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The Oncogenic Fusion Protein EML4-NTRK3 Requires Three Salt Bridges for Stability and Biological Activity

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Zian Jiang

Committee in charge:

Professor Daniel J. Donoghue, Chair
Professor Ulrich Muller
Professor Dong-er Zhang

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The Thesis of Zian Jiang is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

The Oncogenic Fusion Protein EML4-NTRK3 Requires Three Salt Bridges for Stability and Biological Activity

by

Zian Jiang

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Professor Daniel J. Donoghue, Chair

Chromosomal translocation of neurotrophic receptor tyrosine kinases leads to various pediatric cancers. While tyrosine kinase inhibitors such as Larotrectinib and

Entrectinib remain a major course of treatment, relapse of tumor still occur, suggesting the need for new therapeutic targets. This work focuses on a novel translocation identified in cases of Infantile Fibrosarcoma, which contains the coiled-coil multimerization domain of Echinoderm Microtubule-like protein 4 (EML4) and the tyrosine kinase domain of Neurotrophic receptor tyrosine kinase 3 (NTRK3). Activation of EML4-NTRK3 relies on both the tyrosine kinase activity of NTRK3 and the salt bridge stabilization in the coiled-coil domain of EML4. As shown in focus formation assay, the tyrosine kinase activity of NTRK3 is essential for the biological activation of EML4-NTRK3. Furthermore, EML4-NTRK3 activates downstream signaling pathways MAPK/ERK, JAK/STAT3 and PKC/PLCgamma. The importance of the salt bridge interactions within EML4-NTRK3 was shown: disruption of all three salt bridge interactions blocks its downstream activation, biological activity and the ability to heterodimerize with EML4. This work also demonstrated that EML4-NTRK3 resides in the cytoplasm and fails to associate with microtubules. Taken together, these data suggest a therapeutic strategy for Infantile Fibrosarcoma cases bearing EML4-NTRK3 fusion: inhibition of salt bridge interactions within EML4.

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

Megha R. Aepala [a,1], Malalage N. Peiris [a,1], Zian Jiang [a], Wei Yang [a], April N. Meyer [a], Daniel J. Donoghue [a,b,*]

[a] Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA 92093-0367, USA
[b] UCSD Moores Cancer Center, University of California San Diego, La Jolla, CA 92093-0367, USA

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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

* Corresponding author at: Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA 92093-0367, USA.
E-mail address: ddonoghue@ucsd.edu (D.J. Donoghue).
[1] These authors contributed equally to this work.

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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].

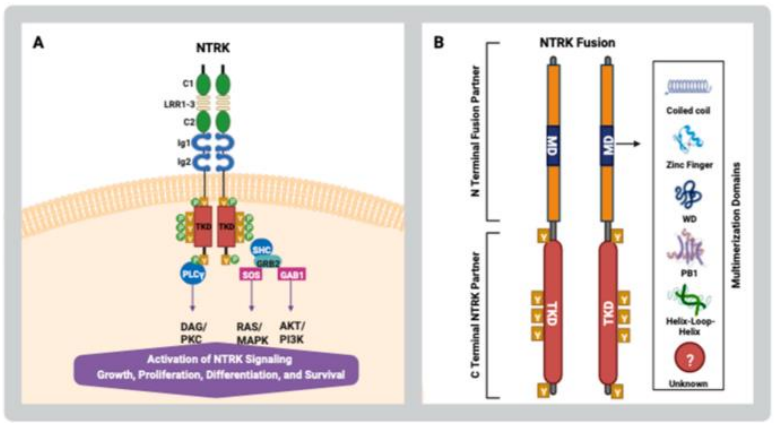

Fig. 1. NTRK Structure and Signaling. (A) Structure of NTRKs. A schematic of an NTRK dimer is shown. The extracellular region of NTRKs is comprised of the Cysteine 1 motif (C1), Leucine Rich Residues motifs (LRR1–3), Cysteine 2 motif (C2), Immunoglobulin-like motifs 1 and 2 (Ig1, Ig2), followed by a 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

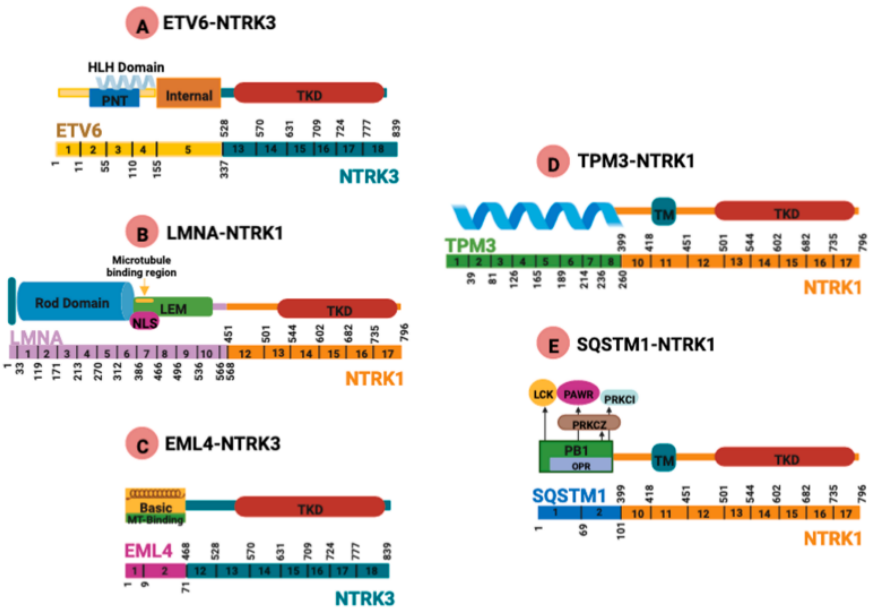

Fig. 2. Significant Pediatric NTRK Fusions. (A) ETV6-NTRK3. A typical ETV6-NTRK3 fusion is shown which includes exons 1–5 of ETV6 fused to exons 13–18 of NTRK3. The N terminal ETV6 fusion partner is a transcriptional repressor and contains a Helix-Loop-Helix (HLH) domain (exons 3–4) which is a DNA binding 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

