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Original Article

Global expression profiling reveals gain-of-function oncogenic activity of a mutated thyroid hormone receptor in thyroid carcinogenesis

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Abstract: Thyroid hormone receptors (TRs) are critical in regulating gene expression in normal physiological processes. Decreased expression and/or somatic mutations of TRs have been shown to be associated several types of human cancers including liver, breast, lung, and thyroid. To understand the molecular mechanisms by which mutated TRs promote carcinogenesis, an animal model of follicular thyroid carcinoma (FTC) ($Thrb^{PV/PV}$ mice) was used in the present study. The $Thrb^{PV/PV}$ mouse harbors a knockin dominant negative PV mutation, identified in a patient with resistance to thyroid hormone. To understand whether oncogenic actions of PV involve not only the loss of normal TR functions but also gain-of-function activities, we compared the gene expression profiles of thyroid lesions in $Thrb^{PV/PV}$ mice and $Thra1^{-/-}Thrb^{-/-}$ mice that also spontaneously develop FTC, but with less severe malignancy. Analysis of the cDNA microarray data derived from microdissected thyroid tumor cells of these two mice showed contrasting global gene expression profiles. With stringent selection using 2.5-fold change (p<0.01) in cDNA microarray analysis, 241 genes with altered gene expression were identified. Nearly half of the genes (n=103: 42.7% of total) with altered gene expression in thyroid tumor cells of $Thrb^{PV/PV}$ mice were associated with tumorigenesis and metastasis; some of these genes function as oncogenes in human thyroid cancers. The remaining genes were found to function in transcriptional regulation, RNA processing, cell proliferation, apoptosis, angiogenesis, and cytoskeleton modification. These results indicate that the more aggressive thyroid tumor progression in $Thrb^{PV/PV}$ mice was not due simply to the loss of tumor suppressor functions of TR via mutation but also, importantly, to gain-of-function in the oncogenic activities of PV to drive thyroid carcinogenesis. Thus, the present study identifies a novel mechanism by which a mutated TRβ evolves with an oncogenic advantage to promote thyroid carcinogenesis.

Keywords: Mutant TR, thyroid cancer, mouse model, microarray, gene expression

Introduction

Thyroid cancer is the most common malignancy of endocrine organs, and its incidence rate is steadily increasing [1]. Prognostic factors of thyroid cancer patients include tumor histological type, tumor size, the presence of lymph node metastasis, extrathyroidal extension, distance metastasis, and the existence of oncogenes [1]. Among these factors, the main cause of mortality in thyroid cancer is due to distant disease with about 50% survival at 3.5 years [2].

Thyroid hormone receptors (TRs), encoded by thyroid hormone receptor a (*THRA*) and b (*THRB*) genes, mediate the action of the thyroid hormone (T3) in embryonic development, cell growth, development, differentiation, and metabolic homeostasis. They are ligand-dependent transcription factors that bind to thyroid hormone response elements (TREs) in the promoter regions of target genes [3]. In view of the vital biological roles of TRs, it is reasonable to expect that their mutations could lead to deleterious effects. Indeed, mutations of the *THRB* gene are known to cause a genetic disease, resistance to thyroid hormone (RTH). However, whether mutations of the *THRB* gene also play a role in cancer development has not been clear.
Loss or reduced expression of the *THRB* gene is reported to be closely associated with human

malignancies such as breast, liver, thyroid, pituitary, colon, and renal cancers [4, 5]. High frequencies of somatic deletions, gene rearrangements, and/or loss of heterozygosity of chromosome 3p where the *THRB* gene is located were detected in many neoplasms [4]. In addition, somatic mutations leading to aberrant TRβ functions were identified in hepatocellular carcinomas [6], thyroid carcinomas [7], renal clear cell carcinomas [8], and pituitary tumors [9]. These observations led to a converging proposition that TRβ could function as a tumor suppressor. Indeed, cell-based studies and xenograft models have demonstrated that TRβ is a suppressor of ras-mediated cell proliferation, transformation, and tumorigenesis [10]. Moreover, TRβ disrupts the mitogenic action of growth factors by suppressing activation of extracellular signal-regulated kinase and phosphatidylinositol 3-kinase (PI3K) signaling pathways to suppress tumor cell invasiveness and metastasis [11].

That TRβ could function as a tumor suppressor is further strengthened by the compelling *in vivo* evidence that mice harboring a TRβ mutation spontaneously develop follicular thyroid carcinoma (FTC) similar to human thyroid cancer ($Thrb^{PV/PV}$ mice; [12, 13]). The PV mutation was identified in a patient with resistance to thyroid hormone (RTH) [14]. It has a frame-shift mutation in the C-terminal 14 amino acids, resulting in the complete loss of T3 binding activity and transcription capacity [15]. The phenotypic manifestation of the $Thrb^{PV/PV}$ mouse is reminiscent of cancer patients with somatic mutations in TRβ which have lost T3 binding and transcriptional capacity [6-8]. Using the $Thrb^{PV/PV}$ mouse, we have shown that PV functions as an oncogene by suppressing the expression and activity of a tumor suppressor, such as the peroxisome proliferator activating receptor γ (PPARγ) [16], and by activating tumor promoters such as cyclin D1, β-catenin, PI3K, AKT, and pituitary tumor transforming gene [17-21]. However, a critical question has been whether the oncogenic activity of PV is due simply to the loss of the wild -type (WT) TR tumor suppressor functions or also results from gain-of-function activities.

To address this question *in vivo*, we took advantage of another mutant mouse that is deficient in all functional WT TRs ($Thra1^{-/-}Thrb^{-/-}$ mouse). In further support of the idea that TRs can function as a tumor suppressor, this mouse, which lacks both TRβ and TRα1, also spontaneously develops FTC [22]. The $Thrb^{PV/PV}$ and $Thra1^{-/-}Thrb^{-/-}$ mice exhibit similarly elevated serum levels of thyroid stimulating hormone (TSH) and thyroid hormones [23], but intriguingly the $Thra1^{-/-}Thrb^{-/-}$ mouse develops FTC with a slower progression and a less aggressive malignant phenotype [22-25]. These observations led us to hypothesize that in addition to the loss of normal tumor suppressor functions of WT TRβ, PV could acquire additional oncogenic activity via gain-of-function through mutation. To test this hypothesis, using cDNA microarrays, we compared gene expression profiles in microdissected thyroid tumor lesions of age- and gender-matched $Thrb^{PV/PV}$ and $Thra1^{-/-}Thrb^{-/-}$ mice. We found that the gene expression profiles in the thyroid tumor cells differed between $Thrb^{PV/PV}$ and $Thra1^{-/-}Thrb^{-/-}$ mice, indicating that PV has acquired additional functions beyond simply the loss of normal TR functions. Thus, the more aggressive thyroid tumor progression in $Thrb^{PV/PV}$ than in $Thra1^{-/-}Thrb^{-/-}$ mice resulted from dual oncogenic activity of PV. The present findings uncover a novel mechanism by which a mutated TRβ could exert deleterious effect, leading to cancer and other diseases.

Materials and methods

Animals

Mice harboring the two mutated alleles of the *Thrb* gene ($Thrb^{PV/PV}$ mice) and mice with double knockout of *Thr* genes ($Thra1^{-/-}Thrb^{-/-}$ mice) were generated as previously described [12, 23]. As with the WT mice, these mutant mice were given normal chow and tap water and housed under 12-h light/12-h dark cycles at 22°C. Thyroid tissues or tumors were dissected out from mice around 10- to 12-months-old and kept frozen at -80°C until further use. The animal protocols used in the study were approved by the Animal Care and Use Committee at NCI.

Laser capture microdissection

Laser capture microdissection was performed on 5- to 8-μm-thick cryosections of histologically proven thyroid tissues of WT, $Thra1^{-/-}Thrb^{-/-}$, and $Thrb^{PV/PV}$ mice by using an Arcturus^{XT} (Arcturus Engineering, Inc.) or Veritas machine (Arcturus Engineering, Inc). Captured cells were further processed for RNA extraction by using a PicoPure kit (Cat. No. 0202, Arcturus Biosciences, Inc) according to the manufacturer's instructions. The extracted total RNA was then amplified with a MessageAmp™II aRNA Amplification

Kit (AM 1751, Ambion). Briefly, 0.1~1.0 ng of RNA was subjected to two rounds of amplification, and enriched aRNA was labeled with biotin-11-UTP for microarray hybridization. The quantity and quality of biotinylated aRNA were analyzed by a Nanodrop (Thermo Scientific) and 2100 Bioanalyzer (Agilent Technologies).

Microarray hybridization and data analysis

For microarray analysis, biotinylated-aRNA from three replicates of each group were used in hybridization of the GeneChip Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA) and scanned on Affymetrix GeneChip scanner 3000. Data were collected using Affymetrix GCOS software. Statistical and clustering analysis was performed with Partek Genomics Suite software using a random multiple access normalization algorithm. Differentially expressed genes were identified with ANOVA analysis. Genes that were up- or down-regulated more than 2.5-fold with a $p<0.01$ were considered significant. Significant genes were analyzed for pathway enrichment and for functional annotation by using Ingenuity Pathway Analysis software (Ingenuity Systems, Inc., Redwood City, CA) and the DAVID bioinformatics database (http://david.abcc.ncifcrf.gov), respectively.

Validation of array analysis by real time RT-PCR

Real time RT-PCR was applied to confirm the differential expression of selected genes from microarray analysis. Briefly, a total 50 ng of RNA extracted from thyroid tissue of *Thra1*$^{-/-}$*Thrb*$^{-/-}$ or *Thrb*$^{PV/PV}$ mice was used in each reaction of the real-time RT-PCR. The reactions were performed with a QuantiTech SYBR RT-PCR kit (Qiagen, Germantown, MD) on LightCycler (Roche, Indianapolis, IN). For the comparison of gene expression between *Thra1*$^{-/-}$*Thrb*$^{-/-}$ or *Thrb*$^{PV/PV}$ mice, three or four samples were tested on each target gene. The Student's *t*-test was performed using GraphPad Prism version 5.00 for Mac (GraphPad Software, San Diego CA).

