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The *ETV6-NTRK3* gene fusion encodes a chimeric protein tyrosine kinase that transforms NIH3T3 cells

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The congenital fibrosarcoma t(12;15)(p13;q25) rearrangement splices the *ETV6* (*TEL*) gene on chromosome 12p13 in frame with the *NTRK3* (*TRKC*) neurotrophin-3 receptor gene on chromosome 15q25. Resultant *ETV6-NTRK3* fusion transcripts encode the helix–loop–helix (HLH) dimerization domain of ETV6 fused to the protein tyrosine kinase (PTK) domain of NTRK3. We show here that ETV6-NTRK3 homodimerizes and is capable of forming heterodimers with wild-type ETV6. Moreover, ETV6-NTRK3 has PTK activity and is autophosphorylated on tyrosine residues. To determine if the fusion protein has transforming activity, NIH3T3 cells were infected with recombinant retroviral vectors carrying the full-length *ETV6-NTRK3* cDNA. These cells exhibited a transformed phenotype, grew macroscopic colonies in soft agar, and formed tumors in severe combined immunodeficient (SCID) mice. We hypothesize that chimeric proteins mediate transformation by dysregulating NTRK3 signal transduction pathways via ligand-independent dimerization and PTK activation. To test this hypothesis, we expressed a series of *ETV6-NTRK3* mutants in NIH3T3 cells and assessed their transformation activities. Deletion of the ETV6 HLH domain abolished dimer formation with either ETV6 or ETV6-NTRK3, and cells expressing this mutant protein were morphologically non-transformed and failed to grow in soft agar. An ATP-binding mutant failed to autophosphorylate and completely lacked transformation activity. Mutants of the three NTRK3 PTK activation-loop tyrosines had variable PTK activity but had limited to absent transformation activity. Of a series of signaling molecules well known to bind to wild-type NTRK3, only phospholipase-Cγ (PLCγ) associated with ETV6-NTRK3. However, a PTK active mutant unable to bind PLCγ did not show defects in transformation activity. Our studies confirm that ETV6-NTRK3 is a transforming protein that requires both an intact dimerization domain and a functional PTK domain for transformation activity. *Oncogene* (2000) **19,** 906–915.

Keywords: ETV6-NTRK3; TEL-TRKC; receptor tyrosine kinase; translocation; chimeric oncoprotein

Introduction

Congenital fibrosarcoma (CFS) is a cellular, mitotically active neoplasm of the soft tissues primarily affecting infants less than 1 year of age. It is a spindle cell sarcoma which may grow to enormous sizes and can cause considerable morbidity or mortality. Although CFS has a high propensity for local recurrence, this tumor is biologically unique among human sarcomas because of its overall survival of 80–90% and metastatic rate of only 10% (Lamballe *et al*., 1993; Obermeier *et al*., 1993a). Cytogenetic analyses of CFS cases have revealed nonrandom gains of chromosomes 8, 11, 17, and 20, with trisomy 11 being the most consistently observed alteration (Adam, 1990; Argyle, 1992; Coffin, 1994; Mandahl, 1989; Sankary, 1993; Speleman, 1989). We have recently identified a novel, recurrent t(12;15)(p13;q25) chromosome rearrangement in CFS that fuses *ETV6* (*TEL*) on 12p13 to *NTRK3* (*TRKC*) on 15q25 (Knezevich *et al*., 1998b). This rearrangement, which can be detected in primary tumor specimens by reverse transcriptase-PCR assays (Knezevich *et al*., 1998b), was present in all CFS cases analysed to date and was not detected in other fibroblastic lesions including benign infantile fibromatosis and adult-type fibrosarcoma (unpublished results). However, we and others have recently detected identical *ETV6-NTRK3* fusion transcripts in the cellular form of congenital mesoblastic nephroma (CMN), a pediatric renal tumor which shows considerable histopathologic and clinical overlap with CFS (Knezevich *et al*., 1998a; Rubin *et al*., 1998).

ETV6 is a member of the ETS family of transcription factors which may play a role in early hematopoiesis and angiogenesis (Baens *et al*., 1996; Edel, 1998; Kwiatkowski *et al*., 1998; O'Connor *et al*., 1998; Poirel *et al*., 1997; Wang *et al*., 1997, 1998). *ETV6* was first identified as the fusion partner of *PDGFRβ* in the t(5;12)(q33;p13) of chronic myelomonocytic leukemia (Golub *et al*., 1994), and has since been identified in a variety of gene fusions associated with hematopoietic malignancies. These include fusions with *CBFA2* (*AML1*) (Golub *et al*., 1995; Romana *et al*., 1995), *ABL* (Papadopoulos *et al*., 1995), *MN1* (Buijs *et al*., 1995), *JAK2* (Lacronique *et al*., 1997; Peeters *et al*., 1997a), *STL* (Suto *et al*., 1997), *MDS1/EVI1* (Peeters *et al*., 1997b), *CDX2* (Chase *et al*., 1999), *BTL* (Cools *et al*., 1999), and *ACS2* (Yagasaki *et al*., 1999). The well characterized *ETV6-CBFA2* (*TEL-AML1*) fusion

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is often accompanied by deletion of the normal *ETV6* allele on chromosome 12 (Cave *et al*., 1997).

NTRK3 encodes the transmembrane surface receptor for neurotrophin-3 (NT-3) and mediates many aspects of growth and development in the central nervous system (Barbacid, 1995a; Conover and Yancopoulos, 1997; Ebadi *et al*., 1997; Ichaso *et al*., 1998; Lamballe *et al*., 1991; McGregor *et al*., 1994). NTRK3 is activated by cell surface ligand-mediated oligomerization, which facilitates autophosphorylation of NTRK3 cytoplasmic tyrosine residues 516, 705, 709, 710, and 820 and subsequent kinase activation (Barbacid, 1994, 1995a,b; Jing *et al*., 1992; Lemmon and Schlessinger, 1994). Several adapter molecules and protein tyrosine kinase (PTK) substrates have been linked to NTRK3 signal transduction. Phosphorylated NTRK3 tyrosine-516 binds the adaptor protein Src-homology/collagen (SHC) via the SH2 domain of the latter (Guiton *et al*., 1995), and is also the site of association with the p85 subunit of phosphoinositol-3′ kinase (PI-3K) (Barbacid, 1994; Hallberg *et al*., 1998; Obermeier *et al*., 1993b). Activated SHC recruits growth factor receptor bound protein 2 (GRB2), an adaptor protein, as well as the RAS guanine nucleotide exchange factor Son-of-sevenless (Sos) to activate the RAS-RAF1 pathway (Bonfini *et al*., 1996; Downward, 1996). Phosphorylated PI-3K mediates activation of the protein kinase B (Akt/PKB) pathway (Kaplan, 1997). Phosphorylated NTRK3 tyrosine-820 binds phospholipase-Cγ (PLCγ) (Lamballe *et al*., 1993; Obermeier *et al*., 1993a), resulting in the activation of protein kinase C (PKC) (Berridge, 1993; Cockcroft and Thomas, 1992; Lee and Rhee, 1995; Nishizuka, 1995). The suc1-associated neurotrophin factor target (SNT) protein was shown to bind to an NTRK3 juxtamembrane KFG sequence and become tyrosine phosphorylated (Peng *et al*., 1995; Rabin *et al*., 1993). More recent studies on fibroblast growth factor (FGF) receptor-1 signaling indicate that phosphorylated SNT can associate with GRB2 and Sos in FGF-stimulated fibroblasts (Kouhara *et al*., 1997; Ong *et al*., 1996). Other molecules known to associate with activated NTRK3 and potentially be involved in NTRK3 signaling include rAPS and SH2-Bβ (Qian *et al*., 1998).

The CFS t(12;15) rearrangement splices the first five exons of *ETV6* to exons 6–8 of *NTRK3*, creating a hybrid gene encoding the ETV6 helix–loop–helix (HLH) protein dimerization domain fused to the terminal 298 amino acids of NTRK3 (Knezevich *et al*., 1998b). These NTRK3 residues include the PTK domain and C-terminus of NTRK3, but do not include the tyrosine-516 residue of NTRK3 which binds SHC and p85, nor the KFG sequence which binds SNT (Knezevich *et al*., 1998b). We hypothesize that expression of the *ETV6-NTRK3* fusion transcript is essential for the development of CFS tumors. Moreover, we propose that the HLH domain of ETV6-NTRK3 facilitates ligand-independent dimerization of the chimeric protein, and that subsequent constitutive PTK activation leads to oncogenesis by dysregulating known or novel NTRK3 signal-transduction pathways. We show here that *ETV6-NTRK3* is an oncoprotein with potent transforming activity in NIH3T3 cells, and that intact HLH and PTK domains are both required for transformation.

