Molecular Endocrinology. First published ahead of print April 24, 2007 as doi:10.1210/me.2007-0040



**Research Article for Molecular Endocrinology**

Full title

# A mutation in *Tpst2* encoding tyrosylprotein sulfotransferase causes dwarfism associated with hypothyroidism

Abbreviated title: *Tpst2* mutation causes hypothyroidism

Nobuya Sasaki, Yayoi Hosoda, Aogu Nagata, Ming Ding, Ji-Ming Cheng, Tomomi Miyamoto, Shinya Okano, Atsushi Asano, Ichiro Miyoshi, and Takashi Agui

*Laboratory of Experimental Animal Science (N.S., Y.H., A.N., S.O., A.A., T.A), Graduate School of Veterinary Medicine, Hokkaido University.*
*Center for Experimental Animal Science (M.D., J.C., T.M., I.M), Nagoya City University Medical School.*

Address all correspondence and requests for reprints to: Professor Takashi Agui
Laboratory of Experimental Animal Science, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku, Sapporo. 060-0818. Phone & FAX: 81-11-706-5106
Email: agui@vetmed.hokudai.ac.jp

Present address for M.D.: Department of Pharmacology, Southern Illinois University School of Medicine, J.C.: Division of Hematology and Oncology, Department of Internal Medicine, Southern Illinois University School of Medicine, S.O.: Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.
A.A.: Laboratory of Veterinary Biochemistry, School of Veterinary Medicine, Faculty of Agriculture, Tottori University.

Keywords: dwarfism, thyroid hypogenesis, tyrosylprotein sulfotransferase, TSH receptor

The abbreviations used are: cM, centimorgan cM; CCK, cholecystokinin; CF-8, coagulation factor VIII; FSHR, FSH receptor; GP1BA, glycoprotein 1b alpha polypeptide; *grt*, growth-retarded; HE, haematoxylin and eosin; RFLP, restriction fragment length polymorphism; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PAS, periodic acid schiff; PSGL1, P-selectin glycoprotein ligand-1; TPST, tyrosylprotein sulfotransferases; TSHR, TSH receptor; WT, wild-type.

Disclosure summary: The authors have nothing to disclose.

## ABSTRACT

The growth-retarded (*grt*) mouse has an autosomal recessive, fetal-onset, severe thyroid hypoplasia related to TSH hyporesponsiveness. Through genetic mapping and complementation experiments, we show that *grt* is a missense mutation of a highly conserved region of the tyrosylprotein sulfotransferase 2 (*Tpst2*) gene, encoding one of the two *Tpst* genes implicated in post-translational tyrosine O-sulfation. We present evidence that the *grt* mutation leads to a loss of TPST2 activity, and TPST2 isoform has a high degree of substrate preference for TSH receptor (TSHR). The expression of TPST2 can restore TSH-TSHR-mediated cAMP production in fibroblasts derived from *grt* mice. Therefore, we propose that the tyrosine sulfation of TSHR by TPST2 is crucial for TSH signaling and resultant thyroid gland function.


## INTRODUCTION

Normal thyroid function is essential for development, growth, and metabolic homeostasis. Permanent congenital hypothyroidism affects about 1:3000-4000 newborns, and is one of the most common preventable causes of mental retardation. In about 90% of all cases, congenital hypothyroidism is the consequence of thyroid dysgenesis such as a small and sublingual thyroid, or no thyroid tissue. Most of these cases appear sporadically, although a few cases of recurring familial thyroid dysgenesis have been described (1). Molecular genetic analyses have identified four thyroid dysgenesis susceptibility genes in humans, *TSHR* (2) and the genes for transcription factors *TTF1* (3, 4), *TTF2* (5) and *PAX8* (6) in pathways crucial for the normal development of the thyroid. Studies of the spontaneous mutation and targeted-disruption of *Tshr* (7), *Ttf1* (8), *Ttf2* (9), and *Pax8* (10) in mice have provided insight into the molecular mechanisms of organogenesis and

thereby form the basis for molecular genetic studies in human patients affected by thyroid dysgenesis. However, mutations in these genes are found in only 5% of patients with thyroid dysgenesis. Therefore, the genetic and pathological mechanisms underlying thyroid dysgenesis are still poorly understood.

The growth-retarded (*grt*) mouse is a spontaneous mutant exhibiting severe primary hypothyroidism and dwarfism controlled by a single recessive locus. The gene responsible for the *grt* has not yet been cloned, nor has its molecular mode of action been determined. However, homozygous *grt* mice exhibit low concentrations of serum T3 and T4, and a compensatory elevation in the level of circulating TSH demonstrates a normal pituitary response (11). In fact, measurements of several additional pituitary hormones revealed no significant differences between normal and *grt* mice. Growth retardation is recovered by the administration of T3, indicating that the *grt* mutation does not affect anterior pituitary function (12). The thyroid gland of *grt* mice is defective in TSH responsiveness, particularly in signaling pathway involving TSH, TSHR, G protein, and adenylate cyclase (13). These results suggest that the growth-retarded phenotype is due to an impairment of thyroid glands in the production of thyroid hormone or a response to TSH. To determine the molecular mechanism of the *grt* phenotype, we mapped the *grt* locus responsible for dwarfism in a 59-centimorgan (cM) region of mouse chromosome 5 (14). No genes responsible for human and rodent dwarfism or thyroid disease have been mapped to this region. In this report, we narrowed the *grt* locus to a $< 0.1$ cM region using 1,084 backcross progenies, sequenced candidate genes located in this region, and finally identified a causative mutation in the tyrosylprotein sulfotransferase 2 (*Tpst2*) gene. Recent papers reported that the posttranslational modification by tyrosine sulfation regulates many important protein-protein interactions and modulates binding affinity and specificity. Mutational analysis has demonstrated that TSHR is sulfated on tyrosines in a motif that is essential for high-affinity binding of TSH and activation of the receptor (15).

In this report, we propose that severe thyroid hypogenesis and consequent dwarfism are due to an impairment of tyrosine sulfation in TSHR by TPST2.

## RESULTS

### Identification of *Tpst2* mutation in *grt* mutants

To identify the gene underlying the *grt* mutant phenotype, we created a high-resolution genetic map of the *grt* locus using 1,084 backcross mice, and analyzed their phenotypes of homozygotes (*grt*/*grt*) or heterozygotes (*grt*/+) by weighing mice at 5 wk of age. Homozygotes of both sexes were small with shortened limbs and tails and weighed approximately 40-60% less of the heterozygotes or wild-type (WT) mice. None of the phenotypes of 1,084 backcross progenies had recombination with a microsatellite marker, *D5Mit24*, indicating a linkage of < 0.1 cM (Fig. 1A). As shown in Fig. 1B, the gene order was from *Cryba4* to *Asphd2* covering the critical region. In this region, the mouse genome database disclosed seven genes, *Cryba4, Crybb1*, *Tpst2*, *Tfip11*, *Srr1*, *Hps4* and *Asphd2*. We examined the expression of these possible candidate genes in multiple tissues including thyroid with RT-PCR; there was no qualitative difference observed (data not shown). We next compared the nucleotide sequences of these genes between normal and affected mice, and identified a distinct mutation in *Tpst2*. The mutation is a single missense mutation with a transversion of C at nucleotide 798 to G, leading to the replacement of a highly conserved histidine with a glutamine at codon 266 in the sulfotransferase domain (Fig. 1B). Sequence alignment of TPST1 and 2 reveals that the histidine residue was highly conserved among TPST family proteins of diverse species (Fig. 2A). In addtion, phenotypically affected mice were homozygous for mutant alleles with PCR-restriction fragment length polymorphism (RFLP) genotyping assay,

whereas normal mice were homozygous for wild type alleles or heterozygous in DW/J mouse colony (Fig. 2B). Furthermore, this mutation was not present in ten other laboratory strains (data not shown). These results suggested that the *Tpst2* mutation is responsible for the dwarfism.

**Expression of *Tpst2* transgene rescues the mutant phenotype**

To confirm this hypothesis, we attempted to complement the *grt* phenotype using transgenic rescue. We produced transgenic mice carrying *Tpst2* cDNA driven by the cytomegalovirus enhancer and chicken actin (CAG) promoter (Fig. 3A). Founder mice expressed the *Tpst2* gene in several tissues including thyroid (Fig. 3B). Crosses between *grt*/+ males with the transgene and *grt*/+ females generated 17 phenotypically affected mice, all of which were negative for the transgene. Twenty normal mice were homozygous for the *grt* mutation, and all were positive for the transgene, demonstrating phenotypic rescue of the dwarfism (Fig. 3C and D). *Tpst2* overexpression had no influence on growth except in *grt*/*grt* mice. Two independent founder lines produced similar results. Next, we investigated the rescue of thyroid function, serum T3, T4, and TSH levels were analyzed in *grt*/*grt* mice with or without transgenes. In *grt*/*grt* mice, serum levels of TSH were elevated 3 to 5-fold above normal, whereas T3 and T4 levels were less than normal. Rescued mutants demonstrated normal serum levels for T3, T4, and TSH (Fig. 3E). Histologically, the mutant thyroid glands showed hypogenesis characterized by poorly developed follicles that were heterogeneous in size with reduced levels of colloid (Fig. 4A middle, some follicles were unreactive with periodic acid schiff (PAS) staining. Interestingly, the thyroids of rescued mutants revealed no abnormalities in thyroid size, follicular structure, or the morphology of cells (Fig. 4A, bottom). The numbers of cells and follicles per square millimeter were in the normal range, and follicle

size was not significantly different from that of normal mice (data not shown).

