Antisense transcription from lentiviral gene targeting linked to an integrated stress response in colorectal cancer cells

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INTRODUCTION

Remarkable advances in gene therapy research have resulted in the successful development of new therapies for clinical use. Here, we explored a gene targeting approach to deplete ephrinB2 from colorectal cancer cells using an inducible lentiviral vector. EphrinB2, a transmembrane ephrin ligand, promotes colorectal cell growth and viability and predicts poor patient survival when expressed at high levels in colorectal cancer tissues. We discovered that lentiviral vector integration and expression in the host DNA frequently drive divergent host gene transcription, generating antisense reads coupled with splicing events and generation of chimeric vector/host transcripts. Antisense transcription of host DNA was linked to development of an integrated stress response and cell death. Despite recent successes, off-target effects remain a concern in genetic medicine. Our results provide evidence that divergent gene transcription is a previously unrecognized off-target effect of lentiviral vector integration with built-in properties for regulation of gene expression.

RESULTS

Conditional EFN2 silencing in colorectal cancer cells

We engineered three short hairpin RNAs (shRNAs) for the conditional depletion of EFN2 (sh-7, sh-596, and sh-647) to target unique sequences of EFN2 mRNA from exon 2, exon 4, and the 3' UTR. The target sequences were the same we had previously used successfully for the constitutive shRNA silencing of EFN2.14 The new conditional shRNAs were designed as Pol II-driven shRNA's in the pTRIPZ

Colorectal cancer is a heterogeneous disease of intestinal stem cells and the second leading cause of cancer-related death in the United States.9,10 On the heels of recent successes in genetic medicine and the prospect of advances to overcome current challenges of genetic cancer targeting in vivo,12 we explored a lentiviral-based gene therapy approach for the reversible depletion of ephrinB2 in colorectal cancer cells. The current study unveils the previously unrecognized occurrence of antisense gene transcription driven by lentiviral integration and expression in the host genome. Since antisense transcripts have built-in regulatory properties with important implications for gene regulation,11–13 we speculate that the current discovery has general implications for current gene therapy approaches, cell infection by DNA-integrating viruses, and investigation of non-coding DNA.

Recently, we and others have found that ephrinB2 and its Eph receptors control colorectal cancer cell growth and survival.5,15 EFN2 (the gene coding for ephrinB2) expression is significantly higher in colorectal cancer than in normal colon, other normal tissues, and many other cancer types, and the probability of survival is significantly greater in colorectal cancer patients with low as opposed to high ephrinB2 in their cancer.5 Ephrin ligands and Eph tyrosine kinase receptors comprise a family of transmembrane proteins that are pivotal regulators of cell function through cell-to-cell communication.5,8

Colorectal cancer targeting linked to an integrated stress response in colorectal cancer cells
self-inactivating lentiviral vector containing a tetracycline-inducible cytomegalovirus (CMV) promoter, a puromycin resistance gene, and a red fluorescent protein (RFP) marker (Figure 1A). After infection of HT29 colorectal cancer cells, puromycin selection (1 μg/mL, 1 week), and doxycycline (Dox) induction (1 μg/mL, 48–96 h; virtually all cells were RFP+), EFNB2 mRNA and protein levels were variably reduced by all shRNAs compared with a non-targeting inducible control and the empty vector (Figures S1A and S1B). HT29 cell proliferation was also variably reduced by all these shRNAs (Figure S1C).

To select cells with the highest degree of ephrinB2 depletion, we cloned HT29 cells transduced with each of the three shRNAs; 169 clones were screened. Cell death was clearly observed in 2/74 sh-7 clones, 1/38 sh-596 clones, and 1/57 sh-647 clones after shRNA activation with Dox (24–72 h). Clone 596-7 transduced with sh-596 targeting the 3′ UTR was distinctive in displaying a virtually complete and selective depletion of ephrinB2 protein after Dox (Figure 1B), prominent cell death (Figure 1C), presence of the cell death marker protein cleaved caspase-3 (Figure 1D), markedly reduced proliferation (Figure 1E), and inability to reach confluency (Figure 1F).

Independent experiments reproducibly confirmed the depletion of ephrinB2 protein and the markedly reduced proliferation in clone 596-7 when the shRNA was expressed (Figures S1D and S1E). Also,
clone 596-7 did not give rise to tumors in immunodeficient NOD-SCID mice fed with Dox-containing chow (beginning 7 days after cell inoculation), but the same cells grew into tumors in control mice (no Dox-containing chow) (Figure 1G). Thus, clone 596-7 was selected for further analysis of the lethal effects of ephrinB2 depletion in colorectal carcinoma cells.

