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| Title | A mutation in Tpst2 encoding tyrosylprotein sulfotransferase causes dwarfism associated with hypothyroidism. |
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| Citation | Molecular Endocrinology, 21(7), 1713-1721 https://doi.org/10.1210/me.2007-0040 |
| Issue Date | 2007-07 |
| Doc URL | http://hdl.handle.net/2115/26433 |
| Type | article (author version) |
| File Information | ME21-7.pdf |



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1 **Research Article for Molecular Endocrinology**

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3 Full title

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

7

8 Abbreviated title: *Tpst2* mutation causes hypothyroidism

9

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29

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

32

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

39

40 Disclosure summary: The authors have nothing to disclose.

1 **ABSTRACT**

2

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

13

14 **INTRODUCTION**

15

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

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

5 The growth-retarded (*grt*) mouse is a spontaneous mutant exhibiting severe
6 primary hypothyroidism and dwarfism controlled by a single recessive locus. The gene
7 responsible for the *grt* has not yet been cloned, nor has its molecular mode of action been
8 determined. However, homozygous *grt* mice exhibit low concentrations of serum T3 and
9 T4, and a compensatory elevation in the level of circulating TSH demonstrates a normal
10 pituitary response (11). In fact, measurements of several additional pituitary hormones
11 revealed no significant differences between normal and *grt* mice. Growth retardation is
12 recovered by the administration of T3, indicating that the *grt* mutation does not affect
13 anterior pituitary function (12). The thyroid gland of *grt* mice is defective in TSH
14 responsiveness, particularly in signaling pathway involving TSH, TSHR, G protein, and
15 adenylate cyclase (13). These results suggest that the growth-retarded phenotype is due to
16 an impairment of thyroid glands in the production of thyroid hormone or a response to
17 TSH. To determine the molecular mechanism of the *grt* phenotype, we mapped the *grt*
18 locus responsible for dwarfism in a 59-centimorgan (cM) region of mouse chromosome 5
19 (14). No genes responsible for human and rodent dwarfism or thyroid disease have been
20 mapped to this region. In this report, we narrowed the *grt* locus to a < 0.1 cM region using
21 1,084 backcross progenies, sequenced candidate genes located in this region, and finally
22 identified a causative mutation in the tyrosylprotein sulfotransferase 2 (*Tpst2*) gene.
23 Recent papers reported that the posttranslational modification by tyrosine sulfation
24 regulates many important protein-protein interactions and modulates binding affinity and
25 specificity. Mutational analysis has demonstrated that TSHR is sulfated on tyrosines in a
26 motif that is essential for high-affinity binding of TSH and activation of the receptor (15).

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

3 4 **RESULTS**

5 6 **Identification of *Tpst2* mutation in *grt* mutants**

7
8 To identify the gene underlying the *grt* mutant phenotype, we created a
9 high-resolution genetic map of the *grt* locus using 1,084 backcross mice, and analyzed
10 their phenotypes of homozygotes (*grt/grt*) or heterozygotes (*grt/+*) by weighing mice at 5
11 wk of age. Homozygotes of both sexes were small with shortened limbs and tails and
12 weighed approximately 40-60% less of the heterozygotes or wild-type (WT) mice. None
13 of the phenotypes of 1,084 backcross progenies had recombination with a microsatellite
14 marker, *D5Mit24*, indicating a linkage of < 0.1 cM (Fig. 1A). As shown in Fig. 1B, the
15 gene order was from *Cryba4* to *Asphd2* covering the critical region. In this region, the
16 mouse genome database disclosed seven genes, *Cryba4*, *Crybb1*, *Tpst2*, *Tfip11*, *Srr1*,
17 *Hps4* and *Asphd2*. We examined the expression of these possible candidate genes in
18 multiple tissues including thyroid with RT-PCR; there was no qualitative difference
19 observed (data not shown). We next compared the nucleotide sequences of these genes
20 between normal and affected mice, and identified a distinct mutation in *Tpst2*. The
21 mutation is a single missense mutation with a transversion of C at nucleotide 798 to G,
22 leading to the replacement of a highly conserved histidine with a glutamine at codon 266
23 in the sulfotransferase domain (Fig. 1B). Sequence alignment of TPST1 and 2 reveals that
24 the histidine residue was highly conserved among TPST family proteins of diverse
25 species (Fig. 2A). In addition, phenotypically affected mice were homozygous for mutant
26 alleles with PCR-restriction fragment length polymorphism (RFLP) genotyping assay,

