NTRK3 Is a Potential Tumor Suppressor Gene Commonly Inactivated by Epigenetic Mechanisms in Colorectal Cancer

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Abstract

NTRK3 is a member of the neurotrophin receptor family and regulates cell survival. It appears to be a dependence receptor, and thus has the potential to act as an oncogene or as a tumor suppressor gene. NTRK3 is a receptor for NT-3 and when bound to NT-3 it induces cell survival, but when NT-3 free, it induces apoptosis. We identified aberrantly methylated NTRK3 in colorectal cancers through a genome-wide screen for hypermethylated genes. This discovery led us to assess whether NTRK3 could be a tumor suppressor gene in the colon. NTRK3 is methylated in 60% of colon adenomas and 67% of colon adenocarcinomas. NTRK3 methylation suppresses NTRK3 expression. Reconstitution of NTRK3 induces apoptosis in colorectal cancers, if NT-3 is absent. Furthermore, the loss of NTRK3 expression associates with neoplastic transformation in vitro and in vivo. We also found that a naturally occurring mutant NTRK3 found in human colorectal cancer inhibits the tumor suppressor activity of NTRK3. In summary, our findings suggest NTRK3 is a conditional tumor suppressor gene that is commonly inactivated in colorectal cancer by both epigenetic and genetic mechanisms whose function in the pathogenesis of colorectal cancer depends on the expression status of its ligand, NT-3.

Introduction

Colorectal cancer (CRC) arises through the accumulation of gene mutations and epigenetic alterations that result in the transformation of normal colon epithelial cells into adenocarcinomas [1]. One of the most common epigenetic changes observed in CRC is the aberrant methylation of CpG islands in the promoter region of genes. Aberrant CpG island methylation is associated with gene silencing and can inactivate tumor suppressor genes in the colon, which promotes tumor formation through the deregulation of various cellular processes including proliferation and apoptosis, among others [1,2].

In order to identify methylated tumor suppressor genes that play a role in the formation of CRC, we conducted a genome-wide screen for methylated genes in colorectal cancers and matched normal colon epithelium tissue samples. Through this screen, we found a set of novel methylated genes, which included neurotrophin tyrosine kinase receptor 3 (NTRK3), a gene that has been found to be hypermethylated in esophageal adenocarcinoma [3]. The identification of methylated NTRK3 in CRC was unexpected given that NTRK3 has been shown to be an oncogene in breast cancer and possibly hepatocellular carcinoma [4,5]. However, NTRK3 has also been shown to be a tumor suppressor gene in neuroblastomas [6]. Thus, our findings raised the question of whether NTRK3 acts as an oncogene or tumor suppressor gene in the pathogenesis of CRC.

NTRK3 is a member of the NTRK neurotrophin receptor family, which includes NTRK1 (TRKα), NTRK2 (TRKB) and NTRK3 (TRKC). NTRK family members and their ligands, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT4/5, are crucial to the development of the nervous system and have poorly defined roles in other tissues [7]. NTRK1 is the receptor for NGF, and NTRK2 preferentially binds BDNF and NT4/5. NT-3 is the only known physiologically relevant ligand for NTRK3. The NTRKs have been shown to play oncogenic roles in certain cancers, such as breast cancer and liver cancer [8]. For example, a fusion of ETV6 (ETS translocation variant 6) to TRKC leads to the constitutive activation of TRKC tyrosine kinase, which promotes tumor formation and progression in human breast carcinoma [9].
Author Summary

NTRK3 is a neurotrophin receptor and appears to be a dependence receptor in certain tissues. NTRK3 has been previously shown to be an oncogene in breast cancer and possibly hepatocellular carcinoma. Through a genome-wide methylation screen, we unexpectedly found that NTRK3 is commonly methylated in colorectal cancers but not in normal colon samples, which led us to assess whether NTRK3 could be a tumor suppressor gene in the colon. We now demonstrate that NTRK3 is frequently methylated in colorectal adenomas and cancers. Induced NTRK3 expression in the absence of its ligand, NT-3, causes apoptosis and suppresses in vitro anchorage-independent colony formation and in vivo tumor growth. Reintroduction of NT-3 releases colon cancer cells from NTRK3-mediated apoptosis, which is consistent with NTRK3 being a dependence receptor in the colon. Finally, somatic mutations of NTRK3 have been observed in primary human colorectal cancer. We provide evidence that a subset of these mutations inactivate tumor suppressor activities of NTRK3. These findings suggest that NTRK3 is a conditional tumor suppressor gene in the colon that is inactivated by both genetic and epigenetic mechanisms and whose function in the pathogenesis of colorectal cancer depends on the expression status of its ligand, NT-3.

However, rather than being classic tyrosine kinase receptors, recent data suggests that NTRK1 and NTRK3 may be dependence receptors [6,10,11]. Dependence receptors are characterized by their ability to induce opposing biological effects depending on the availability of their ligands. In the presence of the receptor’s ligand, a positive cellular differentiation or survival signal is transduced, whereas lack of the ligand results in cleavage of a death-domain peptide and induction of apoptosis [12]. Indirect support for the role of NTRK3 as a dependence receptor and conditional tumor suppressor gene is provided by the observation that NTRK3 is a favorable-prognostic factor in a variety of cancers, such as melanoma [13] and medulloblastomas [14]. These findings suggested that NTRK3 might likewise serve as a conditional tumor suppressor gene in colorectal cancer.

With regards to the possibility that NTRK3 could act as a tumor suppressor gene in the colon rather than as an oncogene, somatic inactivating mutations of NTRK3 have been identified in CRC [15], as well as in other cancers including breast, lung, and pancreatic [16]. These mutations are missense mutations that are predicted to inhibit the function of NTRK3 [16] (See Table S1). Thus, the discovery of mutant, as well as methylated, NTRK3 in CRC suggested the possibility of NTRK3 being a CRC tumor suppressor gene. Consequently, we carried out a series of studies to determine the effect of aberrant DNA methylation on the expression of NTRK3 and to determine if NTRK3 had oncogene or tumor suppressor activities in colorectal cancer cell lines.

