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Novel Downstream Effectors of Mitogenic Sonic Hedgehog Signaling in Cerebellar Development and Sonic Hedgehog Medulloblastoma

By

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Doctor of Philosophy

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M. Hope Robinson

A.S., Pre-Med, Georgia Perimeter College, 2009

B.S., Biological Science, Georgia State University, 2011

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An abstract of
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Abstract

Novel Downstream Effectors of Mitogenic Sonic Hedgehog Signaling in Cerebellar Development and Sonic Hedgehog Medulloblastoma

The most common central nervous system malignancy in children, medulloblastoma arises from neural progenitor cells in the cerebellum and is diagnosed in over 300 children each year in the United States. While the current standard of care has brought the survival rate to ~70%, the treatments often leave patients with long-term negative side effects. The need for therapeutics that are specific, effective, and less toxic requires a greater understanding of the molecular underpinnings of medulloblastoma. Sonic Hedgehog (SHH) medulloblastoma, driven by aberrant activation of the SHH pathway, constitutes ~30% of cases and is the focus of this work. The major components of the SHH pathway have been identified, but there is still much to learn about the downstream effectors.

Pediatric cancers have few genetic mutations, providing a relatively uncluttered background to dissect molecular signaling pathways. At the same time, this paucity of mutations presents few targets and has led researchers to look beyond mutations. Epigenetics, changes in gene expression without changes in DNA sequence, has become an important avenue for research. We have identified Helicase, Lymphoid Specific (HELLS) as an epigenetic factor that is upregulated downstream of the SHH pathway in SHH-N stimulated CGNPs, in the developing murine cerebellum, and in both human and murine SHH MB when compared to non-tumor cerebellar tissue. HELLS upregulation was modulated by inhibition of the interaction between TEAD, a transcription factor, and the oncogenic co-activator YAP1, an established downstream effector of SHH. To ascertain direct regulation of HELLS by YAP/TEAD, a ChIP-qPCR assay was utilized, revealing YAP1 binding to *Hells* upstream DNA. These results indicate YAP1 mediates the SHH induced upregulation of HELLS in our systems. Because epigenetic modifications can be reversed, identification of an epigenetic factor upregulated by the SHH signaling pathway in medulloblastoma could have important implications for future therapeutics, not only in SHH MB but also in the myriad of other cancers with aberrant SHH signaling.

In a separate project, we have identified high levels of HIF1α protein downstream of SHH signaling in CGNPs cultured in normoxic conditions. Rather than increased transcription, our results indicate stabilization of HIF1α, likely through ROS signaling. In addition, we identified increased levels of NOX4 protein, a potent producer of ROS. Taken together, our results demonstrate increased HIF1α protein levels as a result of ROS signaling induced stabilization. Similar to epigenetic regulation, ROS levels can be modulated, presenting an intriguing possibility for therapeutics development.

These studies lay the foundation for further research and may help fill in some of the gaps between SHH signaling and tumor formation. Importantly, both of these avenues of research could result in targeted therapies to treat SHH MB.

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Acknowledgments

Earning a Ph.D. is a difficult thing. It should be. Add the demands of raising teenagers and helping with aging parents, and this difficult thing becomes seemingly impossible at times. Without the help and support of many people, completing this undertaking would have been unattainable.

To my husband: We've been through so much in our 28 years together. I know this educational odyssey of mine has added challenges to an already challenging experience. Your unwavering support has been incredible. You've taken on many of the responsibilities that had been mine with admittedly mixed results, but your willingness to pitch in and ease my load did not go unnoticed. Even when I encountered some bumps in the road that had me losing hope, not once did you ever suggest I should quit. I could not have even considered this road without your love and support. You are the best!

To my children: I know this has not been easy for you. Going from having a full-time stay at home mom who was available to help with whatever you needed, to having a mom who actually needed YOUR help to keep things running could not have been fun. I hope that here at the end of it you are proud of what WE have accomplished as a family. You have become amazing people who are not only competent adults, but also empathetic humans who care about the world around them and seek for ways to make it better. I could not be prouder of each of you!

To my parents and sisters: You can now say we have a doctor in the family – just not THAT kind of doctor! When I started this Ph.D. program, our parents were living independently in a new house they had just built and looking forward to spending more time with their grandchildren. In the six and half years it took me, we lost Dad to cancer and have watched our Mom forget us. The four of you have had to take on more responsibility even while dealing with your own families. Sherry and Leslie, if the two of you had not taken on the lion's share of this responsibility, I would have

discontinued my studies to come help. Because you DID take on so much, I didn't have to. This victory is yours as well! I cannot thank you enough.

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To the Kenney lab members: You all took me in, taught me everything you could, collaborated, commiserated, celebrated (often with cake! Thanks Victor!) and became true friends. I'm so grateful for your friendship. Ahbi and Anshu, your advice, and expertise saved the day more times than I can count. Collaborating with you, Victor has been a blast, and I've learned so much working with you – I already miss not working next to you! Yun Wei, having the opportunity to become your friend is one of the things that made joining the lab such a great decision. I'm excited to watch your career in the future and proud to be your friend!

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You have served on several of my committees, for qualifying exams and my dissertation projects. You've seen me at some of the lowest times and some of the best. You have always had my back and supported me, even when I'm sure you had doubts! I cannot express to you how much that support has meant.

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List of Abbreviations

5mC	5-methylcytosine
Apo	Apocynin
ARID1A	AT-rich interactive domain-containing protein 1A
ARID1B	AT-rich interactive domain-containing protein 1B
BCC	basal cell carcinoma
BCOR	BCL6 co-repressor
BET	Bromodomain and extra terminal domain
BMI1	BMI1 proto-oncogene, polycomb ring finger
BRG1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4; also known as SMARCA4
CDCA7	cell division cycle associated 7
CDKN1A	cyclin-dependent kinase inhibitor 1A (protein - P21CIP1)
CDKN2A	cyclin-dependent kinase inhibitor 2A (proteins - P16INK4A, P19ARF)
ChIP	chromatin immunoprecipitation
CM-H_2DCFDA	(5-(and 6) Chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate
CNGPs	cerebellar granule neuron precursors
CNS	central nervous system
CREBBP	CREB binding protein
DHH	Desert hedgehog
DNA	deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
DNMT3B	DNA methyltransferase 3 beta
DNMTs	DNA methyltransferases
DUOX	dual oxidase
E2F1	E2F transcription factor 1
EGL	external granule layer
EP300	E1A binding protein p300
EZH2	enhancer of zeste 2 polycomb repressive complex 2 subunit
FDA	United States Food and Drug Administration
FOXM1	forkhead box M1
GANT61	GLI antagonist that inhibits GLI1 and GLI2-induced transcription
GLI	glioma-associated oncogene
GLI2	GLI family zinc finger 2
GPS2	G-protein pathway suppressor 2
GS	Gorlin syndrome
H3K4me1	monomethylated histone 3 lysine 4
HATs	histone acetyltransferases
HDAC1	histone deacetylase 1
HDAC2	histone deacetylase 2
HDACs	histone deacetylases
HELLS	helicase, lymphoid-specific; Also known as LSH, PASG, or SMARCA6
HH	Hedgehog

HIF-1	Hypoxia-Inducible Factor-1
HIF1α	Hypoxia-Inducible Factor-1-Alpha
HNSCC	head and neck squamous cell carcinoma
ICF	immunodeficiency, centromeric instability, facial anomalies syndrome
IGL	internal granule layer
IHH	Indian hedgehog
KDM1A	lysine demethylase 1A
KIF7	Kinesin family member 7
LDB1	LIM domain binding 1
LSH	Lymphoid-specific helicase; Also known as HELLS, PASG, or SMARCA6
MAGIC	Medulloblastoma Advanced Genomics International Consortium
MAX	MYC associated factor X
MB	Medulloblastoma
MBCs	cultured primary medulloblastoma cells
MET	MET proto-oncogene, receptor tyrosine kinase
MG132	proteosome inhibitor, carbobenzoxy-Leu-Leu-leucinal
miRNA	microRNA, non-coding ribonucleic acid
MLL2	*KMT2D*, lysine methyltransferase 2D
MLL3	*KMT2C,* lysine methyltransferase 2C
MOL	molecular layer
MRI	Magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSH2	mutS homolog 2; human homolog of the E. coli mismatch repair gene mutS
MYC	MYC proto-oncogene, bHLH transcription factor
MYCL	MYCL proto-oncogene, bHLH transcription factor
MYCN	v-myc avian myelocytomatosis viral related oncogene, neuroblastoma derived
NAC	N-acetyl-L-cysteine; Antioxidant, increases cellular pools of free radical scavengers
NBCCS	nevoid basal cell carcinoma syndrome
N-Cor	nuclear receptor co-repressor
NOX	NADPH oxidase
NOX4	NADPH oxidase 4
OCT4	octamer-binding transcription factor 4; coded for by the gene POU5F1
P16INK4A	cyclin-dependent kinase inhibitor 2A isoform 2 - P16INK4a
P19ARF	cyclin-dependent kinase inhibitor 2A isoform 1 - P19ARF
P21CIP1	cyclin-dependent kinase inhibitor 1 isoform 1 - P21CIP1
PASG	Proliferation-Associated SNF2-like Gene; Also known as HELLS, LSH, or SMARCA6
PC	primary cilium
PCAF/KAT2B	histone acetyltransferase PCAF/*KAT2B*
PHDs	prolyl hydroxylases (PHDs)
PNET	primitive neuroectodermal tumors
POU5F1	POU class 5 homeobox 1, also known as OCT4
PRC1	polycomb repressive complex 1
PTCH1	Patched 1
PTEN	phosphatase and tensin homolog
qRT-PCR	quantitative real-time PCR

RB	retinoblastoma
RMS	rhabdomyosarcoma
RNA	ribonucleic acid
ROS	reactive oxygen species
rpS6	ribosomal protein S6
SAG	Smoothened agonist
SANT2	antagonist of the Smo protein function and an inhibitor of SAG activity
SHH	Sonic hedgehog
SHH-N	recombinantly produced, biologically active N-terminal fragment of SHH
siRNA	small interfering ribonucleic acid
SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4; BRG1
SMARCA6	SWI/SNF2-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 6; Also known as HELLS, LSH, or PASG
SMO	Smoothened
SUFU	Suppressor of fused
SUFU	Suppressor of fused
TAZ	Tafazzin
TEAD	TEA domain transcription factor
TERT	telomerase reverse transcriptase
TET	ten-eleven translocations
TGFB1	transforming growth factor beta-1
TP53	tumor protein p53
TP63	tumor protein p63
TSG	tumor suppressor gene
TSS	transcription start site
UHRF1	ubiquitin-like, containing PHD and RING finger domains 1
VAV1	vav guanine nucleotide exchange factor 1
VHL	Von Hippel-Lindau
WHO	World Health Organization
WNT	Wnt family member 1; wingless/integrated
WT	Wild-type, non-mutated gene
YAP1	Yes-associated protein 1
YB1	Y-box binding protein 1; YBX1
ZIC2	zinc finger protein of cerebellum; Zic family member 2

Chapter 1 Introduction

1.1 Medulloblastoma

1.1.1 Medulloblastoma Overview

Cancers of the central nervous system (CNS) are the most common solid malignant tumors in children [5]. While they were originally classified based on the location and traits of the tumor, histological evaluation has allowed scientists to differentiate between various tumors that occur in the same anatomical space. Initially described and named in the 1920s by Harvey Cushing and Percival Bailey, medulloblastomas occur in the cerebellum or posterior fossa, below the tentorium, which separates the cerebral hemispheres of the forebrain from the cerebellum (See Fig 1.1) [6]. The word cerebellum means "little brain" and while it is smaller than the cerebral cortex, it contains more than half of the neurons of the entire brain and is responsible for coordinating fine motor control movements as well as posture [7]. Histological evaluation of one of these medulloblastomas revealed a tumor composed of small, round cells that appeared blue with hematoxylin and eosin staining [8].

Medulloblastomas (MB) are diagnosed in over 300 children per year in the United States [5]. While there are differences depending on patient age, most MBs are treated with surgical resection, radiation of the brain and spinal cord, and chemotherapy [8]. Patients under three are generally spared radiation therapy as the effects to their developing brains are neurocognitively deleterious [9]. Improvements in multimodal treatment have brought the overall survival rate to ~75% [10]. However, survivors are left with significant negative long-term sequelae including neurocognitive deficits, neuroendocrine disorders, hearing loss, fertility issues, and a lifetime risk of secondary neoplasms, many of which are a result of the current aggressive treatment strategies [8, 10, 11]. All MBs are considered Grade IV tumors [12], but stratification based on clinical characteristics

and risk of recurrence has allowed de-escalation of treatment and better outcomes for some patients [9].

Further advances in survival have plateaued and the need for a better understanding of MB carcinogenesis and maintenance has become critical. While adult tumors have benefitted from precision medicine targeting specific molecular or genetic drivers of tumorigenesis or tumor maintenance, pediatric cancers suffer from a lack of targets due to the paucity of genetic mutations [13]. Additionally, in recent years, many of these sparse mutations in MB have been identified as epigenetic factors (detailed in Section 1.3). As epigenetic regulation is capable of being reversed, this represents a novel horizon for therapeutic development in medulloblastoma [14].

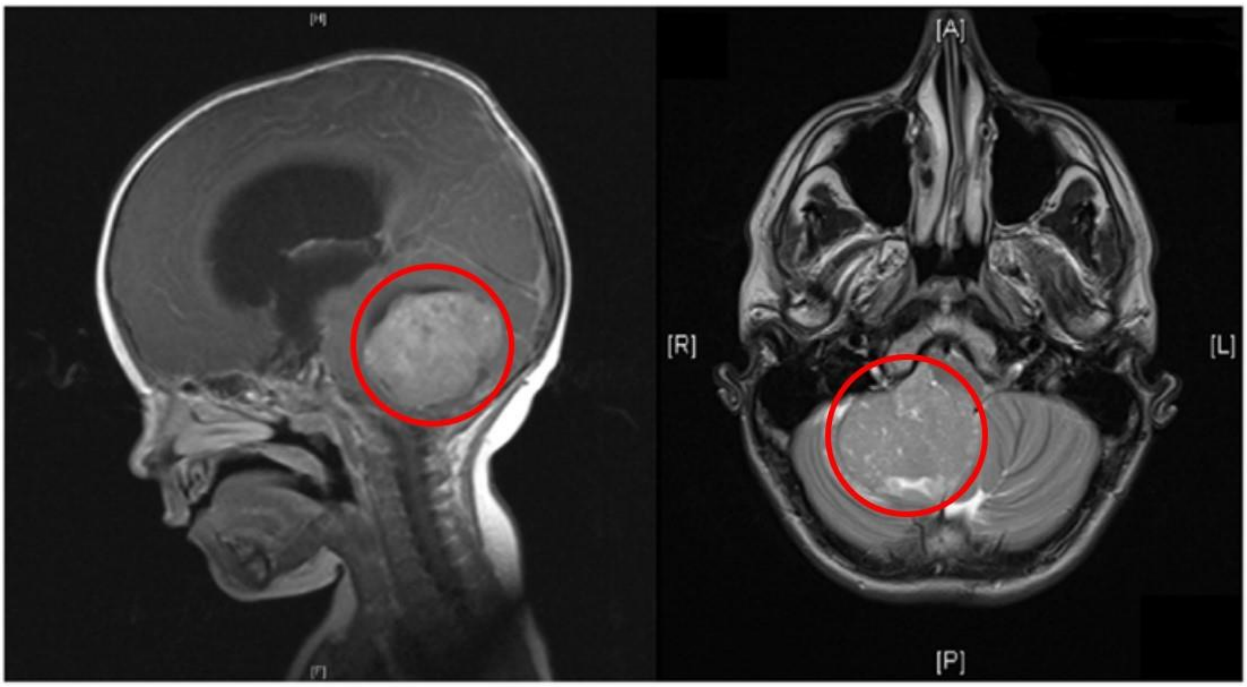


Figure 1.1 Magnetic resonance imaging of a child with medulloblastoma
(adapted from [3])

1.1.2 Medulloblastoma Subgrouping

Historically, MB prognosis and treatment decisions have been based on clinical factors including patient age and success of tumor resection, combined with tumor cell histology [8]. MB can present with different histological variants including classic, large cell/anaplastic, desmoplastic/nodular, or with extensive nodularity [12]. Histological classification is still relevant, even with our more advanced molecular dissections of these tumors, and can aid in treatment decisions. In the last decade, several research groups have made advances in our molecular understanding of medulloblastoma that has resulted in a classification system instituted by the World Health Organization (WHO) in 2016 based on both histology and genetic characteristics [12]. Genetically, or molecularly, MB has been grouped based on transcriptional profiles believed to be critical to the development or maintenance of the tumor. The WHO has outlined the following subgroups: wingless (WNT)-activated; Sonic hedgehog (SHH)-activated, TP53-wildtype; SHH-activated, TP53-mutant; and non-WNT-SHH which comprises the two groups previously called group 3 and group 4 [12]. Within each of these subgroups, histology can vary and can provide prognostic information and guidance for tumor treatment. The various combinations of genetic and histological findings are numerous, but the WHO compiled a list of the most common "integrated diagnoses" with corresponding prognoses which can be found in Table 1 [12].

Genetic profile	Histology	Prognosis
Medulloblastoma, WNT-activated	Classic	Low-risk tumour; classic morphology found in almost all WNT-activated tumours
	Large cell / anaplastic (very rare)	Tumour of uncertain clinicopathological significance
Medulloblastoma, SHH-activated, *TP53*-mutant	Classic	Uncommon high-risk tumour
	Large cell / anaplastic	High-risk tumour; prevalent in children aged 7–17 years
	Desmoplastic / nodular (very rare)	Tumour of uncertain clinicopathological significance
Medulloblastoma, SHH-activated, *TP53*-wildtype	Classic	Standard-risk tumour
	Large cell / anaplastic	Tumour of uncertain clinicopathological significance
	Desmoplastic / nodular	Low-risk tumour in infants; prevalent in infants and adults
	Extensive nodularity	Low-risk tumour of infancy
Medulloblastoma, non-WNT/non-SHH, group 3	Classic	Standard-risk tumour
	Large cell / anaplastic	High-risk tumour
Medulloblastoma, non-WNT/non-SHH, group 4	Classic	Standard-risk tumour; classic morphology found in almost all group 4 tumours
	Large cell / anaplastic (rare)	Tumour of uncertain clinicopathological significance

Table 1: WHO summary of medulloblastoma types most commonly diagnosed with corresponding prognostic information. Reproduced from [12]

Survival statistics vary considerably between subgroups even though treatment protocols are fairly standard across subgroups. In patients with WNT MB, the five-year overall survival (OS) is ~95% [15]. In contrast, Group 3 MB 5-year OS is only ~50%, and the SHH group and Group 4 have intermediate outcomes with ~75% 5-year OS [15]. However, these overall survival statistics fail to represent the tumor heterogeneity found in medulloblastoma accurately. Two reports from separate groups in 2017 supported the four main subgroups of WNT, SHH, Group 3, and Group 4, and further subdivided them [1, 16]. Cavalli et al. extended the four subgroups into 12 by using similarity network fusion on genome-wide DNA methylation and gene expression data and correlated that with clinical and genomic characteristics in a set of 763 primary medulloblastoma samples from the Medulloblastoma Advanced Genomics International Consortium (MAGIC) [1]. In this cohort, appreciable differences in survival were seen in subsets of the SHH MB group and in Group 3 and Group 4 MB [1]. See Figure 1.2 for details from this study.

In the report from Schwalbe et al., analysis of a slightly smaller cohort of 428 pediatric patients resulted in seven reproducible groups based on comprehensive clinical data, pathology review, and molecular profiling including DNA methylation microarray [16]. Importantly, this sample cohort was comprised of children aged 3-16 years and did not include infants under three years of age or adults [16]. As more infants and adults are diagnosed with SHH MB, this study may not be as informative for SHH MB. Additionally, survival data in this study was modeled on a smaller set of patients who had been treated with craniospinal irradiation, and this study reported 10-year overall survival as opposed to 5-year, further complicating comparison between the two studies. Importantly, this study validated the previously defined four main subgroups of MB.

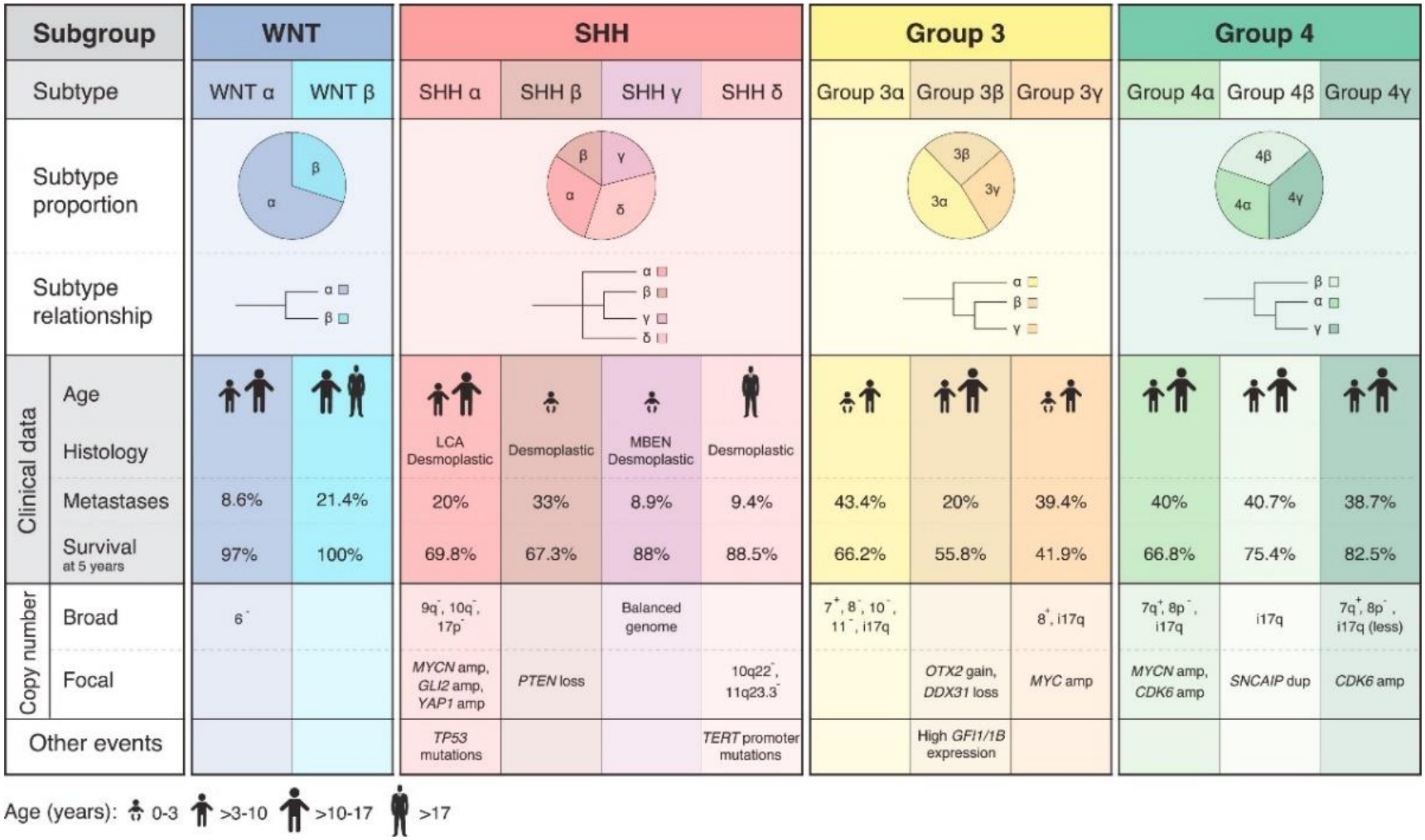


Figure 1.2 Summary of 12 subtypes of medulloblastoma
Cavalli et al confirmed the four main subgroups of MB and further defined 12 subtypes. The SHH MB subgroup was divided into four subtypes as shown in the figure. Reproduced from [1]

The Sonic Hedgehog group of medulloblastoma is the focus of this dissertation work. SHH MB arises from neural progenitor cells in the cerebellum, is characterized by aberrations in the SHH pathway, and constitutes ~30% of all medulloblastomas [17]. SHH MB has a bimodal age distribution with the majority of infant and adult cases falling in the SHH MB group [8, 18]. Mutations in TP53 are observed in 20% of SHH MB, predominantly in childhood cases between the ages of 3 and 16 [1, 17, 19].

Survival in SHH MB is highly correlated with patient age with infants typically faring better (77% for 5- and 10-year OS) than adults (5-year OS 75%; 10-year OS 34%) [18]. However, in the report from Cavalli 2017, a dramatic difference in survival for infants exists in group SHH β (5-year OS 67%) compared to SHH γ (5-year OS 88%) [1]. SHH β infants exhibit higher rates of metastasis (33%), and 25% have a deletion of a tumor suppressor gene called phosphatase and tensin homolog (*PTEN)*, either of which could be responsible for the lower survival outcomes [1]. As infants with SHH MB have traditionally been treated with the same protocol, this finding could be very important, providing critical information to treating physicians and opening a class of targeted treatment options for those infants with PTEN deletion. Overall survival in childhood (aged 3-16 years) SHH MB is highly dependent on *TP53* mutation status as children in one study from Zhukova et al reported that SHH MB with wild-type (WT) *TP53* have a 5-year OS of 81% while children with mutated *TP53* have a 5-year OS of only 41% [19]. The study from Cavalli et al. in 2017 with a larger cohort reported that TP53 mutations were highly enriched in the subtype SHHα with 35% of the patients in this subtype having a mutation in *TP53*. Five-year overall survival in the SHHα group was worse at ~70%, but not as low as the 41% reported in the Zhukova study. These studies provide important distinctions between different tumors and in the future, we expect treatment decisions will be based on molecular diagnoses in addition to clinical and

histopathological factors. In the meantime, research to further characterize these groups and identify vulnerabilities will provide avenues for therapeutic development.

