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► **To cite this version:**

Angela Greco, C. Miranda, M.A. Pierotti. Rearrangements of gene in papillary thyroid carcinoma. *Molecular and Cellular Endocrinology*, 2010, 321 (1), pp.44. 10.1016/j.mce.2009.10.009. hal-00582104

**HAL Id: hal-00582104**

**<https://hal.science/hal-00582104v1>**

Submitted on 1 Apr 2011

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## Accepted Manuscript

Title: Rearrangements of *NTRK1* gene in papillary thyroid carcinoma

Authors: Angela Greco, C. Miranda, M.A. Pierotti

PII: S0303-7207(09)00556-5  
DOI: doi:10.1016/j.mce.2009.10.009  
Reference: MCE 7355

To appear in: *Molecular and Cellular Endocrinology*

Received date: 30-6-2009  
Revised date: 18-9-2009  
Accepted date: 20-10-2009

Please cite this article as: Greco, A., Miranda, C., Pierotti, M.A., Rearrangements of *NTRK1* gene in papillary thyroid carcinoma, *Molecular and Cellular Endocrinology* (2008), doi:10.1016/j.mce.2009.10.009

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**REARRANGEMENTS OF *NTRK1* GENE IN PAPILLARY THYROID CARCINOMA**

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**Abstract**

*TRK* oncogenes are observed in a consistent fraction of papillary thyroid carcinoma (PTC); they arise from the fusion of the 3' terminal sequences of the *NTRK1/NGF* receptor gene with 5' terminal sequences of various activating genes, such as *TPM3*, *TPR* and *TFG*. TRK oncoproteins display constitutive tyrosine kinase activity, leading to *in vitro* and *in vivo* transformation. In this review studies performed during the last 20 years will be summarized. The following topics will be illustrated: a) frequency of *TRK* oncogenes and correlation with radiation and tumor histopathological features; b) molecular mechanisms underlying *NTRK1* oncogenic rearrangements; c) molecular and biochemical characterization of TRK oncoproteins, and their mechanism of action; d) role of activating sequences in the activation of TRK oncoproteins.

**Keywords:** NTRK1, gene rearrangement, papillary thyroid tumor

Papillary thyroid carcinoma (PTC), the most frequent neoplasia originating from the thyroid epithelium, accounts for about 80% of all thyroid cancers (Hedinger *et al.*, 1988). A consistent fraction of PTC (up to 60%) harbors chimeric oncogenes created by chromosomal rearrangements involving prevalently *RET* and, to a less extent, *NTRK1* loci (Pierotti *et al.*, 1996). Recently, the BRAF V600E mutation, increasing its basal kinase activity, has been identified as the most common genetic lesion in PTC with frequency ranging from 29 to 69% (Frattoni *et al.*, 2004; Kimura *et al.*, 2003; Soares *et al.*, 2003).

Both *RET* and *TRK* oncogenes have been discovered in our laboratory by DNA transfection/focus formation assay in NIH3T3 cells, starting from papillary thyroid tumor DNA. Transforming activity correlated with the presence of human *RET* and *NTRK1* sequences in the mouse transfectants DNA (Bongarzone *et al.*, 1989); this provided the basis for the isolation and characterization of the chimeric oncogenes, containing the receptor tyrosine kinase domain preceded by activating sequences from different genes.

*TRK* oncogenes arise from rearrangements of the *NTRK1* gene (also known as *TRKA*) on chromosome 1. *NTRK1* encodes the high affinity receptor for NGF; it exerts a critical role for development and maturation of central and peripheral nervous system and, in addition, it stimulates proliferation of a number of cell types such as lymphocytes, keratinocytes and prostate cells (Di Marco *et al.*, 1993; Djakiew *et al.*, 1991; Otten *et al.*, 1989). *NTRK1* was originally isolated from a human colon carcinoma as a transforming oncogene activated by a somatic rearrangement that fused *TPM3* (non-muscle tropomyosin) gene to the kinase domain of a novel tyrosine kinase receptor (Martin-Zanca *et al.*, 1986). Cloning of the full length gene (Martin-Zanca *et al.*, 1989) and identification of the NGF as a ligand occurred few years later (Kaplan *et al.*, 1991; Klein *et al.*, 1991).

