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MECHANISMS OF HEDGEHOG SIGNALING ACTIVATION IN CANCER DEVELOPMENT

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Mechanisms of Hedgehog Signaling Activation in Cancer Development

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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‘Science is simply common sense at its best, that is, rigidly accurate in observation, and merciless to fallacy in logic’

-Thomas Huxley

ABSTRACT

Hedgehog (HH) signaling has an important role in many physiological processes, and deregulation of this pathway can result in a wide range of malignancies. The aim of this thesis is to identify and evaluate the role of various posttranscriptional mechanisms, including alternative splicing, RNA editing and antisense RNAs, associated with different key components of HH signaling.

In PAPER I, we studied the mechanism of action and biological significance of the carboxy-terminal truncated variant of SUFU, SUFU-ΔC, in rhabdomyosarcoma. Our investigations revealed that SUFU-ΔC mRNA was generally expressed at lower to comparable levels than SUFU-FL mRNA but the protein level of SUFU-ΔC was very low compared with SUFUFL. SUFUΔC could repress GLI2 and GLI1ΔN, but not GLI1FL, transcriptional activity to the same extent as SUFUFL. Co-expression of GLI1-FL with SUFU-ΔC in Hek293 cells indicated that SUFU-ΔC but not SUFU-FL reduced the protein levels of GLI1FL. Confocal microscopy revealed a co-localization of GLI1FL with SUFU-ΔC in aggregate structures. Moreover, knockdown of endogenous SUFU-ΔC with shRNA constructs in RMS13 cells caused an increase in GLI1FL protein levels and up-regulation of Hedgehog signaling targets (*PTCH1-1B* and *PTCH1-1C*).

In PAPER II, we studied the prevalence and impact of GLI1 RNA editing in modulating its oncogenic properties. GLI1 mRNA is edited at nucleotide 2179, which results in adenosine (A) to inosine (I) substitution, leading to a change from Arg to Gly at position 701. This editing event is prevalent (around 50%) in a number of human normal tissues. However, in tumors biopsies and tumor cell lines, the extent of GLI1 editing is reduced. SiRNA mediated knockdown revealed both ADAR isoforms (ADAR1 and ADAR2) are needed for GLI1 RNA editing. Edited GLI1 has a higher capacity to activate most of the transcriptional targets and is less susceptible to inhibition by SUFU. Moreover, the edited GLI1 is less responsive to activation by the Dual-specificity Tyrosine Phosphorylation-regulated Kinase 1A (Dyrk1A) compared with the non-edited GLI1. Finally, we showed that GLI1 editing affects GLI1-dependent cellular growth.

In PAPER III, we unveiled the regulatory mechanisms employed by non-coding transcripts overlapping the *GLI1* gene, *GLI1AS*, in normal development and carcinogenesis. *GLI1AS* is positioned head-to-head with the gene encoding *GLI1*. The expression of the 885-nucleotide, three-exon GLI1AS RNA was consistently lower but concordant with GLI1. SiRNA knockdown of GLI1AS up-regulated GLI1 and increased cellular proliferation. Overexpression of GLI1AS resulted in down-regulation of GLI1 and the GLI1 target genes *PTCH1* and *PTCH2*, and decreased cellular proliferation. ChIP assays indicate a local alteration of chromatin structure via H3K27me3 and H3K4me3 remodeling. We also observed a reduction in RNA polymerase II recruitment at the *GLI1* promoter region upon overexpression of *GLI1AS*, which is in-line with the chromatin-remodeling phenomena. Additionally, GLI1 knockdown reduced GLI1AS, while GLI1 overexpression increased GLI1AS, demonstrating a regulatory feedback loop on GLI1/GLI1AS expression.

In PAPER IV, we analyzed GLI1 target genes, using single molecule RNAseq, employing two complementary approaches, overexpression of GLI1 and edited GLI1 combined with GLI1 depletion using siRNAs. Gene ontology (GO) analysis revealed that GLI1 and edited GLI1 are involved in developmental and metabolic processes, cellular proliferation, KEGG pathways in cancer, basal cell carcinomas and thyroid cancer. Moreover, these candidate target genes were further filtered via the FANTOM5 dataset resulting in 29 targets. Validation of the 20 targets, which have a Spearman correlation > 0.1 with the FANTOM dataset, by qPCR indicated that 15 targets are down-regulated in knockdown experiments with Rh36 rhabdomyosarcoma cells. Additionally, 4 targets (FOXS1, SOSTDC1, LOC100507346 and SOX18) are also up-regulated in overexpression experiments with Rh36 cells. Moreover, knockdown of FOXS1 in Rh36 cells resulted in down-regulation of GLI1, highlighting a FOXS1/GLI1 regulatory loop. Finally, GLI1 knockdown and Smoothened agonist SAG treatment in HH signaling responsive Daoy medulloblastoma cells modulate the expression of 9 out of the 15 targets, including SOSTDC1 and FOXS1.

LIST OF SCIENTIFIC PAPERS

I. Ulrica Tostar, Csaba Finta*, **Mohammed Ferdous-Ur Rahman***, Takashi Shimokawa and Peter G. Zaphiropoulos
Novel mechanism of action on Hedgehog signaling by a Suppressor of Fused carboxy terminal variant
PLoS ONE 2012, 7(5):e37761. *Equal contribution

II. Takashi Shimokawa*, **Mohammed Ferdous-Ur Rahman***, Ulrica Tostar, Enikö Sonkoly, Mona Ståhle, Andor Pivarcsi, Ramesh Palaniswamy and Peter G. Zaphiropoulos
RNA editing of the GLI1 transcription factor modulates the output of Hedgehog signaling
RNA Biology 2013, 10(2):321–333. *Equal contribution

III. Victoria E. Villegas*, **Mohammed Ferdous-Ur Rahman***, Maite G. Fernandez-Barrena, Yumei Diao, Eleni Liapi, Enikö Sonkoly, Mona Ståhle, Andor Pivarcsi, Laura Annaratone, Anna Sapino, Sandra Ramírez Clavijo, Thomas R. Bürglin, Takashi Shimokawa, Saraswathi Ramachandran, Philipp Kapranov, Martin E. Fernandez-Zapico and Peter G. Zaphiropoulos
Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1
Molecular Oncology 2014, 8(5):912-926. *Equal contribution

IV. **Mohammed Ferdous-Ur Rahman**, Yumei Diao, Yuri Vyatkin, Ani Azatyan, Georges St. Laurent, Philipp Kapranov and Peter G. Zaphiropoulos
Global analysis of GLI1 and RNA-edited GLI1 target genes
Manuscript

LIST OF SCIENTIFIC PAPERS (NOT INCLUDED IN THE THESIS)

I. Yumei Diao, **Mohammed Ferdous-Ur Rahman**, Victoria E. Villegas, Malin Wickström, John I. Johnsen and Peter G. Zaphiropoulos
The impact of S6K1 kinase on neuroblastoma cell proliferation is independent of GLI1 signaling
BMC Cancer 2014, 14:600.

CONTENTS

LIST OF ABBREVIATIONS

5-HT	Serotonin
ADAR	Adenosine Deaminase acting on RNA
AS	Alternative splicing
asRNA	Antisense RNA
BACE1	Beta-site APP-cleaving enzyme 1
BCC	Basal cell carcinomas
BOC	Brother of CDO
CDO	CAM-related/downregulated by oncogenes
ChIP	Chromatin immunoprecipitation
CK1	Casein Kinase 1
crRNA	Circular RNA
CSC	Cancer stem cells
CUL3	Cullin 3
DHH	Desert Hedgehog
DNMT3	DNA methyltransferases 3
dsRBD	Double stranded RNA binding domains
Dyrk1A	Dual-specificity Tyrosine Phosphorylation-regulated Kinase 1A
EMT	Epithelial-mesenchyme transition
ER	Endoplasmic reticulum
eRNA	Enhancer RNA
ESC	Embryonic stem cell
EST	Expressed sequence tag
EVC	Ellis-van Creveld syndrome protein
FOXS1	Forkhead box S1
GAS1	Growth arrest-specific 1
GLI	Glioma associated oncogenes
GLI1-FL	GLI1-Full length
GLI1AS	GLI1 antisense transcript
GLIBS	GLI binding site
GO	Gene ontology
GPCR	G-protein-coupled receptor
Gprk2	G protein-coupled receptor kinase 2
GSK3	Glycogen Synthase Kinase 3
H3K27me2/3	Histone H3 lysine 27 di/tri-methylation
H3K4me3	Histone H3 lysine 4 tri-methylation
H3K9me2/3	Histone H3 lysine 9 di/tri-methylation
HH	Hedgehog
HHIP	Hedgehog interacting protein
I	Inosine
IGF2	Insulin like growth factor 2
IGF2R	Insulin-like growth factor 2 receptor
IHH	Indian Hedgehog
INPP5E	Inositol polyphosphate 5-phosphatase E
KEGG	Kyoto Encyclopedia of Genes and Genomes
lncRNA	Long non-coding RNA
LRP2	Low-density lipoprotein receptor-related protein 2

miRNA/miR	Micro RNA
mRNA	Messenger RNA
nt	Nucleotide
ORF	Open reading frame
PKA	Protein Kinase A
PRC	Polycomb repressive complexe
PTCH	Patched
RACE	Rapid Amplification of cDNA Ends
RMS	Rhabdomyosarcoma
SHH	Sonic Hedgehog
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SMO	Smoothened
SOSTDC1	Sclerostin domain containing 1
SOX18	SRY-box 18
SUFU	Suppressor of Fused
SUFU-FL	SUFU-Full length
tRNA	Transfer RNA
Tulp3	Tubby-like protein 3
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
XIST	X inactive specific transcript

1 INTRODUCTION

1.1 HEDGEHOG SIGNALING PATHWAY

The Hedgehog (HH) signaling pathway is a highly conserved signal transduction cascade that was first discovered in the fruit fly (*Drosophila melanogaster*) (Nüsslein-Volhard and Wieschaus, 1980) and subsequently found in vertebrates, including mammals (Amakye et al., 2013; Briscoe and Thérond, 2013; Robbins et al., 2012; Teglund and Toftgård, 2010). HH signaling is involved in a number of developmental and physiological processes (Briscoe and Thérond, 2013; O'Toole et al., 2009; Robbins et al., 2012; Teglund and Toftgård, 2010). Moreover, deregulation in this pathway is known to cause neoplastic transformation (Amakye et al., 2013; Epstein, 2008; O'Toole et al., 2009; Scales and de Sauvage, 2009; Teglund and Toftgård, 2010; Varjosalo and Taipale, 2008). Aberrant activation of the HH signaling pathway is involved in several types of malignant tumors, such as medulloblastoma, rhabdomyosarcoma, basal cell carcinoma, and pancreas, colon, stomach, lung and prostate cancers (Amakye et al., 2013; O'Toole et al., 2009; Scales and de Sauvage, 2009; Teglund and Toftgård, 2010; Tostar et al., 2010). Moreover, deregulation of the HH pathway during embryonic development leads to severe birth defects, including holoprosencephaly (Haas and Muenke, 2010), polydactyly, craniofacial defects and skeletal malformations (Heby-Henricson, 2011; Tostar, 2010). In this summary I will mainly discuss about HH signaling in vertebrates. The major chain of events of HH signaling is summarized in **Figure 1**.

1.1.1 HH Ligand production and secretion

HH proteins are synthesized as precursors of about 45 kDa containing two domains: an amino-terminal (HH-N) domain (19kDa) and a carboxy-terminal (HH-C) domain (25 kDa) (Briscoe and Thérond, 2013; Heby-Henricson, 2011; Teglund and Toftgård, 2010; Tostar, 2010). Precursor HH is subjected to autoproteolytic cleavage at the endoplasmic reticulum (ER) resulting in HH-N containing cholesterol at the carboxy-terminal and HH-C (Briscoe and Thérond, 2013; Mann and Beachy, 2004; Perler, 1998). Although HH signaling is mediated by HH-N, HH-C is important for the autoproteolytic cleavage event (Bürglin, 2008; Heby-Henricson, 2011). HH-N is further modified by the attachment of fatty acid groups (Long et al., 2015), important for proper release, extracellular movement and, hence, for its range of action (Briscoe and Thérond, 2013; Long et al., 2015; Mann and Beachy, 2004; Pepinsky et al., 1998). HH protein can travel up to 300μm in vertebrates (Briscoe and Thérond, 2013; Robbins et al., 2012).

In mammals there are three different HH proteins, Sonic (SHH), Indian (IHH) and Desert (DHH). SHH is broadly expressed and involved in the development of many organs (Briscoe and Thérond, 2013; Kasper et al., 2009, 2009; Teglund and Toftgård, 2010). IHH has been implicated in the regulation of cartilage, bone and gut (Briscoe and Thérond, 2013; Koziel et al., 2004; O'Toole et al., 2009), and DHH is involved in germ-line development (Briscoe and Thérond, 2013; Ingham and McMahon, 2001; Tostar, 2010).