Results

Aberrant methylation of NTRK3 is common in colorectal adenomas and cancers

DNA from CRCs and normal colon mucosa samples was subjected to analysis using Infinium HumanMethylation450 BeadChip arrays (Illumina). After filtering the data as described previously [3], we identified a number of genes that were aberrantly methylated in the CRCs. One of these methylated genes was NTRK3, which was methylated in all the CRCs and in none of the normal colon epithelial samples. In light of the preferential methylation of NTRK3 in CRCs and because of its role as a neurotrophin receptor, which suggested it could have a functional role in the formation of colorectal cancer, we carried out a series of studies to further assess the effect of NTRK3 methylation on CRCs.

The promoter region of NTRK3 (NM_002530) contains a dense CpG island located from nucleotides −96 to +179 relative to the transcription start site (TSS; Figure 1A). After observing methylated NTRK3 in the colorectal cancers run on the HumanMethylation450 arrays, we assessed the methylation status of NTRK3 in a second independent set of normal colon mucosa, colon adenomas, and CRCs using a quantitative methylation-specific PCR assay (qMSP; MethyLight) designed to assess the promoter region of NTRK3. We first established that a Percent of Methylated Reference (PMR) threshold of 13.7% had a specificity of ~90% for cancer vs. normal tissue. Using this PMR threshold, we detected NTRK3 promoter methylation in 67% of colorectal cancers (N = 76) (See Text S1 for methods used to determine optimal PMR. Figure S11 and Table S4). Using this same PMR threshold, NTRK3 promoter methylation was found in 60% of adenomas (N = 55) and 10% of the normal colon samples (N = 98; normal versus cancer: p<0.0001; normal versus adenoma: p<0.0001). The frequency of normal colon mucosa cases adjacent to CRC with NTRK3 promoter methylation did not differ significantly from that observed in the normal mucosa of cancer-free individuals (Table 1). We also assessed the status of NTRK3 in a panel of colon cancer cell lines (N = 9) and found that all the cell lines had methylated NTRK3 using the 13.7% PMR threshold.

In addition, we performed bisulfite sequencing of the promoter region of NTRK3 in representative cases of normal colon epithelium, adenomas, and adenocarcinomas (5 samples/group) and correlated these results with those of the NTRK3 qMSP assay. The bisulfite sequencing results correlated well with the qMSP results (Figure 1B–E).

Methylation of NTRK3 is independent of CIMP status, MSI status, KRAS mutations and BRAFV600E mutations in colorectal cancer

Colorectal cancer can be classified into molecular classes, which include the Microsatellite Unstable ( MSI), Chromosome Unstable (CIN, also known as Microsatellite Stable, MSS), and CpG Island Methylator Phenotype (CIMP) [1]. These classes of CRC appear to have unique pathogenic mechanisms that give rise to the CRCs. We assessed the association of methylated NTRK3 with these classes of CRC and with mutations that are commonly found in CRC. As shown in Table 2, methylated NTRK3 is more frequent in tumors in women, and is independent of CIMP and MSI status, as well as KRAS, BRAFV600E, TP53, PIK3C2 or APC mutations. In addition, as shown in Figure S1, methylated NTRK3 appears to be independent of other genes that are frequently methylated in CRC, such as MLH1, CDKN2A/p16 or RASSF1A.

Methylation of NTRK3 silences NTRK3 expression

As mentioned above, we noticed that all nine of the colon cancer cell lines analyzed carried methylated NTRK3. Consistent with methylation silencing NTRK3 expression, we did not detect NTRK3 mRNA expression in any of these cell lines. Next, the CRC cell lines RKO and HCT116, which carry methylated NTRK3, were treated with 5-aza-2′-deoxycytidine (5-AZA), which inhibits DNA methyltransferase1 (DNMT1), to determine if demethylation of the NTRK3 promoter would induce NTRK3 expression. Following 5-AZA treatment, NTRK3 mRNA expression was induced in both HCT116 and RKO cells (Figure 2A). We next assessed NTRK3 mRNA expression in normal colon

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mucosa samples, colorectal adenomas, and primary colon adenocarcinomas. NTRK3 mRNA expression was significantly lower in colorectal adenocarcinomas and adenomas as compared to the matched normal colon mucosa, which carried unmethylated NTRK3 (Figure 2B). Moreover, the expression of NTRK3 was significantly higher in the primary colon tumors that carry unmethylated NTRK3 compared to the tumors that carry methylated NTRK3 (Figure 2C).

We also assessed NTRK3 protein expression in normal colon mucosa and in adenomas and colorectal cancer by immunostaining. The normal colon mucosa showed heterogeneous membrane and cytoplasmic staining using an anti-NTRK3 monoclonal antibody, whereas almost no expression was detected in most adenoma and cancer cases (N = 30). Among the 20 adenoma and cancer samples, only 6 samples showed weak or moderate NTRK3 expression, while 9 out of 10 normal samples showed strong or moderate NTRK3 expression (Figure 2D). Taken together, these data provide support for the aberrant methylation of the NTRK3 promoter silencing NTRK3 expression in colon neoplasms.

Expression of NT-3 in colon cancer cell lines and primary tissues

Because NTRK3 has been shown to function as a dependence receptor in certain tissues, we also assessed the expression of NTRK3’s ligand, NT-3, in CRC cell lines and primary CRCs. NTRK3’s preferred ligand is NT-3, and NT-3 has been shown to inhibit NTRK3 mediated apoptosis and to induce NTRK3-mediated activation of signaling pathways involved in cell proliferation, apoptosis and motility [6,17]. Therefore, we assessed the expression levels of NT-3 in the panel of colon cancer cell lines previously assessed for methylated NTRK3. No NT-3 expression was detected in RKO, HCT116, FET, Vaco400 and HT-29, whereas NT-3 was expressed at a low (although relatively high level in relation to the other CRC cell lines) in SW480. NT-3 expression was present at low levels in Lovo, LS174T, and AAC1/SB10 (Figure S2A). We next assessed the expression of NT-3 in primary CRC tissues and in matched normal colon mucosa specimens. NT-3 expression was significantly lower in the CRC’s when compared to the normal colon (Figure 3A). Interestingly, we found a direct correlation between NT-3 expression and NTRK3 expression in the normal colon and in the CRC’s (r² = 0.81, Pearson’s correlation P<0.0001), suggesting that the presence of NT-3 relieved the selective pressure to silence NTRK3 (Figure 3B).