Results

Analysis of gene expression profiles in thyroid tumor cells of Thrb$^{PV/PV}$ *and Thra1*$^{-/-}$*Thrb*$^{-/-}$ *mice*

Array data were obtained from laser capture microdissected normal thyrocytes of age-matched male WT mice and thyroid lesions of *Thrb*$^{PV/PV}$ and *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice (n=3 for each genotype of mice). **Figure 1A** shows the results of principal component analysis (PCA) of gene expression profiles from the three groups of mice. The three-dimensional projection of the top three principal components of PCA, capturing 70.09% of total variance, shows clear separation of the three groups (**Figure 1A**). The well-segregated three clusters of data derived from WT, *Thrb*$^{PV/PV}$, and *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice allowed us to compare changes in gene expression due to the effects of PV mutation (compare WT with *Thrb*$^{PV/PV}$ mice) or due to the lack of functional TRs (compare WT with *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice). Subsequent comparison of gene expression profiles between the PV-mediated effects (loss of TR suppressor functions and/or oncogenic activity via gain-of-function) and the effects due to the lack of TR tumor suppressor functions allowed us to sort out the gene expression profiles due to the gain-of-function of PV critical for tumor-promoting activity.

Analysis of array data derived from the Affymetrix GeneChip, representing approximately 14,000 well-substantiated mouse genes, identified 241 genes with a significant difference in fold change >2.5 (adjusted $p<0.01$). Among these genes with altered expression, 97 were up-regulated and 144 were down-regulated in thyroid tumor cells of *Thrb*$^{PV/PV}$ mice as compared with those of *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice. Hierarchical clustering analysis of the top 50 genes with altered expression profiles is shown in the **Figure 1B**. Contrasting expression patterns were clearly evident in the tumor cells between *Thrb*$^{PV/PV}$ and *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice, indicating that the expression of genes mediated by mutant PV in *Thrb*$^{PV/PV}$ mice did not simply reflect from the loss of normal TR functions.

Functional classification of genes with distinct expression in thyroid tumor cells of Thrb$^{PV/PV}$ *mice*

To understand how the distinctly expressed genes were involved in the gain-of-function activity mediated by PV, we analyzed the gene ontology and functional annotation using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/) and Ingenuity Pathway Analysis software (Ingenuity Systems, Inc., Redwood City, CA). In addition, we searched the GO Ontology database, as well as the PubMed data-

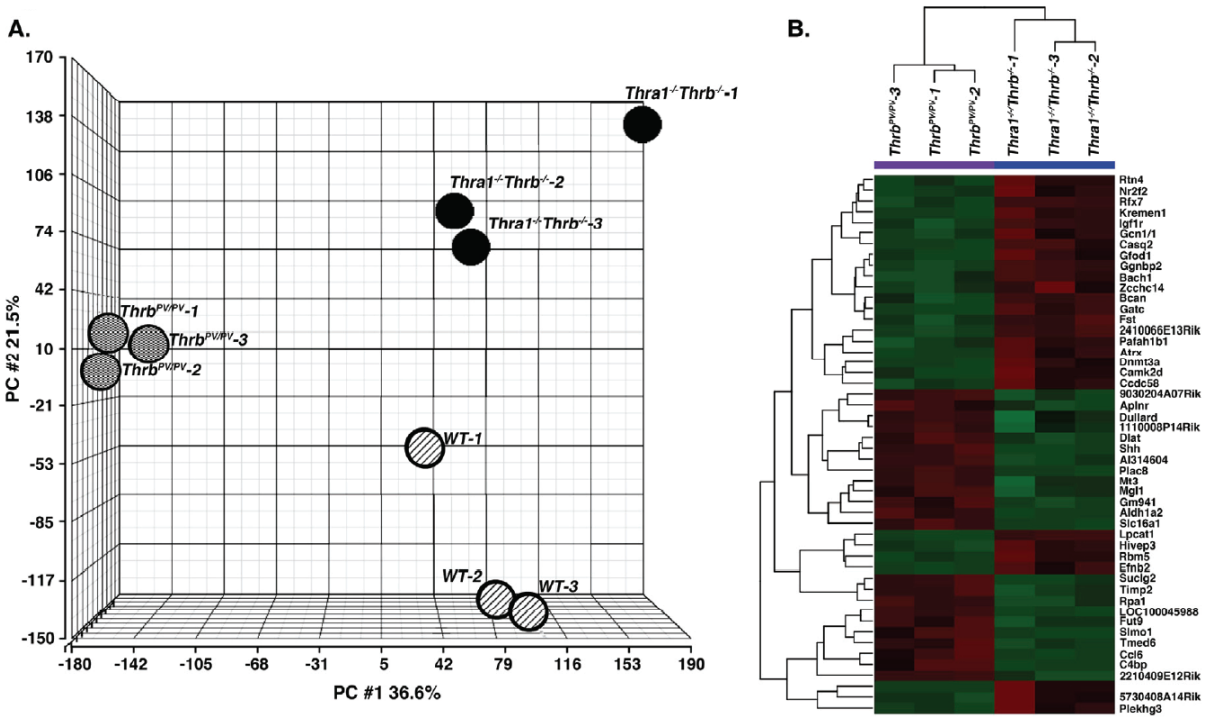


Figure 1. A. Principal Component Analysis (PC) of the gene expression profiles in the microdissected thyroid cells of wild-type (WT) mice and tumor cells of *Thrb*$^{PV/PV}$ and *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice. 3D Projection of the top three principal components of PCA in (**A**) which captures 70.09% of total variance, shows clear separation of the three groups. **B**. Hierarchical clustering (average of Euclidean distance) analysis of the top 50 genes, which were filtered by the adjusted P values <0.01 and a minimum 2.5-fold change in the expression level between between *Thrb*$^{PV/PV}$ mice and *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice.

base, for each gene, using the terms *tumor*, *metastasis*, *invasion*, *thyroid hormone receptor*, *thyroid tumor*, and *metabolism*, to identify the functions of genes. We then grouped the genes in three categories: tumor and/or metastasis-related (**Table 1**), thyroid or thyroid tumor-related (**Table 2**), and other related biological functions (**Table 3**). Since it is not possible within the allowed journal space to detail the cellular functions of each of 241 genes that had altered expression, only the genes that have been well studied in other cancers are highlighted in each category, as shown in the following sections. Readers should refer to the Tables for the genes that they are interested in.

Identification of genes involved in tumorigenesis mediated by PV to drive thyroid tumor progression of Thrb$^{PV/PV}$ *mice*

Among the total 241 genes with altered expression in thyroid tumor cells of *Thrb*$^{PV/PV}$ mice, 100 (41.5%) genes have been reported to be associated with carcinogenesis or tumor metastasis. Of these 100 genes, 49 (49%) were up-regulated (2.5- to 13.1-fold) and 51 were down-regulated (2.6- to 21.3-fold). These genes were originally involved in many different cellular functions including transcriptional regulation, RNA processing, chromatin modification, cell development, cell proliferation, apoptosis, embryonic development, angiogenesis, signaling transduction, and cell adhesion (**Table 1**). At the top of the list with activated expression (**Table 1-a**) are fibrinogen gamma chain (*Fgg;* 13.1-fold), glial cell line derived neurotrophic factor family receptor alpha 1 (*Gfra1;* 11.78-fold), and placenta-specific 8 (*Plac8*; 11.31-fold). Use of real time RT-PCR confirmed mRNA levels were increased in thyroid tumor cells of *Thrb*$^{PV/PV}$ mice as compared with *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice. As shown in **Figures 2A-C**, 5-, 155-, and 4.5-fold increased

Table 1-a. Genes with altered expression having roles in tumorigenesis/metastasis (n=100)

Symbol	Gene name	Accession	Fold change	Tumor-related	Metastasis-related	Type(s)
Fgg	fibrinogen gamma chain	NM_133862	13.1	*		other
Gfra1	glial cell line derived neurotrophic factor family receptor alpha 1	AV221299	11.78	*	*	transmembrane receptor
Plac8	placenta-specific 8	AF263458	11.31	*		other
Slc16a1	solute carrier family 16 (monocarboxylic acid transporters), member 1	NM_009196	9.48	*		transporter
Eid1	EP300 interacting inhibitor of differentiation 1	BC010712	7.78	*		transcription regulator
Ap1s3	adaptor-related protein complex AP-1, sigma 3	AW259574	6.56	*		transporter
Camk2n2	calcium/calmodulin-dependent protein kinase II inhibitor 2	AK013788	6.1	*		other
Shh	sonic hedgehog	AV304616	4.66	*		peptidase
Masp1	mannan-binding lectin serine peptidase 1	AB049755	4.42	*		peptidase
Arsb	arylsulfatase B	BI440651	4.35		*	enzyme
Rassf4	Ras association (RalGDS/AF-6) domain family member 4	AV291679	4.32	*	*	other
Lad1	ladinin	NM_133664	4.28	*		other
Bach2	BTB and CNC homology 2	BB529913	3.99	*		transcription regulator
Usp2	ubiquitin specific peptidase 2	AI553394	3.88	*		peptidase
Adi1	acireductone dioxygenase 1	NM_134052	3.7	*		enzyme
Timp2	tissue inhibitor of metalloproteinase 2	BF168458	3.69	*	*	other
C4bp	complement component 4 binding protein	NM_007576	3.56	*		other
Gng10	guanine nucleotide binding protein (G protein), gamma 10	NM_025277	3.56	*		enzyme
Sirpa	signal-regulatory protein alpha	AB018194	3.53	*		phosphatase
Idh1	isocitrate dehydrogenase 1 (NADP+), soluble	NM_010497	3.44	*	*	enzyme
B4galnt2	beta-1,4-N-acetyl-galactosaminyl transferase 2	AI593864	3.34	*		enzyme
Prkcsh	protein kinase C substrate 80K-H	NM_008925	3.34	*		enzyme
Mt3	metallothionein 3	NM_013603	3.28	*		other
Ccl6	chemokine (C-C motif) ligand 6	AV084904	3.21	*		cytokine
Muc4	mucin 4	AF218265	3.14	*	*	growth factor
Itpr1	inositol 1,4,5-triphosphate receptor 1	NM_010585	2.99	*		ion channel
Prdx3	peroxiredoxin 3	NM_007452	2.83	*		enzyme
Ube2a	ubiquitin-conjugating enzyme E2A, RAD6 homolog (S. cerevisiae)	BG868960	2.83	*		enzyme
Thy1	thymus cell antigen 1, theta	AV028402	2.82	*		other
Fdft1	farnesyl diphosphate farnesyl transferase 1	BB028312	2.81	*		enzyme
Pnkd	paroxysmal nonkinesiogenic dyskinesia	NM_019999	2.79	*	*	other
Sema4d	sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	AV256403	2.78	*	*	other
Slc9a3r1	solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 1	BG066200	2.78	*		other
Uqcrh	ubiquinol-cytochrome c reductase hinge protein	AK019085	2.74	*		
Aebp1	AE binding protein 1	NM_009636	2.73	*		peptidase
Rpa1	replication protein A1	BB491281	2.72	*		other