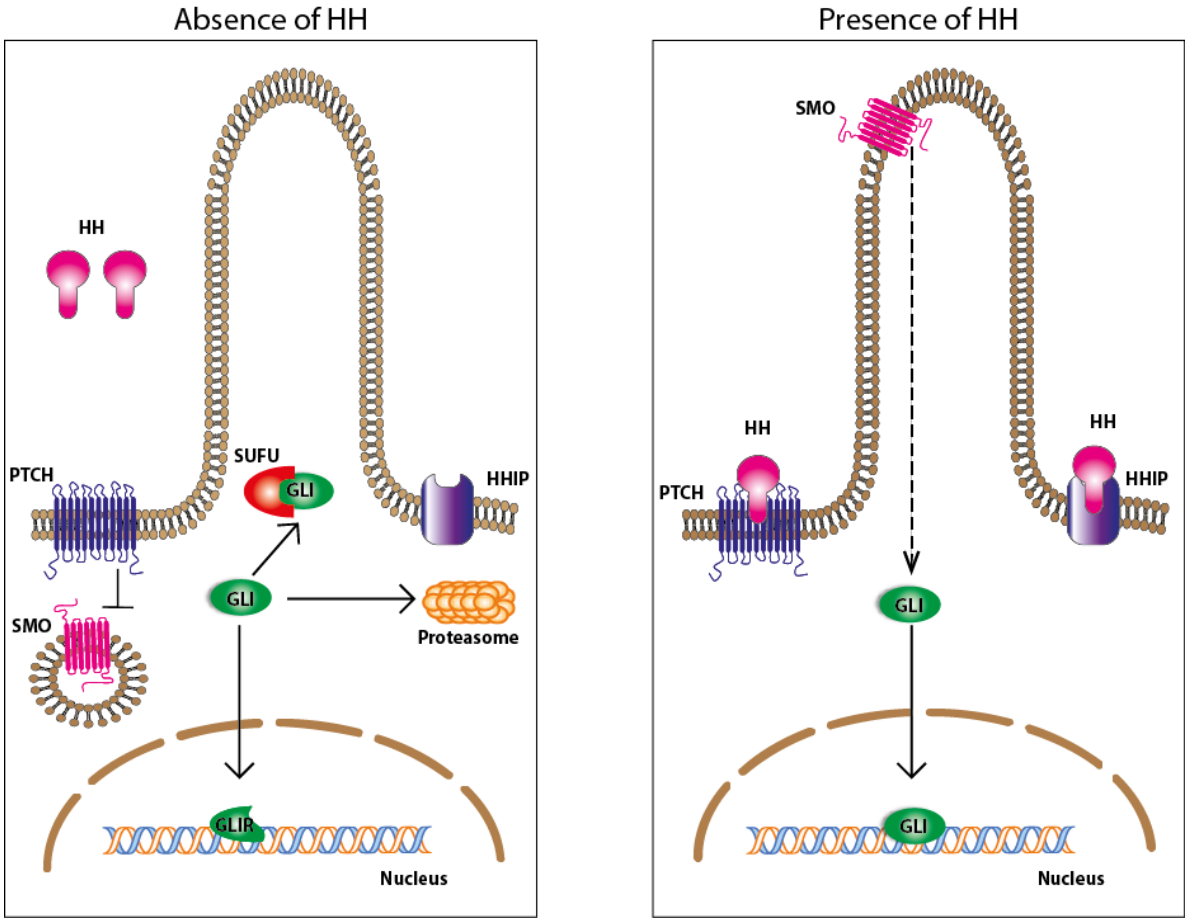

Figure 1: The Hedgehog signaling pathway (simplified). In the absence of Hedgehog ligand (HH), Patched (PTCH) suppresses the function of Smoothened (SMO) and thus shuts off all downstream processing of the HH pathway. Glioma-associated oncogene (GLI) transcription factor is sequestered in the cytoplasm and inactivated by Suppressor of Fused (SUFU). GLI can also be partially degraded into a repressor form (GLI-R), which inhibits GLI targets in the nucleus. HH binding to PTCH receptors relieves SMO from PTCH-mediated suppression, allowing SMO to translocate into the primary cilium. Activated SMO initiates intracellular signal transduction and ultimately activates GLI that acts on Hedgehog target genes.

1.1.2 Reception of the HH signal

In vertebrates, HH proteins interact directly with the twelve-pass transmembrane protein patched (PTCH). There are two *PTCH* homologs in humans, *PTCH1* (ubiquitously expressed) and *PTCH2* (expressed in skin and testis) (Robbins et al., 2012; Teglund and Toftgård, 2010; Tostar, 2010). In vertebrates several cell surface proteins can act as HH co-receptors: CAM-related/downregulated by oncogenes (CDO), brother of CDO (BOC), growth arrest-specific 1 (GAS1) and low-density lipoprotein receptor-related protein 2 (LRP2) (Beachy et al., 2010; Briscoe and Thérond, 2013). Hedgehog interacting protein (HHIP) is another membrane protein known to bind HH, though no downstream signaling is elicited from the HH-HHIP interaction (Tostar, 2010). Therefore, HHIP is a considered as a negative regulator of HH signaling since it competes with PTCH for HH binding (Tostar, 2010).

In the absence of HH signaling PTCH is enriched in and around the primary cilium

(Drummond, 2012; Rohatgi et al., 2007) and inhibits Smoothened (SMO), a seven-pass transmembrane G-protein-coupled receptor (GPCR) (Briscoe and Thérond, 2013; Jiang and Jia, 2015; Robbins et al., 2012; Taipale et al., 2002). The primary cilium is essential for the transmission of HH signaling (Drummond, 2012; Rohatgi et al., 2007) but why this is mandatory is unclear. One possibility is that HH signaling requires a distinct membrane lipid composition; a number of recent studies showed that the ciliary membrane contains phosphatidylinositol 4-phosphate [PI(4)P)], whereas the ciliary base houses phosphatidylinositol 4, 5-biphosphate [PI(4,5)P2] (Garcia-Gonzalo et al., 2015). This differential distributions of lipids are mediated by inositol polyphosphate 5-phosphatase E (INPP5E) and mutations in *INPP5E* have been associated with ciliary dysfunction (Xu et al., 2016). INPP5E restricts the ciliary entry of HH signaling inhibitors, including GPCR 161 (Gpr161) and [PI(4,5)P2] interacting Tubby-like protein 3 (Tulp3) (Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Xu et al., 2016). In the absence of HH, PTCH interacts with and sequesters PI(4)P at the cilia.

Upon binding of HH, PTCH undergoes conformational change and releases PI(4)P. Free PI(4)P directly interacts with SMO through an arginine motif in the SMO C-terminal domain, leading to SMO activation and ciliary localization (Garcia-Gonzalo et al., 2015). SMO is also subjected to phosphorylation by G protein-coupled receptor kinase 2 (Gprk2) and subsequent dimerization (Chávez et al., 2015; Garcia-Gonzalo et al., 2015; Jiang and Jia, 2015; Xu et al., 2016).

1.1.3 Signaling from SMO to GLI

In the absence of HH, SMO is localized to the membranes of intracellular, endocytic vesicles (Robbins et al., 2012; Teglund and Toftgård, 2010; Tostar, 2010). In response to HH signaling in vertebrates, SMO shuttles from these vesicles to the membrane of the primary cilium. Within the cilium, SMO is enriched proximally in association with EVC (Ellis-van Creveld syndrome protein) and EVC2 (Farzan et al., 2008; Robbins et al., 2012). The activation of SMO results in increased recruitment of the negative regulator of HH signaling, Suppressor of Fused (SUFU) and the HH signaling associated transcription factors, Glioma associated oncogenes (GLI2 and GLI3) in the primary cilium (Briscoe and Thérond, 2013; Robbins et al., 2012). The transport of the SUFU-GLI complex through cilia is dependent in part on KIF7 (Briscoe and Thérond, 2013; Robbins et al., 2012). Usually, in the absence of HH signaling GLI makes an inactive complex with SUFU (Cherry et al., 2013); but activated SMO causes dissociation of the GLI–SUFU complex within the cilia. Activated SMO is also known to initiate non-canonical HH signaling outside cilia in the form of GPCR signaling (Robbins et al., 2012). The transcription factor GLI (**PARER II, III and IV**) and the negative regulator SUFU (**PAPER I**) will be discussed in more detail since these are a major focus of the research presented in this thesis.

1.1.4 The GLI transcription factors and their interaction with SUFU

GLI-family proteins are zinc finger transcription factors associated with the HH signaling pathway. Three *GLI* paralogs have been discovered in mammals: *GLI1*,

GLI2 and *GLI3* (Briscoe and Thérond, 2013; Robbins et al., 2012; Teglund and Toftgård, 2010). GLI1 is the first GLI factor, originally identified in glioblastoma containing *GLI1* gene amplification (Kinzler et al., 1987; Vogelstein and Kinzler, 2004). GLI2 and GLI3 are direct effectors of the HH signaling pathway (Kasper et al., 2009; Robbins et al., 2012; Scales and de Sauvage, 2009; Teglund and Toftgård, 2010), whereas GLI1 is induced as a later event to amplify the initial HH signal (Shimokawa et al., 2008, 2013).

In the absence of HH signaling proteolytically cleaved versions of GLI2 and more often GLI3 are produced (GLI2R and GLI3R), which act as transcriptional repressors and bind to the same DNA consensus motif as activated GLI (Briscoe and Thérond, 2013; Hui and Angers, 2011; Robbins et al., 2012). The limited proteasome mediated cleavage of GLI (GLI2 and GLI3) is controlled both by specific GLI phosphorylation and ubiquitination events. Protein Kinase A (PKA), Casein Kinase 1 (CK1), and Glycogen Synthase Kinase 3 (GSK3) are responsible for GLI phosphorylation. In addition, GLI ubiquitination is performed by the E3 ubiquitin ligase, β-TrCP (Briscoe and Th érond, 2013; Hui and Angers, 2011). Another mode of GLI repression is mediated by SUFU, which makes a protein complex with GLI (Briscoe and Thérond, 2013; Hui and Angers, 2011). SUFU-GLI complex is sequestered in the cytoplasm rendering GLI mediated gene activation obsolete. Moreover, SUFU, along with KIF7, has a role in GLIR production (Hui and Angers, 2011; Robbins et al., 2012; Teglund and Toftgård, 2010; Tostar, 2010).

Activation of HH signaling, releases GLI from the repressive grip of SUFU. Released GLI (more specifically GLI2) can undergo further activation by phosphorylation, acetylation or sumoylation and eventually moves to the nucleus to activate target genes including *GLI1* (Briscoe and Thérond, 2013). Moreover, GLI1 can further amplify the signal by inducing itself along with other targets.

The termination of HH signaling is facilitated by proteasome mediated degradation of activated GLI proteins (Hui and Angers, 2011; Robbins et al., 2012). Several studies showed that GLI could be completely degraded by three independent ubiquitin pathways: cullin 3 (CUL3) mediated SPOP E3 ubiquitin ligase (CUL3/SPOP) (Briscoe and Thérond, 2013; Wen et al., 2010), CUL1/β-TrCP (Huntzicker et al., 2006), and Numb/Itch mediated ubiquitination (Di Marcotullio et al., 2006).

1.1.5 GLI targets

GLI transcription factors directly bind to the consensus DNA binding sequence (5´-GACCACCCA-3´) (Hallikas et al., 2006; Kinzler and Vogelstein, 1990). Both activator and repressor forms of GLI bind the same sequence. The canonical GLI targets include *GLI1* (resulting in a positive feedback loop) and components of the HH pathways: *PTCH1*, *PTCH2* and *HHIP* (Shimokawa et al., 2013a; Teglund and Toftgård, 2010). Additional targets of GLI include cyclin D1 (involved in cell cycle) (Amakye et al., 2013; Robbins et al., 2012; Scales and de Sauvage, 2009), N-Myc (cell cycle regulator), Bcl2 (anti-apoptosis pathway), insulin like growth factor 2 (*IGF2*), vascular endothelial growth factor (*VEGF*), angiopoietins and *SNAIL*

(involved in the epithelial-mesenchyme transition (EMT) in cancer metastasis) (Amakye et al., 2013; Briscoe and Thérond, 2013; Heby-Henricson, 2011; Robbins et al., 2012; Scales and de Sauvage, 2009; Teglund and Toftgård, 2010). Moreover, there are reports of GLI targets e.g. E2F1 (involved in cell cycle progression) without canonical GLI binding sites (Pandolfi et al., 2015). Further studies are needed to dissect the broad spectrum of GLI targets **(PAPER IV)**.

1.1.6 HH signaling and cancer

HH signaling is involved in many cellular processes, including cell cycle progression and regulation, apoptosis, cellular, tissue and organ growth, vascularization, and EMT (Briscoe and Thérond, 2013; O'Toole et al., 2009; Robbins et al., 2012; Teglund and Toftgård, 2010). Thus, aberrant activation of HH signaling can lead to carcinogenesis (Amakye et al., 2013; Scales and de Sauvage, 2009). Four basic models have been proposed for HH pathway activation in cancer **(Figure 2)** (Amakye et al., 2013; Kasper et al., 2009; Scales and de Sauvage, 2009; Teglund and Toftgård, 2010).

Ligand independent constitutive activation of HH signaling (Type I) includes:

(i) Loss-of-function mutation of negative regulators of HH signaling (tumor suppressor genes), e.g., loss-of-heterozygosity and/or loss-of-function mutation of *PTCH1* in Gorlin syndrome, basal cell carcinomas (BCCs) (over 73% of the cases), medulloblastomas and rhabdomyosarcomas; loss-of-function mutation of *SUFU* observed in sporadic cases of medulloblastomas, BCC (8%) and Gorlin syndrome; mis-sense mutation of *GLI3* in colorectal and pancreatic cancer (Bonilla et al., 2016; Scales and de Sauvage, 2009; Teglund and Toftgård, 2010).

(ii) Gain-of-function mutation or amplification of positive regulators of HH signaling (proto-oncogenes), e.g. activating mutation of *SMO* in BCC (20%); *GLI1* amplification in glioma, medulloblastomas and rhabdomyosarcomas (Kinzler et al., 1987; Northcott et al., 2009; Roberts et al., 1989; Teglund and Toftgård, 2010); *GLI2* amplification in squamous cell carcinoma (SCC) and medulloblastomas (Amakye et al., 2013).

Ligand dependent autocrine activation of HH signaling (Type II) involves elevated expression of HH protein leading to auto-activation of HH signaling e.g. lung, upper gastrointestinal tract, colorectal, prostate, breast and melanoma tumors (Scales and de Sauvage, 2009).

Ligand dependent paracrine activation of HH signaling (Type III) involves elevated secretion of HH ligands by the tumor cells, which induces HH signaling in stromal cells **(Type IIIa)**. Activated stromal cells, in turn, secrete VEGF, IGF2, Wnt, which drives tumor cell growth (Amakye et al., 2013; Teglund and Toftgård, 2010). Many of the tumor examples listed in **Type II** may also work by paracrine manner (Scales and de Sauvage, 2009). **Type III** can also be reverse paracrine **(Type IIIb)**, where stromal cells secrete HH ligands and activate tumor cells.