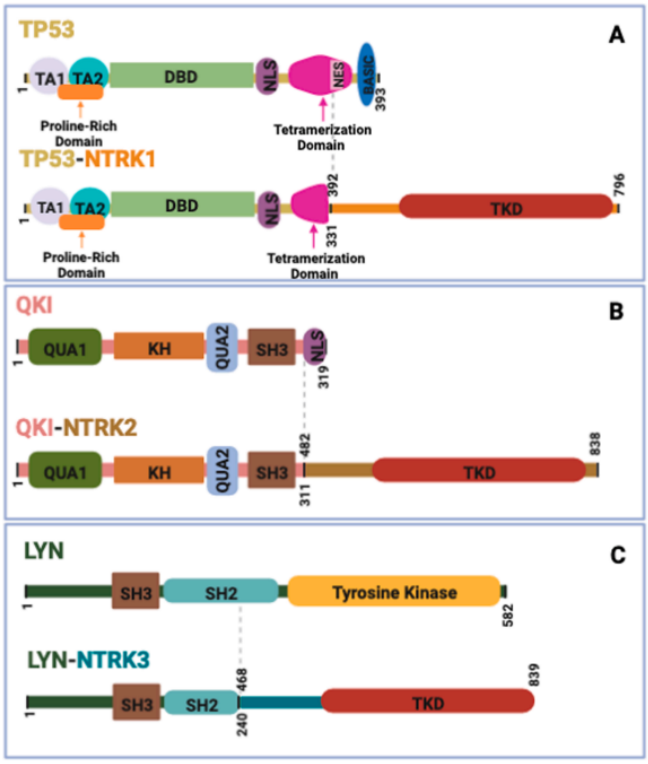

Fig. 3. Beyond Oligomerization. (A) TP53-NTRK1. The N-terminal fusion partner is the well-known tumor suppressor protein Tumor Protein P53 (TP53). TP53 contains 2 transactivation (TA) domains which are important for tumor suppression abilities. The proline rich domain (residues 63–97) serves as docking 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, multikinase 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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Megha R. Aepala is a researcher in the Donoghue group in the Department of Chemistry & Biochemistry at the University of California, San Diego, conducting research on the oncogenic fusion protein SQSTM1-NTRK1 in the Donoghue research group.

Malalage N. Peiris recently completed her doctoral research in the Donoghue group in the Department of Chemistry & Biochemistry at the University of California, San Diego, conducting research on the oncogenic fusion protein BCR-FGFR1. She obtained her Bachelor's degree in Biochemistry and Molecular Biology from the University of California, Davis. She is currently a postdoctoral research studying therapeutic approaches to triple negative breast cancer at Brigham and Women's Hospital in Boston.

Zian Jiang is a native of Shanghai, China. She completed her Bachelor's degree in 2020 at the University of California, San Diego, with a major in Biochemistry/Chemistry. She is currently a graduate student in the Donoghue lab, conducting research on the oncogenic fusion protein EML4-NTRK3.

April N. Meyer is a Staff Research Associate in the Donoghue laboratory at the University of California, San Diego. She earned her Bachelor's degree from the University of California, San Diego in the Department of Chemistry & Biochemistry. Her areas of expertise include molecular biology, FGFR signaling., and NTRK signaling.

Wei Yang is a native of JiangXi, China. He completed his Bachelor's degree in 2020 at the University of California, San Diego, with a major in Chemistry. He is currently a graduate student in the Donoghue lab, conducting research on the oncogenic fusion protein NACC2-NTRK2.

Daniel J. Donoghue is a Professor in the Department of Chemistry & Biochemistry at UC San Diego. He received his graduate training with Phillip A. Sharp at MIT, and post-doctoral training with Tony Hunter at the Salk Institute before joining UC San Diego. He has a lifelong interest in Receptor Tyrosine Kinases and their roles in human cancer.

Aknowledgements

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The thesis author was a co-author of this review but did not perform the research described by the review. The thesis author was responsible for the EML4-NTRK3 and LYN-NTRK3 sections of this review in its entirety. The thesis author also assisted with figures and tables as well. Co-authors include Megha R Aepala, Madalage N Peiris, Yang W, Meyer AN, and Donoghue DJ.

Chapter 2

The Oncogenic Fusion Protein EML4-NTRK3 Requires Three Salt Bridges for Stability and Biological Activity

ABSTRACT

Chromosomal translocations of neurotrophic receptor tyrosine kinases (NTRKs) lead to various pediatric cancers. While tyrosine kinase inhibitors such as Larotrectinib and Entrectinib remain a major course of treatment, relapse of tumor still occur, suggesting the need for new therapeutic targets. This work focuses on a novel translocation identified in cases of Infantile Fibrosarcoma, which contains the coiled-coil multimerization domain of Echinoderm Microtubule-like protein 4 (EML4) and the tyrosine kinase domain of Neurotrophic receptor tyrosine kinase 3 (NTRK3). Activation of EML4-NTRK3 relies on both the tyrosine kinase activity of NTRK3 and the salt bridge stabilization in the coiled-coil domain of EML4. As shown in focus formation assays, the tyrosine kinase activity of NTRK3 is essential for the biological activation of EML4-NTRK3. Furthermore, EML4-NTRK3 activates downstream signaling pathways MAPK/ERK, JAK/STAT3 and PKC/PLCγ. The importance of the salt bridge interactions within EML4-NTRK3 was shown as the disruption of all three salt bridge interactions blocks downstream activation, biological activity and the ability to heteromultimerize with EML4. This work also demonstrates that EML4-NTRK3 is localized in the cytoplasm and fails to associate with microtubules. Taken together,

these data suggest a novel therapeutic strategy for Infantile Fibrosarcoma cases bearing EML4-NTRK3 fusion: inhibition of salt bridge interactions.