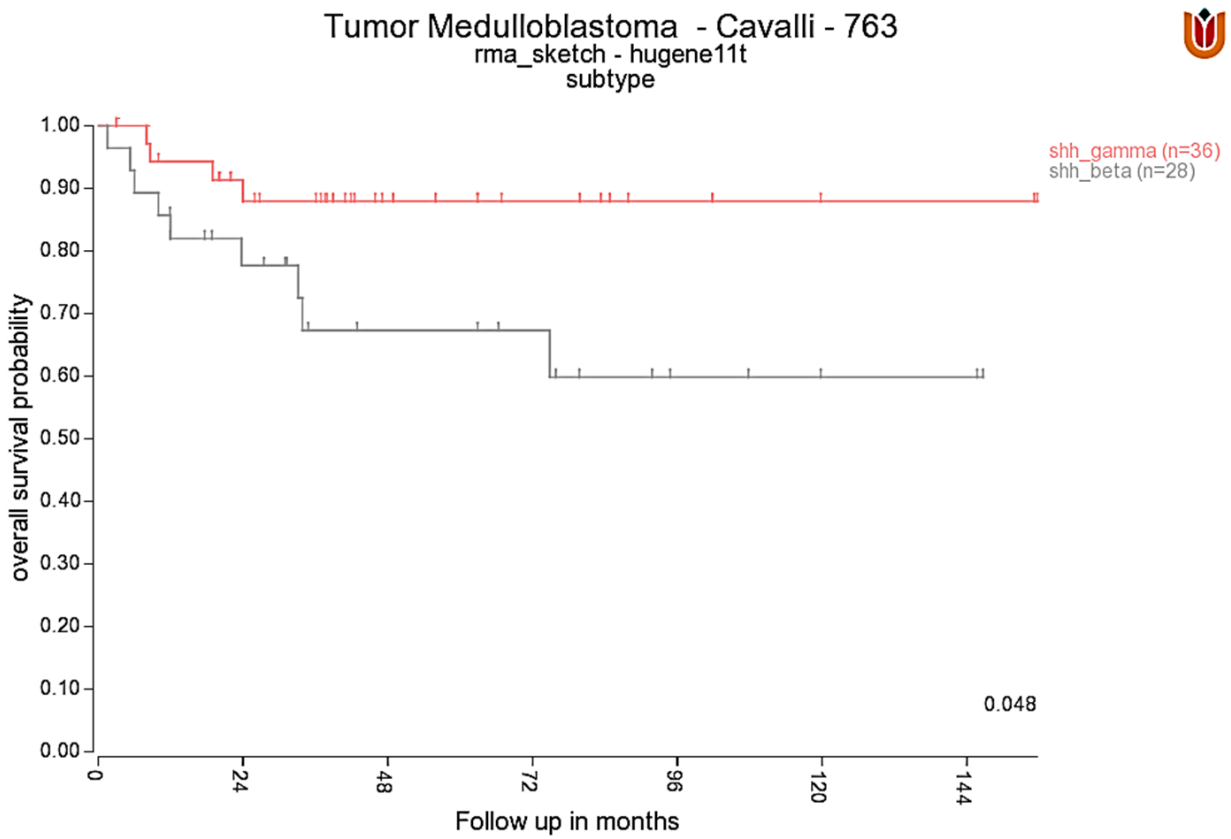


Figure 1.3 Overall survival for infants with SHH MB varies based on tumor subset classification In the Cavalli 2017 dataset, four subtypes of SHH MB were identified, revealing a significant difference in survival for infants with subtype SHH-β (5-year OS 67%) versus SHH-γ (5-year OS 88%). Kaplan-Meier curve created with R2: Genomics Analysis and Visualization Platform. p=0.048; Logrank test as described in Bewick et al Critical Care Vol8 No5 2004, pp.389-94.

As with survival, the mutational landscape of SHH MB is highly age dependent with infants often harboring mutations in Patched 1 (*PTCH1)* or Suppressor of fused (*SUFU)* or the previously mentioned deletions of *PTEN* and adult cases presenting with mutations in telomerase reverse transcriptase (*TERT)* as well as SHH pathway genes [17]. Children frequently are found to have amplifications of GLI family zinc finger 2 (*GLI2)* and v-myc avian myelocytomatosis viral related oncogene, neuroblastoma derived (*MYCN*, also called N-MYC), which is likely to be a consequence of chromothripsis due to *TP53* mutations [17]. To better understand the implications of aberrations in the SHH pathway, an appreciation of the proteins involved and how they interact with each other is essential. Details are provided in section 1.2. In general though, the mutational burden of SHH MB is low, as in most pediatric cancers. This has presented a challenge in this time of genetic profiling of tumors because even with the low rate of genetic mutation, transformation and tumorigenesis still occurs. This lack of targets for research and therapeutics has likely played a part in the recent plateau in survival improvements.

1.2 Sonic Hedgehog

1.2.1 The Hedgehog Pathway

The secreted protein Hedgehog (HH) was originally identified in screenings done in *Drosophila melanogaster* when the appearance of the fruit fly embryo was altered by a null allele of *hh,* leaving the embryo covered in hairs that looked like the spines that cover hedgehogs [20]. In mammals, three homologs were identified and named Sonic hedgehog (SHH), Indian hedgehog (IHH), and desert hedgehog (DHH) [20]. Each of these proteins has roles in embryonic development, and their dysregulation has been found to be a key factor in several congenital disorders [20]. Additionally, aberrant HH signaling is a frequent finding in cancer and mutations that activate pathway components are found in MB as well as in basal cell carcinoma (BCC) and

rhabdomyosarcoma (RMS) [21, 22]. HH signaling has also been implicated in cancers of the lung, pancreas, liver, stomach, colon, prostate, and breast [22].

HH proteins are post-translationally modified and secreted from cells, forming a gradient of HH signaling that dictates developmental outcomes such as the dorsal-ventral axis in neural development and the anterior-posterior axis in limb development [21]. The primary cilium (PC), a cell organelle found on most mammalian cells, is where HH signaling is initiated and is therefore critical to HH pathway signaling [22]. As a ligand, HH binds primarily to the 12-pass transmembrane protein PTCH1. Without HH, PTCH1 is localized to the PC where it constitutively inhibits the activity of another transmembrane protein, the seven-pass G protein-coupled receptor Smoothened (SMO). When HH binds to PTCH1, PTCH1 leaves the PC and SMO inhibition is released, activating downstream pathways through the canonical glioma-associated oncogene (GLI) proteins or through other downstream effectors like N-MYC or Yes-associated protein 1 (YAP1). An SHH pathway schematic is displayed in Figure 1.4.

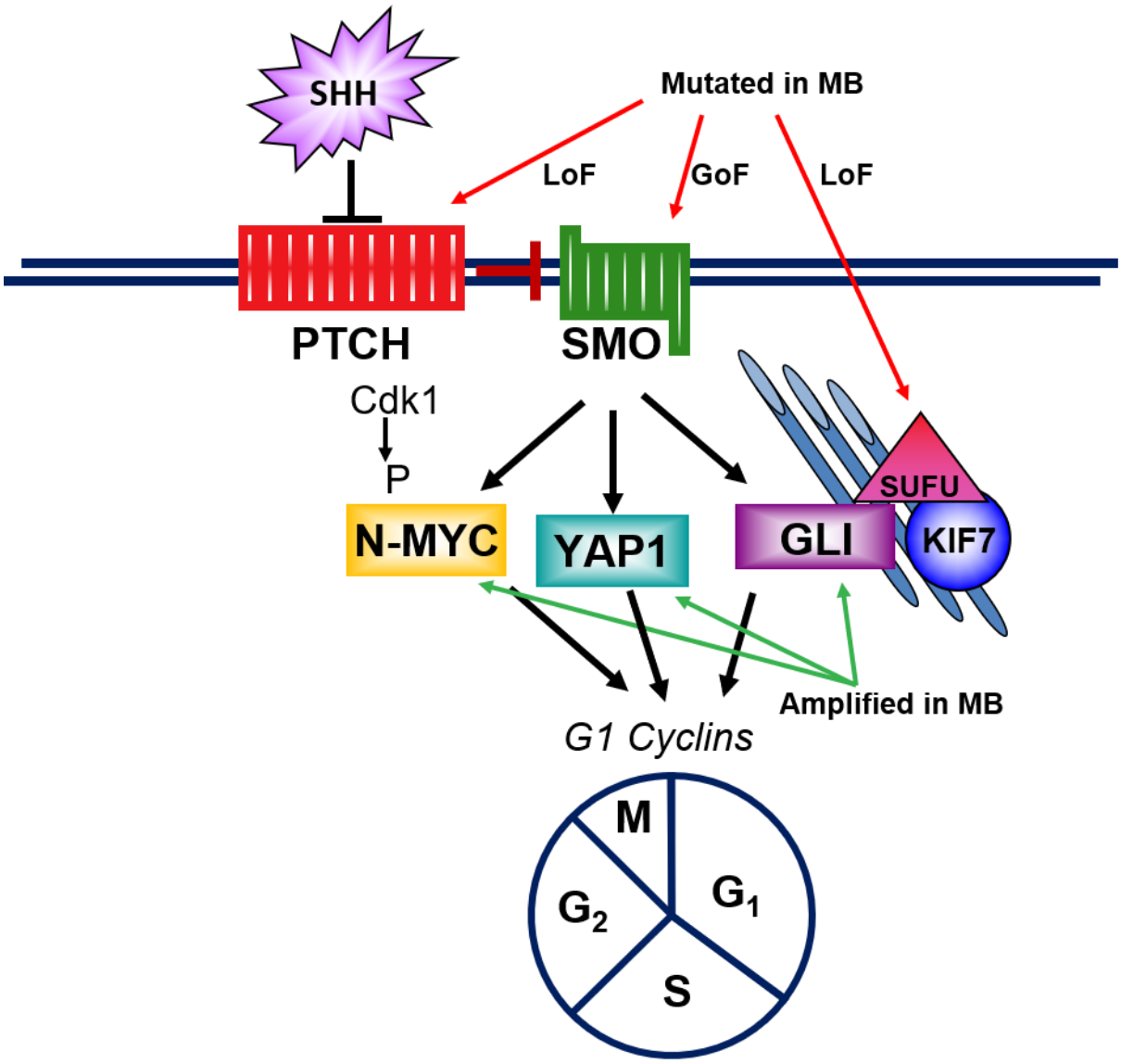


Figure 1.4 Sonic Hedgehog signaling pathway
The secreted ligand Sonic Hedgehog (SHH) binds to and inhibits Patched (PTCH) inhibition of Smoothened (SMO), allowing SMO to set off a signaling cascade through downstream effectors N-MYC (MYCN), Yes-associated protein 1 (YAP1), and the GLI family of proteins. Activation of this signaling pathway generally results in continued cell cycle activity. Mutations in PTCH, SMO, and SUFU have been identified in MB as well as amplifications of the genes for N-MYC, YAP1, and GLI2. Loss of Function (LoF); Gain of Function (GoF)

A major focus of the research in the Kenney lab revolves around identification of the factors downstream of N-MYC, YAP1, and GLI that play roles in cerebellar development and medulloblastoma. Identification of these factors has the potential to provide prognostic information, tailor therapeutic strategies, and offer targets for the development of new therapeutics.

1.2.1.1 GLI

Originally discovered as highly overexpressed in glioma and named glioma-associated oncogene (GLI), GLI family zinc finger transcription factors are considered the canonical downstream effectors of the HH pathway. In mammals, there are three known GLI family members, GLI1, GLI2, and GLI3. GLI1 acts only as a transcriptional activator while the full-length forms of GLI2 and GLI3 are activating, but these proteins can be processed, and the resulting truncated forms of GLI2 and GLI3 can act as transcriptional repressors [22]. HH signaling changes the balance of these proteins and in general GLI2 is the main HH-regulated activator and GLI3 is the main HH-regulated repressor [23]. In the primary cilia, other proteins interact with and affect GLI protein functions. SUFU sequesters GLI proteins in the cytoplasm as well as interacting directly with GLI2 in the nucleus, inhibiting GLI2 activity [22]. Kinesin family member 7 (KIF7) may play a part in the structure of the PC and functions as both a negative and a positive regulator in the SHH pathway. In its role as a negative regulator, it is localized at the base of the PC and prevents aberrant activation of GLI2 without HH signaling [21, 22]. In contrast, when the HH pathway is activated, KIF7 moves to the distal end of the PC where GLI2 and GLI3 are accumulating and plays a role in activating these proteins [21, 22].

1.2.1.2 MYCN

A transcription factor that heterodimerizes with another basic-helix-loop-helix-leucine zipper protein called MYC associated factor X (MAX), MYCN has been implicated in a broad range of tumor types, many of which are nervous system cancers [24]. *Mycn* was found to be upregulated by SHH in cerebellar granule neuron precursors (CGNPs), the cell of origin for SHH MB [25]. Further studies established that *MYCN* and *MYCL1* are both amplified and highly expressed specifically in the human SHH subgroup of MB [26]. While expression of MYCN is high in SHH MB, it does not always correlate with prognosis. However, amplification of MYCN does correlate with a worse prognosis and is associated with more aggressive tumors and tumors with TP53 mutations [26].

1.2.1.3 YAP1

Yes-associated protein 1 (YAP1) is an oncogenic transcriptional coactivator that is part of and normally repressed in the Hippo pathway. As part of the Hippo signaling pathway, YAP1 and its paralog tafazzin (TAZ, coded for by the gene *WWTR1*) are important in development, tissue homeostasis, and many cancers [27, 28]. *YAP1* was identified as a gene that is amplified and highly expressed in SHH MB [29]. In keeping with the conserved mechanisms between development and tumor pathogenesis, *YAP1* expression is also upregulated by SHH stimulation in CGNPs [29]. In fact, YAP1 overexpression promoted the proliferation of CGNPs even in the absence of SHH [29]. Murine tumors with elevated ectopic YAP1 expression grew faster, were radioresistant, and displayed genomic instability, findings that could have implications for recurrence [30]. Additionally, YAP1 expression in murine tumors was highest in the perivascular niche and these tumor cells co-stained for nestin and CD15 indicating these cells may be tumor stem cells, capable of repopulating the tumor after irradiation [29].

1.2.2 Hedgehog in Cerebellar Development

Hedgehog signaling is critically important for normal cerebellar development. In mice, cerebellar development is accomplished primarily after birth, multiplying 1000-fold in volume in 15 days, from a small, nondescript collection of cells to a much larger structure with deep fissures and intricate foliation [31] (Figure 1.5).

While the structure of the cerebellum appears complex, it is a relatively simple layered structure. The developing cerebellum is composed of layers of cells from two different progenitor zones. One of these zones, the ventricular zone, produces neurons, cerebellar interneurons, and Purkinje cells, which secrete SHH. Another area, termed the germinal zone and located in the rhombic lip, generates cerebellar granule neurons, the most prevalent neuron in the mammalian CNS [32]. The expansion of the CGNP population in the external granule layer (EGL) is dependent on the gradient of SHH signaling from the Purkinje cells in the molecular (MOL) layer (Fig 1.6) [33]. CGNPs exit the cell cycle, begin differentiating, and migrate inwards to form the internal granule layer (IGL) which persists into adulthood while the EGL thins and disappears by ~P15 in mice [33]. SHH signaling also controls the pattern of foliation in the cerebellar cortex [33]. Without SHH signaling, the population of CGNPs is dramatically reduced as is the volume of the resulting cerebellum, which also lacks the characteristic foliation pattern [34, 35]. Mature granule cells act as an intermediary, relaying information from the central nervous system to the other cells in the cerebellum. Thus they are critically important to the function of the cerebellum.

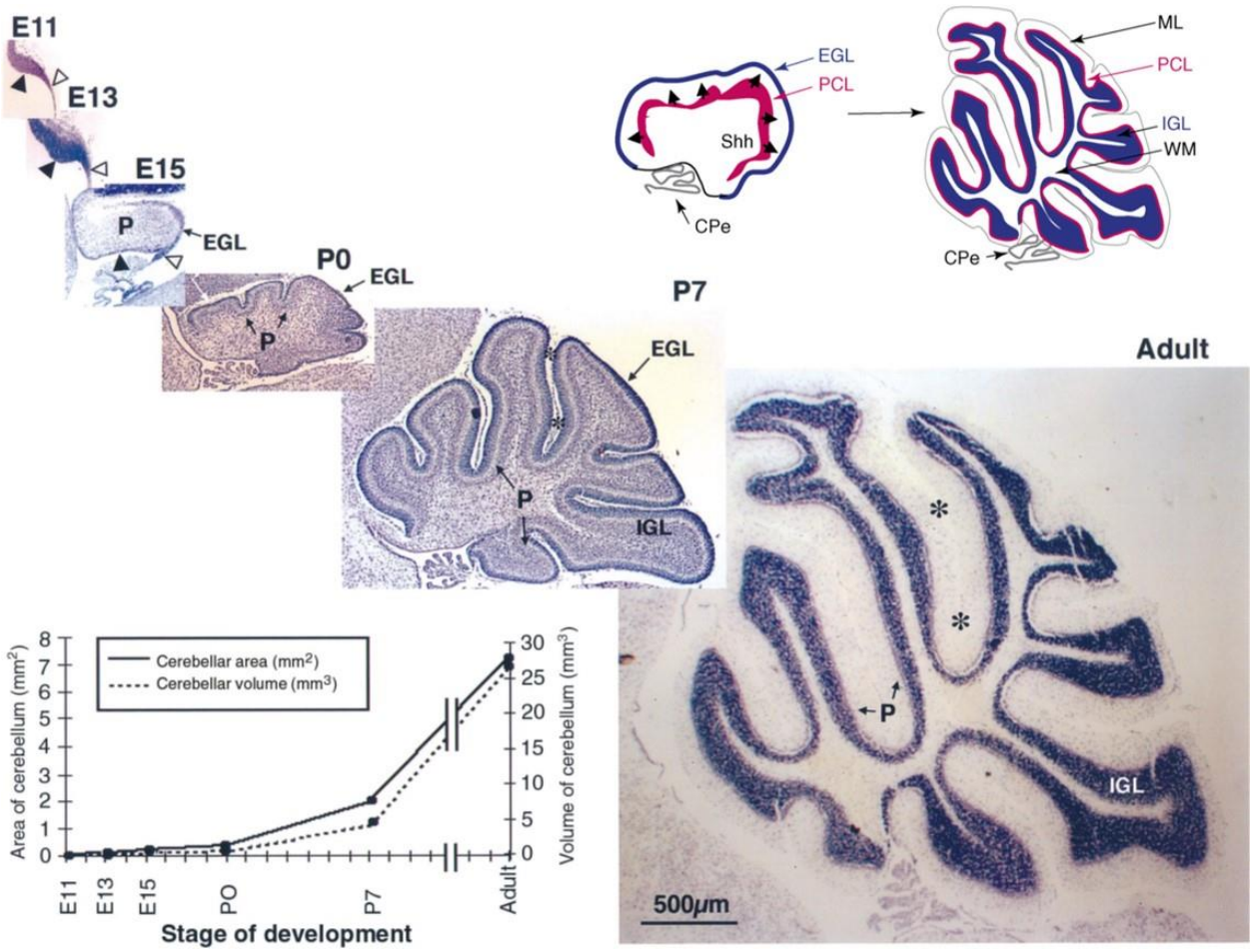


Figure 1.5 Cerebellar development in mice

At birth, the murine cerebellum is very small and lacks the definition seen in the mature cerebellum. Over the course of 15 days, CGNPs in the external granule layer (EGL) undergo massive expansion, begin differentiating, and migrate through the molecular layer (ML) to the internal granule layer (IGL). The rapid expansion of CGNPs is stimulated by Sonic Hedgehog secreted from Purkinje cells in the Purkinje cell layer (P or PCL). White matter (WM); Choroid plexus (CPe). Asterisks denote the primary fissure. Image combined from [31, 36]

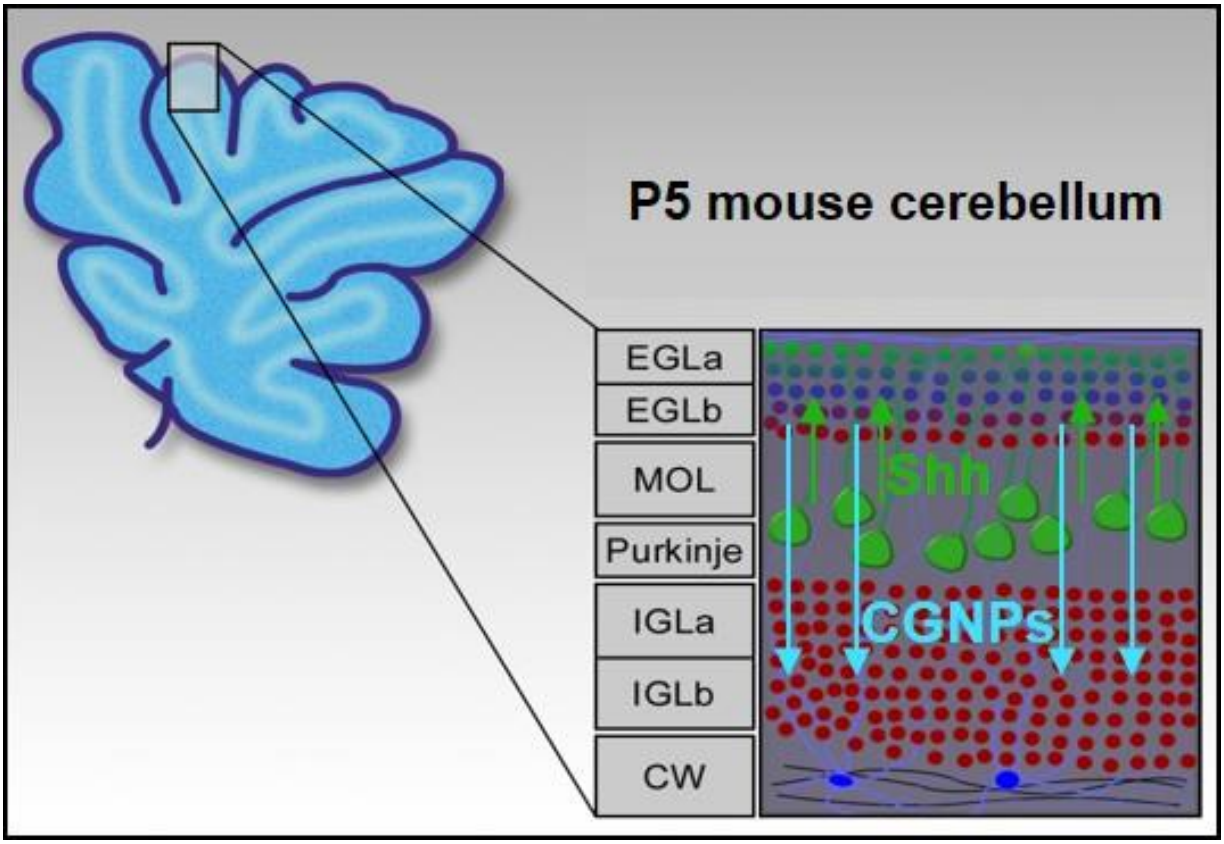


Figure 1.6 Representation of the layers of the cerebellum in day 5 postnatal mice
In 5-day-old mice, the layers of the cerebellum are comprised by: a thick outer layer called the external granule layer (EGL) where CGNPs are rapidly proliferating, the molecular layer (MOL), the Purkinje cell layer which is responsible for secretion of SHH which diffuses towards the EGL, creating a gradient of SHH ligand, the internal granule layer (IGL) where CGNPs migrate and complete differentiating, and cerebellar white matter (CW). Adapted from [4].

1.2.3 Hedgehog in Cancer

As important as SHH signaling is to development of the cerebellum, it is not surprising that unrestrained SHH activity is part of tumorigenesis as developmental pathways are often co-opted in cancer. Aberrant Hedgehog signaling has been implicated in a number of cancers. In MB, RMS, and nevoid basal cell carcinoma syndrome (NBCCS), also called Gorlin syndrome (GS), mutations in SHH components have been identified which cause ligand-independent SHH signaling [34]. Aside from mutations, activated SHH signaling was observed through expression analysis in MB lacking SHH pathway mutation [37]. This indicates there are other ways to activate the SHH pathway. Additionally, there are cancers with SHH ligand-dependent activation of the SHH pathway [34].

The link between HH signaling and cancer was originally discovered in patients with GS, a disorder with inherited loss-of-function mutations in *PTCH1,* predisposing patients to a number of neoplasms, especially BCC [21]. Incidence in GS is increased for MB, meningioma, fetal rhabdomyoma, rhabdomyosarcoma, and fibromas of the ovary and heart [37]. Aberrant HH signaling is also a factor in sporadic BCCs, where there is a high rate of inactivating mutations in *PTCH1* or occasionally there are activating mutations in *SMO* [21, 37].

Aberrant activation of the SHH pathway is what sets SHH MB apart from the other subgroups of medulloblastoma. Mutations are often correlated with patient age. GS patients are at a higher risk for MB due to an inactivating germline mutation of *PTCH1* or *SUFU,* and MB in these patients generally occurs in infancy. Children between the ages of 3 and 16 are more likely to present with mutations in *TP53* (30% of childhood SHH MB) and frequently harbor amplifications of *GLI2* and/or *MYCN* as well, likely as a result of chromothripsis [17]. Li Fraumeni Syndrome (LFS), in which patients have a germline mutation of *TP53,* is also linked to SHH medulloblastoma [19]. Patients

with TP53-mutant SHH MB are recommended for genetic counseling to determine whether they have LFS [38]. Adults with SHH MB have a higher mutational burden in general with mutations often seen in *PTCH1* and *SMO* in addition to mutations in the promoter region of *TERT* [17], which is observed almost exclusively in adult SHH MB [39].

1.2.4 Therapeutic Targeting of Hedgehog

Ideally, all that we have learned about the SHH pathway and the pathogenic role it plays in carcinogenesis will lead to the development of therapeutic agents that are not only specific but also very effective. Efforts to inhibit SHH signaling can be targeted at multiple levels of the SHH pathway and are well reviewed in Wu et al., 2017 [22]. Interference of the binding of the SHH ligand to the receptor PTCH1 is possible via several routes. The SHH protein undergoes critical post-translational modifications (PTMs), and inhibition of the palmitoylation of SHH prevents both autocrine and paracrine SHH signaling [40]. Direct blocking of SHH with antibodies or a small molecule drug called robotnikinin has also been attempted in the laboratory [22]. Since relief of the inhibition of Smoothened activates the SHH pathway, a number of SMO antagonists have been developed and tested both in the laboratory and in clinical trials. Cyclopamine, a compound produced by the corn lily plant, was discovered as a potent inhibitor of SMO activity several decades ago [22]. Since that time, numerous SMO antagonists have been developed and tested. Perhaps the most well-known example is vismodegib, which has been approved by the United States Food and Drug Administration (FDA) for the treatment of BCC [22]. Itraconazole, an antifungal drug, also has SMO inhibitory function, with a mechanism separate from other SMO antagonists [22]. All of these possible inhibitors of the SHH pathway at this level have significant drawbacks, however. The developmental role of the SHH pathway would likely prohibit the use of these inhibitors in infants and concerns of premature osseous fusions would make their use contraindicated in children [17].

Additionally, targeting at the level of SMO will only work in the percentage of patients with aberrations in SMO or PTCH1. For patients with amplification of downstream effectors MYCN, GLI2, or YAP1, SMO inhibition will have no anti-tumor effects. Aside from primary resistance to SMO inhibitors, acquired or secondary resistance has been identified in patients treated with SMO inhibitors who initially exhibited responses to vismodegib, but then experienced relapses [22]. Overall, it is apparent that targeting the SHH pathway at the level of Smoothened will have limited applicability, but may still prove useful in combination treatments.

Targeting the SHH pathway downstream of Smoothened has the likelihood of being more specific in the context of cancer. Direct inhibition of GLI transcription factors has been tested in the pre-clinical setting and two drugs, GANT58 and GANT61, have shown anti-proliferative effects in vitro and anti-tumor effects in vivo [22]. Regulating the levels of GLI proteins through PI3K/mTOR inhibition has also shown some success, particularly in combination with SMO inhibitors [22]. Additionally, epigenetic regulation (discussed in 1.3) of GLI transcription factors has been identified, and inhibition of BRD4, an epigenetic factor that binds to the promoter regions of GLI1 and GLI2, was observed to have anti-tumorigenic effects, even in cells which are SMO inhibitor resistant [41]. Importantly, below the level of GLI, NMYC, and YAP1 in the SHH pathway, there is still much to be discovered, and it is likely that identification of downstream components of the pathway will provide even greater specificity in SHH MB treatment.

