Growth regulation in thyroid development

Shawn Liang

Sahlgrenska Cancer Center

Department of Medical Biochemistry and Cell Biology

Institute of Biomedicine

Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

Gothenburg 2018

Cover illustration: Immunofluorescence image of the developing mouse thyroid gland at E15.5, thyroid lobes in orange with Nkx2-1 and E-cadherin co-staining, esophagus (top), trachea (middle) and parathyroid (far left) by Shawn Liang

The noblest pleasure is the joy of understanding

- Leonardo da Vinci

Abstract

The fundamental aspects of developmental mechanisms that regulate embryonic and postnatal thyroid growth gaining the final size of the gland are still largely undetermined. In embryonic development, various organs and glands are composed of branched structures, designed to maximize efficiency and function. Branching morphogenesis is the developmental process that gives rise to these multicellular tubular networks. This growth process, which involves a range of paracrine and cell-autonomous factors including Fgf10 and Sox9, are utilized by the lung and numerous exocrine glands. In thyroid, an endocrine gland, postnatal growth involving thyroid stimulating hormone (TSH) from the pituitary differs to embryonic growth, which is TSH independent and thus rely on local factors of yet unknown identity. This thesis investigates thyroid growth regulation by Fgf10, Sox9, Shh and mutant Braf in normal (wildtype) and genetically modified mice engineered to constitutively or conditionally delete or express the targeted genes of interest.

In paper I, we show that branching morphogenesis is a key process in glandular development of the embryonic thyroid. Sox9, Fgfr2b and Ki-67 are co-expressed at distal tips of branching epithelial buds. Mesenchymal Fgf10 is crucial for embryonic thyroid growth. The $Fgf10^{-/-}$ mutant thyroid has a normal anatomical shape and uninterrupted functional differentiation but is severely hypoplastic due to defective branching. These findings uncover a novel mechanism of thyroid development in which branching growth generated by reciprocal mesenchymal-epithelial interactions determines final organ size.

Paper II investigates postnatal thyroid growth regulation. This identified growth retardation comprising reduced numbers of Ki-67+ proliferating cells in the thyroid of $Fgf10^{+/-}$ mutant mice. Thyroid growth is rescued postnatally in $Fgf10^{+/-};Shh^{+/-}$ double mutant animals, suggesting Shh regulation over Fgf10 signalling. This demonstrates for the first time gene dosage dependent regulation of postnatal thyroid growth accomplished through reciprocal interactions between Fgf10 and Shh signalling pathways.

In Paper III, we examine effects of conditionally expressed $Braf^{v600e}$ oncoprotein in Nkx2-1+ progenitors on growth and differentiation of the embryonic thyroid. Constitutive activation of MAPK pathway by mutant Braf in thyroid progenitors lead to a global growth response and a 4-fold increase in thyroid size at birth, however without disturbing the natural morphogenesis to a bilobed gland or the differentiation into functional follicular cells. Thyroid specific gene analysis confirmed expression of Tg, Nis, Tpo, $Tshr$ and $Pax8$, suggesting capability of iodination and thyroid hormone

production in mutant embryonic cells. These results indicate that mechanisms of *de novo* thyroid differentiation in mouse embryos resist dedifferentiation as regularly observed in MAPK-activated adult thyroid cells. A potential clinical importance of this novel finding relies on the fact that thyroid tumour cells carrying BRAF^{V600E} mutation are insensitive to radioiodine treatment due to repressed thyroid gene expression.

Sammanfattning på svenska

Organutvecklingen i fostret regleras av molekylära mekanismer som kan orsaka missbildningar om de påverkas negativt av t ex inaktiverande genmutationer, läkemedel och droger. En och samma mekanism kan vara verksam för helt olika organ med vitt skilda funktioner. Så är fallet med njurarna, lungorna och exokrina körtlar där utförsgångarna utvecklas på ett likartat sätt genom en process som kallas "branching morfogenes", vilket närmast kan översättas som ´förgrening på ett stereotypiskt sätt i flera generationer´.

Sköldkörteln (*glandula tyreoidea*) är en endokrin körtel med vilket menas att hormoner bildas och levereras till blodbanan för att via cirkulationen utöva sina effekter på andra ställen i kroppen. Sköldkörtelns föregångare i djurserien under ryggradsdjuren (i protokordaterna) är dock placerad i svalgväggen och kan därmed anses vara utsöndrade som andra exokrina körtlar. När ryggradsdjuren utvecklades för 500 miljoner år sedan konverterades sköldkörteln, för att öka förmågan att koncentrera jod och lagra hormon, till ett endokrint organ genom avknoppning från svalgväggens epitelskikt. Den processen äger fortfarande rum och kan studeras närmare i embryot under organutvecklingen.

Musmodeller efterliknar sköldkörtelns embryonala utveckling hos människa. I avhandlingens första delarbete visas att sköldkörteln bildas från förstadieceller (progenitorer) genom en branching-process som inte bara genererar fler celler utan också påverkar deras organisation i vävnaden innan de hormon-producerande folliklarna bildas. Mekanismen styrs av en tillväxtfaktor (Fgf10) som bildas i bindväven runt sköldkörtelanlaget. I möss som saknar *Fgf10* genen är sköldkörteln en dvärgkopia (15% av den normala körtelstorleken) men ser i övrigt normal ut. I det andra delarbetet visas att Fgf10 också stimulerar sköldkörtelns fortsatta tillväxt och follikelbildning efter födelsen, och att effekten är gen-dosberoende. Vi kan därmed konstatera att Fgf10 reglerar sköldkörtelns finala organstorlek genom en tidigare okänd mekanism varmed tillräckligt många celler bildas och organiseras i folliklar både embryonalt och postnatalt.

Den motsatta effekten av onormalt ökad tillväxt under sköldkörtelns embryonala utveckling studerades i avhandlingens tredje delarbete. Med transgen teknik stimulerades tillväxten av progenitorceller genom aktivering av en signalmolekyl (Braf) vars gen är muterad (Braf^{v600e}) på samma sätt som vid den vanligaste cancerformen (papillär tyreoideacancer) i sköldkörteln och som initierar och driver tumörutvecklingen. Resultaten visar att organutvecklingen fortsätter obehindrat trots att cellerna delar sig oftare och ger upphov till en jättekörtel som är fyra gånger större än normalt. Särskilt intressant är att Braf-muterade embryonala celler har kvar

förmågan att bilda folliklar och uttrycka gener (*Nis*, *Tg* och *Tpo*) som är nödvändiga för upptag av jod och hormonsyntes i sköldkörteln, vilket muterade sköldkörtelceller hos vuxna djur eller tumörceller inte kan. Embryonala celler besitter således en skyddande mekanism som förhindrar förlust av genuttryck vid onkogen aktivering, och som om den kunde återuppväckas i tumörceller skulle kunna göra strålbehandling med radioaktiv jod möjlig.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Liang S**, Johansson E, Barila G, Altschuler DL, Fagman H, Nilsson M.

   A branching morphogenesis program governs embryonic growth of the thyroid gland

   Development, 2018; 145 (2). Pii: dev146829

II. **Liang S**, Fagman H, Nilsson M.

   Thyroid developmental growth depends on Fgf10 gene dosage

   Manuscript

III. **Liang S**, Moccia C, Fagman H, Nilsson M.

   Mutant Braf (Braf^{v600e}) accelerates embryonic thyroid growth without interfering with glandular morphogenesis and *de novo* thyroid differentiation

   Manuscript

x

Content

xiii

Abbreviations

ALSG	Aplasia of lacrimal and salivary glands
AR	Androgen receptor
COPD	Chronic obstructive pulmonary disease
DuOX	NADPH oxidase
E	Embryonic day
EMT	Epithelial to mesenchymal transition
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
Fox	Factors forehead box
H2O2	Hydrogen peroxidase
HSPG	Heparin or heparan sulfate proteoglycan
iFGF	Intracellular Fibroblast growth factor
KO	Knockout
LADD	Lacrimo-auriculo-dento-digital
MAPK	Mitogen-activated protein kinase
MSG	Mouse submandibular gland
NIS	Sodium-iodide symporter
P	Postnatal day
PKA	Protein kinase A
PTC	Papillary thyroid cancer

PLC	Phospholipase C
Shh	Sonic hedgehog
Sox9	Sex-determining Region Y (SRY) box 9
TGF	Transforming growth factor
T3	Triiodothyronine
T4	Thyroxine
TG	Thyroglobulin
TPO	Thyroperoxidase
TRH	Thyrotropin releasing hormone
TSH	Thyroid stimulating hormone
TSHR	TSH receptor
UB	Ultimobranchial bodies
WT	Wild type
YAP	Yes-associated protein

Introduction

The thyroid gland

Anatomy, histology and cells

The human thyroid gland is H-shape and consist of two lobes interconnected by a median tissue called the isthmus [1]. It is positioned anteriorly in the lower neck region, overlying the second to forth tracheal cartilages (Fig. 1A). The simplest form of thyroid anatomy composed of a row of loosely associated follicles spread out along the anterior foregut is observed in zebrafish [2]. Conversely, all vertebrates obtain increasingly sophisticated and encapsulated thyroid glands that are located in the neck region close to the trachea [3]. However, its shape and size varies greatly between vertebrate species. Pigs, turtles and sharks have a single midline thyroid. In comparison, amphibians and birds have two separate distinct thyroid glands, whereas in lizards, mouse and humans two lobes are connected with a bridging isthmus [4].

The functional unit of the thyroid gland are the follicles (Fig. 1B). They are composed of a single layer of epithelial follicular cells or thyrocytes, which constitute varying shape and size. Follicular cells are the major cell type in the thyroid. They possess an apical surface facing the follicular lumen, which is filled with colloid and servers as a repository for prohormone, thyroglobulin (TG) and a basolateral surface oriented towards the circulation resting on a basement membrane [5]. Calcitonin producing parafolliclular C-cells are another important cell type scattered in between the follicles throughout the thyroid parenchyma, predominantly nearing the middle of the lobe. They are most often situated inside of same basement membrane as the thyrocytes. Additional cells such as endothelial and stromal fibroblast cells form an extensive vascular/stromal network that give functional support to the thyroid gland [1].

Thyroid hormone synthesis and regulation

The essential function of the thyroid gland is the synthesis of thyroid hormones such as thyroxine (T4), triiodothyronine (T3) and calcitonin (Fig. 1C). Thyroid hormones have multifaceted functions e.g. in development of the nervous system, somatic growth and homeostatic control of various physiological mechanisms including metabolism. Regulation of gene transcription is accomplished through binding of T3 to thyroid hormone receptors presented in the nucleus of the target cell [6]. Iodide is a key

component of thyroid hormone. From an evolutionary perspective, the thyroid gland appeared in animals living in a low iodide environment turning it into a highly efficient and specialised iodide capturing organ [7]. Trapping and transport of iodide from the blood stream occurs through a specialised sodium-iodide symporter (NIS), which is expressed at the basolateral plasma membrane of the thyrocyte [8]. NIS is functionally coupled to a sodium-potassium ATPase, which generates an ion gradient leading to negatively charged iodide entering the cells and eventually the follicular lumen against its concentration gradient [9]. TG, the prohormone, is synthesised by thyrocytes and secreted apically into the follicular lumen reservoir. Once iodide appears in the lumen, it is oxidised and covalently bonded to tyrosyl residues on TG [10]. The iodination reaction is catalysed by thyroperoxidase (TPO) at the apical plasma membrane in the presence of hydrogen peroxide (H_2O_2), which is produced by NADPH oxidase (DuOX) [11]. TPO also mediates the coupling of iodotyrosines into iodothyronines (T3 and T4), which remain covalently bonded to TG until it is degraded [10]. This is accomplished by iodinated TG that is internalised by micropinocytosis and macropinocytosis, the latter through a process mediated by pseudopods, and hydrolysed into thyroid hormones T3 and T4 through the actions of endosomes and lysosomes, respectively. Thyroid-stimulating hormone (TSH) released from the pituitary gland stimulates uptake of iodinated TG from the follicle lumen and release of thyroid hormone into the circulation [12, 13]. MCT8 expressed at the basolateral plasma membrane facilitates the transport of thyroid hormones out of the cell [14]. Calcitonin is a hypocalcemic hormone that acts as antagonist to parathyroid hormone. It is critical for calcium homeostasis in aquatic animals including fish and amphibians as well as in some mammals such as mice and rats. However, humans born without thyroid gland or patients with a total thyroidectomy do not require any calcitonin substitute [6].

TSH (synonymous to thyrotropin) is released into the blood circulation by the pituitary gland, and serves as the primary stimulus of thyroid hormone synthesis and secretion. It is regulated through a negative-feedback mechanism, which involve the hypothalamus, pituitary and thyroid gland, known as the hypothalamic/pituitary/thyroid axis. Thyrotropin releasing hormone (TRH) is synthesized and released by the hypothalamus. It binds to TRH receptors found in a population of endocrine cells called thyrotropes situated in the anterior pituitary. Circulating TSH finds and binds to the TSH receptor (THSR), a seven-transmembrane G protein-coupled receptor, located on the basolateral membrane of thyroid follicular cells. TSHR stimulates mainly through activation of the cyclic AMP/protein kinase A (PKA) signalling pathway the expression of several crucial thyroid genes including TG, NIS and TPO thereby promoting thyroid hormone synthesis.

Figure 1. Structure and function of the human thyroid gland. *(A) The thyroid gland is composed of two lateral lobes interconnected with an isthmus and is positioned in the anterior lower neck region. hb, hyoid bone; tc, thyroid cartilage; tr, trachea; ca; carotid artery. (B) Schematic cross section of a thyroid lobe. Colloid filled follicular cells varying in size and shape are distributed throughout the lobe, surrounded by network of capillaries. C cells and fibroblasts are closely associated with the follicles. F, follicle; c, C cell; f, fibroblast. (C) Overview of thyroid hormone synthesis. AJ, adherent junction; BM, base membrane; C, C cell; CaSR, calcium-sensing receptor; cd, colloid droplet; DUOX, NADHP oxidase; E, endothelium; H_2O_2, hydrogen peroxide; I^-, iodide; NIS, sodium-iodide symporter; L, follicular lumen; ly, lysosome; T, thyrocyte; T_3 & T_4, iodothyronines; TG, thyroglobulin; TJ, tight junction; TPO, thyroid peroxidase; TSH, thyroid stimulating hormone; TSHR, thyroid stimulating receptor. Reproduced with permission, Development [15].*

During normal homeostasis, TSH-stimulated uptake of iodinated TG from the follicle lumen and release of thyroid hormone into the circulation is balanced with the physiological requirements [12, 13]. Low levels of thyroid hormone feeds back mainly on the pituitary but also the hypothalamus thereby increasing the release of both TSH and TRH leading to normalization of thyroid function [16].