INTRODUCTION

Infantile fibrosarcoma (IFS) is the most common type of non-rhabdomyosarcoma soft tissue tumor and is generally found in infants and children less than 2 years old. The main course of treatment for IFS is surgical excision, after which there is a low chance of metastasis and a high probability of long-term survival (>90%) (1). IFS tumors reside in subcutaneous fat, muscle, fascia and tendons (2) and they are morphologically described as spindle-shaped cells in layers of dilated blood vessels arranged in a herringbone pattern (3). Chromosomal translocations frequently occur in IFS, with the oncogenic fusion ETV6-NTRK3; ETV6-NTRK3 consists of the helix-loop-helix (HLH) domain of ETV6 (ETS variant transcription factor 6) joined to the protein tyrosine kinase domain of NTRK3 (4, 5, 6).

In addition to ETV6-NTRK3, recent studies have identified another recurrent translocation in IFS involving Echinoderm microtubule-associated protein-like 4 (EML4) joined to NTRK3 (3, 7). This novel fusion has been found recurrently in children with IFS and also congenital mesoblastic nephroma ranging from the age of 6 days to 1 year old (8). The EML4-NTRK3 fusions consists of exons 1 and 2 of EML4 joined with exons 14-19 of NTRK3, encompassing the coiled-coil domain of EML4 and the tyrosine kinase domain of NTRK3 (3). The EML4 N-terminal domain has been shown to form a parallel trimeric coiled-coil structure, of which residues 14-44 have

been described in a crystal structure (9). Four salt bridges have been identified as conserved between EML proteins (9), presumed to stabilize the triple helix, and these are conserved in the EML4-NTRK3 fusion. Previous studies have also shown that the expression of EML4-NTRK3 promotes anchorage-independent growth of NIH3T3 cells and induces in vivo tumor formation in NOD scid gamma (NSG) mice (3). In addition to the coiled-coil domain at the N-terminus, EML4 also contains a "Tandem Atypical Propeller domain" (TAPE domain), which includes the "Hydrophobic Motif in EML Proteins" (HELP motif) and WD repeats, and also a microtubule binding motif, but these domains are not preserved in the EML4-NTRK3 fusion.

The fusion partner for both ETV6-NTRK3 and EML4-NTRK3 is NTRK3, or neurotrophic tyrosine receptor kinase, one of a small family of three tropomyosin receptor kinases (10). Overexpression of NTRK3 occurs in a variety of cancers such as glioma (91.8%), thyroid cancer (87%) and breast cancer (82.4%) (11, 12, 13) and is normally activated by the ligand neurotrophin-3 (NT-3). Upon binding of NT-3, NTRK3 can activate various signaling pathways including Ras/MEK/MAPK, PI3K/AKT, JAK2/STAT3 and phospholipase C-gamma (PLCγ) pathways, which can regulate the survival, cell differentiation and apoptosis in the peripheral and central neuronal systems (6, 10).

The study presented here explores the requirements and mechanisms of activation of EML4-NTRK3 and the effects on signaling pathways (MAPK, STAT3, PLCγ) that can lead to cell proliferation, differentiation, survival and adhesion. Importantly, we explore the importance of salt bridge residues in the trimerization

domain of EML4, previously identified in a study of native proteins in the EML family (EML1-4) (9). We show that disruption of the salt bridges leads to a reduction of downstream signaling pathways, reduced biological activity in transformation assays, and reduced multimerization of the fusion protein. We also demonstrate that EML4-NTRK3 localizes diffusely in the cytoplasm, but unlike native EML4 fails to associate with microtubules.

RESULTS

Characterization and biological activity of EML4-NTRK3

EML4 consists of 981 amino acid residues (Uniprot Q9HC35-1) encoded by 23 exons, whereas NTRK3 contains 839 amino acid residues (Uniprot Q16288-1) encoded by 18 exons. Currently, only one EML4-NTRK3 fusion variant has been identified, which joins EML4 at exon 2 to the beginning of NTRK3 at exon 12 (3, 14). Therefore, we constructed this oncogenic fusion, which retains the N-terminal coiled-coil domain of EML4 joined to the tyrosine kinase domain of NTRK3 (Figure 4A). A kinase-dead mutation, K572R, was introduced into both NTRK3 and EML4-NTRK3 to determine whether the transforming ability of EML4-NTRK3 depends on the tyrosine kinase activity of NTRK3 (Figure 4A). These constructs were assayed using NIH3T3 cell transformation assays (15). A previously characterized fusion protein BCR-FGFR1 was used as a positive control (16). EML4-NTRK3 formed slightly lower levels of foci compared to BCR-FGFR1, and interestingly, the foci formed are larger in size compared to BCR-FGFR1 (Figure 4B). Other constructs, including full length NTRK3,

kinase-dead NTRK3 K572R, and kinase-dead EML4-NTRK3 K572R did not form any visible foci (Figure 4B, 4C). As a result, the transforming ability of EML4-NTRK3 clearly relies on the tyrosine kinase activity of NTRK3.

