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Histone deacetylase 6 represents a novel drug target in the oncogenic Hedgehog signaling pathway

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List of Abbreviations

ABC	ATP binding cassette
AD	Alzheimer's disease
AMC	Academic medical centre (Amsterdam)
AP	Alkaline Phosphatase
APS	Ammonium persulfate
ATO	Arsenic trioxide
AurA	Aurora A
BBS	Bardet-Biedl syndrome
BCC	Basal Cell Carcinoma
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
Boc	Brother of Cdo
Boi	Brother of *Ihog*
CALK	Chlamydomonas aurora-like protein kinase
cDNA	Complementary DNA
Cdo	Cell adhesion molecule related/downregulated by oncogenes
Cep70	Centrosomal protein of 70kDa
CFTR	Cystic fibrosis transmembrane conductance regulator
CK1α	Casein Kinase1α
CMB	Classic Medulloblastoma
CML	Chronic Myeloid Leukemia
CMT	Charcot Marie tooth disease
CNS	Central Nervous System
CRD	Cysteine-rich domain
CREB	cAMP response element binding protein
CSC	Cancer stem cells
CTA	Cancer testis antigens
C-Terminus	Carboxy Terminus

DAPI	4’6-Diamidino-2-Phenylindole
DBD	Dynein Binding Domain
Dhh	Desert Hedgehog
Disp	Dispatched
DMEM	Dulbecco’s Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose Nucleic Acid
DNMB	Desmoplastic nodular medulloblastoma
DPBS	Distilled Phosphate Buffer Saline
Dyrk1	Dual specificity tyrosine-phosphorylation-regulated kinase
ECL	Enhanced chemiluminescence
EvC	Ellis van Creveld Syndrome
EMT	Epithelial to mesenchymal transition
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GAGE	G antigen
GANT	Gli antagonist
Gas1	Growth arrest specific protein 1
GGS	Gorlin-Goltz syndrome
Gli	Glioma associated oncogene
Gli-A	Activator form of Gli
Gli FL	Full length of Gli
Gli-R	Repressor form of Gli
GNP	Granule neuron precursors
Gpr	G-protein-coupled-receptor
GSK3	Glycogen synthase kinase 3
HAT	Histone Acetyltransferase
HD	Huntington’s disease
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase inhibitors
HEF1	Human enhancer of filamentation 1

Hh	Hedgehog
Hip	Hedgehog interaction-protein
HRP	Horse radish peroxisidase
HSF	Heat Shock Factor
HSP90	Heat Shock Protein
IFT	Intra Flagellar Transport
IGF	Insulin like Growth Factor
Ihh	Indian Hedgehog
Ihog	Interference hedgehog
IMiDS	Immunomodulatory drugs
IMT	Institute of Molecular Tumour Biology (University of Marburg)
K	Lysine
Kif	Kinesin
LCA	Large cell/anaplastic
MAGE	Melanoma associated antigen
MAPK	Mitogen Activated Protein Kinase
MB	Medulloblastoma
MBEN	Medulloblastoma with extensive nodularity
MDCK	Madin Darby Canine Kidney
MEF	Mouse Embryonic Fibroblast
Min	Minutes
MKS	Meckel-Gruber syndrome
mRNA	Messenger RNA
MS	Mass Spectrometry
NBCSS	Nevoid basal cell carcinoma syndrome
NES	Nuclear Export Signal
NFκB	Nuclear Factor Kappa light chain enhancer of activated B cells
NLS	Nuclear localization signal
NPC1	Niemann-Pick C1 protein
NPHP	Nephronophthisis

N-Terminus	Amino Terminus
OKC	Odontogenic keratocysts
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffer Saline
PC	Primary cilium
PCR	Polymerase Chain Reaction
PD	Parkinson's disease
PDE4D	Phosphodiesterase 4D
PDGF	Platelet derived growth factor
PKA	Protein kinase A
PKD	Polycystic kidney disease
PNET	Primitive neuro ectodermal tumor
PTCH1	Patched1
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real time PCR
RMS	Rhabdomyosarcoma
RNA	Ribose Nucleic Acid
RND	Resistance-nodulation-cell division
RPM	Rounds per minute
RT	Reverse Transcriptase
RT	Room Temperature
SAG	Smoothened Agonist
SANT-1	Smoothened Antagonist
SBMA	Spinal bulbar muscular atrophy
SD	Standard Deviation
SDS	Sodium dodecyl sulphate
SG	Stress granules
Shh	Sonic Hedgehog
siRNA	Short interfering RNA
SLE	Systemic lupus erythematosus
Smo	Smoothened

Sol	Solvent
Spop	Speckle- type POZ
SSD	Sterol-Sensing Domain
SUFU	Suppressor of Fused
TAT	Tubulin acetyl transferase
TBS	Tris-Buffered Saline
TEMED	Tetramethylethylenediamine
TM	Trans membrane
Tregs	T-regulatory cells
TRIS	Tris (hydroxymethyl) aminomethane
TSA	Trichostatin A
TSC	Tumor stem cells
UPS	Ubiquitin proteasome system
UV	Ultraviolet
VIS	Vismodegib
WHO	World Health Organization
WT	Wild type
µ	Micro
µl	Microliter
µg	Microgram

1 Summary

Hedgehog signaling plays a vital role in regulating varied fundamental processes including embryonic development, proliferation, and differentiation. Aberrant hedgehog signaling has been one of the reason for cancers such as Basal cell carcinoma (BCC), rhabdomyosarcoma (RMS) and medulloblastoma (MB). Medulloblastoma, a malignant pediatric brain tumor is one such cancer. Even after the development of impressive Hh pathway antagonists, drug resistance in medulloblastoma has been one of the most waffling issues which require identification of new drug targets.

In the present study, increased histone deacetylase 6 (HDAC6) expression was observed in Hh-driven medulloblastoma and it is crucial for full Hh pathway activation. Interestingly, the stimulatory outcome/s of HDAC6 are partially integrated downstream of primary cilia, a known HDAC6-regulated structure. Further, HDAC6 is also essential for the repression of basal Hh target gene expression. These diverse outcomes are negotiated by HDAC6's impact on Gli2 mRNA and GLI3 protein expression. As a consequence of this intricate interplay with Hh signaling, only a subset of Gli and Smoothened driven genes are regulated by HDAC6 apart from the well-known Hh targets such as *Gli1* or *Ptch1* which was shown by global transcriptome analysis. Overall, survival of medulloblastoma cells was critically compromised by *in vitro* inhibition of HDAC6 and blockade of HDAC6 pharmacologically greatly reduced tumor growth in an *in vivo* allograft model.

In conclusion, the data illustrates the crucial aspects of HDAC6 in regulating the Hh pathway in mammals and encourage novel studies directed towards HDAC6 as a unique drug target in medulloblastoma.

1 Zusammenfassung

Der Hedgehog Signalweg spielt eine wichtige Rolle bei der Regulation verschiedener fundamentaler Prozesse wie der Embryogenese, der Proliferation und der Differenzierung. Ein aberranter Hedgehog Signalweg trägt zur Krebsentstehung im der Lunge, dem Gehirn, der Brust, und der Haut. Das Medulloblastom, ein maligner Hirntumor der im Kindesalter auftritt, ist hierfür ein prominentes Beispiel. Die Resistenzentwicklung gegen etablierte Hedghog Signalweg-Antagonisten stellt ein ernst zu nehmendes Problem dar, welches die Aufklärung neuer pharmakologischer Angriffspunkte verlangt.

In der vorliegenden Arbeit konnte eine erhöhte Histon Deacetylase 6 (HDAC6) Expression in durch Hedgehog Signalweg induziertem Medulloblastom gezeigt werden, welche ausschlaggebend für die vollständige Aktivierung des Signalweges ist. Die stimulatorischen Effekte von HDAC6 sind zum Teil downstream des Primärziliums zu finden, welches eine bekannte durch HDAC6 regulierte Struktur darstellt.Desweiteren ist HDAC6 essentiell für die Repression der basalen Hedgehog Signalweg Aktivität. Diese unterschiedlichen Effekte werden über die Regulation des mRNA Levels von Gli2 und die Protein Expression von GLI3 hervorgerufen. In einer Transkriptomanalyse konnte gezeigt werden, dass abgesehen von bekannten Hedgehog Zielgenen wie GLI1 oder PTCH1 lediglich eine Untergruppe von Gli und Smoothened Zielgenen durch HDAC6 reguliert werden. Zudem beeinträchtigt eine *in vitro* Inhibition von HDAC6 das Überleben von Medulloblastoma Zellen entscheidend und die pharmakologische Blockade von HDAC6 reduziert das Tumorwachstum in einem allogenen *in vivo* Model.

Zusammenfassend zeigen die Daten wichtige Aspekte von HDAC6 bei der Regulation des Hedgehog Signalweges in Säugern auf und legen den Grundstein für neue Studien über HDAC6 als interessanten Angriffspunkt zur Behandlung von Medulloblastoma.

2 Introduction

2.1 The Hedgehog signaling pathway

Proper developmental control, metabolism and tissue homeostasis of multicellular organisms is a fine tuned and highly orchestrated process that depends on regulation of molecular signaling pathways in a spatial and context-dependent manner. The relevance of controlled activation and termination of signal is important to the functioning of all organisms. Despite molecular advances, our knowledge with respect to health and development of higher organisms and the pathways controlling them is sparse. One of the pathways which plays a very important role in development is Hedgehog (Hh) signaling pathway (Teperino, Aberger, Esterbauer, Riobo, & Pospisilik, 2014).

The Hedgehog signal transduction pathway plays a quintessential role in mediating diverse fundamental mechanisms which comprise of cell proliferation, differentiation, survival, patterning, stem cell maintenance and tissue polarity (Gupta, Takebe, & Lorusso, 2010; Varjosalo & Taipale, 2008). The pioneering work of Eric F. Weischaus and Nusslein-Volhard in 1980 led to the discovery of the Hedgehog gene (Nusslein-Volhard & Wieschaus, 1980). In their study, a mutational screen was performed that disrupted the body plan of *Drosophila* larvae. In general, the *Drosophila* larva is normally divided into various segments, the posterior part of each segment is smooth and the anterior part is coated in bristles which are known as denticles. In their mutational screen, they described a group of mutants that affected the segmental patterning. In these mutants known as polarity mutants, the posterior part of each segment did not develop properly or failed to develop resulting in a phenotype which was short and spiky similar to that of a hedgehog which led to the term hedgehog- gene (*hh*) (Ingham & McMahon, 2001; van den Brink, 2007; Varjosalo & Taipale, 2008).

The mammalian Hh ligand family members consist of Desert Hedgehog (Dhh), Indian Hedgehog (Ihh) and the most common Sonic Hedgehog (Shh) (Chiang et al., 1996;

Echelard et al., 1993). In mouse and humans the three hedgehog genes are highly conserved (Marigo et al., 1995).

The hedgehog proteins go through comprehensive and specific post-translational modifications and cleavage events producing a ~45 kDa precursor protein. This precursor protein is autocatalytically cleaved thereby giving rise to a cholesterol modified 19-kDa NH2-terminal fragment (HhNp) and an unmodified 26-kDa COOH terminal fragment (HhC) (J. a Porter, Young, & Beachy, 1996). The most striking feature of Hedgehog proteins is dual lipid modification of the19-kDa NH2- terminal fragment. The modified signalling protein is linked covalently to cholesterol and a palmitate group and is poorly soluble (Brink, 2007). The palmitoylation modification assist hedgehog protein/s to integrate in the cell membrane and play vital role in hedgehog signalling range in a tissue. The 26-kDa COOH terminal fragment acts as a cholesterol transferase and also catalyses the cleavage (Bumcrot, Takada, & McMahon, 1995; J. J. Lee et al., 1994; J. A. Porter et al., 1996; van den Brink, 2007). It was also recently demonstrated that palmitoylation promotes cleavage of amino acids at N-terminal by proteases like ADAM (metalloprotease and disintegrin family member) (Ohlig et al., 2011). This kind of cleavage leads to formation of active Shh multimers. These amino acids residues, if not cleaved, interrupt with the Zn^{2+} coordination sites on adjacent molecules and this region has been shown to interact with Ptch and is known to modulate Shh activity and stability (Bishop et al., 2009; Bosanac et al., 2009; Day et al., 1999; Fuse et al., 1999).

The role of the cholesterol moiety is yet not clear (Lewis et al., 2001; Yina Li, Zhang, Litingtung, & Chiang, 2006; van den Brink, 2007). The Hh proteins have an exclusive feature of travelling to long distances up to 300μm to reach their targets. Dispatched (Disp), a 12- pass transmembrane protein related to the bacterial RND (Resistance-nodulation-cell division) family of transporters is essential for the release of long-range signalling of cholesterol and palmitate modified Hh (Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002).

2.2 The Mechanism of Hedgehog signal Transduction

The transmission of Hedgehog signal takes place upon binding of Hedgehog ligands to 12-span transmembrane receptors coded by genes PTCH1 and PTCH2 (Lisa V. Goodrich, Johnson, Milenkovic, McMahon, & Scott, 1996). The receptors exhibit two extracellular loops which are hydrophilic in nature and negotiate Hedgehog binding (van den Brink, 2007). The 12-pass transmembrane protein, Ptch exhibits homology to bacterial transport proteins belonging to RND (Resistance-nodulation-cell division) family. The Ptch family of proteins consist of RND- derived domain and a sterol-sensing domain (SSD). The RND transport proteins are basically antiporters of proton and are involved in active efflux of various substrates across the cell membrane. These antiporters utilize the physiological proton levels at the cell membrane to pump out various substrates and in return allow the flow of other protons into the cell. The eukaryotic Ptch superfamily includes Dispatched (Disp) and Niemann-Pick C1 protein (NPC1). Dispatched is involved in release of Hh proteins whereas NPC1 is engaged in cholesterol homeostasis (Hausmann & Von Mering, 2009).

The Hh reception by Ptch is further enhanced by the presence of other Hh binding proteins at the cell surface. These additional coreceptors constitute fibronectin type III (FnIII) and immunoglobulin family of membrane proteins Boi (Brother of Ihog) and Ihog (Interference hedgehog) in *Drosophila* and Boc (Brother of Cdo) and Cdo (Cell adhesion molecule related/downregulated by oncogenes) in vertebrates and Gas1 which is a vertebrate specific surface protein (Allen et al., 2011; Beachy, Hymowitz, Lazarus, Leahy, & Siebold, 2010; Izzi et al., 2011).

Apart from Boc, Cdo and Gas1 (Growth arrest specific protein1) vertebrates exhibit a fourth Hh-binding protein known as Hip. Hip has no role in downstream signalling but competes with Ptch for Hh binding (Bosanac et al., 2009; Chuang & McMahon, 1999). Ptch plays a dual role in Hh signalling; on one hand, it is the receptor for Hh and on the other hand it serves as a negative regulator of Hh signal transduction pathway by inhibiting Smo, which is a seven-pass transmembrane protein.

When Hh ligands are absent Ptch localizes to primary cilium (PC) and represses signalling by inhibiting G-protein-coupled receptor (GPCR) (Eggenschwiler & Anderson, 2007) like signal transducer Smoothened (SMO) by entering into the primary cilium (Rohatgi, Milenkovic, & Scott, 2007). The way by which Smo is inhibited by Ptch still remains elusive. It is assumed that repression of Smo by Ptc occurs via yet unidentified small molecule inhibitor (J. K. Chen, Taipale, Young, Maiti, & Beachy, 2002; Taipale, Cooper, Maiti, & Beachy, 2002).

Recently, this assumption has been supported by small molecule inhibitors of Smo that mimic Ptc over-expression functionally (J. K. Chen et al., 2002; Frank-Kamenetsky et al., 2002). These Smo antagonists seem to target the hepta-helical bundle belonging to Smo, the domain which is shown to be affected by Ptch (J. K. Chen et al., 2002) A major understanding into the regulation of Smo surfaced up when it was shown that oxidized cholesterol derivatives (oxysterols) specifically bind to the Cysteine-rich Domain (CRD) of Smo and are involved in activation of Hh pathway. Binding of Oxysterols by the CRD region of Smo can be functionally distinguished from binding of small molecules to the 7TM (Trans membrane) site because deletion of Smo CRD leads to loss of Smo activation by oxysterols but do not alter the activity of agonists and antagonists targeting the 7TM region of Smo. It has been found in a screening that 7-keto-27-OHC and 7-keto-25-OHC both of which are 7-ketocholesterol metabolites activate Hh signaling in a CRD dependent way. The finding that Smo CRD can bind oxysterols and regulate Hh signaling throws some light on the route by which Smo may be modulated by Ptch (McCabe & Leahy, 2015). Recent work also suggests that Smo inhibition by Ptch may be non-stoichiometric (Taipale et al., 2002). It has been demonstrated in *Drosophila* that Ptch might inhibit Hh signalling by modulating the production of phosphatidylinositol 4-phosphate (PI4P), acknowledging that decreasing and increasing levels of PI4P lead to Hh pathway repression and activation (Yavari et al., 2011).

The principal mediators of canonical Hh signalling are the zinc finger containing Gli transcription factors. When Hh ligands are absent Gli-FL (Full length) is cleaved proteolytically by β-TRCP giving rise to N-terminal transcriptional repressor (Gli-R)

(Ramsbottom & Pownall, 2016). In *Drosophila*, there is only one Gli family member, Cubitus interruptus (Ci) whereas vertebrates exhibit three different Gli transcription factors Gli1, Gli2 and Gli3. Among these Gli transcription factors Gli2 and Gli3 function both as repressors and activators. Gli1 functions mainly as an activator and it is also a target gene of Hh signalling (Fig. A1 and A2). Albeit myriad facets of vertebrate Gli-R production remain elusive, Kif7 (Kinesin), Suppressor of Fused (Sufu) and the primary cilium are needed for adequate processing of Gli-FL into Gli-R (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Goetz & Anderson, 2010; Liem, He, Ocbina, & Anderson, 2009; Svärd et al., 2006).

Sufu plays a very important role in stabilizing Gli2/Gli3 FL and retains both the proteins in cytoplasm, thereby preventing its nuclear translocation and activation (Humke, Dorn, Milenkovic, Scott, & Rohatgi, 2010; Tukachinsky, Lopez, & Salic, 2010; C. Wang, Pan, & Wang, 2010; Wilson & Chuang, 2010). Another vital role played by Sufu is the phosphorylation of Gli-FL C-terminal residues by protein kinase A (PKA), which prepares Gli-FL for next round of phosphorylation by Glycogen synthaseβ (GSK3β) and casein kinase1(CK1α) (Kise, Morinaka, Teglund, & Miki, 2009; Tempé, Casas, Karaz, Blanchet-Tournier, & Concordet, 2006). Recognition of phosphorylated Gli2/3-FL by E3 ubiquitin ligase TrCP leads to ubiquitylation and finally the degradation of C-terminal peptides to form Gli-R (Bhatia et al., 2006; Kise et al., 2009; Tempé et al., 2006; B. Wang & Li, 2006).

Protein Kinase A (PKA) is known to play key roles in many biological processes. In Hh receptive cells, PKA is involved in fate specification and in proliferation by attenuating Hh signaling. When the Hh pathway is inactive, even basal levels of active PKA can repress the Hh target genes. The important substrates of PKA are Gli transcription factors which are involved in repression and activation of Hh pathway. PKA is involved in phosphorylation of Gli thereby producing Gli repressors which then lead to repression of Hh target genes. When Hh ligands are present, the pathway is activated producing Gli activators eventually leading to Hh target gene expression. Due to fluctuations in the level of PKA activity, it is important to regulate PKA activity in Hh receptive cells precisely or it can lead to change in fate specification and aberrant proliferation of cells. It is not

very clear how PKA is regulated and still the mechanism remains elusive between various tissues, cell types and organisms. Two different mechanisms have been proposed to address the mechanism; (1) activity of PKA is regulated by cAMP; (2) PKA activity is regulated by protein known as Misty somites (Kotani, 2012). PKA and CKI are involved in regulation of Smo accumulation at the cell surface in response to Hh. It has been shown in *Drosophila* wing disc, blockade of PKA and CKI leads to prevention of Smo accumulation upon Hh induction. Smo is phosphorylated by PKA and CKI at many sites and phosphorylation defective mutants of Smo are unable to accumulate at the cell surface and poorly equipped to transduce Hh signals. At the same time, it has also been shown that variants of Smo mimicking phosphorylation exhibit continuous expression at cell surface and also able to transduce signals (Jianhang Jia, Tong, Wang, Luo, & Jiang, 2004).

Another important player in the Hh pathway is the G-protein-coupled-receptor Gpr161. It plays an important role in Hh signaling by negatively regulating the pathway. The IFT-A complex and Tulp3 are involved in trafficking of Gpr161 to the primary cilia (Pal & Mukhopadhyay, 2015). Expression of Gpr161 is mainly found in neural tube development and is localized in nervous system post mid-gestation period. It has also been found that Gpr161 is localized to cilia in many cultured cells and the ciliary localization is perturbed upon knockdown of Tulp3 and IFT-A complex in these fibroblasts (Mukhopadhyay & Rohatgi, 2014). Gli3 processing defects have also been observed in Gpr161 knockout mutants implying that Gpr161 could be pivotal in modulating this process. Mutational studies in Gpr161 double knock out mutants, have shown that Gli3 processing defects in these mutants are cilia dependent and takes place independent of Smo.

The phenotypic appearance of Gpr161 is like that of Sufu and PKA mutants; anyhow Sufu effects on the Hh pathway takes place independent of primary cilia (M. H. Chen et al., 2009; Humke et al., 2010; Jinping Jia et al., 2009), indicating that Gli3 processing by Gpr161 is modulated via activation of PKA. When Shh is absent, Gpr161 is localized to primary cilium and is involved in promotion of increased levels of cAMP mediated through Gαs activation of adenylyl cyclase. Whereas, when the ligand is present Gpr161

moves away from the primary cilium thereby preventing production of cAMP and leading to pathway activation. Mainly, Gpr161 is involved in Shh signaling by stimulation of ligand, regulating PKA, and roles in primary cilium (Pal et al., 2016).

It is known that Hh signaling is involved in the division of brain cells also known as the granule neuron precursor cells (GNP). The regulation of these cells is tightly controlled but uncontrolled signaling leads to medulloblastoma. Neuropilins which are proteins bind to Semaphorin molecules and lead to activation of Hh signaling and eventually to medulloblastoma. Ge et. al (2015), demonstrated the role of Neuropilins in mice cerebellum and in cultured cells. Their experimental data reveals that phosphodiesterase 4D (PDE4D) which is an enzyme, accumulates at the cell membrane and is promoted by semaphorin3. The enzyme PDE4D upon interaction with neuropilin blocks the function of another enzyme which is normally involved in the inhibition of Hh signaling pathway.

Mice that are deficient in semaphorin3 and neuropilin, the granule neuron precursor cells are unable to divide properly leading to development of abnormal cerebellum. They have also shown that drugs targeting PDE4D are capable of inhibiting tumor growths that are resistant to treatment with Vismodegib. The present PDE4D inhibitors suffer with severe side effects hence need of newer drugs. The findings of Ge et.al (2015), show a novel mechanism in which Hh signaling is regulated and highlights a novel strategy for medulloblastoma treatment (Ge et al., 2015).

When Sufu is absent, Gli2-FL translocates to the nucleus and is converted into Gli2-A (upon phosphorylation by unknown kinase) which is labile and rapidly degraded by cullin-3-based ubiquitin ligase adaptor Spop (M. H. Chen et al., 2009; C. Wang et al., 2010; Q. Zhang et al., 2006, 2009). Apart from Sufu, Kif7 plays a cardinal role in Gli processing, though the exact mechanism is enigmatic but it is thought to recruit PKA, GSK3 and CK1 thereby phosphorylating Gli-FL (Ryan & Chiang, 2012).

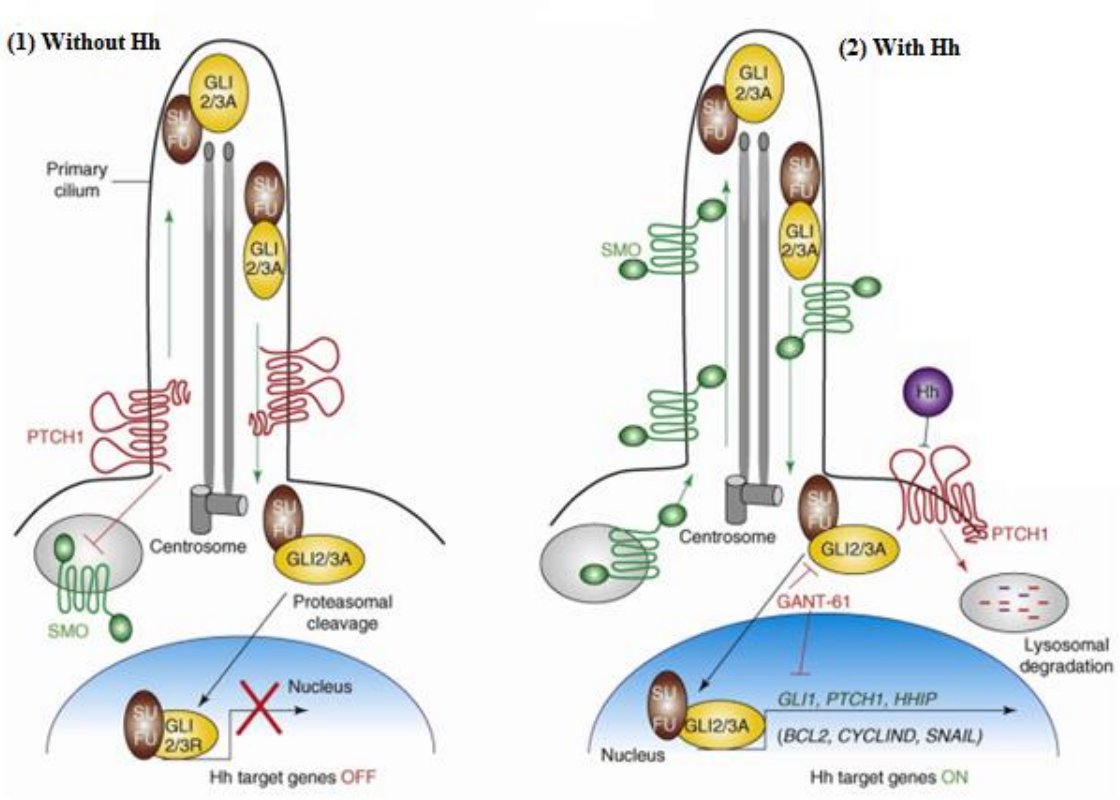


Figure A: The key components of mammalian Hedgehog pathway. (1) When Hh ligands are absent, Patched (PTCH1, 12 - transmembrane protein, shown in red)is present at the primary cilium's base close to the centrosome (grey cylinders) and inhibits Smoothened (SMO, 7-transmembrane protein shown in green) from going into the cilium thereby shutting the pathway off. Activators Gli2/3A; Gli2 and Gli3 (yellow ovals) move up and down the cilium via intraflagellar transport plausibly with negative regulator supressor of fused (SUFU; shown in brown) and are cleaved by proteasome into repressor patterns (GLI2/3) which fail to activate transcription of target genes upon binding to DNA in nucleus.(2) In the presence of Hh ligands(purple spheres),PTCH1 moves out of primary cilium affecting its capability to inhibit SMO which later moves into the primary cilium thereby activating the pathway and preventing cleavage of GLI2 and GLI3. The binding of activated GLI2 and to a small tune GLI3 to GLI promoter sites leads to transcription of target genes, GLI1,PTCH1, HHIP and othe cell specific genes as CYCLINS and SNAIL. Degradation of Hh and PTCH1 takes place in lysosomes. (Image adapted and modified from-Scales & de Sauvage, 2009)

2.3 Ptch1 a Tumor suppressor gene

The *Ptch1* gene codes for the PTCH1 protein and is the main receptor for the SHH signalling pathway. When the Hh ligands are absent, PTCH1 represses smoothened (SMO) and prevents it entry to primary cilium and further transcription of target genes which are important for normal development and growth thereby making it a negative regulator of the SHH pathway (Danwei Huangfu & Anderson, 2006). Gorlin-Goltz syndrome patients have high predisposition to medulloblastoma due to aberrant SHH-

signalling pathway (Fujii & Miyashita, 2014). Gorlin-Goltz syndrome (GGS) is also known as nevoid basal cell carcinoma syndrome (NBCCS), basal cell nevus syndrome and multiple basalioma syndrome (Ramesh, Krishnan, Chalakkal, & Paul, 2015). It is highly penetrant and dominant autosomal disorder. It mainly occurs due to mutation in tumor suppressor gene, *Ptch1*. This gene is located at 9q22, 3-q31. The clinical features of this disease are normally detected between the first and third decades of life. This syndrome affects several organ systems including skin, eye, skeletal, neural as well as reproductive system (Ramesh et al., 2015). The prevalence of this disease is around 1 in 57,000 to 1 in 256,000 inhabitants. The ratio of the disease between male to female is 1:1. In the year 1960 Gorlin and Goltz, characterized the triad which encompasses odontogenic keratocysts (OKC), multiple basal cell carcinoma and bifid ribs when describing the syndrome. Early detection and diagnosis of GGS is pivotal because of the susceptibility of the patients to neoplasms (De Amezaga, Arregui, Nuño, Sagredo, & Urizar, 2008).

2.4 Primary Cilium and Hedgehog Signalling

The primary cilium is a solitary, non-motile, microtubule based structure that rise from cell surface of relatively all cell types in mammals including endothelial, epithelial, stem, muscle cells, neurons as well as connective tissues. (Satir, Pedersen, & Christensen, 2010) It was first termed as “primary cilia” by Sergei Sorokin (Sorokin, 1968). Primary cilia are normally produced during the G1 or quiescence phase of cell cycle (S. Kim & Dynlacht, 2013). Growing information demonstrates that primary cilia are key modulators of varied signalling pathways like Hedgehog (Hh), Wingless (Wnt) and Platelet derived growth factor (PDGF) which are involved in development and tissue homeostasis (Berbari, O’Connor, Haycraft, & Yoder, 2009; Michaud & Yoder, 2006). Defective cilia and its components are the cause of many human developmental disorders and diseases which are known as Ciliopathies (Satir & Christensen, 2007). Ciliopathies encompass a collection of disorders that are linked to genetic mutations encoding abnormal proteins, leading to defective development or functioning of cilia. Some examples of ciliopathies

are Bardet-Biedl syndrome (BBS), Ellis van Creveld syndrome (EvC), Polycystic kidney disease (PKD) and Meckel-Gruber syndrome (MKS) among various others (Waters & Beales, 2011). A number of ciliopathies are associated with Hh signaling. One such example is Nephronophthisis (NPHP) which is a cystic kidney disease and autosomal recessive in origin. Mutations in *NPHP7/GLIS2* were observed in this disease which codes for the Kruppel-like zinc-finger transcription factor "Gli-similar protein 2". It is localized to nucleus and the primary cilia. Knockout mouse model of Glis2 show fibrosis and renal atrophy, these mutant mice displayed upregulation of genes which were important in fibrosis and epithelial-to-mesenchymal transition (EMT). *GLIS2* is closely associated to GLI family of transcriptional regulators and thereby connect NPHP to the Shh signaling pathway, which is known to play central role in tissue patterning and determination of cell fate (Hildebrandt, Attanasio, & Otto, 2009; Wolf & Hildebrandt, 2011).