1.2.5 Hedgehog Model Systems

The Kenney lab utilizes several model systems to study SHH mitogenic signaling. In SHH MB, an ideal model system has been established using the putative cells of origin, CGNPs. These CGNPs can be harvested from neonatal mice and grown in culture with an exogenously added, recombinantly produced, biologically active N-terminal fragment of SHH (SHH-N) to maintain

them in a proliferative state for up to 72 hours. This SHH-N stimulation results in gene expression patterns and phenotypical characteristics that recapitulate SHH driven proliferation in cerebellar development and in medulloblastoma with high fidelity [42, 43]. Using this system, dissection of the downstream effects of SHH signaling can be achieved. Also, genetically engineered mice with aberrant SHH pathway activity in the cerebellum are utilized. Specifically, the *NeuroD2:SmoA1* strain with a point mutation in Smoothened driven by *NeuroD2* expression is used, which results in the development of medulloblastomas that accurately represent human tumors in gene expression signatures and histology [44]. Additionally, *NeuroD2:SmoA1* and *Atoh1:GFP* mice are crossed to generate *NeuroD2:SmoA1;Atoh1:GFP*, providing tumors in which all tumor cells produce GFP protein, facilitating dissection of the tumor from normal tissue [45]. Tumors from both of these mouse models can also be harvested and disassociated, and the cells can be grown in culture for several days, providing a system to evaluate drugs or genetic manipulation in the tumor cell context.

1.3 Epigenetics

This section (1.3 Epigenetics), is edited from a review in preparation with Yun Wei.

1.3.1 What is epigenetics?

In the past, our understanding of the processes governing the initiation, promotion, and progression of cancer was largely based on alterations to the genome. Researchers looked for mutations in DNA that resulted in gains or losses of entire genes or for protein products with loss of function, a gain of function, or altered function. We now know that genomics is only part of a very complex picture. In pediatric cancers, the mutational load is very low, providing few targets for therapeutic development [13]. Epigenetics, heritable changes in gene expression without changes to the DNA sequence, controls the availability of the genome to the transcriptional machinery. These changes can result in very different phenotypical outcomes for cells that are genetically identical and epigenetic alterations have been identified in many cancers, including SHH medulloblastoma [46].

Chromatin is the complex of proteins and DNA that allow dense packaging of eukaryotic DNA into the higher order structure of chromosomes. The basic unit of chromatin is the nucleosome, an octamer of histone proteins with ~147 base pairs of DNA wrapped around it [47] (Fig 1.7). Epigenetic changes are made by covalent modifications to components of chromatin, DNA or histones, which change whether it is available for further modification or transcription [48]. These covalent modifications are added by proteins referred to as “writers.” Some examples of “writers” are DNA methyltransferases (DNMTs) or histone acetyltransferases (HATs). “Erasers” like histone deacetylases (HDACs) remove these marks. “Readers” are proteins with specialized domains that recognize and interpret the epigenetic modifications.

Aside from covalent modifications to DNA and histone proteins, dynamic modulation of the position of DNA around nucleosomes via ATP-dependent chromatin remodelers is another mechanism of epigenetic control of transcription [49]. Chromatin primarily exists in two different states. Heterochromatin is tightly condensed and the genes in these nucleosomes are generally considered inactive, or unavailable for transcription. In contrast, euchromatin is in a more relaxed configuration, creating a more permissive situation for transcription.

All of these mechanisms of epigenetic control have been implicated in SHH MB and are described in the following sections.

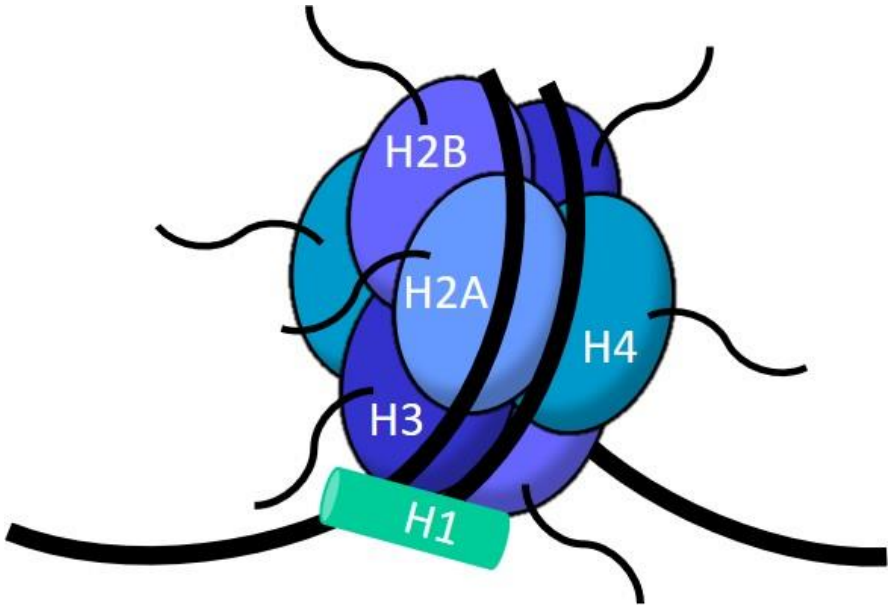


Figure 1.7 Nucleosome schematic
The nucleosome is comprised of two copies each of four different histone proteins (H2A, H2B, H3, and H4) with ~147 base pairs of DNA wrapped around it. The histone linker protein H1 is not part of the octamer but still binds to DNA. Each histone protein has a tail with residues that can be chemically modified.

1.3.2 DNA Methylation

The most often discussed DNA modification is methylation of the pyrimidine base cytosine, especially in childhood cancers [46]. Hypomethylation of DNA may activate transcription of oncogenes and contribute to genome instability [50]. Hypermethylation, particularly of CpG islands in the promoters of genes, may result in gene silencing, a phenomenon seen in tumor suppressor genes (TSGs), which may act as the "second hit" according to Knudson's hypothesis whereby both alleles of a TSG must be lost or inactivated to produce the phenotype [50]. Resolution of cytosine methylation, specifically 5-methylcytosine (5mC), can be accomplished through oxidation via TET (ten-eleven translocations) family enzymes [50]. However, spontaneous hydrolytic deamination of 5mC to thymine also occurs, thereby creating a point mutation [48]. In fact, 50% of the mutations found in p53 are at cytosine methylation sites, mutations of which are found in 20% of SHH medulloblastoma cases [50].

Differential DNA methylation has been described in multiple published reports of SHH MB [1, 16, 51, 52]. Genome-wide methylation arrays have confirmed that the four major MB subgroups have specific DNA methylation patterns [1, 16]. Interestingly, differences were also observed within the SHH subgroup in that the pattern in children was distinct from infants with more genes hypermethylated in children, notably in genes known to be involved in development [51]. Hypermethylation as a mechanism for gene silencing was not found to be a key regulatory feature in a study of 34 human medulloblastoma samples [52]. However, there are a few genes with critical roles in controlling cell proliferation, whose methylation state positively or negatively corresponds with gene expression. Hypomethylation of the gene for vav guanine nucleotide exchange factor 1 (*VAV1)* leads to elevated expression of *VAV1* in mouse and human SHH MB and is associated with poor outcomes [53]. Manipulations of VAV1 levels in human MB cell lines

demonstrated a role for VAV1 in proliferation [53]. Experiments in mice indicated VAV1 involvement in cerebellar development and SHH MB tumor maintenance [53].

Hypomethylation of the oncogenes transforming growth factor beta 1 (*TGFB1)* and MET proto-oncogene, receptor tyrosine kinase (*MET)* was also observed in human SHH MB [51]. The gene for mutS homolog 2 (*MSH2)*, an important component of DNA repair, was found to be hypermethylated in SHH MB samples [51]. Additionally, the genes for *PTCH1* and the zinc-finger protein of the cerebellum family member 2 *(ZIC2),* were reported to be silenced by promoter hypermethylation in medulloblastoma [54]. A more recent study noted that DNA hypermethylation was more prominent in childhood MB when compared to infant cases of MB [16]. Childhood MB corresponds to the SHH-α group which is also known to have a higher proportion of cases with p53 mutations and a worse prognosis [1]. Hypermethylation of specific CpG sites upstream of the transcription start site for telomerase reverse transcriptase (*TERT)* was reported in 36% of SHH MB tumors [55]. As previously mentioned, hypermethylation of CpG islands in the promoter regions of genes usually results in gene silencing. However, DNA methylation status at specific sites in the TERT promoter has been associated with increased expression in several reports [55, 56]. Interestingly, SHH tumors in infants and children showed hypermethylation at this site while adult MB tumors displayed more frequent mutations of the *TERT* gene, but in both cases *TERT* expression was increased [55].

1.3.3 Histone post-translational modification

Histones are central to the structure of chromatin. Each histone has a tail comprised largely of lysine and arginine residues that are prone to modification. These modifications, primarily methylation and acetylation, alter the affinity of DNA for the histone octamer, thereby determining the level of chromatin compaction [57]. Many of the gene mutations in medulloblastoma are found in enzymes that “write,” “erase,” or “read” these marks on histones

[54]. Mutations of histone lysine methyltransferases and demethylases have been identified across subgroups of medulloblastoma without identified subgroup affiliation [58-60]. Mutation of histone acetyltransferases (HATs) or upregulation of histone deacetylases (HDACs) has also been described in medulloblastoma [14]. Specifically, the histone acetylation protein called CREB binding protein (*CREBBP*) and the E1A binding protein p300 (*EP300*) have been identified as mutated in some SHH MB [15]. Aside from mutations, a 2013 study showed that SHH signaling regulates expression and activity of HDACs, specifically HDAC1, 2, 3, and 6 and that this activation is required for CGNP proliferation [54, 61]. Two histone lysine methyltransferases MLL2 (*KMT2D*) and MLL3 (*KMT2C*) have been found to have inactivating mutations in 16% of MB [14]. In a study using SHH MB stem-like cell cultures and a xenograft tumor model, inhibition of the methyltransferase enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) reduced H3K27me3 levels which reduced cell proliferation, induced cellular apoptosis, and delayed tumor growth [62]. The histone demethylase lysine demethylase 1A (KDM1A, also called LSD1), is upregulated in human and mouse models of SHH MB [63]. In the context of *KDM1A* knockdown, Pajtler et al. observed inhibition of migratory capacity in addition to reduced proliferation and increased apoptosis of these cells [63]. Depletion of the histone acetyltransferase PCAF/*KAT2B* in SHH MB cell lines also decreased proliferation and increased apoptosis [64]. Importantly, many of these histone modifiers are components of large complexes that activate or repress transcription. One good example is the nuclear receptor co-repressor (N-CoR) complex, mutations in three members of which were found in SHH MB, namely BCL6 co-repressor (*BCOR*), LIM-domain binding 1 (*LDB1*), and G-protein pathway suppressor 2 (*GPS2*) [15, 65]. As suggested by the name of the complex, it is believed to act as a repressor to gene transcription possibly via HDAC activity and mutations in components of the complex likely results in aberrant transcription [15, 65]. *BMI1*, a member of the polycomb repressive complex 1 (PRC1), is overexpressed in SHH MB and its

transcription is driven in part by GLI family members [66]. BET bromodomain proteins recognize acetylated lysine in histones and therefore serve as epigenetic "readers." Inhibition of BET proteins with JQ1 in transgenic mouse models and patient-derived SHH tumors resulted in decreased tumor cell proliferation even in tumors resistant to SMO inhibitors [41]. The authors found that *GLI1* and *GLI2* transcription are subject to epigenetic control by BET proteins and modulation of these downstream effectors of SHH holds promise, particularly for patients with primary or secondary resistance to SMO inhibitors [41].

1.3.4 Chromatin remodeling

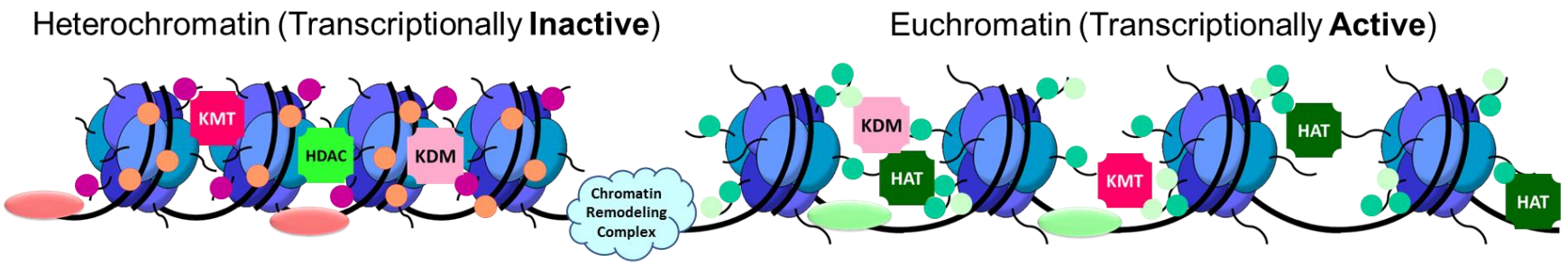
Aside from covalent modifications to DNA or histones, another method of epigenetic control is chromatin remodeling [49, 57]. This process involves molecular machinery that utilizes the energy of ATP hydrolysis to disrupt DNA histone interactions, altering the position of nucleosomes, or exchanging histone protein variants [49]. There are four families of mammalian chromatin-remodeling ATPases that are differentiated based on the binding domains that enable function. These ATPases work in large complexes along with enzymes described above that modify DNA and histone tails. Mutations of several members of SWI/SNF chromatin remodeling complexes have been identified in SHH MB including *ARID1A*, *ARID1B*, and BRG1 (*SMARCA4*) [15]. In another study using a SMO mouse model, the chromatin remodeler BRG1 was shown to coordinate genetic and epigenetic networks in mouse cerebellar development and in murine SHH medulloblastoma [67].

1.3.5 Other epigenetic mechanisms

Lastly, while not acting directly on chromatin, RNA interference by non-coding RNAs called microRNAs (miRNAs) can alter the expression of genes without changing the underlying DNA sequence in SHH MBs as reviewed by Wang et al. in 2018 [68]. Both oncogenic roles and tumor

suppressor roles for miRNAs have been proposed. A group of oncogenic miRNAs termed the miR-17-92 cluster is overexpressed in MB, predominantly in SHH MB [68]. This cluster has been identified as frequently involved in cancer. Experiments knocking out these genes resulted in smaller cerebella and less foliation indicating a crucial role for these genes in cerebellar development which implicates them in tumorigenesis as well [68]. Using a mouse model for SHH MB, researchers observed no tumor development with knockdown of miR-17-92, confirming the involvement of these miRNAs in MB [68]. There are also several examples of miRNAs acting as tumor suppressors. One example is miR-128a which is reported to have decreased expression in MB and is known to regulate BMI1 proto-oncogene, polycomb ring finger (*BMI1*) [68]. Less miR-128a could be part of the reason BMI1 is upregulated in MB, which results in transcriptional inhibition of the *CDKN2A* gene which encodes for two cell cycle inhibitors, p16INK4A and p19ARF [68].

The importance of alterations in normal epigenetic regulation of transcription is becoming increasingly clear. In pediatric cancers, epigenetics may be the path to targeted treatments in tumors that typically have few genetic mutations. Our understanding of these factors and how they function is still a work in progress, but the next few years hold promise for exciting discoveries that will likely change our understanding of cancer and how to treat it. Importantly, epigenetic regulation can be modulated, which increases the likelihood that research in this area can lead to targeted therapeutics.



DNA methylation	**Histone Modifications**	**Chromatin Remodeling**
Me DNA methylation DNA demethylation	**Activating** Ac — Acetylation of H3K9, H3K27, H4 Me — Methylation of H3K4, H3K36 **Repressive** Me — Methylation of H3K9, H3K27	Chromatin Remodeling Complex
Cytosine → 5-Methylcytosine (5mC) **Genes hypomethylated in SHH MB** *VAV1, TGFB1, MET* **Genes hypermethylated in SHH MB** *TERT1, MSH2, PTCH1, ZIC2*	**Histone modifiers mutated or with altered expression in SHH MB:** HAT — **Histone acetyltransferases:** EP300, CREBBP, PCAF/KAT2B HDAC — **Histone deacetylases:** HDAC1,HDAC2, HDAC3, HDAC6 KMT — **Histone lysine methyltransferases:** MLL2 (KMT2D), MLL3 (KMT2C), EZH2 KDM — **Histone lysine demethylases:** KDM1A	**Components of chromatin remodeling complexes mutated or with altered expression in SHH MB:** **SWI/SNF** complexes – ARID1A, ARID1B, BRG1 (SMARCA4)

Figure 1.8 Summary of Epigenetics in SHH Medulloblastoma

Top - Depiction of heterochromatin versus euchromatin and the epigenetic modifications observed in these states. Middle –Three major processes are responsible for epigenetic control of gene expression: DNA methylation, histone modification, and chromatin remodeling. Bottom – Examples of specific epigenetic factors or the effects of epigenetic alterations that have been reported in SHH MB. 5mC: 5-methylcytosine, HAT: histone acetyl transferases, HDAC: histone deacetylases, HMT: histone methyltransferases, HDM: histone demethylases, SWI/SNF: SWItch/Sucrose Non-Fermentable. From a review in preparation with Yun Wei

1.4 HELLS

1.4.1 Helicase, Lymphoid Specific

In 1996, the Muegge group at the National Cancer Institute at Frederick, Maryland cloned a novel putative helicase from murine fetal thymocytes and called it lymphoid-specific helicase (Lsh) as it was only found in early thymus tissue, but not in other organs like the heart, liver, lung, muscle, brain, or kidney [69]. In subsequent publications by their group and others, they proceeded to characterize Lsh, which is now officially known as helicase, lymphoid-specific (*HELLS*), but has also been called Proliferation-Associated SNF2-like Gene (PASG), and SWI/SNF2-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 6 (SMARCA6).

HELLS is a member of the SNF2 family of chromatin remodelers, a group of ATP-dependent enzymes that modify nucleosome organization and position by disrupting histone-DNA interactions [70]. HELLS is an important epigenetic regulator, linking multiple mechanisms to regulate chromatin accessibility and initiate a repressive chromatin environment [71]. Binding of HELLS to DNMT1 and DNMT3B in addition to co-immunoprecipitation of HELLS with HDAC1 and HDAC2 has been reported [72]. Roles for HELLS in epigenetics, senescence, DNA repair, transcription, stem cell maintenance, HOX gene control, development, and meiosis have been proposed [71-77].

Loss of *Hells* in a mouse model resulted in perinatal lethality and global hypomethylation of DNA [78, 79]. Another model used a hypomorphic mutation of *Hells* that allowed for the survival of some mice for several weeks, however, these mice displayed growth retardation, low birth weight, failure to thrive, and a premature aging phenotype that included graying and loss of hair, reduced fat deposition, osteoporosis, and premature death [80]. Increased expression of tumor suppressor genes *CDKN2A* (P16INK4A, P19ARF), *TP53*, and *CDKN1A* (P21CIP1) was observed with

loss of *HELLS* [80]. Experiments with depletion of *Hells* in murine embryonic fibroblasts (MEFs) resulted in reduced proliferation and signs of cell senescence with defects in spindle formation, micronuclei, and increased DNA content [81]. In keeping with a cell senescence phenotype, *Hells*$^{-/-}$ MEFs also showed increased resistance to radiation [80].

Human *HELLS* is located on chromosome 10, while murine *Hells* in on chromosome 19. The *HELLS* gene is composed of 22 exons which results in multiple isoforms [82]. Murine HELLS and human HELLS share 95% protein homology [83]. While HELLS contains the ATP binding and C-terminal helicase domains common to SNF2 chromatin remodeler proteins, it lacks the other domains seen in these proteins, e.g. BRK, PHD, RING finger, chromodomains, or bromodomains [84]. There is also a nuclear localization domain, and HELLS protein is localized to the nucleus (Figure 1.9) [85].

While there are many reports of HELLS functions in various conditions which are described below, there are far fewer reports of HELLS regulation. In human embryonic kidney and human lung fibroblast cell lines, E2F1 binding to the *HELLS* promoter was observed resulting in regulation of HELLS transcription [86]. There are also reports of transcriptional activation of HELLS by E2F1 in gliomas and retinoblastoma [74, 87]. In skin stem cells, HELLS has been reported to be a target of an isoform of TP63 [88]. However, there are no reports of HELLS regulation in medulloblastoma.

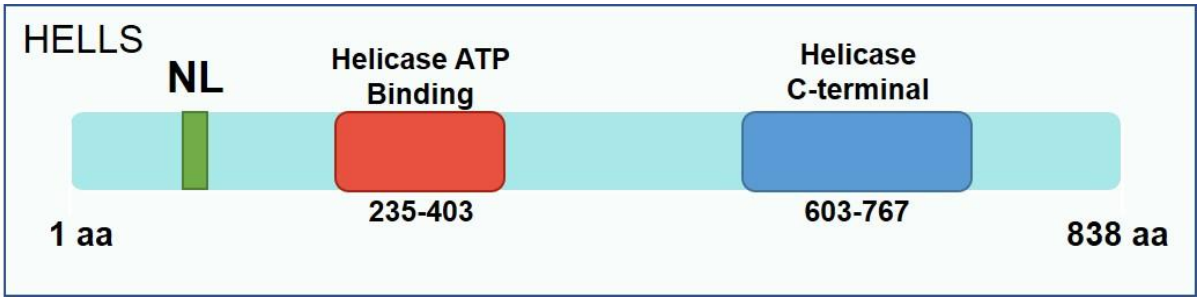


Figure 1.9 Protein structure of human HELLS
In contrast to other members of the SNF2 family of helicase proteins, the HELLS protein contains only the helicase ATP binding and helicase C-terminal domains as well as a nuclear localization signal. Murine and Human HELLS proteins share 95% homology [83]. Modified from [84].

1.4.1.1 HELLS in DNA Methylation

As mentioned previously, HELLS is an important regulator of epigenetics that links different mechanisms of chromatin modification [71]. There are numerous reports regarding HELLS involvement in DNA methylation. Dennis et al. observed genome-wide loss of methylation in the Hells$^{-/-}$ model in spite of normal mRNA levels of DNA methyltransferases which led the authors to hypothesize involvement of HELLS in de novo or maintenance DNA methylation via regulation of chromatin accessibility [79]. In the following years, HELLS association with chromatin was confirmed as well as a direct role for HELLS in centromeric DNA sequence methylation [85]. Zhu et al. reported the requirement of HELLS in de novo DNA methylation, but not in maintenance of previously methylated episomes [89]. Separately, Myant and Stancheva found that HELLS binds to DNMT1 and DNMT3B, acting as a recruiting factor for DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), but that it is not detected as part of a large multiprotein complex [72]. They also reported that HELLS promotes binding of DNA methyltransferases in developmentally programmed DNA methylation which facilitates gene silencing during lineage commitment and differentiation [90]. During development, DNA methylation associated with HELLS was found to silence some specific HOX genes [91].

In a mouse embryonic carcinoma cell line, differentiation was induced with all-*trans* retinoic acid which resulted in silencing of stem cell genes via CpG methylation. This methylation was mediated in part by HELLS, as methylation was reduced and gene silencing was found to be incomplete with knockdown of HELLS [92]. In addition, HELLS was detected upstream of the OCT4 transcription start site [92]. Further studies involved tethering HELLS to the OCT4 locus to observe epigenetic changes induced by HELLS [71]. Ren et al. reported that in addition to gain of DNA methylation in OCT4 enhancer sites, they noted histone modifications and changes in chromatin accessibility resulting in a transcriptionally repressive state with tethering of HELLS [71].

1.4.1.2 HELLS in Histone Modification

As mentioned in the DNA methylation section, HELLS recruits HDAC1 and HDAC2, and this results in changes to the acetylation status of genes [72]. Repression of p16INK4a expression is achieved by the recruitment of HDACs to the *CDKN2A* promoter [77]. Even though HELLS has not been identified as part of a large complex, HELLS associates with components of the polycomb repressive complex 1 (PRC1) and influences PRC-mediated modifications of histones [91]. Formation of monomethylated histone 3 lysine 4 (H3K4me1) at putative enhancers was observed in *Hells*$^{-/-}$ MEFs with concurrent hypomethylation of DNA [93]. Some of these differentially enriched H3K4me1 regions were found at neuronal lineage genes [93].

1.4.1.3 HELLS in Chromatin Remodeling

ATP-dependent chromatin remodeler proteins act as the enzymatic engine of complexes of proteins, but HELLS has not been identified as part of one of these large complexes. In spite of this, studies of the effects of HELLS depletion indicate that ATP dependent nucleosome remodeling in conjunction with cytosine methylation is the primary molecular function of HELLS [94]. In the developmental disease immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome, Jenness et al. identified a functional nucleosome remodeling complex comprised of HELLS and CDCA7 [95]. DNMT3B is recruited to this complex, resulting in increased cytosine methylation and nucleosome remodeling [95]. In these proteomic studies done in Xenopus egg extracts, HELLS alone was unable to remodel nucleosomes, but when part of a bipartite complex with CDCA7, a functional remodeling complex was formed [95].

1.4.2 HELLS in Development and Disease

1.4.2.1 HELLS in Development

HELLS was found to be highly expressed during murine development of the face, limbs, skeletal muscle, heart, and tail [73]. HELLS was one of 40 genes identified as signature genes in a meta-analysis of human embryonic stem cells [96]. Deletion of HELLS in mice resulted in perinatal lethality but apparently normal embryonic development [79]. While development was grossly normal, low birth weight, reduced lymphoid numbers, and renal lesions were noted [79]. A more detailed look at HELLS in murine neural development demonstrated a crucial role for HELLS with reduced growth, increased apoptosis, and defects in self-renewal of neural stem/progenitor cells when HELLS is depleted [83]. Additionally, in the previously mentioned developmental disorder ICF, mutations in the HELLS gene are reported to cause one version of the disorder, ICF syndrome type 4 [97]. Importantly, cerebellar development was not the focus of any of these studies.

1.4.2.2 HELLS in Cancer

HELLS has been implicated in a number of different cancers including leukemia [98, 99], retinoblastoma [74], lung cancer [100], gliomas [87], head and neck cancer [101, 102], breast cancer [103], prostate cancer [104], and skin cancers [88].

High levels of HELLS alternative transcripts were observed in samples of acute myelogenous leukemia and acute lymphoblastic leukemia [98]. In a separate study, mice given hematopoietic progenitor cells lacking HELLS displayed defective hematopoiesis, and some of these mice developed erythroleukemia [99].

