc* transfected *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells cultured with mock transfected cells was set at 1. (E) *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells were mock transfected or transfected with *Shh*, *ShhE90A*, or *ShhH183A*. Luciferase levels in *Gli-luc* transfected *Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$* reporter cells cultured with mock transfected cells was set at 1. All error bars are s.e.m., *p* values (Student T-test, 2 tailed) are indicated were relevant, n>16 (C,E) n>6 (B,D).

A significant distinction between Shh and ShhN is that the ShhN lacks the cholesterol moiety found on autoproteolytically processed Shh, rendering it highly soluble (Yang *et. al.*, 1997). The possibility of distinct differences in activity between the full length and soluble forms led us to test the ability of Shh, ShhE90A and ShhH183A to signal in trans. Reporter cells activated the Hh response pathway when co-cultured with Shh and ShhE90A expressing cells, but not with ShhH183A expressing cells (**Figure 12, D**). This suggests that the cholesterol modification of Shh is able to compensate for the loss of co-receptor binding during non-cell autonomous signaling. The autocatalytic cleavage of ShhH183A is impaired (**Figure 13**) (Goetz *et. al.*, 2006), similar to the pro-peptide Shh mutant that retains its C-terminal domain (ShhC199A), but levels of the N-terminal fragment are difficult to assess as this mutation appears to affect antibody binding.

Cell-autonomous activation of the Hh response may not require extracellular Shh receptors. To address this, we transfected all forms of *ShhN* and *Shh* into *Ptch1*$^{LacZ/LacZ}$*;Ptch2*$^{-/-}$ reporter cells (**Figure 12, A Purple**). Transfection of any form of *ShhN* can activate the Hh pathway significantly in reporter cells (**Figure 12, C**), indicating that cell-autonomous pathway activation does not require Ptch1/2, Boc, Cdo, or Gas1. Transfection of *Shh* into reporter cells results in a small but significant increase of pathway activity ($p<0.001$). In contrast, transfection of *ShhE90A* or *ShhH183A* results in a much greater (5 to 6 fold) induction (**Figure 12, E**), despite the inability of ShhH183A to undergo autoproteolysis (Goetz *et. al*., 2006). Nevertheless, these results indicate that mutations affecting receptor interaction or auto-proteolytic processing result in a greater ability of Shh to activate the Hh pathway cell-autonomously.

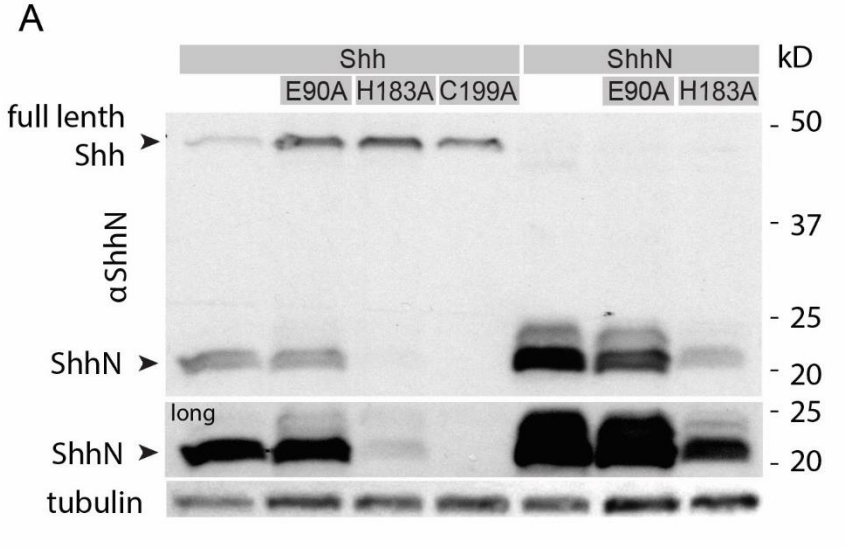

Figure 13 Shh mutants are expressed in Ptch$^{LacZ/LacZ}$;Ptch2$^{-/-}$ cells

Figure 13. Shh mutants are expressed in *Ptch$^{LacZ/LacZ}$;Ptch2$^{-/-}$* cells

(A) Western blot analysis of Shh, ShhE90A, ShhH183A, ShhC199A, ShhN, ShhNE90A, and ShhNH183A in Ptch1$^{LacZ/LacZ;}$Ptch2$^{-/-}$ cells. An antibody directed against the N-terminal domain of Shh was used to assess expression and autoproteolytic processing of each Shh mutant.

4.2.3 Shh mutants can activate the Hh pathway cell-autonomously independent of Shh receptor function *in vivo*

Both canonical and non-canonical co-receptors play central roles in regulation of the Hh response *in vivo*. We assessed to what extent activation of the Hh response pathway relies on the interaction of Shh with Ptch1/2 and the co-receptors in the developing neural tube. We co-electroporated stage 10-11 (Hamburger & Hamilton, 1951) chick neural tubes with *GFP* and constructs coding for *ShhN*, *ShhNE90A*, or *ShhNH183*. We found that all ShhN mutants activate the Hh pathway as assessed by changes in Shh-mediated dorsal-ventral patterning (**Figure 14, A-J**). Forced expression of ShhN, ShhNE90A, and ShhNH183A causes extensive dorsal expansion of the Nkx2.2 and Mnr2 domains (**Figure 14, A-F**), as well as repression of Pax7 (**Figure 14, G-J**). Electroporation of *Shh* or *ShhE90A* in developing chick neural tubes causes an expansion of Nkx2.2 and Mnr2 domains, as well as a repression of Pax7 (**Figure 14, N,O,Q,R,T,U**). Electroporation of *ShhH183A* does not result in extensive activation of the pathway (**Figure 14, P,S,V**).

We stained electroporated neural tubes with the anti-Shh monoclonal antibody 5E1 (Ericson *et. al.,* 1996). 5E1 staining pattern on the (left) side of neural tubes overexpressing ShhN or Shh coincide with GFP expressing cells (**Figure 14, K,W**). The 5E1 staining pattern on neural tubes expressing ShhNE90A or ShhE90A is more profuse than neural tubes expressing ShhN or Shh (**Figure 14, L,X**), and we hypothesize that the inability of Shh to bind the co-receptors provides greater access to the 5E1 epitope. The 5E1 staining pattern on neural tubes expressing ShhNH183A or ShhH183A reveals only endogenous Shh expression in the floor plate (**Figure 14, M,Y**). The lack of staining is caused by the disruption of the 5E1 epitope, which overlaps with Ptch1 binding in ShhNH183A and ShhH183A (Ericson *et. al.,* 1996). The lack of ectopic 5E1 staining in ShhNH183A expressing cells (**Figure 14, M**) demonstrates that the effect on neural tube patterning is solely due to the expression of ShhNH183A itself and not ectopic induction of Shh expression.

Our experimental observations indicate that ShhN can mediate separate non-cell autonomous and cell-autonomous activation events both *in vitro* and *in vivo*. Whereas Shh is a potent inducer of the Hh response *in trans*, *in vitro*, its ability to activate the Hh response cell-autonomously is relatively weak. Interestingly, the introduction of the E90A or H183A mutations in Shh enhances its ability to activate the pathway cell-autonomously.

