Molecular Mechanisms of Thyroid Hormone Action in the Pax8$^{-/-}$ Mouse Model of Congenital Hypothyroidism

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Hiermit versichere ich, dass ich die vorliegende Dissertation selbstständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel verfasst habe. Ich habe die Dissertation nicht als Diplomarbeit oder ähnliche Arbeit verwendet und abgesehen von den angegebenen Teilpublikationen nicht veröffentlicht.

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(Jens Mittag)

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"Nothing in this world that's worth having comes easy"
Bob Kelso, MD. (Scrubs: My Boss's Free Haircut, 4-20)

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Artikel:

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Mittag J, Wistuba J, Luetjens CM, Cooper TG, Yeung C-H, Sandhove-Klawerkamp R, Nieschlag E, Bauer K: „Thyroid Hormone Cannot Restore Male Fertility in Congenital Hypothyroid Pax8$^{-/-}$ Mice"
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Mittag J, Winterhager E, Bauer K, Grümmer R: „Female Congenital Hypothyroid Pax8$^{-/-}$ Mice Are Infertile Despite Thyroid Hormone Replacement Therapy"
88th Annual Meeting, Endocrine Society, Boston, USA 2006

Kurzzusammenfassung

Schilddrüsenhormone spielen eine wesentliche Rolle für die Entwicklung und Homöostase von fast allen Geweben. Dies wird besonders deutlich im Falle des kongenitalen Hypothyroidismus, der in ungefähr 1 von 3600 Geburten auftritt und häufig durch Schilddrüsendys- oder –agenese verursacht wird. Wenn er nicht sofort mit Thyroxin (T4) behandelt wird, führt der kongenitale Hypothyroidismus zu Stoffwechselstörungen, verzögertem Wachstum, neurologischen Schäden und geistiger Retardierung. Die $Pax8^{-/-}$ Maus ist ein ideales Modellsystem für den kongenitalen Hypothyroidismus, da ihr von Geburt an die follikulären Strukturen der Schilddrüse fehlen und sie athyroid aufwächst. Als Folge zeigen diese Mäuse Ataxie, Taubheit, Wachstumsverzögerung sowie eine komplett gestörte Zusammensetzung der Adenohypophyse und sie überleben die Entwöhnungsphase nicht. Ziel dieser Doktorarbeit war die Untersuchung der molekularen Mechanismen einiger dieser Defekte.

Es wurden $Pax8^{-/-}TR\alpha1^{-/-}$ Doppelmutanten erzeugt, um die von einer anderen Arbeitsgruppe aufgestellte Hypothese zu überprüfen, dass der Schilddrüsenhormonrezeptor (TR) α1 in ungebundenem Zustand als Aporezeptor für die Mortalität in athyroiden Mäusen verantwortlich ist (Flamant et al. 2002). Die $Pax8^{-/-}TR\alpha1^{-/-}$ Doppelmutanten wiesen jedoch den gleichen Phänotyp wie $Pax8^{-/-}$ Mäuse auf, inklusive verzögertem Wachstum, gestörter Zusammensetzung der Adenohypophyse und Letalität, woraus geschlussfolgert werden kann, dass die Sterblichkeit kein Resultat der Aporezeptoraktivität des TRα1 ist. Sie wird vermutlich durch die zweite Schilddrüsenhormonrezeptorisoform des TRα Gens, TRα2, verursacht, die in den Doppelmutanten noch exprimiert wird.

Um die gestörte Zusammensetzung der Hypophyse in $Pax8^{-/-}$ Mäusen zu analysieren, wurde eine Mikroarray-Analyse durchgeführt, bei der Gene identifiziert werden konnten, die in dieser Maus im Vergleich zum Wildtyp unterschiedlich exprimiert sind. Unter anderem ist die Expression der Tyrosinhydroxylase in diesen Mäusen stark erhöht, was zu einer verstärkten lokalen Synthese von Dopamin führt, das die Expression von Prolaktin mRNA unterdrückt. Dopamin behindert allerdings nicht die Entwicklung der laktotrophen Zellen, wie durch Experimente mit Dopaminagonisten und –antagonisten gezeigt werden konnte. Weiterhin wurde mit Annexin A5 ein Gen identifiziert, das positiv durch Schilddrüsenhormone reguliert wird und wie die Analyse Annexin A5 defizienter Mäuse ergab, vermutlich die Synthese der Gonadotropine stimuliert.

Während normalerweise alle Symptome des kongenitalen Hypothyroidismus durch Therapie mit T4 reversibel sind, bleiben T4 behandelte $Pax8^{-/-}$ Mäuse beiderlei Geschlechts überraschend unfruchtbar. Die Analyse zeigte, dass T4-behandelte Pax8 defiziente Weibchen keinen Uterus und keine Vaginalöffnung besitzen, während die Männchen in der Epididymis keine Spermien aufweisen. Da sowohl die mRNA Expression der hypophysären Hormone als auch die Serumspiegel der Steroidhormone in $Pax8^{-/-}$ Mäusen durch T4-Behandlung normalisiert werden, scheinen diese Defekte eine direkte Folge der Pax8 Inaktivierung zu sein, zumal Pax8 mRNA in den Epithelien von Uterus und Eileiter, sowie Ductus efferentis und Epididymis nachgewiesen werden konnte. Da ebenfalls eine Pax8 Expression im Epithel menschlicher Endometrien gefunden wurde, ist dringend anzuraten, zu überprüfen, ob Patienten mit einer Pax8 Mutation in ähnlicher Weise betroffen sind.

Schlagworte: Kongenitaler Hypothyroidismus, Hypophyse, Infertilität

Abstract

Thyroid hormones are essential for normal vertebrate development and for the metabolic homeostasis of almost all tissues. The physiological importance of thyroid hormone becomes most evident under the conditions of congenital hypothyroidism, a common disorder occurring in 1 among 3600 newborns. If not treated immediately after birth by thyroxine (T4) substitution, congenital hypothyroidism leads to the syndrome of cretinism that is characterized by metabolic disturbances, severe neurological defects, mental deficiencies and growth retardation. The $Pax8^{-/-}$ mouse is an ideal animal model to study the consequences of congenital hypothyroidism, since these mice are born without thyroid follicular structures and therefore they are completely athyroid in postnatal life. Consequently, Pax8 null mice exhibit ataxia, deafness, growth retardation, a completely distorted cellular composition of the anterior pituitary and they do not survive weaning. The analysis of the underlying molecular mechanisms of some of these symptoms was part of this thesis.

$Pax8^{-/-}TR\alpha1^{-/-}$ double mutants were generated, since it has been speculated that the unliganded thyroid hormone receptor (TR) α1 acting as an aporeceptor might be responsible for the mortality of $Pax8^{-/-}$ mice. These compound mutants exhibited the same phenotype as $Pax8^{-/-}$ mice, including growth retardation, a deranged appearance of the pituitary and death around weaning. Therefore we can conclude that the mortality is not caused by TRα1 aporeceptor activity but rather by the other isoform of the TRα gene, TRα2.

A microarray based analysis was used to identify factors which contribute to the distorted cellular make-up of the pituitary in athyroid mice. Several genes were found to be differentially expressed in these animals, among them tyrosine hydroxylase which was upregulated in $Pax8^{-/-}$ mice, leading to an increased local production of dopamine. This dopamine inhibits prolactin mRNA expression, but not postnatal lactotroph development as demonstrated by the use of dopamine antagonists and agonists. Additionally, annexin A5 was identified as a pituitary gene which is positively regulated by thyroid hormones and seems to stimulate gonadotropin synthesis as revealed by the analysis of annexin A5 deficient mice.

Although usually all symptoms of congenital hypothyroidism can be reversed by thyroid hormone replacement therapy, T4-treated $Pax8^{-/-}$ mice of both genders were infertile. The analysis revealed that Pax8 deficient females entirely lack the uterus and do not have a vaginal opening, whereas male mice do not have any sperm in the epididymis and exhibit testicular atrophy due to a narrowed passage in the efferent ducts. Since neither the mRNA expression of adenohypophyseal hormones nor the serum levels of steroid hormones were impaired in T4-treated mice, this defect seems to be directly related to the inactivation of the Pax8 gene, which was supported by the identification of Pax8 mRNA expression in the epithelium of the uterus and the oviducts as well as the epithelium of the efferent ducts and the epididymis of wildtype mice. Since Pax8 mRNA expression was also demonstrated in human endometrial tissue samples, it remains to be elucidated whether human patients with mutations in the Pax8 gene are similarly affected.

Keywords: Congenital Hypothyroidism, Pituitary, Infertility

Content

1. Introduction

1.1. The Endocrine System

The endocrine system represents a very important communication pathway comparable in complexity to the central nervous or the immune system. It controls essential processes like reproduction, growth, energy consumption, and temperature homeostasis by messenger substances called hormones which are produced by specialized glands and subsequently released into the systemic circulation. These hormones then act on distant target cells by binding to specific hormone receptors.

The anterior pituitary is the master gland of the endocrine system. It influences several target tissues such as the thyroid gland by the secretion of thyroid stimulating hormone (TSH), the mammary gland by the release of prolactin (PRL), liver and bone through growth hormone (GH), the reproductive system by follicle stimulating hormone (FSH) and luteinizing hormone (LH), and the adrenal cortex by adrenocorticotrophic hormone (ACTH). The activity of the anterior pituitary itself is controlled by the hypothalamus which integrates information from higher brain regions. The first hypothalamic hypophysiotropic neuropeptide hormone identified was thyrotropin releasing hormone (TRH), a tripeptideamide with the sequence pyro-Glu-His-Pro-NH_2 (Guillemin 1978; Schally 1978). As the name implies, TRH stimulates the secretion of TSH from the anterior pituitary. Subsequent studies in man (Jacobs et al. 1971) and with cultured rat pituitary tumor cells (Tashjian et al. 1971) then demonstrated that TRH is also effective as a PRL secretagogue. Besides the stimulating effect of TRH, PRL secretion is mainly controlled by tonic inhibition through hypothalamic dopamine (Ben-Jonathan et al. 2001).

In addition to the hypothalamic control, the synthesis and the secretion of most anterior pituitary hormones is also regulated by negative feedback loops, for example TSH, which stimulates synthesis and release of thyroid hormone (TH) from the thyroid gland. TH in turn

inhibits the synthesis of TRH and TSH on the level of the hypothalamus or the pituitary respectively (Fig. 1.1).

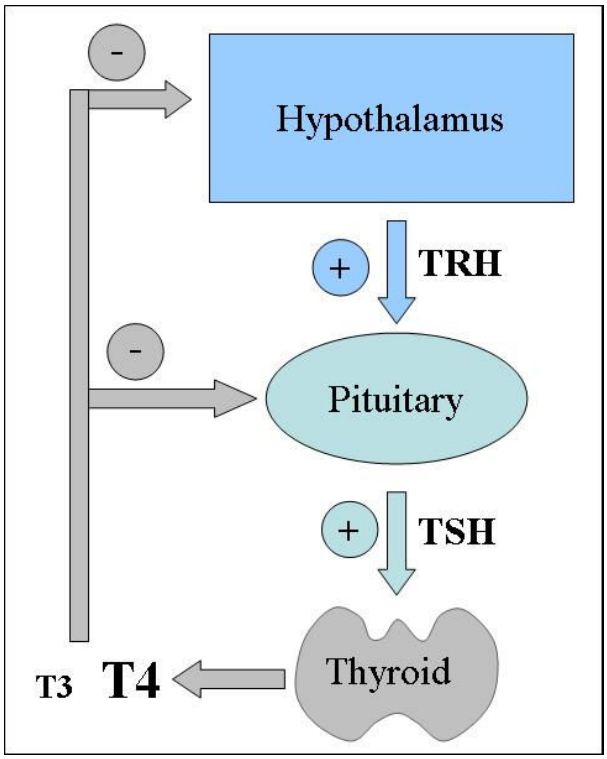


Fig. 1.1: The production of TH is modulated by TRH from the hypothalamus and TSH from the pituitary; both hormones in turn are negatively regulated by TH.

1.2. Thyroid Hormones

THs are essential for proper vertebrate development and the homeostasis of almost all tissues (Porterfield and Hendrich 1993, Oppenheimer and Schwartz 1997, Jones et al. 2005, Bernal 2005). They are involved in the regulation of body temperature and oxygen consumption, the metabolism of lipids and carbohydrates, and they also affect growth, differentiation and development (Yen 2001, Bernal 2005). The secretory products of the thyroid gland are mainly T4 (thyroxine, tetraiodothyronine) and to a lower extent T3 (triiodothyronine). Since T3 is the receptor active form, a conversion of the prohormone T4 is required before any receptor-mediated action on target tissues. The conversion of T4 to T3 is carried out by three enzymes

called deiodinases (D1, D2, and D3) that catalyze either outer (D1 and D2) or inner ring deiodination (D1 and D3) which activates or inactivates TH (Fig. 1.2).

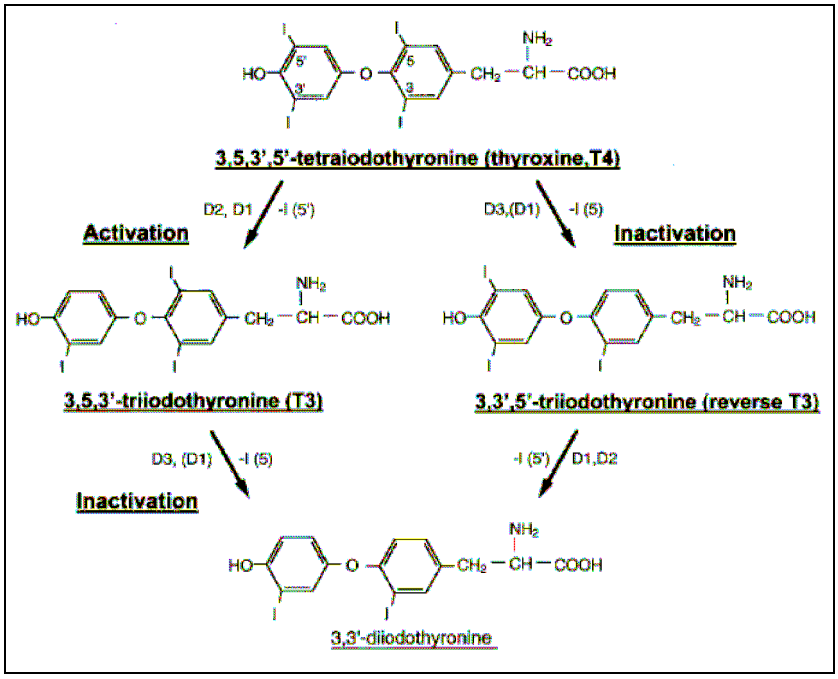


Fig. 1.2: Activation and inactivation of thyroid hormones by the different deiodinases (Bianco et al. 2002)

The majority of TH effects are mediated through the binding of T3 to nuclear hormone receptors that regulate the expression of T3-sensitive genes (Yen et al. 2003). These TH receptors (TRs) are encoded by two different genes, TRα and TRβ, that give rise to eight different splice variants (TRα1, -α2, -Δα1, -Δα2, -β1, -β2, -β3, and –Δβ3).

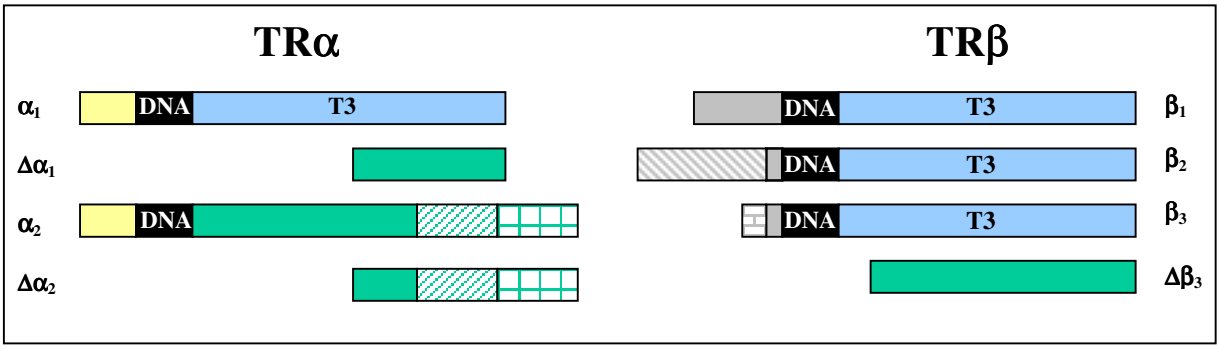


Fig. 1.3: The two different TR genes which give rise to 8 different splice variants. Only four of them are capable of binding DNA (black box labeled DNA) and T3 (blue box labeled T3) (O'Shea and Williams 2002).

However, only four of these isoforms (TRα1, -β1, -β2, and –β3) are considered to act as functional TRs, because they contain a ligand- as well as a DNA-binding domain (O'Shea and Williams 2002). The nonbinding isoforms have been discussed to act as dominant negative antagonists as deduced from in vitro experiments; however, their physiological role in vivo is still poorly defined (Chassande et al. 1997).

Corresponding to the vast array of TH effects on body homeostasis, TRs are found in almost every tissue, with TRα1 more ubiquitously expressed than TRβ isoforms. Mutant mice deficient in the expression of one or both TRs have been generated to assign certain TH effects to specific TR isoforms. Analysis of TRβ deficient mice revealed that this TR plays an important role in cochlear and retina development as well as TSH feedback regulation (Forrest et al. 1996), whereas the phenotype of TRα1 null mouse suggests a predominant role of TRα1 in the regulation of heart rate and body temperature (Wikström et al. 1998). Even mice devoid of all TRs ($TR\alpha1^{-/-}TR\beta^{-/-}$ mice) are surprisingly viable although severely growth retarded and poorly fertile (Göthe et al. 1999). In contrast to the absence of all functional TRs, congenital hypothyroid Pax8 null mice which lack TH as ligand do not survive weaning indicating that unliganded TRs exert lethal aporeceptor-effects in these mice (Mansouri et al. 1998, Flamant et al. 2002).

1.3. Congenital Hypothyroidism

The importance of TH becomes most evident under the conditions of congenital hypothyroidism (CH), a disorder occurring in 1 among 3600 newborns which is mainly caused by thyroid dys- or agenesis (Kopp et al. 2002, De Felice and DiLauro 2004). If not treated immediately after birth with TH, severe forms of CH lead to cretinism, a syndrome characterized by growth retardation, metabolic disturbances and severe neurological deficits (Roberts and Ladenson 2004, Bernal 2005).

Among other genes, CH in humans has been associated with mutations in the paired-box gene 8 (Pax8), which result in severe thyroid hypoplasia (Macchia et al. 1998). Correspondingly, in mice where the Pax8 gene has been deleted, the thyroid gland is completely devoid of TH-producing follicular cells (Mansouri et al. 1998). Thus, these athyroid mice provide an ideal animal model to study the consequences of CH, especially since no further defects have been observed in other tissues expressing Pax8 such as the kidney or some hindbrain regions, which is probably explained by a partial redundancy of the highly homologous Pax2 and Pax5 gene products (Mansouri et al. 1994, Mansouri et al. 1998).

Due to their athyroidism, Pax8 deficient animals exhibit a severe phenotype. They are growth retarded, ataxic, deaf, and do not survive weaning (Mansouri et al. 1998). The lack of TH also affects the appearance of the pituitary gland which shows hypertrophy and hyperplasia of the thyrotrophs, a reduced number of somatotrophs and an almost complete absence of lactotrophs (Friedrichsen et al. 2004). As in humans, all these disturbances can be reversed by the timely institution of TH replacement therapy directly after birth (Larsen et al. 2003, Friedrichsen et al. 2004, Christ et al. 2004, Roberts and Ladenson 2004).

1.4. Aim of this Project

Thyroid hormones play an essential role in various aspects of development, homeostasis and metabolism. The Pax8 null mouse is a valuable animal model to identify the effects of TH action, because it is born without a functional thyroid gland and thus athyroid in postnatal life. The aim of this thesis is to reveal molecular mechanisms underlying TH action using $Pax8^{-/-}$ mice and mouse mutants with deficiencies in TH receptor (TR) genes. The analysis of congenital hypothyroid Pax8 deficient mice should be focussed on three aspects namely the pre-weaning mortality, the completely distorted appearance of the anterior pituitary, and the infertility of TH-substituted $Pax8^{-/-}$ mice.

To investigate the mortality of Pax8 null mice in more detail, the analysis included $Pax8^{-/-}$ $TR\alpha1^{-/-}$ mice which lack TH and TRα1, the only isoform from the TRα gene that is capable of binding T3 and DNA. Since it has been reported before that $Pax8^{-/-}$ mice can be rescued by the complete inactivation of the TRα-gene ($Pax8^{-/-}TR\alpha^{0/0}$ double mutant mice; Flamant et al. 2002), it might be possible to assign the mortality to one specific TRα isoform, depending on the survival of the $Pax8^{-/-}TR\alpha1^{-/-}$ double mutant mice.

The identification of TH dependent factors contributing to the deranged composition of the anterior pituitary should be pursued by comparing the gene expression in pituitaries of wildtype and Pax8 deficient mice using microarray technologies. Genes found to be differentially expressed should be validated by in situ hybridization or quantitative Real-Time PCR and analyzed especially with regard to a possible involvement in the regulation of prolactin mRNA expression and lactotroph development.

Although most defects observed in congenital hypothyroidism are usually normalized by TH treatment, Pax8 null mice of both genders are infertile despite proper thyroxine substitution. It is also part of this study to investigate the cause for this infertility by careful histological and endocrine analysis in order to determine whether this defect is related to disturbances in TH metabolism or directly caused by the inactivation of the Pax8 gene.