In order to determine the mechanism responsible for loss of NT-3 expression, we measured the methylation status of the promoter region of NT3 using an NT3 MSP assay and correlated these results with the NT3 mRNA expression levels. We found that the
colon cancer cell lines lacking NT3 expression have aberrantly methylated NT3, whereas those that express NT3 mRNA carry unmethylated NT3 (Figure S2C). We also found that 5-AZA treatment of two cell lines that carry methylated NT3, HCT116 and RKO, induces the expression of NT3 (Figure S2B). Therefore, we conclude that the methylation of NT3 can repress the expression of NT3.

NTRK3 is a dependence receptor in CRC

NTRK3 has been shown to be a dependence receptor in certain tumors and can trigger caspase-based apoptosis when not bound by NT-3 [6,10]. In the presence of NT-3, NTRK3 induces differentiation, guidance or survival in neurons; however, NTRK3 can alternatively induce apoptotic cell death in the absence of NT-3 in neuroblastoma cells and presumably other cell types [6]. The dependence receptor aspect of the biological effects of NTRK3 suggests it has the potential to be either an oncogene or a tumor suppressor gene, depending on the presence of NT-3. In order to assess the effect of NTRK3 on colorectal cancer, NTRK3 was transfected into the HCT116 (MSI), RKO (CIMP) and HT29 (CIMP/MSS) cell lines, which lack NTRK3 and NT3 mRNA expression (Figure S3A and B). In these cell lines, NTRK3 reconstitution increased caspase activity by 2–3 fold compared to the control vector transfected cells. Furthermore, the addition of NT-3 (100 ng/mL) suppressed apoptosis induced by NTRK3 reconstitution (Figure 4A, B and C). These results were confirmed using an independent assay that assesses apoptosis by detecting apoptosis specific DNA:histone complexes (Cell Death Detection Assay (Roche); Figure S4 A, B and C).

Somatic NTRK3 mutations occur in primary colorectal cancer and can inactivate NTRK3

Somatic mutations of NTRK3 have been identified in primary colorectal cancers [15]. In order to determine the effect of the mutant NTRK3 genes on the behavior of colorectal cancers, we constructed plasmids that express the following NTRK3 mutants: NTRK3-G608S, NTRK3-I695V and NTRK3-L760I [15]. The mutant NTRK3 constructs were then transfected into the CRC cell line RKO. Transfection of NTRK3-L760I into the RKO cells did not induce apoptosis (Figure 5B), but the wild-type NTRK3, NTRK3-G608S or NTRK3-I695V alleles did induce apoptosis. Moreover, inhibition of colony formation by NTRK3 was not induced by NTRK3-L760I, but was induced by NTRK3-G608S and NTRK3-I695V (Figure 5C). These findings demonstrate that NTRK3-L760I inactivates NTRK3 with regards to its apoptosis and colony formation ability. However, the other mutant alleles do not affect the function of NTRK3 and are presumably passenger mutations. (Of note, the mutation status of all the constructs was

| Table 1. NTRK3 promoter methylation in colorectal adenocarcinoma, adenoma and normal colon epithelium. |
|---------------------------------------------------------------|
| Description of Sample | N | Methylated cases (percentage, %) |
|-----------------------|---|-------------------------------|
| Adenocarcinoma        |   | PMR = 13.7†                  |
| Stage I or II         | 18| 12 (66.7%)                   |
| Stage III             | 25| 17 (68.0%)                   |
| Stage IV              | 14| 12 (85.7%)                   |
| Unknown stage         | 19| 9 (47.4%)                    |
| Adenoma               | 55| 33 (60.0%)                   |
| Early adenoma         | 25| 13 (52.0%)                   |
| Advanced adenoma      | 27| 19 (70.4%)                   |
| Unknown stage         | 3 | 1 (33.3%)                    |
| Normal colon epithelium† | 98 | 9 (9.18%)                  |
| CRC patients          | 46| 4 (8.70%)                    |
| Cancer-free patients  | 52| 5 (9.62%)                    |

†Methylation was scored as being present in a case if the PMR >13.7. The PMR threshold was optimized by using ROC analysis on cancer and normal samples with an area under the curve (AUC) of 0.843.

1Cancer-free patients” indicates that the patient did not have colorectal cancer.

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| Table 2. Clinical, pathological, and molecular characteristics of CRCs that carry methylated NTRK3 and unmethylated NTRK3. |
|---------------------------------------------------------------|
| NTRK3 | Methylated | Unmethylated | p |
| All | 25 | 7 | 1 |
| Age |  |  |  |
| >60 | 12 | 4 |  |
| ≤60 | 13 | 3 |  |
| Gender |  |  | 0.03181 |
| Male | 6 | 5 |  |
| Female | 19 | 2 |  |
| Stage | 1 |  |  |
| I or II | 8 | 2 |  |
| III or IV | 17 | 5 |  |
| Tumor Location | 1 |  |  |
| Colon | 22 | 6 |  |
| Rectum | 3 | 1 |  |
| CIMP |  |  | 0.1497 |
| Yes | 8 | 0 |  |
| No | 17 | 7 |  |
| MSI |  |  | 0.5896 |
| Yes | 4 | 2 |  |
| No | 21 | 5 |  |
| KRAS |  |  | 0.6833 |
| Wild-type | 15 | 5 |  |
| Mutant | 10 | 2 |  |
| BRAF |  |  | 0.2964 |
| Wild-type | 19 | 7 |  |
| Mutant | 6 | 0 |  |
| APC |  |  | 0.2964 |
| Wild-type | 22 | 5 |  |
| Mutant | 3 | 2 |  |
| TP53 |  |  | 0.6479 |
| Wild-type | 18 | 4 |  |
| Mutant | 7 | 3 |  |
| PIK3CA |  |  | 0.5523 |
| Wild-type | 21 | 7 |  |
| Mutant | 4 | 0 |  |

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confirmed by direct sequencing, see Figure S5). Also, we did not observe any change in NT-3 expression after transfection with the wild-type or mutant NTRK3 constructs. These findings suggest NTRK3 is a tumor suppressor gene in the colon that can be inactivated by both epigenetic and genetic mechanisms. The identification of both methylated NTRK3 and inactivating NTRK3 mutations in colorectal cancers provides evidence that inactivation of NTRK3 promotes tumor formation in the colon.

