

## Genetic defects, thyroid growth and malfunctions of the TSHR in pediatric patients

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## 1. ABSTRACT

Naturally occurring activating and inactivating mutations of the thyrotropin receptor (TSHR) were found as a molecular cause of diseases in patients suffering from non-autoimmune hyperthyroidism and syndromes of thyrotropin resistance, respectively. These mutations are mostly functionally characterized *in vitro* and therefore, they represent an excellent tool to study structure-function relationships of this G-protein-coupled receptor. In this review, we summarize published germline mutations of the TSHR with focus on 1) the phenotype of (pediatric) patients, 2) potential genotype/phenotype correlations, 3) structural implications for receptor activation and inactivation, 4) the impact on thyroid growth, and 5) finally on aspects of TSHR dimerization. In conclusion, this comprehensive analysis of medical and biological data opens an avenue to understand genetic defects and malfunctions of the TSHR in molecular detail and in their entirety. This knowledge is important to refine our insights in non-autoimmune diseases caused by defects of the TSHR gene and it might help to develop pharmacological means for compensation of uncontrolled thyroid growth.

## 2. INTRODUCTION

Thyroid stimulating hormone (TSH, also called Thyrotropin) is the most important factor for thyroid growth and function (reviewed in (1, 2)). TSH exerts its action via binding to and activating of the TSH receptor (TSHR) a member of the large superfamily of G-protein-coupled receptors (GPCR). These receptors share the common structural feature of a serpentine domain (SD) consisting of seven membrane-spanning alpha-helices (TMHs) that are connected by three intracellular and three extracellular loops (ICLs and ECLs). The N-terminal part of the receptor is extracellular and the C-terminus is intracellular located. The TSHR belongs to the subfamily of glycoprotein hormone receptors (GPHRs) together with the lutropin (LHCGR) and the follitropin receptor (FSHR). Binding of TSH to the large extracellular region of the receptor forces a conformational change in the protein resulting in G-protein activation (reviewed in (3)).

The TSHR is promiscuous with regard to its ability to activate all four G-protein families (4-6). So far activation of the G<sub>s</sub>/adenylyl cyclase pathway seems to be

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of highest importance for thyroid growth and function (2), however, recently it was shown that activation of the  $G_{q/11}$  pathway is obligatory for thyroid growth and thyroid hormone synthesis also (7, 8). The physiological relevance of  $G_i$  and  $G_{12/13}$  activation is poorly understood, although it was recently shown that MAPK activation is dependent on  $G_{13}$  signaling (9). Disturbance of thyroid function, resulting in a hyper- or hypo-functioning thyroid, results in disease conditions due to excess or lack of thyroid hormones, respectively.

Genetic defects in the TSHR were found as molecular causes of non-autoimmune hyperthyroidism (in cases of thyroid hormone excess) and of resistance to TSH (in cases of complete or partial lack of thyroid hormones) (10). In this review, we will focus on previously (reviews (11-13)) and recently published naturally occurring mutations in the TSHR gene leading to gain- or loss-of-function of thyroid hormone production. We will reflect also on the functional characterization of these mutations *in vitro*, which is a helpful tool to improve and to support the molecular diagnosis in patients. Moreover, these mutations are an excellent tool to obtain deeper insights into molecular details of TSHR structure and regulatory mechanisms of inter- as well as intramolecular interactions. This knowledge is necessary to learn more about the mechanisms of receptor activation and inactivation, which is a prerequisite to develop new ideas concerning therapeutic concepts (agents) for potential optimized treatment of thyroid diseases caused by malfunction of the TSHR.

### 3. GENETIC DEFECTS OF THE TSHR

It is known for a very long time that auto-antibodies to the TSHR in patients with Graves disease result in hyperfunction of the thyroid gland (14, 15). Additionally blocking antibodies of the TSHR were found in patients with congenital hypothyroidism (16). Shortly after cloning of the TSHR (17-22) it became obvious that not only immunological reactions lead to malfunction, but also mutations in the coding region were found to be responsible for disturbed thyroid function (23).

The gene of the TSHR is located on chromosome 14q31 (24), and the protein is encoded by 10 exons. Exon 1 to 9 encode for the extracellular region of the TSHR and exon 10 is encoding the serpentine domain and the C-terminal part (reviewed in (25)). Genetic defects of the TSHR include nonsense mutations, missense mutations, frame shift mutations, small deletions and insertions.

The molecular etiology of thyroid dysgenesis is poorly understood and in rare cases mutations in thyroid transcription factors (Pax-8, NKX2.1 and FoxE1) were identified (26-28). So far, mutations in the TSHR gene are the most common genetic cause of congenital hypothyroidism with hypoplasia of the thyroid gland in patients (16) were no further defects like kidney or lung involvement were identified, although also TSHR mutations in these patients are rare.

### 3.1. Somatic mutations

In 1993 the first mutations in the TSHR were found as somatic mutations in toxic thyroid adenomas (23). These mutations result in constitutive activation of the  $G_s$ /adenylyl cyclase signaling pathway (constitutively activating mutations, CAMs). This first report was followed by a huge variety of others, where heterozygous mutations in the TSHR gene were found as the molecular cause of hyperfunctioning adenomas (Figure 1B). The frequency of these somatic mutations varies largely (between below 10% up to 80 % (29)) depending on the study performance, e.g. patients origin and mutation screening procedure. As result of these mutations thyroid hormones in these patients were elevated and TSH levels were suppressed. Furthermore, some rare mutations (e.g. D633H,C (30) in TMH 6, I486F (31) in ECL 1) also activate both the  $G_s$ /adenylyl cyclase and the  $G_{q/11}$  phospholipase C pathway.

No inactivating somatic TSHR mutation has been reported yet and somatic activating TSHR mutations in pediatric patients are extremely rare. Only one case has been reported so far (32). Therefore, this review will focus on germline mutations.

### 3.2. Germline mutations

Shortly after the first report of a somatic CAM in the TSHR gene, germline mutations were found in two large pedigrees with hyperfunctioning thyroid glands (33). These heterozygous CAMs were identified as the molecular cause of non-autoimmune hyperthyroidism and were described in familial as well as sporadic cases. So far, in all carriers of germline TSHR CAMs the thyroid gland was enlarged, which was addressed as an effect of  $G_s$  activation (2).