Results

ETV6-NTRK3 *transforms NIH3T3 cells*

NIH3T3 cells were infected with recombinant retroviruses carrying either *ETV6-NTRK3* cDNA or empty MSCVpac vector as a negative control. The predicted structure of the ETV6-NTRK3 protein encoded by the chimeric cDNA is shown in Figure 1. Positive transformants were obtained by puromycin drug selection, and cell morphology was assessed after at least 1 week of growth. Cells infected with *ETV6-NTRK3* exhibited a dramatically transformed phenotype compared to negative control cells (data not shown). The transformed cells possessed a high nucleus-to-cytoplasm ratio, their cell bodies strongly refracted light, and their growth was not inhibited by contact to other cells. *ETV6-NTRK3*-infected NIH3T3 cells were able to form macroscopic colonies after 16–19 days of culture in soft agar (see Figure 2a,c; Table 1). Colony formation was only slightly less than that observed with retrovirally infected NIH3T3 cells expressing the oncogenic Ewing sarcoma EWS-FLI1 fusion protein (see Table 1). Cells infected with vector alone failed to grow colonies even after 28 days of incubation (see Figure 2b,d) and in subsequent long term growth. Expression of the predicted 73 and 68 kD ETV6-NTRK3 protein doublet in transformed NIH3T3 cells was verified by Western blotting using

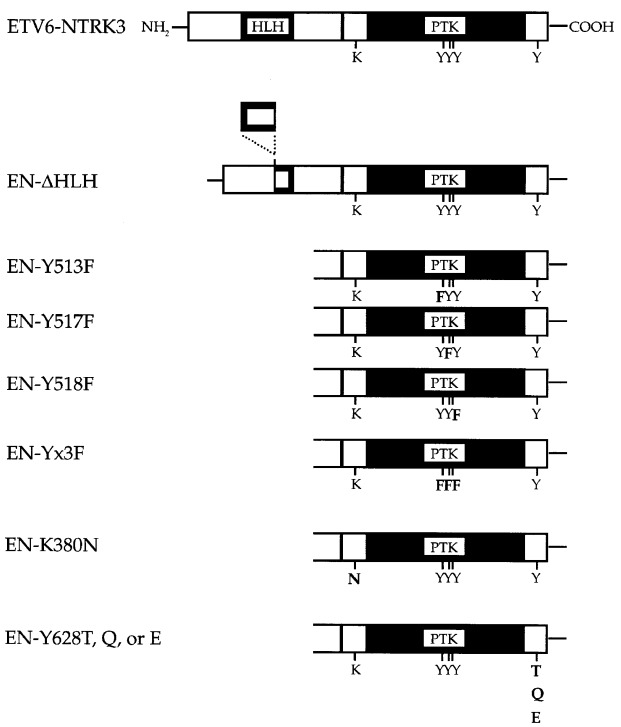

Figure 1 Schematic representation of constructs made by site-directed mutagenesis of ETV6-NTRK3. Non-mutated ETV6-NTRK3 is shown at the top. The HLH-domain deletion mutant, EN-ΔHLH, created by deleting amino acids 64–177 representing part of the ETV6-NTRK3 HLH domain. The three activation-loop tyrosines (Y) sequentially substituted with phenylalanine (F) in mutants EN-Y513F, EN-Y517F, and EN-Y518F, respectively. The activation-loop tyrosine triple mutant, EN-Yx3F, with all three activation-loop tyrosines mutated to phenylalanine. The ATP-binding site, lysine 380 (K), substituted with asparagine (N) in mutant EN-K380N. Tyrosine 628, the PLCγ-binding site, mutated to a threonine, glutamine, or glutamic acid residue in the EN-Y628T, EN-Y628Q, or EN-Y628E mutants, respectively

α-TrkC (C-14), while this doublet was not present in control cells infected with vector alone (see lanes 1 and 7, left panel, Figure 2f). Therefore, *ETV6-NTRK3* encodes a chimeric protein with strong transforming potential in NIH3T3 cells.

ETV6-NTRK3-mediated transformation requires both the ETV6 HLH and the NTRK3 PTK domains

The HLH protein dimerization domain of ETV6 was deleted to assess the potential role of oligomerization in ETV6-NTRK3 transformation. Retrovirally infected NIH3T3 cells expressing the HLH-mutant, EN-ΔHLH (see Figure 1), were morphologically identical to those infected with vector alone; that is, they did not exhibit a transformed phenotype (data not shown). Moreover, in soft agar assays, *EN-ΔHLH*-infected NIH3T3 cells failed to form colonies even after 4 weeks of incubation (see Table 1). These results indicate that the HLH domain is essential for ETV6-NTRK3 transformation of NIH3T3 cells. Protein expression at levels similar to ETV6-NTRK3 was verified by immunoprecipitation using α-TrkC (C-14) antibodies (see lanes 2 and 3, right panel, Figure 2f).

Table 1 Transformation of NIH3T3 cells by EWS-FLI1, ETV6-NTRK3 and ETV6-NTRK3 mutants

Construct	*Morphological appearance*	*Growth in soft agar*[a]
EWS-FLI1	Transformed	215±18
ETV6-NTRK3	Transformed	172±14
Vector	Normal	0
EN-ΔHLH	Normal	0
EN-Y513F	Normal	0
EN-Y517F	Semi-transformed	0[b]
EN-Y518F	Semi-transformed	0[b]
Triple mutant, EN-Yx3F	Normal	0
EN-K380N	Normal	0
EN-Y628Q	Transformed	196±17

[a]Average number of macroscopic colonies per 60-mm diameter soft agar plate. Cells were seeded at a density of 30 000 cells per plate in quadruplicate. [b]Limited microscopic colony growth was observed

To assess the role of the PTK domain in transformation, NIH3T3 cells were infected with recombinant viruses expressing ETV6-NTRK3 proteins with tyrosine-to-phenylalanine mutations in each of the three activation loop tyrosines (designated EN-Y513F, EN-Y517F, and EN-Y518F, respectively) known to be essential for PTK activity (Barbacid, 1994; Lamballe *et al*., 1993; Tsoulfas *et al*., 1993; Valenzuela *et al*., 1993), as well as a mutant in which all three tyrosines were converted to phenylalanine (EN-Yx3F) (see Figure 1). We also constructed a kinase-inactive ETV6-NTRK3 mutant in which the ATP-binding lysine 380 (NTRK3 lysine 572) (Lamballe *et al*., 1991; Mitra, 1991) was converted to asparagine, designated EN-K380N (Figure 1). Transformation activity was assessed by cell morphology and ability to grow in soft agar. NIH3T3

