Hedgehog Signaling in Cancer

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UNIVERSITY OF MIAMI

HEDGEHOG SIGNALING IN CANCER

By

Jun Long

A DISSERTATION

Submitted to the Faculty
of the University of Miami
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the requirements for the degree of
Doctor of Philosophy

HEDGEHOG SIGNALING IN CANCER

Jun Long

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Two major biological questions regarding Hedgehog (HH) signaling were investigated in this dissertation. The first important question is the role of lipid modification in HH ligand function, while the second one is epigenetic regulation in HH-driven cancer. In the second chapter, identification of lipid modifications in HH ligands from different contexts and pertinent functional studies were performed to elaborate the role of lipids in regulating the biological properties of HH. This is the first description of heterogeneous fatty acylation in a fully processed form of HH ligand, and shows that such a profile of lipidation is dynamic in different cellular contexts. These lipid speciated HH ligands were shown to exhibit distinct potency and intracellular trafficking. In the third chapter, an unbiased epigenetic compound screen was performed to identify compounds that attenuate HH-driven luciferase activity. One of these compounds was a bromodomain and extra terminal (BET) protein inhibitor. This inhibitor potently inhibited HH signaling in vitro and in vivo. Mechanistic studies demonstrated that it acted downstream of SMO, mutations of which have led to resistance to an FDA-approved drug currently being used in the clinic. Targeting BET proteins may bypass this mutation-induced drug resistance when treating HH-driven cancer.
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CHAPTER ONE: INTRODUCTION

1. 1 HH ligand

Hedgehog (HH) signaling has been demonstrated to play an essential role in animal development and disease in the past two decades. *hh* gene was identified by embryonic mutagenesis in *Drosophila Melanogaster* (Mohler, 1988). Mutations of the *hh* gene were generally embryonic lethal, leading to a patch of denticles on the ventral surface of embryos. Its mutation in the adult showed a series of defects such as a reduction in size of eyes, loss of distal portions of the antenna and legs and loss of structures in the male and female genitalia. Subsequent studies on cloning and expression of *hh* revealed that it encodes a novel secreted protein (Lee et al., 1992; Tabata et al., 1992). There were several lines of evidence to suggest the role of *hh* gene product in transmitting cell signaling. The amino acid sequence predicted a transmembrane protein that appeared to enter into secretory pathway by a type II secretory mechanism (Lee et al., 1992). Processing of this transmembrane protein released a carboxyl terminal peptide (Lee et al., 1992). This protein was able to be detected beyond producing cells in the embryo (Forbes et al., 1993). Collectively, *hh* encodes a novel signaling peptide to regulate embryonic segmentation and organ patterning of the adult fly.

The homologous *hh* gene in mammalian system was firstly identified in mouse model (Echelard et al., 1993). There are three different members of *hh* gene family: *Sonic hh, Indian hh and Desert hh* (*Shh, Ihh and Dhh*, respectively). Their protein products share 47-51% amino acid identity with *Drosophila* hh. The homology is highest in the amino terminal half of the protein where 62% of amino acid identity is observed between
Comparing of those three homologs indicate that *Ihh* and *Shh* are more closely related to each other (90% amino acid identity in the amino terminal sequence) than to *Dhh* (80% amino acid identity). Comparison of SHH across different species reveals strikingly high level of sequence identity in the amino terminal half of protein. Mouse SHH and chick SHH share 99% identity in the amino terminal half (position 85-266) while mouse SHH and zebrafish Shh share 94% identity. In summary, high conservation of HH amino terminal protein sequence suggests conservational function as a secreted signaling peptides in animal.

SHH is the most extensively studied ligand in the HH signaling pathway (Echelard et al., 1993; Riddle et al., 1993). It was found to be expressed in the notochord, floor plate and zone of polarity activity. These sites are signaling center to regulate the development of central nervous system and limb polarity. SHH was found to be processed into two peptides with molecular masses of approximately 19 kDa (amino termini) and 27 kDa (carboxyl termini) (Bumcrot et al., 1995). The amino terminal peptide were detected in a variety of mouse and chick embryonic tissues. Further studies demonstrated that this amino terminal product regulates differentiation of floor plate cells and motor neuron (Marti et al., 1995; Roelink et al., 1995) and limb patterning (Lopez-Martinez et al., 1995). In addition, *Drosophila* HH was found to be modified by cholesterol in the carboxyl termini of amino terminal fragment via its own carboxyl terminal domain-mediated proteolytic processing (Porter et al., 1996). This cholesterol modified HH signaling peptide was able to be recognized and released by a sterol-sensing domain protein Dispatched (Dispt). In the absence of Dispt, cholesterol modified HH but not
cholesterol-free HH was retained in the HH producing cells (Burke et al., 1999). The cholesterol modification was observed in human SHH as well (Pepinsky et al., 1998). In addition to cholesterol modification, it was discovered that SHH underwent palmitoylation in the first amino acid cysteine via amide bond linkage (Pepinsky et al., 1998). The dual lipid modified SHH is 30 folds more potent than modification-free soluble SHH in C3H10T1/2 alkaline phosphatase induction assay (Kinto et al., 1997). Subsequent potency analysis on human SHH-N with a variety of hydrophobic modification in the amino termini in vitro demonstrated a wide range of EC(50) from 99 nM for the unmodified protein to 0.6 nM for the myristoylated form (Taylor et al., 2001).

The Hedgehog Acyl Transferase (HHAT) is responsible for the palmitoylation of HH (Buglino and Resh, 2008; Chamoun et al., 2001). HH processed form (HH-Np) is the only protein with both palmitoylation and cholesterol modification so far.

The lipid modification of HH plays essential roles in its ligand biology. First of all, dual lipids tether HH into membrane during secretion pathway and direct ligand intracellular trafficking for Dispt-dependent release (Chen et al., 2004; Gallet et al., 2006; Kawakami et al., 2002; Peters et al., 2004). Secondly, dual lipid modification is critical for HH multimerization when released (Zeng et al., 2001). Mutation of the first amino acid cysteine in SHH led to failure of forming multimer (Chen et al., 2004). Exogenous HH-N without cholesterol modification in hh-null Drosophila lost the ability to form large punctate structures, a membrane tethered HH multimers, while HH-Np retained such ability (Gallet et al., 2006). Thirdly, dual lipids are substantial for HH to form a long range signaling in animal development. Loss of either lipid modification caused inability
of HH to form long range signaling (Chen et al., 2004; Lewis et al., 2001). The last but not the least, lipids, especially palmitoylation, can enhance potency of ligand as illustrated by the observation described above.

However the information regarding biogenesis and function of HH protein remains to be limited due to the fact that purification of HH protein was performed in a vastly over-expression system (Pepinsky et al., 1998; Porter et al., 1996; Taipale et al., 2000) and that ligand functional analysis was done using modification-free recombinant protein (Lee et al., 1994; Pathi et al., 2001; Taylor et al., 2001). The endogenous protein remains unknown in terms of lipid modification therefore the biology of lipids in HH function is yet to be fully understood.

1.2 HH signaling

In the context of canonical HH signaling, in the absence of HH ligand, receptors PATCHED 1 (PTCH1) and PATCHED 2 (PTCH2) (Carpenter et al., 1998; Goodrich et al., 1996; Marigo et al., 1996; Motoyama et al., 1998; Nakano et al., 1989), multi-transmembrane proteins, suppress a seven transmembrane protein SMOOTHENED (SMO) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), thereby blocking the activation of downstream signaling. In such state, SMO is excluded out of the primary cilium, a cell surface projection as sensory antenna to transit cell signaling (Singla and Reiter, 2006). On the contrary, PTCH1 is enriched in such organelle (Rohatgi et al., 2007). In the presence of HH ligand, HH ligand binds to its receptor PTCH1 and PTCH2, thus removing inhibition of SMO. In such state, PTCH1 moves out of primary cilium
upon HH binding and SMO is translocated into primary cilium (Aanstad et al., 2009; Corbit et al., 2005; Kovacs et al., 2008; Milenkovic et al., 2009; Rohatgi et al., 2007). Kif7/Costal2, a cilia-associated protein, mediates activated signals to recruit Gli transcription factors in primary cilium (Liem et al., 2009). Upon upstream activation signals, Gli transcription factors are dissociated with Suppressor of Fused (SuFu) and enriched in primary cilium thereafter translocating into nucleus (Humke et al., 2010; Tukachinsky et al., 2010). Activated form of Gli transcription factor binds to target gene promoter and then recruits downstream transcriptional complex to drive target gene transcription.

There are three members of Gli transcription factor family in the vertebrate: Gli1, Gli2 and Gli3 (Hui et al., 1994; Ruppert et al., 1988). Original GLI1 gene was cloned from a human Glioblastoma (Kinzler et al., 1987) and its protein product contains conserved five zinc finger domains and a consensus cysteine-histidine link, typical structures of Kruppel family of zinc finger proteins (Kinzler et al., 1988). The consensus DNA sequence to which Gli binds is a 9-base-pair 5'-GACCACCCA-3' (Kinzler and Vogelstein, 1990). Gli proteins undergo a series of post-translational modifications and proteolytic processing during canonical HH signaling. This regulation requires a number of protein kinases, including protein kinase A, (PKA), glycogen synthase kinase 3 (GSK3) and casein kinase1α (Bhatia et al., 2006; Pan et al., 2006; Tempe et al., 2006; Wang et al., 2000), and the negative regulator SuFu (Svard et al., 2006). GLI2 and GLI3 are both subjected to processing regulation upon HH stimulation while Gli1 processing has not been identified yet. In the absence of HH ligand, GLI2 FL and GLI3 FL are associated with
SuFu in the basal body of primary cilium and subjected to phosphorylation and ubiquitination. Thereafter, they are dissociated with SuFu via an unknown mechanism and then undergo partial degradation into cleaved form GLI2R and GLI3R. GLI2R and GLI3R then traffic out of primary cilium into nucleus where they suppress HH target gene transcription (Humke et al., 2010). In the presence of HH ligand, GLI2 FL and GLI3 FL together with SuFu are trafficking into the tip of primary cilium from basal body. They are differentially phosphorylated to become activated form GLI2A and GLI3A then dissociated with SUFU. Then these activated form of GLI are translocated into nucleus where they activate target gene transcription (Humke et al., 2010).

In the context of non-canonical HH signaling, there are basically three types, I, II and III. For type I, PTCH1 can function as a dependent receptor (Bredesen et al., 2004) to regulate cell apoptosis in a ligand dependent manner (Thibert et al., 2003). In the absence of HH ligand, a dependence-associated receptor C terminal motif in PTCH1 protein can be cleaved by caspases, which exposes a proapoptotic domain (Kagawa et al., 2011; Thibert et al., 2003). This recruits a multiprotein complex required for induction of cell death. Binding of HH ligand dissociates PTCH1 with such proapoptotic complex thus preventing PTCH1-induced cell death (Mille et al., 2009). This ligand-induced inhibition of cell apoptosis cannot be mimicked by either agonist-induced activation of SMO or overexpression of SMO and is independent of Gli transcriptional activity (Chinchilla et al., 2010). PTCH1 can also regulate cell cycle via binding to cyclin B1 and binding of HH ligand can disrupt its association with cyclin B1 (Barnes et al., 2001). The latter
allows cyclin B1 translocation into nucleus then proceeding of cell cycle from G2 to M (Barnes et al., 2001). 

