The role of the aryl hydrocarbon receptor interacting protein (AIP) in pituitary tumorigenesis:

A novel animal model for investigating the role of AIP during embryogenesis and pituitary tumour formation

Thesis presented by:
Mária Hérincs, MBBS

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Under the supervision of:
Prof. Márta Korbonits, MBBS, MD, PhD
Prof. Paul Chapple, PhD

Centre for Endocrinology
William Harvey Research Institute
Barts and The London School of Medicine
Queen Mary University of London

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Statement of originality

I, Dr Mária Hérincs, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below, and my contribution indicated. Previously published material is also acknowledged below.

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Date: 8 November 2020

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Abstract

Pituitary adenomas (PAs) have a 1:1000 prevalence and carry significant morbidity despite their benign nature. Mutations in the AIP (aryl hydrocarbon receptor interacting protein) gene have been unambiguously associated with higher predisposition for PAs. These tumours pose a challenge in treatment due to delayed diagnosis, increased size, earlier onset, aggressive nature and considerable resistance to therapy. The exact mechanism of the tumour formation due to loss of AIP and the role of AIP in pituitary organogenesis is still unknown.

A large-cohort retrospective (225 patients) and prospective (876 patients) clinical study was carried out on the pituitary adenoma patients of a tertiary referral centre in London assessing the prevalence of FIPA (familial isolated pituitary adenomas). 20% of FIPA patients are reported to harbour an AIP mutation, but half of the AIP mutation-positive probands are not aware of a positive family history. This study has shown that active inquiry of family history increases detection of previously unknown family history with nearly 3-fold, enabling the genetic screening of these families for early diagnosis and better customised therapy.

The novel, pituitary- specific, biallelic Aip-knockout murine model (AipFlox/Flox; Hesx1Cre/+) generated within this study allowed for the first time to investigate the role of AIP during the embryonic stages. There is phenotypical difference (enlarged anterior lobe, incomplete fusion of the sphenoid bone) in the pituitaries 17.5 dpc AipFlox/Flox; Hesx1Cre/+ embryos when compared with the wildtype of the same embryonic stage, with no difference in cell lineage determination (dpc 15.5). A decrease in growth hormone and prolactin producing cells is described at terminal differentiation (dpc 17.5), which is intriguing because patients harbouring AIP mutations most commonly present with growth hormone and/or prolactin secreting

PAs. Further research has since been done efficiently using this model focusing on postnatal tumour formation, which is not part of this thesis.

Deregulation of the Hippo signalling components has been increasingly investigated in relation to pituitary tumorigenesis, especially in human hormone secreting PAs, but no study had investigated the role of Hippo signalling in AIP-mediated tumour formation. A bioinformatic-based analysis was performed for the expression of 41 Hippo pathway associated genes in AIP-silenced rat pituitary somatomammotroph GH3 cells, pituitary specific AIP-knockout mice, humans with AIP mutation-positive and AIP mutation-negative familial and sporadic PAs. Both up- and downstream members of the Hippo signalling show significantly altered expression in the different subgroups, which warrants more targeted future studies. Additionally, in the analysed exome and whole genome sequencing of PA patients, variants of several key Hippo pathway components show segregation within families. These data (along with a global phosphorylation analysis) suggest that further studies are needed to investigate the role of Hippo signalling in AIP-mediated tumorigenesis.

Justification of the project

Clinically relevant pituitary adenomas (PAs) are amongst the most common intracranial tumours with their 1:1000 prevalence in the developed world (1, 2). Despite their usual benign nature, they are associated with significant morbidity due to their local compressive effects on nearby vital structures, as well as the severe consequences of hormone over- or underproduction. These features make them important, yet challenging diagnostic targets, as symptoms can be vague and complex, delaying diagnosis and prompt treatment.

Since its initial identification for being responsible for increased PA predisposition, the *AIP* (aryl hydrocarbon receptor interacting protein) has been widely investigated and familial isolated pituitary adenoma (FIPA) has become an emerging clinical entity. Approximately 20% of FIPA patients harbour a mutation in the *AIP*, and the mutations are most prevalent in somatotrophinomas (growth hormone secreting PAs), but mutations have been described in all the other PA types as well (3).

AIP is a tumour suppressor, loss of heterozygosity has been observed in tumour samples. *In vitro* data shows that wildtype AIP attenuates cell proliferation (4), whereas mutant AIP loses this effect, the knockdown of *AIP* leading to increased cell proliferation (5).

To date, the only tumours found to unambiguously associate with *AIP* mutations are PAs, and these tumours are larger, more invasive and aggressive, have an earlier onset and a poorer treatment response compared to those without *AIP* mutation (6).

Elucidating the role of AIP in pituitary tumour formation would lead to more efficient and targeted therapies in this often therapeutically challenging patient cohort.

The novel animal model created within the setting of this PhD project provides us with a tool to elucidate pituitary tumour formation as well as giving us the opportunity to describe the role of AIP during embryogenesis and pituitary tumorigenesis and to characterise *AIP*-mutated tumours *in vivo*.

General aims of the PhD project

- Perform retrospective and prospective study on the pituitary patient population of Barts Health NHS Trust Endocrinology department in terms of family history of pituitary adenoma and other tumours in order to determine the rate of different neoplasms in this patient cohort and to determine the prevalence of FIPA in the cohort
- Investigate whether direct questioning on family history increases the detection rate and prevalence of FIPA in the same patient cohort
- Generate a novel, pituitary tissue-specific Aip-knockout mouse model and establish the colony
- Explore the expression pattern of AIP during murine embryogenesis *in vivo*
- Characterize the animal model during embryogenic stages
- Characterize the animal model in the postnatal stages, explore the tumour formation and general characteristics of the animals
- Investigate the role of the Hippo signalling pathway in AIP-mediated pituitary tumorigenesis

Chapter I.

General overview

The pituitary gland

The pituitary gland (hypophysis) is a small endocrine organ often referred to as the “master gland” of the body since it controls diverse and complex functions of the body. The size of the pituitary is usually below 8 millimetres in the cranio-caudal plane, but it can reach 10-12 millimetres during pregnancy and puberty. Despite the pivotal role of the pituitary, the true superior regulator remains the hypothalamus. The hypothalamic-pituitary axis (HPA) is a key regulator of homeostasis, controlling not only the release of pituitary hormones, but also a range of autonomic and behavioural functions including temperature regulation, food and water intake, sleep, circadian rhythms and mediation of emotion (7).

The pituitary gland ensures that the body is capable of adequate response to changing physiological situations, such as puberty, pregnancy or stress. As the seven hormones (Table 1) secreted to the bloodstream affect most physiological processes, it is hardly a surprise that pathological conditions affecting the gland resulting in hormonal imbalance are of major clinical importance with significant morbidity.

History of the pituitary gland

Etymologically the term *hypophysis* comes from the Greek ὑπό (hupó, “under”) + φύσις (phúsis, “nature”), from φύω (phúō, “to bring forth”), while *pituitary* originates from the latin word *“pituita”* meaning phlegm, as it was believed that it was draining mucus to the nose from the brain (8). Anatomist Andreas Vesalius was the first to use the term “glandula pituitaria”, from which the English name pituitary gland is derived (9).

There have been many wrong turns and false ideas during the process of discovering the pituitary axis, some ideas dating back to ancient China and Egypt. Even though they did not have an explanation, they realized that the removal of ovaries/testes and prolonged lactation were efficient ways of preventing pregnancy. Aristotle described that the brain “was a gland secreting humours” and he was the first to observe that obesity must somehow be linked to female infertility (10), which were - in principle - theories pointing to the right direction.
Giants are mentioned in the Holy Bible on multiple occasions, first in the Book of Genesis. Some suggest that the story of Goliath and David from the Bible - describing Goliath to be a giant whose visual field defect allows David to defeat him - is in fact the first description of the mass effect of a GH secreting pituitary tumour (10, 11). A paper published by Donnelly *et al.* suggests that Goliath’s family tree (Figure 1) and their description in the Bible might make them the first identifiable family with a pituitary disease following an autosomal dominant hereditary pattern, similar to the families carrying a mutation in the *AIP* gene (11).

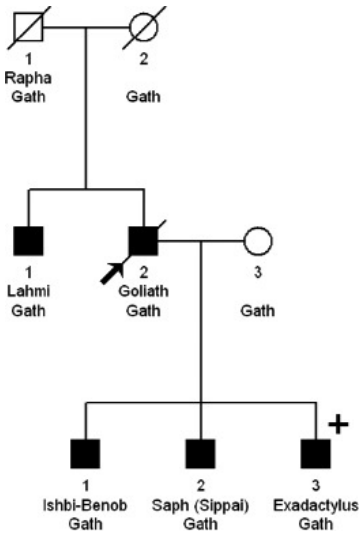


Figure 1. Goliath's Pedigree. Family members with gigantism shaded in black, + symbol indicates (h)exadactyly (11)

Others consider the first note of an endocrine disease to be a portrait of pharaoh Akhenaten (from 1365 BC) depicting the typical signs of acromegaly (12).

Galen of Pergamon, the Greek physician was the first to describe the existence of the gland around 150 AD. He was getting closer to the truth, proposing that its role was to drain the phlegm from the brain (12). The gland's exact role in the endocrine homeostasis, however, remained undiscovered until many centuries later mostly due to a need for more sophisticated research techniques (imaging, laboratory evaluation) as well as due to a general decline in scientific advancement during the dark Middle Ages (10).

It was not until the 18th century that Joseph Lieutaud discovered the pituitary-portal blood system, known today as the hypothalamo-hypophyseal axis. In 1887, Minkowski was the first to make the connection between the expansion of the pituitary gland and various clinical symptoms. In 1892, Vassale and Sacchi

demonstrated that removing the pituitary gland (hypophysectomy) affects the mineral metabolism of the body. Massalongo linked acromegaly to over-functioning of the hypophysis shortly before Cushing discovered the first experimental link between the pituitary and the reproductive organs in 1910 (10).

The real functions of the pituitary gland as an important constituent of the endocrine system were not extensively understood until the latter part of the nineteenth century and the first half of the 20th century.

Anatomy and physiology of the pituitary gland

The pituitary gland (hypophysis cerebri) is a small, bean-shaped organ housed in a bony cavity, the so-called sella turcica of the sphenoid bone at the base of the skull in the midline. The fully developed gland is 0.6 x 0.9 x1.3 cm in size and weighs 0.5-0.6 grams on average (13) and is enveloped by the sellar diaphragm (diaphragma sellae), which is a flat piece of the dura mater, that consists of two layers, the outer (osteo-periostal) and the inner (cerebral) layer.

The gland rests in close proximity to the optic chiasm, which is the reason behind pituitary masses commonly presenting with visual disturbance as a first symptom (bitemporal hemianopia), which often makes pituitary surgery challenging due to potential damage to the chiasm resulting in vision loss. Inferior to the gland is the sphenoid sinus and the most common surgical access to the gland is via this cavity.

The hypophysis is connected to the hypothalamus by the infundibulum (also called the pituitary stalk), which goes through the central opening of the dura mater. The infundibulum is a funnel-shaped structure originating from the tuber cinereum and median eminence of the hypothalamus. It can be divided into the anterior pars tuberalis (often considered to be the part of the pituitary gland itself) and the posterior pars infundibularis. The latter contains the unmyelinated axons of paraventricular and supraoptic neurons (14) (**Figure 2**).

The hypothalamus (the most ventral part of the diencephalon) is the superior regulator of the hypophysis. It controls the release of pituitary hormones, as well as a wide range of autonomic and behavioural functions (temperature regulation, food and water intake, sleep, circadian rhythms and mediation of emotion). The hypothalamus receives both hormonal and neural input from the periphery and the brain and exerts its action via neural as well as humoral pathways. The

hypothalamus is extensively connected to both lobes of the pituitary gland via long portal vessels and axons in the pituitary stalk. The anterior and the posterior lobes are connected via the short portal vessels (15) (**Figure 2***).*

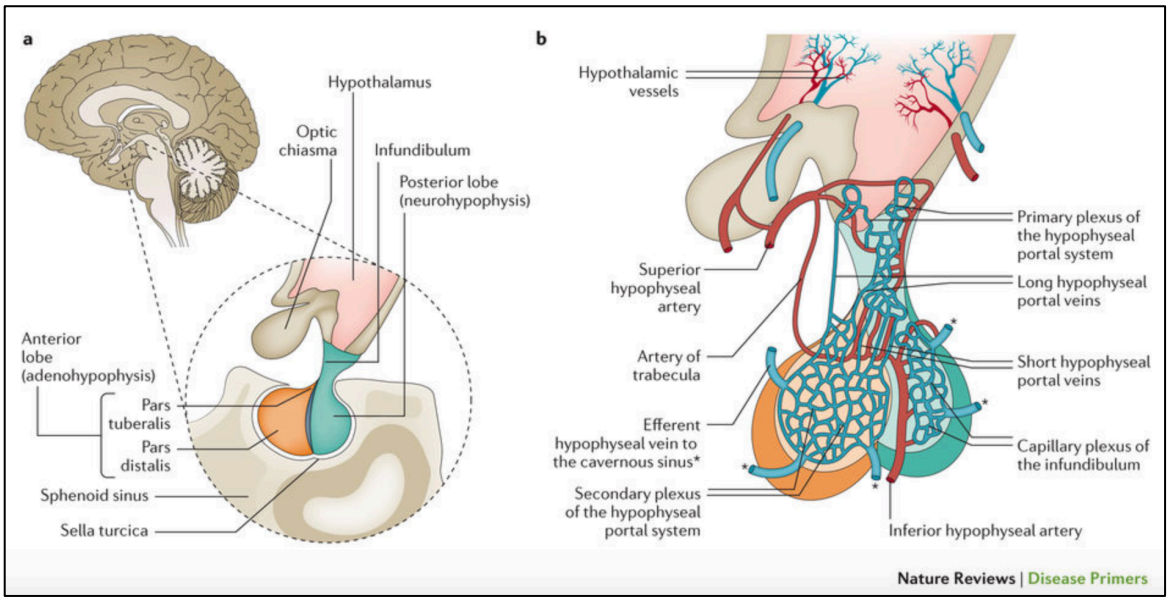


Figure 2. Anatomy and vascularisation of the pituitary gland *(16)*

The pituitary is composed of two anatomically and functionally distinct parts: the **neurohypophysis (posterior lobe)** and the **adenohypophysis (anterior lobe)**, the latter compromising approximately 75% of the whole pituitary. The anterior lobe (AL) is generally smaller in males than in females and in nullipara women (a woman that has not given birth to a viable child) than multipara (a woman that has had more than one pregnancy resulting in birth to a viable child) women. The intermediate lobe (important in many animals) is rudimentary in humans (17). It represents a narrow zone between the adeno- and neurohypophysis potentially containing microscopic remnants of Rathke's cleft (epithelial invagination of the oral ectoderm during embryogenesis) (14).

The **posterior lobe (PL)** does not contain neuroendocrine cells but instead consists of nerve fibres coming from the supraoptic and paraventricular nuclei of the hypothalamus. It also consists of pituicytes, special glial-like cells that do not have secretory functions but form a supportive matrix for the hypothalamic nerve fibres. Hypothalamic nuclei synthesise arginine-vasopressin (also known as antidiuretic hormone) and oxytocin (both 9 amino-acid peptide hormones). These hormones are transported to the posterior pituitary via the infundibulum bound to the neurophysin carrier protein to the ends of the axons, which are located in close proximity to the bloods vessels, enabling rapid release of the hormones to the circulation (15).

Microscopically, the **anterior lobe of the pituitary gland (AL)** has five main cell types organised into cords around venous sinusoids. These cells manufacture six types of hormones to be released into the circulation (**Table 1***)*. Their release is regulated by stimulatory or inhibitory hypothalamic hormones arriving from the hypothalamus via the vessels in the stalk. Sommatomammotroph cells are rare cells that secrete both growth hormone (GH) and prolactin (PRL). The folliculostellatae are agranular, star-shaped, non-hormone producing cells that have been shown to play an important role in cell to cell interaction within the gland (18).

Cell type	Percentage out of all pituitary cells	Hypothalamic regulatory hormone	Secreted hormone	Target organ
Somatotrophs	45-50 %	Growth hormone releasing hormone (GHRH) Ghrelin *Inhibited by somatostatin*	Growth hormone (GH)	liver, muscle, bone, cartilage, adipose tissue
Thyrotrophs	5 %	Thyrotrophin-releasing hormone (TRH)	Thyroid stimulating hormone (TSH)	thyroid gland
Lactotrophs	10-30 %	*Inhibited by dopamine* Thyrotrophin-releasing hormone (TRH)	Prolactin (PRL)	mammary gland
Gonadotrophs	10-15 %	Gonadotrophin-releasing hormone (GnRH)	Follicle-stimulating hormone (FSH) Luteinizing hormone (LH)	ovaries and testes
Corticotrophs	10-20%	Corticotrophin-releasing hormone (CRH) Arginine vasopressin (AVP)	Adrenocorticotrophic hormone (ACTH)	adrenal gland (cortex)

Table 1. Major cell types of the anterior pituitary gland

The endocrine system is primarily controlled by negative feedback loops within the hypothalamic-pituitary-peripheral organ axis. The stimulatory and inhibitory hypothalamic and pituitary hormones are influenced by negative feedback loops from the periphery and the central organs. In the gonadal axis at the time of puberty and ovulation positive feedback exists (14).

Embryology of the pituitary gland in human and mice

The pituitary gland is of dual origin - the anterior lobe (adenohypophysis) deriving from the oral ectoderm and the posterior lobe (neurohypophysis) originating from the neural ectoderm. Much of our data on pituitary organogenesis come from murine model studies, where pituitary development is initiated during neurulation, from 8 days post coitum (dpc) (19). The phylogenetic distribution of homologs of pituitary hormones extends to invertebrates, but the existence of the gland itself is unique to vertebrates (20, 21).

Pituitary morphogenesis in humans commences during the 3rd-4th foetal week and is complete by the 20th gestation week (14).

A thickening of cells in the oral ectoderm form the hypophyseal placode, which gives rise to Rathke's pouch (RP), an upward evagination that extends towards the neural ectoderm. The RP is present in humans by the 3rd gestational week. The anterior pituitary derives from the cells of the anterior wall of the RP. The posterior lobe derives from the floor of the third ventricle. When fused, the sides of the third ventricle form the neural stalk, while the superior floor gives rise to the median eminence (22). The RP constricts at its base and separates from the oral epithelium during gestational week 6-8. Terminal differentiation occurs within the anterior lobe to form the specialised endocrine cell types of the pituitary gland, which is a very precisely regulated process by various transcription factors. The expression of the transcription factors controlling cell-lineage commitment and cell proliferation is a very sensitive process, minor disruptions can result is developmental disorders of the gland or development of pituitary tumours (14).

The timeline and details of murine pituitary embryogenesis is as shown in **Figure 3** (19).

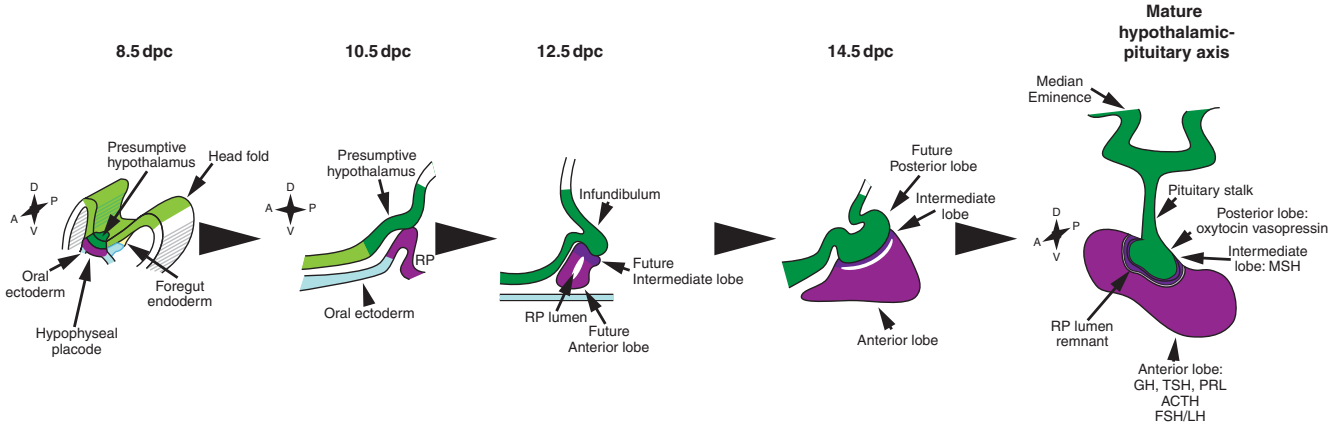


Figure 3. Murine pituitary embryogenesis from 8.5 days post coitum (dpc) *The process is initiated during neurulation with the appearance of the hypophyseal placode at 8.5 dpc, which later invaginates and becomes the Rathke's pouch (RP). The infundibulum at this developmental stage lies within the ventral diencephalon and starts to evaginate towards the RP by 10 dpc and at 12.5 dpc giving rise to the median eminence, the pituitary stalk and the posterior lobe. The anterior and intermediate lobes both derive from the RP. In the mature gland, similar to humans, the anterior pituitary endocrine cells comprise lactotrophs (producing prolactin, PRL), gonadotrophs (producing luteinizing hormone, LH; and follicle stimulating hormone, FSH), thyrotrophs (producing thyroid stimulating hormone, TSH), corticotrophs (producing adrenocorticotrophic hormone, ACTH) and somatotrophs (producing growth hormone, GH). Hypothalamic hormones can reach the gland via the pituitary stalk, oxytocin and vasopressin are secreted directly into the posterior lobe coming from the hypothalamus. The intermediate lobe (rudimentary in humans) is located between the anterior and the posterior lobes, containing melanotrophs (producing melanocyte-stimulating hormones, MSH) (19). In contrast, in humans, the intermediate lobe mostly disappears during embryogenesis.*

Genetics of pituitary development

In mice, the gland will undergo a phase of growth during the initial few weeks of life, characterised by high levels of proliferation, both in progenitors and in endocrine cells. Signals secreted mostly by the ventral diencephalon (VD), are crucial for induction and maintenance of the Rathke's pouch (RP). These include the **bone morphogenetic proteins** (BMPs, firstly in time *Bmp4*, then *Bmp2* and lastly *Bmp7*) that are essential for the development of the pouch and probably also have a role in progenitor proliferation (23).

Fibroblast growth factors (FGFs) are required for activation of cell cascades affecting cell proliferation, survival and motility as well as RP progenitor proliferation (imbalance of these factors causes double RP formation (24)) . Dysregulation of these processes is also linked to cancer formation and to the development of congenital disorders, most importantly Kallmann syndrome (disrupted differentiation of gonadotropin releasing hormone resulting in hypogonadism) (25, 26). Fgf8, Fgf10 and Fgf18 are first detected during RP development from 9.5 dpc, therefore appearing at a slightly later embryonic stage than BMPs (8.5 dpc) (27). *Fgf10* deletion causes the formation of a small apoptotic pouch (28, 29), some data suggest it may be involved in RP invagination as well (30).

Hedgehog ligands, such as **sonic hedgehog (SHH)** are detected in the ventro-medial diencephalon (lateral to the infundibulum) where they are required for the maintenance of the prospective hypothalamus (31). In the absence of SHH in VD, infundibular Bmp4 and Fgf10 patterns are expanded ventrally causing RP duplication as well as failure of invagination and consequently RP cannot progress beyond 10.5 dpc (31). In summary, according to these data a fine-tuned antagonism between BMP and SHH in VD is essential for infundibular morphogenesis, RP positioning and

development. In a novel murine model, conditional deletion of Shh in the anterior hypothalamus resulted in a fully penetrant phenotype characterised by a complete arrest of RP development, with lack of **LIM-homeodomain transcription factor 3 and 4 (Lhx3/Lhx4)** expression in RP epithelium at 9.0 dpc and total loss of pituitary tissue by 12.5 dpc (32). Interestingly in the same study, over-activation of the Shh pathway resulted in severe hyperplasia and enlargement of the Sex determining region Y-box 2 positive (Sox2+) stem cell compartment (32). SHH-expression in the diencephalon is dependent on **Sex determining region Y-box 2 and 3 (Sox2 and Sox3*)*** transcription factors (33).

Molecules of the wingless-type mouse mammary tumour virus (**Wnt)** pathway are not only important postnatally (regulating stem cell population proliferation (34) and in cancer formation (35-38)) but are also present in the VD and RP from 9.5 to 12.5 dpc (39) responsible for correct patterning and indirectly affecting RP shaping. Absence of a particular Wnt molecule, the **Wnt5a** results in more oral ectoderm being recruited to form the RP and extra bifurcations appear (40). Deletion of *WNT4* (expressed exclusively in RP) causes a slight reduction in expression the pituitary-specific transcription factor 1 (**Pit1)** required for emergence of somatotrophs, lactotrophs and thyrotrophs (41). Activation of the Wnt pathway results in loss of the pituitary at 13.5 dpc (41). In contrary to this, two studies have recently supplied convincing data that a constitutively active Wnt pathway causes increased proliferation and tumour formation (34, 42) in mice.

The precise role of the **Notch** signalling pathway in early VD formation is yet to be elucidated, but it is in some way required for infundibular morphogenesis. **Hairy and enhancer of split-1 (Hes1*)***, an important Notch-pathway member, is expressed throughout the VD and total loss of ***Hes1*** results in reduced invagination and later

absence of the posterior lobe (43). Several members of the Notch-pathway have been demonstrated to have a role in progenitor proliferation and cell fate choice, but the data not yet convincing and these studies are slightly contradictory to each other (44-46). Conditional deletion of ***Notch2*** in RP results in down-regulation of the transcription factor Prop1 (47). Notch directly activates **PROP paired-like homeobox 1** (**Prop1*)*** expression, the required pre-step to the **pituitary-specific positive transcription factor 1** (**Pit1)** lineage (detailed later) emergence (46) . All in all, Notch signalling appears to be necessary to prevent differentiation, ensuring the generation of sufficient progenitors, and later to promote emergence of the Pit1 lineage.

A wide array of **transcriptional regulators** is required to be present and functioning during pituitary embryogenesis for adequate cell lineage commitment and proper pituitary development, which is a process very sensitive to even the slightest perturbation.

Homeobox expressed in ES cells 1 (Hesx1) is a homeodomain transcription factor essential for forebrain development, mutations associated with septo-optic dysplasia in humans (48). *Hesx1* null mice display pituitary dysplasia at birth (49) and postnatal lethality due to severe central nervous system (CNS) defects (48). Hesx1 can be found in the RP until 13.5 dpc (50), its expression under the positive control of ***Lhx3*** and ***Pitx2*** (51). It is downregulated by the **beta-catenin/PROP1** complex. Hesx1 represses Prop1 expression, which is crucial for pituitary development progression (52, 53). In, summary, Hesx1 is an important regulator of early pituitary development and its interaction with Prop1 regulates RP development.

Sine Oculis homoeobox transcription factor family protein 3 and 6 (SIX proteins, **Six3** and **Six6)** are known for their role in eye development and regulation of proliferation during tumorigenesis, they are both expressed in VD and RP during embryogenesis (54). Deletion of *Six6* results in hypomorphic pituitary and retina (55), while deletion of *Six3* results in mutants arresting prior to initiation of pituitary development (56). *Hesx1* mutants suggest an interaction of *Six3* with Wnt signalling in RP (49, 57), where *Six3* would serve as a Wnt repressor, but further data is required on this.

The paired-like homeodomain proteins 1 and 2 (**Pitx1** and **Pitx2**) are regulators in the morphogenesis of several organs as well as knowns for their role in mouth development (58). Pitx1 and Pitx2 are present during embryogenesis in the hypothalamus, the RP and later in differentiated endocrine cells (59). They are required for early RP progenitor maintenance (60). Pitx2 seems to be more important at earlier embryonic stages than Pitx1 as RP development is arrested in *Pitx2*$^{-/-}$ mutants, while animals with missing *Pitx1* develop a normal pouch (60). *Pitx2* is a target of Wnt-signalling and it induces (via direct transcriptional activation of cyclins) progenitor proliferation (61). Zhao *et al.* created *Pitx1, Pitx2* double mutants and observed increase in cell apoptosis and absence of Lhx3 expression (62) underscoring that Lhx3 functions downstream of the *Pitx* factors controlling early cell differentiation. Proper Pitx dosage is required throughout development for generating and maintaining enough endocrine cells (63), and it is also required postnatally for thyrotrop function (64). In conclusion, Pitx1 and Pitx2 have many interacting partners, consequently playing a role in a several aspects of pituitary morphogenesis

and maintaining pituitary function postnatally as well.

LIM-homeodomain transcription factor family protein 2 (Lhx2) is expressed in the VD during the formation of the RP plays an important role in infundibulum and posterior lobe formation (65), absence of Lhx2 results in failed invagination of the pituitary stalk and consequent more rostral position of the RP as it cannot expand dorsally (31), endocrine function remains intact due to uninterrupted cell differentiation (65). Lhx3 is first expressed at 9.5 dpc and maintained throughout into the postnatal stages (66). Prop1 activates Lhx3 expression. Conclusively, Lhx3 and Lhx4 are initially required for progenitor maintenance rather than proliferation and art later stages Lhx3 is also required for proper cell differentiation. In *Pitx1, Pitx2* double mutants the absence of Lhx3 expression emphasises that Lhx3 functions downstream of the *Pitx* factors controlling early cell differentiation (62).

SOX2 and **SOX3** are broadly expressed in the central nervous system promoting a proliferative state (67), homozygous *Sox2* null mutants die during embryogenesis. *Sox2* and *Sox3* are present in the VD targeting *Six3 (68)* and *Six6 (69)* as well as *Shh (33).* Loss of *Sox2* results in change to the number of GnRH neurons; hypogonadotrophic hypogonadism in patients harbouring a *Sox2* mutation is therefore likely to be of hypothalamic origin (70).

Sox2 is expressed in the RP, first detected at 9.5 dpc, persists throughout embryogenesis and remains present in the adult pituitary (in stem cell populations – see chapter *Stem cells in the pituitary gland* within this thesis) (70). **Sox9,** a SOXE sub-family member is also expressed in the RP (71). Time-specific *Sox2* deletion at 12.5 dpc causes proliferation with consequent endocrine cell deficit (somatotrophs in

particular) as well as downregulation of Prop1 and Pitx1 (70). In conclusion, Sox2 is required in the RP for progenitor proliferation.

A paired homeodomain transcription factor, ***Prop1*** is the earliest pituitary identity marker, exclusive to the pituitary. Humans harbouring autosomal recessive *Prop1* mutations develop combined pituitary hormone deficiency (72). In mice, loss of *Prop1* results in reduction of Pit1 expression and consequent lack of somatotrophs, lactotrophs, thyrotrophs and also gonadotrophs but uninterrupted Hesx1 expression (73). Prop1 starts to be expressed at 10 dpc and is then maintained throughout pituitary embryogenesis co-existing with Sox2-expressing progenitors (absent in the ones becoming the intermediate lobe). Postnatally, it is rapidly down-regulated and it remains detectable only in a small proportion of Sox2 cells (74, 75). *Prop1* can activate *Pit1* expression via binding to the beta-catenin, as mentioned earlier. *Prop1* is possibly not the sole regulator of this but only a contributor considering that *Prop1* is already expressed from 10 dpc and *Pit1* is first detected at 13.5 dpc (74). In summary, **Prop1** is initially ubiquitously expressed in progenitors and at later stages becomes crucial for anterior lobe development.

In the normal pituitary, emergence of each specific endocrine lineage **(lineage commitment and differentiation**) relies on a lineage-specific transcription factor promoting commitment to a certain lineage while repressing another.

The Pit1 lineage (somatotrophs, lactotrophs, thyrotrophs and somatomammotrophs)

Pit1 starts being expressed from 13.5 dpc and is exclusive to the pituitary (76), its expression activated by *Prop1* - as detailed earlier. It remains present in the terminally differentiated cells regulating the expression of hormones. *Pit1*-mutant murine models revealed that embryogenic lineage commitment does happen without Pit1, however the colony of differentiated cells do not remain present after birth, proposing a role of *Pit1* in postnatal cell survival (77). Both thyrotrophs and gonadotrophs express the alpha GSU (glycoprotein hormone alpha subunit, the common subunit for TSH and gonadotrophins), the earliest (11.5 dpc) marker of endocrine differentiation, therefore the two populations share the early specification stages.

The TPIT lineage (corticotrophs)

Corticotroph differentiation requires the T-box transcription factor (TPIT), which starts to be expressed at 12.5 dpc in the Rathke's pouch and it remains to be expressed in mature corticotrophs (78, 79), mutations causing severe ACTH deficiency due to failure in terminal differentiation (80). TPIT controls Pomc1 expression (similarly to Pitx1) (79). TPIT and SF1 mutually inhibit each other's function, resulting in TPIT blocking gonadotroph emergence (78). Neuro D1 (transcription factor) acts together with Pitx1 induces Pomc1 expression (81).

Gonadotrophs

The earliest gonadotroph marker is the GnRH receptor, which is expressed from 12.5 dpc (partially induced by Lhx3) (82). Gonadotrophs originate from the ventral part of the RP initiating LH and FSH expression at embryonic day 16.5-17.5 in mice (83).

Steroidogenic factor-1 (SF-1) is a factor first transcribed at embryonic day 13.5-14.5 (shortly before LH and FSH appears) and is required for gonadotroph differentiation. SF1-mutated mice have markedly lower FSH and LH levels, gonadal agenesis, infertility and sexual immaturity (84).

Stem cells in the pituitary gland

Stem cells are unspecialized (undifferentiated) cells of a multicellular organism, retaining the ability to divide and give rise to specialized cells, hence contributing towards the body's capability of tissue renewal.

The existence of pituitary stem cells in the adult murine pituitary (42) and human pituitary (85) has been described using genetic tracing experiments and inducible murine models. These stem cells can differentiate into any of the cell types of the pituitary as well as self-propagate *in vitro*, therefore playing a major role in pituitary homeostasis.

Pituitary stem cells (PSCs) have the ability to commit to either of the three major lineages, characterized by the expression of PIT1 (POUF1), TPIT (TBX19) and SF1 (NR5A1) transcription factors. PIT1 positive cells give rise to somatotrophs (expressing GH), lactotrophs (expressing PRL) and thyrotrophs (expressing TSH), TPIT1-positive cells give rise to adenocorticotrophs (expressing ACTH) and melanotrophs, while SF1-positive cells give rise to gonadotrophs (expressing LH and FSH) (42). The homeostasis of the gland and its capacity to react to physiological demand as well as damage to the gland are ensured by the strict and dynamically changing regulation of these cell populations.

The presence of so-called cancer stem cells in pituitary adenomas has been widely reported, both in mice and human samples (86, 87). These cells are multipotent cells within the tumour supposedly having a driving role in tumorigenesis. Cancer stem cells are capable of self-renewal and producing tumour cells in an autonomous manner, meaning the tumours cells are descending from the cancer stem cells directly. Whether cancer stem cells arise from pituitary stem cells is yet unknown.

This discovery opened new possibilities in tumour therapy many believing that more targeted therapies should aim eliminating these cancer stem cells as well as the tumour cells for optimal outcome. However, the challenge is that cancer stem cells are very resistant to anti-proliferative agents and radiotherapy due to their particular molecular properties (88, 89).

Recent studies on adamantinomatous craniopharyngioma (a type of pituitary tumour mostly present in children) mouse models have shown that mutated PSCs promote tumorigenesis in a paracrine manner (42).

Better characterization of the PSC population and its role in tumorigenesis will certainly lead to more targeted and effective therapy, identification of novel biomarkers helping earlier diagnosis and improving the prognosis of PAs.

Vasculature of the pituitary gland

The pituitary gland has a generous blood supply provided by the portal circulation (supplying the anterior lobe) and the arterial circulation (supplying the posterior lobe) (90, 91).

The anterior pituitary has branches descending from the hypothalamus via the infundibulum. A rich network of six arteries supply the stalk and the posterior lobe of the pituitary, three from above and three from below. The anastomoses of these six arteries form the following two networks:

- circuminfundibular anastomosis
- inferior hypophyseal arterial circle

The circuminfundibular anastomosis surrounds and supplies the pituitary stalk, while the inferior hypophyseal arterial circle is responsible for transporting fresh blood to the base of the pituitary gland (90, 91) (**Figure 2**).

Chapter II.

Pituitary adenomas

Pituitary disorders can be broadly divided into the following categories :

- developmental abnormalities and cystic lesions
- vascular disorders
- inflammatory conditions
- miscellaneous alterations
- hyperplasia
- neoplasms (adenomas, carcinomas)

Anatomical variations in the position and shape of the pituitary hold little clinical significance when it comes to the function of the gland but are important for radiologists and neurosurgeons.

Enlargement of the pituitary (hyperplasia) without pituitary tumours

Females commonly have larger pituitaries (up to 1g), especially during or after pregnancy (reaching 10-12 mm) due to the hyperplasia of the lactotroph cells, which is a physiological phenomenon (92). During puberty and menopause, physiological transient enlargement has been observed (93). Prolonged antipsychotic treatment regime correlates with larger pituitary volumes and hyperprolactinaemia is also frequently seen (94). Post-marketing data of antipsychotics show an association with pituitary tumours (95), however the data available so far does not imply more than a slightly increased risk for adenoma development (96). Less commonly, pituitary hyperplasia can be caused by amyloidosis, hemosiderosis, sarcoidosis, tuberculosis, Erdheim-Chester disease or secondary to hypothyroidism (97-99).

Pituitary adenomas

Malignant tumours (pituitary carcinomas), although extremely rarely (0.1-0.2% of all pituitary tumours), but can develop in the pituitary gland carrying high mortality rates, mostly reported on autopsy (100). **Benign tumours** of the gland, pituitary adenomas (**PAs)** can secrete hormones autonomously or remain silent. Although benign and indolent, pituitary adenomas can be locally invasive and destructive, or clinically malignant due to the metabolic consequences of excess hormone secretion, therefore they are clinically important diagnostic and treatment targets. The vast majority of pituitary adenomas occur sporadically, but they are increasingly recognised in a familial setting. Many tumours remain clinically silent and get detected incidentally (incidentalomas) either during brain imaging for other reasons or during autopsy (101, 102).

Pituitary adenomas can occur as part of hereditary syndromes (e.g. Multiple Endocrine Neoplasia Type 1 (MEN1) or Type 4 (MEN4)), or as an isolated (non-syndromic) disorder which may be inherited (e.g. Familial Isolated Pituitary Adenomas (FIPA)), or more commonly (>95%) as non-familial (i.e. sporadic) neoplasms.

Prevalence of pituitary adenomas

Pituitary adenomas used to be considered rare, but recent studies have shown that the prevalence is higher than previously suggested as a result of increased use of imaging techniques and better diagnostic modalities as well as increased awareness of clinicians. They are amongst the most common intracranial tumours with their 1:1000 prevalence in the developed world (1, 2) and the third most common type of intracranial neoplasms after meningiomas and gliomas (103). It has been estimated that they represent approximately 25% of all clinically manifest intracranial neoplasms (104). More details on prevalence are in the next chapter.

Classification of pituitary adenomas

Pituitary adenomas can be classified based on size, clinical manifestation, hormone or cytokeratin expression profile, radiological features, defining somatic mutations and histologic features.

Microadenomas are less than 10 mm in their largest diameter and they are named **macroadenomas** when the largest diameter is 10mm or above (105). When they reach 40 mm or above, they are considered **giant adenomas** (106).