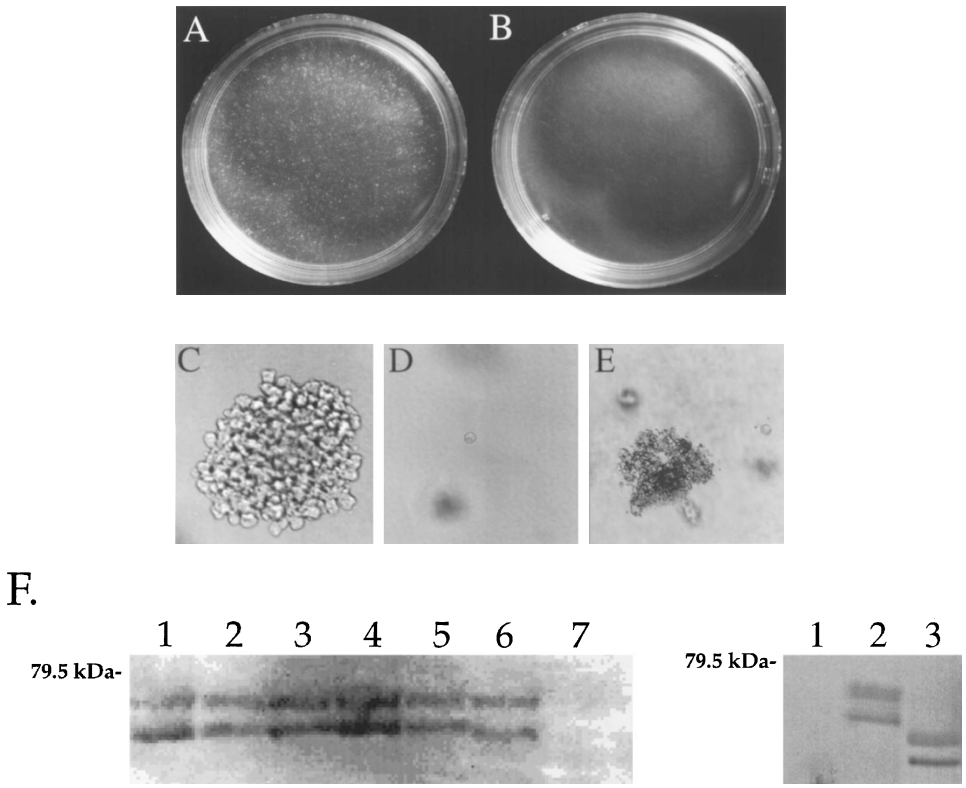

Figure 2 Soft agar assays demonstrating macroscopic colony formation by ETV6-NTRK3 expressing NIH3T3 cells. Puromycin-selected cells were seeded at a density of 30 000 cells in 60-mm plates at a serum concentration of 10%. Similar findings were observed in at least three independent experiments for each construct. (**a**) NIH3T3 cells expressing ETV6-NTRK3 formed macroscopic colonies in soft agar. (**b**) NIH3T3 cells infected with MSCVpac vector alone failed to form macroscopic colonies in the agar. (**c**) Photomicrograph of a typical soft agar colony formed by ETV6-NTRK3-expressing NIH3T3 cells (magnification 200×). (**d**) NIH3T3 cells infected with MSCVpac vector alone grew as single cells in soft agar (magnification 200×). (**e**) NIH3T3 cells expressing EN-Y517F or EN-Y518F mutants exhibited microscopic but not macroscopic colony formation (magnification 200×). (**f**) Western blot analysis of NIH3T3 cells expressing ETV6-NTRK3 and various mutants using α-TrkC (C-14) antibody. Left panel: ETV6-NTRK3, lane 1; EN-Yx3F, lane 2; EN-K380N, lane 3; EN-Y628T, lane 4; EN-Y628E, lane 5; EN-Y628Q, lane 6; and MSCVpac, lane 7. Right panel: MSCVpac, lane 1; ETV6-NTRK3, lane 2; and EN-ΔHLH, lane 3

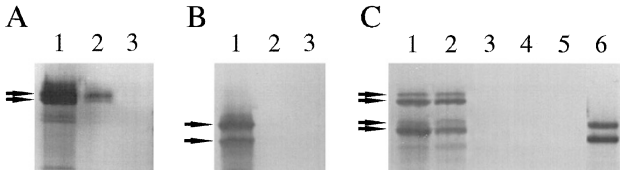

Figure 3 Immunoblot analysis demonstrating HLH-domain dependent homo- and heterodimerization of ETV6-NTRK3. (**a**) Metabolically labeled *in-vitro* translated ETV6-NTRK3 (shown in lane 1 (arrows)) was pulled down with purified GST-ETV6-NTRK3 protein plus glutathione beads (lane 2), but not with glutathione beads alone (lane 3). (**b**) Metabolically labeled *in vitro*-translated EN-ΔHLH (shown in lane 1 by arrows) could not be pulled down with purified GST-ETV6-NTRK3 fusion protein plus glutathione beads (lane 2) nor with glutathione beads alone (lane 3). (**c**) Metabolically labeled ETV6-NTRK3 and wild-type ETV6 were co-*in vitro* translated (shown in lane 1 by arrows) and subjected to immunoprecipitation with α-TrkC antibody plus protein A-sepharose beads (lane 2), or with protein A beads alone (lane 3), demonstrating heterodimerization of ETV6-NTRK3 and ETV6. ETV6 could not be pulled down in these experiments when *in-vitro* translated EN-ΔHLH was substituted for ETV6-NTRK3 using α-TrkC antibodies plus protein A beads (lane 4) or with protein A beads alone (lane 5). It was necessary to use unlabeled EN-ΔHLH was in these experiments due to size overlap with ETV6. A Western blot of the reaction mixture using α-TrkC antibodies demonstrates the presence of the EN-ΔHLH doublet in lane 6

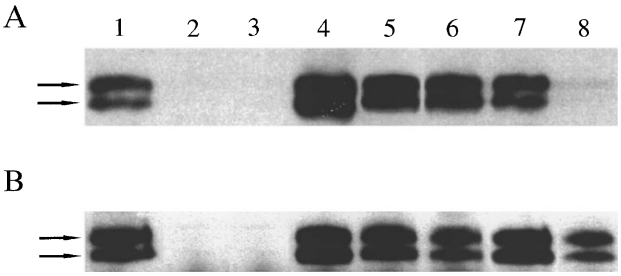

Figure 4 Immunoblot analysis demonstrating ETV6-NTRK3 tyrosine phosphorylation. (**a**) Whole cell lysates were prepared from NIH3T3 cells expressing ETV6-NTRK3 and various mutant constructs and immunoprecipitated with α-TrkC antibody and subsequently immunoblotted with the anti-phosphotyrosine antibody, RC-20. Immunoprecipitates from cells expressing ETV6-NTRK3, EN-Yx3F, EN-Y628T, EN-Y628E, and EN-Y628Q (lanes 1, and 4–7 respectively) demonstrated tyrosine phosphorylation of 73 and 68 kD bands (arrows). NIH3T3 cells expressing MSCVpac, ΔHLH and EN-K380N (lanes 2, 3 and 8, respectively) showed no tyrosine phosphorylation. (**b**) Presence of constructs was confirmed by reprobing the blot in panel **a** with an α-ETV6:HLH antibody (panel **b**) or α-TrkC (not shown)

cells expressing EN-Y513F, EN-Yx3F, or EN-K380N mutants all exhibited a normal phenotype and failed to form microscopic or macroscopic colonies in soft agar when scored after 28 days (see Table 1). Cells expressing the EN-Y517F and EN-Y518F mutants exhibited a semi-transformed phenotype (data not shown); these cells did not form macroscopic colonies in soft agar but did show microscopic abortive-looking colonies after 3 weeks in culture (see Figure 2e and Table 1). The expression of equivalent levels of ETV6-NTRK3 and mutated proteins in each of the cell lines was confirmed by Western blot analysis (see Figure 2f). These results indicate that the NTRK3 PTK region is crucial for transformation of NIH3T3 cells. Moreover, they demonstrate that ETV6-NTRK3 Y513 is essential for transformation while both Y517 and Y518 are required for full transformation.

ETV6-NTRK3 homodimerizes and heterodimerizes with wild-type ETV6

We predicted that ligand-independent dimerization of ETV6-NTRK3 was prerequisite for the PTK-mediated transformation activity of this oncoprotein. To begin to test this hypothesis, we co-incubated purified GST-ETV6-NTRK3 fusion proteins with *in vitro*-translated ETV6-NTRK3 radiolabeled with ^{35}S-methionine. Glutathione beads were able to pull down labeled ETV6-NTRK3, but not in the absence of GST-ETV6-NTRK3 (see lanes 2 and 3, Figure 3a). This indicated that ETV6-NTRK3 was capable of homodimerization. To test if this dimerization was mediated by the ETV6 HLH domain, we co-incubated purified GST-ETV6-NTRK3 fusion proteins and *in vitro*-translated EN-ΔHLH mutant protein radiolabeled with ^{35}S-methionine and co-precipitated with glutathione beads. However, the EN-ΔHLH mutant could not be pulled down with glutathione beads indicating that the HLH domain is required for dimerization with ETV6-NTRK3 (see lane 2, Figure 3b). As expected, the radiolabeled EN-ΔHLH mutant could not be pulled down when co-incubated with glutathione beads alone (see lane 3, Figure 3b). Next we determined whether ETV6-NTRK3 is able to heterodimerize with wild-type ETV6. ETV6-NTRK3 and ETV6 were co-*in vitro*-translated in the presence of ^{35}S-methionine, and then subjected to immunoprecipitation using α-TrkC (C-14) antibody and protein A beads. As shown in Figure 3c (lane 2), ETV6 was co-immunoprecipitated along with ETV6-NTRK3 protein indicating that ETV6-NTRK3 can heterodimerize with ETV6. In contrast, ETV6 could not be pulled down when co-incubated with *in vitro*-translated EN-ΔHLH mutant (see lane 4, Figure 3c). It was necessary to use unlabeled EN-ΔHLH in the latter experiment due to size overlap with ETV6; the presence of EN-ΔHLH in the reaction mixture was confirmed by Western blotting using α-TrkC (C-14) antibody (see lane 6, Figure 3c). These results provide strong evidence that ETV6-NTRK3 undergoes self-association and can heterodimerize with ETV6, and that these interactions are mediated by the ETV6 HLH domain.

ETV6-NTRK3 has PTK activity and undergoes tyrosine autophosphorylation

We wished to test our hypothesis that the ETV6-NTRK3 oncoprotein functions as a PTK. NIH3T3 cells expressing either ETV6-NTRK3, EN-Yx3F, EN-ΔHLH, the EN-K380N kinase inactive mutant, or mutants unable to associate with PLCγ were analysed for tyrosine-specific phosphorylation. Whole cell lysates from cells grown in log phase were subjected to immunoprecipitation with α-TrkC (C-14) antibody, followed by immunoblot analysis with the anti-phosphotyrosine antibody, RC-20. Immunoprecipitates showed tyrosine phosphorylation of ETV6-NTRK3 in transformed cells expressing ETV6-NTRK3 or the PLCγ-binding mutants (see Figure 4a). There was no evidence of ETV6-NTRK3 tyrosine phosphorylation in cells expressing the EN-K380N kinase inactive mutant or the EN-ΔHLH mutant. Interestingly, cells expressing EN-Yx3F also showed ETV6-NTRK3 tyrosine phosphorylation, possibly due to residual PTK activity or phosphorylation by other kinases (see Discussion).

