THE EFFECTOR OF HEDGEHOG SIGNALING, THE TRANSCRIPTION FACTOR GLI1: NOVEL REGULATORY MECHANISMS AND ROLE IN TAMOXIFEN TREATMENT

THESIS FOR DOCTORAL DEGREE (PH.D.)

21/07/2016
UNIVERSIDAD DEL ROSARIO
Victoria Eugenia Villegas Gálvez

European Director:
Peter Zaphiropoulos

Universidad del Rosario Director:
Sandra Ramirez

UNIVERSIDAD DEL ROSARIO
FACULTY OF NATURAL SCIENCES AND MATHEMATICS
SCHOOL OF MEDICINE AND HEALTH SCIENCES
DOCTORATE IN BIOMEDICAL SCIENCES
BOGOTA

DEDICATORIA

A Suecia un país que se robó mi corazón!

ACKNOLEDGEMENTS

First of all, **Universidad del Rosario** for the opportunity to continue studying.

I would like to thank **ERACOL**, the scholarship that made possible to develop my thesis in Karolinska Institute.

This research work and some courses were performed at the Department of Biosciences and Nutrition in **Karolinska Institute** then I would like to express my gratitude to all my co-workers, co-authors and friends who have helped me so much during this period.

My sincere gratitude to my European supervisor, **Professor Peter Zaphiropoulos** for his support throughout my time as a student and push me every day to overcome obstacles in the best way possible.

I am grateful to **Professor Rune Toftgård** for having me in his wonderful group where I enjoyed every moment.

I would like to thank my Colombian supervisor, **Professor Sandra Ramirez** for her continued collaboration and optimism.

My sincere gratitude also goes to my dean, **Professor Mauricio Linares** who has allowed me to continue collaborative work with my European supervisor.

I am grateful to my "unofficial co-supervisor" **Takashi** for his advice and teachings.

Åsa B, **Elin, Agneta**, **Maryam** and **Raoul** for their valuable assistance and collaboration.

My co-workers, co-authors and friends, **Yumei**, **Ferdous**, **Milena** and **Magda Carolina** with whom I have shared problems and better yet, fun times!

I would like to specially thank the **present** and **past members** of the **RTO** and **MKA** group of Karolinska Institute as well as the **Biology Unit Members** of Universidad del Rosario for their cooperation, academic discussions and unforgettable moments.

I would express my gratefulness to **Luisa Matheus** for her commitment to PhD students.

Finally, thanks to my closest friends, **Viviana** and **Stella** and my **Family**, ATP of my life.

TABLE OF CONTENTS

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred in Chapter I and Chapter II

CHAPTER I

Review paper

Villegas VE, Zaphiropoulos PG. Neighboring gene regulation by antisense long non-coding RNAs. Int J Mol Sci. 2015 Feb 3;16(2):3251-66.
doi: 10.3390/ijms16023251 PMID: 25654223

Research paper

Villegas VE, Rahman MF, Fernandez-Barrena MG, Diao Y, Liapi E, Sonkoly E, Ståhle M, Pivarcsi A, Annaratone L, Sapino A, Ramírez Clavijo S, Bürglin TR, Shimokawa T, Ramachandran S, Kapranov P, Fernandez-Zapico ME, Zaphiropoulos PG. Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1. Mol Oncol. 2014 Jul;8(5):912-26. Epub 2014 Mar 22.
doi: 10.1016/j.molonc.2014.03.009 PMID: 24726458

CHAPTER II

Review paper

Rondón-Lagos M, **Villegas VE**, Rangel Nelson, Sánchez Magda Carolina and Zaphiropoulos PG. Tamoxifen resistance: emerging molecular targets. Submitted manuscript end of June 2016.

Research paper

Villegas VE, Rondón-Lagos M, Annaratone L, Castellano I, Grismaldo A, Sapino A, Zaphiropoulos PG. Tamoxifen Treatment of Breast Cancer Cells: Impact on Hedgehog/GLI1 Signaling. Int J Mol Sci. 2016 Feb 27;17(3):308.
doi: 10.3390/ijms17030308 PMID: 26927093

LIST OF FIGURES & TABLES

LIST OF ABBREVIATIONS

ADAR	Adenosine deaminase acting on RNA
ARMS	Alveolar rhabdomyosarcoma
BCC	Basal cell carcinoma
CAM	Chick chorioallantoic membrane
ChIP	Chromatin immunoprecipitation
DHH	Desert HH
ER	Estrogen receptor
ERMS	Embryonal rhabdomyosarcoma
GLI1AS	Antisense strand of the GLI1 gene
GLI	Glioma associated oncogenes
HER	Epidermal growth factor receptor
HH	Hedgehog
HIP	Hedgehog-interacting protein
IARC	International agency for research on cancer
IHH	Indian HH
Ki-67	Proliferation index
LncRNA	Long non-coding RNA
PR	Progesterone receptor
PTCH	Patched
RACE	Rapid amplification of cDNA ends
RMS	Rhabdomyosarcoma
SERM	Selective estrogen receptor
SHH	Sonic HH
siRNA	Small interfering RNA
SMO	Smoothened
SUFU	Suppressor of fused
TAM	Tamoxifen
TNBC	Triple negative breast cancer

ABSTRACT

The Hedgehog (HH) signal transduction cascade is a major pathway involved in embryonic development, cell proliferation, stem cell generation and tissue repair. Consequently, it is not surprising that it also has a crucial role in tumorigenesis, as evidenced by the fact that more than 25% of human cancers have been associated with aberrations of this pathway.

The outcome of HH signaling activation varies depending on the cell type, and may include up-regulation of a variety of cell-specific transcription factors mediating different developmental fate responses. Genes generally induced by HH signaling activity, include *PTCH1* and *PTCH2*, which code for proteins that function as receptors, Hedgehog-interacting protein (HIP) and GLI1, which acts as a transcription factor contributing to the regulation of proliferation and differentiation. In different malignancies the pro-tumoral function of *GLI1* is associated with its increased expression. Thus, understanding the mechanisms influencing GLI1 expression is particularly relevant, as these may represent additional means of constraining the oncogenic capacity of *GLI1*.

In Chapter I, we describe the characterization of an RNA transcript from the antisense strand of the *GLI1* gene, termed GLI1AS, with no potential to code for a long protein, which acts as a negative regulator of GLI1 expression. We provide evidence for capping and polyadenylation of this antisense RNA, suggesting that it is processed similarly to a typical mRNA, even though it lacks the potential to code a protein. Additionally, our data show that GLI1 mRNA expression is higher than GLI1AS across all samples examined, consistent with the results reported for most antisense transcripts with regulatory roles on the corresponding sense gene. Chromatin immunoprecipitation assays supported the notion that GLI1AS acts as an epigenetic modifier, which elicits negative feedback on GLI1 expression via local chromatin remodeling, observations that were in-line with cellular proliferation and chick chorioallantoic membrane (CAM) tumor assays.

In Chapter 2, we present *in vitro* data using a number of different breast cancer cell lines, demonstrating the modulatory effect of tamoxifen (TAM) on cellular proliferation and expression of HH signaling components, in particular GLI1. Our results show that cell lines that express nuclear GLI1 staining after TAM treatment exhibit an increase in cell proliferation compared to control, GLI1 negative cells. These findings could indicate that the HH signaling pathway can be activated by TAM in breast cancer cells, eliciting cellular growth.

Overall, our findings suggest the possibility that these novel regulatory mechanisms may provide clues for possible drug targets that could be an effective therapeutic option in GLI1-dependent tumors.

GENERAL INTRODUCTION

Activation of the Hedgehog (HH) pathway has been implicated in the development of tumors that can be derived from either ligand-dependent or ligand-independent mechanisms. The latter include mutations in the receptor of the HH ligand, the patched homolog 1 (PTCH1) or in the signaling molecule smoothened (SMO), a transmembrane component of the pathway, resulting in constitutively activated HH signaling. Consequently, specific inhibitors of the HH cascade are considered as good targets for cancer therapy. The first known antagonist of the HH pathway to be identified was cyclopamine, a chemical that belongs to the group of steroidal alkaloids, which binds and inhibits SMO (1). Nevertheless, this drug and other SMO antagonists will not be effective in tumors with activation downstream of SMO, such as GLI (Glioma-associated oncogene) amplification/mutation (2). Accordingly, it is necessary to focus on inhibitors and molecular mechanisms that can block this pathway by directly reducing the transcriptional activity of the GLI factors.

In Chapter I special emphasis is given on regulatory controls of the HH pathway that include RNA-based mechanisms, since there is increasing evidence that these may constitute a novel way to modulate the levels of critical signaling molecules. The establishment of an antisense RNA-mediated regulation of GLI1, one of the three GLI factor in humans, using cancer cell lines and the chick chorioallantoic membrane (CAM) assay as a xenograft model were the prominent goals of this research.

In Chapter II the modulatory effect of tamoxifen (TAM) on cellular proliferation and expression of HH signaling components, including the terminal effector of the pathway, the transcription factor GLI1, is demonstrated in an *in vitro* study using a panel of different breast cancer cell lines.

As more than 25% of human cancers have been associated with aberrations of the HH signaling pathway (3–6) efforts for a better clarification of the individual steps in this signal transduction cascade are apparently justifiable. It is anticipated that further dissection of key regulatory events in HH signaling may provide clues for a rational design of inhibitory molecules that could be exploited in future cancer therapeutic approaches.

BACKGROUND

Cancer

Cancer is not just one disease; this name is given to a collection of related diseases, all with one common characteristic, the uncontrolled growth of cells that have lost the normal characteristics of the tissue from which they originate. Normal cells respond to a growth program that is coupled to the needs of the tissue, and die when irreparable damage occurs, with new healthy cells taking their place. Cancer cells accumulate damaging insults and evade control systems, with continuous growth of new cells and accumulation of additional mutations causing significant deregulations in the part of the body where the cancer has started. In addition, these cells begin to demonstrate their ability to colonize other parts of the body (7).

There are more than 100 distinct types of human cancer named from the organs/tissues/type of cell where the cancer originates. Three general classes of cancer types are described:

Carcinomas, the most common type of cancer derived from epithelial cells, which include about 80-90% of human cancers. *Sarcomas* or soft-tissue tumors, from mesenchymal origin, which are rare in humans constituting about 1% of all cancer types. *Leukemias* or *lymphomas,* non-solid tumors from the blood-forming cells and from cells of the immune system respectively, which account for approximately 8% of human malignancies (8).

Despite the variability of the different types of cancer described, in 2000 and in a subsequent update in 2011 certain common characteristics have been highlighted and considered as hallmarks of cancer (9,10). These include:

- *Self-sufficiency in growth signals*. Cancer cells can control their own proliferation through production of growth signals or hyper-activation of receptors.
- *Insensitivity to anti-growth signals*. Malignant cells are able to interrupt or ignore signals that restrain cell growth and proliferation.
- *Evasion of apoptosis*. Tumors avoid normal cell death by increasing the anti-apoptotic response and/or down-regulating the pro-apoptotic program.
- *Limitless replicative potential*. Activation of telomerase in cancer cells, with consequent maintenance of the telomeres and indefinite replication.
- *Sustained angiogenesis*. Tumor cells are able to stimulate the formation of new blood vessels for increased growth and energy needs.
- *Tissue invasion and metastasis*. Cancer cells spread from one organ to another.
- *Reprogramming energy metabolism*. Cells in the interior of a tumor can grow despite oxygen and nutrient deprivation, with this being mediated through adjustments of energy metabolism.