The *TRK* oncogenes isolated from papillary thyroid tumors are reported in Table 1. The most frequent oncogene is *TRK* (Butti *et al.*, 1995), identical to that first isolated from colon carcinoma, and containing sequences from the *TPM3* gene on chromosome 1q22-23 (Wilton *et al.*, 1995). *TRK-T1* and *TRK-T2* are activated by different portions of *TPR* (Translocated Promoter Region) gene on chromosome 1q25 (Greco *et al.*, 1992; Greco *et al.*, 1997; Miranda *et al.*, 1994). *TRK-T3* is activated by TFG (*TRK Fused Gene*), a novel gene on chromosome 3q11-12 (Greco *et al.*, 1995). Details on *TRK* oncoproteins will be reported in the following paragraphs.

Somatic rearrangements of the *NTRK1* gene in PTC are less common than those involving *RET* gene; their frequency does not exceed 12%. The association of *NTRK1* rearrangements with radiation is not clearly defined. No experimental data documenting the association of *TRK* oncogenes with radiation are available. In PTC from patients exposed at young age to radioiodine released from the Chernobyl reactor a high rate of *RET* rearrangements was observed; on the contrary, the frequency of *NTRK1* rearrangements was similar to that of sporadic PTC (Rabes *et al.*, 2000). Analogously, the same frequency of *NTRK1* rearrangements in sporadic PTC was detected in a cohort of PTCs associated with therapeutic ionizing radiation (Bounacer *et al.*, 2000). Analysis of correlation between *NTRK1* rearrangements with clinicopathological features did not produce unequivocal data. The number of PTCs carrying *TRK* oncogenes so far identified is limited, also because in some studies the genotyping of PTCs is restricted to *RET* rearrangements and *BRAF* mutation analysis. In addition, most correlation studies

consider PTCs carrying *RET* and *TRK* rearrangement as a unique group. Bongarzone and coworkers (Bongarzone *et al.*, 1998) reported the association between *RET* and *NTRK1* rearrangements with young age at diagnosis and a less favorable disease outcome, and the lack of association with tumor subtype. Similarly, in a study of PTC from the Polish population the lack of association of oncogenic rearrangements and tumor subtype was reported (Brzezianska *et al.*, 2006). Analysis of the survival rate of thirteen PTC patients with *NTRK1* rearrangement demonstrate a worse prognosis when compared to patients with *RET* rearrangement (15 cases) or without any rearrangement (89 cases) (Musholt *et al.*, 2000).

Experimental evidence suggests that *TRK* oncogenes exert a direct role and represent an early event in the process of thyroid carcinogenesis. Transgenic mice carrying *TRK-T1* oncogene under the control of thyroglobulin promoter (*Tg-TRK-T1* mice) develop thyroid hyperplasia and papillary carcinoma (Russell *et al.*, 2000). Crossing of *Tg-TRK-T1* mice with *p27<sup>kip1</sup>*-deficient mice increased the penetrance of thyroid cancer and shortens the latency period of tumor incidence, indicating that *TRK-T1* needs the cooperation with oncosuppressor genes to transform thyroid epithelium (Fedele *et al.*, 2009).

### Molecular mechanisms underlying oncogenic *NTRK1* rearrangements

Figure 1 shows the chromosomal localization and transcriptional orientation of *NTRK1* and its rearranging partners: *TPM3*, *TPR* on chromosome 1q, and *TFG* on chromosome 3q. The type of chromosomal rearrangement generating *TRK* oncogenes is not documented, since no cytogenetic studies on PTCs carrying *NTRK1* rearrangements are available. A t(1;3)(q21;q11) translocation is most likely responsible for the generation of *TRK-T3* oncogene (*TFG/NTRK1* rearrangement). For *TRK*, *TRK-T1* and *TRK-T2*, involving rearranging genes located on the q arm of chromosome 1, similarly to *NTRK1*, the most likely rearrangement is chromosome inversion; this is supported by the presence in the tumor DNA of the reciprocal products of the rearrangement, and by the evidence that *NTRK1* has transcriptional orientation opposite to that of *TPM3* and *TPR*, as deduced by analysis of sequence data available in public databases (Fig. 1) (Greco *et al.*, 2004).

