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Critical Domains for NACC2-NTRK2 Fusion Protein Activation

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ABSTRACT OF THE THESIS

Critical Domains for NACC2-NTRK2 Oncogenic Activation

By

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Professor Daniel J. Donoghue, Chair

Neurotrophic receptor tyrosine kinases (NTRKs) belong to the receptor tyrosine kinase (RTK) family. NTRKs are responsible for the activation of multiple downstream signaling pathways that regulate cell growth, proliferation, differentiation, and apoptosis. NTRK-associated mutations lead to aberrant activation of these downstream pathways and often result in oncogenesis. This study characterizes the NACC2-NTRK2 oncogenic fusion protein that leads to pilocytic astrocytoma and pediatric glioblastoma. This fusion joins the broad-complex, tramtrack, and bric-a-brac (BTB) domain of Nucleus Accumbens-associated protein 2 (NACC2) with the transmembrane helix and tyrosine kinase domain of NTRK2. This work focuses on identifying domains critical to the fusion protein activity and possible methods to deactivate the fusion. NACC2-NTRK2 is able to transform NIH3T3 cells. Such activity depends on the NTRK2 kinase domain phosphorylation that activates signaling pathways including MAPK,

JAK/STAT, and PLCγ. The activation of the NTRK2 kinase domain relies on the multimerization of the NACC2 BTB domain. A BTB domain charged pocket mutation and a monomer core mutation result in deactivation of the kinase domain and abrogates the ability to transform NIH3T3 cells, suggesting that BTB domain inhibition could be a potential treatment for the NACC2-NTRK2 induced cancer. The undefined region of NACC2 at residues 120-418 is responsible for forming stronger multimers. Once removed, the NACC2-NTRK2 multimer is vulnerable to SDS denaturation and leads to reduced activity of the fusion. Lastly, the removal of the transmembrane helix leads to higher activation of the NACC2-NTRK2 fusion. A protein stability assay indicates that transmembrane deletion prevents NACC2-NTRK2 degradation.

Chapter 1

Nefarious NTRK oncogenic fusions in pediatric sarcomas: Too many to Trk

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Nefarious NTRK oncogenic fusions in pediatric sarcomas: Too many to Trk

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ABSTRACT

Neurotrophic Tyrosine Receptor Kinase (NTRK) genes undergo chromosomal translocations to create novel open reading frames coding for oncogenic fusion proteins; the N-terminal portion, donated by various partner genes, becomes fused to the tyrosine kinase domain of either NTRK1, NTRK2, or NTRK3. NTRK fusion proteins have been identified as driver oncogenes in a wide variety of tumors over the past three decades, including Pediatric Gliomas, Papillary Thyroid Carcinoma, Spitzoid Neoplasms, Glioblastoma, and additional tumors. Importantly, NTRK fusions function as drivers of pediatric sarcomas, accounting for approximately 15% of childhood cancers including Infantile Fibrosarcoma (IFS), a subset of pediatric soft tissue sarcoma (STS). While tyrosine kinase inhibitors (TKIs), such as larotrectinib and entrectinib, have demonstrated profound results against NTRK fusion-positive cancers, acquired resistance to these TKIs has resulted in the formation of gatekeeper, solvent-front, and compound mutations. We present a comprehensive compilation of oncogenic fusions involving NTRKs focusing specifically on pediatric STS, examining their biological signaling pathways and mechanisms of activation. The importance of an obligatory dimerization or multimerization domain, invariably donated by the N-terminal fusion partner, is discussed using characteristic fusions that occur in pediatric sarcomas. In addition, examples are presented of oncogenic fusion proteins in which the N-terminal partners may contribute additional biological activities beyond an oligomerization domain. Lastly, therapeutic approaches to the treatment of pediatric sarcoma will be presented, using first generation and second-generation agents such as selitrectinib and repotrectinib.

1. Introduction

Soft Tissue Sarcoma (STS) represents a rare, heterogenous group of cancers arising in the bone, muscle, fibrous connective tissue, blood vessels, fat, or other supporting tissues [1], typically presenting as tumors within the arms, legs, chest, or abdomen. Pediatric STS represents 7% of all cancer diagnoses for adolescents under the age of 20 [2]. Pediatric cancers typically arise prenatally from embryonal cells derived from embryonic fetal tissue, in contrast to adult sarcomas which originate from epithelial cells [3]. Pediatric STS can be categorized into Rhabdomyosarcoma (RMS) and Non-Rhabdomyosarcoma STS (NRSTS) [4]. RMS is the most prevalent STS with about 400–500 cases in the US annually. NRSTS is an umbrella term used for non-muscular sarcomas, such as fibrosarcoma, hemangiopericytoma, and spindle cell sarcoma, among others [5]. RMS typically presents in the muscles of the limbs, head regions, or urogenital tract; NRSTS, although most commonly occurring in the limbs, can occur anywhere in the body [6].

Chromosomal translocations have been identified in approximately 20% of STS [7]. In fact, gene fusions, such as EWSR1-FLI1, PAX3/7--FOXO1, SS18-SSX1/2/4 have been extensively characterized within pediatric sarcomas including alveolar rhabdomyosarcoma, Ewing Sarcoma, and synovial sarcoma [8]. These translocations are characterized by the presence of one or more potent transcriptional activation domains resulting in target gene dysregulation [9]. Another category of gene fusions involves translocations with receptor tyrosine kinases (RTKs), particularly Neurotrophic Tyrosine Receptor Kinases (NTRKs), resulting in activation of potent tyrosine kinases that sit atop complex signaling cascades. NTRK fusions involving chromosomal rearrangements are identified in 0.34% of pediatric sarcomas, although additional NTRK alterations such as mutation, amplification, and mRNA overexpression

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occur in ~14% of samples [10].

NTRK translocations create a novel hybrid protein containing a multimerizing domain, derived from the translocation partner, fused to the NTRK kinase domain. The NTRK family comprises NTRK1, NTRK2, and NTRK3, originally named TRKA, TRKB, and TRKC – for Tropomyosin Receptor Kinase – due to sequence similarity of their N-terminal domain with a non-muscle tropomyosin [11]. NTRKs promote regulation of cell proliferation, differentiation, apoptosis, and survival of neurons in both the central and peripheral nervous systems [12,13]. Specific neurotrophins bind to these transmembrane kinases, thereby activating multiple downstream signaling pathways such as SHC/RAS/MAPK, PI3K/AKT, and PLC-γ/ PKC (Fig. 1A) [13]. NTRK fusions are consistently over-represented in pediatric STS [12], with NTRK3 fusions most prevalent, followed by NTRK1, and lastly NTRK2. This review focuses on oncogenic NTRK gene fusions in pediatric STS; however, information concerning all currently identified oncogenic NTRK gene fusions, numbering well over 100, is compiled in Supplementary Tables S1–S3.

2. Pediatric sarcomas

NTRK fusions are associated with an important subset of pediatric STS, and are divided into Rhabdomyosarcoma (RMS) and Non-Rhabdomyosarcoma soft tissue sarcoma (NRSTS). Certain medical conditions have been associated with greater likelihood of pediatric STS, including: Li-Fraumeni syndrome, Neurofibromatosis, Beckwith-Wiedemann syndrome, Costello syndrome, and Noonan syndrome [14]. While controversial, there is evidence suggesting that alcohol consumption, narcotics, sun exposure, or chemicals including dioxin, pesticides, solvents, and petroleum products, represent environmental factors that contribute to STS [3].

2.1. Rhabdomyosarcoma

Rhabdomyosarcoma represents an STS of skeletal muscle origin characterized by high grade and increased metastasis, accounting for roughly 3–4% of childhood cancers [15]. The five-year survival rate of RMS is 67% for adolescents under 15 years, and 51% for adolescents between 15 and 19 years [14]. Treatment options may include surgery, radiation therapy, sometimes together with targeted therapy using sunitinib, a small molecule Tyrosine Kinase Inhibitor (TKI) active against multiple RTKs [16]. There are two major histologic subsets of pediatric RMS: alveolar and embryonal, and two minor subtypes: spindle cell/sclerosing and pleomorphic [5,17].

Alveolar RMS (ARMS) is generally a more aggressive subtype of Rhabdomyosarcoma, named for its superficial resemblance to alveolar tissue in the lungs. ARMS accounts for 20–25% of all RMS and typically occurs in the large muscle regions of the leg and arms but, in some cases, in the head and neck regions [18]. ARMS is associated with a poorer prognosis, exhibiting only 25% survival [19]. While translocations that create oncogenic fusion proteins are commonly observed in ARMS, these do not involve NTRKs; rather, the underlying cause is either the t(2;13)(q35;q14) rearrangement encoding the PAX3-FOXO1 fusion protein, or the t(1;13)(p36;q14) translocation, encoding the PAX7-FOXO1 fusion protein [7] Metastatic disease with these specific rearrangements shows a 4-year survival rate of 75% for PAX7-FOXO1 fusions versus 8% for PAX3-FOXO1 [19]. The FOXO family of proteins, also known as forkhead, encode transcription factors involved in tissue homeostasis by modulating downstream functions including apoptosis, cell proliferation, cell cycle arrest, autophagy, and oxidative stress response [20]. Unfortunately, FOXO1 translocations represent challenging drug targets due to their propensity to acquire resistance [21].

Embryonal Rhabdomyosarcoma (ERMS) is the more common subtype of RMS, accounting for about 60% of RMS cases, typically arising in the embryonal skeletal muscle and presenting in the head, neck, and urogenital tract regions [22]. ERMS mostly affects children 0–4 years old, in comparison with ARMS which may present throughout childhood [23]. Unlike ARMS, ERMS is usually not associated with significant chromosomal translocation or rearrangement [24]. Instead, cytogenic studies have linked some cases to CNVs (copy number variations). Genomic CNVs can be caused by either a single event or by an accumulation of chromosomal lesions that result in widescale genome disruption; however, studies have indicated that the RMS cases align more with an accumulation of lesions. Indeed, genomic CNVs are frequently observed in STS, which further complicate treatment options. These CNVs are often numerous, involving amplifications in the PI3K signaling pathway, cell-cycle machinery, additional RTKs, MAPK signaling, RNA regulatory genes, and others [25].

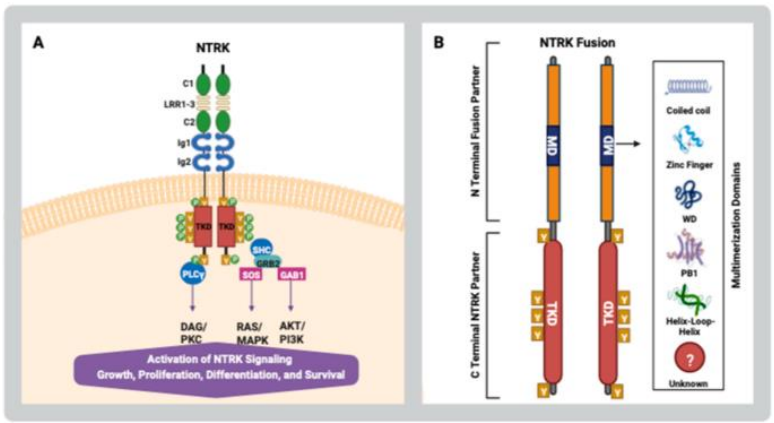

Fig. 1. NTRK Structure and Signaling. (A) Structure of NTRKs. A schematic of an NTRK dimer is shown. The extracellular region of NTRKs is comprised of the Cysteine 1 motif (C1), Leucine Rich Residues motifs (LRR1–3), Cysteine 2 motif (C2), Immunoglobulin-like motifs 1 and 2 (Ig1, Ig2), followed by a Transmembrane Domain and an intracellular Tyrosine Kinase Domain (TKD). Neurotrophins bind to the extracellular domain resulting in dimerization and activation by autophosphorylation of key tyrosine residues in the intracellular TKD, followed by recruitment of adaptor proteins. The adaptor proteins: PLCγ, SHC, GRB2, SOS, and GAB1 activate the downstream pathways of DAG/ PKC, RAS/MAPK, and AKT/PI3K which can lead to growth, proliferation, differentiation, and survival. (B) Activated Oncogenic Fusion. A typical NTRK fusion is presented, containing an N-terminal multimerization domain fused to the C-terminal TKD domain of an NTRK. Key phosphotyrosine residues are shown. The nature of the Multimerization Domain (MD) is variable and includes any of the following: coiled-coil domain, Zinc finger domain, WD domain, PB1 domain, or a Helix-Loop-Helix domain. It should be noted that other uncharacterized multimerization domains may exist.

Lastly, the Spindle Cell/Sclerosing Rhabdomyosarcoma is a relatively new subtype that occurs in 5–13% of RMS [17]. ScRMS is characteristic of micro-alveolar patterns within sclerotic or hyalinized stroma. ScRMS is frequently characterized by a recurrent gene fusion involving transcriptional activators such as VGLL2, TEAD1 or SRF (see Table 1A). Interestingly, these fusion-positive infantile cases of ScRMS display similar behavior as ETV6-NTRK3-driven infantile fibrosarcoma [26]. Meanwhile, the second minor subtype, Pleomorphic RMS, which occurs rarely in pediatric cases, comprises approximately 10% of RMS cases and typically presents in extremities [27].

2.2. Nonrhabdomyosarcoma

Nonrhabdomyosarcoma soft tissue sarcomas (NRSTS) represent 3–3.5% of childhood cancers and, due to their rarity, are less intensively studied [28]. NRSTS can occur in a wide range of regions including the limbs, head, neck, chest, abdomen and pelvis, and occurs in many age groups. The other subtypes of NRSTS display varying epidemiology with higher incidence across adolescence and early adulthood [29]. The histopathology varies but involves cells of mesenchymal origin, such as fibroblasts, smooth muscle cells, and perineural cells [30].