The primary cilium principally consists of an axoneme that is made up of nine doublet microtubules that arise from the basal body and a septin like part at the base of the cilium which restricts access to the body. They are different from motile cilia in many ways. They are deficient in having the central microtubules and the radially distributed spokes that are needed for motility. The number of single microtubules and peripheral doublets are normally used to abbreviate microtubule ciliary axoneme configuration. Motile cilia have a 9+2 configuration, whereas non-motile cilia have a 9+0 configuration. The non-motile primary cilia do not have the key elements needed for ciliary motility which include the central microtubules, proteins surrounding them, inner and outer dynein arms (Satir & Christensen, 2007). The production of primary cilium is tightly regulated to the cell cycle. Trafficking occurs in a microtubule dependent way in the primary cilium and is monitored by multiprotein membrane bound complexes. The transport of proteins that takes place in both motile and primary cilia is known as intra flagellar transport (IFT). The IFT process is important for both maintenance and growth. IFT depends on the fundamental components of cilia like radial spokes, membrane proteins and tubulin. Various retrograde and anterograde molecular motors play vital role in trafficking of these multiprotein complexes from tip to basal body and vice versa (Robbins, Fei, & Riobo,

2012; Satir et al., 2010). When Hh ligands are present, Ptch repression on Smo is relieved and allows Smo to be activated and enters the primary cilium. In *Drosophila*, PKA,CK1 and G protein coupled receptor kinase2 (GRK2) phosphorylate the C-terminal residues which leads to conformational change in Smo and membrane accumulation (Apionishev, Katanayeva, Marks, Kalderon, & Tomlinson, 2005; Yongbin Chen et al., 2010; Jianhang Jia et al., 2004; Lum et al., 2003; Molnar, Holguin, Mayor, Ruiz-Gomez, & de Celis, 2007; Su et al., 2011). The Smo C terminus in vertebrates is quite different from *Drosophila* and does not have phosphorylation sites for PKA but CK1 and GRK2 phosphorylate the Smo C terminal residues which leads to conformational change and translocation to cilium (W. Chen, 2004; Yongbin Chen et al., 2011; Meloni et al., 2006). The Kinesin 2 motor subunit Kif3a and arrestins are required for Smo movement into the cilium upon its phosphorylation. Phosphorylation leads to Smo activation and inhibition of Gli processing. Apart from this, activated Smo leads to conversion of Gli-FL proteins into Gli-A and this is likely achieved by promoting the disassembly of Gli-Sufu complexes in the cilium (W. Chen, 2004; Yongbin Chen et al., 2011; Kovacs et al., 2008; Milenkovic, Scott, & Rohatgi, 2009).

Recently, it has been demonstrated that the Rusc (RUN and SH3 domain) family of proteins play an important regulatory role in Hh signaling. In vertebrates, the family consists mainly of two proteins namely Rusc1 and Rusc2 (Jin et al., 2016). The Rusc1 protein also known as Nesca is shown to be engaged in neurotrophin signal transduction pathway (MacDonald et al., 2012; Sun et al., 2012). Knockdown studies of Rusc1 in *Xenopus* embryos lead to increased Hh signaling amidst development of eye leading to acute ocular defects. Both the proteins (Rusc1/Rusc2) interact with Sufu leading to formation of a heterotrimeric complex with Gli and Sufu. When Hh signaling is activated this heterotrimeric protein complex is dissociated, Rusc2 exits first from the complex eventually leading to disassembly of Gli-Sufu complexes. Overexpression and knockdown studies of Rusc2 in the absence of Sufu has no overall output on Hh signaling indicative of its role in providing stability to the Gli-Sufu complexes. It seems that Hh signaling is inhibited by Rusc2, which binds to Sufu leading to stabilization of Gli-Sufu complexes thereby playing a regulatory role in the complex Hh signaling pathway (Jin et al., 2016). The disassembly of Gli-Sufu complexes leads to translocation of Gli-FL into

the nucleus and which is converted to Gli-A (Tukachinsky et al., 2010) leading to transcription of genes engaged in cell survival, proliferation and differentiation. The kinesin Kif7 is also thought to promote Gli-Sufu disassembly and plays a positive role in Hh signalling (Endoh-Yamagami et al., 2009). Apart from this, genes for negative regulators of Hh pathway like Ptch and Hip are also transcribed to regulate the pathway activity by negative feedback (Ryan & Chiang, 2012). A number of enzymes have been shown to modulate Gli factors namely kinases and HDACs. Kinases like casein kinase I (CKI), glycogen synthase kinase 3 (GSK3) and Protein Kinase A (PKA) participate in regulating Hh pathway. All these three kinases interact with Cos2 and are involved in phosphorylation of homologous domains on Smo and Ci. Ci phosphorylation by CKI, GSK3 and PKA is needed for effective processing of Ci 155 to its repressor form namely Ci75 showing that these kinases have a blocking effect on Hh signaling (Y Chen, Gallaher, Goodman, & Smolik, 1998; Jianhang Jia et al., 2002; Price & Kalderon, 2002). Ci155 accumulates upon loss of phosphorylation by any of these kinases (Jianhang Jia et al., 2002; Price & Kalderon, 2002).

The exact mechanism through which Hh negotiates the switch from negative effect to positive effects of these kinases is still elusive, but it has been suggested that it may be through reorganization of the Smo-Cos2-Fu-Ci complex upon reception of Hh (Aikin, Ayers, & Thérond, 2008). It has been shown that Dyrk1(Dual specificity tyrosine-phosphorylation-regulated kinase), is a kinase involved in regulation of Gli1. Dyrk1 modulates Gli1 activity by phosphorylating it at several serine/threonine sites and has been demonstrated to promote nuclear accumulation and Gli1-based transcription (Mao et al., 2002). Gli proteins transcriptional activity has been shown to be modified by many chromatin remodeling proteins and histone modifying enzymes. It has been demonstrated by Canettieri et al (2010) that acetylation alters Gli1/2 and HDACs class-I are involved in modulation of their transcriptional activity. In the granule cell precursors of cerebellum, Gli transcriptional activity is promoted by Hh signaling via HDAC1 upregulation. The modulation of pathway occurs via REN-Cullin-3 ubiquitin ligase complex which leads to ubiquitination and proteasomal degradation of HDAC1. Interestingly, deacetylation due to overexpression of HDAC1/2 has not been shown to affect Gli3. Histone acetyltransferase (HATs) and cAMP response element binding

protein (CREB) are involved in transcriptional activation by Gli3 (P. Dai et al., 1999), whereas repressor action of Gli3's is negotiated via Ski-based HDAC recruitment (P. Dai et al., 2002). The histone modifying enzymes namely HATs and HDACs alter Gli3 directly or modify Gli3 function through chromatin remodeling has yet to be determined.

2.5 Histone Deacetylases (HDACs)

One of the most cardinal enzymes involved in epigenetic regulation of gene expression and chromatin remodelling are histone modifying enzymes. Chromatin remodelling between 'closed' and 'open' forms have important epigenetic role in regulation of gene expression. Nucleosome remodelling is required for such epigenetic changes to take place, the fundamental units of chromatin. i.e. histones have to be modified for such changes to take effect. A number of histone amino terminal tail modifications are involved which comprise phosphorylation, methylation, acetylation, ribosylation, sumoylation, ubiquitnylation, carbonylation and glycosylation.

One of the important kind of modification is acetylation and is carried out by histone acetyltransferases (HATs) which transfer acetyl groups to lysine residues at amino-terminal on histones. This results in chromatin expansion and greater accessibility for transcription factors to bind to DNA. On the other hand, histone deacetylases (HDACs) remove acetyl group from lysine residues leading to repression of transcription and condensation of chromatin (Nightingale, O'Neill, & Turner, 2006; Roth, Denu, & Allis, 2001; Thiagalingam et al., 2003). 0

It has also been reported that Histone deacetylases (HDACs) play important role in modifying the function of varied type of non-histone proteins, like signal transducing molecules and transcription factors (Drummond, Noble, Kirpotin, & Guo, 2005). For example, it has been shown that HDAC6 plays important role in repression of basal hedgehog target gene expression and the effects are negotiated by HDAC6's impact on Gli2 mRNA and GLI3 protein expression (Dhanyamraju et al., 2015).

2.6 Classification of HDAC

Histone deacetylases (HDACs) in mammals are mainly classified into four classes: class I, IIa, IIb and IV (Fig. B). The classification is based on molecular function and cellular localization (Federico & Bagella, 2011; Lane & Chabner, 2009). HDAC class I are universally expressed and nuclear in localization and includes HDACs 1,2,3 and 8. Knockout investigations have demonstrated that class I HDACs play important role in cell survival and proliferation (Haberland, Montgomery, & Olson, 2009; Marks, 2010). The class II HDACs include 4,5,6,7,9 and10, these HDACs can shuttle to and from nucleus to cytoplasm and vice versa and thought to be tissue restricted. Out of these class II HDACs, 6 and 10 (class IIb) are special due to the presence of two catalytic sites and play role in many different biological functions. Sirtuin family of structurally distinct and NAD+ dependent HDACs belong to class IIb and do not act directly on histones. Finally, class IV includes HDAC11 which is universally expressed. Apart from histone targets, non-histone HDAC targets include NFκB, Ku70, p53, c-Myc, STAT3 and α-tubulin (Federico & Bagella, 2011).

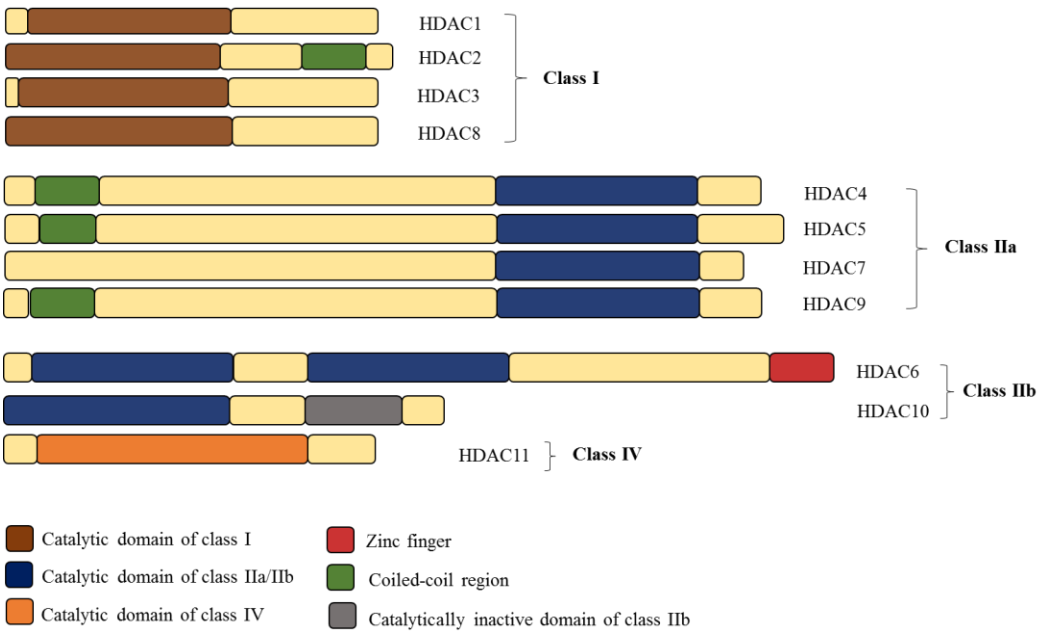


Figure B: Structural and functional domains of HDACs: The image depicts HDAC 1,2,3, and 8 from class I, HDAC 4,5,7 and 9 from class IIa, HDAC 6 and 10 from class IIb and HDAC11 from class IV. HDACs with different structural and functional domains are also shown (Image adapted and modified from Bolden, Peart, & Johnstone, 2006).

2.7 Histone deacetylase 6 - HDAC 6

Histone deacetylase 6 (HDAC6) belongs to class IIb family of HDACs. It has a special place in class II family of HDACs due to presence of two homologous catalytic domains (Fig. C). The overall functional activity of HDAC6 protein is maintained by the two independent catalytic domains (Grozinger, Hassig, & Schreiber, 1999; Verdel & Khochbin, 1999). HDAC6 gene is located on Xp11.23 (http://www.ncbi.nlm.nih.gov/). It is the largest member of HDAC family and has around 1,216 amino acids (Grozinger et al., 1999; Yingxiu Li, Shin, & Kwon, 2013). Mainly, HDACs are localized to nucleus but class II HDACs are special due to their translocation to cytoplasm (de Ruijter, van Gennip, Caron, Kemp, & van Kuilenburg, 2003).

HDAC6 is predominantly localized to the cytoplasm due to the presence of NES (Nuclear export signal) and SE14 motifs (C Boyault, Sadoul, Pabion, & Khochbin, 2007; de Ruijter et al., 2003). But, it has also been demonstrated that a small fraction of HDAC6 localizes to the nucleus (Verdel et al., 2000; Z. Wang et al., 2009). Nuclear localization of HDAC6 is primarily due to the presence of nuclear localization signal (NLS) at the amino terminal end of HDAC6. The interaction of this region with importin helps HDAC6 to shuttle into the nucleus. Intriguingly, heavy acetylation of this region leads to blockage of importin and thereby leads to reduced HDAC6 amounts in the nucleus (Y. Liu, Peng, Seto, Huang, & Qiu, 2012). Detectable amounts of nuclear HDAC6 has also been observed in hematopoietic cells. HDAC6 is associated with nuclear factors and is involved in control of their activity (Gao, Cueto, Asselbergs, & Atadja, 2002; Girdwood et al., 2003; Palijan et al., 2009; Westendorf et al., 2002; Yang & Grégoire, 2005).

Recruitment of HDAC6 to gene promoters and regulation of transcription has also been reported (Z. Wang et al., 2009). Acetylation plays a cardinal role in regulating the nuclear and cytoplasmic functions of HDAC6 (Y. Liu et al., 2012). HDAC 6 encompasses in its c-terminal an exclusive ubiquitin-binding zinc-finger domain (ZnF-UBP domain) and a

dynein binding domain (DBD) (G. M. Cooper & Hausman, 2000). HDAC6 functions as a cortactin, HSP90 and α tubulin deacetylase. It has also been shown that HDAC6 plays a significant role in multiple biological mechanisms which include role in immune synapse formation, cell spreading, cell migration, degradation of stress granules (SG), degradation of misfolded proteins and in viral infections via complex formation with various partner proteins (Yingxiu Li et al., 2013).

The primary substrate of HDAC6 is α tubulin. Tubulin acetylation is carried out by a number of enzymes which include ELP3 (Creppe et al., 2009), GCN5, San15 (Conacci-Sorrell, Ngouenet, & Eisenman, 2010), ARD-NAT1 and αTAT1(Akella et al., 2010; Shida, Cueva, Xu, Goodman, & Nachury, 2010; Topalidou et al., 2012) which are all acetyltransferases. Apart, from these HDAC6 and SirT2 are the deacetylases which are involved in deacetylation of microtubules (Hubbert et al., 2002; North, Marshall, Borra, Denu, & Verdin, 2003).

One of the first well described α-tubulin acetylation was shown to be at ε-amino group of lysine40 in *Chlamydomonas* flagella (L'Hernault & Rosenbaum, 1983; LeDizet & Piperno, 1987). Tubulin acetylation is particularly enhanced in motile and primary cilia and therefore used as markers for the structures extensively (Piperno & Fuller, 1985). Assembly as well as disassembly of primary cilium has been shown to be impacted by acetylation of α-tubulin (Pugacheva, Jablonski, Hartman, Henske, & Golemis, 2007; Shida et al., 2010). α-tubulin acetylation has also been shown to be involved in acceleration of kinesin based transport along the axonal microtubule tracks (Reed et al., 2006). Deletion of TAT2 and MEC17 which are the orthologues of αTAT1 in *C.elegans*, leads to reduction in sensitivity towards touch (Akella et al., 2010; Shida et al., 2010) and also leads to collapse of microtubule architecture in neurons for touch reception (Cueva, Hsin, Huang, & Goodman, 2012; Topalidou et al., 2012).

Knockout of MEC17 which is an orthologue of αTAT1 in zebrafish leads to neuromuscular, developmental disorders (Akella et al., 2010). αTAT1 is one of the

important and major player in acetylation of α-tubulin in mice and is necessary for typical flagellar function of sperm (Kalebic et al., 2013). It has been shown that it plays significant role in oncogenic cell transformation, hence has become a prime target for drug development to treat cancers. Previous work has demonstrated that, inhibition of HDAC6 leads to apoptosis in multiple myeloma cells. In some cancers, HDAC6 is also used as prognostic marker. HDAC6 and HSF1 regulate oncogenic Ras/MAPK signal transduction pathway required for proper tumour growth (C. Dai, Whitesell, Rogers, & Lindquist, 2007; Y. S. Lee et al., 2008a). Its interaction with cortactin regulates motility. HDAC6 contributes to cancer metastasis since its upregulation increases cell motility in breast cancer MCF-7 cells and its interaction with cortactin modulates motility (Sakamoto & Aldana-Masangkay, 2011).

It is engaged in microtubule control and actin dependent cell motility. HSP90, a chaperone protein is also a HDAC6 substrate. Among other important functions; HDAC6 plays a critical role in misfolded protein clearance by autophagy or via generation of aggresomes (Delcuve et al., 2012). Keeping all these functions in view HDAC6 is candidate therapeutic target for treatment of diseases like cancer and neurodegenerative diseases (G. Li, Jiang, Chang, Xie, & Hu, 2011; Sakamoto & Aldana-Masangkay, 2011; Valenzuela-Fernández, Cabrero, Serrador, & Sánchez-Madrid, 2008).

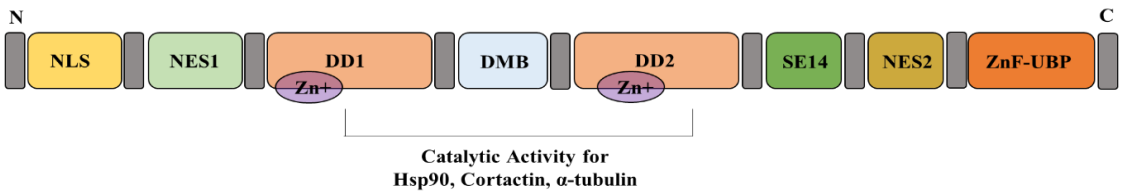


Figure C: Image depicts functional domains of HDAC6. It has two tandem catalytic deacetylase domains (DD1 and DD2). Hsp90, Cortactin andα tubulin are substrates for HDAC6. Nuclear export signal (NES) restricts the accumulation of protein in nucleus. The Ser-Glu tetrapeptide (SE14) part provides a strong platform for the enzyme in the cytoplasm. The binding of dynein and ubiquitin-binding zinc finger domain (ZnF-UBP) is accomplished by linker between both DDs. Zn+ (Zinc) cofactor at active site. Dynein motor binding domain (DMB). Nuclear localization signal (NLS) (Image adapted and modified from (S. N. Batchu et al., 2016).

2.8 Regulation of ciliogenesis by HDAC6

The primary cilium is a microtubule based small antenna like structure and has been shown to play cardinal roles in varied cellular functions like cell growth, cell migration, cell cycle, planar cell polarity, immune response and transactivation. Keeping in view such important and diverse functions of the cilium, any dysfunction in ciliary function leads to diseases known as the ciliopathies (Suizu et al., 2016). In Bardet-Biedl Syndrome (BBS), polycystic kidney disease (PKD) and other cilia related diseases mutations in signaling proteins or cilia related proteins lead to insensitivity to foreign signaling cues leading to hyperplastic growth (Benzing & Walz, 2006; J Pan, Wang, & Snell, 2005; Singla & Reiter, 2006). It has recently been shown that several signaling pathways like Wnt, PDGFαα, hedgehog and other signaling pathways coordinate at cilia (Cano, Murcia, Pazour, & Hebrok, 2004; A. Liu, 2005; Schneider et al., 2005; Simons et al., 2005; Tanaka, Okada, & Hirokawa, 2005).

Even though a large number of ciliary proteins involved in structural and functional roles of cilia have been identified, the current knowledge about cellular machinery regulating cilia resorption and formation is sparse. Regulation of cilia throughout the cell cycle is a very dynamic process. In varied cells, ciliary resorption takes place at mitotic entry and ciliary reappearance post entry to G1. Taking into consideration the important role/s played by cilia in detection and transmitting external cues, cilia shortening and disassembly might play a cardinal role in growth control and aberrant cell growth signals in the external environment (Pugacheva, et al., 2007).

The primary cilium principally consist of an axoneme that is made up of nine doublet microtubules that arise from the basal body and a septin like part at the base of the cilium which restricts access to the body (Satir & Christensen, 2007). Motile flagella of lower eukaryotes like *Chlamydomonas* are related evolutionarily to the cilium. Recently, research focusing on *Chalmydomonas* have started to understand the mechanism of resorption of flagella (Bradley, 2005; Marshall, Qin, Rodrigo Brenni, & Rosenbaum, 2005; Junmin Pan & Snell, 2005; Quarmby, 2004). These investigations found altered

capabilities of the IFT machinery and axonemal destabilization as indicative marks of ciliary disassembly, and suggested the role of CALK (*Chlamydomonas* aurora-like protein kinase) and other kinases as main regulators of ciliary disassembly. The exact mechanism of how CALK and other effectors are activated in disassembly still remains elusive. CALK kinase shares a 55% similarity to that of human Aurora A (AurA) kinase around the protein catalytic domain. Aurora A plays a very important role in humans as a centrosomal kinase regulating mitotic entry via activating Cdk1-cyclin B and other important molecules that are involved in organization of mitotic spindle (Bischoff et al., 1998; Marumoto, Zhang, & Saya, 2005). In varied cancers, it has been observed that AurA is activated or amplified which can be characterized by amplification of centrosome and genomic instability (Anand, Penrhyn-Lowe, & Venkitaraman, 2003; Goepfert et al., 2002; Gritsko et al., 2003).

HEF1 (human enhancer of filamentation 1) is a scaffolding protein and is known to play important roles in migration, attachment & anti-apoptotic cues at focal adhesions (O'Neill et al., 2000). It has been recently demonstrated that AurA and HEF1 interact with each other at the centrosome which is needed for cell progression over mitosis (Pugacheva & Golemis, 2005, 2006). Looking for targets phosphorylated by AurA, Pugacheva et al., (2007) considered acetylated α-tubulin because it has been shown that α-tubulin deacetylation led to microtubule destability in vivo (Matsuyama et al., 2002). It has been demonstrated that Histone deacetylase 6 (HDAC6) plays important role as tubulin deacetylase and effects chemotaxis and mitosis via controlling tubulin stability (Hubbert et al., 2002). Pugacheva et al., (2007) showed that HDAC6 plays an important role in ciliary disassembly mediated by Aurora A. When HDAC6 is depleted in cells, Aurora A activation does not lead to disassembly of cilium pointing that HDAC6 operates downstream of Aurora A. It has also been shown that HDAC6 is phosphorylated by Aurora A in vitro (Pugacheva et al.,2007). The model proposed by Pugacheva et al., (2007) show that external growth factors promote disassembly of cilia via activation of HEF1 expression, which then activates Aurora A. Aurora A eventually phosphorylates HDAC6. Upon phosphorylation HDAC6 destabilizes primary cilium microtubules via deacetylation of axonemal tubulin thereby causing ciliary resorption (Pugacheva et al., 2007) (Fig.D).

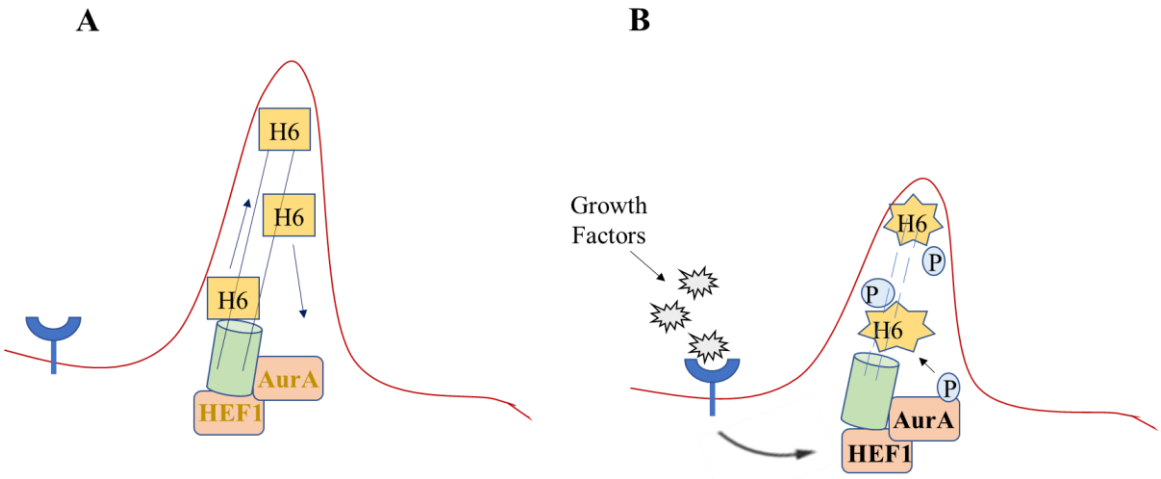


Figure D: Regulation of ciliogenesis by HDAC6: (A) To the basal body of quiescent ciliated cells Aurora A (AurA) and low amounts of HEF1 are localized. (B) Induction of HEF1 by growth factors leads to activation of Aurora A which eventually results in phosphorylation of ciliary HDAC6 (H6), thereby resulting in resorption of cilia. Image adapted and modified from Pugacheva et al., 2007

2.9 Hedgehog signaling and Cancer

The molecular processes/mechanisms leading to aberrant activation of Hh pathway has been one of the main reasons for Hh associated cancers. Three basic models of pathway activation have been proposed (Rubin & de Sauvage, 2006; Scales & de Sauvage, 2009). Type I cancers are those containing activating mutations in Hh pathway which are independent of ligand like Medulloblastoma (MB) and Basal cell carcinoma (BCC). Type II cancers are ligand dependent (autocrine or juxtacrine) mechanisms; which means that Hh is produced and also utilized by same cells or neighboring tumor cells. Type III are ligand dependent paracrine signaling mechanisms in which Hh produced by the cancer cells are collected by stroma which further feed the cells with other signals leading to survival and growth of tumors. (Rubin & de Sauvage, 2006; Scales & de Sauvage, 2009).

2.10 Hedgehog signaling Type I: mutation driven, ligand independent

The very first indication of Hh pathway involvement in cancers was acknowledged when Ptch inactivating mutations were described in a condition known as Gorlin's syndrome

(Hahn et al., 1996; Johnson et al., 1996). Patients suffering with Gorlin's syndrome show up with many BCCs and are at higher risk of developing rhabdomyosarcoma (Muscle tumor) and medulloblastoma (brain tumor). It has also been demonstrated that ligand independent activation of hedgehog pathway (Fig.E1) was seen in most random cases of BCCs (Dahmane, Lee, Robins, Heller, & Ruiz i Altaba, 1997).

In majority of tumors SMO activating mutations (10%) and PTCH inactivating mutations were observed (Xie et al., 1998). Moreover, in 1/3rd of medulloblastoma cases and in rhabdomyosarcomas Ptch and Sufu mutations have led to Hh pathway activation aberrantly (Taylor et al., 2002; Tostar et al., 2006). Increased tumor formation and high cell proliferation have been shown as a result of aberrant Hh signaling. In many different mice models the same observations have been noticed and confirmed. As in Gorlin's syndrome patients, mice carrying heterozygous Ptch mutations are predisposed to medulloblastomas and are sensitive to BCC upon exposure to UV (Ultraviolet) (Aszterbaum, Beech, & Epstein Jr., 1999). Many patients with metastatic BCC are being treated with Hh pathway inhibitors, because these tumors are not dependent on ligand the inhibitors should be targeted at or below the level of Smo in the Hh pathway to be productive (Gupta et al., 2010).

2.11 Hedgehog signaling Type II: Autocrine, ligand dependent

In several tumors like prostrate, liver, breast and brain constant Hh pathway activation has been detected (Gupta et al., 2010) These tumors differ from medulloblastomas and BCC in that they do not display any somatic mutations in the hedgehog pathway. They show aberrant Hh pathway activation in a ligand dependent and autocrine manner (Fig. E2). Several of these tumors exhibit high expression levels of Shh or Ihh and /or ectopic Gli1 and Ptch expression in the epithelial section. Ligand production ectopically by tumor cells or tumor stem cells (TSC) works on surrounding tumor cells or itself leading to its own survival and growth. This autocrine mode of signaling in these tumors can be

inhibited by using Smo antagonists and/or antibodies against Hh pathway (Gupta et al., 2010).

2.12 Hedgehog signaling Type III: Paracrine, ligand dependent

The paracrine mechanism of Hh signaling (Fig.E3) is pivotal for development and in maintaining epithelial structures like the small intestine (Ingham & McMahon, 2001; Theunissen & de Sauvage, 2009; Varjosalo & Taipale, 2008). Epithelium secreted Hh ligands are detected by the mesenchymal stroma cells and lead to stimulation and eventually proliferation in the mesenchyme. When Hh pathway is activated, the mesenchymal cells generate molecules which ultimately feed back to the epithelial cells. Recently it was reported by Yauch et al (2008) that paracrine way of Hh signaling play an important role in improving the tumor microenvironment (Jiang & Hui, 2008; Yauch et al., 2008).