The presence of comparable amounts of immunoprecipitated ETV6-NTRK3 or mutant proteins in the samples was confirmed by re-probing immunoblots with either α-ETV6:HLH antibodies (see Figure 4b), or α-TrkC (C-14) to visualize EN-ΔHLH (data not shown). Similar results were obtained when α-ETV6:HLH instead of α-TrkC (C-14) was used for the initial immunoprecipitation step.

To confirm that ETV6-NTRK3 is capable of auto- (or cross-) phosphorylation, ETV6-NTRK3, EN-ΔHLH, and EN-K380N were each *in-vitro* translated and subjected to immunoprecipitation with α-ETV6:HLH or α-TrkC (C-14), followed by immunoblotting using RC20. This revealed tyrosine phosphorylation of ETV6-NTRK3 but not of EN-ΔHLH or the EN-K380N kinase mutant (data not shown). These results provide evidence that ETV6-NTRK3 is a PTK capable of autophosphorylation, and that this activity requires an intact HLH domain and a functional PTK domain.

ETV6-NTRK3 binds PLCγ but not SHC, GRB2, or PI-3K p85

NTRK3 is known to interact with specific cytoplasmic substrates which become tyrosine phosphorylated upon activation of the NTRK3 PTK domain. These interacting molecules include SHC (Guiton *et al*., 1995), GRB2 (Bonfini *et al*., 1996; Downward, 1996), PI-3K (Barbacid, 1994; Hallberg *et al*., 1998; Obermeier *et al*., 1993b), and PLCγ as well as others (Berridge, 1993; Cockcroft and Thomas, 1992; Lee and Rhee, 1995; Nishizuka, 1995). To determine if these molecules were involved in ETV6-NTRK3 signaling, we tested whether these molecules interacted with the ETV6-NTRK3 fusion protein. Sequence analysis of the ETV6-NTRK3 chimer predicted that the SHC, GRB2, and PI-3K interaction site (corresponding to NTRK3 Y516) is absent from the ETV6-NTRK3 protein due to the position of the fusion point, but that the PLCγ site is retained (Knezevich *et al*., 1998b). However, it was possible that other residues may mediate SHC, GRB2, and PI-3K binding to ETV6-NTRK3. To test this, lysates from ETV6-NTRK3 expressing cells grown in log phase were immunoprecipitated with either α-TrkC (C-14) or α-ETV6:HLH antibodies, and immunoblotted with antibodies against SHC, GRB2, PI-3K, or PLCγ. As predicted by the structure of ETV6-NTRK3, only PLCγ could be coimmunoprecipitated in these experiments (Figure 5). PLCγ was also found to associate with the triple-tyrosine activation loop mutant, EN-Yx3F, but not with the EN-ΔHLH or EN-K380N mutants (Figures 5 and 6). We could find no evidence of an interaction between SHC, GRB2, or PI-3K with ETV6-NTRK3 or any of the mutants, even though these molecules interacted with wild-type NTRK3 in control human medullary thyroid carcinoma cells overexpressing wild-type NTRK3 (Figure 5). These data suggest that, other than PLCγ, ETV6-NTRK3 may interact with signaling molecules that are different than those normally associated with wild-type NTRK3.

To determine the role of PLCγ-binding in the transformation activity of ETV6-NTRK3, NIH3T3 were infected with *EN-Y628T*, *EN-Y628E*, or *EN-Y628Q* recombinant retroviral vectors. The corresponding mutant proteins (see Figure 1) are predicted to lack the ability to bind PLCγ, as phosphorylated ETV6-NTRK3 Y628 corresponds to the known PLCγ binding site in NTRK3 (Y820) (Lamballe *et al*., 1993; Obermeier *et al*., 1993a). All three puromycin-selected mutant-expressing cell lines still appeared fully transformed morphologically and were able to form macroscopic colonies in soft agar after 16–19 days at levels similar to ETV6-NTRK3 (data not shown; Table 1). Immunoblot assays confirmed that the EN-Y628T, EN-Y628E, and EN-Y628Q mutants were unable to associate with PLCγ (see Figure 6). The expression of equivalent levels of ETV6-NTRK3 and mutated proteins in each of the cell lines was confirmed by Western blot analysis (see lanes 4–6, left panel, Figure

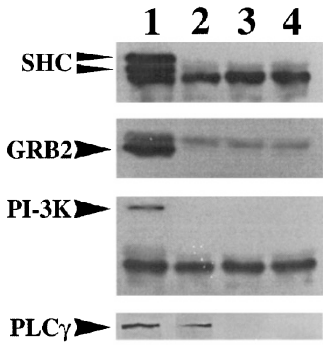

Figure 5 ETV6-NTRK3 interacts with PLCγ but not with SHC, GRB2, or PI-3K p85 subunit. Whole cell lysates were prepared from a human medullary thyroid carcinoma cell line overexpressing wild-type NTRK3 (lane 1), as well as from NIH3T3 cells expressing ETV6-NTRK3 (lane 2), empty MSCVpac vector (lane 3), or EN-K380N (lane 4). Immunoprecipitation was performed with antibodies against the NTRK3 PTK domain followed by immunoblotting with antibodies directed against SHC, GRB2, PI-3K, or PLCγ as indicated. Only wild-type NTRK3 was found to associate with SHC, GRB2, and PI-3K (lanes 1 of top three panels), while both NTRK3 and ETV6-NTRK3 bound PLCγ (lanes 1 and 2 of bottom panel)

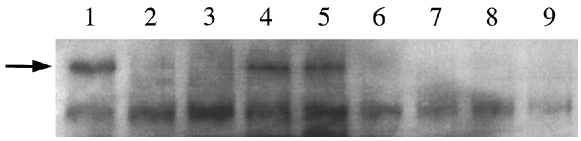

Figure 6 Analysis of PLCγ binding mutants for ability to associate with PLCγ. Whole cell lysates were prepared from control human medullary thyroid carcinoma cells overexpressing wild-type NTRK3 (lane 1), or NIH3T3 cells (lanes 2–9) expressing ETV6-NTRK3 or mutant constructs described in Materials and methods (see Figure 1). Lysates were subjected to immunoprecipitation with antibodies against the NTRK3 PTK domain followed by immunoblotting with α-PLCγ antibodies. Lane 2, empty MSCVpac vector; lane 3, EN-ΔHLH; lane 4, ETV6-NTRK3; lane 5, EN-Yx3F; lane 6, EN-K380N; and lanes 7–9, mutants with Y628 (corresponding to NTRK3 Y820 known to bind PLCγ (Lamballe *et al*., 1993; Obermeier *et al*., 1993a)) mutated to threonine, glutamic acid, or glutamine, respectively. The arrow shows the position of PLCγ

Table 2 Tumor formation in SCID mice

Construct	*Average tumor mass* (g)	P *value*[a]
ETV6-NTRK3	3.15 ($n=5$)	–
MSCVpac vector	0.26 ($n=5$)	0.009
EN-K380N	0.05 ($n=5$)	<0.001

[a]Weights of ETV6-NTRK3-induced tumor masses were compared to those resulting from injection of cells expressing MSCVpac vector or EN-K380N

2f). These findings, coupled with the fact that the EN-Yx3F activation loop mutant also bound PLCγ even though it was non-transforming (see Figure 6 and Table 1), provides compelling evidence that binding and tyrosine phosphorylation of PLCγ does not play a significant role in transformation by the ETV6-NTRK3 oncoprotein.

ETV6-NTRK3-transformed NIH3T3 cells are tumorigenic in SCID mice

The above retrovirally infected NIH3T3 cells expressing either ETV6-NTRK3, the kinase inactive EN-K380N mutant, or vector alone were injected subcutaneously into severe combined immunodeficient (SCID) mice as described in the Materials and methods. ETV6-NTRK3-expressing NIH3T3 cells formed significantly larger tumor masses than cells expressing EN-K380N ($P=0.009$) or vector alone ($P=0.009$). Mice injected with ETV6-NTRK3-expressing cells developed tumors averaging 3.15 g ($n=5$; see Table 2). On the other hand NIH3T3 cells expressing EN-K380N or vector alone did not form significant tumor masses, with the respective average weights of the excised tissues at the sites of injection measuring 0.05 g ($n=5$) and 0.26 g ($n=5$), respectively (see Table 2).