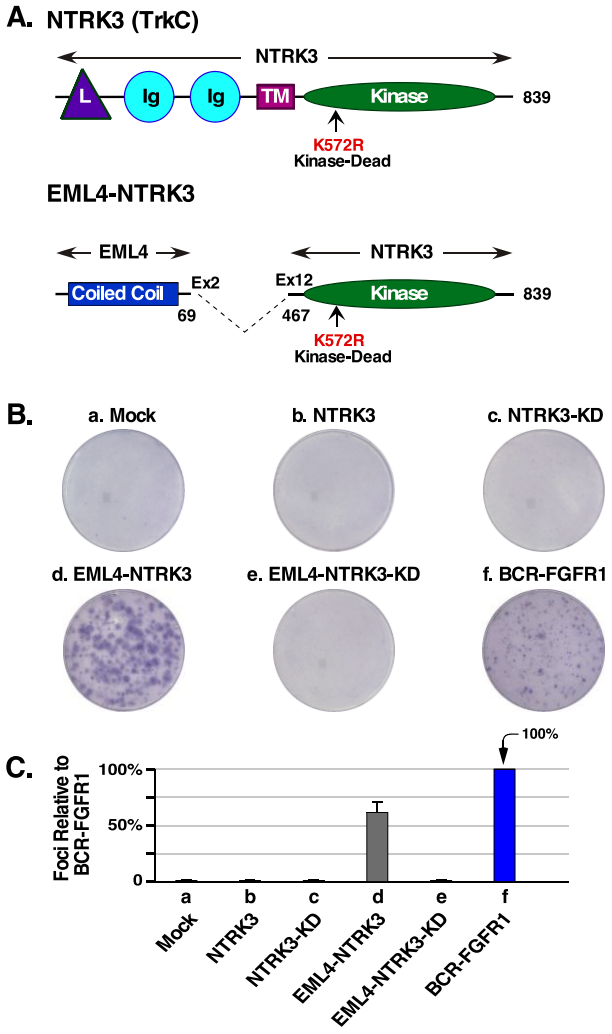

Figure 4: Structure and cell transformation assays of EML4-NTRK3. (A) schematic of NTRK3 and EML4-NTRK3 with K572R kinase dead mutations shown. NTRK3 contains a leucine-rich domain (L), and an extracellular ligand binding domain with immunoglobulin-like domains (Ig), a transmembrane domain (TM), and a tyrosine kinase domain. EML4-NTRK3 contains EML4 exon 2 at the N-terminus fused to the kinase domain of NTRK3 at exon 12. EML4 contributes a coiled-coil domain to the EML4-NTRK3 fusion. (B) Representative plates from NIH3T3 cell transformation assays by EML4-NTRK3, with transfected constructs indicated. (C) The graph shows the number of foci scored, normalized for transfection efficiency, and calculated as a percentage of transformation relative to BCR-FGFR1 -/+ standard error of the mean (SEM). Assays were performed a minimum of three times per each DNA construct.

A short coiled-coil EML4 trimerization domain consists of three salt bridges

The coiled-coil domain of EML4, defined by amino acid residues 1 to 68, was previously shown to assume a parallel triple helix stabilized by three interhelical salt bridges (9). These salt bridges maintain the register of the three parallel alpha helices (Figure 5), and this structure dictates that there are, in fact, nine total salt bridges stabilizing this structure. The side-view crystal structure of the trimeric coiled coil shows the three salt bridges occurring between Arg23 and Glu28 (#1), Arg30 and Glu35 (#2), and Glu37 and Lys42 (#3) (Figure 5B, D). There are 6 heptad repeats in the EML4 coiled-coil domain, in which the heptad positions are conventionally labelled from “a” to “g” (9). In a triple helix, charged amino acid side chains in positions “e” and “g” in one alpha helix have the potential to form electrostatic interactions with those in the neighboring alpha helix, stabilizing the structure of the trimeric coiled-coil, as shown in both the schema and the top-view crystal structure of EML4 coiled-coil domain (Figure 5C, D).

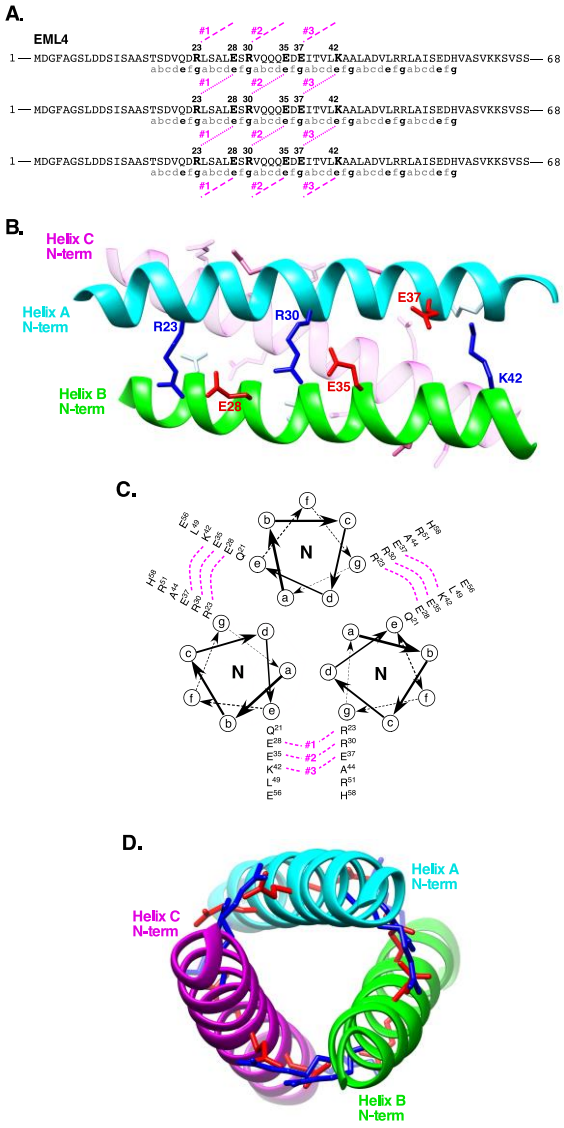

Figure 5: Three salt bridges in the EML4 trimerization domain are required for the stability of the coiled coil structures. (A) The linear sequence showing the salt bridge residues in EML4 trimeric coiled coil. (B) The side-view crystal structure of the EML4 trimeric coiled coil domain is shown (PDB code 4CGC), as viewed using Chimera software. Positive residues R23, R30, K42 are in blue, while negative residues E28, E35, E37 are in red. (C) This shows the parallel heptad repeats of the trimeric coiled coil domain of EML4 with the salt bridge residues in the e and g positions. The interhelical salt bridges are indicated between residues R23 and E28 (#1), R30 and E35 (#2), E37 and K42 (#3). (D) The top-view crystal structure of the EML4 trimeric coiled coil domain is shown, as viewed using Chimera software. Positive residues R23, R30, K42 are in blue while negative residues E28, E35, E37 are in red.