Dnajc15	DnaJ (Hsp40) homolog, subfamily C, member 15	NM_025384	2.7	*		other
Hnf1b	HNF1 homeobox B	AI987804	2.7	*		transcription regulator
Kctd12	potassium channel tetramerisation domain containing 12	BM220945	2.7	*		ion channel
Mobkl3	MOB1, Mps One Binder kinase activator-like 3 (yeast)	AK011829	2.69	*		other
Pigy	phosphatidylinositol glycan anchor biosynthesis, class Y	AK003713	2.65	*		other
Nudt2	nudix (nucleoside diphosphate linked moiety X)-type motif 2	NM_025539	2.64	*		phosphatase
Esr1	estrogen receptor 1 (alpha)	AI646838	2.61	*	*	ligand-dependent nuclear receptor
Mrpl38	mitochondrial ribosomal protein L38	BI135190	2.6	*		other
Prdm2	PR domain containing 2, with ZNF domain	BM226301	2.6	*		transcription regulator
Rnf4	ring finger protein 4	AV045658	2.6	*		transcription regulator
Ccdc56	coiled-coil domain containing 56	C77389	2.58	*		other
Uba1	ubiquitin-like modifier activating enzyme 1	NM_009457	2.53	*		enzyme
Plekha8	pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 8	AI324154	2.52	*		other
Bmp5	bone morphogenetic protein 5	NM_007555	-2.61	*		growth factor
Ggnbp2	gametogenetin binding protein 2	BG078867	-2.67	*		other
Tbl1xr1	transducin (beta)-like 1X-linked receptor 1	BG071620	-2.69	*		transcription regulator
Creb1	cAMP responsive element binding protein 1	AK014391	-2.71	*		transcription regulator
Rbm5	RNA binding motif protein 5	BE446879	-2.74	*	*	other
Mcf2l	mcf.2 transforming sequence-like	AV293368	-2.75	*		other
Lair1	leukocyte-associated Ig-like receptor 1	BB667693	-2.76	*		transmembrane receptor
Abcc5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	BB436535	-2.79	*		transporter
Khdrbs1	KH domain containing, RNA binding, signal transduction associated 1	AV117555	-2.79	*		transcription regulator
Rgnef	Rho-guanine nucleotide exchange factor	BB530298	-2.79	*	*	other
Ccdc68	coiled-coil domain containing 68	AV378320	-2.81	*		other
Birc6	baculoviral IAP repeat-containing 6	BB527646	-2.82	*		enzyme
Fus	fusion, derived from t(12;16) malignant liposarcoma (human)	BE985138	-2.83	*		transcription regulator
Bach1	BTB and CNC homology 1	NM_007520	-2.86	*		transcription regulator
Efnb2	ephrin B2	U30244	-2.87	*		other
Elf2	E74-like factor 2	BC027739	-2.9	*		transcription regulator
Notch2	Notch gene homolog 2 (Drosophila)	AI787996	-2.9	*		transcription regulator
Bnip2	BCL2/adenovirus E1B interacting protein 2	AV144704	-2.93	*		other
Alcam	activated leukocyte cell adhesion molecule	BB534113	-3.05	*	*	other
Kcnq1ot1	KCNQ1 overlapping transcript 1	BG063584	-3.44	*		other
Bcan	brevican	BB335613	-3.59	*		other
Mirhg1	microRNA host gene 1 (non-protein coding)	AK017164	-3.6	*		other
Nfia	nuclear factor I/A	AF326553	-3.6	*		transcription regulator
Sp4	trans-acting transcription factor 4	NM_009239	-3.76	*		transcription regulator
Cpeb3	cytoplasmic polyadenylation element binding protein 3	BB281000	-3.78	*		other
Bmf	BCL2 modifying factor	BB212341	-3.82	*		other
Sfpq	splicing factor proline/glutamine rich (polypyrimidine tract binding	BI738328	-3.92	*		other

	protein associated); similar to PTB-associated splicing factor					
Crtc3	CREB regulated transcription coactivator 3	AI429792	-3.96	*		other
Krit1	KRIT1, ankyrin repeat containing	BI793814	-4.02	*		other
Srd5a1	steroid 5 alpha-reductase 1	AA530749	-4.08	*		enzyme
Vegfc	vascular endothelial growth factor C	NM_009506	-4.17	*	*	growth factor
Cckrs	CDC2-related kinase, arginine/serine-rich	BG070845	-4.25	*		enzyme
Ednrb	endothelin receptor type B	BB451714	-4.35	*		G-protein coupled receptor
Itsn2	intersectin 2	AI326108	-4.36	*		other
Igf1r	insulin-like growth factor I receptor	AV374369	-4.42	*		transmembrane receptor
Nr2f2	similar to COUP-TFI; nuclear receptor subfamily 2, group F, member 2	AI463873	-4.44	*	*	ligand-dependent nuclear receptor
Nmt2	N-myristoyltransferase 2	BB409982	-4.5	*		enzyme
Smc4	structural maintenance of chromosomes 4	BI665568	-4.79	*		transporter
Rtn4	reticulon 4	BG072267	-4.91	*		other
Mtss1	metastasis suppressor 1	BB157298	-5.09	*	*	other
Vps37a	vacuolar protein sorting 37A (yeast)	AK008752	-5.78	*	*	other
Dnmt3a	DNA methyltransferase 3A	BB795491	-5.97	*		enzyme
Git2	G protein-coupled receptor kinase-interactor 2	AK017943	-6.4		*	other
Sdha	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	AK005350	-6.86	*		enzyme
Cspg4	chondroitin sulfate proteoglycan 4	BB377873	-7.13	*	*	other
Cpd	carboxypeptidase D	NM_007754	-8.93	*		peptidase
Bclaf1	BCL2-associated transcription factor 1	BE853331	-10.45	*		transcription regulator
Wnt4	wingless-related MMTV integration site 4	AW047257	-10.61	*		other
Abca3	ATP-binding cassette, sub-family A (ABC1), member 3	BC006932	-11.71	*	*	transporter
Ddx6	DEAD (Asp-Glu-Ala-Asp) box polypeptide 6	BC021452	-15.13	*		enzyme
Sik1	salt inducible kinase 1	AI648260	-21.3	*	*	kinase

Table 1-b. Functional annotation analysis in genes relevant to tumorigenesis/metastasis in *Thrb*$^{PV/PV}$ mice

Function	Count	Genes
Regulation of transcription	22	Khdrbs1,Fus,Crtc3,Bach1,Eid1,Tbl1xr1,Dnmt3a,Bclaf1,Hnf1b,Aebp1,Elf2,Bach2,Creb1,Esr1,Shh,Notch2,Rnf4,Sfpq,Sp4,Prdm2,Nr2f2,Nfia
Regulation of RNA metabolic process	15	Bach1,Fus,E id1,Dnmt3a,T bl1xr1,Aebp1,Hnf1b,Elf2,Bach2,Creb1,Esr1,Rbm5,Shh,Nr2f2,Nfia
Regulation of DNA metabolic process	6	Rpa1,Dnmt3a,Ube2a,Sfpq,Kcnq1ot1,Nfia
Regulation of cell development/differentiation	5	Rtn4,Wnt4,Shh,Thy1,Mt3,Vegfc,Sema4d
Positive regulation of cell proliferation	5	Rpa1,Vegfc,Esr1,Birc6,Shh
Regulation of apoptosis	7	Notch2,Bclaf1,Nudt2,Esr1,Rbm5,Birc6,Bmf
Embryonic development	5	Rpa1,Ube2a,Hnf1b,Birc6,Shh,Nr2f2
Blood vessel morphogenesis/angiogenesis	6	Rtn4,Vegfc,Cspg4,Nr2f2,Shh,Thy1
Signal	26	Arsb,Aebp1,Masp1,Cspg4,Bcan,Timp2,Prkcsh,Shh,Ccl6,Alcam,Ednrb,Igf1r,Wnt4,Fgg,Lair1,Efnb2,Sirpa,Muc4,Thy1,Notch2,Vegfc,C4bp,Gfra1,Sema4d,Cpd,Bmp5
Small GTPase regulator activity	6	Krit1,Git2,Itsn2,Rgnef,Mcf2l,Thy1
Oxidation reduction	7	Adi1,Sdha,Uqcrh,Idh1,Srd5a1,Prdx3,Fdft1
Vesicle-mediated transport	4	Apis3,Itsn2,Sirpa,Shh
Cell adhesion	6	Alcam,Aebp1,Bcan,Sirpa,Muc4,Thy1

Table 2. Thyroid cancer-related genes with altered expression in *Thrb*$^{PV/PV}$ mice (n=19)