2. Materials and Methods

2.1. Experimental Animals and Human Tissue Samples

Animal procedures were approved by the animal welfare committee of the Medizinische Hochschule Hannover. Mice were kept at constant temperature (22°C) and light cycle (12-h light, 12-h dark) and were provided with standard laboratory chow and tap water ad libitum. If indicated, animals were injected with thyroxine (18 ng/g BW sc), bromocryptine (10 µg/g BW ip) or haloperidol (1 µg/g BW ip or sc). Haloperidol (Sigma, Schnelldorf, Germany) was dissolved in 0.9% NaCl containing 0.1 M hydrochloric acid at a final concentration of 1 mg/ml. Bromocryptine was dissolved in 8 mg/ml tartaric acid at a final concentration of 8 mg/ml.

If not stated otherwise, animals were killed by decapitation at postnatal day 21 (P21); tissues isolated quickly, frozen on dry ice, and stored at –80°C until further processing. Serum was obtained from trunk blood by centrifugation and stored at –80°C.

$Pax8^{+/-}$ mice (Mansouri et al. 1998) were obtained from Ahmed Mansouri and Peter Gruss (Göttingen, Germany). $TR\alpha1^{-/-}$ (Wikström et al. 1998) and $TR\beta^{-/-}$ mice (Forrest et al. 1996) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA), $TR\alpha1^{-/-}TR\beta^{-/-}$ double mutant mice were generated by intercrossing these mice. $TRH\text{-}R1^{-/-}$ mice were generated as described previously (Rabeler et al. 2004). Annexin $A5^{-/-}$ mice (Brachvogel et al. 2003) were obtained from Bent Brachvogel and Ernst Poeschl (University of Erlangen, Germany). $TR\alpha2^{-/-}$ mice (Salto et al. 2001) were obtained from Björn Vennström (Karolinska Institute, Stockholm, Sweden). Wildtype controls were either littermates (Pax8, A5) or age- and strain-matched controls of the mice analyzed. Genotyping was performed using standard PCR assays with the primers indicated below.

Endometrial tissue was obtained from women undergoing endometrial biopsy or hysterectomy at the department of Gynecology, University Hospital Essen, Germany. Institutional ethical approval was obtained and all women provided written informed consent.

The stage of the menstrual cycle was confirmed by serum hormone determination using competitive immunoassays for estrogen (Bayer Diagnostics 06792063) as well as progesterone (Bayer Diagnostics 01586287, Leverkusen, Germany) and by histological staging (Noyes et al. 1975). Progesterone concentrations above 1.0 ng/ml were allocated to the secretory phase, samples below that level were allocated to the proliferative phase.

Gene	**Primer (5' – 3')**
TRα1	GGAGTCGACCGAGAAGAGTCAGGA GTATGGGAGCTGCATCTATCCAAG CACTGCATTCTAGTTGTGGT
TRα2	CACTGCATTCTAGTTGTGGT GGACAAGATCGAGAAGAGTCAGGA GAAGGGAAATCTAGGCCAAGGAAC
TRβ	CCCTGGAGGCCAAAGGTCATCAATG GTGCCAGCGGGGCTGCTAAAG GCACAGGGAGGAAGTAGGCTGTTCT
Pax8	GGATGTGGAATGTGTGCGAGG GATGCTGCCAGTCTCGTAG GCTAAGAGAAGGTGGATGAGAG
TRH-R1	GTGCTGTTGAAGCATCTG GACTGTCCTGGCCGTAAC TGAGTGTGGCTTGATTGG
Anxa5	CCTGTACTCTATCACTATCACTGACTGTTAATC CGAGAGGCACTGTGACTGACTTCCCTGGAT GCCAGTTTGAGGGGACGACGACAG GAAGCAATGCTCAGCGCCAGGA

Table 2.1: Primers used for PCR genotyping of the genes indicated

2.2. RNA Isolation, cDNA Synthesis and Microarray Analysis

Total RNA was isolated from pools of 3-5 mouse pituitaries using the Absolutely Microprep RNA Isolation Kit (Stratagene, LaJolla, USA). cDNA synthesis was performed using the Thermoscript RT System (Invitrogen, CA, USA) according to the instructions of the manufacturer. A sample without reverse transcriptase was used as negative control to confirm the absence of genomic DNA. The microarray analysis was done using the U430A gene chip (Affymetrix, High Wycombe, UK) that contains more than 34.000 mouse genes. The hybridization and the read-out of the raw data were carried out by Dr. Ludger Klein-Hitpaß at the Microarray Facility of the University Essen (Germany).

2.3. In Situ Hybridization

Tissues were rapidly removed from the decapitated animals and embedded in Tissue-Tek medium (Sakura Finetek, Torrance, USA) and frozen on dry ice. Sections (16 µm) were cut on a cryostat (Leica, Bentheim, Germany), thaw-mounted on silane-treated slides, and stored at –80°C until further processing. In situ hybridization histochemistry (ISH) was carried out according to Schäfer et al. (Schäfer et al. 1995). Frozen sections were fixed in a 4% phosphate-buffered paraformaldehyde (PFA) solution (pH 7.4) for 1 h at room temperature (RT), rinsed with phosphate buffered saline (PBS) and treated with 0.4% phosphate buffered Triton X100 solution for 10 min. After washing with PBS and water, tissue sections were incubated in 0.1 M triethanolamine (pH 8) containing 0.25% (v/v) acetic anhydride for 10 min. Following acetylation, sections were rinsed several times with PBS, dehydrated by successive washing with increasing ethanol concentrations, and air dried.

Radioactive-labeled probes were generated from cDNA subclones in pGEM Teasy plasmids as listed below. In vitro transcription was carried out according to standard protocols with (^{35}S)-UTP and (^{35}S)-CTP as labeled nucleotides (Melton et al. 1984).

Radioactive cRNA probes were diluted in hybridization buffer (50% formamide, 10% dextrane sulfate, 0.6 M NaCl, 10 mM Tris·HCl pH 7.4, 1x Denhardt's solution, 100 µg/ml sonicated salmon sperm DNA, 1 mM EDTA and 10 mM dithiothreitol) to a final concentration of $5x10^4$ cpm/ml. After application of the hybridization mix, sections were coverslipped and incubated in a humid chamber at 58°C for 16 h. Following hybridization, coverslips were removed in 2x standard saline citrate (SSC: 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0). The sections were then treated with RNAse A (20 µg/ml) and RNAse T_1 (1 U/ml) at 37°C for 30 min. Successive washes followed at room temperature (RT) in 1x, 0.5x and 0.2x SSC for 20 min each and in 0.2x SSC at 65°C for 1 h. The tissue was dehydrated and exposed to Biomax MR Film (Kodak, Sigma Aldrich, Germany) for 48 h. For microscopic analysis, sections were dipped in NTB2 emulsion (Kodak, Integra Biosciences, Germany) and

stored at 4°C. After exposure for 14 days, autoradiograms were developed in D19 (Kodak, Sigma-Aldrich, Germany) for 4 min and fixed in Rapid Fix (Kodak, Sigma-Aldrich, Germany) for 4 min. If required, sections were counterstained with cresyl violet and then photographed under darkfield or brightfield illuminations.

Digoxigenin-labeled probes were generated from cDNA subclones in pGEM Teasy plasmids (Promega, Mannheim, Germany) as listed below using a DIG RNA Labeling Kit (Boehringer, Mannheim, Germany). In vitro transcription was carried out according to standard protocols.

The digoxigenin labeled probes were diluted in hybridization buffer to a final concentration of 5 ng/µl. Hybridization and posthybridization were performed as described above for radioactive in situ hybridization. Sections were then rinsed with P1 (100 mM Tris, 150 mM NaCl, pH 7.5) and incubated for 2 h in blocking solution provided by the manufacturer of the kit. After incubation overnight with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:1000 dilution; Boehringer, Mannheim, Germany), the tissue sections were washed with P1. Staining proceeded for 2 to 6 h in substrate solution containing NBT (nitroblue tetrazolium chloride, 340 µg/ml; Biomol, Hamburg, Germany), X-Phosphate (5-bromo-4-chloro-3-indolyl phosphate, 175 µg/ml; Biomol, Hamburg, Germany), 100 mM Tris, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.0.

cDNA	vector	size	fragment	NCBI accession no	linearized with	RNA polymerase and orientation
GH	pLS	198bp	nt 248-445	U62779	HindIII PstI	T7 (antisense) T3 (sense)
β-TSH	pLS	254bp	nt 190-445	M109902	HindIII PstI	T7 (antisense) T3 (sense)
β-FSH	pLS	880bp	nt 1-879	M36804	HindIII PstI	T7 (sense) T3 (antisense)
prolactin	pLS	184bp	nt 1566-1749	J00769	HindIII PstI	T7 (antisense) T3 (sense)
POMC	pGEM4	471bp	nt 56-526	J00759	XbaI HindIII	T7 (antisense) Sp6 (sense)
β-LH	pLS	457bp	nt 1-457	NM_012858	XbaI BamHI	T3 (antisense) T7 (sense)
deiodinase type II	pGEM Teasy	914bp	nt 131-1045	AF096875	SacII SacI	SP6 (antisense) T7 (sense)
tyrosine hydroxylase	pGEM Teasy	1109bp	nt 616-1724	NM_009377	SpeI SacII	T7 (sense) SP6 (antisense)
dopamine receptor 2	pGEM Teasy	2110bp	nt 31-2140	XM_125162	SpeI AatII	T7 (sense) SP6 (antisense)
pax8 (*)	pGEM Teasy	190bp	nt 887-1177	NM_011040	SacII SpeI	SP6 (antisense) T7 (sense)
AnxA1 (*)	pGEM Teasy	330bp	nt 632-962	NM_010730	SpeI SacII	SP6 (antisense) T7 (sense)
AnxA5 (*)	pGEM Teasy	429bp	nt 529-958	D63423	SpeI SacII	T7 (antisense) SP6 (sense)
AnxA10 (*)	pGEM Teasy	448bp	nt 575-1023	NM_011922	SpeI SacII	T7 (antisense) SP6 (sense)
calbindin 2 (*)	pGEM Teasy	388bp	nt 692-1080	NM_007586	SpeI SacII	T7 (antisense) SP6 (sense)
calsequestrin 2 (*)	pGEM Teasy	564bp	nt 1069-1633	MMU91483	SpeI SacII	T7 (antisense) SP6 (sense)
STAT1 (*)	pGEM Teasy	314bp	nt 167-481	NM_009283	SpeI SacII	T7 (sense) SP6 (antisense)
STAT4 (*)	pGEM Teasy	393bp	nt 2030-2423	NM_011487	SpeI SacII	T7 (antisense) SP6 (sense)

Table 2.2: List of all plasmids with cDNA inserts that were used for probe generation. The plasmids have been generated either during this study (*) or they were generously provided by Dr. Heike Heuer, Dr. Martin Schäfer and Dr. Sönke Friedrichsen.

2.4. Quantitative Real-Time PCR

Quantitative Real-Time PCR was performed using the iCycler iQ Multi-Color Real-Time PCR Detection System and the iQ SYBR Green Supermix (Biorad, Munich, Germany). Cyclophilin was used as housekeeping gene for normalization. The following primers were chosen to generate the PCR-fragments:

Gene	**Primer (5' – 3')**
Cyclophilin	GCAAGGATGGCAAGGATTGA AGCAATTCTGCCTGGATAGC
β-TSH	CCGCACCATGTTACTCCTTA GTTCTGACAGCCTCGTGTAT
prolactin	GCAGTCACCATGACCATGAA AGATTGGCAGAGGCTGAACA
GH	CGCTTCTCGCTGCTGCTCAT GTCCGAGGTGCCGAACATCA
β-FSH	CGCACACCAAGTGGTATTGA AGAGCAATCTGCTGCCATAG
β-LH	AGTACTCGGACCATGCTAGG CAACTCTGGCCGCAGAGAAT
AnxA1	TTCCTCAAGCAGGCCCGTTT TAAGTACGCGGCCTTGATCT
AnxA2	GTCTACTGTCCACGAAATCC TCTCCAGGTGGCCAGATAAG
AnxA3	TCACCTTCGGCTGAGCTTCTG CCTGTTCATACGCTGCTTGG
AnxA4	TCTCCGCACCAGAGGAACTT TTGCTGCTCAGCTCCGACTT
AnxA5	TTGGCGTGTGCATCGGTCCT TGGCGCTGAGCATTGCTTCG
AnxA6	TCTGCTGCAGAACCTGAGAC TCACTATCAGCCGCTCGAAC
AnxA7	CTGCAGGTCAGGAGTCATCT TGTGCAGGTGGCTGTGGATA
AnxA8	CACTGAGCAGAGGCCAACTA CCTCTTGGTGAGCACGTCTA
AnxA9	GTGCTTCACGACGCTGAACC CCACACCTTGGCCTGCGATA
AnxA10	TACCCTGGTTACTTCCTCTC GGCCATACATGCTCTGATAG
AnxA11	GCTGGATAACGTAGCCAACT GGCGGATACATTCCATAAGG
AnxA13	GTCTTATCCAGCAGGACATC TGTTGCTTCGTGTGCATAGG
deiodinase type II	GGAACAGCTTCCTCCTAGAT GGTCTTCTCCGAGGCATAAT
tyrosine hydroxylase	GCTGGAGGATGTGTCTCACT GAGGAGGCATGACGGATGTA
dopa decarboxylase	GACTACAGGCACTGGCAGAT CTGGCGTACCAGTGACTCAA
TRα1	GAGGCTAGGACCCAAGTTCT CCTCCTGACCCTACAGTCAA
TRβ	GTGACACGAGGCCAGCTGAA TAGCAGGACGGCCTGAAGCA

Table 2.3: Primers used to generate cDNA fragments for quantitative Real-Time PCR

2.5. SDS-Polyacrylamide-Gelelectrophoresis and Western Blot

Pools of 3-5 pituitaries of each genotype were homogenized in 50 µl of 100 mM HEPES pH 7.0 and tissue remnants were separated by centrifugation. Subsequently, 10 µl of the supernatant containing the proteins were separated on a 7% SDS-Polyacrylamidegel according to standard protocols. Proteins were then transferred to a nitrocellulose membrane (Optitran BA-S 83; Schleicher and Schuell, Dassel, Germany) by a semi-dry Western Blot protocol. Membranes were then blocked with 3% BSA in PBS. Annexin A5 was detected with a polyclonal rabbit anti-annexin A5 antibody (1:3000; Hyphen, Biomed, Neuville, France), tyrosine hydroxylase with a mouse monoclonal anti-tyrosine hydroxylase antibody (1:1000; Sigma, St. Louis, USA). A rabbit polyclonal anti-β actin antibody (1:5000; abcam, Cambridge, UK) was used for normalization.

Primary antibodies were visualized using a horseradish peroxidase-coupled secondary antibody (1:30000; Promega, WI, USA) and the ECL detection system (Pierce, Rockford, IL, USA).

2.6. Histological Examination and Immunohistochemistry

For immunohistochemistry of mouse cerebella, animals were perfused with 50 ml of ice-cold PBS and the same volume of ice-cold 4% PFA in PBS. The brains were subsequently kept in 4% PFA for 24 h at 4°C, then washed with PBS, and mounted in 4% agarose (w/v in PBS). 70 µm sections of the cerebellum were cut on a vibratome (Leica, Bentheim, Germany) and blocked with 10% normal goat serum in PBS. The free floating sections were then incubated with a polyclonal rabbit anti-annexin A5 antibody (1:100; Hyphen, Biomed, Neuville, France) or a polyclonal mouse anti-calbindin antibody (1:2000; Swant, Switzerland) for 1 h at RT. Primary antibodies were subsequently detected using fluorescent secondary antibodies (1:800; Alexa Fluor 488 or 555, Molecular Probes, Invitrogen, Carlsbad CA, USA).

For histological examinations of the reproductive system, ovaries, oviduct, uterus, cervix, and vagina or pieces of testicular tissue, epididymides, and efferent ducts respectively were dissected, and the tissues were fixed overnight in 4% PFA in PBS at pH 7.2, dehydrated through an ethanol series, and embedded in paraffin. Serial sections of 7 µm were prepared and stained with haematoxylin and eosin.

For immunostaining paraffin sections were deparaffinized and rinsed in PBS containing 0.5% BSA to reduce nonspecific antibody binding. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS for 10 min. After washing in PBS, sections were incubated for 1 h at RT with rabbit-anti-α-smooth muscle actin (1:500; A2547, Sigma, USA or RB-9010, Lab Vision, USA) which was subsequently detected with a biotinylated goat anti-rabbit IgG (1:800; E0432, Dako, Denmark) as secondary antibody. After the addition of streptavidin-coupled horse radish peroxidase, the chromogenic reaction was carried out by incubating the sections with the substrate 3,3‘-diaminobenzidine for 5 min; sections were rinsed in PBS, dehydrated in ascending ethanol concentrations, coverslipped, and recorded with a Zeiss Axiophot photomicroscope. The histological analysis of the female reproductive tract was done in collaboration with PD Dr. Ruth Grümmer and Prof. Dr. Elke Winterhager from the Department of Anatomy, University Essen, Germany. The histology of the male reproductive system was analyzed in collaboration with Dr. Joachim Wistuba and coworkers from the Institute of Reproductive Medicine, University of Münster, Germany.

2.7. Dopamine Radioimmuno Assay

Serum was pooled from 4 animals per genotypes and 300µl were used for radioimmuno assay (RIA). At least six pituitaries, adrenals and hypothalami per genotype were pooled, homogenized in 100µl of 100mM HEPES buffer pH 7.0; the tissue remnants were separated by centrifugation and 50µl of the supernatant were used for RIA and for the determination of

protein content. The RIA was carried out according to the instructions of the manufacturer of the kit (LDN, Nordhorn, Germany).

2.8. Tyrosine Hydroxylase Activity Assay

The anterior parts of 5 pituitaries of each genotype were pooled and homogenized in 50µl of 100mM HEPES pH 7.0. Tyrosine hydroxylase activity was determined using a modified method from Thorolfsson et al. (Thorolfsson et al. 2002). Tissue remnants were separated by centrifugation and 29 µl of the supernatant were incubated at 30°C in 100 mM HEPES pH 7.0 containing 1 µM ^{3}H-tyrosine (55 Ci/mmol; Amersham GE Biosciences, UK), 0.5 mg/ml catalase (Sigma, Schnelldorf, Germany), 100 µM $FeSO_4$ and 50 µM tetrahydrobiopterin (dissolved at 5mM in 500mM DTT; Sigma, Schnelldorf, Germany) in a final volume of 50 µl. Aliquots of 9µl were taken after different periods of time (5, 10, 20 and 60 minutes). The reaction was stopped by adding 91 µl of a slurry of activated charcoal in 1 M hydrochloric acid. The ^{3}H-tyrosine containing charcoal was separated by centrifugation and the ^{3}H-containing water in the supernatant was quantified by liquid scintillation counting. Activity was subsequently normalized according to the total protein content of the samples.

2.9. Hormone Measurements

Serum hormone levels were measured using competitive immuno assays for estrogen (Bayer Diagnostics 06792063, Leverkusen, Germany) and progesterone (Bayer Diagnostics 01586287, Leverkusen, Germany) in the Department of Anatomy, University of Essen, Germany. Serum testosterone was measured by RIA (Chandolia et al. 1991) by the Institute of Reproductive Medicine, University of Münster, Germany.

2.10. Deiodinase Type II Activity Assay

Deiodinase type II activity assays were performed by Prof. Dr. Theo Visser (Erasmus Medical Center, Rotterdam, The Netherlands) as already described (Friedrichsen et al. 2003).

2.11. Statistical Analysis

For statistical analysis the Students' ttest was applied. If not stated otherwise, results are presented as mean ± standard deviation (SD) and the following p-values were considered significant and marked accordingly: $p<0.05$ *, $p<0.005$ ** and $p<0.001$ ***.

3. Results

3.1. Generation and Analysis of Pax8$^{-/-}$TRα1$^{-/-}$ Double Mutant Mice

3.1.1. Development of Pax8$^{-/-}$TRα1$^{-/-}$ Animals

Pax8$^{-/-}$TRα1$^{-/-}$ mice were generated by intercrossing Pax8$^{+/-}$ (Mansouri et al. 1998) and TRα1$^{-/-}$ mice (Wikström et al. 1998). Female and male Pax8$^{+/-}$TRα1$^{-/-}$ mice showed normal fertility and the litter size was unaffected. Like Pax8$^{-/-}$ mice, most Pax8$^{-/-}$TRα1$^{-/-}$ mice were born with a lower body weight compared to their littermates. Postnatal growth of Pax8$^{-/-}$ TRα1$^{-/-}$ mice was clearly retarded (Fig. 3.1) compared to Pax8$^{+/-}$TRα1$^{-/-}$ and TRα1$^{-/-}$ animals that developed more slowly than wildtype controls.

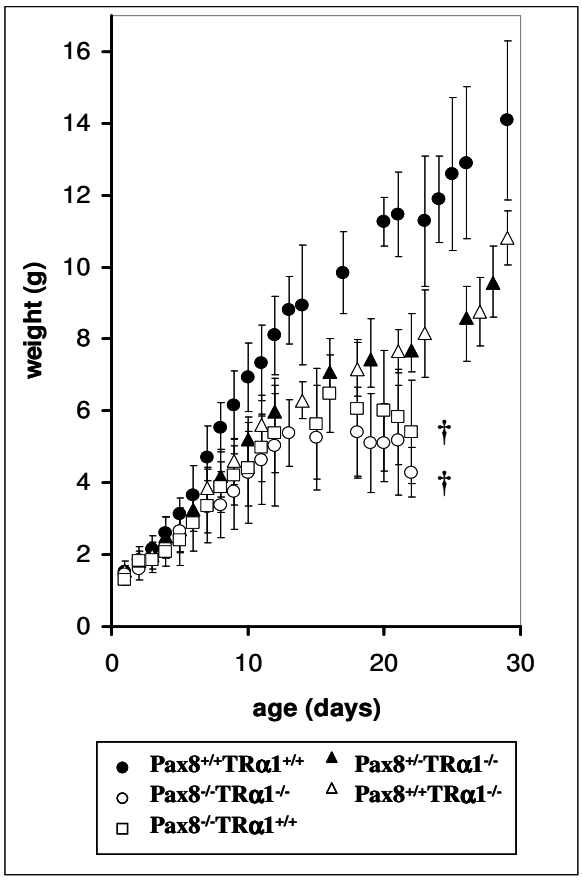


Fig. 3.1: Postnatal development of Pax8$^{-/-}$TRα1$^{-/-}$ double mutant mice in comparison with Pax8$^{-/-}$ mice and controls.