In a mouse model of retinoblastoma (RB), HELLS was found to be upregulated along with UHRF1 (ubiquitin-like, containing PHD and RING finger domains 1), another chromatin remodeler [74]. Benavente et al. found that HELLS was tumorigenic only when upregulated in the tumor cell of

origin and that HELLS upregulation epigenetically mediated the aberrant expression of genes that drive retinoblastoma tumorigenesis [74]. Depletion of HELLS in RB cell lines resulted in significant reductions in colony number [74]. Likewise, cells lentivirally infected with shRNA to HELLS were used for orthotopic xenograft tumors and resulted in delays of tumor formation, smaller tumors, and less overall tumor burden, leading the authors to conclude that targeting HELLS may be a promising treatment strategy [74].

HELLS in non-small cell lung cancer presents an interesting spectrum of expression. Yano et al. reported that while 21% of non-small cell lung cancer samples showed increased expression of HELLS, 53% had similar levels of HELLS when compared to matched normal tissue [100]. Additionally, in contrast to a number of other studies, decreased expression of HELLS was observed in ~26% of tumor tissue [100]. Interestingly, in this study ten splicing variants of HELLS were discovered, some of which were tumor-specific [100].

HELLS is highly expressed in astrocytomas and glioblastomas, and elevated expression is correlated with progression and poor prognosis [87]. When HELLS was overexpressed in a xenograft model, the resulting tumors were larger compared to tumors from cells without ectopic expression of HELLS [87].

Studies in head and neck squamous cell carcinoma (HNSCC) revealed upregulation of HELLS downstream of *FOXM1* and promoter hypermethylation of *CDKN2A*, the gene for the cyclin-dependent kinase inhibitor $p16^{INK4A}$, with a reduction of $p16^{INK4A}$ expression [101, 102]. When they knocked down the expression of *HELLS* in normal human oral keratinocytes using siRNA, they observed increased $p16^{INK4A}$ and decreased expression of *DNMT1* and *DNMT3B* [101].

1.5 In search of novel downstream effectors of SHH

The overarching goal of research in the Kenney lab is to identify and characterize downstream effectors of SHH signaling that are involved in cell cycle control in cerebellar development and in SHH MB. The major players and their functions have been well characterized, but efforts to inhibit the SHH pathway with Smoothened inhibitors have met with limited success. Aberrations downstream of SMO have the potential to be more effective and more specific. As the pathway has been well characterized, SHH MB and mouse cerebellar development provide ideal systems within which we can explore further downstream of GLI, MYCN, and YAP1. Identification of genes and proteins with altered levels of expression in neural precursors with SHH stimulation enables us to tease apart the functional outcomes of SHH signaling. A better understanding of the factors involved has the potential to lead to improved diagnostics, prognostics, and precision therapeutics.

As the mutational burden of SHH MB is quite low, we asked whether any epigenetic factors were being regulated by SHH signaling in the developing cerebellum and in SHH MB. We identified the chromatin remodeler HELLS as a gene with marked upregulation in SHH-N stimulated CGNPs and in both murine and human SHH MB. Chapter 2 details our discovery of YAP1 mediated regulation of HELLS downstream of SHH.

Additionally, an active area of research in the lab has been to elucidate the regulation of HIF1α downstream of SHH signaling in the developing cerebellum and in SHH MB. In Chapter 3, an introduction to this research as well as our results implicating reactive oxygen species signaling in the stabilization of HIF1α in normoxic conditions is provided.

Chapter 2 Upregulation of the Chromatin Remodeler HELLS is mediated by YAP1 in Sonic Hedgehog Medulloblastoma

2.1 Author's Contribution and Acknowledgement of Reproduction

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MHR, RR, and AMK contributed to conception, the design of the study, and methodology. AMK sponsored the study. MHR acquired, analyzed, and interpreted the Western blot, qRT-PCR, and R2: Genomics Analysis and Visualization Platform data. MHR and SL acquired the ChIP data and MHR analyzed and interpreted the ChIP data. HF and MDT analyzed the human patient data. MHR wrote the paper. All authors read the manuscript and approved the final version.

2.2 Abstract

Medulloblastoma (MB) is a malignant pediatric tumor that arises from neural progenitors in the cerebellum. Despite a five-year survival rate of ~70%, nearly all patients incur adverse side effects from current treatment strategies that drastically impact the quality of life. Roughly one-third of MB are driven by aberrant activation of the Sonic Hedgehog (SHH) signaling pathway. However, the scarcity of genetic mutations in MB has led to the investigation of other mechanisms contributing to cancer pathogenicity including epigenetic regulation of gene expression. Here, we show that Helicase, Lymphoid Specific (HELLS), a chromatin remodeler with epigenetic functions including DNA methylation and histone modification, is induced by Sonic Hedgehog (SHH) in SHH-dependent cerebellar progenitor cells and the developing murine cerebella. HELLS is also up-regulated in mouse and human SHH MB. Others have shown that HELLS activity generally results in a repressive chromatin state. Our results demonstrate that increased expression of HELLS in our experimental systems is regulated by the oncogenic transcriptional regulator YAP1 downstream of Smoothened, the positive transducer of SHH signaling. Elucidation of HELLS as one of the downstream effectors of the SHH pathway may lead to novel targets for precision therapeutics with the promise of better outcomes for SHH MB patients.

2.3 Introduction

Medulloblastoma (MB), the most common solid pediatric tumor, is a devastating central nervous system cancer of the cerebellum that is diagnosed in over 300 children in the US each year [5]. While a standard protocol of surgical resection, chemotherapy, and radiation has improved the five-year survival rate for medulloblastoma patients overall, survivors are left with life-long effects including cognitive deficits and an increased risk of secondary tumors [11]. Many of these sequelae are a direct result of the current aggressive treatment strategy that does not yet account for tumor heterogeneity, highlighting the need to develop targeted, personalized treatments that are both highly effective and less toxic than current therapies.

As in most cancers, there is considerable heterogeneity in medulloblastoma. Classification of tumors based on genetic and phenotypic characteristics has resulted in the division of medulloblastoma into four main subgroups, one of which is driven by aberrant Sonic Hedgehog (SHH) pathway activity [18, 105, 106]. The SHH subgroup represents approximately 30% of all medulloblastomas. The overall five-year survival rate for SHH MB is fairly high at ~70%. However, patients lacking functional p53 or with metastasis have a worse prognosis, and recurrence of medulloblastoma is uniformly fatal [38, 107-109]. Recently, the SHH subgroup was further sub-typed into four distinct groups based on clinical features, DNA methylation status, and gene expression [1].

The Sonic Hedgehog pathway is critical for embryological development with important roles in patterning, cell differentiation, and organogenesis. Any defects in the SHH pathway can result in severe malformations [20, 110]. In cerebellar development, the SHH pathway is essential. SHH is secreted by Purkinje cells in the molecular layer, forming a gradient of SHH ligand that drives the proliferative program responsible for expanding the population of cerebellar granule neuron

precursors (CGNPs) in the external granule layer [34]. This program is activated postnatally in mice, and without SHH pathway activity, the population of CGNPs is severely reduced, resulting in a much smaller cerebellum that also lacks proper foliation [34, 35]. Importantly, CGNPs are also believed to be the progenitor cells for SHH MB [111].

Mechanisms driving cell proliferation are often conserved between development and tumorigenesis, so it is not surprising that in SHH medulloblastoma, the same mitogenic pathway responsible for development of the cerebellum is aberrantly activated through mutations of regulators (*PTCH1, SMO, SUFU*) or amplification of downstream effectors (*MYCN, YAP1, GLI*) [18, 105]. Aberrant activation of the SHH pathway, combined with other signaling pathways, results in transformation and proliferation of progenitor cells which ultimately form a tumor [34, 112-114]. Many cancer types other than medulloblastoma have reported SHH pathway activation including cancers of the lung, pancreas, breast, and prostate in addition to basal cell carcinoma, leukemias, and gliomas [115]. Involvement of Sonic Hedgehog in so many neoplasms demonstrates that a better understanding of the pathway and the downstream effectors is an important avenue of cancer research [37, 110].

Identification of downstream components of the SHH pathway is an essential part of dissecting Sonic Hedgehog driven carcinogenesis and tumor maintenance which may lead to the discovery of actionable targets for the development of therapeutics. One of the downstream effectors of SHH that is conserved between cerebellar development and medulloblastoma is Yes-associated protein 1 (YAP1) [29]. YAP1 is a transcriptional co-activator that, in concert with transcription factors such as TEA domain transcription factor (TEAD) family members, regulates expression of genes in response to signals from upstream pathways including the Hippo pathway and the SHH pathway [29, 30, 116-119]. Fernandez et al. previously established that YAP1 is amplified and

highly expressed in SHH MB and in SHH-N stimulated CGNPs [29]. In that study, YAP1 upregulation was shown to mediate proliferation of CGNPs even in the absence of SHH [29]. In an extension of those findings, using an SHH MB mouse model, they demonstrated that tumors with elevated YAP1 expression grew faster and were radioresistant through the upregulation and activation of downstream components that resulted in a bypass of cell cycle checkpoints [30]. Additionally, Dey et al. identified a downstream component of YAP1 called Y-box protein 1 (YB1) that is upregulated in SHH stimulated CGNPs as well as in SHH MB and is required for cell proliferation in both of these systems [120].

While pediatric cancer research has made great strides, there is a basic difference between pediatric cancers and adult cancers that has yet to be fully explained. When adult cancers are analyzed, they tend to possess hundreds or even thousands of mutations. In contrast, pediatric cancers including medulloblastoma consistently display a low mutational load [13, 14, 121]. In the quest to understand how pediatric cancers form with very few genetic changes, researchers have had to look beyond the sequence of nucleotides that make up a gene. Epigenetics, heritable changes in gene expression without changes to the actual DNA sequence, has become an active area of pediatric cancer research [14, 122]. Through methylation of DNA, modifications of histone tails, and chromatin remodeling, epigenetics determines the accessibility of DNA for transcription.

In analyzing a dataset from Paul Northcott comprising 181 SHH medulloblastomas, 65% of the somatic mutations identified were in epigenetic regulators [123] (https://pecan.stjude.cloud/bubble/BT-MB-SHH). Importantly, epigenetic regulation is reversible and could be a targetable approach to improve medulloblastoma treatment outcomes [14]. With this in mind, we analyzed the results of a microarray experiment comparing CGNPs +/- exogenous SHH-N ligand. Expression of several epigenetic factors was increased with the addition of SHH-N

to the cell culture media. One of these was the chromatin remodeler helicase, lymphoid-specific (HELLS). Upregulation of HELLS was also seen in a similar experiment from the Wechsler-Reya lab [124].

HELLS protein, also referred to as LSH, PASG, or SMARCA6 is a member of the SNF2 family of chromatin remodelers, ATP-dependent enzymes that modify nucleosome organization and position by disrupting histone-DNA interactions, thereby regulating access to the DNA [70]. While most members of this family accomplish this as a part of a large complex, HELLS has not been identified as part of such a complex [95]. However, binding of HELLS to DNMT3B and co-immunoprecipitation of HELLS with DNMT1, HDAC1, and HDAC2 has been reported [72]. Roles for HELLS in development, epigenetics, senescence, DNA repair, transcription, stem cell maintenance, *HOX* gene control, and meiosis have been proposed [71, 73-77, 90]. Importantly, homozygous deletion of *HELLS* is perinatally lethal, and a hypomorphic mutation of *HELLS* results in the short-term survival of a few pups which exhibit growth retardation, a premature aging phenotype, and early mortality [79, 80, 83, 84, 90]. *HELLS* is highly conserved with murine *HELLS,* which shares 95% protein homology with human *HELLS* [83]. Aside from the thymus and testis, expression of *HELLS* in normal adult cells is low, and higher expression of *HELLS* in stem cells decreases upon differentiation [82, 83, 92]. While there are many reports of HELLS functions in various conditions, there is very little known about how HELLS is regulated. Transcriptional activation of HELLS by E2F1 in gliomas and in cell lines has been reported and in skin stem cells HELLS has been reported to be a target of an isoform of TP63, but there are no previous reports of HELLS regulation in medulloblastoma [86-88].

With so many possible functions of HELLS that result in alterations in gene expression, knowing it is expressed at higher levels in CGNPs stimulated with SHH-N led us to hypothesize HELLS involvement in cerebellar development and medulloblastoma. Here, we investigated HELLS

expression and regulation in murine cerebellar development and SHH MB. We found significantly higher expression of *HELLS* in the developing murine cerebellum and in mouse SHH MB tumors. We also show evidence that this upregulation is driven by the SHH pathway through the downstream effector YAP1. These novel findings add to our understanding of the downstream activity of the SHH pathway and may help bridge the gap between the downstream effector YAP1 and the unregulated proliferation in SHH MB.

2.4 Results

2.4.1 HELLS expression is upregulated in SHH-N stimulated CGNPs and in the developing murine cerebellum

Cerebellar granule neural precursors, the putative cell of origin for SHH MB, can be isolated from the cerebella of 4-5-day old mice and grown in serum-free culture for 3-4 days before they begin to differentiate, even with SHH in the culture medium. These cells provide an important model as the SHH induced mitogenic signaling and gene expression seen in these cells is similar to that seen during cerebellar development and in SHH MB [4]. Microarray experiments were performed on CGNPs in the presence or absence of SHH-N, the recombinantly produced, biologically active N-terminal fragment of SHH, revealing genes regulated by SHH in our system. These data included a number of epigenetic factors that were upregulated in CGNPs cultured with SHH-N. One of these factors is the chromatin remodeler HELLS. HELLS was also identified as one of the genes upregulated by SHH-N in a 2003 study from the Wechsler-Reya lab [124]. Validation with quantitative real-time PCR (qRT-PCR) confirmed that *Hells* mRNA expression is consistently upregulated in SHH treated CGNPs. A statistically significant 6-fold increase in *Hells* mRNA was observed in CGNPs with SHH-N stimulation while expression returned to basal levels with the addition of either cyclopamine or SANT-2[125], which both directly inhibit Smoothened (SMO)

with different mechanisms of action (Fig. 2.1a). This differential regulation was also demonstrated in HELLS protein levels (Fig. 2.1b).

In mice, the cerebellum at birth is a small cluster of cells, but within 15 days, massive proliferation has taken place as well as foliation, resulting in a fully formed cerebellum [35]. As HELLS regulation seems specific to SHH signaling and this pathway is critical to post-natal cerebellar development, we speculated that HELLS could be part of the SHH proliferative program that is highly active in the cerebellum between postnatal days five and eight (P5-P8). Pooled tissue samples from three separate litters of mice were analyzed for mRNA expression and protein level of HELLS at multiple time points in development. We establish here that HELLS is significantly higher in the P7 murine cerebellum when compared to the P7 cortex. As illustrated in Figure 2.1c, cerebellar *Hells* mRNA levels are highest at P7, while in the cerebral cortex *Hells* mRNA levels are highest at P1, but still lower than in the cerebellum. In keeping with the results seen in expression, HELLS protein levels (Fig. 2.1d) in the cerebellum are highest at P7 (n=3). This timing coincides with highly active SHH signaling, suggesting that HELLS may have a role in SHH dependent postnatal cerebellar development.

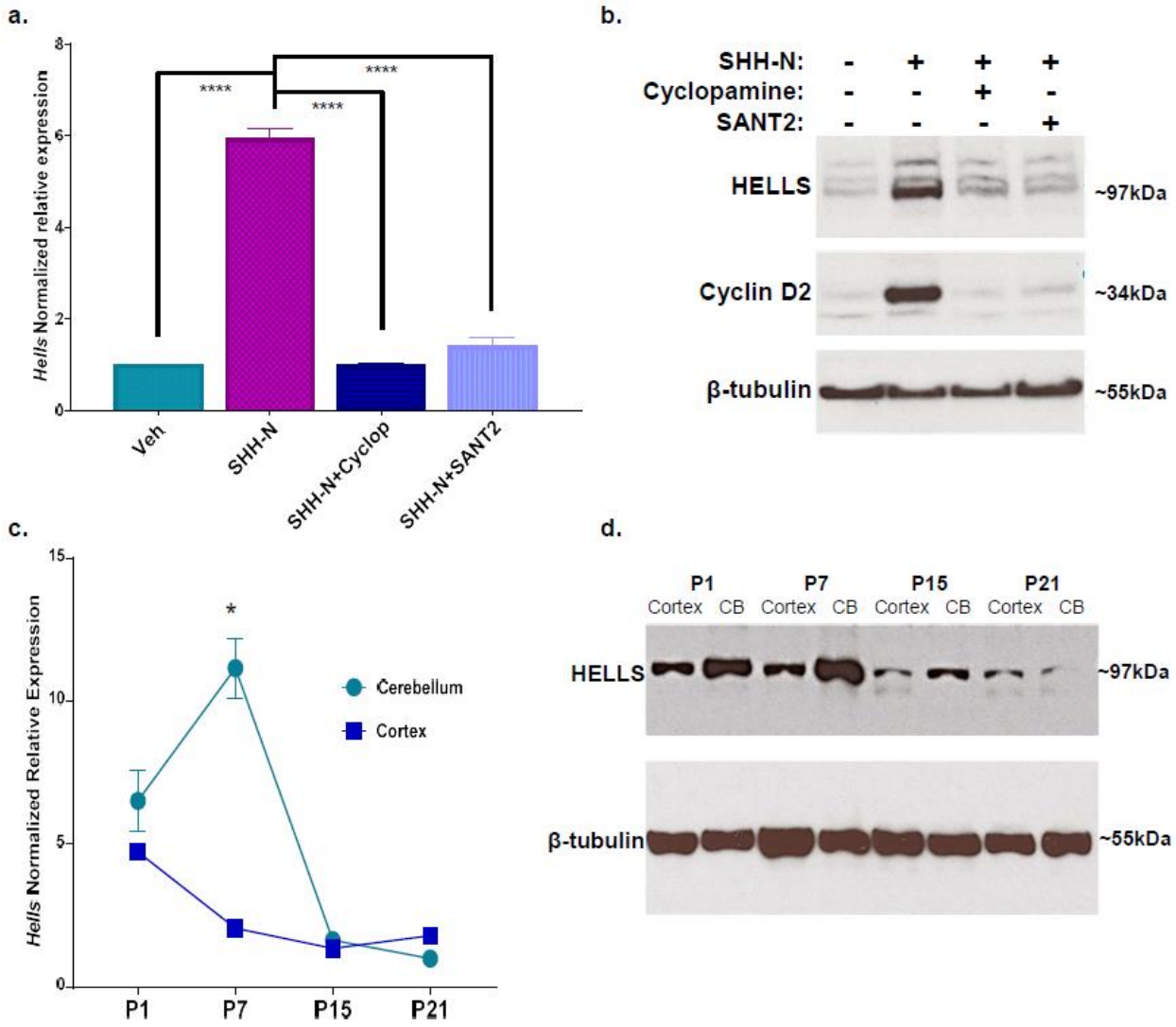

Figure 2.1 HELLS (Helicase, Lymphoid specific) is upregulated in SHH-N stimulated CGNPs and in the developing mouse cerebellum

(a) qRT-PCR analysis of SHH-N stimulated CGNPs show increased *Hells* expression at 48 hours which is abrogated by inhibition of SMO with either cyclopamine or SANT2. Dunnett's Multiple Comparison Test of ANOVA was used. n=3, *P*<.0001; Data represent mean ± S.E.M. (b) Western blot analysis indicates that protein levels of HELLS are similarly elevated in the same experiments depicted in 1a. CyclinD2 is shown as an indicator of SHH signaling [2]. Data are representative of 3 independent replicates. Full-length blots are presented in Supp. Fig. S4. (c) qRT-PCR analysis illustrates *Hells* mRNA levels are significantly higher in the cerebella of P7 mice compared to cortex at a time coinciding with high SHH levels in the cerebellum. Normalization to P21 cerebellum. Two-tailed T-test was used. n=3, *P*=0.0132; Data represent mean ± S.E.M. (d) Protein levels of HELLS are also elevated most significantly in the cerebella of P7 mice during cerebellar development.

2.4.2 Elevated expression of HELLS in Human SHH MB

Developmental pathways such as SHH are often co-opted in cancer, and this is definitely true for SHH MB. Since we observed increased levels of HELLS in SHH stimulated CGNPs and in the developing murine cerebella, we next sought to determine whether *HELLS* is upregulated in human MB. To this end, we queried a database of human medulloblastoma samples originally published in Pugh et al. [126]. As shown in Figure 2.2a, compared to normal cerebellum *HELLS* is expressed at higher levels in all medulloblastomas with the highest expression in the SHH subgroup.

Further analysis of the Cavalli 2017 dataset utilizing the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) allowed us to visualize HELLS expression in the SHH subtypes (Fig. 2.2b). HELLS expression is highest in the SHH α subtype. This group comprises primarily children aged 3-16 years with higher rates of MYCN and GLI2 amplifications as well as TP53 mutations [1]. Consequently, SHH α is the SHH subtype with the worst prognosis [1].

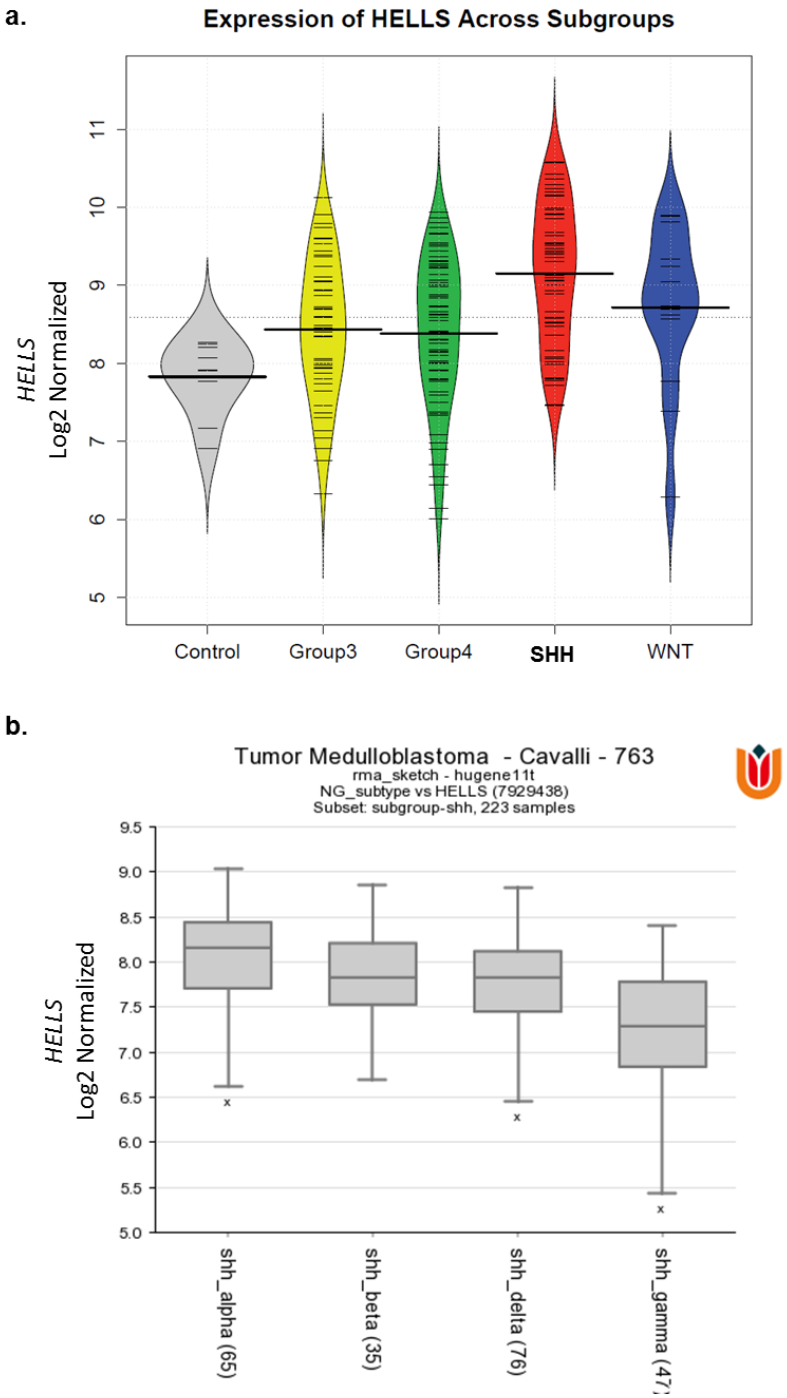

Figure 2.2 HELLS is upregulated in human SHH medulloblastoma
(a) HELLS upregulation was observed across MB subgroups with the highest levels of HELLS gene expression observed in the SHH subgroup of MB. This figure represents data previously published by Pugh et al, 2012 [121]. (b) Further analysis of the Cavalli 2017 dataset utilizing R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl) allowed us to visualize HELLS expression in the SHH subtypes. HELLS expression is highest in the SHH-alpha subset**.**

2.4.3 HELLS levels are increased in SHH murine MB

SHH medulloblastoma is characterized by aberrations in the Sonic Hedgehog pathway so our next question was to determine whether HELLS is upregulated in an SHH MB mouse model. We use the *NeuroD2-SmoA1* model originally developed by Jim Olson's research group [127]. In these mice, a point mutation of the *Smoothened* gene driven by the Neuro D2 promoter results in a SMO protein that is constitutively activated, generating downstream sustained high SHH signaling. Approximately 70% of these mice develop tumors between four and six months of age. Upon symptom onset such as ataxia or other neurological signs, the tumor and adjacent non-tumor cerebellar tissue can be collected for analyses. As shown in Figure 2.3a, a comparison of mouse MB tumors and adjacent non-tumor cerebellum reveals an average 10-fold increase in the expression of *Hells* mRNA in these tumors. This increase can also be seen in the level of HELLS protein from eight separate SHH murine tumors compared to normal adjacent cerebellum (Fig. 2.3b and c).