- *Evading the immune system*. Cancer cells can escape immunological destruction by disabling components of the immune system that have been dispatched to eliminate them.
- *Genome instability*. Accumulation of changes in DNA, mainly in genes that promote or control the cell cycle, generates genetic diversity in the cells, which can lead to the acquisition of the malignant phenotype
- *Inflammation.* Tumor cells promote inflammation, with this tumor-associated inflammation facilitating tumor growth (9,10).

There are two categories of genes that play an important role in triggering cancer, tumor suppressor or growth inhibitory genes and proto-oncogenes, which are involved in promoting growth (11).

Tumor suppressor genes

Tumor suppressor genes have a role in controlling cell growth and division before cells display a cancerous phenotype. Loss of function of these genes contributes to the development of cancer, with the mutant cells dividing in an uncontrolled manner. In cancers, tumor suppressor genes undergo alterations more frequently than oncogenes (11,12).

Proto-oncogenes

Proto-oncogenes are normal genes involved in cell growth and division. Mutant alleles with a gain of function are more active in promoting growth and are called oncogenes. In contrast to the tumor suppressor genes, which restrain cell proliferation, oncogenes actively promote proliferation. Mutation in one allele is often sufficient to initiate cancer development (11).

Glioma-associated oncogene 1

GLI1 was the first member of the Krüppel zinc finger proteins to be identified in humans (13), originally isolated from a glioma tumor. *GLI1* encodes a transcriptional activator involved in developmental processes (14). It is composed of 12 exons, spanning approximately 12 kb of genomic DNA in a centromere to telomere orientation (Figure 1). The translation initiation codon is located in exon 2, and the stop codon in exon 12 (15). *GLI1* transcriptional activation is a general biomarker of the cell's response to HH ligands and it can be used as a diagnostic tool for HH pathway activity. However, several additional signaling cascades have also been implicated in its regulation (16).

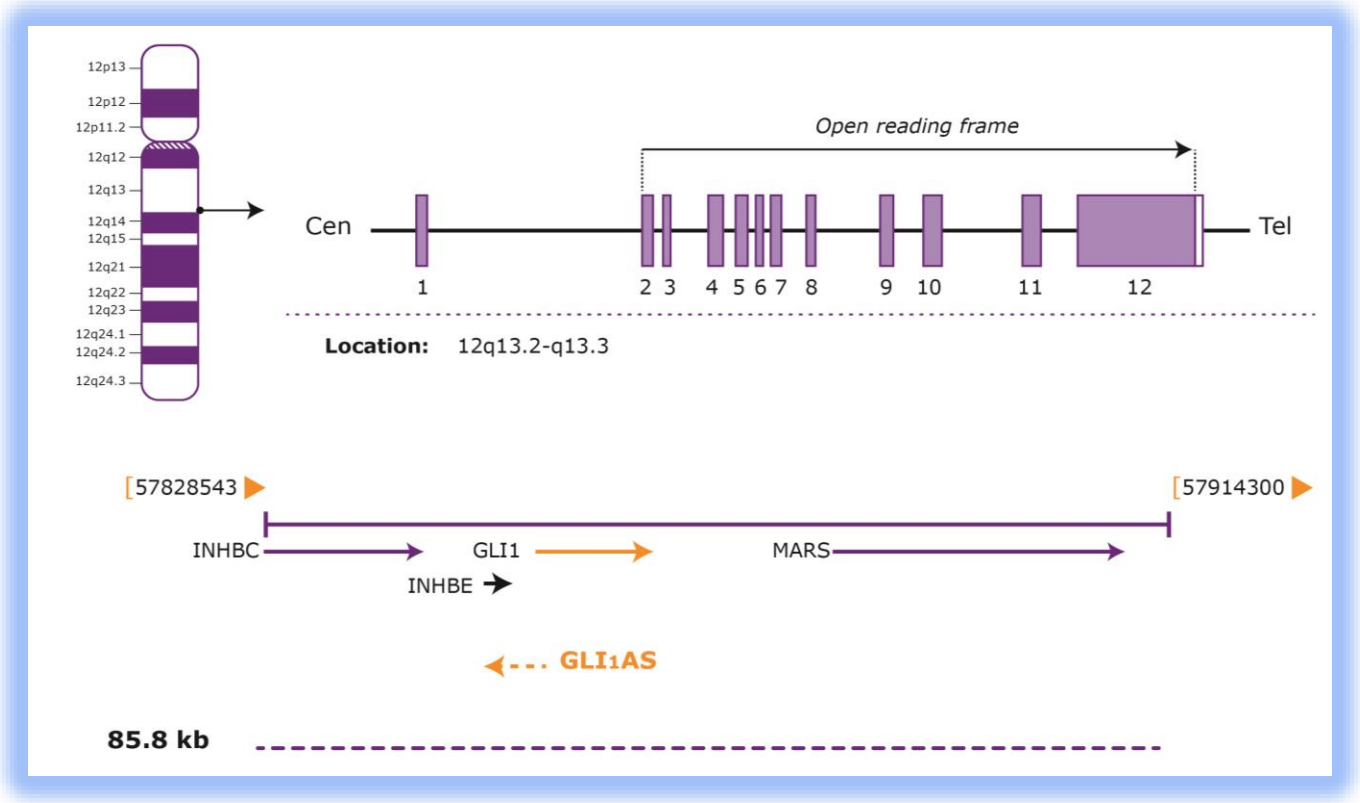

Figure 1. Structure of the *GLI1* gene. This gene is located in chromosome 12q13.2–q13.3, encompasses about 12 kb of genomic sequences and it is composed of 12 exons. Taken and modified from *Atlas of Genetics and Cytogenetics in Oncology and Haematology.*

Hedgehog pathway in cancer

The HH signaling pathway is a major regulator of cell differentiation, tissue polarity, cell proliferation, with crucial roles in embryonic development and tissue repair. Additionally, there is strong evidence that HH signaling has a critical role in tumorigenesis (3,17–19). Deregulation of this pathway through sporadic mutations or other mechanisms, is strongly correlated with various types of cancer (Figure 2). Some examples include: basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, and cancers of the lung, prostate, pancreas, ovarian and breast (5,20–22).

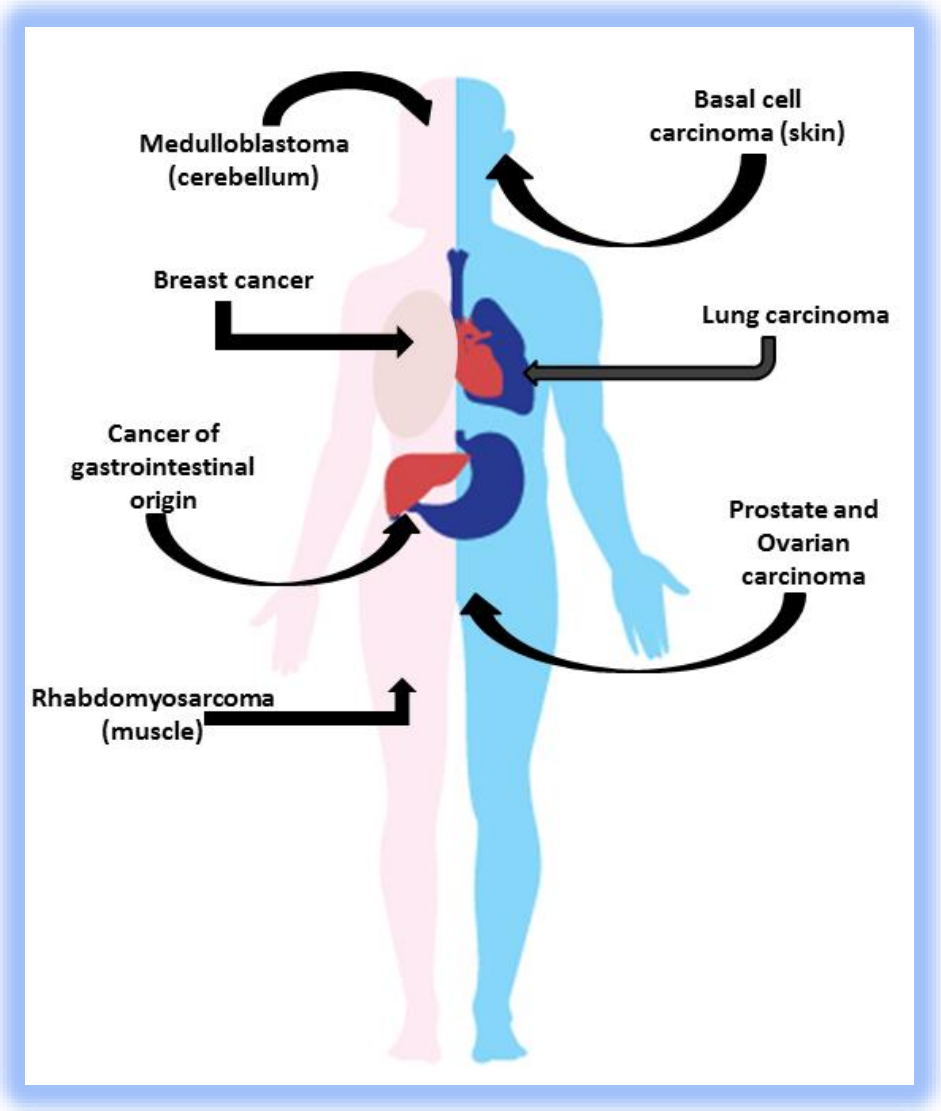

Figure 2. Types of cancer correlated with Hedgehog signaling deregulation. Activation or mutations of this pathway are implicated in skin (basal cell carcinoma), brain (medulloblastoma), rhabdomyosarcoma, gastrointestinal, lung, ovarian, breast and prostate cancers.

Hedgehog signal transduction

The signaling cascade initiates at the transmembrane receptor Patched (PTCH), which responds to the HH ligands. This transmembrane receptor also acts as a repressor of the pathway. Binding of HH ligands to PTCH causes a change in its inhibitory effects on the downstream effector Smoothened, another membrane-associated protein (19,21,23). Release of SMO from PTCH repression allows the initiation of a series of intracellular events that culminate in the activation of the GLI family of zinc finger transcription factors, which are GLI1, GLI2, and GLI3 (24). The GLI proteins can function as both

activators and repressors, with GLI2 and GLI3 indeed possessing repressor and activator domains, however, GLI1 acts only as a transcriptional activator of the HH pathway since it does not contain a repressor domain. *GLI1* is an oncogene and its increased expression is associated with many cancers, including glioma and basal cell carcinoma (21).