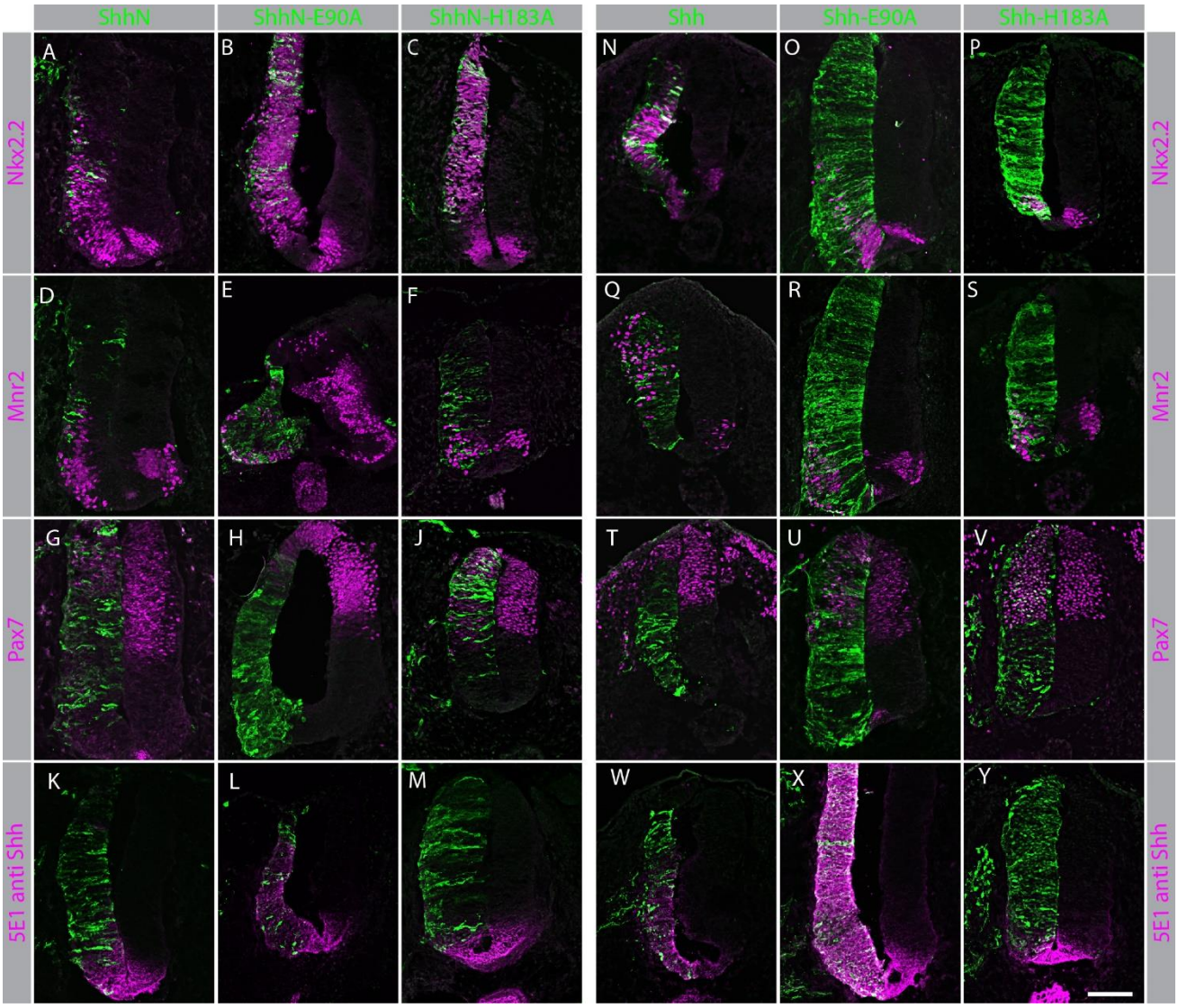

Figure 14 Shh mutants activate the Hh pathway in the neural tube of chicken embryos

Figure 14. Shh mutants activate the Hh pathway in the neural tube of chicken embryos

(A-Y) Cross-sections of stage 20 HH chicken neural tubes co-electroporated with *GFP* and *ShhN* (A,D,G,K), *ShhNE90A* (B,E,H,L), *ShhNH183A* (C,F,J,M), *Shh* (N,Q,T,W), *ShhE90A* (O,R,U,X), *ShhH183A* (P,S,V,Y) labeled in green. Sections are stained with antibodies to Nkx2.2 (A-C, N-P), Mnr2 (D-F,Q-S), Pax7 (G-J,T,V), and Shh (5E1) (K-M,W-Y) labeled in magenta. Scale bar is 100µm (A-Y) except E (200µm).

Chapter 5. Cell-autonomous pathway activation is impeded by the cholesterol modification of Shh but requires the CRD of Smo

5.1 Introduction

Shh becomes modified with a cholesterol on its C terminus during auto-proteolytic processing and with a palmitoleoyl moiety on its N terminus before secretion (Porter *et. al.,* 1996; Lee *et. al.,* 1994; Porter *et. al.,* 1995; Pepinsky *et. al.,* 1998). While both Shh and ShhN can activate the pathway non-cell autonomously or *in trans*, we find a significant difference in their ability to activate the pathway cell-autonomously. The only structural difference between Shh and ShhN is the cholesterol modification, which has been shown to localize Shh to specialized lipid rafts (Chen *et. al.,* 2004). This intriguing discrepancy between the two forms of Shh may reflect a cellular mechanism that prevents cell-autonomous activation of the pathway in endogenous Shh-expressing cells. We next aim to address the effects of Shh lipid modifications on its ability to activate the pathway cell-autonomously.

We have also shown that cell-autonomous activation of the pathway requires Smo. Smo belongs to Class F of the G-protein coupled receptor (GPCR) superfamily defined by Frizzleds (Frz), the canonical receptors of the Wnt signaling pathway (Bhanot *et. al.,* 1996; Kristiansen *et. al.,* 2004), with which Smo shares over 25% sequence identity. Smo and Frzs both contain a conserved Cysteine Rich Domain (CRD), which is required by Frz for Wnt binding and signal transduction (Pei *et. al.,* 2012; Janda *et. al.,*2012). Binding between the CRD of Smo and an endogenous ligand has never been shown; however the Smo CRD can bind to artificially supplied oxysterols, resulting in modulation of the Hh pathway (Myers *et. al.,* 2013; Nedelcu *et. al.,* 2013; Nachtergaele *et. al.,* 2013; Sever *et. al.,* 2016). Although it is unclear whether endogenous oxysterols are ligands for Smo, these results support the idea that the CRD of Smo regulates Hh pathway activity. Structural analysis of the oxysterol binding pocket within zebrafish SmoCRD reveals a binding interface homologous to Frz8 and the palmitoyl moiety of Wnt (Nachtergaele *et. al.,* 2013). These structural parallels suggest that Smo activity may be regulated through an endogenous ligand that can bind its CRD. Other small molecule agonists and antagonists can also modulate Smo activity by binding to its membrane-exposed heptahelical domain, long postulated to be its orthosteric site (Chen *et. al.,*2002; Wang *et. al.,* 2013). However our observations indicate that the heptahelical domain of Smo functions more like an allosteric site that modulates Smo activity rather than an orthosteric site. We aim to address whether the CRD of Smo is the orthosteric site through which it becomes activated by Shh cell-autonomously.

