IDENTIFICATION OF MUTATIONS IN THE *GLI2* GENE IN CPHD (COMBINED PITUITARY HORMONE DEFICIENCY) PATIENTS. FUNCTIONAL ANALYSIS OF THE IDENTIFIED VARIANTS.

By

RANJITH MUNISWAMY

A thesis submitted to

The Department of Health Sciences of the

University of Eastern Piedmont

In Partial fulfilment of the requirements

For the degree of

Doctor of Philosophy

In

Molecular Medicine

Supervisor: Prof. MARA GIORDANO

Coordinator: Prof. EMANUELE ALBANO

YEAR 2016 SEPTEMBER

XXVIII CYCLE

ACKNOWLEDGMENT

My scientific journey was a challenging and invaluable experience. Along the way, I had many people who guided my scientific training, supported my choices as well as helped me in my personal growth. First and Foremost, I would like to thank my supervisor, Prof. Mara Giordano who gave me opportunity to carry out this research. I appreciate all her contribution of time, advice, patience to make my Ph.D. possible. I would like to express my gratitude for St. Paul's Society, Italy for funding my Ph.D.

I wish to express my sincere gratitude to all the members of my group, for providing a wonderful environment to work in. I gratefully acknowledge the contributions of Deepak Babu, Ileana Fusco, Simona Mellone, Antonella Fanelli, to this work. I would like to extend my sincere thanks to Prof. Avanzi, Prof. Lucia Corrado, Ileana Fusco and physician Dr. Giorda for their kindness and timely help in hospital, when I was ill and bed ridden. I would like to thank Nadia Barizzone, Miriam Zuccalà, and all the other present and past members of the laboratory for their kind gesture and moral support.

I am thankful to friends who made my time in Novara, enjoyable. I would like to thank Yogesh Shivakumar, Nausicaä Clemente and Simone Merlin for their friendship and timely help whenever I needed the most. Lastly, I would like to thank my family for all their love and encouragement. I am grateful to my parents for their unconditional support in all my pursuits, to whom I dedicate this thesis.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

GH – Growth Hormone

PRL – Prolactin Hormone

TSH – Thyroid Stimulating Hormone

FSH – Follicle Stimulating Hormone

LH – Luteinizing Hormone

ACTH – Adrenocorticotrophin

POMC – Proopiomelanocortico

MSH – Melanocyte Stimulating Hormone

AVP – Arginine Vasopressin

IGF1 – Insulin-Like Growth Factor 1

IGFBP3 – Insulin-Like Growth Factor Binding Protein 3

MC2R – Melanocortin 2 Receptor

SHH or Shh – Sonic Hedgehog Signaling

BMPS – Bone Morphogenetic Protein

FGF – Fibroblast Growth Factor

WNT – Wingless Pathway

TF – Transcription Factor

OTX2 – Orthodenticle Homeobox

HESX1 – Homeobox Gene Expressed in ES Cells

PITX1 – Paired Like Homeodomain 1

PITX2 – Paired Like Homeodomain 2

CNS – Central Nervous System

POU1F1 – Pou Class 1 Homeobox 1

SOX – Sry-Related HMG Box

NKX2 – NK2-Homeobox-Thyroid Transcript Factor

CPHD – Combined Pituitary Hormone Deficiency

HH – Hypogonadotropic Hypogonadism

DI – Diabetes Insipidus

ONH – Optic Nerve Hypoplasia

ACC – Agenesis of Corpus Callosum

GHD – Growth Hormone Deficiency

TRH – Thyrotropin-Releasing Hormone

GnRH – Gonadotropin-Releasing Hormone

IGHD – Isolated Growth Hormone Deficiency

Hh – Hedgehog Pathway

DHH – Desert Hedgehog

IHH – Indian Hedgehog

Ttv – Tout Velu Family

Sotv – Sister of Ttv

Botv – Brother of Ttv

Disp – Dispatched

Hip – Hh-Interacting Protein

IHog – Interference Hedgehog

Boi – Brother of Ihog

CDO – Cell Adhesion Associated Oncogene Regulated

BOC – Brother of CDO

SMO – Smoothened Gene

Ptc – Patched 1 Gene

Wg – Wingless Proteins

Col – Collagen Coding Gene

Gas1 – Growth Arrest Specific 1 Gene

VEGF – Vascular Endothelial Growth Factor

PDGFRα – Platelet Derived Growth Factor Receptor Alpha

HNFβ3 – Hepatocyte Nuclear Factor 3 Beta

SPOP – Speckle Type BTB/POZ Protein

SuFu – Suppressor of Fused Homolog

Ski – Skinny Hedgehog

Hhat – Hedgehog Acyltransferase

Cos 2 – Costal 2 Gene

Kif7 – Kinase Family 7

STK36 – Serine/Threonine Kinase 36

Dlp – Dally Like Protein

GPC4 – Glypican 4

GPC6 – Glypican 6

Ext 1 – Exostosin 1

Ext2 – Exostosin 2

Ext3 – Exostosin 3

Shf – Shifted Gene

Wif – Wnt Inhibitory Factor

PKA – Protein Kinase A

CK1 – Casein Kinase 1

Sgg – Shaggy Gene

GSK3β – Glycogen Synthase Kinase 3 Beta

Slimb – Supernumerary Limbs

β-TRCP – Beta-Transducin Repeat Containing.

ORF – Open reading frame.

GLI2-FL – *GLI2*-full length.

GLI2-ΔN – *GLI2*-Delta N isoform.

INTRODUCTION:

PITUIARY GLAND

The pituitary gland is a central regulator of growth, reproduction, metabolism and stress responses, and functions to relay signals from the hypothalamus to peripheral organs. It is situated within the sella turcica, a recess in the sphenoid bone, at the base of the brain. The hypothalamus is the principal neural structure regulating homeostasis in vertebrates, coordinating complex signals from other regions of the brain and the periphery. The hypothalamus releases factors that control the endocrine activity of the pituitary cells. [1]

The pituitary gland is formed by the juxtaposition of the adenohypophysis (anterior and intermediate lobes) and the neurohypophysis (posterior lobe). The anterior pituitary consists of five different endocrine cell types secreting six hormones: somatotrophs that secrete growth hormone (GH), lactotrophs that secrete prolactin (PRL), thyrotrophs that secrete thyroid-stimulating hormone (TSH), gonadotrophs that secrete both gonadotrophins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and corticotrophs that secrete adrenocorticotrophin (ACTH). [2]

Somatotrophs are the majority of adenohypophyseal secretory cells comprising nearly 50% of all anterior pituitary cells. Lactotrophs embryologically arise from GH-producing cells, and constitute about 15-20% of the anterior pituitary cell population, although pregnancy and lactation alter the number of maternal lactotrophs. Corticotrophs and gonadotrophs represent 15-20% and 10-15% of anterior pituitary cells, respectively. The thyrotroph is the least common cell type in the anterior pituitary, accounting for less than 10% of the pituitary cell population [3, 4].

The intermediate lobe secretes proopiomelanocortin (POMC), a precursor to melanocyte-stimulating hormone (MSH), and involutes in the adult [2]. The neurohypophysis is formed from

axonal terminals, projecting from two discrete groups of magnocellular neurons in the hypothalamus, surrounded by modified astrocytes called pituicytes. The two hormones secreted by the posterior lobe of the pituitary gland, arginine vasopressin (AVP) and oxytocin, are synthesized in the paraventricular and supraoptic nuclei within the hypothalamus [2, 5].

The hypothalamus is positioned above the pituitary gland in the basal part of the forebrain. The magnocellular neurons, within the paraventricular and supraoptic nuclei in the hypothalamus, produce AVP and oxytocin. Their axons form the hypothalamo-hypophyseal tract, and the hormones are released from the posterior pituitary into the general circulation in response to electrical excitation. The adenohypophysis is anatomically distinct from the hypothalamus. However, parvocellular neurons of the hypothalamus secrete releasing factors that, via a system of hypophyseal portal vessels, act on the endocrine cells of the anterior lobe to stimulate or inhibit the synthesis and secretion of GH, prolactin, TSH, ACTH, and FSH and LH. The infundibulum (or pituitary stalk) carries both the portal blood delivering hypothalamic hormones to the anterior pituitary and the neural tract from the hypothalamic nuclei to the posterior pituitary. It is noteworthy that the optic chiasm lies above the hypophysis and anterior to the pituitary stalk. Thus, any mass lesion of sufficient size in the area of the pituitary gland will cause visual field defects [1, 2 and 4].

Table 1: List of Pituitary Gland Hormones and their Target and Major Functions.

ANTERIOR PITUITARY HORMONES	PRIMARY TARGETS OF ANTERIOR PITUITARY HORMONES	MAJOR FUNCTION OF ANTERIOR PITUITARY HORMONES
Thyroid Stimulating Hormone (TSH, Thyrotropin)	Thyroid Gland	Stimulates secretion of Thyroid Hormones
Follicle Stimulating Hormone (FSH, Gonadotropin)	Ovaries and Testes	Triggers ovulation, secretion of estrogen, progesterone and testosterone
Luteinizing Hormone (LH, Gonadotropin)	Follicles and Testes	Stimulation and maturation of Oocyte and sperm production
AdrenoCortico Tropin Hormone (ACTH, Corticotrophin)	Adrenal Cortex	Secretion of Glucocorticoid
Growth Hormone (GH)	Most Tissues in Body	Growth regulation, metabolism and protein biosynthesis and regulation of Blood Glucose level
Prolactin (PRL)	Mammary Glands	Development and lactation of mammary gland

POSTERIOR PITUITARY HORMONES	PRIMARY TARGETS OF POSTERIOR PITUITARY HORMONES	MAJOR FUNCTIONS OF POSTERIOR PITUITARY HORMONES
Arginine Vasopressin (AVP)	Vascular smooth muscles and kidney	Aquaporin and distal tubes development and water regulation in kidney
Oxytocin	Mammary glands and Uterus	Contraction of Uterus and Milk regulation in mammary gland

The hormones secreted from the anterior pituitary regulate growth, puberty, metabolism, response to stress, reproduction, and lactation, while those from the posterior pituitary are required during parturition and lactation, and regulate water balance see to (Tab.1) [2].

GH stimulates insulin-like growth factor 1 (*IGF1*) gene expression and *IGF1* synthesis in liver and bone, amongst other tissues, acting on growth. GH also regulates the hepatic production of insulin-like growth factor binding protein 3 (*IGFBP3*), acting as a gluco-counterregulatory hormone in metabolism. In muscle, GH increases protein synthesis, while in the adipocyte, GH induces lipolysis [6, 7]. PRL is the major hormone that simulates milk production, it inhibits LH and FSH secretion inducing lactation-related amenorrhea in the postpartum period [5]. ACTH binds with high affinity to the melanocortin 2 receptor (*MC2R*) in the adrenal gland and regulates steroidogenesis [3].

In the male, LH binds to a receptor on testicular Leydig cells and increases the synthesis of testosterone [8], while FSH binds to testicular Sertoli cell and stimulates the production of proteins in the seminal fluid [9]. In females, LH binds to its receptor on ovarian theca cells and stimulates steroidogenesis; FSH stimulates ovarian follicular growth and facilitates generation of estrogen from thecal cells [10]. TSH binds to its receptor on thyrocytes, resulting in an increase in iodine transport, in the expression of thyroperoxidase and thyroglobulin, and ultimately in increased synthesis of thyroid hormones [2, 3].

AVP acts on the V2 receptor in the renal collecting duct and increases water permeability to facilitate water reabsorption, and on V1 receptor in endothelial cells to promote vasoconstriction [3]. Oxytocin acts through its receptor, inducing intracellular calcium release that, in turn, results

in smooth muscle contraction in the uterine myometrial cells and mammary gland myoepithelial cells to cause uterine contraction and milk ejection, respectively [11].

FACTORS INVOLVED IN EMBRYOGENESIS OF PITUITARY GLAND

Pituitary gland is an amalgam of two tissues (Adenohypophysis and Neurohypophysis). Early in gestation a finger like projections of ectoderm grows upward from the upper portion of oropharynx. This protrusion is called *Rathke's pouch* and will develop into the anterior pituitary or adenohypophysis [13].

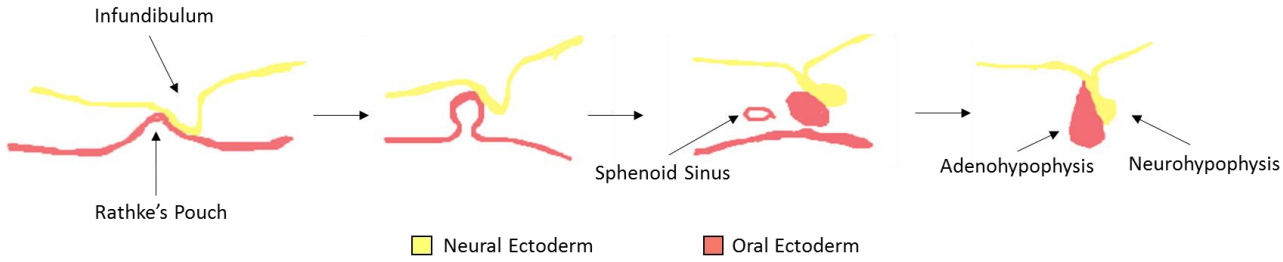


Figure 1: Schematic representation of Rathke's pouch formation and Pituitary Gland Development.

At the same phase that Rathke's pouch is developing, another finger like projections of ectodermal tissue evaginates ventrally from the diencephalon of the developing brain. This extension of the ventral brain tissue will become the posterior pituitary or neurohypophysis. Finally, the two tissues grow into one another and become tightly apposed, but their structure remains distinctly different, reflecting their differing embryological origins (Fig.1) [12].

Pituitary cell proliferation and differentiation are regulated by different transcriptional activators and repressors and by signalling molecules from adjacent regions. The early development of pituitary gland in mammals are similar in embryonic stages, so here we considered mice pituitary gland development for explanation. In the early stage of pituitary development, which corresponds to embryonic days (E) 6.5–10.5 in mice, the extrinsic signalling pathways are activated, including the sonic hedgehog (*Shh*) [17], bone morphogenetic proteins (*Bmps*) [18], fibroblast growth factor (*Fgf*) [18] and wingless (*Wnt*) [19] pathways (Fig.2).

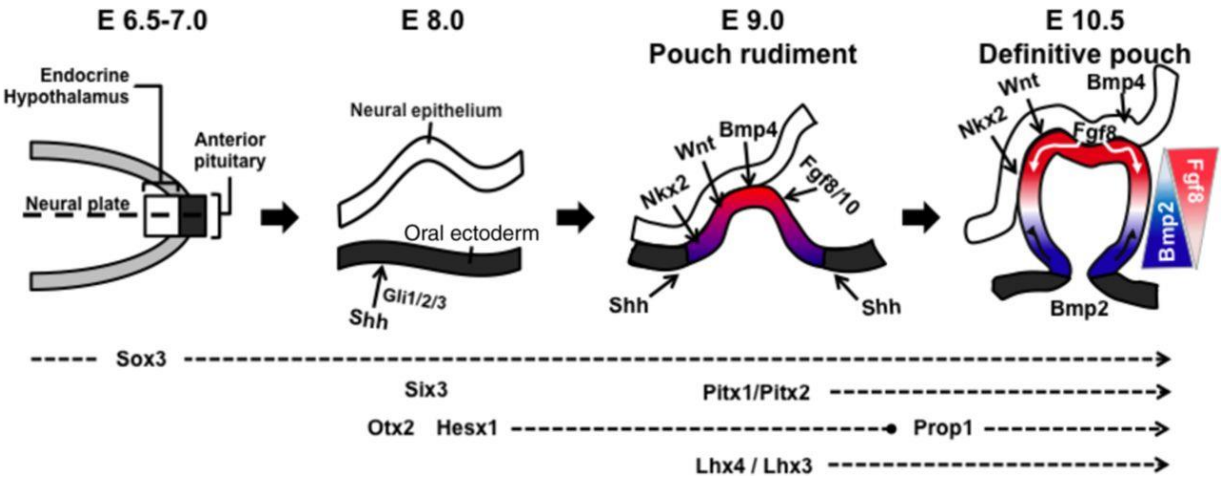


Figure 2: Embryonic days of developing Pituitary Gland.