Next, to assess thyroid-specific gene expression in *grt*/*grt* mice, we performed a semiquantitive RT-PCR analysis. TSH-TSHR signaling is essential for the proliferation and maintenance of the differentiated function of the thyroid follicular cells, but it is not required for early organogenesis and migration of the thyroid anlage (16). TSH-TSHR signaling is essential for the expression of sodium-iodide symporter (*Nis*), thyroperoxidase (*Tpo*) and *Tshr* itself, which are required for thyroid hormone biosynthesis, whereas it is not required for thyroglobulin (*Tgn*) expression (17). These observations are consistent with our results that the expression of *Tshr* and *Nis* is reduced by 3-5-fold, although *Tgn* was normally expressed in *grt*/*grt* thyroids compared with normal controls (Fig. 4B). Previously we have shown that much less iodine is accumulated in the *grt*/*grt* thyroid than in the control mice, although the incorporation of iodine into thyroglobulin, glycosylation, and the intracellular transportation of thyroglobulin are all normal (18). The reduction in the amount of iodine accumulated in the *grt*/*grt* thyroid might be the major cause of the decrease in levels of thyroid hormones. Consistent with the results of histopathology and serum hormone levels, the restoration of *Tpst2* rescued the levels of expression of *Tshr* and *Nis* in the transgenic mutant's thyroids (Fig. 4B). Thus, on the basis of chromosomal mapping, nucleotide sequence analysis, and genetic complementation study, we concluded that the dwarf-causing *grt* mutation was contained in the *Tpst2* gene.

**Loss of enzymatic activity of TPST2 H266Q mutation**

TPST catalyzes the transfer of a sulfuryl group from the universal sulfation substrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to a tyrosyl residue within acidic motifs of proteins that transit the Golgi (Fig. 5A). To characterize the enzymatic

activity, we prepared a peptide array presenting the 15-mer peptide substrates, glycoprotein 1b alpha polypeptide (GP1BA) (19), coagulation factor VIII (CF-8) (20), cholecystokinin (CCK) (21), FSH receptor (FSHR), TSHR (15) and P-selectin glycoprotein ligand-1 (PSGL1) (22), that are known acceptors for tyrosine sulfation, and detects the incorporation of $^{35}$S-labeled sulfate into the substrates. TPST1, TPST2 and TPST2 (H266Q) expression vectors were transfected into HEK293T. Immunoblot analysis indicated that each protein was expressed at similar level in the transfected cells (Fig. 5B). Although HEK293T cell expressed both human isozymes, which were detected by RT-PCR, there was no detectable sulfation of all peptide substrates when lysates of mock transfected cell were used (Fig. 5C). Thus, the basal TPST activity was undetectable level for this assay. Fig. 5C shows that all peptides were sulfated by wild-type TPST2. In contrast, the enzyme activity of mutant TPST2 (H266Q) was reduced to undetectable levels, although the same amount of protein was present on each reaction. Thus, histidine 266 must be a critical residue for TPST activity. We investigated the subcellular distribution of normal and mutant TPST2 tagged with FLAG and the effects of mutant TPST2 on the survival of transfected cells, no changes were found (data not shown). This mutation might affect the affinity of TPST protein for acceptor proteins/PAPS or the enzymatic activity itself. Further mutagenesis and crystallographic studies will be needed to address this issue.

**The tyrosine sulfation of TSHR by TPST2 is crucial for TSH signaling**

In mammals, tyrosine sulfation of proteins is catalyzed by two isozymes; TPST1 and 2. A question arose as to whether the isozyme, TPST1, compensates for a deficiency of TPST2, since the mouse TPST1 has 65% homology with TPST2 at the amino acid level. Previously, Northern analyses indicated that both *Tpsts* were

ubiquitously expressed in various tissues and cell lines (23, 24). However, it has not been determined whether the two TPSTs are co-expressed on cells or tissues. To evaluate tissue expression preferences of *Tpst* isoforms, we examined the expression of the *Tpst1* and *Tpst2* genes in various mouse tissues, the rat thyrocyte cell line FRTL-5 (Fig. 6A), murine primary fibroblasts, NIH3T3 cells (data not shown). Semiquantitative RT-PCR analysis was performed concomitantly for both genes in the same tube on each sample, and revealed that the *Tpst* isoforms co-expressed in the same tissues and cells including thyrocytes, although there are some differences in tissue preference. Further, RT-PCR analyses showed that the *grt* mutation did not affect steady-state transcription of *Tpst1* or *Tpst2* itself in any tissues (data not shown).

In *vitro* studies using synthetic peptide acceptors indicated that the two TPST isoforms differed in substrate preference (24, 25). Inactivation of murine *Tpst1* did not result in any abnormality of the thyroid (26). Therefore, we investigated the substrate specificity of TPSTs. As shown in Fig. 5C and D, TPST1 and 2 similarly sulfated GP1BA, CF-8, CCK and PSGL1. In contrast, the preference of TPST2 was 5-fold that of TPST1 for TSHR peptides, implying that TPST1 cannot compensate for the deficiency of TPST2 activity.

In the thyroid, the TSH-bound TSHR induces coupling of the receptor to the G protein and produces intracellular cAMP through the activation of adenylate cyclase (27). The thyroid gland of the *grt* mutant exhibited a markedly diminished response of TSH both in *vitro* and in *vivo*, although cAMP production was increased following stimulation of the thyroid glands with nonspecific adenylate cyclase activators such as forskolin (12, 13). To obtain further evidence, we performed an *in vitro* TSH signaling assay with primary fibroblasts obtained from *grt*/*grt* mutants. The Lentiviruses encoding *Tpst1*, *Tspt2* and mutant *Tpst2* (H266Q) with *TSHR* cDNA were introduced into *grt*/*grt* fibroblasts, and TSH-induced production of cAMP was determined. The *grt*/*grt*

fibroblasts showed an increase in cAMP production when cultured in the presence of forskolin. Stimulation of cells expressing just TSHR (mock) with TSH resulted in a slight increase in cAMP accumulation (Fig. 6B). The TSH-dependent cAMP accumulation in the cells expressing TPST1 or mutant TPST2 was very low or similar to that of mock-transfected cells. In contrast, in cells expressing TPST2, cAMP accumulation was approximately 2-3 folds higher than that in cells expressing the others. These results strongly suggest that only TPST2 can efficiently mediate the TSH-TSHR signal transduction pathway, whereas TPST1 and TPST2 (H266Q) cannot.

## DISCUSSION

The present study revealed that *grt* mice carried a recessive loss-of-function mutation in *Tpst2* gene. We have demonstrated that TPST2 is required for normal development and function in thyroid. This conclusion is supported by the following evidence: (a) the position of the *Tpst2* gene closely located to *grt* locus and the perfect co-segregation of the mutations with the phenotype. (b) H266 is highly conserved in TPST family among many species. (c) Q266 substitution causes a loss of enzymatic activity. (d) *Tpst2* transgene rescues the mutant phenotypes *in vitro* and *in vivo*. (e) TPST2 shows high specificity for the substrate toward TSHR. Previously site-directed mutagenesis suggested that sulfation of tyrosine 385 of TSHR is required for high-affinity hormone binding and receptor activation by TSH (15). Further, two kinds of *Tshr* mutant mice, spontaneous and targeted mutants, have been reported (16, 17). Both mutants can produce thyroglobulin, however, they show reduced iodine uptake in the thyroid. Similarly, the uptake of iodine is markedly lower in *grt* mice (18). Combining these observations with our data, we propose that sulfation of tyrosine 385 of TSHR by TPST2 is indispensable for the activation of TSH signaling. Since *grt*/*grt* mice are unable

to fully respond to TSH, they develop hypothyroidism and dwarfism. The growth of *grt*/*grt* mice is virtually normal until 2 wk after birth, then is suppressed in the pubertal period; however, it gradually catch up with those of normal mice after approximately one year. This might be because normal rodent chows provide enough thyroid hormone for survival.