The classification based on histochemical stains divides adenomas into acidophilic, basophilic, and chromophobic. This classification is of very limited practical value and has, therefore been almost fully ignored (13).

Based on their clinical behaviour, they can be either **clinically functioning** (hormone-secreting) or **non-functioning** (not hormone-secreting) pituitary adenomas (NFPAs). The majority, approximately two thirds of diagnosed PAs, are hormone secreting (105).

Hardy's classification was developed before the magnetic resonance imaging (MRI) era and slightly modified versions are very commonly used by clinicians (surgeons and medics equally). It is particularly important in preoperative evaluation of the tumours (107, 108). The system is based on radiographic features and consists of the following four grades, dividing pituitaries according to their size and extension to local structures.

- *Grade I:* microadenoma, minimal alteration to sella appearance
- *Grade II:* macroadenoma, sella enlargement or suprasellar extension without bony destruction
- *Grade III:* invasive adenoma, sella erosion and suprasellar growth
- *Grade IV:* strongly invasive adenoma destroying adjacent bony structures with suprasellar outgrowth and invasion of bone, hypothalamus and cavernous sinus

Mooney *et al*. in 2017 have raised concerns regarding the reliability of the Hardy's classification suggesting that editing the measure to a clinically relevant scale simplifies the rating process and may be better suited for preoperative tumour evaluation in the MRI era (108).

The more recently developed **Knosp's classification** is based on the degree of invasion to the cavernous sinus as seen on MRI imaging (109, 110):

- *Grade 0:* no involvement of the cavernous sinus
- *Grade 1:* tumour surpassing the medial tangent, but not the intercarotid line

- *Grade 2:* tumour extending beyond the intercarotid line
- *Grade 3A:* tumour extending lateral to the lateral tangent of the intra and supracavernous internal carotid artery
- *Grade 3B:* tumour extending lateral to the lateral tangent of the intra and supracavernous internal carotid artery into the inferior cavernous sinus component
- *Grade 4:* total encasement of the intracavernous carotid artery

The functional classification (Knosp's classification) is the most widely used within clinical practice. It groups adenomas according to the hormonal syndromes with which they are associated. This classification includes the functioning adenomas and the clinically "silent" or non-functioning adenomas (111).

Recently Trouillas *et al*. proposed a new clinicopathological classification, which takes into account both tumour size and the five IHC subtypes (PRL, GH, FSH/LH, ACTH and TSH) (112). They have set up a grading system based on proliferation and invasion status. The five grades are the following: Grade 1a, non-invasive tumour, Grade 1b, non-invasive and proliferative tumour Grade 2a, invasive tumour Grade 2b, invasive and proliferative tumour Grade 3, metastatic tumour (cerebrospinal or systemic metastases).

Diagnosis and management of pituitary adenomas

Adequate diagnosis and management of pituitary lesions require a multidisciplinary approach and continuous collaboration between the endocrinologist, the general practitioner, the radiologist, the neurosurgeon, the ophthalmologist, the clinical geneticist, the pituitary pathologist, the oncologist, the pituitary nurse specialist, the fertility specialist, the clinical psychologist and the clinical biochemist.

Most patients present with symptoms of mass effect, such as headaches, visual field defects and symptoms of hormone over/underproduction to the general practitioner, less frequently with pituitary apoplexy. Diagnostic approach includes imaging of the sellar region, laboratory evaluations for hormone levels and visual field examination/ophtalmology referral if the lesion involves the optic nerve or the optic chiasm. MRI has been the imaging modality of choice for detecting pituitary lesions since the 1990s (113, 114), while CT nowadays mostly has a supplementary role in the evaluation of changes in bone structure of the sella and calcifications (115). Increasing recognition of pituitary incidentalomas is the “by-product” of modern imaging. They are lesions detected incidentally/accidentally in the pituitary gland during brain imaging for unrelated causes (trauma or symptoms involving the neck or central nervous system) (116). Improvement in technology has not only enabled better and more punctual diagnosis but also the effect of medical/surgical treatment or progression of tumour formation can now be precisely monitored, which is essential for proper patient surveillance. Detailed radiological characterisation of pituitary tumours preoperatively majorly increases the surgical success rate as well as providing help in the differential diagnosis and treatment planning (113). Patient

demographics and their general health status are important issues that need to be always considered when deciding on treatment.

Prolactinomas usually respond well to **dopamine-agonist therapy**, such as bromocriptine or cabergoline. GHomas can be treated with **somatostatin analogues** (e.g. octreotide) and **growth-hormone antagonists.**

Neurosurgery remains the treatment of choice for adenomas causing visual abnormalities due to tumour mass effect or for tumours rapidly growing as well as for the ones not well controlled by pharmacological management. The pituitary is complicated to approach surgically (due to its small size and being surrounded by vital structures) requiring specialised neurosurgical skills. Initially, surgery was performed via the transfrontal route carrying a high mortality and morbidity risk. Cushing developed a transsphenoidal approach through the nose but the technical requirements could not be met at the time, therefore he abandoned the technique (117, 118). Now, with modern equipment and significant development in neurosurgery, the transsphenoidal approach is preferred and routinely performed safely (119).

Radiotherapy is used if medical treatment and/or surgery fail(s), or if the patient develops intolerance to the medication. External beam radiotherapy has been used for more than 50 years, most successfully applied when treating NFPAs. Its use is somewhat limited because of the potential toxicity, gamma knife surgery is regarded as an effective and well-tolerated management strategy (120).

Genetic causes of pituitary adenomas

Germline mutations

Familial pituitary adenomas can be either solely involving the pituitary gland (Familial isolated pituitary adenoma – FIPA) or appear as part of a multi-organ syndrome such as MEN1, MEN4, Carney-complex, DICER1-syndrome, *SDHx* gene-associated syndrome and neurofibromatosis type 1 syndrome (121).

FIPA is defined by the presence of PAs in two or more family members without the presence of other syndromic features (122), this group of patients include (most commonly) patients with a mutation in the ***AIP* gene** (123) (not all of them have family history due to low penetrance). There are FIPA patients with known family history of PAs but yet unknown genetic cause.

Patients with X-linked acrogigantism (XLAG) harbour a mutation of the ***GRP101* gene** resulting in growth hormone excess but without other syndromic features. The disease-causing mutation (de novo duplication on the *GPR101* located on the X chromosome) has been first described by Trivellin *et al.* Patients usually present before the age of 5, sporadically, with GHomas and mixed cell pituitary hyperplasia (124). The exact mechanism how duplicated *GPR101* leads to GH excess needs to be further explored. This is a particularly challenging patient cohort due to the young age of onset, the aggressive symptoms and due to the fact that they show significant resistance to conventional medical treatment (somatostatin analogues, dopamine agonists) (125-127).

MEN1 (multiple endocrine neoplasia) syndrome is a triad of hyperparathyroidism, neuroendocrine tumour and pituitary adenoma (most commonly prolactinomas). Patients harbour a mutation in the *MEN1* gene, encoding a ubiquitously expressed

transcription factor regulating cell-cycle proteins (128, 129). Patients with *CDKN1B* (coding p27) loss of function mutation develop **MEN4 syndrome** involving the same symptom-triad as MEN1 (130). Mutations of other cell cycle regulator-coding genes have also been described to manifest in MEN1-like features: *CDKN2B* (coding p15), *CDKN2C* (coding p18) and *CDKN1A* (coding p21) (131).

Carney complex is diagnosed when endocrine and neuroendocrine tumours manifest along with skin pigmentation, cardiac and cutaneous myxomas (132), mostly (70% of cases) caused by inactivating mutations of the regulatory subunit of protein kinase A (*PRKAR1A*) (133), leading to activation of cAMP signalling (134) and dysregulation of the Wnt pathway (135).

Germline ***DICER1*** mutations have been recently described to cause a syndrome of paediatric pituitary blastomas, thyroid carcinomas, goitres, ovarian sex cord stromal tumours, Sertoli-Leydig cell tumours, pineoblastomas and nasal chondromesenchymal hamartomas. Pituitary blastomas develop at a very early age during childhood (around age of 2), and are mostly aggressive ACTHomas causing severe Cushing-disease (136).

Mutations in ***SDHA-D*** or ***SDHA2F*** are associated with paragangliomas, phaeochromocytomas and PAs (mostly prolactinomas) (137).

Somatic mutations

Heterozygous gain-of-function mutations of the ***GNAS*** gene (coding the $G_S$alpha subunit) disrupt GTPase activity, consequently, increase cAMP synthesis resulting in increased proliferation and excess GH secretion. The mutation is located on the maternal allele (imprinted) and results in the development of GHomas (138-140).

McCune-Albright syndrome is associated with somatic mosaic mutation of ***GNAS.*** The disease is characterised by pituitary hyperplasia with increased GH and/or PRL levels. Adenoma development has been observed too. Per definition, the syndrome further involves polyostotic fibrous dysplasia, precocious puberty and cafe-au-lait spots (141). Somatic mosaicism of ***GPR101*** causes GH excess, but it has only been described in males so far (127, 142). A novel, ***USP8*** somatic gain-of-function mutation was described in ACTHomas recently (143), causing increased POMC levels and ACTH synthesis.

The role of miRNAs in pituitary adenomas

A more detailed understanding of the pituitary epigenome has been facilitated by recent technological advances – genome-wide mapping studies revealed frequent epigenetic changes in pituitary tumours, commonly aberration to the methylation of CpG dinucleotides, modification of histone tails and the expression of microRNAs (144).

MicroRNAs (miRNAs) are small, non-coding RNA molecules capable of acting as posttranslational genetic expression repressors. Apparently around one third of the human genome is target for their regulatory effect (145, 146).

MicroRNA, as a novel target for pituitary adenoma treatment is a very promising opportunity. Altered expression of certain miRNAs (miR-410-3p, miR-200a, miR-

200b, miR-107) has been observed to be characteristic for different pituitary adenoma types (5, 147-149).

FIPA – familial isolated pituitary adenomas

FIPA is characterized by the presence of pituitary adenomas in two or more members of the same family without other syndromic clinical features (150). It is an autosomal dominant disease with variable penetrance accounting for approximately 2.5% of all pituitary adenomas.

Only in 25% of FIPA patients is the causative genetic mutation known. Most FIPA families have 2-5 affected members, in two third of the cases the affected members are first-degree related (151). FIPA patients most commonly develop GHomas or PRLomas - together accounting for 75% of all tumours in FIPA (152). In 90% of FIPA families the affected members have either PRLomas or GHomas and no other adenoma types (151). When all members of the same family have the same pituitary tumour type, that family is called homogenous (60% of reported FIPA families). Members of heterogenous families exhibit two or more different pituitary adenoma types (151).

FIPA patients tend to develop more aggressive pituitary tumours than the sporadic cases (151) and they have an average 4 year younger age at diagnosis when compared to those diagnosed in a non-familial setting (153).

In 90% of FIPA families the so far identified genes associated with pituitary adenomas do not harbour mutations (154), therefore the disease-causing mutation is not known. Two or more pituitary adenomas present within the same family can be coincidental, but most likely these families have mutations in so far undetermined genes. When compared to *AIP* mutated FIPA patients, affected members of these families (with undetermined genetic cause) have later onset of symptoms and generally smaller, less aggressive tumours (155).

AIP mutation-associated FIPA

The *AIP* gene was originally identified by its interaction and inhibition of the hepatitis B-virus X protein 2 (156). *AIP* is a tumour suppressor gene located at 11q13 (155). FIPA associated with mutations in the *AIP* is an autosomal dominant disease exhibiting highly incomplete and variable (mostly low) penetrance (157). This penetrance pattern may result in the affected phenotype skipping one or more generations (158). AIP has three predicted alternative transcripts, two of which encode proteins. AIP is ubiquitously expressed in both mice and human, most dominantly in the heart, brain, kidney and testes (155). The gene is evolutionarily conserved amongst species.

Germline *AIP* mutations have been identified in 15-20% of FIPA cases, having a higher prevalence (30-35%) in families where affected members only develop somatotrophinomas (157). *AIP* mutations can be identified outside of FIPA, in a small subset (approx. 3%) of the sporadic pituitary adenomas (157). Since its first identification of *AIP* as a causative gene in FIPA, more than 90 pathogenic mutations have been identified, including deletions, insertions, segmental duplications, missense, nonsense, promoter and splice-site mutations (155). *De novo* mutation has only been reported in one case about a single patient (159). Somatic *AIP* mutations have never been reported. *AIP* mutation-positive patients have larger and more aggressive tumours and earlier disease onset (155) as well as significantly reduced response to somatostatin-analogue therapy and a much higher chance to require surgery (160). A slight male predominance has also been observed, the reason is yet to be elucidated (6).

Available data strongly suggest that genetic screening of the *AIP* gene is advised in patients with a pituitary adenoma (with no other associated features) who either have a family history of pituitary adenoma, or have a childhood-onset pituitary adenoma or a pituitary somatotroph or lactotroph macroadenoma diagnosed at or below the age of 30 years (155).

AIP and its role in pituitary tumorigenesis

The *AIP* gene is 8075 base pairs and is located on chromosome 11:67,250,505-67,258,579 on the forward strand spanning across 6 exons. It was initially cloned by Kuzhandaivelu *et al.* in 1996 who named it HBV X-associated protein 2 *(XAP-2)* (161).

It is a tumour suppressor gene, as loss of heterozygosity has been observed in tumour samples. Further proving that *AIP* bears the properties consistent with a tumour suppressor role *in vitro* data shows that wild-type AIP attenuates cell proliferation (4) whereas mutant AIP loses this effect, *AIP* knockdown leading to increased cell proliferation (162). AIP is mostly localized in the cytoplasm, less nuclear expression is observed (163, 164).

In the normal pituitary, the AIP protein is expressed in somatotroph and lactotroph cells, although its exact function there is yet to be fully understood (4).
The three-dimensional predicted structure of AIP (165) is shown in **Figure 4**. The C-terminal section is responsible for the interaction with other proteins. In the case of FIPA, it is hypothesized to lose its ability to bind to its binding partners and therefore to stop acting as a tumour suppressor (166, 167).

The exact mechanism how AIP loss of function disrupts the pituitary is still unknown. *AIP* mutated pituitary adenomas show no nuclear AHR (aryl hydrocarbon receptor) staining, indicating that AIP-mediated pituitary tumorigenesis is very unlikely to be driven via increased activation of the AHR signalling pathway (168, 169), further underscored by murine models, where *AHR* deficiency does not result in pituitary problems (170).

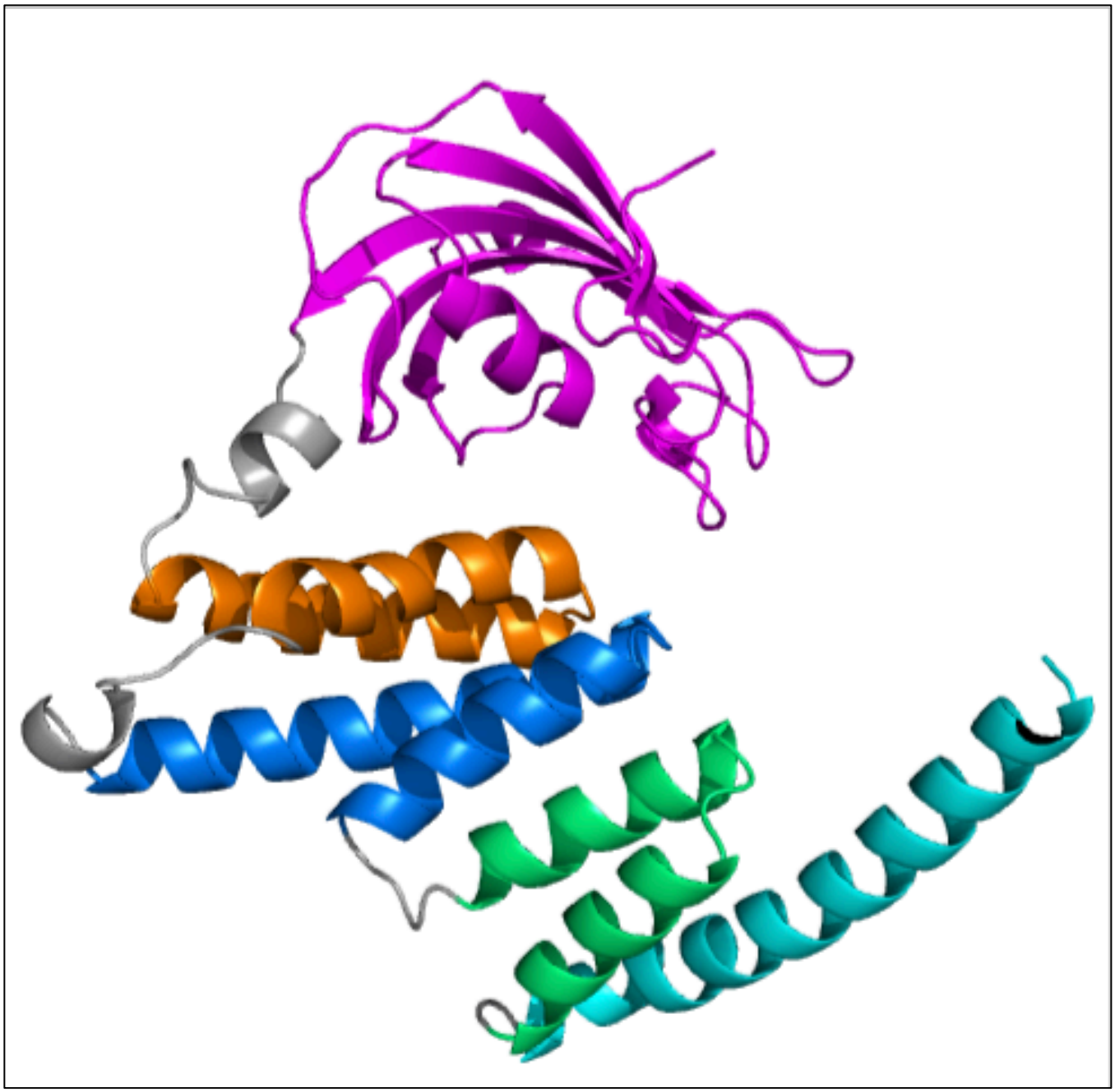


Figure 4. Prediction of the 3D structure of AIP (165) (171).
Main motifs displayed in colour. Pink: ***PPIase domain*** *(amino acids 31-121), Orange:* ***TPR1*** *(amino acids 179-212), Blue:* ***TPR2*** *(amino acids 231-264), Green:* ***TPR3*** *(amino acids 265-298), Turquoise:* ***C-terminal alpha-7 helix*** *(amino acids 300-330)*
Abbreviations: *PPIase: peptidyprolyl cis-trans isomerase, TPR: tetratricopeptide repeat*

Chapter III. Prevalence of familial isolated pituitary adenomas (FIPA) – a clinical study

Background

Genetic screening of the AIP gene is advised in patients with a pituitary adenoma (with no other associated features) who either have a family history of pituitary adenoma, or they have a childhood-onset pituitary adenoma or a pituitary somatotroph or lactotroph macroadenoma diagnosed at or below the age of 30 years (151). In *AIP* mutation positive FIPA families the pituitary adenomas usually occur in 2 or 3 closely related family members, but half of the *AIP* mutation positive probands are not aware of a positive family history. Patients with AIP mutations have larger, more invasive and more aggressive tumours, consequently they are more likely to need surgical intervention (6). More detailed description of FIPA can be found in Chapter II, section *FIPA – familial isolated pituitary adenomas.*

Acromegaly is characterized by sustained elevation of circulating growth hormone (GH) and its tissue mediator, the insulin-like growth factor I (IGF-I). It is associated with increased morbidity and overall mortality mainly due to cardiovascular (cardiomyopathy, hypertension), metabolic (diabetes mellitus), and respiratory diseases (sleep apnoea, chronic hypoxaemia) (172) and increased occurrence of thyroid lesions (173-175) and thyroid carcinoma (176). Without effective IGF-1 and GH-control these patients usually died by the age of 60, mostly due to cardiovascular and cerebrovascular complications (177).

The issue of the risk of benign and malignant neoplasms in acromegaly patients remains a debated topic. Numerous authors have described an increased prevalence of colon adenomas and polyps and colon cancer (174, 178-185). Breast

cancer (186-188) and prostatic cancer (189-192) also has a reported elevated prevalence in the acromegaly patient cohort. Since these early studies, this topic has remained a very debated question with inconsistent results. It is not clear, whether cancer risk is enhanced in active acromegaly due to excessive GH and IGF1 secretion. Over the years, this potential association has been sustained by a number of experimental and animal models, epidemiological data from non-acromegaly populations, and longitudinal and cross-sectional cohorts of patients with acromegaly (193, 194).

Aims

1. Assess the prevalence of FIPA in a tertiary referral centre pituitary patient population
2. Assess the family history of pituitary adenomas

Hypothesis

1. Careful history taking increases the proportion of patients with a family history of acromegaly
2. Active inquiry may reveal previously unknown familial connections in all types of pituitary adenomas
3. PA, especially FIPA patients may be more likely to develop certain other neoplasms

Methods

- Retrospective and prospective analysis on the Barts and the London Hospital NHS Trust (tertiary referral centre) pituitary patient population
- A control group of patients suffering from Graves' disease has been introduced as it has been previously described, that these patients have an increased likelihood to develop certain types of tumours compared to the age and sex-adjusted average patient cohort (195).
- A simple one-page questionnaire on the personal and family history of other tumours is filled out with the patients at their outpatient appointments.

The questionnaire contains:

- age of diagnosis
- age of onset
- items to be circled in personal and family history (Yes/No):
    - micro- or macroadenoma
    - pituitary adenoma types
    - family history of pituitary adenomas
    - 6 major type of tumours (thyroid, colon, prostate, breast, lung and skin malignancy)
    - other type of tumours
- In order to compare the tumour prevalence in the patient cohort with the general population, comparison with the sex- and age-adjusted data of the UK Cancer Registration and the Office for National Statistics was applied
- Young- onset pituitary patients were defined as patients presenting with symptoms before the age of 30 years.

Definitions:	
Young onset disease	diagnosed <30y or at 30y
Positive pituitary family history	at least one family member with pituitary adenoma
Malignancy in family history	at least one family member with any type of malignancy
Positive personal history	Patient with pituitary adenoma also diagnosed with a malignancy
Retrospective study	Data gained exclusively from patient notes - only GHomas
Prospective study	Data gained by directly asking patients and filling out questionnaires

Results

Retrospective and prospective audits were performed on our pituitary patient population in terms of family history of PA where we also aimed to determine the occurrence of neoplasms in these patients, as acromegaly is known to favour their development.

Retrospective data of 225 patients with acromegaly were reviewed; 11 (4.48%) patients were identified with a positive family history (**Figure 5**). Four patients were *AIP* mutation-positive, meaning 36.4 % of patients with a positive family history, 1.78% of all retrospective patients, 9.76% of young onset cases and 66.7% of young-onset cases with positive family history.

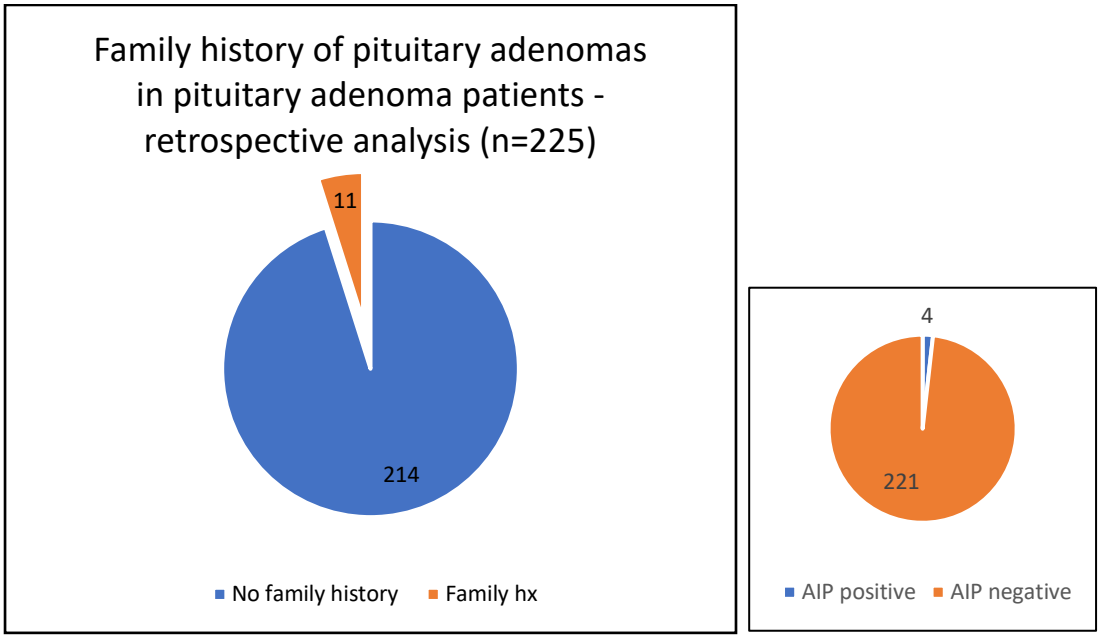


Figure 5. Family history of pituitary adenomas in pituitary adenoma patients - retrospective analysis and prevalence of AIP mutation*. 11 patients (4.48%) were identified out of total 225 to have family history of pituitary adenomas in our retrospective cohort. Four AIP positive patients were identified within the cohort (1.78% of all retrospectively analysed patients, 36.4% of patients with positive PA family history)*

Prospective data gained from active questioning of 876 patients with all types of PAs (**Figure 7**) identified 51 patients (5.82%) with a family history of PA, 40 of these patients were young-onset cases. 6 patients were identified to harbour *AIP* mutation out of all prospectively analysed patients (0.68%).

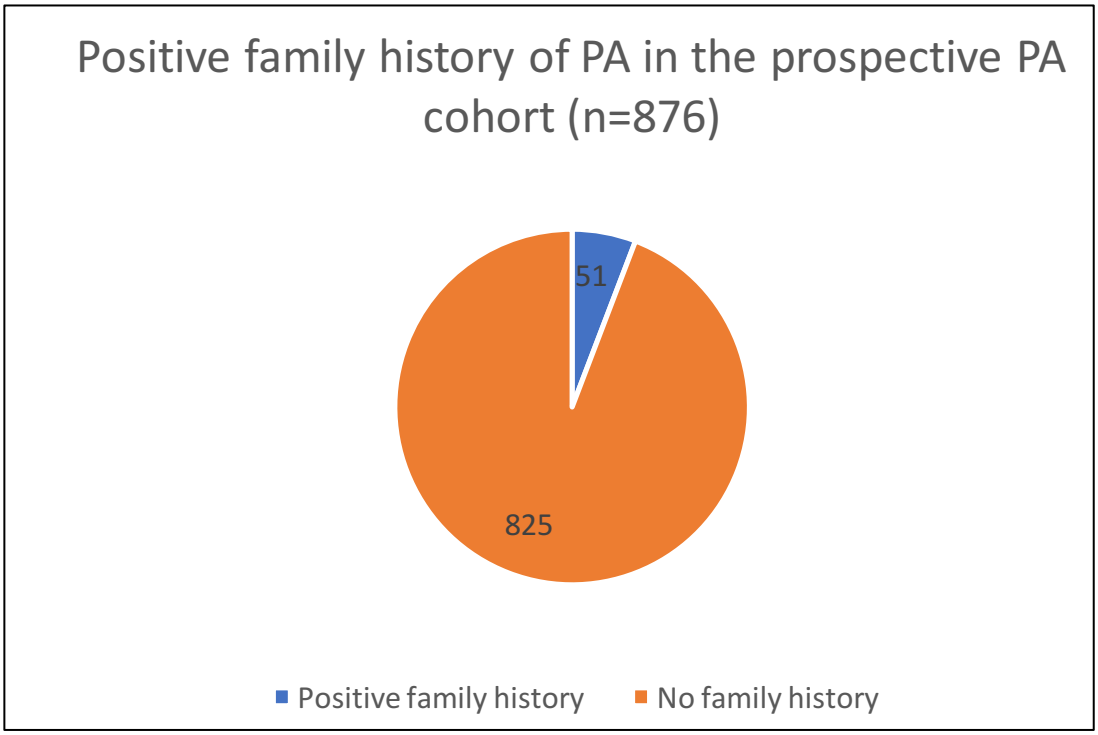


Figure 6. Positive family history of PA in the prospective patient cohort

Prospective data collection revealed 51 patients (5.82%) to have a family history of PA out of a cohort of 876 pituitary adenoma patient, which strongly suggests that careful history taking increases the chance of revealing previously undetected family history of pituitary disease

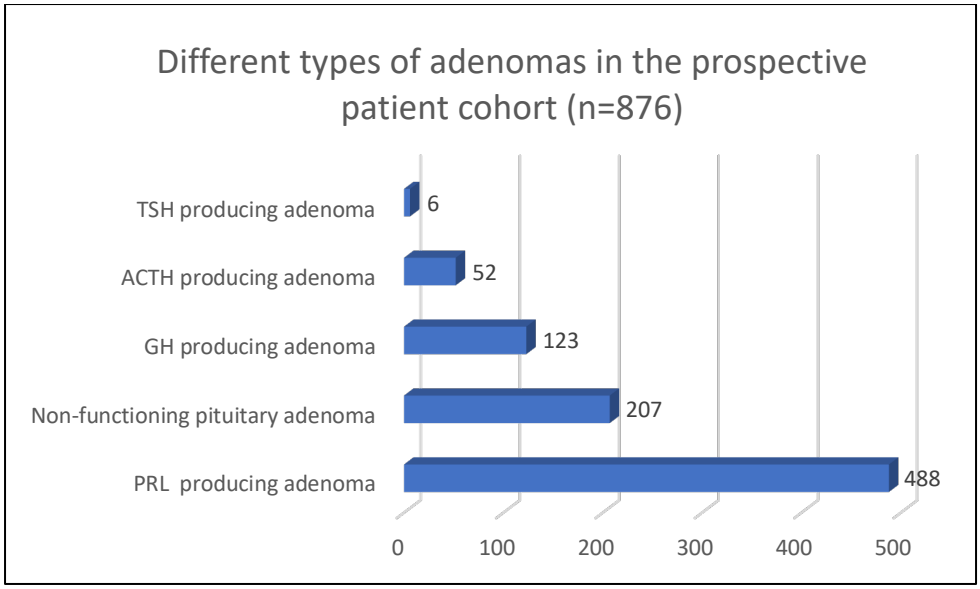


Figure 7. Distribution of pituitary adenoma types in the prospective patient cohort (n=876) *55.7% of all the adenomas were prolactinomas and 23.63% were non-functioning adenomas, which is in keeping with most prevalence studies determining these adenoma types to be most commonly occurring (196) Abbreviations: TSH: thyroid stimulating hormone, ACTH: adrenocorticotrophic hormone, GH: growth hormone, PRL: prolactin*

When focusing on acromegaly, there is a considerable difference in the percentage of patients with family history in the retrospective (overall: 3.6%, young-onset: 17.1%) and prospective (overall: 10.9%, young-onset: 23.5%) acromegaly cohort. Careful history taking increases the detection of family history of PA in acromegaly patients with nearly 3-fold and therefore it is reasonable to suggest that active inquiry may reveal previously unknown familial connections in not only acromegaly, but in all types of PA.

41 patients (18.22%) in the retrospective cohort were identified as **young-onset cases** (patients presenting with symptoms at or below the age of 30). 6 patients of the young-onset patient group had a positive family history, which is 14.63% of the young-onset cases and 2.67% of the total retrospective cohort (**Figure 8**). The average age of onset in the retrospective cohort was 36.3 years, while the average age at diagnosis was 43.96, which delay is well illustrating the difficulty of diagnosis

due to the wide array of symptoms as well as underscores the importance of a better, earlier diagnosis.

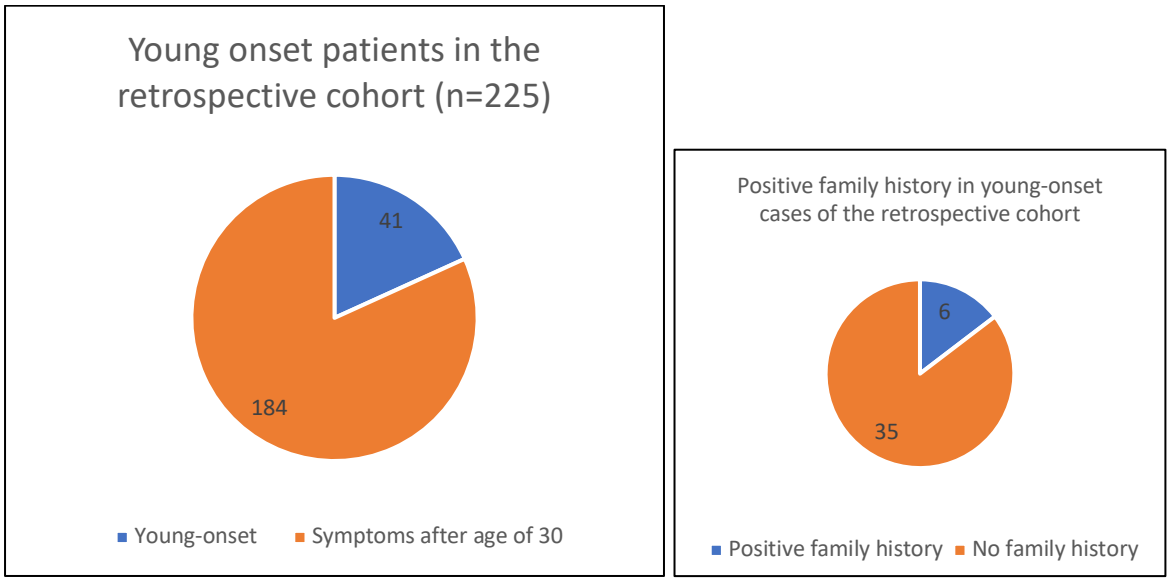


Figure 8. Young onset patients in the retrospective cohort and prevalence of family history in these cases
18.22% of all restrospectively analysed patients presented with symptoms at or below the age of 30, 2.67% of these patients were found to have a positive family history. When examining the young-onset cases separately, these 6 patients with positive family history account for 14.63% of all the young onset cases.

Regarding patients with acromegaly, in the retrospective study 41 (18.2%) and in the prospective study 17 (27%) patients had young-onset disease; 7 (17.1%) young patients in the retrospective and 4 (23.5%) in the prospective study had a family history.

236 (26.9%) patients of the total prospective cohort were young-onset cases, 40 of these 236 cases were identified to have a positive family history (16.94%), which accounts for 4.56% of all prospectively analysed cases.

We questioned whether there might be an increased rate of personal and family history of certain neoplasms in patients with pituitary adenomas.

In the retrospectively analysed cohort 29.95% of patients reported having family history of some sort of neoplasm. There was no difference in the occurrence of the investigated neoplasms (thyroid, colon, prostate, breast, lung and skin malignancy) that would defer from the general population (**Figure 9**), neither did we detect any unpredicted other tumour types in the patients' personal medical history. Important to say that this analysis was made at time of retrospective data collection, no follow up has taken place since. There is a chance that neoplasms have developed in these patients since (due to ageing and other factors), not necessarily at a different rate from that of the general population. Patients potentially not fully aware of the precise medical history of their families must also be taken into consideration when interpreting the results.

Similarly, in the prospective cohort the frequency of the reported family or personal history of neoplasms did not differ from the general population (**Figure 10**) or from the control group (Graves's patients) (**Figure 11**).