Most of the double mutant mice died during the first 2 postnatal weeks and only about one fourth reached the weaning age of 21 days. Even when the litter size was reduced to 4 pups directly after birth, Pax8$^{-/-}$TRα1$^{-/-}$ mice did not survive weaning. By daily administration of T4 from P2 onwards, Pax8$^{-/-}$TRα1$^{-/-}$ mice could be rescued and survived to adulthood, indicating that the mortality of these animals is a direct consequence of their athyroidism.

Survival of athyroid mutant mice (Pax8$^{-/-}$TRα1$^{-/-}$ and Pax8$^{-/-}$) could also be achieved by keeping these mice under a lactating dam for additional 15 days until P36, indicating that a retarded intestinal development might be responsible for the death around weaning when the food situation is changed. By the age of P36 the intestine seems to be readily developed to digest the normal food and the animals can reach adulthood despite their athyroidism.

3.1.2. Serum Thyroid Hormone Levels

Total T3 and T4 are undetectable in serum of Pax8$^{-/-}$ and Pax8$^{-/-}$TRα1$^{-/-}$ animals, confirming that maternal supply is negligible after birth. In Pax8$^{+/-}$TRα1$^{-/-}$ animals, total T4 was slightly reduced in both genders, while T3 serum levels were slightly increased (Table 3.1), with the consequence that the T3/T4 ratio was also increased in these animals.

	T4	T3	T3/T4
Pax8$^{+/-}$TRα1$^{+/+}$ ♂	75.7±13.3	1.00±0.19	1.34
Pax8$^{+/-}$TRα1$^{-/-}$ ♂	61.2±12.5 *	1.29±0.23 **	2.15
Pax8$^{+/-}$TRα1$^{+/+}$ ♀	78.3±15.3	1.04±0.18	1.36
Pax8$^{+/-}$TRα1$^{-/-}$ ♀	67.4±11.9 *	1.31±0.22 ***	1.97
Pax8$^{-/-}$TRα1$^{+/+}$	X	X	
Pax8$^{-/-}$TRα1$^{-/-}$	X	X	

Table 3.1: Thyroid hormone serum levels and T3/T4 ratio in Pax8$^{+/-}$TRα1$^{-/-}$ animals and controls. The athyroid mutants Pax8$^{-/-}$TRα1$^{+/+}$ and Pax8$^{-/-}$TRα1$^{-/-}$ did not show detectable serum levels of T3 or T4. Values are indicated as mean ± SD. *: $p<0.01$; **: $p<0.05$ and ***: $p<0.001$.

In good agreement with the increased T3/T4 ratio D1 liver activity was higher in Pax8$^{+/-}$ TRα1$^{-/-}$ mice (17.19 ± 3.41 pmol/min·mg) compared to controls (10.63 ± 6.53 pmol/min·mg;

p<0.05), whereas no significant difference was observed in the athyroid mouse models (0.99 ± 0.33 pmol/min·mg in Pax8$^{-/-}$ compared to 0.91 ± 0.21 pmol/min·mg in Pax8$^{-/-}$TRα1$^{-/-}$).

3.1.3. Tissue Weight Analysis

As an initial characterization of the Pax8$^{-/-}$TRα1$^{-/-}$ mice, tissue weights were analyzed at P21. When normalized to the corresponding body weights, organ weights differed quite remarkably between the different mutants (Fig. 3.2).

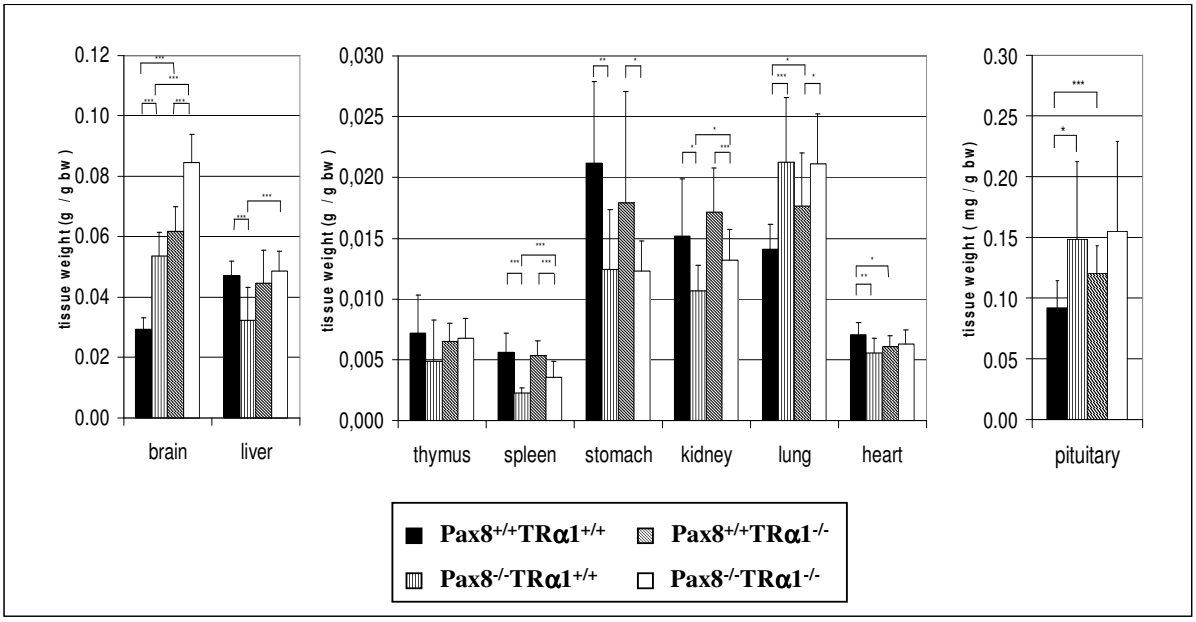


Fig. 3.2: Tissue weights of the different mutant mice after normalization relative to their body weight. (*: p<0.05; **: p<0.005; ***: p<0.001)

In Pax8$^{-/-}$ and Pax8$^{-/-}$TRα1$^{-/-}$ mice, relative organ weights of lung and pituitary were increased, whereas the weights of spleen, stomach and kidney were decreased compared to controls and TRα1$^{-/-}$ animals. Tissue weights of Pax8$^{-/-}$TRα1$^{-/-}$ mice closely resembled those of Pax8$^{-/-}$ animals with one exception: in all mutant mice analyzed, relative brain weight was found to be elevated compared to control animals with the highest weight observed in Pax8$^{-/-}$TRα1$^{-/-}$ mice (a 1.6-fold increase compared to Pax8$^{-/-}$ mice). Even when absolute brain weights were

considered, the relatively small Pax8$^{-/-}$TRα1$^{-/-}$ animals (4.2 g) had a slightly larger brain (0.425 ± 0.047 g) relative to control animals (0.336 ± 0.017 g) with a body weight of 11.1 g. While all organs analyzed appeared macroscopically normal, the pituitaries of the athyroid Pax8$^{-/-}$ and Pax8$^{-/-}$TRα1$^{-/-}$ mice exhibited a glossy/translucent and not an opalescent appearance like those of control and TRα1$^{-/-}$ animals (Fig. 3.3).

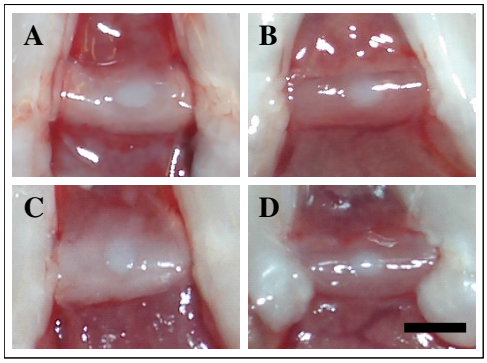


Fig. 3.3: Macroscopical appearance of pituitaries from wildtype (A), Pax8$^{-/-}$ (B), TRα1$^{-/-}$ (C) and Pax8$^{-/-}$TRα1$^{-/-}$ double mutant mice (D). Scale bar 2mm.

3.1.4. Expression Pattern Analysis of Pituitary Hormones

Analysis of pituitary hormone expression by in situ hybridization revealed no differences between control and TRα1$^{-/-}$ mice, neither with regard to the signal intensities nor with regard to the cellular distribution pattern (Fig. 3.4).

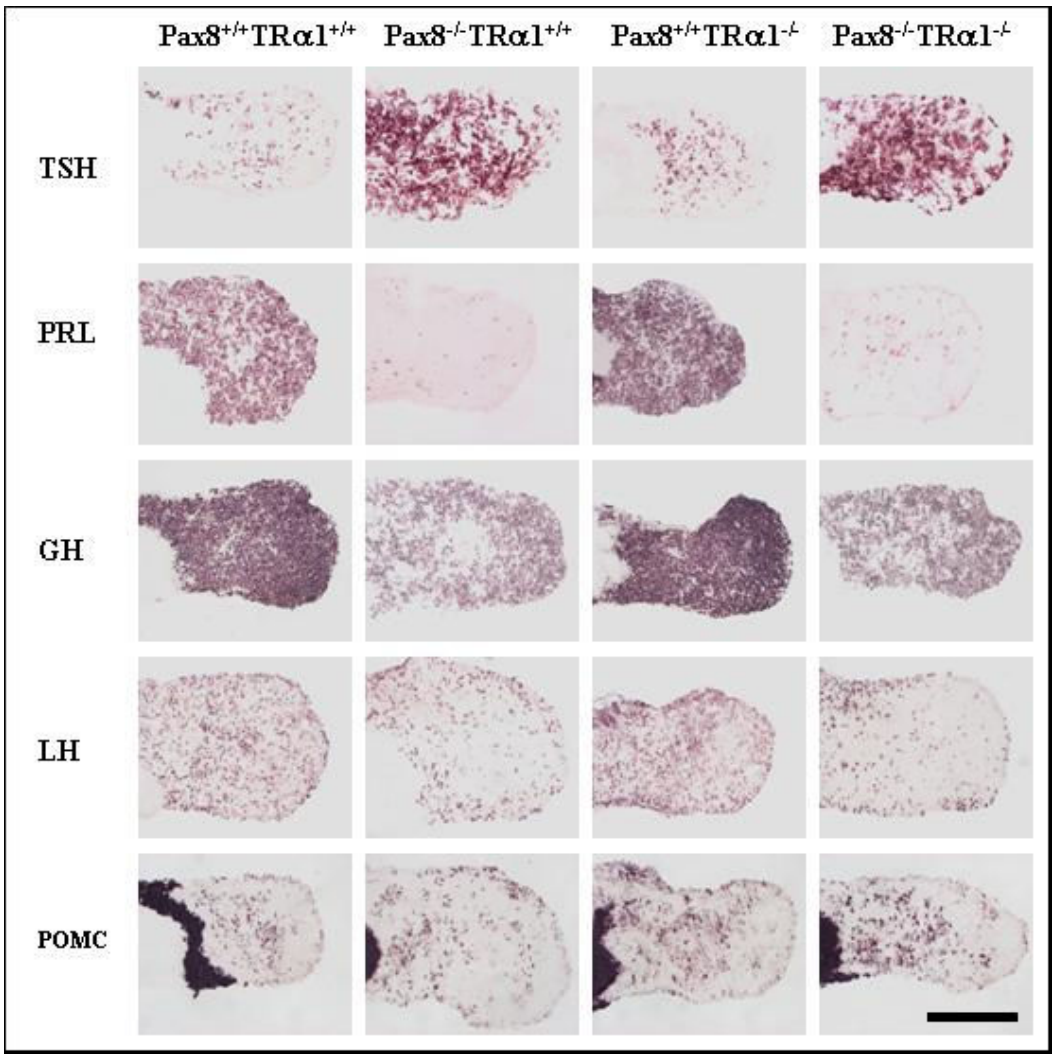


Fig. 3.4: mRNA expression of the pituitary hormones in the different mutant mice as analyzed by in situ hybridization histochemistry. Scale bar 500µm.

In contrast, the cellular composition of the athyroid Pax8$^{-/-}$ and Pax8$^{-/-}$TRα1$^{-/-}$ mice pituitaries was completely deranged. Not only the β-TSH transcript levels but also the number of cells expressing β-TSH mRNA were dramatically increased in both animal models. Lactotrophs

were almost absent in these mice and the numbers of GH and LH mRNA expressing cells were decreased. Only corticotrophs appeared not to be affected by the absence of TH.

Quantitative Real-Time PCR analysis revealed that corresponding to the ISH, β-TSH transcript levels were highly increased whereas prolactin and GH levels were dramatically decreased in the athyroid animals (Fig. 3.5).

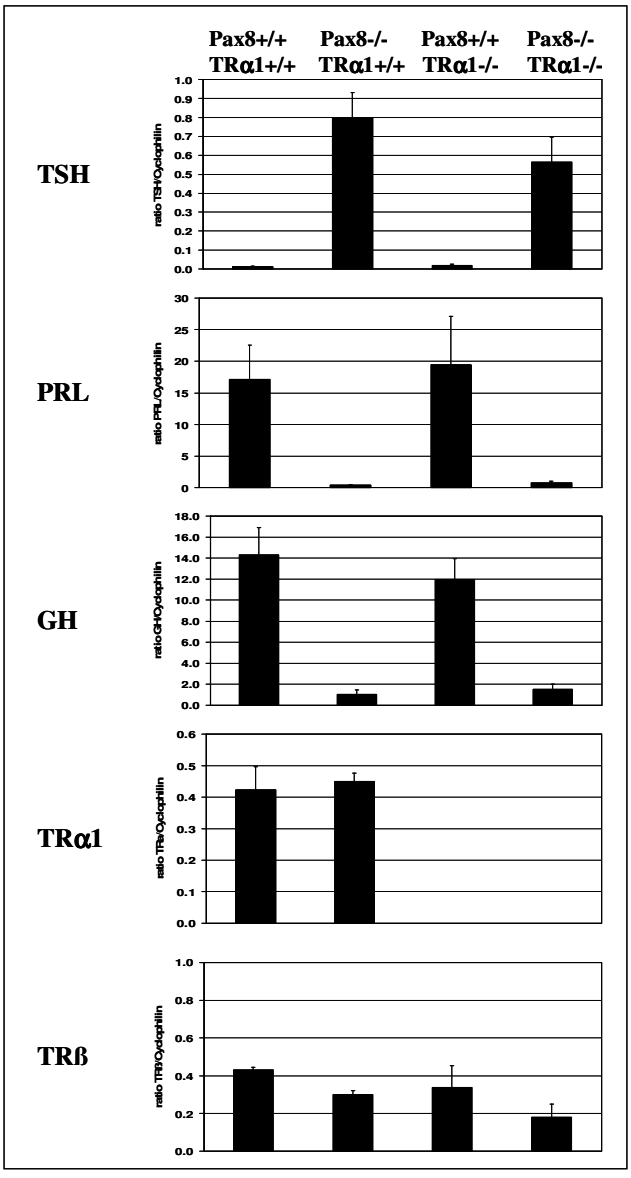


Fig. 3.5: mRNA levels of pituitary genes in the different mutant mice as analyzed by Real-Time PCR.

No obvious changes in transcript levels were observed in the pituitaries of TRα1$^{-/-}$ mice compared to wildtype controls. Again, the Pax8$^{-/-}$TRα1$^{-/-}$ pituitary phenotype closely

resembled that of $Pax8^{-/-}$ animals implying that TRα1 aporeceptor activity is not responsible for the malformation of the pituitary as observed in $Pax8^{-/-}$ mice.

We further employed quantitative Real-Time PCR to assess the transcript levels of the T3 binding TR isoforms. Compared to control animals, TRα1 mRNA levels were not different in $Pax8^{-/-}$ mice. TRβ transcript levels were also quite similar in all four genotypes.

3.1.5. Deiodinase Type II in the Pituitary

In the pituitary, expression of deiodinase type II is known to be negatively regulated by TH and critically involved in mediating the effect of TH on pituitary gene expression. Therefore the regulation and the localization of this enzyme were also analyzed. While D2 mRNA expression could be detected in the thyrotrophic cells by colocalization with TSH mRNA, none of the other endocrine celltypes like somatotrophs, corticotrophs, lactotrophs or gonadotrophs expressed D2 (Fig. 3.6). When quantified, 99% of cells expressing TSH also expressed D2 mRNA, whereas 43% of the D2 positive cells were not positive for TSH, indicating that a subpopulation of non-endocrine cells (probably folliculo-stellate cells) also expressed D2 mRNA.

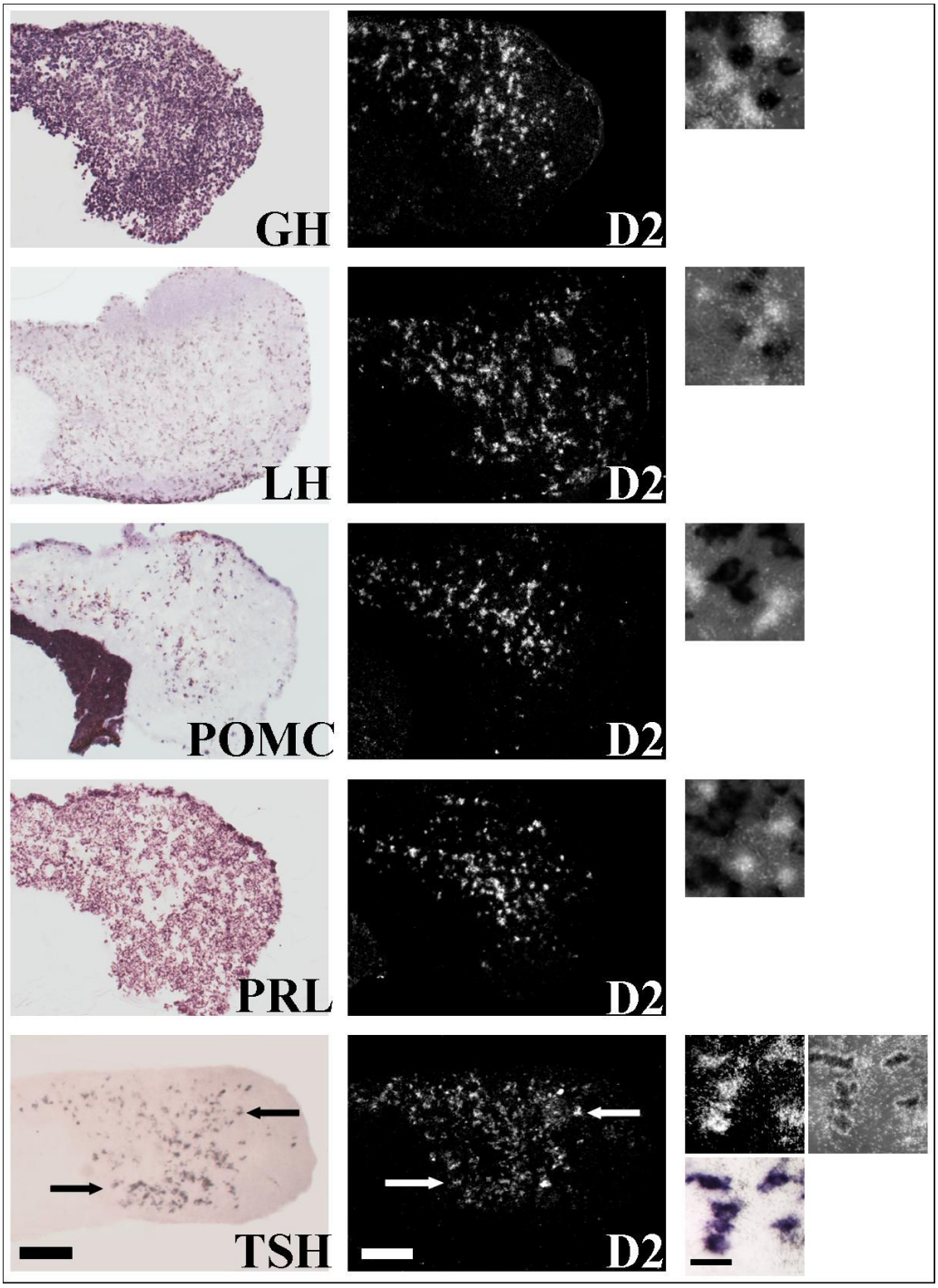


Fig. 3.6: Colocalization of D2 mRNA with hormonal mRNA expression. The left panel shows the mRNA expression pattern of the hormones GH, LH, POMC, prolactin and TSH as assessed by in situ hybridization histochemistry with DIG labeled probes. The middle panel shows the corresponding D2 mRNA expression as analyzed by in situ hybridization with radioactively labeled probes and visualized under darkfield illumination. The arrows indicate identical structures in the TSH and D2 mRNA expression patterns. The right panel shows single cells at higher magnification, either expressing D2 mRNA (white grains) or hormonal mRNA (black spots). Scale bar left panels 200µm, scale bar right panel 20µm.

The regulation of D2 by TRα1 was also analyzed. Real-Time PCR was used to determine the mRNA expression levels, whereas enzymatic activity was analyzed by the turnover of radioactively labeled thyroxine (Fig. 3.7).