The *NTRK1* genomic rearrangements present in the tumor DNA as well as the *NTRK1* locus have been cloned and characterized (Butti *et al.*, 1995; Greco *et al.*, 1995; Greco *et al.*, 1996; Greco *et al.*, 1997). The *NTRK1* gene consists of 17 exons distributed within a 25 kb genomic region (Fig. 2). All the breakpoints fall within a *NTRK1* region of 2.9 Kb showing a GC content of 58.8% (Greco *et al.*, 1993a). They occur within intronic sequences (*TRK*, *TRK-T2*) or exonic sequences (*TRK-T3*, *TRK-T1*) (Fig. 2).

Despite the high frequency of chromosomal rearrangements in PTC (about 60% of the cases), the molecular bases underlying the predisposition of thyrocytes to undergo chromosome rearrangements are not completely understood. The intrinsic capacity of a cell to repair DNA double strand breaks (DSBs) might contribute to chromosomal rearrangements. The *NTRK1* rearrangements are balanced; in fact, in addition to the oncogenic rearrangement containing the 5' portion of the *NTRK1* receptor gene, the reciprocal product (ie: the 5' portion of *NTRK1* fused to the 3' portion of each activating gene) was present in tumor DNA. Sequence analysis of thyroid-specific *TRK* oncogenes

genomic breakpoints revealed the presence of short sequence homology between the rearranging genes (Butti *et al.*, 1995; Greco *et al.*, 1995; Greco *et al.*, 1997) and only small deletion/insertion with respect to the germline sequences. These features suggest the involvement of the Non Homologous End Joining (NHEJ) mechanism in the generation of *TRK* oncogenes. The NHEJ pathway is activated followed DNA damage and is capable to repair DNA DSBs produced by ionizing radiations or carcinogens. It has been reported that thyrocytes respond to ionizing radiations by increasing the DNA end-joining activity, unlike other cell types. Therefore, following DNA damage, thyrocytes would be more prone to DNA repair than to apoptosis, and this would increase the likelihood of gene rearrangements (Yang *et al.*, 1997). In a recent work we have characterized the kinetics of normal human primary thyrocytes to repair DNA DSBs induced by ionizing radiation, thus providing an important step towards the dissection of the link between DNA DSBs repair and thyroid-specific oncogenic rearrangements (Galleani *et al.*, 2009).

Several recent reports have suggested that the spatial proximity of translocation-prone gene loci may favor gene rearrangements, thus proposing spatial genome topology as a contributing factor in the formation of specific cancerous chromosomal translocations. The concept that loci proximity may favor PTC-specific chromosomal rearrangements has been demonstrated for *H4/RET* and *TPR/NTRK1* rearrangements. Nikiforova *et al.* have proposed that in thyroid interphase nuclei the spatial contiguity of *H4* and *RET* loci, both located on chromosome 10, may provide the structural basis for the chromosomal inversion generating the thyroid *H4/RET (PTC1)* oncogene (Nikiforova *et al.*, 2000). Using a different experimental approach we have determined the distance between *NTRK1* and its oncogenic partner *TPR* on nuclei of normal thyrocytes and peripheral blood lymphocytes. We found that the distance between *NTRK1* and *TPR* loci in thyrocytes is reduced with respect to lymphocytes, suggesting that loci proximity may favour thyroid specific *NTRK1* oncogenic rearrangements (Roccatò *et al.*, 2005a) (Fig. 3). Spatial contiguity, which is determined by the chromosome folding in interphase nuclei (Gandi *et al.*, 2006), predisposes to gene rearrangements by placing free DNA ends, produced by ionizing radiation or other DNA damage agents, close to each other within the nuclear volume; this may favor non homologous end joining.

### **NTRK1 receptor and TRK oncoproteins: structure and function**

NTRK1, the high affinity receptor for NGF, is a member of the neurotrophin receptor family which also includes NTRK2 and NTRK3, binding to BDNF and NT3, respectively (Kaplan *et al.*, 1991; Klein *et al.*, 1991).