It was demonstrated by Stevaux et.al (2009) that deletion of Smo by genetic means do not lead to change in Gli1 and Ptch expression in neoplastic ductal cells and do not lead to progression of pancreatic adenocarcinoma (Nolan-Stevaux et al., 2009). All these reports suggest and support the paracrine mode of Hh signaling, in which Hh signaling is activated in surrounding stroma leading to the production of extracellular matrix molecules and soluble components which act on tumor epithelium eventually leading to tumor growth (Theunissen & de Sauvage, 2009). The most efficient way of treating these tumors would be by using a combination therapy; an inhibitor targeting the Hh pathway in stromal cells and other drugs directed towards the tumor cells (Gupta et al., 2010).

2.13 Hedgehog signaling Type III b: Reverse Paracrine signaling

Lately, a “reverse paracrine” mode of signaling (Fig.E4) has also been demonstrated in which stromal cells secrete the Hh and is detected by the tumor cells (Theunissen & de

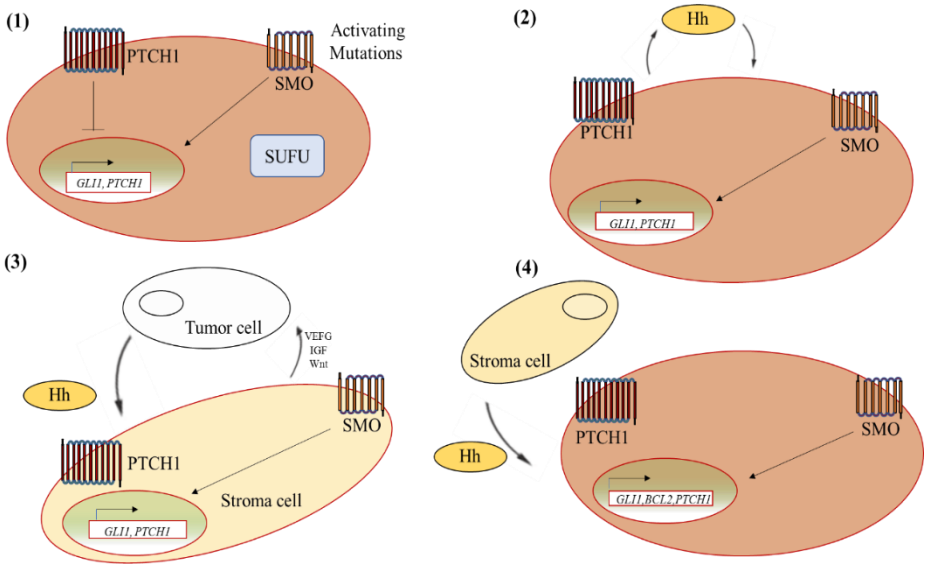
Sauvage, 2009). Till now this mode of signaling has only been observed in hematological malignancies like leukemia, lymphoma and multiple myeloma in which stromal secreted Hh is utilized by cancerous B cells through upregulation of Bcl2, which is an antiapoptotic factor (Dierks et al., 2007; Hegde et al., 2008; Scales & de Sauvage, 2009). In this model, the Hh secreted by the stromal cells is thought to support the environment needed for effective tumor growth and thereby can be used as suitable drug target (Gupta et al., 2010).

2.14 Hedgehog signaling in cancer stem cells

Stem cells are involved in tissue maintenance and have the capacity to form and give rise to new stem cells and are able to differentiate into mature cells of a tissue. Hh signaling plays a pivotal role in activity of stem cells, self-renewal and multiplication of these cells in many tissues (Taipale & Beachy, 2001; Y Zhang & Kalderon, 2001). It is considered that small CSCs populations help tumors to propagate and grow (Fig.E5). These small CSCs have similar properties like that of normal stem cells and are normally regulated by similar kind of signaling factors like that of normal stem cells (Reya, Morrison, Clarke, & Weissman, 2001).

Increasing data conveys that tumor formation and expansion are directly a result of aberrant signaling pathways in stem cells like Wnt, notch, and the Hh pathways (Rubin & de Sauvage, 2006). It has been demonstrated that Hh signaling plays important role in self-renewal and maintenance of CSCs in multiple myeloma, breast and chronic myelogenous leukemia (CML) stem cells (Clement, Sanchez, de Tribolet, Radovanovic, & Ruiz i Altaba, 2007; Dierks et al., 2008; S. Liu et al., 2006; Peacock et al., 2007; Theunissen & de Sauvage, 2009). Dierks et al (2008) demonstrated that CML stem cells (Bcr-Abl driven $Lin^{-}/Sca1^{+}/c\text{-}Kit^{+}$) having a SMO knockout exhibited decreased capacity to form tumors in mice which were irradiated despite the fact that SMOM2 expression led to enhancement (Dierks et al., 2008; Peacock et al., 2007). Moreover, when 5E1 (Hh blocking antibody) and cyclopamine (SMO antagonist) was used both of them led to the

inhibition of CML CSCs in vivo and in vitro. The loss of Smoothened in hematopoietic system of mouse led to diminished induction of chronic myelogenous leukemia by the oncoprotein Bcr-Abl and activated Numb, leading to reduction of CML stem cells. Growth inhibition was observed in imatinib-resistant human and mouse CML by cyclopamine, pointing that Hh signaling can be targeted in the treatment of imatinib-resistant CML (Zhao et al., 2009). The increasing evidence that active Hh signaling plays important role in varied type of CSCs makes it a hopeful target by inhibiting the Hh pathway in these tumors forming CSCs. This can be achieved by using a combination of drug therapies: one targeting the Hh signaling in CSCs and the other for the bulk of the tumor or in combination with radiation (Scales & de Sauvage, 2009). It was shown by Feldmann et al., (2007) in pancreatic cancer cells that GLI expression ectopically leads to greater invasiveness, whereas Hh pathway inhibition leads to Snail downregulation thereby decrease in invasive characteristics (Feldmann et al., 2007). Tumor metastasis has also been shown to be promoted by Hh signaling by playing an active role in epithelial to mesenchymal transition (EMT). EMT consists of converting epithelial cells which are polarized into mesenchymal cells which have invasive and migratory characteristics eventually leading to metastasis. Hh exercises its effects on EMT through down regulation of E-cadherin and upregulation of SNAIL (Karhadkar et al., 2004; Rubin & de Sauvage, 2006).



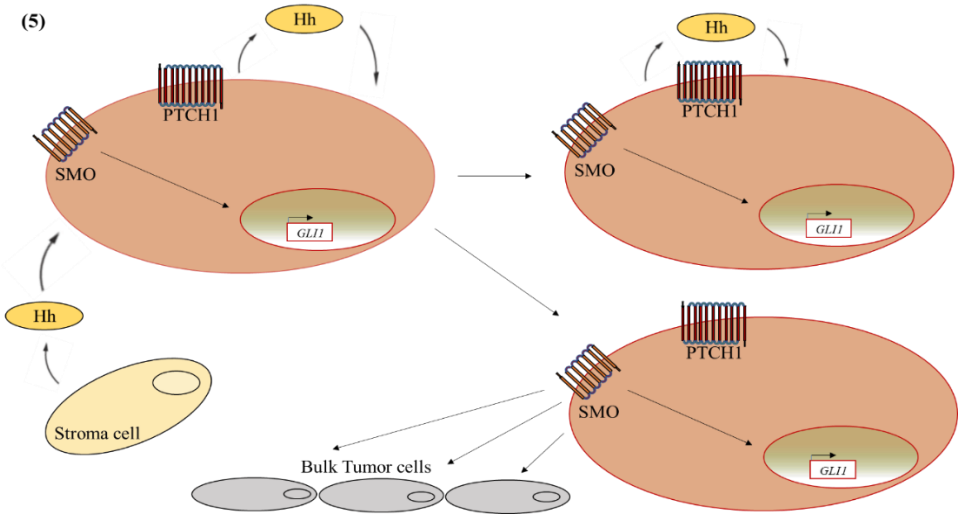


Figure E: Diverse models of Hedgehog signaling pathway: (1) Type I ligand independent cancers contain activating mutations in Smoothened (SMO) or inactivating mutations in Ptch1 (PTCH) which lead to continuous activation of the Hh pathway even when the ligand is not present. (2) Type II are autocrine cancers which are ligand dependent. These cancers produce and use the Hh ligands which eventually leads to tumor survival and growth. (3) Type III are paracrine cancers which are ligand dependent. These cancers produce ligands which are detected and received by stromal cells which lead to activation of pathway in the stroma. The stromal cells in turn feeds back variety of signals like IGF (Insulin-like growth factor), VEGF (Vascular endothelial growth factor), Wnt (Wingless) to the tumors thereby leading to survival and growth. (4) Type IIIb are cancers which exhibit reverse paracrine signaling. Tumors detect and receive Hh ligand secreted by stromal cells thereby leading to activation of pathway in tumor cells via upregulating survival cues. (5) Cancer stem cells (CSCs): Hh ligands produced by stroma or CSCs lead to Hh signaling only in self-renewing CSCs. These CSCs will propagate further and give more CSCs which are Hh pathway dependent or may differentiate into tumor cells which are Hh negative consisting majority of the bulk cells of the tumor. Image adapted and modified from: Scales, S.J. and de Sauvage, F.J. (2009).

2.15 Hedgehog signaling in Central Nervous System (CNS) Tumors

Even though much information regarding Hh signaling in medulloblastoma is known, the role of Hh signaling and its role in other CNS tumors is sparse. Very few cases have been reported showing Smo or Ptch1 mutations in extra cerebellar tumors. In 1987, for the very first time it was shown that Hh signaling plays role in Gliomagenesis, when Gli1 gene was isolated from Glioblastoma cell line (Kinzler et al., 1987). Analysis of several primary tumors like glioblastoma, astrocytoma, oligodendroglioma, PNETs (Primitive neuroectodermal tumor) and other cell lines show constitutive Gli1 expression (Dahmane et al., 2001). It was shown that growth inhibition of several human glioma cell lines was noticed when Cyclopamine (hedgehog pathway inhibitor) was used indicating that these tumors rely upon Hh signaling pathway for their survival and growth (Dahmane et al.,

2001). Recent studies have pointed a role of neural stem cells in brain tumors, the idea emerges from the fact that many primary tumors like oligodendrogliomas and astrocytomas express neural stem cell markers and are capable of undergoing self-renewal as well as undergo differentiation in vitro (Hemmati et al., 2003; Oliver & Wechsler-Reya, 2004; Singh, Clarke, Hide, & Dirks, 2004). As Hh signaling has been demonstrated to be important for neural progenitor cell maintenance in the hippocampus, it is very reasonable to hypothesize that this pathway might also play a role in tumorigenesis outside the cerebellum (Lai, Kaspar, Gage, & Schaffer, 2003; Machold et al., 2003). Anyhow, further research is needed to know if mutations in certain players of the pathway are necessary for tumor initiation or they are just needed for tumor survival and growth. Finally, tumors of the CNS can be targeted by using pharmacological inhibitors (Marie P. Fogarty, Kessler, & Wechsler-Reya, 2005).

2.16 Medulloblastoma (MB)

Medulloblastoma (MB) is one of the most common, highly invasive paediatric brain tumour arising from embryonal cells of the cerebellum. It accounts for around 20% of all paediatric brain tumours (de Bont, Packer, Michiels, den Boer, & Pieters, 2008; Pui, Gajjar, Kane, Qaddoumi, & Pappo, 2011). MB was described initially as cerebellar glioma before Bailey and Cushing named it as medulloblastoma in 1925 (Bailey & Cushing, 1925).

Patients with MB present clinical and slight neurological symptoms that prevail for few months before diagnosis. The symptoms include lethargy, vomiting, headaches, ataxia, cranial nerve defects, facial weakness, hearing loss, ringing in the ears, Parinaud's Syndrome (pupillary defect and upward gaze) and head tilt etc. (Huang & Yang, 2015). MB has been described by pathologists as a heterogenous disease and according to the World Health Organisation (WHO) it has been classified into (1) Classic medulloblastoma and four subtypes based on histological constitution: (2) Nodular or desmoplastic (D/N); (3) Medulloblastoma with extensive nodularity (MBEN); (4)

anaplastic medulloblastoma and (5) large-cell variant (A. J. Gajjar & Robinson, 2014; Louis et al., 2014), the histopathologic features of each class are given below:

2.16.1 (1) Classic Medulloblastoma (CMB)

It is one of the most common and frequent kind of medulloblastoma and accounts for 70-80% of all known cases (Gilbertson & Ellison, 2008; Pizer & Clifford, 2009). It is characterized by circular hyperchromatic nuclei, moderate nuclear pleomorphism (D.W. Ellison, 2002; D. W. Ellison, 2010) and have small tiny sheets of cells exhibiting high nuclear: cytoplasmic ratio when stained with H&E (Hematoxylin and Eosin) (D.W. Ellison, 2002) (Fig.F1). The presence of apoptotic bodies can also be seen in tumor cells along with mitotic figures (D.W.Ellison, 2002; D. W. Ellison, 2010).

2.16.2 (2) Desmoplastic or Nodular (D/N) medulloblastoma

The nodular desmoplastic medulloblastoma (DNMB) accounts for 10-15% of the medulloblastoma cases. It is identified by internodular desmoplasia and the nodules consist of differentiated neurocytic cells (Fig.F2). These differentiated cells express neuronal proteins and exhibit poor growth fraction. The internodular desmoplastic zones are represented by embryonal cells which are undifferentiated and in between the nodules, collagen positive strands can be observed which are reticulin positive (D. W. Ellison, 2010). In this subtype, the degree of nodularity is quite unstable and it has been shown that increased nodularity is linked to better prognosis (McManamy et al., 2007).

2.16.3 (3) Medulloblastoma with extensive nodularity (MBEN)

The histological features of this class are similar to that of the desmoplastic variant. This subtype makes around 1% of medulloblastomas (McManamy et al., 2007). In comparison to desmoplastic tumors, MBENs consists of irregularly shaped large nodules. One of the essential features of this subtype is decreased desmoplastic elements between the nodules (D. W. Ellison, 2010) (Fig.F3).

2.16.4 (4) Large cell medulloblastoma

This medulloblastoma subtype accounts for around 2-4% of the disease (D. Ellison, 2002; McManamy et al., 2007). It consists of groups of cells which are large and round having single nucleolus (Fig.F4). These cells exhibit high mitotic rate and also higher apoptotic rate when compared to other subtypes (McManamy et al., 2003). This subtype has poor prognosis and usually shows up as a metastatic disease (A. Gajjar et al., 2004).

2.16.5 (5) Anaplastic medulloblastoma

Anaplastic medulloblastomas makeup for 10-20% of the disease (Gilbertson & Ellison, 2008). The histopathologic characters of this class include wrapping of cells, paved arrangement of nuclei, cell moulding and nuclear pleomorphism (Brown et al., 2000; Eberhart et al., 2002) (Fig.F5). High apoptotic and mitotic activity can be seen in this class (McManamy et al., 2003). Anaplastic medulloblastomas like that of large cell variant show poor prognosis (Eberhart et al., 2002; McManamy et al., 2003). Due to similar histologic aspects and biological behavior between anaplastic and large cell variant, they are commonly grouped into single large cell/anaplastic (LCA) class in medulloblastoma research (Gilbertson & Ellison, 2008).

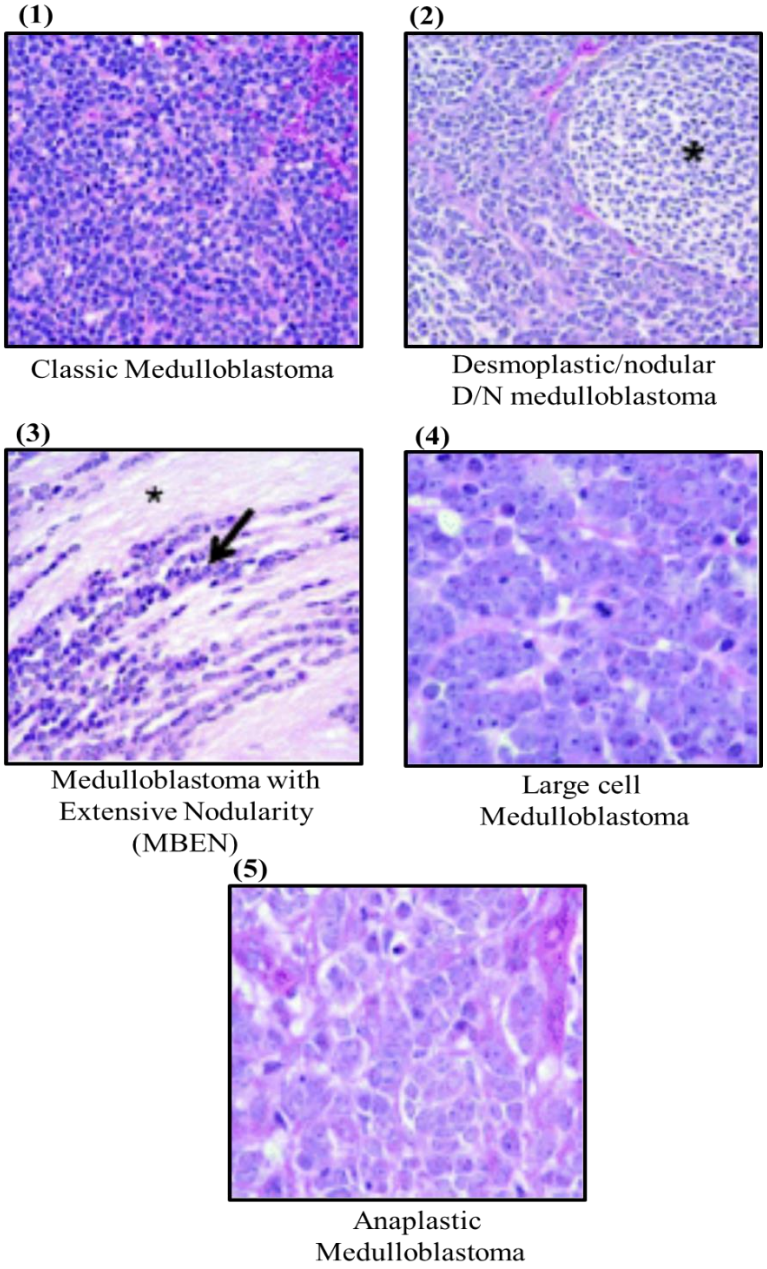


Figure F: Histopathological classification of medulloblastoma: (1) classic, (2) desmoplastic/nodular (D/N), (3) medulloblastoma with extensive nodularity (MBEN), (4) large-cell medulloblastoma, and (5) anaplastic medulloblastoma. Images adapted and modified from (Eberhart, 2011; D. W. Ellison, 2010).

Due to rapid development of novel genomic tools the molecular classification of MB has been possible which is based on genetic alterations and distinct transcriptional profiles. Based on this approach, MB has been categorized into four molecular subtypes: wingless pathway MB (Wnt-MB; subtype 1), sonic hedgehog MB (SHH-MB; subtype 2), MYC-

amplified MB (subtype 3), and heterogeneous (subtype 4) (Cho et al., 2011; Kool et al., 2012; K. K. W. Li, Lau, & Ng, 2013; Northcott, Jones, et al., 2012; Pietsch et al., 2014; Sadighi, Vats, & Khatua, 2012; Thompson et al., 2006) (Fig. G). The Subtype1 (Wnt - MB) is seen in comparable amounts both in children and adults and has a better prognosis. This subtype is characterized by Wnt signalling, MYC expression and monosomy 6 and accounts for around 10% of all medulloblastomas (Northcott, Dubuc, Pfister, & Taylor, 2012). Subtype 2 (SHH-MB) normally affects children below 5 years and adolescents above 16 years. Activating mutations in smoothened (SMO), inactivating mutations in patched1 (PTCH1) and SUFU are commonly seen in this subtype, apart from *MYC* and *GLi2* amplification (Hallahan et al., 2004a) and the frequency of this subtype is around 30% (Northcott, Dubuc, et al., 2012). Subtype3 (MYC-amplified MB) is characterized by amplification of MYC, loss of 5q and gain of chromosome1q and has the poorest prognosis. The frequency of this kind is about 25% (Northcott, Dubuc, et al., 2012). Subtype4 (heterogeneous) is characterized by loss of X chromosome and is more common in males and the prognosis is intermediate (Ransohoff, Sarin, & Tang, 2015) This subtype has the highest frequency of about 35% (Northcott, Dubuc, et al., 2012). In the present thesis, I am mainly interested in aberrant Hh signalling with respect to MB, so the details would be limited to subtype2 (SHH-MB).

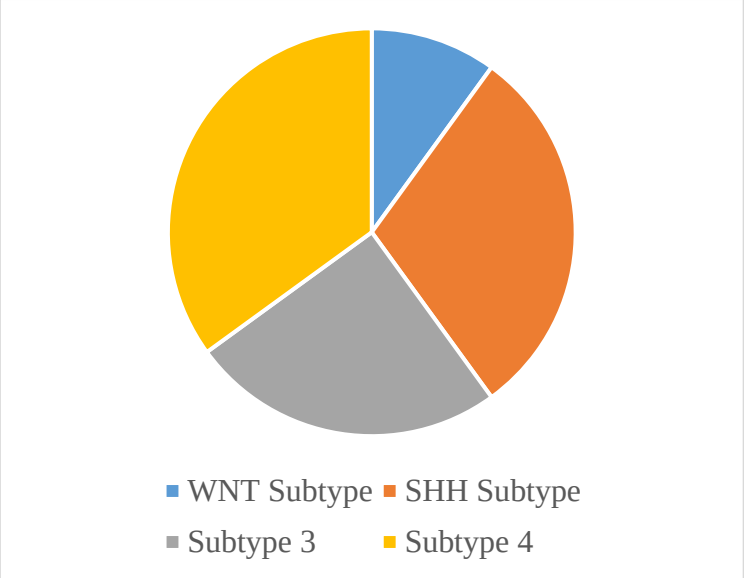


Figure G: The image shows frequency of Medulloblastoma molecular subtypes: WNT (~10%), SHH (~30%), Subtype 3 (~25%) and Subtype4 (~35%). (Image adapted and modified from (Northcott, Dubuc, et al., 2012)

2.17 SHH-Subtype medulloblastoma

The development of normal cerebellum is highly dependent on SHH signaling. The development of normal cerebellum occurs mainly post-birth, amidst which the Granule neuron precursors (GNP) multiply and eventually differentiate into granule neurons which constitute the most abundant cell kind in the brain. *In vitro* and *in vivo* studies have shown that GNP multiplication is mainly regulated by Shh which is derived from Purkinje cells (Vaillant & Monard, 2009). When the Shh ligand is absent, the GNPs do not multiply and directly migrate from external granule cell layer (EGL) to the internal granule cell layer (IGL) of the cerebellum during differentiation into granule neurons thereby producing a small cerebellum. In one of the research studies it was shown that when Shh signaling was blocked by using anti-Shh antibodies in chick cerebellum the size of the cerebellum decreased considerably, pointing the role of Hh signaling in development of normal cerebellum (Dahmane & Ruiz i Altaba, 1999). A number of signals play an active role in retaining the GNPs in the EGL during multiplication which include brain-derived neurotrophic factor (BDNF) and CXCL12 (Borghesani et al., 2002; Klein et al., 2001). Even though the secretion of Shh by purkinje cells is continued even after the development of cerebellum, the GNPs only react for a period of distinct time, pointing at other signaling pathways contributing to differentiation and migration.

Nevertheless, uncontrolled Shh signaling due to mutations in the pathway lead to neoplasia. For example, Gorlin's syndrome patients (also known as Nevoid basal cell carcinoma syndrome) have mutations in *PTCH1* gene leading to continuous activation of the Hh pathway leading to tumors. These patients are predisposed to medulloblastoma and basal cell carcinoma (Johnson et al., 1996). The true role of this signaling pathway became evident when targeted Ptch1 mutations in mice lead to development of medulloblastoma (L V Goodrich, Milenković, Higgins, & Scott, 1997). Mutations in PTCH1 are the most prevalent kind of medulloblastomas arising in this subtype accounting for around 25-30% (PA Northcott et al., 2012). Apart from *PTCH1* mutations; mutations in *SMO, SUFU,* amplification of *SHH, GLI2* and *MYCN* can also be noticed in this subtype showing a clear link between SHH pathway and medulloblastoma (Jones et

al., 2012; Pugh et al., 2012; Robinson et al., 2012). The frequency of genetic abnormalities is tightly linked with age at diagnosis. Mutations in PTCH1 or SUFU are more common in infants and usually are carried in patient's germline. More diverse molecular heterogeneity is seen in children with germline and somatic mutations in TP53 together with PTCH1 mutations, MYCN, GLI2 and SHH amplifications. PTCH1 and SMO mutations can be seen more commonly in adult SHH-subtype medulloblastomas. (Kool et al., 2012). Chromosomal aberrations like loss of 17p,9q and 10q have been observed in this subtype (P A Northcott et al., 2012; Shih et al., 2014). Loss of PTEN and gene amplification of components of the Insulin like growth factor (IGF) signaling have also been observed. The frequency of disease is almost comparable in females and males, though slight higher tumor prevalence is seen in male infants (PA Northcott et al., 2012; Taylor et al., 2012).

The SHH-subtype of medulloblastomas make up about 30% of all medulloblastoma cases (Kool et al., 2012). The current treatment includes chemotherapy, craniospinal irradiation and surgical resection (A. J. Gajjar & Robinson, 2014). Common chemotherapy procedures for MB include carboplatin, cisplatin, cyclophosphamide and vincristine among others but many side effects have been observed which include neurocognitive impairments, hearing loss, endocrine perturbations, cardiopulmonary problems and secondary malignancies (Fossati, Ricardi, & Orecchia, 2009). Recently, SMO inhibitors have shown encouraging results but demonstrate drug resistance due to mutations in SMO and have undermined the initial enthusiasm (Huang & Yang, 2015).

The main therapeutic idea of modulating SMO in the hedgehog pathway came from the alkaloid cyclopamine which represses the Hh pathway (M. K. Cooper, 1998; Incardona, Gaffield, Kapur, & Roelink, 1998; Keeler, 1975). In many cancers cyclopamine has been shown to inhibit the growth of the tumors and it is also used for regulating the hedgehog pathway in cancer research studies. Hyperactivity of Hh signaling is one of the main reasons for basal cell carcinoma (BCC). It has been shown that use of cyclopamine diminishes 66% of UV-B induced BCC in $Ptch^{+/-}$ mice (Athar et al., 2004). Apart from BCC, cyclopamine has also been shown to be effective in suppressing medulloblastoma development in $Ptch^{+/-}$ mice (Sanchez & Ruiz I Altaba, 2005). Due to poor oral solubility

issues cyclopamine cannot be used in clinical development (Lipinski, Hutson, et al., 2008). Several SMO inhibitors are being evaluated now like sonidegib (LDE-225), vismodegib (GDC-0449), LY2940680, PF-04449913 and BMS-833923 which are in advanced clinical trials (Amakye, Jagani, & Dorsch, 2013).

One of the first SMO inhibitor, Vismodegib (GDC-0449) was approved by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic or advanced Basal Cell Carcinoma (BCC). Vismodegib notably reduced the appearance of fresh BCC in treated individuals but led to reappearance upon stoppage of the drug (Huang & Yang, 2015). Apart from Vismodegib, HPI-1 and GANT61 are promising therapeutics which target Gli1 and Gli2 (Hyman et al., 2009; Lauth & Bergström, 2007; Mazumdar et al., 2011) whereas the drugs Saridegib, BMS-833923 and LDE-225 target SMO (Buonamici et al., 2010; M. J. Lee et al., 2012). With respect to SHH medulloblastoma, Kool et al., (2014) explain that patients having mutations upstream of SMO, like PTCH1 or amplifications of SHH are good targets for SMO antagonists but patients exhibiting mutations downstream of SMO like NMYC and GLI2, would not react to drugs against SMO (Kool et al., 2014). Though the initial outcome of SMO inhibitors is promising in SHH driven medulloblastomas but quick drug resistance has been a challenge in treating the patients hence there is a need for development of new SMO inhibitors (Huang & Yang, 2015).

2.18 Drug resistance in medulloblastoma

Drug resistance in medulloblastoma has been quite a waffling issue. Different kinds of drug resistance mechanisms have been observed in medulloblastoma patients and have been linked to different number of genes (Huang & Yang, 2015). In one of the studies, it was demonstrated that radiation surviving medulloblastoma cells were evaluated for genes expressing stem cell behavior which is associated to the capability of a subset of tumor cells to incite metastasis or formation of tumor. In these cells, ATP-binding cassette (ABC) transporter ABCG2, which is related to drug resistance and stem cell behavior was seen to be highly elevated. Radiation surviving cells from the patients when treated with

reserpine and verapamil which are ABC transporter inhibitors led to sensitization of cells to radiation (Ingram et al., 2013). Elevated amounts of ABCG2 are the characteristics of stem cells, and these transporters are involved in efflux of toxic substances from the cells which might include chemotherapeutic substances like drugs (Dean, Fojo, & Bates, 2005).

It was shown that cancer testis antigens (CTA) were found to be highly expressed in cancers like breast cancer and melanoma, these CTAs were also tested with respect to resistance to drugs in medulloblastoma. Expression of G antigen (GAGE) and melanoma-associated antigen (MAGE) which belong to the CTA family of proteins, correlated to chemotherapeutic resistance. When these genes were inhibited, medulloblastoma cells became sensitized to etoposide and cisplatin (Kasuga et al., 2008).

2.19 Mechanisms of resistance to SMO inhibitors

In cancer therapeutic studies, acquired resistance is often a frequent phenomenon. Targeting SMO has been one of the key aspects in treating medulloblastoma but treating patients with acquired resistance against SMO inhibitors has been quite challenging. Four different acquired resistance mechanisms have been put forward in clinical as well as preclinical studies (Amakye et al., 2013).

2.19.1 Resistance to SMO inhibitors due to mutations in SMO

In one of the examples of acquired drug resistance to SMO inhibitors, a patient suffering with metastatic medulloblastoma displayed a missense mutation from G-to-C at 1697 position (Aspartic acid to Histidine-D473H) in Smoothened drug binding pocket and thereby acquired resistance to drug GDC-0449 (Vismodegib). In a mouse model alteration of the same amino acid has led to resistance against GDC-0449 (Vismodegib)

(Yauch et al., 2009). Apart from this particular mutation, mutations at D338N, N223D, D477G, L225R, G457S and S391N also render resistance to SMO inhibitors (Kieran, 2014). In one of the studies with respect to BCC, resistance to SMO inhibition was demonstrated because of the mutation in SMO-G497W. Analysis of this mutation in silico further showed that it leads to conformational change thereby leading to blockage of protein drug entry site (Pricl et al., 2015) (Fig.H1).