3

TSH and thyroid growth

Another role of TSH is the regulation of cell proliferation and growth of the thyroid gland [3]. However, it is known that thyroid organogenesis and functional differentiation is independent of TSH control. In mice, TSH dependent growth takes place postnatally, at a time when thyroid hormone secretion has begun and functional differentiation has been completed [17]. Thus, suggesting that intrinsic thyroid factors and signalling are involved during embryonic thyroid development. The mitogenic activity of TSH is mainly modulated by intracellular cAMP leading to activation of multiple signalling cascade and ultimately thyroid growth [18]. Additionally, TSH can also indirectly promote thyroid cell proliferation by inducing autocrine growth factor and receptor expression [19]. In mice it have been shown that over stimulation of TSH induce thyroid hyperplasia and eventually development of cancer [20]. In Braf induced thyroid cancer mouse models, down-regulation of thyroid hormone genes results in severe hypothyroidism and elevated levels of TSH in turn induce dedifferentiation of thyroid follicular cells and increase malignancy [21]. In humans, high TSH levels are associated with nodular goitre, thyroid cancer and even extrathyroidal extension tumours [22-24].

Thyroid development

Origin of thyroid progenitor cells

Thyroid progenitors of the follicular cell lineage have their origins from the anterior endoderm and form the midline thyroid primordium. They can be first recognized as a placode in the midline of the pharyngeal floor at the base of the future tongue thus forming the thyroid primordium. The thyroid progenitor cells can be discriminated from the rest of the endoderm by expression of four key thyroid transcription factors namely, Nkx2-1, Foxe1, Hhex and Pax8 that may either act individually or in combination depending on developmental stage [25-28]. Although each of the aforementioned transcription factors are expressed in several embryonic tissues, it is only in the thyroid that they are all co-expressed and play essential roles not only in thyroid bud formation but also in the growth and functional differentiation and proliferation of the gland [29]. Loss of any of these genes will inevitably result in athyreosis or severe thyroid hypoplasia [25, 27, 30, 31]. It is still largely unknown how thyroid precursors are recruited to the placode as factors regulating this process have not yet been identified.

Thyroid C cells, the second endocrine cell type, have another embryonic origin. During thyroid organogenesis, the ultimobranchial bodies (UB) are specified as two lateral anlagen from the fourth and fifth pharyngeal pouches in mice and humans, respectively. The paired UB deliver C cell precursors to the embryonic thyroid by fusing with the prospective lateral lobes derived from the midline primordium These deliver progenitor C cells to the midline thyroid primordium [32, 33]. For a long time, it was thought that C cell progenitors originated from neural crest cells. It was first proposed in the 1960s and later strengthened in the 1970s with quail-chick grafting experiments that demonstrated the fate of quail crest cells transplanted to chick embryos providing calcitonin-producing cells to the avian ultimobranchial glands [34-37]. However, recently a study using genetic lineage tracing in mice has overturned this long-standing concept, providing direct evidence that Sox17+ anterior endoderm is the origin of thyroid C cells [38]. Another supporting evidence indicates that embryonic C cells also co-express forkhead box transcription factors Foxa1 and Foxa2, both of which are typically expressed and acting in concert in many foregut endoderm derivatives [38].

Thyroid morphogenesis

Thyroid development is a multiple stage process in which the embryonic thyroid undergoes a series of morphological changes. As mentioned previously, the thyroid is composed of follicular and C cells both originating from the anterior endoderm. The follicular progenitors are specified as one median anlagen in the pharyngeal floor, whilst the UB, which transfers C cell progenitors to the midline primordium, emergence as two lateral primordia in which progenitor cells are specified in the inferior-most pharyngeal pouch. Human and mouse essentially follow the same stages of thyroid morphogenesis but with different timing. Following their specification and budding from the endoderm, these independent primordia migrate caudally, resulting in the fusion of UB with the midline primordium. Subsequently, onset of folliculogenesis including differentiation of progenitors into hormone producing cells, and further proliferation take place in the developing gland [39]. Main stages of thyroid development (Fig. 2) will be described in more detail below.

At embryonic day (E) E8.5-9.5 in mouse (4th week in human), the median thyroid primordium containing follicular progenitors co-expressing Nkx2-1, Foxe1, Hhex and Pax8 can be identified as a placode at the base of the tongue in the most anterior part of the foregut endoderm. The paired UB budding from the fourth pharyngeal pouch are identified one day later. Between E9.5-11.5 in mouse and 4-5th week in human, the thyroid placode gradually thickens prior to budding into the ventral mesenchyme. Eventually the thyroid bud is connected to the pharyngeal endoderm only by a thin

thread. Around E11.5-12.5 in mouse (5-7th week in human) the caudally moving midline primordium moves further away from pharyngeal endoderm finishing budding from the endoderm. After descent to an inferior position in the neck, the developing primordium extends bilaterally and establishes a cap-like shape along the 3rd pharyngeal arch arteries, the beginning of bilobation. By this time, the UB have detached from the fourth pharyngeal pouch and are migrating towards the midline primordium.
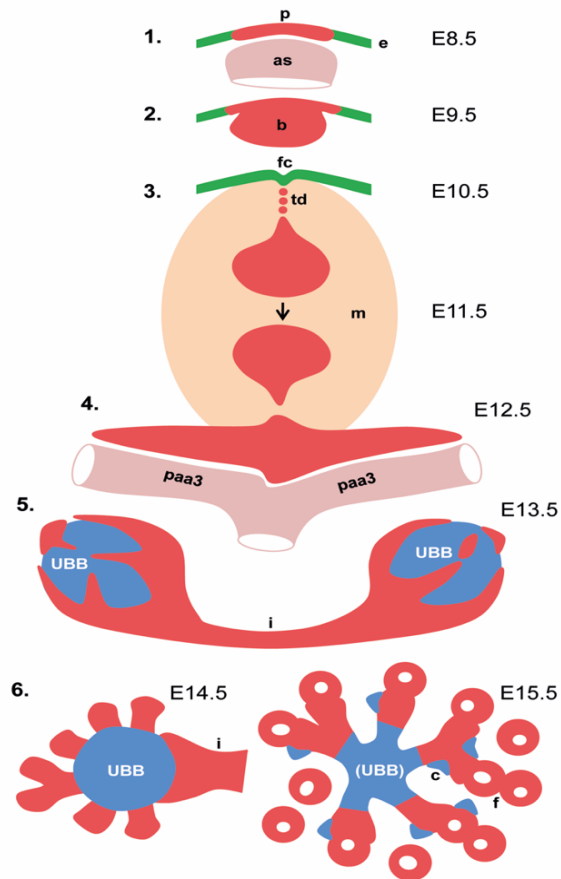

Figure 2. Overview of thyroid development. *(1) Specification of thyroid progenitor cells known as the placode (p) in the pharyngeal floor of the endoderm (e). (2) Thyroid placode gradually thickens prior to budding. (3) Detachment and caudal migration of the thyroid primordium. (4) Bilateral extension of the thyroid primordium along the third pharyngeal arch arteries (paa3). (5) Bilobation of the thyroid primordium and fusion of midline primordium with a pair of lateral ultimobranchial bodies (UBB). Isthmus (i) connects the two thyroid lobes. (6) Branching growth of the thyroid parenchyma. Thyroid progenitor cells differentiate into functional follicular cells (f) and C cell (c) producing TG and calcitonin, respectively. b, thyroid bud; fc, foramen caecum; m, mesenchyme; td, thyroglossal duct. Reproduced with permission, Development [15].*

At E12.5-13.5 in mouse (7-8th week in human), the midline primordium further elongates and meets the UB on both sides after which fusion of the two independent primordial tissues result in the formation of a single unified gland. The fusion process appears to involve engulfment of the entire UB by the midline primordium, but the two progenitor cell populations are not yet mixed. At this stage, the gland consists of two rudimentary lobes positioned on either side of the trachea interconnected by a primitive isthmus. By E15.5, the first signs of *de novo* follicle formation are evident, and many C cell progenitors have migrated out of the centre of the lobes to a more peripheral position. Notably, from this stage both pools of thyroid progenitors synchronously differentiate into follicular cells and C cells producing TG and calcitonin, respectively; before maternal supply supported the embryo/foetus with these hormones. At 17.5 in mouse (12th week in human), the thyroid parenchyma is further enlarged and organized into a network of cord-like structures in which new micro-follicles continue to develop. Each follicle is now surrounded by a rich capillary network, which will assist in the transfer of thyroid hormone from follicles into the blood stream [40] (Fig. 2). The thyroid gland continues to growth postnatally for which TSH-mediated plays an important role [16]. Factors regulating embryonic thyroid growth, a major concern and objective of the thesis work, are currently largely unknown.

Fibroblast growth factors

Fibroblast growth factors (FGFs) consist of a large polypeptide family with pleomorphic properties and functions. They can act both as extracellular proteins that bind to and activate cell surface receptor tyrosine kinases called fibroblast growth factor receptors (FGFRs) as well as intracellular FGFs (iFGFs), which are FGFR independent [41]. Both varieties of FGFs are found throughout the animal kingdom in both invertebrates and vertebrates. They have diverse and fundamental roles both during development and in adult organism. In embryonic development, FGFs are involved in differentiation, proliferation and migration. In adults, FGFs function as homeostatic factors associated with metabolism, tissue maintenance, repair and regeneration, but they also contribute to pathogenesis of cancer [41, 42].

In papers I and II we use the *Fgf10* deficient mouse model to investigate the role of Fgf10 in thyroid development. A general overview of FGFs and some important details on the developmental roles of Fgf10 in mice are provided below.

7

Fibroblast growth factor ligand

In 1973, FGFs were first discovered in the pituitary extracts and subsequently isolated from the brain and pituitary gland [43, 44]. The name refers to their fibroblast stimulating capabilities [45]. In vertebrates, the FGF family presently accounts for 22 members, which are then further divided into 7 subfamilies depending on sequence homology and phylogeny. Most FGFs possess the amino terminal signalling peptide for secretion. However, iFGFs are lacking the signalling peptide and thus remain intracellular. They are involved in e.g. regulation of neuronal and myocardial excitability [46] and as far we know iFGFa do not participate in embryonic development.
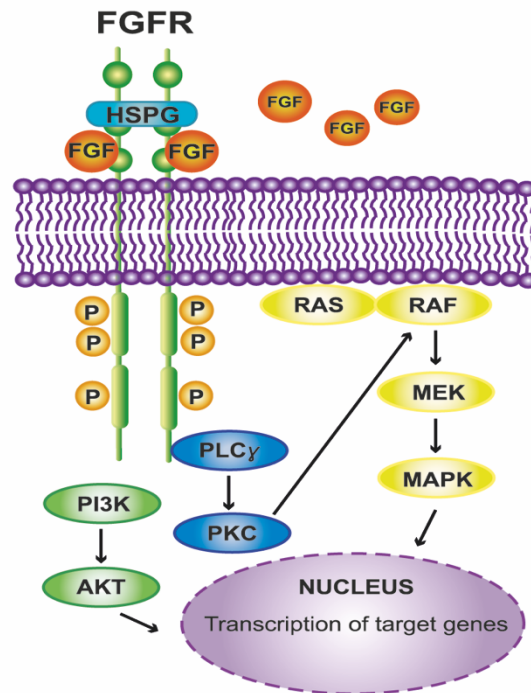
Figure 3. FGF-FGFR signalling pathway. *A simplified illustration showing activation of the FGF-FGFR pathway. Following FGF ligand binding to FGFR, the receptor dimerizes and is activated through multiple autophosphorylations of cytoplasmic kinase domains. Activation of FGFR leads to initiation of multiple downstream signalling pathways, resulting in gene regulation. Heparan sulfate proteoglycan (HSPG) interacting with the FGF-FGFR complex, stabilising ligand-receptor interaction and protect FGF against degradation.*

Variations in the N- and C-terminal sequences provide for different biological roles FGF ligands. An important feature of FGFs signalling involves binding to heparin or heparan sulfate proteoglycan (HSPG) on target cells (Fig. 3). The HSPG interaction which involves both FGF and FGFR, provide protein-protein stabilisation thus increasing ligand affinity for the receptor and aiding in the dimerization of the FGF-FGFR complex. Furthermore, the HSPG binding stabilises and protects FGF against thermal denaturation and proteolytic degradation [42, 47-49].

Fibroblast growth factor receptor

FGF ligands induce their diverse functions through binding to and signalling through tyrosine kinase FGFRs expressed on surface on target cells (Fig. 3). The FGF-FGFR multiplex consists of a symmetrical dimer of two FGF-FGFR-HSPG units, each FGF ligand interacting with both FGFRs and both receptors being in direct contact with each other [50]. The FGFR family consists of four receptors (FGFR1-4), which are differentially expressed across multiple tissues [51]. Each FGFR is made up of an extracellular domain, which contains two or three immunoglobulin-like domains and a heparin binding sequence within the immunoglobulin-domain II, a single transmembrane domain, and an intracellular tyrosine kinase domain [48, 52]. The carboxy-terminal of immunoglobulin-domain III is alternatively spliced and gives rise to two variants, IIIb or IIIc, of FGFR [53]. In general, isoform IIIb is expressed on cells of epithelial linages and IIIc is found on mesenchymal cells [54-56]. Since FGFs are released by both epithelial and mesenchymal cells and the corresponding receptors are expressed on the opposite tissue, reciprocal interactions likely to prevail during development. Following ligand binding, FGFR dimerization promotes receptors activation through multiple autophosphorylation of cytoplasmic kinase domains [57]. Activated FGFR triggers phospholipase C (PLC)1 and FGFR substrate 2 to further activate multiple downstream signalling pathways comprising protein kinase C, Ras-mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)-Akt [58] (Fig. 3).