Chromosomal rearrangements producing active NTRK fusion proteins have been identified as drivers of NRSTS, specifically: LMNA-NTRK1, EML4-NTRK3, ETV6-NTRK3, TPM3-NTRK1, SQSTM1-NTRK1, SPECC1L-NTRK3, TPR-NTRK1, and RBPMS-NTRK3 (see Table 1B) [31]. Interestingly, the ETV6-NTRK3 fusion has been identified as an oncogenic driver in over 90% of Infantile Fibrosarcoma (IFS) cases, a subset of NRSTS [32]. Table 1B summarizes the major oncogenic NTRK fusions identified in pediatric STS.

When managing treatment options, low grade NRSTS tumors are generally treated by surgery alone, but targeted therapies along with radiotherapy are appropriate for more aggressive cases. While targeted therapies are still in need of more clinical testing, crizotinib, larotrectinib, entrectinib, selitrectinib, and repotrectinib are examples of TKIs that exhibit clinical value (see Sections 7 and 8) [29]. Because of the high occurrence of NTRK fusions in NRSTS, NRSTS patients should be screened for NTRK fusion-positive cancers immediately after diagnosis to permit a wider range of treatment options.

3. Three of a kind – the NTRK family

3.1. Structure

The three homologous NTRKs, known as NTRK1, NTRK2, and NTRK3 – historically TrkA, TrkB, and TrkC – all present an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. The extracellular domain contains three leucine-rich 24-residue repeats (LRR1–3), two cysteine-rich clusters (C1 and C2), and two immunoglobulin-like domains (Ig1 and Ig2) (Fig. 1A) [56]. The intracellular kinase domain contains five key tyrosine residues. Three of these residues reside within the activation loop of the kinase domain; phosphorylation within this region promotes activation. Of the remaining two tyrosines, one proximal to the kinase domain and the other distal, their phosphorylation enables docking of adaptor proteins [57]. During normal receptor activation, NTRKs interact with their cognate ligands via the extracellular Ig2 domain, leading to dimerization, autophosphorylation, and activation of signaling pathways [58].

The signal peptide, encoded at the N-terminus of NTRKs and responsible for their membrane insertion as Type I transmembrane proteins, is universally absent in NTRK fusion proteins. This observation dictates, without exception, that NTRK fusion proteins are not expected to be transmembrane proteins; instead their localization within the cell, whether cytoplasmic, nuclear, or associated with a specific structure, will be determined by localization signals present either in the N-terminal fusion partner, or within the retained portion of the NTRK (Fig. 1B). Notably, although some fusion proteins retain the "transmembrane domain" of the NTRK, this is never expected to function in this capacity given the lack of membrane insertion. Whether retention of the "transmembrane domain" in a specific NTRK fusion protein contributes any functional significance remains unclear [57].

Table 1A
Fusions in Pediatric STS: RMS tumors.

Tumor Origin	Type of Tumor	Fusions Identified	Ref.
Striated Muscle	Embryonal (ERMS)	No fusions found	
Striated Muscle	Alveloar (ARMS)	Characteristic of the FOXO and NCOA2 family of fusions	[33, 34]
Striated Muscle	Pleomorphic (PMS)	No fusions found	
Striated Muscle	Spindle Cell/ Sclerosing (SRMS)	Characteristic of the VGLL2 Family fusion proteins	[26]

Table 1B
Fusions in Pediatric STS: NRSTS tumors.

Tumor Origin	Type of Tumor	Fusions Identified	Ref.
Fibrous tissue	Infantile Fibrosarcoma	LMNA-NTRK1 SQSTM1-NTRK1 TPM3–NTRK1 ETV6-NTRK3 EML4-NTRK3 SPECC1L-NTRK3 TPR-NTRK1	[35-38]
Fibrous tissue	Congenital Fibrosarcoma	LMNA-NTRK1 ETV6-NTRK3 EML4-NTRK3	[39-41]
Fibrous tissue	Malignant Fibrous Histiocytoma	Characteristic of the CREB1 and ATF1 family of fusions	[42]
Fibrous tissue	Dermatofibrosarcoma Protuberans	Characteristic of the PDGFB family of fusions	[43]
Peripheral Nerve	Malignant Peripheral Nerve Sheath Tumor	Characteristic of the RET family of fusions	[44]
Fat	Liposarcoma	Characteristic of the DDIT3 family of fusions	[45A]
Muscle (smooth)	Leiomyosarcoma	Characteristic of the PGR family of fusions	[45B]
Muscle (smooth)	Myofibroblastic Tumor	RBPMS-NTRK3 TFG-NTRK3	[38,46]
Synovial Tissue	Synovial Sarcoma	Characteristic of the SSX family of fusions	[47]
Blood vessels	Malignant Hemangiopericytoma	Characteristic of the STAT6 family of fusions	[48]
Blood vessels	Kaposi's Sarcoma	Characteristic of the ORF45 family of fusions	[49]
Cartilage	Chondrosarcoma	Characteristic of the CDX1 and NCOA2 family of fusions	[50]
Bone	Osteosarcoma	Characteristic of the SNRNP25 and CCND3 family of fusions	[51]
Unspecified/ multiple origins	Unspecified Soft Tissue Sarcoma	PDE4DIP–NTRK1 KHDRBS1- NTRK1	[52,53]
Unspecified/ multiple origins	Undifferentiated	DCTN1-NTRK1 TPR-NTRK1	[35,54]
Unspecified/ multiple origins	Infantile Spindle Cell Tumor	MIR548F1-NTRK1 STRN3-NTRK3	[38,55]

3.2. Signaling

Members of the NTRK family promote regulation of neuronal cell proliferation, differentiation, apoptosis, and survival [13]. Activation

occurs in response to neurotrophins: Nerve Growth Factor (NGF) activating NTRK1; Brain-Derived Neurotrophic Factor (BDNF) and Neurotrophin-4 (NT-4) activating NTRK2; and Neurotrophin-3 (NT-3, or NTF3) activating NTRK3 [59]. Ligand binding invariably induces dimerization and autophosphorylation of three key tyrosine residues located within the autoregulatory loop of the kinase domain. Phosphorylation of two other tyrosine residues, which flank the kinase domain at either end, create binding sites for adaptor proteins such as SHC1, PLCγ, GAB1, Dok5, and Dok6 [13,60]. These adaptor proteins activate downstream signaling pathways, such as SHC/RAS/MAPK, PI3K/AKT, and PLC-γ/PKC, that control cell-cycle progression, proliferation, apoptosis, and survival (Fig. 1A) [13,61]. Multiple NTRK splice variants also exist with varying affinities for their corresponding neurotrophin ligands [57]. Some isoforms have been characterized with short inserts that apparently increase affinity for neurotrophins, while truncated NTRK variants have also been identified that lack large parts of the intracellular domain [13,57].

3.3. Biological roles of NTRKs

NTRK1 signaling is activated throughout the central and peripheral nervous system in response to NGF [62], although the neurotrophin NT-3, which generally promotes axonal extension, can also bind and activate NTRK1 with lower affinity [63]. Using the canonical NTRK1 sequence (Uniprot P04629–1), also referred to as TrkA-II, the key activation loop residues which undergo ligand-induced phosphorylation are Y676, Y680, and Y681 [64]. With multiple isoforms, the three most common are TrkA-I, TrkA-II, and TrkA-III, of which TrkA-II (796 residues) is the most abundant. In comparison, TrkA-I lacks exon 9 (residues 393–398) [57] and exhibits enhanced responsiveness to NT-3. TrkA-III lacks exons 6, 7, and 9 in comparison with TrkA-II [57], alterations which render it unable to bind NGF; instead, TrkA-III exhibits spontaneous intracellular ligand-independent activation of PI3K/AKT/NF-kappa-B pathways and an inability to activate the Ras-MAPK signaling cascade [65]. Therefore, the result is a greater level of angiogenic related factors along with a reduced level of neuroblastoma tumor suppressing effects [65].

NTRK2 promotes similar functions as NTRK1, such as regulation, migration, differentiation, survival, and proliferation of neurons, along with an added function of supporting synapse formation and plasticity [66]. NTRK2 is typically activated in response to BDNF binding, but also responds less efficiently to NT-3. Using the canonical NTRK2 sequence (Uniprot Q16620–1), the three key tyrosine residues involved in activation loop phosphorylation are Y702, Y706, and Y707 [57]. A novel human isoform of NTRK2 found predominantly in the nervous system has been designated as TrkB-T-TK, containing an extended exon 22 [67]. The lack of a PLC-γ binding site in TrkB-T-TK suggests an inability to activate PLC-γ/ PKC signaling pathways [67]. Another splice variant, TrkB-N-T1, notable for lacking a functional kinase domain, also exhibits a unique C-terminal 12-amino acid sequence [68]. TrkB-N-T1 activates RhoA (Ras homolog family member A) which is involved in cytokinesis and cellular adhesion [68].

NTRK3 activation results in cell differentiation and the development of proprioceptive neurons, which are mechanosensory neurons located throughout the body [69]. Upon ligand activation by NT-3, NTRK3 exhibits phosphorylation of the activation loop residues Y705, Y709, and Y710 in the canonical isoform (Uniprot Q16288–1) [57]. Multiple NTRK3 isoforms have also been observed, separable into two categories depending upon the presence or absence of a functional kinase domain [70]. The catalytic isoforms of NTRK3 are expressed in neuronal crest cells essential for the development of the peripheral nervous system where they are activated by NT-3, promoting proliferation and differentiation of these neuronal crest cells [70]. In contrast, noncatalytic variants of NTRK3, encoded by truncated isoforms, are primarily found in the CNS and promote differentiation but not proliferation.

3.4. Oncogenic activation of NTRKs

Oncogenic NTRK activation can result from mutation, CNVs, overexpression, and translocations [10]. NTRK fusion proteins are typically created by chromosomal rearrangements forming hybrid genes in which the 5' end of the partner gene is joined to the 3' portion of NTRK1, NTRK2, or NTRK3, containing the tyrosine kinase domain. While NTRK1 and NTRK3 fusions have been well characterized in pediatric STS, NTRK2 fusions have been documented in only two cases of unspecified STS to date: STRN-NTRK2 and RBPMS-NTRK2 [55,71].

Mechanistically, the presence of a multimerization domain is necessary for activation of the fusion oncoprotein. Multimerization can result in: (i) a gain-of-function fusion protein, with overstimulation of downstream signaling; (ii) a loss of normal regulatory mechanisms for wildtype (WT) receptors; (iii) abnormal localization of a constitutively activated kinase; and (iv) altered interactions with novel proteins and pathways [72]. The multimerization domains that have been identified in NTRK fusions include coiled-coil domains, zinc finger domains, WD domains, helix-loop-helix (HLH) domains, and PB1 domains (Fig. 1B) [57,72].

4. Highlights of selected NTRK chromosomal translocations

Chromosomal translocations are estimated to account for approximately 20% of all malignant neoplasms [72], including STS [7]. Often these translocations create an oncogenic fusion protein containing a constitutively activated tyrosine kinase, resulting in aberrant downstream signaling [72]. The identification of fusion-positive pediatric STS increases therapeutic options for these patients and represents an important diagnostic criterion. In this section, we have chosen a small subset of NTRK fusions which epitomize their essential features.

4.1. ETV6-NTRK3

The ETV6-NTRK3 fusion protein (Fig. 2A), resulting from the t (12;15)(p13;q25) translocation, is detected in over 90% of IFS cases [32]. One breakpoint of IFS fuses exons 1–5 of ETV6 to exons 13–18 of NTRK3 [31]. The resulting ETV6-NTRK3 fusion protein consists of the HLH multimerization domain of ETV6 fused to the tyrosine kinase domain of NTRK3 [32]. The HLH domain, comprising exons 3–4 of ETV6, exhibits multiple ETS DNA binding domains on its surface [73]. Another breakpoint characterized in Congenital Fibrosarcoma fuses exons 1–5 of ETV6 to a splice site within exons 6–8 of NTRK3.

ETV6-NTRK3 expression leads to constitutive activation of major NTRK3 signaling pathways including RAS/MAPK and PI3K/AKT [74], and the three tyrosine residues within the NTRK3 activation loop have been shown to be required for full biological activity of ETV6-NTRK3. However, this fusion protein lacks tyrosine 516 of NTRK3 which, when phosphorylated, enables docking of adaptor proteins such as SHC and Dok6 [60]. ETV6-NTRK3 has not been structurally characterized; however, a related fusion protein, TEL-SAM, in which TEL is an alternate name for ETV6, has been extensively characterized and shown to form an open-ended polymeric structure, including the formation of large aggregates [75].

ETV6 (ETS variant transcription factor 6) is a member of the ETS transcription family and is important in developmental embryonic processes [73,74]. Initially discovered as the ETV6-RUNX1 fusion in acute lymphoblastic leukemia, ETV6 has since been identified as a partner in over 30 different oncogenic fusion proteins. Endogenous ETV6 functions as a transcriptional repressor of the tumor suppressor protein stromelysin-1 (MMP-3), with DNA binding mediated by the HLH domain [73].