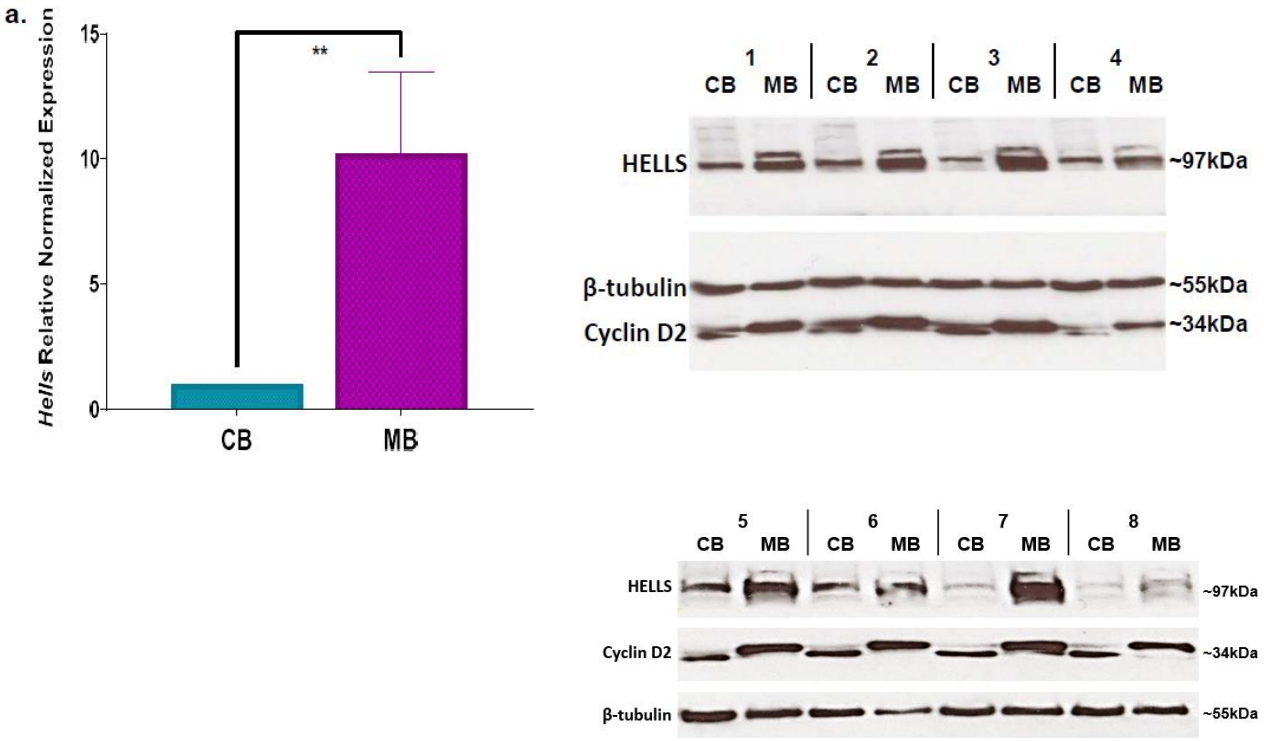

Figure 2.3 HELLS is upregulated in SHH murine medulloblastoma

(a) *Hells* mRNA transcript levels are significantly higher in *NeuroD2:SmoA1* mouse medulloblastoma (MB) than in adjacent non-tumor cerebellum (CB). Wilcoxon matched-pairs signed rank test was used. n=9; *P*=0.0039. Data represent mean ± S.E.M. (b) Protein levels of HELLS are similarly elevated in murine NeuroD2:SmoA1 MB compared to adjacent normal CB. Blots show HELLS levels in eight separate murine MB tumors with adjacent non-tumor CB. Western blots are cropped to show bands of interest clearly. Bands presented together in an image are from the same gel/blot.

2.4.4 HELLS expression is dependent on YAP1 activity

In our experimental systems, both mRNA expression and protein levels of HELLS are modulated by the SHH pathway. Downstream of SMO there is more than one possible effector of the pathway, so we next sought to determine which of these effectors might be implicated in HELLS regulation. The canonical SHH pathway transcriptional program is carried out by GLI proteins, so we started by investigating HELLS regulation by GLI. The GLI family of proteins are transcription factors with targets including *PTCH1, PTCH2,* and *GLI1* [112]. GLI1 and GLI2 have both been implicated in SHH MB and efforts to inhibit them are ongoing [115]. The small molecule GANT61 inhibits both GLI1 and GLI2 at an IC50 of ~5μM in the NIH 3T3 cell line [128]. Using GANT61 at a range of doses in CGNPs and MBCs we observed slight downregulation of HELLS (Figs 2.4a and 2.4c), along with concurrent increases in cleaved caspase 3 (Figs 2.4b and 2.4d) leading us to conclude this downregulation is due to cell death processes as opposed to direct regulation of HELLS by GLI proteins.

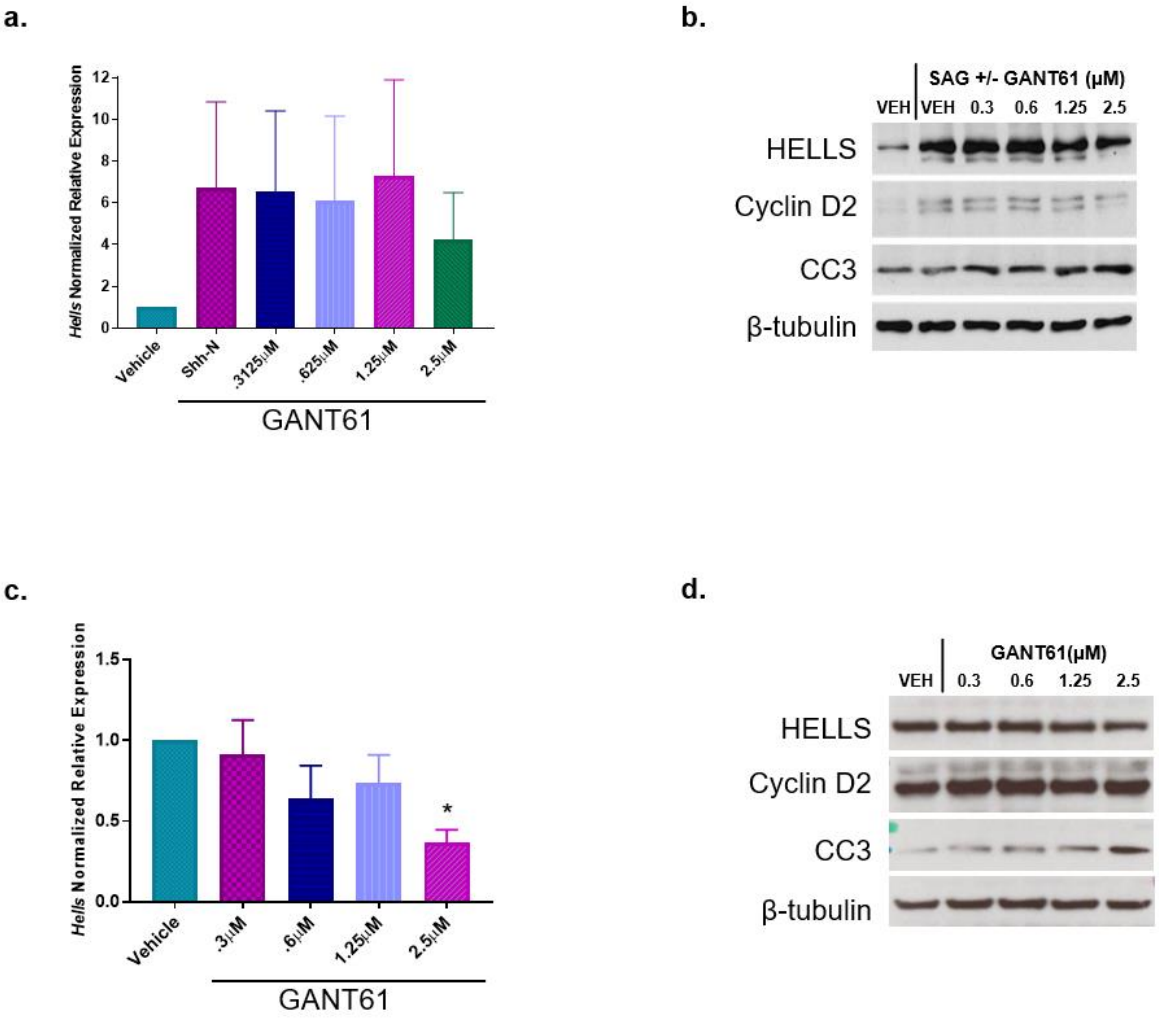

Figure 2.4 HELLS expression and protein levels are slightly decreased with inhibition of GLI1/2 with concurrent increases in cleaved caspase 3

(a) *Hells* mRNA expression in SHH-N or SAG stimulated CGNPs treated with increasing concentrations of the GLI1/2 inhibitor GANT61. n=3 (b) HELLS protein levels in GANT61 treated CGNPs. Blot is representative of 3 biological replicates (c) *Hells* mRNA expression in cultured murine medulloblastoma cells (MBCs) treated with increasing doses of GANT61; n=4, P=0.04 (d) HELLS protein levels in GANT61 treated MBCs.

We next asked whether HELLS may lie downstream of YAP1, a transcriptional coactivator known to be upregulated in SHH MB [29]. TEAD proteins are among the transcriptional partners of YAP1, and the interaction between TEAD and YAP1 can be inhibited with verteporfin, a drug used clinically with light activation to treat macular degeneration [129]. In multiple studies, verteporfin has also been shown to inhibit the YAP1/TEAD interaction without light activation [129-132]. *HELLS* expression in SHH stimulated CGNPs was inhibited in a dose-dependent manner when cells were treated with verteporfin (Fig. 2.5a). This inhibition was also seen at the protein level with no increase in cleaved caspase 3 (CC3) levels (Fig. 2.5b). This suggests verteporfin specifically downregulates HELLS via disruption of the YAP1/TEAD complex as opposed to causing the cells to undergo programmed cell death. To assess verteporfin effects in the context of tumor cells, we cultured medulloblastoma cells (MBCs) dissociated from *NeuroD2:SmoA1* murine tumors. In MBCs, the downregulation of HELLS expression with verteporfin was even more striking (Fig. 2.5c and 2.5d). This downregulation was seen at both the mRNA and protein levels and importantly, the levels of cleaved caspase 3 were not affected. Additionally, while levels of Cyclin D2 as a measure of proliferation did not change with verteporfin treatment in CGNPs, in MBCs there is a concurrent reduction of Cyclin D2 with the reduction of HELLS in response to verteporfin inhibition of the YAP1/TEAD interaction. These experiments suggest regulation of HELLS through YAP1, but cannot tell us whether *HELLS* is a direct target of the YAP1/TEAD transcriptional program.

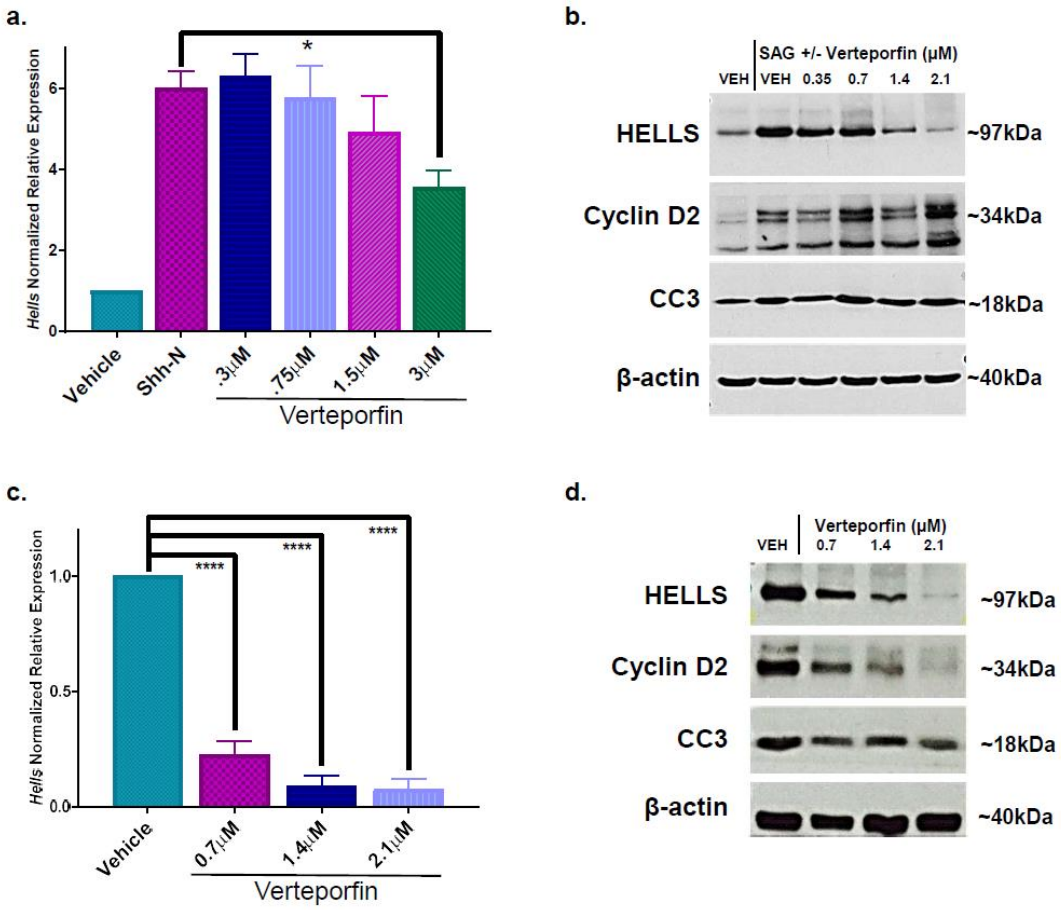

Figure 2.5 HELLS expression and protein levels are modulated with YAP1/TEAD inhibition downstream of SHH signaling

(a) *Hells* mRNA expression in SHH-N or SAG stimulated CGNPs treated with increasing concentrations of the YAP1/TEAD inhibitor verteporfin. Dunnett's Multiple Comparison Test of ANOVA was used. n=3, *P*=0.0435; Data represent mean ± S.E.M. (b) HELLS protein levels in verteporfin treated CGNPs. Blot is representative of 3 replicates. Full-length blots are presented in Supp. Fig. S4. (c) *Hells* mRNA expression in cultured murine medulloblastoma cells (MBCs) treated with increasing doses of verteporfin. Dunnett's Multiple Comparison Test of ANOVA was used. n=3, *P*=0.0001; Data represent mean ± S.E.M. (d) HELLS protein levels in Verteporfin treated MBCs. Western blots are cropped to show bands of interest clearly. Bands presented together in an image are from the same gel/blot.

2.4.5 YAP1/TEAD binding to DNA upstream of HELLS confirms direct regulation of HELLS

To further evaluate possible YAP1 regulation of HELLS downstream of SHH, we carried out chromatin immunoprecipitation (ChIP) followed by qRT- PCR. For these experiments, we utilized the PZp53 murine medulloblastoma cell line, a line derived from a mouse medulloblastoma heterozygous for PTCH1 and null for p53 [133]. First, we verified that these cells expressed HELLS and that HELLS was regulated in a manner similar to what we observed in CGNPs and MBCs (Fig. 2.6). Protein and DNA from cross-linked cells were immunoprecipitated using a YAP1 antibody, and the resulting DNA was queried with primers designed to recognize putative upstream TEAD binding sites as YAP1 itself does not bind directly to DNA. Using the Eukaryotic Promoter Database, putative TEAD binding sites were identified up to 5000 base pairs upstream of the HELLS transcription start site (TSS). As illustrated in Figure 2.7, five different binding sites were significantly enriched for YAP1/TEAD binding. Two of the binding sites are distal to the TSS and may represent binding to an enhancer as the transcriptional activity of YAP1 has been identified as being mediated by binding of TEAD to distal enhancers [117]. The other three sites enriched for YAP1/TEAD are located in a region identified as the HELLS promoter region. These experiments indicate YAP1/TEAD binding to HELLS upstream DNA, suggesting direct regulation of HELLS transcription by YAP1/TEAD in SHH medulloblastoma.

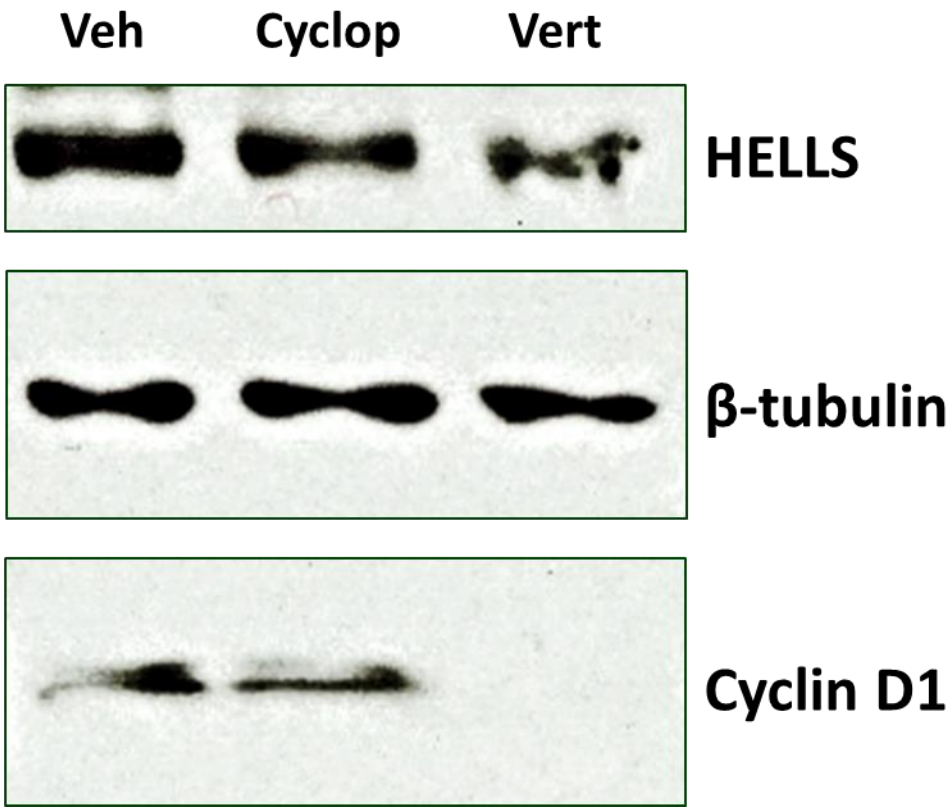

Figure 2.6 HELLS in PZp53 cells +/- cyclopamine +/- verteporfin
HELLS regulation in the murine cell line PZp53 is consistent with the regulation observed in primary mouse CGNPs and MBCs

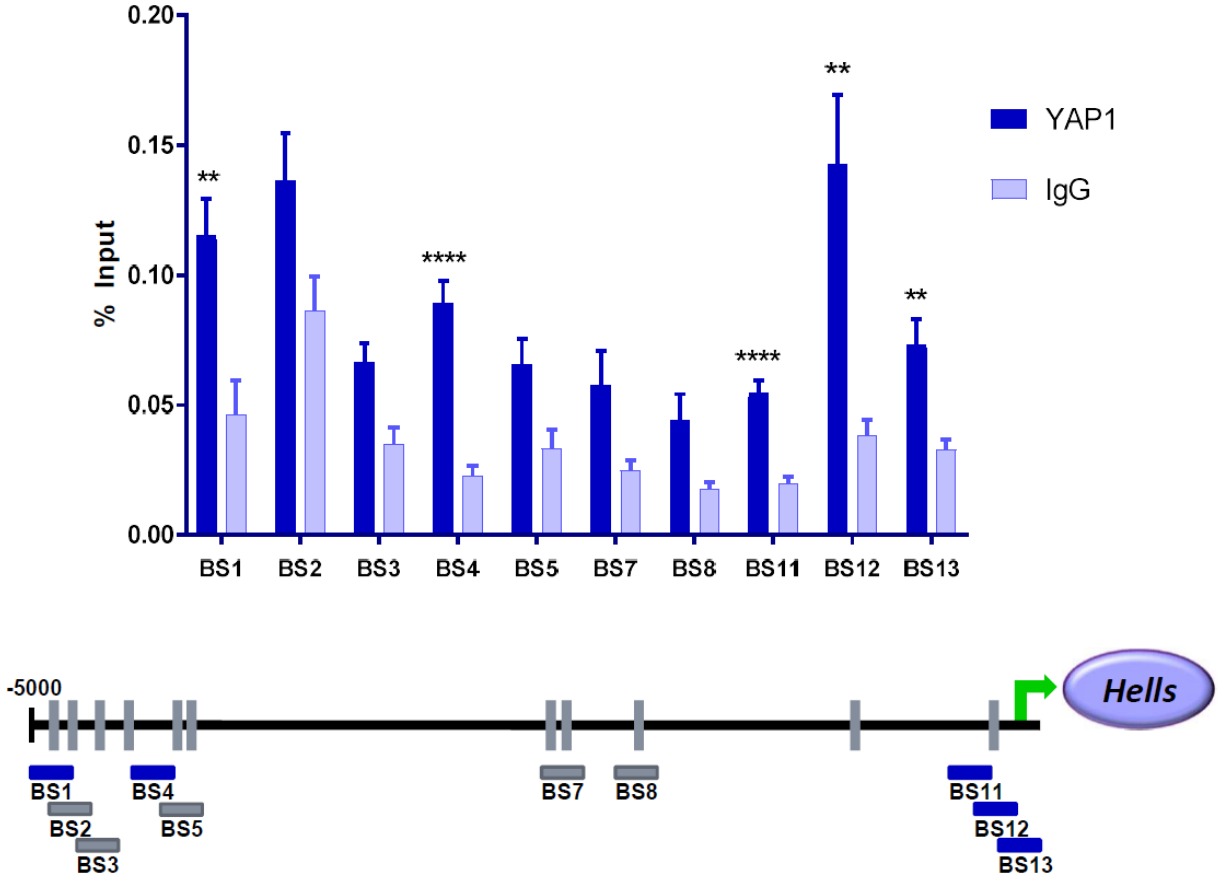

Figure 2.7 Binding of YAP1/TEAD to *Hells* upstream DNA

Putative TEAD binding sites up to 5000bp upstream of *Hells* transcription start site were identified. Tiled primers to those locations were designed and used to interrogate DNA isolated during chromatin immunoprecipitation using a YAP1 antibody. IgG as control. Values are presented as a percentage of input (non-immunoprecipitated chromatin). Data represent mean ± S.E.M. T-tests were used to compare YAP1 to IgG at each putative binding site and the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli was applied to avoid false discovery. n=8 (BS1 *P*=0.005; BS2 *P*=0.053; BS3 *P*=0.013; BS4 *P*=0.00003; BS5 *P*=0.025; BS7 *P*=0.055; BS8 *P*=0.041; BS11 *P*=0.00009; BS12 *P*=0.004; BS13 *P*=0.004)

2.5 Discussion

Here we have identified a novel role for HELLS in SHH dependent mitogenic signaling. In SHH stimulated precursor cells and in SHH MB cell culture, HELLS is upregulated at both the mRNA and protein levels. Analysis of the developing murine brain reveals elevated HELLS expression in the cerebellum compared to the neural cortex in keeping with the timing of high levels of SHH activity and CGNP proliferation. We observe upregulated HELLS expression in both murine and human SHH medulloblastoma. In murine primary cell culture, this upregulation can be abolished by inhibition of the SHH effector YAP1, suggesting regulation through this downstream effector.

Furthermore, chromatin immunoprecipitation indicates direct regulation of HELLS through YAP1. Our results indicate a possible role for HELLS in the proliferative program of Sonic Hedgehog in both development and in medulloblastoma (Fig. 2.8). Indeed, the verteporfin induced decrease in CyclinD2 in MBCs in contrast to in CGNPs links the YAP1/HELLS axis to a requirement for proliferation in tumor cells. This provides a therapeutic advantage because we did not see an effect on proliferation in CGNPs with verteporfin.

Epigenetics is becoming an increasingly important area of cancer research, particularly in pediatric malignancies where the low mutational burden leaves researchers looking for viable targets. Inhibitors of drugs targeting epigenetic factors have been approved for use in several cancers, and clinical trials are ongoing in several other types of cancer [134]. A more complete understanding of the epigenetic underpinnings of medulloblastoma could provide valuable avenues of research for therapeutic interventions that have the potential to be more effective and less harmful than our current non-specific DNA damaging radiation and chemotherapeutics. As a member of a family of chromatin remodelers with putative roles in senescence [77, 80, 135, 136], repression of tumor suppressor genes [77, 80], stem cell control [71, 83, 88, 92], Hox gene control [91], and

DNA repair [137], our identification of HELLS upregulation in medulloblastoma could be an important finding. That this upregulation is a result of SHH pathway activation and HELLS is also upregulated in normal murine cerebellar development adds weight to this theory.

HELLS is known to be an important epigenetic regulator, linking multiple mechanisms for chromatin remodeling including methylation of DNA and modification of histone proteins [71]. In embryological development, HELLS is vital for normal murine development with HELLS null pups dying at or shortly after birth with severe renal lesions [78]. A mouse model with a hypomorphic mutation resulted in some mice surviving, but with growth retardation, premature aging, and early death [80]. Coupled with these phenotypic results in mice with a truncated HELLS protein, upregulation of tumor suppressor genes including *p16INK4a, p21CIP1*, and *p53* was observed, leading to replicative senescence [80]. Less is known regarding HELLS effects in humans, but it is known that mutations of HELLS cause ICF syndrome (immunodeficiency, centromeric instability, facial anomalies) and that HELLS forms a nucleosome remodeling complex with the protein Cell Division Cycle Associated 7 that is defective in this disease [97]. Utilizing the R2 web-based genomics analysis and visualization application to analyze human gene expression in a large cohort of human medulloblastomas from Cavalli 2017 [1] allowed us to evaluate HELLS gene expression correlation in SHH MB. This analysis revealed highly significant correlations for genes involved in the cell cycle and DNA repair (Table 2.1). Developmentally, as cerebellar formation occurs predominantly postnatally in mice the effects of HELLS depletion on that process would be difficult to assess as pups die at birth, but a 2017 report from Yan et al. using neural stem/progenitor cells (NSPCs) showed the importance of HELLS in mouse neural development with reduced growth of these cells, increased apoptosis, and impaired ability of self-renewal [83].

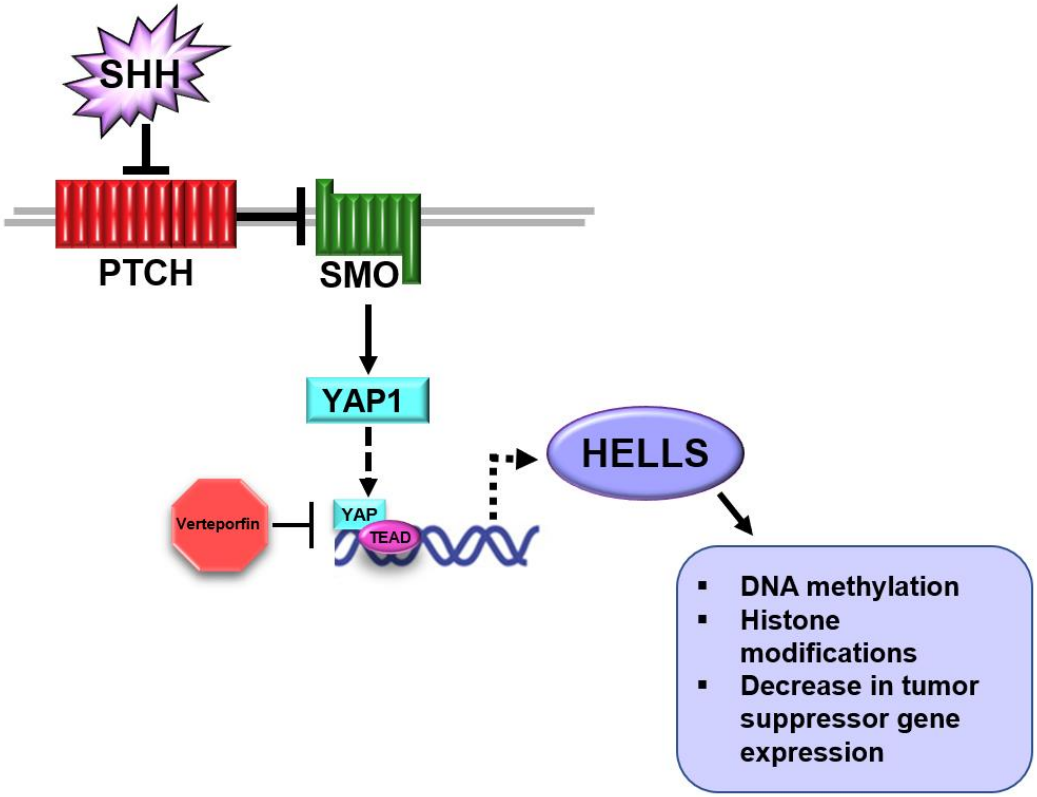

Figure 2.8 Model of SHH induced increase of HELLS
Activation of the SHH pathway leads to upregulation and activation of YAP1, a transcriptional co-activator that functions in partnership with TEAD family transcription factors. We propose that *HELLS* is a YAP1/TEAD transcriptional target. *HELLS* is upregulated in SHH-N stimulated CGNPs and in murine and human SHH MB. This upregulation can be modulated by inhibition of the interaction between YAP1 and TEAD family transcription factors using the drug verteporfin. Chromatin immunoprecipitation indicates YAP1/TEAD binds to DNA upstream of *Hells* suggesting that regulation of *Hells* transcription through YAP1 is direct.