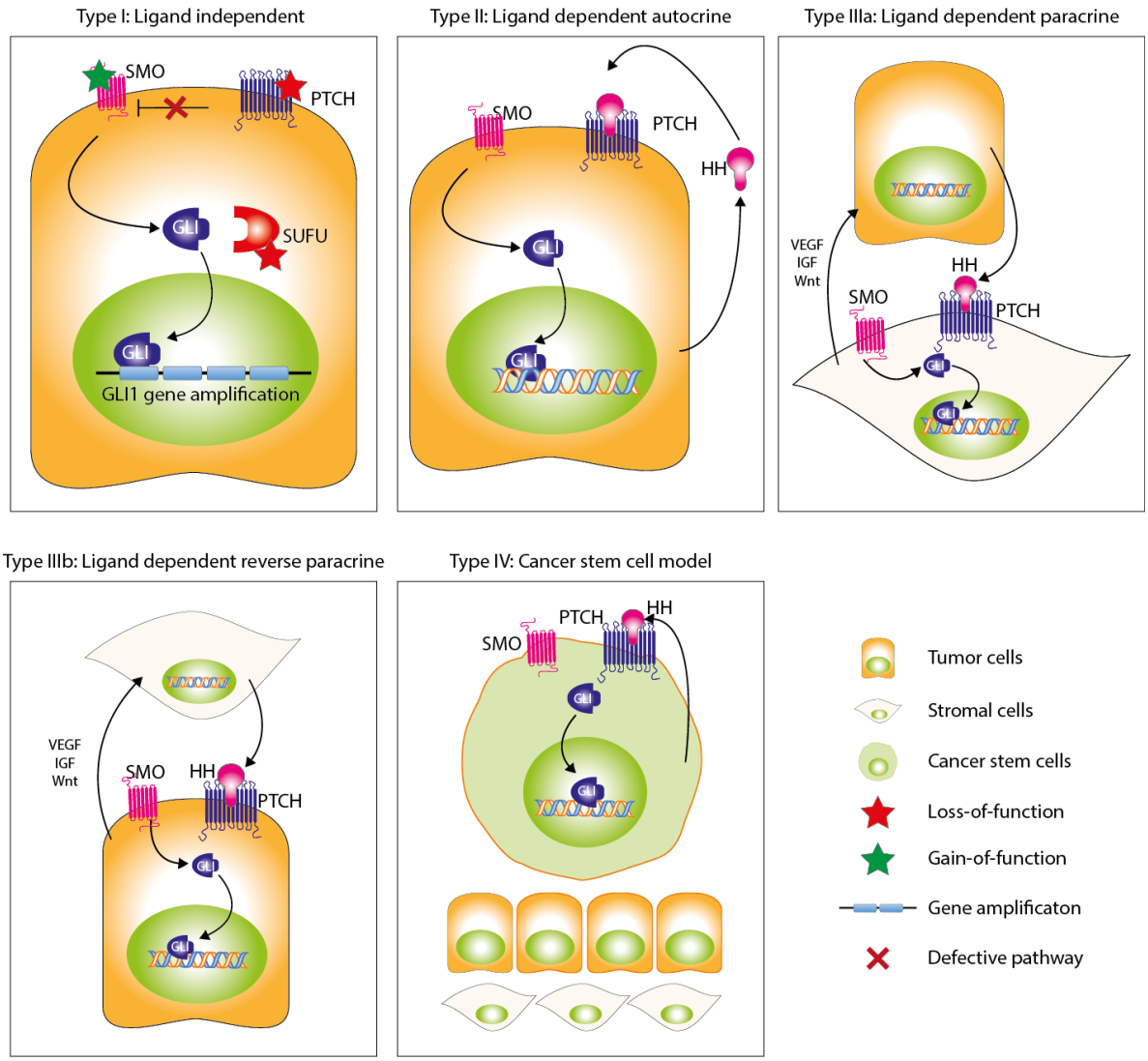

Figure 2: Models of HH signaling pathway activation in cancer. a) Type I ligand independent activation of HH signaling includes loss-of-function mutation affecting negative regulators of HH signaling (*PTCH, SUFU*), gain-of-function mutation in *SMO* and gene amplification of *GLI*. b) Type II autocrine ligand dependent activation of HH signaling where tumor cells secrete HH and activate themselves. c) Type IIIa paracrine ligand dependent activation of HH signaling in stromal cells where HH ligands are produced and secreted by the tumor cells. Activated stromal cells secrete VEGF, IGF and Wnt to induce tumor growth. d) Type IIIb reverse paracrine ligand dependent activation of HH signaling in tumor, where HH ligands are produced and secreted by the stromal cells. e) Cancer stem cell model, where a subset of cancer cells produce HH ligands and terminally differentiate to generate the bulk of tumor cells.

Cancer stem cell (CSC) model (Type IV): This is a variation of type II and III, where only a subpopulation of tumor cells, cancer stem cells (CSC), have self-renewal potential (Scales and de Sauvage, 2009). A number of developmental pathways including HH signaling have been implicated in the CSC tumor model (Merchant and Matsui, 2010). CSCs can produce daughter cells that make up the bulk of the tumor (Amakye et al., 2013).

1.2 ALTERNATIVE SPLICING

Alternative splicing (AS) is one of the most well studied post-transcriptional modifications, which involves alternative selection of splicing sites on precursor messenger RNAs (pre-mRNAs) leading to the production of multiple mature messenger RNAs (mRNAs) (Nieto Moreno et al., 2015). Ultimately, AS results in the diversity in protein production (Irimia and Blencowe, 2012; Nieto Moreno et al., 2015). Although we know about AS events for a while, recent and rapid rise of high throughput transcriptome analysis revealed an ever-increasing number of AS events, especially in higher eukaryotes (Irimia and Blencowe, 2012; Pan et al., 2008). Microarray, EST-cDNA data and especially RNAseq analysis revealed that about 95% of multi-exonic proteins undergo AS (Pan et al., 2008). Most of AS events are species and tissue specific (Irimia and Blencowe, 2012; Pan et al., 2008).

Proteins emerging from AS are associated with a number of physiological processes, including embryonic stem cell (ESC) differentiation (AS of FOXP1 and Sall4) and neurogenesis (AS of Oct2 and REST) (Irimia and Blencowe, 2012). Moreover, AS events are found to be associated with the development, maintenance and progression of different forms of cancer (David and Manley, 2010). AS has been implicated in all the essential carcinogenic hallmarks: anti-apoptosis (AS of Bcl-x) (David and Manley, 2010; Minn et al., 1996), promotion of Warburg type metabolism (AS of pyruvate kinase M), regulation of proto-oncogenes (AS of cyclinD1 and H-Ras), invasion and metastasis (AS of CD44, FGFRs, Rac1, Ron and GLI1) (David and Manley, 2010; Lo et al., 2009).

1.2.1 AS and HH signaling

Splice variations of key components of HH pathway have been identified and characterized, which add complexity and fine-tuning on the HH signaling output. There are several splice variants of *PTCH1*, most of which occur at the 5'-end. *PTCH1* has four distinct first exons (exon 1, 1A, 1B, and 1C), which can alternatively tether to exon 2 resulting in functional diversity, e.g. PTCH1-1B is lowly expressed compared to PTCH1-1 or PTCH1-1A but especially unregulated in BCCs (Shimokawa et al., 2004, 2007). There is an additional splice variant of *PTCH1*, which skips exon 10 but includes a new exon, exon 12b (Uchikawa et al., 2006). *PTCH2* also has couple of splice variants: 3'-end splice variant producing shorter and different C-terminal ends (Zaphiropoulos et al., 1999), and *PTCH2* with skipped exons 9-10 (Rahnama et al., 2004). Internal splicing events have also been reported for *GLI2*, of which one variant showed increased expression in BCCs (Speek et al., 2006; Tojo et al., 2003). In addition, there are two reported *GLI1* splice variants: 1) GLI1ΔN (skips exon 2 and 3), which is functionally subpar in comparison to canonical GLI1 (Palaniswamy et al., 2010; Shimokawa et al., 2008); and 2) tGLI1 (deletion of the entire exon 3 and part of exon 4), which is highly expressed in human glioblastoma multiform (GBM) but barely detectable in normal tissues, and promotes glioblastoma cell migration and invasion (Carpenter and Lo, 2012; Lo et al., 2009). Finally, SUFU-ΔC is the C-terminal splice variant of *SUFU*, which contains a unique exon, exon 10a (contains stop codon) and has a different mechanism of

GLI1 repression; unlike SUFU, SUFU-ΔC reduces the GLI protein level by inducing GLI degradation (Tostar et al., 2012) (**PAPER I**).

1.3 RNA EDITING

RNA editing is broadly defined as a post-transcriptional modification that introduces changes in RNA sequences. The most obvious example of RNA editing is transfer RNA (tRNA) editing, which is crucial for tRNA 3D structure and function (Nishikura, 2010). In this summary, we will mostly focus on adenosine to inosine (A→I) editing, which affects both messenger RNAs (mRNAs) and non-coding RNAs (ncRNAs) transcribed by RNA polymerase II (Farajollahi and Maas, 2010; Nishikura, 2006, 2010, 2016). A→I editing is the most prevalent type of RNA editing in eukaryotes, especially higher organisms including mammals. The enzyme responsible for A→I editing is Adenosine Deaminase acting on RNA (ADAR) (Daniel et al., 2015a; Farajollahi and Maas, 2010; Nishikura, 2006, 2010, 2016) (**Figure 3**).

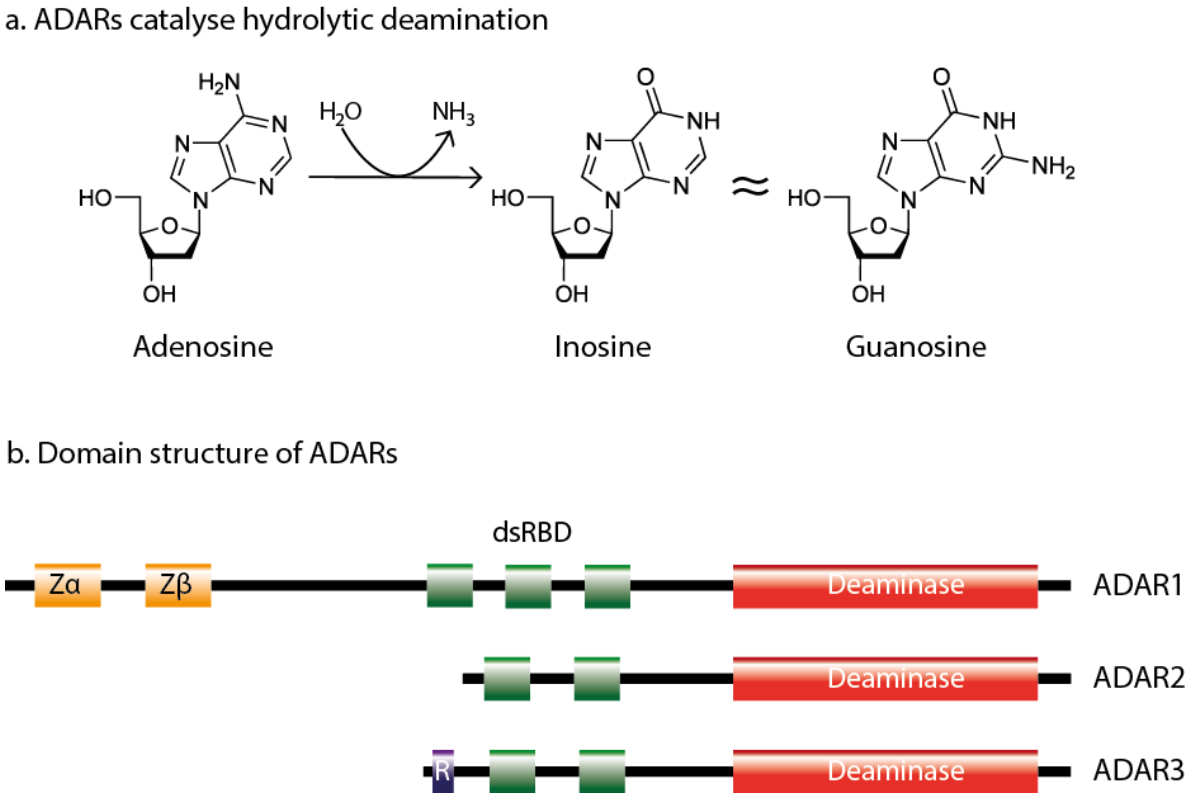

Figure 3: Domain structure and the function of ADARs. a) ADARs catalyze the hydrolytic deamination of adenosine (A) to inosine (I), which is read as a guanosine (G) by the translational machinery. b) All three ADARs (ADAR1, 2 & 3) have a C-terminal catalytic domain and double stranded RNA binding domains (dsRBD). ADAR1 has additional Z-DNA binding domains (Za, Zb) at the N-terminal end. R, arginine/lysine rich domain.

1.3.1 Functional implications of A→I editing

Physiological and pathophysiological implications of A→I editing are widespread and include: amino acid sequence changes, e.g. Arginine (R) to Glycine (G) substitution in GLI1 at position 701 (Shimokawa et al., 2013) and Glutamine (Q) to Arginine (R) substitution in glutamate receptor-2 (GluR-2) at position 607 (Li and Church, 2013);

modification of splicing events by altering splicing junction sequences, e.g. A→I editing in Protein Tyrosine Phosphatase Non-Receptor Type 6 (PTPN6) resulting in retention of an extra intron (Deffit and Hundley, 2016); alteration of microRNA (miRNA) biogenesis by introducing change in the stem-loop structure of precursor miRNAs (pre-miRNA), which can modify the interaction with miRNA biogenesis components, including Dicer, Drosha, DGCR8 and TRBP (Deffit and Hundley, 2016; Nishikura, 2016), e.g. editing of pri-miRNA-142 blocks its processing by the Drosha–DGCR8 complex (Nishikura, 2016; Yang et al., 2006); interference of miRNA-mRNA interactions, e.g. A→I editing at 3'-UTR of the Rho GTPase activating protein 26 (ARHGAP26) disrupts binding of miR-30b-3p and miR-573 preventing gene silencing (Deffit and Hundley, 2016; Kawahara et al., 2007; Wang et al., 2013) (**Figure 4**).

Recent studies showed the importance of A→I editing in mammalian brain development especially in the regulation of neurotransmission, neural differentiation and maturation (Daniel et al., 2015a; Li and Church, 2013). RNA editing is also important in cancer development (Nishikura, 2010, 2016; Paz et al., 2007). Results of a global bioinformatic analysis with Alu sequence RNA editing showed significant hypoediting in various types of human cancers (Paz et al., 2007), which suggest that a reduction of A→I editing may be involved in the pathogenesis of cancer (Paz et al., 2007).

1.3.2 Adenosine Deaminase acting on RNA (ADAR)

ADAR catalyzes the hydrolytic deamination of adenosine (A) to inosine (I) and the translational machinery reads inosine (I) as a guanosine (G) (Deffit and Hundley, 2016; Farajollahi and Maas, 2010; Nishikura, 2010, 2016). The first mammalian ADAR gene, **ADAR1**, was originally discovered in humans (Farajollahi and Maas, 2010; Nishikura, 2016), followed by the identification of two additional homologs, *ADAR2* and *ADAR3*. The adenosine deaminase activity of ADAR1 and ADAR2 has been shown by many groups, but no incident of ADAR3 mediated editing has been reported, although the functional domain in ADAR3 appears to be intact (Farajollahi and Maas, 2010; Nishikura, 2016). The expression of *ADAR1* and *ADAR2* is ubiquitous and mostly present in the nucleus; *ADAR3* is only found in the brain (Farajollahi and Maas, 2010; Nishikura, 2016). Additionally, the ADAR proteins are conserved among vertebrates. Each *ADAR* has several splice variants that differ on intracellular localization, target specificity and extend of enzymatic activity (Deffit and Hundley, 2016; Farajollahi and Maas, 2010; Nishikura, 2016).

The domain structure is very similar amongst the human ADARs (**Figure 3b**). All three ADARs have a single C-terminal catalytic deaminase domain containing Zn-Fe at the catalytic center. They also have multiple double stranded RNA binding domains (dsRBDs), which indicate that the functionality of the ADAR depends on the 3D structure, more specifically the hairpin loop of the target RNA (substrate) (Daniel et al., 2015a; Nishikura, 2016). The longest isoform of ADAR1, p150, has Z-DNA binding domains at the N-terminal end, indicating possible additional functionality or targets specify (Farajollahi and Maas, 2010; Nishikura, 2010) (Figure 3). Inactive

ADAR3 has a 'N-terminal arginine/lysine rich R-domain' of unknown function. The functionality of ADAR1 and ADAR2 depends on dimer formation. ADAR 1 and ADAR2 can form both homodimers (ADAR1-ADAR1 or ADAR2-ADAR2) and an heterodimer (ADAR1-ADAR2), although the ADAR heterodimer is considered inactive by many researchers (Nishikura, 2016).