4.2. LMNA-NTRK1

LMNA–NTRK1 fusions (Fig. 2B), resulting from the translocation t

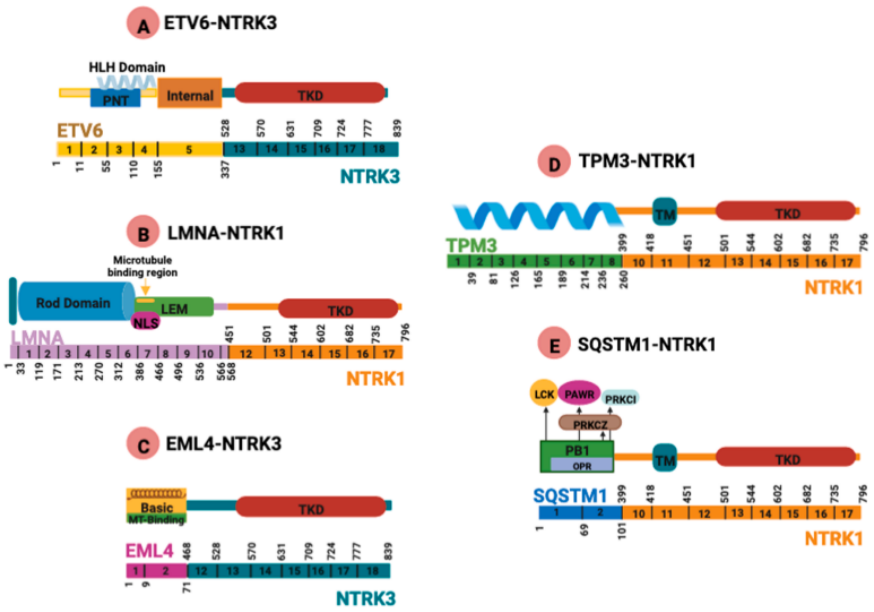

Fig. 2. Significant Pediatric NTRK Fusions. (A) ETV6-NTRK3. A typical ETV6-NTRK3 fusion is shown which includes exons 1–5 of ETV6 fused to exons 13–18 of NTRK3. The N terminal ETV6 fusion partner is a transcriptional repressor and contains a Helix-Loop-Helix (HLH) domain (exons 3–4) which is a DNA binding domain that mediates dimerization. The Pointed (PNT) domain (residues 40–124) is a conserved domain within a subset of ETS transcription factors that mediates polymerization [151-153]. An internal domain (exon 5) is also present, however the role of this domain has not been clearly established. The C terminal NTRK3 partner contains a complete tyrosine domain (TKD) (residues 538–839). (B) LMNA-NTRK1. A typical LMNA-NTRK1 fusion is presented, containing exons 1–10 of LMNA fused to exons 12–17 of NTRK1. The LMNA partner encompasses an N-terminal globular domain (Residues 1–33) which is necessary in lamina assembly [154].The Rod Domain (exons 1–6) allows for multimerization via the formation of an alpha coiled coil, whereas the LEM (LAP2 & Emerin) domain (residues 390–550) plays a role in facilitating binding to BAF (barrier-to-autointegration factor), which binds double stranded DNA and histones. The LMNA fusion partner also contains a Nuclear Localization signal (NLS) (residues 417–422) and a Microtubule-Binding Region (residues 396–436). The C-terminal NTRK1 partner contains the complete tyrosine kinase domain (TKD) (residues 510–781). (C) EML4-NTRK3. The EML4-NTRK3 fusion presented retains exons 1–2 of EML4 fused to exons 12–18 of NTRK3. The N-terminal EML4 fusion partner has a Basic Domain (exons 1–2) which contains a Microtubule (MT) Binding Region and a coiled-coil domain that forms a parallel trimeric coiled-coil [82]. The C-terminal NTRK3 partner contains the complete tyrosine kinase domain (TKD). (D) TPM3-NTRK1. The TPM3-NTRK1 fusion found in Infantile Fibrosarcoma is presented, with exons 1–8 of TPM3 fused to exons 10–17 of NTRK1. The coiled-coil domain spans the entire length of the fusion partner and forms a parallel dimer of coiled coils. The C-terminal NTRK1 partner contains the transmembrane domain (TM) domain (residues 424–439), and the complete TKD domain. (E) SQSTM1-NTRK1. The SQSTM1-NTRK1 fusion is presented, in which exons 1–2 of SQSTM1 are fused to exons 10–17 of NTRK1. The N-terminal SQSTM1 fusion partner, also known as the autophagy receptor p62, contains a Phox and Bem1 (PB1) Domain (residues 1–101) which provides for oligomerization of SQSTM1-NTRK1. Within the PB1 domain there are several interaction sites. The LCK interaction site (residues 2–50) mediates the T- cell receptor (TCR) signaling cascade [155]. Interaction with PAWR (PRKC apoptosis WT1 regulator protein) (residues 50–80) may mediate apoptosis in cancer cells. Residues 43–101 mediate an interaction with the atypical PKCs, PKC-Zeta and PKC-Iota, which functions in PI3K signaling [156]. The Octatricopeptide Repeat (OPR) domain (residues 20–100) functions as an RNA interaction module [157]. The C-terminal NTRK 1 partner contains the transmembrane domain (TM) domain (residues 424–439), and the complete TKD.

(1;1)(q22;q23), have been implicated in several malignancies, including Spitz nevus, colorectal cancer, lung cancer, soft-tissue sarcomas, and a rare case of infantile-onset lipofibromatosis-like neural tumor [36]. One breakpoint found in CIFS (Congenital Infantile Fibrosarcoma) encodes exons 1–10 of LMNA fused to the kinase domain of NTRK1 (exons 12–17) [41].

The LMNA gene, encoding the Lamin A/C proteins, is localized at chromosome 1q22 and encodes a key component of the nuclear lamina involved in nuclear assembly and chromatin organization [76]. During mitosis, the nuclear lamina is disassembled, accompanied by hyperphosphorylation of the constituent lamins causing dissociation into free lamin dimers [77]. Alternative splicing forms Lamin A, encoded by 12 exons, or Lamin C, encoded by 10 exons [78].

The structural basis for lamin assembly at the molecular level depends upon a coiled-coil dimerization domain, arranged in an anti-parallel arrangement of two coiled-coil dimers [72]. The coiled-coil multimerization domain contributed by LMNA leads to the activation of the LMNA-NTRK1 fusion and, in turn, initiates downstream signal transduction cascades [41]. The smallest LMNA-NTRK1 fusion protein found in IFS encodes exons 1–2 of LMNA fused to exons 11–17 of NTRK1 [36]. Exons 1–2 code for an N-terminal globular domain and a partial alpha helical rod domain, suggesting their importance in activation of additional larger LMNA-NTRK1 fusions.

4.3. *EML4-NTRK3*

EML4-NTRK3 fusions (see Fig. 2C) are important in malignancies such as IFS and congenital mesoblastic nephroma [31]. This fusion protein results from the t(2;15)(p21;q24) translocation, identified in pediatric congenital fibrosarcoma, primitive sarcoma, congenital

mesoblastic nephroma, and metastatic spindle cell sarcoma [31]. This fusion protein contains exons 1–2 of EML4 joined to exons 12–18 of NTRK3 [40].

EML4, also known as Echinoderm Microtubule Associated Protein 4, belongs to the conserved family of echinoderm microtubule-associated protein (EMAP)-like proteins. EML4 contains a HELP (Hydrophobic EMAP-Like Protein) motif, encoded by exons 7 and 8, and thirteen WD40 domains spanning from exon 7 to exon 24. Endogenous EML4 is essential for the organization of the mitotic spindle and the proper attachment of spindle microtubules to kinetochores. Interaction between the WD40 repeats of EML4 and the C-terminus of nuclear distribution gene C (NUDC) protein is required for localization of NUDC onto the mitotic spindle during mitotic progression [79]. EML4 also stabilizes microtubules, leading to a reduction in the amount of free tubulin and microtubule growth rate [80].

As the EML4-NTRK3 fusions retain only the N-terminal exons 1–2 of EML4, these normal functions of EML4 described above will be lost in the fusion protein. Notably, EML4 has also been identified fused to Anaplastic Lymphoma Kinase (ALK) in non-small cell lung cancer [81] with several common breakpoints observed [82]. One EML4-ALK fusion, in which exon 2 of EML4 is fused to ALK, displays a similar EML4 breakpoint as EML4-NTRK3. The diffuse cytoplasmic localization of this EML4-ALK reveals that the microtubule binding function of EML4 is not retained in these EML4-ALK fusions [83], suggesting a loss of microtubule binding for EML4-NTRK3 as well.

Similar to other NTRK fusions, EML4 serves as a multimerizing protein partner and contributes an unusual trimeric coiled-coil domain to the EML4-NTRK3 fusion. The consequent multimerization of the NTRK3 domain underlies the constitutive tyrosine kinase activity of EML4-NTRK3 [83]. Expression of EML4-NTRK3 induces transforming and tumorigenic ability of NIH3T3 fibroblast cells [40], activating downstream signaling pathways [84].

4.4. *TPM3-NTRK1*

The TPM3–NTRK1 chimeric oncogene (see Fig. 2D) was originally identified in human colon carcinoma, and results from an intrachromosomal inversion involving nearby genes, TPM3 at 1q21.3, and NTRK1 at 1q23.1 [85]. Additionally, TPM3 has previously been found fused to ALK as a driver of lymphoma [86].

The partner gene, TPM3 (Tropomyosin Alpha-3 Chain) encodes a member of the tropomyosin family of coiled-coil proteins that bind to actin filaments in muscle and non-muscle cells, primarily expressed in slow/Type 1 skeletal fibers [87]. TPM3 exists largely as a parallel dimer of coiled coils, and deletion of this coiled-coil domain reduces its biological activity [88]. The coiled coils of TPM3 also form higher order structures through a four-helix coiled-coil "overlap region," an interleaved bundle that binds the N-terminal fragment of troponin T [89]. This is required for cooperative binding along actin filaments and modulates the cooperative interaction of myosin with the actin filament. This overlap region involves the N-terminus of one dimer of TPM3, interacting with the C-terminal domain of the next dimer of TPM3. In TPM3-NTRK1, this N-terminal domain is retained while the C-terminal domain is not; thus, the ability of the fusion protein to participate in higher order structure formation remains an interesting question.

The TPM3-NTRK1 fusion has been identified in cases of colorectal carcinoma, papillary thyroid carcinoma, and in a case of IFS with Bloom syndrome (chromosomal instability disorder) [85,90]. Sequencing analysis of this IFS case shows that exon 8 of TPM3 was fused to exon 10 of NTRK1. This study suggested that the genomic instability brought on by Bloom syndrome may have resulted in the TPM3-NTRK1 fusion [90].

4.5. *SQSTM1-NTRK1*

The SQSTM1-NTRK1 fusion protein (see Fig. 2E) results from the t (1;5)(q23;q35) chromosomal translocation. In addition to STS, this fusion protein is a known driver for NSCLC [91-93]. In IFS, exon 2 of SQSTM1 is fused to exon 10 of NTRK2, while in NSCLC, exon 6 of SQSTM1 is typically fused to exon 10 of NTRK1 [92]. The smallest SQSTM1-NTRK1 fusion comprises the N-terminal PB1 (Phox and Bem1p) domain of SQSTM1 fused to the tyrosine kinase domain of NTRK1 [92,93]. In SQSTM1, multimerization is mediated by this domain, comprised of a beta-grasp fold of about 80 residues which recurs in many eukaryotic signaling proteins [94]. The PB1 domain drives homo-oligomerization and hetero-oligomerization with other specific PB1 domain-containing proteins, mediated by acidic and basic residues. Although it remains to be confirmed experimentally, it seems probable that the PB1-mediated multimerization of SQSTM1-NTRK1 results in constitutive activation of NTRK1 signaling pathways.

SQSTM1(sequestosome1), also known as the autophagy receptor p62, is associated with a wide range of diseases, including bone and muscle disorders, neurodegenerative and metabolic diseases, and multiple forms of cancer. [95]. SQSTM1 has a unique cellular function as the autophagy receptor for the aggregation of ubiquitinated substrates and their assemblage into the sequestosome, a vesicle which then matures to an autophagosome and undergoes subsequent fusion with the lysosome [96,97]. Autophagy is important in eliminating unwanted cargo and reducing intracellular stressors. SQSTM1 binds to ubiquitinated substrates via the UBA (ubiquitin-associated) domain and delivers them to the phagophore for degradation [96,97]. The PB1 domain allows SQSTM1 to carry out its function as a shuttling protein delivering ubiquitinated substrates to the proteasome [96]. Furthermore, the PB1domain recruits aPKC (atypical protein kinase C), including PKC iota and PKC zeta, which also contributes to NF-κB signaling [97]. Which of these multiple functions associated with p62/SQSTM1 directly contribute to the oncogenicity of SQSTM1-NTRK1 fusions remains to be determined.

5. Beyond oligomerization

All NTRK fusions exploit an oligomerization domain, contributed by the N-terminal fusion partner, which allows constitutive kinase activation and downstream signaling activation. Oligomerization may be facilitated by disparate structural motifs, including coiled-coil domains, PB1 domains, Zinc Finger domains, WD Domains, etc. [98]. Despite the importance of providing a motif for multimerization, the contribution of some fusion partners may go beyond this basic function by bringing an additional biochemical activity that contributes to oncogenic activation. Many of the NTRK fusion proteins, listed in Supplementary Tables S1–S3, potentially fall into this category; we have chosen three potential candidates to discuss briefly: TP53-NTRK1, QKI-NTRK2, LYN-NTRK3, each of which harbors a fusion partner with potentially unique activation mechanisms.

5.1. *TP53-NTRK1*

TP53-NTRK1, which occurs in spitzoid neoplasm [99], has multiple documented breakpoints spanning exons 8–12 of TP53 fused to exon 9 of NTRK1; the fusion with the exon 8 breakpoint of TP53 is presented in Fig. 3A [100]. This fusion protein retains most of the master transcriptional regulator TP53 except for the very C-terminal domains. TP53, also known as Tumor Suppressor P53, causes apoptosis, cell cycle arrest, or differentiation via transcriptional regulation using protein complexes TPB (TATA-binding protein) and NF-Y (Nuclear factor Y). TP53 contains a nuclear localization signal (NLS) as well as a nuclear exclusion signal (NES), resulting in nuclear-cytoplasmic shuttling [99].

