Cholesterol recognition motifs in the transmembrane domain of the tyrosine kinase receptor family: the case for TRKB

Cecilia Cannarozzo, Senem Merve Fred, Caroline Biojone, Plinio C Casarotto*, Eero Castren

Neuroscience Center - HILife, University of Helsinki, Finland

* correspondence:

Plinio Cabrera Casarotto, PhD
plinio.casarotto@helsinki.fi
Neuroscience Center
Haartmaninkatu 8, B233b
PO Box 63
University of Helsinki, Finland

ABSTRACT

Cholesterol is an essential constituent of cell membranes. Recently, the discovery of cholesterol recognition aminoacid consensus (CRAC) on proteins indicated a putative direct, non-covalent interaction between cholesterol and proteins. In the present study, we evaluated the incidence of CRAC motifs, and its inverted version (CARC), in the transmembrane region (TMR) of tyrosine kinase receptor family (RTK) in *Caenorhabditis elegans* (nematode); *Drosophila melanogaster* (fruit fly); *Danio rerio* (zebrafish); *Mus musculus* (mouse), and *Homo sapiens* (human) by using *in silico* methods. We found CRAC motifs across all species analyzed, whereas, CARC motifs, comprising the extracellular side of the TMR, were only found in vertebrates.

On top of its contribution to structural properties of cells, cholesterol (or its' metabolites) regulates a myriad of cellular functions culminating in increased neuronal plasticity in the central nervous system. The tropomyosin-related kinase B (TRKB), a member of RTK family, is also a core participant in neuronal plasticity process. Therefore, focusing on TRKB receptor, we observed high homology in TRKB.TMR and conservation of CARC motifs across multiple organisms. Upon the recognition of conserved CARC motif in the TRKB, we compared the effect of point mutations in CARC on structural changes in the TMR of human TRKB. The alignment of wild-type and mutant TMR indicates small changes across the 5 mutations analyzed (Y434F, Y434C, Y434A, V439K and R428A), indicated by the root-mean-squared deviation (RMSD) values for the superimposed structures, average=2.448Å. The TMR point mutation also impacted the physicochemical properties of the models, measured by solvent accessibility. The mutation of the Y, V or R residues in the CARC motif increased the solvent access in the juxtamembrane portions of the TMR, ranging from 150-500%.

In conclusion, it is plausible to consider a potential role for CARC/CRAC motifs in the function of RTK family TMR. The presently described CARC motif in TRKB.TMR may exert its effect by regulating the receptor capability to interact with the polar heads of membrane phospholipids.

Keywords: cholesterol-recognition motif, TRKB, tyrosine kinase receptor, BDNF

INTRODUCTION

The human brain contains 23% of the body's total cholesterol, mostly in the myelin sheath of oligodendrocytes [1,2]. The blood brain barrier prevents lipoprotein or cholesterol transport to the brain, therefore local *de novo* synthesis takes place. In mouse brain, cholesterol synthesis peaks during the second postnatal week and then decreases significantly, without being influenced by sex or blood cholesterol concentration [3,4]. During early development, neurons produce cholesterol autonomously [4–6]. In later stages, cholesterol is synthesized by glial cells. However, it is not defined whether it is a constant or regulated production [4,7].

Cholesterol, which can be localized on both leaflets of the plasma membrane [8], induces changes in the membrane physical properties such as fluidity [9] and curvature [10] or interacts with transmembrane domain to regulate protein function [11,12]. Cholesterol is a core constituent of micro-domains known as lipid rafts, which serve as signaling platforms for several pathways [13–15]. In the nervous system, cholesterol interaction with membrane proteins influences several crucial events such as exocytosis of synaptic vesicles [16], synaptic activity, connectivity, plasticity, signal transduction, transmission, and cell survival [17–19]. The neurotrophic receptor subfamily is one of the most prominent representatives of tyrosine kinase receptor family (RTK), given its crucial role in regulating neural plasticity [20]. The tropomyosin-related kinase receptors (TRK) comprise three members TRKA, TRKB and TRKC, which are phosphorylated in different tyrosine residues in the intracellular portion upon activation by their high-affinity ligands: NGF, BDNF and NT-3, respectively [21]. TRKA and TRKC (NTRK1 and NTRK3, respectively) are located in lipid rafts, while TRKB (NTRK2) presence in rafts occurs upon BDNF stimulation [22,23]. Functionally, in the absence of ligand, TRKA and TRKC, but not TRKB, induce cell death through a p75NTR-mediated mechanism [20].

