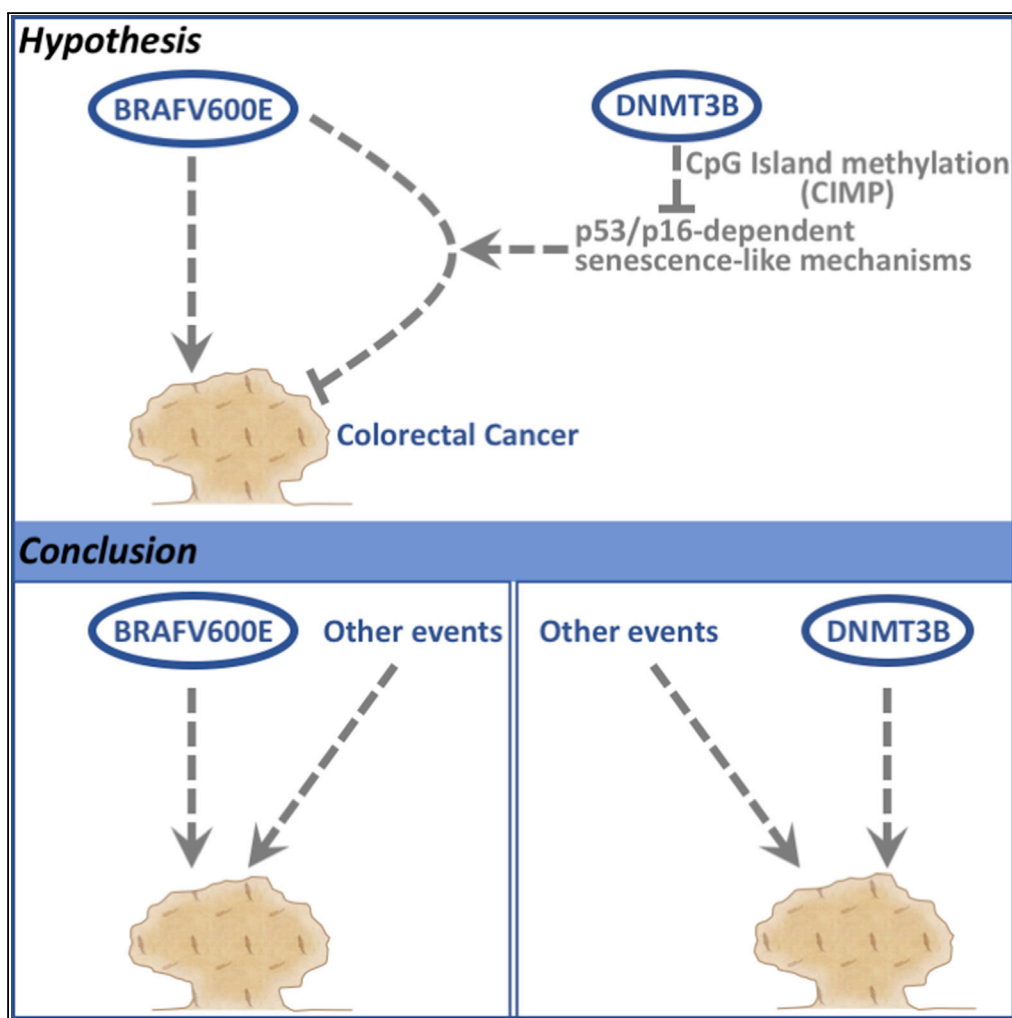


## Article

# DNMT3B Oncogenic Activity in Human Intestinal Cancer Is Not Linked to CIMP or BRAFV600E Mutation



Douglas J. MacKenzie, Neil A. Robertson, Iqbal Rafter, ..., Catrin Pritchard, Karen Blyth, Peter D. Adams

padams@sbpdiscovery.org

#### HIGHLIGHTS

DNMT3B antagonizes BRAFV600E-induced senescence-like state

DNMT3B accelerates BRAFV600E-induced intestinal cancer

Other studies do not support a role for DNMT3B in CIMP and cooperation with BRAFV600E

On balance, BRAFV600E and DNMT3B are unlikely to cooperate in human intestinal cancer

#### DATA AND CODE

AVAILABILITY  
GSE126434

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## Article

# DNMT3B Oncogenic Activity in Human Intestinal Cancer Is Not Linked to CIMP or BRAFV600E Mutation

Douglas J. MacKenzie,<sup>1</sup> Neil A. Robertson,<sup>1</sup> Iqbal Rather,<sup>1,5</sup> Claire Reid,<sup>1</sup> Gintare Sendzikaite,<sup>1</sup> Hazel Cruickshanks,<sup>1</sup> Tony McBryan,<sup>1</sup> Andrew Hodges,<sup>4</sup> Catrin Pritchard,<sup>2</sup> Karen Blyth,<sup>1,3</sup> and Peter D. Adams<sup>1,4,6,\*</sup>

## SUMMARY

Approximately 10% of human colorectal cancer (CRC) are associated with activated BRAFV600E mutation, typically in absence of APC mutation and often associated with a CpG island methylator (CIMP) phenotype. To protect from cancer, normal intestinal epithelial cells respond to oncogenic BRAFV600E by activation of intrinsic p53 and p16-dependent tumor suppressor mechanisms, such as cellular senescence. Conversely, CIMP is thought to contribute to bypass of these tumor suppressor mechanisms, e.g. via epigenetic silencing of tumor suppressor genes, such as p16. It has been repeatedly proposed that DNMT3B is responsible for BRAFV600E-induced CIMP in human CRC. Here we set out to test this by *in silico*, *in vitro*, and *in vivo* approaches. We conclude that although both BRAFV600E and DNMT3B harbor oncogenic potential *in vitro* and *in vivo* and show some evidence of cooperation in tumor promotion, they do not frequently cooperate to promote CIMP and human intestinal cancer.

## INTRODUCTION

In normal cells, the BRAF kinase, encoded by the *BRAF* gene, is a critical effector of cell signaling pathways, most notably the RAS-BRAF-MEK-ERK mitogenic pathway. This proto-oncogenic pathway is activated by genetic mutations at some point along the cascade in most human cancers (Yaeger and Corcoran, 2019). Approximately 10% of human CRC are associated with activated BRAFV600E mutation. Although inhibitors of activated BRAF, such as Vemurafenib, are of some benefit in other cancers harboring BRAFV600E, such as melanoma, in CRC these inhibitors are of limited therapeutic value (Dienstmann et al., 2017). Yet CRC harboring BRAFV600E has a poor prognosis (Samowitz et al., 2005b; Ogino et al., 2009). Thus, it is important to understand the oncogenic mechanisms underlying this disease.

Activated BRAFV600E mutation is typically found in absence of mutation in the *Adenomatous Polyposis Coli* (*APC*) gene, a gene that is mutated and inactivated in ~80% of CRC (Dienstmann et al., 2017). Activated BRAFV600E drives hyperproliferation and neoplasia via the MEK-ERK mitogenic pathway (Lavoie and Therrien, 2015). However, to protect from cancer, normal intestinal epithelial cells are thought to respond to oncogenic BRAFV600E by activation of intrinsic TP53 (p53) and CDKN2A (p16)-dependent tumor suppressor mechanisms (Rad et al., 2013), including cellular senescence (Carragher et al., 2010; Kriegl et al., 2011). In response to acquisition of an activated oncogene, primary human cells can enter a proliferation-arrested senescent state (oncogene-induced senescence [OIS]) that suppresses tumor formation (Michaloglou et al., 2005; Chen et al., 2005; Braig et al., 2005; Collado et al., 2005; He and Sharpless, 2017). Senescent cells also exhibit an altered secretory program, the so-called Senescence Associated Secretory Phenotype (SASP) (Kuilman et al., 2008; Acosta et al., 2008; Krtolica et al., 2001) comprised of pro-inflammatory cytokines and chemokines, which also contributes to tumor suppression by promoting clearance of senescent cells by the immune system (Lujambio et al., 2013; Xue et al., 2007; Kang et al., 2011).

Conversely, CpG island methylator phenotype (CIMP) is thought to contribute to bypass of tumor suppressor mechanisms by epigenetic silencing of tumor suppressor genes, such as *MLH1* and *CDKN2A* (Lao and Grady, 2011). In CRC, BRAFV600E mutation and CIMP are quite tightly linked (Ogino et al., 2009; Weisenberger et al., 2006; Inoue et al., 2015; Nagasaka et al., 2004, 2008; Hinoue et al., 2012; Cancer Genome Atlas

<sup>1</sup>Institute of Cancer Sciences, University of Glasgow, Glasgow, UK

<sup>2</sup>Leicester Cancer Research Centre, University of Leicester, Leicester, UK

<sup>3</sup>Cancer Research UK Beatson Institute, Glasgow, UK

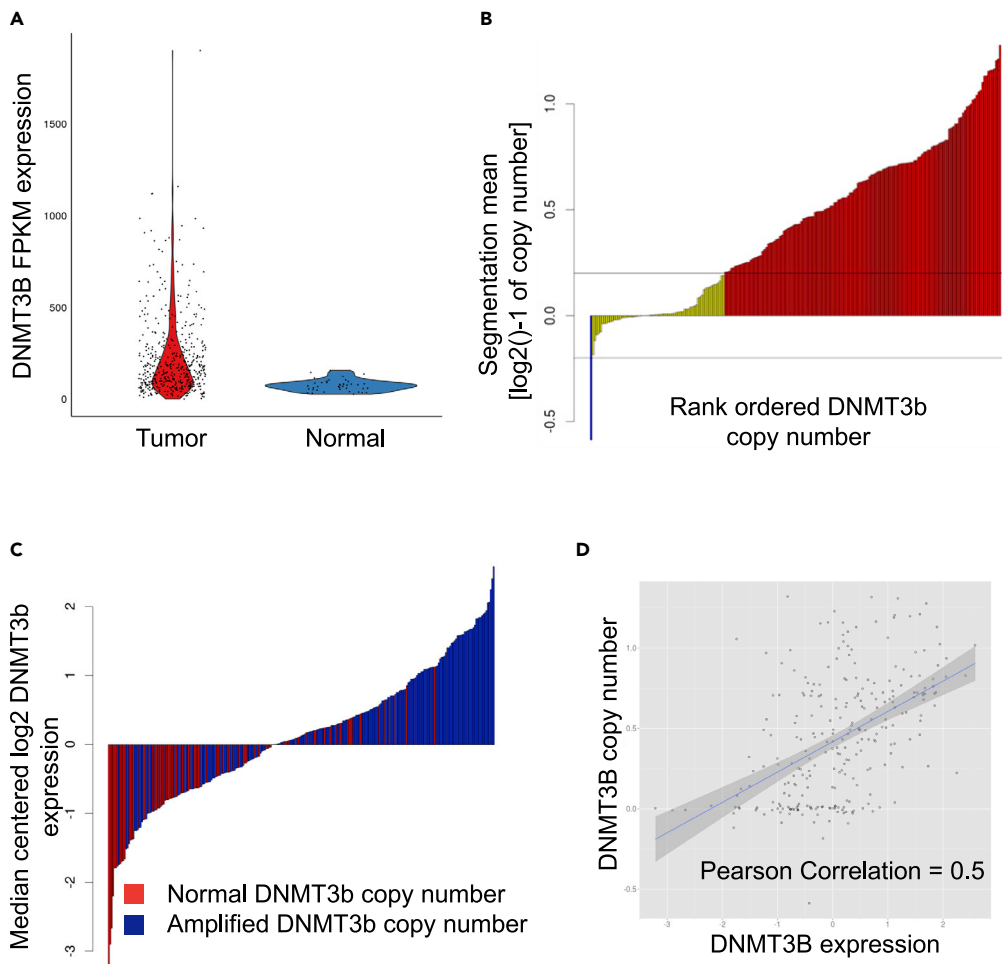
<sup>4</sup>Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA

<sup>5</sup>Present address: Department of Biochemistry, Govt. Degree College Kulgam, University of Kashmir, India

<sup>6</sup>Lead Contact

\*Correspondence: padams@sbdiscov.org  
<https://doi.org/10.1016/j.isci.2020.100838>





**Figure 1. DNMT3B Is Frequently Amplified and Overexpressed in CRC**

(A) Violin plot showing distribution of *DNMT3B* expression levels in 207 CRC tumor samples. Mann-Whitney U test; p value =  $5.785e-10$ .

(B) *DNMT3B* relative copy number across 207 tumor samples. Lines at y axis  $-0.2$  and  $0.2$  indicate the threshold for deletion and amplification, respectively.

(C) Median centered  $\log_2$  *DNMT3B* mRNA expression plot from 207 tumor samples (TCGA) annotated with BRAF mutation status.

(D) The relationship between *DNMT3B* relative copy number and *DNMT3B* mRNA expression in 207 tumor samples.

Network, 2012), leading to speculation that BRAFV600E promotes and/or selects for CIMP *en route* to tumor progression. Because *CDKN2A* encodes p16, a key effector of cellular senescence (He and Sharpless, 2017), CIMP might be expected to suppress OIS.

DNA methyl transferase 3B (DNMT3B) is a so-called *de novo* DNA methyl transferase, capable of methylating CpGs where both CpGs of the palindrome are in the unmethylated state. In normal mouse intestine, inactivation of *Dnmtb* does not have a marked phenotype (Elliott et al., 2016; Lin et al., 2006). However, several studies have suggested that elevated DNMT3B might cooperate with BRAFV600E to drive tumorigenesis and that DNMT3B is responsible for methylation of DNA to generate CIMP (Carragher et al., 2010; Fang et al., 2014; Ibrahim et al., 2011; Noshio et al., 2009). According to one model in which the link between oncogene activation and CIMP can be quite indirect, oncogene-induced senescence and/or other oncogene-activated p53 and p16-dependent tumor suppressor mechanisms provide the selective pressure for DNMT3B and CIMP-mediated silencing of these key tumor suppressor pathways. Consistent with this model, in a mouse model of BrafV600E-driven colon cancer, escape from senescence and tumor progression was linked to increased expression of *Dnmt3b* and methylation and silencing of p16

(Carragher et al., 2010). According to this model, although activated BRAFV600E can increase the selective pressure that favors CIMP, methylation is not directly caused by BRAFV600E but has an inherent tendency to encroach on unmethylated CpG islands during aging (Skvortsova et al., 2019; Ushijima and Suzuki, 2019; Tao et al., 2019). In another model that more directly links oncogenic signaling and CIMP, it has been proposed that BRAFV600E signaling recruits DNMT3B to genes silenced by CIMP via the transcriptional repressor, MAFG, thereby directly promoting CIMP (Fang et al., 2014, 2016).