2.19.2 Resistance to SMO inhibitors via amplification of *Gli2*

A second mode of resistance to SMO inhibitors in a *Ptch*-mutant medulloblastoma mouse model is via *Gli2* amplification. *Gli2* amplification has been reported for both sonidegib and vismodegib resistance (Buonamici et al., 2010; Dijkgraaf et al., 2011). *Gli2* amplification correlated with increase in mRNA expression of *Gli2* and negotiated growth of tumor in a Smo-independent way. Apart from this cyclin D which is one of the Hh target gene is also found to be amplified in tumors resistant to vismodegib likely providing another escape mechanism (Dijkgraaf et al., 2011) (Fig.H2).

2.19.3 Resistance to SMO inhibitors via upregulation of PI3K-AKT pathway

An alternate mechanism of resistance to SMO inhibitors is via PI3K-AKT pathway upregulation which was found by means of gene expression profiling studies in sonidegib sensitive vs resistant tumors (Buonamici et al., 2010). The molecular events and players engaged in PI3K pathway upregulation and its link to GLI activation in resistant tumors is still enigmatic. The upregulation of PI3K pathway was seen in tumors both without and with amplification of *Gli2* (Buonamici et al., 2010). The importance of compensatory PI3K pathway upregulation was shown by the capacity to overcome or delay the rise of resistance in medulloblastoma mouse models by combining BEZ235 (a dual mTOR and PI3K inhibitor) with sonidegib, everolimus (mTOR inhibitor) or buparlisib (BKM120;

PI3K inhibitor) (Buonamici et al., 2010). New reports suggest that crosstalk amidst PI3K-mTOR and Hh signaling pathway along with SMO independent GLI signaling provide additional explanation for this combination (Riobó, Lu, Ai, Haines, & Emerson, 2006; Y. Wang et al., 2012) (Fig.H3).

The current clinical trial study of buparlisib and sonidegib (NCT01576666) might give proof of concept of this treatment in related cancer types. Alternative ways which are worth exploring in combating acquired resistance involve inhibitors of SMO that are effective against resistant mutations and novel combinations with cilial translocation inhibitors or inhibitors of GLI transcription factors. In one of the studies it was shown that Arsenic trioxide (ATO) which is a GLI inhibitor and itraconazole a cilial translocation inhibitor could suppress the growth of tumors in mouse medulloblastoma models exhibiting SMO-D477G resistance (J. Kim et al., 2013). In 23% of advanced basal cell carcinoma patients who are on continuous vismodegib treatment the mechanism of acquired resistance is still unknown (Atwood, Chang, & Oro, 2012; Chang & Oro, 2012). It would be useful to know if the lessons learned in BCC can be applied to MB or vice versa.

2.19.4 Resistance to SMO inhibitors via upregulation of atypical protein kinase (aPKCι/λ)

Atwood et al., (2013) demonstrated that atypical protein kinase (aPKCι/λ) is a GLI activator and described that in SMO inhibitor resistant human basal cell carcinoma the activity of aPKCι/λ is increased when compared to SMO inhibitor sensitive tumors. Blockade of this aPKCι/λ with the help of a myristoylated peptide blocker led to inhibition of mouse SMO inhibitor resistant basal cell carcinoma lines thereby providing an alternative path to overcome resistance to SMO inhibitors (Atwood, Li, Lee, Tang, & Oro, 2013) (Fig.H4).

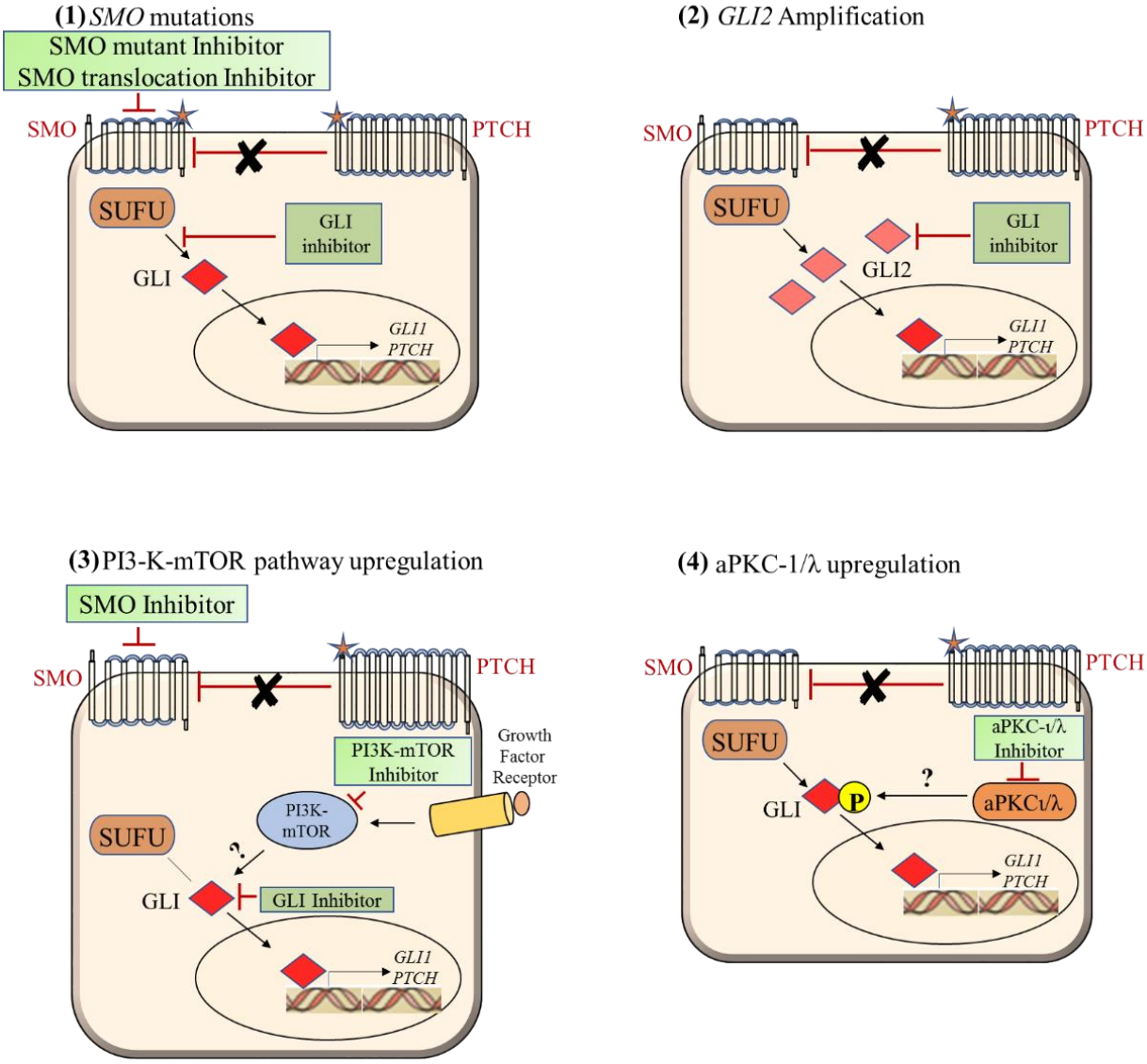


Figure H: Acquired resistance mechanism to inhibitors of SMO and ways to overcome resistance: (1) resistance via mutations in SMO. Resistance can be overcome by using SMO mutant, SMO translocation and GLI inhibitors. (2) via *GLI2* Amplification, GLI inhibitor can be used for overcoming the resistance. (3) resistance via PI3-K-mTOR pathway upregulation, GLI and PI3KmTOR inhibitor can be used. (4) via upregulation of aPKCι/λ and inhibitor of aPKCι/λ can be used for overcoming resistance. (Image adapted and modified from Amakye et al., 2013)

Apart from these different mechanisms of SMO inhibitor resistance, it is also important to consider and investigate other pathways that are interacting with Shh signaling so that the components of these pathways can be used in context of combination therapy for medulloblastoma treatment (Huang & Yang, 2015).

2.19.5 p53

Kool et al., (2014) observed that TP53 was elevated in pediatric medulloblastoma patients (4-17 ages) therefore further investigation is needed to ascertain the role of p53 in medulloblastoma. The status of p53 is critical for prognosis of Shh-medulloblastoma patients and also disease incidence. In one of the cohort studies, it was shown that TP53 plays a pivotal role in survival status of Shh-medulloblastoma patients. They demonstrated that the five-year survival rates varied considerably between 41% to 81% in Shh-subtype medulloblastoma patients with and without TP53 mutations (Zhukova et al., 2013). In mice with single PTCH deletion, the prevalence of medulloblastoma was 14%; this frequency rises to >95% in the presence of loss of p53, and this increase in frequency is specific for loss of p53 (Wetmore, Eberhart, & Curran, 2001). In one of the studies, it was observed that decreased MDM2 levels, a negative regulator of p53 led to reduced Gli1 and Gli2 expression besides small cerebella a distinct characteristic of decreased Shh signaling. Apart from this, Shh signaling in neuronal granule precursor cells stimulated accumulation of MDM2 and reduction of MDM2 blocked tumorigenesis in heterozygous PTCH mutant mice (Malek, Matta, Taylor, Perry, & Mendrysa, 2011). All of these observed results indicate that MDM2 interacts with Shh signaling and plays significant role in promoting medulloblastoma and for that reason MDM2 can be used as a possible target in combination therapy together with Shh signaling inhibitors in treating medulloblastoma (Huang & Yang, 2015).

2.19.6 Atoh1 & Boc

Aberrations in Hh signaling or defects in the components of Hh signaling at the time of cerebellar development leads to medulloblastoma formation. Upon stimulation with Shh granule neuron precursors (GNPs) divide and exit the cell cycle and move towards the direction of cerebellum. Further, these cells start differentiating into neuronal granule cells in the internal granule layer (IGL) of the brain (Huang & Yang, 2015). The

transcription factor Atoh1 is crucial for this process, its expression is limited to proliferating GNPs and completely absent in IGL neurons. Atoh1 protein levels are stabilized by Shh signaling and achieved by blocking its proteasomal degradation via a phosphor-dependent process. Aberrant and continuous Shh signaling leads to elevated levels of Atoh1 thereby transforming GNPs to medulloblastoma cells (Forget et al., 2014). It was demonstrated in one of the studies that Boc which is a Shh binding protein is upregulated in medulloblastomas. Apart from this, Boc inactivation led to decrease in proliferation and progression of early medulloblastoma to advanced stage cancer (Mille et al., 2014). Atoh1 and Boc are both promising targets for combination therapy (Huang & Yang, 2015).

2.19.7 Survivin

Survivin deals with regulation of cell cycle and apoptosis inhibition (Sah, Khan, Khan, & Bisen, 2006). Brun et.al reported a crucial role for survivin in medulloblastoma tumor progression and proliferation. In their study, they described that PTCH mutant medulloblastoma cells expressed elevated levels of survivin. Isolated cells from survivin deleted medulloblastoma tumor cells from mice displayed significantly reduced thymidine incorporation together with cell cycle arrest in the G2/M stage (Brun et al., 2015). Further studies are needed to delineate the role of survivin and its interaction with Shh signaling. Survivin might be a possible target in co-inhibition therapy for treating Shh driven medulloblastoma (Huang & Yang, 2015).

2.19.8 bFGF (Basic Fibroblast growth factor)

Basic fibroblast growth factor (bFGF) signaling seems to possess an inhibitory effect on Shh directed proliferation. In one of the studies, co-application of bFGF with Shh led to complete elimination of Shh-induced proliferation. Additionally, bFGF repressed the expression of Hh target genes like *Gli1, Nmyc* and *Cyclin D1* (M. P. Fogarty,

Emmenegger, Grasfeder, Oliver, & Wechsler-Reya, 2007). Considering these effects of bFGF on Hh signaling, activating bFGF signaling might be an alternative approach in co-targeting Shh-induced medulloblastoma (Huang & Yang, 2015).

Finally, patients belonging to the SHH-subtype of medulloblastoma should be treated with novel strategies due to existing heterogeneity and new therapeutic approaches should be developed for better treatment outcome. Recently, Pei et al., (2016) demonstrated that HDAC inhibitors together with PI3K inhibitors were able to inhibit the growth of *MYC* driven medulloblastoma (subtype 3) (Pei et al., 2016). Thinking in the same line HDAC inhibitors (HDACi) together with other SHH pathway inhibitors might bring about the same effect in SHH driven medulloblastoma.

3 Clinical significance and purpose of this study

Hedgehog (Hh) signalling plays crucial roles in growth, development, cell fate, survival, pattern formation and regulation of several invertebrates and vertebrate organs (Varjosalo & Taipale, 2008). Abnormal Hh signalling during embryogenesis or in the midst of development may lead to severe disorders like polydactyly, skeletal malformations, craniofacial defects and holoprosencephaly (Hill, Heaney, & Lettice, 2003; McMahon, Ingham, & Tabin, 2003; Muenke & Beachy, 2000; Lu Zhang et al., 2006). It has also been reported that altered Hh signalling has been noticed in ciliopathies leading to syndromes like Bardet-Biedl syndrome, Polycystic kidney disease, Kartagener syndrome and retinal degeneration (Kyttälä et al., 2006; J Pan et al., 2005). Basal cell carcinoma (BCC, skin cancer) (Hahn et al., 1996), medulloblastoma (MB) (Berman et al., 2002; L V Goodrich et al., 1997) and rhabdomyosarcoma (Kappler et al., 2004) can be caused by aberrant Hh signalling. Keeping in view the critical and diverse role/s played by Hh signalling, it is also important to treat, manage and cure diseases or disorders caused by aberrant Hh signalling.

Medulloblastoma is one of the type of cancers caused by altered Hh signalling. It encompasses a collection of molecularly and clinically well-defined tumour subgroups that emerge from brainstem or cerebellum (Grammel et al., 2012; Louis et al., 2007; Taylor et al., 2012). It is a neuroectodermal tumour affecting the cerebellum. Even though after development of impressive array of Hh antagonists drug resistance is still an unresolved problem that needs attention. The development of drug resistance is a frequent challenge in cancer drug therapy studies. It has been shown that mutations that are acquired in Hh pathway also lead to drug resistance and further challenges in treating the disease. In one of the examples of acquired drug resistance mechanism, a patient suffering with metastatic medulloblastoma displayed a missense mutation from G-to-C at 1697 position (Aspartic acid to Histidine) in Smoothened drug binding pocket and thereby acquired resistance to drug GDC-0449 (Vismodegib). In one of the mouse models alteration of the same amino acid has led to resistance against GDC-0449 (Vismodegib) (Yauch et al., 2009).

The present study addresses the important role played by histone deacetylase (HDAC6) in Hh signalling and how one can use HDAC6 as a molecular drug target for future therapy against medulloblastoma. It also opens new avenues for future drug molecules which are effective against this class of medulloblastomas.

4 Materials and Methods

4.1 Materials

4.1.1 Laboratory equipment and consumables

Name	Manufacturer
Autoclave	Fedegai Autuclavi Spa (Albuzzano, Italy)
Block Thermostat QBD2	Grant Instruments (Cambridgeshire, UK)
Blotting Paper 0,4mm thick	Hahnemühle FineARt GmbH, (Dassel,Germany)
Cell culture plates (60x15mm,100x20mm,150x20mm)	Sarstedt (Nümbrecht, Germany)
Cell scraper	Sarstedt (Nümbrecht, Germany)
Coverslips (25mm)	Thermo Fisher Scientific (Waltham, USA)
Cryotubes	Thermo Fisher Scientific (Waltham, USA) and Sarstedt (Nümbrecht, Germany)
Cryobox (Cell freezing container)	Thermo Fisher Scientific (Waltham, USA)
Experion Automated Electrophoresis System	BioRad (Hercules, CA, USA)
Falcon tubes (15ml,50ml)	Sarstedt (Nümbrecht, Germany)
Filter tips 10µl,1-20µl,20-200µl,100-1000µl	STARLAB, GmbH (Hamburg, Germany)
Finnpipette	Thermo Fisher Scientific (Waltham, USA)
Fluorescence Microscope DMI3000B with colour camera DFC300FX	Leica (Wetzlar, Germany)
Ice machine	Ziegra (Isernhagen, Germany)
Immobilon®- P Transfer Membranes	Millipore (Billerica, MA, USA)
LaminAir HA2448	Heraeus (Hanau, Germany)
Leica DMR Fluorescence Microscope with Quantifire XI camera	Leica (Solms, Germany) Intas (Göttingen, Germany)
Mini-PROTEAN electrophoresis system	BioRad (Hercules, CA, USA)
Mini Trans Blot Cell tank blot system	BioRad (Hercules, CA, USA)
Multiwell plates for Cell culture (6 well,12well, 24 well,48 well, 96 well)	Greiner Bio-one, Kremsmünster, Austria Sarstedt, Nümbrecht, Germany
Mx3000P/Mx3005P Qpcr system	Agilent (Böblingen, Germany
NanoDrop ND-1000 spectrophotometer	Peqlab (Erlangen, Germany)
Nunc™ MicroWell™ 96-Well Optical-Bottom Plates with Polymer Base	Thermo Scientific (Rochester, NY, USA)

Orion L microplate luminometer	Berthold Detection Systems (Pforzheim, Germany)
Parafilm	Pechiney plastic packaging (Chicago, USA)
Pasteur pipette	Hirschmann Laboratory equipment (Eberstadt, Germany)
PP-microplate with flat bottom (white 96-well)	Greiner bio-one (Frickenhause, Germany)
Serological pipette (5ml,10ml,25ml)	Sarstedt (Nümbrecht, Germany)
SRX-101A table top processor	Konica Minolta (Wayne, NJ, USA)
Super RX film	Fujifilm (Tokyo, Japan)
Syringe filter-0.2µm	VWR (Radnor, USA)
Tabletop Centrifuge Pico™ 17	Thermo Fisher Scientific (Waltham, USA)
Thermofast 96well qPCR plate non-skirted with adhesive sealing sheets	ABgene (Epsom, UK)
Water bath Thermomix ME	B.Braun Melsungen AG (Melsungen, Germany)

4.1.2 Chemicals

Chemicals	Manufacturer
Absolute™ QPCR SYBR Green® Mix	ABgene (Schwerte, Germany)
Acrylamide mix (Rotipherose® 30%)	Roth (Karlsruhe, Germany)
APS	AppliChem (Darmstadt, Germany)
Beetle Juice	PJK (Kleinbittersdorf, Germany)
DMEM (High glucose)	Invitrogen (Carlsbad, CA, USA)
DMSO	AppliChem (Darmstadt, Germany)
DTT	VWR (West Chester, PA, USA)
Ethanol (Rotipuran; 99.8%; p.a.)	Roth (Karlsruhe, Germany)
FBS GOLD	PAA (Pasching, Austria)
Goat Serum (#G6767)	Sigma-Aldrich (St.Louis, MO, USA)
iScript™ cDNA synthesis kit	Bio-Rad (Munich, Germany)
PBS (1x)	Invitrogen (Carlsbad, CA, USA)
Penicillin/Sterptomycin(100x)	PAA (Pasching, Austria)
Trypsin/EDTA(1x)	PAA (Pasching, Austria)
2-Mercaptoethanol	Sigma-Aldrich (St.Louis, MO, USA)

2-Propanol (HiPerSolvChromanorm)	VWR (West Chester, PA, USA)
Methanol	VWR (West Chester, PA, USA)
Milk powder	Roth (Karlsruhe, Germany)
NucleoSpin® RNA II Kit	Macherey-Nagel (Dueren, Germany)
PageRuler™ Prestained Protein Ladder	Thermo Scientific (Waltham, MA, USA)
Passive Lysis Buffer(5x)	Promega (Fitchburg, WI, USA)
Peirce ECL Western Blotting Substrate	Thermo Scientific (Waltham, MA, USA)
Saponin	Fisher Scientific (Schwerte, Germany)
SDS	Roth (Karlsruhe, Germany)
TEMED	Sigma-Aldrich (St.Louis, MO, USA)
TRIS	Acros (Geel, Belgium)
Triton X-100	AppliChem (Darmstadt, Germany)
Vectashield® mounting medium with DAPI	Vector labs (Burlingame, CA, USA)
Water (HiPerSolv Chromanorm for HPLC)	VWR (West Chester. PA, USA)
Renilla Juice	PJK (Kleinbittersdorf, Germany)

4.1.3 Cell lines

Cell lines	Source of Cell lines
ShhL2	ATCC
Hek293A	ATCC
C3H10T1/2	ATCC
NIH 3T3	ATCC
MEF [Smo*]	Wade Bushman
MEF Gli2dN	Wade Bushman
MEF Gli23 $^{-/-}$	Wade Bushman
MEF [SHH]	Wade Bushman
MEF-WT	R. Toftgård (KI, Huddinge, Sweden)
MEF Sufu $^{-/-}$	R. Toftgård (KI, Huddinge, Sweden)

4.1.4 Mouse lines

The mouse line Neuro2-SmoA1 was purchased from JAX (Stock number-008831) and has been described previously (Hallahan et al., 2004b).

4.1.5 Primary Antibodies

Antibody	Host species	Clone	Order No.	Manufacturer
Ac-H3	Rabbit		4499	Millipore
Ac-αTub	Mouse	6-11B-1	T6793	Sigma
β-Actin	Mouse	AC-15		Sigma
GLI1	Mouse		2643	Cell Signalling Technology
GLI2	Goat		AF3635	R&D Systems
GLI3	Goat		AF3690	R&D Systems
HDAC6	rabbit		sc-11420	Santa Cruz
HDAC6	rabbit		7558	Cell signalling Technology
HIP1	goat		AF1568	R&D Systems
Histone H3	rabbit		4499	Cell signalling Technology
Lamin-B	goat		sc-6217	Santa Cruz
(α)-Tub	mouse	DM1A	T6199	Sigma

4.1.6 Secondary Antibodies

Antibody	Manufacturer	Dilution
Anti-goat-HRP	Santa Cruz	1:3300 in 5% milk
Anti-mouse-HRP	NEB	1:3000 in 5% milk
Anti-rabbit-HRP	GE Amersham	1:6000 in 5% milk

4.1.7 Short Interfering RNA(siRNA) Sequences (Targeting mouse genes)

Name	Target Sequence
siGli1_1	GUAAUUACGUUCAGUCGCA
siGli1_2	CCACAAGUCAAUAGCUAUA
siGli1_3	GAAGUCCUAUUCACGCCUU
siGli1_4	GUAACGCUCUGGACUCUCU
siGli2_1	GGAGGGAAGGUACCAUUAU
siGli2_2	CAUCAAGGCUCACACCGGU
siGli2_3	GCAUCACGAUUCUCUAGUC

siGli2_4	GAUCAGAACACGCUAUACU
siHdac6_1	UCUAGAGGGUGGAUACAAU
siHdac6_2	UAAUGGAACUCAGCACAUA
siHdac6_3	CAUCCAAGUCCAUCGCAAA
siHdac6_4	GCGAAAGAGUAGGCACAAU
siSmo_1	CCAAUUGGCCUGGUGCUUA
siSmo_2	GAGCCCACCUCCAGUGAGA
siSmo_3	GGGCAAGACAUCCUAUUUC
siSmo_4	GAGGGUGGCCUGACUUUCU
siCon (Targeting Firefly luciferase; siLuc)	UAAGGCUAUGAAGAGAUAC
siCon (Qiagen's All-Star; siAll)	AAUUCUCCGAACGUGUCACGU
siCon (siMix)	Equimolar mix of siUni, siAll, siLuc
siCon (Sigma's Universal neg. control#1; siUni)	Proprietary

4.1.8 qPCR Primer Sequences

Gene	Species	Sequence (5'→ 3')
Foxj1	murine	for: TTCTGCTACTTCCGCCATGCAGACC rev: TCATCCTTCTCCCGAGGCACTTTGA
Gapdh	murine	for: GGTGTGAACGGATTTGGCCGTATTG rev: CCGTTGAATTTGCCGTGAGTGGAGT
Gli1	murine	for: CCCATAGGGTCTCGGGGTCTCAAAC rev: GGAGGACCTGCGGCTGACTGTGTAA
Gli2	murine	for: TGAGGAGAGTGTGGAGGCCAGTAGCA rev: CCGGGGCTGGACTGACAAAGC
Gli3	murine	for: AAAGCGGGAAGAGTGCCTCCAGGT rev: TGGCTGCTGCATGAAGACTGACCAC
Hdac1	murine	for: AGGAGGGAGAAGGTGGTCGCAAGAA rev: GCAGACCTTGCTCAGGCCAACTTGA
Hdac2	murine	for: CTGGGGACAGGCTTGGTTGTTTCAA rev: GTGTAGCCTCCTCCACCGAGCATCA
Hdac3	murine	for: ACCATGCACCCAGTGTCCAGATTCA rev: CATGGTCGCCATCATAGAACTCATTGG
Hdac4	murine	for: TTCCCAGGAAGTGGAGCACCAGATG rev: AAGGCTGCCAAGTACTCAGCGTCTCC
Hdac5	murine	for: TCGTCCTAGTCTCCGCTGGGTTTGA rev: TGTCATGAGCTGCCTGGTCAAGTGG
Hdac6	murine	for: TCCCTACAGCTTGGGGTTCTCAGCA rev: TCCCCAAATCCTTGTGTCAGCATCA

Hdac7	murine	for: CTTTCTGTGGGCATCCTGGCTGAAG rev: AGGACCAGGGCTGACATCAGAGACG
Hdac8	murine	for: CCACCGAATCCAGCAAATCCTCAAC rev: TTCCACAAACCGCTTGCATCAACAC
Hdac9	murine	for: CTCGGGGTGGACAGTGACACCATTT rev: AGCCAGCTCAATGACACAGCCAACA
Hdac10	murine	for: GAGCCCAGTAGGGGGCCGAATTCTA rev: AGTGGCCTTCGAGAAAGGACCCAGA
Hdac11	murine	for: CCCAAGGCCCCCAACTTATCTCCTC rev: GCCTTGCCTTCTCTCCATCCTGGTC
Hip1	murine	for: TGGCTCCCATCGGCTCTTCATTCTA rev: AGGCTTAGCAGGCCCCTTTCGTCTC
Hsd11b1	murine	for: CGTGTCCATCACTCTCTGTGTCCTTGG rev: TCCTTGGGAGAAGCTTGGGCGTTAAT
Ptch1	murine	for: CGCCTTCGCTCTGGAGCAGATTTC rev: TGAGGAGACCCACAACCAAAAACTTGC
Ptch2	murine	for: CCCGTGGTAATCCTCGTGGCCTCTAT rev: TCCATCAGTCACAGGGGCAAAGGTC
Rasl11b	murine	for: TCATCGGGGACTACGAACGAAATGC rev: ACTGCTCGCTGCAACTCAAGCCATT
Rplp0 (P0)	murine	for: TGCACTCTCGCTTTCTGGAGGGTGT rev: AATGCAGATGGATCAGCCAGGAAGG
GLI1	human	for: TCTGGACATACCCCACCTCCCTCTG rev: ACTGCAGCTCCCCCAATTTTTCTGG
PTCH1	human	for: CCGCCTTCGCTCTGGAGCAGATT rev: TCTGAAACTTCGCTCTCAGCCACAGC
RPLP0 (P0)	human	for: CCTTCTCCTTTGGGCTGGTCATCCA rev: CAGACACTGGCAACATTGCGGACAC

4.1.9 Buffers

10x Blotting Buffer

Tris	30.3
Glycine	144
20% SDS	1.5ml

Add H_2O to 1l

Add 20% methanol when diluting

10x SDS Running Buffer

Tris	30.3
Glycine	144
20% SDS	50ml

Add H_2O to 1l

5x SDS Buffer

Tris-HCl, pH 6.8	250 nM
SDS	10%
Glycerol	30% 30%
2-Mercaptoethanol	5%

10x TBS-T Buffer

Tris, pH-8	25 ml
5M NaCl	30 ml
Tween 20	1 ml

Add H_2O to 1l

Running Gel Solution

Acrylamide Mix	4%
Tris-HCl, pH 6.8	188mM
SDS	0.1%
APS	0.1%
TEMED	0.01%

Resolving Gel Solution

Acrylamide Mix	7-15%
Tris-HCl pH 8.8	188mM
SDS	0.1%
APS	0.1%
TEMED	0.01%

4.1.10 Software

Software	Manufacturer
Adobe reader	Adobe Systems Inc. San Jose, USA
Image J 1.45s	Wayne Rasband, National Institute of health, USA
Leica LAS AF Lite	Leica Microsystems, Wetzlar, Germany
Microsoft office 2013 and 2016	Microsoft Corporation,USA
Mx3000 Analysis software-version 4.10	Agilent Technologies, Santa Clara, USA
Nanodrop 1000 measurement Version 3.7.1	Thermo Fisher Scientific, Waltham, USA
Prism5 and Prism6	GraphPad Software, La Jolla (USA)
Mendeley Reference Manger-1.17.8	Elsevier (USA)

4.2 Methods

4.2.1 Cell Culture

All the cell lines were cultured in Dulbecco's modified Eagles medium (DMEM; high glucose plus glutamine and pyruvate; Invitrogen, Carlsbad, USA) supplemented with 10% Fetal bovine serum (FBS Gold, PAA, Pasching, Austria) and 1% penicillin/streptomycin (PAA, Pasching, Austria) at 37^{o} C with 5% CO_2. Authentication of cell lines was performed by ATCC. All the MEF cell lines were regularly authenticated by functional testing (e.g., loss of *Sufu* in the case of Sufu$^{-/-}$ MEFs). Cell lines were also regularly tested for mycoplasma contamination. Sub-culturing of cells was done by washing the cells twice with DPBS and incubated with 1ml of trypsin/EDTA (PAA, Pasching, Austria) for 2mins and plated in new culture plates with fresh growth medium.

