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Secondary receptors of the Shh pathway

By

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Abstract

Secondary receptors of the Shh pathway

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Astrid Carolina Alfaro

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The Sonic Hedgehog signaling pathway is central in vertebrate development and in several human disease states. Our current understanding of the pathway outlines a straight-forward mechanism whereby the Shh ligand is perceived by the nine-pass transmembrane receptor Ptch1. In its active state Ptch1 represses Smo, a GPCR like protein, which acts as the ultimate on and off switch of the Shh pathway. When Shh binds Ptch1, Ptch1 becomes inactivated and Smo becomes activated, subsequently leading to the downstream activation of the Shh response.

Despite this framework our understanding of how Ptch1-Shh binding leads to Smo activation remains rudimentary. To understand this crucial step of the pathway one must consider that Ptch1 exists within the Shh receptosome. The Shh receptosome, consists of several membrane bound and transmembrane proteins, which bind Shh. Delineating the function of these proteins is important for understanding how receptosome-Shh interactions lead to Shh pathway activation. Boc, Cdo and Gas1 are secondary receptors of Shh that are known agonists of the pathway. Furthermore, all three proteins are suspected to interact with Ptch1 and there collective presence is necessary for activation of the Shh pathway. Ptch2, a paralogue of Ptch1, is dispensable for development, but has been documented to act as a repressor of the Shh pathway.

We sought to further characterize the function of Ptch2, Boc, Cdo, and Gas1 in the context of Ptch1. Through in vitro experiments we have determined that Shh binding to Ptch1; or Boc, Cdo and Gas1 alone is insufficient to potentiate positive Shh signaling, however, Shh binding to Ptch1 alone is sufficient to cause Smo localization to the primary cilia, an event associated with active Shh signaling. Additionally, we have found that Ptch2 functions as a repressor of the Shh pathway in the absence of Ptch1, further suggesting that Ptch2 and Ptch1 share overlapping functions. Moreover, the distinct possibility remains that like other proteins of the Resistance Nodulation Family, Ptch1 and Ptch2 may interact. Thus, the simple model of Ptch1 mediated Shh reception needs to be revised to include the collective activity of Ptch2, Boc, Cdo and Gas1.

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List of Acronyms

AcrB – Acriflavine resistance B protein
ADAMs – A disintigren and metalloproteases
BCC – Basal Cell Carcinoma
BMP – Bone morphogenetic protein
Boc – Brother of Cdo
cAMP – Cyclic adenosine monophosphate
Cdo – Cell adhesion molecule-related/down-regulated by oncogenes
CNS – Central Nervous system
CTD – Carboxy Terminal Domain
Dhh – Desert Hedgehog
Disp1 – Dispatched 1
DMEM – Dulbecco's Modified Eagle's medium
DNA – Deoxyribonucleic acid
Dpp – Decapentaplegic
DRAL – Down-regulated in rhabdomyosarcoma LIM domain protein
EB – Embryoid Bodies
ECD – Extracellular Cysteine-rich Domain
EDTA – Ethylenediaminetetraacetic acid
ELISA – The enzyme-linked immunosorbent assay
En – Engrailed
ER – Endoplasmic reticulum
ERK – Extracellular signal-regulated kinases
FBS – Fetal Bovine Serum
FCS – Fetal Calf Serum
FGF – Fibroblast Growth Factor
Gas1 – Growth Arrest Specific protein 1
GFP – Green Fluorescent Protein
GPCR – G-Protein Coupled Receptor
GPI – Glycosylphosphatidylinositol
HH – Hamburger-Hamilton
Hh – Hedgehog
Hhip – Hedgehog interacting protein
HPE – Holoprosencephaly
HRP – Horse Radish Peroxide
HS – Heparan sulfate
HSPGs – Heparan sulfate proteoglycans
IFE – Interfollicular Epidermis
IFT – Intraflagellar Transport
Ihh – Indian Hedgehog
Ihog –Interference Hedgehog
IP3 – Inositol triphosphate
Iro – Iriqouix
LDA – Ligand Dependent Antagonism

LDS – Lithium Dodecyl Sulfate
LIA – Ligand Independent Antagonism
LIF – Leukemia Inhibitory Factor
MB – Medulloblastoma
MCS – Multiclonal site
MEFs – Mouse Embryonic Fibroblasts
mESCs – Mouse Embryonic Stem Cells
MT – Metallothionein
NBCC – Nevoid Basal Cell Carcinoma
NEB – Neuralized Embryoid Body
NEDD – neural precursor cell expressed developmentally down-
regulated protein 4
NPC1 – Niemann Pick Complex 1
P14P – phosphatidylinositol 4'-monophosphate
PCR – Polymerase Chain Reaction
PFA – Paraformaldehyde
PKA – Protein Kinase A
PLC – Protein Lipase Complex C
PPD – Preaxial polydactyly
PPRL – The Poisonous Plant Research Laboratory
Ptch1 – Patched 1
Ptch1∆L2– Patched 1- Shh binding loop 2 mutant
Ptch1∆L2D499A – Patched 1- Shh binding loop 2 and proton antiporter mutant
Ptch2 – Patched 2
Ptch2∆L2– Patched 2- Shh binding loop 2 mutant
Ptch2∆L2D496A – Patched 2- Shh binding loop 2 and proton antiporter mutant
qPCR – quantitative Polymerase Chain Reaction
RNA – Riboxynucleic acid
RND – Resistance Nodulation Division
RVD – Repetitive Variable Di-residue
SAG – Smoothened Agonist
Shh – Sonic Hedgehog
ShhN – Sonic Hedgehog N- terminal domain
siRNA – small interfering Riboxynucleic acid
Smo – Smoothened
SSD – Sterol Sensing Domain
SUFU – Suppressor of Fused
TALEN – Transcription Activator-Like Effector Nuclease
TGFb – Transforming Growth Factor Beta
UV – Ultraviolet
Wg – Wingless
WT – Wild type
ZPA – Zone of Polarizing Activity

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1 Introduction

Sonic Hedgehog: A signaling molecule

1.1.1 Morphogens in Development

How complex multicellular life arises from single cellular beginnings is a core question of biology. The environments to which cells in a developing embryo are exposed to are central in determining cell fate. The molecular nature and function of these extracellular signals has always been of great interest. Morphogens are a special type of extracellular signaling molecule; cells acquire distinct phenotypes in response to different concentrations of a morphogen. Morphogens instruct cells to acquire a specific fate, either directly or at a distance in a concentration dependent fashion. Usually, graded distribution of a morphogen is established away from a local source, inducing stereotypic cell differentiation. The graded activity of morphogens dictates many of the complex structures that arise during development (Rogers and Schier 2011). Well known morphogens include protein families such as the Bone Growth and promoting factors (BMPs), Wingless (Wg), Fibroblast growth factors (FGFs), Transforming Growth Fibroblast Beta (TGFβ) and Hedgehog (Hh).

1.1.2 Hedgehog proteins

The *Hedgehog* gene was originally discovered in the prominent fly screen of Nullsein-Volhard, which aimed to identify genes involved in fly embryonic patterning (Nüsslein-Volhard and Wieschaus 1980). The hedgehog (*Hh*) gene's namesake comes from the peculiar bristle pattern present in flies lacking *Hh*. Consistent with its role in determining segmental pattern, characterization of *Hh* in the fly revealed Hh was a secreted protein expressed in segmental stripes. Within amniotes there are three hedgehog family genes: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh).
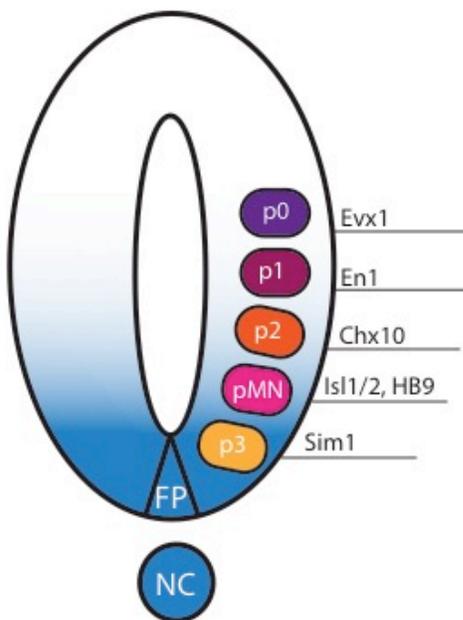
Figure 1. Shh patterns the mammalian neural tube. The Shh gradient emanates from the floor plate, the most ventral aspect of the neural tube, and the notochord, the structure underlying the neural tube. Distinct neuronal progenitors and neurons differentiate based on the amount of Shh they receive. Depicted above are the five neural progenitor domains, from p3-p0 and the terminally differentiated neuronal factors they express Sim-Evx1.

Hedgehog proteins are crucial for developmental patterning both in vertebrates and invertebrates. In Drosophila, the foremost-characterized Hh dependent tissue is the

wing imaginal disc. Within this structure Hh is secreted from the posterior, where it is induced by Engrailed (En), and signals in the anterior compartment at the anterior-posterior compartment boundary. High Hh signaling at the compartment boundary leads to the expression of the protein decapentaplegic (dpp). The dpp domain presents as a stripe, which physically divides the anterior from the posterior compartment (Biehs, Sturtevant, and Bier 1998). This early patterning ultimately turns the wing imaginal disc into a fly wing appendage.

In vertebrates, hedgehog proteins govern the developmental aspects of distinct organs and tissues. Shh is involved in varying signaling centers including the zone of polarizing activity (ZPA) of the early limb buds and the ventral midline of the neural tube. Ihh is thought to play a critical role in vertebrate skeletal morphogenesis and Dhh is involved in gonadal development (Vortkamp et al. 1996; Bitgood, Shen, and McMahon 1996). Of the three Hh molecules, Shh is the most extensively studied due to its involvement in central nervous system (CNS), limb, and gut development, as well as left/right asymmetry (Martí and Bovolenta 2002; Dillon, Gadgil, and Othmer 2003; Fukuda and Yasugi 2002; Babu and Roy 2013). Moreover, Shh is of great interest to humans due to its role in varying cancer disease states, including basal cell carcinoma, and deadly cancers like pancreatic adenocarcinoma, and medulloblastoma (Epstein 2008; Thayer et al. 2003; Y. Lee et al. 2003)

The neural plate, the precursor to the adult CNS, is a tissue derived from ectoderm in response to signals released by the node. The neural plate folds to form the neural tube, which overlies the notochord, an axial mesodermal structure that is a derivative of the node. Patterning along the dorso-ventral axis of the neural tube is mediated by Shh, which is released from the notochord. This initial Shh gradient leads to the upregulation of Shh at the floor plate, the most ventral aspect of the neural tube. Shh released from the notochord and floor plate establish an activity gradient, with the highest concentrations of Shh localized to the most ventral portions of the neural tube, and the lowest close to the dorsal aspect of the developing neural tube, the roof plate. The domain closest to the floor plate receives the highest Shh concentrations, the high level of Shh pathway activation in this domain induces the expression of the ventral transcription factor Nkx2.2 (Jessell 2000; L. Wilson and Maden 2005). In the mid-ventral portions of the neural tube where less Shh is present motor neuronal markers like HBG9 and Isl1/2 are expressed. Even lower concentrations of Shh result in the expression of En1 and Chx10, which define specific classes of interneurons (Figure 1). The absence of Shh pathway activity allows Pax7 expression, which defines the dorsal half of the developing spinal chord (Jessell 2000; L. Wilson and Maden 2005). Shh governed neural patterning is key for the stereotypic patterning of the ventral neural tube.

The Sonic hedgehog-signaling cascade

The central mechanism by which cells respond to Shh is relatively straightforward. In its unliganded state Ptch1 keeps the protein Smoothened (Smo) repressed. Smo is a 7 pass transmembrane protein that is a putative GPCR. Binding of Shh to Ptch1 causes Ptch1 to release Smo from its repressive state; Smo then activates a series of down

stream components that lead to the activation of the Gli proteins. The Glis, thus called because of their initial discovery in Gliomas, are the transcription factor outputs of the pathway there are three Glis 1,2, and 3. These transcription factors ultimately instruct cells to grow, divide or differentiate as dictated by their exposure to Shh ligand (Fuccillo, Joyner, and Fishell 2006; Varjosalo and Taipale 2008).

Ptch1 and Smo are central players in the modulation of the response pathway. Smo acts downstream of Ptch1 and its importance is clear in *Smo-/-* mice, which resemble *Shh -/-* mice, characterized by the absence of an Shh response, evident by severe midline defects. The mechanism by which Ptch1 modulates Smo activity remains unresolved. Ptch1 inhibits Smo in a catalytic manner (Taipale et al. 2002). Ptch1 has been proposed to repress Smo via the trafficking of an inhibitor of Smo. The nature of this inhibitor is discussed in detail, further on.

While the presence of active Ptch1 promotes Smo inactivity it is unclear whether the absence of Ptch1 results in fully activated Smo. The activation of Smo appears to be a
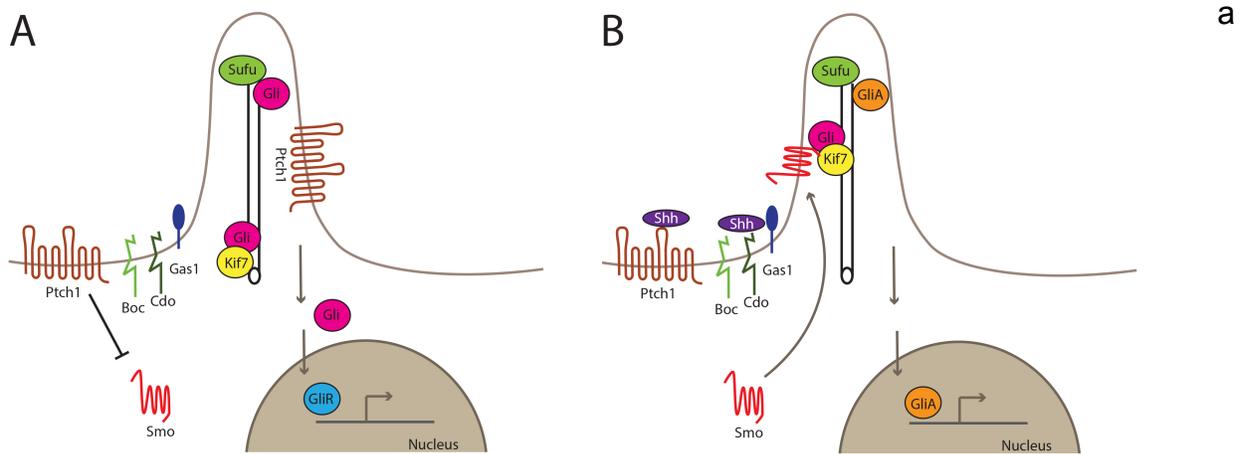
Figure 2. Overview of the Shh transcriptional response. The Shh pathway in its inactive state. Ptch1 holds Smo inactive and outside of the primary cilia. Meanwhile, the SuFu protein within the primary cilia helps direct Gli repressor forms out of the cilia and towards the nucleus, keeping the pathway in an inactive state (A)**.** The Shh pathway in its activated state. Upon binding to Shh Ptch1 moves out of the primary cilia, simultaneously it releases its inhibition of Smo. Smo is then recruited into the primary cilia, SuFu mediates activation of the Gli activator form, which is free to travel to the nucleus and function as a transcription factor (B).

multistep process: translocation of Smo from intracellular vesicles to the cell surface followed by phosphorylation of its C-terminal tail. In drosophila Smo exists as a homodimer through association of its N-terminal regions. Hh binding to Ptch leads to the subsequent phosphorylation of Smo at the C-terminal, the negative phosphates neutralize the positive charge of the basic residues in this cluster allowing the C-terminal tails to dimerize (Fan, Liu, and Jia 2012; Zhao, Tong, and Jiang 2007). Differential phosphorylation of the Smo C-terminal tail leads to graded activation of Smo (Fan, Liu, and Jia 2012). Phosphorylation of the Smo C-tail also reduces its ubiquitination preventing Smo from being targeted for proteasomal and lysosomal degradation (S. Li et al. 2012; Xia et al. 2012). In vertebrates, G-protein coupled

receptor kinase 2 (Grk2) phosphorylates the C-terminal of Smo causing it to associate with β-arrestin and subsequently to Kif3a, this phosphorylation event has also been linked with Smo ciliary accumulation and Shh pathway activation (Meloni et al. 2006). Although Smo, resembles a classical 7TMR GPCR, there is little data suggesting its association to small G-proteins, it is possible that Smo instead signals through a less well understood pathway that employs GRKs and β-arrestin.

Both Smo and Ptch1 traffic in and out of the primary cilium, a major signaling center of the cell (Figure 2). When Ptch1 is in its unliganded state it is localized to the primary cilium. Shh binding to Ptch1 causes its removal from the cilium, subsequently allowing Smo to localize to the primary cilium (Rohatgi, Milenkovic, and Scott 2007). Both surface localized Smo as well as Smo localized to endocytic vesicles can move into the primary cilium(Milenkovic, Scott, and Rohatgi 2009). Cilial localized Smo is thought to allow interactions with downstream Shh pathway components within the organelle and is generally associated with an activated Shh pathway.

The formation and maintenance of the primary cilia is key to a fully active Shh pathway. Screens in mice deficient for the molecular motors that help build and maintain primary cilia reveal phenotypes indicative of perturbed Shh activation. The retrograde and anterograde intraflagellar transport (IFT) complexes, IFT-B and IFT-A are necessary for assembly of cilia and ciliary trafficking. Loss of components of the IFT-B complex results in a loss in cilial structural integrity and a diminished Shh response. (Liem et al. 2012; Hao and Scholey 2009; Taschner, Bhogaraju, and Lorentzen 2012; Danwei Huangfu et al. 2003; Keady et al. 2012).

Kif3a is a microtubular kinesin, which plays a role in the anterograde transport of Smo. *Kif3a -/-* mice display a slight reduction in ventral neural cell types in the neural tube, and in vitro *Kif3A -/-* cells are unable to translocate Smo to the primary cilia displaying a dampened Shh response (Corbit et al. 2005; Danwei Huangfu et al. 2003). Kif3A is thought to directly bind Smo, as it has been successfully coimmunoprecipitated in a complex along with β-arrestin 1 and 2, which are all known to colocalize at the primary cilia (Kovacs et al. 2008). The small GTPase Arl13b is involved in retrograde transport of Smo its loss results in constitutive localization of Smo to the primary cilia and upregulation of the Shh response (Larkins et al. 2011). Clearly localization of Smo to the primary cilium is an important step in the Shh signaling cascade that follows a complicated itinerary involving several components of the intraflagellar trafficking machinery. The mechanism of Smo's ciliary localization remains unresolved, particularly because the loss of retrograde and anterograde cilial motors affect ciliar integrity, and the loss of cilia can cause defects outside the Shh signaling cascade proper.

Interestingly, Smo localization to the primary cilia does not necessarily guarantee pathway activation. Treatment of NIH 3T3 cells with the Smo antagonist cyclopamine causes a conformational change in Smo that allows Smo primary cilia localization but does not result in pathway activation (Rohatgi et al. 2009). Several subsequent studies have showed that Smo localization to the primary cilium is not sufficient for pathway activation (C. W. Wilson, Chen, and Chuang 2009). The loss of ciliary retrograde motor components such as Dync2h1 causes the constitutive localization of Smo to the primary cilium; however, this localization results in a loss of Shh signaling rather than an upregulation of signaling (Firestone et al. 2012; Ocbina and Anderson 2008). It is

plausible that Smo localization to the primary cilia and Smo activation are two distinct steps in Shh pathway activation.

Smo (a putative GPCR) has been shown to interact with G-proteins of the G_i family, inducing activation of the Glis (Riobo et al. 2006; Shen et al. 2013). There is also evidence that the G_{12} family of Gα proteins coupled with Rho GTPase can mediate Shh signaling (Kasai et al. 2004). Active PKA near the primary cilia mediates the processing of full length Gli3 into its repressor form Gli3R, which represses the pathway. A straightforward mechanism by which Smo -G protein coupling could work is by down-regulating cAMP leading to down regulation of PKA activity, and subsequent upregulation of the response. However, disruption of G_{ai} protein activity with pertussis toxin has only a partial inhibitory effect on mouse cells in vitro and in chick neural tubes in vivo (Low et al. 2008; Riobo et al. 2006). All of these factors possibly play into mediation of the Shh transcriptional response.

Loss of SuFu and protein kinase A (PKA), result in activation of the Hh response pathway, indicating that these molecules negatively affect Smo activity. SuFu is an enigmatic protein conserved in both vertebrates and invertebrates that negatively regulates the Hh pathway. It is cytoplasmic and contains few identifiable protein sequence motifs. Mice lacking SuFu are embryonic lethal, presenting severe developmental defects of the neural tube, which are indicative of its repressive role in Shh signaling (Svärd et al. 2006). In contrast, PKA is a well-known and evolutionary conserved repressor of the Shh pathway. In Drosophila Smo is a phosphorylation target of PKA (Jia et al. 2004); however this mechanism is not conserved in vertebrates, instead PKA is known to be involved in processing both Gli2 and Gli3 into their repressor forms, leading to Shh pathway repression(Pan et al. 2006; Pan, Wang, and Wang 2009; Huang, Roelink, and McKnight 2002; Concordet et al. 1996; Tiecke et al. 2007).

The Glis, are a family of zinc finger transcription factors, and are the transcriptional mediators of the Shh pathway. These proteins contain an N-terminal domain that functions as a transcriptional repressor and a C-terminal domain that functions as a transcriptional activator (Aza-Blanc and Kornberg 1999). The activities of the Glis are controlled by regulation of their expression, protein stability and modulation of specific activities via posttranslational modifications. There are three Gli proteins in the Shh signaling cascade. Gli1, the absolute activator of the pathway, Gli2, which is mostly considered a pathway activator and Gli3, which is largely a repressor. Upregulation of the Shh pathway leads to the upregulation of Gli1 (Dai et al. 1999). Inactivity of the Shh pathway causes partial proteolyzation of Gli2 and Gli3, resulting in cleavage, and leaving only the N-terminal Gli2 and Gli3 domains to act as repressors of the pathway. Activation of the pathway prevents accumulation of the repressor forms of Gli2 and Gli3 and instead full length Gli2 and Gli3 accumulate and can appropriately act as pathway activators (Sasaki et al. 1999; Wang, Fallon, and Beachy 2000; Mo et al. 1997).

Not only do Ptch1 and Smo traffic through the cilia, several other components of the pathway travel through this organelle as well including Gli2, Gli3 and SuFu. In vertebrates SuFu is known to associate with and control the stability of full length Gli2 and Gli3 (Cheng and Yue 2008; Humke et al. 2010). In a cell's basal Shh inactivated state, SuFu travels up the primary cilia along with full length Gli2 and Gli3. Within the Sufu-Gli2 and Sufu-Gli3 complexes Glis become hyper phosphorylated in the primary

cilia (M.-H. Chen et al. 2009). This phosphorylation event is mediated by Kif7, a kinesin that is a PKA complex scaffolding protein, CK1 and GSK3, which are enriched at the basal body of the cilium. Once the SuFu-Gli complexes are ubiquitinated, through a Cul1 ubiquitination pathway, Gli's dissociate from SuFu and the full length Glis are partially cleaved by proteasomes at the basal body (Zhang et al. 2009). The cleaved Glis can then translocate to the nucleus and repress the response.

During activation of the pathway Smo moves into the membrane of the, primary cilium and Kif7 travels to the tip of the primary cilium, along with SuFu and the Glis2 and 3. Gli2 and Gli3 become phosphorylated at the tip of the cilia dissociating from SuFu, the new Gli2 and Gli3 modifications cause the proteins to be converted into the activator forms of Gli2 and Gli3 (Jiang 2006). Gli2 and Gli3 then translocate to the nucleus activating the downstream transcriptional response. Although SuFu promotes the formation of the Gli activator forms, the activator forms of Glis can occur in the absence of SuFu as well as in the absence of primary cilia. A function of the primary cilia may be to mediate SuFu function (M.-H. Chen et al. 2009; Jiang 2006).