However, studies into the relationship between BRAFV600E and DNMT3B to this point are incomplete. For example, their associations in human TCGA data and functional interactions in mouse models have not, as far as we are aware, been considered in detail in previous studies. Here we set out to investigate whether BRAFV600E and DNMT3B cooperate in intestinal tumorigenesis by examination of human tumor data, *in vitro* models of senescence, and mouse models of CRC. The results present a mixed story, but are important for the field, and, on balance, lead us to suggest that BRAFV600E mutation and DNMT3B do not frequently cooperate to promote CIMP and human intestinal cancer.

## RESULTS

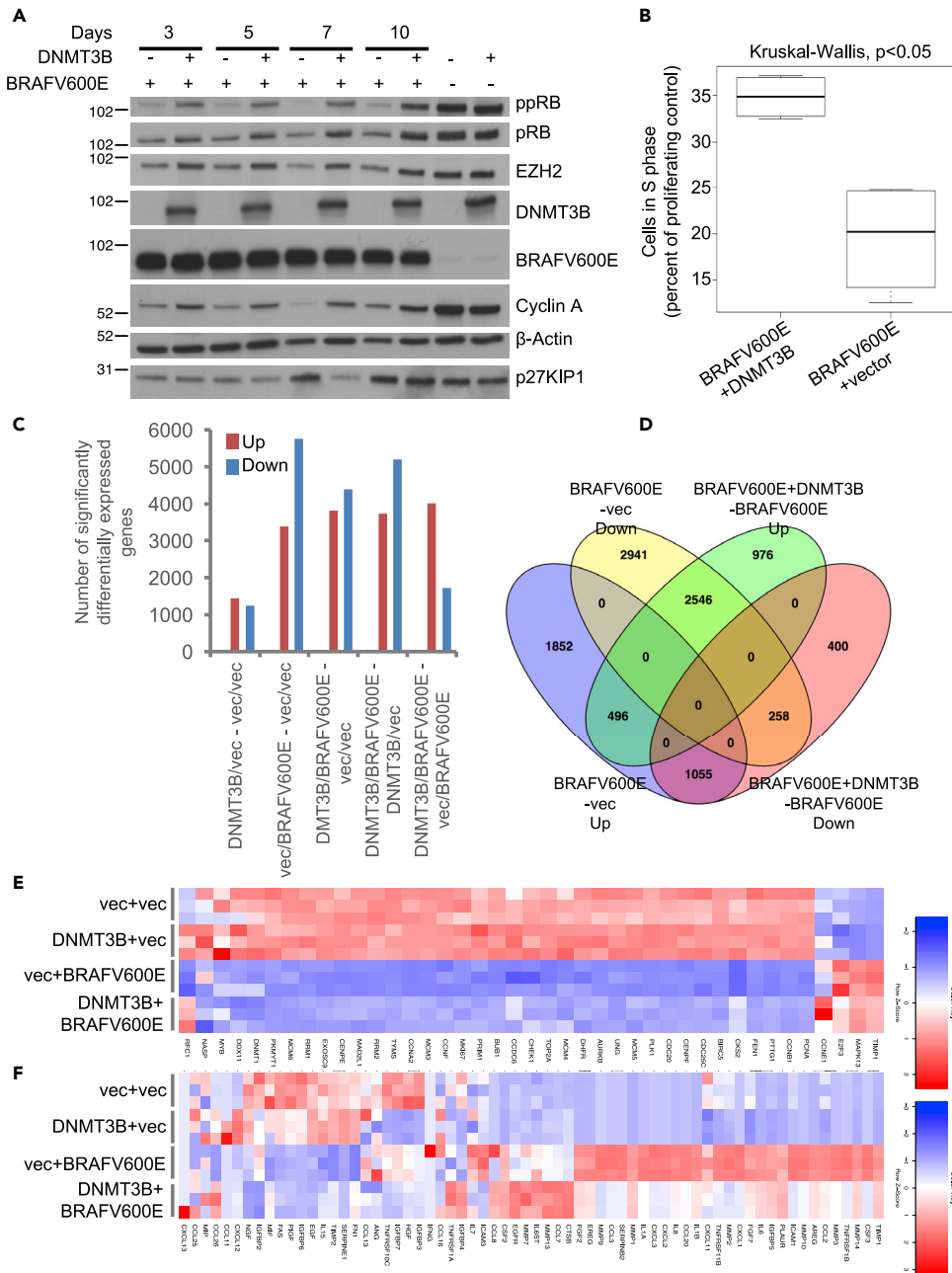
### DNMT3B Is Frequently Amplified and Overexpressed in Human CRC

To begin to probe the putative oncogenic function of DNMT3B in human CRC, we mined human TCGA data examining levels of *DNMT3B* expression and gene copy number. First, we confirmed that, at the mRNA level, *DNMT3B* is expressed at a higher level in human CRC than normal human colon (Figures 1A and S1A–S1C). We also found that the *DNMT3B* gene exhibits increased copy number, indicative of gene amplification, in a substantial proportion of CRC (Figure 1B). Amplification of *DNMT3B* in human CRC is also apparent via Database: [cbioportal.org](http://cbioportal.org) (Gao et al., 2013). Moreover, comparing *DNMT3B* mRNA expression and gene copy number revealed a correlation between *DNMT3B* copy number and mRNA expression (Figures 1C and 1D). These data are consistent with the idea that increased DNMT3B expression can be oncogenic and suggest that increased expression in human CRC is often a result of gene amplification.

### Ectopic Expression of DNMT3B Antagonizes BRAFV600E-Induced Proliferation Arrest and SASP

Previous studies suggested that elevated DNMT3B might cooperate with BRAFV600E to drive tumorigenesis (Carragher et al., 2010; Fang et al., 2014; Ibrahim et al., 2011; Noshio et al., 2009). To investigate this possibility of oncogenic cooperation between BRAFV600E and elevated DNMT3B, we tested functional interactions between the two in an *in vitro* cell culture model. In the absence of a robust normal human colonic epithelial cell model, we turned to primary human fibroblasts that were previously used to model molecular events underlying CIMP in CRC (Fang et al., 2014) and more generally have been a paradigm for investigating mechanisms of oncogene cooperation in human cells (Hahn et al., 1999, 2002). Primary human IMR90 fibroblasts were infected with drug-selectable lentiviruses directing expression of BRAFV600E, DNMT3B, or empty vector as a control. Both proteins were efficiently expressed for up to 10 days post-infection (Figure 2A). When expressed on its own, activated BRAFV600E induced a marked decrease in the percent of cells in S phase of the cell cycle, in line with this oncogene's ability to induce senescence-associated proliferation arrest in primary cells (Michaloglou et al., 2005; Pawlikowski et al., 2013) (Figure S2A). The decline in cells in S phase was accompanied by decreased abundance of cell cycle drivers, cyclin A, and phosphorylated pRB, and, perhaps accounting for decreased phosphorylated pRB through regulation of p16INK4A (McHugh and Gil, 2018), decreased expression of EZH2 (Figure 2A). Ectopic expression of DNMT3B increased the proportion of cells in S phase, proportionately more so in cells ectopically expressing BRAFV600E (Figures 2B and S2A). In cells expressing both BRAFV600E and DNMT3B compared with those expressing BRAFV600E only, this was accompanied by increased cyclin A, EZH2 and phosphorylated pRB, and, at the seven-day time point, a consistent decrease in expression of cell cycle inhibitor, CDKN1B (p27KIP1) (Figure 2A). Expression of DNMT3B alone had no detectable effect on these key cell-cycle regulators (Figure 2A). We conclude that elevated DNMT3B antagonizes BRAFV600E-induced cell-cycle arrest.

To better understand the effect of DNMT3B on BRAFV600E-induced cell-cycle arrest, we infected cells with empty vector, DNMT3B alone, BRAFV600E alone, or the combination and performed RNA-seq. Principal component analysis showed that three replicates of each condition expressed distinct transcriptomes (Figure S2B). Expression of activated BRAFV600E significantly increased and decreased expression of 3403 and



**Figure 2. DNMT3B Suppresses Features of OIS**

(A) Kinetic analysis assessing the impact of ectopic DNMT3B on markers of BRAFV600E-induced cell-cycle exit of primary IMR90 cells, determined by Western blot. MW markers in kDa.

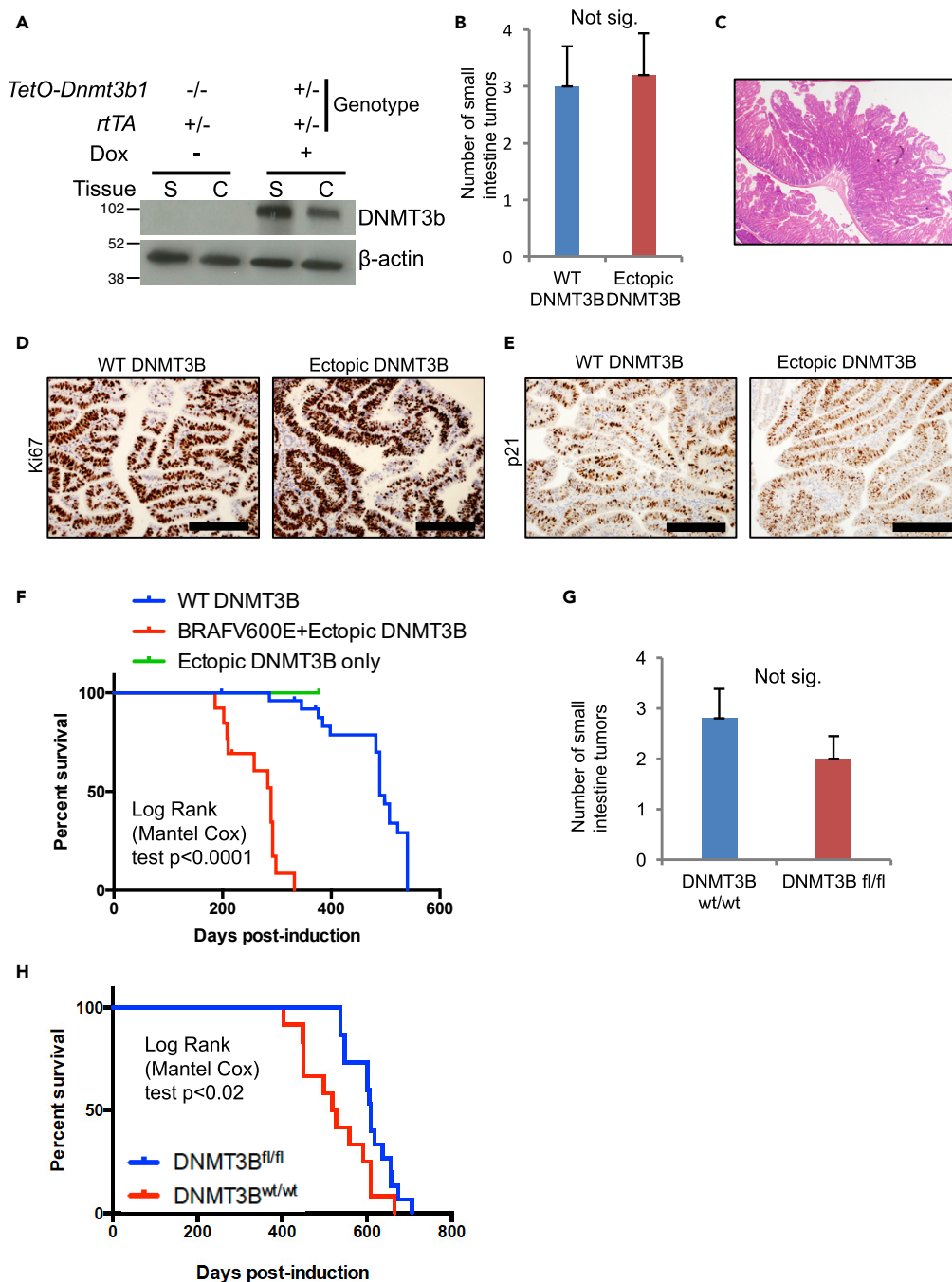
(B) Impact of ectopic DNMT3B on BRAFV600E-induced S-phase exit of primary IMR90 cells, determined by BrdU labeling. Each group  $n = 4$ , from days 3, 5, 7, and 10 post-infection. Middle line = median; box top/bottom = upper and lower quartiles; whiskers 90<sup>th</sup> (upper) & 10<sup>th</sup> (lower) percentile of data. Kruskal-Wallis test,  $p < 0.05$ .

(C) Number of significantly differentially expressed genes between indicated pairwise comparisons of primary IMR90 cells expressing ectopic BRAFV600E and/or DNMT3B and/or empty vectors, determined from RNA-seq FPKM values (Cuffdiff, FDR corrected  $p$  value of  $< 0.05$ ).

(D) Number of significantly up or down genes between indicated pairwise comparisons of primary IMR90 cells expressing ectopic BRAFV600E and/or DNMT3B and/or empty vectors, determined from RNA-seq FPKM values.

(E) Heatmap of expression of significant changed proliferation-associated genes.

(F) Heatmap of expression of significant changed SASP genes.



**Figure 3. DNMT3B Accelerates Intestinal Cancer**

(A) Western blot showing doxycycline-inducible expression of *Dnmt3b* in small intestine (S) and colon (C) of *R26-M2-rtTA;Col1a1-tetO-Dnmt3b1* and *R26-M2-rtTA;WT* mice. MW markers in kDa.

