Supplementary Information

Part I - Microarray analysis.
Part II -Summary of findings in relation to our previous studies.

Part I - Microarray analysis. To understand the global changes in gene expression consequent to p300 deletion, we performed a pilot full human genome microarray analysis, comparing wild-type p300 HCT-116 cells to p300 knockout F5 and D10 cells (+/- butyrate). The microarray data reveal marked differences in gene expression between p300 knockout cells and wild-type p300 cells. Although the two p300 knockout cell lines F5 and D10 exhibit gene expression differences between each other, they exhibit more differences in gene expression when compared to the wild-type p300 HCT-116 cells. These findings were compared to our previous microarray data that examined the gene expression profiles of butyrate-resistant HCT-R cells and other neoplastic colonic cell lines [S1-3], in order to identify genes responsible for butyrate resistance.

The analyses presented here provide information on all genes differentially regulated by a p300 knockout in the mock- and butyrate-treated states. Since we are searching for gene expression differences that underlie butyrate resistance we compared the published here microarray data to these obtained from the comparison of wild-type p300, butyrate-sensitive HCT-116 cells to butyrate-resistant, p300-deficient HCT-R cells. We did consider the fact that HCT-R cells exhibit a higher degree of butyrate resistance than do p300-knockout F5 cells, since gene alterations other than that of p300 also affect resistance [S1]. We also focused on genes that are both (a) differentially regulated by the knockdown of p300, and (b) known targets of Wnt activity in presence and absence of butyrate.

To accomplish this objective, we cross-referenced gene targets identified with previously published microarray data [S3] that compared (+/- butyrate) HCT-116 cells with HCT-116 cells in which Wnt activity is suppressed by a dominant negative inhibitor. Comparison of these data sets and the current ones allowed us to identify the specific genes that are (a) targets of the p300-mediated Wnt signaling, (b) associated with the CRC cell response to butyrate, and (c) differentially regulated in all analyzed p300-deficient cells.

The following is a summary of the changes in gene expression exhibited by p300 knockout CRC cells in the presence or absence of butyrate. We specifically focused on genes that, due to their known function, are most likely associated with the changes in cell signaling and phenotype observed with p300 deficiency. We also compared the differential gene expression between p300 knockout cells (D10 and F5) and HCT-116 cells (-/+ butyrate) to the differential gene expression between HCT-R and HCT-116 cells from a previously published study [S1].

<u>Wnt signaling</u>
Both HCT-R and F5 cells showed repressed hyperactivation of Wnt signaling when exposed to butyrate. Tcf4 (*TCF7L2*) is a major Wnt signaling–controlled transcription factor that binds to beta-catenin and mediates Wnt transcriptional activity in colonic cells. *TCF7L2* was downregulated at the RNA level in all three p300-deficient cells (i.e., HCT-R, D10 and F5 cells). Repressed expression of Tcf4 may contribute to the inhibited Wnt

signaling hyperactivation by butyrate in HCT-R and F5 cells; however, additional factors must contribute to this effect, since D10 cells that also exhibited downregulation of *TCF7L2* expression, did not exhibit the same repression of Wnt signaling hyperactivation. The same observation holds for the Wnt signaling inhibitor Axin 2 (*AXIN2* is itself a Wnt signaling-targeted gene), which was upregulated in all three cell lines and may play a role in suppressing Wnt hyperactivation in HCT-R and F5 cells. Tcf3 (*TCF7L1*) is a Wnt signaling factor that binds beta-catenin; however, Tcf3 is more often associated with repression of Wnt activity, and we have shown [S1] that the overexpression of Tcf3 observed in HCT-R cells (at both the RNA and protein levels) contributes to butyrate resistance in that cell line. Interestingly, the microarray data did not show that *TCF7L1* was consistently overexpressed in D10 or F5 cells; thus, compared to wild-type p300 HCT-116 cells, these two p300 knockout cell lines exhibited a less than two-fold decrease in expression of Tcf3 in the absence of butyrate and a less than two-fold increase in expression in the presence of butyrate. In contrast, mock-treated HCT-R cells exhibited a three-fold increase in Tcf3 expression at the mRNA level compared to HCT-116 cells, and more than a two-fold increase in Tcf3 expression in the presence of butyrate. Thus, the differential Tcf3 expression between D10, F5, and HCT-R cells may in part contribute to the greater degree of butyrate resistance observed in HCT-R vs. D10 or F5 cells.

