Supporting Information

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SI Experimental Procedures

Colorectal Samples. This study is based on 90 colorectal samples from a cohort of 45 patients. To preserve anonymity, a specific ID was attributed to each patient. For each sample, the tumoral tissue was paired with the adjacent normal tissue. The tumor stages (I–IV) were defined according to the TNM status of the tumors (American Joint Committee on Cancer Staging system) (1).

RNA Isolation and Quantitative Real-Time RT-PCR (Q-RT-PCR). To assay TrkC expression in human colorectal samples, total RNA was extracted from biopsies of patients undergoing surgery for colorectal cancer, using the Nucleospin RNAII kit (Macherey-Nagel). Five hundred nanograms of RNA were reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time quantitative RT-PCR (Q-RT-PCR) was performed using a LightCycler 480 (Roche Applied Science) and the FastStart TaqMan Probe Master Mix (Roche Applied Science). Q-RT-PCR was performed using (*i*) probe #43 (Roche Applied Science) and the following primers: TrkC forward 5′-CAACTCTCAAACACGGAGGTC-3′ and reverse 5′CCAGCATGACATCGTACACC-3′; (*ii*) probe #44 (Roche Applied Science) and the following primers: NT-3 forward: 5′-CCCTTGTATCTCATGGAGGATT-3′ and reverse: 5′-TTTCCGCCGTGATGTTCT-3′. The ubiquitously expressed human gene PGK, showing the least variable expression between normal and colorectal tumoral tissues, was used as internal control, using probe # 42 (Roche Applied Science) and the following primers: forward 5′-CTGTGGCTTCTGGCATACCT-3′ and reverse 5′-CGAGTGACAGCCTCAGCATA. For all couples of primers, polymerase was activated at 95 °C for 10 min, followed by 45 cycles of amplification and 30 s of cooling. Moreover, the cohort has been previously validated using 11 other ordinarily housekeeping genes to strengthen the results: RxRα, PPIA, GAPDH, β-actin, Phosphoglycerokinase 1, β2-microglobulin, hypoxanthine ribosyltransferase, TATA-box-binding protein, porphobilinogen deaminase (PBGD), transferrin receptor, and ribosomal protein large P0 (RPLPO).

Laser Capture Microdissection and Q-RT-PCR. Laser capture was performed under direct microscopic visualization using a Arcturus Pixcell II Laser Capture Microdissection system on 12-μm-thick tissue frozen sections prepared from colon tumor and normal biopsies stained with cresyl violet (Ambion LCM staining kit; Life Technologies). Three nanograms of RNA underwent two rounds of linear amplification using the ExpressArt TR*inucleotide* mRNA amplification Nano kit, (AmpTec, Excilone). Reverse transcription of amplified RNA was performed with an iScript cDNA Synthesis kit (Bio-Rad). cDNA synthesis was carried out from 1 μg of amplified cRNA.

Real-time Q-RT-PCR was performed on a LightCycler 2.0 apparatus (Roche), using the Light Cycler FastStart DNA Master SYBER Green I kit (Roche). Q-RT-PCR was performed using the following primers: TrkC forward 5′-AGCTCAACAGCCAGAACCTC-3′ and reverse 5′-AACAGCGTTGTCACCCTCTC-3′. The ubiquitously expressed human PGK gene, showing the least variable expression in colon, was used as an internal control, using the following primers: forward 5′-CTGTGGCTTCTGGCATACCT-3′ and reverse 5′-CTTGCTGCTTTCAGGACCC-3′. For all two couples of primers, polymerase was activated at 95 °C for 10 min, followed by 35 cycles at 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 5 s.

LOH Analysis. The locus of TrkC has been studied with Repeat Masker. Single repeat microsatellites were used as markers to compare normal and tumor tissues using fluorescently labeled primers amplifying the DNAs from normal and tumoral tissue of 30 patients. All primers and PCR conditions are available upon request.

Cell Lines and Treatment. Colorectal cancer cell lines HCT116, SW480, HT29, V9P, Caco-2, Colo320, HCT-15, SW48, SW620, SW116, SW480, ISP1, LS1034, SW837, and HCT8 have been described. The HCT116 double knockout for DNMT1 and DNMT3b (DKO) have been described and were kindly provided by B. Vogelstein (Ludwig Center at Johns Hopkins, Baltimore, MD) (2). HCT116 wild-type and HCT116 DKO colorectal cell lines were maintained in Dulbecco's Minimum Essential Medium (DMEM) (Life Technologies), supplemented with 10% FCS (Lonza). HCT8 colorectal cell line was grown in RPMI (Life Technologies), supplemented with 10% fetal horse serum. HCT116 cells were treated with 5-Aza-2′-deoxycitidine (Sigma-Aldrich) (1–5 μM) 24 h after plating, during 72 h. HCT116 cells were also treated with Histone DeAcetylase inhibitors (HDACi), SAHA and MS275 (Sigma-Aldrich) at a concentration range of 1–5 μM, 24 h after plating, during 24 h. After treatment, cells were harvested and RNA was isolated for gene expression (Q-RT-PCR) analysis, as described above. HCT116 transfected cells were treated with recombinant NT-3 (Abcys) at 10 ng/mL, 24 h after plating.