Structural organization of the Hedgehog pathway

The HH ligands are secreted hydrophobic proteins. Three homologs have been identified in vertebrates, Sonic HH (SHH), Indian HH (IHH) and Desert HH (DHH). The functional differences among these ligands appear to relate to their tissue specificity and their level of expression. These ligands can act in autocrine or paracrine mechanisms and responsive cells can be localized either near or at a distance from the secreting cells (25–27). PTCH1 is a twelve pass transmembrane receptor that indirectly inhibits SMO, a seven pass transmembrane G-protein-coupled-like receptor located on the membrane of intracellular endosomes. In addition to PTCH1, mammals have another HH receptor, PTCH2. All three mammalian HH ligands can bind to both receptors with similar affinity. Suppressor of fused (SUFU) is an additional negative regulator of HH signaling; it binds to all three GLI proteins and may control their processing and/or degradation (28,29). The GLI proteins are zinc finger transcription factors that mediate the transcriptional responses of HH signaling. Generally, in the absence of HH ligands, GLI1 is transcriptionally repressed, whereas GLI3 and possibly GLI2 are proteolytically processed to truncated repressor forms (30–32). HH-interacting protein (HIP) is another negative regulator of the pathway and encodes a membrane glycoprotein that binds to all three HH ligands with an affinity comparable to that of PTCH1. HIP attenuates HH signaling as a result of this ligand binding (33).

Hedgehog targets genes

The HH signaling biological response varies according to the cell type, and includes a vast array of cell-specific transcription factors that mediate different developmental fate responses. Genes generally induced by HH activity include *PTCH1*, *PTCH2*, *HIP* and *GLI1*, which can trigger positive or negative feedbacks on this pathway and modify the strength or duration of the HH signal. Additional HH signaling targets include genes contributing to the regulation of proliferation and differentiation, e.g. *CCND1, CCND2, N-MYC, WNT, PDGFRA, IGF2, FOXM1* and *HES1* (20,34).

GLI1 and its variants

Two splice variants of GLI1 have been identified, GLI1DeltaN and tGLI1 (Figure 3). Similar to wild-type GLI1, these variants are up-regulated by HH signaling activation. GLI1DeltaN has a deletion of 128 amino acids from the N-terminus (35), and tGLI1 a deletion of 41 amino acids, which includes the entire exon 3 and part of exon 4 (36). GLI1DeltaN acts similarly to wild-type GLI1, with a certain cell type specificity, in both normal and cancer

cells. However, tGLI1 is reported to be expressed only in tumor tissues and promotes more aggressive cancer phenotypes (37,38).

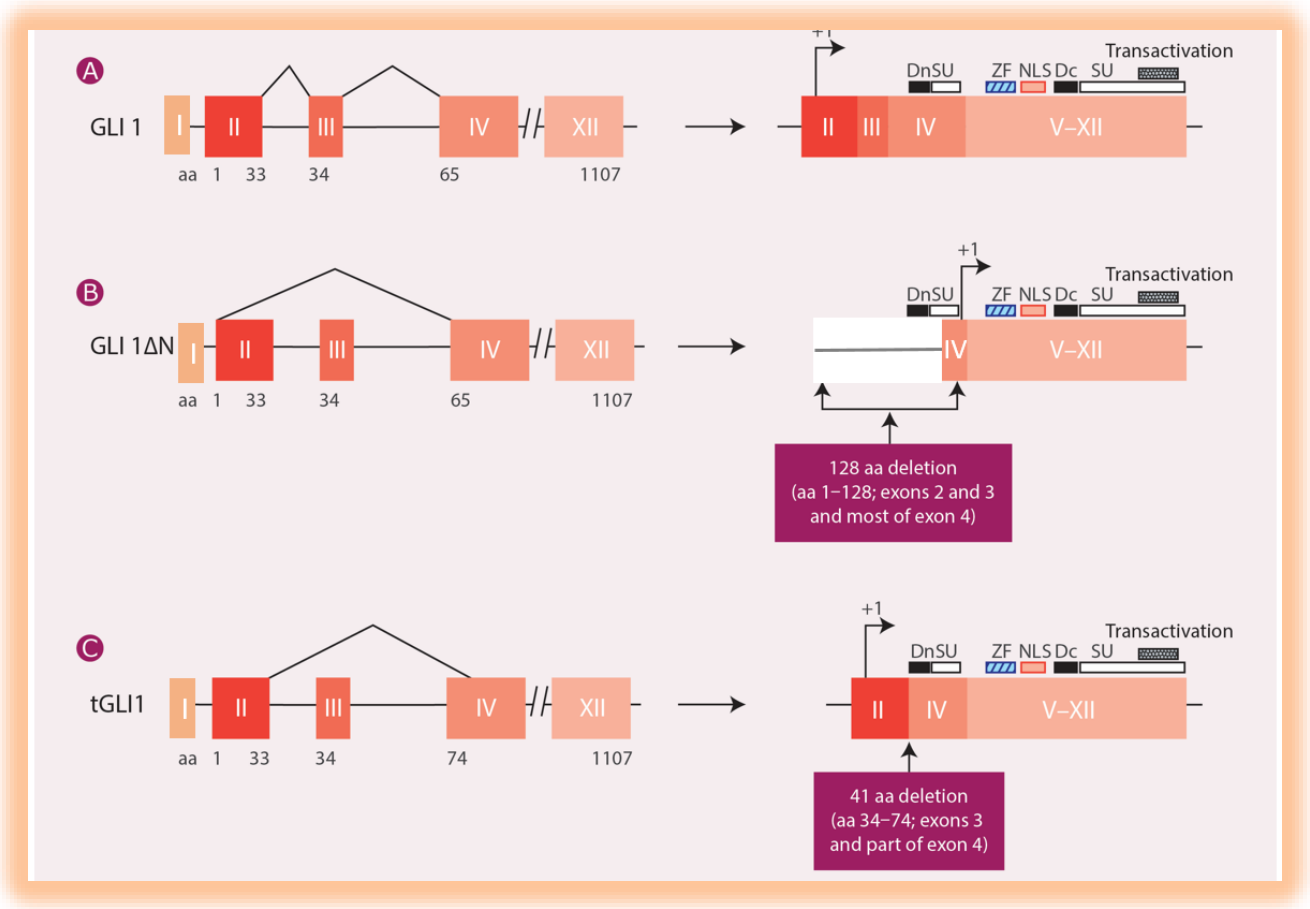

Figure 3. Structure of the human *GLI1* gene and its two splice variants. Full-length GLI1 GLI1DeltaN and tGLI1. Taken and modified from *Carpenter RL & Lo H-W 2012* with permission. Exons are indicated as color boxes while introns are shown by lines. ***GLI1***: glioma-associated oncogene homolog 1 full-length. **GLI1ΔN**: splice variant of GLI1 that has 128 amino acids deleted from the N-terminus as a result of splicing exon 1 directly to exon 4. **tGLI1**: is a product of alternative splicing that lacks 41 amino acids corresponding to the entire exon 3 and part of exon 4 of the GLI1 gene. **Dn** and **Dc**: degron degradation signals. **SU**: SUFU-binding domains. **ZF**: zinc finger domains. **NLS**: the nuclear localization signal. **Transactivation** domain.

GENERAL AIM

The general goal of this thesis work is to delineate regulatory mechanisms through which the HH signaling pathway can operate during carcinogenesis, with special emphasis on the human *GLI1* oncogene, the terminal effector of this signal transduction cascade.

REFERENCES

1. Chen JK. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. Genes Dev [Internet]. 2002 Nov 1; 16 (21):2743–8. Available from: http://www.genesdev.org/cgi/doi/10.1101/gad.1025302

2. Tostar U, Toftgard R, Zaphiropoulos PG, Shimokawa T. Reduction of Human Embryonal Rhabdomyosarcoma Tumor Growth by Inhibition of the Hedgehog Signaling Pathway. Genes Cancer [Internet]. 2010 Sep 1; 1 (9):941–51. Available from: http://gan.sagepub.com/lookup/doi/10.1177/1947601910385449

3. Hanna A, Shevde LA. Hedgehog signaling: modulation of cancer properies and tumor mircroenvironment. Mol Cancer [Internet]. 2016 Dec 18; 15(1):24. Available from: http://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-016-0509-3

4. Gupta S, Takebe N, LoRusso P. Review: Targeting the Hedgehog pathway in cancer. Ther Adv Med Oncol [Internet]. 2010 Jul 1; 2 (4):237–50. Available from: http://tam.sagepub.com/cgi/doi/10.1177/1758834010366430

5. Rubin LL, de Sauvage FJ. Targeting the Hedgehog pathway in cancer. Nat Rev Drug Discov [Internet]. 2006 Dec; 5(12):1026–33. Available from: http://www.nature.com/doifinder/10.1038/nrd2086

6. Lum L. The Hedgehog Response Network: Sensors, Switches, and Routers. Science (80-) [Internet]. 2004 Jun 18; 304 (5678):1755–9. Available from: http://www.sciencemag.org/cgi/doi/10.1126/science.1098020

7. National Cancer Institute USA Gov. web page [Internet]. 2016; (USA). Available from: http://www.cancer.gov/about-cancer

8. Cancer Research UK. web page [Internet]. 2016; (UK). Available from: http://www.cancerresearchuk.org/about-cancer/what-is-cancer/how-cancer-starts

9. Hanahan D, Weinberg RA. The Hallmarks of Cancer. Cell [Internet]. 2000 Jan; 100 (1):57–70. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0092867400816839

10. Hanahan D, Weinberg RA. Hallmarks of Cancer: The Next Generation. Cell [Internet]. 2011 Mar; 144 (5):646–74. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0092867411001279

11. Chial H. Tumor suppressor genes and the two-hit hypothesis. Nature Education. 2008; 1:177. Available from: http://www.nature.com/scitable

12. Morris LGT, Chan TA. Therapeutic targeting of tumor suppressor genes. Cancer [Internet]. 2015 May 1; 121(9):1357–68. Available from: http://doi.wiley.com/10.1002/cncr.29140

13. Kinzler KW, Ruppert JM, Bigner SH, Vogelstein B. The GLI gene is a member of the Kruppel family of zinc finger proteins. Nature [Internet]. 1988 Mar 24; 332(6162):371–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2832761

14. Kinzler KW, Bigner SH, Bigner DD, Trent JM, Law ML, O'Brien SJ, et al. Identification of an amplified, highly expressed gene in a human glioma. Science [Internet]. 1987 Apr 3; 236(4797):70–3. Available from: http://www.ncbi.nlm.nih.gov/pubmed/3563490

15. Genetics A of. Atlas of Genetics and Cytogenetics in Oncology and Haematology. web [Internet]. 2005; Available from: http://atlasgeneticsoncology.org/Genes/GLIID310ch12q13.html

16. Aberger F, Ruiz i Altaba A. Context-dependent signal integration by the GLI code: The oncogenic load, pathways, modifiers and implications for cancer therapy. Semin Cell Dev Biol [Internet]. 2014 Sep; 33:93–104. Available from: http://linkinghub.elsevier.com/retrieve/pii/S108495211400130X