4.2.2 Cryopreservation of Cells

Adherent cells were washed once with 1xPBS and then 1ml of trypsin was added to the plate and incubated at 37^{o}C for 2 mins. The trypsinized cells were resuspended in media and transferred to a sterile falcon tube and centrifuged for 5min at 1000 rpm at 4^{o}C.After centrifugation the supernatant was removed with sterile pasteur pipette. The pelleted cells were quickly resuspended by adding 1ml of freezing media per vial to be frozen. Now, the freezing media with cells were aliquoted into labelled cryovials and then placed in precooled cryobox later the vials were transferred to -80^{o}C and finally to liquid N_2 tank for long term storage.

4.2.3 Thawing of Cells

The cells were removed from liquid Nitrogen (N_2) tank and the vial was rapidly thawed at 37^{o}C in water bath and immediately sprayed down with ethanol and placed in the laminar hood. The vial contents were immediately pipetted to a 50ml falcon containing prewarmed medium and centrifuged at 1000rpm for 5mins at 4^{o}C. The pellet was resuspended in fresh prewarmed medium and pipetted into new 10cm dishes and incubated at 37^o C with 5% CO_2 untill further subculturing.

4.2.4 RNA Isolation

Extraction of total RNA was performed using Nucleospin RNAII kit (Macharey-Nagel) according to manufacturer's protocol. Elution of RNA was performed with 40µl RNase-free H_20 which was different to the protocol. The eluate was reapplied to the RNA column for re-elution. The purity and concentration of RNA extracted was photometrically determined using NanoDrop ND-1000 spectrophotometer (Peqlab).

4.2.5 cDNA Synthesis

Synthesis of cDNA was performed using iScript cDNA Synthesis Kit (Biorad) following the guidelines of manufacturer. In brief, 1µg of total RNA was diluted in 15µl of RNase-free H_20 and incubated with 4µl of iScript reaction mix and 1µl of iScript reverse transcriptase(RT) enzyme using the following protocol: iScript ™

Components	Concentration
iScript ™ Reaction Mix	1 x
iScript ™ Reverse Transcriptase	1 µl
RNA	1 µg

RNase free H_2O	ad 20µl

Time	Temperature
5 mins	25º C
30 mins	42º C
5 mins	85º C
∞	4º C

RNA and cDNA were stored at -20ºC until further analysis. For cDNA synthesis (PTC-200) Peltier Thermal Cycler, MJ Research, Quebec, Canada was used.

4.2.6 Quantitative RT-PCR (qPCR)

qPCR reactions were performed using 10µl of Absolute QPCR SYBR Green Mix (ABGene),0.4µl of forward primer(10µM), 0.4µl of reverse primer (10µM) and 8.2µl RNAase free H_20. 1µl of cDNA was used for the qPCR reaction. qPCR reactions were performed in triplicates on 96-well qPCR plates (ABGene) using either the Mx3000p or Mx3005p qPCR systems(Agilent). The following protocol was used:

Steps	Time	Temperature	Cycle/s
Initialization	15 mins	95 º C	1 x
Denaturation	15 s	95 º C	40 x
Annealing	20 s	65 º C	
Elongation	15 s	72 º C	
Melting curve	60 s	95 º C	1x
	30 s	65 º C	
	30 s	95 º C	

Results were calculated as relative mRNA expression($2^{\Delta\Delta Ct}$) and the data was obtained from at least three independent experiments and are shown as the mean ± SD.

4.2.7 RNAi Transfection

Cells were plated subconfluently and transfection was performed by using 35nmol/L siRNA (Dharmacon SMART pools) on day0 using Lipofectamine RNAiMAX (Invitrogen) as transfection reagent. On day1 the cells were confluent and the siRNA solution was replaced with full growth medium. Cells were further grown for another 48 hours and RNA was isolated on day3.

4.2.8 Luciferase reporter assays

Cells were plated in triplicates and allowed to grow to full confluency in solid white 96-well plates having clear bottom. Consequently, the media was replaced with full growth medium with 100nmol/L SAG along with the indicated compounds for 2 days. Later, the cells were lysed with Passive Lysis Buffer (Promega) and Firefly and Renilla luciferase activities were measured by using an Orion L microplate luminometer (Berthold Detection Systems) with the help of Beetle and Renilla Juice reagents (PJK; Kleinblittersdorf, Germany). The measurement was taken for 5seconds after 2 second's time delay.

4.2.9 Osteogenic Differentiation Assay

Cells were plated in triplicates and allowed to grow to full confluency in 96-well plates. Consequently, the cells were treated with compounds in full growth media for 4 days. Later, the cells were lysed with Passive Lysis Buffer (Promega). The lysate was divided into two parts; one part was used to measure the alkaline phosphatase(AP) activity

(Alkaline Phosphatase Blue Micro well Substrate; Sigma) and the other part was used for quantification of protein (Bio-Rad Protein Assay; Bio-Rad).

4.2.10 Immunofluorescence

The cells were grown sub-confluently on etched glass cover slips (25mm diameter, Thermo Scientific). Cells were washed gently with 1x PBS and then fixed in 4% formaldehyde/PBS for 10mins at room temperature (RT). The cells were then washed twice with 1xPBS at room temperature for 5mins. For permeabilization 0.5% Triton-X100/PBS was used for 5min at RT. Later, the cells were blocked with 10% serum and 0.1% saponin and incubated for 2hrs at RT.The cells were washed once with PBS at RT for 10 mins. Primary antibodies were diluted in 50µl PBS (per cover slip) containing 10%serum and 0.1% saponin and incubated overnight at 4^{o}C. After washing twice with 1xPBS at RT for 5mins, the cells were incubated with secondary antibody conjugated to fluorophores diluted in PBS containing 10%serum and 0.1% saponin at room temperature in the dark for 2hrs. Later, the cells were washed twice with 1xPBS for 5mins and the cells were rinsed with millipore H_2O. Finally, the cells were mouted on glass plates using Vectashield® mouting medium with DAPI (4'6-Diamidino-2-Phenylindole).

4.2.11 Immunohistochemistry

Paraffin embeded and formaldehyde-fixed tissue sections were used for immunohistochemistry and then treated with HDAC6 antibody (1:50) from Santa Cruz Biotechnology(sc-11420).

4.2.12 Cilia formation assay

Cells were plated in low (1%) serum and the compounds were added immediately. Later, the cells were stained with cilia specific antibodies and cilia were counted after 48hrs under the microscope.

4.2.13 Cilia resorption assay

Cells were plated in low (1%) serum. After, 24 hrs the compounds were added in media containing 1% FBS and the cells were stained with cilia specific antibodies and then counted under the microscope.

4.2.14 SDS PAGE and Immunoblotting

The cells were lysed in 400-500µl of SDS loading buffer according to the cell density and plate format. The lysates were then boiled at 95^{o}C for 5 mins and stored at -20^{o}C. The lysates were seperated using the Mini-PROTEAN electrophoresis system (Bio-Rad) for SDS PAGE. 25µl to 30µl of lysates per lane and 10µl of prestained protein ladder were loaded. Seperation of proteins was performed at 10mA in stacking gel and 30mA in the running gel. After seperation of gels, the gels were blotted on Immobilon®- PVDF tansfer membranes (Millipore) by using Mini Trans-Blot® cell tank blot system (BioRad). Blotting was done for 90 min at 350 mA. Later, the blots were blocked in 5% milk/TBS-T for atleast 2hrs and then incubated with primary antibody at 4^{o}C overnight. The blots were washed three times for 10 mins in TBS-T, then the membranes were incubated with HRP-conjugated secondary antibodies for 2hrs at room temperature which was followed by washing the blot for three times for 10 mins at RT. Detection of the signal was

performed by using Pierce ECL western blotting substrate (Thermo Scientific) according to the manufacturer's protocol. Luminescence was visualized by exposing the blots to Fuji Medical RX films and developing using SRX-101A table top machine (KONICA MINOLTA).

4.2.15 Microarray

RNA was isolated from MEF $^{[SHH]}$ cells using Nucleospin RNAII kit (Macharey-Nagel) according to manufacturer's protocol. The RNA integrity was determined using the Experion Automated Electrophoresis system (Bio-Rad). The isolated RNAs from two different experiments were pooled in a 1:1 ratio. Labelling, hybridization and analysis of data was done by the Genomics core facility of IMT (Institute of Molecular Tumour Biology, University of Marburg). Labelling of RNA was performed with the two-colour Quick-Amp Labelling kit(Agilent) and hybridized against Agilent-026655 microarrays.
For normalisation of microarray data "loess" method was used implemented in marray package of Bioconductor/R. Probes from Agilent were assigned to the Ensembl revision70 genome annotations by aligning sequences with a short-read aligner (Bowtie against both, the genome and the transcriptome) as described previously (Adhikary et al., 2011). The data obtained from microarray has been deposited at Array express under the number E-MTAB-2440.

4.2.16 Compound Soubilization

First the compounds were dissolved in 100% DMSO at a concentration of 70mg/mL. Later, the compounds were diluted in 45% (2-hydroxypropyl) -b-cyclodextrin (Sigma #332607) in PBS to get a final concentration of 5mg/mL (1mg/200mL). All animal studies were in approval with institutional and federal state laws.

4.2.17 Statistical Analysis

All the data shown here are the mean of three independent experiments (unless indicated otherwise) ±SD. Calculation of statistical significance was done by applying two-tailed Student *t* test (Microsoft Excel). *, $P < 0.05$; **, $P < 0.005$; *** $P < 0.0005$. Unless stated otherwise, the comparison is between experimental and corresponding control (si Con, DMSO).

5 Results

5.1 In Murine Medulloblastoma HDAC6 is overexpressed

It has been demonstrated earlier that HDAC6 plays an important role in etiology of many different cancers and in the biology of primary cilium (Jacob et al., 2011; Y. S. Lee et al., 2008b; Pugacheva et al., 2007; Sakamoto & Aldana-Masangkay, 2011; Santo et al., 2012) which made me to look into the role of HDAC6 in Hh-driven medulloblastoma. Consistent with a previous report (S. J. Lee et al., 2013), I observed endogenous Hdac6 to be overexpressed in cerebellar tumours arising in SmoA1model. In this SmoA1 mouse model the Shh signaling pathway was precisely activated in precursors of cerebellar granule neurons, which was accomplished by constitutively expressing the active form of *Smo* gene SmoA1 under the control of the NeuroD2 (Neurogenic differentiation 2) promoter that is expressed particularly in cerebellar granule cells; Fig.1A; (Hallahan et al., 2004b; Hatton et al., 2008). Protein expression of HDAC6 in wt animals can be seen mostly in non-tumor Purkinje cells. Contrastingly, granule cell-derived tumour cell nests show high immunoreactivity to HDAC6 (Fig.1B) in the transgenic mice. These observations raised the question regarding functional role for HDAC6 in Hh driven medulloblastoma.

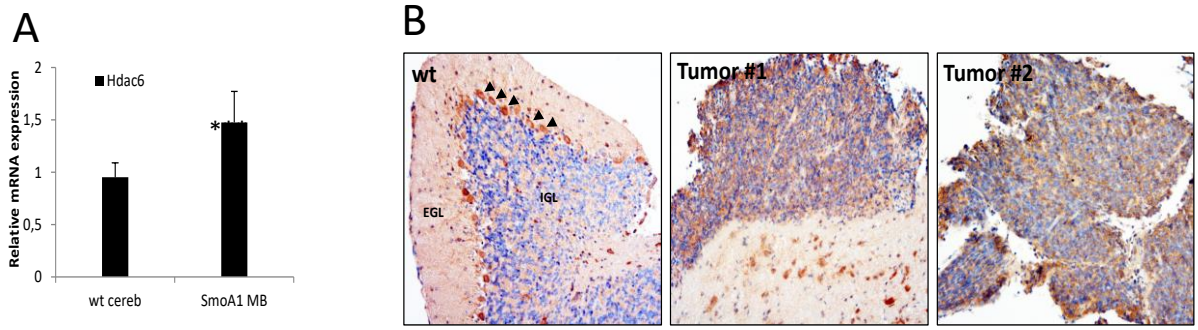
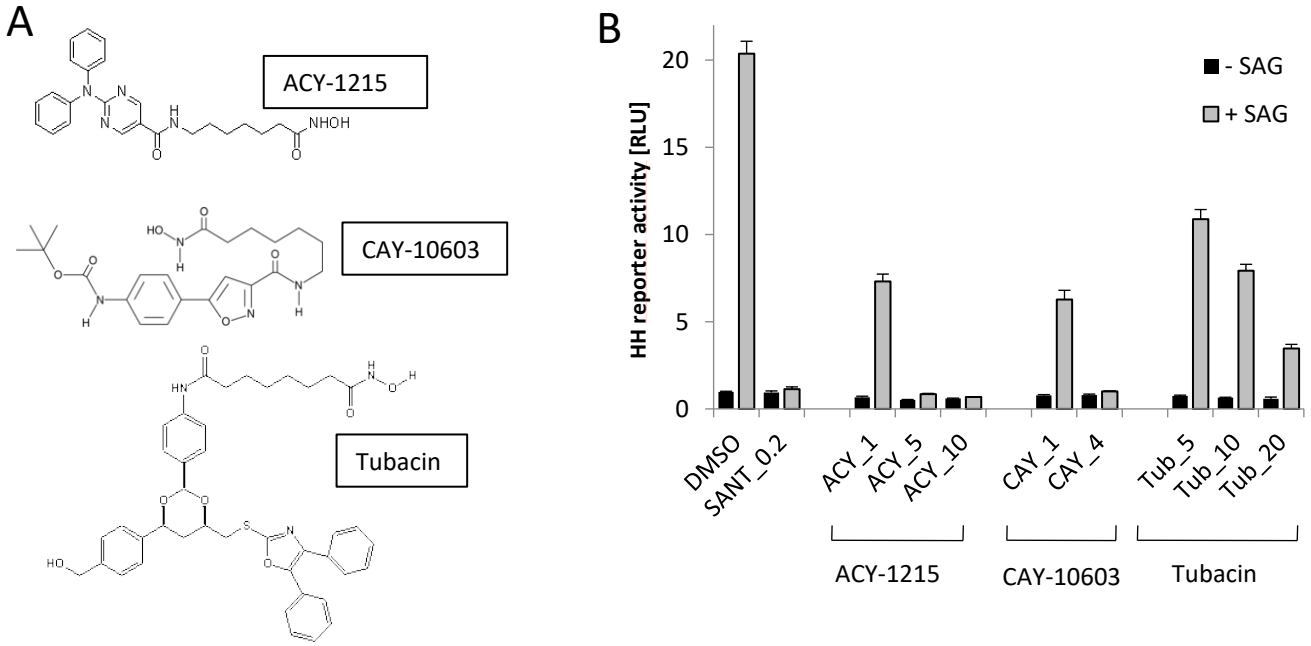


Figure1: In Medulloblastoma mouse model HDAC6 is overexpressed. A, Expression of Hdac6 mRNA in cerebellae of wt mice (~ 4 months old; n=9) and from cerebellae of medulloblastoma mice (SmoA1 mice, n=9). B, Immunohistochemistry of HDAC6 on paraffin-embedded sections from wt cerebellum (left) and cerebellum from SmoA1 medulloblastoma (middle and right). Note: In wt,the purkinje cells (and not the granule cells in the Internal Granule cell Layer) show the highest HDAC6 signal (indicated by arrowheads, brown), whereas in medulloblastoma SmoA1 the cancerous granule cell nests show the highest HDAC6 expression.

5.2 Active Hh signalling is blocked by pharmacological HDAC6 inhibitors

To address the importance of HDAC6 in Hh-induced disease, I first investigated the role of this enzyme in the Hh pathway. For this, I used specific and three structurally distinct HDAC6 antagonists (Tubacin, CAY-10603, and ACY-1215), which had been characterized and described before (Fig. 2A; (Haggarty, Koeller, Wong, Grozinger, & Schreiber, 2003; Kozikowski, Tapadar, Luchini, Ki, & Billadeau, 2008; Santo et al., 2012)). I used these inhibitors to treat fibroblasts as they are one of the major Hh-responsive cell types known and also, they represent a good model system for the analysis of Hh signaling pathway. As demonstrated in Fig. 2B, analysis of ShhL2 cells (NIH 3T3 fibroblast cells which carry a Hh-responsive luciferase construct in their genome stably), shows that induction of Hh pathway by synthetic agonist (SAG; (J. K. Chen et al., 2002) could be dampened by co-applying HDAC6 inhibitors in increasing amounts. The blockade of HDAC6 and its negative effect on endogenous Hh signaling could be seen with all the three compounds pointing against unspecific off-target effects. To preclude the effects of the compounds on ciliogenesis negatively, in compound treated cells I analyzed the presence of primary cilia. In line with earlier reports, HDAC6 helps in stabilizing rather than destabilizing cilia (Jacob et al., 2011; Pugacheva et al., 2007) I could not find any change in ciliary morphology or significant reduction of ciliogenesis in cells treated with HDAC6 inhibitors, regardless of serum concentrations used (Supplementary Fig. S1A-S1C). Apart from this, inhibition of HDAC6 did not lead to gross cytotoxicity (Supplementary Fig. S2A). To analyze further, I checked the effect of inhibition of HDAC6 on Hh driven physiologic process. C3H10T1/2 cells, which are mesenchymal progenitor cells differentiate along osteogenic lineage upon treatment with Hh, a process that can be visualized by staining for Alkaline phosphatase (AP) activity, which is an osteogenic marker protein. As demonstrated in Fig. 2C and D, the treatment with HDAC6 inhibitors CAY-10603 or Tubacin potently antagonized the SAG-induced differentiation of C3H10T1/2 cells. Interestingly, SMO induced cell differentiation was blocked by TSA (a pan-HDAC inhibitor), but also led to an increase of basal induction (Fig. 2D). However, to validate the need of HDAC6 in Hh signaling with a chemical compound free approach, I selectively knocked-down endogenous Hdac6 mRNA with

the help of a pool consisting of four different siRNAs (si Hdac6) constructs in MEF cells stably transfected with SHH (MEF[SHH]; (Lipinski, Bijlsma, Gipp, Podhaizer, & Bushman, 2008)) Transfection of siHdac6 in these constitutively signaling cells led to a clear reduction of expression of the established Hh target genes *Gli1, Ptch1*, and *Ptch2*; suggestive of Hh pathway inhibition (Fig.2E and F). I investigated individual siRNAs present in the pool and could demonstrate that except one, three other RNAi sequences inhibited Hh signaling, in line with my earlier findings (Supplementary Fig.S2B). In addition, the siRNA pool specific for Hdac6 did not lead to reduction in the expression levels of other Hdac family members but only Hdac6 (Supplementary Fig. S2C), confirming that the noticed effects are indeed due to particular interference with Hdac6. Furthermore, siRNA targeting Hdac6 significantly inhibited SAG- induced osteogenic differentiation in C3H10T1/2 cells (Supplementary Fig. S2D-S2F). Lastly, action of HDAC6 was vital for Hh signaling regardless of the serum concentration used (10% FBS; see Fig. 2; 1% FBS; see Supplementary Fig. S3A) and the nature of the ligand (see recombinant SHH instead of SAG in Supplementary Fig. S3B). Collectively, my data demonstrates that HDAC6 operates in the Hh pathway and is needed to attain full pathway activity.



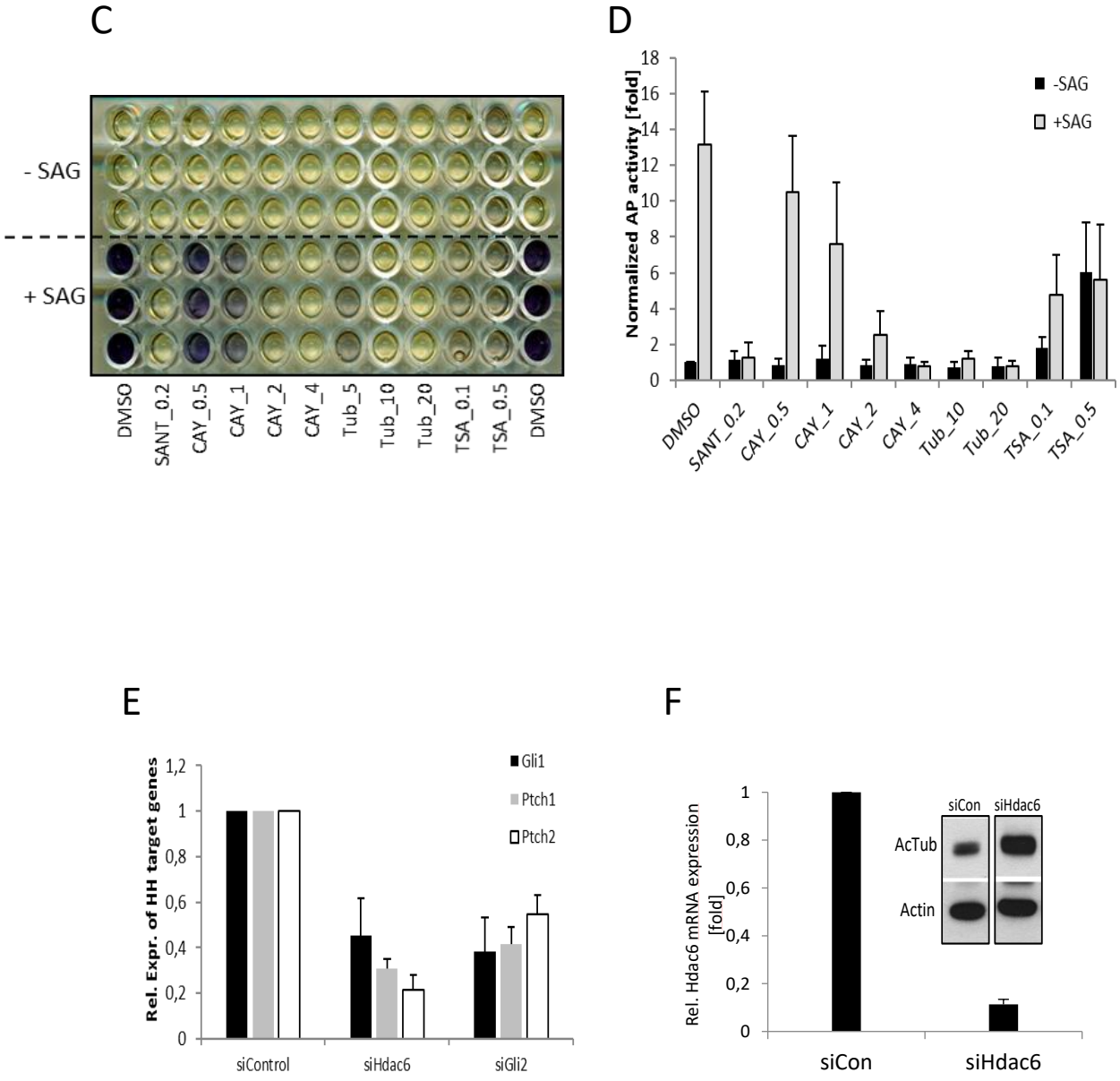


Figure 2: Hh signalling is impaired by targeting endogenous HDAC6. A, HDAC6 inhibitors chemical structure used in this study.B, Reporter luciferase assay using ShhL2 cells. The respective compound concentrations are shown in [mmol/L] following the drug, for example, SANT_0.2 indicates 0.2mmol/L of the SMO antagonist SANT-1 (positive control),C,C3H10T1/2 osteogenic differentiation assay.SAG was used to activate Hh signalling which induces osteogenic differentiation, shown by the blue AP staining. HDAC6 inhibitor/s treatment interferes with this process.D, Osteogenic differentiation assay result quantification shown in C. The mean (±SD) of three biological independent experiments is shown. Note: TSA (pan-HDAC inhibitor) blocks nuclear HDAC activity resulting in AP induction even in the absence of SAG.E, qPCR measurement of Hh target gene expression in (Gli1, Ptch1, and Ptch2) in MEF[SHH] cells transfected with indicated siRNA. RNAi against *Gli2* was used as positive control. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.F, verification of knockdown of *Hdac6* mRNA in MEF $^{[SHH]}$ cells via siRNA.b The inset shows functional depletion of HDAC6 protein indirectly: transfection with siHdac6 leads to increase in the levels of acetylated tubulin (Ac. Tub). The endogenous HDAC6 protein expression levels were too low to be detected by Western blotting using MEF cell lysates.

5.3 Epistatic investigation of HDAC6 effects

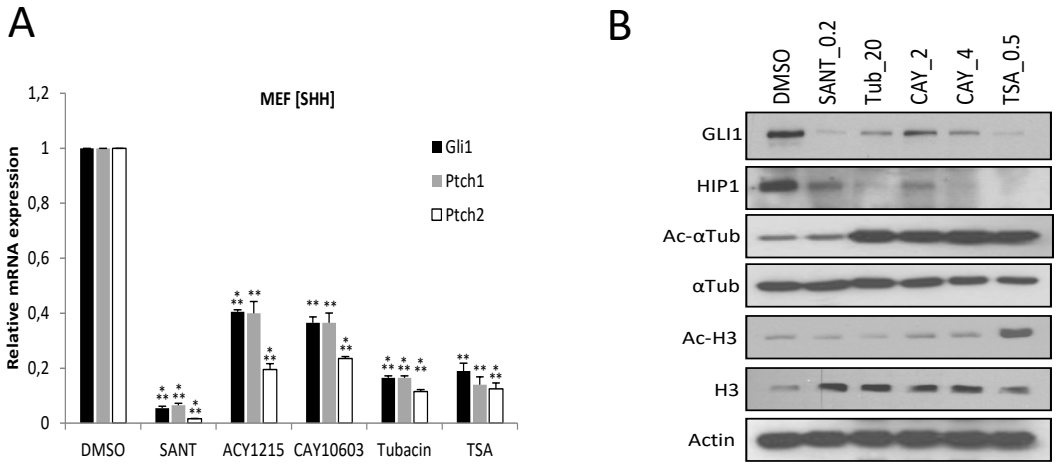
Next, I was keen to know at which stage in the Hh signal transduction pathway the function of HDAC6 would be integrated. For this purpose, I used MEF cell lines that contain activating alterations to trigger Hh signaling at various levels of the pathway and measured the extent of Hh target genes *Gli1, Hip1, Ptch1* and *Ptch2*. As demonstrated in Fig. 3A and B signaling in MEF[SHH] cells (which stimulate Hh signaling at ligand step) could be inhibited by treatment of different HDAC6 antagonists as shown at the mRNA (Fig. 3A) and protein level (Fig. 3B). Once again, neither of the HDAC6 inhibitors used notably led to increase in the levels of acetylated histone H3 (Fig. 3B and Supplementary Fig. S2G), in comparison with Trichostatin A (pan-HDAC inhibitor) arguing for HDAC6-selective mechanisms. Interrupting endogenous HDAC6 activity also inhibited Hh signaling in $Ptch1^{-/-}$ MEFs (Supplementary Fig. S3C) and in $MEF^{[Smo*]}$ (cells stably expressing a dominant active version of Smo); Fig. 3C; (Lipinski, Bijlsma, et al., 2008).

In spite of the fact that blockade could be seen in all cell lines, it looked as if cells bearing wt Smo (i.e., MEF[SHH] and $Ptch1^{-/-}$ MEFs) were more effectively inhibited than cells with mutant Smo $MEF^{[Smo*]}$. Additionally, in $Ptch1^{-/-}$ cells I observed Hip1(Hh target gene) expression was strongly inhibited only if TSA (a pan HDAC inhibitor) was used (Supplementary Fig. S3C). On the other hand, HDAC6 inhibitors and SANT led to a modest reduction in Hip1 levels pointing indirectly to HDAC6 antagonists having no profound result on acetylation of histone as demonstrated earlier. To know if HDAC6 also plays functional role downstream of Smo and Ptch1, I took advantage of MEF cells bearing genetic deletion of *Sufu* gene, leading to strong, but ligand- and receptor-independent constitutive signaling (A. F. Cooper et al., 2005; Svärd et al., 2006). Despite the ineffectiveness of SANT (SMO-selective inhibitor) (J. K. Chen et al., 2002) in these cells, inhibition of HDAC6 by ACY-1215, CAY-10603 or Tubacin led to inhibition of Hh target gene expression, although not alike as in MEF[SHH] cells (Fig. 3D).

Further, confirmation for inhibition of pathway in $Sufu^{-/-}$ cells was also seen upon knockdown of Hdac6 via RNAi (Supplementary Fig. S3D). Lastly, I employed cells with

stable expression of low amounts of an activated mutant of GLI2, the transcription factor determining the terminal steps of the Hh signaling pathway MEF[Gli2dN] cells; (Lipinski, Bijlsma, et al., 2008). As demonstrated in Fig. 3E blockade of HDAC6 also inhibited Hh target gene activity in these cells, whereas SANT the upstream inhibitor was inactive. Once more the repression attained by blockade of HDAC6 was not as marked as in cells with Hh pathway activation at ligand step (e.g., MEF[SHH]). Considering that nuclear HDACs are involved in direct deacetylation and activation of GLI (Canettieri et al., 2010), I was curious to know if identical process applied to HDAC6 too. In Hh luciferase reporter assays the activity of transfected GLI1 was promoted significantly by HDAC1 and HDAC2, HDAC6 remained inactive, despite marked protein expression levels (Fig. 3F).

This outcome shows that HDAC6 does not act possibly through deacetylation of the GLI1 transcription factor directly and probably works in an indirect way. In conclusion, I summarize that HDAC6 regulates Hh signaling positively, likely at two different steps: first, at the receptor level (PTCH1/SMO), and second, at a downstream point in the signaling pathway at the level of transcription factors.