Surprisingly, Dox activation did not reduce EFN B2 mRNA levels in clone 596-7, despite full complementarity of the antisense RNA to the EFN B2 mRNA target sequence (3’ UTR GGACACCTAA T). Kinetic experiments showed that EFN B2 mRNA levels increase beginning 4 h after Dox, peak at 24 h, and nearly normalize by 72 h (Figure 1H). In five independent experiments, EFN B2 mRNA levels increased by 3.2- to 10.1-fold over control after Dox induction, and this increase was detected by primers for EFN B2 products spanning exons 1/2 and 3’ UTR (Figure S1F).

We examined whether the unexpected increase in EFN B2 mRNA levels was attributable to defective mRNA degradation in clone 596-7. An intrinsic deficiency of the shRNA degradation pathway of Dicer, RNA-induced silencing complex (RISC), Argonaute (AGO), and DROSHA was unlikely to be responsible for the failure of clone 596-7 to degrade EFN B2 mRNA, since expression in this clone of three shRNAs (#17, #24, and #36) targeting NNM T (coding for nicotinamide N-methyltransferase) efficiently reduced the specific target mRNA (Figure 1I), and shRNA #17 (sh-17) reduced NNM T protein levels (Figure S1G).

Mechanisms of ephrinB2 protein depletion and cell death in the presence of EFN B2 shRNA

Since activation of the shRNA did not reduce EFN B2 mRNA levels, the ephrinB2 protein reduction induced by activation of the shRNA could not be attributed to reduced transcription. To detect potential translational repression, we performed polysome profiling (Figure 2A). The results showed a reduction of polysome abundance in the Dox-treated 596-7 clone compared with the control, coupled with enrichment of monosome and small and large ribosomal subunits, indicative of reduced mRNA translation. We also examined the activation status of the translation initiation factor 2A (eIF2α), an essential regulator of mRNA translation and protein synthesis. Once phosphorylated at S51, eIF2α triggers a general reduction of mRNA translation. We found that eIF2α becomes phosphorylated at S51 in clone 596-7 after shRNA activation for 2–4 h (Figure 2B).

Together, the results of polysome profiling and the time-dependent phosphorylation of eIF2α indicated that RNA translation was compromised in 596-7 cells after the shRNA was activated.

Phosphorylation (S51) of eIF2α is the core initiator of the integrated stress response (ISR), a complex regulatory network that helps cells adapt to stress or promotes cell death when stress surpasses the cell’s adaptive responses.15–17 Typically, the ISR is associated with a broad reduction in mRNA translation and activation of selective genes. These observations raised the possibility that the ISR was induced by Dox activation of the shRNA in clone 596-7, causing cell death. To evaluate the occurrence of the ISR, we compared the transcriptome of clone 596-7 incubated with or without Dox. Replicate samples of clone 596-7 incubated for 24 h with (n = 4) or without (n = 4) Dox were used as a source of RNA samples (n = 8). Principal-component analysis of annotated RNAs (Figure S2A) and volcano plots visualized substantial and significant change in RNA expression after Dox treatment (Figure 2C).

IFN B1 coding for interferon β1 (IFN-β1), IFN L1 coding for interferon λ1 (IFN-λ1), and the interferon-induced RSAD2, MX2, and TRIM22 genes were among the most significantly (p < 0.001; average counts >10) induced genes in clone 596-7 treated with Dox compared with untreated clone 596-7 (Figures 2C and S2B). The mRNAs encoding the transcriptional regulators ATF3,18 CHOP (C/EBP-homologous protein encoded by DDIT319), and ATF4,20 which play critical roles in transcriptional reprogramming of the ISR,17,21 were also significantly (p < 0.005) increased in clone 596-7 treated with Dox compared with the untreated clone 596-7 (Figure 2D). Expression of GADD34 (encoded by PP1CA), the regulatory subunit of the phosphatase PP1, which is induced by p-eIF2α and de-phosphorylates eIF2α, providing feedback regulation of the ISR,22 was significantly (p < 0.005) reduced in the Dox-treated clone 596-7 (Figure 2D).

We applied Ingenuity Pathway Analysis (IPA) to gain further insight into changes in gene expression induced by Dox in clone 596-7 (see Materials and methods for details). Consistent with the ISR, transcripts linked to signaling pathways for cell death and survival (Figure 2E); cell-cycle control/DNA biosynthesis (Figure S2C); interferon signaling (Figure S2D); metabolic pathways for glycolysis, gluconeogenesis, and fatty acid biosynthesis (Figure S2E); and cytokine-induced
inflammation (Figure S2F) were significantly (p < 0.005) altered in Dox-treated clone 596-7 compared with the untreated control. Thus, induction of the EFNB2 shRNA expression in clone 596-7 induces the ISR, broadly altering the transcriptome of clone 596-7.

eIF2α and the ISR are activated through the engagement of the sensor kinases heme-regulated inhibitor (HRI; gene EIF2AK1), double-stranded RNA-dependent protein kinase (PKR; gene EIF2AK2), PKR-like ER kinase (PERK; gene EIF2AK3), and general amino acid control nondepressible 2 (GCN2; gene EIF2AK3), which contain distinct regulatory domains enabling them to respond to different stress stimuli. We found that PKR, activated by double-stranded RNA-dependent protein kinase (PKR; gene EIF2AK3), which originate at the shRNA vector locus. Several of the host genes where the vector integrated have the potential to globally regulate mRNA transcription and stability, particularly ZRANB2,28 PURB,29 EP400,30 and YTHDC2.31 However, expression of these genes changed by less than 2-fold after vector activation (Table S1). Protein levels of YTHDC2 and PURB changed modestly (Figure S2G). Therefore, a direct contribution of these genes to phenotypic changes in clone 596-7 was not supported by these observations.