5.2 Results

5.2.1 Autoproteolytic cleavage of Shh impedes cell-autonomous activation of Smo

Intrigued by the weak cell-autonomous activation of the Hh response by Shh, we assessed the ability of forms of Shh with varying lipophilic and C-terminal modifications to signal cell-autonomously or *in trans*. We evaluated fully modified Shh, as well as Shh lacking either the cholesterol moiety, the palmitoleoyl moiety, or both, to test whether the presence of lipid

modifications (Porter *et. al.*, 1996; Pepinsky *et. al.*, 1998; Bumcrot *et. al.*, 1995; Gao *et. al.*, 2011) endow Shh with properties beyond membrane association (**Table 2**).

Mutant	Cholesterol	Palmitate	Trans Signaling	Cis Signaling
WT Shh	+	+	+	-
ShhC25S	+	-	-	-
ShhE90A	+	+	+	+
ShhH183A	-	+	-	+
ShhC199A	-	+	-	+
ShhN	-	+	+	+
ShhNC25S	-	-	-	+
ShhNE90A	-	+	-	+
ShhNH183A	-	+	-	+

Table 2 Status of lipid modifications and Trans/Cis signaling of Shh mutants

Table 2. Status of lipid modifications and Trans/Cis signaling of Shh mutants

The presence of the cholesterol and palmitoleoyl modifications of the Shh mutants assessed in this chapter are indicated. The ability of each mutant to mediate trans (non-cell autonomous) and cis (cell-autonomous) signaling are summarized.

We find that the ability of Shh to induce the Hh response *in trans* in reporter cells was unaffected by the absence or presence of its lipophilic modifications, with the exception of ShhNC25S (**Figure 15, B**). The absence of palmitoylation on ShhN results in a diminished ability to induce the Hh response in reporter cells, as observed before (Chen *et. al.*, 2004). Shh with either CD4 or GFP as its C terminus retains the ability to activate the Hh response in reporter cells (**Figure 15, B**) (Yang *et. al*, 1997; Chamberlain *et. al.*, 2008). Consistent with previous observations (Roelink *et. al.*, 1995), we find that a form of Shh unable to undergo auto-proteolytic cleavage (ShhC199A) cannot induce a strong Hh response *in trans*. However, this contradicts reports that full length unprocessed Shh retains juxtacrine signaling activity (Tokhunts *et. al.*, 2010).

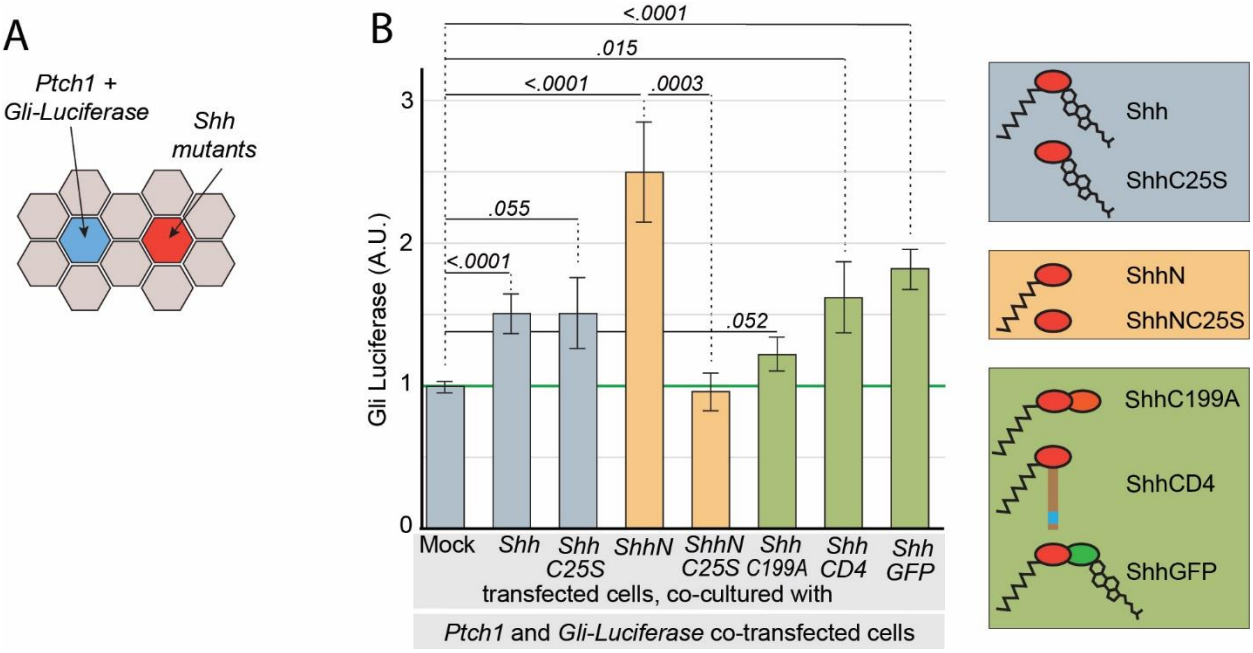

Figure 15 Non-cell autonomous signaling by Shh mutants with varying c-terminal modifications

Figure 15. Non-cell autonomous signaling by Shh mutants with varying c-terminal modifications

(A) Diagram of non-cell autonomous (trans) signaling: *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ reporter cells transfected with *Gli-luc* and *Ptch1* (blue hexagons) were grown in a 1:1 mixed co-culture of *GFP* (Mock) transfected or *Shh* (mutant) transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells (red hexagons). (B) Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$ reporter cells co-cultured with mock, *Shh*, *ShhC25S*, *ShhN*, *ShhNC25S*, *ShhC199A*, *ShhCD4*, or *ShhGFP* transfected Ptch1$^{LacZ/LacZ}$;Ptch2$^{-/-}$ cells. Luciferase levels in *Gli-luc* transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ reporter cells cultured with mock transfected cells. All error bars are s.e.m., p values (Student T-test, 2 tailed) are indicated where relevant, n>4.
Diagram of Shh (mutants) with varying lipophilic C-terminal modifications (right).

The observation that cells expressing Shh are not responsive to their own signal has been made in mammalian cells (García-Zaragoza *et. al.*, 2012), and is supported by our results indicating that Shh is not a strong cell-autonomous inducer of the Hh response. This is in stark contrast to our observation that ShhN is a potent cell-autonomous activator. To evaluate the extent to which the lipophilic modifications of Shh affect its ability to activate the Hh response cell-autonomously, we transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ reporter cells with various forms of lipid-modified Shh (**Figure 16, A**). We find that the palmitoylation of Shh has little effect on its ability to induce a cell-autonomous response, unlike its activity *in trans* (**Figure 16, B**). We find that the presence of cholesterol on Shh significantly decreased its ability to induce the Hh response cell-autonomously (**Figure 16, B**). This decrease in activity is not due to its membrane association, as Shh-CD4 and Shh-GFP fusion proteins are potent cell-autonomous activators of the pathway. Consistent with the observation that forms of Shh with extraneous C termini are able to induce the Hh response cell-autonomously, we found that ShhC199A retains this activating ability (**Figure 16, B**).

