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MECHANISMS ACTING ON HEDGEHOG-GLI PATHWAY AND THEIR THERAPEUTIC POTENTIAL

Ani Azatyan

Karolinska
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Mechanisms acting on Hedgehog-GLI pathway and their therapeutic potential

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Ani Azatyan

Principal Supervisor:
Prof. Peter Zaphiropoulos
Karolinska Institutet
Department of Biosciences and Nutrition

Co-supervisor(s):
Prof. Karl Ekwall
Karolinska Institutet
Department of Biosciences and Nutrition

Doc. Vladimir Bykov
Karolinska Institutet
Department of Oncology-Pathology

Opponent:
Prof. Matthias Lauth
Philipps-University Marburg, Germany
Center for Tumor Biology and Immunology

Examination Board:
Doc. Ning Xu Landén
Karolinska Institutet
Department of Medicine, Division of Dermatology

Prof. Sonia Lain
Karolinska Institutet
Department of Microbiology, Tumor and Cell Biology

Prof. Ann-Kristin Östlund Farrants
Stockholm University
Department of Molecular Biosciences

“I am among those who think that science has great beauty”

- Marie Curie

Abstract

Hedgehog signaling is crucial for diverse aspects of animal development, essential in regulating many cellular processes and is largely implicated in various forms of human cancer. However, many aspects of Hedgehog signaling are not completely understood. This thesis aims to contribute towards a better understanding of the mechanisms acting on Hedgehog-GLI signaling and explore their possible therapeutic potential.

PAPER I. We demonstrate that the small molecule RITA, a p53 activator, downregulates Hedgehog signaling in human medulloblastoma and rhabdomyosarcoma cells via JNK kinase and irrespective of p53. *In vitro* RITA enhanced the anti-proliferative effects of the GLI antagonist GANT61. RITA was more potent than GANT61 in downregulating Hedgehog-GLI signaling in rhabdomyosarcoma subcutaneous xenograft tumors with the dual drug administration almost completely blocking the Hedgehog signaling response *in vivo*, suggesting a certain antagonism of the two drugs. Notably, RITA and GANT61 co-administration decreased cell proliferation and elicited a broader response of pathways involved in cancer cell growth, providing a plausible interpretation for tumor reduction in the absence of Hedgehog signaling downregulation.

PAPER II. We address the possible therapeutic role of Hedgehog-GLI1 signaling for targeting and prognosis of ER-alpha positive breast cancer. We showed that expression of the Hedgehog signaling effector protein GLI1 is higher in tamoxifen resistant relative to tamoxifen sensitive cells. In both cell types GLI1 depletion mitigated cell proliferation and ER-alpha activity, irrespective of estrogen stimulation. Tamoxifen cytotoxicity was enhanced by GANT61 co-treatment, both in tamoxifen resistant and sensitive breast cancer cells, reflecting a crosstalk between ER-alpha and Hedgehog-GLI1 signaling. We have observed a positive correlation between GLI1 and ER-alpha/ER-alpha target gene expression, while high *GLI1* expression was associated with poor distant metastasis-free survival in breast cancer patients.

PAPER III. We identified a signature of GLI1 target genes via a combination of RNA-seq analyses of GLI1 overexpression and depletion datasets supplemented with in-depth validation in human cancer cell lines. Additionally, we found that RNA editing of GLI1 can modulate its effects on GLI1 target genes. Markedly, one of the highly upregulated targets, *FOXS1*, was found to engage in feedback mechanisms limiting the capacity of GLI1 to act as a proliferation factor in medulloblastoma and rhabdomyosarcoma cells. *FOXS1* was both highly expressed and positively correlated with *GLI1* in SHH medulloblastoma, further arguing for the existence of a FOXS1-GLI1 interplay in human tumors.

PAPER IV (Manuscript). In this ongoing work we address the role of circRNAs in the context of Hedgehog signaling activation and Hedgehog-linked SHH medulloblastoma tumors. Via modified RNA-seq protocols we have determined the circRNA transcriptome of Daoy medulloblastoma and human embryonic palatal mesenchyme HEPM cells, following activation of Hedgehog pathway with SHH ligand or Smoothened agonist SAG. In total, 29 selected circRNAs were independently validated by Sanger sequencing and RT-PCR assays. Of these circRNAs, 10 were apparently regulated by Hedgehog signaling activation, however to a much lesser extent compared with known target genes of the pathway, e.g. *GLI1* and *HHIP*. 7 circRNAs had reduced expression in human medulloblastoma tumors in comparison to normal cerebellum, while the linear mRNAs originating from the same genes did not exhibit a reduced expression. These findings highlight distinct regulatory mechanisms acting on the BACH1, CDYL, FKBP8, GLIS1, OGDH, SMARCA5 and ZKSCAN1 circRNAs and deserve further analysis for possible contribution to the development of medulloblastoma.

List of scientific papers

I. **Azatyan A**, Gallo-Oller G, Diao Y, Selivanova G, Johnsen JI, Zaphiropoulos PG
RITA downregulates Hedgehog-GLI in medulloblastoma and rhabdomyosarcoma via JNK-dependent but p53-independent mechanism
Cancer Letters. 2019;442:341-50. doi: 10.1016/j.canlet.2018.11.005

II. Diao Y, **Azatyan A**, Rahman MF, Zhao C, Zhu J, Dahlman-Wright K, Zaphiropoulos PG
Blockade of the Hedgehog pathway downregulates estrogen receptor alpha signaling in breast cancer cells
Oncotarget. 2016;7(44):71580-93. doi: 10.18632/oncotarget.12259

III. Diao Y, Rahman MF-U, Vyatkin Y, **Azatyan A**, St Laurent G, Kapranov P, Zaphiropoulos PG
Identification of novel GLI1 target genes and regulatory circuits in human cancer cells
Molecular Oncology. 2018;12(10):1718-34. doi: 10.1002/1878-0261.12366

IV. **Azatyan A**, Wang T, Darabi A, Siesjö P, Zaphiropoulos PG
Circular RNAs in Hedgehog signaling activation and Hedgehog-mediated medulloblastoma tumors
Manuscript

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List of abbreviations

circRNA	Circular RNA
DHH	Desert Hedgehog
ER	Estrogen receptor
GANT61	GLI antagonist 61
GLI	Glioma-associated oncogene
HHIP	Hedgehog interacting protein
IHH	Indian Hedgehog
JNK/SAPK	JUN amino-terminal kinase/Stress-activated kinase
MAPK	Mitogen-activated kinase
mRNA	Messenger RNA
PTCH	Patched
RITA	Reactivation of p53 and induction of tumor cell apoptosis
ROS	Reactive oxygen species
SAG	Smoothened agonist
SHH	Sonic Hedgehog
SMO	Smoothened
SUFU	Suppressor of fused

1 Introduction

1.1 Hedgehog signaling pathway

Hedgehog pathway is a highly conserved signal transduction cascade, initially discovered in *Drosophila melanogaster* (Nusslein-Volhard and Wieschaus, 1980) and subsequently described in higher organisms, including humans (Ingham and McMahon, 2001). The Hedgehog signaling pathway plays a pivotal role in embryogenesis, embryonic stem cell differentiation and tissue patterning. Later in adult physiology, the pathway is involved in stem cell maintenance, tissue repair and regeneration. Disruption of Hedgehog signaling leads to a variety of developmental abnormalities affecting multiple organ systems (Briscoe and Therond, 2013; Teglund and Toftgård, 2010), including holoprosencephaly (Gorlin, 1995), polydactyly, craniofacial defects and skeletal malformations (Ingham and McMahon, 2001; Jiang and Hui, 2008), whereas its aberrant ectopic activation can lead to tumor formation.

1.1.1 Hedgehog ligands

In mammals, canonical Hedgehog signaling is initiated by one of the three extracellular Hedgehog glycoprotein ligands, i.e Sonic Hedgehog (SHH), Indian Hedgehog (IHH) and Desert Hedgehog (DHH), which are expressed in tissue-specific manner. (Ingham, 2008; Pathi et al., 2001; Teglund and Toftgård, 2010). SHH has been the most studied of the three, as it is broadly expressed and affects the development of many organs, including central nervous system, lungs, teeth, intestines and hair follicles (Altaba et al., 2002; Chiang et al., 1996). IHH is involved in the cartilage, bone, intestine and ovary formation, while DHH regulates germ cell development in testis (Briscoe and Therond, 2013; McMahon et al., 2003; Varjosalo and Taipale, 2008).

All Hedgehog ligands are synthesized as precursor proteins that undergo autocatalytic cleavage and a release of amino-terminal domain containing a signaling peptide (Porter et al., 1995). This results in subsequent modifications of the amino-terminal domain: the covalent attachment of cholesterol moiety to its carboxyl terminus and palmitoylation of its amino terminus, which are required for proper activity, secretion, movement and reception of the Hedgehog ligands (Chen et al., 2004; Ingham and McMahon, 2001; Lewis et al., 2001; Pepinsky et al., 1998). The Hedgehog ligands are secreted as signaling molecules, which function both in nearby and distant cells (Varjosalo and Taipale, 2008).

1.1.2 Hedgehog signaling transduction from cell membrane to GLI

Mammalian Hedgehog signaling depends on primary cilium, a specialized cellular compartment where many of Hedgehog signaling components co-localize (Haycraft et al., 2005). The main pathway components are the 12-span transmembrane receptor Patched (PTCH1, PTCH2), which in the absence of Hedgehog ligands represses the proto-oncogene Smoothened (SMO), thus preventing the downstream signaling. In the “OFF” state Suppressor of fused (SUFU) binds and sequesters Glioma-associated oncogene family of transcription factors (GLI1, GLI2, GLI3), restraining target gene transcription (**Figure 1**).