Shh is not directly involved in Rathke's pouch formation; however, it is required for midline formation, forebrain development, brain lobe determination, eye formation [18, 20, 21 and 22] and *BMP2* expression induction. Mouse embryos that lack *Shh* have pituitary hypoplasia and the optic disc is absent [21]. The *Shh* pathway depends on zinc finger factors, such as *Gli1*, *Gli2* and *Gli3* [17]. Although Shh is not expressed in Rathke's pouch, *Gli* factors are found in the precursor structures of the pituitary. Therefore, it is possible that in response to *Shh* signalling, *Gli* proteins

activate other target genes directly involved in pituitary organogenesis [20]. *Otx2* is another TF that is not expressed in the pituitary tissues themselves [23, 24]. This is a bicoid protein that is important for eye and forebrain formation [24, 25]. *Otx2* is also responsible for *Hesx1* expression regulation [23]. *Hesx1* is the first pituitary-specific TF to be expressed at or before E6.5 [26, 27]. *Hesx1* expression begins in the rostral region and progresses dorsally; the restricted expression of this TF is responsible for Rathke's pouch formation. *Hesx1* is important for midline formation and regulates the expression of other TFs [23, 26, 28 and 29].

The *Pitx1* and *Pitx2* genes are expressed at approximately E9 and participate in the different steps of central nervous system (CNS) organogenesis. *Pitx1* is initially expressed in the first branchial arch, then in the oral cavity, and next in Rathke's pouch [30]. *Pitx1* continues to be expressed in the latter stages of pituitary embryogenesis and participates in cellular differentiation [30, 31]. *Pitx2* is expressed in several organs, including the CNS, forelimbs, lungs, kidneys and tongue. In addition to its role in CNS formation, *Pitx2* appears to be important in the determination of the left–right axis. Similar to *Pitx1*, *Pitx2* continues to be expressed during pituitary cell differentiation and acts synergistically with other TFs to determine pituitary cell types, primarily *Pit1* (*Pou1f1*)-specific cells [30-32].

Similarly, other molecules play relevant roles in the development of the CNS, including the *Soxb1* TFs (*Sox1*, *Sox2* and *Sox3*) [33, 34]. *Sox3* expression begins during early embryogenesis; recent studies have suggested that this gene must be expressed at a constant level because both increases and decreases in its expression are related to pituitary deficiencies and CNS malformations [34]. Some signalling molecules expressed in the infundibulum directly contribute to the induction of pouch invagination, among which *Bmp4* [18] and *Nkx2* are key [35].

Mutant animals lacking any of these factors may develop pituitary absence, malformation or even embryonic lethality [36, 37]. In parallel with the invagination of oral ectoderm, the pituitary precursor cells proliferate and migrate. The *Wnt* [33] and *Shh* [38] pathways are important for proliferation regulation, while the *Bmp* and *Fgf* pathways are required for proliferation and for determining cellular migration [39]. Rathke's pouch formation is complete at approximately E10.5, and the pituitary precursor cells begin to express specific factors that determine their differentiation patterns (Tab.2) [33].

Table 2: Genes involved in embryogenesis of Pituitary Gland with embryonic days.

Pituitary embryogenesis	**Embryonic Day 6.5 – 7.0**	**Embryonic Day 8.0**	**Embryonic Day 9.0**	**Embryonic Day 10.5**
Regulating Genes in Pituitary Gland Embryogenesis	*Sox3*	*Sox3, Shh, Gli1,2,3, Six3, Otx2 and Hesx1*	*Sox3, Shh, Gli1,2,3, Six3, Otx2, Hesx1, Bmp4, Fgf8/10, Wnt, Nkx2, Pitx1/2 and Lhx4/3*	*Sox3, Shh, Gli1,2,3, Six3, Otx2, Hesx1, Bmp4/2, Prop1, Fgf8/10, Wnt, Nxk2, Pitx1/2, Lhx4/3*

DISORDERS AND DEFICIENCIES IN PITUITARY GLAND HORMONES

Deficiency of one or multiple pituitary hormones is defined as Hypopituitarism. Congenital hypopituitarism is a syndrome with a wide variation in severity, age at presentation, from the early neonatal period to later in life (e.g. with abnormal pubertal development), and inheritance. It may manifest as isolated deficiency of GH, ACTH or TSH, hypogonadotropic hypogonadism (HH) or central diabetes insipidus (DI). Alternatively, several pituitary hormone axes may be defective, resulting in combined pituitary hormone deficiency (CPHD) syndromes. The hormonal deficits can be associated with extra-pituitary abnormalities, notably of the eye and midline forebrain, such as optic nerve hypoplasia (ONH), anophthalmia/microphthalmia, agenesis of corpus callosum (ACC) and absence of septum pellucidum [1, 2].

The endocrinopathy can evolve to include other hormonal deficits, necessitating ongoing assessment, as these conditions are often associated with significant morbidity and occasional mortality. Neonates with congenital hypopituitarism may present with nonspecific symptoms, such as hypoglycaemia, lethargy, seizures, failure to thrive, cholestasis and prolonged jaundice, with or without associated developmental defects. Alternatively, they may be initially asymptomatic but at risk of developing pituitary hormone deficiencies over time. Males may present with undescended testes and a micropenis. Growth failure in severe growth hormone deficiency (GHD) can occur early in infancy, while bone maturation may be delayed for the chronological age but this is usually evident later in life. Moreover, neonates with optic nerve hypoplasia and/or midline abnormalities or syndromes known to be associated with hypopituitarism will need, in the first instance, assessment of their endocrine status, as well as long term follow-up even if the initial endocrine investigations are normal. Early diagnosis of hypopituitarism in the neonatal period is difficult due to the immaturity of the hypothalamic-pituitary axis, and the contraindication for

some GH provocation tests at this age. More than 50% of patients with eye/forebrain and pituitary abnormalities have ACTH deficiency, and the resulting cortisol deficiency can be life threatening. Neonates with TSH deficiency may also present with temperature instability [14].

Investigations of hypopituitarism include the use of combined pituitary function and provocative testing of the hypothalamo-pituitary axis. GHD may be confirmed on the basis of low concentrations of *IGF1* and *IGFBP3* in combination with a poor growth rate, while GH provocation tests are contraindicated in children less than one year of age. The diagnosis of TSH deficiency is made in the presence of a low concentration of free thyroxine and basal TSH, and central hypothyroidism is associated with additional pituitary hormone deficiencies in 78% of cases. A thyrotropin-releasing hormone (TRH) test may be useful for the diagnosis of prolactin deficiency. A poor response to gonadotropin-releasing hormone (GnRH) stimulation within the first 12-18 months of life is suggestive of gonadotropin deficiency, which provides a window of opportunity for the early detection of HH, although patients will require repeat investigations at puberty. In neonates, multiple random cortisol measurements may point towards the integrity of the hypothalamo-pituitary-adrenal axis, but requires frequent blood sampling, while hypoglycaemia-inducing tests are contraindicated at this age. The cortisol response to an exogenous ACTH test is safe, but it has a sensitivity of 80%. Once the circadian rhythm has been established, an 08:00 am cortisol, a 24-hour plasma cortisol, and a mean cortisol may represent a more sensitive tool to confirm ACTH deficiency. Finally, early morning paired plasma and urine osmolarities point towards the diagnosis of DI [2, 14].

Neuroimaging also plays an important role in the diagnosis and monitoring of patients with congenital hypopituitarism, as there is a correlation between the neuroradiological abnormalities and the severity and evolution of the endocrinopathy. Signs to look for at the magnetic resonance imaging (MRI) of the brain and pituitary include the size of the anterior pituitary, the presence and location of the posterior pituitary (absent or ectopic/undescended), the presence and morphology of the infundibulum, the presence and morphology of the corpus callosum and septum pellucidum, the appearance of the optic nerves and chiasm, as well as associated brain abnormalities [15]. The risk of hypopituitarism is 27.2 times greater in patients with an undescended posterior pituitary as compared with those with a normally positioned posterior pituitary, and midline forebrain defects are up to 5.2 times more prevalent in patients with CPHD as compared with isolated growth hormone deficiency (IGHD) [14, 15]. The mainstay of treatment of hypopituitarism is replacement therapy with appropriate hormones, which entails the use of subcutaneously administered recombinant human growth hormone, oral hydrocortisone, thyroxine, and intramuscular or transdermal testosterone or estrogen [16].

HEDGEHOG SIGNALING PATHWAY

Hedgehog (*Hh*) signaling is mediated by a group of morphogen ligands: sonic hedgehog (*SHH*), Desert hedgehog (*DHH*) and Indian hedgehog (*IHH*). These are synthesized as precursor proteins that are then processed into two fragments, namely an amino-terminal peptide and a carboxy-terminal peptide. The amino-terminal peptide is responsible for Hh signaling [40, 41]. Both the N- and C-termini of the amino-terminal Hh peptide are modified with lipid moieties, catalyzed in part by the carboxy-terminal peptide [42]. These lipid modifications must either be cleaved such that the secreted ligand is soluble or shielded in a transport mechanism through the bloodstream.

In Drosophila, it has been shown that heparin sulfate glycoproteins called glypicans play a role in the transport of *Hh* ligand [43]. These glypicans can recruit lipophorins, lipoproteins that transport the hydrophobic *Hh* ligand through the bloodstream.

Hh signaling requires intact primary cilium, a microtubule-containing organelle that extends from the surface of nearly all cells in mammalian tissues. Responding to mechanical and chemosensation, primary cilium are localization points for signaling receptors, ion channels and transporters. Acting at the primary cilium, *Hh* morphogens play essential roles in embryogenesis, cell proliferation and tissue development, and stem cell maintenance [44]. Specifically, at the activation of *Hh* signaling, *Hh* morphogens bind to the 12 pass-transmembrane receptor, *Ptach* (*Ptch1*) which is localized to the base of primary cilium, releasing its inhibition of Smoothened (*Smo*), a protein responsible for activating the downstream *Hh* pathway. Once *Smo* is activated, it binds to *Sufu* and induces nuclear translocation of *Hh* pathway transcription regulators, *Gli1* (activator), *Gli2* (activator and repressor) and *Gli3* (repressor) [45, 46]. *Gli1*, *Gli2* and *Gli3*

regulate the expression of downstream targets such as *Gli1*, *Ptch1*, *cyclin D*, and myc involved in cell survival, proliferation and differentiation (Figure 3) [47, 48].

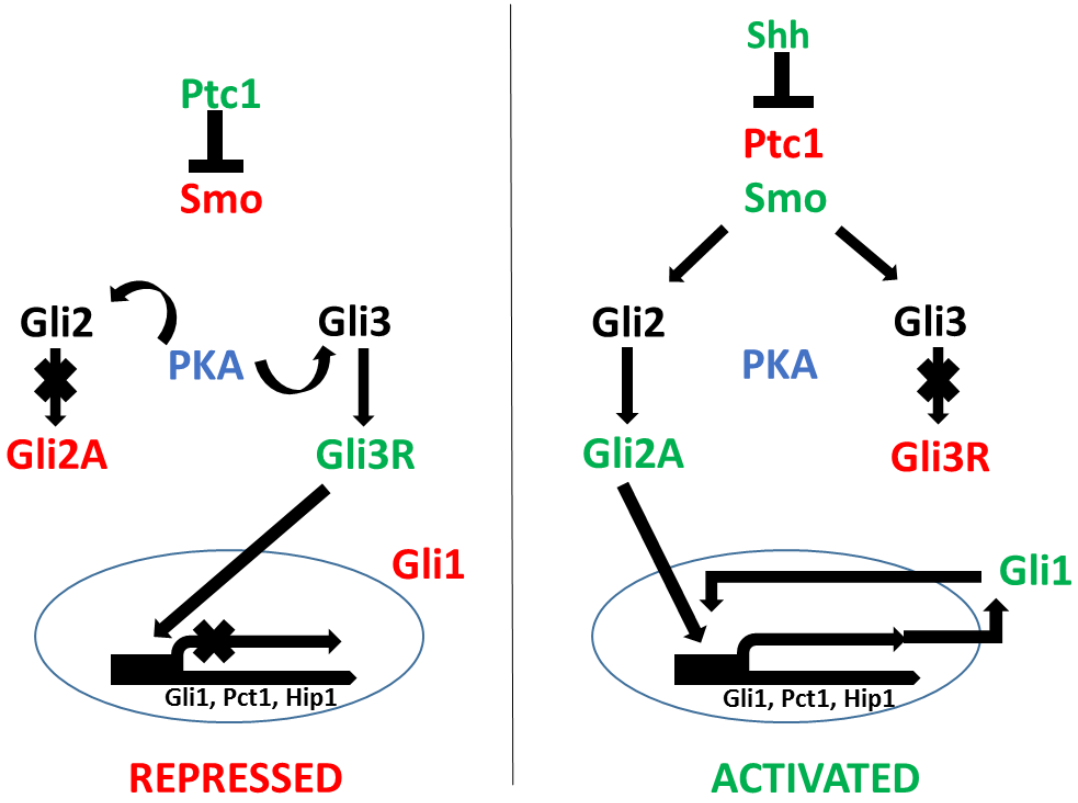


Figure 3: Upstream regulation of the Gli transcription factors and their individual and combined roles in regulating Hh target gene expression. In the absence of the signal, Gli3 functions repressor of the Hh signaling pathway. When there is signaling, repression of Gli3 is relieved and activator form of the Gli2 becomes active and induces target gene activation.

RELEASE AND TRANSPORT OF *Hh* THROUGH TISSUES

Despite its tight membrane association, *Hh* is able to affect patterning of distal tissues, acting directly over a long range in a time- and concentration-dependent manner [49]. The formation of the gradient of *Hh* activity emanating from the secreting cells is facilitated by multiple macromolecules, which control release, transport and sequestration of *Hh. Hh* is released from the secreting cell by *Dispatched* (*Disp*), a conserved protein that shares sequence similarity with transmembrane transporters [50, 51]. Subsequent transport of *Hh* through tissues requires heparin sulfate, as indicated by the failure of *Hh* transport in embryos lacking heparansulfate-synthesizing enzymes of the *EXT/tout velu* (*ttv*) family [51, 52]. The cholesterol modification of *Hh* also affects the range of *Hh* action by affecting its palmitoylation, stability, diffusion and/or transport [53-58]. Several other proteins that affect *Hh* transport and/or shape the *Hh* gradient have been described in different species. For example, in addition to the Hh receptor *Patched* (*Ptc*), which sequesters *Hh* and restricts its range of action in all species analyzed [51, 59], vertebrates have an additional transmembrane protein, *Hh*-interacting protein (*Hip*), which binds to *Hh* proteins and reduces their range of movement [51, 60].