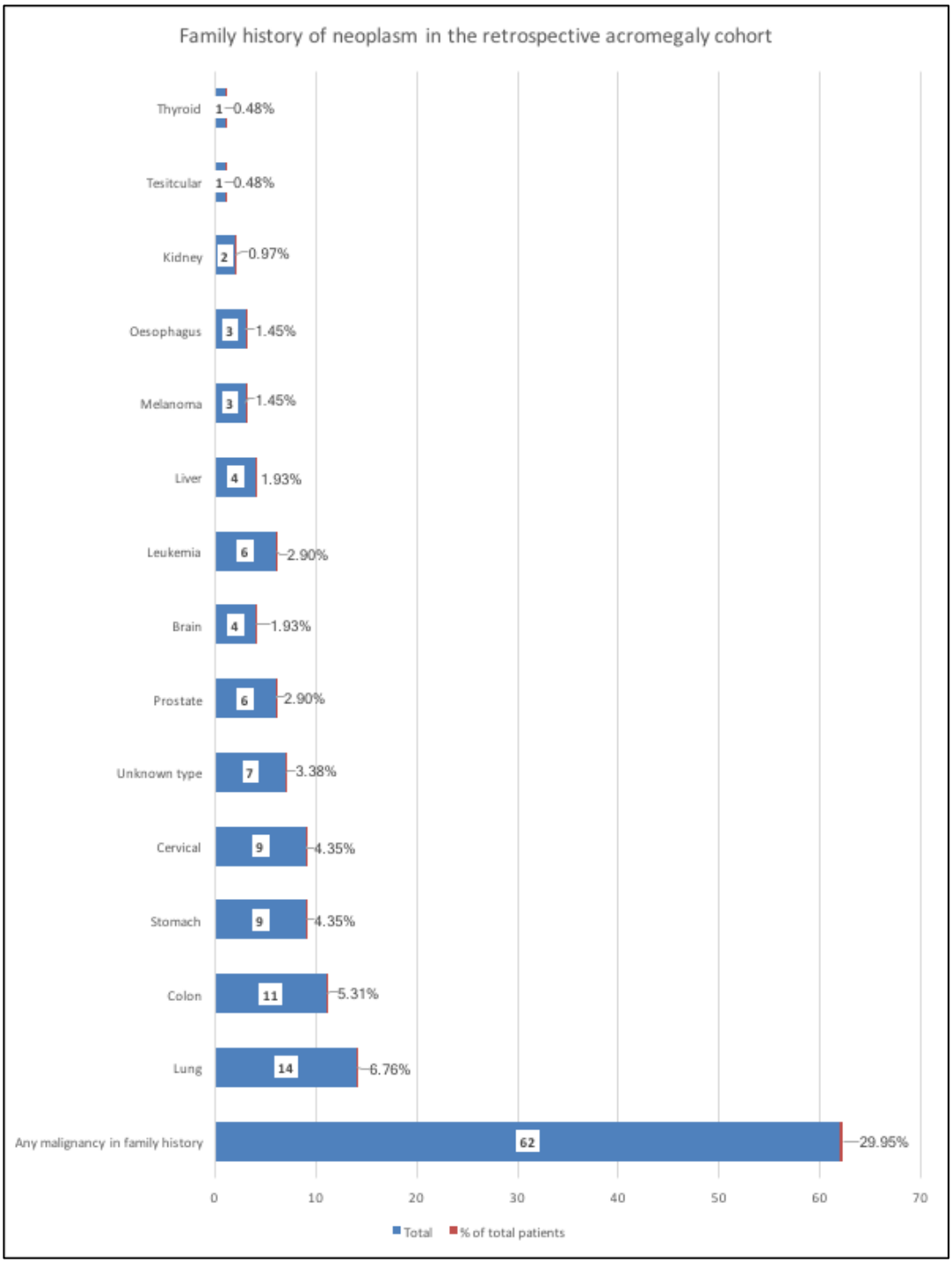


Figure 9. Family history of neoplasms in the retrospectively analysed acromegaly patient cohort

29.65% of all retrospective patients reported a presence of some sort of neoplasm in their family history. Leading types of cancer were in keeping with statistics of the average population. "Unknown type" means patient does not recall what type of cancer

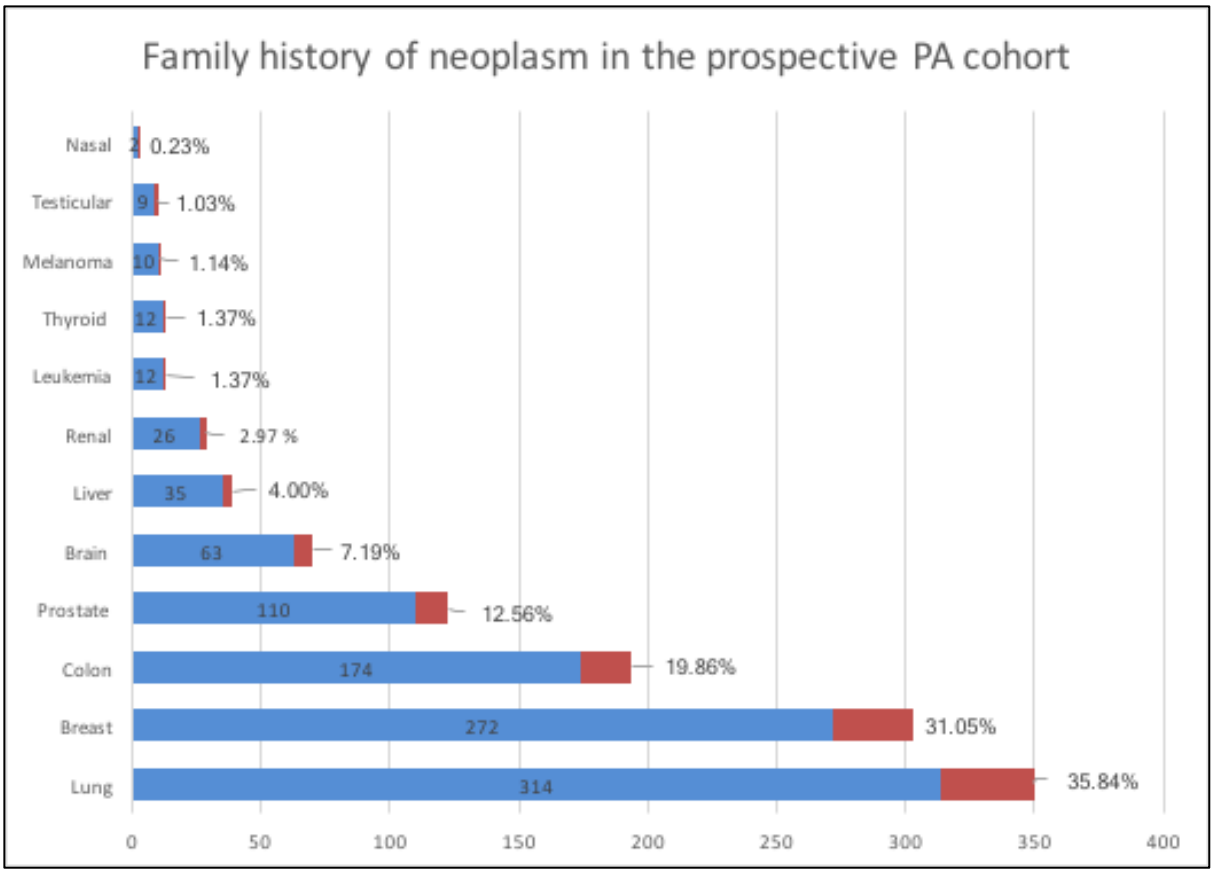


Figure 10. Family history of neoplasm reported in the total prospective cohort of 876 patients *The reported frequency of neoplasms in the family history did not differ from that of the average population*

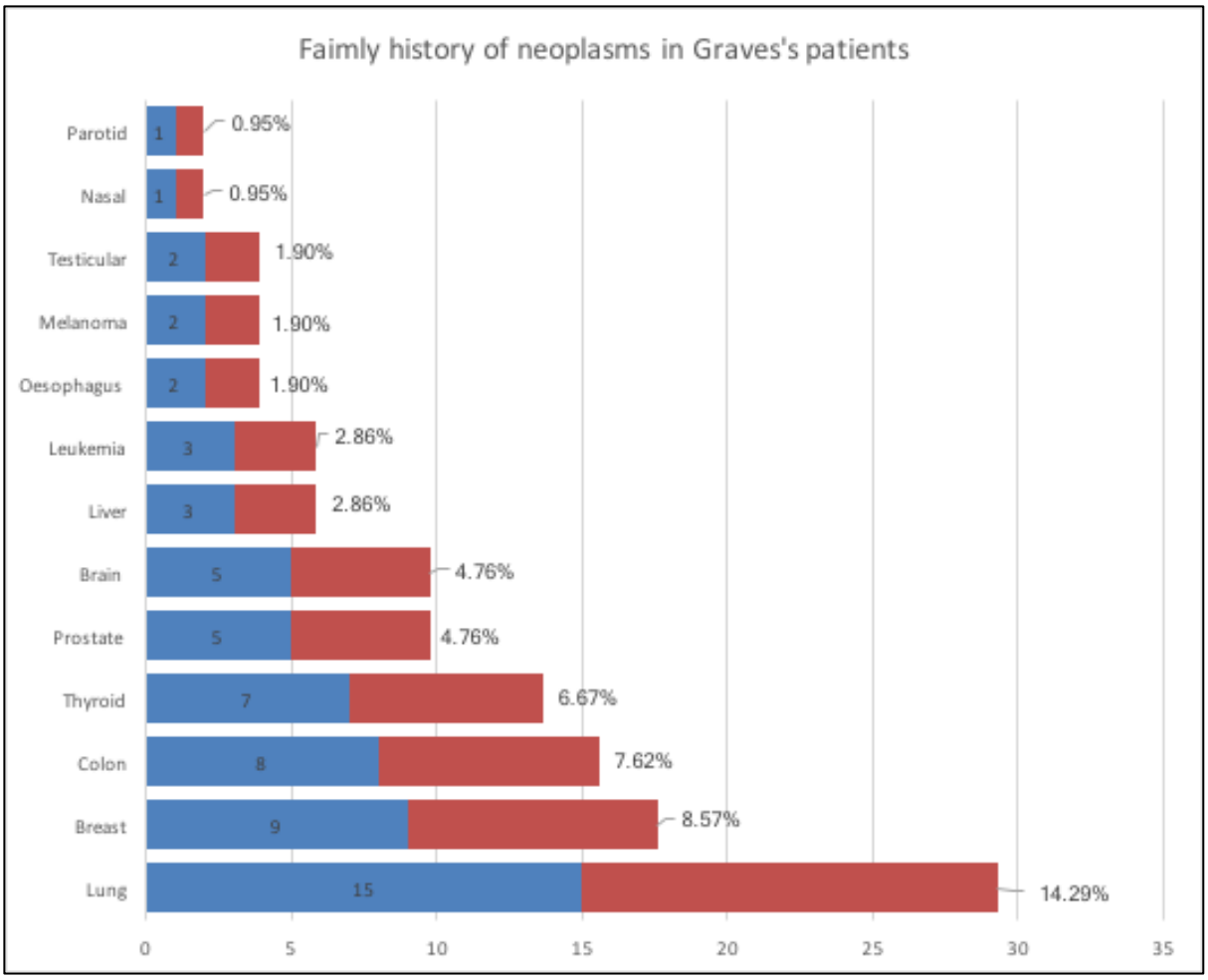


Figure 11. Family history of neoplasms in Graves's patients (control group, n=105 total patients involved)

44 patients of all involved reported positive family history for neoplasm in this patient group. The observed prevalence of the reported malignancies do not differ significantly from the population average, neither from the prospective PA cohort

Discussion

Determining the prevalence of different types of pituitary adenomas in a tertiary referral centre via retrospective (225 patients) and prospective (876 patients) analysis can be considered a respectable cohort size for a single-centre study given the niche nature of pituitary tumours. In the prospective cohort, 55.7% of all the adenomas were prolactinomas and 23.63% non-functioning adenomas, which is in keeping with most prevalence studies (196). The retrospective cohort was only composed of acromegaly patients therefore it could not be analysed from this point of view.

The increased risk of cancer in acromegaly is an ongoing debate. The data analysis is discrepant for a variety of reasons, most importantly it being a rare disease (small patient cohorts). Some studies go back to the middle of 20th century when there was much less effective pharmaceutical hormone control. Only nationwide studies have the appropriate statistical power, further large epidemiologic studies are required to clarify this issue. 29.65% of all acromegaly patients involved in this study reported a presence of some sort of neoplasm in their family history. Leading types of cancer were in keeping with statistics of the average population. I found that in our prospective cohort the frequency of reported family or personal history of neoplasms did not differ from the general population or from the control group of Graves's patients. Limitation to the study could be the fact that patients are self-reporting the neoplasm therefore the reliability can always be doubted. Important to add, that no follow-up happened since, therefore the possibility that patients have developed cancer since (as they aged) cannot be excluded.

I also investigated whether active and targeted questioning would reveal more or previously unknown familial history in pituitary adenoma patients.

In *AIP* mutation-positive FIPA families pituitary adenomas usually occur in 2 or 3 closely related family members, but half of the *AIP* mutation-positive patients are not aware of a positive family history (151). In the retrospective cohort 4.48% of patients were found to have family history, 5.8% in the prospective cohort. Careful history taking increases the detection of family history of PA in acromegaly patients and therefore it is reasonable to suggest that active inquiry may reveal previously unknown familial connections in not only acromegaly, but in all types of PA. Identifying a possible familial pattern is clinically important, not only because putting these patients and their families through for genetic screening is essential but also because they and their families require specialised management and surveillance given the challenging behaviour of AIP-mutated tumours. Patients' anxiety related to their family members being screened is appreciated and understood, yet close monitoring of the family members of the affected patient is encouraged as it enables a possible early diagnosis with better chance for successful therapy.

Chapter IV. Generating *AIP*$^{Flox/Flox}$; *Hesx1*$^{Cre/+}$ mouse model

Background

In mice, the *Aip* gene is located on chromosome 19 and has an exon-intron organization similar to that of the human gene. Murine AIP has 95% identity and 98% similarity to human AIP at the amino acid level (197).

The first *Aip* mouse model was established by Lin *et al*. in 2007, where global homozygous knockout mice (*Aip*$^{-/-}$) died as embryos (the majority of them before the 10-14th embryonic day) due to severe cardiac malformations, such as double outlet right ventricle, ventricular-septal defects and pericardial oedema (198), in accordance with AIP being heavily expressed in the heart. Raitila *et al.* also found global homozygous *Aip* knockout to be lethal in utero. Global heterozygous (*Aip*$^{+/-}$) animals, however, survived and showed a significantly higher tendency to develop pituitary adenomas at around the age of 6 months (78% of the heterozygous animals developed a pituitary tumour compared to 20% observed in wildtype mice) (199). As opposed to humans, where penetrance is low, in these animals, full penetrance was reached by the age of 15 months. The tumours were mainly localized in the pars distalis, which is the equivalent part of the animal to the human anterior pituitary. The majority (88%) of these tumours were GH secreting and had a higher proliferation rate. They also found increased serum IGF-1 levels in the mutant animals (199). A similar global heterozygous *Aip* knockout mouse model (investigating only male animals) had a mild phenotype (200).

A somatotroph-specific *Aip*-knockout mouse model was generated to successfully overcome the in-utero lethality previously observed in the global homozygous knockout mice (197). In this study, by 40 weeks of age, >80% of somatotroph-specific *Aip* knockout mice (sAIPKO) developed growth hormone (GH) secreting

adenomas with physiologic effects similar to that of acromegaly in humans. Interestingly, the expansion of the somatotroph population and ensuing state of GH excess does not manifest until well into murine adulthood, showing a distinct onset at 18 weeks in the sAIPKO mice (197). Even though this model overcame the intrauterine lethality of the conventional homozygous *Aip*-knockout mice of Lin *et al.*, it is GH cell specific, not pituitary tissue specific.

At the time of starting this project (2014) there was no pituitary tissue specific *Aip*-knockout mouse model published. At the time of writing this dissertation (2020), I am not aware of one generated since (apart from my own model detailed in this chapter).

Aims

When generating this novel murine model of pituitary specific, biallelic Aip loss, it was hypothesised that if viable, it would give a better, previously unavailable insight into AIP-related tumour formation.

Obtaining human pituitary samples for research purposes is quite challenging and the amount of sample available for researchers is often heavily restricted. Patients are often on medical therapy prior to surgery, which might influence the reliability of the sample in certain cases.

This novel animal model is hypothesised to have the potential to become an easily maintainable animal model of AIP-related pituitary tumour formation, enabling future studies to look at tumorigenesis from new aspects, such as AIP-related embryogenic events and they can become an excellent tool for exploring therapeutic options *in vivo*.

Pituitary-specific biallelic *Aip* loss could help answer important questions, such as the cell origin of pituitary tumours and why certain cell types are more susceptible to AIP gene dosage. It could further elucidate the role and expression pattern of AIP during pituitary embryogenesis. This mouse model could also be useful in investigating the reasons behind the clinical observation, that patients harbouring an AIP mutation often show very poor response to currently available therapeutic agents as well as have very aggressive adenomas with high proneness to haemorrhage.

Methods

I have gained my Personal License (PIL 70 / 26063) from the Home Office in September 2013 after attending the three-day course and passing the exam. *Aip*$^{Flox/+}$ mice were purchased from the Jackson Laboratory, USA (B6.129-Aiptm2Bra/J, stock number D13195). The *Hesx1*$^{Cre/+}$ animals were kindly donated to us by Prof. Juan Pedro Martinez-Barbera (University College London).
All animals were kept within our biological support unit (BSU animal facility) and were handled in keeping with the Home Office regulations and regulations established in our project license, no procedures were performed that were not pre-authorised in the project license. During the use of animals in experiments and testing we were rigorously following the regulations established in the "*Animals (Scientific Procedures) Act 1986 (ASPA)*, *recently revised to change the European Directive 2010/63/EU on the protection of animals used for scientific purposes".*
Mice were housed under standard conditions of light (12-hour light, 12-hour dark cycle; lights on at 6:00 am) and stable room temperature (22 to 24°C). All animals involved in the study had continuous and unrestricted access to standard rodent chow and water and received standard hygiene care from the staff. They were caged 3-4 animals/cage (identifiable by ear-punch patterns), males and females separated once reached adulthood. Males were kept in individual cages after mating once to avoid animal loss due to aggression and fighting. Breeding females were kept with their pups when littered down unless signs of aggression towards pups were noticed. In case of setting up timed matings, the animals were put together before lights went out in the evening and were separated early in the following morning, when females were checked for presence of vaginal plug to determine if mating has occurred.

In order to maintain the minimum number of animals for a viable colony and for meeting the project's requirements, animals were culled at the earliest possibility with rapid dislocation of the cervical vertebrae.

Weight of the animals were measured on a bi-weekly basis on standard laboratory scales without sedation.

Harvesting and processing embryos

For the purpose of harvesting embryos, pregnant females were culled at the desired timepoints and embryos were carefully removed, processed, embedded in wax, sectioned and fixed on slides as detailed below.

On day 1, freshly harvested embryos were put in 4% paraformaldehyde (PFA) and were kept at 4°C overnight. On day 2 they were twice washed in cold PBS then were dehydrated through series of ethanol washes for 1 hour each concentration (25%, 50%, 70% ethanol overnight). If not used immediately, samples were long-term stored in 75% ethanol. On day 3, samples were washed in 80%, 95% (both for 1 hour) and then 100% ethanol overnight at 4°C. All washes were done on a roller. Embryos were then washed 2x for 45 min in Histo-Clear solution (if 15.5 dpc, first wash at room temperature, second at 60°C prewarmed oven) or 2x for 1 hour in Xylene (if 16.5 dpc or older). Embryos were then put into 50:50 wax:solvent at 60°C for 45 min, then 3x 60 min in wax only at 60°C. The vials were regularly inverted in the over throughout the process. Embedded embryos were then left to cool down and were sectioned.

Genotyping

The animals and embryos were genotyped by PCR method (DNA Releasy (Anachem, DNA Releasy BN0112-1) from ear punches (also used for identifying animals) or tail snips. Each sample was put in 20 ul of DNA Releasy and was placed in a thermal cycler for 75°C for 5 min then 96°C for 2 min and then 20°C hold for 20 min. Samples were then stored at -20°C for later use. For Hesx1-Cre genotyping, we used primers from Sigma (OL39 - 5'TCAGCAAAGCTACAAGGTGAACTG, OL89 - 5'GGAGACAATTCTTTTGTGAAACCCTG, OL91- 5'CCAGAGTGTCTGGCTTCTGTCAC and CreT 5'CAGAAGCATTTTTCCAGGTATGCTC) and 35 cycles were run (94°C for 2 min, 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds) then 72°C for 5 min, products were then run on 2% agarose gel.

For AIP genotyping forward primer (5' CAATCCCCCACTGTCACTTT) and reverse primer (5'TCACCCCTCCCACTGACTAC) were used (Sigma), with the following thermocycler conditions: 92°C for 5 min, then 30 cycles of 92°C for 30 seconds and 72°C for 30 seconds, the final step being 72°C for 5 min. Products were run on 2.5 agarose gel.

ROSA-YFP primers were 5' AAGACCGCGAAGAGTTTGTC, 5' AAAGTCGCTCTGAGTTGTTAT and 5'GGAGCGGGAGAAATGGATATG with the following thermocycler conditions: 3 min at 94°C, 35 cycles of 30 seconds at 94°C, 1 min at 58°C and 1 min at 72°C, followed by 2 min at 72°C. Products were cooled down to 4°C then were run on 3% agarose gel.

Immunohistochemistry

Paraffin-embedded sections were deparaffinised in Histo-Clear for 10 min, rehydrated through ethanol series (100%, 75%, 50% and 25%, in ddH2O) for 2 min each and left to stand in water for 5 min. Slides were then transferred to a glass beaker containing citrate buffer (10 mM citric acid, pH 6.0) in ddH2O previously heated to near its boiling point and subjected to heat-induced epitope retrieval (HIER) for 10 min on a hot plate to maintain the solution hot. After cool down at room temperature, slides were washed 1X PBS-T [0.1% Triton® X-100 (Fisher) in 1X PBS] and incubated for 1 hour with blocking buffer [5% (v/v) Normal Goat Serum (Vector Laboratories) in 1X PBS-T]. 200 µl of primary antibody was applied to each slide. Primary antibodies and their concentrations are listed in Table 2. Slides were covered with parafilm (Bemis) to allow homogenous distribution of the antibody on the slide, and then incubated overnight at 4 °C in a dark wet chamber. Slides were then washed in PBS-T 3 times for 10 min to remove any excess primary antibody. The second wash was performed in 3% hydrogen peroxide [H_2O_2] in PBS-T. Slides were then incubated with biotinylated secondary antibody for 1 hour at room temperature, after which they were washed in PBS-T prior to incubating for 1 hour with VECTASTAIN® Elite® ABC solution (Vector Laboratories). ABC solution was prepared according to the manufacturer's instruction by mixing 2 drops of Reagent A (Avidin DH) and 2 drops of Reagent B (Biotinylated Horseradish Peroxidase H) for every 5 ml of 1X PBS. Slides were washed in PBS-T 2 times prior to final incubation with 3,3′-Diaminobenzidine (DAB) solution (Vector Laboratories). DAB solution was prepared according to the manufacturer's instructions (2 drops of Buffer Solution pH 7.5, 2 drops of Hydrogen Peroxidase solution and 4 drops of DAB stock solution, in 5

ml of ddH2O). When staining was visible, the reaction was interrupted by gently washing the slides under running tap water. Slides were counterstained with 0.1% Gill's III haematoxylin solution, then dehydrated through ethanol series (25%, 50%, 75% and 100% ethanol, in ddH2O). Once dehydrated they were incubated in Histo-Clear for 5 min and then mounted with VectaMount™ Mounting Medium. They were left overnight to dry and then slides were taken under microscope for photography and analysis. Cell counting was performed with Image J, Prism used for statistical analysis.

IGF-1 ELISA

Blood samples were taken by tail bleeding, (3ul) samples were put into EDTA tubes, centrifuged 13000x rpm for 10 min then were stored at -80 °C. Mouse/Rat IGF-1 Quantikine ELISA kit was purchased from R&D systems (catalogue no. MG100). First 50 µL of assay diluent (included in kit) was added to each well, then 50 µL of Standard, Control, or sample was added to the wells. Plate was sealed then incubated at room temperature for 2 hours on a horizontal orbital microplate shaker. Each well was aspirated and washed, repeating the process 4 times for a total of 5 washes. 100 µL of Conjugate was added to each well. Plate was sealed and incubated at room temperature for 2 hours on the shaker. Each well was aspirated and washed, repeating the process 4 times for a total of 5 washes. 100 µL Substrate Solution was added to each well and plate was incubated at room temperature for 30 min on the benchtop while protected from light. 100 µL of Stop Solution was added to each well and plate was analysed at 450 nm by the ELISA plate reader.

***In situ* hybridisation**

Paraffin-embedded sections were pre-treated with DEPC (1ml DEPC per litre of solution, incubate overnight and then autoclave the following day), glass baked at 180°C for 6 hours. 4% paraformaldehyde in 1x PBS was prepared in the fume hood (16g PFA, 40mls 10 x PBS, 200 µl NaOH 10M, made up to 300ml with DEPC water). Wax sections were placed into Histo-Clear twice for 10 min followed by a series of ethanol washes for 2x2 min each (100%, 90%, 70%, 50% and 25% ethanol). Slides were then washed in 1x PBS for 2 min, then 4% paraformaldehyde in 1x PBS 20 min in fume hood, then again in 1x PBS twice for 2 min. Slides were than treated with 20 ug/ml Proteinase K for no longer than 8 min, followed by washing in 4% paraformaldehyde in 1x PBS for 5 min in fume hood then in 1x PBS twice for 2 min. Stir for 10 min and put 1ml acetic anhydride over the slides whist stirring followed by 1xPBS 2x2 min wash and finally dehydrated with same alcohol series (100%, 90%, 70%, 50% and 25% ethanol, 2x2 min each). Slides were left to air-dry at room temperature. Slides were then laid out on aluminium foil and Hybmix (50% formamide, 0.3M sodium chloride, 20mM Tris HCl, 5mM EDTA, 10% Dextran sulphate, 1x Denhardt's, heat to 50°C until dissolved) were applied on them which Hybmix included the 1ul/ml RNAse inhibitor, 0.5mg/ml tRNA and probe at 1:50 dilution. Once mix applied, slides were covered with clean cover slip and were placed into black plastic racks, which were then put into boxes and closed for overnight incubation at 65°C. After this step, racks were removed and placed into 2x SSC (75ml 20x SSC, 675ml H20) at hybridization temperature. All washes performed at hybridization temperature in a glass staining trough in a water bath from this step onwards unless otherwise stated. After 20 min slides were transferred

one at a time to a fresh rack in 2x SSC at hybridization temperature and cover slips were carefully removed. Slides were then washed with Formamide 2 x 20 min (175ml formamide 35ml x20 SSC, 140ml H2O), followed by 2x SSC twice for 30 min and 0.2x SSC twice for 30 min and then were left to cool down at room temperature in 0.2x SSC. For antibody detection, slides were washed with buffer 1 at room temperature for 2 min. (Buffer 1 0.1MTris pH 7.6, 0.15M sodium chloride), followed by the blocking step for an hour (10% foetal calf serum in Buffer, added 1ml per slide and cover the slide with parafilm). Slide were drained and 0.5ml of antibody solution per slide was applied and then slides were covered with parafilm (antibody solution= anti DIG fab antibody from Sigma Aldrich, diluted to 1:1000 in Buffer 1 containing 2% foetal calf serum) and were incubated overnight in humid chamber at 4°C. The DIG-labelled oligonucleotide probes were kindly donated by Dr Carles Gaston-Massuet, originally generated from plasmids obtained from Source Bioscience (201). The next day slides were thoroughly washed with Buffer 1, three times, for 5 min each then washed in 1xBuffer 2, twice for 5 min (Buffer 2. 0.1M Tris-HCl pH 9.5, 0.1M sodium chloride, and 0.05M magnesium chloride). Developing solutions were prepared as per manufacturer's instructions - developing solutions added to Buffer 2/PVA mix (NBT separate solution (Roche), 4.5µl/ml,BCIP separate solution (Roche), 3.5µl/ml). Slides were then placed one at a time into a light proof humid chamber and 0.5ml of developing solution was applied and was incubated at room temperature. Once developed, slides were washed under running water for full removal of developing solution and avoiding over-development. Slides were then placed at 50°C for 15 min to loosen any residual PVA followed by incubation in ethanol series (25% ethanol twice for 1 min, 50 % ethanol twice for 1 min, 75% ethanol twice for 30 seconds,

100% ethanol twice for 30 seconds). Slides were incubated in Histo-Clear for 5 min, then mounted with VectaMount™ Mounting Medium.

Genetic features and genetic crosses

The **Cre-lox system** was originally introduced in the 1980s and has since been widely used in genetics (202). It is based on the ability of the P1 bacteriophage cyclization recombination (Cre) recombinase enzyme gene (*Cre*) to catalyse recombination between pairs of lox sites. Such recombination in a "Cre-lox" mouse can either activate or inactivate the gene of interest – as seen on **Figure 12**.

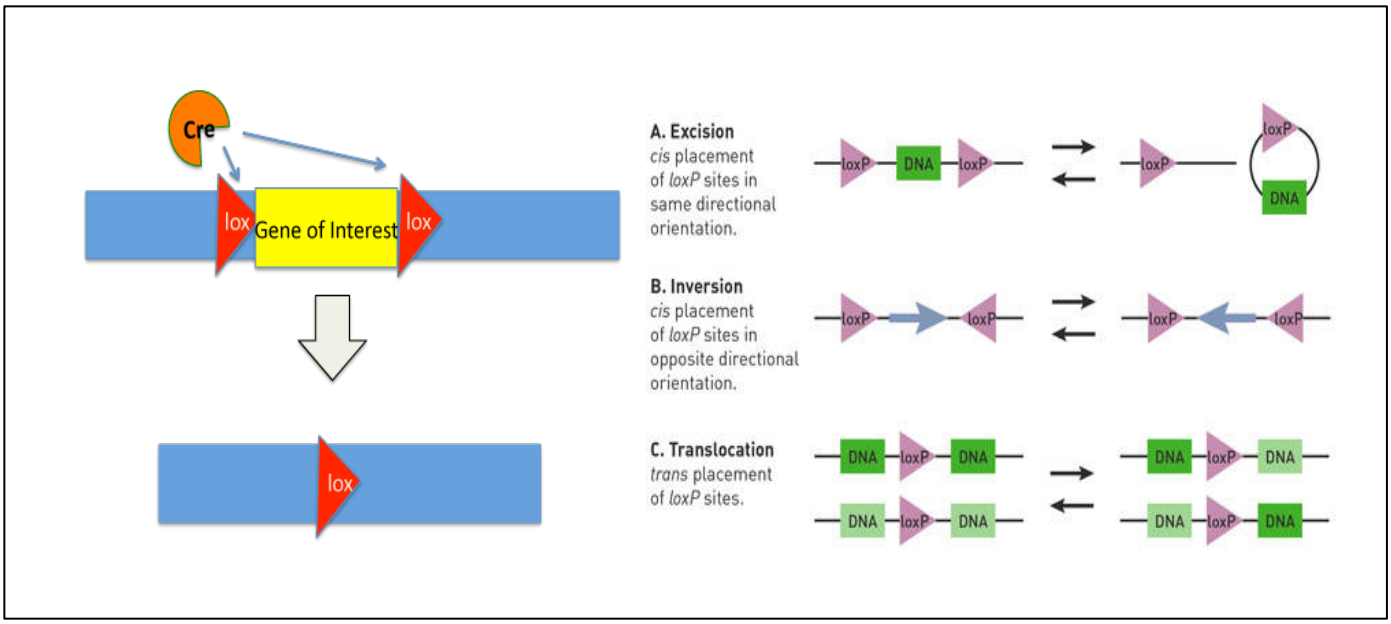


Figure 12. The Cre-lox system
The orientation and location of LoxP sites determine whether the Cre enzyme induces excision, inversion or translocation. Cre and LoxP strains are usually developed separately and then cross them, enabling conditional gene modification depending on which specific promoter the Cre is under (Image from www.jax.org)The genetic features and the crosses required for generating the AipFlox/Flox; Hesx1Cre/+ mouse model are detailed on Figure 13 and Figure 14.

Aip-Floxed (*Aip*$^{Flox/+}$) mice were purchased and transferred from the Jackson Laboratory, USA (B6.129-Aiptm2Bra/J, stock number D13195), five females and five males.

These mice possess *loxP* sites on either side of exons 3 to 6 of the targeted *Aip* gene (203). When these mice are bred to mice expressing *Cre* recombinase under

the *Hesx1* promoter, the resulting offspring will have exons 3 through 6 of *Aip* deleted in the *Cre*-expressing tissue. With this deletion, the *Aip* becomes truncated. We have obtained the pituitary-specific Cre-line, $Hesx1^{Cre/+}$ (204) in order to generate a deletion of *Aip* in a pituitary tissue-specific manner. In these mice, the *Cre* recombinase is under a pituitary specific, *Hesx1* regulatory promoter element.

Firstly, AipFlox/+ mice were intercrossed to obtain AipFlox/Flox animals, at the same time, AipFlox/+ were crossed to Hesx1Cre/+ to obtain Aip Flox/+; Hesx1 Cre/+ (heterozygous tissue specific ablation of Aip). By breeding AipFlox/Flox mice to AIPFlox/+; Hesx1Cre/+ we obtained AipFlox/Flox; Hesx1Cre/+, i.e. pituitary-tissue specific genetic ablation of Aip in the pituitary (Figure 14). At this point the desired genotype was reached

Figure 13 C-D) and we carried on establishing and maintaining a viable colony. Continuous genotyping was performed during crossings, genotypes were confirmed by PCR (

Figure **13**).

Antibodies	Source
Rabbit α-GAPDH	1:500 dilution, Santa Cruz Biotechnology
Rabbit α-AIP	1:500 dilution, Invitrogen
Rabbit α-GSU	1:500 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rabbit α-LHx3	1:300 dilution, Thermo Fisher Scientific
Rabbit α-Pomc1	1:500 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rabbit α-GH	1:500 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rabbit α-PRL	1:500 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rat α-ACTH	1:300 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rabbit α-LH	1:500 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rabbit α-FSH	1:500 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rabbit α-TSH	1:500 dilution, The National Hormone and Peptide Program (NHPP) Harbour-UCLA Medical Centre
Rabbit α-SOX9	1:300 dilution, Sigma-Aldrich
goat anti-rabbit antibody	1:300 dilution, Vector Laboratories, BA-1000
horse anti-goat antibody	1:200 dilution, Vector Laboratories, BA-9500

Table 2. List of antibodies

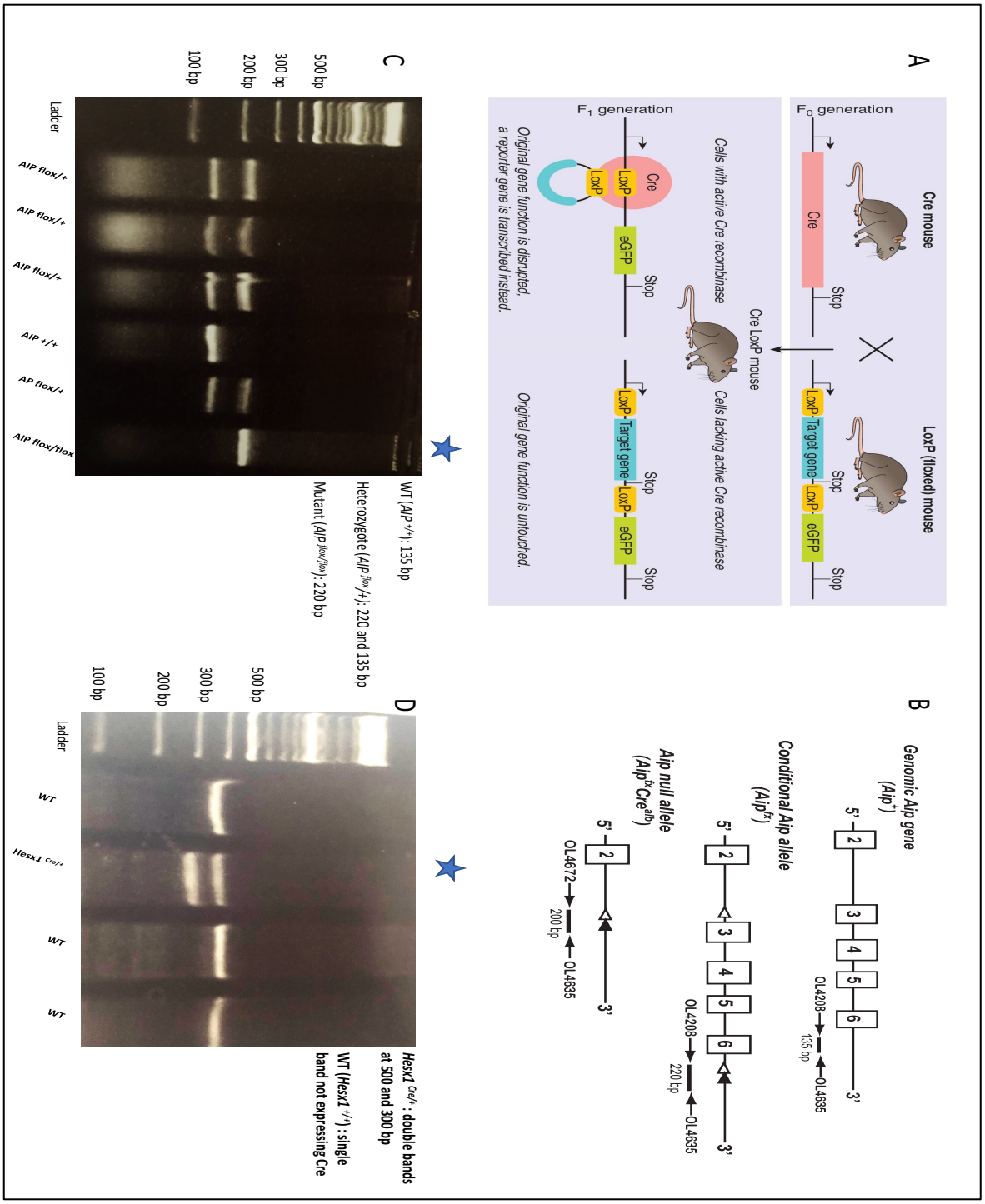


Figure 13. Genetic features of the pituitary-specific Aip-knockout mouse model
A) The CreLox system. In our case the target gene is Aip and the Cre is under the pituitary specific Hesx1 promoter. The gene function of Aip will be therefore disrupted in the Hesx1 cells, but not elsewhere as those cells are lacking the Cre enzyme
B) Genome maps of native mouse Aip gene (Aip^{+}), conditional Aip allele (Aip^{Flox}) and Aip null allele (170, 198). C) PCR genotyping of pups at birth for Aip
D)PCR genotyping of pups at birth for Hesx1
Blue star marks the same animal, showing it has the genotype $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$, which was the experimental goal.

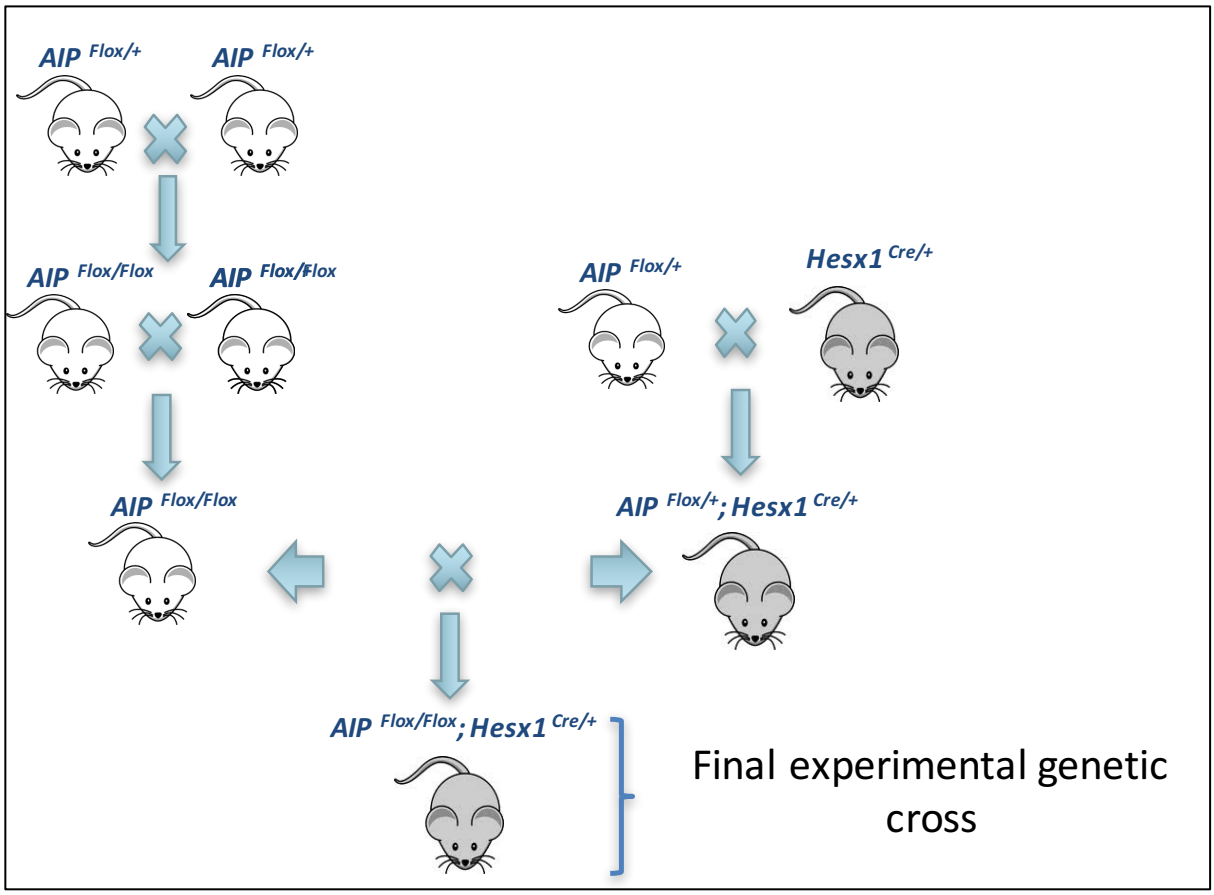


Figure 14. Genetic crosses performed to generate Aip$^{Flox/Flox}$; Hesx1$^{Cre/+}$
Firstly, Aip$^{Flox/+}$ mice were intercrossed to obtain Aip$^{Flox/Flox}$ animals, at the same time, Aip$^{Flox/+}$ were crossed to Hesx1$^{Cre/+}$ to obtain Aip$^{Flox/+}$; Hesx1$^{Cre/+}$(heterozygous tissue specific ablation of Aip). By breeding Aip$^{Flox/Flox}$ mice to Aip$^{Flox/+}$; Hesx1$^{Cre/+}$ we obtained Aip$^{Flox/Flox}$; Hesx1$^{Cre/+}$, i.e. pituitary-tissue specific genetic ablation of Aip in the pituitary

Results

Viable colony established

Aip$^{Flox/Flox}$; *Hesx1*$^{Cre/+}$ animals of both genders are viable and fertile in our BSU animal facility. No embryogenic lethality was observed, litters followed a Mendelian pattern of inheritance. There was no noticeable difference in size or weight of embryos at any examined embryonic stage and no gross external developmental abnormalities were observed.