Symbol	Gene name	Accession No.	Fold change	Tumor-related	Metastasis-related	Thyroid/thyroid tumor-related	Note
Gfra1	glial cell line derived neurotrophic factor family receptor alpha 1	AV221299	11.78	*	*	*	medullary thyroid carcinomas (MTC)- and RET oncogene-related
Chrdl1	chordin-like 1	AV144145	5.54			*	down-regulation in FTC
Shh	sonic hedgehog	AV304616	4.66	*		*	thyroid development
Minpp1	multiple inositol polyphosphate histidine phosphatase 1	AV339366	4.07			*	mutation found in FTC
Thra	thyroid hormone receptor alpha; similar to thyroid hormone receptor	BI076689	3.55			*	
Idh1	isocitrate dehydrogenase 1 (NADP+), soluble	NM_010497	3.44	*	*	*	mutation in FTC and ATC
Slc5a5	solute carrier family 5 (sodium iodide symporter), member 5	AF380353	3.15			*	iodide transporter
Muc4	mucin 4	AF218265	3.14	*	*	*	thyroid tumor
Hnf1b	HNF1 homeobox B	AI987804	2.70	*		*	regulatory factor for TBG genes and nicotinamide N-methyltransferase (NNMT) gene in PTC
Esr1	estrogen receptor 1 (alpha)	AI646838	2.61	*	*	*	polymorphism associated with thyroid cancers
Casq2	calsequestrin 2	NM_009814	-2.66			*	up-regulation in Grave's ophthalmopathy patients
Tbl1xr1	transducin (beta)-like 1X-linked receptor 1	BG071620	-2.69	*		*	co-repressor of TRs
Efnb2	ephrin B2	U30244	-2.87	*		*	diagnostic marker for malignant thyroid tumors
Mirhg1	microRNA host gene 1 (non-protein coding)	AK017164	-3.60	*		*	miR-17-92 cluster in ATCs
Vegfc	vascular endothelial growth factor C	NM_009506	-4.17	*	*	*	thyroid tumor lymphatic vessel formation
Igf1r	insulin-like growth factor I receptor	AV374369	-4.42	*		*	overexpression in human thyroid cancers
Nr2c2	nuclear receptor subfamily 2, group C, member 2	AU066920	-4.49			*	modulator of TR-targeted genes
Sdha	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	AK005350	-6.86	*		*	thyroid tumors
Abca3	ATP-binding cassette, sub-family A (ABC1), member 3	BC006932	-11.71	*	*	*	TTF-1- and SREBP-target gene

Table 3-a. Genes with altered expression in *Thrb*$^{PV/PV}$ mice related to biological functions other than tumorigenesis or metastasis (n=135)

Symbol	Gene name	Accession No.	Fold change	Note(s)
Prpf19	PRP19/PSO4 pre-mRNA processing factor 19 homolog (S. cerevisiae)	BC004070	5.25	ubiquitin ligase activity; mRNA processing; DNA repair
Fam163a	family with sequence similarity 163, member A	BB183509	4.16	aka, neuroblastoma-derived secretory protein (NDSP)
Tmed6	transmembrane emp24 protein transport domain containing 6	NM_025458	4.07	ER transport
Il10rb	interleukin 10 receptor, beta	NM_008349	3.96	interleukin-10 receptor activity
Sc4mol	sterol-C4-methyl oxidase-like	AK005441	3.93	putative function in cholesterol biosynthesis
Slmo1	slowmo homolog 1 (Drosophila)	BB835597	3.82	aka erythroid differentiation and denucleation factor 1; function unknown
Kpna1	karyopherin (importin) alpha 1	U20619	3.80	nuclear import; regulation of DNA recombination
Dlat	dihydrolipoamide S-acetyltransferase	AV336908	3.72	accept acetyl groups formed by the oxidative decarboxylation of pyruvate and transfers them to coenzyme A
Tmem213	transmembrane protein 213	AI315206	3.57	unknown
Mgl1	macrophage galactose N-acetyl-galactosamine specific lectin 1	NM_010796	3.50	sugar binding
Pigx	phosphatidylinositol glycan anchor biosynthesis, class X	BC002202	3.47	GPI anchor biosynthetic process
Ogdh	oxoglutarate dehydrogenase (lipoamide)	BC013670	3.38	a subunit of the 2-oxoglutarate dehydrogenase complex; convert 2-oxoglutarate to succinyl-CoA during the Krebs cycle
Lcmt1	leucine carboxyl methyltransferase 1	NM_025304	3.36	protein modification
Kti12	KTI12 homolog, chromatin associated (S. cerevisiae)	NM_029571	3.29	chromatin modification
Mrps21	mitochondrial ribosomal protein S21	NM_078479	3.24	mitochondrial small ribosomal subunit
Med8	mediator of RNA polymerase II transcription, subunit 8 homolog (yeast)	AK011080	3.13	a subunit of the mediator complex
Grinl1a	glutamate receptor, ionotropic, N-methyl D-aspartate-like 1A	AK002571	3.11	transcriptional regulation (?); maintenance of ER location
Aldh1a2	aldehyde dehydrogenase family 1, subfamily A2	NM_009022	3.06	an enzyme that catalyzes the synthesis of retinoic acid (RA) from retinaldehyde
Atp5j2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	BG794445	3.03	the f subunit of the F0 complex of the proton channel of mitochondrial ATP synthase
Rshl2a	radial spokehead-like 2A; radial spokehead-like 2B	AA544511	3.03	abundantly expressed in tissues rich in highly ciliated cells, such as olfactory sensory neurons with unknown function
Nubp2	nucleotide binding protein 2	AV035368	2.99	microtubule organizing center
Ube2f	ubiquitin-conjugating enzyme E2F (putative)	AK007937	2.99	Nedd8 ligase activity; protein neddylation (ubiquitination-like)
Tmem45a	transmembrane protein 45a	NM_019631	2.98	unknown
Dusp14	dual specificity phosphatase 14	AK009744	2.97	phosphatase
Gstm5	glutathione S-transferase, mu 5	NM_010360	2.94	glutathione transferase activity
Mrps2	mitochondrial ribosomal protein S2	AV031454	2.91	mitochondrial small ribosomal subunit

Hbs1l	Hbs1-like (S. cerevisiae)	BG067949	2.84	translation elongation factor activity
Morn2	MORN repeat containing 2	BF319573	2.84	membrane occupation and recognition nexus (MORN) motif protein in testis; function unknown
Fut9	fucosyltransferase 9	AU067636	2.75	synthesizes the LeX oligosaccharide, which is expressed in organ buds progressing in mesenchyma during human embryogenesis
Nsdhl	NAD(P) dependent steroid dehydrogenase-like	BC019945	2.70	a sterol dehydrogenase involved in the removal of C-4 methyl groups in one of the later steps of cholesterol biosynthesis.
Suclg2	succinate-Coenzyme A ligase, GDP-forming, beta subunit	BF608645	2.70	a GTP-specific beta subunit of succinyl-CoA synthetase
Cisd1	CDGSH iron sulfur domain 1	NM_134007	2.69	a member of the CDGSH domain-containing family and may play a role in the regulation of mitochondrial oxidative capacity
Twf2	twinfilin, actin-binding protein, homolog 2 (Drosophila)	AK002699	2.68	intracellular signaling
Slc35a3	solute carrier family 35, member 3	AW822833	2.67	UDP-GlcNAc transporter
Zscan22	zinc finger and SCAN domain containing 22	BB811893	2.67	transcription regulation
Zcchc17	zinc finger, CCHC domain containing 17	BG962152	2.65	ribonucleoprotein complex
Bat1a	HLA-B-associated transcript 1A	BB145254	2.64	pre-mRNA processing
Coq9	coenzyme Q9 homolog (yeast)	AK004527	2.64	one of several enzymes involved in biosynthesis of CoQ10 and likely functions in modification of the benzoquinone ring
Cacnb3	calcium channel, voltage-dependent, beta 3 subunit	NM_007581	2.64	calcium ion transport
Mrps15	mitochondrial ribosomal protein S15	NM_025544	2.64	mitochondrial small ribosomal subunit
Pcbd1	pterin 4 alpha carbinolamine dehydratase	NM_025273	2.62	phenylalanine hydroxylation; regulate HNF1 dimerization
Socs1	suppressor of cytokine signaling 1	AB000710	2.61	cytokine-mediated signaling pathway; fat cell differentiation.
Ndufa12	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12	BF152811	2.60	an accessary subunit of mitochondrial complex I
Naglu	alpha-N-acetylglucosaminidase (Sanfilippo disease IIIB)	NM_013792	2.53	hydrolase activity, acting on glycosyl bonds
Map6d1	MAP6 domain containing 1	BB762333	2.52	negative regulation of microtubule depolymerization
Srrm1	serine/arginine repetitive matrix 1	BE199719	-2.55	pre-mRNA processing
Etohd2	ethanol decreased 2	BB214299	-2.56	unknown
Mtf2	metal response element binding transcription factor 2	BG066919	-2.57	transcription regulation
Gfod1	glucose-fructose oxidoreductase domain containing 1	BB538651	-2.57	oxidoreductase activity
Zfp871	zinc finger protein 871	BB008634	-2.61	unknown
Atl3	atlastin GTPase 3	BC017138	-2.64	ER/Golgi-bound GTPase
Zfp40	zinc finger protein 40	AI450803	-2.65	transcription regulation
Taf1a	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, A	NM_021466	-2.66	a component of SL1/TIFIB complex involved in the assembly of the preinitiation complex on RNA polymerase I promoter
Ccdc76	coiled-coil domain containing 76	BF147713	-2.75	methyltransferase activity; tRNA processing