Type II non-canonical HH signaling is generally SMO-dependent but Gli-independent. It regulates the actin cytoskeleton through activation of the Rho family of small GTPases (Polizio et al., 2011; Renault et al., 2010; Sasaki et al., 2010) and stimulates calcium release from the endoplasmic reticulum (ER) in spinal neurons through Gi and PLC-γ-catalyzed generation of IP3 and opening of IP3-dependent channels (Belgacem and Borodinsky, 2011). Activation of small GTPases by Hh proteins in SMO-dependent manner was found in epithelial cells, fibroblast and neuron. For example, HH ligand increased the number of dendritic spines in hippocampal neurons through an alternative mechanism (Sasaki et al., 2010). Specifically, inactive Smo prevented Rac1 activation by interacting with the Rac guanine nucleotide exchange factor (GEF) T-lymphoma invasion and metastasis 1 (Tiam1). Shh-mediated activation of Smo dissociated this Smo-Tiam1 complex, which allowed Tiam1 to activate Rac1. The rapid responses of these processes, the lack of Gli-dependent transcriptional activity, and the inability of Gli3R to prevent these responses imply that this is a Gli-independent Hh signaling pathway.

Type III non-canonical HH signaling is Gli-dependent signaling events that are activated through other signaling modules such as RAS and TGF beta signaling. In pancreatic cancer cells, oncogenic KRAS mutant strongly stimulated Gli activity which is ligand independent and occurs downstream of SMO (Ji et al., 2007). The RAS effectors were probably Mek1 or Erk1/2 since Mek1 inhibitors were capable of blocking the RAS effect
but the PI3K-specific inhibitor were not. However, there is lack of evidence to show Erk-mediated phosphorylation of GLI1/GLI2 (Kasper et al., 2006; Riobo et al., 2006a), which suggests indirect activation by RAS signaling. Another signaling cascade that activates Gli1 activity is transforming growth factor beta (TGF beta) pathway (Dennler et al., 2007). TGF beta acted downstream of SMO and induces the expression of GLI2 in a Smad3-dependent way. TGF beta-induced target gene activation was not able to be blocked by cyclopamine. Accordingly, treatment of a cyclopamine resistant pancreatic tumor cell line with an antagonist of the TGF beta receptor reduced GLI2 expression and cell proliferation.

1.3 HH signaling in human diseases

Extensive studies have demonstrated that HH signaling plays critical role in cancer initiation and progression. Many HH target genes, which include MYCN, CCND1, CCND2 and BCL2 (Katoh and Katoh, 2009), promote cell proliferation, cell cycle progression and anti-apoptosis. There are two major types of HH-driven cancers: HH ligand dependent type and HH ligand independent type. For HH ligand dependent cancers, cancer cells require HH ligand to maintain cell proliferation and self-renewal in either autocrine or paracrine manner. Examples are lung cancer, breast cancer, pancreatic cancer, glioblastoma, colon cancer and sarcoma (Bar et al., 2007; Kubo et al., 2004; Singh et al., 2011; Thayer et al., 2003; Varnat et al., 2009; Wang et al., 2012). Among them, there are HH ligand dependent autocrine subtype and HH ligand dependent paracrine subtype. For the autocrine subtype, tumors have elevated expression of HH ligand in either all tumors cells or in a small number of stem cells to support tumor
growth and survival. For the paracrine subtype, it is tumor cells that secret HH ligand to active the HH signaling in stroma cells which in turn feedback tumor cell to boost proliferation and survival.

For HH ligand independent cancers, tumor usually harbors mutations of HH signaling components which can activate downstream signaling without binding of HH ligand. The most representative mutation is \textit{PTCH} inactive mutation, which was initially identified in the rare condition Gorlin’s syndrome (Wolter et al., 1997). Patients with Gorlin’s syndrome developed basal cell carcinomas (BCCs) during their lifetime and were at an increased risk of other tumors including medulloblastoma and rhabdomyosarcoma. Then mutations of several HH signaling components were subsequently identified in majority of sporadic BCC patients (Dahmane et al., 1997). Majority of HH-related mutations were \textit{PTCH} mutation (85%) and 10% were \textit{SMO} mutation (Xie et al., 1998). In addition, the genomic analysis of primary HH-driven medulloblastoma patients identified prevalent \textit{PTCH} mutation across all age groups, \textit{SUFU} mutation in infant group and \textit{SMO} mutation in adult group (Kool et al., 2014).

Mutations of HH signaling components have also been linked with other human diseases. First of all, mutation of SHH ligand is the most common cause of sporadic and inherited holoprosencephaly (HPE), a developmental disorder that is characterized by defective prosencephalon development (Singh et al., 2009). Missense mutations in SHH amino terminal domain can result in low levels of protein expression, defective processing of SHH into its active form and protein with reduced activity. Secondly, Mutations in the
human genes \textit{EXT-1} and \textit{EXT-2}, which are required for the synthesis of heparin sulfate (Lind et al., 1998), lead to impaired IHH signaling in the bone growth. These mutations are strongly associated with multiple exostoses syndrome, frequent skeletal dysplasias or benign bone tumors that can develop into malignant chondrosarcoma (Bellaiche et al., 1998). Thirdly, Grieg cephalopolysyndactyly, Pallister-Hall syndrome, and postaxial polydactyly are three related autosomal dominant disorders that are caused by mutation of \textit{GLI3}. The mutations range from large deletions to premature stops which lead to truncation of GLI3 into partial forms. These truncated forms of GLI3 act as a repressor to suppress target gene expression (Hui and Joyner, 1993; Kang et al., 1997; Radhakrishna et al., 1997; Vortkamp et al., 1991).

1.4 HH pathway inhibitors (HPIs)

The first class of HPIs is the SMO antagonist. The first SMO antagonist, cyclopamine, was isolated from corn lilies which caused teratogenic effects in lambs whose mothers had ingested this plant (Bryden et al., 1971). It was subsequently demonstrated that cyclopamine can inhibit HH pathway by binding to SMO hepta-helical bundle (Chen et al., 2002a). Because of it poor bioavailability and low affinity, optimized derivatives have been synthesized based upon its structure platform (Chen et al., 2002b; Tremblay et al., 2008). There are other SMO antagonist synthesized upon different structure platforms: HhAntag (Frank-Kamenetsky et al., 2002), SANT1–SANT4 (Williams et al., 2003), Cur-61414 (Williams et al., 2003) and vismodegib (Robarge et al., 2009). Among them, vismodegib has been a FDA approved drugs that demonstrated promising effects in clinical trials of basal cell carcinoma (Sekulic et al., 2012; Von Hoff et al., 2009).
However, the acquired resistance has been observed in the BCC treated with vismodegib due to mutations of SMO and other downstream signaling components (Sharpe et al., 2015). Vismodegib has also been tested in an advanced medulloblastoma patient, which showed capacity to abrogate tumor growth in the first two months but lost the efficacy in the third month (Rudin et al., 2009).

The second class of HPIs targets upstream of SMO. One well known example is a monoclonal antibody 5E1 that has been widely applied to inhibit HH signaling in cancer studies (Scales and de Sauvage, 2009) by blocking the binding of HH ligand to PTCH1 receptor (Bosanac et al., 2009). 5E1 was generated in mouse using the rat Shh N-terminal domain as antigen (Ericson et al., 1996). 5E1 binding sites in SHH-N is adjacent to SHH-PTCH1 binding interface and its binding affinity is similar to the one between ligand and receptor (Aanstad et al., 2009; Maun et al., 2010). Another example is RU-SKI 43, a specific hedgehog acyl transferase (HHAT) inhibitor (Petrova et al., 2013), reduced pancreatic cancer cell proliferation and target gene \textit{GLI1} activation through SMO-independent non-canonical signaling (Petrova et al., 2015). Inhibition of fatty acylation of HH ligands by HHAT shRNA also reduced non-small cell lung carcinoma growth \textit{in vitro} and \textit{in vivo} (Rodriguez-Blanco et al., 2013). Attenuation of HH fatty acylation is a potential therapeutic strategy to target HH ligand dependent cancers.

The third class of HPIs targets downstream of SMO. There are major two types: the one targeting GLI protein stability and the other targeting GLI-DNA binding. The first type is focused on modulation of post-translational modification and processing of Gli proteins.
Gli proteins can be subjected to phosphorylation, acetylation, ubiquitination and small ubiquitin-like modification (SUMO), all of which can contribute to protein processing and stability. For example, the degron domain of GLIs has casein kinase-1α (CK1α) mediated phosphorylation sites which promote GLI2 degradation and GLI3 processing into repressive form (Pan et al., 2006; Wang et al., 2000). A FDA-approved anti-pinworm compound pyrvinium, as a novel CK1α-agonist, is a potent inhibitor of HH signaling by reducing the stability of the Gli family of transcription factors (Li et al., 2014). In the presence of pyrvinium, GLI proteins were boosted to be phosphorylated and then degraded, therefore which attenuated HH-driven cancer cell growth in vitro and in vivo. Another example is PI3K/AKT-mediated phosphorylation of Gli which potentiates GLI signaling (Riobo et al., 2006b). A specific PI3-kinase inhibitor LY294002 inhibited Shh-activated Gli-luciferase expression to the same extent as cyclopamine and retained the inhibitory effect when activation of the Gli-luciferase reporter was induced by SmoM2, an oncogenic mutant of Smo (Taipale et al., 2000). The second type is revolving around inhibition of GLI-DNA binding. GANT61 is the best example for this type of inhibitors (Lauth et al., 2007). Treatment with GANT61 led to accumulation of GLI1 in the nucleus and GLI1 transcriptional activity was strongly inhibited. DNA binding of GLI1 in GANT61-treated cells was markedly reduced, which suggested that GANT61 either prevented DNA binding or destabilized the GLI1–DNA complex through unknown post-translational modifications of GLI1.

Collectively, HH signaling plays a critical role in cell proliferation, cell cycle progression and self-renewal to drive and maintain tumorigenesis. In order to develop targeted
therapy against HH-driven cancers, there is an urgent need to have a better understanding of rate-limiting steps in HH signaling such as HH biogenesis and Gli downstream activation.
CHAPTER TWO: THE ROLE OF LIPIDS IN HEDGEHOG FUNCTION

2.1 Summary

Hedgehog (HH) proteins are proteolytically processed into a biologically active form that is covalently modified by cholesterol and palmitate. However, most studies of HH biogenesis have characterized protein from cells in which HH is overexpressed. We purified Sonic Hedgehog (SHH) from cells expressing physiologically relevant levels and showed that it was more potent than SHH isolated from overexpressing cells. Furthermore, the SHH in our preparations was modified with a diverse spectrum of fatty acids on its amino termini, and this spectrum of fatty acids varied dramatically depending on the growth conditions of the cells. The fatty acid composition of SHH affected its trafficking to lipid rafts as well as its potency. Our results suggest that HH proteins exist as a family of diverse lipid-speciated proteins that might be altered in different physiological and pathological contexts in order to regulate distinct properties of HH proteins.
2.2 Methods

Comparison of SHH-Np Levels

Fertile, certified research-grade, pathogen-free eggs (Charles River) were incubated at 37.5°C. At Hamburger-Hamilton (H&H) developmental stage 22, embryos were isolated and the limb buds were resected as previously described (Zeng et al., 2001). Resected buds were further divided into SHH-producing posterior and SHH-negative anterior portions, and lysed by suspension in 1% Triton X-100, 10 mM sodium phosphate, 150 mM NaCl buffer, pH 7.4, supplemented with protease inhibitors (Roche). Immunoblotting was performed using anti SHH-Np polyclonal H-160 antibodies (Santa Cruz).