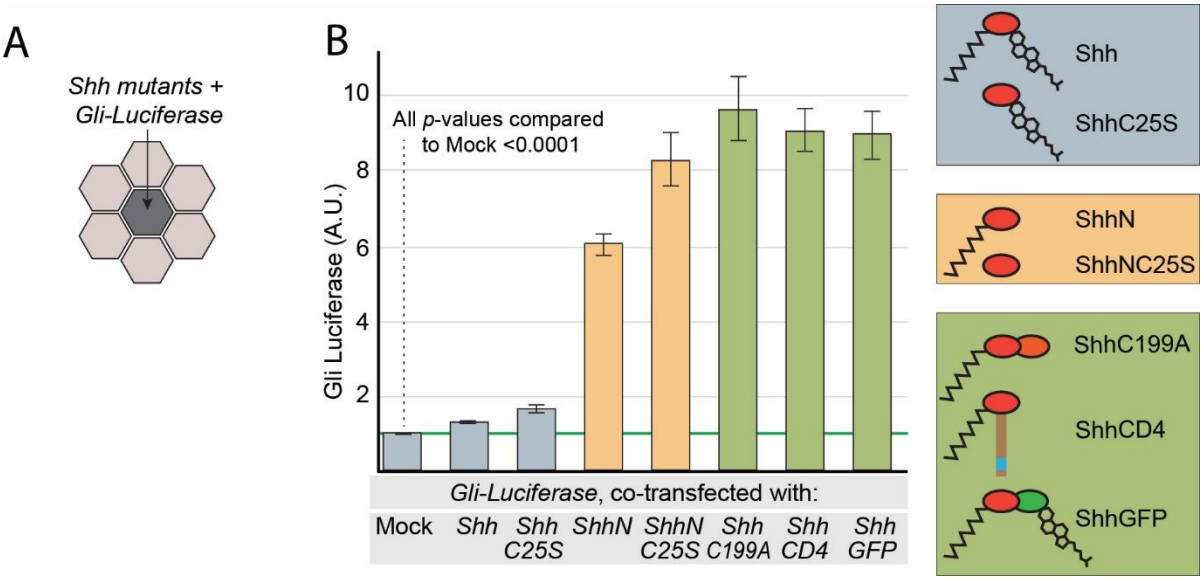

Figure 16 Cell- autonomous signaling by Shh mutants with varying c-terminal modifications

Figure 16. Cell- autonomous signaling by Shh mutants with varying c-terminal modifications

(A) Diagram of cell-autonomous signaling: *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ reporter cells transfected with *Gli-luc* were *GFP* (Mock) transfected or transfected with *Shh* (mutants).

(B) *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ reporter cells were mock transfected or transfected with *Shh*, *ShhC25S*, *ShhN*, *ShhNC25S*, *ShhC199A*, *ShhCD4*, or *ShhGFP*. Luciferase levels in mock transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells was set at 1. All error bars are s.e.m., *p* values (Student T-test, 2 tailed) are indicated where relevant, n>8.

Diagram of Shh (mutants) with varying lipophilic an C-terminal modifications (right).

It appears that an important function of the cholesterol moiety on Shh is to prevent Shh-expressing cells from dramatically upregulating the Hh response pathway cell-autonomously. To test whether the cholesterol modification on Shh alters its intracellular distribution, we stained transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells for Shh and ShhN. We find that Shh is stereotypically localized to the cell membrane, including filopodial extensions (**Figure 17, A**). ShhN is consistently absent from the cell membrane and filopodial structures, and instead occupies a perinuclear location (**Figure 17, B**). This suggests that the cholesterol modification regulates the localization of Shh in expressing cells, possibly preventing co-localization with Smo or interaction with other proteins.

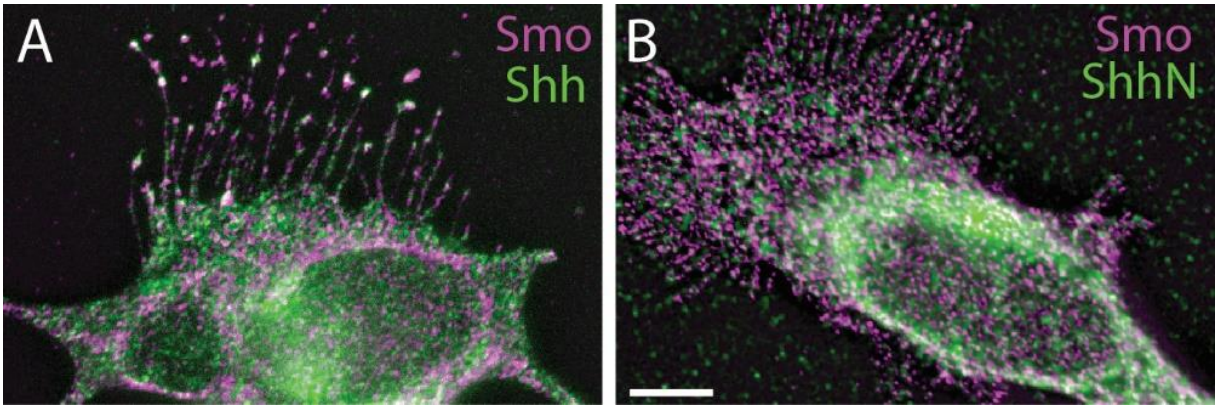

Figure 17 The cholesterol modification of Shh localizes it to the cell membrane and filopodia

Figure 17. The cholesterol modification of Shh localizes it to the cell membrane and filopodia

(A) *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells were transfected with *Shh* and *Smo-myc*. Cells were stained for Shh (5E1)(green) and myc (magenta).
(B) *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cell transfected with *ShhN* and *Smo-myc*. Cells were stained for ShhN (5E1)(green) and myc (magenta).
Scale bar is 20μm.

5.2.2 The CRD of Smo is required for cell autonomous activation by Shh mutants

Frz contains an extracellular CRD, an indispensable domain that binds to Wnt through two distinct binding sites, one of which is a protein-lipid interface (Janda *et. al.*, 2012). As a homolog of Frz, Smo contains a CRD which can bind to sterols (Myers *et. al.*, 2013; Nedelcu *et. al.*, 2013; Nachtergaele *et. al.*, 2013). Previously published results show that the CRD of Smo is not a target for Ptch1-mediated inhibition, however SmoΔCRD appears to be less sensitive to Shh signaling (Myers *et. al.*, 2013; Nachtergaele *et. al.*, 2013; Aanstad *et. al.*, 2009).