Analysis of the host transcriptional landscape revealed the unexpected presence of antisense RNA gene reads (in antisense orientation to the host gene transcript, coming from the opposite DNA strand of host gene sense transcription), which originated at the shRNA vector site of integration in the host genome (Figures 3A, 3B, 4A, and 4B). Whereas the sensor kinases GCN225 and PERK26 do not (Figure 2F), the engagement of the sensor kinases GCN225 and PERK26 do not (Figure 2F).

Table 1. Characterization of shRNA integration in host DNA

| Chromosome number | Vector integration site on chromosome | Gene annotation of vector integration site | NCBI reference α | Gene start β | Gene end | Location γ |
|-------------------|---------------------------------------|-------------------------------------------|-----------------|-------------|---------|----------|
| 1                 | 71,076,570                            | ZRANB2                                    | NM_203350       | 71,063,291  | 71,081,289 | intron   |
| 7                 | 44,881,135                            | PURB                                      | NM_033224       | 44,876,299  | 44,885,330 | exon     |
| 7                 | 15,681,655                            | MEOX2; LOC105375166                     | NM_005924       | 15,611,212  | 15,686,683 | intron   |
| 5                 | 73,643,317                            | ARHGEF                                    | NM_001177693    | 73,626,158  | 73,941,993 | intron   |
| 1                 | 189,437,807                           | FAM5C                                     | NM_190051       | 190,066,796 | 190,446,759 | 660 kb    |
| 2                 | 11,332,375                            | ROCK2                                     | NM_004850       | 11,179,759  | 11,348,330 | intron   |
| 12                | 132,023,419                           | EP400                                     | NM_015409       | 131,949,942 | 132,080,460 | intron   |
| 5                 | 113,567,928                           | YTHDC2                                    | NM_023828       | 113,513,694 | 113,595,285 | intron   |
| 17                | 38,208,780                            | LOC440434                                 | NR_036750       | 38,195,703  | 38,257,192 | intron   |
| 4                 | 68,935,607                            | UGT2A3                                    | NM_024743       | 68,928,463  | 68,951,804 | intron   |

αChromosome in which the shRNA vector has integrated.
βGene coordinates based on human genome 38 (Human hg38 chrX:15560138-15602945 UCSC Genome Browser v422).
γGene annotation of vector integration site in the host genome. Based on the identification of host/vector DNA junctions, we identified 15 integration sites in 11 chromosomes: 12/15 located in introns, 1/15 located in an exon, and 2/15 mapping at distant sites (>65,000 bp) from the neighboring genes and lacking annotation (Table 1). Each of 10 subclones of the 596-7 clone displayed the same 15 integration sites, confirming that 596-7 cells are a clonal population.

Vector-driven antisense transcription in clone 596-7

We examined if vector integration and expression could explain off-target activation of PKR and ISR in clone 596-7 treated with Dox. First, we confirmed by long-range PCR, sequencing, alignment to the vector, and de novo assembly that the full-length provirus (5' to 3' LTR) and the EFNB2-targeting sequences were present in the host DNA (supplemental information). We then mapped vector integration in the host genome. Based on the identification of host/vector DNA junctions, we identified 15 integration sites in 11 chromosomes: 12/15 located in introns, 1/15 located in an exon, and 2/15 mapping at distant sites (>65,000 bp) from the neighboring genes and lacking annotation (Table 1). Each of 10 subclones of the 596-7 clone displayed the same 15 integration sites, confirming that 596-7 cells are a clonal population.

There was no vector integration in the EFNB2 locus. Several of the host genes where the vector integrated have the potential to globally regulate mRNA transcription and stability, particularly ZRANB2,28 PURB,29 EP400,30 and YTHDC2.31 However, expression of these genes changed by less than 2-fold after vector activation (Table S1). Protein levels of YTHDC2 and PURB changed modestly (Figure S2G). Therefore, a direct contribution of these genes to phenotypic changes in clone 596-7 was not supported by these observations.
Figure 3. Antisense transcription from the YTHDC2 and PURB genes and chimeric vector/host RNA reads

(A and B) Vector integration site in the host DNA and the junction between host RNA and vector RNA are marked by the blue vertical lines for the YTHDC2 (A) and PURB (B) genes. The red arrow below the schematic gene structure indicates the sense (5' to 3') direction of gene transcription. Sense (red) and antisense (green) reads by stranded RNA-seq were derived from clone 596-7 treated or not treated with Dox. Each read is representative of four replicate samples. The read scale is indicated on the read panels.