Recently, it has been reported that *Tpst2* (-/-) mice on the 129/Sv background were produced by gene targeting (28). *Tpst2* (-/-) mice showed growth retardation at 4-5 wk of age in both sexes, which resembled that in the *grt* mutants. However, their report could not defined the molecular mechanism of the growth retardation. In our report, we determined *Tpst2* as the gene responsible for the severe thyroid dysgenesis, which relates to TSH hyporesponsiveness through positional cloning and transgenic rescue. Furthermore, we show that TPST2 has high degree of substrate specificity for TSHR, and is essential for TSH-TSHR signaling. Therefore, our report has clarified the crucial role of TPST2 for a particular signaling pathway *in vivo* for the first time. The maximum difference in body weight of *Tpst2* (-/-) mice at 5 wk was 20% less compared with normal littermates. In contrast, DW/J-*grt* mice lost up to 50%. It has been reported that thyroid dysgenesis were displayed in C57BL/6 mice with *Pax8* (+/-)/*Ttf1* (+/-) and not in 129Sv strain (29). Therefore, *Tpst2* (-/-) in the 129 background might be resistant to thyroid dysgenesis. These might represent a spectrum of different degrees of severity of the same underlying molecular defect. In human, most cases of thyroid dysgenesis are sporadic, most patients do not display a clear Mendelian transmission, suggesting the existence of several genetic factors which could contribute to the disorder (30). Therefore, this strain differences should help us to identify modifier gene(s) involved in morphogenesis, growth, and differentiation of the thyroid. The other explanation of phenotypic differences between *Tpst2*-null mutants and *grt* mice could be through the dimerization of TPST proteins, because the many sulfotransferases are homo- and/or heterodimer in

solution (31). The physiological significance of dimerization of cytosolic sulfotransferases is not yet clearly defined. However, it has been reported that both TPST1 and TPST2 form homodimer (23, 32). Therefore, if dimerization is essential for the TPST activity, high expression of TPST2 H266Q mutant may result in a significant decrease in TPST1 activity in a dominant-negative manner. This hypothesis is consistent with the result that the dwarfism of *grt* mice is more severe than that of *Tpst2*-null mice.

Hypothyroidism in human is associated with a marked delay in sexual maturation and development (33). The g*rt* males also demonstrate the severe decrease in testicular weight and the numbers of Leydig cells until 5-8 wk age, whereas they gradually acquire normal structure and function of the testis, finally become fertile at 3-4 months (our unpublished data). Thus, we could cross F1 females with *grt* males to obtain backcrossed progenies. *Tpst2* (-/-) mutant males are infertile at 10 wk of age, although spermatogenesis and mating are normal (28). This result is in agreement with previous reports showing a decrease in sperm motility in rats with hypothyroidism (34, 35). However, it has not been described whether *Tpst2* (-/-) males become fertile after 10 wk or are infertile throughout their life. If *Tpst2* knockout is a phenocopy of the *grt* mutant, male infertility can be due to dwarfism/hypothyroidism and may also gradually acquire fertility after 10 wk of age.

Tyrosine sulfation was estimated to occur in approximately 1% of all tyrosines of the eukaryotic proteome (36). In mammals, approximately 60 proteins have already been identified (37). The known tyrosine-sulfated proteins include certain adhesion molecules, G protein-coupled receptors, coagulation factors, and extra-cellular matrixes, and hormones. In some of these proteins, tyrosine sulfation has been shown to be required for optimal binding, but in many, a functional role of tyrosine sulfation still has been unclear. Thus, although many proteins are possible to be the substrates of TPST2, our current study mainly focuses on TSHR. However, the possibility that the *grt* phenotype

reflects a defect in other proteins as the substrate of TPST2 in downstream of the TSHR could not be excluded. Tyrosine sulfation might also affect enzymatic activity, protein transportation, localization and lifespan, possibly being important for hemostasis, chemotaxis, inflammation, and development, as well as for viral and cancer pathogenesis (38).

In the past several decades, the causative genes of inherited hypothyroidism have been identified in humans and rodents. These genes encode transcription factors, hormones, and their receptors. The post-translational processing in the thyroid has been considered to have a significant role in their correct function. However, these processes have not been shown to be mainly involved in the pathogenesis of thyroid until our reports. Although a mutation of TPST has not been found in human disease, our data suggest that TPST2 may be one of the causative genes for thyroid dysgenesis of unknown origin. Further pathological examination of *grt*, *Tpst1* (-/-) and *Tpst2* (-/-) mutants, and further biochemical examination to find a variety of acceptor proteins will provide new insights into the biological function of tyrosine O-sulfation.

## MATERIAL AND METHODS

### Pedigree material and haplotype analysis

(C57BL/6J × DW/J-*grt*)F1 females were mated with DW/J-*grt* males to obtain backcrossed progenies. Linkage analysis was performed using microsatellite markers, *D5Mit314* (MGI:100402), *D5Mit240* (MGI:93321), *D5Mit24* (MGI:93320) and a single nucleotide polymorphism for *Crybb1* (MGI:104992). Mice were maintained under the control of a 12-h light-dark cycle. Research was conducted according to the Guidelines for the Care and Use of Laboratory Animals of both Graduate School of Veterinary Medicine, Hokkaido University and Nagoya City University Medical School. The

experimental protocol was approved by the Institutional Animal Care and Use Committee of both Graduate School of Veterinary Medicine, Hokkaido University and Nagoya City University Medical School.

**Vector constructions**

The murine *Tpst2*-coding regions (accession number: NM_009419) were amplified by PCR to include the native translation initiation sequence of *Tpst2* with primer pair F/R: ggctggccatgggcctgtcggtgc/tttcacttatcgtcgtcatccttgtaatccgaacttcctaggtgtggggaggtgc. The *Tpst1*-coding region (accession number: NM_013837) was amplified with primer pair F/R: acgtgatatccgttgggaagctgaagcaga/tttcacttatcgtcgtcatccttgtaatcctccacttgctccgtctggg. The sequence coding for the FLAG (FL) peptide YKDDDDK was added to their COOH-terminals and the resultant cDNAs were subcloned in pTriEx 1.1 vector (Novagene) to generate pTriEx/*Tpst1*-FL, pTriEx/*Tpst2*-FL and pTriEx/*Tpst2* (H266Q)-FL.

**Genetic complementation test using transgenic mice.**

pTriEx/*Tpst2*-FL was prepared for microinjection by digestion with *Sal*I and *Swa*I, followed by electrophoresis and purification of the linearized DNA. Transgenic mice were generated by pronuclear injection of the linear transgene into fertilized zygotes prepared from BDF1 mice. RT-PCR analysis of transgene expression in various tissues was performed using primer pair: A/B, tctgactgaccgcgttact/ggccgcatcctccgtgggtt. (Fig. 3A). The transgenic hemizygotes on *grt*/+ were mated to generate the transgenic *grt*/*grt* homozygotes. The following primer pair was used for genotyping transgenic animals: primers C/D, cacactcaagtcatccgtcta/cttgcacgtgtatacagctg (Fig. 3A), which cannot amplify

endogenous *Tpst2* locus due to the long distance between both primers. For genotyping *Tpst2* alleles, PCR and subsequent RFLP analysis using *Eco*NI have been used.

**Hormonal analysis**

To test thyroid function of *grt*/*grt* mice with or without transgenes, blood was collected into tubes containing 1 mg/ml EDTA at the final concentration and kept on ice, and plasma was obtained by centrifugation. Serum was removed after centrifugation and stored at -20°C until analysis. Serum levels of free T3 and T4 were measured using ACTIVE Free T3 or T4 EIA kits (Diagnostic System lab). Serum TSH was determined using RAT TSH ELISA kit (Shibayagi) according to the manufacturer's instructions with minor modification to optimize the signal intensity. Serum samples from *grt* mice needed to be diluted 10 times with PBS (pH 7.4). NIDDK-rat TSH RP-3 was used as the standard.

**mRNA detection methods**

For detecting mutations, total RNA from WT and *grt* tissues were extracted with TRizol and reverse-transcribed with a RT-PCR kit according to the manufacturer's instructions (Invitrogen). For the quantitation of thyroid-specific gene, each mRNA level of murine *Tshr*, *Nis*, *Tpo*, *Tgn* and *Pax8* was determined using specific primers as follows:

*Tshr*-F/R: acctctcttacccgagccactgc/tccaggcgcatggcgaaggtgat,

*Nis*-F/R: cattcccggatcaacctgatggact/tttagagatgaaaaccagcttccg,

*Tgn*-F/R: cagaccgtagtggggctgatgtg/gcatagtcgtctgtggagtgct,

*Tpo*-F/R: attgggaagcagatgaaggctct/gggtgtgtcagatctgcacact,

*Pax*8-F/R: gaatattctggcaatgcctacag/tgtacaccctcagactcatctc,

*Actb*-F/R: tgatggtgggaatgggtcag/gaaggctggaaaagagcctc.

For the quantitation of mRNA for *Tpst1* and *Tpst2*, RT-PCR was performed for both genes in the same tube on each sample concomitantly. Primers were designed so that the sense primer was shared for amplification of both *Tpst1* and *Tpst2* genes. The primers used were: tcacggccatgtcttgtaag (*Tpst1* antisense); gtgctgttctggttcacctg (*Tpst2* antisense) and tgcaggccttcattctggaggtgat (common sense). To determine the optimum PCR amplification conditions in the linear range, three amounts of cDNA (10, 50 and 100 ng) were tested for each sample. Each reaction tube contained 2 µl of 10 × Ex Taq buffer (Takara), 2 µl of 2.5 mM dNTP mixture, 0.1 µl of Ex Taq polymerase (Takara), 1 µl (4 µM) of each antisense primer and 2 µl (4 µM) of common sense primer. The total reaction volume was 20 µl. PCR consisted of denaturation at 94°C for 3 min, 25 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel. All assays were performed in duplicate.