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

5

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

7

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

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

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

21 22 **Loss of enzymatic activity of TPST2 H266Q mutation**

23
24 TPST catalyzes the transfer of a sulfuryl group from the universal sulfation
25 substrate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), to a tyrosyl residue within
26 acidic motifs of proteins that transit the Golgi (Fig. 5A). To characterize the enzymatic

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

20

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

22

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

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

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

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

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

9

10 DISCUSSION

11

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

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

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

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

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

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

1 reflects a defect in other proteins as the substrate of TPST2 in downstream of the TSHR
2 could not be excluded. Tyrosine sulfation might also affect enzymatic activity, protein
3 transportation, localization and lifespan, possibly being important for hemostasis,
4 chemotaxis, inflammation, and development, as well as for viral and cancer pathogenesis
5 (38).

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

16

17 MATERIAL AND METHODS

18

19 Pedigree material and haplotype analysis

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

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

4

5 **Vector constructions**

6 The murine *Tpst2*-coding regions (accession number: NM_009419) were amplified by
7 PCR to include the native translation initiation sequence of *Tpst2* with primer pair F/R:
8 ggctggccatgggctgtcgggtc/ttcacttatcgtcgtcatccttgtaatccgaactcctaggtgtggggaggtgc. The
9 *Tpst1*-coding region (accession number: NM_013837) was amplified with primer pair
10 F/R: acgtgatatccgttgggaagctgaagcaga/ttcacttatcgtcgtcatccttgtaatcctccacttgctccgtctggg.
11 The sequence coding for the FLAG (FL) peptide YKDDDDK was added to their
12 COOH-terminals and the resultant cDNAs were subcloned in pTriEx 1.1 vector
13 (Novagene) to generate pTriEx/*Tpst1*-FL, pTriEx/*Tpst2*-FL and pTriEx/*Tpst2*
14 (H266Q)-FL.

15

16 **Genetic complementation test using transgenic mice.**

17 pTriEx/*Tpst2*-FL was prepared for microinjection by digestion with *SalI* and *SwaI*,
18 followed by electrophoresis and purification of the linearized DNA. Transgenic mice
19 were generated by pronuclear injection of the linear transgene into fertilized zygotes
20 prepared from BDF1 mice. RT-PCR analysis of transgene expression in various tissues
21 was performed using primer pair: A/B, tctgactgaccgcgttact/ggccgcacatcctccgtgggtt. (Fig.
22 3A). The transgenic hemizygotes on *grt*/+ were mated to generate the transgenic *grt*/*grt*
23 homozygotes. The following primer pair was used for genotyping transgenic animals:
24 primers C/D, cacactcaagtcacgtctca/cttgacagtgatatacagctg (Fig. 3A), which cannot amplify

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

3

4 **Hormonal analysis**

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

14

15 **mRNA detection methods**

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

20 *Tshr*-F/R: acctcttaccgagccactgc/tccaggecatggcgaagtgat,

21 *Nis*-F/R: cattcccggatcaacctgatggact/ttagagatgaaaaccagcttccg,

22 *Tgn*-F/R: cagaccgtagtggggctgatgtg/gcatagtcgtctgtggagtgt,

1 *Tpo*-F/R: attggaagcagatgaaggctct/gggtgtgcagatctgcacact,

2 *Pax8*-F/R: gaatattctggcaatgcctacag/tgtacacccctcagactcatctc,

3 *Actb*-F/R: tgatgtgggaatgggtcag/gaaggctggaaaagagcctc.