The TP53-NTRK1 fusion protein includes the transactivation domains TA1 and TA2, the entire DNA binding domain, a proline-rich domain, the NLS, and the tetramerization motif of TP53. Missing from the TP53-NTRK1 fusion are the NES signal and the basic C-terminal domain, which functions as a positive regulator of TP53 tumor suppressor function [101]. Interestingly, the crystal structure of the

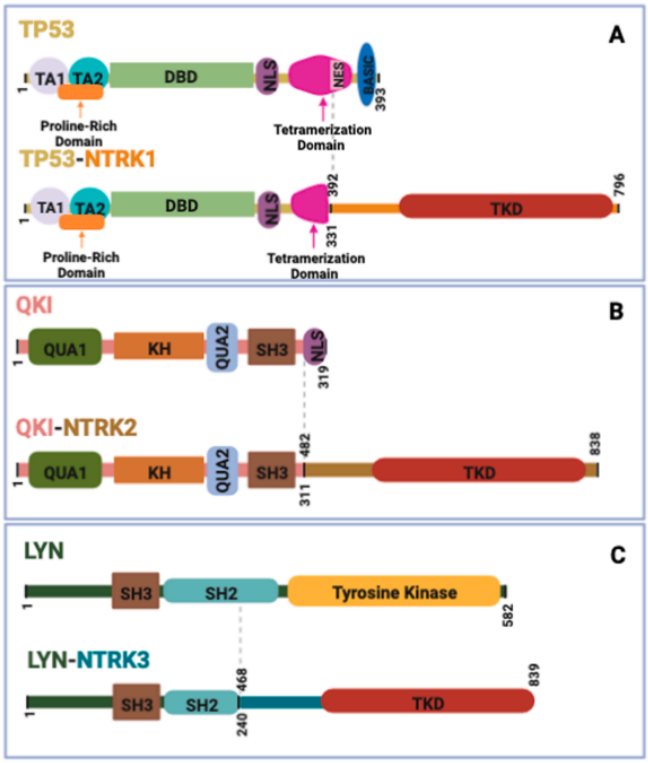

Fig. 3. Beyond Oligomerization. (A) TP53-NTRK1. The N-terminal fusion partner is the well-known tumor suppressor protein Tumor Protein P53 (TP53). TP53 contains 2 transactivation (TA) domains which are important for tumor suppression abilities. The proline rich domain (residues 63–97) serves as docking sites for various signaling proteins playing a role in cell-cell communication and signal transduction [158,159]. The DNA binding domain (DBD) participates in interactions with the tetramerization domain which underlies the mechanism of multimerization. Beyond multimerization, the interactions with the DBD can result in positive regulation or inhibition of apoptosis. The fusion contains a nuclear localization signal (NLS) (Residues 300–323); however, the nuclear export signal (NES) sequence and the Basic Domain (residues 362–393) are not retained [160]. (B) QKI-NTRK2. The fusion QKI-NTRK2 consists of exons1–5 of QKI joined to exons 13–17 of NTRK2. The N-terminal fusion partner QKI (Quaking protein) is composed of several domains involved in different aspects of RNA processing; such as the QUA1 domain (residues 12–69) with a role in alternative splicing; the KH (K homology RNA-binding domain) (residues 69–183) that functions in RNA recognition; and the QUA2 domain (residues 183–204) that modulates RNA-binding specificity [161]. Multimerization appear to be mediated by a region within the QUA1 domain. The QKI-NTRK2 fusion lacks a Nuclear Localization Signal (NLS) from QKI. The C-terminal NTRK2 domain contain the complete TKD (residues 554–823). (C) LYN-NTRK3. The oncogenic fusion LYN-NTRK3 is presented. Like all members of the SFK family, LYN itself contains an SH3 (Src Homology 3) domain, an SH2 (Src Homology 2) domain, and a tyrosine kinase domain. The SH2 and SH3 domain normally play a role in regulation of LYN kinase activity as well as binding to adaptor proteins [162,163]. Although LYN-NTRK3 retains the SH3 domain (residues 133–193) and part of the SH2 domain (residues 199–296), the tyrosine kinase domain of LYN is substituted by the tyrosine kinase domain (TKD) of NTRK3.

DNA-binding domain forms an antiparallel β-sandwich composed of two large loops and a loop-sheet-helix motif, and p53 assumes a tetrameric structure stabilized as a symmetric dimer of dimers which bind cooperatively to DNA [102]. p53 mutants with a functional tetramerization domain will form mixed tetramers which exhibit dominant-negative effects [103], which could also occur with TP53-NTRK1 if localized to the nucleus. Indeed, whether TP53-NTRK1 is localized to the nucleus and bound to DNA poses a fascinating question about this fusion protein, and how the biological properties of TP53-NTRK1 may be affected or enhanced by the TP53-specific functions retained in the fusion remains unexplored.

Also noteworthy is the ability of TP53 to repress PTPN6, an NTRK1-phosphatase that specifically dephosphorylates two key phosphotyrosine residues in NTRK1 [99]. Therefore, TP53 potentially promotes enhanced NTRK1 phosphorylation which in turn promotes activation of downstream pathways. Whether this specific functionality of TP53 is retained in the TP53-NTRK1 fusion remains unexplored.

5.2. QKI-NTRK2

The QKI-NTRK2 fusion protein, shown in Fig. 3B, has been reported in pilocytic astrocytoma [104]. In this fusion, exons 1–6 of QKI, also known as Protein Quaking, are fused with exons 16–21 of NTRK2 [105]. Due to the presence of the entire NTRK2 protein kinase domain and all key phosphotyrosine residues, QKI-NTRK2 potentially activates all downstream NTRK2 pathways. QKI is an RNA binding protein that regulates the expression of multiple oligodendrocyte genes [106], and mediates the regulation of myelination [107]. In addition, QKI is involved in many functions concerned with pre-mRNA splicing, mRNA export, protein translation, mRNA protection and stabilization. Surprisingly, QKI functions as a tumor suppressor as evidenced by its regulation of cancer-related genes such as p27 and β-catenin [108]. Malfunction of QKI results in dysregulations of these mRNAs and eventually may lead to multiple human diseases, including lung cancer, glioblastoma, and neurological diseases [109,110]. The QKI-NTRK2 fusion retains the Qua1 domain that mediates homodimerization, the Qua2 domain involved in RNA interaction, the KH RNA binding domain, and an SH3-binding motif, lacking only the C-terminal domain which encodes an NLS. Conceivably, the absence of this NLS may result in abnormal localization and disruption of normal QKI functions. Future experimentation will be required to determine whether this potential alteration of localization accounts for the oncogenicity of the fusion protein QKI-NTRK2.

5.3. LYN-NTRK3

The LYN-NTRK3 fusion protein, shown in Fig. 3C, is found in spitz tumors [111], an uncommon melanocytic lesion containing large epithelioid and spindled cells. LYN is a member of the Src Family Kinases (SFKs), a group of nine non-receptor tyrosine-protein kinases including Src, Fyn, Lck, Blk, and others, that transmit signals from cell surface receptors and are involved in cell proliferation, differentiation, apoptosis, migration, inflammation, and tumorigenesis [112]. Like all SFKs, LYN contains an N-terminal myristylation signal, SH3 and SH2

regulatory domains of about 100 residues each, and a C-terminal kinase domain. Phosphorylation of a C-terminal regulatory tyrosine Y505 by Csk keeps LYN in an inactive conformation that is stabilized by the interaction between the SH3 domain and a proline-rich sequence between the SH2 and kinase domain [113]. In the active state, upon dephosphorylation of Y508 by phosphatases CD45 and SHP-2, SH2 and SH3 domains interact with various effector proteins to effect kinase activation [114]. Studies of the prototypic SFK, Src, demonstrate that dimerization occurs by the myristylated N-terminal region binding to a hydrophobic pocket in the kinase domain of a dimerization partner [115]. Whether LYN-NTRK3 undergoes dimerization by a similar mechanism is unknown. LYN is involved in the transmission of signals from various receptors such as the B-cell receptor, GM-CSF receptor, FcεR1, Epo-receptor, c-Kit and integrins, and phosphorylates signaling molecules such as immunoreceptor tyrosine-based inhibitory/activation motifs (ITIM/ITAM), PI3K, FAK, PLCγ, STAT5 and MAP kinase [116].

The LYN-NTRK3 fusion is notable for retention of the N-terminal domain, the SH3 domain, and most of the SH2 domain of Lyn, fused to the kinase domain of NTRK3. The LYN-NTRK3 fusion most likely localizes to the plasma membrane by virtue of its myristylation signal, and the possible roles of the regulatory SH2 and SH3 domains in the fusion remain unexplored.

6. Beyond oligomerization: protein-protein interactions

The importance of dimerization and multimerization for constitutive kinase activation among oncogenic fusion proteins has been well documented [72,117] However, as discussed, the partner genes mentioned above may contribute additional oncogenic functionalities beyond multimerization. Recent studies of another oncogenic fusion protein, BCR-FGFR1, responsible for stem cell leukemia/lymphoma [118], have shown that the BCR domain contributes a Grb2 recruitment site, required for oncogenic activation, in addition to the contribution of a coiled-coil multimerization domain [118]. Another example is provided by FGFR3-TACC3, commonly observed in glioblastoma, where the TACC3 domain contributes a multimerization signal required for activity [119,120]. However, FGFR3-TACC3 has also been shown to recruit endogenous TACC3 away from the mitotic spindle where it has an essential function as a motor spindle protein, required for stabilization of the mitotic spindle and accurate chromosome segregation [121]. As a result, FGFR3-TACC3 contributes to the accumulation of mitotic defects and aneuploidy, hallmarks of aggressive cancers.

In summary, a thorough understanding of the diverse biological functions of these NTRK fusion partners may uncover important functional roles, above and beyond multimerization, which contribute to the oncogenicity of each particular fusion protein. The identification of these additional biological functionalities may suggest additional therapeutic routes for inhibition.

7. TKIs as targeted therapies for NTRK fusion-positive cancers

Treatment options for pediatric sarcomas can range from intensive chemotherapy and radiotherapy to targeted therapies. In ARMS, the HDAC inhibitor Entinostat reduced expression of the oncogene, PAX3-FOXO1 [8]. Furthermore, JQ1 +, an inhibitor of the BET family of bromodomain proteins, inhibited EWSR1-FLI1 in Ewing Sarcoma and the SS18-SSX1/2/4 fusion in synovial syndrome [8].

Similarly, several TKIs have demonstrated promising results within NTRK fusion-positive cancers. Cabozantinib, crizotinib, nintedanib, ponatinib, larotrectinib, and entrectinib have all been used to treat NTRK-driven malignancies [122]. By inducing a conformational shift preventing NTRK activation, these inhibitors result in the induction of cellular apoptosis and inhibition of cell proliferation in tumors. Notably, larotrectinib and entrectinib have demonstrated fast, durable results in patients with fewer adverse side-effects [122]. Crizotinib has also yielded positive results in NTRK-driven pediatric STS [123]. Selitrectinib and repotrectinib represent next generation agents developed to target resistance brought on by emergent kinase domain mutations and substitutions of solvent front/gatekeeper residues [122].

7.1. Larotrectinib

Larotrectinib (LOXO-101) is an orally administered ATP-competitive first generation TKI with demonstrated efficacy against NTRK fusion-positive tumors in a wide variety of cancers [124,125]. Larotrectinib has also been used as targeted therapy in NTRK fusion-positive pediatric sarcomas containing notable ETV6-NTRK3 and LMNA-NTRK1 fusions [41,126,127]. Clinical trials on LMNA-NTRK1-driven pediatric STS showed promising results leading to disappearance of the majority of the tumor with limited adverse effects [41,128]. Larotrectinib demonstrates potent and specific inhibition of NTRKs [126], and NTRK fusion tumor studies treated with larotrectinib show targeted inhibition of downstream signaling pathways [129].

Larotrectinib has shown positive results in several pediatric STS clinical cases involving SQSTM1-NTRK1 (IFS), TPM3-NTRK1 (pediatric spindle cell sarcoma), ETV6-NTRK3 (IFS), PDE4DIP-NTRK1 (pediatric intramuscular soft tissue sarcoma), and SPECC1L-NTRK3 (IFS) [37,38,53]. Larotrectinib also led to tumor reduction in ETV6-NTRK3-driven cancers such as IFS [37], infantile glioblastoma [130], Philadelphia chromosome (Ph)-like Acute Lymphoblastic Leukemia (ALL) [131], and pediatric B-cell Acute Lymphoblastic Leukemia [132]. Furthermore, larotrectinib can effectively cross the blood brain barrier and demonstrates activity against NTRK-positive central nervous system tumors [122].

A phase 1 clinical study of larotrectinib in pediatric patients harboring solid tumors with NTRK gene fusions showed a 94% overall response rate. The research group was primarily comprised of pediatric patients with IFS and STS (half metastatic, half localized) [129]. An analysis of 24 patients (NCT NCT02637687O) demonstrated that larotrectinib results in relatively few adverse effects and exhibits a high safety profile [124].

7.2. Entrectinib

Unlike larotrectinib, entrectinib displays broader activity against multiple RTKs [133]. Entrectinib is an oral, ATP competitive, multi-kinase inhibitor [129,133]. A variety of cancers have been targeted with this drug, including NTRK-driven infantile fibrosarcoma, NTRK, ROS1, or ALK-driven pediatric high-grade gliomas, ALK-driven neuroblastoma, and ALK or ROS1-driven inflammatory myofibroblastic tumors [134]. Notably, entrectinib is well tolerated against NTRK gene fusions in solid tumors, including patients with primary or secondary CNS disease [91].

Patients presenting with NTRK fusions, such as ETV6-NTRK3, EML1-NTRK2, TPR-NTRK1, LMNA-NTRK1, and SQSTM1-NTRK1 have all showed drastic improvement in response to entrectinib [91,134,135]. Specifically, entrectinib, showed promising results in acute myeloid leukemia derived patient cells harboring an ETV6-NTRK3 fusion. A rapid reduction of NTRK3 autophosphorylation and decreased phosphorylation of the PLCγ, ERK, and STAT3 signaling pathways was observed [136].

Clinical trials involving entrectinib have produced positive results in patients [91]. Of 17 documented patients harboring either NTRK, ROS, or ALK alterations, 58% showed partial response and 70% experienced disease stabilization; and, one patient exhibited a complete response [133].