**Cell culture, plasmids and expression of transgenes**

*Tpst1*-FL, *Tpst2* (H266Q)-FL, *Tpst1*-FL and *TSHR* cDNA were expressed by the ViraPower Lentivirus expression system (Invitrogen). The primary skin-derived fibroblasts from *grt* mice were grown in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37°C. The expression of these genes were detected with semiquantitative RT-PCR.

**In *vitro* sulfation of peptide arrays**

pTriEx1.1-*Tpst1*-FL, *Tpst2*-FL, and mutant *Tpst2*-FL plasmids were transfected into 1 ×

$10^7$ HEK293T cells using Lipofectamine 2000 (Invitrogen). After 48 h, each cell was homogenized in 500 µl of ice-cold 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, 2 mg/ml aprotinin and 2 mg/ml leupeputin. Expression of each TPST protein in cell lysates (20 µg) was detected by Western blot analysis using a FLAG antibody (Sigma). TPST activity was determined by measuring the transfer of $^{35}$S-sulfate from $^{35}$S-PAPS to Fmoc-based membrane-immobilized 15-mer peptides (39). Peptide arrays, $^{35}$S-PAPS and 50 ml of TPST lysate were accomplished by incubating 50 µl of the buffer {50 mM piperazine-1,4-bis(2-ethanesulfonic acid), pH 6.9, 0.4 mM EDTA, 1 mM Mg-acetate, 200 mM NaCl, 1 mg/ml BSA, 10 mM dithiothreitol} for 1 h at 37°C. After incubation, peptide arrays were washed with 0.1 M Tris-HCl (pH 8.0) twice. The relative amounts of incorporated radioactivity were visualized and quantified with a BAS2500 Bio-Imaging analyzer (Fuji Film). All assays were performed in duplicate.

**Measurement of cAMP**

Amount of cAMP was determined according to the instruction manual of the cAMP enzyme immunoassay kit (Cayman chemical). In brief, $2 \times 10^5$ cells of each clone were seeded in a 6-well tissue culture dish. The cells were starved of serum for 18 h, then serum-free medium and bovine TSH (10 mU/ml) was added and incubated for 60 min. To assess the value for nonspecific stimulation of intracellular cAMP levels, cells were incubated with 10 mM forskolin and 0.2 mM 3-isobutyl-1-methylxanthine (Sigma). Each experiment was repeated 2 times.

**ACKNOWLEDGEMENTS**

We greatly thank Dr. Nagayama Y (Nagasaki University, Japan) for human TSHR cDNA.

We also wish to acknowledge lab members for helpful discussions, especially thanks to A.Y Simon for comments on the manuscript.

## REFERENCES


1. Kopp P 2002 Perspective: genetic defects in the etiology of congenital hypothyroidism. Endocrinology 143:2019-2024
2. Refetoff S 2003 Resistance to thyrotropin. J Endocrinol Invest 26:770-779
3. Lafferty A, Schwarz S, DeFelice M, von Deimling A, van Landeghem F, DiLauro R, Gruters A 2002 Choreoathetosis, hypothyroidism, and pulmonary alterations due to human NKX2–1 haploinsufficiency. J Clin Invest 109, 475–480.
4. Pohlenz J, Dumitrescu A, Zundel D, Martine U, Schonberger W, Koo E, Weiss RE, Cohen RN, Kimura S, Refetoff S 2002 Partial deficiency of thyroid transcription factor 1 produces predominantly neurological defects in humans and mice. J Clin Invest 109, 469–473.
5. Clifton-Bligh RJ, Wentworth JM, Heinz P, Crisp MS, John R, Lazarus JH, Ludgate M, Chatterjee VK 1998 Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. Nat Genet 19:399-401
6. Macchia PE, Lapi P, Krude H, Pirro MT, Missero C, Chiovato L, Souabni A, Baserga M, Tassi V, Pinchera A, Fenzi G, Gruters A, Busslinger M, Di Lauro R 1998 PAX8 mutations associated with congenital hypothyroidism caused by thyroid dysgenesis. Nat Genet 19:83-86
7. Stein SA, Oates EL, Hall CR, Grumbles RM, Fernandez LM, Taylor NA, Puett D, Jin S 1994 Identification of a point mutation in the thyrotropin receptor of the hyt/hyt hypothyroid mouse. Mol Endocrinol 8:129-138
8. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez

FJ 1996 The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev 10:60-69

9. De Felice M, Ovitt C, Biffali E, Rodriguez-Mallon A, Arra C, Anastassiadis K, Macchia PE, Mattei MG, Mariano A, Scholer H, Macchia V, Di Lauro R 1998 A mouse model for hereditary thyroid dysgenesis and cleft palate. Nat Genet 19:395-398

10. Mansouri A, Chowdhury K, Gruss P 1998 Follicular cells of the thyroid gland require Pax8 gene function. Nat Genet 19:87-90

11. Yoshida T, Yamanaka K, Atsumi S, Tsumura H, Sasaki R, Tomita K, Ishikawa E, Ozawa H, Watanabe K, Totsuka T 1994 A novel hypothyroid 'growth-retarded' mouse derived from Snell's dwarf mouse. J Endocrinol 142:435-446

12. Tomita K, Yoshida T, Morita J, Atsumi S, Totsuka T 1995 In vivo responsiveness of thyroid glands to TSH in normal and novel 'growth-retarded' mice: transient elevation in normal mice and impairment in 'growth-retarded' mice. J Endocrinol 144:209-214

13. Kobayashi K, Yamamoto H, Kobayashi T, Machida T 2001 Responses of the thyroid gland to TSH and other thyroid stimulators in the growth retarded (grt) mouse. Zool Sci 18:955–961

14. Agui T, Miyamoto T, Tsumura H, Yoshida T 1997 Mapping of the grt locus to mouse chromosome 5. Mamm Genome 8:944

15. Costagliola S, Panneels V, Bonomi M, Koch J, Many MC, Smits G, Vassart G 2002 Tyrosine sulfation is required for agonist recognition by glycoprotein hormone receptors. EMBO J 21:504-513

16. Postiglione MP, Parlato R, Rodriguez-Mallon A, Rosica A, Mithbaokar P, Maresca M, Marians RC, Davies TF, Zannini MS, De Felice M, Di Lauro R 2002

Role of the thyroid-stimulating hormone receptor signaling in development and differentiation of the thyroid gland. Proc Natl Acad Sci USA 99:15462-15467

17. Marians RC, Ng L, Blair HC, Unger P, Graves PN, Davies TF 2002 Defining thyrotropin-dependent and -independent steps of thyroid hormone synthesis by using thyrotropin receptor-null mice. Proc Natl Acad Sci USA 99:15776-15781

18. Cheng JM, Ding M, Miyamoto T, Fujimori O, Agui T 2002 Investigation of post-transcriptional events of the thyroglobulin in the thyroid gland of the hypothyroid growth-retarded mouse DW/J-grt. Nagoya Med J 45:133-143

19. Dong JF, Li CQ, Lopez JA 1994 Tyrosine sulfation of the glycoprotein Ib-IX complex: identification of sulfated residues and effect on ligand binding. Biochemistry 33:13946-13953

20. Leyte A, van Schijndel HB, Niehrs C, Huttner WB, Verbeet MP, Mertens K, van Mourik JA 1991 Sulfation of Tyr1680 of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. J Biol Chem 266:740-746

21. Vargas F, Frerot O, Tuong MD, Zuzel K, Rose C, Schwartz JC 1985 Sulfation and desulfation of cerebral cholecystokinin. Ann N Y Acad Sci 448:110-120

22. Pouyani T, Seed B 1995 PSGL-1 recognition of P-selectin is controlled by a tyrosine sulfation consensus at the PSGL-1 amino terminus. Cell 83:333-343

23. Ouyang Y, Lane WS, Moore KL 1998 Tyrosylprotein sulfotransferase: purification and molecular cloning of an enzyme that catalyzes tyrosine O-sulfation, a common posttranslational modification of eukaryotic proteins. Proc Natl Acad Sci USA 95:2896-2901

24. Ouyang YB, Moore KL 1998 Molecular cloning and expression of human and mouse tyrosylprotein sulfotransferase-2 and a tyrosylprotein sulfotransferase homologue in Caenorhabditis elegans. J Biol Chem 273:24770-24774

25. Seibert C, Cadene M, Sanfiz A, Chait BT, Sakmar TP 2002 Tyrosine sulfation of CCR5 N-terminal peptide by tyrosylprotein sulfotransferases 1 and 2 follows a discrete pattern and temporal sequence. Proc Natl Acad Sci USA 99:11031-11036

26. Ouyang YB, Crawley JT, Aston CE, Moore KL 2002 Reduced body weight and increased postimplantation fetal death in tyrosylprotein sulfotransferase-1-deficient mice. J Biol Chem 277:23781-23787

27. Dumont JE, Vassart G 1995 Thyroid regulation. In: DeGroot (ed) Endocrinology:543–559

28. Borghei A, Ouyang YB, Westmuckett AD, Marcello MR, Landel CP, Evans JP, Moore KL 2006 Targeted disruption of tyrosylprotein sulfotransferase-2, an enzyme that catalyzes post-translational protein tyrosine O-sulfation, causes male infertility. J Biol Chem 281:9423-9431