(B) Number of small intestine tumors in *VilCreErT2;LSL BrafV600E* mice with and without ectopic *Dnmt3b* expression. Mean with error bars  $\pm$  SEM. Mann Whitney test  $p = 0.98$  (ns)  $n = 5$  vs  $5$ .

(C) Representative small intestine tumor from *VilCreErT2;LSL BrafV600E* mice, similar with or without *Dnmt3b*.

(D) Representative immunohistochemistry images for Ki67 in *VilCreErT2;LSL BrafV600E* mice with and without ectopic *Dnmt3b*. Scale bars are  $200 \mu\text{m}$ .

(E) Representative immunohistochemistry images for p21 in *VilCreErT2;LSL BrafV600E* mice with and without ectopic *Dnmt3b*. Scale bars are  $200 \mu\text{m}$ .

**Figure 3. Continued**

(F) Kaplan-Meier curve comparing survival in *VilCreErT2; LSLBrafV600E* mice with and without intestinal *Dnmt3b* ectopic expression. “DNMT3B only” mice (“*Col1a1-tetO-Dnmt3b1 +/-*”) also shown. Log rank (Mantel-Cox) test,  $p < 0.0001$ .

(G) Total small intestine tumor number in *VilCreErT2; LSLBrafV600E; Dnmt3b<sup>wt/wt</sup>* and *VilCreErT2; LSLBrafV600E; Dnmt3b<sup>fl/fl</sup>* mice. Mann Whitney test  $p = 0.42$ ;  $n = 5$  vs  $5$ . Mean with error bars  $-/+$  SEM.

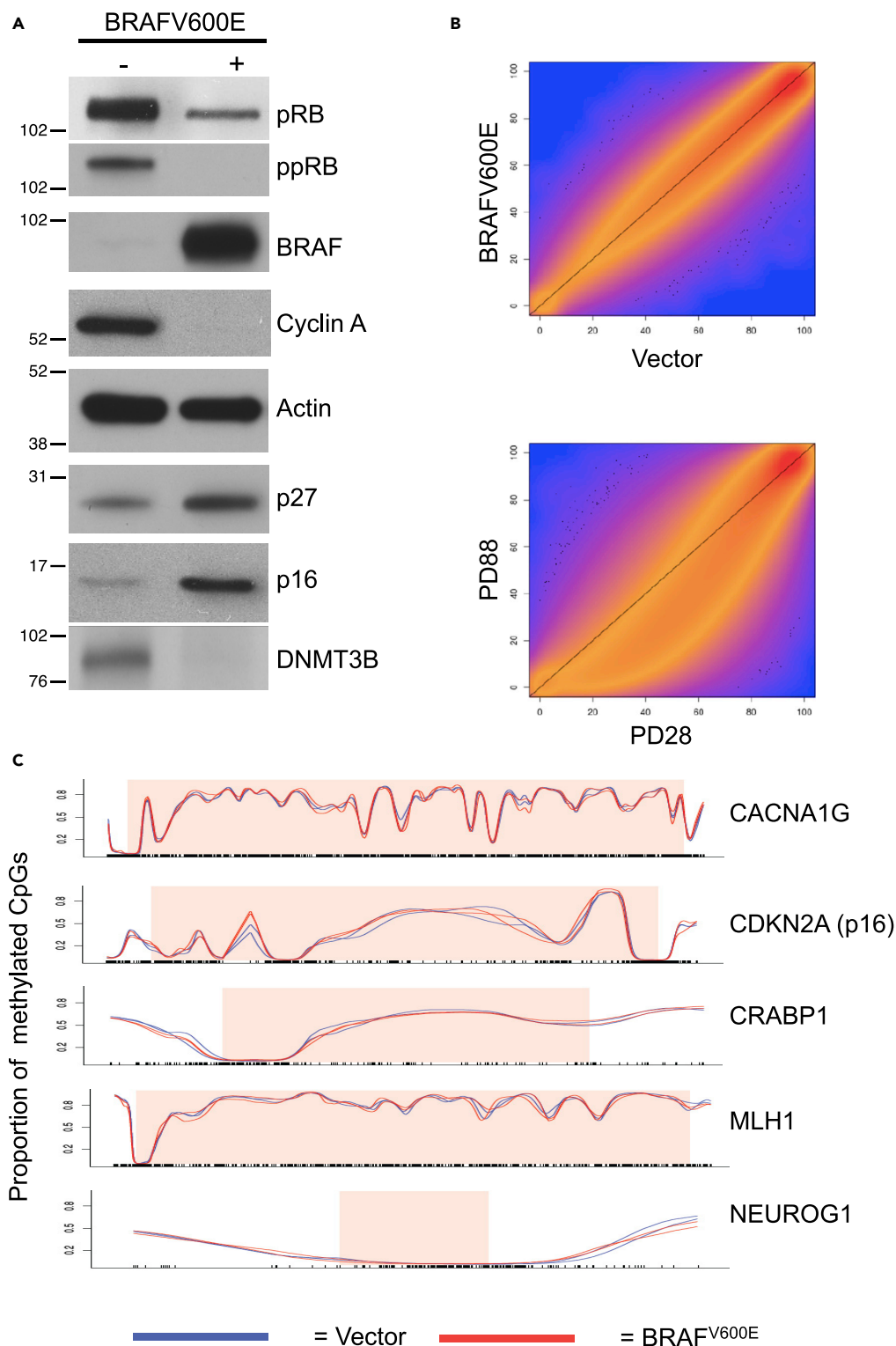
(H) Kaplan-Meier curve of survival in *VilCreErT2; LSLBrafV600E* mice with wild-type or floxed *Dnmt3b*. Log Rank (Mantel-Cox) test,  $p < 0.02$ .

5745 genes, respectively (Figures 2C and 2D). These changes in gene expression include those characteristic of OIS, including repression of cell cycle genes and activation of inflammatory genes indicative of the SASP (Figures 2E and 2F). However, co-expression of DNMT3B antagonized a substantial proportion of the gene expression changes induced by BRAFV600E (Figure 2D) (specifically, 1,055 of genes upregulated by BRAFV600E and 2,546 of genes downregulated by BRAFV600E). Consistent with the previous cell-cycle analyses (Figures 2A, 2B, and S2A), DNMT3B modestly suppressed BRAFV600E-induced repression of many cell proliferation genes (Figures 2E, S2C, and S2D). In addition, DNMT3B markedly suppressed many of the SASP genes induced by BRAFV600E (Figure 2F). DNMT3B-mediated suppression of BRAFV600E-induced repression of cell-cycle genes and activation of SASP was also apparent by GSEA analysis (Figure S2E). In sum, elevated DNMT3B impaired two key tumor suppressive phenotypes induced by BRAFV600E in primary human cells, cell-cycle arrest and SASP. These data show that combined expression of BRAFV600E and DNMT3b could cooperate to promote tumorigenesis.

**Dnmt3b Enhances Tumorigenesis and Decreases Survival in a BrafV600E-Driven Mouse Model of Intestinal Cancer**

To investigate the tumor promoting potential of combined BrafV600E mutation and elevated *Dnmt3b* *in vivo*, we tested the two in an *in vivo* mouse model of intestinal tumorigenesis. A previous study reported that mouse intestinal-specific expression of activated Braf under control of a constitutive villin-Cre recapitulates features of the human serrated pathway of intestinal tumorigenesis (Rad et al., 2013). In an effort to better mimic cancer initiation in human adult tissues, in a modified version of this model, mice expressing a conditional-activated intestinal-specific *BrafV600E* oncogene under the control of tamoxifen-inducible *Villin-Cre-ERT2* were crossed to mice expressing conditional *Dnmt3b* under control of doxycycline inducible rtTA (el Marjou et al., 2004; Linhart et al., 2007; Steine et al., 2011). Transgene expression was induced in 6- to 10-week-old mice, and western blotting confirmed inducible expression of *Dnmt3b* in the small intestine and colon (Figure 3A). Mice were culled when they showed comparable clinical signs of disease. Accordingly, at the time the BrafV600E and BrafV600E/*Dnmt3b* mice were culled, the two groups harbored a comparable number of small intestinal tumors (Figures 3B and 3C); no overt differences in tumor histology were noted between BrafV600E and BrafV600E/*Dnmt3b* tumors. No colonic tumors were identifiable in any mice examined. At this time, we did not detect any overt differences between proliferation and senescence markers in BrafV600E/*Dnmt3b* and BrafV600E mice, in either tumor or histologically normal intestine (Figures 3D, 3E, and S3A and data not shown). Strikingly, however, combined ectopic *Dnmt3b* expression together with activated BrafV600E in the murine intestine resulted in a marked decrease in overall survival compared with BrafV600E alone (Figure 3F). Median post-survival induction in mice expressing BrafV600E and endogenous *Dnmt3b* was 489 days, compared with 289 in mice with ectopic BrafV600E and *Dnmt3b*. Mice expressing ectopic *Dnmt3b* alone did not develop any clinical signs of illness up until 377 days following induction and were culled at this time. Neither intestinal abnormalities nor tumors were identified in these mice, consistent with previous reports (Steine et al., 2011; Linhart et al., 2007). These results suggest that elevated *Dnmt3b* accelerated the accumulation of a lethal burden of BrafV600E-driven intestinal tumors.

To test whether *Dnmt3b* is required for BrafV600E-induced intestinal tumorigenesis, we generated mice harboring a conditionally activated *BrafV600E* oncogene and conditionally inactivated *Dnmt3b<sup>fl/fl</sup>*, both under control of *Villin-Cre-ERT2* (Figure S3B). Similar to the observations with elevated *Dnmt3b*, inactivation of *Dnmt3b* did not significantly affect the number of intestinal tumors at the time of terminal disease (Figure 3G). Again, no differences in tumor histology were noted either. However, inactivation of *Dnmt3b* did significantly extend survival of mice harboring *BrafV600E* (Figure 3H). Consistent with the previous elevated *Dnmt3b* model, these results suggest that abundance of *Dnmt3b* is rate-limiting for acquisition of a lethal burden of tumors in this BrafV600E-induced intestinal cancer model. Overall,



**Figure 4. Ectopic Expression of DNMT3B Does Not Induce CIMP**

(A) Western blot of lysates from human fetal lung fibroblasts (IMR-90) infected with lentivirus directing expression of BRAFV600E or corresponding empty vector. MW markers in kDa.

(B) Scatterplot of CpG methylation in BRAFV600E-infected fibroblasts compared with proliferating “vector” controls (top), and replicative senescent (PD88) compared with proliferating controls (PD28) (bottom).



**Figure 4. Continued**

(C) Smoothened methylation plots of CIMP panel genes in vector and BRAFV600E-expressing cells. Red and blue lines indicate the smoothened average percentage methylation at corresponding CpGs. Individual CpGs are indicated by black "ticks" along the x axis.

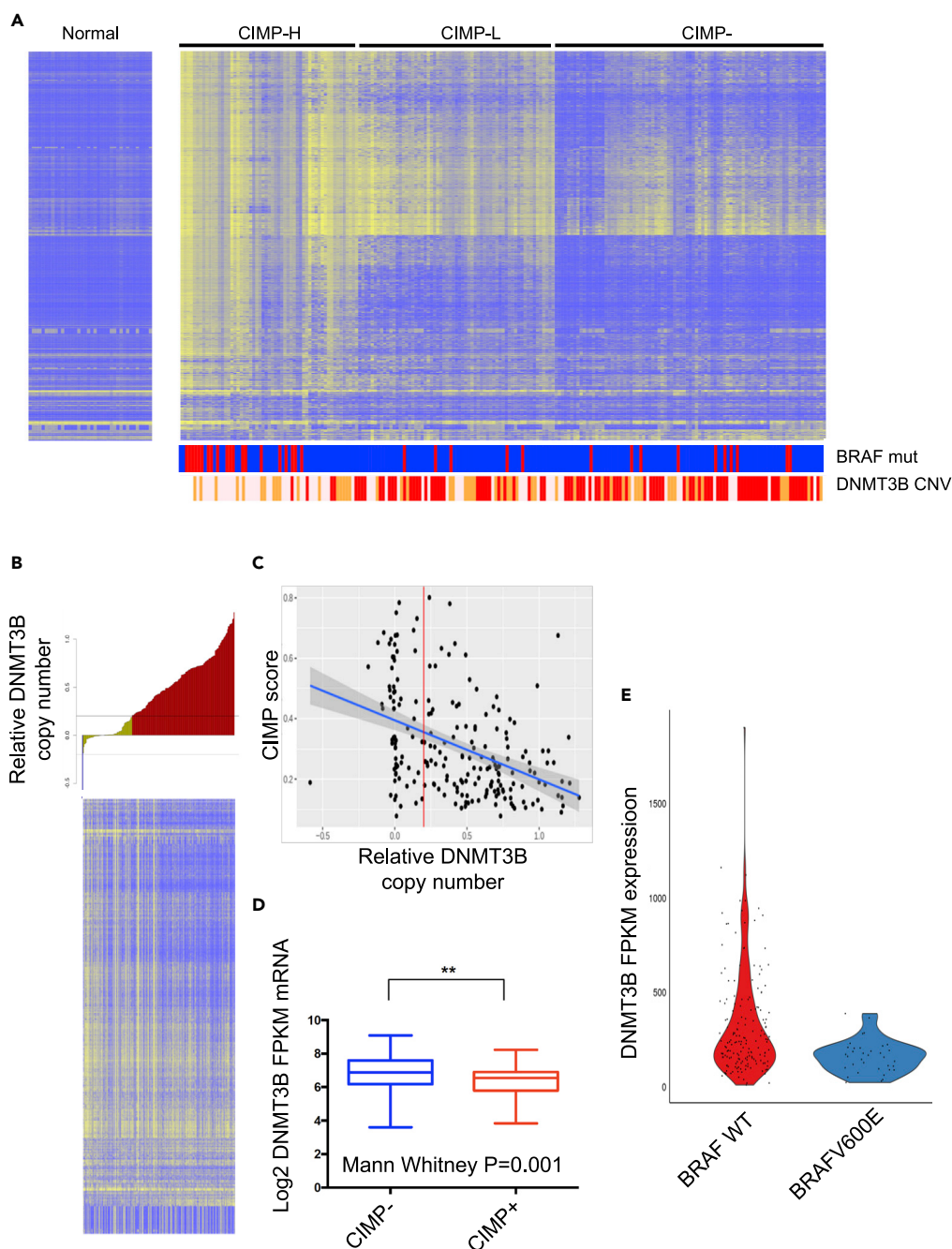
these data support the notion that the combination of BraFv600E and elevated Dnmt3b can be potentially oncogenic in CRC.