In silico models suggest that two cholesterol molecules can interact in a tail-to-tail fashion as transbilayer dimer [24,25] or back-to-back through their flat alpha faces, leaving the beta sides accessible for interactions with proteins [26]. On these target proteins, two consensus motifs with predictive value have been defined [27]: the Cholesterol Recognition Aminoacid Consensus sequence (CRAC) and its ‘inverted’ version - CARC [28,29]. The CRAC linear sequence, from N- to C-terminus, comprises an apolar residue [leucine (L) or valine (V)], one to five amino acids of any kind, an aromatic amino acid [tyrosine (Y) or phenylalanine (F)], one to five amino acids of any kind, and a basic residue [arginine (R) or lysine (K)] [11]. CARC consists of the same algorithmic pattern in the opposite direction, with tryptophan (W) as alternative aromatic residue, with higher affinity for cholesterol than CRAC [11,27]. Several proteins have been identified to contain CRAC/CARC motifs, such as nicotinic acetylcholine (nAchR), type-3 somatostatin,and GABA-A receptors [11,30,31].

Thus, the aim of the present study was to evaluate the incidence of cholesterol interacting motifs (CRAC and CARC) in the RTK family. Given the promiscuous nature of CRAC motifs, we focused on the RTK TMR, where such interaction has a higher probability of occurrence. Transmembrane domains are crucial for proper positioning in the lipid bilayer of biological membranes [11]. Interaction of transmembrane domains of embedded integral proteins with the lipid component of the bilayer provides a diffusion barrier, and contains environment to maintain electrochemical properties [32]. Upon the recognition of CARC in TRKB, we determined the impact of a series of mutations in the transmembrane region physicochemical properties, measured as solvent accessibility to the aminoacid residues, and conformation in the human TRKB.TMR.

METHODS

Data mining

For data mining, we used 144 hand-curated inputs for RTK family (code 2.7.10.1) from UniProt database [33]. The canonical primary structure of TMR (transmembrane domain and the flanking 5 aminoacid residues, from N- and C-terminal) of RTK from each target of human (52 proteins), mouse (51 proteins), zebrafish (14 proteins), fruit fly (12 proteins), and nematode *C. elegans* (15 proteins) database were extracted. The TMR FASTA sequences for each protein was manually screened for the presence of cholesterol recognition alignment consensus (both CRAC and it's inverted version CARC) [8,11]. Next, we searched for putative pathogenic mutations using SwissVar, ClinVar and COSMIC databases [34–36].

Homology of TRKB (and TMR) across species

The homology of TRKB.TMR (and full length sequences) among several species: *D. rerio* (zebrafish), *G. gallus* (chicken), *C. familiaris* (dog), *R. norvegicus* (rat), *M. musculus* (mouse), *P. troglodytes* (chimpanzee), and *H. sapiens* (human), was determined using the align tool in UniProt database [33].

Alignment and solvent accessibility of models

The models of human TRKB.TMR (in this case with extra 10aa residues in each terminal) were generated in the RaptorX server [37], using wild-type (WT) and mutated FASTA sequences. The point mutation Y434F was chosen to exclude only the -OH group, but protect the aromatic ring structure of tyrosine. Y434A mutation was induced to examine the effects of the aromatic ring of tyrosine removal. Finally, Y434C mutation was introduced based on evidence showing the association of this particular change with hyperphagic obesity syndrome, which also causes defective memory, learning, and nociception [38,39]. The V439K mutation was chosen aiming to

change an apolar/neutral to a basic aminoacid residue, and the same reason was considered for the R428A mutation. The models were aligned with WT for comparison, and the root-mean-squared deviation (RMSD) between the backbone carbon structure calculated by the server. The relative solvent accessibility per residues was calculated using PyMOL (v2.0 Schrödinger, LLC), and normalized by WT values.

RESULTS

Data mining

The presence of CRAC motifs were found throughout all the species analyzed [human (11 of 52 proteins), mouse (10 of 51 proteins), zebrafish (2 of 14 proteins), fruit fly (2 of 12 proteins), and *C. elegans* (2 of 15 proteins)] (figure 1), however, the presence of CARC motifs in RTK family was observed only in vertebrates, with 3 in human, 3 in mouse and 2 in zebrafish RTK. None of the proteins analyzed was found to carry CRAC and CARC motifs simultaneously. The ClinVar and COSMIC databases indicated five proteins (see table 1) comprising 8 mutations in the CRAC/CARC domains associated with central nervous system, endocrine disorders or cancer.
Full list of proteins positive to CRAC/CARC are listed in table 1, the full list of proteins examined from each species can be found in the deposited raw data (DOI:10.6084/m9.figshare.7935980).