Purification of SHH-Np

SHH-I cells (Taipale et al., 2000) were washed with PBS, collected by scraping, Dounce homogenized, and centrifuged at 100,000 × g for 1 hr. The resultant pellet was resuspended in buffer A (10 mM sodium phosphate, 350 mM NaCl buffer, pH 7.4.) and recentrifuged, and the membrane-enriched pellet was extracted twice with buffer B (1% Tx-100, 10 mM sodium phosphate, 150 mM NaCl buffer, pH 6.5) by Dounce homogenization and centrifugation at 16,000 × g for 30 min. The supernatants were combined, pH adjusted to 5.0 with 1 M 2-N-morpholino ethanesulfonic acid (MES), and applied to a bulk SP Sepharose Fast Flow resin. This resin was washed once with buffer C (1% Tx-100, 10 mM sodium phosphate, 150 mM NaCl buffer, pH 5.0.), followed by a second wash with buffer D (0.2% Np-40, 10 mM sodium phosphate, 300 mM NaCl buffer, pH 5.5.). SHH-Np was eluted from this resin using buffer E (0.2% Np-40, 10 mM
sodium phosphate, 450 mM NaCl buffer, pH 7.8.). The eluted fractions were adjusted to pH 7.2 with 1 M HEPES, and then passed through a 5E1 monoclonal antibody (mAb) column (Ericson et al., 1996). After the column was washed with buffer F (0.1% Np-40, 10 mM sodium phosphate, 150 mM NaCl buffer, pH 7.4.), the SHH-Np was eluted with buffer G* (0.1% Np-40, 10 mM sodium phosphate, 150 mM NaCl buffer, pH 2.7) and then immediately neutralized with 1 M HEPES, pH 7.4. *To obtain saturating concentrations of SHH-Np, we used a 0.01% Np-40, 10 mM sodium phosphate, 150 mM NaCl buffer, pH 2.7, in 50% FBS to elute SHH-Np.

After SDS-PAGE, the purified SHH-Np was quantified by comparing its concentration against a standard curve of recombinant SHH-II (R&D). This gel was subsequently protein stained using a SilverQuest staining kit (Invitrogen). The optical density of each stained protein was then calculated and compared using ImageJ software (NIH). SHH activity measurements were performed essentially as described previously (Singh et al., 2009), using NIH 3T3 cells expressing an HH reporter gene (Light-II cells). SHH-Np-dependent differentiation and gene expression were assayed using the C3H10T1/2 cell line as previously described (Zeng et al., 2001). All activity measurements were done in triplicate and each experiment was repeated at least three times. The activity data presented are shown as the mean and SD of one representative experiment.

**Mass Spectrometry Analysis**

The identity of the purified SHH-Np was validated by microcapillary liquid chromatography/MS (LC/MS) on a ThermoFinnigan LTQ ion trap mass spectrometer.
For identification of fatty acid modifications, SHH-Np was reduced with 4 mM DTT, alkylated with 15 mM iodoacetamide, and EndoLysC digested. Lipid modified peptides were identified by MS/MS analysis on a LTQ-Orbitrap (Thermo) interfaced with an Eksigent nanoLC-2D HPLC. MS/MS spectra were searched against the SHH protein sequence using a Mascot (v 2.1) error-tolerant search with 20 ppm parent mass accuracy, and Inspect/MS-Alignment run in blind modification search mode (Tanner et al., 2006). All MS/MS spectra peptide assignments were manually verified for peptide assignments.

**Cerebellar Granular Neuronal Precursors culture and BrdU staining**

The isolation and culture of Cerebellar Granular Neuronal Precursors (CGNP) from P5 mice was done following the papain extraction protocol (Lee et al., 2009). Cells were placed in poly-D-lysine coated chamber slides for 2 h in Neurobasal-A-medium plus Glutamax, B27 and KCl 250uM prior to add NP40 (1:300), purified SHH-Np (1:300), tomatidine (10uM) or cyclopamine (10uM). 24 h later BrdU to a concentration 10uM was added and incubated overnight. Cells were fixed in Paraformaldehyde 4 % 1 h.

Immunostaining for BrdU was done as previously described (Fernandez et al., 2010) with minor changes. The Cell Signaling BrdU antibody was used 1:1000 in BSA 5 % overnight and secondary Alexa Fluor 594 used 1:500 was incubated the same way. Nuclei were counterstained with Hoechst 1:10000 (Sigma) and samples were mounted with ProLong (Invitrogen). At least 8 independent fields were counted per experimental condition under the 20x objective of an Olympus IX71 microscope.
**Lipid Treatments**

SHH-I cells were serum deprived for 6–7 hr in 0.5% FBS. Prior to lipid treatment, the cells were washed with PBS once and then maintained in the presence of 100 μM fatty acids or DMSO (vehicle) for 16–18 hr (Liang et al., 2001). For embryonic tissue studies, 10–12 pathogen-free H&H developmental stage 22 chick embryos (Charles River) were collected and posterior fragments of the limb buds were dissected as previously described (Zeng et al., 2001). These posterior tissues were incubated with 100 μM fatty acids or DMSO (vehicle) for 16–18 hr in six-well plates. These tissues were subsequently washed twice in ice-cold PBS and homogenized in a 1% Tx-100, 10 mM sodium phosphate, 150 mM NaCl buffer (pH 6.5).

**OptiPrep Density Gradient Ultracentrifugation**

Cell or tissue extract was mixed with OptiPrep medium to obtain a 40% fraction. Then two other fractions, composed of 25% and 10% OptiPrep medium or 30% and 0% for tissue extract, were sequentially layered on top followed by centrifugation at 120,000 × g, or 160,000 × g for tissue extract, for 21 hr (Bruses et al., 2001; Lisanti et al., 1994; Searls et al., 2004). Fractions from the tubes were collected and subjected to SDS-PAGE analysis.
2.3 Results

**SHH-I Cells Express SHH-Np at Physiological Relevant Level**

SHH-inducible cells (SHH-I) are Human Embryonic Kidney 293 cells that express ectopic mouse full length SHH driven by ecdysone-inducible promoter (Taipale et al., 2000). In the presence of muristerone A, a phytoecdysteroid analog of ecdysone, there was a vast induction of SHH expression compared to vehicle control (Fig.2.1A and 2.6A). In the absence of muristerone A, SHH expression level paralleled to endogenous SHH level from posterior tissue of zone of polarity activity (ZPA) of stage 23-25 chick embryos (Riddle et al., 1993) (Fig.2.1C). Induced and uninduced cell lysate was harvested in Light-II cell growth medium and homogenized by Dounce on the ice. Homogenized cell lysate was titrated to test the activity of SHH-Np in Light-II reporter assay (Fig.2.1B and 2.6B). The activity of SHH-Np was then normalized by the protein abundance quantified by western blot (Fig.2.6A). It showed that uninduced SHH-Np has 10 folds higher potency than induced SHH-Np does. Thus, to understand the biogenesis of SHH-Np, SHH-I cells without induction may be an ideal source to purify protein for further lipid modification analysis.
Figure 2.1. SHH-I Cells Produce Endogenous-like Levels of Potent SHH-Np

(A) An immunoblot showing SHH-Np abundance in SHH-I cells under conditions in which its expression was induced (+) or uninduced (−) with muristerone. SHH-I parental cells, which are not engineered to express SHH, were used as a control (Ctrl). GAPDH was used to verify protein normalization.

(B) An aliquot of SHH-I cellular lysate was tested for SHH-Np-associated activity using the Light-II reporter cell line. SHH-Np activity measurements were carried out in the linear range of this assay (see Figure 2.6B) and these activity results were then normalized to overall SHH-Np levels to determine potency. Error bars represent the SD in one representative experiment. This assay was repeated at least three times.

(C) SHH-Np levels from uninduced SHH-I cells and chick embryo limb buds were compared by immunoblotting. During development, SHH-Np is produced in the posterior portion of limb buds (Post). Here, the anterior (Ant) portion of limb buds serves as a negative control for SHH-Np. As only ~20% of the posterior tissue consists of SHH-producing cells (Riddle et al., 1993), we mixed 20% of SHH-I cell lysate with 80% of
lysate from anterior limb bud tissue for this comparison. Then tissue homogenate and cell lysate were loaded on the gel for immunoblotting. GAPDH was used to verify normalization.

Purification of SHH-Np from SHH-I cells

The purification protocol was optimized to consist of membrane preparation, ionic exchange chromatography and affinity chromatography (Table 2.1). Aliquots of sample from each step were analyzed by silver staining to demonstrate the progression of purity and by immunoblotting to track the flow of SHH-Np during purification (Fig.2.2B). Purified SHH-Np was assayed to determine potency in C3H10T1/2 cells. It showed that EC$_{50}$ of SHH-Np purified from SHH-I cells without induction is 0.3 nM in alkaline phosphatase assay (Fig.2.2C). In contrast, a recombinant SHH-N purified from bacteria showed much higher EC$_{50}$ (10 nM) in such assay. RNA was also collected from such cells to determine potency of SHH-Np in activating HH target gene expression (Fig. 2.2D). The EC$_{50}$ of SHH-Np purified from SHH-I cells without induction in target gene activation is 0.18 nM. This was the most potent form of purified SHH-Np described so far. Purified SHH-Np was also able to stimulate the proliferation of primary cerebellar granular neuron precursor cells (GPCs) (Dahmane and Ruiz i Altaba, 1999), confirming its activity (Fig. 2.7).
Figure 2.2 SHH-Np Purified from Low-Level SHH-Expressing Cells Is Highly Active

(A) SHH-I cells or the SHH-I parental cell line (Ctrl) were Dounce homogenized under isotonic conditions, and total lysate (left panel) was separated by ultracentrifugation at 100,000 × g (right panel) to generate a cytosol-enriched fraction (Cyt) and a membrane-enriched fraction (Mem). These fractions were volume normalized to that of the original cellular lysate and immunoblotted as indicated. Tubulin served as a cytosolic protein control and the Na+/K+ transporter served as a membrane protein control.

(B) Aliquots of the indicated fractions from various steps of SHH-Np purification were separated by SDS-PAGE, followed by visualization of proteins by silver staining (TL, total lysate; S, 100,000 × g supernatant; M, combined 100,000 × g pellet detergent
extract; FT, non-bound material; W, column wash; E, column eluate). Recombinant, unmodified SHH-N is shown as a control (rSHH-N). Electrophoretic retardation of rSHH-N relative to cholesterol-modified SHH-Np was previously noted (Lee et al., 1994).

(C) The indicated amounts of purified SHH-Np were incubated with C3H10T1/2 fibroblasts, which differentiate into osteoblasts in response to SHH (squares: purified SHH-Np; circles: rSHH-N). Alkaline phosphatase activity, which is an indirect, quantitative measurement of this differentiation, was then measured.

(D) The indicated amounts of purified SHH-Np were incubated with C3H10T1/2 fibroblasts, followed by RNA extraction. The levels of Gli1 and GAPDH were then determined by qRT-PCR. Error bars represent the SD in one representative experiment. See also Figure 2.7.

**Heterogeneous Fatty Acylation of SHH-Np**

Purified SHH-Np was subjected to reduction, alkylation and endo lys C digestion for the purpose of Mass Spectrometry (MS) Analysis (Fig.2.3A). The liquid chromatography (LC)-MS/MS using a high-resolution LTQ Orbitrap mass spectrometer was utilized to identify the lipid modification of SHH-Np peptides. The mass/charge ratios obtained during these analyses were cross-referenced against expected unmodified masses of individual peptides, and the MS/MS of modified forms was validated manually (Table 2.2; Fig.2.8A-C). Surprisingly, on the contrary to what has been entrenched, the first amino acid cysteine was modified by a series of fatty acids that varied in terms of length and saturation degree (Fig.2.3B-D). Since induction of SHH expression leads to lower
potency of SHH-Np and fatty acylation has been shown to alter ligand potency, it was hypothesized that the profile of fatty acylation of SHH-Np from induced condition is distinct compared to uninduced condition.