Plasmids, Transfection Procedure, and Reagents. The full-length TrkC has been described (3). The plasmid constructs were transfected using JetPrime transfectant (PolyPlus) following manufacturer's instructions. The construct encoding the luciferase reporter gene under the control of TrkC promoter was a kind gift of P. J. Donovan and H. Fong (Sue and Bill Gross Stem Cell Research Center, University of California, Irvine, CA) and has been described (4).

TrkC Site-Directed Mutagenesis. Mutations were generated on the human TrkC gene using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The mutation E543D was generated with the following primers: forward 5′-GCTGAAGCGAGACCTGGGTGAGGGAG-3′ and reverse 5′-CTCCCTCACCCAGGTCTCGCTTCAGC-3′. The mutation D584E was generated with the following primers: forward 5′-GCCCGGAAGGAATTCCAGAGGGAGG-3′ and reverse 5′- CCTCCCTCTGGAATTCCTTCCGGGC-3′.

Cell Death Assays. A total of 10^5 cells were transfected with TrkC constructs using JetPrime (PolyPlus), and grown in serum-poor medium. Cell death was analyzed 48 h posttransfection: Caspase-3 activity was measured as described (5) using the Ac-DEVD-AFC substrate assay (Biovision, K105-400), whereas total cell death index was measured using the ToxiLight assay from Lonza (LT07-117). The active caspase-3 was detected in immunostaining using an antibody specifically targeting the active form of caspase-3 and not the zymogen (Cell Signaling, #9661).

Protein Detection. HCT116 and HCT116 DKO were lysed in 50 mM Hepes (pH 7.6), 125 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40 in the presence of proteases inhibitors. For Western blotting, we used anti-panTrk (Santa Cruz, sc-139), anti-actin (Chemicon, MAB1501R).

Immunostaining of patient biopsies sections or cell lines was performed with an anti-TrkC antibody (Santa Cruz, sc-117) after

citrate unmasking (pH 7.3, 98 °C for 35 min) and revealed with Novolink kit (Leica). Sections of patient biopsies were counterstained with a Mayer's Hematoxylin coloration.

DNA Isolation. DNA was isolated from 60 samples of normal and tumoral colorectal tissue from patient biopsies. All of the samples were cryogrinded in liquid nitrogen and incubated at 55 °C in DNA extraction buffer containing 0.1 μg/μL of proteinase K for 3 h. After complete digestion, proteinase K was inactivated and DNA precipitated with NaCl, washed with ethanol, and resuspended in water.

Bisulfite Conversion and Analysis of TrkC Promoter Methylation by Pyrosequencing. The methylation status of the promoter of TrkC was examined by pyrosequencing, a highly reliable and quantitative method for the analysis of DNA methylation at multiple CpG sites with built-in internal controls for completeness of bisulfite treatment. Bisulfite treatment was carried out as described (6). Briefly, genomic DNA (0.5–1 μg), from associated normal and tumoral colorectal cancer samples, was treated with EZ DNA methylation-Gold kit (Zymo Research), according to the manufactured protocol. The modified DNA (20–25 ng/μL) was stored at −20 °C until use. A set of primers was designed on an in silico modified DNA sequence, to amplified a GC rich region of the TrkC promoter. DNA amplification was carried out on bisulfite-treated DNA using specific primers and PCR conditions: TrkC Forward 5′-GATTTGGTGATTTTAGTATTATTTTT-3′ and Reverse 5′-AAAAAAAACCTCTACCTTTAAAAC-3′. Modified DNA was amplified in a total volume of 50 μL: 10 μL of PCR was analyzed on agarose gel, and the remaining 40 μL was used in pyrosequencing assay using sequencing primers: 5′-GTTTTTAGAGTTTT-3′ and 5′-TACAAAATCCTTCAA-3′. Pyrosequencing reactions were set up using PyroGold Reagent kit (Biotage) according to the manufacturer's instructions. The methylation levels at the target CpGs were evaluated by converting the resulting programs to numerical values for peak heights and expressed either as the percentage of methylation of individual CpG sites or as the mean of all CpGs analyzed.