Disruptions of EML4 salt bridges abolish cell transforming ability and activation of downstream signaling pathways

The coiled-coil domain of EML4 presents a potential therapeutic target as it contributes to the cell transforming ability, as demonstrated previously in soft agar colony formation assays (3) and in the work presented here. Thus, we investigated the importance of salt bridges in the EML4 coiled-coil domain to determine whether they are essential for the oncogenic activation of EML4-NTRK3. The three salt bridges in the parallel coiled-coil domain of EML4, between Arg23 and Glu28 (#1), Arg30 and Glu35 (#2), and Glu37 and Lys42 (#3) were disrupted by mutagenizing one residue of each pair to the opposite charge to abolish the electrostatic interactions. Each of the resulting single mutants, containing a single disrupted salt bridge designated #1, #2, or #3, exhibited a drastic decrease in cell transforming ability (<20% of EML4-NTRK3) in NIH3T3 cells. By combining mutations in each of the salt bridges, we also constructed mutants with any two of the salt bridges disrupted (#1+#2, #2+#3, #1+#3), as well as a mutant with all three salt bridges disrupted (#1+#2+#3). Each of the double mutants and the triple mutant were devoid of morphological transforming activity (Figure 6A).

Knowing that the salt bridge residues are essential for the cell transforming ability, we were interested in assessing their effects on phosphorylation of the NTRK3 tyrosine kinase domain, reflecting activation of the kinase, and on activation of downstream signaling pathways including MAPK, STAT3, and PLCγ1 in HEK293T cells. Interestingly, phosphorylation of the NTRK3 tyrosine kinase domain for all the single and double salt bridge mutants was similar to that of EML4-NTRK3. However,

phosphorylation of the NTRK3 tyrosine kinase domain was abolished by the triple salt bridge mutant (Figure 6B). With regards to activation of MAPK and PLCγ1 pathways, P-MAPK and P- PLCγ1 levels for the single salt bridge mutants were similar to EML4-NTRK3; however, phosphorylation diminished significantly for each of the double salt bridge mutants (#1+#2, #1+#3, #2+#3) and was completely abolished by the triple mutant (#1+#2+#3). With regard to Stat3 activation, phosphorylation was partially diminished by each of the single mutants but was completely absent for each of the double mutants as well as the triple mutants. These results are generally consistent with a model that activation of EML4-NTRK3 diminishes progressively with greater disruption of the stabilizing salt bridges. However, the P-NTRK3 signal for the single and double mutants, which detects phosphorylated residues in the activation loop at Y709/Y710, clearly shows that these mutations allow for activation loop phosphorylation; however, this may not reflect the totality of tyrosine phosphorylation that occurs in a fully activated EML4-NTRK3 complex in which additional tyrosine phosphorylation may be required for recruiting and activating the downstream signaling proteins MAPK, STAT3 and PLCγ1.

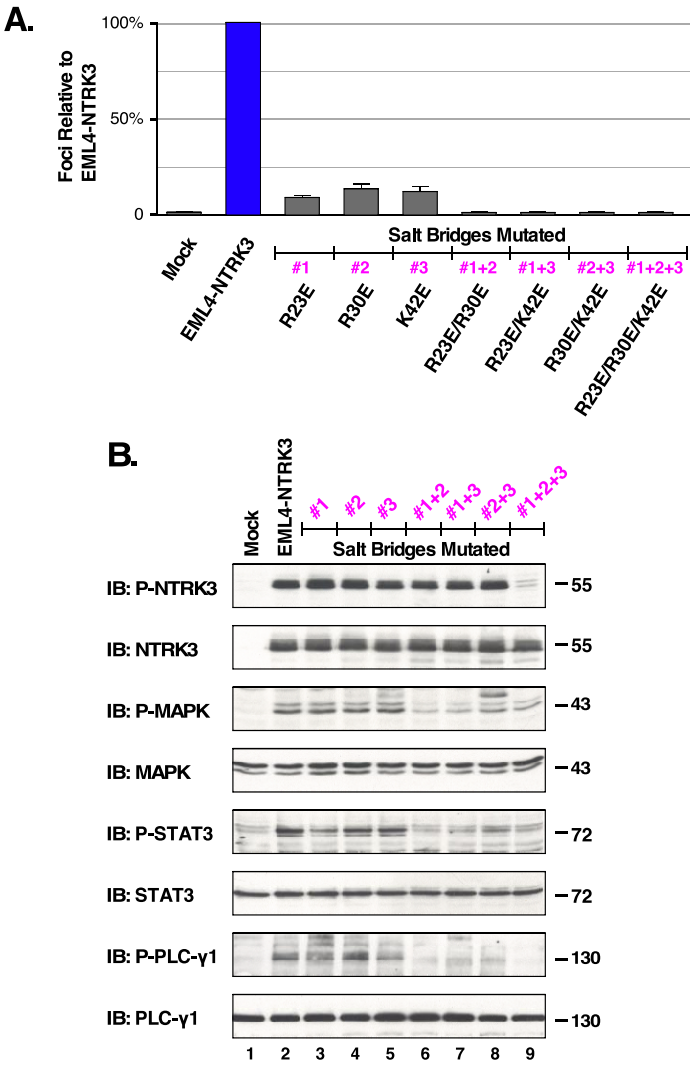

Figure 6: Abrogation of biological activity and downstream activation by salt bridges. (A) The graph shows the results from NIH3T3 cell transformation assays. The number of foci were scored, normalized for transfection efficiency, and calculated as a percentage of transformation relative to EML4-NTRK3 -/+ standard error of the mean (SEM). Assays were performed a minimum of three times per each DNA construct. (B) EML4-NTRK3 and the salt bridge mutants were expressed in HEK293T cells and immunoblotted for phospho-NTRK3 and total NTRK3, phosho MAPK and total MAPK, phospho-STAT3 and total STAT3 and phospho-PLCγ and total PLCγ.