RECEIVING THE *Hh* SIGNAL

The binding of *Hh* to cells is facilitated by two classes of accessory receptor: the glypican-family of cell surface proteoglycans (e.g. dally-like in Drosophila) [61] and the transmembrane proteins *iHog* and *Boi* (CDO and BOC in vertebrates) [62, 63]. *iHog* and *Boi* also increase the binding affinity of *Hh* for the signaling receptor *Ptc*, a 12- span transmembrane protein related to bacterial transmembrane transporters of the resistance-nodulation-division (RND) family. In the absence of *Hh*, *Ptc* catalytically inhibits the activity of the seven-transmembrane-span receptor-like protein

Smoothened (Smo) [64], potentially by affecting localization and/or concentration of a small molecule. *Smo* activity can be modulated by many synthetic small molecules [65]. Of endogenous metabolites, oxysterol derivatives [66] and vitamin D3 derivatives [67] have been suggested to mediate the effects of *Ptc* on *Smo*. Binding of *Hh* to *Ptc* results in loss of *Ptc* activity, and consequent activation of *Smo*, which transduces the *Hh* signal to the cytoplasm [64, 68], ultimately leading to the activation of the *Ci/GLI* family of transcription factors [69, 70 and 71].

TRANSCRIPTIONAL TARGETS OF *Hh* SIGNALING

The *Hh* signaling response is mediated by the binding of the *Ci/Gli1-3* transcription factors to a *Gli*-consensus binding sequence, 'TGGGTGGTC' [72, 73, 74 and 75], in promoter and enhancer regions of target genes. The transcription factors act as both activators and repressors on the transcription of a number of genes that vary between organisms and tissues. There are several examples of graded responses to *Hh* signaling, often in conjunction with other signaling factors, including the establishment of the A/P boundary in wing disks and the tight segmental boundaries in *Drosophila* described above. In addition, several response elements and enhancers, in addition to the *GLI* consensus sequence, may regulate the expression of each specific gene target. In *Drosophila*, *Hh* targets genes include *Dpp, Wg, Ptch, Col* and *En* [76, 77]. Vertebrate targets include components of the pathway, *Ptch*, *Gli1* and *Hip*, as well as several proteins from various protein families including *Bmp* [78] *Hox* [78], *Fgf* [79], *Myc* [80], *Cyclin* [81], *Vegf* [82,83], *Angiopoietin* [83,84], and other proteins including *Pdgfrα* [85], *Bcl-2* [86,87], *Bmi1* [88], *Wnt* [89], *Hes1* [90], *HNF-3b* [75], *Spop* [91]. *Gli-R* represses target genes when *Hh* is absent, and while moderate levels of *Hh* signaling leads to the de-depression these genes, higher levels leads to

transcriptional activation. In mice, a total of 42 genes have two or more *Gli* consensus binding sequences in the enhancer regions (Tab.3) [92].

Table 3: Hedgehog pathway components in Drosophila and vertebrates

DROSOPHILA	**VERTEBRATE**	**FUNCTION**
Hedgehog (*Hh*)	*Shh, Ihh, Dhh*	Secreted signaling ligand activating the *Hh* pathway
Skinny hedgehog (*Ski*)	*Hhat (Skn)*	Acyltransferase involved in *Hh* ligand palmitoylation
Dispatched (*Disp*)	*Disp1, Disp2*	Transmembrane protein involved in *Hh* ligand release
Smoothened (*Smo*)	*Smo*	Positive transmembrane transducer
Patched (*Ptc*)	*Ptch1, Ptch2*	Inhibits *Smo* translocation and activity, and possibly a sterol pump (cholesterol, provitamin D3). Also blocks *Stt4* kinase activity.
Interference hedgehog (*Ihog*), Brother Ihog (*Boi*)	*Cdo, Boc*	*Ptch* co-receptors
Costal-2 (*Cos-2*)	*Kif 7, (Kif family)*	Scaffold for *Ci/Gli* processing, positive and negative roles
Fused (*Fu*)	*Fu (Stk36)*	Positive transducer required for *SuFu* and *Cos2* phosphorylation
Suppressor of fused (*SuFu*)	*SuFu*	Negative regulator of *Ci/Gli* proteins
Cubitus interruptus (*Ci*).	*Gli1, Gli2, Gli3*	Transcriptional activator and repressor of *Hh* target genes
Dally, Dally-like protein (*Dlp*)	*Gpc4, Gpc6*	Heparan-sulfate proteoglycan glypican involved in *Hh* movement and reception
Tout-velu (*Ttv*), Sister of *Ttv* (*Sotv*), Brother of *Ttv* (*Botv*)	*Ext1-3*	*Hspg* glycosylation (polymerization), involved in *Hh* movement
Shifted (*Shf*)	*Wif*	Secreted protein involved in *Hh* movement
Hedgehog-interacting protein (*Hip1*)	*HIP*	Negative regulator of *Hh* movement
Protein kinase A (*Pka*)	*PKA*	*Ci/Gli* and *Smo* phosphorylation, positive and negative regulator
Casein kinase 1 (*CkI*)	*ck1*	*Ci* and *Smo* phosphorylation
Shaggy (*Sgg*)	*Gsk3β*	*Ci* and *Smo* phosphorylation
Supernumerary limbs (*Slimb*)	*β-Trcp*	*F-box* protein substrate recognition subunit of ubiquitin *E3* ligase

There are *Gli* family of genes targets that act in feedback mechanisms on *Hh* pathway activity and the *Hh* protein. While *Gli1* mediates an important positive feedback signal, the expression of *Ptch* and *Hip* reduce the movement of *Hh* ligands and retrains *Hh* signaling in a negative feedback loop. Factors involved in movement and reception of the *Hh* ligands, like *Ihog/Boi*, Cdo/Boc and *Gas1* are down regulated in response to *Hh* signaling, also functioning as negative feedback to *Hh* pathway activation.

HUMAN *GLI* FAMILY

In vertebrates, there are three *GLI* (glioma-associated oncogene homolog) transcription factors, *GLI1*, *GLI2* and *GLI3*, which mediate Hedgehog signalling [93-94]. The three isoforms contain five zinc-finger DNA-binding domains, but their N-terminal domains exhibit important differences. GLI1 functions as a strong transcriptional activator. It lacks a repressor domain found in the N-terminus of *GLI2* and *GLI3*. *GLI1* expression also depends on *GLI2* and/or *GLI3*-mediated transcription since it constitutes a direct target gene of the *Hh* pathway. Compared to *GLI2*, *GLI1* exerts the largest part of activator functions by providing a positive feedback-loop. However, *GLI2* represents the primary downstream activator and is indispensable to initiate activation of target genes of the Hedgehog pathway. Thereby, both factors display identical or very similar DNA binding specificities [95, 97-100]. By contrast, *GLI3* exerts a role as a repressor (Fig.4) [96, 99].

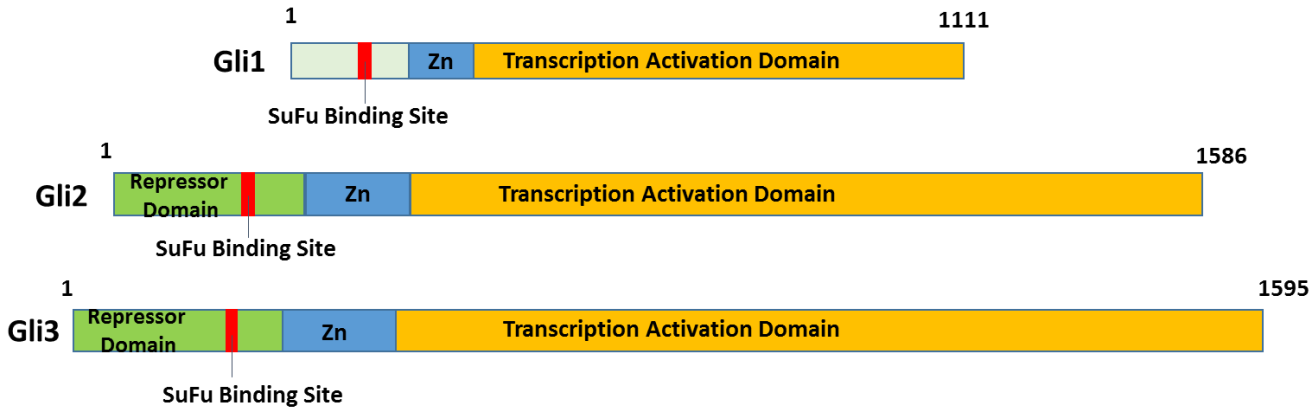


Figure 4: Regulatory domains in Gli Zinc Finger Family proteins.

***GLI2* GENE**

The *GLI2* is a member of *GLI* zinc finger family of transcription factors along with *GLI1* and *GLI3*. These zinc finger transcription factors are characterized by the consensus sequence X3-Cys-X4-Cys-X12-His-X4-His-X3, where X is any amino acid. The zinc finger forms a compact globular structure that contains a β-sheet and an α-helix held together by a central Zinc ion. *GLI2* is specifically recognized and binds to the 5'-GAACCACCCA-3' motif in the target genes.

GLI2 is a 1586 – amino acid protein (197kDa) which is encoded by 13 exons on chromosome 2q14. In addition to the central zinc finger DNA binding domain consisting of 5 fingers, *GLI2* proteins also contains an amino terminal (N-terminal) repressor domain and carboxyl terminal (C-terminal) transactivation domain (Fig.5).

In previous study showed that the region encoding *GLI2* repressor domain is subject to alternative splicing in the gonadal tissues and different cell lines. The alternatively 5' end of *GLI2* mRNA splicing resulted in two major isoforms from skipping exon 3 (*GLI2*Δ3) or exons 4 and 5 (*GLI2*Δ4–5 also known as *GLI2*-ΔN). This both isoforms contain premature translational stop codons in the *GLI2* open reading frame (ORF) starting from exon 2. Translation of *GLI2*Δ3 and *GLI2*Δ4–5 (*GLI2*-ΔN) *in vitro*, initiated from downstream AUG codons, to produce N-terminally truncated proteins [119].

In *GLI*-dependent transactivation assay, expression of *GLI2*-Δ3 induced activation of the reporter gene similar to that of the *GLI2*-full-length construct containing complete ORF. However, expression of the *GLI2*Δ4–5 (GLI2-ΔN) resulted in about 10-fold increase in activation, suggesting that deletion of the major part of repressor domain was responsible for the enhanced activation of *GLI2* protein and study suggested that in addition to proteolytic processing, alternative splicing

may be another important regulatory mechanism for the modulation of repressor and activator properties of *GLI2* protein. [119]

At least five different *GLI2* isoforms are produced by alternative splicing of mRNA known as α (133kDa), β (131kDa), γ (88kDa), δ (86kDa) and *GLI2* full-length [100, 107, 108-110] (Tab.4&5). The Gli2-α also known as*GLI2*-ΔN variant which lacks the N-terminal repressor domain shows a 30-fold higher reporter activity compared with the full length protein *in vitro*.

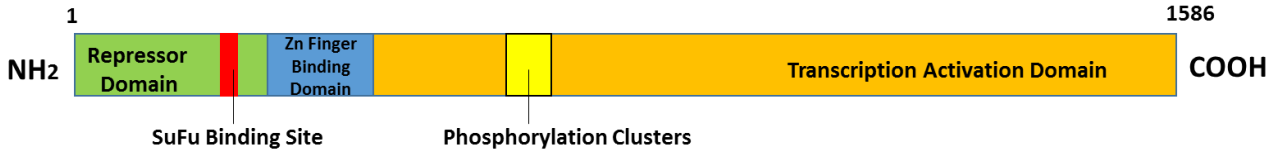


Figure 5: Schematic Representation of GLI2 Gene Full-Length with regulatory Domains.

Table 4: GLI2 Isoforms with missing amino acid positions, length and mass.

***GLI2* ISOFORMS NAME**	**MISSING AMINO ACID POSITIONS**	**LENGTH AND MASS OF ISOFORMS**
GLI2 Alpha or *GLI2*-ΔN	1-328	1258 amino acids & 133kDa
GLI2 Beta	1-328 & 394-410	1241 amino acids & 131kDa
GLI2 Gamma	1-328, 1149-1157 & 1158-1586	829 amino acids & 88kDa
GLI2 Delta	1-328, 394-410, 1149-1157 & 1158-1586	812 amino acids & 86kDa
GLI2 Full-length	No missing	1586 amino acids & 167kDa

Table 5: GLI2 Zinc Finger positions and length of binding site.

ZINC FINGERS NO:	**POSITION OF BINDING SITES**	**LENGTH OF BINDING SITES**
Zinc Finger 1	437 – 464	28
Zinc Finger 2	475 – 497	23
Zinc Finger 3	503 – 527	25
Zinc Finger 4	533 - 558	26
Zinc Finger 5	564 – 589	26

GLI2 **IN THE DEVELOPMENT OF PITUITARY**

As an effector molecule of the sonic hedgehog (*SHH*) signalling pathway, *GLI2* has a fundamental role in the development. Sonic hedgehog is a morphogen expressed in the early steps of pituitary ontogenesis by exerting effects on both proliferation and cell-type determination. *SHH* is expressed in the ventral diencephalon and throughout the oral ectoderm except Rathke's pouch [17, 101]. However, the patched receptor (*PTCH1*) as well as the *GLI* family of zinc finger transcription factors are expressed in the Rathke's pouch, indicating that the developing gland is competent to receive and respond to *SHH* signalling [102].

The *Hh* pathway (Fig.3) is considered as the canonical pathway through which *GLI2* activity is regulated. The Hedgehog ligands binds to and activate the transmembrane receptor called *patched* (*PTCH*). When the *Hh* ligand is absent, *PTCH* exerts a consistent inhibitory effect on transmembrane G-protein coupled receptor *smoothened (SMO).* When the *Hh* ligand is present and binds to *PTCH*, inhibition over *SMO* is released [59, 68]. The *GLI2* transcription factors are bound with *SuFu* which keeps *GLI2* tethered in the cytoplasm [103]. Activated *SMO* triggers the dissociation of *SuFu/GLI2* complex and allowing the nuclear translocation and activation of *GLI2*. This translocation promotes the subsequent DNA binding and transcription of a series of *Hh* pathway target genes.

Multiple studies using knockout mice has been performed to study the importance of *Gli2* in the development. Mice with homozygous loss of function *Gli2* mutation resulted in lethal phenotype later in development while the heterozygous mice developed normally [102].

The phenotypic evaluation of abnormalities in the knock out mice showed severe skeletal abnormalities including absence of vertebral body and intervertebral disc, truncated mandibles

with absent incisors, shortened limbs and sternum, missing tympanic ring bones of the inner ear and severe cleft palate malformations [102]. *Gli2* deficient mice also showed defects in the pituitary development including partial loss of anterior and complete loss of posterior pituitary [104, 105]. These defects were attributed by the loss of expression of *Gli2* target genes *Bmp4* and *Fgf8* [104].

***GLI2* IN CPHD DISEASES**

SHH and to lower extent *GLI2* mutations were initially reported in patients with Holoprosencephaly (HPE), a severe neurological characterized by incomplete or failed forebrain separation, or HPE-like phenotypes with pituitary anomalies and postaxial polydactyly [118]. As the *SHH* pathway is also involved in pituitary development, mutations in *SHH* and *GLI2* have been subsequently searched in CPHD patients. Franca et al [20] reported novel heterozygous frame-shift and nonsense *GLI2* mutations and considerable frequency of missense *GLI2* variants in patients with congenital hypopituitarism without HPE and most of these patients presented with CPHD and an ectopic posterior pituitary lobe.

More recently, individuals with truncating mutations in *GLI2* were reported with the presence of typical pituitary anomalies, polydactyly and subtle facial features rather than HPE [115]. In all the patients so far identified carrying *GLI2* mutations, the pattern of inheritance was dominant with incomplete penetrance and variable phenotype [20, 115].