29. Amendola E, De Luca P, Macchia PE, Terracciano D, Rosica A, Chiappetta G, Kimura S, Mansouri A, Affuso A, Arra C, Macchia V, Di Lauro R, De Felice M 2005 A mouse model demonstrates a multigenic origin of congenital hypothyroidism. Endocrinology 146:5038-5047

30. Castanet M, Lyonnet S, Bonaiti-Pellie C, Polak M, Czernichow P, Leger J 2000 Familial forms of thyroid dysgenesis among infants with congenital hypothyroidism. N Engl J Med 343:441-442

31. Strott CA 2000 Sulfonation and molecular action. Endocr Rev. 27:703-732.

32. Beisswanger R, Corbeil D, Vannier C, Thiele C, Dohrmann U, Kellner R, Ashman K, Niehrs C, Huttner WB 1998 Existence of distinct tyrosylprotein sulfotransferase genes: molecular characterization of tyrosylprotein sulfotransferase-2. Proc Natl Acad Sci USA 95:11134-11139

33. Jannini EA, Ulisse S, D'Armiento M 1995 Thyroid hormone and male gonadal function. Endocr Rev. 16: 443-459

34. Kumar PN, Aruldhas MM, Juneja SC. 1994 Influence of hypothyroidism induced at prepuberty on epididymal lipids and the number and motility of spermatozoa in rats. Int J Androl. 17:262-270

35. Chandrasekhar Y, Holland MK, D'Occhio MJ, Setchell BP. 1985 Spermatogenesis, seminal characteristics and reproductive hormone levels in mature rams with induced hypothyroidism and hyperthyroidism. J Endocrinol. 105:39-46

36. Kehoe JW, Bertozzi CR 2000 Tyrosine sulfation: a modulator of extracellular protein-protein interactions. Chem Biol 7:R57-61

37. Moore KL 2003 The biology and enzymology of protein tyrosine O-sulfation. J Biol Chem 278:24243-24246

38. Huttner WB 1988 Tyrosine sulfation and the secretory pathway. Annu Rev Physiol 50:363-376

39. Reineke U, Volkmer-Engert R, Schneider-Mergener J 2001 Applications of peptide arrays prepared by the SPOT-technology. Curr Opin Biotechnol 12:59-64

## FIGURE LEGENDS

**Figure 1.**

(A) Haplotype map of (C57BL/6J × DW/J-*grt*)F1 × DW/J-*grt* backcrossed progenies. Three microsatellite markers shown from chromosome 5 are those that have been typed on 1,084 backcross DNAs. Open squares represent the homozygous for the DW allele; black squares represent the heterozygous for B6 and DW alleles. The astarisks indicate the affected mice. Values at the bottom of the figure are the number of progenies.

(B) Transcript map of the critical region for the *grt* locus. All genes were screened for mutations. Transcriptional orientations are shown by arrows. Cen, centromeric; Tel, telomeric. In the genomic structure of the mouse *Tpst2* gene, exonic sequences that contribute to coding regions are boxed and shaded in stripes, while untranslated regions are open-boxed. The sulfotransferase domain of TPST2 protein is boxed in gray. A transversion of C to G is observed at position 798 in the affected mice, which causes an amino acid substitution (H266Q).

**Figure 2.**

(A) Multiple alignment of amino acid sequences of TPST1 and 2 in various organisms. *C. elegans* and *D. melanogaster* have only one TPST protein. An arrow indicates highly conserved histidine residue among all proteins in TPST family.

(B) The 1115 bp PCR product from genome was digested with restriction endonuclease *Eco*NI and genetic polymorphism was detected by PCR-RFLP. The wild type fragment was not digested, whereas two fragments of 597 and 518 bp were produced upon *Eco*NI digestion of the *grt* sequence. The *grt*/+ heterozygous shows both band patterns.

**Figure 3.**

(A) The *Tpst2* amplicon was inserted downstream of the CAG promoter and its intron and upstream to the simian virus 40 polyadenylation signal. Arrows indicate the location and orientation of two primer pairs used. Primers A and B were used for detection of the expression of transgene with RT-PCR, whereas primers C and D for genotyping with genomic PCR.

(B) *Tpst2* transgene is expressed in thyroid, brain, kidney, ovary and testis, but not in liver and spleen of transgenic mice.

(C) Transgenic rescue of *grt* phenotypes. Typical *grt*/*grt* animals with (+) or without (-) the CAG-*Tpst2* transgene (TG) at 5 wk of age.

(D) Comparison of weights among each genotype at 5 wk of age. 1; *grt*/*grt* with TG (n = 20), 2; *grt*/*grt* (n = 17), 3; *grt*/+ with TG (n = 30), 4; *grt*/+ (n = 30) 5; WT (+/+) with TG (n = 16), 6; WT (+/+) (n = 14). The weight data were presented by combining those of male and female, because no difference was found between them at 5 wk of age. An asterisk indicates a significant difference against other groups ($P < 0.0001$).

(E) Serum TSH, T3 and T4 values of WT (+/+) (n = 8), *grt*/*grt* (n = 8), and transgenic *grt*/*grt* (TG-*grt*) mice (n = 8). The values are presented as means ± SEM. They were analyzed for statistical significance with Student's t-test and $P < 0.005$ was considered to be significant (*).

**Figure 4.**

(A) Histological analysis. Thyroid glands from 8-wk WT, *grt*/*grt* and TG-*grt* mice were fixed, sectioned, and stained with haematoxylin and eosin (HE) and PAS. The small boxes at 4 × magnification indicate thyroid glands of each genotype. The *grt*/*grt* thyroid is significantly smaller in size. High magnification (40 ×) HE staining showed that the *grt*/*grt* thyroid is marked by a reduction in follicular cell number and replacement of some hypoplastic portion by adipose tissue (arrow). In PAS staining of *grt*/*grt* thyroid gland at

40 × magnification, colloid staining was not present in some lumens, suggesting a decrease in thyroglobulin production and storage. On the other hand, hypogenesis of the thyroids was completely rescued in transgenic *grt*/*grt* mice. (B) Semiquantitative RT-PCR analysis of thyroid-specific genes, *Tgn*, *Pax8*, *Nis*, *Tpo* and *Tshr* from two WT, *grt* and TG-*grt* thyroids each. Beta-actin (*Actb*) was used as internal control. *Tshr* and *Nis* levels were significantly reduced in *grt*/*grt* thyroids.

**Figure 5.**

(A) TPST catalyzes the transfer of sulfate from the universal sulfate donor PAPS to the hydroxyl group of a peptidyltyrosine residue to form a tyrosine O-sulfate ester.

(B) Equal amounts of cell extracts were extracted from HEK293T cells, subjected to Western blot analysis with a FLAG antibody. The bands of approximately 55kDa and 50kDa detected on the blots can be identified as TPST2 (TP2) and TPST1 (TP1), respectively. Actb was used as a loading control.

(C) Tyrosine-sulfation on the peptide array by TPST1 and 2. Each spot contains the 15-mer peptide substrates, GP1BA, CF-8, CCK, FSHR, TSHR, and PSGL1. The same input of each TPST was used for in this assay. The tyrosine sulfation of peptides was revealed by the incorporation of $^{35}S$ into the substrates by autoradiography. The putative sulfation sites are indicated as gray characters. TSHR (Mu) and PSGL1 (Mu) are negative control peptides, in which a mutation from tyrosine to phenylalanine was introduced. TP1, TP2 and TP2 (H266Q) indicate TPST1, TPST2 and mutant TPST2 (H266Q), respectively.

(D) The quantitative evaluation of Fig. 5C. The relative incorporation of $^{35}S$ into peptides was evaluated.