Taken together the transcriptional state, stability, and proteolytic processing of the Glis regulate basal as well as activated Hh activity, depending on the concentration of Hh ligand interpreted by a cell. Chromatin immunoprecipitation studies on Shh signaling centers like the neural tube and limb bud have found thousands of genomic binding sites for Gli3. The ultimate outputs of the Shh pathway depend on the fine tuned balance of Gli activator and repressor activities, where some Shh responsive genes require input from a Gli activator to initiate transcription and others require the removal of Gli inhibitor activity (Vokes et al. 2007; Vokes et al. 2008).

Hedgehog Signaling in Disease: Developmental Abnormalities

The central players of the Hh response have been identified in screens in Drosophila, and it is thus no surprise that the vertebrate homologs of these genes retain their essential role in development. Loss of Hh signaling results in defects that affect midline structures and digit patterning.

1.1.3 Holoprosencephaly

A classic story in Shh signaling and disease is that of the cyclopic sheep. Long before the basic framework of Shh signaling had been deciphered, ranchers in southern Idaho were following a curious problem. In the summer of 1950 their sheep gave birth to a staggering number of cyclopic lambs, up to 25% in some flocks. Fearing strange genetic abnormalities in their stock the farmers kept their cyclopic sheep a secret until the late 50's when they enlisted the help of the Poisonous Plant Research Laboratory (PPRL) in Utah (Binns et al. 1962; Binns et al. 1963). The cause of the cyclopia was not genetic; sheep livestock grazed on pasture in the western mountainsides and often encountered *Veratrum californicum*, a plant producing a steroidal alkaloid that was poisonous when consumed in high amounts. Studies by the PPRL found that sheep grazing on *V.californicum* on the 14th day of gestation gave birth to cyclopic lambs (Binns et al. 1965). In the late 60's the causative agent of cyclopia was purified from

V.californicum, the small molecule antagonist of Smo that we now know as cyclopamine (Van Kampen and Ellis 1972; S. T. Lee et al. 2014).

In development of the early forebrain, a key step is the division of the prosencephalon into two cerebral hemispheres. Cyclopia is a severe and rare form of holoprosencephaly (HPE) a condition where the left and right hemispheres fail to separate; its occurrence is well documented to coincide with Shh mutations. HPE is one the most common developmental aberrations of the forebrain and midface in humans, and is a frequent cause of miscarriage. One out of every 8000 live births presents with some form of HPE. Failure of the early forebrain to divide in varying degrees leads to different forms of HPE. The main symptoms of HPE are facial dymorphism and neurologic impairment. Severe cases of HPE present with cyclopia have extreme neurological deformities and often are stillborn or die in utero. Milder HPE such as microholoprosencephaly is characterized by subtle craniofacial aberrations at the midline, like a single maxillary central incisor, or closely spaced eyes (hypotelorism). Patients with micro-HPE do not demonstrate any neurological impairment (Raam, Solomon, and Muenke 2011; Solomon et al. 2010).

Shh mutations cause a large fraction of autosomal dominant holoprosencephaly, they are detected in 37% of families with autosomal dominant transmission, 18% of familial, and 3.7 % of sporadic HPE (Nanni et al. 1999; Wallis and Muenke 2000). Loss of function mutations in Gas-1 as well as Cdo, which presumably prevent Shh binding to Ptch1 with high efficiency, are also present in HPE patients (Pineda-Alvarez et al. 2012). Ptch1 mutations involved in HPE are gain of functions mutations, both Ptch1, loop1 and 2 mutants, have been identified in HPE cases (Ming et al. 2002; Rahimov et al. 2006; Ribeiro, Murray, and Richieri-Costa 2006). This is consistent with the constitutive repression of Shh activity reported for Ptch1ΔL1 and Ptch1ΔL2 mutants in vitro (Taipale et al. 2002)

Interestingly, familial HPE individuals harboring identical mutations in Shh can exhibit distinct HPE phenotypes. A family case study was conducted with two siblings with a Shh missesnse (Glu256stop) mutation terminating Shh in exon 3, the first sibling presented mild HPE, growing into childhood with small craniofacial abnormalities and few neurological issues, the second sibling presented with severe HPE, dying shortly after birth (Verlinsky et al. 2003). These discrepancies in phenotype are likely due to environmental factors that cause Shh activity to drop below a certain threshold. Studies in chicken have revealed that modifying Shh pathway activity at discrete developmental time points, using cyclopamine, can account for the spectrum of HPE phenotypes (Cordero et al. 2004).

1.1.4 Polydactyly

Along with a number of other morphogens Shh ensures proper digit patterning in vertebrate limbs (Niswander 2003). Shh can regulate formation of the type of and number of digits (Zeller 2004).

Morphogen disruptions in limb patterning can result in a loss or gain of digits. Preaxial polydactyly (PPD) presents itself as an extra digit, a duplication of the thumb or big toe on the anterior side of the limb in humans. In contrast, postaxial polydactyly is characterized by a duplication of the pinky or small toe. PPD is the most frequently

observed congenital hand malformation occurring between 5 and 9 of every 1000 live births, it can occur as an isolated deformity or in conjunction with several syndromes (Zguricas et al. 1999). Shh is expressed in the posterior region of the developing limb bud mesoderm in a transient structure known as the Zone of polarizing activity (ZPA) (Butterfield, McGlinn, and Wicking 2010). Ectopic expression of Shh on the anterior side of the limb bud has been documented in most instances of PPD. Furthermore, most animal models of PPD show expression of Shh both at the anterior and posterior ends of the limb bud (Sharpe et al. 1999).

Familial cases of PPD have mutations in enhancers controlling limb specific expression of Shh (Sagai et al. 2004). Transcriptional repressors and activators negatively and positively control enhancers, of Shh expression in the limb. Mutating residues within enhancers can cause expansion of the range of Shh expression in the limb (Maas and Fallon 2005). Mutations in Shh limb expression enhancers in PPD associated diseases like Werner mesomelic syndrome, a disease characterized by hypo or aplasia of the tibea with PPD or five fingered hands with the absence of thumbs and Haas polysyndactyly have been reported by several groups (Albuisson et al. 2011; Lettice et al. 2012).

Hedgehog Signaling in Disease: Cancer

1.1.5 Cancer
In adults the Hedgehog signaling pathway plays a role in the maintenance and differentiation of adult stem cells particularly in the adult subventricular zone (Gonzalez-Perez 2014), and hair follicle cycle (Millar 2002) in the interfollicular epithelium. The aberrant activation of Hedgehog signaling in adults is involved in various cancers.

Ptch1 is an inhibitor of Smo, and as such is a tumor suppressor gene. The loss of Ptch1 in mice cause increased incidence of medulloblastoma and basal cell carcinoma. This finding led to the realization that a rare syndrome in humans, Nevoid Basal Cell Carcinoma (NBCC) also known as Gorlins Syndrome (Hahn et al. 1996) was caused by the loss of the *Ptch1* allele. NBCC is an autosomal dominant disease, that is characterized by several developmental aberrations and a propensity for neoplasia (Lo Muzio 2008).

Outside of NBCC Shh activity is highly implicated in two major types of cancer: Basal Cell carcinoma (BCC) and Medulloblastoma (MB). BCC, a non aggressive form of skin cancer, arises in the top epidermal layer of the dermis, and often presents itself as pearly pink growths on the skin. It is very amenable to treatment went caught early and tends to not metastasize. BCC is one of the leading causes of cancer and is by far the most commonly diagnosed skin cancer in adults.

MB is a form of brain cancer that is more prevalent in children than adults, and due to its pathology and location is a far more lethal form of cancer than BCC. MB is categorized into four groups with a distinct molecular signature; one of these groups is known to have upregulated Shh. More than 50% of MBs in adults and infants are grouped into this Shh category (Kool et al. 2012).

Mutations of members of the Shh pathway are known causative agents of both of MB and BCC. Ptch1 mutations have been identified in over 90% of BCC tumors, and 30% of MB tumors (Epstein 2008). Loss of heterozygosity of Ptch1 alleles is also commonly reported in BCC (Reifenberger et al. 2005). It is common knowledge that sun exposure increases the chances of skin cancer and this is evident from the Ptch1 mutations found in sporadically occurring BCC. Most sporadic BCC Ptch1 mutations are substitutions, cytosine to thymidine conversions that are correlated with UV exposure. This is further highlighted by *Ptch1 +/-* mice which do not develop BCC, but do develop accelerated BCC in response to UV exposure (Aszterbaum et al. 1999).

In medulloblastoma, Ptch1 mutations are reported in 30% of MB tumors (Raffel et al. 1997). In sporadic samples of MB most identified Ptch1 mutations are truncations (Lindström et al. 2006). Like in BCC Ptch2 appears to modulate MB in the absence of Ptch1. Ptch2 is upregulated in Ptch1 mutant tumors with over 30% of MBs showing increased Ptch2 expression, this upregulated Ptch2 expression is commonly associated with a poor patient prognosis (Y. Lee et al. 2006; Y. Lee et al. 2003). Infrequent Ptch1 mutations are also identified in other forms of cancer including ovarian, and pancreatic cancer, however, the requirement of Ptch1 activity in preventing tumorigenesis in these models is not as well substantiated as in BCC and MB.

Smo gain of function mutations, like SMOM2 (W535L) and SMOM1 (R562Q), which lead to a constitutively upregulated Shh pathway have also been identified in BCC and MB patients. Germline and somatic mutations of SUFU have also been found in MB cases, with a seemingly high frequency of SUFU germline mutations in pediatric medulloblastoma patients (Brugières et al. 2012; Kirk 2012).

Mutations in PTCH, SMO, SUFU, and GLI all represent ligand independent mechanisms that potentiate cancer, however, there are also well-documented mechanisms for Shh ligand dependent potentiation of cancer. The Shh ligand is involved in both autocrine and paracrine signaling in tumor growth. Ligand dependent autocrine Shh signaling occurs when a tumor cell upregulates its Shh ligand proper causing further production of Shh that feeds back into the signaling relay. Several cancers have been identified to use Shh autocrine signaling to maintain tumor growth including melanomas, lung, breast, pancreatic, colorectal, prostate, and gastrointestinal tract cancers (Watkins et al. 2003; Mukherjee et al. 2006; Thayer et al. 2003; Qualtrough et al. 2004; Karhadkar et al. 2004; Berman et al. 2003). In a phenomenon known as paracrine signaling, Shh ligand produced by a tumor with upregulated Shh activity signals to the surrounding stromal tissue (X. Li et al. 2012). Additionally tumors can use reverse paracrine signaling to sustain their growth. In reverse paracrine signaling a tumor upregulates Shh ligand in its surrounding stroma, the ligand produced by the stroma then signals back to the tumor, causing a feedback loop where the Shh pathway is upregulated in the tumor and the stroma resulting in the production of more ligand.

Small molecule antagonists and agonists have long been known to specifically bind Smo, making Smo an obvious and sought after target for cancer therapeutics. Taking advantage of this several pharmaceutical companies have tested small molecule antagonists for treatment of Shh driven cancers. As of 2014 two companies, Genentech and Novartis have formulated small molecule antagonists against Smo, vismodegib and sonidegib, respectively. Vismodegib, also known by its commercial name Everidge,

gained market approval by the Federal Food and Drug Administration in 2012, sonidegib still remains in clinical trials. Making Everidge the first commercially approved drug targeting the Shh pathway it is approved for prescription in patients with advanced stage BCC, as well as metastatic BCC.

Sonic hedgehog ligand production and reception

As is evident in cancers with paracarine signaling, Shh secretion into the extracellular space is key for Shh pathway activation, this is not only true for Shh mediated cancers but also for Shh mediated developmental processes.

Mature Sonic hedgehog is a small glycoprotein of 22 kDa. It is derived from a ~50 kDa protein that is autocatalytically cleaved into N and C terminal fragments. Cleavage of Shh occurs within the endoplasmic reticulum and is followed by degradation of the C terminal fragment (X. Chen et al. 2011). The remaining N terminal segment is the functionally signaling fragment of Hh proteins in both vertebrates and insects (Fietz et al. 1995; Porter et al. 1995). This N terminal fragment has two secondary lipophilic modifications; a cholesterol moiety at its C-terminal end and a palmitoyl at its N-terminal end. These lipophilic modifications dramatically affect the spread and release of Shh.

The lipid moieties of Shh render it as obligatory membrane bound (Y. Li et al. 2006). How a membrane-tethered molecule can nevertheless signal across several cells diameters at a distance remains unclear. Several lines of evidence demonstrate that there are dedicated molecules involved in the secretion of Shh. One of these mechanisms is Shh release by the membrane bound protein Dispatched 1 (Disp1). Disp1 is a 12 pass transmembrane protein that was originally described in Drosophila for its ability to release cholesterol modified Hh (Callejo et al. 2011). Drosophila lacking Disp retained cholesterol modified Hh while releasing cholesterol free Hh (Burke et al. 1999). In vertebrates Disp1 is involved in Shh secretion and Shh spread from Shh source cells. *In vitro* experiments have shown that cells lacking *Disp1* accumulate Shh as contact dependent spread of Shh is limited in a *Disp1 -/-* background (Etheridge et al. 2010; Callejo et al. 2011).

The removal of one or both lipophilic modifications has been proposed as a mechanism to allow Shh transport away from the sites of synthesis. The A disintegrin and metalloproteases (ADAMs) have also been implicated in Shh secretion. Lipidated and membrane tethered Shh is released by ADAM family proteins through a metalloprotease ectodomain induced shedding mechanism (Dierker et al. 2009).

Recent developments have proposed that Shh spreads through cytonemes. Cytonemes are actin based cell protrusions which structurally are like filopodia but mechanistically are proposed to function in cell signaling (Kornberg and Roy 2014). Cytonemes are thought to actively drop off Hh ligand to responding cells or possibly pick up Shh by ligand producing cells. These structures have been described in Chicken embryos and Drosophila (Bischoff et al. 2013; Sanders, Llagostera, and Barna 2013).

Clearly a complex set of mechanisms is involved in the production and secretion of Shh ligand. It is likely that Shh ligand is not secreted passively followed by "free"

diffusion, but that there are several dedicated pathways involved in the release and distribution of Hh molecules in a gradient that forms away from the sources.

1.1.6 Shh Receptors

Ptch1 is the main receptor of the Sonic Hedgehog ligand. Ptch1 is a negative regulator of the Hh response pathway. *Ptch1-/-* mice, which are embryonic lethal mutants have severely perturbed central nervous system (CNS) development (Goodrich et al. 1997) that is characterized by a highly activated Shh response. Ptch1 specifically binds to Shh in a stoichiometric fashion leading to the activation of Smoothened (Smo), a 7 pass transmembrane protein and member of the F Class of G-Protein Coupled Receptors (GPCRs), Ptch1 represses Smo in the absence of Shh ligand.

Amniotes also contain a paralogue of Ptch1 known as Patched 2 (Ptch2). Ptch2 is highly homologous to Ptch1 sharing at least 60% of its protein sequence identity (Smyth et al. 1999). Ptch2 is also a repressor of Smo, but its repression of the Shh pathway has been reported to be less crucial than that of Ptch1. *Ptch2-/-* mice have no embryonic defects but rather display hyperplasia and basal carcinoma phenotypes in adult hood (Nieuwenhuis et al. 2006; Motoyama et al. 1998). *Ptch2-/-* mice with selectively driven *Ptch1-/-* mutations in the dermis have a higher incidence of hyperplasia and quicker advancement of basal cell carcinoma than *Ptch2 -/-* or *Ptch1 -/-* mice (Adolphe et al. 2014).

Several other Shh receptors exist within the context of Ptch1 and Ptch2. Cell adhesion molecule-related/down-regulated by oncogenes (Cdo) and Brother of Cdo (Boc) are cell surface glycoproteins and members of the Fibronectin-Igg superfamily. Cdo and Boc were first associated with Shh in a screen for feedback components of the Shh pathway conducted in somite stage mice (Tenzen et al. 2006). Both Cdo and Boc are positive regulators of the Shh pathway. Their expression is downregulated as a consequence of upregulation of the Shh response pathway. Shh can directly bind to Boc and Cdo, (Tenzen et al. 2006). Sequence analysis of Boc suggests it binds Shh in the same manner as Cdo (McLellan et al. 2008). Mutations in Cdo have been documented in holoprosencephaly (HPE), a human congenital aberration associated with reduced Shh activity that results in defects of the forebrain midline (Bae et al. 2011; Cole and Krauss 2003). Missing Boc alleles contribute to defects in axon guidance in the CNS. Boc has been identified as the functional and necessary Shh receptor of dorsal commissural neurons that cross the midline (Charron et al. 2003; Okada et al. 2006).

Growth arrest specific protein (Gas1) is another direct Shh ligand receptor and like Boc and Cdo is also a positive regulator of the Shh pathway. Gas1 is a glycosyl phosphatidylinositol (GPI)- anchored cell surface protein and is downregulated as a result of activation of Shh pathway activity (Allen et al. 2011). *Gas1 -/-* mouse embryos display mild craniofacial defects that are indicative of reduced Shh pathway activity these midline defects are exacerbated by the loss of Shh alleles, *Gas1-/-; Shh-/-* mice display more severe phenotypes than *Gas1-/-* mice(Allen et al. 2011; Martinelli and Fan 2007).

It has been proposed that Boc, Cdo and Gas1 have partially overlapping and essential roles in mediating the Shh pathway. This is highlighted by the phenotypes found in compound mutants of these genes. While structurally unrelated Cdo and Gas1

mutants display similar although not identical mild craniofacial phenotypes. Both *Cdo -/-* mice and *Gas1-/-* mice present mild neural tube patterning defects, however, *Cdo-/-;Gas1-/-* mice have more pronounced perturbations showing both a lose of Shh floor plate expression and a reduction of ventral neuronal marker expression. Further highlighting the simultaneous need of these three proteins the loss of both Boc and Cdo also has additive effects, *Boc-/-; Cdo-/-* mice have aberrant patterning of the neural tube losing formation of a floor plate and showing a reduction in ventral neuron cell types (Allen et al. 2011). The compound triple mutant *Boc-/-; Cdo-/-; Gas 1-/-* mice present severe holoprosencephaly as well as heart looping defects, and die at E 9.5. While compound mutants, which retain at least one Cdo or Gas1 allele, such as *Boc-/-; Cdo +/-; Gas1+/-* survive to birth and are viable, presenting a phenotype of a double mutant (Tenzen et al. 2006; Allen, Tenzen, and McMahon 2007; Allen et al. 2011; Martinelli and Fan 2007).

Further evidence that these three receptors are essential for maximal pathway activation comes from Boc, Cdo and Gas1's ability to form heterocomplexes. Boc, Cdo, and Gas1 bind Shh in a putative multi-complex with Ptch1 as demonstrated by coimmunoprecipitation of the four receptor proteins, dual receptor complexes appear to form between Ptch1/Boc, Ptch1/Cdo, and Ptch1/Gas1, as no Gas1 molecules are found in either Ptch1/Boc or Ptch1/Cdo coimmunoprecipitations (Izzi et al. 2011). Additionally, a Shh mutant unable to bind Boc, Cdo, and Gas1 which retains its ability to bind to Ptch1 fails to activate the Shh response pathway in NIH 3T3 cells (Izzi et al. 2011) . Whereas Shh binding to Ptch1/2, Boc/Cdo, and Gas1 are all associated with the induction of the Shh response pathway, several molecules have been identified that appear to sequester Shh and inhibit the Shh response.

Hedgehog interacting protein (Hhip) is a single pass transmembrane protein, whose Hh binding loop shares a great resemblance to the Hh binding loop of Ptch1. Hhip has been shown to directly bind Hh, acting as an Hh reservoir and competing for active Shh binding with Ptch1 thus repressing the pathway. It is unknown whether Hhip has any downstream interactions with components of the Shh signaling cascade (Chuang and McMahon 1999; Bosanac et al. 2009). However, Hhip has been demonstrated to inhibit the Shh response cell autonomously as well as non-cell autonomously. Hhip's extracellular domain may be released from cell membranes diffusing to bind Shh ligand on adjacent cells (Kwong, Bijlsma, and Roelink 2014).

Non-canonical Sonic hedgehog signaling

Outside of the transcriptional Gli dictated response several other responses are mediated by Shh.

Non-canonical Shh signaling: Non-Smo mediated non-canonical Shh responses

1.1.7 Ptch1 is a direct dependence receptor

Ptch1 is known to function as a direct dependence receptor that is able to regulate cell survival through Shh (Thibert et al. 2003). Dependence receptors are proteins that regulate cell survival via apoptosis. Dependence receptors promote apoptosis in the absence of their ligand through interactions with caspases and this proapototic activity is inhibited in the presence of ligand (Bredesen, Mehlen, and Rabizadeh 2004). In the absence of Shh, Ptch1 has a C terminal motif that is cleaved by caspases 3,7 and 8 this subsequently promotes apoptosis by exposing its proapoptotic domain (Thibert et al. 2003; Kagawa et al. 2011). A C terminally truncated mutant of Ptch1 that resembles this proapoptotic domain can mediate Ptch1 induced apoptosis in cells even in the presence of Shh. Ptch1 is also found in multiprotein complexes implicated in cell death. The pro-apoptotic C-terminus of Ptch1 can recruit the proapoptotic DRAL complex, which consists of the adaptor protein DRAL and the caspase recruitment domain TUCAN, and caspase 9. Ptch1 can also associate with NEDD 4 an E3 ubiquitinase, NEDD4 ubiquitinates caspase 9 within the proapoptotic DRAL complex allowing caspase mediated apoptosis to occur (Fombonne et al. 2012; Mille et al. 2009). Shh, Dhh and Ihh are documented to have antiapoptotic effects in cells in vitro. The apoptotic activity of Ptch1 lies outside of its interactions with Smo as well as Gli protein transcriptional activation (Chinchilla et al. 2010). Whereas Shh has antiapoptotic effects neither Smo agonists nor activated Smo mutants are able to attenuate apoptosis.

1.1.8 Ptch1 directs cell cycle progression

Non-canonical Hh signaling is also involved in cell proliferation although, it should be noted that canonical Hh signaling is also involved in regulating cell proliferation through transcriptional regulation of cyclins D1 and N-Myc (Dahmane and Ruiz i Altaba 1999). However, Ptch1 is also able to regulate the cell cycle by direct interaction with cyclins, outside of the canonical Hh signaling cascade. Ptch1 can directly bind phosphorylated cyclin B1, the association of these two proteins is regulated by the G2/M checkpoint. If Shh binds Ptch1 it disrupts the Ptch1-cyclinB1 interaction subsequently allowing the translocation of cyclin B1 to the nucleus and the progression of cell division. Biochemical studies have shown this association is dependent on the middle intracellular loop of Ptch1, which can bind phosphomimetic cyclin B1 (Barnes, Heidtman, and Donoghue 2005; Takizawa and Morgan 2000). Truncated Ptch1 mutants unable to interact with cyclin B1 have been identified in basal cell carcinomas suggesting cyclin B1 drives proliferation in these Hh dependent tumors (Barnes, Heidtman, and Donoghue 2005). Furthermore, this interaction is evident *in vivo* in mice harboring mutations in their interfollicular epithelium (IFE). IFE *Ptch1-/-* mice have more nuclear localized cyclin B1 compared to wild type mice as detected by immunostaining (Adolphe et al. 2006)

1.1.9 Ptch1 src kinase interactions

Ptch1 is also involved in a non-canonical signaling cascade involving src kinase. Epithelial mammary fibroblasts display activation of c-src as well as a MEK- dependent activation of ERK1/2 in response to Shh (Chang et al. 2010). ERK1/2 activation by

Ptch1 is Smo independent and may occur by direct interaction of Ptch1 and c-src, as the c-terminus of Ptch1 has been shown to interact with c-src in coimmunoprecipitation assays (M.-H. Chen et al. 2009).