The NTRK1 receptor (Fig. 4) is a glycosylated protein of 140 kDa, comprising an extracellular portion, including Ig-like and Leucine rich domains for ligand binding, a single transmembrane domain, a juxta-membrane region, a tyrosine kinase domain and a C-terminal tail. Following NGF binding, NTRK1 undergoes dimerization and autophosphorylation of five tyrosine residues (Y490, Y670, Y674, Y675, and Y785). Three of these (Y670, Y674, Y675) are in the autoregulatory loop of the tyrosine kinase domain, which controls tyrosine kinase activity, and their phosphorylation results in elevated NTRK1 tyrosine kinase activity. Phosphorylation of the other two tyrosine residues (Y490 and Y785) creates binding sites for several proteins containing PTB or SH2 domains. NTRK1 activates Ras, Rac, phosphatidylinositol 3-kinase (PI3K) and the

Phospholipase C (PLC)- $\gamma$ , and signaling pathways controlled through these proteins, such as the Mitogen Activated Protein Kinase (MAPK). These signaling cascades culminate in the induction of genes mediating NGF effects on proliferation, differentiation and apoptosis (Kaplan *et al.*, 2000).

Several TRK oncoproteins differing in the activating portions have been isolated in our laboratory from thyroid tumors (Fig. 4). The TRK oncogene, identical to that first isolated from colon carcinoma, and containing sequences from the TPM3 gene, is the most frequent TRK oncogene detected in PTC (Butti *et al.*, 1995). TRK-T1 and TRK-T2 derive both from rearrangement between *NTRK1* and *TPR* gene; however, they display different structure, especially in the TPR portion (Greco *et al.*, 1992; Greco *et al.*, 1997). *TRK-T3* is activated by *TFG*, a gene first identified in this rearranged version (Greco *et al.*, 1995). All TRK oncoproteins retain NTRK1 tyrosine kinase domain and the five tyrosine residues crucial for NTRK1 activity. The activating sequences confer to the oncoproteins the following features: i) ectopic expression in thyrocytes; ii) cytoplasmic localization (despite the presence of the transmembrane domain in all of them but TRK-T1); iii) constitutive dimerization, mediated by coiled-coil domains, resulting in constitutive kinase activity.

In experimental models TRK oncoproteins recapitulate the biological effects of the NGF-stimulated NTRK1 receptor: they induce morphological transformation of NIH3T3 mouse embryo fibroblasts, and neuronal-like differentiation of rat pheocromocytoma PC12 cells (Greco *et al.*, 1993b). The mechanisms by which TRK oncoproteins mediate their effects have been in part elucidated. TRK oncoproteins interact to and activate PLC- $\gamma$ , SHC, FRS2, FRS3, IRS1 and IRS2. The tyrosine residue corresponding to Tyr785 of NTRK1 receptor is involved in the recruitment of PLC- $\gamma$ ; mutation of this site did not affect the oncogenic activity. All the other molecules are recruited by the same tyrosine residue (corresponding to Tyr490 of NTRK1), most likely in a competitive fashion. Such interaction site is crucial for oncogenic activity, as its mutation to phenylalanine completely abrogate TRK oncoprotein biological activity. Moreover, by using a SHC dominant-negative mutant unable to recruit GRB2, we showed a crucial role of SHC adaptor in TRK-T3 biological activity. It is worth noting that our studies on TRK oncogenes allowed the identification of novel proteins interacting with NTRK1 kinase, such as IRS1, IRS2 and FRS3 (Miranda *et al.*, 2001; Ranzi *et al.*, 2003; Roccato *et al.*, 2002).

It is worth noting that another member of the neurotrophin receptor family, NTRK3, undergoes oncogenic rearrangement. The ETV6-NTRK3 chimeric oncoprotein, whose mechanism of action is similar to that of TRK oncoproteins, has been detected in different tumor types, thus capable to transform multiple cell lineages (Lannon and Sorensen, 2005).