DKK1 is a Wnt signaling inhibitor, and we have previously shown that overexpression of DKK1 represses the hyperactivation of Wnt signaling by butyrate in CRC cells [S4]. These findings suggest that one mechanism whereby butyrate enhances Wnt signaling is through promotion of canonical Wnt pathway at the ligand-receptor level, even in cells with Wnt signaling deregulated by a mutation [S4]. *DKK1* was mildly upregulated in all three p300-deficient cell lines in absence of butyrate, and slightly repressed in butyrate-treated D10 and F5 cells. Similarly, the Wnt inhibitor *DKK4* was downregulated in butyrate-treated HCT-R, D10, and F5 cells. The simultaneous up- and downregulation of Wnt inhibitors may result in an optimized levels of Wnt activity that lead to cell proliferation, rather than apoptosis [S4]. *TLE1*, which codes for a transcriptional corepressor and tumor suppressor that inhibits Wnt activity, was downregulated in all three p300-deficient cells. Sox13 represses Wnt signaling; therefore it is not surprising that the *SOX13* gene was overexpressed in F5 cells that showed repressed Wnt activity in the presence of butyrate. However, HCT-R cells exhibited downregulation of *SOX13*, suggesting that whereas p300-deficient CRC cells may share a number of mechanisms by which butyrate-induced Wnt activity and apoptosis are downregulated (as shown by the totality of our data), there are cell-type specific differences in gene expression that influence the butyrate-resistant phenotype. Of relevance to butyrate functioning as an HDACi, *HDAC2* was upregulated in butyrate-treated D10 and F5 cells; whereas, *HDAC5* was downregulated in butyrate-treated HCT-R and F5 cells. One difference between HCT-R cells, that have a more pronounced butyrate-resistant phenotype, and D10 and F5 cells, that are partially butyrate resistant, was the expression of *ZNRF3*, which codes for a Wnt activity repressor. *ZNRF3* expression was upregulated in HCT-R compared to wild-type p300 HCT-116 cells (in the presence of butyrate); whereas, D10 and F5 cells showed a modest decline in expression of this gene Thus, the ZNRF3 repressor may contribute to the greater suppression of Wnt activity observed in butyrate-treated HCT-R cells compared to the other p300 deficient CRC cell lines.

Apoptosis and proliferation

Among the three p300 deficient CRC cell lines, HCT-R cells exhibited the greatest degree of butyrate resistance with respect to modulation of Wnt signaling, apoptosis, and proliferation. Whereas all three cell lines (HCT-R, D10, and F5) showed repressed apoptosis when exposed to butyrate, HCT-R and F5 cells also exhibited resistance to the butyrate-induced inhibition of cell proliferation. The anti-apoptotic factor survivin (*BIRC5*) is downregulated by p300 [S5]. Consistent with the known effects of butyrate, survivin expression was downregulated by butyrate in HCT-R, D10, and F5 cells. However, this repression was attenuated in all three p300 deficient cell lines. These findings suggest that one mechanism leading to resistance to butyrate-induced apoptosis is through maintenance of relatively higher levels of survivin. Expression of the pro-apoptotic genes *BAD* and *BIK* was mildly downregulated in mock-treated F5 cells at the RNA level, and BIK was also downregulated in butyrate-treated F5 cells. *BIK* was also downregulated in HCT-R cells; whereas, counterintuitively, BAD was upregulated. Caspases 4, 5, and 10 were downregulated in D10, F5, and HCT-R cells; however, caspases 3 and/or 7 were moderately upregulated in some of these cell lines. In D10 and F5 cells, the cell cycle inhibitor p21 was upregulated, and we have previously observed that increased expression of p21 interferes with apoptosis in CRC cells [S1]. Expression of cytochrome C, which is involved in induction of apoptosis, was downregulated in F5 cells.