Chromatin Immuno-Precipitation (ChIP). Formaldehyde was added to control or treated HCT116 cells to a final concentration of 1%, and the cells were incubated at 37 °C for 10 min. The medium was removed, and the cells were washed in 1 mL of ice-cold PBS containing protease inhibitors (Complete, Roche). Cells were pelleted, resuspended in 0.5 mL of lysis buffer (1% SDS/10 mM EDTA/50 mM Tris·HCl, pH 8.1), and incubated on ice for 10 min. Lysates were sonicated with 30-s cycles using a bioruptor. Debris were removed from samples by centrifugation for 10 min at 15,000 × *g* at 4 °C. An aliquot of the chromatin preparation (30 μL) was set aside and designated as the Input Fraction. Supernatants were diluted 10-fold in dilution buffer (1% Triton X-100/2 mM EDTA/20 mM Tris·HCl, pH 8.1/150 mM NaCl), precleared with protein A Sepharose beads (sigma, previously blocked with salmon sperm DNA and BSA) for at least 2 h at 4 °C. Beads were pelleted by centrifugation, and supernatants were incubated overnight at 4 °C with 5 μg of total H3 (Abcam, ab1791), antiacetylated histone H4 (Millipore, 06–598), or anti-H3K27Me3 Millipore 07-449) antibodies. Protein A Sepharose (40 μL) was added for 2 h at 4 °C. Protein A complexes were centrifuged, washed three times with wash buffer (0.1% SDS/2 mM EDTA, pH 8/150 mM NaCl/20 mM Tris·HCl, pH 8.1/1% Triton X-100) and once with final wash buffer (0.1% SDS/2 mM EDTA, pH 8/500 mM NaCl/20 mM Tris·HCl pH 8.1/1% Triton X-100). Immune complexes were eluted with 450 μL of elution buffer (1% SDS/0.1 M NaHCO3). Samples were treated with RNase A and proteinase K overnight at 65 °C and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. DNA concentration was measured and adjusted using a nanodrop. TrkC promoter specific primers (forward: 5′-GTCTTCACACTCTGTCCCCG-3′ and reverse: 5′-ATTTTTACCCCCGTCGCCAT-3′) were used to carry out Q-PCR on input and ChIP samples using Sybr green (Roche).

Soft Agar Assay. For bottom agar, 2 mL of 1.8% agarose were diluted with 2 mL of 2× DMEM (2× DMEM/20% FBS/0.2% geneticin/500 mg/mL fungizone). A 4-mL volume of bottom agar was plated in a 60-mm tissue culture dish and allowed to harden. Cells were trypsinized and resuspended at 10^5 cells per mL in 2× DMEM. The top agar cell suspensions were composed of 1 mL of cell suspension in 2× DMEM and 1 mL of 0.9% agarose, and were overlaid on dishes containing bottom agar. The final plating concentration was 10^5 cells per dish. Fourteen days later, clones were observed and counted.

Migration Assay. ***Scratch assay.*** Six-well plates were coated with 50 μg/mL poly-L-lysine by incubating the dishes overnight at 4 °C or for 2 h at 37 °C without rotation or shaking. The unbound extracellular matrix (ECM) substrate was removed and the coated dishes blocked with 3 mL of 2 mg/mL BSA for 1 h at 37 °C. Then, the dishes were washed once with PBS and refilled with 3–5 mL of media before plating the cells. HCT116 cells (2×10^6 per well) were plated and transfected with the plasmid constructs. A scratch was realized with a pipet tip 24 h later. The cell migration was monitored for the following 96 h.

Transwell assay. Transwell migration assays were performed using 24-well transwell Boyden chambers (8 μm PET membrane, Falcon). HCT116 cells (5×10^5) were added to the top well resuspended in 500 μL serum-free medium, and the lower well was filled with 800 μL of medium containing 20% FBS. Where applicable, NT-3 or Z-VAD was added to the top well, together with the cells. After 48-h incubation, cells remaining on the upper side of the filter were removed with cotton swabs, and the cells that had migrated to the bottom surface of the filter were counted in 10 different fields per condition. The experiment was repeated three times, and one representative experiment is shown.

