
# 1st HEALING International Meeting

Kolymbari, Crete

June 23-25 2011

## Hh-Gli Signalling in Development, Regeneration and Cancer

# Abstract Book

*Organized by Christos Delidakis and Ariel Ruiz i Altaba for the EU funded HEALING Consortium.*



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Hh-Gli Signalling in Development, Regeneration and Cancer

# Abstract Book

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# Abstract Book

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| | | Deborah Gumucio<br>Hedgehog and the gut: Multiple roles for Hedgehog signaling in intestinal development and homeostasis | **Natalia Riobo**: Sonic Hedgehog activates GTPases Rac1 and RhoA in a Gli-independent manner via coupling of Smoothened to Gi proteins |
| | Isabel Guerrero | | Alberto Gulino<br>Regulators and targets of Hedgehog signaling in stem cells |
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| | **Neha Vyas**: Nanoscale organisation of Drosophila Hedgehog | **Leni Jacob**: Genome-Wide RNAi Screen Reveals Disease-Associated Genes That Are Common to Hedgehog and Wnt Signaling | **Ariel Ruiz i Altaba**: closing remarks |
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| | **Anna Marie Kenney**: The sonic hedgehog target YAP promotes radioresistance and genomic instability in medulloblastoma through IGF2-mediated Akt activation | Barbara Stecca<br>Modulation of Hedgehog-GLI signaling by WIP1 phosphatase in cancer | |
| Dinner | Matt Scott<br>New Puzzles in Hedgehog Signal Transduction | Martine Roussel<br>Positive and Negative Signaling Pathways in Shh-subtype medulloblastomas | 20h |
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| | Drinks under the stars | Drinks under the stars | 22h |

# Talk Abstracts

## Studies in Drosophila of long distance signaling mediated by direct contact

*Kornberg, Tom*

Cytonemes, types of filopodia first identified in the Drosophila wing imaginal disc, were proposed to move signaling proteins between producing and target cells. We now have evidence for several types of cytonemes in the wing and eye imaginal discs and in the trachea, and find that cytonemes are diverse in composition, and exhibit specificity for stimulating ligands and plasticity in orienting to novel sources of ligand. Receptors for ligands have punctal distributions specifically in responding cytonemes, and cytonemes emanating from cells in which signal transduction was blocked did not extend normally. Moreover, puncta containing both ligand and receptor can be found in cytonemes that appear to touch ligand-expressing cells, evidence that cytonemes can ferry signaling proteins. These findings support cytoneme-based movement of signaling proteins as a mechanism for cell-cell communication that transfers controlled amounts of signaling protein in a targeted manner to a specific recipient cell.

## Synergy of Lipoprotein associated and Lipoprotein-free Hedgehog proteins

*Wilhelm Palm*, Marta Swierczynska*, Veena Kumari and Suzanne Eaton*
**equal contribution*

Hedgehog family proteins are covalently linked to both cholesterol and palmitate. Despite this, they are secreted and spread over long distances to pattern developing tissue. In Drosophila, one mechanism leading to Hedgehog release is lipoprotein association. Lipoproteins act as vehicles for the spread of Hedgehog, but also contain lipids that repress the pathway, promoting Smoothened degradation and processing of Ci to its transcriptional repressor form. Here, we show that both human and Drosophila Hedgehog proteins are secreted in lipoprotein associated forms, when lipoproteins are available. In the absence of lipoproteins, both are secreted as non-lipid modified monomers. Although both forms can signal, only lipoprotein-associated Hedgehog reverses pathway inhibition by lipoprotein lipids. Monomeric and lipoprotein-associated Hedgehog act synergistically in both Drosophila wing imaginal discs and in human tissue culture cells to stimulate target gene transcription.

## Signalling via endocytosis - the case of Delta

*Katerina Daskalaki, Kristina Kux, George Tsoumpekos and Christos Delidakis*

Institute of Molecular Biology & Biotechnology, FORTH and Department of Biology, University of Crete

Cell signalling is intimately linked with the trafficking of ligands, receptors and accessory factors. We have studied the role of Delta trafficking in the Notch signalling pathway. Delta is a transmembrane protein and represents one of the two ligands of the Notch receptor in Drosophila. Its extracellular domain (ECD) interacts with the Notch ECD to trigger its activation (when bound in trans – from adjacent cells) or its inhibition (when bound in cis – from the same cell). Subsequent to binding, Notch and Delta are frequently co-localized in endosomes. The postitive activity of Delta requires the presence of one of two E3 ubiquitin ligases, Neuralized or Mindbomb1. We have made mutations in the Dl intracellular domain (ICD) and tested five parameters: (a) interaction with Neur and Mib1, (b) ubiquitylation by these enzymes, (c) ability to signal in two different contexts, (d) subcellular distribution and (e) efficiency of endocytosis. We have identified a docking site for each Ub ligase. The ability of our Dl variants to signal requires that the Ub ligase interaction be intact. In addition to triggering Dl activity, Dl ubiquitylation leads to endocytosis and degradation at the lysosome. Our different Dl variants showed essentially the same distribution among early and late endosomes, suggesting that ubiquitylation does not affect the endosomal route of trafficking, but most likely affects an early endocytic step. Indeed the rate of endocytosis of Dl, measured by a live antibody uptake assay, depends to a large extent on the activity of Neur and Mib1.

## Nanoscale organization of Drosophila Hedgehog

*Vyas N, Goswami D, Manonmani A, Sharma P, Ranganath HA, VijayRaghavan K, Shashidhara LS, Sowdhamini R, Mayor S.*

Hedgehog family of protein remains highly conserved across species and plays a major role in patterning the developing embryos. It is also required for stem cell maintenance in adult tissues as well for several cancer progressions. Hedgehog proteins are post translationally modified by palmitate and cholesterol at the N-terminus and C-terminus of the signaling domain respectively. Across species, mutually exclusive populations of cells produce and respond to Hedgehog, making its release and transport mandatory for signal transduction and hence tissue patterning. Heparan Sulphate Proteoglycans (HSPGs) have been implicated in capture and transport of these proteins several cell diameters away. We have examined the cell surface organization of functional, fluorescently-tagged variants of Hh in living cells. Using optical imaging, FRET microscopy, and mutational studies guided by bioinformatics prediction, we find that Hedgehog displays a hierarchical organization at the cell surface. Starting with a sub-optical resolution oligomers to optically resolvable large punctuate structures. This hierarchy of structural organization appears to be essential for interacting with HSPGs and activation of signaling at a distance from expressing cells.

**A counter-intuitive way of regulating hedgehog expression in the Drosophila wing.**

*Marco Milán*

Trithorax-group and Polycomb-group proteins interact with chromosomal elements, termed PRE/TREs, to ensure a stable heritable maintenance of the transcriptional state of nearby genes. Those regulatory elements that bind both groups of proteins are termed Maintenance Elements (MEs). Some of these MEs maintain the initial activated transcriptional state of a nearby reporter gene through several rounds of mitosis during development. Here we show that expression of hedgehog in the posterior compartment of the Drosophila wing results from the communication between a previously defined ME and a nearby cis-regulatory element termed C enhancer. The C enhancer integrates the activities of the Notch and Hedgehog signalling pathways and drives, already in the early wing primordium, expression to a thin stripe in the posterior compartment that corresponds to the dorsal-ventral compartment boundary. The ME maintains the initial activated transcriptional state conferred by the C enhancer and contributes to expand, by growth, its expression domain throughout the posterior compartment. Communication between the ME and the C enhancer also contributes to repressing gene expression in anterior cells. Most interestingly, we present evidence that enhancers and MEs of different genes are interchangeable modules whose communication is involved in restricting and expanding the domains of gene expression. Our results emphasize the modular role of MEs in regulating gene expression within growing tissues.

## Mechanisms controlling cancer stem cell and metastatic behavior

*A. Ruiz i Altaba*

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Hedgehog (HH)-GLI signaling modulates precursor proliferation in the late embryonic and postnatal brain and controls brain stem cell behavior in stem cell niches. This activity on precursors/stem cells in development and homeostasis seems conserved in other organs. Inappropriate HH-GLI signaling is essential for the growth of a large variety of human cancers, including those of brain, skin, prostate, lung and pancreas. Interestingly, active HH-GLI signaling is also required by glioblastoma and colon cancer and for their self-renewal and survival of their cancer stem cells. Recent data on the role of HH-GLI signaling in colon cancer stem cells and metastatic progression will be presented. A framework for the integration on oncogenic inputs and loss of tumor suppressor function by the Gli code, acting as an information nexus regulating cancer stem cell behavior, will also be discussed.

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Varnat F, Siegl-Cachedenier I, Malerba M, Gervaz P, Ruiz i Altaba A. (2010). Loss of WNT-TCF addiction and enhancement of HH-GLI1 signalling define the metastatic transition of human colon carcinomas. EMBO Molecular Medicine 2, 440-57

Lorente-Trigos A, Varnat F, Melotti A, Ruiz i Altaba A. (2010) . BMP signaling promotes the growth of primary human colon carcinomas in vivo. J Mol Cell Biol. 2,318-32.

## A cell-autonomous requirement for Hedgehog signaling in small cell lung cancer

*Kwon-Sik Park[1,2*], Luciano G. Martelotto[5*], Martin Peifer[6], Martin L. Sos[6,7], Anthony N. Karnezis[9], Moe R. Mahjoub[2,3], Katie Bernard[1,2], Jamie Conklin[1,2], Anette Szczepny[5], Jing Yuan[10], Ribo Guo[10], Beatrice Ospina[10], Jeanette Falzon[12], Samara Bennett[12], Tracey J Brown[12], Ana Markovic[11], Wendy L. Devereux[11], Cory A. Ocasio[4], James K. Chen[4], Tim Stearns[2,3], Roman K. Thomas[6,7,8], Marion Dorsch[10], Silvia Buonamici[10], D. Neil Watkins[5], Craig D. Peacock[11*] and Julien Sage[1,2*].*

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[*] *Equal contribution*

Small cell lung carcinoma (SCLC) is a deadly neuroendocrine subtype of lung cancer with a 5-year survival rate of less than 10%. More than 200,000 people die from this disease every year worldwide, with treatment options for patients having remained basically unchanged for the past 25 years. Expanding on preliminary observations in tumor cell lines and a few primary tissue samples, we have examined the role of the Hedgehog (Hh) signaling pathway in the tumorigenesis of SCLC

We found active Hh signaling in tumors that arise in a mouse model of human SCLC, developed around the fact that the RB and p53 tumor suppressor genes are mutated in most, if not all cases of human SCLC. Constitutive activation of the Hh pathway using a mutant allele of Smo was sufficient to enhance SCLC development in vivo in this mouse model. Conversely, genetic deletion of Smo in tumor cells suppressed tumorigenesis, indicating that activation of the Hh pathway plays a role in initiation and development of SCLC in this mouse model. Experiments with cell lines derived from these mouse tumors indicate that the activation of Hh signaling in SCLC cells is independent of the lung parenchyma. Furthermore, pharmacological blockade of Hh signaling inhibits the growth of SCLC cells ex vivo and in vivo, indicating that activation of the Hh pathway is also necessary for the maintenance of the tumorigenic phenotype. Intriguingly, we report that blockade of Hh signaling in primary human SCLC xenografts and parallel cell lines inhibits clonogenicity and tumor recurrence following treatment with first-line chemotherapeutic agents.

Thus, the Hh pathway plays an intrinsic role in the initiation and maintenance of SCLC, with pre-clinical models suggesting that combination of Hh pathway inhibitors and conventional chemotherapy may benefit SCLC patients.