Expression of cell cycle/proliferation genes was also deregulated in the cells. *CDK6* and *CCNE2*, whose products are involved in cell cycle progression, exhibited upregulated expression in HCT-R, D10, and F5 cells. *CCNT2* was upregulated in F5 cells. The following genes, whose products enhance cell proliferation/cell cycle progression, were overexpressed at the RNA levels in HCT-R, D10, and F5 cells in the presence of butyrate: *TTK, CDCA2*, *CDC25A*, *CDK1*, *CDCA3*, *CCNA2*, *CCNB1*, and *CCNB2*. Overexpression of these genes may assist in promoting continued proliferation of butyrate-resistant HCT-R cells and F5 cells in the presence of butyrate. However, additional factors may be required for butyrate-resistant proliferation since D10 cells, which also overexpress these genes, do not exhibit statistically significant differences in proliferation in the presence of butyrate compared to wild-type p300 HCT-116 cells. One difference in this respect is that *CDCA5*, whose product is important for sister chromatid cohesion, was upregulated in HCT-R and F5, but not D10, cells.

EMT, Notch, and other cell signaling

Vimentin was overexpressed, and E-cadherin repressed, in p300 knockout cells F5 and D10, consistent with their more pronounced EMT-like phenotype. This pattern of gene expression was also observed in the butyrate-resistant, p300-deficient HCT-R cell line [S1], suggesting connections between p300 deficiency, EMT, and butyrate resistance. Consistent with this, the EMT-activator ZEB1 was overexpressed in all three cell lines. Claudin-4, which is downregulated at the invasive front of CRCs, exhibited repressed expression in HCT-5, D10, and F5 cells. Other genes involved in EMT and tumor invasion were upregulated in the D10 and F5 cells, including *TGM2*, *THBS1*, and *NANOS1* (which itself is normally downregulated by E-cadherin). *TGM2* and *NANOS1* were also upregulated in HCT-R cells; however, *THBS1* was downregulated in this cell line upon exposure to butyrate. Cyclin G2 (*CCNG2*) inhibits EMT by repressing Wnt activity; this gene was expressed at higher levels in LT97 cells compared to metastatic SW620 cells,

which is consistent with the SW620 line already have undergone EMT. Interestingly, F5 cells expressed higher levels of cyclin G2 at the RNA level than D10 cells.

Surprisingly, the tumor suppressor *NGFR* was upregulated in all three p300 deficient, butyrate-resistant cell lines [S1]. Expression of *GAREM*, whose product promotes MAPK/ERK signaling, was downregulated in D10 and F5 cells. *NAAC2*, whose product promotes p53 stability, was upregulated in D10 and F5 cells, and in HCT-R cells exposed to butyrate. Microarray data also revealed that the histone acetylase PCAF, which associates with CBP and p300, and promotes drug resistance, was upregulated in all three p300 deficient cell lines: HCT-R, D10, and F5.

Additional colonic signaling pathways may be affected by p300 downregulation. For example, expression of the Notch signaling pathway inhibitor DLK2 was upregulated in HCT-R cells, and in the p300 knockout D10 and F5 cells. *JAG2,* encoding a Notch ligand, was downregulated in all three cell lines. The Notch targets *HES1* and *HES2* were upregulated in HCT-R cells; *HES1* was upregulated in D10 cells and *HES2* was upregulated in F5 cells. *SMARCA2*, encoding for a chromatin remodeling factor, was downregulated in all three p300 deficient cell lines in the presence of butyrate, suggesting more global effects on gene transcription control. The expression of SIRT1, which codes for a histone deacetylase that represses p300 transactivation, was upregulated in D10 and F5 cells.