17. Beachy PA, Karhadkar SS, Berman DM. Tissue repair and stem cell renewal in carcinogenesis. Nature [Internet]. 2004 Nov 18; 432(7015):324–31. Available from: http://www.nature.com/doifinder/10.1038/nature03100

18. Hooper JE, Scott MP. Communicating with Hedgehogs. Nat Rev Mol Cell Biol [Internet]. 2005 Apr; 6(4):306–17. Available from: http://www.nature.com/doifinder/10.1038/nrm1622

19. Ingham PW. Hedgehog signaling in animal development: paradigms and principles. Genes Dev [Internet]. 2001 Dec 1; 15(23):3059–87. Available from: http://www.genesdev.org/cgi/doi/10.1101/gad.938601

20. Stecca B, Ruiz i Altaba A. Context-dependent Regulation of the GLI Code in Cancer by HEDGEHOG and Non-HEDGEHOG Signals. J Mol Cell Biol [Internet]. 2010 Apr 1; 2(2):84–95. Available from: http://jmcb.oxfordjournals.org/cgi/doi/10.1093/jmcb/mjp052

21. Teglund S, Toftgård R. Hedgehog beyond medulloblastoma and basal cell carcinoma. Biochim Biophys Acta - Rev Cancer [Internet]. 2010 Apr; 1805(2):181–208. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0304419X10000065

22. Ruiz i Altaba A. Hedgehog Signaling and the Gli Code in Stem Cells, Cancer, and Metastases. Sci Signal [Internet]. 2011 Nov 22; 4(200):pt9–pt9. Available from: http://stke.sciencemag.org/cgi/doi/10.1126/scisignal.2002540

23. Merchant JL. Hedgehog signalling in gut development, physiology and cancer. J Physiol [Internet]. 2012 Feb 1; 590(3):421–32. Available from: http://doi.wiley.com/10.1113/jphysiol.2011.220681

24. Carpenter D, Stone DM, Brush J, Ryan A, Armanini M, Frantz G, et al. Characterization of two patched receptors for the vertebrate hedgehog protein family. Proc Natl Acad Sci U S A [Internet]. 1998 Nov 10; 95(23):13630–4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/9811851

25. Tukachinsky H, Kuzmickas RP, Jao CY, Liu J, Salic A. Dispatched and Scube Mediate the Efficient Secretion of the Cholesterol-Modified Hedgehog Ligand. Cell Rep [Internet]. 2012 Aug;2(2):308–20. Available from: http://linkinghub.elsevier.com/retrieve/pii/S2211124712002203

26. Scales SJ, de Sauvage FJ. Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. Trends Pharmacol Sci [Internet]. 2009 Jun; 30(6):303–12. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0165614709000698

27. Varjosalo M, Taipale J. Hedgehog: functions and mechanisms. Genes Dev [Internet]. 2008 Sep 15; 22(18):2454–72. Available from: http://genesdev.cshlp.org/cgi/doi/10.1101/gad.1693608

28. Zaphiropoulos PG, Undén AB, Rahnama F, Hollingsworth RE, Toftgård R. PTCH2, a novel human patched gene, undergoing alternative splicing and up-regulated in basal cell carcinomas. Cancer Res [Internet]. 1999 Feb 15; 59(4):787–92. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10029063

29. Shimokawa T, Rahnama F, Zaphiropoulos PG. A novel first exon of the Patched1 gene is upregulated by Hedgehog signaling resulting in a protein with pathway inhibitory functions. FEBS Lett [Internet]. 2004 Dec 3; 578(1-2):157–62. Available from: http://doi.wiley.com/10.1016/j.febslet.2004.11.006

30. Pan Y, Bai CB, Joyner AL, Wang B. Sonic hedgehog Signaling Regulates Gli2 Transcriptional Activity by Suppressing Its Processing and Degradation. Mol Cell Biol [Internet]. 2006 May 1; 26(9):3365–77. Available from: http://mcb.asm.org/cgi/doi/10.1128/MCB.26.9.3365-3377.2006

31. Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell [Internet]. 2000 Feb 18; 100(4):423–34. Available from: http://www.ncbi.nlm.nih.gov/pubmed/10693759

32. Gerhardt C, Leu T, Lier JM, Rüther U. The cilia-regulated proteasome and its role in the development of ciliopathies and cancer. Cilia [Internet]. 2016 Dec 10; 5(1):14. Available from: http://www.ciliajournal.com/content/5/1/14

33. Beachy PA, Hymowitz SG, Lazarus RA, Leahy DJ, Siebold C. Interactions between Hedgehog proteins and their binding partners come into view. Genes Dev [Internet]. 2010 Sep 15; 24(18):2001–12. Available from: http://genesdev.cshlp.org/cgi/doi/10.1101/gad.1951710

34. Katoh Y, Katoh M. Hedgehog target genes: mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. Curr Mol Med [Internet]. 2009 Sep; 9(7):873–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19860666

35. Shimokawa T, Tostar U, Lauth M, Palaniswamy R, Kasper M, Toftgard R, et al. Novel Human Glioma-associated Oncogene 1 (GLI1) Splice Variants Reveal Distinct Mechanisms in the Terminal Transduction of the Hedgehog Signal. J Biol Chem [Internet]. 2008 May 23; 283 (21):14345–54. Available from: http://www.jbc.org/cgi/doi/10.1074/jbc.M800299200

36. Lo H-W, Zhu H, Cao X, Aldrich A, Ali-Osman F. A Novel Splice Variant of GLI1 That Promotes Glioblastoma Cell Migration and Invasion. Cancer Res [Internet]. 2009 Sep 1; 69(17):6790–8. Available from: http://cancerres.aacrjournals.org/cgi/doi/10.1158/0008-5472.CAN-09-0886

37. Carpenter RL, Lo H-W. Identification, Functional Characterization, and Pathobiological Significance of GLI1 Isoforms in Human Cancers. In 2012. p. 115–40. Available from: http://linkinghub.elsevier.com/retrieve/pii/B9780123946225000067

38. Cao X, Geradts J, Dewhirst MW, Lo H-W. Upregulation of VEGF-A and CD24 gene expression by the tGLI1 transcription factor contributes to the aggressive behavior of breast cancer cells. Oncogene [Internet]. 2012 Jan 5; 31(1):104–15. Available from: http://www.nature.com/doifinder/10.1038/onc.2011.219

CHAPTER I

THE EFFECTOR OF HEDGEHOG SIGNALING, THE TRANSCRIPTION FACTOR GLI1, IS REGULATED BY A NON-CODING ANTISENSE RNA

1.1 INTRODUCTION

Antisense transcripts are a prominent and complex class of RNAs that are transcribed from the opposite DNA strand of a protein-coding gene and overlap, in part, with the corresponding mRNA. Antisense RNAs can encode proteins, but more often represent non-coding transcripts. There is growing evidence supporting the functional role of non-coding RNAs; this is exemplified by the HOTAIR RNA, which has no protein-coding potential, yet promotes breast cancer invasiveness and metastasis (1).

The marker of HH signaling activation, the transcription factor GLI1, is apparently controlled by an antisense transcript, which highlights additional regulatory mechanisms that can modulate the expression of this key oncogenic player. In this context, the finding of the GLI1 antisense (GLI1AS) RNA mediated regulation of the human *GLI1* oncogene opens up new perspectives on the already sophisticated control mechanisms of GLI1 expression and its implications in carcinogenesis. These observations pinpoint on the role of RNA-based regulatory mechanisms in orchestrating the expression of a gene and fine-tuning its expression with respect to external signals. Additionally, these research efforts provide evidence for the contribution of the RNA transcriptome as a regulatory component of protein expression in the human genome. Consequently, a better understanding of the role of non-coding RNA transcripts as "controlling elements" for gene expression, with possible implications for organismal complexity and diversity, is an outcome of our data.

The research presented in this chapter is focused on Hedgehog signaling, a major pathway implicated in many human cancers, including rhabdomyosarcoma, medulloblastoma and breast cancer. The results highlight the identification of a non-coding RNA that is antisense to the *GLI1* gene, which appears to regulate the expression levels of the GLI1 mRNA.

1.2 AIMS OF THE RESEARCH

Characterize GLI1AS, the antisense transcript to the human *GLI1* gene, and delineate its possible impact on the expression of the *GLI1* gene.

- ✓ Determine the expression of GLI1AS in different rhabdomyosarcoma cell lines and clinical samples.
- ✓ Characterize the GLI1AS transcript.
- ✓ Determine whether the expression of GLI1 and GLI1AS are correlated with each other and delineate a possible mechanism of interaction between the transcripts.
- ✓ Evaluate the biological significance of the GLI1AS transcript in rhabdomyosarcoma cells, taking also advantage of the CAM tumor assay.

1.3 MATERIALS AND METHODS

The materials and methods employed in the study carried out are described in the research paper (enclosed 1.8). The aim of this section is to detail specific methodological issues and to give more descriptive information of some techniques.

Human rhabdomyosarcoma cell lines

Rhabdomyosarcoma (RMS) is a relatively rare form of cancer (soft tissue sarcoma) most common in children and represents approximately 3% of all childhood cancers. Based on histological criteria RMS is divided into two main classes, embryonal rhabdomyosarcoma (ERMS), which is found in about 60% of cases, and alveolar rhabdomyosarcoma (ARMS) found in about 20% of cases, and the remaining percentage for other types of rhabdomyosarcoma less frequent. At the time of diagnosis, rhabdomyosarcoma is presented as a disseminated disease in 20% of the patients (2). Some studies have reported that human rhabdomyosarcoma cell lines and biopsy specimens overexpress *SHH, DHH, IHH, PTCH1, SMO, GLI1, GLI2 and GLI3* (3-5). This overexpression of HH pathway components was the main criterion for choosing RMS cell lines as a study model.

Alveolar rhabdomyosarcoma *RMS13* cell line was purchased from ATCC (Manassas, VA). The cell line was established from bone marrow cells of a child with rhabdomyosarcoma (6).

Embryonal rhabdomyosarcoma cells lines. *Rh36,* derived from a paratesticular relapse in a 15-year-old male (7) was a kind gift from P. Houghton (St. Jude Children's Research Hospital, Memphis, TN). *CCA* was a kind gift from P.L. Lollini (University of Bologna, Italy) This cell line was derived from the biopsy of a "vesical" recurrence of embryonal RMS in an 8-year-old Caucasian male (6,8). The *RD* cell line was directly derived from biopsy specimens of a 7-year-old female with a pelvic RMS (9), and was purchased from ATCC (Manassas, VA).