The subcellular localization of EML4-NTRK3 is distinct from either parental proteins

We next wished to investigate the cellular localization of EML4-NTRK3 in NIH3T3 cells. EML4 is known to localize in the cytoplasm and associates with microtubules (9), while NTRK3 is a Type I integral membrane protein (18). Using indirect immunofluorescence, we first confirmed the membrane localization of NTRK3. For this purpose, we inserted a FLAG tag at the beginning of the N-terminal extracellular domain of NTRK3, immediately after the signal peptide cleavage site. Without permeabilizing the membrane, indirect immunofluorescent staining was observed only on the periphery of the cell, clearly delineating the plasma membrane (Figure 7A). However, following membrane permeabilization, staining was observed diffusely throughout the cell (Figure 7B). We also examined the localization of EML4 by double staining cells for Myc-tagged EML4 and ß-tubulin, which confirmed the previously reported microtubule association of EML4 (9) (Figure 7C-E).

To determine the localization of the fusion protein EML4-NTRK3, permeabilized cells were stained for the FLAG tag, located at the N-terminus of EML4-NTRK3, which showed diffused cytoplasmic staining (Figure 7F). Double staining of the same cell with antibodies recognizing ß-tubulin showed little evidence of colocalization of EML4-NTRK3 with microtubules (Figure 7G, H). Thus. we conclude that the N-terminal EML4 coiled-coiled domain present in the fusion protein EML4-NTRK3 is not sufficient for microtubule association.

Given that the EML4-NTRK3 fusion protein loses the N-terminal signal peptide of NTRK3 and, therefore, should lack the ability to undergo membrane insertion, we anticipated that EML-NTRK3 would exhibit a cytoplasmic localization. This was confirmed by the absence of immunofluorescent staining in non-permeabilized cells (Figure 7I); however, after permeabilization and staining of the same cell with a second antiserum to detect NTRK3, EML4-NTRK3 was observed to be exclusively cytoplasmic (Figure 7J,K). Using antiserum to detect the FLAG-tagged EML4 domain of EML4-NTRK3 in permeabilized cells, a similar diffuse staining was observed throughout the cytoplasm (Figure 7L).

Considered together, these results demonstrate that EML4-NTRK3 does not undergo membrane insertion, contrary to its parent NTRK3; nor does it undergo microtubule association, in contrast to its parental EML4. Rather, it presents as a diffusely localized cytoplasmic protein which is largely excluded from the nucleus.

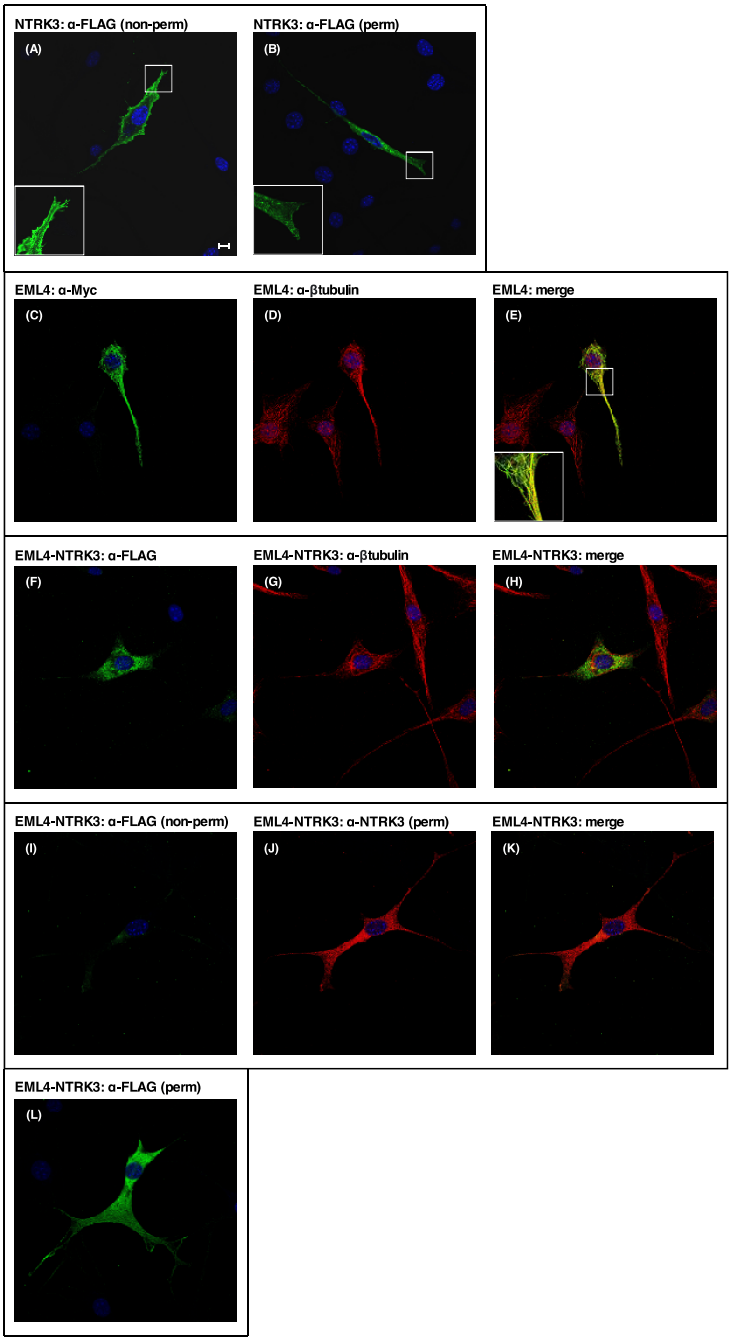

Figure 7: Subcellular localization of EML4-NTRK3. (A-B) Membrane localization of full-length NTRK3. (C-E) Full-length EML4 co-localizes and associates with microtubules. (F-H) EML4-NTRK3 does not associate with microtubules. (I-L) EML4-NTRK3 localizes in the cytoplasm.