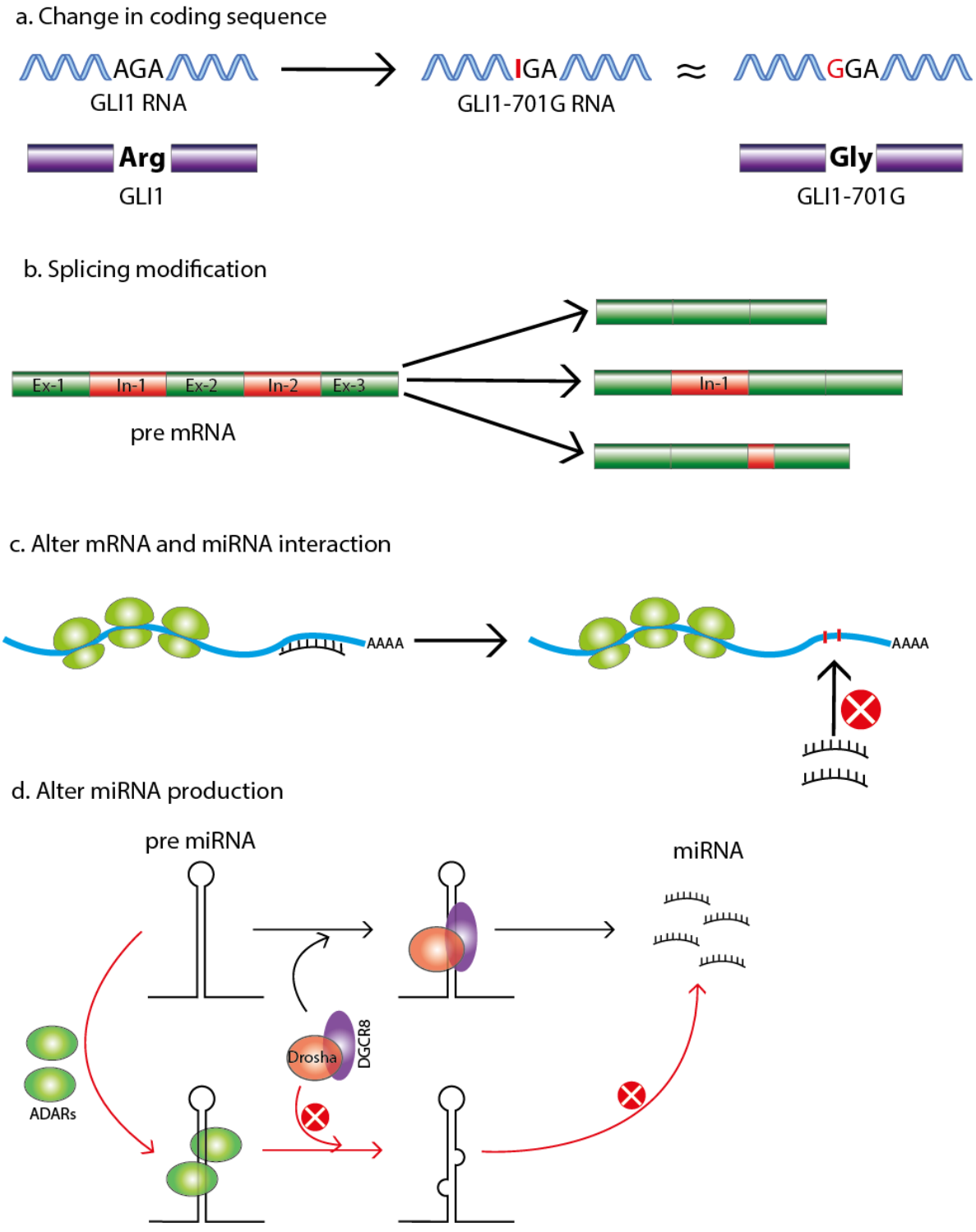

Figure 4: Functional implication of RNA editing. a) RNA editing in the exons can result in coding sequence change e.g. Arg to Gly at position 701 of GLI1. b) RNA editing can either diminish or introduce splice sites resulting in splicing variations. c) RNA editing at the 3' UTRs can alter the interaction between mRNA and miRNA. d) RNA editing can affect

miRNA processing by altering pre-miRNA secondary structures.

ADAR targets must have a stem loop structure of >20 nucleotides (Daniel et al., 2015a). The extent of editing depends on both the size of the loop and the number of mismatches within the loop (Daniel et al., 2015b). Moreover, most of the editing targets are preferentially edited by either ADAR1 or ADAR2, e.g., glutamate receptor-2 (GluR-2) is specifically edited by ADAR2; functional serotonin (5-HT) receptor-2C has five editing events, two of which are specifically edited by ADAR1 (Burns et al., 1997; Li and Church, 2013). Some targets can be edited by both ADAR1 and 2, e.g., GLI1 editing at 701 (Shimokawa et al., 2013), three of the five specific editing events in 5-HT receptor-2C (Li and Church, 2013; Marcucci et al., 2011; Peng et al., 2006).

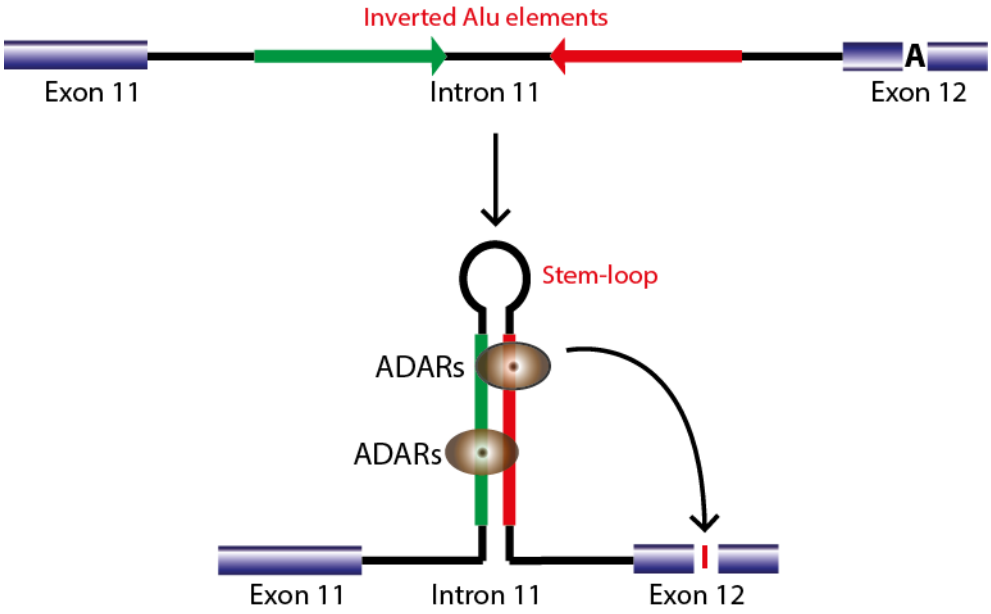

Figure 5: Mechanism of RNA editing of GLI1. Inverted Alu sequence present in intron 11 of the GLI1 pre-mRNA can produce a stem-loop structure. ADARs can bind the stem-loop and introduce A-to-I editing at a specific A residue at exon 12.

1.3.3 A→I editing site specificity

A→I editing of a target sequence is very specific. Moreover, the extent of editing can also vary. A recent transcriptome analysis of the brain showed that different RNA editing target sites can have different editing efficiency (Li et al., 2009), e.g., in human brain the editing efficiency of GLI1 at position 701 is about 50% (Shimokawa et al., 2013), meaning that 50% of the GLI1 mRNA in the brain is edited. Recent studies showed that in humans both the specificity and extent of A→I editing is directly related to the presence of inverted Alu elements in introns and in UTRs (untranslated regions) (Daniel et al., 2014, 2015a, 2015b). Inverted Alu elements produce stem-loop structures, which attract the recruitment of ADARs; ADARs can, in turn, edit intronic Alu stem-loop and/or adjacent exons resulting in changes in the exonic sequence and abolish or introduce new splice sites (Daniel et al., 2015b). Human GLI1 is edited at exon 12 and contains inverted Alu elements at intron 11; on the contrary mouse GLI1, which has only one Alu element at intron 11 and incapable

of producing a stem-loop, is not edited (Daniel et al., 2014) (**Figure 5**). Even if inverted Alu elements directed RNA editing can explain the approximate location of A→I editing events, still the exact sequence position of an editing event can not be rationalized, e.g., why GLI1 is edited at position 701, why not any other adenosines (A) surrounding position 701?

1.4 LONG NON-CODING RNA

Long non-coding RNAs (lncRNAs), as the name implies, are diverse class of long (~200nt) RNAs, which have no long open reading frame (ORF) and are not translated into proteins (Ulitsky and Bartel, 2013; Villegas and Zaphiropoulos, 2015; Wang and Chang, 2011; Zhao et al., 2015). LncRNAs are a new class of RNA, which are increasingly appreciated due to the rapid emergence of high-throughput transcriptome sequencing. It is estimated that the prevalence of lncRNAs can be as much as 90% of the total transcriptome, whereas protein coding mRNAs only account for roughly 2% (Bánfai et al., 2012; Esteller, 2011; Fatica and Bozzoni, 2014; Quinn and Chang, 2016). The most well established example of lncRNAs is the X inactive specific transcript (XIST) eliciting X chromosome inactivation (Penny et al., 1996). lncRNAs are involved in a number of biological processes including RNA mediated genome imprinting by chromatin modification (Fatica and Bozzoni, 2014; Geisler and Coller, 2013; Pelechano and Steinmetz, 2013; Villegas and Zaphiropoulos, 2015), RNA degradation (Gong and Maquat, 2011) or stabilization (Faghihi et al., 2008; Fatica and Bozzoni, 2014; Geisler and Coller, 2013), translational repression and allosteric regulation of enzymatic activity (Geisler and Coller, 2013). LncRNAs are also found to be associated with cellular differentiation, development and disease (Esteller, 2011; Fatica and Bozzoni, 2014). Even though the biogenesis of lncRNAs is well established and has no considerable difference from protein coding mRNAs (Quinn and Chang, 2016), little is known about their functional role and mechanism of action.

1.4.1 Classification of lncRNAs

LncRNAs can be classified based on their location in the genome or mode of action. According to location lncRNAs are broadly divided into two groups; intergenic lncRNAs, sandwiched between two protein coding genes; and overlapping RNAs, which overlap with an often protein coding gene (Pelechano and Steinmetz, 2013; Quinn and Chang, 2016; Villegas and Zaphiropoulos, 2015). The overlap is more often than not in a head-to-head arrangement; but in rare occasions tail-to-tail or head-to-tail arrangements can also be found (Mahmoudi et al., 2009; Villegas and Zaphiropoulos, 2015). Antisense RNAs, defined as overlapping head-to-head or tail-to-tail, influence the expression of sense RNAs, which are often protein coding, by modulating their transcription rate, mRNA stability, transport or translation efficiency (Mahmoudi et al., 2009; Pelechano and Steinmetz, 2013; Villegas and Zaphiropoulos, 2015; Villegas et al., 2014; Yap et al., 2010). According to the literature, about 70% of transcripts have antisense partners (Pelechano and Steinmetz, 2013). The level of expression of antisense RNAs is often very low compared with the sense transcript (Pelechano and Steinmetz, 2013; Villegas and

Zaphiropoulos, 2015; Villegas et al., 2014).

In addition, lncRNAs can be divided into several groups based on their biological roles. Firstly, transcriptional regulatory lncRNAs are nuclear lncRNAs involved in transcriptional regulation of target genes as transcription activators or repressors (Blackledge et al., 2015; Fatica and Bozzoni, 2014; Holoch and Moazed, 2015). This type of lncRNAs can act in *cis* and/or *trans* (Blackledge et al., 2015; Fatica and Bozzoni, 2014; Holoch and Moazed, 2015). A second group of lncRNAs intervenes at post-transcriptional and translational levels (Geisler and Coller, 2013; Ulitsky and Bartel, 2013; Villegas and Zaphiropoulos, 2015). Their functions range from influencing the stability of target RNAs to modulating the translational outcome. Unlike transcriptional regulatory lncRNAs, post-transcriptional regulatory lncRNAs can function both in the nucleus and the cytoplasm (Geisler and Coller, 2013). Finally, a vast majority of lncRNAs have no known function and may be considered as products of transcriptional noise (Fatica and Bozzoni, 2014; Quinn and Chang, 2016).

1.4.2 LncRNA-mediated transcriptional regulation

As mentioned earlier, nuclear functional lncRNAs can module transcriptional outcome in *cis* and/or *trans. Cis*-acting nuclear lncRNAs can be intergenic or overlapping to the target gene (Holoch and Moazed, 2015; Quinn and Chang, 2016). Moreover, they can act both as repressors or activators (Blackledge et al., 2015; Holoch and Moazed, 2015). LncRNAs often work as scaffolds for chromatin remodeling complexes and via RNA-DNA interactions with their target gene, promote either transcriptional repression or activation (Blackledge et al., 2015; Pelechano and Steinmetz, 2013; Wang and Chang, 2011) (**Figure 6**).

Transcriptional repressor lncRNAs (**Figure 6a and 6b**) often employ chromatin imprinting complexes, like DNA methyltransferases 3 (DNMT3) (Fatica and Bozzoni, 2014; Rinn et al., 2007; Zhao et al., 2008), the polycomb repressive complexes (PRC1and PRC2), histone H3 lysine 9 (H3K9) methyltransferase, resulting in an increase of DNA and histone methylation especially at promoters, e.g. histone H3 lysine 9 di/tri-methylation (H3K9me2/3) and/or H3 lysine 27 di/tri-methylation (H3K27me2/3) (Fatica and Bozzoni, 2014; Rinn et al., 2007; Zhao et al., 2008). Example of *cis*-acting repressor lncRNAs include X inactive specific transcript (XIST) (Nozawa et al., 2013; Penny et al., 1996; Schoeftner et al., 2006; Zhao et al., 2008), AIRN, antisense to *IGF2R* (insulin-like growth factor 2 receptor RNA) gene (Latos et al., 2012; Sleutels et al., 2002) and GLI1AS, antisense to *GLI1* (Villegas et al., 2014) (**PAPER III**). The most well studied *trans*-acting repressor lncRNA is the HOXA transcript antisense RNA (HOTAIR), which represses the expression of *HOXD* genes (Rinn et al., 2007; Wang and Chang, 2011). HOTAIR can work as a scaffold to recruit two different distinct repressive complexes, PRC2 and the H3K4 demethylating complex KDM1A–coREST–REST on the same genomic region (Fatica and Bozzoni, 2014; Rinn et al., 2007; Tsai et al., 2010). A hallmark of repressor lncRNAs is the increase of promoter methylation, H3K9me2/3 and H3K27me2/3, and the reduced recruitment of RNA polymerase II at the locus of

action (Fatica and Bozzoni, 2014; Holoch and Moazed, 2015; Wang and Chang, 2011).