Chick chorioallantoic membrane (CAM) assay

The chick chorioallantoic membrane (CAM) is naturally immunodeficient and highly vascularized, making it an ideal system for the study of tumor growth (Figure 1). Additionally, the CAM assay is a facile technique, inexpensive and can be easily handled (10).The chorioallantoic membrane is an extra-embryonic organ formed at day 4-5 of development by the fusion of the allantois and the chorion. Then, at 7-8 days, the chorioallantoic membrane expands beneath the egg shell and eventually envelops the entire embryo. Between day 7 and the stage of hatching at day 20, the chorioallantoic membrane serves as the respiratory organ of the embryo. The highly vascularized nature of the CAM promotes the formation of solid, human-like tumors (11,12).

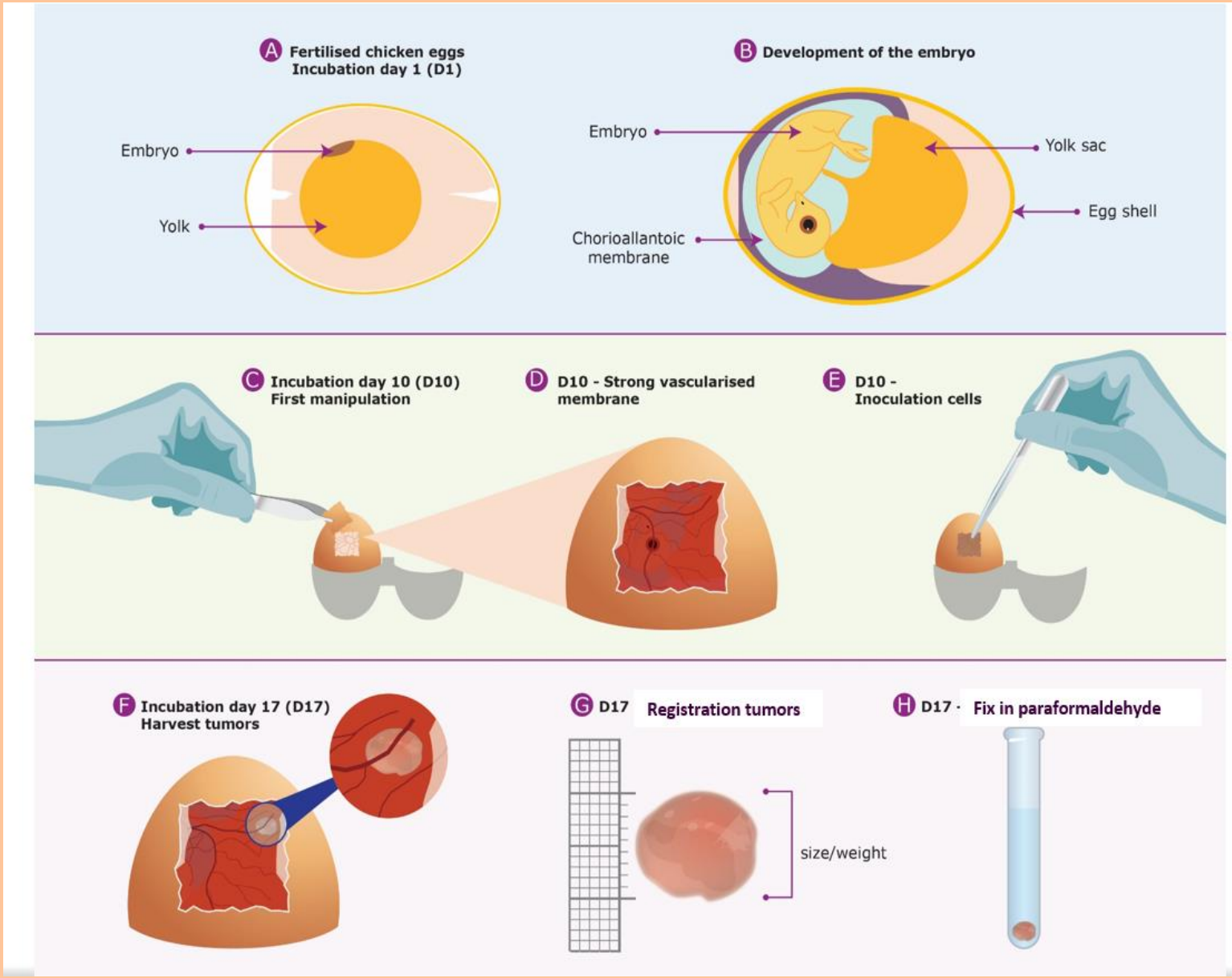
A Fertilised chicken eggs
Incubation day 1 (D1)
Embryo
Yolk
B Development of the embryo
Embryo
Yolk sac
Egg shell
Chorioallantoic membrane
C Incubation day 10 (D10)
First manipulation
D D10 - Strong vascularised membrane
E D10 -
Inoculation cells
F Incubation day 17 (D17)
Harvest tumors
G D17
Registration tumors
size/weight
H D17 -
Fix in paraformaldehyde

Figure 1. **Overview of the CAM tumor assay.** Procedure: **A.** Day 1-fertilized chicken eggs were placed into a humidified incubator at 37 °C without CO2. **B&D.** The embryo develops accompanied by an increase in vascularity. **C.** Day 10- first manipulation. A small hole was opened and **E.** transfected cells were applied on top to the CAM. The eggs were sealed and returned to the incubator for an additional 7 days. **F.** On day 17, the tumors were collected, **G.** weighed and measured. **H.** All tumors were fixed in 4% paraformaldehyde, embedded in paraffin and processed for sectioning. Additionally, the tumors were stained with hematoxylin and eosin and further analyzed by standard light microscopy in order to ensure the presence of tumor tissue.

1.4 RESULTS AND DISCUSSION

In this study, an RNA transcript from the antisense strand of the *GLI1* gene, termed GLI1AS, with no potential to code for a long protein, was characterized. This appears to act as a negative regulator of GLI1 expression using different experimental approaches, which include a combination of *in vitro* and *in vivo* assays.

By *in silico* analysis of EST databases a transcript, GLI1AS, flanking the *GLI1* promoter, antisense of *GLI1* gene in a *head to head* orientation was identified. The use of Rapid Amplification of cDNA Ends (RACE) in *Rh36*, *CCA* and *RMS13* cancer cells determined the sequence of GLI1AS RNA, which is composed of 885-nucleotides, three exons, is 5´ capped and 3´ poly (A) tailed, but without any long open reading frame. Polyadenylation of GLI1AS was also confirmed by cDNA synthesis in the presence or absence of oligo (dT) primers in RMS13 cells.

Expression analysis of GLI1AS in *PC3, 22Rv1 PANC1, A549, AGS, D283Med, RMS13, RD, Rh36*, and *CCA* cancer cell lines show co-regulation between GLI1 and GLI1AS. Additionally, it was found that the expression of GLI1AS was lower compared to GLI1.

In order to find out possible biological functions of GLI1AS, its subcellular localization was determined using nuclear/cytoplasmic fractionation of rhabdomyosarcoma cell lines, followed by RNA isolation and real-time RT/PCR. These results revealed that the unspliced GLI1AS RNAs are preferentially retained in the nucleus, while the spliced GLI1AS RNAs are transported to the cytoplasm. GLI1AS expression was also evaluated in a panel of basal cell carcinomas (BCCs), a tumor type characterized by increased GLI1 levels. It was found that GLI1AS levels were lower than GLI1 and more pronounced in the nine BCCs compared to the eight normal skin samples analyzed, showing an apparent co-regulation with GLI1 expression. Moreover, the levels of GLI1 and GLI1AS were analyzed in breast cancer with similar results as previously seen in BCC tumor samples and cancer cell lines. It is of interest that GLI1 mRNA expression levels were higher than GLI1AS across all samples examined, consistent with the results reported for most antisense transcripts with regulatory roles on the corresponding sense gene.

In order to investigate whether the GLI1AS transcript could regulate GLI1 expression, small interfering RNA (siRNA) knockdown assays were performed in *Rh36* and *CCA* cells. Knockdown of GLI1AS in these cells resulted in a significant increase in GLI1 expression whereas siRNAs targeting of GLI1 resulted in a decrease of GLI1AS levels. Consistent with these results it was found that GLI1AS depletion increased cell proliferation, while depletion of the GLI1 decreased proliferation (it should be emphasized that in different malignancies the pro-tumoral function of GLI1 is associated with its increased expression). To examine whether the endogenous modulation of GLI1 and GLI1AS levels in *Rh36* cells had an impact on tumor growth, the CAM xenograft model was used. GLI1 knockdown

treatment of *Rh36* cells with the siRNAs decreased their capacity to form tumors in this model, on the other hand an increased tumor weight was observed following knockdown GLI1AS.

To determine the impact of increased levels of the GLI1AS RNA on GLI1, expression constructs of full-length GLI1AS, a 5´ segment of GLI1AS and a 3´ segment of GLI1AS in the pCMV5 vector were generated. The 5´ and the 3´ GLI1AS constructs did not elicited major changes in the GLI1 mRNA levels but the full-length GLI1AS construct conferred an almost 10-fold reduction of the GLI1 mRNA. Additionally, the full-length but not the 5´ or the 3´ constructs decreased the expression of the GLI1 protein and down-regulated the well-established GLI1 target genes, *PTCH1* and *PTCH2*. These findings suggest that the complete GLI1AS RNA sequence/structure is needed to elicit regulatory effects on GLI1. Additionally, GLI1AS overexpression conferred a major reduction in *Rh36* cellular proliferation.

To examine whether the repressive effects of GLI1AS on GLI1 expression are gene specific, the mRNA levels of *INHBE*, a gene positioned tail-to-tail to GLI1AS and of an unrelated gene on another chromosome, *ADAR2*, were also analyzed. ADAR2 levels were not changed by GLI1AS overexpression, however, *INHBE* expression was reduced, albeit not to the same extent as to that seen for GLI1. This finding rules out the possibility that *INHBE* is a GLI1 target gene.

In order to address the role of epigenetic modifications in eliciting the GLI1 and INHBE down-regulation by GLI1AS overexpression, chromatin immunoprecipitation assays on transfected *Rh36* cells were performed. Overexpression of GLI1AS increased the recruitment of repression marker H3k27me3 in the *INHIBE/GLI1AS/GLI1* genomic region as well as elicited a reduction in RNA polymerase II recruitment at the GLI1 promoter region. These results suggest that GLI1AS acts as an epigenetic modifier that represses gene expression at its locus.

1.5 CONCLUSIONS

The function of most non-coding antisense transcripts remains unknown. A certain proportion of these may constitute transcriptional noise; however, there is a growing number of examples with a regulatory impact that is physiologically significant. Contributing to increased knowledge on the function of these transcripts, a non-coding RNA located *head-to-head* with the gene encoding the *GLI1*, a transcriptional effector of multiple cancer-associated signaling pathways was identified in this study. This non-coding RNA, GLI1AS, elicits negative feedback on GLI1 expression via local chromatin

remodeling (Figure 2), with concomitant effects on cellular proliferation, providing therefore evidence for a novel non-coding antisense RNA with biological relevance.