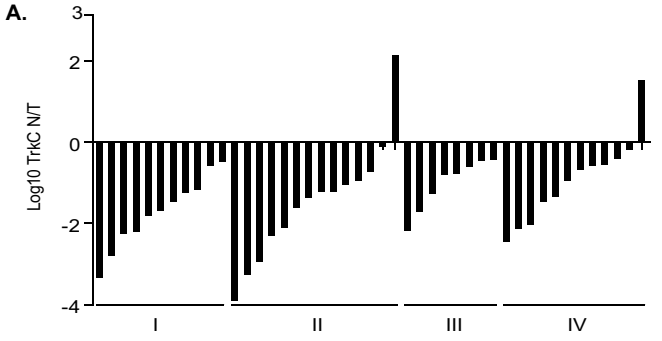
Chicken Model for Colorectal Cancer Progression. HCT116 cells were transfected with plasmids 48 h after plating, treated or not with NT-3 (10 ng/mL) and harvested 24 h posttransfection. HTC 116 transfected cells (5×10^6) were suspended in 25 μL complete medium and 25 μL of matrigel with or without NT-3 (10 ng/mL). These cells were seeded on 10-d-old (day 10) chick chorioallantoic membrane (CAM) previously locally injured with a cotton swab. On day 17, tumors were resected and the area was measured with AxioVision Release 4.6 software (Carl Zeiss).

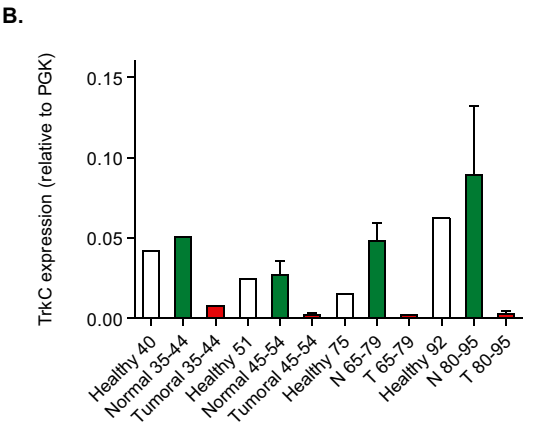
TUNEL Labeling of Tumor Sections. To monitor apoptosis on primary tumors, they were fixed on 4% PFA, cryoprotected by overnight treatment with 30% sucrose, and embedded in Cryomount (Histolab). TUNEL staining was performed on tumor cryostat sections (Roche Applied Science Diagnostics), and nuclei were stained with Hoechst (Sigma-Aldrich).

MAPK Pathway Activation. Cells transfected with the different TrkC mutants were lysed on ice in the following buffer (50 mM Tris, pH 7.5/1 mM EDTA/1mM EGTA/0.5 mM Na_3VO_4/0.1% β-mercaptoethanol/1% Triton X-100/50 mM sodium fluoride/5 mM sodium pyrophosphate/10 mM β-glycerophosphate/0.1 mM PMSF). Proteins were then analyzed by Western blot using an anti-Erk antibody (Cell Signaling) and an anti-phospho Erk antibody (Cell Signaling).

Statistics. Data presented are representative of at least three independent experiments, data are mean ± SEM. Statistical significance of differences between means was assessed by a Mann–Whitney test for paired values. Categorical data were analyzed with the χ^2 test. All statistical tests were two-sided. *P* values of less than 0.05 were considered to be statistically significant.

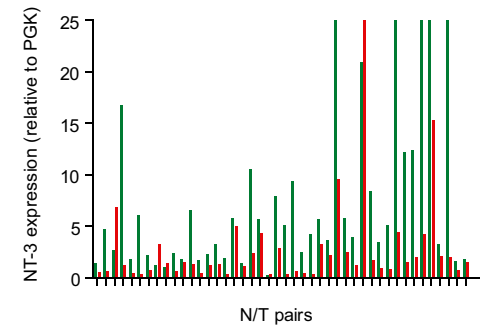
1. Compton CC, et al. (2012) *AJCC Cancer Staging Atlas: A Companion to the Seventh Editions of the AJCC Cancer Staging Manual and Handbook* (Springer-Verlag, Heidelberg), 2nd ed.
2. Rhee I, et al. (2002) DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature* 416(6880):552–556.
3. Tauszig-Delamasure S, et al. (2007) The TrkC receptor induces apoptosis when the dependence receptor notion meets the neurotrophin paradigm. *Proc Natl Acad Sci USA* 104(33):13361–13366.
4. Fong CT, et al. (1989) Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: Correlation with N-myc amplification. *Proc Natl Acad Sci USA* 86(10):3753–3757.
5. Bouzas-Rodriguez J, et al. (2010) Neurotrophin-3 production promotes human neuroblastoma cell survival by inhibiting TrkC-induced apoptosis. *J Clin Invest* 120(3): 850–858.
6. Tost J, Dunker J, Gut IG (2003) Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. *Biotechniques* 35(1):152–156.