It has to be considered that *GLI2* is a large and highly polymorphic gene with several rare variations reported in the exome server database (http://evs.gs.washington.edu/EVS/). Thus especially for the missense variants it is quite difficult to assess the pathogenicity in the absence of functional studies.

AIM:

AIM

The aim of this study was to determine the frequency of *GLI2* mutations in a cohort of Italian CPHD patients that resulted negative for mutations in other causative genes encoding pituitary transcription factors (*PIT1, PROP1, HESX1, LHX3*, and *LHX4*). Moreover, in the case of missense mutations, to discern between polymorphic variants and causative mutation we settled a series of *in-vitro* functional study aimed to evaluate the modifications induced by the different variants on the transcriptional *GLI2* activity.

SUBJECTS:

SUBJECTS

One hundred and thirty-six CPHD patients were recruited based on the following criteria:

1) They presented with a clinical and hormonal evidence of childhood-onset GH deficiency combined with at least one other pituitary defect in the absence of an identified cause of hypopituitarism (e.g. cerebral tumors, cranial trauma, documented asphyxia, or other injuries at delivery).

2) Mutations in the coding sequences of genes associated with multiple pituitary hormone dysfunctions (*PIT1*, *PROP1*, *HEXS1*, *LHX3*, and *LHX4*) had been previously excluded.

Mean height SDS for chronological age was calculated using the criteria of Tanner-Whitehouse method [111]. The mean height of the patients at diagnosis was −2.26 SDS ± 2.3 sd. Morphological evaluation of the hypothalamus-pituitary area and/or of the central nervous system was performed in 136 patients by magnetic resonance imaging, using precontrast coronal spin echo T1-weighted images followed by postgadolinium T1-weighted imaging. Among the 136 CPHD index cases, 8 (5.8%) were the probands of pedigrees with more than one affected individual (familial cases). Four patients were born from consanguineous parents but they were considered as sporadic cases since they were the only affected subject in their families. The mean height of these patients at diagnosis was -2.81 ±1.83 SDS and the mean delay in bone age relative to chronological age was 2.57± 2.36 years. GHD was present in all the patients, TSH deficiency in 78.6% (107/136) and ACTH deficiency in 61% (83/136). Thirty-nine subjects were Prepubertal at the time of diagnosis. Among the remaining 97 subjects that could be evaluated in terms of pubertal age, 81 (83.5%) presented with FSH/LH deficiencies. Eight male patients presented neonatal micropenis and/or cryptorchidism. Five patients (3.5%) had diabetes insipidus. We obtained MRI data from 101

patients (74% of the total). Among these, abnormalities (ectopy of the neurohypophysis, pituitary hypoplasia and empty sella) were found in 81 (80%) subjects; in particular, anterior pituitary hypoplasia or aplasia was the most frequent abnormality and was present in 61 patients (60.4%), while pituitary stalk interruption and/or neuropediatric ectopia were observed in 35 patients (34.6%), 16 of them presenting both abnormalities. Eleven patients (10.8%) presented also extra-pituitary abnormalities such as SOD, other midline defects or cerebellar abnormalities.

Patients or parents of the patients under 18 years of age gave their written informed consent to participate to this study, which was approved by the local ethical committee of each contributing auxological center.

MATERIALS AND METHODS:

SCREENING *GLI2* GENE

The entire coding region of *GLI2* (13 exons and exon-intron boundaries) was PCR amplified from peripheral blood genomic DNA by 18 couple of primers designed for separate fragments (Tab.6). The PCR products were visualized on a 2% Agarose gel and purified using Exo/SAP-IT enzymatic PCR clean up system (Affymetrix). The Purified products were then sequenced with Big Dye Terminator kit (Applied Biosystems, Foster City, CA) and automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Table 6: To examine all coding regions of GLI2 gene 18 pairs of following primers designed.

EXONS	FORWARD PRIMERS	REVERSE PRIMERS
EXON 1	5'-TGGGTTTGGGCTCAGTGT-3'	5'-CCTCTTCGCCCTCCATAAAC-3'
EXON 2	5'-TGGCTGCTCTTGCTATGAAA-3'	5'-GCAGGAGATGTGGCTGAGG-3'
EXON 3	5'-CATGTTGGTTTTGGGGTCTT-3'	5'-GACCAAGGCTGAGGAGTTGA-3'
EXON 4	5'-CCAGGTGTGCATTTCTCTCTG-3'	5'-TTGTCCCCAAAAGAAACAGC-3'
EXON 5	5'-CCTTGCAGGCTCTTCCTATC-3'	5'-TCTTTCTCCTCGGGTCAAAA-3'
EXON 6	5'-TGGGCAAGGTTCTCTCTGTC-3'	5'-CTTAGCATGAGCTGGCAGTG-3'
EXON 7	5'-TGTGCGGAGAGATCCTAGAG-3'	5'-TTCACCACCAAGGGTACAGC-3'
EXON 8	5'-TTCCCCACAGCACTTCGAT-3'	5'-TCCAGCCCCTTCTGTCTAGT-3'
EXON 9	5'-GACAGCAGGGGGTGGTCT-3'	5'-CCACCTCCAAACATGATCC-3'
EXON 10	5'-GGTTGGAGCAGAGCAGAGAA-3'	5'-GGCACCTGGCTATCTACTGG-3'
EXON 11	5'- CGTGGGTAGCTTCAGGAGAA -3'	5'-GATATCGCTGTGCCCCTAGA-3'
EXON 12	5'-GCCTGTGCAGGCCTAGAG-3'	5'-GTGGGTGCCAGCCTAGTTG-3'
EXON 13.1	5'-GTGTTGCAAGCCCTCTTCTC-3'	5'-AGTGGCTGCCGCGTACTT-3'
EXON 13.2	5'-AGCAGTACAGCCTGCGGGCCAAGTA-3'	5'-CTCCATCGCCACGTTCTCGCT-3'
EXON 13.3	5'-CTTCCACAGCACCCACAAC-3'	5'-CCTTGCGGACTGTAGCCC-3'
EXON 13.4	5'-GCAGTGGAATGAGGTGAGCT-3'	5'-GATGGCTCTGCTGTGGGTAG-3'
EXON 13.5	5'-CCCTCAGCAGACAGAAGTGG-3'	5'-GTACATGTGGATCTGGCCGT-3'
EXON 13.6	5'- CAGTCAGGAAACAGCAGAGG-3'	5'-GGAAAAAGACAAGACAGCTGGA-3'

GENOMIC DNA EXTRACTION

Genomic DNA was extracted from whole blood samples using salting out method based on Miller et al. [112].

POLYMERASE CHAIN REACTION

The PCR reaction was carried out with the GoTaq Flexi DNA polymerase (Promega) in a 15µl reaction volume, with touchdown protocol from 65^0 C to 55^0 C annealing temperatures. The initial denaturation at 94°C for 5 min, 20 cycles consisting of 30s denaturation at 94° C, 30s annealing at higher temperature 65° C and 30s extension at 72° C, followed by second cycle consisting 25 cycles of denaturation at 94^0 C for 30s, annealing at lower temperature 55^0 C and extension at 72^0 C, followed by a final extension at 72°C for 7 minutes and cooled to 4^0C (Tab.7&8).

Table 7: Reagents used for Polymerase chain reaction fragments 1-18 except 4 and 13.2.

REAGENTS	INITIAL CONCENTRATIONS	REACTION MIX
Go Taq® Flexi Buffer	5X	3µl
MgCl2	25mM	0.9µl
dNTPs	2.5mM	1.2µl
Primers F+R	10pmol/µl	1.2µl
Go Taq® (Promega)	5U/µl	0.06µl
DNA	50ng/µl	1µl
H2O	-	7.64µl
Volume Total	15µl	15µl

Table 8: Thermal cycle conditions for fragments 1 – 18 except fragment 4 and 13.2.

STEPS	TEMPERATURES	TIME	NUMBER OF CYCLES
Initial Denaturation	94°C	5 Minutes	1 Cycle
Denaturation	94°C	30 Seconds	20 cycles
Annealing (High Temp.)	65°C	30 Seconds	
Extension	72°C	30 Seconds	
Denaturation	94°C	30 Seconds	25 cycles
Annealing (Low Temp)	55°C	30 Seconds	
Extension	72°C	30 Seconds	
Final Extension	72°C	7 Minutes	−
Rest	4°C	∞	−

The exon 4 and exon 13.2 fragment which is GC rich region we modified the PCR reaction conditions by adding 5% DMSO and making final reaction volume to 20µl, with touchdown protocol from 64^0 C, 61^0 C, 58^0 C and 57^0 C annealing temperatures. The initial denaturation at 94^0 C for 2mins, 3 cycles consisting of 10s denaturation at 94^0 C, 10s annealing at higher temperature 64^0 C and 30s extension at 72^0 C, followed by second, third and fourth cycles consisting 3 cycles of denaturation, annealing and extension at 61^0 C, 58^0 C and 57^0 C, followed by final extension at 72^0 C for 5mins and cooled to 4^0 C (Tab.9&10).

Table 9: Reagents used for Polymerase chain reaction fragments 4 and 13.2.

REAGENTS	INITIAL CONCENTRATIONS	REACTION MIX
Go Taq® Flexi Buffer	5X	4µl
MgCl2	25mM	1.2µl
dNTPs	2.5mM	1.6µl
Primers F+R	10pmol/µl	2µl
DMSO	5%	1.2µl
Go Taq® (Promega)	5U/µl	0.06µl
DNA	50ng/µl	1µl
H2O	-	8.94µl
Volume Total	20µl	20µl

Table 10: Thermal conditions for Fragment 4 and 13.2.

STEPS	TEMPERATURES	TIME	NO OF CYCLES
Initial Denaturation	94°C	2 Minutes	1 Cycle
Denaturation	94°C	10 Seconds	3 Cycles
Annealing (1st Temp)	64°C	10 Seconds	
Extension	72°C	30 Seconds	
Denaturation	94°C	10 Seconds	3 Cycles
Annealing (2nd Temp)	61°C	10 Seconds	
Extension	72°C	30 Seconds	
Denaturation	94°C	10 Seconds	3 Cycles
Annealing (3rd Temp)	58°C	10 Seconds	
Extension	72°C	30 Seconds	
Denaturation	94°C	10 Seconds	40 Cycles
Annealing (4th Temp)	57°C	10 Seconds	
Extension	72°C	30 Seconds	
Final Extension And rest at 4°C.	72°c	5 Minutes	–

EXO/SAP PURIFICATION

After the reaction all the PCR products were visualized on a 2% agarose gel and purified using Exo/SAP-IT enzymatic PCR clean up system (Affymetrix) (Tab.11).

Table 11: Reagents used for Exo/SAP purification.

REAGENTS	THERMAL CONDITIONS	VOLUMES
PCR Product	37°C – 15 Mins 80°C - 15 Mins 4°C - ∞	1µl
Exo/SAP mix		5µl
Total Volume		6µl

SEQUENCING

The purified products were directly sequenced in the forward or reverse direction with Big Dye Terminator kit (Applied Biosystems) and analyzed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) (Tab.12)

Table 12: Reagents used for Sequencing Reaction.

REAGNETS	THERMAL CONDITIONS				VOLUMES
Purified PCR products	Steps	Temp	Time	Cycles	2µl
Primer (3.2µM)	Denaturation	96°C	15 secs		1µl
Big Dye Terminator Mix	Annealing	50°C	5 secs	25	1µl
H2O	Extension	60°C	4 mins		6µl
	Rest	4°C	∞		

SITE DIRECTED MUTAGENESIS

The *GLI2*-cDNA incorporated pCS2-MT expression vectors used for site directed mutagenesis is commercial available. Size of pCS2-MT is 4.3KB and size of *GLI2*-cDNA is 4.8KB totally 9.1KB plasmid size. Two kinds of construct were used for study one pCS2-GLI2FL (Addgene plasmid #17648) and another one pCS2-GLI2ΔN (Addgene plasmid #17649) [100]. *GLI2*ΔN isoform is activated form of *GLI2* gene and its expression is higher than that of *GLI2*-FL construct in Luciferase assay.

Variation p.Y575H:

We generated mutagenized *GLI2*-FL and *GLI2*-ΔN constructs by using Stratagene QuikChange® Site-Directed Mutagenesis Kit and mismatch complementary primers containing desired mutation.

Primers used for Mutagenesis:

Forward GLI2_575HisFN 5'-CTGCACCAAGAGA**C**ACACAGACCCCAGCTC-3'

Reverse GLI2_575HisRN 5'-GAGCTGGGGTCTGTGT**G**TCTCTTGGTGCAG-3'

Table 13: Reagents used for Mutagenesis for p.Y575H

REAGENTS	VOLUMES
BUFFER (10x)	5µl
dNTPs	6µl
MgSO4	1µl
Forward Primer (10mM)	1.5µl
Reverse Primer (10mM)	1.5µl
PCR Enhancer (10x)	5µl
Pfx polymerase (2.5 U/µl)	1µl
H2O	27µl
Plasmid DNA	2µl (50ng/µl)

Table 14: Thermal conditions for Mutagenesis of p.Y575H

CYCLE STEPS	TEMP.	TIME	NUMBER OF CYCLES
Initial Denaturation	98^0 C	5 mins	1
Denaturation	98^0C	10 secs	25
Annealing	53^0C	1 min	
Extension	68^0C	9 mins (1 min/kb)	
Rest	10^0C	∞	-

Variation p.A593V:

We generated mutagenized *GLI2*-FL and *GLI2*-ΔN constructs by using Stratagene QuikChange® Site-Directed Mutagenesis Kit and mismatch complementary primers containing desired mutation.

Primers used for Mutagenesis:

Forward GLI2_593ValF 5'-GGTCCACGGCCCAGATG**T**CCACGTCACCAAGAAGC 3'

Reverse GLI2_593ValR 5'-GCTTCTTGGTGACGTGG**A**CATCTGGGCCGTGGACC 3'

Table 15: Reagents used for Mutagenesis for p.A593V

REAGENTS	VOLUMES
BUFFER (10x)	5µl
dNTPs	6µl
MgSO4	1µl
Forward Primer (10mM)	1.5µl
Reverse Primer (10mM)	1.5µl
PCR Enhancer (10x)	5µl
Pfx polymerase (2.5 U/µl)	1µl
H2O	27µl
Plasmid DNA	2µl (50ng/µl)

Table 16: Thermal conditions for Mutagenesis of p.A593V

CYCLE STEPS	TEMP.	TIME	NUMBER OF CYCLES
Initial Denaturation	94^0 C	5 mins	1
Denaturation	94^0C	15 secs	25
Annealing	58^0C	1 min	
Extension	68^0C	9 mins (1 min/kb)	
Final Extension	68^0C	2 mins	1
Rest	10^0C	∞	-

Variation p.P386L:

We generated mutagenized *GLI2*-FL construct by using New England BioLabs® Q5 Site-Directed Mutagenesis Kit and mismatch complementary primers containing desired mutation.

