Targeting bivalency de-represses Indian Hedgehog and inhibits self-renewal of colorectal cancer-initiating cells

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In embryonic stem cells, promoters of key lineage-specific differentiation genes are found in a bivalent state, having both activating H3K4me3 and repressive H3K27me3 histone marks, making them poised for transcription upon loss of H3K27me3. Whether cancer-initiating cells (C-ICs) have similar epigenetic mechanisms that prevent lineage commitment is unknown. Here we show that colorectal C-ICs (CC-ICs) are maintained in a stem-like state through a bivalent epigenetic mechanism. Disruption of the bivalent state through inhibition of the H3K27 methyltransferase EZH2, resulted in decreased self-renewal of patient-derived C-ICs. Epigenomic analyses revealed that the promoter of Indian Hedgehog (IHH), a canonical driver of normal colonocyte differentiation, exists in a bivalent chromatin state. Inhibition of EZH2 resulted in de-repression of IHH, decreased self-renewal, and increased sensitivity to chemotherapy in vivo. Our results reveal an epigenetic block to differentiation in CC-ICs and demonstrate the potential for epigenetic differentiation therapy of a solid tumour through EZH2 inhibition.
The cancer stem cell model posits that a subset of tumour cells, cancer stem cells or cancer-initiating cells (C-IC), are endowed with the capacity for self-renewal, which is characterised by the ability to initiate and sustain tumour growth in xenotransplantation assays\(^5\). C-ICs have been described in multiple cancer types, including colorectal cancer (CRC), and are often associated with chemotherapy resistance and disease recurrence\(^6\). In CRC, an intestinal stem cell signature of the tumour correlated with decreased patient survival and predicted relapse\(^7\), further stressing the clinical relevance of stemness in this disease. The original model of C-ICs as static entities at the apex of a cellular hierarchy has been challenged by evidence of phenotypic plasticity involving interconversion from non-C-IC to C-IC states in response to both intrinsic and extrinsic stimuli\(^8\). Such reprogramming of cellular identity within a tumour cell population raises the question of whether C-ICs retain the capacity to terminally differentiate into non-C-ICs. Thus, further work is required to understand the molecular mechanisms that drive C-ICs to irreversibly exit the stem-like state, and whether differentiation therapy represents a clinical opportunity to target solid tumour C-ICs.

Recent evidence indicates that epigenetic regulation of transcriptional programmes is a key driver of self-renewal capacity, the defining feature of the C-IC state. In the context of CRC, epigeneticists have been shown to contribute to the C-IC state by modulating key pathways such as Wnt signalling, where high activity has been shown to designate the colorectal cancer-initiating cell (CC-IC) population\(^9\). One of the initial examples of an epigenetic regulator influencing CRC growth was LSD1, an enzyme catalyzing the demethylation of H3K4me1/2 and H3K9, which contributes to CC-IC self-renewal through downregulation of the Wnt pathway antagonist DKK1\(^9\). Another example is Bmi1, a subunit of the PRC1 complex, which binds to H3K27me3 to repress transcription and has been well documented for its role in tumorigenicity. Bmi1 inhibition reduced self-renewal of CC-ICs by decreasing Wnt pathway activity through reduction of β-catenin levels\(^7\). These initial studies point to the importance of epigenetic regulators in maintaining CC-IC self-renewal through their contribution to Wnt pathway activation. However, a key question that remains is whether CC-ICs can be reprogrammed through targeting epigenetic modifiers to alter cell fate specification and drive terminal differentiation.

To better understand how epigenetic regulation defines and maintains the CC-IC state, we used a collection of epigenetic chemical probes\(^8\) to interrogate a panel of patient-derived CRC models. We identified Enhancer of Zeste Homologue 2 (EZH2), as a key contributor to the CC-IC state. EZH2 is the catalytic subunit of Polycomb Repressive Complex 2 (PRC2) which tri-methylates H3K27, and is upregulated in multiple cancer types including CRC, where its expression level correlates with worse prognosis\(^9,10\). EZH2 expression levels have also been shown to be higher in C-ICs from a broad range of tumours, including breast, ovarian and leukaemia\(^11\).

EZH2 has an established role in maintaining embryonic stem cells (ESCs) in an undifferentiated state through repression of lineage commitment and differentiation genes\(^12,13\). In ESCs, the promoters of genes involved in differentiation are often found in a bivalent state, defined by the simultaneous presence of both the activating histone mark H3K4me3 and the repressive histone mark H3K27me3. The presence of both marks results in silencing of developmental programmes and maintenance of pluripotency\(^14\). These bivalent genes are thought to be poised for expression upon loss of repressive H3K27me3, which triggers transcription of differentiation programmes\(^12,14\). Therefore, as the catalytic subunit of PRC2, EZH2 plays a key role in maintaining bivalency at lineage commitment genes in ESCs.

Initial work in cancer suggests that bivalency may be playing a similar role in the context of cancers with activating mutations in EZH2, such as germinal centre B cell lymphomas, where H3K27me3-mediated repression of bivalent promoters results in suppression of differentiation\(^15\). In contrast, the role of bivalency in tumours that overexpress EZH2 but lack activating mutations has not been established. Instead, a number of other bivalency-independent mechanisms have been identified in a tissue-specific manner. In the context of CRC, previously published work using commercially available CRC cell lines showed that EZH2 inhibition resulted in downregulation of the Wnt/β-catenin pathway and decreased CC-IC tumour-initiating capacity and mammosphere formation\(^16\). Despite extensive research on EZH2 in cancer, there is currently no evidence showing that similar to ESCs, EZH2 represses differentiation programmes in C-ICs through maintenance of bivalency. If bivalency is maintaining the C-IC state through repression of differentiation programmes, it will be important to determine whether, similar to ESCs, this state is reversible upon loss of H3K27me3. Furthermore, a better understanding of the role of bivalency in the context of cancer could lead to novel epigenetic therapeutic strategies to promote C-ICs to terminally differentiate.

Here, we identify a role for EZH2 in maintaining the CC-IC state, in part, through repression of the bivalent promoter of Indian Hedgehog (IHH), a member of the Hedgehog pathway and a canonical marker of colonocyte differentiation in normal intestinal development. Our results demonstrate that, similar to ESCs, CC-ICs depend on bivalency to maintain self-renewal through transcriptional repression of a lineage-specific differentiation gene, and that this state can be targeted through EZH2 inhibition.