## The sonic hedgehog target YAP promotes radioresistance and genomic instability in medulloblastoma through IGF2-mediated Akt activation

*Africa Fernandez-L[1], Massimo Squatrito[1], Paul Northcott[2], Eric C. Holland[1], Michael D. Taylor[2], Zaher Nahlé[3], and Anna Marie Kenney[1, 3]*

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Radiation therapy is the standard of care for many cancers, including the pediatric brain tumor medulloblastoma, the most common solid malignancy of childhood. Radiation leads to long-term side effects, while radio-resistance contributes to tumor recurrence. In Sonic hedgehog-driven mouse medulloblastomas, radio-resistant cells occupy the peri-vascular niche. These cells are capable of proliferation after irradiation and may function as tumor re-populating cells, driving recurrence. They express Yes-associated protein (YAP), which we have previously identified as a Sonic hedgehog (Shh) target markedly elevated in Shh pathway-associated medulloblastomas in human patients. Here we report that YAP accelerates tumor growth and confers radio-resistance, promoting on-going proliferation of medulloblastoma cells and cerebellar progenitor cells after radiation. YAP activity enables cells to enter mitosis with un-repaired DNA through driving IGF2 expression and Akt activation, which results in ATM/Chk2 inactivation and abrogation of cell cycle checkpoints. Our results establish a central role for YAP in counteracting radiation-based therapies and driving genomic instability, and indicate the YAP/IGF2/Akt axis as a therapeutic target in medulloblastoma.

## New Puzzles in Hedgehog Signal Transduction

*Tyler Hillman, Brian Feng, Jun Ni, Wei-Meng Woo, Ljiljana Milenkovic, Anthony Oro, James Chen, and Matthew Scott*

Stanford University School of Medicine

The development of numerous tissues and organs depends on Hedgehog (Hh) protein signals that influence gene expression in target cells. Defective Hh signaling leads to birth defects and cancer. We are investigating Hh signal transduction and gene regulation mechanisms in the context of cultured fibroblasts and cerebellum development and tumorigenesis. Reception and transduction of the Hh protein signal has many unique features, including the importance of primary cilia as a Hh signal transduction organelle. Primary cilia, immotile organelles found on most cells, have been implicated in several signaling pathways and we have observed direct binding of Hh protein to them. We find that the Ptc receptor protein is localized in cilia, where it prevents accumulation of the Smo seven-transmembrane domain protein. Binding of Hh to Ptc causes departure of both from the cilium, allowing entrance of Smo. Smo in the cilium is able to activate Gli transcription factors, which in turn control target gene expression. Using tagged proteins, and mutants that affect primary cilia, we are exploring the mechanisms of protein trafficking and target gene activation. We have tested the effect of inhibiting cilia formation in mouse cerebellum on the rate of tumorigenesis and found that tumor cells are addicted to the presence of cilia. We have performed a RNAi screen in cultured cells for genes required for Hedgehog signal transduction and identified neuropilins as important factors.

## Hedgehog and the gut: Multiple roles for Hedgehog signaling in intestinal development and homeostasis.

*Deborah L. Gumucio, PhD. Professor Department of Cell and Developmental Biology*

Center for Organogenesis; and Center for Computational Medicine and Bioinformatics, University of Michigan Medical Center, Ann Arbor, MI, USA, 48109-2200.

In the normal developing and adult intestine, hedgehog ligands are manufactured by epithelial cells and act on target cells in the underlying stroma. Though this strictly paracrine mode of signaling appears simple in terms of signal flow, the downstream effects of hedgehog signals are felt by multiple stromal cell types and the phenotypes elicited by alterations in hedgehog signaling at the tissue level reflect the combinatorial responses of all of these target cell types. Additionally, hedgehog-mediated modulations in phenotype or gene expression within the target cell populations more often than not result in changes in the secretion of other signaling molecules by these target cells. These secondary eventshave additional consequences for the target cells themselves as well as for by-stander cells (those uninvolved in the initial hedgehog signaling event). Moreover, these secondary changes can elicit tertiary effects in the signaling milieu (and so on). Since the use of therapeutic inhibitors of hedgehog signaling is well underway to treat cancers and since localized increases in hedgehog signaling could potentially promote injury repair in some tissues, an important immediate goal is to begin to deconvolute the complex downstream events of hedgehog signaling at the tissue level, in order to identify the primary events. Such knowledge will be important both for our ability to monitor and predict potential side effects of treatment and for the further development of more specific therapeutics that target specific cell populations or specific molecular targets of hedgehog signaling. To this end, our laboratory is working to: a) identify the target cell populations that receive the primary hedgehog signal in the developing and adult intestine; b) elucidate the phenotypic and gene expression changes elicited by the hedgehog signal in these cell types. I will present two examples of this work, examining the effects of hedgehog signaling on villus development in the fetal intestine and on smooth muscle differentiation in the adult intestine.

## Crosstalk between MAP kinase and Hedgehog pathways via direct phosphorylation of Gli proteins

*A. Jane Bardwell, Elizabeth. A. Gordon, Thomas C. Whisenant, and Lee Bardwell**

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There is emerging evidence that cross-talk between the Hedgehog-Gli pathway and mitogen-activated protein kinase (MAPK) signaling pathways may be relevant to development and cancer (reviewed in [1, 2]). However, the molecular mechanism(s) of this cross-talk are unclear. We recently exploited the observation that MAPKs briefly attach to many of their substrates before phosphorylating them to develop a computational tool (D-finder) that searches genome databases for MAPK-docking sites [3]. Among the novel substrates predicted by D-finder and subsequently verified biochemically were the human Gli1 and Gli3 transcription factors. Both ERK and JNK-family MAPKs bind to Gli1/3 via the predicted docking site, and phosphorylate multiple nearby target residues. These MAPK target phosphosites lie in a putative "hotspot" for the regulation of Gli activity. We will report progress on phosphosite mapping and mutagenesis, as well as functional studies on the regulatory consequences of MAPK-dependent Gli phosphorylation. Our work is the first to provide evidence that MAPKs bind to and directly phosphorylate Gli proteins.

[1] Lauth M & Toftgard R (2007) Cell Cycle 6: 2458–2463.

[2] Stecca B & Ruiz I Altaba A (2010) J Mol Cell Biol 2:84-95.

[3] Whisenant TC… & Bardwell L (2010) PLoS Comput Biol. 6 pii: e1000908.

**Direct regulation of Neogenin1 in granular cell precursors (GCP) through the Sonic Hedgehog (Shh) pathway: A key mechanism in both cerebellar development and tumorigenesis.**

*Luis A. Milla[1], Víctor Cornejo[1], Brandon Wainwight[1] and Verónica Palma[1]*

[1] Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile; [2] Institute for Molecular Bioscience, University of Queensland, Australia.

The canonical Shh/Gli pathway plays multiples roles during embryonic development and adulthood. By using different genomic approaches, we have recently uncovered several new direct and indirect Shh targets. One of these targets, Neo1, classically known as a Netrin1 receptor and recently involved in many processes during Central Nervous System (CNS) development, has emerged as an interesting candidate. Here, we used Chromatin Immunoprecipitation (ChIP) in mouse embryonic CNS and luciferase reporter assays to demonstrate *in vivo* and *in vitro* direct binding of Gli transcription factors to the *neo1* promoter. Neo1 is expressed in CNS, namely rostrocaudal migratory stream, cortex, and cerebellum. In particular in the developing cerebellum, its expression is located in the proliferative outer External Germinal Layer (EGL). The Shh pathway activation seems to be relevant to drive and regulate Neo1 expression and, more importantly, to contribute thereby to GCP proliferation since EGL precursors that undergo active migration and differentiation do not longer express the Neo1 marker. Taken together, our results show that Neogenin1 is regulated by the canonical Hh signaling in the CNS, and may play a prominent role in the development of medulloblastoma, an observation essential for improving anticancer pharmacology.

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## Genome-Wide RNAi Screen Reveals Disease-Associated Genes That Are Common to Hedgehog and Wnt Signaling

*Leni S. Jacob, Xiaofeng Wu, Michael E. Dodge, Chih-Wei Fan, Ozlem Kulak, Baozhi Chen, Wei Tang, Baolin Wang, James F. Amatruda and Lawrence Lum*

The Hedgehog (Hh) and Wnt signal transduction pathways are master regulators of embryogenesis and tissue renewal and represent anticancer therapeutic targets. Using genome-wide RNA interference screening in murine cultured cells, we established previously unknown associations between these signaling pathways and genes linked to developmental malformations, diseases of premature tissue degeneration, and cancer. From biochemical and cell biological studies of these disease-associated genes, we uncover novel mechanisms of Hh and Wnt pathway regulation. We identified functions in both pathways for the multitasking kinase Stk11 (also known as Lkb1), a tumor suppressor implicated in lung and cervical cancers. Stk11 loss prompted disassembly of the primary cilium, a cellular organizing center for Hh pathway components. Shortening of the primary cilia in turn accelerates the proteolytic processing of the transcriptional regulator Gli3 into a repressor molecule thereby dampening Hh pathway response. Loss of Stk11 also induced aberrant cell autonomous signaling through the Wnt pathway. Chemicals that targeted the Wnt acyltransferase Porcupine or that restored primary cilia length by inhibiting the tubulin deacetylase HDAC6 (histone deacetylase 6) countered deviant pathway activities driven by Stk11 loss. Our study demonstrates that Stk11 is a critical mediator in both the Hh and the Wnt pathways, and that functional genomics based approaches to dissect cell-fate determination pathways may support the development of targeted therapeutic strategies.

## SHH and the Dependence Receptor notion

*Patrick Mehlen*

Apoptose, Cancer et Développement. UMR INSERM 1052 CNRS 5286, Centre Léon Bérard, Université de Lyon. 28 rue Laennec, 69008 Lyon. France.

Dependence receptors are receptors that display two totally different signal transductions depending on ligand availability. If in the presence of ligand, these receptors transduce a positive signal leading to differentiation/proliferation/migration, in the absence of ligand these receptors are not inactive but rather induce an active process of cell death. Thus, such receptors create cellular states of dependence on their respective ligands by inducing apoptosis when unoccupied by ligand. This growing family of such bi-functional receptors now includes p75ntr, DCC, UNC5H1-4, neogenin, some integrins and tyrosine kinase receptors RET, EPHA4, ALK, TrkA and TrkC. We demonstrated some years ago that Patched is such a dependence receptor: Ptc triggers apoptosis in the absence of Shh while Shh presence inhibits the pro-apoptotic activity of Ptc. We have proposed that this pro-apoptotic activity is crucial for the adequate development of the nervous system and for the tumor suppressive activity of Ptc. We will show here mechanistic data that support the view that Ptc triggers apoptosis by recruiting a caspase-activating complex allowing initiator caspase-9 activation. We will also present new data on a novel SHH dependence receptor and will propose alternative anti-cancer therapeutic strategy based on activation of apoptosis via this novel dependence receptor.

## Epithelial Expression of Gli2 Mediates Epithelial Proliferation in the Gastric Antrum of the Gastrin Null mice

*Merchant Juanita L., Saqui-Salces Milena, Waghray Meghna*

Departments of Internal Medicine and Molecular and Integrative Physiology, University of Michigan

Gastrin null mice develop hyperplastic/metaplastic changes in the gastric antrum by 9 months of age. We reported previously that the metaplastic changes correlate with an increase in follistatin, a molecular target of Gli2 but not Gli1. Follistatin is an activin/BMP antagonist. Moreover, follistatin expression emerges in the proliferative zone of the gastric antrum and is highly expressed in gastric cancers.

**Aim:** We therefore queried whether the increase in follistatin might be due to epithelial expression of Gli2.

**Methods:** The Shh-LacZ and Gli2-LacZ reporter mice were each placed on the gastrin null genetic background then sacrificed between 7 and 12 months after birth.

**Results:** The Shh reporter mice on a wild type background exhibited strictly epithelial LacZ expression occasionally in a few scattered antral glands. Shh expression in the gastric corpus occurs in all cell types (Gastroenterology, 2010). The Gli2 reporter mice exhibited strong LacZ expression in the mesenchyme (typically myofibroblasts and scattered smooth muscle cells). However, on the gastrin null background where there was antral hyperplasia, LacZ expression in the Shh reporter mice exhibited increased expression in the deep antral glands that were typically positive for the mucous neck cell marker TFF2 (trefoil factor 2). By comparison, intense LacZ expression in the Gli2 reporter mice occurred in the same deep antral glands.

**Conclusions:** Both Shh and Gli2 expression increases in the deep antral glands of the hyperplastic /metaplastic gastric antrum. The deep antral glands have previously been shown to contain Lgr5+ cells, a marker for both the antral and intestinal stem cells. Thus, we concluded that epithelial Gli2 expression correlates with follistatin expression in the hyperplastic/metaplastic antrum and contributes to the antral proliferation. Since Shh expression also increases in these glands, the increase in Gli2 expression might represent activation of canonical Hh signaling; however, we cannot exclude a role for non-canonical signaling the Gli2 expression pattern.