Discussion

The CFS t(12;15)(p13;q25) chromosomal rearrangement creates a fusion gene encoding the ETV6 HLH domain linked to the tyrosine kinase domain of NTRK3 (Knezevich *et al*., 1998b). Several other chimeric proteins involving the ETV6 HLH domain fused to a PTK domain have been described in human hematopoietic malignancies (Golub *et al*., 1994; Janssen *et al*., 1995; Lacronique *et al*., 1997; Peeters *et al*., 1997a). As these fusion proteins have each been shown to be capable of transforming hematopoietic cells *in vitro*, we wanted to determine whether ETV6-NTRK3 has transformation activity. We chose to test this activity in NIH3T3 fibroblastic cells as the *ETV6-NTRK3* gene fusion was originally identified in CFS, which is thought to be of fibroblastic derivation (Knezevich *et al*., 1998b). Interestingly, an *ETV6-NTRK3* gene fusion identical to the one described for CFS was recently observed in an adult patient with AML (Eguchi *et al*., 1999), although transformation studies were not reported. Our experiments demonstrated that ETV6-NTRK3 is a potent oncoprotein, as NIH3T3 cells expressing this molecule were morphologically transformed and readily grew macroscopic colonies in soft agar. Colony formation was of the same order of magnitude as NIH3T3 cells expressing the Ewing sarcoma EWS-FLI1 molecule, known to be a strong transforming protein (May *et al*., 1993). Moreover, ETV6-NTRK3 expressing NIH3T3 cells were tumorigenic in SCID mice, which was not observed with cells expressing the PTK inactive EN-K380N mutant. These findings indicate that ETV6-NTRK3 is a strong oncoprotein, and is the first demonstration, to our knowledge, of an ETV6 fusion protein with transforming activity in NIH3T3 cells.

We found that ETV6-NTRK3 transformation activity requires an intact HLH domain and that ETV6-NTRK3 is capable of homodimerization, both of which could be abrogated by deletion of the HLH domain. Moreover, absence of this domain eliminated ETV6-NTRK3 tyrosine phosphorylation as well as detectable PTK activity. We therefore hypothesize that the ETV6 HLH domain mediates ligand-independent homodimerization leading to autophosphorylation and activation of the ETV6-NTRK3 PTK. The ETV6 HLH domain has been shown to be required for homodimerization and subsequent autophosphorylation in fusions with *ABL* in AML (Golub *et al*., 1996) and with *PDGFRβ* in CMML (Carroll *et al*., 1996). Moreover, ETV6 HLH-domain dependent dimerization has been demonstrated in fusions with *Jak2* in ALL (Lacronique *et al*., 1997) which leads to constitutive Jak-Stat signaling (Ho *et al*., 1999). ETV6-NTRK3 tyrosine phosphorylation most likely represents autophosphorylation activity of the chimeric protein based on several findings. First, mutation of the lysine 380 ATP-binding site of ETV6-NTRK3 (mutant EN-K380N), corresponding to NTRK3 lysine 572 (Lamballe *et al*., 1991; Mitra, 1991), completely abolished tyrosine phosphorylation of the fusion protein. Second, the non-dimerizing EN-ΔHLH mutant was not tyrosine phosphorylated in NIH3T3 cell lysates. Third, *in-vitro* translated ETV6-NTRK3 became tyrosine phosphorylated, while both EN-ΔHLH and the EN-K380N kinase inactive mutant were not tyrosine phosphorylated upon *in vitro* translation (data not shown).

EN-K380N did not transform NIH3T3 cells in our studies, indicating an essential role for functional PTK activity in ETV6-NTRK3 transformation. This is corroborated by detailed analysis of the PTK-encoding region of *ETV6-NTRK3* fusion transcripts. Naturally occurring NTRK3 isoforms have been described which have 14, 25, or 39 amino acid inserts within the PTK domain adjacent to tyrosine autophosphorylation sites (reviewed in Barbacid, 1994). These isoforms have low intrinsic PTK activity, fail to tyrosine phosphorylate SHC, SNT, and PLCγ, and are defective in the biological responses they elicit (Guiton *et al*., 1995; Gunn-Moore *et al*., 1997; Lamballe *et al*., 1993; Tsoulfas *et al*., 1996). We have found that *ETV6-NTRK3* transcripts in CFS tumor cells lack these kinase inserts, consistent with the fully active PTK being involved in CFS-associated *ETV6-NTRK3* gene fusions and in contrast to what was claimed in a recent report (Eguchi *et al*., 1999). Consistent with this is the observation that the CFS-associated ETV6-NTRK3 protein is able to bind PLCγ (see Figure 6).

To further investigate the role of tyrosine phosphorylation, we analysed the effects of tyrosine-to-phenylalanine mutations of the three predicted PTK activation loop tyrosines of ETV6-NTRK3, Y513, Y517 and Y518 (corresponding to NTRK3 Y705, Y709 and Y710, respectively) on NIH3T3 transformation. The activation loop tyrosines of wildtype NTRK3 reside within a conserved YSTDYYR sequence of the PTK domain and are essential for PTK activation (Barbacid, 1994). The function of the first conserved tyrosine (Y705) has yet to be determined, while Y709 and Y710 are thought to be responsible for NTRK3 autophosphorylation (Barbacid, 1994; Lamballe *et al*., 1993; Tsoulfas *et al*., 1993; Valenzuela *et al*., 1993). Simultaneous mutation of all three tyrosines in ETV6-NTRK3 resulted in a non-transformed phenotype. Similarly, expression of the EN-

Y513F mutant resulted in morphologically normal NIH3T3 cells and absence of macroscopic colonies in soft agar, indicating that this tyrosine is essential for transformation. EN-Y517F- or EN-Y518F-expressing cells each had a semi-transformed phenotype and exhibited markedly attenuated soft agar colony formation (see Figure 2e). Therefore, while both appear to be required for full transformation, there may be redundancy between these two tyrosines in ETV6-NTRK3 mediated transformation.

Interestingly, we observed varying but consistent tyrosine phosphorylation of either EN-Yx3F, EN-Y513F, EN-Y517F, or EN-Y518F in cells expressing these proteins (see Figure 4 and data not shown), which is in contrast to analyses of NTRK1 (TRKA) where ligand-induced autophosphorylation of NTRK1 markedly decreases when the three activation loop tyrosines are mutated individually or in pairs (Cunningham *et al*., 1997). There are several potential explanations for these findings. First, it is possible that some level of autophosphorylation is maintained in the mutants (possibly through phosphorylation of Y628 and/or other tyrosines of ETV6-NTRK3), but that recruitment of essential substrates for transformation is abrogated by these mutations. Second, these mutant proteins may become tyrosine phosphorylated by other PTKs present in NIH3T3 cells, but still incapable of mediating essential phosphorylations required for transformation. A third possibility is that ETV6-NTRK3 phosphorylation does not play a role in its oncogenicity, and that other functions of the molecule are involved. More studies are necessary to confirm the relationship between the three activation loop tyrosines and ETV6-NTRK3 phosphorylation. Taken together, however, our results are most consistent with a model in which ETV6-NTRK3 is a constitutively active PTK capable of autophosphorylation, although we have not absolutely ruled out that ETV6-NTRK3 is a substrate for another PTK.

NT-3 induced PTK activation of wild-type NTRK3 results in the direct association and tyrosine phosphorylation of several downstream signaling molecules including SHC, GRB2, the PI-3K p85 subunit, and PLCγ (Barbacid, 1994) as well as SNT (Peng *et al*., 1995; Rabin *et al*., 1993). This leads to activation of multiple pathways involved in NTRK3 signaling, including the RAS-RAF1 pathway (Bonfini *et al*., 1996; Downward, 1996), PI-3K activation of the cell survival mediator, Akt/PKB (Barbacid, 1994; Hallberg *et al*., 1998; Kaplan, 1997; Obermeier *et al*., 1993b), and the PKC pathway (Berridge, 1993; Cockcroft and Thomas, 1992; Lee and Rhee, 1995; Nishizuka, 1995). However, the binding site for SHC/GRB2 and PI-3K p85, NTRK3 tyrosine-516 (Hallberg *et al*., 1998), is lost from the NTRK3 portion of the ETV6-NTRK3 fusion protein due to the position of the breakpoint (Knezevich *et al*., 1998b). In keeping with this, we were unable to demonstrate any association of either SHC, GRB2, or PI-3K p85 with ETV6-NTRK3. However, this does not rule out a role for the RAS-RAF1 pathway in ETV6-TRK3 mediated transformation. It is possible that SHC (and therefore the RAS/RAF1 pathway) might be activated indirectly without binding to the fusion oncoprotein. Alternatively, other adaptor molecules may be involved in RAS activation; several newly described molecules have been shown to interact with activated NTRK3 and to be potentially involved in NTRK3 signaling, including rAPS and SH2-Bβ (Qian *et al*., 1998). Recent reports have also described interactions between the related receptor, NTRK1, and the GRB2-associated binder-1 docking protein (Gab1) (Holgado-Madruga *et al*., 1997), and between NTRK1 and caveolin in PC12 cells exposed to NGF (Bilderback *et al*., 1999). Their potential involvement in ETV6-NTRK3 signaling is currently being studied.