Fibroblast growth factor 10

FGF10 signalling is extensively involved in both early embryonic development and in organogenesis. The *Fgf10* gene was originally discovered in rat embryos [59]. *FGF10* encodes a secreted protein containing 215 amino acids that has been identified in all vertebrates, including humans and mice [60, 61]. Genetic deletion of the *Fgf10* gene in mice results in death shortly after their birth due to lung agenesis [62]. Importantly, Fgf10 belongs to the paracrine FGF family. Accordingly, due to a high affinity for

HSPG Fgf10 or any other members of the paracrine family typically act close to their origin of expression, thus signalling in order to regulate epithelial and mesenchymal interactions within the immediate microenvironment. As will be discussed in more detail, employing this mode of action FGF10 is essential for branching morphogenesis in multiple organs during embryonic development [62-64]. FGF10 secreted from the mesenchyme, signals to epithelial FGFR2 of the IIIb splice variant [59, 65]. The strong specificity in this response is demonstrated in *FGFR2* knockout (KO) mice [66, 67], which essentially reproduce the phenotype of *Fgf10* null mutant [62, 63, 68]. The multiple roles of Fgf10 in development and disease will be focused on more details below.

Fgf10 in embryonic development

Utilising the *Fgf10* deficient mouse model, numerous studies have demonstrated that Fgf10 signalling is essential for development of a broad variety of major organs and tissues, including thyroid [61]. Most if not all endodermal organs are affected, and in particular lung development provides understanding of the great importance of Fgf10 as a morphogen. Fgf10 is expressed in the lung mesenchyme during early lung development. Although in *Fgf10* KO mice lung development is severely impeded, the initial specification and formation of the lung primordium still follows a normal development pattern. However, Fgf10 is found to be crucial for outgrowth of left and right primary lung buds, survival of epithelial progenitors and, to be discussed in more detail below, branching of the lung epithelium [62, 68]. Interestingly, overexpression of Fgf10 can lead to the rescue of *Fgf10* null lung phenotype [69]. The embryonic liver in the *Fgf10$^{-/-}$* mice show a less prominent malformation with signs of dysgenesis due to impaired hepatoblasts proliferation [70]. FGF10 is also involved in early stages of prostatic budding and epithelial proliferation during later stages of prostate development, with prostatic agenesis seen in *Fgf10* KO animals [71]. In pancreas development, both dorsal and ventral buds are normally formed in *Fgf10* null mutants, but both epithelial branching morphogenesis and differentiation are retarded primarily due to failure of epithelial progenitor cell proliferation in the pancreatic bud [72]. In salivary gland development, epithelial progenitors form a network of ducts and adopt branching morphogenesis. Fgf10 expressed by neural crest derived mesenchyme surrounding the gland is a key component in this process, as indicated by growth arrest at the early placode stage resulting in a severely hypoplastic salivary gland in null mutants [73, 74]. During embryonic limb formation, Fgf10 is crucially involved in the initiation and proximal-distal growth of the limb bud. It is expressed in the lateral plate mesoderm and induces Fgf8 expression through Fgfr2b in superficial ectodermal cells. Fgf8 in turn induces growth of the underlying mesoderm thus forming a positivity feedback loop, which facilitates cell proliferation and growth of the limb bud [75-77].

Notably, animals with *Fgf10* deleted fail to develop limbs, therefore Fgf10 acts as a key morphogen and regulator of limb formation [62].

Earlier studies have reported thyroid agenesis, meaning no thyroid anlage was originally formed, in both *Fgf10* and *Fgfr2IIIb* deficient mice [61, 67]. However, in a recent study on various *Fgf10* mutants including *Fgf10*$^{-/-}$ a severely hypoplastic unilateral thyroid remnant was observed [78]. Based on these findings, in paper I we decided to extensively characterise thyroid development in *Fgf10* null mice. Since this study concerned novel findings of branching morphogenesis this important mechanism for normal organogenesis will be introduced to some extent.

Fgf10 in branching morphogenesis

Numerous mammalian organs and glands are composed of branched structures that are designed for exceptional efficiency and function, e.g. to increase parenchymal size with a preserved conducting system. This involves a developmental process known as branching morphogenesis that dichotomously will give rise to multicellular tubular networks. During embryonic development, branching morphogenesis is thus crucial for the formation of exocrine glands but also other organs as the lungs and urinary system [79]. Most developmental processes are regulated by a range of paracrine and autocrine signalling factors. Branching morphogenesis is no different with FGF10 being one of the major signalling factors operating in a wide range of developing organs. Although variations exist in the signalling factors controlling branching growth, the overall branching pattern is similar in different organs, thus adopting a common program that is likely to be conserved among species.

Lung

During early lung development, lung buds form tree-like tubular networks, its growth is characterised by a repeated process of elongation, terminal bifurcation and lateral budding [80]. Fgf10 expression is highly concentrated in the distal mesenchyme of the bud tip during elongation, along the sides of the bud tip during terminal bifurcation, and in the mesenchyme bordering the elongating bud during lateral budding. This indicates the importance of Fgf10 in directional outgrowth of the endoderm-derived progenitors and induction of multiple buds through lateral budding and proliferation of epithelial cells [68, 81]. Genetic deletion of either *Fgf10* ligand or its receptor *Fgfr2* contributed to a reduction of epithelial branches, as well as enhanced cell death, indicating the significance of Fgf10-Fgfr2 signalling in lung epithelial branching and growth [82]. Branching growth has been shown to be negatively regulated by sonic hedgehog (Shh), expressed in the distal epithelium where lung branching occurs [83].

11

Shh at the lung bud tip will gradually downregulate Fgf10 expression in the mesenchyme adjacent to the elongating bud tip thus regulating bud growth. Furthermore, Shh may also regulate the shape of the bud tip by controlling both quantity and placement of mesenchymal Fgf10 expression as well as expression of Fgfr2b in the bud epithelium [83-85]. Deletion of Shh contributed to the loss of mesenchymally expressed downstream targets of Shh, thus terminating branching morphogenesis and arresting lung development at a rudimentary bud stage [85]. A recent study in mice has demonstrated that loss of *Patched-1*, encoding the Shh target receptor, in lung mesenchyme contributes to defective lobe formation due to a branching defect [86]. Notably, reduced of Fgf10 expression in the distal mesenchyme is also evident in *Patched-1* mutant lung [86]. Together, these results show Shh-Patched-1 signalling regulates downstream Fgf10 expression and further strengthening the concept that both Fgf10 and Shh interactively play crucial roles in lung branching morphogenesis.

Submandibular gland

Mouse submandibular gland (SMG) development is initiated around E11.5 with the pre-bud stage followed by budding stage at E12.5, in which the oral epithelium thickens and grows into the mandibular arch mesenchyme thus forming the SMG bud. As the epithelium proliferates the SGM primordium turns into a solid elongated epithelial stalk. Subsequently, by E13.5 onwards, bud tip branching generates an epithelial branching network that will ultimately give rise to the ductal systems of the fully developed SMG [87, 88]. Fgf10-Fgfr2b signalling is essential during SMG development, as it induces both epithelial cell proliferation and branching. In both *Fgf10* and *Fgfr2b* deficient mice, the MSG is severely hypoplastic, with only a remaining rudiment reminiscent to the earliest initial bud stage indicating bud tip branching is totally absent. Furthermore, *Fgf10* and *Fgfr2b* haplodeficiency resulted in salivary branching hypoplasia [74]. These findings highlight the importance of Fgf10-fgfr2b signalling acting in a dose dependent manner during development. A recent study has indicated that Sex-determining Region Y (SRY) box 9 (Sox9) is expressed in the distal epithelium during bud stage as well as during branching morphogenesis [73]. Ablation of *Sox9* gene in the SMG epithelium leads to reduction in proliferation and absence of branching. Moreover, mesenchymal Fgf10 positively regulates Sox9 through the MAPK signalling pathway [73].

Pancreas

The pancreatic gland is specified from the primitive gut endoderm forming dorsal and ventral pancreatic anlagen. As the two anlagen proliferate from bud stage, they will undergo branching morphogenesis and differentiation, ultimately fusing with each other to form one single composite gland [89]. During early pancreatic development in mice, Fgfr2b and its high-affinity ligand Fgf10 are expressed in the non-differentiated epithelium and the mesenchyme that surrounds the pancreatic buds, respectively [72, 90]. High expression levels of Fgf10 and Fgfr2b coincide with rapid growth of the epithelial buds during branching morphogenesis. In $Fgf10^{-/-}$ mouse embryos, both pancreatic buds exist and elongation of the epithelium is initiated, as is the differentiation of endocrine and exocrine cells. However, the epithelium bud size is greatly reduced and branch-like epithelial structures are entirely absent, as a result of significant decrease in epithelial cell proliferation [72].

Fgf10 in disease

Hereditary diseases

In human, *FGF10* haploinsufficiency caused inactivating mutations leads to several congenital anomalies including aplasia of lacrimal and salivary glands (ALSG), autosomal dominant lacrimo-auriculo-dento-digital (LADD) and chronic obstructive pulmonary disease (COPD) [91-93]. ALSG is an autosomal dominant inherited disorder that is characterised by aplasia, atresia or hypoplasia of the lacrimal and salivary glands, which is caused by a heterozygous nonsense mutation in exon 1 of the *FGF10* gene [91]. A heterozygous misssense mutation in exon 1 of *FGF10* gene contributes to the LADD syndrome, which is a hereditary disorder characterised by lacrimal duct hypoplasia, cup-shaped ears and dental and digital anomalies, [93]. COPD patients display structural changes of the lung epithelium leading to remodelling of the airways and obstruction of airflow. Since Fgf10 signalling in crucial for lung development as well as epithelial renewal, it is likely that *FGF10* haploinsufficiency caused reduced lung function in a *FGF10* gene dosage dependent manner [92].

Cancer

Fgf10 has been implicated in several types of human cancer, such as pancreatic, breast and prostate carcinomas. Additionally, skin tumorigenesis and ameloblastoma have been associated with aberrant Fgf10 signalling. Pancreatic cancer is the fourth leading cause of death malignancy in the western world [94]. Studies have shown that FGFR2

expression stems from cancer cells, whereas surrounding stromal cells express FGF10, i.e. reminiscent of the expression pattern in embryonic development. High expression of FGFR2 is detected in patients with poor prognosis, and FGF10 has been found to induce both migration and invasion of cells with FGFR2-IIIb expression, thus indicating a strong stromal-epithelial interaction through FGF10-FGFR2b mediated signalling in the tumours [95]. In breast cancer, both FGF10 and FGFR2 are overexpressed with the FGFR2-IIIb isoform being more abundant. Interestingly, somatic *FGFR2* mutations have been identified in breast tumours. This comprises heterozygous missense mutations in exon 14 encoding the tyrosine kinase domain 2 and in the immunoglobulin-like domain 2. Both mutations have been observed to strongly increase tyrosine the kinase activity of FGFR2. Furthermore, tyrosine autophosphorylation were detected in FGFR2-IIIb mutants resulting in increased tyrosine kinase activity [96, 97]. In multifocal prostate adenocarcinoma, paracrine FGF10 secretion by the tumour stroma gives rise to increased androgen receptor (AR) expression in the malignant epithelial cells [98]. It has been reported that AR gene is upregulated in ~30% of human prostate cancers, a mechanism associated with androgen independence, which results in more aggressive tumour metastasis phenotype [99, 100]. In addition, enhanced expression of FGF10 also resulted in the development of differentiated multifocal prostate adenocarcinoma [98]. Ameloblastoma is a benign epithelial tumour of the jaw, which distinguished by local invasion and high recurrence. FGF10 were found to stimulate ameloblastoma cell line proliferation through the mitogen-activated protein kinase (MAPK) pathway [101]. Squamous cell carcinoma is a common skin cancer, which can be induced by deletion of epidermal tumour suppressor gene Pten in mouse models. In skin tumourgenesis, deletion of Pten in keratinocytes results in increased FGF10 protein level [102, 103]. Elevated FGF10 expression were also found in poorly differentiated human squamous cell carcinoma samples acting in concert with diminished levels of PTEN expression. Additionally, Fgf10-Fgfr2 signalling induces epidermal hyperplasia and papilloma formation leading to epidermal tumourgenesis in Pten null mice [104]. Together, these findings indicate FGF10 signalling as a key mechanism in skin tumourgenesis through downstream PI3K-mTOR pathway. Presently, Fgf10 has not been studied in human thyroid cancer. However, a significant reduction of FGFR2 expression has been observed in both thyroid follicular adenoma and papillary carcinoma and have been suggested as a potential maker for early thyroid cancer diagnosis [105]. Consistent with the previous finding, forced expression of FGFR2 in thyroid epithelial cancer cells lead to diminished invasiveness *in vitro* and reduced metastasis and tumour growth *in vivo* [106].

Sonic hedgehog

In the early 1980s, the hedgehog gene was originally identified in *Drosophila* for its importance in segmental development [107]. In mammals, the hedgehog family consists of three ligands, sonic hedgehog (Shh), desert hedgehog and indian hedgehog. They are secreted as both as mitogens and morphogens, playing fundamental roles in embryonic patterning, growth and organ morphogenesis. The hedgehog signalling pathway consist of Patched-1 (a 12 transmembrane receptor), Smoothened (Smo; a G-protein coupled receptor like several transmembrane protein) and three transcription factors (GLI1, GLI2 and GLI3) [108]. In the absence of hedgehog protein, membrane expressed Patched-1 receptor exerts inhibitory an effect and prevents activation of the co-receptor Smo from entering the primary cilium and preventing GLI from translocating to the nucleus and activating gene expression. Upon secretion of extracellular hedgehog, it binds and activates membrane Patched-1 expressed on the target cell, which reduces the inhibition on Smo leading to its activation and subsequent translocation into the primary cilium. Further signal transduction into the nucleus activates of GLI transcription factors inducing gene expression [109].