The metastatic tumor promoter *CD24* was overexpressed at the RNA level in the three p300 knockout cell lines; however expression of *MALAT*1, whose product enhances cancer cell proliferation, migration, and invasion was upregulated in D10 and F5 cells, and significantly downregulated in HCT-R cells. The tumor suppressor *CDKN2A* was overexpressed in all three p300 deficient cell lines in the presence of butyrate. *RET*, a proto-oncogene, whose product has paradoxically been suggested to act as a tumor suppressor in CRC [S6], was markedly upregulated in the F5 cell line. *JUNB*, an oncogene that codes for a transcription factor, was downregulated in D10 and F5 cells; *JUND* was also downregulated in the two cell lines. *BMP4,* whose product promotes differentiation and apoptosis in colonic cells, was downregulated in HCT-R, D10, and F5 cells, possibly contributing to the butyrate-resistant phenotype. *BMP7*, whose product enhances invasiveness and protects against apoptosis in breast cancer cells, was upregulated in HCT-R and F5 cells. It is possible that BMP7 plays a similar anti-apoptotic role in p300 deficient CRC cells. *FOXF1*, which codes for a transcription factor and tumor suppressor, was upregulated in all three p300 deficient cell lines. *KLK6* was upregulated in F5 cells, but downregulated in HCT-R and D10 cells. *CD44* was upregulated in butyrate-treated F5 cells, and downregulated in untreated HCT-R cells. In addition, *PBK*, whose product enhances resistance to anoikis, was also upregulated in all three p300 deficient cell lines. c-Myc expression s stimulated by p300, and as expected, c-*myc* expression was repressed in the p300 deficient HCT-R [S1], D10, and F5 cells.

The following genes were upregulated at the RNA level in all three p300 deficient cell lines: *DCLK1*, *FGF9*, and *SP5*. *DCLK1* codes for a putative tumor stem cell marker; altered expression of this gene may indicate that p300 deletion leads to a more stem cell-like phenotype. *SP5* is a Wnt signaling-targeted gene that encodes for a transcription factor; the upregulation of *SP5* expression in p300 deficient cells suggests that this gene is a specific target of CBP-Wnt activity. *PAIP2B* and *TDRD3* expression was upregulated in F5 cells only; while *RASA4* expression was upregulated in F5 cells and downregulated

in HCT-R cells. *S100A14* was downregulated in all three cell lines; the product of this gene is a putative tumor suppressor and its downregulation enhances cell cycle progression in esophageal cancer cells. *PLA2G10* was downregulated in butyrate-treated HCT-R and F5 cells; whereas *RND3* was downregulated in butyrate-treated F5 cells and upregulated in butyrate-treated HCT-R cells. *MBNL2* was upregulated in butyrate-treated F5 cells only. Counterintuitively, *MSX1* was upregulated in all three cell lines; the product of this gene represses cell cycle progression, and may act as a tumor suppressor. *RRM2* was upregulated in all three cell lines in the presence of butyrate.

Other genes whose products of which control cell growth were also upregulated differentially. For example, in the presence of butyrate *DHFR* was upregulated in all three p300 deficient cell lines; whereas, *TK1* was upregulated in butyrate-treated F5 cells. Circadian rhythm deregulation may contribute to cancer, and the gene *CRYZC1*, whose product is involved in circadian control, was upregulated in HCT-R and F5 cells. *NXF2*, which codes for a RNA export factor, was downregulated in all three cell lines, possibly affecting gene expression through mRNA transport. *ERBB2*, whose product activates signaling pathways such as MAPK and PI3K, was upregulated in all three p300 deficient cell lines. *TXK,* whose product is involved in PI3K and Akt signaling, was downregulated in D10 cells, and to a lesser extent in F5 cells. Gastrin (*GAST*), which promotes CRC cell growth, was downregulated in all three cell lines.

Choline metabolism. A previous array analysis on p300 knockout HCT-116 cells, concentrating on metabolic genes, ascertained that these cells have a derangement of choline metabolism [S7]. Similar to these previous results, the p300 knockout D10 and F5 cells overexpressed certain genes involved in choline metabolism (e.g., *PLAA, PCYT1A, CHPT1*). Interestingly, these genes were not differentially modulated (two-fold or greater) in HCT-R cells [S1], suggesting that the p300 deficient lines may differ in aspects of phenotype other than butyrate resistance.