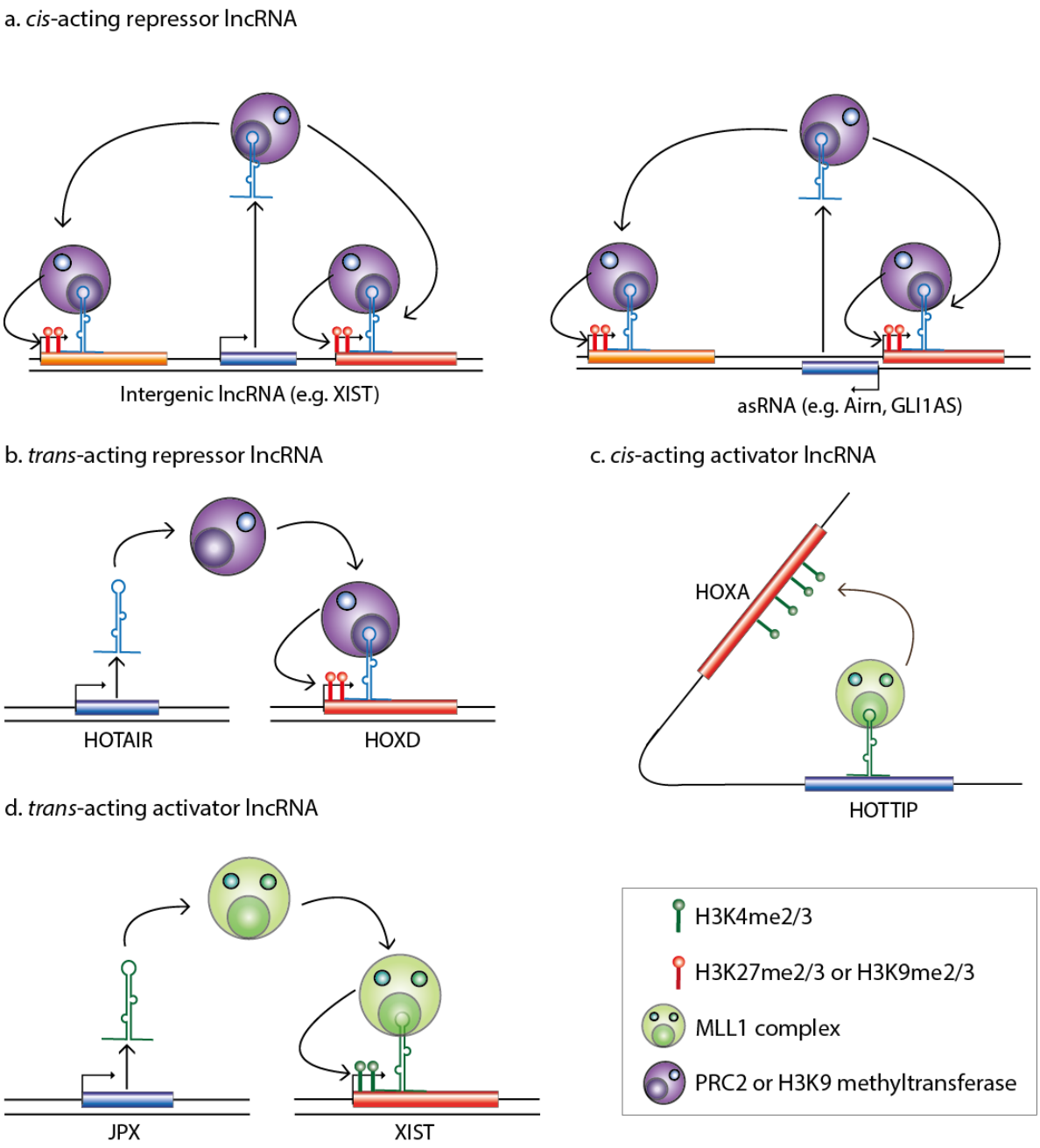

Figure 6: Chromatin modification by lncRNAs. LncRNAs can act as transcription repressors by recruiting repressor protein complexes like PRC2 and H3K9-methyltransferace to *cis* or *trans* targets (a and b). LncRNAs can also act as transcriptional activators (c and d).

Transcriptional activator lncRNAs (**Figure 6c and 6d**) are less studied compared with repressor lncRNAs. *Cis*-acting transcriptional activator lncRNAs, also known as enhancer RNAs (eRNAs) (Ørom et al., 2010), can activate neighboring genes by recruiting chromatin activation complexes, including the MLL1 complex, a multi-protein complex that mediates both histone H3 trimethylation at lysine 4 (H3K4me3) and histone H4 acetylation at lysine 16 (H4K16ac), which are associated with transcriptionally active genes (Bertani et al., 2011; Fatica and Bozzoni, 2014; Wang

et al., 2011). An example of an eRNA is HOTTIP, which is encoded at the distal 5' end of the *HOXA* locus (Wang et al., 2011). HOTTIP eRNA binds at the *HOTTIP* locus and brings it closer to *HOXA* genes by facilitating chromosome looping. HOTTIP eRNA recruits MLL1 complex at the *HOXA* locus, which results in an increase of H3K4me3 leading to the recruitment of RNA polymerase II (Wang et al., 2011). We do not know much about *trans*-acting eRNAs; a rare example of a *trans*-acting eRNA is JPX, which binds to the transcriptional repressor CTCF, inhibiting its binding to the *XIST* promoter, thus activating XIST transcription (Sun et al., 2013).

1.4.3 Post-transcriptional and translational regulation elicited by lncRNAs

LncRNAs also have complex post-transcriptional and translational modes of action and are known to influence target RNAs by affecting their splicing pattern, RNA stability, RNA editing, translational efficiency and last but not least, may function as sponges for microRNA (miRNA) (**Figure 7**) (Fatica and Bozzoni, 2014; Geisler and Coller, 2013; Pelechano and Steinmetz, 2013; Ulitsky and Bartel, 2013; Villegas and Zaphiropoulos, 2015; Wang and Chang, 2011).

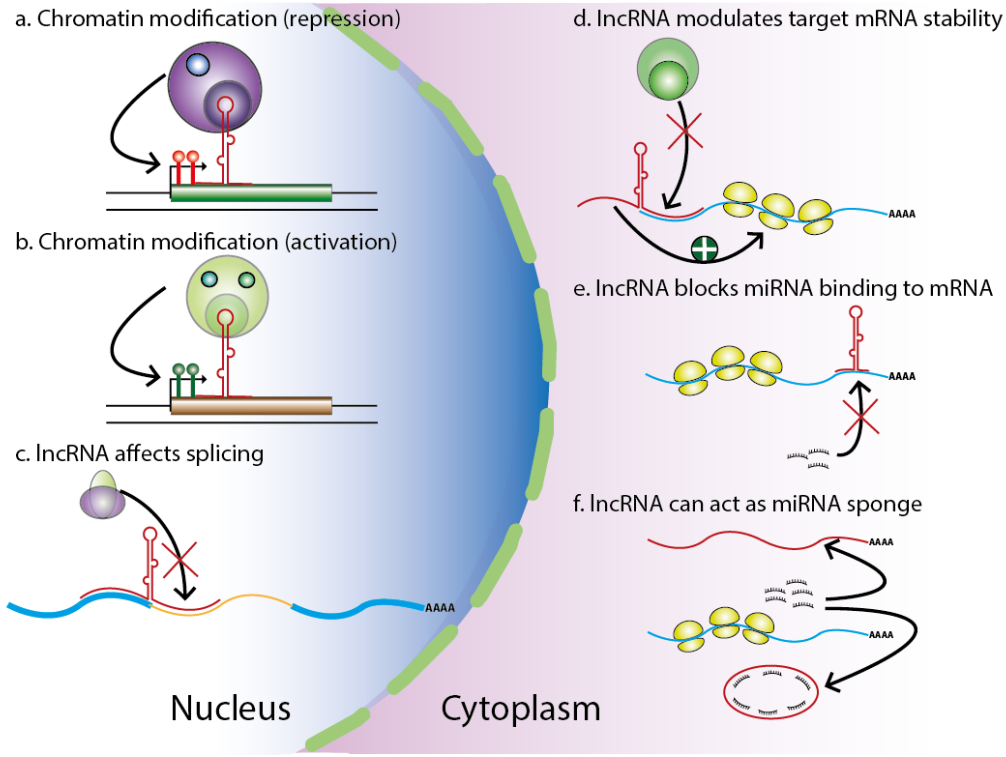

Figure 7: Function and compartmentalization of lncRNAs. Nuclear lncRNAs can induce chromatin modification (a and b) and affect splicing events of target mRNA (c). Cytoplasmic lncRNAs can increase the stability of target mRNAs by inhibiting mRNA degradation machinery (d), alter mRNA-miRNA interactions (e) or can act as miRNA sponge (f).

LncRNA affects splicing: Antisense RNAs can form complete or partial RNA-RNA duplexes with overlapping target mRNAs, which can inhibit the recruitment of the splicing machinery leading to alternative splicing of target mRNA, e.g., 'natural antisense transcript against MYC' inhibits MYC intron-1 splicing (Krystal et al., 1990). RNA-RNA duplexes also have the potential to affect target mRNA editing events by

inhibiting ADAR (adenosine deaminase acting on RNA) (Geisler and Coller, 2013).

LncRNA modulates target mRNA stability: RNA-RNA duplex formation with an antisense lncRNA can affect the stability of the target sense mRNA. Wrap53, the antisense RNA to p53, increases p53 mRNA stability. siRNA mediated knockdown of wrap53 dramatically reduce the stability of p53 (Mahmoudi et al., 2009).

LncRNA affects miRNA binding to target mRNA: LnRNA and target mRNA RNA-RNA duplexes can rescue miRNA mediated translational repression, e.g. an RNA-RNA duplex between BACE1AS (antisense lncRNA to BACE1) with BACE1 (beta-site APP-cleaving enzyme 1) mRNA stabilizes BACE1 mRNA by blocking miRNA (miR-485-5p) induced repression (Faghihi et al., 2008, 2010). Moreover, lncRNAs can also act as cytoplasmic sponges for miRNAs, indirectly increasing mRNA stability, e.g. PTEN-pseudogene antisense transcript can act as a sponge for miRNAs targeting PTEN mRNA (Poliseno et al., 2010). An additional recent example of an miRNA sponge is the CDR1AS/CiRS7 non-coding circular RNAs (crRNAs), which acts as sponge for miR-7 (Memczak et al., 2013).

LncRNA mediated translational up-regulation/down-regulation: Antisense UCHL1 lncRNA up-regulates UCHL1 protein production via a SINE2B repeat element-mediated translational up-regulation (Carrieri et al., 2012). On the other hand, some lncRNA-mRNA duplexes can increase target mRNA decay, e.g. down-regulation of SERPINE1 (Gong and Maquat, 2011).

2 AIMS OF THE THESIS

The general goal of my PhD study is to dissect the HH signaling pathway for a better understanding of its role in carcinogenesis. More specifically, we are interested to identify and evaluate the role of various posttranscriptional mechanisms, including alternative splicing, RNA editing and antisense RNAs, in modulating the HH signaling output.

Specific aims:

1) Investigate the mechanism of action and biological significance of SUFU splice variants in rhabdomyosarcoma.

2) Unveil the regulatory mechanisms employed by non-coding transcripts overlapping the GLI1 in normal development and carcinogenesis.

3) Determine the prevalence and impact of GLI1 RNA editing events in modulating its oncogenic properties for carcinogenic growth.

3 RESULTS AND CONCLUSIONS

3.1 PAPER I

Novel mechanism of action on hedgehog signaling by a suppressor of fused carboxy terminal variant

SUFU protein (also known as SUFU-Full length) is 484 amino acid long (Stone et al., 1999). Apart from SUFU-Full length (SUFU-FL), a carboxy-terminal deleted isoform was identified in human, known as SUFU-ΔC, which is 433 amino acid long (Stone et al., 1999). These two isoforms of SUFU are the result of splicing variation. The *SUFU* gene (chromosome 10q24-25) contains 12 exons (Grimm et al., 2001; Stone et al., 1999) and in SUFU-FL all 12 exons are included but SUFU-ΔC incorporates only the first 10 exons and then uses a stop codon from a unique exon 10a (present in intron 10). Thus, SUFU-ΔC shares identical amino acid sequence with SUFU-FL up to amino acid 432 and identical nucleotide sequence up to base pair 1442 (Stone et al., 1999; Tostar et al., 2012).

Our investigations revealed that SUFU-ΔC mRNA was generally expressed at lower levels than SUFU-FL in all cell types tested except in the lung cell line, GI117. Moreover, we detected comparable level of SUFU-ΔC and SUFU-FL mRNA in the rhabdomyosarcoma (RMS) cell lines, CCA and RMS13. However, the protein level of SUFU-ΔC was very low compared with SUFU-FL in RMS cell lines, which indicates that SUFU-ΔC protein may be unstable. Additionally, heterologous expression of the SUFU variants in the Hek293 cell line was in agreement with a reduced stability of SUFU-ΔC relative to SUFU-FL in human RMS cells. Moreover, treatment with the proteasome inhibitor MG-132 conferred a selective increase of both the endogenous and exogenous SUFU-ΔC but still not reaching the levels of SUFU-FL, suggesting the involvement of additional mechanisms, possibly lysosomal autophagy (Gao et al., 2010).

Biochemical analysis upon overexpression revealed that SUFU-ΔC could repress GLI2 and GLI1ΔN, but not GLI1FL, transcriptional activity to the same extent as SUFU-FL. SUFU and GLI1 bind to each other using both N-terminal and C-terminal regions of the two proteins (Dunaeva et al., 2003; Merchant et al., 2004). Since SUFU-ΔC could not act as an equally efficient repressor as SUFU-FL, a role of the last 51 amino acids of SUFU in the repression on GLI1FL may be suggested. Moreover, under conditions of activated HH signaling SUFU-ΔC was more effective than SUFU-FL in inhibiting GLI1ΔN. Importantly, co-expression of GLI1FL with SUFU-ΔC in Hek293 cells indicated that SUFU-ΔC but not SUFU-FL reduced the protein levels of GLI1-FL. These findings suggest that the mechanism of inhibition of GLI1FL by SUFU-ΔC may be fundamentally different from SUFU-FL, and could involve an increased degradation of the GLI1FL protein. Interestingly, the levels of GLI2 remained unchanged in the same transfection setting, highlighting the specificity of the GLI1FL and SUFU-ΔC interaction. Additionally, the levels of endogenous GLI1FL in RMS13 cells were investigated following transfection of the

SUFU variants. SUFU-ΔC, in contrast to SUFU-FL, conferred a detectable reduction of the GLI1FL levels. Moreover, the proteasome inhibitor MG-132 increased the GLI1FL protein in both SUFU-ΔC and SUFU-FL transfected cells. Additionally, confocal microscopy revealed a co-localization of GLI1FL with SUFU-ΔC but not SUFU-FL in aggregate structures.