Loss of NTRK3 leads to perturbed MAPK signaling pathway

As shown in the experiments above, NTRK3 can act as a tumor suppressor gene in colon cancer cell lines and can induce apoptosis in CRC cell lines through the activation of caspase 3 or caspase 7. We next assessed the signaling pathways that are affected by NTRK3, which have been shown to include the MAPK/Erk, NF-

kB and PI3K/Akt pathways, to determine if they may be mediating NTRK3-induced apoptosis in the colon cancer cell lines [18]. We initially assessed the activation status of the MAPK/Erk pathway in the HCT116 and RKO cell lines. HCT116 and RKO cells reconstituted with NTRK3 show increased activation of the MAPK/Erk pathway as determined by increased phospho-Erk1/2 expression. This increase in p-Erk1/2 was accompanied by increased caspase3/7 activity. The NTRK3-induced apoptosis was inhibited by the MAPK inhibitor U0126 (Figure 6A and B). In order to confirm that increased p-Erk1/2-induced apoptosis was specific to NTRK3, we used 16% FBS as an extracellular stimulus to induce increased p-Erk1/2. Not surprisingly, cells treated with 16% FBS showed significant increased p-Erk1/2, which was not accompanied by increased apoptosis (Figure S6). Since the addition of NT-3 inhibited apoptosis induced by NTRK3 expression, we assessed whether the introduction of NT-3 affected the activation status of the MAPK/Erk pathway. Interestingly, the addition of NT-3 decreased NTRK3 protein expression and decreased p-Erk1/2 levels (Figure S7). These findings suggest that at least part of NTRK3's...
Figure 3. NT3 expression and the relationship of NTRK3 and NT3 mRNA expression in primary colon tissues. A. NT3 mRNA expression is significantly lower in both the adenoma and adenocarcinoma samples compared to the matched normal colon epithelium (both p < 0.01) (Please see methods for units on Y axis). B. There is a direct correlation between NT3 and NTRK3 expression in normal colon and adenocarcinomas (r² = 0.81, Pearson’s correlation P < 0.0001). The units on the Y-axis are relative expression units and each bar on the X-axis represents one sample. The bar graphs are aligned so that the samples correspond in the upper and lower graphs. doi:10.1371/journal.pgen.1003552.g003

Suppression of NTRK3 induces transformed behavior in colon epithelial cells

Since NTRK3 is frequently methylated in colorectal adenomas, we carried out a series of studies to determine if loss of NTRK3 could induce transformed behavior in normal colon epithelial cells. We knocked down the expression of Ntrk3 in an immortalized murine colon epithelial cell line (YAMC) and then assessed the cells for transformed behavior using a soft agar colony formation assay. As shown in Figure 7, the knockdown of Ntrk3 was ~80% as measured by RT-PCR, and this level of knockdown promoted anchorage independent growth in the YAMC cells. Of note, the parental YAMC cells grow slowly in soft agar. These findings suggest that loss of NTRK3 could be an early event in CRC formation.

NTRK3 suppresses the tumorigenic behavior of colon cancer cell lines both in vitro and in vivo

We also performed studies on anchorage independent growth and tumor xenograft formation in established CRC cell lines to assess the putative tumor suppressor role of NTRK3 in CRC. First, we assessed the effect of NTRK3 expression on soft agar colony formation in HCT116 (MSI), RKO (CIMP) and HT29 (CIMP/ MSS) cells. Transfection of full-length NTRK3 induced a nearly 5- to 10-fold reduction in colony number of HCT116 (Figure 8A), RKO and HT29 cells (Figure S9A and B). We also assessed the tumor-suppressor activity of NTRK3 in xenografts in immunodeficient nu/nu nude mice. NTRK3 reconstitution significantly suppressed tumor growth compared to xenografts containing a control vector (Figure 8B). Twenty-one days after subcutaneous injection of the cells, the mice were sacrificed and the tumors were excised and measured. We found that both the size and weight of the NTRK3-expressing tumors were significantly reduced compared to the control xenografts (Figure 8C and Figure S10). NTRK3 expression in the xenografts from the cells transfected with NTRK3 was confirmed by IHC (Figure 8D). Taken together, these results provide support for a tumor suppressor role for NTRK3 in CRC.

Discussion

The aberrant methylation of CpG islands in the promoter regions of genes is a common event in many cancers [19]. The average colon cancer genome contains 1,000–3,000 abnormally methylated genes [20]. In many cases, the aberrant methylation of these genes can silence the expression of tumor suppressor genes and consequently promote tumor formation. However, it is also apparent that the hypermethylation of many genes in cancer has no effect on the expression of the methylated gene and does not influence tumor formation. This later class of methylated genes is felt to represent passenger events in tumorigenesis [20]. Through a genome-wide screen for methylated genes in colon cancers, we identified methylated NTRK3 in colon adenomas and adenocarcinomas. Methylated NTRK3 was found in 67% of colorectal adenocarcinomas and 60% of adenomas. With regards to the functional significance of this epigenetic alteration, we found that the aberrant methylation of NTRK3 suppressed NTRK3 expression, which suggested NTRK3 might act as a tumor suppressor gene in colon cancer. Our findings are in contrast to other studies in breast cancer that have demonstrated that NTRK3 is oncogenic [4]. These opposing results appear to be a consequence of NTRK3 being a dependence receptor, which means that it can induce proliferation when it binds its ligand, NT-3, but induces apoptosis when NT-3 is not available [6]. Because NT-3 is expressed in the colon epithelium but not in colon neoplasms, our
findings suggest that silencing of NTRK3 releases colon cancer cells from NTRK3-mediated apoptosis. These findings suggest that NTRK3 might function as a novel conditional tumor suppressor gene in CRC.