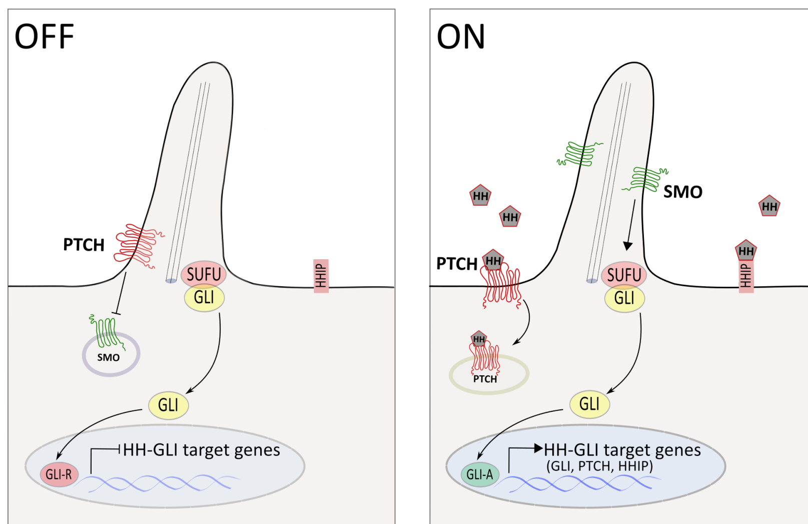

Figure 1. A simplified schematic model of the Hedgehog pathway in vertebrates.

(Left panel) In the absence of Hedgehog ligands (HH), PTCH represses SMO activity keeping the intracellular signal transduction at the "OFF" state. Here, full-length GLI proteins undergo sequential phosphorylation and proteolytic truncation, processing into repressor forms (GLI-R), predominantly GLI3. **(Right panel)** Binding of HH results in loss of PTCH activity, consequent activation and relocation of SMO from intracellular vesicles to cilium, and finally, recruitment and activation of full-length GLI proteins. Cytoplasmic sequestration of GLI by SUFU is released. Activated full-length GLI proteins (GLI-A), predominantly GLI1 and GLI2, enter the nucleus and promote transcription of target genes.

At the cell membrane, Hedgehog ligands can directly interact with PTCH, which in unbound state generally is localized at the base of the cilium. Of two PTCH homologs, PTCH1 is expressed ubiquitously, whereas PTCH2 is preferentially expressed in skin and testis (Carpenter et al., 1998; Hahn et al., 1996a). In addition, Hedgehog ligands can bind to membrane co-receptors CAM-related/downregulated by oncogenes (CDO), Brother of CDO (BOC) and Growth arrest-specific 1 (GAS1), which depending on the tissue context, usually enhance binding of the Hedgehog ligands to PTCH, and cooperate in promotion of Hedgehog signaling (Allen et al., 2011; Cardozo et al., 2014; Izzi et al., 2011). Another co-receptor, Hedgehog interacting protein (HHIP) acts as a negative regulator by competing for Hedgehog ligand binding with PTCH (Beachy et al., 2010).

Binding of extracellular Hedgehog ligands to PTCH results in internalization and endosomal degradation of PTCH-ligand complex, releasing the inhibitory effects of PTCH on SMO, a G protein-coupled receptor protein (Murone et al., 1999). It is not yet fully clear how PTCH regulates SMO activity. However, current view suggests that PTCH does not physically interact with SMO, but given its similarity to bacterial proton-driven efflux pumps, may rather function as a transporter of various SMO-regulating small molecules across the cytoplasmic membrane (Hasanovic et al., 2018; Taipale et al., 2002). In line with this, naturally occurring molecules that can be transported by PTCH, such as oxysterols and phosphatidylinositol 4-phosphate were shown to activate, while vitamin D3 was suggested to repress SMO activity (Bijlsma et al., 2006; Corcoran and Scott, 2006; Yavari et al., 2010).

Activation of SMO also depends on phosphorylation by G protein-coupled receptor kinase 2 (Teglund and Toftgård, 2010).

In response to Hedgehog signaling in vertebrates, SMO shuttles from intracellular vesicles to the membrane of the primary cilium, resulting in recruitment and activation of the GLI transcription factors (Haycraft et al., 2005). Here, several proteins, including SUFU, KIF7, regulate GLI function (Teglund and Toftgård, 2010). In the absence of Hedgehog signaling, SUFU makes an inactive complex with GLI, preventing its translocation into nucleus, while activated SMO causes disassociation of SUFU-GLI complex within the primary cilia. Upon activation, GLI factors translocate to the nucleus and promote transcription of Hedgehog target genes, including *GLI1* itself, *PTCH* and *HHIP* (Briscoe and Therond, 2013; Haycraft et al., 2005; Ingham, 2008; Kasper et al., 2006) (**Figure 1**). PTCH and HHIP provide a negative feedback loop restricting both auto- and paracrine spreading of the Hedgehog ligands, while GLI1 acts as an ultimate amplifier of the initial Hedgehog signal.

Additional targets of the Hedgehog-GLI pathway include FOXA2, which in turn induces the SHH activity and is involved in neural development (Mavromatakis et al., 2011); cyclin D1 and MYCN, involved in cell-cycle progression and regulation; BCL2, which can be increased upon Hedgehog signaling activation and is involved in cell survival; SNAIL, Insulin-like growth factor (IGF2) and Vascular endothelial growth factor (VEGF), involved in epithelial-mesenchymal transition in cancer metastasis (Amakye et al., 2013; Everson et al., 2018; Pak and Segal, 2016; Varjosalo and Taipale, 2008). The broad spectrum of genes that are regulated by Hedgehog signaling is continuously being discovered. In this perspective, in **PAPER III**, we have identified new GLI1 target genes using a combination of RNA-seq and follow-up molecular validation techniques in human cancer cell lines.

1.1.3 The GLI transcription factors

The GLI proteins contain five Zinc-finger motifs of which Zinc-finger 4 and 5 bind to a 9 base pair DNA binding consensus sequence 5'-GACCACCCA within the GLI target genes (Kinzler and Vogelstein, 1990). GLI1 was the first known GLI factor, initially identified in glioblastoma (Kinzler et al., 1987). GLI1 acts as an activator of the pathway and is found only in a full-length activator form, while GLI2 and GLI3 can also be partially processed into truncated repressor forms (Aberger and Ruiz, 2014; Teglund and Toftgård, 2010; Yang et al., 2010). GLI1 is considered as a main effector of Hedgehog signaling which is capable of upregulating its own gene expression, resulting in a positive feedback loop (Briscoe and Therond, 2013). Thus, expression of GLI1 is a reliable indicator of Hedgehog pathway activity (Shimokawa et al., 2008).

Some studies argue for a prominent role of GLI2 in Hedgehog signaling activation and subsequent tumor formation, which unlike GLI1, could be triggered independent of SHH (Bai et al., 2002; Bai and Joyner, 2001; Park et al., 2000). Moreover, transgenic mice bearing homozygous *Gli1* hypomorphic or null mutations were shown to be phenotypically normal, while mice with similar mutations in *Gli2* died soon after birth having abnormalities in

various tissues, including spinal cord and lungs (Bai and Joyner, 2001; Park et al., 2000). Interestingly, *Gli1* and *Gli2* double homozygous null mutants have more severe developmental defects, suggesting their overlapping roles (Bai et al., 2002; Bai and Joyner, 2001; Park et al., 2000). Several studies also indicate certain compensatory roles of GLI1 and GLI2 functions (Bai and Joyner, 2001; Kimura et al., 2005; Lipinski et al., 2006). For example, when *Gli1* cDNA was knocked into the *Gli2* locus, it was able to rescue all Shh signaling defects in *Gli2*$^{-/-}$ mice (Bai and Joyner, 2001). It is noteworthy that, although these gene misexpression studies can greatly clarify the functions of GLI proteins, they may not necessarily fully reflect the normal developmental situations.

Regulation of the GLI factors involves a multitude of mechanisms including post-transcriptional modifications, such as alternative splicing (Shimokawa et al., 2008), RNA editing (Shimokawa et al., 2013), post-translational modifications including acetylation, phosphorylation, ubiquitination, and cytoplasmic-nuclear shuttling (Briscoe and Therond, 2013; Mirza et al., 2019). For example, in the absence of Hedgehog ligand, full length GLI proteins (mainly GLI3) are phosphorylated by Protein kinase A (PKA), Glycogen synthase kinase 3-beta (GSK3-β), Casein kinase 1 (CK1), leading to proteolytic cleavage of GLI into carboxy-terminally truncated repressor form (Teglund and Toftgård, 2010; Yang et al., 2010).

It has been shown that GLI1 can be RNA-edited by Adenosine deaminase acting on RNA (ADAR) via deamination of the Adenosine at nucleotide position 2179, converting it to Inosine. This Adenosine to Inosine substitution of the mRNA molecule results in a codon change from Arginine to Glycine at position 701 of the GLI1 protein, resulting in subsequent changes in its functional activity. As a result, edited GLI1 can exhibit reduced sensitivity towards SUFU, slightly higher transcriptional activity, but reduced effectivity at promoting cellular growth (Shimokawa et al., 2013). It is worth noting, that we have further addressed the cell-context specific differences or similarities of GLI1 and edited GLI1 target genes in **PAPER III**.

1.1.4 Regulation of Hedgehog pathway by non-Hedgehog signals

The Hedgehog pathway can also be activated by so-called “non-canonical” mechanisms, which are Hedgehog ligand-independent. This mode of activation is downstream of PTCH/SMO and culminates in the activation of the GLI transcription factors.

Several signaling cascades that can promote tumorigenesis, e.g. Transforming growth factor beta (TGF-beta), Epidermal growth factor receptor (EGFR), Mitogen-activated kinases (MAPK), K-RAS, PI3K/AKT/mTOR, Wingless (WNT) pathways were shown to converge and activate Hedgehog signaling. Additionally, crosstalk between Hedgehog signaling components and tumor suppressors, such as p53, PTEN, PCAF have also been demonstrated (Aberger and Ruiz, 2014; Mazza et al., 2013; Stecca and Ruiz i Altaba, 2009) (**Figure 2**).