### **Role of activating sequences in TRK oncogenic activation**

The capability of *TPM3*, *TPR* and *TFG* to activate chimeric tyrosine kinase oncogenes is not restricted *TRK* oncogenes; in fact, they have been found fused to other kinase genes. Both *TPM3* and *TFG* were reported to fuse to *ALK* receptor gene in anaplastic large cell lymphoma (Hernandez *et al.*, 2002; Lamant *et al.*, 1999); moreover, *TFG* is rearranged with *NOR1* in extraskeletal myxoid chondrosarcoma (Hisaoka *et al.*, 2004). *TPR* was first identified in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-



transformed HOS cells as part of the *MET* oncogene, fused to the TK domain of the hepatocyte growth factor receptor gene (Park *et al.*, 1986); subsequently it was detected fused to *raf* during the transfection of a rat hepatocarcinoma (Ishikawa *et al.*, 1987). Interestingly, *TPR* and *TFG* were first identified in rearranged, oncogenic versions. *TPM3* gene encodes a non-muscle tropomyosin isoform. *TPR* gene encodes a large protein of the nuclear pore complex; recent studies have shown that TPR is a phosphorylated protein involved in mRNA export, through the formation of complexes with different interacting proteins (Green *et al.*, 2003; Shibata *et al.*, 2002). *TGF* encodes a protein of still unknown function.

Despite the diversity in structure and function, all NTRK1 activating proteins are ubiquitously expressed and contain coiled-coil domains that promote protein dimerization/multimerization. Coiled-coil domains are characterized by heptad repeats with the occurrence of apolar residues preferentially in the first (*a*) and fourth (*d*) positions (Lupas *et al.*, 1991; Lupas, 1996). This confers to the proteins the capability to fold into  $\alpha$ -helices that are wound into a superhelix. In Fig. 5 the coiled-coil domains detected in TRK activating sequences by sequence analysis with the COIL program (Berger *et al.*, 1995) are shown. TPM3 contains numerous, overlapping coiled-coil domains. Several coiled-coil domains are present in TPR, and two of them fall in the region contained in MET and TRK-T1 oncogenes. It has been reported that mutations within the first coiled-coil domain drastically reduces MET transforming activity (Rodrigues *et al.*, 1993). TFG contains a single coiled-coil domain, of approximately three heptads, shorter than typical coiled-coil domains. However, the presence of a hydrophobic residue in position *a*, would increase the strength of association, despite the reduced length of the domain (Greco *et al.*, 1995).

Studies on receptor and non receptor tyrosine kinase chimeric oncogenes have demonstrated that the importance of activating genes is related to the coiled-coil domains which mediate dimerization leading to constitutive tyrosine kinase activity. With respect to TRK oncogenes this concept has been demonstrated for the coiled-coil domain contained in the TFG portion of TRK-T3 oncogene. By using mutants carrying deletion or point mutations at critical leucine residues we have demonstrated that TFG coiled-coil domain plays a crucial role in TRK-T3 oncogenic activation by mediating oncoprotein complexes formation, an essential step for tyrosine kinase activation (Greco *et al.*, 1998). The TFG coiled-coil domain is predicted to fold into trimers. By size-exclusion chromatography we have demonstrated that the wild type TRK-T3 protein is part of high molecular weight complex, compatible with the assembly of six oncoproteins molecules, or the presence other proteins (Roccatò *et al.*, 2003).

An attractive hypothesis is the possibility that activating sequences may contribute with other functions, beside dimerization. In addition, since the oncogenic rearrangements disabled one allele of the activating gene, the reduced activity of the corresponding protein could play a role in thyroid carcinogenesis. In this respect TFG, involved in TRK-T3 oncogene, represents an attractive model for at least three reasons: 1) it codes for a novel protein whose function remains to be unveiled; 2) its portion contained in TRK-T3 display a single, short coiled-coil domain, corresponding to 14% of the aminoacid sequence; 3) it might interact with other proteins (see later).

After initial isolation in our laboratory as part of the *TRK-T3* oncogene, the normal *TFG* counterpart was cloned and characterized (Mencinger *et al.*, 1997) (Fig. 6).