Expression of AIP during pituitary embryogenesis

AIP starts to be expressed in mice at 9.5 dpc ubiquitously (**Figure 15**), but most prominently in the pituitary, heart and testes (156). The role of AIP in embryogenesis and its function in the normal adult pituitary is yet unknown.

Pituitary-specific loss of AIP expression in *Aip*$^{Flox/Flox}$; *Hesx1*$^{Cre/}$*mice*

We have confirmed the embryonic, pituitary tissue-specific loss of *Aip* in our model with immunohistochemistry in *Aip*$^{Flox/Flox}$; *Hesx1*$^{Cre/+}$ 15.5 dpc embryos, where genotype of the same embryo was confirmed with PCR (**Figure 16**). When comparing the pituitary glands of the mutant *Aip*$^{Flox/Flox}$; *Hesx1*$^{Cre/+}$ 15.5 and 17.5 dpc embryos to the wildtype at the same embryonic stage, the difference in AIP expression in the gland is obvious (**Figure 17**). The hypophysis of the tissue-specific mutant animals shows a phenotype difference, an enlarged anterior lobe with incomplete sphenoid bone fusion (wholemark of pituitary enlargement). Phenotype difference to a certain extent was observed in other mutant embryos as well, but not

in all of them.

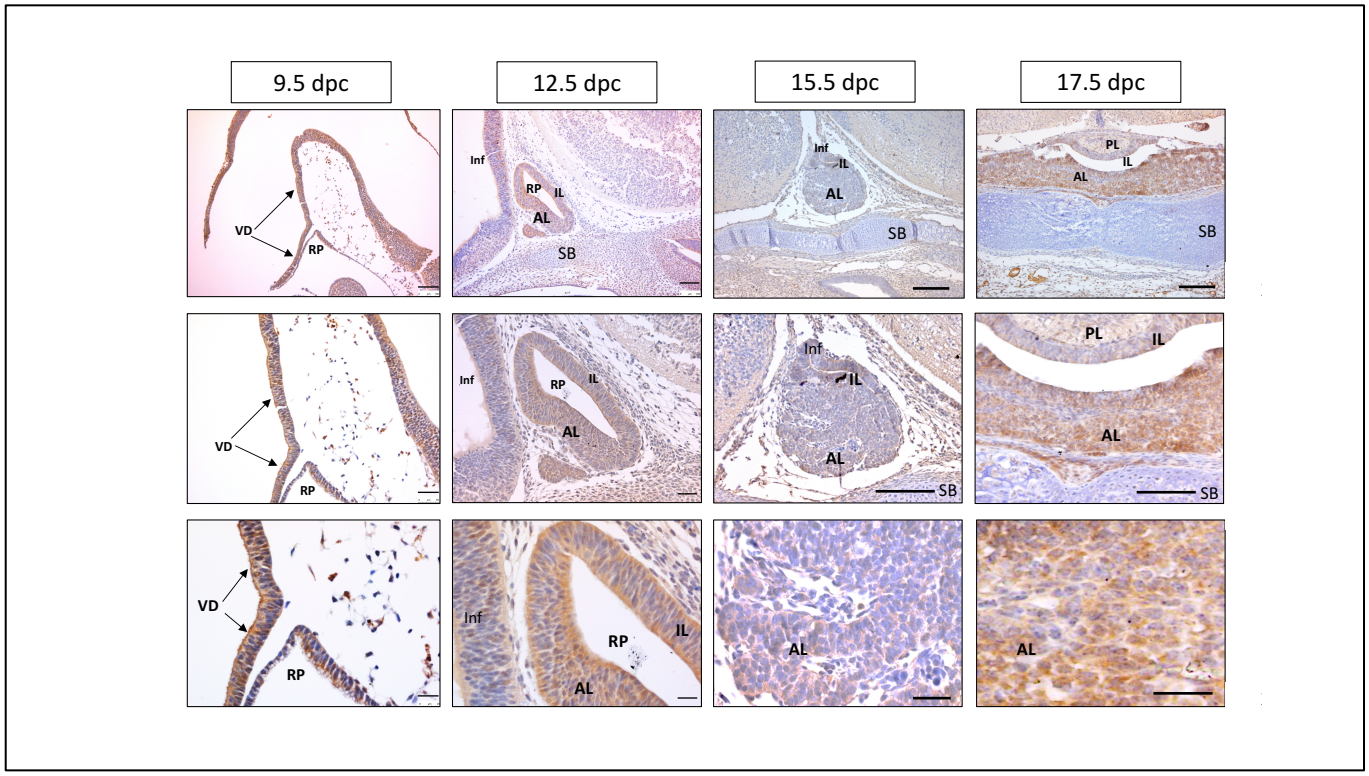


Figure 15. Expression of* AIP *during pituitary embryogenesis

AIP immunohistochemistry demonstrating expression of AIP *at 9.5 dpc, 12.5 dpc, 15.5 dpc and 17.5 dpc wildtype mouse embryos. AIP is present throughout pituitary development, first expressed at 9.5 dpc. Detailed explanation of pituitary developmental stages are shown on* ***Figure 3****. Scale bars, first and second columns 100 µm, 50 µm, 25 µm, third column 200 µm, 100 µm, 40 µm, fourth column 200 µm, 100 µm, 40 µm*

Abbreviations: VD: ventral diencephalon, RP: Rathke's pouch, Inf: infundibulum, IL: intermediate lobe of pituitary gland, AL: anterior lobe of pituitary gland, PL: posterior lobe of pituitary gland, dpc: days post coitum, SB: sphenoid bone.

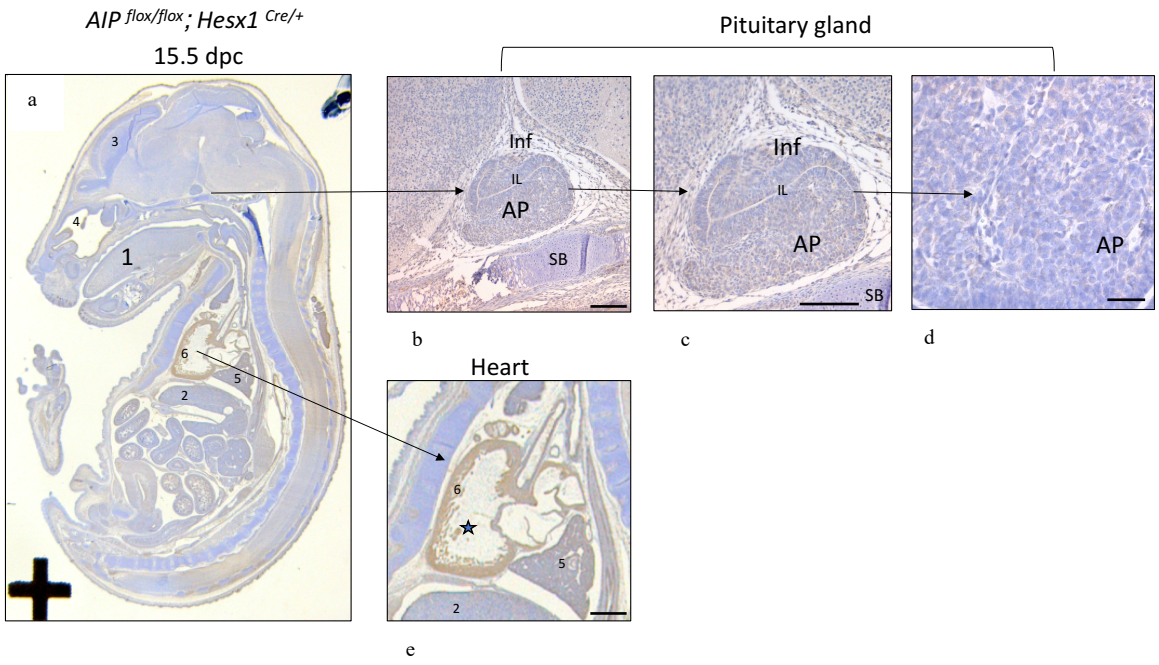


***Figure 16.* AIP *immunohistochemistry of Aip$^{Flox/Flox}$; Hesx1$^{Cre/+}$15.5 dpc embryo shows pituitary tissue-specific loss of* AIP**

AIP *is not expressed in the pituitary gland but very strongly expressed in the heart and the rest of the embryo, star marks strong positive* AIP*-staining.*
The genotype of the same embryo was confirmed with PCR.
Sagittal view. 1: tongue 2: liver 3: cerebrum 4: olfactory epithelium 5: lung.
Abbreviations: dpc: days post coitum, AP: anterior pituitary, Inf: infundibulum, SB: sphenoid bone, IL: intermediate lobe of pituitary. Scale bars: 200 µm in b and c, 40 µm in d, 1000 µm in e

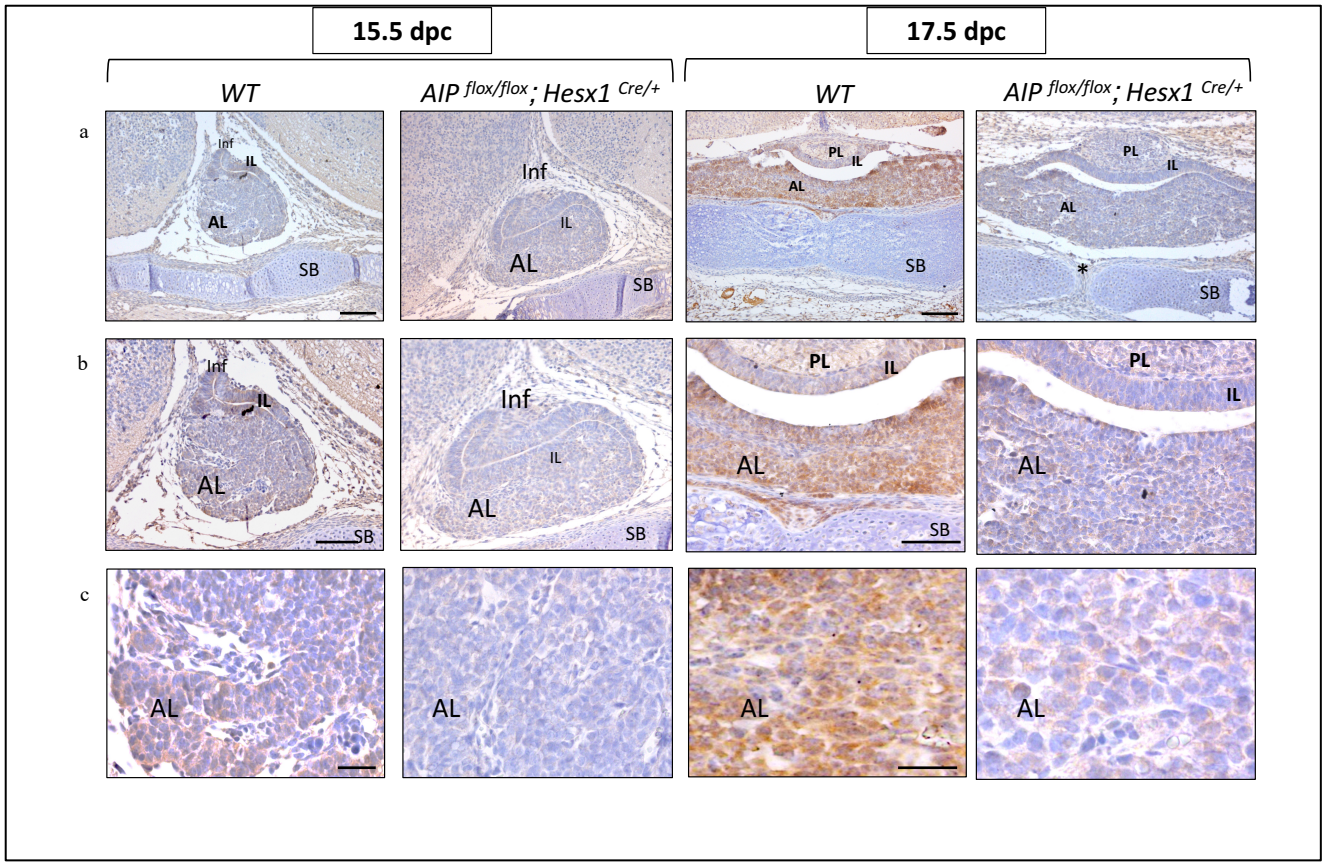


Figure 17. Loss of AIP expression in the pituitary gland at 15.5 and 17.5 dpc
*AIP immunohistochemistry showing AIP is not expressed in the pituitary gland of $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ (tissue-specific mutant) animals when compared to age-matched wildtypes (WT). Note that in the 17.5 dpc $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ the sphenoid bone (SB) is not fully fused (marked with *) as it would be expected by this embryonic stage, which is a sign of pituitary enlargement. The chunkier pituitary gland of the mutant animal is a phenotype we did not find to have 100% penetrance, as shown on other figures. Scalebars lines a and b: 200 µm, line c: 40 µm.*

***Abbreviations**: Inf: infundibulum, IL: intermediate lobe of pituitary gland, AL: anterior lobe of pituitary gland, PL: posterior lobe of pituitary gland, SB: sphenoid bone, WT: wild-type, dpc: days post coitum*

Cell lineage determination – early commitment markers

In situ hybridization at 15.5 dpc showed no difference in the expression of early commitment markers (alpha GSU, Lhx3, Pit1 and POMC1 – detailed description is in Chapter I) in all 3 genotypes, which means the pituitary specific *Aip* knockout animals go through cell lineage determination and reach the phase of terminal differentiation without disturbance (**Figure 18**). SOX9 expression remains intact as well (**Figure 19**).

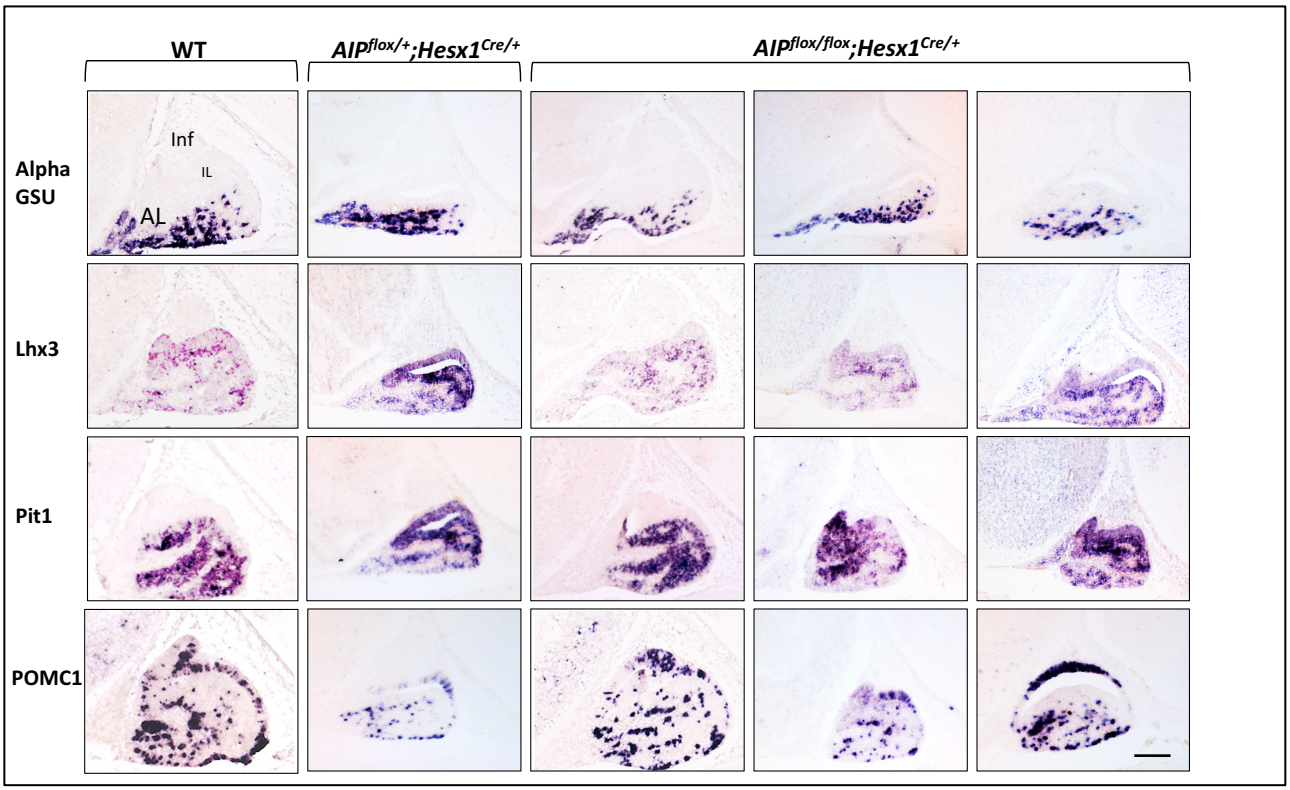


Figure 18. Early commitment markers in murine pituitary gland at 15.5 dpc

In situ hybridization showing no difference in expression pattern of early commitment markers (alpha GSU, Lhx3, Pit1 and POMC1) at embryonic day 15.5 in pituitary glands of wildtype, heterozygous mutant ($Aip^{Flox/+}$; $Hesx1^{Cre/+}$) and homozygous mutant ($Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$) mice (n=4 in all genotypes). This shows that cell lineage commitment is unaffected by loss of AIP in both the homozygous and the heterozygous mutant. Also noted the different pituitary phenotypes of $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ mice already present at this embryonic stage (slightly deformed, enlarged anterior lobe). Scale bar represents 200 µm.

***Abbreviations**: AL: anterior lobe of the pituitary gland, WT: wildtype, Inf: infundibulum, IL: intermediate lobe of the pituitary gland, dpc: days post coitum.*

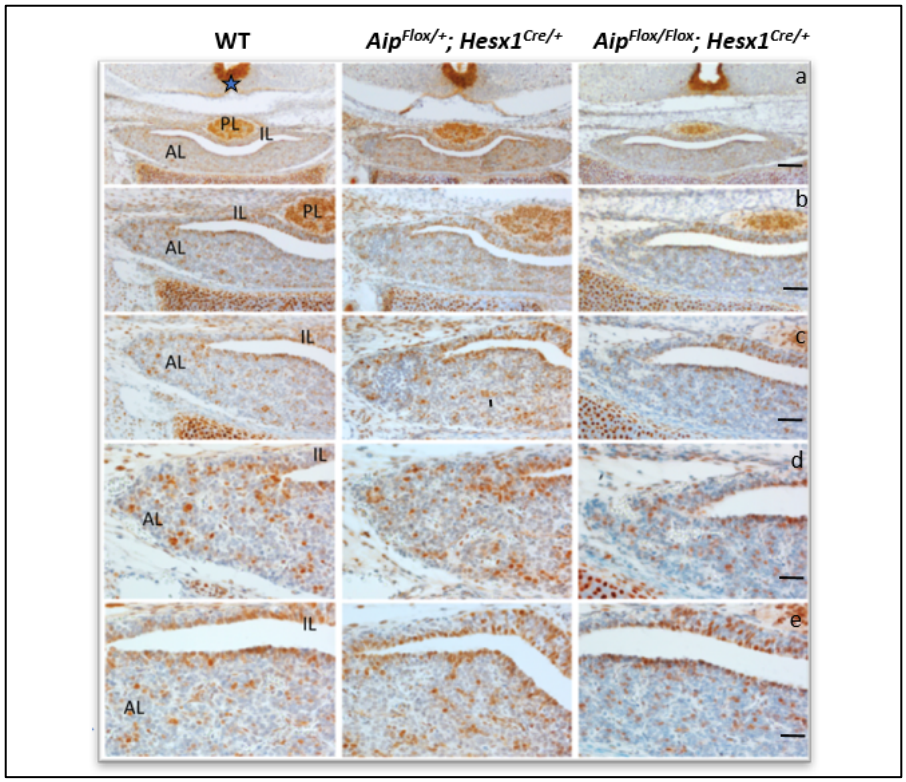


Figure 19. SOX9 expression in the murine pituitary gland at 17.5 dpc
SOX9 immunohistochemistry showing no difference in WT, heterozygous mutant and homozygous mutant animals

Abbreviations*: AL: anterior lobe of the pituitary gland, WT: wildtype, IL: intermediate lobe of the pituitary gland, PL: posterior lobe of the pituitary gland, green asterisk: immunostaining in tanycytes lining the third ventricle, dpc: days post coitum. Scale bars a: 200 µm, b: 100 µm, c: 75 µm, d: 50 µm, e: 50 µm*

Terminal differentiation to hormone producing cells

Terminal differentiation starts at 15.5 dpc and is complete by 17.5 dpc and it is the process of early progenitor cells becoming differentiated pituitary cells, secreting one of the major pituitary hormones (ACTH, GH, PRL, FSH, TSH, LH). The process is detailed in *Chapter I - Genetics of pituitary development*. In summary, the Pit1 lineage gives rise to the somatotrophs (GH), lactotrophs (PRL) and TSH, the Tpit to the ACTH producing cells and the SF1 to the LH and FSH.

At 17.5 dpc the homozygous mutant animals show a decrease in GH expression when compared to wildtype, implying the Pit1 lineage is affected by the loss of *Aip*– needs further elucidation how. It was hypothesised that perhaps the cells that do not become GH producing cells remain stuck in the previous phase (Pit1). We performed Pit1 immunohistochemistry at 17.5 dpc stage to see if there are more Pit1 cells in homozygous mutant e17.5 pituitaries, but the experiment showed no difference, our hypothesis therefore does not explain the GH reduction in mutants. Heterozygous mutant animals show somewhat reduced expression of both PRL and GH, but this difference is non-significant (**Figure 20**).

The other lineages appear intact in both the homozygous and the heterozygous mutants as demonstrated by immunohistochemistry for ACTH, LH, FSH and TSH. (**Figure 21**)

As mentioned previously, phenotypical differences were observed in the homozygous mutants but to different extent. There was enlargement of the anterior pituitary in majority of the cases (to different degree) and all but one mutant animals

had incomplete sphenoid bone fusion, which is a typical sign of pituitary enlargement as shown on **Figure 20** and **Figure 21**.

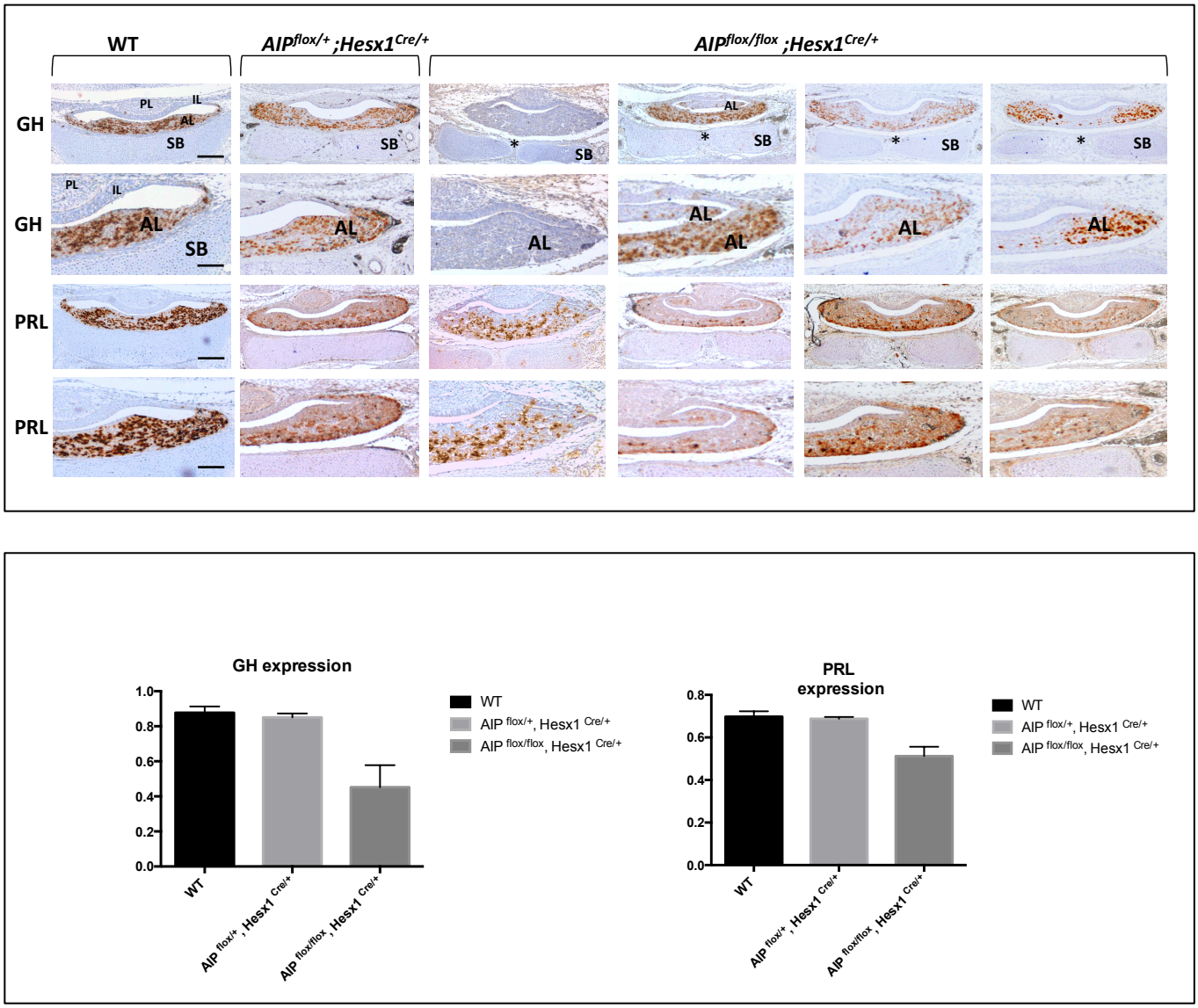
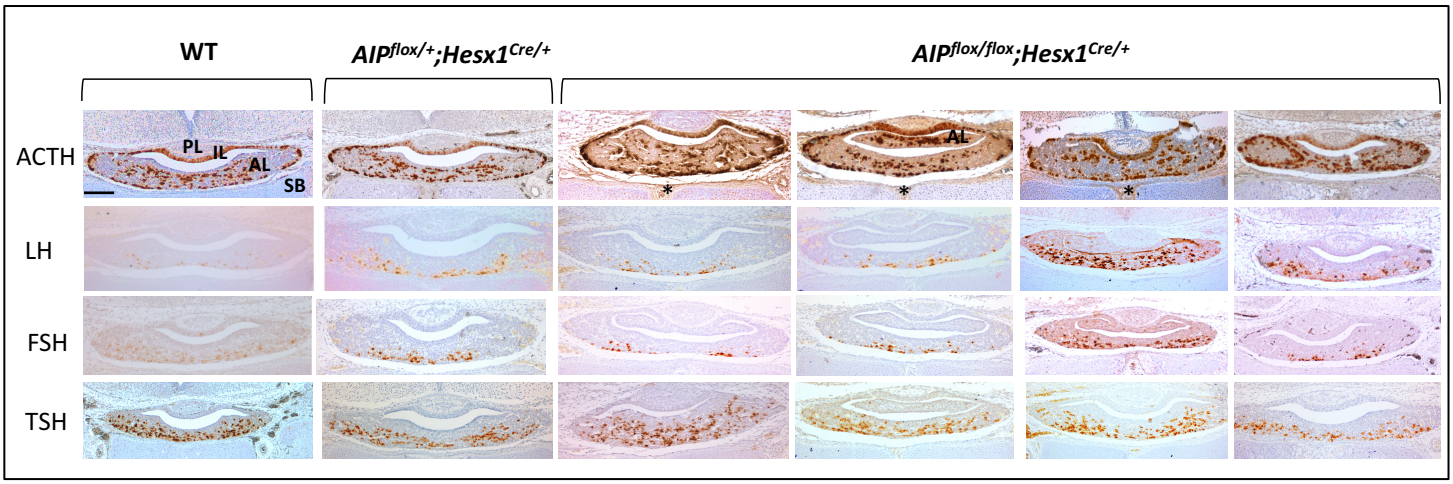


Figure 20. GH and PRL expression at 17.5 dpc

*Immunohistochemistry for GH and PRL in 17.5 dpc mice pituitaries showing significantly reduced GH expression in homozygous mutant animals ($AIP^{Flox/Flox}$, $Hesx1^{Cre/+}$) when compared to WT and heterozygous mutant ($AIP^{Flox/+}$, $Hesx1^{Cre/+}$), (one-way ANOVA, p-value <0.05, 4 samples per genotype). Reduced, but not statistically significant PRL expression in the homozygous mutant is demonstrated (one-way ANOVA, p-value 0.06, 4 samples per genotype). The panel also demonstrates the different phenotypes of the homozygous mutant animals – all showing signs of pituitary enlargement (not quantified) with anterior lobe enlargement as well as incomplete sphenoid bone fusion (marked with *), however to different extent. Scalebar line 1 is 200 µm, line 2 is 100 µm, line 3 is 200 µm, line 4 is 100 µm.*

Abbreviations: WT; wildtype, GH: growth hormone, PRL: prolactin, AL: anterior lobe of the pituitary gland, IL: intermediate lobe of the pituitary gland, PL: posterior lobe of the pituitary gland, SB: sphenoid bone



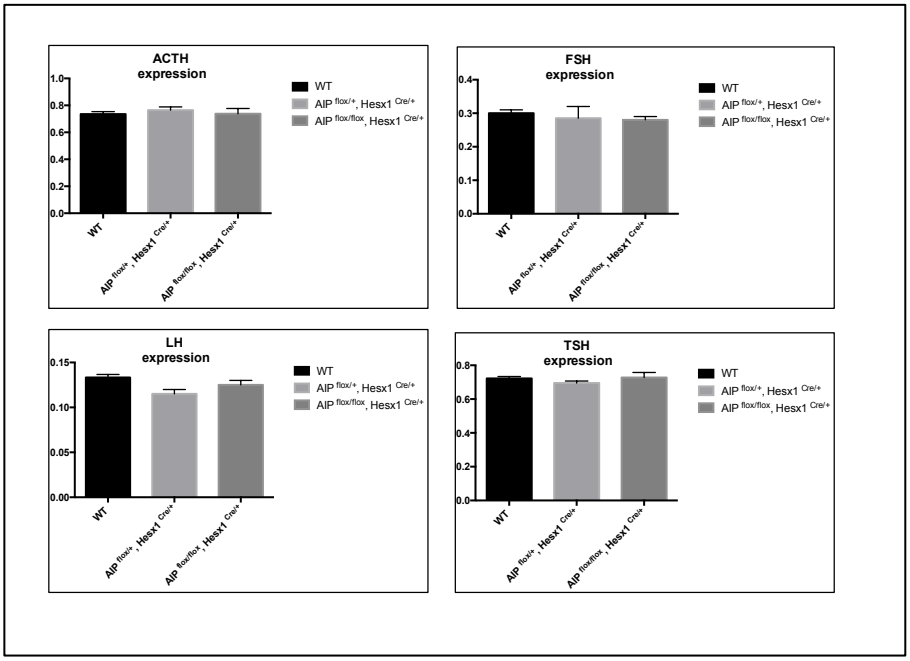


Figure 21. ACTH, LH, FSH and TSH expression at e17.5
*Immunohistochemistry for ACTH, FSH, LH and TSH in 17.5 dpc mice pituitaries showing no difference (one way ANOVA, p-value > 0.05, 4 samples per genotype) in expression pattern in homozygous mutant animals (AIP$^{Flox/Flox}$, Hesx1$^{Cre/+}$) when compared to WT and heterozygous mutants (AIP$^{Flox/+}$, Hesx1$^{Cre/+}$). The panel also demonstrates the phenotype variety of the homozygous mutant animals – all showing signs of pituitary enlargement with marked anterior lobe enlargement as well as incomplete sphenoid bone fusion (marked with *), however to different extent.* ***(n=4).*** *Scalebar 200 µm.*

Abbreviations: WT; wildtype, FSH: follicle stimulating hormone, LH: luteinizing hormone, TSH: thyroid stimulating hormone, ACTH: adrenocorticrotoph hormone, AL: anterior lobe of the pituitary gland, IL: intermediate lobe of the pituitary gland, PL: posterior lobe of the pituitary gland, SB: sphenoid bone

Postnatal characterisation of the *Aip*$^{Flox/Flox}$; *Hesx1*$^{Cre/+}$ mouse

Homozygous *Aip* knockout mice in both genders were becoming larger from week 10, reaching statistically significant difference by week 12, without significant difference in naso-anal length (**Figure 22**). Circulating IGF-1 levels were determined with ELISA from tail blood showing significantly higher levels in AIP knockout animals from week 28 (**Figure 23**). A different investigator's results using my model correspond with mine, showing significance as early as 8 weeks.

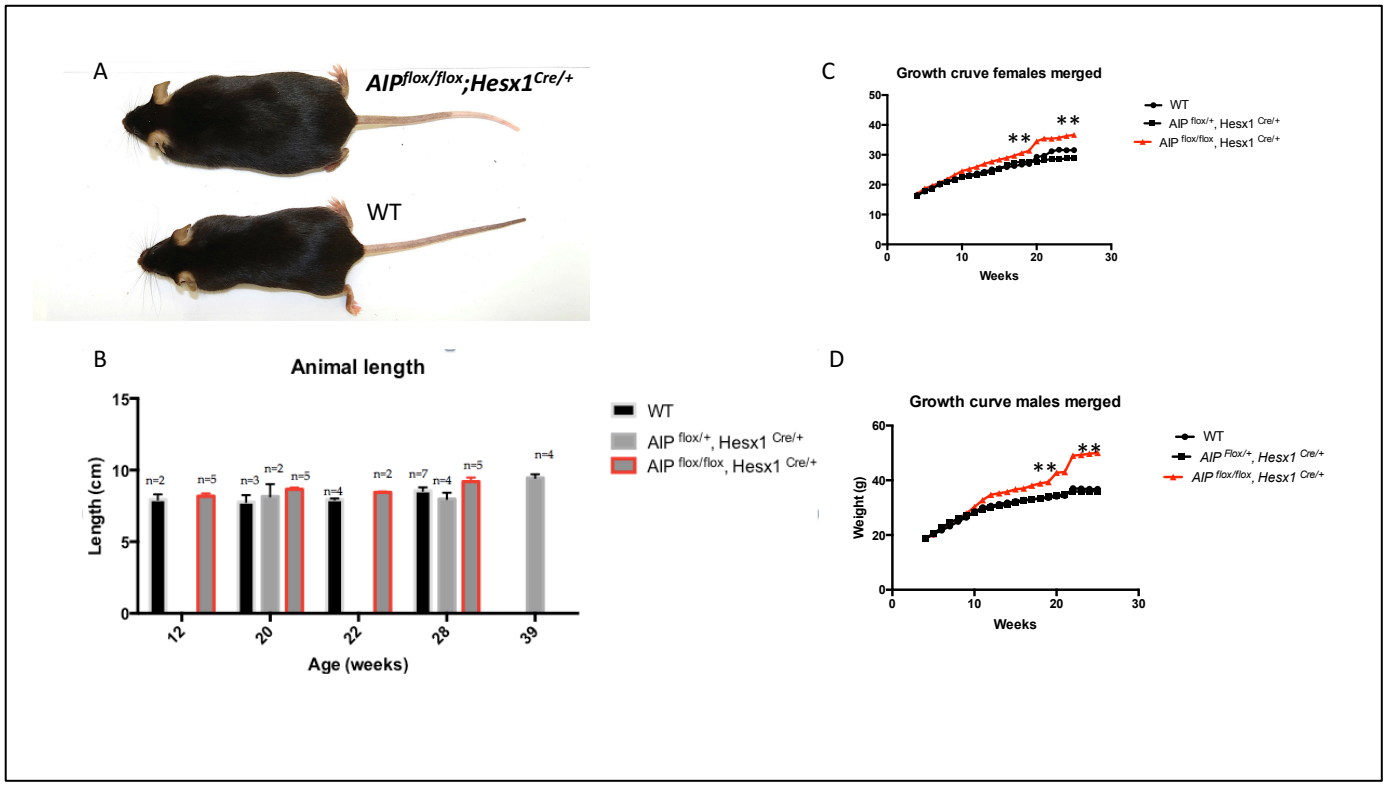


Figure 22. Characterisation of length and weight of the *Aip*$^{Flox/Flox}$*; Hesx1*$^{Cre/+}$ *animals postnatally*

A) *photograph of a 31-week old Aip*$^{Flox/Flox}$*; Hesx1*$^{Cre/+}$ *animal demonstrating little difference in naso-anal length but visible weight difference compared to its age-matched wildtype (WT) littermate* ***B)*** *Serial naso-anal measurements of animals of all three phenotypes (WT, Aip*$^{Flox/Flox}$*; Hesx1*$^{Cre/+}$*, Aip*$^{Flox/Flox}$*; Hesx1*$^{Cre/+}$ *) until week 28, and some homozygous mutants until week 39 revealed no length or weight difference between the animals (one-way ANOVA, data represented as mean ± SD, p-value > 0.05)* ***C and D)*** *Bi-weekly weight measurements resulted in growth curves showing significant weight differences in both genders when comparing WT and heterozygous animals to their age and sex-matched Aip*$^{Flox/Flox}$*; Hesx1*$^{Cre/+}$ *littermates (one-way ANOVA, p-value <0.05).*

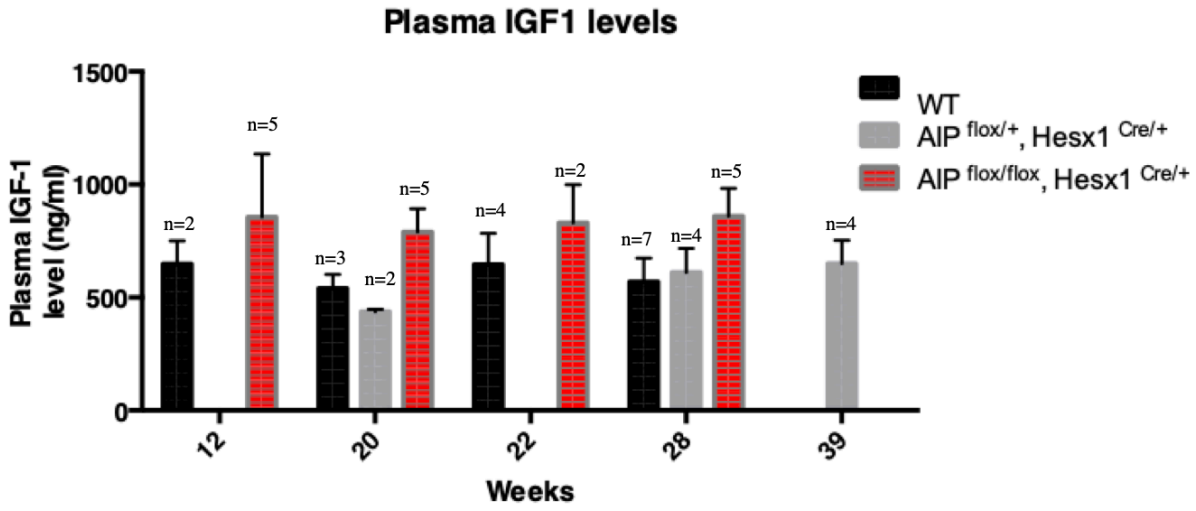


Figure 23. Plasma IGF-1 levels *Aip knockout animals show elevated plasma IGF-1 levels at week 28 when compared to their littermates (one-way ANOVA, data represented as mean ± SD, p=0.0037).*

Generating *Aip*$^{Flox/Flox}$; *ROSA*$^{YFP/+}$; *Hesx1*$^{Cre/+}$ mice and *Aip*$^{Flox/Flox}$; *Sox2*$^{CreERT2/+}$ mice

In order to generate a deletion of *Aip* in a time and tissue specific manner (inducible, pituitary-specific model), *Aip*$^{Flox/Flox}$ mice were crossed to Sox2-CreER^{T2} animals (donated to us by Dr Carlos Gaston-Massuet) as shown on **Figure 24.** In the Sox2-CreER^{T2} knockin animals the Sox2 open reading frame is replaced with a CreER^{T2} fusion gene, were the Cre-recombinase is fused with triple-mutant ER, which does not bind to its natural ligand, but it does to tamoxifen (a synthetic oestrogen ligand). Therefore, the Cre-recombination in these animals is inducible with tamoxifen meaning the deletion of the Floxed sequence (*Aip)* only takes place in the Cre-expressing cells of the offspring when tamoxifen is administered (204).