Butyrate transport, metabolism, and *MDR*1 expression. Not surprisingly, mock-treated HCT-R cells downregulated the expression of *SLC16A1* and *SLC16A3*, which encode the butyrate uptake transporters MCT1 and MCT4. Interestingly, p300 knockout D10 and F5 cells which exhibit butyrate resistance despite never being selected for such resistance through incubations with increasing butyrate concentrations, also demonstrated downregulated expression of these genes. Thus, in mock-treated D10 and F5 cells, and in butyrate-treated F5 cells, *SLC16A1* expression was downregulated at the mRNA level; whereas, *SLC16A3* was downregulated in these cell lines in absence and presence of butyrate. Altered butyrate uptake could contribute to butyrate-resistance; however, it does not explain (a) cross-resistance to other HDACis as exhibited by HCT-R cells, [S4], or (b) the resistance of D10 and F5 cells to butyrate without these cells being previously selected for growth in presence of the agent. One possibility is that in p300 knockout cells, the lack of p300 activity downregulates butyrate uptake transporters, although the HDACi cross-resistant phenotype of HCT-R cells suggests a broader effect involving modulation of histone deacetylase vs. histone acetylase activity and Wnt signaling. Furthermore, it has been reported that HCT15 CRC cells, which are p300 negative, exhibit resistance to butyrate by upregulating, not downregulating, the butyrate transporter SLC16A1. In HCT15 cells, *ACADS* (SCSD) and *ACADM* (MCAD), which encode for

acyldehydrogenases that catalyze the initial step in butyrate beta-oxidation, are upregulated. *ACADS* was not upregulated in the cell lines analyzed in our study; whereas, *ACADM* was upregulated in D10, F5, and butyrate-treated HCT-R cells. Therefore, butyrate-resistant cells may exhibit increased expression of some enzymes involved in butyrate metabolism; however, there is no clear association between butyrate-resistance, cross-resistance to other HDACis, p300 status, and butyrate uptake/metabolism. The product of the *ABCG2* gene, BCRP, has been identified as a butyrate efflux transporter in intestinal cells; however, expression of *ABCG2* at the mRNA level was not detected in p300 wild-type or p300 knockout HCT-116 cell lines. In HCT-R cells, this expression was low with no significant upregulation. In summary, the microarray data do not support altered butyrate efflux as a substantial contributor to the butyrate resistance phenotype.

Chemoresistance to a variety of pharmacological agents has been linked to activated expression of multidrug resistance 1 (MDR1 from the *ABCB1* gene). Our array data revealed that MDR1 was overexpressed in the HCT-R line [39]; however, it was not expressed at detectable levels in D10 and F5 butyrate-resistant cells.

Comparison to butyrate-modulated Wnt signaling-targeted genes. It has been established that survivin expression is upregulated by CBP-Wnt activity and suppressed by p300-Wnt activity, expression of cyclin D1 is upregulated by CBP-Wnt activity and unaffected by p300, and expression of c-Myc is upregulated by p300-Wnt activity. As described above, microarray analysis revealed that, as expected, p300 deficient cells exhibited higher levels of survivin expression in the presence of butyrate than the parental p300 wild-type HCT-116 cells. This suggests that p300 deficient cells acquire a degree of butyrate resistance through the loss of p300-mediated repression of survivin expression, leading to decreased levels of apoptosis after exposure to butyrate. As expected, the data show that c-Myc expression was downregulated in p300 deficient cells.