We found that ETV6-NTRK3 is able to bind and tyrosine phosphorylate PLCγ, as predicted by the presence of carboxy-terminal Y628 in the chimeric protein. This residue corresponds to NTRK3 Y820, which is known to bind PLCγ (Lamballe *et al*., 1993; Obermeier *et al*., 1993a). However, expression of three different ETV6-NTRK3 constructs with mutations of Y628 to threonine, glutamic acid, or glutamine,

Table 3 Primer sequences used in site-directed mutagenesis and sequence analysis

Primer	*Comments*	*Sequence (5′-3′)*
TEL114	*ETV6* nt 114-133	GAC GCC ACT TCA TGT TCC AG
TEL352	*ETV6* nt 352-373	GGT GAT GTG CTC TAT GAA CTC C
TEL541	*ETV6* nt 541-560	CCT CCC ACC ATT GAA CTG TT
TEL541rev	*ETV6* nt 541-560	AAC AGT TCA ATG GTG GGA GG
TEL701	*ETV6* nt 701-720	AGA ACA ACC ACC AGG AGT CC
PS2	*NTRK3* nt 2304-2324	GTA ATG CAC TCA ATG ACC TC
Trk1	*NTRK3* nt 2414-2431	TCT CCT TGA TGT TCA ACC
Trk2	*NTRK3* nt 1821-1838	CCG CAC ACT CCA TAG AAC
Trk3	*NTRK3* nt 1601-1620	CC TCT TAA TGT GCT GCA CAT
μ1164F		GTG GCT GTG AAC GCC CTG AAG GAT CCC
μ1164R		GGG ATC CTT CAG GGC GTT CAC AGC CAC
μ1562F		C GGC ATG TCC AGA GAT GTC TTC AGC ACG G
μ1562R		C CGT GCT GAA GAC ATC TCT GGA CAT GCC G
μ1574F		GTC TAC AGC ACG GAT TTT TAC AGG GTG GGA GG
μ1574R		CC TCC CAC CCT GTA AAA ATC CGT GCT GTA GAC
μ1577F		C AGC ACG GAT TAT TTC AGG GTG GGA GGA CAC
μ1577R		GTG TCC TCC CAC CCT GAA ATA ATC CGT GCT G
μtyrx3F		TCC AGA GAT GTC TTC AGC ACG GAT TTT TTC AGG GTG GGA GGA CAC
μtyrx3R		GTG TCC TCC CAC CCT GAA AAA ATC CGT GCT GAA GAC ATC TCT GGC
Y628T-for		G GCC ACC CCA ATC ACC CTG GAC ATT CTT GGC
Y628T-rev		GCC AAG AAT GTC CAG GGT GAT TGG GGT GGC C
Y628E-for		G GCC ACC CCA ATC GAG CTG GAC ATT CTT GGC
Y628E-rev		GCC AAG AAT GTC CAG CTC GAT TGG GGT GGC C
Y628Q-for		G GGC ACC CCA ATC CAG CTG GAC ATT CTT GGC
Y628Q-rev		GCC AAG AAT GTC CAG CTG GAT TGG GGT GGC C

respectively, each transformed NIH3T3 cells to the same degree as non-mutated ETV6-NTRK3 (Table 1), even though there was no detectable association of these mutant proteins with PLCγ in immunoprecipitation experiments. These results provide preliminary evidence that ETV6-NTRK3-induced transformation does not require PLCγ activation and thus is likely not mediated through the PKC pathway. In keeping with this interpretation, the activation loop tyrosine mutant EN-Yx3F also bound PLCγ even though it was non-transforming (Figure 6). More detailed analyses are required to test the role of PKC activation in ETV6-NTRK3 transformed cells.

An intriguing possibility is that ETV6-NTRK3 recruits components of potentially novel signaling pathways not involving RAS-RAF1 activation or functioning downstream of RAS-RAF1. We are currently evaluating the role of the RAS-RAF1 pathway in ETV6-NTRK3 signaling as well as searching for novel ETV6-NTRK3 associating molecules. Interestingly, we observed *in vitro* heterodimerization between ETV6-NTRK3 and wild-type ETV6. It is possible that binding of ETV6-NTRK3 to ETV6 depletes levels of the latter, which has been hypothesized to function at least in part as a tumor suppressor (Poirel *et al*., 1998). ETV6 is known to be a phosphoprotein (Poirel *et al*., 1997), and so may be a substrate for ETV6-NTRK3; however, we found no evidence of ETV6 tyrosine phosphorylation by ETV6-NTRK3 in these studies (data not shown). Alternatively, heterodimerization with other HLH-containing molecules may lead to the recruitment and activation of, as yet, unidentified signaling pathways. Subcellular localization studies suggest that ETV6-NTRK3 resides predominantly in the cytoplasm, but it is also observed at low levels in the nucleus (unpublished results). Further studies are required to determine the significance of these findings.

Materials and methods

Generation of full-length ETV6-NTRK3

Full-length *ETV6-NTRK3* was constructed by splicing a partial *ETV6-NTRK3* fragment to wild-type *ETV6*. The partial *ETV6-NTRK3* fragment was isolated by digesting a 3′-RACE PCR product obtained from a CFS tumor (Knezevich *et al*., 1998b), cloned into pCR™2.1 (Invitrogen), with *Pvu*II and *Not*I. This fragment contained nucleotides 826 to 1033 of *ETV6* cDNA fused in-frame to the 3′-end of *NTKR3* cDNA at nucleotide 1601. Full-length *ETV6*, cloned into pGEM-3Z (a generous gift of P Marynen, University of Leuven, Leuven, Belgium), was digested with *Eco*RI and *Pvu*II to generate a fragment containing the first 825 base pairs of *ETV6* cDNA. These two products were directionally ligated into pBluescript II KS at the *Eco*RI and *Not*I sites to produce the 2106-bp *ETV6-NTRK3* fusion cDNA. All restriction endonucleases were obtained from New England Biolabs. Full-length clones were screened by DNA sequencing and PCR using TEL541 and Trk2 primers. All primer sequences are described in Table 3. PCR obtained the expected 731-bp product using the following conditions: 94°C for 1.5 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension of 72°C for 10 min. DNA sequencing used *ETV6* primers TEL352 and TEL701, *NTRK3* primers Trk1 and Trk3, and standard primers T3 and T7. All sequencing was performed on an ABI373A DNA Sequencer (Applied Biosystems) and analysed using DNA-STAR™ software.

ETV6-NTRK3 HLH-deletion mutant (EN-ΔHLH)

A fusion protein mutant lacking a functional ETV6 helix–loop–helix (HLH) domain was constructed by using *Bsp*MI and *Eco*NI to delete a large portion of the HLH domain (ETV6 amino acid residues 64–117). This generated a 1950-bp *EN-ΔHLH* construct which lacked nucleotides 193–351 of *ETV6* (Figure 1). Clones were screened by PCR using TEL114 and TEL541rev primers (Table 3), followed by cDNA sequencing to confirm correct sequences and orientation.

Site-directed mutagenesis

The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) was used to introduce point mutations within the *NTRK3* portion of the 2106-bp fusion cDNA in pBluescript II KS. Three primer sets (μ1562F/μ1562R, μ1574F/μ1574R, and μ1577F/μ1577R; Table 3) were used in the site-directed mutagenesis (SDM) of the three activation loop tyrosines (Y705, Y709, and Y710 of NTRK3) (Barbacid, 1994; Lamballe *et al*., 1993; Tsoulfas *et al*., 1993; Valenzuela *et al*., 1993) to phenylalanines, resulting in EN-Y513F, EN-Y517F, and EN-Y518F mutants, respectively. An ETV6-NTRK3 triple Y513F, Y517F, and Y518F mutant (called EN-Yx3F), was made using μtyrx3F and μtyrx3R primers (Figure 1; Table 3). An ETV6-NTRK3 PTK-inactive mutant replacing the ATP-binding lysine 380 (NTRK3 K572) (Lamballe *et al*., 1991; Mitra, 1991) with asparagine, designated EN-K380N, was made using μ1164F and μ1164R primers (Figure 1; Table 3). Finally, ETV6-NTRK3 tyrosine 628 (NTRK3 tyrosine 820) was converted to either a threonine (EN-Y628T), glutamic acid (EN-Y628E), or glutamine (EN-Y628Q) residue to ablate this amino acid as the putative binding site for phospholipase-Cγ (Lamballe *et al*., 1993; Obermeier *et al*., 1993a). Three sets of primer pairs were employed to create these respective mutants: Y628T-for/Y628T-rev, Y628E-for/Y628E-rev, and Y628Q-for/Y628Q-rev (Figure 1; Table 3). SDM-PCR conditions for all primers were as follows: 95°C for 30 s, followed by 12 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 10 min 15 s. DNA sequencing of the inserts was used to confirm the point mutations; sequences using standard primer T7 and *NTRK3* primer PS2 were obtained and analysed as described above.