(legend continued on next page)
Since the antisense reads originated in all eight cases from the sites of shRNA integration and increased in number after the shRNA was induced by Dox, we focused on the vector (Figure S3E). Analysis of the data based on mapping to a hybrid reference (inclusive of the shRNA plus the Hg38 genome) revealed the presence of chimeric reads, i.e., reads containing vector-derived and host-derived RNA sequences. All chimeric reads at each of the eight integration sites contained vector reads transcribed in the 5’ to 3’ orientation (5’ LTR to 3’ LTR) and host gene reads in the antisense orientation (Figures 3A, 3B, 4A, 4B, and S3A–S3D). This suggested that the vector in the 5’ to 3’ orientation drives aberrant transcription of the host gene predominantly when activated by Dox. Focusing on the vector integration junctions, we captured vector-host chimeric RNA splicing events from cryptic splice donors within the vector to the host splice acceptor (Figures 3A, 3B, 4A, 4B, and S3A–S3D). In most cases, the chimeric reads at each DNA integration site showed the presence of two to four splice junction sites. Most splicing events followed the standard GT-AG mRNA processing rule for major class introns, whereas others were previously identified atypical splice sites (Figures 3A, 3B, 4A, 4B, and S3A–S3D). Since the 5’ LTR and 3’ LTR vector regions function as transcriptional promoters, and the RFP, UBC, and woodchuck hepatitis posttranscriptional regulatory element (WPRE) sequences are 3’ of the CMV promoter (Figure S3E), these results indicate that the vector drives antisense host gene transcription and splicing, generating chimeric vector/host transcripts.

When all sense and antisense chimeric reads from 596-7 cells with or without Dox were combined, we confirmed that antisense chimeric reads are significantly more abundant than sense chimeric reads and that antisense reads are more abundant in Dox-treated cells compared with untreated cells. In addition, we observed that the antisense chimeric reads contain predominantly the same small set of vector reads mapped to a few vector loci (Figure 4C).

These results provide evidence that vector integration in clone 596-7 drives antisense transcription at most integration sites after the shRNA is induced by Dox.

Vector-induced antisense transcription in other colorectal cancer cell clones
To evaluate whether divergent transcription was unique to clone 596-7, we examined 9 additional clones among 38 that we derived from the parental HT29 cells infected in bulk with the sh-596 vector. The 9 clones (clones 1–4, 6, and 8–11) differed from clone 596-7 at the mRNA and protein levels of ephrinB2 targeted by shRNA-596, in the presence of the death cell marker cleaved caspase-3, and in the expression levels of IFNBI, IFNLI, and the shRNA (Figures 5A and 5B). Mapping the shRNA integration in the host DNA identified 13 to 30 integration sites in each of the clones (Figure 5C). Few integration sites were shared among the clones (Figure 5D).

Clones 596-1, 596-6, and 596-11, along with independent samples of clone 596-7, were selected for stranded RNA-seq based on their differences from one another and the originally evaluated clone 596-7. We found that all three clones display chimeric antisense reads originating from the shRNA integration sites in the host DNA, predominantly after Dox treatment (Figures 6A–6C: 6A, 596-6; 6B, 596-1; and 6C, 596-11; and Figures S4A, S4B, and S5). Each chimeric read had evidence of cryptic splicing from the shRNA splice donor to the host splice acceptor (Figures 6A–6C, S4A, S4B, and S5).

Overall, antisense reads were detected in 7/30 shRNA insertion sites of clone 596-1, in 6/17 insertion sites of clone 596-6, and in 6/18 insertion sites of clone 596-11. Clone 596-11 differed from the other clones, 596-1, 596-6, and 596-7, in showing a similar number of sense and antisense chimeric reads that were more numerous in Dox-treated cells compared with the untreated cells (Figures 7A–7D). In addition, the normalized antisense read number was lower in clones 596-1, 596-6, and 596-11 compared with clone 596-7 (results from repeat RNA-seq with independent sample) (Figure 7E). Overall, these results indicate that antisense transcription is a common event among HT29 cell clones infected with the shRNA.

To gain additional insight into the roles of vector integration, shRNA expression and the presence of antisense reads, we analyzed data from clones 596-1, 596-6, 596-7, and 596-11, which possess variable numbers of integrated vector copies, variable levels of shRNA expression, and variable numbers of antisense reads. Analysis of correlation using the non-parametric Spearman’s test showed no direct correlation between vector integrated copy number and relative number of antisense reads (Figure 7F) but a moderately strong correlation between relative shRNA expression level and relative antisense read number (r = 0.4; Figure 7G). Overall, these results show that antisense transcription is a common event among HT29 cell clones infected with the shRNA and suggest that the abundance of antisense reads directly correlates with expression levels of the integrated vector, rather than the number of integrated copies of the vector.