Non-canonical Shh signaling: Axon guidance and migration

Yet another key non-canonical Shh activity is the induction of actin based cytoskeletal changes, which can be grouped as Shh migrational/axon guidance responses. Unlike the responses discussed above, Shh mediated cytoskeletal changes involve a non-canonical Shh responses channeled through Smo. It has been shown *in vitro* in mouse embryonic fibroblasts (MEFs) that Shh mediated migration does not involve the activity of the Ptch1 receptor nor does it require the presence of primary cilia (Bijlsma, Damhofer, and Roelink 2012). Recent work in our lab has shown *Ptch1-/-* MEFs are able to migrate towards a Shh gradient established *in vitro* in a trans well migration chamber (Alfaro et al. 2014). The Smo mutant, SmoΔCLD, is unable to translocate to the primary cilia and results in an inactive transcriptional Shh response, however SmoΔCLD can mediate migration of *Smo-/-* MEFS towards a Shh source (Bijlsma et al. 2007; Bijlsma, Damhofer, and Roelink 2012). It has been demonstrated in MEFs that the formation of actin stress fibers and the migratory response is regulated through G-proteins and IP3 as well as the small GTPase's Rac and Rho. Stimulation of RhoA and Rac1 in fibroblasts is required for MEF migration towards Shh (Polizio et al. 2011).

It is also well known that Shh functions as an axon guidance cue. Dorsal commissural interneurons guide their axons towards the ventral side of the neural tube through the activity netrins and Shh (Charron et al. 2003). Dorsal commissural neuron guidance occurs through the activity of the Boc receptor, in a Smo dependent manner (Okada et al. 2006). Cytoskeletal rearrangements induced by Shh do not require active transcription and are instead thought to be mediated by Src kinase activity (Yam and Charron 2013). A dorsal commissural neuron exposed to Shh can turn towards a Shh source within minutes indicating that this response does not require transcription and by extension does not require Gli activity. Over expression of the Gli3 repressor form in these neurons does not interfere with Shh axon guidance. In contrast interference of Src and Fyn kinases using pharmacological inhibitors halts axon turning towards Shh. Other neurons are also documented to guide via Shh including retinal ganglion cells. All documented Shh mediated axon guidance responses, thus far are Smo dependent and Gli independent.

Ca^{2+} is a likely secondary messenger in the noncanonical Shh axon guidance/migration response. Calcium transients in response to Shh have been reported in the neural tube of *Xenopus*. Explants of *Xenopus* neural tubes show spikes of Ca^{2+} in response to Smo agonists, and this Ca^{2+} response is attenuated by repression of G-alpha proteins through the addition of pertussis toxin or Smo antagonists. It is possible that Smo activation causes Gi protein activation, which leads to activation of a PLC producing IP3 (what is IP3) that increases the levels of intracellular Ca^{2+}. This plausible mechanism is corroborated by the observation that addition of the Smo agonist SAG causes the accumulation of IP3.

Ca^{2+} dependent signaling could be a pathway for Shh regulation of many diverse physiological responses (Belgacem and Borodinsky 2011). In recent studies Shh has also been shown to rewire cellular metabolism in muscle and brown fat cells via a Ca^{2+} mediated mechanism (Teperino et al. 2012).

Patched 1 and Patched 2: Structure and Function

1.1.10 Ptch1 Structure

Ptch1 is a 12 pass transmembrane protein, encoded by 23 exons; its key domains are the cytoplasmic N-terminal domain (aa residues 1-86), two large extracellular loops (aa residues 108-422 and 756-1013), one large intracellular loop (residues 585-734), and a cytoplasmic C-terminal tail (residues 1162-1434). Ptch1 is a member of the Resistance Nodulation Division (RND) family of proteins. RND proteins are large multitopic proteins that have the ability to transport small molecules across membranes. Bacterial proteins of the RND family are well characterized and have proton motive force efflux pumps that are known to move hydrophobic and amphipathic compounds and metals.

1.1.11 Ptch1 Function: Shh binding and Smo repression

How Ptch1 works is relegated to two distinct functions: (1) its ability to bind and act as a reservoir for Shh ligand and (2) its ability to repress Smo, whether these are entirely separate activities remains unknown. Deletion of either of the two large extracellular loop results in a Ptch1 mutant unable to bind Shh in Xenopus oocytes *in vitro* (Marigo et al. 1996). The second large extracellular loop is essential for Shh pathway regulation and binding in vivo. Ptch1ΔL2 mutants are missing the second large extra cellular loop and are insensitive to Shh, and maintain Smo in an inactive state. Ptch1ΔL2 overexpression causes constitutive cell autonomous repression of the Shh pathway, it is a dominant inhibitor (Briscoe et al. 2001; Taipale et al. 2002).

Ptch1 negatively affects the Shh response in two ways, ligand independent antagonism (LIA) and ligand dependent antagonism (LDA). LIA refers to Ptch1s repression of Smo in the absence of Shh ligand. LDA occurs when Ptch1 protein is upregulated in response to an active Shh response. The accumulation of the Ptch1 receptor is thought to inhibit the spread of Shh ligand thus shortening the range of the Shh gradient. Ptch1ΔL2 cell autonomously represses Smo, however, it is unable to cell non-autonomously repress Shh signaling since it is unable to bind Shh ligand. This has been demonstrated in vivo, as expression of Ptch1ΔL2 causes the abnormal spread of Shh to dorsally located cells in the chick neural tube (Briscoe et al. 2001). Thus, Ptch1ΔL2's constitutive cell-autonomous repression is proposed to stem from its inability to bind Shh ligand.

1.1.12 Ptch1 Function: proton driven efflux pump

Like other proteins of the RND family, Ptch1 has an evolutionary conserved aspartic acid motif in its 4th transmembrane domain (TM-4), which encodes for the activity of its proton motive force efflux pump. Notably the proton motive efflux pump,

lies within the SSD domain (aa residues 424-584). To functionally pump molecules RND proteins must trimerize (Kuwabara and Labouesse 2002; Tseng et al. 1999). In other RND proteins closely related to Ptch1 such as Disp1 and Niemann-Pick Type 1 (NPC1), mutations of the aspartic acid motif disrupt protein function. NPC1 is involved in lipid storage disorders, mutations in NPC1's proton motive efflux pump can promote or inhibit NPC1's ability to move cholesterol out of lysosomes and late endosomes (Millard et al. 2005). Documented mutation of the aspartic motif in TM-4 of Ptch1 causes a reduction in Ptch1's ability to repress Shh pathway activity. In Drosophila missense mutations in Ptch1's proton driven efflux pump result in mutants that are able to bind Shh but have a reduced ability to repress Smoothened (Hime et al. 2004; Martín et al. 2001; Strutt et al. 2001). Expression of these Ptch mutants in Drosophila cells that normally do not receive Hh ligand causes activation of downstream Hh targets, suggesting that Ptch-proton driven efflux pump mutants can activate Hh signaling even in the absence of ligand (Strutt et al. 2001). In *Ptch1-/-* MEF luciferase reporter assays, proton motive efflux pump Ptch1 mutants have a diminished ability to repress Shh pathway activity compared to wild type Ptch1 (Taipale et al. 2002).

The importance of the proton driven efflux pump is highlighted by Ptch1ΔL2D499A (Alfaro et al. 2014). Ptch1ΔL2D499A, is a mutant with two mutations, mutation of the second large extra cellular loop and mutation of the proton motive efflux pump. When Ptch1ΔL2D499A is overexpressed in chicken neural tube in vivo the resulting neural tube has no obvious defects, thus the D499A mutation abrogates Ptch1ΔL2's constitutive repressor activity. Notably, the Ptch1D499A mutant shows a decrease in Shh pathway repression in vitro in *Ptch1-/-* MEFs whereas in vivo there is no indication that it interferes with repression of the Shh response. Thus endogenous Ptch1 is unaffected by Ptch1D499A.

1.1.13 Trimerization of RND proteins

Ptch mutants can function as dominant negatives with endogenous WT Ptch because, like other RND proteins, Ptch can trimerize. In fact, many RND proteins form homotrimers a conformation that is necessary for protein activity. Trimerization of bacterial RND proteins leads to the formation of a complex that is able to pump small molecules across membranes; structural dysfunction of this trimer abolishes pumping activity (Kim, Nagore, and Nikaido 2010). Similarly Drosophila Ptch has been shown to trimerize (Lu, Liu, and Kornberg 2006). The CTD domain of Ptch, which controls its localization and half-life, has been identified as an oligomerization domain that on its own is able to trimerize and is essential for Ptch1 function (Lu, Liu, and Kornberg 2006). However, the CTD is not the key trimerization residue as Ptch1ΔCTD mutants are still able to trimerize, it is likely that Ptch trimerization is mediated by several domains including several of its transmembrane motifs. The bacterial RND protein AcrB has extensive contacts amongst its transmembrane domains; making this a likely possibility in Ptch1. Trimerization of mammalian Ptch1 has not been described. However, it is strongly suggested by experiments in *Ptch1 -/-* MEFs were co-expression of the truncated C- and N- terminal portions of the Ptch1 molecule as two separate fragments restores Ptch1 Smo inhibition. Expression of either C- or N-terminal Ptch1 fragments on

there own does not restore Ptch1 Smo inhibition (Bailey et al. 2002). These observations suggest that the C and N terminal halves of Ptch1 can interact to form a functional protein and that trimerization of Ptch1 molecules is highly probable.

1.1.14 Ptch1 sterol pumping

The SSD domain is necessary for Ptch1 function; however, it is unclear whether it serves any activity outside encoding the proton driven efflux motif. Proteins containing a SSD domain are in large part involved in cholesterol homeostasis and a link between cholesterol sterols and Ptch1 activity has long been suspected. Ptch1's ligand, Shh, has sterol modifications, however, it is well documented that Shh without cholesterol modifications is still perceived by Ptch1 (Burke et al. 1999; Lewis et al. 2001). Ptch1's relationship with sterols may be indicative of its vesicular trafficking abilities. In Drosophila, expression of Ptch1SSD mutants results in Ptch1 trafficking defects, whereas wild type Ptch1 can localize to cytoplasmic endosomes and the cell surface, Ptch1SSD molecules are highly enriched in cytoplasmic endosomes. Over expression of Ptch1SSD also causes an accumulation of Smo (Martín et al. 2001).

It is possible that functional Ptch1 causes trafficking of Smo to a compartment where it is targeted for degradation or where Smo cannot be activated. Inhibition of Ptch1 through Hh binding leads to the accumulation of Smo at the plasma membrane (J P Incardona et al. 2000). Ptch has been shown to use lipids derived from lipoproteins to destabilize Smo on the basolateral membrane in the Drosophila wing imaginal disc. It has been proposed that Ptch achieves this by reorganizing the lipid composition of endosomes through which Smo travels, and that sterol derivatives of lipophorin recruited by Ptch negatively regulate Smo activity (Khaliullina et al. 2009). The idea that lipophorin derived lipids are involved in pathway regulation is further confirmed by evidence that depletion of phosphatidylinositol-4-phosphate (P14P) by Ptch represses Smo activity. While an increase in P14P stabilizes Smo at the plasma membrane as well as increases Hh signaling (Yavari et al. 2010).

Depletion of cholesterol has negative effects on Shh signaling. In fibroblasts without functional cholesterol biosynthesis, responses to exogenous Shh are reduced. In the absence of cholesterol the reduction of Shh responsiveness stems from a Ptch1 and Smo interaction. *Ptch1-/-* MEFs, which have a constitutively activated Shh response due to the absence of Ptch1, have decreased activation of the Shh response in the absence of cholesterol. Whereas, cells expressing an activated mutant of Smo, which activates the Shh response, show no reduction in pathway activation in the absence of cholesterol (Cooper et al. 2003). These results indicate that the loss of cholesterol affects a step between or upstream of the Ptch1 Smo interaction.

Ptch1-mediated repression of Smo has been proposed to involve a steroidal molecule. Non-autonomous Ptch1-Shh pathway inhibition has been suggested to occur via cholesterol derivatives. 3β-hydroxysteroids, like 7-DHC and its derivative vitamin D3 have been previously shown to inhibit the Shh pathway and have been suggested to be released by Ptch1. Vitamin D3 competes for binding to Smo with cyclopamine and antagonizes Smo activity, suggesting that it could be the inhibitory sterol that Ptch1 releases to inhibit Smo (Bijlsma et al. 2006). When it was discovered that cyclopamine, a steroidal alkaloid derived from *Veratrum Californicum* (J P Incardona et al. 1998; S. T. Lee et al. 2014), repressed the Shh response, it was believed to occur through the SSD

of Ptch1. Subsequently it was found that cyclopamine directly interacted with Smo, and was unlikely to be involved in Ptch1s sterol trafficking abilities. Nevertheless, Ptch1's ability to move sterols in order to inhibit Smo remains plausible. Ptch1 has been shown to not only bind to cholesterol derived molecules but also promote cellular efflux of fluorescently labeled cholesterol derived molecules in vitro (Bidet et al. 2011)

While the identity of the putative inhibitory sterol of Smo remains unknown recent work has described oxysterols that directly bind Smo to cause Shh pathway activation. Most recently oxysterols have been shown to bind the extracellular (ECD) cysteine rich domain of Smo and modulate activation of the Shh response (Nedelcu et al. 2013).

1.1.15 Patched 2

Ptch2 is a large multitopic protein, which like Ptch1 belongs to the RND family and stoichiometrically binds Shh as well as Ihh, and Dhh (Carpenter et al. 1998). It shares 56% of its sequence identity to Ptch1.   The largest structural differences between Ptch1 and Ptch2 are Ptch2's truncated N and C terminal and the hydrophilic region between transmembrane domains 6 and 7. The truncated C terminal of Ptch2 renders the molecule more stable than Ptch1 (Kawamura et al. 2008). In biochemical experiments measuring the half life's of Ptch1 and Ptch2, Ptch2 is found to have a longer half life than Ptch1, swapping the C terminal domains between the two proteins reverses the stability of the two proteins (Kawamura et al. 2008). Ptch2 is also a repressor of Smo, however its repression of the Shh pathway is not comparable to that of Ptch1 in many studies (Nieuwenhuis et al. 2006; Rahnama, Toftgård, and Zaphiropoulos 2004). Nevertheless, recent studies have shown that in luciferase assays Ptch2 is able to inhibit the pathway at levels comparable to Ptch1. The discrepancies on Ptch2's relative strength in Shh pathway repression may have arisen from *in vitro* artifacts, however this remains to be resolved. However Ptch2 displays other similarities to Ptch1, for example mutation of the proton motive efflux domain of Ptch2 reduces its ability to inhibit Shh pathway activity as measured by a Gli luciferase reporter (Holtz et al. 2013). Similarly to Ptch1, Ptch2 is upregulated in response to Shh pathway activation and can localize to the primary cilium (Holtz et al. 2013;Rahnama, Toftgård, and Zaphiropoulos 2004) as well as coimmunoprecipitate with Boc, Cdo, and Gas1 likely forming a multiprotein complex. Whether Ptch1 and Ptch2 interact has yet to be addressed.

Despite these similarities, Ptch1 and Ptch2s roles do not entirely overlap this is highlighted by the phenotype of *Ptch2 -/-* mice. *Ptch2-/-* mice are viable unlike *Ptch1-/-* mice, which are embryonic lethal. Furthermore *Ptch2-/-* mutants show no developmental defects nor propensity to tumorignesis (Y. Lee et al. 2006; Nieuwenhuis et al. 2006). The activity of Ptch2 only becomes clear in compound mutants of Ptch1. Mutation of Ptch2 in both *Ptch1 -/-* and *Ptch1+/-* backgrounds has been shown to promote the progression of cancer disease states in several studies. While *Ptch1+/- ;Ptch2-/-* mice have no gross phenotypes, tumorignesis is greatly affected by the status of Ptch2. The cumulative loss of Ptch2 alleles in *Ptch1-/-* mice causes a great reduction in tumor latency (Y. Lee et al. 2006). Other studies suggest that Ptch2's true role is in homeostasis of the adult skin. Mice carrying a hypomorphic allele of Ptch2 *Ptch2^{tm1}/Ptch2^{tm1}* are viable and fertile much like *Ptch2-/-* mice. Interestingly adult male *Ptch2^{tm1}*

/Ptch2^tm1 mice develop skin lesions with alopecia and epidermal hyperplasia, suggesting Ptch2 plays a role in maintaining the aging mammalian skin (Nieuwenhuis et al. 2006).

Ptch1 -/- mice display mild BCC phenotypes with epidermal cells invading the dermis of the IFE, subsequent loss of Ptch2 in the IFE causes a rapid progression of BCC with gross invasion of dermal epithelial cells into the underlying mesoderm. This data suggests that Ptch2 functions as a gatekeeper in the progression of Basal cell carcinoma. Furthermore, compared to the loss of Ptch1 alone, loss of both Ptch1 and Ptch2 causes greater defects in lineage specification and differentiation of the epidermis (Adolphe et al. 2014).

A recent study identified Ptch2s role in neural tube development by creating *Ptch1-/-* mutant mice with Ptch1 reconstituted under a metallothionein promoter (MT). The MT-*Ptch1;Ptch1-/-* mice produce enough Ptch1 to prevent the embryonic lethal effects and gross deformities caused by the complete loss of Ptch1 while the metallothionein promoter is unresponsive to Shh pathway activation. Therefore, Ptch1 in MT-*Ptch1;Ptch1-/-* mice is not upregulated in response to Shh activation and its expression is kept at low levels. Under these conditions the function of Ptch2 becomes evident. MT-*Ptch1;Ptch1-/-;Ptch2-/-* mice have perturbed neural patterning indicative of activation of the Shh response. In the absence of Ptch2 inhibition and with little Ptch1 expression the neural tubes of MT-*Ptch1;Ptch1-/-;Ptch2-/-* mice display an expansion of the ventral neural progenitors Nkx2.2 and reduction of dorsal markers like Pax7 (Holtz et al. 2013).

Aims of this project

Clearly both Ptch1 and Ptch2 play repressive roles in the Shh signaling cascade. However, it has not been conclusively demonstrated whether Ptch1 and Ptch2 have overlapping roles, or possibly even function together. Further complicating the reception of Shh ligand is the existence of several co-receptors, of which Boc, Cdo, and Gas1 are known to have necessary and overlapping functions in Shh pathway activation. It is the broad goal of this thesis to address how reception of Shh by Ptch1, Ptch2, and Boc, Cdo, and Gas1 results in pathway activation by addressing the following questions (1) Can the individual contributions of Ptch1, Boc, Cdo, and Gas1 to pathway activation be identified (2) Does Ptch2 function as a repressor of the Shh signaling pathway in the absence of Ptch1.

2 Materials and Methods

Cell culture

HEK 293T were obtained from the ATCC cell repository. *Smo-/-* fibroblasts (gift of Dr. Taipale), *Ptch1-/-* and wild type MEFs (gift of Dr. Scott) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 2mM Glutamine, and 50 U/ml Penicillin/Streptomycin. Serum starvation media was composted of DMEM supplemented with 0.05% 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.

Expression vectors

pcDNA3.1 vector was obtained from Invitrogen. *Prk7ShhNWT was a gift from Genentech. pBABE:A1SMOWT:GFP was a kind gift from Matt Scott. Ptch1Dloop2* was a gift from Dr. Thomas Jessell (Columbia University). The Gli-luciferase reporter and the Renilla control were a gift from Dr. H. Sasaki(Sasaki et al. 1997). *Boc* and *Cdon* constructs were a gift from Dr. Krauss (Mount Sinai School of Medicine). *Ptch1* was a gift of Dr. Scott (Stanford University). *Ptch2* was obtained from Thermo Scientific. Shh binding mutants, Shh-NE90A, Shh-NH183A, and Shh-NR154E were constructed using Quickchange mutagenesis with the primer pairs outlined in Table1. *Ptch1* and *Ptch2* channel mutants were created by Quikchange mutagenesis (Stratagene). In the *Ptch2* mutant the aspartic acids residues at positions 469 and 470 were changed to alanines.

Production of ShhN supernatant from HEK293T cells

Plasmid prK75 carrying Shh-N E90A, Shh-N WT, or Shh-N H183A were separately transfected into HEK293T cells at 90% confluency using Lipofectamine (Invitrogen) as described in its standard proprietary protocol. 24 hrs post transfection, transfection complexes were removed from cells and replaced with DMEM supplemented with 0.05% FBS. Cells were subsequently cultured in this minimal media for 72 hrs, supernatants were collected and spun at 2.3 rpm too remove residual cell bodies. Supernatants were quantified using ELISA against recombinant ShhN (R&D sciences).

Neural induction of AB1 mESCs

AB1s mESC cell lines were neuralized as previously described. AB1's were trypsinized and serum inactivated and spun down 750,000 cells were counted and plated into free suspension in a tissue culture untreated 60 mm petri dish. Cells in petri dishes were aggregated in suspension into Embryoid bodies (EBs) for 24 hrs in DFNB. On day 2 of aggregation EBs were removed and resuspended into fresh DFNB media supplemented with Retinoic Acid under constant rotation at approximately 1 rpm. On the third day of aggregation EBs were removed into fresh DFNB supplemented with RA as well as Shh-N WT supernatants, or mutant SHHN supernatants, or empty construct control supernatant at nM, under constant rotation. EBs were subsequently cultured in DFNB, with RA and ShhN induction conditions for 72 hrs. Approximately 5uM of ShhN sup derived from 293T HEK was added to EB cultures following addition of retinoic acid. EBs were formed and cultured for a total of 5 days.

Neural induction *of Ptch1-/-;Ptch2-/-;Ptch1+/-* and Ptch1-/- mESCs

mESCs were neuralized using established procedures (Wichterle et al., 2002). NEBs were cultured in the presence of α-Shh (5E1) or α-Myc (9E10) conditioned in DFNB medium at 1:5 for the duration of the experiment. NEBs were harvested after 5 days in culture, fixed and stained for Isl1/2 and Nkx2.2, or Pax7(Kawakami et al. 1997).NEBs were mounted in Fluormount and quantified for number of positive nuclei.

Luciferase assays

Luciferase assays were conducted using Light II and *Ptch1-/-* MEFs. Light II cells were plated at subconfluency into 12 well dishes in 10%FBS in DMEM, approximately after 3 days in culture cells were superconfluent. Subsequently the superconfluent Light II cells were switched into a serum starvation media, 0.05% FBS in DMEM supplemented with ShhN enriched 293T sup at 1:10. Light II cells were simultaneously starved and induced for 48 hrs, following this period cells were lysed into 1XPLb and processed for a luciferase assay using a standard protocol (Promega).
Ptch1-/- MEFS were plated at subconfluency into 12 well dishes and transfected with Luciferase and Renilla expression vectors according to a standard protocol (Promega) in 10% FBS in DMEM. These transient transfections were performed using Effectene (Qiagen,Hildern,Germany). DNA was used at 1:15 DNA/Effectene. *Ptch1-/-* MEFS were transfected with Ptch1 mutant constructs, Gli-luciferase, and CMV Renilla. CMV Renilla and Gli- Luciferase constructs were used at a 1:1000 ratio. Cells were incubated with transfection complexes for at least 24 hrs, and subsequently fed fresh 10%FBS in DMEM. Following transfection the cells were cultured for at least 72 hours to reach confluency cells were then switched into a serum starvation media, 0.05% FBS in DMEM supplemented with ShhN enriched 293T sup at 1:10. Following 48 hrs of induction and starvation the cells were lysed and luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

Stable A1:SMO:GFP;*Smo-/-* cell lines

pBABE:A1SMOWT:GFP was transfected into the viral packaging cell line GP+E-86 (ref here). Viral supernatant was collected from the cells and spun down at 2.3 rpm, and filtered using a 0.4 um syringe. Subsequently the viral supernatant was added to Smo-/- MEFS and cells were cultured for 24 hrs. After infection had been established for 24hrs cells were switched into fresh media, 10% FBS in DMEM to recover. 24 hrs following recovery cells with genomic integration of the pBABE:A1SMOWT:GFP plasmid were selected for using 2.5ug/ml puromycin. Following selection cells were split, cloned, and screened for SMOWTGFP and Smo mutant GFP expression.

Smo cilial localization assays

pBABE:A1SMOWT:GFP ; Smo -/- MEFs were plated onto glass cover slides in 24 well plates and subconfluency, and cultured in complete media for several days. Upon reaching confluency the cells were switched into starvation media for 12 hrs. Cells were then fixed and immunostained.