Purification and LC/MS analysis of SHH-Np from other cellular conditions (10 % serum with muristerone A, serum deprived with muristerone A and serum deprived plus myristic acid with muristerone A) identified that the fatty acylation profile is substantially dynamic (Fig. 2.3B-D). In the context of 10% serum without muristerone A, there was approximately 30% of SHH-Np modified with unsaturated fatty acids. In the context of 10% serum with muristerone A, there was roughly 70% of SHH-Np modified with unsaturated fatty acids. In the context of serum deprivation with muristerone A, there was almost 90% of SHH-Np modified with unsaturated fatty acids. In the context of serum deprivation plus myristic acids with muristerone A, there was still about 90% SHH-Np modified with unsaturated fatty acids although myristic modified one was enriched by 4-5 folds. The major trend is that serum deprivation and induction of SHH can cause predominant lipid modification consisting of unsaturated fatty acids. In addition, there was no unmodified SHH-Np detected by LC/MS, which excludes the alternate hypothesis that differential potency is due to level of fatty acylation.
Figure 2.3. Fatty Acid Speciation of SHH-Np Is Dependent on the Cellular Context

(A) A schematic showing the procedure used to identify the fatty acid modification on SHH-Np.

(B–D) Pie charts showing the relative abundance of lipid species identified on SHH-Np that was purified and isolated under three different cellular contexts: 10% FBS without muristerone induction of SHH expression, 10% FBS and muristerone induction of SHH expression, and serum deprivation and muristerone induction of SHH expression.

See also Figure 2.8 and Table 2.2.

Lipid Speciation alters HH lipid raft localization \textit{in vitro}

The hydrophobic properties of the various fatty acid modifications we observed on SHH-Np vary over a 500-fold range (Table 2.2), suggesting that they would alter the biological properties of SHH-Np. Further, unsaturated fatty acids, such as those found on SHH-Np, are known to segregate away from lipid rafts (Levental et al., 2010), where HH proteins are thought to enrich as part of their regulated intracellular movement (Callejo et al.,
2011; Creanga et al., 2012; Mao et al., 2009; Rietveld et al., 1999; Taipale et al., 2000).

To test this hypothesis, we altered the fatty acid composition of media used with cells or chick limb bud explants expressing SHH, and then measured various properties of the resulting SHH-Np. Such lipid-doping experiments have previously been used to alter the covalent lipid modifications of numerous proteins (Hashimoto et al., 2004; Liang et al., 2001; Wolven et al., 1997) before determining changes in their biological function. We therefore incubated serum-deprived SHH-I cells with saturated C14:0, C16:0, C18:0, or unsaturated C16:1 fatty acids, and examined the levels of SHH-Np in both cell lysates and secreted from those cells into conditioned media. Although we did not observe changes in the absolute levels of cell-associated SHH-Np when cells were doped with different fatty acids, consistent with previous reports (Bumcrot et al., 1995), we did observe differences in the secretion of SHH-Np forms from cells incubated with different fatty acids (Fig.2.4A).

Because lipid raft localization is thought to be a prerequisite for the secretion of HH proteins (Callejo et al., 2011; Creanga et al., 2012; Rietveld et al., 1999), we asked whether the various fatty acid-modified SHH-Np forms would differentially localize to lipid rafts. Therefore, we fractionated the cellular lysates of cells incubated with various fatty acids over an OptiPrep gradient to isolate lipid raft-enriched fractions (Fig.2.4B and data not shown). Incubation of uninduced SHH-I cells with any of the tested saturated fatty acids (C14:0, C16:0, or C18:0) increased the percentage of SHH-Np that was enriched in lipid rafts (Fig.2.4C). In contrast, incubation of SHH-I cells with palmitoleate (C16:1) reduced the percentage of SHH-Np that was enriched in lipid rafts.
Figure 2.4. Fatty Acid Speciation of SHH-Np Alters Its Lipid Raft Enrichment

(A) An immunoblot of cell lysates and conditioned media from SHH-I cells incubated in the presence of indicated fatty acids. GAPDH served as a normalization control for cellular lysates. The same volume of conditioned media was subjected to TCA precipitation prior to loading.

(B) Lysates from SHH-I cells incubated with the indicated lipids or DMSO control were separated over an OptiPrep gradient to isolate a lipid-raft-enriched fraction. Fractions from these various OptiPrep density gradients were resolved by SDS-PAGE and then analyzed by immunoblotting for SHH-Np, GAPDH as a cytoplasmic protein marker, or
the lipid raft marker flotillin. Note that flotillin localization did not change with various lipid additions.

(C) Quantification of SHH-Np lipid raft enrichment from cells incubated with the indicated fatty acids. Error bars represent the SEM of three independent experiments; p values ≤ 0.05 are considered statistically significant and are indicated by an asterisk.

**Lipid Speciation alters endogenous HH lipid raft localization**

Similar experiments were performed on anterior or posterior chick limb bud explants. Consistent with our SHH-I cell-based observations, treatment of the explants with unsaturated C16:1 resulted in decreased SHH-Np localization to lipid rafts in posterior tissue (Fig.2.5A). Although incubation of tissue explants with saturated fatty acids had no effect on steady-state SHH-Np levels, increased levels of tissue SHH-Np were observed upon palmitoleoyl (C16:1) incubation. The mRNA levels of SHH were unchanged by incubation with different fatty acids (data not shown). These results are consistent with decreased secretion of palmitoleoyl (C16:1)-modified SHH-Np, resulting in increased retention in posterior limb bud tissue. We further measured the activity of SHH-Np from these tissues and normalized this activity to their relative abundance (Fig.2.5B and 5C). This analysis showed that incubation of posterior limb bud fragments with C16:1 reduced SHH-Np activity.
Figure 2.5. Modification of SHH-Np with Distinct Fatty Acids Alters Its Functionality

(A) Lysates from embryonic limb bud explants exposed to different fatty acids were separated over an OptiPrep density gradient to isolate the lipid raft fraction. Various gradient fractions were resolved by SDS-PAGE and then analyzed by immunoblotting for SHH-Np. Caveolin-1 was used as a lipid raft marker, and GAPDH served to label non-lipid-raft-associated subcellular fractions. Treatment with saturated fatty acids did not change the SHH-Np localization pattern compared with the DMSO control (data not shown).
(B) Upper panel: an immunoblot of limb bud lysates shows the effect of lipid modifications on SHH-Np levels. Lower panel: the lysates contain similar amounts of total protein, as indicated by total protein silver staining.

(C) The potency of SHH-Np was determined by incubating lysates from treated limb buds with Light-II cells and then normalizing this activity to SHH-Np levels. Error bars represent the SEM of three independent experiments; p values $\leq 0.05$ are considered statistically significant and are indicated by an asterisk.

Figure 2.6. Induction of SHH-I cells produces large amounts of lower activity SHH-Np.

(A) An immunoblot of lysates from SHH-I cells induced with (+) or without muristerone (-). (B) Aliquots of cellular lysate both from muristerone induced (open square) and uninduced (colored square) SHH-I cells, were incubated with Light-II reporter cells to
assay SHH-Np activity. We estimated the linear portion of this assay to be between 2-5 ml of lysate (see Figure 1B). Error bars represent the SD in one representative experiment.

**Figure 2.7. Granule neuron precursor cell proliferation assay**

The ability of purified SHH-Np to induce the proliferation of primary granular neuron precursor cells is measured here, using a BrdU incorporation assay. The use of the SHH pathway antagonist cyclopamine validates this assay as SHH dependent. Tomatidine, an inactive analog of cyclopamine, is used as a negative control. Error bars represent the SD in one representative experiment.
Figure 2.8. Sequencing and modifications of the amino-terminal SHH-Np peptides.

A representative analysis of the amino-terminal SHH peptide is depicted here (left panels). These peptides were obtained by subjecting purified SHH-Np to endoproteinase Lys C digestion followed by LC-MS/MS analysis. A chromatographic peak corresponding to the sequenced peptide is also shown (right panels). Ions corresponding to backbone fragmentations and parent neutral losses are labeled, resulting in the indicated coverage over the peptide sequence, indicated by brackets for C-terminal y ions (⊥) and N-terminal b-ions (⊤), corresponding to cleavage at the backbone amide bond. A spectrum of fatty acid modified amino-terminal peptides of SHH-Np were identified in a similar manner. In this example, the amino-terminal peptide is modified by palmitate (Palm) (A), stearoyl (B) and myristoyl (C).
Table 2.1 Purification table for SHH-Np from uninduced SHH-I cells.

<table>
<thead>
<tr>
<th>Purification Steps: Fraction</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total activity (unit)</th>
<th>Potency (unit/mg)</th>
<th>Yield (%)</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 Plates (150mm each)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MEMBRANE PURIFICATION</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Lysate: TL</td>
<td>70ml</td>
<td>700</td>
<td>70000</td>
<td>100</td>
<td>100</td>
<td>0.005</td>
</tr>
<tr>
<td>Membrane Extracts: M/2</td>
<td>120ml</td>
<td>180</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ION EXCHANGE CHROMATOGRAPHY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined Sepharose Elute</td>
<td>120ml</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFFINITY CHROMATOGRAPHY</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5EI Load: 5EI</td>
<td>130ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5EI Flow Through: 5EIFT</td>
<td>150ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5EI Elute: 5EI (one peak fraction)</td>
<td>0.005</td>
<td>70000</td>
<td>1,400,000</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Obtained by multiplying protein concentration with volume of samples. Protein concentration was measured using BCA protein assay and BSA as the standard (except 5EI sample which is quantified by silver staining using recombinant SHH-N standards).

* Obtained by following steps: 1. Luminescence of HH-induced firefly luciferase activity and TK-driven renilla luciferase activity were measured in the presence of the same volume (1ul) of total lysate and 5EI elutes after 24 hours incubation; 2. Firefly luciferase activity was normalized by renilla luciferase activity then this normalized luciferase activity for total lysate sample was set as 1 unit/ul activity; 3. normalized luciferase activity for 5EI elutes was 70 folds higher compared to total lysate so that 5EI elutes had 70 units/ul activity; 4. Multiplying these numbers with total volume generated total activity.

* Obtained by comparing band intensity of SHH-Np immunoblot from aliquots of each step of purification to band intensity of SHH-Np from an aliquot of the first step of total lysate.

* Obtained by following steps for SHH-Np from total lysate: 1. 50 ug of total lysate protein was loaded in the SDS-PAGE as well as filtered recombinant SHH-N standards; 2. amount of SHH-Np from total lysate was quantified to be 2.5 ug by comparing band intensity of SHH-Np to band intensity of recombinant SHH-N standards; 3. purity is calculated by dividing 2.5 ug with 50 ug, which turned out to 0.005% in percentage. For SHH-Np from the 5EI elutes it was determined by silver staining shown in the Fig. 2.2B and set as 100%.

Table 2.2. Fatty acid modifications identified on purified SHH-Np.