Ankrd50	ankyrin repeat domain 50	BM119343	-2.81	unknown
Prrc1	proline-rich coiled-coil 1	BB481914	-2.81	unknown
Trim41	tripartite motif-containing 41	BE685711	-2.82	ligase activity
Clasp1	CLIP associating protein 1	BB190028	-2.83	cell cycle-related; microtubule-binding
Zcchc14	zinc finger, CCHC domain containing 14	BB223737	-2.83	cell communication; phosphoinositide binding
Impa1	inositol (myo)-1(or 4)-monophosphatase 1	AV154049	-2.87	inositol phosphate dephosphorylation
Rapgef4	Rap guanine nucleotide exchange factor (GEF) 4	AK004874	-2.87	calcium ion-dependent exocytosis; cAMP-mediated signaling
Atrx	alpha thalassemia/mental retardation syndrome X-linked homolog (human)	BF165715	-2.93	DNA helicase; chromatin remodeling
Dgkq	diacylglycerol kinase, theta	BB818538	-2.94	GPCR signaling; co-regulator of NR-targeted gene transcription
Rbm26	RNA binding motif protein 26	AV134514	-2.96	pre-mRNA processing
Fst	follistatin	NM_008046	-3.00	BMP signaling pathway
Tcf20	transcription factor 20	AW552808	-3.05	transcription regulation
Tcfe2a	transcription factor E2a	AF352579	-3.06	transcription regulation; histone acetylation
Ash1l	ash1 (absent, small, or homeotic)-like (Drosophila)	BG694892	-3.11	chromatin remodeling; cell junction
Gatc	glutamyl-tRNA(Gln) amidotransferase, subunit C homolog (bacterial)	AI452045	-3.11	mitochondrial tRNA modification
Stc2	stanniocalcin 2	AF031035	-3.12	hormone activity
Epb4.1	erythrocyte protein band 4.1	AI606195	-3.14	actin cytoskeleton organization
Gm5124	predicted gene 5124	BB049966	-3.21	unknown
Sbno1	sno, strawberry notch homolog 1 (Drosophila)	BB147192	-3.22	hydrolase activity
Sfrs12	splicing factor, arginine/serine-rich 12	AV012790	-3.24	pre-mRNA processing
Neu1	neuraminidase 1	AI649303	-3.26	lysosomal hydrolase activity; acting on glycosyl bonds
Npc2	Niemann Pick type C2	BB556874	-3.27	lysosome-located protein; cholesterol and lipid transport from lysosome to other cell compartment (?)
Scaper	S phase cyclin A-associated protein in the ER	AV319713	-3.27	unknown
Cugbp1	CUG triplet repeat, RNA binding protein 1	BI412951	-3.29	pre-mRNA processing; embryonic development
Heatr7a	HEAT repeat containing 7A	BB142087	-3.30	cellular component (?)
Cflar	CASP8 and FADD-like apoptosis regulator	BE284491	-3.30	anti-apoptosis
Slc4a4	solute carrier family 4 (anion exchanger), member 4	BE655147	-3.33	sodium ion transport
Gmeb1	glucocorticoid modulatory element binding protein 1	BB039426	-3.35	transcription regulation
Phf21a	PHD finger protein 21A	BB094173	-3.35	chromatin modification
Fgfr1op2	FGFR1 oncogene partner	AB041650	-3.36	unknown
Spnb2	spectrin beta 2	BM213516	-3.37	actin filament capping; SMAD protein nuclear translocation
Myh9	myosin, heavy polypeptide 9, non-muscle	BM121854	-3.39	cell morphology; cell-cell adhesion; establishment of meiotic spindle localization
Daam1	dishevelled associated activator of morphogenesis 1	BB794633	-3.39	actin-binding protein; non-canonical Wnt mediated planar cell polarity (PCP) signaling pathway (?)

Sorbs1	sorbin and SH3 domain containing 1	BB372866	-3.40	signaling molecule in insulin-stimulated glucose uptake (?)
Olfml2a	olfactomedin-like 2A	AW489058	-3.40	extracellular matrix organization
Trove2	TROVE domain family, member 2	BQ176653	-3.41	transcription from RNA polymerase III promoter
Serinc3	serine incorporator 3	BM239368	-3.41	induction of apoptosis
Lpcat1	lysophosphatidylcholine acyltransferase 1	BG068664	-3.42	surfactant phospholipid synthesis
Cgn	cingulin	AK018143	-3.45	apical junction complex
Pcmtd2	protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing 2	BM117243	-3.54	protein methyltransferase
Phip	pleckstrin homology domain interacting protein	BB473157	-3.55	insulin receptor signaling pathway; negative regulation of apoptosis
Dnaja2	DnaJ (Hsp40) homolog, subfamily A, member 2	BB324466	-3.81	protein folding
Pafah1b1	platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	L25109	-3.81	actin cytoskeleton organization; establishment of mitotic spindle orientation
Scfd1	Sec1 family domain containing 1	BB200692	-3.82	regulation of ER to Golgi vesicle-mediated transport
Plaa	phospholipase A2, activating protein	BB532258	-3.93	inflammatory response; phospholipase A2 activator activity
Gdpd5	glycerophosphodiester phosphodiesterase domain containing 5	BC024955	-3.95	glycerol metabolism; it catalyzes degradation of glycerophosphocholine (GPC)
Tmcc1	transmembrane and coiled coil domains 1	BB470329	-3.95	unknown
Xylb	xylulokinase homolog (H. influenzae)	BB431728	-3.95	energy metabolism (?)
Flnb	filamin, beta	BM206272	-4.13	cytoskeleton; stress fiber; acting binding
Eif4g3	eukaryotic translation initiation factor 4 gamma, 3	BM212250	-4.13	translation regulation
Apobec2	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 2	AI666693	-4.17	pre-mRNA processing
Sfrs18	splicing factor, arginine/serine-rich 18	BG277020	-4.22	nuclear speck
Phf7	PHD finger protein 7	AK005673	-4.31	unknown
Atp6v1h	ATPase, H+ transporting, lysosomal V1 subunit H	AI849595	-4.33	protein transport
Lnpep	leucyl/cystinyl aminopeptidase	BE850004	-4.34	proteolysis; cell-cell signaling
Saps3	SAPS domain family, member 3	AK018652	-4.57	regulation of phosphoprotein phosphatase activity
Rfx7	regulatory factor X, 7	BB148972	-4.60	DNA-dependent transcriptional regulation
Fnbp4	formin binding protein 4	BG091626	-4.67	cellular component (?)
Stx17	syntaxin 17	AK018158	-4.69	vesicle-mediated transport
Ino80d	INO80 complex subunit D	BE197105	-4.87	chromatin remodeling
Slc12a9	solute carrier family 12 (potassium/chloride transporters), member 9	BB668140	-5.00	ion transporter
Klhdc2	kelch domain containing 2	BB105408	-5.16	transcrioptional co-repressor
Rhbdl2	rhomboid, veinlet-like 2 (Drosophila)	AV378451	-5.17	membrane protease
Rnf125	ring finger protein 125	BB667823	-5.18	ligase activity
C1qtnf3	C1q and tumor necrosis factor related protein 3	NM_030888	-5.24	an adipokine of the C1q/TNF molecular superfamily; secreted by the adipocytes of mice and humans
Nipbl	Nipped-B homolog (Drosophila)	BG070859	-5.24	cell cycle; embryonic development
Abca4	ATP-binding cassette, sub-family A (ABC1),	NM_007378	-5.47	ATPase, photon-transduction

	member 4			
Fhdc1	FH2 domain containing 1	BB736539	-5.62	actin cytoskeleton organization
Rpgrip1	retinitis pigmentosa GTPase regulator interacting protein 1	AK015037	-5.69	eye photoreceptor cell development
Mef2c	myocyte enhancer factor 2C	BB280300	-5.72	transcription regulation
Cnot10	CCR4-NOT transcription complex, subunit 10	BB297961	-5.73	a subunit of the CCR4-NOT complex; transcriptional repression; mRNA metabolism
Plekhg3	pleckstrin homology domain containing, family G, member 3	BB280013	-5.78	Rho guanyl-nucleotide exchange factor activity
Kremen1	kringle containing transmembrane protein 1	BB373408	-5.89	Wnt receptor signaling pathway
Ccdc58	coiled-coil domain containing 58	BG064532	-6.29	mitochondrion component (?)
Fam13a	family with sequence similarity 13, member A	BB745929	-6.34	unknown
Klf7	Kruppel-like factor 7 (ubiquitous)	BB524597	-6.70	transcriptional co-activator
Eif2s2	eukaryotic translation initiation factor 2, subunit 2 (beta)	BG066754	-6.84	translation initiation complex
Kctd14	potassium channel tetramerisation domain containing 14	AW553424	-7.20	potassium ion transport
Senp7	SUMO1/sentrin specific peptidase 7	BM238538	-8.52	acts as a SUMO-2/3-specific protease that is likely to regulate the metabolism of poly-SUMO-2/3
Camk2d	calcium/calmodulin-dependent protein kinase II, delta	BG074866	-9.58	calcium ion transport; cell cycle-related
Snhg3	small nucleolar RNA host gene (non-protein coding) 3	BI082172	-17.34	unknown

Table 3-b. Summary of functions in genes with altered expression in *Thrb*$^{PV/PV}$ mice

Biological function	Count	Genes
Transcriptional regulation	19	Apobec2,Atp6v1h,Cnot10,Dgkq,Gmeb1,Ino80d,Klf7,Klhdc2,Kti12,Med8,Mtf2,Phf21a,Rfx7,Taf1a,Tcf20,Tcfe2a,Trove2,Zfp40,Zscan22
Intracellular non-membrane-bounded organelle	17	Dgkq,Taf1a,Twf2,Mrps15,Mrps21,Myh9,Zcchc17,Flnb,Mrps2,Atrx,Cgn,Nubp2,Spnb2,Epb4.1,Clasp1,Pafah1b1,Map6d1
Metabolism	17	Aldh1a2,Atp5j2,Cisd1,Coq9,Dlat,Fut9,Gdpd5,Lpcat1,Ndufa12,Npc2,Nsdhl,Ogdh,Pcbd1,Suclg2,Sc4mol,Sorbs1,Xylb
Cytoskeleton	13	Dgkq,Twf2,Nubp2,Cgn,Spnb2,Epb4.1,Pafah1b1,Clasp1,Myh9,Flnb,Map6d1,Fhdc1,Daam1
Ribonucleoprotein complex	9	Prpf19,Trove2,Mrps15,Bat1a,Srrm1,Mrps21,Zcchc17,Mrps2,Sfrs12
Protein catabolic process	7	Rnf125,Lnpep,Med8,Socs1,Ube2f,Myh9,Senp7
Translation	6	Eif4g3,Mrps15,Hbs1l,Eif2s2,Mrps21,Mrps2
Intracellular transport	5	Stx17,Spnb2,Pafah1b1,Myh9,Kpna1
Tight junction	5	Cgn,Spnb2,Epb4.1,Ash1l,Myh9
mRNA processing	5	Prpf19,Apobec2,Bat1a,Srrma,Sfrs12
Calmodulin binding	4	Spnb2,Camk2d,Myh9,Map6d1
Methyltransferase	4	Ccdc76,Lcmt1,Pcmtd2,Ash1l
Cell-cell junction	4	Sorb1,Cgn,Ash1l,Myh9
Negative regulation of protein complex disassembly	3	Spnb2,Clasp1,Map6d1
Nuclear speck	3	Sfrs18,Dgkq,Srrm1
Cell signaling	11	Dgkq,Dusp14,Fst,Il10rb,Kremen1,Phip,Plaa,Plekhg3,Saps3,Socs1,Twf2
Transporter	11	Abca4,Atp6v1h,Cacnb3,Camk2d,Kctd14,Kpna1,Rapgef4,Scfd1,Slc12a9,Slc35a3,Slc4a4,