4 For the quantitation of mRNA for *Tpst1* and *Tpst2*, RT-PCR was performed for both
5 genes in the same tube on each sample concomitantly. Primers were designed so that the
6 sense primer was shared for amplification of both *Tpst1* and *Tpst2* genes. The primers
7 used were: tcacggccatgtcttgaag (*Tpst1* antisense); gtgctgttctggttcacctg (*Tpst2* antisense)
8 and tgcaggccttcattctggaggtgat (common sense). To determine the optimum PCR
9 amplification conditions in the linear range, three amounts of cDNA (10, 50 and 100 ng)
10 were tested for each sample. Each reaction tube contained 2 μ l of 10 \times Ex Taq buffer
11 (Takara), 2 μ l of 2.5 mM dNTP mixture, 0.1 μ l of Ex Taq polymerase (Takara), 1 μ l (4
12 μ M) of each antisense primer and 2 μ l (4 μ M) of common sense primer. The total reaction
13 volume was 20 μ l. PCR consisted of denaturation at 94°C for 3 min, 25 cycles of
14 denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30
15 sec and a final extension at 72°C for 5 min. The PCR products were electrophoresed on
16 1.5% agarose gel. All assays were performed in duplicate.

17

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

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

24

25 **In vitro sulfation of peptide arrays**

26 pTriEx1.1-*Tpst1*-FL, *Tpst2*-FL, and mutant *Tpst2*-FL plasmids were transfected into 1 \times

1 10^7 HEK293T cells using Lipofectamine 2000 (Invitrogen). After 48 h, each cell was
2 homogenized in 500 μ l of ice-cold 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1
3 mM EDTA, 1% TritonX-100, 0.1 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin,
4 2 mg/ml aprotinin and 2 mg/ml leupeptin. Expression of each TPST protein in cell
5 lysates (20 μ g) was detected by Western blot analysis using a FLAG antibody (Sigma).
6 TPST activity was determined by measuring the transfer of 35 S-sulfate from 35 S-PAPS to
7 Fmoc-based membrane-immobilized 15-mer peptides (39). Peptide arrays, 35 S-PAPS and
8 50 ml of TPST lysate were accomplished by incubating 50 μ l of the buffer {50 mM
9 piperazine-1,4-bis(2-ethanesulfonic acid), pH 6.9, 0.4 mM EDTA, 1 mM Mg-acetate, 200
10 mM NaCl, 1 mg/ml BSA, 10 mM dithiothreitol} for 1 h at 37°C. After incubation, peptide
11 arrays were washed with 0.1 M Tris-HCl (pH 8.0) twice. The relative amounts of
12 incorporated radioactivity were visualized and quantified with a BAS2500 Bio-Imaging
13 analyzer (Fuji Film). All assays were performed in duplicate.

14

15 **Measurement of cAMP**

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

23

24 **ACKNOWLEDGEMENTS**

25

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

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

1

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15

1 **FIGURE LEGENDS**

2

3 **Figure 1.**

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

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

16

17 **Figure 2.**

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

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

25

26 **Figure 3.**

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

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

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

10 (D) Comparison of weights among each genotype at 5 wk of age. 1; *grt/grt* with TG (n =
11 20), 2; *grt/grt* (n = 17), 3; *grt/+* with TG (n = 30), 4; *grt/+* (n = 30) 5; WT (+/+) with TG (n
12 = 16), 6; WT (+/+) (n = 14). The weight data were presented by combining those of male
13 and female, because no difference was found between them at 5 wk of age. An asterisk
14 indicates a significant difference against other groups (P < 0.0001).

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

19

20 **Figure 4.**

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

1 40 × magnification, colloid staining was not present in some lumens, suggesting a
2 decrease in thyroglobulin production and storage. On the other hand, hypogenesis of the
3 thyroids was completely rescued in transgenic *grt/grt* mice. (B) Semiquantitative
4 RT-PCR analysis of thyroid-specific genes, *Tgn*, *Pax8*, *Nis*, *Tpo* and *Tshr* from two WT,
5 *grt* and TG-*grt* thyroids each. Beta-actin (*Actb*) was used as internal control. *Tshr* and *Nis*
6 levels were significantly reduced in *grt/grt* thyroids.