## Functional analysis of Hh target genes in neural progenitors in the retina

*Valerie Wallace*

The Hedgehog signalling pathway is a critical growth and differentiation in brain development and deregulation of this pathway causes congenital malformations and cancers in humans. Our laboratory uses the neural retina, a tractable model of CNS development, to investigate the mechanism of Hh-mediated growth control in neural progenitor cells. In the developing retina, Sonic hedgehog is secreted by ganglion cell neurons and signals to the pool of multipotent progenitor cells (PCs) to control cell cycle progression, self-renewal and cell fate. These functions are mediated, in part, through Gli2-dependent regulation of Hes1 that is independent of Notch signalling. Here we describe the identification of a novel set of temporally regulated Gli target genes and the function of Gli2 interacting proteins in neural progenitor development.

## Modulation of Hedgehog-GLI signaling by WIP1 phosphatase in cancer

*Barbara Stecca*

Laboratory of Tumor Cell Biology, Core Research Laboratory – Istituto Toscano Tumori (CRL-ITT), Florence, Italy

The Hedgehog-GLI (HH-GLI) signaling pathway plays a critical role in regulating growth and tissue patterning during embryogenesis and in controlling stem cell renewal and proliferation. When aberrantly activated the HH-GLI pathway contributes to cancer development in several tissues, including the skin. We have previously shown that the tumor suppressor p53 negatively regulates GLI1 and suggested that a p53-dependent phosphatase might regulate GLI1 activity (1). We have identified the oncogenic WIP1 phosphatase as a novel positive regulator of HH-GLI signaling. WIP1 is a nuclear Ser/Thr phosphatase expressed at low levels in most normal tissues. WIP1 expression is increased in a p53-dependent manner in response to genotoxic stresses and it is amplified/overexpressed in several cancer types. We will present evidence that WIP1 enhances GLI1, by increasing its transcriptional activity and nuclear localization, and that the two proteins physically interact. Modulation of GLI1 by WIP1 depends on its phosphatase activity, but does not require the main WIP1 substrate p53. More importantly, WIP1 is essential for the maintenance of HH-GLI-induced cancer stem cell self-renewal and growth *in vitro* and in controlling HH-GLI-driven xenograft growth *in vivo.* These results suggest a critical role of the WIP1 phosphatase in sustaining activation of HH-GLI1 signaling. The therapeutic implications of these findings will also be discussed.

(1) Stecca B. and Ruiz i Altaba A. (2009). A GLI1-p53 inhibitory loop controls neural stem cell and tumor cell numbers. *EMBO J.* 28:663-76.

## Positive and Negative Regulation of Hedgehog signaling in Medulloblastomas

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Medulloblastoma (MB) is the most common malignant pediatric tumor of the central nervous system that arises in the cerebellum. Human medulloblastomas have been classified molecularly by gene expression profiling into four distinct subgroups [Thompson et la., 2006; Northcott et al., 2010; Cho et al., 2010]: (1) the SHH-subtype (~25%) which harbors mutations that induce constitutive activation of the Sonic Hedgehog (SHH)/Patched (PTCH) signaling pathway; (2) the WNT-subtype (~15%) that consists of mutations in the WNT pathway; (3) the MYC-subtype characterized by high expression levels of the MYC oncogene (MYC) and (4) another subtype with no specific genetic anomalies as yet identified. We have modeled the SHH-subtype of medulloblastoma in mice by deleting either Trp53 and Cdkn2c or one copy of Ptch1+/- and Cdkn2c. Using these mouse models and an orthotopic transplant approach in cortices of recipient mice, we showed that Mycn and the microRNA-17~92 cluster collaborate with p53 or Ptch1 loss to induce SHH-subtype medulloblastoma. In contrast we found that signaling via the Bone Morphogenic Proteins (BMP) 2,4,7 antagonizes Shh-induced proliferation by inducing tumor cells differentiation. We will present our current efforts to inhibit Shh-subtype medulloblastoma development with antagomiRs to miR-17~92 and small molecule activators of the BMP4 signaling pathway.

## Sonic Hedgehog activates GTPases Rac1 and RhoA in a Gli-independent manner via coupling of Smoothened to $G_i$ proteins.

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Our group recently established that Smoothened (SMO) functions as a GPCR with selectivity towards Gi (*PNAS 103(33): 12607-12; 2006*), an activity that is required for Gli activation in NIH 3T3 cells. In the present study we sought to evaluate the role of SMO-$G_i$ coupling in fibroblast migration as a paradigm of non-canonical Hh signaling.

Using NIH 3T3 cells and $Ptc1^{+/-}$, $Ptc1^{-/-}$ and $SMO^{-/-}$ MEFs we demonstrated the relevance of SMO activity on spontaneous and Shh-stimulated migration in wound-healing assays. Our results show that fibroblast migration in response to Shh is strictly dependent on SMO and independent of Gli transcriptional activity. Remarkably, we found that Shh strongly stimulates the small Rho GTPases Rac1 and RhoA within 1-5 minutes. Activation of the small GTPases was sensitive to cyclopamine and abolished by pre-treatment with pertussis toxin (PTX), which prevents $G_i$ proteins activation by SMO, and by LY294002, a phosphoinositide-3 kinase (PI3K) inhibitor. These cyclopamine-sensitive responses are present under non-stimulated conditions in $Ptc1^{-/-}$ MEFs, and are significantly attenuated in $SMO^{-/-}$ cells. Indeed, a C-tail deleted mutant of SMO was able to rescue activation of Rac1 and RhoA and cell migration in SMO-deficient MEFs. This mutant, however, cannot restore Gli activation.

In conclusion, our data indicate that SMO signals to the small G proteins RhoA and Rac1 via a $G_i$ protein- and phosphoinositide-3 kinase (PI3K)-dependent mechanism, and that all these components are required for Shh-induced cell migration. Moreover, our findings underscore the role of SMO-$G_i$ coupling in non-canonical Hh signaling.

## Regulators and targets of the Hedgehog signaling in stem cells

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Hedgehog (Hh) pathway has a pivotal function in development and tumorigenesis, processes sustained by stem cells (SCs). We observed that one of the Hh pathway that sustains post-natal neural SCs is the transcription factor Nanog that controls stemness acting as a key determinant of both embryonic SC self-renewal and differentiated somatic cells reprogramming to pluripotency, in concert with the loss of the oncosuppressor p53. Nanog is highly expressed, together with Gli1, in SCs from postnatal cerebellum and medulloblastoma, and acts as a critical mediator of Hh-driven selfrenewal, through the binding to and transactivation of Nanog-specific cis-regulatory sequences by Gli1 and Gli2, both in mouse and human SCs. Hh regulation of Nanog does not require the Hh-downregulated p53 that is known to inhibit Nanog Our data reveal a mechanism for the function of Hh in the control of stemness that represents a crucial component of an integrated circuitry determining cell fate decision and involved in the maintenance of cancer SCs.

To this regard, we have identifie a number of regulatory mechanims of Hh/Gli function. First, acetylation of Gli1 and Gli2 proteins is a critical transcriptional switch, by inhibiing Gli transacting function. Such a mechanism is regulated by a novel family of HDAC1 inhibitors (KCASH) composed of *REN/KCASH1, KCASH2 and KCASH3*, that bind to the E3 ligase Cul3 complex, leading to the degradation of HDAC1 and consequent hyperacetylation and inhibition of the Gli proteins. Secondly, Gli1 is proteolitycally degraded by the Numb/Itch complex, through the Numb-induced conformational change of Itch enabling its binding and ubiquitination of Gli1.

Finally, I will also discuss novel mechanisms of signal transduction from Hedgehog/Smo activation.

## In vitro and in vivo characterization of a novel Hedgehog signaling antagonist in human glioblastoma cell lines

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Glioblastoma multiforme (GBM) is composed of heterogeneous and genetically different cells which are highly invasive and motile. The standard chemotherapeutic agent, temozolomide, affects GBM cell proliferation but is unable to prevent tumor recurrence. Hedgehog pathway activation has been reported to be relevant in GBM and different pharmacological pathway modulators have been identified. However, the study of Hedgehog pathway modulation in GBM cell lines has been often hampered by the apparent lack of expression of key pathway components in adherent, monolayer cultures. We report that by growing a commercially available recurrent GBM cell line (DBTRG-05MG) without serum in presence of growth factors; we obtained a less differentiated cell population, growing in suspension as neurospheres, in which the Hedgehog pathway is activated. Furthermore, the Hedgehog expression pattern found in DBTRG-05MG neurospheres is similar to primary stem-like cells derived from recurrent GBM patients as observed by qRT-PCR and Western Blot analysis. In this study, we report the specific effect on Hedgehog/GLI pathway of our novel Smoothened receptor antagonist (SEN450) in neurosphere growing cells (3D proliferation, protein modulation and Immunofluorescence). Furthermore we compared SEN450 effect to that of well known benchmark compounds such as GDC-0449, N-[3-(1H-benzimidazol-2-yl)-4-chlorophenyl]-3,5-dimethoxybenzamide (Curis compound) and Cyclopamine. Finally, we showed that SEN450 is both anti-proliferative on its own and further reduces tumor volume after temozolomide pre-treatment in a mouse xenograft model using DBTRG-05MG NS cells. Altogether our data indicate that the Hedgehog pathway is not irreversibly switched off in adherent cells but can be re-activated when exposed to well-defined culture conditions. Even more important, our observations could encourage clinical testing of Smoothened antagonists for GBM.

# Poster Abstracts

## The role of Langerhans cells in development and treatment of skin cancer

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In the white population skin cancers like melanoma and basal-cell carcinoma (BCC) are the most common types of cancer. Superficial BCC can be successfully treated by topical application of Aldara cream, a 5% formulation of the TLR7 agonist Imiquimod. In most BCC patients treated with Aldara a strong immune response is observed and tumor regression is achieved within a few weeks.

We have previously shown that topical Aldara application leads to tumor regression in a mouse melanoma model. Treatment success correlated with the percentage of infiltrating plasmacytoid dendritic cells (pDCs). Additionally, Aldara treatment leads to activation and reversible emigration of Langerhans cells (LCs) from the epidermis. We therefore addressed the question, if the presence of Langerhans cells has any impact on skin tumor progression and whether they play any role in mediating the Imiquimod effect. For that purpose we employed *Langerin-DTR:EGFP* transgenic mice, which allow to ablate LCs upon Diphteria Toxin treatment. These mice were intradermally injected with B16F10 melanoma cells and depleted of LCs before starting with Aldara treatment. Although the absence of LCs inhibited the recruitment of $CD8\alpha^+$ T cells and $CD4^+$ DCs in response to Aldara treatment, our results demonstrate that LCs are not required for inducing the Imiquimod-mediated antitumor response. We are also employing a BCC mouse tumor model (*Rosa26-SmoM2-YFP x K5Cre$^{ERT}$*) and preliminary results show that, in contrast to the melanoma model, Aldara does not lead to tumor regression after 14 days of treatment. We are currently repeating these experiments and extending the treatment time and will additionally study if LCs contribute to the Aldara-mediated antitumor response in this BCC model.