C.

Microdissected tumors (n=8) with downregulation of TrkC greater than:		
10 fold	50 fold	100 fold
25%	25%	50%



E.

	Cell line	TrkC (10^{-3}mol)
Colorectal cancer	HCT 116	0
	SW480	0
	HT29	0
	V9P	0
	Caco2	0
	Colo320	0
	HCT 15	0
	LoVo	0
	SW48	0
	SW620	0.12
	SW116	0.13
	SW480	0.17
	ISP1	0.21
	LS1034	0.23
	SW837	0.24
	HCT 8	0.49
Neuroblastoma	CLB-Ge2	57.2
	IMR32	1300.0
Breast cancer	MDA-MB-436	742.0

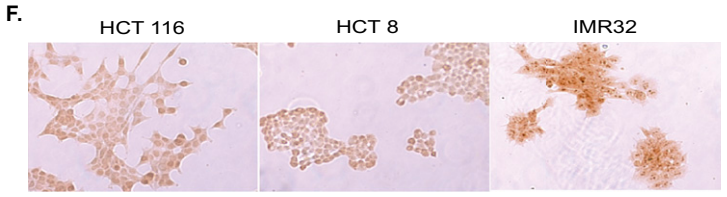


Fig. S1. TrkC expression is lost in colorectal tumors. (*A–C*) Quantitative real-time RT-PCR was performed using total RNA extracted from normal (N) and tumoral (T) tissues with specific human TrkC and NT-3 primers. PGK was used as housekeeping gene. (*A*) The ratio of TrkC expression in normal tissue on tumoral tissue (log T/N) is presented for each patient, the patients being ordered by different stages (I to IV) defined by the TNM status of the tumors and by decreased loss of TrkC in tumors. (*B*) Comparison between TrkC expression in normal (green bar) and tumoral tissue (red bar) from patients and TrkC expression in age-matched tissue from healthy colon (white bar). The range of age of each group of patients is indicated in abscissa. (*C*) The level of TrkC down-regulation observed after laser microdissection of tumor samples versus normal samples is indicated. (*D*) The expression levels of NT-3 (x10^{-3} mol) in 45 colorectal tumors (Tumoral) and corresponding normal tissues (Normal) are given as a ratio between NT-3 and PGK, the internal control. (*E*) TrkC expression (x10^{-3} mol) was measured in several colorectal, breast and neuroblastoma cancer cell lines, by Q-RT-PCR, using PGK as internal control. Number of molecules was calculated by 2exp(ΔCp), where ΔCp is TrkC minus PGK Cp. (*F*) Immunostaining of TrkC protein was performed on IMR32, HCT116, and HCT8 cells using a diaminobenzidine/peroxydase revelation.