7.3. Crizotinib

Crizotinib (Xalkori) is another oral, ATP-competitive TKI. Although less characterized for pediatric sarcoma, positive results have been described. Crizotinib was initially characterized and FDA approved as a treatment option for patients with ALK rearrangements specifically in

cases of locally advanced or metastatic NSCLC [36]. In fact, crizotinib was identified as more effective than standard chemotherapy in patients that were previously treated for ALK-driven NSCLC [137]. However, research has assessed that crizotinib may have a similar IC50 against ROS1, ALK, and NTRK rearrangements in vitro [41]. In addition, crizotinib showed durable results against NTRK-driven cancers [36]. To date, crizotinib has been characterized in the following NTRK fusions: MPRIP-NTRK1, IRF2B2-NTRK1, KHDRBS1-NTRK1, LMNA-NTRK1, ETV6-NTRK3 [36,41,123]. In one case of LMNA-NTRK1 fusion-positive metastatic IFS, the patient achieved complete response after approximately 31 months of treatment [36]. In another study, crizotinib treatment resulted in nearly complete tumor regression for a CIFS patient harboring LMNA-NTRK1 [41]. Lastly, in a nonpediatric case of unclassified mesenchymal sarcoma harboring KHDRBS1-NTRK1, the patient showed a complete response after 3 months on this therapy [52].

Crizotinib is currently undergoing Phase III clinical trials (NCT03874273) for treatment in adolescent Inflammatory Myofibrolastic Tumors (IMT), a type of soft tissue sarcoma. The results from Phase II showed that in a group of 14 participants who had unresectable, relapsed or refractory IMT, 86% of cases responded to treatment with crizotinib. Moreover, 36% received a complete response while 50% received a partial response.

Screening for NTRK gene fusions should be considered a priority following diagnosis to determine which patients may benefit from therapy with entrectinib, larotrectinib, or crizotinib. Specifically, for infantile and early adolescent pediatric patients that are more sensitive to harsher chemotherapy and radiation, screening for NTRK gene alterations could prove to be lifesaving with fewer adverse effects.

8. Overcoming resistance: next generation TKIs

Resistance to first-generation NTRK inhibition can eventually occur after administration of pan-TRK Inhibitors such as entrectinib and larotrectinib [125]. Primary analysis of the efficacy of larotrectinib revealed acquired resistance involving substitution in the solvent-front (NTRK1 G595R or NTRK3 G623R) or gatekeeper residue (NTRK1 F589L), as well as a mutation in the DFG motif which plays an important role in the regulation of kinase activity (NTRK1 G667S or NTRK3 G696A) [125]. Notably, several patients exhibited multiple mutations, demonstrating that an individual can develop more than one NTRK mutation [125]. Acquired entrectinib resistance was also documented within a case of metastatic colorectal carcinoma harboring LMNA-NTRK1(NTRK1 G595R and G667C) and in a case of analog secretory carcinoma harboring ETV6-NTRK3 (NTRK3 G623R). As a result, second generation agents such as selitrectinib and repotrectinib were designed to target acquired resistance from first generation NTRK inhibitors.

8.1. Selitrectinib

Selitrectinib (BAY 2731954, LOXO-195) is a second generation orally administered NTRK inhibitor which targets specific TKI-resistant point mutations [138], and which has showed efficacy in Phase I/II trial of acquired resistance from prior TKI administration [54]. In one report, an adult patient with LMNA-NTRK1-positive colorectal cancer developed a TKI-resistant mutation (G595R) following treatment with larotrectinib; however, treatment with selitrectinib significantly reduced tumor volume and expression of LMNA-NTRK1 [139]. Similarly, in another case, a patient suffering from TPM3-NTRK1-driven cancer developed resistance after administration of larotrectinib; however, following the appearance of the G595R mutation, the patient was successfully treated with selitrectinib [140]. Another TKI resistant mutation (G623R) was observed in the case of an ETV6-NTRK3 fusion in infantile fibrosarcoma; after administration of selitrectinib, tumor size again exhibited a significant decrease [139]. While the detailed mechanism of selitrectinib has not been described, it is speculated that selitrectinib accommodates the bulky arginine side chain in the solvent front without steric clashes and is predicted to better accommodate DFG substitutions in NTRK1/2/3, in comparison with larotrectinib [139].

8.2. Repotrectinib

Another second-generation agent, repotrectinib (TPX-0005), is an orally available potent ATP- competitive inhibitor that targets solvent-front mutations and inhibits downstream effectors. Repotrectinib has been used with ROS1, ALK, and NTRK TKI-resistance [141]. Structural and mechanical insights of repotrectinib were categorized by in silico evaluation [141]. Similar to selitrectinib, repotrectinib was reported to accommodate large, bulky, and positively charged arginine side chains which were no longer sterically hindered by the solvent front [141]. Therefore, treatment with second generation NTRK inhibitors has been successful in combating acquired resistance in NTRK fusion-positive pediatric sarcomas.

9. Discussion

NTRK fusion proteins allow constitutive NTRK activation in the absence of the cognate ligand, leading to nonscheduled activation of multiple downstream intracellular signaling pathways including SHC/RAS/MAPK, PI3K/AKT, and PLC-γ/PKC that control cell-cycle progression, proliferation, apoptosis, and survival.

A fascinating question to consider: what causes these chromosomal translocations? Translocations are a subset of rearrangements resulting from multiple double-stranded breaks (DSBs) in chromosomal DNA [72,142]. Chromosomal translocations are critically important in cancer, resulting in the creation of oncogenic gene fusions or, by bringing genes closer to enhancer or promoter elements, leading to altered expression [142]. The widespread release of radioactive material from the Chernobyl Nuclear Power Plant, in 1986, subsequently led to large clusters of patients suffering from papillary thyroid carcinoma (PTC) who exhibited specific translocations involving RET, another RTK, and to a lesser extent NTRK1 [143-145]. Chromosomal translocations appear to be influenced by nuclear architecture; several studies of radiation-induced thyroid cancers in Chernobyl victims suggest the importance of spatial proximity in the creation of specific translocations. In this model, large-scale chromosome folding within interphase nuclei leads to nonrandom positioning of gene pairs, increasing the probability of specific intrachromosomal translocations in response to DSBs [146,147]. Furthermore, activation-induced cytidine deaminase, which plays a pivotal role in immunoglobulin class switch recombination and somatic hypermutation, may underlie some translocation-driven cancers [148]. Other recent work demonstrates that chromosome ends produced by DSBs can freely move over large distances within the nuclear space; domains containing these ends are able to cluster, and this juxtaposition of ends can result in translocations. These observations are consistent with a dynamic "breakage-first" hypothesis requiring that DSB-containing chromosomal domains are mobile, in contrast to a "contact-first" hypothesis [149,150]. Clearly, more research is required to understand the frequent emergence of specific translocations which may present in only a limited selection of cancers.

Due to the plethora of NTRK oncogenic fusions described, there has been significant progress as reflected in ongoing clinical trials of several TKIs, which are typically ATP-competitive inhibitors, particularly larotrectinib and entrectinib. However, cases of acquired resistance have necessitated development of next-generation TKIs, such as selitrectinib and repotrectinib. These inhibitors have brought about large-scale tumor shrinkage, and reduce the emergence of gatekeeper, solvent-front, and compound mutations, which often accompany TKI-resistance.

Typical treatments for pediatric sarcomas involve chemotherapy and radiotherapy, which unfortunately can have many adverse effects, highlighting the need for more targeted therapies for pediatric cases. Overall, the studies presented in this review suggest that screening for

NTRK gene rearrangements should be prioritized to provide the greatest flexibility of treatment options.

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Author contributions

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Conflicts of interest

There are no conflicts of interest to disclose.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cytogfr.2022.08.003.

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The thesis author was responsible for the QKI-NTRK2 section of this review in its entirety. The thesis author also assisted with figures, tables, the conclusion, and other sections as well. Co-authors include Megha R. Aepala, Malalage N. Peiris, Zian Jiang, April N Meyer, and Daniel J Donoghue.

Chapter 2

Critical Domains for NACC2-NTRK2 Oncogenic Activation

ABSTRACT

Neurotrophic receptor tyrosine kinases (NTRKs) belong to the receptor tyrosine kinase (RTK) family. NTRKs are responsible for the activation of multiple downstream signaling pathways that regulate cell growth, proliferation, differentiation, and apoptosis. NTRK-associated mutations lead to aberrant activation of these downstream pathways and often result in oncogenesis. This study characterizes the NACC2-NTRK2 oncogenic fusion protein that leads to pilocytic astrocytoma and pediatric glioblastoma. This fusion joins the broad-complex, tramtrack, and bric-a-brac (BTB) domain of Nucleus Accumbens-associated protein 2 (NACC2) with the transmembrane helix and tyrosine kinase domain of NTRK2. This work focuses on identifying domains critical to the fusion protein activity and possible methods to deactivate the fusion. NACC2-NTRK2 is able to transform NIH3T3 cells. Such activity depends on the NTRK2 kinase domain phosphorylation that activates signaling pathways including MAPK, JAK/STAT, and PLCγ. The activation of the NTRK2 kinase domain relies on the multimerization of the NACC2 BTB domain. A BTB domain charged pocket mutation and a monomer core mutation result in deactivation of the kinase domain and abrogates the ability to transform NIH3T3 cells, suggesting that BTB domain inhibition could be a potential treatment for the NACC2-NTRK2 induced cancer. The undefined region of NACC2 at residues 120-418

is responsible for forming stronger multimers. Once removed, the NACC2-NTRK2 multimer is vulnerable to SDS denaturation and leads to reduced activity of the fusion. Lastly, the removal of the transmembrane helix leads to higher activation of the NACC2-NTRK2 fusion. A protein stability assay indicates that transmembrane deletion prevents NACC2-NTRK2 degradation.

INTRODUCTION

NTRK (neurotrophic tropomyosin receptor kinases), or TRK, is a subclass of transmembrane receptor tyrosine kinase (RTK) proteins that are expressed in neuronal tissues (1). NTRK1, NTRK2, and NTRK3 are three members of the NTRK family. NTRKs share similar protein structures. Each NTRK contains an extracellular ligand binding domain, a transmembrane domain, and an intracellular kinase domain (2). Each NTRK is activated by its corresponding extracellular ligand (3). As a ligand binds to the extracellular domain of NTRK, it induces NTRK dimerization, followed by rapid autophosphorylation of tyrosine residues in the intracellular kinase domain (4). Once activated, NTRK is able to turn on multiple cell survival, proliferation, and apoptosis-related intracellular signaling pathways, including RAS/MAPK, PLCγ, and JAK/STAT3 pathways (5). NTRK point mutations or NTRK chromosomal translocation events often lead to aberrant activation of the kinase domain, resulting in carcinogenesis due to the constant activation of downstream signal (6).

Since the discovery of the first NTRK fusion protein in 1982 (7), numerous studies have been conducted to discover the mechanisms for the oncogenic activation of NTRK fusion proteins. Inhibitors have been developed against the NTRK kinase domain and yielded

promising therapeutic effects (8). This study focuses on Nucleus Accumbens-associated protein 2 - neurotrophic tropomyosin receptor kinase 2 (NACC2-NTRK2) fusion protein. The first NACC2-NTRK2 (ex4: ex13) fusion was discovered in pilocytic astrocytoma (9), which is a WHO grade 1 tumor, with over 90% of 10-year survival rate. Later, another variant fusion exon 4: exon15 was discovered in pediatric glioblastoma (10), which is a rare WHO grade IV tumor.

In the NACC2-NTRK2 fusion, residue 1-418 of NACC2 is fused with NTRK2. This sequence contains the BTB domain (residue 20-120), an undefined region (residue 121-350), and part of the BEN domain (residue 351-448). The 5' fusion partner Nucleus Accumbens-associated protein 2 (NACC2) is a transcription repressor that regulates the transcription of E3 Ubiquitin ligase Mouse Double Minute 2 (MDM2). NACC2 has two ~~major~~ functioning domains: a broad-complex, tramtrack, and bric-a-brac (BTB) domain that is responsible for NACC2 dimerization and recruitment of Nucleosome Remodeling and Deacetylase (NuRD) transcriptional regulator complex to the MDM2 promotor (11), a BEN domain that recognizes a specific DNA sequence (12). The BTB domain is an evolutionarily conserved domain that is possessed by a variety of proteins (13), and most of the BTB domain-containing proteins are zinc finger proteins that serve as transcriptional regulators (14). BTB domains are responsible for various protein-protein interactions, including self-association, hetero-multimerization, and transcriptional factor interactions (15). BTB domain contains multiple alpha helices. Once dimerized, these helices in two BTB domain monomers form a tidily intertwined dimer interface, with a charged pocket formed by 4 charged residues in the center of the interface (16). Disruption of the charged pocket or dimer formation results in a dysfunctional BTB domain (17). In other BTB domain containing fusion-positive cancers, such disruption of BTB

domain also abrogates oligomer formation and fusion protein functions (18).

This is the first study that characterizes the activation mechanism of NACC2-NTRK2 fusion, and the functions of each domain that exists in this fusion protein. We demonstrate that the fusion protein activates the downstream signaling pathways of NTRK2 independently of Brain-Derived Neurotrophic Factor (BDNF) ligand. Disruption of the BTB domain by either mutation in the charged pocket or the monomer core leads to a reduction in multimer formation and abrogates the NIH3T3 transformation activity of the fusion protein. To investigate the function of the NACC2 undefined region (residue 121-418), we constructed a new fusion that contains only the NACC2 BTB domain (residue 1-120) and the NTRK2. Results show that this clone shows weaker activation but is still biologically active. Further multimerization studies indicate that the reduced activation results from the formation of an SDS-sensitive fusion multimer. Furthermore, we investigated the differences between the variant that causes pilocytic astrocytoma and the variant causing glioblastoma. We also show that the transmembrane helix of the NTRK2 regulates the activity of the fusion protein. By deleting the transmembrane helix in the fusion, we observed elevations of all downstream signal activities and biological activity of the fusion, suggesting the TM region is an important regulatory domain in the fusion.