In-ovo Electroporations

Fertilized *Gallus gallus* eggs (Petaluma farms) were kept under refrigeration at 16 degrees C for storage. Subsequently eggs were incubated at 36 degrees C for approximately 36 hrs until reaching Hamburger-Hamilton (HH) Stage 10 (Meyer and Roelink 2003). Eggs were taped and windowed, and demembraned, India ink was injected into the egg yolk beneath the embryo for contrast, 4-6 ug/ul of DNA was caudally injected into the neural tube of embryos and electroporated using sliver electrodes and a electroporated set at 25. Embryos were incubated for another 24 or 48 hours following electroporation. Embryos were then fixed in 4% PFA, sunk in a 1M Sucrose solution and frozen and mounted into Tissue-Tek® O.C.T.™ Compound (Sakura) and sectioned using a cryotome.

Immunohistochemistry

Antibodies for mouse Pax7, HB9, Nkx2.2 (745-A5), Shh (5E1) and Myc (9E10) were obtained from the Developmental Studies Hybridoma Bank, used at 1:10 in staining. Guinea pig α-Isl1/2 was a gift from Dr. Thomas Jessell (Columbia University) used at 1:10,000. Antibodies against GFP were Rabbit anti GFP (Invitrogen) used at 1:1000. Mouse- anti Acetylated tubulin antibodies were from Sigma, and used at 1:1000. In all experiments, Alexa488- or Alexa568- or Alexa647- conjugated secondary antibodies were from Invitrogen used at 1:1000.

Reporter Gene Assays for β-Galactosidase

mESCs were neuralized using established procedures as previously described (Wichterle et al., 2002). After aggregation and addition of retinoic acid NEBs were cultured in 5nM of ShhN WT conditioned HEK293T supernatant or an equivalent volume of control, empty vector, conditioned HEK293T supernatant. NEBs were collected after 5 days in culture and lysed into a standard lysis buffer (100 mM Potassium Phosphate, pH 7.8, 0.2 % Triton X-100). *Ptch1-/-* MEFs were subconfluently plated and were allowed to grow to confluence before switching to a low serum medium (0.5% FCS) and ShhN, SAG, or cyclopamine was added for another 24 hours when cells were lysed. Lysates were analyzed using the Galacto-Light™ chemiluminescence kit (Applied Biosciences) to measure levels of LacZ expression.

RT-PCR

RNA isolation was performed using Trizol (Invitrogen) according to the manufacturer's recommendations. For cDNA synthesis, SuperScript III (Invitrogen) was used on 1 µg RNA. PCR was performed using ReddyMix (Thermo Scientific).

Lentiviral transductions

HEK293T cells were transfected with *psPAX2* and *pMD2.G* helper plasmids and *pLKO.1* clones from the Sigma TRC1.0 shRNA library using FuGene HD (Roche, Basel, Switzerland). Following virus production, supernatant was filtered, and *Ptch1-/-* MEFs were transduced using 1:1 supernatant with 5 µg/mL polybrene (Sigma), transduced cells were selected with 1 µg/mL puromycin. Knockdown was verified by RT-PCR.

Western blotting

Cells were lysed using LDS sample buffer (Invitrogen) and subjected to SDS-PAGE. Proteins were transferred to PVDF membranes, blocked with 5% milk/Tris-buffered saline with 0.1% Tween-20 (TBS-T), and incubated in 9B11 a-Myc 9B11 (Cell Signaling Technology, Danvers, MA) at 1:5,000, or a-FLAG M2 (Sigma) at 1:2,000. Appropriate HRP-conjugated secondary antibodies were used at 1:5,000. Proteins were visualized using a FujiFilm LAS 4000 imager.

RNA transient transfections

Transient DNA transfections were performed using Effectene (Qiagen, Hilden, Germany). DNA was used at a 1:15 ratio of DNA/Effectene. Cells were incubated with transfection complexes for 16h. For RNA transfections, 100 nm siRNA was transfected using 5 µL DharmaFect 3 (Dharmacon, Lafayette, CO) in OptiMem (Invitrogen).

Chemotaxis assay

Migration assays were performed as previously described (Biljsma et al.,2007). Cells were labeled with 10uM CellTracker Green (Invitrogen) according to manufacturer's protocol. After labeling, cells were detached with 5mM EDTA, resuspended in serum free medium,and transferred into FluoroBlok Transwell inserts (BD Falcon) at approximately 5 X 104 cells per insert. Chemoattractant was added to the bottom compartments of the Transwell plates and GFP- spectrum fluorescence in the bottom compartment was measured in a Syngergy HT plate reader (BioTek, Winooski, VT) every 2 min for 99 cycles (approximately 3 hours).

TALENS

The pCTIGTALEN expression vector was generated by cloning the *BglII/SacI* digested TALEN ORF fragment of pTAL4 into the MCS of pIRES2-eGFP. After sequencing to confirm correct RVD architectures in pCTIG, constructs were further modified by replacing the *SacI/BsrG*I IRES:eGFP fragment in the pCTIG backbone with IRES:PuroR or IRES:HygroR fragments from pQCXIP and pIRES-hyg3, respectively, using PCR with primers containing *SacI* and *BsrGI* sites. Each pair of TALEN constructs targeting a locus was modified so that one construct co-expressed HygroR and the other PuroR, conferring transient resistance to both hygromycin and puromycin.

TALEN constructs targeting mouse *Shh* and *Ptch2* were designed using Golden Gate cloning (Cermak et al., 2011) into the pCTIG expression vector. The following repeat variable domain architectures were generated: *Shh*: 5' TALEN: NN HD HD HD HD NN NN NN HD NG NN NN HD HD NG NN NG, 3' TALEN: NN HD HD NN HD HD NG HD NG NG NG HD HD NI NI NI HD. *Ptch2*: 5' TALEN: NN NN HD NG NG HD NN NI NN HD NG NG NI HD NG NG HD, 3' TALEN: NG HD NG NN NN NI NG HD HD NG NN HD NI HD HD HD HD

mESCs were transfected with paired TALEN constructs using Lipofectamine 2000. 1 day after transfection, cells were passaged into ES medium containing 100 µg/mL Hygromycin and 0.5 µg/mL Puromycin and cultured for 4 days. Selective medium was then removed and surviving mESC colonies were isolated, expanded and genotyped by sequencing PCR products spanning the TALEN binding sites.

Genotyping

PCR screening was performed on cell lysates using primers flanking the *Shh* and *Ptch2* TALEN binding sites: *Shh:* (5') TGGGGATCGGAGACAAGTC and (3') TCTGCTCCCGTGTTTTCCT, *Ptch2*: (5') AAGGCACAGGGAAAGAGAGTT and (3') ACTTGCCTAGCTTGCACAATG. PCR products were sequenced using Sanger sequencing. Samples with mixed signals indicative of small INDEL mutations were TOPO cloned into PCR2.1 and sequenced to confirm allele sequences. A

Ptch1-/-;Shh+/- mESC clone harboring a 5bp deletion in *Shh* exon 1 was validated and re-transfected with *Shh* TALENs. A *Ptch1-/-;Shh-/-* clone heteroallelic for 5bp and 4bp deletions with predicted stop codons in exon 1 was characterized for its response to ShhN. A *Ptch1-/-;Ptch2-/-* clone was characterized with a 5bp deletion in exon1 of *Ptch2*

3 Involvement of Boc, Cdo, and Gas 1 in Shh signaling

Introduction

The Shh pathway inhibitor Patched1 is part of the receptor complex (receptosome) of Shh, collection of cell surface proteins that bind Shh. Boc, Cdo, and Gas1 are part of the Shh receptosome. These Shh binding proteins play semi-redundant and essential roles in positively potentiating Shh responses. Boc, Cdo, and Gas1 fine-tune the Shh response as agonists along side Ptch1. This is highlighted by their roles in diseases associated with incorrect regulation of the Shh response pathway. HPE, which results from a lack of Shh signaling, is present in individuals with Boc and Cdo mutations that disrupt Shh binding (Bae et al. 2011).

Single mutants of these proteins like *Boc-/-, Cdo -/-,* or *Gas1-/-* mice generally do not present any critical developmental phenotypes. However, *Boc-/-;Cdo-/-;Gas1-/-*

Mutant	Primers
Shh-N E90A	E90AFOR- 5′ ATA TTT AAG GAT GCG GAA AAC ACG GGA GCA GAC CGG 3′ E90AREV- 5′TCC CGT GTT TTC CGC ATC CTT AAA TAT GAT GTC GGG 3′
Shh-N H183A	H183AFOR- 5′ CAA AGC TCA CAT CGC CTG TTC TGT GAA AG 3′ H183AREV- 5′ CTT TCA CAG AAC AGG CGA TGT GAG CTT TG 3′
Shh-N H183A	R154EFOR-5′ACG TCC GAC GAG GAC CGC AGC AAG TAC GGC ATG CTG 3′ R154EREV-5′ GTA CTT GCT GCG GTC CTC GTC GGA CGT GGT GAT GTC 3″

Table 1. Primers used to mutate Shh-N into binding mutants against Boc, Cdo, Gas1, and or Ptch.

mice have severe disruptions in neural tube patterning that resemble the lack of Shh activity present in *Shh-/-* and *Smo -/-* mice (Allen et al. 2011; Kang, Zhang, and Krauss 2007). How these three proteins can compensate for one another is unclear, Boc and Cdo are both closely related proteins of the Igg/Fibronectin family, and their Shh binding modes are predicted to be very similar based on co-crystallization data of Cdo and Shh, and sequence analysis of Boc. Both Boc and Cdo interact with Shh via one of their fibronectin domains. In contrast, Gas1 is a GPI linked protein that belongs to the GFRα

family and is unrelated to Boc or Cdo (Baloh et al. 2000). Despite these differences Boc, Cdo, Gas1 and Ptch1 require an intact Shh Ca^{2+} coordination site for proper binding (McLellan et al. 2008).

It is generally accepted that Boc, Cdo, and Gas1 positively potentiate the Shh response in the presence of Ptch1. Although there are competing reports suggesting that Cdo can compete with Ptch1 for Shh binding (McLellan et al. 2008). How Boc, Cdo, and Gas1 coordinate with Ptch1 to potentiate the Shh response is unclear. All four proteins have been co-immunoprecipitated in what is thought to be a heterocomplex (Izzi et al. 2011). Furthermore, Shh mutants that retain Ptch1 binding but are unable to bind Boc, Cdo, and Gas1, are unable to activate a Shh response in a Gli mediated luciferase assay in NIH 3T3 cells (Izzi et al. 2011).

It is possible that Boc, Cdo, and Gas1 facilitate Shh binding to Ptch1. In other words, Ptch1 in the absence of Boc, Cdo and Gas1 is blind to the presence of Shh. While Shh binding to Boc, Cdo and Gas1 has been shown to be crucial for activation of the Shh pathway, it has not been addressed whether Ptch1 actively binds Shh in the absence of these proteins. It is plausible that Shh binding to Boc, Cdo, and Gas1 leads to a conformational change in Shh that renders the protein accessible for Ptch 1 binding.

Yet another plausible explanation is that direct downstream signaling from Boc, Cdo, or Gas1 along with Ptch1 is necessary to activate the response. Thus Shh would have to bind to some critical number of these four receptors to elicit a response. Signaling components lying directly downstream of Boc, Cdo, or Gas1 in the canonical Shh pathway have not been identified but very likely function through Smo. However, this idea is refuted by Ihog, the invertebrate homolog of Cdo. When Ihog is expressed without its intracellular domain it is still able to potentiate Hh signaling in the fly, suggesting the role of Ihog and by extension Boc and Cdo in signaling is to bind Shh extracellulary and not to transmit a signaling response (Yao, Lum, and Beachy 2006). Nevertheless, it should be noted that while Cdo and Ihog are homologous molecules their mechanisms for Shh binding are very distinct, thus there downstream functions may also not be identical (McLellan et al. 2008).

Smo travels to the cilium upon losing Ptch1's repressive activity, but Smo localization to the primary cilium does not necessarily coincide with an active Smo protein or activated Shh Pathway (Rohatgi, Milenkovic, and Scott 2007). It is possible that Smo is activated in a two-step mechanism, whereby Shh binding to Ptch1 causes relocalization of Smo and a secondary step results in the activation of Smo, possibly mediated by Boc, Cdo, and Gas1. Previous observations in our lab that *Ptch1-/-* cell lines retain responsiveness to Shh and SAG corroborate the presence of a two step model, whereby derepression of Smo in the absence of Ptch1 does not result in a fully activated Shh response (Alfaro et al. 2014). Furthermore, the necessity of Shh binding to Boc, Cdo, Gas1 and Ptch1 for Shh pathway activation might be reflective of a two-step activation model. Whereas Ptch1 binding to Shh is sufficient to release its repression over Smo, Shh binding to Ptch1, Boc, Cdo and Gas1 is needed to completely activate Smo and subsequently the transcriptional Shh response.

Results

Shh-N E90A does not elicit a Shh transcriptional response

To further address this question we mutagenized soluble Shh-N at surface-exposed evolutionary conserved residues that were previously implicated in Shh ligand function. The mutagenized residues were also chosen based on plausible interactions with Ptch1, Boc, Cdo, or Gas1 as determined by co-crystallization of these proteins with Shh (Fuse et al. 1999; Bosanac et al. 2009; McLellan et al. 2008; Ohlig et al. 2011). Because most of our Shh mutants were in sides chains reported to be highly exposed in solvent, we expected the folded protein structures of our mutant proteins would not be affected (Hall et al. 1995; Fuse et al. 1999). We chose residues that were characterized as essential for either Boc, Cdo, and Gas1 binding only or essential for Ptch1 binding only using past co-crystallization experiments with Shh. Our chosen mutants included Shh-N H183A and the previously published mutants Shh-N E90A, and Shh-N R154E, Shh-N WT was mutagenized using the primers listed in Table 1. Shh-N mutants and their receptor binding partners are outlined in Table 2.

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

Table 2. Receptor binding partners of Shh-N WT, Shh-N E90A, and Shh-N H183A. Shh-N WT binds Ptch1, Boc, Cdo, and Gas1. Shh-N E90A binds Ptch1 but has deficient Boc,Cdo,Gas1 binding. Shh-N H183A has deficient Ptch1 binding and retains binding to Boc, Cdo, and Gas1.

Ultimately the mutants were compared on their ability to activate the Shh pathway in a standard Light II assay in NIH 3T3's, where Shh activity is quantified by the amount of luciferase protein produced from a gene element under the control of the Gli1 promoter. Shh-N mutants were expressed in HEK 293T cells and cultured in low serum media for collection of supernatants for subsequent experiments, and the amount of Shh-N protein in each supernatant was quantified using ELISA (enzyme linked immuno-sorbent assay). Under standard starvation conditions and induction by Shh-N E90A supernatants or Shh-N WT supernatants, Shh-N E90A was unable to elicit Shh pathway activity to the degree of Shh-N WT (Figure 3C). Our results corroborated
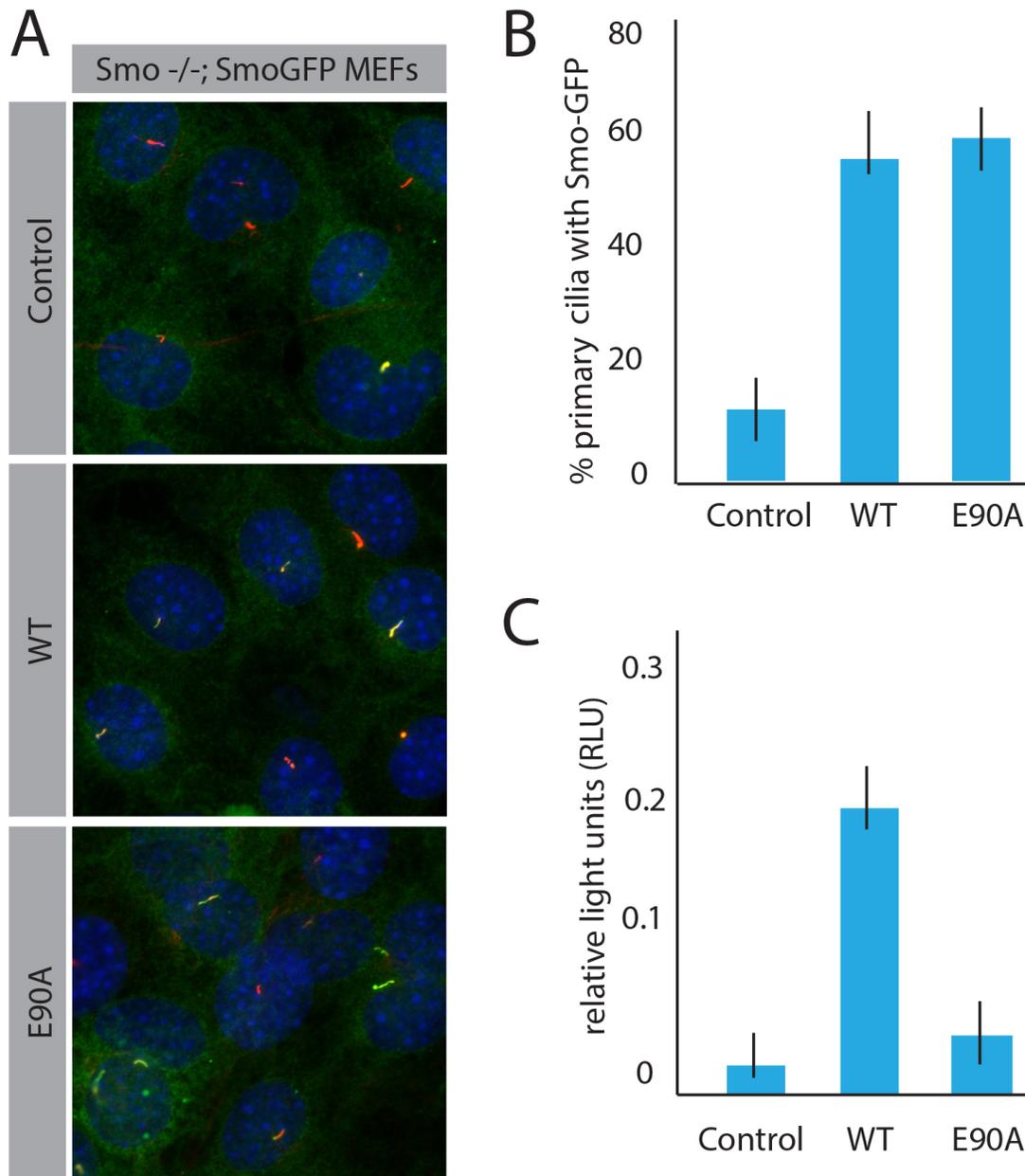
Figure 3. Shh-N E90A does not elicit a transcriptional response in a Light II assay but does cause Smo localization to the primary cilium Smo -/- A1:SmoGFPMEFs cultured with 293T supernatant conditioned with Shh-N E90A, Shh-N WT, or control. Cilial localization of Smo is caused by Shh-N WT and Shh-N E90A. (A) Levels of cilial localization by Shh-N WT and Shh-N E90A are similar (B). A luciferase assay conducted in NIH 3T3 cells, the level of luciferase detected (denoted by relative light units) is indicative of upregulated pathway activity. Shh-N WT upregulates luciferase whereas Shh-N E90A and control supernatant does not (C). Shown is the mean ± SEM n≥60 cilia per condition; Control vs. WT and Control vs. E90A $p < 0.01$. Relative light units (RLU) are values derived from the amount of luciferase produced divided by the value of a transfection control. Luciferase assays were performed in triplicate and each condition was performed in quadruplicate. Control vs. WT and WT vs. E90A had $p < 0.05$.

previous data published for Shh-N mutant E90A, which was nonfunctional in a luciferase assay and in C3T101/2 chondrocyte induction (Izzi et al. 2011). The glutamic acid residue in Shh-N E90A is an evolutionarily conserved amino terminal domain residue that is surface exposed, this has been determined by exposure of side chains in solvent (Hall et al. 1995). Co-crystallization of Shh and Cdo revealed their interface involves a hydrophilic region of Shh and a hydrophobic region of Cdo, as well as a hydrophobic region of Shh that contains residue E90. E90 is also one of six side chains involved in Shh calcium coordination, this calcium binding site in Shh-N has been previously described as important for interactions with Cdo and Gas1's Shh binding site, Fibronectin 3 (McLellan et al. 2008). Furthermore residue E90, along with several other residues, has been implicated in the formation of Shh multimers (Ohlig et al. 2011). To further test this residues necessity for functional Shh ligand activity, we tested Shh-N E90A in a neural differentiation assay.

Differentiation of neuronal precursors into motor neurons is driven by Shh in the ventral domain of the neural tube. In vitro, embryonic stem cells can be aggregated into embryoid bodies (EBs) and differentiated into motor neurons using Shh in a similar process. Exposure of aggregate EBs in the presence of Shh-N WT results in the expression of transcription factor Isl1/2 a marker of terminally differentiated motor neurons. Aggregated neural precursors posterioirized by retinoic acid and cultured in Shh-N E90A or Shh-N WT, revealed that E90A was impaired in activating Shh activity in comparison to ShhN-WT. In addition, fewer Isl1/2 positive nuclei resulted in EBs

A
B
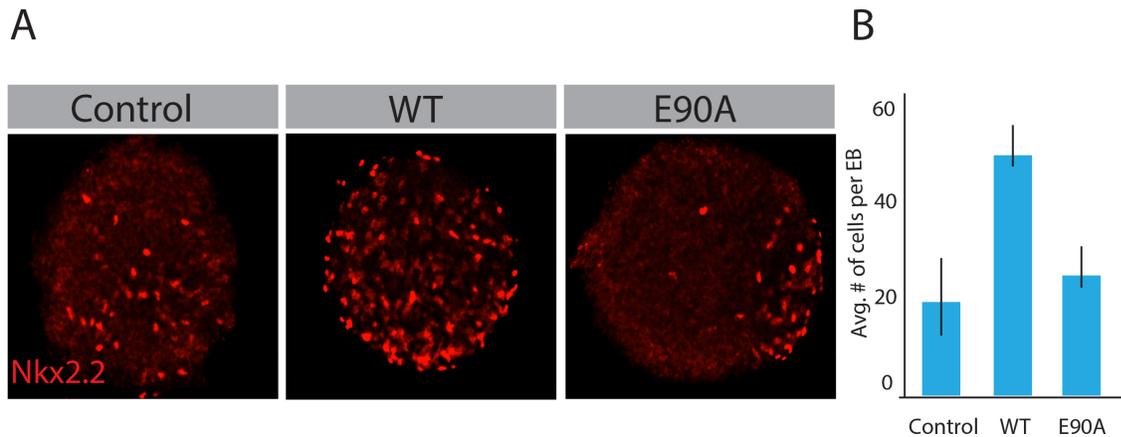
Figure 4. Shh residue E90 is important for proper Shh pathway induction. Ptch+/+ EBs cultured with Shh-N E90A vs. Shh-N WT and Control supernatants (A). Shh-N E90A does not upregulate Isl1/2 to the same degree as Shh-N WT. Levels of Isl1/2 in EBs cultured with Shh-N E90A are similar to those cultured with control mock supernatant (B). Shown is the mean ± SEM n≥20 EBs per condition; Control vs. WT and Control vs. E90A p<0.05.

cultured with ShhN-E90A as compared to ShhN-WT (Figure 4 A, B). Similar results were obtained in embryoid bodies derived from *Ptch1-/-* ES cells, although the difference in Is1/2 cells was not statistically significant between Shh-N E90A and Shh-N WT conditions (data not shown).

To test our hypothesis that Shh elicits a two-step activation mechanism, we questioned whether Shh-N E90A's inability to mediate the Shh response would reflect

itself in a Smo localization phenotype. Surprisingly, although Shh-N E90A was unable to elicit a transcriptional response in either a luciferase or neuralization assay that was comparable to Shh-N WT, it was able to cause localization of Smo:GFP to the primary cilium at levels comparable to Shh-N WT (Figure 3A,B). This activity is comparable to that observed with cyclopamine, a small molecular antagonist of Smo that causes Smo to localize to the primary cilia in NIH 3T3 cells, while simultaneously suppressing the Shh response (Rohatghi et al. 2007). Smo localized to the primary cilium via Shh-N E90A could represent a subpopulation of Smo that is uninhibited by the activity of Ptch1 but not fully competent to elicit a Shh response. Shh binding to Ptch1 may represent the first step of Smo activation, allowing it to enter the primary cilium where Smo requires Shh binding to Ptch1, Boc, Cdo, and Gas1 to become fully activated and mediate activation of the Shh transcriptional response.