The following genes which were previously shown to be upregulated by butyrate in a Wnt activity-dependent manner [S3] also exhibited increased expression in p300 deficient HCT-R [S1] and/or D10 and/or F5 cells: *AXIN2*, *DKK1*, *VIM*, *NANOS1*, *HES1*, *HES2*, *SP5*, and *MSX1*. It is therefore likely that these genes are specific CBP-Wnt targets and/or are normally repressed by p300-Wnt activity. This control by CBP- and/or p300-mediated Wnt activity may be direct or indirect, although some of these genes are already known direct Wnt activity targets. The pro-apoptotic *BAD* gene is of significant interest since (a) this gene is upregulated by butyrate in a Wnt signaling-dependent manner [S3], and (b) its expression was downregulated in F5 cells. Therefore, *BAD* is a candidate p300-Wnt target gene whose expression may mediate the putative positive effects of p300-Wnt activity on apoptosis. *TTK* and *DHFR* are upregulated by butyrate in a Wnt-dependent manner [S3]; however, these genes were upregulated in all three p300 deficient cell lines by butyrate. This suggests that these genes are normally repressed by p300-Wnt activity (and perhaps upregulated by CBP-Wnt activity); p300 deficiency, therefore, enhances their expression. *JAG2* is downregulated by butyrate in a Wnt activity-dependent manner [S3]; expression of this gene was also downregulated in all three p300 deficient cells.

Summary. In summary, comparative microarray analyses have allowed preliminary identification of genes, whose expression might be specifically modulated by CBP-Wnt vs. p300-Wnt activity, with possible downstream physiological consequences (e.g., butyrate resistance, resistance to clinically relevant HDACis) in CRC cells.

Part II – Summary of findings in relation to our previous studies. The methodological approach and findings of the current study will now be put into the perspective of our previous work involving butyrate upregulation of Wnt signaling and consequent effects on CRC cell physiology [S1-S4 and references therein], as well as our previous findings on the role of p300 on butyrate-modulated Wnt signaling [S8] and our review on the relationship between butyrate/fiber, Wnt signaling, and CRC risk [S9].

The most significant novel aspect of the methodological approach of our current study is the use of CRC cells that specifically have inactivation of p300 expression. In this manner, all effects of p300, including and especially p300-Wnt signaling, are specifically abrogated in these cells, allowing us to narrowly interrogate the role of p300 in butyrate resistance (i.e., Wnt signaling and CRC cell physiology). This contrasts with earlier studies that examined butyrate resistant CRC cells (HCT-R) developed in our laboratory [S4,S8] and the HCT-15 cell line that had naturally developed p300 inactivation (and a degree of butyrate resistance) as a result of the *in vivo* process of oncogenesis in the tumor from which it was derived [S8 and references therein]. These cells naturally possess a wide variety of other changes that influence cell signaling and gene expression other than the downregulation of p300. Thus, comparing those cell lines to butyrate-sensitive HCT-116 cells introduces a wide variety of variables that would obscure the purely p300-related effects of interest in our current manuscript.

Likewise, previous microarray studies compared effects of butyrate on HCT-116 cells in the presence of normal Wnt activity and with that activity artificially suppressed [S3], or compared gene expression of HCT-R cells to the HCT-116 cells from which they were derived [S1]. The former study was comprehensive, but in no way specifically restricted to effects of p300; the latter study, dealing with HCT-R cells, had all the confounding variables of cell signaling and gene expression mentioned above. Thus, evaluating the specific effects of knockout of p300, and of reintroduction of p300 in the "rescue" cells, is a methodological advance over previous work with respect to the role of p300 in butyrate resistance. Therefore, our present paper allows us to ask to what degree is p300 expression required for sensitivity to butyrate, and our findings indicate that p300 expression is indeed one factor required for a complete (i.e., HCT-116 cell-like) response to that agent with respect to Wnt signaling and apoptosis.

In our analysis of past and present microarray data, we compared findings from p300 knockout cells to the HCT-R line and discovered considerable similarities. Of particular interest is the finding that all of these butyrate resistant cell lines exhibit upregulation of gene expression markers of EMT, concomitant with downregulation of p300 These data suggest connections between p300 downregulation, butyrate resistance, and metastatic progression; this line of inquiry would be a fruitful topic for investigation in future studies.