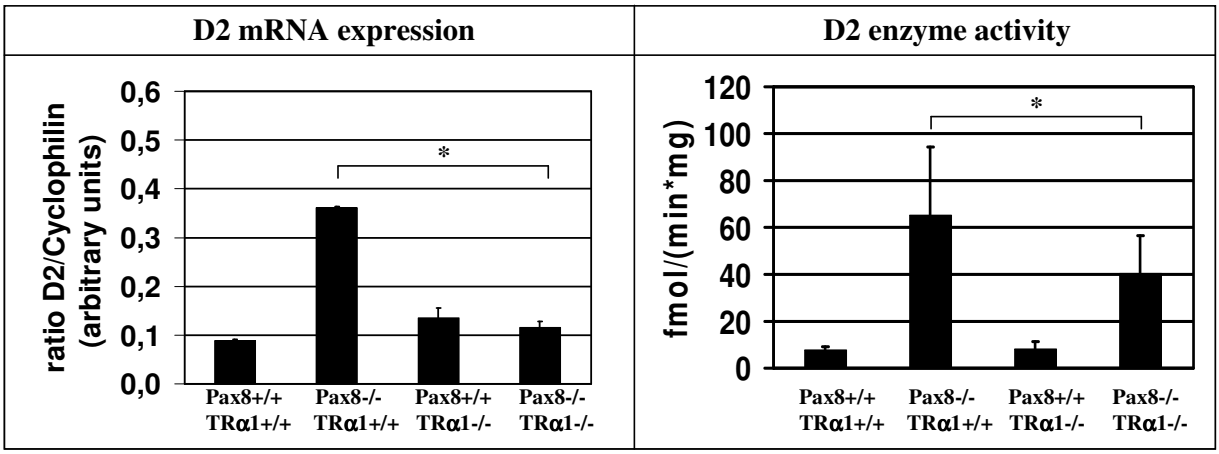


Fig. 3.7: Deiodinase type II mRNA levels as analyzed by Real-Time PCR and the enzyme activity in pituitaries of the different mutant mice. (*: $p<0.05$)

While no significant difference was observed between wildtype and TRα1$^{-/-}$ animals neither in mRNA expression nor in enzyme activity, D2 mRNA levels were upregulated in athyroid Pax8$^{-/-}$ mice as expected. Surprisingly this upregulation of D2 mRNA was abolished in Pax8$^{-/-}$ TRα1$^{-/-}$ double mutant mice. Since D2 activity is posttranslationally negatively regulated by a non-genomic effect of T4, D2 enzyme activity was higher in these mice when compared to wildtype and TRα1$^{-/-}$ animals, although mRNA levels were not different. However, D2 enzyme activity was still significantly reduced in Pax8$^{-/-}$TRα1$^{-/-}$ animals when compared to Pax8$^{-/-}$ mice, indicating that the posttranslational regulatory mechanism cannot fully compensate for the reduction of D2 mRNA levels in the double mutants.

3.2. Identification of Thyroid Hormone Regulated Factors in the Pituitary

3.2.1. Prolactin mRNA Expression and Lactotroph Development in Pax8$^{-/-}$ Mice

As already described, Pax8 deficient animals exhibit a reduced mRNA expression of prolactin as well as a dramatically reduced number of lactotrophs in the anterior pituitary (Friedrichsen et al. 2004). TH replacement therapy from P3 until P21 restores these deficits as analyzed by ISH, indicating that both effects are a direct consequence of the athyroid conditions in Pax8 null mice (Fig. 3.8). The reduction in the lactotrophic cell population was additionally confirmed using radioactive labeled probes for dopamine receptor 2 which is exclusively expressed on these cells (Inoue et al. 1999).

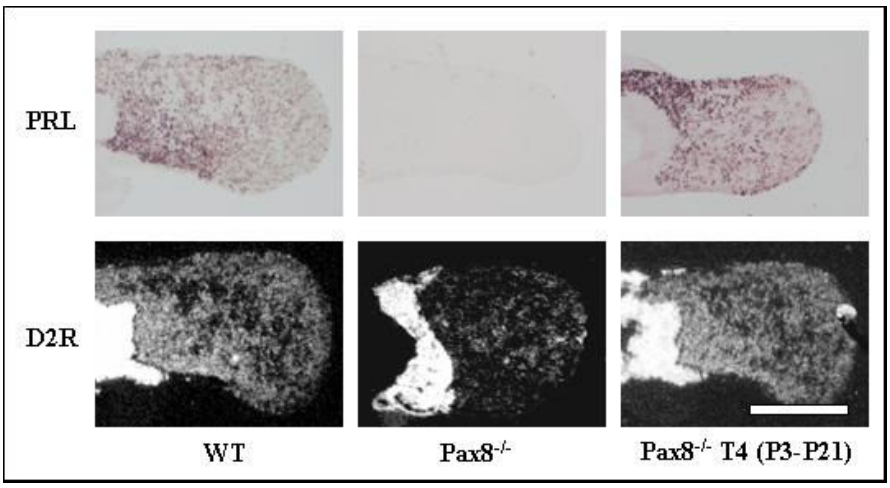


Fig. 3.8: In situ hybridization showing the mRNA expression of prolactin (PRL) and dopamine receptor 2 (D2R) in wildtype (WT), untreated and T4 treated Pax8$^{-/-}$ mice. Scale bar 500µm.

In good agreement with previous observations (Friedrichsen et al. 2004), a complete restoration of the lactotroph population requires treatment with TH from P3-21 (Fig. 3.8), while prolactin mRNA expression in the remaining lactotrophs is already restored after a 3 day administration of physiological TH doses (Fig. 3.9C) (Friedrichsen et al. 2004).

To analyze postnatal lactotroph development in Pax8 null mice in more detail, these animals were injected at the age of 3, 6 and 12 weeks for 3 consecutive days with physiological doses of TH to increase the prolactin mRNA to a detectable level. Animals at 6 and 12 week of age

were obtained by prolonged weaning as described above (see 3.1.1) and were thus completely athyroid during postnatal life until the injections. Lactotroph distribution was then analyzed by ISH for prolactin mRNA (Fig. 3.9).

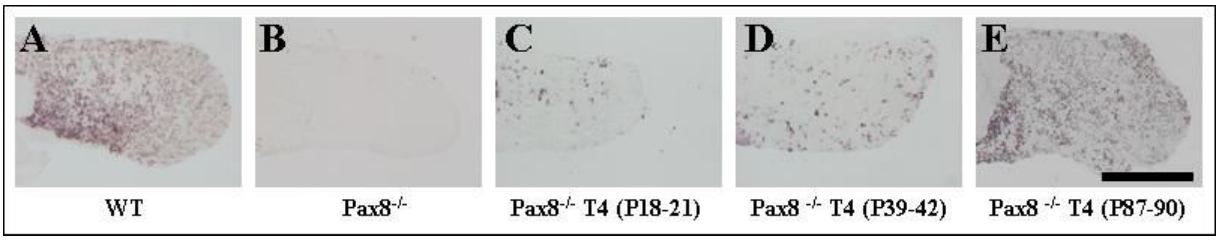


Fig. 3.9: In situ hybridization for prolactin mRNA in P21 wildtype (A) and Pax8 null (B) mice as well as Pax8 deficient animals at the age of 3 (C), 6 (D) and 12 (E) weeks which were treated for 3 days with physiological doses of TH. Scale bar 500µm.

As expected, prolactin mRNA expression was not detectable in untreated Pax8$^{-/-}$ mice (Fig. 3.9B) and even after treating these animals with T4 for 3 days, only few lactotrophs were observed (Fig. 3.9C). In Pax8 deficient animals at 6 weeks of age that were also treated with T4 for 3 days (Fig. 3.9D), the number of lactotrophs was already increased compared to the Pax8 null mice at 3 weeks of age. At 12 weeks of age, the number of lactotrophs was further increased (Fig. 3.9E) reaching almost the level of wildtype mice at 3 weeks of age (Fig. 3.9A). This result suggests that under athyroid conditions the development of lactotrophs is not completely abolished but only delayed. Untreated Pax8 null mice throughout all ages did not show any detectable prolactin mRNA (data not shown).

3.2.2. Differential Gene Expression in Pituitaries of Pax8 Deficient Animals

In order to identify factors that are regulated by TH and might be involved in modulating prolactin mRNA expression or lactotroph development by auto- or paracrine mechanisms, the mRNA expression profile of pituitaries from wildtype and Pax8 deficient animals was analyzed using the Affymetrix Gene Chip 430A. The comparison was done in duplicates with the Affymetrix Micorarray Analysis Software and revealed that the expression level of 11.3% of all genes analyzed was significantly changed. In average 5.6% of the genes were down- and 5.7% were upregulated in Pax8$^{-/-}$ mice.

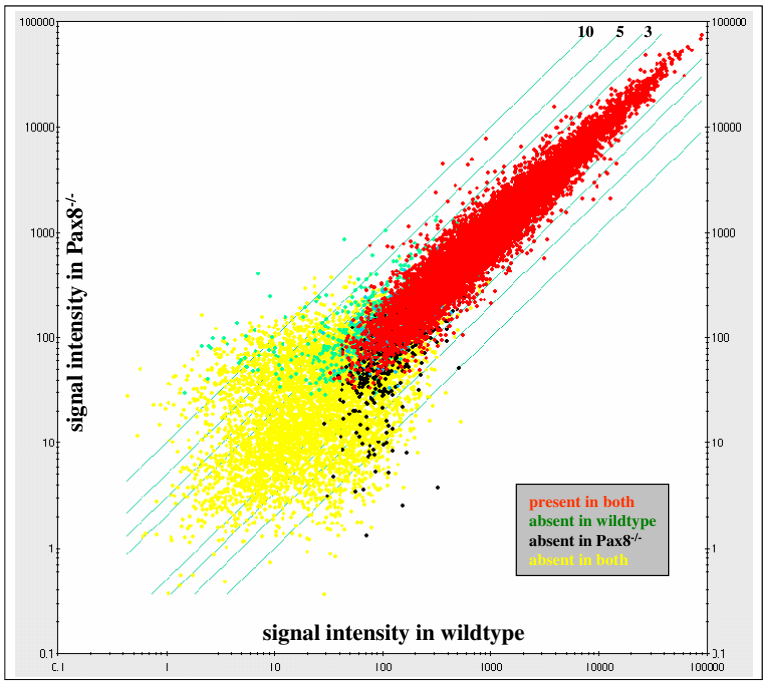


Fig. 3.10: Scatterplot showing the expression levels of >34.000 genes in pituitaries of wildtype and Pax8 deficient mice. Green lines indicate a 2, 3, 5 and 10-fold change in gene expression between wildtype and mutant mice. Gene expression was considered absent when mRNA levels were not significantly above background. Red spots mark genes that were expressed in both genotypes, green spots indicate genes only expressed in the wildtype, black spots symbolize genes only expressed in pituitaries of Pax8 null mice and yellow spots mark genes that were not significantly expressed in mouse pituitaries.

Many of the genes with significant changes in both arrays were either of unknown function such as some RIKEN cDNA clones, or the expression of these genes was already known to be

changed in pituitaries of Pax8 null mice such as the genes encoding β-TSH, TRH-R1, GH, PRL, β-FSH, β-LH or dopamine receptor 2. Due to the limitations of a PhD thesis, it was impossible to validate all genes differentially expressed in the pituitary of Pax8 deficient animals. Thus, eight genes which were considered to be of biological significance in the context of this study were chosen for further investigation (Table 3.2).

1st experiment				2nd experiment				mean		
mRNA wildtype		mRNA Pax8$^{-/-}$		mRNA wildtype		mRNA Pax8$^{-/-}$		change -fold	error (SD)	gene name
69,1	A	361	P	103,8	A	574,6	P	5,4	0,2	Calcitonin Gene Related Peptide α
465,1	P	2831,1	P	75	A	4723,8	P	35	28,4	Annexin A10 (Spot 1)
4,5	A	139,3	P	1,9	A	238,7	P	78	47,3	Annexin A10 (Spot 2)
431,1	P	883	P	616,9	P	1174,7	P	2	0,1	Annexin A1
10837	P	7884,1	P	11449,7	P	4719,8	P	0,6	0,2	Annexin A5
358,1	P	4553,7	P	182,6	A	2983,5	P	15	1,8	Tyrosine Hydroxylase
978	P	1226,4	P	591,3	P	2087,3	P	2,4	1,1	Neuromedin B
308,1	P	2414,5	P	219,8	A	1225,2	P	6,7	1,1	Calsequestrin 2
44,5	A	876,3	P	196,9	A	1308,9	P	13	6,5	Calbindin 2
899	P	617,1	P	1346,4	P	607,3	P	0,6	0,1	STAT 1
167,4	P	552,5	P	17,9	A	638,2	P	19,5	16,2	STAT 4

Table 3.2: Differentially regulated genes that were chosen for further analysis (values show the relative expression level in arbitrary units after normalization with the overall signal intensity; A: absent, P: present).

3.2.3. Validation of Differential mRNA Expression by In Situ Hybridization

To confirm the results from the microarray analysis, cDNA fragments of the candidate genes were cloned and subsequently used for the generation of ISH probes. While mRNA expression of calcitonin-gene related peptide and neuromedin B could not be detected by this technique, mRNA expression of the other genes was in good agreement with the microarray data revealing an upregulation of Annexin A1, A10, tyrosine hydroxylase, STAT4, calbindin 2 and calsequestrin 2 as well as a downregulation of Annexin A5 and STAT1 in athyroid Pax8 null mice (Fig. 3.11).

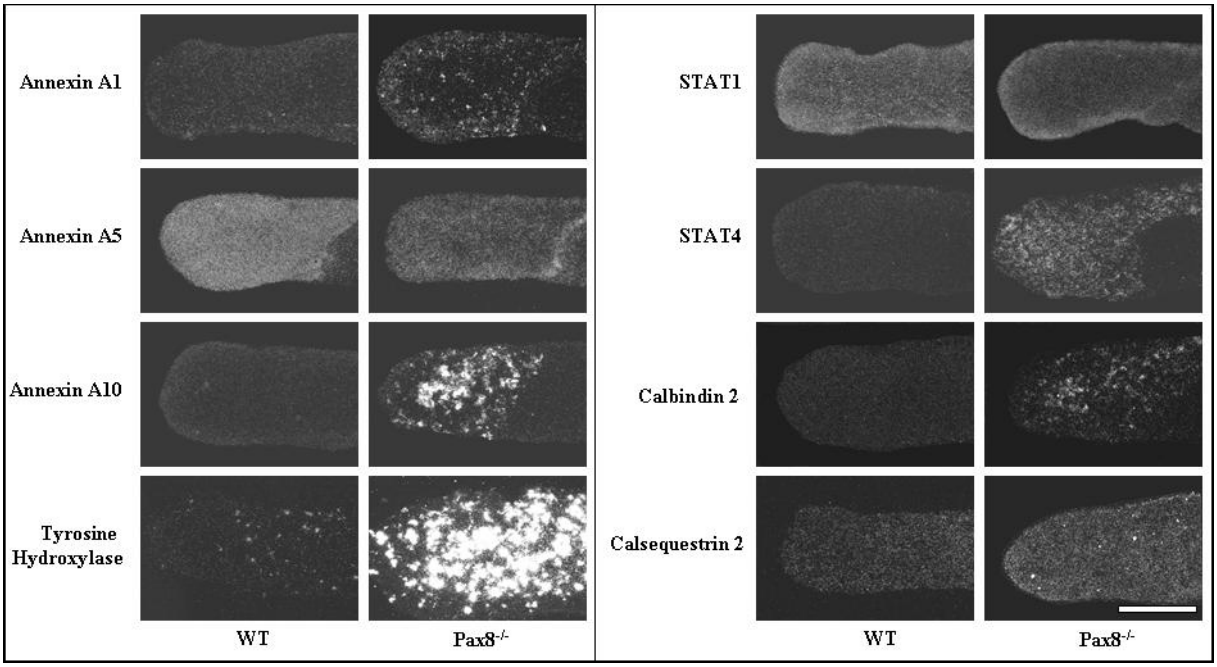


Fig. 3.11: Validation of the microarray data by in situ hybridization histochemistry showing the mRNA expression of chosen candidate genes in pituitaries of wildtype and Pax8 deficient mice. Scale bar 500µm.

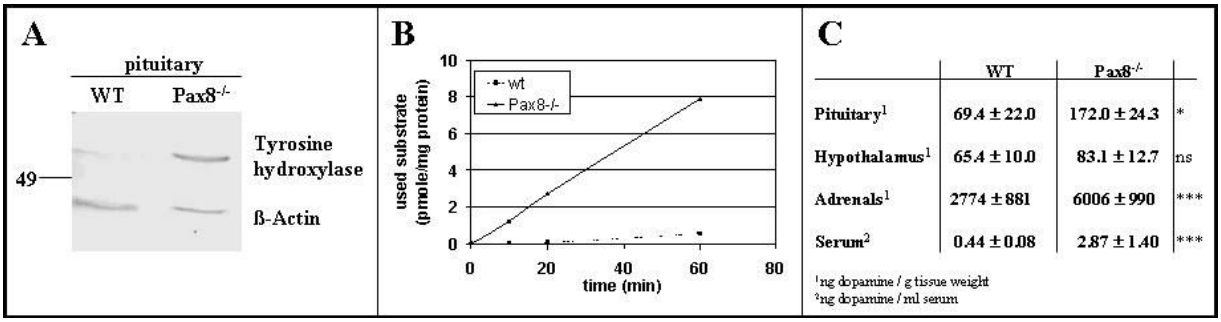
3.2.4. Tyrosine Hydroxylase as a Potential Candidate for Lactotroph Inhibition

Since dopamine is the decisive factor in the control of lactotroph proliferation and prolactin secretion (Ben-Jonathan et al. 2001), tyrosine hydroxylase seemed to be the most promising candidate among these genes, because this enzyme catalyzes the committed step in dopamine synthesis (Kumer et al. 1996).

The increase of tyrosine hydroxylase mRNA in anterior pituitaries of Pax8 null mice was additionally confirmed by quantitative Real-Time PCR revealing a 20-fold increase at the mRNA level, whereas mRNA expression of dopa decarboxylase, the second enzyme necessary for dopamine synthesis, was not increased. No transcripts were detected for enzymes that are required for the production of epinephrine from dopamine like dopamine-β-hydroxylase or phenylethanolamine-N-methyl-transferase (PNMT) (data not shown).

To analyze whether tyrosine hydroxylase is indeed expressed at the protein level, a western blot analysis was performed. While in pituitaries of wildtype mice only a faint band of

tyrosine hydroxylase was detected by this technique, athyroid Pax8 deficient animals revealed a distinct band of this protein (Fig. 3.12A). To confirm the enzymatic activity of the tyrosine hydroxylase protein, an activity assay was performed by measuring the in vitro conversion of ^{3}H-labeled tyrosine (Fig. 3.12B). The results demonstrated that pituitaries of Pax8$^{-/-}$ but not of wildtype mice had the capacity to actively synthesize dopamine.



	WT	Pax8$^{-/-}$	
Pituitary[1]	69.4 ± 22.0	172.0 ± 24.3	*
Hypothalamus[1]	65.4 ± 10.0	83.1 ± 12.7	ns
Adrenals[1]	2774 ± 881	6006 ± 990	***
Serum[2]	0.44 ± 0.08	2.87 ± 1.40	***

[1]ng dopamine / g tissue weight
[2]ng dopamine / ml serum

Fig. 3.12: Analysis of tyrosine hydroxylase protein content in the anterior pituitary by Western Blot analysis (A) and enzymatic activity assay (B). The analysis revealed an increased amount of enzymatically active tyrosine hydroxylase under athyroid conditions. The dopamine levels in pituitary, hypothalamus, adrenals and serum were then determined by radioimmuno assay (C). (*:p<0.05; ***:p<0.001; ns: not significant)

The analysis of the dopamine content by radioimmuno assay revealed an increased dopamine content in anterior pituitaries of Pax8$^{-/-}$ mice (Fig. 3.12C), indicating that the increase of tyrosine hydroxylase in this tissue indeed lead to higher dopamine synthesis. An increase of dopamine in the serum and the adrenals of Pax8 null mice was also detected, which is not easily explained.

The dopamine content in the hypothalamus was not different in Pax8 deficient animals compared to wildtype controls, which is in good agreement with an unaltered tyrosine hydroxylase mRNA expression in hypothalamic areas of these mice as assessed by ISH (Fig. 3.13A), indicating that the hypothalamus is not the cause for the higher dopamine content in the pituitary.

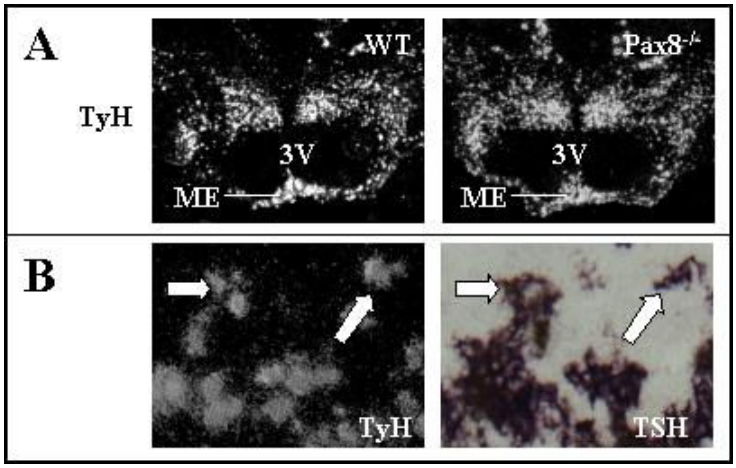


Fig. 3.13: Tyrosine hydroxylase mRNA expression in the hypothalamus of wildtype (WT) and Pax8 deficient animals (A) as assessed by radioactive in situ hybridization (ME: median eminence, 3V: 3rd ventricle). In anterior pituitaries of Pax8 null mice (B), radioactively labeled probes for tyrosine hydroxylase (TyH) mRNA colocalize with DIG-labeled probes for β-thyroid stimulating hormone (TSH) as analyzed by double in situ hybridisation (white arrows indicate identical cells).