**Results**

**Targeting EZH2 reduces growth of patient-derived CC-ICs.** Utilising a previously established serum-free, growth factor-enriched spheroid culture that enriches for CC-ICs in patient-derived CRC samples\(^17\), we screened a collection of 22 selective chemical probes that inhibit epigenetic regulatory proteins using viable cell count as a readout (Supplementary Figure 1a). Each probe was used at a single concentration aiming for an estimated cellular IC\(_{50}\) concentration\(^18\) (Fig. 1a, Supplementary Table 1). Three chemical probes reduced the growth of a patient-derived CC-IC enriched culture, POP92, by more than 50% (Fig. 1a). These growth inhibitory probes included the BET Bromodomain BRD2/3/4 inhibitor JQ1, an antagonist of the Bromodomains of BRPF1/2/3, and the EZH1/2 inhibitor UNC1999\(^19\). The epigenetic probes screen was repeated in a broader range of CRC samples grown as patient-derived organoids (PDO), a system recently shown to maintain the heterogeneity of primary patient samples\(^20\) (Supplementary Table 2). We consistently observed strong growth suppression, with both BET and EZH2 inhibitors (Fig. 1b). Given the important role of EZH2 in maintaining self-renewal of ESCs through silencing of differentiation programmes, we focused on investigating EZH2 as a potential driver of CC-IC self-renewal and tumour growth. We next confirmed a dose-dependent reduction in growth of the PDOs (Supplementary Figure 1b-c) and CC-IC enriched cultures (Fig. 1c) upon treatment with UNC1999, whereas the chemically similar, but inactive compound UNC2400\(^19\) showed no effect. The decrease in cell growth following UNC1999 was concomitant with the decrease in cellular levels of H3K27me3 and H3K27me2, with an IC\(_{50}\) of 3 μM (Fig. 1d and Supplementary Figure 1d).

Additional EZH2 inhibitors such as GSK343 and GSK126 are available and have greater selectivity for EZH2 over EZH1\(^21\). Treatment with GSK343 and GSK126 had a similar reduction in viable cell count compared with UNC1999 across three CC-IC
enriched models, supporting the role of EZH2, in maintaining growth of CC-ICs (Supplementary Figure 1e). Furthermore, genetic knockdown of EZH2 using two different shRNAs also reduced the growth of CC-IC enriched cultures (Fig. 1e–f), in line with the results obtained with the EZH2 inhibitors. Importantly, following EZH2 knockdown CC-IC enriched cultures showed no further reduction in the number of viable cells in the presence of UNC1999 (Fig. 1g). Taken together, this confirms that the growth inhibitory effect of UNC1999 is a consequence of EZH2 inhibition.

**Fig. 1** EZH2 inhibition suppresses growth in patient-derived 3D colon cancer models. a Viable cell count of a patient-derived spheroidal culture, POP92, treated with chemical probes for the indicated epigenetic targets for 7 days. Data shown are mean calculated as percentage of DMSO control (n = 4, ± SEM, one-way ANOVA). b Epigenetic screen performed on six patient-derived organoid models. Data are represented as percentage of DMSO control. c Two patient-derived (POP92 and POP66) and one commercial cell line (LS174T) grown in spheroid CC-IC enriching cultures were treated with UNC1999 (left) and its negative control UNC2400 (right) for 7 days, and viable cell count was measured and normalised to control. Data shown are mean (n = 4, ± SD, two-way ANOVA). d Viable cell count of a patient-derived spheroidal culture, POP92, a

**Targeting EZH2 reduces CC-IC growth and self-renewal.** Previous reports have shown that EZH2 is highly expressed in cancer compared with normal tissue. To assess EZH2 levels in CRC, we analysed EZH2 and H3K27me3 immunohistochemistry staining from a tumour microarray of 283 patient samples. Staining for both EZH2 and H3K27me3 was significantly higher in CRC compared with normal intestinal tissue (Fig. 2a, b). The self-renewal properties of C-ICs have been shown to contribute to disease recurrence. We therefore investigated the percent recurrence in patients with top and bottom quintiles of EZH2 expression. We observe a significantly greater proportion of disease recurrence in patients with tumours expressing high levels of EZH2 (31%) compared with the tumours exhibiting low levels of EZH2 expression (14%) (Fig. 2c). Using TCGA data and a published gene expression signature for colon crypt stem cells and differentiated colonocytes, we found that EZH2 mRNA levels in CRC samples are positively correlated with a large subset of genes, specifically expressed in the colon crypt stem cells (Fig. 2d). In contrast, EZH2 expression is inversely correlated with the majority of genes, specifically expressed in the top of the crypt,
which represents the differentiated colonocyte compartment (Fig. 2e)\(^3\). Altogether, these data indicate that EZH2 expression correlates with the colonic stem cell compartment and a higher incidence of disease recurrence.

To better understand how UNC1999 treatment affects growth of CC-ICs, we performed cell cycle and apoptosis analyses on UNC1999-treated POP92 spheres (Supplementary Figure 2a–c). We observed a 9.2% increase in G1 and a 38% decrease in S phase, but no significant change in apoptosis or necrosis. We further investigated whether the growth arrest in the G1/S phase following UNC1999 treatment might reflect an exit from the CC-IC state. To this end, we tested UNC1999 on two patient-derived spheroid models stably expressing a TCF/LEF GFP reporter of Wnt activity, a well-established marker of CC-ICs\(^5\). UNC1999 treatment resulted in a decrease of the Wnt-high expressing cells, indicating a reduction in the CC-IC fraction (Fig. 2f, Supplementary Figure 2d–h). The reduction in Wnt reporter activity was also observed with GSK343, another EZH2 inhibitor (Supplementary Figure 2f–g). Finally, to determine whether transient EZH2 inhibition resulted in an irreversible functional decrease in CC-ICs, in vitro limiting dilution assays (LDAs) were carried out for three CC-IC enriched samples. The cells were pretreated with UNC1999 for 7 days, after which the inhibitor was removed and viable cells were seeded at limiting dose in serum-free medium lacking the inhibitor (Fig. 2g). Treatment with UNC1999 resulted in a two- to fourfold decrease in the frequency of sphere-initiating cells (Fig. 2h–j). Thus, transient inhibition of EZH2 results in an irreversible reduction in CC-ICs.