Retroviral vectors

cDNAs encoding wild-type or mutated forms of ETV6-NTRK3 were inserted into the retroviral vector MSCVpac (Hawley *et al*., 1994) at the *Eco*RI site. Plasmid DNA of the retroviral vectors was transfected into the BOSC23 ecotropic retroviral packaging cell line (Pear *et al*., 1993), using calcium phosphate precipitation as described (Pear *et al*., 1993). The retrovirus-containing supernatants were collected from the BOSC cells 48 h after transfection and used to infect NIH3T3 cells.

Soft agar assays

Soft agar assays were performed according to established protocols (May *et al*., 1993). NIH3T3 cells infected with recombinant retroviruses carrying either empty vector, *ETV6-NTRK3*, or one of the above mutants were plated in quadruplicate at a density of 30 000 cells per 6-cm tissue culture dish. The NIH3T3 cells were suspended in soft agar (1 × IMDM, 10% calf serum, and 0.7% agar noble). Macroscopic colonies were counted (in quadruplicate) and the plates were photographed 16 to 19 days after plating, and they were observed for up to 28 days for macroscopic colony growth.

SCID mice studies

Pathogen free female C.B-17 scid/scid (SCID) mice, 5–6 weeks old, tested for leakiness, were obtained from

Bomholtgard Breeding and Research Center (Ry, Denmark) and were injected subcutaneously (s.c.) into the left lower flank with 10^7 NIH3T3 cells transfected with control vector, a ETV6-NTRK3 kinase-deficient mutant (EN-K380N) or ETV6-NTRK3 ($n=5$/group). The animals were housed in laminar flow racks and microisolator cages under specific pathogen free conditions and received autoclaved food and water. SCID mice were evaluated for tumor growth and organ involvement 11 days after injection, a time point at which tumor growth in the ETV6-NTRK3 group necessitated the termination of the experiment. Statistical significance of differences in tumor weight in the respective groups was evaluated using the Mann-Whitney *U*-Test. *P* values of <0.05 were considered to be of statistical significance.

Production of GST-ETV6-NTRK3 fusion proteins

Glutathione-S-transferase (GST) ETV6-NTRK3 fusion proteins expressed in Sf9 cells were generated from full-length *ETV6-NTRK3* cDNA using the BaculoGold Expression and Purification kit (Pharmingen), following the manufacturer's protocols. Recombinant protein was purified by glutathione column chromatography for subsequent protein studies.

Antibodies

Antibodies used for immunoprecipitation included α-TrkC (C-14) (Santa Cruz Biotechnology) (2 μg/ml) and α-ETV6:HLH (a gift of Dr P Marynen, University of Leiden) (1 : 500 dilution). Antibodies used for immunoblot analysis included α-phosphotyrosine (RC20-Horse Radish Peroxidase conjugated [1 : 2500]), α-SHC [1 : 250], α-PI-3K [1 : 5000], α-GRB2 [1 : 5000], α-PLCγ1 [1 : 1000] (all from Transduction Laboratories) and α-SH2Bβ [1 : 15000] (a gift of Drs L Rui and C Carter-Su, University of Michigan, Ann Arbor, MI, USA).

Immunoprecipitation and immunoblotting

Lysates of PBS-washed NIH3T3 cells expressing the above constructs were prepared, immunoprecipitated and analysed by immunoblotting as previously described (Obermeier *et al*., 1993b). Briefly, T25-flasks containing 70–90% confluent cells were lysed in 1 ml of lysis buffer (1.5 mM $MgCl_2$, 150 mM NaCl, 50 mM HEPES, 10 mM NaF, 10 mM $Na_4P_2O_7$, 2 mM Na_3VO_4, 2 mM EDTA, 2 mM $NaMoO_4{\cdot}2H_2O$, 10% Glycerol, 0.5–1.0% Nonidet P-40, Leupeptin (10 μg/ml), Aprotinin (10 μg/ml), and freshly prepared PMSF (250 μM)) on ice for 15 min. Lysates were cleared by centrifugation at 12 000 *g* for 10 min prior to immunoprecipitation for 2 h with the relevant antibody. Washed immunoprecipitates were then mixed with Laemmli buffer and electrophoresed overnight on 10–15% SDS-polyacrylamide gels according to standard methods. Electrophoresed proteins were transferred to Immobilon-P (Millipore) prior to immunoblot analysis with the indicated antibodies. Proteins were visualized with ECL (Amersham) according to the manufacturer's protocols.

Oligomerization studies

To determine whether ETV6-NTRK3 heterodimerizes with wildtype ETV6, both ETV6 and ETV6-NTRK3 were co-translated with the TNT T7/T3 Coupled Reticulocyte Lysate System (Promega) according to the manufacturer's instructions. Complexes were immunoprecipitated as described previously (Lacronique *et al*., 1997) with α-TrkC (C-14) or α-ETV6:HLH antibodies. Immunoprecipitates were analysed by immunoblotting with α-ETV6:HLH or α-TrkC (C-14), respectively, and visualized as described above. To test for ETV6-NTRK3 homodimerization, 5 μg of purified GST-ETV6-NTRK3 recombinant protein was mixed with ^{35}S-Methionine-labeled (Amersham) *in vitro* translated ETV6-NTRK3, immunoprecipitated with glutathione beads, and electrophoresed as above. The resolving gel was dried and exposed to XAR-5 film for 16 h.

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Accession number
The updated full-length *ETV6-NTRK3* cDNA sequence is deposited in Genbank under accession number AF041811.

References

Adam LR, Davison EV, Malcolm AJ, Pearson ADJ and Craft AW. (1990). *Cancer Genet. Cytogenet.,* **48,** 193–198.
Argyle JC, Tomlinson GE, Stewart D and Schneider NR. (1992). *Arch. Pathol. Lab. Med.,* **116,** 972–975.
Baens M, Peeters P, Guo C, Aerssens J and Marynen P. (1996). *Genome Res.,* **6,** 404–413.
Barbacid M. (1994). *J. Neurobiol.,* **25,** 1386–1403.
Barbacid M. (1995a). *Curr. Opin. Cell Biol.,* **7,** 148–155.
Barbacid M. (1995b). *Ann. NY Acad. Sci.,* **766,** 442–458.
Berridge MJ, (1993). *Nature,* **361,** 315–325.
Bilderback TR, Gazula VR, Lisanti MP and Dobrowsky RT. (1999). *J. Biol. Chem.,* **274,** 257–263.
Bonfini L, Migliaccio E, Pelicci G, Lanfrancone L and Pelicci PG. (1996). *Trends Biochem. Sci.,* **21,** 257–261.
Buijs A, Sherr S, van Baal S, van Bezouw S, van der Plas D, Geurts van Kessel A, Riegman P, Lekanne Deprez R, Zwarthoff E and Hagemeijer A, *et al*. (1995). *Oncogene,* **10,** 1511–1519.
Carroll M, Tomasson MH, Barker GF, Golub TR and Gilliland DG. (1996). *Proc. Natl. Acad. Sci. USA,* **93,** 14845–14850.
Cave H, Cacheux V, Raynaud S, Brunie G, Bakkus M, Cochaux P, Preudhomme C, Lai JL, Vilmer E and Grandchamp B. (1997). *Leukemia,* **11,** 1459–1464.
Chase A, Reiter A, Burci L, Cazzaniga G, Biondi A, Pickard J, Roberts IA, Goldman JM and Cross NC. (1999). *Blood,* **93,** 1025–1031.
Cockcroft S and Thomas GM. (1992). *Biochem. J.,* **288,** 1–14.
Coffin CM, Jaszcz W, O'Shea P and Dehner LP. (1994). *Pediatric Pathology,* **14,** 133–150.
Conover JC and Yancopoulos GD. (1997). *Rev. Neurosci.,* **8,** 13–27.
Cools J, Bilhou-Nabera C, Wlodarska I, Cabrol C, Talmant P, Bernard P, Hagemeijer A and Marynen P. (1999). *Blood,* **94,** 1820–1824.