Mini Ontology Analysis				
Group	InSet	Total	%	pval
All	3378	8836	38.20%	1
DNA repair	122	192	63.50%	**5.30E-13**
TF	284	783	36.30%	0.26
apoptosis	239	595	40.20%	0.33
cell cycle	281	467	60.20%	**1.70E-22**
development	552	1519	36.30%	0.13
diff	226	639	35.40%	0.14
drugtarget	423	1098	38.50%	0.84
kinase	274	623	44.00%	**3.10E-03**
membrane	1692	4691	36.10%	**2.30E-03**
sign transd	1033	2932	35.20%	**8.40E-04**
transcription regulator Act	432	1162	37.20%	0.46
transcriptional repressor Act	82	175	46.90%	0.02

Tumor Medulloblastoma - Cavalli - 763 - rma_sketch - hugene11t
subgroup-shh 223 of 763 samples, transform_2log, PresCalls>=1
Sourcegene=HELLS(7929438)
6818 combinations meet your criteria (3381 / 3437)
11656 combinations did not meet **p<0.01** as R p-value and **1** as minimal # of PresentCalls
p-value correction for multiple testing: False Discovery Rate
'R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl)'

Table 2.1: *HELLS* gene expression correlation in SHH MB Using R2, a web-based application for genomics analysis, we evaluated the correlation between *HELLS* expression and the expression of other genes in a human dataset from Cavalli et al 2017. Focusing on the 223 SHH MB samples in their dataset, we observe *HELLS* expression is most positively correlated with genes involved in DNA repair and cell cycle. There is a positive correlation for genes in the kinase, membrane, and signal transduction groups as well, but with less significance.

In SHH-N stimulated CGNPs and in normal murine cerebella with endogenous SHH expression, HELLS upregulation is observed in these highly proliferative cells. As HELLS is known to suppress inhibitors of the cell cycle[80], it is possible that increased HELLS levels in these cells promotes continued cell cycle activity and proliferation. Future work to dissect HELLS activity in these conditions may enable a better understanding of HELLS role in cerebellar development.

Involvement of HELLS has been reported in a number of different cancers including leukemia [98], retinoblastoma [74], lung cancer [100], gliomas [87], head and neck cancer [101, 102], breast cancer [103], prostate cancer [104], and skin cancers [88]. In some of these cancers, mutations are present resulting in increased HELLS levels or activity, however, in other cases, HELLS levels are decreased, or its function is abrogated. HELLS regulation of epigenetic factors is itself tightly regulated, and alterations in either direction result in deleterious effects [78, 99, 104]. Expression of HELLS in non-tumor cerebellar cells is low or non-existent, making HELLS an attractive, tumor-specific target for research. That HELLS expression in MBCs was abrogated to a greater extent than in CGNPs with lower doses of verteporfin seems to confirm the tumor-specific importance of HELLS.

YAP1 overexpression in SHH MB allows cancer cells to bypass cell cycle checkpoints by upregulating and activating downstream components [30]. While some of those components have been discovered, there are others yet to be identified. Our finding of YAP1 directed HELLS upregulation in SHH MB suggests a role for HELLS regulated chromatin remodeling in SHH medulloblastoma. As other epigenetic regulators have been targeted for cancer treatment with varying degrees of success, future studies to determine HELLS role in medulloblastoma may lead to new avenues of research and eventually to new therapeutic interventions.

2.6 Methods

Animal studies

All animal studies were carried out in accordance with the Emory University Institutional Animal Care and Use Committee guidelines under Dr. Kenney's approved IACUC protocol #2003395 "Interactions between signaling pathways in the developing brain and medulloblastoma." *NeuroD2:SmoA1* mice were purchased from Jackson Labs (008831); CD-1 mice were purchased from Charles River; *NeuroD2:SmoA1* and *Atoh1:GFP* mice were crossed to generate *NeuroD2:SmoA1;Atoh1:GFP*, in which all tumor cells produce GFP protein [45]. For studies involving HELLS in murine brain development, pooled tissue samples from three separate litters of CD-1 mice were used.

Cell Culture

CGNPs were isolated and cultured as previously published [2]. Briefly, cerebella from 5-day old CD-1 mice were harvested, cells were disassociated into a single cell suspension, plates were coated with poly-DL-ornithine, and cells were plated in DMEM-F12/N2 with or without recombinant SHH-N at 3μg/mL or SAG at 200nM to activate the SHH pathway.

MBCs were isolated from *NeuroD2:SmoA1* and *NeuroD2:SmoA1;Atoh1:GFP* murine tumor tissue. Tumors were disassociated into single cell suspensions using incubation with Papain/DNase, filtered through a cell strainer, and then separated by centrifugation through 35/65% Percoll (GE Healthcare) and cells at the interphase were collected for plating. MBC plates were coated with poly-D-lysine (Sigma) and Geltrex (Gibco) and cells were grown in Neurobasal medium with B27 supplement (without vitamin A), Glutamax, sodium pyruvate, and 1% penicillin-streptomycin (all from Gibco).

The PZp53 cell line was used for ChIP experiments. This cell line is derived from a $Ptc^{+/-}/p53^{-/-}$ murine medulloblastoma. The SHH pathway is constitutively active in this cell line and it is useful

in evaluating downstream effects on the pathway. PZp53 cells were cultured in DMEM with 10% FBS.

Drug treatment of cells was performed 24 hours after cells were plated and cells were allowed to grow another 48 hours in the presence of vehicle or drug. Cyclopamine (R&D Systems) was utilized at 1µg/mL. SANT2 (Alexis BioChemicals) was used at a concentration of 100nM. GANT61 (A kind gift from Dolores Hambardzumyan; synthesized at Memorial Sloan Kettering) was resuspended in ethanol to a concentration of 10mM and stored at -80°C until ready for use. Verteporfin (Sigma) was dissolved in DMSO to a concentration of 1.4mM and stored at -20° and then thawed in a 37° water bath just prior to use. GANT61 and verteporfin treatments were used at the indicated doses.

RNA extraction and qRT-PCR

RNA extraction from cells and tissues was performed according to the protocol provided by Genecopeia. Briefly, cells or tissues were homogenized in Trizol (ThermoFisher), phase separation was carried out using chloroform and centrifugation, and RNA was precipitated with alcohol washes. RNA was quantified with a Nanodrop 1000. The protocol for cDNA first strand synthesis was complete using the High-Capacity cDNA Reverse Transcription Kit from Applied Biosystems. Primer for *Hells* was purchased from GeneCopoeia, Inc. Rockville, MD (Primer ID: Mm-QRP-21432) Primers for controls *Gli1* (Unique Assay ID: qMmuCID0026119), *Gli2* (Unique Assay ID: qMmuCID0005725), *CyclinD2* (Unique Assay ID: qMmuCID0023538), *B2M* (Unique Assay ID: qMmuCID0040553), and *GusB* (Unique Assay ID:qMmuCID0046361) were purchased from Bio-Rad. Quantitative real-time PCR (qRT-PCR) was done in triplicate in 96 well plates using the SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) and the C1000 Touch™ Thermal Cycler with the CFX96™ Optical Reaction Module from BioRad. At least 3 biological replicates were

performed for each experiment and the results were processed for statistical analysis using the BioRad CFX Manager™ software and Prism (GraphPad).

Protein preparation and immunoblotting

Proteins were isolated from cells and tissues using the following methods. Cells from cell culture were washed with phosphate-buffered saline and collected in lysis buffer and processed as previously outlined [2]. Protein content was determined using the BioRad protein assay. Equal amounts of whole protein lysate were separated on SDS-polyacrylamide gels and transferred to activated polyvinylidene fluoride membranes (Millipore) Western blotting was done in keeping with standard protocols. Primary antibodies used were: anti-HELLS (ABD41, Millipore), anti-Cyclin D2 (sc-593, Santa Cruz), anti-Cyclin D1 (NBP2-32840, Novus Biologicals), anti-β-actin (4970S, Cell Signaling Technology), anti-β-tubulin (T4026, Sigma), and anti-cleaved caspase-3 (9661L, Cell Signaling Technology). Secondary antibodies conjugated to horseradish-peroxidase were: anti-mouse (715-035-150, Jackson Immuno Research), and anti-rabbit (31460, Pierce, Life Technologies). Blots were developed using Pierce ECL reagents and chemiluminescence was detected by exposing membranes to GE-Amersham film.

ChIP

Chromatin immunoprecipitation was done using the SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) from Cell Signaling Technologies. PZp53 cells were cultured to confluency, crosslinked with formaldehyde, and YAP1(D8H1X) XP Rabbit mAb (#14074 Cell Signaling Technology) was used to precipitate DNA bound to the YAP1/TEAD complex. Normal Rabbit IgG (#2729 CST) was used as a negative control and Histone H3 (D2B12) XP Rabbit mAb (#4620 CST) was used as a positive control for immunoprecipitation. After un-crosslinking and isolation of DNA, quantitative real-time PCR (qRT-PCR) was performed. Input 2% samples were also evaluated. Putative TEAD1 binding sites up to 5 kb upstream of the *Hells* gene were identified

using the Eukaryotic Promoter Database (https://epd.vital-it.ch/index.php) and tiled primer sets were designed to hybridize to these binding sites using PCR Tiler (http://pcrtiler.alaingervais.org/PCRTiler). Primer sequences were ordered from Eurofins (www.eurofinsgenomics.com) and are listed in Table 2.1. Immunoprecipitation was done three separate times with multiple samples immunoprecipitated each time and qRT-PCR was done on each set of samples (IP for YAP1, HistoneH3, and IgG).

Name	Sequence	Corresponds to murine *Hells* upstream DNA
Hells-1F	ACCACAGAGCCTGGGACA	-4896
Hells-1R	GGCCAGACACGCATACCC	-4896
Hells-2F	CTGAGATTGGCAACTTGGTGT	-4815
Hells-2R	CCCACTTTCTAGCAGGGACA	-4815
Hells-3F	TTGACCTTTTGTCCCCCTTT	-4699
Hells-3R	AGAGTTTGGAAGCAGTCTGAGC	-4699
Hells-4F	GGCATGCATCACCACACC	-4699, -4310
Hells-4R	CCAGCCTCATCAGCCACA	-4699, -4310
Hells-5F	CACCACACTGGGAGACCTG	-4310, -4264
Hells-5R	CCTTTGGGATGTGTTCTAAGCA	-4310, -4264
Hells-7F	CAGGTCGGCTTCGAACACT	-2391, -2333
Hells-7R	AATGACTTACACTTAGCCAGGCTTT	-2391, -2333
Hells-8F	GGAGAACTGATGTTGCCCAAA	-1958
Hells-8R	CAGGGTTCCTAAATGTCCACTG	-1958
Hells-11F	CAACTCGGGACCATCATTAAATA	-131
Hells-11R	GAGGGATGCGTTAAGCCTTT	-131
Hells-12F	GCGACATTCAAGGCTGGAG	-131
Hells-12R	CGGCTCACGAGATTTGGA	-131
Hells-13F	AGCGCGTCCAAATCTCGT	-131
Hells-13R	AAATTCGCGCGCTTCTCT	-131

Table 2.2 Primers for ChIP-qPCR

Statistical Analysis

Graphs were made using GraphPad Prism 7 (GraphPad Software) and were analyzed using ANOVA and two-tailed t-tests unless otherwise noted. For Figure 1A, 4A, and 4C, we used Dunnett's Multiple Comparison Test of ANOVA, which compares all columns versus the control column. For Figure 3A, Wilcoxon matched-pairs signed rank test was used to compare each matched pair of

tumor and non-tumor cerebellum. Data for Figure 5 was analyzed using multiple T-tests to compare YAP1 to IgG at each putative binding site and then the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli was applied to avoid false discovery.

(*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$; (****) $P < 0.0001$; (no asterisk) not significant.

2.7 Acknowledgments

The authors wish to thank Dolores Hambardzumyan for the kind gift of GANT61, Krishanthan Vigneswaran for guidance on ChIP, Victor Maximov for statistical analysis and manuscript assistance, and members of the Kenney lab for helpful discussion, technical advice, and proofreading.

Chapter 3 Reactive Oxygen Species Signaling Promotes HIF1α Stabilization in Sonic Hedgehog-Driven Cerebellar Progenitor Cell Proliferation

3.1 Author's Contribution and Acknowledgement of Reproduction

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* Equal contributions as second authors

Author contributions: Designed and carried out experiments, analyzed results, prepared figures, wrote and edited manuscript: NE, CRP, MHR, VM. Oversaw study, designed experiments, analyzed results, designed and edited figures, wrote/edited manuscript: AMK. The authors declare no conflict of interest.

3.2 Abstract

Medulloblastoma is the most common solid pediatric malignancy of the central nervous system. The current standard of care leaves patients with long-term side effects that severely impair quality of life, driving us to investigate mechanisms promoting tumor growth in order to find better treatments for these patients. The Sonic Hedgehog (SHH) subgroup of medulloblastoma is proposed to arise from SHH-dependent cerebellar granule neuron precursors (CGNPs). Using primary CGNP cultures, a well-established model for SHH-driven proliferation, we have investigated pathways whose activity drives cancer-associated phenotypes and thus might represent novel therapeutic targets. Here we show that SHH-treated CGNPs and mouse SHH medulloblastomas feature high levels of Hypoxia-Inducible Factor-1-Alpha (HIF1α), which is known to promote glycolysis, stemness, and angiogenesis. In CGNPs cultured under normoxic conditions, HIF1α is post-translationally stabilized in a manner dependent upon reactive oxygen species (ROS) and NADPH oxidase (NOX), both are which are also upregulated in these cells and mouse SHH medulloblastoma. Inhibition of NOX activity resulted in HIF1α destabilization and reduced levels of Cyclin D2, a marker of CGNP proliferation. Taken together, our findings suggest that future approaches promoting HIF1α destabilization through NOX inhibition could be therapeutically relevant in medulloblastoma, enabling de-escalation of toxic radiation and chemotherapy.

3.3 Introduction

Medulloblastoma (MB) is the most common solid pediatric malignancy of the central nervous system (CNS). MBs/primitive neuroectodermal tumors (PNETs) account for nearly 25% of pediatric CNS neoplasms [138-141]. The current standard of care consists of surgery, chemotherapy, and craniospinal radiation. This harsh regimen results in a "cure" rate of approximately 70%, but survivors are beset with permanent, long-term side effects, including cognitive impairment, seizures, premature aging, and increased susceptibility to cancer [142]. Development of novel molecular targeted therapies is critical to provide an improved quality of life for survivors and to reduce the incidence of recurrence, which is lethal. Arising in the posterior fossa, or cerebellum, medulloblastomas can be divided into four genetically and histologically distinct subclasses [143]. Approximately 30% of all cases of medulloblastoma are characterized by perturbations in the expression of Sonic hedgehog (SHH) pathway components [105]. In particular, MBs occurring in the youngest patients are primarily identified as SHH MBs. Elucidating the processes underlying early cerebellar development and aberrant SHH signaling is crucial in understanding medulloblastoma formation and developing targeted therapies featuring minimal side effects as well as permitting de-escalation of toxic radiation and chemotherapy.

Cerebellar granule neuron precursors (CGNPs), neural progenitors arising in the rhombic lip, are the proposed cells of origin for SHH MB [32, 144, 145]. At birth, the cerebellum consists of three layers: the external granule layer (EGL) where CGNPs first reside, the molecular layer (MOL) where Purkinje neurons localize, and the internal granule layer (IGL), where CGNPs ultimately translocate. SHH ligand is secreted by the Purkinje neurons and is required for CGNPs to undergo rapid proliferation in the EGL before migrating through the MOL to the IGL, where they terminally

differentiate and mature into glutamatergic interneurons [2, 146]. Binding of SHH to its receptor Patched (PTCH) activates target genes in CGNPs that drive proliferation and inhibit differentiation [145, 147, 148]. Importantly, many of these target genes have also been implicated in SHH medulloblastoma, including *MYCN*, *GLI1*, and *YAP1* [29, 43, 149].

Hypoxia-Inducible Factor-1 (HIF-1) is a heterodimeric helix-loop-helix transcription factor which acts as a master regulator of gene expression in response to tumor oxidative stress across a broad array of cancers [150-152]. HIF-1-driven gene expression has been extensively studied in cancer and regulates oxidative stress-induced metabolic reprogramming (increased glycolysis), stemness, angiogenesis, cell survival, invasion, metastasis, and therapy resistance [153-159]. The rapid degradation of Hypoxia-Inducible Factor-1-Alpha (HIF1α) is the rate-limiting step in HIF-1 activation, and its stabilization has been an attractive area of research and a target for the development of novel cancer therapies [160, 161].

Most research on HIF1α is done in regard to oxygenation status. Conventionally, under normoxic conditions, prolyl hydroxylases (PHDs) hydroxylate HIF1α, which is then sequestered and marked for proteasomal degradation by the Von Hippel-Lindau (VHL) complex, a tumor suppressive E3 ubiquitin ligase [162, 163]. HIF1α is stabilized under hypoxic conditions when its hydroxylation-driven degradation is inhibited. Interestingly, PHDs can be inhibited by reactive oxygen species (ROS) which interfere with the ability of PHDs to tag HIF1α for degradation, leading to HIF1α stabilization independent of hypoxia [164-170]. Because PHD-mediated hydroxylation of HIF1α requires ferrous iron (Fe^{2+}) and elevated ROS within the cell favors the conversion to ferric iron (Fe^{3+}), ROS can hinder PHD function [171].

Potential regulators of ROS within proliferating CGNPs include the NADPH Oxidase (NOX) family of transmembrane proteins given their well-established enzymatic production of cellular ROS and

the extensive literature describing them as essential modulators of signal transduction across various cell types [172, 173]. The NOX family of proteins uniquely produce ROS as their primary function [174]. Currently, five NOX homologs and two so-called dual oxidase (DUOX) enzymes featuring peroxidase activity in addition to oxidase function have been identified in humans; they vary in the amount/type/timing of ROS production and their organ-specific expression [175]. NOX4 is the only member of the NOX family to display constitutive activation [176-178]. Preliminary studies in the Kenney lab have identified upregulation of NOX4 in human SHH MB [personal communication with Anshu Malhotra, Kenney Lab]. Of note, PHD inhibition/HIF1α accumulation resulting from NOX activity has been studied in other cancer models [179]. Extensive work has been done analyzing the relationship between NOX4 and HIF1α-driven tumor progression [172, 173, 180, 181]. Little is known of how ROS, NOX, and HIF interact during cerebellar development and in medulloblastoma, wherein the cells are rapidly proliferating and exhibit a glycolytic phenotype [182, 183].

We used primary CGNP cultures to investigate potential interactions between SHH signaling and HIF1α. Our results indicate that mitogenic SHH signaling upregulates HIF1α, but not at the mRNA level. Rather, we found that HIF1α is stabilized under non-hypoxic conditions. Along with sustained cell cycle progression, SHH pathway activation also increased ROS production in CGNPs. Our findings support NOX4-produced ROS downstream of SHH as a key contributor to HIF1α stabilization. Taken together, these observations suggest a role for ROS signaling in SHH-driven proliferation, including HIF1α stabilization. Moreover, these results also suggest potential therapeutic applications in medulloblastoma for targeting ROS production, which would destabilize HIF1α and promote cell cycle exit.

3.4 Results

3.4.1 HIF1α is Upregulated at the protein level in SHH-treated CGNPs.

We first aimed to determine the level of HIF1α in SHH-stimulated CGNPs, since these cells feature certain phenotypes attributable to HIF activity, including glycolysis and expression of stem cell markers such as nestin. To this end, CGNPs from P5 mice were harvested and vehicle- or SHH-treated for 48hrs. Whole cell protein lysates were generated and western blotting was performed according to standard methods (see materials/methods). CGNPs that were exposed to SHH displayed higher levels of HIF1α protein compared to vehicle-treated CGNPs and CGNPs that were treated with both SHH and the Smoothened inhibitor cyclopamine (Fig.3.1A). Cyclin D1 is used as a proliferation marker and confirms SHH pathway activation in CGNPs. Quantitative RT-PCR experiments revealed there was no biologically significant change in *HIF1α* gene expression compared to *Gli1* as a positive control. (Fig. 3.1B) In parallel, protein lysates collected from *NeuroD2:SmoA1* mouse medulloblastomas and analyzed by Western blot showed a striking increase in HIF1α protein in the tumor compared to adjacent cerebella (Fig. 3.1C).

As we observed no change in mRNA levels of *HIF1α*, we sought to determine changes at the translational level of control. We, therefore, treated CGNPs with a combination of SHH and the mTOR inhibitor rapamycin and observed a decrease in HIF1α over time (Fig. 3.2). Reduced phosphorylation of ribosomal protein S6 (rpS6) serves as confirmation of mTOR inhibition. This finding is consistent with previous work regarding mTOR and HIF1α, indicating that SHH-induced HIF1α elevation occurs post-transcriptionally [184].

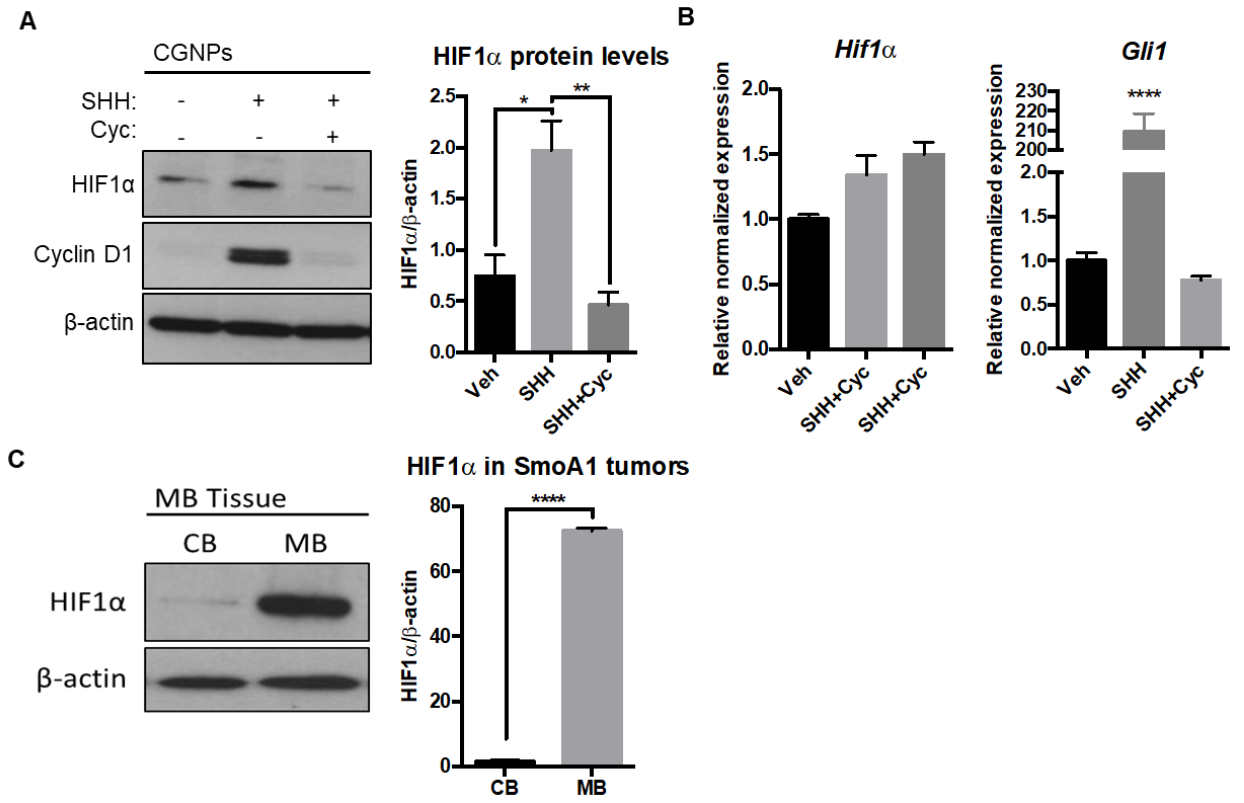

Figure 3.1 HIF1α protein is up-regulated in SHH-treated CGNPs and SHH medulloblastoma
(A) CGNPs were harvested from P5 WT mice and incubated for 48 hours in the presence of exogenous Veh, SHH, or SHH+Cyclopamine (Cyc.). HIF1α levels were detected by immunoblot and quantified using densitometry (ImageJ). Cyclin D1 levels served as a positive control of SHH pathway activation. (B) *Hif1α* transcripts are not significantly elevated in SHH treated CGNPs in three sets of cDNA generated from separate CGNP RNA lysates. *Gli1* is used a positive control. (C) HIF1α levels in mouse medulloblastoma (MB) and adjacent cerebellum (CB) were detected by immunoblot and quantified using densitometry (ImageJ). Data are shown as mean ± S.E.M., N=3, *P<0.05, **P<0.01, ****P<0.0001.

