Supplementary Information

Materials and Methods

TOPFlash/FOPFlash assays. TOPFlash is a luciferase reporter that contains a minimal *fos* promoter coupled to Tcf-binding sites upstream of a modified firefly luciferase gene. FOPFlash is similar, except that the Tcf-binding sites are mutated and non-functional. Thus, FOPFlash, which is a control reporter, yields expression from the minimal *fos* promoter only; whereas, TOPFlash yields expression from the minimal *fos* promoter and the Wnt activity-sensitive Tcf sites. Therefore, the ratio of expression from TOPFlash to expression from FOPFlash (T/F) provides a precise measurement of canonical Wnt-specific transcriptional activity. These plasmids were each cotransfected with the *Renilla* luciferase plasmid pRLTK, which controls for transfection efficiency. The transfections were carried out with Lipofectamine 2000 in 96-well plates with 25,000 cells/well, plated 24 hours earlier. The dual luciferase system of Promega was used with a luminometer to measure expression levels in light units.

UPF1 CRISPR activation. This system (Santa Cruz Biotechnology) uses a deactivated Cas9 (dCas9) nuclease to activate the target gene via *VP*64 and *HSF*1 transactivation domains. This is a three-plasmid system; stable transfection utilizes blasticidin, puromycin, and hygromycin as selection agents. A control plasmid set with a non-specific guide sequence is the negative transfection control. These plasmid sets were transfected, and selection commenced 48 hr later. Transfections were performed with Lipofectamine 2000 in 6-well plates with 200,000 cells/well, plated 24 hours earlier.

NMDI-14/Ataluren: NMDI-14 was used at 50 μM, a concentration shown to effectively upregulate NMD reporter expression at the mRNA level in U2OS cells [31], while not inducing significant cellular toxicity after a 48 hr incubation [31]. In pilot experiments, we observed that 10 μM Amlexanox [21] did not increase expression from the NMD reporter in HCT-116 cells (data not shown); thus, 50 μM of this agent was evaluated in this experimental protocol.

Results

Amlexanox Western blot data: The expression of the Wnt co-factors CBP and p300 are not affected by treatment with Amlexanox (Fig. S1).

NMDI-14 and Ataluren data: Unexpectedly, treatment with NMDI-14, either alone or in conjunction with Ataluren, decreased, not increased, expression from the NMD reporter (Fig. S2A). This suggests that this agent is potentiating, not inhibiting, NMD in HCT-116 cells. NMD reporter expression was reduced both by NMDI-14 alone ($P < 0.03$) and by the combination of NMDI-14 and Ataluren ($P < 0.001$). Ataluren alone, which is a read-through activator only, did not exhibit altered expression from the reporter.

With respect to apoptosis (measured by caspase 3/7 activity), NMDI-14 increases HCT-116 cell apoptosis when applied alone, although levels of butyrate-

induced apoptosis are not increased; thus the fold up-regulation of apoptosis by butyrate is reduced in the presence of NMDI-14 (Fig. S2B).

Importantly, Ataluren, alone or in conjunction with NMDI-14, completely abrogates HCT-116 cell apoptosis (Fig. S2B), similar to the anti-apoptotic effects exhibited by Amlexanox (Fig. 2D). Because of the similar action of Ataluren on apoptosis compared to Amlexanox, we evaluated how treatment with Ataluren affects basal and butyrate-induced Wnt signaling in the HCT-116 line. While Amlexanox increases basal Wnt activity (Fig. 2B), Ataluren reduced basal Wnt activity by 29% ($P < 0.005$) (Fig. S2D). Ataluren suppressed the overall levels of butyrate-induced Wnt signaling (Fig. S2C) by more than two-fold ($P < 0.005$). This contrasts with Amlexanox, which did not significantly change overall levels of Wnt activity in combination with butyrate (Fig. 2A). With respect to the fold-induction of Wnt activity by butyrate, which associates with butyrate-induced apoptosis, Ataluren reduced fold-induction of Wnt activity (Fig. S2E) by 35% (at the borderline of statistical significance at $P = 0.053$); Amlexanox treatment resulted in a greater suppression of the fold-induction of Wnt activity by butyrate (Fig. 2C).