## Atoh1/Math1 function in collaboration with Shh activation during medulloblastoma formation

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Medulloblastoma (MB), a tumor of the cerebellum is the most common malignant pediatric tumor of the central nervous system. MBs are classified in 4 subgroups by gene profiling, two of which have mutations in the Wnt (15%) and Sonic hedgehog (Shh)/Patched (25%) pathways, while no known mutations have been found for the other two subgroups. All patients, regardless of the MB subgroup are treated with conventional therapy that includes surgery, radiation and systemic chemotherapy. One third of patients die from MB and existing therapies for medulloblastoma have severe side effects, which make finding new drugs a priority. In the course to find new therapeutics strategy to target medulloblastoma, my previous studies showed that MB tumor cells treated with Bone Morphogenic Proteins (BMPs) 2, 4 and 7 fail to proliferate and instead differentiate [Zhao*, Ayrault* et al., 2008]. Moreover, tumor cells from murine MBs expressing a retrovirus encoding BMP failed to give rise to MB formation in vivo. Similar results were obtained when tumor cells were pretreated with BMP-4 during three days in culture, followed by their re-inoculation into recipient mice. All together, these studies highlight BMPs as potent inhibitors of MB. Thus, in the effort to gain a deeper understanding of BMP-dependent mechanism, the proneural basic helix-loop-helix (bHLH) transcription factor Atoh1 was found rapidly degraded via the proteasome during cell cycle arrest in tumor cells. In addition, the overexpression of Atoh1 in GNPs and tumor cells overcomes the BMPs' inhibitory effects. More interestingly, unlike many reports showing that Atoh1 is required for cell differentiation, my recent work uncovered a role for Atoh1 in MB formation. Indeed granule neuron progenitors (GNPs) purified from 7 days old (P7) wild type mice and co-infected with retroviruses expressing Atoh1 and Gli1 induced tumors with complete penetrance within the first 50 days after injection in the cortices or the cerebellum of naïve recipient animals. Taken together, these recent studies uncovered a critical role for Atoh1 in medulloblastoma development and demonstrated that Atoh1, in collaboration with Gli1, a Shh-dependent transcription factor, transforms wild type granule neuron progenitors into MB-initiating cells [Ayrault et al., 2010].

Given these findings, our group is focused on the understanding of mechanisms that govern Atoh1's normal cerebellar development and medulloblastoma formation using a multidisciplinary approach. More specifically, we aim to:

1/ Understand the nature of the mechanisms regulating Atoh1.

2/ Understand the role of Atoh1 in normal cerebellar development and tumorigenesis in vivo.

To address these key questions we are developing adapted tools in the area of biochemistry, cell biology and mouse models. We anticipate that characterization of Atoh1 regulation and function both in vitro and in vivo will provide novel insights into Atoh1 as a potential therapeutic target in the treatment of medulloblastoma.

Ayrault O, Zhao H, Zindy F, Qu C, Sherr CJ, and Roussel MF. (2010) Atoh1 Inhibits Neuronal Differentiation and Collaborates with Gli1 to Generate Medulloblastoma-Initiating Cells. Cancer Research, 70, 5618-27.

Zhao H*, Ayrault O*, Zindy F, Kim JH and Roussel MF. (2008) Post-transcriptional down regulation of Atoh1/Math1 by bone morphogenic proteins suppresses medulloblastoma development. Genes and Development. 22, 722-7.

## Sonic Hedgehog and lung cancer

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Lung cancer is the cancer causing the most important number of deaths in men all over the world. However, no effective treatments exist currently and the 5-year survival rate is only 14% for patients with treatment. Hedgehog signalling pathway (Hh) has been recently identified as key player in human cancers including tumours of the skin, brain, prostate and pancreas. Recent but contradictory studies report an expression of Shh in lung cancer cells. In order to elucidate the role of Shh in lung tumorigenesis, we investigated the effect of Shh in lung cancer cell proliferation using different approaches. First, we blocked Shh pathway with cyclopamine, a specific inhibitor of the G-protein coupled receptor Smoothened. When treated with cyclopamine, lung cancer cells showed a significant decrease in cell proliferation. This effect was seen in A549 lung adenocarcinoma cells and in H520 lung squamous carcinoma cells. In a second approach, we used small interference RNAs (siRNA) to silence the three human responding transcription factors of the Shh signaling pathway: Gli1, Gli2 and Gli3. Upon a specific and important reduction in RNA levels of Gli and Gli2, a decrease in cell proliferation was found. On the contrary, the siRNA of Gli3 showed not effect in cell proliferation. Taken together, these results show that Shh signaling pathway plays a role in regulating lung cancer cell proliferation, being Gli1 and Gli2 the main regulators. The effect of Shh in lung cancer cell growth appear to be modulated, in part, by cyclin D1 and cyclin D2, as the expression of cyclin D2 and cyclin D1 decreased significantly when Gli1 and Gli2 were silenced. The expression of Gli transcription factors was then assessed in human cancer tissues by RT-qPCR. The RNA levels of Gli1 and/or Gli2 was found to be higher in some tumors compared with the non-tumor lung tissue from the same patient. Further analysis in tissue coming from these patients will be performed in order to characterize the expression of Shh components in the different cells of these tumors. In summary, Shh pathway was found to play a positive role in lung cancer cell proliferation, through Gli1 and Gli2. The levels of these transcription factors were up-regulated in some human lung cancer tissues, showing that Shh pathway can be (re-)activated in lung cancer cells. The cellular and molecular mechanisms leading to Shh signaling in lung cancer will need further investigations.

## Sonic Hedgehog is required for the activation of Smoothened in the absence of Patched[1]

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The receptors Patched1 (Ptch1) and Smoothened (Smo) regulate the response to Sonic Hedgehog (Shh). Unlike most receptors, the loss of Ptch1 causes an upregulation of Shh pathway activity and the explanation for this observation has been that Ptch1 acts as an inhibitor of Smo. This inhibition is released by the binding of Shh to Ptch1, and Smo is free to signal to downstream signaling molecules. This long-standing model implies that Ptch1 is the key receptor for Shh, and that Shh binding to Ptch1 is the switch for pathway activation.

We demonstrate here, by using assays with high temporal resolution such as Ca2+ mobilization and Transwell migration setups, that the absence of Ptch1 is not sufficient to elicit full Smo activation as was previously assumed, and that the presence of Shh is still required to activate the Shh response in a Ptch1-/- background. Knockdown of Cdo/Boc strongly diminished this response, suggesting Cdo/Boc to be involved in the fast responses to Shh in the absence as well as presence of Ptch1.

We propose a revised and expanded model for activation of the Shh response, in which the initial binding of Shh to Ptch1 does not activate the pathway, but results in a redistribution of Smo, rendering the cells competent to respond to Shh. Subsequently, Shh can activate Smo independently of Ptch1 by acting through Boc/Cdo to induce the Shh response.

## Localization and interaction dynamics of Gli1, SuFu and GSK3β in HEK293 cells

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The Hedgehog-Gli (Hh-Gli) signalling pathway plays a crucial role in embryonic development, but is mainly inactive in adult tissues. Impaired signalling during development can lead to various developmental malformations. Aberrant activation of this pathway in adult tissues has been implicated in the development of cancers in various organs.

Although the basics of the signalling pathway are well known there are mechanisms regulating the pathway that are not fully understood yet. For example, the interactions between the transcription factor Gli1 and its regulators Suppressor of Fused (SuFu) and GSK3β. The mechanism controlling the association of the SuFu/Gli1 complex to microtubules, the role of GSK3β in activated cells or the transport dynamics of these proteins in and out of the primary cilium. To fully understand the functioning of this pathway, it is essential to establish the localization and interaction dynamics between its components.

We used human embryonic kidney cells (HEK293) as a model, since they have adequate expression of pathway genes. Our results revealed an interesting pattern. Gli1, SuFu and their regulator GSK3β co-localized in highly specific cytoplasmic spots of positive staining. These spots also stained positively for γ-tubulin, a centrosomal marker, suggesting that these proteins accumulate in the centrosome, which gives rise to basal bodies of the primary cilia. In our hands the primary cilia were undetectable under tested conditions. After adding exogenous Shh protein the accumulation of these proteins in the centrosomes decreased and translocation of Gli1 to the nucleus was observed.

On the other hand, Ptch and Shh also co-localized in the cytoplasm but not in the centrosome. Gli3, the transcription repressor, showed cytoplasmic localization as well, in the region similar to that of Ptch and Shh.

We have shown an interesting pattern of interactions between the effector of the Hh-Gli signalling pathway, Gli1, and its regulators, SuFu and GSK3β, positioning them all in the same organelle, the centrosome, demonstrating that even without primary cilia these proteins accumulate in specific regions, centrosomes, suggesting that that is where the regulatory processes between them take place.

## Investigating the role of Sortilin in Sonic hedgehog trafficking

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Sonic hedgehog (Shh) is a secreted morphogen that plays a critical role in patterning and growth control in many tissues, most notably parts of the central nervous system. Intra-axonal transport is emerging as an important mechanism for long-range Shh signaling in the central and peripheral nervous system, but the mechanisms of this transport remain largely unknown. Using a GST-based affinity screen, with Shh peptide as bait and rat-brain microsomal fraction as the prey, we identified Sortilin (Sort) as a novel candidate Shh-interacting protein. Emerging evidence suggests that Sort is a diverse sorting receptor, specializing in targeting its ligands to endosomes, lysosomes, and the regulated secretory pathway, making it an attractive novel interacting partner for Shh. To test the characteristics of this interaction two Sort perturbation approaches were used: ectopic expression of a dominant negative, truncated Sort (tSort) and RNAi-mediated Sort knockdown in primary neurons. In fibroblasts and primary neurons, co-expression of Shh with tSort caused a disruption in normal reticular Shh distribution, sequestering it in a perinuclear pattern. In addition, it appears that the effects of Sort on Shh distribution require the Shh cholesterol modification. Surprisingly, knockdown of endogenous Sort in primary neurons increases the level of Shh protein on the plasma membrane and in neural processes, and also resulted in an increase in overlap between Shh and SV2 (synaptic vesicle) puncta relative to shScrambled controls. Taken together, these results suggest that Sortilin functions as a negative regulator of Shh transport in neurons.

## Genetic analysis of Hh/Gli and EGFR interactions in Lgr5+ hair follicle stem cells

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Skin stem cells (SC) marked by Lgr5 expression maintain hair follicle (HF) homeostasis and upon wounding, contribute to epidermal healing processes in mice. Lgr5+ cells harbour an active Hedgehog (Hh)/Gli pathway and intesively proliferate in anagen and during skin regeneration. Constitutive activation of Hh signaling in Lgr5+ SC leads to BCC-like lesions in the mouse skin, supporting the theory of a HF SC origin of BCC. Wounding accelerates and enhances tumor formation in this model.

In our project we investigate the cross talk between the Hh/Gli and EGFR signaling pathway in Lgr5+ HF stem cells. Our previous studies have shown that both pathways synergize in oncogenic transformation. Here we use a mouse model, which expresses tamoxifen-inducible cre recombinase under the Lgr5 promoter (Lgr5cre) to activate Hh/Gli signaling via expression of constitutively active Smo (SmoM2) and at the same time to delete EGFR in Lgr5+ HF stem cells. Skin biopsies were taken from mice at different time points and analyzed for tumor development. Lgr5cre, SmoM2, EGFR +/+ and Lgr5cre, SmoM2, EGFR +/flox mice developed epidermal hyperplasia and BCC-like lesions, which were more pronounced

in the wound area. By contrast, Lgr5cre, SmoM2, EGFR flox/flox mice showed some signs of abnormal hair follicles but notably, only very few BCC-like tumors.

Preliminary results also suggest that EGFR function may be crucial for the migration of Lgr5+ progeny to the wound site as both pharmacological and genetic inhibition of EGFR decrease the number of Lgr5cre-LacZ traced cells in the wound while not decelerating the wound healing process itself. These preliminary data point to an important requirement of HH-EGFR cooperation in stem cell activation and stem-cell derived cancer development.

## Identification and characterization of KCASH2 and KCASH3, two novel suppressors of Hedgehog activity in Medulloblastoma

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Medulloblastoma (MB) is the most common pediatric malignant brain tumor, and arises from aberrant cerebellar precursors development, a process mainly controlled by Hedgehog (Hh) signaling pathway. Histone deacetylase HDAC1 has been recently shown to modulate Hh, deacetylating its effectors Gli1/2 and enhancing their transcriptional activity. HDAC represents therefore a potential therapeutic target for Hh-dependent tumours, but still little information is available on the physiological mechanisms of HDAC regulation. The putative tumor suppressor REN$^{KCTD11}$ acts through ubiquitination-dependent degradation of HDAC1, thereby affecting Hh activity and medulloblastoma growth.

We describe here two REN$^{KCTD11}$ homologues, defining a new family of proteins named KCASH, as "KCTD containing, Cullin3 Adaptor, Suppressor of Hedgehog". Indeed, the novel genes (KCASH2$^{KCTD21}$ and KCASH3$^{KCTD6}$), share with REN$^{KCTD11}$ a number of features, such as a BTB domain required for the formation of a Cullin3 ubiquitin ligase complex and HDAC1 ubiquitination and degradation capability, suppressing the acetylation-dependent Hh/Gli signaling. Expression of KCASH2 and -3 is observed in cerebellum while epigenetic silencing and allelic deletion is observed in human medulloblastoma. Rescuing KCASHs expression reduces the Hedgehog-dependent medulloblastoma growth, suggesting that loss of members of this novel family of native HDAC inhibitors is crucial in sustaining Hh pathway mediated tumorigenesis. Accordingly, they might represent a promising class of endogenous "agents" through which this pathway may be targeted.