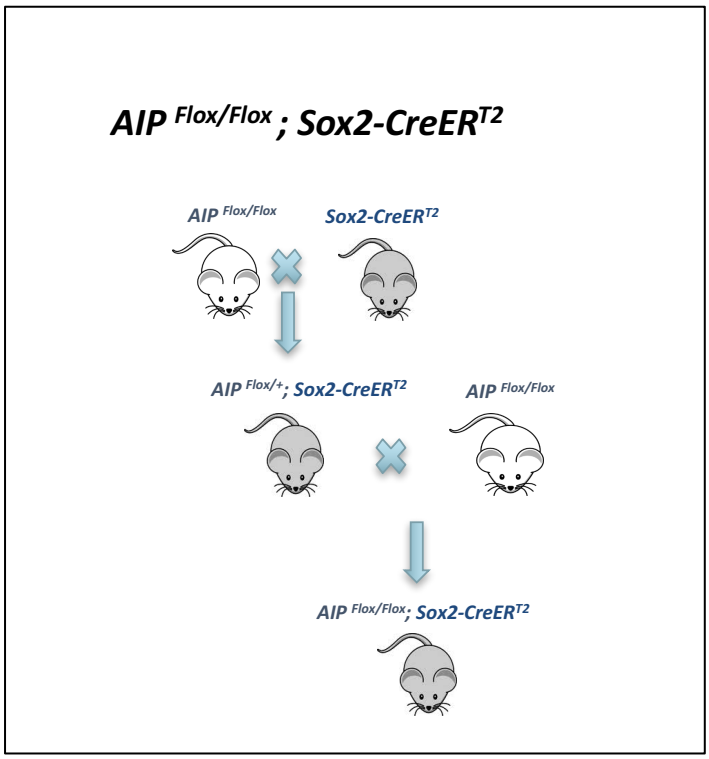


Figure 24. Generation of AIP$^{Flox/Flox}$*; Sox2*^{CreERT2} *animals. Firstly, AIP*$^{Flox/Flox}$ *mice were crossed to Sox2-CreER*^{T2} *animals to obtain AIP*$^{Flox/+}$*; Sox2-CreER*^{T2}*. By breeding AIP*$^{Flox/Flox}$ *mice to AIP*$^{Flox/+}$*; Sox2-CreER*^{T2} *we obtained AIP*$^{Flox/Flox}$*; Sox2*^{CreERT2}*, i.e. inducible pituitary-tissue specific genetic ablation of AIP in the pituitary.*

AIP$^{Flox/Flox}$ mice were also bred to *ROSA26*$^{YFP/YFP}$ animals and the previously described *Hesx1*$^{Cre/+}$ mice (donated to us by Dr Carles Gaston-Massuet). These R26-stop-YFP mutant mice have a loxP-flanked STOP sequence followed by the Yellow Fluorescent Protein gene (YFP) inserted into the Gt(ROSA)26Sor locus. When bred to mice expressing Cre-recombinase, the STOP sequence is deleted and YFP expression is observed in the Cre-expressing tissue(s) of the double mutant offspring, enabling the lineage of such cells in embryos, young, and adult mice at desired time point. Through crosses as detailed on **Figure 25**, we obtained *Aip*$^{Flox/Flox}$*; ROSA*$^{YFP/+}$*; Hesx1*$^{Cre/+}$ animals, which animals have yellow-fluorescent, pituitary specific *Aip*$^{-/-}$ cells. I have initial crosses to make this model inducible, by crossing these animals to the Sox2-CreER^{T2} colony.

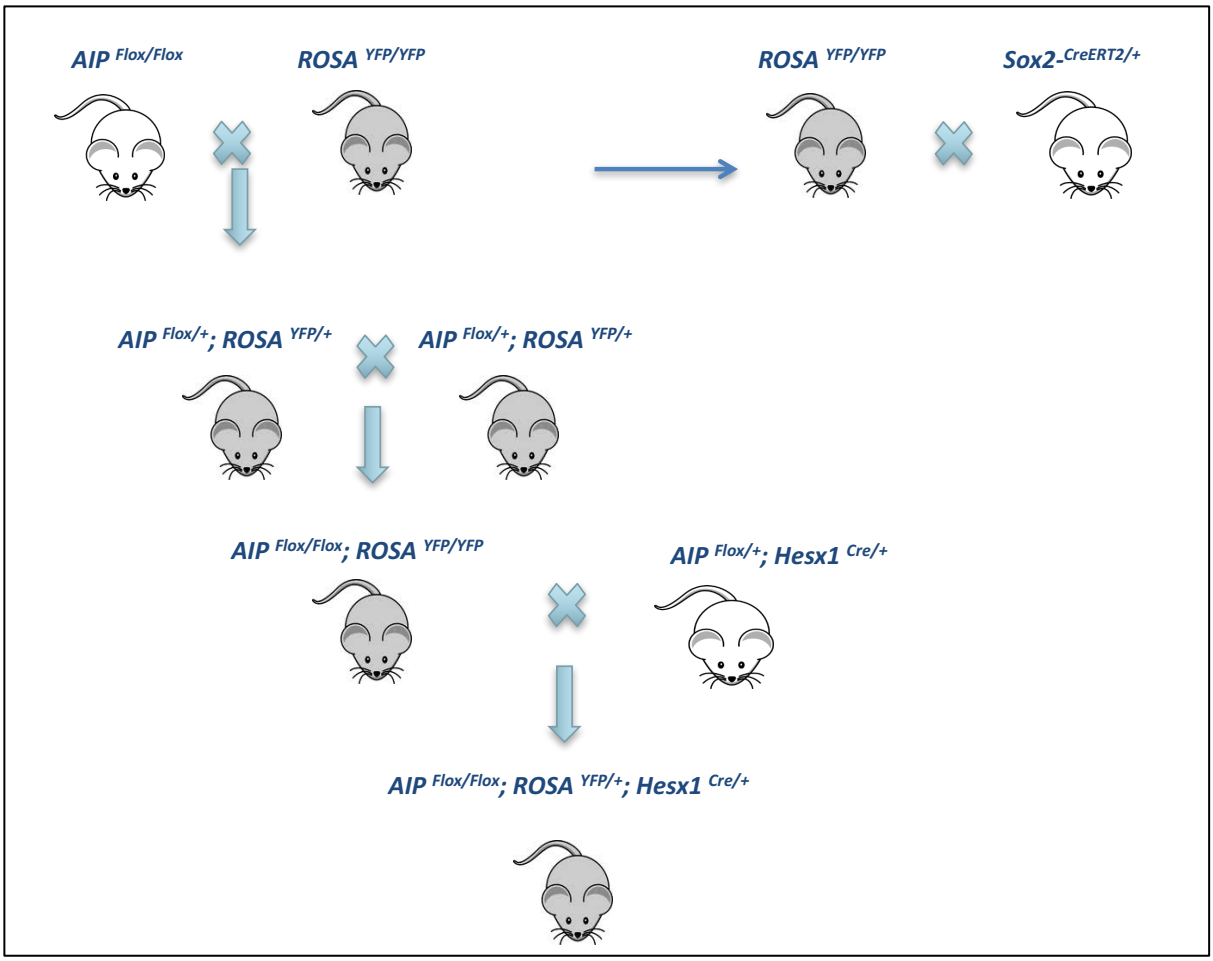


Figure 25. *Generation of AIP$^{Flox/Flox}$;**AIP$^{Flox/Flox}$; ROSA$^{YFP/+}$;Hesx1$^{Cre/+}$**animals.*

Firstly, AIP$^{Flox/Flox}$ mice were crossed to ROSA26$^{YFP/YFP}$ animals to obtain AIP^{Flox} ROSA26$^{YFP/+}$. By intercrossing these animals, we obtained AIP$^{Flox/Flox}$; ROSA$^{YFP/YFP}$, which were then crossed with AIP$^{Flox/+}$; Hesx1$^{Cre/+}$ to obtain the AIP$^{Flox/Flox}$; ROSA$^{YFP/+}$; Hesx1$^{Cre/+}$ animal.

Discussion

Obtaining human pituitary adenoma samples for research purposes is very challenging. If the patient undergoes surgery, the size of the tumours is relatively small, therefore the amount of tissue that can be spared for research (if any) is very limited. Often these patients undergo radiotherapy or pharmacological therapy before the surgery, which can influence the tissue quality and reliability. To obtain *AIP* mutation-positive human pituitary tumour samples are even more challenging, as genetic status usually not know for the first person in the family, only after a second, and even then, genetic testing results often only available after the surgery, so prospective collection of fresh tissue is only available in a next family member or at a repeated surgery of the known family members.

Global homozygous knockout of *Aip* in mice is lethal in the embryonic stage due to severe cardiac malformations (198, 199). For this reason, a viable, tissue specific *Aip*-knockout animal was important to generate and since became a truly novel and powerful tool for further research into the role of AIP in pituitary formation and tumorigenesis.

I have generated $Aip^{Flox/Flox}$; $ROSA^{YFP/+}$; $Hesx1^{Cre/+}$ and $Aip^{Flox/Flox}$; $Sox2^{CreERT2/+}$ mouse models. The $Aip^{Flox/Flox}$; $ROSA^{YFP/+}$; $Hesx1^{Cre/+}$ mice enable deletion of *Aip* in a time and tissue specific manner (induced by tamoxifen), while the $Aip^{Flox/Flox}$; $ROSA^{YFP/+}$; $Hesx1^{Cre/+}$ animals have yellow-fluorescent, pituitary specific $Aip^{-/-}$ cells, enabling lineage tracing of the pituitary. I have generated, but not used these animals for my research project.

Aip is known to be expressed from 9.5 dpc (156) ubiquitously, but given the non-tissue specific homozygous embryonic lethality (198, 199) this is the first study examining the effect of homozygous loss of *Aip in utero*.

The loss of *Aip* in the pituitary, but not in other organs in our $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ mice was obvious on immunostaining of embryos at 15.5 dpc and 17.5 dpc. The global homozygous *Aip* knockout animal in previous studies died by embryonic day 14 (198, 199), this model overcame the severe cardiac complications that caused lethality and the $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ were born at normal time and were undistinguishable from their wildtype littermates. In Lin *et al.*'s global homozygous knockout model, the homozygous embryos were paler, had decreased vessel calibre compared to the wildtype and heterozygous embryos and there was no viable litter (198).

There is phenotypical difference (enlarged anterior lobe, incomplete fusion of the sphenoid bone) in the pituitaries 17.5 dpc $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ embryos when compared with the wildtype of the same embryonic stage. The penetrance of this abnormality is not a 100% and the differences manifest with various intensity. Some of the heterozygous ($Aip^{Flox+/}$; $Hesx1^{Cre/+}$) animals show some degree of phenotypical change, but less so than their homozygous littermates.

With regards to cell lineage determination, there is no observed difference at 15.5 dpc embryos between the three examined genotypes, they all seem to reach the terminal differentiation stage (15.5 to 17.5 dpc) in a completely normal way.

In the terminal differentiation stage, when the pituitary cells become dedicated hormone producing cells, the $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ animals show a decrease in growth hormone and prolactin producing cells, implying the Pit1 lineage could be affected. I explored whether the cells "get stuck" at the earlier, *Pit1* stage and are

unable to progress, but *Pit1* staining at 17.5 dpc showed no difference between the mutant and the wildtype pituitary. The fact that the Pit1, and then consequently the growth hormone and prolactin secretion could be directly affected by loss of *Aip* (already at an embryonic stage) is particularly intriguing in the light of the fact that we know that in humans, patients harbouring *AIP* mutations usually present in childhood and the most common tumours in this cohort are growth hormone and/or prolactin secreting (3, 6, 150).

$Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$ mice in both genders were larger from week 10, reaching statistically significant difference by week 12, circulating IGF-1 levels showing significantly higher levels in *Aip* knockout animals from week 20. A different investigator's (within our group) results using my model correspond with mine, but with a larger cohort showing significance as early as 8 weeks. The animals survive well into adulthood and are fertile. Other researchers within our group carried on investigating these animals at later adult stages and observed tumour development mirroring the human phenotype (Mistry, A. – unpublished). Raitila *et al.* in her heterozygous, non-tissue specific knockout model showed a significantly higher tendency to develop (mostly GH secreting) pituitary adenomas at around the age of 6 months, mainly localized in the pars distalis, which is the equivalent part of the animal to the human anterior pituitary, that we see affected in our model as early as embryonic day 17.5 (199).

This animal model has ultimately become an easily accessible tool for investigating the role of AIP in pituitary tumorigenesis and opened up various new avenues for future research.

Chapter V.

The role of Hippo signalling in AIP-mediated pituitary tumorigenesis

Background

The Cancer Genomic Atlas has categorised the Hippo signalling pathway as one of the eight most important pathways altered in human cancers (205). Its role in pituitary tumours is unclear and in this Chapter I analysed data on Hippo pathway members regarding familial and sporadic pituitary tumorigenesis.

The control mechanisms of organ size have long been a central question of biological research, yet our understanding of the exact molecular mechanisms is still far from complete. The discovery of an inhibitory phosphorylation cascade, the Hippo pathway (also known as the Salvador-Warts-Hippo pathway) has shed some light on this early on by suggesting a crucial role in organ size control (206, 207).

The Hippo pathway is an evolutionarily highly conserved serine/threonine kinase cascade first discovered at the turn of 21st century in fruit fly (*Drosophila melanogaster)* (206, 207).

The core components of the Hippo pathway are the Ste20-like kinases 1/2 (MST1/2) that form heterodimers with Salvador Family WW Domain Containing Protein 1 (SAV1). MST1/2 then phosphorylates SAV1 and the Large tumour suppressor kinase 1/2 (LATS1/2 kinase). LATS1/2 then directly phosphorylates the YAP (yes-associated protein) and TAZ (coded by the WW domain–containing transcription regulator protein 1 gene *WWTR1*) which inhibits their localization to the nucleus (Figure 26). Phosphorylated YAP/TAZ then are sequestered in the cytoplasm, resulting in YAP/TAZ inhibition. Further phosphorylation of YAP/TAZ leads to ubiquitination and degradation (208).

YAP and TAZ are transcriptional co-regulators lacking DNA-binding domains, whose primary binding partners are the TEAD family of transcription factors (TEAD1-4). When active, the Hippo pathway restricts tissue growth and cell proliferation by phosphorylating and inhibiting the YAP/TAZ. When the pathway is inactive, YAP/TAZ and translocated into the nucleus, where they bind to the TEADs to induce cell proliferation. Without YAP/TAZ, TEAD represses the expression of target genes (208) (Figure 26).

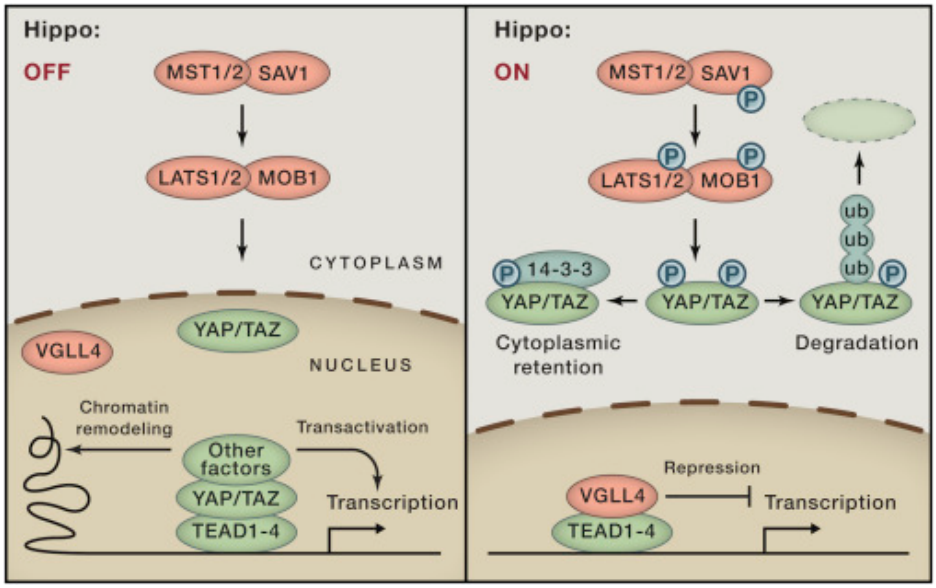


Figure 26. Hippo signalling. Left panel: when Hippo signalling is off, YAP/TAZ enter the nucleus, compete with VGLL4 for TEADs, and recruit other factors to induce gene transcription. YAP/TAZ may bind proximal promoters or distal enhancers of target genes to induce transcription. Right panel: when Hippo signalling is on, YAP/TAZ are phosphorylated by LATS1/2 on multiple sites, resulting in interaction with 14-3-3 and cytoplasmic retention; phosphorylation also leads to YAP/TAZ poly-ubiquitination and degradation. VGLL4 interacts with TEADs and represses target gene transcription (209).

The Hippo pathway regulation is not static in either ON or OFF status, but rather it is dynamically changing between these two states depending on regulatory influences. YAP/TAZ is under constant and rapid phosphorylation and dephosphorylation, and while phosphorylation is by LATS1/2, much less is known about the regulation of the phosphatases (210).

The role of the Hippo pathway in controlling organ size (211, 212) by regulating cell growth, proliferation and apoptosis has since been established, and the pathway has been an area of research. The pathway has attracted interest from the clinical side as it has been shown to play a role in tumorigenesis and angiogenesis in multiple malignancies (213, 214), elements of the pathway rapidly emerging as promising anti-cancer targets (215-217) in oncotherapy.

Given the diverse roles of the pathway, it is rather unsurprising that the pathway is associated with a range of human malignancies (**Table 3**). Generally speaking, many cancers show high expression of YAP and TAZ, therefore silencing them is heavily investigated as a therapeutic possibility (218).

More recently, the role of the Hippo pathway in angiogenesis has been suggested, vessel formation being a determining component in malignant tumour and metastasis formation. As angiogenesis is requisite for cancer metastasis and tumour growth, in many human tumours increased angiogenesis correlates with poor prognosis and poor survival (219, 220).

While the pathway's disease-causing role has – understandably – mostly been focused on cancer, it is worth noting that the many components have been implicated in multiple other pathologies, the major ones of which I have summarised in **Table 4.** In these diseases, the exact mechanisms and role of the Hippo pathway remains to be further explored on a molecular level. Very recently it has been suggested, that the Hippo pathway could play a determining role in viral diseases caused by the Zika-virus, Hepatitis B, Epstein-Barr virus, Kaposi sarcoma-associated herpesvirus and human papillomavirus (221).

Cancer type	Suggested mechanism	Reference
Non-small cell lung cancer (NSCLC)	overexpression of YAP and TAZ LATS2 downregulation	Chen *et al.*, 2015 (222) Malik *et al.*, 2018 (223)
Breast cancer	YAP deficiency LATS1, LATS2 deregulation	Chen *et al.*,2014 (224) Li *et al.*, 2017 (225) Wei *et al.*, 2018 (226)
Gastric cancer	Overexpression of YAP	Lam-Himlin *et al.*, 2006 (227) Hu *et al.*, 2014 (228)
Oesophageal cancer	TAZ overexpression	Sun *et al.*, 2014 (229)
Hepatocellular cancer	overexpression of MST1/2 YAP1 overexpression	Wang *et al.*, 2015 (230) Mao *et al.*, 2014 (231)
Renal cancer	YAP overexpression	Philips *et al.*, 2014 (232)
Colorectal cancer	YAP, TAZ, TEAD overexpression	Liang et l., 2014 (233) Wierzbicki *et al.*, 2015 (234)
Chronic myeloid leukaemia Acute pro-myelocytic leukaemia	YAP overexpression	Li *et al.*, 2016 (235) Chen *et al.*, 2017 (236)
Osteosarcoma	TEAD1 and YAP1 overexpression	Chai *et al.*, 2017 (237) Basu-Roy *et al.*, 2016 (238)
Rhabdomyosarcoma	YAP overexpression	Tremblay *et al.*, 2014 (239)
Angiosarcoma	YAP overexpression	Tsuneki *et al.*, 2017 (240)

Table 3. Malignancies associated with the deregulation of the Hippo pathway

Pathology	Suggested mechanism	Reference
Cataract formation	YAP activation	Kresak and Walsh, 2016 (241)
Sveinsson chorioretinal atrophy	loss of YAP activity	Fossdal *et al.*, 2004 (242)
Coloboma	Yap mutation	Williamson *et al.*, 2014 (243)
Autosomal recessive primary immunodeficiency	MST1 mutation	Nehme *et al.*, 2012 (244)
Arrhythmogenic cardiomyopathy	Hippo activation	Chen et al,.2014b (245)
Stanford type A aortic dissection	decreased YAP protein	Jiang *et al.*, 2016 (246)
Pulmonary hypertension	elevated YAP protein	Wang *et al.*, 2018 (247)
Atherosclerosis	elevated YAP protein	Wang *et al.*, 2018 (247)
Polycystic kidney disease	nuclear accumulation of YAP	Cai *et al.*, 2018 (248)
Nonalcoholic steatohepatosis	elevated TAZ protein	Wang *et al.*, 2016 (249)
Sjogren syndrome	mislocalisation of TAZ	Enger *et al.*, 2013 (250)
Sporadic amyotrophic lateral sclerosis	increased MST1 activity	Lee *et al.*, 2013 (251)
Keratoconus corneas	decreased LATS2, TEAD2 and TEAD4	Kabza *et al.*, 2017 (252)
Huntington's disease	activation of MST1, decrease in nuclear YAP	Mueller *et al.*, 2018 (253)

Table 4. Non-cancerous pathologies associated with the Hippo pathway

Hippo signalling in the pituitary gland and in pituitary tumorigenesis

There is a lot left to be elucidated, but there is already enough convincing evidence that the Hippo cascade is important in pituitary organogenesis and homeostasis.

An early study in 1999 by St John *et al.* reported pituitary hyperplasia in the anterior lobe of *Lats1*-deficient mice (no tumours were noted) but lower serum hormone-levels were measured (254), first implying that the Hippo pathway could play a role in pituitary organ development.

In 2016 Lodge *et al.* were the first to prove that the Hippo signalling cascade components are detectable during all stages of embryonic pituitary development and postnatally as well (255, 256). The genes encoding the core pathway kinases (*Mst1* and *Mst2*) and *Lats1* are strongly expressed throughout murine pituitary development, *Lats2* is almost non-detectable (255, 257, 258), making *Lats1* the most likely main kinase upstream of *Yap/Taz* in murine pituitary organogenesis. *Tead2* is the highest expressed out of the four *Tead* genes encoding the pathway's transcription factors; therefore it is currently considered to be the most important downstream pathway regulator in the developing pituitary (255).

Loss of *Lats1* and *Lats2* results in significant tissue overgrowth during gestation, suggesting a growth restricting role of the Hippo pathway during pituitary development (255). Loss of *Lats1* postnatally on its own is enough to lead to tumour formation in the anterior and intermediate lobe in murine models (256), with identical tumours developing if deletion is performed embryonically.

In adult mice, the Hippo pathway is required to maintain the population of SOX2+ cells in the pituitary. *Lats1/2* kinases control proliferation of SOX2+ progenitors and their progression to the committed pituitary cell lineages (256). Loss of *Lats1* drive deregulation of SOX2+ pituitary stem cells, generating highly proliferative non-

functioning tumours, where genetic lineage tracing proves SOX2+ cells to be the cell of origin of these tumours (256).

The two key downstream effectors of the Hippo cascade, YAP and TAZ are active in the normal human adult and foetal pituitary, providing evidence for the pathway's presence in the gland from the 14th gestational week to the adult stage (259). Recapitulating the findings in mice (256), the YAP/TAZ expression pattern is reminiscent of SOX2 in the human gland in both embryos and the postnatal hypophysis (259) and protein ubiquitination has been acknowledged as an important posttranslational modification in pituitary adenomas and ubiquitin proteomics analysis of human pituitary adenoma and normal pituitary tissue revealed the Hippo pathway to be of interest (260).

A significant decrease of YAP/TAZ in human hormone-secreting pituitary tumour tissue when compared to normal pituitary and NFPA implies that the Hippo kinases may have a role in promoting a hormone-secreting tumour type (259). This observation supports the *Lats1*$^{-/-}$ mice, which were found to have low serum pituitary hormone levels despite their hyperplastic pituitaries (254). *In vitro* knockdown of *Lats1* decreases anterior pituitary hormone synthesis, proving a role for the Hippo cascade in repressing endocrine differentiation (259). Given that management of pituitary tumours heavily relies on reducing hormone hypersecretion and its damaging effects, this makes the pathway particularly intriguing for further investigation in pituitary adenomas.

There is no published data directly exploring the role of Hippo signalling in *AIP*-mediated pituitary tumorigenesis.

Methods

Wang et al. categorised the main members of the Hippo pathway into the following two groups (261)(Table 5), which nomenclature I keep throughout this chapter:

1. **Hippo Core genes** (Figure 27): curated from literature, 19 genes that function mainly through the Hippo pathway as Hippo core genes

2. **Hippo Target genes** (Figure 27): manually curated 22 downstream target genes of the Hippo pathway from the literature and experimental data, these 22 genes represent a robust index that can effectively capture Hippo pathway activity

Core Hippo genes	Target Hippo genes
FRMD6	F3
WWC1	ASAP1
YAP1	GADD45A
MST1/STK4	ARHGEF17
TAOK1	IGFBP3
TAOK2	CRIM1
TAOK3	TGFB2
NF2	RBMS3
SAV1	CYR61
STK3/MST2	CTGF
MOB1A	AMOTL2
MOB1B	PTPN14
LATS2	DOCK5
WWTR1/TAZ	NT5E
TEAD1	FJX1
TEAD3	AXL
DCHS1	MYOF
TEAD4	FOXF2
LATS1	CCDC80
	NUAK2
	LATS2
	ANKRD1

Table 5. Core and target Hippo genes (as determined by (261)

I have performed a search with a systematic approach for the Hippo core and target genes in the following datasets:

1. Gene expression profiling of *Aip* silenced rat pituitary somatomammotroph GH3 cells
2. RNAseq analysis on pituitary specific *Aip*-knockout (*Aip*-KO) mice (samples obtained from the mice generated within this PhD project)
3. Gene expression profiling of *AIP* mutation-positive, *AIP* mutation-negative familial and sporadic pituitary adenomas (human)
4. Exome and whole genome sequencing of pituitary adenoma patients

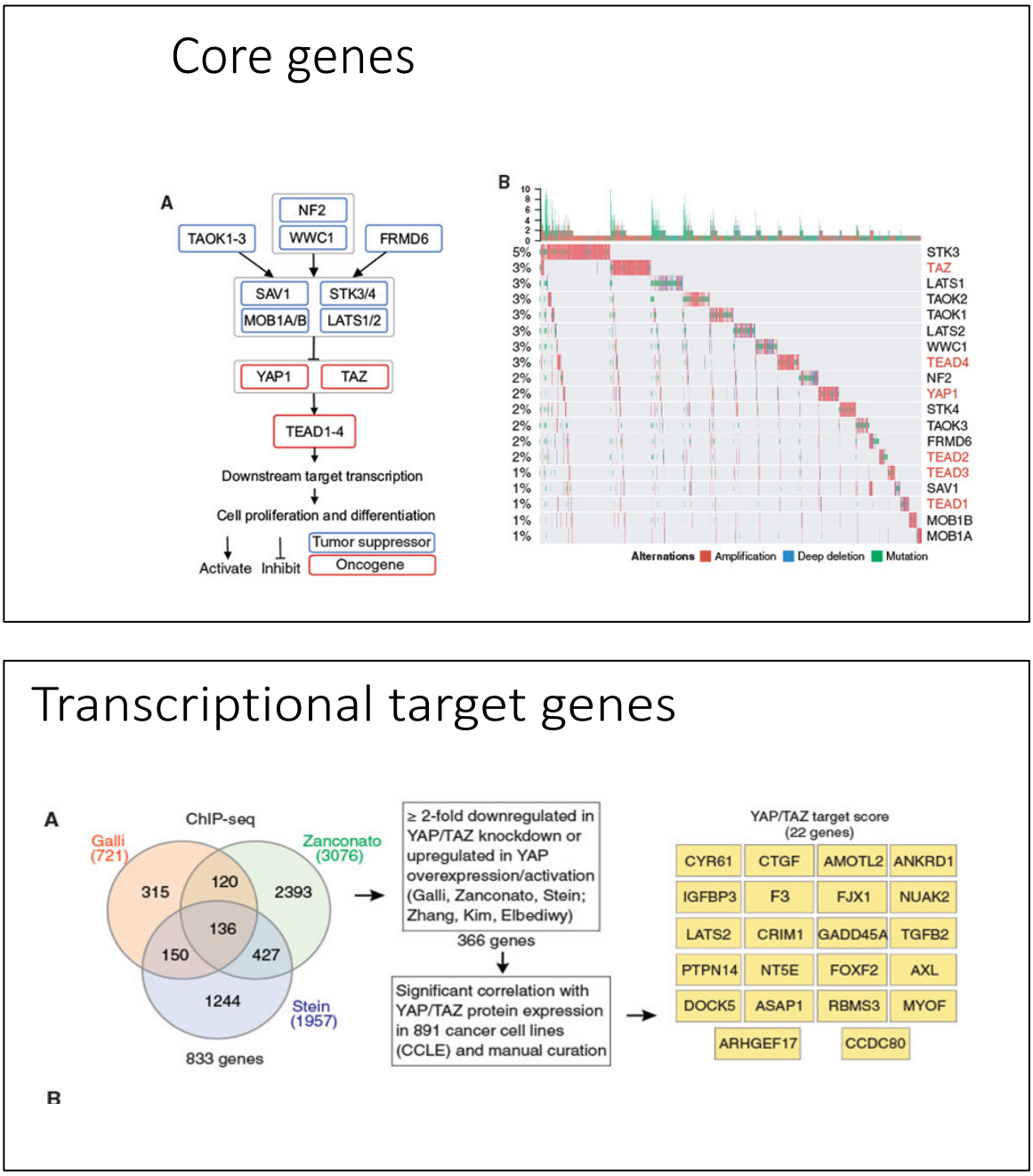


Figure 27. ***Hippo core and target genes as determined by Wang et al****. (261). The 19 core genes were manually curated from literature research, these genes were determined as mainly functioning through the Hippo pathway, therefore characterise its core component well. The 22 target genes were curated from literature research as well as Wang et al. own analysis, where they described that these 22 transcriptional target genes represent a robust index that can effectively capture Hippo pathway activity. Image from (261)*

1. Gene expression profiling of *Aip* silenced rat pituitary somatomammotroph GH3 cells

To reveal the potential molecular mechanisms of AIP in pituitary tumorigenesis, our lab had previously profiled rat pituitary GH3 cells with silenced *Aip* using Rat Genome 230 2.0 Arrays (Affymetrix). The gene expression profiles of *Aip* siRNA (n=4) samples were compared with the gene expression of non-targeting siRNA (n=4) samples and we identified genes that had an altered expression in the *Aip*-silenced cells at a statistically significant level (adjusted p-value <0.05, n=8). I have systematically searched this database for the core and target Hippo genes to assess whether they are differentially expressed when *Aip* is knocked down *in vitro*.

2. RNAseq analysis on pituitary specific *Aip*-knockout (*Aip*-KO) mice

The following comparative datasets were analysed:

1. 3-week-old *Aip*-KO (n=6) mice versus 3-week-old wildtype mice (n=5)
2. 15-month-old *Aip*-KO (n=7) mice versus 15-month-old wildtype mice (n=6)
3. 15-month-old *Aip*-KO (n=7) mice versus 3-week-old *Aip*-KO mice (n=6)
4. 15-month-old wildtype (n=6) mice versus 3-week-old wildtype mice (n=5)

The genotypes of the animals were: *Aip*-KO ($Aip^{Flox/Flox}$; $Hesx1^{Cre/+;}$ $R26^{YFP/+}$ pituitary specific homozygote *Aip*-knockout) and wildtype ($Aip^{+/+}$; $Hesx1^{Cre/+}$; $R26^{YFP/+}$). The $Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$; $R26^{YFP/+}$ animals are not only pituitary specific *Aip*-knockouts, but also have yellow-fluorescent, pituitary specific $Aip^{-/-}$ cells, enabling FACS-sorting. RNA had been extracted from the FAC-sorted mouse pituitary cells, the samples were then sent for RNA sequencing (Otogenics).

The bioinformatic RNAseq analysis was performed by James Nicholson. Softwares used: Kallisto used for alignments and counts read in with Tximport, DESeq2 used

for differential gene expression analysis, TopGO and cluster profiler used for Gene Ontology enrichment analysis, Gage and cluster profiler used for KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis.

3. Gene expression profiling of *AIP* mutation-positive, *AIP* mutation-negative familial and sporadic pituitary adenomas (human)

For obtaining the human pituitary tumour gene expression data, our research group had previously performed transcriptional profiling of on *AIP* mutation-positive familial and sporadic pituitary adenomas and normal pituitaries (Table 6) (n=25) on Affymetrix Human Gene Chip HG-U133 Plus 2.0 array(262). A double cut-off of false discovery rate <0.05 and fold change of ≥2 was used to identify differentially expressed genes. Microarray data have been deposited to the National Center for Biotechnology Information's Gene Expression Omnibus (accession number GSE63357).

I have searched for the core and target Hippo genes in the following comparative datasets:

1. Sporadic NFPA vs normal pituitary
2. Sporadic GHoma vs normal pituitary
3. *AIP* mutation-positive GHoma vs normal pituitary
4. *AIP* mutation-positive GHoma vs sporadic GHoma
5. *AIP* mutation-negative GHoma vs sporadic GHoma
6. *AIP* mutation-positive GHoma vs *AIP* mutation-negative GHoma

Sample ID	**Diagnosis**	***AIP* mutation status**
F26M2	Acromegaly	positive
F142M2	Gigantism	positive
F35M7	Acromegaly	positive
F34M2	Gigantism	positive
F27M1	Acromegaly	positive
Sp763	Acromegaly	positive
F23M1	NFPA	negative
F65M1	NFPA	negative
F73M1	Acromegaly	negative
F84M2	Acromegaly	negative
F68M1	Acromegaly	negative
1244	Acromegaly	negative
C98	Acromegaly	negative
926	Acromegaly	negative
1138	Acromegaly	negative
1233	NFPA	negative
1213	NFPA	negative
956	NFPA	negative
514	NFPA	negative
730	NFPA	negative
NP56	Normal pituitary	
NP52	Normal pituitary	
NP77	Normal pituitary	
NP55	Normal pituitary	
NP35	Normal pituitary	

Table 6. Human samples used for gene expression profiling of AIP mutation-positive and negative familial and sporadic pituitary adenomas (the samples containing the letter "F" in their ID are cases with a positive family history of pituitary adenoma, while the rest of the patients had no known family history of pituitary adenoma) and normal pituitaries (n=25)

4. Global phosphorylation analysis of Aip-KO mouse embryonic fibroblast cells by mass spectometry

A global phosphorylation analysis of *Aip-KO* mouse embryonic fibroblasts (MEFs) cells by mass spectrometry was carried out. Using *p*-value < 0.05, 2,156 phosphopeptides were identified. Ingenuity Pathway Analysis (IPA) on this dataset was performed (the global analysis was part of Dr Sayka Barry's project, who kindly shared the Hippo-relevant data to add to this thesis).

5. Exome and whole genome sequencing of pituitary adenoma patients

The samples used for this analysis are detailed in **Table 7** and **Table 8**. In the *AIP* mutation-negative exome sequencing samples (n=78) we used 4 acromegaly families (1 to 3 members each, n=6), 8 prolactinoma families (2 to 7 members, n=24), 4 ACTH families (2 members each, n=8), 5 NFPA families (1 to 3 members each and one tumour-tissue pair, n=11) and sporadic prolactinoma cases (n=25) as well as a sporadic TSH/FSH secreting tumour patient. Additionally, a pituitary hyperplasia patient and his unaffected mother and father were sequenced; later we recognised that this patient had mosaic *GPR101* duplication (127) . In the *AIP* mutation-negative whole genome sequencing samples (n=9), for acromegaly samples we analysed 3 families, 1 member from each family (n=3), for prolactinoma samples we analysed 3 families, 1 member from each family (n=3) and for NFPA samples we analysed 3 families, 1 member from each family (n=3).

For next generation sequencing our group used exome sequencing via 'Otogenics', Kings College Genome centre and Whole Genome sequencing by 'Complete Genomics'. At Otogenics exomes were captured by Agilent Human All Exon 50Mb assay and sequenced on the Illumina HiSeq 2000 platform. The exome sequence

reads were aligned to the HG19 reference genome. Variants were called by GATK and Picard tools with at least 30X coverage. At Kings College exomes were captured by Agilent Human All Exon 50Mb assay and sequenced on the Illumina HiSeq 2000 platform. The exome sequence reads were aligned to the HG19 reference genome, minimum 30X coverage. Whole Genome sequencing by 'Complete Genomics': sequences were aligned to the human reference genome (HG19) and analysed using Complete Genomics's pipeline. Subsequent variants were then filtered using different filters: zygosity, frequency in 5400 exomes, 1000 genomes (CEU population) and 69 publicly available whole genomes (sequenced by Complete Genomics)with at least 40X coverage.

I have manually searched in the dataset for variants of the core and target Hippo genes. The analysis was then further filtered for the heterozygote variants, keeping the depth of coverage criteria above 5. The variant was categorised rare when minor allele frequency was equal or below 1% (0.01). The variant was considered to be segregating within a family when it was found be present in 2 or more members from the same family.