Discussion

Relevance of our study to the findings of El-Bchiri *et al*.: The work of El-Bchiri *et al*. [6], utilizing siRNA knockdown of UPF1 in HCT-116 cells, demonstrated significant changes in gene expression resulting from NMD inhibition. UPF1-mediated NMD correlated to a poor prognosis for CRC, due to impaired host immune recognition of the cancer cells. The mechanism proposed for these observations was that more efficient NMD prevented the expression of immunogenic peptides, particularly from MSI+ CRC. Our findings show that overexpression of UPF1 enhanced Wnt signaling hyperactivation by butyrate and also increased CRC cell apoptosis; these effects are likely due to the greater NMD induced by UPF1 overexpression. Therefore, under different conditions, NMD may influence colonic carcinogenesis in an opposing manner. On the one hand, NMD can promote carcinogenesis and impede immune responses. On the other hand, NMD may enhance the protective activity of butyrate against CRC, possibly relevant in the context of a high-fiber diet. MSI+ CRCs have been reported to overexpress UPF1 compared to normal tissues [6], and we have shown [10,17 and refs. therein] that the MSI+ line HCT-116 is highly sensitive to Wnt signaling hyperactivation by butyrate. This is consistent with the enhanced sensitivity of the cells to butyrate when NMD is increased via enforced overexpression of UPF1 (Fig. 3). Therefore, the widespread changes in gene expression due to NMD may enhance the efficacy of butyrate against CRC, partially off-setting the negative effects of NMD on tumor progression and immune surveillance.

Amlexanox as an HDACi: Amlexanox has been reported to have some HDACi activity [33]; this may explain the ability of this agent to upregulate basal Wnt activity (Fig. 2B), since HDACis hyperactive Wnt signaling [summarized in refs. 10,17]. However, the fact that Amlexanox represses, rather than potentiates, Wnt hyperactivation by butyrate (Fig. 2C), as well as apoptosis (2D), suggests that the NMD-dependent action of Amlexanox in HCT-116 cells is dominant over other functions, such as that of an HDACi. This is

supported by the observation that the NMD activator UPF1 has opposite effects on Wnt hyperactivation and apoptosis as compared to Amlexanox (Figs. 2,3).

NMDI-14 and Ataluren findings: We evaluated whether the NMD inhibitor NMDI-14 [31] and/or the PTC read-through activator Ataluren [21] influence expression of the NMD reporter in HCT-116 cells, and whether any observed change in NMD efficiency (and/or read-through) influences the main physiological metric under consideration in our study: basal and butyrate-induced apoptosis, as measured by caspase 3/7 activity, in HCT-116 CRC cells.

We have previously demonstrated lack of efficacy of a lower concentration of Ataluren on NMD reporter activity in HCT-116 cells (pilot experiments; data not shown). NMDI-14 has not been demonstrated to inhibit NMD in HCT-116 cells; the published study investigating NMDI-14 used HCT-116 cells as a negative control as they are wild-type for the p53 target that is mutated with a PTC in other cell lines [31]. Therefore, that neither agent, nor the combination of these two agents, upregulates NMD expression in this cell line is not unexpected. However, the observation that NMDI-14 actually decreases expression from the NMD reporter in HCT-116 cells was unexpected and is a finding that needs to be further investigated in future in-depth studies. It has been reported that NMDI-14 functions by interfering with the association of the factors UPF1 and SMG7, which is required for canonical NMD [31]. It is possible that in certain cell types, under certain experimental conditions, the agent may not inhibit UPF1/SMG7 association and may possibly even potentiate the association and subsequent NMD activity. Investigating whether this occurs in HCT-116 cells under the conditions utilized in our experimental approach is also a possibility that should be investigated in the future, but which is beyond the scope of our present small-scale pilot study.

Importantly, the apoptotic data (Fig. S2B) demonstrate that Ataluren, a specific activator of PTC read-through, abrogates HCT-116 cell apoptosis, as measured by caspase 3/7 activity, both in the presence and absence of butyrate, similar to what was observed with Amlexanox. This suggests that the activity of Amlexanox of suppressing HCT-116 cell apoptosis is likely primarily dependent on PTC read-through activity. The effects of Ataluren on apoptosis are dominant to those of NMDI-14, which enhances apoptosis when utilized alone, although it does not further enhance butyrate-induced apoptosis. Cotreatment with Ataluren and NMDI-14 results in the same suppression of apoptosis as does Ataluren alone.

Given that Ataluren treatment results in similar effects on HCT-116 cell apoptosis as does Amlexanox, we compared the effects of these agents on basal and butyrate-induced Wnt signaling. While Amlexanox enhances basal Wnt activity (Fig. 2B), Ataluren suppresses this activity. One possibility for this difference is that PTC read-through activators may in general inhibit Wnt activity, but the additional HDACi activity possessed by Amlexanox [33] stimulates basal Wnt signaling. Our work [10-18 and refs. therein] has demonstrated that HDACis stimulate Wnt activity in CRC cells possessing mutations in this pathway that cause deregulated signaling.