Although somatic mutations of NTRK3 that are predicted to inactivate function have been observed in CRC, NTRK3’s role as a tumor suppressor gene in CRC has not been clearly demonstrated to date [15]. In the present study, we have provided evidence that NTRK3 can have conditional tumor suppressor activities in CRC. A similar role for NTRK3 in neuroblastomas has recently been shown [6]. Reconstitution of NTRK3 in the absence of NT-3, the ligand for NTRK3, induced caspase-related apoptosis and cell death in the colon cancer cell lines RKO, HT29 and HCT116. We found that the effects on apoptosis could be suppressed by the treatment of the NTRK3 expressing cell lines with NT-3. Perhaps most importantly, NTRK3 inhibited colony formation in soft agar colony formation assays and suppressed the growth of tumor xenografts, which are hallmark in vitro effects of tumor suppressor genes. In addition, we have shown that the naturally occurring NTRK3-L760I mutation impairs NTRK3’s ability to induce apoptosis and suppress anchorage independent growth. These findings suggest that NTRK3 is a CRC tumor suppressor gene that is inactivated by both genetic and epigenetic mechanisms.

The demonstration of NTRK3 as a potential conditional tumor suppressor gene in the colon suggests NTRK3 may be the latest member of a class of dependence receptors that suppress colon cancer formation. Other conditional tumor suppressor genes identified in CRC and other cancers, include DCC, UNC5C, p75NTR and MET [12,21]. The dependence receptor model purports that some receptors induce different biological effects on cells depending on whether they are in a ligand-bound or ligand-free state. These receptors can induce caspase-mediated apoptosis in the absence of ligand, but induce proliferation when bound by their ligands. Therefore, one of the critical aspects of this study is the assessment of the expression of the NTRK3 ligand NT-3 in the colon. NTRK3’s preferred ligand, NT-3, was found to be substantially suppressed in both colorectal adenomas and adenocarcinomas, presumably secondary to hypermethylation of the NT3 promoter region. It is plausible that the loss of NT-3 expression precedes the loss of NTRK3, which would create a clonal survival advantage for those CRC cells that silence NTRK3. Our studies suggest that inactivation of NTRK3 occurs early in the polyp→cancer sequence and that it contributes to the transformation of colon epithelial cells.

With regards to the results of our studies, it is also important to consider the effects of loss of NT-3 and NTRK3 in the context of the entire neurotrophin receptor and ligand families because cross-talk between the ligand and receptor family members can occur. It has been shown that a precursor of NT-3, proNT-3, can activate p75NTR and that NT-3 can activate NTRK1 or NTRK2, although this happens with low efficiency [18]. However, despite the potential for cross-talk, we did not observe any effects on colon cancer cells that lacked NTRK3 after being treated with NT-3. Therefore, our findings suggest that NTRK3 is the primary and perhaps only receptor for NT-3 in the colon and in colon neoplasms.

When bound to NT-3, NTRK3 functions as a typical receptor tyrosine kinase. Its activation is stimulated by neurotrophin-mediated dimerization and transphosphorylation of an activation loop tyrosine [22]. The major pathways activated by the NTRKs are MAPK, PI3K and PLC-γ1, among others [18,22,23]. Activation of the NTRKs and p75NTR promote activation of NF-κB, and p75NTR can activate the JNK pathway [18,24,25]. Previous studies have demonstrated that the activation of the MAPK and PI3K pathways by NTRK3 promotes cell differentiation, which in turn affects tumor progression [4,23]. In this study, we also found that NTRK3 expression can activate the MAPK pathway. However, in this context the activation of ERK1/2 appears to be involved in the apoptotic response in colon cancer cells. There is a possibility that the MAPK activation we observed in this setting is an indirect effect of NTRK3 and a consequence of unopposed activation of p75NTR [18]. Our studies...
do not allow us to exclude this possibility, although even if such a mechanism was present, it would not change the interpretation of NTRK3 as being a colorectal cancer tumor suppressor gene.

In summary, we have identified NTRK3 as a novel conditional tumor suppressor gene in the colon that is inactivated by epigenetic and genetic mechanisms. We have provided evidence that NTRK3 can trigger apoptosis and inhibit tumor growth in the absence of its ligand NT-3 and that these effects are reversed by

Figure 5. Assessment of caspase activity and colony formation after reconstitution with mutant NTRK3 in RKO. The three mutant NTRK3 constructs contain NTRK3 mutants found in primary human CRC. A. The expression of reconstituted wild-type NTRK3 (WT), NTRK3-G608S (G608S), NTRK3-I695V (I695V), and NTRK3-L760I (L760I) was confirmed by western blotting. B. Apoptosis was assessed by normalized caspase 3 and 7 activity in the RKO cell line 48 hours after transfection with WT, G608S, I695V, and L760I NTRK3 constructs. C. Soft agar colony formation was assessed in stably-transfected RKO cells after 2 weeks. Results are plotted as the mean colony numbers from three independent experiments. L760I did not induce apoptosis or suppress colony formation, whereas WT, G608S, and I695V did. Thus, the G608S and I695V mutations appear to be passenger mutations. GUS was used as the control vector to normalize for nonspecific effects of the transfection on apoptosis. The asterisks indicate statistically significant differences (p<0.05; 2-sided Mann-Whitney rank sum test). doi:10.1371/journal.pgen.1003552.g005