**UNC1999 reduces tumour growth and self-renewal in vivo**. To determine whether EZH2 inhibition in vivo affects tumour growth, we treated two CRC patient-derived xenograft models...
Fig. 3 Inhibition of EZH2 reduces tumour growth and CC-IC frequency in vivo. **a** Dosing schedule for UNC1999 in vivo. Mice are dosed for 11-day cycles of 9 continuous days followed by a 2-day break. **b-c** Tumour volume measurements of POP92 and POP66. Mice were treated with vehicle or UNC1999 at 300 mg per kg of body weight for 20 days (**b**) or 25 days (**c**). Data shown are n = 37 tumours (**b**, vehicle), n = 42 (**b**, UNC1999), n = 20 (**c**, vehicle) or n = 30 (**c**, UNC1999) ± SEM, with two-way ANOVA. **d** Images of tumours from POP92 collected in (**b**). **e** Tumour weight measurement from xenografts collected in (**b**, **c**). Data are mean of n = 37 tumours (POP92 vehicle), n = 42 (POP92 UNC1999), n = 20 (POP66 vehicle) or n = 30 (POP66 UNC1999), ± SEM, Student’s t-test. **f** Representative western blots showing the reduction of H3K27me3 signal over total H3 normalised to the vehicle control. Data shown are means ± SEM of three independent experiments, with Student’s t-test. **g** Relative H3K27me3 (%). **h** Schematic of the serial in vivo limiting dilution assay performed in (**i, j**). The first passage LDA was performed using POP92 tumours collected in (**b**). Mice were treated for 20 days with UNC1999 at 300 mg/kg, tumours were collected, prepared as single cells and serially diluted and reinjected into primary recipient mice without further treatments (**i**). The second passage LDA (**j**) was performed upon growth of tumours in (**i**): samples were collected, prepared as single cells and reinjected into secondary recipient mice without further treatments (**j**). Data in (**i, j**) are shown as mean ± 95% confidence interval, frequency and probability were computed using ELDA software. stars represent P values: *P < 0.05, **P < 0.01, ***P < 0.001. Source data are provided as a Source Data file.

with vehicle control or UNC1999 at 300 mg/kg for 11-day cycles of 9 consecutive days of treatment followed by 2 days of break, which was well tolerated by the mice (Fig. 3a and Supplementary Figure 3a–b). We observed a significant reduction in both tumour volume and tumour weight in both models during the time course of treatment (Fig. 3b–e). This effect was concomitant with a decrease in H3K27me3 (Fig. 3f, g and Supplementary Figure 3c). Histological analysis revealed a modest increase in necrosis in UNC1999-treated xenografts as well as a decrease in the proliferation marker Ki67 (Supplementary Figure 3c–d). To specifically assess the effect of EZH2 on the CC-IC population, we performed the gold standard assay for enumerating C-ICs, two serial passage in vivo LDAs on POP92 xenografts (Fig. 3h–j). Using the UNC1999-treated xenografts from the experiment shown in Fig. 3b, we serially transplanted cells in limiting dilution into primary recipient mice (1st passage LDA), and the resultant tumours subsequently into secondary recipient mice (2nd passage LDA) (Fig. 3i). The secondary transplantation was performed 52 days after the last exposure to UNC1999. These serial passage LDAs showed a significant fourfold reduction in tumour-initiating...
frequency upon primary passage (Fig. 3i) and an even stronger
ninefold reduction in tumour-initiating frequency upon secondary
passage (Fig. 3j). Collectively, our results show that EZH2
inhibition targets CC-IC self-renewal, resulting in CC-IC
exhaustion as shown by the reduction in tumour initiation in the
in vivo LDA.

The differentiation gene IHH is bivalently marked in CC-ICs.
To gain mechanistic insights into how EZH2 inhibition affects
CC-ICs, we performed RNA-seq to compare control and
UNC1999-treated POP92 CC-IC enriched cultures. A total of 50
genes were significantly downregulated as a consequence of EZH2
inhibition (Fig. 4a), including many cell cycle regulators, con-
sistent with the observed phenotype of growth suppression
(Supplementary Figure 4a). A larger group of 333 genes showed
significantly increased expression after UNC1999 treatment
including genes associated with gene ontology (GO) terms for
metabolism, stress response and innate immune response (Sup-
plementary Data 1, Supplementary Figure 4b). These data are
consistent with previously reported cancer-specific upregulation
of the Type III interferon pathway25, and reduced expression of
cell cycle genes26 upon EZH2 inhibition. Interestingly, Gene Set
Enrichment Analysis (GSEA) uncovered a significantly decreased
enrichment for the Colon Crypt signature in UNC1999-treated
cells (Fig. 4b). We also observed that CDX2, an intestinal dif-
ferentiation marker27, was upregulated following UNC1999
(Supplementary Data 1). Taken together, these data suggest
that UNC1999 might induce exit of the stem-like state by upregulating
a differentiation programme.

In ESCs, the repression of bivalently marked genes that control
lineage commitment and differentiation is partially regulated by
EZH212,13. In the context of cancer, Beguelin et al. previously
showed that EZH2 suppresses differentiation of germinal centre B
cells by establishing bivalent chromatin domains at promoters of
differentiation and proliferation checkpoint genes15. To test
whether differentiation genes are also held in a bivalent state in
CC-ICs, we performed H3K4me3 and H3K27me3 ChIP-seq on
POP92 CC-IC enriched cultures. We then assessed whether the
expression of these bivalent genes was altered following
UNC1999 treatment. This analysis identified 43 genes with
H3K27me3-positive promoters (defined as 2.5 kb upstream and
0.5 kb downstream of the transcription start site) that were
upregulated by UNC1999 treatment (Fig. 4a, Supplementary
Figure 4c–e, Supplementary Table 3). A total of 20 promoters
were bivalently marked (defined by the presence of directly
overlapping H3K27me3 and H3K4me3 ChIP-seq signals), and a
subset of 38 genes harboured a mixture of non-overlapping
H3K27me3 and H3K4me3 regions within the promoter region
(Fig. 4a, Supplementary Table 3). Conversely, none of the genes
downregulated following UNC1999 treatment harboured
H3K27me3 in their promoter. Gene set enrichment analyses
combining the ChIP-seq and RNA-seq experiments show that
promoters harbouring H3K27me3, mixed or bivalent marks, are
more likely to be upregulated following UNC1999 treatment, with
bivalent genes showing the strongest enrichment (Fig. 4c,
Supplementary Figure 4f). In contrast, no significant enrichment
was observed in promoters marked with H3K4me3 alone.
Importantly, GO term analysis of the set of H3K27me3-marked
and bivalent genes which are upregulated following UNC1999
treatment showed significant enrichment for cellular differentia-
tion, which is in line with our observed sustained reduction in
CC-IC self-renewal in vivo (Fig. 4d).