Shh signalling in embryonic development

In vertebrates, Shh is a crucial signalling component during embryonic development [108]. It provides vital mitogenic and morphogenic signallings for growth and differentiation in multiple organs and glands during organogenesis. In early mouse embryonic development, Shh is expressed in the endodermal tracheal diverticulum, oesophagus and tracheal primordia, which is essential in foregut morphogenesis [110]. As mentioned earlier, Shh is expressed in the undifferentiated distal epithelium of the developing lung and influences lung branching morphogenesis through negative regulation of Fgf10 signalling. In the $Shh^{-/-}$ mouse model, lung morphogenesis is terminated at the primary bud stage due to defective branching [85]. Overexpression of Shh increases proliferation and interrupts branching process, indicating that local Shh signalling is required for lung branching morphogenesis [83]. Interestingly, in the *Shh* null lung, expression of Fgfr2b remains unchanged, however Fgf10 were abundantly expressed in the mesenchyme next to the lung epithelium, which is contrasted by pockets of highly localised mesenchymal Fgf10 expression observed in WT counterparts [85]. This, demonstrates the influence of Shh on Fgf10 spatial expression in the distal mesenchyme during initial stages of lung organogenesis. Pancreatic development is also regulated by Shh, where its signal acts as a repressor of pancreatic growth [111]. Forced expression of Shh interrupts pancreatic organogenesis and transforms the pancreatic mesoderm into smooth muscles and intestinal mesenchyme [112]. Interestingly, at E18.5, whilst the *Shh* null mouse

embryos display drastically reduced body mass due to multitude of developmental defects, the pancreatic mass and number of endocrine cells still remain similar to its WT counterparts, indicating that pancreatic growth is not affected by *Shh* deletion [111].

In thyroid development, animals with *Shh* KO mutants display thyroid dysgenesis [113]. The thyroid primordium is specified and initially develops normally but fails to separate into a bilobed gland. Nevertheless, follicular cells within the single thyroid mass have completed functional differentiation and are expressing TG. Furthermore, ectopic differentiated follicular buds were observed in developing trachea, suggesting that Shh signalling not only mediate tracheal morphogenesis but act as a repressor against inappropriate differentiation of progenitor cells [113]. Although postnatal thyroid growth is TSH dependent, however intrinsic growth modulating factors from within the gland is still unknown. In Paper II, we explore this aspect of postnatal thyroid growth.

BRAF^{V600E} oncogenic mutation

The RAS-RAF-MEK-ERK-MAP kinase signalling pathway is a conserved cascade that mediates signals from cell membrane to the nucleus in order to regulate cellular homeostasis including proliferation, differentiation, and survival [114, 115]. This pathway is constitutively activated due to somatic mutations in key genes in approximately 30% of all human cancers [116]. BRAF is a serine/threonine protein kinase, expressed at high levels in hemopoietic cells, neurons and testes [117]. Furthermore, it is also the main isoform found in thyroid follicular cells [118]. In human cancers, BRAF is frequently activated through somatic point mutation within the kinase domain. It is estimated that mutated *BRAF* gene is found in 7% of all cancers [119]. More than 40 BRAF mutations have been identified in a variety of human cancers. The clinically most important is a single base substitution resulting in valine to glutamic acid replacement at amino acid residue 600 (V600E) that encodes a constitutively active BRAFV600E oncoprotein with a 500-fold gain in kinase activity and accounts for 90% of all BRAF mutations [119]. BRAFV600E is expressed in a variety of human cancers including melanoma and carcinomas in colon, lung and thyroid [120].

The BRAF kinase domain is a bilobed structure separated by a catalytic cleft. In its inactive conformation, the conservative DFG motif located in the activation segment form hydrophobic interactions with residues in the ATP binding site thus blocking the

16

catalytic cleft from binding to ATP or the substrate. Activation of the kinase domain through phosphorylation of the activation segment leads to destabilisation of hydrophobic interactions and shift the DFG motif to its active state, exposing the catalytic cleft for subsequent MEK phosphorylation and activation. The BRAF^{V600E} mutation interrupts and destabilises hydrophobic amino acid interactions within the activation segment causing the DFG motif to shift to the active conformation and thereby exposing the catalytic cleft. This results in constitutively active BRAF^{V600E} that has a higher affinity for and more efficiently phosphorylates MEK [120, 121].
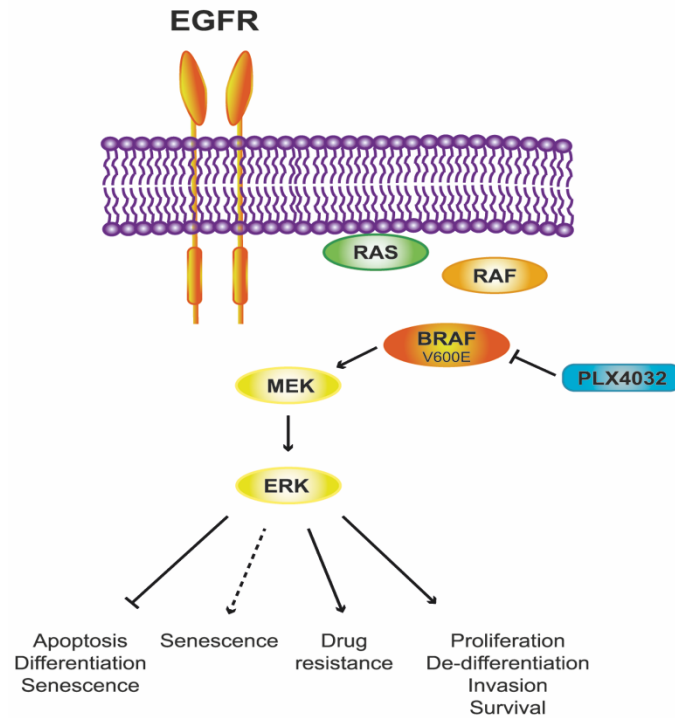

Figure 4. BRAF^{V600E} activation of the MAPK signalling pathway in thyroid cancer. *A simplified schematic diagram showing constitutively active BRAF^{V600E} oncoprotein activating the MAPK signalling cascade, resulting in both inhibition and stimulation of multiple cellular processes. The potent BRAF^{V600E} inhibitor, vemurafenib (PLX4032) are used to treat PTC patients who have developed resistance against radioactive iodine therapy.*

BRAF mutation in papillary thyroid cancer

Papillary thyroid cancer (PTC) derived from epithelial follicular cells is the most common tumour type consisting of 80-85% all thyroid malignancies [122]. *BRAF^{V600E}* oncogene is the most prevalent mutation in poorly differentiated PTCs and occurs in 37-50% of patients with PTC [123, 124]. It has been associated with poor prognosis characterised by extensive lymph node metastases, extrathyroidal invasion, high risk recurrence and poor survival, especially in patients that are resistant to radioactive iodine [125, 126] (Fig. 4). A fundamental treatment for thyroid cancer patients is radioactive iodine therapy, which relies on that efficient iodide uptake tumour cells that retain NIS expression. Many BRAF^{V600E} induced PTC tumours exhibit significant downregulation of NIS, which is mediated by an autocrine loop involving BRAF induced transforming growth factor (TGF)-beta, thereby rendering radioiodine ineffective [125, 127]. TGF-beta has also been shown to induce epithelial to mesenchymal transition (EMT), typically characterised by loss of E-cadherin expression and gaining a mobile phenotype. EMT in PTC subsequently leads to increased levels of migration and invasion of tumour cells [125]. Phase I and II clinical trials using a potent kinase inhibitor, vemurafenib (PLX4032), to mutant BRAF in PTC patients refractory to radioiodine treatment have shown promising results that have contributed to anti-tumour activities [128, 129]. However, a common problem is acquired drug resistance that limits the use of single treatment with vemurafenib or other RAF inhibitors [130] (Fig. 4).

Tumours generated in mouse models with thyroid specific expression of Braf^{v600e} closely resemble the phenotype in BRAF^{V600E} induced human PTC. These models generate dedifferentiated tumours that are characterised by loss of both follicular cell architecture lacking colloid, EMT induced invasion and extrathyroidal dissemination. Notably, abolished expression of thyroid specific genes including *Nis*, *Tg* and *Tpo* in response to constitutive activation of the MAPK pathway results in severe hypothyroidism and superstimulation by elevated TSH levels, which is a confounding factor that modifies tumorigenesis [131-133]. Nonetheless, specific MAPK inhibitors such as PLX4720 and PD0325901 that are antagonists of BRAF^{V600E} and MEK, respectively, have been associated with partial tumour regression as well as restoring iodine incorporation capacity of Braf mutant follicular cells, thus making them at least transiently acquiescent to radio iodine therapy [131]. In a recent study, oncogenic Braf^{v600e} was constitutively activated in the developing embryonic pituitary gland. The hyperactivation of MAPK pathway lead to increased proliferation and growth of the gland, however functional differentiation of progenitor cells was impaired [134]. In Paper III, we investigated the significance of conditional Braf^{v600e} activation on growth and differentiation in the developing embryonic thyroid.

18

Aims

The overall aim of this thesis is to elucidate mechanisms involved in growth regulation of the developing thyroid gland.

Specific aims for the papers included are:

Paper I: To characterise thyroid growth and functional differentiation in $Fgf10^{-/-}$ mice during embryonic development and to investigate factors involved in the branching morphogenesis.

Paper II: To investigate the effects of gene dosage dependent regulation in thyroid growth using $Fgf10$ haploinsufficient mice.

Paper III: To examine the impacts of constitutively active $Braf^{v600e}$ oncogene on growth and differentiation during embryonic thyroid development.

19

Materials and methods

Experimental animals

Targeted disruption of *Fgf10*, *Shh* and *Braf^CA* as well as PCR genotyping of genomic DNA have been described previously [62, 135, 136]. Generation and genotyping of *Nkx2-1Cre* and *TgCreER^T2* transgenic lines have been described [137, 138]. **Paper I**: *Fgf10^{+/-}* mice were bred to generate *Fgf10* null, heterozygous and homozygous mutant embryos and wild-type siblings that were at E9.5, E12.5, E15.5 and E18.5. *NkxCre2-1Cre*, *WntCre*, *Sox2Cre*, *Sox9^{flox}* and *ROSA^{mTmG}* (hereafter called *mTmG*) mice were obtained from Jackson laboratory. *Nkx2-1Cre* were crossed with *Sox9^{flox}* and *mTmG* to generate *Nkx2-1Cre;Sox9^{fl/+}* and *Nkx2-1Cre;sox9^{fl/fl}* and *Nkx2-1cre;mTmG* embryos and pups, respectively. **Paper II**: E18.5 and P10 experimental animals were obtained from mating of *Fgf10^{+/-}* or *Shh^{+/-}* and *Fgf10^{+/-}* with Shh+/-. **Paper III**: Embryos were obtained from mating of *Nkx2-1Cre* with *Braf^CA*, to generate *Nkx2-1Cre; Braf^{CA/+}* and *TgCreER^T2* with *Braf^CA*, to generate *TgCreER^{T2};Braf^{CA/+}* embryos of E9.5, E10.5, E12.5, E5.5 and E18.5, as well as adult mice. All animals in were maintained on a C57BL/6 background. E0.5 was determined on the morning upon detection of vaginal plug. Yolk sac or animal tails were used for DNA extraction and PCR genotyping. Three or more independent experiments on embryos obtained from different litters were analysed for each genotype and developmental stage (unless otherwise stated). AKM6 at the Clinical Research Centre, Karoliska University Hospital (Huddinge, Sweden) was employed for re-derivation of Fgf10^{+/-} mice before transfer to the local animal facility. All animal experiments were approved by the local ethics committee at the University of Gothenburg.

Immunohistochemistry and immunofluorescence

Embryos were dissected and fixed in 4% paraformaldehyde overnight at 4°C, washed in PBS at pH 7.3 for 3x5 min prior to overnight incubation in 30% sucrose at 4°C, embedding in Tissue Tek compound (Sakura, Zoeterwoude, The Netherlands) and storage at -80°C. Sagittal serial sections at 10 μm for E9.5 and E10.5 and transversal for E12.5, E15.5, E18.5 were sectioned using a cryostat and collected on Superfrost Plus glass slides (Mentzel, Gläser, Germany). Sections were permeabilized for 20 min with 0.1% Triton X-100 in PBS then blocked using 2% normal donkey serum (Jackson ImmunoResearch Laboratories) in PBS for 1 hour before overnight incubation with primary antibodies (see below) in blocking buffer at 4°C. After washing with 0.1% Triton X-100 in PBS for 3x5min, secondary antibodies diluted in blocking buffer was added to incubate sections for 1 hour followed by 0.1% Triton X-100 in PBS wash for

3x5min and 30 min incubation with streptavidin-FITC, both at room temperature. Sectioned were then counterstained with DAPI (Sigma Aldrich) and mounted using Fluorescence Mounting Medium (Dako). Images were acquired using a Zeiss Axioscope 2 Plus Fluorescence microscope equipped with a Nikon DS-Qi1Mc camera and processed with NIS Element Imaging and ImageJ software.

Immuno-reagents

The following primary antibodies were used for immunohistochemical staining: rabbit anti- Nkx2-1 (1:1000; PA0100, EAtlab srls); rabbit anti-Fgf10 (1:500; ABN44, EMD Millipore); rabbit anti-Fgfr2 (1:5000; ab10648, Abcam); goat anti-hSOX9 (1:500; AF3075, R&D Systems); rat anti E-cadherin (ECCD-2; 1:500; 205604, Calbiochem); rabbit anti-ki-67 (1:100; ab15580, Abcam); rabbit anti-Tg (1:3000; A0251, Dako); rabbit anti-Pax8 (kindly provided by Roberto di Lauro, Universita di Napoli Federico II, Naples, Italy); rabbit anti-calcitonin (1:500; A0576, Dako). Secondary antibodies used include: Rhodamine Red-X-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories); Rhodamine Red-X-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories); biotin-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories); biotin-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories) and followed by Streptavidin-FITC (Dako).

Morphometry

Serial sections co-immunostained with anti-Nkx2-1 and anti E-cadherin surrounding the entire thyroid placode at E9.5, thyroid primordium at E12.5 and embryonic thyroid gland at E15.5 and E18.5 were utilized to assess thyroid size. Images were captured in a Zeiss Axioscope 2 Plus fluorescence microscope equipped with a Nikon DS-Qi1Mc camera and processed with NIS Element Imaging software. For E9.5, all Nkx2-1-positive cells of the thyroid placode were counted. For E12.5, perimeter of the thyroid primordium was encircled in each section and the area calculated. For E15.5 and E18.5, due to the greater organ size only Nkx2-1/E-cadherin-positive parenchyma in every fourth section was encircled for area calculations. Data acquire from E12.5, E15.5 and E18.5 were then used for 3D reconstructions using WinSURF software version 4.3, which allows the relative volumes of objects to be determined and compared. For E18.5, the parathyroids and trachea were also reconstructed based on E-cadherin staining in the same sections.