Primers used for Mutagenesis:

5’ Phosphorylated Primers:

Forward GLI2-386Leu-FN 5’-GAGGGCCTGCGGCTGGCCTCCCCTCTGG-3’

Reverse GLI2-386Leu-RN 5’-AGGCTCGGTCTTGACCTTGCTGCGCTTG-3’

Table 17: Reagents used for Mutagenesis for p.P386L

REAGENTS	VOLUMES
Q5 BUFFER (5x)	5µl
d-NTP’S (10mM)	0.5µl
Forward Primer (10mM)	1.25µl
Reverse Primer (10mM)	1.25µl
GC Enhancer	5µl
Q5 Hot Start polymerase (0.02 U/µl)	0.25µl
H2O	10.75µl
Plasmid DNA	2 µl (1 ng/µl)

Table 18: Thermal conditions for Mutagenesis of p.P386L

CYCLE STEPS	TEMP.	TIME	NUMBER OF CYCLES
Initial Denaturation	98^0C	30 secs	1
Denaturation	98^0C	10 secs	25
Annealing	72^0C	45 secs	
Extension	72^0C	8 mins	
Final Extension	72^0C	5 mins	1
Rest	10^0C	∞	-

The PCR product obtained by this site directed mutagenesis is linear product to circularize the PCR product we performed T4 ligation reaction.

Table 19: Reagents and thermal conditions for T4-Ligation.

REAGENTS	VOLUMES	INCUBATION TIME & TEMP.
T4 ligation buffer	2µl	16^0C for Overnight
T4 ligase	1µl	
H2O	12µl	
Site directed mutagenesis product	5µl	
Total Volume	20µl	

BACTERIAL TRANSFORMATION:

After the reaction, the products were digested with DpnI. DH5α competent cells were transformed with the different mutagenized constructs and grown on Luria Broth/ampicillin media. After selecting the correct clones by colony PCR, the plasmid DNA was isolated using Miniprep and Maxiprep kit (QIAGEN). The desired mutations were confirmed by sequencing.

CELL CULTURE AND TRANSFECTION:

NIH-3T3 mouse fibroblast cell line was used for the transfection experiments [113, 114]. The stock culture was grown in DMEM High Glucose (Gibco-Life Technologies) each supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin in 5% CO2 at 37°C. A day before transfection $1x10^5$ cells were seeded into each well of a 24-well tissue culture plate in 500µl CGM (Complete Growth Medium). The wells were previously treated with 1:10 dilution Poly-L-lysine solution (Sigma Aldrich) to allow the cells to completely adhere to the plate surface. At 70%-90% confluency, cells were tranfected Mutagenized plasmid constructs, reporter plasmid, and as internal control pRL-TK renilla plasmids with Lipofectamin 2000 transfection reagent (Life Technologies). Green Fluorescent protein construct was used to test transfection efficiency. Whole cell lysate was collected for Luciferase assay after 48hrs of transfection and all assays were performed in triplicate.

CELL LYSATE

After 48 Hrs completely medium removed and washed with PBS solution then 1x PLB solution added to the respective wells and plate incubated at 37°C for 15 mins. The detached cells along with PLB collected and centrifuged at 13,000 rpm for 5 mins. Supernatant was transferred to another Eppendorf tube which is debris free used for assay.

RESULTS:

RESULTS:

We identified 5 mutations in 5 subjects in a cohort of 136 patients with CPHD (3.6%). All the following mutations (Fig.6) were at the heterozygous state:

1) p.P386L (c.1157 C>T) falls within the repressor domain.
2) p.Y575H (c.1723 T>C) is within the Zinc Finger Binding domain.
3) p.A593V (c.1778 C>T) is located at the junction of Zinc Finger Binding Domain and Transactivation Domain.
4) p.V1111Gfs*19 (c.3332delT) is located in the Transactivation domain. It is a single nucleotide deletion of a T that causes a frameshift and the presence of a premature stop codon 19 amino acids downstream. This mutation removes 475 amino acids of the activator domain.
5) p.R1226* (c.3676 C>T)is located in the Transactivation domain. Also in this case the C-terminal part of the activator domain is removed [360 amino acids] (Fig.6 – Tab.20).

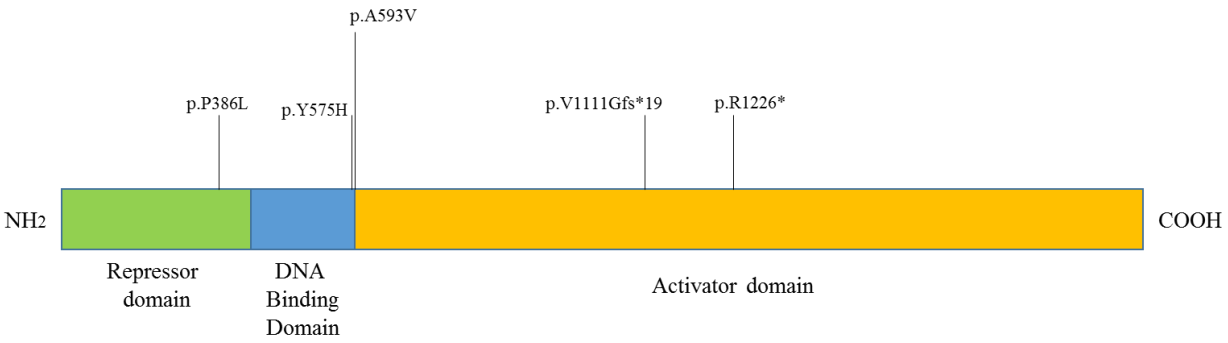


Figure 6: Schematic representation of mutation's found in this study on GLI2 Gene.

In table 20 are reported the characteristics of the identified mutation and the results of *in-silico* analysis performed by PolyPhen, PROVEAN and SIFT prediction tools for the missense mutations. All the three mutations are predicted as probably damaging.

Table 20: Summary of Gli2 mutation Identified in this study, Allele Frequency Data from dbSNP

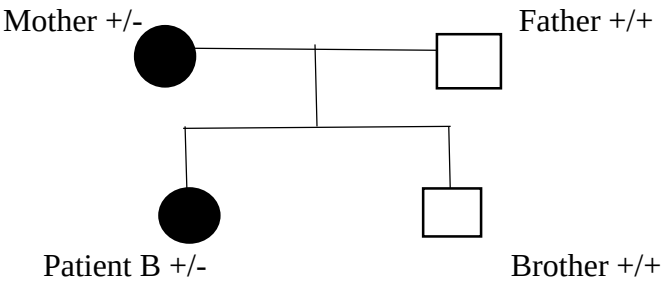
Amino acid Position	**Nucleotide Position**	**dbSNP ID**	**Allele Frequency (%)**	**PolyPhen**	**PROVEAN**	**SIFT**
P386L	c.1157 C>T	rs757467621	0.0001%	Probably damaging	Probably damaging	NA
Y575H	c.1723 T>C	rs763503195	NA	Probably damaging	Probably damaging	Probably damaging
A593V	c.1778 C>T	rs771880068	0.0001%	Probably damaging	Probably damaging	NA
R1226*	c.3676 C>T	NR	-	-	-	-
V1111Gfs*19	c.3332delT	NR	-	-	-	-

PATIENTS DETAILS:

Here are reported the data of the patients carrying a mutation at the time of the diagnosis (in parenthesis is indicated the mutation for each patient).

Patient A (p.P386L) – This patient was a 11.3 years old boy born at term with appropriate for gestational age. The parents were non-consanguineous with normal stature. The height at the diagnosis was -2.6 SDS. The patient showed GH, TSH and ACTH deficiency and no extra pituitary features (Tabl.21).

Patient B (p.Y575H) - This patient was a 9.4 years old boy born at term with no perinatal complications. The bone age was delayed by 1.8 years at diagnosis and the height was -2.2 SDS. The parents of the patient were non-consanguineous. The father was normal whereas the mother was short stature and presented with polydactyly but, not evaluated for pituitary hormone levels. The patient showed GH, TSH and ACTH deficiency. Patient's Pituitary and cerebral imaging showed anterior pituitary hypoplasia. Extra pituitary manifestations were, polydactyly, cranio facial abnormalities and hypercholesterolemia. As expected the mutation was inherited from the affected mother (Fig.7 – Tab.21).



Wild type Mother and Mutated patient - ● Wild type Father and Brother - □

Figure 7: Family tree of Patient B.

Patient C (p.A593V) - This patient was a female born at term and appropriate for gestational age. It was a sporadic case and was born from a non-consanguineous parents. The height at the time of the diagnosis was -2.1 SDS. The patient showed GH, TSH, ACTH, LH, and FSH deficiency. This patient did not show extra pituitary features. The DNA of the parents was not available (Tab.21).

Patient D (p.R1226*) - This patient was a 3 years male, born at term with appropriate for gestational age, the parents were non-consanguineous with normal stature (their DNA was not available). The height at the diagnosis was -1.8 SDS. The patient showed GH, TSH, and ACTH deficiency. The pituitary and cerebral imaging showed anterior pituitary hypoplasia (Tab.21).

Patient E (p.V1111Gfs*19) - This case was a female born from non-consanguineous parents, and was a sporadic case. The patient height was appropriate for gestational age and at term. The condition was identified at the age of 10.9. The height at the time of diagnosis was -1.6 SDS. The patient showed GH, TSH, ACTH, LH, and FSH deficiency. Pituitary and cerebral imaging of the patient showed anterior pituitary hypoplasia. Other pituitary manifestations included congenital malformation syndrome, myopia and intellectual disabilities The analysis on the parent's DNA revealed that this mutation was *"de novo"* as none of them carried it (Tab.21).

Table 21: Clinical characteristics of CPHD patients identified in this study with GLI2 Mutation.

Patient ID	**Patient A**	**Patient B**	**Patient C**	**Patient D**	**Patient E**
Mutation	p.P386L	p.Y575H	p.A593V	p.V1111Gfs*19	p.R1226*
Sex	Male	Male	Female	Female	Male
Sporadic/familial	Sporadic	Familial	Sporadic	Sporadic	Sporadic
Consanguinity Yes/No	No	No	No	No	No
Birth data		At term, AGA	At term, AGA	At term, AGA	At term, AGA
Age at diagnosis, year	11.3	9.4	28	10.9	3
Height SDS at diagnosis	-2.6	-2.2	-2.1	-1.6	-1.8
Bone age delay at diagnosis, year		1.8	NA	NA	NA
GH	D	D	D	D	D
TSH	D	D	D	D	D
ACTH	D	D	D	D	D
LH, FSH		PP	D	D	PP
PRL		NA	NA	NA	NA
Pituitary and cerebral imaging		APH, EP	NA	APH, EP	APH, EP
Other Clinical Characteristics		Polydactyly, Craniofacial abnormalities and Hypercholesterolemia	NA	Congenital Poly-malformative syndrome, mental retardation and myopia.	NA

AGA – Appropriate for gestational age, NA – Not available, D- Deficiency of the evaluated pituitary axis, PP- Prepubertal status at diagnosis and APH – Anterior pituitary hypoplasia.

FUNCTIONAL ANALYSIS:

To evaluate the role of the three missense mutations identified in CPHD patients a functional test (dual luciferase assay) was performed. For the nonsense and frameshift mutations no assay was settled as they are expected to produce C-terminal truncated non-functional protein. Two types of plasmids, one bearing the *GLI2*-cDNA and the other bearing the luciferase reporter gene, were used.

The plasmids bearing the *GLI2*-cDNA were of two types: one with the *GLI2*-Full length cDNA another with *GLI2*-ΔN cDNA, lacking the repressor NH2-terminal domain, both inserted in the pCS2-MT vector (Fig.8). These two types of cDNA correspond to two different isoforms of the *GLI2* protein as previously mentioned (see introduction). In vertebrate the *GLI2* gene undergoes alternative splicing to produce different isoforms, of which *GLI2*-ΔN is the most important in the *SHH* pathway as it acts as an activator whereas the *GLI2*-full length gene (that maintains the repressor domain) acts as a repressor.

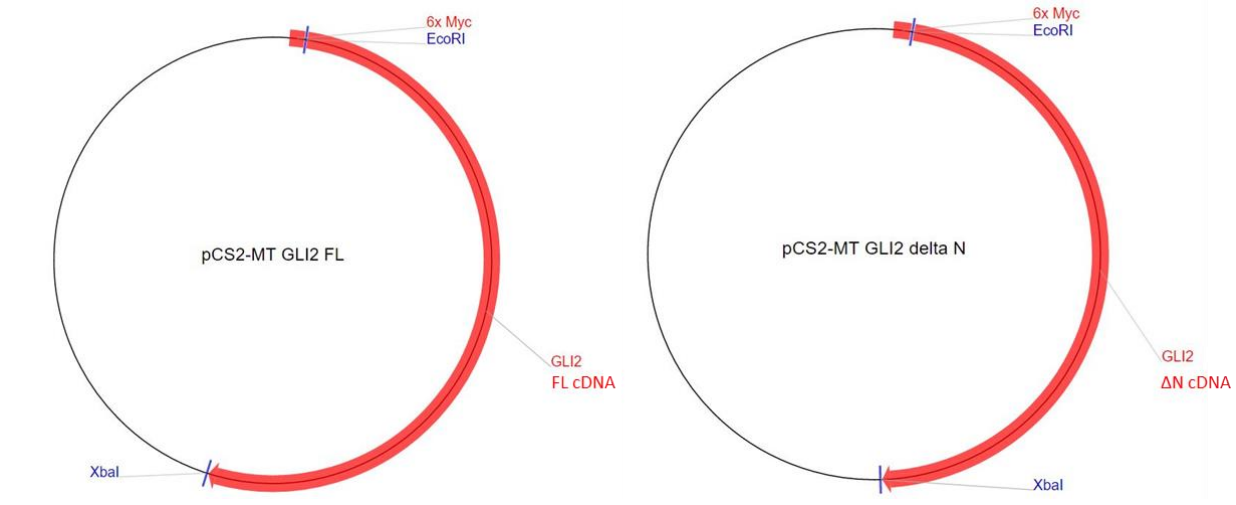


Figure-8: pCS2-MT expression vectors bearing GLI2-cDNA full length and GLI2-ΔN cDNA.

The reporter plasmid construct has a backbone of pδ51 LucII pGL4.10 bearing 8x3'- *GLI* binding site from the Hnf3β floor plate enhancer upstream of the chicken δ-crystallin minimal promoter [75] (Fig.9).

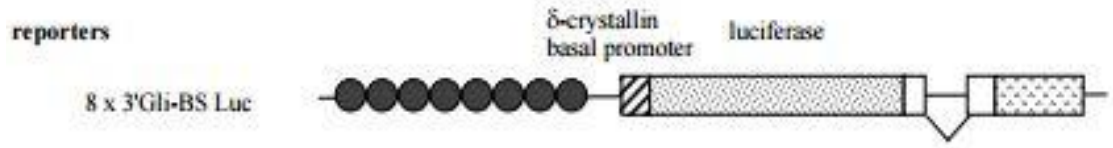


Figure-9: Reporter plasmid with backbone of pδ51 LucII pGL4.10 bearing 8x3'- GLI-BS.

The 8-fold repetition of the *GLI* binding site in the reporter plasmid increases the transcriptional activity. The binding of the *GLI2* to the corresponding binding sites on reporter plasmid drives the LucII gene transcription and the luminescence correlated to functional activity of the assayed *GLI2* protein. The pRL-TK Renilla luciferase reporter gene was used as an internal control.

To investigate the role of each mutation, the three missense mutations were inserted into the wild type *GLI2*-cDNA (full length and ΔN) by site-directed mutagenesis. The activity of the mutagenized plasmids was then compared with the wild-type plasmid.