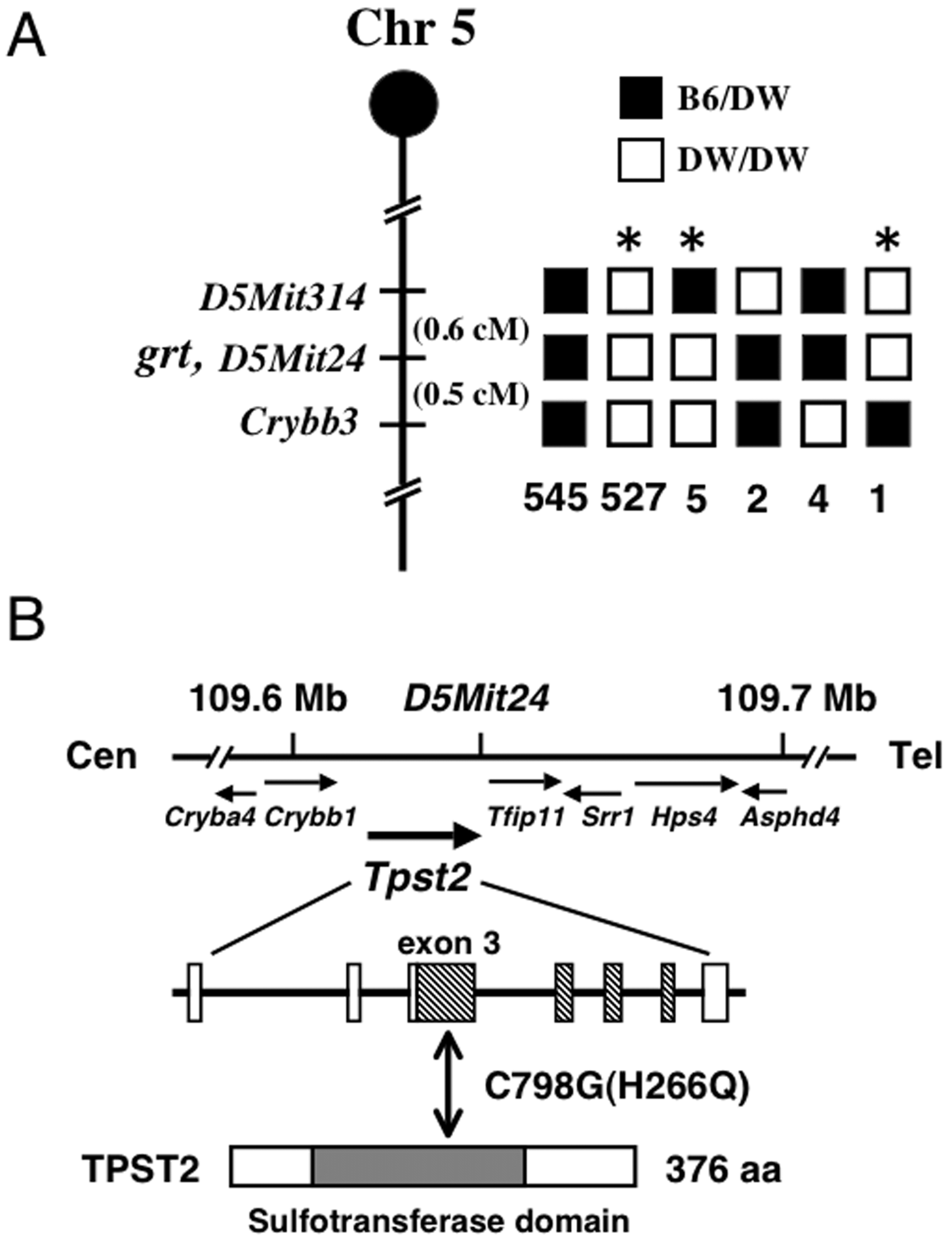
**Figure 6.**

(A) *Tpst1* and *Tpst2* expression in various mouse tissues. Semiquantitative RT-PCR analysis shows ubiquitous expression of both *Tpst1* and *Tpst2*. *Tpst1* (563 bp) and *Tpst2* (741 bp) were concomitantly amplified in the same tube of each sample. Thyroid and thyrocyte cell lines expressed both genes.

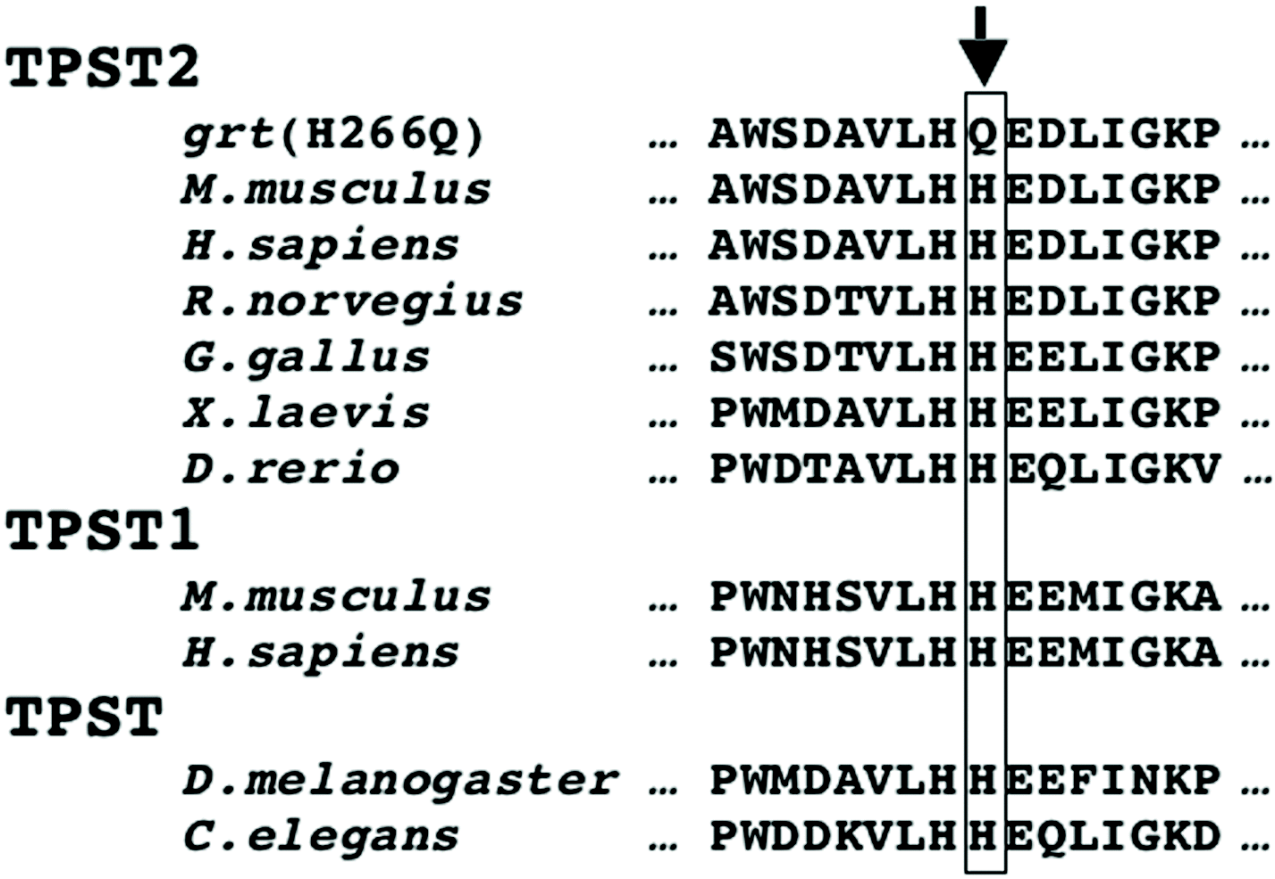
(B) Rescue of TSH-mediated cAMP production in *grt*/*grt* fibroblasts by TPST2 restoration in response to TSH stimulation. Plus (+) or minus (-) indicates the presence or absence of TSH (10 mU/ml) in the medium. Lentiviruses encoding the TSHR and each TP1, TP2 and TP2 (H266Q) were infected in *grt* cells, alone or in combination. Each bar represents the mean ± SEM.

(C) The expression of each gene in *grt*/*grt* fibroblasts was confirmed by RT-PCR. The expressions of transgenes increased to approximately two-three fold more than that of endogenous *Tpst* mRNAs.

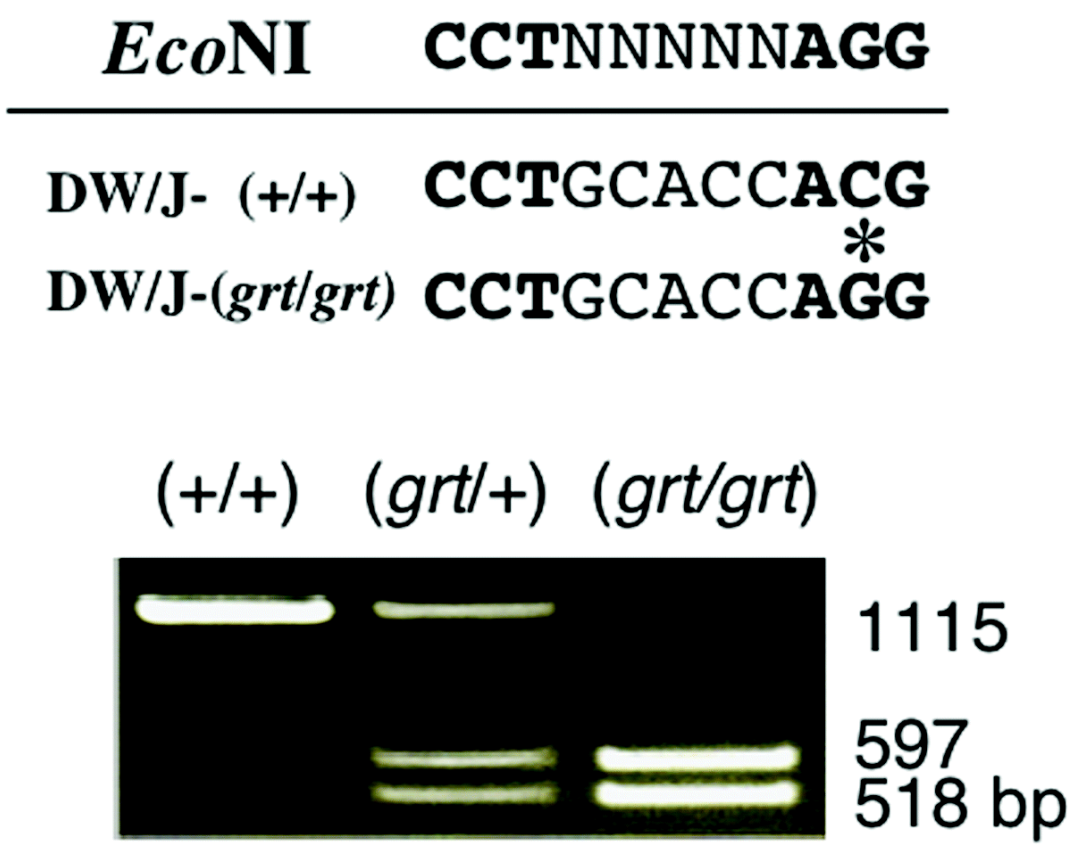
(D) The expression of TPST proteins in *grt*/*grt* fibroblasts were evaluated by Western blotting with FLAG antibody. Mock indicates the untransfected-*grt*/*grt* fibroblasts. Each protein was expressed at similar level in the transfected cells.