*TFG* gene is ubiquitously expressed in human adult tissues and it is conserved among several species, including *C. elegans*. Human *TFG* encodes a 400 aminoacids protein of apparent molecular weight of 50 kDa showing punctuate cytoplasmic distribution compatible with the capability to form aggregates (Fig. 6). In addition to the coiled-coil domain, the TFG protein contains putative phosphorylation sites for PKC and CK2, glycosilation sites, as well SH2- and SH3- binding sites. Several of these sites are identical in TFG proteins from different species, indicating that the protein might be involved in basic cell processes (Mencinger *et al.*, 1999). The TFG N-terminal portion contains a PB1 domain (Roccatto *et al.*, 2003), a protein module mediating protein interaction (Nakamura *et al.*, 2003; Terasawa *et al.*, 2001).

In the past years we studied the role of TFG sequences outside the coiled-coil domain in TRK-T3 oncogenic activation. On the whole our studies demonstrate that the regions outside the coiled-coil domain give a great contribution to TRK-T3 activation (Roccatto *et al.*, 2003). When deleted, complexes formation is unaffected; however transforming activity is reduced to different extent. More detailed information was provided by studies employing point mutants: transforming activity was significantly reduced by mutating the Tyr 33 residue within a putative SH2- binding motif, whereas it was abrogated by the mutation of the conserved Lys 14 residue within the PB1 domain.

We then focused our effort on the identification of proteins partners of TFG by characterizing interactions either suggested by the presence of consensus sequences within TFG, or identified by a yeast two hybrid system approach. Our studies led to the discovery of two important cellular pathways in which TFG is involved: 1) TFG is a novel SHP-1 auxiliary docking protein which participates to the downregulation of phosphatase activity (Roccatto *et al.*, 2005b); 2) TFG is involved in NF- $\kappa$ B activation, through the interaction with NEMO and TANK (Miranda *et al.*, 2006).

Additional information on TFG possible physiological function has been provided by recent studies. By mass spectrometry analysis TFG was identified a src interacting protein; experiments in living cells and *in vitro* kinase assay demonstrated that TFG is a src kinase substrate (Amanchy *et al.*, 2008). TFG has been recently identified as a protein capable to correct the trafficking defect of the Cystic fibrosis transmembrane regulator mutant (F508del-CFTR), which causes cystic fibrosis (Trzcinska-Daneluti *et al.*, 2009). A report on TFG-1, the *C. elegans* TFG ortholog, has shown that it is capable to suppress apoptosis, and it exerts an essential role for normal cell-size control (Chen *et al.*, 2008).

More studies will be required to dissect how the TFG functions so far identified may contribute to thyroid carcinogenesis, as well as to define precisely the TFG physiological role.

## Conclusions

Although occurring less frequently than *RET* rearrangements and *BRAFV600E* mutation, rearrangements of *NTRK1* provide a useful model for studying the molecular basis of thyroid carcinogenesis. Studies performed almost exclusively in our laboratory have defined the mechanisms responsible for *NTRK1* oncogenic rearrangements, and identified the molecular players involved in the process of TRK oncogenes transformation. Studies on *TRK* activating sequences have led to the identification of a novel gene, whose product is involved in important cellular functions. Although further

investigation is still required, studies on *TRK* oncogenes contributed to pave the way for the comprehension of the molecular mechanisms of thyroid carcinogenesis.

#### Acknowledgements

This work is supported by AIRC (Associazione Italiana Ricerca Cancro), ACC (Alleanza Contro il Cancro), Ministry of Health. The Authors thanks Mrs. Cristina Mazzadi for secretarial help.

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## Figure legends

**Figure 1.** Ideogram of chromosomal 1 and 3 showing the localization of *NTRK1*, *TPM3*, *TPR* and *TFG* genes. The arrows indicate the transcriptional orientation.

**Figure 2.** Genomic structure of human *NTRK1* gene. Lines indicate introns; boxes represent exons; the open box indicates an alternative exon. Translation initiation (ATG) and termination (TAG) codons are indicated. Each arrow represents a tumor case carrying *NTRK1* oncogenic rearrangement. (Adapted from Greco *et al.*, 1996).

