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Tcf3 and cell cycle factors contribute to butyrate resistance in colorectal cancer cells

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ABSTRACT

Butyrate, a fermentation product of dietary fiber, inhibits clonal growth in colorectal cancer (CRC) cells dependent upon the fold induction of Wnt activity. We have developed a CRC cell line (HCT-R) that, unlike its parental cell line, HCT-116, does not respond to butyrate exposure with hyperactivation of Wnt signaling and suppressed clonal growth. PCR array analyses revealed Wnt pathway-related genes, the expression of which differs between butyrate-sensitive HCT-116 CRC cells and their butyrate-resistant HCT-R cell counterparts. We identified overexpression of Tcf3 as being partially responsible for the butyrate-resistant phenotype, as this DNA-binding protein suppresses the hyperinduction of Wnt activity by butyrate. Consequently, Tcf3 knockdown in HCT-R cells restores their sensitivity to the effects of butyrate on Wnt activity and clonal cell growth. Interestingly, the effects of overexpressed Tcf3 differ between HCT-116 and HCT-R cells; thus, in HCT-116 cells Tcf3 suppresses proliferation without rendering the cells resistant to butyrate. In HCT-R cells, however, the overexpression of Tcf3 inhibits Wnt activity, and the cells are still able to proliferate due to the higher expression levels of cell cycle factors, particularly those driving the G_1 to S transition. Knowledge of the molecular mechanisms determining the variable sensitivity of CRC cells to butyrate may assist in developing approaches that prevent or reverse butyrate resistance.

1. Introduction

Clinical studies have demonstrated that a high intake of dietary fiber reduces the risk of colon cancer [1,2]. *In vivo* studies have supported a protective role for butyrate [3–5], a fermentation product of dietary fiber and a histone deacetylase inhibitor (HDACi) that induces differentiation and apoptosis of CRC cells. We have reported that sodium butyrate (NaB) inhibits clonal cell growth in CRC cells to an extent dependent upon the hyperactivation of canonical Wnt activity [6,7].

Wnt activity results from the accumulation of active beta-catenin, which can associate with DNA-binding Tcf factors, and stimulate transcriptional activity from target genes [8–12]. The constitutive activation of Wnt signaling, due to mutations in the *APC* and *beta-catenin* genes, promotes tumorigenesis in the colon [8–12]. However, relatively high levels of Wnt signaling result in apoptosis [13–15]; for example, butyrate hyperactivates Wnt transcriptional activity in CRC cells [6,7,15], and this hyperactivition leads to repressed cell proliferation and enhanced apoptosis.

Some Wnt pathway factors are predominantly repressive; for example, Tcf3 is associated with transcriptional repression of Wnt signaling-targeted genes during the differentiation of embryonic stem cells [16–18]. Repressive factors such as Tcf3 may block the ability of butyrate to augment Wnt activity, and therefore interfere with the preventive action of dietary fiber.

We have developed a butyrate-resistant CRC cell line (HCT-R), which grows at 5 mM butyrate, a physiologically relevant concentration at which the parental HCT-116 cells exhibit repressed growth and high levels of apoptosis [7]. The butyrate-resistant phenotype of HCT-R cells is maintained for at least one month in the absence of butyrate exposure, suggesting that this cell line exhibits permanent genetic changes compared to parental HCT-116 cells. HCT-R cells are also cross-resistant to other, structurally distinct HDACis, such as TSA, MS-275, SAHA, and LBH589, all of which, similar to butyrate, upregulate Wnt activity and repress CRC cell growth [7,19]. Resistance to the growth suppressive effects of these HDACis is associated with inhibited hyperactivation of Wnt signaling [7,19], a finding that underscores the importance of Wnt activity in the butyrate-resistant phenotype of HCT-R cells.

Here we report that the repressed induction of Wnt signaling in butyrate-treated HCT-R cells is partially due to higher Tcf3 expression levels, and that Tcf3 knockdown increases cell sensitivity to the Wnt-stimulating and growth suppressive effects of butyrate. Interestingly, Tcf3 overexpression in butyrate-sensitive HCT-116 cells does not render these cells resistant to this agent, and instead suppresses cell proliferation. However, butyrate-treated HCT-R cells proliferate in the presence of elevated Tcf3 levels due to the overexpression of cell cycle promoting genes. Therefore, targeting

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the expression of Tcf3 and cell cycle factors could reverse butyrate resistance, and enhance the efficacy of CRC preventive or therapeutic approaches utilizing HDACis.