Shh-N H183A does not elicit a Shh transcriptional response

In contrast a Shh mutant, such as Shh-N H183A, which is unable to bind Ptch1 but retains its ability to bind Boc, Cdo, and Gas1, would be predicted to both be unable to elicit the Shh transcriptional response and Smo translocation to the primary cilium. Residue H183 lies in the Zinc coordination site of Shh as well as Dhh, the Zn coordination site has been positively identified as the binding site of Ptch1 as well as Hhip in Shh in several studies (Bosanac et al. 2009; Bishop et al. 2009). A mutation at residue H183 is not only predicted to disrupt Shh binding to Shh and Hhip, it also disrupts Shh interactions with anti-5E1 (Bosanac et al. 2009). As expected Shh-N H183A was unable to elicit a transcriptional Shh response in Light II cells and simultaneously was unable to uninhibit Smo. Shh-N H183A did not cause Smo translocation to primary cilium in cells expressing A1:SMOWTGFP (Figure 5A, B, C).

Mutant Shh-N R154E was created to function as a negative control in our experiments. Residue R154 has been mapped as crucial for binding of all four proteins Ptch1, Boc, Cdo, and Gas1. Residue R154 lies in the hydrophilic region of Shh that interacts with Cdo, is an amino terminal domain Shh residue and has been implicated in Shh multimerization, the basic amino acid has been described to make contacts with negatively charged sulfate residues in a possible Shh multimerization mechanism mediated by HSPGs (Ohlig et al. 2011; Ohlig et al. 2012). The mutation of the positive Arginine side chain to the negatively charged Glutamic acid in Shh-N R154E would predictably disrupt its interactions with all four receptors. Previously published reports of this mutant showed it was unable to elicit a Shh response in both Light II assays or in chondrocyte differentiation assays (Izzi et al. 2011). Surprisingly, Shh-N R154E caused expression of Isl1/2 in neural differentiated EBs and caused translocation of Smo to the primary cilia at a rate much higher than Shh-N WT (data not shown). Curiously, primary cilia in *Smo-/-* MEFs cultured under Shh-N R154E were even longer than those cultured under Shh-N WT (data not shown).
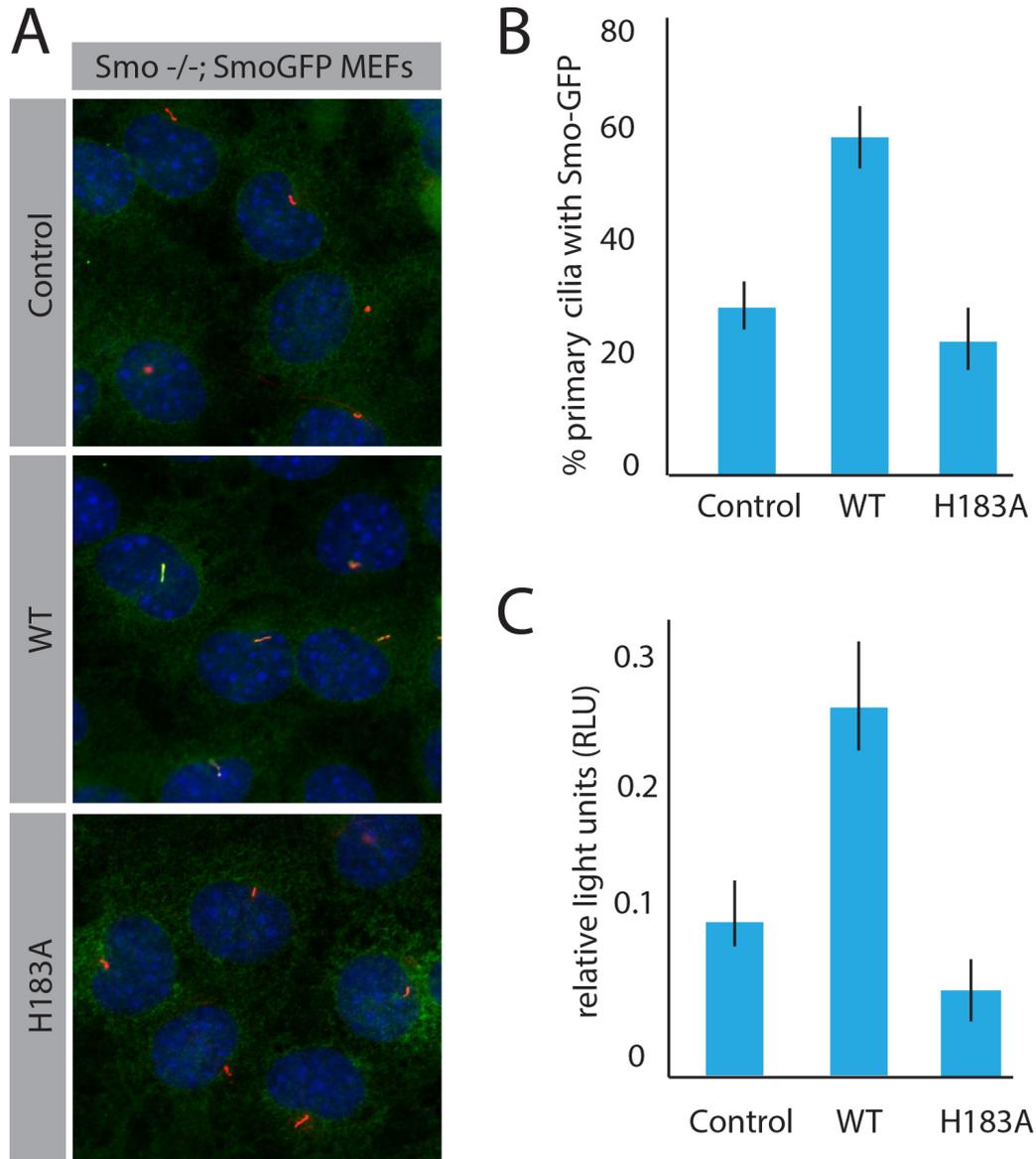
Figure 5. Shh-N H183A is unable to elicit a Shh transcriptional response in a Light II assay or in a Smo localization assay Smo -/-: SmoGFP:MEFs cultured with 293T supernatant conditioned with Shh-N H183A, Shh-N WT, or mock pCDNA (A). Cilial localization of Smo is caused by Shh-N WT and Shh-N H183A. Levels of cilial localization by Shh-N H183A are much lower than Shh-N WT, and are similar to control conditions (B). A luciferase assay conducted in NIH 3T3 cells, the level of luciferase detected (denoted by relative light units) is indicative of upregulated pathway activity (C). Shh-N H183A fails to upregulate the Shh response in this assay. Shown is the mean ± SEM n≥60 cilia per condition; Control vs. WT and H183A vs. WT p<0.01. Relative light units (RLU) are values derived from the amount of luciferase produced divided by the value of a transfection control. Luciferase assays were performed in triplicate and each condition was performed in quadruplicate. Control vs. WT and WT vs. H183A had

WT	E90A	H183A

Nkx2.2
GFP
Isl 1/2
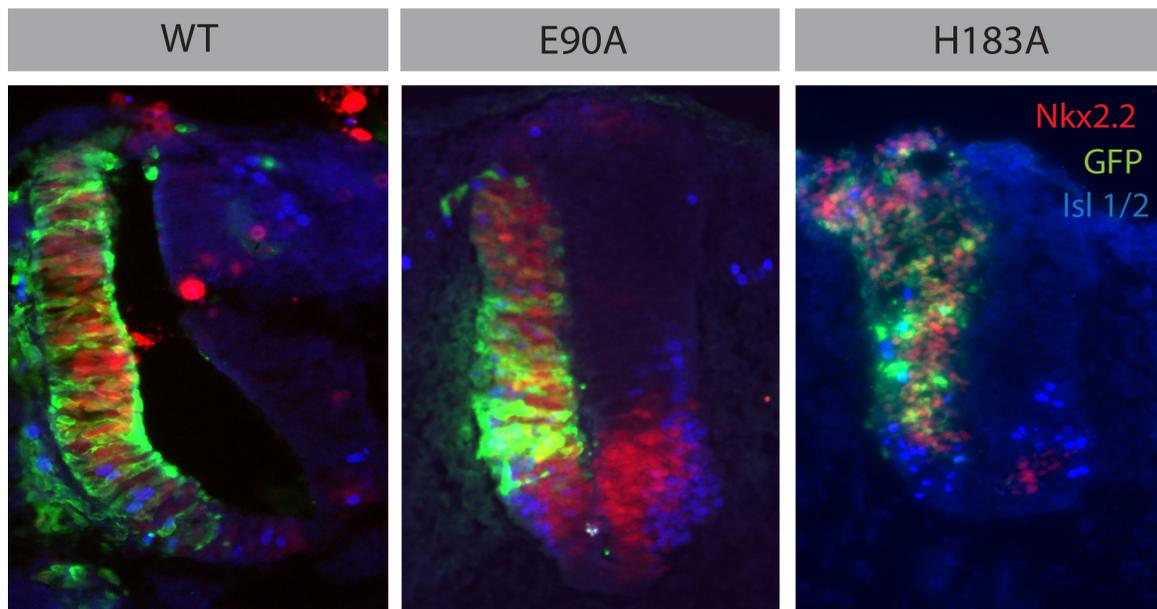
Figure 6. Electroporation of Shh-N mutants in vivo leads to an upregulation of the Shh response. Co-electroporation of Shh-N WT, Shh-N E90A, or Shh-N H183A with GFP causes cell autonomous activation of the Shh response and a dorsal expansion of the ventral marker Nkx2.2. Shown above are representative sections of each electroporation in chicks at stage HH20. Nkx2.2., GFP, and Isl ½ are shown in red, green and blue, respectively. An n≥2 chick embryos were analyzed for each condition.

Shh-N E90A and H183A function comparably to Shh-N WT in vivo

Finally, Shh mutant constructs were co-electroporated into chick neural tubes, along with GFP as a marker, in order to assess their function in vivo. Mutant ShhN-E90A's effects on the Shh response *in vitro* had been previously published but its effects *in vivo* have never been observed. In contrast to the *in vitro* results, all Shh-N mutants electroporated into the chick neural tube were able to elicit a Shh response comparable to Shh-N WT (Figure 6). All mutants electroporated displayed a marked upregulation of the ventral neuronal markers, Nkx2.2, far into the dorsal aspects of the neural tube. This activation of the Shh response was cell-autonomous only being observed in cells expressing GFP, which also expressed the co-electroporated Shh-N mutant. Thus, whereas Shh-N E90A, and Shh-N H183A where unable to elicit a Shh response in cells *in vitro*, they were able to elicit a Shh response *in vivo*.

There are several reasons why this discrepancy is possible. It is unclear from the current *in vivo* data whether the effects observed are autonomous or non-cell autonomous. All the effects observed *in vivo* are cell autonomous as all GFP expressing cells, which contain a Shh-N construct, also express Nkx2.2. It is also unclear whether cells lacking GFP also upregulated Nkx2.2 which would be indicative of a non-autonomous upregulation of the Shh response. It should be noted that the *in vivo* chick electroporation experiments can be used to observe a Shh-N mutants effects in cis or trans. In contrast, the assays performed *in vitro* only observe a Shh-N mutants' ability to potentiate the Shh response in cis. Thus, the presentation of these mutants to their respective receptors may differ between cis and trans signaling.

4 The Shh response in *Ptch1-/-* cells is ligand dependent

Introduction

Shh signaling is regulated by the interaction between Ptch1 (Marigo et al., 1996; Stone et al., 1996) and Smo (Marigo et al. 1996; Murone, Rosenthal, and de Sauvage 1999). Shh binding to Ptch1 releases the Ptch1-mediated inhibition of Smo (Taipale et al., 2002). Smo then localizes to the cell surface (John P Incardona, Gruenberg, and Roelink 2002) and subsequently to the primary cilium (Milenkovic et al., 2009) where it mediates the activation of the Shh response (Corbit et al. 2005; D. Huangfu and Anderson 2005; Rohatgi, Milenkovic, and Scott 2007) This model explains the widespread activation of the Shh response observed in the absence of Ptch1 (Goodrich et al., 1997).

Drosophila genetics strongly supports the canonical model of Hh signaling by demonstrating that the loss of Ptch is epistatic to the loss of Hh (Bejsovec and Wieschaus 1993). In amniotes there are two *Ptch* homologs, *Ptch1* and *Ptch2,* and of these two genes *Ptch1* appears to be the most important. The loss of *Ptch1* results in an embryonic lethal phenotype characterized by the widespread upregulation of the Shh response, including extensive induction of Shh expression and ventral identity in the developing neural tube (Goodrich et al., 1997). In contrast, *Ptch2*-/- mice are fertile and viable, but develop skin abnormalities characterized by basal cell hyperplasia (Nieuwenhuis et al., 2006). Since these data suggested that the functions of Ptch1 and Ptch2 are largely non-overlapping, *Ptch1*-/- cell lines have been used extensively for their high level of cell-autonomous activation of the Shh response. For example, neuralized cells derived from *Ptch1*-/- mouse embryonic stem cells (mESCs) acquire a phenotype typically associated with the induction of the Shh response without the inclusion of Shh in the medium (Crawford and Roelink, 2007). This is consistent with a ligand-independent induction of the Shh response in cells devoid of Ptch1. Similarly, *Ptch1*-/- fibroblasts have been widely studied for having a constitutively upregulated Shh response (Taipale et al. 2000).

In contrast to Ptch1, few studies have focused on Ptch2 because, as mentioned above, unlike Ptch1, Ptch2 is dispensable for embryonic development. Nevertheless, recent work elegantly shows that Ptch2's activity is masked in Ptch1's presence, and that Ptch2, like Ptch1, is involved in ligand dependent inhibition of Shh patterning in the early neural tube (Holtz et al. 2013). Ptch1 is known to participate in both cell autonomous ligand independent antagonism (LIA) and cell non-autonomous ligand dependent antagonism (LDA). Whether Ptch2 has ligand independent inhibition activity or overlapping functions with Ptch1 is unclear as conflicting reports exist on Ptch2's ability to inhibit the Shh pathway *in vitro* as measured by Shh responsive luciferase assays.

The functional disparities between Ptch1 and Ptch2 can be attributed to several factors. For example, the distinct developmental necessity of Ptch1 compared to Ptch2 could be explained by temporal and spatial expression during development. Ptch1 may be expressed earlier and at higher concentrations than Ptch2, which would explain why Ptch1 is more necessary for development.. Comparative expression analysis of Ptch1 and Ptch2 has shown that Ptch2 is diffusely expressed in the developing neural tube

and that Ptch1 is generally expressed at higher levels than Ptch2 throughout the embryo (Y. Lee et al. 2006; Motoyama et al. 1998). Ptch2 may also have specific tissue restricted functions as other studies report that Ptch2 is highly expressed in the skin and testes in comparison to Ptch1, this tissue specific expression of Ptch2 corroborates the idea that it has been coopted as a receptor for Dhh, a Hh ligand crucial for germ line development in the testes. (Carpenter et al. 1998).

It is also plausible that a sequential expression pattern could exist whereby Ptch1 expression occurs before Ptch2. Shh signaling is present as early as the node and induction of the floor plate, where Shh expression mediates further Shh expression, Ptch1 may be expressed in these early developmental processes (J.-B. Charrier et al. 2002; Placzek, Jessell, and Dodd 1993). Thus, in Ptch1's absence, Shh signaling could be perturbed very early on in development, therefore, Ptch2 expression at later stages would not restore proper embryonic development. This could explain the *Ptch1-/-* mouse phenotype and why Ptch2, a protein with similar presumed functions to Ptch1, is a poor substitute in Ptch1's absence.

It is known that both Ptch1 and Ptch2 play roles in ventral neural tube patterning, Holtz et al. 2013 generated *MT-Ptch1;Ptch1-/-;Ptch2-/-* mice, in these mice Ptch2 expression is absent and Ptch1 is expressed under a metallothionein (MT) promoter, keeping Ptch1 expression at low levels and non-responsive to Hh signaling. *MT-Ptch1;Ptch1-/-;Ptch2-/-* mice have expanded ventral neural progenitors as a result of Ptch2's absence indicating that, like Ptch1, Ptch2 functions as a repressor of the Shh pathway (Holtz et al. 2013). In summary, with low levels of Ptch1, Ptch2's activity in the neural tube becomes evident ((Holtz et al. 2013). Interestingly, while *MT-Ptch1;Ptch1-/-;Ptch2-/-* mice demonstrate that both Ptch2 and Ptch1 are expressed in the neural tube and that Ptch2 repression can mediate Shh patterning in the neural tube at stage E10, MT-*Ptch1;Ptch1-/-;Ptch2-/-* mice do not address whether Ptch1 and Ptch2 have completely overlapping functions or if they are simultaneously expressed at comparable levels throughout development. Therefore, it is still an open question why *Ptch1-/-* mice are embryonic lethal despite the presence of Ptch2. It is possible that the expression of these two proteins does not coincide early enough to compensate for each other's activities. Detailed characterization of Ptch1 versus Ptch2 expression throughout development is needed to confirm this idea.

Additionally, it is possible that Ptch1 and Ptch2 do not have entirely overlapping functions and that their biochemical activities are not comparable. Structurally, both proteins are very similar, sharing 56% sequence homology, and sharing similar SSD and Shh binding domains (Carpenter et al. 1998; Motoyama et al. 1998; Smyth et al. 1999). Both are also confirmed to have similar binding affinities to Shh ligand (Carpenter et al. 1998; Marigo et al. 1996). Biochemically, the largest difference between Ptch1 and Ptch2 is their half-lifes, Ptch2 has a longer half-life than Ptch1. In contrast to Ptch1, the N and C terminal domains of Ptch2 are truncated. Ptch2's stability is derived from its shorter C terminal domain (CTD). Swapping the C terminal domains of Ptch1 and Ptch2 causes Ptch2 to lose its stability and Ptch1 to gain stability with a longer observed half-life (Kawamura et al. 2008). It is possible that Ptch2's stability makes it a less dynamic responder of the pathway compared to Ptch1. It has been hypothesized that Ptch1s longer CTD region may have been recruited or conserved during evolution for a unique function in Shh signaling. Previously it was proposed that

the CTD was involved in Ptch-Smo interactions, however, whether Ptch and Smo interact remains contentious and thus the role of the CTD remains undefined.

Moreover, the relative strength of Ptch2's inhibition of the pathway has come into question in several *in vitro* Shh luciferase driven experiments showing contradictory results. For example, a Ptch2 promoter luciferase construct that was highly responsive to Shh in *Ptch1-/-* MEFs was downregulated by overexpression of Ptch1 but not Ptch2 (Rahnama, Toftgård, and Zaphiropoulos 2004). Two additional studies observed that a Gli luciferase reporter with tandem Gli 1 binding sites expressed in *Ptch1-/-* MEFs or NIH 3T3's, respectively, was downregulated equally in response to either Ptch1 or Ptch2 overexpression (Nieuwenhuis et al. 2006; Holtz et al. 2013). Additionally, qPCR of Gli1 and Gli2 expression in *Ptch1-/-* MEFs demonstrated that both Ptch1 and Ptch2 could down-regulate Gli1 and Gli2 expression, equally. Thus, experiments conducted both *in vitro* and *in vivo* have pointed to Ptch2's ability to repress the Shh pathway (Zhulyn et al. 2015). The discrepancies between these studies could be attributed to technical differences in reporter constructs, plasmids, and cell types. In the epidermis where both Ptch1 and Ptch2 are expressed, it has been observed that Ptch1 is upregulated in response to transgenic expression of Smo-M2, whereas Ptch2 remains unresponsive. Additionally, in C3H10T1/2 and Ref52 cells Ptch1 is upregulated in response to Shh stimulation, whereas Ptch2 is not (Carpenter et al. 1998).These discrepancies could be explained by cell type specific regulation mechanisms of Ptch1 and Ptch2, and could explain the differences observed in luciferase experiments in the literature.

In this study we further attempt to determine if Ptch1 and Ptch2 have overlapping roles by determining if Ptch2 represses Shh signaling in Ptch1's absence and if Ptch1 and Ptch2 mutants display similar functions *in vivo* and in *vitro*.

Results

The proton-driven antiporter activity of Ptch1 can mediate the inhibition of Smo

Ptch1 is a putative member of the Resistance, Nodulation and Division (RND) family of proton-driven antiporters (Taipale et al., 2002). This transporter family shares a conserved aspartic acid residue in the fourth trans-membrane region (Van Bambeke, Balzi, and Tulkens 2000). Mutating this residue in other members of the RND family, including Disp1 (Etheridge et al., 2010), results in dominant-negative molecules that are able to inhibit the antiporter function of normal endogenous proteins. Expressing a Ptch1 allele lacking antiporter activity (Ptch1D499A) (Taipale et al., 2002) in the chick neural tube does not recapitulate the loss of Ptch1 function in mouse embryos (Goodrich et al., 1997), since we did not observe an increase in Shh activation as assessed by changes in Shh-mediated dorsoventral patterning (Figure 7 A,B). On occasion we did find some cells expressing Pax7 ectopically, indicating a minor loss of Shh signaling (Figure 8). We attribute this to the ability of Ptch1D499A to sequester Shh away from endogenous Ptch1, leading to both an autonomous and non-autonomous inhibition of Shh signaling.

For members of the RND family to act as dominant negatives, they must retain the ability to form trimers (Hiroshi Nikaido and Takatsuka 2009).It remains a possibility

that the electroporated mouse Ptch1 cannot form trimers with endogenous chicken Ptch1. We
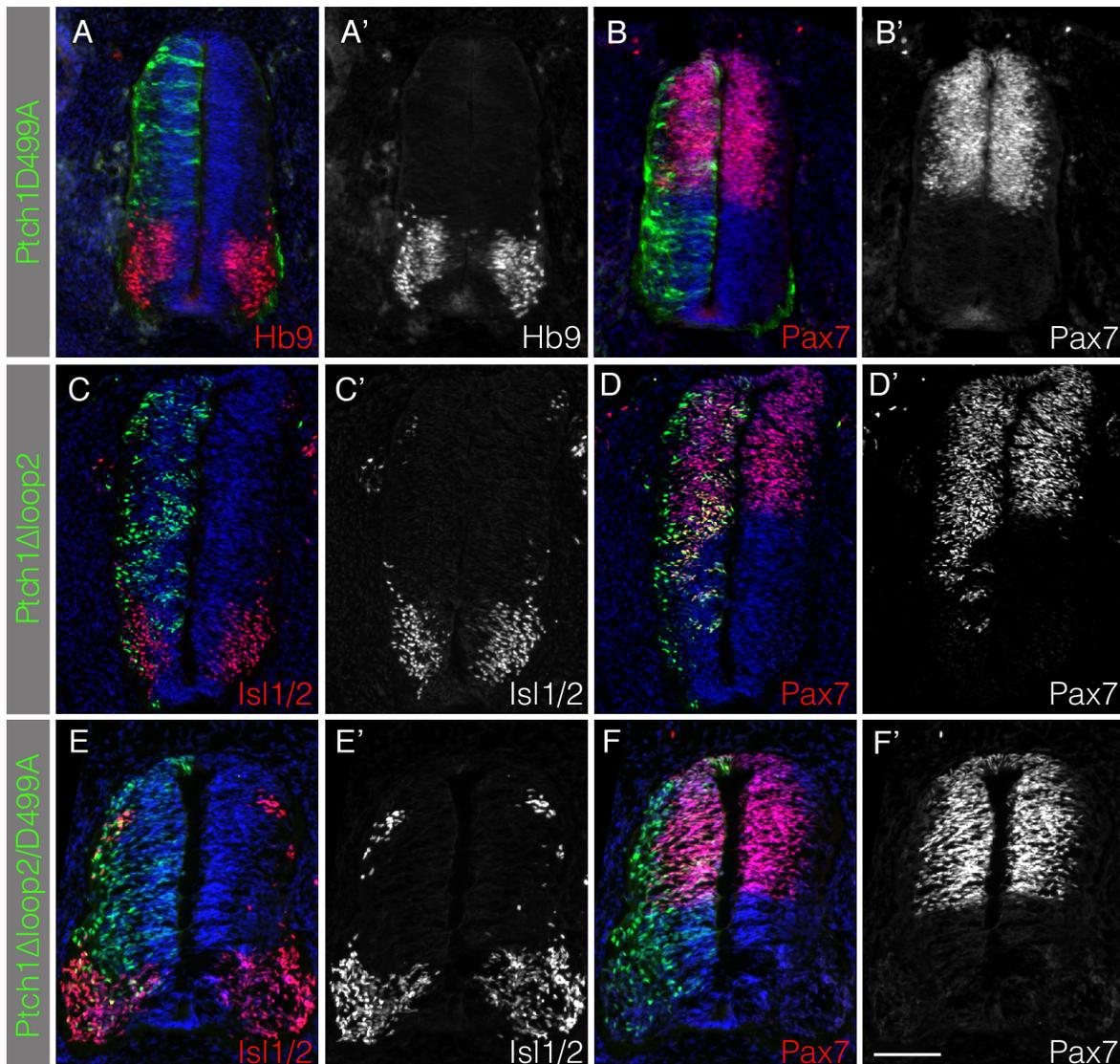
Figure 7. Inhibition of Smo is mediated by the proton-driven antiporter activity of Ptch1 Cross sections of stage 20 HH chicken neural tubes electroporated with *pMES-mPtch1D499A* (A, B), *pCIG-mPtch1Δloop2* (C, D) and *pCIG-mPtch1Δloop2/D499A* (E, F) are labeled in green. Sections are stained with antibodies to Hb9 (A), Islet1/2 (C, E), or Pax7 (B, D, F) as labeled in red or represented in the corresponding gray scale image ('), and DAPI nuclear stain is labeled in blue. Scale bar (F') is 50μm.

therefore tested if chicken Ptch1 lacking antiporter activity was able to induce the Shh response, after misexpression in the developing neural tube. Again we observed little effect on neural tube patterning (Figure 8), indicating that suppressing the proton-driven antiporter activity of Ptch1 has little effect on the Shh response. The inability of Ptch1D499A to apparently act a dominant-negative inhibitor of endogenous Ptch1 raises the question if the proton-driven antiporter activity is important to regulate the Shh response at these stages of development.