Figure 6. NTRK3 expression induces phosphorylation of Erk1/2 (Thr202/Tyr204), and the inhibition of phosphorylation of Erk1/2 correlates with decreased apoptosis induced by NTRK3. HCT116 cells were transfected with either GUS or NTRK3, and then 24 hours after the transfection, were treated with the MAPK inhibitor U0126 (10 μM) for an additional 24 hours. A. NTRK3 mediated phosphorylation of Erk1/2 is inhibited by U0126. B. U0126 reverses the caspase activation induced by NTRK3 expression in HCT116 cells. DMSO was used as a vehicle control. The asterisks indicate statistically significant differences (p<0.05; 2-sided Mann-Whitney rank sum test). Caspase activity was normalized to the GUS vector transfected HCT116 cells treated with DMSO. doi:10.1371/journal.pgen.1003552.g006

In summary, we have identified NTRK3 as a novel conditional tumor suppressor gene in the colon that is inactivated by epigenetic and genetic mechanisms. We have provided evidence that NTRK3 can trigger apoptosis and inhibit tumor growth in the absence of its ligand NT-3 and that these effects are reversed by
the addition of NT-3. We also showed that suppression of NTRK3 can induce transformed behavior in immortalized colon epithelial cells. Our studies provide further insight into the complex relationship between NTRK3 and NT-3 in cancers as well as dependence receptor biology in the colon. This class of tumor suppressor genes may offer new therapeutic strategies in the colon.

**Materials and Methods**

**Cell lines, tissues and nucleic acid extraction**

All studies in this manuscript have been approved by the FHCRC IRB committee and the IACUC committee. The studies of human tissues were all done on anonymous samples. The IRB protocol covering this study is IRB 1989 and is available upon request.

Nine human colorectal cancer cell lines (SW480, Vaco400, LS174T, HT29, Vaco576, RKO, Vaco503, HCT116 and Lovo) representing the spectrum of CRC molecular subtypes [MSI, CIN (aka MSS) and CIMP] were used. The cell lines were either purchased from ATCC or were kindly provided by Sanford Markowitz (Case Western Reserve University School of Medicine and Case Medical Center, Cleveland, OH). All cell lines had their identity confirmed by DNA genotyping. Some of the cell lines were treated with the DNMT1 inhibitor (5 μM) 5-aza-2'-deoxycytidine (5-AZA; Sigma) in the experiments in this study.

Primary tissue samples used in the methylation array studies (N = 8 CRC’s) were obtained from the ColoCare CRC cohort study (Fred Hutchinson Cancer Research Center, Seattle, WA) and from healthy individuals undergoing screening colonoscopy at the University of Washington Medical Center (Seattle, WA) (N = 6). Detailed information on these samples is shown in Table S2.

Formalin-fixed, paraffin-embedded (FFPE) and fresh-frozen colon neoplasms and normal colon tissue samples were obtained from the pathology archives at Vanderbilt University Medical Center (Nashville, TN), the Department of Veterans Affairs Tennessee Valley Health Care System, Meharry Medical Center (Nashville, TN), and the University Hospital of Cleveland (Cleveland, OH) following IRB approved protocols at each institution. Colorectal cancers, colon adenomas, and adjacent normal tissue samples were also provided by the Cooperative Human Tissue Network. In total, these samples included 52 cases of histologically normal colonic mucosa from individuals without cancer or inflammatory bowel disease (IBD) and 25 samples of histological normal colonic mucosa from individuals who had undergone colon resection for CRC or colon adenomas.

DNA and RNA were extracted from these samples as previously described [26].

**Methylation array studies**

These studies were conducted using Infinium HumanMethylation450 BeadChip arrays (Illumina) with DNA from CRCs (N = 8) and normal colon epithelium samples from cancer-free individuals (N = 6). Specific details regarding the platform, sample preparation and data filtering strategies have been described in our previous studies [3].

**Sodium bisulfite conversion of genomic DNA**

Bisulfite conversion of DNA was performed as described previously [27,28].

**Bisulfite sequencing**

For sequencing, bisulfite-converted DNA was PCR amplified, and the amplicons were then subjected to direct sequencing. The primers are described in detail in Table S3. The sequencing was conducted as described previously [27].

**Quantitative methylation-specific PCR**

Quantitative methylation-specific PCR (qMSP; MethyLight) was performed using an ABI Prism 7700 detection system (Applied
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Figure 8. NTRK3 suppresses in vitro soft agar colony formation (A) and tumor xenograft growth (B–D) of colon cancer cell lines. A. The GUS and NTRK3 stably transfected HCT116 cells were grown in soft agar for 2 weeks. Results are plotted as the mean colony numbers from three independent experiments. The asterisks indicate statistically significant differences ($p<0.05$; two-sided student $t$ test). Representative fields depicting colonies of HCT116 cells grown in soft agar are shown. B. The growth of the HCT116-NTRK3 and HCT116-GUS xenograft tumors in the nu/nu mice was measured over 3 weeks. The mean tumor volume is indicated, and the asterisks indicate statistically significant differences between the mean tumor volume of the NTRK3-reconstituted and control tumors (two-sided student $t$ test). C. Tumors were removed 21 days after subcutaneous injection. The final tumor weight in NTRK3-expressing tumors was significantly less than the weight of the control tumors ($p=0.0021$, $n=10$). D. Representative tumors are shown (scale bar: 5 mm) along with confirmatory NTRK3 immunostaining.

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Biosystems). Detailed methods are provided in the Text S1 as well as in previous publications [27]. The primers and probes targeting NTRK3 are described in detail in Table S3.

Molecular characterization of colorectal neoplasms

The CpG Island Methylator Phenotype (CIMP) status and Microsatellite instability (MSI) status of a subset of the colorectal neoplasms were determined as described previously [27]. The gene mutation status of KRAS, BRAF, APC, TP53 and PIK3CA was assessed by using the qBiomarker Somatic Mutation PCR System Arrays/Human Colon Cancer (Qiagen) following the manufacturer’s protocol.