Prominent among these bivalently marked differentiation
genes is Indian Hedgehog (IHH), showing a threefold increase
in expression following UNC1999 treatment (Fig. 4d–f,
Supplementary Figure 4g). IHH is one of three Hedgehog pathway
ligands in addition to Sonic (SHH) and Desert (DHH). IHH is the
primary Hedgehog ligand expressed in the intestine, where it plays
a key role in repressing the Wnt pathway and promoting
differentiation28,29. Therefore, IHH was a strong candidate
mediator of the decreased self-renewal capacity of CC-ICs
following UNC1999 treatment. Using ChIP-qPCR, we confirm
that the promoter of IHH is bivalently marked, and whereas
H3K27me3 decreases following treatment with UNC1999,
H3K4me3 remains unchanged (Supplementary Figure 4h–i).
Comparing our list of bivalently marked genes with three
published lists of bivalently marked genes from ESCs revealed
that the promoter of IHH is also bivalently marked in ESCs12,30,31.
This highlights a potential common epigenetic mechanism
regulating lineage commitment for both CC-ICs and ESCs.

To assess whether any of the UNC1999-upregulated genes
reflect activation of the IHH pathway, we assessed whether any
DNA-recognition motifs for the HH pathway were enriched in
cis-regulatory elements (CREs) of transcriptionally accessible
chromatin in CC-IC cultures. ATAC-seq analysis of
POP92 spheroid cultures revealed 35 CREs in accessible
chromatin regions. Among these CREs, the DNA-recognition
motif for GLI transcription factors, the downstream transcriptional
factors of the HH pathway, was significantly enriched (~fourfold
increase in abundance of the motif in active CREs versus the null
expectation (Fig. 4g, Supplementary Data 2). Moreover, RUNX
and SMAD DNA recognition motifs were also enriched in the
CREs in open chromatin regions of CC-IC cultures (Fig. 4g).
RUNX2 and SMADs transcription factors were previously shown
to cooperate with IHH in other models32. SMAD1/5/8 are the
downstream mediators of the bone morphogenic protein (BMP)
pathway, which is known to cooperate in IHH-induced
differentiation in intestinal stem cells39. Taken together, our data
show that the IHH pathway, as well as known cooperators of
IHH, are transcriptionally upregulated upon UNC1999 treatment
in CC-IC enriched cultures.

Exogenous IHH reduces self-renewal of CC-ICs. IHH is a
known regulator of normal intestinal and colon development, but
its function in CC-ICs is unknown. To determine whether IHH
plays a role in CC-IC self-renewal, we first confirmed our RNA-seq
and ChIP-seq findings in three CC-IC enriched models. ChIP-PCR
for H3K27me3 confirmed that the IHH promoter is repressed in
all three models (Supplementary Figure 5a), and mRNA levels of
IHH increased by two- to fourfold after 7 days of treatment with
UNC1999 (Fig. 5a), while the other IHH ligands SHH and DHH,
remain unchanged following treatment (Supplementary Fig-
ure 5b). To assess whether the increase in IHH results in activation of
the HH pathway, POP92 cells were treated with either
UNC1999 or using direct stimulation of the HH pathway using
recombinant IHH. Both treatments led to an increase in BMP4 and
GLI1 expression, two key Hedgehog target genes, suggesting that
the UNC1999-induced increase in IHH also leads to increased
activation of the HH pathway (Fig. 5b). Furthermore, markers of
differentiation, such as FABP2 and CDX2, were upregulated
following treatment with UNC1999 or recombinant IHH (Fig. 5c),
whereas the stem cell markers Ki67, Oct4 and Nanog decreased in
both IHH recombinant and UNC1999-treated CC-ICs, consistent
with a decreased stem-like state (Fig. 5d). In line with these results,
we observed a significant increase in CDX2 protein in UNC1999-
treated xenografts compared with vehicle (Supplementary
Figure 5c–d). Similar to our results with UNC1999, treatment with
recombinant IHH also resulted in a decrease in Wnt reporter
activity and Wnt target genes (Supplementary Figure 5e–g). This is
consistent with a previous study looking at overexpression of IHH

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in a CRC cell line\(^{28}\). These data combined with our in vitro and in vivo LDAs showing decreased CC-IC numbers confirm that CC-ICs are being driven to a more differentiated-like state following EZH2 inhibition.

To determine the effect of IHH on CC-IC self-renewal, we performed an LDA on three CC-IC enriched models pretreated with recombinant IHH (Fig. 5e). A significant ~1.8-fold decrease in CC-IC frequency was observed in the IHH pretreated group,