## Control of human cancer stem cell behavior and tumor growth by a GLI1-NANOG regulatory loop: essential role of NANOGP8

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Previous work has shown that the HEDGEHOG-GLI signaling pathway is required for glioblastoma multiforme (GBM) growth and stemness (Clement et al, 2007). Moreover, HH-GLI signaling regulates the expression of an ES cell-like stemness signature that includes NANOG, OCT4 and SOX2. To test the functionality of this signature and possible ES-like state we focused on the function of NANOG in GBM, and how it may interact with HH-GLI pathway.

We found that, in vitro, NANOG regulates proliferation and clonogenicity of gliomaspheres, and CD133+ cell proliferation. The knock down of NANOG via RNAi prevented cells to participate in the tumor in orthotopic xenografts, demonstrating that NANOG is essential for GBM tumorigenicity in vivo.

Since NANOG protein can be produced from the NANOG gene (located on chr12) and from a retrogene, NANOGP8 (located on chr15) we tested the relative contributions of each gene in GBMs. We show that all GBM samples tested predominently expressed NANOGP8. This is also found preferentially expressed in CD133+ cells. The specific knock down of NANOG by targeting NANOGP8 leads to a decrease in cell proliferation in vitro, but also prevents affected cells from participating in tumor formation in vivo. NANOGP8 is thus an essential GBM gene.

We also show that NANOG expression is regulated by HH-GLI and identified a positive loop between NANOG/P8 expression and HH-GLI activity. Moreover, NANOG and GLI act in a negative regulatory loop with p53. These results together with the finding that NANOG function is epistatic to HH-GLI activity in vivo allows us to propose the existence of a key GLI1-NANOG node that controls GBM growth and stemness. We suggest that this node operates in other tumor types.

Zbinden M, Duquet A, Lorente-Trigos A, Ngwabyt SN, Borges I, Ruiz i Altaba A. NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. EMBO J. 2010 Aug 4;29(15):2659-74.

Clement V, Sanchez P, de Tribolet N, Radovanovic I, Ruiz i Altaba A. HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity.

Curr Biol. 2007 Jan 23;17(2):165-72.

Stecca B and Ruiz I Altaba A. A GLI1-p53 inhibitory loop controls neural stem cell and tumour cell numbers. EMBO J. 2009 Mar 18;28(6):663-76

## Hedgehog-EGFR cooperation response genes determine the oncogenic phenotype of basal cell carcinoma and tumor-initiating pancreatic cancer cells

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Precise regulation of the Hedgehog/GLI signaling pathway is an important prerequisite for proper embryonic development and tissue homeostasis. By contrast, inappropriate activation plays a critical role in the initiation and growth of a number of human malignancies. Our group has recently identified cooperative interactions of two clinically relevant pathways, the Hedgehog (HH)/GLI and the EGFR pathway (Kasper, M., H. Schnidar, et al. (2006). Mol Cell Biol 26(16): 6283-6298.; Schnidar, H., M. Eberl, et al. (2009). Cancer Res 69(4): 1284-1292.).

The molecular downstream effectors mediating this synergistic interaction of HH/GLI and EGFR pathway were largely unknown. Using time resolved gene expression analysis we could identify a set of "cooperation response genes" (CRG) that determine the malignant phenotype of HH/GLI dependent pancreatic cancer and basal cell carcinoma cells. Furthermore, by using specific pharmacological inhibitors and RNAi mediated knockdown of HH/GLI and EGFR pathway components and/or CRG, we show that these cooperative interactions promote the tumor initiating capacity of putative pancreatic cancer stem cells. These data identify EGFR signaling as valid drug target in HH/GLI driven cancers such as BCC and support the concept of combined treatment by concomitant administration of HH/GLI, EGFR or EGFR-HH CRG antagonists to prevent the growth of HH/GLI-dependent cancers.

## Poster Abstract HEALING International Meeting

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Though Hh and Notch signalling are active in a subgroup of medulloblastoma, it was shown that medulloblastoma with active hedgehog sig nalling in the mouse model can also develop in the absence of Notch signalling. The exact role of Notch in medullo blastoma is still not completely clear. Analysing the interaction of GLI and Notch intracellular domain (NICD1 and NICD2) mediated activation in DAOY cells, we show cooperative Hh and Notch target gene activation in the induction of selected genes which are known as targets of Notch. Luciferase assays support this observation. Conversely, overexpression of NICD1 or NICD2 appears to lead to dimin ished activation of Hh target genes. Kinetics , expression levels and synergy of target gene activation were analysed.

## Deciphering complex cellular signaling networks between Hh/GLI and other pathways implicated in cancer development and progression

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Hedgehog (Hh) signaling plays a pivotal role in many different developmental processes. Besides this, the pathway is needed for stem cell proliferation and tissue repair in the adult organism. A growing amount of evidence points out that a malfunction of the pathway caused by persistent activation or inappropriate reactivation can lead to cellular hyperproliferation and to malignant diseases such as basal cell carcinoma, lung, prostate and pancreatic cancer.

Hence, a precise understanding of the exact molecular mechanisms and dynamics controling the different functions of Hh/GLI is essential. The expression of specific target genes underlying the regulation of Hh signaling is controlled by GLI transcription factors, the downstream effectors of the pathway. Recently, it was proven, that the final pattern of GLI targets can be influenced by numerous other signal cascades like MAPK or PI3K/AKT. However, it still remains to be undeceived how these different signals are crosslinked to modulate the final outcome of gene expression. Therefore, we employed transcriptomic and proteomic tools to investigate the molecular interactions of Hh/GLI with other regulatory networks leading to cancer. Technically, Illumina Chip assays and protein microarrays were used to generate a sound set of quantitative and time-resolved data on the level of transcriptome and proteome, respectively. By now, several genes and proteins co-regulated by an interplay between Hh/GLI and receptor tyrosine kinase induced signaling were identified. Further experiments are ongoing to shed light on the physiological impact of this crosstalk. Additionally, the data obtained from gene expression analysis and protein microarray based studies is integrated into a computational model of Hh signaling. This will allow us to predict how a cell reacts under specific conditions. Finally, the interplay between biological experiments and computational modeling will be used to improve the search for drug targets which may help to overcome cancers induced by aberrant Hh/GLI signaling.

**Ectodysplasin and Wnt pathways are required for salivary branching morphogenesis.**

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The developing submandibular salivary gland (SMG) is a well studied model for tissue interactions and branching morphogenesis. Its development shares similar features with other ectodermal appendages such as hair and tooth. The ectodysplasin (Eda) pathway is essential for the formation and function of a number of ectodermal organs. Mutations in the signaling components of the Eda pathway lead to a human syndrome known as hypohidrotic ectodermal dysplasia (HED) characterized by missing and malformed teeth, sparse hair, and reduced sweating. HED patients suffer also from dry mouth due to reduced saliva flow. In order to understand the underlying mechanism, we analyzed salivary gland development in mouse models with altered Eda pathway activities. We found out that Eda regulates growth and branching of the SMG via transcription factor NF-κB in the epithelium, and that the hedgehog pathway is an important mediator of Eda/NF-κB. We also sought whether a similar reciprocal interplay between the Eda and Wnt/β-catenin pathways known to operate in other skin appendages, functions in developing SMG. Surprisingly and unlike in developing hair follicles and teeth, canonical Wnt signaling activity did not co-localize with Edar/NF-κB in salivary gland epithelium. Instead, we noted high mesenchymal Wnt activity and show that ablation of mesenchymal Wnt signaling either in vitro or in vivo compromised branching morphogenesis. We also provide evidence suggesting that the effects of mesenchymal Wnt/β-catenin signaling are mediated, at least in part, through regulation of Eda expression.

## Cancer Pathway Discovery in Mouse Model of Medulloblastoma

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Objective: Aberrant Hedgehog (Hh) target gene activation causes a significant percentage of medulloblastomas (MB), the most common pediatric brain tumor. In our ptch +/- ;Math1-GFP mouse model of Hh dependent MB, 15% of mice develop MB, however, >90% develop pre-tumor lesions that do not progress to MB. This is a unique pre-tumor model that can be used to identify additional genetic changes contributing to tumorigenesis. Materials and Methods: DNA was prepared from MB and normal surrounding cerebellar tissue from ptch +/- ;Math1-GFP mice, and used for whole genome sequencing. The data were analyzed, aligning the mate-pair reads to the reference mouse genome (NCBI Build 37, mm9). Chromosomal structural variants (insertions, deletions, duplications, inversions, and translocations) were detected in each sample separately. Variants that appeared in the tumor samples but not in the corresponding normal samples were selected, as tumor-specific variants are likely to be relevant to MB development. These data are being compared with human and mouse MB gene expression data, to derive new hypotheses about genes contributing to the development of MB. Results: We identified fifteen distinct genetic mutations that occur in MBs and not in normal surrounding cerebella.

Conclusion: The identification of combinations of genetic lesions that can trigger or sustain MB will guide hypotheses about tumor development, paving the way for new and more effective medical therapies. We will proceed to test these hypotheses in our mouse model, ptch+/-; Math1-GFP, which most accurately represents the human pediatric disease.

## Loss of Trp53 Promotes Medulloblastoma Development but not Skin Tumorigenesis in Sufu Heterozygous Mutant Mice

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Sporadic basal cell carcinoma (BCC) of the skin is the most commonly diagnosed human cancer among Caucasians. These tumors typically carry genetic alterations in components of the hedgehog (HH) signaling pathway. Previously, we generated a knockout mouse with a loss-of-function mutation in suppressor of fused (Sufu), an essential repressor of the pathway downstream of the Hh ligand cell surface reception. Embryos homozygous for the mutated Sufu allele die at E9.5 with severe cephalic and neural tube defects. Mice heterozygous for the mutation develop a skin phenotype at four to six months of age that includes lesions similar to basaloid follicular hamartomas. The purpose of the current study was to test the possibility that the simultaneous loss of the tumor suppressor gene, Trp53, would aggravate the Sufu skin phenotype into full-blown BCCs, since Trp53 loss is known to enhance the growth of other Hh-driven tumors. In fact, mutations in the human TP53 gene often coexist with mutations in the HH signaling pathway in human BCCs. Consistent with previous reports, medulloblastomas and rhabdomyosarcomas developed in $Sufu^{+/-}$;$Trp53^{-/-}$ mice. However, the characteristic $Sufu^{+/-}$ skin phenotype was not altered in the absence of Trp53, and showed no changes in latency, multiplicity, cellular phenotype or proliferative capacity of the basaloid lesions. This finding demonstrated a differential, tissue-specific sensitivity to Sufu and Trp53 tumor suppressor gene loss, suggesting that Hh and p53 pathway cooperativity is intimately linked to stemness, developmental stage and the degree of proliferative activity in specific cell types.

## Characterization of a mouse model of wound-induced tumour formation

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Mice that constitutively overexpress MEK1 specifically in the differentiated layers of the epidermis provide a mouse model of wound-induced tumour formation. The skin of these mice exhibits epidermal hyperproliferation and chronic inflammation [1]. When a full thickness wound is made in the back skin up to 80% of wounds form a benign tumour (papilloma) within 30 days. Once formed, the tumours rarely regress and some progress to low-grade squamous cell carcinomas (keratoacanthomas). Previous work in the lab has established that tumour formation is dependent on recruitment of an inflammatory infiltrate, and that γδT cells and macrophages are involved [2]. We are currently looking into the inflammatory mechanisms that are involved in tumour formation to be able to pinpoint crucial signaling pathways involved in tumour initiation.