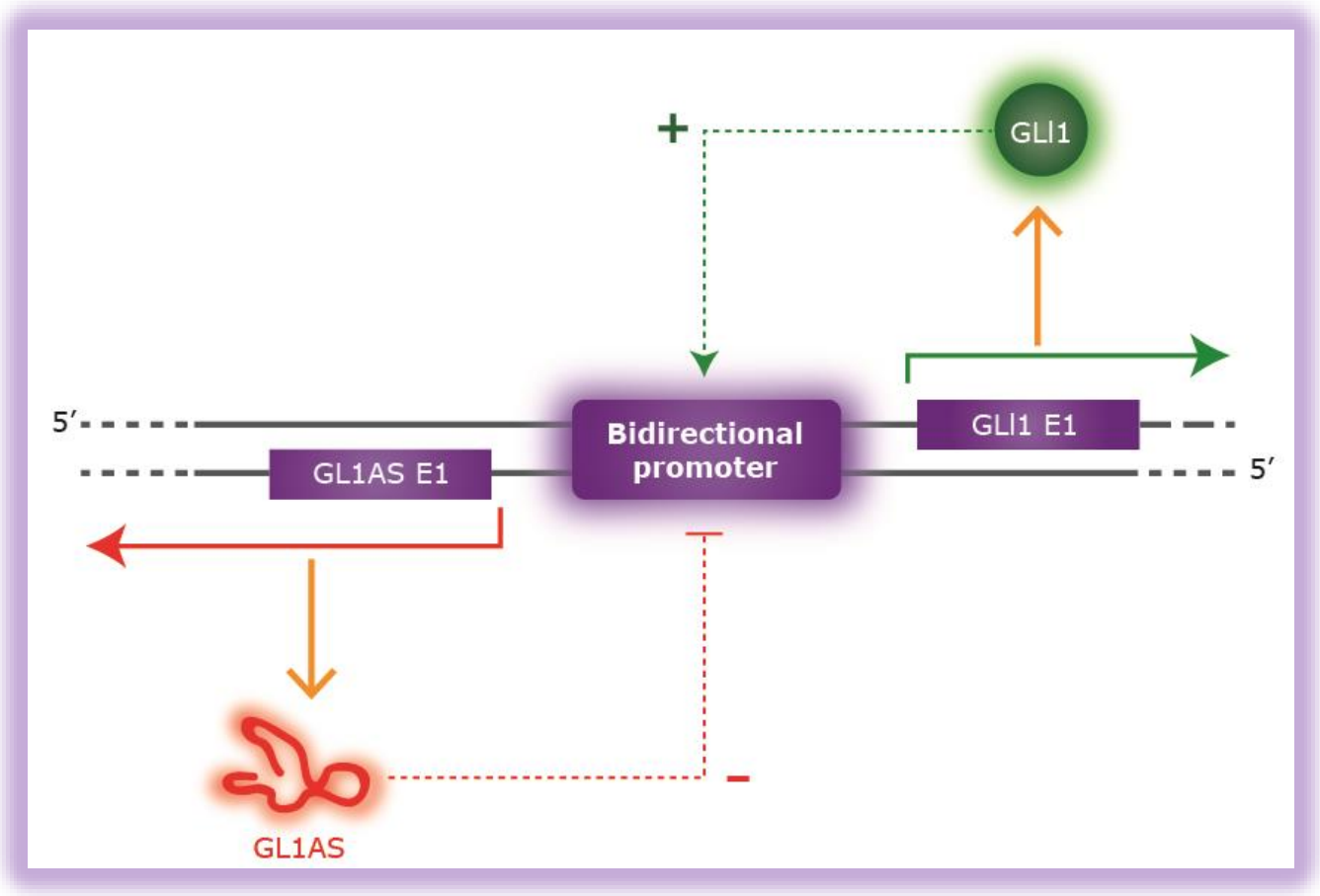

Figure 2. Proposed mechanism for the interplay of the GLI1AS and GLI1 regulatory effects. A non-coding RNA, originating from the antisense strand of the human *GLI1* gene (GLI1AS), which elicits negative feedback on GLI1 expression via local chromatin remodeling.

1.6 REFERENCES

1. Gupta S, Takebe N, LoRusso P. Review: Targeting the Hedgehog pathway in cancer. Ther Adv Med Oncol [Internet]. 2010 Jul 1; 2(4):237–50. Available from: http://tam.sagepub.com/cgi/doi/10.1177/1758834010366430

2. Ognjanovic S, Linabery AM, Charbonneau B, Ross JA. Trends in childhood rhabdomyosarcoma incidence and survival in the United States, 1975-2005. Cancer [Internet]. 2009 Sep 15; 115(18):4218–26. Available from: http://doi.wiley.com/10.1002/cncr.24465

3. Setoguchi T. Pharmacological inhibition of the Hedgehog pathway prevents human rhabdomyosarcoma cell growth. Int J Oncol [Internet]. 2011 Jun 14; Available from: http://www.spandidos-publications.com/10.3892/ijo.2011.1076

4. Oue T, Yoneda A, Uehara S, Yamanaka H, Fukuzawa M. Increased expression of the hedgehog signaling pathway in pediatric solid malignancies. J Pediatr Surg [Internet]. 2010 Feb; 45(2):387–92. Available from: http://linkinghub.elsevier.com/retrieve/pii/S002234680900880X

5. Zibat A, Missiaglia E, Rosenberger A, Pritchard-Jones K, Shipley J, Hahn H, et al. Activation of the hedgehog pathway confers a poor prognosis in embryonal and fusion gene-negative alveolar rhabdomyosarcoma. Oncogene [Internet]. 2010 Dec 2; 29(48):6323–30. Available from: http://www.nature.com/doifinder/10.1038/onc.2010.368

6. Hinson ARP, Jones R, Crose LES, Belyea BC, Barr FG, Linardic CM. Human Rhabdomyosarcoma Cell Lines for Rhabdomyosarcoma Research: Utility and Pitfalls. Front Oncol [Internet]. 2013;3. Available from: http://journal.frontiersin.org/article/10.3389/fonc.2013.00183/abstract

7. Houghton PJ, Morton CL, Tucker C, Payne D, Favours E, Cole C, et al. The pediatric preclinical testing program: Description of models and early testing results. Pediatr Blood Cancer [Internet]. 2007 Dec;49(7):928–40. Available from: http://doi.wiley.com/10.1002/pbc.21078

8. De Giovanni C, Nanni P, Nicoletti G, Ceccarelli C, Scotlandi K, Landuzzi L, et al. Metastatic ability and differentiative properties of a new cell line of human embryonal rhabdomyosarcoma (CCA). Anticancer Res [Internet]. 9(6):1943–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/2516717

9. McAllister RM, Melnyk J, Finkelstein JZ, Adams EC, Gardner MB. Cultivation in vitro of cells derived from a human rhabdomyosarcoma. Cancer [Internet]. 1969 Sep; 24(3):520–6. Available from: http://www.ncbi.nlm.nih.gov/pubmed/4241949

10. Li M, Pathak RR, Lopez-Rivera E, Friedman SL, Aguirre-Ghiso JA, Sikora AG. The In Ovo Chick Chorioallantoic Membrane (CAM) Assay as an Efficient Xenograft Model of Hepatocellular Carcinoma. J Vis Exp [Internet]. 2015 Oct 9;(104). Available from: http://www.jove.com/video/52411/the-ovo-chick-chorioallantoic-membrane-cam-assay-as-an-efficient

11. Lokman NA, Elder ASF, Ricciardelli C, Oehler MK. Chick Chorioallantoic Membrane (CAM) Assay as an In Vivo Model to Study the Effect of Newly Identified Molecules on Ovarian Cancer Invasion and Metastasis. Int J Mol Sci [Internet]. 2012 Aug 10;13 (12):9959–70. Available from: http://www.mdpi.com/1422-0067/13/8/9959/

12. Tostar U, Toftgard R, Zaphiropoulos PG, Shimokawa T. Reduction of Human Embryonal Rhabdomyosarcoma Tumor Growth by Inhibition of the Hedgehog Signaling Pathway. Genes Cancer [Internet]. 2010 Sep 1;1(9):941–51. Available from: http://gan.sagepub.com/lookup/doi/10.1177/1947601910385449

1.7 REVIEW ARTICLE ABSTRACT

Int. J. Mol. Sci. **2015**, *16*, 3251-3266; doi:10.3390/ijms16023251

OPEN ACCESS
International Journal of
Molecular Sciences
ISSN 1422-0067
www.mdpi.com/journal/ijms

Review

Neighboring Gene Regulation by Antisense Long Non-Coding RNAs

Victoria E. Villegas [1,2,*] and Peter G. Zaphiropoulos [1]

[1] Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge 14183, Sweden; E-Mail: peter.zaphiropoulos@ki.se

[2] Faculty of Natural Sciences and Mathematics & Doctoral Program in Biomedical Sciences, Universidad del Rosario, Bogotá 11001000, Colombia

* Author to whom correspondence should be addressed; E-Mail: victoria.villegas@urosario.edu.co; Tel.: +571-297-02-00 (ext. 4029); Fax: +571-297-02-20.

Academic Editor: Martin Pichler

Received: 17 November 2014 / Accepted: 22 January 2015 / Published: 3 February 2015

Abstract: Antisense transcription, considered until recently as transcriptional noise, is a very common phenomenon in human and eukaryotic transcriptomes, operating in two ways based on whether the antisense RNA acts in *cis* or in *trans*. This process can generate long non-coding RNAs (lncRNAs), one of the most diverse classes of cellular transcripts, which have demonstrated multifunctional roles in fundamental biological processes, including embryonic pluripotency, differentiation and development. Antisense lncRNAs have been shown to control nearly every level of gene regulation—pretranscriptional, transcriptional and posttranscriptional—through DNA–RNA, RNA–RNA or protein–RNA interactions. This review is centered on functional studies of antisense lncRNA-mediated regulation of neighboring gene expression. Specifically, it addresses how these transcripts interact with other biological molecules, nucleic acids and proteins, to regulate gene expression through chromatin remodeling at the pretranscriptional level and modulation of transcriptional and post-transcriptional processes by altering the sense mRNA structure or the cellular compartmental distribution, either in the nucleus or the cytoplasm.