All the mutagenized and the wild-type expression plasmids bearing either full length *GLI2*-cDNA or ΔN were co-transfected with the 8x3'-*GLI* BS reporter plasmid in NIH-3T3 mouse fibroblast cells. The assay was performed after 48 hours from the whole cell lysate. The luminescence obtained for the mutated and wild type constructs were normalized with the internal control renilla luciferase signal and the activity of the mutated constructs was reported as percentage with respect to the wild-type. The GLI2-ΔN induces much higher reporter activity compared with GLI2-full length in NIH-3T3 cells. (Fig.10)

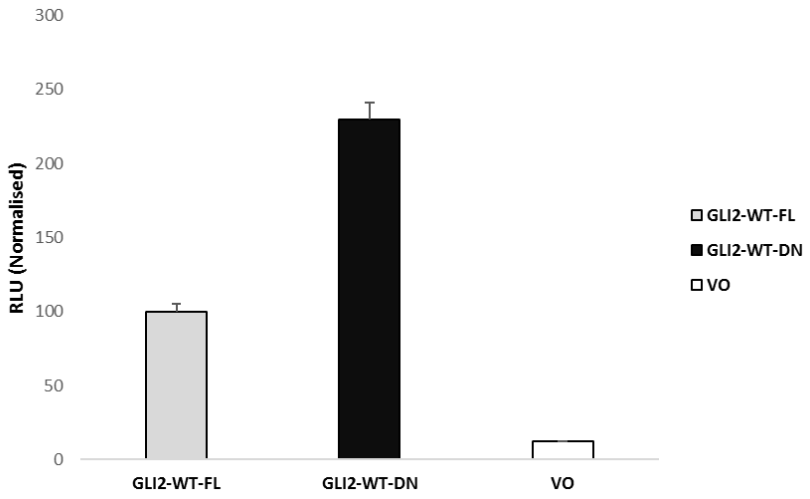


Figure-10: Comparison of reporter activity between GLI2ΔN and GLI2 Full length.

The first set of experiments was performed by co-transfecting one type of *GLI2*-cDNA plasmid, either mutagenized or wild-type, with the reporter plasmids. Obviously for the p.P386L mutation, located in the repressor domain the assay was not performed for the *GLI2*-ΔN construct because it lacks repressor domain. (Fig.11)

None of the plasmid bearing the full length cDNA showed a significant difference in the luciferase activity in comparison to the wild-type. Consequently, none of the mutants influence the repressor activity of *GLI2* including the mutation located within the N-terminal repressor domain, namely p.386L.

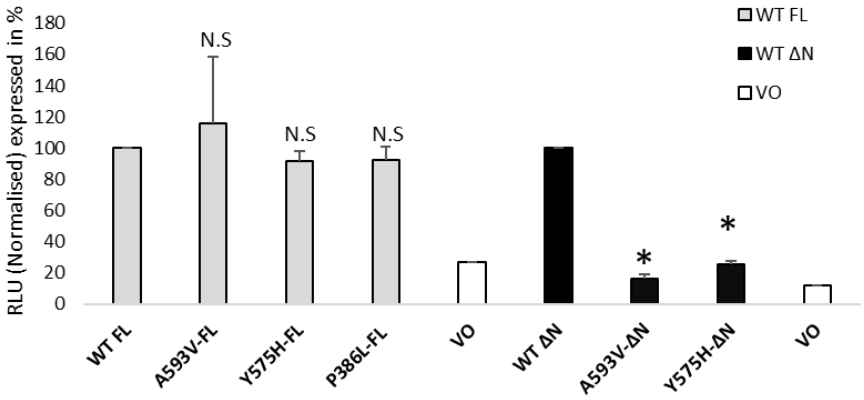


Figure-11: Transcription activity of mutagenized and wild-type bearing the either full length GLI2-cDNA or ΔN constructs.

Expression vectors for mutated *GLI2* and wild types proteins (WT-FL - Full length and WT-ΔN - N-terminally truncated) were transiently co-transfected into NIH-3T3 cells with a luciferase reporter gene under the control of an 8-fold repeat of the Hnf3β *GLI*-binding site. Promoter activity was assayed by measuring luciferase activity 48 hours after transfection. Negative controls (VO - empty vector without promoter) received equivalent amounts of empty vector expression. Activity measured as relative light units (RLUs) are the mean of at least triplicate assays and presented in percentage. RLUs normalized and compared with activity of the corresponding WT construct. Significance levels are indicated as follows: *, $P<0.05$; N.S., Not significant.

On the contrary the transcriptional activity was significantly reduced for the two mutants p.A593V and p.Y575H (of about 75% and 84% in comparison to the wild-type, respectively) when tested in the plasmid containing the *GLI2* cDNA lacking the repressor domain.

In another set of experiments all the *GLI2*-cDNA full length and ΔN constructs were co-transfected with an equal amount of wild-type *GLI2* plasmid.

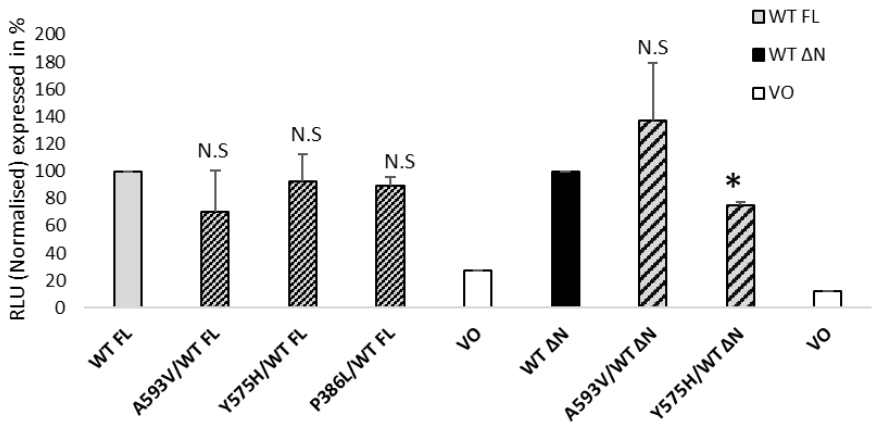


Figure 12: Transcriptional activity of co-transfected mutated construct with wild type construct.

Expression vectors for mutated *GLI2* and wild types proteins (WT FL - Full length and WT ΔN - N-terminally truncated) were transiently co-transfected into NIH-3T3 cells with a luciferase reporter gene under the control of an 8-fold repeat of the Hnf3β *GLI*-binding site. Promoter activity was assayed by measuring luciferase activity 48 hours after transfection. Negative controls (VO - empty vector without promoter) received equivalent amounts of empty vector expression. Activity measured as relative light units (RLUs) are the mean of at least triplicate assays and presented in percentage. RLUs normalized and compared with activity of the corresponding WT construct. Significance levels are indicated as follows: *, $P<0.05$; N.S., Not significant.

This was to reproduce *in vitro* the heterozygous condition of the patients that carry the 50% of the mutated alleles. The transcription activity of the p.386P/386L was reduced of only 11% with respect to the wild type which and it was not significant (Fig.12).

In case of p.593A/593V the transcriptional activity was not significantly changed both with the *GLI2*-FL and *GLI2*-ΔN plasmids. Only the *GLL2*-ΔN bearing the p.575H mutation still maintained a reduced activity when co-transfected with the wild-type with a reduction of about 25%.

DISCUSSION:

DISCUSSION:

The *GLI2* protein is one of the member of the *GLI* family of proteins and has both activation and repressive functions depending upon the isoform. *GLI2* is a critical member in the downstream of sonic hedgehog signaling pathway. Heterozygous loss of function mutations are associated with holoprosencephaly like features characterized by abnormal anterior pituitary formation and hypopituitarism with inappropriately divided forebrain [22]. Recently, *GLI2* mutations have also been detected in patients with congenital hypopituitarism without holoprosencephaly [20, 120].

In the present study we recruited 136 CPHD patients for *GLI2* gene screening. Five mutations have been identified in 5 subjects (Tab.20) all at the heterozygous state. One was a frameshift, one a nonsense and three were missense mutations.

The frameshift and nonsense mutations (p.V1111Gfs*19 and p.R1226*, respectively) were both within the C-terminal transactivation domain. They both are predicted to generate proteins lacking considerable portion of the C-terminal transactivation domain. It has been previously demonstrated that the *GLI2* transactivation domain has transcriptional activity and constructs carrying C-terminal deletions exhibited undetectable transcriptional activity *in vitro* (100). More interestingly, these constructs when cotransfected with a wild-type *GLI2* construct revealed a strong dominant negative effect on wild type *GLI2* [100]. Many of the previously identified *GLI2* mutations include frameshift and nonsense mutations and were proven to be pathogenic with a dominant negative effect [115]. Thus it is conceivable that the two truncating mutations detected in our patients might have the same effect and we did not further consider them for the functional studies.

The *in-silico* analysis (PROVEAN, SIFT, and POLYPEHN) tools predicted that the three missense mutations identified in our study (p.P386L, p.Y575H, p.A593V) to be pathogenic.

As p.Y575H and p.A593V fall within the binding site, they are supposed to interfere with the DNA binding activity were as the p.P386L included in the repressor domain could affect the repressor activity of GLI2. In the evaluation of the intrinsic transcriptional activity of the *GLI2* mutations p.Y575H and p.A593V showed a significant reduction of the transcriptional activity was only observed with the *GLI2*-ΔN. This is likely due to location of mutations and these mutations does not interfere in the repressive activity of N-terminal domain.

The p.P386L variant did not show any change in the full length *GLI2* plasmid. Thus we can hypothesize that this variation does not have an important role in the repressive activity of the *GLI2* protein.

The missense mutations are identified in heterozygous state to reproduce *in vitro* the heterozygous condition of the patients that carry the 50% of the mutated alleles the co-transfection was performed. The transcription activity of the p.386P/386L was modestly reduced but not significant. In case of p.593A/593V the transcriptional activity was not significantly changed both with the *GLI2*-FL and *GLI2*-ΔN plasmids. Only the *GLL2*-ΔN bearing the p.575H mutation still maintained a reduced activity when co-transfected with the wild-type with a reduction of about 25%. In co-transfection assay none of the variants exhibited dominant negative effect. The results were significant only for the p.575H mutation. However, this situation might be different *in-vivo* in presence of additional co-factors.

The role of p.Y575H and p.A593V in *GLI2*-ΔN, can be better understood by analyzing the position of the mutations in the protein crystal structure. The crystal structure of the Zinc finger 4 and 5 reveals an extensive base contacts in a conserved 9 base-pair region. The tyrosine at position 575 is critical in making phosphodiester bonding and alanine at 593 critical in overwound region (Fig.13). Substitution in the carboxyl terminal portion of α-helix affects the arrangement of subsequent linkers transactivational domain [116]. From, the crystal structure it is evident that the mutation p.Y575H impacts the phosphodiester bonding in finger 5 and this substitution is likely to causes vulnerable β-sheet backbone to backbone contact between DNA and protein. The p.A593V mutation which is in overwound region also alters the DNA-protein complex conformation of next linkers from transactivation domain. (Fig.13)

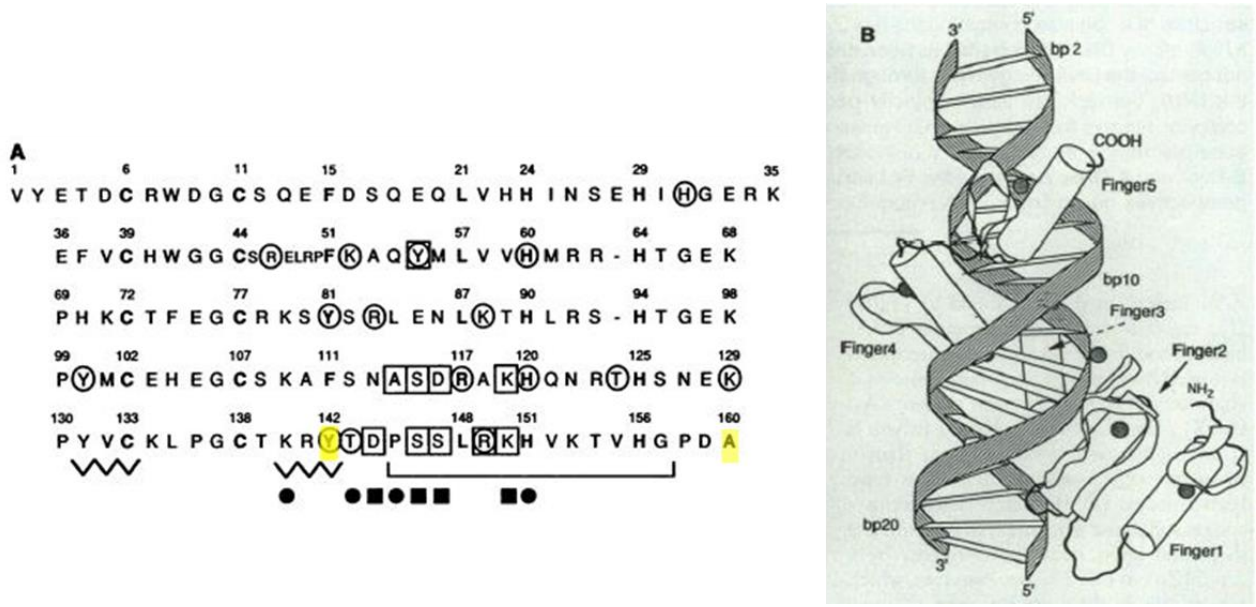


*Figure 13 – (**A**) Sequence of the GLI zinc finger domain and the DNA-binding site. The five zinc fingers of GLI are align to show the conserved residues and secondary structures. The approximate position of the α-helix is underlined, and that of β-sheets is indicated by zig-zag lines. The position of mutations identified in our study is highlighted in color. (**B**) Sketch of DNA-Zn finger complex showing orientation of fingers.*

CONCLUSION:

CONCLUSION:

The screening of the *GLI2* gene in CPHD patients revealed five heterozygous mutations with a mutation frequency of 3.6%. The assay performed on the 3 missense mutations indicated that those located within the DNA-binding site (namely, p.Y575H and p.A593V) lead to a reduction in the transcription activity in the *GLI2*-ΔN, the isoform with transcriptional activation properties. As the *GLI2* gene is highly polymorphic with many missense mutations of uncertain significance functional assay should be always performed especially when a molecular diagnosis is requested to give a more precise response to the patients.