Homology of TRKB (and TMR) across species

Upon the identification of CARC motif in TRKB (human and mouse: REHLSVYAVVV; zebrafish: RVAVYIVV), the homology between TRKB.TMR sequences of several species (human, chimpanzee, mouse, rat, dog, chicken and zebrafish), listed in table 2, was examined using UniProt [33]. Over 90% of homology was found in both TRKB.TMR and full length TRKB of human, chimpanzee, mouse, rat and dog. The homology results of paired comparison between the species analyzed are organized in figure 2, where green gradient highlights the percentage of similarity between the sequences. For comparative purposes, we also determined the homology of the full length TRKB among those species. The homology results of paired comparisons are also organized in figure 2, with red gradient highlighting the percentage similarity between sequences.

Alignment and solvent accessibility of models

We proceeded with human TRKB.TMR to analyze the solvent accessibility following point mutations on tyrosine, valine or arginine in this region. Based on the assessment of solvent accessibility, we detected a prominent increase in the exposed surface to water (ranging from 150-500%) at the two juxtamembrane regions (figure 3a-e). On the other hand, the point mutations induced small morphological changes when the wild-type and mutant models were aligned, as

indicated by the RMSD values (figure 3f-j). The relative solvent accessibility used in the present study is an indication of the surface exposure of the aminoacid residues in a polypeptide sequence [40]. Although simple, this parameter indicates putative conformational alterations in proteins that may translate into functional changes. Thus, even mild/neutral mutations such as Y to F in the CARC motif alters the physicochemical properties of the TMR.

DISCUSSION

In the present study, we evaluated the incidence of cholesterol-recognition motifs in the receptor tyrosine-kinase family (RTK) from mouse, human, zebrafish, fruit fly, and nematode *C. elegans*. We found that while the *bona fide* CRAC, located in the C-terminal portion of transmembrane domain [11], is found in all the species analyzed, its inverted version - CARC - was only observed in vertebrates. Furthermore, we found that TRKB, a tyrosine-kinase receptor crucial for neuronal plasticity, contains a CARC sequence in the TMR. This motif is conserved across different species, and point mutations of the tyrosine, valine or arginine residues alter the solvent accessibility of the juxtamembrane regions of TRKB.

Cholesterol can interact with membrane proteins in several ways, one of its most prominent effect involves a direct post-translational modification on members of the Hedgehog pathway; particularly described in *Drosophila sp.* [41,42]. In this model organism, cholesterol is also found to regulate membrane depolarization through transient receptor potential (TRP) channels [43], and serves as a precursor for ecdysteroids, which in turn regulate several steps of the fly development [44]. In nematodes, such as *C. elegans*, cholesterol is only obtained from diet, although these worms can modify the basic steroid structure into derivatives [45]. In both organisms, cholesterol appears to play a major role as signaling molecule with post translational modifications of proteins as the main mechanism [46].

Although neurons synthesize the absolute minimum necessary cholesterol, glial production and release of lipoproteins supply neuronal demand during development and in adulthood [47]. In particular, apolipoprotein E (APOE) is synthesized primarily by astrocytes and glial cells [4,48]. Glia-derived cholesterol stimulates synapse formation and synaptic efficacy [49,50]. In the presynaptic plasma membrane, the cholesterol-rich lipid rafts are necessary for SNARE-dependent exocytosis of vesicles with high content of cholesterol. At the postsynaptic level, such rafts organize the disposition of receptors, protein scaffold and signaling cascades [49,50]. Importantly, cholesterol removal from neuronal cultures impairs exocytosis of synaptic vesicles [16], synaptic transmission [18] and neuronal viability [19]. In addition, it induces clustering of AMPA receptors, and hinders NMDA-induced long-term potentiation (LTP) in the hippocampus [51,52].