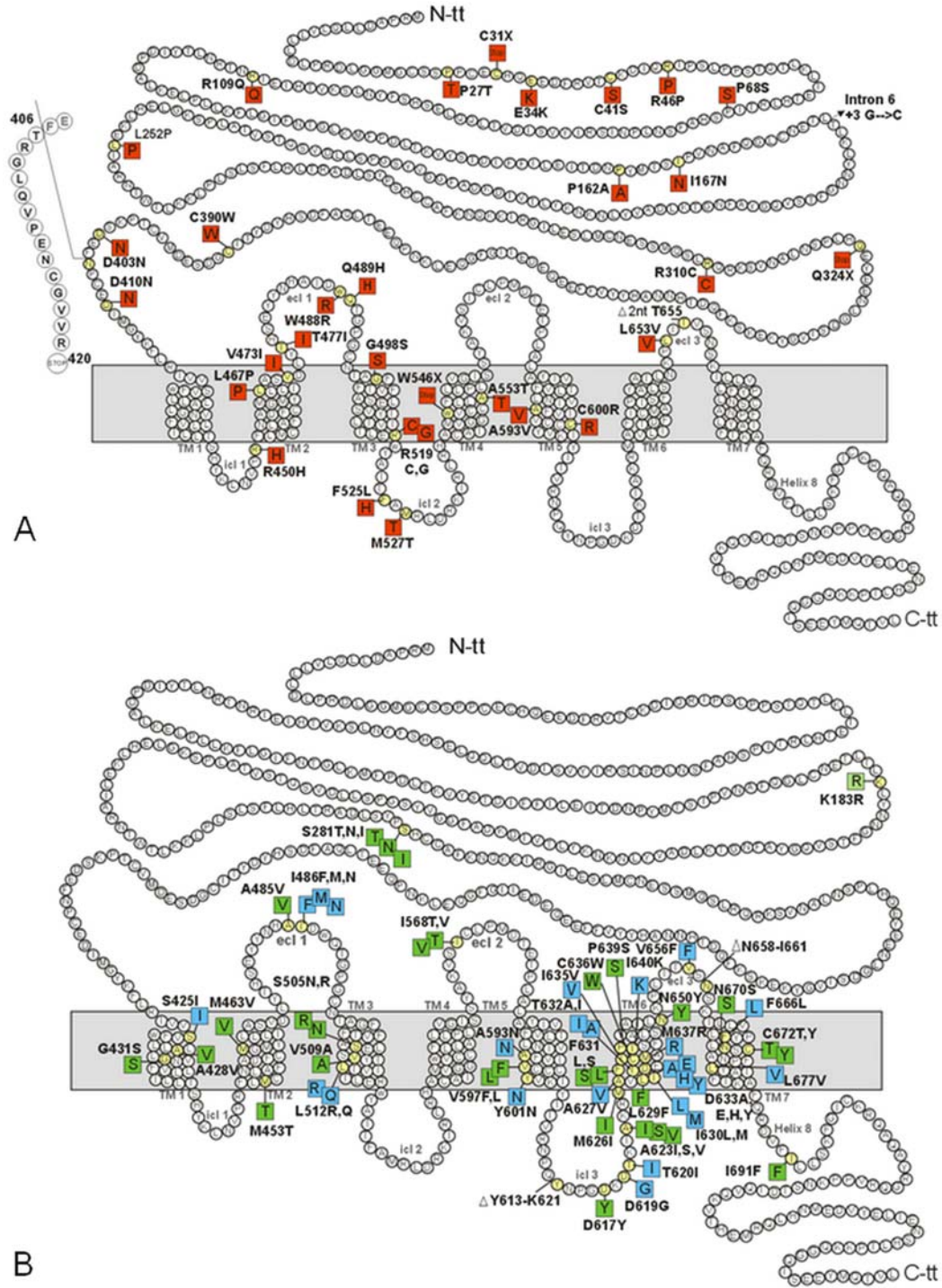
Besides CAMs, inactivating mutations in the TSHR gene were found also. These mutations occur homo- or compound heterozygously and result in hyperthyrotropinemia or congenital hyperthyroidism in the mutation carriers (34, 35). Clinical and functional aspects of inactivating and activating TSHR mutations will be discussed in the following sections.

## 4. INACTIVATING TSHR MUTATIONS AS THE MOLECULAR CAUSE OF HYPERTHYROTROPINEMIA AND CONGENITAL HYPERTHYROIDISM

### 4.1. Clinical aspects

The first patients in whom inactivating TSHR mutations were found are three siblings suffering from hyperthyrotropinemia (36). In these patients the thyroid gland was normal in size and position and thyroid hormone levels were in the normal range, a condition referred to as fully compensated resistance to thyroid hormones. The patients were compound heterozygous carriers of two mutations in exon 6 of the TSHR gene, which both result in a partial loss-of-function (37). Based on these findings, it was likely that more drastic mutations with complete loss-of-function might lead to congenital hypothyroidism. Indeed, the first patient with congenital hypothyroidism

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**Figure 1.** Two-dimensional schemes of the TSHR with highlighted known naturally occurring loss- and gain-of-function mutations. Amino acids are indicated in the one-letter code. Positions of mutations are highlighted in yellow circles. A) Mutated amino acids that lead to inactivation of the receptor are indicated in red quads. B) Mutations that lead to constitutive activation of the receptor are indicated in green quads. The mutation K183R has no constitutive activity and leads to increased receptor sensitivity and activation by hCG during pregnancy (itemized in light green quad). Somatic mutations are depicted in blue quads. Amino acid substitutions that result in a stop codon are marked by the letter X. Deletions are symbolized as delta ( $\Delta$ ). Structural parts of the receptor are designated as follows: TM = transmembrane helix; ecl = extracellular loop; icl = intracellular loop; N-tt = N-terminal tail; C-tt = C-terminal tail. For references see table 1, and website: [www.ssfa-gphr.de](http://www.ssfa-gphr.de), section "Pathogenic Mutations" / "Tabulated overview of GPHR mutations".

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**Table 1.** Summary of germline TSHR mutations

Structural localisation	wt	Position	Mutation	References	Thyroid	Patients phenotype	Occurrence	Functional characterization
A. Overview of reported naturally occurring germline gain-of-function mutations identified in patients with non-autoimmune hyperthyroidism that are characterized by an increase in basal cAMP accumulation. If functional data were only available from the same mutation found in toxic thyroid adenomas this reference is also indicated.								
LRR6	K	183	R	(66)	small diffuse goiter	gestational hyperthyroidism	familial, heterozygous	SE: wt; B: wt; cAMP: b - wt; s - wt; increased CG sensitivity
hinge-region	S	281	N	(10), (120)	goiter	congenital non-autoimmune hyperthyroidism	sporadic, heterozygous	SE: i; B: d; cAMP: b - e; s - wt; PI: b - wt; s - wt
TMH1	A	428	V	(121), (122)*	increased	neonatal hyperthyroidism	sporadic, heterozygous	* SE: d; cAMP: b - e; s - wt;
	G	431	S	(106)	enlarged	non-autoimmune hyperthyroidism	familial, heterozygous	SE: nr; B: e; cAMP: b - e; s - wt; PI: b - e; s - wt
TMH2	M	453	T	(123), (34)	diffuse goiter nodule	neonatal hyperthyroidism	sporadic, familial, heterozygous	SE: wt; cAMP: b - e; s - d; PI: b - wt; s - wt
	M	463	V	(124)	diffuse hyperplasia	hyperthyroidism	familial, heterozygous	SE: d; B: e; cAMP: b - e; s - wt; PI: b - wt; s - d
ECL1	A	485	V	(51)	goiter	autosomal dominant non-autoimmune hyperthyroidism	familial, heterozygous	SE: i; cAMP: b - e; PI: b - wt; s - i
TMH3	S	505	N	(125)	no enlargement at 6 months of age, enlarged under treatment	neonatal hyperthyroidism	sporadic, heterozygous	SE: wt; B: wt; cAMP: b - e; s - wt; PI: b - wt; s - wt
			R	(53), (100)	goiter	hyperthyroidism	familial, heterozygous	B: wt; cAMP: b - e; s - d; PI: b - wt; s - d
	V	509	A	(33)	toxic thyroid hyperplasia	non-autoimmune hyperthyroidism	familial, heterozygous	SE: nr; B: d; cAMP: b - e;
ECL2	I	568	V	(57)	enlarged	non-autoimmune hyperthyroidism	familial, heterozygous	SE: wt; B: wt; cAMP: b - e; s - d; PI: b - wt; s - wt
	I	568	T	(126), (31)*	goiter	congenital non-autoimmune hyperthyroidism	sporadic, heterozygous	* SE: d; B: d; cAMP: b - e; s - wt; PI: b - wt; s - d
TMH5	V	597	L	(127)	enlarged	neonatal hyperthyroidism	sporadic, heterozygous	SE: i; B: d; cAMP: b - e; s - wt; PI: b - wt; s - i
			F	(128)	goiter	non-autoimmune hyperthyroidism	familial, heterozygous	SE: i; cAMP: b - e; s - wt
ICL3	D	617	Y	(58)	diffuse goiter	hyperthyroidism	familial, heterozygous	SE: d; cAMP: b - e; s - wt; PI: b - wt; s - wt
TMH6	A	623	V	(55), (129)*	goiter	congenital hyperthyroidism	familial, heterozygous	* B: wt; cAMP: b - e; s - wt; PI: b - wt; s - wt
	M	626	I	(130)	enlarged	non-autoimmune hyperthyroidism	familial, heterozygous	SE: i; B: d; cAMP: b - e; s - d;
	L	629	F	(131)	goiter	congenital non-autoimmune hyperthyroidism	familial, heterozygous	SE: nr; B: wt; cAMP: b - e; s - wt
	F	631	S	(132)	goiter	hyperthyroidism	familial,	SE: d;