7

8 **Figure 5.**

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

11 (B) Equal amounts of cell extracts were extracted from HEK293T cells, subjected to
12 Western blot analysis with a FLAG antibody. The bands of approximately 55kDa and
13 50kDa detected on the blots can be identified as TPST2 (TP2) and TPST1 (TP1),
14 respectively. *Actb* was used as a loading control.

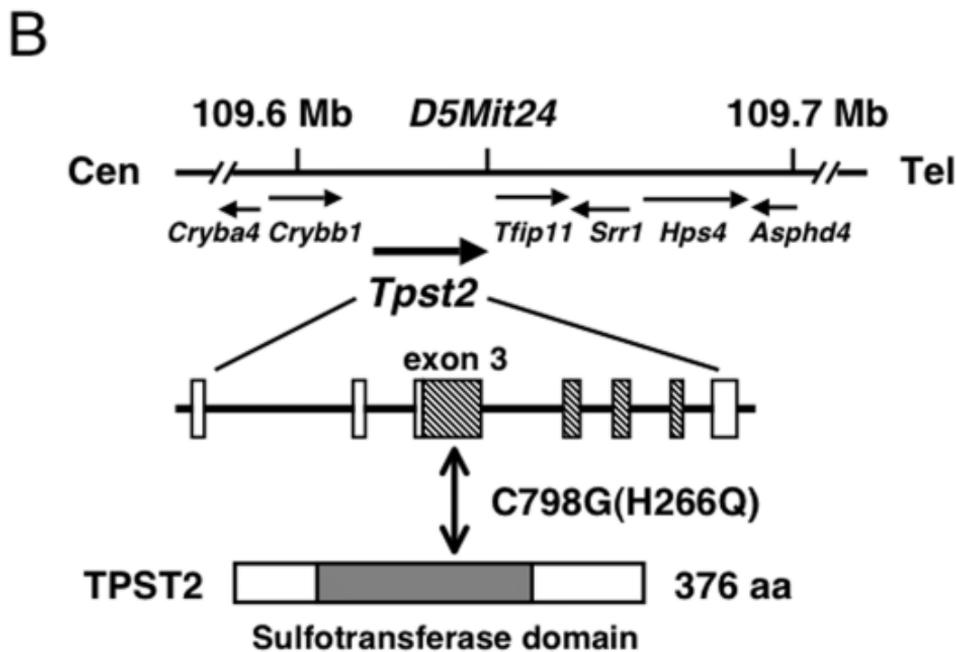
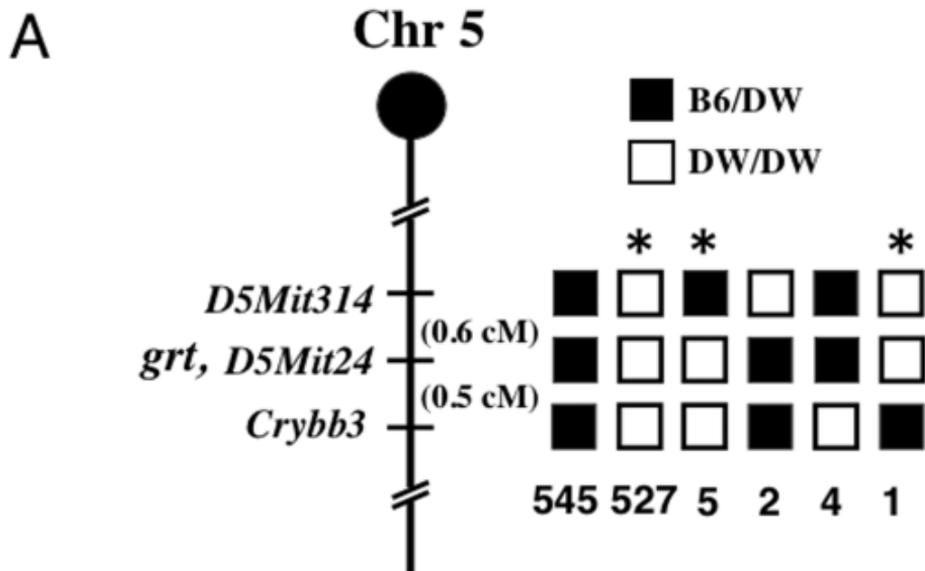
15 (C) Tyrosine-sulfation on the peptide array by TPST1 and 2. Each spot contains the
16 15-mer peptide substrates, GP1BA, CF-8, CCK, FSHR, TSHR, and PSGL1. The same
17 input of each TPST was used for in this assay. The tyrosine sulfation of peptides was
18 revealed by the incorporation of ³⁵S into the substrates by autoradiography. The putative
19 sulfation sites are indicated as gray characters. TSHR (Mu) and PSGL1 (Mu) are negative
20 control peptides, in which a mutation from tyrosine to phenylalanine was introduced. TP1,
21 TP2 and TP2 (H266Q) indicate TPST1, TPST2 and mutant TPST2 (H266Q),
22 respectively.