21

Gene expression analysis

RNA extraction and reverse transcription

After mice were sacrificed, thyroid glands were dissected and immediately stored at -80°C in RNAlater (Thermo Scientific). Frozen tissues were mechanically disrupted and homogenized by metal bead in lysis buffer using TissueLyser II (Qiagen) at 25-30 Hz for 5min. RNA was extracted using the RNeasy mini kit (Qiagen) according to manufacturer's instructions. The final amount of RNA was determined by measuring the absorbance at 260nm (A260) in a spectrophotometer (NanoDrop1000; Thermo Scientific). cDNA synthesis was performed using TATAA GrandScript cDNA Synthesis Kit (TATAA Biocenter). Briefly, 2μl 5X TATAA GrandScript RT Reaction Mix, 0.5μl TATAA GrandScript RT Enzyme and 2.5μl nuclease-free water were added to the sample for a final volume of 10μl. The following thermal program was used: 22°C for 5 min, 42°C for 30min, 85°C for 5min on a T100 Thermal Cycler (Bio-Rad).

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed with CFX384 Touch real-time cycler (Bio-Rad). Each 6μl reaction contained 1× TATAA SYBR GrandMaster Mix (TATAA Biocenter), 400nM of each primer and 2μl cDNA. The temperature profile was 95°C for 1min followed by 50 cycles of amplification (95°C for 3sec, 58°C for 30s and 72°C for 10sec). The formation of expected PCR products was confirmed by agarose gel electrophoresis and all samples were analysed by melting curve analysis.

Statistics

For estimation of thyroid size changes, the mean volume of mutant glands was calculated as a percentage of the mean control volume of WT glands. Statistical analysis of thyroid volume estimates was determined using Student's t-test with $P \leqslant 0.05$ considered to be significant. RT-qPCT data in graphs is shown mean ± SEM of three independent experiments. The t-test was performed with $P \leq 0.05$ considered to be significant. All quantitative data and statistical analyses were assembled using GraphPad Prism version 6 (GraphPad Software).

Results

Paper I

Fgf10 deficiency results in thyroid hypoplasia

Fgf10 have been implicated in the regulation of branching morphogenesis in several organs derived from endoderm. Previous studies on thyroid development did not pay attention to this mechanism but reported that that the thyroid is missing in *Fgf10* homozygous mutant mice. Therefore, we reinvestigated the involvement of Fgf10 in thyroid morphogenesis in this mutant.

A thyroid bud was indeed formed and there were no significant alterations of thyroid primordium size or number of Nkx2-1$^+$ progenitor cells between *Fgf10$^{+/+}$* and *Fgf10$^{-/-}$* embryos in early organogenesis taking place between E9.5 and E12.5 (Fig. 6A-F,I, paper I). However, a few days later, growth of the prospective thyroid lobes was significantly inhibited generating a gland with one-fifth of the normal size in *Fgf10* deficient animals (Fig. 6G-I, paper I). 3D reconstruction confirmed a severely hypoplastic gland and further showed it yet had a normal shape (Fig. 6J, paper I).

Fgf10 stimulates both branching morphogenesis and folliculogenesis in the embryonic thyroid

Growth and expansion of the thyroid parenchyma forming the thyroid lobes followed a conspicuous branching pattern. In the tiny thyroid in *Fgf10$^{-/-}$* embryos, branching growth was much restricted resulting in short and undersized epithelial cords (Fig. 6G,H, paper I). This was accompanied by reduced number of Ki67+ cells diminished accumulation of proliferating cells in the distal tips of branches.

Thyroid transcription factor Pax8 was ubiquitously expressed throughout thyroid development also during folliculogenesis (Fig. 8A-C, paper I). However, in Fgf10$^{-/-}$ thyroids the total number of Pax8$^+$ cells were greatly reduced and they were found in diminutive follicle like structures (Fig. 8E-E´´´, paper I). Undersized follicles contained few cells, occasionally only two, surrounding its lumen (Fig. 8F,F´, paper I), suggesting incomplete recruitment of follicular cells due to inhibited proliferation.

23

Together these findings indicate that Fgf10 is required for thyroid branching growth of distal progenitors and maturation and growth of emerging follicles.

Sox9 expression in thyroid development during budding branching morphogenesis and differentiation

Sox9, a key transcription factor that is heavily involved in embryonic organogenesis, was not previously studied in the thyroid. Since Sox9 more recently is assigned a regulatory role in branching morphogenesis, this led us to investigate whether Sox9 is expressed in thyroid development and in particular during branching growth.

Sox9 was weakly expressed confined to the cytoplasm in primordial cells in the thyroid placode (Fig. 1A′,A′′, paper I). One day later nuclear staining of Sox9 was observed in the emerging thyroid bud (Fig. 1B′,B′′, paper I). As the midline thyroid enclosed the Sox9 negative UB, thyroid progenitors showed medium to high Sox9 expression (Fig. 1C-D″). During thyroid branching, Sox9 positive cells were highly concentrated at the distal ends of branching parenchyma (Fig. 1E-G, paper I). Subsequently, during differentiation and folliculogenesis, the Sox9 expression pattern persisted in follicular cells with same trend as earlier, diminishing in the central parenchyma and strongly expressed in the lobe periphery (Fig. 2C-F, paper I). In Fgf10$^{-/-}$ embryos, Sox9 expression maintained in the hypoplastic thyroid but was more evenly distributed also in the interior of lobe.

Taken together, our data suggested that Sox9 is a new thyroid developmental regulator that likely participates already in bud stage, possibly facilitating in bud growth. Later on, Sox9 may contribute to branching morphogenesis and overall embryonic thyroid growth. We also investigated a possible thyroid phenotype in *Nkx2-1Cre;Sox9$^{fl/fl}$* mice. Although Sox9 expression was abolished in most cells there was still evidence of distal Sox9+ cells in the mutant thyroid parenchyma. Notably, branching growth was not significantly altered by conditional deletion of *Sox9* in Nkx2-1+ progenitors, and similar results were obtained from mutant lungs of the same animals. It was therefore not possible to elucidate more precisely the function of Sox9 in thyroid development, or whether Sox9 might also contribute to thyroid differentiation and folliculogenesis.

Paper II

Embryonic and postnatal thyroid growth is impeded in *Fgf10* haploinsufficient mice

Since Fgf10 regulates lung development gene dose-dependently, heterozygous *Fgf10* null mutant mice were investigated for a possible phenotype.

In late thyroid organogenesis, embryonic thyroid size in *Fgf10* heterozygous mutants was reduced to 65% in comparison to WT littermates (Fig. 1A-D, paper II). We further examined $Fgf10^{+/-}$ pups postnatally at P10. The size of the thyroid lobes was slightly smaller in the mutant compared to the control mice. However, the most noticeable difference was that $Fgf10^{+/-}$ mutant lobes had a significant reduction in the number of Ki-67+ proliferating cells (Fig. 2A-B´,3, paper II).

This indicated that embryonic thyroid growth is regulated by Fgf10 in a gene dosage-dependent manner, and that Fgf10 maintains this influence on the thyroid during postnatal lobe enlargement.

Shh signalling modifies *Fgf10* dependent thyroid growth

Shh strongly influences early thyroid development non-cell autonomously presumably by affecting the growth of surrounding mesenchymal tissues. It also impairs fusion with the UB. However, although thyroid progenitors do not express Shh its expression take place in a minority of cells at the end of development when the lobes enlarge [139], correlating to Fgf10 induced parenchymal growth. We generated a double heterozygous $Fgf10^{+/-};Shh^{+/-}$ mice in order to determine whether Shh might regulate Fgf10 signalling during postnatal growth of the thyroid.

There were essentially no difference in terms of gross anatomy of the lobes between compound *Fgf10* and *Shh* haploinsufficient mice and WT controls. However, the number of Ki-67+ follicular cells was significant increased (Fig. 2, paper II). Moreover, Ki-67+ proliferating cells were evenly distributed throughout the lobe rather than positioned in the periphery as in WT animals (Fig. 2C,C´, paper II). Quantification of Ki-67+ cells indicated that diminished proliferation in $Fgf10^{+/-}$ null mutants can be restored by monoallelic loss of Shh (Fig. 3, paper II). Shh thus regulates thyroid development also postnatally.

Paper III

Conditional *Braf*^{v600e} activation in thyroid progenitors induces hyperplasia of the embryonic thyroid gland

In a recent gain-of-function study in mice, the constitutive active oncogene *Braf*^{v600e} was reported to induce hyperplastic growth, abnormal morphogenesis and loss of endocrine lineage differentiation in the developing pituitary gland [134]. By recombined mice carrying the floxed *Braf*^{v600e} allele with *Nkx2-1Cre* mice we generated a mouse model with conditional mutant Braf expression in embryonic thyroid progenitor cells.

The number of Nkx2-1+ cells in the thyroid placode did not differ between Braf mutants and its WT littermates (Fig. S1A,B, paper III). However, as the thyroid bud emerged a substantial increase in Nkx2-1+ cells were observed (Fig. S1C,D, paper III), indicating early onset of oncogene activation. This trend of accelerated growth continued during further thyroid development coinciding with the major peak of progenitor cell proliferation. As a result, the thyroid primordium was considerably larger in *Braf* mutant mice then in age-matched controls both before bilobation (Fig. 1A,B, paper III) and subsequently as the lateral lobes were formed (Fig. 1 C-F´´, paper III). 3D reconstruction further showed that the shape and position of the Braf mutant thyroid was similar to the WT orthotopic gland, although the size was increased 4-fold at all instances from E12.5 onwards (Fig. 1G,H, paper III).

These findings indicated that thyroid progenitors can be precociously triggered to multiply in response to targeted expression of *Braf*^{v600e} oncoprotein. However, despite accelerated embryonic thyroid growth resulting in a severely hyperplastic gland the natural course of organogenesis is not obstructed. This suggests that the morphogenetic program is tightly controlled restricting or buffering the action of inappropriate signalling of the MAPK pathway.

Mutant Braf alters the pattern of thyroid branching

As found in Paper I, branching morphogenesis is a crucial growth phase in lobe enlargement characterized by proliferation of distal epithelial progenitor cells. We decided to elucidate whether this growth pattern was altered in *Nkx2-1Cre;Braf*$^{CA/+}$ embryos.

26

In *Braf* mutant thyroids, the enlarged primordium showed traces of branching morphogenesis already during bilateral elongation prior to lobe formation (Fig. 3A paper III). Ki67+ cells were abundantly expressed and evenly distributed (Fig. 3A paper III). Furthermore, Sox9 was upregulated and comprised most if not all embryonic thyroid epithelial cells (Fig. 3D-D´´, paper III), which thus differed from the Ki-67/Sox9 pattern in the WT thyroid (Fig. 3C-C´´, paper III).

These observations suggested that mutant Braf may promote thyroid growth also by modifying the branching pattern of thyroid morphogenesis.

Braf mutant thyroid progenitors differentiate into functional follicular cells

Since mutant Braf or constitutive activation of MAPK signaling by other means results in dedifferentiation of adult thyroid cells, we investigated whether conditional expression of $Braf^{600e}$ in thyroid progenitors might interfere with mechanisms required for thyroid differentiation in the embryo.

Normally, mouse thyroid progenitor cells started to express TG approximately at E15, which was reflected by the presence of TG in the cytoplasm of most cells except in the distal epithelial tips were branching growth occurred (Fig. 4A-A´´, paper III). At E18.5, TG accumulated in the lumen of numerous follicles distributed throughout the gland (Fig. 4B-B´´, paper III). Remarkably, the expression pattern of TG was almost identical in the hyperplastic gland in $Nkx2-1Cre;Braf^{CA/+}$ mice Braf mutant thyroid (Fig. 4C-C´´, paper III). Thus, Braf mutant thyroid progenitors developed into follicles filled with TG, the only difference being on average a smaller follicle and lumen size in mutants (Fig. 4D-D´´, paper III).

Thyroid specific gene expression was examined and compared in $Nkx2-1Cre;Braf^{CA/+}$ and WT pups at P0. This showed similar transcript levels of *Pax8*, *Tpo* and *Tshr* and moderately decreased expression of *Tg* and *Nis* in mutants (Fig. 5A,B, paper III). Similar results were obtained in $TgCreER2;Braf^{CA/+}$ newborn mice in with Cre was embryonically induced at E14.5. In contrast, conditional activation of Braf in young adult $TgCreER2;Braf^{CA/+}$ mice at P30 promptly repressed all thyroid specific genes investigated (Fig. 6, paper III). Taken together, these results indicated that embryonic thyroid cells resist dedifferentiation induced by oncogenic Braf.

Discussion

Proliferation of thyroid progenitor cells leading to growth of the thyroid epithelium during development has not yet been fully elucidated. To understand the molecular mechanisms behind thyroid growth is a key step in recognizing causes behind diseases including thyroid dysgenesis, the most common cause of congenital hypothyroidism, and thyroid cancer. The work in this thesis could potentially provide some insights into these diseases.

In **paper I**, we presented a novel growth specific mechanism that governs branching morphogenesis during mouse embryonic thyroid development. The concept of branching growth involving Fgf10 during organogenesis has been well established in several branching organs and glands [68, 72-74]. Our findings in **paper I** indicate that the branching program stimulated by mesenchymal Fgf10 is also an essential process in the growth of embryonic thyroid during development. Thyroid branching of progenitor cells transforms solid primordium, expanding into cord-like parenchyma thus facilitating subsequent folliculogenesis to all areas of the lobe. Genetic deletion of *Fgf10* resulted in a hypoplastic thyroid gland, due to significantly reduced proliferation of thyroid progenitors during branching growth. A significant decrease in Ki-67+ cells was observed at rudimentary branch tips resulting in short undersized epithelial cords. This mirrors observations found in other branching organs including lung, pancreas and submandibular glands, which indicate that Fgf10 stimulation is required for proliferation of epithelial cells leading to branch elongation and further branching [59, 62, 68, 72, 74]. These results suggest that mesenchymal Fgf10 act directly as a mitogen, stimulating progenitor cell proliferation and branching growth of the embryonic thyroid in mice.