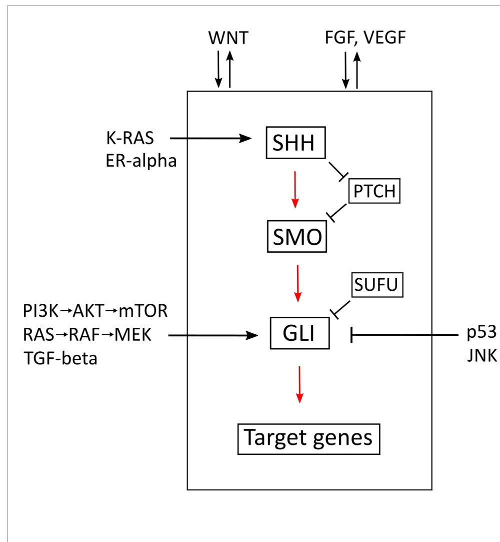

Figure 2. A schematic diagram representing the crosstalk of Hedgehog and other signaling pathways.

WNT signaling or growth factor FGF, VEGF pathways can crosstalk with Hedgehog signaling during tissue development or orchestrate tumorigenesis. SHH levels can be regulated by K-RAS or ER-alpha. Depending on the cellular context and upstream activating events, GLI levels can be regulated by RAS family of GTPases, e.g. PI3K/AKT, RAF/MEK, JNK/MAPK which can act as powerfull oncogenes in various human tumors. TGF-beta ligands can induce GLI expression independent of SMO. The p53 pathway can antagonise Hedgehog-GLI.

Abbreviations. WNT: wingless, FGF: fibroblast growth factor, VEGF: vascular endotelial growth factor, PI3K: phophoinositide 3-kinase, AKT: protein kinase B, MEK: mitogen-activated protein kinase kinase, JNK: JUN amino-terminal kinase, TGF: transforming growth factor.

1.1.4.1 JNK kinase mediated regulation of Hedgehog-GLI

JUN amino-terminal kinases (JNK1, JNK2, JNK3), also known as stress-activated protein kinases (SAPK), are components of the major group of MAPK (Coffey, 2014). JNKs are activated by dual phosphorylation on Threonine and Tyrosine in the conserved tripeptide motif by the members of MAPK kinase group of protein kinases. Upon activation, JNKs phosphorylate Serine and Threonine residues of various target substrates, contributing to the regulation of cell proliferation and apoptosis, orchestrating both tumor suppressing and activating responses (Davis, 2000). While JNK1 and JNK2 are expressed ubiquitously, JNK3 is rather abundant in the brain. The JNKs are key players in the developing and adult brain, where different isoforms and splice variants of JNKs are differentially expressed (Brecht et al., 2005; Coffey, 2014).

Several studies indicated interactions between JNK proteins and the Hedgehog-GLI pathway (Amable et al., 2014; Laner-Plamberger et al., 2009; Liu et al., 2015). For example, basic fibroblast growth factor was shown to block SHH signaling in granule cell precursors and medulloblastoma tumor cells *in vitro* and *in vivo* in mice by predominantly JNK-mediated mechanisms (Fogarty et al., 2007). Additionally, the increased JNK1 phosphorylation induced by H_2O_2 treatment was correlated with decreased expression of Hedgehog signaling genes and GLI1 activity in fibroblast cells (Shiohama et al., 2014). In another study, Whisenant et al. predicted a new MAPK docking-site on GLI3 protein (residues 281-300), and by mass spectrometry have further confirmed that phosphorylation of Serine 343 on GLI3 is JNK1-3-mediated and docking-site-dependent. Computational analysis also pinpointed highly similar docking-sites on GLI1 and GLI2. In particular, they found a homologue docking-site on GLI1 (residues 72-91) mediating the binding of JNK1-3 to GLI1,

which promotes phosphorylation of target sites within GLI1 (residues 68-232) and specifically, Serine 130 (Whisenant et al., 2010).

1.1.4.2 p53 tumor suppressor mediated regulation of Hedgehog-GLI

The paramount role of p53 as a tumor suppressor is well known, and the majority of cancers carry inactivating mutations of p53 or de-regulation of the p53 pathway (Soussi and Wiman, 2015; Zawacka-Pankau and Selivanova, 2015).

Several studies suggested a negative regulatory loop between Hedgehog and p53 signaling, although yet little is known about the mechanisms underlying this interplay (Mazza et al., 2013; Stecca and Ruiz i Altaba, 2009). p53 has been suggested to negatively regulate the activity, nuclear localization and levels of GLI1 in neural stem cells and brain tumor cells, while GLI1 knockdown increased protein levels of p53 and active phospho-serine15 p53 in primary glioblastoma cells (Stecca and Ruiz i Altaba, 2009). This negative regulatory loop can be also maintained by competitive p53 and GLI1 binding to the coactivator TATA-box binding protein associated factor 9 (TAF9) (Yoon et al., 2015). Another possible mechanism is the Hedgehog signaling-mediated phosphorylation and activation of MDM2, which promotes p53 ubiquitination and inhibits p53-mediated apoptosis (Abe et al., 2008). Additionally, it was suggested that p53 can activate a novel *PTCH1* homolog (*PTCH53*), resulting in suppression of Hedgehog signaling (Chung et al., 2014).

Given the importance of p53 as a crucial tumor suppressor and the evidence of a negative regulatory loop between Hedgehog and p53 signaling, we were inspired to restore the p53 function in Hedgehog-dependent tumor cells (**PAPER I**).

1.2 Hedgehog signaling in human cancers

Inappropriate activation of Hedgehog signaling has been linked to hyperproliferation and cancer development, including medulloblastoma, rhabdomyosarcoma, basal cell carcinoma, cancers of the lung, stomach, pancreas, colon and prostate (Aberger and Ruiz, 2014; Altaba et al., 2002; Amakye et al., 2013; Ng and Curran, 2011; Pietrobono et al., 2019; Skoda et al., 2018; Teglund and Toftgård, 2010), accounting for about 25% of human cancer deaths (Lum and Beachy, 2004). In this thesis we will mainly focus on medulloblastoma, rhabdomyosarcoma and breast cancer.

1.2.1 Hedgehog pathway activity and oncogenic drivers

Mechanisms linking constitutive activation of the Hedgehog pathway to tumorigenesis include ligand-independent mutations of key players of the pathway, ligand-dependent autocrine and paracrine activation, and finally cancer stem cell-driven activation of Hedgehog signaling.

The first evidence of oncogenesis and mutation-driven mechanism in the Hedgehog pathway was observed in the nevoid basal cell carcinoma syndrome, also known as Gorlin syndrome, with inherited loss of function mutations in one allele of the *PTCH1* gene. Somatic

inactivation of the other *PTCH1* allele was demonstrated to result in development of basal cell carcinoma (Hahn et al., 1996b; Johnson et al., 1996). Interestingly, these loss of function heterozygous mutations of *PTCH1* were also shown to lead to the development of primitive neuroectodermal tumors of cerebellum, medulloblastomas (Raffel et al., 1997; Yang et al., 2008) and rhabdomyosarcomas (Tostar et al., 2006). Loss of function mutations of *SUFU*, another negative regulator of the Hedgehog pathway, have been observed in basal cell carcinoma, medulloblastoma, rhabdomyosarcoma (sporadic mutations) and Gorlin syndrome-associated childhood medulloblastoma (germline mutations) (Lee et al., 2007; Smith et al., 2014; Taylor et al., 2002; Tostar et al., 2006).

Another significant driver of Hedgehog-dependent tumorigenesis are gain of function mutations and/or amplifications of positive regulators of Hedgehog signaling. Such are activating mutations of *SMO* in basal cell carcinoma and medulloblastoma (Reifenberger et al., 1998; Xie et al., 1998), *GLI1* amplifications in gliomas, medulloblastomas and rhabdomyosarcomas, and *GLI2* amplifications in medulloblastoma (Amakye et al., 2013; Kool et al., 2014; Nilsson et al., 2000; Roberts et al., 1989).

The complete relevance of ligand-dependent activation of Hedgehog signaling as a major driver of cancer is less clear, since it is more complex and involves multiple intrinsic and extrinsic factors, including the tumor itself, tumor microenvironment and adjacent tissues, as well as various possible multiplex crosstalks between these compartments. Ligand-dependent autocrine activation of the Hedgehog pathway through elevated expression of Hedgehog ligands was demonstrated in cancers of lung, breast, stomach and prostate (Amakye et al., 2013).

Ligand-dependent paracrine activation involves increased secretion of Hedgehog ligands by the tumor cells, which further expand and trigger Hedgehog pathway activation in the tumor microenvironment, composed of surrounding normal and tumor supporting stromal cells. In turn, activated stroma stimulates growth of the tumor by various signaling mediators, including Insulin-like growth factor, WNT, Interleukin-6 and Vascular endothelial growth factor. This type of signaling may play a role in solid tumor types, which rarely harbor mutations in Hedgehog genes, e.g. ovarian, pancreatic and colorectal cancers (Yauch et al., 2008).

Finally, Hedgehog signaling is believed to contribute to the maintenance and determination of cancer stem cells in breast, colon, pancreatic cancer and glioblastoma (Bar et al., 2007; Merchant and Matsui, 2010). Considering the role of Hedgehog signaling in embryogenesis and cell fate determination, and the fact that later in normal adult tissues Hedgehog signaling is mainly active in precursor cells, it has been argued that the Hedgehog-associated tumors can be derived from precursor and stem cells (Ruiz i Altaba et al., 2002).

1.2.2 Hedgehog signaling in medulloblastoma

Medulloblastoma is a highly aggressive class of brain tumors, with highest occurrence among childhood brain tumors. Medulloblastoma originates from cerebellar progenitor cells and

exhibits considerable genomic heterogeneity, which defines distinct clinical subsets of this disease. Currently four large molecular subgroups of medulloblastomas are recognized: WNT (10%), SHH (30%), group 3 (25%) and group 4 (35%) (Juraschka and Taylor, 2019; Rusert et al., 2014). Conventional therapies of medulloblastomas are implemented through invasive methods such as surgery, radiotherapy and high-dose cytotoxic chemotherapy. Targeted therapy based on molecular classification of the tumor could improve the disease risk and minimize general toxicity of the treatment.