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							heterozygous	cAMP: b - e; s - wt; PI: b - wt, s - i
			L	(54)	goiter	non-autoimmune hyperthyroidism	sporadic, heterozygous	B: wt; cAMP: b - e; s - wt; PI: b - wt; s - wt
	C	636	W	(108)	normal	non-autoimmune hyperthyroidism	familial, heterozygous	SE: wt; B: d; cAMP: b - e; s - wt; PI: b - wt; s - i
	P	639	S	(133), (134)*	goiter	thyrotoxicosis, mitral valve prolapse	familial, heterozygous	* SE: nr; B: d; cAMP: b - e
ECL3	N	650	Y	(100)	goiter	hyperthyroidism	familial, heterozygous	B: wt cAMP: b - e; s - wt; PI: b - wt; s - e
TMH7	N	670	S	(100)	goiter	hyperthyroidism	familial, heterozygous	B: wt; cAMP: b - e; s - d; PI: b - wt; s - i
	C	672	Y	(33)	toxic hyperplasia thyroid	non-autoimmune hyperthyroidism	heterozygous	B: d; cAMP: b - e; s - d; PI: b - wt; s - d
Helix 8	I	691	F	(135)	nr	non-autoimmune hyperthyroidism	familial, heterozygous	nr
<b>B. Naturally occurring germline loss-of-function mutations identified in patients with euthyroid hyperthyrotropinemia and congenital hyperthyroidism.</b>								
N-terminal region	P	27	T	(49), (50)	normal	congenital hypothyroidism (CH), subclinical hypothyroidism	heterozygous	SE: d; B: d; cAMP: b - wt; s - d;
	C	31	X	(136)	hypoplastic	Subclinical hypothyroidism	familial, heterozygous	nr
	E	34	K	(49), (50)	normal	subclinical hypothyroidism (SH), hyperthyrotropinaemia	heterozygous	SE: d; B: d; cAMP: b - wt; s - d;
LRR 0	C	41	S	(38), (137)	normal	elevated TSH (thyrotropin resistance)	familial, compound heterozygous, heterozygous	SE: 0%
	R	46	P	(49), (50)	normal	subclinical hypothyroidism (SH), hyperthyrotropinaemia	heterozygous	SE: i; B: 0%;
LRR1	P	68	S	(36)	hypoplastic or normal	mild hyperthyrotropinemia	compound heterozygous, heterozygous	SE: wt; cAMP: b - d; s - wt;
LRR 3	R	109	Q	(39)	normal	persistent hyperthyrotropinemia	familial, compound heterozygote	SE: i; B: d; cAMP: b - wt; s - d
		+3 G to C intron 6		(104)	very hypoplastic	Congenital hypothyroidism	sporadic, compound heterozygous	-
LRR 5	P	162	A	(38), (138) (37),	normal	hyperthyrotropinemia	familial, homo- and heterozygous, or compound heterozygous	SE: wt; B: i; cAMP: b - wt; s - d;
	I	167	N	(138)	normal	hyperthyrotropinemia	familial, compound heterozygous	SE: i; B: 0%;
LRR 9	L	252	P	(139)	normal	slight hyperthyrotropinemia	familial, heterozygous	SE: i; B: i; cAMP: b - i; s - i;
hinge-region	R	310	C	(140)	normal	elevated TSH (thyrotropin resistance)	familial, homozygous, heterozygous	SE: nr; B: i; cAMP: b - e; s - i;
	Q	324	Stop	(38)	normal	hyperthyrotropinemia	familial, compound heterozygous	-
	C	390	W	(38), (35)	normal, hypoplasia	elevated TSH (thyrotropin resistance) congenital hypothyroidism	familial, compound heterozygous	B: d; cAMP: b - wt; s - d;
	D	403	N	(49), (50)	normal	subclinical hypothyroidism (SH), hyperthyrotropinemia	heterozygous	SE: d; B: d; cAMP: b - wt; s - d;

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		406-420	Del + Ins, Trunc	(35)	hypoplasia	congenital hypothyroidism	familial, compound heterozygous	SE: nr; B: d; cAMP: b -d; s - d; PI: b -nr ; s -nr
	D	410	N	(38)	normal	elevated TSH (thyrotropin resistance)	familial, compound heterozygous	B: wt; cAMP: b - d; s - d;
TMH 2	R	450	H	(102), (141), (142)	normal	thyrotropin resistance	familial, compound heterozygous, homozygous	SE: d; B: d; cAMP: b - wt; s - d; PI: b - wt; s - 0%
	L	467	P	(137)	normal	elevated TSH levels (thyrotropin resistance)	familial, heterozygous	SE: i; B: nr; cAMP: b - d; s - i;
	V	473	I	(142)	normal	elevated TSH levels (thyrotropin resistance)	compound-heterozygous	SE: wt; B: wt; cAMP: b - wt; s - wt; PI: b - wt; s - 0%
	T	477	I	(143)	normal	congenital hypothyroidism	familial, heterozygous, homozygous	SE: i; B: i; cAMP: b - d; s - i; PI: b - wt; s - i
ECL1	W	488	R	(49), (50)	normal	subclinical hypothyroidism (SH), hyperthyrotropinaemia	heterozygous	SE: i; B: 0%
	Q	489	H	(43)	no thyroid tissue, but detectable TG levels	hypothyroidism	familial, homozygous	SE: wt; B: d; cAMP: b - wt; s - i;
TMH 3	G	498	S	(102)	normal	thyrotropin resistance	familial, compound heterozygous	SE: 0%
	R	519	C, G	(142)	normal	thyrotropin resistance	familial, compound-heterozygous	SE: d; B: i; cAMP: b - wt; s - i; PI: b - wt; s - 0%
ICL 2	F	525	L	(38)	normal	elevated TSH (thyrotropin resistance)	familial, compound heterozygous, heterozygous	B: wt; cAMP: b - i; s - i;
	M	527	T	(49), (50)	normal	subclinical hypothyroidism (SH), hyperthyrotropinaemia	heterozygous	SE: d; B: d; cAMP: b - wt; s - d;
TMH 4	W	546	stop	(39), (38)	enlarged gland, normal	hyperthyrotropinemia	sporadic, familial, compound heterozygous	-
	A	553	T	(103)	hypoplasia	congenital hypothyroidism	familial, homozygous,	SE: i; B: i; cAMP: b - d; s - d; PI: b - wt; s - i
TMH 5	A	593	V	(144)	normal	mild congenital primary hypothyroidism, subclinical hypothyroidism	familial, homozygous	-
	C	600	R	(137)	hypoplasia	elevated TSH levels (thyrotropin resistance)	familial, compound heterozygous, heterozygous	SE: i; cAMP: b - i; s - i;
ECL 3	L	653	V	(8), (36)	hypoplastic or normal-sized thyroid gland	mild hyperthyrotropinemia	familial, homozygous, heterozygous	SE: wt; B: wt; cAMP: b - i; s - wt; PI: b - wt; s - i
	T	655	Del, Trunc	(104), (137)	normal	hyperthyrotropinemia	sporadic, heterozygous	-

SE: surface expression, B: binding; b: basal, s: maximal stimulation, i: impaired (less than 30% compared to wild type), d: decreased (less than 70% compared to wild type), wt: similar to wild type, e: enhanced (more than 170% compared to wild type), nr: not reported, Del: deletion, Trunc: truncation, Ins: insertion, TG: thyroglobulin, \*functional data from reports of similar somatic mutants at the same position.

was a compound heterozygous carrier of a frameshift mutation leading to a complete loss-of-function and a

missense mutation, C390W. The thyroid gland of the patient was located normally but hypoplastic (35).

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Noteworthy, this mutation resulted in partial loss-of-function of TSHR signaling when it was studied *in vitro* in a COS-7 cell overexpressing system. The functional effect of this mutation was even more severe when the mutation was investigated in a stable CHO-K1 cell line that reflects better the physiological level of TSHR expression. These findings demonstrate the difficulties to transfer results of functional *in vitro* characterization to the phenotype of patients. In often used heterologous overexpressing systems the degree of TSHR expression is much higher than in thyrocytes (34), which could lead to signal transduction levels that are not so severely affected as under physiologically relevant conditions. In addition to difficulties in the assessment of data of functional *in vitro* characterization, the phenotype of the patients carrying comparable TSHR mutations can vary between hyperthyrotropinemia and congenital hypothyroidism (35, 38). Reasons for this could be multi-factorial ranging from environmental factors to genetic susceptibility. After the first reports many more homo-, hetero- and compound heterozygous mutations were described as the molecular cause of different degrees of thyroid hormone resistance (Figure 1A, for references see Table 1).