2. Materials and methods

2.1. Plasmids, siRNA, cell lines, transfection, luciferase assay, reagents

pTOPFlash and pFOPFlash were obtained from Hans Clevers (UMC Utrecht, Utrecht, Netherlands), pcDNA3Tcf3 expression vector was obtained from Brad Merrill (University of Illinois, Chicago). HCT-116 cells were obtained from ATCC. siRNA to Tcf3 (sc-36618) and control siRNAs (sc-37007 and sc-44230) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HCT-R cells were derived from HCT-116 cells as described [7]. Stable transfection was performed as described [15] and selected with 500 μg/ml G418. Transfections with lipofectamine and Wnt activity assays with the TOPFlash/FOPFlash luciferase reporter system [9,10] were performed as described [6,7,15]. Sodium butyrate was from Sigma (St. Louis, MO) and PD03329991 was from Selleckchem (Houston, TX).

2.2. Nucleofection

We utilized standard protocols; program D-32 was used to nucleofect HCT-116 and HCT-R cells ($1–2 \times 10^6$ cells/well in a 6-well plate), with 1–2 μg of DNA or 175 pmol siRNA.

2.3. RNA isolation, SA Biosciences Wnt PCR array

To determine the expression of genes related to the Wnt signaling pathway, we utilized the Wnt signaling pathway PCR array service of SA Biosciences (Qiagen, Valencia, CA). RNA isolation and data analyses were performed as described [19].

2.4. Clonogenics, cell proliferation

Clonogenic assays were performed as described [6,7]. Cell proliferation was assayed with the Quick Cell Proliferation Assay kit of BioVision (Milpitas, CA), according to manufacturer's instructions.

2.5. Co-immunoprecipitation and Western blotting

Protein isolations, co-immunoprecipitations, and Western blotting were performed as described [7]. Antibodies were from Santa Cruz Biotechnology: beta-catenin (sc-53483), CtBP1 (sc-11390), cyclin D2 (sc-181 or sc-593), Tcf3 (sc-8635), Tcf4 (sc-8632), TLE1 (sc-13368), TLE2 (sc-9122). When necessary, background was adjusted for optimized band visualization.

2.6. Genus Biosystems total human genome microarray analysis

After treatment with or without 5 mM NaB (17.5 h), cells were washed with 1 × PBS, scraped into PBS and pelleted; the pellets were snap frozen in liquid nitrogen and sent to Genus Biosystems (Northbrook, IL) for RNA extraction and microarray analyses (Agilent human whole genome oligo microarray).

2.7. Statistics

Students *T*-test was utilized, with statistical significance set at $P < 0.05$.

3. Results

3.1. Tcf3 knockdown in HCT-R cells enhances the effects of butyrate on Wnt activity and clonal growth

Compared to parental butyrate-sensitive HCT-116 cells, butyrate-resistant HCT-R cells exhibit repressed induction of Wnt activity in the presence of butyrate [7]. Therefore, we utilized PCR array methodology to determine changes in the expression of 84 genes involved in Wnt signaling. The analyses were performed with RNA isolated from HCT-R and HCT-116 cells exposed to mock or 5 mM NaB treatment [19]. Data analysis revealed that compared to HCT-116 cells, *Tcf3* mRNA is upregulated in HCT-R cells 3.45-fold in the absence of butyrate and 5.87-fold in the presence of butyrate ($P < 0.05$). Western blot analyses confirmed this increased Tcf3 expression (Fig. 1A).

Since Tcf3 can repress canonical Wnt signaling [16–18], increased Tcf3 expression in HCT-R cells may contribute to the butyrate-resistant phenotype. We hypothesized that *Tcf3* knockdown would reverse the suppressed induction of Wnt activity in butyrate-treated HCT-R cells. Therefore, the resulting enhanced Wnt hyperactivation would result in efficient repression of HCT-R cell growth by butyrate. Knockdown of Tcf3 levels (Fig. 1B) increased Wnt activity in HCT-R cells (Fig. 1C), particularly in the presence of butyrate ($P < 0.005$). Tcf3 knockdown also enhanced repression of clonal growth in butyrate-treated HCT-R cells ($P < 0.005$) (Fig. 1D), consistent with our previous report that greater Wnt hyperactivation leads to less cell growth [7].