23 (D) The quantitative evaluation of Fig. 5C. The relative incorporation of ³⁵S into peptides
24 was evaluated.

25

26 **Figure 6.**

- 1 (A) *Tpst1* and *Tpst2* expression in various mouse tissues. Semiquantitative RT-PCR
2 analysis shows ubiquitous expression of both *Tpst1* and *Tpst2*. *Tpst1* (563 bp) and *Tpst2*
3 (741 bp) were concomitantly amplified in the same tube of each sample. Thyroid and
4 thyrocyte cell lines expressed both genes.
- 5 (B) Rescue of TSH-mediated cAMP production in *grt/grt* fibroblasts by TPST2
6 restoration in response to TSH stimulation. Plus (+) or minus (-) indicates the presence or
7 absence of TSH (10 mU/ml) in the medium. Lentiviruses encoding the TSHR and each
8 TP1, TP2 and TP2 (H266Q) were infected in *grt* cells, alone or in combination. Each bar
9 represents the mean \pm SEM.
- 10 (C) The expression of each gene in *grt/grt* fibroblasts was confirmed by RT-PCR. The
11 expressions of transgenes increased to approximately two-three fold more than that of
12 endogenous *Tpst* mRNAs.
- 13 (D) The expression of TPST proteins in *grt/grt* fibroblasts were evaluated by Western
14 blotting with FLAG antibody. Mock indicates the untransfected-*grt/grt* fibroblasts. Each
15 protein was expressed at similar level in the transfected cells.



A

TPST2

| | | | |
|----------------------|--------------|----------|--------------|
| <i>grt</i> (H266Q) | ... AWSDAVLH | Q | EDLIGKP ... |
| <i>M. musculus</i> | ... AWSDAVLH | H | EDLIGKP ... |
| <i>H. sapiens</i> | ... AWSDAVLH | H | EDLIGKP ... |
| <i>R. norvegicus</i> | ... AWSDTV | LH | HEDLIGKP ... |
| <i>G. gallus</i> | ... SWSDTV | LH | HEELIGKP ... |
| <i>X. laevis</i> | ... PWMDAVLH | H | EEELIGKP ... |
| <i>D. rerio</i> | ... PWDTAVLH | H | EQELIGKV ... |

TPST1

| | | | |
|--------------------|----------|------|--------------|
| <i>M. musculus</i> | ... PWNH | SVLH | HEEMIGKA ... |
| <i>H. sapiens</i> | ... PWNH | SVLH | HEEMIGKA ... |

TPST

| | | | |
|------------------------|--------------|---|--------------|
| <i>D. melanogaster</i> | ... PWMDAVLH | H | EEFINKP ... |
| <i>C. elegans</i> | ... PWDDKVLH | H | EEQLIGKD ... |