To further verify that the antiporter function of Ptch1 can regulate Smo independent of its CRD, we co-transfected either *Smo* or *SmoΔCRD* with mutant forms of *Ptch1* into *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ reporter cells. Transfection of either *Smo* or *SmoΔCRD* in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells raised the level of pathway activity (**Figure 18**). Co-transfection of *Smo* or *SmoΔCRD* with forms of Ptch1 that contain an intact antiporter domain, *Ptch1* or *Ptch1ΔL2*, lowered Hh pathway activity caused by *Smo* overexpression. Co-transfection of *Smo* or *SmoΔCRD* with forms of Ptch1 that contain mutations within the antiporter domain, *Ptch1D499A* and *Ptch1ΔL2-D499A*, failed to lower Hh pathway activity caused by *Smo* overexpression (**Figure 18**). This confirms that overexpressed Smo, even lacking its CRD, remains subject to the inhibitory effects of the proton antiporter activity of Ptch1 (Myers *et. al.*, 2013).

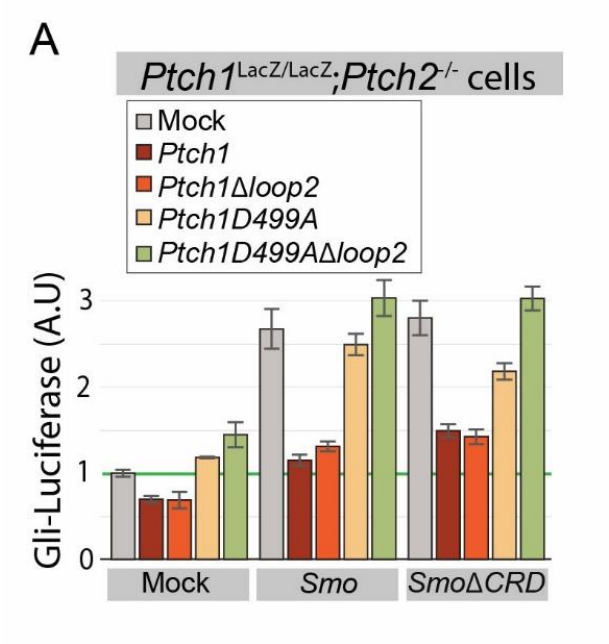

Figure 18 The CRD of Smo is not a target of Ptch1-mediated inhibition

Figure 18. The CRD of Smo is not a target of Ptch1-mediated inhibition

(A) *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells were co-transfected with *Gli-luc* and *GFP* (Mock), *Ptch1*, *Ptch1ΔL2*, *Ptch1D499A*, or *Ptch1ΔL2-D499A*; or *Gli-luc*, *Smo*, and *GFP*, *Ptch1*, *Ptch1ΔL2*, *Ptch1D499A*, or *Ptch1ΔL2-D499A*; or *Gli-luc*, *SmoΔCRD* and *GFP*, *Ptch1*, *Ptch1ΔL2*, *Ptch1D499A*, or *Ptch1ΔL2-D499A*. Luciferase levels in *Gli-luc* transfected *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells was arbitrarily set at 1. All error bars are s.e.m., p values (Student T-test, 2 tailed) are indicated were relevant, n>12.

We assessed the ability of the SmoΔCRD to activate the Hh response in the developing neural tube. Electroporation of *Smo* in stage 10-11 chick neural tubes did not have a significant effect on the Hh response (**Figure 19, A-C**). However, electroporation of *SmoΔCRD* resulted in a cell-autonomous activation of the Hh response pathway, indicating that the CRD regulates the level of Smo activity (**Figure 19, D-F**). The activity of SmoΔCRD is subject to Ptch1 regulation *in*

vivo as well, as co-electroporation of *Smo*Δ*CRD* with *Ptch1*Δ*L2*, reversed the phenotypic effects of *Smo*Δ*CRD* expression (**Figure 19, G-I**). SmoΔCRD has been shown to constitutively localize to the primary cilium, the cellular compartment where it mediates the transcriptional Hh response (Aanstad *et. al.*, 2009). Entry into the cilium is required for pathway activation as electroporating a form of *Smo*Δ*CRD* that cannot localize to the primary cilium(*Smo*Δ*CRD-CLD)* (Aanstad *et. al.*, 2009; Corbit *et. al.*, 2005), did not result in ectopic activation of the pathway *in vivo* (**Figure 19, J-L**).

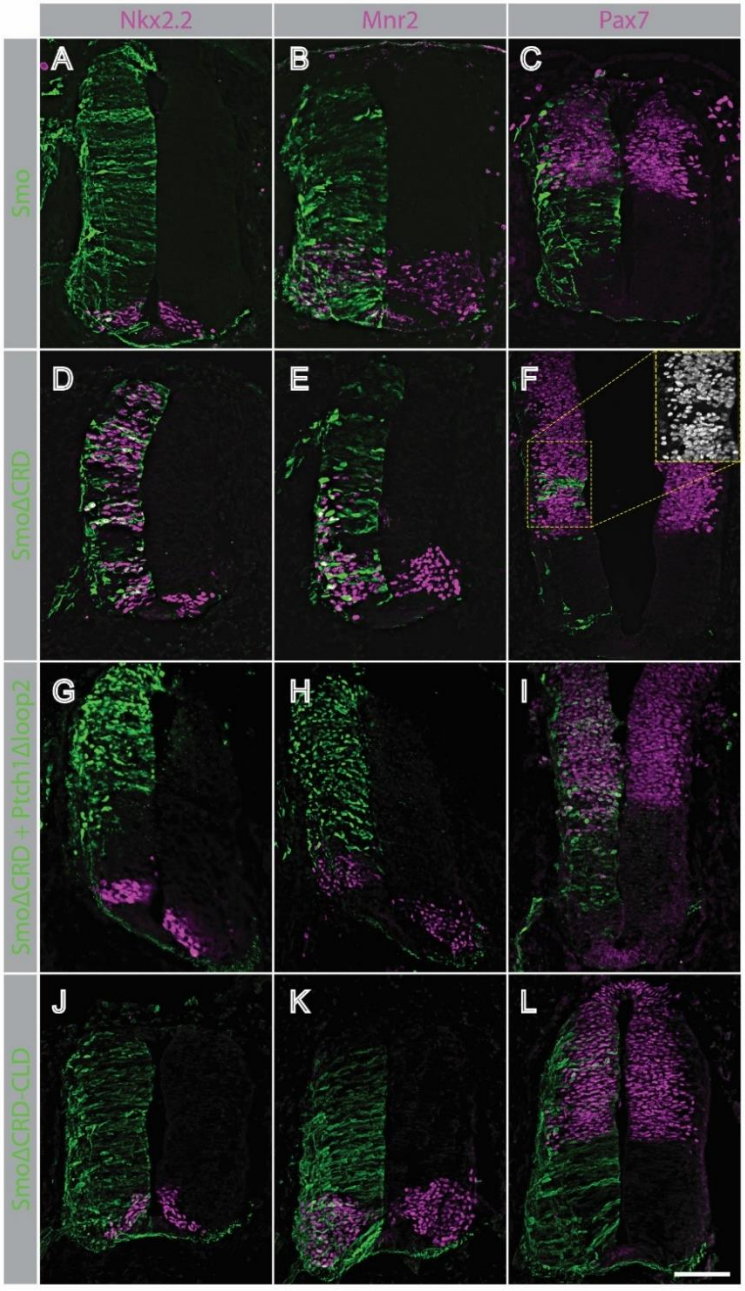

Figure 19. The activity mediated by SmoΔCRD *in vivo* requires localization to the primary cilium

(A-L) Cross-sections of stage 20 HH chicken neural tubes electroporated with *Smo* (A-C), *Smo*Δ*CRD* (D-F), *Smo*Δ*CRD* + *Ptch1*Δ*L2* (1:1) (G-I), or *Smo*Δ*CRD-CLD* (J-L) labeled in green. Sections are stained with antibodies to Nkx2.2 (A, D, G, J,), Mnr2 (B, E, H, K), and Pax7 (C, F, I L) labeled in magenta. Insert in I shows Pax7 staining. Scale bar is 100µm.