**Figure 3.** *Top:* Two-color FISH of peripheral blood lymphocytes (PBL) and normal thyroid cells with the *TPR* probe (red spots) and the *NTRK1* probe (green spots). Nuclei were DAPI counterstained. *Bottom:* Frequency histograms of 3D-maximized distances between *TPR* and *NTRK1* signals. Each graph represents the sum of the two cases of thyrocytes (THY) and peripheral blood lymphocytes (PBL). (Adapted from Roccatto *et al.*, 2005a).

**Figure 4.** Schematic representation of NTRK1 receptor and TRK oncoproteins. C: cysteine-rich domain; L: leucine-rich domain; Ig: immunoglobulin-like domain; TM: transmembrane; JM: juxtamembrane region; TK: tyrosine-kinase domain. In the oncoprotein schemes the coiled-coil domains are indicated in green, the PB1 domain in purple.

**Figure 5.** Prediction of coiled-coil domain in TPM3, TPR and TFG proteins with the use of Paircoil program (Berger *et al.*, 1995).

**Figure 6.** TFG interactions and functions. In the TFG scheme the PB1 and coiled-coil domains are indicated.

Table 1

Oncogenic rearrangements of NTR1 in PTC

ONCOGENE	ACTIVATING GENE	
	GENE NAME	CHROMOSOME LOCALIZATION
TRK	TPM3	1q31
TRK-T1	TPR	1q25
TRK-T2	TPR	1q25
TRK-T3	TGF	3q12