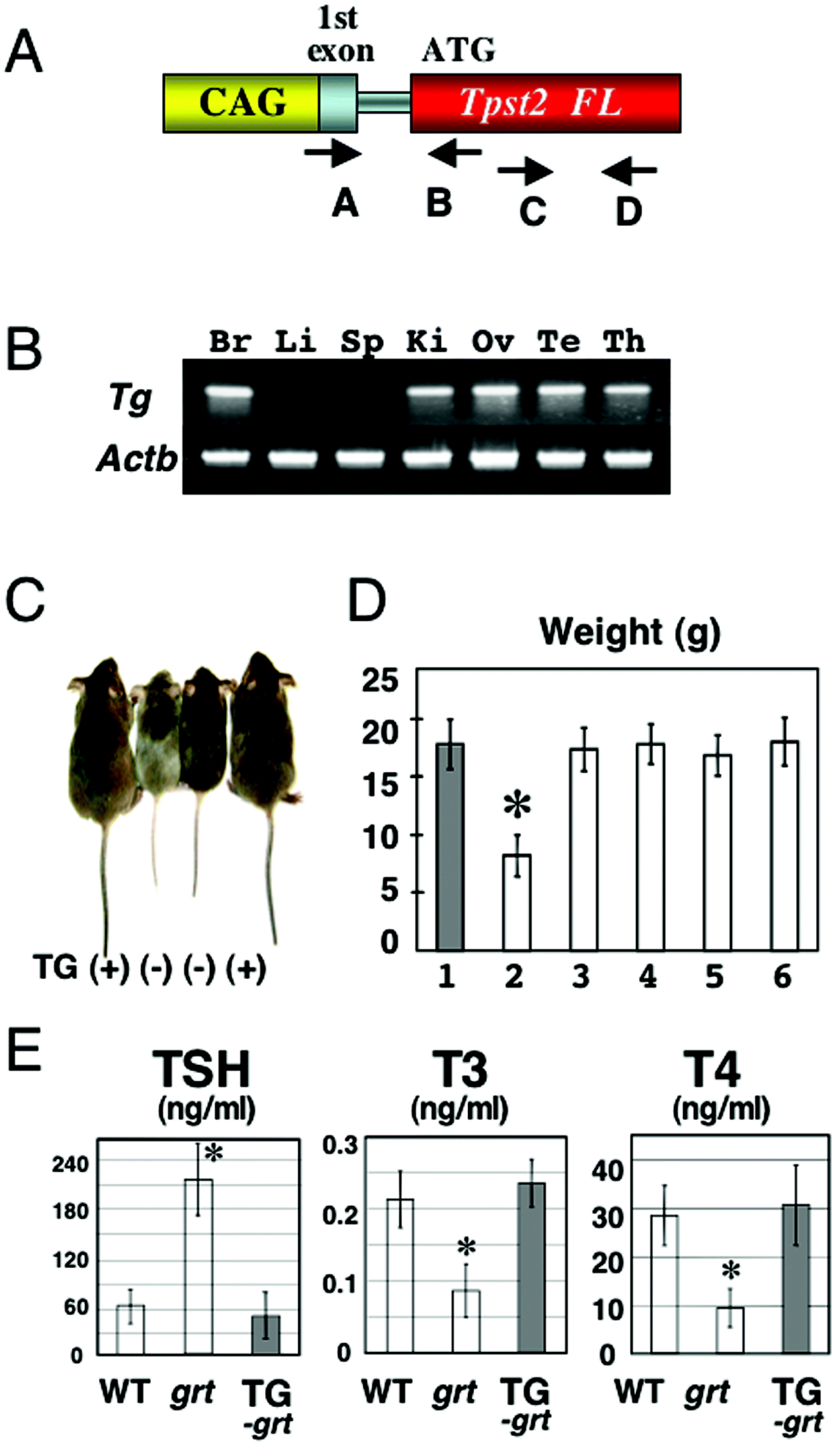

A
Chr 5
B6/DW
DW/DW
D5Mit314
(0.6 cM)
grt, D5Mit24
(0.5 cM)
Crybb3
545 527 5 2 4 1
B
109.6 Mb
D5Mit24
109.7 Mb
Cen
Tel
Cryba4 Crybb1
Tfip11 Srr1 Hps4 Asphd4
Tpst2
exon 3
C798G(H266Q)
TPST2
376 aa
Sulfotransferase domain