DISCUSSION
Here, we report a previously unrecognized outcome of lentiviral DNA integration that holds important implications for lentivirus-based
gene targeting and more broadly for understanding regulation of the human genome. We found that some lentiviral integration events in the host human DNA drive divergent host gene transcription generating antisense reads. This divergent transcription is induced by the activated shRNA readthrough into the host DNA, generating chimeric vector/host transcripts. Since divergent transcripts have built-in properties for regulation of sense gene transcripts, the current results uncover a previously unrecognized consequence of lentiviral gene integration in mammalian cells with potentially important functional implications. In the current study, the cell clone with the highest levels of antisense transcription among those investigated was unique in revealing a death phenotype emerging from an ISR, consistent with the idea that vector-induced antisense reads activate the sensor kinase PRK and ISR, perhaps through formation of antisense/sense RNA duplexes. Consistent with this possibility, dsRNA generated by synthesized sense and antisense SNORD113 RNA activated the sensor PKR kinase.13

In eukaryotes, gene transcription from each DNA strand initiates at non-overlapping sites, each marked by a separate transcription start site associated with a distinct RNA polymerase binding event. Promoter regions in prokaryotes and eukaryotes are bidirectional, but despite this potential for bidirectional transcription, promoters are biased toward transcription of sense reads while suppressing antisense transcription through various mechanisms. When generated, antisense non-coding RNAs may simply represent noise from open chromatin and be rapidly degraded. However, several functions have been suggested, including an evolutionary role by promoting de novo gene formation and other functions.

Integration into the host DNA is a key step in the life cycle of retroviruses and is essential for lentiviral vector function. A drawback to retroviral vectors is that integration can subvert host gene transcription in a variety of ways. As originally shown with ALV, a proportion of viral transcripts fail to be correctly polyadenylated at the correct LTR site, resulting in readthrough transcripts of adjacent genes and chimeric vector/host RNA reads. This divergent transcription is induced by the shRNA vector, a gene therapy vector derived from murine leukemia virus (MLV) conferred clonal growth advantage to T lymphocytes, resulting in cases of human T cell leukemia.

Antisense transcription originating from the integrated pro-virus were uncovered in MLV-induced lymphomas and in T cells naturally infected with HTLV, but not in HIV-infected T cells, despite the presence of considerable aberrant host gene transcription. It is possible that antisense transcription in HIV-infected T cells leads to cell death, preventing detection. To our knowledge, antisense transcription has not been previously reported with lentiviral vectors. In part, this may be attributable to the more recent introduction of RNA-seq platforms that track RNA strand derivation; to dilutional effects from using polyclonal, rather than clonal, cell populations; and to the use of an inducible rather than a constitutive system where cells with antisense reads would be potentially eliminated and not detected. However, the high frequency of divergent transcription we detected and diverse functions attributed to antisense non-coding RNAs suggest a need for analysis of this off-target effect from integrating vectors.

In conclusion, we identify a previously unappreciated feature of lentiviral vector integration, namely, the occurrence of antisense transcription resulting in the generation of antisense reads, shedding new light on the off-target landscape surrounding lentiviral gene integration.

MATERIALS AND METHODS

Cells and culture
The HT29 human colorectal carcinoma cell line (ATCC; HTB-38) was grown in McCoy's 5A medium (Corning; 10-050-CV), with 10% fetal bovine serum (FBS) (Sigma-Aldrich; F2442) and 1% penicillin-streptomycin (Gibco; 15140-122). A cell clone from HT29 transduced in bulk with EFNB2-shRNA-596 lentivirus (clone 596-7) was selected with puromycin (Gibco; A11138-03; 3 μg/mL; 9 days) and maintained in tetracycline-free FBS (Corning; 35-075-CV) and 3 μg/mL puromycin. The monoclonality of clone 596-7 was verified by virus integration site mapping.

pTRIPZ-EFNB2-Tet-on shRNA cloning, lentivirus production, and gene silencing
The human EFNB2 shRNA\textsuperscript{mTR} cassette, designed as a miR-30-based shRNA vector, was cloned into the XhoI and EcoRI sites of the pTRIPZ vector (Thermo Scientific Open Biosystems; RHS4750). Lentiviruses were generated by transfecting 293T cells with Lipofectamine 2000 (Thermo Fisher, 11668), the shRNA-encoding plasmid, the pMD2.G plasmid (Addgene; 12259; 4.8 μg), and the psPAX2 plasmid (Addgene; 12260; 9.6 μg). Lentivirus-containing supernatant was harvested (72 h), concentrated (ultracentrifugation at 19,500 rpm for 150 min), and stored at −80°C until use. Lentiviral