**Activated BRAFV600E Repressed Expression of DNMT3B and Failed to Induce a CIMP Phenotype**

According to a previously proposed model, activated BRAFV600E signaling recruits DNMT3B to genes silenced by CIMP via the transcriptional repressor, MAFG (Fang et al., 2014, 2016). This model was based, in part, on the ability of BRAFV600E to increase expression of MAFG and decrease expression of CIMP target gene *MLH1* in primary human fibroblasts (Fang et al., 2014). To extend this line of investigation, we ectopically expressed activated BRAFV600E in primary human fibroblasts and assessed its impact on DNMT3B and CIMP. As shown previously (Michaloglou et al., 2005), ectopic expression of BRAFV600E induced proliferation arrest and upregulation of markers of senescence (Figures 2A and 4A). However, surprisingly, activated BRAFV600E triggered downregulation of DNMT3B expression (Figure 4A). Next, we set out to ask whether oncogenic BRAFV600E is able to induce methylation changes characteristic of CIMP. Primary human fibroblasts were again infected with a lentivirus directing expression of BRAFV600E. Seven days after infection, the cells were harvested, DNA prepared, and subject to WGBS to map the DNA methylome. Principal Component Analysis poorly separated the control and BRAFV600E-expressing cells (Figure S4A). Consistent with this, although we reported previously that replicative senescent (RS) cells undergo a marked global hypomethylation (Cruickshanks et al., 2013), BRAFV600E-expressing cells showed only a very small overall gain in methylation (Figure S4B). A scatterplot of CpG methylation in control versus BRAFV600E-expressing cells also revealed no substantial differences, in contrast to a comparison of proliferating and replicative senescent fibroblasts, which revealed marked hypomethylation in the latter (Figure 4B) (Cruickshanks et al., 2013). The relative similarity of vector and BRAFV600E cells, compared with proliferating versus replicative senescent, was underscored by whole chromosome difference plot (Figure S4B). This revealed the marked hypomethylation of lamin-associated domains (LADs) in replicative senescent cells reported previously (Cruickshanks et al., 2013), but this was absent from BRAFV600E cells (Figure S4B). Indeed, although many significantly differentially methylated individual CpGs and regions (DMRs) were detected between proliferating and RS cells, very few were detected between control and BRAFV600E-expressing cells (Figure S4C). Although increasing the window size of candidate DMRs decreased the number of DMRs detected in replicative senescent cells due to merging of small DMRs, varying the size of candidate DMRs did not majorly affect the number of DMRs detected in BRAFV600E cells (Figure S4C). Most relevant to a candidate CIMP-like phenotype, BRAFV600E-expressing cells did not gain methylation at CpG islands whose methylation is diagnostic of CIMP, including *MLH1*, *CACNA1G*, *CDKN2A*, *CRABP1*, and *NEUROG1*; this is in contrast to replicative senescent cells (Figure 4C) (Cruickshanks et al., 2013). Taken together, these results do not support a role for BRAFV600E in direct induction of CIMP via DNMT3B, at least in fibroblasts.

**BRAFV600E Mutation Is Neither Necessary nor Sufficient for CIMP, and BRAFV600E Mutations and CIMP Are Both Linked to a Low Expression of DNMT3B**

In light of these functional data, we turned again to human TCGA data to shed more light on the potential interactions between BRAFV600E and DNMT3B in human CRC. First, we clustered patient samples based on methylation values of all probes at CpG islands showing significant variance across the human TCGA CRC datasets. As expected, this separated out human CRC into at least three groups, one of relatively high methylation at CpG islands, CIMP high (CIMP-H), and CIMP low (CIMP-L) and non-CIMP (Hinoue et al., 2012; Cancer Genome Atlas Network, 2012) (Figure 5A). As expected, and as reported previously (Hinoue et al., 2012; Cancer Genome Atlas Network, 2012; Weisenberger et al., 2006; Samowitz et al., 2005a), CIMP-H tumors associated with *BRAFV600E* mutation (Figure 5A) (2.16-fold enrichment,  $p < 0.001$ ). Somewhat surprisingly, however, CIMP-H tumors were anti-correlated with *DNMT3B* copy number amplification (Figure 5A) (0.21-fold enrichment,  $p < 0.001$ ). The same relationship was apparent when we analyzed only samples with microsatellite instability (MSI+), known to be enriched in CIMP (Guinney et al., 2015) (Figure S5A). To examine more closely the relationship between CIMP and *DNMT3B* amplification, we rank-ordered all human CRC samples by the degree of *DNMT3B* amplification (which correlates with expression



**Figure 5. Human *BRAFV600E*/CIMP Tumors Express Low Levels of *DNMT3B***

(A) Clustering of TCGA human CRC based on Illumina 450K DNA methylation array data (all CpGs showing variation  $>0.2$  StdDev). *BRAF* mutation and *DNMT3B* amplification status are shown below (red = mutated/amplified).

(B) CpG probes (y axis of the heatmap) clustered based on increasing *DNMT3B* copy number (x axis of the heatmap).

(C) Relationship between *DNMT3B* copy number and methylation of CIMP biomarker genes (*CACNA1G*, *RUNX3*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, *CDKN2A*). Red line marks the boundary between non-amplified and amplified *DNMT3B*. Each black dot indicates the mean methylation score in the indicated probes in one patient sample. Y axis—mean methylation, x axis—relative copy number.

(D) Boxplot of *DNMT3B* FPKM mRNA expression in human colorectal carcinoma TCGA patients subdivided by CIMP status. Mann Whitney test,  $p = 0.001$ .

(E) Violin plot showing distribution of *DNMT3B* expression levels in 207 CRC tumor samples, separated according to *BRAF* WT or *BRAFV600E* mutant.  $p$  value = 0.0001.

(Figures 1C and 1D)) and compared with CIMP. Amplification of *DNMT3B* was not associated with CIMP and, indeed, the tumors with the most overt CIMP appeared to associate with relatively low *DNMT3B* copy number (Figure 5B). Next, we directly compared CIMP score with *DNMT3B* amplification or expression. This confirmed an inverse relationship between CIMP and both *DNMT3B* amplification and expression (Figures 5C, 5D, and S5A). Finally, we directly compared *DNMT3B* expression and *BRAFV600E* mutation. This showed that *DNMT3B* expression is higher in those tumors with wild-type *BRAF* (Figures 5E, S5C, and S5D). In sum, this analysis of human TCGA confirms the strong association between *BRAF* mutation and CIMP-H but does not support a marked association between amplification and increased expression of *DNMT3B* and either *BRAF* mutation or CIMP-H and so challenges the idea that elevated *DNMT3B* contributes to *BRAFV600E*-induced tumorigenesis and associated CIMP in humans.

## DISCUSSION

Approximately 10% of human colorectal cancers harbor a *BRAFV600E* mutation, which has been demonstrated to act as a founder mutation for an alternative serrated pathway of colorectal carcinogenesis (Cancer Genome Atlas Network, 2012; Fransen et al., 2004; Vaughn et al., 2011; Giannakis et al., 2016; Carragher et al., 2010; Rad et al., 2013). However, on its own, both *in vitro* and *in vivo*, activated oncogenic *BRAFV600E* induces OIS, an established tumour-suppressive mechanism (Michaloglou et al., 2005; Carragher et al., 2010; Rad et al., 2013; Dankort et al., 2007). Consistent with a potent *BRAFV600E*-induced tumor suppressor mechanism in humans, activating *BRAFV600E* mutations are detectable in 62%–70% of hyperplastic colonic polyps: lesions traditionally considered to harbor no oncogenic potential (Yang et al., 2004; Mesteri et al., 2014). Furthermore, the existence of an OIS barrier in human serrated pathway carcinogenesis is supported by the published *in situ* data (Kriegl et al., 2011).

It is clear, therefore, that additional genetic and/or epigenetic events are required to promote neoplastic transformation in the serrated pathway, which if untreated (e.g. by endoscopic polypectomy) eventually culminates in the development of invasive carcinoma. CIMP is thought to promote tumorigenesis by bypass of tumour-suppressor mechanisms, such as silencing of *CDKN2A/INK4A* (Kriegl et al., 2011; Carragher et al., 2010). It has been recognized for some time that there is an extremely close relationship between *BRAFV600E* mutations and CIMP-H in human colorectal cancer (Ogino et al., 2009; Weisenberger et al., 2006; Inoue et al., 2015; Nagasaka et al., 2004, 2008; Hinoue et al., 2012; Cancer Genome Atlas Network, 2012). Although this association was initially correlative, it has recently been proposed that *BRAFV600E* can directly induce CIMP through the *de novo* methyltransferase, *DNMT3B* (Fang et al., 2014, 2016). Separately, elevated *DNMT3B* expression has previously been linked to the development of CIMP in both murine and human colorectal neoplasia (Carragher et al., 2010; Ibrahim et al., 2011; Noshu et al., 2009; Steine et al., 2011). Furthermore, *DNMT3B* has been demonstrated to have an oncogenic function in murine colon cancer (Lin et al., 2006; Linhart et al., 2007). Thus, the emerging dogma is of a model in which neoplastic transformation in *BRAFV600E*-mutant colorectal serrated lesions may be contributed to by the ability of this oncogene to induce a CpG island methylator phenotype, mediated by *DNMT3B*.

There are, however, several obvious paradoxes. Firstly, only a minority of colorectal lesions harboring an activating *BRAFV600E* mutation eventually progress to invasive carcinoma (Yang et al., 2004; Mesteri et al., 2014). Secondly, CIMP develops gradually with serrated pathway progression (Inoue et al., 2015). Thirdly, as many as 40% of CIMP-H tumors do not harbor an activating *BRAFV600E* mutation. Finally, approximately 10% of patients with an activating *BRAFV600E* mutation are CIMP-L or CIMP-negative (Ogino et al., 2009). Thus, a simple, linear model by which *BRAFV600E* directly induces CIMP cannot be fully reconciled with the disease biology. We therefore set out to comprehensively examine the relationships between an activated *BRAFV600E* oncogene, *DNMT3B*, and CIMP in colorectal cancer by multiple approaches.

Because activated *BRAFV600E* is a potent inducer of OIS (Michaloglou et al., 2005; Carragher et al., 2010; Rad et al., 2013; Dankort et al., 2007), we initially set out to test the idea that elevated *DNMT3B* can bypass *BRAFV600E*-induced OIS. Indeed, ectopic expression of *DNMT3B* together with *BRAFV600E* partially suppressed two phenotypes characteristic of *BRAFV600E*-induced OIS, namely proliferation arrest and, most notably, SASP. Extending these results *in vivo*, conditional inducible and knockout mouse models showed that expression of *Dnmt3b* is rate-limiting for accumulation of a lethal burden of *BrafV600E*-driven tumors in mouse intestinal epithelium. The molecular mechanisms underlying these phenotypes remain to be established. Conceivably, suppression of SASP gene expression by *DNMT3B* could depend on suppression

of BRAFV600E signaling, *de novo* methylation of SASP genes, or another mechanism. Regardless of the specific mechanism, these *in vitro* and *in vivo* lines of evidence support an oncogenic function for DNMT3B in cooperation with BRAFV600E.

However, our other analyses do not support a role for DNMT3B cooperating with activated BRAFV600E via its induction of CIMP. Since activated BRAFV600E has been proposed to directly potentiate DNMT3B-mediated CIMP and silencing of tumor suppressor genes (Fang et al., 2014, 2016), we wondered whether BRAFV600E-induced OIS might simultaneously sow the seeds of its own destruction, in the form of DNMT3B-mediated CIMP. However, in contrast to replicative senescent cells, in OIS BRAFV600E efficiently repressed expression of DNMT3B, and we observed very few BRAFV600E-induced DNA methylation changes. Finally, a closer examination of human TCGA data suggests that BRAFV600E and DNMT3B are unlikely to cooperate in tumorigenesis, and DNMT3B is unlikely to mediate CIMP. Analysis of data accessible through cBioportal, including TCGA and the largest study of 1,134 samples (Yaeger et al., 2018; Gao et al., 2013), also indicates that BRAFV600E mutation and DNMT3B amplification tend to be mutually exclusive. Taken together, these *in vitro* and *in silico* studies suggest that in BRAFV600E-driven CRC, elevated DNMT3B is likely not the cause of CIMP, and BRAFV600E and DNMT3B are unlikely to cooperate in progression of this disease. In summary, the data presented herein challenge the current model of the relationships between BRAF, DNMT3B, and CIMP in human colorectal cancer, whereby DNMT3B contributes to CIMP in association with BRAFV600E mutation and, in the most extreme form of the model, that mutant-BRAFV600E drives CIMP via DNMT3B. By multiple approaches, *in vitro* and by *in silico* analysis of TCGA, we have demonstrated that BRAFV600E mutation is associated with and can cause repression of DNMT3B. Furthermore, although DNMT3B overexpression and somatic copy number amplification are common features of human colorectal cancer, they are inversely correlated with CIMP. In functional *in vitro* studies, we obtained no evidence that DNMT3B is a driver of CIMP. Thus, although BRAFV600E and DNMT3B both harbor oncogenic potential, they do not appear to cooperate to induce CIMP and do not appear to cooperate frequently in human colorectal cancer by any mechanism.