<table>
<thead>
<tr>
<th>Modification</th>
<th>Saturation</th>
<th>10% FBS Uninduced</th>
<th>10% FBS Induced</th>
<th>Serum deprived Induced</th>
<th>Hydrophobicity (Log P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitoyl</td>
<td>(C16:0)</td>
<td>58.0</td>
<td>37.4</td>
<td>N/D</td>
<td>7.15</td>
</tr>
<tr>
<td>Palmitoleyl</td>
<td>(C16:1)</td>
<td>29.9</td>
<td>3.4</td>
<td>72.2</td>
<td>6.64</td>
</tr>
<tr>
<td>Stearoleyl</td>
<td>(C18:1)</td>
<td>5.0</td>
<td>55.9</td>
<td>N/D</td>
<td>7.70</td>
</tr>
<tr>
<td>Myristoleyl</td>
<td>(C14:1)</td>
<td>3.2</td>
<td>N/D</td>
<td>25.9</td>
<td>5.57</td>
</tr>
<tr>
<td>Stearoyl</td>
<td>(C18:0)</td>
<td>3.2</td>
<td>3.3</td>
<td>N/D</td>
<td>8.22</td>
</tr>
<tr>
<td>Myristoyl</td>
<td>(C14:0)</td>
<td>0.7</td>
<td>N/D</td>
<td>1.9</td>
<td>5.09</td>
</tr>
</tbody>
</table>
Fatty acid modifications of SHH-Np, along with respective saturation state, relative abundance of speciated SHHNp and hydrophobicity values (LogP), are shown in the table. The purified SHH-Np was isolated from SHHI cells grown under three different cellular contexts: 10% FBS without muristerone induction of SHH expression, 10% FBS and muristerone induction of SHH expression, and serum deprivation and muristerone induction of SHH expression. N/D means not detected above 1%.
CHAPTER THREE: EPIGENETIC REGULATION IN HH-DRIVEN CANCER

3.1 Summary

Epigenetic enzymes modulate signal transduction pathways in different biological contexts. We reasoned that epigenetic regulators might modulate the Hedgehog (HH) signaling pathway, a main driver of cell proliferation in various cancers including medulloblastoma. To test this hypothesis, we performed an unbiased small-molecule screen utilizing an HH-dependent reporter cell line (Light2 cells). We incubated Light2 cells with small molecules targeting different epigenetic modulators and identified four histone deacetylase inhibitors and a bromodomain and extra terminal domain (BET) protein inhibitor (I-BET151) that attenuate HH activity. I-BET151 was also able to inhibit the expression of HH target genes in Sufu−/− mouse embryonic fibroblasts, in which constitutive Gli activity is activated in a Smoothened (Smo)-independent fashion, consistent with it acting downstream of Smo. Knockdown of Brd4 (which encodes one of the BET proteins) phenocopies I-BET151 treatment, suggesting that Brd4 is a regulator of the HH signaling pathway. Consistent with this suggestion, Brd4 associates with the proximal promoter region of the Gli1 locus, and does so in a manner that can be reversed by I-BET151. Importantly, I-BET151 also suppressed the HH activity-dependent growth of medulloblastoma cells, in vitro and in vivo. These studies suggest that BET protein modulation may be an attractive therapeutic strategy for attenuating the growth of HH-dependent cancers, such as medulloblastoma.
3.2 Methods

Assays

Light2 cells (Taipale et al., 2000) were cultured in DMEM supplemented with 10% newborn calf serum, 1% penicillin/streptomycin, 0.2 μg/μl G418, and 0.1 μg/μl Zeocin. Sufu<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) were a gift from Dr. Rune Tofgard and were cultured in DMEM supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Compound screening was performed in a 96-well plate format. The compounds were titrated by Hanks' balanced salt solution and added to Light2 cells at different concentrations in the presence of the Smo agonist SAG. Twenty-four hours later, cells were lysed in passive lysis buffer, and luciferase activity was determined as described previously (Singh et al., 2009). All compounds were screened in the presence of 0.5% newborn calf serum. The selected compounds (positive “hits”) were purchased from Tocris and assayed individually. To assay gene expression, RNA was extracted from cells using an RNeasy kit (Qiagen), converted into cDNA (Applied Biosystems), and subsequently analyzed using real-time PCR, utilizing TaqMan probes as per the manufacturer's instructions (Life Technologies). The Brd4 siRNA SMARTpool as well as non-target control siRNA (Dharmacon) was transfected into Sufu<sup>−/−</sup> MEFs using Lipofectamine 2000 (Life Technologies) for 72 h. ChIP analyses were performed by cross-linking cells with 1% formaldehyde, quenching this reaction with glycine, and then resuspending in an SDS buffer (Weaver et al., 2014). This lysate was then sonicated to yield chromatin fragments of ~300–800 bp. These chromatin fragments were immunoprecipitated with Brd4 antibodies (Bethyl Laboratories) or IgG control, reverse cross-linked at 65 °C, and treated with RNase A and proteinase K, and then the relevant
region of the Gli1 locus was amplified by quantitative PCR. CSC viability was assayed by quantitating trypan blue (Life Technologies) exclusion using the Bio-Rad automatic cell counter TC20TM.

**Mice and Drug Administration**

All mice were handled in accordance with the policies of the University of Miami Institutional Animal Care and Use Committee. Spontaneous medulloblastomas from Ptch1+/− mice (The Jackson Laboratory) were subcutaneously grafted onto CD-1 nude mice (Charles River Laboratories). A subset of the tumors from these allografted mice was harvested and enzymatically digested using a papain dissociation system (Worthington). The resulting cell suspension was maintained in CSC selection medium containing Neurobasal, GlutaMAX, B27, N2, EGF, FGF, and Pen Strep media (Life Technologies) (Singh et al., 2004). A subset of the cohort of mice bearing these allografted medulloblastomas was treated with 30 mg/kg of I-BET151 in DMSO, by daily intraperitoneal injection, once the tumors reached a size of ~200 mm3. Tumor size was measured daily, using the formula: \( V = \pi \times L \times W \times W/6 \), where \( V \) is tumor volume, \( W \) is tumor width, and \( L \) is tumor length (Tomayko and Reynolds, 1989). Medulloblastoma tumors were subsequently harvested and then processed for H&E staining or HH target gene expression. Statistical analysis was determined by Student's two-tailed t test, unless otherwise stated. p values \( \leq 0.05 \) were considered statistically significant.
3.3 Results

I-BET151: a bromodomain and extra terminal protein inhibitor that potently inhibits Gli-driven luciferase activity.

To screen for inhibitors of epigenetic modulators that attenuate HH activity, we used an established fibroblast cell line (Light2 cells) that stably expresses an HH-dependent firefly luciferase reporter gene, as well as a control Renilla luciferase gene driven by the TK promoter. These cells were activated with the Smo agonist SAG to induce luciferase activity, along with varying concentrations (0, 0.5, 1, 2, or 10 μM) of 60 distinct, well characterized epigenetic enzyme inhibitors, a number of which are in various stages of clinical evaluation (Table 1). As a positive control, we used the clinically approved Smo antagonist vismodegib. DMSO-treated cells, and cells not stimulated with SAG, were used as negative controls. Ideally, relevant inhibitors would significantly attenuate firefly luciferase expression, but have minimal effects on Renilla luciferase expression at equipotent doses. Based on the dose-dependent effects of these inhibitors on Light2 cells (Fig. 3.1A and data not shown), five inhibitors were selected for further evaluation. Four of these inhibitors were distinct histone deacetylase inhibitors, and one was the BET inhibitor I-BET151. We purchased four of the epigenetic inhibitors identified in our screen (one was not commercially available) and performed more extensive dose-response curves (see Fig. 3.1B for their structures). All four of these inhibitors attenuated HH signaling, and did so in a dose-dependent manner (Fig. 3.2). As histone deacetylase inhibitors have been previously implicated in regulating HH activity (Canettieri et al., 2010; Lee et al., 2013), for the remainder of this study, we focus solely on the BET bromodomain inhibitor I-BET151.
FIGURE 3.1. A screen for epigenetic inhibitors of HH signaling.

A Light2 reporter cell line, engineered to express a Gli site-driven luciferase reporter gene and TK-driven control luciferase gene, was treated with the Smo activator SAG (100 nM). These cells were subsequently utilized to screen a panel of 60 small-molecule inhibitors targeting various epigenetic regulators.

A, four different concentrations of inhibitors were used: 0.5, 1, 2, and 10 μM, and results for 2 μM are shown. The mean responses to these various inhibitors (top dashed line) and
two standard deviations of this mean (bottom dashed line) are shown. Columns 1–60 represent individual epigenetic enzyme inhibitors, whereas columns 61–63 represent DMSO, vismodegib (100 nM), and unstimulated activity, respectively. The positive hits from this screen are highlighted by blue asterisks or a red asterisk (I-BET151).

B, chemical structures of small-molecule HH inhibitors.

**I-BET151 attenuates Gli1 expression**

A number of key components of the HH signaling pathway have already been identified (Fig. 3.3A), and their importance in various human pathologies has been underscored by their common mutation frequencies (Teglund and Toftgard, 2010). In general, HH binds to its core receptor protein Ptc1, inducing the derepression of the seven-transmembrane protein Smo (Robbins et al., 2012). Activated Smo ultimately regulates the Gli family of transcription factors, via alterations in protein half-life, via an array of post-translational modifications, and through its association with the negative regulator Sufu. The Sufu/Gli association is destabilized via trafficking through the primary cilium, a microtubule-enriched organelle required for canonical HH signaling. To identify the components of the HH signal transduction pathway disrupted by IBET-151, we treated Light2 cells with SAG and different doses of I-BET151 and then quantitated the expression levels of Ptc1, Smo, Gli2, or Gli1 (Fig. 3.3B). Although the expression levels of these four signaling components were affected by I-BET151, the IC50 of I-BET151 for reducing Gli1 levels was most similar to that observed for attenuating SAG-induced luciferase activity (Fig. 3.2).
FIGURE 3.2. Validation of four distinct epigenetic HH inhibitors.

Four of the five inhibitors selected from the screen shown in Fig. 1 were individually purchased and then reanalyzed using SAG-activated Light2 cells. In these dose-response curves, Gli site-driven luciferase activity is normalized to cell number. Error bars represent the S.E. of three independent experiments. TSA, trichostatin A; SAHA, suberanilohydroxamic acid.
FIGURE 3.3. I-BET151 attenuates Gli1 expression.

A, a model of the HH signaling pathway, showing pivotal positive (green) and negative (red) regulators.

B, Light2 cells stimulated with SAG (100 nM) were treated with the indicated doses of I-BET151, and the expression levels of various HH signaling components were determined.

Error bars represent the S.E. of three independent experiments.

I-BET151 acts downstream of SMO to attenuate Gli1 expression

The majority of HH inhibitors described to date bind to and attenuate the activity of Smo, including the Food and Drug Administration-approved small-molecule vismodegib (Teglund and Toftgard, 2010). One convenient way to identify those inhibitors that act downstream of Smo is to screen them using Sufu−/− MEFs as loss of Sufu results in the Smo-independent activation of Gli proteins. We therefore treated Sufu−/− MEFs with two different doses of I-BET151 and then quantitated the expression levels of Ptch1, Smo,
Gli2, or Gli1. I-BET151 attenuated the expression of these pivotal HH signaling components in $Sufu^{-/-}$ MEFs in a manner similar to its effect in SAG-stimulated Light2 cells (Fig. 3.4A), and once again the expression of Gli1 was the most sensitive to I-BET151. Consistent with $Sufu^{-/-}$ MEFs harboring a Smo-independent activation of Gli proteins, vismodegib was unable to attenuate Gli activity in these cells. We also examined the expression of several oncogenes and cell cycle-related genes that are commonly attenuated by I-BET151. Consistent with I-BET151 functioning through the attenuation of multiple signaling nodes (Chaidos et al., 2014; Dawson et al., 2011), the expression of c-Myc, P21, and Cdk4 was inhibited in a similar manner to Gli1 (Fig. 3.4B). We also took advantage of the constitutive Gli1 expression of $Sufu^{-/-}$ MEFs to examine its dependence on the I-BET151 target Brd4. We knocked down Brd4 expression using a validated Brd4 siRNA SMARTpool or a control siRNA. Brd4 knockdown resulted in a ~60% reduction of Gli1 levels, similar to the reduction in expression of Brd4 itself (Fig. 3.5A). Thus, consistent with I-BET151 acting specifically in these cells to inhibit Brd4 function, knockdown of Brd4 also attenuated Gli1 expression.
FIGURE 3.4. I-BET151 acts downstream of Smo in the HH pathway.

A, Sufu$^{-/-}$ MEFs harbor a constitutively active HH signaling pathway, activated downstream of Smo. These MEFs were treated with either I-BET151 (100 nM or 500 nM) or vismodegib (100 nM) for 24 h, and the expression levels of various HH signaling components was determined.
B, Sufu^{−/−} MEFs were treated with 0.5 μM I-BET151 for 8, 16, or 24 h. RNA was collected and subjected to quantitative RT-PCR. The expression of the indicated gene products was then normalized to that of GAPDH. Error bars represent the S.E. of three independent experiments. p values ≤ 0.05 are considered statistically significant and indicated by an asterisk.