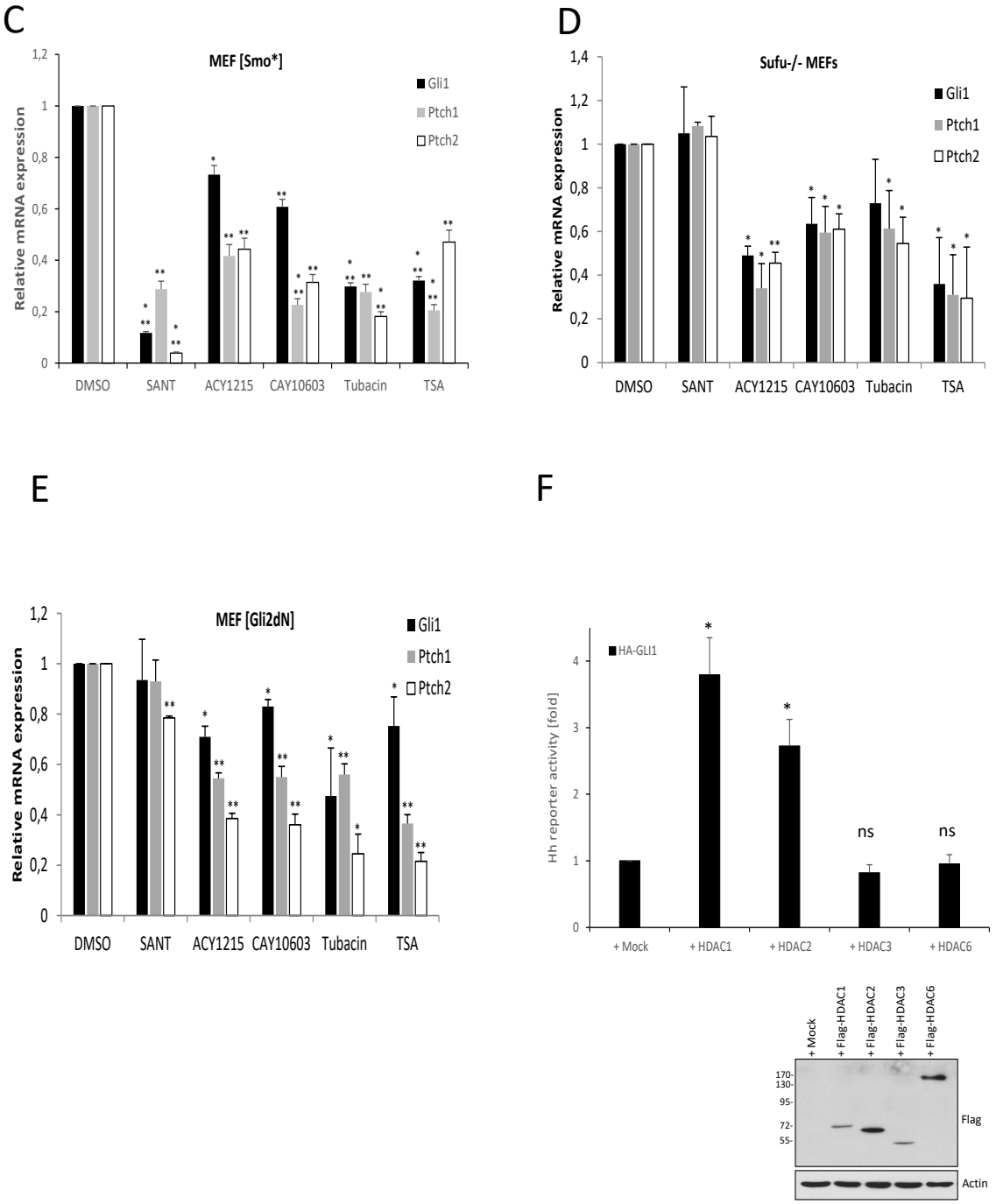


Figure3: HDAC6 epistatic analysis in the Hh cascade. Qpcr analysis of Hh target gene expression (Gli1, Ptch1 and Ptch2) in cells treated for 48 hours to different inhibitors (SANT, 0.2 mmol/L; ACY-1215, 10 mmol/L; CAY-10603, 4 mmol/L; Tubacin, 20 mmol/L; TSA, 0.5 mmol/L). A, Expression of Hh target genes in MEF$^{[SHH]}$ cells. B, Analysis of protein expression in MEF$^{[SHH]}$ treated with the indicated compounds via western blotting. Note that nuclear histone H3 acetylation is increased only by treatment with TSA but not with HDAC6 inhibition with CAY-10603 or Tubacin. C, Expression of Hh target genes in MEF[Smo*] cells. D, Expression of Hh target genes in Sufu$^{-/-}$ MEF cells. E, Expression of Hh target genes in MEF$^{[Gli2dN]}$ cells. F, Hh reporter assay in Hek293T cells transfected with various (Flag-tagged) HDAC constructs or HA-GLI1 plus empty vector control (mock). The Western blot analysis shows the protein expression of HDAC proteins in this assay. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ns, not significant.

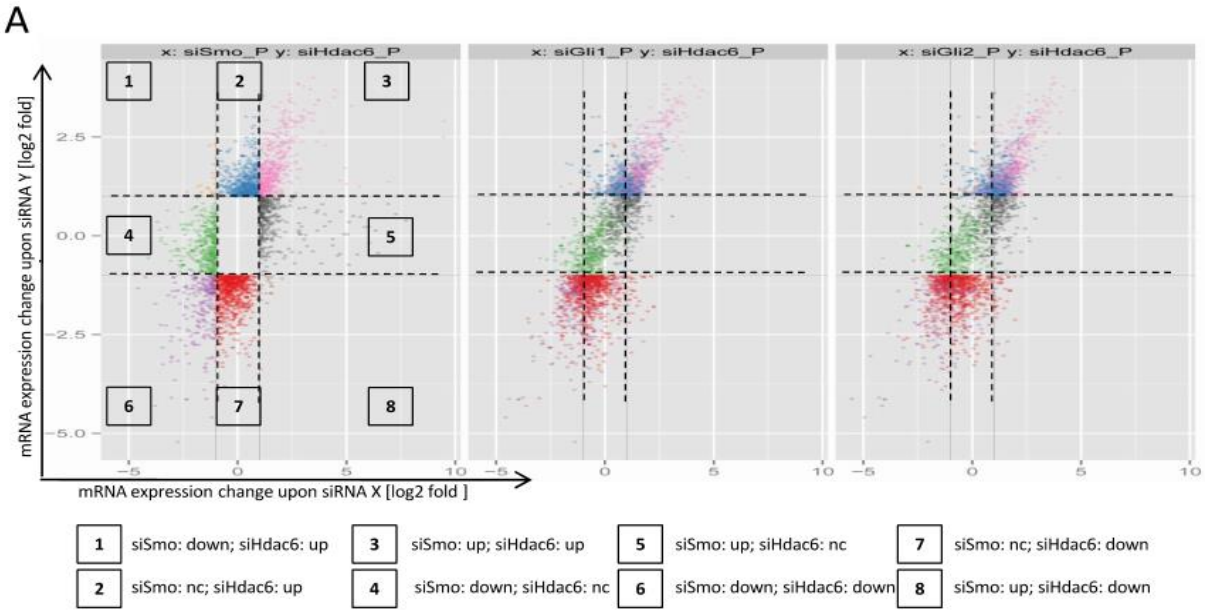
5.4 Global investigation of HDAC6 blockade

After having noticed the opposing effects of inhibition of HDAC6 in distinct Hh assays, I was keen to examine the broad impact on the Hh-activated transcriptome. For this purpose, I treated MEF[SHH] cells with RNAi directed against Hdac6 or with control siRNA (targeting Firefly luciferase, siLuc). As reference constructs, I employed siRNA directed against Gli*1, Gli2* or *Smo*. RNA isolated from these cells was used in microarray analysis. To correlate differences in global expression pattern between siHdac6 and siSmo transfected samples, scatter plot was used to show the data. On the grounds of noticed changes in gene expression, I grouped the transcripts into eight distinct classes, namely: (1) si Smo: down; siHdac6: up, (2) si Smo: no change; siHdac6: up, (3) si Smo: down; siHdac6: up, (4) si Smo: down; siHdac6:no change, (5) si Smo: up; siHdac6:no change, (6) si Smo: down; siHdac6: down, (7) si Smo: no change; siHdac6: down, (8) si Smo: up; siHdac6: down. These eight classes can be noticed as color coded dots on the scatter plot in Fig. 4A.

To explore the behavior of these genes, a subsequent comparison was done with siGli1/2 conditions, the transcripts were transferred (and their respective color) into siGli1/2 scatter plots (Fig. 4A, middle and right). Correlation of the expression differences induced on siHdac6 and siSmo transfection disclosed that, as predicted, the established and well-known canonical Hh target genes (e.g., *Ptch1/2*) come into group 6 (Fig. 4A, left, purple dots) and were down regulated by both the siRNAs. Not surprisingly, regulation of these genes was in the same way by siGli1/2 (purple and pink dots in Fig. 4A, middle & right). Intriguingly, genes that were governed in opposite directions by siSmo and siHdac6 (groups 8 and 1, brown and orange colored dots) were fundamentally absent. Further, an intriguing bunch of genes fell into group7 and was down regulated by siHdac6, but was not influenced by siSmo. Anyhow, a considerable part of these genes (red-colored dots in Fig. 4A) was shifted to the left in siGli1/2 plots, signifying that they were governed by Gli factors and Hdac6, but not by Smo. Lastly, group of genes exist that were not regulated by Hdac6 but were governed by Smo (groups 5 and 4 (gray and green spots) in Fig. 4A). Interestingly, a sizable fraction of these genes was not governed by either

Gli1/2. Considering that not all the *Smo*-governed genes were influenced by Gli1 and Gli2 (and vice versa), I assigned typical set of genes termed as the “Hh signature.” This gene set accomplished the following rigorous criteria: genes must have been (i) regulated in siSmo samples to that of siControl and (ii) Additionally, siSmo-controlled genes had to be governed in the same way by either siGli1/2 transfection. This led to a group of genes consisting of 820 transcripts ("Hh signature"). Analyzing the Hh gene signature with that of the genes regulated upon Hdac6 knockdown uncovered 56% of the Hh signature were influenced by loss of Hdac6 (Fig. 4B). The obtained expression data were verified by qPCR analysis of exclusive genes shown to be regulated by only “*Gli1*” and by "*Gli1* and *Hdac6*". As demonstrated in Fig.4C, in MEF[SHH] cells the canonical hedgehog target genes namely *Ptch1*, *Ptch2*, and *Hip1* all of them were down regulated by siHdac6 as well as by siGli1 transfection. In comparison, the expression of *Rasl11b*, *Foxj1* and *Hsd11b1* were particularly influenced by *Gli1*, but not by *Hdac6*, showing that Hdac6 influences a sub group of *Gli* target genes, as shown by the microarray experiment.

In summary, function of HDAC6 affected more than half of the genes involved in the Hh signature, but did not influence all the Hh signature genes. Strikingly, HDAC6 also have an effect on the regulation of genes, which are *Smo*-independent yet *Gli*-dependent, pointing at some amount of downstream pathway regulation by HDAC6 (Dhanyamraju et al., 2015).



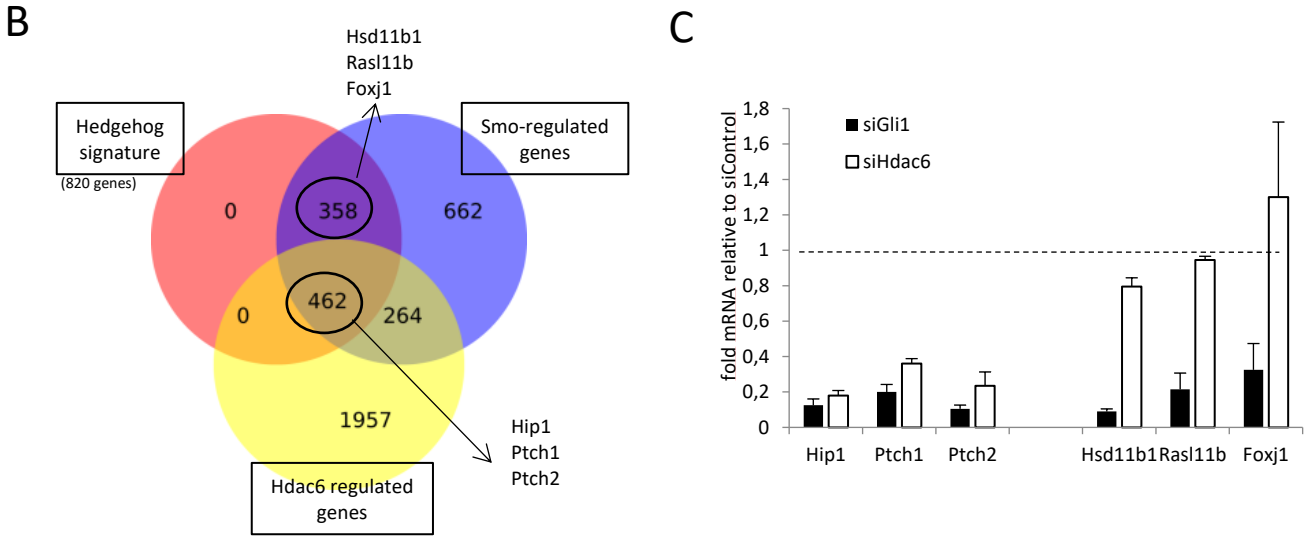


Figure 4: Global gene expression analysis. A, scatter plot representation showing mRNA expression changes in MEF[SHH] cells transfected with shown siRNA sequences (e.g., si Smo_P indicates four different siRNA pools targeting Smo). Left, Comparison between siHdac6 and si Smo samples. Genes fall under eight different color-coded sections based on their change in expression. Due to defined cutoff of at least two-fold the center of the scatter plot is empty. Middle, comparison between siHdac6 and siGli1 samples. Genes are shown on the left.nc, no change. B, Venn diagram showing MEF[SHH] microarray data categorized into "Hh signature," "Smo-regulated," and "Hdac6-regulated" genes. By definition, there is a full overlap between the Hh signature and the Smo-regulated gene set. C, Verification of expression data derived from microarray experiment via quantitative real-time PCR in A. MEF[SHH] cells were used.

5.5 HDAC6 and its dichotomous effect on Hh signaling

To know more about the main molecular association between Hh and HDAC6, I concentrated on GLI2 and GLI3; the key downstream signal transducers in the pathway. Since HDAC6 had been shown to play role in degradation of protein and clearance (Bazzaro et al., 2008; Cyril Boyault et al., 2006, 2007; Hebron et al., 2013; J.-Y. Lee et al., 2010; Pandey, Batlevi, Baehrecke, & Taylor, 2007).

I asked if inhibition of HDAC6 would alter the levels of protein or processing of GLI2/3. Reduction of overall amounts of GLI2 full-length protein including the amount of GLI3 activator (GLI3A) and repressor (GLI3R; Fig. 5A) was observed upon treatment of NIH3T3 fibroblasts with inhibitors of HDAC6; ACY-1215 and CAY-10603. At the level of mRNA, expression of Gli2 was reduced, but the levels of Gli3 mRNA was stable (Fig. 5B). These results imply that HDAC6 takes part in GLI3 protein stabilization and also

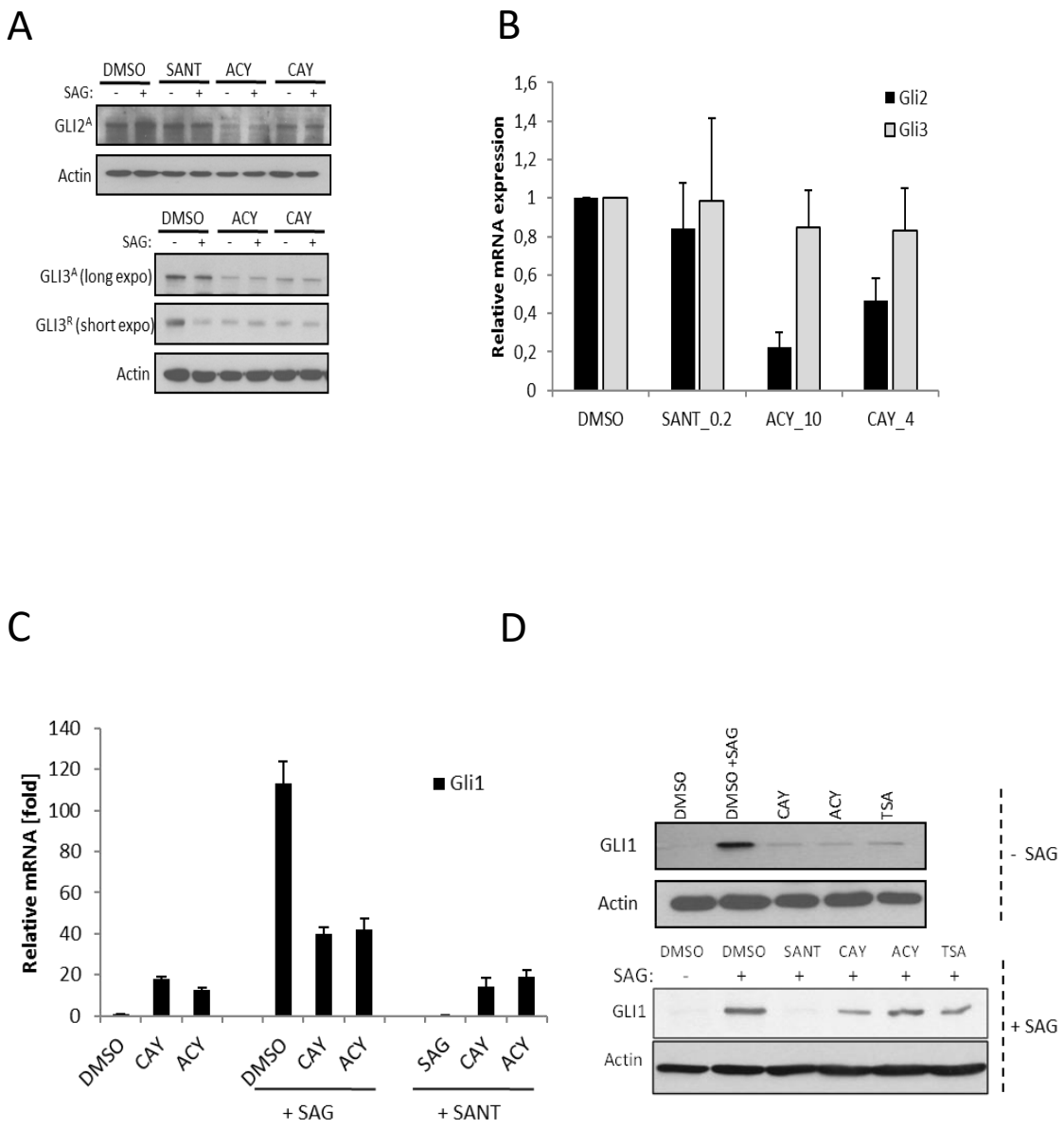
plays a role in transcriptional control of Gli2 expression (Knockdown experiments of siHdac6 and/or knockout studies of Hdac6 would throw further light on impact of Hdac6 on Gli3 protein stabilization and its role in transcriptional control of Gli2 expression).

Considering that inhibition of HDAC6 decreased the GLI3R form, I hypothesized that this could lead to derepression of Hh target gene expression in unstimulated cells. Consequently, I examined expression of Gli1 in wt MEF cells treated with HDAC6 inhibitors. As demonstrated in Fig.5C, treatment with HDAC6 inhibitors ACY-1215 or CAY-10603 certainly activated the Hh pathway even in the absence of SAG or Hh ligand. The observed target gene expression could not be reverted by the co-application of SANT (SMO antagonist), which was capable of blocking SAG- induced signaling (Fig. 5C). Contrary to the latter finding but in agreement with my earlier observations (Figs. 2 and 3), inhibition of HDAC6 also decreased the maximal target gene expression induced by SAG. Comparable data were seen when Ptch1 expression was determined (Supplementary Fig. S4).

Furthermore, the increase in the levels of Gli1 transcription by inhibitors of HDAC6 could also been shown at the protein level in non-stimulated cells. In accordance with the qPCR results (Fig. 5C), blockade of HDAC6 led to a decrease of maximal protein levels of GLI1 in SAG-induced cells (Fig. 5D). To ascertain if the increase in Hh signaling by antagonists of HDAC6 was negotiated via GLI factors or via GLI independent mechanisms, I employed cells with impaired functional *Gli2* and *Gli3* genes (double knockout Gli*23*$^{-/-}$ MEFs;(Lipinski, Bijlsma, et al., 2008)). Treating these cells with inhibitors of HDAC6 disclosed that increase of Gli1 observed in wt cells was notably decreased in the absence of Gli2 and Gli3 (Fig.5E), implying specifically that HDAC6-mediated effects on the protein levels of GLI3R might be crucial. Regardless, positive effects mediated via Gli1 may be required for ligand-independent Hh gene activation as well.

Consequently, I decreased endogenous *Gli1* amounts by RNAi based knockdown of the residual *Gli1* expression in Gli *23*$^{-/-}$ cells. As demonstrated in Fig.5F, transfection with siGli1 decreased the Hh target gene activation (*Ptch1* and *Ptch2*) in comparison to siControl cells, arguing also for the need of Gli1 activator function (Fig. 5F). Altogether,

I noticed a dichotomous role of HDAC6 inhibitors on Hh signaling pathway. On one hand, inhibition of HDAC6 leads to reduction of maximal Hh target gene activity; while on the other, HDAC6 interference activates basal target gene transcription. I speculate that the first effect may be caused due to the decreased Gli2 mRNA expression upon exposure to HDAC6 inhibitors, whereas the latter observation possibly involves the decreased GLI3R protein together with GLI1 activator functions.



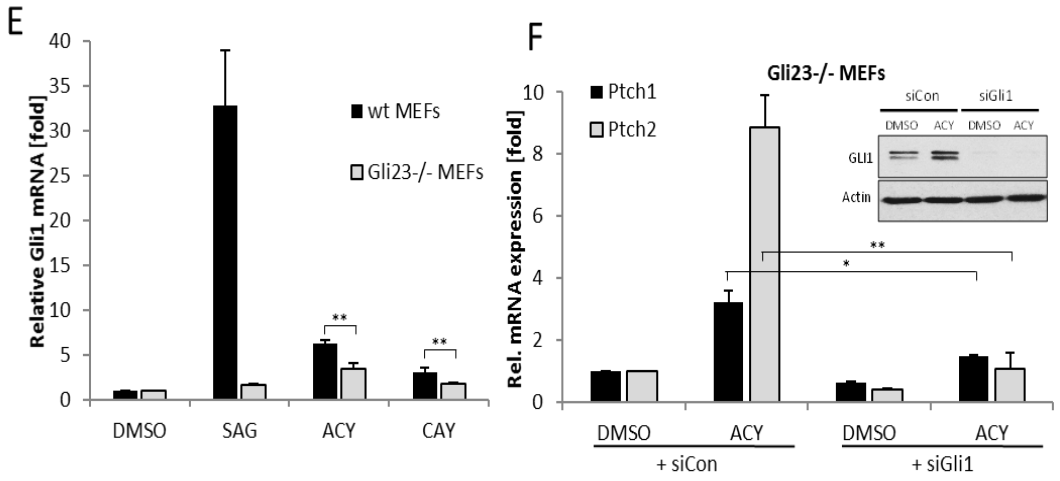


Figure 5: Molecular connection between Hh signalling and HDAC6. A, top, Expression of endogenous Gli2A (full-length Gli2) in NIH3T3 cells. As a loading control Actin was used. Bottom, Protein expression of endogenous Gli3A (full-length Gli3) and Gli3R (truncated repressor form) expression in NIH3T3 cells treated with the indicated compounds for 2days (ACY-1215,10mmol/L; CAY 10603,4mmol/L; SANT,0.2mmol/L; SAG,0.1mmol/L) B, Expression of Gli2 and Gli3Mrna in NIH 3T3 cells treated with indicated compounds for 2days ACY-1215, 10 mmol/L; CAY-10603, 4 mmol/L; SANT, 0.2 mmol/L) C, Expression of Gli1 mRNA in wt MEFs treated with the indicated inhibitors(concentrations as in A) D, Expression of endogenous Gli1 protein in wt MEFs treated with indicated compounds (concentrationsasinA;TSA,0.5mmol/L) E, Expression of Gli1 mRNA in wt MEFs in comparison with Gli1 expression in Gli23$^{-/-}$ MEFs, both treated with shown inhibitors(concentrations as given in A). The samples were set to 1 for comparison. F, Expression of Ptch1and Ptch2 mRNA in Gli23$^{-/-}$ MEFs transfected with siRNA controls or with siRNA against Gli1. Cells were treated with DMSO or with 10 mmol/L ACY-1215 for 2 days. The inset depicts an immunoblot confirming the Gli1 knockdown in these cells. *, $P < 0.05$; **, $P < 0.005$.

5.6 Inhibition of HDAC6 pharmacologically has repressive effects on growth of *in vivo* medulloblastoma

Next, I was interested to investigate whether an HDAC6 directed approach could in principle be exploited as a future therapy option in the treatment of medulloblastoma. To investigate this matter, I treated primary mouse medulloblastoma cells (MB99-1cells derived from the SmoA1 mouse model; (Hallahan et al., 2004a; Hatton et al., 2008) with increasing amounts of HDAC6 inhibitors in culture and measured live cells by the help of Cell Titer assays (Fig. 6A-C). As demonstrated in Fig. 6A-C, I noted marked decrease in cell number upon 2 days of HDAC6 inhibition that correlated with increase in acetylation of tubulin [$r^2 = 0.927$ (ACY-1215) and $r^2 = 0.989$ (CAY-10603) for inhibition of cell growth vs acetylation of tubulin]. The observed cytotoxicity was seen in mouse medulloblastoma cells. Notably, and in accordance with my earlier data, acetylation of

histone was not increased by inhibitors of HDAC6 used and did not correlate with inhibition of growth [r^2= 0.377 (ACY-1215) and r^2= 0.686 (CAY-10603) for inhibition of cell growth vs. acetylation of histone H3; Fig. 6A-C]. These data provoked me to investigate the in vivo effects of HDAC6 inhibition via small molecules.

For this purpose, I took s.c. allografts from primary SmoA1 medulloblastoma cells (MB99-1 cells). For the generation of s.c. allografts, SmoA1 medulloblastoma primary cells were retrieved directly by shearing and mincing the tumor portions. Later, these cells which were obtained from the cerebellum were mixed in a ratio of 1:1 with Matrigel and introduced subcutaneously into C57BL/ 6J mice for expansion. Eventually, after few cycles of this *in vivo* passaging, cells were collected and combined with Matrigel (1/3rd of the volume) and introduced subcutaneously into 15 C57BL/ 6J mice (Bai, Staedtke, Rudin, Bunz, & Riggins, 2015; Metcalfe et al., 2013; G. Y. Wang et al., 2011) which were 3.5 months old and weighed 20g. After 7 days, all the injected mice had perceptible subcutaneous tumors of around 25mm$^{2.}$ This procedure was performed mainly to generate adequate tumor possessing mice for drug treatment and analysis (Metcalfe et al., 2013). After tumors, had reached a perceptible volume, mice (in groups of 5) were injected peri tumoral s.c. with ACY-1215 or solvent or with 50 mg/kg of either Vismodegib (GDC-0449, an FDA-approved SMO antagonist as a positive control). Control (solvent-treated) tumors increased greatly in size over a 12-day period, while ACY-1215 and Vismodegib treated tumors remained small (Fig.6D). This was also seen in the mean tumor weight of dissected tumors taken at the end of the experiment (Fig.6E). Markedly, no signs of toxicity were seen and all the drugs were well tolerated by animals.

Analysis of the tumor tissue immunohistologically disclosed that ACY-1215-treated tumors particularly had elevated levels of cleaved caspase-3, a sign of apoptosis (Fig. 6F), in spite of the fact that both the drugs (ACY-1215 and Vismodegib) repressed activity of Hh pathway in the in vivo allografts, measured by decreased Hh target gene activity (Supplementary Fig. S5).

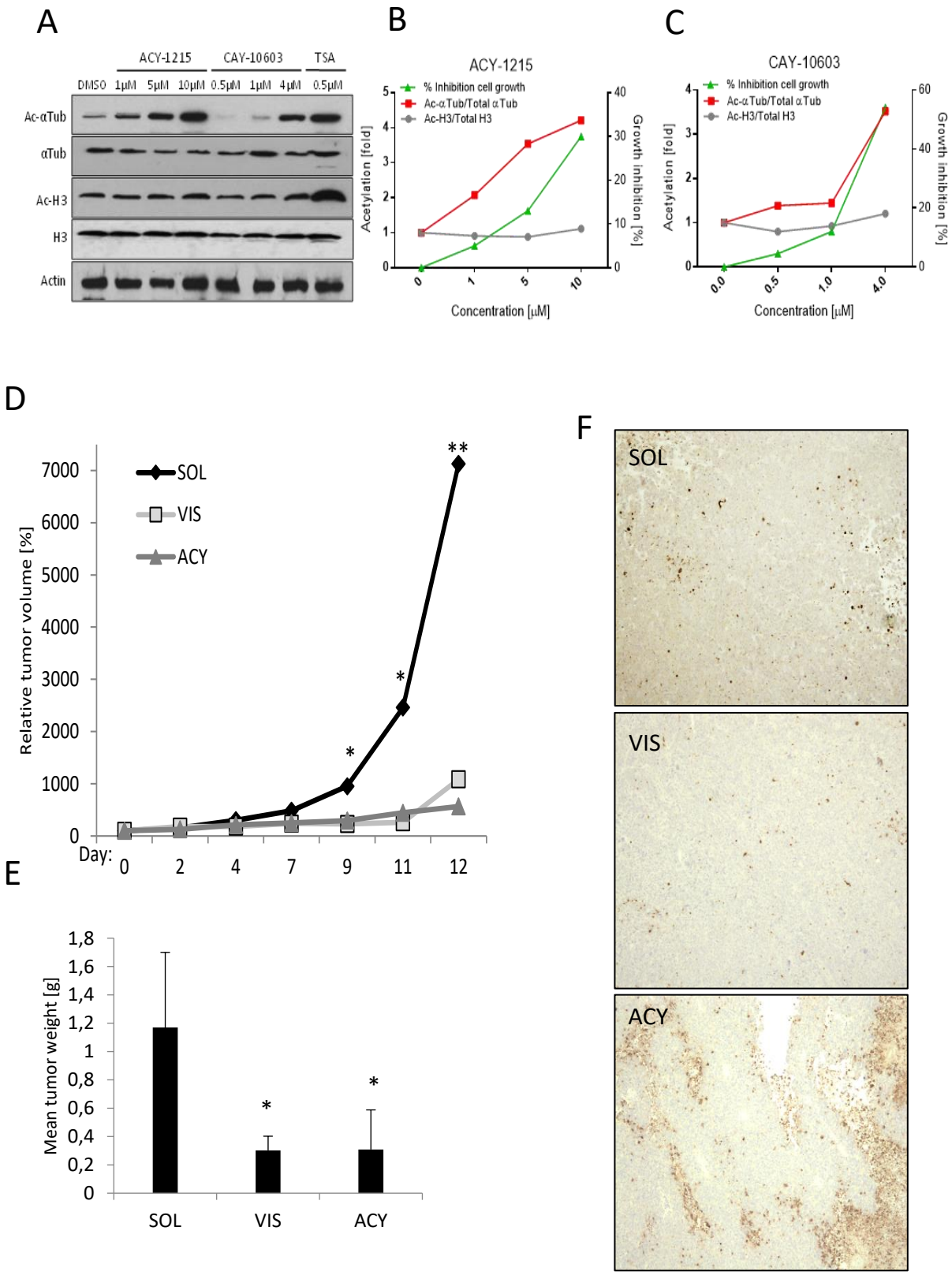


Figure 6: In vivo effects of HDAC6 inhibition pharmacologically. A, immunoblot from MB-99 cells treated with indicated compound concentrations for 2 days. Note that tubulin acetylation is affected by HDAC6-selective inhibitors but histone H3 acetylation is not affected, whereas TSA (the pan HDAC inhibitor) increases both B and C, quantified results shown in A. In addition, the data contain figures on cell growth (CellTiter assay; Promega) measured after 2

days of incubation with indicated compounds. D, in vivo allograft: tumor volume change relative to day 0 (volume ¼ 100%). Shown is the mean of 5 mice, E, mean tumor weight (g) of resected allografts on day 12. F, cleaved caspase-3 immunohistochemistry (brown) on allograft tissue sections taken on day 12. SOL, solvent; VIS, Vismodegib; ACY, ACY-1215. *, P < 0.05; **, P < 0.005.