Ptch1Dloop2, a deletion mutant of Ptch1 that is unable to bind Shh is a potent inhibitor of the Shh response. Consistent with an earlier observation (Briscoe et al., 2001), we found that expression of Ptch1Dloop2, had a strong cell-autonomous inhibitory effect on the Shh response (Figure 7C,D). To assess if this effect is mediated by its antiporter activity we expressed a Ptch1 allele that was unable to bind Shh but also lacks antiporter activity, Ptch1Dloop2/D499A. Ptch1Dloop2/D499A had no effect on Shh activity based on the lack of ectopic cell autonomous Pax7 induction, and only mildly inhibited motor neuron induction, as determined by Isl1/2 expression (Figure 7E,F).
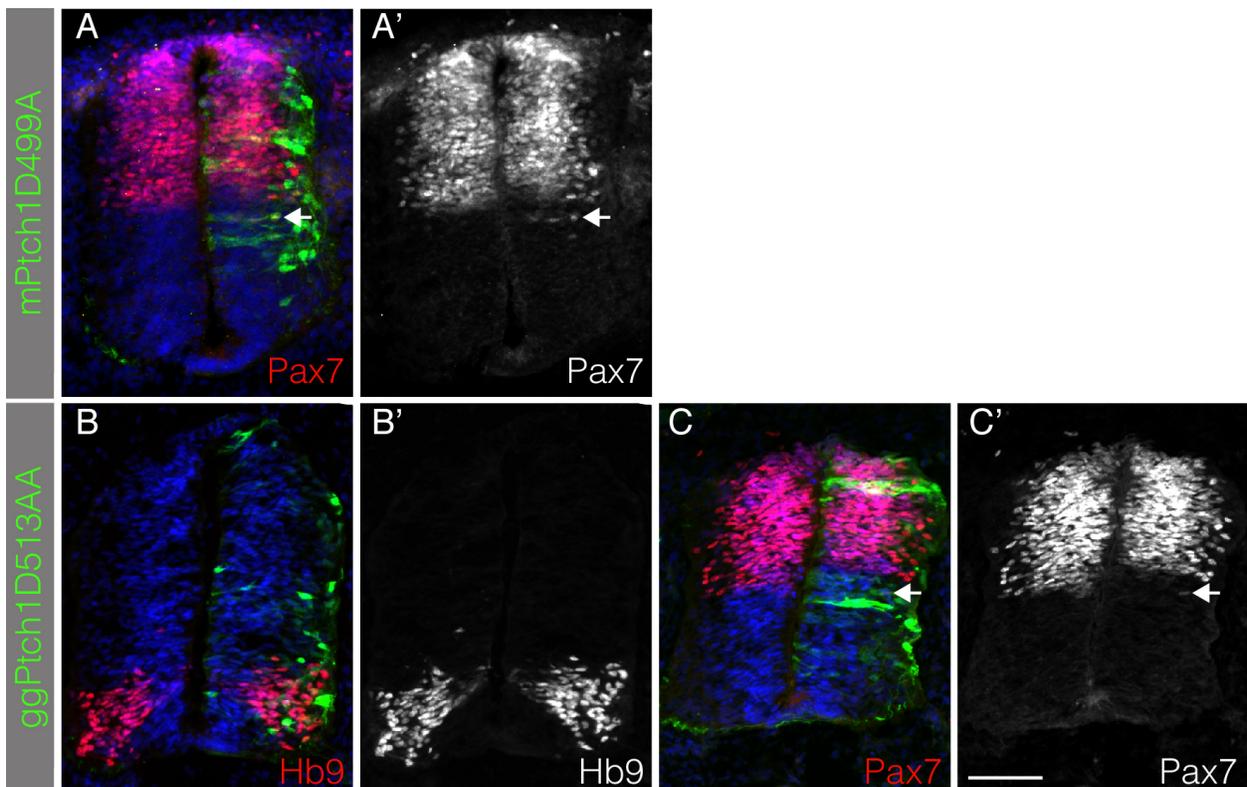
Figure 8. Overexpression of mPtch1D499AA and ggPtch1D513A mutants. Cross-sections of chicken neural tubes electroporated with *pMES-mPtch1D499A* (A) and a chicken antiporter Ptch1 mutant, *pMES-ggPtch1D513A* (B). Electroporated cells are labeled in green (GFP) and sections are stained with antibodies to Pax7 (A, C), Hb9 (B) as labeled in red or represented in the corresponding gray scale image ('), and DAPI nuclear in blue. Arrows in A, A', C, and C' indicate a cell autonomous induction of Pax7 expression by the Ptch1 antiporter mutants. Scale bar ~50 μm (C') is 50μm.

The dramatic difference between the strong inhibition of the Shh response by Ptch1Dloop2 and the mild effects of Ptch1Dloop2/D499A demonstrates that the proton-driven antiporter activity is critical for Smo inhibition by Ptch1Dloop2. Importantly, the loss of repressive activity of Ptch1 did not automatically result in the cell-autonomous activation of the Shh response, indicating that Ptch1Dloop2/D499A is not a strong inhibitor of endogenous Ptch1 function.

To further assess Ptch1ΔL2 and Ptch1ΔL2D499A's function. An *in vivo* assay was created to test Ptch1ΔL2 or Ptch1ΔL2D499A's ability repress an activated Shh response. Ptch1ΔL2 or Ptch1ΔL2D499AA were co-electroporated along with SmoM2 into the neural tube of HH stage 10 chick embryos. SmoM2, is a mutant form of Smo that is a strong activator of the Shh response (Xie et al. 1998). Whereas, Ptch1ΔL2 was able to repress SmoM2's Shh pathway activation, Ptch1ΔL2D499AA was unable to repress this activity. As seen in (Figure 9A), co-electroporation of Ptch1ΔL2 and SmoM2 resulted in a slight expansion of the Pax7 domain, indicative of a down-regulation of the Shh pathway. Thus, any SmoM2 induced upregulation of the Shh
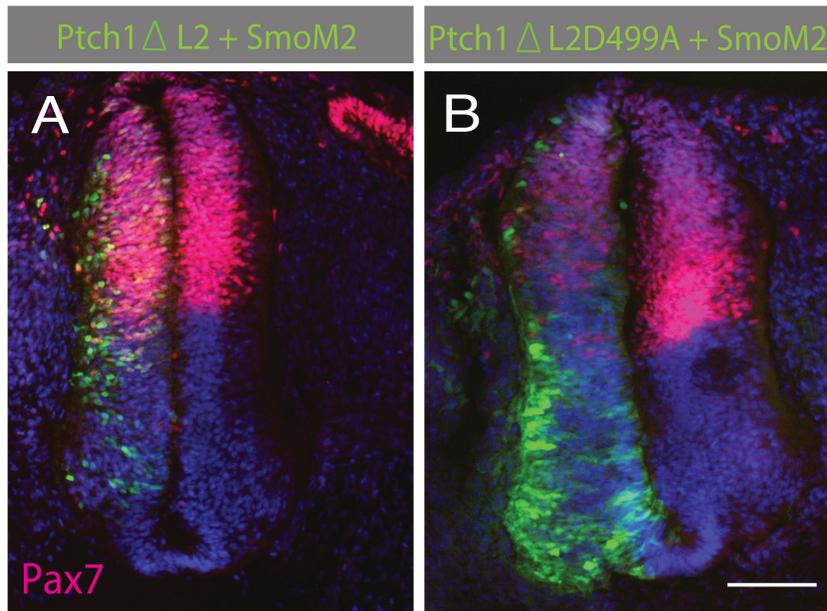
Figure 9. Co-electroporation of Ptch1ΔL2 and Smo-M2 or Ptch1ΔL2D499AA and Smo-M2 into HH stage 10 chick embryos. (A) Co-electroporation of Ptch1ΔL2 and Smo-M2 results in expansion of the Pax7 domain, nuclei expressing Pax7 are shown in magenta. (B) Co-electroporation of Ptch1ΔL2D499A and Smo-M2 results in repression of Pax7 expression, patches of nuclei positive for Ptch1ΔL2D499A and Smo-M2 are negative for Pax7 expression, nuclei expressing Pax7 are shown in magenta. Scale bar is 50μm. An n≥4 chick embryos was observed.

pathway was successfully repressed by Ptch1ΔL2. In contrast, co-electroporation of Ptch1ΔL2D499A and SmoM2 resulted in a downregulation of Pax7 expressing nuclei in the Pax7 domain, indicating positive Shh pathway activation (Figure 9B). Collectively, this data confirms (1) that functional Ptch1-Shh binding is necessary for inactivation of Ptch1's repressive activity and (2) that Ptch1's proton-motive force channel is necessary for Ptch1's repressive activity. To assess the activities of the Ptch1 mutants in the absence of endogenous Ptch1 activity, we expressed them in *Ptch1-/-* immortalized mouse embryonic fibroblasts (MEFs)
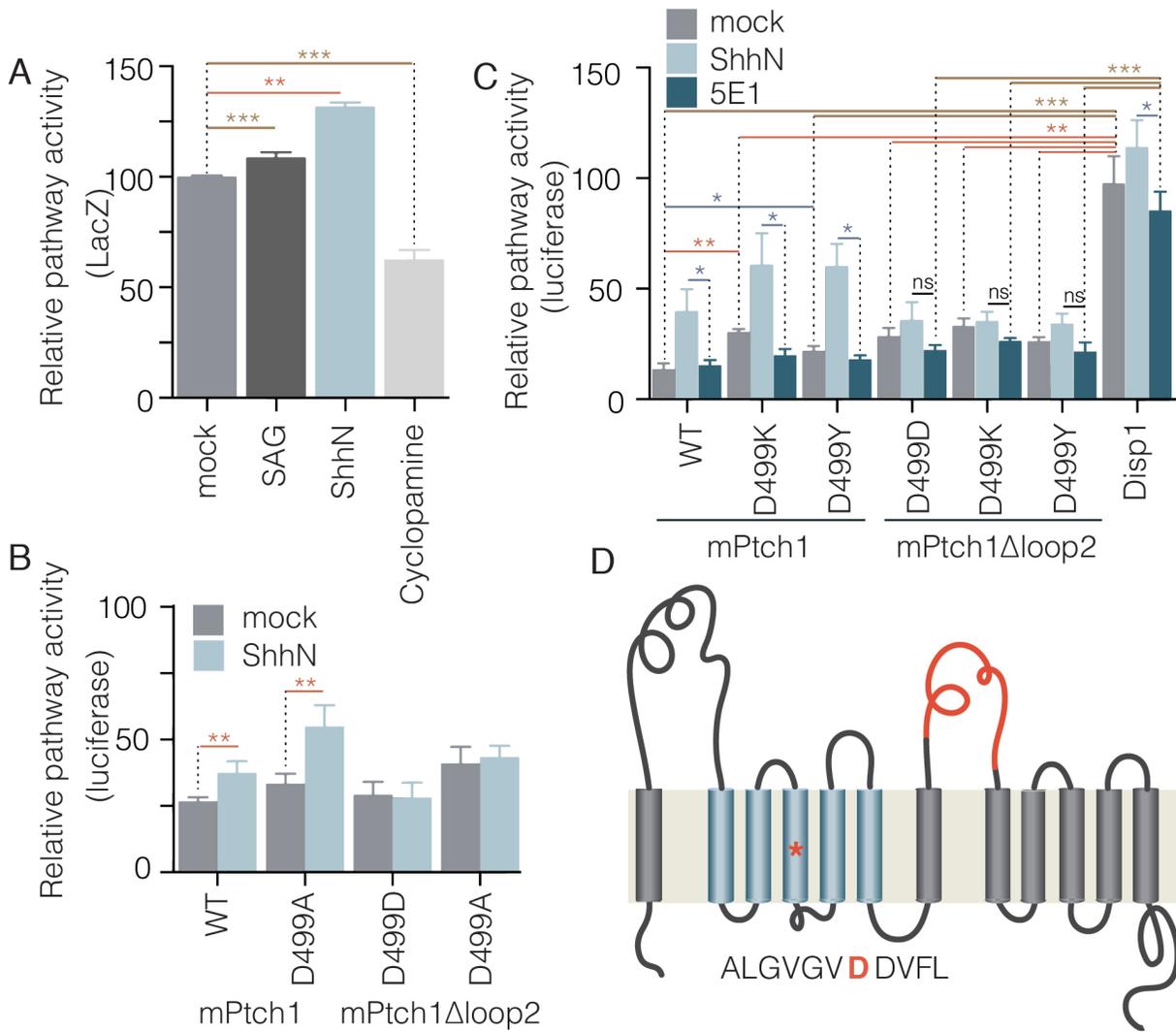
Figure 10. The Shh-binding loop2 of Ptch1 can mediate the Shh response in *Ptch1*-/- fibroblasts independent of the proton-driven antiporter activity.

(A) After *Ptch1*-/- MEFs were grown to confluence, cells were cultured overnight in low serum medium and treated with ShhN conditioned medium, 200 μM SAG, or 1 μM cyclopamine. Cells were lysed and LacZ activity was assed by determining β-galactosidase levels. Data show mean ± SEM from 3 experiments performed in triplicate. (B, C) *Ptch1*-/- MEFs were co-transfected with Ptch1, Ptch1 mutants, or Disp1 as control vector, and a *Gli-luciferase* reporter and *CMV-Renilla*. When transfected cells reached confluence, cells were cultured overnight in low serum and treated with control conditioned medium (mock), ShhN conditioned medium, or 5E1 conditioned medium. Cells were lysed the next day and luciferase activity was measured. Data are shown relative to control (cells transfected with *Disp1*) and treated with control conditioned medium (mock); mean ± SEM from 3 experiments performed in duplicate. In B and C, levels were normalized to the induction level measured in the *Disp1* transfected cells (100). Statistical significance was tested by ANOVA for all forms of Ptch1 vs. Disp1; panel B, one way ANOVA p=0.0015; panel C, two way ANOVA p<0.0001. Relevant pair-wise Student's t-tests are indicated. For A, B, C *, p<0.05; **, p<0.01; ***, p<0.005. (D) Schematic diagram of Ptch1 mutants. The aspartic acid reside labeled in red denotes the antiporter mutation in Ptch1 which is located in the sterol sensing domain labeled in blue. The Shh binding domain located in loop2 is the second large extracellular loop between TM domains 7 and 8.

Ptch1-/- MEFs have an autonomously upregulated Shh response (Taipale et al., 2000) that can be measured due to the integration of the *LacZ* gene into the *Ptch1* locus (Goodrich et al., 1997). We found that SAG, a Smo agonist, further induce Shh pathway activity in the *Ptch1*-/- MEFs, while cyclopamine reduced Shh pathway activity (J. K. Chen et al. 2002; Taipale et al. 2002) (Figure 10A). This indicates that despite the absence of Ptch1, Smo can be activated or inhibited in these cells. The addition of ShhN (a truncated and soluble form of Shh) also increased the Shh response, indicating that there is a Ptch1-independent response to Shh.

In line with their abilities to inhibit Smo, we found that expression of Ptch1 and Ptch1Dloop2 decreased the autonomous Shh response, relative to control transfection with Disp1, which was normalized to 100 (Figure 10B). In these experiments we measured the Shh response by co-transfecting a construct in which luciferase is driven by a Shh-inducible promoter (Taipale et al., 2002). Furthermore, whereas *Ptch1*-/- cells expressing Ptch1 were responsive to ShhN, cells expressing Ptch1Dloop2 were unresponsive (Figure 10B), consistent with the inability of Ptch1Dloop2 to bind Shh, mirroring our observations *in vivo* (Figure 10C,D). For comparison, Ptch1+/+ MEFs were assayed (control 100%±18 *vs.* ShhN 239%±52). In line with their abilities to inhibit Smo, we found that expression of Ptch1 and Ptch1Dloop2 decreased the autonomous Shh response (Figure 10C). To test if the downregulation of the Shh response pathway required the antiporter activity of Ptch1, we expressed the antiporter mutant and found an increase of the autonomous activation of the Shh response as compared to wild type Ptch1 (Figure 8B,C). Nevertheless, these Ptch1 mutants repressed Smo to a much greater degree than the negative control, Disp1. Moreover, cells expressing Ptch1 antiporter mutants retained their sensitivity to ShhN (Figure 10B). This demonstrates that Smo inhibition can be regulated independently of Ptch1 antiporter activity. Combining mutations that antagonize both the proton-driven antiporter activity of Ptch1 as well as Shh binding in the same molecule resulted in forms of Ptch1 that blocked the response to ShhN in *Ptch1*-/- cells (Figure 10B). We expanded this experiment using different mutations in the putative proton pore, replacing the critical aspartic acid with a lysine or tyrosine residue (Ptch1D499K and Ptch1D499Y), and combined these mutations with the Shh binding deletion (Ptch1Dloop2/D499K and Ptch1Dloop2/D499Y). To address the ligand dependency we treated these cells with ShhN or 5E1, a Shh specific monoclonal antibody. Similar to Ptch1D499A, we found that cells expressing Ptch1D499K or Ptch1D499Y retained their ability to respond to ShhN, but mutants combining the antiporter activity mutations with the loop2 deletion resulted in forms of Ptch1 that were unable to mediate the Shh response in *Ptch1*-/- cells, but nevertheless inhibited Smo as compared to our control, Disp1 (Figure 10C)*.*

These results raise the question of how forms of Ptch1 that are unable to bind Shh and repress Smo can nevertheless still inhibit the Shh response. Since these experiments were performed in *Ptch1*-/- cells, Ptch1Dloop2/D499X mutant alleles must inhibit the Shh response independent of endogenous Ptch1. They also support the notion that Shh can induce Smo activity via a mechanism that does not involve Ptch1 antiporter activity.

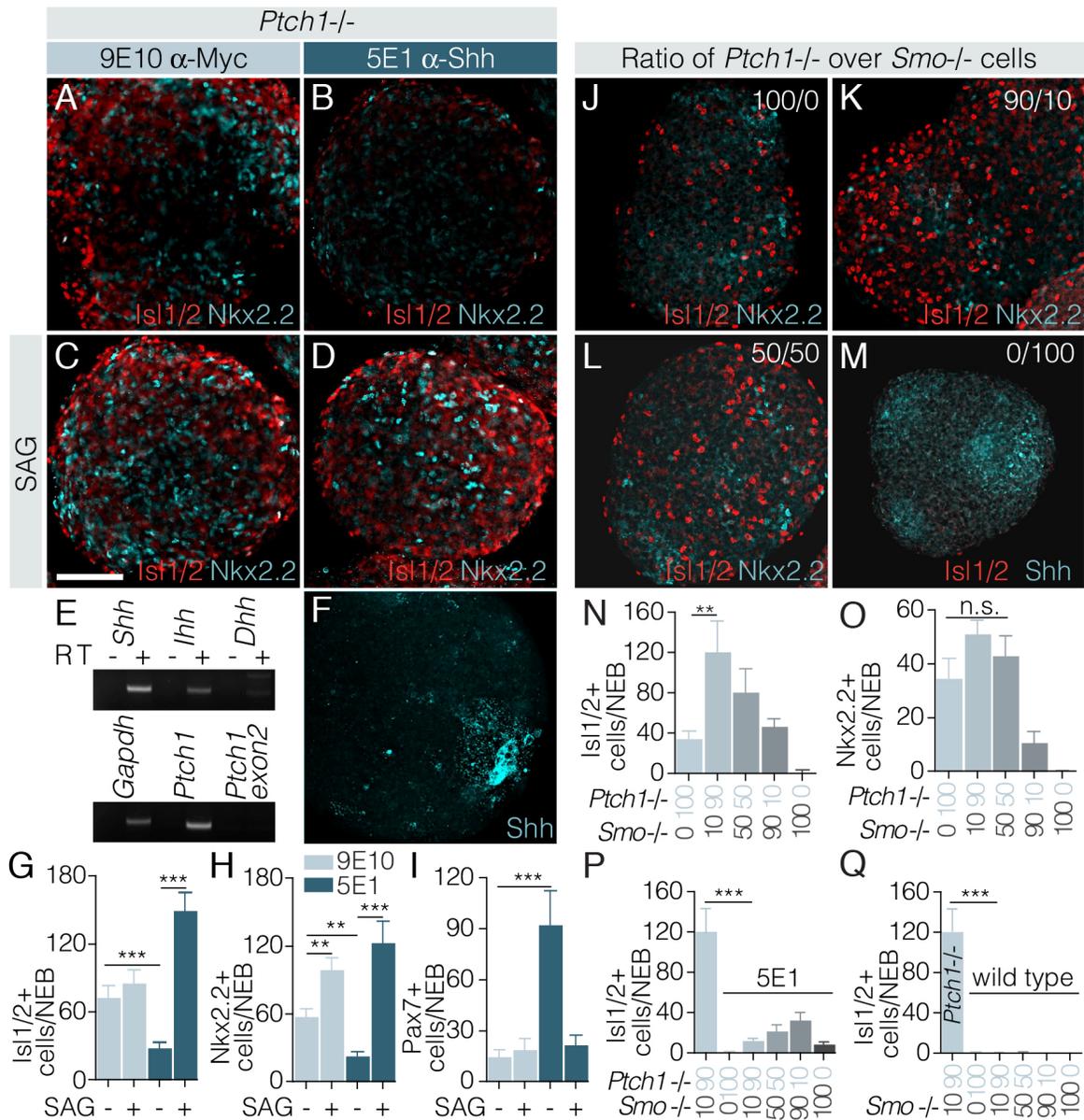**Neuralized *Ptch1*-/- embryonic stem cells remain Shh- dependent for the induction of ventral cell types**

Mouse embryonic stem cells (mESCs), aggregated in defined medium containing retinoic acid, form neuralized embryoid bodies (NEBs) that closely resemble the early

caudal neural tube (Wichterle et al. 2002). Consistent with the inhibitory role of Ptch1 on Smo, we have shown that in the absence of exogenous Shh, *Ptch1*-/- NEBs have higher expression levels of Shh-induced differentiation markers than wild type NEBs. Smo is required for the Shh response and in concordance with this observation we found that *Smo*-/- NEBs cannot respond to Shh(Crawford and Roelink 2007). To determine if endogenously produced Shh is responsible for the induction of Shh-mediated differentiation in the absence of *Ptch1*, *Ptch1*-/- NEBs were cultured in the presence of the Shh-blocking antibody 5E1 (Ericson et al. 1996) or an α-Myc antibody (9E10) (Chan et al. 1987)as a control. After 5 days in culture, NEBs were analyzed for expression of Isl1/2 and Nkx2.2, transcription factors that are induced by activation of the Shh response (Briscoe et al. 1999) and Pax7, which is inhibited by Shh signaling (Ericson et al., 1996). In *Ptch1*-/- NEBs cultured with 5E1, Isl1/2 and Nkx2.2 expression was reduced compared to the 9E10 treated *Ptch1*-/- NEBs (Figure 11A, B, G, H). This loss of ventral cell types was concomitant with an increase of Pax7 expression, further demonstrating that the upregulation of the Shh response in *Ptch1*-/- cells is not due to an autonomous loss of Smo inhibition, but is at least in part dependent on the presence of Shh in the NEBs (Figure 11I). Both RT-PCR and immunofluorescence showed abundant Shh expression in *Ptch1*-/- NEBs (Figure 11E, F). Moreover, in the absence of Ptch1 function, the number of cells expressing Isl1/2 and Nkx2.2 was increased by the Smo agonist SAG (Chen et al., 2002), and the number of cells expressing Pax7 was suppressed, regardless of the presence of 5E1 (Figure 11C, D, G, H), indicating that even in absence of Ptch1, Smo was not fully activated.