3.2. Transcriptional cofactors interacting with Tcf3 in HCT-116 and HCT-R cells

Complexes of beta-catenin and the Tcf4 protein mediate Wnt transcriptional activity in colonic cells [8–12]. We performed co-immunoprecipitations (co-IP) to determine whether Tcf3 associates with beta-catenin in HCT-116 and HCT-R cells. We posited that in HCT-R cells Tcf3 does not associate with beta-catenin, and therefore likely represses the expression of Wnt target genes through competition with active Tcf4/beta-catenin complexes [16–18,20–22]. Our co-IP data support this scenario (Fig. 2). As expected, Tcf4 is complexed with beta-catenin. Consistent with previous findings, levels of Tcf4/beta-catenin complexes are higher in HCT-116 compared to the HCT-R cells [7]. Importantly, Tcf3 does not complex with beta-catenin in either cell line (Fig. 2A), consistent with the possibility that overexpressed Tcf3 represses Wnt activity.

The repressive role of Tcf3 may involve association with the transcriptional corepressors TLE1/2 and/or CtBP1 [20]. Examining the interaction of Tcf3 with each of these corepressors in both cell lines revealed the association of Tcf3 with TLE2 (Fig. 2B). Furthermore, a minimal degree of association of Tcf4 with TLE2 was detected following a sufficiently long exposure, as a slight enrichment of immunoprecipitated TLE2 signal was observed in comparison with the IgG background (Fig. 2B). In contrast, we did not detect binding of the corepressors CtBP1 or TLE1 with either of the two Tcf proteins (data not shown).

3.3. Overexpression of Tcf3 in HCT-116 cells represses butyrate-induced Wnt activity but does not influence clonal cell growth

Our findings suggest that high levels of Tcf3 repress Wnt activity and contribute to butyrate resistance in HCT-R cells. Therefore, we hypothesized that exogenous overexpression of Tcf3 in parental HCT-116 cells would render the cells butyrate resistant. To evaluate this possibility, we measured Wnt activity in the presence of