The transcription factor Sox9 has emerged as an essential factor in branching morphogenesis during lung development and functions downstream of the Fgf-Fgfr signalling [140]. In pancreas development, mesenchymal Fgf10 is required in order to maintain Sox9 expression in epithelial cells, which in turn will promote Fgfr2 expression needed to promote growth and maintain the pancreatic progenitor state [141]. In salivary organogenesis, from bud stage and throughout branching growth, Sox9 is expressed in the distal epithelium. Furthermore, Fgf10 positively regulate Sox9 expression through Erk signaling that induces distal progenitor proliferation and branching [73]. We observed strong Sox9 expression already at an early bud stage and becoming highly concentrated in proliferating distal progenitors during branching morphogenesis. Expression of Sox9 during thyroid organogenesis therefore resembles expression patterns of other organs during development. However, its expression is

28

not dependent on Fgf10 signaling, suggesting a different mode of regulation of Sox9 in the embryonic thyroid as compared to other foregut derivatives.

A number of human cancers have been linked to FGF10-FGFR2IIIb signalling. In thyroid cancer, FGFR2IIIb gene expression have been evaluated using patient samples [105]. Its expression has been found to be upregulated in normal and hyperplastic thyroid tissue. In contrast, FGFR2IIIb expression is dramatically downregulated in both follicular adenoma and papillary carcinoma. Taken together, these observations suggest that downregulation of FGFR2IIIb might be a feature of early stage of thyroid carcinogenesis and could potentially be used an early thyroid cancer diagnostic marker [105]. Pancreatic cancer cells express FGFR2, whilst surrounding stroma cells show FGF10 expression. Stromal FGF10 mediated signaling through epithelial FGFR2IIIb and induces migration and invasion in tumour cells [95]. Mesenchymal FGF10 expression induce upregulation of androgen receptor leading to more aggressive tumourigenesis [98-100]. Although no studies have been done on the role of FGF10 in thyroid cancer, our analysis of Fgf10 mediated growth in the developing thyroid could also be relevant to thyroid cancer development.

Findings in **paper II** indicated that *Fgf10* gene dosage regulates cell proliferation of thyroid progenitors in a graded manner. Embryonic and postnatal thyroid growth in mice were both restricted by *Fgf10* haploinsufficiency, resulting in diminished numbers of Ki67+ proliferating cells and a decreased thyroid lobe size. During submandibular gland development, *Fgf10* heterozygous mutant mice exhibited branching hypoplasia, with fewer ducts and terminal buds resulting in a hypoplastic gland [74]. Later, at an early postnatal age, a reduction of salivary gland size has been reported. In addition, $Fgf10^{+/-}$ mutants suffered from hyposalivation [142]. In lung development, $Fgf10^{+/-}$ mutants display epithelial branching and structural defects [143]. Together, these data suggest that the Fgf10 induced growth signal might be finely tuned in a gene dosage dependent manner possibly as a means to adapt the level of cell proliferation in a spatiotemporal context during the different stages of organ development.

Shh null mutants exhibit thyroid dysgenesis embryonic thyroid development. Specification of the thyroid primordium takes place but it fails to bifurcate. Furthermore, functional differentiation is completed in the single thyroid mass [113], which thus partly reminds of the *Fgf10* null mutant phenotype. During lung branching morphogenesis, Fgf10 signaling is negatively regulated by Shh [83]. Mesenchymal *Fgf10* expression adjacent to branching bud tips is gradually downregulated by Shh, thereby regulating bud growth [85]. We therefore investigated whether a negative feedback loop involving Shh could be involved in Fgf10-mediated growth of the embryonic thyroid. Since Shh is not expressed in early thyroid progenitor cells and

only a small subpopulation of epithelial cells possesses Shh expression after differentiation [113], we decided to examine a possible interaction of Shh and Fgf10 postnatally. Using a double heterozygous mutant *Fgf10*$^{+/-}$*;Shh*$^{+/-}$, we showed that simultaneous monoallelic deletion of *Fgf10* and *Shh* significantly increased the Ki-67 proliferation index in thyroid parenchyma as compared to *Fgf10* haploinsufficient and age-matching control mice. This result suggests that Shh plays a crucial role in the negative regulation Fgf10-Fgfr2b signalling during postnatal thyroid growth. This differs fundamentally from the growth-promoting effect of the Shh pathway in advanced thyroid cancer [144-146].

Paper III showed how *Braf*^{v600e} oncogene, conditionally activated in early thyroid development, exerts a pronounced but yet restricted growth effect on the embryonic thyroid gland. In human PTC, the *BRAFV600E* oncogene is the most common mutation occurring in 37%-50% of all patients [123, 124]. It is correlated with poor prognosis and characterised by lymph node metastases, extrathyoidal invasion, high risk recurrence and poor survival [125, 126]. In embryos, the thyroid bud in *Braf*^{v600e} mutants is substantially larger in size than in WT counterparts. This trend continues throughout development, with mutant Braf accelerating thyroid growth by 4-fold resulting in a dramatically enlarged gland but still maintaining its normal anatomical shape as well as the ability of progenitor cells to differentiate into functional follicular cells. There were no signs of adenoma development. Embryonic thyroid cells thus seem to response differently to mutant Braf that adult neoplastic cells featuring an abnormal growth pattern.

Developmental effects of oncogenes have been studied before. Global expression of *Braf*^{v600e} in mice is embryonic lethal at E7.5 whereas embryos with mosaic expression of mutant Braf survive until late gestation but die before birth [147]. In lung development, conditional activation of *Braf*^{v600e} oncogene by *Shh* promoter induces abnormal airway development that is initiated by MAPK signalling [148]. Expression of *Kras* in lung progenitor cells leads to suppression of alveolar differentiation in late development [140]. In a recent study, conditional activation of *Braf*^{v600e} in the embryonic pituitary gland resulted in prominent augmentation of MAPK-ERK activity, accelerated proliferation and maintenance of a Sox2$^+$ progenitor cell pool. However, most progenitors failed to differentiate into hormone producing cells, indicating that hyperactive proliferation of progenitor cells impedes terminal differentiation of the same cells [134].

For a long time, radioactive iodine has been the preferred treatment for thyroid cancer patients. Its uptake into the follicular cells is mediated by NIS. Constitutively active *BRAFV600E* in PTC induce downregulation of NIS thereby rendering radioactive iodine treatment ineffective [125, 127]. Animal models that induce PTC show close resemblance of human tumour phenotype, exhibiting dedifferentiated tumours, characterised by loss of follicular structure and colloid production as well as down regulation of thyroid specific genes including *Nkx2-1*, *Pax8*, *NIS*, *TG*, *TPO* and *TSHr* [131-133]. We showed here that thyroid differentiation genes including *Tg* and *Nis* are induced and expressed initially at normal or nearly normal levels in embryonic thyroid cells co-expressing mutant Braf. In contrast, transcript levels of all thyroid differentiation genes were gradually supressed postnatally by mutant Braf. Taken together, our findings suggest that constitutively active *Braf^{v600e}* oncogene leading to hyperactive MAPK signalling is differentially regulated during thyroid development versus in adult follicular cells. The developmental program in embryonic thyroid presently harbours unknown regulatory mechanisms, which are able to overcome dedifferentiation induced as a consequence by oncogenic *BRAF^{v600e}* activation in human PTC.

An integrated view on embryonic growth and organ size

Currently, it is surprisingly how little it is known about mechanisms that control final size of organs. However, the Hippo signalling pathway, which was first discovered in *Drosophila*, has been identified as a regulator of organ size [149]. In adult mouse liver, overexpression of the transcription factor Yes-associated protein (YAP) involved in the Hippo pathway results in four-fold increase of liver size due to increased cell numbers [150]. Interestingly, termination of YAP overexpression rapidly reverses the liver back to its normal size, suggesting intrinsic mechanisms controlling organ size may be reversibly suppressed [151]. Other studies have reported that conditional inactivation of Hippo pathway kinases Mst1 and Mst2 in adult mouse liver dramatically increase its size [152-154]. They are thought to be important in maintaining hepatocyte quiescence and restrict postnatal growth [152-154]. In pancreatic development, YAP is initially expressed throughout the developing pancreas but then becomes confined to only exocrine cells following differentiation [155]. In contrast to the liver, conditional Mst1/2 KO in pancreas resulted in a reduced sized gland with disorganized exocrine tissue [155]. YAP overexpression in embryonic mouse pancreatic progenitors induced expansion of ductal cells and simultaneously impaired differentiation of acinar cells [156]. Together, these studies show differential regulation of organ size by the Hippo signalling pathway that likely exerts its effects in an organ-specific context. A putative role of Hippo as a determinant of thyroid development is presently unknown. Nonetheless, the work in this thesis provide

evidence and insight into both intrinsic (cell-autonomous) and extrinsic (non-cell-autonomous) factors that regulate embryonic and postnatal thyroid growth and ultimately the size of the gland.

Conclusions

Paper I

Branching morphogenesis is a major driving force during embryonic thyroid growth in mice. Mesenchymal Fgf10, signalling through epithelial Fgfr2b receptors in conjunction with a key transcription factor, Sox9, are critical for thyroid branching growth. The *Fgf10* null thyroid have defective branching epithelium leading to a severe hypoplastic gland although progenitor cell differentiation remains unaffected, generating functional follicular cells. Expression of Sox9 in proliferating Fgfr2b positive thyroid epithelial progenitors strongly mirror its pattern of expression in classical branching tissues. Here, we demonstrate a new mechanism, involving the Fgf10-Sox9 program, which helps to promote branching morphogenesis during embryonic thyroid development.

Paper II

Fgf10 regulates thyroid growth in a gene dosage dependent manner. Growth of the gland is hampered in *Fgf10* heterozygous mutants during both embryonic and postnatal development due to reduced thyroid epithelial cell proliferation. Monoallelic loss of *Shh* restores diminishing thyroid cell proliferation in $Fgf10^{+/-}$ mutants during postnatal growth thus suggesting that Shh imposes negative control on Fgf10-Fgfr2b signalling.

Paper III

Conditional activation of the $Braf^{v600e}$ oncogene in developing mouse thyroid confers hyperplasia of the orthotopic gland whilst concurrently maintaining intact functional differentiation of Braf mutant progenitors into thyroid follicular cells. Accelerated progenitor cell proliferation induced in early development does not impair the developmental program of thyroid morphogenesis.

33

Acknowledgement

Well…this is it, the big book is almost completed and one of the most important chapter still needs to be written. For many, this chapter is most likely to be written when the brain has seized to a complete halt and I am no different. So therefore, I apologizes in advance to people that I forget to mention below.

Here we go…first and foremost I would like to thank **Mikael Nilsson**, my main supervisor for many years. A big thank you to you for giving me the opportunity to complete my PhD studies under your supervision. It has not been all smooth sailing and there have been many hurdles throughout this journey, but somehow, we always manage to pull through. I really appreciate the "no worries" attitude and the chilled-out approach from you, as well as the freedom that you have given me to run different projects, some turned out to be quite alright, whilst others have failed miserably. Thank you for your guidance and for placing your trust and confidence in my abilities. It has been an honour and pleasure working with you during this time. I thank you from the bottom of my heart.

A big thank you goes to you **Henrik**, my co-supervisor**,** you have always been extremely helpful with my work. You always think a few steps ahead and bring suggestions to solve the numerous issues that I have encountered in my projects. I admire the knowledge and enthusiasm that you have for science, it truly inspires me.

My wonderful friends from the Nilsson lab, **Therese**, you are a cornerstone of our lab, without you managing the lab, our research will halt to an immediate stop. Thank you for helping me with my experiments, you never say no to any of my requests around the lab, without your help and support I would not be writing this book right now. Also, thank you for choosing the best Julbord every xmas. **Ellen** and **Elin**, you guys are awesome, always helpful either around the lab or at EBM and always carrying big smiles. Thank you, guys, for bringing fika and ice cream (Ellen, you make bloody awesome homemade ice cream). I am sorry that I sometimes mixed up your names, I know you guys hate it (from the evil looks you give me). **Carmen**, our newest and youngest lab member. Although we have only known each other for a short period of time, sometimes I feel that we are old friends. I admire your talents and skills in the lab, keep up with the good work.

I would like to express my gratitude to past and present friends and colleagues from Sahlgrenska Cancer Center, EBM and administrators at Institute of Biomedicine, who have helped me over the years and have been there for leisurely chats and work related discussions. **Sohelia**, **Ka-Wei**, **Mattias**, **André**, **Tajana**, **Azbija**, **Lydia, Berglind, Maryam**, **Sandra**, **Ágota**, **Karin Larsson**, **Anna Wenger**, **Susanna**, **Anna**

Danielsson, **Kristell**, **Paul**, **Daniel Andersson, Anna Nordin**, **Pernilla Gregersson**, **Christin Karlsson**, **Yvonne Arvidsson**, **Murali**, **Jonna Sjöholm**, **Carina Petersson and Andrea Frateschi**.

I also would like to thank **Somsundar, Camilla**, **Roger**, **Malin**, **Pontus**, **Tony** and **Kitti** who have become good friends of mine over the years. It is always awesome hanging out with you guys, doesn't matter if is it curry night, Liseberg, Thai food or dumpling. Thank you for your support, you guys are great.

Anna Linder, the most generous, caring, compassionate human being I have ever meet in my life. And not to mention an amazing mother. Pep talk, moral support, helping with the thesis, you name it and she will do it for you. Remember, life has its ups and downs but no matter what it throws at you, I know you can overcome it! YOU CAN DO IT! You are awesome and you are the best!

My beautiful daughter **Alice**, you are a bundle of joy. Who have ever thought that such a little person can bring so much joy and happiness to one's life. No amounts of words could ever describe the love I have for you.

Last but not least, I would like to thank my mom and dad for their unconditional love. I'm forever grateful. I love you both very much.