### Limitations of the Study

Of course, there are limitations to these conclusions drawn in part from “negative data” that we have been unable to address over the time frame of this study. First, although BRAFV600E does not induce detectable methylation changes characteristic of CIMP in a bulk population of cells, we cannot exclude the possibility that it does so in a very small subset of cells that is then prone to senescence escape, clonal outgrowth, and tumorigenesis. Even so, comparison of our WGBS data reported here for OIS with our previous studies in RS suggest that CIMP is more likely to arise in RS cells linked to other cumulative molecular stresses, e.g. telomere shortening, than in OIS cells (Cruickshanks et al., 2013). Second, our analysis of human TCGA data does not preclude an association between DNMT3B expression and BRAFV600E at very early stages of disease, prior to detection and inclusion of tumors in the TCGA study. Although these limitations may go some way toward reconciling our conclusions with those of previous studies, it is important to note that prior functional studies most directly linking DNMT3B to CIMP have been performed in established colon cancer cell lines *in vitro* (Fang et al., 2014). Despite these limitations to our study, we feel that, on balance, the weight of evidence does not support a role for DNMT3B and BRAFV600E cooperating in intestinal cancer.

### METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

### DATA AND CODE AVAILABILITY

RNA-seq and DNA methylation data is available at <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126434>. The accession number for the data reported in this paper is GSE126434: secure token for access exqpcwshxsfdgd).

### SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2020.100838>.

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## AUTHOR CONTRIBUTIONS

D.J.M. performed the bulk of the experiments. I.R., C.R., and H.C. assisted with experiments. N.A.R., G.S., and T.M. performed computational data analysis. C.P. advised on experiments and generated the conditional BRAFV600E allele. K.B. advised on mouse models. A.H. in the SBP Bioinformatics Core provided statistical support. P.D.A. and D.M. conceived the study. P.D.A. wrote the manuscript. All authors edited and approved the manuscript.

## DECLARATION OF INTERESTS

The authors have no competing interests to declare.

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## Supplemental Information

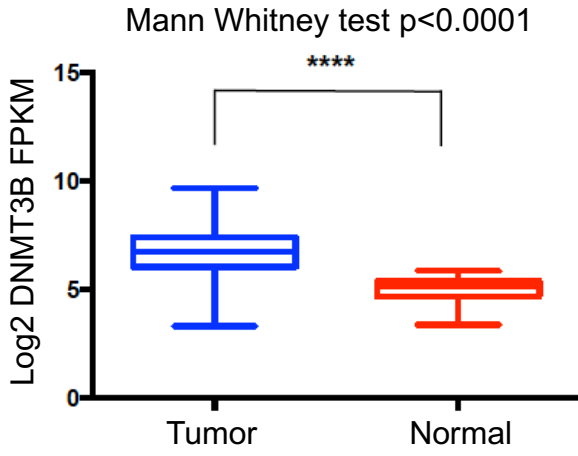
### ***DNMT3B* Oncogenic Activity in Human**

### **Intestinal Cancer Is Not Linked**

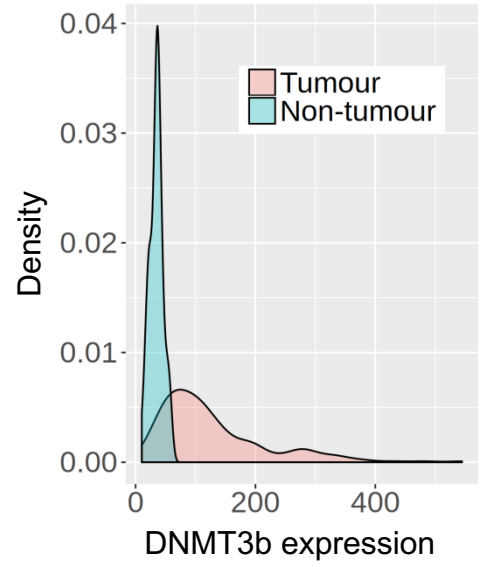
### **to CIMP or *BRAFV600E* Mutation**

**Douglas J. MacKenzie, Neil A. Robertson, Iqbal Rather, Claire Reid, Gintare Sendzikaite, Hazel Cruickshanks, Tony McBryan, Andrew Hodges, Catrin Pritchard, Karen Blyth, and Peter D. Adams**

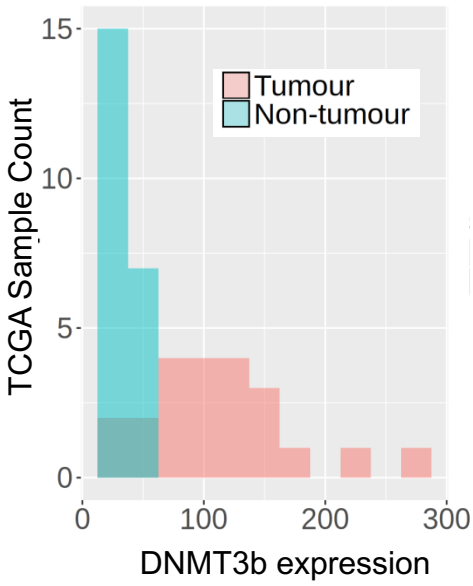
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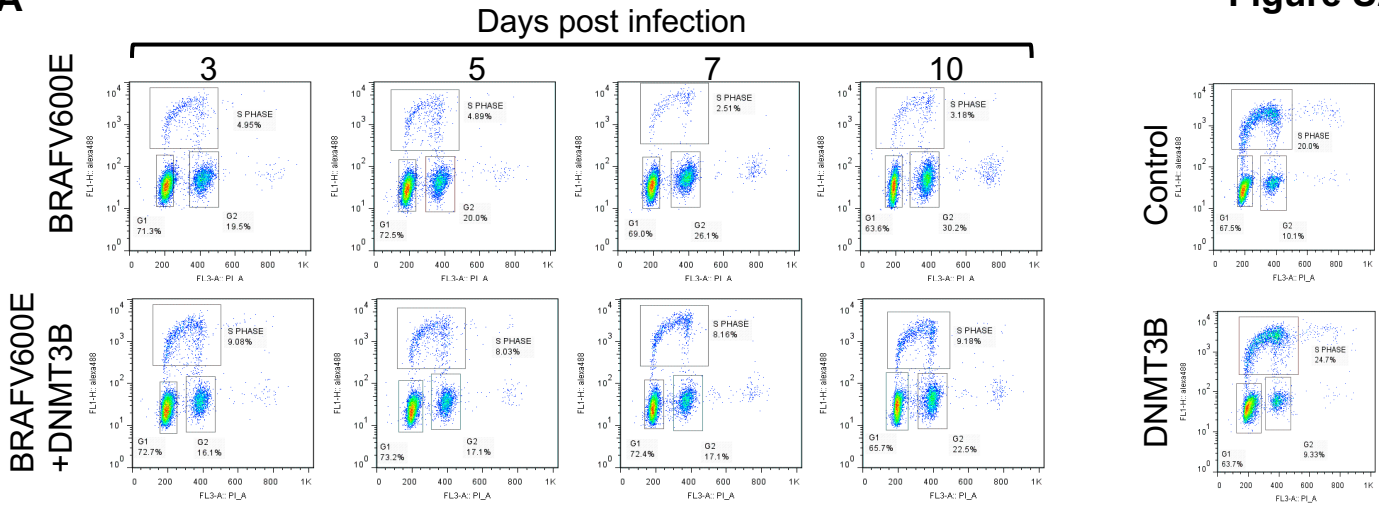


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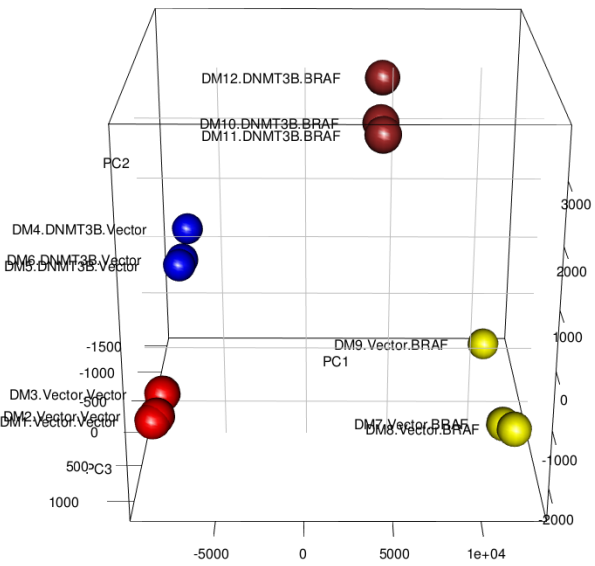




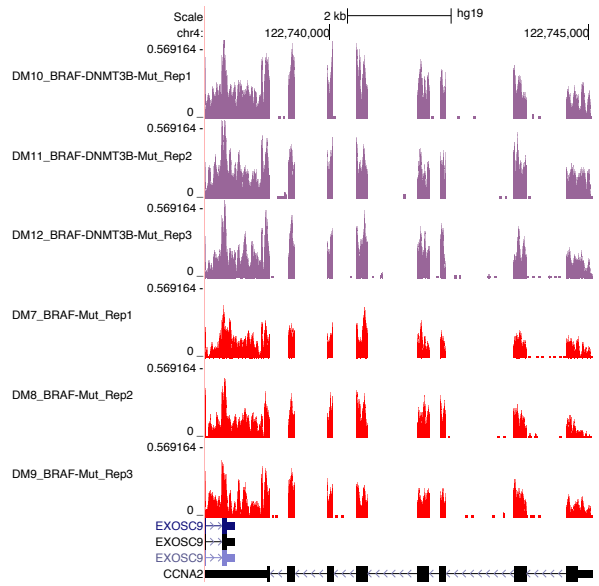
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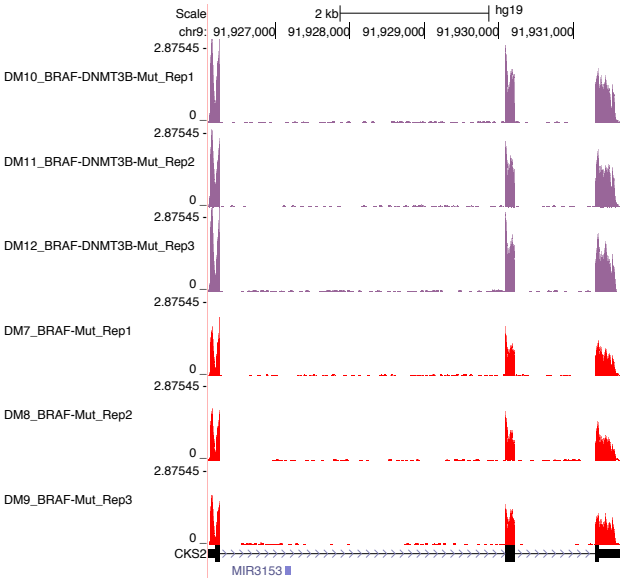
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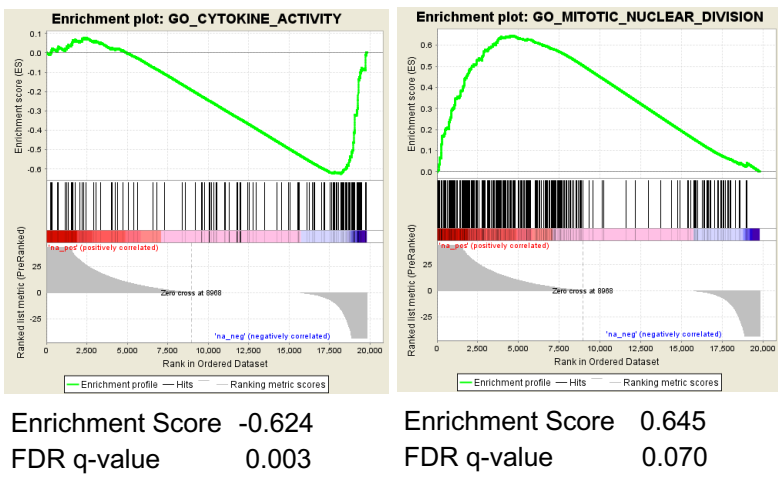
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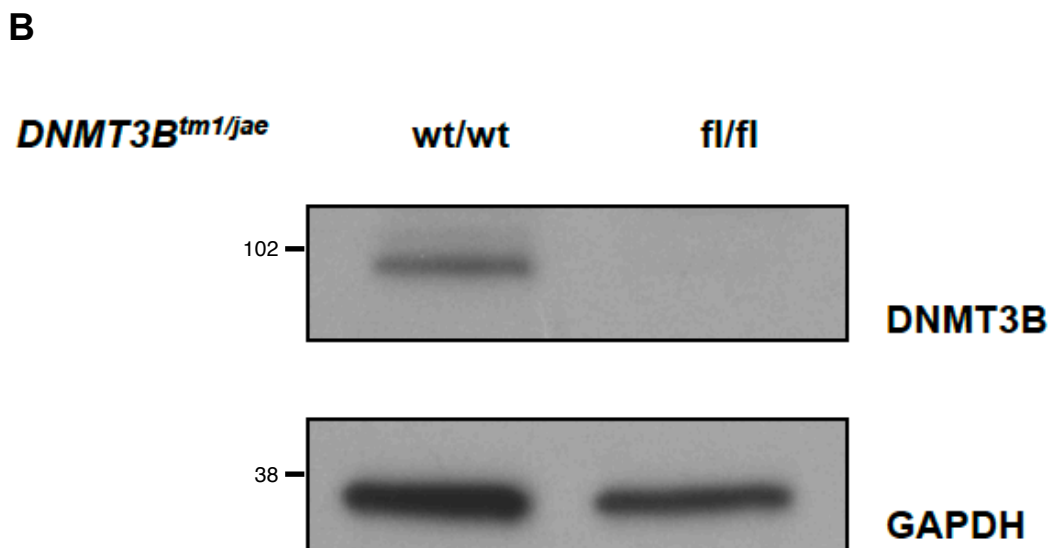
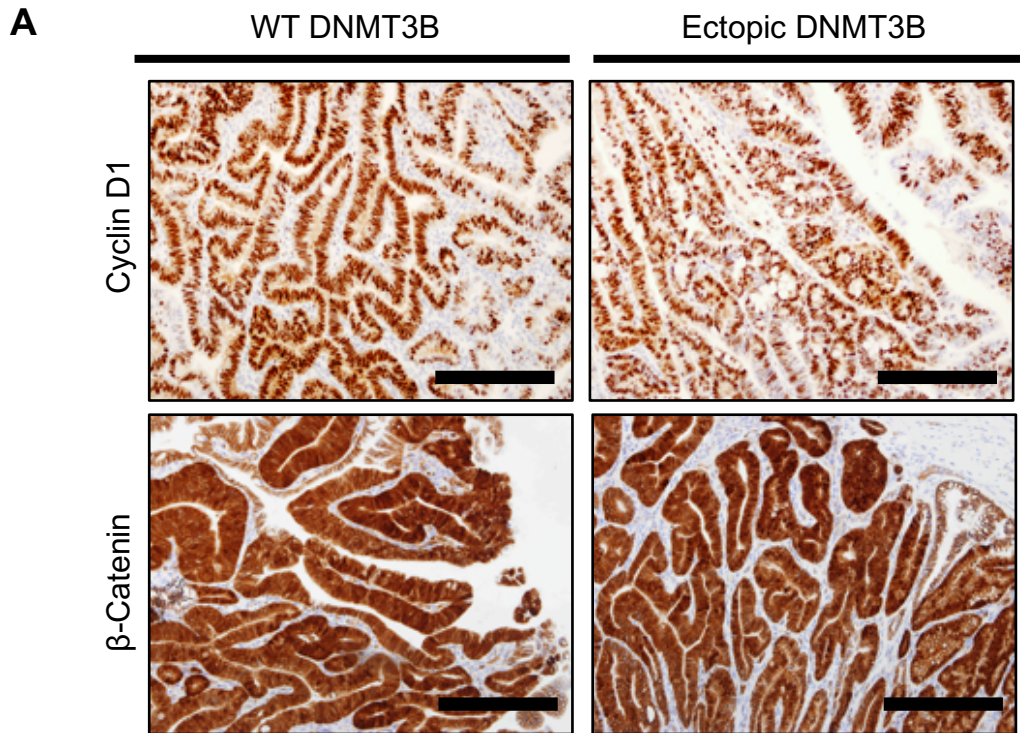