### 4.2. Molecular and structural aspects of receptor inactivation

The wild type amino acids of loss-of-function mutants are involved in the maintenance of important TSHR properties like correct protein folding, the intrinsic signaling-capability, or the predisposition for interaction with TSH and G-protein. Molecular reasons for aberrant signaling activity (partial or complete loss-of-function) of the TSHR caused by mutations can be grouped in the following categories:

1. Most of the known inactivating mutations lead to intracellularly retained TSHRs. This is the case for all nonsense and frame shift mutations, but also for mutations that result in complete misfolding of the receptor (Table 1B).
2. Mutations that maintain the receptor in the TSH unoccupied state and hinder a shift to the active conformation after TSH binding. These mutations are located in the N-terminus (e.g. D403N), serpentine domain (e.g. D460N, TMH2), or the ECLs (e.g. Q489H, ECL1) of the receptor (for references see Table 1).
3. Mutations that are incompatible with TSH binding. These mutations are extremely rare and they are located in the extracellular Leucine-rich repeat domain (LRRD) (8, 39). Most likely they directly influence TSH binding by disruption of potential intermolecular contact interfaces between TSH and TSHR.
4. Mutations at positions (Figure 1A) at the intracellular loops and intracellular transitions to the helices of the TSHR (e.g. R450H, TMH2; or M527T, ICL2), which are potentially important for receptor/G-protein contact.

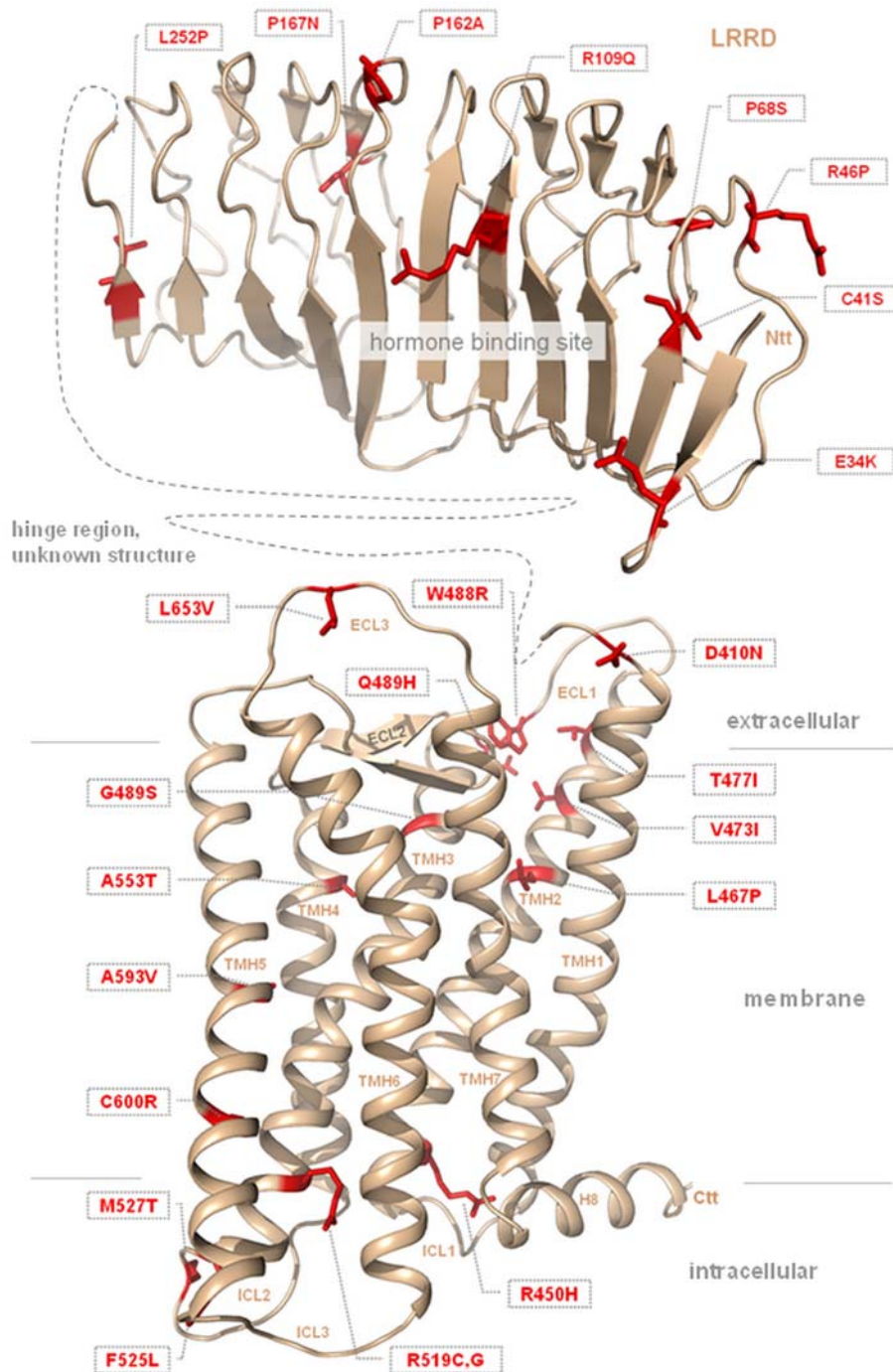
The most drastic phenotype is loss-of-function caused by mutations leading to intracellularly retained

TSHRs (nonsense mutations, frameshift mutations, missense mutations). In terms of partial loss-of-function it has to be assumed that functional properties of the receptor are partially preserved. This could be explained by the recently published finding of a multiply induced activation of the TSHR (40), which means, the full activation process is characterized by modification of the basal conformation at several spatially diversely located activation points. In a reverse conclusion, an interruption of “multi-component” signaling by single substitutions only leads to a locally restricted and therefore partial inactivation. Interestingly, mutations with a decreased basal activity (inverse agonistic mutations) are also under discussion to cause malfunction of GPCRs (like the TSHR) with a loss-of-function phenotype (41, 42).

Mapping of known loss-of-function mutations and the available 3-dimensional model of the TSHR reveal (Figure 2) that the majority of substitutions are localized in peripheral spatial regions like the LRRD, ECLs, and ICLs. We conclude that the loops and the extracellular part of the TSHR are most important for receptor trafficking, cell-surface expression, intermolecular contacts, and stabilization of the active conformation. The relevance and sensitivity of this “functional package” is exemplarily demonstrated by the recently reported mutation Q489H in extracellular loop 1 (ECL1), leading to high receptor expression levels on the cell surface and is able to bind TSH. However, it is suggested by the authors of this recent study that this mutation leads to a receptor that bypasses the endoplasmatic reticulum and golgi apparatus control system and reaches the cell surface as an immature receptor that impedes complete glycosylation and abolishes intramolecular cleavage. This altogether blocks the molecular changes to reach an active conformation (43).