I-BET151 abrogates BRD4 occupancy in the Gli1 locus

As Brd4 interacts with chromatin-associated acetylated histones to regulate target gene transcription (Itzen et al., 2014), we tested the hypothesis that I-BET151 treatment attenuates Gli1 transcription by reducing Brd4 occupancy of the Gli1 locus. Consistent with this hypothesis, we found that Brd4 associates with the proximal regulatory region of the Gli1 locus, near the transcriptional start site. Interestingly, I-BET151 treatment of cells decreased the occupancy of Brd4 on this region of the Gli1 locus (Fig. 3.5B). We also noted that the effects of I-BET151 on Gli-driven luciferase activity could be overcome by the constitutive expression of a human GLI1 cDNA, which lacks the proximal regulatory region of the Gli1 locus that Brd4 enriches on (Fig. 3.5C). As a control, we also show that I-BET151 still attenuated the expression of endogenous mouse Gli1. Together, these results suggest that I-BET151 abrogates Gli1 transcription by reducing the association of Brd4 with the proximal regulatory region of the Gli1 locus.
FIGURE 3.5. I-BET151 reduces Brd4 occupancy of the Gli1 locus.

A, knockdown of Brd4 inhibits HH target gene expression. 50 nM Brd4 siRNA or scramble siRNA was transfected into Sufu−/− MEFs for 72 h. RNA was subsequently extracted for quantitative PCR to determine the expression of the indicated genes.

B, schematic of the mouse Gli1 locus from −10,000 bp to +500 bp, relative to the transcription start sites (TSS) shown. Brd4 occupancy was analyzed by ChIP-quantitative PCR in Sufu−/− MEFs treated with 0.5 μM I-BET151 and then normalized to a control ChIP performed using rabbit IgG.
**C**, a plasmid expressing human GLI1 from a CMV promoter was transfected into Light2 cells and then treated with 0.1 or 0.5 μM I-BET151 48 h later. After 24 h of treatment, firefly luciferase activity was measured and normalized to Renilla luciferase activity, followed by isolation of RNA to measure the expression of the indicated HH target genes.

Error bars represent the S.E. of three independent experiments. p values ≤ 0.05 are considered statistically significant and indicated by an asterisk. NS, not significant.

**I-BET151 attenuates HH-driven medulloblastoma growth in vivo**

To test the ability of I-BET151 to attenuate the growth of an HH-driven cancer, we used a *Ptch1*+/−-derived medulloblastoma mouse model (Goodrich et al., 1997). Spontaneous tumors from this well characterized medulloblastoma model were used to isolate CSCs, which were maintained as a non-adherent spheroid culture, and to establish a transplantable allograph model (Ward et al., 2009). Such CSC lines harbor a constitutively activated HH pathway and considerable Gli1 expression and proliferate in a Smo-dependent manner (Ward et al., 2009). Treatment of CSCs with I-BET151 reduced their viability within 3 days of treatment and did so in a dose-dependent manner (Fig. 3.6A). Moreover, the effect of I-BET151 on CSCs was positively associated with decreased Gli1 levels (Fig. 3.6B). To determine the effect of I-BET151 on cancer growth in vivo, randomized nude mice were implanted with *Ptch1*+/−-derived medulloblastoma tissue and treated with I-BET151 or vehicle once the tumors were ~200 mm3 in size. Although the tumors in the vehicle-treated mice grew rapidly, the growth of the tumors in the I-BET151-treated mice was significantly attenuated (Fig. 3.6C and D). Importantly,
the body weight of the I-BET151-treated mice was not statistically different from that of the vehicle-treated mice (Fig. 3.6E). Further, consistent with I-BET151 attenuating tumor growth through inhibition of HH signaling, I-BET151 reduced the levels of the HH target gene Gli1 relative to the tumors in the vehicle-treated mice (Fig. 3.6F).

**FIGURE 3.6. I-BET151 abrogates HH activity-driven medulloblastoma growth.**

Medulloblastoma cancer stem/progenitor cells established from a Ptch1+/− medulloblastoma were treated as follows.

**A**, treated with the indicated doses of I-BET151 for 3 days prior to determining cell viability. Cell viability was measured by trypan blue staining and then quantitated.

**B**, twenty-four hours after I-BET151 treatment, Gli1 expression was quantified. Ptch1+/− medulloblastoma tissue was subcutaneously implanted into immunodeficient mice (n = 5 for vehicle group and n = 6 for experimental group). When the tumor size reached ∼200 mm3, the mice were treated with 30 mg/kg of I-BET151 or DMSO.

**C**, tumor volume was measured daily.
D, hematoxylin and eosin staining was performed to identify differences in histology between vehicle- and I-BET151-treated tumor tissue.

E, body weights of the I-BET151- or vehicle-treated mice were measured at the indicated times.

F, Gli1 gene expression in tumor tissue from the I-BET151- or vehicle-treated mice. Error bars represent the S.E. of three independent experiments, unless otherwise indicated. p values \( \leq 0.05 \) are considered statistically significant and indicated by an asterisk.
CHAPTER FOUR: DISCUSSION

4.1 Lipid speciation of SHH-Np

- **Biogenesis of lipid-speciated SHH-Np**

My work on identification of lipid speciation of SHH-Np elicits a fundamental question: what causes heterogeneous fatty acylation of SHH-Np? There are many precedents of heterogeneous fatty acylation of proteins. Retina proteins were reported to be subjected to post-translational modification which is composed of a spectrum of fatty acids (C12:0, C14:0, C14:1, C14:2) in the amino termini (Bereta and Palczewski, 2011; Dizhoor et al., 1992; Johnson et al., 1994; Neubert et al., 1992; Sanada et al., 1995). N-myristoyltransferase (NMT), a ubiquitous and essential enzyme in eukaryotes, mediates the catalysis of attachment of fatty acid substrates to modified proteins (Wright et al., 2010). Among these retina proteins, the catalytic subunit of cyclic adenosine monophosphate (cAMP)-dependent protein kinase was demonstrated to be fatty acylated in a tissue-specific manner (Johnson et al., 1994). Specifically, the profile of N-acylation of this kinase varies upon tissue types. Only myristate was found at the N termini of catalytic subunits from brain and heart tissue, whereas the retina-derived subunits were heterogeneously fatty-acyl modified in a manner similar to recoverin and transducin (Neubert et al., 1992; Sanada et al., 1995). Besides retina proteins, Fyn, as one member of Src kinase family, was also reported to be modified by a spectrum of fatty acids when it was co-overexpressed with NMT in COS-1 cells (Liang et al., 2001). Notwithstanding, whether such heterogeneous fatty acylation of Fyn exists in vivo remains unknown. These previous discoveries of lipid speciation of signaling proteins strongly suggest there is a general biological mechanism to account for such dynamic modifications.
It is likely that the selectivity of HHAT gives rise to differential abundance of lipid speciated SHH-Np, assuming HHAT is the only acyl-transferase for SHH-Np fatty acylation. *In vitro* fatty acylation studies of SHH-N by purified HHAT did suggest different affinity of fatty acyl-CoA as substrates for modification of SHH-N by HHAT (Buglino and Resh, 2008). Specifically, HHAT had the highest affinity for decanoyl (C10:0) CoA and lowest affinity for steroyl (C18:0) CoA and palmitoleoyl (C16:1) CoA. The difference between them was around 10 folds. However, *in vitro* data was not in accordance with lipid profile from purified SHH-Np protein in my studies and other’s. My works demonstrated that more than 90% of SHH-Np was modified by fatty acids (C16:0, C16:1 and C18:0). Only a small percentage of SHH-Np was modified by short chain fatty acids (C14:0). There is still a likelihood that HHAT has different selectivity of substrates in cell than *in vitro* conditions. However, when *SHH* was induced with or without serum deprivation, lipid profile of SHH-Np turned out to be extremely dynamic and distinct from the condition when *SHH* was not induced. If SHH-Np fatty acylation is solely dependent on the specificity of HHAT, there should not be such dramatic changes of profile in different cellular contexts. Taken together, the role of specificity of HHAT remains controversial in determining lipid profile of SHH-Np.

Alternatively, the availability of fatty acids determines the profile of SHH-Np modification whereas HHAT is a promiscuous enzyme to utilize substrates (or has low selectivity against substrates). In the case of retina proteins, there was a substantial difference of composition of acyl-coenzyme profiles between the retina and brain (Bereta
and Palczewski, 2011). Higher ratio of C14:1 and C14:2 CoA versus C14:0 CoA was found in the retina tissue compared to brain tissue. The abundance of C14:2 and C14:1 bound to the catalytic subunit of cAMP-dependent protein kinase were predominant over the C14:0 modified one in the retina tissue (Johnson et al., 1994). On the contrary, only C14:0 was found in this catalytic subunit of cAMP-dependent protein kinase in the brain tissue (Johnson et al., 1994). The lipid modification profile of protein may simply mirror the availability of fatty acids substrates in distinct tissues. In the case of SHH-Np, the availability of fatty acyl CoA can be regulated by both exogenous fatty acids and endogenous lipid metabolism. It is likely that when SHH-I cells were growing in the 10% fetal bovine serum and \( SHH \) is expressed at low level, the most available fatty acyl CoA is C16:0 which is also the second most abundant fatty acids in fetal bovine serum (Stoll and Spector, 1984). When SHH-I cells were growing in serum deprivation and/or \( SHH \) is induced at vast level, unknown endogenous lipid metabolism is altered so that unsaturated fatty acyl CoAs become predominant substrates for HHAT. HH protein is known to act as modulator to lipid metabolism by canonical and non-canonical signaling (Lee et al., 2015; Teperino et al., 2012). Absence of exogenous fatty acids and up-regulation of SHH expression may trigger flux of lipid metabolism in cells, which yields differential availability of fatty acyl CoA for SHH-Np modification. In addition, C16:0 is the only fatty acids found in endogenous SHH-Np purified from a small scale of chick embryos (data not shown). This is consistent with the fact that C16:0 is the second most abundant fatty acids in the egg yolk (Cherian et al., 2002). However, there is a lack of solid evidences to show alteration of lipid metabolism in cells, which yields differential availability of fatty acyl CoA for SHH-Np modification. 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• Function of Lipid-speciated SHH-Np

**HH ligand potency**

Lipid modification is essential for HH function including ligand potency, intracellular trafficking and long range signaling. Hydrophobicity has been shown to modulate SHH-N ligand potency (Taylor et al., 2001). EC$_{50}$ of SHH-N can range from 100nM (unmodified) to 0.6 nM (myristoylated form) in alkaline phosphatase (AP) assay. The trend is that the more hydrophobic lipid is, the more potent SHH-N is. In my work, EC$_{50}$ of SHH-Np in AP assay was around 0.2 nM when purified from SHH-I without induction. This the by far the most potent form of SHH-Np. When SHH-I cells was induced, SHH-Np potency was 10 folds less than when uninduced. 70-80% of SHH-Np purified from uninduced SHH-I cells were modified by saturated fatty acids (majority of species is C16:0) while 70-80% of SHH-Np purified from induced SHH-I cells were modified by unsaturated fatty acids (majority of species is C16:1). The difference of hydrophobicity among these different fatty acids is up to 1000 folds according to Log P value of each fatty acid. The differential hydrophobicity of fatty acids found in SHH-Np may account for change of SHH-Np potency. Incubation of chick embryonic limb bud tissue with excess of unsaturated fatty acids (C16:1) did attenuate SHH-Np potency in my work.