Figure 4. Antisense transcription from the VPS52 and ABCC4 genes and chimeric vector/host RNA reads
(A and B) Vector integration site in the host DNA and the junction between host RNA and vector RNA are marked by the blue vertical lines for the VPS52 (A) and ABCC4 (B) genes. The red arrow indicates the sense (S′ to S) direction of gene transcription. Sense (red) and antisense (green) reads from the VPS52 (A) and ABCC4 (B) genes were derived from clone 596-7 treated or not treated with Dox. Each read is representative of four replicate samples. The bar graphs in (A) and (B) reflect the quantification of chimeric antisense reads from cells treated (orange bars) or not (blue bars) with Dox. The results reflect the means of four samples. The vector-derived sequences within the chimeric reads are mapped to the vector sequence joining to the host antisense DNA strand. The hybrid vector-host reads reveal splicing from the splice vector donor (S′ LTR and UBC) into the host splice acceptor site; the dotted lines indicate the shRNA-host splicing junctions. (C) Quantification of all chimeric sense (host and vector-derived reads are in the sense orientation) and antisense (host-derived reads are in the antisense orientation and vector reads are in the sense orientation) reads coming from all 15 sites of vector integration in the host DNA. The vector component of the hybrid reads is mapped to the vector sequence.
Figure 5. Characterization of HT29 cell clones infected with ephrinB2 shRNA 596

(A) Relative mRNA levels of EFNB2, shRNA, IFNB1, and IFNL1 in each of the indicated clones infected with shRNA 596, with or without Dox, for 24 h. Values are normalized to mRNA levels in clone 7 without Dox. The results reflect the means of triplicate measurements. Error bars show the standard deviation. (B) Protein levels of ephrinB2, cleaved caspase-3, and GAPDH in HT29 clones infected with EFNB2 shRNA 596, with or without treatment with Dox for 24 h. Immunoblotting results are shown. (C) Number and location of vector integration sites in each of the HT29 clones infected with EFNB2 shRNA 596-7. (D) Shared DNA integration sites among 10 HT29 clones infected with EFNB2 shRNA 596.

Table D

| 596 clone no. | Chromosome no. | Vector Integration site | Gene annotation | NCBI reference | Gene start | Gene end | Location |
|---------------|----------------|-------------------------|-----------------|---------------|-----------|---------|----------|
| 596-7         | 1              | 189437807               | FAM5C           | NM_1909051    | 190097658 | 190479404| 660kb away |
| 596-5         | 1              | 190036918               | FAM5C           | NM_1909051    | 190097658 | 190479404| 61kb away  |
| 596-11        | 1              | 161853048               | TAF6            | NM_007348     | 161766320 | 161964070| Intronic  |
| 596-11        | 1              | 161853053               | TAF6            | NM_007348     | 161766320 | 161964070| Intronic  |
| 596-10        | 1              | 161814412               | TAF6            | NM_007348     | 161766320 | 161964070| Intronic  |
| 596-7         | 2              | 11332375                | ROCK2           | NM_004850     | 11179759 | 11348330| Intronic  |
| 596-6         | 2              | 11227156                | ROCK2           | NM_004850     | 11179759 | 11348330| Intronic  |
| 596-1         | 3              | 17450074                | TBC1D5          | NM_00134381   | 17157162 | 18444817| Intronic  |
| 596-6         | 3              | 17420188                | TBC1D5          | NM_00134381   | 17157162 | 18444817| Intronic  |
| 596-10        | 8              | 109611472               | ABRA            | NM_139166     | 106759483| 106770244| 141kb away |
| 596-8         | 8              | 108878913               | ABRA            | NM_139166     | 106759483| 106770244| 109kb away |
| 596-11        | 11             | 129038432               | ARHGA32         | NM_001142685  | 129865060| 129273324| Intronic  |
| 596-11        | 11             | 129163378               | ARHGA32         | NM_001142685  | 129865060| 129273324| Intronic  |
| 596-4         | 17             | 82577414                | VOXX2           | NM_004514     | 82519713 | 82646862| Intronic  |
| 596-1         | 17             | 82572052                | VOXX2           | NM_004514     | 82519713 | 82646862| Intronic  |

a Chromosome where the shRNA vector has integrated.
b Human Genome 38 is used to map (bp) vector integration in the genome.
c Gene where the vector has integrated; when the integration occurred outside a gene, the closest gene is listed and its distance from the vector site of integration is noted.
d NCBI (National Center for Biotechnology Information) reference sequence accession number.
e Gene coordinates based on human genome 38 (Human hg38 chrX:15560139-15602345 UCSC Genome Browser v422)
f Vector integration relative to gene; when vector is outside gene boundaries the distance from the nearest gene is noted.
particles containing control shRNA (SHC002) or NNMT shRNA (TRCN0000315817, -24 and -36) (all from MilliporeSigma) were used to infect clone 596-7.