Individual ID	Diagnosis
F20M1	Acromegaly
F20M2	Acromegaly
F20M7	Acromegaly
F10M5	Acromegaly
F28M3	Acromegaly
F66M2	Acromegaly
F151M1	NFPA
F151M2	NFPA
F151M3	Unaffected (Obligate carrier)
F151M1 (tumour DNA)	NFPA
F171M1	NFPA
F171M2	NFPA
F171M3	Unaffected (Obligate carrier)
F171M4	Meningioma
F97M2	NFPA
F17M1	NFPA
F65M4	NFPA
F207M2	ACTH (Cushing's)
F207M1	ACTH (Cushing's)
F191M2	ACTH (Silent pituitary corticotroph adenoma)
F191M1	ACTH (Silent pituitary corticotroph adenoma)
F185M2	ACTH (Cushing's)
F185M1	ACTH (Cushing's)
F147M2	ACTH (Cushing's)
F147M1	ACTH (Cushing's)
F31M1	Prolactinoma
F3M3	Prolactinoma
F86M3	Prolactinoma
F86M4	Prolactinoma
F86M5	Prolactinoma
F86M6	Prolactinoma
F86M8	Prolactinoma
F86M9	Prolactinoma
F199M1	Prolactinoma
F199M2	Prolactinoma
F239M1	Prolactinoma
F239M2	Prolactinoma
F243M1	Prolactinoma
F243M2	Prolactinoma
F243M3	Prolactinoma
F243M6	Prolactinoma

Individual ID	Diagnosis
F243M7	Prolactinoma
F243M8	Prolactinoma
F243M9	Prolactinoma
F253M2	Prolactinoma
F253M3	Prolactinoma
F277M1	Prolactinoma
F277M2	Prolactinoma
F277M3	Prolactinoma
Sp108	Prolactinoma
Sp158	Prolactinoma
Sp165	Prolactinoma
Sp190	Prolactinoma
Sp198	Prolactinoma
Sp25	Prolactinoma
Sp269	Prolactinoma
Sp271	Prolactinoma
Sp295	Prolactinoma
Sp329	Prolactinoma
Sp340M1	Prolactinoma
Sp422	Prolactinoma
Sp448	Prolactinoma
Sp474	Prolactinoma
Sp476	Prolactinoma
Sp493	Prolactinoma
Sp495	Prolactinoma
Sp534	Prolactinoma
Sp554	Prolactinoma
Sp634	Prolactinoma
Sp637	Prolactinoma
Sp638	Prolactinoma
Sp660	Prolactinoma
Sp87M1	Prolactinoma
Sp92	Prolactinoma
Sp95M1	Pituitary hyperplasia, *GPR101* mosaic mutation
Sp95M3	unaffected
Sp95M2	unaffected
Sp266	TSH/FSH secreting adenoma

Table 7. AIP mutation-negative exome sequencing samples (n=78).

Sample ID	Diagnosis
F10M1	Acromegaly
F28M1	Acromegaly
F66M1	Acromegaly
F31M2	Prolactinoma
F3M2	Prolactinoma
F86M1	Prolactinoma
F97M1	NFPA
F17M2	NFPA
F65M3	NFPA

Table 8. AIP mutation-negative whole genome sequencing samples (n=9). For the acromegaly samples we analysed 3 families, 1 member from each family (n=3). For the prolactinoma samples we analysed 3 families, 1 member from each family (n=3). For the NFPA samples we analysed 3 families, 1 member from each family (n=3).

Results

1. Gene expression profiling of *Aip* silenced rat pituitary somatomammotroph GH3 cells

Hippo target gene, *F3* (coagulation factor III) is upregulated *in vitro* (logFC 0.59, adjusted p value=0.005) in the *Aip*-knockdown GH3 cells when compared to non-targeting siRNA cells.

None of genes was found to be differentially expressed in this dataset.

2. RNAseq analysis on pituitary specific *Aip*-KO mice

From the core genes**,** the tumour suppressor *Frmd6* (FERM domain containing 6) in the *Aip*-KO mice at 15 months is downregulated (log2fold change -1.54, adjusted p ≤0.05, n=6) compared to 3-week-old *Aip*-KO mice, but no other comparative datasets in the *Aip*-KO mice showed different expression of *Frmd6*. From the target genes, *F3* (coagulation factor III) is downregulated in the mutant animals when comparing 15-month-old *Aip*-KO to 3-week-old *Aip*-KO (log2fold change -3.07, adjusted p≤0.004, n=6), but the same downregulation can be seen when comparing wildtype animals of the same age (log2fold change -2.49, adjusted p≤0.02, n=6) (Figure 28).

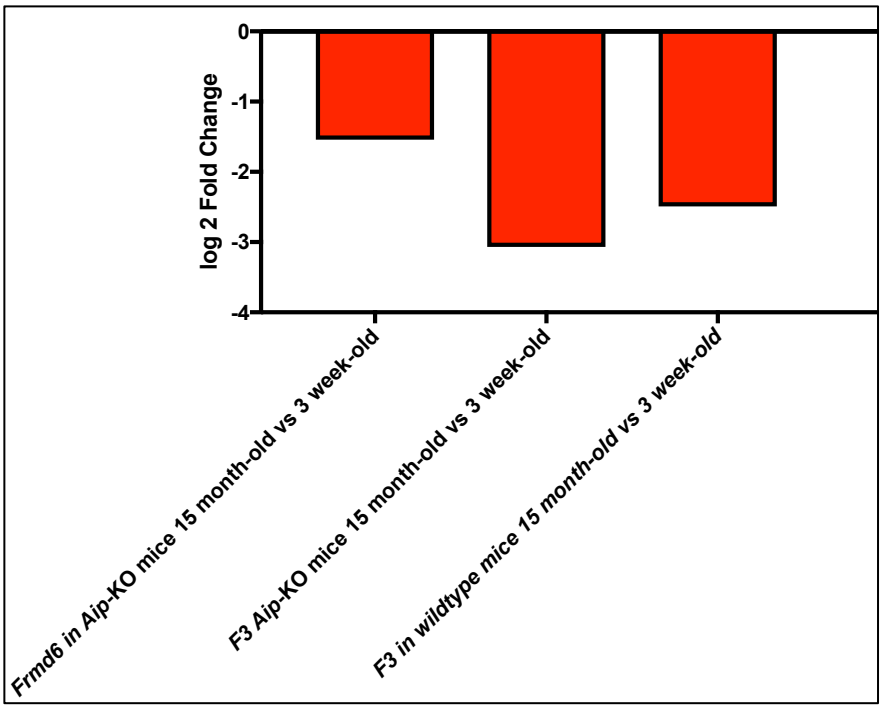


Figure 28. Hippo core and target gene expression in the Aip-KO mice (RNAseq) Frmd6 in the Aip-KO mice at 15 months is downregulated compared to 3-week-old Aip-KO mice. F3 is downregulated in the mutant animals when comparing 15-month-old Aip-KO to 3-week-old Aip-KO, downregulation can also be seen when comparing wildtype animals of the same age (absolute log2fold change >1.5, adjusted p≤0.05, n=6)

3. Gene expression profiling of *AIP* mutation-positive, *AIP* mutation-negative familial and sporadic pituitary adenomas (human)

NFPA versus normal pituitary

From the core genes, when comparing NFPA to normal pituitary tissue, I found that there is a higher expression for *SAV1* in NFPA when compared to normal pituitary. Lower expression is observed for the following genes in NFPA versus the normal pituitary: *WWC1, FRMD6, MST1, LATS2, YAP1* and *TAZ* (Figure 29***)*** (absolute logfold change >2, adjusted p≤0.05). From the target genes, *IGFBP3, LATS2, PTPN14* and *F3* were found to be downregulated in NFPA when compared with normal pituitary tissue (Figure 30) (absolute logfold change >2, adjusted p≤0.05*)*.

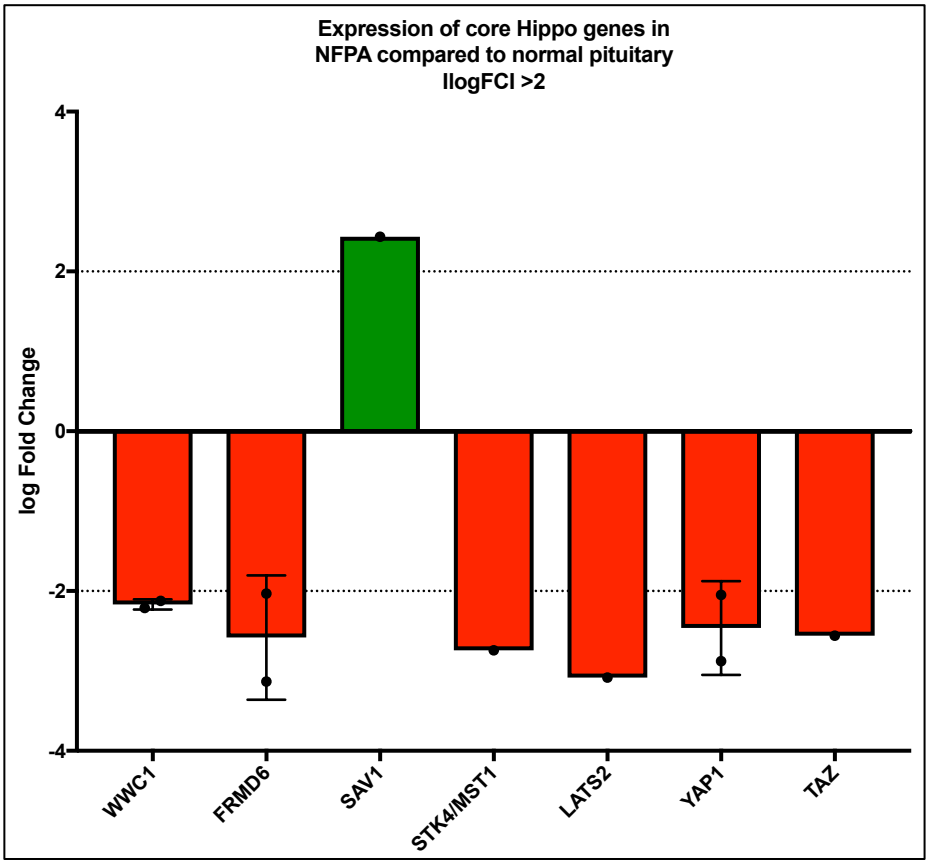


Figure 29. Differentially expressed Hippo core genes in NFPA compared to normal pituitary tissue. Dotted line marks absolute log fold change criteria. Green: expression up, red: expression down

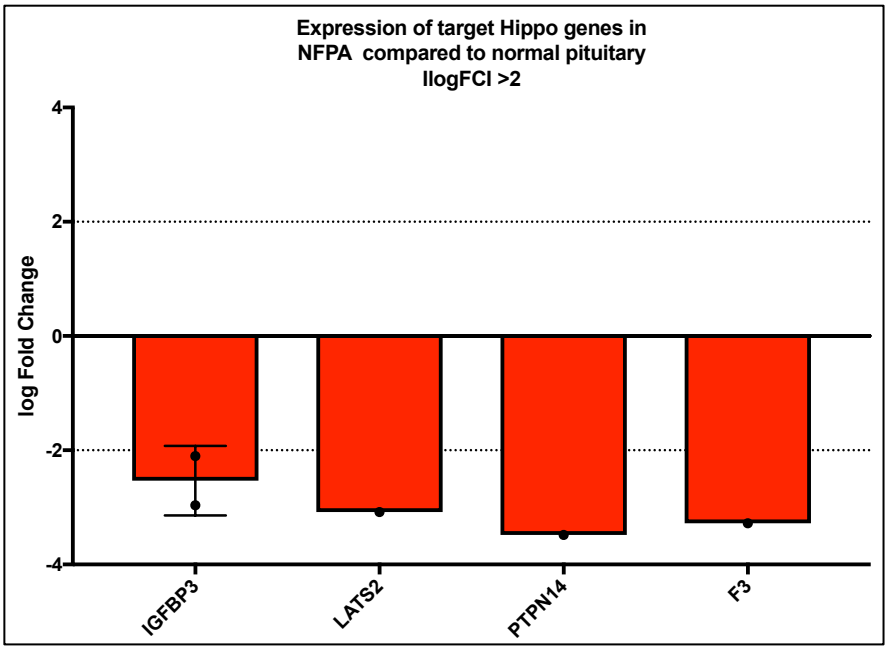


Figure 30. Differentially expressed Hippo target genes in NFPA compared to normal pituitary tissue. Dotted line marks absolute log fold change criteria. Red: expression down.

Sporadic GHoma versus normal pituitary

From the core genes, *LATS2, YAP1*, and *TAZ* were found to be downregulated in sporadic GHoma when compared to normal pituitary (absolute logfold change >2, adjusted p≤0.05) (Figure 31). Amongst the target Hippo genes, *IGFBP3, LATS2, CRIM1* were found to be downregulated in sporadic GHoma when compared to normal pituitary tissue (absolute logfold change >2, adjusted p≤0.05) (Figure 32).

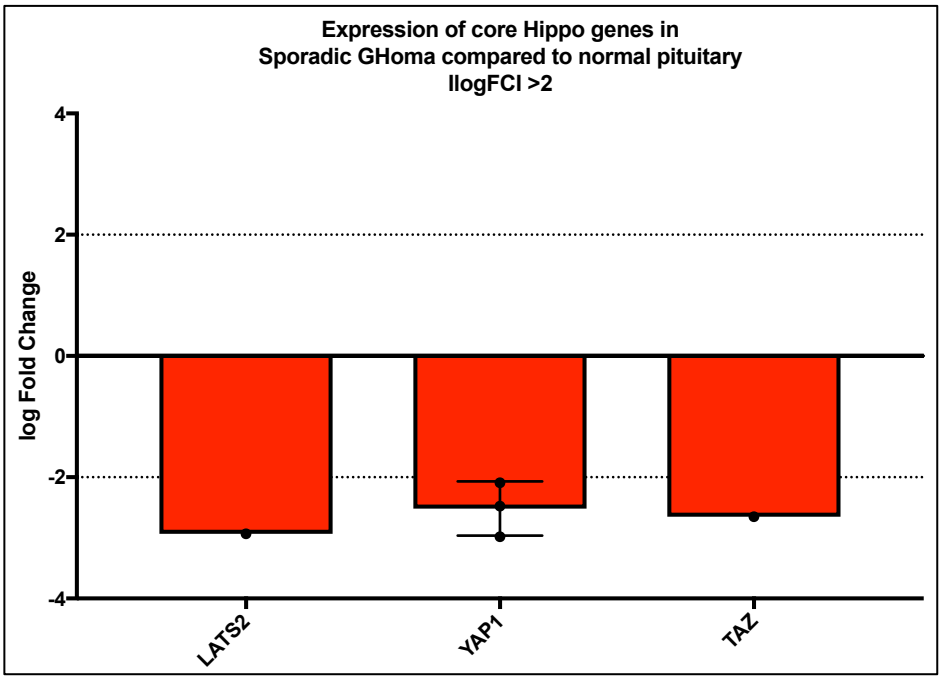


Figure 31. Differentially expressed Hippo core genes in sporadic GHomas compared to normal pituitary tissue. Dotted line marks absolute log fold change criteria. Red: expression down

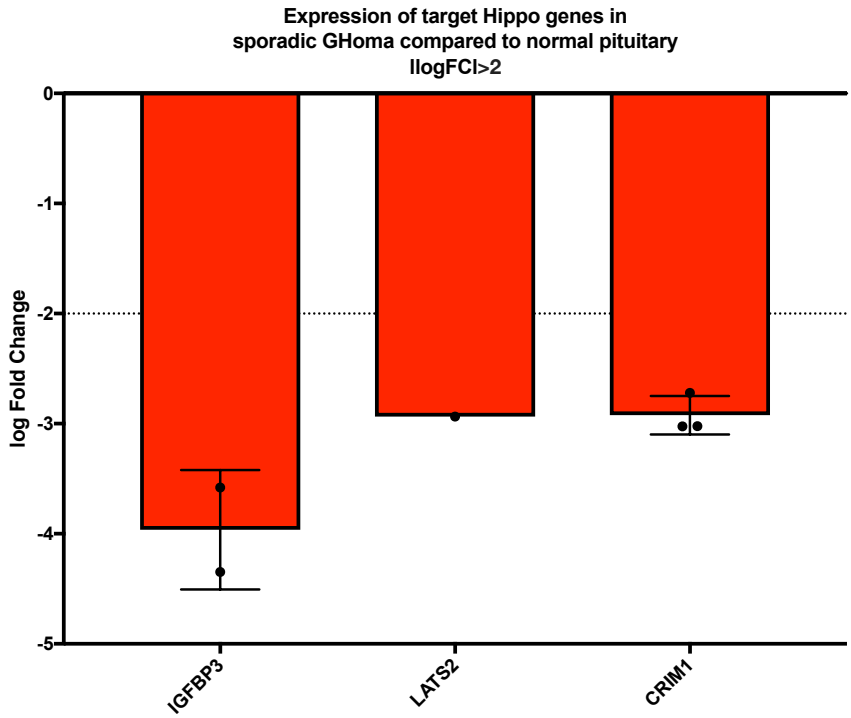


Figure 32. Differentially expressed Hippo target genes in sporadic GHomas compared to normal pituitary. Dotted line marks absolute log fold change criteria. Red: expression down

<u>*AIP* mutation-positive GHoma vs normal pituitary</u>

Out of the core genes, *WWC1* and *YAP1* are downregulated in *AIP* mutation-positive GHomas when compared to normal pituitary tissue (absolute logfold change >2, adjusted p≤0.05) (Figure 33). From the target genes, *IGFBP3* was found to be downregulated, while *TGFB2* upregulated in *AIP* mutation-positive tumours when compared to normal pituitary (absolute logfold change >2, adjusted p≤0.05) (Figure 34).

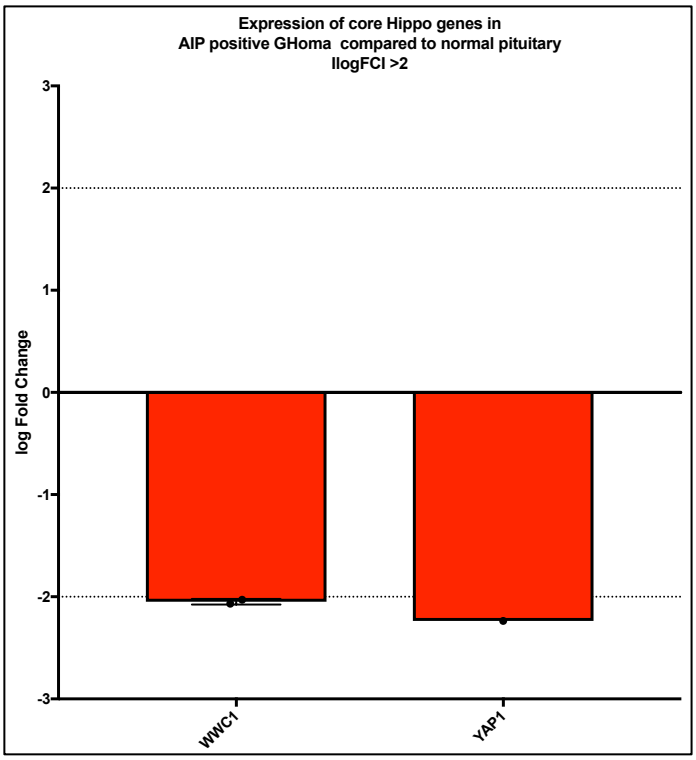


Figure 33. Differentially expressed core Hippo genes in AIP mutation-positive GHomas compared to normal pituitary tissue. Dotted line marks absolute log fold change criteria. Red: expression down

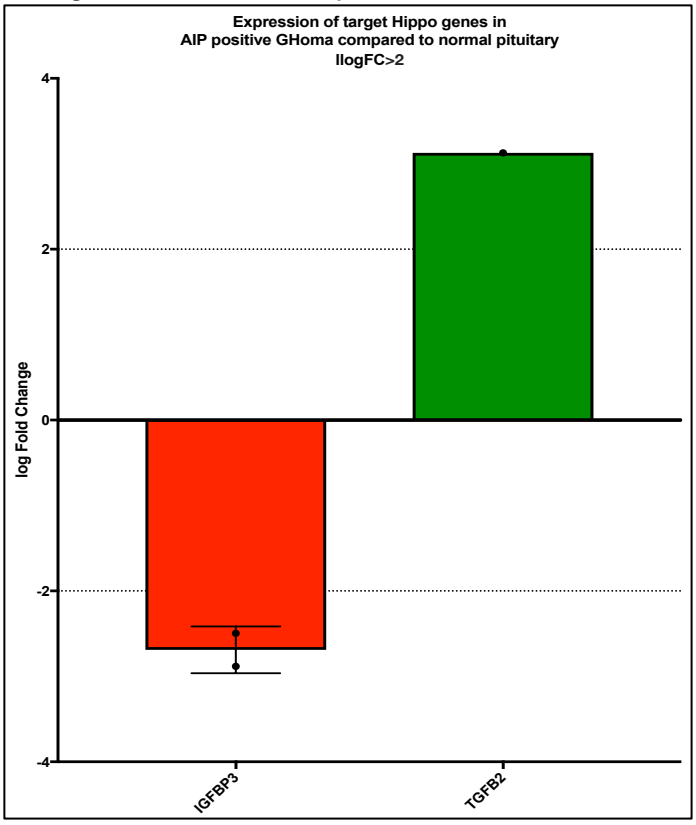


Figure 34. Differentially expressed Hippo target genes in AIP mutation-positive GHomas compared to normal pituitary. Dotted line marks absolute log fold change criteria. Green: expression up, red: expression down

***AIP* mutation-positive GHoma vs sporadic GHoma**

None of the analysed core and target Hippo genes were found to be differentially expressed at a statistically significant level in *AIP* mutation-positive GHomas compared to sporadic GHomas.

***AIP* mutation-negative GHoma vs sporadic GHoma**

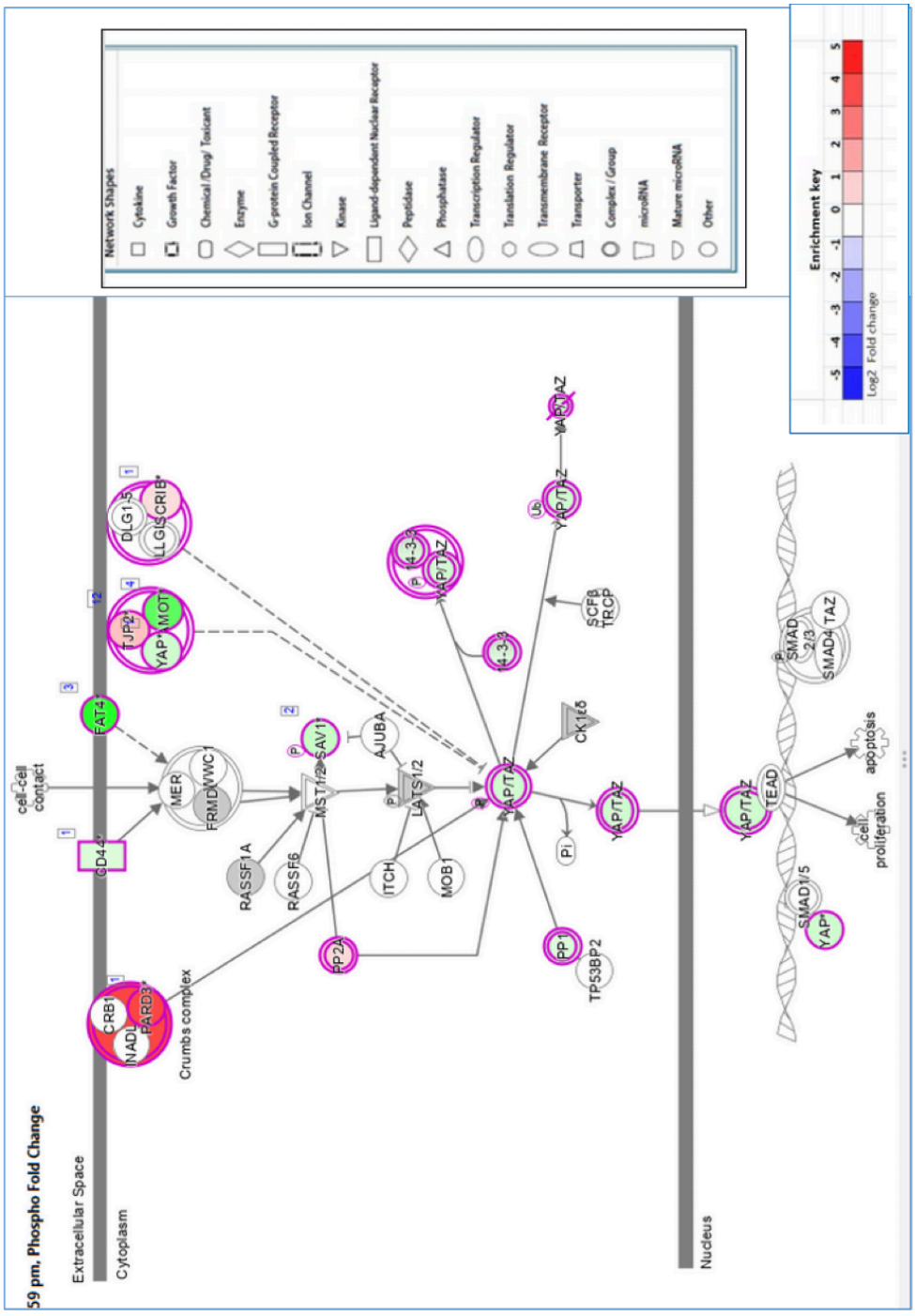
None of the core or target Hippo genes were detected to be differentially expressed in this comparative dataset.

***AIP* mutation-positive GHoma vs *AIP* mutation-negative GHoma**

None of the core Hippo genes were differentially expressed, however from the target genes, *TGFB2* was found to be upregulated in *AIP* mutation-positive GHomas compared to the *AIP* mutation-negative GHomas (logFC 2.82, adjusted p=0.01).

4. Global phosphorylation analysis of Aip-KO mouse embryonic fibroblast cells by mass spectrometry and IPA analysis

Changes in phosphorylation levels represent activities of signalling pathways in cellular protein networks. Using p-value < 0.05, 2,156 phosphopeptides were identified. Ingenuity Pathway Analysis (IPA) on this dataset revealed the Hippo pathway as one of the significantly deregulated pathways (*Figure 35*). When comparing *Aip-KO* mouse embryonic fibroblasts cells to WT mouse embryonic fibroblast cells several upstream and downstream targets of the Hippo pathway show deregulation. From the pathway components identified in the previous experiments, LATS2, SAV1 and YAP 1 show hypophosphorylation. (This dataset was provided to me by Dr Sayka Barry, who assisted with the analysis as well.)



Protein name	Peptide/Phosphorylation site	MEFS Alp-KO Vs MEFS WT fold change
Angiomotin	Amot(M1088):Amot(S1083);	
Angiomotin	Amot(M1088):Amot(T1084);	
Angiomotin	Amot(S607);	
Angiomotin	Amot(S602);	
Angiomotin-like protein 2	Amot(S235);	
CD44 antigen	Cd44(M745):Cd44(M761):Cd44(S742);	
CD44 antigen	Cd44(S742);	
Protocadherin Fat 4	Fat4(T2760-2774_no_mod);	
Protocadherin Fat 4	Fat4(S336-354_no_mod);	
Protocadherin Fat 4	Fat4(S4873);	
Serine/threonine-protein kinase LATS2	Lats2(S72-581_no_mod);	
MOB kinase activator 1A	Mob1b(T35):Mob1a(T35);	
Partitioning defective 3 homolog	Pard3(S378):Pard3(S383);	
Partitioning defective 3 homolog	Pard3(S954);	
Protein phosphatase 1 regulatory subunit	Ppp1r12a(S852);	
Protein phosphatase 1 regulatory subunit	Ppp1r12a(S902);	
Protein phosphatase 1 regulatory subunit	Ppp1r12a(T305);	
Protein phosphatase 1 regulatory subunit	Ppp1r12a(Y900);	
Serine/threonine-protein phosphatase	Ppp2ca(S331-342_no_mod);	
Protein salvador homolog 1	Sav1(S137);	
Protein salvador homolog 1	Sav1(Y134);	
Protein scribble homolog	Scrib(S1206):Scrib(S1209):Scrib(S1211);	
Protein scribble homolog	Scrib(S503):Scrib(S506);	
Tight junction protein ZO-1	Tjp1(M888):Tjp1(S885);	
Tight junction protein ZO-1	Tjp1(M888):Tjp1(S885);	
Tight junction protein ZO-1	Tjp1(M888):Tjp1(Y895);	
Tight junction protein ZO-1	Tjp1(M888):Tjp1(Y895);	
Tight junction protein ZO-1	Tjp1(S912);	
Tight junction protein ZO-1	Tjp1(T868):Tjp1(Y863);	
Tight junction protein ZO-2	Tjp2(1131-1152_no_mod);	
Tight junction protein ZO-2	Tjp2(M883):Tjp2(M889):Tjp2(S898);	
Tight junction protein ZO-2	Tjp2(S1002);	
Tight junction protein ZO-2	Tjp2(S139):Tjp2(S140);	
Tight junction protein ZO-2	Tjp2(S145);	
Tight junction protein ZO-2	Tjp2(S895);	
Tight junction protein ZO-2	Tjp2(S898):Tjp2(Y893);	
Tight junction protein ZO-2	Tjp2(S992);	
Tight junction protein ZO-2	Tjp2(T1130):Tjp2(Y1134);	
Tight junction protein ZO-2	Tjp2(T442);	
Tight junction protein ZO-2	Tjp2(T446);	
Tight junction protein ZO-2	Tjp2(T882):Tjp2(S885);	
Tight junction protein ZO-2	Tjp2(Y1127):Tjp2(Y1134);	
Transcriptional coactivator YAP1	Yap1(R91):Yap1(S94);	
Transcriptional coactivator YAP1	Yap1(R91):Yap1(S94);	
Transcriptional coactivator YAP1	Yap1(S112);	
Transcriptional coactivator YAP1	Yap1(S94):Yap1(T104);	
14-3-3 protein beta/alpha	Ywhab(S2-K3_no_mod);	
14-3-3 protein epsilon	Ywhae(M33);	
14-3-3 protein epsilon	Ywhae(T208);	

Figure 35. Ingenuity Pathway Analysis (IPA) on Aip-KO mouse embryonic fibroblast cells by mass spectrometry.
A global phosphorylation analysis of Aip-KO mouse embryonic fibroblast cells (MEFs) shows key Hippo pathway elements to be significantly deregulated. Entries are shown from blue to red to denote decreased or increased, respectively (see enrichment key for heatmap). Asterisks denote the p-value of the differences (by t-test of log transformed data assuming unequal variances), p-value < 0.05 was used.
On the heatmap, red represents hyperphosphorylation, meaning phosphorylation of a given protein at multiple phosphorylation sites leading to altered function, while blue means hypophosphorylation (phosphorylated to a less than normal extent, or less than fully).

5. Exome and whole genome sequencing of pituitary adenoma patients

The samples used for this analysis are in Table 7 and Table 8, detailed in the Methods section of this chapter.

I found 11 core and 15 target genes to have variants in the whole exome sequencing data pool, but not in the whole genome sequencing data (Table 9).

Core Hippo gene	Variant in genome seq.		Target Hippo gene	Variant in genome seq.
FRMD6	no		F3	yes
WWC1	yes		ASAP1	yes
YAP1	yes		GADD45A	no
MST1/STK4	yes		ARHGEF17	yes
TAOK1	no		IGFBP3	yes
TAOK2	yes		CRIM1	yes
TAOK3	yes		TGFB2	no
NF2	no		RBMS3	no
SAV1	no		CYR61	no
STK3/MST2	no		CTGF	no
MOB1A	yes		AMOTL2	yes
MOB1B	no		PTPN14	yes
LATS2	yes		DOCK5	yes
WWTR1/TAZ	yes		NT5E	yes
TEAD1	no		FJX1	yes
TEAD3	yes		AXL	yes
DCHS1	yes		MYOF	yes
TEAD4	no		FOXF2	yes
LATS1	yes		CCDC80	yes
			NUAK	yes

Table 9. Core and target Hippo gene variants in the whole exome sequencing of pituitary adenoma patients

The data was then manually further filtered for keeping only heterozygote variants with depth of coverage (DP) criteria set at 5 or above.

Core genes

Considering variants that segregate with the disease within the familial cases and have a minor allele frequency under 1%, the following genes were identified: *WWC1, YAP, TAZ/* All of the variants of *WWC1* were single nucleotid variants (Table 10). The segregating *WWC1* variants were detected in families with NFPA and prolactinoma patients. *WWC1* also had variants in sporadic prolactinoma cases.

YAP was found to have only one rare (MAF under 1%) heterozygote variant (nonsynonymous single nucleotid variant) segregating within a family (3 members: F151M1 – NFPA patient, F151M2 – NFPA patient and F151M3 – obligate carrier). No other *YAP* variant was found in any other family, nor was this variant detected in other sporadic pituitary adenoma patients or families (*Table 10*).

WWTR1 (coding for TAZ) was the only gene to have a heterozygote variant (MAF under 1%) resulting in a stop-gain mutation. This variant was found in both sporadic prolactinoma patients (Sp269, Sp534, Sp634, Sp158, Sp422, Sp448) and familial prolactinoma patients (F243M3, F86M9). The same variant was detected in other tumour types as well: in two familial acromegaly patients from two separate families (F10M5, F20M1) and segregating in two members of a familial NFPA family (F151M1 and M2)(Table 10).

Gene	Chromosome	Variant	Patient ID	Patient's disease
WWC1	5	nonsynonymous SNV	Sp269	Prolactinoma
			Sp340M1	Prolactinoma
			Sp634	Prolactinoma
			F239M1	familial isolated pituitary adenoma - Prolactinoma
			F239M2	familial isolated pituitary adenoma -Prolactinoma
			F243M1	familial isolated pituitary adenoma - Prolactinoma
			F277M2	familial isolated pituitary adenoma- Prolactinoma
			F277M3	familial isolated pituitary adenoma- Prolactinoma
			Sp108	Prolactinoma
			Sp474	prolactinoma
			F86M3	familial isolated pituitary adenoma - Prolactinoma
			F86M5	familial isolated pituitary adenoma - Prolactinoma
			F86M6	familial isolated pituitary adenoma - Prolactinoma
			F86M8	familial isolated pituitary adenoma - Prolactinoma
			F171M1	familial isolated pituitary adenoma- NFPA
			F171M2	familial isolated pituitary adenoma- NFPA
			F171M3	Unaffected in NFPA family (Obligate carrier)
			F171M4	Unaffected in NFPA family
			F10M5	familial isolated pituitary adenoma - acromegaly
			F28M3	familial isolated pituitary adenoma - acromegaly
			Sp95M2	unaffected
			Sp95M3	unaffected
		nonsynonymous SNV	Sp474	prolactinoma
			F191M1	familial isolated pituitary adenoma - Prolactinoma
			F191M2	familial isolated pituitary adenoma - Prolactinoma
		nonsynonymous SNV	Sp638	prolactinoma
			F253M3	prolactinoma
			F277M1	familial isolated pituitary adenoma - Prolactinoma
			F277M2	familial isolated pituitary adenoma - Prolactinoma
			F277M3	familial isolated pituitary adenoma - Prolactinoma
			F86M6	familial isolated pituitary adenoma - Prolactinoma
			F86M9	familial isolated pituitary adenoma - Prolactinoma
			Sp95M1	Pituitary hyperplasia
			Sp95M3	unaffected
		nonsynonymous SNV	Sp638	prolactinoma
			F253M3	familial isolated pituitary adenoma - Prolactinoma
			F277M1	familial isolated pituitary adenoma - Prolactinoma
			F277M2	familial isolated pituitary adenoma - Prolactinoma
			F277M3	familial isolated pituitary adenoma - Prolactinoma
			F86M6	familial isolated pituitary adenoma - Prolactinoma
			F86M9	familial isolated pituitary adenoma - Prolactinoma
			Sp95M1	tuitary hyperplasia (Child is affected with pituitary hyperplasia)
			Sp95M3	unaffected
WWTR1	3	stopgain	Sp269	Prolactinoma
			Sp534	Prolactinoma
			Sp634	Prolactinoma
			F243M3	familial isolated pituitary adenoma - Prolactinoma
			Sp158	prolactinoma
			Sp422	prolactinoma
			Sp448	prolactinoma
			F86M9	familial isolated pituitary adenoma - Prolactinoma
			F151M1	familial isolated pituitary adenoma- NFPA
			F151M2	familial isolated pituitary adenoma- NFPA
			F10M5	familial isolated pituitary adenoma - acromegaly
			F20M1	familial isolated pituitary adenoma - acromegaly
			F199M1	familial isolated pituitary adenoma - Prolactinoma
			F199M2	familial isolated pituitary adenoma - Prolactinoma
YAP1	11	nonsynonymous SNV	F151M1	familial isolated pituitary adenoma- NFPA
			F151M3	Unaffected in NFPA family (Obligate carrier)
			F151M2	familial isolated pituitary adenoma- NFPA

Table 10. Core hippo genes with variants that segregate (minimum two members/family) and have a minor allele frequency under 1%, showing all detected

variants and the familial and sporadic patients they were detected in. SNV: single nucleotid variant

Target genes

Considering variants that segregate and have a minor allele frequency under 1%, the following genes were identified: *ARHGEF17, IGFBP3, AXL, MYOF, FOXF2, CCDC80*. All of these genes were found to have variants causing nonsynonymous SNVs in sporadic cases (Table 11).

A variant in *ARGHEF17* segregating in four members of a prolactinoma family (F86) and another variant of the gene in two members of another prolactinoma family (F277). A third variant of the gene was found segregating in two members of a prolactinoma family (Sp95M1 and M3) (Table 11).

A nonsynonymous SNV of *IGFBP3* was detected in four members of a prolactinoma family (F243) (Table 11). The same family (F243) has an *AXL* nonsynonymous SNV variant in two of its members (Table 11). Two separate *MYOF* nonsynonymous SNV causing variants were found segregating in two different families: two members of a prolactinoma family (F199) and two members of an ACTH family (F147) (Table 11).

FOXF2 has a segregating variant in three members of F86, a prolactinoma family, which family also has a segregating *ARGHEF17* variant. *CCDC80* has two segregating variants (SNVs), F243 has three members with one of the variants and F277 has three members with the other variant (both prolactinoma families) (Table 11).

Gene	Chromosome	Variant	Patient ID	Patient's disease
ARHGEF17	11	**nonsynonymous SNV**	**Sp340M1**	Prolactinoma
			F243M1	familial isolated pituitary adenoma - Prolactinoma
			F277M1	familial isolated pituitary adenoma - Prolactinoma
			F277M3	familial isolated pituitary adenoma - Prolactinoma
		nonsynonymous SNV	**Sp95M1**	Prolactinoma
			Sp95M3	Prolactinoma
		nonsynonymous SNV	**F86M3**	familial isolated pituitary adenoma - Prolactinoma
			F86M6	familial isolated pituitary adenoma - Prolactinoma
			F86M9	familial isolated pituitary adenoma - Prolactinoma
			F86M4	familial isolated pituitary adenoma - Prolactinoma
IGFBP3	7	**nonsynonymous SNV**	**F243M1**	familial isolated pituitary adenoma - Prolactinoma
			F243M2	familial isolated pituitary adenoma - Prolactinoma
			F243M6	familial isolated pituitary adenoma - Prolactinoma
			F243M7	familial isolated pituitary adenoma - Prolactinoma
			F171M1	familial isolated pituitary adenoma - NFPA
AXL	19	**nonsynonymous SNV**	**F243M1**	familial isolated pituitary adenoma - Prolactinoma
			F243M7	familial isolated pituitary adenoma - Prolactinoma
MYOF	10	**nonsynonymous SNV**	**F147M1**	familial isolated pituitary adenoma - ACTH
			F147M2	familial isolated pituitary adenoma - ACTH
FOXF2	6	**nonsynonymous SNV**	**F86M3**	familial isolated pituitary adenoma - Prolactinoma
			F86M9	familial isolated pituitary adenoma - Prolactinoma
			F86M4	familial isolated pituitary adenoma - Prolactinoma
			F20M2	familial isolated pituitary adenoma - acromegaly
CCDC80	3	**nonsynonymous SNV**	**F277M1**	familial isolated pituitary adenoma - Prolactinoma
			F277M2	familial isolated pituitary adenoma - Prolactinoma
			F277M3	familial isolated pituitary adenoma - Prolactinoma
			F151M2	familial isolated pituitary adenoma - NFPA
			F31M1	familial isolated pituitary adenoma - Prolactinoma
		nonsynonymous SNV	**Sp340M1**	Prolactinoma
			F243M3	familial isolated pituitary adenoma - Prolactinoma
			F243M6	familial isolated pituitary adenoma - Prolactinoma
			F243M9	familial isolated pituitary adenoma - Prolactinoma

Table 11. Target hippo genes with variants that segregate (minimum two members/family) and have a minor allele frequency under 1%, showing all detected variants and the familial and sporadic patients they were detected in. SNV: single nucleotide variant

Discussion

Deregulation of the mammalian Hippo signalling components has been implicated in the formation of both benign and malignant tumours, murine studies disturbing the Hippo pathway have shown hyperplasia and tumorigenesis in various tissues (263-265). The Hippo pathway plays an essential part in wide range of human cancers (266) (e.g. lung, breast, colorectal, liver, gastric, pancreatic, renal cancer, retinoblastoma and leukemia (215, 267)), Hippo pathway-targeted drugs have been identified to improve cancer mortality and morbidity (268), components of the pathway are considered to be promising anticancer targets.