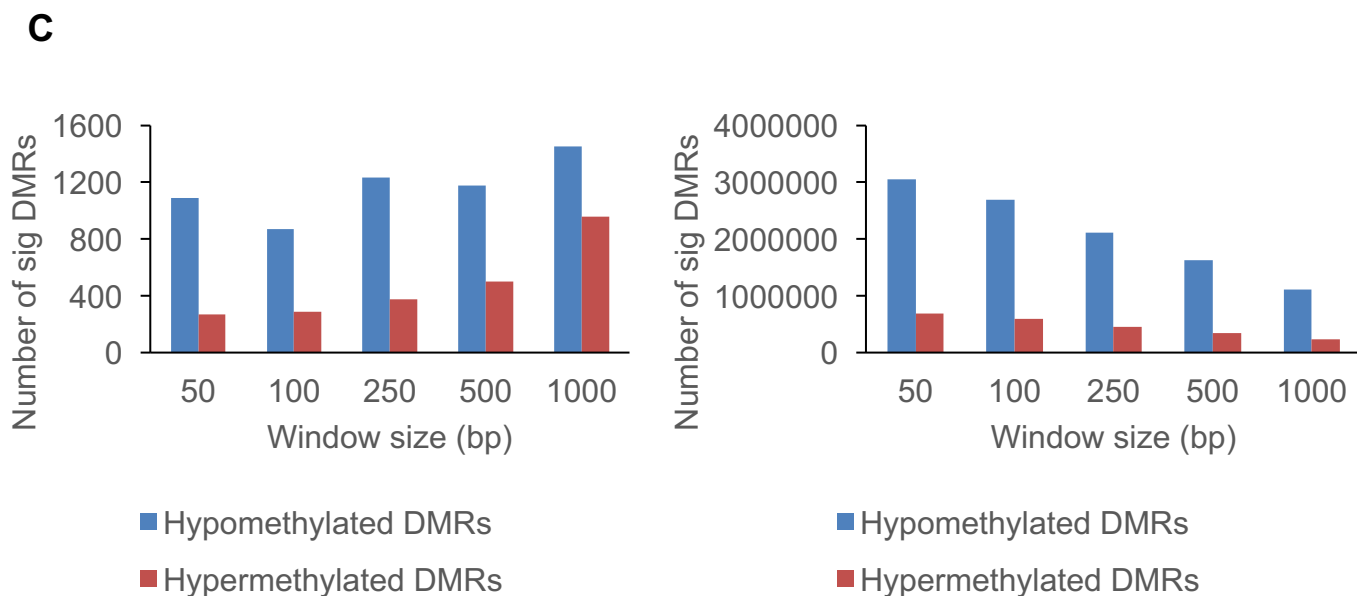
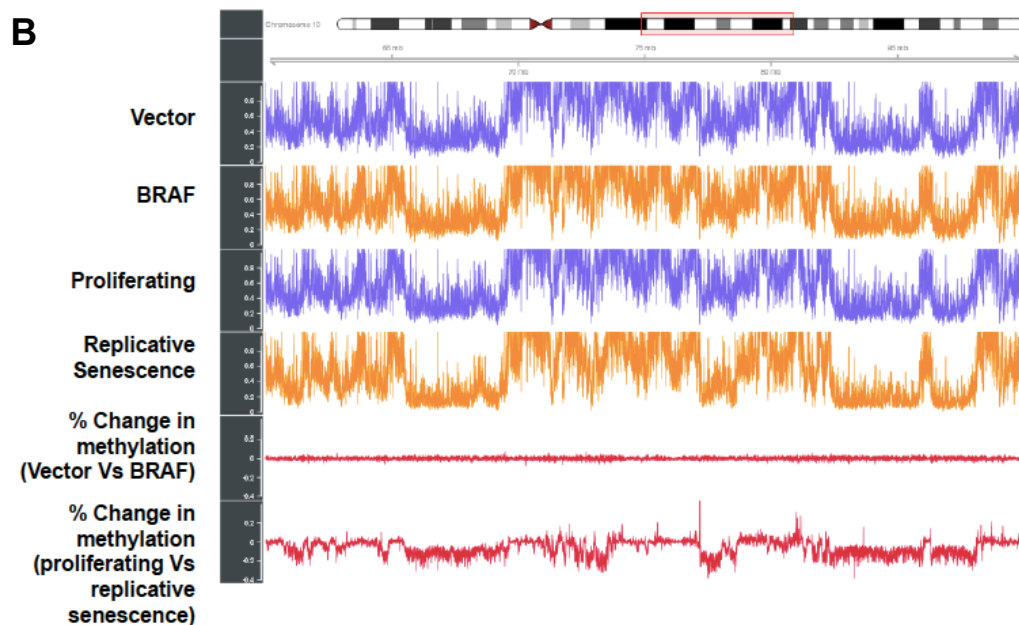
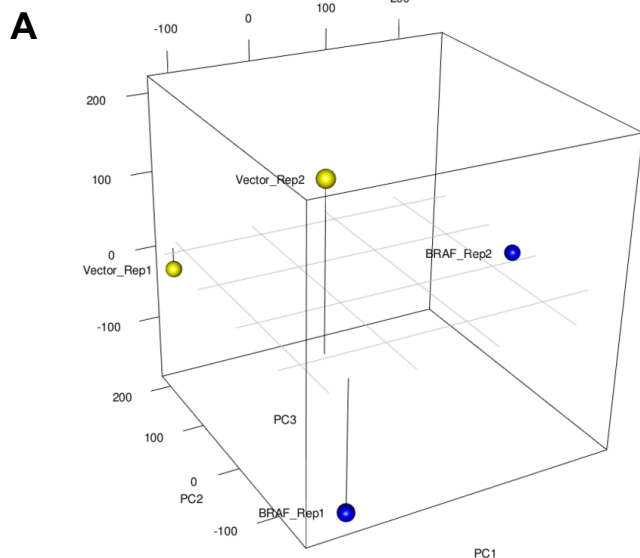
**D**

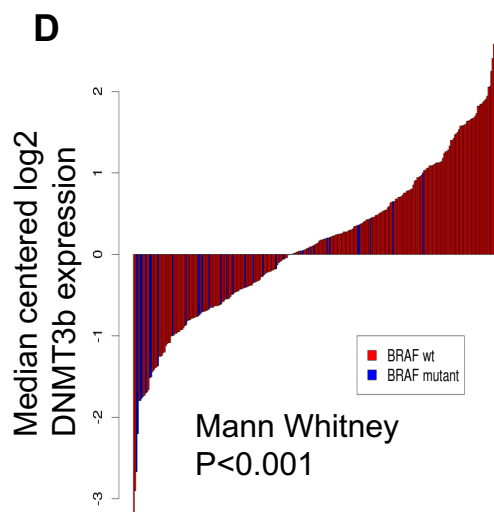
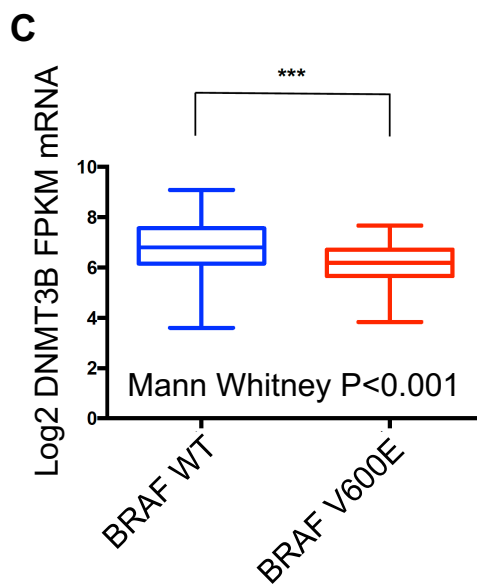
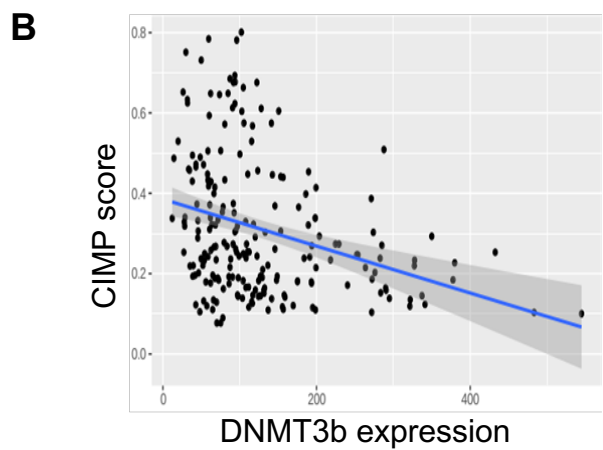
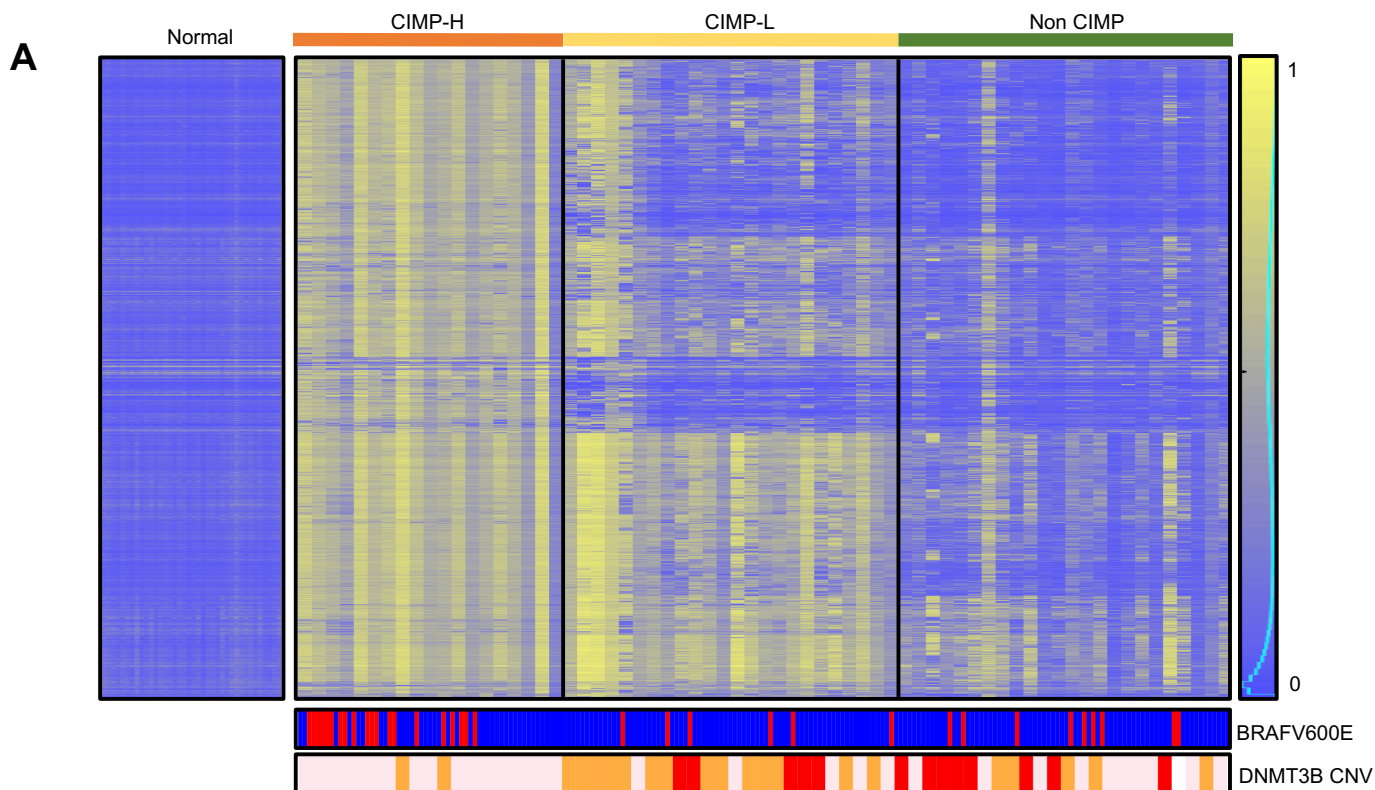


**E**









**Figure S1 (Related to Figure 1).** **A** Boxplot of relative DNMT3B mRNA expression in 22 human colorectal adenocarcinoma samples (TCGA) and matched non-tumor (surrounding normal) samples. **B** Density plot of DNMT3B expression distribution in 207 human colorectal adenocarcinoma samples (TCGA) and 43 available non-tumour samples. **C** A histogram of DNMT3B mRNA expression in 22 tumor and matched non-tumor (surrounding normal) samples.