Ataluren sharply inhibited (more than two-fold) the levels of butyrate-induced Wnt activity (Fig. S2C); in contrast Amlexanox/butyrate cotreatment did not result in significantly different levels of Wnt activity compared to butyrate alone. One hypothesis to explain these findings is that PTC read-through activators tend to suppress Wnt

activity levels in the presence of butyrate as is observed with Ataluren, but this effect is counterbalanced in the case of Amlexanox because of the HDACi activity of this agent.

It is the fold-induction of Wnt activity, rather than the overall levels of this activity, by butyrate that is associated with butyrate-induced apoptosis [10-18 and refs. therein]. Amlexanox and Ataluren both suppress this fold-induction but do so in different manners. Thus, Amlexanox treatment upregulates basal Wnt activity while keeping the final levels of butyrate-induced Wnt activity constant, while Ataluren results in a modest decrease in basal Wnt activity and a much greater decrease in the levels of butyrate-induced Wnt activity; in both cases, a lower degree of fold-induction is achieved. Our work [10-18 and refs. therein] suggests that is the scale of the fold-induction, and not the specific manner, in which the induction is achieved, that determines apoptosis. This interpretation is consistent with findings that suggest that Weber's Law applies to Wnt signaling, in that the physiological effects of Wnt signaling result from the fold-change, rather than the absolute levels, of Wnt activity [34].

Thus, it is possible that the common effect of suppressed fold-induction of Wnt activity by butyrate exhibited by both Amlexanox and Ataluren is in part associated with the ability of the agents to abrogate butyrate-induced HCT-116 cell apoptosis. However, we observed that both agents abrogate basal levels of HCT-116 cell apoptosis in the absence of butyrate, despite the finding that these two agents differ in their effects on basal Wnt activity in untreated cells. This would suggest that the effects of these agents on apoptosis are at least in part Wnt-independent, although the possibility remains that Wnt-dependent effects of these agents on apoptosis occur in the presence of butyrate. For both the Wnt-dependent and Wnt-independent effects, a likely mechanism is the increased expression of PTC-containing genes, as Amlexanox and Ataluren are (a) unrelated agents, (b) have similar effects on apoptosis, and (c) share PTC read-through activity. Further investigation is required to confirm and explore the association between PTC read-through and CRC cell apoptosis, and to identify gene targets that may mediate this effect. Further, it is likely that low level expression of these PTC-containing genes is sufficient for the suppression of HCT-116 cell apoptosis, considering the equal efficacy of Ataluren and Amlexanox in abrogating apoptosis. Thus, despite the greater effectiveness of Amlexanox in enhancing expression from the NMD reporter (Figs. 1, S2A), which models the expression of PTC-containing genes, both agents equally abrogate HCT-116 cell apoptosis.

We note that differences between Amlexanox and Ataluren with respect to Wnt activity can have consequences for colonic cells independent of apoptosis. For example, this can include altered expression of physiologically-relevant Wnt target genes, modulation of decisions of proliferation vs. differentiation, and promotion or suppression of epithelial to mesenchymal transition. Considering the importance of fold-induction in Wnt signaling [10-18,34], we note that the suppressive effect of Amlexanox on induction was more robust than that of Ataluren, possibly because the former combines NMD inhibition with read-through activation; whereas, the latter is a specific read-through activator. Amlexanox is expected to more effectively enhance expression of PTC-containing genes, an effect evident in the ability of Amlexanox to enhance expression from the NMD reporter (Fig. 1), which Ataluren did not do (Fig. S2A). Expression of such genes may be required for the suppression of butyrate-induced Wnt

hyperactivation, and further studies are required to evaluate this possibility and identify the target genes of interest.

Figures and Figure Legends

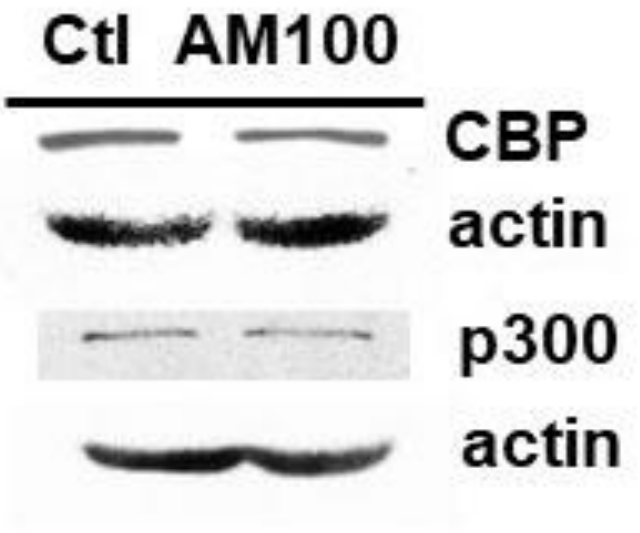

Fig. S1. Western blot data for Amlexanox-treated HCT-116 cells. HCT-116 cells were mock-treated (Ctl) or treated with 100 μM Amlexanox (AM100) for 48 hr. Protein lysates (100 μg) were assayed by Western blotting as described in Fig. 3A. Representative data are shown.