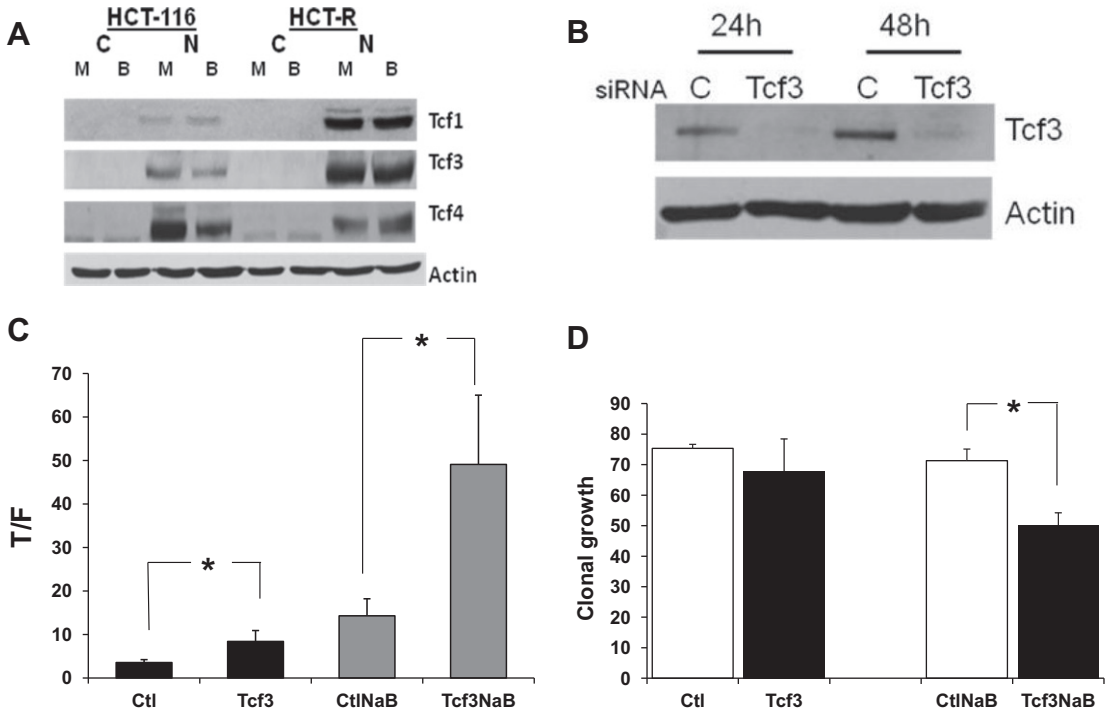

Fig. 1. Overexpressed Tcf3 in HCT-R cells mediates butyrate resistance. (A) Cytoplasmic (C) and nuclear (N) protein fractions from mock (M) and 5 mM NaB (B) treated HCT-116 and HCT-R cells were analyzed by Western blotting using antibodies against Tcf1, Tcf3, Tcf4, and Actin (control). (B) *Tcf3* silencing in HCT-R cells. *Tcf3* or control siRNAs were nucleofected, and cells were analyzed by Western blotting for expression levels of Tcf3 at 24 and 48 h. (C) Modulation of Wnt activity by *Tcf3* silencing. HCT-R cells in a 96-well plate were transfected (Lipofectamine 2000) with TOP/FOPFlash reporter vectors, pRLTK (to normalize transfection efficiency), and control (Ctl) or *Tcf3* siRNA. Cells were mock treated or exposed to 5 mM sodium butyrate [NaB] overnight (17 h). The ratio of TOPFlash to FOPFlash reporter activity [T/F] is shown; data are from three independent experiments. Bars, SDs. (D) *Tcf3* silencing decreases clonal growth of butyrate-treated HCT-R cells. HCT-R cells were nucleofected with Ctrl or *Tcf3* siRNA, and each batch of cells was plated onto 2 wells of a 6-well plate. At 48 h post-nucleofection the cells were exposed to mock treatment or 5 mM NaB for 17 h. On day three cells were counted and plated at low cell density to allow for clonal growth. At 14 days the colonies were fixed, stained with crystal violet, and counted. Clonal growth is the number of colonies formed from 100 plated cells. Data are from three independent experiments. Bars, SDs. *Statistical significance.

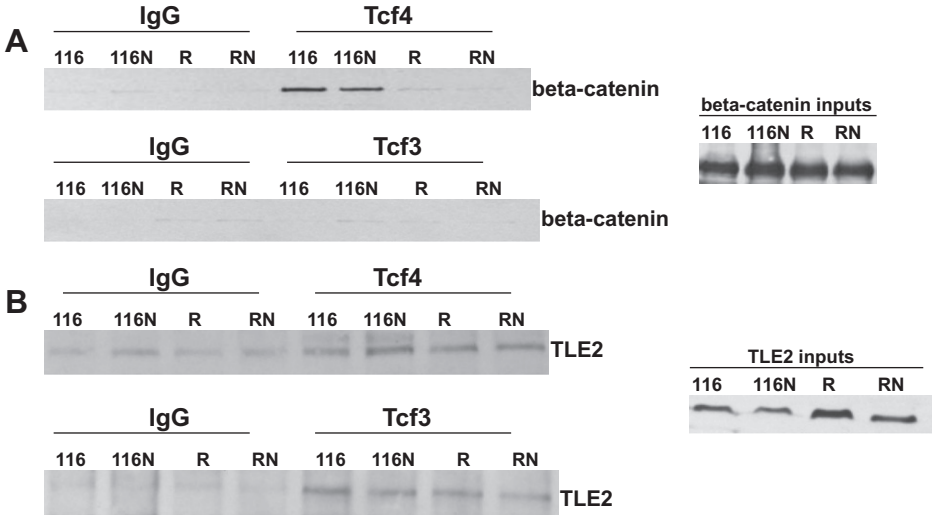

Fig. 2. Tcf3 cofactors in HCT-116 and HCT-R cells. Cells were exposed to mock or butyrate treatment as described in Fig. 1. Co-immunoprecipitations were performed with nuclear protein fractions (12). Western blots were probed for beta-catenin (A) or TLE2 (B). Representative experiments are shown. 116 (HCT-116), R (HCT-R), N (5 mM NaB).

exogenous Tcf3 (Fig. 3A and B). Consistent with a repressive role of Tcf3, we observed that Tcf3 overexpression in butyrate-treated HCT-116 cells results in Wnt activity levels less than one-fifth ($P < 0.02$) of those in control transfected cells.

Since Tcf3 suppresses the hyperinduction of Wnt signaling in butyrate-treated HCT-116 cells (Fig. 3A), we expected that Tcf3 overexpression would enhance clonal growth of butyrate-treated HCT-116 cells. However, overexpression of Tcf3 did not interfere with the ability of butyrate to suppress HCT-116 clonal growth. Thus, butyrate decreased clonal growth to a similar extent in the presence or absence of exogenously expressed Tcf3 (Fig. 3C).