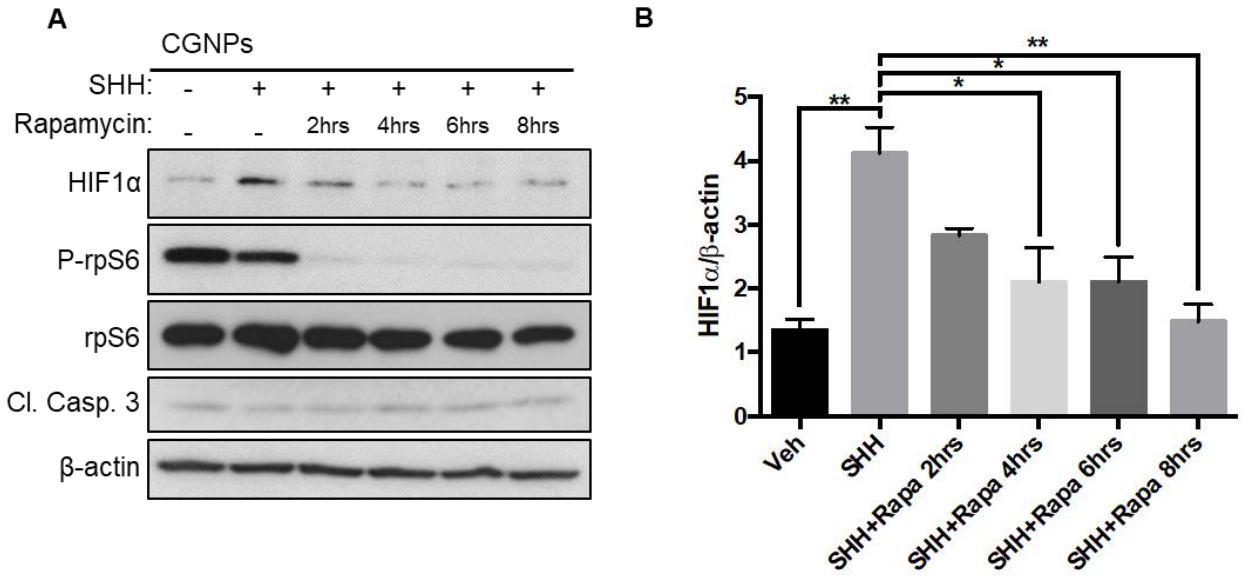

Figure 3.2 HIF1α is post-translationally up-regulated in SHH-treated CGNPs

(A) Primary CGNPs were harvested from P5 WT mice and incubated for 48 hours in the presence or absence of exogenous SHH. HIF1α levels were detected by immunoblot analysis after treatment with rapamycin (10nM) for indicated time intervals. Decreased phosphorylation of rpS6 served as a positive control of mTOR inhibition with rapamycin. Cleaved caspase 3 levels were used to assess cell death. (B) Densitometry quantification of HIF1α levels depicted in (A). Data are shown as mean ± S.E.M., N=3, *P<0.05, **P<0.01, ****P<0.0001.

3.4.2 SHH Increases ROS Production in CGNPs

Because of HIF1α's known interactions with oxygen regulation, we decided to investigate its relationship to reactive oxygen species when the SHH pathway is activated. To test the hypothesis that ROS activity is modified in response to SHH, we measured the relative concentration of ROS in CGNPs treated with exogenous SHH compared to the levels of ROS in the vehicle-treated CGNPs. We proceeded to thoroughly optimize a ROS assay using (5-(and 6) Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Life Technologies). We also employed propidium iodide to gate out dead and dying cells according to the strategy in Supplemental Figure 1. SHH treated cells consistently demonstrated a larger population of cells with high ROS levels compared to vehicle or SHH+Cyclopamine treated cells. (Fig. 3.3). Combined with previous data, ROS induction resulting from SHH signaling follows the same pattern of increased HIF1α protein levels due to SHH stimulation, suggesting a role for ROS in HIF1α stabilization that we then further validated by treatment with the ROS scavenger N-acetyl-L-cysteine (NAC).

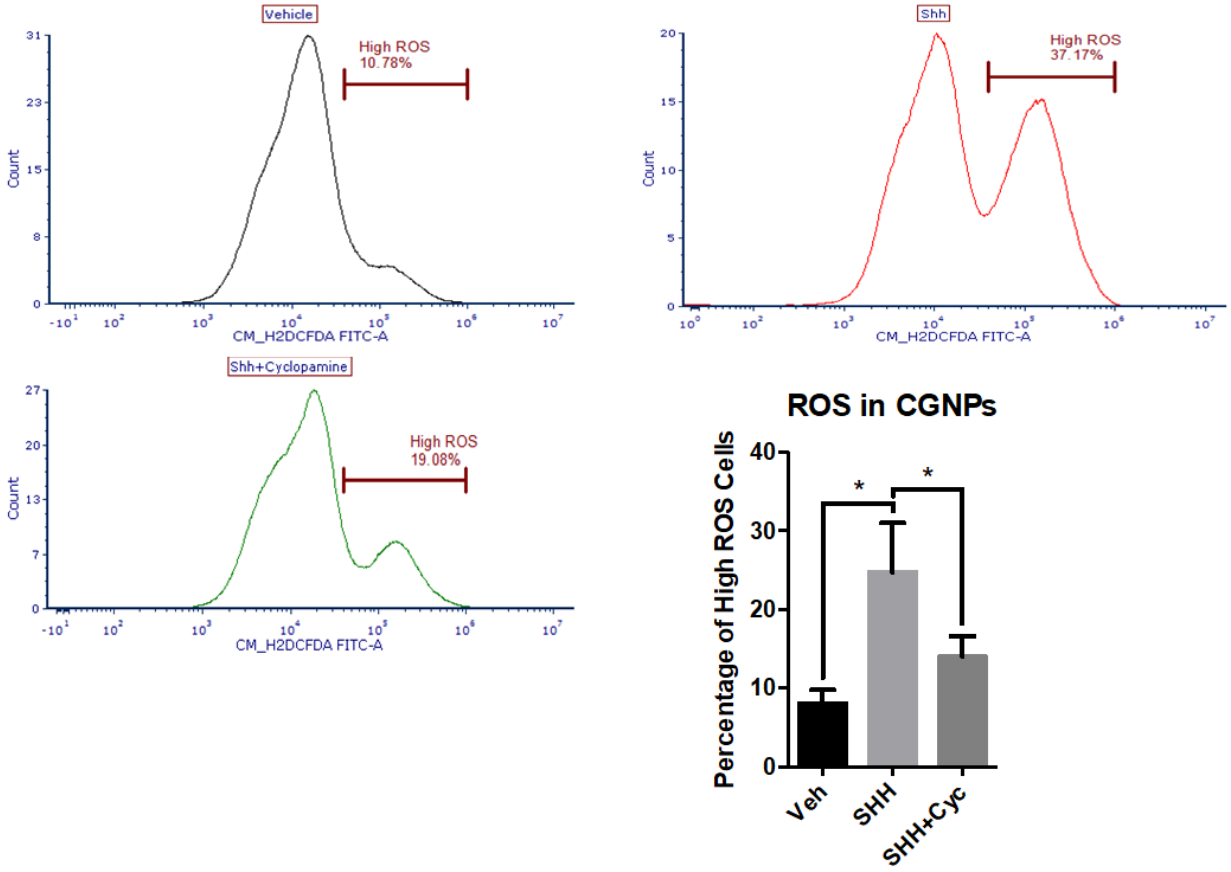

Figure 3.3 Reactive Oxygen Species are elevated in SHH-treated CGNPs

CGNPs were harvested from P5 WT mice, treated with Veh, SHH, or SHH+Cyclopamine (SHH+Cyc), and collected after 48 hours. They were then stained with CM-H_2DCFDA and propidium iodide as described in materials and methods and analyzed by flow cytometry. Histograms from a representative experiment are shown with a marker indicating the percentage of high ROS events in each condition. Data are shown as mean ± S.E.M., Ratio paired two tailed t test, N=3, *P<0.05.

3.4.3 Scavenging of ROS reduces HIF1α stabilization in SHH-treated CGNPs

With evidence linking SHH to higher levels of intracellular ROS production in CGNPs, we decided to scavenge these radicals using the antioxidant N-acetyl-L-cysteine (NAC) *in vitro* and measure effects on HIF1α stabilization. CGNPs were cultured in the presence or absence of exogenous SHH (see materials/methods) in serum-free medium for 36 hours before NAC (10mM) was added and then replenished every six hours for the indicated total time points. (Fig. 3.4) All cells were collected, lysed, and analyzed via western blot. SHH treatment alone showed expected high levels of HIF1α and Cyclin D1 protein, but levels of both diminished with NAC treatment over time (Fig. 3.4). These data support the involvement of SHH induced ROS signaling in HIF1α hypoxia-independent regulation in our systems.

3.4.4 NADPH Oxidase 4 is Upregulated in SHH-Treated CGNPs.

We next focused on identifying the source of ROS and decided to investigate the NADPH Oxidase (NOX) family of proteins as they are potent producers of non-mitochondrial ROS. After investigating several NADPH Oxidases reported to be in the cerebellum, we found NOX4 up-regulated in CGNPs downstream of SHH at the protein level, but not at the mRNA level (Fig. 3.5A, 3.5B). This relationship to SHH is further supported by our observation that NOX4 is elevated in NeuroD2-SmoA1 mouse medulloblastoma when compared to adjacent cerebellum. (Fig. 3.5C)

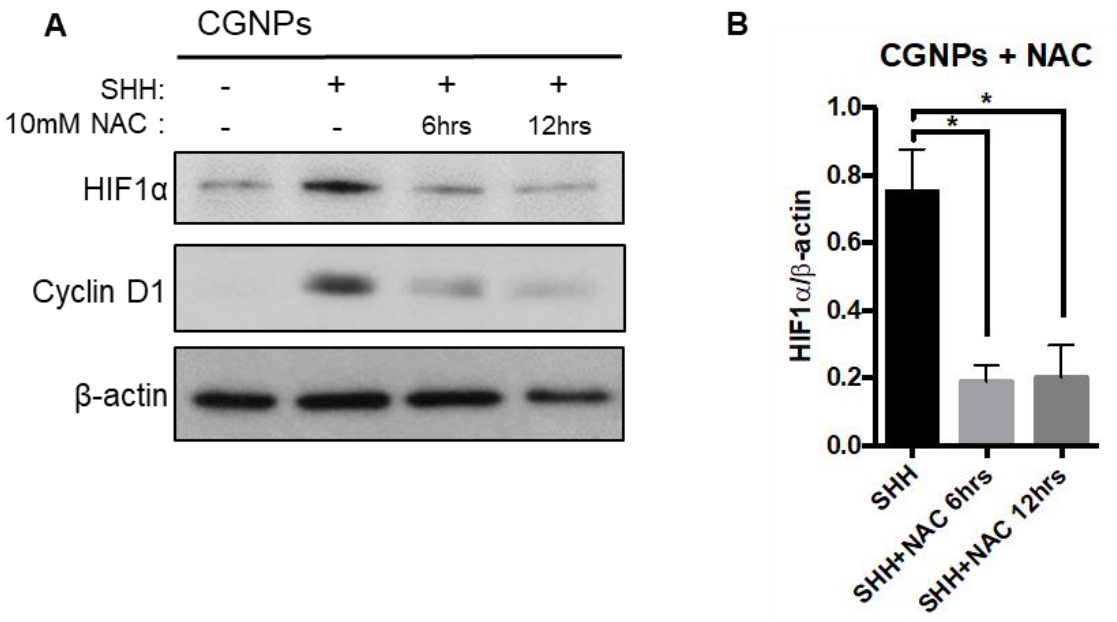

Figure 3.4 ROS scavenger N-acetyl-L-cysteine (NAC) reduces HIF1α protein in SHH-treated CGNPs

CGNPs were harvested from P5 WT mice and incubated for 48 hours in presence or absence of exogenous SHH. Experimental wells were treated with SHH and 10mM NAC every 6 hours for up to 12 hours. The cells were lysed and analyzed by Western blot and HIF1α levels were measured by densitometry (ImageJ). Data are shown as mean ± S.E.M., N=3, *P<0.05.

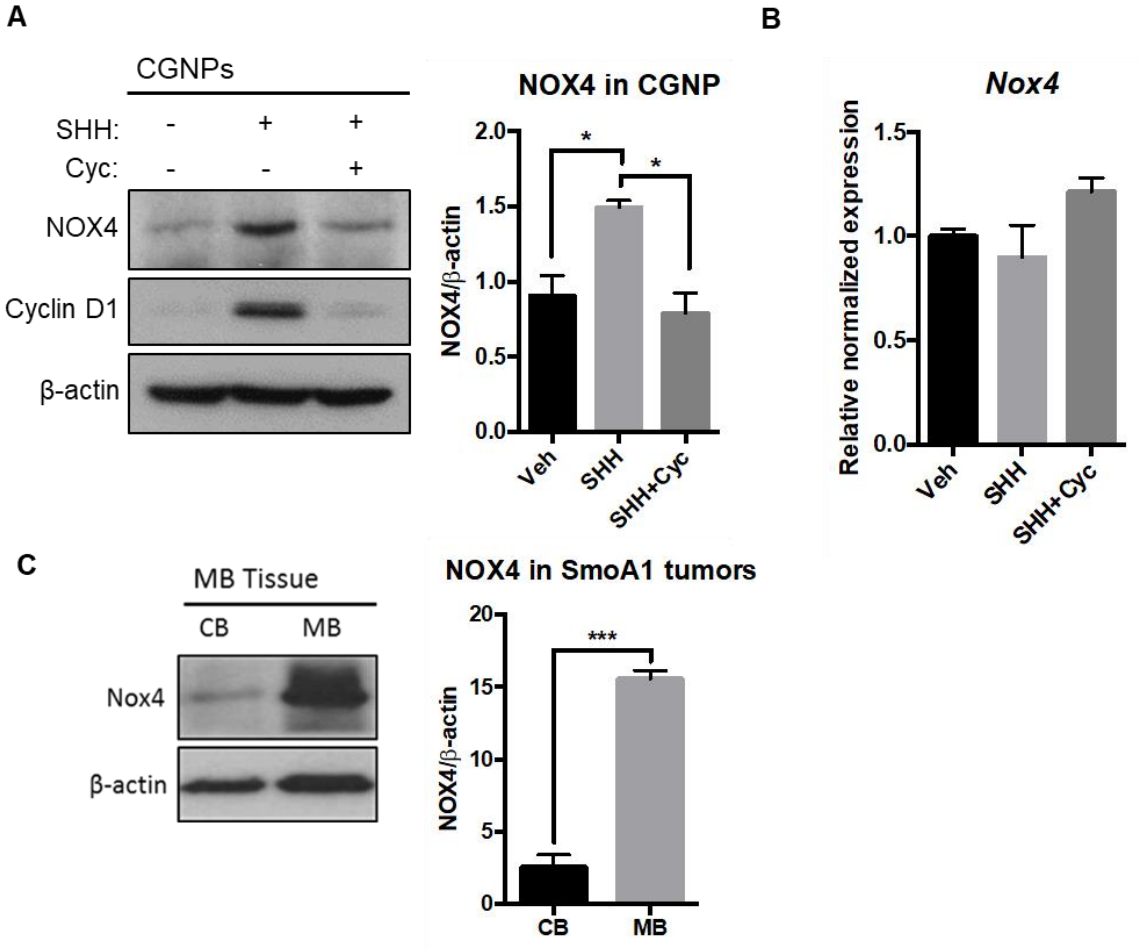

Figure 3.5 NOX4 protein is elevated in SHH-treated CGNPs and mouse medulloblastoma
(A) CGNPs were harvested from P5 WT mice and incubated for 48 hours in the presence of exogenous Veh, SHH, or SHH+Cyclopamine (Cyc.). NOX4 protein levels were detected by Western blot and quantified by densitometry (ImageJ). Cyclin D1 levels served as a positive control of SHH pathway activation. (B) *Nox4* gene expression in CGNP lysates was assessed by qPCR as described in the methods. (C) NOX4 protein levels in adjacent cerebellum (CB) and tumor (MB) tissue from *NeuroD2:SmoA1* mice. Data are shown as mean ± S.E.M., N=3, *P<0.05, ***P<0.001.

3.4.5 NOX Regulates HIF1α at the Protein Level and Appears Necessary for Proliferation

We next wished to determine whether NOX inhibition, like ROS scavenging, would be sufficient to destabilize HIF1α. To this end, we used the NADPH Oxidase inhibitor apocynin to see if SHH related NOX4 activity could be linked to HIF1α's dependency on ROS. We found HIF1α to be destabilized with several commonly used concentrations of apocynin. (Fig. 3.6) Additionally, we found SHH induced proliferation was attenuated in response to apocynin as shown by the reduction in Cyclin D2 at the protein level and *Gli1* at the mRNA level.

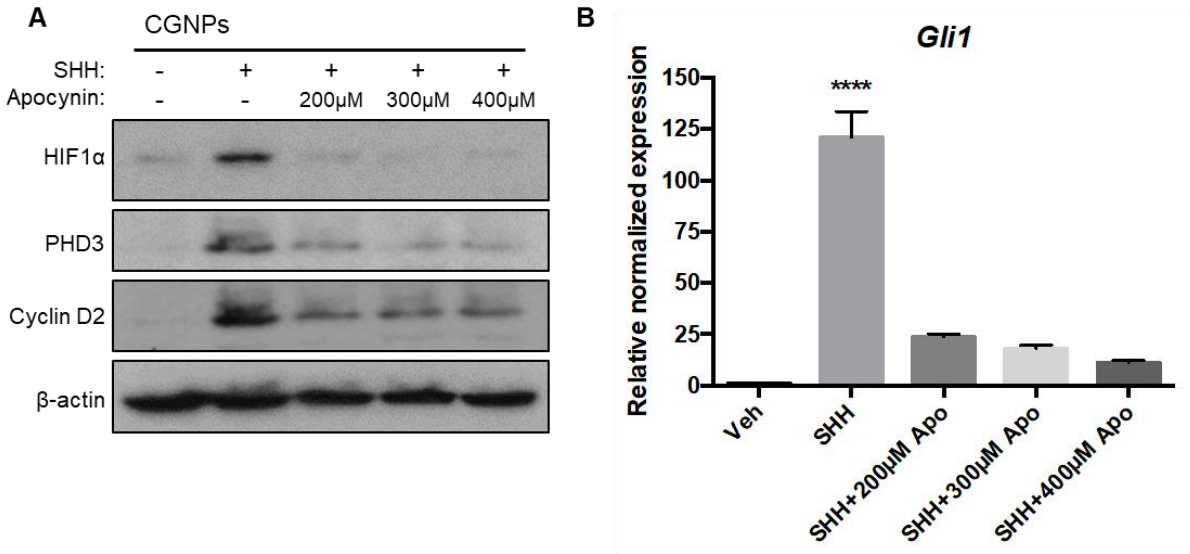

Figure 3.6 NOX4 regulates HIF1α protein and is necessary for proliferation
CGNPs were harvested from P5 WT mice and incubated for 48 hours in the presence of exogenous Veh, SHH, or SHH+Apocynin (Apo.). Several SHH treated wells were treated with increasing doses of the NADPH oxidase inhibitor apocynin for 12 hours prior to lysis. (A) Western blot analysis of lysates obtained from the experiment. (B) qPCR analysis of RNA extracted from cells in the same experiment show a subsequent reduction in cell proliferation as demonstrated by *Gli1* expression. Fold inductions are shown with vehicle as the control. Data are shown as mean ± S.E.M., N=3, ****P<0.001

3.5 Discussion

Medulloblastoma is the most common CNS malignancy among children, with approximately 30% of these tumors belonging to the Sonic Hedgehog molecular subclass. Although the current standard of care leads to a relatively high "cure" rate (~70%), patients are left with a myriad of neurological and developmental deficits; this indicates a substantial need for the development of novel molecular targeted therapies that would allow for reduction or even elimination of chemo- and radiation therapy. Investigating regulation of molecules driving cancer phenotypes, such as inhibited differentiation, stemness, Warburg metabolism, and angiogenesis may lead to the identification of such therapeutic targets. In this context, HIF1α makes an attractive candidate due to the central role it plays in these hallmarks of cancer [153-159]. Although HIF1α has been studied in the context of neural stem cells, it has not been explored in cerebellar progenitor cells, which have a defined cell fate yet express certain stem cell markers [185-187]. Recently it has been reported that HIF1α is upregulated two-fold in medulloblastoma, but this study was limited in scope and did not include molecular subgroup specification [188].

To reliably recapitulate the cell signaling activity and gene expression observed in spontaneous tumors arising from aberrant SHH signaling, we employed an *in vitro* cerebellar granule neuron precursor (CGNP) primary culture system that allows us to closely study SHH mitogenic signaling and its downstream interactions/effectors. We first examined HIF1α status in these cells and found that SHH treatment resulted in HIF1α stabilization, in the absence of mRNA increase. HIF1α protein was also elevated in mouse SHH medulloblastoma samples, consistent with a conservation of Shh targets in normally proliferating CGNPs and medulloblastoma cells. Since CGNPs reside adjacent to the meninges, which houses a highly oxygenated vascular network, we wished to investigate hypoxia-independent factors contributing to HIF1α stability. Accumulation of intracellular ROS has been shown to hinder the process by which HIF1α is degraded,

independent of the oxygenation state [164-171, 189, 190]. These reports are consistent with our finding that SHH pathway induction in CGNPs results in a significant increase in ROS levels along with marked HIF1α protein stabilization. Experiments aimed at directly sequestering ROS used the antioxidant N-acetyl-L-cysteine (NAC). Administering NAC to SHH-treated CGNPs resulted in diminished HIF1α levels.

The current literature supports the NADPH oxidase (NOX) family of proteins as being a significant regulator of intracellular ROS production in a multitude of cancers [172-175, 191, 192] and their activity has been linked to HIF1α stabilization [172, 173, 179-181]. Indeed, we observed upregulation of NOX4 in CGNPs and mouse SHH medulloblastomas; there was no change in other NOX family members (data not shown). Taken together, our results indicate a potential role for NOX-generated ROS in hypoxia-independent HIF1α stabilization. Preliminary findings and other studies suggest that NOX4, one of the five human NOX homologs, could be of particular interest within our paradigm [193-200].

Overall, our work supports a role for intracellular ROS in hypoxia-independent HIF1α stabilization in SHH-driven cerebellar progenitor cell proliferation and medulloblastoma (Fig.7). These studies have important translational implications for developing reagents targeting HIF1α stabilization, such as novel antioxidant therapies, which could supplement the current standard of care and promote improved quality of life for medulloblastoma survivors.

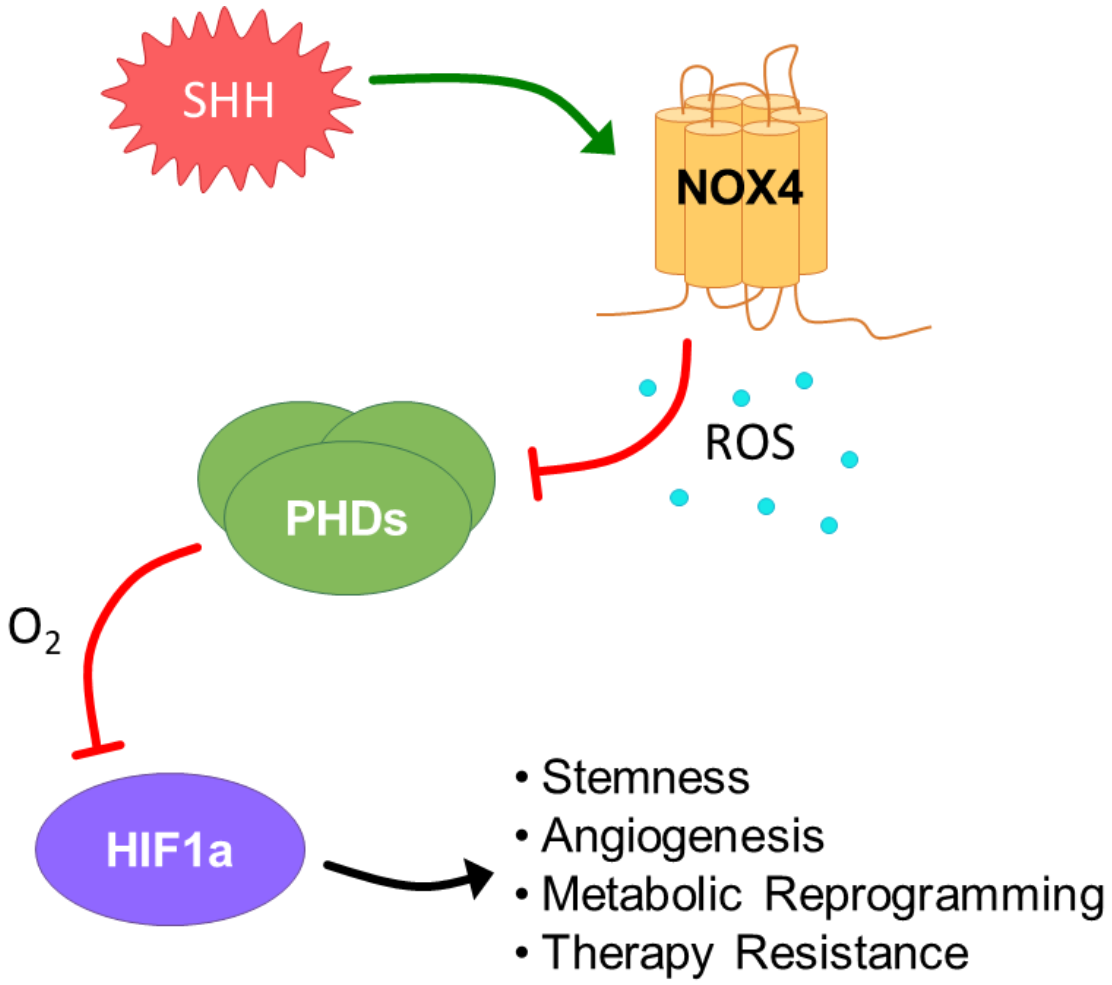

Figure 3.7 NOX4 and ROS promote HIF1α stabilization
Schematic illustrating how NOX4 and ROS activity results in HIF1α protein accumulation in cells with elevated SHH activity.

3.6 Materials and Methods

Animal Studies

Harvest of cerebellar granule neuron precursors from P4 or P5 neonatal wild-type mice and preparation of cerebella for cell culture or histological analysis were carried out in compliance with the Emory University Institutional Animal Care and Use Committee guidelines as was the harvest of tissue from NeuroD2-SmoA1 tumor-bearing mice. Mice used were not discriminated based on sex.

Cerebellar Granule Neuron Precursor Culture. CGNP cultures were established as previously described [2]. Cells were plated on poly-DL-ornithine (Sigma) pre-coated plates. Where indicated, SHH N-terminal fragment was used at a concentration of 3µg/mL and cyclopamine (R&D Systems) was used at 1µg/mL. Cells were seeded in 10% FBS N2 media for two hours before being switched to serum-free N2 media. Where indicated, N-acetyl-L-cysteine (NAC, Sigma), apocynin (Cayman Chemical), or rapamycin (Sigma) was administered in serum-free media at the stated concentration for the given duration leading up to 48hrs of total culture. Notably, due to NAC's short half-life, it was supplemented every six hours during treatment.

Protein Collection and Immunoblotting

Cells or tissue were collected, washed once with 1x PBS, then re-suspended in complete lysis buffer. Whole cell lysates were generated as previously described [2]. To preserve HIF1α protein, special care was taken during collection with cells washed in ice-cold PBS and then collected by scraping in complete lysis buffer over ice. The Bradford assay and the Coomassie Plus protein assay reagent (Thermo Scientific) were used to estimate protein concentrations. 20µg of protein from each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% or 10% gels and transferred to activated Immobilon PVDF membranes (Millipore). Western blotting was carried out according to standard protocols. Primary antibodies

used include: anti-HIF1α (Novus Biologicals), anti-Nox4 (Abcam), anti-Cleaved Caspase-3 (Cell Signaling), anti-rpS6 (Cell Signaling), anti-cyclin D1 (Abcam), cyclin D2 (Santa Cruz), anti-Phospho-rpS6 (Cell Signaling), and anti-β-actin (Sigma). Horseradish peroxidase-conjugated secondary antibodies used were donkey anti-mouse (Jackson ImmunoResearch Inc) and goat anti-rabbit (Pierce). Western blots were developed using ECL reagent (Thermo Scientific) and then exposing membranes to GE-Amersham chemiluminescence film for varying periods of time to achieve optimal saturation. Quantification of relative protein levels was done by densitometry using ImageJ software (NIH).