F3 is upregulated *in vitro* in the *Aip*-knockdown GH3 cells when compared to non-targeted control cells. *F3*, as a receptor of factor VII/VIIa, plays an important role in coagulation and its high expression has been reported in human pituitary adenomas, significantly correlating with the formation of cysts and haematomas in these tumours (269). This is correlating with the fact that patients with *AIP* mutation-positive tumours are more prone to apoplexy (3, 270) than the *AIP* mutation-negative ones. We have seen downregulation of *F3* in human NFPA tumours when compared to normal pituitary. In our murine model, *F3* is downregulated in the 15-month-old *Aip*-KO when compared 3-week-old *Aip*-KO, but equally when comparing the wildtypes of the same time points. One possibility to explain these findings could be that F3 may play an important role in the pituitary during embryogenesis, but less so at later stages. It is an interesting finding that needs validation at the protein level as well as further analysis on larger samples.

*Frmd6 (*a tumour suppressor at upstream of the Hippo signalling pathway) in this project was found to have a lower expression in the *Aip*-KO mice at 15 months when compared to 3-week-old *Aip*-KO mice, which could be interesting given that the animals by 15 months of age develop pituitary tumours. It was also found to be downregulated in sporadic NFPAs when compared to normal pituitary, but not expressed differentially when comparing human tumours of different *AIP* mutation status. In the literature, FRMD6 was found to serve as a tumour suppressor of human breast cancer cells (271) and *FRMD6* knockdown induces the epithelial–mesenchymal transition (EMT) in mammary epithelial cells (272). This corresponds with our research group's human pituitary tumour transcriptome data, the EMT pathway being one of the most significantly altered pathways in *AIP* mutation-positive tumours (262).

The pathway's main kinases, *Yap* and *Taz* were not found to be differentially expressed in the RNAseq analysis on pituitary specific *Aip*-knockout (*Aip*-KO) mice. In the human pituitary tumour gene expression data, *YAP* is downregulated in *AIP* mutation-positive GHomas when compared to the normal pituitary tissue, which is consistent with Xekouki *et al.*, i.e. the Hippo kinases may have a role in promoting a hormone-secreting tumour type over a non-functioning adenoma. In our data, it does appear to be downregulated in NFPA, but we only analysed NFPA versus normal pituitary, the more relevant analysis here would be NFPA versus other pituitary tumour types. When compared to normal pituitary, YAP is downregulated in both sporadic and *AIP*-positive GHomas. When *AIP* positive GHomas were compared to sporadic GHomas and to *AIP* negative GHomas, we did not detect altered expression. Important to note, that YAP can be both oncogenic and tumour

suppressive (by either triggering apoptosis or inhibiting Wnt signalling (273). *YAP* was found to have only one rare heterozygote variant (nonsynonymous single nucleotid variant) segregating within a family (3 members: F151M1 – NFPA patient, F151M2 – NFPA patient and F151M3 – obligate carrier) in our exome sequencing data.

Lats1 (the main kinase upstream of *Yap/Taz* in murine pituitary organogenesis) was not differentially expressed in the *Aip*-KO mice, despite the fact that loss of *Lats1* has been shown to lead to tumour formation in the anterior and intermediate lobe in murine models (256). Loss of *Lats1* and *Lats2* also results in significant tissue overgrowth during gestation, suggesting a growth restricting role of the Hippo pathway during pituitary development (255). I found *LATS1* to be downregulated in NFPA and sporadic GHoma when compared to normal pituitary, which is logical given the role detailed above, however it was not picked up to have altered expression in human tumours with different *AIP* mutation status.

When comparing *AIP* mutation-positive to *AIP* mutation-negative GH secreting human tumours, I only detected *TGFB2* to be upregulated. High *TGFB2* expression has been observed in various malignancies, and recently, it was one of the genes identified to be upregulated in *AIP* mutation-positive somatotroph adenomas in a different study from our research group (262). No Hippo components were shown to have altered expression when comparing *AIP* mutation-positive GHomas to sporadic GHomas.

WWC1 (also known as *KIBRA*) is an established tumour suppressor and one of the regulators of the Hippo pathway and has been linked to several human malignancies

(leukemia, prostate cancer, breast cancer) (274). It activates LATS1/2 kinases by stimulating their phosphorylation, while also promoting YAP phosphorylation (275). *WWC1* nonsynonomous SNV variants were detected in prolactinoma and NFPA families as well as sporadic prolactinoma patients in our data. We also found *WWC1* downregulated in *AIP* mutation-positive GHomas when compared to normal pituitary tissue, also downregulated in NFPA compared to normal pituitary tissue.

One of the main kinases *WWTR1 (TAZ)* was the only gene to have a heterozygote variant resulting in a stop-gain mutation. This variant was found in both sporadic and familial prolactinoma patients and familial NFPA patients. We also found *TAZ* to be downregulated in sporadic GHoma when compared to normal pituitary tissue.

As one of the main pathway transcriptional coactivators, it plays a key role in transcription activation via the TEADs. A significant decrease of YAP/TAZ in human hormone-secreting pituitary tumour tissue when compared to normal pituitary and NFPA suggest that the Hippo kinases may have a role in promoting a hormone-secreting tumour type (259), and our data corresponds with these findings, however need to be validated further.

ARHGEF17 (Rho Guanine Nucleotide Exchange Factor 17) is a Hippo signalling regulator, is a Rho GTPase with a role in maintenance of actin cytoskeleton organization and focal adhesion (276). In human tumour samples and cancer cell lines the knockdown of *ARHGEF17* is in positive correlation with *YAP's* transcriptional activity. I found that *ARHGEF17* had 3 variants, each of them segregating in prolactinoma families.

Insulin-like growth factor binding protein-3 (IGFBP-3) is a p53 tumor suppressor-regulated protein. Loss of IGFBP-3 expression has been associated with a variety of cancers (notably lung, colon, ovarian) promoting tumorigenesis and cancer

progression as well as resistance to radiotherapy and chemotherapy (277). I found a nonsynonymous SNV of *IGFBP3* in four members of a prolactinoma family. *IGFBP3* was downregulated in *AIP* mutation-positive GHomas compared to normal pituitary. AXL (AXL receptor tyrosine kinase) is receptor tyrosine kinase that is a key downstream target driving an oncogenic role. It has been suggested, that AXL directly regulates YAP activity, by phosphorylating it (278). We found a variant (SNV) of this gene segregating in one of our prolactinoma families.

Two separate *MYOF (*myoferlin*)* nonsynonymous SNV causing variants were found segregating in two different families: two members of a prolactinoma family and two members of an ACTH family. MYOF expression is heavily regulated by YAP and TAZ, as they are required for the de-repression of MYOF (279). High expression of myoferlin has been described in breast cancer (280), anti-myoferlin molecules currently being investigated as cancer drugs.

FOXF2 (Forkhead box 2) is a mesenchymal regulator, its underexpression is associated with poor prognosis of patients with breast cancer (281), it is a potential tumour suppressor in prostate cancer as well (282). I found a variant of *FOXF2* segregating in a prolactinoma family. *CCDC80* (coiled-coil domain containing 80) is a direct target of YAP/TAZ. It has two segregating variants (SNVs) in two prolactinoma families. CCDC80 expression is selectively induced by LATS1/2 deletion *in vitro*, loss of CCDC80 resulting in inhibiting the growth inhibitory effect of LATS1/2 loss *in vitro* (283).

In summary, in the exome and whole genome sequencing of pituitary adenoma patients, which included a cohort of *AIP* mutation-negative FIPA cases, variants of several key components of the Hippo pathway (both up- and downstream) show

segregation within families. The majority of segregating variants are in prolactinoma and NFPA families.

In conclusion, the above findings imply a role for the alteration of the Hippo pathway in pituitary tumorigenesis, supporting our initial hypothesis. We do see dysregulation of key pathway components both *in vitro* and *in vivo.*

It is challenging to comment on whether the pathway could play a significant role in AIP-mediated tumorigenesis solely based on this data, but it strongly suggests its role in pituitary tumorigenesis is worth further exploring.

Chapter VI

General discussion

While pituitary tumours are usually benign, their associated high morbidity makes them a clinically important target. Pituitary adenomas are the only identified tumours being associated with mutations in the *AIP* gene to date (20% of FIPA patients harbour an *AIP* mutation), dominantly growth hormone secreting adenomas with mutations described in all other adenoma types as well. The ongoing efforts towards elucidating the role of the AIP protein in pituitary organo- and tumorigenesis is supported by the desire to treat this otherwise challenging patient group (larger, more aggressive and less therapy resistant tumours) with more tailored and targeted therapeutical methods.

This PhD thesis investigates the role of AIP in pituitary tumorigenesis from three different perspectives: clinical, experimental and bioinformatical.

The clinical study presented as a part of this thesis was performed in a pituitary tertiary referral centre, aiming to determine the prevalence of pituitary adenomas via prospective and retrospective analytic methods. It had a special focus on determining family history in these patients hoping to detect more, previously not recognised FIPA families. The potentially incomplete recollection, information provided by patients regarding their family history was a recognised limitation of this study, however hard to overcome. To improve the quality of self-reporting, every patient involved in the prospective analysis was talked to at clinics in person by either myself or the consultant endocrinologist and we talked through the questionnaire instead of them filling it out without a doctor's guidance. Every patient was asked the same set of questions from the questionnaire and was given ample

time to consider their answer to try make the conditions as homogenous as possible. The tertiary referral status of the hospital where the study was carried out could be considered as a potential bias. Bias can occur at any phase of research and is recognised to be nearly always present (to some level) in published clinical studies. While evidence-based medicine's key element is a critical approach to potential bias, it is of equal importance to cautiously judge whether it significantly influences the core conclusions of a study. One of the observations of this analysis was that careful history taking increases the chance of detecting family history in PA patients. When discussing this, the possibility of selection / channelling bias must be addressed. Due to the low overall prevalence of pituitary adenomas in the general population and the fact that they often go undiagnosed, without making the study multi-centre and involve numerous non-tertiary institutions, it would have been impossible to enrol a reasonable number of patients from anywhere but a tertiary referral centre. A much larger scale, multi-centre analysis involving multiple non-tertiary units was way beyond the scope of this PhD project but would be something of immense benefit. This potential bias, I believe, does not significantly affect the core conclusions of the study as it corresponds with what we observe in clinical studies and guidelines when it comes to the importance of thorough history taking as well as with most clinicians' everyday experience (284-286).

Screening patients and their family members (affected and unaffected) for PA related genetic mutations in order to identify carriers and undiagnosed PA patients helps early diagnosis (with better treatment outcome) and provides better monitoring. Admittedly, this could induce stress in these patients and their families, but in our clinical experience, the consenting families have a positive approach to screening and consider it to be overall more beneficial than the anxiety it may trigger (287,

288). Prospectively diagnosed patients with AIP mutation-positive pituitary tumours show better clinical outcomes and more favourable response to therapy when compared to patients diagnosed at time of clinical symptoms present (154), which further demonstrates the benefit of genetic screening in this patient group.

Obtaining human pituitary samples for research is highly challenging, many of these limiting factors (low prevalence, commonly medical therapy without surgical intervention, small tumour sample that is shared with pathology) are near impossible to overcome.

A well-established murine model that would give previously unavailable insight into AIP-related pituitary tumour formation had been lacking prior to this project. Earlier mouse models of loss of Aip faced challenges such as in utero lethality (global homozygous *Aip*-knockout model (198, 199)) due to severe cardiac malformations. This difficulty was overcome by a global heterozygous knockout model however these animals were not pituitary tissue specific but went on to develop pituitary adenomas in adulthood (full penetrance by age 15 months) (199). Probably the most important achievement of this PhD project was generating a viable mouse model of pituitary-specific biallelic loss of Aip (*$Aip^{Flox/Flox}$; $Hesx1^{Cre/+}$)* giving a previously unavailable insight into embryonic changes due to the loss of the Aip protein. This thesis was the first to describe *in utero* changes due to homozygous loss of *Aip*, both phenotypical changes to the pituitary (enlarged anterior lobe by e17.5) and changes in hormone expression at the terminal differentiation stage (decrease in growth hormone and prolactin producing cells). Intriguingly this change happens without any observed change to the Pit1 lineage and is of clinical importance as we know that in humans patients with *AIP* mutations usually present with childhood onset adenomas,

which are most commonly growth hormone and/or prolactin secreting (3). Postnatally the *Aip$^{Flox/Flox}$; Hesx1$^{Cre/+}$* mice were larger with higher circulating IGF-1 levels.

The mouse colony established within this PhD project has since been used by other members of our research group for further studies on postnatal tumour formation and effects of therapeutic agents on pituitary tumours. These further studies have shown that the AipFlox/Flox;Hesx1Cre/+ murine model in fact mirrors the human disease characteristics (large body size, enlarged internal organs – most prominently the heart -, high circulating IGF-1 levels). At 15 months postnatally the functional pituitary tumour penetrance is 85%.

Using the inducible version of the model (AipFlox/Flox;Sox2CreERT2/+) generated within this PhD project (it deletes Aip upon administration of tamoxifen exclusively in the Sox2-expressing cells, forming the pituitary stem-cell niche) they found no increased body size or IGF-1 rise up to 18 months when induced at 3 or 8 weeks postnatally, however 50% of the animals show ectopic PIT-1, growth hormone and prolactin-expressing cells in the intermediate lobe of the pituitary gland (both in the AipFlox/Flox;Hesx1Cre/+ embryos and tamoxifen-injected AipFlox/Flox;Sox2CreERT2/+ adults). When tamoxifen is injected into pregnant mice, it also causes the above embryonic intermediate lobe abnormalities, further strengthening my original findings in the AipFlox/Flox;Hesx1Cre/+ embryos. Further studies confirmed that some of the tumours observed in the adult animals arise from these altered cell groups of the intermediate lobe. Data from our AipFlox/Flox;Hesx1Cre/+ model shows cardiac abnormalities, therefore proving to be an effective model to study cardiac disease in acromegaly*. (A. Mistry et al, BES 2021 conference paper & unpublished data)*

Available published data supports that the Hippo cascade is important in pituitary organogenesis and homeostasis, potentially playing an important part in pituitary tumorigenesis (254-256). Until the submission of this thesis, there was no published data exploring the role of Hippo signalling in *AIP*-mediated pituitary tumorigenesis. Bioinformatic analysis performed found both *in vitro* (AIP-silenced rat pituitary somatomammotroph GH3 cells) and *in vivo* (pituitary specific AIP-knockout mice generated within this project, human samples from patients with AIP mutation-positive and AIP mutation-negative familial and sporadic pituitary adenomas) evidence for deregulation of key upstream and downstream Hippo pathway components. Additionally, analysis of exome and whole genome sequencing data from PA patients showed variants of key components of the Hippo pathway (both up- and downstream) segregating within families. Global phosphorylation analysis of pituitary specific AIP-knockout mouse embryonic fibroblasts cells (MEFs) by mass spectrometry strengthens the previously discussed findings as Ingenuity Pathway Analysis (IPA) on this dataset revealed the Hippo pathway as one of the significantly deregulated pathways, which shows these changes to be present at the protein level.

It is admittedly premature to conclude at this stage that the Hippo pathway would play a significant role in AIP-mediated tumorigenesis, but this data provides a strong enough basis for further research.

In conclusion, this PhD project has investigated the role and significance of AIP in pituitary tumorigenesis from the clinical, molecular and bioinformatical aspect while generating and utilising a novel mouse model that has been shown to be a valuable tool in AIP-related pituitary research since. Hopefully this murine model will

contribute towards the development of more targeted and individualised therapy for these patients as well as continue to help explore the specific role of AIP in the embryonic and postnatal pituitary gland.

Bibliography

1. Daly AF, Rixhon M, Adam C, Dempegioti A, Tichomirowa MA, Beckers A. High prevalence of pituitary adenomas: a cross-sectional study in the province of Liege, Belgium. J Clin Endocrinol Metab. 2006;91(12):4769-75.
2. Fernandez A, Karavitaki N, Wass JA. Prevalence of pituitary adenomas: a community-based, cross-sectional study in Banbury (Oxfordshire, UK). Clin Endocrinol (Oxf). 2010;72(3):377-82.
3. Hernandez-Ramirez LC, Gabrovska P, Denes J, Stals K, Trivellin G, Tilley D, et al. Landscape of Familial Isolated and Young-Onset Pituitary Adenomas: Prospective Diagnosis in AIP Mutation Carriers. J Clin Endocrinol Metab. 2015;100(9):E1242-54.
4. Leontiou CA, Gueorguiev M, van der Spuy J, Quinton R, Lolli F, Hassan S, et al. The role of the aryl hydrocarbon receptor-interacting protein gene in familial and sporadic pituitary adenomas. J Clin Endocrinol Metab. 2008;93(6):2390-401.
5. Trivellin G, Butz H, Delhove J, Igreja S, Chahal HS, Zivkovic V, et al. MicroRNA miR-107 is overexpressed in pituitary adenomas and inhibits the expression of aryl hydrocarbon receptor-interacting protein in vitro. Am J Physiol Endocrinol Metab. 2012;303(6):E708-19.
6. Korbonits M, Storr H, Kumar AV. Familial pituitary adenomas - who should be tested for AIP mutations? Clin Endocrinol (Oxf). 2012;77(3):351-6.
7. Amar AP, Weiss MH. Pituitary anatomy and physiology. Neurosurg Clin N Am. 2003;14(1):11-23, v.
8. Jedidi H, Jedidi Z, Beckers A. [The history of the pituitary gland: evolution of mental representation and concepts over time]. Rev Med Liege. 2014;69(2):89-93.
9. Toni R. Ancient views on the hypothalamic-pituitary-thyroid axis: an historical and epistemological perspective. Pituitary. 2000;3(2):83-95.
10. Atkinson AB. From then to now: lessons from developments in our understanding of the pituitary gland. Ulster Med J. 2010;79(2):89-94.
11. Donnelly DE, Morrison PJ. Hereditary Gigantism-the biblical giant Goliath and his brothers. Ulster Med J. 2014;83(2):86-8.
12. Kaplan SA. The pituitary gland: a brief history. Pituitary. 2007;10(4):323-5.
13. Asa SL. Pituitary Histopathology in Man: Normal and Abnormal. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA)2000.
14. Larkin S, Ansorge O. Development and Microscopic Anatomy of the Pituitary Gland. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA)2000.
15. Corradi PF, Corradi RB, Greene LW. Physiology of the Hypothalamic Pituitary Gonadal Axis in the Male. Urol Clin North Am. 2016;43(2):151-62.
16. Karaca Z, Laway BA, Dokmetas HS, Atmaca H, Kelestimur F. Sheehan syndrome. Nat Rev Dis Primers. 2016;2:16092.
17. McNicol AM. A study of intermediate lobe differentiation in the human pituitary gland. J Pathol. 1986;150(3):169-73.
18. Vankelecom H. Non-hormonal cell types in the pituitary candidating for stem cell. Semin Cell Dev Biol. 2007;18(4):559-70.
19. Rizzoti K. Genetic regulation of murine pituitary development. J Mol Endocrinol. 2015;54(2):R55-73.
20. Patthey C, Schlosser G, Shimeld SM. The evolutionary history of vertebrate cranial placodes--I: cell type evolution. Dev Biol. 2014;389(1):82-97.

21. Schlosser G, Patthey C, Shimeld SM. The evolutionary history of vertebrate cranial placodes II. Evolution of ectodermal patterning. Dev Biol. 2014;389(1):98-119.
22. Cohen LE, Radovick S. Molecular basis of combined pituitary hormone deficiencies. Endocr Rev. 2002;23(4):431-42.
23. Davis SW, Camper SA. Noggin regulates Bmp4 activity during pituitary induction. Dev Biol. 2007;305(1):145-60.
24. Bharti K, Gasper M, Bertuzzi S, Arnheiter H. Lack of the ventral anterior homeodomain transcription factor VAX1 leads to induction of a second pituitary. Development. 2011;138(5):873-8.
25. Chung WC, Tsai PS. Role of fibroblast growth factor signaling in gonadotropin-releasing hormone neuronal system development. Front Horm Res. 2010;39:37-50.
26. Smith N, Quinton R. Kallmann syndrome. BMJ. 2012;345:e6971.
27. Treier M, O'Connell S, Gleiberman A, Price J, Szeto DP, Burgess R, et al. Hedgehog signaling is required for pituitary gland development. Development. 2001;128(3):377-86.
28. Ohuchi H. [Roles for FGF-FGFR signaling during vertebrate development]. Hum Cell. 2000;13(4):169-75.
29. Ohuchi H, Hori Y, Yamasaki M, Harada H, Sekine K, Kato S, et al. FGF10 acts as a major ligand for FGF receptor 2 IIIb in mouse multi-organ development. Biochem Biophys Res Commun. 2000;277(3):643-9.
30. Friedl P, Gilmour D. Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol. 2009;10(7):445-57.
31. Trowe MO, Zhao L, Weiss AC, Christoffels V, Epstein DJ, Kispert A. Inhibition of Sox2-dependent activation of Shh in the ventral diencephalon by Tbx3 is required for formation of the neurohypophysis. Development. 2013;140(11):2299-309.
32. Carreno G, Apps JR, Lodge EJ, Panousopoulos L, Haston S, Gonzalez-Meljem JM, et al. Hypothalamic sonic hedgehog is required for cell specification and proliferation of LHX3/LHX4 pituitary embryonic precursors. Development. 2017;144(18):3289-302.
33. 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(3):585-96.
34. Gaston-Massuet C, Andoniadou CL, Signore M, Jayakody SA, Charolidi N, Kyeyune R, et al. Increased Wingless (Wnt) signaling in pituitary progenitor/stem cells gives rise to pituitary tumors in mice and humans. Proc Natl Acad Sci U S A. 2011;108(28):11482-7.
35. Blauwkamp TA, Nigam S, Ardehali R, Weissman IL, Nusse R. Endogenous Wnt signalling in human embryonic stem cells generates an equilibrium of distinct lineage-specified progenitors. Nat Commun. 2012;3:1070.
36. Chai R, Kuo B, Wang T, Liaw EJ, Xia A, Jan TA, et al. Wnt signaling induces proliferation of sensory precursors in the postnatal mouse cochlea. Proc Natl Acad Sci U S A. 2012;109(21):8167-72.
37. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. Cell. 2012;149(6):1192-205.
38. Willert K, Nusse R. Wnt proteins. Cold Spring Harb Perspect Biol. 2012;4(9):a007864.
39. Potok MA, Cha KB, Hunt A, Brinkmeier ML, Leitges M, Kispert A, et al. WNT signaling affects gene expression in the ventral diencephalon and pituitary gland growth. Dev Dyn. 2008;237(4):1006-20.
40. Cha KB, Douglas KR, Potok MA, Liang H, Jones SN, Camper SA. WNT5A signaling affects pituitary gland shape. Mech Dev. 2004;121(2):183-94.

41. Olson LE, Tollkuhn J, Scafoglio C, Krones A, Zhang J, Ohgi KA, et al. Homeodomain-mediated beta-catenin-dependent switching events dictate cell-lineage determination. Cell. 2006;125(3):593-605.
42. Andoniadou CL, Matsushima D, Mousavy Gharavy SN, Signore M, Mackintosh AI, Schaeffer M, et al. Sox2(+) stem/progenitor cells in the adult mouse pituitary support organ homeostasis and have tumor-inducing potential. Cell Stem Cell. 2013;13(4):433-45.
43. Aujla PK, Bora A, Monahan P, Sweedler JV, Raetzman LT. The Notch effector gene Hes1 regulates migration of hypothalamic neurons, neuropeptide content and axon targeting to the pituitary. Dev Biol. 2011;353(1):61-71.
44. Raetzman LT, Ross SA, Cook S, Dunwoodie SL, Camper SA, Thomas PQ. Developmental regulation of Notch signaling genes in the embryonic pituitary: Prop1 deficiency affects Notch2 expression. Dev Biol. 2004;265(2):329-40.
45. Monahan P, Rybak S, Raetzman LT. The notch target gene HES1 regulates cell cycle inhibitor expression in the developing pituitary. Endocrinology. 2009;150(9):4386-94.
46. Zhu X, Zhang J, Tollkuhn J, Ohsawa R, Bresnick EH, Guillemot F, et al. Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. Genes Dev. 2006;20(19):2739-53.
47. Nantie LB, Himes AD, Getz DR, Raetzman LT. Notch signaling in postnatal pituitary expansion: proliferation, progenitors, and cell specification. Mol Endocrinol. 2014;28(5):731-44.
48. Dattani MT, Martinez-Barbera JP, Thomas PQ, Brickman JM, Gupta R, Martensson IL, et al. Mutations in the homeobox gene HESX1/Hesx1 associated with septo-optic dysplasia in human and mouse. Nat Genet. 1998;19(2):125-33.
49. Gaston-Massuet C, Andoniadou CL, Signore M, Sajedi E, Bird S, Turner JM, et al. Genetic interaction between the homeobox transcription factors HESX1 and SIX3 is required for normal pituitary development. Dev Biol. 2008;324(2):322-33.
50. 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. Development. 1996;122(1):41-52.
51. Chou SJ, Hermesz E, Hatta T, Feltner D, El-Hodiri HM, Jamrich M, et al. Conserved regulatory elements establish the dynamic expression of Rpx/HesxI in early vertebrate development. Dev Biol. 2006;292(2):533-45.
52. Dasen JS, Martinez Barbera JP, Herman TS, Connell SO, Olson L, Ju B, et al. Temporal regulation of a paired-like homeodomain repressor/TLE corepressor complex and a related activator is required for pituitary organogenesis. Genes Dev. 2001;15(23):3193-207.
53. Dasen JS, Rosenfeld MG. Signaling and transcriptional mechanisms in pituitary development. Annu Rev Neurosci. 2001;24:327-55.
54. Larder R, Clark DD, Miller NL, Mellon PL. Hypothalamic dysregulation and infertility in mice lacking the homeodomain protein Six6. J Neurosci. 2011;31(2):426-38.
55. Li X, Perissi V, Liu F, Rose DW, Rosenfeld MG. Tissue-specific regulation of retinal and pituitary precursor cell proliferation. Science. 2002;297(5584):1180-3.
56. Lavado A, Lagutin OV, Oliver G. Six3 inactivation causes progressive caudalization and aberrant patterning of the mammalian diencephalon. Development. 2008;135(3):441-50.
57. Sajedi E, Gaston-Massuet C, Signore M, Andoniadou CL, Kelberman D, Castro S, et al. Analysis of mouse models carrying the I26T and R160C substitutions in the transcriptional repressor HESX1 as models for septo-optic dysplasia and hypopituitarism. Dis Model Mech. 2008;1(4-5):241-54.
58. Soukup V, Horacek I, Cerny R. Development and evolution of the vertebrate primary mouth. J Anat. 2013;222(1):79-99.

59. Lanctot C, Lamolet B, Drouin J. The bicoid-related homeoprotein Ptx1 defines the most anterior domain of the embryo and differentiates posterior from anterior lateral mesoderm. Development. 1997;124(14):2807-17.
60. Charles MA, Suh H, Hjalt TA, Drouin J, Camper SA, Gage PJ. PITX genes are required for cell survival and Lhx3 activation. Mol Endocrinol. 2005;19(7):1893-903.
61. Ai D, Wang J, Amen M, Lu MF, Amendt BA, Martin JF. Nuclear factor 1 and T-cell factor/LEF recognition elements regulate Pitx2 transcription in pituitary development. Mol Cell Biol. 2007;27(16):5765-75.
62. Zhao Y, Morales DC, Hermesz E, Lee WK, Pfaff SL, Westphal H. Reduced expression of the LIM-homeobox gene Lhx3 impairs growth and differentiation of Rathke's pouch and increases cell apoptosis during mouse pituitary development. Mech Dev. 2006;123(8):605-13.
63. Suh H, Gage PJ, Drouin J, Camper SA. Pitx2 is required at multiple stages of pituitary organogenesis: pituitary primordium formation and cell specification. Development. 2002;129(2):329-37.
64. Castinetti F, Brinkmeier ML, Gordon DF, Vella KR, Kerr JM, Mortensen AH, et al. PITX2 AND PITX1 regulate thyrotroph function and response to hypothyroidism. Mol Endocrinol. 2011;25(11):1950-60.
65. Zhao Y, Mailloux CM, Hermesz E, Palkovits M, Westphal H. A role of the LIM-homeobox gene Lhx2 in the regulation of pituitary development. Dev Biol. 2010;337(2):313-23.
66. Sheng HZ, Moriyama K, Yamashita T, Li H, Potter SS, Mahon KA, et al. Multistep control of pituitary organogenesis. Science. 1997;278(5344):1809-12.
67. Sarkar A, Hochedlinger K. A gutsy way to grow: intestinal stem cells as nutrient sensors. Cell. 2011;147(3):487-9.
68. Lee B, Song H, Rizzoti K, Son Y, Yoon J, Baek K, et al. Genomic code for Sox2 binding uncovers its regulatory role in Six3 activation in the forebrain. Dev Biol. 2013;381(2):491-501.
69. Lee B, Rizzoti K, Kwon DS, Kim SY, Oh S, Epstein DJ, et al. Direct transcriptional regulation of Six6 is controlled by SoxB1 binding to a remote forebrain enhancer. Dev Biol. 2012;366(2):393-403.
70. Jayakody SA, Andoniadou CL, Gaston-Massuet C, Signore M, Cariboni A, Bouloux PM, et al. SOX2 regulates the hypothalamic-pituitary axis at multiple levels. J Clin Invest. 2012;122(10):3635-46.
71. Fauquier T, Rizzoti K, Dattani M, Lovell-Badge R, Robinson IC. SOX2-expressing progenitor cells generate all of the major cell types in the adult mouse pituitary gland. Proc Natl Acad Sci U S A. 2008;105(8):2907-12.
72. de Graaff LCG. PROP1-Related Combined Pituitary Hormone Deficiency. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Stephens K, et al., editors. GeneReviews((R)). Seattle (WA)1993.
73. Ward RD, Raetzman LT, Suh H, Stone BM, Nasonkin IO, Camper SA. Role of PROP1 in pituitary gland growth. Mol Endocrinol. 2005;19(3):698-710.
74. Yoshida S, Kato T, Susa T, Cai LY, Nakayama M, Kato Y. PROP1 coexists with SOX2 and induces PIT1-commitment cells. Biochem Biophys Res Commun. 2009;385(1):11-5.
75. Yoshida S, Kato T, Yako H, Susa T, Cai LY, Osuna M, et al. Significant quantitative and qualitative transition in pituitary stem / progenitor cells occurs during the postnatal development of the rat anterior pituitary. J Neuroendocrinol. 2011;23(10):933-43.

76. Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG, et al. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev. 1990;4(5):695-711.
77. Ward RD, Stone BM, Raetzman LT, Camper SA. Cell proliferation and vascularization in mouse models of pituitary hormone deficiency. Mol Endocrinol. 2006;20(6):1378-90.
78. Pulichino AM, Vallette-Kasic S, Tsai JP, Couture C, Gauthier Y, Drouin J. Tpit determines alternate fates during pituitary cell differentiation. Genes Dev. 2003;17(6):738-47.
79. Lamolet B, Pulichino AM, Lamonerie T, Gauthier Y, Brue T, Enjalbert A, et al. A pituitary cell-restricted T box factor, Tpit, activates POMC transcription in cooperation with Pitx homeoproteins. Cell. 2001;104(6):849-59.
80. Pulichino AM, Vallette-Kasic S, Couture C, Gauthier Y, Brue T, David M, et al. Human and mouse TPIT gene mutations cause early onset pituitary ACTH deficiency. Genes Dev. 2003;17(6):711-6.
81. Lavoie PL, Budry L, Balsalobre A, Drouin J. Developmental dependence on NurRE and EboxNeuro for expression of pituitary proopiomelanocortin. Mol Endocrinol. 2008;22(7):1647-57.
82. Wen S, Ai W, Alim Z, Boehm U. Embryonic gonadotropin-releasing hormone signaling is necessary for maturation of the male reproductive axis. Proc Natl Acad Sci U S A. 2010;107(37):16372-7.
83. Shima Y, Zubair M, Komatsu T, Oka S, Yokoyama C, Tachibana T, et al. Pituitary homeobox 2 regulates adrenal4 binding protein/steroidogenic factor-1 gene transcription in the pituitary gonadotrope through interaction with the intronic enhancer. Mol Endocrinol. 2008;22(7):1633-46.
84. Zhao L, Bakke M, Parker KL. Pituitary-specific knockout of steroidogenic factor 1. Mol Cell Endocrinol. 2001;185(1-2):27-32.
85. Zhang S, Cui Y, Ma X, Yong J, Yan L, Yang M, et al. Single-cell transcriptomics identifies divergent developmental lineage trajectories during human pituitary development. Nat Commun. 2020;11(1):5275.
86. Chen L, Ye H, Wang X, Tang X, Mao Y, Zhao Y, et al. Evidence of brain tumor stem progenitor-like cells with low proliferative capacity in human benign pituitary adenoma. Cancer letters. 2014;349(1):61-6.
87. Donangelo I, Ren SG, Eigler T, Svendsen C, Melmed S. Sca1(+) murine pituitary adenoma cells show tumor-growth advantage. Endocrine-related cancer. 2014;21(2):203-16.
88. Li L, Bhatia R. Stem cell quiescence. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17(15):4936-41.
89. Abdullah LN, Chow EK. Mechanisms of chemoresistance in cancer stem cells. Clinical and translational medicine. 2013;2(1):3.
90. Gibo H, Hokama M, Kyoshima K, Kobayashi S. [Arteries to the pituitary]. Nihon rinsho Japanese journal of clinical medicine. 1993;51(10):2550-4.
91. Stanfield JP. The blood supply of the human pituitary gland. Journal of anatomy. 1960;94:257-73.
92. Elster AD, Sanders TG, Vines FS, Chen MY. Size and shape of the pituitary gland during pregnancy and post partum: measurement with MR imaging. Radiology. 1991;181(2):531-5.
93. Aquilina K, Boop FA. Nonneoplastic enlargement of the pituitary gland in children. J Neurosurg Pediatr. 2011;7(5):510-5.

94. Pariante CM, Dazzan P, Danese A, Morgan KD, Brudaglio F, Morgan C, et al. Increased pituitary volume in antipsychotic-free and antipsychotic-treated patients of the AEsop first-onset psychosis study. Neuropsychopharmacology. 2005;30(10):1923-31.
95. Szarfman A, Tonning JM, Levine JG, Doraiswamy PM. Atypical antipsychotics and pituitary tumors: a pharmacovigilance study. Pharmacotherapy. 2006;26(6):748-58.
96. Molitch ME. Atypical antipsychotics and pituitary tumors. J Clin Psychopharmacol. 2012;32(6):741-2.
97. Rubin MR, Bruce JN, Khandji AG, Freda PU. Sarcoidosis within a pituitary adenoma. Pituitary. 2001;4(3):195-202.
98. Zia-Ul-Hussnain HM, Farrell M, Looby S, Tormey W, Javadpour M, McConkey S, et al. Pituitary tuberculoma: a rare cause of sellar mass. Ir J Med Sci. 2017.
99. Manaka K, Makita N, Iiri T. Erdheim-Chester disease and pituitary involvement: a unique case and the literature. Endocr J. 2014;61(2):185-94.
100. Yoo F, Kuan EC, Heaney AP, Bergsneider M, Wang MB. Corticotrophic pituitary carcinoma with cervical metastases: case series and literature review. Pituitary. 2018.
101. Chrisoulidou A, Alexandraki KI, Kita M, Tsolakidou K, Papanastasiou L, Samara C, et al. Incidence of pituitary incidentalomas in patients with adrenal adenomas. Exp Clin Endocrinol Diabetes. 2014;122(1):15-9.
102. Daly AF, Burlacu MC, Livadariu E, Beckers A. The epidemiology and management of pituitary incidentalomas. Horm Res. 2007;68 Suppl 5:195-8.
103. Scheithauer BW, Gaffey TA, Lloyd RV, Sebo TJ, Kovacs KT, Horvath E, et al. Pathobiology of pituitary adenomas and carcinomas. Neurosurgery. 2006;59(2):341-53; discussion -53.
104. Larkin S, Ansorge O. Pathology and Pathogenesis of Pituitary Adenomas and Other Sellar Lesions. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA)2000.
105. Kovacs K, Horvath E, Vidal S. Classification of pituitary adenomas. J Neurooncol. 2001;54(2):121-7.
106. Garcea G, Lloyd TD, Gescher A, Dennison AR, Steward WP, Berry DP. Angiogenesis of gastrointestinal tumours and their metastases--a target for intervention? Eur J Cancer. 2004;40(9):1302-13.
107. Hardy J. Transsphenoidal Hypophysectomy. J Neurosurg. 2007;107(2):459-71.
108. Mooney MA, Hardesty DA, Sheehy JP, Bird CR, Chapple K, White WL, et al. Rater Reliability of the Hardy Classification for Pituitary Adenomas in the Magnetic Resonance Imaging Era. J Neurol Surg B Skull Base. 2017;78(5):413-8.
109. Ensenat J, Ortega A, Topcewski T, Vilalta J, Obiols G, Mesa J, et al. [Predictive value of the Knosp classification in grading the surgical resection of invasive pituitary macroadenomas. A prospective study of 23 cases]. Neurocirugia (Astur). 2006;17(6):519-26.
110. Zielinski G, Podgorski JK. [Prognostic value of the Knosp scale in trans-sphenoidal surgery for pituitary adenomas]. Neurol Neurochir Pol. 2002;36(1):69-82.
111. Al-Shraim M, Asa SL. The 2004 World Health Organization classification of pituitary tumors: what is new? Acta Neuropathol. 2006;111(1):1-7.
112. Trouillas J, Roy P, Sturm N, Dantony E, Cortet-Rudelli C, Viennet G, et al. A new prognostic clinicopathological classification of pituitary adenomas: a multicentric case-control study of 410 patients with 8 years post-operative follow-up. Acta Neuropathol. 2013;126(1):123-35.
113. Buchfelder M, Schlaffer SM. Modern imaging of pituitary adenomas. Front Horm Res. 2010;38:109-20.
114. Elster AD. Modern imaging of the pituitary. Radiology. 1993;187(1):1-14.