Differential fatty acylation may alter SHH-Np ligand binding affinity to PTCH receptor, however distinct *in vitro* modifications of SHH-N exhibited no difference of PTCH1 binding affinity (Taylor et al., 2001). If there is no difference in Ptch receptor binding, differential fatty acylation may alter SHH-Np ligand binding affinity to HH co-receptors
including Boc, Gas-1 and Cdo. These co-receptors were demonstrated to promote activation of HH signaling by forming a complex together with HH-PTCH (Allen et al., 2011; Izzi et al., 2011). It has been documented that SHH binding to co-receptors/receptors was enhanced by divalent ions (Bishop et al., 2009; McLellan et al., 2008). There is a likelihood that lipids have differential capacity to help recruiting divalent ions for ligand binding to its receptors/co-receptors. Deprivation of divalent ions will be able to test if differential potency disappear when comparing SHH-Np from uninduced and one from induced.

**HH intracellular trafficking**

A fully processed SHH-Np undergoes signal peptide cleavage, fatty acylation, auto-proteolysis, cholesterol modification and secretion through a highly orchestrated intracellular trafficking from ribosome to cytoplasmic membrane (Kornberg, 2011). Lipid raft, a cell membrane niche composed of sterols and sphingolipids and inclusive of a specific subset of membrane proteins (Levental et al., 2010), serves as isolated platform for cell signaling including membrane receptors anchorage and protein secretion (Brown and London, 1997; Simons and Ikonen, 1997). Post-translation modification by saturated lipids recruits both peripheral and transmembrane proteins to rafts whereas short, unsaturated, and/or branched hydrocarbon chains prevent raft association (Levental et al., 2010). Our findings suggest that one consequence of fatty acid speciation is the regulation of its intracellular trafficking, with decreased localization of SHH-Np modified with unsaturated fatty acids to lipid rafts. This decreased localization is likely the result of unsaturated fatty acids lacking the compactness required to enrich in lipid
raft compartments of cellular membranes, although such fatty acid doping experiments likely result in the production of a number of different fatty acylated species. However, a simple differential localization of SHH-Np proteins to the lipid raft or non-lipid raft compartments of cellular membranes could arise solely by regulating the degree of fatty acid saturation on SHH-Np. Since HH-Np was enriched and secreted in the lipid raft, it is reasonable to hypothesize that differential lipid raft localization gives rise to differential ligand secretion due to the HH lipid modification. This hypothesis was, at least partially, supported by our lipid doping studies where saturated fatty acids did enhance SHH-Np secretion in the conditioned medium.

**HH long range signaling**

HH proteins not only act as local morphogen but also are distributed further away from producing cells to regulate distal cell fate and tissue/organ development. Hitherto, there are multiple models to illustrate the mechanism of HH long range signaling, including multimerization, lipo-protein association and cytonemes. Our lab previously identified a freely diffusible form of SHH-Np from conditioned medium of cells and endogenous SHH producing tissue, whose molecular weight is 6 times larger than monomer SHH-Np (Zeng et al., 2001). This SHH-Np multimers is conceived to be able to distribute further in tissue presumably because multimerization of SHH-Np could reduce the hydrophobicity of lipid modified protein by which receiving cells capture and internalized. It is attempting to hypothesize that lipid speciation plays a substantial role in HH multimerization to modulate long range signaling. If this is true, it would be expected to observe dramatic change of HH multimers between induced SHH-I cells and...
uninduced SHH-I cells since lipid profile for these two conditions were drastically
distinct. However, isolation of multimers from these two condition (data not shown)
failed to show significant difference. This suggests lipid speciation is circumstantial for
SHH-Np multimerization.

On the other hand, a lipo-protein associated form of HH was identified in both
Drosophila and mammalian cell line in the other lab (Palm et al., 2013). Particularly, in
Drosophila, the lipo-protein associated HH is distributed in a hormone-like fashion to
senses nutrition availability and then modulates lipid metabolism systematically
(Rodenfels et al., 2014). This hormone-like HH mediated metabolic changes coordinate
growth rate and development timing in Drosophila. Similar lipoprotein-associated
Hedgehog family proteins also exist in mammals (Palm et al., 2013). Further, SHH
signaling can also regulate glucose and fat metabolism, in both canonical and non-
canonical signaling pathways (Lee et al., 2015; Teperino et al., 2012). Based on such
observations we speculate that distinct lipid speciated forms SHH-Np might also function
in such a hormone-like manner. In such a model, distinct forms of speciated SHH would
be produced in response to fluctuations in lipid metabolism and would then preferentially
act to modulate metabolic pathways. Further investigations need to be performed to
examine the lipid modifications of secreted SHH-Np in the circulatory system. The
ultimate query is whether SHH-Np can exert a hormone-like function to regulate a
systemic response of the human body to its nutrient environment.
Alternatively, HH protein has been reported to exert long range signaling through cytonemes (Kornberg, 2014). Cytonemes are specialized types of signaling filopodia that are actin-based (Kornberg and Roy, 2014). They extend from both the apical and basal surfaces of polarized cells (Hsiung et al., 2005) and they transport signaling proteins between source and target cells (Kornberg, 2014). In *Drosophila*, cytonemes extending from Hh-producing cells as well as Hh-receiving cells have been implicated in Hh transport and signaling (Bilioni et al., 2013; Bischoff et al., 2013; Callejo et al., 2011). In addition, there is a piece of evidence that cytonemes also exert similar function to transport HH from producing cells to target cells in chicken limb buds (Sanders et al., 2013). In the fly imaginal disk, a population of HH proteins is subjected to an intracellular trafficking from apical side of polarized producing cells to basal membrane and then transported to receiving cells via cytonemes. In the chicken limb buds, HH protein is distributed in the form of a particle that remains associated with the producing cell via cytonemes that span several cell diameters. A major limitation of this model in HH long range signaling is relatively short “long range signaling” within local tissue, compared with previous models. Bringing this model to the scope of my work, the finding of lipid speciated SHH suggested that distinct lipid modifications play role in sorting subpopulation of HH proteins into such cytonemes-mediated long range signaling. Being discussed in the previous section, lipid speciation does have a promising role in regulating HH intracellular trafficking aptly illustrated by lipid raft studies upon lipid doping. Further investigation will be necessitated to confirm if such modulation occurs in the cytoneme.
All three model do not have exclusive roles in explaining HH long range signaling and may co-exist/cooperate at differential contexts. Identification of heterogeneous fatty acylation of SHH-Np shed light on the new level of regulation in HH ligand function. Lipid speciated SHH-Np undergoes differential intracellular trafficking into subsets of membrane domain for secretion and long range signaling, whose potency is regulated by specific lipid modification (Fig.4.1). In the homeostatic condition, SHH proteins are modified as mixture of heterogeneous fatty acylated SHH-Np. Saturated fatty acid species drive SHH-Np trafficking into the lipid raft for long range signaling and unsaturated fatty acids species segregate SHH-Np away from lipid raft. In the pathological condition (diabetes and cancers), SHH proteins are predominantly modified as mixture of unsaturated fatty acylated SHH-Np. Unsaturated fatty acid species segregate SHH-Np away from lipid raft for local acting. Saturation degree of fatty acids may contribute to potency, intracellular trafficking and long range signaling.
**Figure 4.1: A proposed model of speciated SHH biogenesis.** Under the physiological conditions, a family of lipid speciated SHH-Np undergoes differential intracellular trafficking to produce long range signaling and local acting signaling. Specific subpopulation of SHH-Np for long range signaling display high potency and are lipoprotein associated forms for circulation in the serum. Under the circumstance of pathology, SHH proteins are predominantly modified by unsaturated fatty acids and secreted for cell signaling in local tissue/organ, which exhibit low potency and non-lipoprotein associated form.

4.2 Epigenetic regulation in HH-driven cancer

- **Resistance of SMO antagonist vismodegib in HH-driven cancers**

Vismodegib, a small molecular compound targeting SMO, was identified as a potent inhibitor of HH pathway, as aptly demonstrated by successful tumor remission of medulloblastoma allograft in mice with dosage of 12.5 mg/kg (Robarge et al., 2009). Thereafter soon, it was administrated in an advanced medulloblastoma patient who experienced a rapid regression of tumor after two months of treatment but then developed quick relapse a month later (Rudin et al., 2009). Further mechanistic studies identified an amino acid substitution at a conserved aspartic acid residue of SMO that had no effect on Hh signaling but disrupted the ability of vismodegib to bind SMO for pathway inhibition (Yauch et al., 2009). Acquired mutations of SMO can serve as a mechanism of drug resistance in HH-driven cancers. Then a few years later, a comprehensive genomic analysis of primary HH-driven medulloblastoma identified several downstream mutations
of HH signaling components including $SMO$, $SUFU$, $GLI2$ and $MYCN$ which predicted the future resistance to vismodegib treatment (Kool et al., 2014).

Basal cell carcinoma is another type of well-established HH-driven cancer. Vismodegib was tested in several phase I clinical trials of basal cell carcinoma (Sekulic et al., 2012; Von Hoff et al., 2009). The safety, pharmacokinetics and responses of metastatic or locally advanced basal-cell carcinoma to this drug were assessed, which demonstrated that vismodegib had mild dose limiting toxicity and anti-tumor activity for both locally advanced and metastatic basal cell carcinoma. Then it was approved by the U.S. Food and Drug Administration to treat adult patients with basal cell carcinoma in 2012. However, similar to medulloblastoma case, $SMO$ mutations were subsequently reported to, at least partially, account for resistance to vismodegib (Brinkhuizen et al., 2014; Pricl et al., 2015). More global genomic analysis of resistant basal cell carcinoma patients revealed that there were not only novel $SMO$ mutations but also downstream signaling components mutations harbored in patients which included but not limited to $SUFU$ and $GLI2$ (Sharpe et al., 2015).

The goal of my studies is to explore ways to bypass clinically observed mutations of SMO and to overcome vismodegib resistance when treating HH-driven cancers. My work has identified that BET inhibitor I-BET151 can potently attenuate HH-driven cancer cell growth in vitro and in vivo by virtue of acting downstream of vismodegib target SMO. Direct modulation of Gli1 transcription also bypassed other mutations downstream of SMO such as $SUFU$ and $GLI2$. Current clinical studies of BET inhibitors in non-HH-
driven cancer patients reported mild toxicity and plasma concentration of drug roughly equivalent to in vitro concentration that is effective to suppress Gli expression (phase I clinical trials ongoing). There is a strong potential for testing BET inhibitor in clinical trial for HH-driven cancers.

- **Epigenetic regulation**

Epigenetic modulators regulate gene expression as four different roles: writer, eraser, reader and remodeler. As writers, epigenetic modulator add functional groups to histone and DNA to either enhance or inhibit target gene expression. Examples are histone acetyl transferase, histone methyl transferase and DNA methyl transferase. Histone acetylation typically loosens up histone-DNA interaction and then allows recruitment of transcriptional complex to initiate transcription. Histone methyl transferase exerts dual role in target gene expression. Histone lysine 4 methylations were associated with activation of gene transcription while histone lysine 27 methylation were associated with gene silencing (Kouzarides, 2007). As erasers, epigenetic modulators remove functional groups from DNA and histone to reverse modulation by writers. Epigenetic “readers” bear the capacity to recognize histone and DNA modification then transmitting signals to downstream effectors. A good example is a family of bromodomain and extra terminal (BET) proteins: BRD2, BRD3, BRD4 and BRDT. BRD2 and BRD3 act as scaffold to recruit distinct transcriptional complex in a tissue-specific manner (Belkina et al., 2014; Gamsjaeger et al., 2011; Hnilicova et al., 2013; Lamonica et al., 2011; Tsume et al., 2012). BRD4 and BRDT are able to recognize histone acetylation by bromodomains and then recruit P-TEFb and RNA polymerase II to regulate elongation of target gene
transcription (Gaucher et al., 2012; Jang et al., 2005; Yang et al., 2005). As to remodeler, ATP-dependent chromatin remodeling complexes specifically recognize histones modifications, unwrap, mobilize, exchange or eject the nucleosome through ATP hydrolysis, subsequently recruiting a transcriptional machinery to nucleosomal DNA (Owen-Hughes, 2003). SWI/SNF family of chromatin remodeling complexes is the most intensive studied remodeler and highly conserved across eukaryotes and prokaryotes.