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## Indian hedgehog signalling regulates cell proliferation, differentiation and p53 function in skin tumours

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In mammalian skin, *Sonic hedgehog (Shh)* induces hair follicle formation and cyclic hair regeneration by controlling proliferation and morphogenesis. Indian hedgehog (Ihh), another Hh ligand, also activates the Hh pathway in the epithelium of the skin. *Ihh* is expressed in sebaceous glands, hair follicle associated epidermal appendages, which secrete sebum to lubricate and protect the skin. Interestingly, components of the Hh pathway are up-regulated and activity of the pathway is increased upon differentiation of human sebocytes in vitro. The *in vivo* function of Ihh for development and homeostasis of the skin and patho-physiological conditions has not been identified yet.

To investigate the function of Ihh signalling for skin development and tumour formation, we generated epidermis specific *Ihh* knockout *($Ihh^{EKO}$)* mice. Our results indicate that the absence of Ihh significantly inhibits proliferation in early skin development. However, morphogenesis of sebaceous glands, hair follicles and the interfollicular epidermis was not disturbed.

Next, we examined the role of Ihh signalling for skin tumour formation utilising various mouse tumour models. Here we show that in sebaceous tumours, Ihh significantly stimulates tumour growth and induces sebocyte differentiation. Furthermore, in squamous epidermal tumours Ihh signalling promotes proliferation and regulates p53 protein levels. Our results clearly demonstrate that regulation of p53 is a general function of Ihh in keratinocytes and that Ihh plays an important role in patho-physiological conditions of the skin.

## Active wound healing can accelerate tumor formation

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** These authors contributed equally.*

Active wound healing is a well-established risk factor for development of epithelial-derived skin tumors. Basal cell carcinomas (BCCs) are the most common skin cancers displaying a number of features reminiscent of hair follicle (HF)-derived cells and are dependent on deregulated Hedgehog (Hh)/GLI signaling. We used three different mouse models to investigate the effect of wounding on the development of BCC: mice overexpressing the positive regulator of the Hh-pathway GLI1 in basal cells under the keratin 5 promoter (K5–GLI1), mice with an inactivated negative regulator of Hh, patched 1 ($Ptch1^{fl/fl}$) in cells that expressed K5 ($K5Cre–Ptch1^{fl/fl}$) and mice where oncogenic mutations were introduced only in Lgr5-expressing hair follicle stem cells ($Lgr5Cre\text{-}Ptch1^{fl/fl}$). The results show that an active wound healing process can increase both the number and size of the tumors. The increase in tumor size is likely due to a general increase in cell proliferation taking place in association with wound healing whereas the increase in number can be attributed to the recruitment of cells emigrating from the hair follicle and having potential to initiate tumor formation. Moreover, the study shows that only full-thickness wounds, but not superficial wounds, were sufficient to recruit and integrate hair follicle cells and their progeny into the IFE. *The study concludes that, as a result of the wound healing process, hair follicle stem cells and their progeny acquire the ability to initiate tumor formation outside their natural habitat.*

## Hh-Gli signaling in ovarian carcinoma

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Hh-Gli signaling is implicated in ovarian carcinoma, but its role in initiation and growth of the tumor is not well defined.

We detected expression of *PTCH1, SMO, GLI1, SHH , SUFU,* and ß-catenin genes in ovarian carcinoma. Also Ptch, Smo, Gli1 and Shh protein expression was found, while Ptch1 was shown to be associated with FIGO stage. Also, we found correlation of Hh-Gli signaling pathway genes *PTCH1, SMO* and *SUFU. PTCH1* was found correlated with *BRCA1* and *BRCA2,* and *BRCA1* with survivin.

Primary culture of high grade ovarian cancer (gradus III) shows down regulation of *PTCH1, SMO, GLI1, SUFU* and *ß-catenin* genes with higher doses of cyclopamine (7,5 µM). Also, proliferation of the cells was blocked 48-72 h with cyclopamine (dose response 0,5 – 7,5 µM) or curcumin (between 15 - 30 µM) but was increased with Shh (in doses 2,5 - 7,5 µM).

In SKOV3 ovarian cancer cell line we detected *PTCH1, SMO, GLI1, SHH , SUFU, c-Myc* and ß-catenin gene expression and Ptch1, Smo, Gli1 and Shh protein expression. Proliferation of these cells was blocked with cyclopamine or curcumin (24-72 h) and addition of Shh haven't shown increased proliferation.

Gli1 transfected SKOV3 cells have higher *PTCH1, SMO, GLI1, GLI2* and *SUFU* expression, but not *GLI3*. Also, siRNA *PTCH* down regulates *PTCH1* expression. Interestingly, upon addition of Shh within 4 h, transwell cell migration assay showed an increase in migration capability of the cells, suggesting pathway activation has a role in migration, and possibly metastases.

Those results might have implications in search for new molecular targets for ovarian carcinoma.

## Biochemical studies of Kif7 in the mammalian Hedgehog signalling pathway

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The Hedgehog (Hh) family of secreted signalling proteins is important for the embryonic development of metazoans and tissue homeostasis in adults. Disruption of the Hh signalling cascade has been found to contribute to neural tube disorders, limb malformations, and multiple forms of cancer. Kinesin family member 7 (Kif7) is a negative regulator of the mammalian Hh pathway, but its function within the pathway is still not well understood, but is thought to contribute in the conversion of Gli protein from transcriptional activators to repressors. A better understanding of the functional roles of Kif7 and its interaction with other regulators and effectors within the Hh pathway is important for our understanding of the Hh signalling. Here, biochemical approaches are utilized to study the properties of endogenous protein complexes containing Kif7 (size exclusion fractnation), and to identify and characterize novel interactors of Kif7 (LC-MS/MS) that are likely contributing to its function. From preliminary data, biochemical analyses of Kif7 endogenous complexes suggest that at steady state Kif7 exists in large protein complexes that contain the Gli2 transcription factor. Further comparative proteomic analysis by LC-MS/MS of Kif7 showed that Kif7 interacts with a novel interactor Liprin-alpha (PPFIA1). Our preliminary observations suggest that the knock down of PPFIA1 affect Kif7 translocation to the tip of primary cilia during pathway activation a process previously associated with the activity of Kif7. More experiments are needed to confirm and support the above observations. Overall, given the implications of Hh signalling in developmental and cancer diseases, a better understanding of the regulators of the pathway and their dynamic interactions with downstream effectors is important.

## Role of diverse signalling pathways in neuroblast maintenance and proliferation in Drosophila

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*Institute of Molecular Biology & Biotechnology, FORTH and Department of Biology, University of Crete*

Hedgehog (Hh) signalling plays an important role in development and its perturbation is associated with tumorigenesis in many mammalian tissues, including the CNS. Since many of the signalling cascades are conserved across species, we were interested to look for Hh role in Drosophila neuroblasts (NBs). NBs are stem cell like precursors, which divide asymmetrically to self-renew and generate a ganglion mother cell (GMC), which divides once to generate neurons. NB divisions must be tightly regulated to maintain homeostasis. We are analyzing NB and differentiated cell type markers in mutants of key components of Hh and other signalling pathways, such as Notch, Dpp/BMP, JAK-STAT, in order to understand the role of these pathways in maintaining NB homeostasis during larval CNS development. From expression profiling studies we know that these pathways are expressed in the larval CNS.

Preliminary mosaic analysis for loss of function (LOF) mutants in the reception of each of the Hedgehog, Notch and Dpp/TGFβ signals did not seem to affect the NBs' identity or their differentiation ability. JAK-STAT pathway disruption resulted in NB division defects with partial penetrance and is being pursued further.

The inability of lof Notch mutations to perturb NB proliferation is somewhat of a paradox, since several other pieces of accumulated evidence point to a pro-proliferation role of this signalling pathway in larval neurogenesis. This raises the interesting possibility of signalling pathway redundancy in this process. We are using double mutant combinations to address this question. Preliminary evidence (controls pending) suggests that Hh – BMP double disruption may have a severe effect on NB proliferation, whereas Notch-BMP disruption does not seem to cause any defects.

## Analysis of JUN/AP1 function in Hedgehog/GLI-induced skin cancer

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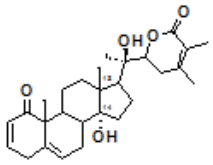
The Hedgehog (HH)/GLI signaling pathway has been implicated in the development and growth of numerous human malignancies such as basal cell carcinoma (BCC), medulloblastoma or prostate and pancreatic cancer. A growing body of evidence suggests that control of oncogenic HH signaling involves interactions with other pathways frequently activated in human malignancies. In line with these findings, we have shown that Epidermal Growth Factor Receptor (EGFR) signaling synergizes with Hedgehog/GLI in transcriptional activation of selected GLI target genes and in oncogenic transformation via activation of the AP1 transcription-factor cJun *in vitro*. Here we analysed the *in vivo* role of Jun/Ap1 and Hedgehog/Gli cooperation in mouse models of basal cell carcinoma. We demonstrate that RNAi mediated inhibition of Jun/Ap1 in murine BCC cells decreases tumor growth in allograft experiments. However, genetic deletion of cJun in wild-type keratinocytes does not interfere with SmoM2/Gli driven tumor development in BCC mouse models, but rather leads to enhanced tumor formation. We will discuss possible mechanisms that may account for the enhanced tumor phenotype of Jun deficient mice, including alterations in epidermal cytokine expression profiles and attenuation of oncogene-induced senescence processes.

## Studies towards the synthesis of Withanolide K

*Laura Manicassamy, Dr. Thorsten Genski (AnalytiCon Discovery GmbH, Potsdam, Germany), Pr. Richard Taylor (Department of Chemistry, University of York, UK)*

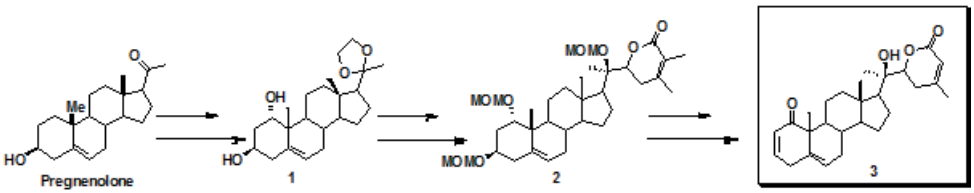


Withanolide K

Embryonic patterning pathways, such as Wnt, HH [1] and NOCHT, play critical roles in human cancer. Inhibition of these pathways can lead to tumor cell proliferative arrest and death. We have therefore started to investigate natural small molecules that have anti-tumoral effects in vitro.

We have focused our attention on Withanolides, a class of natural compounds known to show anticancer activity in vitro and characterised by an ergostane type steroids structure in which C22 and C26 are appropriately oxidised in order to form a □-lactone ring [2]. Withanolide K is a member of this class extracted from Withania sp which has not been studied in detail.

In the course of our synthetic studies towards the framework of the steroid Whitanolide K, we became interested in the influence of the C14 hydroxyl group on the biological activity of the target. In order to determine this role, the total synthesis of the molecule lacking this hydroxyl group is sought first. (Scheme 1).




Scheme 1

Herein the efforts to transform the commercially available pregnenolone into molecule 1 will be reported. Then further synthetic manipulations of this molecule 1 upon which the lactone formation [3] should lead to the synthesis of 3.

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## Mapping GLI interactions

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Several intracellular signaling pathways have been implicated in the control of neurogenesis in vertebrates, notably including the the Shh-Gli pathway (e.g. Lai et al., 2003; Palma and Ruiz i Altaba, 2004; Palma et al., 2005).

Gli proteins are obligatory mediators of canonical Hh signals but it is not clear how they act and interact. It has been proposed that the crucial step in a cell's response to HH signals is the overall balance of positive and negative Gli functions: the Gli code (Ruiz i Altaba 1997; Ruiz i Altaba 1998). We have previously shown that Gli proteins are part of a combinatorial network of cooperative interactions that regulates Gli function and target gene expression (Van Nguyen et al., 2005). The ability of this protein network to act in a context-dependent manner is most probably reliant upon the availability of interacting cofactors and varying Gli-Gli interactions. In this sense, the Gli code may depend on the types of cooperative interactions present and the types of factors that dock on or interact with the network. The cooperative and combinatorial function of Gli proteins could also be critical in cancer (e.g. Ruiz i Altaba et al., 2004) as endogenous Gli1 and Gli3 have been shown to be required for the induction of frog epidermal and neural tumors by exogenous GLI1 (e.g. Dahmane et al.,1997; Dahmane et al., 2001). To further analyse how this network works we are using biomolecular fluorescence complemementation to analyse and map such interactions in vivo. We will present a our current efforts to this end.