REFERENCES:

1. Kelberman D, Rizzoti K, Lovell-Badge R, Robinson IC, Dattani MT. Genetic regulation of pituitary gland development in human and mouse. Endocr Rev. 2009**;** 30: 790-829. Review.
2. Mehta A, Dattani MT. Developmental disorders of the hypothalamus and pituitary gland associated with congenital hypopituitarism. Best Pract Res Clin Endocrinol Metab. 2008; 22: 191-206.
3. Cheung CC, Lustig RH. Pituitary development and physiology. Pituitary. 2007; 10: 335-50.
4. Pelletier G, Robert F, Hardy J. Identification of human anterior pituitary cells by immunoelectron microscopy. J Clin Endocrinol Metab. 1978; 46: 534-542.
5. Nussey SS, Whitehead SA. Endocrinology an Integrated Approach. Taylor & Francis. 2001.
6. Carter-Su C, Schwartz J, Smit LS. Molecular mechanism of growth hormone action. Annu Rev Physiol. 1996; 58: 187-207.
7. Veldhuis JD, Roemmich JN, Richmond EJ, Rogol AD, Lovejoy JC, Sheffield-Moore M, Mauras N, Bowers CY. Endocrine control of body composition in infancy, childhood, and puberty. Endocr Rev. 2005; 26: 114-46.
8. McFarland KC, Sprengel R, Phillips HS, Köhler M, Rosemblit N, Nikolics K, Segaloff DL, Seeburg PH. Lutropin-choriogonadotropin receptor: an unusual member of the G protein coupled receptor family. Science. 1989; 245: 494-9.
9. Matsumoto AM, Karpas AE, Paulsen CA, Bremner WJ. Reinitiation of sperm production in gonadotropin-suppressed normal men by administration of follicle-stimulating hormone. J Clin Invest. 1983; 72: 1005-1015.
10. Adashi EY. Endocrinology of the ovary. Hum Reprod. 1994; 9: 815-827.
11. Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. Physiol Rev. 2001; 81: 629-683.
12. R. Bowen. Pathophysiology of the endocrine system. March 20, 2003.
13. Debora Cristina de Moraes, Mario Vaisman, Flavia Lucia Conceicao and Tania Maria Ortiga-Carvalho. Pituitary development: a complex, temporal regulated process dependent on specific transcriptional factors. J Endocrinol. 2012; 215: 239-245.
14. Alatzoglou KS, Dattani MT. Genetic forms of hypopituitarism and their manifestation in the neonatal period. Early Hum Dev. 2009; 85: 705-12.
15. Mehta A, Hindmarsh PC, Mehta H, Turton JP, Russell-Eggitt I, Taylor D, Chong WK, Dattani MT. Congenital hypopituitarism: clinical, molecular and neuroradiological correlates. Clin Endocrinol (Oxf). 2009; 71: 376-82.
16. Kelberman D, Dattani MT. The role of transcription factors implicated in anterior pituitary development in the aetiology of congenital hypopituitarism. Ann Med. 2006; 38: 560-77.
17. Treier M, O'Connell S, Gleiberman A, Price J, Szeto DP, Burgess R, Chuang PT, McMahon AP & Rosenfeld MG. Hedgehog signaling is required for pituitary gland development. Development. 2001; 128: 377–386.
18. Ericson J, Norlin S, Jessell TM & Edlund T. Integrated FGF and BMP signaling controls the progression of progenitor cell differentiation and the emergence of pattern in the embryonic anterior pituitary. Dev. 1998; 125: 1005–1015.

19. Rizzoti K & Lovell-Badge R. Early development of the pituitary gland: induction and shaping of Rathke's pouch. Rev Endocr Metab Disord. 2005; 6: 161–172.
20. Franca MM, Jorge AA, Carvalho LR, Costalonga EF, Vasques GA, Leite CC, Mendonca BB & Arnhold IJ. Novel heterozygous nonsense GLI2 mutations in patients with hypopituitarism and ectopic posterior pituitary lobe without holoprosencephaly. J Clin Endocrinology Metab 2010; 95: E384–E391.
21. Zhao L, Zevallos SE, Rizzoti K, Jeong Y, Lovell-Badge R & Epstein DJ. Disruption of SoxB1-dependent sonic hedgehog expression in the hypothalamus causes septo-optic dysplasia. Dev Cell. 2012; 22: 585–596.
22. Roessler E, Du YZ, Mullor JL, Casas E, Allen WP, Gillessen-Kaesbach G, Roeder ER, Ming JE, Ruiz i Altaba A & MuenkeM. Loss-of-function mutations in the human GLI2 gene are associated with pituitary anomalies and holoprosencephaly-like features. PNAS. 2003;100: 13424–13429.
23. Diaczok D, Romero C, Zunich J, Marshall I & Radovick S. A novel dominant negative mutation of OTX2 associated with combined pituitary hormone deficiency. J. Clin. Endocrinol. Metab**.** 2008; 93: 4351–4359.
24. Gorbenko Del Blanco D, Romero CJ, Diaczok D, de Graaff LC, Radovick S & Hokken-Koelega AC. A novel OTX2 mutation in a patient with combined pituitary hormone deficiency, pituitary malformation and an underdeveloped left optic nerve. Eur J Endocrinol. 2012; 167: 441–452.
25. Schilter KF, Schneider A, Bardakjian T, Soucy JF, Tyler RC, Reis LM & Semina EV. OTX2 microphthalmia syndrome: four novel mutations and delineation of a phenotype. Clin. Gen. 2011; 79: 158–168.
26. Hermesz E, Mackem S & Mahon KA. Rpx: a novel anterior-restricted homeobox gene progressively activated in the prechordal plate, anterior neural plate and Rathke's pouch of the mouse embryo. Dev. 1996; 122: 41–52.
27. Brickman JM, Clements M, Tyrell R, McNay D, Woods K, Warner J, Stewart A, Beddington RS & Dattani M. Molecular effects of novel mutations in Hesx1/HESX1 associated with human pituitary disorders. Dev. 2001; 128: 5189–5199.
28. Diaczok D, DiVall S, Matsuo I, Wondisford FE, Wolfe AM & Radovick S. Deletion of Otx2 in GnRH neurons results in a mouse model of hypogonadotropic hypogonadism. Mol. Endocrinol. 2011; 25: 833–846.
29. Reynaud R, Albarel F, Saveanu A, Kaffel N, Castinetti F, Lecomte P, Brauner R, Simonin G, Gaudart J, Carmona E. Pituitary stalk interruption syndrome in 83 patients: novel HESX1 mutation and severe hormonal prognosis in malformative forms. Eur J Endocrinol. 2011; 164: 457–465.
30. Drouin J, Lamolet B, Lamonerie T, Lanctot C & Tremblay JJ. The PTX family of homeodomain transcription factors during pituitary developments. Mol Cell Endocrinol. 1998; 140: 31–36.
31. Tremblay JJ, Lanctot C & Drouin J. The pan-pituitary activator of transcription, Ptx1 (pituitary homeobox 1), acts in synergy with SF-1 and Pit1 and is an upstream regulator of the Lim–homeodomain gene Lim3/Lhx3. Mol Endocrinol. 1998; 12: 428–441.
32. Lamolet B, Pulichino AM, Lamonerie T, Gauthier Y, Brue T, Enjalbert A & Drouin J. A Pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx hemeproteins. Cell. 2001; 104: 849–859.

33. Yako H, Kato T, Yoshida S, Inoue K & Kato Y. Three-dimensional studies of Prop1-expressing cells in the rat pituitary primordium of Rathke's pouch. Cell Tissue Res. 2011; 346: 339–346.
34. Woods KS, Cundall M, Turton J, Rizotti K, Mehta A, Palmer R, Wong J, Chong WK, Al-Zyoud M, El-Ali M. Over- and underdosage of SOX3 is associated with infundibular hypoplasia and hypopituitarism. Am. J. Hum. Genet. 2005; 76: 833–849.
35. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM & Gonzalez FJ. The T/ebp null mouse: thyroid-specific enhancer binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev. 1996; 10: 60–69.
36. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL & German MS. Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Dev. 1998; 125: 2213–2221.
37. Nasonkin IO, Ward RD, Bavers DL, Beuschlein F, Mortensen AH, Keegan CE, Hammer GD & Camper SA. Aged PROP1 deficient dwarf mice maintain ACTH production. PLoS One. 2011; 6 e28355.
38. Fernandez-Rodriguez E, Quinteiro C, Barreiro J, Marazuela M, Pereiro I, Peino R, Cabezas-Agricola JM, Dominguez F, Casanueva FF & Bernabeu I. Pituitary stalk dysgenesis-induced hypopituitarism in adult patients: prevalence, evolution of hormone dysfunction and genetic analysis. Neuroendocrinology. 2011; 93: 181–188.
39. Kato Y, Kimoto F, Susa T, Nakayama M, Ishikawa A & Kato T. Pituitary homeodomain transcription factors HESX1 and PROP1 form a heterodimer on the inverted TAAT motif. Mol Cell Endocrinol. 2010; 315: 168–173.
40. Ingham, P.W.; McMahon, A.P. Hedgehog signaling in animal development: Paradigms and principles. Genes Dev. 2001;15: 3059–3087.
41. Hardy, R.Y.; Resh, M.D. Identification of N-terminal residues of Sonic Hedgehog important for palmitoylation by Hedgehog acyltransferase. J. Biol. Chem. 2012; 287; 42881–42889.
42. Porter, J.A.; Young, K.E.; Beachy, P.A. Cholesterol modification of hedgehog signaling proteins in animal development. Science 1996; 274: 255–259.
43. Eugster, C.; Panakova, D.; Mahmoud, A.; Eaton, S. Lipoprotein-heparin sulfate interactions in the Hh pathway. Dev. Cell. 2007; 13: 57–71.
44. Satir, P.; Pedersen, L.B.; Christensen, S.T. The primary cilium at a glance. J. Cell Sci. 2010; 123: 499–503.
45. Merchant, M.; Vajdos, F.F.; Ultsch, M.; Maun, H.R.; Wendt, U.; Cannon, J.; Desmarais, W.; Lazarus, R.A.; de Vos, A.M.; de Sauvage, F.J. Suppressor of fused regulates Gli activity through a dual binding mechanism. Mol. Cell. Biol. 2004; 24: 8627–8641.
46. Chen, W.; Ren, X.R.; Nelson, C.D.; Barak, L.S.; Chen, J.K.; Beachy, P.A.; de Sauvage, F.; Lefkowitz, R.J. Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. Science 2004; 306: 2257–2260.
47. Rahnama, F.; Shimokawa, T.; Lauth, M.; Finta, C.; Kogerman, P.; Teglund, S.; Toftgard, R.; Zaphiropoulos, P.G. Inhibition of GLI1 gene activation by Patched1. Biochem. J. 2006; 394: 19–26.
48. Katoh, Y.; Katoh, M. Hedgehog target genes: Mechanisms of carcinogenesis induced by aberrant hedgehog signaling activation. Curr. Mol. Med. 2009; 9: 873–886.

49. Stamataki, D., Ulloa, F., Tsoni, S. V., Mynett, A. and Briscoe, J. A gradient of Gli activity mediates graded Sonic Hedgehog signaling in the neural tube. Genes Dev. 2005; 19: 626-641.
50. Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K. A., Dickson, B. J. and Basler, K. Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. Cell 1999; 99: 803-815.
51. Zhu, A. J. and Scott, M. P. Incredible journey: how do developmental signals travel through tissue. Genes Dev. 2004; 18: 2985-2997.
52. The, I., Bellaiche, Y. and Perrimon, N. Hedgehog movement is regulated through tout veludependent synthesis of a heparan sulfate proteoglycan. Mol. Cell. 1999; 4: 633-639.
53. Callejo, A., Torroja, C., Quijada, L. and Guerrero, I. Hedgehog lipid modifications are required for Hedgehog stabilization in the extracellular matrix. Dev. 2006; 133: 471-48.
54. Dawber, R. J., Hebbes, S., Herpers, B., Docquier, F. and van den Heuvel, M. Differential range and activity of various forms of the Hedgehog protein. BMC Dev. Biol. 2005; 5: 21.
55. Gallet, A., Ruel, L., Staccini-Lavenant, L. and Therond, P. P. Cholesterol modification is necessary for controlled planar long-range activity of Hedgehog in Drosophila epithelia. Dev. 2006; 133: 407- 418.
56. Lewis, P. M., Dunn, M. P., McMahon, J. A., Logan, M., Martin, J. F., St-Jacques, B. and McMahon, A. P. Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1. Cell. 2001; 105: 599-612.
57. Li, Y., Zhang, H., Litingtung, Y. and Chiang, C. Cholesterol modification restricts the spread of Shh gradient in the limb bud. Proc. Natl. Acad. Sci. USA, 2006; 103: 6548-6553.
58. Mann, R. K. and Beachy, P. A. Novel lipid modifications of secreted protein signals. Annu. Rev. Biochem. 2004; 73: 891-923.
59. Chen, Y. and Struhl, G. Dual roles for patched in sequestering and transducing Hedgehog. Cell. 1996; 87: 553- 563.
60. Chuang, P. T. and McMahon, A. P. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature, 1999; 397: 617-621.
61. Lum, L., Yao, S., Mozer, B., Rovescalli, A., Von Kessler, D., Nirenberg, M. and Beachy, P. A. Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. Science. 2003; 299: 2039-2045.
62. Tenzen, T., Allen, B. L., Cole, F., Kang, J. S., Krauss, R. S. and McMahon, A. P. The cell surface membrane proteins Cdo and Boc are components and targets of the Hedgehog signaling pathway and feedback network in mice. Dev. Cell. 2006; 10: 647-656.
63. Yao, S., Lum, L. and Beachy, P. The ihog cell surface proteins bind Hedgehog and mediate pathway activation. Cell, 2006; 125: 343-357.
64. Taipale, J., Cooper, M. K., Maiti, T. and Beachy, P. A. Patched acts catalytically to suppress the activity of Smoothened. Nature. 2002; 418: 892-897.
65. Chen, J. K., Taipale, J., Young, K. E., Maiti, T. and Beachy, P. A. Small molecule modulation of Smoothened activity. Proc. Natl. Acad. Sci. USA, 2002; 99: 14071-14076.
66. Corcoran, R. B. and Scott, M. P. Oxysterols stimulate Sonic hedgehog signal transduction and proliferation of medulloblastoma cells. Proc. Natl. Acad. Sci. USA. 2006; 103: 8408-8413.

67. Bijlsma, M. F., Spek, C. A., Zivkovic, D., van de Water, S., Rezaee, F. and Peppelenbosch, M. P. Repression of Smoothened by Patched-Dependent (Pro-) Vitamin D3 Secretion. PLoS Biol. 2006;4: e282.
68. Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H. The tumour suppressor gene patched encodes a candidate receptor for Sonic hedgehog. Nature. 1996; 384; 129-134.
69. Zhang, C., Williams, E. H., Guo, Y., Lum, L. and Beachy, P. A. Extensive phosphorylation of Smoothened in Hedgehog pathway activation. Proc. Natl. Acad. Sci. USA. 2004; 101: 17900-17907.
70. Matise, M. P. and Joyner, A. L. Gli genes in development and cancer. Oncogene, 1999; 18: 7852-7859.
71. Methot, N. and Basler, K. An absolute requirement for Cubitus interruptus in Hedgehog signaling. Dev. 2001; 128: 733-742.
72. Kinzler KW, Ruppert JM, Bigner SH, Vogelstein B. The GLI gene is a member of the Kruppel family of zinc finger proteins. Nature. 1988; 332: 371-374.
73. Ruppert JM, Kinzler KW, Wong AJ, Bigner SH, Kao FT, Law ML, Seuanez HN, O'Brien SJ, Vogelstein B. The GLI-Kruppel family of human genes. Mol Cell Biol. 1988; 8: 3104-3113.
74. Kinzler KW, Vogelstein B. The GLI gene encodes a nuclear protein which binds specific sequences in the human genome. Mol Cell Biol. 1990; 10: 634-642.
75. Sasaki H, Hui C, Nakafuku M, Kondoh H. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenic and can respond to Shh in vitro. Dev. 1997; 124: 1313-1322.
76. Methot N, Basler K. Suppressor of fused opposes hedgehog signal transduction by impeding nuclear accumulation of the activator form of Cubitus interruptus. Dev. 2000; 127: 4001-4010.
77. Methot N, Basler K. Hedgehog controls limb development by regulating the activities of distinct transcriptional activator and repressor forms of Cubitus interruptus. Cell. 1999; 96: 819-831.
78. Roberts DJ, Johnson RL, Burke AC, Nelson CE, Morgan BA, Tabin C. Sonic hedgehog is an endodermal signal inducing Bmp-4 and Hox genes during induction and regionalization of the chick hindgut. Dev. 1995; 121: 3163-3174.
79. Laufer E, Nelson CE, Johnson RL, Morgan BA, Tabin C. Sonic hedgehog and Fgf-4 act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. Cell. 1994; 79: 993-1003.
80. Oliver TG, Grasfeder LL, Carroll AL, Kaiser C, Gillingham CL, Lin SM, Wickramasinghe R, Scott MP, Wechsler-Reya RJ. Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. Proc Natl Acad Sci USA. 2003; 100: 7331-7336.
81. Duman-Scheel M, Weng L, Xin S, Du W. Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature. 2002; 417: 299-304.
82. Chen W, Tang T, Eastham-Anderson J, Dunlap D, Alicke B, Nannini M, Gould S, Yauch R, Modrusan Z, DuPree KJ, Darbonne WC, Plowman G, de Sauvage FJ, Callahan CA. Canonical hedgehog signaling augments tumor angiogenesis by induction of VEGF-A in stromal perivascular cells. Proc Natl Acad Sci USA. 2011; 108: 9589-9594.