Since it is difficult to directly visualize cholesterol binding motifs and crystallize the

transmembrane domains containing such sequences, two consensus motifs with predictive value have been defined through *in silico* methods [27]: CRAC and its 'inverted' version - CARC [28,29]. The non-covalent binding of cholesterol to specific motifs in proteins has been the focus of various recent studies. For example, cholesterol modulates docking of NMDA receptors into lipid rafts [53], and the function of vanilloid receptors (TRPV1, a member of the TRP family) [54], thus interfering in synaptic plasticity. Increased cholesterol concentration enhances the plasticity and the flexibility of 5HT1a dimers and adrenergic receptors [55,56]. Given the opposed dispositions of CARC and CRAC motifs, it is possible to assume the co-existence of both in the same transmembrane domain, and their potential interaction with two cholesterol molecules in a tail-to-tail configuration [27]. However, none of the analyzed TMR of RTK family members in the present study displayed co-existing CARC and CRAC motifs.

Interestingly, we only observed the occurrence of CARC motifs on zebrafish, mouse and human RTK family, and TRKB.TMR is highly conserved among vertebrates, similarly to full length TRKB. However, the TRKB.TMR CARC sequence from chicken differs in the juxtamembrane residue from the other species compared (see table 2). It is plausible to consider two optional scenarios, the role of R/K (charged, basic residues) is fulfilled by glutamate (E), which is also charged at pH 7, although negatively; or by glutamine (N), which is not charged but carries a basic amino group. Additionally, for the second possibility it is also necessary to relax the proposed '5-residue rule' between the Y and the juxtamembrane residue [57], since N is located 6 residues apart from the central Y.

As mentioned above, TRKB plays a crucial role in several aspects of neural plasticity. The activation of this receptor is associated with the reopening of visual critical period [58], and the formation, retention and recall of memory [59,60]. Increased activity of TRKB is linked to the occurrence of epilepsy, while blockade of such signaling normalizes the condition [61,62]. Whole genome sequencing performed on patients with developmental and epileptic encephalopathy (DEE) links a *de novo* missense variant of TRKB (*NTRK2*) to the disease pathology [38]. Patients carrying cysteine at residue 434 instead of tyrosine, located inside TRKB CARC motif, were observed to have a similar phenotype varying from severe global developmental delay or intellectual disability to visual and feeding impairments [38]. Therefore, it is possible to speculate that such Y434C mutation, interfering with TRKB-cholesterol interaction, leads to impairment of adequate TRKB activity and function.

Neurotrophins induce homodimerization of TRK monomers to activate downstream kinases [63]. However, through a p75NTR-dependent mechanism, TRKA and TRKC receptors may induce cell death in the absence of their ligands, NGF or NT-3, respectively. On the other hand, TRKB does not act as death-inducing receptor when coupled to p75NTR [64]. In this sense, it is

suggested that TRKA and TRKC interaction with p75NTR is a necessary step for moving the complex to lipid rafts, where the p75NTR-dependent death signal is triggered through its cleavage by gamma secretase [20]. TRKB was not observed to display such co-occurrence with p75-NTR in lipid rafts. The association of TRKA and TRKC with p75NTR in rafts involves the transmembrane domain of these receptors, given that a chimeric TRKB carrying TRKA transmembrane sequence requires BDNF for survival signal [20]. Curiously, p75NTR possesses a classical CRAC domain in its TMR: VVVGLVAYIAFKR; conserved in human, mouse, rat and chicken.

TRKB is found in lipid rafts only upon activation by BDNF [22]. Interestingly, when cholesterol is sequestered, TRKB translocation to lipid rafts is impaired, and BDNF-dependent potentiation is prevented [22]. However, loss of cholesterol in hippocampal cultures is associated with increased baseline activity of TRKB [65]. These opposite outcomes might be due to a differential modulation exerted by cholesterol, depending on the challenge to TRKB receptor (basal vs BDNF-stimulated), cell type/origin and stage of differentiation. Another possibility poses that cholesterol affects TRKB activity in a bell-shaped manner, where higher as well as lower concentrations of cholesterol impedes, instead of promoting, TRKB phosphorylation. In fact, the decrease of cholesterol levels by beta-cyclodextrin was found to differentially modulate the neurite growth of hippocampal and cortical cultured neurons. In hippocampal cells, the decrease of cholesterol levels induces an increase in neurite length and number, while no effect was observed in cortical cells. Interestingly, cultures of hippocampal cells show higher levels of cholesterol than the cortical counterparts [66]. Altogether, it is a provocative idea to consider TRKB as a 'sensor' of cholesterol levels in the cell membrane via CARC. Thus, TRKB would trigger the synaptic maturation or neurite growth only if the cholesterol levels are ideal for such changes. This assumption, however, remains to be tested.