ShRNA mediated knockdown SUFU-ΔC in RMS13 cells resulted in an increase of GLI1FL protein but not mRNA levels. SUFU-ΔC knockdown also resulted in the transcriptional up-regulation of the Hedgehog signaling targets, PTCH1-1B and PTCH1-1C. The knockdown experiments indicate that endogenous SUFU-ΔC can modulate HH signaling activity by reducing GLI1FL protein levels, and this is different to the mechanism of action of SUFU-FL.

In conclusion, our study suggests the presence of novel regulatory controls in the HH signaling pathway, which are elicited by the distinct mechanism of action of the two alternative spliced SUFU proteins.

3.2 PAPER II

RNA editing of the GLI1 transcription factor modulates the output of Hedgehog signaling

A previous high throuput screening revealed that RNA editing is a frequent post-transcriptional event and a large number of mRNAs, including GLI1, are edited in the brain (Li et al., 2009). In our study, we confirmed that GLI1 mRNA is edited at nucleotide 2179, resulting in adenosine (A) to inosine (I) substitution. This A to I substitution leads to a change from Arginine to Glycine at position 701 of the GLI1 protein. The prevalence of this GLI1 editing event is about 50% in a number of human tissues including cerebellum (both fetal and adult), skin, pancreas, ovary and colon. However, in the corresponding tumor cell lines the extent of GLI1 editing was negligible. Moreover, the reduction in RNA editing of GLI1 was not limited to cancer cell lines but also occurred in BCC biopsy specimens compared to control skin samples. These findings indicate that GLI1 RNA editing is a normal physiological event, which is disrupted during cancer development. This is in-line with previous findings highlighting a global down-regulation of RNA editing in cancer cells (Paz et al., 2007). GLI1 RNA editing in normal cells implies that this post-transcriptional modification may act as a “protective barrier” that has to be overcome in the process of tumorigenesis.

In order to address the role of ADARs in GLI1 editing we performed siRNA mediated knockdown experiments in neuroblastoma cell lines (SK-N-AS, SH-SY5Y, SK-N-BE(2) and SK-N-SH). The level of GLI1 editing was reduced upon the knockdown of ADAR1 or ADAR2, which indicates that both ADAR isoforms are needed for GLI1 RNA editing. One scenario that could provide a mechanistic interpretation of this finding may be the formation of ADAR1/ADAR2 heterodimers that are catalytically active on this substrate.

Biochemical experiments showed that edited GLI1, GLI1-701G, has a higher

capacity to activate most of the transcriptional targets tested, including 12xGLIBS-luc and mPtch1-1B-luc reporters. In addition, GLI1-701G is less susceptible to inhibition by the negative regulator of HH signaling, SUFU.

Confocal microscopy analysis revealed no diferences in edited and non-edited GLI1 cellular localization. Moreover, the GLI1-701G was less responsive compared with GLI1 to activation elicited by the Dual-specificity Tyrosine Phosphorylation-regulated Kinase 1A (Dyrk1A). Finally, we showed that GLI1 editing affected GLI1-dependent cellular growth.

In summary, our findings demonstrated that post-transcriptional modification of GLI1 at the level of RNA editing modulates the biological outcomes of HH signaling.

3.3 PAPER III

Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1

In this project, using *in silico* analysis of EST databases we have identified a polyadenylated non-coding transcript, GLI1AS, on the opposite (antisense) strand of the *GLI1* gene in a head-to-head orientation. Rapid Amplification of cDNA Ends (RACE) in Rh36, CCA and RMS13 cancer cells determined the sequence of GLI1AS RNA, which is 885-nucleotide long and has three exons.

Expression analysis of GLI1AS and GLI1 in different cancer cell lines (PC3, 22Rv1 PANC1, A549, AGS, D283Med, RMS13, RD, Rh36, and CCA) showed concordant regulation between GLI1 and GLI1AS. Moreover, the expression of GLI1AS RNA in cancer cells lines, BCC and breast tumor samples was consistently lower but concordant with GLI1 mRNA expression. Splicing pattern analysis using GLI1AS exonic and intronic PCR primer sets revealed that splicing of intron 1 is more frequent than intron 2, meaning that intron 2 is retained in the final transcript more often than intron 1, suggesting a possible functional implication of the retained sequences. Analysis of RNA expression in nuclear/cytoplasmic fractionations of RMS cells (Rh36, CCA and RMS13) revealed that unspliced GLI1AS RNAs are preferentially retained in the nucleus, whereas spliced GLI1AS RNAs are transported to the cytoplasm.

In order to address possible biological roles of GLI1AS, we performed siRNA-mediated knockdown in Rh36 and CCA cells. Knockdown of GLI1AS resulted in GLI1 expression up-regulation. Moreover, GLI1AS knockdown induced cell proliferation of Rh36 cells. Since proliferation of Rh36 cells is GLI1 dependent (Tostar et al., 2010), the effect of GLI1AS on cell proliferation may be mediated through changes of GLI1 expression. Reciprocally, transfection of siRNAs targeting GLI1 in the Rh36 and CCA cells resulted in a decrease of GLI1AS levels. Thus, depletion of one member of the GLI1/GLI1AS pair has opposing effects on the other partner; GLI1 knockdown reduces GLI1AS but GLI1AS knockdown increases GLI1.

To examine whether the endogenous modulation of GLI1 and GLI1AS levels in Rh36 cells has an impact on tumor growth, the CAM xenograft model was used (Tostar et al., 2012; Villegas et al., 2014). Treatment of Rh36 cells with GLI1 siRNAs decreased their capacity to form tumors in this model. On the other hand an increased tumor weight was observed following treatment with GLI1AS siRNAs.

We also performed GLI1AS overexpression analysis in Rh36 cells to validate the GLI1AS knockdown experiments. Overexpression of GLI1AS resulted in the down-regulation of both GLI1 RNA and protein expression, as well as of the GLI1 target genes *PTCH1* and *PTCH2*. Moreover, the expression of the *INHBE* gene, which is tail-to-tail with *GLI1AS*, was also reduced, while the expression of the unrelated *ADAR2* gene was unaffected. GLI1AS overexpression also reduced Rh36 cellular proliferation. It is interesting to note that overexpression of either the "5'-terninal half" or the "3'-terninal half" of GLI1AS did not affect GLI1 expression or the proliferative capacity of the transfected Rh36 cells. Consequently, in order to function as a GLI1 repressor the complete RNA sequence of GLI1AS is needed, which suggests to a possible 3D folding of GLI1AS, acting as a scaffold to recruit repressor protein complexes, similarly to other lncRNAs (Latos et al., 2012; Poliseno et al., 2010; Rinn et al., 2007).

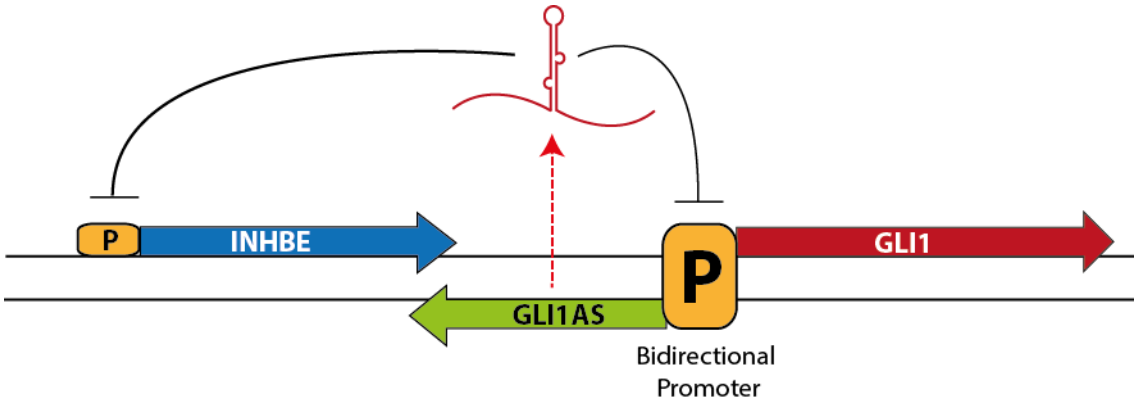

Figure 8: Proposed mechanism for the GLI1AS mediated transcriptional repression of the *GLI1* and *INHBE* genes. The *INHBE GLI1AS* and *GLI1* genes are indicated by arrows. 'P' highlights promoter regions.

In order to address the GLI1AS scaffold hypothesis we performed chromatin immunoprecipitation (ChIP) assays using antibodies against chromatin remodeling markers. Overexpression of GLI1AS increased the recruitment of repression marker, H3K27me3, in the *INHBE*/*GLI1AS*/*GLI1* genomic regions. H3K27me3 is often associated with the recruitment of polycomb repressive complex 2 (PRC2). This finding is in agreement with the observations on other non-coding RNA mediated repression mechanisms (Johnsson et al., 2013; Modarresi et al., 2012). We also observed a reduction in RNA polymerase II recruitment at the *GLI1* promoter region upon overexpression of GLI1AS, which is in-line with the chromatin-remodeling phenomena. Surprisingly, GLI1AS overexpression also increased the chromatin activation marker H3K4me3 in the *INHBE*/*GLI1AS*/*GLI1* locus.

Activation of HH signaling by the Smoothened agonist SAG in Daoy medulloblastoma cells increased not only GLI1 but also GLI1AS expression. Similarly, treatment of pancreatic adenocarcinoma PANC1 cells with TGFβ, a GLI1 inducer in this cellular context, increased not only GLI1 but also GLI1AS expression.

In conclusion, our study showed an additional layer of GLI1 regulation, apart from splice variations and GLI1 RNA-editing, which is mediated by a non-coding RNA.

3.4 PAPER IV

Global analysis of GLI1 and RNA-edited GLI1 target genes

Even though GLI1 is a transcription factor associated with HH signaling and acts as an oncogene (Nilsson et al., 2000), very little is known on the GLI1 targets, apart from a few target genes, including GLI1, PTCH1, PTCH2 and HHIP. In this study, we analyzed the GLI1 global targets by overexpressing both canonical and edited GLI1, as well as by knocking down GLI1 expression. We focused at both common and differentially regulated targets of edited/non-edited GLI1.

Single molecule RNAseq was used following overexpression/depletion in the rhabdomyosarcoma Rh36 cell line. Genes with reciprocally changed expression in the overexpression/depletion experiments were considered as likely true target genes. Combining the selected genes of the GLI1 or the GLI1-701G overexpression with those of the GLI1 depletion resulted in 477 and 480 genes regulated by non-edited/edited GLI1, respectively, 197 of which were common in the two datasets. Gene ontology (GO) analysis using the common 197 genes revealed that both edited and non-edited GLI1 are associated with cellular proliferation, which is in-line with our previous findings (Shimokawa et al., 2013). Non-edited/edited GLI1 are also linked to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways in cancer, BCC and thyroid cancer.

Candidate target genes were further filtered via the FANTOM5 dataset of global gene expression in 833 tissues (FANTOM Consortium and the RIKEN PMI and CLST (DGT) et al., 2014) resulting in 29 targets. Validation of the 20 targets, which have a Spearman correlation > 0.1 with the FANTOM dataset, by qPCR indicated that 15 targets (excluding PTCH1) are down-regulated in biological replicates of knockdown experiments in Rh36 cells. Additionally, out of these 15, 4 targets (FOXS1, SOSTDC1, LOC100507346 and SOX18) are also up-regulated in biological replicates of overexpression experiments in Rh36 cells.

Moreover, knockdown of FOXS1 in the same cells resulted in down-regulation of GLI1, highlighting a FOXS1/GLI1 regulatory loop.

Finally, GLI1 knockdown and Smoothened agonist SAG treatment in HH signaling responsive Daoy medulloblastoma cells modulate the expression of 9 out of the 15 targets, including SOSTDC1 and FOXS1.

In conclusion, this study identified and validated novel non-edited/edited GLI1 target genes using both overexpression and knockdown.

4 FUTURE PROSPECTIVE

4.1 ELABORATING ON THE MECHANISM OF GLI1AS

In PAPER III we successfully showed the transcriptional repressor function of GLI1AS on *GLI1* and the neighboring gene, *INHBE* employing the chromatin-repressing marker H3K27me3. The next logical step would be to dissect the mechanism of action of GLI1AS even further. Based on the findings of PAPER III, it is quite reasonable to speculate on the involvement of PRC2 in the GLI1AS mediated transcriptional repression process. A member of possible experimental approaches can be employed to address the of involvement of PRC2 including, 1) the effect of siRNA knockdown of the components of the polycomb repressive complex PRC2 (siEZH1, siEZH2 and siEED) on GLI1AS mediated repression; 2) DZNep (3-Deazaneplanocin A, HCl, a well known inhibitor of the polycomb component EZH2) treatment of Rh36 cells upon overexpression of GLI1AS; and finally, 3) RNA ChIP or PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) (Hafner et al., 2010; Spitzer et al., 2014) using antibodies against components of PRC2.

In PAPER III, we also showed the importance of the complete sequence of the GLI1AS lncRNA in the *GLI1* and *INHBE* repression process, which may imply the significance of the 3D folding of GLI1AS. A number of recent publications demonstrated that lncRNAs can work as scaffolds to recruit chromatin modifiers at loci of interest (Johnsson et al., 2013; Latos et al., 2012; Rinn et al., 2007). In light of all these finding, it is important to address critical regions of GLI1AS by deletion analysis and clarify possible DNA binding motifs of GLI1AS using modern techniques, including PAR-CLIP and/or ChIRP (Chromatin Isolation by RNA Purification) (Chu et al., 2011). Moreover, a recent article (Holdt et al., 2013) suggested that the Alu elements of ANRIL, a non-coding RNA, confer its ability to recognize global targets. Interestingly, intron 2 and exon 3 of GLI1AS are mainly composed of Alu sequences. Consequently, analyzing the possible functionality of the GLI1AS Alu elements might shed light on the mode of action of GLI1AS. Last but not least, a global analysis can also be performed to identify possible *trans*-targets of GLI1AS.

4.2 DIFFERENTIAL TARGET OF EDITED AND NON-EDITED GLI1

In PAPER IV, we analyzed the GLI1 and GLI1-701G common targets. It would be interesting to address targets differentially regulated by edited/non-edited GLI1 and validate them by qRCR analysis. Moreover, to address the direct targets of edited/non-edited GLI1, GLI1 ChIP-seq (Chromatin Immunoprecipitation followed by DNA sequencing) can be performed using Rh36 cell line over-expressing edited/non-edited GLI1. Another important step could be address the role GLI1 editing in the SHH-responsive human medulloblastoma cell line, Daoy. Endogenous GLI1 editing (Arginine to Glycine at position 701) can be introduced in Daoy cells using CRISPR/Cas9 (Ran et al., 2013) technology. This could be followed by RNA-

seq and ChIP-seq upon SHH signaling induction to assess differences in the HH signaling response depending on the editing status of endogenous GLI1.