Fig. 4 EZH2 Inhibition de-represses the colonic differentiation gene \textit{IHH}. \textbf{a} RNA-seq heatmap of \(\log_2\) fold change for all genes significantly upregulated (red in top panel) or downregulated (blue in bottom panel) following UNC1999 treatment in POP92 cells. Lower bars underneath the heatmap indicate ChIP-seq data for POP92 cells in the absence of UNC1999. The presence of H3K4me3 peaks (green), H3K27me3 peaks (pink) or regions where both marks directly overlap (Bivalent; purple) in promoter regions (2.5 kb upstream of TSS, 0.5 kb downstream of TSS) is shown (prior to UNC1999 treatment) for all genes whose expression is significantly changed after UNC1999 treatment (negative binomial, FDR-corrected \(q < 0.05\)). RNA-Seq was performed in \(n = 3\) biological replicates for each condition, ChIP-seq peaks called in 2 separate replicates were used for each mark. \textbf{b} GSEA performed on the differentially regulated transcripts from RNA-seq data with respect to the Colon Crypt signature (FWER, \(p < 0.03\)). \textbf{c} GSEA performed on 30 randomly selected sets of 200 genes with H3K4me3 (green), H3K27me3 (pink), mixed (blue) or bivalent (purple) marks in their promoters. Distribution of normalised enrichment scores are shown along with the percentage of runs which were significantly enriched among upregulated genes following UNC1999 treatment (\(p < 0.25\), none were significant among downregulated genes). \textbf{d} Enriched biological process GO terms within all genes differentially upregulated following UNC1999 treatment with H3K27me3 marks in their promoter. Matrix shows which differentially expressed genes are present in each GO category, barplot shows \(-\log_2\) FDR-corrected \(q\)-value. Genes with bivalently marked promoters are highlighted in purple. \textbf{e} Boxplot of differential expression of IHH following UNC1999 treatment (mean of \(n = 3\)), error bars show max and min estimated FPKM values, Student’s \(t\) test. \textbf{f} Representative ChIP-Seq tracks for H3K27me3 and H3K4me3 showing read counts over the IHH locus inclusive of the promoter region. \textbf{g} Transcription factor motif analyses performed on the predicted CREs (defined using ATAC-Seq) of UNC1999-differentially upregulated genes using homer, relative to a background of all ATAC-Seq peaks. **\(p < 0.01\)
Inhibition of the HH pathway reduces the efficacy of UNC1999. To further assess whether de-repression of IHH is one of the major consequences of EZH2 inhibition in our model, we co-treated POP92 spheroids with UNC1999 and Vismodegib, an FDA-approved Smoothened (SMO) antagonist, which inhibits the signalling of all three Hedgehog ligands SHH, IHH and DHH. Our data shows that inhibition of the HH pathway using Vismodegib reduces the efficacy of UNC1999 (Fig. 5i). Moreover, a 65% shRNA knockdown of IHH significantly rescued the effect of UNC1999 treatment on both the growth as well as self-renewal capacity of CC-ICs in vitro, further supporting the predominant role of IHH in mediating the UNC1999 phenotype (Fig. 5g, h, Supplementary Figure 6a, b). Importantly, knockdown of IHH also reduces the efficacy of UNC1999 in vivo, limiting the decrease in tumour growth by 50% (Fig. 5i and Supplementary Figure 6b). Altogether, our results uncover IHH as a novel target gene held in a bivalent state by EZH2 in CC-ICs and provide evidence for a previously unobserved link between EZH2, regulation of IHH expression and CC-IC self-renewal. Furthermore, we show that CC-ICs remain responsive to normal cues of differentiation from IHH, which leads to decreased self-renewal capacity and increased differentiation.
UNC1999 treatment in vivo increases chemosensitivity. Chemosensitivity of CC-ICs has emerged as an important cellular property that enables tumours to recur following cytoreductive therapy. We hypothesised that the decrease in CC-IC frequency and more differentiated state of the CC-IC enriched cultures following UNC1999 would result in increased sensitivity to 5-fluorouracil (5-FU), a standard of care chemotherapeutic agent in CRC. In agreement, we observe a threefold increased sensitivity towards 5-FU in the UNC1999-pretreated CC-IC enriched models in vitro (Fig. 6a). We carried out an in vivo study using a sequential dosing regimen starting with 5 days of UNC1999 to allow epigenetic reprogramming to occur, followed by 5-FU every other day for 5 days, using sub-optimal dosing regimen for both treatments to prevent toxicity in mice (Fig. 6b and Supplementary Figure 7a). No significant change in tumour growth was observed using 5-FU alone at 15 mg/kg, while UNC1999 decreased tumour volume by 40%. However, the combination of UNC1999 and 5-FU resulted in a 70% decrease in tumour volume and weight (Fig. 6c–e). A 40% reduction of H3K27me3 was observed in both UNC1999 alone and co-treated tumours (Fig. 6f and Supplementary Figure 7b). Analysis of the tumours show large necrotic areas in the combination group, as well as increased cleaved Caspase 3 staining (Supplementary Figure 7c).

To determine the effect of combination therapy on the CC-IC fraction, we carried out an in vivo LDA using xenograft cells from mice co-treated with UNC1999 and 5-FU. This revealed a fourfold decrease in the tumour-initiating capacity in cells isolated from co-treated tumours compared to control or 5-FU alone, and a 1.8-fold decrease for the sub-optimal dosing of UNC1999 (Fig. 6g). Therefore, combining 5-FU with UNC1999 further decreases tumour growth and increases the elimination of the CC-IC fraction. Moreover, xenograft mRNA analysis showed an increase in IHH as well as the differentiation markers FABP2 and CDX2 in the UNC1999 group but not in the combination,
suggesting that the sequential dosing of UNC1999, followed by 5-FU, pushes CC-ICs towards a differentiated-like state, which in turn are targeted by 5-FU (Supplementary Figure 7d–f).

**Discussion**

**EZH2** has an established role in regulating pluripotency and maintaining self-renewal in ESCs through repression of lineage commitment and differentiation genes that are maintained in a bivalent state. Loss of the repressive histone mark H3K27me3 at bivalent genes results in the rapid transcriptional initiation of lineage-specific differentiation programmes, indicating that ESCs are poised to undergo differentiation. Although much is published on the role of EZH2 in maintaining C-ICs in a broad range of solid tumours, the mechanisms identified are diverse, with most publications attributing the effect on self-renewal to methylation of non-histone targets, recruitment of DNA methyltransferases and/or direct binding to other proteins. Whether EZH2’s canonical role in ESCs contributes to maintaining the C-IC state has never been established. Our results uncover an epigenetic block to differentiation in CC-ICs that can be overcome by targeting the bivalent state of key developmental genes. Furthermore, we functionally demonstrate that CC-ICs retain the capacity to respond to a normal cue of cell fate specification mediated by IHH, a key driver of normal colonocyte differentiation. These findings have broad implications for the C-IC field as we demonstrate a way to target C-ICs through de-repression of bivalently marked differentiation genes. Our results highlight the importance of identifying other, tissue-specific bivalently marked differentiation genes in C-ICs from other disease models, and how re-expression of these genes through epigenetic modulation could trigger C-ICs to exit the stem-like state.

Literature on the role of bivalency in C-ICs remains very limited. Initial work studying plasticity in human basal breast C-ICs demonstrated that non-C-ICs are plastic populations that can switch to a C-IC state through maintaining the ZEB1 promoter in a bivalent configuration. In response to TGFβ, the ZEB1 promoter converted from a bivalent to active chromatin configuration, and as a result non-C-ICs converted to C-ICs. Aiden et al. showed that poorly differentiated Wilms tumours share an epigenetic landscape with ESCs, observing similar bivalent chromatin modifications at a subset of promoters in both cell types. In ovarian cancer, bivalent genes that were identified in ESCs and one ovarian tumour sample were found to be expressed at lower levels in ovarian C-ICs compared with non-C-ICs, where C-ICs were defined by the side-populations. Although the authors showed that bivalent genes in ESCs are more repressed in C-ICs, the potential functional consequence of the increased repression was not explored. To date, our work is the first study to functionally demonstrate that the bivalent state of key developmental gene promoters maintains C-ICs locked in an epigenetically maintained stem-like state.