SHH plays critical roles in controlling the development of cerebellum, influencing cell proliferation, differentiation and migration. SHH medulloblastomas are known to arise from cerebellar granule neuron progenitors derived from the external granule cell layer, cochlear nuclei of the brainstem, and also neural stem cells residing in the subventricular zone (Altaba et al., 2002; Northcott et al., 2012b). Amplifications and mutations of Hedgehog pathway components (*PTCH1*, *GLI2*, *GLI1*, *SMO*) are common drivers of SHH medulloblastomas. Loss of function mutations of *PTCH1* are found in about 20% of sporadic medulloblastomas (Raffel et al., 1997). Mutations in *SMO* and *SUFU* are less frequent (Taylor et al., 2002; Zurawel et al., 2000), although activation of *SMO* results in early cerebellar hyperproliferation and high rate of medulloblastoma formation (Hallahan et al., 2004; Hatton et al., 2008).

Amplifications of *GLI2* and another oncogene *MYCN* are also considered as common drivers of SHH medulloblastomas (Dahmane et al., 2001; Kool et al., 2014; Northcott et al., 2012b). GLI1 has been suggested to induce expression of the SNAIL1 transcription factor, which in turn regulates *MYCN*, thus mediating the transition of oncogenic cerebellar granule neuron progenitor cells into medulloblastoma (Wanshura et al., 2011). In mouse model studies, Kimura et al. have demonstrated that medulloblastoma formation was significantly reduced in $Ptch1^{+/-}$, $Gli1^{-/-}$ mice, i.e. in the absence of functional GLI1. Interestingly, $Ptch1^{+/-}$, $Gli1^{+/-}$ and $Ptch1^{+/-}$, $Gli1^{-/-}$ mice had higher expression of *Gli2* (Kimura et al., 2005). These observations indicate that first, GLI1 plays a crucial role in formation of medulloblastoma tumors, and second, increased GLI2 expression may compensate for the reduced level of GLI1, further regulating target genes responsible for tumor formation in these mice.

In addition, SHH medulloblastomas can also harbor recurrent mutations in the p53 gene. Medulloblastoma formation is dramatically accelerated in double knockout mice with heterozygous defective PTCH ($Ptch1^{+/-}$) and homozygous defective p53 ($p53^{-/-}$), where almost 95% of these mice develop medulloblastoma by the 12 weeks of age (Wetmore et al., 2001). Possible mechanism for this increased tumor frequency could be the interruption of the GLI1-p53 negative regulatory loop in neural precursor cells (Stecca and Ruiz i Altaba, 2009). In fact, p53 gene mutations are particularly enriched in SHH medulloblastomas (Zhukova et al., 2013), and patients with p53 mutated SHH medulloblastomas are at very high risk of reduced survival and almost always fail therapy (Ramaswamy et al., 2016). Thus, PTCH1 loss of function provides a stronger selective advantage in medulloblastoma development, and loss of p53 activity results in substantial enhancement of tumors.

Overall, among Hedgehog-linked tumors, medulloblastoma is one of the well-understood from the Hedgehog pathway perspective. Still, better understanding of the biology of medulloblastoma and approaches focusing on selective targeting of key drivers of the disease, such as Hedgehog-components and p53, could provide promising therapeutic outcomes.

1.2.3 Hedgehog signaling in rhabdomyosarcoma

Rhabdomyosarcoma originates from the skeletal muscle precursor cells, is highly malignant and is the most common soft tissue sarcoma in childhood (Walsh and Hurt, 2008). Two major histological subtypes are known: embryonal and alveolar rhabdomyosarcomas. Sporadically occurring rhabdomyosarcomas are characterized with hyperactive Hedgehog signaling, which is highlighted by elevated *GLI1* and *PTCH1* expression (Roberts et al., 1989; Zibat et al., 2010), and loss of heterozygosity of *PTCH1* and/or *SUFU*, and *HHIP* (Tostar et al., 2006; Uhmann et al., 2005). Other studies also support the involvement of Hedgehog signaling in embryonal rhabdomyosarcoma tumors with frequent loss of chromosomal regions corresponding to *PTCH1* 9q22 and gain of the *GLI1* locus at 12q13-15 (Bridge et al., 2000).

Like medulloblastomas, rhabdomyosarcoma development and frequency can also depend on the genetic background. In experimental mouse models homozygous removal of the p53 gene on the background of activated Hedgehog signaling can increase the incidence of rhabdomyosarcomas (Teglund and Toftgård, 2010).

1.2.4 Hedgehog signaling in breast cancer

Breast cancer is the leading cause of cancer-related deaths and the most common cancer in women (Harbeck et al., 2019). It is a heterogeneous disease and molecular profiling of breast cancer tumors is generally defined by the expression of estrogen and progesterone hormone receptors, human epidermal growth factor receptor 2 (HER2) tyrosine kinase and the nuclear marker of cell proliferation Ki-67. Due to the high heterogeneity of breast cancer subtypes, the treatment response to therapeutic agents varies, suggesting deregulation of other oncogenes (Riobo-Del Galdo et al., 2019).

Possible crosstalk between Hedgehog signaling activation and the development of breast cancer has been widely studied (Flemban and Qualtrough, 2015; Hui et al., 2013). Transgenic mice with conditional overexpression of GLI1 were shown to develop mammary tumors (Fiaschi et al., 2009; Hui et al., 2013). Several studies have indicated activation of Hedgehog signaling and increased GLI1 expression in subsets of breast cancers and cell lines (Bhateja et al., 2019; Mukherjee et al., 2006; Riobo-Del Galdo et al., 2019). It was suggested that increased GLI expression and activated Hedgehog signaling may drive estrogen-dependent stem cell development and epithelial-mesenchymal transition in estrogen receptor-alpha (ER-alpha) positive breast cancer cells (Sun et al., 2014). Moreover, Hedgehog signaling activity and GLI1 nuclear translocation was shown to be increased by estrogen stimulation, enhancing invasiveness of breast cancer cells (Souzaki et al., 2011; Visbal et al., 2011).

Additionally, non-canonical activation of Hedgehog signaling through PI3K/AKT pathway has been suggested to mediate tamoxifen resistance in breast cancer cells. In this study, tamoxifen resistant cells had elevated levels of GLI1, SMO and increased GLI1 transcriptional activity, compared to tamoxifen sensitive cells. Moreover, Hedgehog signaling inhibition suppressed the growth of tamoxifen resistant breast cancer cells *in vitro* and *in vivo* mouse xenografts (Ramaswamy et al., 2012). In **Paper II,** we have further investigated the mechanisms underlying the role of Hedgehog-GLI signaling in breast cancer and the possible role of GLI1 as a therapeutic target.

1.3 Therapeutic approaches for Hedgehog-activated tumors

Hedgehog signaling can be targeted at many levels. The strategies to suppress aberrantly activated Hedgehog signaling in different cancers have focused on inhibition of key players of the "canonical" and "non-canonical" Hedgehog pathway. Currently, these include extracellular blockade of Hedgehog ligands, inhibition of SMO or repression of GLI action in the cells.

1.3.1 SMO inhibitors

Early inhibitors of the Hedgehog pathway were mainly focused on targeting SMO. Cyclopamine is the most extensively studied SMO inhibitor (Chen et al., 2002). It was initially isolated from corn lily and demonstrated high efficiency in preclinical studies, but failed clinical development due to poor pharmacokinetic features and general toxicity (Amakye et al., 2013). Since then, efforts to improve the chemical stability and efficacy of cyclopamine have led to the identification of its more potent derivatives and analogs, such as saridegib (IPI-926), vismodegib (GDC-0449) and sonidegib (LDE-225). In fact, vismodegib was the first Hedgehog pathway targeting drug approved by the U.S. Food and Drug Administration (FDA) for locally advanced and metastatic basal cell carcinoma (Sekulic et al., 2012). Later in 2015, sonidegib was also approved for locally advanced basal cell carcinoma (Migden et al., 2015). Together with other derivatives (IPI-926, BMS-833923, PF-04449913, LY2940680) (Jimeno et al., 2013; LoRusso et al., 2011; Rodon et al., 2014), these drugs are currently undergoing clinical trials for other cancers, including medulloblastoma, colorectal and advanced pancreatic cancer (Amakye et al., 2013; Cortes et al., 2019; Pak and Segal, 2016).

However, cases of cross-resistance or acquired resistance against SMO inhibitors have already been reported in basal cell carcinoma and medulloblastoma (Chang and Oro, 2012; Jimeno et al., 2013; Rudin et al., 2009). Acquired resistance to SMO inhibitors has mainly been linked to mutations in *SMO* (Atwood et al., 2015; Rudin et al., 2009; Sharpe et al., 2015). Moreover, resistant tumors often harbor mutations in other Hedgehog pathway genes, such as mutations in *SUFU*, amplification or activation in the downstream regulators *GLI2* and *GLI1* (Dijkgraaf et al., 2011; Metcalfe and de Sauvage, 2011). Additionally, tumor drivers beyond the Hedgehog pathway genes may also explain some of the clinically

heterogenic responses to SMO inhibition, demonstrated in basal cell carcinoma and medulloblastoma (Bonilla et al., 2016; Kool et al., 2014).

Preclinical studies have also revealed complications associated with toxicities of SMO inhibitors, including taste disturbance, alopecia, muscle spasms, anorexia, fatigue, bone and dental toxicities (Amakye et al., 2013; Kimura et al., 2008). Moreover, administration of higher doses of vismodegib does not enhance its effects, but rather escalates systemic toxicity. Higher dose application of sonidegib, saridegib and BBS-833923 instigate specific adverse effects on the levels of liver enzymes, serum lipase and creatine kinase (reviewed in (Amakye et al., 2013)).

Due to complications with acquired resistance and toxicity, inhibition of SMO alone may not provide sufficient therapeutic benefits. Instead, combinations with other agents may lead to more beneficial outcomes. Notably, since the GLI proteins are the terminal effectors of Hedgehog signaling, their effective inhibition can be particularly attractive. The increased awareness of SMO-independent GLI activation further highlights the importance of targeting GLI.