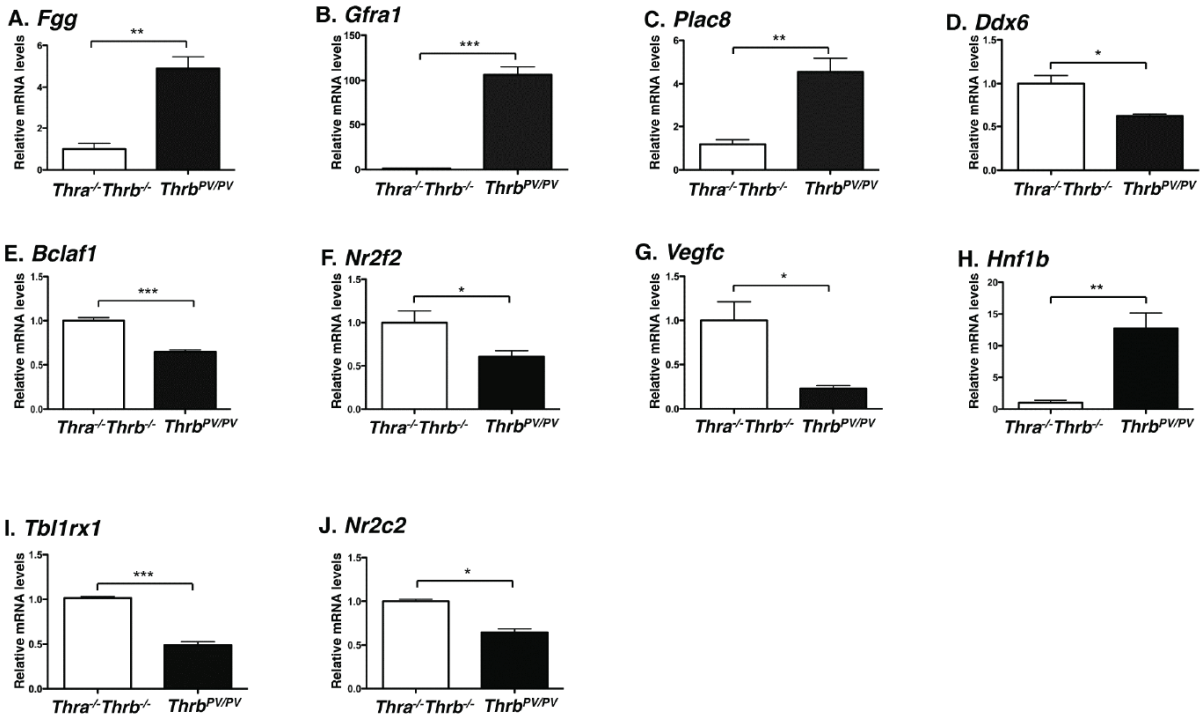


Figure 2. Validation of altered gene expression in the thyroids of $Thrb^{PV/PV}$ and $Thra1^{-/-}Thrb^{-/-}$ mice by real time RT-PCR. Total RNA was extracted from the mouse thyroid at the age of 9-12 months, and real time RT-PCR was performed as described in Materials and Methods. Fold of changes to the mRNA level of $Thra1^{-/-}Thrb^{-/-}$ mice is shown. An "*" indicates $p<0.05$; "**", $p<0.01$; and "***", $p<0.001$, by the Student's *t* test.

expressions were found for *Fgg, Gfra*, and *Plac8*, respectively. The expressions of several relatively down-regulated genes including DEAD (Asp-Glu-Ala-Asp) box polypeptide 6 (*Ddx6*), BCL2-associated transcription factor 1 (*Bclaf1*), and nuclear receptor subfamily 2, group F, member 2 (COUP-TFII, *Nr2f2*) were also examined by real time RT-PCR. As shown in **Figures 2D-F**, 45%, 50%, and 50% repressions of the mRNA expression were found for *Ddx6*, *Bclaf1,* and *Nr2f2*, respectively. These results show a concordance in gene expression as determined by RT-PCR and microarray analyses.

It is important to note that the 100 genes, 45% of the total 241 genes with altered expression mediated by PV exhibited diverse and broad cellular functions relevant to tumorigenesis and metastasis as revealed by the functional annotation (**Table 1-b**). These functions include transcription, RNA and DNA metabolic processes, cell development, differentiation, proliferation, and apoptosis. Moreover, genes involved in embryogenesis, angiogenesis, and cell adhesion were also detected. Thus, PV exerts extensive effects via gain-of-function on critical cellular functions to drive carcinogenesis.

Uncovering genes specifically affected by PV via gain-of-function in the thyroid

Microarray analyses uncovered 11 genes involved in thyroid carcinoma that exhibited altered expression in thyroid tumor cells of $Thrb^{PV/PV}$ mice (**Table 2**). Among these genes, increased expression of the *GFRA1* gene was reported in medullary thyroid carcinoma [26]. Consistent with this observation, microarrays also showed an increase in its expression (11.78-fold; **Table 2**) in the thyroid lesions of $Thrb^{PV/PV}$ mice as compared with $Thra1^{-/-}Thrb^{-/-}$ mice. Array analysis identified another thyroid cancer-related gene, *Minpp1* (multiple inositol polyphosphate histidine phosphatase 1), which is a phosphatase with an overlapping function with that of PTEN. Its gene localization (10q23.3) is near to the PTEN gene. Loss of heterozygosity in this locus is frequent in follicu-

lar thyroid tumors [27]. Intriguingly, the expression of this gene was up-regulated (4.07-fold) according to the array analysis. Another key thyroid cancer-related gene, *Vegfc* (vascular endothelial factor C), was also identified by the array analysis. Vegfc is a key regulator in stimulating proliferation of lymphatic vessels and thus is related to lymphatic metastasis of tumors [28]. Moreover, it is known that serum VEGF (s-VEGF) and VEGF-c (s-VEGF-C) are elevated in patients with recurrence of papillary thyroid cancer (PTC) and correlated significantly with the presence of nodal metastases and advanced tumor stages [29, 30]. Paradoxically, decreased expression of the *Vegfc* gene was detected in the tumor cells of $Thrb^{PV/PV}$ mice as compared with $Thra1^{-/-}Thrb^{-/-}$ mice (**Table 2**). Array analysis indicated a 4.17-fold decrease, a finding confirmed by RT-PCR (4.5-fold decrease; **Figure 2G**). In addition, **Table 2** also shows that several transcriptional co-regulators of TR or TR-targeted genes, such as the HNF1 homeobox B gene (*Hnf1b*; 2.7-fold up-regulation), transducin (beta)-like 1X-linked receptor 1 (*Tbl1xr1;* 2.69-fold down-regulation), and nuclear receptor subfamily 2, group C, member 2 (TR4, *Nr2c2;* 4.49-fold down-regulation), also showed altered expression in the tumor cells of $Thrb^{PV/PV}$ mice. The expressions of the *Hnf1b, Tbl1xr1,* and *Nr2c2* genes were confirmed by RT-PCR, as shown in **Figure 2H**, **I** and **J** with 10-fold up-regulation; 2.2-fold and 2-fold down-regulation, respectively.

Alteration in the expression of genes with diverse functions collaborates with the PV oncogenic actions

In addition to the genes involved in tumorigenesis or metastasis shown in **Tables 1** and **2**, array analysis identified 135 genes (56% of total genes) with altered expression related to other biological functions. This large group includes 45 genes that were up-regulated (ranging from 2.52- to 5.25-fold) and 90 genes that were down-regulated (ranging from 2.55- to 17.34-fold). These 135 genes have broad and diverse cellular functions (see **Table 3-a**) including transcription regulation, cytoskeleton remodeling, molecule transport, cell signaling, RNA processing, protein translation, protein-protein interaction, cell signaling, and metabolism (**Table 3-b**). That PV could invoke the changes in the expression of an extensive array of genes with diverse cellular functions would suggest that its oncogenic actions would require that genes not be exclusively involved in tumorigenesis, but also collaborate with genes involved in other cellular functions. How some of these genes could network with each other to carry out the oncogenic functions of PV via gain-of-function is further elucidated in the next section.

Gene interaction network analysis implicates involvement of multiple signaling pathways

Via Ingenuity Pathway Analysis software, 25 networks that could function coordinately to bring out the tumorigenic phenotypic manifestation were predicted from the genes identified with altered expression. With the highest score of gene correlation, the top four networks are shown in **Figure 3**. In Network 1, a group of genes related to tissue development such as lymphoid tissue formation was linked (**Figure 3A**). Among them, we noted *Vegfc*, *Nr2f2*, and ephrin B2 (*Efnb2*). *Vegfc* and *Nr2f2* are known to be essential in lymphangiogenesis whereas *Efnb2* has been related to lymph node metastasis [31, 32]. All of these genes showed a reduced expression level in the thyroid of $Thrb^{PV/PV}$ mice, which might account for the observations that in $Thrb^{PV/PV}$ mice the metastatic target sites (lung and heart) are via the vascular system, rather than via lymphatic pathways. However, Network 1 shows an increased mRNA level of metallothionein 3 (*Mt3*) which may contribute to the angiogenesis by encoding a small peptide that can significantly induce *Vegf* mRNA and protein expression in mouse brain capillary endothelial cell line bEND.3 cells during hypoxia [33]. Gene-like bone morphogenetic protein 5 (*Bmp5*) in Network 1 is associated with cardiovascular system development, and its transcription is under the regulation of Rbm5 (RNA-binding motif protein 5), a tumor suppressor whose mechanism involves potentiating apoptosis and inhibition of the cell cycle [34]. The coordinated down-regulation of *Bmp5* and *Rbm5* outlined in Network 1 would suggest that the repression of Rbm5 could lead to decreased apoptosis and increased tumor cell proliferation of thyroid tumor cells.