Genome-wide profiling of ESCs and cancer cell lines or bulk tumour samples shows that bivalently marked promoters of differentiation genes in ESCs gain aberrant DNA hypermethylation in CRC cell lines. This suggests that these differentiation genes are permanently silenced in CRC, which would render loss of H3K27me3 irrelevant. However, we demonstrate that C-ICs retain the capacity to exit the stem-like state upon inactivation of EZH2 resulting in the loss of H3K27me3. One possible explanation for the conflicting results is that cancer cell lines grown in adherent conditions in the presence of serum show significantly fewer bivalent regions than normal tissue or ESCs, suggesting that our findings on the role of bivalency in CC-IC self-renewal may have been made possible through the use of patient-derived, CC-IC enriched 3D spheroid/organoid models. It is also plausible that the role of DNA methylation in permanently silencing bivalently marked promoters may be tumour-specific or associated with poorly differentiated cancers. Therefore strategies that combine EZH2 inhibitors together with DNA methylation inhibitors may be beneficial to optimally target silenced differentiation programmes.

IHH is one of the canonical functional markers responsible for colonocyte differentiation. In the adult colon, IHH is expressed by terminally differentiated colonocytes, and has been shown to restrict Wnt signalling to the bottom of the colon crypts as well as restricting the size of the crypts. Furthermore, specific knockout of Ihh in murine models promotes the expansion of the stem cell compartment, indicating that Ihh plays a central role in driving intestinal stem cell differentiation. Several reports studying IHH in the context of normal small intestinal and/or colon tissue, provide evidence that Hh signalling is paracrine, where the stromal cells respond to IHH produced by the enterocytes. Whether IHH signalling in normal colon and CRC might arise from the various models used in the studies, such as normal small intestine or colon, different tumour stages, and cancer cell line models. It is also likely that a unique subset of cells within a tumour may respond differently to IHH, such as the CC-IC subset. When we antagonise canonical HH signalling through treatment with Vismodegib, we observe a dramatic reduction of the efficacy of UNC1999, which demonstrates that HH signalling is a key consequence of EZH2 inhibition in patient-derived CC-ICs. In line with our observations, it has been shown that treatment with Vismodegib or IHH knockout mouse models lead to increased tumour occurrence and larger tumours. A recent clinical trial was carried out in CRC using Vismodegib, and showed no added benefit over standard-of-care alone. Our data supports a distinct role for IHH in driving CC-ICs to exit the stem-like state. Thus, our results may explain, in part, the failure of Vismodegib in clinical trials of CRC; Vismodegib prevents cellular signalling by all three Hedgehog ligands including IHH, which based on our findings, would favour the CC-IC state.

In our models, targeting bivalency through EZH2 inhibition significantly reduced CC-IC self-renewal; however, we note that it did not fully eradicate the CC-IC population. One potential reason for this could be incomplete removal of the H3K27me3 mark in tumour cells using UNC1999, as we observed that the decrease in tumour size was approximately proportional to the decrease of the mark. Therefore, pharmacological agents with greater exposure at the tumour may be more efficacious. We also demonstrate that in vivo treatment with UNC1999 followed by 5-FU, resulted in a statistically significant reduction in tumour growth and CC-IC frequency, as compared with either compound given alone. EZH2 inhibitors are emerging from phase I studies, none of which included CRC, and there is great interest in identifying the best patient populations and clinical strategies for their future clinical development. Our work shows that EZH2 inhibitors could be incorporated as a part of novel adjuvant therapy combinations in the context of CRC. We hypothesise that by treating with UNC1999, IHH was de-repressed, resulting in differentiation of CC-ICs and as a result increased sensitivity to 5-FU. Currently there is a paucity of literature on whether bivalency is playing a role in determining response to chemotherapy. However, a study published in ovarian cancer showed that bivalently marked ESC genes are expressed at much lower levels.
in chemo-resistant ovarian cancer cells as compared with matched chemo-sensitive cells, albeit the functional significance of this finding remains to be determined31. A better understanding of the effect of disrupting bivalent promoters in C-ICs in response to standard of care chemotherapy could lead to novel therapeutic strategies.

Taken together, our results identify that similar to ESCCs, CC-ICs maintain key lineage specification genes in a bivalent state. Moreover, we functionally demonstrate that CC-ICs remain responsive to a normal differentiation cue, IHH, upon loss of CC-IC fraction through disrupting the bivalent state of key differentiation genes. Our findings will lead to novel therapeutic strategies aimed at identifying and targeting bivalently marked differentiation genes in cancer, and specifically C-ICs.

Methods

Colonelctal cancer patient-derived xenografts. Human CRC tissue was obtained with informed patient consent, as approved by the Research Ethics Board at the University Health Network (UHN) in Toronto, Canada. To establish and maintain PDX models, cells from freshly dissociated CRC tissue or freshly thawed cells previously frozen xenograft samples53 were mixed (1:1) with high concentration non-tumorigenic human fibroblasts (Human Embryonic Fibroblasts, HFF-1). Samples were then sonicated in a 1:1 mixture of Dulbecco’s Modified Eagle’s Medium/F-12 (DMEM/F-12) and Matrigel (Corning). Tissue culture-treated 384-well plates with T.nano (NimbleGen) were infected with TCF/LEF reporter lentivirus following the manufacturer’s instructions. The cells were scanned using a 3DHistech Panoramic 250 Flash II slide scanner using a ×40 objective. The IHC score of the TAMs was assessed manually and confirmed by a pathologist. The percentage of positive cells (P) was assessed and scored as follows: 0 (0–10% positive cells); 1 (10–25% positive cells); 2 (26–50% positive cells); 3 (51–75% positive cells); and 4 (≥76% positive cells). The overall staining intensity (I) was scored as: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The final IHC score was calculated by multiplying the quantity of positive cells P (0–4) by the staining intensity I (0–3), ranging from 0 to 12.

Cell cycle and apoptosis/necrosis. For PI cell cycle analysis, cells were pretreated with 0.5 µM of UNC1999 or 5 µM, trypsinized, washed and fixed in ice-cold ethanol, permeabilized with 0.5% Triton X-100, and stained using 1:1 mixture of Propidium iodide (50 µg/mL, Invitrogen) and Hoechst 33342 was added at a concentration of 20 µg/mL (Invitrogen) for 30 min. Cells were analysed using MACSQuant VYB flow cytometer (Miltenyi) and analysed with FlowJo (version 10) software. Apoptosis/necrosis using Annexin V-FITC and propidium iodide was performed following the manufacturer’s instructions (Annexin V-FITC apoptosis detection kit, Sigma). Cells were analysed on a BDF LS II (BD Biosciences).