1.3.2 GLI inhibitors

The most promising targets downstream of SMO are the GLI transcription factors. Compared to the various SMO inhibitors, currently there are fewer drugs in the market targeting the GLI factors. Examples of early GLI inhibitors are itraconazole and arsenic trioxide, which have been approved by FDA, and both entered clinical trials for basal cell carcinoma (Kim et al., 2013). More recently identified small molecule Glabrescione B was shown to directly bind to GLI1 and impair GLI1 transcriptional activity by interfering with GLI1-DNA interaction and resulting in inhibition of the Hedgehog signaling in medulloblastoma, basal cell carcinoma and pancreatic tumor cells *in vitro* and *in vivo* mouse xenografts (Infante et al., 2015; Ingallina et al., 2017). In a cell-based screen for small molecule inhibitors of GLI, two other selective drugs (GANT61 and GANT58) have been discovered to suppress GLI1 and GLI2-mediated gene transactivation (Lauth et al., 2007). Further investigation of the relatively more potent agent GANT61 has proven its high efficacy in suppressing Hedgehog signaling-dependent growth in different cancer cell types and respective animal models, including rhabdomyosarcoma (Tostar et al., 2010), neuroblastoma (Wickström et al., 2013), prostate (Lauth et al., 2007), pancreatic (Fu et al., 2013), hepatocellular (Wang et al., 2013), breast (Benvenuto et al., 2016; Neelakantan et al., 2017) and small cell lung (Huang et al., 2014) cancers.

Initially GANT61 was proposed to induce possible modification of GLI1 protein, without altering DNA binding of GLI1 (Lauth and Toftgard, 2007). In line with this, later it has been suggested that GANT61 binds to the GLI1 protein in a groove between Zinc-finger 2 and 3, at sites E119 (1 hydrogen bond) and E167 (2 hydrogen bonds), distinct from the GLI1 DNA binding region. Interestingly, these binding sites are conserved between GLI1 and GLI2, which can explain the inhibitory effect of GANT61 also on GLI2 (Agyeman et al., 2014;

Calcaterra et al., 2018). Lauth et al. also proposed a possible high instability of GANT61 molecule in acidic and aqueous solutions, which rapidly disassociates into a diamine (GANT61-D) and a benzaldehyde (GANT61-A) substructures. Notably, only the diamine but not the aldehyde derivative was capable of inhibiting Hedgehog signaling with a comparable efficacy compared with parental GANT61 molecule (Lauth et al., 2010). In addition, a recent study confirmed that in physiological conditions GANT61 hydrolyses into GANT61-A and GANT61-D, but with GANT61-D exhibiting slightly higher bioactivity in mediating GLI1 expression levels and expressing highest affinity to GLI1 Zinc-finger 1 and 2 (Calcaterra et al., 2018).

1.3.3 RITA as an inhibitor of Hedgehog-GLI signaling

Misregulation of the p53 pathway exhibited by inactivating recurrent mutations in the p53 gene or a p53-null background can dramatically enhance tumorigenesis. In Hedgehog-activated cancers, such as medulloblastoma and rhabdomyosarcoma, in addition to targeting Hedgehog signaling, strategies aiming at restoring p53 function may provide certain advantages. Restoration of mutant p53 activity is especially attractive, since mutant p53 proteins are usually selectively highly expressed and accumulated in tumors, thus targeting mutant p53 principally should not affect normal tissues (Selivanova and Wiman, 2007). A number of small molecules reactivating mutant p53 have been identified, including PRIMA-1 (Bykov et al., 2002), RITA (Issaeva et al., 2004), APR-246 (Bykov and Wiman, 2014).

Small molecule NSC652287, later named RITA (reactivation of p53 and induction of tumor cell apoptosis) was identified as a potential tumor suppressor (Nieves-Neira et al., 1999; Rivera et al., 1999). It was discovered in a cell-based screening assay, which differed only in the p53 status of the cells analyzed, thus this inhibitory drug was claimed to exert its effects in a p53-dependent manner, through downregulation of a number of oncogenes and activation of survival genes, resulting in cancer cell apoptosis and growth suppression in a dose-dependent manner (Grinkevich et al., 2009; Issaeva et al., 2004; Nieves-Neira et al., 1999; Nikulenkov et al., 2012). It was suggested that RITA binds to the amino-terminus of p53, blocking its interaction with MDM2, a negative regulator of p53 (Issaeva et al., 2004). Later it was demonstrated that RITA can induce DNA damage and interact with Thioredoxin reductase 1 (TrxR1), leading to ROS induction (Hedström et al., 2009). ROS-dependent JNK activation was suggested as one possible mechanism of RITA action (Hedström et al., 2009; Shi et al., 2014; Weilbacher et al., 2014). Notably, RITA was found to be an efficient activator of both wild type and mutant p53 in a panel of neuroblastoma cell lines (Burmakin et al., 2013). Additionally, it is possible that nonmalignant cells (fibroblast, peripheral blood mononuclear cells) are substantially less sensitive to RITA compared to tumor cells, making RITA a valuable tumor-selective drug (Saha et al., 2010; Weilbacher et al., 2014).

Intriguingly, further studies suggested p53-independent effects of RITA, questioning its selective binding to p53 (Krajewski et al., 2005) and suggesting that p53 might be dispensable for RITA activity, as its effects are largely mediated through induction of DNA damage (Wanzel et al., 2016) or inhibition of mRNA translation by inducing phosphorylation

of the alpha subunit of the eukaryotic translation initiation factor 2 (elF2-alpha) (Ristau et al., 2019). Indeed, it was reported that RITA could inhibit the growth and induce senescence of head and neck cancer cells, irrespective of the p53 status (Chuang et al., 2014). Weilbacher et al. demonstrated that whereas p53 has a central role for RITA-mediated effects in wild type p53 cells, neither p53, nor the other two homologs of p53 (p63 or p73) are essential for the RITA response in mutant or p53-null cells. They also suggested, that RITA-induced apoptosis is predominantly mediated by JNK/SAPK and p38 MAPK pathways (Weilbacher et al., 2014).

Still very little is known about RITA transport into the cells and the question why different cells respond variably to RITA is still open. This might be due to differential uptake, accumulation and metabolism of the drug, but the exact mechanisms that impinge on RITA activity are a matter of further investigation.

In **PAPER I,** we have reported a p53-independent action of RITA and showed that RITA can effectively downregulate Hedgehog signaling *in vitro* and suppress rhabdomyosarcoma-cell xenograft tumor growth *in vivo*. In addition, we have addressed the combinatorial effects of RITA and GANT61 in medulloblastoma and rhabdomyosarcoma cells *in vitro* and *in vivo*.

1.4 Circular RNAs

Circular RNAs (circRNAs) in eukaryotes were identified few decades ago (Cocquerelle et al., 1993; Nigro et al., 1991; Zaphiropoulos, 1996) and until recently have been largely neglected as rare and non-functional by-products of the splicing machinery. However, with the advent of next generation sequencing they have been re-discovered as a new class of ubiquitously expressed and biologically functional non-coding RNA molecules.

1.4.1 Biogenesis

circRNAs are unique by their secondary structure and are generated from canonical splice sites through alternative splicing, when the 3' end of a downstream exon "back-splices" to the 5' end of an upstream exon producing a covalently closed loop (**Figure 3**). The back-splicing is catalyzed by the canonical spliceosome machinery and can be facilitated by several factors. The presence of intronic inverted repeats is one of the prevalent features of circRNAs. Introns flanking the circularized exons appear to be enriched with Alu repetitive elements, which can significantly contribute to circRNA biogenesis (Jeck et al., 2013; Liang and Wilusz, 2014). Additionally, tissue specific exon circularization can be facilitated by RNA-binding proteins, such as the splicing factors muscleblind in *Drosophila* and Quaking (Ashwal-Fluss et al., 2014; Conn et al., 2015). An exon skipping event during linear splicing, via the formation of a lariat structure encompassing the skipped exons, can also contribute to circRNA biogenesis (Barrett et al., 2015; Zaphiropoulos, 1997). On the other hand, the RNA editing enzyme

ADAR1 that binds RNA in double stranded regions can act as an inhibitor of circRNA production by destabilizing the pairing between complementary motifs present in the flanking introns of circularizing exons (Rybak-Wolf et al., 2015).

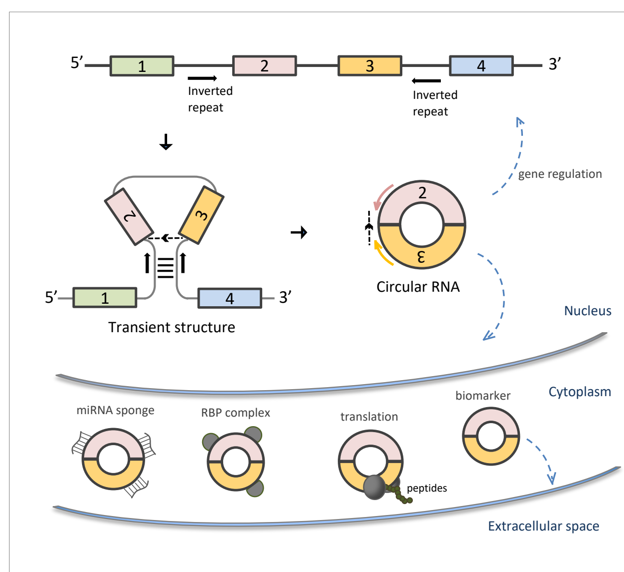

Figure 3. Circular RNA biogenesis.
Genes produce not only linear mRNAs, but also circular RNAs. Circularization occurs via back-splicing of exons. In this illustration, exon 3 back-splices to exon 2 to produce a circular RNA. This is facilitated by inverted repeats in the introns flanking the back-spliced exons.
In the nucleus, circular RNAs can regulate their parental gene activity. In the cytoplasm, circular RNAs can act as miRNA sponges and limit their interaction with cognate mRNAs. They can also function as sponges or decoys for RNA-binding proteins (RBP) and thus regulate their activity. Circular RNAs can be translated giving rise to unique peptides. Finally, circular RNAs can serve as diagnostic and prognostic biomarkers.