In Network 2, genes involved in DNA replication, DNA repair, and cell signaling were crosslinked (**Figure 3B**). In our previous study, we discovered chromosomal aberrations in cell lines derived from thyroid tumors of $Thrb^{PV/PV}$ mice. These cell lines exhibit abnormal karyotypes and a variety of structural chromosomal aberrations, including common recurrent transloca-

Figure 3A. Network 1: Tissue development and lymphoid tissue structure and development

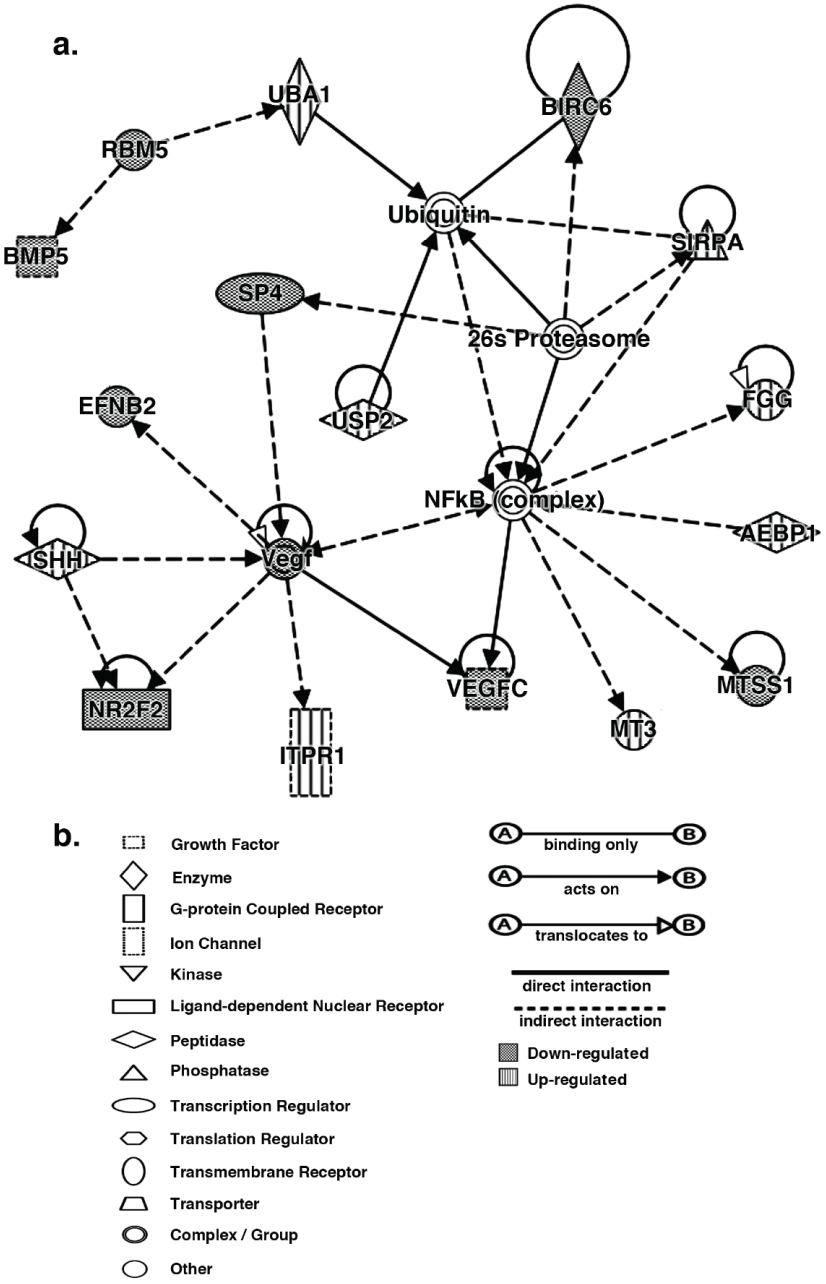


Figure 3. Network prediction of differentially expressed genes in thyroid tumor cells between *Thrb*$^{PV/PV}$ and *Thra1*$^{-/-}$*Thrb*$^{-/-}$ mice. The four top-scoring networks (**A-D**) are displayed graphically. As explained in the legend (**A-b**), different shapes represent different types of proteins while the relationships between proteins are indicated by the solid bar (direct interaction), dotted bar (indirect relationship), or arrow (acting on). The increased (symbol with vertical lines) and decreased expression (hatched symbol) is marked as indicated in the legends (**A-b**). (Refer to next page for Figure 3B, C and D).

tions and deletions, raising the possibility that induction of chromosomal instability may contribute to the thyroid carcinogenesis in *Thrb*$^{PV/PV}$ mice [35]. There is also an aberrant accumulation of a critical mitotic checkpoint protein, Pttg1 (pituitary tumor-transforming gene 1), that helps hold sister chromatids together before entering anaphase, and thus impedes mitotic progression in cells expressing PV [36]. From Network 2, we uncovered additional genes (*Smc4*, *Sfpq*, *Fus*, *Pafah1b1*, *Rpa1*) that play critical roles in DNA replication, cell cycle regulation, maintenance of chromosome stability, and/or DNA repair, which were also deregulated in *Thrb*$^{PV/PV}$ mice. Smc4 (structural maintenance of chromosomes 4) is an essential component of the condensin complex. The Smc complex is vital for a wide range of processes including chromosome structure and dynamics, gene regulation, and DNA repair [37]. Sfpq (splicing factor proline/glutamine rich) has functions in pre-mRNA processing [38], transcriptional regulation, and DNA repair by complexes with its paralog named NONO/p54nrb or RAD51D/RAD51C/XRCC2 during different DNA repair events [39, 40]. Depletion of SFPQ can lead to increased chromosomal aberrations and substantial sister chromatid cohesion defects [40]. Fus (fusion, derived from t (12;16) malignant liposarcoma gene), an oncogenic RNA binding protein, is also related to DNA repair as evidenced by the fact that

Fig.3B. Network 2: DNA replication, DNA repair, and cell signaling

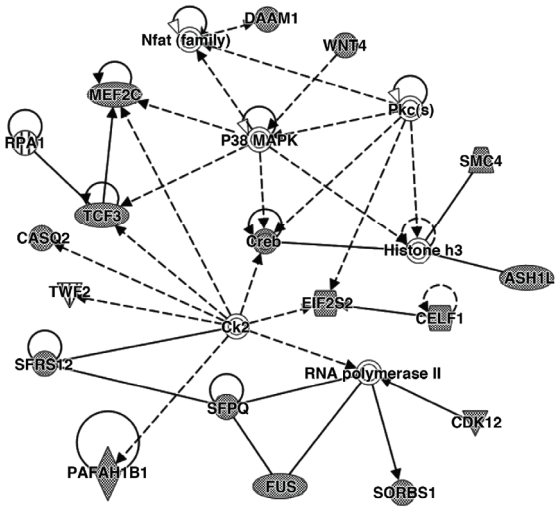


Fig. 3C. Network 3: Cancer-related signaling pathways

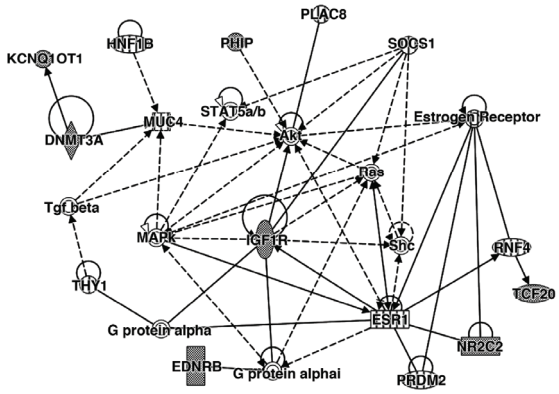
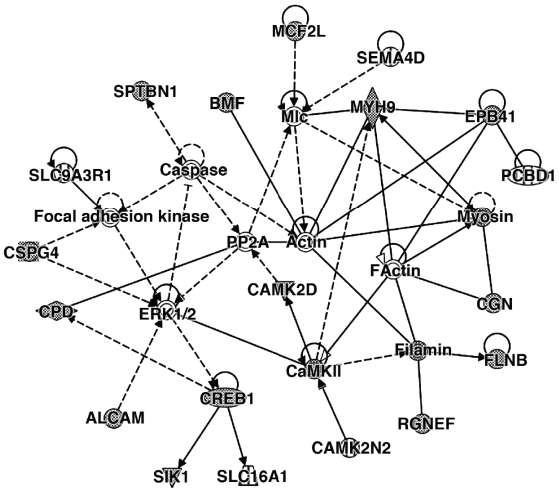


Fig. 3D. Network 4: Cytoskeleton organization, molecular transport, and small molecule biochemistry



mice lacking the protein FUS are hypersensitive to ionizing radiation and FUS is a phosphorylation target of the ATM (ataxia-telangiectasia mutated) signaling pathway in DNA repair [41-43]. Pafah1b1 (platelet-activating factor acetylhydrolase, isoform 1b, subunit 1) plays a pivotal role in microtubule regulation and mitotic spindle orientation of neuroepithelial stem cell proliferation [44]. Remarkably, expression of these four genes was decreased in a coordinated manner in *Thrb*$^{PV/PV}$ mice. This reduction could further weaken the chromosomal stability in *Thrb*$^{PV/PV}$ mice. Replication protein A (Rpa1), a major eukaryotic ssDNA binding protein, is required for cell viability and plays essential roles in DNA replication, repair, and recombination [45]. A higher RPA expression has been reported in breast and colon cancers as a factor in promoting cell proliferation during tumor growth and progression [46, 47]. In *Thrb*$^{PV/PV}$ mice, the mRNA level of *Rpa1* was also higher than in *Thra*$^{-/-}$*Thrb*$^{-/-}$ mice. In this network, we also noted several key regulators in the Wnt/b-catenin signaling pathway, including Wnt4 (wingless-related MMTV integration site 4) and Daam1 (dishevelled associated activator of morphogenesis 1), that could crosstalk with the p38 MAPK pathway. Previously, we showed dysregulation of both signaling cascades (Wnt/b-catenin and p38 MAPK) in thyroid carcinogenesis of *Thrb*$^{PV/PV}$ mice [17, 48]. However, it was unknown whether these two pathways could be linked via PV to affect thyroid carcinogenesis. Thus, the possibility that the two pathways can crosstalk could potentially open a new area of study to gain further insights into thyroid carcinogenesis.