Figure 19 The activity mediated by SmoΔCRD in vivo requires localization to the primary cilium

The CRD of Smo is not a target of Ptch1 inhibitory activity, which raises the question of whether the CRD of Smo is required for the cell-autonomous activation of the Hh response we observe after transfection of Shh mutants. We used *Smo*$^{-/-}$ fibroblasts to measure the effect on Luciferase activity after transfecting mutant forms of *Shh* with and without *Smo*. Mutant forms of ShhN were not useful in this assay as they caused a Smo-independent induction of Gli-Luciferase activity that was resistant to vismodegib (data not shown). We were able to assess the role of Smo in cell-autonomous pathway activation using Shh, ShhE90A, and ShhH83A. Transfection of all forms of *Smo* and *Smo*Δ*CRD* cause a minor activation of the Hh response in *Smo*$^{-/-}$ cells (**Figure 20, A**). Co-transfection of *Smo* or *Smo*Δ*CRD* with *Shh* into *Smo*$^{-/-}$ cells had little effect, further demonstrating that Shh poorly induces the Hh response cell-autonomously. We have shown that ShhE90A and ShhH183A are potent cell-autonomous inducers of the Hh response in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells. Consistent with this observation, we find that co-transfection of *Smo* with *ShhE90A* or *ShhH183A* caused a strong, cell-autonomous activation of the Hh response (**Figure 20, A**). This activation can be blocked by vismodegib, demonstrating that this event is Smo-dependent (**Figure 20, B**). In contrast, co-transfection of *Smo*Δ*CRD* with either *ShhE90A* or *ShhH183A* did not result in cell-autonomous activation, demonstrating that the CRD is required to mediate cell-autonomous activation of the Hh pathway by ShhE90A or ShhH183A (**Figure 20, A**).

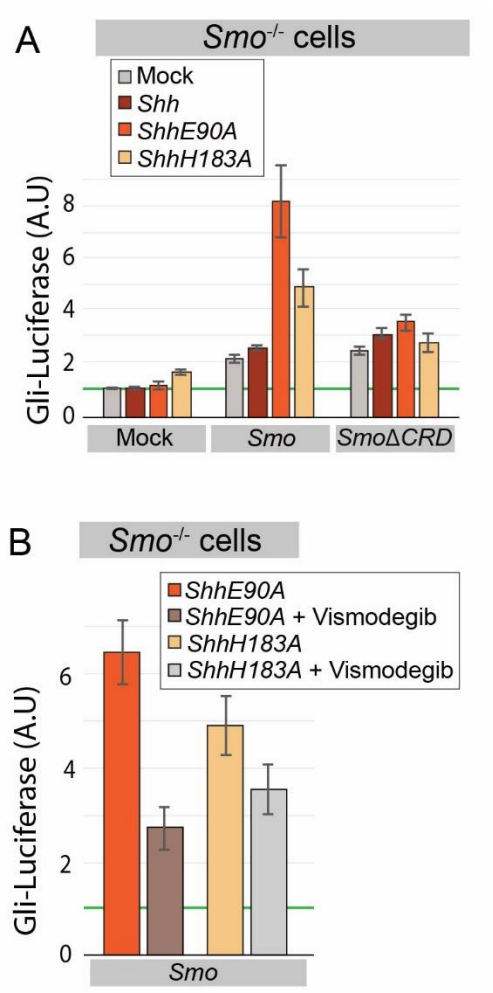

Figure 20 Cell-autonomous pathway activation requires the CRD of Smoothened

Figure 20. Cell-autonomous pathway activation requires the CRD of Smoothened

(A) *Smo*$^{-/-}$ cells co-transfected with *Gli-luc* and *GFP* (Mock), *Shh*, *ShhE90A*, or *ShhH183A*; or *Gli-luc*, *Smo* and *GFP*, *Shh*, *ShhE90A*, or *ShhH183A*; or *Gli-luc*, *Smo*Δ*CRD* and *GFP*, *Shh*, *ShhE90A*, or *ShhH183A*. Luciferase levels in mock transfected *Smo*$^{-/-}$ cells was arbitrarily set at 1. All error bars are s.e.m., n>12.

(B) *Smo*$^{-/-}$ cells were co-transfected with *Gli-luc*, *Smo* and *ShhE90A* or *ShhH183A* and cultured with vismodegib (100 nM) or vehicle control. Luciferase levels in mock transfected *Smo*$^{-/-}$ cells cultured in vehicle control was arbitrarily set at 1 (not shown). All error bars are s.e.m., n>7.

Chapter 6. Discussion and Future Directions

6.1 Discussion

Despite the evidence that Ptch1 is an inhibitor of Smo (Goodrich *et. al.,* 1997), we find that the genetic loss of Ptch1/2 does not necessarily result in constitutive Smo activation. This observation clashes with the current model of Hh pathway activation, which postulates that Ptch1/2-mediated inhibition is central in the regulation of Smo. It has long been accepted that the absence of Ptch1/2-mediated inhibition always results in the activation of Smo. *Ptch1*$^{LacZ/LacZ}$ mice have a dramatic upregulation of Hh pathway activity, resulting in widespread expression of Shh in the developing neural tube, leaving open the possibility that Hh pathway activation in *Ptch1*$^{LacZ/LacZ}$ mice is Shh-dependent. Our observations raise a central question of how Smo becomes activated in the absence of Ptch1/2-mediated inhibition.

Our results establish that Ptch1 plays two distinct and functionally separable roles in the regulation of Smo activity. We confirm that Ptch1 is a potent inhibitor of Smo via its proton antiporter activity. We also show that Ptch1, independent of this inhibitory function, plays a central role in the activation of the Hh response pathway. *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells fail to activate the Hh response when presented with extracellular ShhN unless transfected with *Ptch1*. This activity is cell-autonomous and appears to be involved in the recognition of Shh in the extracellular space, as deletion of the Shh binding domain of Ptch1 results in a molecule that cannot restore sensitivity to extracellularly supplied ShhN. Ptch1 internalizes Shh during signaling and then co-localizes with Smo in an intracellular compartment (Incardona *et. al.*, 2000; Incardona *et. al.,* 2002). These events support the idea that Ptch1 is required to transport Shh from the extracellular space and subsequent delivery to Smo, resulting in its activation. We propose a model in which Ptch1 sets the level of Smo sensitivity in a Shh-dependent manner by raising its activation threshold, while it performs an activating role by delivering extracellular Shh to Smo. This type of graded regulation may be required to properly pattern complex cell types within developing tissues, such as the neural tube.