Based upon our observations of the growth characteristics of Tcf3-transfected HCT-116 cells, we hypothesized that overexpression of Tcf3 blocks HCT-116 cell proliferation (compare clonal

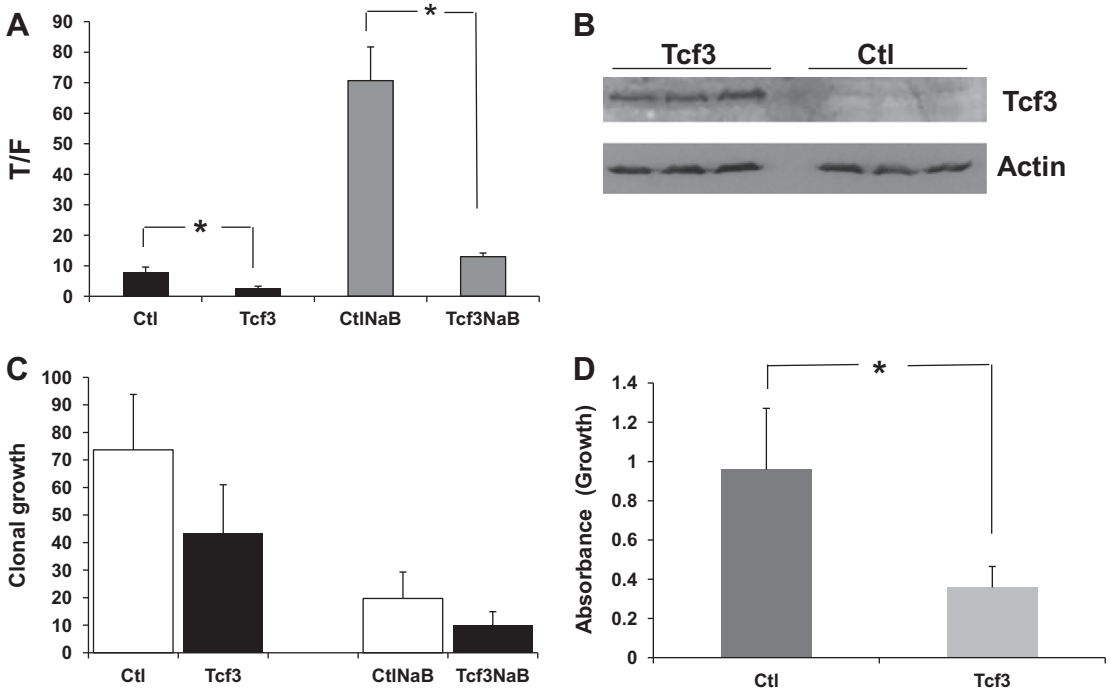

Fig. 3. Exogenous Tcf3 expression in HCT-116 cells. (A) Cells in a 96-well plate were transfected (Lipofectamine 2000) with TOP/FOPFlash reporter vectors, pcDNA3 control (Ctl) or pcDNA3-Tcf3 (Tcf3) expression vector, and pRLTK. Medium was changed 5 h post-transfection, and cells were mock- or 5 mM NaB-treated 17 h. Data are from three independent experiments. (B) Western blot analysis of exogenous Tcf3 in HCT-116 cells. Replicate samples from Tcf3 and pcDNA3 (Ctl) transiently transfected cells are shown. (C) Lack of significant alteration of clonal growth of Tcf3-expressing HCT-116 cells. HCT-116 cells were nucleofected with Tcf3 expression vector or control vector. Each batch of cells was split (plated) onto 2 wells of a 6-well plate. On day two the cells were exposed to mock or 5 mM butyrate treatment for total of 17 h. On day three cells were counted and plated at different cell density for clonal growth. At 14 days the colonies were fixed, stained with crystal violet solution, and counted. Data are from eight independent experiments. (D) Repression of HCT-116 cell proliferation by overexpressed Tcf3. HCT-116 cells were nucleofected with pcDNA3 control [Ctl] vector or Tcf3 expression vector and plated at 25,000 cells/well in a 96-well plate. Medium was changed every 24 h and after 72 h, proliferation was assayed as described in Section 2. Data are from five independent experiments. Bars, SDs. *Statistical significance.

growth of control and Tcf3-transfected cells, Fig. 3C). Repression of cell proliferation by Tcf3 would prevent the increased clonal cell growth expected from the suppression of Wnt hyperactivation. Consistent with this hypothesis, we determined that exogenous expression of Tcf3 inhibits HCT-116 cell proliferation, resulting in a 2.7-fold reduction ($P < 0.005$) of cell growth (Fig. 3D).