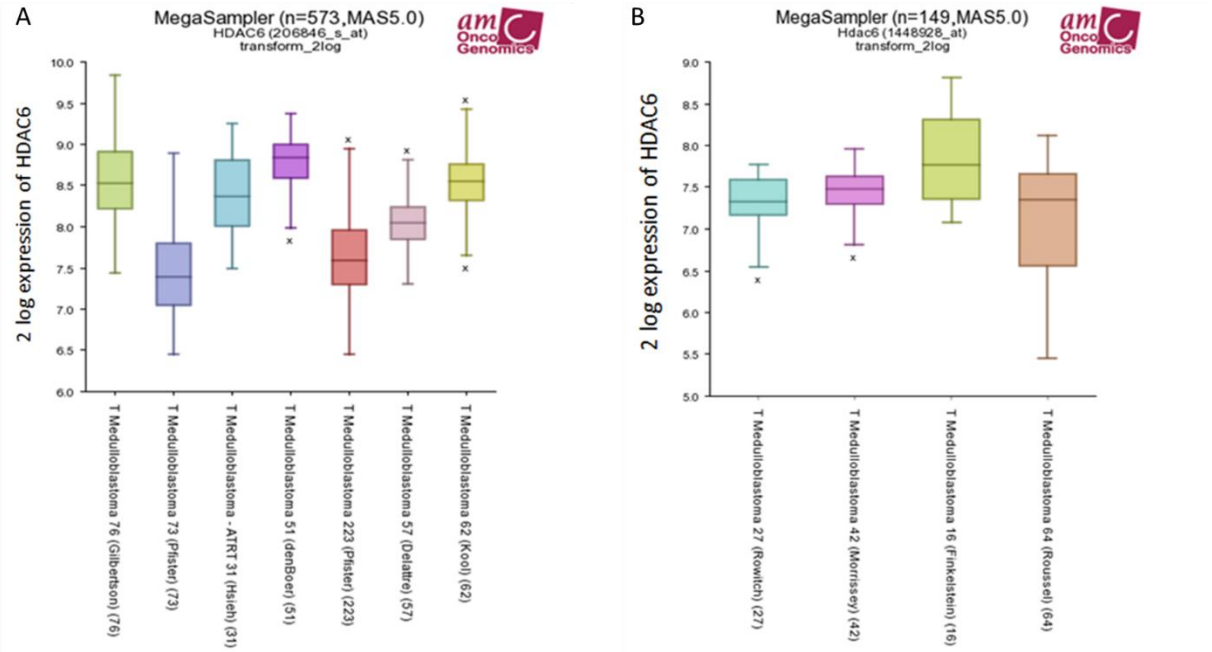
5.7 Analysis of HDAC6 expression using publicly available datasets

To further strengthen my observations and data, I took advantage of publicly available databases. For this purpose, I used R2 (http://r2.amc.nl) which is a Genomics and visualization platform developed by Jan Koster from the department of Oncogenomics, Academic Medical Center (AMC), Netherlands. Firstly, I analyzed the expression of *HDAC6* in seven different human medulloblastoma datasets deposited in R2 database; namely Kool (62 samples), delattre (57 samples), Pfister (223 samples), den Boer (51 samples), Hsieh (31 samples), Pfister (73 samples), Gilbertson (73 samples) altogether containing 570 samples (Fig.7A). Though the expression of *HDAC6* was seen in all the seven human medulloblastoma datasets, it should be mentioned that there was quite a variation between and within the datasets and a particular dataset could not be associated with higher or lower expression of HDAC6. In all the graphs, the cross mark on the top represent outlier/s at the higher end of expression and cross mark under represent outlier/s at the lower end of expression.

Next, I went on to check the expression of HDAC6 in four different mouse medulloblastoma datasets in R2 database; Roussel (64-samples), Finkelstein (16-samples), Morrissey (42-samples), Rowitch (27-samples) (Fig.7B). Also, in these four mouse medulloblastoma datasets the expression of *HDAC6* was quite variable but the highest HDAC6 expression was observed in samples from Finkelstein dataset. It was previously shown by Lee et al., (2013) that *HDAC6* expression was higher in tumor medulloblastomas (Lee et al., 2013) and also recently Dhanyamraju et al., (2015) have demonstrated higher *HDAC6* expression in SmoA1 mouse medulloblastoma model. Further, I was interested to know if *HDAC6* is expressed more in mouse or human medulloblastomas, for that I compared *HDAC6* expression between human medulloblastoma datasets (Fig.7A) and mouse medulloblastoma datasets (Fig.7B).

Among the two datasets HDAC6 expression was found to be slightly higher in human medulloblastoma datasets when compared to that of mouse medulloblastoma datasets.

After, this I was keen to know the expression of *HDAC6* in different medulloblastoma molecular subgroups (Wnt group, Shh group, Group 3 and Group 4). For this, I chose five datasets (molecular classification available only for these five datasets) based on the molecular classification of medulloblastoma, namely Gilbertson (73 samples- Fig.7C), Pfister (223-samples- Fig 7D), Northcott (103 samples- Fig 7E), Shirsat (19 samples- Fig 7F) and Cavalli (763 samples-Fig 7G). *HDAC6* expression could be seen in all the four subgroups and in all the five datasets but it was quite variable. Upon close observation, I could see *HDAC6* expression slightly higher in Group 4 belonging to Gilbertson (Fig 7C), Pfister (Fig 7D), Shirsat (Fig 7F) and Cavalli (Fig.7G) datasets but the same could not be seen in Northcott dataset (Fig 7E).



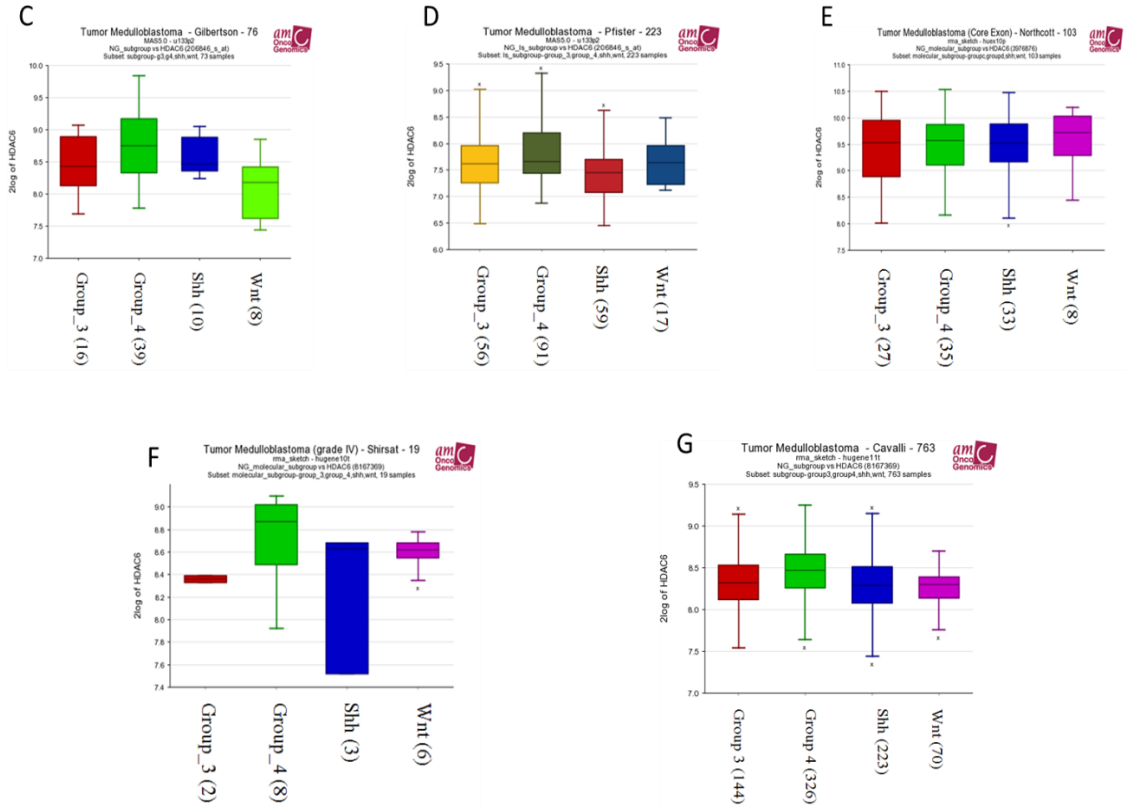


Figure 7: Analysis of HDAC6 expression using publicly available datasets. (A) HDAC6 expression in seven different human medulloblastoma datasets with a total of 573 samples (Kool-62-samples, delattre-57-samples, Pfister-223-samples, den Boer-51-samples, Hsieh-31-samples, Pfister-73-samples, Gilbertson-73-samples). (B) HDAC6 expression in four different mouse medulloblastoma datasets with a total of 149 samples (Roussel-64 samples, Finkelstein-16 samples, Morrissey-42-samples, Rowitch-27-samples). (C, D, E, F, and G) HDAC6 expression in four different medulloblastoma subtypes (Wnt group, Shh group, group3 and group4) from five different human medulloblastoma datasets (C-Gilbertson-73 samples, D-Pfister-223 samples, E-Northcott-103 samples, F-Shirsat-19 samples, and G-Cavalli-763 samples with a total of 1181 samples). Cross mark (x) on the top represent outlier/s at the higher end of expression and cross mark under represent outlier/s at the lower end of expression.

6 Discussion

HDAC6 belongs to class IIb family of HDACs. HDAC6 is predominantly localized to cytoplasm due to the presence of NES (Nuclear export signal) and SE14 motifs (C Boyault et al., 2007; de Ruijter et al., 2003). HDAC6 functions as HSP90, cortactin and α tubulin deacetylase. HDAC6 has been demonstrated to play cardinal role in formation of immune synapse, cell spreading and migration, degradation of misfolded proteins and stress granules (SG). It has also been shown that it takes part in viral infections by forming complexes with distinct protein partners (Yingxiu Li et al., 2013). The primary substrate of HDAC6 is α tubulin by which it modulates vesicular transport and microtubule dynamics (S. Chen, Owens, Makarenkova, & Edelman, 2010; Jim P Dompierre et al., 2007; Zilberman et al., 2009). HDAC6 is involved in modulation of diverse signalling pathways such as TGF-β-Notch pathway (Deskin, Lasky, Zhuang, & Shan, 2016), RAS/MAPK signaling cascade (Sakamoto & Aldana-Masangkay, 2011), ERK-MAPK signal transduction pathway among others. It has also been demonstrated that HDAC6 plays an important role in Hh signaling pathway (Dhanyamraju et al., 2015). HDAC6 plays a critical role in misfolded protein clearance by autophagy or via generation of aggresomes (Delcuve et al., 2012). Furthermore, HDAC6 plays important role as a modulator of gene transcription (P. B. Chen et al., 2013; Ozaki, Wu, Sugimoto, Nagase, & Nakagawara, 2013; Palijan et al., 2009; Westendorf et al., 2002).

Keeping in view the plenty of functions in which HDAC6 is engaged, it is startling to note that *Hdac6* null mice are healthy and viable and exhibit a subtle phenotype (Yu Zhang et al., 2008). In the present study, I observed HDAC6 to be overexpressed in Hh-initiated medulloblastoma and I speculated if HDAC6 plays a functional role in this pathway. Indeed, HDAC6 overexpression has been seen in medulloblastoma before, however no connection to the Hh signalling pathway was made. In publicly available datasets, the expression of HDAC6 in mouse medulloblastoma was also found to be high (Fig.7B). Interestingly, these authors saw no impact of Tubastatin, a known HDAC6 inhibitor on granule cell proliferation or allograft growth (Lee et al., 2013), which

considerably may be due to low concentrations used. Higher concentrations of HDAC inhibitors were required for prominent Hh inhibition in my hands pointing that full blockage of HDAC6 is needed to affect the Hh system. In spite of using higher concentrations of inhibitor, the compounds particularly inhibited HDAC6 but displayed no marked effect on histone acetylation levels. I should bring up that, my allograft system suffers from its synthetic nature and that direct compound injections can possibly lead to high local inhibitor concentrations, still I tried to bypass the issue by altering the injection sites and by not directly injecting into the tumour. It is vital to specify here that modern HDAC6 inhibitors can pass through the blood-brain barrier and consequently be tested under more physiological settings in autochthonous medulloblastoma mouse models in future (Jochems et al., 2014). Interestingly, these modern HDAC6 inhibitors have been shown to carry antidepressive characteristics, an aspect that have been linked to Hh inhibition in the past (Lauth et al., 2010).

Altogether, my data provides insight into HDAC6 and its role in Hh signalling, on one hand HDAC6 is needed for full pathway activation. Secondly, HDAC6 is required for complete repression of target gene expression under basal Hh signalling levels in the absence of ligands. This complicated interaction between negative and positive functions may explain the subtle phenotype in *Hdac6* knockout mice. It has been demonstrated by Zhang et al (2008) that mice deficient in *Hdac6* are normal, fertile and viable. Increase in global tubulin acetylation was found in these mice. *Hdac6* knock out mice displayed slight increase in bone mineral density and mild defective immune response. Moreover, in differentiation assays using C3H10T1/2 cells and in reporter assays with ShhL2 cells induction of ligand-independent Hh signalling was not seen indicating either cell type difference or that the HDAC6 activated derepression of Hh target genes is not adequately strong enough for some biological processes to occur.

However, it is important to point out that in the allograft model, mice getting Vismodegib a SMO antagonist presented with alopecia (hair loss) at the cutaneous injection sites (Supplementary Fig. S6). Indeed, this is not surprising as Hh signalling is well known to

promote hair growth. The hair loss observed in mice treated with Vismodegib was not seen in the ACY-1215 treated group, regardless of the fact that the Hh pathway was blocked. Analysis of microarray data revealed that about half of the SMO/GLI-regulated genes are also influenced by HDAC6 and one of the probable reason for no hair loss might be that HDAC6 does not influence or play role in affecting the Hh target genes needed in hair cell biology. The said observation needs further investigation as it might be used to prevent undesirable side effects related to blocking of Hh-dependent physiological mechanisms. Further, an unanswered question at present is if HDAC6 inhibition influences SMO-dependent, but GLI-independent noncanonical signalling (Bijlsma, Damhofer, & Roelink, 2012; Chinchilla, Xiao, Kazanietz, & Riobo, 2010). I speculate that the effect of HDAC6 on Hh signalling might be a result of acetylation of tubulin and is not due to direct deacetylation of GLI proteins by HDAC6.

A number of tubulin post translational modification have been demonstrated which include acetylation, phosphorylation, polyglycylation, polyglutamylation, polyamination, detyrosination and deglutamylation (Janke, 2014). For example, it has been shown that HDAC6 is involved in deacetylation at Lys-40 of α-tubulin (Hubbert et al., 2002). Recently, Liu et.al, (2015) mapped lysine acetylation sites on tubulin by a mass spectrometry (MS) method. They compared acetylated peptides from HDAC6 knockout mice and wild-type mice and identified six new deacetylation sites on α-tubulin (K370, K60 and K394) and β-tubulin (K58, K154 and K103) which are likely carried by HDAC6 thereby adding new list of sites deacetylated by HDAC6. This would further strengthen our knowledge of how HDAC6 is able to regulate multiple cellular functions and microtubule stability.(N. Liu et al., 2015).

Tubulin post translational modifications have been shown to influence microtubule based motor protein-driven transport. As Hh signalling depends very much on IFT transport toward and from the primary cilia (Corbit et al., 2005; Dorn, Hughes, & Rohatgi, 2012; D Huangfu et al., 2003), meddling with these mechanisms may, consecutively, inhibit the specifically coordinated Hh signalling. Intrinsically, sustained and elevated levels of tubulin acetylation may functionally look like defects in cilia like those normally induced

by loss-of- function of IFT components (Loss of important IFT components like Ift88 and Kif3a which are involved in IFT transport and involved in ciliogenesis lead to elevated levels of post translational modifications including hyper-acetylation of microtubules, high levels of α-tubulin acetyl-transferase activity and altered microtubule stability (Berbari et al., 2013)). In fact, certain aspects of my HDAC6 related data shown here, such as ligand-independent activation of pathway and reduced GLI3R levels are consistent with previously reported defects on cilia (Cervantes, Lau, Cano, Borromeo-Austin, & Hebrok, 2010; Han et al., 2009; Haycraft et al., 2005; Ho et al., 2013; Wong et al., 2009). Shi et al., (2015) showed that centrosomal protein of 70kDa (Cep70) is involved in stability of microtubules by increasing acetylation of tubulin and via interacting with HDAC6. Cep70 interacts with HDAC6 in cytoplasm and colocalizes with it. Using pulldown experiments and immunoprecipitation they demonstrated that Cep70 interacts with HDAC6 physically via the amino terminal peptide fragment that accommodates the coiled-coil domains and the amino terminal Cep70 fragment is needed for regulating tubulin acetylation. They also reason that Cep70 by binding to deacetylase domains of HDAC6 might inhibit the deacetylase activity thereby increasing the levels of acetylation of tubulin and stability of microtubules. It would therefore be of interest to know if Cep70 is involved in ciliogenesis via regulating HDAC6 or via modulating acetylation of tubulin (Shi et al., 2015).In *Sufu*$^{-/-}$ cells, in which signalling is independent of cilium (M. H. Chen et al., 2009; Jinping Jia et al., 2009), inhibition of HDAC6 negatively impacts Hh pathway, pointing that other intracellular transport mechanisms perhaps are needed downstream of primary cilia and SMO. An alternative picture would be that the nuclear HDAC6 fraction may impinge on transcriptional complexes controlling Hh target gene expression, such as Gli2 transcription. Principally, I could show the specificity of the compounds for HDAC6 at multiple instances in my work. Firstly, induction of Hh pathway with the help of SAG (which is smoothened agonist) in ShhL2 could be blocked by using HDAC6 specific inhibitors namely ACY-1215, Tubacin and CAY-10603. The blockage of Hh signaling with all three inhibitors points at the specificity of these inhibitors and rule against *off-target* effects (see figure 2B). I go on to show in a physiologic process driven by Hh, differentiation of mesenchymal progenitor cells; C3H10T1/2 cells into osteogenic lineage by SAG could be inhibited by usage of Tubacin and CAY-10603 (see figure 2C and D). Interestingly, SMO activated cell

differentiation was blocked by pan HDAC inhibitor TSA but also led to an increase in basal induction of Alkaline phosphatase activity (see figure 2D) suggesting that nuclear HDAC family members suppress basal Hh signaling and further highlights the precision of the HDAC6 inhibitors used in the study. Further, I used MEF[SHH] cells which stimulate Hh signaling pathway at the ligand step. In these cells Hh signaling pathway could be inhibited by using different HDAC6 antagonists (Tubacin, ACY-1215, CAY-10603) which could be determined by measuring Hh target genes (*Gli1, Ptch1* and *Ptch2*) at the mRNA level (see figure 3A) and at the level of protein (GLI1, HIP1) (see figure 3B). Once more, neither of the used HDAC6 inhibitors led to increase in the levels of acetylated histone H3 (see figure 3B and supplementary figure S2G) in comparison to TSA arguing for HDAC6-selective mechanisms. Further, I used MEF[Smo*] which expresses dominant active version of Smo (see figure 3C) and Ptch1$^{-/-}$ MEFs (see figure S3C) in which blockade of endogenous HDAC6 activity by ACY-1215, CAY-10603 and tubacin led to decrease in Hh signaling pathway which was determined by measuring the target gene levels of *Gli1, Ptch1* and *Ptch2* in MEF[Smo*] and *Gli1, Ptch2* and *Hip1*in Ptch1$^{-/-}$ MEFs. Additionally, in *Sufu*$^{-/-}$MEFs which signal constitutively independent of ligand and receptor HDAC6 inhibition with ACY-1215, Tubacin and CAY-10603 led to inhibition of Hh target gene expression namely *Gli1*, *Ptch1*and *Ptch2* (see figure 3D). Lastly, I used MEF[Gli2dN] cells which stably express low amounts of an activated mutant of GLI2, the principal transcription factor determining the terminal steps in Hh signaling pathway. Even in these cells HDAC6 inhibition with ACY-1215, Tubacin and CAY-10603 led to inhibition of Hh target genes; *Gli1*, *Ptch1* and *Ptch2* (see figure 3E). In conclusion, I could show the specificity of HDAC6 inhibitors in different cell lines and the effect of HDAC6 inhibitors on Hh signaling pathway. Recently, it has been demonstrated that at immunologic synapse cilia-like processes play important role (de la Roche et al., 2013), a cellular structure that had been linked to HDAC6 previously (Serrador et al., 2004). However, additional data is required to show the function of HDAC6 in immunologic synapse-triggered Hh signalling.

In several tumours HDAC6 has been shown to possess oncogenic activity (Aldana-Masangkay et al., 2011; Gradilone et al., 2013; Jochems et al., 2014). Due to these findings and the need of HDAC6 to obtain maximal Hh signalling, I investigated the *in*

vivo effect of an HDAC6-based therapy in an allograft model of medulloblastoma. Blockade of HDAC6 displayed marked outcome with respect to survival of medulloblastoma cells and was as potent as Vismodegib (positive control) in vivo. Considering that ACY-1215 (not with Vismodegib) led to extensive and robust apoptosis, prolonged exposure to the treatment might even lead to eradication of tumor completely.

It should be mentioned here that ACY-1215 (ricolinostat) the HDAC6 specific inhibitor is presently under second phase of clinical trials for treatment of patients with lymphoid malignancies and multiple myeloma. Apart from ACY-1215, ACY-241 which is also a HDAC6 specific inhibitor is into phase-I clinical trials for treatment of multiple myeloma (S.N.Batchu, et al., 2016). The advantage of ACY-1215 over other known inhibitors is that it is less toxic to T-cells and mononuclear cells in blood (Santo et al., 2012). Most of the knowledge on ACY-1215 has been from combination therapy where it is used along with inhibitors of proteasome like bortezomib (Amengual et al., 2015; Dasmahapatra et al., 2014; Mishima et al., 2015; Santo et al., 2012). The complication involved with proteasome inhibition is it leads to misfolded protein accumulation in aggresomes and it is speculated that blockage of aggresome formation via inhibition of HDAC6 may bring about more synergistic benefit together with inhibition of proteasome (S. N. Batchu et al., 2016). In a mouse model of multiple myeloma, combination therapy consisting of ACY-1215 and bortezomib improved survival and delayed growth of tumor (Santo et al., 2012). The same effect was also seen in a diffuse large B-cell lymphoma xenograft model (Amengual et al., 2015). In another study, ACY-1215 along with carfilzomib (proteasome inhibitor) inhibited formation of aggresomes and led to increased apoptosis in multiple myeloma cells (Mishima et al., 2015). It has also been shown that, for the treatment of multiple myeloma ACY-1215 has been used together with immunomodulatory drugs (IMiDs) (T Hideshima et al., 2015). Two immunomodulatory drugs pomalidomide and lenalidomide are under clinical evaluation. In the same lines, it has been demonstrated that ACY-241 is presently undergoing clinical evaluation and is in phase I a/b for treatment of refractory multiple myeloma. It is used as a monotherapy or as a combination therapy with low-dose dexamethasone or pomalidomide (S. N. Batchu et al., 2016).

Though the most prominent HDAC6 inhibitors have been Tubastatin A, ACY-1215 and Tubacin, several other inhibitors of HDAC6 have also been developed like hydroxamic acids having aryl alanine which are effective at very low micromolar concentrations (Schäfer et al., 2008, 2009). Lately, many different molecules based on thilolate (Itoh et al., 2007), mercaptoacetamide (Kozikowski et al., 2007), sulfamide (Wahhab et al., 2009), and trithiocarbonate (Dehmel et al., 2008) are being developed which can specifically inhibit HDAC6. The major issue with HDAC inhibitors is most of the inhibitors are built on common structural basis hence have broader activity and not very specific for a particular HDAC. Inks et al (2012) screened a library containing 1280 compounds and identified five novel molecules having HDAC inhibitory activities. One of the identified molecules was NSC-95397 which was selective for HDAC6. Based on the structure of NSC-95397 several daughter compounds were synthesized and one such compound was NQN-1 which inhibited HDAC6 at 5.5μM concentration and had minimum inhibitory effect on other HDACs (Inks, Josey, Jesinkey, & Chou, 2012).

Keeping in view the diverse biological roles played by HDAC6 it is not unexpected that it is involved in various diseases like neurodegenerative diseases, cardiovascular, kidney diseases and mood disorders among others (S. N. Batchu et al., 2016). For example, it has been demonstrated in a neurodegenerative disease model of *Drosophila*, spinal bulbar muscular atrophy (SBMA), when ubiquitin-proteasome system (UPS) was impaired, HDAC6 aided in compensatory autophagy (Pandey, Nie, et al., 2007).

HDAC6 has also been shown to play a role in Parkinson's disease (PD). Loss of cells producing dopamine and accumulation of Lewy bodies (abnormal aggregates of protein) which are mainly composed of α-synuclein protein are the characteristics of Parkinson's disease. HDAC6 in *Drosophila* assists in formation of inclusion bodies and secures dopaminergic neurons from the damaging effects of α-synuclein (Du et al., 2010) and lewy bodies are highly enriched for HDAC6 in brain section of patients suffering from PD (Kawaguchi et al., 2003). Taken together these data indicate that upregulation of HDAC6 in PD might be a protective response indirectly implying that inhibition of HDAC6 therapeutically may slow down disease progression (Yan, 2014).

Huntington's disease (HD) is another neurological disease in which HDAC6 has been shown to play a role. HD is caused due to expansion of glutamine repeat sequence in the huntingtin gene, this also leads to protein aggregation and misfolding of protein (Hatters, 2008). It has been shown that the resultant mutant protein incites neuronal toxicity via destabilization of microtubules (Trushina et al., 2003). Inhibition of HDAC6 in HD might lead to increase in α-tubulin acetylation and thereby improved neuronal transport (J. P. Dompierre et al., 2007; Guedes-Dias et al., 2015). Charcot-Marie-Tooth disease (CMT) is another example where inhibition of HDAC6 have shown positive results. Progressive muscle wasting and loss of sensation are the characteristics of CMT. In one of the subtypes of CMT, known as CMT2 missense mutations in gene coding for heat shock protein 27 has been shown (HSPB1) (Evgrafov et al., 2004) which leads to reduced α-tubulin acetylation and axonal transport defects upsetting peripheral nerves. Treatment of mice having mutations in HSP27 with Tubastatin-A reversed the axonal transport defects (d'Ydewalle et al., 2011).

The role of HDAC6 in Alzheimer's disease (AD) is being studied comprehensively (Ling Zhang, Sheng, & Qin, 2013). The most important character of AD is the generation of neurofibrillary tangles that are composed of aggregates of hyper-phosphorylated Tau protein (Mandelkow & Mandelkow, 1998). The aggregation and phosphorylation of Tau protein into aggresomes is principally dependent on SE14 of HDAC6 and Tau microtubule binding domain (Ding, Dolan, & Johnson, 2008). It has been reported that Tau is HSP90's client protein (Karagöz et al., 2014) and the levels of HDAC6 correspond to tau protein burden and a reduction in the expression levels of HDAC6 favoring tau protein clearance possibly through inducing acetylation of HSP90 (Cook et al., 2012). HDAC6 null mutation in *Drosophila* salvaged tau induced defects of microtubule (Xiong et al., 2013). Apart from this the levels of HDAC6 were high in postmortem brain tissue samples from Alzheimer patients (Ding et al., 2008). Recently, two different groups have demonstrated cognition improvement in mouse model of Alzheimer's disease upon inhibition of HDAC6 (Selenica et al., 2014; Ling Zhang et al., 2014). Though a number of studies show HDAC6's role in Alzheimers disease, its precise role is not yet clear (Sri N Batchu et al., 2016). Apart from neurodegenerative diseases HDAC6 has also been

shown to be associated with mood disorders. For example, it has been demonstrated that functional loss of HDAC6 in rodents has antidepressant kind of effect (Espallergues et al., 2012; Fukada et al., 2012; J. B. Lee et al., 2012). One of the plausible reason for this might be that HDAC6 is involved in negotiation of glucocorticoid receptor and HSP90, inhibition of HDAC6 or knockdown inhibits receptor translocation to the nucleus in neuronal cells (Espallergues et al., 2012). Recently, two specific and novel brain penetrating HDAC6 inhibitors ACY-775 and ACY-738 were demonstrated to exhibit exploration-enhancing effects in mice (Jochems et al., 2014).

It has been shown that HDAC6 is involved in cystic diseases in both kidney (Mergen et al., 2013) and liver (Gradilone et al., 2014). For example, in polycystic kidney disease (PKD), PKD1 mutations lead to HDAC6 upregulation which ultimately leads to growth of cysts. Tubacin treatment inhibited proliferation of cystic cells, reduced the levels of cyclic AMP and activated cystic fibrosis transmembrane conductance regulator (CFTR) chloride currents in Madin darby canine kidney cells (MDCK). Therefore, HDAC6 serves as a promising target in PKD (Cebotaru et al., 2016).