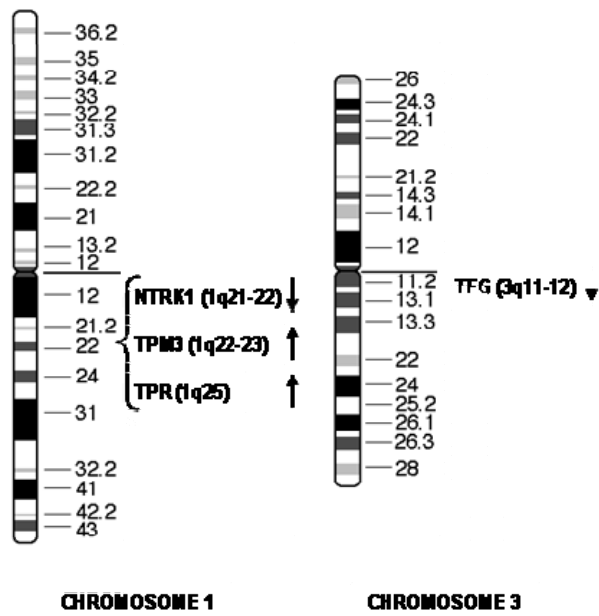


Figure 2

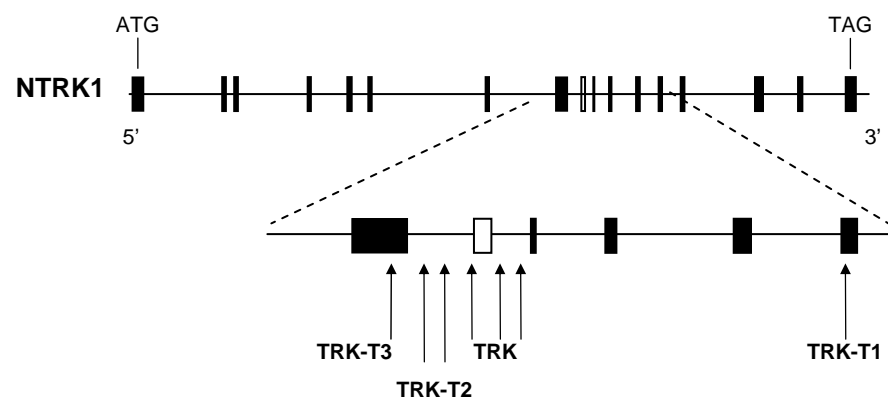


Figure 3

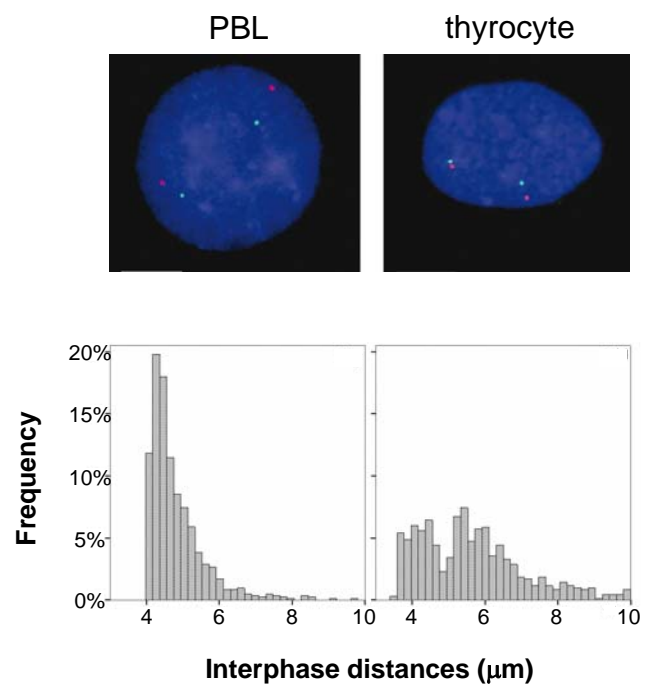


Figure 4

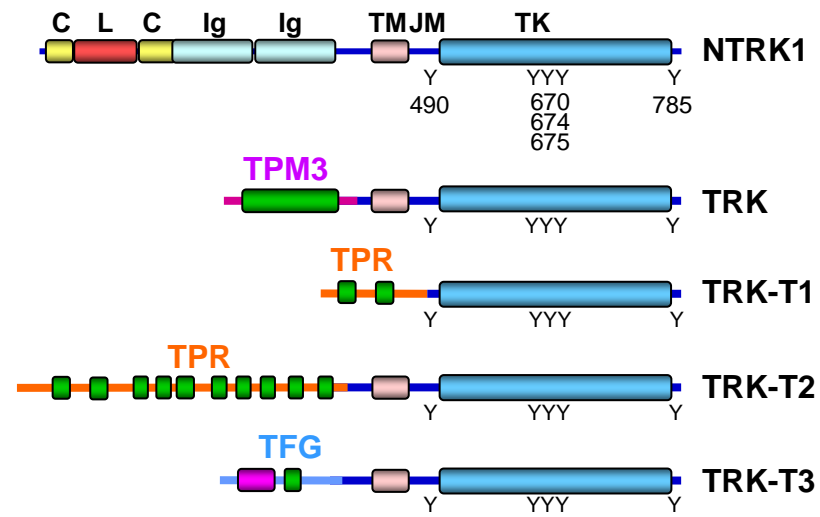


Figure 5

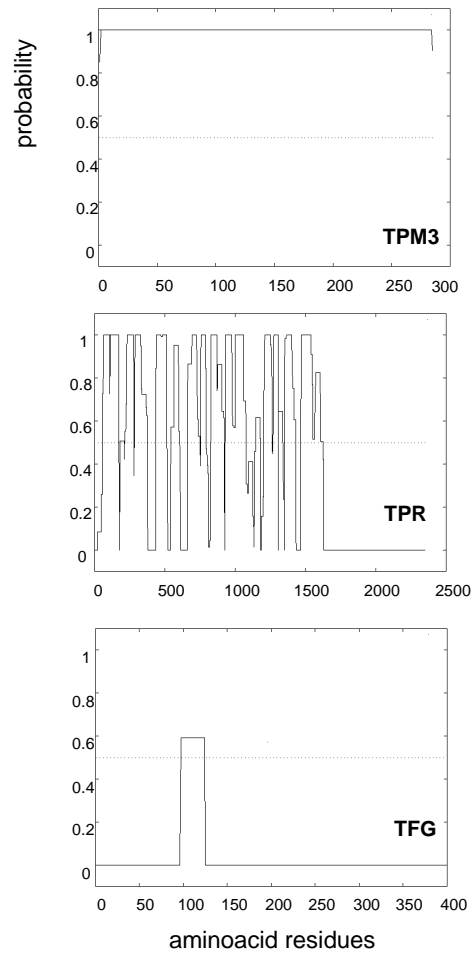


Figure 6