TCF/LEF GFP Wnt reporter assays. POP92 and POP66 were infected with TCF/LEF promoter-driven GFP reporter lentivirus following the manufacturer’s instructions (Qiagen), or with FOP-mutated TCF/LEF negative control (Addgene). Reporter cells were pretreated for 7 or 10 days with UNC1999 or GSK343 at 3 µM, or recombinant IHH (5 ng/mL) (Biolegend) for 10 days. Cells were trypsinized, washed and resuspended in PBS with Sytox blue viability dye, and acquired using the MacsQuant VYB (Miltenyi Biotec). Data were normalised to the top 10% brightest population in the DMSO control.

Tissue microarray and immunohistochemistry. The patient tissue microarray slides were generated by the University Health Network Biobank, obtained with informed patient consent, as approved by the Research Ethics Board at the University Health Network in Toronto. TMA and xenograft slides were stained using E2H2 antibody (Cell Signaling Technologies, Cat nb#3264, 1/100 dilution) and H3K27me3 (Cell Signaling Technologies, Cat nb#9735, 1/200 dilution) following the manufacturer’s instructions. The slides were scanned using a 3DHistech Panoramic 250 Flash II slide scanner using a ×40 objective. The IHC score of the TAMs was assessed manually and confirmed by a pathologist. The percentage of positive cells (P) was assessed and scored as follows: 0 (0–10% positive cells); 1 (10–25% positive cells); 2 (26–50% positive cells); 3 (51–75% positive cells); and 4 (≥76% positive cells). The overall staining intensity (I) was scored as: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). The final IHC score was calculated by multiplying the quantity of positive cells P (0–4) by the staining intensity I (0–3), ranging from 0 to 12.

Limiting dilution assays in vitro. Cells were pretreated with 3 µM of UNC1999 or recombinant IHH (5 ng/mL) (Biolegend) for 10 days, before being trypsinized and seeded in serial dilutions of 1000, 100, 10 and 1 cell per well of a 96-well plate. Cells were incubated for 4–6 weeks at 37 °C in the absence of additional treatment, and every well was assessed for presence or absence of sphere. The data were analysed using the online Extreme Limiting Dilution Analysis (ELDA) Bioinformatics tool38.

RT-qPCR. RNA was extracted using RNeasy kit (Qiagen) following the manufacturer’s instructions. cDNA was generated using iScript Advanced (BioRad), and qPCR was set up using PowerUP SYBR green qPCR mastermix (Thermo Fisher) followed by a time curve PCR (BioRad iCycler system) (BioRad). Housekeeping genes TBP and/or 18S were used to normalise the data. Primer sequences are listed in Supplementary Table 4.
In vivo UNC1999 dosing and limiting dilution assays. All animal experiments were reviewed and approved by the Animal Care Committee at the University Health Network in Toronto. For UNC1999 in vivo dosing experiments, UNC1999 was synthesized in the Jin Laboratory. C17 female scid/scid mice were injected with 3 × 10⁵ CC-IC enriched cells subcutaneously at two tumours per animal. UNC1999 was prepared at 300 μg/kg in 0.5% carboxymethylcellulose and 0.1% Tween, and administered to the mice when the tumours reached around 150 mm³. The treatments were performed once daily by oral gavage, for 9 consecutive days and a 2-day break, for a total of 21 to 25 days (until vehicle reached maximum size), or for 5 consecutive days and 5 days break for the combination experiment in Fig. 6. Body weights were measured every day over the course of treatment, and tumour growth was monitored by caliper measurements every 2–3 days until endpoint was reached.

For in vivo LDA, tumours were collected from the UNC1999 dosing experiments, digested, counted for viable cells using Trypan blue exclusion, and injected into NOD/SCID–γc− mice (NSG) mice at a limiting dose (10000, 1000, 100 and 10 γc− mice) at 30−40−300−3000 live cells per tumour. Tumour formation was monitored over the course of 4 months, and stem cell frequencies determined using the online Extreme Limiting Dilution Analysis bioinformatics tool55.

ChIP-seq. ChIP-qPCR and RNA-seq. ChIP was carried out with POP92 spheroids. Briefly, Dynabeads A and G were incubated with anti-H3K27me3 antibody (Abcam ab6002), anti-H3K4me3 antibody (Abcam ab8580), rabbit IgG isotype control or mouse IgG isotype control for 6 h. In the meantime, cells were fixed with 1% formaldehyde for 10 min, washed with PBS/1% BSA and then with PBS. Fixed cells were lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1), incubated on ice for 10 min and sonicated using a 4 °C waterbath sonicator 5 cycles of 30 s ON and 30 s OFF, incubated on ice for 10 min and sonicated using a 4 °C waterbath sonicator at 16,000 rpm (Bioruptor), transferred to new tubes. Fixed cells were washed with PBS/1% BSA and then with PBS. Fixed cells were lysed in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl pH 8.1), incubated on ice for 10 min and sonicated using a 4 °C waterbath sonicator 5 cycles of 30 s ON and 30 s OFF, incubated on ice for 10 min and sonicated using a 4 °C waterbath sonicator at 16,000 rpm (Bioruptor), transferred to new tubes. Ninety percent of the cross-linked chromatin was incubated overnight along with antibody–dynabeads complexes, whereas 10% was stored at 4 °C as an input. Thereafter, samples were washed and decross-linked overnight with a decross-linking buffer (1% SDS and 0.1 M NaHCO₃) at 65 °C. Samples were purified using MinElute PCR purification kit (Qiagen) and processed for qPCR or sequencing, respectively. The qPCR experiments were set up using PowerUP SYBR green qPCR mastermix (Thermo Fisher) and run on a CFX384 Touch Real-Time PCR detection system (Biorad). Primer sequences are listed in Supplementary Table 4. For sequencing, fragments from 240–360 bp were selected using the PippinHT system (Sage Science). Libraries were prepared using the TruPlex DNA-Seq Kit, following the manufacturer’s instructions (Rubicon Genomics), and sequenced using Illumina HiSeq 2500 or 2000 V4 chemistry.

RNA-seq was performed on POP92 cells pretreated with UNC1999 at 3 μM for 7 days. Cells were collected and RNA was extracted using Qiagen RNeasy kit. RNA concentration and quality was assessed using a Bioanalyzer (Agilent). Sample library preparation was performed using Illumina TruSeq Stranded mRNA sample preparation kit. Sequencing was performed on Illumina NextSeq500 using 75-cycle paired-end protocol and multiplexing.