In Network 3, genes participating in the cancer-related signaling pathways are shown. Several key nodes are Akt, Mapk, Ras, and IGF1R. Aberrant activation of the signaling involving these key regulators has been shown to be associated with human follicular thyroid carcinoma. Consistent with human cancer, PI3K-Akt is also activated during thyroid carcinogenesis of *Thrb*$^{PV/PV}$ mice [18, 19]. One of the genes with altered expression identified in Network 3 that merits further investigation is estrogen receptor (ER). A long-standing question in the understanding of human thyroid cancers is the preponderance of female patients in the ratio of 3- or 4-to-1 over male patients. Network 3 shows the extensive down-stream regulators and effectors of the PV-activated ER signaling. These ER downstream

regulators need to be further studied to understand the gender disparity in thyroid cancer.

Network 4 shows sets of genes grouped by their common functions in cytoskeleton organization, molecular transport, and small molecule biochemistry (**Figure 3D**). In this group, several genes related to cytoskeleton structure or cell adhesion (e.g., erythrocyte protein band 4.1, *Epb4.1*, MCF.2 cell line derived transforming sequence-like, *Mcf2l*, myosin, heavy polypeptide 9, non-muscle, *Myh9*, filamin, beta, *Flnb*, cingulin, *Cgn*) were identified. The differential expression of these actin-related genes might give insights into how cellular behaviors in vascular invasion differed between $Thrb^{PV/PV}$ and $Thra1^{-/-}Thrb^{-/-}$ mice.

Discussion

We have previously shown that both $Thrb^{PV/PV}$ and $Thra1^{-/-}Thrb^{-/-}$ mice spontaneously develop FTC similar to human thyroid cancer [13, 22]. However, it is important to point out that FTC is more aggressive in $Thrb^{PV/PV}$ than $Thra1^{-/-}Thrb^{-/-}$ mice. The size of thyroid tumors is larger and the pathological progression is faster with a higher frequency of metastasis. This observation is intriguing in view of the fact that $Thrb^{PV/PV}$ mice harbor a mutated TRβ that was expected to function as a dominant negative mutant, leading only to the loss of normal TR suppressor functions. The phenotypic manifestation of thyroid tumors in $Thrb^{PV/PV}$ mice was expected to be similar to that of $Thra1^{-/-}Thrb^{-/-}$ mice that have lost TR suppressor functions due to deficiency in TRs. That FTC is more aggressive in $Thrb^{PV/PV}$ than in $Thra1^{-/-}Thrb^{-/-}$ mice prompted us to test the hypothesis that PV, in addition to the loss of normal TR functions, could also act via gain-of-function. Indeed, global gene expression profiling of the microdissected thyroid tumor cells from the two groups of age- and gender-matched mutant mice shows contrasting expression patterns of 241 genes. Although the majority of the genes with contrasting expression are involved in tumorigenesis and metastasis, the remaining genes play key roles in broad and diverse cellular functions. These findings indicate that PV, in addition to acting as a mutant that has lost normal TR functions, could also act via gain-of-function to promote thyroid carcinogenesis.

Extensive analysis of the gene expression data using gene ontology, functional annotation, and gene network prediction has provided new insights into the molecular signaling pathways that lead to a more aggressive thyroid malignancy in $Thrb^{PV/PV}$ mice than in $Thra1^{-/-}Thrb^{-/-}$ mice. Previously, we identified several pathways including PI3K/Akt, Wnt/β-catenin, and p38 MAPK signaling that are dysregulated in $Thrb^{PV/PV}$ mice [17, 19, 48]. In $Thra1^{-/-}Thrb^{-/-}$ mice, activation of PI3K/Akt signaling was also observed [22]. The present studies showed that different subsets of effectors/regulators were implicated in the activation of the same pathways that could further modulate the magnitudes of the responses. The different subsets of genes could also function in different pathways to regulate the extent or alter the outcome of signaling. *Gfra1* was one such gene uncovered in the present studies. Its mRNA expression was highly elevated in the thyroid tumors of $Thrb^{PV/PV}$ mice (**Table 1-a** and **Figure 2B**). This gene encodes a glycosylphosphatidylinositol (GPI)-linked co-receptor of proto-oncogenic receptor tyrosine kinase RET (rearranged during transfection) in mediating extracellular signal from glial cell line-derived neurotrophic factors (GDNFs) via Ras/MAPK, PI3K/Akt, and PLCγ pathways [49, 50]. Gfra1 also directly complexes with neural cell adhesion molecule (NCAM) and activates Fyn kinase to regulate cell migration, neuronal morphology, and synapse formation [51]. Gfra1 can also mediate cell adhesion uniquely in a ligand-dependent manner [52]. In addition, Gfra1 can be shed from the cell surface by the action of membrane-associated phospholipases. The soluble Gfra1 stably binds to GDNF to act at a distance to promote neuronal survival and neurite outgrowth on RET-expressing neurons/axons [53, 54]. In thyroid cancers, increased expression level of *Gfra1* or constitutive activation of RET signaling has been reported due to the rearrangement of *RET* (*RET/PTC*) in papillary thyroid cancer (PTC) or medullary thyroid carcinoma patients [26, 55]. This raises the possibility that a highly increased expression level of *Gfra1* in $Thrb^{PV/PV}$ mice could be involved in the activation of the RET signaling in these mice. If this possibility proves to be true in future studies, $Thrb^{PV/PV}$ mice would become a promising preclinical animal model in testing novel RET inhibitors for thyroid cancer treatment.

In addition to the identification of Gfra1 as a novel modulator of Ras/MAPK, PI3K/Akt signaling in thyroid carcinogenesis of $Thrb^{PV/PV}$ mice, we also detected the highly activated expression of *Shh* (sonic hedgehog) that could also

function to modulate the PI3K/Akt and Wnt/β-catenin pathways. *Shh* encodes a signaling peptide functioning via the Patched (Ptc)-Smoothened (Smo) receptor complex and glioblastoma (Gli) family of transcription factors in controlling cell proliferation and differentiation. The Shh pathway crosstalks with PI3K-Akt and Wnt/β-catenin pathways at multiple levels and coordinates developmental transitions in mammals [56]. In thyroid, knockout of the *Shh* gene leads to hemiagenesis of thyroid and ectopic expression [57]. In addition to Shh, several Shh-signaling-related genes such as *Aldh1a2*, *Mtss1*, and *Nsdhl* were also identified in the analysis. Aldh1a2 (aldehyde dehydrogenase 1 family, member A2) catalyzes the synthesis of retinoic acid, which regulates early mouse embryonic forelimb development by controlling sonic hedgehog signaling [58]. The increased mRNA level of *Aldhla2* (3.7 fold, **Table 3-a**) found in $Thrb^{PV/PV}$ mice may enhance the activated Shh signaling. Another gene that regulates hedgehog signaling is *Nsdh1* (*N*ADH sterol dehydrogenase-like), which was found to be up-regulated (2.7-fold, **Table 4-a**) in $Thrb^{PV/PV}$ mice as well. This gene encodes a sterol dehydrogenase involved in the removal of C-4 methyl groups in one of the later steps of cholesterol biosynthesis. In *Nsdhl*-deficient mice, the abnormality of placental development is related to the hedgehog signaling pathway [59]. This may be due to the decrease of sterol synthesis that directly affects one or more proteins (including Shh) associated with sterols. This could also be due to the alteration of the properties of the plasma membrane which may secondarily affect Shh signaling pathway [60, 61]. Another gene involved in Shh signaling is *Mtss1* (metastasis suppressor 1). *Mtss1* is a direct target gene of Shh-Gli signaling and its product can potentiate Gli-dependent transcription [62]. Mtss1 is also able to bind monomeric actin and behaves as a tumor suppressor in cell proliferation [63, 64]. In our study, the expression of *Mtss1* (5-fold; **Table 1-a**) is not consistent with the increased mRNA level of *Shh* in $Thrb^{PV/PV}$ mice, which suggests that *Mtss1* may not be regulated by Shh in thyrocytes but affects thyroid tumorigenesis via its tumor suppressor function.

That thyroid tumors of $Thrb^{PV/PV}$ mice are larger than those of $Thra1^{-/-}Thrb^{-/-}$ mice could be understood further from the identification of several down-regulated genes encoding pro-apoptotic factors (*Bclaf1*, *Bmf*, and *Bnip2*) related to Bcl2 in the present studies. The suppression of apoptosis by lowering the expression of these pro-apoptotic molecules sustains malignant cell proliferation in thyroids of $Thrb^{PV/PV}$ mice. Thus, while the proliferation of thyroid tumor cells of $Thrb^{PV/PV}$ mice and $Thra1^{-/-}Thrb^{-/-}$ mice is stimulated, the decreased apoptosis due to the suppression of apoptotic activity in thyroid tumor cells of $Thrb^{PV/PV}$ mice gains additional proliferation advantage. The contribution of the apoptotic pathways to the more aggressive thyroid malignancy due to PV via the gain-of-function suggests new therapeutic strategies that would use these apoptotic genes as molecular targets.

Ever since the identification of dominantly negative TRβ mutations in RTH patients, the molecular mechanisms by which these mutants function in the pathogenesis of RTH have been extensively investigated. While it has long been accepted that these TRβ mutations act in a dominant negative fashion to interfere with the functions of WT TRs, whether they could also function via gain-of-function was less extensively explored. The contrasting gene expression profiles in the thyroids of $Thrb^{PV/PV}$ and $Thra1^{-/-}Thrb^{-/-}$ mice clearly show that PV can act beyond the dominant negative mode. It is possible that this gain-of-function of PV can also operate in other target tissues besides the thyroid, thereby further contributing to the pathogenesis of RTH. The verification of this possibility awaits further studies.

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