To identify the cellular localization of tyrosine hydroxylase in the anterior pituitary, a colocalization study was performed by double ISH with radioactively labeled probes for tyrosine hydroxylase and DIG-labeled probes for β-TSH. As shown in Fig. 3.13B, most of the thyrotrophic cells also express tyrosine hydroxylase mRNA.

To confirm the regulation of tyrosine hydroxylase mRNA expression by TH, P18 Pax8$^{-/-}$ mice were treated with physiological doses of thyroxine for 3 days. This treatment lead to an almost complete reduction of tyrosine hydroxylase mRNA expression and to the mRNA expression of prolactin in a few cells (Fig. 3.14), suggesting that the reduction of tyrosine hydroxylase causes decreased dopamine production.

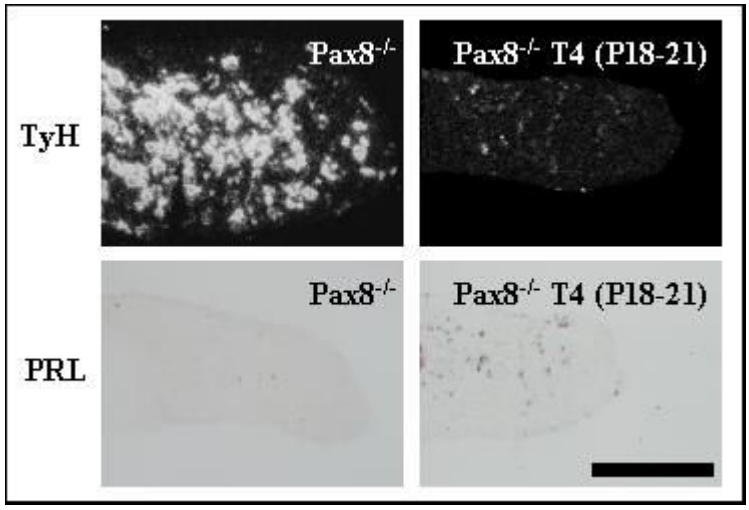


Fig. 3.14: Rapid regulation of tyrosine hydroxylase mRNA expression by TH. After a 3 day treatment of Pax8 deficient mice with physiological doses of T4, tyrosine hydroxylase mRNA was drastically reduced and prolactin transcripts (PRL) were detected in a few lactotrophs only. Scale bar 500µm.

To investigate whether the increased local dopamine production in the anterior pituitary of athyroid mice is responsible for the retardation in lactotroph development and the reduction of prolactin mRNA expression, $Pax8^{-/-}$ mice were treated with the dopamine antagonist haloperidol from P3 until P21 to abolish dopamine action (Fig. 3.15B). As revealed by ISH, prolactin mRNA expression was only detectable in a few cells and the number of lactotrophs was still dramatically reduced compared to wildtype controls (Fig. 3.15A).

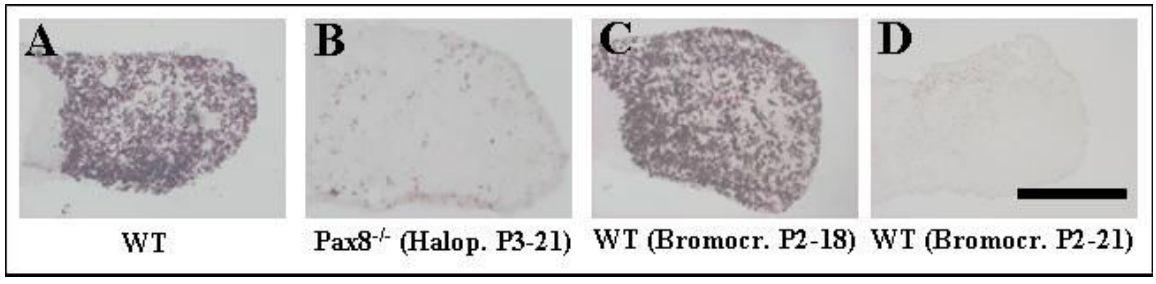


Fig. 3.15: In situ hybridization histochemistry showing prolactin mRNA expression in pituitaries of wildtype (WT), bromocryptine treated wildtype and haloperidol treated Pax8 deficient animals. Scale bar 500µm.

Conversely, to mimic the high dopamine levels in Pax8 deficient animals, wildtype mice were treated with the dopamine agonist bromocryptine from P2 until P21 and analyzed at P21. Pituitaries of these mice did not show any detectable prolactin mRNA expression (Fig. 3.15D), indicating that the bromocryptine treatment was carried out properly. Subsequently, wildtype mice were treated with bromocryptine from P2 until P18 and the pituitaries were analyzed at P21. ISH revealed that prolactin transcript levels were almost normalized in these mice within three days. Surprisingly, the number of lactotrophs was not affected (Fig. 3.15C), which suggests, that lactotroph development cannot be blocked by this dopamine agonist.

Since hypothyroidism is known to induce hyperprolactinemia in adult humans, the situation in adult hypothyroid mice was also analyzed. For this, Pax8 deficient animals were treated from P2 onwards with T4 for 40 days and then left without treatment for 10 or 40 days to render them hypothyroid again. The hypothyroidism was confirmed by an upregulation of β-TSH as analyzed by ISH (Fig. 3.16A-C).

As expected, this experimental manipulation caused an increased prolactin mRNA expression in these animals (Fig 3.16D-F). Interestingly, tyrosine hydroxylase mRNA was also increased in the hypothyroid animals (Fig. 3.16G-I), indicating that the dopamine production of the thyrotrophs is not sufficient to reduce prolactin transcript levels effectively in these mice.

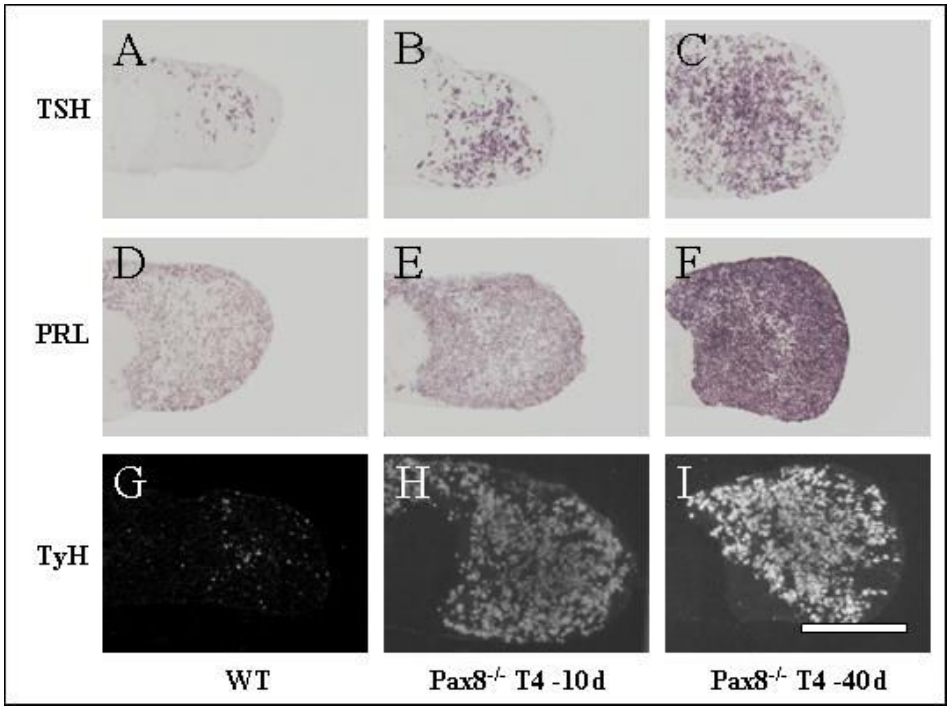


Fig. 3.16: In situ hybridization showing the mRNA expression of β-TSH, prolactin (PRL) and tyrosine hydroxylase (TyH) in pituitaries of wildtype mice at 80 days of age and Pax8 deficient animals that were treated with thyroxine for 40 days and the left without treatment for 10 or 40 days respectively. Scale bar 500µm.

In addition, untreated Pax8 null mice at 6 months of age were also analyzed with regard to their prolactin mRNA expression. These mice were obtained by prolonged weaning as described above (see 3.1.1). Without T4 treatment mRNA expression of prolactin was very low in these mice (Fig. 3.17E), while TSH mRNA was drastically upregulated (Fig. 3.17A) and GH mRNA levels were strongly decreased (Fig. 3.17J). As expected, TSH as well as GH mRNA expression were effectively increased by treatment of Pax8 deficient animals with T4 for 4 days (Fig. 3.17B and K), but not when $Pax8^{-/-}$ mice were treated with haloperidol for 4 weeks (Fig. 3.17C and L). In contrast, prolactin mRNA expression was increased in these mice after thyroxine or haloperidol treatment (Fig. 3.17F, G and H), indicating that dopamine inhibits prolactin mRNA expression in a thyroid hormone dependent manner.

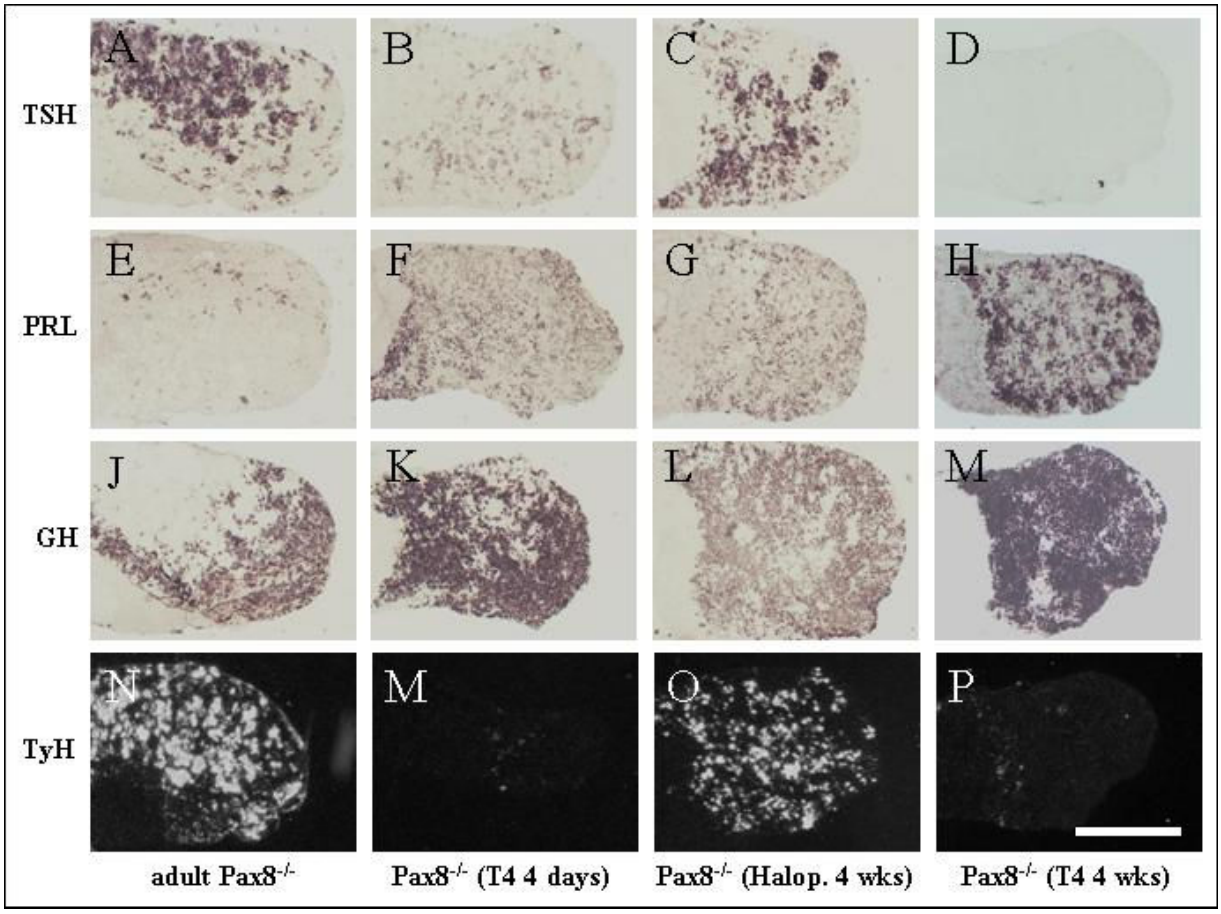


Fig. 3.17: In situ hybridization analysis showing mRNA expression of β-TSH, prolactin (PRL), growth hormone (GH) and tyrosine hydroxylase (TyH) in pituitaries of 6-months-old untreated Pax8$^{-/-}$ mice and Pax8$^{-/-}$ animals that were treated with T4 or haloperidol for the indicated period of time. Scale bar 500µm.

Since an increased stimulation of lactotrophic cells by upregulated hypothalamic TRH is one cause for hyperprolactinemia in humans under hypothyroid conditions (Ceccatelli et al. 1992), TRH-R1$^{-/-}$Pax8$^{-/-}$ double knockout mice were generated by intercrossing TRH-R1$^{-/-}$ (Rabeler et al. 2004) and Pax8$^{+/-}$ mice (Mansouri et al. 1998).

As in the experiment above, these double mutant mice were injected from P2 onwards with T4 for 40 days and then left without treatment for additional 40 days to render them hypothyroid. As in Pax8 deficient mice (Fig. 3.16C), TSH mRNA expression levels were highly increased in pituitaries of TRH-R1$^{-/-}$Pax8$^{-/-}$ double mutants with thyrotrophs exhibiting clear signs of hyperplasia and hypertrophy (Fig. 3.18D). Since the hypothalamic signal TRH

cannot be transmitted to the pituitary in TRH-R1 deficient animals, this result strongly indicates that the distorted condition of the thyrotrophs in athyroid mice is not caused by an overstimulation of the thyrotrophs via TRH but rather by the lack of the negative feedback through TH.

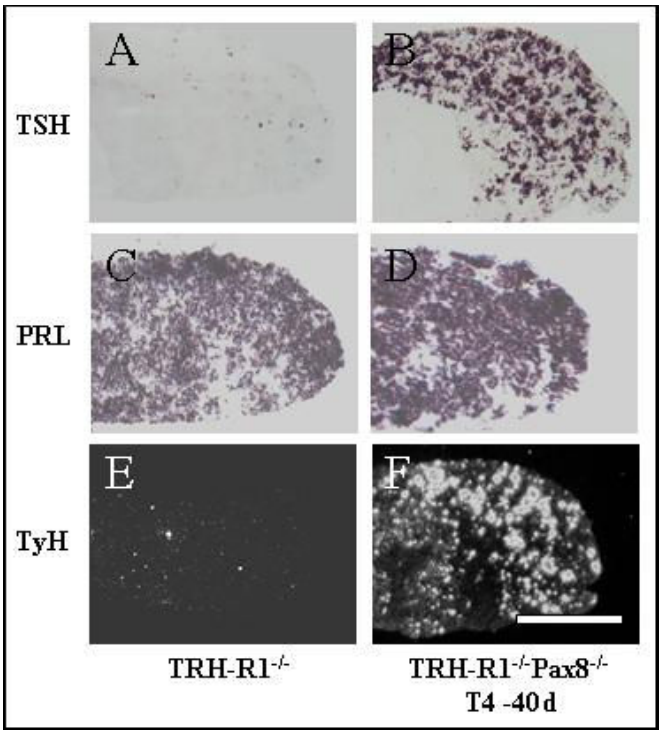


Fig. 3.18: In situ hybridization analysis showing mRNA transcript levels of β-TSH, prolactin (PRL) and tyrosine hydroxylase (TyH) in adult TRH-R1 deficient animals and TRH-R1$^{-/-}$Pax8$^{-/-}$ double mutant mice that were treated with thyroxine for 40 days and then kept without treatment for additional 40 days. Scale bar 500µm.

Compared to TRH-R1 deficient controls (Fig. 3.18C), prolactin mRNA expression was not affected in the experimentally manipulated TRH-R1$^{-/-}$Pax8$^{-/-}$double mutant mice (Fig. 3.18D) suggesting that the increased prolactin mRNA levels in hypothyroid adult Pax8 deficient mice (Fig. 3.16F) were indeed caused by an increased TRH stimulus. As expected, tyrosine hydroxylase mRNA was also increased in the TRH-R1$^{-/-}$Pax8$^{-/-}$ double mutants (Fig. 3.18F). Most interestingly, the observed increase in tyrosine hydroxylase did not lead to a suppression of prolactin mRNA expression in these mice in contrast to the situation in Pax8$^{-/-}$ animals at 3 weeks of age, which did not show significant expression of prolactin mRNA (Fig. 3.9B, p.27).

3.2.5. Role of Annexin A5 in the Anterior Pituitary

To investigate the role of annexin A5, another candidate gene found to be upregulated in the pituitaries of Pax8 deficient mice, annexin A5 deficient animals were analyzed with regard to their mRNA expression of the pituitary hormones by ISH (Fig. 3.19).

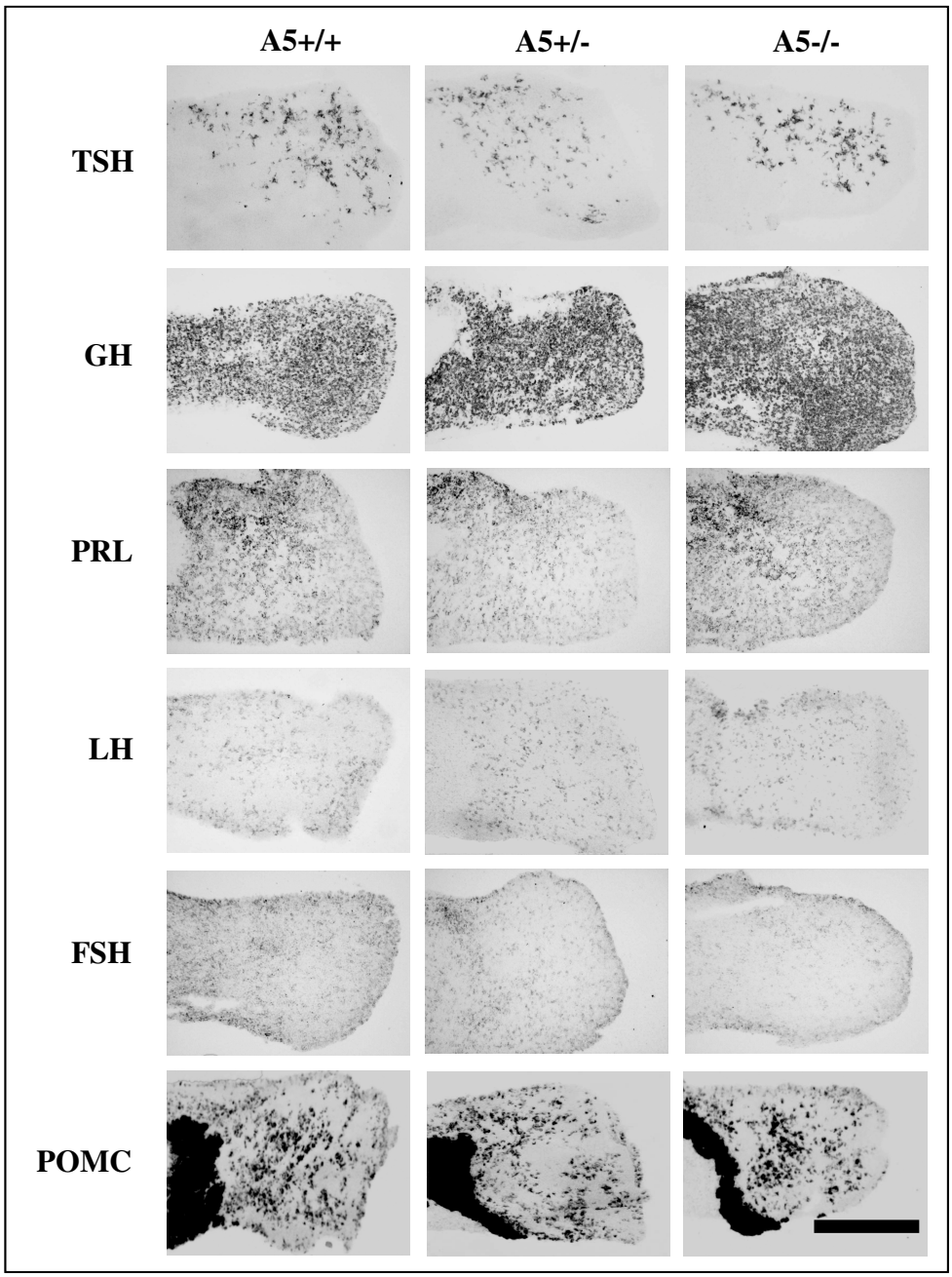


Fig. 3.19: In situ hybridization analysis showing the mRNA expression of β-TSH, growth hormone (GH), prolactin (PRL), β-LH, β-FSH and proopiomelanocortin (POMC) in pituitaries of wildtype ($A5^{+/+}$), heterozygous ($A5^{+/-}$) and annexin A5 deficient mice ($A5^{-/-}$). Scale bar 500µm.

The analysis by ISH revealed no obvious differences neither with regard to the number of cells expressing β-TSH, GH, prolactin, or POMC transcripts, nor with regard to the signal intensity. The mRNA expression of β-LH and β-FSH showed a slight reduction in the number of cells expressing these hormones in the A5 deficient animals. To further quantify this observation, quantitative Real-Time PCR was used (Fig. 3.20), which showed a minor but significant reduction by half of β-FSH mRNA levels in the pituitaries of $A5^{-/-}$ mice. Transcript levels of all other hormones were not significantly changed in A5 deficient mice.