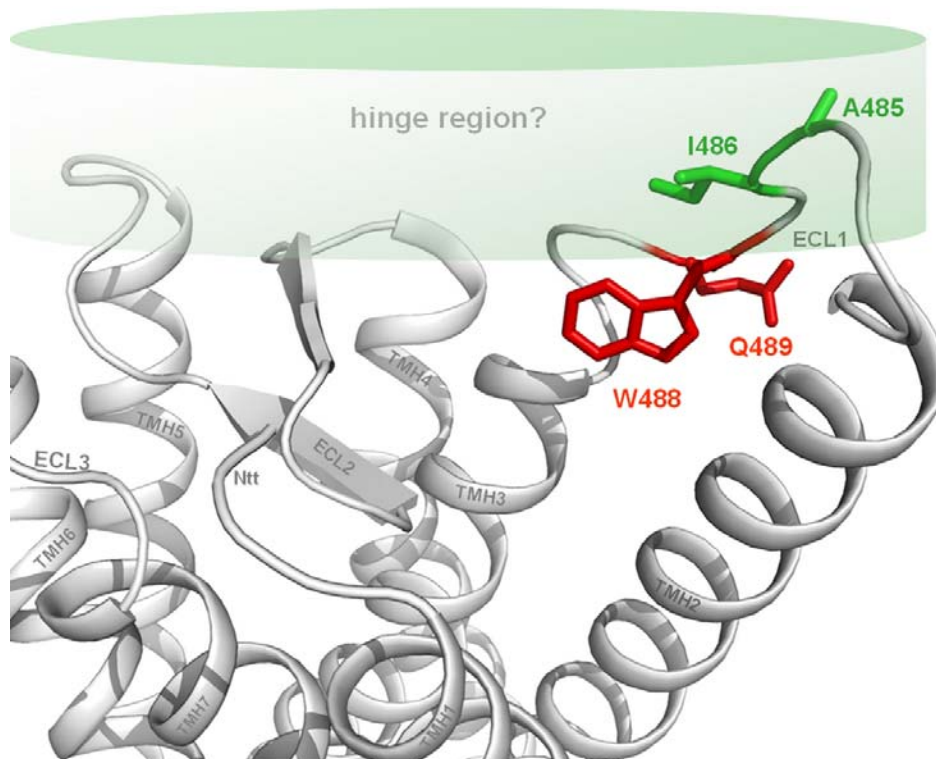
The ECL1 of TSHR has been found to be important for keeping the receptor in its basal state (40, 44). This finding was underlined by data obtained with the closely related FSHR (45). Moreover, the ECL1 is extracellularly exposed and should be a determinant of assumed interaction between the serpentine domain and the hinge region (40, 46-48). Such interactions are involved in regulation of basal TSHR activity and in control of the transition between basal and activated receptor conformations. Mutation Q489H is localized in the middle part of ECL1 and is one out of several identified naturally occurring mutations in ECL1 (Figure 3): inactivating mutations - W488R, Q489H (43, 49, 50), activating mutations - A485V, I486M,F,N (31, 51, 52). While W488 most likely stabilizes ECL1 and the inactivating mutation W488R disturbs the folding of ECL1, Q489 might establish contacts to an interaction partner in spatial proximity that should be located in the hinge region according to the exposed location of ECL1. This interaction might be relevant for signaling related processes like stabilization of the activated TSHR conformation or justification of the receptor components serpentine domain/hinge region/LRRD to each other. In contrast, the mutation Q489H is unable to keep the molecular functions of the wild type side-chain. These conclusions are supported by mutagenesis studies at the ECL1, where the Q489A

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**Figure 2.** Naturally occurring loss-of-function (inactivating) single substitutions of the TSHR mapped to the 3-dimensional structure. In this representation positions of naturally occurring inactivating single substitutions observed for the TSHR are mapped directly to the 3-dimensional model (TSHR backbone) of the serpentine domain and the crystal structure of the TSHR Leucine-rich repeat domain (LRRD) (119). Shown are the side-chains of wild type amino acids as sticks (red). Based on this spatial representation it becomes obvious that most of the published inactivating mutations are localized in peripheral receptor parts like the intra- or extracellular loops (ICLs, ECLs). Interestingly, in the transmembrane helices (TMH) 6 and 7 no inactivating mutations for TSH-induced activation have been observed so far. These two helices are hot-spots for constitutively activating mutations (Figure 4). For the extracellular hinge region that connects the LRRD and the serpentine domain no structure or structural homology model is available yet. For references see table 1A. Deletions in the TSHR leading to inactivation (35, 104) are not highlighted in the homology model.





**Figure 3.** Four recently identified naturally occurring mutations are localized in ECL1. The inactivating mutations W488R, Q489H (43, 49, 50) and activating mutations: A485V, I486M,F;N (31, 51, 52) are indicators for the high importance of extracellular loop 1 (ECL1) for regulation of signaling activity, receptor folding and signal transduction. While W488 most likely stabilizes ECL1 and mutation W488R disturbs the folding of ECL1, Q489 might establish hydrophilic contacts to an interaction partner in spatial proximity. This partner should be located in the hinge region according to the exposed location of ECL1. This interaction might be relevant for signaling related stabilization of the activated TSHR conformation or justification of the receptor components serpentine domain/hinge region/LRRD to each other. In contrast, the mutation Q489H is unable to keep the molecular properties of the wild type side-chain. The activating mutations A485V and I486M,F,N (positions of activating mutations in green) provide hints that tight hydrophobic interactions are essential to keep the ECL1 in its conformation, crucial for the basal state. Mutations in ECL1 potentially lead to a loss of such interactions.

mutation and different W488 side-chain variations always lead to TSHR inactivation (40). The mechanism of CAMs at residues A485 and I486 will be discussed in more detail in section 5.2.

## 5. ACTIVATING GERMLINE MUTATIONS

### 5.1. Clinical aspects

The first patients diagnosed with a constitutive activating mutation as the molecular cause of non-autoimmune hyperthyroidism came from two large pedigrees from Belgium, known as the Reims and Nancy family (33). Already in this study it became obvious that severity and onset of disease vary between members of one family. It was speculated that environmental factors and the genetic background are responsible for this phenotypic variability (33). In addition to familial cases, sporadic cases were described (53, 54). There is some overlap between mutations found in toxic thyroid nodules or adenomas and as germline mutations. In the beginning it was assumed that these somatic mutations could be also found in severe sporadic cases. But over time this assumption did not hold true as these somatic mutations

were also found in familial cases (54-56). For treatment options, the identification of a heterozygous TSHR mutation is of extreme importance if the subsequent functional characterization affirms the constitutive TSHR activation caused by the mutation. For patients, especially children, in whom hyperthyroidism can only be poorly controlled by anti-thyroid drugs, total thyroidectomy is the treatment of choice to avoid long-term sequela and complications like premature craniosynostosis (10). However, thyroidectomy is not necessary in all patients (51, 57, 58) as in the younger patients hyperthyroidism can be controlled by anti-thyroid drug treatment.

For activating mutations a correlation of the degree of constitutive  $G_s$  activation *in vitro* and the phenotype of the patient is not appropriate. One reason for this is the heterologous overexpression cell system standardly used for functional *in vitro* characterization of mutated receptors. Comparison of cAMP (cyclic adenosine monophosphate) accumulation in COS-7 cells and different thyroid cell systems demonstrated a discrepancy between cAMP formation between thyroid and non-thyroid cell

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lines indicating that the cellular context is important for the biological effects of the mutations (59).

### 5.2. Molecular and structural aspects of TSHR activation by mutation

The TSHR is characterized by a physiologically relevant basal (ligand independent) signaling activity (between 5-10 percent compared to TSH induced maximum of activation), which can be increased by mutations of single amino acids or deletions of receptor portions (60, 61). As described in section 5.1., this increase of basal activity can cause disease conditions like described for many other GPCRs (62-64). Two principle molecular reasons of non-autoimmune TSHR activation by mutation which lead to gain-of-function phenotypes can be distinguished:

- (1) constitutive activation of the TSHR in absence of the endogenous ligand TSH,
- (2) abrogated specificity for glycoprotein-hormones (reviewed in (65)).

For abrogated glycoprotein-hormone specificity one mutation, K183R in the LRRD, is reported so far (66) that displays promiscuous ligand binding (binding affinity for CG is increased). In contrast, somatic and germline mutations that constitutively activate the TSHR are published in around 70 reports (Figure 4). What are the molecular mechanisms of TSHR activation caused by mutations? Two possible modes of molecular action for CAMs are under discussion (67): 1. CAMs disrupt an interaction network important for stabilization of the basal conformation (most of the reported CAMs), or 2. substituted side-chains create new interactions (wild type amino acids are not involved in the maintenance of the basal state) and thereby shift the equilibrium between the inactive and active states towards the activated conformation (e.g. Y601N (68, 69)).

It can be assumed, that the majority of the CAMs are acting according to scenario 1. Examples are two positions of naturally occurring CAMs in the ECL1 (Figure 3): A485V, I486M,F;N (31, 51, 52). These side-chain substitutions and related constitutive TSHR activation suggest that tight hydrophobic interactions of the wild type amino acids are essential to keep the ECL1 in its conformation, which is crucial for maintenance of the basal state (40, 44). The exposed extracellular location of ECL1 in combination with its functional importance (see also section 4.4.) designate this loop as a candidate for potential interaction with the hinge region. Interestingly, naturally occurring CAMs in the ECLs or the hinge region have been reported only for the TSHR, but not for the closely related FSHR and LHCGR. Characterization of CAMs observed for the TSHR at corresponding positions of the FSHR or the LHCGR could help to answer the question of similarities or differences in resulting mutant properties and could provide further mechanistic and evolutionary insights into the group of GPHRs.