We also compared our present microarray findings to the previous analysis that identified the cohort of Wnt target genes modulated by butyrate [S3]. This comparison revealed that a number of genes that are upregulated by butyrate in a Wnt activity-

dependent manner exhibit differential expression with p300 deficiency. A number of such genes, which are involved in cell signaling pathways and/or EMT, are upregulated by deficient p300 expression: *AXIN2, DKK1, VIM, NANOS1, HES1, HES2, SP5*, and *MSX1*.

Of particular interest is the pro-apoptotic *BAD* gene, which is also a target of butyrate-induced Wnt signaling. *BAD* is downregulated in F5 cells, a p300 knockout line that exhibits a significant degree of butyrate resistance. This finding suggests that loss of *BAD* expression in p300 deficient CRC cells is one mechanism whereby these cells develop resistance to the apoptosis-inducing effects of butyrate. Thus, upregulating *BAD* expression may in part reverse the butyrate resistant phenotype, a hypothesis that can be explored in future studies. In addition, relatively higher levels of the anti-apoptotic factor survivin is expressed in the presence of butyrate in p300 deficient cells, a finding consistent with survivin expression being repressed by p300-Wnt signaling [S5]. Thus, the relative levels of pro- and anti-apoptotic factors contributes to butyrate resistance that results from p300 deficiency.

We had previously demonstrated that HCT-R cells that are butyrate resistant do not express p300 [S8]. In addition HCT-15 cells that are naturally p300 deficient are partially butyrate resistant, particularly with respect to effects of butyrate on cell proliferation; however, HCT-15 cells are more sensitive to the apoptosis-inducing effects of butyrate than are HCT-R cells [S8]. We have established that other factors, such as the overexpression of Tcf3, contributes to the high degree of butyrate resistance observed in the HCT-R cell line [S1]. The p300 knockout cells did not exhibit the same degree of upregulation of Tcf3 as do HCT-R cells; this may in part be why the p300 knockout cells (Fig. 1) do not exhibit the same degree of butyrate resistance as do HCT-R cells.

Of the two knockout lines, F5 cells are more butyrate resistant than are D10 cells, primarily due to the greater resistance of F5 cells to the anti-proliferative effects of butyrate (Fig. 1). F5 cells also exhibit less butyrate-induced Wnt signaling than do D10 cells (Fig. 1); while HCT-R and HCT-15 cells exhibit less butyrate-induced Wnt signaling than do butyrate sensitive HCT-116 cells. The rescue cells, in which p300 expression of F5 cells is reintroduced, exhibit a partial restoration of butyrate sensitivity (Fig. 2), underscoring the importance of p300 for the response of CRC cells to butyrate. We note that our previous study [S8] demonstrated that exogenous overexpression of p300 in HCT-116 cells (that endogenously express p300), as well exogenous p300 overexpression in p300 deficient HCT-R and HCT-15 cells, increased butyrate-induced Wnt signaling. That finding is consistent to what is observed with the p300 rescue cells in the present study; reintroduction of p300 into F5 knockout cells enhances butyrate-induced Wnt hyperactivation, which is likely linked to the greater sensitivity of the rescue cells to the effects of butyrate on cell physiology (Fig. 2).

Thus, taking all the data together, sensitivity to butyrate among these cell lines can be ranked as: HCT-116>p300 rescue>HCT-15>D10 p300 knockout>F5 p300 knockout>HCT-R. The combination of p300 deficiency and Tcf3 overexpression accounts for the low butyrate sensitivity (and hence high butyrate resistance) of the HCT-R cell line. However, our present study clearly demonstrates that p300 deficiency alone accounts for the preponderance of these effects.