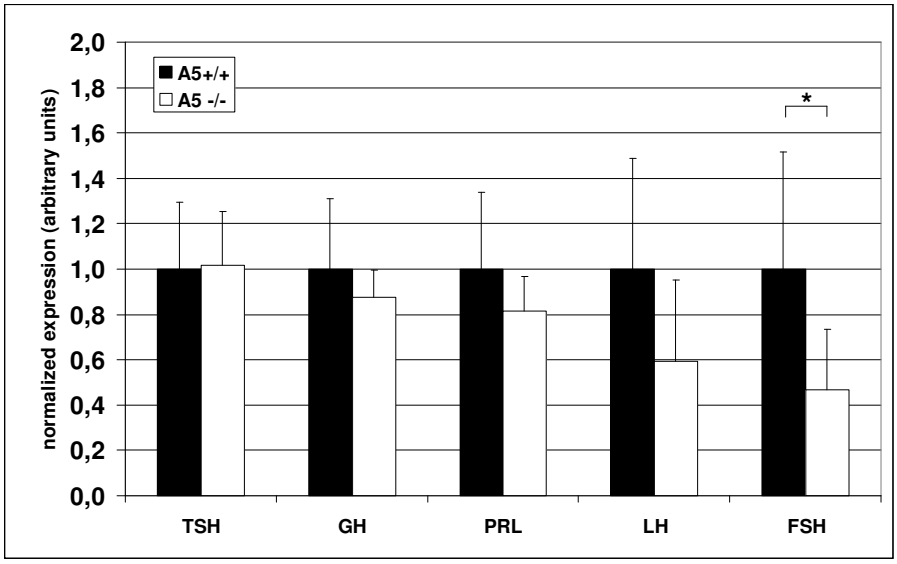


Fig. 3.20: mRNA expression of the pituitary hormones β-TSH, growth hormone (GH), prolactin (PRL), β-LH and β-FSH in wildtype ($A5^{+/+}$) and annexin A5 deficient animals ($A5^{-/-}$) as analyzed by quantitative Real-Time PCR. (*:$p<0.05$)

3.3. Analysis of the Infertility in T4-Treated Pax8$^{-/-}$ Mice

3.3.1. T4-Substituted Pax8$^{-/-}$ Mice Are Infertile

As a consequence of their athyroidism, Pax8$^{-/-}$ mice die around weaning time and therefore they can only be obtained by mating heterozygous Pax8$^{+/-}$ animals. In the attempt to overcome this disadvantage and to set up homozygous breeder pairs, congenital hypothyroid Pax8$^{-/-}$ mice were treated with T4 from P2 onwards in the expectation that they will survive to adulthood and become fertile, since TH replacement therapy generally restores the deficits observed in CH (Friedrichsen et al. 2004, Christ et al. 2004). Although T4-treated Pax8$^{-/-}$ animals of both genders developed similarly to wildtype littermates, they did not reproduce when caged together with fertile wildtype mice of the opposite gender.

3.3.2. Expression of Pituitary Hormones in T4-Substituted Pax8$^{-/-}$ Mice

Untreated Pax8$^{-/-}$ mice exhibit a dramatically distorted cellular composition of the anterior pituitary with hypertrophy and hyperplasia of the thyrotrophs, an almost complete absence of lactotrophs and a drastically reduced number of somatotrophs (Friedrichsen 2004). Therefore the mRNA expression of β-TSH, prolactin, GH, β-LH, β-FSH and POMC was analyzed by ISH in T4-substituted Pax8$^{-/-}$ mice of both genders in order to assess whether their infertility might be caused by an impaired expression of these hormones. Compared to wildtype littermates, no obvious differences neither in the mRNA expression of the hormones analyzed nor in the cellular composition of the gland were found in T4-treated Pax8$^{-/-}$ mice (Fig. 3.21). This was even true for the expression of β-TSH which is tightly controlled by negative feedback of TH, thus illustrating the euthyroid status of the T4-substituted animals.

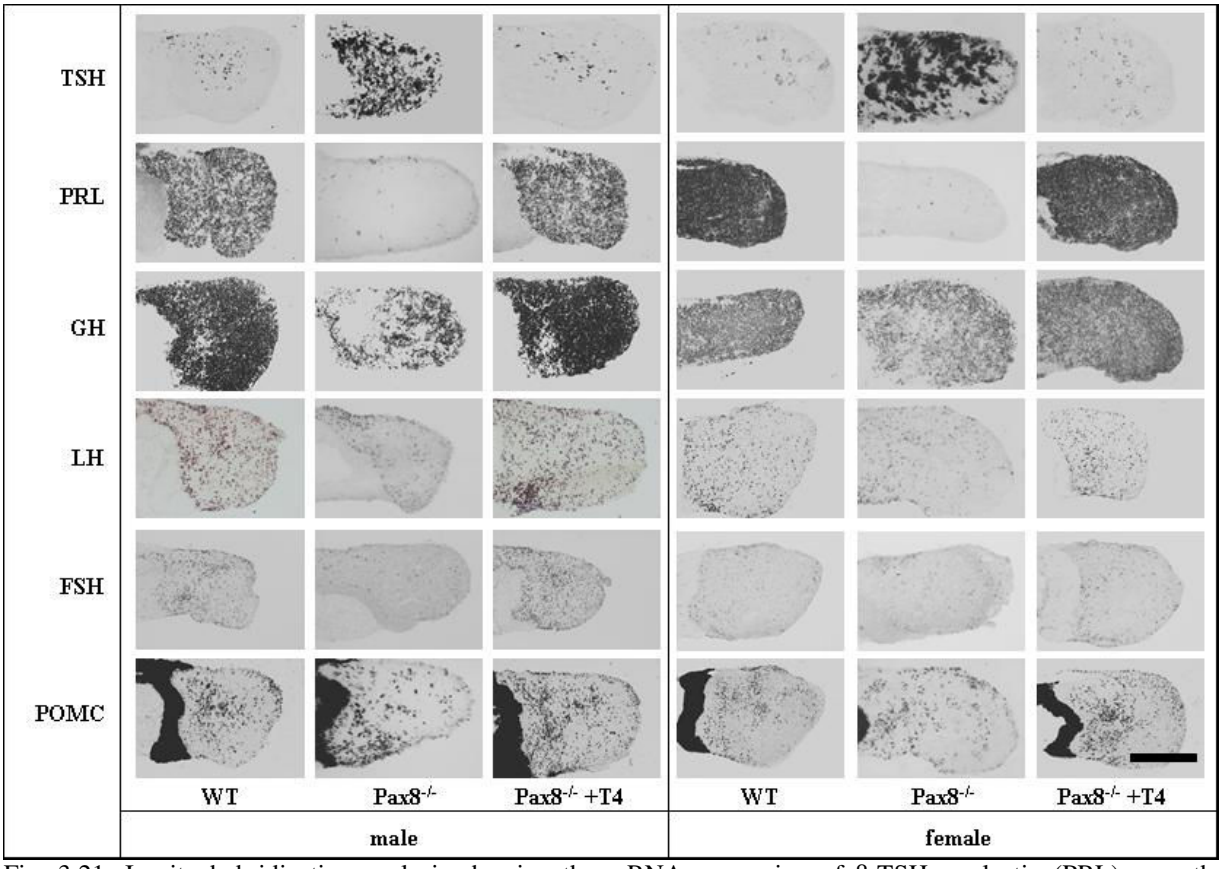


Fig. 3.21: In situ hybridization analysis showing the mRNA expression of β-TSH, prolactin (PRL), growth hormone (GH), β-LH, β-FSH and proopiomelanocortin (POMC) in pituitaries of wildtype (WT), Pax8$^{-/-}$ and T4-treated Pax8$^{-/-}$ mice of both genders. Scale bar 500µm.

3.3.3. Initial Analysis of the Male Reproductive System

As expected from the normal gonadotropin mRNA expression in T4-treated male Pax8$^{-/-}$ mice, serum and intratesticular testosterone levels, although quite variable, were not significantly different between all groups of adult animals (Fig. 3.22A and B).

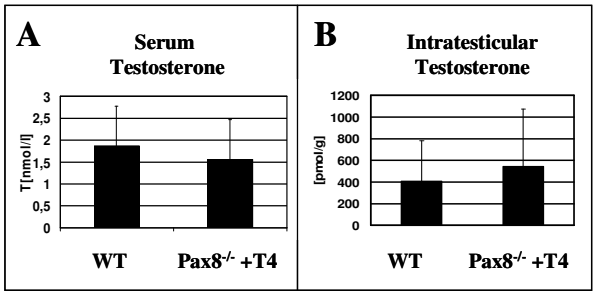


Fig. 3.22: Serum (A) and intratesticular testosterone levels (B) in wildtype (WT) and T4-treated Pax8$^{-/-}$ mice as determined by radioimmuno assay.

The body weight as well as the weight of the epididymides and the accessory sex glands of adult T4-treated Pax8$^{-/-}$ males were not different when compared to control animals, but testis weight exhibited a significant reduction by 35% (Fig. 3.23).

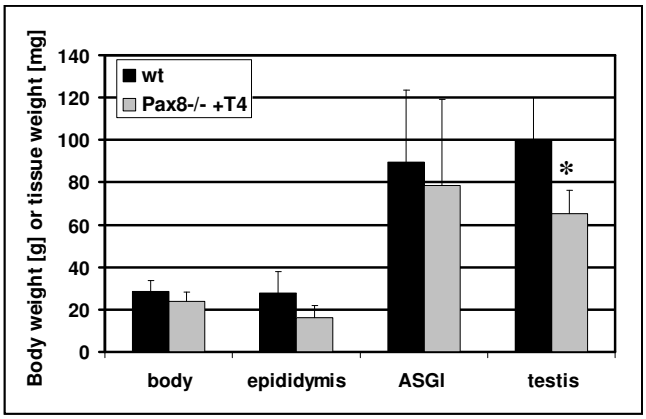


Fig. 3.23: Body weight and weight of the epididymis, accessory sex glands (ASGl) and the testis of wildtype and T4-treated adult Pax8$^{-/-}$ male mice. (*:p<0.05)

3.3.4. Histology of the Testis and Quantification of Spermatogenesis

To investigate the testicular phenotype, cross sections of the testis were analyzed histologically. Wildtype controls showed qualitatively normal spermatogenesis in the testicular cross-sections with elongated spermatids in almost all tubules (Fig. 3.24A). In contrast, testis from T4-treated Pax8 deficient animals revealed focally atrophic regions with tubules showing arrested spermatogenesis and a variable degree of disorganization (Fig. 3.24B).

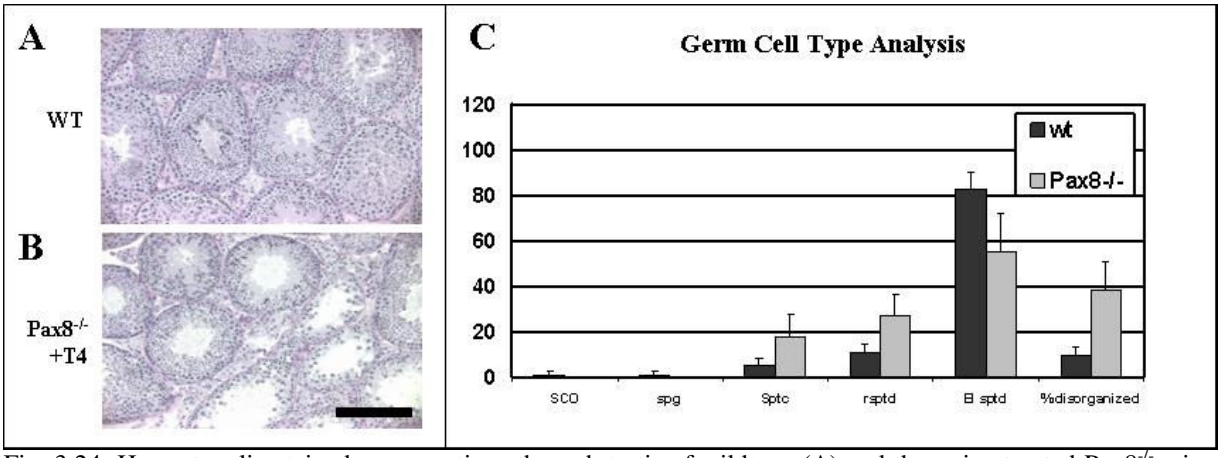


Fig. 3.24: Haematoxylin stained cross-sections through testis of wildtype (A) and thyroxine treated Pax8$^{-/-}$ mice (B). Scale bar 50µm. Quantification of the testicular spermatogenesis in these mice revealed a high degree of disorganization in T4-treated Pax8 deficient animals (C). (SCO: Sertoli Cells Only; spg: spermatogonial; sptc: spermatocytes; rsptd: round spermatids; El sptd: elongated spermatids).

Subsequent quantification of the spermatogenic progress revealed an increased number of tubules with an arrest of the spermatogenic process at the level of spermatocytes or round spermatids in T4-treated Pax8 deficient mice, while the number of tubules containing elongated spermatids as the most advanced germ cell type was decreased compared to wildtype controls (Fig. 3.24C).

3.3.5. Analysis of the Epididymis and the Efferent Duct

Whereas in wildtype animals the epididymal lumen was full of spermatozoa, the epididymis of adult T4-treated Pax8$^{-/-}$ mice did not contain any sperm (Fig. 3.25A). One out of the three Pax8 deficient mice analyzed showed unilateral absence of the efferent ducts and the epididymis up to the vas deferens.

An abnormality in epithelial development was observed in the proximal corpus of the epididymis where the epithelium in Pax8 null mice was taller than in other regions and in the same region of the wildtype. In the more distal regions, especially in the cauda, large vacuoles were frequently found in the epithelium of the Pax8 null mice (Fig. 3.25A; arrows).

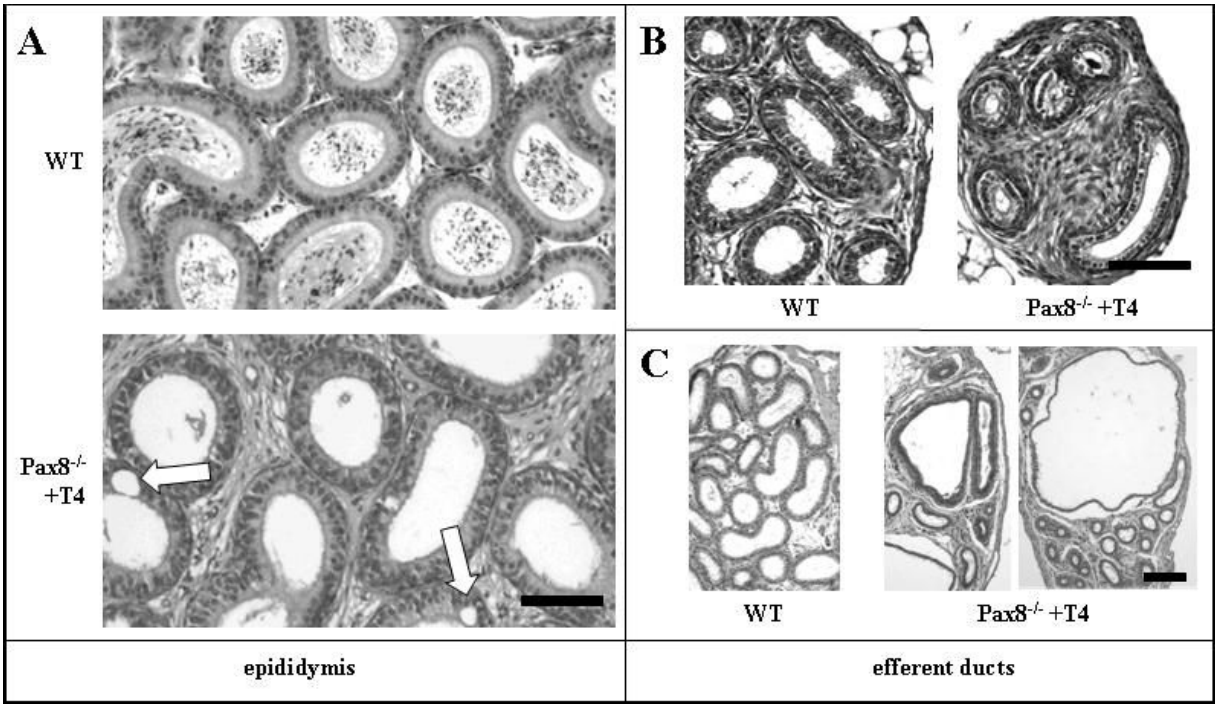


Fig. 3.25: Cross-sections through the epididymis (A) and efferent ducts (B and C) of wildtype (WT) and T4-treated Pax8$^{-/-}$ mice. The white arrows indicate the large vacuoles found in the epididymal epithelium of T4-treated Pax8 null animals. Scale bars 50µm.

Histological examination of the efferent ducts revealed a narrowed lumen in Pax8$^{-/-}$ animals with more extensive connective tissue between the tubules (Fig. 3.25B). Additionally, dilations of the efferent ducts sometimes with extremely large lumen and flattened low epithelium were observed in T4-treated Pax8 deficient animals (Fig. 3.25C).

To obtain additional insights for an involvement of TH signalling in reproductive functions, TRα1$^{-/-}$TRβ$^{-/-}$ mice were also analyzed with regard to their fertility. Histological analysis revealed no differences when the reproductive system was compared with wildtype mice; the epididymal tubules were full of spermatozoa and those matured and stored in the cauda of the epididymis showed the same percentage of motility in both phenotypes. Detailed measurement of sperm kinematics using computerized analysis (Yeung et al. 2002) revealed a slight but significant decrease in the swimming velocities of the spermatozoa from TRα1$^{-/-}$ TRβ$^{-/-}$ double mutants (Table 3.3).

	WT	TRα1$^{-/-}$TRβ$^{-/-}$	
% Motility	43.3 ± 4.2	43.9 ± 6.9	
VCL (µm/s)	141 ± 2	124 ± 2	*
VAP (µm/s)	93.6 ± 6	71.3 ± 3	*
VSL (µm/s)	80.7 ± 13.6	57.6 ± 5.1	
LIN (%)	59.2 ± 10.1	46.1 ± 3.1	
ALH (µm)	9.1 ± 1.4	8.1 ± 0.3	

Table 3.3: Motility analysis of mature spermatozoa from the cauda epididymidis of wildtype and TRα1$^{-/-}$TRβ$^{-/-}$ double mutant mice. The following kinematic parameters were measured: curvilinear (VCL), averaged path (VAP) and straight-line (VSL) velocities, linearity of swim-path (LIN) and amplitude of lateral head displacement (ALH). Values are mean ± SEM. (*:$p<0.05$)

The presence of motile sperm in the epididymis of TRα1$^{-/-}$TRβ$^{-/-}$ double mutant mice indicates that the defects observed in male Pax8 deficient animals are not related to disturbances in TH metabolism.

3.3.6. Ovarian Histomorphology and Steroid Production

T4-substituted female Pax8$^{-/-}$ mice exhibited normal ovarian morphology as shown by the presence of different stages of follicle development similar to wildtype controls (Fig. 3.26A). Besides mature follicles, corpora rubra and corpora lutea could also be seen (data not shown) giving proof of ovulation. Serum hormone levels of 17β-estrogen and progesterone did not show any significant difference in adult T4-substituted Pax8$^{-/-}$ mice when compared to wildtype controls (Fig. 3.26B).

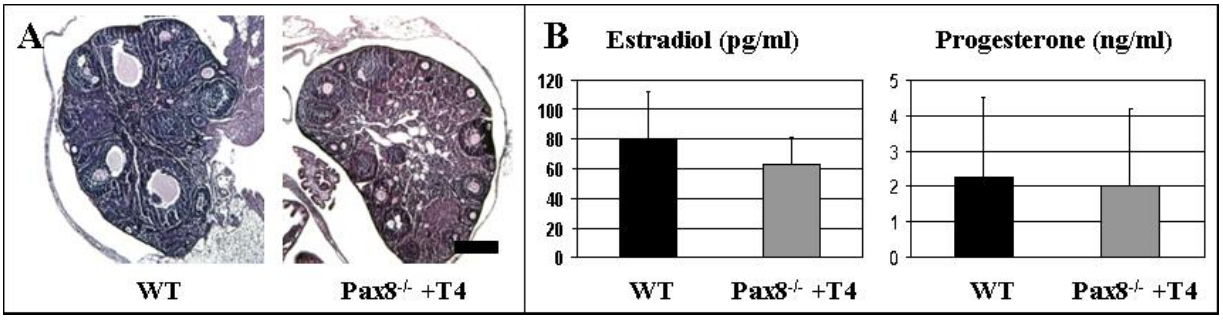


Fig. 3.26: Ovarian morphology of wildtype and thyroxine treated Pax8$^{-/-}$ mice in (A), serum levels of estradiol and progesterone in (B). Scale bar 300µm.

3.3.7. Evaluation of the Female Reproductive Tract

T4-substituted Pax8$^{-/-}$ mice at seven weeks as well as at six months of age exhibited a vagina closed by a lucent membrane (Fig. 3.27A). Following laparatomy, ovarian gross morphology was inconspicuous, but the two uterine horns were absent. Instead, only a very thin ligament could be seen. An obvious manifestation of a dilated, fluid-filled fallopian tube, a so-called hydrosalpinx, had developed in 75% of mice at seven weeks and in 85% of mice at six months after birth (Fig. 3.27A).