The distribution of naturally occurring gain-of-function mutations in the TSHR provides insights in the interrelation between structural and functional aspects of

signaling mechanisms. CAMs of the TSHR are cumulatively localized in the centre of the transmembrane helix-core (Figure 4). The helices 3, 6 and 7 are hot-spots for CAMs. Several of the sensitive wild type amino acids interact directly with each other, e.g. A593 (TMH5) with V509 (TMH3) (70). Breakage of interactions or specific modifications at these positions releases the TSHR in its active conformation, which is confirmed in numerous mutagenesis studies (30, 71-75). In conclusion, the basal receptor conformation is constraint by interactions between residues at the helices with preferences for certain helices and regions in the central core of the helical-bundle. For activation of the TSHR these constraints must be released, which leads to structural shifts between receptor components (LRRD/hinge region/serpentine domain) and also between particular helices like TMH5, TMH6, and TMH7.

## 6. DIMERIZATION OF THE TSHR

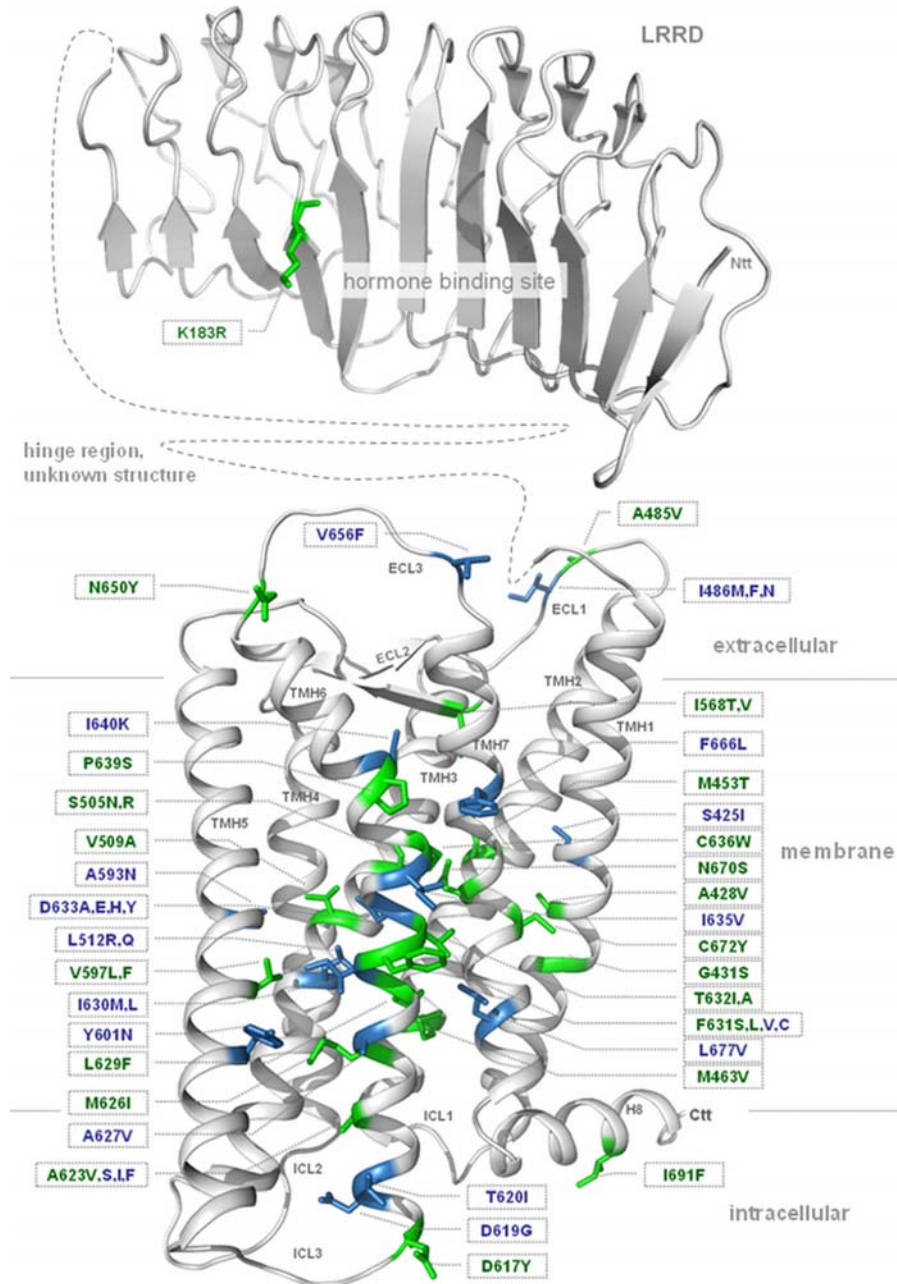
Di- or oligomerization is an accepted structural and functional feature of G-protein-coupled receptors and has been shown for numerous GPCRs (76, 77). Dimerization of GPCRs can affect processes like trafficking to the cell surface, ligand binding, ligand-induced signaling and ligand-induced internalization. Moreover, as GPCRs are potent targets for therapeutical intervention, homo- and heterodimerization with unrelated GPCRs have to be taken into account (78).

For the TSHR first reports of potential organization in oligomers were revealed by the use of different antibodies raised against the N-terminus, the midportion and the C-terminus of the ectodomain (79). By using a fluorescence based technology (fluorescence resonance energy transfer, FRET) TSHR dimerization was first shown after expression in Chinese hamster ovary cells in which TSHR is expressed at a more physiologically relevant density (80). The effect of TSH binding for dimer formation seems to be controversial (80, 81) as it was first shown that TSH binding inhibits receptor oligomerization, however the degree of inhibition was low (80) and this finding was not reproduced in a second study (81).

Detailed characterization of TSHR di- or oligomerization by bioluminescence resonance energy transfer (BRET) and homogenous time resolved fluorescence (HTRF) point to the important role of the heptahelical domain of the receptor for intermolecular protein-protein interaction. The extracellular part might modulate the interaction (81). TSHR di-oligomerization seems to be constitutive and occurs early in the endoplasmic reticulum (like also shown for the homologous LHCGR and FSHR (82, 83)) and is discussed to be obligatory for proper receptor expression (84).

For some patients who are diagnosed with TSH insensitivity only one heterozygous mutation could be identified (36). No second mutation in the coding region of the TSHR could be identified. One could speculate that the second mutation might be located in the promoter or the intronic region. However, dominant-negative effects for

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**Figure 4.** Naturally occurring activating gain-of-function single substitutions of the TSHR mapped to the 3-dimensional structure. This overview of naturally occurring gain-of-function mutations mapped on the 3-dimensional TSHR structure (backbone) simplifies recognition of spatially tight clusters of amino acids sensitive for constitutive activation. Side chains of residues where germline gain-of-function mutations are known are green, positions of somatic constitutively activating mutations (CAMs) are colored in blue. For a few positions occurrence of germline and somatic mutations are reported (not highlighted). It can be observed that for the human TSHR constitutively activating mutations (CAMs) are cumulatively localized in the centre of the transmembrane helix core. By contrast, inactivating mutations are found mainly in the peripheral TSHR parts like the loops of the extracellular hinge region connecting the LRRD (crystal structure (119)) and the serpentine domain (model of the serpentine domain based on the crystal structure of rhodopsin) no structure or structural homology model is available yet. The helices 3, 6 and 7 are hot-spots for CAMs. Several of the sensitive wild type amino acids interact directly with each other and maintain the basal TSHR conformation. Interestingly, substitution K183R in the Leucine-rich repeat domain (LRRD) leads to promiscuous hormone binding (increased affinity for CG). For references see table 1 and website: [www.sfsa-gphr.de](http://www.sfsa-gphr.de), section “Pathogenic Mutations” / ‘Tabulated overview of GPHR mutations’. Deletions in the ECL3 and the ICL3 leading to TSHR activation (60, 61) are not highlighted in the TSHR model.