Keywords: regulatory RNA; antisense transcription; long non-coding RNAs; gene regulation

1.8 RESEARCH ARTICLE ABSTRACT

MOLECULAR ONCOLOGY 8 (2014) 912–926

available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/molonc

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

Victoria E. Villegas[a,b,3], Mohammed Ferdous-Ur Rahman[a,3], Maite G. Fernandez-Barrena[c,4], Yumei Diao[a,4], Eleni Liapi[a], Enikö Sonkoly[d], Mona Ståhle[d], Andor Pivarcsi[d], Laura Annaratone[e], Anna Sapino[e], Sandra Ramírez Clavijo[b], Thomas R. Bürglin[a], Takashi Shimokawa[a,1], Saraswathi Ramachandran[a,2], Philipp Kapranov[f], Martin E. Fernandez-Zapico[c], Peter G. Zaphiropoulos[a,*]

[a]Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden
[b]Faculty of Natural Sciences and Mathematics & Doctoral Program in Biomedical Sciences, Universidad del Rosario, Bogotá, Colombia
[c]Schulze Center for Novel Therapeutics, Mayo Clinic, Rochester, MN, USA
[d]Unit of Dermatology and Venereology, Department of Medicine, Karolinska Institutet, Solna, Sweden
[e]Department of Medical Sciences, University of Turin, Turin, Italy
[f]St. Laurent Institute, One Kendall Square, Cambridge, MA, USA

ARTICLE INFO

Article history:
Received 16 November 2013
Received in revised form
18 February 2014
Accepted 11 March 2014
Available online 22 March 2014

Keywords:
Non-coding RNA
Gene expression
Transcription
Chromatin
GLI1
Cancer

ABSTRACT

Non-coding RNAs are a complex class of nucleic acids, with growing evidence supporting regulatory roles in gene expression. Here we identify a non-coding RNA located head-to-head with the gene encoding the Glioma-associated oncogene 1 (GLI1), a transcriptional effector of multiple cancer-associated signaling pathways. The expression of this three-exon GLI1 antisense (GLI1AS) RNA in cancer cells was concordant with GLI1 levels. siRNAs knockdown of GLI1AS up-regulated GLI1 and increased cellular proliferation and tumor growth in a xenograft model system. Conversely, GLI1AS overexpression decreased the levels of GLI1, its target genes *PTCH1* and *PTCH2*, and cellular proliferation. Additionally, we demonstrate that GLI1 knockdown reduced GLI1AS, while GLI1 overexpression increased GLI1AS, supporting the role of *GLI1AS* as a target gene of the GLI1 transcription factor. Activation of TGFβ and Hedgehog signaling, two known regulators of GLI1 expression, conferred a concordant up-regulation of *GLI1* and *GLI1AS* in cancer cells. Finally, analysis of the mechanism underlying the interplay between GLI1 and GLI1AS indicates that the non-coding RNA elicits a local alteration of chromatin structure by increasing the silencing mark H3K27me3 and decreasing the

1.9 SUPPLEMENTARY INFORMATION ABOUT RESEARCH ARTICLE

The supplementary material of the article "*Identification of novel non-coding RNA-based negative feedback regulating the expression of the oncogenic transcription factor GLI1"* is enclosed and can also be found at:

http://dx.doi.org/10.1016/j.molonc.2014.03.009

CHAPTER 2

THE HEDGEHOG/GLI1 SIGNALING PATHWAY AND ITS ROLE IN TAMOXIFEN TREATMENT OF BREAST CANCER CELLS

2.1 -INTRODUCTION

According to the International Agency for Research on Cancer (IARC), GLOBOCAN program, breast cancer is the second most common cancer in the world and, by far, the most frequent among women both in more and less developed regions (1). This disease originates from the epithelial cells of the normal mammary gland and relates to a wide variety of risk factors, including genetic predisposition, exposure to estrogens and amplification of the *HER2* gene. Additionally, it is characterized by high heterogeneity reflected in different cell type compositions and proliferation abilities (2,3). Accordingly, breast cancer patients with the same clinical diagnostics can exhibit variable clinical outcomes and treatment responses, most likely due to the intrinsic characteristics of the cells that form the bulk of the tumor, complicating the predictions of an optimal therapeutic strategy (4-6).

There are several criteria currently used to classify breast cancer, with molecular techniques focusing on gene expression profiles being instrumental in the characterization of breast cancer subtypes that better predict the response to therapy. The main subtypes are based on the expression of estrogen receptor (ER), progesterone receptor (PR), epidermal growth factor receptor 2 (HER2) amplification and the Ki-67 labeling index, a marker of cell proliferation (7,8).

Luminal A. This type includes tumors that are ER positive and PR positive, but negative for HER2 and have a low Ki-67 proliferation index. Luminal A breast cancer has a good prognosis and also benefits from anti-estrogen therapy. *MCF-7, T47D* and *ZR-75-1* breast cancer cells are of this subtype.

Luminal B. This type includes tumors that are ER positive, PR and HER2 positive or negative, with high Ki-67 proliferation index. Luminal B breast cancers are likely to benefit from chemotherapy and may also benefit from hormonal therapy and treatments targeting HER2. An example of this subtype is the *BT-474* breast cancer cell line.

HER2 positive. This type includes tumors that are ER and PR negative, but HER2 positive, with a high Ki-67 proliferation index. This subtype is resistant to endocrine therapy and treatment is focused in targeting HER2. *SKBR-3* and *JIMT-1* breast cancer cells are examples of this subtype.

Basal-like. This type, which is also called triple-negative, includes tumors that are ER, PR and HER2 negative, with a high Ki-67 proliferation index. Basal-like breast cancers are likely to benefit from chemotherapy. The breast cancer cell line *BT-20* belongs to this subtype (7,8).

Breast cancer studies are often using established cell lines in order to achieve a better understanding of the cellular and molecular processes underlying tumorigenesis. Gene expression analysis in these lines can provide evidence regarding the molecular mechanisms leading to cell transformation and allow the clarification of possible modulatory pathways in endocrine response (2). In the case of the current research efforts, this is highlighted by the modulation of components of HH signaling by treatment with TAM.

The information obtained from these cell line studies on the HH signaling / TAM interplay and the impact of the presence or absence of ER/HER2 could contribute to a better understanding of the carcinogenic process and endocrine resistance. Our investigations, coupled with recent evidence on the role of *GLI1* in response to TAM treatment, lead us to consider the expression of this transcription factor as a biomarker on the prognosis and the response to endocrine therapy.

2.2 AIMS OF THE RESEARCH

- ✓ Investigate the effect of TAM on the expression of components of the HH signaling pathway in different breast cancer cell lines.
- ✓ Determine whether TAM treatment modulates the expression pattern of the terminal effector of the pathway, the transcription factor GLI1, depending on the status of ER/HER2 in a panel of breast cancer cell lines.

2.3 MATERIALS AND METHODS

The materials and methods employed in this study are described in the research paper (enclosed 2.8). The aim of this section is to specify the characteristics of the cell lines used in this research.

Cell lines and culture

Description of the cellular characteristics related to the presence of estrogen receptors, ER positive or negative, progesterone receptor, PR positive or negative, HER2 amplification and the Ki-67 labeling index, a marker of cell proliferation of the breast cancer lines used in this study.

Molecular subtypes	Molecular markers	Cell lines
Luminal A	ER positive and PR positive HER2 negative Ki-67 low	*MCF-7* *T47D* *ZR-75-1*
Luminal B	ER positive PR and HER2 positive or negative Ki-67 high	*BT-474*
HER2 positive	ER and PR negative HER2 positive Ki-67 high	*SKBR-3* *JIMT-1*

Table 1. Molecular subtypes of the breast cancer cell lines used in this study (7,8). Breast cancer subtypes are based on the expression of ER, PR, HER2 amplification and Ki-67 labeling index.

2.4 RESULTS AND DISCUSSION

In this study, the impact of TAM administration on breast cancer cell lines was investigated. Specifically, the role of canonical, SMO-/SHH-dependent HH signaling and non-canonical, SMO-/SHH-independent HH signaling was addressed. The experimental data showed that TAM treatment significantly inhibited cell proliferation in *MCF7* cells at 24, 48, and 96 h, while in *T47D* cells, the inhibition of proliferation as not as pronounced, reaching significance only at 24 h and 48 h. In contrast to *MCF7* and *T47D* cells, TAM induced a significant increase in the proliferation of *ZR-75-1* cells at 24 h and 96 h after treatment. Similar results were observed in *BT474* cells, where TAM induced a significant increase in the proliferation after 24 and 96 h of treatment. In the ER-/HER2+ SKBR3 and

JIMT-1 cell lines the TAM effect was variable at different time points. Taken together the data obtained provide evidence that TAM treatment reduced the proliferation of the ER+/HER2− cell lines *MCF7* and *T47D* whereas the *ZR-75-1* and *BT474* cell lines increased their proliferation following TAM administration. Compared to *MCF7* and *T47D* these two cell lines have a more aggressive profile.

Data on the mRNA expression indicated that the most sustained activation of GLI1 was seen with the *ZR-75-1* cells, and the most sustained activation of SMO and SHH with the *BT474* cells. The GLI1 protein expression detected by immunohistochemistry was variable among the different cell lines analyzed. All untreated cell lines, except *T47D* and *SKBR3* cells, were negative for GLI1 expression. The cells that most clearly show a nuclear translocation of GLI1, an event characterizing activation of Hedgehog signaling, were *ZR-75-1* and *BT474*, the same cells that exhibited the most sustained increases in the mRNA expression of HH signaling components. Cell lines that express nuclear GLI1 staining after TAM treatment exhibit an increase in cell proliferation compared to control, GLI1 negative cells. In the SKBR3 cell line, the immunohistochemical data showed an increase in expression of GLI1 following TAM treatment, with this correlating to the mRNA expression only at 24 h. The *JIMT-1* cell line also increased its proliferation by TAM, but at 24 and 48 h.

These findings indicate that the TAM-dependent increase in the proliferation of the ER+ *ZR-75-1* and *BT474* cells is in-line with the increased GLI1 expression in the nucleus. In the ER- *SKBR3* and *JIMT-1* cell lines, the observed pattern is different. Increased proliferation appears to relate with decreased GLI1 expression, as assessed by combining immunohistochemical and mRNA detection. For the ER+ *MCF7* and *T47D* cells, there is no consistent correlation of GLI1 expression and proliferation changes. Worth noting is the lack of detectable SHH expression in *ZR-75-1* and *T47D* cells, with this observation implicating non-canonical HH signaling in these two cellular settings.

2.5 CONCLUSIONS

Our conclusion based on the experimental results suggests that *GLI1* could be a new prognostic marker in breast cancer, thereby supporting the use of combined therapies involving HH pathway inhibitors and endocrine treatment. It is important to note that the suggested therapeutic inhibitors should be at the level of GLI1 factors, as these would block pathway activity regardless of whether this was elicited by canonical or non-canonical signaling.