83. Lavine KJ, White AC, Park C, Smith CS, Choi K, Long F, Hui CC, Ornitz DM. Fibroblast growth factor signals regulate a wave of Hedgehog activation that is essential for coronary vascular development. Genes Dev, 2006; 20: 1651-1666.
84. Pola R, Ling LE, Silver M, Corbley MJ, Kearney M, Blake PR, Shapiro R, Taylor FR, Baker DP, Asahara T, Isner JM. The morphogen Sonic hedgehog is an indirect angiogenic agent upregulating two families of angiogenic growth factors. Nat Med. 2001; 7: 706-711.
85. Nery S, Wichterle H, Fishell G. Sonic hedgehog contributes to oligodendrocyte specification in the mammalian forebrain. Dev. 2001; 128: 527-540.
86. Cayuso J, Ulloa F, Cox B, Briscoe J, Marti E. The Sonic hedgehog pathway independently controls the patterning, proliferation and survival of neuroepithelial cells by regulating Gli activity. Dev. 2006; 133: 517-528.
87. Bigelow RL, Chari NS, Unden AB, Spurgers KB, Lee S, Roop DR, Toftgard R, McDonnell TJ. Transcriptional regulation of bcl-2 mediated by the sonic hedgehog signaling pathway through gli-1. J Biol Chem. 2004; 279: 1197-1205.
88. Leung C, Lingbeek M, Shakhova O, Liu J, Tanger E, Saremaslani P, Van LM, Marino S Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas. Nature. 2004; 428: 337-341.
89. Mullor JL, Dahmane N, Sun T, Altaba A. Wnt signals are targets and mediators of Gli function. Curr Biol. 2001; 11: 769-773.
90. Ishibashi M. Molecular mechanisms for morphogenesis of the central nervous system in mammals. Anat Sci Int. 2004; 79: 226-234.
91. Zhang Q, Zhang L, Wang B, Ou CY, Chien CT, Jiang J. A hedgehog-induced BTB protein modulates hedgehog signaling by degrading Ci/Gli transcription factor. Dev Cell. 2006; 10: 719-729.
92. Hallikas O, Palin K, Sinjushina N, Rautiainen R, Partanen J, Ukkonen E, Taipale J. Genome-wide prediction of mammalian enhancers based on analysis of transcription-factor binding affinity. Cell. 2006; 124: 47-59.
93. Jacob, J. and J. Briscoe. Gli proteins and the control of spinal-cord patterning. EMBO Rep. 2003; 4: 761-5.
94. Ruiz i Altaba, A., C. Mas, and B. Stecca. The Gli code: an information nexus regulating cell fate, stemness and cancer. Trends Cell Biol. 2007; 17: 438-47.
95. Pan, Y. Bai CB, Joyner AL, Wang B. Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Mol Cell Biol. 2006; 26: 3365-77.
96. Wang, B., J.F. Fallon, and P.A. Beachy. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell. 2000; 100: 423-34.
97. Bai, C.B. and A.L. Joyner. Gli1 can rescue the in vivo function of Gli2. Dev. 2001; 128: 5161-72.
98. Eichberger, T., Sander V, Schnidar H, Regl G, Kasper M, Schmid C, Plamberger S, Kaser A, Aberger F, Frischauf AM. Overlapping and distinct transcriptional regulator properties of the GLI1 and GLI2 oncogenes. Genomics. 2006; 87: 616-32.
99. Litingtung, Y., Dahn RD, Li Y, Fallon JF, Chiang C. Shh and Gli3 are dispensable for limb skeleton formation but regulate digit number and identity. Nature. 2002; 418: 979-83.

100. Roessler, E., Ermilov AN, Grange DK, Wang A, Grachtchouk M, Dlugosz AA, Muenke M. A previously unidentified amino-terminal domain regulates transcriptional activity of wild-type and disease-associated human GLI2. Hum Mol Genet. 2005; 14: 2181-8.
101. Treier M, Gleiberman AS, O'Connell SM, Szeto DP, McMahon JA, McMahon AP, Rosenfeld MG. Multistep signaling requirements for pituitary organogenesis in vivo. Genes Dev. 1998; 12: 1691-1704.
102. Mo R, Freer AM, Zinyk DL, Crackower MA, Michaud J, Heng HH, Chik KW, Shi XM, Tsui LC, Cheng SH, Joyner AL, Hui C. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Dev. 1997; 124: 113-123.
103. Dunaeva M, Michelson P, Kogerman P, Toftgard R. Characterization of the physical interaction of Gli proteins with SUFU proteins. J Biol Chem. 2003; 278: 5116-5122.
104. Wang Y, Martin JF, Bai CB. Direct and indirect requirements of Shh/Gli signaling in early pituitary development. Dev Biol. 2010; 348: 199-209.
105. Park HL, Bai C, Platt KA, Matise MP, Beeghly A, Hui CC, Nakashima M, Joyner AL. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Dev. 2000; 127: 1593-1605.
106. Jia J, Jiang J. Decoding the Hedgehog signal in animal development. Cell Mol Life Sci. 2006; 63: 1249-1265.
107. Tanimura A., Dan S., Yoshida M. Cloning of novel isoforms of the human Gli2 oncogene and their activities to enhance tax dependent transcription of the human T-cell leukemia virus type 1 genome. J. Virol. 1998; 72: 3958-3964.
108. Varjosalo M., Bjorklund M., Cheng F., Syvanen H., Kivioja T., Kilpinen S., Sun Z., Kallioniemi O., Stunnenberg H.G., He W.W., Ojala P., Taipale J. Application of active and kinase-deficient kinome collection for identification of kinases regulating hedgehog signaling. Cell. 2008; 133: 537 – 548.
109. Maloverjan A., Piirsoo M., Michelson P., Kogerman P., Osterlund T. Identification of a novel serine/threonine kinase ULK3 as a positive regulator of Hedgehog pathway. Exp. Cell Res. 2010; 316: 627-637.
110. Cherry A.L., Finta C., Karlstrom M., Jin Q., Schwend T., Astorga-Wells J., Zubarev R.A., Del Campo M., Criswell A.R., de Sanctis D., Jovine L., Toftgard R. Structural basis of SUFU-GLI interaction in human Hedgehog signalling regulation. Acta Crystallogr. D. 2013; 69: 2563-2579.
111. Tanner JM, Whitehouse RH, Cameron N, Marshall WA, Healy WA, H G. Assessment of skeletal maturity and prediction of adult height (TW2 method). San Diego, CA: Academic Press 1988.
112. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids. Res. 1988; 16: 1215.
113. Taipale J, Chen JK, Cooper MK. Effects of oncogenic mutations in smoothened and Patched can be reversed by cyclopamine. Nature. 2000; 406: 1005-1009.
114. Vila G, Theodoropoulou M, Stalla J. Expression and function of sonic hedgehog pathway components in pituitary adenomas: evidence for a direct role in hormone secretion and cell proliferation. J Clin Endocrinol Metab. 2005; 90: 6687-6694.
115. G.M.C. Flemming, J. Klammt, G. Ambler, Y. Bao, W.F. Blum, C. Cowell, K. Donaghue, N. Howard, A. Kumar, J. Sanchez, H. Stobbe, and R.W. Pfaffle. Functional characterization of a Heterozygous Gli2 missense Mutation in patients with Multiple Pituitary Hormone Deficiency. J Clin Endocrinol Metab. 2013; 98: 567-575.

116. Pavletich NP, Pabo CO. Crystal structure of a five-finger GLI-DNA complex: new perspective on zinc fingers. Science. 1993; 261: 1701-1707.
117. Sasaki .H, Nishizaki. Y, Hui C, Nakafuku M, Kondoh H. Regulation of Gli2 and Gli3 activities by an amino-terminal repression domain: implication of Gli2 and Gli3 as primary mediators of Shh signaling. Dev. 1999; 126: 3915-3924.
118. H S Heussler, M Suri. Sonic Hedgehog. Mol Pathol. 2003; 56: 129-131.
119. Mart Speek, Olga Njunkova, Illar Pata, Eola Valdre and Priit Kogerman. A potential role of alternative splicing in the regulation of the transcriptional activity of human *GLI2* in gonadal tissues. BMC Mol Biol. 2006; 7: 13.
120. Kelly A Bear, Benjamin D Solomon, Sonir Antonini, Ivo J P Arnhold, Marcela M França, Erica H Gerkes, Dorothy K Grange, Donald W Hadley, Jarmo Jääskeläinen, Sabrina S Paulo, Patrick Rump, Constantine A Stratakis, Elizabeth M Thompson, Mary Willis, Thomas L Winder, Alexander A L Jorge, Erich Roessler, Maximilian Muenke. Pathogenic mutations in GLI2 cause a specific phenotype that is distinct from holoprosencephaly. J. Med. Genet. 2014; 51: 413–418.

APPENDIX I:
TRANSFECTION DATA

Transfection data for Variation p.A593V:

	PLASMIDS (µl)	GLI B.S 8X3 (µl)	pRL-TK (µl)	LP 2000 (µl)	D-MEM (µl)	INCUBATION TIME 37^0 C	PLB (µl)
WT FL	18	3	2	6	150	48 Hrs	300
A593V FL	5	3	2	6	150	48 Hrs	300
WT ΔN	19	3	2	6	150	48 Hrs	300
A593V ΔN	7.5	3	2	6	150	48 Hrs	300
pGL4.10	3	-	2	6	150	48 Hrs	300
GFP	2	-	-	6	150	48 Hrs	300
Neg C	-	-	-	-	150	48 Hrs	300

Concentration of Plasmid extracted:

CONSTRUCTS EXTRACTED	CONCENTRATIONS
WILD TYPE FL	85 ng/µl
A593V FL	318 ng/µl
WILD TYPE ΔN	81 ng/µl
A593V ΔN	203 ng/µl
pRL-TK Renilla	40 ng/µl
GLI B.S 8X3	380 ng/µl
pGL4.10	500 ng/µl
GFP	512 ng/µl

Co-Transfection data for variation p.A593V:

	P 1 (µl)	P 2 (µl)	GLI B.S 8X3 (µl)	pRL-TK (µl)	LP 2000 (µl)	D-MEM (µl)	INCUBATION TIME 37^0 C	PLB (µl)
WT FL	18	-	3	2	6	150	48 Hrs	300
A593V FL/WT FL	6.5	9	3	2	6	150	48 Hrs	300
WT ΔN	19	-	3	2	6	150	48 Hrs	300
A593V ΔN/WT ΔN	8.1	9.5	3	2	6	150	48 Hrs	300
pGL4.10	3	-	-	2	6	150	48 Hrs	300
GFP	2	-	-	-	6	150	48 Hrs	300
Neg C	-	-	-	-	-	150	48 Hrs	300

Concentration of Plasmid extracted:

CONSTRUCTS EXTRACTED	CONCENTRATIONS
WILD TYPE FL	85 ng/µl
A593V FL	116 ng/µl
WILD TYPE ΔN	81 ng/µl
A593V ΔN	92 ng/µl
pRL-TK Renilla	40 ng/µl
pGL4.10	500 ng/µl
GLI B.S 8X3	380 ng/µl
GFP	512 ng/µl

Transfection data for variation p.Y575H:

	PLASMIDS (µl)	GLI B.S 8X3 (µl)	pRL-TK (µl)	LP 2000 (µl)	D-MEM (µl)	INCUBATION TIME 37^0 C	PLB (µl)
WT FL	18	7.5	2	6	150	48 Hrs	300
Y575H FL	8	7.5	2	6	150	48 Hrs	300
WT ΔN	13	7.5	2	6	150	48 Hrs	300
Y575H ΔN	10.5	7.5	2	6	150	48 Hrs	300
pGL4.10	3	-	2	6	150	48 Hrs	300
GFP	2	-	-	6	150	48 Hrs	300
Neg C	-	-	-	-	150	48 Hrs	300

Concentration of Plasmid extracted:

CONSTRUCTS EXTRACTED	**CONCENTRATIONS**
WILD TYPE FL	85 ng/µl
Y575H FL	191 ng/µl
WILD TYPE ΔN	116 ng/µl
Y575H ΔN	144 ng/µl
pRL-TK Renilla	40 ng/µl
pGL4.10	500 ng/µl
GLI B.S 8X3	134 ng/µl
GFP	512 ng/µl

Co-Transfection data for variation p.Y575H:

	P 1 (µl)	P 2 (µl)	GLI B.S 8X3 (µl)	pRL-TK (µl)	LP 2000 (µl)	D-MEM (µl)	INCUBATION TIME 37^0 C	PLB (µl)
WT FL	18	-	7.5	2	6	150	48 Hrs	300
Y575H FL/WT FL	4	9	7.5	2	6	150	48 Hrs	300
WT ΔN	13	-	7.5	2	6	150	48 Hrs	300
Y575H ΔN/WT ΔN	5.2	6.5	7.5	2	6	150	48 Hrs	300
pGL4.10	3	-	-	2	6	150	48 Hrs	300
GFP	2	-	-	-	6	150	48 Hrs	300
Neg C	-	-	-	-	-	150	48 Hrs	300

Concentration of Plasmid extracted:

CONSTRUCTS EXTRACTED	CONCENTRATIONS
WILD TYPE FL	85 ng/µl
Y575H FL	191 ng/µl
WILD TYPE ΔN	116 ng/µl
Y575H ΔN	144 ng/µl
pRL-TK Renilla	40 ng/µl
pGL4.10	500 ng/µl
GLI B.S 8X3	134 ng/µl
GFP	512 ng/µl

Luciferase assay details:

Reagents	Volume
Whole Cell Lysate	20 µl
LAR II	100 µl
STOP & GLO	100 µl
Fluorescence captured time 10 sec	

Transfection data for variation p.P386L:

	P 1 (µl)	P 2 (µl)	GLI B.S 8X3 (µl)	pRL-TK (µl)	LP 2000 (µl)	D-MEM (µl)	INCUBATION TIME 37^0C	PLB (µl)
WT FL	9.2	-	3	3	6	150	48 Hrs	180
P386L FL	5.3	-	3	3	6	150	48 Hrs	180
P386L FL/WT FL	2.6	4.6	3	3	6	150	48 Hrs	180
pGL4.10	3	-	-	3	6	150	48 Hrs	180
GFP	2		-	-	6	150	48 Hrs	180
Neg C	-		-	-	-	150	48 Hrs	180

Concentration of Plasmids Extracted:

CONSTRUCTS EXTRACTED	CONCENTRATIONS
WILD TYPE FL	164 ng/µl
P386L FL	284 ng/µl
pRL-TK Renilla	50 ng/µl
GLI B.S 8X3	500 ng/µl
pGL4.10	500 ng/µl
GFP	512 ng/µl

Luciferase assay details:

Reagents	Volume
Whole Cell Lysate	5 µl
LAR II	25 µl
STOP & GLO	25 µl
Fluorescence captured time 10 sec	