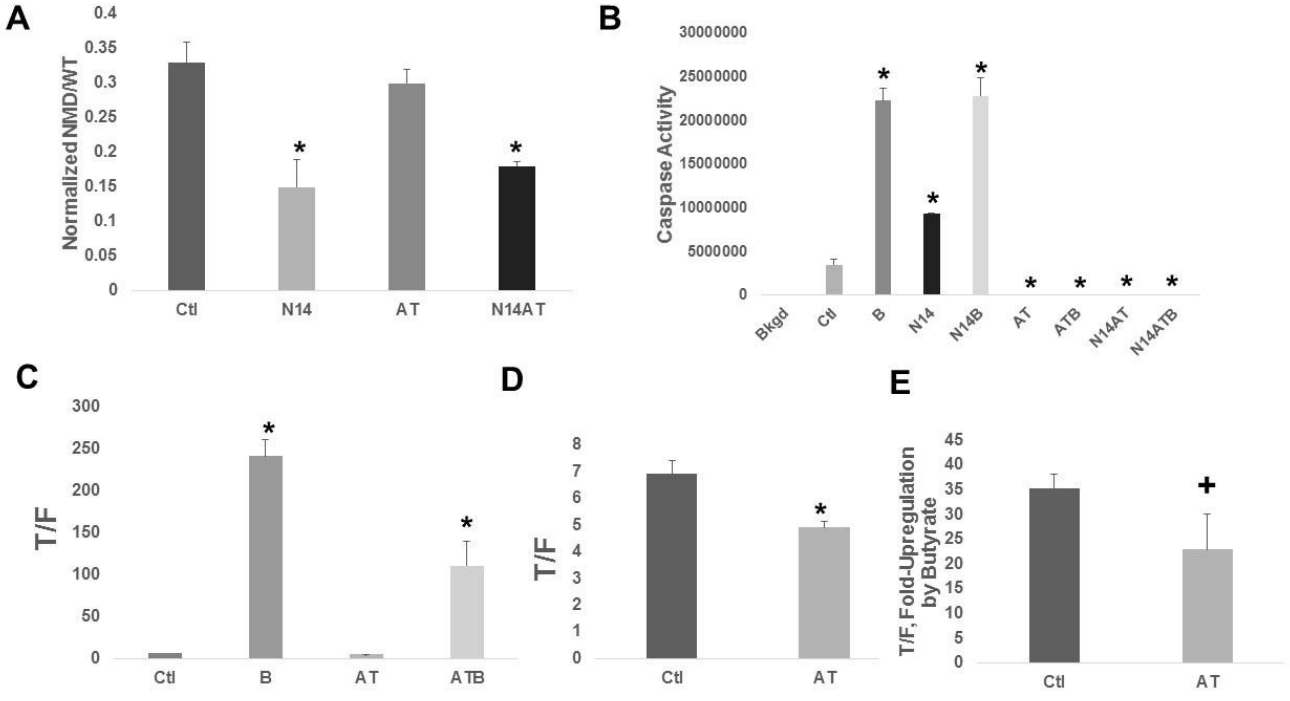

Fig. S2. Effects of NMDI-14 and Ataluren in HCT-116 CRC cells. HCT-116 CRC cells were cotransfected with wild-type or NMD (PTC) reporter vectors and a control

vector for normalization and were then treated with 50 μM NMDI-14 (N14), 50 μM Ataluren (AT), both agents (N14AT), or mock treated (Ctl) for 48 hr. Cells were assayed for luciferase expression, measuring normalized readings for the NMD (PTC) reporter divided by that from the wild-type reporter. Data from three independent experiments. Bars, SDs. (B) Apoptosis assays were performed as previously described [11-15]; cells were treated as in (A) with the addition in some cases of 5 mM butyrate [labeled with "B"]. Data from three independent experiments. Bars, SDs. (C) Wnt signaling data with Ataluren and butyrate, performed similar to that described in Fig. 2 [using 50 μM Ataluren (AT) and/or 5 mM butyrate (B)]. Bars, SDs. (D) Basal Wnt activity from the data of (C). Bars, SDs. (E) Fold-induction of Wnt activity by butyrate, from the data of (C). Bars, SDs.