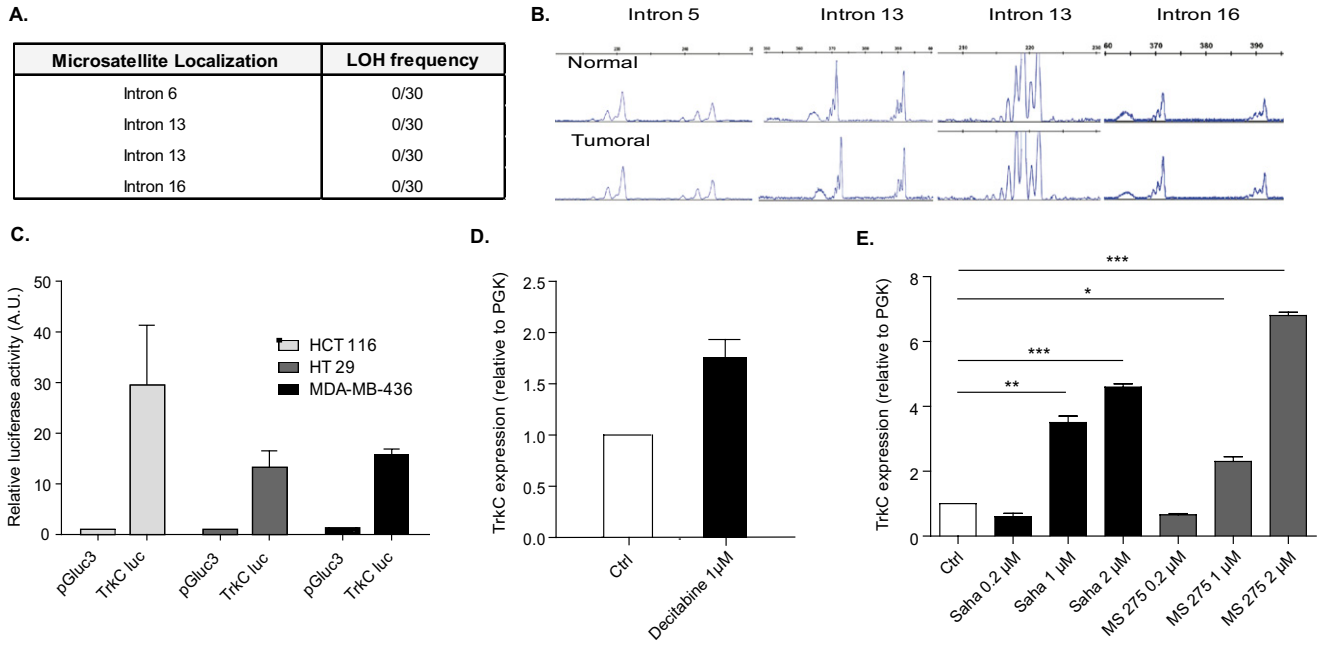


Fig. S2. Absence of LOH at the *TrkC* locus and TrkC re-expression in cancer cell lines. (*A* and *B*) LOH analyses were performed in a panel of 30 pairs tumors and matched normal tissues as described in *Experimental Procedures*. A representative pictogram of microstallite sequence amplification is shown in *B*. (*C*) A construct encoding the luciferase reporter gene fused to TrkC promoter was transfected into HCT116 and HT29 colon cancer cell lines and as a control into breast cancer cells, MDA-MB-436. The corresponding empty vector pGluc3 was used as control. Luciferase activity was measured in the various cell lines. (*D* and *E*) HCT8 cells were treated for 72 h with decitabine (*D*) or for 24 h with Saha or MS 275 (*E*) at the indicated concentrations. TrkC expression was measured by Q-RT-PCR, using PGK as internal control. Data represent mean ± SEM $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-sided Mann–Whitney test, compared with control.