## Abstract

*Zaher Nahle*

Deregulation of the Rb/E2F tumor suppressor complex and aberrantion of Sonic hedgehog (Shh) signaling are documented across the spectrum of human malignancies. Exaggerated *de novo* lipid synthesis is also found in certain highly proliferative, aggressive tumors. Here, we show that in Shh-driven medulloblastomas, Rb is inactivated and E2F1 is upregulated, promoting lipogenesis. Extensive lipid accumulation and elevated levels of the key lipogenic enzyme fatty acid synthase (FASN) mark those tumors. In primary cerebellar granule neuron precursors (CGNPs), proposed Shh-associated medulloblastoma cells-of-origin, Shh signaling triggers E2F1 and FASN expression, whereas suppressing fatty acid oxidation (FAO), in a smoothened-dependent manner. Importantly, E2F1 is required, both *in vivo* and *ex vivo*, for FASN expression and CGNP proliferation, and E2F1 knockdown impairs Shh-mediated FAO inhibition. Furthermore, pharmacological blockade of Rb inactivation and/or lipogenesis inhibits CGNP proliferation, drives medulloblastoma cell death and extends survival of medulloblastoma-bearing animals. These findings identify a novel mechanism where Shh signaling links cell cycle progression directly to lipid synthesis, through E2F1-dependent regulation of lipogenic enzymes. This work, pertinent to the etiology of tumor metabolism in neurological malignancies, also underscores the key role of the Shh→E2F1→FASN axis in regulating lipid synthesis in cancers, and as such its value as a global therapeutic target in hedgehog-dependent and/or Rb-inactivated tumors

## Mammary gland tumour f ormation in nulliparous GLI1 expressing transgenic mice

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Up regulation of the Hedgehog pathway effector GLI1 in breast cancer correlates with unfavourable overall survival. The Hedgehog pathway has a role in the regulation and maintenance of CD44 positive breast cancer stem cells. Skin and intestinal stem cells express the orphan G protein coupled receptor (GPCR) LGR5. We show th at the cells of the basal cell layer of the large mammary ducts are Lgr5 positive . Lgr5 is also expressed in mammary gland tumours induced in conditionally transgenic mice expressing GLI1 in the mammary gland. Previously, we have shown that multiparous con ditional transgenic mice (MMTVrtTA;TREGLI1) expressing GLI1 develop hyperplastic lesions and tumours. Hyperplastic lesions and palpable mammary gland tumours also develop in nulliparous transgenic mice, after long term low level GLI1 expression. Both solid and acinar adenocarcinomas develop in GLI1 expressing nulliparous mice, even within the same mammary gland. The expression of the stem cell marker CD44 is increased in the mammary ducts as well as the tumours in the GLI1 expressing mice. The GLI 1 induced mammary gland tumours are cytokeratin 5 (K5) and cytokeratin 6 (K6) positive. Taken together these data indicate that long term low level expression of GLI1 induces formation of mammary gland tumours with a basal character and that GLI1 expressio n affects the mammary gland stem cells. The orphan GPCR Lgr5 is expressed in the basal cell layer of the large mam mary ducts and might include a mammary stem cell population.

## Hedgehog Can Be Secreted in Lpp-Associated and Lpp-Free Forms

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Hedgehog (Hh) carries two lipid modifications that confer a high affinity to cellular membranes, but is nevertheless secreted in soluble complexes that move through the extracellular space. Long-range Hh signaling in *Drosophila* imaginal tissues requires association with the lipoprotein Lipophorin (Lpp), whose scaffolding protein is homologous to human apolipoprotein B. Short-range signaling does not require Lpp, suggesting that long and short-range Hh signaling might depend on different forms of secreted Hh. Hh is released into the hemolymph, making the *in vivo* secretion of Hh accessible to biochemical analysis. Using a series of Hh mutants, we show that Hh lipid modifications are required for Lpp association, while not being required for Hh secretion. While wild-type hemolymph Hh quantitatively associates with Lpp, Hh variants lacking the cholesterol moiety are partially secreted in a Lpp-free form. Similarly, when Lpp levels are limiting, either due to Lpp knockdown or Hh overexpression, Hh is released in a Lpp-free form. Lpp-free Hh is of high density and has a molecular weight corresponding to a Hh dimer. Furthermore, Lpp-free Hh is less hydrophobic than Lpp-associated Hh and has the same electrophoretic mobility as Hh variants that lack the cholesterol moiety. Together, this strongly suggests that if Lpp levels are limiting, Hh is released from cellular membranes by cleavage of the cholesterol anchor. We previously showed that Lpp lipids repress the Hh signaling pathway by inducing the degradation of full-length $Ci_{155}$; conversely, Lpp depletion stabilizes $Ci_{155}$ in the whole wing disc. We now establish that Lpp-associated Hh delivered through the hemolymph can stabilize $Ci_{155}$ in the wing disc, suggesting that it neutralizes the repressive effects of Lpp lipids. However, this accumulation of $Ci_{155}$ is not sufficient to activate target gene expression. We currently define the signaling activity of Lpp-free Hh in the presence and absence of Lpp.

## Senescence in Human Basal Cell Carcinoma

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Oncogene-induced senescence (OIS) has an important role in tumour biology as it represents a physiological response that restricts the progression of benign tumours into their malignant counterparts. Full malignancy is associated with the loss of important tumour suppressor genes including *RETINOBLASTOMA* and/or *TP53*.

Basal cell carcinoma (BCC) of the skin is the most common skin tumour form and is associated with mutational inactivation of the *PTCH1* tumour suppressor gene (and less frequently oncogenic activation of *SMOOTHENED*). Although BCC does not appear to stem from precursor lesions and is relatively stable at the genomic level, we sought to determine if these unique tumours display any characteristics of OIS.

Human BCCs were positive for Senescence-associated β-galactosidase (SA-β-gal) activity (pH 6.0). Interestingly, SA-β-gal activity was observed in stromal cells surrounding the tumour islands but only weakly in the tumour epithelium; this may be due to UV irradiation which is known to induce senescence in cultured fibroblasts and may also account for why BCCs are difficult to culture *in vitro.* Both tumour epithelium and stroma also expressed known markers of senescence including DCR2, SHARP2 (DEC1) as well as the cell cycle inhibitors p15, p16 and p21. Moreover,

To determine if OIS is associated with Hedgehog signalling in BCC, we employed a novel *in vitro* model of BCC created through *PTCH1* suppression in human immortalised NEB1 keratinocytes. NEB1-shPTCH1 cells are viable and proliferative (albeit more slowly than control NEB1-shCON cells) although they do not display SA-β-gal activity they express higher levels of senescence markers. These data suggest that senescence in BCC is associated with Gli expression and may explain why BCC rarely metastasise.

## Regulation of human SOX14 gene expression by SHH signaling pathway

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*SOX14/Sox14* gene is a member of *SOX* gene family and its expression is restricted to a limited population of neurons in the developing brain and spinal cord in mouse and chick. The expression of *Sox14* gene in spinal cord explants was found to be regulated by *Sonic hedgehog* (SHH) in dose dependent manner. SHH signaling pathway enables downstream transcription factors to regulate expression of target genes involved in the control of developmental processes including embryonic patterning, CNS development, cell survival and growth, vascularization and tumorogenesis. Specific objective of this study has been to investigate the regulation of human *SOX14* gene expression by members of SHH signaling pathway in tumor cell lines. We have identified the positive control element within the *SOX14* promoter and provided the first evidence that *Forkhead transcription factor A2* (FOXA2), is involved in the up-regulation of *SOX14* gene expression in HepG2 and U87MG cell lines. By functional analysis we have demonstrated that mutation in FOXA2 binding site reduced the *SOX14* promoter reporter construct activity, while FOXA2 over-expression increased endogenous SOX14 protein expression. Further, we have shown that human *SOX14* gene expression is GLI1 depended in U87MG cells and SHH-N dependent in U87MG and HepG2 cell lines. By siRNA silencing of *FOXA2*, we have demonstrated that up-regulation of endogenous *SOX14* gene expression by SHH is, at least in part, mediated by FOXA2. The presented data provide the initial insight into molecular mechanism underlying regulation of *SOX14* gene expression mediated by SHH signaling pathway.

## The role of Gli in Hedgehog dependent neural progenitor proliferation

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The Sonic hedgehog (Hh) signaling pathway is a critical regulator of growth and patterning in a wide variety of tissues and organs, including the brain. Perturbation of this pathway has been linked to the progression of various types of cancers and developmental abnormalities.

The retina is a tractable model system to study the effects of both Shh signaling and neuronal differentiation. The retina is a relatively simple tissue, comprised of six neuronal and one glial cell type that are derived from a common pool of multipotential retinal progenitor cells (RPCs). During development, Shh is secreted from post-mitotic retinal ganglion cells (RGC) and signals to retinal progenitor cells (RPCs). Ectopic activation of the Hh pathway, both *in vivo* and *in vitro*, increases RPC proliferation, while loss of activity reduces RPC proliferative capacity. At later stages of retinal development, Shh signaling is required for the development of bipolar and Müller glial cells.

How this important signaling pathway affects these different developmental processes is largely unknown, but is likely to involve the downstream mediators of the Hh signaling pathway, the Gli transcription factors. In the retina, Hh target gene expression and proliferation require Gli2, but not Gli1. The goal of this research is to investigate how Gli2 is regulated during retinal development and it is hypothesized that the regulation of Gli2 is a key control point for Hh mediated neural progenitor proliferation.

To determine the expression pattern of Gli2 during retinal development, *in situ* hybridization was performed on tissue harvested at key time points during retinal development. Gli2 message is initially expressed in the developing neuroblast layer at embryonic stages becoming restricted to a thin band in the inner nuclear layer, likely the Müller glia, late postnatally. Similarly, Gli2 protein is detectable only at early stages of retinal development, suggesting that down regulation of Gli2 may be associated with neuronal differentiation.

To evaluate the effects of gain of function for Gli2, Gli2-GFP fusion proteins were transfected into primary retinal explant cultures. Ectopic Gli2 expression in results in increased proliferation, but is undetectable at later stages of the culture. Surprisingly, ectopic expression of Gli2 *in vitro* results in only minimal Shh target gene induction, but strongly activates a Gli-dependent luciferase reporter, suggesting that additional regulators may impact the ability of ectopic Gli2 to induce target genes.

Based on results thus far, restricting Gli2 activity might be a mechanism utilized by RPCs to activate the differentiation program and forced expression overcomes this process, resulting in proliferation.

## Shh activity regulates the neural stem character of neuroepithelial cells

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During the development of the nervous system, neural progenitor cells either stay in the pool of proliferating stem cells or exit the cell cycle and enter the differentiation pathway, in the process called neurogenesis. Both intrinsic and extrinsic factors determine the fate of a neural progenitor cell after mitosis to control that, both daughter cells remain as progenitors (symmetric P-P division), one daughter cell enters the differentiation pathway (asymmetric P-N division) or both daughter cells differentiate (symmetric N-N division).

Early experiments performed in our laboratory provide evidence that the Shh pathway promotes the proliferation and survival of neuroepithelial stem cells. In addition we demonstrated that, in the developing nervous system, both the Wnt and Shh pathways genetically interact to control the expression the D-type cyclins and the progression of the G1 phase of the cell cycle. Based on these data, we are currently investigating the impact of Shh pathway on cell-fate decision by forcing neural progenitors to cycle and studying the consequences on specification and differentiation programs. In the chick spinal cord, we observed that keeping neural progenitor cells cycling by forcing the expression of the D-type cyclins is not sufficient to retain them as stem cells in the progenitor domain (ventricular zone). Furthermore, cycling cells located in the mantle zone do not retain markers of neural progenitor cells such as Sox2 but progress through neuronal differentiation, acquire neural markers and extend axonal projections. These findings indicate that maintaining neural progenitor cells in proliferation is insufficient to prevent differentiation or to alter the initiation of the neurogenic program. However, we show that activation of the Shh pathway by either introduction of the dominant-negative form of PKA (dnPKA) or the activated version of the Gli3 transcription factor (Gli3A), is sufficient to cause overgrowth in the ventricular zone without causing a dramatic inhibition of primary neurogenesis. Quantitative analysis showed however a significant increase in progenitor cell markers suggesting that Shh activity might regulate the maintenance of the progenitor state of neuroepithelial cells.