Cell culture, plasmid constructs and transfection

Human CRC cell lines (SW480, Vaco400, LS174T, HT29, Vaco576, RKO, Vaco503, HCT116, and Lovo) were grown in Dulbecco’s Modified Eagle media (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). The YAMC (Young Adult Mouse Colon) cell line was a kind gift from Dr. Robert H. Whitehead (Vanderbilt University School of Medicine, Nashville, TN) and was cultured as described previously [29]. To investigate the effects of re-expression of NTRK3, HCT116 and RKO cells were incubated for 72 hours with 5 μM 5′-AZA (Sigma). The media was replaced every 24 hours with fresh 5′-AZA. After 72 hours of treatment, the cells were washed twice with PBS and then grown in drug-free media for another 72 hours before harvesting.

The full-length NTRK3 cDNA (VHO54159, Invitrogen) was subcloned into the pDEST27 Vector (Invitrogen) to create pDEST27-NTRK3. The correct orientation of the insert was confirmed by restriction digest.

The pDEST27-based plasmids containing NTRK3-G608S, NTRK3-L695V and NTRK3-L760I were constructed based on the wild-type NTRK3 expression vector by using the GENEART site-direct mutagenesis system (Invitrogen) following the manufacturer’s protocol. Successful mutagenesis was confirmed by direct sequencing (Figure S5). The sequencing primers are described in Table S3.

pGIPz-based short hairpin RNA (shRNA) constructs specifically targeting mouse Nbk3 mRNA were purchased from Thermo Scientific/Open Biosystems Mouse shRNA Mir Libraries maintained by the Genomics Shared Resource Core at the Fred Hutchinson Research Center (FHCRC, Seattle, WA). The details regarding the target regions of each shRNA are shown in Table S3. All 3 shRNA constructs were confirmed by direct sequencing (Table S3). Lentivirus containing the shRNA constructs were generated by co-transfecting pGIPz-shRNA with the packaging plasmid pPAX2 and the envelope plasmid pMD2.G (kindly provided by Michael Davis, FHCRC, Seattle, WA) into 293T packaging cells. Virus supernatant was filtered through a 0.2-μm filter and stored at −80°C until use. The virus was filtered and then transferred to PVDF membranes. Antibodies used were purchased from Cell Signaling Technology, anti-phospho-Akt (Ser473, #4060), anti-phospho-NF-kB (Ser536, #3265), anti-
phospho-Erk1/2 (Thr202/Tyr204, #4370), anti-phospho-Smad1/Smad5 (Ser463/465/Smad5, Ser463/465/Smad5) (Ser426/420) (#9511), anti-phospho-Smad2/Smad3 (Ser465/467, #3101), anti-phospho-Smad3 (C25A9, Ser125/125, #9520), anti-Smad2/3 (#3102), anti-Tek/C (C44H5, #5376), anti-BMPR2 (#6979), and Epithelial-Mesenchymal Transition (EMT) antibodies, anti-N-Cadherin (#4061), anti-Vimentin (D21H3, #5741), anti-E-Cadherin (24E10, #3195), anti-ZO-1 (D7D12, #6193), anti-Snail (C15D3, #3879). Immune reactive proteins were then visualized by incubating the PVDF membranes with ECL plus detection reagents, followed by imaging of chemiluminescence on an imager (X-ray film-based).

Tumor xenograft studies
The care and use of the mice was conducted following protocols approved by the Fred Hutchinson Cancer Research Center IACUC. The IACUC protocol covering this work is IACUC 1624 and is available upon request. All IACUC protocols at the FHCRC require that animal suffering is eliminated unless required for the studies, in which case extensive justification is required.

Three to four week-old female athymic nu/nu mice were obtained from Harlan Laboratories. The mice were housed for one week in a pathogen-free animal facility prior to tumor cell injection. 1 x 10⁶ GUS or NTRK3 stably transfected HCT116 cells in 200 μL DMEM and Matrigel (1:1 mix) (BD Biosciences) were injected subcutaneously into the right flank of each mouse. The tumor sizes were measured using a caliper, and the tumor volume was calculated as follows: 0.5(length x width²) [6]. The mice were assessed every three days and sacrificed at three weeks after injection.

Statistical analyses
Receiver operating characteristic (ROC) curves and area under the curve (AUC) for NTRK3 methylation frequency of primary tissues were constructed on the basis of methylation levels. The Chi-squared test was used to compare the frequency of methylated NTRK3 between cancer and normal samples. The Fisher’s exact test was used to test the association between the NTRK3 methylation status and clinical/molecular characteristics of CRC patients. Student t test or analysis of variance (ANOVA) was used to analyze the RT-PCR data. The Mann-Whitney rank sum test was used to analyze the data obtained from the cell death and apoptosis assays. Statistical analysis was performed using SPSS 15.0 software. All p values are two-sided, and a p value<0.05 was considered statistically significant.

Supporting Information

Figure S1 Methylated NTRK3 occurs independently of other methylated genes that commonly occur in CRC. A heat map that shows the relative methylation levels of NTRK3, MLH1, CDKN2A/p16, and RASSF1A genes in the 8 CRC cases run on the HumanMethylation450 arrays is displayed. There is no significant correlation between methylated NTRK3 and methylation of any of these genes. The methylation levels are quantified as follows: “1” stands for 100% methylated; “0” represents 100% unmethylated DNA. The red color represents higher methylation levels (towards “1”) whereas green represents lower methylation levels (towards “0”). (TIF)

Figure S2 NT3 mRNA expression is suppressed by methylation in colorectal cancer cell lines. A. There is no NT3 expression in RKO, HCT116, FET, Vaco400 and HT-29 cells. Low-level NT3 expression is apparent in SW480, Lovo, LS174T, and AAC1/ SB10. The expression level is substantially less than that observed in the normal colon, which was on average 3.0±0.66 (Figure 3). B. NT3 mRNA expression in HCT116 and RKO cells is induced by treatment with the DNMT1 inhibitor 5-aza-2-deoxycytidine (5-AZA) when compared to treatment with the vehicle alone (“Mock”). C. NT3 methylation status of colorectal cancer cell lines as determined by methylation specific PCR (mMSP) demonstrates that cells with no NT3 mRNA expression carry methylated NT3, whereas cell lines that express NT3 have unmethylated NT3. These levels of expression are very low. (Methyl: universal methylated control DNA; Unmethyl: universal unmethylated control DNA; H2O: no template control). M = methylated, U = unmethylated. (TIF)