Cell growth and imaging
Cell proliferation was measured by [³H]thymidine incorporation and Incucyte imaging (Essen BioSciences) as described.¹¹ Differential interference contrast (DIC) and red fluorescence images were done with an Olympus inverted microscope (Olympus; IX51).

Mouse xenografts
All mouse experiments were conducted in compliance with protocols approved by the National Cancer Institute’s Institutional Animal Care and Use Committee (ACUC, Bethesda, MD, USA). Groups of female NOD/SCID mice 8 to 11 weeks of age (Charles River Laboratories; strain code 394) were inoculated subcutaneously into the flank with human colorectal carcinoma tumor cells (10⁶ cells/mouse). Conventional or Dox chow (BioServ, S3888) was provided 7 days after cell inoculation when a tumor was visible in all mice. Tumor volume (V) was calculated from caliper measurements as V = D(d²)/2, where D and d are respectively the longest and shortest perpendicular dimensions.

Immunoblotting
After cell washing (1× PBS; Thermo Fisher, 10010-049), cell lysates were prepared in 1× RIPA buffer (Thermo Fisher, 89900) with 1× protease inhibitor cocktail (Thermo Fisher, 78430), 1× phosphatase inhibitor (Thermo Fisher, 1862495), and 5 mM EDTA. They were incubated (1 h at 4°C) and centrifuged (13,200 rpm, 12 min) and the protein concentration was measured (BCA method; Thermo Fisher, 23227). Then the samples were separated by SDS-PAGE (NuPage 4%–12% Bis-Tris gels; Thermo Fisher, NP0321) with 1× MOPS running buffer (Thermo Fisher, NP0001). After transfer to nitrocellulose, the membranes were blocked (1 h, room temperature) in 1× Tris-buffered saline (TBS) buffer (Quality Biological, 351-086-101) with 0.1% Tween 20 (Sigma, P1379) and 5% skim milk (Lab Scientific; M0841). After being washed (30 min in 1× TBS with 0.1% Tween 20, TBST), the membranes were incubated (18 h, 4°C) with primary antibody in 3% BSA in 1× TBST, washed (30 min, 1× TBST), and incubated (1 h, room temperature) with the appropriate horseradish peroxidase-linked secondary antibody in 1× TBST with 5% skim milk. A list of antibodies used is shown in Table S2. After being washed (1× TBST; 1 h, room temperature), the blots were developed using the ECL Prime (GE LifeSciences, RPN2232) reagent, and images were acquired with an LAS4000 (GE LifeSciences) or Amersham Imager 680 (GE LifeSciences) and the bands were quantified (ImageJ).

Real-time RT-PCR and RNA sequencing
Total RNA was extracted using the RNeasy kit (Qiagen; 74106); 1 μg RNA was used to synthesize cDNA with the QuantiTect reverse transcription kit (Qiagen; 205311). RNA integrity (RIN) was evaluated using the Bioanalyzer 2100 (Agilent Technologies). A list of primers is shown in Table S3. mRNA expression was measured (7900HT Fast Real-Time PCR system, Applied Biosystems) using the FastStart Universal SYBR Green Master Mix (ROX) (Roche, 0491394001). Relative mRNA expression was calculated using the ΔΔCt method [ΔCt = Ct[gene of interest] − Ct[housekeeping gene]; ΔΔCt = ΔCt[treated sample] − ΔCt [untreated sample]]. Ct (cycle threshold) values were calculated by SDS 2.4.1 software (Applied Biosystems). For RNA-seq, RNA samples had a score higher than 9.9. A stranded total RNA library was prepared according to the Illumina library prep protocol (Illumina; document 100000004099 v.00). Pair-end stranded RNA-seq was performed by the Sequencing Facility at the Frederick National Laboratory for Cancer Research using Illumina Nextseq (596-7, 596-7 + Dox) and NovaSeq 6000 SP (596-1, -6, -7, and -11). Differentially expressed genes were analyzed for pathway enrichment using IPA (https://digitalinsights.qiagen.com/). Normalized counts per million trimmed mean of M values (CPM_TMM) counts (average of four samples, average counts >2; fold change [plus versus minus Dox > 3 or < −3] [fold change = Dox+/Dox−, when Dox+ is greater than Dox−; fold change = −(Dox−/Dox+), when Dox+ is no greater than Dox−]; fold change p < 0.005) were analyzed by IPA “core analysis.” Gene lists from IPA canonical pathways were used for heatmaps.