Disruption of three salt bridges abrogates heterodimerization with EML4

The activation of receptor tyrosine kinase fusion proteins is dependent on the multimerization of the N-terminal fusion partner; thus we next investigated the requirements of multimerization of EML4-NTRK3 by examining the interactions between the EML4-NTRK3 and EML4. We hypothesized that EML4-NTRK3 can associate with EML4 coiled-coil domain, and the salt bridge mutants can disrupt this association by abrogating the electrostatic interactions between the corresponding alpha helices in the coiled-coil domain.

We co-transfected EML4-NTRK3 or the salt bridge mutants #1 (R23E), #1+2 (R23E and R30E), #1+2+3 (R23E, R30E, K42E) with EML4-Myc into HEK293T cells. Using immunoprecipitation, we pulled down EML4-Myc with c-Myc antibody and immunoblotted for NTRK3. As a result, EML4-NTRK3 and EML4-NTRK3 salt bridge mutant #1 showed strong association with EML4. The association was reduced when disrupting 2 salt bridge interactions and was completely abolished through disruption of all 3 salt bridge interactions, as seen with salt bridge mutant #1+2+3 (Figure 8A). The data illustrated that the hetero-oligomerization between EML4-NTRK3 and EML4 requires the salt bridge electrostatic interactions in the coiled-coil domains and that the salt bridge residues function collaboratively to maintain the interactions and the stability of the coiled-coil trimerization domain.

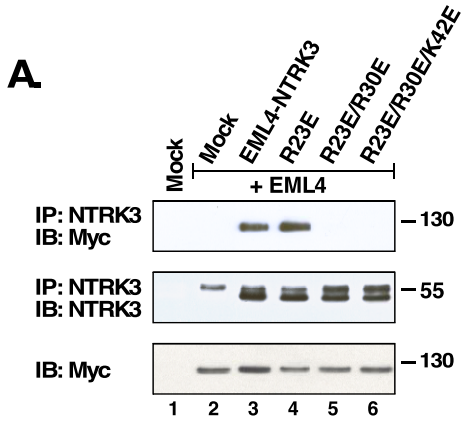

Figure 8. Abrogation of hetero-multimer formation by disruption of salt bridge interactions. (A) EML4-NTRK3, along with salt bridges mutants R23E (#1), R23E and R30E (#1+2) and R23E, R30E, K42E (#1+2+3) were immunoprecipitated with NTRK3 and immunoblotted for Myc and NTRK3.

Discussion

Mis-regulated tyrosine kinases have been an important aspect of personalized medicine and through characterizing these tyrosine kinase fusion proteins, more therapeutic targets can be identified to overcome the resistance resulted from tyrosine kinase inhibitor treatments. Neurotrophic receptor tyrosine kinase 3 (NTRK3) is essential in regulating neuronal survival and fusion with a N-terminal partner gene such as ETV6-NTRK3 has been identified in various tumor types including Infantile Fibrosarcoma and glioma (10). First discovered in 2015 in a case of Infantile Fibrosarcoma (3), the fusion protein EML4-NTRK3, comprised of the coiled-coil domain of EML4 and the tyrosine kinase domain of NTRK3, was found in several cases in Infantile Fibrosarcoma, Congenital Mesoblastic Nephroma, Mammary Analog Secretory Carcinoma and Secretory Breast Carcinoma (8).

Characterization and downstream activation of EML4-NTRK3

In this study, we further characterize EML4-NTRK3 biologically and biochemically. From the focus formation assays in NIH3T3 cells, we demonstrate that the oncogenicity of EML4-NTRK3 relies on the kinase activity of NTRK3. EML4-NTRK3 possesses transforming activity, while EML4-NTRK3 K572R is unable to transform NIH3T3 cells (Figure 4). Through our analysis of downstream signaling, we show that EML4-NTRK3 activates ERK/MAPK, JAK/STAT3 and PKC/PLCγ pathways (Figure 6), confirming the oncogenicity of the fusion protein.

Previous studies showed that the N-terminus of EML4 can associate with the microtubules (9), which are associated with cancer cell proliferation and metastasis (18). Using indirect immunofluorescence, we verified the ability of EML4 to bind to microtubules, while EML4-NTRK3 localizes diffusely in the cytoplasm and does not maintain tubulin-like structures (Figure 7), which demonstrates that the N-terminal coiled-coil domain is not sufficient for microtubule association and functions as a multimerization partner, which is vital in activating the kinase of NTRK3.

Novel therapeutic targets of EML4-NTRK3 cancers

The treatment for Infantile Fibrosarcoma mostly involves surgical resection and TKI treatment. From the data presented, we identified two potential therapeutic targets for EML4-NTRK3 induced Infantile Fibrosarcoma based upon the N-terminus of EML4, which are disruption of electrostatic interactions between the salt bridge residues that stabilize EML4 coiled-coil domain and the introduction of small molecule peptides that mimic the EML4 oligomerization domain.

A previous study has shown the requirement of salt bridges for the transforming ability and downstream signaling pathways of the fusion protein, ETV6-NTRK3, which is also found in Infantile Fibrosarcoma (19). The crystal structure of EML4 was solved, with the interhelical salt bridges identified (9). However, whether the electrostatic interactions between the salt bridges are required to stabilize and activate the EML4-NTRK3 fusion had not been investigated. From the data above, we found that disruption of all three salt bridges #1+#2+#3 (R23E, R30E, K42E) completely abrogates transforming activity of EML4-NTRK3, activation of the NTRK3 receptor, and activation of ERK/MAPK, JAK/STAT3 and PKC/PLCγ pathways (Figure 6). Disruption of either one or two salt bridge interactions still maintains some activation of downstream signaling and transforming ability. In the multimerization assay, disruption of all three salt bridges #1+#2+#3 (R23E, R30E, K42E) prevents the association of EML4-NTRK3 with EML4, while abrogating salt bridge #1 (R23E) and #1+#2 (R23E and R30E) still maintains heterodimerization (Figure 8). Taken together, these results indicate that the three salt bridges can cooperate to preserve the stability of the EML4 coiled-coil domain, multimerization ability and oncogenic activity of EML4-NTRK3. An example of targeting salt bridges is found in Influenza A nucleoprotein. The nucleoprotein can trimerize and within the trimerization, there is a highly conserved salt bridge between E339 and R416. Inhibitors are designed to block the interactions between those residues, preventing the nucleoprotein from trimerization. The inhibitors tested exhibit better potencies than commercially available neuraminidase inhibitors for H1N1 influenza virus strains (24).