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some mutations are likely also. This has been shown recently for partially inactivating TSHR mutations. These mutations (C41S, L467P, C600R) were identified in patients with autosomal dominant inheritance of TSH resistance (85). For all mutations intracellular trapping of the wild-type/mutant complex was demonstrated. This early TSHR dimerization could be an explanation for the dominant occurrence of heterozygous inactivating TSHR mutations (36, 85) that lead to a mild form of hyperthyrotropinemia. Moreover, the problems in correlation of genotype (functional characteristics of a mutation) and the phenotype of the patients (congenital hypothyroidism or hyperthyrotropinemia) may also be due to the fact, that GPCRs are not only able to homo-dimerize, but also to hetero-dimerize with other (unrelated) GPCRs, in particular in overexpression systems. Therefore, it might be valuable to test partial loss-of-function mutations in human thyrocytes, in which the TSHR is expressed at physiologically relevant levels.

The formation of tetrameric complexes has been shown for beta-2 adrenergic receptors (86). If this also holds true for the TSHR, five different variants of tetrameric complexes could be formed consisting of: 1. wild-type receptors only, 2. mutant receptors only, 3. one wild-type and three mutant receptors, 4. two wild-type and two mutant receptors or 5. three wild-type and 1 mutant receptor. The consequences of this mixture *in vivo* and *in vitro* should be responsible for signaling properties that also depend on interacting molecules like chaperones which could strongly differ between thyroid follicular cell and *in vitro* cell systems.

So far, only few inactivating mutations were examined in co-transfection experiments showing complementation of two inactivating mutations (81). For heterozygous constitutively activating mutations such interaction has not yet been investigated, however, it is also likely that activating mutation influence wild-type receptor function.

Interestingly, for the LHCGR it is demonstrated that a signaling inactive LHCGR mutant (that is trafficked normally to the plasma membrane) decreases the signaling of the wild type or the constitutively active LHCGR due to receptor heterodimerization (87).

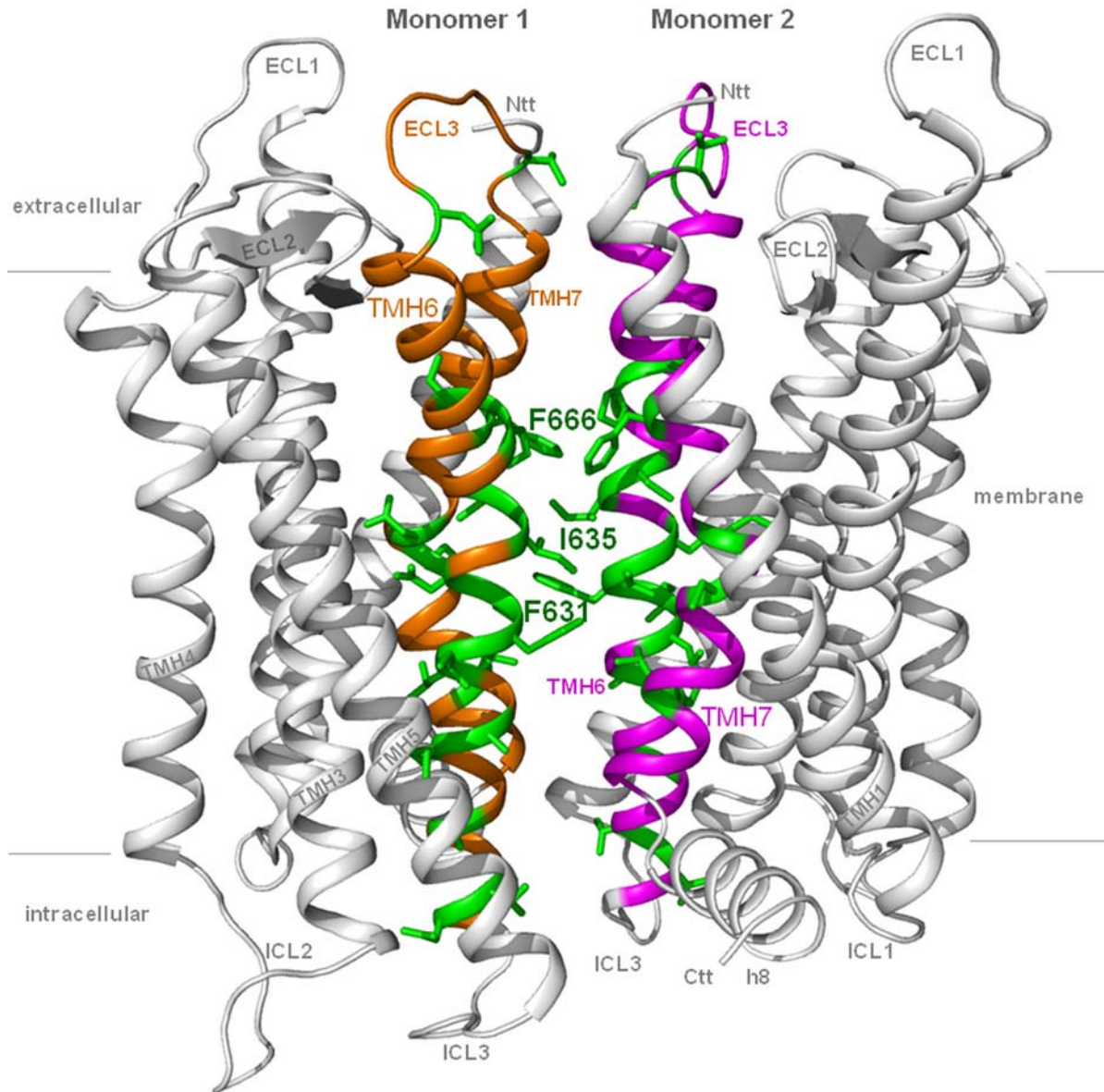
### 6.1. Hypothetical TSHR dimer-interface

The FSHR is like the TSHR a member of the homologous GPHRs (88) and fragments of this gonadotrophic receptor were crystallized in 2005 (89-91). This structure of the LRRD is complexed with the hormone FSH and provided deep insights in the interaction between receptor and the glycoprotein hormone (92). Interestingly, in this crystal structure two LRRD monomers were arranged in a dimer conformation. In consequence, contacts between the extracellular regions of two receptor monomers in a dimer were discussed to be of importance for dimerization of GPHRs (90-92). Indeed, this hypotheses (but not the suggested participating detailed amino acids like Y110) for the FSHR has been refined by current studies of Guan and co-workers (83), who showed both the

extracellular region and the serpentine domain as competent for oligomer-arrangements. A similar potential for oligomerization is also assumed for the extracellular and transmembrane regions of the LHCGR (93). For the TSHR interactions between the transmembrane regions are responsible to stabilize the dimer contact and the extracellular parts might modulate the interaction (81). Anyway, the detailed contacts between the monomers in dimer-oligomer arrangements and the functional significance of TSHR oligomers are still under discussion (80, 84, 94, 95).

For TSHR, FSHR, LHCGR and the Leucine-rich repeat containing G-protein-coupled receptors (LGRs) 7 and 8 (binding of peptide-hormones relaxins 1 to 3 and insulin-like 3/relaxin-like factor) an additional hint for dimer-arrangement and functionality is given by the fact that these receptors can be trans-activated by ligand binding (81, 96, 97) and negative cooperative effects were observed ((81, 98, 99), reviewed in (3)). This mechanism of receptor activation allows the conclusion of at least close distances between receptor monomers.

With respect to naturally occurring mutations one phenomenon is still unexplained and should be included in the discussion of TSHR dimer-arrangement and the dimer-interface (contact(s)). In detail, a microdomain of 5 consecutive amino acid positions (L629, I630, F631, T632, D633) were CAMs have been identified is localized in TMH6 (Figures 1 and 4). Because these residues are in an alpha-helix, where one turn is constituted by 3.6 amino acids per definition, at least one residue side-chain must point into the membrane. Homology models of the TSHR favour the side-chain of F631 to stick into the membrane. Furthermore, a similar observation can be made for I635 one turn above F631, but also for F666 at TMH7 (Figure 5). At these positions, naturally occurring CAMs are known. The question arises, how the increased basal activity by mutation can be explained in these particular cases? The side-chains do not interact directly with other amino acids and therefore mutations can not lead to a disruption (released basal conformation) or to constitution (stabilization of the activated conformation) of constraining interactions. In our here suggested hypothetical dimer arrangement of the TSHR with a main contact-interface between TMH6 and TMH7 these residues would interact with each other (Figure 5). Naturally occurring mutations F631V, I635V (TMH6), or F666L (TMH7) should interrupt hydrophobic and aromatic interactions constraining this potential dimer-interaction by introduction of shorter or non-aromatic side-chains. In a reverse interpretation, TMH6 and TMH7, which are known to be strongly involved in receptor activation are constraint in this dimer-arrangement and a release of such constraint could participate in TSHR activation. This suggestion is underlined by the extraordinary high number of CAMs in TMH6 (Figure 4). Additionally, ECL3 connects TMH6 and TMH7 and should be involved in this dimer-model by forming intermolecular interactions between receptor monomers. Therefore, known gain-of-function mutations like N650Y (100), V656F (60), or deletion del658-661 (101) probably modify this proposed dimer-interface also.