Finally, how do these findings fit into our overarching hypothesis about the role of butyrate on normal and aberrant colonic physiology as has been previously discussed [S9]? The importance of Wnt hyperactivation for the effects of butyrate on CRC cell

apoptosis and proliferation is supported by our present study, with the additional finding that the p300-mediated component of that Wnt hyperactivation is particularly important. Our previous review [S9] emphasized that while butyrate/fiber has been clearly linked to reduced CRC risk, there have been a number of studies that have been inconsistent about this finding. We speculated that tumor-to-tumor differences in Wnt hyperactivation, analogous to CRC cell lines that differ in their response to butyrate, may in part explain these inconsistencies. Thus, while many CRCs may be sensitive to butyrate/fiber, not all are; some CRCs are butyrate resistant. Our current findings suggest that these differences may in part be due to variable p300 expression. As we note in the Discussion of the current manuscript, some CRC patients have tumors exhibiting repressed expression of p300; thus, our findings here may in part explain why study-to-study differences in the butyrate-CRC link have been observed.

We also noted that other factors influence whether butyrate/fiber affects CRC risk, including that of the timing of exposure; thus butyrate most likely is most effective in suppressing the earliest stages of colonic neoplasia [S9]. It is possible that some of the early colonic neoplasms that escape control by butyrate/fiber are those that have developed butyrate resistance, in part by p300 deficiency. Other factors, such as patient-to-patient variation in gut microbiota could affect the amount of butyrate produced for a given amount of dietary fiber consumed. The levels of exposure to butyrate over time may affect the development of butyrate resistance; thus, chronic exposure to low levels of butyrate, insufficient to induce apoptosis of colonic neoplastic cells, may select for butyrate resistant cells, and this resistance may in part be mediated by suppressed expression of p300.

In summary, our current findings, utilizing the targeted approach of p300 knockdown and p300 rescue, support and extend previous findings, provide new evidence for the fundamental importance of p300 in the effects of butyrate, and provide further understanding for previous paradoxical findings in the literature with respect to the link between butyrate/fiber, Wnt signaling, and CRC risk.

Supplementary References

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Figures and Figure Legends (supplementary)

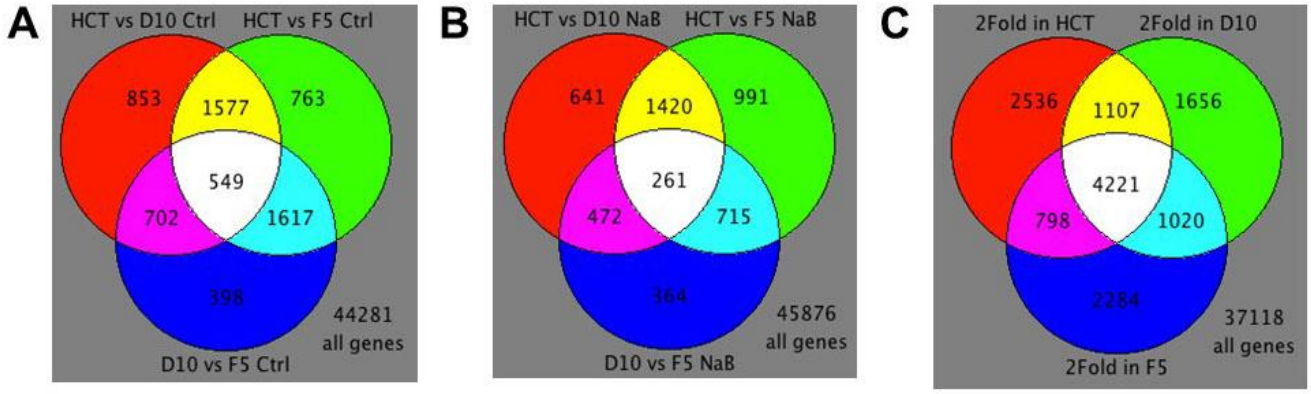

Fig. S1. Microarray analysis, HCT-116 vs. D10 vs. F5. Venn diagram comparisons of differentially expressed genes (>2 fold) across the three cell lines. (A) Comparisons between mock-treated cells. (B) Comparisons between butyrate-treated cells. (C) mock- vs. butyrate-treated gene expression for each cell line. Butyrate treatment was carried out at 5 mM for 17.5 hr.