Figure S3 NTRK3 mRNA and protein expression induced by NTRK3-transfection of cell lines that carry methylated NTRK3. HCT116, RKO and HT29 cells carry methylated NTRK3 and have no detectable NTRK3 mRNA expression (A) or protein expression (B) when transfected with the mock conditions (Mock) or GUS control vector (GUS). NTRK3 mRNA and protein expression can be detected in all three cell lines after transfection with the vector expressing NTRK3 (NTRK3). The bands seen in the western blot are the expected sizes for NTRK3 (100 kd and 145 kd). (TIF)

Figure S4 Assessment of apoptosis after reconstitution of NTRK3 in HCT116 (A), RKO (B) and HT29 (C). NTRK3 induces cell death in HCT116 (MSI), RKO (CIMP) and HT29 (MSS) cells, and NT-3 (100 ng/ml) inhibits this effect in all three cell lines. DMSO treatment was used as a control for nonspecific effects. HCT116, RKO and HT29 carry methylated NTRK3. The asterisks indicate statistically significant differences, p<0.05 as determined by a 2-sided Mann-Whitney rank sum test. All values were normalized to the GUS transfected, DMSO treated cells. (TIF)

Figure S5 Direct sequencing results of mutant NTRK3 constructs (based on somatic mutations G608S, I695V, and L7601 found in human colon cancer samples). The arrow indicates the mutated basepair and confirms the success of the site-directed mutagenesis used to generate the mutant. A. Sequencing results for NTRK3-G608S mutant. B. Sequencing results for NTRK3-I695V mutant. C. Sequencing results for NTRK3-L7601 mutant. (TIF)

Figure S6 Inhibition of MAPK activity after NTRK3 reconstitution in RKO cells decreases NTRK3 induced apoptosis. Caspase activity in the CRC cell line RKO was quantified 48 hours after transfection with NTRK3 using the Caspase-Glo 3/7 assay. The selective MAPK/ERK pathway inhibitor, U0126 (10 μM), significantly decreases caspase activity in the RKO cell line transfected with NTRK3. 16% FBS (30 minute treatment) was used to stimulate the cells before protein harvest as a positive control for ERK activation. All experiments were performed in triplicate, and the results shown are fold changes compared to the empty vector control. The asterisks indicate statistically significant differences, p<0.05 as determined by a 2-sided Mann-Whitney rank sum test. (TIF)

Figure S7 NT-3 suppresses the expression of reconstituted NTRK3 and suppresses ERK1/2 phosphorylation in the colon cancer cell line HCT116. HCT116 cells were transfected with either GUS or NTRK3 followed by treatment with NT-3 (100 ng/
mL for 24 hours) 24 hours after transfection. The phosphorylation of Erk1/2 induced by NTRK3 expression is effectively blocked by NT-3, presumably in part through suppression of NTRK3 protein expression. (TIF)

**Figure S8** NTRK3 transfection does not alter the TGF-β, BMP signaling pathways or contribute to epithelial-mesenchymal transition (EMT) in the colorectal cancer cell line RKO treated with or without NT-3. RKO cells were transfected with either GUS or NTRK3 as indicated. Protein lysates were collected after NT-3 (100 ng/ml) treatment for 24 hours. TGF-β (represented by p-Smad2/3) and BMP (represented by p-Smad1/5/8 and BMPR2) signaling pathways were not affected by reconstitution of NTRK3. None of the EMT markers (N-Cadherin, Vimentin, E-Cadherin, ZO-1 and Snail) was significantly altered by transfection with NTRK3 regardless of NT-3 treatment. Actin was used as a loading control. (TIF)

**Figure S9** NTRK3 suppresses in vitro soft agar colony formation in RKO (A) and HT29 (B) cells. The GUS and NTRK3 stably transfected RKO (A) and HT29 (B) cells were grown in soft agar for 2 weeks. Results are plotted as the mean colony numbers for three independent experiments. The asterisks indicate statistically significant differences. (*p*<0.05; two-sided student *t* test). (TIF)

**Figure S10** Representative tumor xenografts dissected from nu/nu mice injected with HCT116 colon cancer cells transfected with NTRK3 or the control vector (GUS). The NTRK3 transfected tumors are smaller and appear less vascular when compared to the control tumor xenografts. (TIF)

**Figure S11** ROC analysis to determine the optimal percentage of methylated reference (PMR) for methylated NTRK3 that detects cancer specific levels of NTRK3 methylation. An ROC curve was constructed by plotting sensitivity vs. 1-specificity comparing adenocarcinomas (*n* = 76) to normal colon samples (*n* = 98). Area under the curve (AUC) for the sample set was 0.843 (95% CI0.781–0.904). (TIF)

| Table S1 | Predicted effect of naturally occurring somatic mutations of NTRK3 in colorectal cancer. (DOCX) |
| Table S2 | Clinical characteristics of the samples analyzed with the HumanMethylation450 array. (DOCX) |
| Table S3 | Primer and Probe Sequences for NTRK3 reagents. (DOCX) |
| Table S4 | Results of ROC analyses of the methylation levels in colon adenocarcinomas detected by MethylLight. (DOCX) |

**Text S1** Supplemental Methods. Description of methods used for studies whose data resulted in supplemental data and additional detailed description of methods used for studies in main text of manuscript. (DOCX)

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**Author Contributions**

Conceived and designed the experiments: WMG YL AMK SK JW SDM. Performed the experiments: YL SK JDL AMK. Analyzed the data: YL SK JDL AMK PW WMG SDM. Contributed reagents/materials/analysis tools: SDM JDL WMG YL SMM. Wrote the paper: YL WMG JW SMM AMK SDM.

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