Assay for Transposase-Accessible Chromatin (ATAC-Seq). To determine the chromatin accessibility of POP92 cells, an Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-Seq) was performed. In total, 30,000 live cells were washed with PBS and lysed for 5 min on ice using a lysis buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.1% IGEPAL CA-630). After isolating crude nuclei, samples were treated for 30 min at 37 °C with Tn5 transposase, and then the DNA was purified using MinElute PCR Purification Kit. Transposed DNA fragments were amplified using specific adapters followed by purification with MinElute PCR Purification Kit. Fragments from 240–360 bp were selected in the PippinHT system. The quality of the library and its DNA concentration were assessed by Bioanalyzer instruments and sequenced using Illumina HiSeq 2500 sequencer, V4 chemistry.

NGS data processing and analysis. RNA-Seq reads were aligned to the hg19 human reference genome using Bowtie256 (2.0.5) and TopHat57 (2.0.8) using default settings. Cufflinks58 (2.1.1) was used to compute and normalise read counts, and call differentially expressed genes. ChIP-Seq and ATAC-Seq reads were aligned to the hg19 human genome using bwa using default settings, all reads with a quality score of less than 30 were removed, along with all reads mapped to regions marked as belonging to the hg19 blacklist59, chrM, chrY and unlocalized sequences. Picard tools (http://broadinstitute.github.io/picard/) (1.84) was used to mark all duplicate reads. MACS260 (2.0.1) was used to call enriched peaks and calculate the observed fold enrichment over background. Peaks identified in both of two Chip-Seq replicates for each condition were retained. Promoter areas as defined by −2.5 kb − 0.5 kb from the TSS, were first identified. Bivalent promoters were then defined as those having H3K4me3 and H3K27me3 Chip-seq peaks that did not overlap within the promoter region. Promoters that contained both H3K4me3 and H3K27me3 peaks that did not overlap were classified as “mixed”.

The GSEA tool61 was used to assess whether UNC1999 treatment decreased enrichment for the Colon Crypt signature. Overall, 10,000 permutations of gene sets were used to compare the UNC1999 and DMSO treated samples. P-values were corrected for family-wise error rate. GSEA was also used to compare co-regulated genes with H3K27me3, H3K4me3 or both marks present at their promoters were enriched in one RNA-Seq condition. Due to the large number of genes harbouring each mark, 30 random subsets of 200 genes were obtained and GSEA performed on each subset. The distribution of normalised enrichment scores was plotted as a boxplot. The least significant GSEA result was presented for each, as well as the distribution of enrichment scores.

For all differentially expressed genes, potential cis-regulatory elements (CREs) associated with those genes were identified via correlation between their promoters and DNASE hypersensitive sites via Thurmam et al.53. The catalogue of all merged CREs called as accessible in any ATAC-Seq sample were considered putative CRC-accessible CREs. Home3 (4.9) was used to identify enriched transcription factor recognition motifs at these putative CRC-accessible CREs against a background of all ATAC-Seq peaks called in at least one replicate.

Statistical analyses. All measurements were taken from distinct samples/biological replicates. With the exception of the Limiting Dilution assays and epigenome analyses, the statistical analyses were performed using Graphpad Prism 6. For LDAs (Figs. 2h–j, 3i, 5h, 6g) the statistical significance were obtained through ELDA bioinformatics tool55. Unless otherwise indicated in the figure legends, the statistics analyses were performed as follow: one-way ANOVA with Dunnet multiple test correction (Fig. 1a), one-way ANOVA with Bonferroni multiple test correction (5f, 6f, Supplementary Figure 2g, Supplementary Figure 4g, Supplementary Figure 5g, Supplementary Figure 6a, Supplementary Figure 6c, d, Supplementary Figure 7d–j). Two-way ANOVA with Bonferroni multiple test correction (Figs. 1c, 1e, 1g, 2f, 2c, 2e, 2g, 2a, 5b, 5d, 5f, 6f, Supplementary Figure 1b, Supplementary Figure 1e, Supplementary Figure 2a, Supplementary Figure 2c, Supplementary Figure 2e, Supplementary Figure 5a, b). All Student’s t test were performed as two-tailed (Figs. 1d, 2h, Supplementary Figure 3c, e, d, Supplementary Figure 5d).

Statistical analysis of qPCR data was performed in GraphPad Prism. Values were obtained from at least three biological replicates as indicated in each respective figure legend, performed in four technical replicates and were normalised to 18S and/or 185 housekeeping genes.

Ethics. Approval for this study was given by the Research Ethics Board at the University Health Network (UHN), Toronto, Canada (REB 10-0705-TE to CAOB). Patient tissue was obtained by UHN with informed patient consent as per UHN’s Research Ethics Board guidelines. This study complies with all the relevant regulations for animal testing and research, as reviewed and approved by the Animal Care Committee at the University Health Network in Toronto, Canada (AUP 2781 to CAOB).

Data availability. All the data for RNA-seq, ATAC-seq and ChIP-seq are available through GEO (peak tracks, GSE113176 and in the European Genome-Phenome Archive (raw sequencing data, EGA500001003003). Source data underlying Figs. 1–3, 5–6, and Supplementary Figure 1–7 are provided as Source Data file. Gating strategy for Flow Cytometry experiments, as well as uncropped images of Western Blots are provided in the Source Data file. A reporting summary for this Article is available as a Supplementary Information file. All the patient samples (tumours and organoids) are available through the Princess Margaret living biobank upon request.

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Author contributions
C.H.A., C.A.O.B., M.L., D.D.D.C., E.L.-F. and A.M. designed the project. E.L.-F., C.H.A. and C.A.O.B. designed experiments, analysed and interpreted the data. E.L.-F. performed the majority of experiments, Y.W. carried out the in vivo studies, T.M. performed the ChIP-seq experiments, and G.M.L. performed EdU/Hoechst cell cycle experiments. S.D., C.Z. and C.L. provided technical assistance. A.M. performed the bioinformatics analyses. A.P. performed IHC necrosis quantification. A.Ma and J.J. provided UNC1999 for the study. J.H. and B.G.W. provided patient-derived organoid samples. C.H.A., C.A.O.B. and E.L.-F. wrote the paper. A.M., M.L., D.D.D.C. and J.H. provided critical evaluation of the paper. All authors read and approved the final version of the paper.

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