**Figure S2 (Related to Figure 2).** **DNMT3B suppresses features of OIS.** **A** Kinetic analysis assessing the impact of ectopic DNMT3B on BRAFV600E-induced S-phase exit of primary IMR90 cells, determined by BrdU labelling. **B** PCA analysis of RNA-seq from Figure 2C-F. **C** RNA-seq reads at CCNA2 gene in BRAFV600E (bottom) and BRAFV600E+DNMT3B (top) samples. **D** RNA-seq reads at CKS2 gene in BRAFV600E (bottom) and BRAFV600E+DNMT3B (top) samples. **E** GSEA analysis of RNA-seq data showing downregulation of cytokine activity and increased mitotic nuclear division gene sets by DNMT3B.

**Figure S3 (Related to Figure 3).** **DNMT3B accelerates intestinal cancer.** **A** Representative immunohistochemistry images for cyclin D1 and b-catenin in *VilCreErT2;LSLBRAFV600E* mice with and without ectopic DNMT3B. Scale bars are 200 $\mu$ M. **B** Western blot of DNMT3B in *VilCreErT2;LSLBRAFV600E* mice with wild-type or floxed DNMT3B. MW markers in kDa.

**Figure S4 (Related to Figure 4).** **Ectopic expression of DBNMT3B does not induce CIMP.** **A** Principal component analysis was performed on both biological replicates of

BRAFV600E (BRAF) and proliferating (vector) controls. **B** Fractional methylation of a representative region of chromosome 10 in vector control cells, BRAFV600E-infected cells (BRAF), proliferating cells and replicative senescent cells. Bottom two tracks show the subtraction of BRAFV600E-control and replicative senescent – proliferating cells. **C** Number of hypo and hypermethylated DMRs in BRAFV600E-infected cells (left) and replicative senescent cells (right).

**Figure S5 (Related to Figure 5). Human BRAFV600E/CIMP tumors express low levels of DNMT3B.** **A** Clustering of TCGA human MSI+ (MSI-L and MSI-H) CRC based on Illumina 450K DNA methylation array data (all CpGs showing variation  $> 0.2$  StdDev). BRAF mutation and DNMT3B amplification status are shown below (red = mutated/amplified). **B** Relationship between DNMT3B FPKM expression and methylation of CIMP biomarker genes (CACNA1G, RUNX3, IGF2, MLH1, NEUROG1, CRABP1, CDKN2A). Each black dot indicates the mean methylation score in the indicated probes in one patient sample. Y-axis – mean methylation, x-axis – relative FPKM expression. **C** Boxplot of log<sub>2</sub> DNMT3B FPKM mRNA expression and BRAFV600E mutation status from 207 human colorectal adenocarcinoma samples (TCGA). Mann Whitney test  $p = 0.0002$ . **D** Median centred log<sub>2</sub> DNMT3B mRNA expression plot annotated by DNMT3B copy number status.

## **Materials and Methods**

### Cell culture

Human female fetal lung fibroblasts (IMR-90) were grown in DMEM supplemented with 10% FBS, 2mM L-glutamine, 10units/mL Penicillin G and 10µg/ml streptomycin. Cells were cultured in 10cm plates in a humidified atmosphere with 3% O<sub>2</sub> and 5% CO<sub>2</sub>. Passaging of cells was performed at 70-80% confluency. Culture media were aspirated, and the cells lavaged twice with sterile phosphate-buffered saline to remove remaining traces of culture media. Trypsinisation was then performed with 1mL of 1% trypsin in phosphate-buffered EDTA solution for 3 minutes at 37°C. With cell detachment confirmed by visual inspection with a microscope, the trypsin solution was neutralised by the addition of fresh culture media (supplemented with FBS) to the plate. Cells were then resuspended in culture media, passaged to fresh plates, and returned to the incubator.

### Lentivirus infections

Cells were infected with lentiviruses directing expression of DNMT3B1 and/or BRAFV600E. Viruses were made from pLenti6-puro-DNMT3B1 (or pLenti6-puro as control) and/or HIV-CS-CG-blast-BRAFV600E (or HIV-CS-CG-blast as control). HIV-CS-CG-blast plasmids were a gift of Dr. Daniel Peeper (NKI).

12x10<sup>6</sup> HEK-293T cells were seeded in 25 mL of culture medium to TC175 flasks 20 hours prior to transfection. Cells were approximately 70% confluent for transfection.

Immediately prior to transfection, 13mL of culture medium was aspirated from flasks, and the cells returned to the incubator. Lipofectamine 2000 was selected as a transfection agent for all lentivirus production. 120µL of transfection agent were added dropwise to 1.5mL of sterile

DMEM in a 15mL centrifuge tube, gently agitated and incubated for five minutes at room temperature. Concurrently, packaging plasmids (5µg plpVSVG and 8µg psPAX2) were combined with 20µg of vector genome in 1.5mL of sterile DMEM in a separate 15mL centrifuge tube. Thereafter the DNA and transfection reagent solutions were combined, gently agitated and incubated for 20 minutes at room temperature. The transfection solution was then added to the prepared HEK-293T flasks to yield a total final transfection volume of 15mL. Following transfection, HEK-293T cells were returned to the incubator for six hours, following which the transfection solution was aspirated, and a fresh 15mL of culture medium added to the flask. For all transfections, a separate green fluorescent protein (GFP) control transfection was performed in parallel to confirm transfection efficiency.

After a period of twenty hours following transfection, GFP controls were examined to confirm transfection efficiency. Lentiviral supernatant was then aspirated from transfected HEK-293T cells, reserved and stored at 4°C. A further 15mL of culture medium was added to the HEK-293T cells and returned to the incubator for a further twenty hours. The second lentiviral supernatant fraction was then combined with the first, centrifuged at 3000RPM in a table-top centrifuge, and filtered through a 0.45µM low-protein binding PVDF filter prior to concentration. Filtered viral supernatants were transferred to sterile 38.5mL polypropylene centrifuge tubes (Beckman 326823) and weights equalised by the addition of sterile PBS. Ultracentrifugation was performed at 47,000 x g in an SW-28 Beckman- Coulter ultracentrifuge for two hours at 10°C. Following centrifugation, the supernatant was discarded by gentle inversion, and the viral pellet resuspended in 100µL of sterile PBS overnight at 4°C, prior to being aliquoted and stored at -80°C.



The multiplicity of infection (MOI) for lentiviral preparations was calculated empirically by seeding cells to a 6-well plate twenty-four hours prior to transduction. Culture medium was then aspirated from each well, replaced with 1mL of fresh culture medium supplemented with polybrene at a final concentration of 8 $\mu$ g/mL, and the cells returned to the incubator for four hours prior to transduction. Concentrated lentivirus was then added to each well at 0, 1, 2, 4, 8 and 16  $\mu$ L/mL, and returned to the incubator for sixteen hours. Following transduction, the infection medium was removed from each well, and replenished with 2mL of fresh culture medium supplemented with the appropriate drug selection as determined by the construct design. The minimum volume of lentiviral construct required to yield 100% cell survival following drug-selection was then employed for all subsequent experiments. All experiments involving lentivirus were performed in their entirety under drug selection.

#### Cell cycle analysis by flow cytometry

Cell cycle analysis by fluorescence-activated cell sorting (FACS) was performed by combined 5-bromo-2'-deoxyuridine (BrdU) and propidium iodide (PI) staining. Cells were incubated with BrdU at a final concentration of 25 $\mu$ M for 4-24 hours, after which the culture medium was aspirated, the cells washed in PBS and trypsinised before being resuspended in culture medium. Following resuspension, cells were pelleted, washed in PBS and pelleted. Cell pellets were resuspended in 200 $\mu$ L of PBS at 4°C, and fixation achieved by the addition of 2mL of ice-cold ethanol prior to gentle vortexing, and storage at 4°C for a minimum of 12 hours prior to further analysis. Following fixation, cells were pelleted and resuspended in 1mL of PBS before the addition of 1mL of 4N HCl. Cells were then incubated for 15 minutes at room temperature, pelleted and the supernatant aspirated. Cells were then resuspended in 1mL of PBS, pelleted and

resuspended in 1mL of PBT. The cells were then pelleted, before being resuspended in 200 $\mu$ L of anti-BrdU antibody diluted 1:40 in PBT. Cells were incubated with primary antibody for one hour at room temperature, following which they were pelleted, washed with 1mL PBT and then pelleted before being resuspended in 200 $\mu$ L of Alexa-Fluor® 488 goat anti-mouse antibody, diluted 1:40 in PBT. Cells were incubated in the dark with secondary antibody at room temperature for one hour, following which they were pelleted, and resuspended in 1mL of PBS containing propidium iodide at a final concentration of 10 $\mu$ g/mL and RNase A at a final concentration of 250 $\mu$ g/mL. FACS analysis was performed using the FACSCalibur analyser, and subsequent analysis performed using FlowJo software.

### Western blotting

Protein lysates were prepared in 1x sample buffer (62.5mM Tris-HCl pH6.8, 2% SDS, 10% glycerol, 0.1M DTT, 0.01% bromophenol blue). Media was aspirated from tissue culture plates, and the cells washed in 10mL of sterile PBS. The PBS was then aspirated, and the cells scraped into boiled 1x sample buffer using a plastic scraper and transferred to an Eppendorf tube. The contents were vortexed vigorously, aspirated through an 18-gauge needle, and boiled for five minutes at 100 °C. Lysates were then centrifuged for 5 minutes in a bench-top microcentrifuge at 12,000 x g, and flash-frozen in a dry-ice ethanol bath. Protein lysates were stored at -80 °C. The protein concentration of lysates in 1 x sample buffer was determined using the Qubit® protein assay kit. Pre-diluted BSA standards provided with the kit were used to calibrate the fluorometer prior to use, with the addition of 1 $\mu$ L of 1x sample buffer to each of the three protein standards. Quantification was undertaken in triplicate for all samples, and a mean protein concentration calculated. Electrophoresis of proteins was undertaken using pre-cast SDS-PAGE gradient gels.

Protein lysates were boiled, and 25µg of total protein loaded per well. Sample volumes were equalised using 1x sample buffer. Electrophoresis was undertaken using the Bio Rad Mini-PROTEAN® Tetra cell system. Loaded gels were assembled in a Mini-PROTEAN® Tetra cell, which was filled with 1x SDS-PAGE running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3), and protein separation achieved by application of a constant current of 200 V. The total electrophoresis time was varied depending on the molecular weight of the peptide of interest. Following electrophoresis, proteins were immobilised to polyvinylidene fluoride (PVDF). Prior to transfer, membranes were soaked in methanol for 30s with gentle agitation, rinsed with dH<sub>2</sub>O, and placed in cold 1 x blotting buffer (25mM Tris pH 8.3, 192 mM glycine, 0.01% SDS, 20% methanol). A transfer cassette was assembled, incorporating the gradient gel and PVDF membrane, and placed in a Mini Trans-Blot® tank containing 1x blotting buffer. Protein transfer was achieved by application of a constant current of 100V, 350mA for 1 hour. Antibodies used : β-actin, Sigma A1978, WB 1:200,000. BRAF, Santa Cruz SC5284, WB 1:200. Cyclin A, Santa Cruz SC751, WB 1:500. DNMT3B, Imgenex IMG184A, WB (mouse) 1:1000 and Santa Cruz SC10236, WB (human) 1:500. EZH2, Cell Signalling 5246, WB 1:1000. GAPDH, Cell Signalling 14C10, WB 1:2000. p16INK4A, BD Biosciences BD51-1352GR, WB 1:1000. p27KIP1, Cell Signalling 2552s, WB 1:1000. ppRb (Ser807/811), Cell Signalling 9308s, WB 1:1000. Total Rb, Cell Signalling 9309s, WB 1:1000.