1.4.2 Detection

Apart from the lack of a poly(A) tail, another key characteristic of circular transcripts is the presence of a back-spliced junction, which makes them distinguishable from their linear counterparts. Because of these unique features, circRNAs are lost in standard RNA-seq profiling or even in PCR assays that are usually enriched for poly(A) RNAs and detect forward-spliced junctions. Thus, to identify circRNAs some additional modifications of standard protocols need to be implemented. These include depletion of ribosomal RNA by methods such as riboZero techniques, and treatment with RNAse R, a ribonuclease that degrades linear but not circRNAs. Additionally, reverse transcription (RT) should be done using random hexamers instead of oligo(dT) primers (Szabo and Salzman, 2016).

Once a circRNA enriched preparation is subjected to RNA-seq, specific bioinformatic algorithms based on detection of back-spliced junction have to be used. Such algorithms are constantly being developed and updated, for example CIRI (https://sourceforge.net/projects/ciri-full/) and CIRCexplorer (https://circexplorer2.readthedocs.io/en/latest/) (Hansen et al., 2016). A few important factors to consider are the mapper (i.e. TopHat, STAR, Bowtie) and the associated stringency criteria employed by different algorithms, as their false-positive rates can vary considerably (Kristensen et al., 2019).

The information about genomic positions of back-spliced junctions can be used for experimental validations of computationally predicated circRNAs. RT-PCR based assays are

most frequently used, where divergent primers are designed to amplify the back-spliced junction on random hexamers primed cDNA. Additionally, Sanger sequencing validation of the resulting PCR product can confirm the presence of a back-spliced junction. Quantitative qPCR assays can be used to determine the abundance of a circRNA transcript relative to a housekeeping gene, while droplet digital PCR can determine the absolute concentrations using the ratio of positive to negative droplets. Northern blotting can have certain advantages, as it does not include RT or amplification steps, although here the procedures are more time-consuming and large quantities of starting RNA material are required. It is also possible to visualize circRNAs in cells or tissue samples by RNA *in situ* hybridization with oligonucleotide probes (Kocks et al., 2018; Kristensen et al., 2019; Li et al., 2018). In all these settings, great care should be taken to differentiate the circular transcript from the linear mRNAs originating from the same parental gene.

1.4.3 Functions

In contrast to early views, there is now a compelling evidence that circRNAs are stably and abundantly expressed in eukaryotic cells, approximately 2-4% of the total mRNA (Szabo and Salzman, 2016), and in some cases even more abundant than the mRNAs originating from the same gene (Jeck et al., 2013; Salzman et al., 2012). circRNA transcripts can be ubiquitously expressed in a cell and tissue-specific manner and regulate various biological processes (Ji et al., 2019; Maass et al., 2017; Memczak et al., 2013; Salzman et al., 2012; Vo et al., 2019), including brain function (Piwecka et al., 2017; Rybak-Wolf et al., 2015).

In general, circRNAs are expected to have different functions from their linear counterparts. Even though compared to mRNAs, circRNAs are generally expressed at lower levels, it is likely that a co-transcriptional competition may exist between back-splicing and canonical forward-splicing, especially for loci that extensively produce circRNAs (Kristensen et al., 2019).

circRNAs can act as molecular traps for microRNAs (miRNAs) and inhibit the bioavailability of miRNAs to interact with their target mRNAs. miRNAs are known to regulate gene expression by suppressing translation and enhancing exonucleolytic degradation of mRNAs. The increased stability of circRNAs with no free 5’ or 3’ ends makes them inherently resistant to miRNA-mediated exonucleolytic degradation and fits well with a role in sequestering miRNAs (Ebbesen et al., 2017; Yu et al., 2018).

circRNAs can interact with RNA-binding proteins and possibly function as scaffolds for protein interactions (Ebbesen et al., 2017; Feng et al., 2019; Xia et al., 2019). Such an example is circFOXO3 derived from the tumor suppressor gene *FOXO3*, which possesses binding sites for MDM2, the negative regulator of p53, and inhibits MDM2-dependent FOXO3 degradation while facilitating MDM2-induced p53 degradation (Du et al., 2017).

While most of the exonic circRNAs are localized in the cytoplasm, some exon-intron circRNA transcripts generated from intronic lariats are restricted to the nucleus where they can interfere with the transcription of their parental genes. For example, some such circRNA

transcripts were shown to regulate RNA Polymerase II-mediated transcription (Bose and Ain, 2018; Ebbesen et al., 2017).

Although circRNAs are generally considered as a non-coding class of RNA molecules, their predominant accumulation in the cytoplasm may support a cap-independent translation of circRNAs with an AUG start codon and an internal ribosomal entry sites (Zeng et al., 2017).

circRNAs can be present not only inside the cell but also circulate in the extracellular plasma, blood and saliva (Memczak et al., 2015) or be transported by exosomes (Li et al., 2015). Such tumor-derived circRNAs can be promising diagnostic biomarkers.

1.4.4 Circular RNAs in cancer

Owing to their high stability, circRNAs accumulate in the cytoplasm, particularly in cells with low proliferation rates. For example, circRNAs are rather abundant in the brain and in developing neurons (Maass et al., 2017; Piwecka et al., 2017; Rybak-Wolf et al., 2015), while they are predominantly downregulated in cancer (Bachmayr-Heyda et al., 2015; Kristensen et al., 2018; Lv et al., 2018). It is yet not fully clear whether circRNAs are preferentially expressed in cells with low proliferation rates or their abundancy is diluted out by high cell-proliferation rates before reaching steady state levels (Holdt, 2018; Sharova et al., 2009).

A growing number of studies report hundreds of circRNAs deregulated in distinct human cancers, including colorectal (Hsiao et al., 2017), hepatic (Yu et al., 2018), prostate (Chen et al., 2019), bladder cancer (Li et al., 2017) and tumors of central nervous system (Kristensen et al., 2018; Lv et al., 2018; Vo et al., 2019). In this thesis we aim to study the role of deregulated circRNAs in the context of Hedgehog-activated medulloblastoma tumors and how they may impact the development of this cerebellar cancer (**PAPER IV**, manuscript).

2 Aims of the thesis

General aim:

Hedgehog signaling is one of the major pathways involved in both normal and neoplastic development. This thesis aimed at the elucidation of mechanisms that regulate Hedgehog-GLI signaling activity contributing towards a better understanding of Hedgehog-mediated cancer development.

Specific aims:

I. Investigate the role of small molecular drug RITA in downregulation of Hedgehog-GLI signaling in medulloblastoma and rhabdomyosarcoma.

II. Investigate the role of GLI1 as a therapeutic target for estrogen receptor positive breast cancer.

III. Global analysis of GLI1 target genes and their role in biological processes mediated by GLI1.

IV. Address the role of a new class of RNA molecules, circular RNAs, in the context of activated Hedgehog signaling and the Hedgehog subtype of medulloblastoma.

3 Results and conclusions

3.1 PAPER I

RITA downregulates Hedgehog-GLI in medulloblastoma and rhabdomyosarcoma via JNK-dependent but p53-independent mechanism

Medulloblastoma and rhabdomyosarcoma are heterogeneous and aggressive classes of childhood tumors with subtypes characterized by abnormally activated Hedgehog signaling. Additionally, frequent defects in p53 signaling are known to dramatically enhance these tumors (Juraschka and Taylor, 2019; Northcott et al., 2012a; Roberts et al., 1989; Teglund and Toftgård, 2010; Tostar et al., 2010).

In this study, we mainly focused on two human tumor cell lines of distinct origin and p53 background: Rh36 rhabdomyosarcoma cells with wild type p53 and Daoy medulloblastoma cells carrying a homozygous Cysteine to Phenylalanine mutation in codon 242 of the p53 gene (Metzger et al., 1991; Saylors et al., 1991).

Small molecule RITA (Issaeva et al., 2004) effectively downregulated Hedgehog signaling irrespective of the p53 status (wild type or mutant) in the cells or p53 depletion via siRNAs. This was demonstrated in three different cellular contexts, Daoy, Rh36 and MCF7 breast cancer cells (wild type p53), where RITA was capable to downregulate expression of typical Hedgehog target genes (*GLI1*, *GLI2*, *PTCH1*, *PTCH2*). Moreover, RITA was able to reduce Hedgehog signaling activity even in the context of pathway activation by Smoothened agonist SAG or GLI1 plasmid overexpression in Daoy cells. Interestingly, in mouse embryonic fibroblast cells where the negative regulators of Hedgehog signaling, the PTCH1 (*Ptch*$^{-/-}$) and SUFU *(Sufu*$^{-/-}$*)* are eliminated, RITA was rather inefficient at downregulating Hedgehog target genes, and only at very high concentrations induced a detectable reduction in the *Ptch*$^{-/-}$ cells. Noteworthy that in line with this, RITA did not induce p53, nor was it able to effectively suppress the growth of several mouse cell lines in a previously published study (Issaeva et al., 2004).

These effects of RITA are apparently elicited downstream of SMO, as siRNA depletion of SMO did not confer major changes in the response of the Hedgehog target genes. Notably, qPCR analysis of p53 target genes remaining mostly unchanged, and Western blot analysis of p53 protein levels remaining unchanged upon RITA treatment, further supported p53-independent effects of RITA, in line with other studies (Krajewski et al., 2005; Wanzel et al., 2016; Weilbacher et al., 2014). Nor RITA effects were mediated by the two other p53 homologs, p63 and p73, as their siRNA depletion did not affect downregulation of Hedgehog target gene expression by RITA.

Suppression of Hedgehog signaling by RITA was mediated by ROS-independent activation of JNK kinase, as inhibition of JNK but not of ROS accumulation fully reverted RITA's impact on Hedgehog target genes. Here, first, ROS accumulation was prevented by different

antioxidants (resveratrol and NAC) and second, JNK activity was inhibited by the small molecule SP600125 in Daoy and Rh36 cells. Western blot analysis confirmed that the protein levels of the JNK isoforms were elevated by activating phosphorylation upon RITA treatment in Daoy cells.