To demonstrate that *Ptch1*-/- cells can respond to Shh delivered *in trans*, we generated mixed NEBs composed of varying ratios of *Ptch1*-/- and *Smo*-/- mESCs (Figure 11J-O). Neuralized *Smo*-/- mESCs are unable to respond to Shh itself but this particular clone expresses Shh (Figure 11M). Shh derived from these *Smo*-/- cells induced Nkx2.2 and Isl1/2 expression in *Ptch1*-/- cells. This induction could be blocked by the inclusion of 5E1 demonstrating that this induction is mediated by Shh (Figure 11P). Wild type mESCs did not display an induction of their Shh response when co-aggregated with *Smo*-/- mESCs (Figure 11Q). It appears that the concentration of Shh provided by the *Smo*-/- cells within the NEB is not sufficient to activate the response in wild type cells. These results demonstrate that *Ptch1*-/- mESCs are more sensitive to Shh than wild type mESCs, but nevertheless remain dependent on the ligand for full induction of the Shh response. Based on our results using blocking antibodies, we wanted to further address the requirement of Shh in cells by creating genetic nulls.

Ptch1-/-;Shh-/- cells respond to exogenous Shh
To determine if endogenous Shh mediates the Shh response in *Ptch1*-/- cells, we made mutations in the *Shh* locus of *Ptch1*+/- and *Ptch1*-/- mESCs using

Figure 11. Activation of the Shh response in *Ptch1*-/- mESCs is induced by Shh

(A-D) Embryoid bodies (NEBs) derived from *Ptch1*-/- mESCs were neuralized with 1 µM retinoic acid (RA) in the presence of 1:5 α-Shh 5E1 supernatant (B, D), control α-Myc 9E10 supernatant (A, C). (C, D) 200 nM SAG was added. Nkx2.2 and Isl1/2 expression was assessed by immunofluorescence after 6 d. (E) RT-PCR analysis for indicated transcripts was performed on RNA isolated from *Ptch1*-/- NEBs. (F) Shh expression was assessed by immunofluorescence using 5E1. (G-I) Numbers of Isl1/2+ (G), Nkx2.2+ (H), or Pax7+ (I) cells per NEB were quantified. Shown is mean ± SEM; n≥20; ***, p<0.005; **, p<0.01. (J-M) *Ptch1*-/- mESCs were mixed with *Smo*-/- mESCs at indicated ratios. Derived NEBs were neuralized with 1 µM retinoic acid and after 7 d, Nkx2.2, Isl1/2, and Shh expression was assessed. (N) Number of Isl1/2$^+$ or (O) Nkx2.2$^+$ cells per NEB was quantified. (P) *Ptch1*-/- mESCs were mixed with *Smo*-/- mESCs, neuralized, and treated with 1:5 α-Shh 5E1 supernatant. Isl1/2 expression was assessed. (Q) Wild type (AB1) mESCs were mixed with *Smo*-/- mESCs, neuralized, and Isl1/2 expression was assessed. Shown is mean ± SEM n≥20; **, p<0.01.

transcription activator-like effector nucleases (TALENs) (Cermak et al., 2011) directed against an amino-terminal coding sequence of *Shh*. The *Shh-/-* clones were detected by sequencing of PCR products surrounding the area targeted by the TALENs. The clones used for subsequent experiments had small deletions in both *Shh* alleles that caused premature stop codons, resulting in protein products truncated soon after the signal sequence. NEBs derived from these cells were grown in the absence or presence of 5nM ShhN and the induction of Nkx2.2 positive cells was assessed.

In NEBs derived from both the *Ptch1+/-;Shh-/-* and *Ptch1-/-;Shh-/-* cells we observed a significant induction of Nkx2.2 positive cells by ShhN (Figure 12A,B). In addition, we found that the *Ptch1* promoter, as measured by the induction of LacZ was induced in the *Ptch1-/-;Shh-/-* cells in response to ShhN (Figure 12C). These results

Figure 12. *Ptch1-/-;Shh-/-* cells respond to Shh (A) Embryoid bodies (EBs) derived from *Ptch1-/-;Shh-/-* mESCs were neuralized with 1 µM retinoic acid in the absence (mock) or presence of ShhN. At day 5 the EBS were stained for Nkx2.2. (B) the Shh mediated induction of Nkx2.2 was quantified in *Ptch1+/-;Shh-/-* and *Ptch1-/-;Shh-/-* neuralized EBs. Positive cells per EB were counted. Shown is mean ± SEM; n≥20; ***, p<0.005. (C) The ShhN-mediated induction of LacZ driven by the Ptch1 promoter was measured in *Ptch1+/-;Shh-/-* and *Ptch1-/-;Shh-/-* EBs. The average of 5 experiments is shown, ± SEM; ***, p<0.005; **, p<0.01.

show that cells without Ptch1 are sensitive to Shh, and that the upregulation of the Shh response in *Ptch1-/-* cells is at least in part mediated by endogenous Shh. To further assess the mechanism of Ptch1-independent signaling, we tested the ability of *Ptch1-/-* cells to migrate towards a localized source of Shh. This Shh chemotaxis response is fast, and independent of transcription and the primary cilium (Bijlsma et al., 2012). Since this response does require Smo, it assesses more directly upstream events in the Shh response.

Shh chemotaxis is unaffected in the absence of Ptch1, but remains dependent on Cdon and Boc

We tested the migratory response of *Ptch1-/-* and *Ptch1+/+* immortalized MEFs in a modified Boyden chamber assay (Figure 14). Shh chemotaxis of *Ptch1+/+*
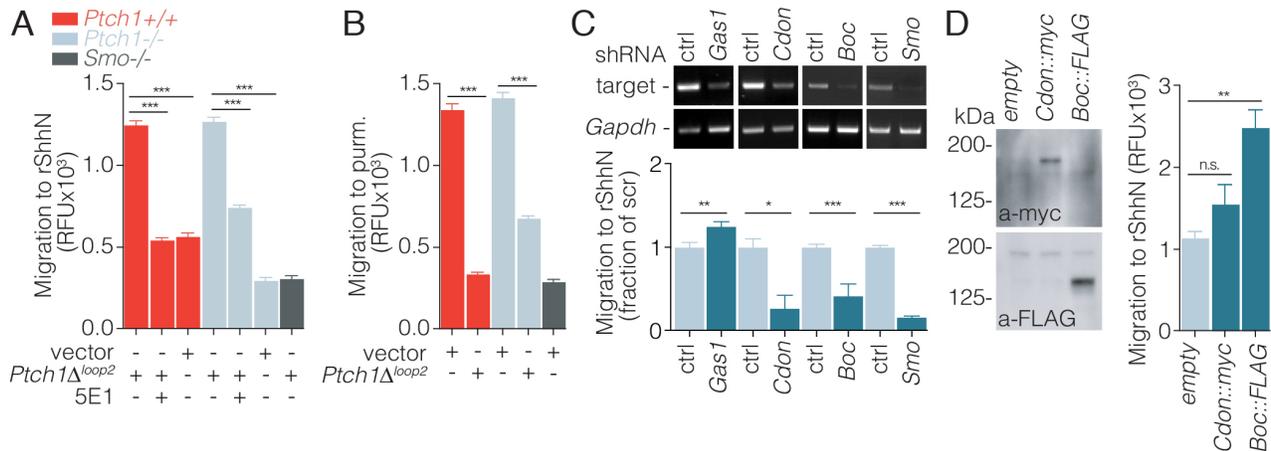
Figure 13. Fibroblast chemotaxis to Shh does not require Ptch1, but is sensitive to Ptch1-mediated inhibition
(A) *Ptch1+/+* and *Ptch1-/-* MEFs were transfected with vector or *Ptch1Δloop2*, and net migration to 5 nM ShhN was assessed in the absence or presence of 5E1. Vector transfected *Smo-/-* MEFs were included as a control. For technical and quantitative details, see Experimental Procedures. Shown is net migration from 6 experiments, ± SEM; ***, p<0.005. (B) As for panel A, using 2μM purmorphamine. Purmorphamine was used rather than SAG, as it is a more consistent Smo agonist in chemotaxis experiments. (C) *Ptch1-/-* MEFs were stably transduced with shRNA constructs against indicated genes or non-silencing controls (ctrl). RT-PCR was performed to assess knockdown efficiency, and net migration to ShhN was assessed. Shown is average net migration from 3 experiments, ± SEM; *, p<0.05; ***, p<0.005. Statistical significance was assessed by Student's t-test. (D) *Ptch1-/-* MEFs were stably transduced with indicated constructs and Western blot analysis was performed to assess expression levels. Subsequently, net migration of transduced MEFs to ShhN was measured. Shown is net migration from 3 experiments, ± SEM; **, p<0.01.

cells does not require transcription (Bijlsma et al., 2008), but is dependent on Smo. The migratory response to ShhN and purmorphamine of *Ptch1-/-* cells was very similar to that of *Ptch1+/+* cells (Figure 13A, B), and this assay thus provides us with a robust Ptch1- independent response to Shh. The migration was specific towards ShhN, since it was inhibited by the inclusion of 5E1 in the upper and lower compartments of the Boyden chamber. Migration to both ShhN and purmorphamine could be ablated by expressing Ptch1Dloop2 or performing the assay in a Smo deficient background (Figure 13A, B). These results indicate that Ptch1 is not required for migration of MEFs towards sources of Shh, further supporting the notion that Ptch1 is not required for cell to respond to Shh.
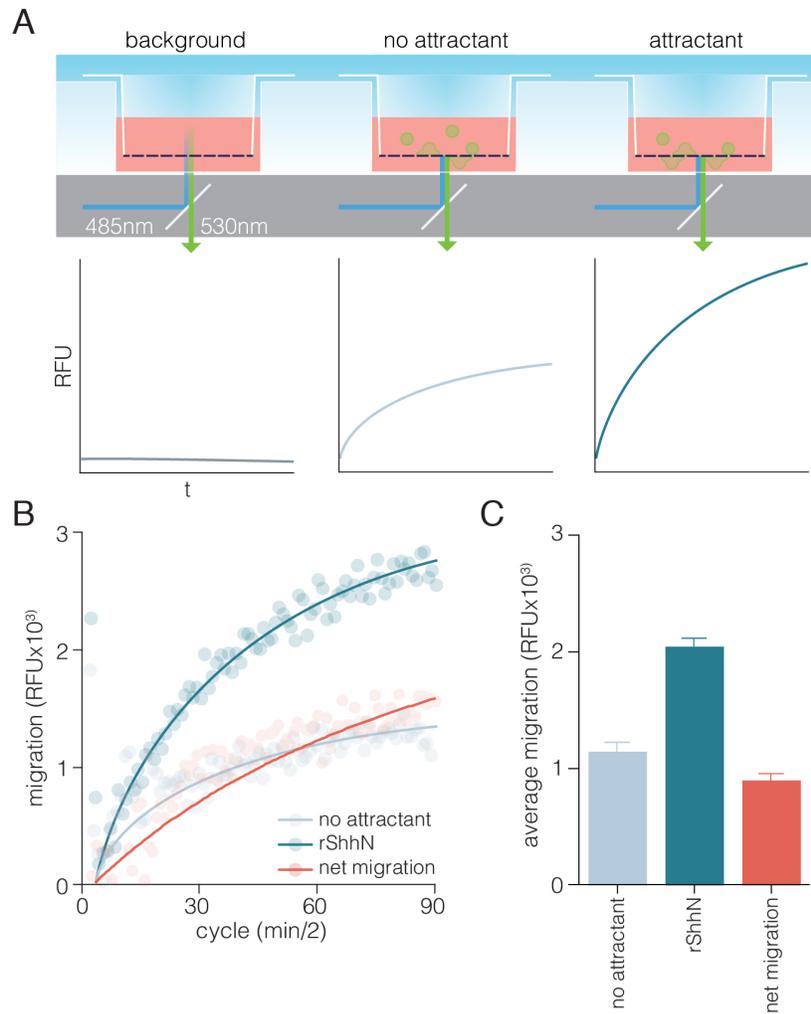
Figure 14. Summary of the modified Boyden chamber migration assay and analysis of results

(A) A Fluoroblok Transwell plate and insert setup is used to measure fluorescence from labeled cells that have migrated through a fluorescence blocking membrane with 8 μm pores. Background fluorescence is measured in time from a well containing medium and these values are subtracted from all other measurements. The 'no attractant' control measures basic cell movement (i.e. movement other than that towards the chemoattractant) for every cell type, transfectant, or other experimental condition. These values are then subtracted from those obtained in the presence of chemoattractant in the bottom compartment of the Transwell setup to yield the specific migration towards a given attractant. Representation as formula; net migration = $(RFU_{attractant}-RFU_{BKG})-(RFU_{no\ att}-RFU_{BKG})$. (B) An example of a chemotaxis experiment using rShhN is shown. Background values are already subtracted, and starting points of migration are set to y=0. To yield specific net migration (indicated in red), values for the no attractant control are subtracted from the chemotaxis towards 5 nM recombinant ShhN. Note that line curves are fitted, rather than plotted means for clarity. (C) The average of migration measured over time as shown in panel B plotted as bar graphs.

We next examined what receptors could potentially perceive Shh in *Ptch1*-/- cells. Several Shh binding proteins such as Gas1, Cdon, and Boc have been proposed to function as co-receptors acting in conjunction with Ptch1 (Allen et al., 2011; Izzi et al., 2011; Tenzen et al., 2006). We tested if these molecules mediated the Shh response in the absence of Ptch1. Stable *Gas1* knockdown in *Ptch1*-/- MEFs did not affect Shh chemotaxis, but stable knockdown of *Cdon* and *Boc* diminished this response (Figure 13C). This effect was confirmed using a transient silencing strategy (Figure 15).
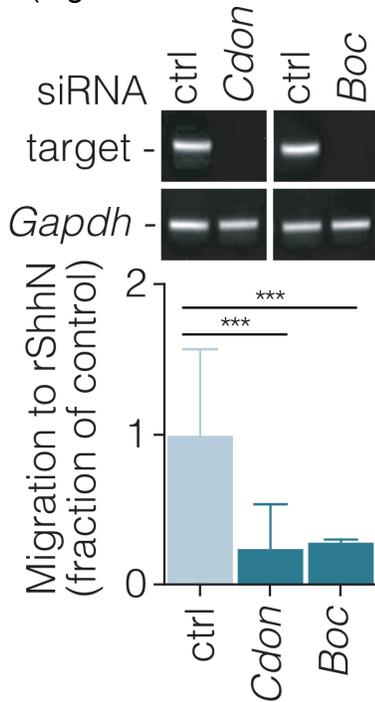
Figure 15. Fibroblast chemotaxis to rShhN requires Cdon and Boc *Ptch1*-/- MEFs were transiently transfected with siRNA against *Cdon, Boc,* or scrambled control and knockdown was assessed by RT-PCR. Net migration to ShhN was assessed as for Figure 3. Shown is average net migration from 3 experiments, ± SEM; *, p<0.05; ***, p<0.005. Statistical significance was assessed by Student's t-test.

Ptch1-/- MEFs stably expressing Cdon or Boc showed an increased chemotactic response to ShhN (Figure 13D). These experiments indicate that the related Shh (co-)receptors Cdon and Boc mediate Shh chemotaxis even in the absence of Ptch1. Since Boc and Cdon are thought to form complexes with Ptch1 and Shh (Izzi et al., 2011), it is a distinct possibility that that they can also form such complexes with Ptch2.

Ptch1-/-;*Ptch2*-/- NEBs have a higher level of Shh pathway activation than *Ptch1*-/- NEBs

To assess if Ptch2 is required for the Ptch1-independent response, we mutated the *Ptch2* locus in *Ptch1*-/- mESCs and found that in NEBs derived from *Ptch1*-/-;*Ptch2*-/- cells, the Shh response was higher than in the *Ptch1*-/- and *Ptch1*+/- NEBs (Figure 16A-C). This indicates that the Shh response in *Ptch1*-/- cells is inhibited by Ptch2. The *Ptch1*-/-;*Ptch2*-/- NEBs had more Nkx2.2+ cells and fewer Isl1/2+ cells than the *Ptch1*-/- NEBs. We conclude that cells in *Ptch1*-/-;*Ptch2*-/- NEBs acquire an even more ventral phenotype resulting in a loss of the number of motorneurons induced. We indeed found that in *Ptch1*-/-;*Ptch2*-/- NEBs the Shh-inducible *Ptch1* promoter was considerably more active than in *Ptch1*-/- and *Ptch1*+/- NEBs (Figure 16D). We were unable to further alter the Shh pathway activation level by inclusion of the Smo agonist SAG (Figure 16D). This indicates that in the absence of both Ptch1 and Ptch2, Smo activation via its heptahelical domain is saturated (Chen et al., 2002). It is thought that Ptch1, via its proton driven antiporter activity re-localizes a sterol that inhibits Smo at this heptahelical site, and our results thus indicate that Ptch2 could also fulfill this role.

Further mutation of *Ptch1*-/- and *Ptch1*-/-;*Ptch2*-/- cells revealed that the responses observed in these cells are Smo mediated. phenotype from one of high Shh

activity to Shh unresponsiveness. Both cell lines were responsive to Smo inactivation, displaying an upregulation of Pax7 upon Smo deletion, establishing that the phenotypes observed in these cell lines were Shh pathway dependent (Figure 17F, H). These results indicate that Ptch2 functions as a repressor and that Ptch2's repressive activity is evident in the absence of Ptch1. Additionally, these results suggest that Ptch1 and Ptch2 have overlapping functions.
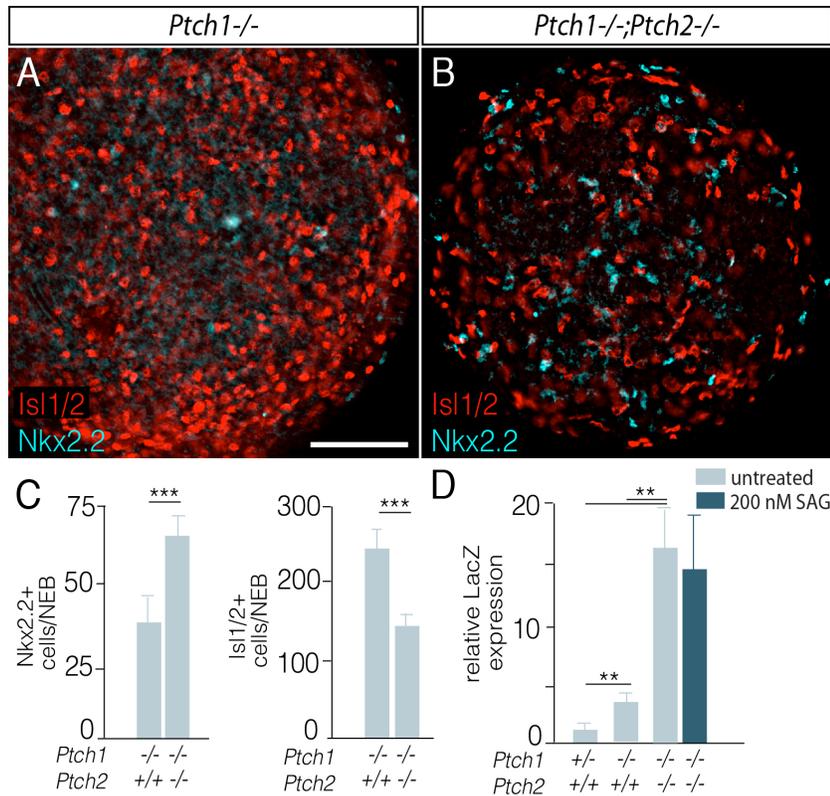
Figure 16. *Ptch1-/-;Shh-/-* cells respond to Shh (A**)** Embryoid bodies (EBs) derived from *Ptch1-/-;Shh-/-* mESCs were neuralized with 1 µM retinoic acid in the absence (mock) or presence of ShhN. At day 5 the EBS were stained for Nkx2.2. (B) the Shh mediated induction of Nkx2.2 was quantified in *Ptch1+/-;Shh-/-* and *Ptch1-/-;Shh-/-* neuralized EBs. Positive cells per EB were counted. Shown is mean ± SEM; n≥20; ***, p<0.005. (C) The ShhN-mediated induction of LacZ driven by the Ptch1 promoter was measured in *Ptch1+/- ;Shh-/-* and *Ptch1-/-;Shh-/-* EBs. The average of 5 experiments is shown, ± SEM; ***, p<0.005; **, p<0.01.

Figure 17. Compound Ptch1, Ptch2, and Smo mutants display activation or down-regulation of the Shh response accordingly. Using TALEN guided genome editing mouse embryonic stem cells (mESCs) with compound mutations were produced. Pax7 expression is shown in red, Nkx2.2 expression is shown in green. mESCs shown are *Ptch1-/-;Ptch2-/-*, (B,F), *Ptch1-/-* (A,E), *Ptch1-/-;Smo-/-* (C,G), *Ptch1-/-;Ptch2-/-;Smo-/-* (D,H). *Ptch1-/-;Ptch2-/-;Smo -/-* . Number of Pax7 (red bars) or Nkx2.2 (green bars) positive nuclei counted per EB (I). Data shown are the average of two separate experiments with an n= 20 EBs per mESC phenotype. The differences observed between Pax7 and Nkx2.2 for each phenotype shown are statistically significant as determined by a student's t-test p<0.05. Scale bar is 20 μm.