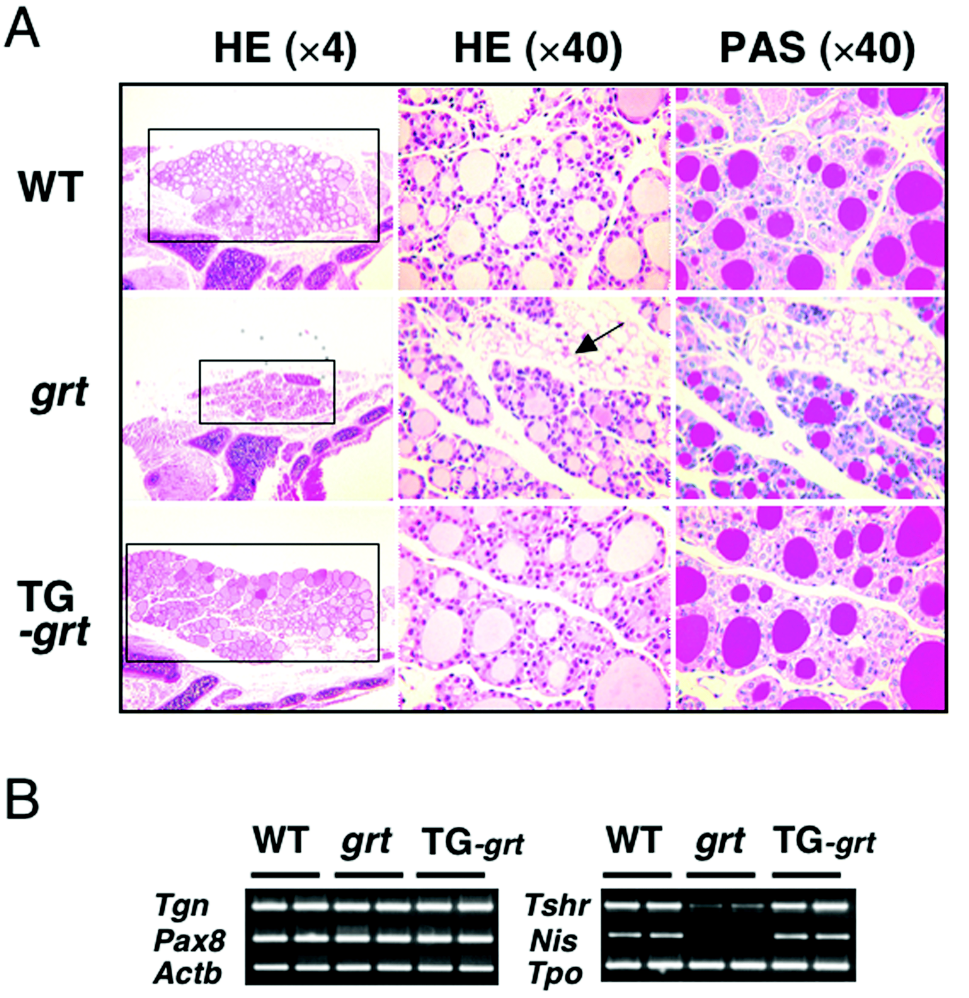
A

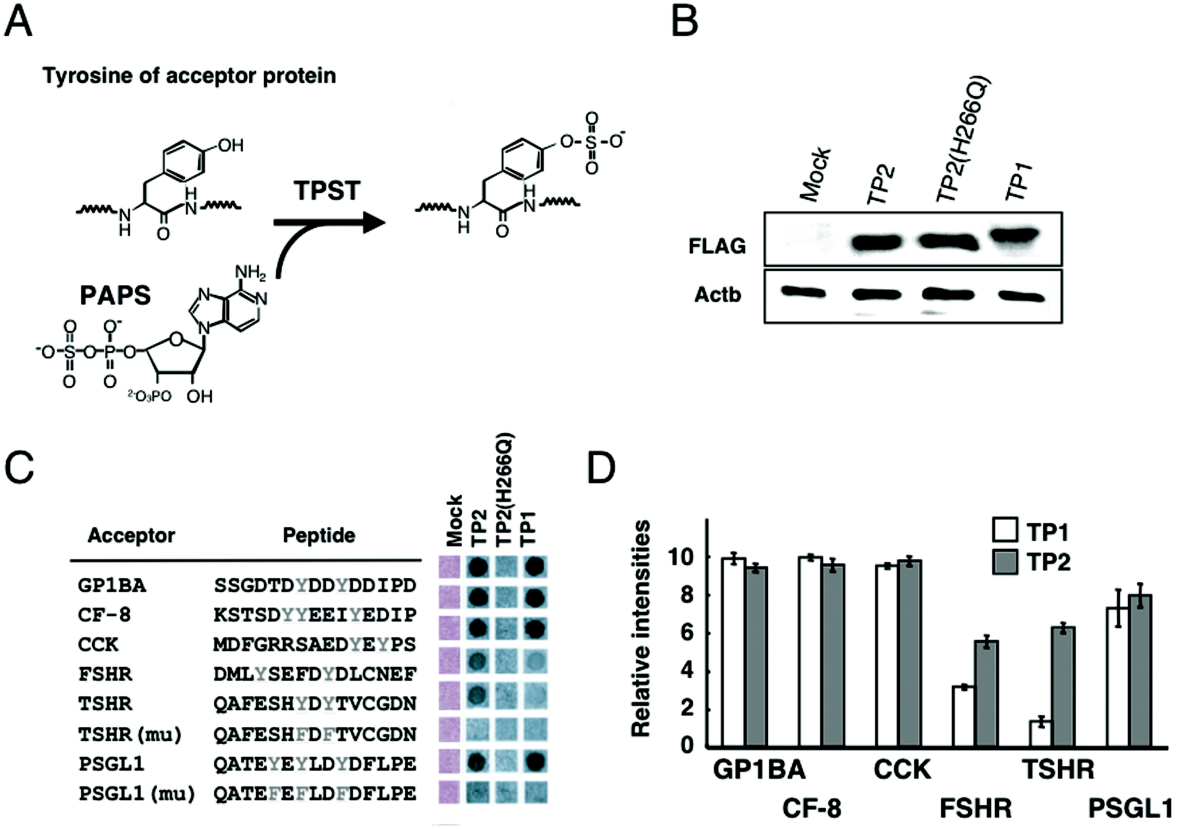

TPST2
grt(H266Q) … AWSDAVLHQEDLIGKP …
M.musculus … AWSDAVLHHEDLIGKP …
H.sapiens … AWSDAVLHHEDLIGKP …
R.norvegius … AWSDTVLHHEDLIGKP …
G.gallus … SWSDTVLHHEELIGKP …
X.laevis … PWMDAVLHHEELIGKP …
D.rerio … PWDTAVLHHEQLIGKV …
TPST1
M.musculus … PWNHSVLHHEEMIGKA …
H.sapiens … PWNHSVLHHEEMIGKA …
TPST
D.melanogaster … PWMDAVLHHEEFINKP …
C.elegans … PWDDKVLHHEQLIGKD …

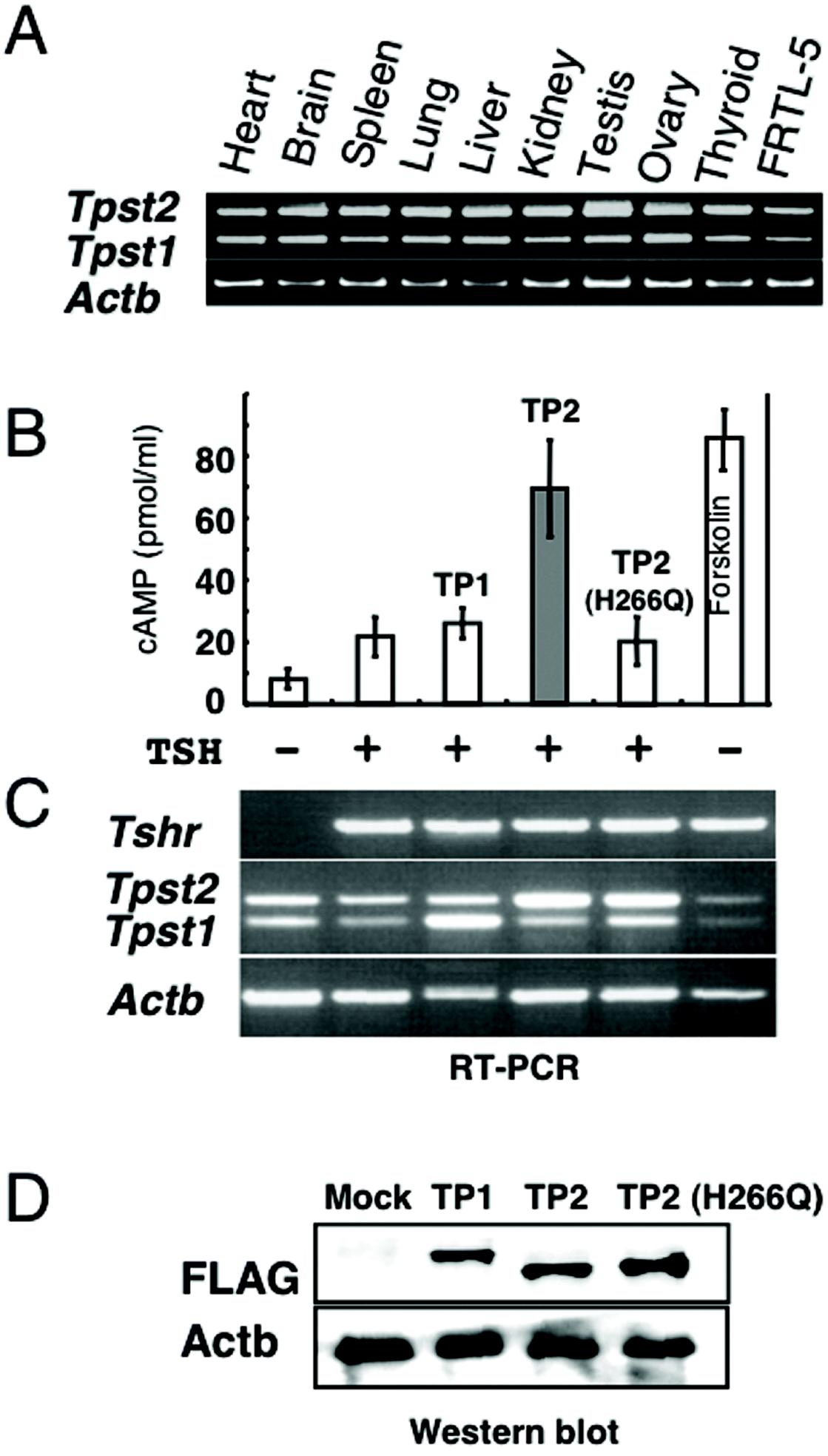
B


EcoNI CCTNNNNNNAGG
DW/J- (+/+) CCTGCACCACG
*
DW/J-(grt/grt) CCTGCACCAGG
(+/+) (grt/+) (grt/grt)
1115
597
518 bp


A
1st exon
ATG
CAG
Tpst2 FL
A
B
C
D
B
Br Li Sp Ki Ov Te Th
Tg
Actb
C
TG (+) (-) (-) (+)
D
Weight (g)
25
20
15
10
5
0
1 2 3 4 5 6
E
TSH (ng/ml)
240
180
120
60
0
WT grt TG -grt
T3 (ng/ml)
0.3
0.2
0.1
0
WT grt TG -grt
T4 (ng/ml)
40
30
20
10
0
WT grt TG -grt


A
HE (×4)
HE (×40)
PAS (×40)
WT
grt
TG
-grt
B
WT
grt
TG-grt
WT
grt
TG-grt
Tgn
Pax8
Actb
Tshr
Nis
Tpo


A
Tyrosine of acceptor protein
TPST
PAPS
B
Mock
TP2
TP2(H266Q)
TP1
FLAG
Actb
C
Acceptor
Peptide
Mock
TP2
TP2(H266Q)
TP1
GP1BA SSGDTDYDDYDDIPD
CF-8 KSTSDYYEEIYEDIP
CCK MDFGRRSAEDYEYPS
FSHR DMLYSEFDYDLCNEF
TSHR QAFESHYDYTVCGDN
TSHR (mu) QAFESHFDFTVCGDN
PSGL1 QATEYEYLDYDFLPE
PSGL1 (mu) QATEFEFLDFDFLPE
D
Relative intensities
TP1
TP2
0
2
4
6
8
10
GP1BA
CF-8
CCK
FSHR
TSHR
PSGL1


A
Heart
Brain
Spleen
Lung
Liver
Kidney
Testis
Ovary
Thyroid
FRTL-5
Tpst2
Tpst1
Actb
B
cAMP (pmol/ml)
0
20
40
60
80
TP1
TP2
TP2 (H266Q)
Forskolin
TSH
- + + + + -
C
Tshr
Tpst2
Tpst1
Actb
RT-PCR
D
Mock
TP1
TP2
TP2 (H266Q)
FLAG
Actb
Western blot