Compared to the DNA damaging agents Doxorubicin and Oxaliplatin, RITA had distinct cytotoxic readouts in cell viably assays and differential impact on Hedgehog signaling. Worth noting is that two other p53 activating drugs, nutlin-3a and APR-246 (PRIMA-1Met) (Bykov and Wiman, 2014), were rather ineffective in downregulating Hedgehog target genes, again, highlighting the distinct action of RITA on Hedgehog signaling.

RITA, in combination with GANT61, a small molecule inhibitor of Hedgehog signaling, elicited more pronounced reduction of tumor cell proliferation than individual drug treatments assessed by *in vitro* cell proliferation and apoptosis assays. This was not fully reflected in the *in vivo* Rh36 cell subcutaneous xenograft studies in nude mice, as the combinatorial and individual administrations of RITA and GANT61 elicited comparable reductions of tumor growth. However, the dual drug administration reduced within-group variation, seen in tumor volume measurements throughout the experiment and downregulated tumor cell proliferation seen in tumor immunohistochemical analysis of Ki-67 positively stained cells. Surprisingly, in Daoy cell subcutaneous xenograft mice, GANT61 administration did not reduce the tumor growth or Hedgehog signaling gene expression.

Detailed qPCR and RNA-seq expression analysis of the individual R36 cell xenograft tumors indicated significant Hedgehog signaling downregulation in the RITA treatment group, minimal downregulation in the GANT61 treatment group and no downregulation in the combination group, suggesting a certain antagonism of the two drugs *in vivo*.

3.2 PAPER II

Blockade of the Hedgehog pathway downregulates estrogen receptor alpha signaling in breast cancer cells

Anti-estrogen treatment, exemplified by tamoxifen, is a well-established therapy for ER-alpha positive breast cancer, however development of drug resistance critically limits the effectiveness of the treatment.

Expression of the key markers of Hedgehog signaling activity, i.e. GLI1 and PTCH1 was higher in tamoxifen resistant LCC2 cells than in parental tamoxifen sensitive MCF7 cells. Noteworthy, ER-alpha target genes *ADORA1* and *pS2* were upregulated in tamoxifen resistant cells, despite the comparable ER-alpha mRNA and protein expression. These suggests that in tamoxifen resistant cells higher Hedgehog signaling activity correlates with high ER-alpha activity.

siRNA depletion of GLI1 decreased ER-alpha expression levels and cell proliferation in both cell lines, although to a lesser extent than in the case of siRNA-mediated ER-alpha depletion.

These observations are in line with the significance of ER-alpha in breast cancer cells (Thomas and Gustafsson, 2011), but still indicate that GLI1 can modulate proliferation in tamoxifen sensitive and resistant cell lines.

siRNA depletion of GLI1 reduced ER-alpha protein levels, with concomitant reduction of ER-alpha activity, assessed with Estrogen Response Element luciferase reporter assay in both tamoxifen resistant and sensitive cells and irrespective of estrogen presence, suggesting an interplay of GLI with ER-alpha signaling.

To address the functional consequences of GLI1 and ER-alpha interplay, we demonstrated that in the context of ER-alpha activation with estrogen, GLI1 depletion elicited a consistent reduction in the mRNA expression of *ER-alpha* itself and its target genes *ADORA1*, *IL20* and *pS2*, as well decreased ER-alpha binding at the promoter region of *pS2* in chromatin immunoprecipitation assay. Likewise, GLI1 depletion decreased ER-alpha protein levels irrespective of estrogen. However, exogenous expression of GLI1 did not increase the mRNA and protein levels of ER-alpha, implying that although GLI1 may regulate ER-alpha transcriptional activity when ER-alpha signaling is on, the impact of GLI1 on ER-alpha is more complicated than a typical direct GLI1 target (for example PTCH1). Noteworthy, we were unsuccessful at detecting convincing GLI1 binding sites on ER-alpha gene (*ERS1*) promoter or possible direct GLI1/ER-alpha interactions in immunoprecipitation assays.

To address possible therapeutic application of Hedgehog-GLI1 and ER-alpha interplay, we showed that inhibition of GLI with GANT61 could sensitize breast cancer cells to tamoxifen treatment. First, we demonstrated that GANT61 treatment can reduce MCF7 and LCC2 cell viability in a dose dependent manner. Furthermore, GANT61 co-administration with tamoxifen further suppressed the growth of MCF7 and LCC2 cells to administration of only tamoxifen, and this was irrespective of estrogen stimulation.

Gene expression analysis revealed that *GLI1* expression positively correlates with the expression of ER-alpha gene *ESR1* and the ER-alpha target genes *pS2* and *GREB1* in a previously published dataset of breast cancer samples from 286 individuals (Wang et al., 2005). Additionally, high GLI1 expression predicted worse distant metastasis-free survival in breast cancer patients with grade I ER-alpha positive breast cancer (Gyorffy et al., 2010). These findings suggest that the GLI1 may be a new candidate for therapeutic targeting and prognosis in ER-alpha positive breast cancer.

Taken together, in this work we have demonstrated that tamoxifen cytotoxicity can be enhanced by blockade of the Hedgehog pathway, reflecting a crosstalk between ER-alpha and GLI1 signaling, both in tamoxifen resistant and sensitive breast cancer cells.

3.3 PAPER III

Identification of novel GLI1 target genes and regulatory circuits in human cancer cells

GLI1 is a transcription factor, terminal effector and activator of Hedgehog signaling, it acts as an oncogene and its upregulation directly correlates with Hedgehog signaling activity. Yet very little is known about the GLI1 target genes, apart from a few targets, including *GLI1*, *PTCH1*, *PTCH2* and *HHIP*. The expression and activity of GLI1 as a transcription factor are regulated by both transcriptional and post-transcriptional modifications, increasing the functional diversity of GLI1 and affecting GLI1-dependent biological outcomes. One such modification is Adenosine to Inosine editing of the GLI1 mRNA at nucleotide 2179, leading to a change from Arginine to Glycine at position 701 of the GLI1 protein, which was shown to modulate the activity of GLI1 (Shimokawa et al., 2013). In this study we identified multiple new GLI1 and edited GLI1 (GLI-701) target genes using a combination of different genomic surveys and subjected them to in-depth validation in human cancer cell lines.

Rhabdomyosarcoma Rh36 cells were transfected with siRNA targeting GLI1 or expression plasmids overexpressing GLI1 or GLI1-701G and subjected to single molecule Helicos RNA-seq. The analysis of overexpression/depletion RNA-seq datasets allowed the selection of putative target genes. Combining the upregulated genes with a Z score > 2 in the GLI1 and GLI1-701G overexpression datasets and the downregulated genes with a fold change > 1.2 in the GLI1 depletion dataset, resulted in a final list of 29 genes. Gene Ontology analysis of this 29-gene list revealed multiple genes encoding for functions potentially involved in downstream effects of GLI1 signaling, justifying their further validation. Thus, five genes, *PTCH1*, *FOXS1*, *SOSTDC1*, *PLAT* and *ENC1* were validated at least in 6 out of 7 independent qPCR-assay-based tests performed on both rhabdomyosarcoma Rh36 and medulloblastoma Daoy cells, these were: GLI1 depletion, GLI1 and GLI1-701G overexpressions in Rh36 and Daoy cells, Smoothened agonist (SAG) treatment in Daoy cells.

Both in Rh36 and Daoy cells, genes that were preferentially or exclusively regulated by GLI1 and GLI-701G were observed. Two of these genes *TMEM158* and *DNMT3B* were commonly regulated in the two cell lines, while the remaining three and six unique to Rh36 and Daoy respectively, highlighting context-specific effects of GLI1 editing.

One of the most highly upregulated targets of GLI1, *FOXS1*, was found to engage in feedback mechanism that limits the GLI1 cellular effects. Here, first we knocked out GLI1 in the Hedgehog signaling-responsive Daoy cells using CRISPR/Cas9 technology, generating two different GLI1 defective subclones. Next, we activated Hedgehog signaling in these cells with administration of SAG. These experiments concluded that *FOXS1* upregulation by SAG requires functional GLI1. On the other hand, FOXS1 siRNA depletion resulted in increased *GLI1* and *HHIP* expression in both Daoy and Rh36 cellular contexts. FOXS1 depletion also promoted Daoy and Rh36 cellular proliferation, arguing that the increased expression of *FOXS1*, elicited by GLI1 upregulation, acts in a negative feedback constraining GLI1 activity.

Luciferase reporter and immunoprecipitation assays suggested a potential mechanism on the interplay between FOXS1 and GLI1, as FOXS1 was found to interact with GLI1 and block GLI1 activity.

Moreover, *FOXS1* was both highly expressed and positively correlated with *GLI1* in medulloblastoma samples of the SHH subgroup, based on a previously published dataset of a large medulloblastoma cohort of 392 samples (Downing et al., 2012) that included all four medulloblastoma subtypes. This further argues for the existence of FOXS1/GLI1 interplay in human tumors and its clinical relevance. Consistently, high *FOXS1* expression predicted longer relapse-free survival in breast cancer in another previously published dataset (Lanczky et al., 2016). Consequently, FOXS1 may have tumor suppressive properties and its upregulation in tumors could be a marker of good prognosis.

3.4 **PAPER IV** (Manuscript)

Circular RNAs in Hedgehog signaling activation and Hedgehog-mediated medulloblastoma tumors

There is overwhelming evidence that circRNAs are deregulated in increasing number of cancers (Vo et al., 2019). However, there is no comprehensive information on deregulated circRNAs in medulloblastoma and how this may impact the development of this cerebellar cancer. In this manuscript we addressed the role of circRNAs in the context of Hedgehog signaling activation and Hedgehog-linked medulloblastoma tumors.