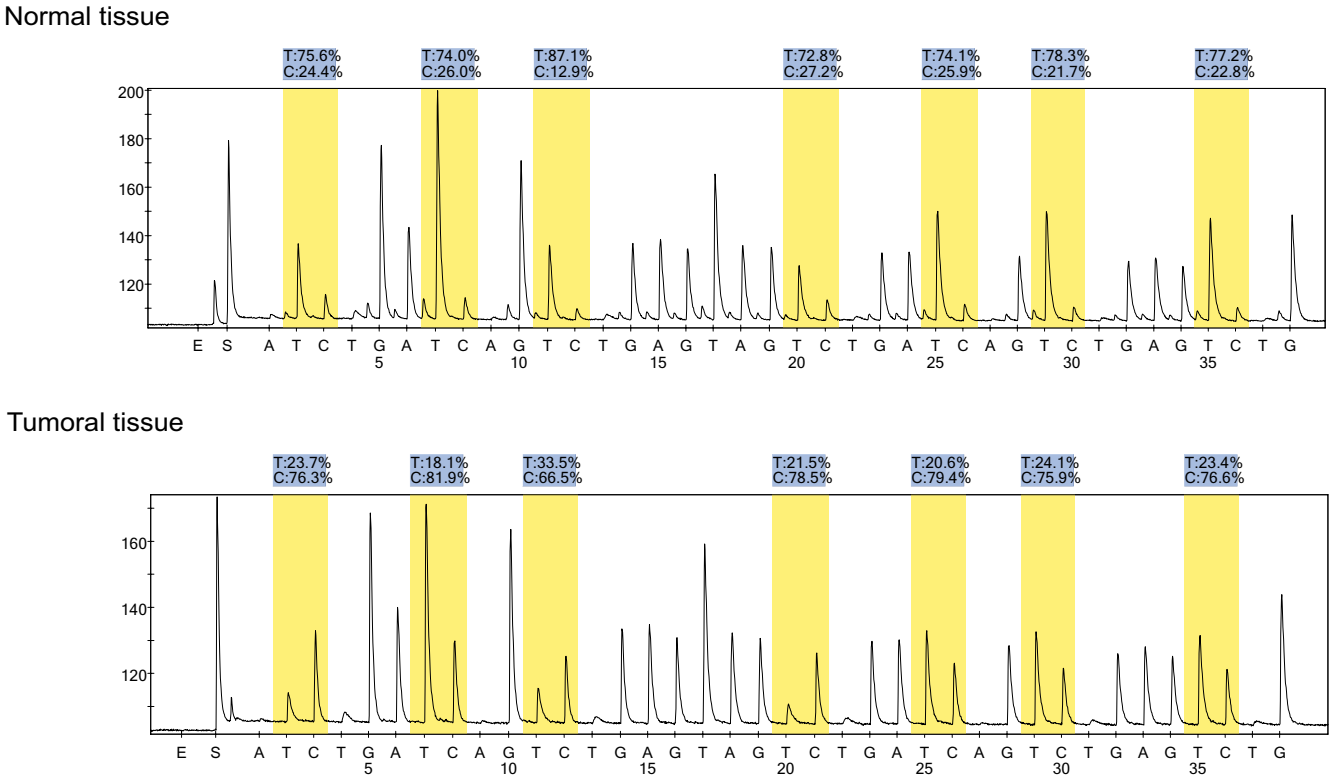


Fig. S3. Methylation of *TrkC* promoter. Representative pyrograms obtained for a pair of matched normal and tumoral samples. The sequence below the pyrograms indicates the sequentially added nucleotides. The yellow regions indicate the analyzed C/T sites; the proportion of C and T detected are provided as percentage values. This proportion corresponds to the ratio of methylated (C) and nonmethylated (T) DNA strands.