3.4. Overexpression of cell cycle factors in HCT-R cells

Our *Tcf3* silencing data indicate that Tcf3 suppresses Wnt activity hyperinduction and counteracts the inhibitory effect of butyrate on the clonal growth of HCT-R cells (Fig. 1). However, exogenous Tcf3 expression in HCT-116 cells does not counteract the effect of butyrate on clonal growth, since Tcf3 overexpression itself inhibits cell proliferation (Fig. 3). Therefore, the next logical question is: how do HCT-R cells proliferate in the presence of high Tcf3 levels? We posited that differential gene expression maintains HCT-R cell growth in the presence of elevated Tcf3, and thus performed human genome microarray analyses to compare three cell lines: control (pcDNA3)-transfected HCT-116 cells, Tcf3-transfected HCT-116 cells, and HCT-R cells. Each cell line was exposed to mock treatment or 5 mM butyrate. Butyrate-treated HCT-116 cells stably transfected with Tcf3 express the protein at levels comparable to those in HCT-R cells (Figs. 1A and 4A).

Tcf3- and control–transfected HCT-116 cells exhibit a similar decrease in cell proliferation ($P < 0.001$ and $P < 0.005$, respectively) in the presence of butyrate (Fig. 4B). In contrast, proliferation of HCT-R cells is relatively unaffected by butyrate (Fig. 4B). These data confirmed our previous observation that while Tcf3 overexpression is necessary for butyrate-resistance (Fig. 1), it is not sufficient, since HCT-116 cells that stably overexpress Tcf3 do not exhibit the same degree of butyrate resistance as HCT-R cells (Fig. 4B).

The full human genome array analyses of the three cell lines identified a large number of genes differentially regulated by butyrate by at least two-fold (Fig. 4C). Table 1 lists a number of such genes that are involved in cell cycle progression. *Cyclins* (A, B, D, E, F forms), *CDC*25 A,B,C, and cyclin-dependent kinases (*cdks* 1,2,4,6) are overexpressed in HCT-R cells.

For example, *Cyclin D2* is a Wnt signaling-targeted gene required for tumorigenesis in mouse models of intestinal cancer [23]. Consistent with the microarray data, HCT-R cells exhibit higher levels of cyclin D2 protein compared to Tcf3-expressing stable transfectants, which in turn exhibit higher levels compared to pcDNA3 transfected HCT-116 cells (Fig. 4D). HCT-R cells also exhibit higher levels of cyclin D2 protein compared to parental HCT-116 cells (Fig. 4D). The cdks associated with cyclin D2 (cdk4,6), which promote the G_1 to S transition, are also overexpressed in HCT-R cells (Table 1). Since butyrate induces G_1 cell cycle arrest, the activity of cyclin D/cdk complexes likely enhances proliferation of butyrate-resistant HCT-R cells.

PD0332991 is a specific inhibitor of cdk4,6, repressing the activity of the cyclin D/cdk complexes that likely contribute to butyrate resistance. Therefore, we hypothesized that treatment with PD0332991 would partially sensitize HCT-R cells to the growth repressing effects of butyrate. PD0332991 represses HCT-R cell growth both in the absence ($P < 0.05$) and presence ($P < 0.003$) of butyrate (Fig. 4E, left). Importantly, exposure to both butyrate and PD0332991 reduces HCT-R cell proliferation by 36% while butyrate alone suppresses cell growth by only 10.4%, a greater than three-fold difference ($P < 0.05$) (Fig. 4E, right). These data support the hypothesis that, in addition to Tcf3, the expression of cell cycle