References

1. Dumont, J.E., et al., *Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors.* Physiol Rev, 1992. **72**(3): p. 667-97.
2. Alt, B., et al., *Analysis of origin and growth of the thyroid gland in zebrafish.* Dev Dyn, 2006. **235**(7): p. 1872-83.
3. Maenhaut, C., et al., *Ontogeny, Anatomy, Metabolism and Physiology of the Thyroid*, in *Endotext*, L.J. De Groot, et al., Editors. 2000, MDText.com, Inc.: South Dartmouth (MA).
4. Gorbman, A., *Some aspects of the comparative biochemistry of iodine utilization and the evolution of thyroidal function.* Physiol Rev, 1955. **35**(2): p. 336-46.
5. Rousset, B., et al., *Chapter 2 Thyroid Hormone Synthesis And Secretion*, in *Endotext*, L.J. De Groot, et al., Editors. 2000, MDText.com, Inc.: South Dartmouth (MA).
6. Boelaert, K. and J.A. Franklyn, *Thyroid hormone in health and disease.* J Endocrinol, 2005. **187**(1): p. 1-15.
7. Venturi, S., et al., *Environmental iodine deficiency: A challenge to the evolution of terrestrial life?* Thyroid, 2000. **10**(8): p. 727-9.
8. Dai, G., O. Levy, and N. Carrasco, *Cloning and characterization of the thyroid iodide transporter.* Nature, 1996. **379**(6564): p. 458-60.
9. Eskandari, S., et al., *Thyroid Na+/I- symporter. Mechanism, stoichiometry, and specificity.* J Biol Chem, 1997. **272**(43): p. 27230-8.
10. Dunn, J.T. and A.D. Dunn, *Update on intrathyroidal iodine metabolism.* Thyroid, 2001. **11**(5): p. 407-14.
11. De Deken, X., et al., *Characterization of ThOX proteins as components of the thyroid H(2)O(2)-generating system.* Exp Cell Res, 2002. **273**(2): p. 187-96.
12. Ericson, L.E., *Exocytosis and endocytosis in the thyroid follicle cell.* Mol Cell Endocrinol, 1981. **22**(1): p. 1-24.
13. Rousset, B. and R. Mornex, *The thyroid hormone secretory pathway--current dogmas and alternative hypotheses.* Mol Cell Endocrinol, 1991. **78**(1-2): p. C89-93.
14. Di Cosmo, C., et al., *Mice deficient in MCT8 reveal a mechanism regulating thyroid hormone secretion.* J Clin Invest, 2010. **120**(9): p. 3377-88.
15. Nilsson, M. and H. Fagman, *Development of the thyroid gland.* Development, 2017. **144**(12): p. 2123-2140. doi: 10.1242/dev.145615
16. Yen, P.M., *Physiological and molecular basis of thyroid hormone action.* Physiol Rev, 2001. **81**(3): p. 1097-142.

17.   Postiglione, M.P., et al., *Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15462-7.

18.   Kimura, T., et al., *Regulation of thyroid cell proliferation by TSH and other factors: a critical evaluation of in vitro models.* Endocr Rev, 2001. **22**(5): p. 631-56.

19.   Cocks, H.C., et al., *Thyroid stimulating hormone increases angiogenic growth factor expression in rat thyrocytes.* Clin Otolaryngol Allied Sci, 2000. **25**(6): p. 570-6.

20.   Schaller, R.T., Jr. and J.K. Stevenson, *Development of carcinoma of the thyroid in iodine-deficient mice.* Cancer, 1966. **19**(8): p. 1063-80.

21.   Franco, A.T., et al., *Thyrotrophin receptor signaling dependence of Braf-induced thyroid tumor initiation in mice.* Proc Natl Acad Sci U S A, 2011. **108**(4): p. 1615-20.

22.   Fiore, E., et al., *L-thyroxine-treated patients with nodular goiter have lower serum TSH and lower frequency of papillary thyroid cancer: results of a cross-sectional study on 27 914 patients.* Endocr Relat Cancer, 2010. **17**(1): p. 231-9.

23.   Gerschpacher, M., et al., *Thyrotropin serum concentrations in patients with papillary thyroid microcancers.* Thyroid, 2010. **20**(4): p. 389-92.

24.   Haymart, M.R., et al., *Higher serum TSH in thyroid cancer patients occurs independent of age and correlates with extrathyroidal extension.* Clin Endocrinol (Oxf), 2009. **71**(3): p. 434-9.

25.   De Felice, M., et al., *A mouse model for hereditary thyroid dysgenesis and cleft palate.* Nat Genet, 1998. **19**(4): p. 395-8.

26.   Mansouri, A., K. Chowdhury, and P. Gruss, *Follicular cells of the thyroid gland require Pax8 gene function.* Nat Genet, 1998. **19**(1): p. 87-90.

27.   Martinez Barbera, J.P., et al., *The homeobox gene Hex is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation.* Development, 2000. **127**(11): p. 2433-45.

28.   Thomas, P.Q., A. Brown, and R.S. Beddington, *Hex: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors.* Development, 1998. **125**(1): p. 85-94.

29.   Parlato, R., et al., *An integrated regulatory network controlling survival and migration in thyroid organogenesis.* Dev Biol, 2004. **276**(2): p. 464-75.

30.   Clifton-Bligh, R.J., et al., *Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia.* Nat Genet, 1998. **19**(4): p. 399-401.

31.   Kimura, S., et al., *The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary.* Genes Dev, 1996. **10**(1): p. 60-9.

32. Grevellec, A. and A.S. Tucker, *The pharyngeal pouches and clefts: Development, evolution, structure and derivatives.* Semin Cell Dev Biol, 2010. **21**(3): p. 325-32.

33. Hazard, J.B., *The C cells (parafollicular cells) of the thyroid gland and medullary thyroid carcinoma. A review.* Am J Pathol, 1977. **88**(1): p. 213-50.

34. Le Douarin, N. and C. Le Lievre, *[Demonstration of neural origin of calcitonin cells of ultimobranchial body of chick embryo].* C R Acad Sci Hebd Seances Acad Sci D, 1970. **270**(23): p. 2857-60.

35. Pearse, A.G. and A.F. Carvalheira, *Cytochemical evidence for an ultimobranchial origin of rodent thyroid C cells.* Nature, 1967. **214**(5091): p. 929-30.

36. Pearse, A.G. and J.M. Polak, *Cytochemical evidence for the neural crest origin of mammalian ultimobranchial C cells.* Histochemie, 1971. **27**(2): p. 96-102.

37. Polak, J.M., et al., *Immunocytochemical confirmation of the neural crest origin of avian calcitonin-producing cells.* Histochemistry, 1974. **40**(3): p. 209-14.

38. Johansson, E., et al., *Revising the embryonic origin of thyroid C cells in mice and humans.* Development, 2015. **142**(20): p. 3519-28.

39. Fagman, H. and M. Nilsson, *Morphogenesis of the thyroid gland.* Mol Cell Endocrinol, 2010. **323**(1): p. 35-54.

40. Fagman, H., L. Andersson, and M. Nilsson, *The developing mouse thyroid: embryonic vessel contacts and parenchymal growth pattern during specification, budding, migration, and lobulation.* Dev Dyn, 2006. **235**(2): p. 444-55.

41. Ornitz, D.M. and N. Itoh, *Fibroblast growth factors.* Genome Biol, 2001. **2**(3): p. Reviews3005.

42. Szebenyi, G. and J.F. Fallon, *Fibroblast growth factors as multifunctional signaling factors.* Int Rev Cytol, 1999. **185**: p. 45-106.

43. Gospodarowicz, D., *Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth.* Nature, 1974. **249**(453): p. 123-7.

44. Gospodarowicz, D., J. Weseman, and J. Moran, *Presence in brain of a mitogenic agent promoting proliferation of myoblasts in low density culture.* Nature, 1975. **256**(5514): p. 216-9.

45. Gospodarowicz, D. and J. Moran, *Effect of a fibroblast growth factor, insulin, dexamethasone, and serum on the morphology of BALB/c 3T3 cells.* Proc Natl Acad Sci U S A, 1974. **71**(12): p. 4648-52.

46. Itoh, N. and D.M. Ornitz, *Evolution of the Fgf and Fgfr gene families.* Trends Genet, 2004. **20**(11): p. 563-9.

47. Hacker, U., K. Nybakken, and N. Perrimon, *Heparan sulphate proteoglycans: the sweet side of development.* Nat Rev Mol Cell Biol, 2005. **6**(7): p. 530-41.

48. McKeehan, W.L., F. Wang, and M. Kan, *The heparan sulfate-fibroblast growth factor family: diversity of structure and function.* Prog Nucleic Acid Res Mol Biol, 1998. **59**: p. 135-76.

49. Ornitz, D.M., *FGFs, heparan sulfate and FGFRs: complex interactions essential for development.* Bioessays, 2000. **22**(2): p. 108-12.

50. Schlessinger, J., et al., *Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization.* Mol Cell, 2000. **6**(3): p. 743-50.

51. Hughes, S.E., *Differential expression of the fibroblast growth factor receptor (FGFR) multigene family in normal human adult tissues.* J Histochem Cytochem, 1997. **45**(7): p. 1005-19.

52. Mohammadi, M., S.K. Olsen, and O.A. Ibrahimi, *Structural basis for fibroblast growth factor receptor activation.* Cytokine Growth Factor Rev, 2005. **16**(2): p. 107-37.

53. Miki, T., et al., *Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene.* Proc Natl Acad Sci U S A, 1992. **89**(1): p. 246-50.

54. Gilbert, E., et al., *Control of BEK and K-SAM splice sites in alternative splicing of the fibroblast growth factor receptor 2 pre-mRNA.* Mol Cell Biol, 1993. **13**(9): p. 5461-8.

55. Scotet, E. and E. Houssaint, *The choice between alternative IIIb and IIIc exons of the FGFR-3 gene is not strictly tissue-specific.* Biochim Biophys Acta, 1995. **1264**(2): p. 238-42.

56. Yan, G., et al., *Exon switching and activation of stromal and embryonic fibroblast growth factor (FGF)-FGF receptor genes in prostate epithelial cells accompany stromal independence and malignancy.* Mol Cell Biol, 1993. **13**(8): p. 4513-22.

57. Mohammadi, M., et al., *Identification of six novel autophosphorylation sites on fibroblast growth factor receptor 1 and elucidation of their importance in receptor activation and signal transduction.* Mol Cell Biol, 1996. **16**(3): p. 977-89.

58. Dailey, L., et al., *Mechanisms underlying differential responses to FGF signaling.* Cytokine Growth Factor Rev, 2005. **16**(2): p. 233-47.

59. Ohuchi, H., 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): p. 643-9.

60. Berg, T., et al., *Fibroblast growth factor 10 is critical for liver growth during embryogenesis and controls hepatoblast survival via beta-catenin activation.* Hepatology, 2007. **46**(4): p. 1187-97.

61. Emoto, H., et al., *Structure and expression of human fibroblast growth factor-10.* J Biol Chem, 1997. **272**(37): p. 23191-4.

62. Sekine, K., et al., *Fgf10 is essential for limb and lung formation.* Nat Genet, 1999. **21**(1): p. 138-41.

63. Min, H., et al., *Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless.* Genes Dev, 1998. **12**(20): p. 3156-61.

64. Sakaue, H., et al., *Requirement of fibroblast growth factor 10 in development of white adipose tissue.* Genes Dev, 2002. **16**(8): p. 908-12.

65. Yu, K. and D.M. Ornitz, *FGF signaling regulates mesenchymal differentiation and skeletal patterning along the limb bud proximodistal axis.* Development, 2008. **135**(3): p. 483-91.

66. De Moerlooze, L., et al., *An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis.* Development, 2000. **127**(3): p. 483-92.

67. Revest, J.M., et al., *Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4.* Dev Biol, 2001. **231**(1): p. 47-62.

68. Bellusci, S., et al., *Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung.* Development, 1997. **124**(23): p. 4867-78.

69. Volckaert, T., et al., *Localized Fgf10 expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors.* Development, 2013. **140**(18): p. 3731-42.

70. Tagashira, S., et al., *Cloning of mouse FGF10 and up-regulation of its gene expression during wound healing.* Gene, 1997. **197**(1-2): p. 399-404.

71. Donjacour, A.A., A.A. Thomson, and G.R. Cunha, *FGF-10 plays an essential role in the growth of the fetal prostate.* Dev Biol, 2003. **261**(1): p. 39-54.

72. Bhushan, A., et al., *Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis.* Development, 2001. **128**(24): p. 5109-17.

73. Chatzeli, L., M. Gaete, and A.S. Tucker, *Fgf10 and Sox9 are essential for the establishment of distal progenitor cells during mouse salivary gland development.* Development, 2017. **144**(12): p. 2294-2305.

74. Jaskoll, T., et al., *FGF10/FGFR2b signaling plays essential roles during in vivo embryonic submandibular salivary gland morphogenesis.* BMC Dev Biol, 2005. **5**: p. 11.

75. Ohuchi, H., et al., *The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor.* Development, 1997. **124**(11): p. 2235-44.

76. Sasak, H., et al., *Identification of cis-elements regulating expression of Fgf10 during limb development.* Int J Dev Biol, 2002. **46**(7): p. 963-7.

77. Tickle, C. and A. Munsterberg, *Vertebrate limb development--the early stages in chick and mouse.* Curr Opin Genet Dev, 2001. **11**(4): p. 476-81.

78. Teshima, T.H., S.V. Lourenco, and A.S. Tucker, *Multiple Cranial Organ Defects after Conditionally Knocking Out Fgf10 in the Neural Crest.* Front Physiol, 2016. **7**: p. 488.

79. Iber, D. and D. Menshykau, *The control of branching morphogenesis.* Open Biol, 2013. **3**(9).

80. Metzger, R.J., et al., *The branching programme of mouse lung development.* Nature, 2008. **453**(7196): p. 745-50.

81. Hirashima, T., Y. Iwasa, and Y. Morishita, *Mechanisms for split localization of Fgf10 expression in early lung development.* Dev Dyn, 2009. **238**(11): p. 2813-22.

82. Abler, L.L., S.L. Mansour, and X. Sun, *Conditional gene inactivation reveals roles for Fgf10 and Fgfr2 in establishing a normal pattern of epithelial branching in the mouse lung.* Dev Dyn, 2009. **238**(8): p. 1999-2013.

83. Bellusci, S., et al., *Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis.* Development, 1997. **124**(1): p. 53-63.