We have characterized the circRNA transcriptome of two Hedgehog signaling inducible cell lines, medulloblastoma Daoy and non-cancerous HEPM (human embryonic palatal mesenchyme) cells in the context of Hedgehog signaling activation. This has been achieved by administration of purified SHH ligand or the small molecule SAG, an agonist of SMO co-receptor, and the subsequent upregulation of the key markers of Hedgehog signaling activity, *GLI1* and *HHIP* was assessed in qPCR assays. Next, total RNA from treated and untreated cells was divided into two fractions to enrich for either linear mRNAs (poly(A) selection) or circRNAs (ribosomal RNA depletion and RNase R treatment), and subjected to Illumina RNA-seq. Specific bioinformatical pipelines (CIRCexplorer2/TopHatFusion) were implemented to identify circRNA reads generated by back-splicing of exons (Zhang et al., 2016), the marker of exonic RNA circles.

RNA-seq analysis of the mRNA fraction confirmed the Hedgehog pathway upregulation seen in qPCR assays, as the key markers of Hedgehog pathway activation, *GLI1*, *HHIP*, *PTCH1* were among the most upregulated genes upon SHH/SAG treatments in both Daoy and HEPM cellular contexts.

RNA-seq analysis of the circRNA fraction resulted in a list of 29 abundantly expressed circRNAs, which were independently validated by Sanger sequencing and in PCR assays, using divergent primers detecting the back-spliced junction of the RNA circle. 10 of these circRNAs were the top most abundantly expressed in Daoy and HEPM cells, 10 the most

abundant and differentially expressed in SAG/SHH treatments of Daoy and HEPM cells, 3 abundant circRNAs derived from genes involved in Hedgehog signaling, and 6 circRNAs from the top 50 in Daoy medulloblastoma cells that were differentially expressed in normal cerebellum, based on publicly available circBase database (www.circbase.org, (Rybak-Wolf et al., 2015)).

Interesting to note, all but one of the 10 circRNAs apparently differentially expressed in Daoy or HEPM RNA-seq datasets were downregulated in the context of Hedgehog signaling activation. To our surprise, the qPCR analysis of the differentially expressed circRNAs did not show consistent differences in the expression compared to control. This might be due to the small expression differences (2-fold or 1.5 fold) of circRNA transcripts in the RNA-seq data and the relative stability (24-48 hr half-life) of circRNAs, which would make difficult the detection of changes within the 48-72 hr timeframe of SAG/SHH treatments. In fact, this provides a possible explanation as to why fewer differentially expressed circRNAs were detected compared to differentially expressed mRNAs (Enuka et al., 2016). Notably, in both cellular contexts, the expression of circRNAs was lower than that of mRNAs originating from the same parental gene, with no correlation detected between circular and concordant linear reads. This is consistent with a previous study (Vo et al., 2019) and indicative of distinct mechanisms involved in back-splicing compared to forward-splicing.

Next, we independently validated the 29 circRNAs in two medulloblastoma cell lines (Daoy, UW-228), SHH-medulloblastoma tumor sample, and compared the expression of these circRNAs to that in normal cerebellum (three independent cerebellar samples, from 5 different individuals). Again, most of the circRNAs were significantly downregulated in medulloblastoma in comparison to cerebellum, with none being upregulated and consistent with previous studies, as circRNAs appear to be preferentially downregulated in cancers (Bachmayr-Heyda et al., 2015), including medulloblastoma (Lv et al., 2018). The correlation of expression in cerebellum and in medulloblastoma of several circRNAs with their corresponding linear counterparts was particularly low. Specifically, the BACH1, CDYL, FKBP8, GLIS1, OGDH, SMARCA5 and ZKSCAN1 circRNA expression was significantly downregulated in medulloblastoma versus cerebellum, while the expression of their linear counterparts was unchanged. This indicates that these circRNAs are products of differently regulated splicing events and distinct mechanisms are involved in their expression, which would be consistent with a possible functional impact.

4 General discussion and future perspectives

Hedgehog signaling is vital for diverse aspects of animal development and essential in regulating many cellular processes, including tumorigenesis. Notably, the role of the GLI1 transcription factor is crucial in regulating Hedgehog pathway activity, as it acts as a target gene, terminal effector, signal amplifier and a direct marker of the pathway activity.

Cancer is a heterogeneous disease and numerous factors and signaling pathways are involved in its initiation and progression. Thus, therapeutic approaches aiming at controlling a single aspect of tumor development are unlikely to result in a sustained effect. Sporadic mutations, development of resistance and side effects are common complications urging for novel treatment strategies. Identification and context-specific targeting of new target genes, feedback mechanisms and interactors that may affect signaling activity in Hedgehog-mediated tumors are of relevance.

In PAPER III, we identified numerous targets of GLI1, whose potential mechanistic roles in Hedgehog-GLI1 activation are worth further studying. We found that *FOXS1*, a gene encoding a transcription factor previously implicated in nervous system development (Montelius et al., 2007) is involved in negative feedback loop, limiting the GLI1 cellular effects. It is possible that a high FOXS1 to GLI1 ratio rather than just high FOXS1 levels can better predict a positive outcome in GLI1-dependent tumors, and we show such correlation for SHH group medulloblastomas, prostate and breast cancers. Remarkably, *FOXS1* was upregulated more than 3-fold in the Daoy medulloblastoma cell line upon Hedgehog signaling activation with SHH ligand (Paper IV), while two additional prominent targets, *SOSTDC1* and *SOX18* were upregulated in HEPM cells. The functional role of the FOXS1/GLI1 regulatory loop in vivo can be further studied in existing mouse models with inactivated FOXS1 ($Foxs1^{+/ß\text{-gal}}$ mice, (Heglind et al., 2005)) and GLI-Luciferase transgenic mice with overactivated and traceable GLI1 (Kimura et al., 2008).

In PAPER I, while aiming at investigating the potential inhibitory role of p53 activation on Hedgehog signaling, we came across novel Hedgehog-inhibitory properties of RITA that occur irrespective of p53. These inhibitory functions of RITA could be studied in additional cancer cell contexts other than medulloblastoma and rhabdomyosarcoma. It would be interesting to investigate whether RITA can directly bind to GLI1/2, for example in cellular thermal shift assays (CETSA) and whether a competition between RITA and GANT61 exist for binding to GLI1/2 proteins. Furthermore, to address (or rule out) the possibility of unspecific DNA damage response of the cells to RITA, we could perform additional Western blot analysis in Daoy and Rh36 cells and follow changes in protein levels of DNA damage markers, such as PARP, gamma-H2AX upon RITA treatment and compare those effect with known DNA damaging agents, i.e. Doxorubicin and Oxaliplatin.

In PAPER II, we have shown that GLI1 can be a potential therapeutic target and a diagnostic marker in breast cancer. Moreover, in combinatorial treatments the GLI antagonist GANT61 could further sensitize breast cancer cells to tamoxifen treatment. This could be worth

examining in animal xenograft models, as apparently, *in vivo* synergistic/additive effects of drug combinations might not fully reflect the *in vitro* scenarios. Especially, since such a variability occurred with GANT61 and RITA co-treatments in PAPER I. Next, since we have shown that GLI1 depletion can downregulate expression and transcriptional activity of ER-alpha, reduce ER-alpha recruitment on the promoter of its target gene *pS2*, further efforts to investigate a possible GLI1-ER-alpha interaction would be of relevance. Particularly, such interaction can be studied in chromatin immunoprecipitation assays following GLI1 overexpression via the adenoviral system used in Paper III.

In PAPER IV, we have addressed the role of a new class of biomolecules, circRNAs, in the context of Hedgehog activation and Hedgehog-linked medulloblastoma. The vast majority of human transcripts represent non-coding sequences and advances of transcriptome sequencing made it possible to study numerous novel classes of RNAs, including circRNAs. Given the increasing body of evidence linking circRNAs expression with human biology, e.g. cancer development and brain function, we aim to extensively address the circRNAs role in medulloblastoma development, with a primary focus on the SHH subtype of medulloblastomas.

In this manuscript, we generated a candidate list of abundantly and/or differentially expressed circRNAs that were first identified in RNA-seq datasets of Hedgehog-inducible human cell lines (Daoy medulloblastoma and HEPM non-cancerous cells) and then independently validated these circRNAs by Sanger sequencing, PCR and qPCR assays in SHH subtype medulloblastoma cells (Daoy, UW-228), SHH medulloblastoma tumor and normal cerebellum samples. Currently, we are functionally addressing the impact of these circRNAs on cellular growth *in vitro* via depletion/overexpression experiments in Daoy and UW-228 cells. This is achieved by designing siRNAs that target the back-sliced junction, which is present in circRNAs, but absent in the corresponding linear mRNAs. Moreover, circRNAs will be overexpressed using commercially available specialized expression vectors (Barrett and Salzman, 2016). Afterwards, *ex vivo* xenografts could be used to address the effects of circRNA depletion/overexpression in the context of a growing tumor (Villegas et al., 2014).

Additionally, it is possible to further study the mechanism of selected circRNAs action by identifying these circRNAs interactors. Protein-circRNA and nucleic acids-circRNA interactions can be studied using the recently developed techniques for circRNA precipitation (Han et al., 2017) and chromatin isolation by RNA purification (Chu and Chang, 2018). Moreover, potential miRNAs that can interact with selected circRNAs can be identified using bioinformatic analysis and later validated by biochemical methods.

Next, prompted by the above data (PAPER IV, manuscript), which suggests that circRNAs may indeed be deregulated in medulloblastoma, we have already planned to engage in a more comprehensive analysis of circRNAs using a large collection of human tissue samples. Here, an expanded list of circRNAs that are differentially expressed in medulloblastomas versus normal cerebellum will be identified. In fact, we already have generated RNA-seq data of 6 medulloblastoma tumors (of which at least 3 are SHH medulloblastomas) and 3 normal

cerebellum samples. Moreover, we are in the final stage of receiving a large number of additional human medulloblastoma samples of the SHH subgroup, four of which are progressive tumors.

Assuming that these analyses result in a robust list of deregulated circRNAs, we plan to extend our efforts to the other three medulloblastoma subgroups, i.e. WNT, group 3 and group 4. It is anticipated that such analysis will pinpoint the similarities and the differences in circRNA expression during the development of all medulloblastoma subgroups, possibly providing a basis for targeted approaches aiming at constraining the growth of this cerebellar tumor.

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