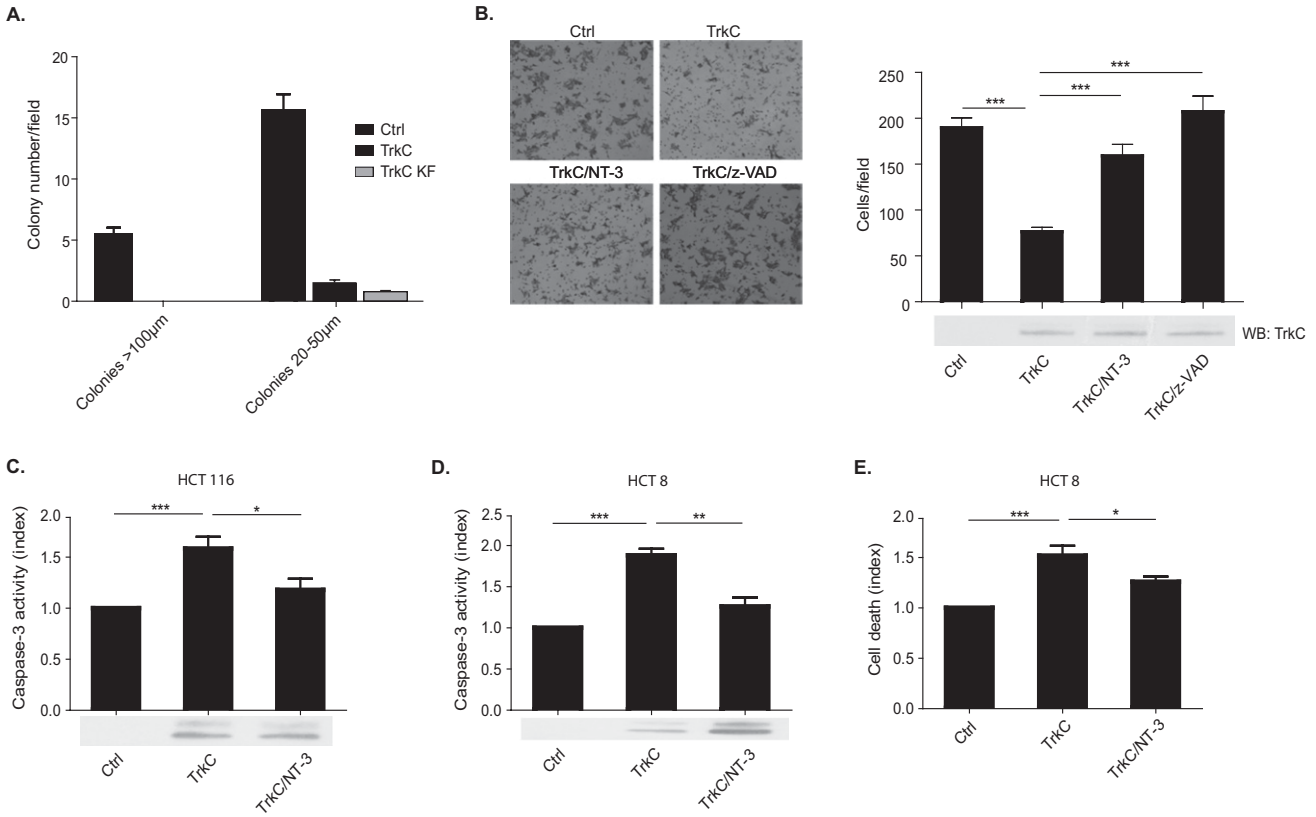


Fig. S4. TrkC limits anchorage-independent growth and migration and induces apoptosis. (*A*) Control, TrkC and TrkC KF overexpressing HCT116 cells were grown in soft agar for 2 wk. The number of colonies was counted in five random fields and the average number per field was calculated. Data represent mean ± SEM $*P < 0.05$, $***P < 0.001$, two-sided Mann–Whitney test, compared with control. The size of colonies for each condition is shown. (*B*) Transwell assay was performed on HCT116 cells expressing control vector and TrkC with or without NT-3 or Z-VAD-fmk addition. The migration was stopped at 48 h, and the migrated cells were counted (*Right*). Representative photographs are shown (*Left*). (*C*) TrkC induces cell death in HCT116 cells as shown by caspase-3 activity. (*D* and *E*) TrkC induces cell death in HCT8 cells as shown by caspase-3 activity (*D*) and Toxilight assay (*E*). Cell death is reversed by addition of NT-3 (10 ng/mL). Data represent mean ± SEM $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-sided Mann–Whitney test.

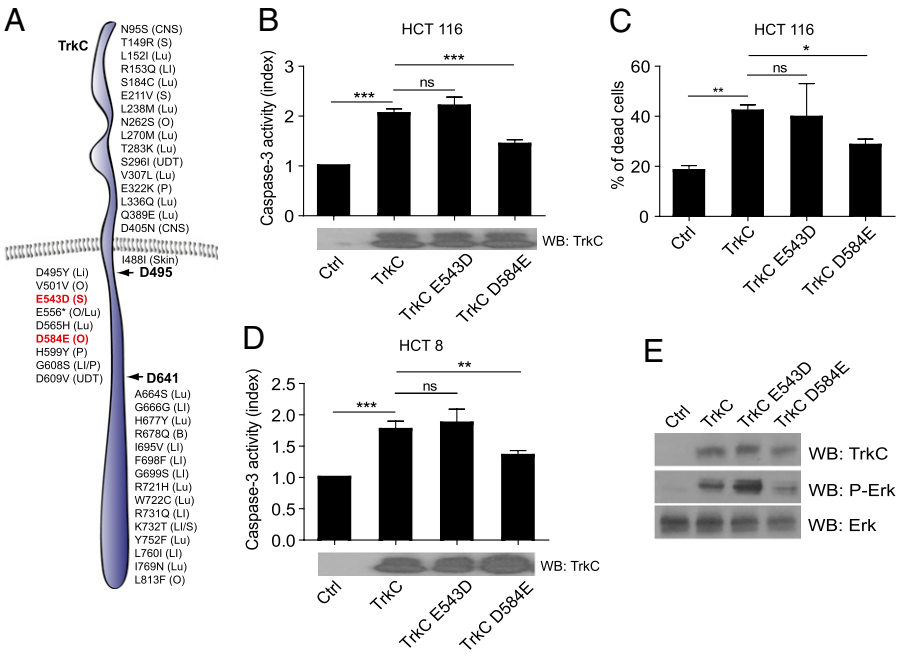


Fig. S5. Mutations of TrkC in cancers. (*A*) TrkC receptor is represented. Mutations identified in patients with various cancers are reported (www.sanger.ac.uk/perl/genetics/CGP/cosmic?action=gene&ln=NTRK3). The caspase cleavage sites D495 and D641 are shown, they define the killer fragment of TrkC. Mutations studied in are labeled in red. The localization of the cancer with the detected mutation is indicated. CNS, central nervous system; Lu, lung; O, ovary; P, pancreas. Mutations detected in cancers of the gastrointestinal tract are indicated in bold (LI, large intestine; S, stomach; UDT, upper digestive tract). (*B–E*) Control, TrkC, and TrkC mutants (TrkC E543D and TrkC D584E) were overexpressed in HCT116 and HCT8 cells. (*B* and *C*) TrkC induces cell death in HCT116 cells, as well as TrkC E543D but not TrkC D584E, as shown by caspase-3 activity (*B*) and trypan blue counting (*C*). (*D*) TrkC induces cell death in HCT8 cells, like TrkC E543D but not TrkC D584E, as shown by caspase-3 activity. Data represent mean ± SEM $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, two-sided Mann–Whitney test. (*E*) Phospho-Erk-1/2 immunoblot is shown as an indication of Erk-1/2 activation.