Polysome profiling
 Cultures of clone 596-7 (~90% confluency) were incubated (10 min) in medium containing 100 μg/mL cycloheximide (BioVision; 1041), washed with PBS, trypsinized, washed with ice-cold PBS containing 100 μg/mL cycloheximide, and lysed in polysome lysis buffer (20 mM Tris [pH 7.2], 130 mM KCl, 15 mM MgCl₂, 0.5% [v/v] NP-40, 0.2 mg/mL heparin, protease inhibitors [Thermo Fisher], RNA Sin [Promega], 2.5 mM DTT, 0.5% deoxycholic acid, and 100 μg/mL cycloheximide). Cell lysates clarified by centrifugation (8,000g, 10 min, 4°C) were loaded onto a 10–50% linear sucrose gradient (10 mM Tris [pH 7.2], 60 mM KCl, 15 mM MgCl₂, 1 mM DTT, 0.5% [v/v] NP-40, 0.1 mg/mL heparin) and centrifuged at 200,000g, 2 h at 4°C, in an SW41 Ti rotor (Beckman). After centrifugation, the contents were collected as fractions by a fractionator (BioComp Instruments) and optical density of the fractions was measured by UV profiling (254 nm), as described.⁵⁶

Lentiviral vector integration
Lentiviral vector integration mapping was performed at the CCR Genomics Technology Laboratory (Frederick National Laboratory

Figure 6. Antisense reads detected in distinct HT29 cell clones
(A–C) Sense and antisense reads from the SETD1B gene in clone 596-6 (A), from the SCFD1 gene in clone 596-1 (B), and from the JPH1 gene in clone 596-11 (C). Vector integration site in the host DNA and the chimeric host-vector RNA junction are marked by the blue vertical lines. The direction of sense gene transcription is indicated by the red arrow below the schematic gene structure. The read scale is 0–200 for all SCFD1 and JPH1 reads and 0–250 for all SETD1B reads. Quantification of chimeric antisense reads is displayed in the bar graphs (orange bars reflect reads from cells treated with Dox and blue bars reflect reads from cells not treated with Dox). The results reflect the means of duplicate samples. The vector-derived sequences within the chimeric reads are mapped to the shRNA sequence. Splicing events from the vector splice donor (5′ LTR, 3′ LTR, or GFP) into the host splice acceptor site are identified; the dotted lines indicate the shRNA-host splice junctions. See also Figure S4.
for Cancer Research), as described. Briefly, integration sites were amplified using a linker-mediated PCR protocol. Primers for the 3' and 5' LTR sequences of the vector were used to selectively amplify the host/shRNA-vector DNA junctions. The 3' LTR and 5' LTR primer sequences are shown in Table S3. Libraries were generated from genomic DNA from shRNA-vector-infected clones 596-1, 596-6, and 596-11 using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina. One microgram of DNA was randomly fragmented and a T-linker unique molecular identifier (UMI) sequence tag was ligated to the ends of the sheared DNA to distinguish independent DNAs with the same integration site. Paired-end sequencing was performed on an Illumina MiSeq and sequencing data were analyzed by a bioinformatics pipeline.

Figure 7. Antisense gene transcription in HT29 cell clones
(A–D) Quantification of all chimeric sense and antisense reads from all sites of vector integration in clones 596-1 (A), 596-6 (B), 596-7 (C), and 596-11 (D) with or without Dox. The vector hybrid reads are mapped to the vector sequence. (E) Quantification of all chimeric antisense reads from all DNA sites of vector integration in each of the clones 596-1, 596-6, 596-7, and 596-11 with or without Dox. The results reflect the means of two separate sequencing results and are displayed as normalized counts/10^6 reads. (F) Correlation between integrated vector copy number and relative antisense read number (antisense reads/10^6 reads) in clones 596-1, 596-6, 596-7, and 596-11. (G) Correlation between relative shRNA expression (shRNA/RLP30 × 10^3) and relative antisense read number (antisense reads/10^6 reads) in clones 596-1, 596-6, 596-7, and 596-11. Correlation between two variables was calculated by Spearman’s rank correlation coefficient.
Bioinformatics
Adapter-trimmed sequencing reads were aligned to host + shRNA (hybrid) genome using STAR aligner. RSEM was used to create a gene-level count matrix. Downstream analyses were then performed using custom scripting; details of the bioinformatics analyses are in the supplemental information.

Statistical analysis
Results are presented as means ± standard deviations. Unpaired two-tailed Student’s t test was used for analysis of two groups with homoscedastic distribution. p values of less than 0.05 were considered statistically significant. The non-parametric Spearman’s rank correlation coefficient was used to measure correlation between two variables; r values were used to estimate the strength of correlation.

DATA AVAILABILITY
The data associated with this study are presented in the paper or in the supplemental information. The sequencing read data generated in this study have been submitted to the GEO repository at NCBI (https://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE186796. All codes used in this paper are available at https://github.com/kopardev/cCBR1060.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.05.029.

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AUTHOR CONTRIBUTIONS
G.T. conceptualized and supervised the study; T.H. designed and executed most experiments; M.P. performed some experiments; V.K. and P.J. performed bioinformatic analysis; M.D., H.O., X.F., and D.L. provided intellectual input and interpreted data; G.T. wrote the manuscript with T.H. and it was edited by all authors. All authors have seen and approved the final manuscript prior to submission.

DECLARATION OF INTERESTS
All authors declare no competing interests.

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