RESULTS

NACC2-NTRK2 promotes ligand-independent kinase and downstream activation

NACC2-NTRK2 contains the NACC2 BTB dimerization domain, the NTRK2 transmembrane helix, and the NTRK2 tyrosine kinase domain. The activation of NTRK2 kinase relies on dimerization induced by BDNF ligand binding to the extracellular domain (Figure 4A). To understand whether activation of the NTRK2 kinase domain leads to the oncogenicity of NACC2-NTRK2 fusion protein, NTRK2, NACC2, NACC2-NTRK2, and NACC2-NTRK2 (K572R) kinase-dead (KD) were transfected into NIH3T3 cells. We included BCR-FGFR1 fusion protein, which was proven to be oncogenic in previous studies, as a positive control (19). The contact inhibition feature of NIH3T3 cells allows the discovery of oncogenes, and the #foci/colony ratio can serve as a quantitative measurement of fusion oncogenicity. Through the transformation assay, wild-type NACC2, NTRK2, and NACC2-NTRK2 (KD) showed no transformation activity, whereas the wild-type NACC2-NTRK2 fusion showed high transformation activity (Figure 4B, 4C).

Next, we tried to elucidate the downstream signaling activity result by NACC2-NTRK2 activation. NTRK2, NACC2, NACC2-NTRK2, and NACC2-NTRK2 (KD) were transfected into HEK293T cells that were previously utilized to characterize downstream activities of FGFR3-TACC3 fusion (20). NTRK2 (KD) was included as a negative control. We analyzed the NACC2-NTRK2 induced activation of MAPK, PLCγ, and JAK/STAT pathways that were proven to be activated by wild-type NTRK2 kinase phosphorylation (21). Cells were collected and lysed in RIPA, and probed with corresponding antibodies. Activation of downstream

pathways was only observed in lysates overexpressed with NACC2-NTRK2 (Figure 4D). No activity is observed in NTRK, NACC2, and fusion K572R. These data together suggest that an kinase domain is required for NACC2-NTRK2 induced downstream activation and transformation of NIH3T3 cells.

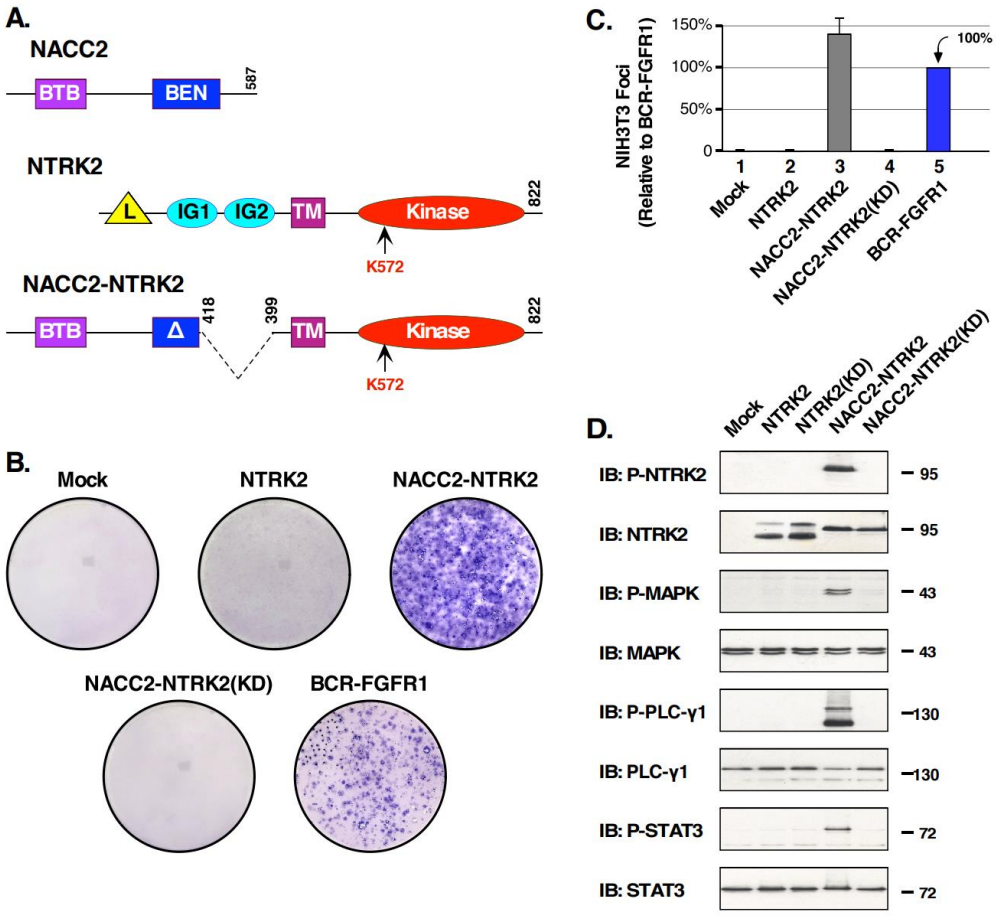

Figure 4. NACC2-NTRK2 exhibits biological activity in cell transformation and cell signaling assays. (A) Schematic of NACC2, NTRK2, and NACC2-NTRK2 fusion with K572R kinase dead mutation (KD). (B) NIH3T3 cell transformation by NTRK2, NACC2-NTRK2, NACC2-NTRK2 KD. BCR-FGFR1 is included as positive control. (C) Foci formed were counted. Foci/G418 ratios were calculated as percentage of BCR-FGFR1 transformation. (D) Lysates expressing NTRK2, NACC2-NTRK2, and NACC2-NTRK2 KD were immunoblotted with phospho-NTRK, total NTRK2 expression, phospho-MAPK, total MAPK expression, phospho-PLCγ1 (upper band), total PLCγ1 expression, phospho-STAT3, and total STAT3 expression. Antibodies used are listed in the Materials and Methods section.

NACC2 BTB domain is responsible for fusion multimerization and activation

After confirming the oncogenic activation of the NACC2-NTRK2 fusion protein, we tried to elucidate the function of the N-terminal fusion partner NACC2. In the fusion, the entire BTB domain (residue 20-120), part of the BEN domain (residue 351-418), and a large uncharacterized region between these two domains are retained. Thus, our next part of the study focuses on the BTB domain. We tried to characterize the relationship between the BTB domain, fusion oligomerization, and fusion kinase activity. We also seek to identify some important regulatory residues that once disrupted can result in the deactivation of the fusion. Currently, there is no detailed structure-function analysis of the NACC2 BTB domain available. However, the BTB domain is an evolutionarily conserved domain that is possessed by various protein families in different species (22). Thus, we used the PLZF BTB domain structure as a template for the NACC2 BTB domain (16). The PLZF BTB domain and NACC2 BTB domain are highly similar in both structure and sequence (Figure 5A, 5B), thus mutations in conserved residues that disrupt multimerization in PLZF could potentially disrupt multimerization of the NACC2 BTB domain (Figure 5C). We introduced charged pocket (D31N R45Q) and monomer core (Y86A) mutations into the BTB domain of NACC2-NTRK2 fusion protein and tested their ability of multimerization.

The NACC2-NTRK2 WT and BTB domain mutants were either transfected or co-transfected with wild-type NACC2 into HEK293T cells. Cells were collected and lysed in E1A lysis buffer. Samples were prepared in non-reducing sample buffer and resolved with SDS-PAGE. Through this protocol, extra bands above the fusion protein and NACC2 bands were observed (Figure 5D, left panel). The size of the multimer signal suggested that the NACC2

BTB domain potentially formed a tetramer. Such signal does not exist with samples that were prepared with sample buffer containing β-mercaptoethanol (Figure 5D, right panel). These are bands of NACC2 and NACC2-NTRK2 fusion oligomers. Both D31N/R45Q and Y86A mutations showed a significant reduction in homo-multimer formation.

In addition to this, a hetero-multimer formation assay that utilized Co-Immunoprecipitation was carried out for the same DNA constructs. Lysates were precleared with Protein-A-Sepharose beads and pulled down with NTRK2 antibody, then probed with NACC2 antibody. The ability of the fusion BTB domains to interact with the NACC2 BTB domain indicated the multimerization ability of the fusion BTB domains. When compared to the NACC2-NTRK2 WT, Both D31N/R45Q and Y86A mutants showed less interaction with the NACC2 BTB domain (Figure 6A), which corresponded with the homo-multimerization assay.

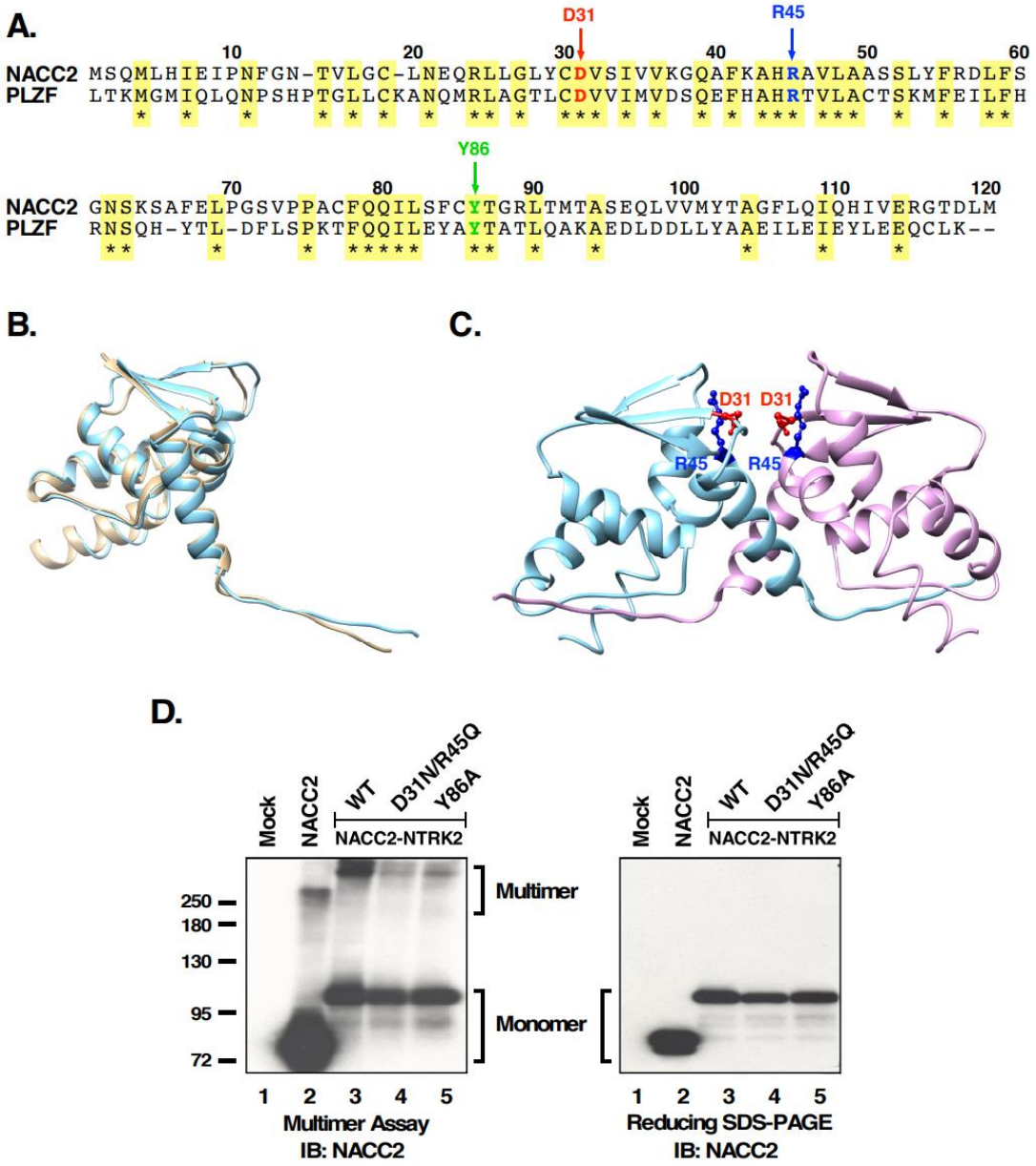

Figure 5. The BTB domain of NACC2 is responsible for multimerization of NACC2-NTRK2. (A) Sequence alignment between NACC2 BTB domain and PLZF BTB domain reveals several conserved residues that are critical for PLZF dimerization, including D31/R45 charged pocket residues and Y86 monomer core residue (B) 3D structural alignment between NACC2 BTB domain (blue) and PLZF BTB domain (brown). (C) NACC2 BTB domain dimer structure proposed from PLZF dimer (PDB ID: 1BUO). Blue and Purple stand for two monomers of NACC2 BTB domain. (D) Samples expressing NACC2, NACC2-NTRK2, NACC2-NTRK2 D31N/R45Q, NACC2-NTRK2 Y86A were prepared in reducing sample buffer (left panel) and non-reducing sample buffer (right panel). Samples were resolved in large SDS-PAGE. Multimer bands of NACC2 and NACC2-NTRK2 were observed in the non-reducing condition (upper bands).

NACC2-NTRK2 fusion activity relies on NACC2 BTB domain multimerization

The extracellular ligand BDNF induces dimerization of NTRK2, resulting in auto-phosphorylation of the NTRK2 kinase domain. In the fusion protein, such dimerization is usually carried out by the N-terminal fusion partner multimerization (23). As the multimerization assay reveals that disruption of the NACC2 BTB domain results in inhibition of oligomer formation, we probed downstream signaling activities of BTB domain mutation mutants using Western blotting. NACC2:NTRK2 WT was included as a positive control. Results showed that all BTB domain mutants, including D31N/R45Q, Y86A, and BTB deletion have similar downstream signaling patterns. These mutants had lower kinase activity when compared to the NACC2-NTRK2. Mutations significantly reduced STAT3 and PLCγ phosphorylation, whereas MAPK activation remained similar to the NACC2-NTRK2 WT fusion (Figure 6B).

The NIH3T3 cell transformation assay showed that all BTB domain mutants were completely biologically inactive. These data demonstrate that by disrupting the oligomerization of the BTB domain, the activation of the NACC2-NTRK2 fusion protein kinase domain can be reduced. As a result of such deactivations, the mutant fusions can no longer transform NIH3T3 cells and are no longer oncogenic (Figure 6C).