Besides protein based epigenetic modulators, there are also emerging DNA based and RNA based epigenetic elements identified in the past a few years, which include non-coding RNA and super-enhancers. Super-enhancers is a research hot spot relevant to my studies. The definition of super-enhancers is relatively new and controversial. Generally speaking, super-enhancers are a class of regulatory elements with high enrichment for the binding of transcriptional coactivators, specifically Mediator (Med1) (Loven et al., 2013; Whyte et al., 2013). Super-enhancers are identified based upon following methods: (1) master regulators ChIP seq (Med1, H3K27ac, BRD4, Oct4, Sox2 and Nanog) to identify enhancer; (2) Enhancers within 12.5 kb of each other were grouped to define a single entity spanning a genomic region; (3) The grouped enhancer entities and the remaining individual enhancers were then ranked by the total background-normalized level of Med1 signal within the genomic region (Pott and Lieb, 2015). Landscape of super-enhancers is cell line/organ specific and highly associated with pluripotency genes in stem cells and oncogenes in cancer cells (Chapuy et al., 2013; Hnisz et al., 2013; Loven et al., 2013). Particularly, super-enhancers in several different tumors were strongly enriched for binding of BRD4 (4-6 folds higher density than regular enhancers) (Chapuy et al., 2013;
Loven et al., 2013). BRD4 inhibition rendered selective sensitivity of those cancer cells, although BRD4 is ubiquitously expressed and essential for transcription. Although my work showed that I-BET151 can abrogate the BRD4 occupancy in the \( \text{Gli1} \) proximal promoter region, the functionally relevant target region might be ignored in terms of \( \text{Gli1} \)-associated super-enhancers. It remains unknown as to the \( \text{Gli1} \)-associated super-enhancers in HH-driven medulloblastoma. The future experiment would be identification of super-enhancers in HH-driven medulloblastoma to understand the mechanism by which BET inhibition preferentially target \( \text{Gli1} \) transcription.

- **Epigenetic dysregulation in HH-driven cancers**

Many HH-driven cancers, including medulloblastoma, undergo substantial epigenetic dysregulation, which cooperates oncogenic mutations at various progression stages. GLI1 function was reported to be downregulated by the chromatin remodeling protein SNF5, through its interaction with Gli1-regulated promoters. SNF5 was found to be inactivated in human malignant rhabdoid tumors, coincident with the activation of the Hh-Gli1 pathway (Jagani et al., 2010). In addition, GLI1 and GLI2 are acetylated proteins and their HDAC-mediated deacetylation promotes transcriptional activation. There is a positive feedback loop where HH activation induces upregulation of HDAC1. This feedback is cut off by HDAC1 degradation through an E3 ubiquitin ligase complex, which is absent in human medulloblastoma (Canettieri et al., 2010). Recently, genomic analysis of primary HH-driven medulloblastoma patients revealed a series of mutations of epigenetic modifiers across all four different categories (Kool et al., 2014). This intrigued me to investigate the epigenetic modulation in HH-driven cancers. Our work
identified that *Gli1* transcription was able to be targeted by modulation of BRD4 (BET inhibitor and siRNA knockdown) and BRD4 is an essential component of HH signaling (data not shown). This finding lends support to the significant role of epigenetic regulation in HH-driven cancers. Targeting epigenetic regulators is a promising strategy to treat HH-driven cancer.

In addition, when screening those epigenetic compounds for inhibition of HH signaling, there were also three other histone deacetylase inhibitors attenuating HH-driven luciferase reporter activity: SAHA, TSA and M344. Further test of these three compounds in MEF Sufu<sup>-/-</sup> revealed that they appeared to be effective in attenuating expression of several HH signaling components (*Gli1, Gli2, Smo*). Treating medulloblastoma stem cells with these HDACs inhibitors also exhibited efficacy in reducing stem cell proliferation and self-renewal. Due the fact that these compounds are pan-HDAC inhibitors (inhibiting many isoforms of HDACs) and HDACs isoforms have distinct function, it is difficult to interpret these results. However, it is certain that modulation of HDACs appears to be another option to target HH-driven cancers.

- **BRD4 in HH-driven cancers**

BRD4 has been extensively studied in a variety of cancers (Belkina and Denis, 2012). As described before, BRD4 acts as an epigenetic reader to recognize histone acetylation and regulate transcription elongation of target gene. Its essential role in gene transcription may render disadvantage to being a druggable target. Notwithstanding, identification of super-enhancers associated with oncogenes lend strong support to the rationale of
targeting BET proteins. Chromatin Immunoprecipitation (ChIP)-sequencing identified that BRD4 not only occupied at promoter and enhancer regions but also are highly enriched in large stretched chromatin areas called “super-enhancers” characterized by H3K27ac ChIP-seqencing (Hnisz et al., 2013). The intensity of BRD4 occupancy on super-enhancers was about 4-6 folds higher than on promoter and enhancer regions. Application of BET inhibitors in a wide spectrum of stem cell and cancer cells were able to substantially remove the occupancy of BRD4 in the super-enhancers and to abrogate transcription of key genes for stem cell maintenance and oncogenesis (Loven et al., 2013). The distribution of super-enhancers is cell/tissue specific and context dependent (Adam et al., 2015; Whyte et al., 2013). During cell differentiation, landscape of super-enhancer undergoes dramatic change, where specific super-enhancers disappear and other emerge. In vitro and in vivo culture conditions also modulate the profile of super-enhancers (Adam et al., 2015). In the case of diseases, profiles of super-enhancers are drastically different across different cell line and differentiation origins (Hnisz et al., 2013). Generally, it is believed that a few key transcriptional factors (TFs) govern the distribution of super-enhancers where several clusters of TFs binding sites reside. Especially cancer cells rely on the addiction of distinct key TFs to drive super-enhancer associated oncogenes expression which are critical for cell proliferation and metastasis. Modulation of these key TFs expression is able to re-model super-enhancers landscape. The theory of super-enhancers characterized by BRD4 enrichment provides solid rationale on selectivity of targeting BRD4 in cancer cells versus normal cells. Mild toxicity of clinical trials of BET inhibitors in hematological cancers supported selective targeting BRD4 in patients.
In HH-driven cancers, the role of BRD4 has not been discussed extensively. Our work of discovery of I-BET151 to attenuate HH-driven cancers, together with another group utilizing another BET inhibitor JQ1 to modulate HH signaling transcriptional output (Tang et al., 2014), is conducive to understand the function of BRD4 in HH-driven cancer. Not only I-BET151 inhibitor but also RNAi mediated knockdown of BRD4 led to attenuation of Gli1 expression. This suggested an essential role of BRD4 in Gli1 transcription. Induction of HH signaling pathway in a HH-responsive cell line (Light-II) was able to induce BRD4 enrichment in the first intron of Gli1 locus as well as enrichment of active chromatin marks (H3K27ac and H3K4me3) (data not shown). And this induction was GLI2/3 dependent as aptly supported by the observation that HH failed to induce these enrichment in Gli2 -/- Gli3 -/- MEFs (data not shown). Co-immunoprecipitation experiment showed physical interaction between BRD4 and GLI2 when Gli2 was overexpressed in 293T cells (data not shown). There preliminary results imply that BRD4 is an important transcriptional co-activator to boost HH target gene transcription.

In addition, the discovery of enhancer RNAs (eRNAs) may be able to, at least partially, explain the function of BRD4 in target gene transcription. eRNAs are transcription product of enhancer DNA sequence belonging to the family of non-coding RNA. eRNAs contribute to enhancer-mediated transcriptional activation of neighborhood coding genes by presumable mediation of chromosomal looping between enhancer and transcription start sties and facilitating RNA Pol II recruitment to the promoter of the target gene (Lam
et al., 2014). Knocking down of eRNAs substantially abrogates function of enhancers. Super-enhancer associated eRNAs has recently been discussed in inflammation (Hah et al., 2015). When it comes to HH-driven cancers, it remains unknown as to super-enhancer associated eRNAs and its potential role in HH signaling. Whether modulation of BRD4 alters the super-enhancer associated eRNA output appears to be an interesting biological question. Presumably, loss of BRD4 in super-enhancer may lead to concomitant loss of eRNAs thereby downregulating the function of super-enhancer. To test this hypothesis, RNA-seq will be utilized to measure eRNAs output upon BRD4 modulation. Re-introduction of plasmid that expresses super-enhancer sequence will then be performed to rescue target gene transcription led by inhibition or knockdown of BRD4.

4.3 Conclusions

Based upon my works on SHH biogenesis and epigenetic regulation in HH-driven cancers, my conclusions are listed below: 1). SHH-Np undergoes a heterogeneous fatty acylation in its amino terminus and this lipid speciation of SHH-Np is dynamic in a cell context dependent manner; 2) a BET protein inhibitor I-BET151 suppresses HH signaling downstream of SMO and attenuates HH-driven cancer growth in vivo. Regarding SHH-Np lipid speciation, differential lipid raft localization and potency of SHH-Np were observed upon exogenous fatty acids incubation. When it comes to I-BET151, this inhibitor attenuates HH target gene Gli1 transcription, at least partially, in a manner that remove BRD4 in Gli1 locus.
4.4. Future directions

Future works on SHH-Np lipid speciation will be carried out in several aspects: molecular mechanisms by which lipid speciation of SHH-Np occurs, identification of lipid modifications in secreted SHH-Np protein and functional studies of SHH lipid speciation. Being discussed in previous section, modulation of key HH processing proteins (HHAT, DISP and SCUBE2) by virtue of siRNA mediated knockdown and exogenous over-expression helps understanding what accounts for such a heterogeneous fatty acylation. Ligand potency and secretion efficiency (secreted SHH-Np/total SHH-Np) will be evaluated upon modulations described above. In addition, fatty acyl-CoA composition will be examined when SHH protein are expressed in different cellular contexts. This may provide an alternative explanation of lipid speciation of SHH-Np.

Secondly, purification works on secreted SHH-Np from different cellular contexts will be performed to investigate lipid modifications of SHH-Np when it is secreted and transported outside of cells. This will test previously described model where a specific group of lipid speciated SHH-Np is secreted in a lipoprotein associated form to act in a hormone like fashion. Thirdly, functional studies of lipid speciated SHH-Np will be carried out such as HH ligand-receptor binding affinity and HH-mediated modulation of metabolism. Distinct lipids are expected to modulate HH potency by regulating ligand-receptor binding affinity and are speculated to dictate SHH ability to trigger HH-induced metabolic rewiring.

Future works on epigenetic regulation in HH-driven cancers will be mainly carried out to validate rationales of targeting BRD4 in HH-driven cancers and to develop diagnostic
markers for epigenetic treatment in HH-driven cancer. For the former, since cancer cells addict to oncogene expression driven by its associated super-enhancers, targeting BRD4 is able to substantially attenuate oncogene expression and render susceptibility to cancer cells versus normal cells. Then it is particularly important to identify HH signaling-associated or Gli-associated super-enhancers in HH-driven cancer cells, which supports the rationality of selective targeting BRD4. For the latter, assuming there is a strong potential of targeting BRD4 in HH-driven cancer, it is important to develop diagnostic markers for selection of patients to receive such a treatment. The expression level of BRD4, cellular distribution of BRD4 and HH target gene expression pattern in HH-driven cancer cells as well as tissues from patients will be evaluated systematically to identify appropriate cellular markers. The ultimate goal is to translate our discovery of BRD4 targeting in pre-clinical studies into clinical usage.
REFERENCES