2.6 REFERENCES

1. GLOBOCAN. Lyon, France: International Agency for Research on Cancer. 2013; Available from: Available from: http://globocan.iarc.fr, accessed on day/month/year

2. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. Breast Cancer Res [Internet]. 2011;13 (4):215. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3236329&tool=pmcentrez&rendertype=abstract

3. Rivenbark AG, O'Connor SM, Coleman WB. Molecular and cellular heterogeneity in breast cancer: Challenges for personalized medicine. Am J Pathol [Internet]. 2013;183(4):1113–24. Available from: http://dx.doi.org/10.1016/j.ajpath.2013.08.002

4. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AAM, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature [Internet]. 2002;415(6871):530–6. http://www.ncbi.nlm.nih.gov/pubmed/11823860\nhttp://www.nature.com/doifinder/10.1038/415530a

5. Sacco K, Grech G. Actionable pharmacogenetic markers for prediction and prognosis in breast cancer. EPMA J [Internet]. 2015;6(1):15. Available from: http://www.epmajournal.com/content/6/1/15

6. Eroles P, Bosch A, Alejandro P??rez-Fidalgo J, Lluch A. Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. Cancer Treat Rev [Internet]. 2012;38(6):698–707. Available from: http://dx.doi.org/10.1016/j.ctrv.2011.11.005

7. Kittaneh M, Montero AJ. Profiling for Breast Cancer : A Comprehensive Review. Biomark Cancer. 2013;5:61–70.

8. Liu Z, Zhang X-S, Zhang S. Breast tumor subgroups reveal diverse clinical prognostic power. Sci Rep [Internet]. 2014;4:4002. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24499868

2.7 REVIEW-MANUSCRIPT

Review

Tamoxifen resistance: emerging molecular targets

Milena Rondón-Lagos[1*†], Victoria Eugenia Villegas[2,3*†], Nelson Rangel[1,2,3], Magda Carolina Sánchez[2] and Peter G. Zaphiropoulos[4]

[1]Department of Medical Sciences, University of Turin, Turin – Italy; srondonl@unito.it; nrangel@unito.it

[2]Faculty of Natural Sciences and Mathematics, Universidad del Rosario, Bogotá - Colombia; victoria.villegas@urosario.edu.co; mc.sanchez@urosario.edu.co

[3]Doctoral Program in Biomedical Sciences, Universidad del Rosario, Bogotá - Colombia

[4]Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge 14183, Sweden; peter.zaphiropoulos@ki.se

*Correspondence: srondonl@unito.it; Tel.: +390116334127 (ext. 4388); Fax: +390116635267
victoria.villegas@urosario.edu.co; Tel.: 57-1-297-0200 (ext. 4029); Fax: +57-1-297-0200

†These authors contributed equally to this work.

Abstract: 17β-Estradiol (E_2) plays a pivotal role in the development and progression of breast cancer. As a result, blockade of the E_2 signal through either tamoxifen (TAM) or aromatase inhibitors (AIs) is an important therapeutic strategy to treat or prevent estrogen receptor (ER) positive breast cancer. However, resistance to TAM treatment is the major obstacle in endocrine therapy. This resistance occurs either *de novo* or is acquired later after an initial beneficial response. The underlying mechanisms for TAM resistance are probably multifactorial and remain largely unknown. Considering that breast cancer is a very heterogeneous disease and patients respond differently to treatment, the molecular analysis of TAM's biological activity could provide the necessary framework to understand the complex effects of this anti-estrogen drug in target cells. Moreover, this could explain at least in part, the development of cellular resistance and indicate an optimal therapeutic option.

This review highlights the implications of TAM in breast cancer as well as the role of receptors/signal pathways (G protein–coupled estrogen receptor (GPER), androgen receptor (AR) and Hedgehog (HH) signaling pathway) recently suggested to be involved in the development of TAM resistance. GPER, AR and HH signaling are emerging as novel therapeutic targets and prognostic indicators for breast cancer, based on their ability to mediate estrogenic signaling in different cell types, including ER-positive or -negative breast cancer.

Keywords: Tamoxifen; Breast Cancer; GPER; ERs, AR; HH signaling pathway; Endocrine resistance

2.8 RESEARCH ARTICLE

International Journal of
Molecular Sciences

MDPI

Article

Tamoxifen Treatment of Breast Cancer Cells: Impact on Hedgehog/GLI1 Signaling

Victoria E. Villegas [1,2,3,*,†], Milena Rondón-Lagos [3,4,†], Laura Annaratone [4], Isabella Castellano [4], Adriana Grismaldo [3], Anna Sapino [4,5] and Peter G. Zaphiropoulos [1]

1 Department of Biosciences and Nutrition, Karolinska Institutet, Huddinge 14183, Sweden; peter.zaphiropoulos@ki.se
2 Doctoral Program in Biomedical Sciences, Universidad del Rosario, Bogotá 11001000, Colombia
3 Faculty of Natural Sciences and Mathematics, Universidad del Rosario, Bogotá 11001000, Colombia; srondonl@unito.it (M.R.-L.); mabel.grismaldo@urosario.edu.co (A.G.)
4 Department of Medical Sciences, University of Turin, Via Santena 7, 10126 Turin, Italy; laura.annaratone@unito.it (L.A.); isabella.castellano@unito.it (I.C.); anna.sapino@unito.it (A.S.)
5 Institute for Cancer Research and Treatment (IRCC), Strada Provinciale 142, 10060 Candiolo, Italy
* Correspondence: victoria.villegas@urosario.edu.co; Tel.: +57-1-297-0200 (ext. 4029); Fax: +57-1-297-0200
† These authors contributed equally to this work.

Academic Editor: William Chi-shing Cho
Received: 31 December 2015; Accepted: 22 February 2016; Published: 27 February 2016

Abstract: The selective estrogen receptor (ER) modulator tamoxifen (TAM) has become the standard therapy for the treatment of ER+ breast cancer patients. Despite the obvious benefits of TAM, a proportion of patients acquire resistance to treatment, and this is a significant clinical problem. Consequently, the identification of possible mechanisms involved in TAM-resistance should help the development of new therapeutic targets. In this study, we present *in vitro* data using a panel of different breast cancer cell lines and demonstrate the modulatory effect of TAM on cellular proliferation and expression of Hedgehog signaling components, including the terminal effector of the pathway, the transcription factor GLI1. A variable pattern of expression following TAM administration was observed, reflecting the distinctive properties of the ER+ and ER− cell lines analyzed. Remarkably, the TAM-induced increase in the proliferation of the ER+ ZR-75-1 and BT474 cells parallels a sustained upregulation of GLI1 expression and its translocation to the nucleus. These findings, implicating a TAM-GLI1 signaling cross-talk, could ultimately be exploited not only as a means for novel prognostication markers but also in efforts to effectively target breast cancer subtypes.

Keywords: GLI1; TAM resistance; breast cancer; Hedgehog signaling; cellular proliferation

2.9 SUPPLEMENTARY INFORMATION ABOUT RESEARCH ARTICLE

Supplementary materials about "*Tamoxifen Treatment of Breast Cancer Cells: Impact on Hedgehog/GLI1 Signaling.*" can be found at:

http://www.mdpi.com/1422-0067/17/3/308/s1

Int. J. Mol. Sci. 2016, *17*, x; doi:

Tamoxifen Treatment of Breast Cancer Cells: Impact on Hedgehog/GLI1 Signaling

Victoria E. Villegas, Milena Rondón-Lagos, Laura Annaratone, Isabella Castellano, Adriana Grismaldo, Anna Sapino and Peter G. Zaphiropoulos

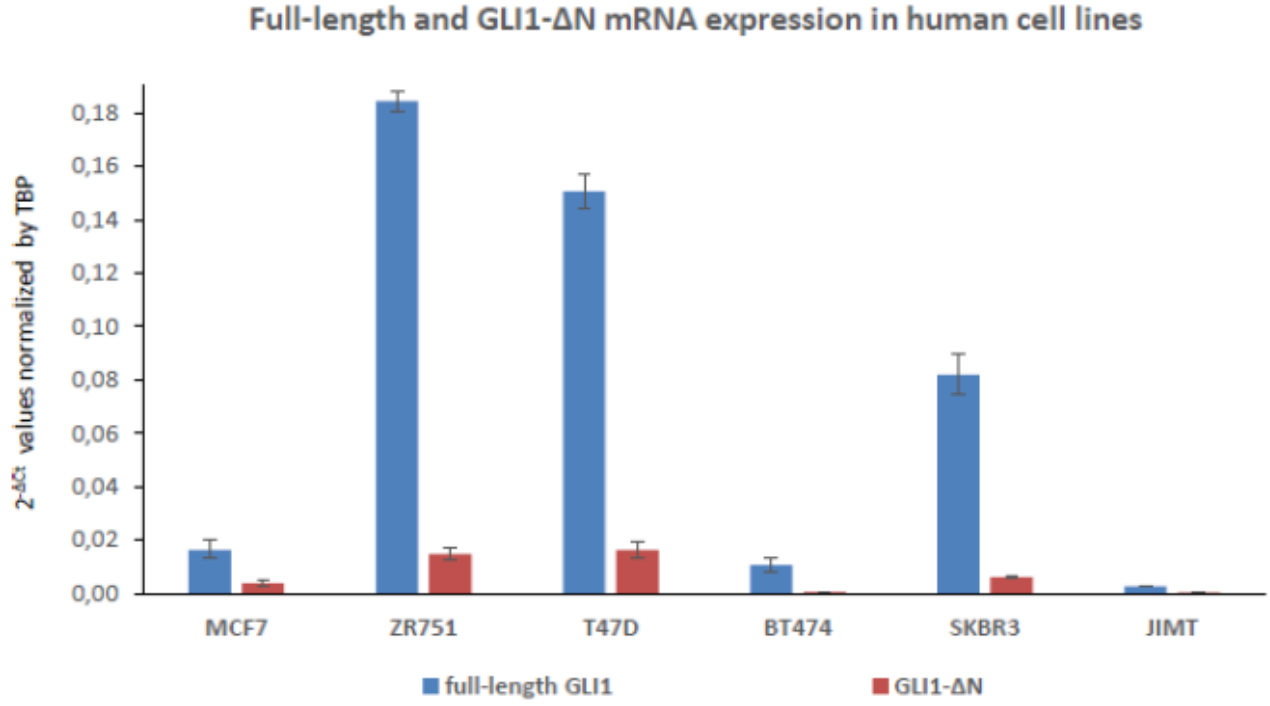

Figure S1. Endogenous expression of full-length GLI1 and GLI1-ΔN variants in human breast cancer cells lines. mRNA levels were measured using real-time RT-PCR and TBP was used as an internal control.

GENERAL CONCLUSION AND PERSPECTIVES

Cancer is a heterogeneous disease characterized by the presence of a mosaic of distinct morphological and phenotypic profiles. This tumor heterogeneity introduces significant challenges in designing effective treatment strategies, since potential targets genes can be involved not only in tumor progression but may also modulate the response to therapy. As more than 25% of human cancers are associated with aberrations of the HH signaling pathway, a better delineation of its key regulatory steps may provide clues for a rational design of inhibitory molecules that could be exploited in future therapeutic approaches.

The results of these two studies demonstrate the multifaceted role of the activation of the HH signaling pathway in the development of cancer. These findings can be considered as a basis for further research that could include HH signaling components as clinical markers of disease progression and may also suggest optimal therapeutic strategies.

Inhibitors of the HH signaling cascade are thought to be good targets for cancer therapy. Specifically, SMO antagonists are currently widely used, but these drugs will not be effective in tumors with activation downstream of SMO, such as *GLI* amplification/mutation. *GLI1* is a marker of activation of HH signaling, however additional pathways via non-canonical routes can impinge on GLI1 expression and activity. Hence, it makes sense to focus on mechanisms that block HH signaling by directly reducing the transcriptional activity of *GLI1*. New pharmacological strategies based on controlling the GLI1 antisense and sense transcripts may be developed to achieve an effective reduction of the capacity of *GLI1* to act as a transcription factor.