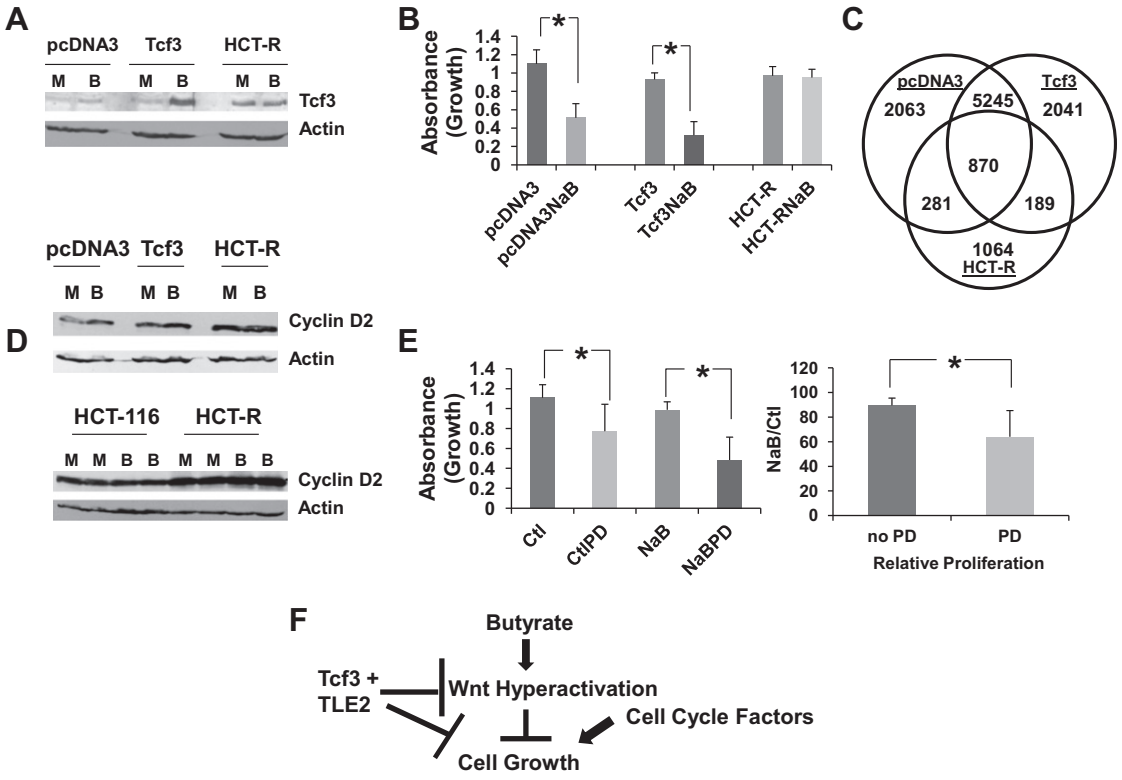

Fig. 4. Cell cycle factors contribute to butyrate resistance. (A) Western blot analysis of Tcf3 expression levels in mock (M) and butyrate (B) treated (5 mM, 17 h) cells. Analyzed cell lines include HCT-R cells and HCT-116 cells stably transfected with pcDNA3 (control) or pcDNA3-Tcf3 (Tcf3). (B) Cell proliferation analyses of the three cell lines described in (4A). Cells were exposed to mock or 5 mM butyrate (NaB) treatment for 17.5 h, and assays were performed as described in Fig. 3. Data are from four independent experiments. (C) Venn diagram showing distribution of differentially expressed genes modulated by butyrate two-fold or more. (D) Western blot analyses of cyclin D2 expression, comparing HCT-R cells to stable transfectants (top) and HCT-116 cells (bottom), in the presence (B) or absence (M) of butyrate. (E) Proliferation assays were performed as in Fig. 3D, and absorbance readings normalized to an internal control. Cells were treated, or left untreated, with 10 μM PD0332991 and/or 5 mM NaB, for 72 h. Left, absorbance growth readings for mock (Ctl) and butyrate (NaB) treated cells, in the absence or presence of PD0332991 (PD). Right, relative proliferation of butyrate treated HCT-R cells compared to mock cells, in the presence (PD) or absence (no PD) of PD0332991. Data are from five independent experiments. Bars, SDs. *Statistical significance. (F) Schematic showing mechanisms of butyrate resistance. Butyrate-induced Wnt hyperactivation suppresses cell growth and is inhibited by Tcf3 and TLE2. However, Tcf3 also suppresses cell growth. Overexpression of cell cycle factors promotes cell growth, allowing cells with high expression levels of Tcf3 to proliferate. Thus, overexpression of both Tcf3 and cell cycle factors allows HCT-R cells to evade Wnt hyperactivation and proliferate in the presence of butyrate. Other mechanisms of butyrate resistance [7] may contribute as well.