Our data indicates increased solvent accessibility to the juxtamembrane regions of mutant TRKB in all the residues analyzed. The changes varied from 150-500% above the levels found in wild-type sequence. The increase in the hydrophilicity of juxtamembrane could implicate a decrease in the BDNF-induced movement of TRKB towards the lipid raft compartments, where the changes in TMR orientation may be altered, due to membrane thickness [67,68].

According to *in silico* analysis of cholesterol interaction with CARC motifs, the Y to F, or Y to W mutations do not interfere with the motif interaction with cholesterol. However, these analyses were performed in multipass or multi-subunits transmembrane receptors, such as nicotinic cholinergic [27] or GPCR receptors [55,56]. Given that TRKB, as well as virtually all members of the RTK family, possess a single transmembrane domain, small changes in this region may impact their function, while not detected in the multipass proteins or to the direct interaction with cholesterol. The exception was found to be Q19238 (Putative tyrosine-protein kinase F09A5.2)

from *C. elegans*, with 2 transmembrane domains, although none is CRAC/CARC-positive.

The insights in the present study could serve as a primary step for experimental testing of the impact of mutations in CRAC/CARC motifs in TMR of RTK family. However, we are limited by only considering the role of CRAC/CARC motifs in the TMR of RTKs. Given the promiscuous properties of these motifs, it is plausible to assume multiple false positive CRAC/CARCs alongside proteins, making the data mining and putative *in silico* or *in vitro* analysis difficult to perform. Therefore, more studies focused on refining the algorithms for detecting these motifs are necessary.