Systemic lupus erythematosus (SLE) is an autoimmune disease and it has been demonstrated that inhibition of HDAC6 leads to reduction of SLE by inhibiting immune complex negotiated glomerulonephritis, inflammatory cytokine production and by enhancing splenic Tregs (T-regulatory) cells. Furthermore, inhibition of HDAC6 led to increase in early pre and pro B cell percentage indicating that manipulation of HDAC6 leads to decrease in SLE disease status by altering differentiation of abnormal B and T cells (Regna et al., 2016)

The role of HDAC6 in relationship with various cancers have been widely studied. It has been shown that HDAC6 is upregulated in many type of cancers (Bazzaro et al., 2008; Bradbury et al., 2005; Saji et al., 2005; Sakuma et al., 2006; Z. Zhang et al., 2004). Role of HDAC6 and its role in formation of aggresome and its relationship to cancer has been deeply investigated. Cancer cells gather misfolded protein at a much rapid rate than non-cancerous cells, for survival of these cancer cells disposal of misfolded proteins via the

aggresome or UPS pathway is needed (Rodriguez-Gonzalez et al., 2008). Inhibition of UPS with the help of proteasome inhibitors and the aggresome pathway with the help of HDAC6 inhibitors might prevent misfolded protein disposal thereby harmful to cancerous cells (Teru Hideshima et al., 2005). It was demonstrated by Lee et al., (2008) that HDAC6 is upregulated in cells that are transformed by Ras oncogene. They also showed that HDAC6-deficient mice and cells are resistant to Ras directed oncogenesis indicating that HDAC6 helps in Ras activation and downstream pathways like MAPK and PI3K pathways. The authors further went on to show that HDAC6 is needed for cancer cells to gain the capacity to resist anoikis thereby assisting in tumor metastasis and invasion (Geiger & Peeper, 2007; Y. S. Lee et al., 2008a).

Cortactin is a well-known substrate of HDAC6 (X. Zhang et al., 2007). Upregulation of Cortactin has been shown in many cancers and it plays cardinal role in tumor invasiveness by aiding cell motility (Weaver, 2008). It aids in cancer cell motility via formation of invadopodia and involved in degradation of extracellular matrix (Castro-Castro, Janke, Montagnac, Paul-Gilloteaux, & Chavrier, 2012; Rey, Irondelle, Waharte, Lizarraga, & Chavrier, 2011; Weaver, 2008).

In several studies, HDAC6 has been demonstrated to be involved in tumor cell invasion and formation of cortactin based invadopodia (Arsenault, Brochu-Gaudreau, Charbonneau, & Dubois, 2013; Castro-Castro et al., 2012; Rey et al., 2011). It was demonstrated by Dhanyamraju et al., (2015) that in SHH driven medulloblastoma, endogenous levels of HDAC6 expression was high and HDAC6 is involved in modulation of Hh signaling. Pharmacological inhibition of HDAC6 led to reduction in growth of tumor in an allograft model and they propose HDAC6 as a unique and potential drug target for treatment of Shh driven medulloblastoma (Dhanyamraju et al.,2015). Recently it was shown that expression levels of deacetylases HDAC1 and HDAC2 were elevated in SHH driven medulloblastoma (Canettieri et al., 2010; Coni et al., 2017). Specific inhibition of these two deacetylases by genetic knock down or by pharmacological means leads to positive outcome in mouse models of SHH driven medulloblastoma. They go on to show that, mocetinostat (MGCD0103) which is specific inhibitor of HDAC1/2 is also a powerful Hh inhibitor and brings about its effect by acetylating Gli1 at lysine 518.

Treating SHH-MB mouse models with mocetinostat leads to slow down of tumor growth by increasing tumor cell apoptosis and reduction in proliferation thereby prolonging survival of mouse. They also propose that inhibition of HDAC1/2 along with HDAC6 inhibition (Dhanyamraju et al., 2015) in SHH driven MB may bring more synergistic effects (Coni et al., 2017b). Apart from these, the role of HDAC6 has also been shown in breast and ovarian cancers (Sri N Batchu et al., 2016) and modulation of HDAC6 can be helpful in disease prognosis. HDAC6 also has been demonstrated to play role in platelet biology (Sadoul et al., 2012) and inhibition of HDAC6 leads to improvement in cardiac function in elderly people (Ferguson & McKinsey, 2015).

Keeping in view, such diverse roles played by HDAC6 in various biological and pathobiological processes it is important to modulate its function. Pharmacological modulation of HDAC6 is one of the options that can be useful. Though the functions of HDAC6 could be modulated by pharmacological means there are limited options and further research into HDAC6 modulators with higher specificity and effectivity at lower concentrations should be the subject of future studies. Importantly, I was able to establish an important role played by HDAC6 in modulating mammalian Hh signaling thereby making HDAC6 a unique therapeutic target for treating Hh- directed malignancies.

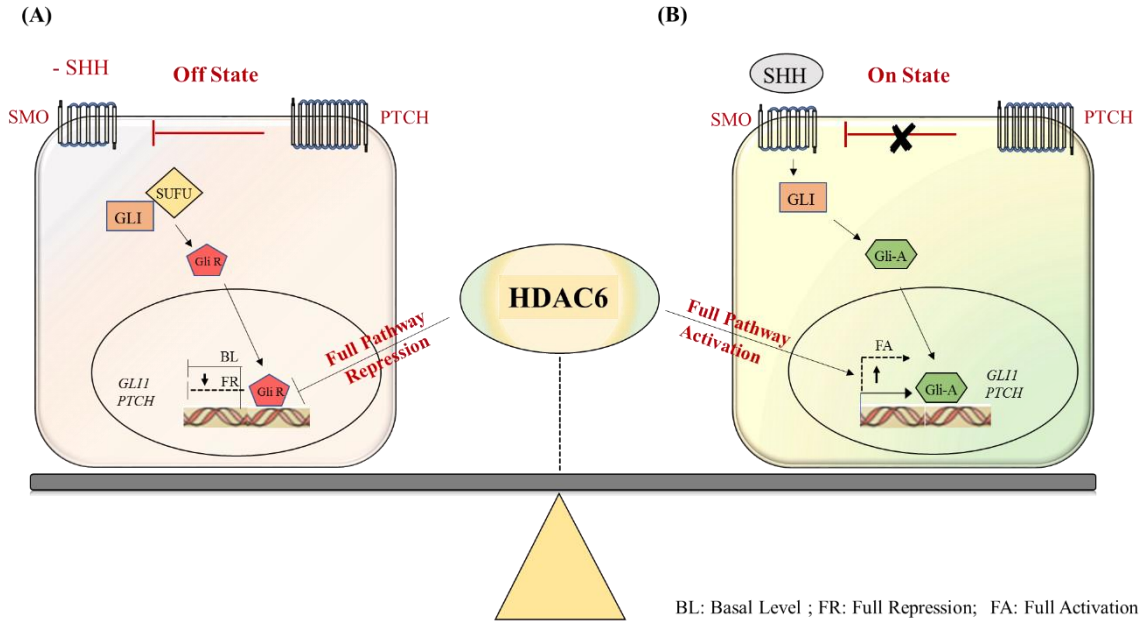


Figure I: Dual role of HDAC6 in Hedgehog signaling: (A) When the ligands are absent; basal Hh signaling is completely repressed by the help of HDAC6. (B) When the ligands are present; HDAC6 is needed for activation of full pathway.

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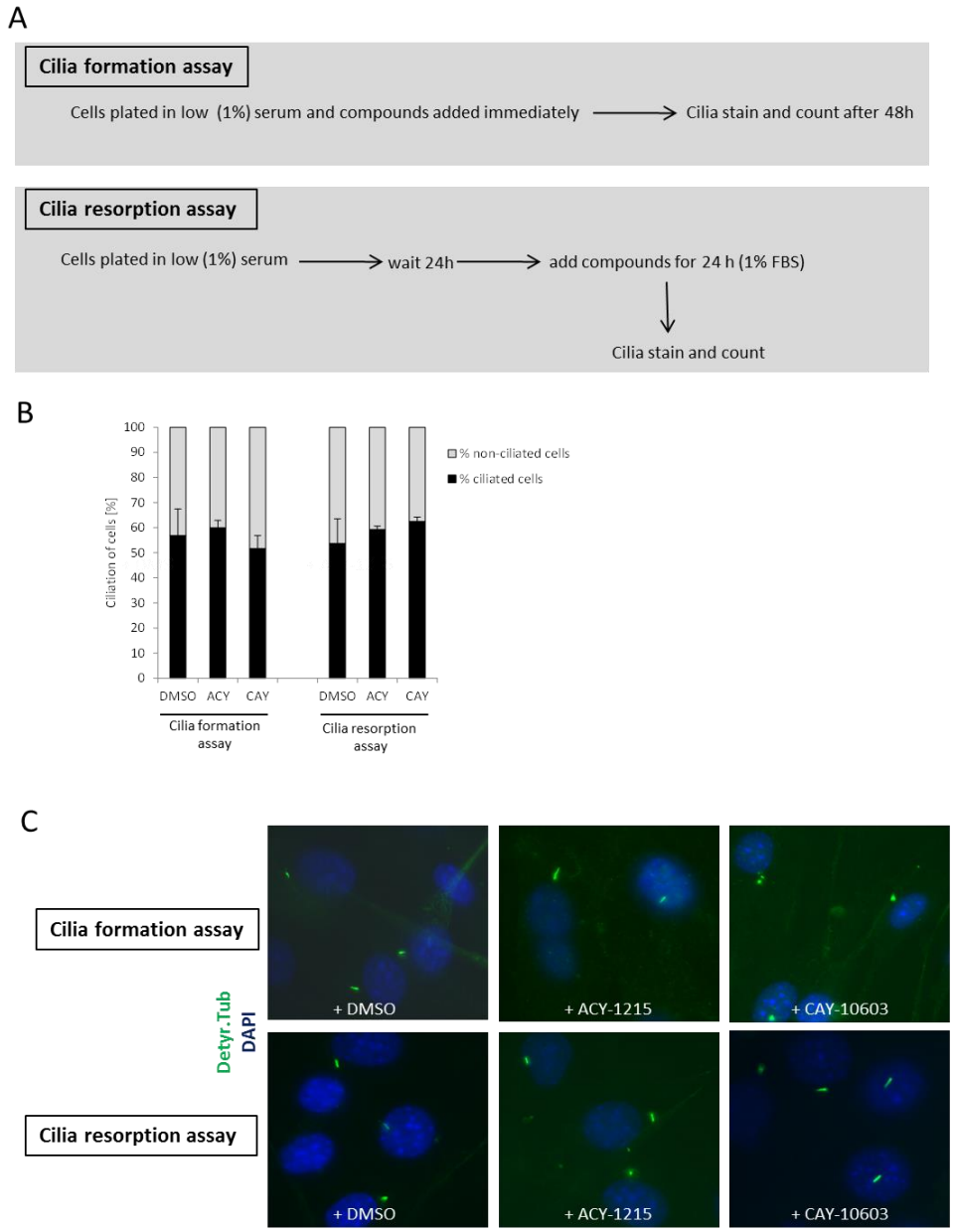
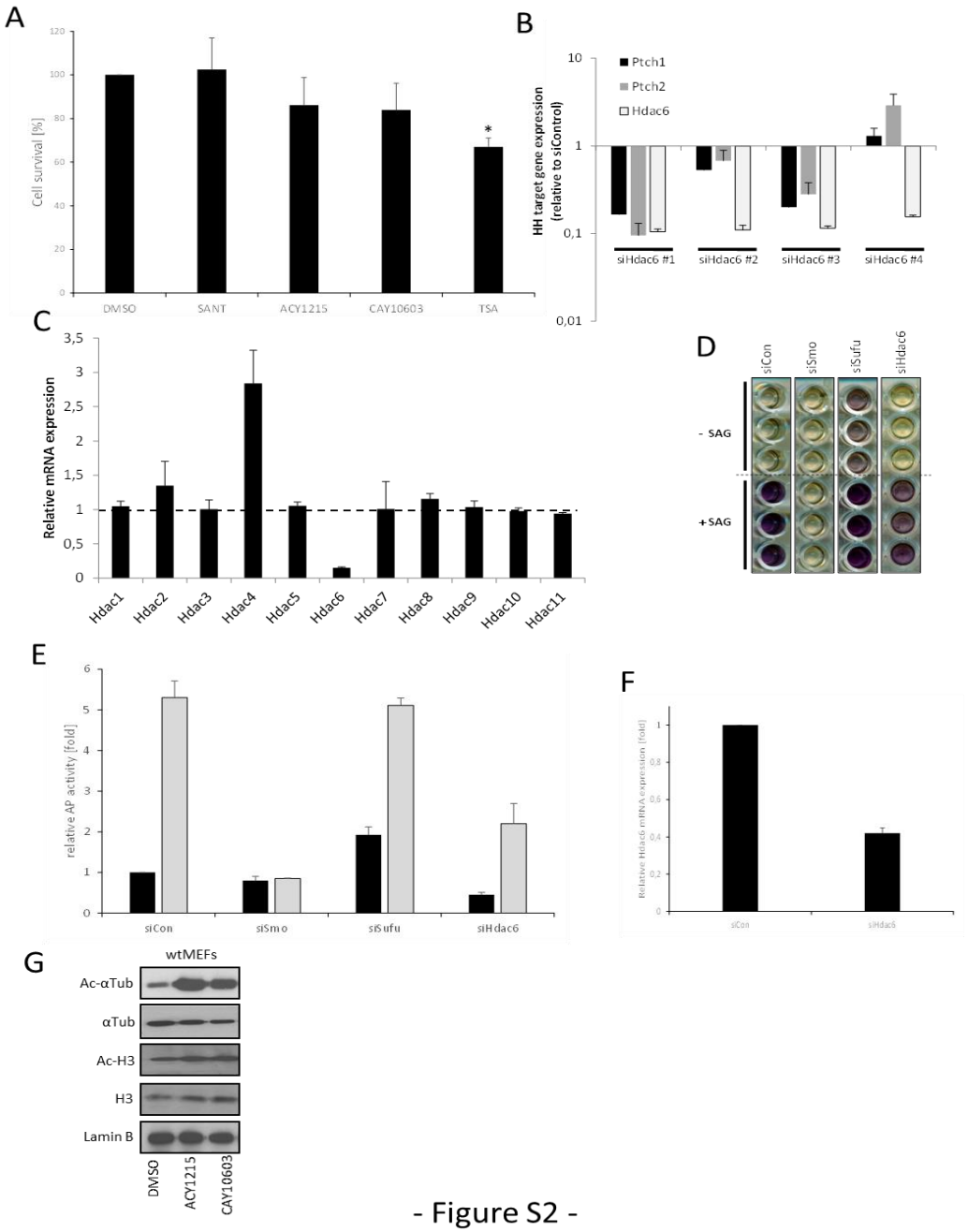
8 Appendix

8.1 Supplementary figures



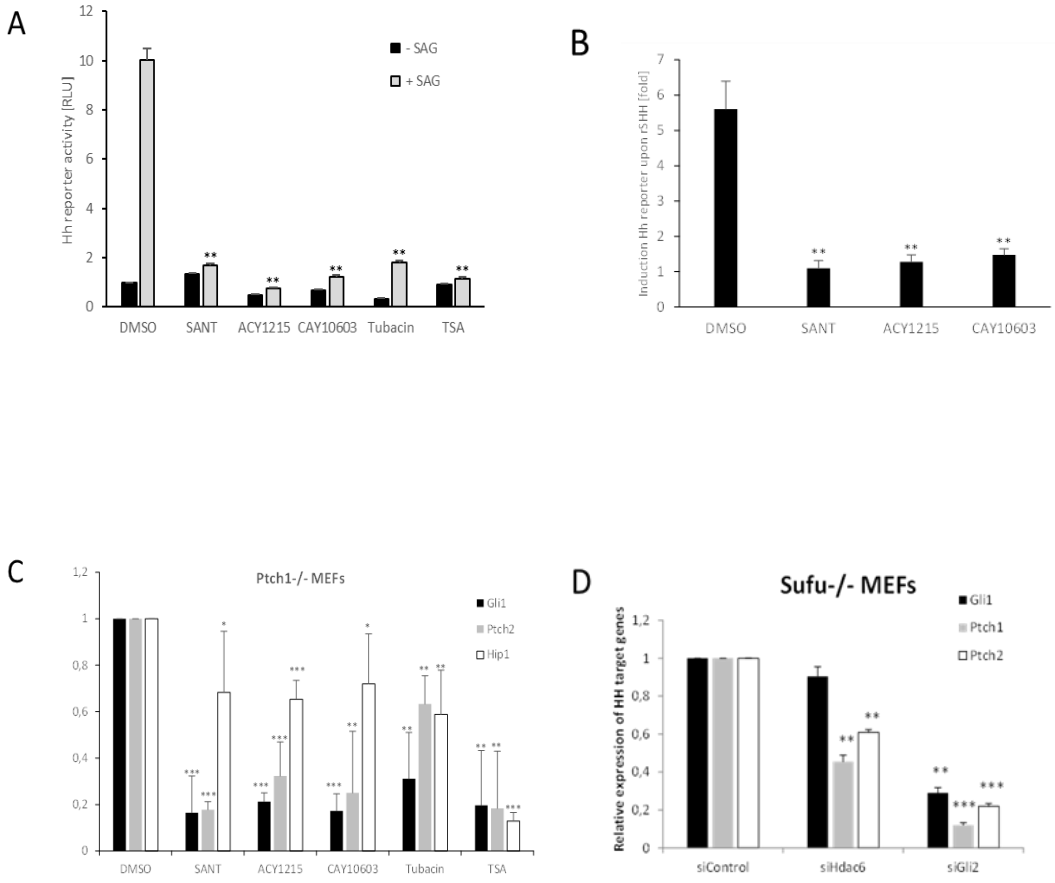
- Figure S1-

Figure S1: Lack of significant cilia effects due to blockade of HDAC6 (A) Experimental description of two approaches performed. The 'Cilia formation assay' analyzed inhibition of HDAC6 effects during formation of primary cilia. The 'cilia resorption assay' investigated whether blockade of HDAC6 resulted in loss (resorption) of pre-established primary cilia. (B) Primary cilia quantification as determined by detyrosinated tubulin staining in treated ShhL2 cells. The mean of three independent experiment is shown with at least 100 counted cells in each experiment. Compound conc. as in (B). (C) Representative micrographs of treated ShhL2 cells as shown in (C). Green: Detyrosinated tubulin; blue: DAPI,



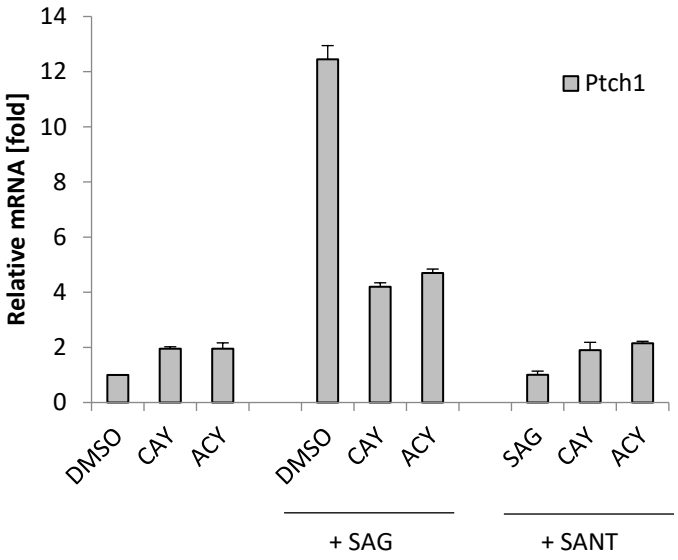
- Figure S2 -

Figure S2: *Hdac6* siRNA sequences validation (A) Cell titer assay measuring cytotoxicity of the inhibitors used in this study. NIH3T3 cells were grown in full growth medium for 2 days with the indicated compounds. Only TSA (pan-HDAC inhibitor) led to a significant decrease in viable cells. (B) Targeting hdac6 in MEF$^{[SHH]}$ cells via four single siRNAs (contained in the SMARTpool) and its validation. Out of the four siRNAs three result in Hh pathway repression as measured by a reduction in Hh target gene expression (*Ptch1, Ptch2*). I speculate some degree of *off*-target activity with respect to the fourth RNAi sequence which counteracts the expected reduction in Hh target gene expression. Alternatively, the efficiency of knock-down might be insufficient for inhibitory effects on Hh target genes. (C) Transfection of si Hdac6(pool) in MEF$^{[SHH]}$ cells and its effects on expression levels of other HDAC family members. (D) C3H10T1/2 cells transfected with indicated pools of siRNA sequences followed by SAG induction, figure shows Alkaline phosphatase staining in blue. (E) Experimental quantification of (D). AP= Alkaline phosphatase. (F) Knock-down efficiency of Hdac6 mRNA via RNAi in the cells shown in (D) and (E). (G) Wildtype MEFs treated for 48h with 10 µM ACY-1215 or 4 µM CAY-10603 shows increased tubulin acetylation (Ac-αTub), but has no effect on acetylation of nuclear histone H3.



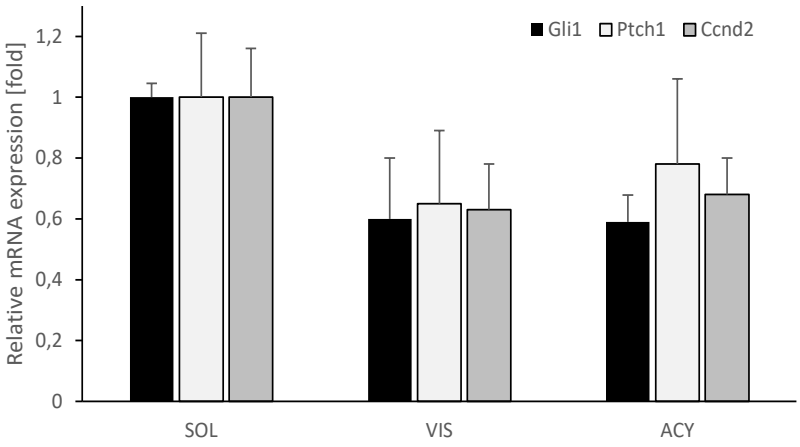
- Figure S3 -

Figure S3: Reduction in Hh signaling due to Hdac6-specific inhibition (A) ShhL2 were treated for 48h with the indicated compounds in low serum conditions (1% FBS). SANT: 0.2 µM; ACY-1215: 10 µM; CAY-10603: 4 µM; Tubacin: 20 µM; TSA: 0.5 µM. (B) ShhL2 cells were treated for 48h with the indicated compounds in the presence/absence of recombinant human SHH ligand (0.4µg/ml final conc. of SHH C24II), R&D Systems, #1845-SH). Fold induction shown by SHH over the corresponding sample without SHH. Concentrations of compound as in (A). (C) Expression of Hh target genes in *Ptch1*$^{-/-}$ MEFs as measured by qPCR. Treatment time was 2days. Concentrations of compound as in (A). (D) Expression of Hh target genes in *Sufu*$^{-/-}$ MEFs transfected with siRNA against *Hdac6 or Gli2* (positive control)



-Figure S4-

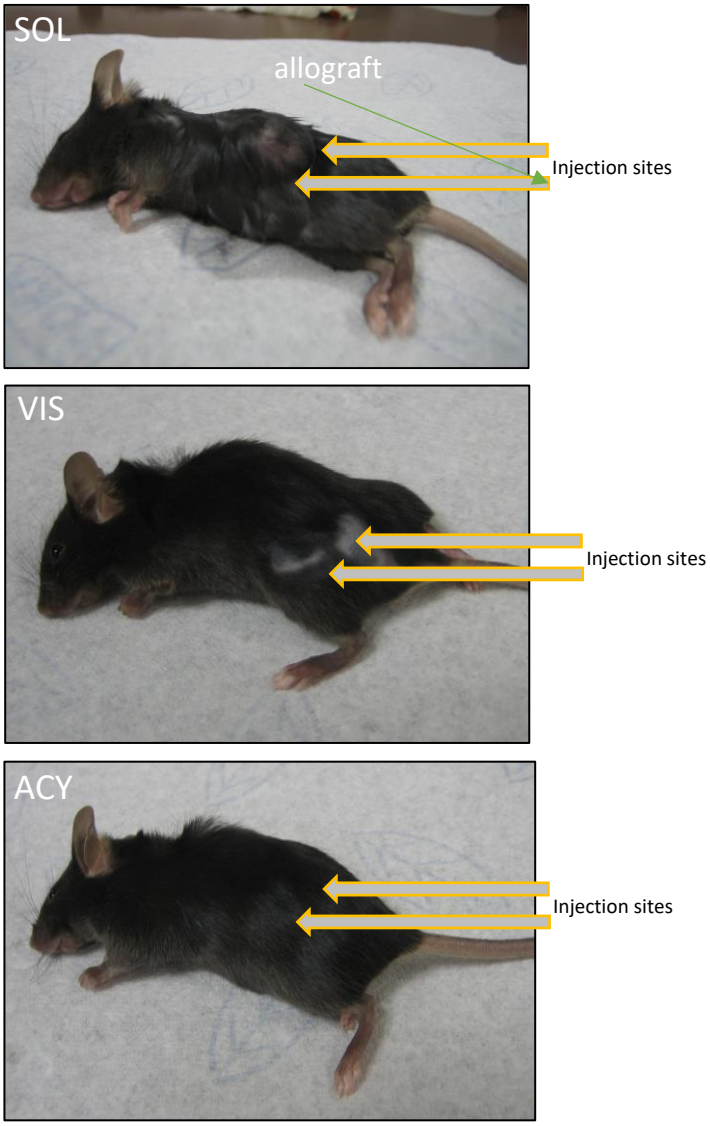
Figure S4: Inhibition of HDAC6 and its dichotomy on Hh target gene expression (A) Expression of *Ptch1* mRNA in wildtype MEFs treated with DMSO, ACY-1215 (10 µM) or CAY-10603 (4 µM) for 48 h. Note that *Ptch1* expression is increased with HDAC6 inhibitors even in the presence of SMO antagonist SANT. However, maximum SAG induction is reduced by HDAC6 inhibition.



- Figure S5-

Figure S5: Inhibition of HDAC6 *in vivo* and its effect on *Gli1* expression (A) Analysis of Hh target gene expression via quantitative real time PCR in allograft tumors taken on 12th day. SOL: Solvent; VIS: Vismodegib; ACY: ACY-1215. mRNA expression of *Ptch1* is reduced but is not statistically significant.

A



- Figure S6 -

Figure S6: Alopecia phenotype in treated mice (A) Alopecia is seen in Vismodegib (VIS) treated allograft mice (picture taken on 12th day). Note the hair loss is not seen in solvent (SOL)- or in ACY-1215 (ACY) treated mice.

8.2 Curriculum Vitae

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Education

High School major in Biology: 1997-1999
Bachelor's in Microbiology: 1999-2002
Master's in Biotechnology: 2002-2004
Diploma in Bioinformatics: 2004-2005
Master's in Molecular Biology: 2005-2007
Master's thesis: "Characterization of peroxisomal Endorphin Localization"
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Doctoral dissertation at the Institute of Molecular Biology and Tumour Research, Centre for Tumor and Immunobiology/Zentrum für Tumor und Immunbiologie
2013 (Jan) -2017 (November)- Philipps University Marburg, Germany
(Hedgehog Signal Transduction Group - PD.Dr. Matthias Lauth)

8.3 List of Academic teachers

My academic teachers/lecturers/mentors were the ladies and gentlemen from University of Skövde, Sweden, Medical university of Vienna, Austria and Kakatiya University, India. From Sweden- Prof. Kjell-Ove Holmström, Prof.Mikael Ejdebäck, Prof.Patric Nilsson, Prof.Abul Mandal, Dr. Anna-Karin Pernestig, Dr. Maria Svensson. From Austria-Prof.Johannes Berger and Dr. Markus Kunze. From India- Prof. K. Subash, Prof. A. Sadanandam, Prof. N. Rama Swamy, Prof. S. Girisham, Prof. Singaracharya, Prof. S.M. Reddy, Dr. T. Christopher, Dr. A. Venkateshwar.Rao.

8.4 Publications

DYRK1B blocks canonical and promotes non-canonical Hedgehog signalling through activation of the mTOR/AKT pathway. Singh R, Dhanyamraju PK, Lauth M. Oncotarget. 2016 Nov 26.

Corrigendum: Identification of a novel actin-dependent signal transducing module allows for the targeted degradation of GLI1. Schneider P, Bayo-Fina JM, Singh R, Dhanyamraju PK, Holz P, Baier A, Fendrich V, Ramaswamy A, Baumeister S, Martinez ED, Lauth M. Nat Commun. 2015 Oct.

Histone deacetylase 6 represents a novel drug target in the oncogenic Hedgehog signalling pathway. Dhanyamraju PK, Holz PS, Finkernagel F, Fendrich V, Lauth M. Mol Cancer Ther. 2015 Mar.

The Yes-associated protein controls the cell density regulation of Hedgehog signaling. Tariki M, Dhanyamraju PK, Fendrich V, Borggrefe T, Feldmann G, Lauth M Oncogenesis. 2014 Aug.

8.5 Acknowledgements

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I would like to take this opportunity to especially thank my entire family. I owe each and every bit of my life to my mother who stood by me and supported me throughout difficult times. You mean everything to me and I thank almighty god for bestowing me with a mother like you. Words cannot express how grateful I am to my sister (Vasavi) and my brother-in-law (Venkata) for encouraging and supporting me for everything. Never to forget all the inputs that you gave me time to time which kept me grounded. My love to my nieces Keerti and Kavya for cheering me up making me laugh during times of stress. I would also like to thank my dear wife (Suneetha) and my daughter Kritika for making life easy for me and for standing by me during difficult times. I appreciate all the help and love that you showered on me during these years.

Last but not least I am grateful to almighty God for making things possible for me.

8.6 Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel “Histone deacetylase 6 represents a novel drug target in the oncogenic Hedgehog signalling pathway” im Institut für Molekularbiologie und Tumorforschung (IMT) unter Leitung von PD.Dr. Matthias Lauth ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Ich versichere, dass ich sämtliche wörtlichen oder sinngemäßen Übernahmen und Zitate kenntlich gemacht habe.

Mit dem Einsatz von Software zur Erkennung von Plagiaten bin ich einverstanden.

Vorliegende Arbeit wurde veröffentlicht.

Marburg, 03-11-2017
Ort, Datum, Unterschrift