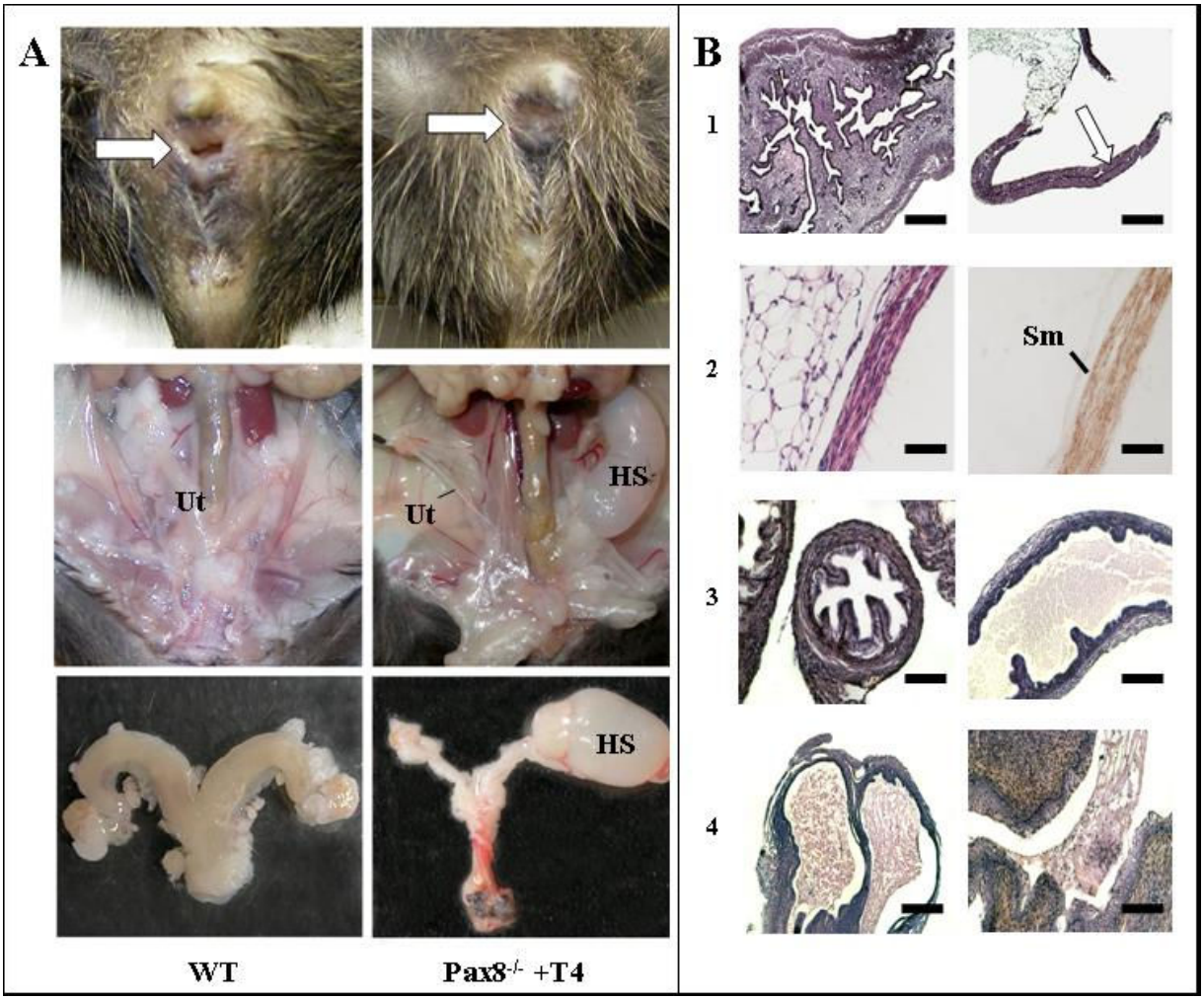


Fig. 3.27: Histology of the female reproductive tract of wildtype and thyroxine treated Pax8$^{-/-}$ mice analyzed macroscopically (A), showing the closed vaginal opening (arrow, upper panel), the ligament-like uterus (Ut) and the hydrosalpinx in Pax8 deficient animals (HS, lower panel). Microscopic inspection (B) shows a wildtype uterus compared with the small tissue remnants of Pax8 deficient mice in (B-1); these tissue residues exhibited the histological appearance of smooth muscle cells (Sm) which could be confirmed by staining for α-smooth muscle actin (B-2). (B-3) shows a normal fallopian tube of T4-substituted Pax8$^{-/-}$ mice and segments dilated by a hydrosalpinx revealing a flattened mucosal layer. (B-4) shows sections of the cervix and the vagina in T4-treated Pax8$^{-/-}$ mice (Scale bar in B1, B4 right: 400 µm; in B2: 150 µm; in B3: 300 µm; in B4 left: 125 µm).

The lack of a normal uterus in T4-substituted Pax8$^{-/-}$ mice was confirmed by histological examination which revealed only small remnants of ligament-like tissue surrounded by fat (Fig. 3.27B-1). These ligaments considerably differed in size compared to wildtype mice. Histologically, this residual tissue revealed the morphology of smooth muscle cells which

could be confirmed by staining for α-smooth muscle actin, pointing to a myometrial origin. The endometrial compartment and the formation of a lumen were missing (Fig. 3.27B-2).

In contrast to the uterus, the oviduct of T4-treated Pax8$^{-/-}$ mice appeared macroscopically normal and revealed no obvious differences compared to wildtype mice (Fig. 3.27B-3 left), except those segments which were dilated by the hydrosalpinx (Fig. 3.27B-3 right). Here, an extended fluid filled lumen lined by a flattened mucosal layer was observed. Interestingly, the cervices were normally developed showing the typical wall morphology of this organ. Due to the lack of the natural outflow of secreted fluid via the vaginal opening, however, dilated fluid-filled lumina could be observed also in the cervix (Fig. 3.27B-4 left). The vagina itself had developed normally showing an appropriate differentiation into a squamous epithelium with underlying submucosa and smooth muscle cell layers organized in a loose arrangement together with connective tissue (Fig. 3.27B-4 right).

3.3.8. Pax8 mRNA Expression in the Reproductive System

To determine whether the reproductive disturbances observed in T4-treated Pax8$^{-/-}$ animals of both genders could be directly related to the inactivation of the Pax8 gene, ISH was used to identify Pax8 transcripts in the reproductive system of adult wildtype mice. In male mice, strong mRNA expression was observed in the epithelium of the epididymis and with weaker intensity in the efferent ducts whereas no signal could be found in the testis (Fig 3.28).

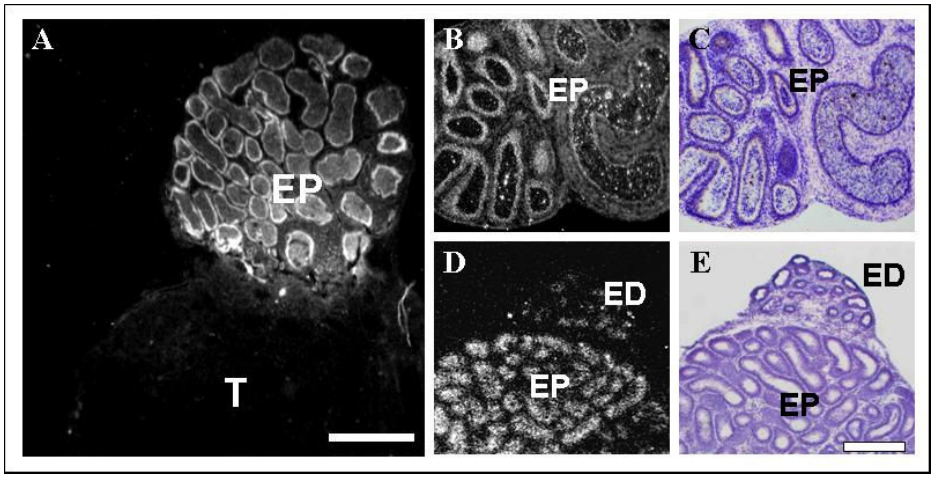


Fig. 3.28: In situ hybridization analysis showing Pax8 mRNA expression in the male reproductive system of adult wildtype mice (A). Higher magnifications of the epididymis (EP) and the efferent duct (ED) are shown in (B) and (D) with the corresponding cresyl violet staining (C and E). No expression was observed in the testis (T). Scale bar left 1mm; right 500µm.

In the female reproductive system of premature 3-week-old as well as sexually mature wildtype mice, Pax8 transcripts were detected in the entire uterine epithelium as well as in the luminal epithelium of the oviduct, the cervix, and the vagina, but not in the ovaries (Fig. 3.29).

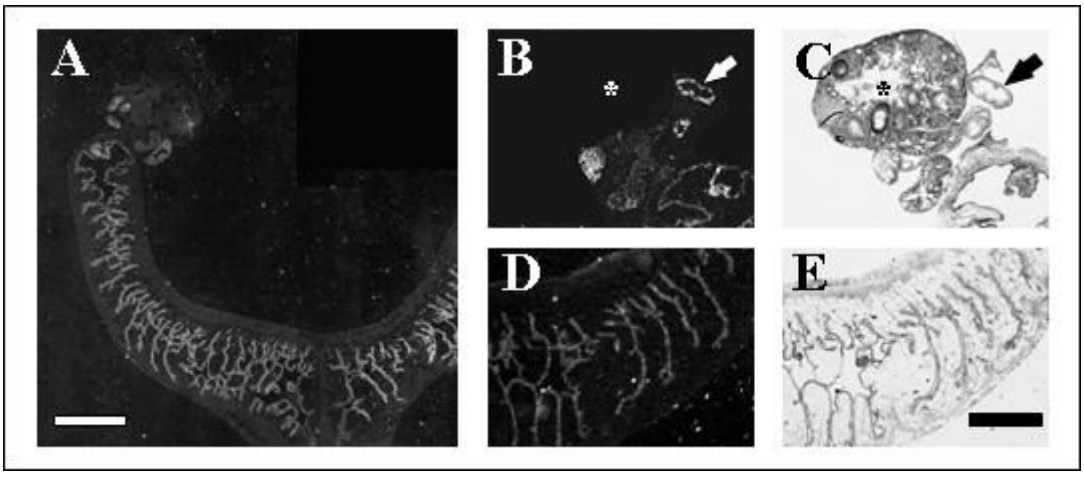


Fig. 3.29: mRNA expression of Pax8 in the female reproductive tract of wildtype mice as assessed by radioactive ISH (darkfield pictures: overview in A, higher magnifications in B and D) with corresponding cresyl violet staining (brightfield pictures: C and E). The epithelium of the oviducts (arrow) and the uterus showed Pax8 mRNA transcripts, but the ovaries did not exhibit Pax8 mRNA expression (asterisk). Scale bar left 3mm, right 1mm.

To further investigate whether Pax8 gene expression might also play a role in the human reproductive system, Pax8 mRNA expression was analyzed in human endometrial tissue samples (Fig. 3.30). Corresponding to the situation in the mouse, strong signals of Pax8 transcripts were observed in the luminal and glandular epithelium of the human endometrium in the secretory (Fig. 3.30A) or the proliferative phase (Fig. 3.30C) of the cycle.

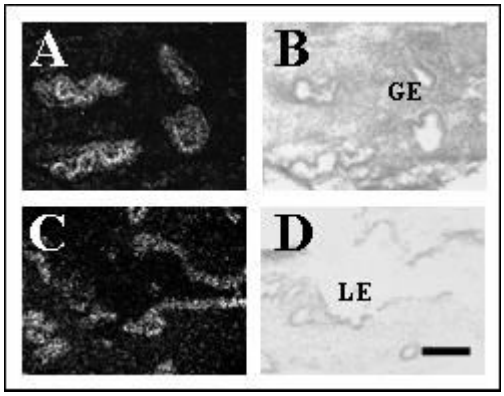


Fig. 3.30: In human endometrial samples of the secretory (A) and proliferative (C) phase, Pax8 mRNA expression was detected by radioactive ISH in the glandular (GE) and luminar epithelium (LE) of the endometrium. Corresponding cresyl violet stainings of neighbouring sections are shown under brightfield illumination in (B) and (D). Scale bar 150µm.

4. Discussion

4.1. Pax8 Deficient Mice Cannot be Rescued by the Inactivation of TRα1

$Pax8^{-/-}TR\alpha1^{-/-}$ mice were generated by breeding $Pax8^{+/-}TR\alpha1^{-/-}$ mice. Since $Pax8^{+/-}$ mice are not different from wildtype controls (Friedrichsen et al. 2004), it was not surprising that $Pax8^{+/-}TR\alpha1^{-/-}$ animals developed like $TR\alpha1^{-/-}$ mice and exhibited the same characteristics. Although it was reported that TH levels are reduced only in $TR\alpha1^{-/-}$ mutant males (Wikström et al. 1998), slightly altered serum T4 and T3 levels were observed in both genders. This is in good agreement with other reports (Amma et al. 2001, Dellovade et al. 2000). Phenotypically $Pax8^{-/-}TR\alpha1^{-/-}$ double knockout mice closely resembled $Pax8^{-/-}$ animals implying that the lack of TH mainly determines the phenotype, which is characterized by severe growth retardation and death around weaning. These defects could be prevented by treatment with TH in both athyroid animal models alike.

In $Pax8^{-/-}TR\alpha1^{-/-}$ and $Pax8^{-/-}$ mice, the absence of TH similarly affected the relative tissue weight of most organs while the absence of TRα1 caused only minor defects. In brain, not only the absence of TH but also the absence of TRα1 led to an increased relative tissue weight. This is not too surprising since in contrast to TRβ, TRα1 is known to be already expressed during early embryonic stages where it might exert a suppressive function on tissue development (Schreiber et al. 2001). Since $Pax8^{-/-}$ animals lack the stimulatory effects of TH as a differentiation factor (Porterfield and Hendrich 1993), it could be speculated that the increased brain mass may be the result of a prolonged proliferation phase during development.

With regard to the pituitary, cellular composition and mRNA levels of the anterior pituitary hormones were not different in $TR\alpha1^{-/-}$ and $Pax8^{+/-}TR\alpha1^{-/-}$ mice and comparable to control animals indicating that TRα1 is of minor importance in pituitary development and hormonal regulation. Correspondingly, pituitaries of $Pax8^{-/-}TR\alpha1^{-/-}$ mice closely resembled those of

Pax8$^{-/-}$ mice (Friedrichsen et al. 2004). Both mutants exhibited a dramatically distorted cellular composition namely severe hypertrophy and hyperplasia of thyrotrophs and an almost complete absence of lactotrophs.

The lack of TH is also the decisive factor determining the mortality of the athyroid animals, since these mice could be rescued by TH treatment. This contrasts with the fact that the absence of all T3 binding receptors has no lethal consequences as shown in TRα1$^{-/-}$TRβ$^{-/-}$ compound mutants (Göthe et al. 1999), which are viable and survive to adulthood. Astonishingly enough, Pax8$^{-/-}$ mice can be rescued by the inactivation of the entire TRα gene, which has been taken as an indication that the mortality of the athyroid animals is the consequence of a lethal TRα1 aporeceptor activity (Flamant et al. 2002). This is not supported by the fact that Pax8$^{-/-}$TRα1$^{-/-}$ mice do not survive weaning. Since these mice lack TRα1, this receptor isoform cannot exert any negative effects as an aporeceptor. It rather indicates that the TRα2 isoform, still expressed in the compound mutants, might be involved, although it is unable to bind T3 (Wikström et al. 1998). It could also be speculated that TRΔα2 might play a role in postweaning survival because TRα$^{-/-}$ mice in which TRα1 and TRα2 have been deleted and only the Δα isoforms are expressed (Fraichard et al. 1997, Gauthier et al. 2001), show a more severe phenotype than TRα1$^{-/-}$ or TRα$^{o/o}$ mutants including early death after weaning. However, the physiological functions of the Δα isoforms are still enigmatic, they lack DNA binding properties and have not yet been detected at the protein level. In contrast, TRα2 is indeed expressed as protein in vivo and is able to bind to DNA as a heterodimer with RXR (O'Shea 2002). Moreover, TRα2 is also known to act as a dominant negative inhibitor of TRE driven transcription and is considered as a weak antagonist although not as potent as unliganded TRs (Tagami et al. 1998, Macchia et al. 2001).

Together with the fact that Pax8$^{-/-}$TRα1$^{-/-}$ mice can be rescued by the administration of TH, this finding suggests a hypothetical model implying a complex formation of TRα2 with

another TR isoform capable of binding T3. Such a complex involving an unliganded isoform like TRβ in the Pax8$^{-/-}$TRα1$^{-/-}$ mouse or TRα1 in the Pax8$^{-/-}$TRβ$^{-/-}$ animals might be responsible for the mortality of these mutants around weaning time. This model would also explain why TRα1$^{-/-}$TRβ$^{-/-}$ mice that still express TRα2 can survive to adulthood because these compound mutants are devoid of a T3 binding TR isoform (Göthe et al. 1999). The analysis of the mortality in Pax8$^{-/-}$TRα2$^{-/-}$ double mutant mice, which is currently under investigation, might help to solve this puzzle.

The Pax8$^{-/-}$TRα1$^{-/-}$ mouse is also a valuable animal model to identify genes that are predominantly regulated by TRα1 as shown by the example of deiodinase type II gene expression. The mRNA of this gene is highly upregulated under the athyroid conditions of the Pax8$^{-/-}$ mouse, but this upregulation is at least partially abolished in Pax8$^{-/-}$TRα1$^{-/-}$ animals, indicating that unliganded TRα1 is important for this process. With regard to the enzymatic activity the abrogated increase in Pax8$^{-/-}$TRα1$^{-/-}$ animals is less pronounced than in Pax8$^{-/-}$ mice, because deiodinase type II activity is mainly negatively regulated posttranslationally by T4 and both mice lack this regulatory mechanism (Gereben et al. 2000). However, in light of embryonic development, when TRα1, but not TRβ, is expressed already at early stages and TH availability is still limited, it may be speculated, that the TRα1 aporeceptor activity induces deiodinase type II mRNA expression. This notion is supported by the fact that in some tissues the expression of deiodinase type II precedes the response to the differentiating effects of TH as shown in Xenopus laevis (Cai et al. 2004). With regard to the hypothalamus-pituitary-thyroid axis the upregulation of D2 under athyroid conditions is even more important, since D2 is absolutely required for TH action in pituitary thyrotrophs as demonstrated by its localization in these cells.

4.2. Mediators of Thyroid Hormone Action in the Anterior Pituitary

TH is important for proper pituitary function and maintenance. This becomes most evident in the congenital hypothyroid Pax8 null mouse which exhibits a completely deranged cellular composition of the anterior pituitary such as hypertrophy and hyperplasia of the thyrotrophs and the almost complete absence of lactotrophs cells (Friedrichsen et al. 2004). Whereas the former effect is easily explained by the missing negative feedback of TH, the cause for the latter is not easily explained, since TH dependent factors interfering with lactotroph activity and development have not been identified yet.

In order to search for putative mediators of TH action in the pituitary, we compared the mRNA expression profile from wildtype and Pax8 deficient animals at 3 weeks of age by microarray analysis. Several genes were found to be differentially expressed, among them tyrosine hydroxylase, the rate-limiting enzyme of dopamine synthesis. Since dopamine is known to be the major regulator of prolactin synthesis and secretion (Ben-Jonathan et al. 2001), the role of this gene in the pituitary with regard to the regulation by TH was analyzed in further detail.

While the existence of tyrosine hydroxylase in the anterior pituitary was already postulated by some investigators (Schussler et al. 1992, Schussler et al. 1995), others failed to detect enzymes for dopamine synthesis in this tissue (Saavedra et al. 1975, Bäck et al. 1987). Regarding the low expression level and activity of tyrosine hydroxylase found in pituitaries of euthyroid wildtype mice, it is still uncertain whether this enzyme contributes to the dopamine level under physiological conditions. However, under hypothyroid or athyroid conditions as in Pax8 deficient mice, tyrosine hydroxylase located in pituitary thyrotrophs does contribute to an increased dopamine level in this tissue and thus to a stronger suppression of prolactin mRNA expression. This suppression could be abolished by the treatment with the dopamine antagonist haloperidol, confirming that dopamine is indeed the factor responsible for the

reduced prolactin transcript levels in these mice. The inhibiting influence of dopamine seems to be much more potent than the increased TRH stimulus on lactotrophic cells, since adult $Pax8^{-/-}$ and TRH-$R1^{-/-}Pax8^{-/-}$ animals that were treated with TH until P40 and then rendered hypothyroid exhibit high levels of prolactin mRNA implying that in these mice tyrosine hydroxylase and subsequent dopamine production are not sufficient to reduce prolactin transcripts effectively. The cause for this surprising observation remains unknown and warrants further investigations.

Although treatment with the dopamine antagonist haloperidol restored the prolactin transcript levels in the few lactotrophs present in Pax8 deficient mice, it did not normalize the number of lactotrophic cells. Surprisingly, treatment of wildtype mice with the dopamine agonist bromocryptine also did not interfere with lactotroph development, although bromocryptine is known to be a very potent inhibitor of lactotroph proliferation and is frequently used in the treatment of human prolactinomas (Ben-Jonathan et al. 2001, Spinas and Fischli 2001). From these observations it can be concluded that in mice early postnatal development of lactotrophs is not critically influenced by dopamine, but severely dependent on proper TH supply. The identification of the unknown factor, that strongly regulates lactotroph development in a TH dependent manner, remains to be elucidated.

The hypothesis that hyperprolactinemia in adult hypothyroid humans (Roberts and Ladenson 2003) and increased prolactin transcript levels in adult hypothyroid mice can be caused by an increased TRH stimulus, could be supported by the analysis of generated TRH-$R1^{-/-}Pax8^{-/-}$ double mutant mice, that did not exhibit increased prolactin mRNA expression levels when rendered hypothyroid at 6 weeks of age. Although both cell types, lactotrophs and thyrotrophs, cannot respond to increased hypothalamic TRH in these mice missing TRH-R1, pituitary thyrotrophs of TRH-$R1^{-/-}Pax8^{-/-}$ animals still exhibited hyperplasia and hypertrophy. The result indicates that, in contrast to the lactotrophs, the increased TRH signalling cannot be

responsible for the overstimulation of the thyrotrophs under athyroid conditions. This contrasts with observations made in the $TRH^{-/-}TR\beta^{-/-}$ double mutant mouse (Nikrodhanond et al. 2006), that were taken as an indication that TRH is absolutely required for TSH synthesis. Whereas the loss of TRβ is at least partially compensated by TRα1 in the mouse, the negative feedback loop through TH is entirely abolished in $Pax8^{-/-}TRH\text{-}R1^{-/-}$ double mutants. Thus, these mice are a better animal model to analyze thyrotroph activity than $TRH^{-/-}TR\beta^{-/-}$ compound mutants.