Cunningham ME, Stephens RM, Kaplan DR and Greene LA. (1997). *J. Biol. Chem.,* **272,** 10957–10967.
Downward J. (1996). *Cancer Surv.,* **27,** 87–100.
Ebadi M, Bashir RM, Heidrick ML, Hamada FM, Refaey HE, Hamed A, Helal G, Baxi MD, Cerutis DR and Lassi NK. (1997). *Neurochem. Int.,* **30,** 347–374.
Edel MJ. (1998). *Anticancer Res.,* **18,** 4505–4509.
Eguchi M, Eguchi-Ishimae M, Tojo A, Morishita K, Suzuki K, Sato Y, Kudoh S, Tanaka K, Setoyama M, Nagamura F, Asano S and Kamada N. (1999). *Blood,* **93,** 1355–1363.
Golub TR, Barker GF, Bohlander SK, Hiebert SW, Ward DC, Bray-Ward P, Morgan E, Raimondi SC, Rowley JD and Gilliland DG. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 4917–4921.
Golub TR, Barker GF, Lovett M and Gilliland DG. (1994). *Cell,* **77,** 307–316.
Golub TR, Goga A, Barker GF, Afar DE, McLaughlin J, Bohlander SK, Rowley JD, Witte ON and Gilliland DG. (1996). *Mol. Cell. Biol.,* **16,** 4107–4116.
Guiton M, Gunn-Moore FJ, Glass DJ, Geis DR, Yancopoulos GD and Tavare JM. (1995). *J. Biol. Chem.,* **270,** 20384–20390.
Gunn-Moore FJ, Williams AG and Tavare JM. (1997). *Biochem. J.,* **322,** 193–198.
Hallberg B, Ashcroft M, Loeb DM, Kaplan DR and Downward J. (1998). *Oncogene,* **17,** 691–697.
Hawley RG, Lieu FH, Fong AZ and Hawley TS. (1994). *Gene Ther.,* **1,** 136–138.
Ho JM, Beattie BK, Squire JA, Frank DA and Barber DL. (1999). *Blood,* **93,** 4354–4364.
Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R and Wong AJ. (1997). *Proc. Natl. Acad. Sci. USA,* **94,** 12419–12424.
Ichaso N, Rodriguez RE, Martin-Zanca D and Gonzalez-Sarmiento R. (1998). *Oncogene,* **17,** 1871–1875.
Janssen JW, Ridge SA, Papadopoulos P, Cotter F, Ludwig WD, Fonatsch C, Rieder H, Ostertag W, Bartram CR and Wiedemann LM. (1995). *Br. J. Haematol.,* **90,** 222–224.
Jing S, Tapley P and Barbacid M. (1992). *Neuron,* **9,** 1067–1079.
Kaplan DR. (1997). *Curr. Opin. Cell Biol.,* **9,** 213–221.
Knezevich SR, Garnett MJ, Pysher TJ, Beckwith JB, Grundy PE and Sorensen PH. (1998a). *Cancer Res.,* **58,** 5046–5048.
Knezevich SR, McFadden DE, Tao W, Lim JF and Sorensen PH. (1998b). *Nat. Genet.,* **18,** 184–187.
Kouhara H, Hadari YR, Spivak-Kroizman T, Schilling J, Bar-Sagi D, Lax I and Schlessinger J. (1997). *Cell,* **89,** 693–702.
Kwiatkowski BA, Bastian LS, Bauer Jr TR, Tsai S, Zielinska-Kwiatkowska AG and Hickstein DD. (1998). *J. Biol. Chem.,* **273,** 17525–17530.
Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M, Berthou C, Lessard M, Berger R, Ghysdael J and Bernard OA. (1997). *Science,* **278,** 1309–1312.
Lamballe F, Klein R and Barbacid M. (1991). *Cell,* **66,** 967–979.
Lamballe F, Tapley P and Barbacid M. (1993). *EMBO J.,* **12,** 3083–3094.
Lee SB and Rhee SG. (1995). *Curr. Opin. Cell Biol.,* **7,** 183–189.
Lemmon MA and Schlessinger J. (1994). *Trends Biochem. Sci.,* **19,** 459–463.
Mandahl S, Heim S, Rydholm A, Willen H and Mitelman F. (1989). *Cancer Genet. Cytogenet.,* **40,** 137–139.
May WA, Lessnick SL, Braun BS, Klemsz M, Lewis BC, Lunsford LB, Hromas R and Denny CT. (1993). *Mol. Cell. Biol.,* **13,** 7393–7398.
McGregor LM, Baylin SB, Griffin CA, Hawkins AL and Nelkin BD. (1994). *Genomics,* **22,** 267–272.
Mitra G. (1991). *Oncogene,* **6,** 2237–2241.
Nishizuka Y. (1995). *FASEB J.,* **9,** 484–496.
O'Connor HE, Butler TA, Clark R, Swanton S, Harrison CJ, Secker-Walker LM and Foroni L. (1998). *Leukemia,* **12,** 1099–1061.
Obermeier A, Halfter H, Wiesmuller KH, Jung G, Schlessinger J and Ullrich A. (1993a). *EMBO J.,* **12,** 933–941.
Obermeier A, Lammers R, Wiesmuller KH, Jung G, Schlessinger J and Ullrich A. (1993b). *J. Biol. Chem.,* **268,** 22963–22966.
Ong SH, Goh KC, Lim YP, Low BC, Klint P, Claesson-Welsh L, Cao X, Tan YH and Guy GR. (1996). *Biochem. Biophys. Res. Commun.,* **225,** 1021–1026.
Papadopoulos P, Ridge SA, Boucher CA, Stocking C and Wiedemann LM. (1995). *Cancer Res.,* **55,** 34–38.
Pear WS, Nolan GP, Scott ML and Baltimore D. (1993). *Proc. Natl. Acad. Sci. USA,* **90,** 8392–8396.
Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P, Monpoux F, Van Rompaey L, Baens M, Van den Berghe H and Marynen P. (1997a). *Blood,* **90,** 2535–2540.
Peeters P, Wlodarska I, Baens M, Criel A, Selleslag D, Hagemeijer A, Van den Berghe H and Marynen P. (1997b). *Cancer Res.,* **57,** 564–569.
Peng X, Greene LA, Kaplan DR and Stephens RM. (1995). *Neuron,* **15,** 395–406.
Poirel H, Lacronique V, Mauchauffe M, Le Coniat M, Raffoux E, Daniel MT, Erickson P, Drabkin H, MacLeod RA, Drexler HG, Ghysdael J, Berger R and Bernard OA. (1998). *Oncogene,* **16,** 2895–2903.
Poirel H, Oury C, Carron C, Duprez E, Laabi Y, Tsapis A, Romana SP, Mauchauffe M, Le Coniat M, Berger R, Ghysdael J and Bernard OA. (1997). *Oncogene,* **14,** 349–357.
Qian X, Riccio A, Zhang Y and Ginty DD. (1998). *Neuron,* **21,** 1017–1029.
Rabin SJ, Cleghon V and Kaplan DR. (1993). *Mol. Cell. Biol.,* **13,** 2203–2213.
Romana SP, Mauchauffe M, Le Coniat M, Chumakov I, Le Paslier D, Berger R and Bernard OA. (1995). *Blood,* **85,** 3662–3670.
Rubin BP, Chen CJ, Morgan TW, Xiao S, Grier HE, Kozakewich HP, Perez-Atayde AR and Fletcher JA. (1998). *Am. J. Pathol.,* **153,** 1451–1458.
Sanakary S, Dickman PS, Wiener E, Robichaux W, Swaney WP, Malone PS and Gollin SM. (1993). *Cancer Genet. Cytogenet.,* **65,** 152–156.
Speleman F, Dal Cin P, De Potter K, Laureys G, Roels HJ, Leroy J and van den Berghe H. (1989). *Cancer Genet. Cytogenet.,* **39,** 21–24.
Suto Y, Sato Y, Smith SD, Rowley JD and Bohlander SK. (1997). *Genes Chromosomes Cancer,* **18,** 254–268.
Tsoulfas P, Soppet D, Escandon E, Tessarollo L, Mendoza-Ramirez JL, Rosenthal A, Nikolics K and Parada LF. (1993). *Neuron,* **10,** 975–990.
Tsoulfas P, Stephens RM, Kaplan DR and Parada LF. (1996). *J. Biol. Chem.,* **271,** 5691–5697.
Valenzuela DM, Maisonpierre PC, Glass DJ, Rojas E, Nunez L, Kong Y, Gies DR, Stitt TN, Ip NY and Yancopoulos GD. (1993). *Neuron.,* **10,** 963–974.
Wang LC, Kuo F, Fujiwara Y, Gilliland DG, Golub TR and Orkin SH. (1997). *EMBO J.,* **16,** 4374–4383.
Wang LC, Swat W, Fujiwara Y, Davidson L, Visvader J, Kuo F, Alt FW, Gilliland DG, Golub TR and Orkin SH. (1998). *Genes Dev.,* **12,** 2392–2402.
Yagasaki F, Jinnai I, Yoshida S, Yokoyama Y, Matsuda A, Kusumoto S, Kobayashi H, Terasaki H, Ohyashiki K, Asou N, Murohashi I, Bessho M and Hirashima K. (1999). *Genes Chromosomes Cancer,* **26,** 192–202.