### Immunohistochemistry

Immunohistochemistry was performed by the histology service at the Beatson Institute for Cancer Research, Glasgow. Following embedding in paraffin, 4µm sections were cut using a microtome. Sections were first dewaxed by immersion in xylene for ten minutes, followed by

rehydration through serially graded ethanol solutions. Tissues were then washed in deionised water for 5 minutes prior to antigen retrieval. Antigen retrieval was achieved by immersion of sections in boiled 1x pH6 sodium citrate antigen retrieval buffer for 25 minutes, and sections then allowed to cool for 30 minutes, prior to a single wash in Tris-buffered Tween. Endogenous peroxidases were then quenched by immersion in a solution of 2% (v/v) hydrogen peroxide in methanol for 15 minutes. Following quenching of endogenous peroxidases, samples were washed with Tris-buffered Tween. Samples were then blocked by immersion in Tris-buffered Tween 1% BSA for 30 minutes. Following blocking, sections were incubated at room temperature for 30 minutes with primary antibody prior to 2 washes in Tris-buffered Tween. Samples were then incubated with an appropriate HRP-linked secondary antibody for a further 30 minutes at room temperature prior to a further 2 washes in Tris-buffered Tween. Samples were then developed with 3,3'-diaminobenzidine tetrahydrochloride for 10 minutes, washed in deionised water for 1 minute, and counterstained for 7 minutes with Haematoxylin Z. Sections were then "blued" by submersion for 1 minute in Scott's water, washed for a further 1 minute in deionised water then dehydrated through serially graded alcohols prior to mounting and coverslip application. Antibodies used :  $\beta$ -catenin, BD Biosciences 610154, IHC 1:1200. Cyclin D1, Dako M3642, IHC 1:50. DNMT3B, Imgenex IMG184A, IHC 1:300. Ki67, Thermo Fisher RM9106, IHC 1:200.  $\gamma$ H2AX, Cell Signalling 9718, IHC 1:50. IL1 $\alpha$  R&D Systems AF400, IHC 1:100. p21WAF, CNiO Institute T3413, IHC 1:4. Sox9, Millipore AB5535, IHC 1:700

#### Whole genome bisulfite sequencing

Whole genome bisulfite sequencing was performed by the Beijing Genomics Institute (BGI, Shenzhen, China). Following DNA quantification and assessment by agarose electrophoresis,

genomic DNA was sent for bisulfite sequencing. Briefly, DNA was fragmented by sonication to a mean size of approximately 100-300bp, followed by “endblunting”, the addition of dA to the 3'-end and adapter ligation (the latter allows assessment of the efficiency of bisulfite conversion). Bisulfite-conversion was then performed using the Zymo EZ DNA methylation-Gold kit, which converts unmethylated cytosines to uracils. Bisulfite-treated DNA was then subjected to 90bp paired-end sequencing using an Illumina HiSeq-4000 machine. The WGBS analysis pipeline has been described previously, and is summarised below (Cruickshanks et al., 2013). The quality of sequenced reads was first tested using FastQC (version 0.10.0). Thereafter, adapters and low-quality sequence tails were excluded using trim-galore (version 0.3.0). Alignment of sequence reads to UCSC (hg19) genome was performed using Bismark (version 0.10.1) based on the Bowtie2 aligner (version 2.1.0) (Krueger and Andrews, 2011). The methylation status of each aligned sequence tag was then inferred by comparison to the unconverted reference genome. Potential duplicate reads (defined as those reads for which both ends of a given fragment align to the same genomic position on the same strand) were then removed to control for PCR bias. Furthermore, the exclusion of reads with >3 methylated cytosines in a non-CpG context controlled for incomplete bisulfite-conversion. Methylated cytosines were then identified by aggregation of processed reads on a per CpG basis, followed by the collapsing of CpG dyads into a single score for the cytosine on the forward strand. Differentially-methylated CpGs were then identified using a two-tailed Fisher's exact test. Only those CpGs with  $\geq 10$  reads within each condition tested were considered for statistical analysis. False-positives were controlled at a rate of 5% by means of FDR-correction of p-values using the Benjamini-Hochberg FDR function. Global percentage methylation was determined by division of the total number of methylated counts by the total number of methylated and unmethylated

counts in the entire data set, followed by multiplication by 100 for each biological condition. Differentially-methylated regions (DMRs) were identified using 500bp sliding-windows. At each window, DMR significance was determined using Fisher's exact test, together with a  $\chi^2$  test of heterogeneity between biological replicates for each condition tested. Multi-sample correction was performed using the Benjamini-Hochberg FDR function, and DMRs defined as those windows with an FDR-corrected p-value  $< 0.05$  and nonsignificant heterogeneity between biological replicates ( $\chi^2$  test  $p > 0.05$ ). Percentage methylation at any particular DMR was then calculated by division of the total number of methylated cytosines by the total number of methylated and unmethylated cytosines, multiplied by 100. Genomic feature overlaps were calculated on a per-base pair basis between two data sets, and the genomic average expected overlap calculated using a permutation test. For composite methylation profiling, the midpoints of a series of regions of interest were taken and used as a base. The area around the midpoint of each feature was then split into 100bp windows spanning 2.5kb upstream and downstream of this central position. The average methylation proportion was then calculated for each window for every genomic feature. A global mean was then taken for each window across all features to aggregate a composite of the mean methylation per window across all probed genomic features. Smoothed methylation plots were generated using the BSmooth algorithm from the bsseq package. Methylation levels were kernel-smoothed, and plotted against a range of DMR and annotated features in bed format (Hansen et al., 2012).

### RNA-seq

RNA was extracted from adherent cells using the RNeasy Mini Kit according to the manufacturer's protocol. In summary, cell pellets were lysed on ice into buffer RLT

supplemented with  $\beta$ -mercaptoethanol. Cell lysates were then homogenised by centrifugation in QiaShredder columns. RNA isolation was then performed using RNeasy mini kit columns, with residual DNA removed by on-column treatment with DNaseI. Following wash steps, RNA was eluted into RNase-free distilled water, and stored at  $-80^{\circ}\text{C}$  prior to subsequent analysis. Prior to sequencing, RNA quantification was performed using the Qubit RNA HS assay kit, and qualitative assessment performed using the Agilent RNA ScreenTape assay according to the manufacturer's instructions. RNA-sequencing was performed in the sequencing facility at the Beatson Institute for Cancer Research, Glasgow. RNA was prepared for sequencing using the TruSeq RNA sample preparation kit. RNA is first purified, fragmented and reverse transcribed to produce cDNA. Following the removal of any remaining RNA, single stranded cDNA is converted to double stranded cDNA. Blunt-end DNA fragments are then generated. The subsequent addition of an "A" base to blunt ends prepares them for ligation to the sequencing adapters, which possess a "T" base overhang at the 3' end, thus providing a complementary overhang for adapter ligation. Adapters possess sequencing primer hybridisation sites for single, paired-end and multiplex reads. PCR is then employed to selectively enrich for those DNA fragments in possession of an adapter molecule on both ends (12 cycles only to prevent skewed representation in the library) and then libraries validated using the Agilent DNA 1000 kit. Libraries were then sequenced using the Illumina GAIIX sequencer at the Beatson Institute for Cancer Research, Glasgow. Paired-end reads were aligned to the human genome (hg19) using a splicing-aware aligner (TopHat2) (Kim et al., 2013). Only unique reads were retained. Reference splice junctions were provided by a reference transcriptome (Ensembl build 73) and novel splicing junctions defined by the detection of reads that spanned exons not in the reference annotation. True read abundance at each transcript isoform was assessed using HTSeq (Python)

before determination of differential expression using CuffDiff, from which differential expression and splicing can be derived (Trapnell et al., 2013). Significance was determined using an FDR corrected p-value of  $<0.05$ . Heatmaps were created in R using the ggplots package.

### TCGA data analysis

All tiers of TCGA data, including Illumina Methylation arrays, RNA-seq, copy-number analysis, microsatellite instability (MSI) and mutational (signatures and processed MAF variant files) data was downloaded from the legacy TCGA data access portal (now at <https://portal.gdc.cancer.gov/>). We processed the data to ensure that all participants featured in subsequent analyses had all aforementioned tiers of data available by merging sample identifiers; leaving a sample size of 207 participants.

To classify CIMP positive patients, we reduced our methylation matrix by including only high variance probes ( $\sigma \geq 0.2$ ) and those that featured in CpG islands. We then clustered the remaining probes initially using the “fastcluster” R package and then using RPMM, before classifying participants as CIMP-H, CIMP-L or non CIMP by cutting the cluster dendrogram to select those appropriate samples. Successive copy-number and mutational heatmaps utilized the initial CIMP clustering.

Focal copy-number amplification or deletion events were classified where the segmentation mean  $[\log_2()-1]$  of copy-number surpassed 0.2 or -0.2 respectively, with regression analyses performed using the “lm” function from R-Core. MSI status was called as per TCGA data standards (and as performed by the TCGA consortium): microsatellite-stable (MSS), low level MSI (MSI-L) if less than 40% of markers were altered, and high level MSI (MSI-H) if



more than 40% of the markers were altered (per

[http://bioinformaticsfmpr.github.io/TCGAbiolinks/clinical.html#microsatellite\\_data](http://bioinformaticsfmpr.github.io/TCGAbiolinks/clinical.html#microsatellite_data)).

### In vivo work

All experiments were carried out in accordance with the requirements of the UK Home Office guidelines under the auspices of Personal License I6C161323, and Project Licence to PDA 70/8354. Across all experiments, approximately equal numbers of male and female mice were used (46 male, 50 female). Mice were typically 8-12 weeks old at time of transgene induction. No effects of sex were observed on the results. Routine husbandry of all colonies, including nutrition, hydration, setting up of matings, weaning and ear-notching for genotyping was undertaken by Biological Services technicians at the Beatson Institute for Cancer Research. Mice were fed a standard diet with water *ad libitum*. The following transgenic alleles were used in this study: Tg(Vil-cre/ERT2)23Syr VilCreErT2, Owen Sansom Laboratory (JAX 020282). Braftm1Cpri LSLBRAFV600E, Catrin Pritchard Laboratory. B6;129S4-DNMT3Btm1Jae/Mmnc (Dnmt3bfl/fl), MMRRC. B6.Cg-Gt(ROSA)26Sortm1(rtTA\*M2)Jae/J R26-M2-rtTA, Jackson Laboratory. B6.Cg-Coll1a1tm9(tet0-DNMT3b\_i1)Jae/J Coll1a1-tetO-DNMT3B1, Jackson Laboratory. Routine genotyping was undertaken at weaning (6 weeks age). Ear notches were obtained from mice by technical staff in the Beatson Institute for Cancer Research animal facility. Automated PCR genotyping of mouse strains was undertaken by Transnetyx.

*VilCreEr<sup>T2</sup>,<sup>LSL</sup>Braf<sup>V600E</sup> +/-;R26-M2-rtTA +/-;Coll1a1-tetO-Dnmt3b1 +/-* cases (n=13) were induced by a single intraperitoneal injection of 80mg/kg tamoxifen and 0.5mg/mL of doxycycline hyclate in 1% sucrose (administered *ad libitum* in the drinking water and changed three times weekly). Wild-type DNMT3B (i.e. endogenous expression) controls (n=26) for this

experiment were either of the same genotype as cases (i.e. *VilCreEr<sup>T2</sup>; LSLBraf<sup>V600E</sup> +/-; R26-M2-rtTA +/-; Colla1-tetO-Dnmt3b1 +/-*), or wild-type for either *R26-M2-rtTA* or *Colla1-tetO-Dnmt3b1*. Control mice were induced by a single intraperitoneal injection of 80mg/kg tamoxifen, but were administered 1% sucrose in the drinking water without the addition of doxycycline. As additional controls, “DNMT3B only” mice (n=31) were also generated. “DNMT3B only” controls were a mixture of *VilCreEr<sup>T2</sup>; R26-M2-rtTA +/-; Colla1-tetO-Dnmt3b1 +/-*, induced in the same fashion as cases and *VilCreEr<sup>T2</sup>; LSLBraf<sup>V600E</sup> +/-; R26-M2-rtTA +/-; Colla1-tetO-Dnmt3b1 +/-* mice, induced with doxycycline but not tamoxifen. Both “DNMT3B only” control genotypes were therefore “*Braf* wild type”.

Mice were monitored for clinical signs of illness, including weight loss, hunching and pedal pallor (anaemia). Mice were sacrificed when they exhibited >20% weight loss, or exhibited two or more clinical signs of illness. Mice were culled by exposure to carbon dioxide gas in a rising concentration in accordance with the requirements of the Animals (Scientific Procedures) Act 1986. Death was confirmed by confirmation of permanent cessation of the circulation and dislocation of the neck. The skin was shaved and prepared with ethanol. The abdomen was then opened through a midline laparotomy, and the abdominal viscera carefully inspected for evidence of tumour formation or metastatic disease. The stomach was divided at the gastro-oesophageal junction, and the small intestine dissected from its mesentery from gastro-duodenal junction to the ileo-caecal junction. The colon was separately dissected from its mesentery, and dissection continued to the pelvis where it was amputated at the anorectal junction. Both the small intestine and colon were flushed with PBS, before being opened longitudinally in their entirety with iris scissors. Following harvest of intestinal tissues, a full necropsy was undertaken to exclude other pathology. For tumour scoring and routine haematoxylin and eosin staining,

tissues were fixed in Methacarn (60% Methanol, 30% chloroform, 10% glacial acetic acid) for 20 hours. Thereafter, the intestine and colon were wound into a “Swiss roll” in 10% neutral buffered formalin and processed for histology. For immunohistochemistry, the small intestine and colon were “pinned out” in a paraffin wax dish containing 10% neutral buffered formalin. After 24 hours, the intestine and colon were wound into a “Swiss roll” in 10% neutral buffered formalin, and processed for histological analysis by the histology service at the Beatson Institute for Cancer Research, Glasgow.

### Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc. La Jolla, California, USA). We used non-parametric tests, such as Mann Whitney U and Kruskal Wallis, to avoid making assumptions about the probability distribution of the data. Details of statistical tests used in individual experiments are detailed in the respective figure legends. Statistical analysis of RNA-sequencing is described in more detail in the description of these methods above. Several different statistical tests were used in this manuscript. When comparing expression levels, we have selected the Mann-Whitney U test where samples are independent and randomly selected from the subpopulation and this also accounts for the complex multimodality of the distributions of RNA expression data. Correlation testing of continuous variable was considered using the Pearson correlation test. Where we have tested the significance of cell-cycle stage with BRAF status *in vitro*, we used the Kruskal-Wallis test - of ranked and nominal variables where the normality assumption is not satisfied. Quantifying differential expression in the RNA-sequencing experiment was conducted using CuffDiff.

### Data and Software Availability

RNA-seq and DNA methylation data is available at

<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126434> (GSE126434 : secure token for access exqpcwysfxsfdgd)

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