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Figures and tables

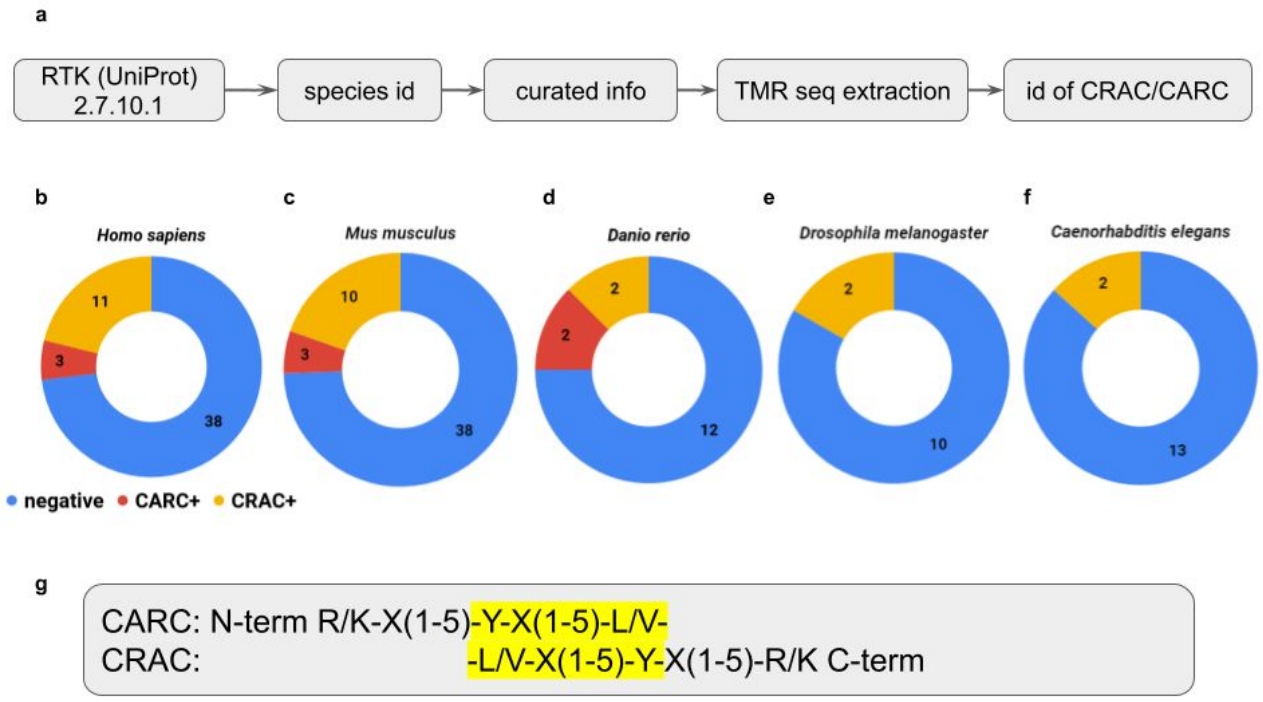

Figure 1. (a) workflow of data mining. The TMR (transmembrane sequence +5aa residues from each N- and C-terminal sides) of curated entries found in UniProt using the code for tyrosine kinase receptor family (2.7.10.1) were blasted against the library of cholesterol recognition and alignment consensus combinations. The incidence of CRAC and CARC motifs in (b) human, (c) mouse, (d) zebrafish, (e) fruit fly and (f) *C. elegans* TMR of RTK family members. (g) library of CRAC and CARC sequences (all the combinations used can be found in the stored data). The yellow highlighted region of the sequences must be embedded into the cell membrane.

Table 1. CRAC and CARC(*)-positive proteins

Unipro ID	Protein name	Mutation in CRAC/CARC
H. sapiens		
Q9UM73	ALK tyrosine kinase receptor	
Q16620*	BDNF/NT-3 growth factors receptor	Y434C
P29320	Ephrin type-A receptor 3	I564V
P11362	Fibroblast growth factor receptor 1	
P22455	Fibroblast growth factor receptor 4	
P08069	Insulin-like growth factor 1 receptor	
P06213	Insulin receptor	F978Y
P07333	Macrophage colony-stimulating factor 1 receptor	L536V, Y540S, K541T, K543M
P10721	Mast/stem cell growth factor receptor Kit	
O15146	Muscle, skeletal receptor tyrosine-protein kinase	
Q15303	Receptor tyrosine-protein kinase erbB-4	
Q12866	Tyrosine-protein kinase Mer	I518V
Q01974*	Tyrosine-protein kinase receptor ROR2	
P34925*	Tyrosine-protein kinase RYK	
M. musculus		
P97793	ALK tyrosine kinase receptor	
P15209*	BDNF/NT-3 growth factors receptor	
Q60750	Ephrin type-A receptor 1	
P29319	Ephrin type-A receptor 3	
P16092	Fibroblast growth factor receptor 1	
Q03142	Fibroblast growth factor receptor 4	
P15208	Insulin receptor	
Q60751	Insulin-like growth factor 1 receptor	
P09581	Macrophage colony-stimulating factor 1 receptor	
P05532	Mast/stem cell growth factor receptor Kit	
Q61006	Muscle, skeletal receptor tyrosine-protein kinase	
Q9Z138*	Tyrosine-protein kinase transmembrane receptor ROR2	
Q01887*	Tyrosine-protein kinase RYK	
D. rerio		
Q9I8N6	Macrophage colony-stimulating factor 1 receptor	
Q8JFR5	Mast/stem cell growth factor receptor kita	
B8JLJ1*	Tyrosine-protein kinase receptor (Ntrk2a)	
A0A0R4ILA2*	Tyrosine-protein kinase receptor (Ntrk2b)	
D. melanogaster		
P09208	Insulin-like receptor	
P83097	Putative tyrosine-protein kinase Wsck	
C. elegans		
P34891	Receptor-like tyrosine-protein kinase kin-15	
G5EGK5	Tyrosine-protein kinase receptor cam-1	

UniProt ID	NTRK2	human	chimpanzee	mouse	rat	dog	chicken	zebrafish
Q16620	human		100.00	93.92	93.55	98.30	77.13	60.43
A0A2J8MRP9	chimpanzee	100.00		93.92	93.55	98.30	77.13	60.43
P15209	mouse	91.18	91.18		98.54	93.80	76.49	60.39
Q63604	rat	94.12	94.12	97.06		93.55	76.49	60.15
E2RKA1	dog	97.06	97.06	94.12	91.18		77.49	60.68
Q91987	chicken	41.18	41.18	44.12	44.12	41.18		61.09
A0A0R4ILA2	zebrafish 2b	35.29	35.29	35.29	35.29	35.29	50.00	

Figure 2. Percent of homology of full length (red) and TMR (green) of TRKB. The aa sequence of full length and TMR of TRKB (NTRK2) from different species were verified for homology in UniProt database.