**Figure 5.** Hypothetical dimer arrangement of the TSHR with a main contact-interface between TMH6 and TMH7. Five consecutive amino acids (L629, I630, F631, T632, D633) for which gain-of-function CAMs have been observed are localized in TMH6. Because these residues are in an alpha-helix, where one turn is constituted by 3.6 amino acids per definition, at least one residue side-chain point into the membrane. Homology models of the TSHR favour the side-chain of F631 to stick into the membrane. Similar observation can be made for I635 one turn above F631, but also for F666 at TMH7 in a TSHR monomer. In consequence, the side-chains do not interact directly with other amino acids and therefore, mutations cannot lead to a released basal conformation or to constitution of an activated conformation. In a hypothetical dimer arrangement of two TSHR monomers with a contact-interface between TMH6 and TMH7 (orange TSHR 1, magenta TSHR 2) exactly these residues would interact to each other. Naturally occurring activating mutations like I635V or F631V should interrupt tight hydrophobic and aromatic interactions constraining this potential dimer-interaction by introduction of shorter or non-aromatic side-chains. In a reverse interpretation TMH6 and TMH7, which are known to be strongly involved in receptor activation would be constraint in this dimer-arrangement. A release of such constraint should participate in TSHR activation.

However, without experimental evidence and exact knowledge of the *in vivo* dimer-interface the question of a relation between TSHR activation and complete interruption of the dimer-contact, a dimer-rearrangement, or dimer-constitution can not be answered yet.

## 7. TSHR MUTATIONS AND THYROID GROWTH

The first identified inactivating TSHR mutations led to a phenotype with a normal size of the thyroid gland

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(37). This was due to the fact that high TSH levels in the patients can overcome the partial inactivation of the mutation resulting in normal function of the thyroid gland. If high TSH levels can not rescue inactivating mutations, congenital hypothyroidism with hypoplasia of the thyroid gland occurs (43). This hypoplasia can be mild (102) or could result in apparent athyrosis (103, 104). For inactivating mutations, effects on cell surface expression, ligand binding and Gs activation were investigated only because higher amounts of TSH for activation of Gq/11 are needed (see Table 1). Interestingly, one TSHR mutation was identified in three siblings who suffer from congenital hyperthyrotropinemia and increased radio-iodine uptake. The identified homozygous L653V mutation showed normal cell surface expression and ligand binding, as well as normal basal cAMP and IP accumulation levels. Strikingly, TSH-induced EC50 levels for cAMP were slightly enhanced and TSH-induced IP levels were markedly reduced (8). This was the first report that demonstrated the important role of the Gq/11 pathway in thyroid hormone production, because the TSH levels needed to overcome the defect of TSHR signaling are much higher than for the slight defect in Gs/adenylyl cyclase signaling (8).

So far, all reported CAMs have been identified in patients with non-autoimmune hyperthyroidism and goiter. Growing of the thyroid gland was assumed to be the result of constitutive activation of the Gs/adenylyl cyclase pathway (2). However, it has been shown that constitutive activation of the Gs/adenylyl cyclase pathway is not sufficient to generate toxic thyroid nodules or adenomas (105). This could be also true for goiter formation in patients carrying constitutively activating germline TSH mutations pointing to more complex mechanisms in goiter formation.

Only a few mutations constitutively activate the Gq/11-mediated pathway. The activation of both signaling pathways did not lead to a more severe phenotype than Gs activating mutations alone (106). So far, only a few mutations with reduced or impaired IP formation were reported (see Table 1). For these mutations a reduction in cell surface expression or maximal binding properties could be responsible for reduction of bTSH-stimulated IP accumulation. For mutations M463V, A485V, I568T, F631S, C672Y an effect of IP impairment, measured in vitro studies, on goiter formation in vivo is not clear, but can not be excluded. This is also the case for two TSHR mutations (S505R, N670S) that are expressed on the cell surface comparable to wild-type receptor, but are characterized by a decreased IP accumulation. In general, for the above mentioned mutations the onset of goiter formation is unclear.

Furthermore, attention on the physiological relevance of the Gq-mediated IP signaling pathway was drawn in previous studies (107). The recent view on thyroid growth probably has to be reconsidered now, because it was shown in 2007 that mice lacking G<sub>q/11</sub> proteins suffer from hypothyroidism and are protected of goitrogenous thyroid growth (7). In this line, we detected a patient with mild non-autoimmune hyperthyroidism. The child only presented with

elevated thyroid hormone and repressed TSH levels. The size of the thyroid gland was normal and anti-thyroid drug treatment was not considered for a long time. Functional characterization of the identified mutation (C636W) showed the expected constitutive activity of the G<sub>s</sub> pathway, however, the G<sub>q/11</sub> pathway induced by TSH was nearly completely inactivated (108) although cell surface expression and binding properties were not affected. We here suppose that loss-of-function of the G<sub>q/11</sub> pathway might participate in the mild phenotype of the patient and the current lack of goiter (age 11.5 years). Therefore, this condition should be termed non-autoimmune, non-goitrogenous hyperthyroidism. To verify the hypothesis that a lack of G<sub>q/11</sub> activation should protect from uncontrolled thyroid growth, more young patients with a similar phenotype have to be investigated.

## 8. CONCLUDING REMARKS

The knowledge concerning medical and biological aspects related to the thyrotropin receptor has been exponentially accumulated in the last decades, also the role of the TSHR in non-thyroidal tissues (109, 110). This includes occurrence and prevalence of naturally occurring mutations, phenotypes and understanding of diseases related to TSHR malfunctions, but also mechanisms of signaling processes at this protein are of high interest (1, 2, 14, 15, 25, 63, 64, 93, 111). Regulation of TSHR function is studied intensively and a few findings can be generalized for other GPCRs characterized by similar structural and functional features like elevated basal signaling activity or multiple and synergistic signal induction (40). Understanding of structural features causally related to functional properties is a prerequisite and of high interest for optimized treatment of patients. The investigation of antibodies (agonistic, antagonistic, inverse agonistic (14, 15, 112-114)) and the development of allosterically bound synthetic drug-like agonists and antagonists for the TSHR (115-117) are topics of high priority in this field (118). Major goals are the understanding of TSHR signaling and the specific pharmacological modulation of signaling pathways.

## 9. ACKNOWLEDGEMENT

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**Abbreviations:** GPHR, glycoprotein hormone receptor; LHCGR, lutropin/choriogonadotropin receptor, lutropin receptor; FSHR, follicle stimulating hormone receptor, follitropin receptor; TSHR, thyroid stimulating hormone receptor, thyrotroin receptor; bTSH, bovine thyroid stimulating hormone; LH, luteinizing hormone; CG, choriogonadotropin; FSH, follicle stimulating hormone; GPCR, G-protein coupled receptor; TMH, transmembrane helix; ECL1/2/3, extracellular loops 1/2/3; ICLs 1/2/3, intracellular loops 1/2/3; LRRD, Leucine-rich repeat domain; SD, serpentine domain; CAM, constitutively activating mutation; wt, wild type; N-tt, N-terminal tail; C-tt, C-terminal tail; cAMP, cyclic adenosine monophosphate; IP, Inositolphosphat; FRET, fluorescence resonance energy transfer; BRET, bioluminescence resonance energy transfer; HTRF, homogenous time resolved fluorescence; COS-7, *Cercopithecus aethiops* (CV1 Origin SV-40); CHO, Chinese hamster ovary; Leucine-rich repeat containing G-protein-coupled receptors, LGRs

**Key Words:** Thyrotropin Receptor, Naturally Occurring Mutations, Pathogenic Mutations, Germline Mutations, Somatic Mutations, Dimerization, Glycoprotein Hormone Receptors, Review

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