Table 1
Fold change in gene expression at the mRNA level, as measured by differential gene expression in pcDNA3 or Tcf3-transfected HCT-116 cells and butyrate-resistant HCT-R cells. Several gene targets are shown, with the fold-change in expression between cell lines in the absence (left) or presence (right) of 5 mM butyrate (NaB). pcDNA3 stable (pc), Tcf3 stable (Tcf3), HCT-R cells (HCTR), A, absent; NDR, not differentially regulated between any of the cell line combinations at a range of two-fold or greater.

No NaB				NaB		
Gene	Tcf3/ pc	HCTR/ pc	HCTR/ Tcf3	Tcf3/ pc	HCTR/ pc	HCTR/ Tcf3
CCNA2		NDR		0.94	6.03	6.39
CCNB1		NDR		0.83	4.36	5.26
CCNB2		NDR		1.04	3.68	3.54
CCND1		NDR		0.81	4.16	5.11
CCND2	A	107.80	112.86	8.70	67.54	7.76
CCNE2	0.78	2.81	3.59	0.80	3.38	4.22
CCNF		NDR		1.05	3.02	2.86
CDC25A	0.68	1.47	2.17	0.96	7.50	7.83
CDC25B	1.01	2.26	2.24	0.89	9.43	10.62
CDC25C		NDR		1.07	3.32	3.11
CDK1		NDR		0.96	6.74	7.03
CDK2		NDR		1.08	2.38	2.08
CDK4		NDR		1.10	2.27	2.07
CDK6		NDR		0.92	4.00	4.35

factors required for the G_1 to S transition promote butyrate resistance in HCT-R cells.

4. Discussion

Hyperinduction of Wnt activity mediates the effects of butyrate on CRC [7,15]; therefore, we hypothesized that butyrate resistance is accompanied by altered expression of genes related to Wnt signaling. In this study, we have identified a role for the Wnt signaling factor Tcf3 in butyrate resistant HCT-R cells. Elevated levels of Tcf3 in HCT-R cells inhibit the hyperactivation of Wnt activity, allowing for cell growth in the presence of butyrate (Fig. 1).

Whereas Wnt signaling-targeted promoters are induced by Tcf4/beta-catenin complexes in CRC cells, Tcf3 factors seem not to associate with beta-catenin [21]. In fact, Tcf3 competes with Tcf4/beta-catenin complexes for binding to Wnt signaling-targeted promoters, and thus inhibits Wnt transcriptional activity [21]. Further, Tcf3, in the absence of bound beta-catenin, associates with corepressors such as TLE2 to inhibit gene expression [20–22]. Our data, which show that Tcf3 does not associate with beta-catenin, but does bind to the transcriptional corepressor TLE2 (Fig. 2), support a repressive role for the elevated levels of Tcf3 in HCT-R cells.

Our findings suggest that (a) elevated levels of Tcf3 are necessary to prevent hyperactivation of Wnt signaling by butyrate (Figs. 1 and 3), (b) high levels of Tcf3 repress cell proliferation (Fig. 3D), and (c) HCT-R cells bypass the anti-proliferative activity of Tcf3 (Figs. 1 and 4). The array data suggest that HCT-R cells

proliferate despite high endogenous levels of Tcf3 by upregulating factors involved in cell cycle progression (Table 1). Further, since butyrate induces G_1 cell cycle arrest, it is not surprising that factors which promote the G_1 to S transition (e.g. cyclin D factors and cdk4,6) are overexpressed in HCT-R cells; thus, repression of cyclin D/cdk activity with PD0332991 increased the sensitivity of HCT-R cells to the growth inhibitory effects of butyrate (Fig. 4E).

In summary, high levels of Tcf3 have opposing effects. By repressing butyrate-induced Wnt hyperactivation, Tcf3 favors HCT-R cell clonal growth in the presence of butyrate (Fig. 1); however, high levels of Tcf3 repress proliferation of parental HCT-116 cells (Fig. 3). Therefore, as outlined in the schematic in Fig. 4F, butyrate resistance in HCT-R cells is in part due to (a) Tcf3-mediated repression of Wnt signaling hyperactivation, and (b) higher expression of cell cycle factors that compensate for the anti-proliferative effects of Tcf3. Cyclin D/cdk4,6 activity also allows HCT-R cells to proliferate (Fig. 4E), by overcoming butyrate-induced cell cycle arrest.

These findings provide potential molecular targets for prevention or reversal of butyrate resistance in CRC. Since the effects of butyrate are mimicked by synthetic HDACis [7,19], knowledge of how to overcome butyrate resistance would assist in the design of an effective anti-CRC therapy based upon HDACis.

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