84. Lebeche, D., S. Malpel, and W.V. Cardoso, *Fibroblast growth factor interactions in the developing lung.* Mech Dev, 1999. **86**(1-2): p. 125-36.

85. Pepicelli, C.V., P.M. Lewis, and A.P. McMahon, *Sonic hedgehog regulates branching morphogenesis in the mammalian lung.* Curr Biol, 1998. **8**(19): p. 1083-6.

86. Ho, U.Y. and B.J. Wainwright, *Patched1 patterns Fibroblast growth factor 10 and Forkhead box F1 expression during pulmonary branch formation.* Mech Dev, 2017. **147**: p. 37-48.

87. Jaskoll, T. and M. Melnick, *Submandibular gland morphogenesis: stage-specific expression of TGF-alpha/EGF, IGF, TGF-beta, TNF, and IL-6 signal transduction in normal embryonic mice and the phenotypic effects of TGF-beta2, TGF-beta3, and EGF-r null mutations.* Anat Rec, 1999. **256**(3): p. 252-68.

88. Jaskoll, T., et al., *Embryonic submandibular gland morphogenesis: stage-specific protein localization of FGFs, BMPs, Pax6 and Pax9 in normal mice and abnormal SMG phenotypes in FgfR2-IIIc(+/Delta), BMP7(-/-) and Pax6(-/-) mice.* Cells Tissues Organs, 2002. **170**(2-3): p. 83-98.

89. Edlund, H., *Pancreatic organogenesis--developmental mechanisms and implications for therapy.* Nat Rev Genet, 2002. **3**(7): p. 524-32.

90. Hart, A., S. Papadopoulou, and H. Edlund, *Fgf10 maintains notch activation, stimulates proliferation, and blocks differentiation of pancreatic epithelial cells.* Dev Dyn, 2003. **228**(2): p. 185-93.

91.   Entesarian, M., et al., *FGF10 missense mutations in aplasia of lacrimal and salivary glands (ALSG).* Eur J Hum Genet, 2007. **15**(3): p. 379-82.

92.   Klar, J., et al., *Fibroblast growth factor 10 haploinsufficiency causes chronic obstructive pulmonary disease.* J Med Genet, 2011. **48**(10): p. 705-9.

93.   Rohmann, E., et al., *Mutations in different components of FGF signaling in LADD syndrome.* Nat Genet, 2006. **38**(4): p. 414-7.

94.   Li, D., et al., *Pancreatic cancer.* Lancet, 2004. **363**(9414): p. 1049-57.

95.   Nomura, S., et al., *FGF10/FGFR2 signal induces cell migration and invasion in pancreatic cancer.* Br J Cancer, 2008. **99**(2): p. 305-13.

96.   Reintjes, N., et al., *Activating somatic FGFR2 mutations in breast cancer.* PLoS One, 2013. **8**(3): p. e60264.

97.   Stephens, P., et al., *A screen of the complete protein kinase gene family identifies diverse patterns of somatic mutations in human breast cancer.* Nat Genet, 2005. **37**(6): p. 590-2.

98.   Memarzadeh, S., et al., *Enhanced paracrine FGF10 expression promotes formation of multifocal prostate adenocarcinoma and an increase in epithelial androgen receptor.* Cancer Cell, 2007. **12**(6): p. 572-85.

99.   Chen, C.D., et al., *Molecular determinants of resistance to antiandrogen therapy.* Nat Med, 2004. **10**(1): p. 33-9.

100.   Feldman, B.J. and D. Feldman, *The development of androgen-independent prostate cancer.* Nat Rev Cancer, 2001. **1**(1): p. 34-45.

101.   Nakao, Y., et al., *Fibroblast growth factors 7 and 10 are involved in ameloblastoma proliferation via the mitogen-activated protein kinase pathway.* Int J Oncol, 2013. **43**(5): p. 1377-84.

102.   Ridky, T.W. and P.A. Khavari, *Pathways sufficient to induce epidermal carcinogenesis.* Cell Cycle, 2004. **3**(5): p. 621-4.

103.   Suzuki, A., et al., *Keratinocyte-specific Pten deficiency results in epidermal hyperplasia, accelerated hair follicle morphogenesis and tumor formation.* Cancer Res, 2003. **63**(3): p. 674-81.

104.   Hertzler-Schaefer, K., et al., *Pten loss induces autocrine FGF signaling to promote skin tumorigenesis.* Cell Rep, 2014. **6**(5): p. 818-26.

105.   Redler, A., et al., *Fibroblast growth factor receptor-2 expression in thyroid tumor progression: potential diagnostic application.* PLoS One, 2013. **8**(8): p. e72224.

106.   Guo, M., et al., *FGFR2 isoforms support epithelial-stromal interactions in thyroid cancer progression.* Cancer Res, 2012. **72**(8): p. 2017-27.

107.   Nusslein-Volhard, C. and E. Wieschaus, *Mutations affecting segment number and polarity in Drosophila.* Nature, 1980. **287**(5785): p. 795-801.

108. Ingham, P.W. and A.P. McMahon, *Hedgehog signaling in animal development: paradigms and principles.* Genes Dev, 2001. **15**(23): p. 3059-87.

109. Rohatgi, R., L. Milenkovic, and M.P. Scott, *Patched1 regulates hedgehog signaling at the primary cilium.* Science, 2007. **317**(5836): p. 372-6.

110. Litingtung, Y., et al., *Sonic hedgehog is essential to foregut development.* Nat Genet, 1998. **20**(1): p. 58-61.

111. Hebrok, M., et al., *Regulation of pancreas development by hedgehog signaling.* Development, 2000. **127**(22): p. 4905-13.

112. Apelqvist, A., U. Ahlgren, and H. Edlund, *Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas.* Curr Biol, 1997. **7**(10): p. 801-4.

113. Fagman, H., et al., *Genetic deletion of sonic hedgehog causes hemiagenesis and ectopic development of the thyroid in mouse.* Am J Pathol, 2004. **164**(5): p. 1865-72.

114. Dhillon, A.S., et al., *MAP kinase signalling pathways in cancer.* Oncogene, 2007. **26**(22): p. 3279-90.

115. Lavoie, H. and M. Therrien, *Regulation of RAF protein kinases in ERK signalling.* Nat Rev Mol Cell Biol, 2015. **16**(5): p. 281-98.

116. Garnett, M.J. and R. Marais, *Guilty as charged: B-RAF is a human oncogene.* Cancer Cell, 2004. **6**(4): p. 313-9.

117. Daum, G., et al., *The ins and outs of Raf kinases.* Trends Biochem Sci, 1994. **19**(11): p. 474-80.

118. Soares, P., et al., *BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC.* Oncogene, 2003. **22**(29): p. 4578-80.

119. Davies, H., et al., *Mutations of the BRAF gene in human cancer.* Nature, 2002. **417**(6892): p. 949-54.

120. Cantwell-Dorris, E.R., J.J. O'Leary, and O.M. Sheils, *BRAFV600E: implications for carcinogenesis and molecular therapy.* Mol Cancer Ther, 2011. **10**(3): p. 385-94.

121. Peyssonnaux, C. and A. Eychene, *The Raf/MEK/ERK pathway: new concepts of activation.* Biol Cell, 2001. **93**(1-2): p. 53-62.

122. Xing, M., *Molecular pathogenesis and mechanisms of thyroid cancer.* Nat Rev Cancer, 2013. **13**(3): p. 184-99.

123. Fugazzola, L., et al., *Correlation between B-RAFV600E mutation and clinico-pathologic parameters in papillary thyroid carcinoma: data from a multicentric Italian study and review of the literature.* Endocr Relat Cancer, 2006. **13**(2): p. 455-64.

124. Xing, M., *BRAF mutation in thyroid cancer.* Endocr Relat Cancer, 2005. **12**(2): p. 245-62.

125. Riesco-Eizaguirre, G., et al., *The oncogene BRAF V600E is associated with a high risk of recurrence and less differentiated papillary thyroid*

carcinoma due to the impairment of Na+/I- targeting to the membrane. Endocr Relat Cancer, 2006. **13**(1): p. 257-69.

126. Xing, M., et al., *BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer.* J Clin Endocrinol Metab, 2005. **90**(12): p. 6373-9.

127. Romei, C., et al., *BRAFV600E mutation, but not RET/PTC rearrangements, is correlated with a lower expression of both thyroperoxidase and sodium iodide symporter genes in papillary thyroid cancer.* Endocr Relat Cancer, 2008. **15**(2): p. 511-20.

128. Brose, M.S., et al., *Vemurafenib in patients with BRAF(V600E)-positive metastatic or unresectable papillary thyroid cancer refractory to radioactive iodine: a non-randomised, multicentre, open-label, phase 2 trial.* Lancet Oncol, 2016. **17**(9): p. 1272-82.

129. Kim, K.B., et al., *Clinical responses to vemurafenib in patients with metastatic papillary thyroid cancer harboring BRAF(V600E) mutation.* Thyroid, 2013. **23**(10): p. 1277-83.

130. Montero-Conde, C., et al., *Relief of feedback inhibition of HER3 transcription by RAF and MEK inhibitors attenuates their antitumor effects in BRAF-mutant thyroid carcinomas.* Cancer Discov, 2013. **3**(5): p. 520-33.

131. Chakravarty, D., et al., *Small-molecule MAPK inhibitors restore radioiodine incorporation in mouse thyroid cancers with conditional BRAF activation.* J Clin Invest, 2011. **121**(12): p. 4700-11.

132. Charles, R.P., et al., *Mutationally activated BRAF(V600E) elicits papillary thyroid cancer in the adult mouse.* Cancer Res, 2011. **71**(11): p. 3863-71.

133. Knauf, J.A., et al., *Targeted expression of BRAFV600E in thyroid cells of transgenic mice results in papillary thyroid cancers that undergo dedifferentiation.* Cancer Res, 2005. **65**(10): p. 4238-45.

134. Haston, S., et al., *MAPK pathway control of stem cell proliferation and differentiation in the embryonic pituitary provides insights into the pathogenesis of papillary craniopharyngioma.* Development, 2017. **144**(12): p. 2141-2152.

135. Dankort, D., et al., *A new mouse model to explore the initiation, progression, and therapy of BRAFV600E-induced lung tumors.* Genes Dev, 2007. **21**(4): p. 379-84.

136. St-Jacques, B., et al., *Sonic hedgehog signaling is essential for hair development.* Curr Biol, 1998. **8**(19): p. 1058-68.

137. Undeutsch, H., et al., *A mouse model with tamoxifen-inducible thyrocyte-specific cre recombinase activity.* Genesis, 2014. **52**(4): p. 333-40.

138. Xu, Q., M. Tam, and S.A. Anderson, *Fate mapping Nkx2.1-lineage cells in the mouse telencephalon.* J Comp Neurol, 2008. **506**(1): p. 16-29.

139. Jessica Westerlund, L.A., Therese Carlsson, Henrik Fagman and Mikael Nilsson, *Misguided Migration of C Cell Precursors to Extra-Thyroidal Locations Related to Defective Pharyngeal Pouch Development in Shh Deficient Mice.* Cell & Developmental Biology, 2013. **2**(4): p. 129-138.

140. Chang, D.R., et al., *Lung epithelial branching program antagonizes alveolar differentiation.* Proc Natl Acad Sci U S A, 2013. **110**(45): p. 18042-51.

141. Seymour, P.A., et al., *A Sox9/Fgf feed-forward loop maintains pancreatic organ identity.* Development, 2012. **139**(18): p. 3363-72.

142. May, A.J., et al., *Salivary Gland Dysplasia in Fgf10 Heterozygous Mice: A New Mouse Model of Xerostomia.* Curr Mol Med, 2015. **15**(7): p. 674-82.

143. Chao, C., et al., *Impact of embryonic Fgf10 expression deficiency on embryonic mouse lung development and repair following hyperoxia injury.* Mol Cell Pediatr. 2015 Dec;2(Suppl 1):A14. doi:10.1186/2194-7791-2-S1-A14.

144. Bian, X.H., et al., *Expression and clinical significance of Shh/Gli-1 in papillary thyroid carcinoma.* Tumour Biol, 2014. **35**(10): p. 10523-8.

145. Bohinc, B., G. Michelotti, and A.M. Diehl, *Hedgehog signaling in human medullary thyroid carcinoma: a novel signaling pathway.* Thyroid, 2013. **23**(9): p. 1119-26.

146. Nelson, K.K., et al., *Expression of the sonic hedgehog pathway molecules in synchronous follicular adenoma and papillary carcinoma of the thyroid gland in predicting malignancy.* Surgery, 2010. **148**(4): p. 654-60; discussion 660.

147. Mercer, K., et al., *Expression of endogenous oncogenic V600EB-raf induces proliferation and developmental defects in mice and transformation of primary fibroblasts.* Cancer Res, 2005. **65**(24): p. 11493-500.

148. Tang, N., et al., *Control of mitotic spindle angle by the RAS-regulated ERK1/2 pathway determines lung tube shape.* Science, 2011. **333**(6040): p. 342-345.

149. Zhao, B., et al., *The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version.* Genes Dev, 2010. **24**(9): p. 862-74.

150. Camargo, F.D., et al., *YAP1 increases organ size and expands undifferentiated progenitor cells.* Curr Biol, 2007. **17**(23): p. 2054-60.

151. Dong, J., et al., *Elucidation of a universal size-control mechanism in Drosophila and mammals.* Cell, 2007. **130**(6): p. 1120-33.

152. Lee, K.P., et al., *The Hippo-Salvador pathway restrains hepatic oval cell proliferation, liver size, and liver tumorigenesis.* Proc Natl Acad Sci U S A, 2010. **107**(18): p. 8248-53.

153.   Lu, L., et al., *Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1437-42.

154.   Song, H., et al., *Mammalian Mst1 and Mst2 kinases play essential roles in organ size control and tumor suppression.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1431-6.

155.   George, N.M., et al., *Hippo signaling regulates pancreas development through inactivation of Yap.* Mol Cell Biol, 2012. **32**(24): p. 5116-28.

156.   Gao, T., et al., *Hippo signaling regulates differentiation and maintenance in the exocrine pancreas.* Gastroenterology, 2013. **144**(7): p. 1543-53, 1553.e1.