We demonstrate that the transcriptional Hh response pathway can be cell-autonomously activated in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells when transfected with *ShhN*. This activation event occurs independent of the canonical Shh (co-)receptors, an obligatory component of non-cell autonomous signaling, but remains dependent on Smo. In Drosophila, the genetic loss of *Ptc* is epistatic to the loss of *Hh* (Bejsovec *et. al.,* 1993), suggesting that Ptch-independent regulation of Smo does not play a major role during Drosophila development. Alternatively, the molecules involved in Drosophila Smo activation may be common and may be found in cells devoid of *Ptc*. Furthermore, there is evidence that cells in the posterior compartment of the wing imaginal disc retain Hh responsiveness independent of Ptc function, suggesting an alternate mechanism of Smo activation in Drosophila (Ramirez-Weber *et. al.,* 2000). There are many lines of evidence that suggest the Hh response pathway has diverged significantly between Drosophila and vertebrates.

Our data also demonstrate that the CRD of Smo is required for cell-autonomous pathway activation. While SmoΔCRD is responsive to Ptch1-mediated inhibition, it is unable to work synergistically with ShhE90A or ShhH183A to activate the Hh pathway cell-autonomously. The idea that the CRD of Smo may be a target of Shh-mediated activation is supported by previously

reported decreased sensitivity of SmoΔCRD to Shh *in vitro* and *in vivo* (Myers *et. al.*,2013; Nachtergaele *et. al.*,2013; Aanstad *et. al.*,2009). These findings support the notion that the CRD is the true orthosteric site of Smo activation, while the heptahelical domain is an allosteric site for non-competitive Smo inhibition. Importantly, we observe Smo activation in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells after *ShhN* transfection, demonstrating that orthosteric activation does not necessarily involve a cargo of Ptch1/2.

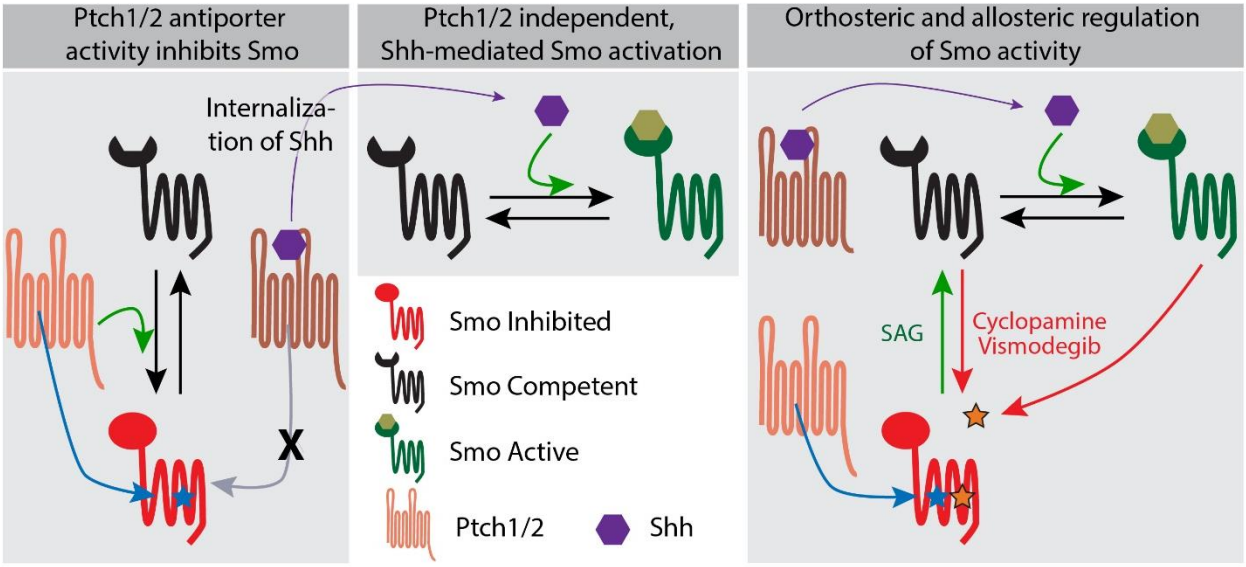

Figure 21 Model of orthosteric and allosteric regulation of Smo activity

A bewildering observation is that transfection of *ShhN* in *Smo*$^{-/-}$ cells results in an increase of Gli-mediated Luciferase levels. This increase in Luciferase levels is unaffected by the addition of vismodegib, which confirms that the activity measured is Smo-independent. The receptor(s) that mediate this activity are not known, however regulation of Gli transcription factors by the Wnt pathway has been observed *in vitro* and *in vivo* (Borycki *et. al.*, 2000; Mullor *et. al.*, 2001). While to identification of alternate Shh receptors is not the focus of this dissertation, this anomaly should be addressed in the future.

Transfection of fully lipid modified Shh results in a significantly reduced cell-autonomous activation of the Hh response in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells as compared to forms of Shh lacking the cholesterol moiety. Because Shh requires Dispatched1 (Disp1) to be secreted from a cell, while ShhN does not (Ma *et. al.*,2002), we hypothesize that the cholesterol modification of Shh allows its secretion by Disp1 while preventing Shh interacting with Smo in the same cell. We find that transfection of Shh with membrane anchors, *ShhCD4* and *ShhGFP*, had an effect similar to *ShhN* transfection, indicating that simple membrane association is not sufficient for regulation. Because fusion proteins such as ShhCD4 and ShhGFP behave differently than Shh, previous data on Shh fusion proteins in the literature should be reassessed.

Expression of ShhC199A and ShhH183A results in forms of Shh that cannot be processed and consequently lack the cholesterol modification, providing an explanation as to why these form of Shh can activate the Hh response cell-autonomously. Previous observations demonstrate a role for full length unprocessed Hh ligands in juxtacrine signaling (Tokhunts *et. al.*, 2010; Pettigrew *et. al.*,2014), however our *in vitro* and *in vivo* data show that the unprocessed Shh ligands ShhC199A and ShhH183A are not strong inducers of the Hh response *in trans*. The observation that the E90A and H183A mutations confer Shh with the ability to activate the pathway cell-autonomously suggests that, along with the cholesterol modification, interaction with Shh-binding proteins may play a role in preventing pathway activation in Shh expressing cells. Our finding that various Shh mutations can lead to unregulated cell-autonomous pathway activation, along with the observation that cells lacking Ptch1/2 activity do not necessarily have an active Hh response, have large implications for Hh-mediated diseases.

Together with results recently obtained from our lab (Roberts *et al.*, 2016, manuscript submitted), a model emerges in which in the absence of Shh, Ptch1/2 inhibits Smo through an allosteric site. When Shh is present, it can bind to a receptor complex involving the ligand-binding domain (L2) of Ptch1/2. We hypothesize that this event releases the allosteric inhibition and initiates a distinct activation event. The receptor complex can internalize the Shh ligand, resulting in its co-localization with Smo where it mediates orthosteric activation of (dis-inhibited) Smo through the CRD. This mediation may not occur through direct binding of Shh to Smo. Although Smo was initially considered the putative receptor for Hh when it was discovered in Drosophila, there has been no evidence supporting the binding of Hh and Smo. One possibility is that Shh and Smo interact within a multi-protein complex where they do not bind directly. While many questions about cell-autonomous Hh activation by Shh are left to be explored, a central question is how Shh and Smo interact. A multidisciplinary approach of biochemistry, genetics, and bioinformatics may be required to solve this enduring puzzle.