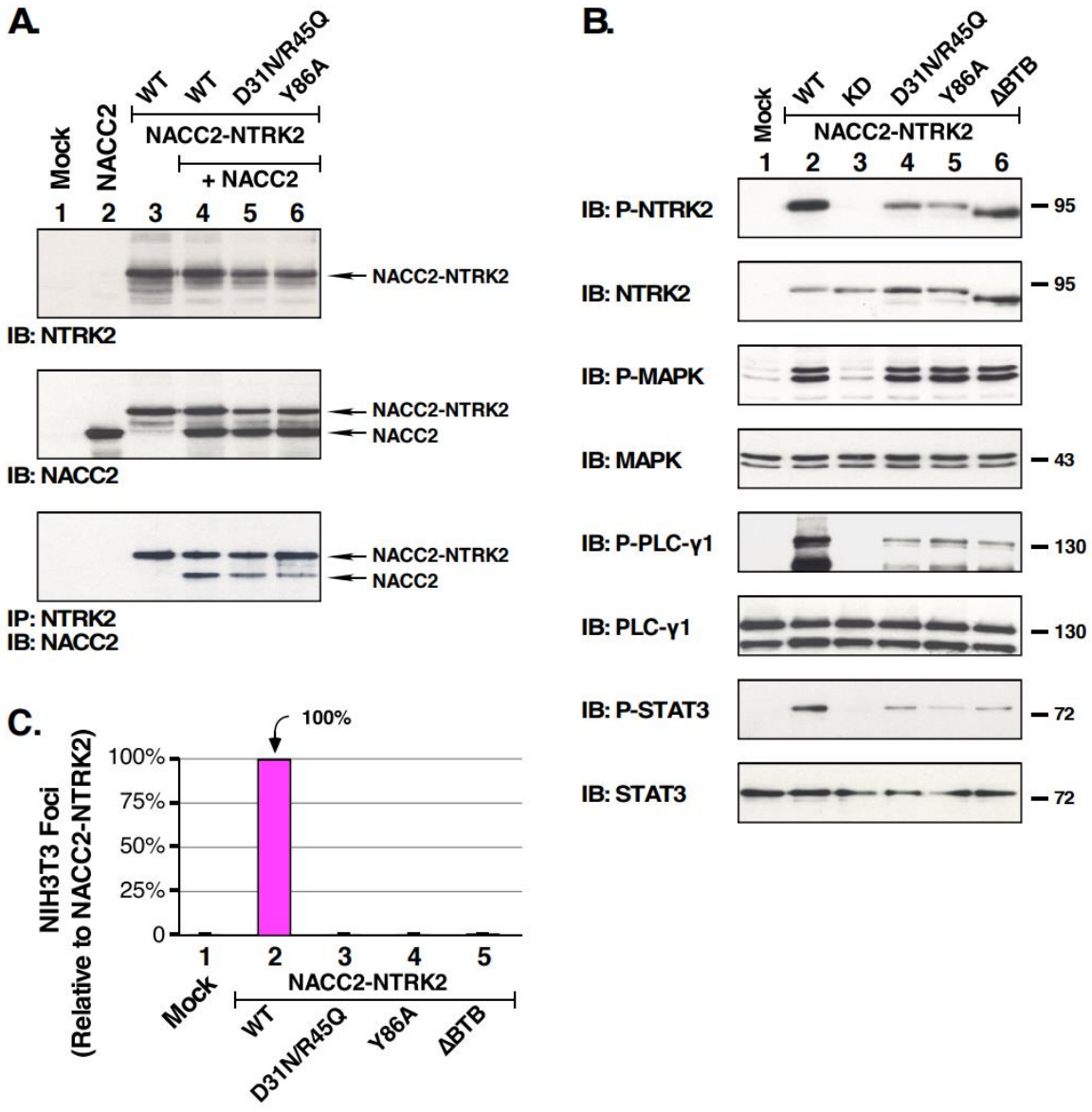

Figure 6. The BTB domain of NACC2 is required for biological activity of NACC2-NTRK2. (A) Lysates expressing NACC2 (1st lane), NACC2-NTRK2 (2nd lane), co-expressing NACC2 and NACC2-NTRK2 (3rd lane), NACC2 and NACC2-NTRK2 D31N/R45Q (4th lane), NACC2 and NACC2-NTRK2 Y86A (5th lane) were immunoblotted with NTRK2 (1st panel) and NACC2 (2nd panel). The same set of lysates were immunoprecipitated with NTRK2 antibody and immunoblotted with NACC2 antibody (3rd panel). (B) Lysates expressing NACC2-NTRK2 and BTB domain mutants were immunoblotted for kinase activation and downstream signaling activity. (C) NIH3T3 transformation assay for BTB domain mutants. The number of foci formed were normalized and compared with the NACC2-NTRK2 (WT)

Biological activity assay for BTB:NTRK2 reveals that NACC2 residues 121-418 stabilizes NACC2-NTRK2 multimerization via covalent interactions

To investigate the possible role of other undefined regions in NACC2 (residues 121-418), we constructed a shorter fusion protein BTB:NTRK2 that contains only the BTB domain (residues 1-120). We performed the downstream activation blotting, multimerization assay, and biological activity assay for this DNA construct. Results showed that the shorter fusion expressed the same amount of phosphorylation activity in the NTRK2 kinase, MAPK, and PLCγ signaling, whereas the STAT3 phosphorylation signal of the shorter fusion is weaker than the wild-type fusion (Figure 7B). NIH3T3 transformation assay indicates that the BTB-NTRK2 posseses only 25% transformation acitivity (Figure 7E).

Interesting results were observed in the multimerization assay. Two forms of dimerization assay were conducted. In the first part of the multimerization assay, samples were prepared in non-reducing sample buffer and resolved in large SDS-polyacrylamide gel. No multimer band of the BTB:NTRK2 fusion was observed in this expression blot (Figure 7C). The second part of the assay utilized co-IP. Cells were co-transfected with the shorter fusion and NACC2. Samples were pulled down with NTRK2 antibody and probed for NACC2 expression. NACC2 interaction was observed in both wild-type fusion and BTB:NTRK2 (Figure 7D). These results indicated that the BTB domain alone contributes to the oncogenic activation of the NACC2-NTRK2 fusion since no activation was observed in the BTB-deleted mutant fusion. NACC2 residues 121-418 might be responsible for the stabilization of the fusion protein multimer through possible covalent interaction. Since the NACC2-NTRK2 multimer was able to survive the SDS denaturation but vulnerable for reduction, whereas the BTB:NTRK2 protein multimer

was completely denatured into a monomer by SDS alone.

Figure 7

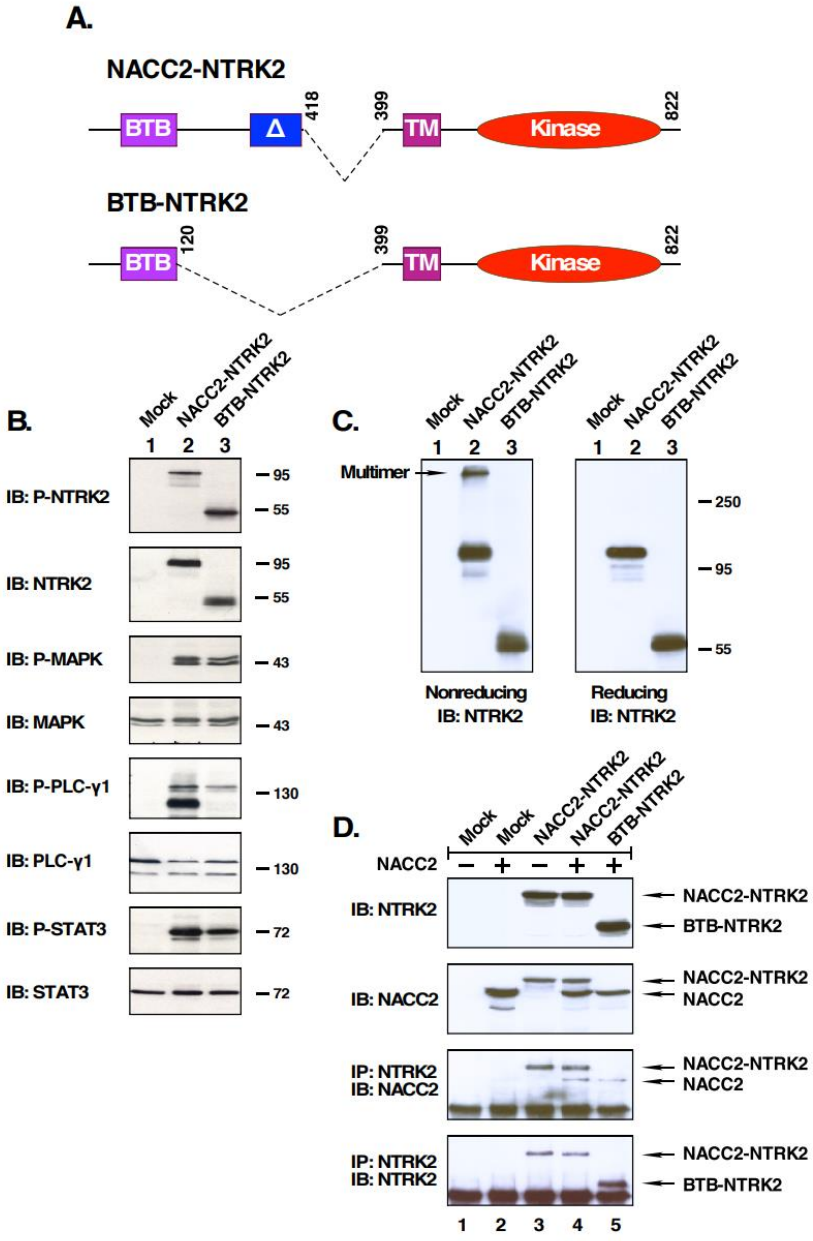

Figure 7 NACC2 residues 121-418 provides potential covalent stabilization for NACC2-NTRK2 multimer. (A)Scheme of BTB-NTRK2. (B) Downstream activation by BTB-NTRK2. (C) Homo multimerization of BTB-NTRK2 under non-reducing (left) condition and reducing (right) condition. (D) Hetero-multimerization assay of BTB-NTRK2. Lysates and IP samples were probed with NACC2 and NTRK2 antibodies.

NTRK2 transmembrane helix deletion induces higher activation by stabilizing the fusion protein

An isoform of NACC2-NTRK2 fusion was observed in a case of pediatric glioblastoma (10). We tried to investigate why such isoform led to more severe cancer by constructing a Δ431-455 transmembrane deletion (ΔTM) fusion, we assayed for its downstream signaling activity and transformation ability. Results indicated that the NACC2-NTRK2 ΔTM induced increased activation in the NTRK kinase, JAK/STAT3, and PLCγ signaling (Figure 8B). NIH3T3 transformation assay showed that NACC2-NTRK2 ΔTM had about 125% transformation activity compared with NACC2-NTRK2, which was higher than the NACC2-NTRK2 WT (Figure 8E). We performed a student's t-test for these data. A p-value of 0.09 proved that these two sets of data were independent.

Data suggest that the TM deletion variant does lead to higher grade of fusion activation. We seek explanations for the extra activity of the TM deletion variant. We performed a protein stability assay for the NACC2-NTRK2 WT and ΔTM. Cycloheximide was applied to HEK293T cells expressing NACC2-NTRK2 WT and ΔTM. Cycloheximide is able to block protein translation and the rate of protein degradation can be used as a reference of protein stability. Cells were treated with 300ng/ml of cycloheximide for 0, 5, 10, and 20 hours before collecting. Results showed that after the addition of cycloheximide, the expression level of the wild-type fusion decayed over time whereas the level of TM deletion mutant remained unchanged (Figure 8C). Thus, the removal of the transmembrane helix slows the proteasomal degradation of NACC2-NTRK2 ΔTM. The increased oncogenicity can potentially result from the more persistent activity of NACC2-NTRK2 ΔTM.