B

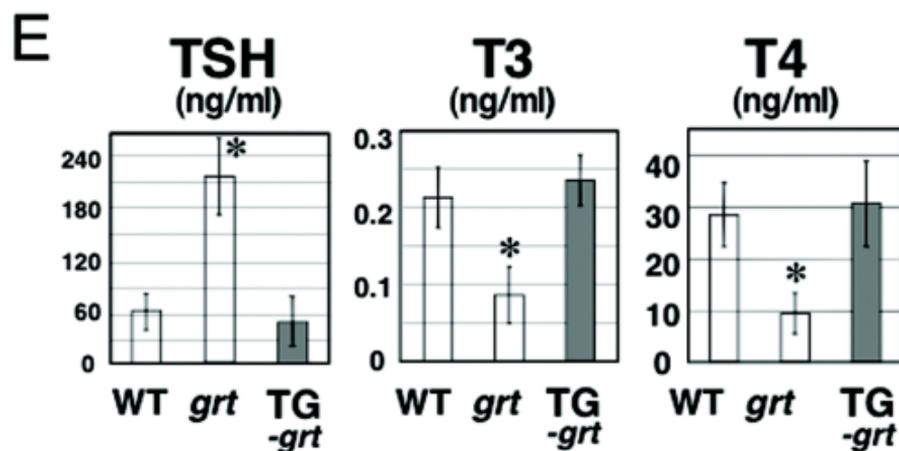
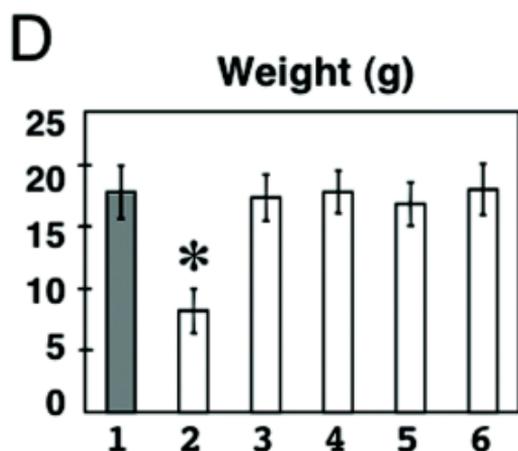
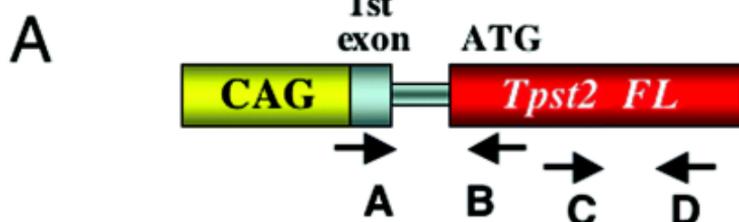
*Eco*NI CCTNNNNNAGG