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6 REFERENCES

Amakye, D., Jagani, Z., and Dorsch, M. (2013). Unraveling the therapeutic potential of the Hedgehog pathway in cancer. Nat Med *19*, 1410–1422.

Bánfai, B., Jia, H., Khatun, J., Wood, E., Risk, B., Gundling, W.E., Kundaje, A., Gunawardena, H.P., Yu, Y., Xie, L., et al. (2012). Long noncoding RNAs are rarely translated in two human cell lines. Genome Res. *22*, 1646–1657.

Beachy, P.A., Hymowitz, S.G., Lazarus, R.A., Leahy, D.J., and Siebold, C. (2010). Interactions between Hedgehog proteins and their binding partners come into view. Genes Dev. *24*, 2001–2012.

Bertani, S., Sauer, S., Bolotin, E., and Sauer, F. (2011). The noncoding RNA Mistral activates Hoxa6 and Hoxa7 expression and stem cell differentiation by recruiting MLL1 to chromatin. Mol. Cell *43*, 1040–1046.

Blackledge, N.P., Rose, N.R., and Klose, R.J. (2015). Targeting Polycomb systems to regulate gene expression: modifications to a complex story. Nat. Rev. Mol. Cell Biol. *16*, 643–649.

Bonilla, X., Parmentier, L., King, B., Bezrukov, F., Kaya, G., Zoete, V., Seplyarskiy, V.B., Sharpe, H.J., McKee, T., Letourneau, A., et al. (2016). Genomic analysis identifies new drivers and progression pathways in skin basal cell carcinoma. Nat. Genet. *48*, 398–406.

Briscoe, J., and Thérond, P.P. (2013). The mechanisms of Hedgehog signalling and its roles in development and disease. Nat Rev Mol Cell Biol *14*, 416–429.

Bürglin, T.R. (2008). The Hedgehog protein family. Genome Biology *9*, 241.

Burns, C.M., Chu, H., Rueter, S.M., Hutchinson, L.K., Canton, H., Sanders-Bush, E., and Emeson, R.B. (1997). Regulation of serotonin-2C receptor G-protein coupling by RNA editing. Nature *387*, 303–308.

Carpenter, R.L., and Lo, H.-W. (2012). Hedgehog Pathway and GLI1 Isoforms in Human Cancer. Discov Med *13*, 105–113.

Carrieri, C., Cimatti, L., Biagioli, M., Beugnet, A., Zucchelli, S., Fedele, S., Pesce, E., Ferrer, I., Collavin, L., Santoro, C., et al. (2012). Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. Nature *491*, 454–457.

Chávez, M., Ena, S., Van Sande, J., de Kerchove d'Exaerde, A., Schurmans, S., and Schiffmann, S.N. (2015). Modulation of Ciliary Phosphoinositide Content Regulates Trafficking and Sonic Hedgehog Signaling Output. Developmental Cell *34*, 338–350.

Cherry, A.L., Finta, C., Karlström, M., Jin, Q., Schwend, T., Astorga-Wells, J., Zubarev, R.A., Del Campo, M., Criswell, A.R., de Sanctis, D., et al. (2013). Structural basis of SUFU–GLI interaction in human Hedgehog signalling regulation. Acta Crystallographica Section D Biological Crystallography *69*, 2563–2579.

Chu, C., Qu, K., Zhong, F.L., Artandi, S.E., and Chang, H.Y. (2011). Genomic Maps of Long Noncoding RNA Occupancy Reveal Principles of RNA-Chromatin Interactions. Molecular Cell *44*, 667–678.

Daniel, C., Silberberg, G., Behm, M., and Öhman, M. (2014). Alu elements shape the primate transcriptome by cis-regulation of RNA editing. Genome Biology *15*, R28.

Daniel, C., Lagergren, J., and Öhman, M. (2015a). RNA editing of non-coding RNA and its role in gene regulation. Biochimie *117*, 22–27.

Daniel, C., Behm, M., and Öhman, M. (2015b). The role of Alu elements in the cis-regulation of RNA processing. Cell. Mol. Life Sci. *72*, 4063–4076.

David, C.J., and Manley, J.L. (2010). Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. Genes Dev. *24*, 2343–2364.

Deffit, S.N., and Hundley, H.A. (2016). To edit or not to edit: regulation of ADAR editing specificity and efficiency. WIREs RNA *7*, 113–127.

Di Marcotullio, L., Ferretti, E., Greco, A., De Smaele, E., Po, A., Sico, M.A., Alimandi, M., Giannini, G., Maroder, M., Screpanti, I., et al. (2006). Numb is a suppressor of Hedgehog signalling and targets Gli1 for Itch-dependent ubiquitination. Nat. Cell Biol. *8*, 1415–1423.

Drummond, I.A. (2012). Cilia functions in development. Current Opinion in Cell Biology *24*, 24–30.

Dunaeva, M., Michelson, P., Kogerman, P., and Toftgard, R. (2003). Characterization of the Physical Interaction of Gli Proteins with SUFU Proteins. Journal of Biological Chemistry *278*, 5116–5122.

Epstein, E.H. (2008). Basal cell carcinomas: attack of the hedgehog. Nat Rev Cancer *8*, 743–754.

Esteller, M. (2011). Non-coding RNAs in human disease. Nat Rev Genet *12*, 861–874.

Faghihi, M.A., Modarresi, F., Khalil, A.M., Wood, D.E., Sahagan, B.G., Morgan, T.E., Finch, C.E., Iii, G.S.L., Kenny, P.J., and Wahlestedt, C. (2008). Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of β-secretase. Nat Med *14*, 723–730.

Faghihi, M.A., Zhang, M., Huang, J., Modarresi, F., Van der Brug, M.P., Nalls, M.A., Cookson, M.R., St-Laurent, G., and Wahlestedt, C. (2010). Evidence for natural antisense transcript-mediated inhibition of microRNA function. Genome Biol. *11*, R56.

FANTOM Consortium and the RIKEN PMI and CLST (DGT), Forrest, A.R.R., Kawaji, H., Rehli, M., Baillie, J.K., de Hoon, M.J.L., Haberle, V., Lassmann, T., Kulakovskiy, I.V., Lizio, M., et al. (2014). A promoter-level mammalian expression atlas. Nature *507*, 462–470.

Farajollahi, S., and Maas, S. (2010). Molecular diversity through RNA editing: a balancing act. Trends in Genetics *26*, 221–230.

Farzan, S.F., Ascano Jr., M., Ogden, S.K., Sanial, M., Brigui, A., Plessis, A., and Robbins, D.J. (2008). Costal2 Functions as a Kinesin-like Protein in the Hedgehog Signal Transduction Pathway. Current Biology *18*, 1215–1220.

Fatica, A., and Bozzoni, I. (2014). Long non-coding RNAs: new players in cell differentiation and development. Nat Rev Genet *15*, 7–21.

Gao, C., Cao, W., Bao, L., Zuo, W., Xie, G., Cai, T., Fu, W., Zhang, J., Wu, W., Zhang, X., et al. (2010). Autophagy negatively regulates Wnt signalling by promoting Dishevelled degradation. Nat. Cell Biol. *12*, 781–790.

Garcia-Gonzalo, F.R., Phua, S.C., Roberson, E.C., Garcia III, G., Abedin, M., Schurmans, S., Inoue, T., and Reiter, J.F. (2015). Phosphoinositides Regulate Ciliary Protein Trafficking to Modulate Hedgehog Signaling. Developmental Cell *34*, 400–409.

Geisler, S., and Coller, J. (2013). RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nat Rev Mol Cell Biol *14*, 699–712.

Gong, C., and Maquat, L.E. (2011). lncRNAs transactivate STAU1-mediated mRNA decay by duplexing with 3′ UTRs via Alu elements. Nature *470*, 284–288.

Grimm, T., Teglund, S., Tackels, D., Sangiorgi, E., Gurrieri, F., Schwartz, C., and Toftgård, R. (2001). Genomic organization and embryonic expression of Suppressor of Fused, a candidate gene for the split-hand/split-foot malformation type 3. FEBS Lett *505*, 13–17.

Haas, D., and Muenke, M. (2010). Abnormal sterol metabolism in holoprosencephaly. Am. J. Med. Genet. *154C*, 102–108.

Hafner, M., Landthaler, M., Burger, L., Khorshid, M., Hausser, J., Berninger, P., Rothballer, A., Ascano, M., Jungkamp, A.-C., Munschauer, M., et al. (2010). PAR-CliP--a method to identify transcriptome-wide the binding sites of RNA binding proteins. J Vis Exp.

Hallikas, O., Palin, K., Sinjushina, N., Rautiainen, R., Partanen, J., Ukkonen, E., and Taipale, J. (2006). Genome-wide Prediction of Mammalian Enhancers Based on Analysis of Transcription-Factor Binding Affinity. Cell *124*, 47–59.

Heby-Henricson, K. (2011). The Role of Suppressor of Fused in Development and Tumorigenesis in the Mouse (PhD thesis, Inst för biovetenskaper och näringslära / Dept of Biosciences and Nutrition).

Holdt, L.M., Hoffmann, S., Sass, K., Langenberger, D., Scholz, M., Krohn, K., Finstermeier, K., Stahringer, A., Wilfert, W., Beutner, F., et al. (2013). Alu elements in ANRIL non-coding RNA at chromosome 9p21 modulate atherogenic cell functions through trans-regulation of gene networks. PLoS Genet. *9*, e1003588.

Holoch, D., and Moazed, D. (2015). RNA-mediated epigenetic regulation of gene expression. Nat Rev Genet *16*, 71–84.

Hui, C.-C., and Angers, S. (2011). Gli proteins in development and disease. Annu. Rev. Cell Dev. Biol. *27*, 513–537.

Huntzicker, E.G., Estay, I.S., Zhen, H., Lokteva, L.A., Jackson, P.K., and Oro, A.E. (2006). Dual degradation signals control Gli protein stability and tumor formation. Genes Dev. *20*, 276–281.

Ingham, P.W., and McMahon, A.P. (2001). Hedgehog signaling in animal development: paradigms and principles. Genes Dev. *15*, 3059–3087.

Irimia, M., and Blencowe, B.J. (2012). Alternative splicing: decoding an expansive regulatory layer. Current Opinion in Cell Biology *24*, 323–332.

Jiang, K., and Jia, J. (2015). Smoothened regulation in response to Hedgehog stimulation. Front Biol (Beijing) *10*, 475–486.

Johnsson, P., Ackley, A., Vidarsdottir, L., Lui, W.-O., Corcoran, M., Grandér, D., and Morris, K.V. (2013). A pseudogene long-noncoding-RNA network regulates PTEN transcription and translation in human cells. Nat Struct Mol Biol *20*, 440–446.

Kasper, M., Jaks, V., Fiaschi, M., and Toftgård, R. (2009). Hedgehog signalling in breast cancer. Carcinogenesis *30*, 903–911.

Kawahara, Y., Zinshteyn, B., Sethupathy, P., Iizasa, H., Hatzigeorgiou, A.G., and Nishikura, K. (2007). Redirection of Silencing Targets by Adenosine-to-Inosine Editing of miRNAs. Science *315*, 1137–1140.

Kinzler, K.W., and Vogelstein, B. (1990). The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. Mol. Cell. Biol. *10*, 634–642.

Kinzler, K.W., Bigner, S.H., Bigner, D.D., Trent, J.M., Law, M.L., O'Brien, S.J., Wong, A.J., and Vogelstein, B. (1987). Identification of an amplified, highly expressed gene in a human glioma. Science *236*, 70–73.

Koziel, L., Kunath, M., Kelly, O.G., and Vortkamp, A. (2004). Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. Dev. Cell *6*, 801–813.

Krystal, G.W., Armstrong, B.C., and Battey, J.F. (1990). N-myc mRNA forms an RNA-RNA duplex with endogenous antisense transcripts. Mol. Cell. Biol. *10*, 4180–4191.

Latos, P.A., Pauler, F.M., Koerner, M.V., Şenergin, H.B., Hudson, Q.J., Stocsits, R.R., Allhoff, W., Stricker, S.H., Klement, R.M., Warczok, K.E., et al. (2012). Airn transcriptional overlap, but not its lncRNA products, induces imprinted Igf2r silencing. Science *338*, 1469–1472.

Li, J.B., and Church, G.M. (2013). Deciphering the functions and regulation of brain-enriched A-to-I RNA editing. Nat Neurosci *16*, 1518–1522.

Li, J.B., Levanon, E.Y., Yoon, J.-K., Aach, J., Xie, B., LeProust, E., Zhang, K., Gao, Y., and Church, G.M. (2009). Genome-Wide Identification of Human RNA Editing Sites by Parallel DNA Capturing and Sequencing. Science *324*, 1210–1213.

Lo, H.-W., Zhu, H., Cao, X., Aldrich, A., and Ali-Osman, F. (2009). A Novel Splice Variant of GLI1 That Promotes Glioblastoma Cell Migration and Invasion. Cancer Research *69*, 6790–6798.

Long, J., Tokhunts, R., Old, W.M., Houel, S., Rodgriguez-Blanco, J., Singh, S., Schilling, N., Capobianco, A.J., Ahn, N.G., and Robbins, D.J. (2015). Identification of a Family of Fatty-Acid-Speciated Sonic Hedgehog Proteins, Whose Members Display Differential Biological Properties. Cell Reports *10*, 1280–1287.

Mahmoudi, S., Henriksson, S., Corcoran, M., Méndez-Vidal, C., Wiman, K.G., and Farnebo, M. (2009). Wrap53, a natural p53 antisense transcript required for p53 induction upon DNA damage. Mol. Cell *33*, 462–471.

Mann, R.K., and Beachy, P.A. (2004). Novel lipid modifications of secreted protein signals. Annu. Rev. Biochem. *73*, 891–923.

Marcucci, R., Brindle, J., Paro, S., Casadio, A., Hempel, S., Morrice, N., Bisso, A., Keegan, L.P., Del Sal, G., and O'Connell, M.A. (2011). Pin1 and WWP2 regulate *GluR2* Q/R site RNA editing by ADAR2 with opposing effects: Pin1 and WWP2 regulate ADAR2. The EMBO Journal *30*, 4211–4222.

Memczak, S., Jens, M., Elefsinioti, A., Torti, F., Krueger, J., Rybak, A., Maier, L., Mackowiak, S.D., Gregersen, L.H., Munschauer, M., et al. (2013). Circular RNAs are a large class of animal RNAs with regulatory potency. Nature *495*, 333–338.

Merchant, A.A., and Matsui, W. (2010). Targeting Hedgehog--a cancer stem cell pathway. Clin. Cancer Res. *16*, 3130–3140.

Merchant, M., Vajdos, F.F., Ultsch, M., Maun, H.R., Wendt, U., Cannon, J., Desmarais, W., Lazarus, R.A., de Vos, A.M., and de Sauvage, F.J. (2004). Suppressor of fused regulates Gli activity through a dual binding mechanism. Mol. Cell. Biol. *24*, 8627–8641.