Expression of Ptch2 antiporter mutants induces the Shh response in vivo

We assessed whether the proton-driven antiporter activity of the Ptch1 paralogue Ptch2 is involved in regulating the Shh response in vivo. Ptch2 has been shown to modulate the Shh response in mouse embryos (Holtz et al., 2013). Whereas Ptch1Dloop2 misexpression in chick embryos causes a significant cell autonomous inhibition of the Shh response (Figure 7), we have been unable to find any autonomous inhibitory effects of Ptch2Dloop2 (not shown), suggesting that the inhibitory action of Ptch2 in the developing neural tube is less important than that of Ptch1, consistent with
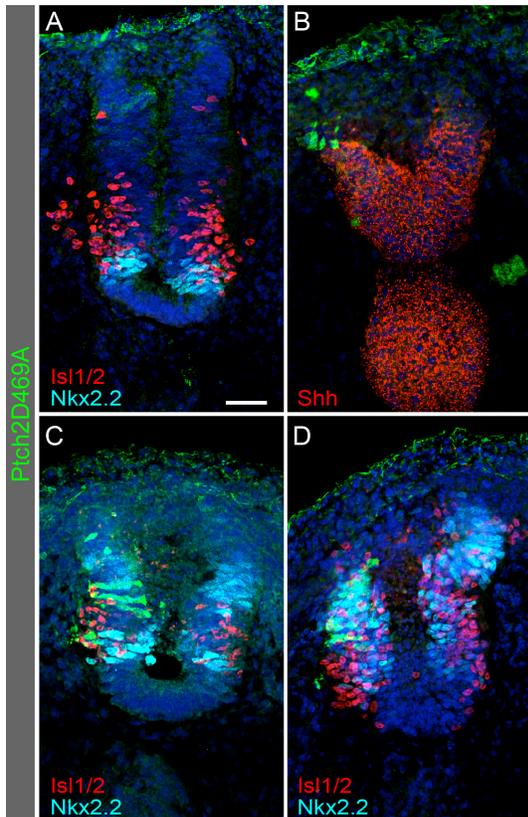
Figure 18. Expression of Ptch2 antiporter mutants causes widespread activation of the Shh response. (A-D) H&H stage 10 embryos were electroporated with *Ptch2D496A*. Electroporated cells are labeled in green (GFP). (A) At most A/P levels the patterning of the neural tube is normal as assessed by Isl1/2 (red) and Nkx2.2 (cyan) expression. (B) At some caudal levels the domain of Shh expression (red) is increased, although most Shh expressing cells do not express *Ptch2D496A.* (C-D) Similarly at some A/P levels neural tube patterning is severely disrupted as visualized by the expression of Nkx2.2 bilaterally dorsal to the normal domain of Nkx2.2 expression (C) or NkX2.2 positive cells (D) dorsal to the Isl1/2 domain (red). A and C show nearby sections, B and D show nearby sections from a different embryo. Scale bar is 10µm.

the normal development of *Ptch2*-/- embryos. To create a dominant negative allele of Ptch2, we mutated the aspartic acid analogous to the one in Ptch1 to alanine, Ptch2D469A. Like Ptch1D499A, expression of Ptch2D469A, did not cause cell autonomous changes in Shh induced patterning (Figure 18A). Co-expression of Ptch2D469A and Ptch1D499A also failed to affect neural tube patterning cell-autonomously (not shown). However, in 4 out of twenty 20 embryos electroporated with *Ptch2D469A* we found widespread upregulation of Shh expression (Figure 18B) and of the Shh response (Figure 18C,D). These embryos were characterized by a bilateral, additional Nkx2.2 domain, localized dorsal to the normal domain (Figure 18C), or by the widespread expression of Isl1/2 and Nkx2.2 (Figure 18D). The induction of the Shh response was largely non-cell autonomous, and it is likely that the ectopic induction of Nkx2.2 and Isl1/2 is a consequence of the expanded domain of Shh expressing cells. This phenotype bears striking resemblance to the phenotype observed in *Ptch1*-/- mouse embryos, which might indicate that Ptch2D469A can inhibit Ptch1 function *in trans*.

We now demonstrate that *Ptch1*-/- fibroblasts display Shh chemotaxis indistinguishable from wild type cells, indicating that *Ptch1*-/- is not required to mediate this Shh response. Furthermore, we show that upregulation of the Shh response in neuralized embryoid bodies (NEBs) derived from *Ptch1*-/- mESCs is dependent on endogenously expressed Shh by mutating the *Shh* locus in *Ptch1*-/- mESCs, and by treating these cells with a Shh-blocking antibody. The role of Ptch2 in mediating the Shh response in the absence of Ptch1 was further supported by the observation that *Ptch1*-/-;*Ptch2*-/- cells cannot respond to activators of the Shh response, and that expression of a dominant negative Ptch2 mutant results in an activation of the Shh response. Together these results demonstrate that the Shh responses observed in *Ptch1*-/- cells can be mediated by Ptch2.

5 Discussion

Despite the identification of several components of the Shh receptosome, little is known about the molecular details that connect these components. Ptch1 is the Shh pathway's main receptor, however, it functions in the presence of several other receptors. For instance, Boc, Cdo, and Gas1 are all secondary receptors with overlapping and necessary functions in the Shh pathway. Interestingly, loss of Boc, Cdo, or Gas1 in isolation does not result in any, pronounced, adverse Shh related defects. *Boc-/-* and *Cdo-/-* mice display forms of microholoprosencephaly and *Gas1-/-* mice display craniofacial abnormalities, all phenotypes associated with reduced Shh signaling (Cole and Krauss 2003; Allen, Tenzen, and McMahon 2007). However, compound mutants like *Boc-/-, Cdo-/-, Gas1-/-* mice, display phenotypes consistent with a severe loss of Shh activity that parallel those of *Shh-/-* mice (Allen et al. 2011). More curious still is the function of Ptch2, a closely related paralogue of Ptch1. Ptch1 and Ptch2 are presumed to have similar functions, nevertheless, their vivo phenotypes are strikingly different *Ptch1-/-* mice are embryonic lethal, whereas, *Ptch2-/-* mice display no overt developmental defects. Determining the exact role of secondary receptors like Boc, Cdo, Gas1, and Ptch2 is key to elucidating how Shh receptor interactions lead to the activation of Smo, the central step of Shh pathway activation.

Shh binding to Ptch1 or Boc, Cdo and Gas1, alone, is insufficient for Shh pathway activation in vitro

We used Shh-N binding mutants to further address Boc, Cdo, and Gas1's role in the Shh response both *in vivo* and *in vitro*. Shh-N E90A, a Shh ligand with altered specificity, retains its Ptch1 binding but has deficient Boc, Cdo and Gas1 binding. Using two separate *in vitro* experiments we corroborated the previously observed effects of Shh-N E90A from Izzi. et al., further suggesting that this Shh variant binds Ptch1 but does not bind Boc, Cdo and Gas1 (Izzi et al. 2011). In both luciferase assays (Light II cells) and in neuralization assays (mESCs), Shh-N E90A was unable to upregulate Shh signaling comparably to Shh-N WT. We inferred that Shh-N E90A's inability to elicit a Shh response was due to its Boc, Cdo and Gas1 binding deficiency, suggesting that binding of Shh to Ptch1 is insufficient to activate the Shh-pathway.

In contrast, to Shh-N E90A, Shh-N H183A harbors a mutation in the Ptch binding domain of Shh, but retains all known binding residues for Boc, Cdo, and Gas1 (Bosanac et al. 2009; Kavran et al. 2010; Maun et al. 2010; Ohlig et al. 2011). As expected, the Shh-N H183A mutant did not activate the Shh response in the Light II cell assays demonstrating that Shh binding to Boc, Cdo, and Gas1 alone is also insufficient for activation of Gli mediated transcription.

Boc, Cdo, and Gas1 and Ptch1 pull down collectively in co-immunoprecipitation experiments (Izzi et al. 2011). It has been shown that these proteins form distinct Boc/Ptch1, Gas1/Ptch1, Cdo/Gas1 complexes, the existence of these specific Ptch1/co-receptor pairs suggests that distinct co-receptors have been coopted for specific functions in the pathway (Izzi et al. 2011; Bae et al. 2011). What the function of these

multi receptor complexes is and what the interactions between these four receptors are have not been conclusively demonstrated.

One possibility is that the collective binding of these receptors to Shh causes the correct combination of downstream responses for Smo activation. How Ptch1 binding to Shh leads to Smo activation remains a central and contentious question in the Shh field. Whether Ptch1 physically interacts with Smo has been addressed in several studies, yielding contradictory results(Marigo et al. 1996; Stone et al. 1996). Boc and Cdo are transmembrane proteins with intracellular domains that could interact with down stream components of the Shh pathway. Boc for example, has been clearly linked to signaling downstream to Src kinases in Shh mediated axon guidance (Yam and Charron 2013). Gas1 is a GPI linked protein, with no intracellular domain, however, it has been recently identified as a key component of non-canonical Shh signaling, along with G_i proteins and Smo in enteric neurons (Jin et al. 2015).

Additionally, it is possible that Hh binding to Boc, Cdo or Gas1 primes Shh for Ptch1 binding. However, it has been demonstrated that Ihog and Boi, the invertebrate homologs of Boc and Cdo, elicit Hh signaling via Ptch1, however, they do not assist Ptch1 in Shh binding. Cells expressing Ptch and lacking Ihog/Boi retained their ability to sequester Hh ligand. In contrast, Ihog/Boi were unable to sequester and retain Hh ligand in the absence of Ptch1 (Camp et al. 2014). Additionally, the importance of Ptch1 and co-receptor interactions are highlighted by Cdo mutants with perturbed Ptch1 binding, as these Cdo mutants cannot support Shh-dependent gene expression (Bae et al. 2011). Taken together, this suggests that co-receptor and Ptch1 interactions are crucial for Shh binding to multi-receptor complexes and furthermore that Shh binding to Boc/Ptch1, Cdo/Ptch1 or Gas1/Ptch1 complexes is necessary for pathway activation.

Ptch1, Boc, Cdo, and Gas 1 binding and Smo ciliary translocation

The basic framework of the Shh pathway is often thought to be sequential. In its active state Ptch1 represses Smo, upon Shh binding, Ptch1 is inactivated Smo becomes uninhibited, subsequently, Smo translocates to the primary cilium where it interacts with downstream components of the Shh pathway, ultimately causing Gli-mediated transcription. We attempted to address where Boc, Cdo and Gas1 fit into this basic framework. Smo localization to the primary cilium is an important step of the Shh pathway that often coincides with pathway activation. Cells co-cultured with Shh-N WT, localize Smo-GFP to the primary cilium. Shh-N H183A does not cause Smo cilial localization, however, and surprisingly, Shh-N E90A elicited Smo localization to the primary cilium.

I propose a model to explain these observations, whereby the effects of Ptch-1/Shh binding and Boc, Cdo, Gas1/Shh binding can be separated. Ptch1 binding to Shh may be necessary for Smo translocation to the primary cilium, and Shh-N E90A retains its ability to bind Ptch1, therefore, this binding may be sufficient to deactivate Ptch1's repression over Smo. This is an interesting possibility because it suggests that binding to Ptch1 is insufficient to complete the activation of the Shh response but is sufficient to complete part of this process. It will be interesting to see if Boc, Cdo, or Gas1 mediate Ptch1 localization to the primary cilium. Prior studies of the invertebrate homolog of Boc

and Cdo, Ihog, have shown that Ihog overexpression causes a dramatic relocalization of Ptch1 to the cell surface, however, Boc, and Gas1 have been shown to play no part in Ptch1's relocalization to the plasma membrane (Zheng et al. 2010; Izzi et al. 2011).

In total, we have demonstrated that Smo localization to the primary cilium does not necessarily correlate with an active Shh response, this phenomena has been previously reported in NIH 3T3 cells exposed to cyclopamine, an antagonist of the Shh pathway, which causes localization of Smo to the primary cilium (Rohatgi et al. 2009) and have shown that Shh binding to Ptch1 or Boc, Cdo, and Gas1 alone is insufficient to elicit a Shh response *in vitro*.

Shh-N binding mutants function in vivo

Surprisingly, when overexpressed *in vivo*, all Shh-N binding mutants caused Shh-N mediated gene upregulation. Not only did Shh-N E90A and H183A activate Shh signaling, they did so in a way that was indistinguishable from wild type Shh-N. A clear caveat is that our *in vitro* experiments only address signaling in trans. Shh signaling *in vivo* is known to consist of both autonomous (cis) and non-autonomous signaling (trans). Potentially, the presentation of Shh-N mutants in cis or trans is an important factor for pathway activation.

Interestingly, preliminary experiments in *Ptch1-/-;Ptch2-/-* MEFs, support this idea. Overexpression of Shh-N E90A and Shh-N H183A in *Ptch1-/-;Ptch2-/-* MEFs potentiates a Gli driven Shh response, in luciferase assays (data not shown). These results mirror what is observed *in vivo*, where overexpressed Shh-N E90A, and H183A can upregulate Shh signaling autonomously. This may indicate that (1) although the Shh-N mutants are deficient in binding to Ptch1 or Boc, Cdo, and Gas1, respectively, simultaneous interaction with these proteins is not necessary for autonomous Shh pathway activation, (2) The autonomously expressed Shh-N binding mutants by-pass, the Shh binding requirements encountered at the cell surface and can activate the pathway in some intracellular compartment or (3) The phenotypes observed *in vitro* may be a result of artifacts caused by the use of Shh-N supernatants produced through overexpression in 293T's.

It is worth revisiting the common practice of using Shh-N ligand produced via over expression. In its un-cholesterol modified state, Shh is soluble from the cell membrane, and easily collected in a cell supernatant for *in vitro* experiments. There are several inherent problems in using Shh ligand produced from overexpression in 293Ts. Shh-N E90A and Shh-N H183A were confirmed to run at the expected size via Western blot (data not shown), however, it is unclear if these mutants harbored other inherent issues. High levels of protein overexpression can lead to problems in protein production within the cell, causing properly misfolded or modified proteins to be produced.

Furthermore, what is observed *in vivo* could indicate a difference in levels of Shh-N concentration *in vivo* versus *in vitro*. In vitro experiments were conducted with a standard concentration of Shh-N 5 nM. In contrast, it is difficult to determine the concentration of Shh-N presented to cells in the neural tube in vivo. It is possible that the Shh-N mutant concentrations used in-vitro were not sufficient to cause a Shh response. Additionally, conducting a titration in vitro may have revealed that these

mutants in fact where functional but required a much higher active concentration to function comparably to Shh-N WT. Thus, Shh-N E90A and Shh-N H183A could be functional hypomorphs, which can potentiate the Shh response, but can only do so at much higher concentrations than their WT counterpart.

Future experiments should revisit the discrepancy observed in vivo versus in vitro by conducting assays to differentiate between Shh-N mutant signaling in cis versus trans. While it could be difficult to address this issue in detail in vivo, using the chick electroporation model, an *in vitro* experiment would be feasible. Responding Light II cells (responders) could be co-cultured with cells stably expressing specific Shh-N mutants (non-responders), this experimental setup would best replicate trans signaling in the chick neural tube. This experimental avenue could be taken a step further by making stable cell lines expressing full-length Shh mutants. Using TALENS *Shh-/-;ShhE90A* and *Shh-/-;ShhH183A* cells could be produced and used to assay signaling in cis and in trans.

Additionally, Shh-N mutants could be assayed in cell backgrounds devoid of Ptch1, Ptch2, Boc, Cdo and Gas1. Luciferase experiments in *Ptch1-/-;Ptch2-/-;Boc-/-;Cdo-/-;Gas1-/-* cells would truly address the necessity of Boc, Cdo, and Gas1 in activating the Shh pathway. If *Ptch1-/-;Ptch2-/-;Boc-/-;Cdo-/-;Gas1-/-* retain there ability to respond to Shh it is likely this response would be mediated through Smo or some unknown receptor.

Ptch2 represses Shh signaling in the absence of Ptch1

Our results demonstrate that the loss of Ptch1 function is not always sufficient to cell-autonomously initiate maximal Smo-dependent Shh responses, and that Ptch2 mediates the residual responsiveness retained in *Ptch1-/-* cells. In flies, based on the embryonic cuticular phenotype, Ptch is epistatic to Hh, and Smo is epistatic to Ptch, consistent with a cell autonomous activation of Smo in the absence of Ptch. The phenotype of *Ptch1-/-* mouse embryos is also consistent with a cell autonomous activation of Smo, although this issue is clouded by the widespread induction of Shh (Goodrich et al., 1997).

The induction of Shh is in part responsible for the upregulation of the Shh response in the absence of Ptch1. This is evident by 5E1-mediated blockade of endogenous Shh ligand in *Ptch1-/-*-neuralized embryonic bodies (NEBs), which results in the loss of ventral cell types, presumably by preventing Shh binding to its receptors. This notion is further supported by NEBs derived from *Ptch1-/-;Shh-/-* mESCs, which can respond to exogenous ShhN. The ability of *Ptch1-/-* NEBs to respond to endogenous ligand highlights the importance of Shh receptors distinct from Ptch1 within these cells. These results indicate that the interpretation of the phenotype of *Ptch1-/-* embryos is incomplete. Our results predict that the phenotype of *Ptch1-/-;Shh-/-* embryos will be different from *Ptch1-/-* embryos, and that this difference can be attributed to Ptch2-mediated Shh signaling.

Ptch1-/- MEFs also retain the ability to respond to Shh, both transcriptionally and via cell migration. While Shh chemotaxis is very similar in *Ptch1-/-* and wildtype MEFs, the Shh-induced transcriptional response of *Ptch1-/-* MEFs is weaker than that of

Ptch1+/+ MEFs. It is possible that Ptch1-independent signaling is more efficient in mediating the migratory than the transcriptional response. Boc and Cdon are Shh co-receptors required both for the transcriptional response (Allen et al., 2011) as well as neural path finding to Shh (Izzi et al., 2011). It is conceivable that like the transcriptional response, Ptch2 can mediate Shh chemotaxis. Boc and Cdon have been proposed to make a tripartite complex with Ptch1 and Shh (Izzi et al., 2011). The Boc and Cdon requirement for Shh chemotaxis in *Ptch1*-/- MEFS suggests that they may also form complexes with Shh and Ptch2.

Possible interactions of Ptch1 and Ptch2

As a member of the RND family of proton-driven antiporters, Ptch1, like Drosophila Ptch (Lu et al., 2006), is expected to function as a trimer, mediating its transporter activity via a rotatory mechanism (Hiroshi Nikaido and Takatsuka 2009; H Nikaido and Zgurskaya 2001).In the absence of endogenous Ptch1 as a trimerization partner, the Ptch1 paralog Ptch2 could fulfill this role. Like Ptch1, Ptch2 expression is upregulated in response to Shh resulting in a significant overlap in their expression domains (Holtz et al., 2013; Resende et al., 2010). This leaves open the possibility that Ptch1 and Ptch2 can form heterotrimers (Rahnama et al., 2003), and that Ptch1/2 heterotrimers in which Ptch1 subunits lack the Shh binding loop cannot mediate the Shh response. RND heterotrimerization is not without precedent. MdtB and MdtC, two bacterial RND proteins that are encoded within a single operon, must be co-expressed in order for drug efflux (a measure of activity) to occur. MdtB and MdtC share 45% sequence identity, which is much less than the 56% sequence identity shared between Ptch1 and Ptch2, further, supporting the possibility that Ptch1 and Ptch2 could also form heterotrimers. The Mdt complex is an $MdtB_2C_1$ heterotrimer. Importantly, mutating the proton translocation pathway of MdtB blocked transporter activity, while the analogous mutation in MdtC did not affect the activity of the trimer (Kim et al., 2010). This result indicates that subunits of RND heterotrimers can contribute different activities to the trimer, and that the proton driven antiporter activity is not required to be active in all three subunits.

The observation that Ptch1Dloop2D499A both inhibits Smo activity in *Ptch1*-/- cells and is insensitive to regulation by Shh would support the notion that in Ptch1/2 heterotrimers, Smo inhibition is mediated by Ptch2 subunits. This is further supported by the observation that Ptch2D469A expression can activate the Shh response when expressed in vivo. An interpretation of this result is that the high levels of expression reached in electroporation drive the formation of Ptch1/2 heterotrimers in which the Ptch2 subunits fail to mediate proton driven antiporter activity and thus prevent the heterotrimers from inhibiting Smo. Together these observations support the model in which the Ptch1 and Ptch2 subunits of a Ptch1/2 heterotrimer mediate distinct activities. Ptch1, via its Shh binging loop2 imparts Shh sensitivity upon the heterotrimers, independent of its proton-driven antiporter activity. Ptch2 on the other hand is not particularly sensitive to Shh, but mediates the antiporter activity.

The non-cell autonomous activation of the Shh response resulting from Ptch2D496A expression in the developing chick neural tube is consistent with the predicted role of Ptch2 on Smo activity. Very strong activation of the Shh response can

result in the induction of Shh expression (Ericson et al., 1996), and consistent with this, we find that expression of Ptch2D496A results in an ectopic or expanded population of Shh expressing cells. It is likely that Shh released from these Shh expressing cells mediates the subsequent ectopic induction of Nkx2.2 and Isl1/2 on both sides of the neural tube. The induction of Shh expression could explain the apparent cell non-autonomous effects of Ptch2D496A expression.

The origin of these ectopic Shh-expressing cells remains unclear but their presence indicates incorrect patterning of the neural tube. Shh-mediated induction of Shh expression occurs in the node(J. B. Charrier et al. 1999; J.-B. Charrier et al. 2002), and soon after when the nascent notochord induces the floor plate (Placzek et al., 1993). The high levels of Ptch1 and Ptch2 expression around the node (Resende et al. 2010) might render this structure particularly sensitive to the consequences of Ptch2D496A overexpression, and explain the nature of the phenotype observed. It is striking that Ptch2D469A overexpression causes a phenotype reminiscent of the loss of Ptch1 in mouse embryos (Goodrich et al., 1997).

Overlapping functions of Ptch1 and Ptch2

In summary, our results reveal that the upregulation of the Shh response in *Ptch1*-/- cells is in part mediated by Shh and we propose that Ptch2 acts as a Shh receptor. The function of Ptch2 becomes more apparent in the absence of Ptch1. Since *Ptch2*-/- mice are viable and fertile, it is obvious that the role of Ptch1 in the regulation of Smo activity is greater than that of Ptch2. Ptch1 can compensate for the loss of Ptch2, but not *vice versa* (Rahnama et al., 2003). Thus whether Ptch1 and Ptch2 have overlapping roles has not been conclusively demonstrated. Nevertheless, the increased tumor incidence in *Ptch1*+/- mice lacking one or two *Ptch2* alleles (Lee et al., 2006; Smyth et al., 1999) is most easily explained by the ability of Ptch2 to regulate Smo activity in the absence of Ptch1. The modulation of Ptch2 activity by Shh provides a simple explanation for why tumors in *Ptch1*+/- mice often occur at known locations of Shh signaling, such as the skin and the cerebellum (Goodrich et al., 1997; Stone et al., 1996), since we predict that loss of function of the normal *Ptch1* allele does not render these cells completely ligand independent.

Further indications that the functions of Ptch1 and Ptch2 are not entirely overlapping comes from the observation that in *ptc1*-/-;*ptc2*-/- zebrafish embryos a more extensive upregulation of the Hh response is observed than in *ptc1*-/- embryos (Koudijs et al. 2008). In mouse embryos without Ptch2, and with Ptch1 expressed off a constitutively active and Shh-insensitive promoter, a mild upregulation of the response is observed. Subsequent loss of the Shh antagonist Hhip results in a strong upregulation of the Shh response (Holtz et al., 2013), indicating that Ptch2 provides ligand-dependent feedback on the Shh response. However, neither the mouse mutants described by Holtz et al. (due to the presence of constitutively expressed Ptch1), nor the *ptc1*-/-;*ptc2*-/- zebrafish (due to the partial genome duplication) address the consequence for the Hh response in the complete absence of Ptch activity.

The question remains to what degree the observed activation of the Shh response in cells without Ptch1 is ligand dependent *in vivo*. The phenotype of

Shh-/-;*Ptch1*-/- embryos is not yet known, but any slight modification of the *Ptch1*-/- phenotype due to the loss of Shh could be attributable to Shh signaling via Ptch2 (Lee et al., 2006). Similarly, comparing early phenotypes of *Ptch1*-/- and *Ptch1*-/-;*Ptch2*-/- embryos could demonstrate further roles of Ptch2 when Ptch1 is absent. Additionally the responsiveness or unresponsiveness of *Ptch1*-/-;*Ptch2*-/- cells has not been conclusively demonstrated. Preliminary in vitro experiments show that *Ptch1*-/-;*Ptch2*-/- cells respond to endogenous overexpression of Shh-N (data not shown). How *Ptch1*-/-;*Ptch2*-/- cells remain responsive is interesting considering that Patched1/2 and at least one Boc, Cdo, and Gas1 molecule is thought to be required for pathway activation. Production of cell lines that are *Ptch1-/-;Ptch2-/-;Boc-/-Cdo-/-;Gas1-/-,* would address if (1) *Ptch1-/-;Ptch2-/-* are still Shh responsive via Boc, Cdo,and Gas or (2) that *Ptch1-/-;Ptch2-/-;Boc-/-Cdo-/-;Gas1-/-,*are still Shh responsive, indicating Shh pathway activation occurs via an unknown receptor or possibly directly through Smo.

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