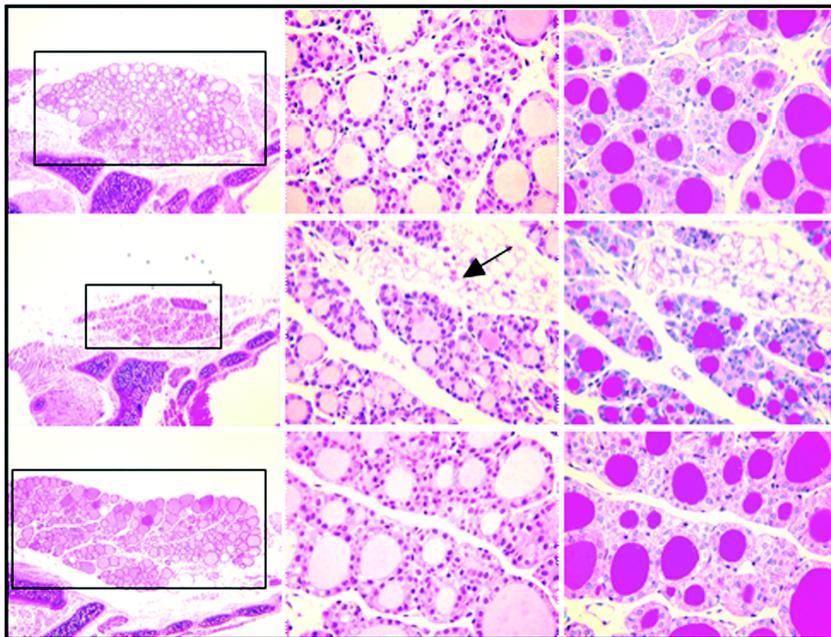
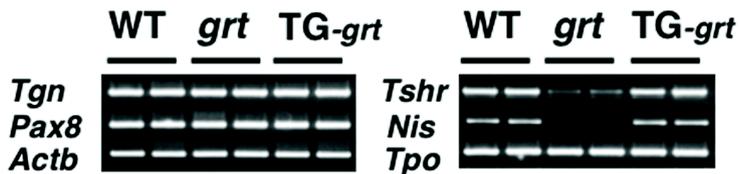
DW/J- (+/+) CCTGCACCACG

DW/J- (*grt/grt*) CCTGCACCAGG*

(+/+) (*grt*/+) (*grt/grt*)

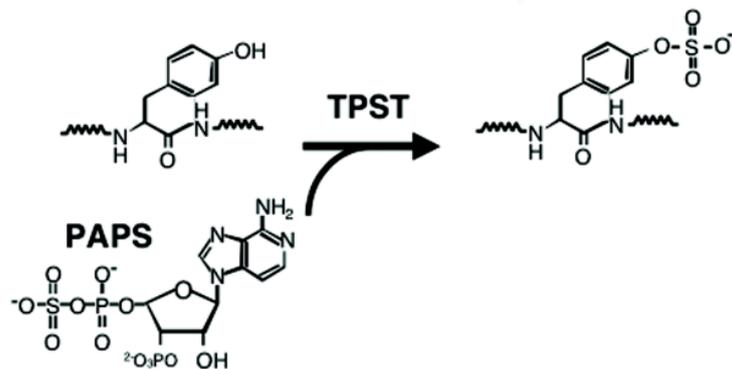




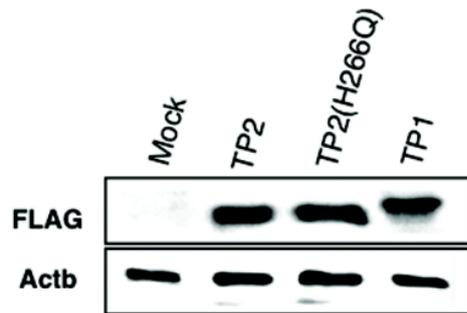
A**HE (×4)****HE (×40)****PAS (×40)****WT*****grt*****TG-*grt*****B**

A

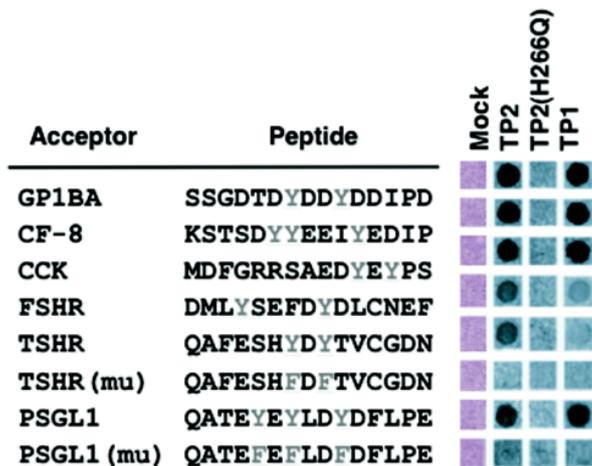
Tyrosine of acceptor protein



B



C



D