Table 2. CARC-containing sequences (red) in TRKB.TMR among vertebrate species.

UniProt	Species	TMR sequence
Q16620	*H. sapiens*	TGREHLSVYAVVVIASVVGFCLLVMLFLLKLARH
A0A2J8MRP9	*P. troglodites*	TGREHLSVYAVVVIASVVGFCLLVMLFLLKLARH
P15209	*M. musculus*	SNREHLSVYAVVVIASVVGFCLLVMLLLLKLARH
Q63604	*R. norvegicus*	TNREHLSVYAVVVIASVVGFCLLVMLLLLKLARH
E2RKA1	*C. familiaris*	SGREHLSVYAVVVIASVVGFCLLVMLFLLKLARH
Q91987	*G. gallus*	ENEDSITVYVVVGIAALVCTGLVIMLIILKFGRH
A0A0R4ILA2	*D. rerio*	PLEDRVAVYIVVGIAGVALTGCILMLVFLKYGRS

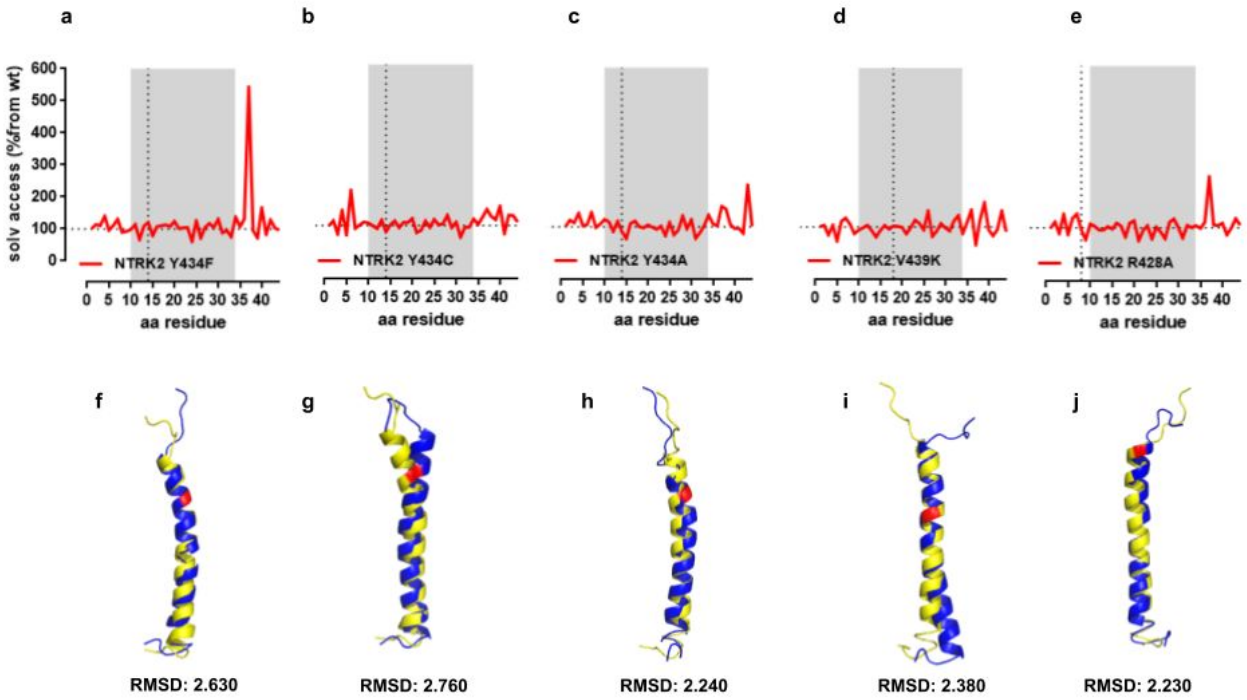

Figure 3. The aa sequences of human TRKB TMR (wild-type and carrying a point mutation the CARC motif) were submitted to RaptorX server. The solvent accessibility to the modeled TMRs and alignment of mutant models against wild-type TMR with the root mean squared deviation (RMSD, values expressed in Å) between the atoms in the backbone of the chains for (a,f) Y434F, (b,g) Y434C, (c,h) Y434A, (d,i) V439K and (e,j) R428A mutations. Gray boxes indicate the TM domain, dotted vertical line indicates the location of point mutation. Yellow: TMR of wild-type; blue: TMR of mutant; red: site of mutation.

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