Minn, A.J., Boise, L.H., and Thompson, C.B. (1996). Bcl-x(S) anatagonizes the protective effects of Bcl-x(L). J. Biol. Chem. *271*, 6306–6312.

Modarresi, F., Faghihi, M.A., Lopez-Toledano, M.A., Fatemi, R.P., Magistri, M., Brothers, S.P., van der Brug, M.P., and Wahlestedt, C. (2012). Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. Nat. Biotechnol. *30*, 453–459.

Nieto Moreno, N., Giono, L.E., Cambindo Botto, A.E., Muñoz, M.J., and Kornblihtt, A.R. (2015). Chromatin, DNA structure and alternative splicing. FEBS Letters *589*, 3370–3378.

Nilsson, M., Undèn, A.B., Krause, D., Malmqwist, U., Raza, K., Zaphiropoulos, P.G., and Toftgård, R. (2000). Induction of basal cell carcinomas and trichoepitheliomas in mice overexpressing GLI-1. PNAS *97*, 3438–3443.

Nishikura, K. (2006). Editor meets silencer: crosstalk between RNA editing and RNA interference. Nat Rev Mol Cell Biol *7*, 919–931.

Nishikura, K. (2010). Functions and Regulation of RNA Editing by ADAR Deaminases. Annual Review of Biochemistry *79*, 321–349.

Nishikura, K. (2016). A-to-I editing of coding and non-coding RNAs by ADARs. Nat Rev Mol Cell Biol *17*, 83–96.

Northcott, P.A., Nakahara, Y., Wu, X., Feuk, L., Ellison, D.W., Croul, S., Mack, S., Kongkham, P.N., Peacock, J., Dubuc, A., et al. (2009). Multiple recurrent genetic events converge on control of histone lysine methylation in medulloblastoma. Nat Genet *41*, 465–472.

Nozawa, R.-S., Nagao, K., Igami, K.-T., Shibata, S., Shirai, N., Nozaki, N., Sado, T., Kimura, H., and Obuse, C. (2013). Human inactive X chromosome is compacted through a PRC2-independent SMCHD1-HBiX1 pathway. Nat. Struct. Mol. Biol. *20*, 566–573.

Nüsslein-Volhard, C., and Wieschaus, E. (1980). Mutations affecting segment number and polarity in Drosophila. Nature *287*, 795–801.

Ørom, U.A., Derrien, T., Beringer, M., Gumireddy, K., Gardini, A., Bussotti, G., Lai, F., Zytnicki, M., Notredame, C., Huang, Q., et al. (2010). Long noncoding RNAs with enhancer-like function in human cells. Cell *143*, 46–58.

O'Toole, S.A., Swarbrick, A., and Sutherland, R.L. (2009). The Hedgehog signalling pathway as a therapeutic target in early breast cancer development. Expert Opin. Ther. Targets *13*, 1095–1103.

Palaniswamy, R., Teglund, S., Lauth, M., Zaphiropoulos, P., and Shimokawa, T. (2010). Genetic variations regulate alternative splicing in the 5' untranslated regions of the mouse glioma-associated oncogene 1, Gli1. BMC Molecular Biology *11*, 32.

Pan, Q., Shai, O., Lee, L.J., Frey, B.J., and Blencowe, B.J. (2008). Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nat Genet *40*, 1413–1415.

Pandolfi, S., Montagnani, V., Lapucci, A., and Stecca, B. (2015). HEDGEHOG/GLI-E2F1 axis modulates iASPP expression and function and regulates melanoma cell growth. Cell Death Differ. *22*, 2006–2019.

Paz, N., Levanon, E.Y., Amariglio, N., Heimberger, A.B., Ram, Z., Constantini, S., Barbash, Z.S., Adamsky, K., Safran, M., Hirschberg, A., et al. (2007). Altered adenosine-to-inosine RNA editing in human cancer. Genome Research *17*, 1586–1595.

Pelechano, V., and Steinmetz, L.M. (2013). Gene regulation by antisense transcription. Nat Rev Genet *14*, 880–893.

Peng, P.L., Zhong, X., Tu, W., Soundarapandian, M.M., Molner, P., Zhu, D., Lau, L., Liu, S., Liu, F., and Lu, Y. (2006). ADAR2-Dependent RNA Editing of AMPA Receptor Subunit GluR2 Determines Vulnerability of Neurons in Forebrain Ischemia. Neuron *49*, 719–733.

Penny, G.D., Kay, G.F., Sheardown, S.A., Rastan, S., and Brockdorff, N. (1996). Requirement for Xist in X chromosome inactivation. Nature *379*, 131–137.

Pepinsky, R.B., Zeng, C., Wen, D., Rayhorn, P., Baker, D.P., Williams, K.P., Bixler, S.A., Ambrose, C.M., Garber, E.A., Miatkowski, K., et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. J. Biol. Chem. *273*, 14037–14045.

Perler, F.B. (1998). Protein Splicing of Inteins and Hedgehog Autoproteolysis: Structure, Function, and Evolution. Cell *92*, 1–4.

Poliseno, L., Salmena, L., Zhang, J., Carver, B., Haveman, W.J., and Pandolfi, P.P. (2010). A coding-independent function of gene and

pseudogene mRNAs regulates tumour biology. Nature *465*, 1033–1038.

Quinn, J.J., and Chang, H.Y. (2016). Unique features of long non-coding RNA biogenesis and function. Nat Rev Genet *17*, 47–62.

Rahnama, F., Toftgård, R., and Zaphiropoulos, P.G. (2004). Distinct roles of PTCH2 splice variants in Hedgehog signalling. Biochemical Journal *378*, 325–334.

Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protocols *8*, 2281–2308.

Rinn, J.L., Kertesz, M., Wang, J.K., Squazzo, S.L., Xu, X., Brugmann, S.A., Goodnough, L.H., Helms, J.A., Farnham, P.J., Segal, E., et al. (2007). Functional Demarcation of Active and Silent Chromatin Domains in Human HOX Loci by Noncoding RNAs. Cell *129*, 1311–1323.

Robbins, D.J., Fei, D.L., and Riobo, N.A. (2012). The Hedgehog Signal Transduction Network. Sci. Signal. *5*, re6–re6.

Roberts, W.M., Douglass, E.C., Peiper, S.C., Houghton, P.J., and Look, A.T. (1989). Amplification of the gli gene in childhood sarcomas. Cancer Res. *49*, 5407–5413.

Rohatgi, R., Milenkovic, L., and Scott, M.P. (2007). Patched1 regulates hedgehog signaling at the primary cilium. Science *317*, 372–376.

Scales, S.J., and de Sauvage, F.J. (2009). Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. Trends in Pharmacological Sciences *30*, 303–312.

Schoeftner, S., Sengupta, A.K., Kubicek, S., Mechtler, K., Spahn, L., Koseki, H., Jenuwein, T., and Wutz, A. (2006). Recruitment of PRC1 function at the initiation of X inactivation independent of PRC2 and silencing. EMBO J. *25*, 3110–3122.

Shimokawa, T., Rahnama, F., and Zaphiropoulos, P.G. (2004). A novel first exon of the Patched1 gene is upregulated by Hedgehog signaling resulting in a protein with pathway inhibitory functions. FEBS Letters *578*, 157–162.

Shimokawa, T., Svärd, J., Heby-Henricson, K., Teglund, S., Toftgård, R., and Zaphiropoulos, P.G. (2007). Distinct roles of first exon variants of the tumor-suppressor Patched1 in Hedgehog signaling. Oncogene *26*, 4889–4896.

Shimokawa, T., Tostar, U., Lauth, M., Palaniswamy, R., Kasper, M., Toftgård, R., and Zaphiropoulos, P.G. (2008). Novel Human Glioma-associated Oncogene 1 (GLI1) Splice Variants Reveal Distinct Mechanisms in the Terminal Transduction of the Hedgehog Signal. Journal of Biological Chemistry *283*, 14345–14354.

Shimokawa, T., Rahman, M.F.-U., Tostar, U., Sonkoly, E., Ståhle, M., Pivarcsi, A., Palaniswamy, R., and Zaphiropoulos, P.G. (2013). RNA editing of the GLI1 transcription factor modulates the output of Hedgehog signaling. RNA Biology *10*, 321–333.

Sleutels, F., Zwart, R., and Barlow, D.P. (2002). The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature *415*, 810–813.

Speek, M., Njunkova, O., Pata, I., Valdre, E., and Kogerman, P. (2006). A potential role of alternative splicing in the regulation of the transcriptional activity of human GLI2 in gonadal tissues. BMC Molecular Biology *7*, 13.

Spitzer, J., Hafner, M., Landthaler, M., Ascano, M., Farazi, T., Wardle, G., Nusbaum, J., Khorshid, M., Burger, L., Zavolan, M., et al. (2014). PAR-CLIP (Photoactivatable Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation): a step-by-step protocol to the transcriptome-wide identification of binding sites of RNA-binding proteins. Meth. Enzymol. *539*, 113–161.

Stone, D.M., Murone, M., Luoh, S., Ye, W., Armanini, M.P., Gurney, A., Phillips, H., Brush, J., Goddard, A., de Sauvage, F.J., et al. (1999). Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli. J. Cell. Sci *112 (Pt 23)*, 4437–4448.

Sun, S., Del Rosario, B.C., Szanto, A., Ogawa, Y., Jeon, Y., and Lee, J.T. (2013). Jpx RNA activates Xist by evicting CTCF. Cell *153*, 1537–1551.

Taipale, J., Cooper, M.K., Maiti, T., and Beachy, P.A. (2002). Patched acts catalytically to suppress the activity of Smoothened. Nature *418*, 892–897.

Teglund, S., and Toftgård, R. (2010). Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer *1805*, 181–208.

Tojo, M., Kiyosawa, H., Iwatsuki, K., Nakamura, K., and Kaneko, F. (2003). Expression of the

GLI2 oncogene and its isoforms in human basal cell carcinoma. British Journal of Dermatology *148*, 892–897.

Tostar, U. (2010). Hedgehog signaling in Rhabdomyosarcoma: Role of GLI factors and splice variants in signal transduction (PhD thesis, Inst för biovetenskaper och näringslära / Dept of Biosciences and Nutrition).

Tostar, U., Toftgård, R., Zaphiropoulos, P.G., and Shimokawa, T. (2010). Reduction of Human Embryonal Rhabdomyosarcoma Tumor Growth by Inhibition of the Hedgehog Signaling Pathway. Genes Cancer *1*, 941–951.

Tostar, U., Finta, C., Rahman, M.F.-U., Shimokawa, T., and Zaphiropoulos, P.G. (2012). Novel Mechanism of Action on Hedgehog Signaling by a Suppressor of Fused Carboxy Terminal Variant. PLoS ONE *7*, e37761.

Tsai, M.-C., Manor, O., Wan, Y., Mosammaparast, N., Wang, J.K., Lan, F., Shi, Y., Segal, E., and Chang, H.Y. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science *329*, 689–693.

Uchikawa, H., Toyoda, M., Nagao, K., Miyauchi, H., Nishikawa, R., Fujii, K., Kohno, Y., Yamada, M., and Miyashita, T. (2006). Brain- and heart-specific Patched-1 containing exon 12b is a dominant negative isoform and is expressed in medulloblastomas. Biochemical and Biophysical Research Communications *349*, 277–283.

Ulitsky, I., and Bartel, D.P. (2013). lincRNAs: Genomics, Evolution, and Mechanisms. Cell *154*, 26–46.

Varjosalo, M., and Taipale, J. (2008). Hedgehog: functions and mechanisms. Genes & Development *22*, 2454–2472.

Villegas, V.E., and Zaphiropoulos, P.G. (2015). Neighboring Gene Regulation by Antisense Long Non-Coding RNAs. International Journal of Molecular Sciences *16*, 3251–3266.

Villegas, V.E., Rahman, M.F.-U., Fernandez-Barrena, M.G., Diao, Y., Liapi, E., Sonkoly, E., Ståhle, M., Pivarcsi, A., Annaratone, L., Sapino, A., et al. (2014). Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1. Molecular Oncology *8*, 912–926.

Vogelstein, B., and Kinzler, K.W. (2004). Cancer genes and the pathways they control. Nat. Med. *10*, 789–799.

Wang, K.C., and Chang, H.Y. (2011). Molecular Mechanisms of Long Noncoding RNAs. Molecular Cell *43*, 904–914.

Wang, K.C., Yang, Y.W., Liu, B., Sanyal, A., Corces-Zimmerman, R., Chen, Y., Lajoie, B.R., Protacio, A., Flynn, R.A., Gupta, R.A., et al. (2011). A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature *472*, 120–124.

Wang, Q., Hui, H., Guo, Z., Zhang, W., Hu, Y., He, T., Tai, Y., Peng, P., and Wang, L. (2013). ADAR1 regulates ARHGAP26 gene expression through RNA editing by disrupting miR-30b-3p and miR-573 binding. RNA *19*, 1525–1536.

Wen, X., Lai, C.K., Evangelista, M., Hongo, J.-A., de Sauvage, F.J., and Scales, S.J. (2010). Kinetics of hedgehog-dependent full-length Gli3 accumulation in primary cilia and subsequent degradation. Mol. Cell. Biol. *30*, 1910–1922.

Xu, Q., Zhang, Y., Wei, Q., Huang, Y., Hu, J., and Ling, K. (2016). Phosphatidylinositol phosphate kinase PIPKIγ and phosphatase INPP5E coordinate initiation of ciliogenesis. Nat Commun *7*, 10777.

Yang, W., Chendrimada, T.P., Wang, Q., Higuchi, M., Seeburg, P.H., Shiekhattar, R., and Nishikura, K. (2006). Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat Struct Mol Biol *13*, 13–21.

Yap, K.L., Li, S., Muñoz-Cabello, A.M., Raguz, S., Zeng, L., Mujtaba, S., Gil, J., Walsh, M.J., and Zhou, M.-M. (2010). Molecular Interplay of the Noncoding RNA ANRIL and Methylated Histone H3 Lysine 27 by Polycomb CBX7 in Transcriptional Silencing of INK4a. Molecular Cell *38*, 662–674.

Zaphiropoulos, P.G., Undén, A.B., Rahnama, F., Hollingsworth, R.E., and Toftgård, R. (1999). PTCH2, a Novel Human Patched Gene, Undergoing Alternative Splicing andUp-regulated in Basal Cell Carcinomas. Cancer Res *59*, 787–792.

Zhao, J., Sun, B.K., Erwin, J.A., Song, J.-J., and Lee, J.T. (2008). Polycomb proteins targeted by a short repeat RNA to the mouse X chromosome. Science *322*, 750–756.

Zhao, Y., Li, H., Fang, S., Kang, Y., Wu, W., Hao, Y., Li, Z., Bu, D., Sun, N., Zhang, M.Q., et al. (2015). NONCODE 2016: an informative and valuable data source of long non-coding RNAs. Nucl. Acids Res. gkv1252.