Annexin A5 was another factor differentially expressed in pituitaries of wildtype and Pax8 null mice. In contrast to the other annexins identified in the microarray analysis, A5 is already highly expressed in wildtype mice and detectable at the protein level, pointing to a functional role in the anterior pituitary not only under pathological conditions. To further test this hypothesis, annexin A5 deficient mice were analyzed with regard to the mRNA expression of adenohypophyseal hormones. The TH sensitive genes β-TSH and GH were found to be unaffected by the lack of A5, indicating normal TH metabolism in these mice. Prolactin mRNA was also not altered, although it had been reported that in vitro exceedingly high concentrations of A5 inhibit the TRH stimulus on lactotrophs (Kawaminami et al. 1992, Kawaminami et al. 2004).

Interestingly, mRNA levels of the gonadotropins were reduced in $A5^{-/-}$ animals, which is in good agreement with the report suggesting that A5 is involved in the GnRH mediated stimulation of gonadotrophs (Kawaminami et al. 2002). The finding that the loss of A5 leads to decreased gonadotropin production corresponds well with the observation that A5 augments gonadotropin secretion in vitro (Kawaminami et al. 2002). However, the reduced gonadotropin mRNA expression does not seem to impair the reproductive capacity of these mice (Brachvogel et al. 2003).

In summary, two genes have been identified so far, that are involved in mediating TH action in the anterior pituitary. Tyrosine hydroxylase, upregulated under athyroid conditions, seems to suppress prolactin synthesis by locally increasing dopamine production, whereas annexin A5, downregulated in congenital hypothyroid $Pax8^{-/-}$ mice, might possibly modulate gonadotropin release. Some other differentially regulated genes could also be promising candidates for further investigations especially with regard to the lactotroph development. Calcitonin-gene-related peptide (see Table 3.2, p.29) has already been associated with inhibition of prolactin synthesis and lactotroph proliferation in vitro (Ren et al. 2001). The best approach to investigate an involvement in pituitary development and maintenance in vivo of course would be to analyze mice with a targeted deletion of this gene (Lu et al. 1999).

4.3. Infertility of T4-Treated Pax8 Deficient Mice

Since TH replacement therapy, if instituted immediately after birth, usually prevents all symptoms resulting from CH (Cassio et al. 2003, Roberts and Ladenson 2004, Larsen et al. 2003), it was surprising that $Pax8^{-/-}$ mice of both genders were unable to reproduce despite proper T4 substitution. As a first approach to identify the reasons for this infertility, the hormonal situation in these mice was analyzed. The normal mRNA expression of all pituitary hormones especially β-TSH and GH confirmed a proper supply with TH, since these genes are highly sensitive to changes in TH metabolism (Yen et al. 2001, Friedrichsen et al. 2004).
Normal serum testosterone as well as normal estrogen and progesterone levels also indicated an unimpaired hormonal situation in male and female mice respectively. No endocrine differences were found in the T4-treated $Pax8^{-/-}$ animals, confirming that the hormonal status is completely restored by TH replacement therapy. Taken together, these observations indicate that the infertility in Pax8 deficient animals is not related to any hormonal imbalance, but rather might be a direct consequence due to the lack of the Pax8 gene.

4.3.1. Infertility of Male T4-Treated Pax8 Deficient Mice

For comparison with Pax8 null mice lacking TH as signalling molecule, TRα1$^{-/-}$TRβ$^{-/-}$ double mutant male mice (Göthe et al. 1999) were analyzed in order to identify possible defects due to impaired TH signalling. The presence of motile spermatozoa in these mice supports the hypothesis that the absence of sperm from the epididymal lumen in T4-treated Pax8$^{-/-}$ mice is not related to disturbances in TH metabolism. The impaired velocity of spermatozoa observed in TRα1$^{-/-}$TRβ$^{-/-}$ animals corresponds well with previous reports showing decreased sperm motility in hypothyroid rats (Kumar et al. 1994, Chandrasekhar et al. 1985).

The situation in Pax8 deficient mice which completely lack sperm in the epididymal lumen is obviously more severe than in TRα1$^{-/-}$TRβ$^{-/-}$ animals. The efferent ducts of Pax8 null mice exhibit a thickened connective tissue which narrows the lumen and hampers the passages of secretions. Together with the histological observations of a distension of the proximal efferent ducts, this indicates that the physical capacity of the post-testicular excurrent ducts might not be sufficient to transmit all the testicular fluid to the epididymis. The increasing testicular fluid secretion occurring at puberty leads to a gradual build-up of pressure within the testis which damages the seminiferous epithelium and leads to an arrest in spermatogenesis comparable to the situation in mice with ligations of the efferent duct (Ross et al. 1974) or in mice deficient in the estrogen receptor α which are unable to absorb water in these ducts (Korach et al. 1994).

In mammals, the male reproductive system except for the testis is derived from the mesonephric or Wolffian duct, which degenerates in the female embryo due to the absence of testosterone (MacLaughlin et al. 2001). In mice, around embryonic day (E) 13.5, the derivatives of this mesonephric duct start forming the efferent ductules that connect the rete testis with the upper part of the epididymis which itself is derived from the most rostral mesonephric tubules (Brune et al. 1999). Pax8 expression is already detectable at E9.5 in the

developing Wolffian duct and the kidney and later at E15.5 also in the epididymis (Mansouri et al. 1994, Bouchard et al. 2004).

In contrast to Pax2-deficient mice, which entirely lack kidneys and genital tracts (Torres et al. 1995), the anterior-posterior patterning is well established in male $Pax8^{-/-}$ mice which display an unaffected differentiation of the Wolffian duct into efferent duct, epididymis and vas deferens. Thus, the loss of Pax8 can be compensated for by other factors such as Pax2 in the early developmental events of the male urogenital system, but Pax8 seems to play a pivotal role for the proper morphogenesis of the efferent duct and the epididymis in later developmental stages.

Since in adult mice Pax8 is still expressed in the epithelium of epididymis and efferent ducts, it might have an additional function not only for the development but also for the maintenance of these tissues, making it a putative target for male contraception studies.

As an additional conclusion of this study, one might suggest to analyze congenital hypothyroid male patients with mutations in the Pax8 gene, since like $Pax8^{-/-}$ mice they might be similarly affected. These patients would be good candidates for Intracytoplasmatic Sperm Injection (ICSI) therapy which can be performed with elongated testicular spermatids.

4.3.2. Infertility of Female T4-Treated Pax8 Deficient Mice

The female reproductive tract in mammals is derived from the Müllerian duct, which degenerates at E13.5 in the male embryo due to the effects of the Anti-Müllerian-Hormone (MacLaughin et al. 2001). In humans, this reproductive tract development is not affected by fetal hypothyroidism (Krassas et al. 2000), whereas in rats, it has been associated with smaller ovaries and follicles, an underdeveloped uterus and vagina as well as delayed vaginal opening (Choksi et al. 2003). Since $Pax8^{-/-}$ animals are born by euthyroid $Pax8^{+/-}$ mothers, fetal thyroid hormone supply is not seriously affected in these mice, which is in good agreement with the fact that the ovaries of the T4-treated Pax8 deficient animals are normal; however, the uterus is absent and vaginal opening does not occur at all, indicating that this severe developmental impairment like in male mice relates also to the lack of the Pax8 gene during development.

In addition to the expression in various tissues such as the thyroid gland, inner ear, pro-, meso- and metanephros, the cloaca and the mid-hindbrain boundary region during embryogenesis (Bouchard et al. 2002, Plachov et al. 1990), Pax8 expression has also been found in the epithelium of the Müllerian duct (Vainio et al. 1999), which differentiates into the oviducts, uterus, cervix and the upper portion of the vagina along the anterioposterior axis. In rodents, the epithelial invaginations of the mesonephros which form the Müllerian duct occur around E11.5 and extend posteriorly reaching the cloaca at ~E13.5 (Kobayashi and Behringer 2003) then forming a duplex uterus and dual cervix (Spencer et al. 2005). The different segments of the female reproductive tract exhibit distinct morphologies and cytoarchitecture (Cunha 1975), and the postnatal establishment of uterine histoarchitecture forming the different elements of the uterine wall, that are endometrium, myometrium and perimetrium, is not completed until two weeks after birth (Cunha 1976, Vernerova et al. 1993, Kurita et al. 2001). Pax8 seems to play an important role in this postnatal differentiation of the Müllerian duct forming an adequate histoarchitecture of the uterus since Pax8 null mutant

mice exhibit only remnants of myometrium and completely lack endometrial structures. Interestingly, the appropriate development of the oviduct, cervix and vagina is not affected.

Patterning events required for differentiation from the Müllerian derivatives occur in both the anterioposterior and radial axes (Spencer et al. 2005). Obviously, anterioposterior patterning establishing histologically distinct segmental boundaries between the oviducts and the uterus as well as between the uterus and cervix is not disturbed in T4-treated Pax8$^{-/-}$ mice, but these animals display defects in radial patterning establishing the tissue-specific uterine morphology.

In addition to the lack of a functional uterus, adult T4-substituted Pax8 deficient mice also lack a vaginal opening although the vaginal development is normal. In wildtype mice, the opening of the vagina occurs at about 6 to 7 weeks of age, around the onset of sexual maturity (Green 1975). It is known that developmental defects in the caudal sections of the urogenital tract can result in an imperforate vagina which may lead to the accumulation of secretion products and subsequently in a marked distention of the vagina (Jubb et al. 1985). The persistence of an imperforate vagina in the mouse is believed to be inherited in a recessive manner (Gruneberg 1952), and the role of Pax8 in this developmental step is not known yet. Alternatively, one could also speculate that certain factors secreted by the ovaries or the uterus itself are necessary for vaginal opening and the delivery of these factors is impaired by the lack of the uterus.

Obviously, the lack of Pax8 expression can be compensated for by other factors during fetal development of the oviduct, cervix and vagina, but not in the postnatal development of the uterine histoarchitecture. Although Pax8 has been shown to be expressed in the entire Müllerian duct (Mansouri et al. 1998, Kobayashi and Behringer 2003), the absence of the Pax8 gene leads to a lack of the mucosal layer, the endometrium, only in the uterus, but not in the other tissues developing from this embryonal structure.

Redundantly acting transcription factors could be responsible for the normal development of these other parts of the reproductive tract. Coexpression of Pax2 with Pax8 has already been demonstrated in the developing Müllerian duct as well as in the kidney (Mansouri et al. 1998; Kobayashi and Behringer 2003), and an overlapping gene function has been shown for Pax2 and Pax8 during development of the mouse urogenital system (Bouchard et al. 2002, Hans et al. 2004). This compensation obviously does not apply for uterine development. While embryonic development of the Müllerian duct does not seem to be disturbed in Pax8 deficient mice, the degeneration of the Müllerian duct during embryogenesis in Pax2 null mutants cannot be compensated for by Pax8 leading to a complete lack of the uterus and oviducts in these mice (Torres et al. 1995). However, one may speculate that Pax2 can at least partly compensate for Pax8 during the development of the oviduct, cervix and vagina, but the exact molecular mechanisms of Pax8 in this process remain unknown.

Prenatal organogenesis as well as postnatal morphogenesis of the reproductive system is obviously a complex, multifactorial process. Pax8 seems to be an important player in the genetic pathway leading to female reproductive tract development. Similar to mouse embryonic development, Pax8 is also expressed in various structures of the human embryo such as the thyroid anlage, the otic vesicle, the mid-hindbrain boundary and in the metanephric blastema and derivatives (Trueba et al. 2005). Mutations of Pax8 found in human patients have been associated with the pathogenesis of thyroid dysgenesis and hypoplasia (Macchia et al. 1998, Meeus et al. 2004, Villain et al. 2001). Nowadays these disorders do not lead to symptoms of cretinism since congenital hypothyroid patients are identified in early postnatal screenings and adequately treated with T4. However, female patients with Pax8 mutations have not yet been analyzed with regard to fertility and the development of their uterus. The observation that Pax8 is expressed not only in the uterine epithelium of mice but also in human tissue samples seems to indicate that the development of the endometrium might also be impaired in these women, which warrants further investigation.

5. Summary

Thyroid hormones are essential for normal vertebrate development and for the metabolic homeostasis of almost all tissues. The physiological importance of thyroid hormone becomes most evident under the conditions of congenital hypothyroidism (CH), a common disorder occurring in 1 among 3600 newborns. If not treated immediately after birth by thyroid hormone substitution, CH leads to a syndrome called cretinism that is characterized by metabolic disturbances, severe neurological defects, mental deficiencies and growth retardation. The $Pax8^{-/-}$ mouse is an ideal animal model for congenital hypothyroidism, since it is born without thyroid follicular structures and thus this mouse is completely athyroid in postnatal life. Consequently, these mice exhibit ataxia, deafness, growth retardation, a completely distorted cellular composition of the anterior pituitary and they die around weaning. The analysis of the underlying molecular mechanisms of some of these symptoms was the aim of this thesis.

In order to analyze the cause for the mortality, $Pax8^{-/-}TR\alpha1^{-/-}$ mice were generated by intercrossing $Pax8^{+/-}$ and $TR\alpha1^{-/-}$ mice. This was of special interest, since Flamant et al. recently reported that $Pax8^{-/-}$ mice can be rescued by the inactivation of the entire thyroid hormone receptor α gene ($Pax8^{-/-}TR\alpha^{0/0}$) (Flamant et al. 2002). The observation was taken as an indication for a lethal aporeceptor activity of TRα1, the only isoform of the TRα gene that is considered as a functional TR, because it is able to bind T3 as well as DNA. However, as shown in this study, $Pax8^{-/-}TR\alpha1^{-/-}$ mice exhibit the same phenotype as $Pax8^{-/-}$ mice, including growth retardation, deranged pituitary appearance and death around weaning. Therefore it could be excluded that TRα1 is responsible for the death around weaning, suggesting that the mortality is most likely associated with the TRα2 isoform. Due to the fact, that $Pax8^{-/-}$ as well as $Pax8^{-/-}TR\alpha1^{-/-}$ mice could be rescued by thyroxine treatment, it was assumed, that TRα2 has to interact with a functional unliganded TR isoform to cause death in these animals.

To identify factors, which contribute to the distorted cellular make-up of the pituitary in athyroid mice, a microarray based analysis was performed. Several genes were found to be differentially expressed in pituitaries of $Pax8^{-/-}$ mice compared to wildtype animals, among them tyrosine hydroxylase, which is transcriptionally and functionally upregulated in $Pax8^{-/-}$ mice, leading to an increase in the local production of dopamine. Consequently, prolactin mRNA expression is inhibited in these animals. However, as demonstrated by the use of dopamine antagonists and agonists, postnatal lactotroph development is not impaired. Since in humans hypothyroidism is often associated with hyperprolactinemia, adult thyroxine treated Pax8 deficient mice were made hypothyroid by stopping thyroxine treatment. By this manipulation it could be shown that in contrast to the young P21 animals, hypothyroidism causes an increase in prolactin mRNA expression. In contrast, after the induction of hypothyroidism, adult TRH-$R1^{-/-}Pax8^{-/-}$ double mutant mice did not show an upregulation of prolactin transcript levels, suggesting that the increase in prolactin transcript levels under hypothyroid conditions is caused by an increased TRH stimulus. It remains unknown, why the increase in tyrosine hydroxylase, which was also found in pituitaries of adult hypothyroid mice, is not sufficient to suppress prolactin mRNA expression, and why the increased TRH stimulus in young $Pax8^{-/-}$ mice does not cause increased prolactin mRNA expression in these animals.

Besides tyrosine hydroxylase, annexin A5 was also identified as a pituitary gene which is positively regulated by thyroid hormones. In good agreement with reported in vitro data, the loss of annexin A5 seems to impair gonadotropin synthesis also in vivo. This was demonstrated by the analysis of A5 deficient mice which revealed a decreased mRNA expression of β-FSH. The expression of the other pituitary hormones was found not to be affected by the lack of A5 and an involvement of A5 in the TRH mediated regulation of prolactin, as reported by other investigators, could not be confirmed.

Some other genes were also identified to be differentially expressed in Pax8 null mice, like calcitonin-gene-related peptide or STAT1 and STAT4. Whether these genes also contribute to the deranged appearance of the pituitary under athyroid conditions remains to be elucidated.

Since usually all symptoms of congenital hypothyroidism can be reversed by thyroid hormone replacement therapy, it was very surprising that $Pax8^{-/-}$ mice of both genders were infertile despite proper TH treatment. The analysis revealed that Pax8 deficient females lack the entire uterus and do not have a vaginal opening and Pax8 deficient male mutants do not have any sperm in the epididymis. With age, these mice develop considerable atrophy of their testis due to a narrowed passage in the efferent ducts. Since the hormonal situation was not impaired in thyroxine treated Pax8 null mice, these defects seem to be directly related to the inactivation of the Pax8 gene. This notion is supported by the identification of Pax8 mRNA expression in the epithelium of the uterus and the oviducts as well as the epithelium of the efferent ducts and the epididymis of wildtype mice. Taken together, these observations indicate an important role for Pax8 during the development of the uterus and the efferent ducts. Since Pax8 mRNA expression was also demonstrated in human endometrial tissue samples, it remains to be elucidated whether human patients with mutations in the Pax8 gene are similarly affected.

Taken together, we were able to elucidate the underlying molecular mechanisms of some defects observed under the conditions of congenital hypothyroidism using the $Pax8^{-/-}$ animal model namely the mortality of these animals as well as the reduced prolactin and gonadotropin mRNA expression in the anterior pituitary of these mice. By analyzing the infertility of T4-treated Pax8 deficient mice, we identified Pax8 as a novel key player in the development of the male and female reproductive system.

6. References

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7. Non-Standard Abbreviations

ACTH	adrenocorticotrophic hormone
bw	bodyweight
CH	congenital hypothyroidism
cpm	counts per minute
D1	deiodinase type I
D2	deiodinase type II
D3	deiodinase type III
EDTA	ethylene diamine tetra acetic acid
FSH	follicle stimulating hormone
GH	growth hormone
LH	luteinizing hormone
min	minute
nt	nucleotide
Pax	paired box gene
POMC	proopiomelanocortin
PRL	prolactin
RT	room temperature
SDS	sodium duodecyl sulphate
T3	triiodthyronine
T4	tetraiodthyronine, thyroxine
TH	thyroid hormone
TR	thyroid hormone receptor
TRH	thyrotropin releasing hormone
TRH-R1	thyrotropin releasing hormone receptor 1
TSH	thyroid stimulating hormone

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Publications

Rabeler R*, Mittag J*, Geffers L*, Ruther U, Leitges M, Parlow AF, Visser TJ, Bauer K: "Generation of Thyrotropin-Releasing Hormone Receptor 1-Deficient Mice as an Animal Model of Central Hypothyroidism" Molecular Endocrinology 2004 Jun;18(6):1450-60

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Mittag J, Winterhager E, Bauer K, Grümmer R: "Pax8 is Involved in Uterine Development" submitted for publication

Wistuba J*, Mittag J*, Luetjens CM, Cooper TG, Yeung CH, Nieschlag E, Bauer K: "Malformation of the Efferent Ducts in Thyroxine-Substituted Pax8-Deficient Mice Results in Partial Testicular Atrophy and Absence of Spermatozoa From the Epididymis" submitted for publication

Mittag J*, Oehr W*, Heuer H, Brachvogel B, Poeschl E, Bauer K: "Annexin A1, A5 and A10 are Putative Mediators of Thyroid Hormone Action in the Anterior Pituitary" manuscript in preparation

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Poster Presentations

Mittag J, Heuer H, Friedrichsen S, Visser TJ, Bauer K: „Deiodinase Type II is Located in Pituitary Thyrotrophs and Regulated by Thyroid Hormone Receptor TRα1“
12th International Congress of Endocrinology (ICE), Lisbon 2004

Mittag J, Heuer H, Friedrichsen S, Visser TJ, Bauer K: „Deiodinase Type II is Regulated by Thyroid Hormone Receptor TRα1 and Located in Pituitary Thyrotrophs“
Deutsche Gesellschaft für Endokrinologie (49. Symposium), Münster 2005

Mittag J, Friedrichsen S, Heuer H, Polsfuss S, Visser TJ, Bauer K: „Athyroid Pax8$^{-/-}$ Cannot be Rescued by the Inactivation of Thyroid Hormone Receptor α1“
87th Annual Meeting, Endocrine Society, San Diego 2005

Mittag J, Winterhager E, Bauer K, Grümmer R: „Thyroid Hormone Cannot Restore Female Fertility in Congenital Hypothyroid Pax8$^{-/-}$ Mice“
Deutsche Gesellschaft für Endokrinologie (50. Symposium), Essen 2006

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Deutsche Gesellschaft für Endokrinologie (50. Symposium), Essen 2006

Mittag J, Winterhager E, Bauer K, Grümmer R: „Female Congenital Hypothyroid Pax8$^{-/-}$ Mice Are Infertile Despite Thyroid Hormone Replacement Therapy“
88th Annual Meeting, Endocrine Society, Boston 2006

Oral Presentations

Mittag J: „The Pax8$^{-/-}$ Mouse as an Animal Model of Congenital Hypothyroidism“, 6th Young Active Research Meeting, Deutsche Gesellschaft für Endokrinologie, Münster Oktober 2004

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