115. Orija IB, Weil RJ, Hamrahian AH. Pituitary incidentaloma. Best Pract Res Clin Endocrinol Metab. 2012;26(1):47-68.
116. Vasilev V, Rostomyan L, Daly AF, Potorac I, Zacharieva S, Bonneville JF, et al. MANAGEMENT OF ENDOCRINE DISEASE: Pituitary 'incidentaloma': neuroradiological assessment and differential diagnosis. Eur J Endocrinol. 2016;175(4):R171-84.
117. Aron DC. The path to the soul: Harvey Cushing and surgery on the pituitary and its environs in 1916. Perspect Biol Med. 1994;37(4):551-65.
118. Landolt AM, Novoselac M. Modification of the Cushing speculum used for transsphenoidal pituitary surgery. Technical note. J Neurosurg. 1975;42(1):108-10.
119. Schuss P, Hadjiathanasiou A, Klingmuller D, Guresir A, Vatter H, Guresir E. Transsphenoidal pituitary surgery: comparison of two sellar reconstruction techniques and their effect on postoperative cerebrospinal fluid leakage. Neurosurg Rev. 2018.
120. Sheehan JP, Starke RM, Mathieu D, Young B, Sneed PK, Chiang VL, et al. Gamma Knife radiosurgery for the management of nonfunctioning pituitary adenomas: a multicenter study. J Neurosurg. 2013;119(2):446-56.
121. Caimari F, Korbonits M. Novel Genetic Causes of Pituitary Adenomas. Clin Cancer Res. 2016;22(20):5030-42.
122. Daly AF, Beckers A. Familial isolated pituitary adenomas (FIPA) and mutations in the aryl hydrocarbon receptor interacting protein (AIP) gene. Endocrinol Metab Clin North Am. 2015;44(1):19-25.
123. Vierimaa O, Georgitsi M, Lehtonen R, Vahteristo P, Kokko A, Raitila A, et al. Pituitary adenoma predisposition caused by germline mutations in the AIP gene. Science. 2006;312(5777):1228-30.
124. Trivellin G, Daly AF, Faucz FR, Yuan B, Rostomyan L, Larco DO, et al. Gigantism and acromegaly due to Xq26 microduplications and GPR101 mutation. N Engl J Med. 2014;371(25):2363-74.
125. Naves LA, Daly AF, Dias LA, Yuan B, Zakir JC, Barra GB, et al. Aggressive tumor growth and clinical evolution in a patient with X-linked acro-gigantism syndrome. Endocrine. 2016;51(2):236-44.
126. Beckers A, Lodish MB, Trivellin G, Rostomyan L, Lee M, Faucz FR, et al. X-linked acrogigantism syndrome: clinical profile and therapeutic responses. Endocr Relat Cancer. 2015;22(3):353-67.
127. Rodd C, Millette M, Iacovazzo D, Stiles CE, Barry S, Evanson J, et al. Somatic GPR101 Duplication Causing X-Linked Acrogigantism (XLAG)-Diagnosis and Management. J Clin Endocrinol Metab. 2016;101(5):1927-30.
128. Marini F, Falchetti A, Luzi E, Tonelli F, Maria Luisa B. Multiple Endocrine Neoplasia Type 1 (MEN1) Syndrome. In: Riegert-Johnson DL, Boardman LA, Hefferon T, Roberts M, editors. Cancer Syndromes. Bethesda (MD)2009.
129. Papaconstantinou M, Maslikowski BM, Pepper AN, Bedard PA. Menin: the protein behind the MEN1 syndrome. Adv Exp Med Biol. 2009;668:27-36.
130. Tonelli F, Giudici F, Giusti F, Marini F, Cianferotti L, Nesi G, et al. A heterozygous frameshift mutation in exon 1 of CDKN1B gene in a patient affected by MEN4 syndrome. Eur J Endocrinol. 2014;171(2):K7-K17.
131. Milne TA, Hughes CM, Lloyd R, Yang Z, Rozenblatt-Rosen O, Dou Y, et al. Menin and MLL cooperatively regulate expression of cyclin-dependent kinase inhibitors. Proc Natl Acad Sci U S A. 2005;102(3):749-54.
132. Bosco Schamun MB, Correa R, Graffigna P, de Miguel V, Fainstein Day P. Carney complex review: Genetic features. Endocrinol Diabetes Nutr. 2018;65(1):52-9.

133. Liu Q, Tong D, Liu G, Yi Y, Zhang D, Zhang J, et al. Carney complex with PRKAR1A gene mutation: A case report and literature review. Medicine (Baltimore). 2017;96(50):e8999.
134. Salpea P, Stratakis CA. Carney complex and McCune Albright syndrome: an overview of clinical manifestations and human molecular genetics. Mol Cell Endocrinol. 2014;386(1-2):85-91.
135. Almeida MQ, Muchow M, Boikos S, Bauer AJ, Griffin KJ, Tsang KM, et al. Mouse Prkar1a haploinsufficiency leads to an increase in tumors in the Trp53+/- or Rb1+/- backgrounds and chemically induced skin papillomas by dysregulation of the cell cycle and Wnt signaling. Hum Mol Genet. 2010;19(8):1387-98.
136. Solarski M, Rotondo F, Foulkes W, Priest JR, Syro LV, Butz H, et al. DICER1 gene mutations in endocrine tumors. Endocr Relat Cancer. 2018.
137. Denes J, Swords F, Rattenberry E, Stals K, Owens M, Cranston T, et al. Heterogeneous genetic background of the association of pheochromocytoma/paraganglioma and pituitary adenoma: results from a large patient cohort. J Clin Endocrinol Metab. 2015;100(3):E531-41.
138. Vortmeyer AO, Glasker S, Mehta GU, Abu-Asab MS, Smith JH, Zhuang Z, et al. Somatic GNAS mutation causes widespread and diffuse pituitary disease in acromegalic patients with McCune-Albright syndrome. J Clin Endocrinol Metab. 2012;97(7):2404-13.
139. Mantovani G, Lania AG, Spada A. GNAS imprinting and pituitary tumors. Mol Cell Endocrinol. 2010;326(1-2):15-8.
140. Freda PU, Chung WK, Matsuoka N, Walsh JE, Kanibir MN, Kleinman G, et al. Analysis of GNAS mutations in 60 growth hormone secreting pituitary tumors: correlation with clinical and pathological characteristics and surgical outcome based on highly sensitive GH and IGF-I criteria for remission. Pituitary. 2007;10(3):275-82.
141. Dean L. McCune-Albright Syndrome. In: Pratt V, McLeod H, Dean L, Malheiro A, Rubinstein W, editors. Medical Genetics Summaries. Bethesda (MD)2012.
142. Iacovazzo D, Caswell R, Bunce B, Jose S, Yuan B, Hernandez-Ramirez LC, et al. Germline or somatic GPR101 duplication leads to X-linked acrogigantism: a clinico-pathological and genetic study. Acta Neuropathol Commun. 2016;4(1):56.
143. Reincke M, Sbiera S, Hayakawa A, Theodoropoulou M, Osswald A, Beuschlein F, et al. Mutations in the deubiquitinase gene USP8 cause Cushing's disease. Nat Genet. 2015;47(1):31-8.
144. Farrell WE. Epigenetics of pituitary tumours: an update. Curr Opin Endocrinol Diabetes Obes. 2014;21(4):299-305.
145. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell. 2005;120(1):15-20.
146. Denzler R, McGeary SE, Title AC, Agarwal V, Bartel DP, Stoffel M. Impact of MicroRNA Levels, Target-Site Complementarity, and Cooperativity on Competing Endogenous RNA-Regulated Gene Expression. Mol Cell. 2016;64(3):565-79.
147. Grzywa TM, Klicka K, Rak B, Mehlich D, Garbicz F, Zielinski G, et al. Lineage-dependent role of miR-410-3p as oncomiR in gonadotroph and corticotroph pituitary adenomas or tumor suppressor miR in somatotroph adenomas via MAPK, PTEN/AKT, and STAT3 signaling pathways. Endocrine. 2019;65(3):646-55.
148. Denes J, Kasuki L, Trivellin G, Colli LM, Takiya CM, Stiles CE, et al. Regulation of aryl hydrocarbon receptor interacting protein (AIP) protein expression by MiR-34a in sporadic somatotropinomas. PLoS One. 2015;10(2):e0117107.

149. Wang Y, Yin X, Zhao L, Li S, Duan J, Kuang R, et al. MicroRNA-200b inhibits pituitary tumor cell proliferation and invasion by targeting PKCalpha. Exp Ther Med. 2017;14(2):1706-14.
150. Stiles CE, Korbonits M. Familial Isolated Pituitary Adenoma. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA)2000.
151. Beckers A, Aaltonen LA, Daly AF, Karhu A. Familial isolated pituitary adenomas (FIPA) and the pituitary adenoma predisposition due to mutations in the aryl hydrocarbon receptor interacting protein (AIP) gene. Endocr Rev. 2013;34(2):239-77.
152. Igreja S, Chahal HS, King P, Bolger GB, Srirangalingam U, Guasti L, et al. Characterization of aryl hydrocarbon receptor interacting protein (AIP) mutations in familial isolated pituitary adenoma families. Hum Mutat. 2010;31(8):950-60.
153. Daly AF, Tichomirowa MA, Petrossians P, Heliovaara E, Jaffrain-Rea ML, Barlier A, et al. Clinical characteristics and therapeutic responses in patients with germ-line AIP mutations and pituitary adenomas: an international collaborative study. J Clin Endocrinol Metab. 2010;95(11):E373-83.
154. Marques P, Caimari F, Hernandez-Ramirez LC, Collier D, Iacovazzo D, Ronaldson A, et al. Significant Benefits of AIP Testing and Clinical Screening in Familial Isolated and Young-onset Pituitary Tumors. J Clin Endocrinol Metab. 2020;105(6).
155. Beckers A, Daly AF. The clinical, pathological, and genetic features of familial isolated pituitary adenomas. Eur J Endocrinol. 2007;157(4):371-82.
156. Meyer BK, Pray-Grant MG, Vanden Heuvel JP, Perdew GH. Hepatitis B virus X-associated protein 2 is a subunit of the unliganded aryl hydrocarbon receptor core complex and exhibits transcriptional enhancer activity. Mol Cell Biol. 1998;18(2):978-88.
157. Chahal HS, Chapple JP, Frohman LA, Grossman AB, Korbonits M. Clinical, genetic and molecular characterization of patients with familial isolated pituitary adenomas (FIPA). Trends Endocrinol Metab. 2010;21(7):419-27.
158. Cooper DN, Krawczak M, Polychronakos C, Tyler-Smith C, Kehrer-Sawatzki H. Where genotype is not predictive of phenotype: towards an understanding of the molecular basis of reduced penetrance in human inherited disease. Hum Genet. 2013;132(10):1077-130.
159. Stratakis CA, Tichomirowa MA, Boikos S, Azevedo MF, Lodish M, Martari M, et al. The role of germline AIP, MEN1, PRKAR1A, CDKN1B and CDKN2C mutations in causing pituitary adenomas in a large cohort of children, adolescents, and patients with genetic syndromes. Clin Genet. 2010;78(5):457-63.
160. Tichomirowa MA, Barlier A, Daly AF, Jaffrain-Rea ML, Ronchi C, Yaneva M, et al. High prevalence of AIP gene mutations following focused screening in young patients with sporadic pituitary macroadenomas. Eur J Endocrinol. 2011;165(4):509-15.
161. Kuzhandaivelu N, Cong YS, Inouye C, Yang WM, Seto E. XAP2, a novel hepatitis B virus X-associated protein that inhibits X transactivation. Nucleic Acids Res. 1996;24(23):4741-50.
162. Chahal HS, Trivellin G, Leontiou CA, Alband N, Fowkes RC, Tahir A, et al. Somatostatin analogs modulate AIP in somatotroph adenomas: the role of the ZAC1 pathway. J Clin Endocrinol Metab. 2012;97(8):E1411-20.
163. Ma Q, Whitlock JP, Jr. A novel cytoplasmic protein that interacts with the Ah receptor, contains tetratricopeptide repeat motifs, and augments the transcriptional response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. J Biol Chem. 1997;272(14):8878-84.
164. Meyer BK, Petrulis JR, Perdew GH. Aryl hydrocarbon (Ah) receptor levels are selectively modulated by hsp90-associated immunophilin homolog XAP2. Cell Stress Chaperones. 2000;5(3):243-54.

165. Linnert M, Haupt K, Lin YJ, Kissing S, Paschke AK, Fischer G, et al. NMR assignments of the FKBP-type PPIase domain of the human aryl-hydrocarbon receptor-interacting protein (AIP). Biomol NMR Assign. 2012;6(2):209-12.
166. Trivellin G, Korbonits M. AIP and its interacting partners. J Endocrinol. 2011;210(2):137-55.
167. Tahir A, Chahal HS, Korbonits M. Molecular genetics of the aip gene in familial pituitary tumorigenesis. Prog Brain Res. 2010;182:229-53.
168. Beckers A, Jaffrain-Rea ML, Daly AE. [Genetics of familial pituitary adenomas]. Bull Acad Natl Med. 2009;193(7):1557-70; discussion 70-1.
169. Jaffrain-Rea ML, Angelini M, Gargano D, Tichomirowa MA, Daly AF, Vanbellinghen JF, et al. Expression of aryl hydrocarbon receptor (AHR) and AHR-interacting protein in pituitary adenomas: pathological and clinical implications. Endocr Relat Cancer. 2009;16(3):1029-43.
170. Lin BC, Nguyen LP, Walisser JA, Bradfield CA. A hypomorphic allele of aryl hydrocarbon receptor-associated protein-9 produces a phenocopy of the AHR-null mouse. Mol Pharmacol. 2008;74(5):1367-71.
171. Morgan RM, Hernandez-Ramirez LC, Trivellin G, Zhou L, Roe SM, Korbonits M, et al. Structure of the TPR domain of AIP: lack of client protein interaction with the C-terminal alpha-7 helix of the TPR domain of AIP is sufficient for pituitary adenoma predisposition. PLoS One. 2012;7(12):e53339.
172. Pivonello R, Auriemma RS, Grasso LF, Pivonello C, Simeoli C, Patalano R, et al. Complications of acromegaly: cardiovascular, respiratory and metabolic comorbidities. Pituitary. 2017;20(1):46-62.
173. Wolinski K, Stangierski A, Gurgul E, Brominska B, Czarnywojtek A, Lodyga M, et al. Thyroid lesions in patients with acromegaly - case-control study and update to the meta-analysis. Endokrynol Pol. 2017;68(1):2-6.
174. Wolinski K, Stangierski A, Dyrda K, Nowicka K, Pelka M, Iqbal A, et al. Risk of malignant neoplasms in acromegaly: a case-control study. J Endocrinol Invest. 2017;40(3):319-22.
175. Gasperi M, Martino E, Manetti L, Arosio M, Porretti S, Faglia G, et al. Prevalence of thyroid diseases in patients with acromegaly: results of an Italian multi-center study. J Endocrinol Invest. 2002;25(3):240-5.
176. Tita P, Ambrosio MR, Scollo C, Carta A, Gangemi P, Bondanelli M, et al. High prevalence of differentiated thyroid carcinoma in acromegaly. Clin Endocrinol (Oxf). 2005;63(2):161-7.
177. Jenkins PJ. Cancers associated with acromegaly. Neuroendocrinology. 2006;83(3-4):218-23.
178. Rokkas T, Pistiolas D, Sechopoulos P, Margantinis G, Koukoulis G. Risk of colorectal neoplasm in patients with acromegaly: a meta-analysis. World J Gastroenterol. 2008;14(22):3484-9.
179. Brunner JE, Johnson CC, Zafar S, Peterson EL, Brunner JF, Mellinger RC. Colon cancer and polyps in acromegaly: increased risk associated with family history of colon cancer. Clin Endocrinol (Oxf). 1990;32(1):65-71.
180. Gordon MB, Nakhle S, Ludlam WH. Patients with Acromegaly Presenting with Colon Cancer: A Case Series. Case Rep Endocrinol. 2016;2016:5156295.
181. Ituarte EA, Petrini J, Hershman JM. Acromegaly and colon cancer. Ann Intern Med. 1984;101(5):627-8.
182. Jenkins PJ. Acromegaly and colon cancer. Growth Horm IGF Res. 2000;10 Suppl A:S35-6.

183. Ritter MM, Richter WO, Schwandt P. Acromegaly and colon cancer. Ann Intern Med. 1987;106(4):636-7.
184. Robles Perez R, Muino Miguez R, Rodriguez de Castro E, Gonzalez Ramallo V. [Acromegaly and cancer of the colon]. Med Clin (Barc). 1987;88(8):344-5.
185. Shimazaki H, Ueno T, Takeda R, Kurumadani H, Nakagawa M. [Two cases of acromegaly with colon cancer (author's transl)]. Nihon Shokakibyo Gakkai Zasshi. 1981;78(3):724-7.
186. Tagliafico A, Calabrese M, Tagliafico G, Resmini E, Martinoli C, Rebora A, et al. Increased mammographic breast density in acromegaly: quantitative and qualitative assessment. Eur J Endocrinol. 2011;164(3):335-40.
187. Marova EI, Bazarova EN, Bronshtein MI, Rozhinskaia L, Karetnikov Iu P. [Primary thyroid and breast cancers in a patient with pituitary adenoma and acromegaly]. Sov Med. 1983(7):119-22.
188. Ballon HC, Gomberg C, Wilansky DL. Acromegaly and carcinoma of the breast. Can Med Assoc J. 1960;82:1268-70.
189. Colao A, Marzullo P, Spiezia S, Ferone D, Giaccio A, Cerbone G, et al. Effect of growth hormone (GH) and insulin-like growth factor I on prostate diseases: an ultrasonographic and endocrine study in acromegaly, GH deficiency, and healthy subjects. J Clin Endocrinol Metab. 1999;84(6):1986-91.
190. Colao A, Marzullo P, Spiezia S, Lombardi G. Acromegaly and prostate cancer. Growth Horm IGF Res. 2000;10 Suppl A:S37-8.
191. Correa LL, Lima GA, Paiva HB, Silva CM, Cavallieri SA, Miranda LC, et al. Prostate cancer and acromegaly. Arq Bras Endocrinol Metabol. 2009;53(8):963-8.
192. Kumar S, Yadav RN, Gupta P, Gaspar BL, Kakkar N, Verma A, et al. Prostatic hyperplasia in acromegaly, a myth or reality: a case-control study. Eur J Endocrinol. 2015;172(2):97-106.
193. Colao A, Ferone D, Marzullo P, Lombardi G. Systemic complications of acromegaly: epidemiology, pathogenesis, and management. Endocr Rev. 2004;25(1):102-52.
194. Clayton PE, Banerjee I, Murray PG, Renehan AG. Growth hormone, the insulin-like growth factor axis, insulin and cancer risk. Nat Rev Endocrinol. 2011;7(1):11-24.
195. Popovic V, Damjanovic S, Micic D, Nesovic M, Djurovic M, Petakov M, et al. Increased incidence of neoplasia in patients with pituitary adenomas. The Pituitary Study Group. Clin Endocrinol (Oxf). 1998;49(4):441-5.
196. Ezzat S, Asa SL, Couldwell WT, Barr CE, Dodge WE, Vance ML, et al. The prevalence of pituitary adenomas: a systematic review. Cancer. 2004;101(3):613-9.
197. Gillam MP, Ku CR, Lee YJ, Kim J, Kim SH, Lee SJ, et al. Somatotroph-Specific Aip-Deficient Mice Display Pretumorigenic Alterations in Cell-Cycle Signaling. J Endocr Soc. 2017;1(2):78-95.
198. Lin BC, Sullivan R, Lee Y, Moran S, Glover E, Bradfield CA. Deletion of the aryl hydrocarbon receptor-associated protein 9 leads to cardiac malformation and embryonic lethality. J Biol Chem. 2007;282(49):35924-32.
199. Raitila A, Lehtonen HJ, Arola J, Heliovaara E, Ahlsten M, Georgitsi M, et al. Mice with inactivation of aryl hydrocarbon receptor-interacting protein (Aip) display complete penetrance of pituitary adenomas with aberrant ARNT expression. Am J Pathol. 2010;177(4):1969-76.
200. Lecoq AL, Zizzari P, Hage M, Decourtye L, Adam C, Viengchareun S, et al. Mild pituitary phenotype in 3- and 12-month-old Aip-deficient male mice. J Endocrinol. 2016;231(1):59-69.

201. Gaston-Massuet C, McCabe MJ, Scagliotti V, Young RM, Carreno G, Gregory LC, et al. Transcription factor 7-like 1 is involved in hypothalamo-pituitary axis development in mice and humans. Proc Natl Acad Sci U S A. 2016;113(5):E548-57.
202. Sternberg N. Bacteriophage P1 site-specific recombination. III. Strand exchange during recombination at lox sites. J Mol Biol. 1981;150(4):603-8.
203. Nukaya M, Lin BC, Glover E, Moran SM, Kennedy GD, Bradfield CA. The aryl hydrocarbon receptor-interacting protein (AIP) is required for dioxin-induced hepatotoxicity but not for the induction of the Cyp1a1 and Cyp1a2 genes. J Biol Chem. 2010;285(46):35599-605.
204. Andoniadou CL, Signore M, Sajedi E, Gaston-Massuet C, Kelberman D, Burns AJ, et al. Lack of the murine homeobox gene Hesx1 leads to a posterior transformation of the anterior forebrain. Development. 2007;134(8):1499-508.
205. Sanchez-Vega F, Mina M, Armenia J, Chatila WK, Luna A, La KC, et al. Oncogenic Signaling Pathways in The Cancer Genome Atlas. Cell. 2018;173(2):321-37 e10.
206. Harvey KF, Pfleger CM, Hariharan IK. The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell. 2003;114(4):457-67.
207. Wu S, Huang J, Dong J, Pan D. hippo encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with salvador and warts. Cell. 2003;114(4):445-56.
208. Ma S, Meng Z, Chen R, Guan KL. The Hippo Pathway: Biology and Pathophysiology. Annu Rev Biochem. 2019;88:577-604.
209. Yu FX, Zhao B, Guan KL. Hippo Pathway in Organ Size Control, Tissue Homeostasis, and Cancer. Cell. 2015;163(4):811-28.
210. Manning SA, Dent LG, Kondo S, Zhao ZW, Plachta N, Harvey KF. Dynamic Fluctuations in Subcellular Localization of the Hippo Pathway Effector Yorkie In Vivo. Curr Biol. 2018;28(10):1651-60 e4.
211. Zhang L. Control of growth and beyond: a special issue on Hippo signaling. Acta Biochim Biophys Sin (Shanghai). 2015;47(1):1.
212. Halder G, Johnson RL. Hippo signaling: growth control and beyond. Development. 2011;138(1):9-22.
213. Kim J, Kim YH, Kim J, Park DY, Bae H, Lee DH, et al. YAP/TAZ regulates sprouting angiogenesis and vascular barrier maturation. J Clin Invest. 2017;127(9):3441-61.
214. Neto F, Klaus-Bergmann A, Ong YT, Alt S, Vion AC, Szymborska A, et al. YAP and TAZ regulate adherens junction dynamics and endothelial cell distribution during vascular development. Elife. 2018;7.
215. Zanconato F, Cordenonsi M, Piccolo S. YAP/TAZ at the Roots of Cancer. Cancer Cell. 2016;29(6):783-803.
216. Nakatani K, Maehama T, Nishio M, Goto H, Kato W, Omori H, et al. Targeting the Hippo signalling pathway for cancer treatment. J Biochem. 2017;161(3):237-44.
217. Buning H, Hacker UT. Inhibitors of Angiogenesis. Adv Exp Med Biol. 2016;917:261-85.
218. Chai Y, Xiang K, Wu Y, Zhang T, Liu Y, Liu X, et al. Cucurbitacin B Inhibits the Hippo-YAP Signaling Pathway and Exerts Anticancer Activity in Colorectal Cancer Cells. Med Sci Monit. 2018;24:9251-8.
219. Moriya J, Minamino T. Angiogenesis, Cancer, and Vascular Aging. Front Cardiovasc Med. 2017;4:65.
220. Rajabi M, Mousa SA. The Role of Angiogenesis in Cancer Treatment. Biomedicines. 2017;5(2).
221. Wang Z, Lu W, Zhang Y, Zou F, Jin Z, Zhao T. The Hippo Pathway and Viral Infections. Front Microbiol. 2019;10:3033.

222. Chen HY, Yu SL, Ho BC, Su KY, Hsu YC, Chang CS, et al. R331W Missense Mutation of Oncogene YAP1 Is a Germline Risk Allele for Lung Adenocarcinoma With Medical Actionability. J Clin Oncol. 2015;33(20):2303-10.
223. Malik SA, Khan MS, Dar M, Hussain MU, Shah MA, Shafi SM, et al. Molecular Alterations and Expression Dynamics of LATS1 and LATS2 Genes in Non-Small-Cell Lung Carcinoma. Pathol Oncol Res. 2018;24(2):207-14.
224. Chen Q, Zhang N, Gray RS, Li H, Ewald AJ, Zahnow CA, et al. A temporal requirement for Hippo signaling in mammary gland differentiation, growth, and tumorigenesis. Genes Dev. 2014;28(5):432-7.
225. Li C, Wang S, Xing Z, Lin A, Liang K, Song J, et al. A ROR1-HER3-lncRNA signalling axis modulates the Hippo-YAP pathway to regulate bone metastasis. Nat Cell Biol. 2017;19(2):106-19.
226. Wei C, Wang Y, Li X. The role of Hippo signal pathway in breast cancer metastasis. Onco Targets Ther. 2018;11:2185-93.
227. Lam-Himlin DM, Daniels JA, Gayyed MF, Dong J, Maitra A, Pan D, et al. The hippo pathway in human upper gastrointestinal dysplasia and carcinoma: a novel oncogenic pathway. Int J Gastrointest Cancer. 2006;37(4):103-9.
228. Hu X, Xin Y, Xiao Y, Zhao J. Overexpression of YAP1 is correlated with progression, metastasis and poor prognosis in patients with gastric carcinoma. Pathol Oncol Res. 2014;20(4):805-11.
229. Sun L, Chen F, Shi W, Qi L, Zhao Z, Zhang J. Prognostic impact of TAZ and beta-catenin expression in adenocarcinoma of the esophagogastric junction. Diagn Pathol. 2014;9:125.
230. Wang C, Zhu ZM, Liu CL, He XJ, Zhang HY, Dong JH. Knockdown of yes-associated protein inhibits proliferation and downregulates large tumor suppressor 1 expression in MHCC97H human hepatocellular carcinoma cells. Mol Med Rep. 2015;11(6):4101-8.
231. Mao B, Hu F, Cheng J, Wang P, Xu M, Yuan F, et al. SIRT1 regulates YAP2-mediated cell proliferation and chemoresistance in hepatocellular carcinoma. Oncogene. 2014;33(11):1468-74.
232. Philips GK, Atkins MB. New agents and new targets for renal cell carcinoma. Am Soc Clin Oncol Educ Book. 2014:e222-7.
233. Liang K, Zhou G, Zhang Q, Li J, Zhang C. Expression of hippo pathway in colorectal cancer. Saudi J Gastroenterol. 2014;20(3):188-94.
234. Wierzbicki PM, Rybarczyk A. The Hippo pathway in colorectal cancer. Folia Histochem Cytobiol. 2015;53(2):105-19.
235. Li H, Huang Z, Gao M, Huang N, Luo Z, Shen H, et al. Inhibition of YAP suppresses CML cell proliferation and enhances efficacy of imatinib in vitro and in vivo. J Exp Clin Cancer Res. 2016;35(1):134.
236. Chen M, Wang J, Yao SF, Zhao Y, Liu L, Li LW, et al. Effect of YAP Inhibition on Human Leukemia HL-60 Cells. Int J Med Sci. 2017;14(9):902-10.
237. Chai J, Xu S, Guo F. TEAD1 mediates the oncogenic activities of Hippo-YAP1 signaling in osteosarcoma. Biochem Biophys Res Commun. 2017;488(2):297-302.
238. Basu-Roy U, Han E, Rattanakorn K, Gadi A, Verma N, Maurizi G, et al. PPARgamma agonists promote differentiation of cancer stem cells by restraining YAP transcriptional activity. Oncotarget. 2016;7(38):60954-70.
239. Tremblay AM, Missiaglia E, Galli GG, Hettmer S, Urcia R, Carrara M, et al. The Hippo transducer YAP1 transforms activated satellite cells and is a potent effector of embryonal rhabdomyosarcoma formation. Cancer Cell. 2014;26(2):273-87.

240. Tsuneki M, Kinjo T, Mori T, Yoshida A, Kuyama K, Ohira A, et al. Survivin: A novel marker and potential therapeutic target for human angiosarcoma. Cancer Sci. 2017;108(11):2295-305.
241. Kresak JL, Walsh M. Neurofibromatosis: A Review of NF1, NF2, and Schwannomatosis. J Pediatr Genet. 2016;5(2):98-104.
242. Fossdal R, Jonasson F, Kristjansdottir GT, Kong A, Stefansson H, Gosh S, et al. A novel TEAD1 mutation is the causative allele in Sveinsson's chorioretinal atrophy (helicoid peripapillary chorioretinal degeneration). Hum Mol Genet. 2004;13(9):975-81.
243. Williamson KA, FitzPatrick DR. The genetic architecture of microphthalmia, anophthalmia and coloboma. Eur J Med Genet. 2014;57(8):369-80.
244. Nehme NT, Schmid JP, Debeurme F, Andre-Schmutz I, Lim A, Nitschke P, et al. MST1 mutations in autosomal recessive primary immunodeficiency characterized by defective naive T-cell survival. Blood. 2012;119(15):3458-68.
245. Chen SN, Gurha P, Lombardi R, Ruggiero A, Willerson JT, Marian AJ. The hippo pathway is activated and is a causal mechanism for adipogenesis in arrhythmogenic cardiomyopathy. Circ Res. 2014;114(3):454-68.
246. Jiang WJ, Ren WH, Liu XJ, Liu Y, Wu FJ, Sun LZ, et al. Disruption of mechanical stress in extracellular matrix is related to Stanford type A aortic dissection through down-regulation of Yes-associated protein. Aging (Albany NY). 2016;8(9):1923-39.
247. Wang L, Xia J, Li J, Hagemann TL, Jones JR, Fraenkel E, et al. Tissue and cellular rigidity and mechanosensitive signaling activation in Alexander disease. Nat Commun. 2018;9(1):1899.
248. Cai J, Song X, Wang W, Watnick T, Pei Y, Qian F, et al. A RhoA-YAP-c-Myc signaling axis promotes the development of polycystic kidney disease. Genes Dev. 2018;32(11-12):781-93.
249. Wang X, Zheng Z, Caviglia JM, Corey KE, Herfel TM, Cai B, et al. Hepatocyte TAZ/WWTR1 Promotes Inflammation and Fibrosis in Nonalcoholic Steatohepatitis. Cell Metab. 2016;24(6):848-62.
250. Enger TB, Samad-Zadeh A, Bouchie MP, Skarstein K, Galtung HK, Mera T, et al. The Hippo signaling pathway is required for salivary gland development and its dysregulation is associated with Sjogren's syndrome. Lab Invest. 2013;93(11):1203-18.
251. Lee JK, Shin JH, Hwang SG, Gwag BJ, McKee AC, Lee J, et al. MST1 functions as a key modulator of neurodegeneration in a mouse model of ALS. Proc Natl Acad Sci U S A. 2013;110(29):12066-71.
252. Kabza M, Karolak JA, Rydzanicz M, Szczesniak MW, Nowak DM, Ginter-Matuszewska B, et al. Collagen synthesis disruption and downregulation of core elements of TGF-beta, Hippo, and Wnt pathways in keratoconus corneas. Eur J Hum Genet. 2017;25(5):582-90.
253. Mueller KA, Glajch KE, Huizenga MN, Wilson RA, Granucci EJ, Dios AM, et al. Hippo Signaling Pathway Dysregulation in Human Huntington's Disease Brain and Neuronal Stem Cells. Sci Rep. 2018;8(1):11355.
254. St John MA, Tao W, Fei X, Fukumoto R, Carcangiu ML, Brownstein DG, et al. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. Nat Genet. 1999;21(2):182-6.
255. Lodge EJ, Russell JP, Patist AL, Francis-West P, Andoniadou CL. Expression Analysis of the Hippo Cascade Indicates a Role in Pituitary Stem Cell Development. Front Physiol. 2016;7:114.
256. Lodge EJ, Santambrogio A, Russell JP, Xekouki P, Jacques TS, Johnson RL, et al. Homeostatic and tumourigenic activity of SOX2+ pituitary stem cells is controlled by the LATS/YAP/TAZ cascade. Elife. 2019;8.

257. Avruch J, Zhou D, Fitamant J, Bardeesy N, Mou F, Barrufet LR. Protein kinases of the Hippo pathway: regulation and substrates. Semin Cell Dev Biol. 2012;23(7):770-84.
258. Mitsiadis TA, Cantu C, Jimenez-Rojo L. Editorial: Signaling Pathways in Developing and Pathological Tissues and Organs of the Craniofacial Complex. Front Physiol. 2018;9:1015.
259. Xekouki P, Lodge EJ, Matschke J, Santambrogio A, Apps JR, Sharif A, et al. Non-secreting pituitary tumours characterised by enhanced expression of YAP/TAZ. Endocr Relat Cancer. 2019;26(1):215-25.
260. Qian S, Zhan X, Lu M, Li N, Long Y, Li X, et al. Quantitative Analysis of Ubiquitinated Proteins in Human Pituitary and Pituitary Adenoma Tissues. Front Endocrinol (Lausanne). 2019;10:328.
261. Wang Y, Xu X, Maglic D, Dill MT, Mojumdar K, Ng PK, et al. Comprehensive Molecular Characterization of the Hippo Signaling Pathway in Cancer. Cell Rep. 2018;25(5):1304-17 e5.
262. Barry S, Carlsen E, Marques P, Stiles CE, Gadaleta E, Berney DM, et al. Tumor microenvironment defines the invasive phenotype of AIP-mutation-positive pituitary tumors. Oncogene. 2019;38(27):5381-95.
263. Chen Q, Zhang N, Xie R, Wang W, Cai J, Choi KS, et al. Homeostatic control of Hippo signaling activity revealed by an endogenous activating mutation in YAP. Genes Dev. 2015;29(12):1285-97.
264. Cai J, Zhang N, Zheng Y, de Wilde RF, Maitra A, Pan D. The Hippo signaling pathway restricts the oncogenic potential of an intestinal regeneration program. Genes Dev. 2010;24(21):2383-8.
265. Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, et al. Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell. 2007;130(6):1120-33.
266. Han Y. Analysis of the role of the Hippo pathway in cancer. J Transl Med. 2019;17(1):116.
267. Zanconato F, Battilana G, Cordenonsi M, Piccolo S. YAP/TAZ as therapeutic targets in cancer. Curr Opin Pharmacol. 2016;29:26-33.
268. Tao Y, Cai F, Shan L, Jiang H, Ma L, Yu Y. The Hippo signaling pathway: an emerging anti-cancer drug target. Discov Med. 2017;24(130):7-18.
269. Nishi T, Goto T, Takeshima H, Hamada K, Tada K, Saito Y, et al. Tissue factor expressed in pituitary adenoma cells contributes to the development of vascular events in pituitary adenomas. Cancer. 1999;86(7):1354-61.
270. Xekouki P, Mastroyiannis SA, Avgeropoulos D, de la Luz Sierra M, Trivellin G, Gourgari EA, et al. Familial pituitary apoplexy as the only presentation of a novel AIP mutation. Endocr Relat Cancer. 2013;20(5):L11-4.
271. Visser-Grieve S, Hao Y, Yang X. Human homolog of Drosophila expanded, hEx, functions as a putative tumor suppressor in human cancer cell lines independently of the Hippo pathway. Oncogene. 2012;31(9):1189-95.
272. Angus L, Moleirinho S, Herron L, Sinha A, Zhang X, Niestrata M, et al. Willin/FRMD6 expression activates the Hippo signaling pathway kinases in mammals and antagonizes oncogenic YAP. Oncogene. 2012;31(2):238-50.
273. Yuan M, Tomlinson V, Lara R, Holliday D, Chelala C, Harada T, et al. Yes-associated protein (YAP) functions as a tumor suppressor in breast. Cell Death Differ. 2008;15(11):1752-9.
274. Stauffer S, Chen X, Zhang L, Chen Y, Dong J. KIBRA promotes prostate cancer cell proliferation and motility. FEBS J. 2016;283(10):1800-11.

275. Yu J, Zheng Y, Dong J, Klusza S, Deng WM, Pan D. Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. Dev Cell. 2010;18(2):288-99.
276. Mitin N, Rossman KL, Currin R, Anne S, Marshall TW, Bear JE, et al. The RhoGEF TEM4 Regulates Endothelial Cell Migration by Suppressing Actomyosin Contractility. PLoS One. 2013;8(6):e66260.
277. Cai Q, Dozmorov M, Oh Y. IGFBP-3/IGFBP-3 Receptor System as an Anti-Tumor and Anti-Metastatic Signaling in Cancer. Cells. 2020;9(5).
278. Saab S, Chang OS, Nagaoka K, Hung MC, Yamaguchi H. The potential role of YAP in Axl-mediated resistance to EGFR tyrosine kinase inhibitors. Am J Cancer Res. 2019;9(12):2719-29.
279. Zemke NR, Gou D, Berk AJ. Dedifferentiation by adenovirus E1A due to inactivation of Hippo pathway effectors YAP and TAZ. Genes Dev. 2019;33(13-14):828-43.
280. Zhang W, Zhou P, Meng A, Zhang R, Zhou Y. Down-regulating Myoferlin inhibits the vasculogenic mimicry of melanoma via decreasing MMP-2 and inducing mesenchymal-to-epithelial transition. J Cell Mol Med. 2018;22(3):1743-54.
281. Wang QS, Kong PZ, Li XQ, Yang F, Feng YM. FOXF2 deficiency promotes epithelial-mesenchymal transition and metastasis of basal-like breast cancer. Breast Cancer Res. 2015;17:30.
282. Hirata H, Ueno K, Shahryari V, Deng G, Tanaka Y, Tabatabai ZL, et al. MicroRNA-182-5p promotes cell invasion and proliferation by down regulating FOXF2, RECK and MTSS1 genes in human prostate cancer. PLoS One. 2013;8(1):e55502.
283. Pan WW, Moroishi T, Koo JH, Guan KL. Cell type-dependent function of LATS1/2 in cancer cell growth. Oncogene. 2019;38(14):2595-610.
284. Rich EC, Burke W, Heaton CJ, Haga S, Pinsky L, Short MP, et al. Reconsidering the family history in primary care. J Gen Intern Med. 2004;19(3):273-80.
285. Saul RA, Trotter T, Sease K, Tarini B. Survey of family history taking and genetic testing in pediatric practice. J Community Genet. 2017;8(2):109-15.
286. Wood ME, Rehman HT, Bedrosian I. Importance of family history and indications for genetic testing. Breast J. 2020;26(1):100-4.
287. Boguslawska A, Korbonits M. Genetics of Acromegaly and Gigantism. J Clin Med. 2021;10(7).
288. Denes J, Korbonits M. The clinical aspects of pituitary tumour genetics. Endocrine. 2021;71(3):663-74.