Taken together, we assessed the importance of salt bridge interactions within the coiled-coil domain of EML4 in the biological activity of the fusion EML4-NTRK3. By proving the disruption of salt bridge interactions leads to abrogation of downstream signaling cascades and multimerization abilities, we concluded that inhibition of these salt bridge interactions can serve as a novel target for TKI resistant EML4-NTRK3-positive sarcomas.

MATERIALS AND METHODS

DNA Constructs

The EML4 plasmid (pCMV6-Entry-EML4) was purchased from Origene (Watertown, MA, USA) and the NTRK3 plasmid (pDONR223-NTRK3) was purchased from Addgene (Watertown, MA, USA). A stop codon (TGA) and an XbaI site were added to the 3' end of the NTRK3 coding region by site-directed mutagenesis. Using restriction sites EcoRI and XbaI, the two genes were subcloned into the vector pcDNA3. A ClaI site was introduced by PCR site-directed mutagenesis after residue S68 in EML4 and before amino acid G462 in NTRK3. The internal ClaI and the XbaI sites were used to subclone exons 12-18 of NTRK3 into the EML4 pcDNA3, creating a fusion breakpoint of EML4 exon 2 fused to NTRK3 exon 12. All the salt bridge point mutations described were introduced by PCR site-directed mutagenesis. The constructs flag-NTRK3 and flag-EML4-NTRK3 were made using PCR site-directed insertions (20). Using EcoRI and XbaI, NTRK3 and EML4-NTRK3 constructs were subcloned

into the pLXSN vector (15) for NIH3T3 transformation assays. The flag-EML4 1-68 clone, encoding only the N-terminal coiled-coil domain of EML4, was derived from flag-EML4-NTRK3 using an adapter to introduce a stop codon after residue S68 of EML4, which also deleted the remainder of EML4-NTRK3. The flag-EML4 1-68 mut clone, containing the triple salt bridge mutations, was constructed similarly.

Antibodies and reagents

Antibodies were obtained as follow: NTRK3 (Abcam ab181560), P-NTRK (Cell Signaling 4621S), MAPK (Cell Signaling 9102), P-MAPK (Cell Signaling 4370), STAT3 (Cell Signaling 9139), P-STAT3 (Cell Signaling 9145), PLCγ (Santa Cruz sc-81), P-PLCγ (Cell Signaling 2821), Flag M2(Sigma F3165), Myc (Santa Cruz sc-40 9E10), Myc (Cell Signaling 2276, 9B11), β-tubulin (Santa Cruz sc-9104). Reagents for Enhanced Chemiluminescence (ECL), including Horseradish peroxidase (HRP) anti-mouse (NA931V) and HRP anti-rabbit (NA934V) were obtained from Cytiva. Reagents for fluorescence microscopy included Alexa Fluor 488 donkey anti-mouse (A21202 Invitrogen), Alexa Fluor 594 goat anti-rabbit (A11012 Invitrogen), Hoescht 33342 (Tocris Bioscience 5117), and Prolong Gold anti-fade reagent (Invitrogen, Carlsbad, CA, USA). Other reagents included G-418 Sulfate (Fisher Scientific), Lipofectamine 2000 (Invitrogen), and Protein A-Sepharose (Sigma P3391).

Cell transfection, immunoprecipitation, immunoblot analysis

HEK293T cells were grown in 10% FBS, 1% Pen/Strep and were seeded at 1 x 10^6 cells per 10 cm plate. Cells were transfected with 2-5 µg of the EML4-NTRK3 pcDNA3 constructs using calcium phosphate transfection at 3% CO_2 for 16 h, moved

to 10% CO_2 for 7 h, and starved with FBS-free DMEM for 17 h before harvest (21). 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, 1 mM sodium orthovanadate, 1 mM PMSF and 10 mg/mL aprotinin [10 mg/mL]. Protein concentrations were determined by Lowry Assay and 20-40 µg of protein was loaded on either 10% or 12.5% SDS-PAGE gels for immunoblot analysis (21). After electrophoresis, proteins were transferred to Immobilon-P PVDF membranes (Millipore, Burlington, MA, USA) at 30 V for 120 min. Membranes were blocked in 5% bovine serum albumin (BSA)/0.1% Tween 20-TBS or 5% nonfat milk/0.1% Tween 20-TBS.

For immunoprecipitation, cells were lysed in E1A Lysis Buffer [250 mM NaCl, 50 mM HEPES, 5 mM EDTA and 0.1% NP-40] and total protein concentration was measured by Lowry assay. 300 µg protein was incubated overnight at 4°C before the addition of Protein A-sepharose beads (Sigma P3391). After rocking for 2 h at 4°C, complexes were washed with E1A wash buffer [125 mM NaCl, 50 mM HEPES, 0.2% NP-40 and 5 mM EDTA] 5 times for 10 min each. Complexes were disrupted by adding equal volume of 2× sample buffer and boiling for 5 minutes and analyzed on 10% SDS-PAGE.

Focus Assay

NIH3T3 cells were seeded at 4 x 105 cells per 60 mm plate (21). The cells were transfected with 10 µg of pLXSN plasmid DNA using Lipofectamine 2000 and fed with 10% CS-DMEM 24 h after transfection. The cells were re-fed with 2.5% CS-DMEM

every 3-4 d before fixation. Cells were fixed with methanol, stained with Geimsa after 14 d and the Geneticin (G418)-resistant colonies were scored to determine the transfection efficiency (21). Numbers of foci were counted, normalized by transfection efficiency, and quantitated relative to a positive control (BCR-FGFR1) +/- standard error of the mean (SEM).

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