RT-qPCR

CGNP lysates were collected in TRIzol (Life Technologies) and RNA was extracted according to the manufacturer's protocol. cDNA was then synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. 20ng of cDNA was added per well with the Bio-Rad SsoAdvanced Universal SYBR Green Supermix and appropriate primer assay for the gene of interest. Primer assays purchased from BioRad were for mRNA transcripts of *Gli1*, *Hif1α*, and *Nox4,* and the endogenous controls *Actb* and *Gusb.* The manufacturer's protocol was followed and qPCR was performed on a Bio-Rad CFX96 thermocycler with the fluorescent detection module. The analysis was done according to the $\Delta\Delta C_T$ method of measuring relative gene expression. The runs were analyzed on the Bio-Rad CFX manager software and three replicate runs were combined into gene studies adhering to MIQE standards.

Reactive Oxygen Species Assay

CGNPs were cultured as stated above in six-well plates. At 48 hours, CGNPs were washed in warm 1x PBS and harvested by trypsinization (250µL of 0.05% trypsin). 10% FBS N2 media was added to stop trypsinization and cells were transferred to 1.5mL tubes where they were washed in warm 1x PBS, pelleted, and re-suspended in warm 1x Hank's Balanced Salt Solution (HBSS) and

transferred to black 1.5mL tubes where 2µL of 10µM (5-(and 6) Chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Life Technologies) solution were added. Samples were placed in a cell incubator [(37 °C), high relative humidity (95%), and controlled CO_2 level (5%)] in the dark for 45 min. CGNPs were re-suspended by pipetting, propidium iodide was added to a concentration of 1µg/mL, and 200µL aliquots were added to a 96-well plate and analyzed by flow cytometry on a Beckman Coulter Cytoflex at the Emory University Pediatrics Flow Cytometry Core. CM-H_2DCFDA was analyzed on the FITC channel and propidium iodide was analyzed on the PerCP channel. All conditions in each run were subject to the same gain settings. Once the CGNP population was gated in and doublets gated out, the remaining propidium iodide positive population denoting dead cells was excluded. 5000 events were then selected from the remaining live population in each condition and a marker for the "High ROS" population was created for each experimental condition covering the same range of signal strength in the FCS Express 6 software (De Novo Software).

Statistical Analysis

Statistical analysis was performed using Prism 6 (GraphPad Software Inc.) or FCS Express 6 (De Novo Software) and Bio-Rad CFX manager for flow cytometry and qPCR analyses respectively.

3.7 Acknowledgments

This work was supported by: NINDS R01NS061070 (AMK) and NCI Winship Cancer Institute P30 Center Grant CA138292 (AMK). We thank Nicholas Eyrich's Cancer Biology 4+1 Master's thesis committee (Mala Shanmugam and Keith Wilkinson) for valuable feedback on the project and Pablo Eyrich Freile for technical assistance.

3.8 Additional Preliminary Findings

The preceding sections 3.2 through 3.7 are reproduced from our submission to Molecular and Cellular Biology. There is additional work regarding the mechanism of stabilization that was the bulk of my experimental effort on this project. While we didn't feel these results were convincing enough to include in our submission, they are intriguing.

HIF1α protein stabilization

As described in the introduction, in the presence of oxygen HIF1α is hydroxylated, ubiquitinated, and then undergoes proteasomal degradation [162, 163]. Hydroxylation at prolines 402(P402) or 564 (P564) is accomplished through the action of prolyl-4-hydroxylases (PHDs) which marks the protein for ubiquitination by the von Hippel-Lindau (VHL) protein [201]. Ubiquitinated HIF1α is then rapidly degraded [201]. Stabilization of HIF1α can be accomplished in hypoxia due to PHDs requirement for oxygen, but other mechanisms for HIF1α stabilization are known that do not depend on hypoxia (reviewed in Wigerup et al 2016) [201]. Our hypothesis is that downstream of SHH signaling increased levels of ROS generated by NOX4 inhibit the function of PHDs, leading to accumulation of HIF1α as depicted in Figure 3.7.

If our hypothesis is correct, we should be able to assess the hydroxylation status of HIF1α and we would expect to see less hydroxylation of P402 and/or P564 as a result of SHH signaling. Efforts to demonstrate this required many months of optimization. As HIF1α is degraded rapidly subsequent to hydroxylation, experimental conditions had to be altered. The addition of MG132, a proteasome inhibitor, in the cell culture media allowed us to visualize the levels of accumulated HIF1α both with and without SHH-N. The antibody for HIF1α provided a much cleaner blot only after using it once so we changed our Western blot procedures to allow for freezing and reusing

our primary antibodies. In spite of our best efforts, the antibodies currently available for hydroxylated HIF1α proved to be inconsistent and provided fairly “dirty” blots.

Even with these difficulties, we were able to discern differences in the hydroxylation status of HIF1α as shown in Figure 3.8. With the addition of SHH-N, the relative levels of HIF1α protein increase while at one hour of MG132 we saw very little change in the levels of hydroxylated HIF1α (h- HIF1α) at P564. At four hours of the proteasome inhibitor, the reduction in h-HIF1α was less pronounced, but still there when densitometry was performed and the relative level of h- HIF1α was divided by the relative level of HIF1α. To further our investigation of HIF1α hydroxylation, we treated CGNPs with the NOX inhibitor apocynin for four hours prior to collection. The reduction in h-HIF1α that we observed with SHH-N was reversed with the addition of apocynin, more so at four hours of proteasome inhibition than at one hour (Fig 3.9). These results reinforce our hypothesis, but the difficulty of trying to assess hydroxylation status and the inconsistent results provided by the hydroxylated HIF1α antibody meant we did not include these findings in our paper. It is possible that a better antibody will be developed in the future which could facilitate this portion of the research. Densitometry was performed with ImageJ. Calculations and chart creation were done with Microsoft Excel.

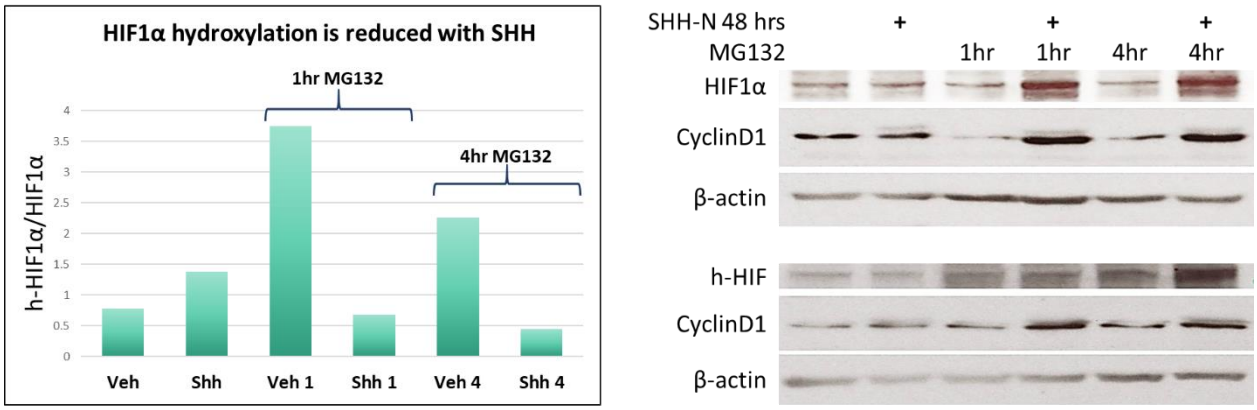

Figure 3.8 HIF1α hydroxylation is reduced with SHH

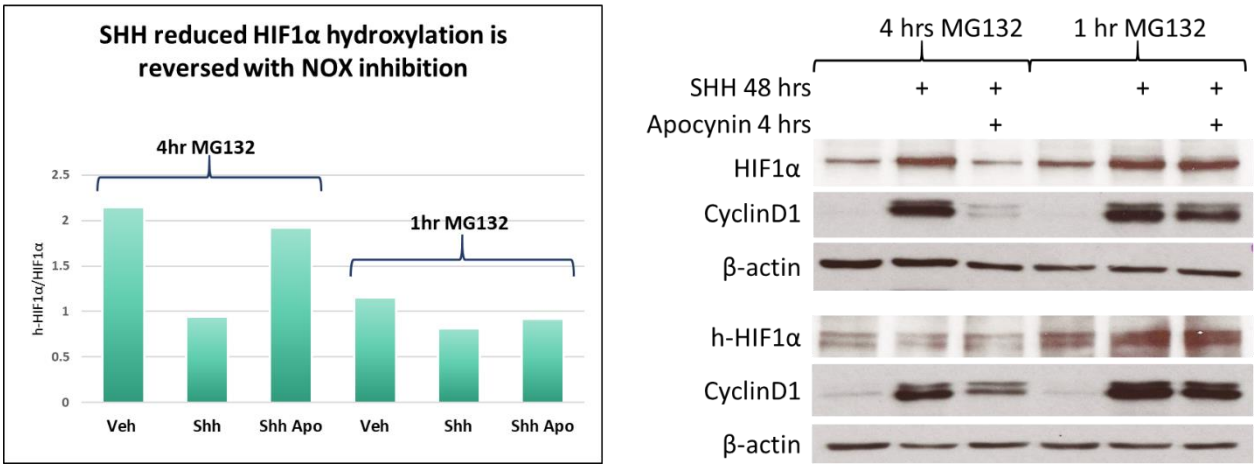

Figure 3.9 Inhibition of NOX reverses SHH induced reduction of hydroxylated HIF1α

Chapter 4 Summary and Future Directions

While pediatric cancers are fortunately very rare, they provide a unique opportunity for researchers to study specific molecular pathways in a relatively uncluttered background. Pediatric cancers, in general, have very low rates of genetic mutations, yet cancers still develop. Utilizing CGNPs in addition to a transgenic mouse model of SHH MB and primary tumor cell culture we have endeavored to identify and characterize downstream effectors of the SHH pathway. While we and others have made great progress in understanding the SHH pathway, what lies between the effector proteins GLI, MYC-N, and YAP1 and the dysregulated cell cycle is still an area of active research. The work detailed in this dissertation attempts to elucidate two different downstream effects of SHH pathway activation. Identification of HELLS, a chromatin remodeler, as an epigenetic factor that is upregulated in SHH-N stimulated CGNPs and in human and mouse SHH MB is described in Chapter 2. Additionally, stabilization of HIF1α protein downstream of SHH signaling in SHH stimulated CGNPs is explored in Chapter 3. It is hoped that the work here will provide a foundation for further research that may lead to better diagnostics, prognostics, or therapeutics for pediatric SHH MB.

4.1 Upregulation of the epigenetic factor HELLS downstream of SHH signaling in cerebellar development and in SHH MB

4.1.1 Summary of findings

The low rate of genetic mutations in the pediatric cancer SHH MB allows dissection of the SHH pathway without the confounding factors of the hundreds of other mutations seen in adult cancers. However, the paucity of mutations has also left researchers with fewer targets for therapeutic development. The advent of epigenetics, heritable changes in gene expression without changes to DNA sequence, has provided an important avenue of discovery, particularly in pediatric cancers. Hypermethylation of DNA leading to silenced gene expression or

deacetylation of histone tails at specific lysine residues resulting in repressive chromatin have been identified as tumor-specific in medulloblastoma (See 1.3 for details). A better understanding of the mechanisms that create and maintain cancer is important in and of itself, but the fact that epigenetics are reversible and can be exogenously modulated makes understanding epigenetics in medulloblastoma an exciting path to treatments that have the potential to be both effective and have less long-term negative side effects.

Our exploration into possible epigenetic factors downstream of SHH signaling led us to the chromatin remodeler HELLS which we found to be upregulated in SHH stimulated CGNPs. Looking at endogenous levels of HELLS in mouse cerebellar samples at postnatal days 1, 7, 15, and 21, we confirmed that HELLS is highly expressed in the developing mouse cerebellum during the time when cerebellar progenitor cells are undergoing extensive SHH induced proliferation. HELLS expression in the cerebellum was at a much higher level than that in the murine cerebral cortex during the same time period indicating a possible important role for HELLS in cerebellar development.

As developmental pathways are often co-opted in cancer, we next questioned whether HELLS expression was upregulated in human SHH MB. Our finding that HELLS is upregulated in all human medulloblastoma subgroups compared to normal cerebellum and is highest in the SHH and WNT subgroups could prove to be valuable diagnostic or prognostic information. HELLS is highly expressed in human SHH MB, so we wanted to confirm HELLS expression in our *NeuroD2:SmoA1* mouse model of SHH MB and found it be markedly induced in tumor tissue compared to adjacent non-tumor cerebellum at both the mRNA and protein level.

Our next question was to ask which downstream effectors of SHH could be responsible for HELLS upregulation. GLI transcription factors and YAP1 are both downstream of SHH signaling and have

commercially available inhibitory drugs. Using these inhibitors, we found that blocking the interaction between YAP1 and its transcriptional partner TEAD resulted in downregulation of HELLS, suggesting YAP1 regulation of HELLS. In CGNPs and MBCs, we observed a reduction in the levels of HELLS transcript and protein with the addition of the YAP/TEAD interaction inhibitor, verteporfin. In MBCs the reduction in *Hells* transcript levels was striking at lower doses than seen with CGNPs which may indicate greater susceptibility to YAP1 inhibition in the tumor setting. This could be an important finding as HELLS or other downstream targets of a YAP1 transcription program could provide tumor-specific targets in patients. We then sought to determine whether YAP1 regulation of HELLS was direct. Using ChIP, we found enrichment of YAP1 binding upstream of the transcription start site for HELLS. Interestingly, while three of the significant binding sites were in the promoter region, two other sites were nearly 5,000 base pairs upstream of the start site, suggesting YAP1 is binding to an enhancer. As YAP1 has been reported to function in transcriptional control through activation of enhancers, this also supports our findings (Stein 2015, Galli 2015 http://dx.doi.org/10.1016/j.molcel.2015.09.001). Taken together, our data establishes upregulation of HELLS in cerebellar development and in SHH MB downstream of SHH signaling. Identification of an epigenetic factor regulated by YAP1 downstream of SHH signaling presents an intriguing new avenue of research. Further research to determine SHH induced HELLS function in cerebellar development and SHH MB could lead to better diagnostics, prognostics, and ultimately, to therapeutic options with more specificity.

4.1.2 Discussion and Future Directions

While the work presented in this dissertation establishes the upregulation of HELLS downstream of SHH signaling in CGNPs, cerebellar development, and in mouse and human SHH MB, many questions remain. Our finding that this upregulation is mediated by YAP1 is important, particularly in terms of tumor development and maintenance. It has been established previously that YAP1

expression is upregulated in SHH MB and in WNT MB [29], in keeping with our finding that HELLS levels were highest in SHH and WNT MB. Inhibition of the interaction between YAP1 and TEAD resulted in a dose-dependent downregulation of HELLS in CGNPs and in MBCs, with a greater effect seen in the primary tumor cells. The presence of YAP1 bound to consensus sequences upstream of the HELLS transcription start site confirms YAP1 involvement in the transcriptional regulation of HELLS.

Inhibition of YAP1 with verteporfin reduced levels of HELLS, but it was not completely abrogated suggesting inhibition was incomplete or that HELLS is regulated by more than one transcriptional program. There are reports of E2F1 regulation of HELLS including a report of E2F1 binding to the *HELLS* promoter in human embryonic kidney and human lung fibroblast cell lines and another in osteosarcoma cell lines [86, 202]. Additionally, there are E2F1 consensus sequences identified in the promoter regions of the genes for both human and murine HELLS. There are no reports of E2F1 regulation of *HELLS* in cerebellar development or in MB. Bhatia et al. established that E2F1 is strongly induced by SHH-N stimulation in CGNPs [182, 203]. Similar to the experiments performed to investigate YAP1 regulation of HELLS, it would be interesting to compare levels of E2F1 during development and in tumors followed by suppression of E2F1 activity in CGNPs and MBCs. Drugs to inhibit cyclin-dependent kinases that normally phosphorylate the tumor suppressor protein retinoblastoma (Rb) have shown promise in preclinical studies, effectively limiting proliferation and activating apoptosis [204]. Phosphorylation of the Rb protein results in the release of E2F family transcription factors which drive cell cycle and promote the proliferation of cells [204]. Evaluation of the effects on HELLS mRNA expression and protein levels with E2F1 inhibition may indicate E2F1 regulation of HELLS in our systems. In support of this, a recent report in osteosarcoma cell lines demonstrated downregulation of HELLS with a CDK4/6 inhibitor called palbociclib [202]. Chromatin immunoprecipitation to determine E2F1 enrichment on the

upstream regulatory portion of the *HELLS* gene could also provide valuable insight. It is possible, if not likely, that both E2F1 and YAP1 regulate transcription of *HELLS*. HELLS regulation may be context dependent with E2F1 or YAP1 taking a more predominant role in HELLS regulation during cerebellar development or in tumorigenesis. As inhibition of YAP1 had a greater effect on HELLS levels in MBCs, we theorize that YAP1 may be more important for driving *HELLS* transcription in the setting of tumorigenic dysregulation of proliferation downstream of SHH signaling. Both YAP1 and E2F1 have numerous transcriptional targets, and transcription factors have been notoriously difficult to target therapeutically. As HELLS may be downstream of both YAP1 and E2F1, it may provide a more specific therapeutic target for SHH MB.

Perhaps the most important question in this research is this: What is HELLS function in cerebellar development and in SHH MB? To answer this question will require depletion of *Hells* in our systems, a challenging endeavor. Others have successfully depleted *Hells*, but usually in cell lines [74, 77, 92, 97, 101, 137, 202, 205, 206]. Currently, the cell lines available for SHH MB do not recapitulate tumor-specific gene expression as well as SHH-N stimulated CGNPs. However, CGNPs start to differentiate after ~3 days in culture even with additional SHH-N so this limited culture time in combination with the difficulty of infecting primary cells presents substantial challenges to knocking down any gene and our efforts were unsuccessful. Other possibilities for depletion of HELLS include Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR), but the protocol for CRISPR generally takes far longer than the three days we are able to culture CGNPs or MBCs. The mouse model from the Muegge group results in the death of *Hells*-/- pups at birth, and as cerebellar development occurs postnatally, this model is not suitable. An inducible model for *Hells* depletion may be the best option and would be worth generating going forward. If knockdown can be achieved in our system(s), initial experiments will include analysis of the effects on proliferation, survival, apoptosis, senescence, and orthotopic tumor development. If

phenotypic effects are observed, molecular analysis of changes in the expression of cancer-related genes would be the logical next step. As epigenetic factors have the potential to alter the expression of a multitude of genes, using large-scale analysis such as a cancer gene panel in Nanostring™ could provide targets for further analysis. For example, we may observe changes in the expression of tumor suppressor genes or in drivers of proliferation. To evaluate the direct involvement of HELLS in the regulation of these genes, chromatin precipitation of HELLS followed by sequencing (ChIP-seq) or ChIP-PCR could be performed. To extend these findings, we could evaluate DNA methylation and histone modification status of genes with HELLS depletion. Lastly, as HELLS is proposed to be a chromatin remodeler, it would be interesting to compare chromatin accessibility with and without HELLS using the assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq).

Of particular interest, many of the published reports of HELLS function involve modulation of stem cell genes and genes for cyclin-dependent kinase inhibitors (CDKi) which are implicated in senescence [71, 74, 77, 80, 83, 92, 101, 135]. Both of these avenues of research would be valuable to our understanding of SHH MB. In 2016, the Charron group published their findings proposing that evasion of cell senescence is responsible for the progression of SHH MB [207]. Specifically, Tamayo-Orrego et al. proposed that either mutation of TP53 or inactivation of the CDKi p16INK4A is a critical step in SHH MB development. As depletion of HELLS results in a senescence phenotype and HELLS has been reported to be involved in epigenetically altering the expression of *CDKN2A*, the gene for p16INK4A in several studies, investigating the function of upregulated HELLS in our systems in regard to p16INK4A levels could help fill in some of the gaps in this mechanism. Importantly, HELLS is believed to act either by recruitment of DNMTs which methylate the CDKN2A promoter or recruitment of HDACs which results in a repressive chromatin state at the locus for CDKN2A [72, 77, 80, 89]. Aside from p16INK4A, other CDKi have been identified as genes

with upregulated expression upon HELLS depletion. Analysis of these tumor suppressor genes in SHH MB in the presence or absence of HELLS in our systems could also provide valuable information.

While pediatric cancers are rare, they are devastating for the afflicted children and their families with long-term, life-changing sequelae often resulting from the current standard of care. In terms of genetic mutations, pediatric cancers are relatively "quiet," but cancer still develops. Understanding how this occurs has been challenging for many reasons, but the discovery of epigenetics has opened new pathways of discovery. Our finding of an epigenetic chromatin remodeler that is upregulated downstream of SHH signaling in SHH MB could have important implications for the development of treatments for SHH MB. Future work to elucidate the role of HELLS in cerebellar development and in SHH MB may lead to a better understanding of the interplay between genetic alterations in the SHH pathway and the downstream epigenetic alterations that can result in widespread gene expression changes.

4.2 Stabilization of HIF1α downstream of SHH signaling

4.2.1 Summary of findings

To investigate HIF1α in SHH medulloblastoma, we utilized the CGNP cell culture system that recapitulates the gene expression and cell signaling exhibited by spontaneously formed SHH MB tumors. In this system, the addition of SHH-N to cell culture resulted in what appeared to be an increase of HIF1α protein. When we assessed the mRNA levels, however, there was no increase in transcription of *Hif1a* leading us to the conclusion that rather than an increase in transcription of HIF1α, there is increased translation and stabilization of the protein. Importantly, these cells are grown in non-hypoxic conditions, an environment which should result in rapid degradation of HIF1α. There are other reports of HIF1α stabilization in normoxia, typically in stem cells [186].

Consistent with recapitulated signaling and expression in SHH stimulated CGNPs and SHH MB, HIF1α protein levels were also stabilized in our mouse SHH MB model. As CGNPs are grown in normoxia, we next sought to determine hypoxia-independent mechanisms for HIF1α stabilization. Reactive oxygen species (ROS) are known to inhibit HIF1α degradation, and we observed a significant increase in ROS levels with SHH pathway activation in CGNPs. In keeping with ROS inhibition of HIF1α degradation, when we added an antioxidant to SHH stimulated CGNPs we observed a decrease in HIF1α levels. Our next course of action was to determine the source of ROS in SHH stimulated CGNPs. The NOX family of proteins have been identified as robust producers of ROS so we assessed levels of NOX in our SHH stimulated CGNPs. NOX4 was found to be upregulated in SHH CGNPs at the protein level when compared to control CGNPs. As with HIF1α levels, NOX4 levels were also elevated in our SHH MB mouse model compared to adjacent non-tumor cerebellum. As increased ROS levels inhibit degradation of HIF1α by inhibiting the function of PHDs, we hypothesized that we would be able to see a decrease in the hydroxylation of HIF1α. The effort to visualize this decrease in hydroxylated HIF1α was my main contribution to this work. Unfortunately, after months of optimization, we determined the antibodies currently available to assess hydroxylated HIF1α provide inconsistent and difficult to interpret results. While a definitive answer as to whether HIF1α is hydroxylated in the presence or absence of SHH signaling would add to the weight of our hypotheses, based on what is known about HIF1α degradation, we can infer that there is less hydroxylation of HIF1α with SHH signaling as the protein is stabilized. Taken together, our finding of normoxic HIF1α stabilization downstream of SHH signaling could be important to our understanding of SHH MB tumorigenesis and maintenance and may provide a path to development of future therapeutics.

4.2.2 Discussion and Future Directions

HIF1α is known to contribute to the Hallmarks of Cancer, and upregulated levels of HIF1α protein in cancer patient biopsies is correlated with a decrease in survival [163, 201]. Therefore, increased levels of HIF1α protein in SHH stimulated CGNPs, the precursor cells of SHH MB, is an intriguing finding. HIF1α has been reported to be upregulated in many different cancer types even though only 50-60% of solid tumors contain regions of hypoxia [208]. This discrepancy may be crucial in gaining a better understanding of tumorigenesis and tumor maintenance as the transcriptional program of activated HIF is extensive.

Recurrence of SHH medulloblastoma is universally fatal as we have little to offer therapeutically once first-line treatments have failed. Identification and characterization of the cells that survive initial treatment and are capable of reconstituting the tumor is perhaps the best hope for these patients. It is known that the perivascular niche is a hub for MB tumor stem cells that survive radiation [209, 210]. In addition, HIF1α expression has been observed in neural stem/progenitor cells [187]. In unpublished data, we have observed co-expression of HIF1α and the stem cell marker nestin in cells adjacent to the vasculature [Bobby Bhatia and Nick Eyrich]. Future studies aimed at characterizing these HIF1α expressing stem cells in the SHH MB perivascular niche, which is presumably a normoxic environment, could provide important insights into recurrence and open new avenues of treatment.

4.3 Next Challenges

While the outlook for SHH MB patients has improved dramatically since the time of Bailey and Cushing, we have reached a point where further improvements to the standard first-line strategy of surgical resection, chemotherapy, and radiation are unlikely. Going forward, improvements to survival and to long-term outcomes are expected to come from the application of molecular

subgrouping data and new therapeutics developed from identification of novel, tumor-specific targets. In SHH MB, the focus in the Kenney lab is to identify downstream effectors of the SHH signaling pathway that play roles in tumorigenesis, tumor maintenance, or survival of tumor stem cells.

Epigenetics has become an active area of cancer research and may be vitally important in pediatric cancers which have few mutations. In SHH MB, a few epigenetic factors have been identified as mutated, but our finding of upregulation of the chromatin remodeler HELLS downstream of SHH signaling without a mutation to the *HELLS* gene may provide a specific target for therapeutic development. HELLS primarily functions to create a repressive chromatin state, and many reports indicate downregulation of tumor suppressor genes in tumors with high levels of HELLS. If this is true for SHH MB and if a specific inhibitor could be developed, targeting HELLS in these tumors in conjunction with standard therapies could provide synergetic killing of cancer cells and allow for de-escalation of therapy. Much research will have to be done before that possibility exists, but the identification of a possible target is the first step.

The dismal outlook for patients with recurrence of SHH MB warrants increased research of the cells that survive our standard therapies. Radiation resistant stem cells have been identified in the perivascular niche. In preliminary studies, we have observed HIF1α in cells in the perivascular niche, colocalizing with the stem cell marker nestin. Combined with our finding of HIF1α stabilization in SHH-N stimulated CGNPs, likely as a result of elevated ROS produced by NOX4, there may be an opportunity to specifically target SHH MB stem cells by modulating ROS levels or targeting NOX4 or HIF1α itself.

The future of SHH MB treatment will likely involve targeting the specific attributes of each individual tumor. What we currently know regarding SHH MB subtypes will be incorporated with

genotyping and expression data to provide a clear path to a cure. As we look forward to this, it is our responsibility to identify and characterize these possible targets so that therapeutics can be developed. HELLS and HIF1α may have essential roles in SHH MB and with further research may become important targets in the fight to find more effective, less toxic treatments for SHH MB patients. It is hoped that the work outlined in this dissertation will provide the foundation for that research.

Chapter 5 References

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