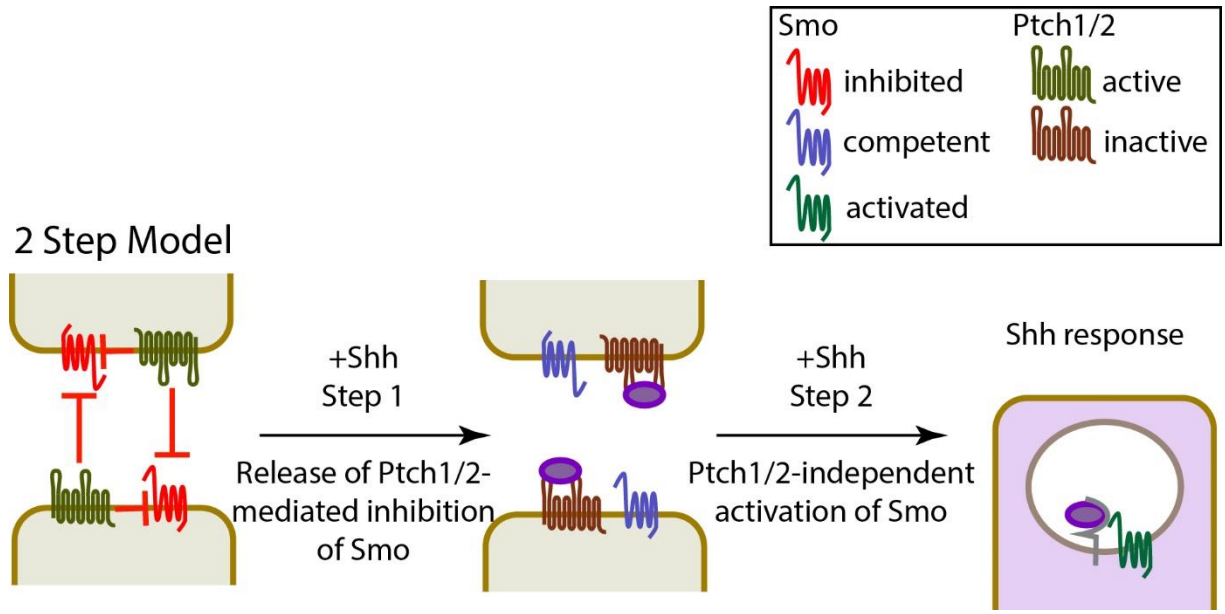

Figure 22 Model of Shh-mediated Smo activation

6.2 Future Directions

This dissertation addresses the Smo-dependent canonical responses mediated by Shh in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells. It is of great interest to learn whether *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells still retain Smo-dependent non-canonical responses to Shh. Of particular interest is whether cells devoid of Ptch1/2 function can still migrate, or sort, towards a source of Shh. It has been shown that *Ptch1*$^{-/-}$ cells can migrate towards Shh sources in modified boyden-chambers (Bijlsma *et. al.*, 2012), however it is unknown whether Ptch2 mediates this response in the absence of Ptch1. Using the *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ fibroblasts to study the migrational response would clarify which receptors are involved in non-canonical responses. Moreover, it has been shown that cells expressing similar neuronal markers can sort themselves out in NEBs similar to cells in the neural tube *in vivo*. Thus studying the ability of *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ mESC to sort in a well-established system like NEBs would be highly informative.

If *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$ cells retain their ability to sort or migrate towards a source of Shh, then the roles of the co-receptors should be analyzed in this context. While we have evidence that the co-receptors are not involved in direct cell-autonomous activation of Smo during the canonical response, it is possible that they may play a role in activating Smo during the non-canonical response. Evidence supporting this hypothesis includes the observation that Boc functions as a receptor for Shh during commissural neuron axon guidance, a process that requires Smo (Okada *et. al.*, 2006). The next logical step in this project would be to analyze whether *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$; *Boc*$^{-/-}$;*Cdo*$^{-/-}$;*Gas1*$^{-/-}$ mESCs retain their ability to sort in an NEB system. Additionally, it would be highly beneficial to establish a *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$; *Boc*$^{-/-}$;*Cdo*$^{-/-}$;*Gas1*$^{-/-}$ fibroblast line and analyze their ability to migrate towards a Shh source using systems like Boyden-chambers or Dunn chambers. Furthermore, it would be useful to use this fibroblast cell line to confirm that these cells activate the pathway cell-autonomously in response to *ShhN* transfection.

One distinct possibility is that the canonical Shh receptors as well as the co-receptors are dispensable for the non-canonical responses. If this is the case, then Smo may act as a true GPCR during migration or cell-sorting, and in that context may be able to interact with extracellular Shh. If this is the case, perhaps Smo requires its own co-receptor on the cell surface. Smo is homologous to Frz, and Frz requires the co-receptors LRP5/6 for Wnt binding. Megalin, or LRP2, has been shown to bind to Shh in the extracellular space, and there is a possibility that is may act as a co-receptor for Smo. Additionally, the requirement of the CRD of Smo for non-canonical responses should be evaluated. A *Ptch1*$^{-/-}$;*Ptch2*$^{-/-}$;*Smo*$^{-/-}$ fibroblast cell line should be established, and the ability of SmoΔCRD to mediate migration should be assessed. Additionally, it would be useful to confirm that SmoΔCRD in *Ptch1*$^{-/-}$;*Ptch2*$^{-/-}$;*Smo*$^{-/-}$ fibroblasts is unable to mediate the cell-autonomous transcriptional response after Shh transfection.

Another area of focus should be to further evaluate whether the co-receptors are involved in clearing Shh from the extracellular space. A role for the co-receptors in the sequestration of Shh has not been extensively studied, however we provide evidence that neural tubes ectopically expressing forms of Shh which cannot bind to the co-receptors (ShhE90A/ShhNE90A) stain profusely with the Shh antibody 5E1. This may indicate that forms of Shh that cannot be bound by the co-receptors remain in the extracellular space and are accessible to the antibody. This

would elucidate dual roles for the co-receptors in the Hh pathway: a cell-autonomous positive role and a non-cell autonomous negative role. The effects of Shh ligand sequestration in *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$; *Boc*$^{-/-}$;*Cdo*$^{-/-}$;*Gas1*$^{-/-}$ mESCs should be assessed and compared to wt mESCs, and *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$ mESCs. It would be particularly interesting to assess the effects of removing Hhip from the genome of *Ptch1*$^{LacZ/LacZ}$;*Ptch2*$^{-/-}$;*Shh*$^{-/-}$; *Boc*$^{-/-}$;*Cdo*$^{-/-}$;*Gas1*$^{-/-}$ mESCs. Hhip has been shown to be a very powerful non-cell autonomous inhibitor of the Hh pathway through Shh ligand sequestration, and the combinatorial effects of Hhip and the co-receptors should be evaluated.

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