## CYCLOPAMINE AND TAMOXIFEN SHOULD NOT BE USED TOGETHER FOR TREATMENT OF BREAST CANCER

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It has been demonstrated that the Hedgehog signaling pathway may be one of the targets for treatment of breast cancer. Therefore, we decided to test the effects of combined treatment of Tamoxifen, a commonly used breast cancer therapeutic which inhibits estrogen receptor, toghether with Cyclopamine, the Hedgehog pathway inhibitor.

First, we determined the effect of Tamoxifen on two different breast cancer cell lines: MCF-7, which is estrogen receptor positive (ER+), and SkBr3, which is estrogen receptor negative (ER–). As expected, Tamoxifen caused a decrease in cell viability, which was more pronounced in ER+ cell line, compared to ER– cell line. Hedgehog signaling pathway proteins (Ptch, Smo, Gli1, Shh, SuFu) were detected on both mRNA and protein level in both cell lines, although Gli1 gene expression was barely detectable in ER– cell line.

Cyclopamine treatment of these cells caused a decrease in viability, which was more pronounced in the ER+ cell line. On the other hand, induction of the pathway with exogenous Shh protein induced proliferation of these cells, with a more pronounced effect on ER– cell line.

Combined treatment with Cyclopamine and Tamoxifen demonstrated an unexpected result in ER+ cell line: the survival of the cells was dramatically increased compared to either treatment alone, suggesting that the combination of these two drugs somehow enables cell survival. This effect was the opposite in ER– cell line, where combined treatment reduced cell survival. Combined treatment induced the Hedgehog pathway gene expression in ER+ cells, and downregulated it in ER– cell line. Transwell migration assay showed a similar effect on ER+ cell line, with reduced migration with either Cyclopamine or Tamoxifen alone, which recovers to control levels when these two compounds are used together.

These results suggest that the combined treatment with Tamoxifen and Cyclopamine may be very dangerous to use in patients, especially patients with ER+ tumors. Further research in this area is vital before any application in clinic.

## A genome-wide search for novel Hedgehog signalling regulators involved in Cubitus interruptus (Ci) processing

*Spencer Spratt, Satoshi Hasegawa, Umesh Gangishetti, Michiko Arai, Yifei Wang, Marco Tsui and Mary Ann Price.*

Hedgehog (Hh) signalling is a highly conserved signal transduction pathway used again and again during development and in the adult of multicellular animals. Its inappropriate activation is implicated in numerous cancer types in humans. Cells respond to Hh primarily through altered transcription of specific target genes using members of the Gli family of zinc finger transcription factors. In Drosophila the signal is mediated by a single Gli family member, Cubitus interruptus (Ci). In the absence of Hh, full-length Ci ($Ci^{FL}$/Ci-155) is processed by partial proteasomal degradation into a shorter form ($Ci^{R}$/Ci-75) that represses some target genes. In the presence of Hh, processing is blocked and $Ci^{FL}$ is converted to a transcriptional activator ($Ci^{ACT}$). This can lead to target gene activation via derepression or activation by $Ci^{ACT}$. Still, few genes are known to be involved in Ci processing and we do not fully understand the mechanism. Thus, we have undertaken a genome-wide double-stranded RNA interference (RNAi) screen that tries to directly identify regulators of Ci processing. Using a stable Drosophila Kc cell line expressing the Firefly-Ci-Renilla luciferase reporter construct, we identified over 200 candidate genes. Here we describe this screening methodology and our approaches to validate candidates using both cell culture and in vivo Drosophila techniques.

## Inhibition of Cytosolic Phospholipase $A_2$ as a Novel Mechanism Suppressing Cell Cycle Progression and Tumorigenicity of Lung Carcinoma Cells

*S.Sundarraj and S.Kannan*

Cytosolic phospholipase $A_2$ produces free arachidonic acid and lysophospholipids contributes to the production of eicosanoids and platelets activating factors, we show that the expression and activity of the cytosolic phospholipase $A_2\alpha$ in lung cancer cell lines. Blocking $cPLA_2\alpha$ activity with the pharmacological inhibitor wyeth-1 induces cell cycle arrest in G2/M phase. Inhibition of $cPLA_2\alpha$ activity also leads to modest increases in apoptosis of lung cancer cells. The G2/M phase accumulation is accompanied by increased levels of the cell cycle regulators, cyclin E but no cyclin D1 expression. Interestingly, the G2/M phase arrest is released by supplementing the growth factors, LPL (L-$\alpha$-lysophosphatidyl choline). However, inhibition of $cPLA_2\alpha$ activity with wyeth-1 effective in repressing growth factor stimulated proliferation of lung cancer cells through s phase arrest. $cPLA_2\alpha$ treated with wyeth-1 inhibited cell proliferation in culture and tumorigenicity of lung cancer cell lines in nude mice. These results indicate an essential role for $cPLA_2$ in cell cycle progression and tumorigenesis of lung carcinoma cells.

## Lipoproteins in mammalian Sonic hedgehog release and signaling

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Hedgehog (Hh) proteins, due to modifications by cholesterol and palmitic acid, have high affinity towards cellular membranes yet can be secreted and transported over many cell diameters. Mechanisms responsible for secretion and spreading of these hydrophobic morphogens are still not fully understood.

Our group has shown that in *Drosophila melanogaster* lipoproteins help to mobilize Hh from the plasma membrane and are required for its long range transport. We have also shown that lipoproteins can actively influence Hedgehog signaling pathway. However, whether lipoproteins can influence mammalian Hh pathway in a similar manner has not been elucidated.

Here, we show that Sonic hedgehog (Shh), one of three mammalian Hh counterparts, can be secreted from the human cells in monomeric or lipoprotein-associated forms. These forms differ in terms of their lipid modifications, with monomer lacking, and lipoprotein-associated form possessing cholesterol modification. Shh can be secreted on all vertebrate lipoprotein classes, and sterolation, but not palmitoylation, is required for this process. The association with lipoproteins most likely depends on interaction with heparan sulfate proteoglycans. Moreover, we show that the mechanism responsible for secretion of Hh proteins on lipoproteins is conserved between mammals and fruit fly.

We also show that monomeric and lipoprotein-associated forms of Shh have different signaling properties. Signaling of monomeric Shh is inhibited by lipoproteins, whereas lipoprotein-associated protein can signal efficiently. However, these two forms, when present together, can potentiate the action of the each other. Moreover, we show that the mechanism responsible for the inhibitory action of lipoproteins on the Hh signaling pathway is conserved between mammals and flies.

## Functional G-protein coupling of the human Smoothened receptor

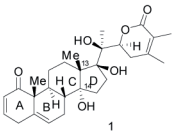
*Tanja Barbic, Axel Meyer, Jeroen van Bergeijk*

The Hedgehog pathway is highly active during embryogenesis but becomes silent in adulthood. High constitutive signaling through Smoothened receptor (Smo) has been associated with different tumors, e.g. medulloblastoma and basal cell carcinoma. In adolescence distinct activation can be observed during regenerative events mediating proliferation, migration and differentiation of stem cells. Smo has been the major target for therapeutic intervention and anti-tumor drug development. Since most studies regarding Hedgehog signaling have been carried out in arthropod models and significant species differences have been reported, we focused on the potential for G-protein-coupling of the human GPCR-like Smoothened (hSmo) receptor. Here, we report coupling of hSmo to the human Gα-subunit hGαi3 but not to hGαz using different membrane preparations of CHO cells . We demonstrate a concentration-dependent and significant inhibition of GTP-Eu recruitment to the receptor using Cyclopamine as a naturally occurring Smo antagonist. Since Smo exhibits intrinsic activity, applying the Smo agonist SAG 1.3 increased GTP-Eu binding only to a minor extend. Tomatidine, a steroidal alkaloid structurally similar to Cyclopamine but non-active on hedgehog signaling did not exhibit an effect on GTP-Eu recruitment. Next, we studied effects of pathway activation in a human mesenchymal cell line. Application of recombinant human Sonic hedgehog caused a decrease in cellular levels of the second messenger cAMP. Likewise, overexpression of hSmo lowered cAMP levels temporarily. Changes of intracellular Ca2+ levels upon Smo stimulation indicating Gαq coupling were not observed. These results clearly indicate that the human 7-transmembrane receptor Smo signals via hGαi -protein, cAMP and most probably protein kinase A offering new targets for therapeutic intervention. This work has been partially funded by the German Ministry for Research and Education (BMBF) and by Abbott GmbH & Co.KG.

## Synthetic studies on anti-cancer compounds from Withania sp.

*Lucia Vitellozzi, Dr. Thorsten Genski (AnalytiCon Discovery GmbH, Potsdam, Germany) Prof. Richard Taylor (University of York, UK)*

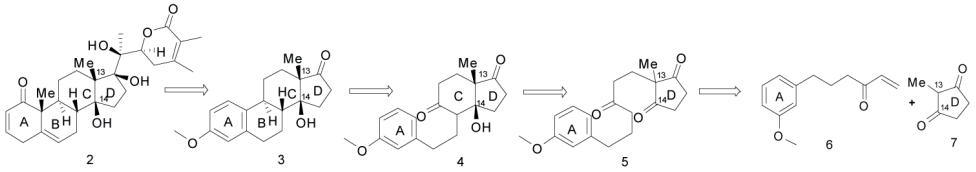
Embryonic patterning pathways, such as WNT, HH and NOTCH, play critical roles in human cancer. Inhibition of these patways can lead to tumor cell proliferative arrest and death [1]. We have therefore sterted to investigate natural small molecules that have anti-tumoral effects in vitro.



1

We have focused our attention on Withanolides, a class of natural compounds known to show anticancer activity in vitro and characterised by an ergostane type steroids structure in which C22 and C26 are appropriately oxidised in order to form a δ-lactone ring [2]. Withanolide K **(1)** is a member of this class extracted from *Withania sp.* which has not been studied in detail.

In order to evaluate the SAR of this class of compound, a total synthesis of Withanolide K analogue **(2)** is being performed (Scheme 1).



*Scheme 1*

Our first efforts are focused on the evaluation of the biological activity of compound **2** with the C13 methyl group and the C14 hydroxyl group being cis to each other. The synthesis of the bicycle CD was inspired by the Hajos-Parrish-Wiechert reaction [3].

Further structural modifications of Withanolide K **(1)** will be considerated in future.

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[2] Glotter, E. *Natural product report, 1991*, 415.

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**Sonic hedgehog (Shh) pathway regulates axonal elongation in hippocampal neurons through a non-canonical dependent signaling pathway which modulates RhoGTPases activity.**

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The Shh canonical pathway acts through the Patched1 (Ptc1) and Smoothened (Smo) membrane proteins to trigger an intricate cytoplasmic transduction machinery. Recent evidence suggests that Shh proteins exert additional functions mediated by Smo acting as G-coupled receptor referred to as "non-canonical". In this context, signaling is independent of Gli-mediated transcription but still occurs through Ptc1-Smo and is cyclopamine and purmorphamine sensitive (antagonist and agonist of the Shh pathway, respectively). The non-canonical pathway has been observed during axonal guidance in the optic chiasm and during the migration of commissural axons in the spinal cord in vertebrates, but the underlying cellular mechanisms have not been described yet. Axonal guidance occurs during the development of the nervous system and depends on both attractive and repulsive guidance cues, which are sensed in a specialized region of the axon, the growth cone. These cues may induce actin cytoskeleton remodeling, through the monomeric GTPases RhoA, Rac1 y cdc42. Axon establishment in cultured hippocampal neurons has been very well stereotyped, and is a widely used system model to support cytoskeleton changes occurring during neuronal differentiation. Here we evaluated the non-canonical Shh pathway contribution to development of neuronal polarity in hippocampal cells. Pharmacological treatments mimicking gain- and loss-of-function for Shh were evaluated in the context of axonal elongation. Purmorphamine was able to increase RhoA activity. Conversely, cyclopamine increased both LIMK and cofilin phosphorylation, read-outs of Rac1 activation. These molecular changes were paralleled with differential effects upon axonal elongation, suggesting a novel role for the non-canonical Shh signaling pathway in axon specification and elongation in hippocampal neurons.

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