Figure 8

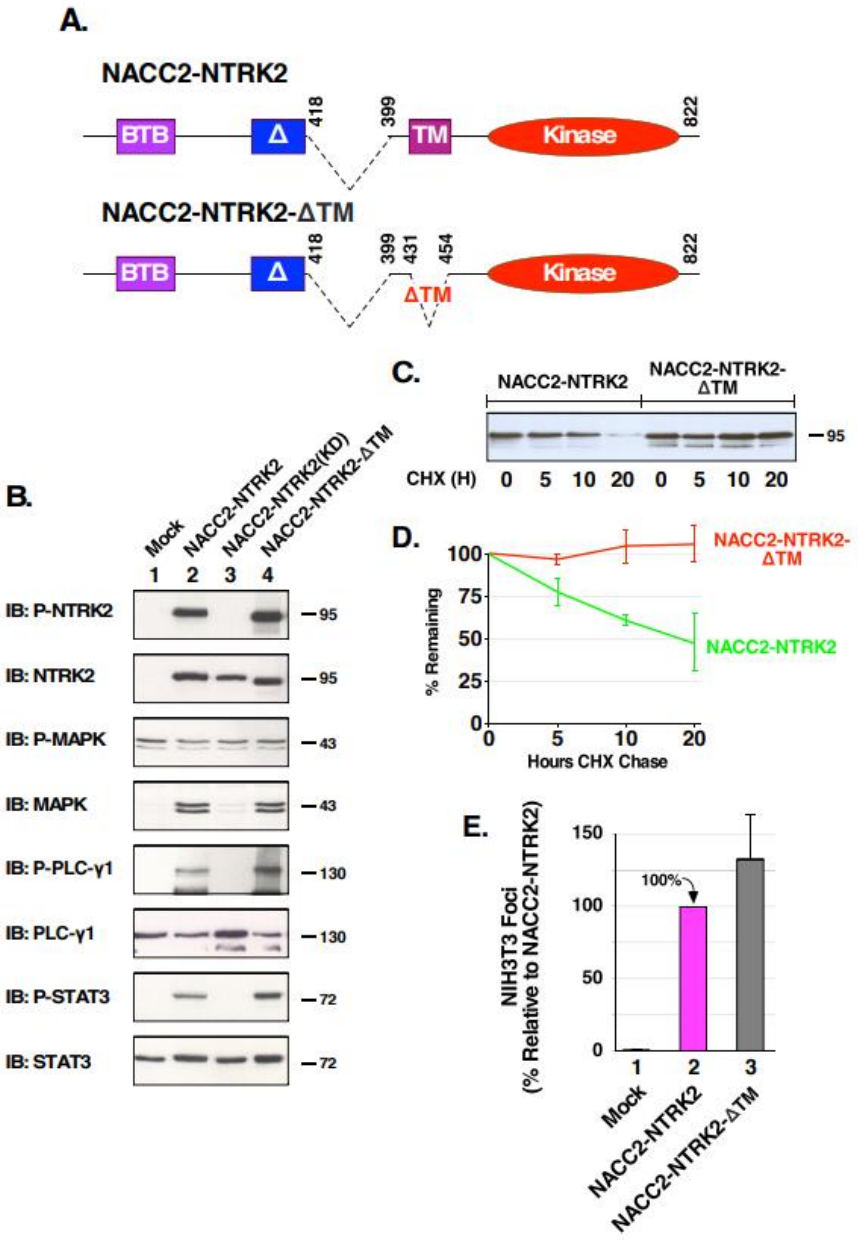

Figure 8 NACC2-NTRK2 (ΔTM) promotes higher activation by stabilizing the fusion protein. (A)Scheme of NACC2-NTRK2 ΔTM. (B)Downstream activations by Mock (1st lane), NACC2-NTRK2 (2nd lane), NACC2-NTRK2 (K572R) (KD) (3rd lane), NACC2-NTRK2 (ΔTM) (4th lane). (C) Cycloheximide Chase assay for NACC2-NTRK2 (lane 1 to 4) and NACC2-NTRK2 (ΔTM) (lane 4-8). (D)Band intensity quantification by imageJ. 0 hour band intensity is set as 100% (E) NIH3T3 transformation assay for NACC2-NTRK2 (ΔTM). The number of foci formed were normalized and compared with the NACC2-NTRK2.

DISCUSSION

Since the discovery of the first NTRK fusion in the 20th century, more NTRK fusions were identified in multiple cancer types, especially in rare cancer types (24). The first kinase-targeting pan-TRK inhibitor Larotrectinib was developed in 2013 and approved in 2018. Treatment with Larotrectinib yielded significant results in NTRK fusion-positive cancers. To overcome the drug resistance arising from NTRK mutations such as TrkC G623R solvent front mutation (25), second-generation TRK inhibitors are under development (8). In this study, we present that the BTB domain of the N-terminal fusion partner NACC2 is a potential drug target that can also be utilized to overcome drug resistance.

Characterization of NACC2-NTRK2 activation

Our experiments show that in the NACC2-NTRK2 fusion protein, the N-terminal BTB domain multimerization induces constitutive activation of the C-terminal tyrosine kinase domain. Our downstream signaling analysis showed that NACC2-NTRK2 kinase activation is able to trigger the phosphorylation of downstream signaling proteins including ERK/MAPK, PLCγ1, and JAK/STAT, and is able to transform NIH3T3 cells in focus formation assay (figure 4). The NACC2-NTRK2 (K572R) kinase-dead mutant and all BTB domain mutants have weaker downstream activation. These mutants are also unable to transform NIH3T3 cells, indicating that the NACC2-NTRK2 oncogenic activation not only relies on the normal activity of the kinase domain but also requires an intact BTB domain (Figure 4-6).

TM deletion induces higher activation by preventing proteasomal degradation

The first case of NACC2-NTRK2 ex4:ex13 fusion was identified in pilocytic astrocytoma

(9). In 2021, a case report (10) filed another NACC2-NTRK2 ex4:ex15 fusion in glioblastoma. The transmembrane helix of NTRK2 was found missing in this novel fusion event. Thus we constructed a transmembrane helix deletion fusion NACC2-NTRK2 (ΔTM). Results show that the TM deletion leads to increased downstream activation and a higher ratio of NIH3T3 cell transformation (Figure 8B, E). Further experiments show that the TM deletion has a longer half-life time than the NACC2-NTRK2 ex4:ex13 fusion in cycloheximide assays. After 20 hours of cycloheximide treatment, the ex4:ex13fusion started decaying at 10 hours whereas the level of the TM deletion remained almost unchanged (Figure 8C). These data show that the NTRK2 transmembrane helix regulates NACC2-NTRK2 degradation, suggesting that the transmembrane helix may be a potential degron sequence (31). By removing the transmembrane helix, the fusion protein becomes resistant to proteasomal degradation and results in more persistent kinase activation and eventually leads to higher transformation activity.

BTB domain as a druggable target

Treatment of NTRK fusion-positive cancers currently relies on Trk inhibitors. In this study, we demonstrate that disrupting the normal functioning of the NACC2 BTB domain could be an alternative therapeutic approach.

The BTB domain is an evolutionary conserved domain that can be found in various zinc finger proteins. The structure and function of BTB domains have been well characterized (26), but its role in NTRK fusion proteins has not been investigated. Here we present that the NACC2-NTRK2 induced signaling activity and cell transformation can be inhibited by disruption of the BTB domain of NACC2. As shown by the focus assay results of NACC2-

NTRK2 (D31N/R45Q), (Y86A), and (ΔBTB) mutants, disruption of the BTB domain charged pocket and monomer core abrogates the cell transformation activity (Figure 6 C). In our multimerization study, NACC2-NTRK2 (D31N/R45Q) charged pocket mutation and NACC2-NTRK2 (Y86A) monomer core mutation also show reduced homo- and hetero-multimerization, as well as reduced downstream activities (Figure 5-6). These data suggest that the BTB domain of the NACC2-NTRK2 fusion protein is a potential drug target. Also, since the BTB domain is evolutionarily conserved, disruption of a motif in BTB domain may inhibit activities of multiple BTB domain proteins. In another BTB domain containing fusion protein PLZF/Retinoic Acid Receptor alpha (RAR alpha) that causes Acute Myeloid Leukemia (AML), similar mutations result in inactive fusion-positive cells (18). Thus, a novel inhibitor that targets these conserved motifs may be beneficial for multiple BTB domain protein associated cancers.

Precedent of BTB domain inhibitors can be found in another BTB domain containing oncogene. B-cell lymphoma 6 (BCL6). Two types of inhibitors for this protein have been developed. BI-3802 inhibitor lead to BCL6 dimer degradation (27), and BI-3812 prevents BCL6 dimer formation (28). Despite limitations in bioavailability, in vitro analysis proved their efficacy (29). Thus, the BTB domain is a druggable domain and a novel inhibitor targeting the NACC2-NTRK2 BTB domain charged pocket or monomer core can be an alternative treatment method to overcome possible tyrosine kinase inhibitor (TKI) resistance.

The study of a shorter form of the fusion protein BTB-NTRK2 reveals another interesting aspect of NACC2 in the fusion protein. In the homo multimerization experiment, protein samples were prepared in non-reducing sample buffer and resolved in SD-PAGE. The NACC2-NTRK2 fusion and BTB domain point mutants were still able to multimerize whereas the

homo-multimerization of BTB-NTRK2 was not observed (Figure 7C). However, the co-IP experiment proved that BTB-NTRK2 was able to interact with another BTB domain at the same level as the NACC2-NTRK2 (Figure 7D). Also, BTB-NTRK2 focus assay results showed that the BTB domain alone is able to mediate downstream activation and transform NIH3T3 cells, but the transformation activity is reduced. These data put together indicate that the NACC2 residues 121-418 provide structural support for the NACC2 multimer through covalent interactions, making it resistant to SDS denaturation. Since such a multimer is sensitive to β-mercaptoethanol reduction, that structure support may be a disulfide bond. Furthermore, such structural support can lead to higher activation of NACC2-NTRK2 fusion protein, but the structural support alone cannot activate the fusion. Thus, a possible mechanism for NACC2-NTRK2 multimerization and activation may be: the BTB domain is responsible for self-associating into correct conformation, creating correct docking sites for downstream signaling proteins. The rest of NACC2 is responsible for locking the multimer together to make the multimer stronger, resulting in higher oncogenic activation.

Through this study, we briefly characterized the functions of every domain in the NACC2-NTRK2. The BTB domain (1-120) is responsible for fusion multimerization, the rest of NACC2 (121-418) is responsible for the stabilization of the multimer, the NTRK2 kinase domain is responsible for the oncogenic activation of the fusion, and lastly, the removal transmembrane helix results in greater stability of the fusion protein and higher grade activation. Point mutations in the NACC2 BTB domain abrogate the activity of the NACC2-NTRK2 fusion protein. Thus, patients may benefit from a combined treatment of TKI inhibition and BTB domain inhibition. These findings suggest that characterizations of oncogenic fusion

proteins are important for the discovery of novel therapeutic methods and allowed the development of novel treatments in the battle against cancers.

MATERIALS AND METHODES

DNA constructs

NACC2 gene (SC319661) and NTRK2 gene (RG221838) were purchased from Origene (Rockville, MD, USA). To subclone both genes in pCDNA3 vector, an XbaI restriction site was introduced to 3' ends of both NACC2 and NTRK2 by quick-change PCR mutagenesis. NACC2 and NTRK2 were subcloned into pCDNA3 vector by internal EcoRI site and XbaI site. A ClaI restriction site was introduced at residue V417 of NACC2 and Y397 of NTRK2. NACC2 and NTRK2 were joint together via ClaI and XbaI double digestion, gene clean, and ligation. Point mutations (D31N/R45Q, Y86A, K572R) were introduced by site-directed PCR. Flag tag insertions and domain deletions (ΔBTB, Δ121-418, and ΔTM) were introduced by PCR site-directed insertions (30). For NIH3T3 transformation assay, DNA constructs were moved into PLXSN vector using EcoRI and XbaI restriction sites.

Antibodies and reagents

Antibodies were purchased as following: NACC2 (Bethyl A304-991A), P-NTRK (Cell Signaling 4621S), NTRK2 (Invitrogen PA5-86241), P-MAPK (Cell Signaling 4370), MAPK (Cell Signaling 9102), P-PLCγ (Cell Signaling 2821), PLCγ (Santa Cruz sc-81), P-STAT3 (Cell Signaling 9145), STAT3 (Cell Signaling 9139), flag (Sigma F3165), β-Actin (Cell Signaling 4967), Alexa Fluor 488 donkey anti-mouse (A21202 Invitrogen), Alexa Fluor 594 goat anti-rabbit (A11012 Invitrogen). ECL reagent, HRP anti-mouse (NA931V) and HRP anti-rabbit (NA934V) secondary antibodies were purchased from Cytiva.

Other reagents were purchased as following: Hoescht 33342 (Tocris Bioscience 5117),

Prolong Gold anti-fade reagent (Invitrogen), G-418 Sulfate (Fisher Scientific), Lipofectamine 2000 (Invitrogen), Protein A-Sepharose (Sigma P3391), Cycloheximide (Fisher J66901.03).

Transfection and immunoblotting

HEK293T cells were grown in 1%Pen/Strep and 10%FBS. $1x10^6$ cells were seeded in a 10cm plate. 5µg of pCDNA3 DNA constructs were transfected using CaCl2 transfection. Cells were incubated in 3% CO_2 for 14-18 hours, recovered in 10% CO_2 for 6-8 hours, and then starved with 0%FBS media for 14-16 hours. Cells were lysed in RIPA buffer [50 mM Tris HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM NaF] with 1 mM sodium orthovanadate, 1 mM PMSF and 10 mg/mL aprotinin. Lysate concentrations were determined by Lowry Assay. Samples were resolved by SDS-PAGE and transferred to Immobilon-P PVDF membranes (Millipore, Burlington, MA, USA). Membranes were blocked with either 5% BSA in 0.1% TBS-T or 5% nonfat milk in 0.1%TBS-T.

Immunoprecipitation and multimerization assay

DNA constructs were transfected into HEK293T cells using $CaCl_2$ transfection and collected with E1A buffer [250 mM NaCl, 50 mM HEPES, 5 mM EDTA and 0.1% NP-40]. Total protein concentrations were measured by Lowry Assay. For multimerization assay, protein samples were made with either reducing sample buffer (50mM Tris-Cl, 10% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) or non-reducing sample buffer (4% SDS,10mM NaPO4 pH7.0, 20% Glycerol, 0.08% Bromophenol blue). Samples were resolved in large SDS-PAGE. For immunoprecipitation, 300µg of total protein was filled up to 1mL with E1A wash buffer [125 mM NaCl, 50 mM HEPES, 0.2% NP-40 and 5 mM EDTA]. IP samples were precleared with Protein A-Sepharose for 3 hours at 4°C, and then

incubated with antibody overnight at 4°C. Protein A-Sepharose was added to each sample to pull down the complex. Beads were washed with E1A wash buffer 5 times. 30µL of reducing sample buffer were added to each sample. Samples were resolved by SDS-PAGE.

Focus Assay

4 x 10^5 NIH3T3 cells were seeded in 60mm plate and transfected with 10µg of pLXSN DNA constructs using Lipofectamine 2000. Cells were fed with 10%CS-DMEM 24 hours after transfection and refed with 2.5%CS-DMEM every 4 days. After 14 days, cells were fixed with methanol and stained Geisma.

Cycloheximide chase

1x10^6 HEK293T cells were seeded in a 10cm plate. 2-5µg of pCDNA3 DNA constructs were transfected into cells using $CaCl_2$ transfection. Cells were incubated in 3% CO_2 for 14-18 hours, recovered in 10% CO_2 for 6-8 hours, and then treated with 300µg of cycloheximide per ml of media (3mg per plate). Cycloheximide was dissolved in pure EtOH to a concentration of 100mg/ml. Cycloheximide was added to the cells at different time points. Cells were harvested at the same time and lysed in RIPA.

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